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Universidad del País Vasco
Euskal Herriko Unibertsitatea

Study of the epigenetic cellular memory induced by morphine: a *Next Generation Sequencing* approach

Morfinak eragindako zelulen arteko memoria epigenetikoaren azterketa, Bigarren Belaunaldiko Sekuentziazioaren bitartez

Doctoral Thesis
Doktore Tesia

Iraia Muñoa Hoyos
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“Plotting is the art of doing science”

Biostars Handbook,
Bioinformatics data analysis guide

HITZAURREA

Jarraian aurkezten den tesia, **Euskarazko Nazioarteko Tesi** bat da, hori dela eta, eskuliburu hau euskaraz eta ingelesez idatzita dago zati berdintsuetan. Zehazki “Laburpena”, “Hipotesia eta Helburuak”, eta “Ondorioak” atalak bi hizkuntzetan idatzita daude. Baina “Sarrera”, “Material eta Metodoak” eta “Eranskinak” Euskaraz soilik daude idatzita, eta “Emaitzak”, “Eztabaida” eta “Bibliografia” ordea, ingelesez azaltzen dira. Aipatzekoa da sarrerako 1.5.2.2. atalean ingelesez idatzitako errebisio artikulu bat gehitzen dela, tesian zehar burututakoa eta aldizkari zientifiko batean argitaratua dagoena.

FOREWORD

The thesis presented below is an **International PhD written in Basque** that is why this manual is written in Basque and in English in similar parts. Specifically, "Summary", "Hypothesis and Aims," and "Conclusions" are written in two languages. However, "Introduction", "Materials and Methods" and "Appendix" are only written in Basque, and "Results", "Discussion" and "References" explained in English. It is noteworthy that in the section 1.5.2.2. of the introduction, a review article is included in English, which was written during the PhD period and published in a scientific journal.

LABURPENA
SUMMARY

LABURPENA

Epigenetikak, kanpo faktoreek edo inguruneak norbanakoan izan dezaketen eragina aztertzen du. Azken urteetan, zenbait ikerketek erakutsi dutenez, ingurumenak eragin zuzena izan dezake norbanako helduaren ezaugarri fisiologikoetan. Hala ere, kanpo faktoreek umekian eta garapen goiztiarrean izan dezaketen eraginari buruz, ezer gutxi deskribatu da. Geneen adierazpenarekin duten elkarteratzea dela eta, histonen aldaketak informazio epigenetikoaren eragile nagusien artean aurkitzen ditugu, eta memoria epigenetikoan paper garrantzitsua izan dezaketela iradoki da. Hala ere, naiz eta histonen aldaketa espezifikoek transkripzio egoerekin erlazioa duten, epe luzean zehar mantendu daitekeen herentziaren mekanismoa ez da oraindik ere argitu.

Hau guztia aztertzeko eta kanpo estimulu eredu bezala morfina erabiliz, bigarren belaunaldiko sekuentziazio teknikak erabili dira kromatina eta transkriptomika analisiak burutzeko. Bertatik H3K27me3 marka epigenetiko errepresiogilearen desdoitze orokor bat hauteman dugu, epe luzeko geneen adierazpen aldaketak sortuz, saguen ama zelula enbrionikoetan. Zehazki morfinaren tratamendu kronikoak eragindako efektua CpG irletan ugariak diren sustatzaileetan, inpronta genomikodun geneen metilazioaren erregulazio eremuetan, lncRNA-etan eta elementu errepikakorretan aurkitu ditugu. Aipatzekoa da, H3K27me3-ren eta bere erregulazio konplexuaren (Polycomb repressive complex 2 edo PRC2), auto erregulazio mekanismo bat identifikatu dugula morfinaren presentzian. Horrez gain, morfinak eragindako H3K27me3-ko aldaketek bi gene espezifikotan sortzen dituen adierazpen aldaketak hauteman ditugu, Bmp4 eta Smchd1 hurrenez hurren, hauek parte hartzen duten prozesuak guztiz eraldatuz. Bmp4 enbrioi goiztiarren garapenean eta jatorrizko hozi zelulen sorreran parte hartzen duen hazkuntza faktorea da eta morfinaren ondorioz, bi prozesu horien azkartzea eragiten duela hauteman dugu. Bestalde, Smchd1, X kromosomaren inaktibazioaren mantentze lanetan haritzen da eta gure sisteman morfinaren eraginez erreprimituta aurkitu dugu X kromosomaren birraktibazioa azkartuz.

Doktoretza tesi honetan lortutako emaitza hauek, kanpo faktoreek sortutako zelulen arteko memoria epigenetikoaren mekanismo molekularrei buruzko informazio baliagarriaz hornitzen gaituzte, belaunaldiz belaunaldiko oinordekotza epigenetikoa ulertzeko oinarri gisa. Hori dela eta, ezagutza hori heredatu daitezkeen aldaketa epigenetikoen identifikaziorako baliagarria litzateke etorkizunean

SUMMARY

Epigenetic is the study of how environmental factors or external stimuli can affect the individual. In the last years, many research groups have described that environmental factors have direct impact in physiological traits of adult individuals. However, little is known about the effect of external stimuli in the fetus, and the consequences that those stimuli can produce in the early stages of the developmental process. Histone modifications are thought to act as contributors of epigenetic information, because of their strong association with gene expression, and their potential role in transcriptional memory. Nevertheless, although specific histone modifications correlate with transcriptional status, the mechanism involved in the long-term inheritance of gene expression patterns is unclear.

In order to explore this and using morphine as an external stimulus model, chromatin analysis and transcriptomic approaches, through next generation sequencing techniques, revealed a global deregulation of H3K27me3 epigenetic repressive mark, which could produce long term gene expression changes in mouse embryonic stem cells. Specifically, the effect produced by morphine chronic treatment is perceived in CpG enriched promoters, imprinting control regions and differentially methylated regions of imprinted clusters, lncRNAs and repetitive elements. Interestingly we provide a self regulatory mechanism of H3K27me3 and its regulatory complex, Polycomb Repressive Complex 2 (PRC2). Moreover, we have identified morphine induced H3K27me3 modifications in the expression of two specific genes, Bmp4 and Smchd1 respectively, producing different changes in the processes they regulate. Bmp4 is involved in early embryo development and primordial germ cells differentiation, processes that are accelerated after morphine exposure. Furthermore, Smchd1 is the responsible of X chromosome inactivation maintenance, which is repressed in our study model, thus reactivating prematurely the X chromosome.

The results obtained in the following PhD thesis, could provide with valuable information for a better comprehension of molecular mechanisms underlying epigenetic cellular memory produced by external factors, as a basis to understand the epigenetic transgenerational inheritance. In that way, this information could be essential for the identification of heritable epigenetic modifications.

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1. SARRERA

INTRODUCTION

1. SARRERA

1.1 EPIGENETIKA

"Epigenetika" hitza XX. mendearen hasieran erabili zen lehenengo aldiz, genetika eta garapenaren biologia uztartzeko helburuarekin. Conrad Waddington enbriologoak, "epi-" aurrizkia erabiliz, gene sekuentziaren gainetik zeuden mekanismo batzuen existentzia iradoki zuen (Waddington CH 1942), hain zuzen ere, zelulen berariazko identitatearen eskuratze eta mantentze prozesuan parte hartzen zutenak (Holliday R 2006). Zehazki Waddington-ek proposatutako ereduak, zelula anitzeko organismoek ingurunearekin elkarrekintza burutzen zutela adierazten zuen, bertatik fenotipoak sortzeko, garapen prozesua gidatuz. Hau da, inguruneak garapen prozesuan, parte hartze aktiboa zuela iradoki zuen. Aitzitik, garai hartan geneek herentzian izan zezaketen parte hartzea oraindik ez zen ezagutzen (Waddington CH 1942; Goldbert eta lank. 2007).

Gaur egun, epigenetikak "DNA-ri itsasten zaizkion molekula kimikoen aldaketa heredagarriak aztertzen ditu, beti ere DNA-ren sekuentzian aldaketarik egin gabe" (Goldberg eta lank. 2007; Bird 2007). Aldaketa hauek, zeluletan edo organismo batean geneen adierazpena erregulatu dezakete. Honek bere barne hartzen ditu zelula batetik ondorengoetara transmititzen den geneen adierazpena, zelulen desberdintzean gerta daitezkeen geneen adierazpenaren asaldadurak eta inguruneak edo kanpo faktoreek geneen adierazpenaren aldaketan izan dezaketen eraginaren ikerketa (The epigenome network of excellence, 2009).

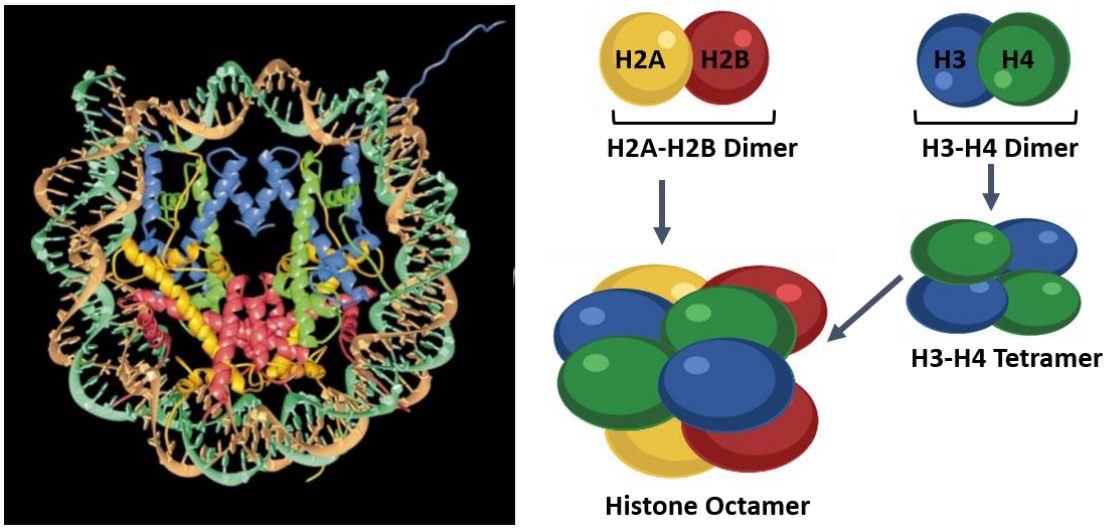
Nukleoan DNA, histona proteinekin, RNA-rekin eta histonak ez diren beste proteinekin (transkripzio faktoreak kasu), bilduta egon ohi da, antolatutako egitura bat osatuz "kromatinaren zuntza" izena duena. Kromatinaren osagaiak aldaketa epigenetikoen baitan daude. Marka kimiko horiek transkripzioko makinariak interpretatuko ditu, jarraian geneen adierazpen egokia bultzatzeko. Garrantzitsua da aipatzea, marka kimiko horiek gehitu edo ezabatzen dituzten entzimen aktibitatea ere, aldaketa epigenetikoen menpe egon daitekeela. Izan ere, organismoen garapenean zehar, desberdintze prozesuaz gain, zelula bakoitza hainbat faktore ezberdinen menpe egon ohi da, seinale mitotiko edo apoptotikoak, DNA-ren konponketa, metabolitoen erabilgarritasuna, droga menpekotasuna edo ingurune kimiko orokorra besteak beste. Faktore hauek guztiak gene espezifikoen piztuera edo isiltzea behar izaten dute, aldaketa horiek epe motz edo luzerako mantenduz (zelula ziklo bat baino gehiago). Hori dela eta, zelulek eta bertako kromatina zuntzak bere ingurunea hauteman behar dute, gene garrantzitsuen transkripzio maila bertara egokitzeko eta funtzio egokiak mantentzeko (Bird eta Tweedie, 1995). Guzti honekin interesgarria litzateke, kromatina zuntzak ingurunearen hautemate prozesua nola burutzen duen aztertzea eta honek genomaren jardueran zer nolako eragina izan dezakeen ulertzea, bai epe motzean edota epe luzean ere (Turner BM 2009).

1.2. KROMATINAREN ANTOLAKETA ETA BERE ALDAKETAK

Zelula eukariotetan informazio genetikoa nukleoan biltzen da. Nukleoaren diametroa mikra gutxikoa den arren, DNA-ren molekulak bi metroko luzera izan dezake. Hori dela eta, zelularen beharren arabera DNA zuntzak maila ezberdinetako trinkotze prozesuak jasaten ditu (DNA biluzitik, metafaseko kromosomaraino), lan horretan espezializatutako proteinen laguntzarekin (Grunstein M eta lank. 1992). Proteina hauek bi motakoak izan daitezke, histonak edota histonak ez diren beste hainbat proteina. DNA eta inguruan biltzen zaizkion proteinen konplexuari "kromatina zuntza" deitzen zaio eta hau antolaketa maila ezberdinetan deskribatu izan da.

1.2.1. Kromatinaren antolaketa

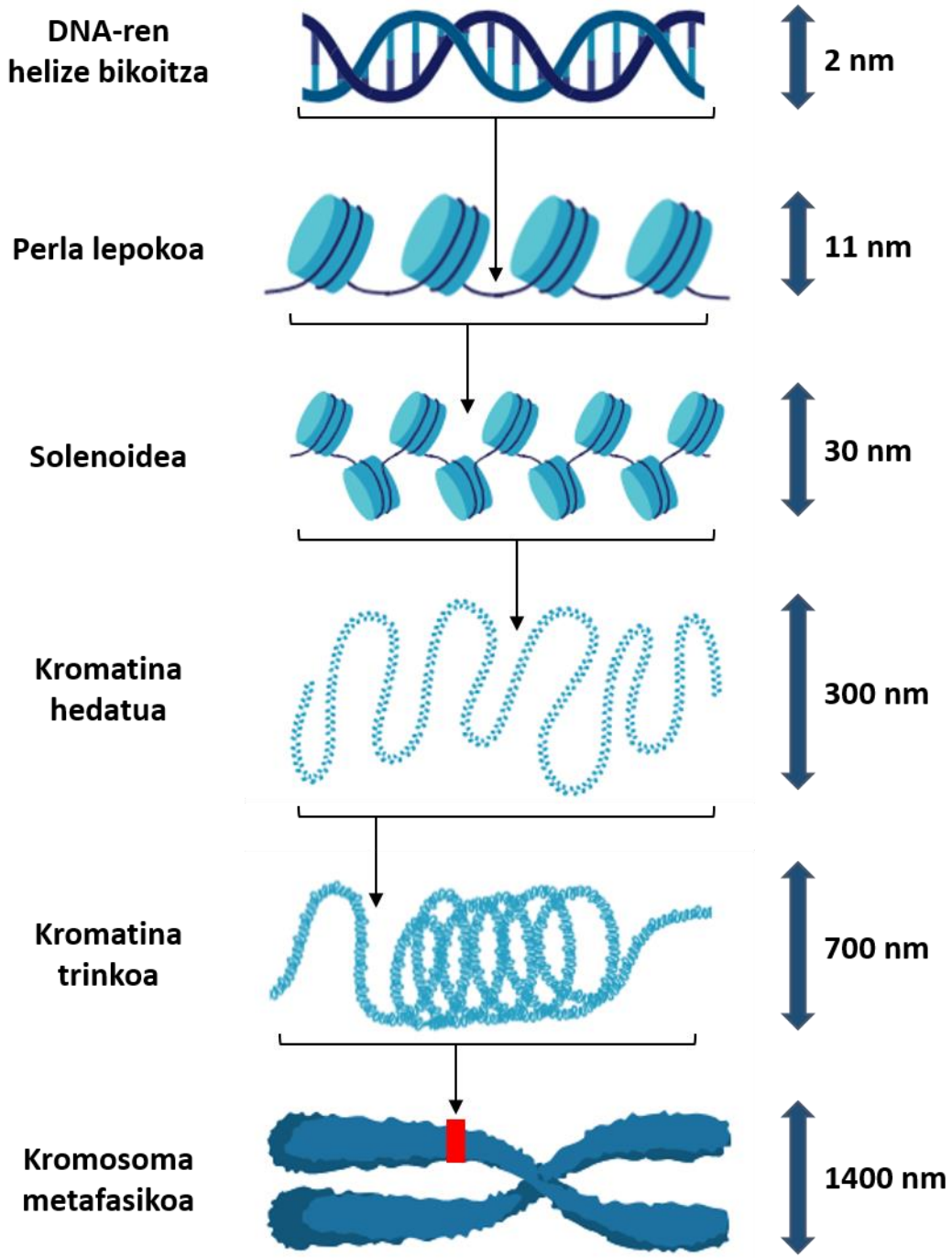
Kromatinaren oinarrizko antolaketa nukleosoma izenez ezagutzen da eta hau histonaz osatutako disko itxurako egitura bat da, zeinari DNA zuntza biribilkatzen zaion. Nukleosoma bakoitzean zehazki 8 histona aurkitzen dira, nukleosomaren bihotza osatuz eta baita organismoaren araberako DNA zati bat, histonen oktameroari bira bat eta hiru laurden ematen dizkiona. 8 histonako oktameroa, histona bakoitzeko 2 kopiak osatzen dute, 2 H2A, 2 H2B, 2 H3 eta 2 H4 (Luger eta lank. 1997) (1.1.Irudia). Histonak proteina basikoak dira (lisinan eta argininan oso ugariak dira), eta ezaugarri horrengatik, erraz elkartzen dira izaera negatiboa duten azido nukleikoekin. Honez gain, bostgarren histona bat ere deskribatu izan da, H1 histona, nukleosomaren bihotzeko histonen oktameroaren kanpoaldean biltzen dena, nukleosomaren egitura finkatzeko eta egonkortzeko. Izan ere, hainbat ikerketek erakutsi dutenez, nahiz eta H1 histona kopuru urria duen kromatinazko zuntzak oraindik ere trinkotzeko ahalmena izan dezakeen (Hansen eta lank. 1989), beharrezkoa da garapen prozesu egokirako (Fan eta lank. 2003) eta baita adibidez metafaseko kromosomen trinkotze mailara iristeko (Maresca eta Heald 2006). Esan bezala histonak beraien artean elkartu egiten dira, konplexu trinko bat sortuz, baina histona bakoitzaren eremu bat (amino muturreko eremua), konplexutik irteten da, histonen isatsak sortuz. Azkenik, nukleosomen artean DNA zati askeak geratzen dira (ingelesezko *DNA linker* deiturikoak), kromatinazko zuntzari malgutasuna ematen diotenak. *DNA linker* hauek nukleosoma bakoitzaren posizioa zehazten dute kromatina zuntzean zehar eta beraz, kromatinaren funtzioan faktore garrantzitsu bat kontsideratzen dira. Izan ere, nukleosomaren funtzioa alde batetik, DNA antolatu eta paketatzea da eta bestetik nukleosomen posizioaren bidez, DNA zuntzaren eskuragarritasuna edo bertara iristeko erraztasuna erregulatzea da, geneen adierazpena doitzeko asmoarekin. Honen erakusle da legamian egin zen ikerketa bat, non geneen adierazpena, transkripzioaren hasiera inguruko nukleosomaren betetze mailarekiko alderantziz proportzionala zela deskribatu zen (Lee eta lank. 2007). Nukleosomen posizioa zehazki zerk mugatzen duen, oraindik ere ikertzeko dagoen gai bat da, baina argi dago, prozesuan DNA-ren metilazioaren, kromatinaren eraldatze entzimen, histonen modifikazioen eta transkripzio faktoreen parte hartzea (Arya eta lank. 2010).



1.1 Irudia. Nukleosomaren eredua. Ezkerrean kristalezko egitura (X izpien difrakzioz burutua) eta eskuman nukleosomaren barne egiturako osagaien eskema. Nukleosoma H3-H4 histonen tetrameroaz osatuta dago, (H3 urdinez eta H4 berdez) eta baita bi H2A-H2B dimeroz ere (2HA horiz eta 2HB gorriz). (Luger K eta lank. 1997; Allis eta lank. 2006, eraldatua).

Zuntz nukleosomikoa kromatinaren lehenengo paketatze maila da, baina zelula gehienetan kromatina kondentsatuago agertu ohi da. Bigarren antolaketa mailan nukleosomak ordenatuta ageri dira, elkarrengandik oso gertu egitura laburragoa eta lodiagoa sortzeko, 30 nm-ko kromatinazko zuntza hain zuzen ere (Thoma eta lank. 1979). 30 nm-ko zuntz hau oso malgua da eta nukleosomak gertuago edo urrunago egotearen arabera, zuntza trinkoago edo lasaiago egon daiteke. Hori dela eta kromatinazko zuntza egitura dinamiko bat dela esan ohi da. Prozesua deskribatzeko bi mekanismo proposatu dira: alde batetik, H1 histonaren loturak eraginda, nukleosomak bata besterantz bultzatzen dituela zig-zag itxura sortuz edota “perlazko lepoko” itxurarekin alderatu den egitura osatuz (Olins eta Olins, 1974). Eta bestetik, histonen isatsen parte hartzearen bitartez, dirudienez ondoan dauden nukleosomak isatsen bidez zuzenean elkartu daitezke eta (Allan J eta lank. 1982). Era batera edo bestera, zuntzak sortzen duen bira bakoitzeko 6 nukleosoma biltzen dira, solenoidea izeneko egitura osatuz.

Solenoidearen egitura era berean biribilkatua ager daiteke trinkotze maila handiago batean, 300 nm-ko kromatinazko zuntza osatuz (Widom 1986). Eta era berean 300 nm-ko zuntza bere baitan biribilkatu daiteke kromatidak osatuz. Behin kondentsinen laguntzarekin kromatida ahizpak batzean kromosomak sortzen dira (1.2. Irudia). Hala era maila altueneko kromatinaren egitura osatzeko eta egonkortzeko mekanismoak ez daude guztiz argi gaur egun. Adibidez, metafasean dagoen kromosomaren osaera mekanismoan H1-aren fosforilazioa, topoisomerasa II entzima eta kondentsina konplexuen parte hartzea deskribatu da, baina oraindik ez da argitu nola ainguratzen den zuntz kromatikoaren egitura horretan (Belmont 2006).



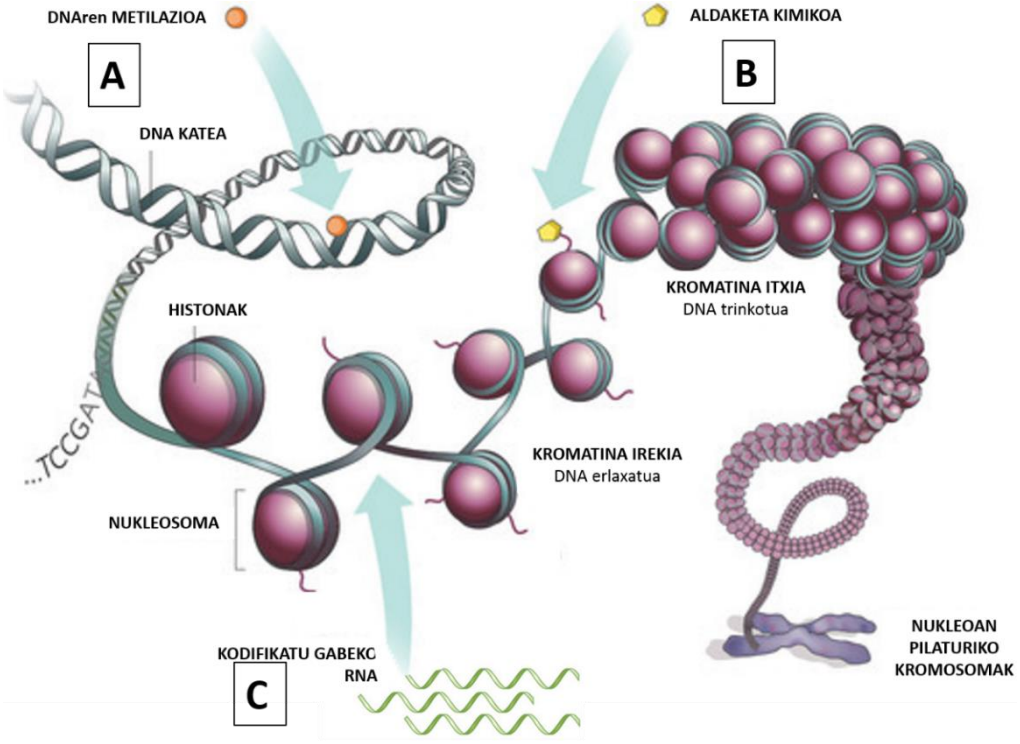
1.2. Irudia. Kromatinaren antolaketa osatzen duten trinkotze maila ezberdinak. Kromatinaren antolaketan identifikatu diren trinkotze maila ezberdinak, DNA-ren helize bikoitza, "perladun lepokoa" zuntza, 30 nm, 300 nm, 700 nm-ko zuntza eta kromosoma metafasikoa dira. BioRender tresnarekin sortutako irudia.

Nukleoan bi kromatina moten bereizketa lehenengo aldiz 1928. urtean deskribatu zen (Heitz E 1928): Alde batetik, heterokromatina edota eremu kondentsatuak (zelularen %10-a izatera iritsi daitezkeenak), eta bestetik eukromatina edo kondentsazio maila baxuko eremuak. Heterokromatina kromosoma guztietan ager daiteke hainbat eremutan. Eremu horietan gene gutxi egon ohi da, eta hori dela eta klasikoki onartu egin da alderantzizko erlazioa dagoela heterokromatinaren presentziaren eta gene adierazpenaren artean. Honela, heterokromatina erabili izan da genoman zehar isildutako edo transkripzio gabeko eremuak izendatzeko eta bestalde, eukromatina erabili da, kromatina irekia edo transkripzio eremuak adierazteko. Teoria

oraindik ere konplexuagoa bihurtu zen, heterokromatina bi zatitan banandu zenean, heterokromatina konstitutiboa edota, funtzioaren eta geneen adierazpenaren arabera kondentsazio-maila ezberdinak erakusten dituena, hala nola zentromeroak eta telomeroak. Eta heterokromatina fakultatiboa edo, DNA errepikakorrez eratuta eta beti kondentsatuta dagoena. Azken hauek zelulen araberakoak dira eta desberdintze prozesuen bitartez erregulatzen dira (Dimitri eta lank. 2005). Heterokromatina fakultatiboaren adibide bat ugaztunetan emeen X kromosoma inaktiboa izango litzateke. Aipatzekoa da, transkripzioa ez dela prozesu konstante bat, hori dela eta kromatina zuntzeko eremu espezifikoek konformazio egitura ezberdinak osatzen dituzte, kromatina zuntza dinamikoa den heinean, zelularen beharretara egokitzeko (Dimitri eta lank. 2009).

1.2.2. Kromatinaren aldaketak

Aurretik aipatu bezala, epigenetikaren oinarriek DNA-ren sekuentzia eraldatu gabe DNA-n ematen diren aldaketa kimikoak aztertzen dituzte, "aldaketa epigenetiko" bezala ezagutzen direnak (Jaenish R eta Bird A 2003). Hauek, eragina dute transkritoen egonkortasunean, DNA-ren kondentsazioan, nukleosomaren kokatzean, kromatinaren trinkotzean eta nukleoaren antolaketan. Ikerketa askotan marka epigenetikoek, geneen adierazpenarekin erlazionatu dituzte eta beste batzuetan marka epigenetiko espezifikoek transkripzioa piztu edota itzaltzeko joera dutela azaldu da. Ondorioz, argi dago kromatinaren funtzioa ez dela DNA-ren paketatze edo trinkotze soila, baizik eta geneen adierazpena erregulatzen duen mekanismoa baizik (Lennartsson A eta lank. 2009). Gaur egun, aldaketa epigenetikoei dagokienez hiru mekanismo ezberdin ezagutzen eta ikertzen dira (1.3. Irudia): DNA-ren metilazioa, histonen aldaketak (histonen bariante ezberdinak ere kontuan hartuz), eta kodifikatu gabeko RNA molekulak (ingelesezko *non-coding RNA* edota ncRNA) (Kieffer JC eta lank. 2007).



1.3. Irudia. Deskribaturiko aldaketa epigenetikoak. A) DNA-ren metilazioa, B) Histonen aldaketak, C) Kodifikatu gabeko RNA. Marx V 2012, moldatua

1.2.2.1. DNA-ren metilazioa

Aldaketa epigenetikoen artean, DNA-ren metilazioa da gaur egun gehien aztertu dena (Holliday eta lank. 1975). Zelula eukariotetan DNA-ren metilazioa, 5. Karbonoko zitosinan metilo talde bat gehituz (5-mC) gertatzen da, batez ere CpG dinukleotidoetan. Hala ere, CpG irlen inguruan, CpG dentsitate gutxiagoko eremuak daude (~2kb) "Shore" izenarekin ezagutzen direnak, eta ehun espezifikoetako DNA-ren metilazioaren zati haundi bat bertan gertatzen da (Doi AI eta Park H eta lank. 2009). Gainera, enbrioi zeluletan CpG dinukleotidoetatik kanpo eman diren metilazioak deskribatu izan dira (Ramsahoye eta lank. 2000). DNA-ren metilazioak epe luzeko geneen isiltzea sortzen du garapenean zehar (Klose RJ eta Bird AP 2006; Bestor TH eta Bourchis D 2004; Parveen P eta lank. 2014), histonen aldaketen patroietan eraginez eta baita transkripzioaren aktibazioarekin erlazionatutako lotura faktoreak eragotziz. Estuki lotua dago, inpronta genomikoa (Law JA eta Jacobsen 2010; Reik W eta lank. 2007; Borgel J eta lank. 2010) eta X kromosomaren isiltzearekin (Nestevora TB eta lank. 2008). Aipatzekoa da bestalde, ugaztunen genomaren %70 osatzen duten elementu transposagarrien (TE) isiltze mekanismoa burutzen duela, genomaren egonkortasuna mantentzeko (Goodier eta Kazazian 2008; Bestor TH eta Bourchis D 2004). Gainera, 5-mC marka epigenetiko egonkorra kontsideratzen da, izan ere, behin sortu eta finkatzen denean, DNA-aren erreplikazioan zehar transmititu daitekeelako zelula alabetara (Lande-Diner L eta Cedar H 2005).

Genoman zehar DNA-aren metilazioaren banaketa aldakorra da, izan ere, badira eremu txikiak CG-z oso aberatsak direnak. CpG isla deituriko eremu hauek geneen 5' muturrean kokatzen dira eta genearen sustatzailearen eta lehen exoiaren parte izan ohi dira. Saguaren genoman adibidez, CpG irlak genomaren %1-a besterik ez diren arren, geneen erdia baino zertxobait gutxiagotan aurkitzen dira (Cross eta Bird 1995; Fatemi eta lank. 2005). Honen harira, metilazio prozesuaren mekanismoa azaltzeko hainbat bide ezberdin proposatu dira CpG irlei lotuta, ikerketek aurrea egin ahala. Lehenengo, DNA-ren metilazioak CpG irlez osaturiko sustatzaileei espezifikoki batzen zaizkien transkripzio faktoreen lotura eragozten duela proposatu da (Tate eta Bird 1993; Campanero eta lank. 2000). Bigarrenez, DNA-ren metilazioak nukleosomaren antolaketa aldatu dezakeela deskribatu da, geneen sustatzaileetan isiltze faktoreak batzea ahalbidetuz, transkripzio makinariara batu beharrean (Kass eta lank. 1997). Hirugarrenez, baliteke DNA-ren metilazioak faktore espezifikoak biltzea horrela transkripzio faktoreen lotura eragozteko eta beraz, geneen isiltze prozesua bideratzeko (Lewis eta lank. 1992; Lopez-Serra eta Esteller 2008).

Ugaztunetan DNA-ren metilazioaren mantentze lana eta finkapena hiru DNA metiltransferasek (Dnmt) burutzen dute. Zelula ama enbrionikoetan eta zelula somatikoetan Dnmt1 aritzen da metilazio lanetan, batez ere hemimetilazioarekin erlazionaturik. Izan ere, erreplikazio osteko hemimetilatutako DNA ezagutzen du eta metilazio patroi hori zelula alabaren katean kopiatzen du. Hori dela eta, mantenu lanetan aritzen den metiltransferasa bezala ere ezagutzen da (nahiz eta *de novo* metilazioan ere aurkitu izan den (Jeltsch A eta Jurkowska RZ 2014)). Obuluetan, oinarrizko Dnmt1-aren ordez, obulu espezifikoa den Dnmt1o entzima aktibatzen da garapen goiztiarrean zehar inpronta genomikoaren mantenua burutzeko (Latham eta lank. 2008; Bestor TH 2000; Sharif J. eta lank. 2007). Dnmt2 ez da funtsezkoa DNA-ren metilazioa gertatzeko edota saguaren garapenerako baina, dirudienez, tRNA molekulen metiltransferasa gisa aritzen da (Goll eta lank. 2006). Hirugarren DNA metiltransferasa Dnmt3-a da eta honek splicing bidezko hainbat bariante ditu. Horien artean, Dnmt3a eta Dnmt3b, *de novo* metilazio prozesuan oso garrantzitsuak dira eta garapeneko etapa goiztiarretan metilatu gabe dagoen DNA-ren

metilazioaz arduratzen dira. Hori dela eta, aktibitate handia dute ama zelula enbrionikoetan, baina zelulen desberdintze prozesuarekin batera beraien parte hartzea ere gutxiagotu egiten da (Okano eta lank. 1999). Dnmt3L-a azken bi hauen oso antzekoa da, eta metilazioaren efizientzia hobetzen laguntzen du. Gainera oso garrantzitsua da, saguen garapenean zehar amaren eta aitaren inpronta genomikoa finkatzeko (Latham eta lank. 2008; Arnaud eta lank. 2006; Bourchis eta lank. 2001; Hata eta lank. 2006), baita hozi zelula lerroetan ere (Sakai eta lank. 2004). Bestalde, DNA-ren metilazioa, marka epigenetikoetan egonkorrena den arren, posible da zenbait mekanismoren bitartez ezabatzea: 1) Demetilazio pasiboa, non 5-mC metilazio marka ez den mantentzen zelula alabetan eta beraz, erreplikazio zikloetan zehar galdu egiten den. 2) Demetilazio aktiboa, non TET entzimek (ingelesezko *ten eleven translocation enzymes*), 5-mC-tik, 5-hidroximetil zitosinara (5-hmC), 5-formil zitosinara (5-fC) eta 5-kaboxil zitosinara (5-caC) bihurtzen duten. Ondoren erreplikazio zikloetan zehar pasiboki galduko lirateke edota aktiboki ordezkatuak izango lirateke eraldatu gabeko C batekin, basearen eszisio konponketa bidez (Kholi RM and Zhang Y 2013). Beraz, guzti honek adierazten duenez, DNA-ren metilazio egoera prozesu aktibo eta pasiboen bitartez oso zuzendua dago (Jeltsch A and Jurkowska RZ 2014; Bagci H and Fisher AG 2013).

1.2.2.2 Histonen aldaketak

Lau dira nukleosomako oktameroa osatzen duten histonak (H2A, H2B, H3 eta H4) eta beraien N-muturreko isatsetan modu ezberdinetan jasotzen dituzte aldaketa epigenetikoak. Masa espektometria bitartez 30 aldaketa baino gehiago identifikatu diren arren ezagunenak edo ikertuenak jarraian zerrendatzen dira: azetilazioa (Sterner DE and Berger SL 2000), metilazioa (Zhang Y eta Reinberg D 2001), fosforilazioa (Nowak SJ eta Corces VG 2004), ubikitinazioa (Shilatifard A 2006), deaminazioa (Cuthbert GL eta lank. 2004), sumoilazioa (Nathan D eta lank. 2006), ADP-ribosilazioa (Hassa PO eta lank. 2006) eta prolinaren isomerizazioa (Cuthbert GL eta lank. 2004) besteak beste (Tessarz P eta Kouzarides T 2014). Gainera, aldaketa hauek histonen isatsetako aminoazido ezberdinetan kokatzen dira, orokorrean lisina, arginina, serina eta treonina hondakinetan. Zehazki, lisinek azetilazioa, ubikitinazioa edo sumoilazioa jaso dezakete, serinak eta treoninak fosforilatu egiten diren bitartean. Metilazioa aldiz lisina eta argininetan gertatu ohi da eta maila ezberdinetan, hondakin hauek mono-, di- edota tri-metilatu egin daitezkeelako (Kouzarides 2007). Beraien funtzioa konkretua oraindik ere guztiz argituta ez dagoen arren, histonen isatsetan ematen diren aldaketa ezberdinek, banaka edo taldeka jarduten dute (Berger SL 2007), histonen kodigo bat osatuz, kromatinaren egitura edo antolaketan aldaketak sortzeko gaitasunarekin (Strahl BD eta lank. 2000; Luger K eta lank. 1997). Horrela, kromatinaren egitura eukromatinara edo heterokromatinara bihurtzen dute, histonen eta DNA-ren arteko lotura aldatuz eta ondorioz transkripzioan eraginez (Mersfelder eta Parthun 2006; Kouzarides T 2007). Zehazki, kromatinaren konformazio aldaketa horien eragile nagusiak metilazioa eta azetilazioa direla deskribatu da (Cosgrove MS eta lank. 2005).

Histonen hondakinen metilazioak geneen transkripzioa piztu edo isiltzeko gaitasuna du. Histonen metilazioak ez du histonen karga aldatzen eta ondorioz, lisinen metilazioa iraupen luzeko marka kontsideratzen da, oso egonkorra delako eta mitosiaren ostean irauten duela frogatu delako (Margueron eta lank. 2005). Metilazioa histonen entzima metiltransferasen bitartez burutzen da (KMT) eta lisina espezifikoetan gertatu ohi da: H3-K4, H3-K36 eta H3-K79 lisinen metilazioak geneen adierazpena pizten du; eta H3-K9, H3-K27 eta H4-K20 lisinen

metilazioa geneak isilarazteko marka gisa deskribatu da (Kouzarides eta lank. 2007). Gainera aipatzekoa da H3-K9 eta H3-K27 lisinen trimetilazioak, marka epigenetiko errepresibo bezala kontsideratzen direla eta hortaz, kromatinaren trinkotzean eta transkripzioaren errepresio mekanismoetan parte hartzen dutela, heterokromatina sortuz (Peterson eta lank. 2004). Kromatina dinamikoa den heinean, histonen metilazioak ezabatu egin daitezke, lisina demetilasen (KDM) funtzioari esker. Horrela, zelulek metilazio patroien erregulazio zorrotza gidatzen dute, metilazioa prozesua, zelulen memoria heredagarriaren zigilua da eta (Shi eta lank. 2004). Arginina hondakinen metilazioa arginina metiltransferasek (PRMT) burutzen dute eta geneen adierazpenaren erregulazioan garrantzia handiko marka bezala deskribatu da, transkripzioa piztu (Chen eta lank. 1999) edo itzali (Pal eta lank. 2004) dezakeelako, metilazioa jasotzen duen hondakinaren arabera.

Orokorrean, histonen azetilazioa, kromatinaren transkripzio aktiboa duten eremuekin erlazionatu izan da. Hiperazetilazioak edota azetilo taldeak gehitzeak, histonen lisinen karga positiboa neutralizatzen du. Honek, positiboki kargaturiko histonen eta negatiboki kargaturiko DNA-ren erakarpena gutxitzen du bien arteko lotura ahulduz eta ere berean, DNA-ren sekuentzia agerian utziz, transkripzio faktoreak eta RNA polimerasa batzea ahalbidetuz (Tse eta lank. 1998). Bestalde, hipoazetilazioak kontrako joera sortuko luke (Turner BM eta lank. 2000). Gainera, transkripzioaren erregulazioaz gain, kromatinaren erreplikazioan, errekonbinazioan eta DNA-ren konponketan ere parte hartzen duela erakutsi da (Kimura eta lank. 2005). Azetilazioa histonen entzima azetiltransferasen bitartez burutzen da (KAT) eta lisina espezifikoetan gertatu ohi da: H4-K8, H4-K9, H4-K12 eta H4-K14. Marka hau ezabatu egin daiteke histona deazetilasen bidartez (HDAC).

Fosforilazioa, serina eta treonina hondakinetan gertatu ohi da eta kromatinaren trinkotzean eragina dute histonen isatsetan karga negatiboak gehitzen dituzte eta, horrela beraien karga neutralizatu eta DNA-rekiko afinitatea ahulduz. Deskribatu izan denez, histonen fosforilazioak, berehalako geneen adierazpenaren erregulazioan du eragina *in-vitro* egindako ikerketetan (Sultan FA eta lank. 2011). Bestalde "DNA-ren kalte seinaleztapenen" aktibazioaren ostean gertatzen den prozesu bezala ere deskribatu da, DNA-ren konponketa erraztuko lukeen kromatinaren antolamenduan parte hartzea iradokiz (Foster eta Downs 2005). Histonen fosforilazio prozesua entzima kinasa eta fosforilasen bitartez burutzen da, hala nola, Aurora-B, IKK-alfa eta PP1 entzimak.

Ubikitinizazioa, lisina hondakinetan gertatzen da, Ubikitina (Ub) izeneko entzimaren bidez. Prozesua maila ezberdinetan gerta daiteke, mono- edo poli-Ub eran hain zuzen ere (Bhaumik eta lank. 2007; Weake and Workman 2008). Prozesu ugarirekin erlazionatu izan da, proteinen degradazioa, DNA-ren konponketa, zelula zikloaren kontrola, estresarekiko erantzuna, zelulen desberdintze prozesua, erribosomen eta peroxisomen biogenesia, infekzio biralak, degradazio neuronal eta muskularra eta transkripzioa besteak beste (Jason eta lank. 2002; Finley eta Chau 1991; Jennissen 1995). Histonen ubikitinazioa ere ezabatu egin daiteke eta maila ezberdinak ubikitina askearen eta aktibitate entzimatikoaren menpe daude. Adibidez, zelula eukariotetan, H2A eta H2B histonen ubikitinazioa da ugariena (Jason eta lank. 2002), nahiz eta ugaztunetan bakoitzetik lisina bakarra ubikitinatzen den, 119 eta 120 lisinak hurrenez hurren (Osley 2006). Honenbestez, H2A histonaren ubikitinazioa transkripzioaren isiltzearen eta DNA-ren kaltetzearekin erlazionatu izan da (Wang eta lank. 2004; Zhou eta lank. 2008b; Cao eta lank.

2005; Zhou eta lank. 2008a). H2B histonaren ubikitinazioa aldiz, DNA-ren konponketa prozesuekin eta proteinen degradazioarekin erlazionatu da, eta nola ez, geneen adierazpenaren erregulazioarekin ere, horretarako beste histonen aldaketekin interakzio bat sortuz (Weake eta Workman 2008; Bhaumik eta lank. 2007; Jason eta lank. 2002).

Aipatzekoa da ere, nukleosomako oktameroa osatzen duten lau histonetaz gain, badirela beste hainbat histonen aldaera, erreplikazio prozesutik kanpo biltzen direnak baina gertakari epigenetiko osagarri kontsideratzen direnak (Sarma K and Reinberg D 2005). Hauek marka epigenetikoen mantentze lanetarako ezin bestekoak dira eta orokorrean, aurretik aipatutako lau histonen antzekoak dira baina zenbait aldaketekin (Malik HS and Henikoff S 2003; Henikoff S and Ahmad K 2005). Adibidez, zelula eukariotetan H3 eta H2A histonek bere barianteak dituzte, kromatinaren egoera ezberdintzeko ahalmena dutenak (Jin eta lank. 2005): H2A.X-a fosforilatua azaldu ohi da bi kateko DNA-ren hausturetan (Celeste A eta lank. 2002; Celeste A eta lank. 2003; Fernandez-Capetillo O 2003), H2A.Z eta H3.3 transkripzio aktiboko eremuetan aurkitzen diren bitartean (Ausio J and Abbott DW 2002; Redon C eta lank. 2002; Fernandez-Capetillo O eta lank. 2004; Hendzel M and Davie J 1990; Tagami H eta lank. 2004; Ahmad K and Henikoff S 2002). Bestalde, Macro2A-k heterokromatinaren osaeran parte hartzen du (Angelov D eta lank. 2003) eta CENP-A kromosomen zentromeroetan kokatzen da (Jin eta lank. 2005; Palmer DK eta lank. 1991; Palmer DK eta lank. 1987; Blower MD eta Karpen GH 2001; Buchwithz BJ eta lank. 1999; Howman EV eta lank. 2000; Stoler S eta lank. 1995). H2B histonaren bariantea, TH2B izenekoa testikuluetan aurkitu izan da (Zalensky AO eta lank. 2002), eta fosforilazio eremu espezifikoak ditu. H1 histonak bariante ezagun asko dauzka, horien artean H1A, H1B (Hendzel MJ eta lank. 2004; Fantz DA eta lank. 2001) eta H1t, H1t2 eta HILS1 testikuluetako espezifikoak direnak. Beraien lana zelulen polaritatea ezartzea eta hozi zeluletan funtzio erregulatzailea burutzea lirateke (Martianov I eta lank. 2005; Kimmins S eta Sassone-Corsi P 2005). Azkenik, H4 histonaren bariante ezagunik ez da deskribatu (Akhamanova A eta lank. 1996; Kamakaka RT eta Biggins S 2005). Beraz, histonen barianteen gaiaren inguruan oraindik ere ikerketa asko burutu ez diren arren, eta beraien funtzioak zeintzuk diren finkatuta ez dauden arren, argi dago erregulazio epigenetikoan parte hartzen dutela eta dirudienez geneen adierazpen patroien oinordekotzan ere funtzio garrantzitsu bat izan dezaketela eman da aditzera (Henikoff eta lank. 2004).

1.2.2.3. Kodifikatu gabeko RNA (ncRNA)

Azkenik, epigenetikaren inguruan, gaur egun indarra hartzen ari den beste ikerketa arlo bat kodifikatu gabeko RNA edo ncRNA-rena da (ingelesezko *non coding RNA*). ncRNA molekulak, RNA funtzionalak izan arren, ez dira proteina espezifikoetara itzultzen. Azken urteotan argitu denez, batez ere "giza genomaren proiektua"-ren laguntzaz, ugaztunen genomako %2-a bakarrik itzultzen da proteinara (Collins F eta lank. 2004; Alexander RP eta lank. 2010). Beraz, nahiz eta zelulen DNA gehiena RNA-ra transkribatzen den (%75), zati handi batek ez du proteinarik kodifikatzen, proportziorik gehiena ncRNA egoeran mantenduz (Sandberg K eta lank. 2013). Gainera, molekula hauek geneen adierazpenaren erregulazioan garrantzitsuak izan daitezke batez ere geneak isiltzerako orduan. Hori dela eta eragina izan dezakete, besteak beste kromosomen dinamikan, kromatinaren egituraren mantentzean eta eraldaketan, genomaren egonkortasunean, aldaketa epigenetikoen sustapenean, memoria, inpronta genomikoan, DNA-ren metilazioan epigenetikoan (Baulcombe D eta lank. 2004; Mattick JS eta lank. 2005; Zhou eta lank. 2010), eta hainbat gaixotasunen sorreran, hala nola minbizia, gaitz neurologikoa eta gaitz

kardiobaskularrak (Esteller M 2011; Ohsawa R eta lank. 2013; Fatica A eta Bozzoni I 2013; Mercer TR eta lank. 2009; Cech TR eta Steitz JA 2014; Frias-Lasserre D eta lank. 2017). ncRNA-k orokorrean bi talde nagusitan banatzen dira, kodifikatu gabeko RNA luzeak (>200 nukleotido) eta motzak (<200 nukleotido).

- Kodifikatu gabeko RNA luzeak edo lncRNA: hauek intergenikoak, intronikoak, kontrako noranzkodunak edo gainjarritako noranzko biko transkritoak izan daitezke, eremu intergenikoetatik sortzen dira, ugaztunen genoman milaka molekula izanez (Carninci P eta lank. 2005). Aditzera eman denez, lncRNA-k ez dira zitoplasmara garraiatzen mRNA-rekin gertatzen den bezala, horren ordez, nukleoan mantentzen dira (Mao YS eta lank. 2010), funtzio nuklear garrantzitsuak izan ditzaketela iradokiz. Izan ere, aldaketa epigenetikoetan parte hartzen dutela deskribatu da, kromatinaren eraldaketan laguntzen duten konplexuetan, RNA molekuletara lotzeko eremuak aurkitu direlako (Sun Y eta lank. 2005). Horrela lozi espezifikoetako kromatinaren antolaketa eta geneen adierazpena aldatzen dute (Esteller M 2011), adibidez histona ezberdinetan aldaketak sortuz (Rinn JL eta lank. 2007).

Ugaztunetan aurkitu zen lehenengo lncRNA geneen adierazpena isiltzeko ahalmena zuena Xist izenekoa izan zen. Xist-ek, eme ugaztunetan X kromosomaren inaktibazioan parte hartzen du (McCarrey JR eta Dilworth DD 1992), DNA-ren metilazioa bultzatuz eta errepresio histona markak finkatu daitezen eraginez, horrela X kromosoma isiltzea lortuz (Thorvaldsen eta lank. 2006). Xist beraren adierazpenaren erregulazioa beste ncRNA batek burutzen du Tsix izenekoa eta Xist-arekiko osagarria dena (Lee JT eta lank. 1992). Isildutako X kromosoman Xist aktibo mantentzearen eta aktibatutako kromosoman Tsix aktibatzen duten mekanismoak gaur egun ez dira oraindik ere guztiz argitu, baina dirudienez, Tsix-ek heterokromatinaren sorrera bultzatzen duen zenbait entzima biltzen ditu Xist genearen sustatzailean eta horrela bere adierazpena ekiditen du (Lee JT 2012; Froberg JE eta lank. 2013).

Bestalde, lncRNA-ek inpronta genomikodun geneen adierazpenaren erregulazioan parte hartze garrantzitsu bat dute. Inpronta genomikoa, fenomeno genetiko bat da non zenbait gene jatorri parental espezifikoaren bitartez adierazten diren (Swales eta Spears 2005; Ideraabudullah eta lank. 2008; Reik W eta lank. 2001; Ferguson Smith eta lank. 2001), hau da, gene espezifiko batzuen isiltze prozesua da, gurasoetako batetatik jasotako kopia kromosomalean. Prozesu hau, oso kontserbatua da ugaztunetan, primateetan, karraskarietan, hausnarkarietan eta martsupialetan (Monk eta lank. 2006; Lucifero eta lank. 2006; Umlauf eta lank. 2004; Vu eta lank. 2006). Gaur egun, 150 inpronta genomikodun gene inguru ezagutzen dira, eta garapen enbrionikoan zehar fetuaren eta plazentaren osatzean garrantzi handiko papera dutela deskribatu da (Brannan eta lank. 1999; Ferguson-Smith eta lank. 2001; Sleutels eta lank. 2002; Arnaud and Feil 2005; Fowden eta lank. 2006; Nafee eta lank. 2008). Honen harira, Inpronta prozesuaren erregulatzaile bezala 200 lncRNA baino gehiago deskribatu dira (Adalsteinsson BT eta Ferguson-Smith 2014; Barlow DP and Bartolomei MS 2014).

- Kodifikatu gabeko RNA motzak edo sRNA-k aldiz, hiru azpitaldetan banatzen dira, microRNA-k (miRNA), interferentziazko RNA motzak (siRNA) eta PIWI molekulekin lotzen diren RNA-k (piRNA) (Sandberg K eta lank. 2013). sRNA-k hereokromatinaren mantenuarekin erlazionatu dira (Mirouze M 2012; Siomi MC eta lank. 2011) eta baita geneen isiltzearekin ere

(Elbashir SM eta lank. 2001), horrela gaixotasunak sortzen dituzten geneen erregulaziorako itu terapeutiko garrantzitsuak bilakatu direlarik (Mraheil MA eta lank. 2010).

Hauek guztietatik gehien ikertu direnak mikroRNA-k dira, 19-24 pb-ko tamaina dutenak, eta proliferazio, hantura, fibrosi eta desberdintze prozesuen erregulazioarekin erlazionatzen direnak (Esteller M eta lank. 2011). Orokorrean miRNAk beraien gene ostalariarekin batera transkribatzen dira, proteina kodifikatzen duen genearen eta miRNA-aren arteko erregulazio parekidea bermatuz (Mraz M eta lank. 2012). Erregulaziorako makinaria RISC konplexuak burutzen du (ingelesezko *RNA-induced silencing complex*), non miRNA-rekin elkartzean, miRISC konplexua sortzen duen eta hau isildu behar den mRNA-aren 3' UTR-ra gidatuko duen. miRNA bat mRNA bati lotzean bere sintesia inhibitu egiten da, mRNA-ren ezegonkortzea eta degradazioa dela eta, edo transkripzioaren inhibizioa dela eta (Stroynowska-Czerwinska A eta lank. 2014; Bazzini AA eta lank. 2012). Ondorioz miRNA-k zein gene aktibatu edo isildu gidatzen dute.

siRNA-k bi kateko RNA molekula luzeetan dute beraien jatorria, non kate luze horiek Dicer endoribonukleasak moztu egiten dituen siRNA txikiak sortuz (Ketting RF eta lank. 2001). siRNA-k mRNA ezagutu egiten dute sekuentzien osagarritasunagatik eta ondorioz, mRNA horien degradazio prozesua hasten da, konplexu bat osatuz, siRISC konplexua (Agrawal N eta lank. 2003). siRISC konplexuak Argonauta proteina bat dauka, RNasa aktibitatearekin eta beraz ezagututako mRNA-ra lotu eta degradatu egiten du (Tomari Y and Zamore PD 2005). siRNA-k, geneen adierazpenaren erregulazioan parte hartzen dute, batez ere TE-n isiltzearekin erlazionatzen direlarik (Cogoni C eta lank. 1996).

Erregulazio epigenetikora zuzendutako beste sRNA mota bat, piRNA-k dira (Stuwe E eta lank. 2014), Miwi/PIWIL1, Mili/PIWIL2 eta Miwi2/PIWIL4 proteina taldeaz osatuak saguan. Izan ere, beraien funtzioa gauzatu ahal izateko piRNA-ek piwi proteinekin elkarlanean aritzen dira.Zenbait ikerketa taldek zelula espermatikoetan deskribatu dituzte, transkripzio ondorengo TE-en adierazpenaren isiltzearekin erlazionatu dituzte hozi zeluletan (52). piRNA-en sorrera ezberdina da miRNA edo siRNA-ekin alderatzen baditugu. Aurreko kasuetan ez bezala, piRNA-etan ez da DICER nukleasadun konplexurik sortzen (Stuwe E eta lank. 2014), eta bere mekanismoa burutzeko bi bide proposatu dira (Ghildiyal M eta Zamore PD 2009). Bi kasuetan amarengandik jasotako piRNA biltegietan dauden piRNA-k patroi gisa erabiliz sortzen direla iradoki da: 1) alde batetik, piRNA aitzindarietatik erauziz (Brennecke J eta lank. 2008); eta 2) bestetik, piwi proteinen laguntzaz "Ping-Pong" bezala ezagutzen den prozesuan (Brennecke J eta lank. 2007; Gunawardane LS eta lank. 2007; Olivieri D eta lank. 2012). Bi kasuetan, piRNA-k sortu ondoren piwi proteinen laguntzaz histonen metilazioan haritzen diren entzimak biltzen dira, horrela heterokromatinaren eraketa ahalbidetuz (Gu T eta Elgin SC 2013).

1.3. HISTONEN ALDAKETAK ETA TRANSKRIPZIOA

Histonetan aldaketak ezarri edo kendu egiten dira geneen adierazpena piztu edo itzaliz, antolamendu berezi baten bitartez histona kodea osatuz. Gainera histonen aldaketak sortzen dituzten entzima espezifikoen taldea oso handia eta era askotarikoa da. Histonetan gertatzen diren marka epigenetikoak, hortaz, prozesu dinamiko baten emaitza dira, kontrako erreakzioak katalizatzen dituzten bi entzimek burutuak.

1.3.1. Histonen aldaketen antolamendua. histona kodea

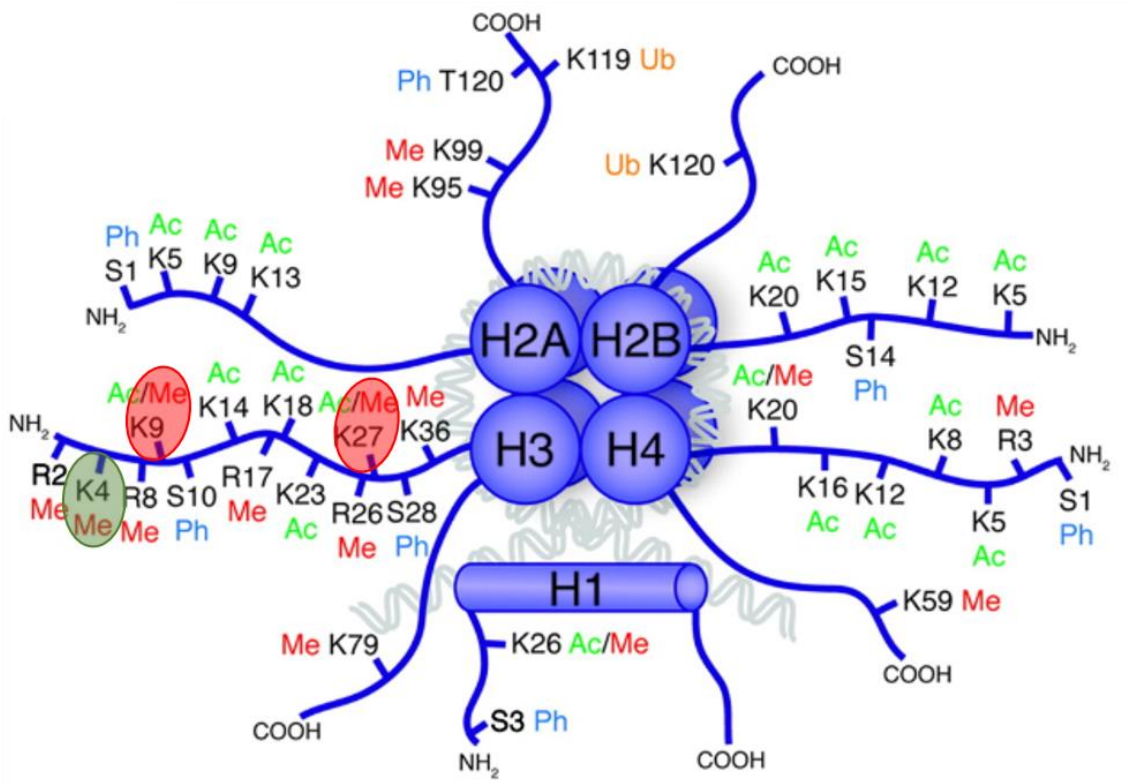
Azken urteetako teknika berriek gene espezifikoen adierazpen egoeraren eta bertara lotutako histonen identifikazioa burutzea ahalbidetu dute (Lee eta Mahadevan 2009). Honen harira, Histona Kodea izeneko hipotesi bat eman da aditzera, non aurretik aipatu bezala, DNA-n kodifikatutako informazioaren transkripzioa, histonetan ematen diren aldaketa kimikoen bitartez erregulatzen dela azaltzen den (Turner 2000; Jenuwein T eta Allis C 2001). Gainera, hipotesi horren barruan, histonetan proteina zehatzen loturarako domeinu espezifikoak ere ikertzen dira. Izan ere, aurretik aipatu ditugun histonak erregulatzen dituzten entzimetan badira eboluzioan zehar kontserbatu diren proteina domeinuak, histona espezifikoetara lotzeko ahalmena erakutsi dutenak. Horrela, histonen aldaketak, histonen eta DNA-ren arteko lotura handitu edo gutxitzeaz gain, proteina edo entzima espezifiko hauek biltzeko seinale ere direla adierazi da, jarraian kromatinaren antolamendua aldatuz, transkripzioa areagotu edo isiltzeko. Laburbilduz, histonen erregulaziorako entzima horiek histona kodea irakurtzeko ahalmena dute beraien espezifikotasuna dela eta (Lall 2007).

1.1. Taula. Histonen kodearen laburpen bat, histonen aldaketa ikertuenekin (Dong X eta Weng Z 2013; Kimura H 2013; Zaho Y eta Garcia BA 2015, moldatua eta osatua).

Histona	Mota	Histona	Mota
H3K4me1	Aktibatzaile[2]	**H3K79me1**	Aktibatzaile[8]
H3K4me2	Aktibatzaile[1]	**H3K79me2**	Aktibatzaile[1;8]
H3K4me3	Aktibatzaile[4;5]	**H3K79me3**	Biak[1;8]
H3K9me1	Aktibatzaile[1]	**H4K20me1**	Aktibatzaile[1]
H3K9me2	Errepresiogile[7]	**H2BK5me1**	Aktibatzaile[1]
H3K9me3	Errepresiogile[1]	**H2BK5me3**	Errepresiogile[7]
H3K27me1	Aktibatzaile[1]	**H3K9ac**	Aktibatzaile[4]
H3K27me2	Errepresiogile[7]	**H3K14ac**	Aktibatzaile[4]
H3K27me3	Errepresiogile[1]	**H3K27ac**	Aktibatzaile[3]
H3K36me3	Errepresiogile[1]	**H3K122ac**	Aktibatzaile[6]
[1]Barski A eta lank. 2007 [2]Benevolenskaya EV 2007 [3]Creyghton MP 2010 [4]Koch CM eta lank. 2007		[5]Liang G 2004 [6]Pradeepa MM eta lank. 2016 [7]Rosenfeld JA eta lank. 2009 [8]Steger DJ eta lank. 2008	

Histonen aldaketen atalean gainetik azaldu bezala, histonetako lisina hondakinen azetilazioak, lisinen karga positiboa neutralizatzen du eta era berean, DNA eta histonen arteko lotura ahultzen du, DNA-ren eskuragarritasuna handituz transkripzioa burutzen duten proteinekiko (Tse eta lank. 1998; Robinson eta lank. 2008; Shogren-Knaak eta lank. 2006; Loyola eta Almouzni 2004). Hipoazetilazioa gertatzen den eremuetan ordea, histonek transkripzio faktoreak biltzeko ahalmena galtzen dute eta transkripzioaren isiltzea gertatzen da (Loyola eta Almouzni 2004; Grewal eta Jia 2007). Bestalde, histonen lisina hondakinen metilazioa aldakorragoa da DNA-ren eskuragarritasuna azaltzerako orduan. Adibidez, H3K4 eta H3K36 histonen metilazioak transkripzioa areagotzen dutela adierazi da (Barski eta lank. 2007; Pokholok eta lank. 2005), H3K4-ren demetilazioa transkripzioaren isiltzearekin erlazionatu den bitartean. H3K9 eta H3K27 histonen metilazioa geneen adierazpenaren isiltzearekin erlazionatu da, nahiz eta metilo kopuruen arabera transkripzioaren pizgarri bezala aritu daitezkeen (Rosenfeld JA eta lank. 2009; Barski eta lank. 2007). Beraz, histona kodearen arabera (1.1. Taula), inguruko geneen adierazpena era batekoa edo bestekoa izango da (Kimura H 2013; Dong X eta Weng Z 2013).

Baina histona kodea ez da taulan azaltzen den bezain sinplea, izan ere, oinarrizko lau histonetako bakoitzak aldi berean aldaketak izan ditzakelako hondakin ezberdinetan eta hondakin batean baino gehiagotan (1.4. Irudia). Beraz, histona kodea oso konplexua da milaka konbinazio ezberdin gerta daitezke eta, zelula bakoitzeko nukleosoma bakoitzean. Honen adibide da, hainbat eta hainbat lanetan azaltzen den bezala H3K4me3 eta H3K27me3 histonen aldaketak elkarrekin aurkitu izan direla sustatzaile batzuetan, "Eremu Bibalente" bezala izendatu direnak (Roh eta lank. 2006; Bernstein eta lank. 2006; Mikkelsen eta lank. 2007; King AD eta lank. 2016). Histonen aldaketa indibidualak aztertu dituzten lan esperimental asko dago erabilgarri gaur egun (Wang M eta lank. 2010; Dong X eta Weng Z 2013). Pausu bat aurrera eginez, hainbat ikerketa burutu dira histonen aldaketa gutxi batzuetan zehar patroi ezberdinak mantentzen direla argitzeko (Wang Z eta lank. 2008; Janssen KA eta lank. 2017), hala ere, histonen aldaketa guztien sarea osatzetik oso urruti gaude oraindik ere.



1.4. Irudia. Nukleosoma osatzen duten histonen isatsetako N eta C muturreko aldaketa epigenetikoak. Irudiak barne hartzen ditu deskribatutako, metilazioa, azetilazioa, ubikitinazioa, eta fosforilazioa. Gorriz metilazioak ageri dira, berdez azetilazioak, urdinez fosforizalioak eta laranjaz ubikitinazioak. Bestalde, borobil gorriarekin tesian azterturiko marka epigenetiko errepresiogileak ageri dira eta borobil berdearekin marka epigenetiko aktibatzaileak. (Tollervey JR eta Lunyak VV 2012, moldatua).

1.3.2. Histonen aldaketak sortzen dituzten entzimak

Aurretik aipatu bezala, histonek metilazioa, azetilazioa, fosforilazioa, ubikitinazioa, deaminazioa, sumoilazioa, ADP-ribosilazioa eta prolinen isomerizazioa jasan dezakete besteak beste (Fischle W eta lank. 2003). Hala ere, prozesu hauek guztietatik, epigenetikaren arloan gehien aztertu diren histonen aldaketak metilazioa eta azetilazioa izan dira eta honenbestez, baita aldaketa hauek sortzen dituzten entzimak ere.

1.2. Taula. KMT-en familietako entzimak biltzen dituen taula. (Dillon SC eta lank. 2005; Allis eta lank. 2007, moldatua)

KMT Familia	Entzimak	Nomenklatura internazionala	Histona itua
SUV39	Suv39h1	KMT1A	H3K9
	Suv39h2	KMT1B	H3K9
	G9a/Ehmt2	KMT1C	H3K9
	GLP/Ehmt1	KMT1D	H3K9
	Setdb1/ESET	KMT1E	H3K9
	Setdb2/Clld8	KMT1F	H3K9
Set1	Mll1/HRX	KMT2A	H3K4
	Mll2/Wbp7	KMT2B	H3K4
	Mll3/Halr	KMT2C	H3K4
	Mll4/Arl	KMT2D	H3K4
	Mll5	KMT2E	H3K4
	Setd1a/Nsccn1	KMT2F	H3K4
	Setd1b	KMT2G	H3K4
SET2	Ash1l	KMT2H	H3K4
	Setd2	KMT3A	H3K36
	Nsd1	KMT3B	H3K36 / H4K20
SMYD	Smyd2	KMT3C	H3K36 / H3K4
	Smyd1	KMT3D	H3K4
	Smyd3	KMT3E	H3K4
SET2	Nsd2 (WHSC1)	KMT3F	H3K27
	Nsd3 (WHSCL1)	KMT3G	H3K27 / H3K4
Others	Dot1l	KMT4	H3K79
Others	Setd8	KMT5A	H4K20
SUV4-20	Suv4-20h1	KMT5B	H4K20
	Suv4-20h2	KMT5C	H4K20
EZ	Ezh1	-	H3K27
	Ezh2	KMT6	H3K27
Others	Sedt7 (Set7/9)	KMT7	H3K4
RIZ	Riz (Prdm2)	KMT8	H3K9

1.3. Taula. KDM-en familia ezberdinetako entzimak biltzen dituen taula. (Allis eta lank. 2007, moldatua)

Domeinuak	Entzimak	Nomenklatura internazionala	Histona itua
Amine oxidase	Lsd1	KDM1A	H3K4me1/me2; H3K9
	Lsd2	KDM1B	H3K4me1/me2
Jumonji	Jhdm1a/Fbxl11	KDM2A	H3K36me1/me2
	Jhdm1b/Fbxl10	KDM2B	H3K36me1/me2; H3K4me3
	Jmjd1a(Jhdm2a	KDM3A	H3K9me1/me2
	Jmjd1b/Jhdm2b	KDM3B	H3K9?
	Jmjd1c	KDM3C	H3K9?
	Jmjd2a/Jhdm3a	KDM4A	H3K9me2/me3; H3K36me2/me3; H1K26me2/me3
	Jmjd2b	KDM4B	H3K9me3
	Jmjd2c	KDM4C	H3K9me3; H3K36me3
	Jmjd2d	KDM4D	H3K9me2/me3
	Jarid1a/Rbbp2	KDM5A	H3K4me2/me3
	Jarid1b/Plu1	KDM5B	H3K4me/me2/me3
	Jarid1c/Smcx	KDM5C	H3K4me2/me3
	Jarid1d/Smcy	KDM5D	H3K4me2/me3
	Utx	KDM6A	H3K27me2/me3
	Jmjd3	KDM6B	H3K27me2/me3
	Uty/Hydb	Kdm6C	H3K27me3 (male specific)

Histonen isatsen metilazioaren egoera, entzima familia handi baten baitan dagoen prozesua da. Lisina hondakinen metilazioa, lisina metiltransferasen bitartez (KMT) burutzen da. Hauek SET domeinuak izaten dituzte eta S-adenosil-metionina-tik (SAM) eratorritako metilo taldeen transferentzia egiten dute histonen isatsetara. Era berean, metilo talde hori metionina sintetasa entzimak lortzen du, metionina zikloan zehar, jatorria folato molekulan izanik. Beraz, bere erabilgarritasuna dietarekin eta folato kontsumoarekin erlazionatu daiteke, histonen aldaketa hauek kanpo faktoreekin lotuz (Loenen 2006). KMT-ekin jarraituz, SET domeinua duten entzimak zazpi familia orokorretan banatzen dira (Dillon SC eta lank. 2005), sekuentzien artean duten homologia eta burutzen duten funtzioaren arabera (1.2.Taula): SUV39, SET1, SET2, EZ, RIZ, SMYD, eta SUV4-20. Salbuespena DOT1 familia da, non bertako kideek K79 lisina metilatzen duten H3 histonan, baina estrukturalki SET domeinua izan gabe (Feng Q eta lank. 2002; Ng HH eta lank. 2002). Laburbilduz, histonen lisina hondakin espezifikoen metilazioa, itzulpen prozesuaren osteko aldaketa epigenetikoa da, geneen adierazpena erregulatzen duena eta hainbat konplexuren bilketa martxan jartzeko markatzaile dena, kromatinaren berrantolaketa gidatuz (Dillon SC eta lank. 2005). Urte askotan zehar histonen metilazioa itzulezina zela pentsatu izan da, 2004. urterarte (Shi Y eta lank. 2004). Hori dela eta, lisinen metilazioa, esan bezala, aldaketa epigenetiko dinamiko bat da, metilo taldeak kentzen dituzten entzimak ere identifikatu direlako. Hauek lisina demetilasak (KDM) dira eta beraien funtzioa betetzeko Fe^{2+} erabiltzen dute kofaktore bezala, kasu honetan ere entzimak nutriente espezifikoen kontsumoaren eta oinarrizko metabolismo prozesuaren menpe jarriz (Young JI eta lank. 2015). KDM-ek hainbat domeinu ezberdin dituzte: SWIRM1 domeinua (Tochio N eta lank. 2005), histona ezberdinetan aurkitu dena; Jumonji domeinua (Mosammaparast N eta lank. 2010; Aprelikova O eta lank. 2016), batez ere kofaktoreen lotura eremua dena; PHD-finger domeinua

(Mosammaparast N eta lank. 2010), metilatutako peptidoak batzeko beharrezkoa; Zinc-finger (Mosammaparast N eta lank. 2010), DNA-ren lotura eremua eta amina oxidasa eremua (Mosammaparast N eta lank. 2010), katalitikoki eremu aktiboa. Hauek ere hainbat familiatan banatzen dira: KDM1, KDM2, KDM3, KDM4, KDM5 eta KDM6 (1.3. Taula).

Arginina hondakinen metilazioari buruz informazio gutxiago dagoen arren, prozesuan parte hartzen duten entzimak identifikatuak izan dira. Entzima hauek peptidilarginin metiltransferasak dira (PRMT) eta argininen monometilazioa burutzen dute. PRMT-ak H3, H4 eta H2A histonetan gehitzen ditu metilo taldeak, geneen adierazpenaren erregulazioan parte hartu dezaketela iradokiz (Bedford 2007). Bestalde, argininen demetilazio prozesuari buruz ez da informazio zehatzik argitaratu.

Aipatu beharra dago, epigenetika arloan egindako hainbat ikerketek minbizia histonen metilazioarekin erlazionatu dute, hainbat geneen adierazpenaren alterazio edota tumoreen ezabatzaile diren geneen bidez hain zuzen (Huang S 2002; Chen F eta lank. 2010). Argitu beharra dago histonen metilazioen eta minbiziaren arteko lotura hori ez dela zuzena, baina hainbat ikerketek minbiziaren garapenean laguntzen duela aditzera eman dute (Espino PS eta lank. 2005; Hamamoto R eta lank. 2004; Albert M and Helin K 2010). Hori dela eta, KMT eta KDM-en aktibitatea erregulatzen duten hainbat molekula, entsegu klinikoetan edota minbiziaren terapietan erabiltzen hasi dira (Mund eta Lyko 2010; Morera L eta lank. 2016; Liu Q eta Wang MW 2016).

Histonen isatsetako lisina hondakinen azetilazioa, histona azetil transferasek burutzen dute (KAT), eta azetilo taldea ezabatu berriz, histona deazetilasek (HDAC) egiten dute (1.4.Taula eta 1.5. Taula). KAT-ak eta HDAC-ak ere, talde ezberdinetan banatu dira, sekuentziaren homologia edota betetzen duten funtzio biologikoaren arabera. KAT-en kasuan, bi taldetan banatzen dira, zelularekiko posizioaren arabera (Lee KK eta Workman JL 2007), A taldea KAT nuklearrek osatzen dute eta batez ere geneen adierazpena erregulatzen dute nukleosomako histonetan azetilazioak burutuz (Weaver R 2007). B taldea aldiz, KAT zitoplasmatikoek osatzen dute eta hauen funtzioa sortu berri diren histonen azetilazioa gauzatzea da, hauek nukleosomaren parte bihurtu aurretik. Era berean talde horietako KAT-ak hainbat familiatan banatzen dira: GNAT, p300/CBP, MYST, SCR eta organismo eukariotetan aurkitu diren beste HAT batzuk (Lee KK eta Workman JL 2007). KAT guztiek azetil koentzima A (CoA) erabiltzen dute azetilo taldearen emaile gisa, pirubatoaren dekarboxilazio zikloan jatorria duena. Azetil-CoA-ren erabilgarritasuna beraz, glukosaren metabolismoaren menpe dago (Spriet eta Heigenhauser 2002). Aipatu beharra dago, KAT ezberdinak oso espezifikoak ez izan arren, histona edota lisina hondakin konkretuekiko lehentasuna erakusten dutela (Marmorstein 2001). Bestalde, HDAC-ak beraien arteko berdintasunetan eta laguntzaile bezala duten kofaktoretan oinarrituta, bi taldetan banatu izan dira, klasikoa eta sirtuinen familia (Haberland eta lank. 2009). Era berean familia klasikoan lau talde bereizten dira (I, IIa, IIb, IV) eta hauek guztiak azetilo taldearen hartzaile gisa Zn^{2+} behar dute. Familia klasikoan ordea, talde bakarra dago (III) eta bertako HDAC entzimek NAD^{+}-ren menpekoak dira (Yang eta Seto 2007). Hauek ere ez dute histona edo lisina konkretuekiko afinitate berezirik adierazten, baina hainbat zelula lerrotan eta saguetan garatu diren *knockout*-ek adierazi dutenez, hainbat isoforma ehun ezberdinetan adierazten dira garapen prozesuko momentu ezberdinetan (Haberland eta lank. 2009).

1.4. Taula. **KAT-en familietako entzimak biltzen dituen taula.** (Mund eta Lyko, 2010, moldatua).

KAT Familiak	Entzimak	Nomenklatura internazionala	Histona itua
GNAT	Hat1	KAT1	H4K5/H4K12
	Gcn5	KAT2A	H3K9/H3K14/H3K18; H2B; H4K8
	Pcaf	KAT2B	H3K9/H3K14/H3K18; H2B
P300/CBP	Crebbp	KAT3A	H2AK5; H2BK12/H2BK15
	Ep300	KAT3B	H2AK5; H2BK12/H2BK15
Other	Taf1	KAT4	H3; H4
MYST	Plip/Tip60	KAT5	H4K5/H4K8/H4K12/H4K16; H2A
	Myst3/Moz	KAT6A	H3K14
	Myst4/Morf	KAT6B	H3K14
	Myst2/Hbo1	KAT7	H4K5/H4K8/H4K12; H3
	Myst1/Mof	KAT8	H4K16
GNAT	Elp3	KAT9	H3
Other	Gtg3c4/TFIIIC90	KAT12	H3K9/H3K14/H3K18
SCR	Ncoa1/Src1	KAT13A	H3; H4
	Ncoa3/Actr	KAT13B	H3; H4
	Ncoa2/Src2	KAT13C	H3; H4
Other	Clock	KAT13D	H3; H4

1.5. Taula. HDAC-en familietako entzimak biltzen dituen taula. (Mund eta Lyko, 2010, moldatua).

HDAC familiak	Entzima	Kokapen zelularra
I	HDAC1	Nukleoa
	HDAC2	Nukleoa
	HDAC3	Nukleoa
	HDAC8	Nukleoa/Zitoplasma
IIa	HDAC4	Nukleoa/Zitoplasma
	HDAC5	Nukleoa/Zitoplasma
	HDAC7	Nukleoa/Zitoplasma/ Mitokondrioa
	HDAC9	Nukleoa/Zitoplasma
IIb	HDAC6	Batez ere zitoplasma
	HDAC10	Batez ere zitoplasma
III	SIRT1	Nukleoa
	SIRT2	Batez ere zitoplasma
	SIRT3	Mitokondrioa
	SIRT4	Mitokondrioa
	SIRT5	Mitokondrioa
	SIRT6	Nukleoa
	SIRT7	Nukleoa
IV	HDAC11	Nukleoa/Zitoplasma

Azetilazio mailak erregulatzen dituzten entzimak ere orokorrean hainbat azpiunitaterekin batera sortutako konplexuetan osatzen dute beraien funtzioa eta hainbat gaixotasunekin erlazionatu dira. Zenbait prozesu biologikotan duten parte hartzea dela eta, proliferazioa, hanturazko erantzuna, ikasketa eta memoria, endekapen neuronala edota infekzio birikoak kasu, erabilera terapeutikoan oso ikertuak izan dira. Izan ere, Huntington-en gaitza (Klein G eta Vand Woude GF 2002), Hutchinson Gilford progeria (Krishnan V eta lank. 2011), garuneko ataxia (Cvetanovic

M eta lank. 2012), esklerosi lateral amiotrofikoa (Wang WY eta lank. 2013) edota hainbat minbizi mota (Oike T eta lank. 2012), KAT eta HDAC ezberdinen erregulazio okerrarekin erlazionatu dira. Honen harira, minbiziarekin erlazionatuta, KAT inhibitzaile asko identifikatu dira, hainbat produktu natural, kurkumina, garzinola edo azido anakardikoa barne (Dekker eta Haisma 2009; Oike T eta lank. 2012). Bestalde KAT-ak aktibatzen dituzten molekulak ere aurkitu dira (Selvi eta lank. 2010).

1.3.3. Histonen aldaketak sortzen dituzten entzimen erregulazioa

Histonen metilazioa areagotu edo gutxitu egiten dituzten entzimak hainbat azpiunitaterekin batera osatutako konplexuetan burutzen dute beraien funtzioa. Honen harira, tesi honetan aztertu diren histonen aldaketak erregulatzen dituzten entzimak sakonago deskribatuko dira jarraian: H3K27me3 marka errepresiogilearekin erlazionatutako Polycomb taldeko proteina konplexua, H3K4me2/3 marka aktibatzailearekin elkarreraginetan aritzen diren Trithorax proteina konplexua, eta H3K9me2 marka errepresiogilearen metilazioan parte hartzen duen G9a/GLP konplexua.

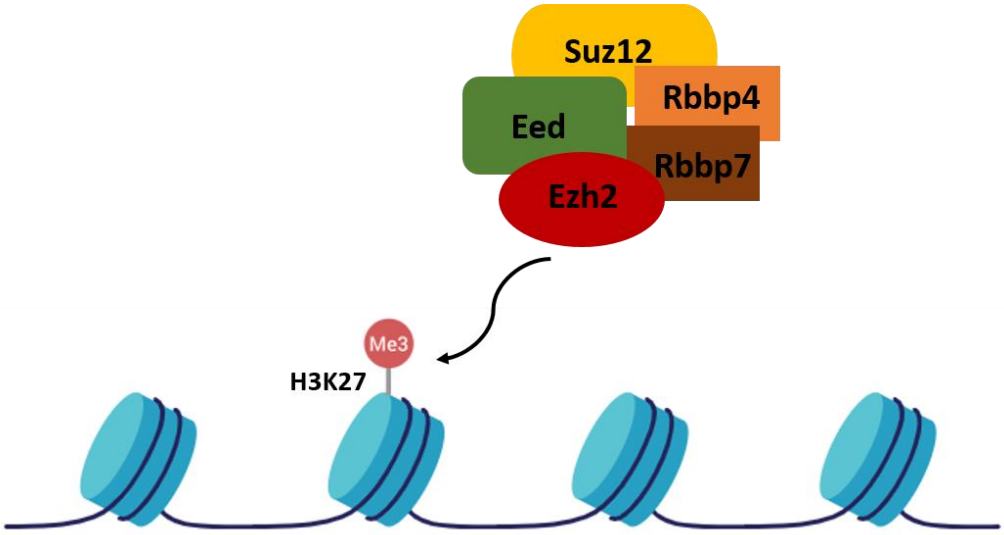
1.3.3.1. Polycomb taldeko proteinak (PcG) eta H3K27me3

Polycomb taldeko proteinak (PcG), eboluzioan zehar kontserbatu diren kromatinaren erregulazioa burutzen duten entzima ikertuenak dira, batez ere, garapenean zehar memoria epigenetikoaren finkatzea eta mantenuan duten funtzio garrantzitsuagatik, eta gene adierazpenaren isiltzearekin erlazionatuta daude. (Beisel eta Paro, 2011; Kerppola 2009; Margueron eta Reinberg, 2011; Ringrose eta Paro 2004; Schuettengruber eta Cavalli, 2009; Schuettengruber eta lank., 2007; Simon eta Kingston 2009; Simon eta Tamkun, 2002; Schwartz eta Pirrotta, 2007). PcG taldeko proteinak bi konplexu nagusitan banatzen dira, Polycomb konplexu errepresiogilea 1 eta 2 (PRC1 eta PRC2) (Lund eta van Lohuizen 2004). PRC1 konplexua hainbat azpiunitatez osatua dago, hala nola, Cbx2, Cbx4, Edr1, Edr2, Ring1, Rnf2, Rnf10, Bmi1 eta Znf134 (Lund eta van Lohuizen 2004). Konplexu honetako Bmi1 eta Ring1B azpiunitate nagusienak, batez ere histonen ubikitinazioarekin dute erlazioa (adibidez H2AK119 histona (Wang H eta lank. 2004; Cao R and Zhang Y 2004; Li eta lank. 2006; Stock eta lank. 2007)). Bestalde, aditzera eman denez, PRC1 konplexuak kromatinaren trinkotzea bideratzen du eta PcG bidezko epe luzerako memoria epigenetikoa bultzatzen du (Schuettengruber B eta lank. 2007). Azkenik, PRC1 konplexuaren bidez burututako H2AK119ub1-ak PRC2 konplexua kromatinara biltzen duela ere erakutsi da, PRC1 eta PRC2 konplexuen arteko elkarlana frogatuz (Cooper S eta lank. 2014).

Hala ere, orokorrean H3K27me3 histona aldaketaren metilazioa PRC2 konplexuak burutzen du (1.5. Irudia), lau azpiunitatez osatua dagoena: SET domeinua duen H3K27 metiltransferasa Ezh2-a (edo bere homologoa den Ezh1) eta Eed, Suz12 eta histonaren txaperona den Rbbp7/4 proteinak (Cao R eta lank. 2002; Margueron eta Reinberg, 2011). PRC2 konplexuko aktibitate katalitikoa, Ezh azpiunitateek burutzen dute, zeinek H3K27-are dimetilazio eta trimetilazioa gauzatzeko ahalmena duten. Baina hala ere, Eed eta Suz12 azpiunitateak beharrezkoak dira Ezh azpiunitateen metiltransferasa funtzioa katalitikoa betetzeko eta egonkortzeko (Margueron R eta lank. 2009; Pasini D eta lank. 2004), eta Rbbp7/4 proteina histonara lotzeko proteina da. Aipatu bezala, Ezh azpiunitateak bi proteina homologo ditu, Ezh1 eta Ezh2. Hauen funtzionamendua argitzeko egin diren ikerketetan erakutsi denez, nahiz eta PRC2 konplexuko

beste azpiunitateekiko elkarreragintza biek erakusten duten eta biak diren H3K27 metilatzeko gai (Shen X eta lank. 2008), Ezh1-ek metiltransferasa aktibitate baxuagoa du eta baita lotura une gutxiago ere (Margueron eta lank. 2008; Son J eta lank. 2013). Gainera Ezh2, hazten dabiltzan ehunetan aurkitu izan da, Ezh1 berriz desberdindutako ehunetan aurkitu den bitartean, transkripzioa isilaraziz eta kromatina zuntzaren trinkotzea burutuz (Margueron eta lank. 2008; Visser HP eta lank. 2001; Stojic L eta lank. 2011; Mousavi K eta lank. 2012). Honenbestez, ikerketa hauek ondorioztatzen dute bi azpiunitate homologo hauek funtzio nahiko espezifikoak dituztela, izan ere, Ezh1-ren funtzioa, H3K27-ren metilazioa eta transkripzioaren isiltasuna konpentsatzeko mekanismoa izan liteke, batez ere Ezh2 ez dagoenean (Margueron eta lank. 2008; Shen X eta lank. 2008). Honen adierazle dira bi azpiunitateen ezabatzearekin batera egin diren zenbait lan, non Ezh1 ezabatutako saguak bizigaiak diren bitartean (Ezhkova E eta lank. 2011), Ezh2 ezabatutako saguak hil egiten diren umetokian ezarri ondorengo garapen enbrionikoan, gastrulazio akatsak sortzen dira eta (O'Carroll D eta lank. 2001). PRC2 konplexuaren funtzionamendu egokirako Ezh2/1 azpiunitateez gain, Eed eta Suz12 proteinak beharrezkoak direla ere erakutsi da, izan ere, azken bi hauen ezabatzeak, H3K27me3-an jaitsiera globala sortzen du, eta baita mESC-en izaera definitzen duten gene espezifikoen errepresioaren itzultzea ere, eremu bibalenteak barne (Azuara V eta lank. 2006; Boyer LA eta lank. 2006; Pasini D eta lank. 2007). Gainera interesgarria da argitzea, PRC2 konplexuko azpiunitate bakoitzaren ezabatzeak (Ezh2, Eed eta Suz12 barne, baina esan bezala Ezh1 kanpo) umetokian ezarri ondorengo enbrioiaren garapenean gastrulazio arazoak sortzen dituela eta beraz hilgarriak direla (O'Carroll D eta lank. 2001; Pasini D eta lank. 2004; Faust C eta lank. 1995), baina ezabatze horiek mESC-tan egitenez gero, zelula horiek oraindik ere bizigarriak direla eta proliferazioan jarraitzeko ahalmena dutela (Shen X eta lank. 2008; Pasini

D eta lank. 2007; Chamberlain SJ eta lank. 2008; Leeb M eta lank. 2010). Azken honek PRC2 konplexuaren garrantzia berretsiko luke zelula amen desberdintze prozesuan.



1.5. Irudia. H3K27 histonaren trimetilazioa PRC2 konplexuaren bidez. PRC2 konplexua osatzen duten azpiunitateak azaltzen dira. (Harikumar A and Meshorer E 2015, moldatua).

PRC2 konplexuak geneen sustatzaileetara hurbiltzeko prozesuan, CpG irlek parte hartzea beharrezkoa da, hainbat ikerketek erakutsi dutenez mESC-tan PRC2 biltzen zaien sustatzaile

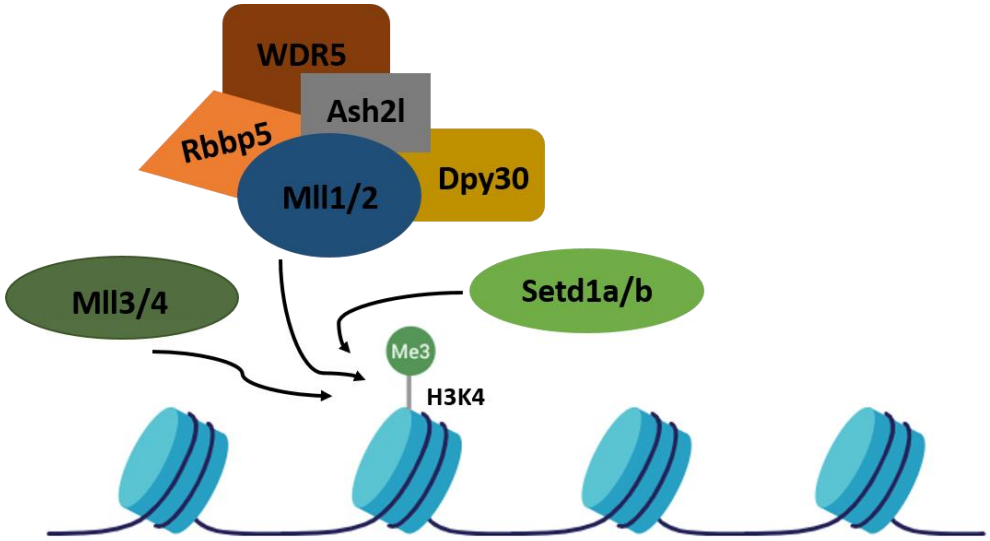
gehienek CpG irlak dituzte eta (Tanay A eta lank. 2007; Ku M eta lank. 2008). Honen adibide da Ku M eta lankideen lana, non mESC-tan Ezh2 2461 sustatzailerekin lotzen dela argitzen den, eta horietatik %88-a CpG irletan aberatsak direla adierazten duen. Interesgarria da aipatzea, mESC-tan badaudela zenbait CpG irla non PRC2-a ez den lotzen. Horren zergatia ondorengoa da, badirudi irla espezifiko horiek adierazpen handia duten transkripzio faktoreen lotura gune direla, orokorrean CpG irlak dituzten sustatzaileak izaera bibalentea hartzen dutela iradokiz, transkripzio faktore aktibatzaileak agertzen diren arte (Ku M eta lank. 2008). Beraz, PRC2 konplexua biltzeko CpG irlen garrantzia deskribatua dagoen arren, biltze prozesua bera oraindik ere ez dago guztiz argi, batez ere, PRC2 konplexuko azpiunitate batek ere ez duelako CxxC metilatu gabeko eta DNA-ra biltzeko eremurik. Hori dela eta, zenbait biltze mekanismo proposatu dira PRC2 konplexuarekin elkarreraginean aritzen diren hainbat proteinarekin (Voigt P eta lank. 2013). 1) Sekuentzia espezifikoak diren DNA-ri lotzen zaizkion transkripzio faktoreekin elkarrekintza. PRC2 konplexuarekin batera lan egiten duten transkripzioaren zenbait errepresiogile (hala nola, Yy1, Rest eta Snail), CpG irletan aberatsak diren sustatzaileetara biltzeko makinaria bezala proposatu dira (Herranz N eta lank. 2008; Dietrich N eta lank. 2012; Arnold P eta lank. 2013). 2) ncRNA taldeko molekulekin elkarrekintza. Adibidez, Xist lncRNA-k, emeen X kromosomaren isiltzean duen parte hartzeagatik ezaguna denak, Ezh2-arekin batera elkarrekintzan aritzen da X kromosoma inaktibatzerako garaian PRC2 konplexua bertara biltzeko (Plath K eta lank. 2003). Beste hainbat lncRNA ere proposatu dira lozi espezifikoetan PRC2 konplexua biltzen lagunduz, horien artean HOTAIR (Rinn JL eta lank. 2007) eta Kcnqot1 (Pandey RR eta lank. 2008) dira ikertuenak. Gainera, CpG-z aberatsak diren sustatzaileen eremuen inguruan, 50-200 nukleotido tarteko transkrito motzak aurkitu dira (adibidez Repeat A) PRC2 konplexuarekin batera lan egiten dutenak (Kanhere A eta lank. 2010; Zaho J eta lank. 2010), lncRNA motzen parte hartzea ere deskribatuz. Hala ere, ikerketa hauek ncRNA molekulen eta PRC2 konplexuaren artean elkarreragina dagoela argitzen duten arren, badirudi ez litzatekela makinaria martxan jartzeko bide nagusia. Beraz, aipatu beharra dago arlo honetan asko dagoela oraindik ere ikertzeko. Hori dela eta, gaur egun ez dago argi ncRNA-ek zer nola burutzen duten beraien funtzioa PRC2 konplexua sustatzaile bibalenteetara biltzerako orduan (Brockdorff N 2013). 3) Histonen aldaketetara biltzen diren proteina osagarriekin edo laguntzaileekin elkarlana. Aurretik aipaturiko proteina guztiez gain, PRC2 konplexua elkarlanean aritzen da, drosophila organismoko Polycomb-like (PCL)-aren hiru ugaztun homologoekin: PCL1, PCL2 eta PCL3-a (PHF1, MTF2 eta PHF19 ere deituak hurrenez hurren). Proteina hauek, besteak beste bi PHD finger eta Tudor eremuak dituzte, zeinak adibidez H3K36me2/me3 marken loturak bideratzen dituzten. Hauek era berean, gene aktiboen eremu kodifikatzaileetan aberastuak izaten diren, baina baita sustatzaile bibalente batzuetan ere (Ballare C eta lank. 2012; Brien gl eta lank. 2012; Musselman CA eta lank. 2012; Cai L eta lank. 2013). mESC-tan proteina osagarri hauetako PCL2-aren ezabatzeak, pluripotentzia faktoreen gainadierazpen bat sortzen dute eta era berean desberdintze prozesuan akatsak (Walker E eta lank. 2010). Honek, PCL proteina osagarriek, PRC2 konplexua biltzerako orduan paper garrantzitsu bat izan dezaketela iradokitzen du. Ildo beretik, mESC-tan, PCL3-aren adierazpenaren jaitsierak, Suz12-ren lotura ahultzen du sustatzaile bibalenteetan, era horretan zelulen berriztatzean eta proliferazioan arazoak sortuz (Hunkapiller J eta lank. 2012). Azkenik, aipatu beharra dago PRC2 konplexua H3K27me3 molekula berara lotzen dela, sustatzaile eremuen egonkortasunean lagunduz (Margueron R eta lank. 2009; Hansen KH eta lank. 2008). 4) GC-etan aberatsak diren sekuentzietara lotzen diren proteina osagarriekin elkarrekintza. Aebp eta Jarid2 proteinak, ez dira DNA-ra era espezifikoan

lotzen baina afinitatea dute GC-etan aberatsak diren sekuentziekiko. Hori dela eta, parte hartzaile bezala proposatu dira CpG-ak dituzten sustatzaile bibalenteetan PRC2-a biltzen (Peng eta lank. 2009; Shen eta lank. 2009; Leeb M eta lank. 2010; Kim H eta lank. 2009; Li G eta lank. 2010; Landeira D eta lank. 2010; Pasini eta lank. 2010). Bestalde, Aebp eta Jarid2 PRC2 konplexuaren aktibitate katalitikoa bultzatzeko gai dira. Izan ere, gai honen inguruan burutu den ikerketa batek, Jarid2-ren beharra azaltzen du, PRC2 eta Ezh2-ren arteko lotura gauzatu dadin nukleosometan, baina ez PRC2-a eta Ezh1-aren arteko lotu gertatzeko (Son J eta lank. 2013). Ikerketa honek era berean Ezh2 eta Jarid2-ren adierazpen mailaren jaitsiera deskribatu du zelulen desberdintze prozesuan (Son J eta lank. 2013). Honen harira, PRC2 konplexuaren funtzionamenduaren eredu bat proposatu dute, non PRC2-Ezh1-ek eta PRC2-Ezh2-k funtzio ezberdinak dituzten mESC-tan, zelula ama helduetan eta desberdindutako zeluletan. Zehatzago esanda, zatitzen ari diren mESC-tan, Jarid2-ak PRC2-Ezh2 kromatinaren inguruan biltzen duela deskribatu dute, bere metiltransferasa aktibitatearen bitartez H3K27me3 marka eremu horri gehitzeko. Bitartean, PRC2-Ezh1-ek kromatinaren trinkotzeaz arduratzen dela iradoki dute. Ama zelula helduetan, Jarid2-aren maila baxuak ageri diren arren, oraindik ere PRC2-Ezh2-a kromatinara biltzeko ahalmena duela adierazi dute, H3K27me3 marka epigenetikoa gehituz PRC2-Ezh1-aren laguntzarekin. Baina, guztiz desberdindutako zeluletan Jarid2-ren eta Ezh2-ren maila baxuak dituztenez, PRC2-Ezh1-aren metiltransferasa aktibitate ahula H3K27me3-ren mantenurako nahikoa den arren, ez da gai H3K27me3 marka berririk gehitzeko (Son J eta lank. 2013). Laburbilduz, mekanismo askoren elkarlana behar da PRC2-a eta CpG-etan aberatsak diren sustatzaile bibalenteen arteko lotura egin eta H3K27me3-aren erregulazio zuzena burutzeko.

1.3.3.2. Trithorax (TrxG) taldeko proteinak eta H3K4me2/me3

PcG konplexuak ez bezala, Trithorax taldeko proteinak (TrxG), geneen adierazpenaren aktibazioarekin lotuta daude. H3K4 histonaren metilazioa, aurreko ataletan aipatu bezala, SET domeinua duten entzima familiek burutzen dute, Set1a eta Set1b barne eta baita, TrxG taldeko proteinak ere Mll1, Mll2, Mll3, Mll4. H3K4 hauetako metiltransferasa bakoitza WRAD konplexuarekin uztartzen da, beraien egonkortasuna eta aktibitate katalitikoa bultzatuz. WRAD konplexua hainbat azpiunitatez osatuta dago, hala nola, WDR5, RbBP5, Ash2l eta Dpy30 (Ernst P eta lank. 2012, Couture JF eta lank. 2013) (1.6. Irudia). Aditzera eman denez Set1a/b-ren konplexuen helburua H3K4me3 pilatzea den bitartean (Shilatifard A 2012), Mll1/Mll2 entzimak H3K4-ren trimetilazioaren arduradun nagusitzat deskribatu dira, batez ere sustatzaile bibalenteetan (Hu D eta lank. 2013; Denissov S eta lank. 2014) Mll2-ren papera ezinbestekoa izanik. Izan ere, dirudienez Set1a/b konplexuak sustatzaile bibalenteen metilazio makineriatik kanpo geratzen dira (Denissov S eta lank. 2014). Aipatzekoa da WRAD konplexuko edozein azpiunitate ezabatuz gero, H3K4me3-ren metilazio mailan jaitsiera nabarmen bat gertatzen dela (Wysocka J eta lank. 2005; Dou Y eta lank. 2006; Jiang H eta lank. 2011; Wan M eta lank. 2013), Set1a/b taldeko metiltranferasen aktibitatea bultzatzerako orduan, WRAD konplexuaren paper garrantzitsua agerian utziz. Bestalde, Mll1/Mll2 konplexuen funtzioa ezinbestekoa da enbrioien garapenaren bideragarritasuna bermatzeko, izan ere bi entzimetako bat ezabatzeak enbrioi goiztiarren heriotza dakar (Yu BD eta lank. 1995; Glaser S eta lank. 2006). Gainera aipatzekoa da, Mll2-aren urritasunak adibidez, mESC-en pluripotentzia arriskuan jartzen ez duen arren, zelula horien proliferazioan, biziraupenean eta ezberdintzean jaitsiera nabarmen bat sortzen duela, zelula motaren denbora eta koordinazioa konprometituz (Lubitz S eta lank. 2007). WRAD

konplexuko azpiunitate diren WDR5 eta Ash2l-ren ezabatzeak mESC- ren pluripotentzian akats latzak sortzen dituen bitartean (Wan M eta lank. 2013; Ang YS eta lank. 2011), Dpy30 azpiunitatearen jaitsierak mESC-ren zori zelularraren okertzea dakar, nahiz eta proliferaziorako ahalmena kaltetzen ez den (Jiang H eta lank. 2011). Laburbilduz, ikerketa hauek guztiek, SET1a/b familiako metiltransferasek, H3K4-ren metilazioan parte hartze ezinbestekoa dutela adierazten dute, batez ere mESC-en zelula ama izaeraren funtzioetan, baina baita sustatzaile bibalenteak ez diren eremuetan ere parte hartzen dutela egiaztatzen dute.

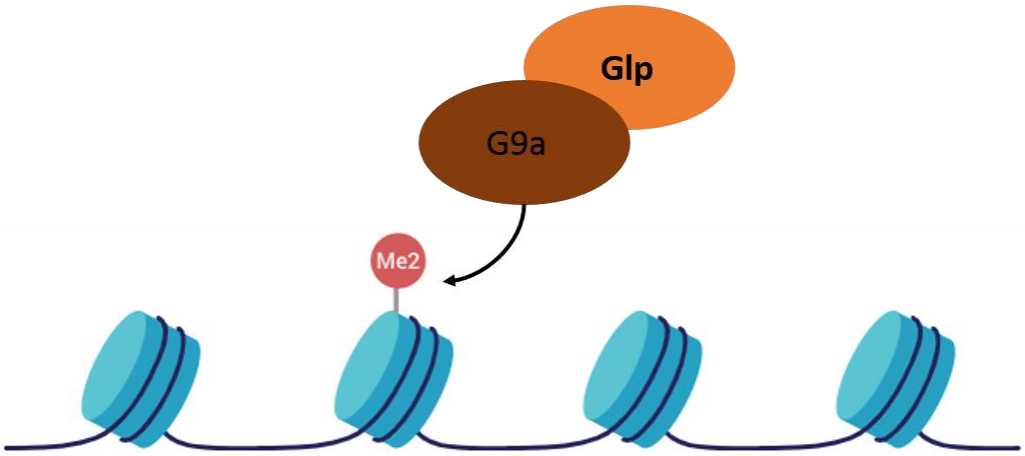


1.6. Irudia. H3K4 histonaren trimetilazioa Mll1/2 konplexuaren bidez. Mll1/2 konplexua osatzen duten azpiunitateak azaltzen dira. (Harikumar A and Meshorer E 2015, moldatua).

PcG konplexuetan bezala, H3K4 histonaren metilazioa burutzeko, Mll1/Mll2 barne dituzten konplexuak CpG irlen inguruan bildu behar dira, eta horretarako hainbat mekanismok parte hartzen dute, era indibidualean edota elkarrekin, geneen edo zelularen ingurunearen arabera. Lehendabizi, Mll1 eta Mll2-k DNA-ra biltzeko CxxC domeinua dute, zeinak metilatu gabeko CpG eremuak espezifikoki ezagutzen dituen (Birke eta lank. 2002; Bach C eta lank. 2009). Honek MLL-ak metilatu gabeko CpG irletara lotzea sustatzen du, eta gainera CxxC domeinuek, MLL konplexuak erreprimituta dauden geneetatik urruntzen ere laguntzen dute DNA-ren metilazioaren bitartez, aktibazio faltsu edo okerra saihesteko. Mll1/2 konplexuak kromatinara biltzeko beste era bat, DNA-ra espezifikoki lotzen diren transkripzio faktoreekin elkarreraginez izan daitekeela ere argitu da, (hala nola, Oct4 (Ang YS eta lank. 2011), estrogenoaren hartzaileak (Bach C eta lank. 2009), NFE2 (Demers C eta lank. 2007)), eta baita lncRNA-kin (adibidez, HOTTIP (Wang KC 2011)) edota TET/OGT proteinek MLL konplexuaren HCF1 azpiunitatearekin egiten duten loturaren bitartez ere (Capotosti eta lank. 2011). Izan ere TET/OGT proteinak CpG irletan aberastuak agertzen dira eta beraz, DNA-ren demetilazio prozesuarekin erlazionatu dira, ondorioz H3K4me3-aren trimetilazio funtzioa DNA-ren demetilazioarekin lotuz (Deplus R eta lank. 2013; Delatte B eta lank. 2013). Azkenik Mll1/2 konplexuek RNA polimerasa II-arekin egiten duten lotura (Ser5 domeinu fosforilatuaren bitartez), Mll konplexuen eta sustatzaile bibalente eta aktiboen arteko loturaren egonkortasunaren bermatzearekin erlazionatuta egon daitekeela ere iradoki da (Milne TA eta lank. 2005).

1.3.3.3. G9a/GLP proteinak eta H3K9me2

G9a eta bere homologoa den GLP metiltransferasek osatzen duten taldeak, H3K9me2 marka errepresiogilearen arduraduna da mESC-tan (Wen B eta lank. 2009). Gainera, badirudi G9a entzima hainbat geneen adierazpena erreprimitzearekin erlazionatuta dagoela bai mESC-tan eta baita zelula ama multipotente eta desberdinduetan ere (Collins R and Cheng X 2010), epe luzeko memoria epigenetikoa finkatuz (Balemans eta lank. 2012; Gupta-Agarwal eta lank. 2012; Kramer eta lank. 2011; Schaefer eta lank. 2009). G9a eta GLP proteinek Suv39h familiako metiltransferasen SET eremua dute beraien funtzioa gauzatu ahal izateko (Collinseta lank. 2008). H3K9me2 saguen enbrioi goiztiarraren garapenerako nahitaezkoa den heinean, batez ere, genoman zehar geneetatik hurbil aurkitzen da eta baita megabase luzeetan zeharreko kromatinazko blokeetan hedatuta ere. Honek, funtzio estrukturalak gauzatzen dituela iradokitzen du, zelula lerroen garapenean memoria epigenetikoa mantenduz (Went eta lank. 2009). Era berean, G9a eta GLP ere beharrezkoak dira garapeneko lehen faseetako errepresio epigenetikoa gauzatzeko, bien jaitsierak saguetan enbrioiak hiltzea dakar eta (Tachibana M eta lank. 2002; Tachibana M eta lank. 2005; Esteve PO eta lank. 2005). Hala ere, G9a/GLP konplexuaren eta H3K9me2 arteko mekanismoa ez da oraindik guztiz argitu (1.7. Irudia).



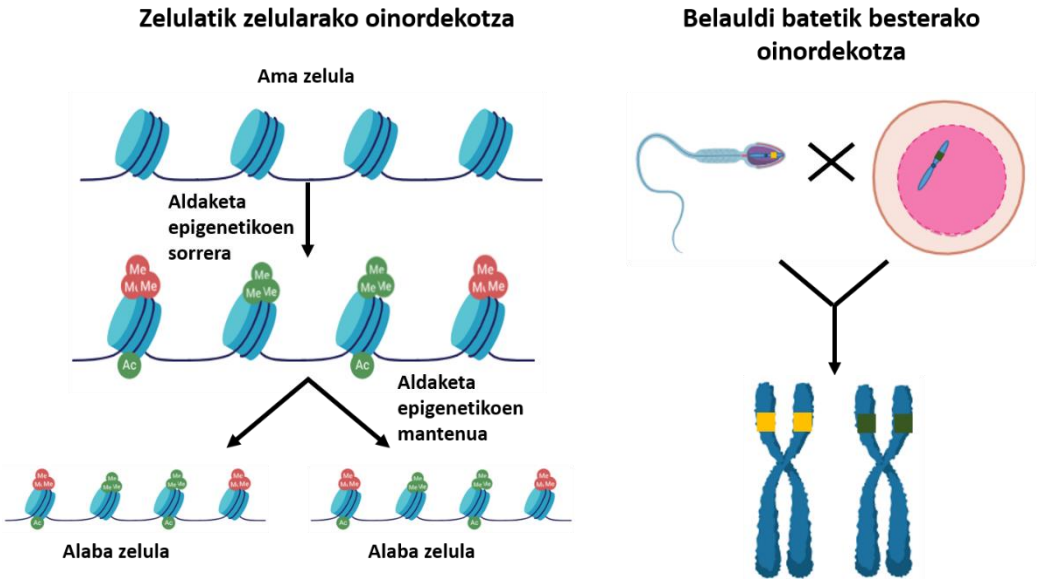
1.7. Irudia. Irudia. H3K9 histonaren trimetilazioa G9a/Glp konplexuaren bidez. G9a/Glp konplexua osatzen duten azpiunitateak azaltzen dira

G9a-k eta GLP-k metiltransferasa funtzioa era independientean burutzen duten arren eta substratu berdinak dituzten arren (*in-vitro* bada ere), H3K9 metilatuaren mailak beheraka egiten du G9a edo GLP-aren ezabaketa egiten bada (Ogawa eta lank. 2002; Tachinaba eta lank. 2005; Weiss eta lank. 2010). Dirudienez, mESC-tan G9a-k eraginda H3K9me2 markarekin erreprimitutako geneak nukleoaren periferian kokatzen dira batez ere (Yokochi eta lank. 2009). Ikerlari batzuen ustetan, G9a eta GLP entzimek heterodimeroak osatuz burutuko lukete beraien funtzioa (Tachibana eta lank. 2005; 2008), nahiz eta G9a-ren funtzioa GLP-arena baino garrantzitsuagoa izan. Dirudienez, G9a-k Glp-arekin heterodimeroa sortzeaz gain, Wiz izeneko molekula (*multi zinc finger* eremua duena) batekin ere sortzen du lotura, konplexuaren egonkortasuna bermatuz (Ueda eta lank. 2006). Gainera, GLP edo Wiz proteinen ezabatzeak, G9a-aren mailan ere jaitsiera bat sortzen dute (Tachibana eta lank. 2005; Ueda eta lank. 2006), hori dela eta beraien arteko elkarlana baieztatuz. Interesgarria da aipatzea, G9a-k H3K9-aren mono- eta dimetilazioa burutzeaz gain, hainbat ikerketek H3K27-aren metilazioarekin ere

erlazionatu dutela bai *in-vitro* eta baita *in-vivo* ereduetan ere (Tachibana M eta lank. 2001; Chaturvedi CP eta lank. 2009; Wu H eta lank. 2011), honen harira sustatzaile bibalenteen metilazioan parte hartu dezakeela iradokiz. Hala ere, beste zenbait ikerketek geneen errepresioa burutzeko, G9a/Glp konplexuaren eta PRC2 konplexuaren arteko elkarlana deuseztatu egin dute, izan ere, frogatua izan da G9a/Glp konplexua ez dela PRC2-arekin biltzen ez mESC-tan ezta jatorrizko zeluletan ere (Shen X eta lank. 2008; Chaturvedi CP eta lank. 2012). Gainera, ez da G9a eta sustatzaile bibalenteen arteko loturarik deskribatu mESC-tan (Shen X eta lank. 2008). Azkenik garrantzitsua da aipatzea, azken aldian burututako zenbait ikerketek G9a entzima beste transkripzio faktore batzuekin batera aktibatzaile gisa aritu daitekeela iradoki dutela, aurretik esandako guztiaren harridurarako (Poulard eta lank. 2017; Purcell eta lank. 2011; Shankar eta lank. 2013; Pang KKL eta lank. 2019).

1.4. MEMORIA EPIGENETIKOA

Epigenetikaren deskribapenak adierazten duen bezala, DNA-ren sekuentzian mutaziorik edo aldaketarik sortu gabe gertatzen diren geneen adierazpenaren derregulazioak aztertzen ditu, mitotikoki edo meiotikoki heredagarriak izan daitezkeenak, nahiz eta sortu dituen seinalea presente ez egon (Riggs AD 1996; Daxinger L eta lank. 2010). Honek fenotipoan ematen den aldaketa dakar, baina genotipoa eraldatu gabe. Izan ere, jakina da aldaketa epigenetikoak ehunen eta zelulen desberdintze prozesua gidatu dezaketela, azaleko , burmuineko, gibeleko etab.-eko zelulak sortuz. Behin zelula desberdinduen ezaugarriak finkatzen direnean, zelula motaren identitate propioa eraikiz, hauek zelulatik zelulara transmititzen dira, zelulen arteko memoria garatuz, nahiz eta horretarako mekanismoa ez dagoen horren argi. Hori dela eta, histonen aldaketak eta DNA-ren metilazioa bezalako aldaketa epigenetikoek, DNA-ren erreplikazioa eta zatiketa prozesua gainditu edo biziraun dezaketela uste da (Ptasne M 2007), ondorengo zeluletara helaraziz. Hala ere, belaunaldietan zehar transmititzen den kromatinaren egoeraren nondik norakoa edota histonen aldaketak edo DNA-ren metilazio patroiak guztiz kopiatzen diren, argitu gabeko zalantzak dira.



1.8. Irudia. Oinordekotza epigenetikoaren motak. Ezkerrean zelulatik zelularako oinordekotza epigenetikoa azaltzen da eta eskuman berriz, belaunaldi batetik besterako oinordekotza epigenetikoa. (Mills BB eta lank. 2016, moldatua).

Aipatu bezala, memoria edo oinordekotza epigenetikoa bi talde nagusitan banatzen da: 1) memoria epigenetiko zelularra deritzona, zelula amatik zelula alabetara transmititzen dena mitosi prozesuaren bitartez, eta 2) belaunaldiz belaunaldiko memoria epigenetikoa, gurasoen eta ondorengoen artean gertatzen den oinordekotza, meiosi prozesuaren bitartez gauzatzen dena (1.8. Irudia). Mitosia eta meiosian zehar informazio genetikoaren ibilbidea asko ikertu den bitartean, informazio epigenetikoarekin zer gertatzen den ez dago horren argi, eta orokorrean aldakorragoa izan ohi da, aldaketa epigenetikoen motaren eta espeziearen arabera. Hala ere, arlo honetan egin diren ikerketek ondorioztatu dutenez, organismoen garapenean zehar badaude informazio epigenetikoaren ibilbidearen inguruan aurkitu diren patroi batzuk nola mantendu edo eraldatzen diren azaltzen dutenak.

1.4.1. Memoria epigenetiko zelularra eta bere transmisioa

Zelulen arteko memoria epigenetikoa, zelulen izaera epigenetikoaren mantenua hartzen du bere baitan, adibidez desberdintze prozesuaren osteko zelula moten marka epigenetiko espezifikoak (Ng RK eta Gurdon JB 2008). Izaera epigenetiko horrek zelulen identitatea definitzen du, izan ere banakoaren zelula guztiek informazio genetiko berdina duten bitartean, marka epigenetiko ezberdinak izan ohi dituzte (Chen T eta Dent SY 2014), zelula motaren arabera espezifikoak direnak. Hori dela eta, garrantzitsua da, zelula ametatik zelula alabetara bitarteko zatiketa horretan izaera epigenetikoa heredatzea, zelula mota horretako identitate zelularra mantendu dadin. Izaera epigenetikoa mitosian mantentzen den arren, zelulen desberdintzean informazio epigenetikoak aldaketak jasaten ditu, eta baita ingurune edo kanpo faktoreen eraginez ere (Chen T eta Dent SY 2014; Becker C eta Weigel D 2012).

1.4.1.1. DNA-ren metilazioa

Aldaketa epigenetikoetatik, DNA-an ematen diren metilazioen oinordekotza nahiko bakuna da. Metilazioak DNA-ri era kobalentean lotuta daudenez, berarekin batera transmitituko dira mitosian zehar. Hala ere, marka indibidualen oinordekotza sinplea den arren, patroi espezifikoen oinordekotza hainbat zatiketa ziklotan zehar bermatzea prozesu konplexuagoa da. DNA-ren erreplikazioan, eredua den kateak DNA-ren marka daraman basea mantendu egingo du, baina sortu berri den kateak ez du marka eramango, beraz DNA hemimetilatua sortzen da. Dagokion metilazioa gehitzen ez bazaio sortu berri den kateari, hurrengo zatiketa zikloan alde batetik, metilazio gabeko DNA molekula sortuko da, eta bestetik, kate bakarra metilatua duen beste DNA molekula bat. Horrela, DNA-ren metilazio patroiak heredatu ahal izateko, sortzen diren DNA kateak eraldatu ahal izateko makinaria egokiaren presentzia beharrezkoa da.

Ugaztunetan, 5mC markak CpG irletan gertatu ohi dira. Hauek leku bereziak dira, non kate bateko zitosinak metilatu daitekeen beste zitosina bat daukan ondoko katean (eremu simetrikoak). Gainera, sortu berri diren kateetan DNA-ren metilazioak gehitzeko, lan hori burutzen duten metiltransferasa bereziak daude. Dnmt1-ek DNA-ren metilazioa kate berrian itsasten dute, marka epigenetikoa zatiketa zikloetan transmititzea posible eginez era egonkor batean (Jeltsch A eta Jurkowska RZ 2014; Hermann eta lank. 2004). Beraz, Dnmt1-a presente dagoen bitartean, DNA-ren metilazioa mitosia eta meiosian zehar transmititzea posiblea da. CpG-etatik kanpo, DNA-ren metilazio patroiak mantentzea konplexuagoa da, kasu horietan ez direlako zenbait zitosina eremu simetrikoetan kokatuak egoten. Hori dela eta, 5mC marka

bakoitza berrezarri egin behar izaten da zatiketa ziklo bakoitzaren ostean *de novo* metiltransferasaren laguntzarekin (He X-J eta lank. 2011).

1.4.1.2. Histonen aldaketak

Histonen aldaketak, zelulatik zelulara transmititzeko ahalmena duten marka epigenetiko bezala deskribatu dira (Campos EI eta lank. 2014; Rivera C eta lank. 2014), eta guztietatik transmisiorako histona aldaketa egonkorrena lisinen metilazioa da. Izan ere, zenbait lisinen metilazioen bataz besteko bizitza orduetatik egunetaraino luzatu daiteke (Zee BM eta lank. 2010), histonen azetilazio edo fosforilazioak minutuak irauten dituzten bitartean (Chestier A and Yaniv M 1979; Jackson V eta lank. 1975). Baina ala ere DNA-aren metilazioarekin ez bezala, histonen aldaketen prozesuaren inguruan eskura dagoen informazioa mugatua da. DNA-ren erreplikazio prozesuan nukleosomak askatu eta histonak DNA molekulatik kanporatuak izaten dira (MacAlpine DM and Almouzni G 2013). Hori dela eta, DNA-ren erreplikazioak histonen aldaketen galtzea dakar berarekin, askotan sortutako DNA kateetan histona berriak gehitzen direlako (aldaketarik gabekoak). Hala ere, erreplikazio prozesuaren osteko histona zahar eta berrien arteko banaketa nola gertatzen den eztabaida gaia da gaur egun. Deskribatua izan da nukleosomako histonak H3-H4 tetrameroetan eta H2A-H3B dimeroetan banatzen direla behin DNA molekulatik askatzerakoan (Campos EI eta Reinberg D 2009). H3-H4 histonetan egin diren hainbat ikerketen ondorioz, hiru banaketa eredu proposatu dira (Annunziato AT 2005; Annunziato AT 2013; Probst eta lank. 2009): 1) histona zaharrak zoriz banatzea bi DNA kateetan zehar, 2) histona zaharren erdia kate batera banatzea eta beste erdia beste katera, eta 3) eredu asimetrikoa, H3 eta H4 tetrameroak kate batera edo bestera mugitzea. Ondoren zelula amatik eratorritako eta zelula alabetan banatutako histonak, histona berriekin osatuko lirateke (De Koning L eta lank. 2007). Ondorioz, erreplikazioaren ostean sintetizatu berri den DNA-k histonen aldaketen nahasketa bat izango luke, alde batetik zelula amen jatorrizko histonen aldaketekin eta bestetik aldaketarik gabeko histona sortu berriekin.

Histonen aldaketen patroiekin epe luzeko oinordekotza finkatzeko, alde batetik, erreplikazio ostean histonen aldaketak sortzen dituzten entzimak azkar bildu behar dira beharrezko marka epigenetikoak gehitzeko, eta beste aldetik, DNA kate berrietan sortu diren histonek, ama zelulatik datozen histonen aldaketak jasotzea beharrezkoa da (Loyola A eta lank. 2006). Prozesu hau era zuzenean gauzatzeko nahitaezkoa da, entzima konplexu espezifikoak sekuentzia konkretuetara biltzea. Mitosian zehar, histonen aldaketak zelula batetik hurrengoetara transmititzeko prozesua, hauek sortzen dituzten entzimen bilketan oinarritzen da. Aurreko ataletan deskribatu dugun bezala, adibidez H3K9 histonaren aldaketa sortzeko G9a/Glp konplexua beharrezkoa da (Wen B eta lank. 2009), H3K27-aren metilazio marka burutzeko PRC2-a biltzen da (Cao R eta lank. 2002; Margueron eta Reinberg, 2011) eta H3K4-ren metilaziorako Mll konplexuen hurbilketaren bidez burutzen da (Hu D eta lank. 2013; Denissov S eta lank. 2014). Honetaz aparte, konplexu horien proteina osagarriak, ncRNA-ak edota elementu errepikakorrak zelulen zatiketaren ostean kromatinara biltzeko lagungarri izan daitezkeela ere proposatu da. Aipatu beharra dago, oinordekotza epigenetikoa CpG irlekin estuki lotuta dagoela, hori baitda PRC2 konplexuaren azpiunitateak gehien lotzen diren eremua (Ku eta lank. 2008). Saguen fibroblasto enbrionikoetan egindako ikerketa batean, PRC2 konplexuko azpiunitateen eta kromatinaren arteko lotura konstantea deskribatu da mitosiaren fase guztietan, nahiz eta H3K27-ren metilazio aldaketak zikloaren G1 faserarte ez diren berrezartzen (Xu M eta lank.

2011). Hala ere, PRC1-aren Bmi1 azpiunitatea, espero bezala, mitosian zehar oso ahula bihurtzen da eta berriro ere azaltzen da H3K27me3 inguruan G1 fasean (Aoto eta lank. 2008). H3K4 metilazioaren entzimen kasuan, Mll entzimek mitosiaren ostean aktibazio azkarra bultzatzen dute eta Mll konplexuaren osagarri diren Rbbp5 eta Ash2l proteinen presentzia deskribatu da M fasean zehar. Gainera Mll entzimen jaitsierarekin gene ituen aktibazio motelagoa ematen dela ikusi da mitosiaren ostean (Blobel eta lank. 2009). Azkenik, H3K9me2-aren mailak S fasearen ostean guztiz finkatzen dira (Xu M eta lank. 2011), marka epigenetiko honen berrezartzea garrantzi handikoa dela agerian utziz (Esteve PO eta lank. 2006). Hala ere, aipatu beharra dago, badaudela hainbat lan erreplikazio ondorengo kate berriaren histonen aldaketak kate zaharrekoekin alderatu dituztenak eta bertan deskribatzen denez, H3K9me2-ak bi kateetan balio antzekoak biltzen dituen arren S fasearen ostean, H3K27me3-an kate berriko balioak nabarmen txikiagoak direla eta G1 faserarte ez direla berrezartzen (Xu M eta lank. 2011).

1.4.1.3. ncRNA-k

Aurreko ataletan aipatu bezala ncRNA-en ikerketa, arlo berri samarra da epigenetikaren barruan eta azken lanetan, zelulen nortasunean, memoria zelularrean eta oinordekotza epigenetikoan, paper garrantzitsu bat izan dezaketela iradoki da (Mirouze M 2012). ncRNA-en taldeko lncRNA-k eta sRNA-etako miRNA-k, siRNA-k eta piRNA-k deskribatu dira memoria epigenetikoari lotura (Zamore PD eta Haley 2005).

Aurretik aipatu bezala, nahiz eta lncRNA-ek zeluletan duten funtzioa garrantzitsua dela iradoki den, gutxi batzuk bakarrik deskribatu dira (Mercer TR eta lank. 2009), hori dela eta oraindik ere funtzio konkretuak ez dira guztiz argitu. Hainbat ikerketek, zelulako oinarrizko prozesuekin lotu dituzte, hala nola, kromatinaren eraldaketa, transkripzioa, transkripzio ondorengo prozesatzea, zelulen arteko garraioa, X kromosomaren isiltzea, inpronta prozesua eta honen oinordekotza epigenetikoa (Wilusz JE eta lank. 2008; Ponting CP eta lank. 2009; Chen LL eta Carmichael GG 2010; Hannon GJ eta lank. 2006; Mercer TR eta lank. 2009). Azken bi prozesuei erreferentzia eginez, alde batetik deskribatua izan da X kromosomaren isiltzearen ostean, erreprimitutako kromosomaren egoera mitosian zehar mantendu egiten dela (Jonkers I eta lank. 2008). Hori dela eta, X kromosomaren inaktibazioa zelulatik zelulara gertatzen den lncRNA-en oinordekotza epigenetikoaren adibide bat da. Inpronta genomikoari dagokionean, aipatu dugu lncRNA batek bere inguruko geneak isildu ditzakeela interferentzia burutuz, horrela inpronta geneen kluster edo familia bat erregulatuz. Horrela, lncRNA-k ama zeluletatik alabetara banatu egiten dira zatiketa zelularrean eta beraz, batez ere inpronta genomikodun geneen klusterretan nortasun epigenetiko konkretuak mantendu ditzake Adalsteinsson BT and Ferguson-Smith 2014; Barlow DP eta Bartolomei MS 2014).

sRNA-ak berriz aurretik aipatu bezala, gene espezifikoen transkripzioaren isiltzean parte hartzen dute eta baita heterokromatinaren mantenuan ere (Mirouze M 2012; Siomi MC eta lank. 2011). Aldaketa epigenetikoen transmisioan sRNA talderik aztertuena miRNA-ena da. Gene espezifikoen adierazpen mailan duten eragina eta hauen profila aldatzeko duten ahalmena dela eta, funtzio oso garrantzitsua dute zelulen nortasuna finkatzearen prozesuan. Hori dela eta, miRNA-k zein gene aktibatu edo isildu gidatzen dute eta hauek heredatzeak beraz, memoria epigenetikoa garatu dezake, zelulatik zelulara transmititu daitekeena (Stuwe E eta lank. 2014). Duten egonkortasunaren arabera, heredatutako miRNA-k zelula ametako geneen adierazpen

patroi beretsuak ezarriko dituzte zelula alabetan, saguen kasuan deskribatu den bezala (Gapp K eta lank. 2014). Zehatzago esanda, mRNA eta isiltzen duen miRNA, zelula alabetan proportzio berdinetan banatzen diren bitartean, ama zelulako geneen adierazpen profila, zelula alabetan ere mantentzen da (Ptashne M 2013). sRNA-en beste talde bat siRNA-ena da, zeintzuk inaktibazio mekanismo bezala deskribatu diren (Tomari Y and Zamore PD 2005; Watanabe T eta lank. 2006). Adibidez, legamian burutu diren azken ikerketa batzuek, erakutsi dute nola siRNA eta H3K9 metilatua elkarlanean aritu daitezkeen nortasun epigenetikoaren babesle gisa eta marka epigenetikoen ezabatzearen aurka. Honek, oinordekotza epigenetikoaren sistemaren parte direla iradokitzen du (Yu R eta lank. 2018). Hala ere, azaldutako sistema saguan ere gertatzen den oraindik ez da argitu. Hori dela eta, miRNA-k zein gene aktibatu edo isildu gidatzen dute eta miRNA-k heredatzeak beraz, memoria epigenetikoa garatu dezake, zelulatik zelulara eta belaunaldi batetik bestera transmititu daitekeena (Stuwe E eta lank. 2014). Duten egonkortasunaren arabera, heredatutako miRNA-k zelula ametako geneen adierazpen patroi beretsuak ezarriko dituzte zelula alabetan, saguen kasuan deskribatu den bezala (Gapp K eta lank. 2014). Zehatzago esanda, mRNA eta isiltzen duen miRNA, zelula alabetan proportzio berdinetan banatzen diren bitartean, ama zelulako geneen adierazpen profila, zelula alabetan ere mantentzen da (Ptashne M 2013). sRNA-en hirugarren talde garrantzitsuena piRNA-ena da, eta aurretik aipatu bezala, orokorrean ar eta emeen hozi zeluletan burutzen dute beraien funtzioa (Siomi MC eta lank. 2001; Houwing S eta lank. 2007; Thomson T and Lin H 2009). Hori dela eta oinordekotza epigenetikoarekin duten erlazioa belaunaldiz belaunaldiko oinordekotza epigenetikoaren atalean (1.4.2.4. atalean) jorratuko da sakonago.

1.4.2. Belaunaldiz belaunaldiko memoria epigenetikoa eta bere transmisioa

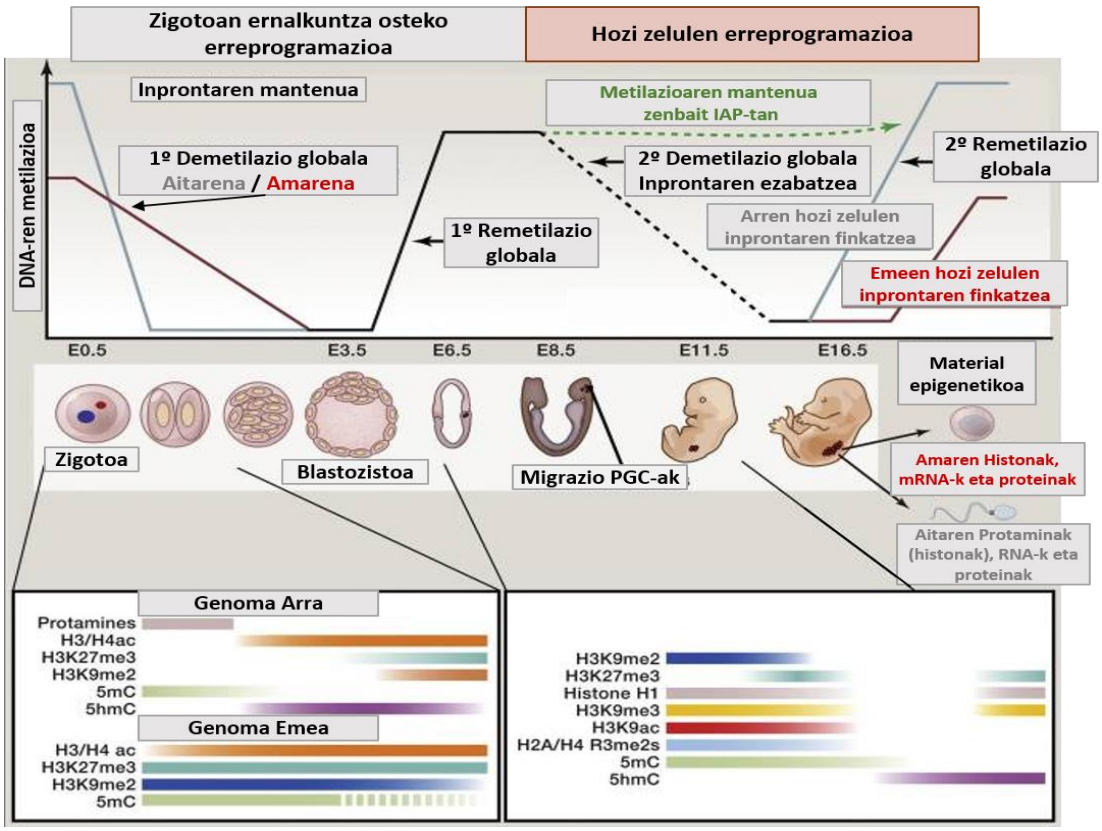
Gurasoetatik ondorengoetara transmititzen den informazio epigenetikoaren kasua ezberdina da eta gertatzen denaren ebidentzia nahikoa dagoen arren, mekanismoaren inguruan eskura dagoen informazioa urria da (Gapp eta lank. 2014; Skinner 2014; Babenko eta lank. 2015; Bohacek eta Mansuy 2015; Szyf 2015; van Otterdijk eta Michels 2016; Ambeskovic eta lank. 2017; Pang eta lank. 2017; Yeshurun eta Hannan 2018). Gametoetan gertatzen diren aldaketa epigenetikoek bakarrik izango dute ondorengo belaunaldien fenotipoan eragiteko ahalmena. Izan ere, mitosian zehar heredatu daitezkeen aldaketa epigenetikoak sortzen badira hozi zeluletan, meiosian ere transmititzea posible izango da ondorengo belaunaldietan zehar. Hala ere, belaunaldi batetik ondorengorako trantsizioa gauzatzeko, orokorrean marka epigenetiko guztien ezabatzea beharrezkoa izaten da (Bond DM eta Finnegan EJ 2007). Informazio epigenetiko parentalaren ezabatzeak desberdindu gabeko eta izaera totipotentea duen zigotoaren osaera ahalbidetzen du, garapen zuzena bermatuz (Mitalipov S eta Wolf D 2009). Aipatzekoa da, ezabatze hori kasu askotan ez dela guztiz zuzena, akats batzuek direla eta marka epigenetikok iraun dezaketelako, eta ondorioz, ondorengo belaunaldiaren geneen adierazpenean eragina izan (Campos EI eta lank. 2014; Heard E eta Martienssen R 2014).

1.4.2.1. Informazio epigenetikoa garapenean zehar (Erreprogramazio prozesua)

Enbrioi goiztiarraren garapeneko zelula pluripotenteek marka epigenetiko bilduma bat izan ohi dute (Buszaczak M eta Spradling aC 2006; Leeb M eta Wutz A 2012), desberdintze prozesuan zehar aldatu egiten dena, zelula espezifiko bakoitzak bere profil epigenetikoa eraikitzeko (Chen

T eta Dent SY 2014). Orokorrean zelulen arteko marka epigenetikoak mitosian zehar mantendu egiten dira, zelula alabek, zelula amen marka epigenetiko eta fenotipiko berdina erakutsiz.

Aipaturiko zelula somatikoetan ez bezala, belaunaldi batetik hurrengokorako trantsizioa burutzen duten hozi zeluletan, eta horien elkartzetik sortuko den zigotoan beharrezkoa da gurasoetan pilatutako marka epigenetiko guztiak ezabatzea, zigoto horrek berriro ere izaera pluripotentea berreskuratzeko (Feng S eta lank. 2010). Funtsean, ezabatze prozesu hori "erreprogramazio" (ingelesezko *reprogramming*) izenaz ezagutzen da. Ugaztunetan erreprogramazioa bi momentu ezberdinetan ematen da jadanik aipatu dugun bezala (Reik W eta lank. 2001; Feng S eta lank. 2010) (1.9. Irudia): 1) zigotoan, ernalkuntzaren ostean hasi eta garapen goiztiarrean bukatzen da; 2) jatorrizko hozi zeluletan (ingelesezko *primordial germ cells* edo PGC). Erreprogramazioaren lehenengo fasea zigotoan gertatzen da, non hozi zelulen profil epigenetiko espezifikoa, informazio epigenetiko enbrionikoaz ordezkatzen den, zelula izaera pluripotentera itzuliz (Messerschmidt DM eta lank. 2014), hau da, gurasoen hozi zeluletan pilatutako marka epigenetikoak ezabatu egiten dira. Adibidez, saguan lehenengo erreprogramazio honek, informazio epigenetiko osoaren ezabatzea dakar, besteak beste histonen aldaketak eta DNA-ren metilazio guztia borratuz bai amaren eta aitaren genoma osoan zehar, inpronta genomikodun geneetan izan ezik, jatorrizko informazio parentala mantenduz (Messerschmidt DM eta lank. 2014). Prozesu hau guztiz beharrezkoa da enbrioaren garapen egokia eman dadin eta desberdintze prozesua aurrera doan heinean, izaera pluripotentearen profil epigenetikoa (Bao S eta lank. 2009; Papp B eta Plath K 2013), zelula mota espezifikoen profilera aldatuz joango da. Bigarren erreprogramazioa PGC-etan gertatzen da, hauen migrazioaren aurretik, behin zelula helduak bihurtzen direnean DNA-ren metilazio profila eta histonen aldaketak ia guztiz ezabatuz, inpronta parentala barne. Bestalde, emeetan isilduta zegoen X kromosoma berriro ere piztu egiten da. Behin marka epigenetiko guztien ezabatzearen ostean, hozi zelulen profil epigenetiko propioa ezartzen da bi sexuen artean ezberdina dena, eta inpronta genomidun geneak barne dituena (Messerschmidt DM eta lank. 2014). Ondorioz, hozi zelulen garapenean, informazio epigenetiko somatiko parentala ezabatzen da, hozi zelula espezifikoen profil epigenetikoa finkatzeko.



1.9. Irudia. Saguaren garapenean gertatzen den erreprogramazio prozesua. Lehendabizi zigotoaren erreprogramazioa gertatzen da, justu ernalkuntzaren ostean hasten dena eta blastozisto garairarte irauten duena. Gametoetan pilatutako marka epigenetikoen ezabatzea emango da, gurasoetatik eratorritako inprontan izan ezik. Bigarrenez, jatorrizko hozi zelulen edo PGC-en erreprogramazioa ematen da migrazioarekin batera, non demetilazio globala gertatzen den inprontaren ezabatzea barne. Bukatzeko, hozi zeluletan inpronta espezifiko parentala ezartzen da. (Heard E eta Martienssen RA 2014, moldatua)

Hau guztia kontuan izanda, gurasoetatik ondorengo belaunaldietara aldaketa epigenetikoak transmititu ahal izateko, beharrezkoa da beraz, erreprogramazio prozesua gainditzea. Hala ere, nahiz eta prozesua oso eraginkorra den, aipatu bezala hainbat akats nabarmendu dira, belaunaldi batetik ondorengoetarako oinordekotza epigenetikoa gidatuz. Adibidez, genomako hainbat eremu espezifiko deskribatu dira batez ere DNA-ren desmetilazioa neurri batean gainditu dezaketenak, hala nola, elementu errepikakorrak (Hajkova P eta lank. 2008; Lee J eta lank. 2002) eta inpronta genomikodun geneak (Lane N eta lank. 2002; Borgel J eta lank. 2010; Reik W eta lank. 2001; Nakamura T eta lank. 2012). Zehazki, bai zigotoan zein PGC-etan elementu errepikakorren taldeko muturreko errepikapen luzeak dituzten erretrotransposoiak (ingelesezko *long terminal repeats* edo LTR), A partikula intrazisternalak barne (ingelesezko *intracisternal A particle* edo IAP), demetilazioa eragozteko ahalmena dute (Hajkova P eta lank. 2008; Lee J eta lank. 2002). Beste ikerketa batzuek, metilazioaren mailaz-mailako ezabatzea deskribatu dute zenbait inpronta genomikodun geneetan edota LINE1 eta IAP familietako erretrotransposoietan (Lee J eta lank. 2002). Bestalde zigotoan ematen den erreprogramazioan, Nnat (*neuronatin*), H19 eta Peg10 (*Paternally expressed 10*) bezalako inpronta genomikodun geneak partzialki demetilatuak bakarrik agertu direla azaldu da (Lane N eta lank. 2003), eta inprontadun eremuetako metilazio bereizgarria duten tokietan (ingelesezko *differentially methylated regions*), demetilazioa eragotzia izan daitekeela (Lee J eta lank. 2002; Lane N eta lank. 2003; Borgel J eta lank. 2010; Reik W eta lank. 2001). Aipatzekoa da baita ere, Stella bezalako faktoreek inprontadun geneak babesten dituztela azaldu da, demetilazio prozesuan

zehar, H19, Rasgrf1 (*RAS protein specific guanine nucleotide-releasing factor 1*), Peg1, Peg3 eta Peg10 (*Paternally expressed genes*)

Guzti honekin, erreprogramazioa eragozteko eta gainditzeko ahalmena duten marka epigenetikoak, belaunaldiz belaunaldiko oinordekotza epigenetikoa sortuko dute (Sharma A 2015; Daxinger L eta Whitelaw E 2012), naiz eta aldaketa horiek babesten dituzten mekanismoak oraindik ere argitzeko dauden (Sharma A 2015; Daxinger L and Whitelaw E 2012).

1.4.2.2. DNA-ren metilazioa

Belaunaldi batetik besterako DNA-ren metilazio marken transmisioan, CpG irletatik kanpo ematen diren metilazioek garrantzia handia dutela deskribatu da. Hala ere, prozesua nola burutzen den oraindik ere ez dago argi, izan ere ugaztunen kasuan zelula enbrionikoetara mugatzen da eta (Ramsahoye BH eta lank. 2000). Gaur egungo ikerketek bi transmisio bide deskribatzen dituzte besteak beste. Lehenengoak, histonen aldaketekin egindako elkarlanaren bitartez gertatu daitekeela azaltzen du, besteak beste H3K4-ren metilazioak DNA metiltransferasen lotura guneak inhibitu ditzakeelako (Ooi SK eta lank. 2007). Ondorioz, transmisio bide hau gauzatu dadin, histonen aldaketen oinordekotza egonkor bat beharrezkoa litzateke. Bigarrengo transmisio bidea, piRNA molekulen (ingelesezko *P-element induced wimpy testis-interacting RNA*) makinariaren bitartez izango litzateke, non piRNA-k *de novo* DNA metiltransferasa gidatuko lukeen (Kuramochi-Miyagawa eta lank. 2004; Aravi AA eta lank. 2007). Hau horrela gertatzeko, beharrezko izango litzateke piRNA heredatzea, DNA-ren metilazioa ondorengo belaunaldietan bermatzeko, era honetan, piRNA-en garrantzia iradokiz.

1.4.2.3. Histonen aldaketak

Histonen aldaketak, belaunaldiz belaunaldi transmititzeko ahalmena duten marka epigenetiko bezala deskribatu dira (Campos EI eta lank. 2014; Rivera C eta lank. 2014). Kasu honetan ere, zelula batetik ondorengoetara ematen diren mekanismoen parekoak gerta daitezkeela proposatu den arren, emeen eta arren arteko makinariak ezberdinak izango lirateke. Izan ere, espezie askotan (saguak barne), espermatozoideetako kromatina, zelula somatikoetako kromatinaren oso ezberdina da. Espermatozoideetan histona gehienak protaminengatik ordezkatzen dira (Rathke C eta lank. 2014), horrela DNA-ren %10-a bakarrik mantenduz histonei lotuta gizakian eta %1-a saguetan, besteak beste. Hori dela eta, espermatozoideen bidez histonen aldaketa kopuru oso txikia bakarrik transmititu daitezke, eta beharrezko diren histona berriak zigotoan gehitzen dira, baina histona berri hauen aldaketak nola ezartzen diren oraindik ere ez da guztiz argitu. Ikerketa batzuen ondorioz, histonen aldaketak sortzen dituzten entzimak beraien artean, DNA-ren metilazioa burutzen duten entzimekin (Blomen and Boonstra 2011) eta sRNA-ekin elkarlana egin dezaketela iradoki dute (Fritsch L eta lank. 2010; Rountree MR eta lank. 2000; Cho S eta lank. 2014), eta era honetan guztien artean histonen aldaketen oinordekotza epigenetikoa finkatuko litzateke belaunaldi ezberdinetan zehar.

1.4.2.4. ncRNA-k

ncRNA-etan, lncRNA-en artean belaunaldiz belaunaldi transmititzen den oinordekotza epigenetkoaren aldetik, geroz eta gehiago dira kanpo faktoreek eraginda, ondorengo belaunaldietan azaltzen diren hainbat aldaketen eragileak ncRNA-k direla baieztatzen duten ikerketak. Adibidez, estresarekin (Rodgers AB eta lank. 2013; Short AK eta lank. 2016; Gapp K

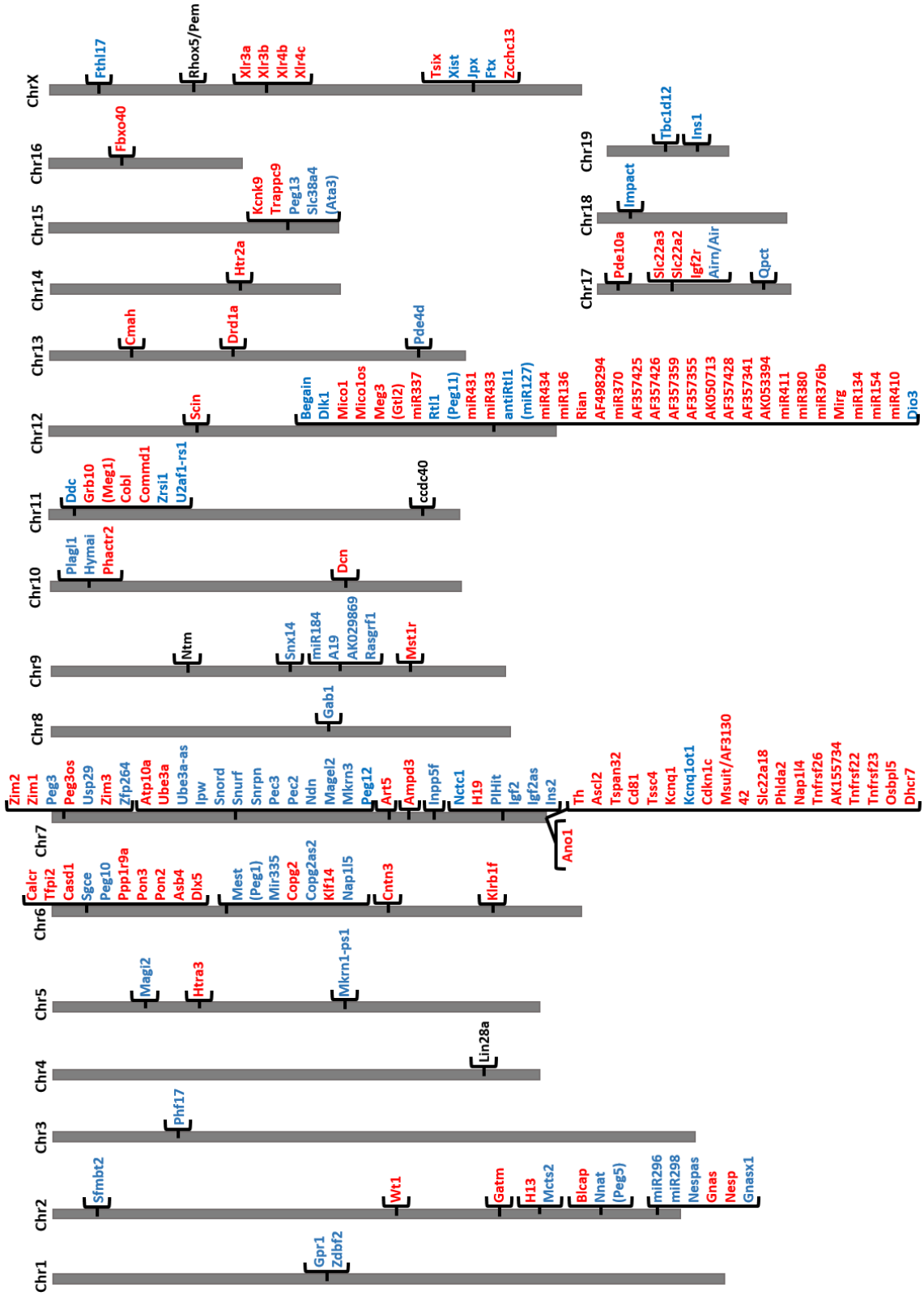
eta lank. 2018), dietarekin edota ariketa fisikoarekin (De Castro Barbosa T eta lank. 2016; Fullstron T eta lank. 2016; Grandjean V eta lank. 2015; Benito E eta lank. 2017; Schuster A eta lank. 2016), lotzen dituzte eta arren espermatozoideen bitartez transmititzen direla deskribatu dute. Hala ere, belaunaldiz belaunaldi igarotzen den oinordekotza epigenetikoaren mekanismoaren inguruan eskura dagoen informazioa oso mugatua da eta gurasoengandik jasotako RNA multzo bezala transmititzen direla iradoki da.

sRNA-en barneko miRNA-en taldearen kasuan, zelulatik zelularako memoria epigenetikoaren garapenean parte hartzeaz gain, belaunaldi batetik bestera meiosiaren bitartez transmititzeko ahalmena dutela ere deskribatu da (Stuwe E eta lank. 2014). Horren adibide dira giza espermatozoideetan aurkitu diren miRNA-k, garapen goiztiarrean gene espezifikoen erregulazioan funtzio garrantzitsuak dituztela iradokiz (Ostermeier GC eta lank. 2005), eta zehazki miRNA-34c, saguen enbrioiaren lehen zatiketan beharrezkoa dena (Liu W eta lank. 2012). Azken adibide honek, argi erakusten du, obuluetako eta espermatozoideetako miRNA-k duten garrantzia, gurasoen informazio epigenetikoak ondorengoen geneen adierazpenean eragiterako garaian, oinordekotza epigenetikoa sortuz. siRNA-etan berriz, beraien funtzioa esan bezala TE-ekin erlazionatzen da batez ere hozi zeluletan (Tomari Y and Zamore PD 2005; Watanabe T eta lank. 2006). Landareetan deskribatu denez, siRNA-k TE-n adierazpenaren isiltze mekanismoan parte hartzen dute hozi zeluletan eta errepresio hau belaunaldietan transmititu daiteke gametoen bitartez (Autran D eta lank. 2011). Hori dela era, ernalkuntza prozesuan zigotoak gurasoen informazio genetikoa jasotzeaz gain, TE-n isiltzeaz arduratzen diren siRNA multzo bat ere jasotzen du (Mirouze M 2012). Bestalde, siRNA-k belaunaldietan zehar DNA-ren metilazio patroien mantenuan garrantzi handikoak izan daitezkeela ere deskribatu da, gurasoetatik ondorengoetarako informazio epigenetikoaren oinordetza prozesua erraztuz (Johannes F eta lank. 2009; Reinders J eta lank. 2009; Teixeira FK eta lank. 2009). Hala ere, siRNA-etako belaunaldiz belaunaldi transmititzen den oinordekotza epigenetikoaren adibide hauek, landareetan aztertu dira, eta beraz, ugaztunetan gertatzen diren mekanismoak oraindik ere aztertu gabe daude. Azkenik piRNA-eri dagokionez, zenbait ikerketa taldek erakutsi dutenez naiz eta burmuinean funtzio garrantzitsuak egotzi zaizkien (Lyengar BR eta lank. 2014), batez ere zelula espermatikoetan aurkitzen dira. Honi lotuta, hozi zelulen geneen adierazpenaren eta TE-en erregulatzaile bezala aritzen dira eta baita zelula amen pluripotentziaren mantenuan ere (Siomi MC eta lank. 2001; Thomson T and Lin H 2009). Orokorrean talde edo klusterretan biltzen dira genoman zehar (Brennecke J eta lank. 2007; O'Donnell KA eta lank. 2007), eta eme zein arretan aurkitu ohi dira (Houwing S eta lank. 2007). Zizareetan, kromatinaren estrukturan eragina dutela deskribatu da, histonen marka errepresiboak sortuz (Luteijn Mj eta lank. 2012), batez ere H3K9me3-rekin erlazionatuz. Behin kromatinaren estrukturan aldaketak sortu ondoren isiltze mekanismo hau hurrengo belaunaldietara era egonkorrean transmititzen dela iradoki da (Bagijn MP eta lank. 2012), naiz eta zehaztasunik oraindik ez den deskribatu. Eulietan bestalde, hozi zeluletako TE-en isiltzearekin erlazionatu dira, piRNA mekanismoek TE-en genomako txertatzea eragozteko makinaria bezala jardunez. Behin hozi zeluletan funtzio hori burututa, hurrengo belaunaldietan heredatzen dira, amarengandik jasotako piRNA-en biltegia osatuz zigotoan (Muerdter F eta lank 2012; Khurana JS eta lank. 2011).

1.4.2.5. Inpronta genomikoa duten geneak

Aurreko atalean aurkeztu dugun bezala, inpronta genomikoa, fenomeno genetiko bat da non zenbait gene jatorri parental espezifikoaren bitartez adierazten diren. (Swales eta Spears 2005; Ideraabudullah eta lank. 2008; Reik W eta lank. 2001; Ferguson Smith eta lank. 2001). Aipatu beharra dago, inpronta genomikoa gametogenesian zehar finkatzen dela. Ondoren zigotoa sortzen den momentuan, erreprogramazio prozesuaren bitartez gurasoengandik jasotako marka epigenetiko guztiak ezabatu egiten dira, baina, inpronta genomikodun geneek prozesu hau gainditzea lortzen dute, era honetan gurasoen marka epigenetikoak enbrioiaren garapenean mantenduz (Ideraabdullah eta lank. 2008; Latham KE 1995). Jarraian, enbrioiaren eta organismo helduaren ehun somatikoetan inpronta hori irakurri egiten da. Eta bestalde, hozi zeluletan, bigarren erreprogramazioa ematen denean, orduan bai inpronta genomikodun geneetako marka epigenetikoak ezabatu egiten direla eta sexuan oinarritutako inpronta patroia bakarrik berrezartzen dira (Lucifero D eta lank. 2002; Davis TL eta lank. 1999; Ueda T eta lank. 2005). Hau da, orokorrean ugaztunek inprontadun bi genoma jasotzen dituzte, bat amarengandik eta beste aitarengandik, baina bakarra transmititzen diote ondorengo belaunaldiari, arrak edo emeak direnaren arabera. Azken arrazoi honengatik, ugaztunetan inpronta genomikodun geneak dira belaunaldietan zeharreko transmisioaren ebidentzia nagusiena, bigarren erreprogramazio prozesua bakarrik eragotzi behar dutelako (van Otterdijk eta Michels 2016).

Inpronta genomikoak, gurasoen aleloen arteko adierazpen maila ezberdina sortzen du, eta alelo aktibo eta inaktiboa, ingurune berdinean batera mantentzen direnez, bien arten aldaketa epigenetiko ezberdinak pilatu daitezkeela iradoki da. Izan ere, amaren edo aitarengandik heredatutako DNA-ren ezberdintasunak markatzeko makinaria bat beharrezkoa da, eta DNA-ren metilazioa edota kromatinaren egoeran aldaketak izendatu dira horren sortzaile. Honen arrazoia, DNA-ren metilazioak eta histonen aldaketak inpronta duten aleloen artean bereizten dutela da, inprontadun geneen eremuaren eskuragarritasunean eragina izanez (McEwen KR eta Ferguson-Smith AC 2010; Barlow DP 2011). Horrela, inprontaren prozesuan kanpo faktoreek izan dezaketen eragina, belaunaldi batetik bestera transmititu daitekeen oinordekotza epigenetikoaren gakoetako bat izan daiteke, eta gainera kanpo faktoreek sortutako aldaketa epigenetikoak (histonen aldaketetan, DNA-ren metilazioan eta ncRNA-etan), inpronta genomikoaren erregulazioan eragina izan dezakete.



1.10. Irudia. Saguaren inrpontadun geneen mapa genomikoa. Inprontadun geneen distribuzio genomikoa irudikatzen da geneen klusterrak taldekatuz. Gorriz amaren adierazpena duten geneak ageri dira eta urdinez aitaren adierazpena duten geneak. www.geneimprint.com eta www.mousebook.org-etik osatua eta moldatua.

Orokorrean inprontadun geneak klusterretan biltzen dira eta elementu erregulatzaileak elkarbanatzen dituzte (Paulsen eta lank. 1998; Paulsen eta lank. 2000; Engemant eta lank. 2000) (1.10. Irudia). Ezaugarri esanguratsuena, gene horiek izaten duten DNA sekuentzia bereziak dira, alelo bakarrean oso metilazio trinkoa izaten dutenak eta metilazio hori mantendu egiten da zelulen zatiketan zehar (Tremblay KD eta lank. 1997). Eremu hauek DMR bezala ezagutzen dira (ingelesezko *differentially methylated regions*), eta aleloen arteko erregulazio funtzioan beharrezkoak dira (Thorvaldsen JL eta lank. 1998; Wutz A eta lank. 1997; Liu J eta lank. 2005; Kantor B eta lank. 2004; Fitzpatrick G 2002; Yoon BJ eta lank. 2002; Takada S eta lank. 2002; Coombes C eta lank. 2003). Kluster bakoitzeko erregulazioan agintzen duen DMR nagusiari ICR izena jarri zaio (ingelesezko *imprinting control region*), eta hauek guztietan pilatzen dira DNA-ren metilazioak, histonen aldaketak eta histonen aldaketak sortzen dituzten entzimak marka epigenetikoen bidez, adierazpen parental espezifikoa ezartzeko (Delaval eta Feil 2004; Ideraabdullah eta lank. 2008).

Gaur egun inpronta prozesuaren inguruan burutu diren ikerketa guztiak geneen adierazpenaren aldaketetan oinarritu dira, erregulazio eremuen edo ICR/DMR-en metilazioekin erlazionatuz. Teknika bioinformatikoen laguntzaz inprontadun geneen eremuetan CpG irlak, sekuentzia errepikakorrak eta TE-ak ezaugarri komun bat izan daitezkeela iradoki da (Paoloni-Giacobino A eta lank. 2007; Hutter B eta lank. 2006; Bestor TH and Bourchis 2004). Hala ere, oraindik ere ez dago ezaugarri genomikorik, inprontadun geneak inpronta gabeko geneetatik bereiztuko dituena, ondorioz, inprotaren mekanismoa eta honen erregulazioa ez da argitu. Prozesua era sinplean azalduz, adierazten diren inprontadun geneak ez dira DNA-ren metilazioaz markatuak egongo eta erreprimituta daudenak ordea metilauak izango dira (Fowden eta lank. 2006). Metilazioa hau, hozi zeluletako *de novo* DNA metiltransferasek burutzen dute (DNmt3a eta Dnmt3l) (Kaneda M eta lank. 2004; Hata K eta lank. 2002), honen ondorioz, inprontadun geneen metilazioaren inguruko ikerketa asko, Dnmt hauek beraien funtzioa nola burutzen duten argitzen saiatu izan dira, *de novo* DNA metiltransferasek ez baitdute sekuentzia espezifikoetan lan egiten (Smallwood SA eta lank. 2011; Davis TL eta lank. 2000). Beste alde batetik, ncRNA-k inprontadun geneen erregulazioan parte hartzen dutela ere deskribatu da (Reik W eta lank. 2005; Weaver JR eta Bartolomei MS 2013). Adibidez, inpronta eremu bateko ICR-ak eta lncRNA-ren arteko elkarrekintzak, Piwi makinariaren laguntzarekin Dnmt3a eta Dnmt3l-a biltzen ditu, metilazioa burutzeko (Watanabe T eta lank. 2011). Hala ere, honen guztiaren mekanismo zehatza ez da bere osotasunean argitu.

Gainera, DMR/ICR-etan DNA-ren bidezko inpronta markak finkatzerako orduan kromatinak izan ditzazkeen aldaketak zein eragin izan dezakeen ikertu duten lanak oso mugatuak dira. Baina, histonen aldaketek ere inprontadun geneen adierazpenaren erregulazioan parte hartzen dutela azaldu da, izan ere metilatutako ICR-ak H3K9me3 eta H4K20me3-rekin erlazionatzen dira, metilatu gabeko eremuak H3K4me2 eta H3ac-rekin erlazionatzen diren bitartean (Delaval eta lank. 2007). H3K27me3 marka epigenetiko errepresiboa eta PRC2 konplexua zenbait inpronta eremutan aurkitu izan dira mESC-tan, erreprimitutako aleloari lotuta, besteak beste amarengandik jasotako Kcnqlotl-an (Umlauf D eta lank. 2004; Verona RI eta lank. 2008) edota aitarengandik jasotako Ascl2 eta Cdknlc-an (Lewis A eta lank. 2004). Bestalde H3K27me3-aren eta Snrpn inpronta eremuaren arteko erlazioa ere deskribatua izan da (Verona RI eta lank. 2008; Han L eta lank. 2008), baina hau inpronta eremu guztietara zabaldu ezin den mekanismoa dela

ere argitu dute. H3K4me2-a, *de novo* metiltransferasaren funtzioarekin erlazionatu dira (Ciccone DN eta lank. 2009), zehazki H19-Igf2 klusterrean, inpronta eremu espezifikoak babestuz metilazioa ekiditeko (Jia eta lank. 2007; Ooi eta lank. 2007;Lee eta lank. 2010; Singh P eta lank. 2013). H3K9-ren metilazio ezberdinak marka errepresiboa izan daitezkeen heinean, inprontadun geneen erregulazioarekin ere erlazionatu izan dira zenbait lanetan (Lewis A eta lank. 2004; Umlaur F et la. 2004; Verona RI eta lank. 2008; Singh P eta lank. 2011). G9a entzima metiltransferasa ere inprontadun geneekin erlazionatua izan da, zehazki Airn genearekin bere klusterreko geneak erregulatzeko (Nagano eta lank. 2008) eta Kcnql genearekin, non G9a-ren jaitsierak Kcnql-ren klusterrean inprontan akatsak sortzen dituen, era berean H3K9 histonaren metilazioa ere gutxituz (Wagschal eta lank. 2008). Azkenik, G9a/Glp konplexuak inprontadun geneen babesle papera ere jorratu dezakeela ikusi da *de novo* DNA metiltransferasak bilduz (Zhang T eta lank. 2016). Adibide hauek guztiek, argi uzten dute inpronta genomikoaren erregulazioa DNA-ren metilazio eta histonen aldaketen bitartez osatutako sare handi baten bidez burutzen dela, naiz eta gaur egun eskura dagoen informazioa oraindik ere oso mugatua den. Hala ere, argi dagoen gauza bakarra da, inpronta eremu edo kluster bakoitzak bere funtzionamendu propio edo espezifikoa duela, eta beraz, banan-banan aztertu behar direla.

1.4.2.6. Elementu errepikakorrak

Tesiaren sarrera osoan zehar hainbat alditan aipatu dira elementu errepikakorrak aldaketa epigenetiko eta memoria zelularrarekin erlazionatuta daudela. Hauek, genoman zehar behin eta berriz errepikatzen diren DNA edo RNA sekuentziak dira. Organismo askotan DNA genomikoaren kopuru handi bat errepikakorra da, ugaztunetan %70 ingurura iritsiz (Koning eta lank. 2011). Dirudienez, funtzio garrantzitsua dute kromosomen egituraren antolaketan, zenbait generen adierazpenean (inpronta genomikodun geneak barne) eta garapenean zehar (Kidweell MG eta lank. 2000; Lander ES eta lank. 2001; Waterston RH eta lank. 2002; Jaenishc R and Bird A 2003; Feschotte C 2008; Ting eta lank. 2011; Zhu eta lank. 2011).

Orokorrean DNA elementu errepikakorrak bi taldetan banatzen dira: 1) bata bestearen ondoan kokatuak egon daitezkeen elementuak, tandem errepikapenak osatuz eta 2) era zabalean tartekatutako elementu errepikakorrak. Horrela base kopuru eta kopia kopuru aldakorra izan dezakete batetik milioietaraino (Batze rand deininger 2002; Jurka eta lank. 2007; Kim eta lank. 2008; Britten 2010; Hua-van eta lank. 2011). Tandem errepikapenen taldean, Sateliteak, errepikapen sinpleak (*Simple-repeats*), konplexutasun gutxiko errepikapenak (*Low complexity repeats*), RNA errepikapenak eta besteak ditugu. Sateliteak, errepikapen tandem luzeak dira eta zentromeroen osagarri nagusia izateaz gain, heterokromatina konstitutiboaren parte dira (Guenatri M eta lank. 2004; Wong AK eta Rattner JB 1988 Waterston RH eta lank. 2002). Bestalde, tartekatutako elementu errepikakorrak, elementu mugikorrak dira, hau da, DNA TE-ek eta erretrotransposoiek osatzen dute talde hau (Solyom eta Kazazian 2012). Era berean bigarrengo hauek beste bi azpitaldetan banatzen dira, muturreko errepikapen luzeak dituzten erretrotransposoiak (LTR) edota ez dituztenak (non-LTR). LTR-ak, zelula ostalaritik irtetzeko ahalmena galdu duten erretrobirus endogenoek osatzen dituzte. Non-LTR-ak aldiz, tartekako elementu luzeek (LINEs) eta motzek (SINEs) osatzen dute. Aipatu beharra dago, batez ere, TE-ak ugaztunen genomako aldaketa epigenetikoen iturri bezala identifikatu izan dituztela (Ekram eta lank. 2012), oinordekotza epigenetikoarekin erlazionatuz (Faulkner 2011). Hori dela eta, elementu errepikakorren egoera epigenetikoaren azterketa, garrantzi handiko arloa ari da

bilakatzen azken aldian, zenbait histonen aldaketa, DNA metilazio eta ncRNA-ren mekanismoen patroiak deskribatuz (Slotkin RK eta Martienssen R 2007; Maksakova IA eta lank. 2008).

Aktibo dauden TE-ak oso mutagenikoak dira, geneetan txertatu, splicing prozesua aldatu, kromosometan errekonbinazio akatsak sortu eta genoma birrantolatu dezaketelako (Girard L eta Freeling 1999). Hori dela eta zenbait mekanismo epigenetiko deskribatu dira TE-ak erreprimituta mantentzeko zelulen memorian zehar. Aurretik azaldu dugun bezala, mekanismo nagusia interferentziazko RNA molekulen bidezkoa da, RISC konplexua osatuz (Sijen T eta Plasterk RH 2003; Watanabe T eta lank. 2006; Yang N eta Kazazian HH 2006; Lippman Z eta lank. 2003), adibidez LTR taldeko, IAP, ERV-L eta ERV-K azpifamilietan (Svoboda eta lank. 2004). Hala ere, prozesua bere osotasunean ez da guztiz argitu. Histonen aldaketen artean, LTR taldeko ERV-K eta ERV1 azpifamiliak H3K9me3 eta H4K20me3 markekin erlazionatu izan dira, ERV-L eta ERVL-MaLR azpifamiliek H3K27me3-ren aberastea erakutsi duten bitartean (Maksakova IA eta lank. 2009; Dong KB eta lank. 2008; Mikkelsen TS eta lank. 2007; Gendrel AV eta lank. 2002; Martesn JH eta lank. 2005; Day DS eta lank. 2010). Bi kasuetan marka errepresiogileak diren arren, lehengoak heterokromatina konstitutiboarekin du erlazioa eta bigarrenak aldiz PRC2 konplexuarekin. Izan ere, deskribatua izan da G9a entzimaren mutazioak TE-en birraktibazioa dakarrela mESC-tan (Martesn JH eta lank. 2005), H3K9me3-ren jaitsiera emanez, H3K27me3-ren igoerarekin batera, bien arteko konpentsazio mekanismo bat iradokiz (Peters AH eta lank. 2003). Azkenik DNA-ren metilazioa ere proposatu da TE-en errepresiogile modura (Liang eta lank. 2002; Kato eta lank. 2007), batez ere Dnmt1-a, bere jaitsierak TE-en mailen igoera nabarmena sortzen du eta (Walsh CP eta lank. 1998). Genoma osoko metilazioa ikertu izan dute eta ondorioztatu da LINE, LTR, DNA elementuak eta Satelliteak direla DNA-ren metilazioaren bidez erregulatzen diren elementu errepikakorren talde nagusiak. Dnmt3 eta Dnmt3l ere hainbat elementu errepikakorren metilaziorako beharrezko entzima bezala deskribatu dira hozi zeluletan (Bourchis D eta Bestor TH 2004). Aldaketa epigenetiko guztiek elementu errepikakorren errepresioan parte hartzea, batez ere hauen aktibazioaren aurkako babes sistema bat izan daiteke. Hala ere, aktibaziorako erregulazio espezifikoa ere baimentzen da hozi zeluletan (Peaston eta lank. 2003), zelula enbrionikoetan (Kano eta lank. 2009) eta garapen berantiarrean (Muotri eta lank. 2005; 2010).

Bestalde, aldaketa epigenetikoen bitartez erregulatuak izateaz gain, azaldu dugun moduan, beraiek ere zenbait geneen adierazpenean erregulazio epigenetikoa gauzatu dezakete. Adibidez enbrioiaren garapenean eta obulu helduetan, hostalari diren hainbat generen ordezko sustatzaile bezala aritu daitezke (Peaston AE eta lank. 2004). Bestalde, TE-ek inguruko geneen adierazpenaren erregulazioa bakarka egin dezakete, LINE1-aren kasuan deskribatu den bezala (Yang N eta Kazazian HH 2006), edo TE-en familia oso batek batera zenbait gene talde erregula ditzake (Matienssen R eta Baron A 1994). Aipatu beharra dago TE-en aktibitatea kanpo faktoreen menpe dagoela (Capy P eta lank. 2000), ondorioz, erregulatzen dituzten geneengan epe luzerako adirazpen aldaketak sortuz. Azkenik inprontadun geneen erregulazioaren partaide direla ere aipatu dugu, izan ere alelo bakarreko adierazpena duten geneekin estuki lotuta daude. Adibidez, ugaztunetan IAP-a eta LINE1-a hipometilatuta ageri dira emeen hozi zeluletan, horrela hainbat generen adierazpena handituz (Peaston AE eta lank. 2004). Espermatozoideetan aldiz ez da hipometilazio hori aurkitu. Bestalde aitarengandik jasotako hainbat inprontadun gene LINE1 elementuarekin erlazionatzen dira, SINE-rik deskribatu ez den bitartean gene horien

sustatzaileetan (Allen E eta lank. 2003; Greatlly JM 2002). Honek guztiak, TE-ek aitaren edo amarengandik jasotako inprontadun geneetan era ezberdinetan eragin dezaketela iradoki du, eta jarrera hau batez ere IAP, LINE eta SINE-etan ikertu da (Walter J eta lank. 2006). Hala ere, TE-ek inprontadun geneak erregulatzeko baliatzen duten mekanismoa oraindik ere aurkitzeko dago.

1.5. INGURUNEA ETA EPIGENETIKA

Aldaketa epigenetikoak gertakari arrunt eta berezkoak izan daitezke aurretik argitu dugun bezala, baina azken aurkikuntzek azaldu dutenez, kanpo faktore edo inguruneak ere, bizi estiloa barne, aldaketa epigenetikoak sortu ditzakete, geneen adierazpenean eragin zuzena izanez. Ideia honek Jean Baptiste Lamarck zientzialariak bere garaian proposatutako teoria babesten du, izan ere, Lamarck-en hitzetan “eskuratutako ezaugarrien oinordetzak” eboluzioa gidatzen zuen. Lamarck-en teoria honek, Darwinen hautaketa naturalaren eboluzioaren teoriaren aurka indarra galdu zuen arren, eskuratutako ezaugarrien transmisioaren kontzeptuak gaur egun bere garrantzia berreskuratu du eboluzioaren teoriaren aldaketa bultzatuz. Horrela ingurunearen epigenetika, Lamarck-en eboluzio proposamenaren mekanismo molekulartzat kontsideratu daikete, inguruneak fenotipoan eragina izan dezakeela baieztatuz, hau era berean heredagarria izanez (Lamarck 1802; Calabi 2001; Burkhardt 2013).

Honen harira, eta aurretik aipatu dugun bezala, jakina da aldaketa epigenetikoak ehunen eta zelulen desberdintze prozesua gidatu dezaketela, azaleko , burmuineko, gibeleko etab.-eko zelulak sortuz; edo kanpo faktoreen eraginez, aldaketa epigenetikoek efektu kaltegarriagoak izan ditzaketela azkenik minbizia bezalako gaixotasunak sortuz. Hori dela eta “ingurune epigenetika” garrantzitsua bilakatzen ari da medikuntza arloko ikerketetan (Reamon-Buettner SM eta lank. 2008). Honenbestez, aldaketa epigenetikoak itu garrantzitsua dira, inguruneak edo kanpo faktoreek geneen adierazpenaren erregulazioaren bitartez, funtzio fisiologikoetan izan dezaketen eragina ulertzeko.

1.5.1. Kanpo faktoreak

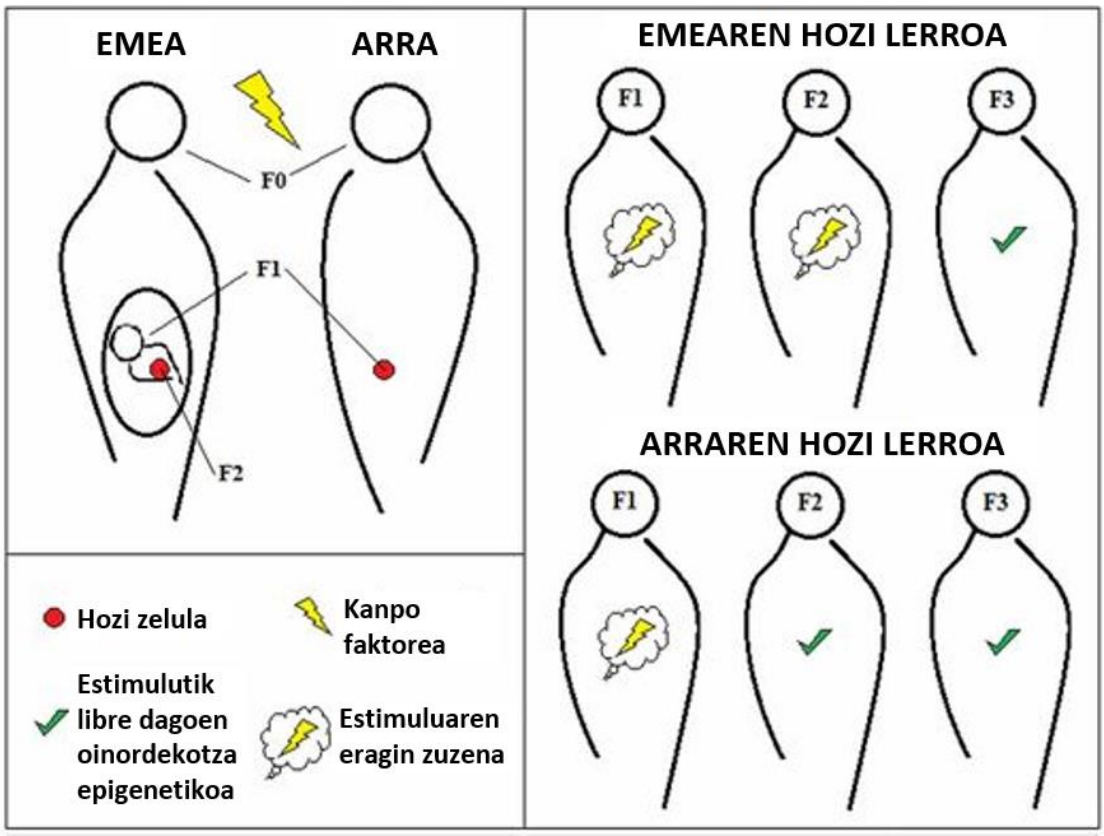
Azkenaldiko aurkikuntzarik harrigarrienek erakutsi dutenez, inguruneak edo kanpo faktoreek aldaketa epigenetikoak sortu ditzakete, horrela inguruneko estimuluak mekanismo epigenetikoen bitartez, kromatinaren aldaketa egonkor bilakatuz eta era berean geneen adierazpena piztu edo isilduz (Jaenisch R eta Bird A 2003), naiz eta efektua sortu duen estimulua desagertu. Honen harira, zenbait ikerketek deskribatu dute ingurune edo kanpo faktoreek zenbait aldaketa epigenetiko sortu ditzaketela, hala nola, dietak (Whitelaw NC 2003), estresak (Skinner MK 2014), jarduera fisikoak, aldaketa klimatikoak, toxiko ezberdinen kontsumoak, tabakok edota menpekotasuna sortzen duten drogak (Yohn NL eta lank. 2015). Norbanakoaren garapenean zehar ematen diren kanpo faktoreen esposizioak, patroi epigenetiko espezifikoen egonkortzea dakar, fenotipo ezberdinak sortuz, momentuan edo garapeneko heldutasunean. Izan ere, aurretik aipatu dugun bezala, kanpo faktoreen eragina, berauek presente daudenean azaldu daiteke edota hauek bukatu ondoren (Cortessi VK eta lank. 2012; Skinner MK eta lank. 2012: Skinner MK eta lank. 2014; Guerrero-Bosargna eta Skineer MK 2012; Heard E eta Martienssen RA 2014).

Kasu askotan aldaketa epigenetikoak belaunaldietan zehar ere transmititu daitezke, ikerketa epidemiologikoen bidez erakutsi den bezala, gurasoak zenbait substantziaren menpe jarri ostean belaunaldietan zehar mantentzen diren efektuak deskribatu dira eta (Bell JT eta Spector TD 2011). Honen adibide dira, 40. hamarkadan Herbehereetan gertatu zen gosetea eta hauen ondorengoek agerian utzi zuten obesitatea, glukosarekiko intolerantzia eta bihotzeko gaixotasunak (Painter eta lank 2008; Lumey eta lank. 2011; Veenendaal eta lank. 2013); Norvegiako Overkalix kohortean janari urri edo ugariaren baliabideak sortu zuten ondorengoen bizi-itxaropenaren luzatzea edota diabetesak sortutako hiltze arriskua, hurrenez hurren (Kaati eta lank. 2002; Bygren eta lank. 2001); Holokaustoan bizirik iraun zutenen traumaren transmisioa ondorengo belaunaldietan (Vaage eta lank. 2011; Kellermann 2013), etab. Belaunaldietan transmititzen den oinordekotza epigenetikoari buruzko lehenengo aurkikuntzak "vinclozin" fungizidaren inguruko azterketen bitartez identifikatu ziren, izan ere, aldaketa egonkor eta heredagarriak sortzen zituen zenbait belaunaldietan zehar (Anway eta lank. 2005). Geroztik, hainbat eta hainbat kanpo faktore deskribatu dira aitaren edo amarengandik transmititutako fenotipoaren adibide gisa, hala nola, jaio aurreko estresa (Morgan eta Bale 2011), dieta (Kaati eta lank. 2002; Dunn eta Bale 2009; Champagne 2010; Ng eta lank. 2010; Vassoler eta lank. 2014), alkoholak (Govorko D eta lank. 2012), nikotinak (Zhu J eta lank. 2012) edota opioideak (Byrnes JJ eta lank. 2011; He eta lank 2006; Novikova eta lank. 2008; Vassoler eta lank. 2014; Byrnes EM 2005), nahiz eta ondorengo belaunaldiak substantzia edo kanpo faktore horren menpe ez egon (Saab B eta Mansuy I 2013).

Aipatu bezala, aldaketa epigenetikoak zelulatik zelulara transmititu daitezen, mitosi prozesuan zehar mantendu behar dira (Campos eta lank. 2014), eta belaunaldi batetik hurrengora transmititu ahal izateko berriz, hozi lerroan gertatu eta meiosian zehar iraun behar dute, nahiz eta aldaketa epigenetikoak sortu dituzten kanpo faktoreak presente ez egon (Sharma eta Rando 2017). Horrez gain, bi kasuetan erreprogramazioaren bi olatuetan ezabatu gabe mantendu behar dira heredagarriak izateko (Heard E eta Martienssen RA 2014). Beraz, kanpo faktoreek eraginda aldaketa epigenetiko berriak sortu eta erreprogramazioan zehar mantentzen badira, ondorengo belaunaldiek epigenomaren patroi eraldatuak heredatuko dituzte. Hala ere, garapenean kanpo faktoreen eragina erreprogramazio prozesuan zehar nola mantentzen den ez da argitu, eta sortutako aldaketa epigenetiko horiek norbanako helduan edo ondorengo belaunaldietan izan dezaketen eragin espezifikoa edo sortu ditzaketen gaixotasunak zeintzuk izan daitezkeen, ikertzen jarraitzeko arlo garrantzitsu bat da gaur egun (Dolinoy DC eta lank. 2007; Bohacek eta Mansuy 2013; Moosavi A eta Ardekani AM 2016).

Belaunaldiz belaunaldi ematen den oinordekotza epigenetikoaren inguruan, puntu garrantzitsu bat, aldaketa epigenetikoak denboran zehar egonkorrak izatean datza. Horrela, kanpo faktoreen eragina bi talde handitan banatu ohi da (1.11. Irudia): jaio aurreko esposizioa, hau da, haurdun dauden emakumeetan gertatzen dena; eta esposizio parentala, haurdunaldia izan aurretik gertatzen dena hozi zeluletan (Heard eta Martienssen 2014). Jaio aurreko esposizioan, kanpo faktoreek uteroko fetuan eta bere hozi zeluletan izango dute eragina. Hori dela eta jaioberrian (F1) eta bere ondorengo zuzenetan (F2) mantentzen diren aldaketa epigenetikoak ez dira transgenerazionalak kontsideratuko kanpo faktoreen esposizio zuzena izan dutelako. Hurrengo belaunaldietan mantentzen diren aldaketa epigenetikoak aldiz (F3 eta ondorengoak), aldaketa transgenerazional kontsideratuko dira kanpo faktoreen esposiziotik kanpo daude eta. Bestalde

esposizio parentalaren kasuan, kanpo faktoreen eraginez sortutako aldaketa epigenetikoak gurasoen hozi zeluletan emango dira eta beraz, transgenerazionala kontsideratuko da F2 belaunaldian.



1.11. Irudia. Belaunaldietan zehar mantentzen den oinordekotza epigenetikoa. Haurdun dauden emakumeengan (F0) erasan dezaketen kanpo faktoreek zuzenean eragiten diote lehenengo belaunaldiari (F1) eta baita bigarren belaunaldia osatuko duten bere hozi zelulei (F2). Hori dela eta, F3 belaunaldian mantentzen diren aldaketak bakarrik kontsideratzen dira belaunaldietan zeharreko oinordekotza epigenetiko purua. Arren hozi zeluletan berriz, kanpo faktoreen eragin zuzena lehenengo belaunaldian bakarrik sortu dezakete, beraz, F2 belaunaldian mantentzen diren aldaketek, belaunaldietan zehar mantentzen den oinordekotza epigenetikoa osatuko dute (Lacal I eta Ventura R2018, moldatua)

1.5.2. Sistema opioidea

Sistema opioidea, komunikazio sistema bat da, zeinak fisiologian garrantzi handia duten prozesu zelular asko erregulatzen dituen. Tradizionalki, minaren eta plazerraren erregulazioarekin erlazionatua izan da nerbio sistema zentralean eta pixkanaka, garapeneko hainbat prozesu fisiologikoekin erlazionatzen joan da (Subiran eta lank. 2011).

1.5.2.1. Sistema opioidearen ezaugarriak eta funtzionamendua

Hartzaile opioideak, Gαi/Gαo proteinetara akoplaturiko hartzaileen taldearen parte dira (GPCR) eta 7TM egitura daukate, hau da, guztiek domeinu N- extrazelular bat, 7 mintzarteko domeinu eta bukaerako buztan C-terminala dituzte. Hartzaileek pizten dituzten seinaleztapen bidezidor intrazelularren artean adenilato ziklasaren inhibizioa, boltai menpeko kaltzio kanalen inaktibazioa, potasio kanalen aktibazioa, kaltzio erreserben mobilizazioa edota MAPK-en transdukzio bideen estimulazioa daude (Waldhoer eta lank. 2004). Hiru hartzaile opioide mota dira orokorrean deskribatu direnak: mu opioide-hartzailea (μ= mu, morfinarekiko duen

loturarengatik aurkitu zena; MOR); delta opioide-hartzailea (δ= delta, saguaren hodi deferentean identifikatu zenez "*deferens*" hitzetik eratorria, DOR) eta kappa opioide-hartzailea (κ= kappa, ketoziklazozinagatik identifikatu zena, KOR) (Lord eta lank. 1997). Bestalde, opioide-hartzaileekin alderatuta oso antzekoa den beste hartzaile opioide bat ere deskribatu izan da aurreko hiruekin batera: nozizeptina lotzen duen opioide-hartzailea (NOR) (Mollereau eta lank. 1994; Wick eta lank. 1994). Guztien artean, %60-ko antzekotasuna ageri dute sekuentzia osoa konparatzen baldin bada eta %73-76-koa mintzaren zeharreko aldeetan (Minadi eta Satoh 1995).

Honen harira, barne sistema opioidea, hartzaile opioideez, barne-peptido opioideez (BPO) eta andeakuntza-prozesuetan parte hartzen duten entzimez osatuta dago. BPO-en eraketari buruz egin diren ikerketek, nerbio sistema zentralean aurkitu daitezkeen peptido ia guztiak (karnosina dipeptidoa eta glutation tripeptidoa izan ezik) neuronetako erribosometan molekula aitzindari peptidiko edo pre-pro-proteina bezala sintetizatzen direla deskribatu dute (Siegel eta lank. 1993). BPO-en aitzindariak sortzen dituzten hiru geneak ondorengoak dira: 1) Proentzefalina (PENK), nerbio sistema zentralean eta muin adrenalean sintetizatzen dena (Comb eta lank. 1982) eta zazpi peptido opioide sortu ditzakeena, ezagunena entzefalina izanik; 2) Propiomelanokortina (POMC), β -endorfina bezalako BPO-ak sortzeaz gain hormona adrenikortikotropikoa (ACTH), α eta β -lipotropina (LPH) eta α eta β-melanotropina (MSH) ere sortzen dituena (Nakanishi eta lank. 1979); eta 3) Prodinorfina (PDYN), A eta B dinorfinak eta β-neo-endorfina bezalako peptido opioideak sortzen dituena (Kakidani eta lank 1982).

BPO-en jarduera hartzaile opioideekiko loturari esker gertatzen da eta bere ekintza zenbait entzimek inaktibatzen dute. Gaur egun, 20 BPO ezagutzen dira eta ugaztunetan metentzefalina, Leu-entzefalina, B-endorfina, Dinorfina A, Dinorfina B, α-neoendorfina, Nozizeptina eta Endomorfina deskribatu dira (Florez 1998). BPO-ek euren hartzaileengatik afinitate aldagarria erakusten dutela ere eman da aditzera (Subiran eta lank. 2011). Adibidez, met eta leu-entzefalinek, DOR-ekiko afinitate altua aurkezten duten arren, MOR-i ere batzeko gai dira afinitate gutxiagorekin. Bestalde B-endofinek, MOR eta DOR-ekiko antzeko afinitatea erakusten dute eta dinorfinak KOR-i lotzen zaizkio. Peptido opioideen inaktibazioa, peptidasa izeneko entzimek andeakuntza metabolikoaren bitartez burutzen dute, hau da biologikoki aktiboak diren peptidoak apurtuz eta era horretan zatiki inaktiboak sortuz funtzionatzen dute (Iversen 1978). Zehazki, N aminopeptidasa eta 24:11 endopeptidasa dira peptido opioideak *in-vivo* degradatzen dituzten enzimak (Subiran eta lank. 2011).

Aurretik aipatu dugun bezala, hartzaile opioideak G proteinetara loturiko hartzaileen familiaren parte dira eta zenbait erantzun zelular induzitzen dituzte G proteinen inhibitzaileen Gαi/Gαo azpiunitateen ekintzaren bitartez (Koch eta Hollt, 2008). Horien artean, behin hartzailea aktibatzen denean, adibidez neuronetan adenilato ziklasaren inhibizioa gertatzen da cAMP-aren mailak murriztuz eta PKA-ren aktibazioa inhibituz. Ondorioz zelula barneko eta nukleoko proteina askoren fosforilazio-egoeran eragiten du eta transkripzio prozesu askoren aldaketak gerta daitezke, hauek opiazeoekiko tolerantziaren edota abstinentzia sindromearen prozesuen eragile bihurtuz (Lorenzo eta lank. 1999). Horrez gain, Gαi azpiunitateak boltai-menpeko kaltzio kanaleak inaktibatu eta potasio kanaleak aktibatzen ditu (Law eta lank. 2000). Bestalde opioideek, opioide hartzaileen fosforilazioa sortu ondoren MAPK bidezko seinaleztapen bidezidorraren aktibazioa sustatzen dute, ERK, p38 edota JNK bezalako proteinen aktibazioa

bultzatzen dute (Al-Hasani eta Bruchas 2011; Law eta lank. 2000). ORL1-ean berriz, ERK-ren aktibazioa, PLC-ren aktibazioaren bitartez gertatzen da (Fukuda eta lank. 1997; Lour eta lank. 1998). Azkenik, Gβγ azpiunitateek Cβ fosfolipasaren aktibazioa bultzatzen dute eta inositol trifosfato bidezko kaltzioaren mobilizazioaren erregulazioaren bidez, proteina kinasa C aktibatzen dute (Chan eta lank. 1995; Tsu eta lank. 2002). Hala ere, ez dago argi zein den Gβγ azpiunitatearen inplikazioa nozizeptinak bideratutako PLCβ-ren aktibazioan (New and Wong, 2002).

Erantzun molekularra kontuan hartzen badugu, MOR eta DOR-en aktibazioa gertatzen denean, zelulen mintzaren hiperpolarizazioa eta jarduera bioelektriko neuronalaren inhibizioa eragiten da. Horren ondorioz, potasio kanalak irekitzen dira eta agonisten bidezko zelula barneko kaltzioaren askapena sustatzen da (Williams eta Clouet 1982). KOR aktibatzean ordea, tentsioaren menpeko kaltzio kanalen inaktibazioa gertatzen da, kaltzioa neurona barrura sartzea ekidinez eta neurotransmisoreen askapena inhibituz (Lorenzo eta lank. 1999; Surprenant eta lank. 1990). Honen guztiaren ondorioz, nerbio ehunean aktibitate bioelektrikoaren inhibizioa eta neurotransmisoreen askapenaren inhibizioa eragiten da.

1.5.2.2. Sistema opioidea eta Epigenetika

Azaldu dugun bezala, peptido opioideen funtzio ezagunenak nerbio sistema zentralean minaren ezabapenarekin edo analgesiarekin erlazionatu izan badira ere, gaur egun argi dago organismo osoan zehar funtzio fisiologiko desberdinak betetzen dituztela, beraien eragina opioide motaren eta aktibatutako hartzailearen araberakoa izanik (Fabbri eta lank. 1989). Adibidez, burmuinaren garapenean zein birsorkuntzan eta plastikotasunean (memoria eta ikaskuntza bezalako funtzioetan); funtzio sentsorialen erregulazioan; elikadura portaeran; depresioa edo antsietatea bezalako arazoen modulazioan; heste, giltzurrun eta gibel funtzioetan; odol presioaren eta erantzun kardiobaskularraren modulazioan; arnasketaren modulazioan (arnasketa urritasuna eta erantzun termoerregulatzailea eraginez); erantzun immuneen modulazioan; lokomozioaren eta aktibitate orokorraren erregulazioan; eta ugal funtzioaren erregulazioan (Bodnar and Klein, 2004). Bestalde, sistema opioidea, aldaketa epigenetikoen bidezko erregulazioaren menpe dagoela deskribatu da (Muñoa eta lank. 2015). Horren ondorioz, eta aurrekoari jarraituz, aipaturiko kontestu fisiologiko guztietan aldaketa epigenetikoek sortutako transkripzio eta transkripzio ondorengo erregulazioa ondo ulertzea beharrezkoa da gaur egun.

Honen harira, hartzaile opioideetan eta peptido opioideetan orain arte deskribatu diren aldaketa epigenetikoen bilaketa bibliografiko bat egin da tesian zehar, ingelesezko artikulu eran argitaratua izan dena *Journal of Cellular Biochemistry* aldizkarian.

The Epigenetic Regulation of the Opioid System: New Individualized Prompt Prevention and Treatment Strategies

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ABSTRACT

The most well-known physiological effect associated with opiod system is their efficacy in pain reduction or analgesia, although their effect on a variety of other physiological and physiophatological functions has become apparent in recent years. This review is an attempt to clarify in more detail the epigenetic regulation of opioid system to understand with more precision their transcriptional and posttranscriptional regulation in multiple physiological and pharmacological contexts. The opioid receptors show an epigenetic regulation and opioid peptide precursors by methylation, chromatin remodeling and microRNA. Although the opioid receptor promoters have similarity between them, they use different epigenetic regulation forms and they exhibit different pattern of expression during the cell differentiation. DNA methylation is also confirmed in opioid peptide precursors, being important for gene expression and tissue specificity. Understanding the epigenetic basis of those physiological and physiopathological processes is essential for the development of individualized prompt prevention and treatment strategies. J. Cell. Biochem. 116: 2419–2426, 2015.

KEY WORDS: EPIGENETIC; METHYLATION; CHROMATIN REMODELING; MIRNA; OPIOIDS; DRUG ADDICTION.

The opioid system is a biological communication system for which activity is mediated by the so-called endogenous opioid peptides (EOPs). Pharmacologically, it has been described three opioid receptors (ORs): mu opioid receptor (MOR), delta opioid receptor (DOR) and kappa opioid receptor (KOR), which belong to the 7-transmembrane, G protein-coupled receptors super-family [Law et al. 2004]. Later it was discovered the fourth OR, the nociceptin receptor (NOP). The comparison of the amino acid sequence and nucleotides has established that four genes are highly conserved in their homolog coding exons (73–100%), which are located in the middle of each gene. That exon codifies the 7-transmembrane domain, suggesting that four ORs come from the same ancestor gene. Also it has a single intron in the coding region. However, the four ORs diverge in their amino-end, which is produced out from the cellular surface, and also in the carboxyl-end which is extended in the intracellular space (9–20% of conservation) [Neer 1995]. Those slight differences explain specificity between the ligand union pattern, pharmacological effects and transduction signaling pathway of each OR [LaForge et al. 2000]. But, despite their conserved structure, it has been proved that each gene has a single regulatory pathway and shows a different expression pattern [Wei and Loh 2002]. These genes are Oprm1, Oprd1, Oprk1, and Oprl1. cDNA alignment studies with the genomic DNA and mRNA processing have established the existence of several isoforms or variants produced by each OR, by means of the use of alternative splicing, alternative

promoters (Oprm1, Oprk1), alternative polyadenylation sites (Oprk1) or inclusions in non-coding exons [Wei and Loh 2002]. The endogenous opioid peptides, endorphins, enkephalins, dynorphins and orphanin/nociceptins, are derived from precursors encoded by proopiomelanocortin (POMC), proenkephalin (PENK), prodynorphin (PDYN) and nociceptin/orphanin FQ (PNOC), respectively [LaForge et al. 2000; Levran et al. 2012]. However, biological approaches have demonstrated that in mammals, these peptides are grouped in three major types of opioid peptides: endorphins, enkephalins and dynorphins [Koneru A et al. 2009]. Recent studies have shown that there are more endogenous opioid peptides which do not belong to these three major types and which precursors are not yet known. These two endogenous opioid peptides are tetrapeptides, endomorphin-1 (TyrPro-Tr p-Phe-Nh2) and endomorphin-2 (Tyr-Pro-Phe-Phe-NH2) [Fichna eta lank., 2007]. Although their better-known function of the endogenous opioid peptides is to suppress pain (analgesia), currently other different physiological functions have been reported. The system is involved in brain development, regeneration and plasticity phenomena, taking part even in higher functions as memory and learning; sensory functions regulation; production of changes in eating behavior; modulation of mental illnesses such as anxiety or depression and mood; gastrointestinal, renal and hepatic functions; modulation of cardiovascular response and blood pressure; modulating the breathing, causing deficiencies in respiratory and thermoregulatory responses; modulating of immunological response, particularly immune-suppression; regulating the movement and general activity; and finally in the regulation of reproductive function [Bodnar, 2007; Subiran et al. 2011]. This review tries to clarify the epigenetic mechanisms of opioid system, focusing in more detail on methylation patterns, chromatin remodeling, and microRNA regulation, to understand with more precision their transcriptional and posttranscriptional regulation in multiple physiological and pharmacological contexts.

THE EPIGENETIC REGULATION OF OPIOID RECEPTORS

Four ORs are subjected to epigenetic regulation since they maintain several features, making them susceptible to be controlled by this machinery. 1) All of the genes are rich in CpG islands and hence, they can be highly methylated. 2) Their promoters show several types of modifications in different cellular stages or culture conditions. 3) Chromatin remodeling occurs in their promoters producing changes in their expression patterns in the differentiation process [LaForge et al. 2000].

MU OPIOID RECEPTOR (OPRM1)

MOR is encoded by the gene Oprm1, which has an important role in clinical effects of opiates, such as analgesia, tolerance development, and physical dependency under long drug treatment. MOR is one of the four ORs receiving further action by endogenous opioids, opiates and opioid analgesic drugs and also by exogenous opioid drugs as follows methadone, heroin and morphine [Kreek et al. 2002]. Certain numbers of splicing variants have been named: they are variants of MOR mRNA, which are modified in their 5’ UTRs and are differentially regulated at transduction level

[Song et al. 2009]. This receptor uses two closely positioned promoters, the distal promoter and the proximal promoter, which is responsible for most of the activity of the gene. Both of them belong to TATA-less type promoter, which is rich in CGs and has several regulatory elements. Lots of transcription factors have been examined as transcription regulators of the activity of Oprm1: Sox2 activators (Sry-like high-mobility-group box gene), Sp1 (specificity protein 1), iGA (Sp1 on an inverted GA motif), PCBP (poly C binding protein), and CREB (cyclic adenosine monophosphate response element binding protein). In contrast, the repressors are, Oct-1 (octamer-1), PU.1 (PU box binding on a 34-bp silencer region), PARP1 (polyADPribose polymerase 1 on a double-stranded poly-C sequence), Sp3 (two TFs binding to the 50 UTR specificity protein 3) and REST (repressor element-1 silencing transcription factor) [Hwang et al. 2009; Wei and Loh, 2011]. The Oprm1 promoter is heavily methylated in the CpG islands, in the undifferentiated embryonic carcinoma cells, thus the Oprm1 is silenced. The silenced Oprm1 can be activated in two ways, the first one by decreasing the expression of the methyl CpG binding protein 2 (MeCP2) and the second one by adding a histone acetylation inducer, that is the trichostatin A (TSA) [Lin et al. 2008]. In addition, DNA methylation on the Oprm1 can be reduced adding an artificial demethyilation agent such as the 50 -Aza-20 -deoxycytidine (5-Aza-C), but it is not clear which type of endogenous signal triggers the DNA demethylation on the promoter of undifferentiated cells. Other studies further provided that a higher-order chromatin conformational remodeling of the Oprm1 promoter occurs during neuronal differentiation in embryonic carcinoma cells [Hwang et al. 2010]. Together with the silencing of Oprm1 in pre-differentiated cultures, the promoter region is also organized into an ordered nucleosome. Whereas neuronal differentiation occurs by the retinoic acid (RA) induction, nucleosomes of the promoter region change their positions, and they start to recruit specific chromatin remodelers, which remodel the promoter and activate the gene. In that way, the activation or silencing of Oprm1 is correlated with histone modification, as in brain studies is observed, that to achieve Oprm1 activation, it is necessary the hyperacethylation of H3 and H4 [Song et al. 2009]. Recent studies have demonstrated that in term of non coding RNAs, there are some miRNAs linked with epigenetic regulation: miR-23b, miR-339, and Let-7 miR. On the one hand, miR-23b interacts with the 3’ UTR of Oprm1 through the K box motif (5’ -UGUGAU-3’), which is a conserved sequence. That interaction blocks the attachment between Oprm1 mRNA and the polysomes, hence, the transduction is arrested and MOR expression is canceled at protein level [Wu et al. 2013]. On the other hand, the binding of miR-339 with Oprm1 in the 3’ UTR, suppress the receptor expression which is recovered after the addition of miR-339 inhibitor or mutating the binding target [Hwang et al. 2012]. Finally, the last miRNA discovered in Oprm1 regulation is Let-7 miR, which also regulates opioid tolerance. Let-7 miR binds to its target in the Oprm1 3’ UTR and represses its expression, sequestering Oprm1 mRNA to P-bodies and finally leading to translational repression. Thus, binding between Oprm1 transcript and polysomes is decreased in a Let-7 dependent way [He et al. 2010]. (Table I)

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J. Cell. Biochem, 116(11):2419-26

TABLE I. The Epigenetic Mechanisms Involved in the Opioid Receptors Gene Expression

	OPRM1	OPRD1	OPRK1	OPRL1
SILENCING				
Regulatory elements	Oct1, PU.1, PARP1, Sp3, REST	Sp3, IK	IK	--
Metilation mechanisems	Metilation MeCp2	Metilation MeCP2	--	--
Histon modifications	--	Histone deacetilases H3, H4	H3K9me2	--
MiroRNA	miR-23b, miR-339, Let-7	--	--	--
ACTIVATION				
Regulatory elements	Sox2, Sp1, iGA, PCBP, CREB,	STAT6, Sp1, Ets-1, USF, NF-kB, AP2	c.Myc, Sp1, AP2	--
Metilation mechanisems	Hipometilation MeCp2	Hipometilation MeCp2	--	Hipometilation
Histon modifications	Histone acetilases H3, H4	Demetilation of H3K9me3 by NGF/PI3K signalling	H3K4me2	--
MiroRNA	--	--	--	--

DELTA OPIOID RECEPTOR (OPRD1)

DOR, which is encoded by the gene Oprd1, is involved in the modulation of addition, affective state, pain perception, and analgesia. It has a single promoter, TATA-less and is rich in CGs. Although some studies have shown that Oprd1 initiates its transcription in two adjacent sites, there is no study, which confirms that alternative splicing [Wang et al. 2003]. Studies of transcriptional regulation of DOR have focused on activating factors, such as, STAT6, Ik (Ikaros), Sp1/Sp3, Ets-1 (E-twenty-six 1), USF (upstream stimulatory factor), NF-kB, and AP2 [Wang et al. 2003; Wei and Loh, 2011].

In studies using embryonic carcinoma cells, the Oprd1 is constitutively active in undifferentiated cells, but it becomes inactive in the neuronal differentiation [Wang et al. 2003]. It has been shown a heavy DNA methylation on Oprd1 promoter in neural crest-derived cells, where it is not expressed and a demethylation in neuroblastoma cells, where Oprd1 gene is highly expressed, displaying an inverse correlation between both cell types. In addition, 5-Aza-C treatment on the culture generates an increased Oprd1 expression in neural crest-derived cells [Wang et al. 2005].

Through different studies, scientists have been trying to establish a connection between promoters methylation state and chromatin modifications, concluding that, this connection is important to regulate Oprd1 expression. In lack of methylation, Oprd1 promoter in neuroblastoma cells maintains its accessibility if comparing with partially methylated Oprd1 promoter in neural crest-derived cells [Wang et al. 2005]. It suggests that the silencing induced by the methylation generates a modification in the chromatin structure limiting the accessibility to the promoter

during the transcription. For example, Oprd1 promoter methylation contributes to MeCP2. In consequence, the histone deacetylase can access to the chromatin, generate decreased levels of H3 and H4 acetylation in Oprd1 promoter region and change the chromatin structure. In contrast, when Oprd1 promoter is completely methylated, it is correlated with low acetylation level in H3 [Wang et al. 2005]. Furthermore, other studies show the signaling role of fosfatidilinositol 3-kinase (PI3K) in the regulation of H3K9 state during neuronal differentiation induced by the nerve growth factor (NGF). That is to say, NGF/PI3K signaling is involved in demethylase activity of H3K9me3 in rat adrenal pheochromocytoa cells changing from a chromatin repressive mark to a activating mark [Chen et al. 2008]. (Table I)

KAPPA OPIOID RECEPTOR (OPRK1)

KOR is codified by Oprk1, and has an important role in a wide range of physiological systems, such as, pain regulation, drug abuse addiction, neuroendocrine regulation, cardiovascular functions, breathing, temperature regulation, nourishment behavior, and ability in stress response [Bruchas et al. 2010; Knoll and Carlezon 2010]. This OR also modulates the effect of opiates, cocaine and other drugs, through the modulation of the basal level and dopaminergic tone induced by drugs [Knoll and Carlezon 2010]. Several alternative splicing variants have been named. Some of them are disturbed in the 5'UTR of Oprk1 mRNA and they are differentially regulated at transduction level. Others are disrupted in the 3'UTR and they are differentially regulated at stability level or RNA transport [Hu et al. 2002]. In different studies, at least six mRNA variants have been validated and they are generated from the same gene but by the use of two alternative promoters (P1 and P2, TATA-less and rich in GCs) and two alternative polyadenylation sites. Besides the inclusion of one non-coding exon is described (upstream), where the alternative splicing occurs to produce different 5'UTRs [Lu et al. 1997]. The transcription factors, which regulate this gene, include three positives, the proto-oncogene c-Myc, Sp1 and activating protein (AP2) and one negative, Ikaros (IK) [Wei and Loh 2011].

Oprk1 is constitutively active and highly expressed in embryonic carcinoma cells in proliferative state, and once the cells complete that differentiation induced by RA, Oprk1 expression decreases. Because there is no evidence on charges in methylation pattern, it has been suggested that the acquisition of chromatin repressive marks are the main epigenetic regulators of Oprk1 expression [Chen et al. 1999; Bi et al. 2001]. In proliferative embryonic carcinoma cells that are in differentiation, the P1 promoter of Oprk1 has a totally accessible chromatin conformation, while, the promoter P2 shows a totally inaccessible chromatin [Lu et al. 1997]. The transcription factors, such as, Sp1 can bind promoter P1 and activate transcription. However, after embryonic carcinoma cell differentiation, the chromatin structure gets ordered and organized, and in that way Sp1 loses the possibility to bind [Park et al. 2005]. Studies using the embryonic carcinoma cells have revealed a biphasic pattern, which shows that Oprk1 expression is stated again later in differentiated cell populations. This reactivation is explained through the action of transcription factor NGF, because differentiated cells start to express NGF-binding receptors. The NGF binding transmits signals to activate AP2 transcription factor to bind this latter to P2

promoter, inducing changes in the Oprk1 promoter epigenetic marks from silenced H3-K9-me2 to activated H3-K4-me2 [Park et al. 2008]. (Table I)

NOCICEPTIN RECEPTOR (OPRL1)

NOP, which is encoded by opioid receptor like 1 gene (Oprl1), is a G protein coupled receptor with high homology to opioid receptors Oprm1, Oprd1, and Oprk1 [Mollereau et al. 1994; Wick et al. 1995]. It shares many structural traits with other OR genes, especially in terms of primary structure (60% homology), yet its pharmacological profile in not opioid. Anatomic data reveal that the Oprl1 receptor is widely expressed in the brain, spinal cord, and peripheral nervous system, and is found in areas involved in various processes: pain and sensory perception, memory, stress, motricity and hormonal regulation [Mollereau et al. 1994]. Oprl1 mRNA is expressed as two alternatively spliced forms, which differ only in their 5'UTRs [Wick et al. 1995]. It consists of five exons with a TATA-box in its 5' flanking region, and the protein coding region starts in exon 2 and ends in exon 4.

Regarding epigenetic regulation, it is the less studied receptor. A recent study on environmentally regulated gene expression shows, that Oprl1 gene together with other candidate genes is epigenetically regulated by the methylation of CpG islands [Zhang et al. 2013a]. It reports a hypermethyation in the CpGs of the Oprl1 gene, and an elevated overall methylation levels in the promoter region too, in a changing environment exposure, leading to changes in gene transcription and an increased risk for other disorders [Zhang et al. 2013a].

Others have worked with different epigenetic modifications, as histone methylation, highlighting that the reduction of mRNA levels of the Oprl1 gene are supported by the decrease of H3K4me3 and the increase of H3K27me3, it is said, a decreased level of the activating marker and the increased level of the repressive mark [Caputi et al. 2014]. To the date, there is no evidence of research in miRNA regulation of this gene. (Table I)

THE EPIGENETIC REGULATION OF OPIOID PROTEIN PRECURSORS

The endogenous opioid peptides are derived from precursors encoded by POMC, PENK, PDYN, and PNOC [Tseung 1995]. The importance of the peptide processing enzymes is evident from studies examining the forms of the opioid peptides in different tissues, because each one has different bioactive properties [Tseung 1995].

PROOPIOMELANOCORTIN GENE (POMC)

The pro-opiomelanocortin (Pomc) gen, encodes a cDNA spanning 3 exons and 2 introns. The sequence covers four activating transcription factor binding sites (FoxO1, STAT3, Sp1, NF-kB) and one inhibiting transcription factor binding site (nGRE) [Plagemann et al. 2009]. Pomc is a precursor polypeptide, which is cleaved in a tissue specific fashion by prohormone convertases to yield a variety of bioactive peptides, including a-melanocortin stimulating hormone (a-MSH), b-lipotropin (b-LPH), b-endorphin and adrenocorticotropin (ACTH). These two latter, are the principal components of the hypothalamic-pituitary-adrenal axis. b-endorphin and met-enkephalin are created from b-LPH and they both are the most powerful opioids. These peptides play diverse roles in pathophysiology, including obesity, depression, skin pigmentation, adrenal

development and regulation of the HPA axis. In other tissues, including the hypothalamus, placenta and epithelium, all potential cleavage sites may be used to produce peptides responsible for energy homeostasis, pain, perception, melanocyte stimulation and immune responses.

Pomc-derived peptides actively regulate drug-related behaviors [O'Malley et al. 2002; Levran et al. 2012]. It has been suggested that there is a variable tissue specific CpG island mehtylation, and that it has important implications for gene expression. Some studies revealed 2 CpG islands within the mouse Pomc gene locus [Gardiner-Garden and Frommer 1994]. The called CpG island 1, flanking the Pomc transcription start site, which is highly tissue-restricted, and the CpG island 2, approximately 5 kb downstream, comprising the third exon of the Pomc gene, which is weakly active in many tissues [Lavender et al. 1991]. In ACTH-secreting tumors and Pomc expressing cell lines, Pomc is unmethylated at the pituitary-specific promoter region. In contrast, in non-ACTH-secretion tumors, this region is heavily methylated. In addition, Pomc is heavily methylated at the same region in a number of normal ACTH-non-expressing tissues including: pancreas, spleen, lung, testes, and peripheral blood leukocytes [Lavender et al. 1991]. Moreover, studies in rodents have shown that Pomc DNA methylation can be altered by environmental conditions. For example, in a neonatal model of obesity, the hypothalamus revealed hypermethylation of CpG dinucleotides in the Pomc promoter, and stressor factors elevate Pomc mRNA levels in the pituitary [Wu et al. 2014]. The DNA methylation appears to be responsible for controlling Pomc gene expression by recruiting MeCP2 to silence the gene. MeCP2 is phosphorylated at serine 438 and generates dissociation from the Pomc promoter. As a result of that, the lack of MeCP2 prevent the binding of co-repressor complex such as histone deacetylase 2 (Hdac2) and DNA (cytosine-5)-methyltransferase 1 (Dnmt1) at the promoter losing the capacity to maintain of DNA methylation pattern during cell replication [Wu et al. 2014].

On the other hand, other epigenetic mechanism seems to increase H3K9 acetylation levels in late-gestation ewe fetal hypothalami, although it shows no change in the expression of Pomc mRNA [Stevens et al. 2010]. Some studies have been carried out on betaendorphin that reduces its production in POMC neurons, by decreasing levels of activation histone marks H3K4, aceH3K9, and pH3S10 and increasing the levels of the repressive histone mark H3K9 [Bekdash et al. 2013].

In the case of non coding RNA regulation, different studies have demonstrated that miR-375 dramatically inhibits Pomc expression both at the gene and protein levels by targeting mitogen activated protein kinase 2 (MAP2K8) and mediating the Corticotropin releasing factor signaling pathway in AtT-20 cells [Zhang et al. 2013b]. Finally, a recent study has highlighted, that Dicer-derived miRNAs are essential for survival and maintenance of Pomc expressing neurons during post-natal and early adulthood, suggesting that the deletion of Dicer controls Pomc gene expression [Schneeberger et al. 2012]. (Table II)

PROENKEPHALIN GENE (PENK)

PENK is a large precursor, which is processed through the action of proprotein convertase 1 (PC1) and proprotein

convertase 2 (PC2) to produce several peptide sequences: 4 met-enkephalin copies and 1 leu-enkephalin, met-enkephalin-Arg6-Phe7, met-enkephalin-Arg6-Gly2-Leu8, sin enkephalin copy, and C- or N- terminally extended variants of these peptides [Goumon et al. 2000]. The gene consists of four exons separated by three introns, and there are also identificated several repetitive DNA sequences within and flanking the gene. PENK-derived peptides act on MOR and DOR to produce rewarding actions of substances of abuse in different brain regions, including the ventral tegmental area and nucleus accumbens (NAc) [Levran et al. 2012]. They are also involved in analgesia, responses to stress and pain and regulation of appetite and sleep [Kieffer and Gaveriaux-Ruff 2002]. The structural organization of the Penk gene exhibits similarities to the organization of the Pomc gene suggesting that two opioid peptide precursors may have arisen by duplication from a common ancestral gene.

Data shows that Penk expression is specific for cell-type and tissue-compartment and that this expression can be regulated by environmental factors, such as, natural sunlight, salt water bathing, ultraviolet A irradiation and certain pathologies (psoriatic) [Nissen et al. 1999; Slominski et al. 2011]. Previous studies reveled that approximately 80–90% of the CpG dinucleotides occurring in the Penk gene are concentrated at the 5’ and 3’ ends, with a nonrandom distribution [McClelland and Ivarie 1982]. Furthermore, some CpG sites have been shown, by analysis of genomic DNA, to be methylated in a tissue specific fashion suggesting a control of the gene expression [Comb and Goodman 1990]. DNA methylation may also control the level of Penk expression since the methylation of CpG dinucleotides within the promoter inhibits its expression. In addition, that methylation affects a site located within a binding site for the AP-2 transcription factor. Thus, the methylation inhibits Penk expression by inhibition of AP-2 binding site [Comb and Goodman 1990].

The studies of histone methylation have demonstrated the regulation of Penk expression by repressive marks, such as the methylation of H3K9me2 in upstream regions of the gene, typically enriched at peri-centromeric heterochromatin and sites of repressed chromatin; and by activating marks such as the methylation of H3K4me3 across the Penk promoter [Tomasiewicz et al. 2012]. This suggests that the distinct epigenetic profiles during the different cellular periods may allow the Penk to respond differentially to similar environmental cues.

At the miRNA level, it has been suggested an interaction between Penk and miR-29c [Slominski et al. 2011], but there is too little information on this topic in current studies. (Table II)

PRODYNORFHIN GENE (PDYN)

PDYN is the precursor for the next opioid peptides 3 leu-enkephalin sequences, and other bigger peptides such as alpha- and betaneoendorphins, dynorphin A and dynorphin B, which act as endogenous ligands for the Oprk1 [Levran et al. 2012]. Dynorphin peptides reduce basal and drug-induced dopamine levels in different areas of the dopaminergic, nigrostriatal, and mesolimbic, mesocortical systems. Expression of Pdyn is increased by cocaine behavior in anxiety tests demonstrating the anxiogenic role of prodynorphin-derived peptides [Wittmann et al. 2009]. Pdyn contains four exons: exon 1 and 2 encode the 5’UTR, exon 3 encodes a signal peptide,

and exon 4 encodes the dynorphin peptides and has multiple transcription start sites located in exons 1 and 4 and introns 1 and 2 [Nikoshkov et al. 2005; Tejeda et al. 2012]. It has been identified some alternatively spliced Pdyn transcripts, which contribute to dynorphin/ Oprk1 system diversity [Kimura et al. 2006]. Several potential transcription factor binding sites within the Pdyn promoter have been reported to play a role in regulating Pdyn expression. A polymorphic 68-bp tandem repeat polymorphism (rs35286281) that contains a putative AP-1, a site at _156 in the proximal promoter; and _2745 microsatellite [Babbitt et al. 2010], and a calcium sensitive transcription repressor DREAM (downstream regulatory element antagonist modulator) that binds to the regulatory element (DRE) locate in the 5' UTR within exon 1 [Yuferov et al. 2011].

Epigenetic factors, mainly DNA methylation, play an important but still unknown role in modulation of Pdyn expression. It has been analyzed the DNA methylation patterns of three CpG-rich regions of the Pdyn, a CpG island, cluster A in the proximal promoter and cluster B in coding exon 4 [Yuferov et al. 2011]. Those results have suggested that the CpG island is implicated in tissue- or cell-specific regulation of gene expression, while the CpG cluster A may be associated to regulation of basal activity of the Pdyn [Yuferov et al. 2011]. In addition, the decrease in association of the methyl DNA binding protein MeCP2; to the promoter of Pdyn suggests the possibility that alterations in DNA methylation may be concomitant with altered Pdyn transcription [Reed et al. 2012].

To study the chromatin modifications of the Pdyn, human neuroblastoma SH-SY5Y cells model is used by several groups, which is endogenously expressing the opioid system genes. It is shown that there is a relationship between chromatin modifications and Pdyn expression, thus epigenetic changes may precede gene transcription [D'Addario et al. 2011]. It has been demonstrated that there is an increase in H3K27me3 and a decrease in H3K4me3 and H3K9Ac in promoter when the gene expression is repressed [D'Addario et al. 2011]. In contrast, there is an increase in H3K4me3 and H3K9Ac together with a return on unmethylated H3K27me3 when the Pdyn expression back to normal levels after the original repression [D'Addario et al. 2013]. This hypothesis is also supported in the same study by temporal changes in RNA polymerase II (RNAPII) recruitment and activation consistent with epigenetic changes [D'Addario et al. 2013].

On the other hand, a gene encoding a long non-coding RNA, AK090681, is transcribed from the opposite strand of Pdyn, and both are separate but overlapping transcription units. Some studies show that this gene appears to be actively transcribed in human embryonic stem cells while Pdyn does not. Interestingly, the promoter of AK090681 contains a CpG island which methylation status may correlate with that of H3K27, suggesting also an epigenetic regulation[Tejeda et al. 2012]. (Table II)

PRONOCICEPTIN GENE (PNOC)

PNOC is a precursor for 3 peptides, 1 nociceptin/orphanin FQ copy, 1 nocistatin copy and one prepronociceptin copy and it is the endogenous agonist of the NOP, another opioid receptor type in terms of primary structure, but without an *in-vitro* opioid pharmacological profile [Mollereau et al. 1996]. The overall structure and organization of the Pnoc codifying gen is highly homolog of those codifying Pomc, Pdyn, and Penk, it suggest a common

evolutionary ancestor. Consists in four exons, exon 1 constitutes the majority of the 5'UTR, exon two contains the translational start site and the signal peptide, exon 3 contains the coding region for the multiple bioactive peptides and exon 4 encodes the 3'UTR and poliadenylation signal. The promoter region upstream of exon 1 contains several regulatory sites including cAMP response elements and glucocorticoid receptor binding sites [Xie et al. 1999; Arjomand and Evans 2001], while other study shows the presence of NFkB-binding site too [Wiggins et al. 2010]. Some reports show a pattern of splicing variants: in the 3'-end of exon 3, which result in elongated and conserved C-terminal of the precursor protein, and other 2 spliced variants in the exon 2 [Arjomand and Evans 2001]. This gene is a modulator of pain sensation and it is essentially expressed in nerve tissues, brain and spinal cord, where its distribution correlates with the localization of Orl1 transcripts [Nothacker et al. 1996].

There are few results about epigenetic regulation in Pnoc gene. A very recent study has demonstrated a consistent increase in DNA methylation in the Pnoc promoter, suggesting that epigenetic regulation might affect its expression level upon environmental changes such as diet manipulation [Di Bonaventura et al. 2013]. Selective epigenetic changes in histone modifications, similar to Pdyn, are reported for the Pnoc gene. It seems to be an inverse relationship between the repressive mark H3K27me3 and the activating mark H3K9Ac, in the Pnoc promoter [D'Addario et al. 2013]. This fact has suggested that there is a long-term maintenance of epigenetic chromatin state, which determines accessibility for transcription factors, and that might be still present even when the histone modifications are decreasing [D'Addario et al. 2013]. It confirms that, at least for the Pnoc gene, there is a transient genomic memory triggered by histone modifications occurring immediately at exposure.

There is no evidence of epigenetic control at miRNA level in this gene. Therefore, this is a field to focus future studies. (Table II)

TABLE II. The Epigenetic Mechanisems Involved in the Opioid Protein Precursors Gene Expression

	POMC	PENK	PDYN	PNOC
Silencing				
Regulatory elements	nGRE	--	DREAM	---
Metilation mechanisems	Metilation MeCp2	Metilation MeCp2	Metilation MeCp2	Metilation
Histon modifications	--	H3K9 me2	H3K27me3	H3K27me3
MiroRNA	miR-375	miR-29c	--	--
Activation				
Regulatory elements	FoxO1, STAT3 Sp1, NF-kB	AP2, Sp1, CREB	AP1	CREB, NF-kB
Metilation mechanisems	Hipometilation	--	H3K4me3, H3K9ac	H3K9ac
Histon modifications	H3K9ac	H3K4 me3	--	--
MiroRNA	--	--	--	--

CONCLUSION

Different physiological functions have been reported for the opioid system. Discovering and validating the complete biological roles of these three genes will require extensive studies of each in multiple physiological and pharmacological contexts and, in particular, studies that define with more precision their transcriptional and posttranscriptional regulation. In this context, the opioid receptors show an epigenetic regulation. DNA methylation is confirmed in the case of Oprm1, Oprd1, and Oprl1, while the chromatin remodeling is reported in four of the receptors. Although four promoters have similarity between them, they use different epigenetic regulation forms and they exhibit different pattern of expression during the cell differentiation. Moreover, the opioid peptide precursors are also epigenetically regulated. DNA methylation is confirmed in four of them, being important for gene expression and tissue specificity. Histone methylation is also present in four precursors, which suggest the possible genomic memory acquisition at least in Pnoc.

In spite of that, these genes interact with environment factors that are increasingly recognized to be complicated by intertwined biological processes, including temporal and spatial controls that must underlie the activity of any drug receptor in the whole organism. Consequently, future studies of opioid system genes must go beyond the action of transcription factors to include factors affecting their epigenetic states, physiological contexts, and posttranscriptional control to develop individualized prompt prevention and treatment strategies.

ACKNOWLEDGMENTS

We would like to thank to Basque Government and Merck Serono for supporting this work. Iraia Mu~noa and Itziar Urizar were supported by fellowship from Basque Government and UPV/EHU respectively. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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1.5.2.3. Morfinaren belaunaldien arteko eragin epigenetikoa

Kanpo faktore edo inguruneak geneen adierazpenaren erregulazioarekin uztartzen dituzten mekanismo epigenetikoak, menpekotasuna sortzen duten drogekin erlazionatuak izan dira hainbat ikerketatan. Adibidez, opioideen gehiegizko erabilerak DNA-ren metilazioan aldaketak sortzen dituela eman da aditzera (Chorbov VM eta lank. 2011; Oertel BG eta lank. 2012; Zhang H eta lank. 2012; Nielsen DA eta lank. 2009; Nielsen DA eta lank. 2010; Ebrahimi G eta lank. 2017).

Substantzia opioideen artean morfina aurkitzen dugu, sistema opioidearen parte dena. Morfina opioaren osagarri aktiboena da eta bere balio terapeutikoaz gain, hainbat albo ondorio ere deskribatu dira (Subiran eta lank. 2011). Aipatu beharra dago, azken aldian asko direla morfina eta menpekotasuna sortzen duten beste droga batzuek belaunaldietan zehar transmititzen diren aldaketa epigenetikoen sorrerarekin erlazionatu dituzten ikerketa lanak (Yohn NL eta lank. 2015). Zehazki morfinak analgesiaren tolerantziarekin erlazionatuta dago eta portaeraren aldaketan du eragina ondorengo belaunaldietan (Byrnes EM 2005, Hammoud SS eta lank. 2009; Gapp K eta lank. 2014; Byrnes JJ eta lank. 2011; Gilardi F eta lank. 2018). Gainera morfinarekin tratatutako arratoien ondorengo belaunaldietan, portaeran, dopaminaren seinaleztapen bidezidorrean, plastikotasun sinaptikoan eta egitura neuronaletan aldaketak nabarmendu dira (Yohn NL eta lank. 2015; Byrnes JJ eta lank. 2013; Cicero eta lank. 1991; Sarkaki eta lank. 2008; Vyssotski 2011). Aipatzekoa da, morfinarekin trataturiko emeen ondorengo belaunaldian sexu espezifikoak diren aldaketak hauteman direla emozioan eta baita morfinarekiko sentikortasunean. Alde batetik ondorengo belaunaldiko emeek antsietate moduko portaera erakutsi dute. Beste aldetik, ondorengo belaunaldiko arretan morfinarekiko analgesiaren garapena deskribatu da tratamentu motzetan eta analgesiarekiko tolerantziaren garapena tratamendu kronikoetan (Byrnes EM 2005; Hammoud SS eta lank. 2009; Gapp K eta lank. 2014; Byrnes JJ eta lank. 2011). Honen harira, estruktura neuronalean ere deskribatu izan dira belaunaldietan zeharrreko aldaketak F0 belaunaldian tratatutako arretatik F2 belaunaldiraino mantendu direnak (Byrnes JJ eta lank. 2013).

Orokorrean opioideen gehiegizko erabilera %43-60 bitartean oinordekotza genetikoaren bitartez azaldu daitekeen arren (Ho MK eta lank. 2010), belaunaldietan zehar eraldatutako fenotipoen transmisioa ezin da genetikaren bidez bakarrik adierazi. Hau guztia dela eta, argi dago morfinak bezalako opioide batek, belaunaldi batetik ondorongoetara transmititzen diren aldaketa sortzen dituela, baina horretarako gertatzen diren mekanismoei buruzko informazioa oso urria da gaur egun (Muñoa eta lank. 2015). Oraindik ez da aurkitu morfinaren tratamendu kronikoak eraginda, belaunadietan zehar mantentzen den epigenomaren aldaketarik.Hala ere, morfinaren eraginez belaunaldi bakarrean sortutako aldaketa epigenetikoak deskribatu dituzten zenbait lan argitaratu dira azken hamarkadan, nahiz eta oso urriak diren. Adibidez, nukleo *accumbens*-etan eta burmuineko beste hainbat eremutan histonen aldaketak nabaritu dira (Maze eta Nestler 2011; Sun eta lank. 2012), H3K9me2 marka errepresiboan besteak beste, zenbait geneen adierazpenaren igoeroarekin erlazionatua izan dena (Rice eta Allis 2001). Erretrotransposoien familiako LINE-1-ak, hainbatetan drogen menpekotasun prozesuan parte hartzen duela deskribatu da (Maze eta lank. 2011; Prak eta Kazazian 2000), eta histonen metilazio gutxitua adierazten dute nukleo *accumbens*-etan morfinaren tratamenduaren ostean (Sun eta lank. 2012). Histoenen azetilazioa ere kaltetua agertzen da morfinaren

tratamenduarekin, adibidez arratoietan H3K14 histonarenazetilazia handitu egiten da nukleo accumben-etan eta amigdala basolateralean (Sheng eta lank. 2011; Wang eta lank. 2014b). Bestalde, DNA-ren metilazio patroietan ere morfinak eragindako aldaketak deskribatu dira belaunaldi bakarrean (Nielsen eta lank. 2008; Chorbov eta lank. 2011; Doehring eta lank 2013; Trivedi eta lank. 2014). Aipatu beharra dago ncRNA-k ere funtzio garrantzitsua dutela drogen menpekotasunaren ondorioz sortutako gaixotasunen garapenean (Bali KK eta lank. 2014; Briana AB eta lank. 2014), eta batez ere opioideekin erlazionatu dira (Toyanna K eta lank. 2017; Zehng H eta lank. 2012). Bukatzeko, MOR, DOR eta KOR hartzailearen presentzia arren ugal aparatuan eta emeen obozitoetan deskribatu izan dira, ar eta emeen gametoak opioide endogeno eta exogenoen hartzaile direla adieraziz (Albrizio eta lank. 2006; Agirregoitia eta lank. 2012; Estomba & Muñoa-Hoyos eta lank. 2016). Beraz, gametoetan hartzaide opioideen presentziak, belaunaldietan zeharreko oinordekotza epigenetikoa gerta daitekeela iradokitzen du.

Guzti hau dela eta, morfinak sortutako eta denboran zehar mantendutako aldaketa epigenetikoen oinarrizko mekanismoen identifikazioa oso garrantzitsua da, zelulen arteko memoria epigenetikoa finkatzeko nahiz eta estimulua presente ez egon. Era horretan bakarrik izango da posilbe, belaunaldietan zehar transmititzen diren aldaketa epigenetikoen mekanismoa ulertu ahal izatea.

2. HIPOTESIA ETA HELBURUAK

HYPOTHESIS AND AIMS

2. HIPOTESIA ETA HELBURUAK

Inguruneak herentzian eragina duela, mendeetan zehar irudikatu den pentsamendu baten parte da. Guzti hau, Conrad Had Waddington-ek adierazi zuen lehenengo aldiz XX. mendearen hasieran, Joan-Baptist Lamarck-ek iradoki zuenari jarraituz (Burbank L 1906; Whitelaw E 2015). Azken urteetako ikerketek, gai honi buruzko eztabaida berriz ere zabaldu dute, izan ere, gero eta gehiago dira gizakion fenotipoa geneetako informazioaz bakarrik erregulatzen denaren ideia baztertzen dutenak eta inguruneak eragindako erregulazio mekanismoak existitzen direla diotenak. Hau da "epigenetikak" aztertzen duena, zoriz edo ingurune faktoreek eraginda (nutrizioa, estresa, toxikoen kontsumoa (Turner BM 2011; Jaenisch R and Bird A 2003)), geneen adierazpena kontrolatzeko martxan jartzen den mekanismoa, beti ere nukleotidoen sekuentzian inolako aldaketarik sortu gabe. Halaber, kontzeptu honek, aldaketa epigenetikoak heredagarriak izan daitezkeela hartzen du bere baitan, zelulen identitatea finkatuz (Holliday R et al. 2006). Hori guztia dela eta, aldaketa epigenetikoak egiazkoak kontsideratzeko, alde batetik transkripzio prozesuan eragina izan behar dute eta bestetik, ziklo zelularrean heredatuak izan behar dira, baita sortu dituen seinalea desagertzen denean ere. Ildo honetatik jarraituz, nahiz eta zehazki histonen eraldaketak transkripzio prozesuarekin maiz erlazionatu diren, kanpo faktoreen eraginez berauetan sortzen diren eta behin estimulua bukatuta denboran zehar mantentzen diren aldaketa epigenetikoen mekanismoa zein den, oraindik ere argitu gabe dago. Honetaz gain, landare eta animalia batzuetan, kanpo faktoreek oinordekotza epigenetikoa sortu dezaketela deskribatu da zenbait ikerketa lanetan (Heard E eta Martienssen RA 2014). Baina ugaztunen kasuan ez dago argi zein den aldaketa epigenetikoak finkatzeko gertatzen den prozesua, ezta belaunaldietan zehar oinordekotza epigenetikoa pizten duen mekanismoen funtzionamendua.

Bestalde, morfina opioaren konposatu aktiboena da. Bere balio terapeutikoa onartua dago minaren aringarri modura (Subiran N et al. 2011), naiz eta erabilerengatik hainbat albo ondorio kaltegarri deskribatu diren. Are gehiago, azken aldiko zenbait ikerketek erakutsi dutenez, morfinak eta baita mendekotasuna eragiten duten beste droga batzuek, belaunaldi batetik bestera igarotzen den oinordekotza genetikoa sortzen dute, drogen gehiegizko erabileraren desoreka ondorengoetara transmitituz (Yohn NL et al. 2015; Byrnes EM et al. 2005; Hammoud SS et al. 2009; Gapp K et al. 2014; Byrnes JJ et al. 2011; Byrnes JJ et al. 2013). Opioideen gehiegizko erabileraren eragin genetikoa %43 eta %60 bitartekoa den arren (Ho MK et al. 2010), genetikak ez ditu guztiz azaltzen, behin drogaren esposizioa bukatuta, ondorengoetan heredatzen diren aldaketa fenotipikoak. Hori dela eta, aldaketa epigenetikoek belaunaldiz belaunaldi transmititzen diren gertakari horien atzean ezkutatzen diren mekanismoetan parte hartze garrantzitsu bat izan dezaketela iradoki da.

Honenbestez, prozesu konplexu hau ulertzeko, lehenik eta behin zelulen zatiketa prozesuan mantentzen diren aldaketa epigenetikoak eta horretarako mekanismoak identifikatzea garrantzitsua litzateke. **Bada, ideia honi jarraituz, kanpo faktoreek sortutako aldaketa epigenetikoak aztertzea baliagarria litzateke memoria epigenetikoaren oinarriak finkatzeko**. Horrela, belaunaldiz belaunaldiko oinordekotza epigenetikoaren mekanismoak ulertu eta transmititu daitezkeen aldaketa epigenetikoekiko itu terapeutikoak garatzen laguntzeko.

Arestian aipatzen diren aurrekariak kontuan hartuta, doktore tesi honetan ondorengo helburuak lantzen dira:

1. HELBURUA: Morfinaren tratamendu kronikoak sortzen dituen aldaketa epigenetikoak identifikatzea. Zehazki, lehenengo atal honetan proposamen garrantzitsuena transkripzionalki eta histonen aldaketa mailan, morfinaren tratamenduarekiko sentikorrak diren geneak aurkitzea izan da

2. HELBURUA: Morfinaren tratamendu kronikoak sortzen duen memoria epigenetikoa aztertzea. Konkretuki, aurreko helburuan lortutako hautagaietan oinarrituz burutu zen bigarrengo atal hau, non transkripzio eta histonen aldaketa mailak ziklo zelularrean zehar mantentzen dituzten geneak identifikatu diren.

2. HYPOTHESIS AND AIMS

The notion that the environment influences heredity has figured prominently in evolutionary thinking for centuries, first by Conrad Waddington at the beginning of XX century, following what Joan-Baptist Lamarck postulated time before (Burbank L 1906; Whitelaw E 2015). At present, the old debate about potential environmental effects on heredity has been reopened. In fact, the idea that the human phenotype is regulated only by information from genes is changing dramatically because there are mechanisms that provide regulatory information to the genome. These mechanisms are known as "Epigenetics", changes that can arise sporadically or can be induced by environmental factors (such as nutrition, stressors or toxins (Turner BM 2011; Jaenish R and Bird A 2003)), which are capable to regulate gene expression, without altering its primary nucleotide sequence. However, this term also includes the expectation that these marks are inherited as they are strongly suspected to define cell identities (Holliday R et al. 2006). Thus, if epigenetic modifications are truly epigenetic marks, they should therefore be causative for transcription and be inherited throughout the cell cycle, although when the signal(s) that induced them has disappeared. In this sense, specific histone modifications have been widely correlated with transcription, however, it has not been clear so far the mechanism from where external factors can induce epigenetic changes that can be maintained over time in the absence of the stimulus. Furthermore, in plants and in some animals, transgenerational epigenetic inheritance induced by environmental factors is well documented and relatively common (Heard E and Martienssen RA 2014). Nevertheless, in mammals the process to establish the epigenetic changes and the underlying machinery, which switches the transgenerational epigenetic inheritance, is still in doubt.

Moreover, morphine is the most active component of opium. The therapeutic value of opioids for pain relief is well established (Subiran N et al. 2011), despite a considerable number of adverse side effects. Interestingly, some recent studies describe that not only morphine but also other addictive drugs induce a transgenerational inheritance that can explain the prevalence of drug abuse disorders on the offspring (Yohn NL et al. 2015; Byrnes EM et al. 2005; Hammoud SS et al. 2009; Gapp K et al. 2014; Byrnes JJ et al. 2011; Byrness JJ et al. 2013). Although genetic vulnerability to opiate abuse is between 43% and 60% (Ho MK et al. 2010), genetics does not completely explain the inheritance of altered phenotypes in several generations of offspring following the initial drug exposure. Thus, epigenetic modifications could provide a mechanism underlying these altered phenotypes across generations.

Therefore, to understand this complex process, first of all, it could be of vital importance to identify epigenetic changes and the mechanisms, which are maintained during cell division cycle. **Following this idea, to study epigenetic changes induced from exposure to external factors could be helpful to establish the bases of epigenetic cellular memory.** Hence, we could completely understand the mechanisms of epigenetic transgenerational inheritance and this information could be useful to develop new therapeutic targets for those inherited phenotypes, traits or diseases.

Taking into account the background of the research, the aims of this doctoral thesis are the following:

1. AIM: To identify the epigenetic changes induced by chronic morphine treatment. Specifically, the main purpose in this section was to identify target genes, which were sensitive to morphine treatment at transcriptional and histone modification level.

2. AIM: To study the epigenetic memory generated by morphine chronic treatment, once the stimulus was absent. Explicitly, the results were based following the candidate s obtained on the previous aim, and identifying those whose transcription changes and histone modifications were maintained through cell cycle.

3. MATERIAL ETA METODOAK
MATHERIALS AND METHODS

3. MATERIAL ETA METODOAK

Atal honetan tesian zehar erabilitako material, lagin zein teknika desberdinak deskribatzen dira. Aipatzekoa da emaitzen atalean, egindako esperimentuen deskribapen labur bat eman eta irudiak gehitzen direla ulermena errazteko.

3.1. MATERIALAK

3.1. Taula. Teknika desberdinak burutzeko erabili diren gailuen zerrenda.

Gailua	Etxe Komertziala
Zelulen hazkuntzarako CO2-dun inkubagailua	MCO-15AC, Sanyo
Fluxu laminar bertikaleko Kanpaia	F72600350, Faster Bio 48
Errotore baskulantedun zentrifuga	Heraeus MegaFuge 40R, Thermo
Mikroskopio alderantzikatua	Axioscope EclipseTS100, Nikon
Mikroskopio fase-kontrasteduna	NiconEclipse 50i-H550S, Nikon
Mikroskopioari atxikitako kamera	D07739, Jenoptik
Lupa Mikroskopioa	Olympus Sz51
Ur-bainu termostatikoa	TBN-06/100, ICT SL
Nitrogeno likidorako tankea	GT-40, Air Liquide
Mr Frosty edukiontzi izoztzailea	Thermo Fisher Scientific
ARE Heating Magnetic Stirrer	VELP Sicentifica
Timer Clock ordularia	LabBox
Balantza	Mettler AE200
Heraeus Fresco 21 mikrozentrifuga	Thermo Fisher Scientific
Zelula homogenizatzailea	D-1 30170, MICCRA
Heidolph GIRALT homogeneizagailua	Heidolph
FLUOstar OPTIMA fluorimetroa	BMG Labtech
Elektroforesi eta transferentzia euskarriak	Bio-Rad
PowerPac Basic Power Supply Energia hornidura	Bio-Rad
Hotcold S Inkubagailu lehorra	Selecta
Duomac 1030 balantzina	Heidolph
Mini-PROTEAN Tetra Vertical Electrophoresis	BioRad
Criteion Blotter	BioRad
Chemidoc XRS sistema	Bio-Rad
NanoDrop ND-1000 espektrofotometroa	Thermo Fisher Scientific
Termozikladoreak Mycycler	Life technologies, BioRad
PerkinElmer CFX96 Real Time detekzio sistema	BioRad
Plaka zentrifuga	Sorvall RT 7
Bortize nahasgailua	$2x^3$ VELP Scientifica
AccuBlock Digital Dry Bath termoblokea	Labnet international Inc.
Labnet Mini Labroller-a	Labnet International
Thermomix irabiagailua	Eppendorf
Magnetic Rack imana	NVIGEN
Soniprep 150 Sonikatzailea	Soniprep&Process Timer MSE

3.2. Taula. Erabilitako erreaktiboen zerrenda.

Erreaktiboak	Erreferentzia	Etxe komertziala
PBS 10X	BE17-517Q	Lonza
TrypLE Express Enzyme (1x)	12605028	ThermoFisher
Txerriaren azaleko gelatina	G189	Sigma-Aldrich
Knock Out DMEM hazkuntza medioa	10829-1018	Gibco
KSR	10828-028	Gibco
Sodio pirubatoa	S8636	Sigma-Aldrich
Aminoazido ez esentzialak (NEAA)	M7145	Sigma-Aldrich
Penizilina/estreptomizina antibiotikoa	P4333	Sigma-Aldrich
L-Glutamina	G7513	Sigma-Aldrich
B-Mercaptoetanol-a	146250	Sigma-Aldrich
GKF_3 inhibitzailea	BX33140	StemCell
MEK ½ inhibitzailea	04-0006	Stemgent
LIF inhibitzailea	ESG1107	Sigma-Aldrich
Morfina hidrokloruroa		Alcaliber S.L
DMSO	SU01502500	Scharlau
DMEM/F12 hazkuntza medioa	H3BE12-741F	Lonza
N_2	17502-048	Gibco
Insulina	16634-50MG	Sigma-Aldrich
Apotranferrina	T1428	Sigama-Aldrich
Progesterona	P0130	Sigma-Aldrich
Putreszina	51799	Sigma-Aldrich
Sodium selenite	S5261	Sigma-Aldrich
Neurobasal hazkuntza medioa	21103-049	Invitrogen
B27	17504-044	Gibco
Activin A	120-14E	Preprotech
bFGF	13256-029	Invitrogen
Fibronectina	FC010	Millipore
Bmp4	5020-BP	R&D Systems
Bmp8	7540-BP	R&D Systems
SCF	455-MC	R&D Systems
EGF	2028-EG	R&D Systems
PMSG hormona	G4527	Sigma-Aldrich
hCG hormona	C8554	Sigma-Aldrich
Hialuronidasa	H1136	Sigma-Aldrich
EmbryoMax KSOM hazkuntza medioa	MR-020P-5F	Merck-Millipore
Parafinazko olioa	18512-1L	Sigma-Aldrich
Hepes	H3375	Sigma-Aldrich
KCl	P9541	Sigma-Aldrich
EGTA	E4378-25	Sigma-Aldrich
EDTA	1084180250	Merck
Sukrosa	131621.1211	Panreac
TritonX-100	X100	Sigma-Aldrich
Glizerola	G7757	Sigma-Aldrich
DTT	DTT-RO	Sigma-Aldrich
Azetona	BPA181	Fisher Chemicals
cOmplete, Mini, EDTA-free Protease inhibitor Cocktail	04693159001	Roche
PhosSTOP	04906845001	Roche
Pierce BCA Protein Assay Kit	23225	Thermo Scientific
Precision Plus Protein Dual Color Standars	1610374	BioRad
30% Acrilamida/Bis Soluzioa 29:1	1610156	BioRad
N,N,N',N'-Tetrametiletileneadiamina TEMED	T9281	Sigma-Aldrich
Amonio persulfatoa (PSA)	A3678	Sigma-Aldrich

Behitik eratorritako serumeko albumina (BSA)	A7906	Sigma-Aldrich
Trizma Base	T1503	Sigma-Aldrich
SDS	142363.1209	PanReac
Bromofenol urdinaren sodio gatza	B5525	Sigma-Aldrich
Glizina	G8898	Sigma-Aldrich
MetOH	141091.1214	PanReac
NaCl	31414	Sigma-Aldrich
Luminola	A8511	Sigma-Aldrich
Azido p-kumarikoa	C9008	Sigma-Aldrich
H_2O_2	H1009	Sigma-Aldrich
TRizol erreaktiboa	15596026	ThermoFisher Scientific
Kloroformoa $CHCl_3$	CL01981000	Scharlau
Biologia Molekularreko Isopropanola	A3928	Panreac AppliChem
Analisirako etanol absolutua	121086	Panreac AppliChem
DEPC-rekin trataturiko ur ultrapurua	750023	ThermoFisher Scientific
Midori Green	MG40	Nippon Genetics Europe GmbH
EZ Load 100 PCR Molecular Ruler	170-8353	BioRad
Agarosa	118282B	BioRad
iScript cDNA sintetizatzeko Kita	1708891	BioRad
iTaq Universal SYBR Green Supermix	1725121	BioRad
Formaldehido	141328.1211	Panreac
LiCl	L9650-100G	Sigma Aldrich
NP40	492916	Calbiochem
Sodio deoxikolatoa	D6750	Sigma-Aldrich
Sodio azetatoa	W302406	Sigma-Aldrich
Sodio fosfato monobasikoa	S8282	Sigma-Aldrich
Sodio bikarbonatoa	S5761	Sigma-Aldrich
Azido azetiko glaziala	211008.1211	Panreac
RNAse	EN0531	ThermoFisher
Proteinase K	25530049	ThermoFisher
Ampure XP bola magnetikoak	A63880	Bechman Coulter
DynaBeads bola magnetikoak	10003D	Invitrogen

3.3. Taula. Laborategian prestatu diren soluzioen zerrenda.

Soluzioa	Osagaiak
Kargatzeko tanpoia 4X	50ml: 250mM Tris-HCl pH 7.5, 40% glizerola, 4% SDS, %0.025 Bromofenol urdina p/b, %5 DTT p/B.
"A Indargetzailea"	100 mM Hepes pH 7.8, 150 mM KCl, 10 mM EGTA, 10 mM EDTA, 3.2M Sukrosa, %3 Triton X-100, 1 M DTT.
"Gatz baxuko Indargetzailea"	100 mM Hepes pH 7.8, 150 mM KCl, 10 mM EGTA, 10 mM EDTA, 80% Glizerola, 1M DTT.
"Gatz altuko Indargetzailea"	100 mM Hepes pH 7.8, 4 M KCl, 10 mM EGTA, 10 mM EDTA, %80 Glizerola, 1 M DTT.
Tris-HCl 1M pH 8.8	1L: 121.14 g Tris-Base, HCl pH 8.8-an kokatzeko.
Tris-HCL 0.5M ph 6.8	1L: 62.07 g Tris-Base, HCl pH 6.8-an kokatzeko.
Sodio dodezil sulfatoa (SDS) %10	10 g SDS 100 ml H_2O-tan.
Persulfato amonikoa (PSA)	1 g persulfato amoniko 10 ml H_2O-tan
Proteina Elektroforesi indargetzailea - 10X	250 mM Tris-Base pH 8.3, %1 SDS, 2M Glizina.
Transferentzia Indargetzailea - 10X pH 8.3	250 mM Tris-Base, %0.25 SDS, 1.92M Glizina. *Diluitzean, %20 metanol gehitu behar zaio.
Blokeo soluzioa	Blotto, %5 BSA.
Garbiketa Indargetzailea-a (Blotto)	20 mM Tris, 150 mM NaCl, %0.1 Triton X-100.
Kimioluminiszentzia areagotzailea (ECL)	1.25 mM luminol, 0.2 mM azido p-kumarikoa, 0.1 M Tris-HCl Ph 8.5.

	*Erabiltze-egunean, nahasturara %0.3 H_2O_2 (%30) gehitu behar zaio.
RNA/DNA TAE Elektrofores indargetzailea -10X	1L: 242 gr Tris-Base, 57.1 ml azido glaziala, 0.5 M EDTA.
"A Kromatina Garbiketa Indargetzailea"	10 mM Hepes pH 8, 10 mM EDTA, 0.5 mM EGTA, 200 mM NaCl, %0.25 Triton X-100.
"B Kromatina Garbiketa Indargetzailea"	10 mM Hepes pH 8, 1 mM EDTA, 0.5 mM EGTA, 200 mM NaCl, %0.01 Triton X-100.
"Sonikazio Indargetzailea"	%1 Triton X-100, 25mM Tris pH 8, 2 mM EDTA, 150 mM NaCl.
"Kromatina Disolbatzeko Indargetzailea"	Sonikazio Indargetzailea + %7.5 Glizerola.
Sodio Azetato 3M pH 7.0	1L: 246 g Sodio azetato anhidridoa, Azido azetiko glaziala pH 7.0-an kokatzeko.
"I IP Indargetzailea"	20 mM Tris-HCl pH 8, 2 mM EDTA, 150 mM NaCl, %0.1 SDS, %1 Triton X-100.
"II IP Indargetzailea"	20 mM Tris-HCl pH 8, 2 mM EDTA, 500 mM NaCl, %0.1 SDS, %1 Triton X-100.
"III IP Buffer Indargetzailea"	10 mM Tris-HCl pH 8, 1 mM EDTA, 0.25 M LiCl, %1 NP40, %1 Na Deoxikolatoa.
"TE/NaCl Indargetzailea"	10 mM Tris pH 8, 1 mM EDTA, 50 mM NaCl.
Sodio Bikarbonatoa 0.1M pH 10.1	1L: 8,401 g Sodio bikarbonatoa.
Tris 2M pH 6.0	1L: 242.28 g Tris-Base, HCl pH 6-an kokatzeko.
"TE Indargetzailea"	1 mM Tris pH 8, 50 mM NaCl.

3.2. LAGINAK

Ikerketa proiektu honetan erabilitako laginak jarraian deskribatzen dira.

3.2.1 Zelulak

Ikerketa proiektu honen "*in-vitro*" garapenean sagu enbrioien barne masatik eratorritako zelula amen (mESC) lerro zelular komertziala erabili ziren (Mouse Oct4-GFP ES Cells, PCEMM08, PrimCells LLC.). Espezifikoki zelula lerro honek GFP proteina fluoreszentea adierazten du OCT4 genearen promotorearen menpe.

Zelulak hazteko oinarri bezala gelatina erabili zen, %0,1-eko kontzentrazioan hazkuntza plakari gehitua bere oinarri guztia estali arte eta 20 minutuz erreakzionatzen utzita. Ondoren, soberan zegoen gelatina deuseztu eta zelulak gehitu zitzaizkien. mESC-ak Knock Out Serum DMEM hazkuntza medioan kultibatu ziren, hainbat osagai gehituta: %15 KSR (ingelesezko *KnockOut Serum Replacement*, hazkuntzan zehar zelula amen pluripotentzia mantentzeko, behi umekiaren sueroaren edo FBS-aren ordezko osagaia), %1 Na Pyruvate, %1 ez funtsezko aminoazidoak, %1 penizilina-estreptomizina, %1 L-Glutamina eta %0.07 B-Mercaptoetanol-a, gehi hainbat hazkuntza faktore, 10 mM PD0325901, 30 mM CHIR99021 eta 1000 U/ml LIF (ingelesezko *leukaemia inhibitor factor*). Zelulak egunez egun hazten, 37ºC-tan eta %5-eko CO2-dun inkubagailuan mantendu ziren. Zelulak hazkuntzan mantentzeko P6 plaketatik, 75 edo 175 cm2-rainoko fraskoak erabili ziren, bi egunetik behin hazkuntza media aldatu zitzaien eta %80-90-eko konfluentziara heltzean, fraskoz aldatu ziren.

Zelulak altxatzeko, zelulei hazkuntza medioa kendu eta PBS-arekin garbitu ondoren, tripsina gehitu zitzaien eta giro tenperaturan (RT) inkubatu zen 2-3 minutuz. Tripsinaren efektua inaktibatzeko, hazkuntza medioa gehitu zitzaien plakei (erabilitako bolumenaren bikoitza), eta

nahasketa 15 ml-ko edukiera zuten falcon tubuetan jaso zen. Ondoren zelulak 1600 rpm-tara (biraketa minutuko) zentrifugatu ziren 3 minutuz, hodiaren beheko ertzean sedimentatuz. Azkenik, zelulen jalkina medio berrian birsuspenditzen zen, aurretik gelatinarekin trataturiko frasko berrian hazten jartzen zelarik.

3.2.1.1. Zelula lerroen tratamendu kronikoa aldaketa epigenetikoak aztertzeko

Gure sistema ereduan kanpo estimulu batek sortzen dituen aldaketa epigenetikoak aztertu ahal izateko morfina erabili zen. Horretarako, zelulak %50 konfluente zeuden momentuan, zelulei hazkuntza medio berria jarri zitzaien morfina gehituz 10 μM-eko kontzentrazioan eta 24 orduz. Kontrol zelulei morfina gehitu ordez %0.9 (p/v)-ko sodio kloruroa gehitu zitzaien, hau da, serum fisiologikoa. Behin tratamendua bukatuta, petri plaketan zelulen hazkuntza medioa kendu, PBS-arekin garbitu eta tripsinarekin jasotzen ziren. Jarraian zelulak eppdorf-etan bildu eta ondorengo esperimentuetarako erabiltzen ziren.

3.2.1.2. Zelula lerroen tratamendu kroniko memoria epigenetikoa aztertzeko

Gure sistema ereduan kanpo estimulu batek sortzen duen memoria epigenetikoa aztertu ahal izateko aurreko tratamendu berbera erabili zen morfinarekin. Hala ere, tratamendua hasi eta 24 ordutara, zelulen erdia soilik jaso zen, ondorengo esperimentuetan erabiltzeko (P1 taldea), eta beste erdia, berriz ere plakeatu egin zen, aurretik gelatinarekin trataturiko plaka berrietan, eta medio berri/garbia gehituz. Horrela bi egun gehiago mantendu ziren hazten, hau da, tratamendua bukatu eta 48 ordutara, zelula hauek tripsinarekin tratatu eta jaso egin ziren. Berriz ere, zelulen erdia gorde egin zen ondorengo esperimentuetan erabiltzeko (P2 taldea), eta beste erdia, berriz ere plakeatu zen, aurretik gelatinarekin trataturiko plaka berrietan, eta medio berri/garbia erabiliz. Kasu honetan ere beste bi egunez mantendu ziren zelulak hazten, hau da, tratamendua bukatu eta 96 ordutara, zelula hauek tripsinarekin tratatu eta jaso egiten ziren. Azken zelula hauek ere, ondorengo esperimentuetarako gorde ziren beraien osotasunean (P3 taldea).

3.2.1.3. Zelula lerroen desberdintzea jatorrizko hozi zelulak osatzeko

Kontrol eta Morfinazko tratamendua jasotako mESC-ak, jatorrizko hozi zeluletara desberdintzeko erabilitako prozesua, 2011.urtean Katsuhiko Hayashi eta lankideek Cell aldizkarian argitaratu zuten protokoloa jarraituz egin zen. Laburki, mESC-k hainbat hazkuntza faktoreren eraginez, bFGF (ingelesezko *basic fibroblast grow factor*) eta Aktibin A, epiblasto antzeko zeluletan (EpiLC-s) desberdintzen dira. EpiLC-ek aldiz jatorrizko hozi zelulen antzeko zelulak (PGCLC) *in-vitro* osatzeko gaitasuna dute, Bmp 4, Bmp 8, SCF, EGF eta LIF (ingelesezko *bone morphogenetic protein 4/8, stem cell factor* eta *epidermal grow factor*) gehituz, garapenaren prozesuko laugarren egunaren ostean. Prozesuaren amaieran lortu ziren PGCLC zelulak *in-vivo* sortzen diren jatorrizko hozi zelulen (PGC) parekoak dira, geneen adierazpen, egoera epigenetiko eta espermatozoide ugalkorrean desberdintzeko ahalmenean, beraz, *in-vitro* lan egiteko eredu egokia dira. mESC-tatik EpilCL-tara desberdintzeko, plaken oinarria giza plasmatik eratorritako fibronektinarekin estali ziren eta mESC-ak N2B27 hazkuntza medioan kultibatu ziren, hainbat osagai gehituta: %1 KSR. gehi aurretik aipaturiko hazkuntza faktoreak bFGF (12ng/ml) eta Activin A (20 ng/ml). Zelulak egunez egun hazten, 37ºC-tan eta %5-eko Co2-dun inkubagailuan mantendu ziren eta hazkuntza medioa egunero aldatu zen. mESC-tik EpiLC-rako desberdintze prozesuak 2-3 egun iraun zituen eta jarraian bigarren desberdintzea martxan

jarri zen EpiLC-tik PGCLC-ra. Horretarako zelulak flotazio baldintzapean hazi ziren eta erabilitako hazkuntza medioari hainbat osagai gehitu zitzaizkion: %15 KSR, 0.1 mM ez funtsezko aminoazidoak, 1 mM sodio pirubatoa, 0.1 mM B-Merkaptoetanola, 100U/ml penizilina, 0.1 mg/ml streptomizina, 2 mM L-glutamina, gehi aurretik aipaturiko hazkuntza faktoreak, 500 ng/ml Bmp4, 500 ng/ml Bmp8, 100 ng/ml SCF, 50 ng/ml EGF eta 1000U/ml LIF. EpiLC-tik PGCLC-rako desberdintze prozesuak beste 3 egun inguru iraun zituen.

3.2.2. Animaliak

Ikerketa proiektu honen "*in-vivo*" garapenean, Swiss Webster (SW) ereduko saguak (mus musculus espeziea) erabili ziren, Euskal Herriko Unibertsitateko SGIKER animaliategiak ekoiztutakoak.

Prozedura esperimental guztiak Europar Batasunak ikerketa eta animaliekin esperimentazioa egiteko ezarrita dituen arauen arabera gauzatu ziren (2010/63/EU). Gainera saguekin egindako prozesu guztiak Euskal Herriko Unibertsitateko animaliekin esperimentazioa egiteko etika batzordeak baimendu zituen (CEBA) (CEEA/339/2013/SUBIRAN CIUDAD eta M20/2016/142).

Erabilitako sagu guztiak, ar zein emeak, 2 hilabeteko adinarekin jaso ziren eta Euskal Herriko (UPV/EHU) animaliategian mantendu ziren bananduak, hamarnakako taldetan argi eta iluntasuneko 12 orduko zikloekin, janaria eta ura edozein momentutan eskuragarri zutelarik.

3.2.2.1. Tratamendu kronikoa

Saguekin lanean hasi aurreko astean egunean behin 10-15 minutuko tarte batez, saguekin kontaktua landu zen, hau da, ukitu, altxatu, eskuetan hartu… egiten ziren, era horretan ikerlariaren ekintzetara eta usainera ohitzeko. Honek saguen tratamendu prozesuan zehar, hauen estresa nabarmen gutxitu zuen.

Saguei egin zitzaien morfinazko tratamendu kronikoaren administrazioa, Crain eta Shen-ek 1995.urtean deskribatutako protokoloa jarraituz burutu zen. Animaliei, morfina hidrokloruroa larruazalpean injektatu zitzaien birritan, 12 orduko denbora-tarteetan (8:00 eta 20:00), 4 egunetan zehar eta egunetik egunera dosien zenbatekoa handituz (20, 30, 40 eta 50 mg/kg). Tratamenduaren 5.egunean saguek morfinazko injekzio bakarra jaso zuten larruazalpean (10 mg/kg) 8:00tan. Talde kontrolari, morfinazko injekzioak eman ordez, %0.9 (p/v)-ko sodio kloruroa gehitu zitzaien, hau da, serum fisiologikoa.

Morfinazko tratamenduaren efizientzia aztertzeko, "Plater Beroaren Analgesia Test-a" (ingelesezko *Hot Plate Analgesia Test*) gauzatu zen. Honen bestez, morfinazko azken dosiaren ostean, tratatu eta tratatu gabeko saguak, ar eta emeak, banan-banan, 60º-tan zegoen plaka bero baten gainean jarri ziren eta min-erreflexua neurtu zitzaien, salto egin arte zenbat denbora igarotzen zen kontuan hartuz (Ripoll N eta lank. 2006; Tzschentke T eta lank. 2007). Behin morfinazko tratamenduak eragina zuela egiaztatu ondoren, animaliak dislokazio zerbikalaren bitartez hil ziren eta sistema biologiko bakoitzeko organoak batu ziren, proteina ezberdinen (histonen modifikazioak) eta geneen adierazpen (histonen modifikazioen gene erregulatzaileak) mailak aztertu ahal izateko.

3.2.2.2. Sagu emeen hiperestimulazio obarikoa

Sagu emeetan hiperestimulazio obarikoa bi proposamenetarako erabili zen. Lehendabizikoa, morfinazko tratamenduarekin batera burutu zen, esperimentuan parte hartzen zuten eme guztien obulazioa sinkronizatu eta egoera hormonala kontrolatzeko asmoz. Bigarrengoa, emeek ernalduko zituzten enbrioien kopurua handitzeko eta era horretan saguen enbrioiak aztertuz burutu ziren esperimentuetan ahalik eta animalia gutxien sakrifikatu behar izateko (3 R-en printzipioa).

Kanpo hiperestimulazio obariko hormonala burutzeko bi hormona injektatzen zaizkie sagu emeei, injekzio intraperitonealaren bitartez: lehenengoa, umedun behorraren gonadotropina suero edo PMSG (ingelesezko *Pregnant Mare Serum Gonadotropin*), hormona folikuloestimulatzaile edo FSH hormonaren analogoa dena eta beraz, hazte prozesuan dauden obozito guztiak errekrutatuko dituena; eta bigarrena giza gonadotropina korionikoa edo hCG (ingelesezko, *human Chorionic Gonadotropin*), hormona luteinizantea edo LH hormonaren analogoa dena eta beraz, obulazioa eragiten duena, xiringatu eta 14-15 ordutara. Sagu eme bakoitzak PMSG-aren 5IU eta hCG-aren 5IU-ko injekzioa jaso zituen 48h tartearekin. Hiperestimulazio obarikoa morfinazko tratamenduarekin batera burutu zen kasuetan PMSG-aren injekzioa morfinazko tratamenduaren hirugarren egunarekin bateratu zen, eta hCG-aren injekzioa morfinazko tratamenduaren azken egunarekin. Saguen enbrioien garapenaren azterketa burutzeko ordean tratamenduaren hasiera PMSG-aren injekzioarekin, enbrioien erauzketa baino 76 ordu inguru lehenago egin zen, hCG-aren injekzioa berriz, enbrioien erauzketa baino 25-27 ordu inguru lehenago burutu zen.

3.2.2.3. Ar eta emeen gurutzaketa

Enbrioien erauzketa burutu ahal izateko, sagu ar eta emeen gurutzaketa egin behar izan genuen. Aurretik esan bezala ahalik eta animalia gutxien sakrifikatu behar izateko, sagu emeek hiperestimulazio obarikoa jaso zuten PMSG eta hCG hormonen tratamenduaren bidez. Bibliografian deskribatua dagoen bezala, sagu emeak elkarrekin bilduta mantenduz gero denbora tarte batez, animaliategian dauden bezala, Lee-Boot efektua jasan dezakete (Van der Lee eta Boot-ek 1956), hau da gernuan askatzen duten feromona baten bitartez ziklo estralaren atzerapena jasan dezakete, anestroan sartuz eta azkenik desagerraraziz. Fenomeno hau zuzentzeko, aipaturiko Whitten efektua sortarazten da (W. K. Whitten 1956; W. K. Whitten 1957), sagu ar baten gernuaz bustitako zerrautsa gehitzen zaio emeen kaiolei, arren gernuan dagoen beste feromona baten bidez emeen ziklo estrala errekuperatu eta sinkronizatzeko. Horrela, gure kasuan lehenengo hormonaren injekzioaren ostean, Whitten efektua gauzatu zen sagu arren gernuaren bitartez, emeen ziklo estralaren sinkronizazioa lortzeko hormonen tratamenduaren bukaerara iritsi orduko.

Hiperestimulazio obarikoaren bigarren injekzioaren egun berean, egunaren azken orduan sagu ar bakoitzeko, bi eme gehitu ziren animaliategiko kaioletan eta bertan utzi ziren gabe guztian zehar sagu arrek emeak estali zitzaten (saguak gaueko animaliak direnez, orokorrean estalketa gauan zehar gertatu ohi da). Hurrengo eguneko lehenengo orduan, emeak estalduak izan zirela frogatzeko, tapoi baginala zutela aztertu zen eta berriz ere banandu ziren sagu arretatik. Tapoi baginalak estalketa adierazten du baina, ez du esan nahi ernalketa gertatu denik. Hala ere, saguek duten ugalketa-tasa handiagatik, orokorrean, estalketa gertatzen denean, ernalketa ere gertatzen dela ondorioztatu daiteke.

3.2.2.4. Embrioien erauzketa eta kultiboa

Enbrioien erauzketa hasi aurretik, erauzketa prozesurako eta garapenean hazteko plakak prestatu ziren gutxienez prozesua hasi baino 4 ordu lehenago, horrela plaketako medioa gasifikatu eta atenperatzeko. Sakrifikatu beharreko sagu eme bakoitzeko erausketa plaka bana prestatu zen eta bertan, 60 μl-ko hialuronidasa tanta bat, eta KSOM hazkuntza medioaren 40 μl-ko tanta bat eta 20 μl-ko bi tanta prestatu ziren. Ondoren tanta guzti hauek ez desegiteko parafinazko olio kapa batekin estali ziren. Beste alde batetik enbrioien erauzketa egun bakoitzeko bi hazkuntza plaka prestatu ziren, kontrol eta morfinazko tratamendua jasoko zuten enbrioiak ezberdintzeko. Bertan 10 μl-ko 9 tanta KSOM hazkuntza medio gehitu ziren eta parafinazko olioaz estali. Esan bezala, behin plakak prestatuta inkubadorean sartzen ziren gasifikatu eta atenperatzeko, 37ºC-tan eta %5CO2-arekin.

Argitu beharra dago enbrioiak zelula bakarrean eraузteko, injekzio, gurutzatze eta erauzketa orduak ondo zenbatu beharra dagoela. Hori dela eta garrantzitsua da, PMSG-aren injekzioa, erauzketa baino 76 ordu inguru lehenago egitea; hCG-aren injekzioa bitartean 48 ordu inguru igarotzea, hau da, erauzketa baino 24-27 ordu lehenago egitea gehienez. Emeen sakrifizioa denbora tarte hori baino lehenago egiten bada, oraindik ere ernalketa gertatu ez izana gertatu daiteke eta beraz obozitoak metafase II-an aurkitzea. Bestalde emeen sakrifizioa denbora tarte hori baino beranduago egiten bada, cumulus oophorus izeneko zelula folikularren masa, sakabanatua aurkituko dugu obidukto guztian zehar.

Behin momentu egokia zehaztuta, eme bakoitzaren sakrifizioa eta enbrioien erauzketa banan-banan egin zen. Lehendabizi saguei lokadura zerbikala egin zitzaien, sakrifikazio metodo bezala. Ondoren, animalien sabela ebaki egin zen, sabel paretako azala eta muskuluak baztertuz eta horrela umetokia eta obarioak bistaraziz. Jarraian obarioa eta umetoki ertzaren artean kokatzen den obiduktuaren muturrak moztu eta erauzketarako prestatutako plakan jarri zen, hialuronidazari zegokion tantanean. Obozito eta espermatozoideen arteko ernalketa egon bada obiduktuak anpulu antzeko bat erakusten zuen, enbrioiak igarotzen ari ziren puntu espezifikoan. Laborategiko pintzekin anpula hori ziztatu eta obidukto barneko enbrioi zelulak cummuluko zeluletan bilduak, kontu handiz kanporatu ziren. Enbrioiak hialuronidasan uzten dira denbora tarte motz batez RT-ran, cumulus-eko zelulak desegin eta enbrioiak garbi-garbi geratu ziren arte. Behin enbrioiak garbiak zirela, plastikozko kapilareen laguntzaz, enbrioi guztiak hialuronidasako tantanetik, KSOM medio garbia zegoen tantanera pasatu ziren. Bertan hainbat garbiketa egin zitzaizkien enbrioiei eta hurrengo tantanera pasatu ziren, enbrioien egoera morfologikoa aztertzeko eta benetan zigotoak zirela baieztatzeko. Lortutako enbrioi egoki guztiak kontatu eta hazkuntza plaketako KSOM mediodun tantanetan banatu ziren 4-5 enbrioietako taldeetan.

Enbrioien kultiboa aurrera eramateko egunero lehenengo orduan medio garbiaren plakak prestatu ziren, enbrioien hazkuntza medioa aldatu aurretik plakak gasifikatu eta atenperatzeko denbora izateko. Enbrioiak egunero garapenean aurrera jarraitzen zutela frogatzeko aztertu egiten ziren mikroskopioan, eta egunero hazkuntza medioa aldatu zitzaien garapenaren azken egunera iritsi arte. Erauzketa gauzatu eta 24 ordura, enbrioiak bi zelulatan banatuta aurkitzen zirela frogatu zen eta bildutako enbrioien erdiari morfinazko tratamendua egin zitzaien KSOM hazkuntza medioaren tantanei 10μM morfina gehituta, 24 ordutan zehar. Beste enbrioien erdiari morfinaren ordez, %0.9 (p/v)-ko sodio kloruroa gehitu zitzaien, 24 orduz. Tratamendua bukatuta, enbrioien garapena 4-6 zelulatan zegoela frogatu eta enbrioiak hazkuntza medio

garbia zuten plaketan aldatu ziren plastikozko kapilareak erabiliz. Azkenik enbrioiak garapenean mantendu ziren 24-48 ordu gehiago, blastozisto mailara iritsi ziren arte, non enbrioien kopurua zenbatu, morfologia aztertu eta bildu egin ziren eppendorf banatan eta -20ºC-tan izoztu ziren ondorengo esperimentuetan erabiltzeko.

3.3. METODOAK

Jarraian tesian zehar erabilitako metodologia deskribatzen da.

3.3.1. Western Blot (WB)

Western Blot edo immunoblot teknikak, edozein proteina nahasketan intereseko proteinaren detekzioan oinarritzen da, antigorputz espezifikoen bitartez (Towbin H eta lank. 1979). Horrela, intereseko proteinaren identifikazioa lortzeaz gain, kuantifikazio erlatiboa ere azter daiteke.

3.3.1.1. Proteinaren erauzketa

Erauzketa prozesua laginen araberakoa izan da, horregatik, kultibo zeluletatik eta saguen organoetatik eratorritako proteinaren erauzketan pausu ezberdinak jarraitu ziren.

Alde batetik, zelulen proteinaren erauzketa prozesurako, argitu beharra dago zelula osoaren metodoa erabili zela. Horretarako, petri plaketako hazkuntza medioa kendu eta PBS-arekin garbitu ondoren, zelulak tripsinaren mililitro (ml) bat gehituz eta giro tenperaturan (RT) inkubatuz, altxatu ziren. Tripsinaren efektua inaktibatzeko, hazkuntza medioa gehitu zitzaien plakeri (erabilitako bolumaren bikoitza), eta nahasketa 15 ml-ko edukiera zuten falcon hodietan jaso zen. Zelulak 1600 rpm-tara (biraketa minutuko) zentrifugatu ziren 3 minutuz, tubuaren beheko ertzean prezipitatuz. Ondoren, gainjalkina deuseztatu eta zelulen pelleta birritan garbitu zen ml bat PBS erabiliz, 1600 rpm-tara zentrifugatuz 3 minutuz. Garbiketa horien tartean, 10 mikrolitro (µl) jaso ziren lagin bakoitzaren zelula kopurua zenbatzeko, Neubauer ganberak erabiliz, etxe komertzialak gomendatzen dituen pausuak jarraituz. Jarraiki, lagin bakoitza $1 \cdot 10^3$ zelula/µl-ko kontzentraziora doitu ziren eta proteasa eta fosfatasa inhibitzaileak (1X), DTT(1X) eta karga tanpoia (4X) gehitu zitzaien.

Saguen organoen kasuan, nukleoko eta zitoplasmako proteinaren erauzketa burutu izen gatz maila altuko bufferrak erabiliz. Hasteko organo bakoitzaren 50 mg pisatu eta bisturi batekin zatikatu ostean, 1.5 ml-ko edukiera zuten eppendorfetan bildu ziren, “A Indargetzailea”-ren 500 µl gehitu zitzaizkien eta potter homogeneizagailuaren bitartez homogeneizatu ziren. Ondoren, nahasketa 45-60 minutuz izotzetan mantendu zen astintzen. Ondoren, laginak 4ºC-tan 2000 rpm-tara zentrifugatu ziren 7 minutuz. Gainjalkina, frakzio zitoplasmatikoa, jaso eta -20ºC-tan gorde zen. Bestalde, pelleta birritan garbitu zen “Gatz baxuko Indargetzailea”-aren 100 µl erabilita, bi kasuetan 2000 rpm-tara zentrifugatuz 7 minutuz 4ºC-tara eta gainjalkina deuseztuz. Segidan, pelleta “Gatz baxuko Indargetzailea” 1x eta “Gatz altuko Indargetzailea” 2X-etan disolbatu zen eta nahasketa 45 minutuz izotzetan mantendu zen astintzen. Azkenik, nahasketa 12000 rpm-tara zentrifugatu zen 20 minutuz 4ºC-tara eta gainjalkina, frakzio nuklearra eppenderf berri batean jaso zen.

Proteinaren kuantifikazioa burutu aurretik gomendagarria da gatz maila altuen protokoloa erabiliz erauzitako laginen proteinen azetonazko prezipitazioa gauzatzea, gatz kopuru hauek

kuantifikazioa oztopatzen dutelako. Beraz, jasotako laginen proteina frakzio nuklearreri azetona gehitu zitzaien (4X) eta -20ºC-tan gorde ziren gau osoan zehar (ON). Hurrengo goizean, laginak 13000 rpm-tara zentrifugatu ziren 5 minutuz 4ºC-tan, eta gainjalkina deuzestu ondoren pelleta lehortzen utzi zen. Azkenik laginen frakzio nuklearraren pelleta proteasa eta fosfatasa inhibitzaile (1X) eta PBS-an disolbatu ziren.

3.3.1.2. Proteinaren kuantifikazioa

Erauzitako proteina homogeneizatuen kontzentrazioa neurtzeko azido bizinkoninikoaren (BCA) metodo fotometrikoa erabili zen (Smith PK eta lank. 1985). Hau, peptido loturek soluzioko Cu2+ ioiak erreduzitzeko duten gaitasunean oinarritzen da, zein Cu+ bihurtzean, azido bizinkoninikoaren bi molekularekin lotzen den more koloreko konposatua sortuz. Konposatu honek 562nm-ko uhin luzeran daukan absorbantziak, proteinen kontzentrazioa neurtzea ahalbideratzen du, proteina kontzentrazio ezaguneko soluzioekin eraikitako zuzenaren bitartez. Kontzentrazio ezaguneko proteina, BSA (ingelesetik Bovine serum albumin) erabili zen, ur destilatuan disolbatuta hurrengo kontzentrazioetan zuzena eraikitzeko: 0, 0.2, 0.4, 0.6, 0.8 eta 1 mg/ml. Metodoaren detekzio mailaren barruan lan egiteko, zelulen zein saguen organoen lagin guztiak uretan diluitu ziren. Erreakzioa, P96 plaketan burutu zen. Hala, proteina estandarraren eta lagin bakoitzaren 12.5 µl, azido bizinkoniniko/CuSO4 50:1 disoluzioaren 100 µl-rekin inkubatu ziren 37ºC-tan 30 minutuz. Lagin bakoitzarentzat 3 aldiz errepikatu zen erreakzioa, baina bakarrik saguen organoen laginekin.

Absorbantzia neurtzeko, FluoStar OPTIMA mikroplaka irakurgailua erabili zen, laginen absorbantzia 562nm-tan neurtuz, absorbantziaren balioak proteina mailarekin zuzenki erlazionatzeko. Estandarraren absorbantzia balioetatik osatutako zuzenaren bitartez laginen kontzentrazioa kalkulatu zen µgr/µl unitatetan. Behin lisatu bakoitzaren proteina kontzentrazioa kalkulatuta PBS eta fosfatasa eta proteasa inhibitzaileekin diluitu ziren, konparatu beharreko lagin guztiek proteina totalaren eduki berdina izan zezaten. Horrez gain, laginen lisatu bakoitzari 4X karga tanpoia edo LB (ingelesetik loading buffer) gehitu zitzaion, azkenean amaierako kontzentrazioa 1X izan zedin. Ondoren, bai zelulen zein saguen organoen laginak 10 minutuz 95ºC-tan berotu ziren termoblokean, proteinen desnaturalizazioa ahalbidetzeko, gelean kargatu aurretik.

3.3.1.3. WB-aren prozedura

WB-a gauzatzeko lehenengo pausua, elektroforesirako akrilamidazko gelak egitea izan zen. Gelen osagaiak eta proportzioak gel banatzailerako eta gel kontzentratzailerako 3.4. Taulan biltzen dira hurrenez hurren. Gelen akrilamida proportzioa, ikergai ziren proteinen pisu molekularren arabera aukeratu zen, eta lan honetan histonen modifikazio ezberdinak aztertu zirenez (17kDa-neko proteinak), %12-ko akrilamida kontzentraziodun gelak osatu ziren.

3.4. Taula. WB teknikan, elektroforesi bidezko proteinen banaketarako erabilitako gelak egiteko erreaktiboak eta hauen proportzioak.

Gel banatzailea		Gel kontzentratzailea	
H_2O	Bolumena bete arte	**H_2O**	Bolumena bete arte
Tris 1M pH=8.8	%25 (bol)	**Tris 0.5 M pH=6.8**	%25 (bol)
Bis Akrilamida %30	%40 (bol)	**Bis Akrilamida %30**	%13.3 (bol)
SDS %10	%1 (bol)	**SDS %10**	%1 (bol)
PSA %10	%0.5 (bol)	**PSA %10**	%0.5 (bol)
Temed	5 µl	**Temed**	5 µl

Laginen proteinen banaketa elektroforetikoa burutzeko, aurretik desnaturalizatutako laginak akrilamidazko gelean kargatu ziren pisu molekular ezaguneko markatzaile estandarrekin batera, eta 100V-tako potentzial diferentzia aplikatu zitzaien BioRad etxeko Mini PROTEAN Tetra elektroforesirako gailuan, proteinak tamainaren arabera banatu ziren arte. Energia iturri gisa etxe komertzial bereko PowerPac HC Power Supply erabili zen. Elektroforesian proteinak banatu ondoren, proteinak 0,22 µm-ko porodun PVDF mintz batera transferitu ziren Mini Trans-Blot III-aren euskarriak erabilita. Transferentzia sandwicha itxurako muntaia erabiliz burutu zen, kanpoaldetik barrualderako ordenean, kuxinak, 3 whatman paper geruza, gel-a eta PVDF mintza erabiliz. Honen ondorioz eta korrontea aplikatuz, proteinak mintzera leku aldatzen dira. Transferentzia tenperatura hotzean gauzatu zen, Mini Trans Blot Cell unitatea erabiliz, 100V-etan eta ordu betez utziz.

Transferentzia egin eta gero, PVDF mintza %5-eko BSA-dun blokeo soluzioarekin ordu betez inkubatu zen RT-n, antigorputz primarioaren espezifikotasuna bermatu eta lotura ez espezifikoak ekiditeko. Ondoren, mintza antigorputz primarioarekin 4ºC-tan inkubatu zen O.N. Esperimentu ezberdinetan zehar erabilitako antigorputz bakoitzaren diluzio eta inkubazio denborak 3.5.taulan laburbiltzen dira. Hurrengo goizean, mintzari antigorputz primarioa kendu, Blotto tanpoiarekin 5 minutuko hiru garbiketa egin zitzaizkion, antigorputz soberakina deuseztatzeko eta zegokion HRPari (ingelesetik Horseradish peroxidase) konjugaturiko antigorputz sekundarioarekin (1:1000) inkubatu zen ordu batez giro tenperaturan (3.5.taulan). Jarraiki, blottorekin 5 minutuko beste hiru garbiketa egin ziren mintza errebelaziorako prestatzeko.

3.5. Taula. Tesi honetan erabili diren antigorputz primario eta sekundarioen zerrenda. WB: Western Blot; ON: over night; RT: Giro tenperatura; ChIP: Kromatinaren immunoprezipitazioa.

Antigorputz primarioak	**Erreferentzia**	**Etxe komertziala**	**Ostalaria**	**Diluzioa**	**Inkubazio denbora**
Anti-H3K27me3	A2363	AbClonal	Untxia	1:1000 (WB)	4ºC-tan, ON
Anti-H3K9me2	A2359	AbClonal	Untxia	1:1000 (WB)	4ºC-tan, ON
Anti-H3K4me2	A2357	AbClonal	Untxia	1:1000 (WB)	4ºC-tan, ON
Anti-H3K4me3	A2356	AbClonal	Untxia	1:1000 (WB)	4ºC-tan, ON
Anti- β-Actin–Peroxidase	A3854	Sigma-Aldrich	Sagua (monoklonala)	1:25000 (WB)	RT, 1 ordu
Antigorputz sekundarioak	Erreferentzia	Etxe komertziala	Ostalaria	Diluzioa	Inkubazio denbora
2º Anti-untxia HRP	Sc-2004	Santa Cruz Biotech	Ahuntza	1:1000	RT, 1 ordu

Antigorputz sekundarioan konjokaturiko HRParen aktibitatearen neurketaren bidez, mintzaren errebelazioa burutzeko, ECL substratu kimioluminiszentea erabili zen. Izan ere, ECL substratuak, antigorputz sekundarioei atxikitako HRP entzimarekin erreazkionatzean, argia emititzen du eta kimioluminiszentzia hori detektatzeko BioRad etxeko ChemiDoc XRS+ sistema eta Quantity One softwarea erabili ziren.

Azkenik, proteinen detekzioen emaitzen kauntifikazio erlatiboa Image J softwarea erabiliz gauzatu zen. Software honek, background-a kendu eta lagin bakoitzaren banda(k) housekeeping gene batekiko normalizatzeko aukera ematen du. Kasu guztietan housekeeping genea β-Aktina izan zen. Horrela, lagin ezberdinen banden tamaina eta intentsitatea kontutan hartuta, ratioa

kalkulatu zen, eta jarraian, kontrol eta morfinarekin trataturiko taldeen arteko ratio erlatiboa kalkulatu zen.

3.3.2. Transkriptomaren analisia

Bai zelula zein saguen organoetan, morfinaren eraginez sortutako gene adierazpena neurtzeko, transkriptomaren analisian oinarritutako zenbait teknika erabili ziren: 1) polimerasaren kate-erreakzio kuantitatiboa edo PCR-a (ingelesezko *Polymerase Chain Reaction*), 2) Fluidigm PCR nanofluidikoaren sistema eta 3) RNA-ren sekuentziazioa. PCR-a, DNA-aren anplifikazio esponentziala eta kuantifikazio erlatiboa bateratzen dituen metodoa da, polimerasa entzimarekin, intereseko oligonukleotidoen kate osagarriaren sintesiaren bitartez burutzen dena. Fluidigm PCR nanofluidikoaren sistemaren bidez, itu geneen adierazpena eta kuantifikazio absoltua gauzatzen da. Azkenik RNA-ren sekuentziazioaren bitartez,

3.3.2.1. RNA-ren erauzketa

Kultibo zeluletatik eta saguen organoetatik eratorritako RNA totalaren erauzketa TRIzol erreaktiboaren bitartez burutu zen. Laginen homogeneizatuak prestatzerako orduan, pausu ezberdinak burutu ziren, zelulak izan edo saguen organoak izan, jarraian deskribatzen den bezala.

Zelulak petri plaketan hazi ziren. Lehendabizi, plakako hazkuntza medioa kendu eta PBS-arekin garbitu ondoren, zelulak tripsinaren ml bat gehituz eta RT-n inkubatuz, altxatu ziren. Tripsinaren efektua inaktibatzeko, hazkuntza medioa gehitu zitzaien plakeri (erabilitako bolumaren bikoitza), eta nahasketa 15 ml-ko edukiera zuten falcon hodietan jaso zen. Zelulak 1600 rpm-tara zentrifugatu ziren 3 minutuz, hodiaren beheko ertzean prezipitatuz. Ondoren, gainjalkina deuseztatu eta zelulen pelleta birritan garbitu zen PBS-a erabiliz, 1600 rpm-tara zentrifugatuz 3 minutuz. Jarraiki, zelulen pelletari TRizol erreaktiboaren ml bat gehitu zitzaion homogeneizazioa burutzeko, nahasketa gora eta behera pipeteatu zen hainbat alditan eta RT-n 5 minutuz inkubatu zen.

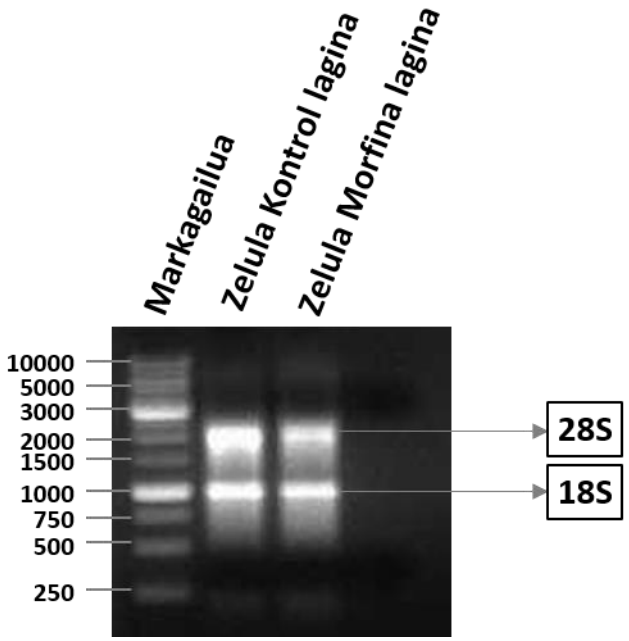
Saguen organoen kasuan, organo bakoitza 1.5 ml-ko edukiera zuten eppendorfetan bildu ziren eta TRizol erreaktiboaren ml bat gehitu zitzaien eta 800rpm-tara potter homogeneizagailuaren bitartez homogeneizatu ziren. Ondoren, nahasketa 5 minutuz inkubatu zen RT-n.

Behin laginen homogeneizatua gauzatuta, jarraitutako pausuak berdinak izan ziren bai zelula zein saguen organoen laginetan. Horrela, homogeneizatuari 200 µl kloroformo gehitu zitzaizkion eta 15 segundoz bortize bidez nahastu ondoren, 5 minutuz inkubatu zen RTn. Ondoren, aurretik 4ºC-tara girotutako zentrifugan, 12000 g-tara (indar zentrifugo erlatiboa) zentrifugatu zen 15 minutuz, homogeneizatua goiko fase urtsu garden, interfase eta behealdeko fase arrosan banatuz. RNA-dun fase urtsua 1,5 ml-ko eppendorf berri batera transferitu eta 500 µl isopropanolekin nahastu zen. Gutxienez ordubetez, -20ºC-tan inkubatu ostean, RNA 12000 g-tara 10 minutuz zentrifugatuz prezipitatu zen. Gainjalkina deuseztatu eta RNA pelleta birritan garbitu zen, %75eko purutasuneko etanolarekin eta 7500 g-ko zentrifugazio bitartez 5 minutuz. Etanola deuseztatu, RNA garbia lehortzen utzi eta nukleasarik gabeko uretan disolbatu zen, 60ºC-tan 10-15 minutuz inkubatuz. RNAren kontzentrazioa eta kalitatea Nanodrop espektrofotometroaren bidez neurtu ziren.

3.3.2.2. RNAren kontzentrazioa, purutasuna eta integritatearen neurketa

Erauzitako RNAren kuantifikazioa NanoDrop izeneko espektrofotometroaren bidez egin zen. Laginaren RNAren kontzentrazioa neurtzeaz gain, absorbantzia ere neurtu zen eta uhin-luzera desberdinen arteko ratioa kalkulatu zen laginen purutasuna zehazteko (proteina eta beste era bateko kutsatzaileen presentzia aztertzeko). RNA purutasuna onartzeko NanoDrop-ak kalkulaturiko A260/280 eta A260/230 ratioen balioak ~2 ingurura hurbildu behar dira.

Beste alde batetik, RNAren integritatea zuzena zen frogatzeko, laginak 70ºC-tan berotu ziren 5 minutuz eta ondoren, midori green zuen (azido nukleikoen tindatzailea, etidio bromuroaren ordezkoa) %1-eko agarosa gelean kargatu ziren. Elektroforesia 125V-ra burutu zen eta RNA-ren integritatearen adierazle diren 18S eta 28S banda erribosomalak, BioRad etxeko Chemidoc XRS+ sistemaren bitartez aztertu ziren argi ultramorea erabiliz (3.1.Irudia).



3.1.Irudia. RNA-ren integritatearen irudikapena. 28S eta 18S bana erribosomalak bistakoak dira bi laginetan.

3.3.2.3. Alderantzizko transkripzioa

Laginen DNA osagarriaren (cDNA) sintesia burutzeko iScript cDNA Synthesis Kit komertziala erabili zen. Laburki, 1 µg RNA, 1 µl iScript alderantzizko traskriptasa eta 4 µl 5X iScript erreakzio nahasketarekin, MyCycler termoziklagailuan inkubatu zen hurrengo zikloetan zehar: 5 minutu 25°C-etan, 30 minutu 42°C-etan eta 5 minutu 85°C-etan. Amaieran, cDNAren kontzentrazioa 50 ng/µl-takoa zen.

3.3.2.4. Polimerasaren kate-erreakzio kuantitatiboa (RT-qPCR)

Lehendabizi, intereseko geneen mRNA-ren detekziorako hasle espezifikoak (primer) diseinatu ziren Primer3 (v 0.4.0; Koressar T eta Remm M 2007) softwarea erabiliz (Untergasser eta lank. 2012). Hautaturiko hasleen sekuentziaren ezaugarriak Integrated DNA technologies (IDT) etxearen OligoAnalyzer Tool, softwarearekin analizatu ziren, hauen egokitasuna baieztatuz. Gainera, hasleen efizientzia eta espezifikotasuna hurrenez hurren kurba patroiak eta disoziazio kurbak erabiliz analizatu ziren. 3.6. Taulak hasle guztien sekuentziak jasotzen ditu. Bukatzeko

"Blast-Basic local alignment search tool" → "Nucleotide Blast" aukera erabili zen hasleak intereseko geneekiko espezifikoak zirela baieztatzeko (Altschul SF eta lank. 1990).

3.6. Taula. RT-qPCR teknika burutzeko erabili ziren hasleen sekuentzia. mRNA: intereseko geneen mRNA-ren detekziorako hasle espezifikoak. ChIP: intereseko geneen sustatzaileen eremurako hasle espezifikoak.

Izena	Genea	Sekuentzia (5' → 3')
Enhancer Of zeste 2 polycomb repressive xomplex 2 subunit	*Ezh2* (mRNA)	(f) AGAATGTGGAGTGGAGTGGTG (r) CAGTGGGAACAGGTGCTATG
Embryonic ectoderm development	*Eed* (mRNA)	(f) CCACAAATACGCCAAATGC (r) CAAACACCAGAGGGTCTCCT
AE binding protein 2	*Aebp2* (mRNA)	(f) GCGAAGAGAAAGGAGGAATCT (r) TCCAACAGCAAGGCAGTATCT
SUZ12, polycomb repressive complex 2 subunit	*Suz12* (mRNA)	(f) ACAGAAGCCAGAGACGACCT (r) GGAGCCATCATAACACTCATTG
Euchromatic histone lysine methyltransferase 2	*G9a* (mRNA)	(f) TCGGAACAAAGAAGGAGACAC (r) ATTGACACAGGGGATGGGTA
Euchromatic histone lysine methyltransferase 1	*Glp* (mRNA)	(f) GATGGATGGAGATTCAGAGGA (r) CTTTTCCGAGCAGGTTTGAT
Lysine methyltransferase 2A	*Mll1* (mRNA)	(f) GCAGGCACTTTGAACATCCT (r) TTATGGGGACAGAGGTCAGG
RB binding protein 5, histone lysine methyltransferase complex subunit	*Rbbp5* (mRNA)	(f) AGATTGCGACCAGAGGTTTC (r) ATCCGAGTCATCGTCTACCG
SET domain containing 1A	*Setd1a* (mRNA)	(f) CAGACGGGCTTGTAGATTCC (r) GTGGGGGTAGGAGAGGGATA
SET domain containing 1B	*Setd1b* (mRNA)	(f) CCAGCAGCACAGAGAGTGAG (r) GCCTCGGGTTGATTTTACTG
CXXC finger protein 1	*Cfp1* (mRNA)	(f) ACAGCAACACCTGAGCCACT (r) AGGAACGGGGACTCTTCT
Bone morphogenetic protein 4	*Bmp4* (mRNA)	(f) AGGAGGAGGAGGAAGAGCAG (r) TTGAAGAGGAAACGAAAAGCA
Sirtuin 1	*Sirt1* (mRNA)	(f) GACGCTGTGGCAGATTGTTA (r) GCAAGGCGAGCATAGATACC
Structural maintenance of chromosomes flexible hinge domain containing 1	*Smchd1* (mRNA)	(f) TGGATAGGACAGTAGCCGAGA (r) ATTGCTTCCCCCTTTTTGTT
Glyceraldehyde-3-phosphate dehydrogenase	*Gapdh* (mRNA)	(f) TATGACTCCACTCACGGCAAATT (r) TCGCTCCTGGAAGATGGTGAT
Actin beta	*Actb* (mRNA)	(f) GGGCTATGCTCTCCCTCAC (r) CACGCACGATTTCCCTCT
Pyruvate carboxylase	*Pcx* (mRNA)	(f) CAACACCTACGGCTTCCCTA (r) TCCACAAACAACGCTCCAT

Hasleak liofilizatuta jaso ziren, spin bitartez zentrifugatu eta 100 µM-eko kontzentraziora doitu ziren, behar beste nukleasarik gabeko uretan disolbatuz (stock disoluzioa). Ondoren, lanerako diluzioak prestatu ziren, gene bakoitzaren Forward edo Reverse stock disoluzioen hogei mikrolitro 180 µl nukleasarik gabeko urarekin nahastuz. Stock nahiz lanerako diluzioak hozkailuan mantendu ziren -20ºC-tan.

Intereseko geneen adierazpena modu semi-kuantitaboan analizatu zen Real Time Polimerase Chain Reaction bidez, eta detekziorako iTaq Universal SYBR Green Supermix zunda fluoreszentea erabili zen. Erreakzio bakoitzaren 3 erreplika burutu ziren, P96 plakaren putzu bakoitzean, 20ng cDNA-tik abiatuta eta 10 µl-ko bolumenean. Mixaren osagaien proportzioak honakoak izan ziren: 5 µl iTaq SYBR Green Supermix 2X, 0.15 µl Forward primer-a, 0,15 µl Reverse primer-a, 0.7 µl nukleasarik gabeko ura eta 4 µl cDNA (5ng/µl). Jarraiki, plaka denbora tarte laburrez

zentrifugatu eta BioRad etxe komertzialeko CFX96 Real Time PCR detekzio termoziklagailuan sartu zen, primer-en ezaugarrietara egokituriko protokoloarekin. Tenperatura eta denbora zikloak ondorengoak izan ziren: desnaturalizazioa 10 minutu 95ºC-etan; hibridazioa 20 segundo 95ºC-etan eta luzapena minutu 1, 59ºC-etan 39 zikloz; eta azkenik fusio kurba (melting curve) 5 segundo 65ºC-etan.

Emaitzen analisirako, gene bakoitzaren adierazpena bost esperimentu independentetan neurtu zen eta errepliken arteko desbiderapen estandarra 0.2 unitate baino txikiagoa zen kasuetan bakarrik hartu zen kontutan. Hau horrela, gene adierazpenaren kuantifikazio erlatiboa $2^{-\Delta\Delta Ct}$ metodoa erabiliz gauzatu zen (Livak KJ eta Schmittgen TD 2001), hau da, termoziklagailuan lortutako Ct balioak $2^{-\Delta\Delta Ct}$ bihurtu ziren kontrol eta morfinarekin trataturiko taldeen arteko kuantifikazio erlatiboa lortzeko.

3.3.2.5. Gene adierazpen analisia Fluidigm qPCR nanofluidikoaren sistemarekin

Intereseko gene bakoitza RT-qPCR bidez banan-banan aztertu ordez, Fluidigm qPCR nanofluidikoa erabili genuen gene talde handiak batera aztertzeko. Horrela, Fluidigm-eko Biomark HD Nanofluidic qPCR sistema, GE 48.48 Array Dinamikoaren sistemarekin uztartu zen, Euskal Herriko Unibertsitateko, Genomika eta Proteomikako zerbitzuak eskaintzen duen zerbitzua erabiliz (https://www.ehu.eus/es/web/sgiker/gene-adierazpenen-zerbitzuak). Honek, zenbait lagin ezberdinetan, hainbat gene ezberdin aztertzeko aukera eman zigun. Laburki, zelulen eta animalien organoetako Trizol erreaktiboaren bidezko RNA-ren erauzketa; kontzentrazioa, pututasuna eta integritatearen neurketa; eta alderantzizko transkripzioa aurreko ataletan deskribatutako prozedura berberak jarraituz burutu ziren (3.3.2.1/2/3 atalak). Horren ostean geneen adierazpen aztertzeko, gene espezifikoen sustatzaile bikoteko 100 μM erabili ziren eta detekzioa EvaGreen erabiliz egin zen.

Emaitzen analisirako, aurreko atalean adierazi bezala, lortutako Ct balioak $2^{-\Delta\Delta Ct}$ bihurtu ziren kontrol eta morfinarekin trataturiko taldeen arteko kuantifikazio erlatiboa lortzeko.

3.3.2.6. RNA-ren sekuentziaziorako laginen prestaketa

RNA-ren sekuentziazioak, bigarren belaunaldiko sekuentziazio teknikak (ingelesezko *Next Generation Sequencing*) erabiltzen ditu geneen presentzia eta adierazpenaren kuantifikazioa aztertzeko transkriptoma osoan era global batean. Lagina prestatzeko erabili zen teknika Trizol erreaktiboaren bitartez buruturiko RNA-ren erauzketa izan zen eta 3.3.2.1 atalean azaltzen dena eta horren ondoren kontzentrazioa, purutasuna eta integritatea ere neurtu ziren 3.3.2.2 atalean deskribatzen den bezala. Sekuentziazioa eta bere analisi bioinformatikoa berriz 3.3.5 ataletik aurrera azaltzen da.

3.3.3. Kromatinaren analisia

Kromatinaren analisia, kromatinaren inmunoprezipitazio teknikaren bidez (ChIP) burutu zen, proteina eta DNA-ren arteko elkarrekintza aztertzean oinarritzen dena. Ondoren gure intereseko proteinetara, hau da histonetara, lotutako DNA-ren analisia bi era ezberdinetara egin zen: 1) ChIP-RT-qPCR bitartez eta 2) Sekuentziazioarekin. ChIP-RT-qPCR-arekin itu gene espezifikoek histonarekin loturarik duten aztertu daiteke. Bestalde, ChIP sekuentziazioaren bitartez, intereseko proteinek genomako zein eremu espezifikori uztartzen zaizkion identifikatu

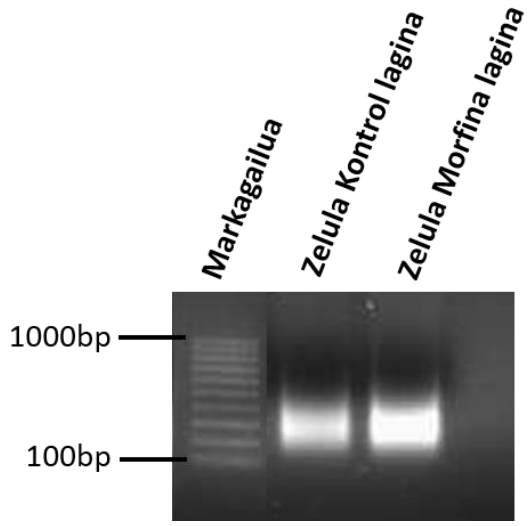
daiteke, hala nola, geneen sustatzaile eremuak, exoiak, CpG irlak etab., eta beraz, intereseko proteinaren itu espezifikoak ezagutu daitezke (Philippe Collas, 2010).

3.3.3.1. Zelulen finkapena, lotura gurutzatua (Doble crosslink)

ChIP teknika zelulekin bakarrik erabili izan da tesian zehar, beraz behin zelulak plaketatik jaso ostean, eta PBS-arekin 3 garbiketa egin ostean, zelulak finkatu egin ziren lotura gurutzatua edo ingelesezko *doble cosslink*-a burutuz. Horretarako, zelulak 15 ml-ko falcon hodi batera pasatu ziren eta bertan PBS-aren 10 ml-rekin nahastu ziren, cross-linking soluzioaren 1ml gehituz (%1 formaldehidoaz osatua). Nahasketa 10 minutuz inkubatu zen RT-n biraka. 10 minutuak igaro eta berehala, 1.1 ml Glizina 2.5 M gehitu zitzaion, hodia gora eta behera mugituz guztia ondo nahasteko eta izotzetan inkubatu zen 3 minutuz, cross-link-a inaktibatzeko. Ondoren, zelulak 4ºC-tara girotutako zentrifugan, 1500 rpm-tara zentrifugatu ziren 5 minutuz eta gainjalkina deuseztatu zen. Zelula pelleta birritan garbitu zen PBS 10 ml-rekin eta azkenik 1.5 ml-ko edukierako eppendorf batean bildu zen. Puntu honetan protokoloarekin aurrera jarraitu zitekeen edota laginak -20ºC-tara gorde zitezkeen.

3.3.3.2. Kromatinaren erauzketa

Cross-link-a burutu ondoren zelulen pelleta 4 ºC-tan zegoen “A Kromatina Garbitzaile Indargetzailea” eta fosfatasa eta proteasa inhibitzaileez osaturiko soluzioaren 1 ml-rekin nahastu zen, eta 5 minutuz inkubatu zen 4ºC-tara biraka. Ondoren, nahasketa 2000rpm-tara zentrifugatu zen 5 minutuz, 4º-tan eta gainjalkina deuseztatu zen. Pelleta 4ºC-tan zegoen “B Kromatina Garbitzaile Indargetzailea” eta fosfatasa eta proteasa inhibitzaileez osaturiko soluzioaren 1 ml-rekin nahastu zen, berriz ere 5 minutuz inkubatu zen 4º-tara biraka. Jarraian nahasketa 4ºC eta 2000 rpm-tara zentrifugatu zen 5 minutuz eta gainjalkina deuseztatu zen. Hurrengo pausuan pelletari “Sonikazio Indargetzailea+SDS %0.1” eta fosfatasa eta proteasa inhibitzaileez osaturiko soluzioaren 500µl gehitu zitzaion eta zelula laginak sonikatu egin ziren Soniprep 150 sonikatzailean. Hainbat proba egin ondoren, 200-500 bp-ko kromatina fragmentuak lortzeko erabilitako sonikazio protokoloa osatu zen, 20 segundu ON eta 20 segundu OFF-eko 60 ziklorekin. Sonikazio prozesuan zehar, laginetan burbuilak sortzea ekidin zen eta tenperatura hotzean gauzatu zen, laginak izotzez inguratuta mantenduz. Kromatinaren fragmentuak tamaina zuzenean zeudela egiaztatzeko, midori green zuen %2-ko agarosa gelak erabili ziren. Laginak bertan kargatu eta elektroforesia 125V-tara burutu zen, eta fragmentuak, BioRad etxeko Chemidoc XRS+ sistemaren bitartez aztertu ziren argi ultramorea erabiliz (3.2.Irudia) .



3.2.Irudia. Kromatinaren sonikazioaren ondorengo fragmentuen irudikapena. Fragmentuak 200 eta 500 bp inguruan ageri dira.

Bestalde, erauzitako kromatinaren kontzentrazioaren neurketa NanoDrop espektrofotometroaren bidez egin zen, eta gainera laginaren purutasunaren A260/280 ratioa kasu honetan 1.6 ingurukoa zela baieztatu zen proteinaren presentzia zela eta.

Jarraian sonikatutako kromatinaren lagin bakoitzari %5-eko glizerol-a zeraman "Kromatin Disolbatzeko Indargetzailea"-ren 2 bolumen gehitu zitzaizkion eta nahasketa 10 minutuz zentrifugatu zen 13200 rpm-tara 4ºC-tan. Bertatik, gainjalkina eppendorf berri batean berreskuratu genuen eta era honetan kromatinazko metaketa disolbaezinak deuseztatu ziren. Puntu honetan, lagin bakoitzeko %10 Input-a osatzeko banandu zen eppendorf berri batean, eta gainerakoaren heren bat IgG-a osatzeko banandu zen eppendorf berri batean. Konparaketa berdinerako erabili ziren Kontrol eta Morfina laginetatik eratorritako IgG-aren zatiak elkartu egin ziren, horrela kantitate berbera izateko kasu guztietan. Puntu honetan protokoloarekin aurrera jarraitu zitekeen edota laginak -80ºC-tara gorde zitezkeen.

3.3.3.3. Input-aren prestaketa

Behin Input-a lagin orokorretik banandu ondoren, lehenengo, aurretik buruturiko Crosslink-a desegin zen (Reverse Crosslink deiturikoa), kromatina osatzen duten proteina eta DNA-ren arteko loturak apurtzeko. Horretarako input-aren 100 µl-ko, pH 7 zuen 10 ul 3M NaAzetato eta %10-eko 10 µl SDS gehitu zitzaizkion eta 3 orduz 65ºC-tan edo 55ºC-tan ON inkubatu zen. Ondoren, 0.5mg/ml-ko kontzentraziodun RNAseA-ren 2 µl gehitu zitzaizkion eta ordubetez 37ºC-tan inkubatu zen, input-ean egon zitekeen RNA kutsatzaile guztia ezerezteko. Jarraian, 10mg/ml-ko kontzentraziodun Proteinase K-ren 10 µl gehitu zitzaizkion eta 3 orduz 65ºC-tan edo 55ºC-tan ON inkubatu zen, Input-ean zegoen proteina guztia desegiteko. Bukatzeko laginak -20ºC-tan gorde ziren Input-eko DNA-ren purifikazioa gauzatu arte.

3.3.3.4. Antigorputz eta Beads-en prestaketa

Kromatinaren immunoprezipitazioa gauzatzeko, lagin bakoitzeko DynaBeads izeneko bola magnetikoen 30 µl erabili ziren. Bola magnetikoak eppendorf-etn kokatu eta imanen laguntzaz DynaBeads-en Bufferra kendu zitzaien. Ondoren, pH 8 zuen 500 ul 100mM sodio fosfato hotzarekin bi garbiketa egin zitzaizkion, likidoak kentzerako orduan imanarekin lagunduz. Gero,

bola magnetikoak pH 8 zuen 300 µl 100mM sodio fosfato eta %0.5 BSA-n disolbatu ziren eta immunoprezipitatu nahi zen proteinaren aurkako antigorputza gehitu zitzaion. Gure kasuan H3K27me3, H3K4me2 eta H3K4me3 proteinen aurkako antigorputzak erabili ziren esperimentu ezberdinetan zehar eta beti ere antigorputz bakoitzeko 3.7. Taulan zehazten diren µg-ak erabili ziren bola magnetikoekin nahastuta lagin bakoitzeko. Aipatzekoa da, kontrol negatibotzat untxiaren-IgG inespezifikoak erabili zirela eta antigorputzak bola magnetikoekin konjugatzeko garaian, beste eppendorf bat prestatu zela 5µg IgG ere DynaBeads-ekin konjugatzeko. Konjugazioa ON burutu zen, biraka eta 4ºC-tara.

Konjugazioa bukatu ostean, imanen laguntzaz soberako antigorputza eta IgG-a kendu eta bolak birritan garbitu ziren pH 8 zuen 500 µl 100mM sodio fosfatoarekin. Bukatzeko, aurreko sodio fosfatoaren 10 µl gehitu zitzaizkien bola magnetikoen lagin bakoitzari eta izotzetan utzi ziren kromatina gehitu arte.

3.7. Taula. Tesi honetan erabili diren antigorputz primario eta sekundarioen zerrenda. WB: Western Blot; ON: over night; RT: Giro tenperatura; ChIP: Kromatinaren immunoprezipitazioa.

Antigorputz primarioak	Erreferentzia	Etxe komertziala	Ostalaria	Diluzioa	Inkubazio denbora
Anti-H3K27me3	07449	Millipore	Untxia	4 µg (ChIP)	4ºC-tan, ON
Anti-H3K4me2	A2357	AbClonal	Untxia	5µg (ChIP)	4ºC-tan, ON
Anti-H3K4me3	Ab8580	abcam	Untxia	5µg (ChIP)	4ºC-tan, ON

3.3.3.5. Immunoprezipitazioa (IP)

Lehenik eta behin, aurretik sonikaturiko kromatina laginak desizoztu ziren: esperimentu bakoitzeko Kontrol lagin bat, Morfina lagin bat eta IgG-a osatzeko aurreko biekin laginekin osaturiko nahasketa. Kromatina lagin bakoitzeko 500 µg, aurreko pausuan prestaturiko bola magnetiko eta antigorputzaren konjugatuari gehitu zitzaion eta nahasketa ON inkubatu zen biraka, 4ºC-tan.

Inkubazioa igarota, imanaren laguntzaz, kromatina soberakinak kendu ziren eta gorde egin ziren (ingelesezko *Unbound* izenarekin, -80ºC-tan). Jarraian bola magnetikoak garbitu egin ziren birritan "I.IP Indargetzailea" eta fosfatasa eta proteasa inhibitzaileez osaturiko soluzioaren 500 µl-rekin 10 minutuz inkubatuz 4ºC-tan. Ondoren beste bi garbiketa egin ziren "II.IP Indargetzailea" eta fosfatasa eta proteasa inhibitzaileez osaturiko soluzioaren 500 µl-rekin 10 minutuz inkubatuz biraka eta 4ºC-tan. Gero beste behin garbitu zen "III.IP Indargetzailea"-aren 500 µl erabiliz eta 10 minutuz inkubatuz biraka eta 4ºC-tan. Azkenik bi garbiketa gehiago burutu ziren pH 8 zuen "TE/NaCl Indargetzailea"-aren 500 µl gehituz bola magnetikoei, biraka eta 4ºC-tan 10 minutuz inkubatuz. Bigarren garbiketa hasi aurretik laginak eppendorf berrietara aldatu ziren.

3.3.3.6. Eluzioa

Kromatina eta antigorputzaren konplexuak bola magnetikoetatik askatzeko, nahasketari %1 SDS eta pH 10.1 zuen 0.1M sodio bikarbonatorekin osatutako soluzioaren 50 µl gehitu zitzaizkion, pH alkalinoak bola magnetiko eta antigorputzaren arteko lotura askatzea ahalbidetzen du eta. Nahasketa 15 minutuz inkubatu zen RT-n eta makina irabiagailu batean, askatze prozesua errazteko. Bola magnetikoak imanarekin eutsi ziren eta kromatina eta antigorputz konplexuak

eppendorf berri batean bildu ziren. Prozesua bigarrengo aldi batez errepikatu zen eta bildutako lagina aurrekoarekin elkartu zen eppendorf berberean, 100 μl total batuz.

3.3.3.7. Crosslink-aren desegitea

Hurrengo pausua kromatina eta antigorputz konplexuaren alderantzizko Crosslink-a burutzea izan zen, DNA-ren eta proteinen arteko loturak desegiteko. Horretarako, lagineko 100 μl-ko, 4 μl 5M NaCl eta 5 μl 0.2M EDTA gehitu zitzaizkion eta 3 orduz inkubatu zen 65ºC-tan edo 55ºC-tan ON.

3.3.3.8. DNA-ren purifikazioa

DNA-ren purifikazioa gauzatzeko, lagin bakoitzeko kromatinaren 100 μl-ko, 4 μl pH 6 zuen 2M Tris Buffer-a gehitu zitzaien eta baita 0.5 mg/ml-ko kontzentrazioa zuen RNAseA-ren 2 μl, eta 37ºC-tan inkubatu ziren 30 minutuz, laginetan egon zitekeen RNA kutsatzaile guztia ezerezteko. Ondoren 10 mg/ml-tan zegoen Proteinase K-ren 1 μl gehitu zitzaien eta gutxienez 2 orduz inkubatu zen 55ºC-tan, laginetako proteina guztia desegiteko (ON ere utzi zitekeen). Bukatzeko, laginak Input-aren laginarekin batera gorde ziren -20º-tan, DNA-ren berreskuratzea egin arte.

3.3.3.9. DNA-ren berreskuratzea

Input-aren eta kromatinaren laginen DNA berreskuratzeko eta sekuentziatu ahal izateko behar den purutasuna lortzeko, Agencourt AMPure XP bola magnetikoak erabil ziren. Lagin bakoitzeko 100 μl-ko, 180 μl AMPure gehitu ziren eta pipetarekin nahastu ziren gora eta beheraka 10 aldiz eraginez. Ondoren, nahasketa 10 minutuz inkubatu zen RT-n. Imanaren laguntzaz soberakinak deuseztatu egin ziren eta birritan 70% Etanolaren 500 μl-rekin garbiketak egin ziren. Bigarrengo garbiketaren ostean, imanaren laguntzaz soberakinak baztertu eta bola magnetikoak 5 minutuz lehortzen utzi ziren. Jarraian, bola magnetikoak eta DNA banandu egin ziren “TE Indargetzailea”-ren 50 μl gehituz eta 5 minututan zehar makina irabiagailuan inkubatuz RT-n. Gero, imanen laguntzaz bola magnetikoak harrapatu eta DNA eppendorf berri batean berreskuratu genuen. Badaezpada, bigarrengo aldiz errepikatu genuen prozesua, baina berreskuratutako lehenengo eta bigarrengo DNA laginak banandurik mantendu genituen, lehenengoaren kontzentrazioa altua izango zelako eta bigarrengoa berriz, bestelako probetarako erabiliko zelako. Bukatzeko, berreskuratutako DNA-ren kuantifikazioa NanoDrop espektrofotometroaren bidez egin zen.

3.3.3.10. ChIP-RT-qPCR

Lehendabizi, intereseko geneen DNA sekuentziaren detekziorako hasle espezifikoak diseinatu ziren. Kasu honetan, geneen sustatzaileen eremurako hasle espezifikoak diseinatu ziren. Horretarako mRNA-ren sekuentzia erabili ordez, DNA sekuentzia erabili zen. Horretarako, UCSC Genome Browser-eko “View” → “DNA” aukeran, genearen hasieratik +/-1000kb-ko sekuentzia identifikatu zen eta Primer3 (v 0.4.0) softwarea erabiliz, hasleen diseinua burutu zen (Untergasser eta lank. 2012), aurreko kasuan bezala. 3.8. Taulak hasle guztien sekuentziak jasotzen ditu. Bukatzeko “Blast-Basic local alignment search tool” → “Nucleotide Blast” aukera erabili zen hasleak intereseko geneekiko espezifikoak zirela baieztatzeko (Altschul SF eta lank. 1990).

3.8. Taula. RT-qPCR teknika burutzeko erabili ziren hasleen sekuentzia. mRNA: intereseko geneen mRNA-ren detekziorako hasle espezifikoak. ChIP: intereseko geneen sustatzaileen eremurako hasle espezifikoak

Izena	Genea	Sekuentzia (5' → 3')
TATA binding protein	*Tbp* (ChIP)	(f) CTACCGTGAATCTTGGCTGTAAAC (r) AATCAACGCAGTTGTCCGTGGC
Chromosome 1	Chrm1 (ChIP)	(f) GCCTTCTCCGAAAACAATGCCG (r) TTCCGAGTGGAGCGAGCATGTA

Hasleak liofilizatuta jaso ziren, spin bitartez zentrifugatu eta 100 µM-eko kontzentraziora doitu ziren, behar beste nukleasarik gabeko uretan disolbatuz (stock disoluzioa). Ondoren, lanerako diluzioak prestatu ziren, gene bakoitzaren Forward edo Reverse stock disoluzioen hogei mikrolitro 180 µl nukleasarik gabeko urarekin nahastuz. Stock nahiz lanerako diluzioak hozkailuan mantendu ziren -20ºC-tan.

Intereseko geneen adierazpena modu semi-kuantitaboan analizatu zen Real Time Polimerase Chain Reaction bidez, eta detekziorako iTaq Universal SYBR Green Supermix zunda fluoreszentea erabili zen. Erreakzio bakoitzaren 3 erreplika burutu ziren, P96 plakaren putzu bakoitzean, 20ng cDNA-tik abiatuta eta 10 µl-ko bolumenean. Mixaren osagaien proportzioak honakoak izan ziren: 5 µl iTaq SYBR Green Supermix 2X, 0.15 µl Forward primer-a, 0,15 µl Reverse primer-a, 0.7 µl nukleasarik gabeko ura eta 4 µl cDNA (5ng/µl). Jarraiki, plaka denbora tarte laburrez zentrifugatu eta BioRad etxe komertzialeko CFX96 Real Time PCR detekzio termoziklagailuan sartu zen, primer-en ezaugarrietara egokituriko protokoloarekin. Tenperatura eta denbora zikloak ondorengoak izan ziren: desnaturalizazioa 10 minutu 95ºC-etan; hibridazioa 20 segundo 95ºC-etan eta luzapena minutu 1, 59ºC-etan 39 zikloz; eta azkenik fusio kurba (melting curve) 5 segundo 65ºC-etan.

Emaitzen analisirako, gene bakoitzaren adierazpena bost esperimentu independentetan neurtu zen. Hau horrela, histonarekiko genearen aberastasun mailaren kuantifikazioa inputaren portzentaiaren metodoa erabiliz gauzatu zen. Zehazki, inputaren portzentaiaren analisiarekin hasierako materialaren kopuruarekiko erlatiboa zen, histonaren antigorputzarekin imunoprezipitaturiko DNA kopuruaren kantitatea kalkulatu zen. Behin, Ct balioei inputarekiko doiketa eginda, balio horiek, $2^{-\Delta\Delta Ct}$ bihurtu.

3.3.3.11. ChIP-Sekuentziazioa

Behin ChIP-a burutu eta bertako DNA-ren berreskuratzea egin ostean, DNA fragmentu hauek sekuentziatu genituen, horrela, aurretik zehaztu bezala, gure intereseko proteinak ziren histonak, genomako zein eremu espezifikori uztartzen zitzaizkion identifikatu ahal izateko. Jarraian 3.3.5 eta 3.3.6 ataletan sekuentziazio prozesua laburbiltzen da eta baita sekuentzien analisi bioinformatikoa ere.

3.3.4. Datuen analisi estatistikoa

Tesi honetan aurkezten diren western blot eta era guztietako RT-qPCR tekniketatik eratorritako emaitzen estatistika Student T Proba erabiliz burutu zen eta estatistikoki esanguratsuak kontsideratu ziren $p<0.05$ (*), $p<0.01$ (**) eta $p<0.001$ (***) araua jarraituz.

3.3.5. Sekuentziazioa

Ingelesezko *Next Generation Sequencing* (NGS) edo bigarren belaunaldiko sekuentziazioa, gaur egun, azido nukleikoen sekuentziazio masiboa burutzeko erabiltzen diren teknologia guztiak biltzen dituen hitza da.

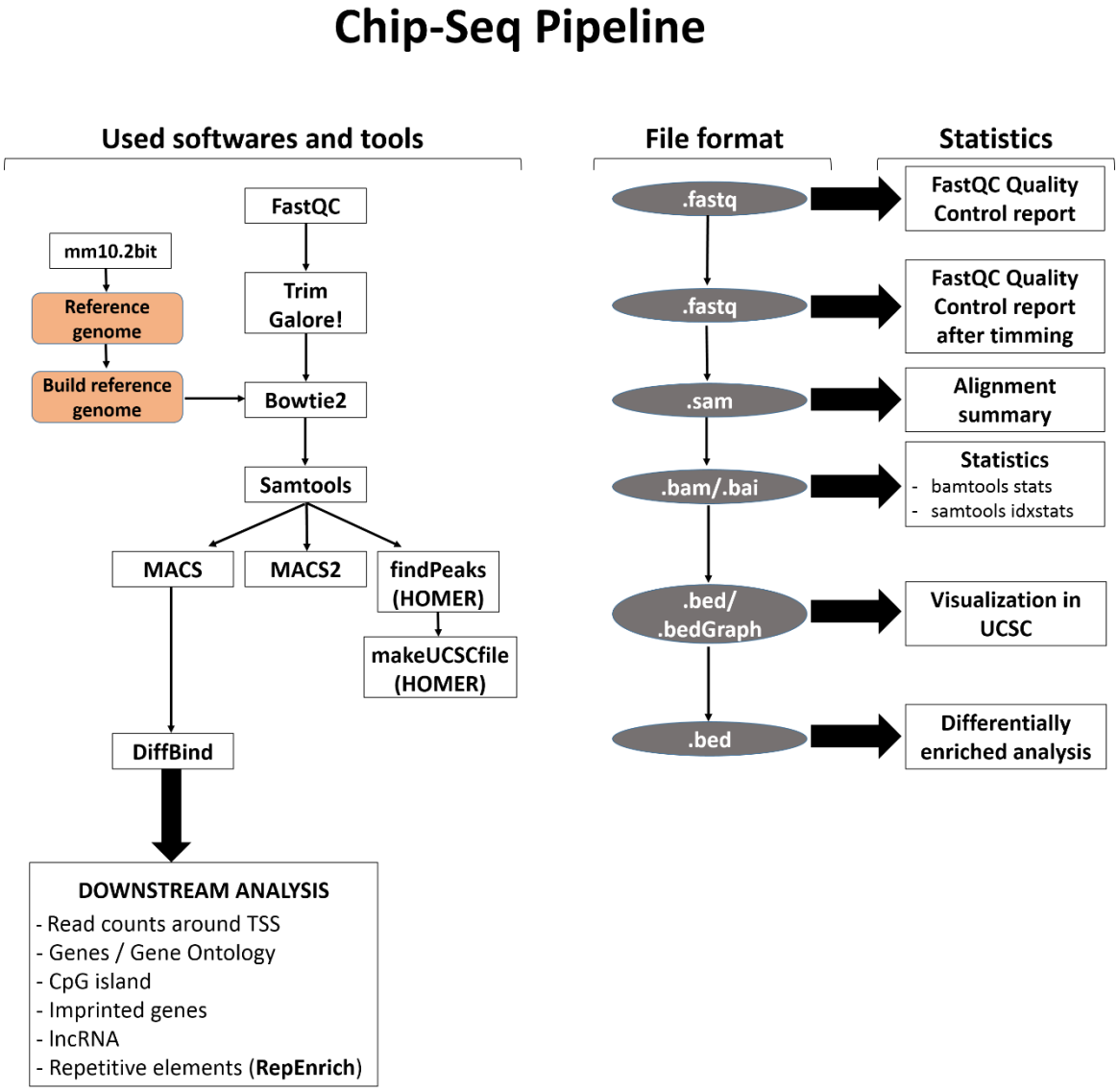
Laburki, sekuentziazio prozesuarekin hasi haurretik DNA eta RNA laginen kalitatezko kontrola egin ohi da, non laginen DNA edo RNA kontzentrazioa eta fragmentuen tamainaren kalitatea neurtzen diren. Ondoren sekuentziatu nahi diren laginen anplifikazio bat egiten da eta prozesu honi laginen liburutegien prestaketa deitzen zaio. Erabiltzen den sekuentziazio plataformaren arabera prozesua ezberdina izaten da. Gure lagin guztietan Illumina HiSeq sekuentziazio plataformak erabili ziren eta beraz, liburutegia sortu edo laginen anplifikazio prozesua burutzeko, DNA edo RNA fragmentuen ertzetan egokitzaile batzuk gehitu zitzaizkien ondoren PCR bitartez laginak anplifikatzeko. Egokitzaileak kasu guztietan TruSeq protokoloak jarraituz gehitu ziren (https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/samplepreps_truseq/truseqchip/truseq-chip-sample-prep-guide-15023092-b.pdf; https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/samplepreps_truseq/ truseq-stranded-mrna-workflow/truseq-stranded-mrna-workflow-reference-1000000040498-00.pdf). Jarraian liburutegien kalitatezko kontrola egin zen, laginen kantitatea eta tamaina aztertuz, eta bukatzeko sekuentziazioa burutu zen.

Gure laginen kasuan liburutegien prestaketa eta sekuentziazio prozesua, kanpo zerbitzuek eskaintzen dituzten sekuentziazio plataformetan egin ziren: H3K27me3 ChIP-a eta RNA denbora ezberdinetako laginen (P1, P2 eta P3) liburutegien prestaketa eta sekuentziazioa Bartzelonako CRG Ikerketa Zentroko Genomika unitatean (Centre of Genomic Regulation) burutu zen (http://www.crg.eu/en/core/programmes-groups/genomics-unit) eta H3K4me2 eta H3K4me3 ChIP-en liburutegien prestaketa eta sekuentziazioa Derioko CiCbioGune Ikerketa Zentroko Genomika unitatean (https://www.cicbiogune.es/research/platforms/genome#instrumentation) gauzatu zen. Aipatzekoa da, aurretiaz zehaztu den bezala, bi zentroetan TruSeq egokitzaileak erabili zirela liburutegien sorrerarako eta Illumina HiSeq 2500 plataforma erabili zela sekuentziazio prozesua gauzatzeko. Lagin guztietan mutur bakarreko sekuentziazioa edo SE (ingelesezko *single end*) burutu zen (DNA zatiak mutur bakarretik sekuentziatuz 5'-tik 3'-ra) eta ezberdintasun bakarra, Bartzelonako CRG-ra bidalitako laginetan sekuentziazioaren irakurketa tamaina 50 nukleotidokoa izan zela, CiCbioGuneko laginetan irakurketa tamaina 51 nukleotidokoa izan zen bitartean. Bestalde, zehaztu beharra dago, RNA-seq esperimentuko laginetan PolyA-n aberasturiko hautaketa egin zela horrela RNA mezulariak bakarrik anplifikatuz. Gainera RNA-seq esperimentuko 6 laginetako bakoitzetik bi erreplika izateaz gain, makinako bi errail edo lerrotan (ingelesezko *Lane*) sekuentziatu ziren (P1_rep2 laginean izan ezik, lagin hau lane bakarrean sekuentziatu zen), 50 mila *read*-eko sekuentziazio minimoa burutuz. Indibidualki mantendu ziren analisi osoan zehar, lerrokatze ostean elkartu genituen arte.

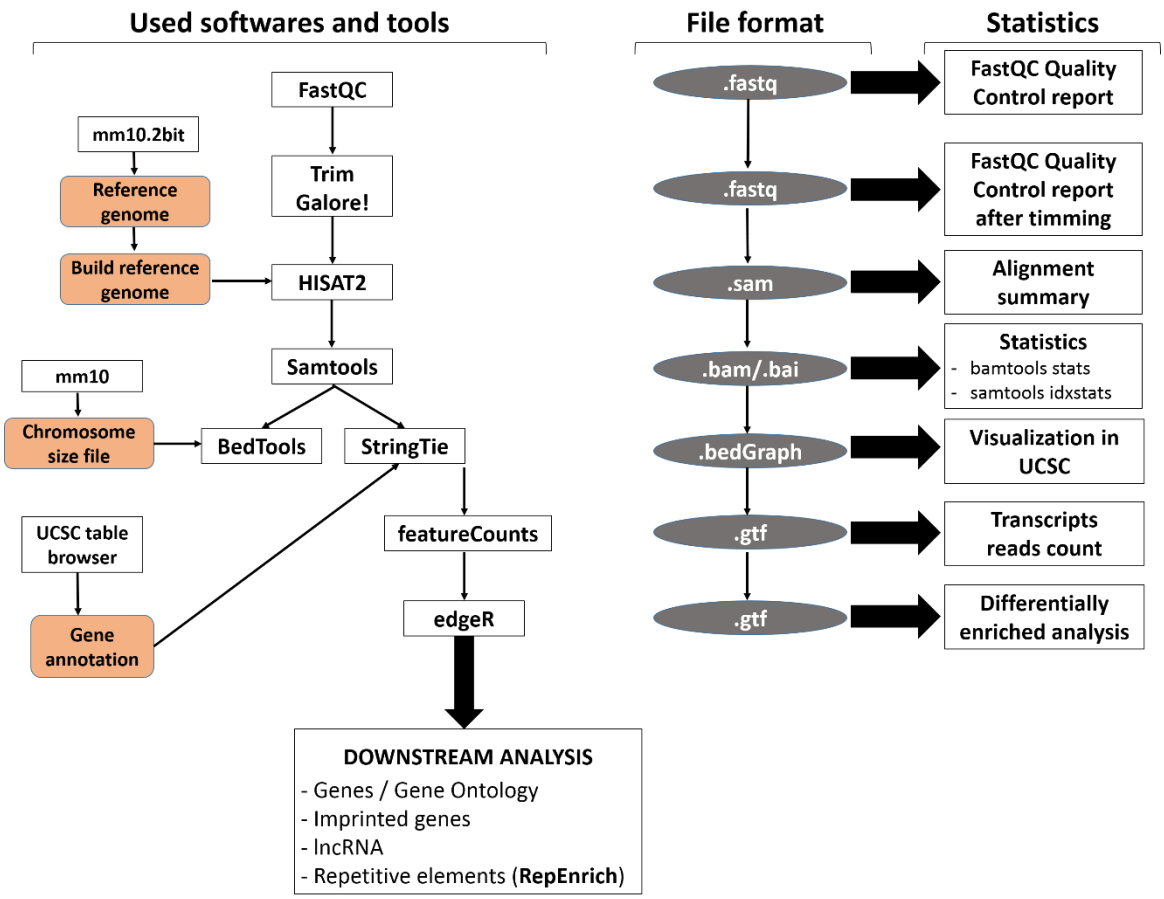
3.3.6. Sekuentziatutako datuen analisi bioinformatikoa

Sekuentziatutako ChIP-Seq eta RNA-Seq datuen analisi bioinformatikoa behetik gorako prozesu bat da, bi pausu orokorretan banatzen dena, behin sekuentziaturiko datu gordinak jaso ostean: lehenengoa, DNA edo RNA fragmentuen sekuentzia motzak (ingelesezko *read*-ak) datu gordinak osatzen dituztenak, genoma osoko sekuentziara mapatu behar dira puzzle antzeko bat osatuz, hau da, *read* bakoitza genomako puntu konkretu batean kokatuz. Eta bigarrengoa, sekuentziaturiko laginen informazio biologikoaren erauzketa izango litzateke, esperimentu batetik bestera eta nahien arabera asko aldatu daitekeena eta hainbat eta hainbat programaren laguntzaz burutzen dena. Analisi bioinformatikoa osatzen duten argibide eta erabili beharreko programak uztartzen dituen protokoloari ingelesezko *pipeline* deitzen zaio. Interneten asko dira prozesua burutzeko eskuragarri dauden sowftware libreak, programatzeko era ezberdinen bitartez osatuak (R, Komando lerroa, Linux, Perl etab.), eta zein programa erabili azaltzen duten blog, foro eta web orrialdeak. Honela, tesiaren helburuetako bat, talde honetan lehendabiziko aldiz, ChIP eta RNA sekuentziazioen pipeline bat osatzea izan da komando lerroan, ahalik eta informazio gehien eskuratzeko.

Gure laginen analisia burutzeko, Euskal Herriko Unibertsitateak eskaintzen duen ARINA plataformaren Katramila zerbitzaria eta bertako kalkulu noduluak erabili genituen. Bestalde laginen ChIP-seq eta RNA-seq esperimentuak saguen zelula ama enbrionikoetan gauzatu zirenez, analisian zehar erabili zen erreferentziazko genoma saguarena izan zen (mm10). Eta analisian zehar jarraitutako pipelinea ondorengo irudietan laburbiltzen da (3.3.Irudia eta 3.4.Irudia), bertan protokoloan zehar erabili ziren software edo tool-en izenak ageri dira, hauei dagozkien fitxategien formatuak, eta pausu bakoitzean gauzaturiko analisi estatistikoak.



3.3.Irudia. ChIP-Seq esperimentuaren analisi bioinformatikoan jarraitutako pipeline edo protokoloa. Irudian ezkerretik eskumara, pausu ezberdinetan erabilitako software edo tresna guztien izenak ageri dira, ondoren programa hauei dagozkien fitxategien formatuak azaltzen dira eta azkenik eskuma aldean pausu bakoitzeko analisi estatistikoa ere laburtzen da.



3.4.Irudia. RNA-Seq esperimentuaren analisi bioinformatikoan jarraitutako pipeline edo protokoloa. Irudian ezkerretik eskumara, pausu ezberdinetan erabilitako software edo tresna guztien izenak ageri dira, ondoren programa hauei dagozkien fitxategien formatuak azaltzen dira eta azkenik eskuma aldean pausu bakoitzeko analisi estatistikoa ere laburtzen da.

3.3.6.1. Datuen formatua

Datu genomikoak gordetzeko hainbat formatu proposatu izan dira, eta gure laginetako sekuentziazio tekniken analisi bioinformatikoan formatu horietako asko erabili ziren. Izan ere, analisiko pausu bakoitzean datuak formatu jakin batean sartu ziren eta beste batean atera. Laburbilduz, datu gordinak .fastq formatuan jaso ohi dira, lerrokatze datuak .sam/ .bam/ .bai formatuan bihurtzen dira eta irudikatze prozesuaren datuak .bed/ .bedGraph/ .gtf/ .bigwig fitxategietan bihurtzen dira. I. Eranskinean analisi bioinformatikoan zehar erabili ziren datuen formatu guztien ezaugarriak zehazten dira.

3.3.6.2. Kalitate kontrola

Sekuentzien datuak jaso eta lehenengo pausua .fastq fitxategietan kalitatezko kontrol orokor bat egitea izan zen, beraien kalitatea ebaluatzeko eta sekuentzietatik aterako ziren emaitzetaz fidatu ahal izateko. Horretarako, "***FastQC***" erabili genuen, kalitatezko kontrola irudikatzeko zabalki onartua dagoen software bat, Babraham Institutuak garatua (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) (Andrews S 2010). ***FastQC***-k bere txostenak burutzeko, datu guztien azpimultzo bat aztertzen du eta bertako emaitzak datu multzo osora estrapolatzen ditu. Txosteneko informazioarekin, sekuentziazio prozesuan arazo teknikorik eman den jakin daiteke. Komando lerroan ***FastQC*** erabiltzeko era honakoa da: seqfile 1… seqfileN, azterturiko fitxategiak.

```
> fastqc seqfile1 seqfile2 .. seqfileN
```

FastQC analisia hainbat modulutan banatzen da jarraian azaltzen direnak eta atal interesgarrienen emaitzak bakarrik erakusten diren arren lagin guztietatik lortu ziren emaitzak "Eranskina" atalean aurkezten dira:

- Laburpena *(Summary)*

Txosteneko lehenengo atalean, analisian zehar ebaztu diren moduluen emaitza orokorrak laburtzen dira, emaitzak normalak (zeinu berdea), apur bat arraroak (zeinu laranja) edo ezohikoak (zeinu gorria) diren adieraziz. Hala ere, nabarmentzekoa da nahiz eta modulu batzuetan zeinu laranja edo gorriak azaldu, honek ez duela esan nahi datuak nahitaez okerrak direla, baizik eta ezohiko datuak direla, laginen izaera biologikoa dela eta. Beraz, analisiko hurrengo pausuetan arreta jartzeko balio dute. Guk aztertu genituen laginek orokorrean, modulu guztietan emaitza normalak izan zituzten (II. Eraskinean 2.1.Irudiak ChIP-seq esperimentuko laginak erakusten ditu eta II. Eranskinean 2.2.Irudiak RNA-seq esperimentuko laginen laburpena biltzen du). Hala ere, baziren gutxi batzuk moduluetan zehar ezohiko balioak azaltzen dituztenak (II. Eranskineko 2.1.Irudian H3K4me3 lagineri dagozkienak). Dena den, analisi bioinformatikoarekin aurrera jarraitu genuen sekuentziatutako lagin guztiekin.

- Oinarrizko estatistikak (*Basic Statistics*)

Modulu honetan aztertutako fitxategiaren oinarrizko informazioaren deskribapen bat azaltzen zaigu: fitxategiaren izena eta formatua, kalitatezko baloreak, prozesatutako sekuentzien kopurua, kalitate txarreko sekuentzia kopurua, sekuentzien tamaina eta sekuentzien %GC-a (II. Eranskineko 2.3 eta 2.4.Irudiak).

- Sekuentzien base bakoitzeko kalitatea (*Per base sequence quality*)

Analisi honek, .fastq fitxategiko posizio bakoitzeko base guztien kalitatezko baloreen ikuspegi orokorra adierazten du. Kalitatearen azterketa egiteko, .fastq dokumentuen ezaugarrietan azaldutako kalitatezko kodeak (Qscore) erabiltzen ditu eta kalitatezko estatistika txostenetan bihurtzen ditu kutxa diagrama bitartez (Y ardatzean). Qscore horiek, nukleotido bakoitza izendatzerakoan egon daitekeen errorearen probabilitatea adierazten dute eta ondorengo formularen bitartez kalkulatzen dira: Q=log-10(P). Horren arabera, nukleotido baten izendapenari Qscore=10 egokitzeak, %10-eko errorea adierazten du eta Qscore=30 egokitzeak, 1000 alditik behin nukleotidoaren izendapena okerra izateko probabilitatea dagoela adierazten du, hau da, prozesuaren doitasuna %99,9-koa dela. Izan ere Qscore-an 30-eko baloreak, errorerik gabeko prozesu bat adierazten du eta erreferentzia-puntutzat hartzen da NGS-an (B. Ewing eta P. Green 1998).

Sortzen den grafikoaren Y ardatza hiru zatitan banatzen da (II. Eranskinean 2.5. Irudiak, ChIP-seq esperimentuko laginak erakusten ditu eta II. Eranskinean 2.6.Irudiak RNA-seq esperimentuko laginen laburpena biltzen du), kalitate oneko baseen izendapena (berdea), zentzuzko kalitatezko izendapena (laranja) eta kalitate urriko izendapenak (arrosa). Kutxa horiek datuen %50 adierazten dute eta errore barrek ezohizko baloreen %75-a. Gure ChIP-seq eta RNA-seq laginen kasuan, sekuentzia guztiak kalitate oneko baseen izendapena egin zela ikus dezakegu, grafikoetako kutxak orokorrean zati berdean irudikatzen direlako. Modulu honetan ematen diren akatsak orokorrean, kalitate urriko Qscore-ei dagozkie, sekuentziazio prozesu luzeen ondorioz, egokitzaileen erabileragatik aldi berean sekuentziatzen diren laginetan edota sekuentziazio prozesuan burbuilak sortu izanaren ondorioz.

- Sekuentzien lauza bakoitzeko kalitatea (*Per tile sequence quality*)

Read bakoitzaren jatorria adierazten duen prozesuko lauzak daude kodifikatuta modulu honetan. Sortzen den mosaiko antzeko grafikoak, Qscore-ak base guztietan zehar ikusteko aukera ematen du, sekuentziazio prozesuko atal konkretu batean kalitatearen galerarik eman den ikusteko. Grafikoak kalitatearen batezbesteko balioaren desbideratzea adierazten du, non kolore hotz edo urdinek kalitate oneko posizioak adierazten dituzten eta bero edo gorriek kalitate gutxiko posizioak adierazten dituzten da (II. Eranskinean 2.7. Irudiak, ChIP-seq esperimentuko laginak erakusten ditu eta II. Eranskinean 2.8. Irudiak RNA-seq esperimentuko laginen laburpena biltzen du). Orokorrean H3K27me3 laginek eta RNA-seq lagin guztiek, guztiz emaitza egokiak ageri zituzten, baina H3K4me2 ta H3K4me3 laginen emaitzetatik ondorioztatu genuen, sekuentziazioan zehar kalitate gutxiko hainbat posizio gertatu zirela, kolore gorrixkarekin adierazita. Grafiko honetan ematen diren akatsen zergatia, sekuentziazio prozesuan sortutako burbuilak, sekuentziazio makinako errailetan egon daitekeen zikinkeria arrastoak edota prozesuan erabilitako laginaren gainkarga izan daitezke.

- Sekuentzia bakoitzeko kalitatezko balioak (*Per sequence quality scores*)

Modulu honetan, sekuentzia bakoitzeko *read*-en batez besteko kalitatea neurtzen da, hau da, orokorrean kalitatezko balio altuak edo baxuak diren adierazten da. X ardatzean kalitatezko balioak azaltzen dira, eta Y ardatzean kalitate hori duten *read*-en kopurua azaltzen da. Gure laginetan orokorrean, sekuentzia guztiek kalitatezko balio altuak zituzten, 37-38 balioetan pilatuak (II. Eranskinean 2.9. Irudiak, ChIP-seq esperimentuko laginak erakusten ditu eta II. Eranskinean 2.10.Irudiak RNA-seq esperimentuko laginen laburpena biltzen du). Azterketa honetan ezohiko balioak, sekuentziazio prozesuan zehar, egokitzaileen erabileraren ondorioz gertatzen dira.

- Sekuentzia bakoitzeko base edukia (*Per base sequence content*)

Grafiko honetan, fitxategi bakoitzeko baseen proportzioa azaltzen da. Orokorrean lau baseen proportzio berdina espero da sekuentziatutako fitxategi bakoitzean, beraz, grafikoan azaltzen diren lau lerroak bata bestearekiko paraleloak izan beharko lirateke. Nabarmentzekoa da, liburutegi konkretu batzuk, *read*-en hasieran alborapen bat sortzen dutela. Honek ez du sekuentzia osoa arriskuan jartzen, baina hainbat nukleotidoen aberastea dakar *read*-en 5' muturrean. Arazo hau, alborapen tekniko bezala sailkatu dezakegu, eta ez da konponduko sekuentzien egokitzaileen mozketaren bitartez. Ondorioz, ez ditu ondorengo analisi bioinformatikoko pausuak oztopatuko baina modulu honen emaitza ezohikoa bihurtu daiteke (zeinu gorria). Hori da gure laginekin gertatzen zena (II. Eranskinean 2.11. Irudiak, ChIP-seq esperimentuko laginak erakusten ditu eta II. Eranskinean 2.12. Irudiak RNA-seq esperimentuko laginen laburpena biltzen du). Orokorrean H3K27me3 eta RNA-seq laginei dagozkien grafikoan, laginek *read*-en hasierako alborapena irudikatzen zutela ikusi dezakegu nahiz eta guztiz lagin arruntak izan. H3K4me2 eta H3K4me3 laginei dagozkien grafikan berriz, alborapen hori ez zen hain nabaria baina, baseak irudikatzen zituzten lerroak ez ziren horren paraleloak, beraz, azken bi lagin hauen emaitzak ezohikotzat sailkatu ditzakegu.

- Sekuentzia bakoitzeko GC edukia (*Per sequence GC content*)

Modulu honek, sekuentzia osoaren GC edukia neurtzen du eta ohiko banaketa edo distribuzioa duen eredu batekin konparatzen du. Liburutegi arrunt batean, ohiko distribuzioa duen GC edukia esperoko dugu, tontor nagusia GC edukiaren balio orokorrarekin bat datorrena. Itxura ezberdineko distribuzioak, kontaminazioa duen liburutegi bat adierazi dezake. Tontor zorrotzek edo distribuzio lauak, kutsatzaile espezifikoen adierazgarri dira, tontor zabalek aldiz, espezie ezberdinen kontaminazioa erakusten dute. Honen harira, gure laginetan ikusi genuen ((II. Eranskinean 2.13. Irudiak, ChIP-seq esperimentuko laginak erakusten ditu eta II. Eranskinean 2.14. Irudiak RNA-seq esperimentuko laginen laburpena biltzen du), H3K27me3 laginek eta RNA-seq laginek ereduaren itxurarekin guztiz bat zetozela, eta H3K4me2 eta H3K4me3 laginak aldiz tontor zorrotzez osatua zeudela, ereduaren itxurarekin alderatuz ezberdintasunak erakutsiz, sekuentziazio prozesuan arazo edo kutsatzaileren bat izan zenaren seinale.

- Base bakoitzeko N edukia (*Per base N content*)

Sekuentziazio prozesuan zehar, base baten izendapena konfiantza osoz egiten ez denean, A, T, G edo C bat idatzi ordez N bat idazten da, eta modulu honek "N" guztien izendapenaren proportzioa kalkulatzen du. Orokorrean balio baxua izaten du azterketa honek, baina balio altuek, baseen izendapenerako datuen interpretazio desegokia adieraziko lukete. Honetarako arrazoirik arruntena, sekuentziazio kalitatearen galera izan ohi da. II.Eranskineko 2.15. eta 2.16. Irudietan azaltzen den bezala (ChIP-seq eta RNA-seq esperimentuetako laginak hurrenez hurren, ez dirudi gure laginetan N izendapenik egon zenik.

- Sekuentzien luzeeraren distribuzioa (*Sequence length distribution*)

Grafiko honek, sekuentziaturiko *read* edo zatien luzeraren distribuzioa adierazten du. Orokorrean *read* guztien luzera 50 nukleotidokoa izan zen, aurretik adierazi bezala (II. Eranskinean 2.17. Irudiak, ChIP-seq esperimentuko laginak erakusten ditu eta II. Eranskinean 2.18. Irudiak RNA-seq esperimentuko laginen laburpena biltzen du), CiCbioGunen sekuentziatu ziren laginetan izan ezik, non *read*-en luzera 51 nukleotidokoa izan zen.

- Sekuentzien bikoizketa maila (*Sequence duplication level*)

Liburutegi arrunt batean, sekuentzia gehienak behin bakarrik azalduko dira. Bikoizketa maila baxu batek, sekuentzien estaldura maila handia adieraziko dute, baina bikoizketa maila altu bat orokorrean PCR-an sortutako gain anplifikazioaren adierazle izan daiteke. Modulu honetan liburutegi osoko sekuentzia bakoitzaren bikoizketa maila neurtzen da, eta grafikoan, bikoizketa maila ezberdinetako sekuentzia kopuruak azaltzen dira. Marra urdinak, sekuentzia guztiak biltzen ditu eta bikoizketa mailaren distribuzioa irudikatzen du. Marra gorriak berriz, sekuentzia guztietatik, zenbat diren desberdinak azaltzen du, hau da, behin bikoiztutako sekuentziak kenduta, geratzen diren bakar horien kopurua. Grafikoaren gainean dagoen portzentajeak bikoiztutako sekuentziak ezabatuz gero geratuko litzatekeen sekuentzien ehunekoa adierazten du. Azterturiko laginetan emaitza arruntak izan genituen (II. Eranskinean 2.19. Irudiak, ChIP-seq esperimentuko laginak erakusten ditu eta II. Eranskinean 2.20. Irudiak RNA-seq esperimentuko laginen laburpena biltzen du), baina bikoizketa maila oso altua ageri zen H3K4me2 laginetan eta batez ere H3K4me3 laginetan. Aipatu bezala, bikoizketak sortzearen arrazoi nagusia, liburutegiko anplifikazio prozesuan sorturiko PCR artefaktuak izango lirateke edota bikoizketa biologikoak. Modulu honetako azterketan ezohiko balioek, sekuentziazio gaitasunaren ekoizpen okerraren seinale dira. Hala ere, aipatu beharra dago, RNA-seq teknikatik sortutako liburutegietan bikoizketa maila, gain-espresatutako edo azpi-espresatutako transkritoen adierazle dela. Eta antzeko zerbait gertatzen da ChIP-Seq teknikatik sortutako liburutegietan, nahiz eta kasu honetan bikoizketa mailak baxuagoak izan ohi diren.

- Gain-irudikatutako sekuentziak (*Overrrepresented sequences*)

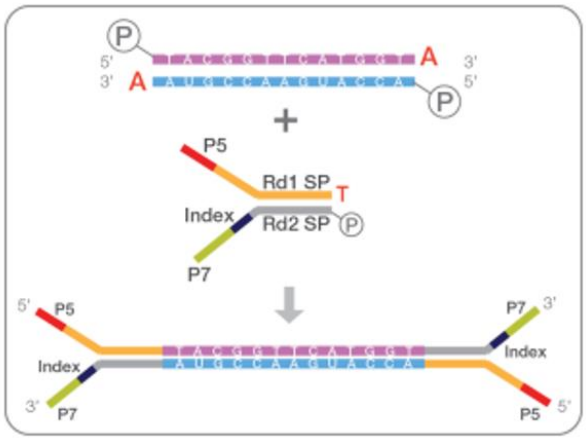
Sekuentziatutako liburutegietan, askotariko sekuentziak aurkitzen dira eta orokorrean, ugaritasunagatik ez da bat ere ez nabarmentzen. Sekuentzia baten gain-espresio deigarriak, biologikoki oso garrantzitsua dela adierazi dezake, edota liburutegian kontaminazio bat dagoela. Modulu honetan beraz, sekuentzien totaletik %0.1 baino gehiagoagotan azaltzen diren sekuentzien zerrenda bat irudikatzen da. Gainera errepikatzen diren nukleotidoen sekuentzia horiek, ohiko kutsatzaileen datu base batekin konparatzen ditu. Askotan kutsatzaile horiek, sekuentziazioan erabili diren egokitzaileak izaten dira eta modulu honetan detektatzen baditu, egokitzaileen izena ere adieraziko digu. Hori da azterturiko laginekin gertatu zitzaiguna ChIP-seq esperimentuaren kasuan (II. Eranskinean 2.21. Irudiak, ChIP-seq esperimentuko laginak erakusten ditu), izan ere, ***FastQC*** analisiak adierazten zuenez sekuentziazio prozesuan zehar TruSeq kit-eko egokitzaileak erabili ziren. RNA-seq laginetan ordea, nahiz eta sekuentziazioa burutu duen zerbitzuak egokitzaileak erabili zituela adierazi zigun, ***FastQC*** analisiko atal honetan ez zen egokitzaileen presentziarik aurkitu, detektatu ezinezko kantitate edo portzentaia baxuetan zeudenaren erakusle.

- Egokitzaileen edukia (*Adapter content*)

Aurretik aipatu dugun bezala, Illumina sekuentziazio plataformak erabiltzen direnean, liburutegien prestakuntzan, lagin bat baino gehiago elkartu ohi dira, eta sekuentziazio prozesuan ezberdindu ahal izateko lagin bakoitzari egokitzaile espezifiko bat gehitzen zaio. Modulu honetan, Illumina etxe komertzialeko sekuentziazio egokitzaile erabilienak detektatzen dira batez ere, eta analisi bioinformatikoko ondorengo pausuetan egokitzaile horiek ezabatu behar diren edo ez jakiteko balio du. Gerta liteke, ohiko egokitzaileak erabili ez izana gure laginetan bezala eta beraz, nahiz eta grafiko honetan tontorrik ez ikusi egokitzaileen presentzia izatea (II. Eranskinean 2.22. Irudiak, ChIP-seq esperimentuko laginak erakusten ditu eta II. Eranskinean 2.23.Irudiak RNA-seq esperimentuko laginen laburpena biltzen du).

3.3.6.3. Egokitzaileen mozketa

Bigarrengo pausua, egokitzaileen mozketa gauzatzea izan zen. Egokitzaileak, DNA-azko oligonukleotido artifizialak dira, aurretik aipatu bezala, Illumina sekuentziazio plataformek prozesua burutzeko beharrezkoak dituztenak. Illuminaren liburutegia prestatzerako orduan, egokitzaileak DNA sekuentzieri itsasten zaizkie (3.5.Irudia), era horretan lagin ezberdinak batera sekuentziatu ahal izateko. Egokitzaileak 30 base inguruko zati laburrak izaten dira, *read*-en 5’ eta 3’ muturretan kokatzen direnak eta horrek askotan sekuentziazio Q-Score balioa beharrezkoa baino txikiagoa izatea dakar. Gainera, ***FastQC*** analisiko gain-irudikatutako sekuentzien moduluan, hainbat egokitzaile erabili zirela ere ikusi genuen. Beraz, analisi bioinformatikoarekin jarraitu aurretik erabakirik zuzenena egokitzaileak moztu edo ezabatzea izan zen, ondorengo pausuetan arazorik ez izateko eta batez ere sekuentzien lerrokatze kalitatea hobetzeko.



3.5.Irudia. Egokitzaileak DNA *read*-eri itsasteko prozesua. Illuminako "TrueSeq adapter" protokoloko irudiaren egokitzapena.

Egokitzaileak mozteko erabili zen tresna "***Trim Galore!***" izan zen, kalitatea automatizatu, egokitzaileak moztu eta kalitatezko kontrola berriz egiteko balio duen lanabesa, Babraham Insitutoak garatutakoa (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore) (Krueger F 2012). Tresna honek liburutegia sortzeko erabili zen "TruSeq adapter" egokitzaileak hauteman, ezabatu eta FastQC analisia berriz ere egitea ahalbidetzen du. "TruSeq adapter" egokitzaileen zerrenda, Illuminako web orrian dago erabilgarri ondorengo estekan: https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/experiment-design/illumina-adapter-sequences-1000000002694-09.pdf (20 eta 21. orrialdeetan).

Komando lerroan Trim_Galore! erabiltzeko era honakoa da: -a, moztu nahi genuen egokitzaile sekuentzia zehatza "AGATCGGAAGAGCACACGTCTGAACTCCAGTCA" (Illumina web orriko gomendioa jarraituz, https://support.illumina.com/bulletins/2016/12/what-sequences-do-i-use-for-adapter-trimming.html); -q, egokitzaileak moztu eta kalitate urriko muturrak ere mozteko; -e 0.05, gehienez onartzen den errore proportzioa; --length 50, aukeratutako tamaina baino motzagoak bihurtzen diren *read*-ak ezabatzeko aukera, --fastqc, FastQC analisia berriz ere egiteko aukera egokitzaileen mozketa burutu ondoren; --gzip, sortu ziren fitxategi berriak konprimatzeko aukera; filename(s), azterturiko fitxategiak.

```
> trim_galore [options] <filename(s)>
```

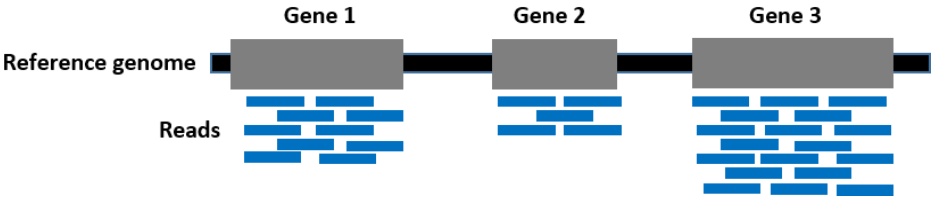
Behin, egokitzaileen mozketa amaituta prozesuaren txosten bat sortu zen, non besteak beste, laginen *read* kopurua, egokitzaileak zituzten *read* kopurua eta behin egokitzaileak moztuta, luzeera urria erakutsi zuten *read*-ak zehaztu ziren (III. Eranskinean 3.1. Taula eta 3.1 Irudia). Ikus dezakegun bezala, sekuentziazio prozesuan egokitzaileak erabili ziren, nahiz eta ez ziren gure laginetan kopuru handian aurkitu.

Azaldutako aurreko bi pausuak berdinak dira era guztietako sekuentzientzat, baina lerrokatzea eta puntu honetatik aurrerako pausu guztiak, aztertzen hari garen sekuentziekiko espezifikoak izan ziren, hau da, ChIP-seq sekuentziak izan edo RNA-Seq sekuentziak izan, pausu eta tresna ezberdinak erabili ziren analisi bioinformatikoa gauzatzeko.

--ChIP-Seq--

3.3.6.4. ChIP-Seq sekuentzien lerrokatzea

Behin kalitatezko analisia eta egokitzaileen mozketa prozesuak burututa, hurrengo pausua lagin bakoitzeko sekuentziak erreferentziazko genomari lerrokatzea izan zen. Ezinbesteko urrats honekin, sekuentzietako *read*-ak genomako posizio espezifiko batera esleitzen dira, erreferentziazko genoma gida bezala erabiliz (3.6.Irudia). Horrela sekuentzietako *read* bakoitzari genomako posizio edo koordenatu bat uztartzen zaio puzzle bateko pieza bat izango balitz bezala.



3.6.Irudia. Laginetako *read*-ak erreferentziazko genomarekin lerrokatzea.

Lerrokatzea gauzatzeko lanabes asko daude gaur egun, sekuentziazio plataformetako elkarteek sortuak, edo software libreko beste hainbeste Interneteko erabiltzaileek sortuak. ChIP-Seq laginetarako erabilienak, ***BWA*** (Li H eta Durbin R 2009), ***Bowtie1*** (Langmead B eta lank. 2009) eta ***Bowtie2*** (Langmead B eta Salzberg S 2012; Langmead eta lank. 2018) dira, lerrokatzea osatzeko Burrows-Wheeler Transformazioa (BWT) erabiltzen dutenak (Burrows M eta Wheeler D 1994). Gure laginetarako ***Bowtie2*** software-a aukeratu genuen, lerrokatze prozesua era sentikor eta bizkorrago batean egiten duelako memoria gutxiago erabiliz eta eguneratuen dagoen software-tako bat delako (http://bowtie-bio.sourceforge.net/bowtie2/manual.shtml).

Aipatu beharra dago, lerrokatzea burutzeko aukeratutako erreferentziazko genoma saguaren genomaren azken bertsio egonkorra izan zela: 2011ko abenduko (GRCm38/mm10) saguaren genomaren muntaia (mm10, Genome Reference Consortium Mouse Build 38 (GCA_000001635.2)). Azken hau, Mouse Genome Sequencing Consortium eta National Center for Biotechnology Information (NCBI), arteko elkarlanean ekoiztu zen.

Lerrokatze prozesuarekin lanean hasi aurretik, aukeratutako erreferentziazko genoma indexatu behar izan genuen, horrela ***Bowtie2***-k *read*-ak bizkorrago bildu eta lerrokatu ahal izateko. Horretarako, UCSC genomako nabigatzailetik, saguaren genoma deskargatu genuen (http://hgdownload.soe.ucsc.edu/goldenPath/mm10/bigZips/). Fitxategi honetan saguaren genomaren sekuentzia osoa biltzen da 2bit formatuan (mm10.2bit). .2bit fitxategitik genomaren informazioa .fasta formatuan erauzteko "***twoBitToFa***" tresna (http://hgdownload.cse.ucsc.edu/admin/exe/linux.x86_64/) erabili genuen komando lerroa jarraian adierazten da: input.2bit, saguaren genomaren fitxategia; output.fa, emaitza fitxategia:

```
> twoBitToFa input.2bit output.fa
```

Erreferentziazko genoma indexatzeko, ***Bowtie2*** softwareko "***bowtie2-build***" aukera espezifikoa erabili genuen, aurreko pausuan erauzitako genomaren .fa fitxategiarekin, komando lerro honen bitartez: -f, genomaren fitxategia .fasta formatuan zegoela adierazteko aukera; <reference_in>, genomaren fitxategia; <bt2_index_base>, ematiza fitxategia.

```
> bowtie2-build [options]* <reference_in> <bt2_index_base>
```

Komando honen bitartez erreferentziazko genomaren aurkibide antzeko bat sortu genuen, .bt2 formatuan zeuden 6 fitxategik osatua. Hauek guztiak beharrezkoak ziren lagin bakoitzaren *read*-ak erreferentziazko genomarekin lerrokatzerako orduan.

Ondoren, lagin bakoitzeko lerrokatzea burutu genuen komando lerroaren bitartez, ondorengo aukerekin: -q, lerrokatu nahi diren fitxategiak .fasta edo .fq formatuan daudela adieraztea; -x, erreferentziazko genomaren fitxategia; -U, lerrokatu nahi diren fitxategiak; -S, emaitza fitxategiaren izena.

```
> bowtie2 [options]* -x <bt2-idx> {-1 <m1> -2 <m2> | -U <r>} [-S <sam>]
```

Behin prozesua bukatuta, ***Bowtie2*** software-ak lerrokatze maila laburtzen duen txosten bat ere sortzen du. Bertan, ***Bowtie2***-k lerrokatzeko erabili dituen *read* kopuruak, 0 aldiz, behin eta behin baino gehiagotan lerrokatu ziren *read*-ak eta lerrokatze proportzio orokorra azaltzen dira zehaztuta, lagin bakoitzeko (IV.Eranskina 4.1 Taula). Lerrokatze maila orokorra begiratuz gero, ikusi genuen ChIP-seq laginek lerrokatze proportzio egokia izan zutela, azken bi sekuentziak izan ezik. Hauetan lerrokatze maila %20 eta %45 bitartean kokatu zen, oso lerrokatze maila urria. Izan ere, ***FastQC*** analisiak aurreikusi zigun bi lagin hauekin nolabaiteko arazo bat izan zela sekuentziazio prozesuan zehar. Beraz, emaitza hauek ikusita erabaki genuen H3K4me3 histonaren laginen sekuentzien analisia bertan behera utzi eta aurrerago esperimentua berregin eta berriz ere sekuentziatzea, emaitza aberatsago batzuk lortzeko helburuarekin.

Ondoren, lerrokatze fitxategien formatua aldatu genuen .bam formatura, aldez aurretik azaldu bezala .sam fitxategiaren kopia binarioa izanik, memoria gutxiago okupatu eta beraz, lan egiteko errazagoa delako. .sam/.bam fitxategiak manipulatzeko "***Samtools***" programa erabili genuen (http://samtools.sourceforge.net/) (Li H eta lank. 2009; Li HA eta lank. 2011). Programa honek .sam formatuekin lan egiteko aukera ematen digu: .bam formatura aldatu, hauek ordenatu, nahastu, indexatu etab. .sam/.bam fitxategien arteko aldaketa gauzatzeko hurrengo komandoa erabili genuen, ondorengo aukerekin: -b, emaitza fitxategia .bam formatuan lortzeko aukera; -S, hasierako fitxategia .sam formatuan zegoela adierazteko aukera; -o, emaitza fitxategiaren izena; in.sam, hasiera fitxategia .sam formatuan.

```
> samtools view [options] <in.bam>|<in.sam>|<in.cram> [region ...]
```

Ondoren .bam fitxategiko posizio datuak antolatu genituen, kromosoma zenbakiaren arabera lehenengo eta bigarrenez posizio koordenatuak ezarriz. .sorted.bam fitxategiak sortzeko erabili genuen komando lerroa hau izan zen: -o, emaitza fitxategia; in.bam, hasiera fitxategia .bam formatuan.

```
> samtools sort [options...] [in.bam]
```

Azkenik, .bam fitxategiaren manipulazioarekin bukatzeko, .sorted.bam fitxategia indexatu genuen, hurrengo komando lerroa erabiliz: -b, .bai formatuko aurkibidea sortzeko aukera; in.bam, hasiera fitxategia .sorted.bam formatuan; out.index, emaitza fitxategia.

```
> samtools index [-bc] [-m INT] <in.bam> [out.index]
```

ChIP-Seq sekuentzien analisi bioinformatikoaren protokolo ezberdinek, Samtools lan-tresna erabiltzea gomendatzen dute lerrokatzearen estatistiko ezberdinak egiteko, ***Bowtie2*** software-

ak egindako laburpen txostenaren osagarriak izan daitezkeenak. Era berean, -sorted.bam eta .bai fitxategiek, informazioa era egokian mantentzen dutela baieztatzeko balio zigun.

Horietatik lehendabizikoa, .sorted.bam fitxategiak lerrokatze prozesutik gordetzen zituen estatistikoak aztertzea izan zen. Hauek samtools programaren "***bamtools stats***" tresnaren bitartez lortu genituen, ondorengo komando lerroaren bitartez: -in<filename>, hasierako fitxategiaren izena; >output.txt, datu estatistikoak biltzen dituen fitxategia.

```
> bamtools stats [-in <filename> -in <filename> ... | -list <filelist>]
[statsOptions]
```

.sorted.bam fitxategitik eratorritako datu estatistikoak (IV. Eranskina 4.2. Taula), aurreko datuekin alderatu daitezke, lerrotze mailak %96-97 balioetan mantentzen direla ikusteko.

Hurrengo analisi estatistikoa .sorted.bai fitxategitik eratorri genuen, non lerro bakoitzean kromosoma ezberdinetan lerrokatutako *read* kopurua azaltzen zen. Erabilitako komando lerroa honakoa izan zen: in.bam, hasierako fitxategiaren izena; >output.txt, emaitzekin sortutako fitxategia.

```
> samtools idxstats <in.bam>
```

Sortutako .txt fitxategiko emaitzekin kromosoma bakoitzeko *read* kopurua irudikatzen zuen grafikoa egin genuen, kromosoma guztietan zehar hainbat milioiko *read*-en aberastea genuela baieztatuz (IV. Eranskina 4.1 Irudia).

Lerrokatzeko azken analisi estatistikoa "***Picard***" programaren bitartez egin genuen "***CollectAlignmentSummaryMetrics***" aukera erabilita, (https://broadinstitute.github.io/picard/). Programa hau erabili ahal izateko lehendabizi erreferentziazko genomaren aurkibide propio bat sortu behar izan genuen, ondorengo komando lerroaren bidez: R, genomaren fitxategia ***Bowtie2***-arekin erabili genuena; O, sortutako fitxategia.

```
> picard CreateSequenceDictionary R=reference.fasta O=reference.dict
```

Ondoren, analisi estatistikoa burutu genuen, hurrengo komando lerroaren bitartez: INPUT, hasierako fitxategia; OUTPUT, emaitza fitxategia; REFERENCE_SEQUENCE, genomaren fitxategia.

```
> picard CollectAlignmentSummaryMetrics R=reference_sequence.fasta
I=input.bam O=output.txt
```

Picard programak lerrokatze prozesuari buruzko estatistiko ugari aztertzen ditu bere analisian. Guzti horietatik guretzat esanguratsuenak aukeratu genituen: berriz ere, *read* kopurua, lerrokatze *read*-ak eta lerrokatze maila deskribatzen dira, baina baita desdoitze maila (erreferentziazko genomarekin bat ez datorren base kopuruaren tasa), errore maila (lerrokaturiko *read*-etatik erreferentziazko genomarekin bat ez datozen base kopurua) eta INDEL maila (txertatze eta ezabatze gertaeren kopurua lerrokatutako 100 baseko). Guzti hauek maila oso baxuetan aurkitu genituen, beraz, ondorioztatu genuen gure sekuentziak era egokian lerrokatu zirela erreferentziazko genomara (IV. Eranskinaren 4.3. Taula).

3.3.6.5. ChIP-Seq Tontorren antzematea eta irudikapena

Chip-Seq sekuentzien analisi bioinformatikoaren hurrengo pausua eta garrantzitsuena, proteinaren (gure kasuan histonen) loturaren ondorioz esanguratsuki aberastutako genomaren toki edo posizioak (inglesezko *Binding sites*) antzematea izan zen (ingelesezko *Peak calling* bezala ezagutzen den prozesua). Toki edo posizio horiei "tontor" deitu diegu, eta immunoprezipitaturiko proteina genomari lotzen zaion tokia adierazten dute. Urteetan zehar hainbat programa garatu dira tontorren antzematea gauzatzeko, eta duela gutxi horietako 30-en alderaketa argitaratu zuten artikulu zientifiko batean (Thomas eta lank. 2016). Bertan deskribatzen duten bezala, orokorrean transkripzio faktoreek, tontor mehe eta zorrotzak agertzen dituzte. Histonek eta batez ere heterokromatinari lotzen direnak aldiz, tontor lodiagoak azaltzen dituzte guztiz definitu gabeak. Beste aldetik, lan zaila da estatistikoki esanguratsuak diren tontorrak identifikatzea.

Gure kasuan hiru tontor izendatzaile programa ezberdin erabili genituen, histonen modifikazioetarako parametroak propio ezarrita. Programa horiek, ***MACS*** (Model-based Analysis for ChIP-Seq, Zhang eta lank. 2008), ***MACS2*** (Model-based Analysis for ChIP-Seq version 2.0, Zhang eta lank. 2008) eta ***findPeaks*** (HOMER suite, Heinz S eta lank. 2010) izan ziren. Laburki, MACS programak dituen algoritmoek genomaren konplexutasuna neurtzen dute, ChIP eremu aberastuak estatistikoki esanguratsuak diren edo ez aztertzeko, horrela lotura eremuen erresoluzioa hobetuz (http://liulab.dfci.harvard.edu/MACS/). ***MACS2*** programa aldiz, aurrekoaren bertsio berrituagoa da eta tontor zorrotzen edo lodien arteko bereizketa burutzea ahalbidetzen du (-broad aukera) (https://github.com/taoliu/MACS/). Azkenik, ***HOMER*** plataformak findPeaks programa du bere barnean tontorren antzematearen analisia egiteko (http://homer.ucsd.edu/homer/ngs/peaks.html#Variable_length). Azterketa egiteko aukeran dituen 7 moduluen artean, histonen eremuak aztertzeko espezifiko bat eskaintzen du (-histone aukera), edota zabalera aldakorreko eremuak bilatu ditzake gure datuen arabera moldatu daitezkeenak (-variable aukera).

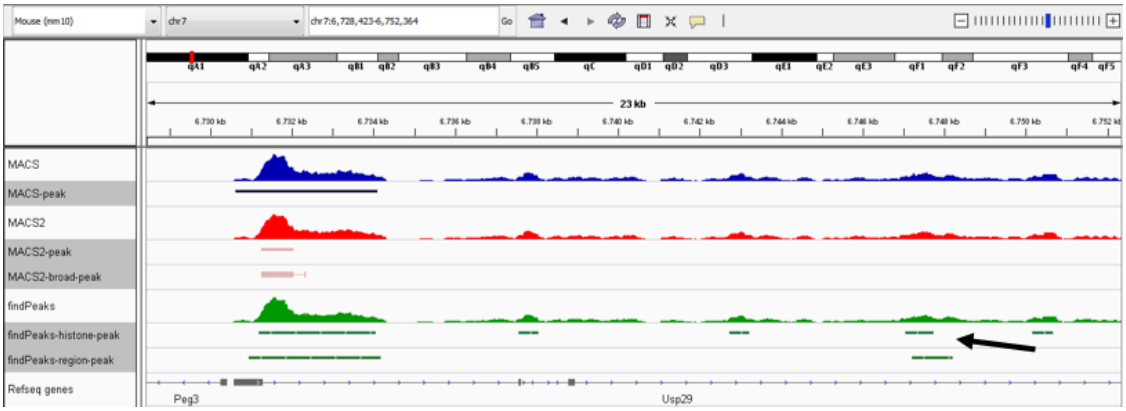
Guztiak martxan jarri ostean, alde batetik, detektaturiko tontorren eremuak mugatzen dituzten koordenatuak biltzen dituzten .bed fitxategiak alderatu genituen (3.9.Taula) eta beste aldetik, genomaren nabigatzaileetan irudikatzeko .bedGraph fitxategiak ere konparatu genituen (3.16.Irudia), emaitza egokienak identifikatzeko asmoarekin. Honenbestez, .bed fitxategiak aztertuz, ikusi genuen ***MACS*** eta ***MACS2***-ko aukera ezberdinen artean lagin bakoitzeko lortu genuen tontor kopurua maila beretsuan zegoela. Baina, ***HOMER*** plataformako findPeaks trenak detektaturiko kopurua aldiz, hainbat bider handiagoa zen, azken honetan tontorrak identifikatzeko erabilitako parametroak baliokideak ez zirela ondorioztatuz. Gainera, ***MAC***s eta ***MACS2***-ko aukeretan tontorren autemate prozesuan igoera bat antzeman genuen, bi programen arteko algoritmoen ezberdintasunek tontorren kopurua aldarazten zutela ondorioztatuz.

3.9.Taula. Tontorren autematearen emaitzen laburpena. Tontorren autematea burutzeko erabilitako tresna bakoitzetik eratorritako tontor kopurua azterturiko laginetan.

FITXATEGIAK	MACS	MACS2	MACS2-BROAD PEAK	FINDPEAK-HISTONE	FINDPEAK-VARIABLE
c_27me3_rep1	10305	13232	13167	121905	49635
c_27me3_rep2	13778	18447	19030	167181	72026
m_27me3_rep1	8681	10277	10870	113973	48486
m_27me3_rep2	12282	15595	16442	165416	69519
c_me2	3884	3960	3557	144753	20708
m_me2	5492	3062	3271	125279	9412

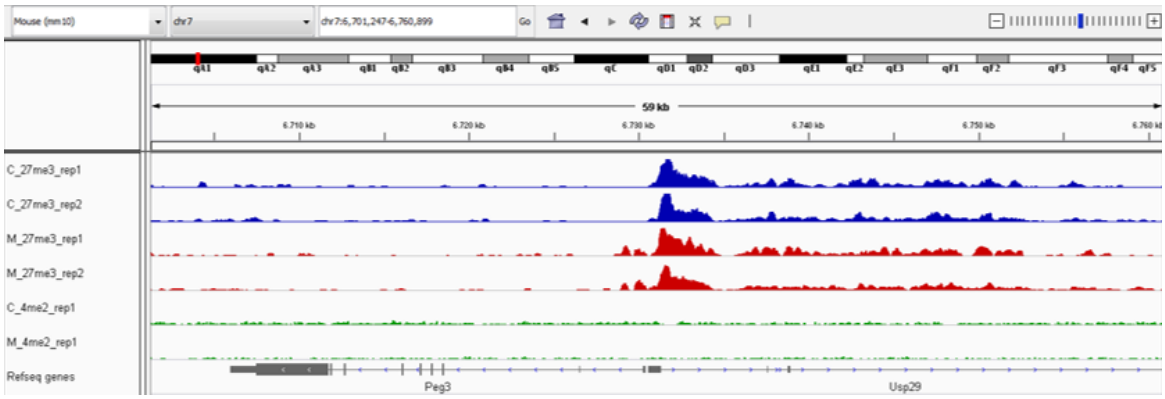
Hala ere, guzti hauetatik tontorren detekzio egokiena zeinek egin zuen erabakitzeko, aipatu bezala, emaitzak genomaren nabigatzailean irudikatu genituen, zer nolako aberastasun patroiak adierazten zituzten aztertzeko (.bedGraph fitxategiekin) eta baita tontorren antzematea era egokian egin zen egiaztatzeko (.bed fitxategiekin). Ondorengo irudian (3.7.Irudia), gure sekuentzietako baten .bedGraph eta .bed fitxategi guztiak ikusi ditzakegu, tontorren identifikazio egokiena ***MACS*** tresnarekin egin zela ondorioztatuz. Izan ere, ***MACS***-etik lortutako .bed fitxategiko tontorrak ziren, .bedGraph fitxategiko tontor eremura hobekien egokitzen zirenak, tontor eremu osoan zehar hedatuz eta kutsadura baztertuz.

Azterketa honen ondorioz, ***MACS*** tresnaren emaitzak erabili genietuen sekuetziazioaren analisi bioinformatikoan aurrera jarraitzeko, aipatu bezala, tontorren eremuan behar bezala hedatzen zirelako eta kutsadura edo atzealdeko zara izan zitezkeen tontorrak bereizten zituelako.



3.7.Irudia. Tontorren antzematearen emaitzen irudikatzea genoma nabigatzailean. Tontorren antzemate prozesuan erabilitako tresna guztien emaitzak azaltzen dira lagin bakar batean (.bedGraph eta .bed fitxategiak). Goitik behera, MACS eta MACS-en tontor fitxategia (urdinez), MACS2 eta MACS2-ren tontor fitxategiak, berariazko parametroekin eta –broad aukerarekin (gorriz), findPeaks eta findPeaks-en tontor fitxategiak, histonen parametro bereziekin eta tontor aldakorretarako gomendaturiko parametroekin (berdez) eta erreferentziazko genoma (grisez). Gezi beltza kutsadura edo atzealdeko zarata biltzen duen tontor okerra da.

Gainera, tontorren irudikapenaren bitartez, sekuentziek profil egokiak zituzten ala ez ikusi konprobatu genuen. Horretarako genoma nabigatzailean lagin guztien .bedGraph fitxategiak kargatu genituen (3.8.Irudia). H3K27me3 histonaren kasuan, ikusi genuen sekuentziek tontorren profil nahiko garbiak azaltzen zituztela, histonarekiko aberastasun uneak agerian utziz. Eta baita bi erreplikak nahiko antzekoak zirela bai kontrol laginetan eta baita morfinarekin trataturiko laginetan. H3K4me2 kasuan, ez genuen tontorrik aurkitu, ez irudian azaltzen den genomaren eremuan ezta arakatu genituen genomako beste edozein eremutan ere. Emaitza hori dela eta, erabaki genuen H3K4me2 histonaren laginen sekuentzien analisia bertan behera utzi eta aurrerago esperimentua berregin eta berriz ere sekuentziatzea, emaitza aberatsago batzuk lortzeko helburuarekin.



3.8.Irudia. MACS programarekin tontorren antzematearen emaitzen irudikatzea genoma nabigatzailean. Tontorren antzemate prozesuan erabilitako MACS tresnaren emaitzak azaltzen dira, azterturiko sekuentzietan (.bedGraph fitxategiak). Goitik behera, H3K27me3 kontrol laginak (urdinez), H3K27me3 morfinarekin trataturiko laginak (gorriz), H3K4me2 kontrol eta morfinarekin trataturiko laginak (berdez) eta erreferentziazko genoma (grisez).

Behin lagin ezberdinetako tontorrak antzemanda, lagin bakoitzeko bi erreplikak komunean zituzten tontorrak identifikatu ziren, eta erreplika komun horiek bakarrik erabili ziren ondorengo azterketetan. Horretarako, R plataformako "***Bioconductor***" software-ak eskaintzen duen ChIPpeakAnno (Zhu, Gazin eta lank. 2010) tresna erabili genuen "***findOverlapsOfPeaks***" funtzioa erabilita.

3.3.6.6. ChIP-Seq lotura bereizgarridun tontorren antzematea

MACS programaren bitartez identifikaturiko tontorrak aztertzeaz gain, lan honetan lotura bereizgarridun tontorrak ere antzeman ziren (ingelesezko, *Differentially expressed*). Horretarako, ez daude lan tresna ugari erabilgarri Interneten, eta gainera ez dago jarraitu daitekeen protokolo zehatzik. Gure kasuen R plataformako Bioconductor softwar-eak (https://bioconductor.org/) eskaintzen digun pakete bat erabili genuen lotura bereizgarridun tontorren antzematearen analisia burutzeko, "***DiffBind***" paquete hain zuzen ere (Stark R eta Brown G 2011; Ross-Innes CS eta lank. 2012; http://www.bioconductor.org/packages/release/bioc/html/DiffBind.html). DiffBind-ek, identifikaturiko tontor eremuak, lerrokatutako *read*-en datuekin biltzen ditu, DNAren aberastasuna neurtzeko azterturiko proteinan. Horretarako 4 pausu jarraitzen ditu:

- Tontorren irakurketa: lehenengo pausu honetan, esan bezala ***MACS*** programarekin lortutako tontorrak, lerrokatzean sortutako .bam fitxategiekin elkartu ziren, lagineko erreplika guztien kontsentsu tontor zerrenda bat osatuz, *dba* aukera erabiliz.
- *Read*-en zenbatzea: behin lagineko erreplika guztien kontsentsu tontot zerrenda osatu ondoren, programak lerrokatzeko .bam fitxategiak aztertzen ditu tontor bakoitzean ageri diren *rea*d-ak zenbatzeko. Emaitza bezala afinitate matrize bat sortzen da, *read*-en balio normalizatuekin protinarekin lotura dagoen eremu bakoitzeko. Honetarako `dba.count` aukera erabiltzen da.
- Lotura bereizgarridun tontorren analisia: laginen artean, estatistikoki esanguratsuak diren lotura bereizgarridun tontorren identifikazioa gauzatzen du. Horretarako kontraste bat ezartzen da laginak bi taldetan banatuz (`dba.contrast` aukera erabiliz), eta estatistika gauzatzeko "***edgeR***" pakete estatistikoa erabiltzen du (TMM normalizazioarekin, ingelesezko *trimmed mean of Mvalues* (Robinson eta Oshlack,

2010), identifikatutako lotura bereizgarri bakoitzari p eta FDR balio bat emanez (*dba.analyze* aukera erabiliz).

- Amaierako txostena: bertan lortutako lotura bereizgarridun tontorren emaitzak azaltzen dira adierazgarritasunaren balioarekin eta datu estatistikoekin.

Gure kasuan, kontraste talde bezala Kontrol eta Morfina taldeak erabili ziren eta analisi estatistikoan zehar "block" aukera erabili genuen, egun ezberdinetan batutako errepliken arteko lotura kontuan izateko. Normalizatutako datuen distribuzio edota osagai nagusien analisiaren (ingelesezko *Principal Component Analysis* edo PCA) grafikak aztertu genituen, TMM metodoa erabili ondoren datuek distribuzio normalagoa erakusten zutela ondorioztatuz. Gainera PCA grafikoaren bitartez, kontrol eta morfina laginen erreplika biologikoen trinkotasuna ikusi genuen eta tratamenduaren ondoren laginen bi profil ezberdinak agerikoak zirela ere identifikatu genuen (emaitzen ataleko ……. Irudian agertzen dira grafiko hauek).

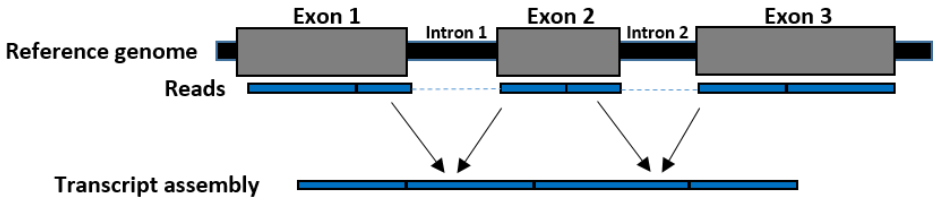
3.3.6.7. ChIP-seq tontorren izendatzea

Jarraian, aurreko bi pausuetan antzemandako tontor orokorren zerrenda eta lotura bereizgarridun tontorren zerrenda, gene espezifikoekin eta ezaugarri genomikoekin korrelazioan jarri genituen, horretarako R plataformako Bioconductor softwareak eskaintzen duen ***ChIPseeker*** (Yu G eta lank. 2015) lanabesa erabiliz. Aipatu beharra dago, bertan mm10 saguaren genoma erabili zela erreferentziazko genoma bezala, UCSC-tik eratorria. Zehazki, ChIPseeker tresnak eskaintzen duen "***annotatePeak***" funtzioa erabili genuen eta gure laginetako tontor bakoitzarekin bat zetorren promotore, 5'UTR, 3'UTR, exoi, introi, genearen amaierako eremua eta gene arteko eremuak identifikatu ziren, eta baita ezaugarri genomiko horietatik hurbilen aurkitzen zen genearen izena ere. Bestalde, promotoreen eremuei buruzko informazioa ezagutzeko, transkripzioaren hasiera puntuen (TSS) inguruan (+/- 3000bp), gure laginen *read*-ek sortzen zuten antolamendu profilak identifikatu genituen "***plotAvgProf***" funtzioa erabiliz.

--RNA-Seq--

3.3.6.8. RNA-Seq sekuentzien lerrokatzea eta irudikapena

Transkriptomaren analisiak funtzio desberdinak ditu, orokorrean bi taldetan banatuak: alde batetik, transkrito berrien aurkikuntza eta lerrokatzea, eta bestetik RNA-ren kuantifikazioa. ChIP-seq teknikarekin lortutako sekuentziak erreferentziazko genomarekin lerrokatzea lan erraza den arren, DNA genomikoa posizio konkretu batean kokatzean besterik ez datzalako; RNA-seq sekuentziak lerrokatzea prozesu konplexuagoa da. Izan ere, RNA-seq teknikan RNA mezulariaren molekulak aztertzen dira, bertako introiak "splicing" prozesuaren bitartez ezabatu direnak. Hori dela eta, izan liteke *read* asko jarraian dauden bi exoiekin bat etortzea eta beraz, introietan zehar hedatuta azaltzea, 10000bp edo gehiagoko tarte batez bereiztuta (3.9.Irudia). Exoi aniztasun honen kasua sekuentzia guztiko >%35-tan eman daiteke. Ondorioz, exoi aniztasuna gauzatzen duten *read*-ak lerrokatzea prozesu korapilatsu batean bihurtzen da.



3.9. Irudia. RNA-Seq laginetako *read*-ak erreferentziazko genomarekin lerrokatzea, splicing prozesua ahalbidetuz, transkritoak osatzeko.

Lerrokatzea gauzatzeko lanabes asko daude gaur egun, eta horietatik erabilienak ***topHat2*** (Kim D eta lank. 2013), ***STAR*** (Dobin A eta lank. 2013) eta ***Hisat2*** (Kim D, Langmead B eta Salzberg SL 2015; Pertea M eta lank. 2016) dira. TopHat2 oso motela da lerrokatzeak gauzatzerako orduan eta STAR programak aldiz memoria behar handiak izaten ditu lerrokatzea burutzeko. Hisat2 softwareak aldiz, memoria gutxiago erabilita, lerrokatze azkarra egin ahal izateko, aurkibide sistema berria proposatzen du, Burrows-Wheeler Transformazioa (BWT) erabiliz (Burrows M eta Wheeler D 1994). Izan ere, bi aurkibide mota sortzen ditu lerrokatzea egiterako orduan: genoma osoko aurkibide orokor bat eta aurkibide lokal txiki ugari. Honen bestez, gure laginetarako Hisat2 software-a aukeratu genuen, lerrokatze prozesuan exoi eta introien arteko splicing-na kontuan izaten duelako era bizkorrago batean eta memoria gutxiago erabiliz (https://ccb.jhu.edu/software/hisat2/index.shtml).

Lerrokatzea burutzeko aukeratutako erreferentziazko genoma, ChIP-seq sekuentziak lerrakatzerakoan erabili genuen berdina izan zen GRCm38/mm10. Eta lerrokatze prozesuarekin lanean hasi aurretik, kasu honetan ere, erreferentziazko genoma indexatu behar izan genuen, horretarako, UCSC genomako nabigatzailetik, saguaren genoma deskargatuz eta .fa formatura bihurtuz (3.3.6.4. atalean).

Erreferentziazko genoma indexatzeko, Hisat2 softwareko "***hisat2-build***" aukera espezifikoa erabili genuen, genomaren .fa fitxategiarekin, komando lerroaren bitartez: <reference_in>, genomaren fitxategia; <ht2_index_base>, emaitza fitxategiaren izena.

```
> hisate2-build [options]* <reference_in> <ht2_index_base>
```

Komando honen bitartez erreferentziazko genomaren aurkibidea sortu genuen, .ht2 formatuan zeuden 8 fitxategik osatua. Hauek guztiak beharrezkoak dira lagin bakoitzaren *read*-ak erreferentziazko genomarekin lerrokatzerako orduan. Ondoren, lagin bakoitzeko lerrokatzea burutu genuen komando lerroaren bitartez: --dta, sortutako transkritoak hurrengo pausuetan irakurri ahal izateko aukera; -q, lerrokatze fitxategiak .fasta edo .fq formatuan zeudela adierazteko aukera; -x, erreferentziazko genomaren fitxategia; -U, lerrokatu nahi genituen fitxategien izenak; -S, emaitza fitxategiaren izena.

```
> hisat2 [options]* -x <ht2-idx> {-1 <m1> -2 <m2> | -U <r>} [-S <sam>]
```

Behin prozesua bukatuta, Hisat2 software-ak lerrokatze maila laburtzen duen txosten bat ere sortzen du. Bertan, Hisat2-k lerrokatzeko erabilitako *read* kopuruak, 0 aldiz, behin eta behin baino gehiagotan lerrokatu diren *read*-ak eta lerrokatze proportzio orokorra azaltzen dira zehaztuta, lagin bakoitzeko (V. Eranskinean 5.1 Taula). Lerrokatze maila orokorra begiratuz gero, ikus dezakegu orokorrean RNA-seq laginek lerrokatze proportzio egokia izan zutela, %93-94 bitartekoa.

Jarraian, lerrokatze fitxategien formatua aldatu genuen .sam formatutik .bam formatura, aurretik aipatu bezala samtools tresna erabilita. Bestalde, sekuentziazioaren atalaren hasieran aipatu bezala, RNA-seq esperimentuko laginak bi lane ezberdinetan sekuentziatu ziren. Guk laginen datu horiek era indibidualean mantendu genituen lerrokatzea burutu arte, lerrokatzean ahalik eta emaitza hoberenak lortzeko asmoz. Baina behin puntu honetara iritsita, laginetako errepliken bi lane-ak elkartu egin genituen, horretarako berriz ere samtools programa erabiliz. Zehazki, kasu honetan "***samtools merge***" aukera erabili genuen, lerrokatzean osatu genituen errepliken lane bakoitzeko .bam fitxategiak batzeko, hurrengo komandoa erabiliz: <out.bam>, emaitza fitxategiaren izena; <in1.bam>…<inN.bam>, nahasteko .bam fitxategien izenak.

```
> samtools merge [-nurlf] [-h inh.sam] [-b <bamlist.fofn>] <out.bam> <in1.bam>
[<in2.bam> ... <inN.bam>]
```

Ondorengo ChIP-seq laginen kasuan deskribatutako komando lerro berberak erabiliz, .bam fitxategiak ordenatu egin genituen lexikografikoki (samtools sort aukera), .sorted.bam formatura pasatuz eta azkenik .sorted.bam fitxategien aurkibideak ere sortu genituen (samtools index aukera).

RNA-seq laginetan ere lerrokatzearen analisi estatistiko batzuk gauzatu genituen, ChIP-seq-aren kasuan erabilitako lan tresna berdinekin. Lehenik eta behin ***bamtools stats*** erabili genuen erreplika bakoitzean nahastutako bi lane-etako datuekin eratutako .sorted.bam fitxategietan lerrokatzearen datu estatistikoak biltzeko (V. Eranskinean 5.2 Taula). Ikusi dezakegunez, bi lane-tako informazioa nahasteak lerrokatze maila altua mantentzea ekarri zuen.

Jarraian .sorted.bai fitxategietatik eratorritako datu estatistikoak bildu genituen, "***samtools idxstats***" aukeraren bitartez, kromosoma ezberdinetan lerrokatutako *read* kopurua egokia zen aztertzeko (V. Eranskinean 5.1. Irudia). Era honetan egiaztatu genuen, lagin guztietan *read*-en estaldura egoki bat mantentzen zela kromosoma guztietan zehar, eta beraz, analisiarekin aurrera jarraitzea egokia zela.

Lerrokatzeko azken analisi estatistikoa ***Picard CollectAlignmentSummaryMetrics*** aukerarekin egin genuen, aurretik azaldu bezala. RNA-seq laginekin ere, *read* kopurua, lerrokatze *read*-ak eta lerrokatze maila, desdoitze maila, errore maila eta INDEL maila aztertu genituen (V. Eranskina 5.3. Taula). Azken hiruek, maila oso baxuetan aurkitu genituen, beraz, ondorioztatu genuen gure sekuentziak era egokian lerrokatu zirela erreferentziazko genomara.

Froga estatistiko guztiak egin ondoren, RNA-seq esperimentuko datuen irudikapenerako fitxategiak prestatu genituen, "***bedtools***" programaren "***genomecov***" aukera erabiliz (https://bedtools.readthedocs.io/en/latest/content/tools/genomecov.html). Honek, .bam fitxategia .bedGraph fitxategian bihurtzen du, horrela gure laginek genoman duten estaldura edo aberastasuna ikusi ahal izateko genoma nabigatzaileetan. Lan hau burutzeko beharrezko komando lerroa honakoa da: -ibam, bihurtu nahi dugun fitxategiaren izena; -bg, bedGraph fitxategia sortzeko aukera; -g, erreferentziazko genoma; -split, geneen introietan azaltzen zaigun informazioa ez ikusarazteko aukera.

```
> bedtools genomecov [OPTIONS] -i <bed/gff/vcf> -g <genome>
```

3.3.6.9. RNA-Seq lerrokatzeen mihiztatzea eta zenbaketa

RNA-seq analisiaren pausu garrantzitsuenetako bat lerrokatzeen mihiztatzean datza, hau da, erreferentziazko genomarekiko puzzle itxuran lerrokatu diren *read* horiek batu egiten dira, geneen isoforma guztiak berreraikiz eta isoforma horien ugaritasun erlatiboa kalkulatzen da prozesu honetan. Pausu hau burutzeko hainbat eta hainbat programa dauden arren, guk "***StringTie***" aukeratu dugu, Johns Hopkins Unibertsitateak garatua (Pertea M eta lank. 2016), lehenengo, .sorted.bam fitxategietan lerrokatutako *read*-ak gene lozietan taldekatzen dituena eta ondoren lokus bakoitzean bertako datuak azaltzeko behar beste isoforma mihiztatzen dituena (https://ccb.jhu.edu/software/stringtie/index.shtml?t=manual). Gainera prozesua burutzeko erabiltzen duen algoritmoak lan azkarragoa egiten du beste programa batzuekin konparatuta. StringTie erabiltzen hasi aurretik, UCSC Genoma Nabigatzaileak eskaintzen duen "***Table Browser***" tresnarekin, mm10 saguaren erreferentziazko genomaren datuak bildu genituen .gtf formatuan, horrela *read*-en mihiztatze prozesuan gidari bezala erabili ahal izateko. Honenbestez, mihiztatze prozesua burutzeko erabilitako komando lerroa honakoa izan zen: -G, erreferentziazko genomaren fitxategia; -o, sortutako fitxategiaren izena; <aligned_reads.bam>, .sorted.bam fitxategien izena.

```
> stringtie <aligned_reads.bam> [options]*
```

Prozesu honen ostean, lortu ziren fitxategietan, gure laginetan identifikaturiko transkrito guztien informazioa biltzen zen, adibidez, genomako posizio zehatza, genearen izena etab. Baina transkrito horietako bakoitzaren ugaritasuna aztertzeko, amaierako analisian konparatu nahi ziren laginen fitxategi guztiak nahasten zituen bilduma bat sortzea beharrezkoa zen. Izan ere, bilduma hori, konparatu nahi ziren laginetako gene eta isoforma guztiez osatuta egongo zen eta horrek ondorengo transkrito bakoitzaren ugaritasunaren zenbaketa erraztuko luke. Horretarako, "***StringTie --merge***" aukera erabili genuen ondorengo komando lerroaren bitartez: -G, erreferentziazko genomaren fitxategia; -o, emaitza fitxategia.

```
> stringtie --merge [Options] { gtf_list | strg1.gtf ...}
```

Jarraian, lagin bakoitzeko lerrokatzeen mihiztatze datuekin eta sortu berri genuen bilduma fitxategiekin gene bakoitzeko isoformen ugaritasuna zenbatu genuen R plataformako "***Bioconductor***" software-ak eskaintzen duen "***subread***" pakete estatistikoko "***featureCounts***" softwarea erabiliz (Liao Y eta lank. 2014). Tresna hau, Walter and Eliza Hall Bioinformatics, Ikerketa Medikoko Institutuan sortu zen eta lerrokatutako *read*-ak zenbatzeko erabili ohi da, ezaugarri genomikoekin erlazionatuz, adibidez, exoiak, sustatzaileak, genen gorputza etab. Gure kasuan erabili genuen komando lerroa ondorengoa izan zen: -a, aurreko pausuan sortutako bilduma fitxategia ; -o, sortuko den emaitza fitxategia; input_file1,2,3…, konparatu nahi genituen fitxategi guztiak.

```
> featureCounts [options] -a <annotation_file> -o <output_file> input_file1 [input_file2]
```

Ondorioz, taula bat lortu genuen, non konparatu nahi genituen laginetan, geneen transkrito bakoitzak zuen ugaritasun balioa adierazten zen.

3.3.6.10. RNA-Seq adierazpen bereizgarridun transkritoen antzematea

RNA-seq esperimentuetako helburu orokorra adierazpen bereizgarria duten geneak identifikatzea da lagin ezberdinen artean edota tratamenduen efektua ikusteko. Horretarako ezinbestekoa da gene bakoitzaren adierazpena zuzen kuantifikatzea ondoren adierazpen mailak laginen artean alderatzeko inolako akatsik egin gabe. Datuen normalizazioari buruz, hainbat eta hainbat lan daude metodo ezberdinak konparatzen dituztenak laginen erreplika teknikoak eta biologikoak kontuan hartuta. Lehendabizi proposatu zen metodoa RPKM normalizazioa izan zen (ingelesezko *Reads Per Kilobase per MIllion mappable reads*), zeinak lerrokatutako milioi bat *read*-eko kilobase bakoitzeko *read*-en kopurua hartzen duen kontutan (Mortazavi A eta lank. 2008). Horrela geneen RPKM balioak laginen artean konparagarri bihurtzen dira, geneen adierazpen erlatiboaren ranking antzeko bat sortuz. Geroztik, metodo ezberdin asko garatu dira, hala nola, milioiko dauden transkritoak kontuan hartzen dituena edo TPN (inglesezko Tags/Transcirpts per million; Wagner GP eta lank. 2012), lerrokatutako milioi bat *read*-eko kilobase bakoitzeko zatiak kontuan hartzen dituena edo FPKM (inglesezko Fragments Per Kilobase of transcript per Milion mapped *read*s; Trapnell C eta lank. 2010), errail bakoitzeko goiko kuartileko zuzenketa metrikoa edo UQUA erabiltzen duena (ingelesezko *per-lane upper quartile correction metric*; Bullard JHeta lank. 2010) edota balioen batazbeztekoarekin lana egiten duena edo TMM (ingelesezko *trimmed mean of M values*; Robinson eta Oshlack 2010).

Bestalde, adierazpen bereizgarria kalkulatzeko orduan algoritmo ezberdinak garatu dira. Hasieran Poisson-en eredua erabiltzen bazen ere, gaur egun eredu desegokitzat jotzen da. Horren ordez, eredurik erabilienak distribuzio binomial negatiboa duten eredu parametrikoak dira hainbat aldaketekin, adibidez edgeR (Robinson MD eta lank. 2010), DESeq (Ander eta Huber 2010) edota bayseq (Hardcastle TJ eta Kelly KA 2010). Oraindik ere ez dago jarraitu beharreko protokolo zehatzik ezta algoritmo zuzenena zein den argituko duen pipeline-rik (Bullard JH eta lank. 2010; McGettigan PA 2013), eta beraz, gure kasuan edgeR erabiltzea erabaki genuen bioinformatika arloan duen harrera ona dela eta. "***edgeR***" (https://bioconductor.org/packages/release/bioc/html/edgeR.html), R plataformako "Bioconductor" software-ak eskaintzen duen pakete estatistiko bat da, esan bezala, distribuzio binomial negatiboa erabiltzen duena geneen adierazpen bereizgarria kalkulatzeko.

Lanean hasteko "***featureCounts***" lan tresnarekin lortutako geneen transkritoen ugaritasun balioen taula erabili genuen eta edgeR-ren erabilera gida jarraitu genuen. Aipatzekoa da laginak TMM ereduarekin normalizatu zirela eta baita, egun ezberdinetan batutako errepliken arteko lotura kontuan izan genuela, kasu honetan "batch" eran agertzen dena jarraitu genuen erabilera gidan. Honenbestez, normalizazioaren ondoren datuen distribuzioan egokiak zirela eta PCA-k konparaketa guztietan kontrol eta morfina laginen bereizketa garbia zela baieztatu genuen (emaitzen ataleko ……. Irudian agertzen dira grafiko hauek).

3.3.6.11. Ezaugarri genomikoen izendatzea

Behin ChIP-seq analisiko tontor orokorren eta lotura bereizgarria zuten tontorren zerrenda geneekin izendatu ondoren eta RNA-seq esperimentuko adierazpen bereizgarria zuten geneen zerrenda lortuta, lehendabizi bi esperimentuetako emaitzekin, lagin bakoitzeko gene taldeen funtzio biologikoak aztertu ziren. Gaur egun, hainbat eta hainbat tresna daude eskuragarri, eta guk Gene Ontology Consortium (GO, http://geneontology.org/) lanabesa erabili genuen geneen ontologia burutzeko. Horrela, gene zerrenda bakoitzari zegokion funtzio biologikoen

informazioa jaso genuen (Ashbuner eta lank. 2000; GO Consortium 2017), aberastasun horren balioa Fisher-ren test zehatzarekin ebatziz eta Bonferroniren zuzenketa ezarriz ($p<0.05$). gene espezifikoekin ondoren.

Ondoren, lortutako geneen zerrendetako datu guzti horiek, ezaugarri espezifikoekin korrelazioan jarri genituen, CpG irlak, inpronta genomikodun geneak, kodifikatu gabeko RNA luzeak (lncRNA) edota elementu errepikakorrak identifikatuz besteak beste.

- CpG irlak

Gure ChIP-seq laginetako tontorretan, CpG irlak aztertzeko erreferentziazko zerrenda berri bat sortu behar izan genuen CpG irlen koordenatuen datuak biltzeko. Honenbestez, UCSC-ko "***Table Browser***" funtzioaren bitartez, mm10 saguaren genomaren ezaugarrien taldea "Expression and Regulation" aukerarekin sortu genuen zerrenda. Ondoren CpG irlen inguruko eremuak mugatu ziren <2kb distantziara "Shore" eremuak izendatuz, <4kb distantziara "Shelves" eremuak izendatuz eta geratzen zen genomako eremua "Open Sea" bezala izendatuz. Behin CpG irlen erreferentziazko zerrenda sortu ondoren, gure laginen tontorrekin korrelazioan jartzeko, "BEDTools" (Quinlan and Hall 2010) tresnak eskaintzen duen "***intersect***" aukera erabili genuen (https://bedtools.readthedocs.io/en/latest/content/tools/intersect.html). "***Bedtools***" tresna erabiltzeko komando lerroa honakoa da: -wa, konparatu genituen lehen fitxategiaren datuak mantentzeko aukera; -wb, konparatu genituen bigarren fitxategiaren datuak mantentzeko aukera; -a, konparaketako lehen fitxategiaren izena; -b, konparaketako bigarren fitxategiaren izena; >output, emaitza fitxategia.

```
> bedtools intersect [OPTIONS] -a <bed/gff/vcf/bam> -b <bed/gff/vcf/bam>
```

Honenbestez, gure laginen tontorren koordenatuen eta erreferentziazko zerrenda horien koordenatuen gainjartze puntuak identifikatu genituen eta era berean, zein CpG irlari zegozkien identifikatu genuen. Emaitza hauek "Gazta diagrama"-ren bitartez irudikatu ziren.

- Inpronta genomikodun geneak

Bestalde, ChIP-Seq laginetako tontorretan eta RNA-Seq analisiko geneen zerrendan, inpronta genomikodun geneak aztertzeko, berriz ere erreferentziazko zerrenda berri bat sortu behar izan genuen, gene hauen koordenatuen datuak biltzeko. Zeregin hori burutzeko, hainbat informazio iturri erabili ziren: the Geneimprint database (http://geneimprint.com), Catalogue of Parent of Origin Effects (http://igc.otago.ac.nz) eta hainbat eta hainbat arikulu (Peters J eta Beechey C 2003; Tunster JS eta lank. 2013; Autuoro JM eta lank. 2014). Inpronta genomikodun geneen zerrenda bat osatu ostean, UCSC-ko "***Table Browser***" futzioa erabili genuen, koordenatuak jasotzeko. Horrela, "identifiers(names/accesions)" aukeran osaturiko geneen zerrenda itsatsi zen, eta azkenik amaren edo aitaren aldeko adierazpena zuten geneen kategoria eskuz gehitu zen. Aipatzekoa da, inpronta genomikodun geneen informazioa oraindik eta osoagoa izateko, Imperial College London Unibertsitateko Petra Hajkova garapeneko epigenetikan katedradunak, gene hauen DMR/ICR koordenatuen zerrenda elkarbanatu zuela gurekin. Behin inprota genomikodun geneen erreferentziazko zerrendak sortu ondoren, ChIP-Seq laginen tontorrekin eta RNA-Seq geneekin korrelazioan jartzeko, "***BEDTools intersect***" (Quinlan eta Hall 2010) erabili genuen CpG irlekin egin genuen komando lerro berbera jarraituz. Era honetan, gure laginen

tontorren koordenatuekin eta RNA-seq esperimentuko transkritoekin bat zetozen inpronta genomikodun geneak identifikatu genituen eta emaitzak "Gazta diagrama"-ren bitartez irudikatu ziren.

- lncRNA-k

Jarraian, lncRNA-k aztertzeko, hirugarrengo erreferentziazko zerrenda bat sortu behar izan genuen. Kasu honetan, zerrenda guk osatu ordez, deskribatuta dauden gizakia eta saguen genomako lncRNA guztiak biltzen dituen datu base bat aurkitu genuen: LncRBase (Chakraborty S eta lank. 2014; http://bicresources.jcbose.ac.in/zhumur/lncrbase/index.html). Saguaren kasuan 83201 sarrera ditu eta orokorrean lncRNA transkritoen oinarrizko informazioa biltzen du, hainbat ezaugarrirekin, adibidez, kokapen genomikoa, ncRNA txikiekiko gainjartzea (ingelesezko *small non conding RNA* edo sncRNA), erlazionatutako elementu errepikakorrak eta inpronta genomikodun geneak, eta lncRNA-en sustatzaileari buruzko informazioa. Guk bertako zerrenda erabili genuen, ChIP-Seq laginen tontorrekin korrelazioan jartzeko, "***BEDTools intersect***" (Quinlan and Hall 2010) bitartez. Behin ChIP-Seq esperimentuen lncRNA bereizgarrien zerrendak genituenean, lortutako emaitzak "Gazta diagrama" bidez irudikatu genituen. RNA-seq laginei dagokienez, ez ziren lncRNA-k aztertu, izan ere, sekuentziazio prozesuan PolyA hautaketa erabili genuenez RNA mezulariaren informazioa bildu genuen, hau da, exoiekiko aberastasuna bakarrik. Beraz, lncRNA-k aztertuz gero, ez genuke informazio guztia edukiko, lncRNA exonikoak identifikatuko genituzke, lncRNA intronikoak ordea ez.

- Elementu errepikakorrak

Gure laginen tontorrean aberasturiko elementu errepikakorrak identifikatzeko jarraituriko prozesua ezberdina izan zen. Elementu errepikakorren izaera dela eta, hau da, leku batean baino gehiagotan mapeatzen direla (ingelesezko *multiple mapped reads*), protokolo orokorra aldatu beharra dago eta elementu hauen analisirako espezifikoki sortutako programak erabili. Gure kasuan RepEnrich2 programa erabili genuen (Criscione SW eta lank. 2014; https://github.com/nerettilab/RepEnrich2), non *read*-ak bi taldetan banatzen diren, lerrokatze bakarra edo anitza duten arabera. Lerrokatze bakarreko *read*-ak elementu errepikakorrekin gainjartzen dira eta lerrokatze anitza dutenak elementu errepikakorren azpifamiliekin erlazionatzen dira. Horrela programak, jarraipen bat egiten dio *read* bakoitza lerrokatzen den elementu errepikakor guztiei eta sistematikoki ugaritasunaren zenbaketa burutzen da.

Elementu errepikakorren analisiarekin hasteko, lehenengo pausua erreferentziazko zerrenda osatzea izan zen, kasu honetan ere USCS-ko "***Table Browser***" funtzioa erabiliz lortu genuena. Bertan mm10 saguaren genomaren ezaugarrien taldean "***Variation and Repeats***" aukeratu genuen, "***RepeatMasker***" zerrenda osatzeko, genomako elementu errepikakorren kokapenaren datuekin. Ondoren ChIP-seq eta RNA-seq analisi orokorretan ***Bowtie2*** eta ***hisat2*** tresnekin egin genuen bezala, lerrokatze prozesuko erreferentziazko genoma indexatu egin genuen, horretarako elementu errepikakorren erreferentziazko zerrenda eta "***RepEnrich2_setup.py***" aukera erabiliz. Agindu hau gauzatzeko jarraitu genuen komando lerroa honako izan zen: <reference_RepeatMasker>, UCSC-tik lortu genuen elementu errepikakorren erreferentziazko zerrenda; <reference_genome>, mm10 genomaren fitxategia; --is_bed, TRUE edo FALSE aukerak elementu errepikakorren erreferentzia zerrenda .bed formatuan zegoela adierazteko.

```
> RepEnrich2_setup.py <reference_RepeatMasker> <reference_genome> --is_bed
```

Behin elementu errepikakorren erreferentziazko zerrenda indexatu ondoren, hau berau lerrokatze prozesuan erabili genuen, lagin guztien .fastq fitxategiekin, aurretik aipaturiko ***Bowtie2*** tresna erabiliz eta komando lerro berberarekin. Jarraian, lerrokatzetik sortutako .sam fitxategiak, protokolo orokorreko pausuak jarraituz, .bam formatura pasatu, ordenatu .sorted.bam formatuan eta aurkibidea .bai formatuan egin genituen, "samtools" programa erabiliz. Jarraian, lerrokatze bakarreko *read*-ak eta lerrokatze anitza zuten *read*-ak banandu genituen "***RepEnrich2_subset.py***" aukera erabiliz, hurrengo komando lerroaren bidez: <in.bam>, aztertu nahi genuen fitxategia; --mapq-value, lerrokatze bakarreko *read*-ak, lerrokatze anitzeko *read*-ekin elkartzeko balioa, ***Bowtie2*** tresnaren kasuan gomendagarria 30 izanik; --pairedend, TRUE edo FALSE aukerak sekuentziazio prozesua mutur bietako edo bakarrekoa izan zen adierazteko.

```
> RepEnrich2_subset.py <in.bam> --mapq-value –-pairedend
```

Behin lerrokatze bakarreko eta anitzeko fitxategiak sortu ondoren, "***RepEnrich2.py***" funtzio nagusia erabili genuen elementu errepikakorren ugaritasunaren zenbaketa burutzeko. Horretarako, komando lerro hau erabili genuen: <reference_genome>, mm10 genomaren fitxategia; <sample_multimap>, aurreko pausuan sortutako lerrokatze anitzeko fitxategia; <sample_unique>, aurreko pausuan sortutako lerrokatze anitzeko fitxategia; --is_bed, TRUE edo FALSE aukerak elementu errepikakorren erreferentzia zerrenda .bed formatuan zegoela adierazteko; --pairedend, TRUE edo FALSE aukerak sekuentziazio prozesua mutur bietako edo bakarrekoa izan zen adierazteko.

```
> RepEnrich2.py <reference_RepeatMasker> <sample_multimap> <sample_unique> --
is_bed --pairedend
```

Beste fitxategi batzuen artean, sortu zen garrantzitsuena elementu errepikakorren ugaritasuna laburbiltzen zuena izan zen. Jarraian fitxategi hau protokolo orokorrean erabilitako "***edgeR***" programaren bitartez aztertzeko, lehenengo balioak normalizatuz (TMM) eta ondoren adierazpen bereizgarria zuten elementu errepikakorrak identifikatuz analisi estatistikoaren bitartez (VI. Eranskinaren 6.1 eta 6.2. Irudiak). Buakatzeko, adierazpen bereizgarria zuten elementu errepikakorren familia ezberdinak "Gazta diagrama" bidez irudikatu genituen.

4. RESULTS

EMAITZAK

4. RESULTS

4.1 EPIGENETIC CHANGES INDUCED BY MORPHINE IN mESCs

4.1.1. *In-vitro* global changes in histone modifications induced by chronic morphine treatment in mESC

Literature has widely shown how excessive use of opioids generates changes in DNA methylation pattern over the genome (Chorbov VM et al. 2011; Oertel BG et al. 2012; Zhang H et al. 2012; Nielsen DA et a. 2009; Nielsen DA et al. 2010; Ebrahimi G et al. 2017). However, little is described about histone modification changes related to chronic opioids use. Therefore, our first aim was to elucidate the global changes induced by chronic morphine treatment in mESC *in-vitro*. For that purpose, mESC were chronically treated with morphine (24h, 10μM, Figure 4.1), and then, the global levels of repressive histone modification H3K27me3 and H3K9me2; and active histone modification marks H3K4me3 and H3K4me2 were performed by immunoblotting.

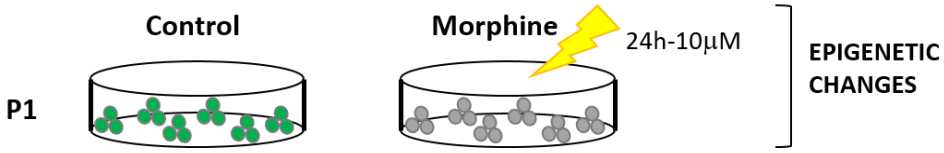


Figure 4.1. Scheme of mESC culture for *in-vitro* epigenetic changes determination.

As figure 4.2A shows, morphine induced global changes in histone levels at both repressive and active marks, H3K27me3 and H3K4me2 led to a down-regulation on histone levels, while H3K9me2 and H3K4me3 showed an up-regulation after morphine treatment, being all of them statistically significant (Figure 4.2B). No immunoreactivity was observed in negative controls omitting primary antibody before secondary antibody (data not shown). Since every histone modification has a specific regulatory complex, which is the responsible for the methylation process, we next evaluated the impact of morphine on different subunits of each histone modification regulatory complex. Changes on gene expression of different components belonging to polycomb repressive complex 2 (PRC2), G9a/GLP complex, Mll1 complex and Setd1 complex, involved in the methylation of H3K27me3, H3K9me2 and H3K4me3/me2 respectively, were evaluated (Figure 4.2C). We observed a significant decrease in the gene expression of main components of PRC2 complex upon 24h morphine treatment, including the catalytic subunit *Ezh2* and another core component *Eed*. Regarding G9a/GLP complex, morphine led to an increase at gene expression level of both analyzed subunits. The catalytic subunit of Mll1 complex, *Mll1*, and the other studied component *Rbbp5* however, presented not significant changes on gene expression after morphine treatment. *Setd1a*, one of the catalytic subunits of Setd1 complex and responsible of the methylation of H3K4me2/me3, as well as Cfp1 finger protein produced an increase in gene expression level.

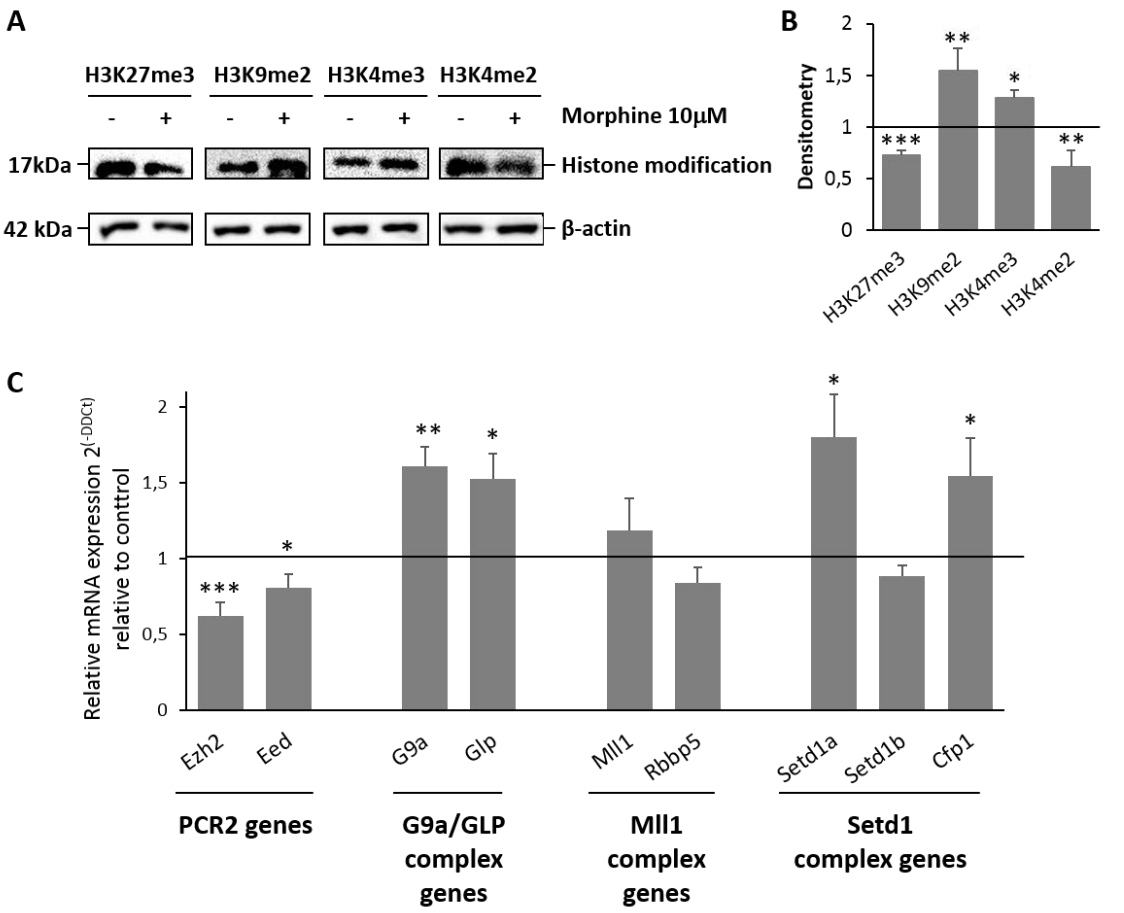


Figure 4.2. Effect of chronic morphine treatment on histone modifications in mESC *in-vitro*. (A) Western blot analysis of H3K27me3, H3K9me2, H3K4me3, H3K4me2 after morphine treatment for 24h. β-actin was used as loading control. (B) Densitometry of H3K27me3, H3K9me2, H3K4me3, H3K4me2 levels measured by Image J software. (C) RT-qPCR analysis of genes belonging to each histone modification methylation complex. Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) was used as housekeeping gene. Acquired Ct values were normalized respect to the control sample using 2^{ddCT}. Statistical significance in B and C was determined by Student-T test (* p<0.05; **p<0.01; ***p<0.001. Sample size n=5.

4.1.2. *In-vivo* histone modification changes induced by chronic morphine treatment in male and female mice.

Since mESC are a pluripotent cells able to drive any type of cells, our next goal was to evaluate the impact of morphine on chromatin conformation by histone modifications at variety of organs from different biological systems. Swiss mice at 8–10 weeks old were chronically treated by morphine, as has been previously described (Crain and Shen 1995; Hai-Yu Yang eta al. 2014) (Figure 4.3). Mice were injected morphine hydrochloride subcutaneously twice daily at 12h intervals (8:00 and 20:00), for 4 days with increasing doses on each day (20, 30, 40, 50 mg/kg). On day 5, all animals received a single subcutaneous injection of morphine (10mg/kg) at 8:00. Normal saline was used instead of morphine treatment to prepare the control group. Morphine treatment was overlapped with an ovarian hyperstimulation treatment in female mice in order to synchronize the ovulation phase and control the hormonal state of females. Female mice received a hormone treatment, based on 5IU PMSG combined with 5IU hCG in 48h interval. Treatment efficacy was confirmed by "Hot Plate Analgesia Test". Both, treated male and female mice increased the latency to pain-reflex behavior compared to control ones when animals were placed in a heat-conductive (around 60ºC) surface (Ripoll N. et al. 2006; Tzschentke, T et al.

2007) (Figure 4.3). Finally, the animals were killed by cervical dislocation and organs from different biological systems were collected.

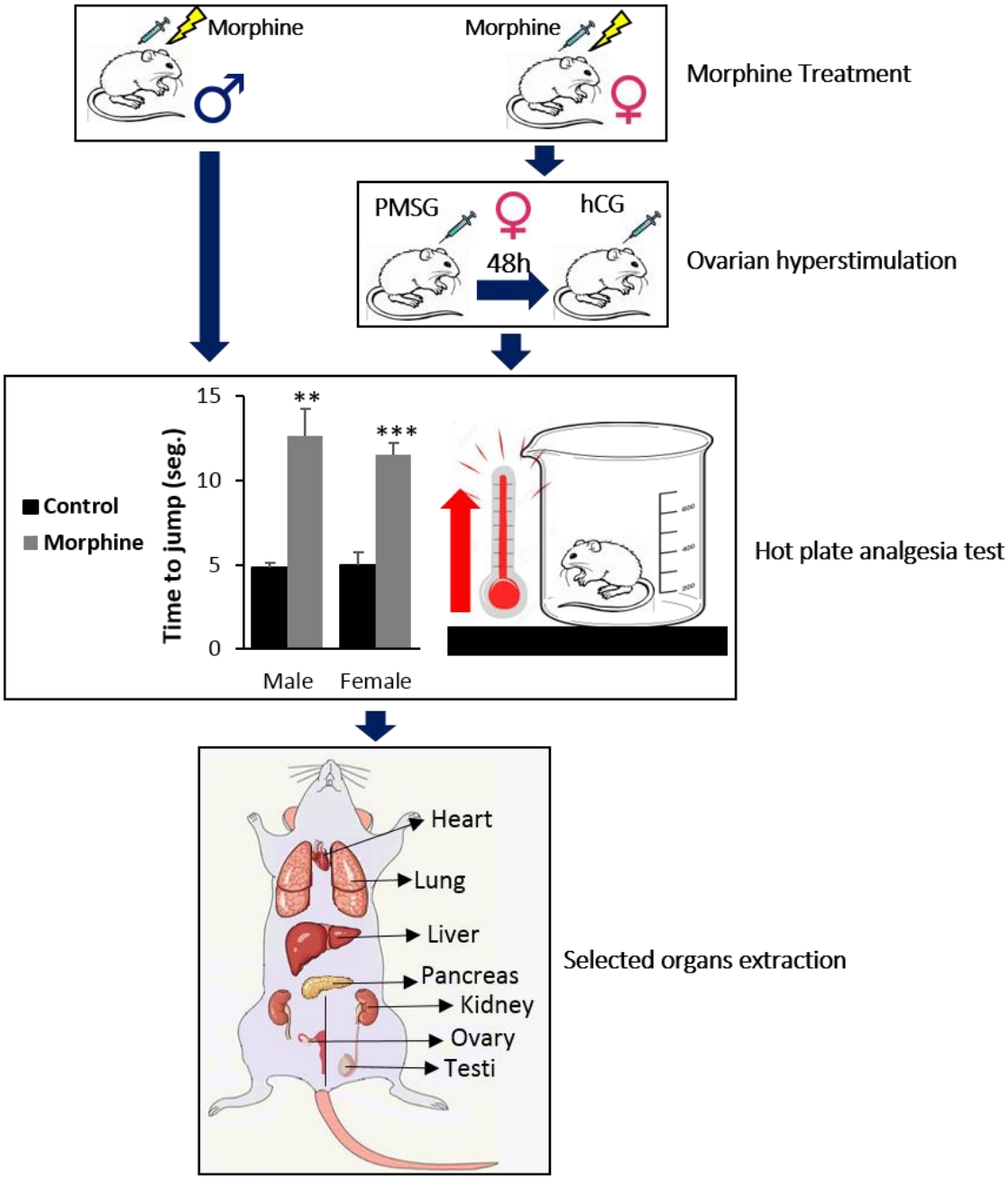


Figure 4.3. *In-vivo* experimental design for the study of the epigenetic changes induced by morphine in male and female mice. Hot plate analgesia test carried out to measure morphine effect in male and female mice *in-vivo*. Statistical significance was determined by Student-T test (* $p<0.05$). Sample size n=5

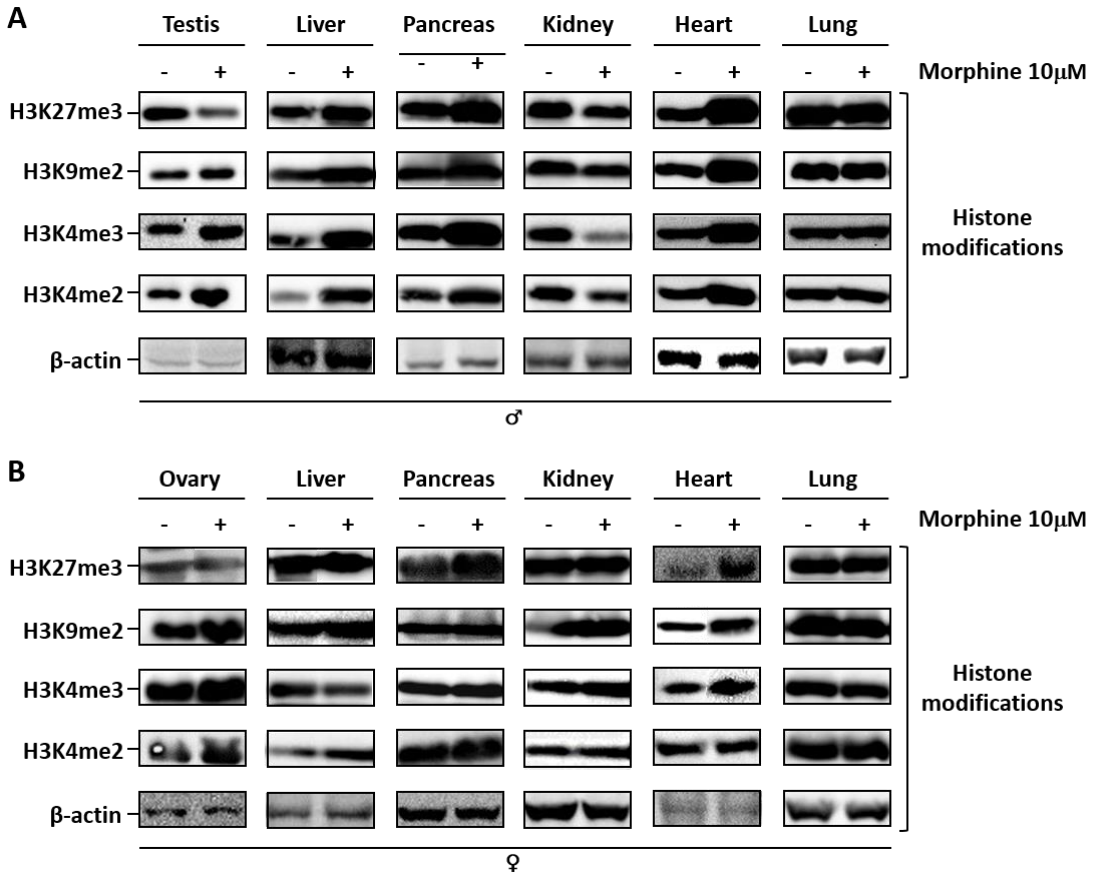


Figure 4.4. Effect of chronic morphine treatment on histone modifications in female mice *in-vivo*. (A) Western blot analysis of H3K27me3, H3K9me2, H3K4me3, H3K4me2 after chronic morphine treatment for male mice organs (testis, liver, pancreas, kidney, heart and lung). (B) Western blot analysis of H3K27me3, H3K9me2, H3K4me3, H3K4me2 after chronic morphine treatment for female mice organs (ovary, liver, pancreas, kidney, heart and lung). β-actin was used as loading control. Sample size n=5.

Figure 4.4A and B show morphine altered histone levels in both male and female mice organs. In male mice, morphine led to a global down-regulation in repressive histone mark H3K27me3 and a global up-regulation in H3K9me2, H3K4me3 and H3K4me2 in testis (Figure 4.4A). In contrast, we observed a sharp increase in all studied histone mark levels in liver, pancreas and heart after morphine exposure while a significant decrease was reported in kidney. In lung, however, morphine did not produce any effect on histone modification levels. In female (Figure 4.4B), a global decrease was observed in H3K27me3 in ovary, while H3K9me2, H3K4me3 and H3K4me2 were increased after morphine treatment. Moreover, in female liver we observed a global upregulation of the studied histone modifications except for H3K4me3, whose histone level was decreased. In pancreas, we found an upregulation in H3K27me3 and H3K4me2 levels; however, H3K9me2 and H3K4me3 seem not to be affected by morphine treatment. Furthermore, in female kidney and heart samples, morphine effect upregulated all histone levels. Finally, regarding to female lung we did not observed any change after morphine chronic exposure.). Protein expression was normalized by using β-actin and as negative control primary antibody was omitted before secondary antibody, abolishing the fluorescent staining pattern

Additionally, comparing both male and female results, in general terms it was evident that morphine generated more striking changes in male tissues, while female changes were slight. Surprisingly, both tissues in reproductive system, testis and ovaries, showed same histone

modification levels in studied four histones. In contrast, tissues belonging to digestive system in the majority of the histone modifications followed same pattern of uregulation. Furthermore, kidney sample showed a totally reverse pattern between male and female samples in all histone modifications after morphine treatment. Finally heart tissues, seem to match the upregulated pattern in both, male and female samples. To sum up, most of the studied tissues showed histone modification changes after chronic morphine treatment in both males and females, except lung, which was not affected by the treatment in any histone modification (Figure 4.4A, B).

4.1.3 Effect of chronic morphine treatment on global transcriptome in mESC by mRNA-Sequencing.

To identify morphine sensitive target genes we carried out a transcriptomic analysis by mRNA-Seq. For that purpose, cDNA library synthesis was performed by using Poly A selection to amplify only mRNA information, with a minimum of 50 millions of reads for each of the two replicate per sample.

PCA plot confirmed distinguishable differences between control and morphine treated duplicate samples and read count distribution plot showed a correct data normalization (TMM normalization, correcting for library size) (Figure 4.5A, B). Considering significant gene differences with $p<0.05$ and $FDR<0.05$ (Figure 4.5C), a total of 932 differentially expressed genes (DEGs) were identified after 24h morphine treatment), being 386 of them up-regulated genes and 546 down-regulated genes (Figure 4.5D). Functional enrichment analysis revealed several biological functions related to metabolism, cell division, DNA repair, gene expression or signaling (Figure 4.5E). Unexpectedly, it is worthy to mention that we also found DEGs related to female gamete generation process after chronic morphine treatment.

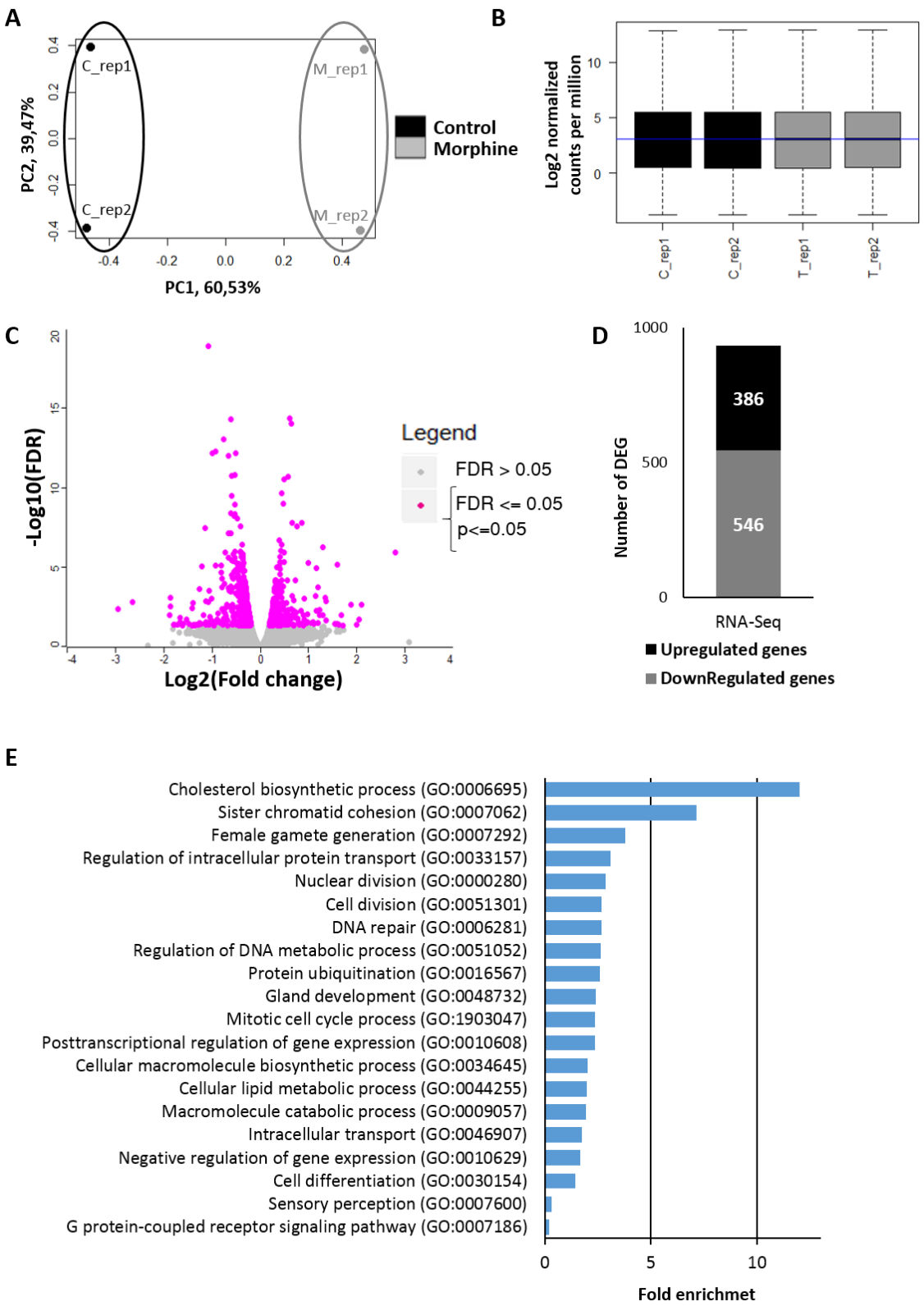


Figure 4.5. Transcriptomic analysis after chronic morphine treatment in mESC. (A) Principal component analysis plot of control and morphine treated samples. (B) Boxplot of read-counts from control and morphine treated samples from RNA-seq experiment. (C) Volcano plot of differential expressed genes (DEGs) showing significant genes ($p<0.05$ and $FDR<0.05$) in purple and not significant in grey. (D) Bar chart of DEGs. (E) Gene Ontology analysis showing the top biological functions, performed with the criteria of Bonferroni corrected for $p<0.05$.

4.1.3.1. Morphine induced gene expression changes at imprinted genes and repetitive elements

Defects in the erasure of epigenetic information do occur during reprogramming *in-vivo*, which may lead to transgenerational epigenetic inheritance. It has been reported that several imprinted genes, long non coding RNA and retrotransposons escape from reprogramming, being proposed as potential important mechanism for epigenetic memory and transgenerational epigenetic inheritance (S. Biliya & A. Bulla 2010; S. D. van Otterdijk & K. B. Michels 2016; A. P. Jason de Koning et al. 2011; E.E. Eichler et al 2001; H. H. Kazazian 2004). Therefore, once to have an overview of the global transcriptomic impact of morphine on mESCs, we next focused our study on analysing its effect on imprinted genes and repetitive elements. We did not consider the analysis of lncRNA, because after doing the PolyA selection in the library preparation step before sequencing, we only amplified mRNA information, that is to say only exonic information. That is why we could not have complete information of DE lncRNAs, as we could not identify the intronic ones.

So, to gain insights into how morphine modifies the expression of imprinted genes and repetitive elements, we next analyzed the differentially expressed genomic features (DEs), by RNA-Seq. Only differentially enriched regions with $p<0.05$ and $FDR<0.05$ were considered. Surprisingly, only 4 imprinted genes were differentially expressed after morphine treatment, which 3 of them were maternally expressed (*Mirg, Dhcr7 and Ube3a*) and 1 was unknowingly expressed (*Rhox5)* (Figure 4.6A). It is worthy to mention that *Ubea3* is the only gene with downregulated gene expression. Finally, we identified 32 DE repetitive elements compared to control sample (Figure 4.6B). LTR, endogenous retroviruses ERVK and ERV1 subfamilies (52%), RNA repeats with rRNA and tRNA subfamilies (16%) and LINE (10%) are the most represented morphine-sensitive repetitive elements, showing mainly a decrease on its expression after morphine exposure.

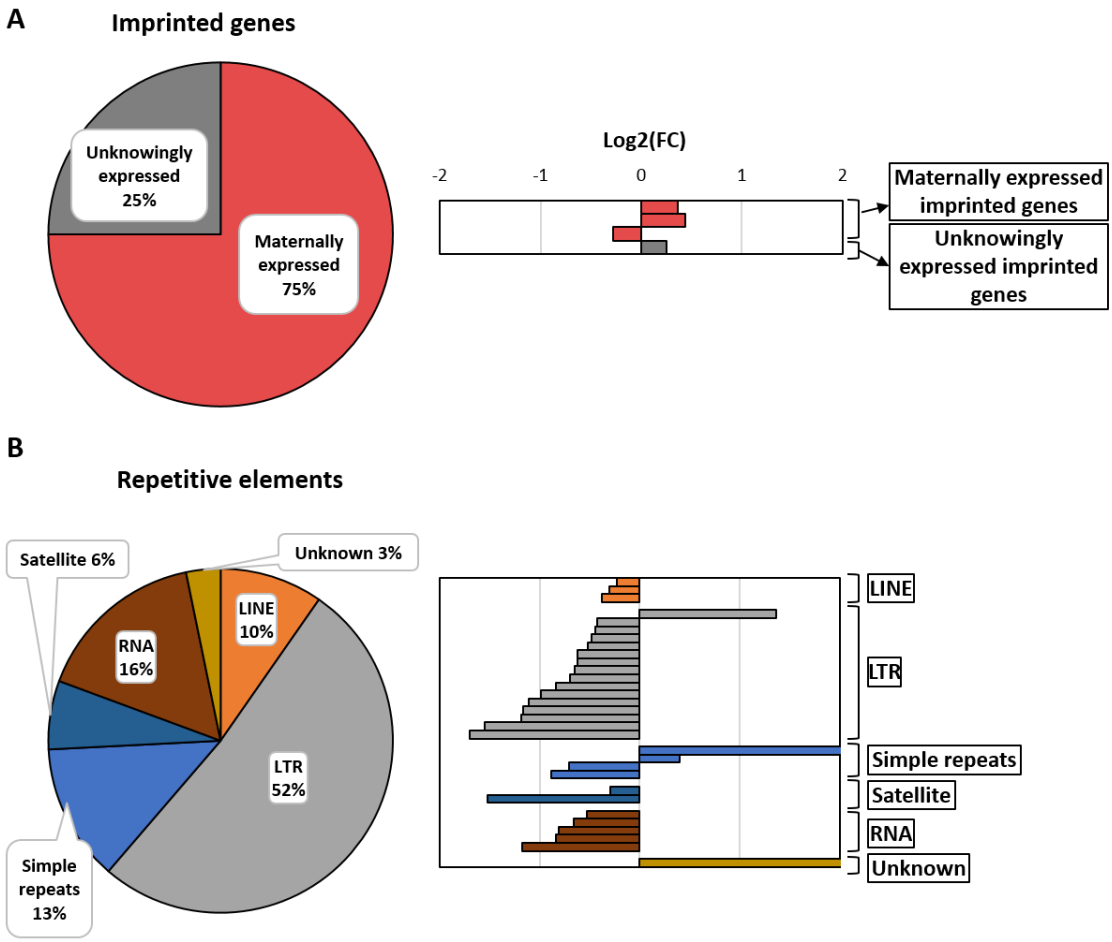


Figure 4.6. DE genomic features after morphine treatment in mESC. (A) Maternally and unknowingly expressed imprinted genes, DE after morphine treatment. (B) Repetitive elements families DE after morphine treatment. In all of the cases, the number next to each class name corresponds to the number of DE subtype ($p< 0.05$ and FDR<0.05). Each studied feature is presented next to the expression fold-change heatmap between control and morphine treated samples.

4.1.4 Effect of chronic morphine treatment on histone modification in mESC by Chip-Sequencing.

Due to the fact that histone modifications may play an important role in epigenetic cellular memory induced by chronic morphine treatment, our next aim was to study more in deep the genome-wide distribution of H3K27me3, H3K4me3 and H3K4me2 epigenetic marks by chromatin immunoprecipitation technique, with subsequent High-throughput Sequencing analysis (ChIP-Seq). Chromatin immunoprecipitation was performed by using rabbit monoclonal H3K27me3, H3K4me3 and H3K4me2 antibodies (Millipore REF: 07449; AbClonal A2357 and Abcam Ab8580, respectively) and, in parallel assays, non-specific rabbit IgG was used as a negative control. Prior to library preparation, pull–down specificity was also confirmed by qPCR, measuring an enrichment of mentioned histone modifications at Chrm1 gene, which tends to be related to silenced regions of the genome and *Tata Binding Site* (*Tbp*) gene's promoter, which tends to be related to active regions in the genome (Figure 4.7). They were used as positive or negative controls for studied repressive and active histone marks. Specifically, Chrm1 served as positive control and TBP as negative control in H3K27me3 repressive histone mark, and Chrm1 served as negative control and TBP as positive control in H3K4me3/me2 active histones mark.

After library preparation, samples were sequenced and the reads (with a minimum of 50 million of reads per replicate) quality was verified using FASTQC analysis (II Eranskina). During the bioinformatics analysis, we realized that H3K4me3 samples alignment levels were poor enough to obtain trustable results (explanation on 3.3.6.4. Materials and Methods section and IV. Apendix Table 4.1). That is why we decided not to follow analyzing them.

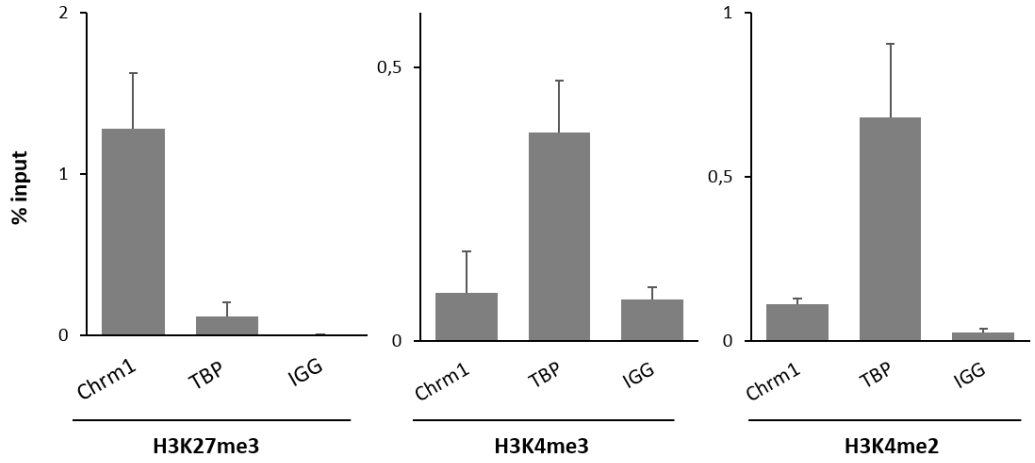


Figure 4.7. H3K27me3, H3K4me3 and H3K4me2 ChIP specificity validation. ChIP-RT-qPCR amplification of negative (Tbp promoter region) and positive (Chrm1 gene) controls and rabbit IgG. Acquired Ct values were analyzed respect to the input sample**.**

To evaluate the effect of morphine on histone distribution, we first analyzed histone binding sites (BSs) that appeared and disappeared after the chronic morphine treatment. BSs annotation was performed as following: 10.30 m-fold, 300 bandwidth and p-value cutoff as $10^{\wedge-5}$ settings with the aim of calling high-confidence binding sites. Only the BSs present in both duplicates of each sample were considered for further analyses. At this point of the bioinformatics analysis the resulted total number of peaks for H3K4me2 were low, concluding that the sequences had a poor enrichment for the histone modification. Considering this, we did not follow analyzing it and the presented results are uniquely focused on H3K27me3.

Spearman correlation performed in H3K27me3 samples confirmed the reproducibility of the two biological replicates (Figure 4.8A). Morphine led to a decrease in the number of BSs enriched by H3K27me3 after morphine treatment (Figure 4.8B). 8065 BSs were annotated in control sample, while 6899 BSs were identified after chronic morphine treatment. Morphine induced more significant down-regulation of H3K27me3 BSs at distal intergenic regions and introns. Remarkably, morphine produced an increase on H3K27me3 enrichment at promoters (Figure 4.8C, D, E). The distribution plot of BSs around transcription start site (TSS; +/- 3000bp) of the nearest genes (Figure 4.8D) and a heatmap (Figure 4.8E) confirmed the increase on H3K27me3 enrichment around TSS regions in treated mESCs.

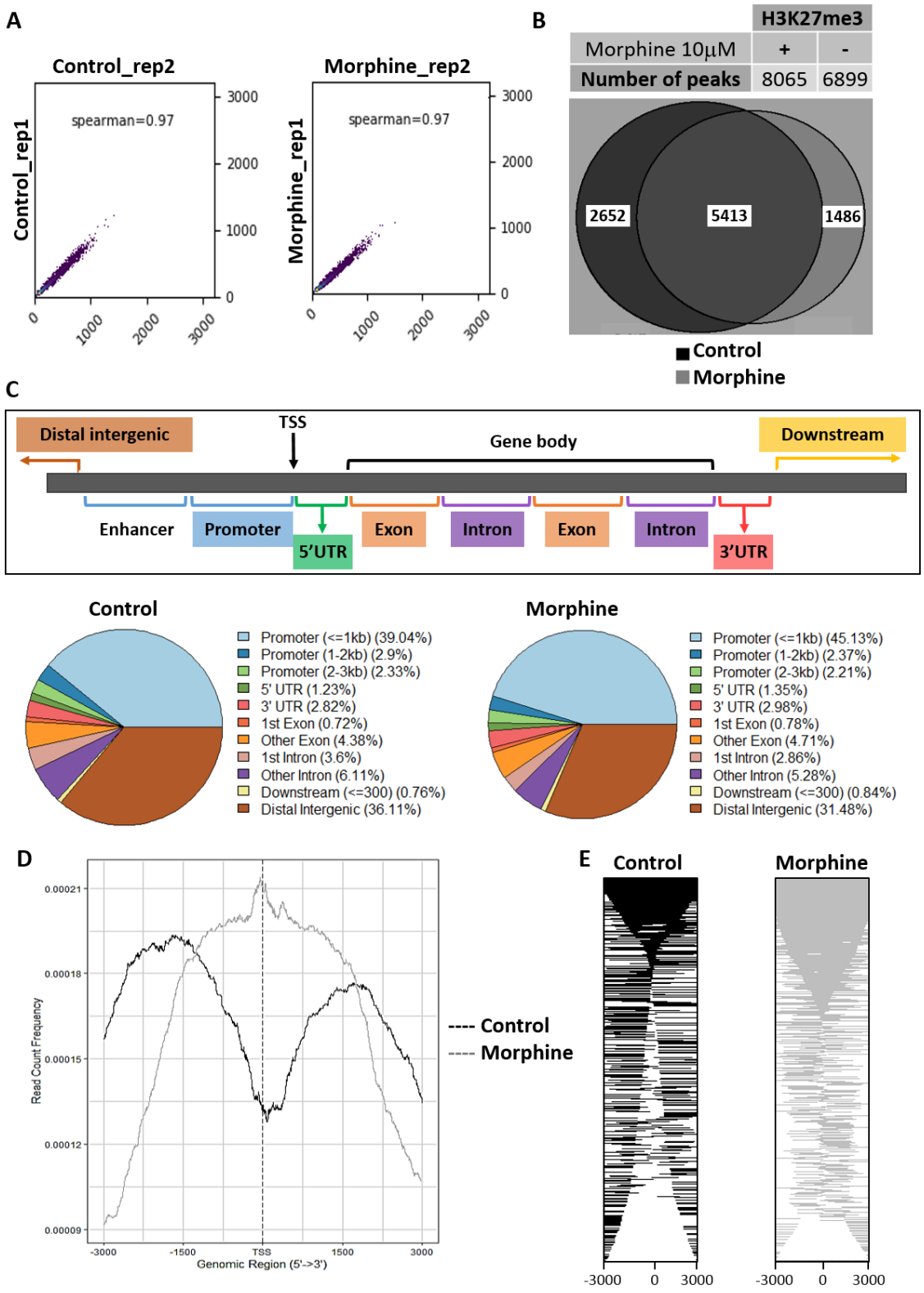
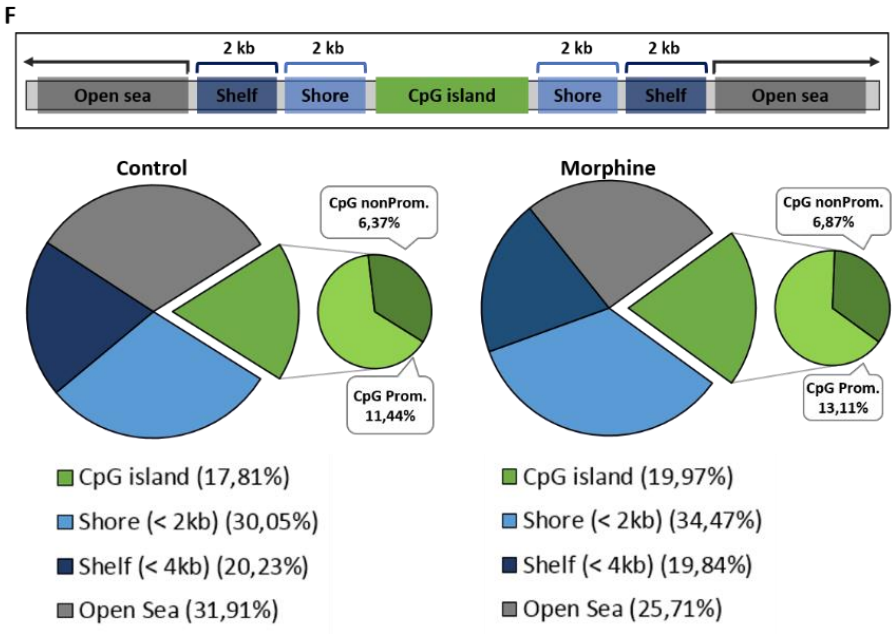


Figure 4.8. Chip-Sequencing analysis of H3K27me3 after chronic morphine treatment. (A) Spearman correlation scatterplot for replicate samples. (B) Total number of peaks in control and morphine treated samples. Venn diagram of H3K27me3 BSs. (C) Pie-chart showing genomic feature distribution of H3K27me3 peaks: promoter (divided in <=1kb, 1-2kb and 2-3kb), 5'UTR, 3'UTR, exon (1st and others) intron (1st and others), downstream of the gene end and intergenic region. D) Distribution of the H3K27me3 peaks around +-3 kb from TSS regions. (E) Heatmap representation of H3K27me3 peaks at +-30kb around the TSSs in control and morphine treated samples.

(Legend continued on next page)



(F) Pie-chart showing CpG feature distribution of H3K27me3 peaks in CpG island (belonging to promoter or non-promoter region +1kb from TSS), Shore (<2kb), Shelf (<4kb) and Open sea (the rest of the genome) regions.

Control sample displayed the common feature of the TSS centered plot with a sharp dip in H3K27me3 around the TSS, while morphine treated sample resulted in a big peak showing the increase of H3K27me3 enrichment (Figure 4.8D). Due to the fact that CpG islands have been implicated in polycomb recruitment and therefore in H3K27me3 modification (Li et al. 2017; Liefke R and Shi Y 2015; Riising EM et al. 2014), we analyzed changes induced by morphine at CpG islands and flanking features (Figure 4.8F). CpG islands and shore regions were enriched after morphine treatment, while open sea regions were decreased. Interestingly, we observed a remarkable increase at promoters regions with high density of CpG islands.

Aiming to elucidate morphine effect on histone distribution, we evaluated not only the impact on histone BSs (Figure 4.8), but also on the differential histone binding sites (DBSs) (Figure 4.9). We next focused our attention on distinguishing quantitative increases or decreases of H3K27me3 histone modification after morphine treatment. Principal component analysis (PCA) was used to give a deeper insight into how samples are associated, confirming that treated samples are very distinguishable from control ones (Figure 4.9A). Read count distribution plot showed a correct data normalization (TMM normalization, correcting for peaks library size) (Figure 4.9B) and DBSs analysis was carried out considering only differentially enriched regions with $p<0.05$ and $FDR<0.05$ (Figure 4.9C). Thereby, 1028 DBSs where identified, 595 of them showing a histone up-regulation after morphine treatment and 433 of them a down-regulation. (Figure 4.9D). Specifically, the majority of DBSs sensitive to chronic morphine treatment were distributed at promoter regions (62,89%) (Figure 4.9E) with high density of CpG islands (Figure 4.9F).

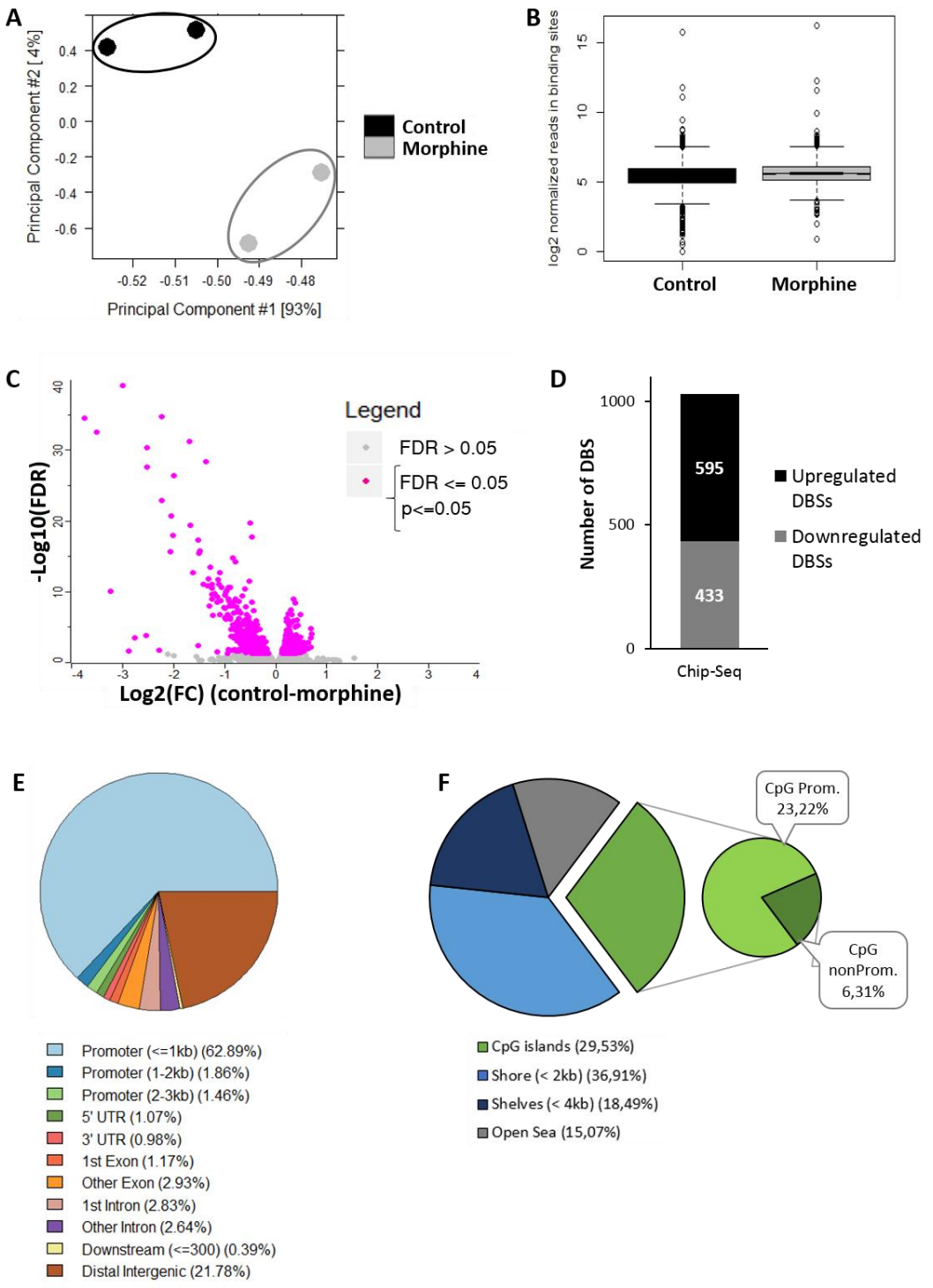


Figure 4.9. DBSs analysis. (A) Principal component analysis plot of control and morphine treated samples. (B) Boxplot of normalized read-counts from control and morphine treated samples. (C) Volcano plot DBSs and their closest genes: in purple genes, significant genes (p<0.05 and FDR<0.05), in grey not significant. (D) Bar chart of the numbers of DBSs found to be upregulated or downregulated after 24h morphine treatment. (E) Pie-chart showing genomic feature distribution of H3K27me3 DBSs: promoter (<=1kb, 1-2kb and 2-3kb), 5'UTR, 3'UTR, exon (1st and others) intron (1st and others), downstream of the gene end and intergenic region. (F) Pie-chart showing CpG feature distribution of H3K27me3 peaks in CpG island (belonging to promoter or non- promoter region +1kb from TSS), Shore (<2kb), Shelf (<4kb) and Open sea (the rest of the genome) regions.

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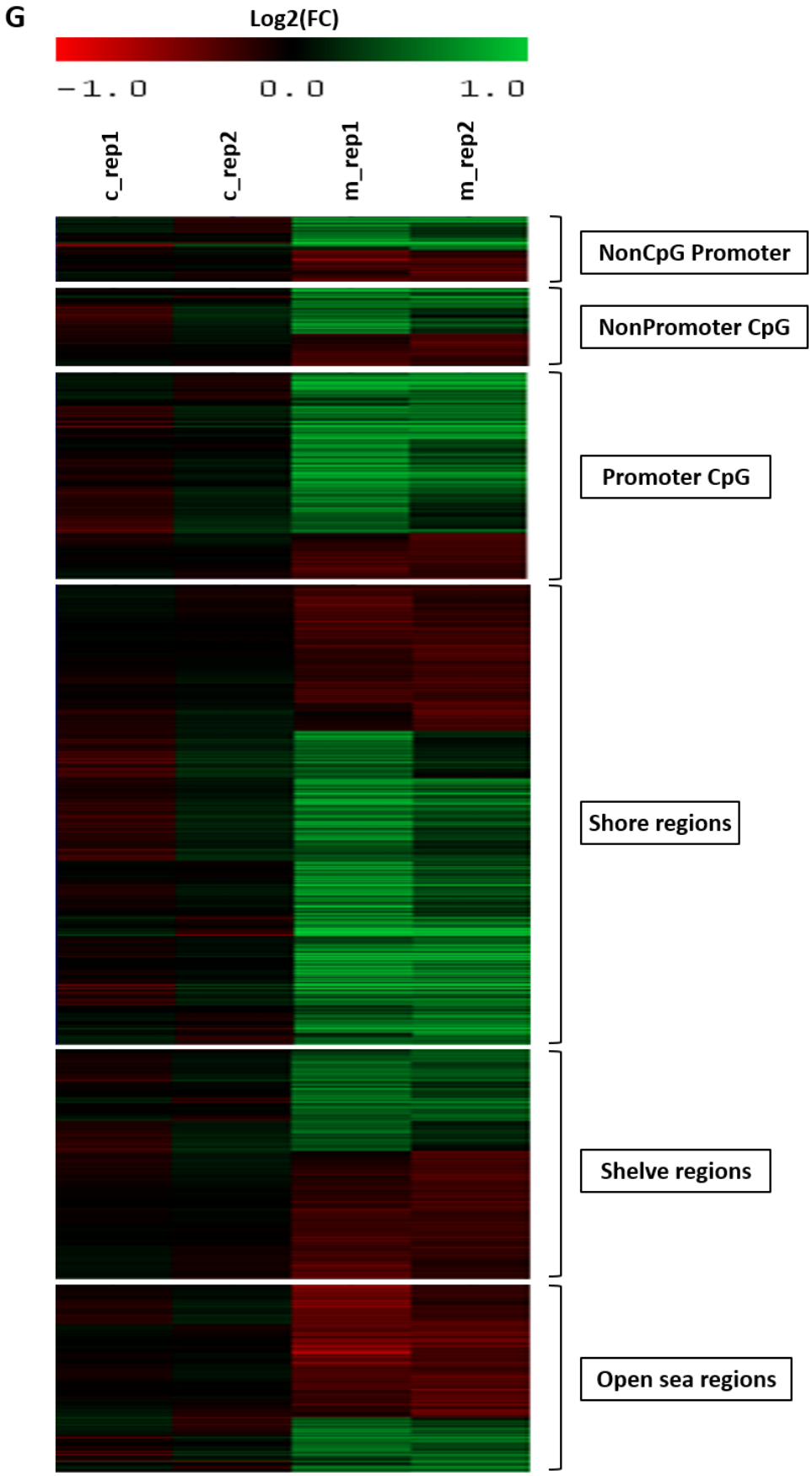

G) Heatmaprepresentation of Log2(FC) values of DBSs in control and treated samples related to promoters, CpG island and surrounding features.

Heatmap analysis confirmed the presence of both up-regulated and down-regulated DBSs at the different genomic features surrounding CpG islands such as shores, shelves and open sea regions (Figure 4.9G). Specifically, morphine produced an up-regulation of H3K27me3 enrichment in the majority of the DBSs at promoters, being this increase more evident at high density of CpG islands promoters (Figure 4.9G).

Chip-Seq analysis, therefore, revealed that morphine is able to regulate chromatin conformation by H3K27me3 histone modification. Chronic morphine treatment led to a global genome down regulation of H3K27me3 levels in mESC, together with a specific increase in the histone enrichment at CpG rich promoter regions, indicating that morphine might mainly affect the gene expression of those particular genes, modifying the H3K27me3 enrichment.

To understand in which biological functions morphine was involved, the target closest genes regulated by H3K27me3 from BSs and DBSs were annotated to perform a Gene Ontology (GO) analysis using The Gene Ontology Resource from GO Consortium (Figure 4.10). Regarding BSs, 6129 genes were annotated from control sample peaks and 5597 genes from morphine ones (Figure 4.10A). Functional enrichment analysis was conducted using only the specific genes annotated from each sample, 1389 in control and 857 in morphine treated samples. In control sample H3K27me3 enriched genes were involved in developmental processes, mainly in nervous system, and in basic cellular functions. In contrast, genes enriched to H3K27me3 in morphine treated sample were involved mostly in basic cellular processes, homeostasis, as well as in the regulation of metabolic processes (Figure 4.10B). On the other hand, 980 genes were annotated form DBSs (Figure 4.10C). 575 genes showed an up-regulation on histone enrichment, which were related to cell differentiation, cell migration and nuclear functions. Meanwhile 405 genes mainly involved in nervous system function and cellular development showed a down-regulation on H3K27me3 enrichment after morphine treatment (Figure 4.10D).

Since changes of H3K27me3 at the promoter level may directly influence on gene transcription, our next aim was to analyze more in deep the biological function of histone enriched genes at promoter level after morphine treatment (Figure 4.11). First, 3045 genes were annotated from BSs in control sample and 3028 in morphine treated sample (Figure 4.11A). GO analysis was performed with specific annotated genes of each sample, 554 and 537 in control and morphine samples respectively. As we have previously observed, control samples showed a histone enrichment of genes related to developmental processes mainly focused in neuron system, cell differentiation and basic cellular processes. Genes enriched in morphine samples, however, were involved in basic cellular functions such as signaling, homeostasis and locomotion as well as in developmental processes (Figure 4.11B). Concerning genes annotated form DBSs at promoters (Figure 4.11C), 465 genes showed an up-regulation on histone enrichment, being involved in cellular differentiation and organ development, cell movement, signaling and nuclear function (Figure 4.11D). Meanwhile, morphine induced a down-regulation on histone enrichment of 177 genes at promoter level, which were mainly involved in cellular transport activity and multicellular organism processes regulation.

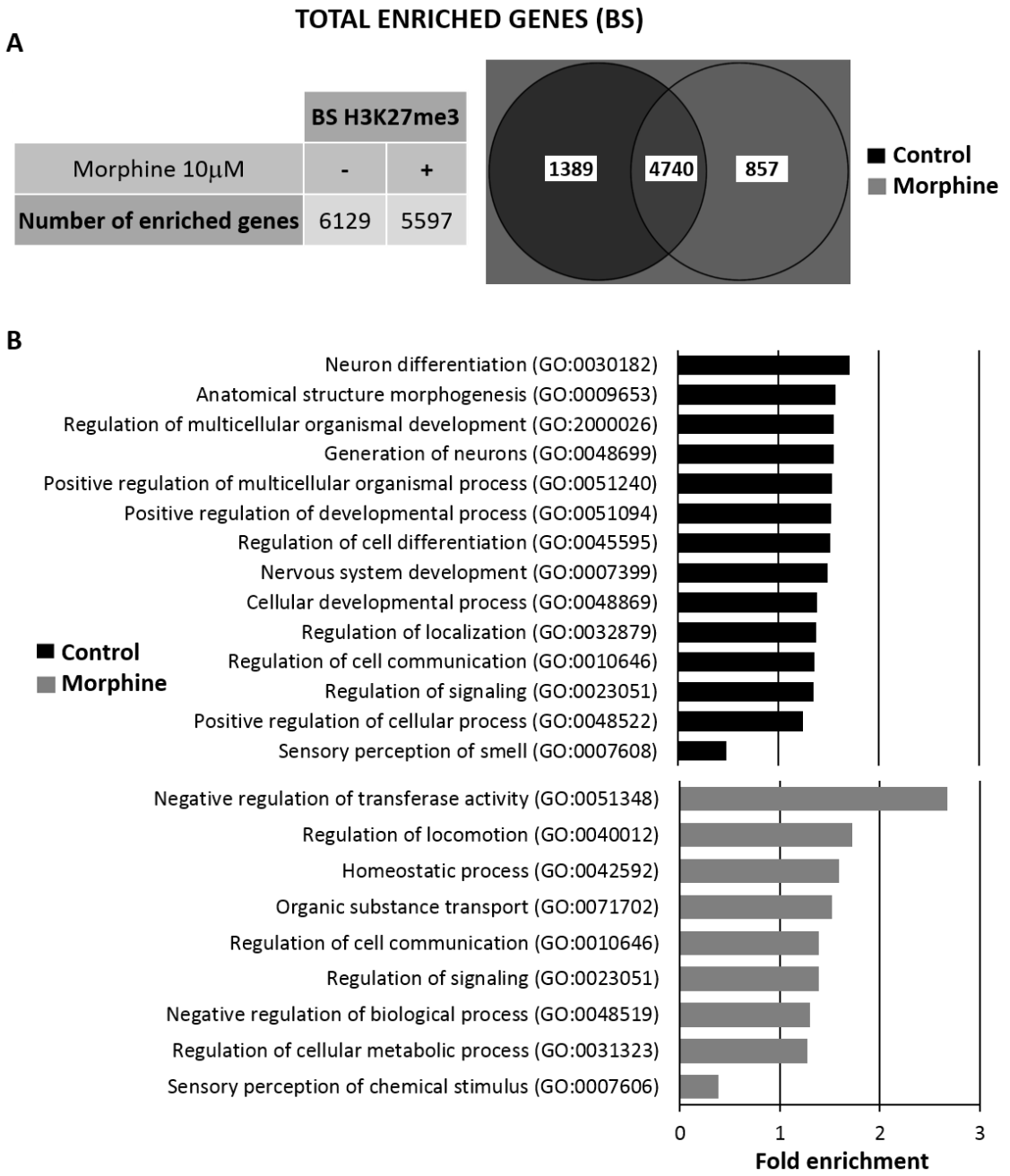

Figure 4.10. Gene Ontology analysis of H3K27me3 BSs and DBSs closest genes. (A) Number of target closest genes annotated from H3K27me3 BSs and Venn Diagram showing the overlap H3K27me3 enriched genes after morphine treatment. (B) Functional enrichment analysis showing the most indicative biological functions. Statistical analyses Bonferroni corrected for p<0.05. *(Legend continued on next page)*

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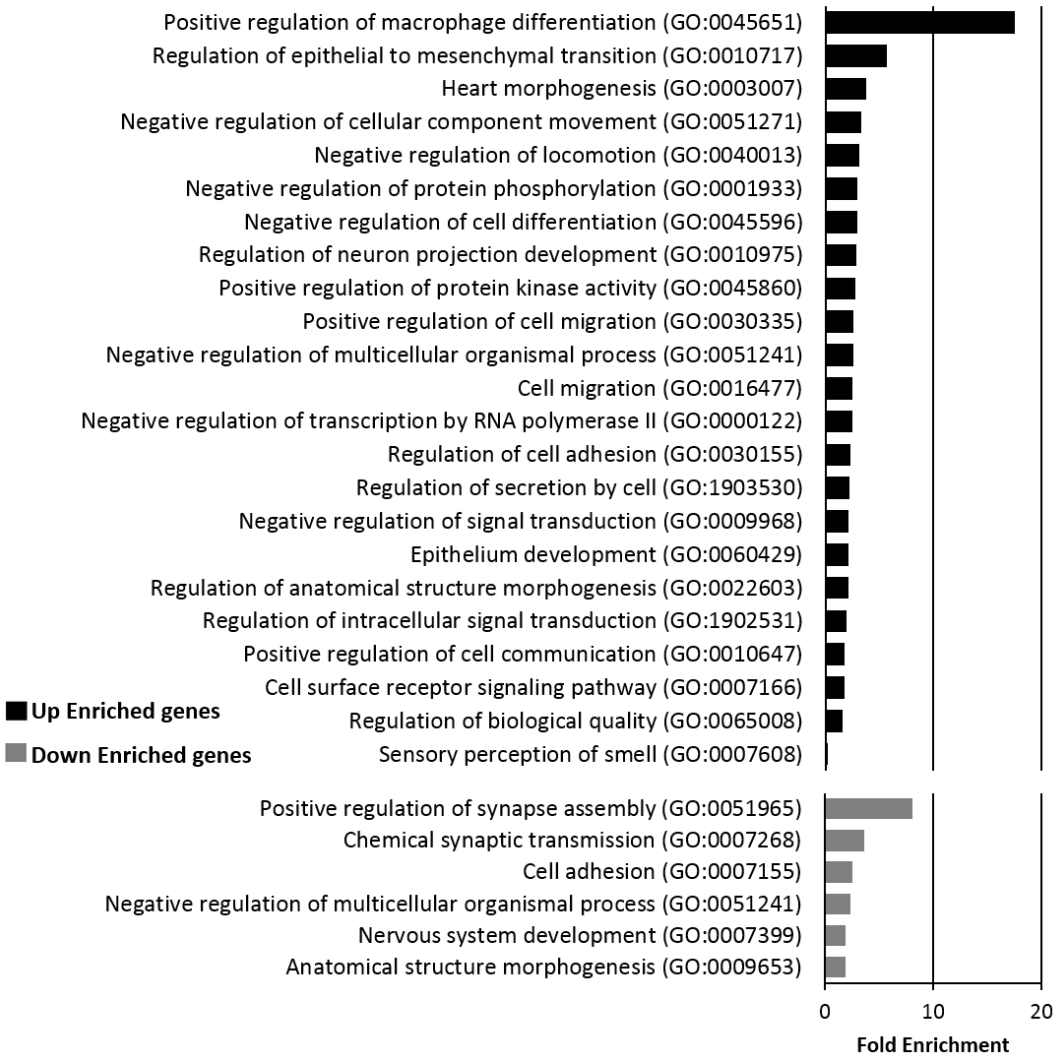
TOTAL ENRICHED GENES (DBS)

	DBS H3K27me3	
Morphine 10μM	**Up**	**Down**
Number of enriched genes	575	405

D



(C) Summary of DBSs associated number of genes in control or treated samples and treated samples. (D) GO analysis of H3K27me3 DBSs closest genes, showing the top biological functions. GO analysis was performed with the criteria of Bonferroni corrected for $p<0.05$ in B.

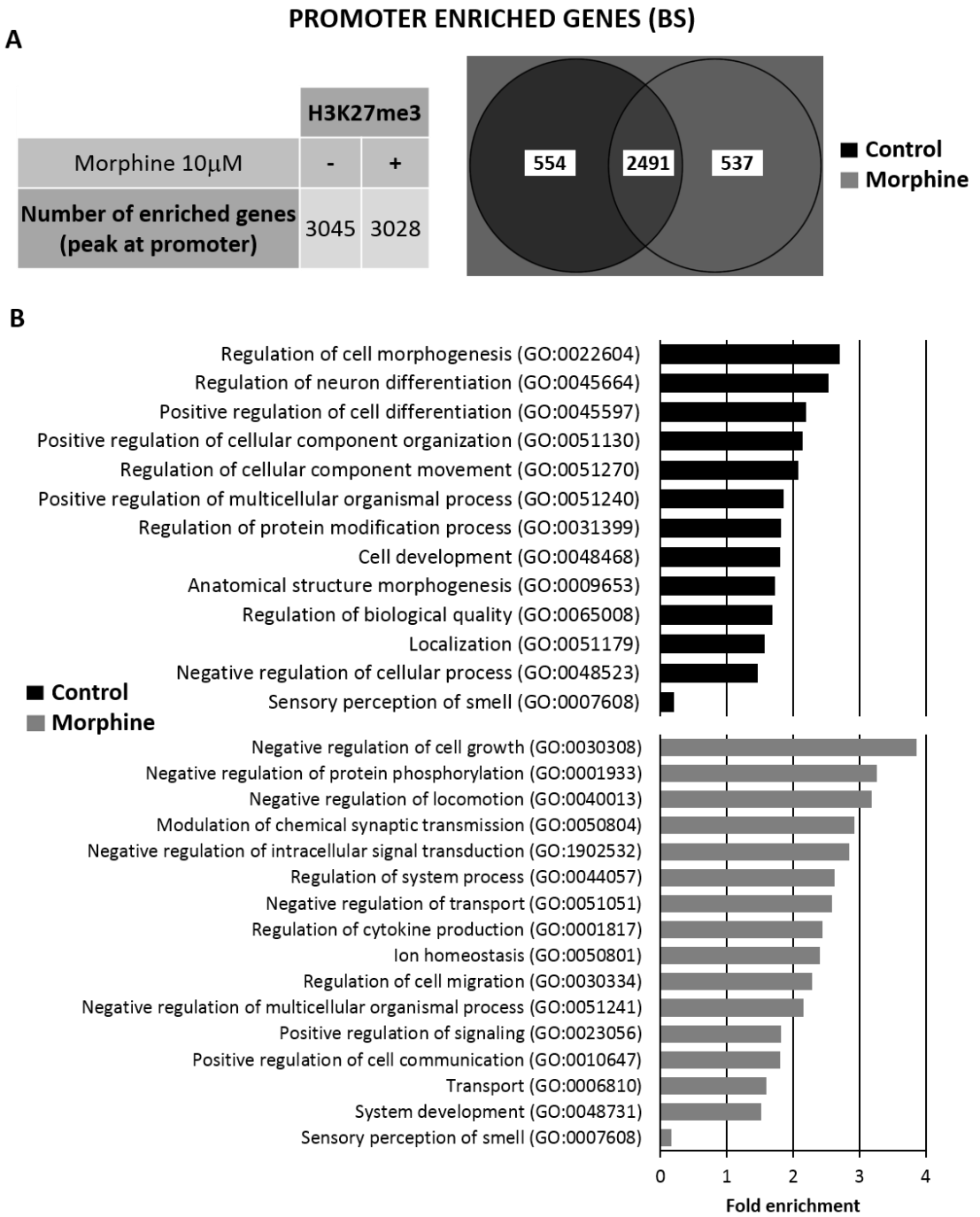


Figure 4.11. Gene Ontology analysis of H3K27me3 BSs and DBSs closest genes enriched specifically at promoter level. (A) Summary of promoter region associated number of DBs genes in control or treated samples and treated samples and Venn Diagram showing the overlap H3K27me3 enriched genes at promoter level after morphine treatment. (B) GO analysis of H3K27me3 BSs closest genes at the promoter level, showing the top biological functions. GO analysis was performed with the criteria of Bonferroni corrected for $p<0.05$ in B

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PROMOTER ENRICHED GENES (DBS)

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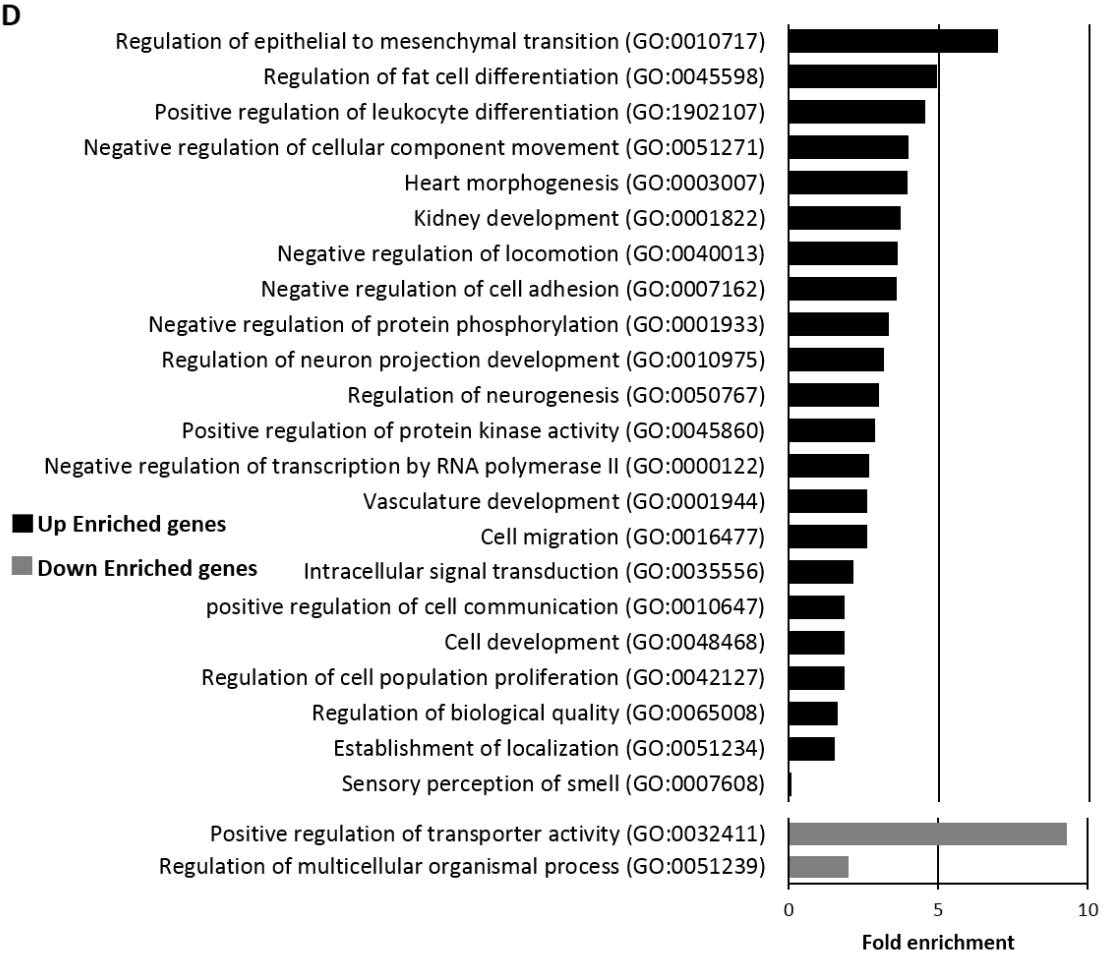
	DBS H3K27me3	
Morphine 10μM	**Up**	**Down**
Number of enriched genes (peak at promoter)	465	177

D



(C) Summary of DBSs associated number of genes in control or treated samples and treated samples. (D) GO analysis of H3K27me3 DBSs closest genes, showing the top biological functions. GO analysis was performed with the criteria of Bonferroni corrected for $p<0.05$ in B.

4.1.4.1. Morphine induced changes on H3K27me3 distribution at imprinted genes

To define morphine induced changes on the H3K27me3 at imprinting genes (Figure 4.12), to create a reference gene list., we collected information from different sources: the Geneimprint database (http://geneimprint.com) or the Catalogue of Parent of Origin Effects (http://igc.otago.ac.nz), together with several scientific publications (Peters J and Beechev 2003; Tunster JS et al. 2013; Autuoro JM et al. 2014). This list consists in a total of 165 genes, composed by 96 maternally, 64 paternally and 5 unknowingly expressed genes.

Although, we did not observe any differences at global percentages of histone BSs at paternally (41%), maternally (56%) and unknowingly (3%) expressed genes between control and morphine treated samples (Figure 4.12A), a total of 10 imprinting genes showed a differentially H3K27me3 enrichment after morphine treatment when analyzing differentially histone enrichment binding sites (DBSs) (Figure 4.12B). Only differentially enriched regions with $p<0.05$ and $FDR<0.05$ were considered. Specifically, four of the DBSs retaliated genes were paternally expressed genes: *Pde4d, Begain, Snrpn/Snurf* and Nespas/Gasxl, and six were maternally expressed genes: *Htr2, Phactr2, Tnfrsf22, Atp10a, Nesp* and Gnas. Apart from *Htr2, Phactr2* and *Begain*, morphine increased the H3K27me3 enrichment at the rest of imprinting genes. DBSs were confirmed by gene landscapes visualized in the UCSC Genome Browser (Figure 4.12C). Specifically, morphine induced an up-regulation on paternally imprinting *Pde4d* at both CpG islands and on maternally imprinting *Tnfrsf22* at promoter. Meanwhile, the H3K27me3 enrichment showed morphine induced down-regulation on *Htr2a* gene also at promoter region. Remarkably, morphine induced histone changes on *Gnas, Nesp*, and *Nespas/Gnasxl* genes, belonging to the same GNAS cluster (Figure 4.12D). We observed a slight increase on histone enrichment at *Gnas, Nesp* and *Nespas/Gnasxl* gene promoters, which are also CpG enriched regions. Surprisingly, *Gnas* promoter sensitive to morphine correspond to ICR and DMR regions important for the expression of genes under the cluster control. In addition, morphine produced histone enrichment changes on genes belonging to SNRPN cluster such as *Atp10a, Snrpn* and *Snurf.* UCSC Genome Browser landscapes showed H3K27me3 enrichment increase at *Atp10a,* and *Snurf* promoter region after morphine treatment and at *Snrpn* along the gene body. Similar to GNAS cluster, morphine induced changes at *Snurf* promoter that corresponded to the cluster ICR (Figure 4.12D).

Next, we focused our attention on other imprinted genes previously described as genes that can escape the reprogramming process and thus, which can maintain the methylation pattern, being potential candidates for the epigenetic memory and the transgenerational epigenetic inheritance (Lee J et al. 2002; Lane N et al. 2003; Reik W et al. 2001; Borgel J et al. 2010; Nakamura T et al. 2012). Landscapes visualized on UCSC genome Browser showed an increase on H3K27me3 enrichment in maternally expressed genes *Meg3* and slightly in *H19* and also in most of paternally expressed genes, such as *Peg1, Peg3* and *Peg10* genes (Figure 4.12E). Rasgrf1 did not change the enrichment of H3K27me3 after morphine treatment. Interestingly, H3K27me3 enrichment was increased specifically at promoter regions after morphine treatment in *Meg3, Peg1, Peg3* and *Peg10*. Moreover, *Peg1* and *Peg10* promoter region corresponded with the DMR region, while the promoter region in *Peg3* was at the same time ICR region of the cluster controlled by *Peg3*. Regarding maternally expressed genes, we observed H3K27me3 enrichment increase out of the promoter regions, which surprisingly were located at the ICR of the clusters controlled by *Meg3* and *H19*. Chip-Seq analyses reported a different genomic distribution of epigenetic mark H3K27me3 at ICR/DMR of imprinted genes by morphine exposure in mESC *in-vitro*, providing an epigenetic mechanism that might modify imprinting pattern by external exposures such as morphine.

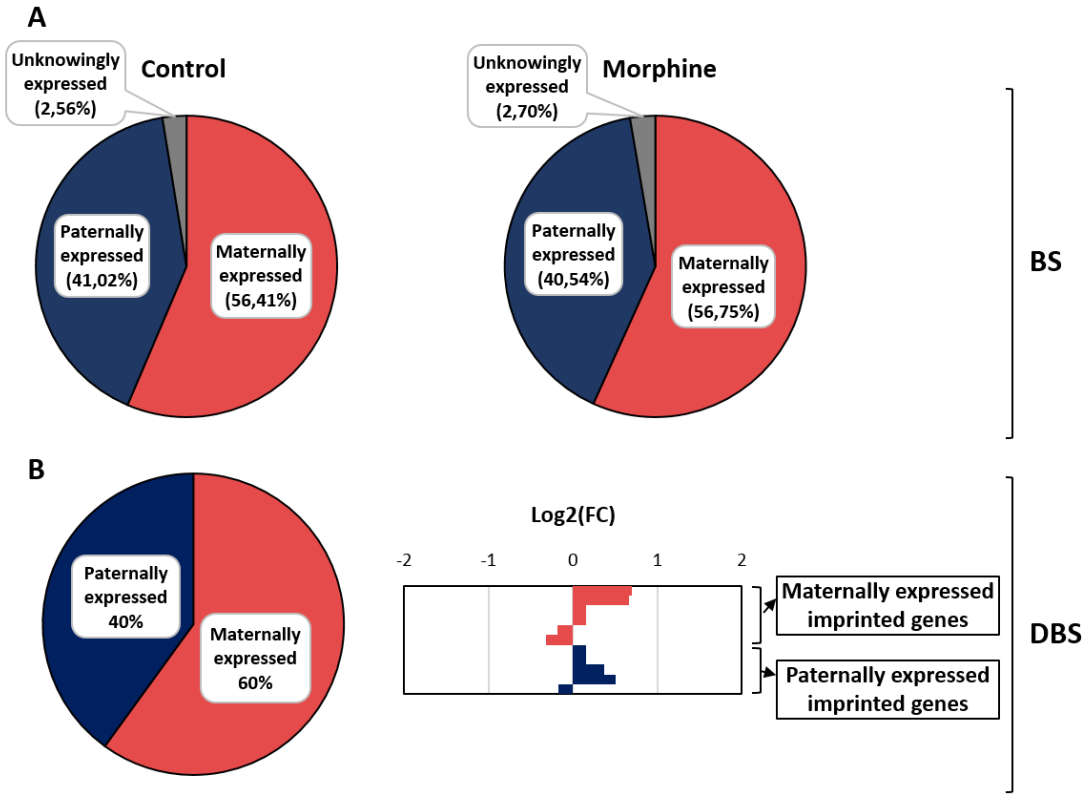


Figure 4.12. H3K27me3 enrichment distribution at imprinted genes. (A) Pie-chart showing imprinted genes feature distribution of H3K27me3 BSs. (B) Pie-chart showing imprinted genes feature distribution of H3K27me3 DBSs and enrichment fold-change heatmap between control and morphine treated samples. In both cases the distribution is composed by maternally, paternally and unknowingly expressed genes groups.

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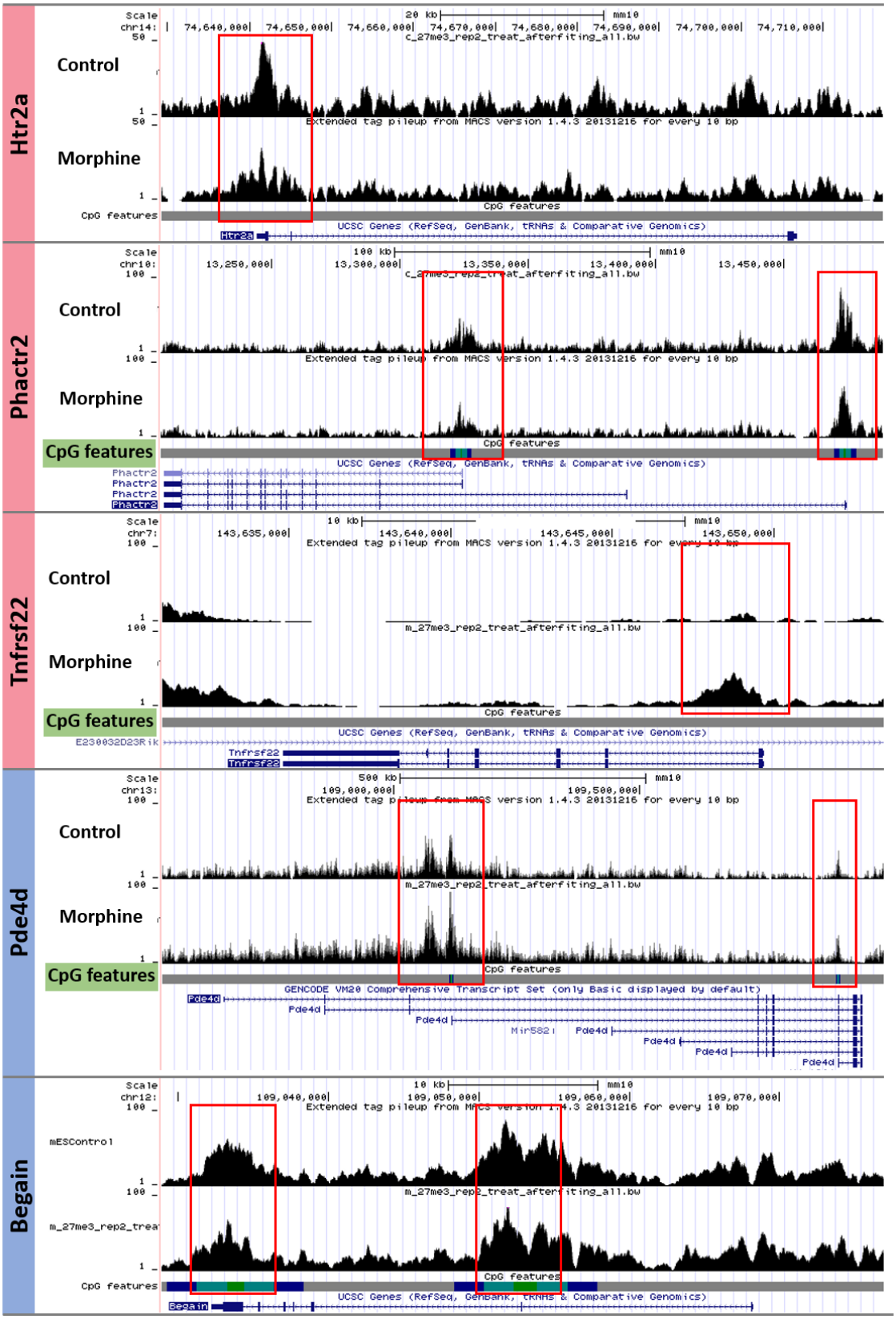

(C) ChIP-Seq landscapes of DBSs for H3K27me3 around maternally expressed genes Htr2a, Phactr2 and Tnfrsf22 and paternally expressed genes Pde4d and Begain displayed on the UCSC Genome Browser.

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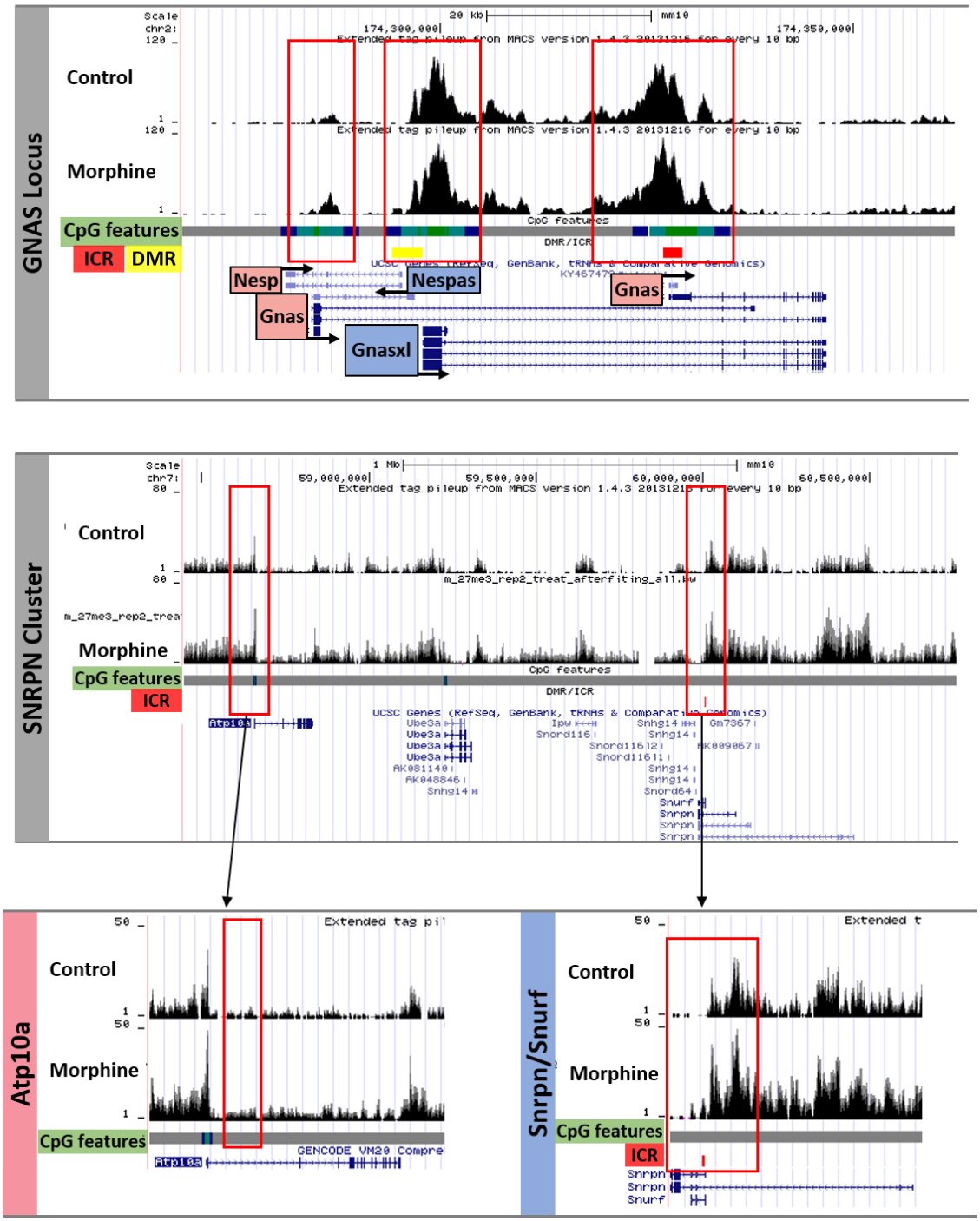

(D) ChIP-Seq landscapes of DBSs for H3K27me3 around GNAS and SNRPN imprinted clusters on the UCSC Genome Browser. In GNAS cluster, maternally expressed Nesp and Gnas and paternally expressed genes Nespas and Gnasxl are specified displayed. In SNRPN cluster, maternally expressed Atp10a and paternally expressed Snrpn and Snurf are detailed.

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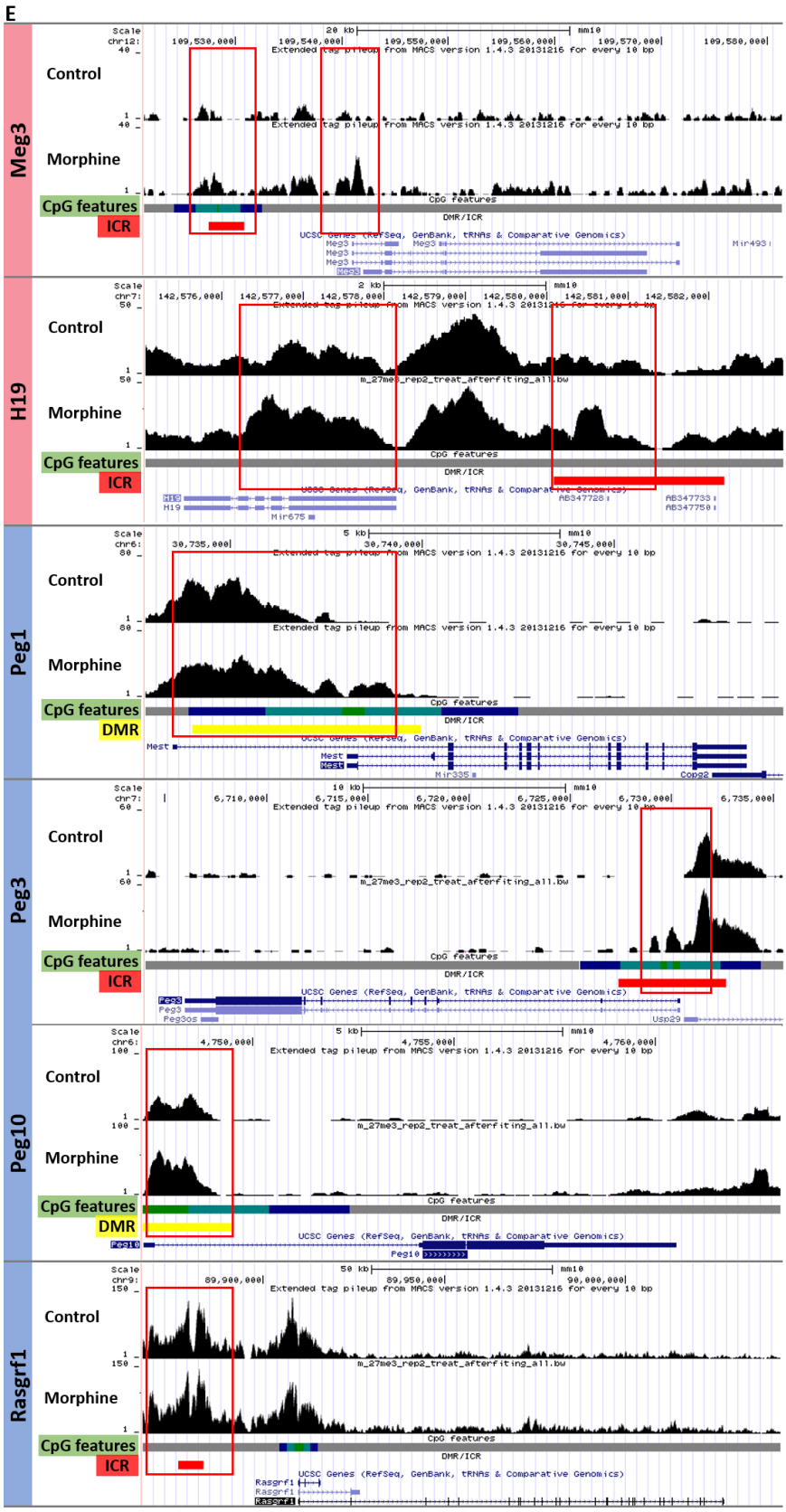

(E) ChIP-Seq landscapes of DBSs for H3K27me3 around candidate genes, maternally expressed genes Meg3 and H19 andpaternally expressed genes Peg1, Peg3, Peg10 and Rasgrf1. For all the landscapes of the figure, CpG features track was composed by CpG islands in green, Shores in light blue, Shelves in dark blue and Open sea in grey. DMR/ICR track was composed by ICR in red and DMR in yellow. Red boxes point out the enrichment change location.

4.1.4.2. Morphine induced changes on H3K27me3 distribution at lncRNA

Next, we evaluated changes induced by morphine on the H3K27me3 (Figure 4.13) at lncRNA. For that purpose, we used the lncRNA reference genome list from LncRBase database (Chakraborty S et al. 2014; http://bicresources.jcbose.ac.in/zhumur/lncrbase/index.html). This source provides 83201 entries for mouse genome, summarizing basic information about lncRNA transcripts.

Morphine did not have any effect on H3K27me3 BSs distribution at the fourteen lncRNA subtypes analyzed (Figure 4.13A). Global proportions of the most represented lncRNA subtypes, such as processed transcript not containing an ORF (PT, 20%), intergenic lncRNAs (LI, 20%), intron overlapping lncRNA (IO, 19%) and antisense lncRNAs (AN, 13%), as well as the proportions of the less represented subtypes were similar in control and morphine treated mESC.

Surprisingly, the most evident fold changes were observed at lncRNA genomic features. 1261 lncRNA subtypes were found to display significant differential enrichment after morphine treatment (Figure 4.13B), being almost all of them sensitive to morphine treatment. Differential histone enrichment induced by morphine was observed principally at the intron overlapping lncRNAs (IO, 20%), processed transcript which contain an ORF (PT, 20%), subtypes intergenic lncRNAs (LI, 19%) and antisense lncRNAs intersecting any exon of a protein-coding locus on the opposite strand (AN, 16%). Specifically, morphine caused mainly an up-regulation on histone enrichment, which was confirmed by UCSC Genome Browser landscape visualization (Figure 4.13C) and reporting the highest fold changes induced by morphine in those genomic features.

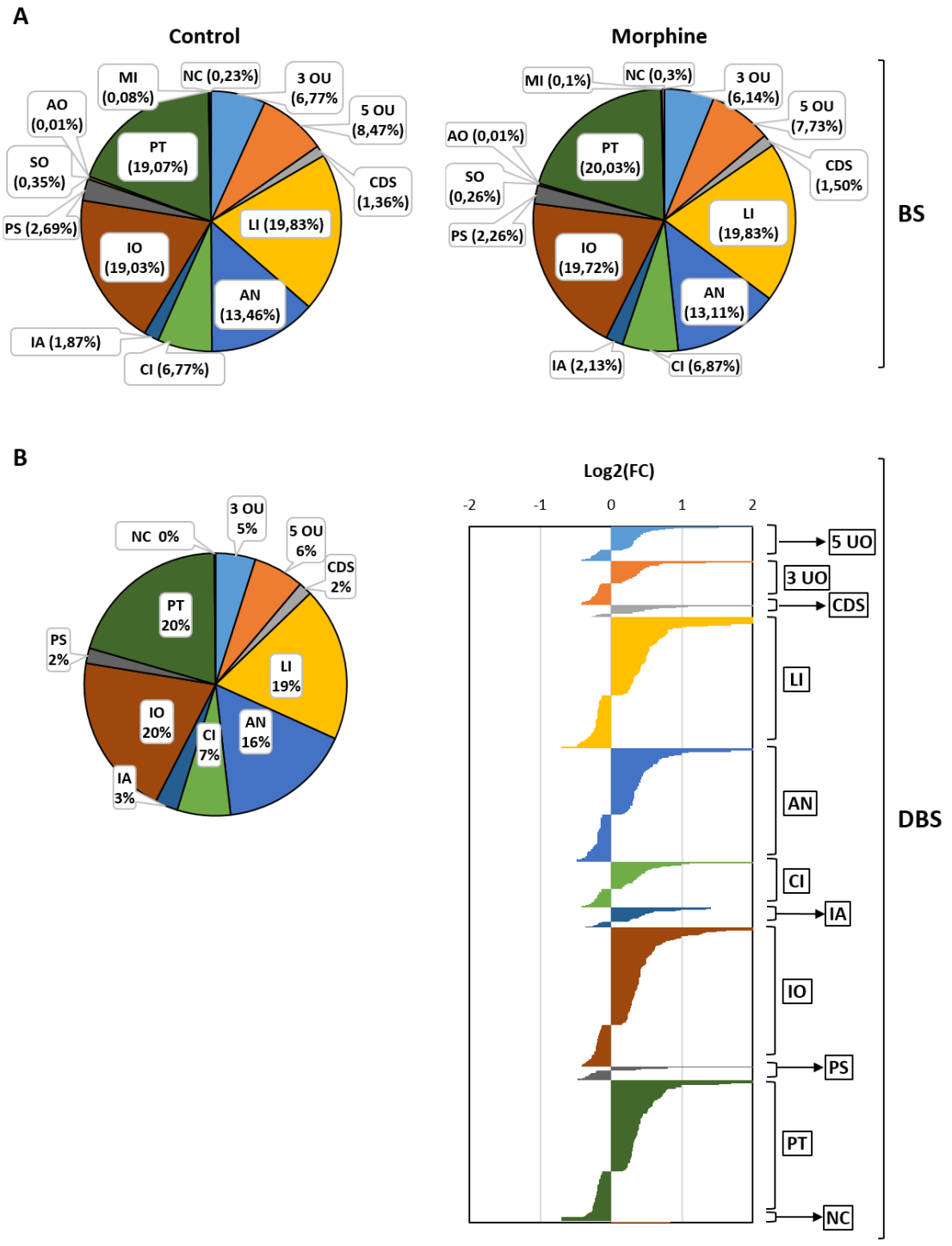

Figure 4.13. H3K27me3 enrichment distribution at lncRNAs. (A) Pie-chart showing lncRNAs subtype feature distribution of H3K27me3 BSs. (B) Pie-chart showing lncRNAs feature distribution of H3K27me3 DBSs and enrichment fold-change heatmap between control and morphine treated samples. In both cases the distribution is composed by 3UO, 3/UTR overlapping lncRNAs. 5UO, 5/UTR overlapping lncRNAs. CDS, CDS overlapping lncRNAs. LI, Intergenic (linc) lncRNAs transcribed from in between two gene loci. AN, Antisense lncRNAs intersecting any exon of a protein-coding locus on the opposite strand. CI, Completely Intronic transcripts, but do not intersect any exons. IA, Intronic Antisense lncRNAs that completely overlap with an intron in the opposite strand. IO, Intron Overlapping lncRNA. PS, Pseudogene transcripts. SO, Sense Overlapping lncRNAs containing a coding gene in its intron on the same strand. AO, Ambiguous ORF transcripts. PT, Processed Transcript not containing an ORF. MI, miscRNA from the Ensembl transcript dataset. NC, Non coding transcripts not falling in any of the above mentioned categories.

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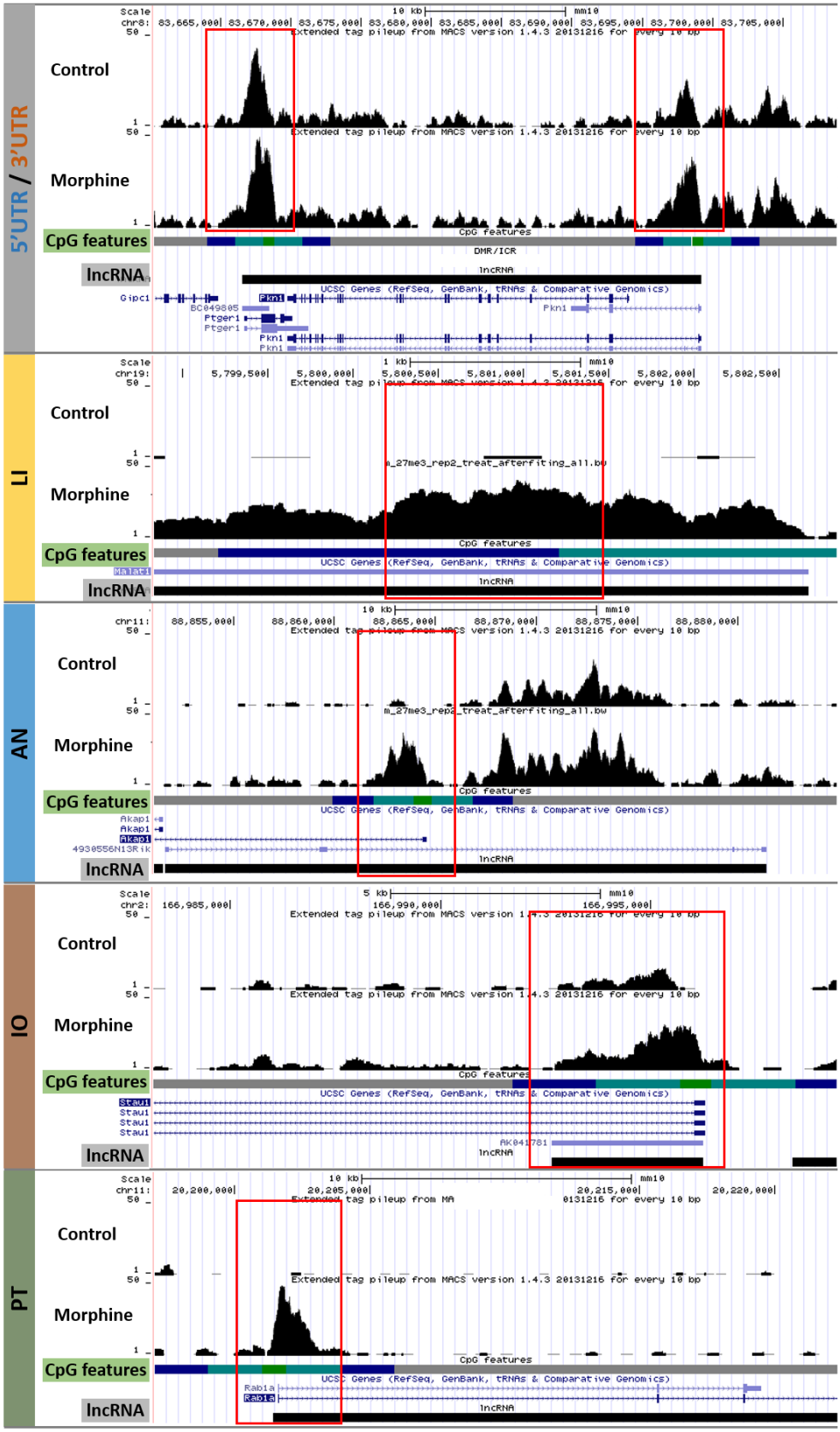

(C) ChIP-Seq landscapes of DBSs for H3K27me3 around lncRNAs, 5'UTR, 3'UTR, LI, AN, IO and PT lncRNAs displayed on the UCSC Genome Browser. LncRNA track was composed by different lncRNA families represented in black. CpG features track was composed by CpG islands in green, Shores in light blue, Shelves in dark blue and Open sea in grey.

4.1.4.3. Morphine induced changes on H3K27me3 distribution at repetitive elements

Regarding repetitive elements, we used Table Browser facility which belongs to UCSC Genome Browser, and created a reference genome list from RepeatMasker Database, for interspersed repeats and low complexity DNA sequences.

When analyzing H3K27me3 BSs distribution at repetitive elements such as LINE (42%), LTR (24%) or SINE (17%) we realized that they were also not affected by morphine (Figure 4.14A).

In spite of that, 499 of repetitive elements showed differential histone enrichment after morphine treatment after DBSs identification, principally at LTR (63%), simple repeats (17%), RNA (9%) and DNA (8%) repeats classes (Figure 4.14B). Remarkably, morphine was no effect on SINE subfamilies and only very few LINE repeats were differentially enriched after the treatment. Apart from DNA and LINE repeats, we mainly observed an up-regulation of histone enrichment at different repetitive subfamilies after morphine treatment, which were also visualized in UCSC Genome Browser (Figure 4.14C). Specifically, most of the LTR subfamilies sensitive to morphine belonged to endogenous retroviruses (ERVK and ERV1), being IAP a potential important repetitive element related to transgenerational epigenetic inheritance (Nakamura T et al. 2012). According to the Fold Changes observed, Simple repeats and RNA repeats showed an up-regulation of the H3K27me3 enrichment, being tRNA the most representative subfamily.

Our results showed that morphine induced differentially enriched distribution of H3K27me3 at specific imprinted genes, lncRNAs and repetitive elements induced by morphine in mESC *in-vitro*, importants for cellular epigenetic memory and transgenertionl epigenetic inheritance.

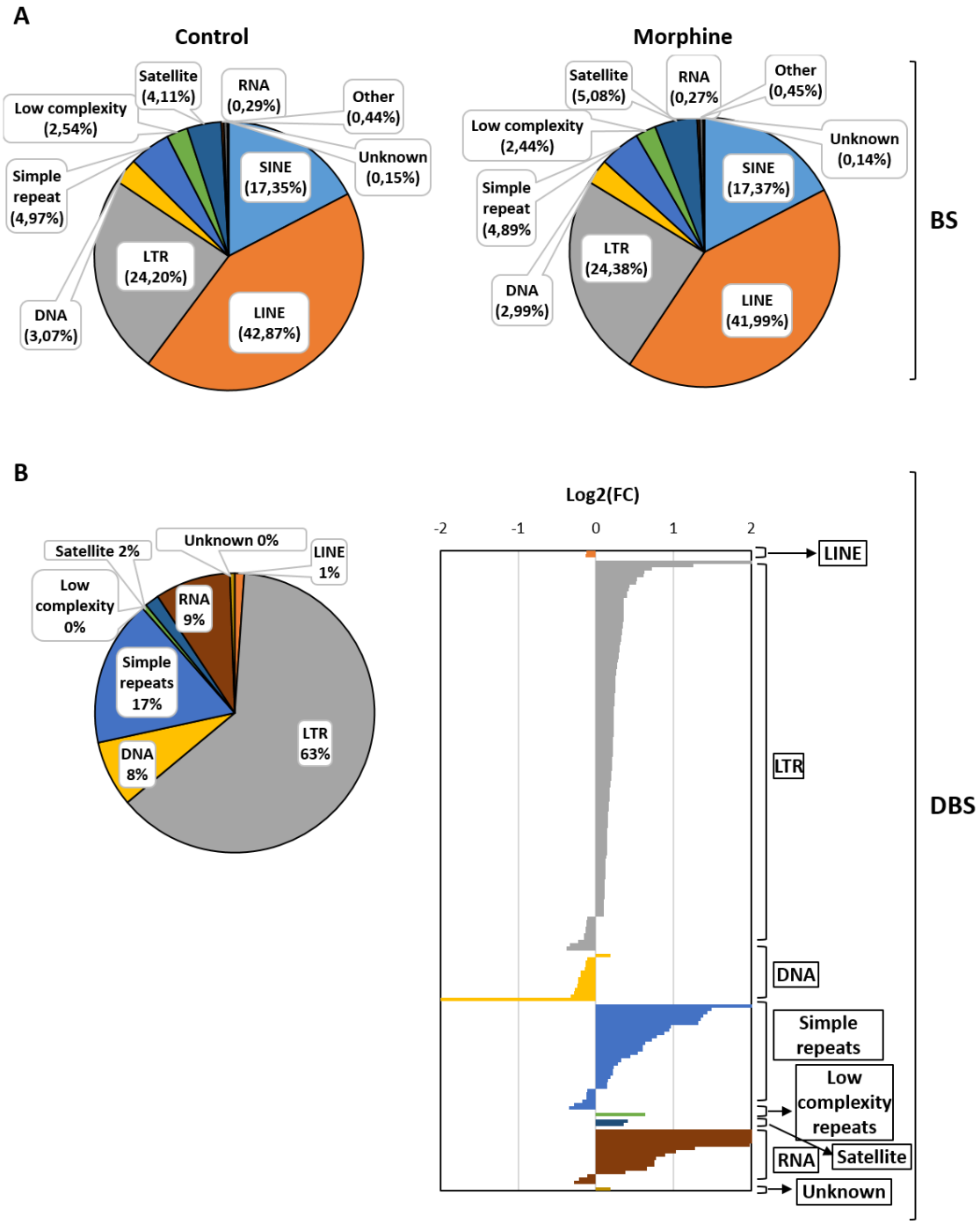

Figure 4.14. H3K27me3 enrichment distribution at repetitive elements. (A) Pie-chart showing repetitive elements class group feature distribution of H3K27me3 BSs. (B) Pie-chart showing repetitive elements feature distribution of H3K27me3 DBSs and enrichment fold-change heatmap between control and morphine treated samples. In both cases, the distribution is composed by repetitive elements class group: SINEs, LINEs, LTRs, DNA repeats, Simple repeats, Low complexity repeats, Satellite repeats, RNA repeats, Other repeats (including Rolling Circles) and Unknown repeats.

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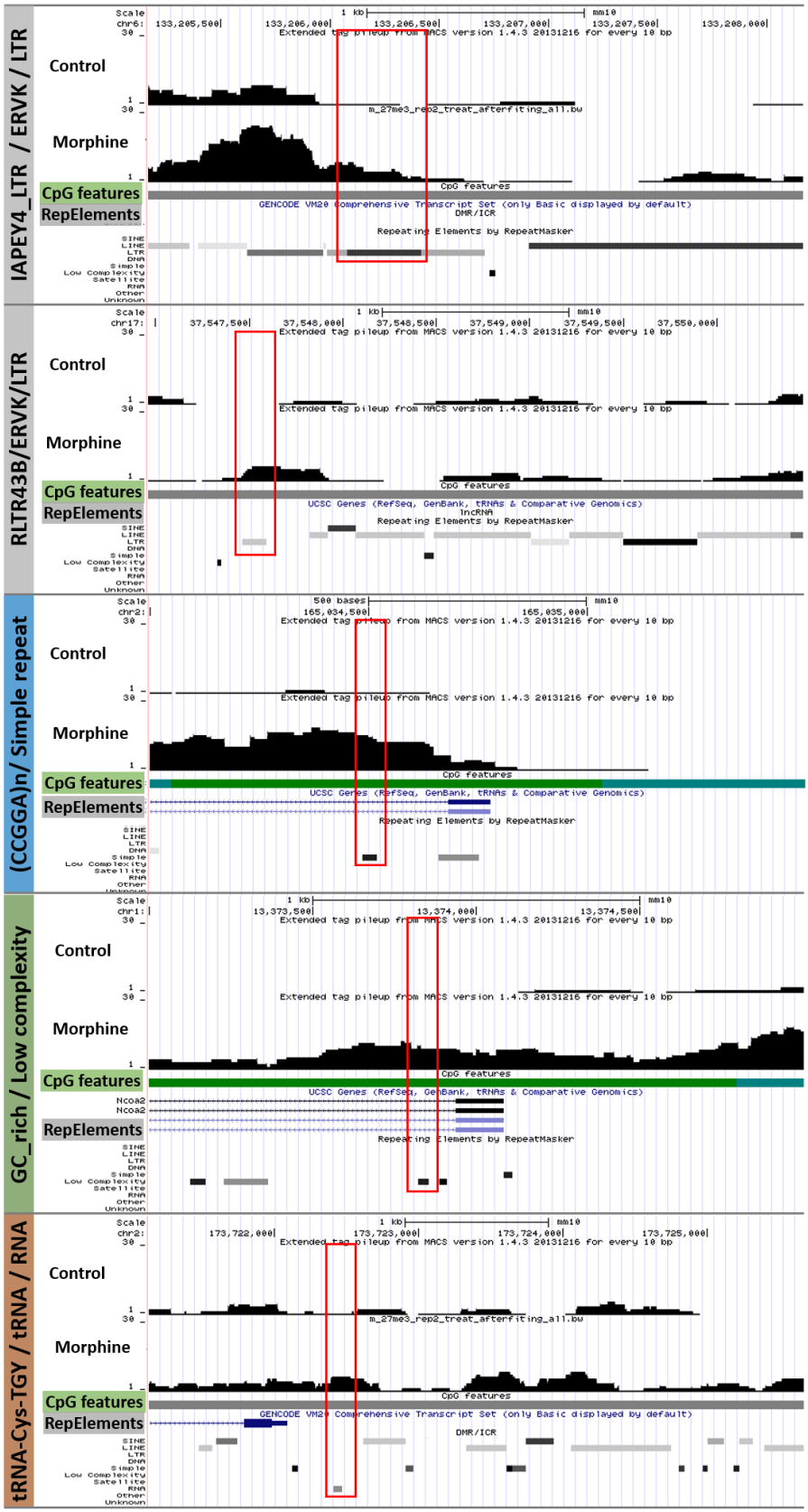

(C) ChIP-Seq landscapes of DBSs for H3K27me3 around LTRs, Simple repeats, Low complexity repeats and RNA repeats groups from repetitive elements displayed on the UCSC Genome Browser. Repetitive elements track was composed by different repetitive elements groups represented in black-grey scale. CpG features track was composed by CpG islands in green, Shores in light blue, Shelves in dark blue and Open sea in grey.

4.1.5 Integrative analyses between *RNA-Sequencing* and *Chip-Sequencing* data.

Aiming to understand the epigenetic mechanism important for transcriptomic deregulation after chronic morphine treatment, an integrative analysis was performed between Chip-Seq and RNA-Seq data (Figure 4.15A). Venn diagram showed 125 genes sensitive to morphine in common between both transcriptomic and Chip-Seq analyses (Figure 4.15B). Next, a functional enrichment analysis was carried out to elucidate the biological functions commonly linked between chromatin conformation and gene expression deregulation after morphine exposure. Genes related to apoptosis, neurogenesis, metabolic processes and gene expression changed at both H3K27me3 distribution and gene expression after morphine treatment (Figure 4.15C).

Because histone changes at the promoter can directly influence on gene transcription, we reanalyzed the integrative analysis filtering out genes at promoter level, to understand how morphine can epigenetically modified gene expression. Specifically, 1733 genes annotated from H3K27me3 BSs and DBSs and 932 DEGs analysis (Figure 4.16A) were considered for Venn diagram analysis, identifying 62 genes in common (Figure 4.16B). GO analysis (Figure 4.16C) indicated that chronic morphine treatment was involved in similar biological functions such as apoptosis, metabolism and development.

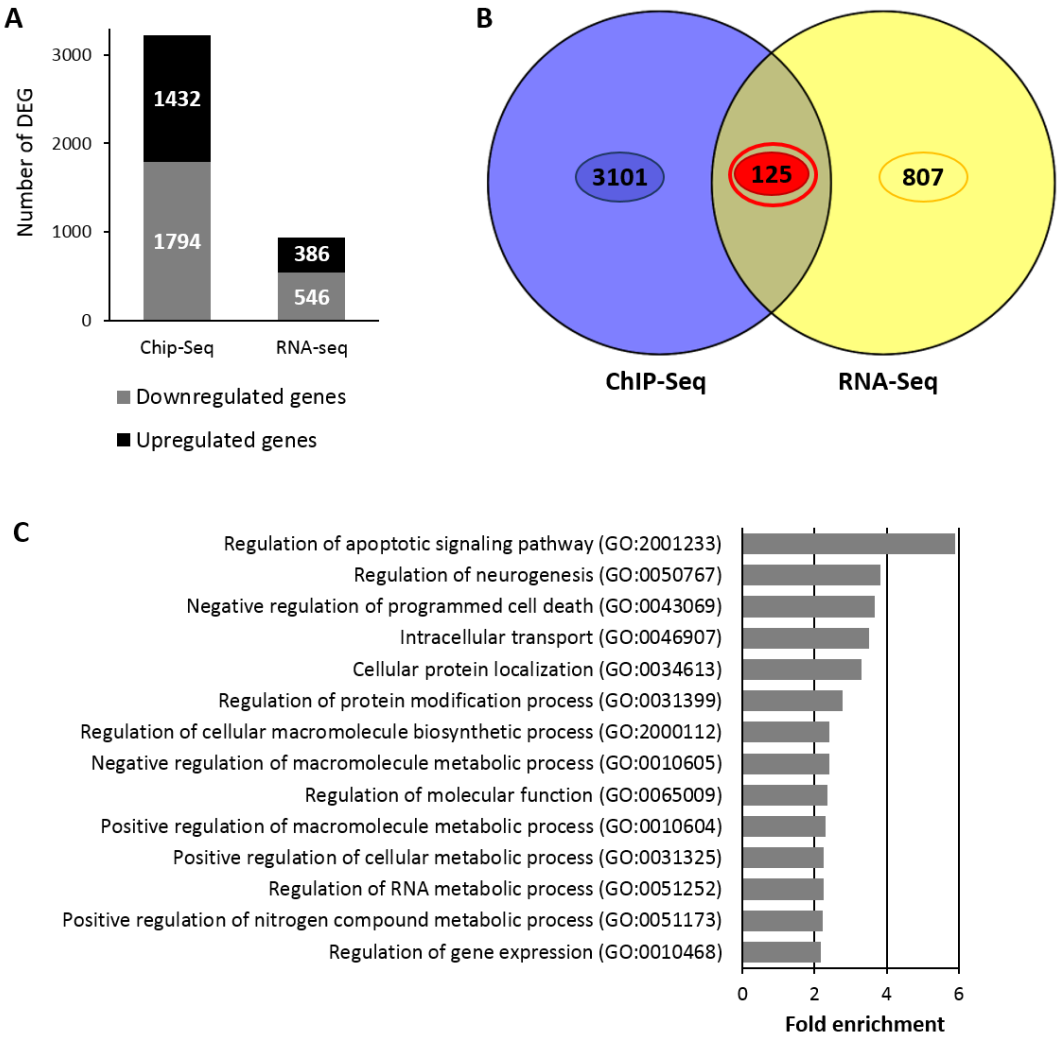

Figure 4.15. Integrative analyses with H3K27me3 Chip-seq and RNA-seq data (A) Numbers of annotated genes form H3K27me3 BSs and DBSs, and DEGs after 24h morphine treatment. (B) Venn diagram showing the overlap between H3K27me3 BSs and DBSs, and RNA-seq DEGs after chronic morphine treatment. (C) Gene Ontology analysis showing the top biological functions, performed with the criteria of Bonferroni corrected for $p<0.05$.

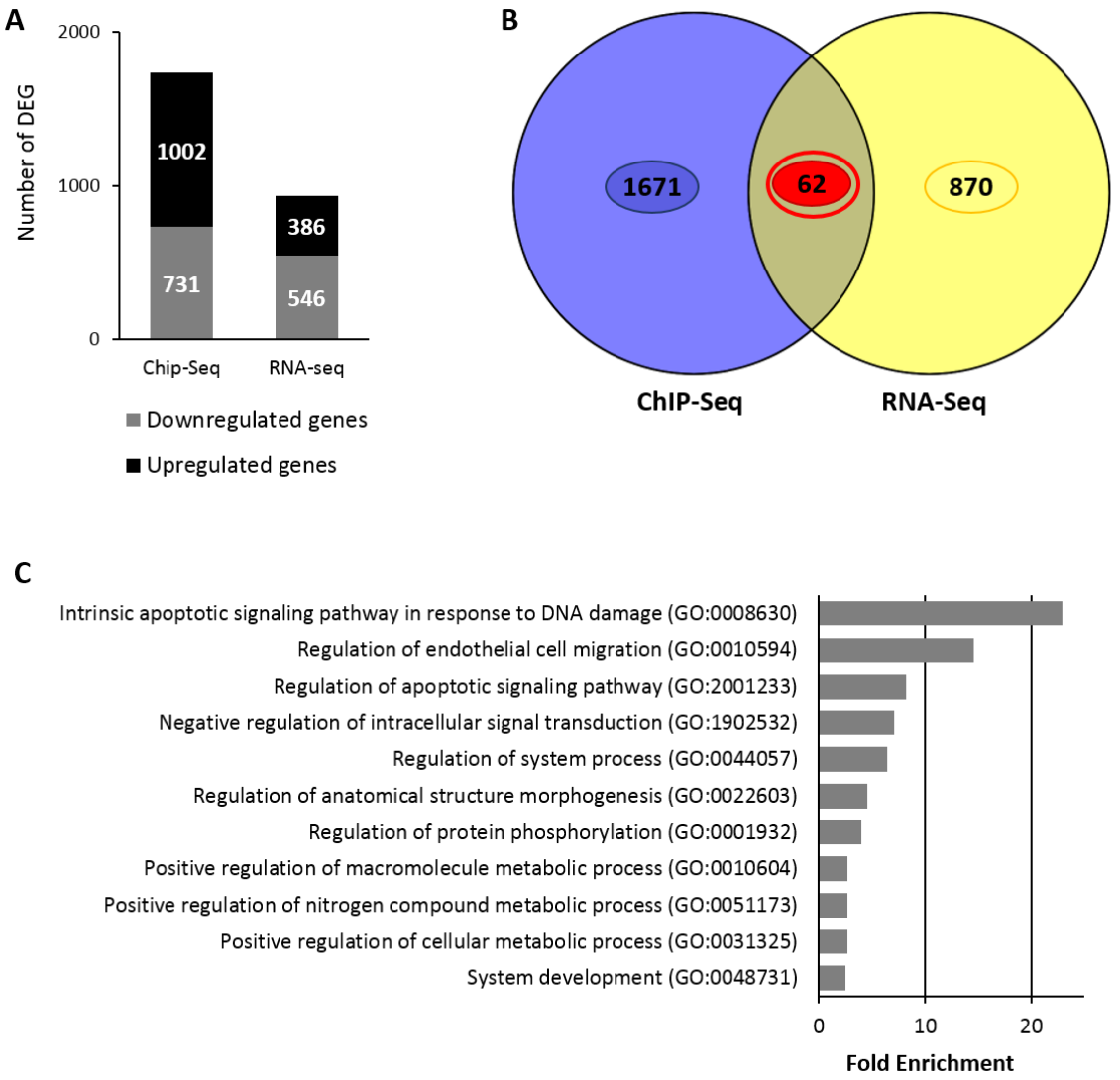

Figure 4.16. Integrative analyses with H3K27me3 Chip-seq and RNA-seq data (A) Numbers of annotated genes form H3K27me3 BSs and DBSs at promoter level and DEGs after 24h morphine treatment. (B) Venn diagram showing the overlap between H3K27me3 BSs and DBSs, and RNA-seq DEGs after chronic morphine treatment. (C) Gene Ontology analysis showing the top biological functions, performed with the criteria of Bonferroni corrected for $p<0.05$.

Due to the fact H3K27me3 is a repressive histone mark, we only considered those genes that link between chromatin conformation and gene expression to elucidate how morphine impact on gene expression through changes on H3K27me3 enrichment at promoter level. As Table 4.1 shows, morphine induced a decrease on H3K27me3 enrichment at promoter level together with an increased on gene expression only in *Bmp4* gene, a growth factor related to embryonic developmental processes. On the other hand, we identified 16 genes, which were increased on H3K27me3 enrichment at promoter region and were decreased on gene expression. Specifically, we found genes related to nuclear functions, such as *Atad5, Cnot6l, Med13, Suz12, Sirt1, Mga* and *Tead1;* metabolism, such as *Kdm1b, Lpgat1, Man2a1, Pykfive, Psme4*; and others such as *Slc12a2* and a RIKEN cDNA gene 1700001L05RIK.

Table 4.1. Genes identified by interactive analysis between Chp-Seq and RNA-Seq data

ChIP-Seq BSs and DBSs Down + RNA-Seq Up		
Bmp4	Bone morphogenetic protein 4	Embryonic developmental process
ChIP-Seq BSs and DBSs Up + RNA-Seq Down		
Atad5	ATPase family, AAA domain containing 5	DNA damage response
Smchd1	Structural maintenance of chromosomes (SMC) hinge domain containing 1	Epigenetic silencing by regulating chromatic architecture
Cnot6l	CCR4-NOT transcription complex, subunit 6-like	Gene expression
Med13	Mediator complex subunit 13	Gene Expression and transcription regulation
Suz12	Suppressor of zeste 12 PRC2 subunit	DNA and chromatin silencing through PRC2
Sirt1	Sirtuin 1	Acetylation and chromatin regulation
Mga	MAX gene associated	DNA binding transcription factor activity (MYC-MAX)
Tead1	TEA (DNA binding region) domain family member 1	Transcriptional factor involved in Hippo signaling pathway
Kdm1b	Lysine (K)-specific demethylase 1B	Histone lysine methylation regulation
Lpgat1	Lysophosphatidylglycerol acyltransferase 1	Glycerophospholipid metabolism
Man2a1	Mannosidase 2, alpha 1	Glycosyl hydrolase activity in N-glycans metabolism
Pgap1	Post-GPI attachment to proteins 1	Glycosylphosphatidylinositol (GPI) metabolism
Pikfyve	Phosphoinositide kinase, FYVE type zinc finger containing	Lipid and protein kinase activity, phosphatidylinositol metabolism
Psme4	Proteasome (prosome, macropain) activator subunit 4	Recognition of acetylated histones and promotion of core histones degradation
Slc12a2	Solute carrier family 12, member 2	Ion transport and neuropathic pain signaling
1700001L05Rik	RIKEN cDNA 1700001L05 gene	Other

4.1.5.1. Morphine is involved in *in-vitro* early embryo development and PGCs formation by regulating Bmp4 growth factor.

The interactive analysis identified *Bmp4* gene as a morphine target. Both RNA-Seq and Chip-seq analyses reveled that morphine is able to increase *Bmp4* gene expression by a down-regulation on H3K27me3 enrichment at promoter level (Figure 4.17A) and this results was validated by RT-PCR and Chip-qPCR (Figure 4.17C). BMP4 is a member of the bone morphogenetic protein family, which acts mainly through Smad-dependent pathway (the canonical pathway). BMP4 initiates the signal transduction cascade, where the R-Smads (Smad1/5/7) are phosporylated and then associated with the co-Smad (Smad4). Finally, this complex is translocated to the nucleus and associates with coactivators or corepressors to regulate gene expression, such as Prdm1 and Prdm14 genes for germ cell lineage formation (Yamaji M et al. 2008; Wang RN et al. 2014; Lochab AK and Extavour CG 2017). In fact, BMP4 is part of evolutionarily highly conserved superfamily of the transforming growth factor-beta that seems to be involved early in embryo development and PGC differentiation in mice (Lawson 1999; Yasuhide Ohinata et al. 2009; Graham 2014; Nicolas C. Rivron et al 2018; R. Michael Roberts et al. 2018). First, we analyzed the RNA-seq and ChIP-seq landscapes of all those mentioned genes to identify whether all the signaling pathway was affected or not (Figure 4.17A). Furthermore, we compared the observed differences with the counts per million (CPM) values boxplot from the RNA-seq (Figure 4.17B), realizing that some of the components of the R-Smad could be affected by morphine treatment, specifically at Smad5.

In line with BMP4 related functions, we next focused our attention on how morphine impacts on *in-vitro* early embryo development and PGC formation. Zygotes were recovered from the oviduct of mated animals and were cultured until two-cell-embryos stage. They were chronically treated with morphine during 24h and *in-vitro* cultured up to the blastocyst stage in the absence of morphine (Figure 4.17D). Although morphine did not induce any morphological changes on *in-vitro* embryo development, we reported an increase on blastocyst rate after morphine treatment. As Figure 4.17D shows, the percentage of embryos reaching the blastocyst stage were approximately 30% higher in morphine treated embryos than in control ones ($p<0.05$), suggesting that external morphine exposure may affect early embryo development.

Because BMP4 is also required for the generation of PGC in mouse embryo, we next aim to elucidate the effect of morphine on *in-vitro* PGC formation (Figure 4.17E). For that purpose, OCT4-GFP reporter mESCs, were chronically treated with morphine. Then, treated- and non-treated mESCs were used to differentiate PGC-Like cells (PGCLCs) via epiblast cell formation, as Hayashi et al. have described (Hayashi et al. 2011). Since OCT4 expression is known to increase during *in-vitro* and *in-vivo* PGC formation (Kehler J et al. 2004), the obtained PGCLCs were identified measuring OCT4-GFP fluorescence by FACS, with an efficiency of 5%. Morphine did not induce any morphological changes neither on *in-vitro* Epiblast Like Cells nor on PGCLCs. According to the results obtained from blastocysts analysis, morphine treatment caused an increase in the percentage of reaching PGCLCs. Actually, the recovered PGCLC amount was almost 5 times bigger ($p<0.05$) in cells from morphine treated mESCs than in control cells. These results confirmed that morphine had an influence on both processes, early embryo development and PGCs formation that are known to be regulated by BMP4 signaling.

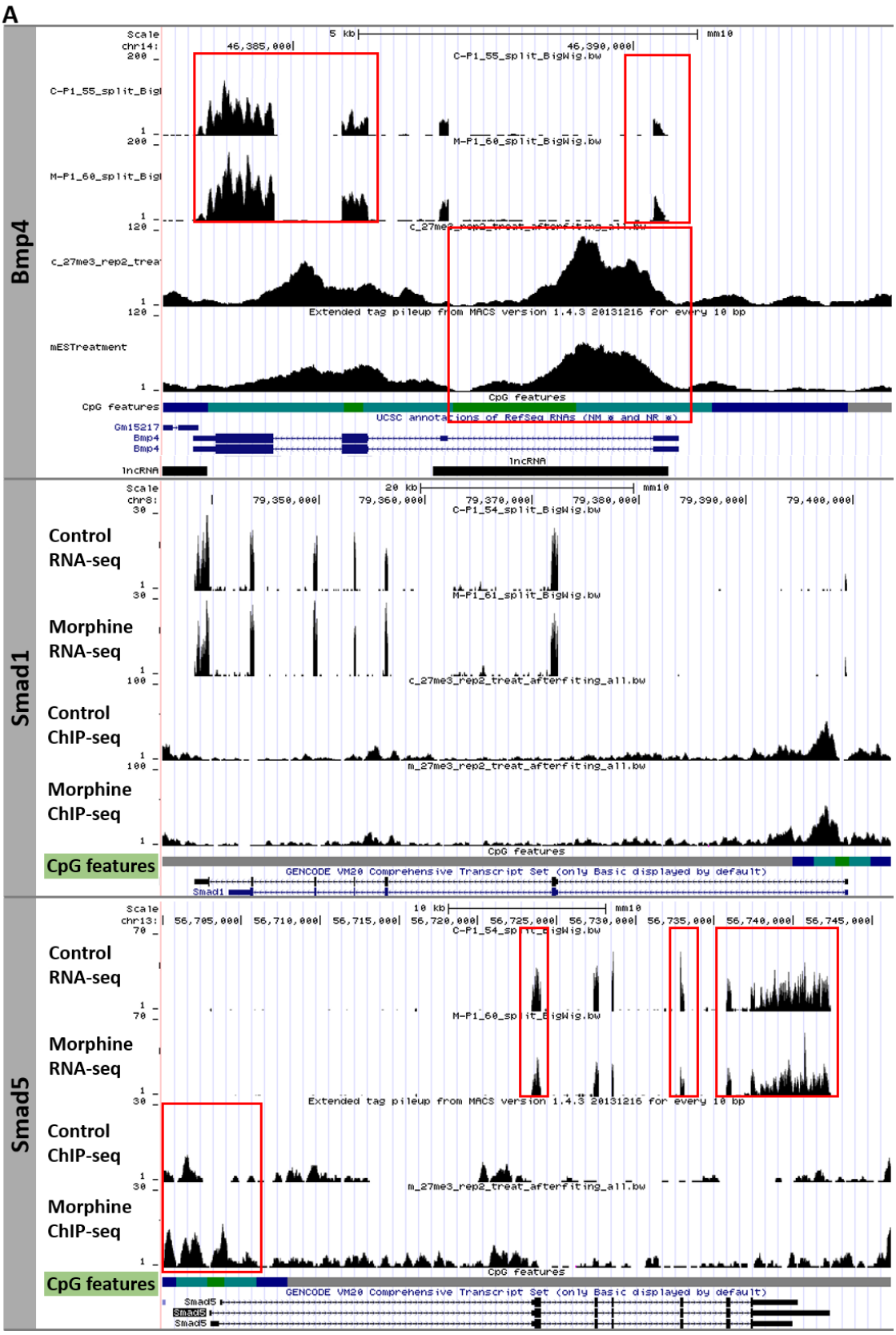

Figure 4.17. RNA-seq and H3K27me3 Chip-seq integration study around Bmp4 gene, and related involvement in embryonic development. (A) RNA-seq and H3K27me3 ChIP-seq track transitions enriched at promoter level, for *Bmp4* gene, and its signaling pathway components, *Smad1, Smad5, Smad7, Smad4, Prdm1* and *Prdm14*.

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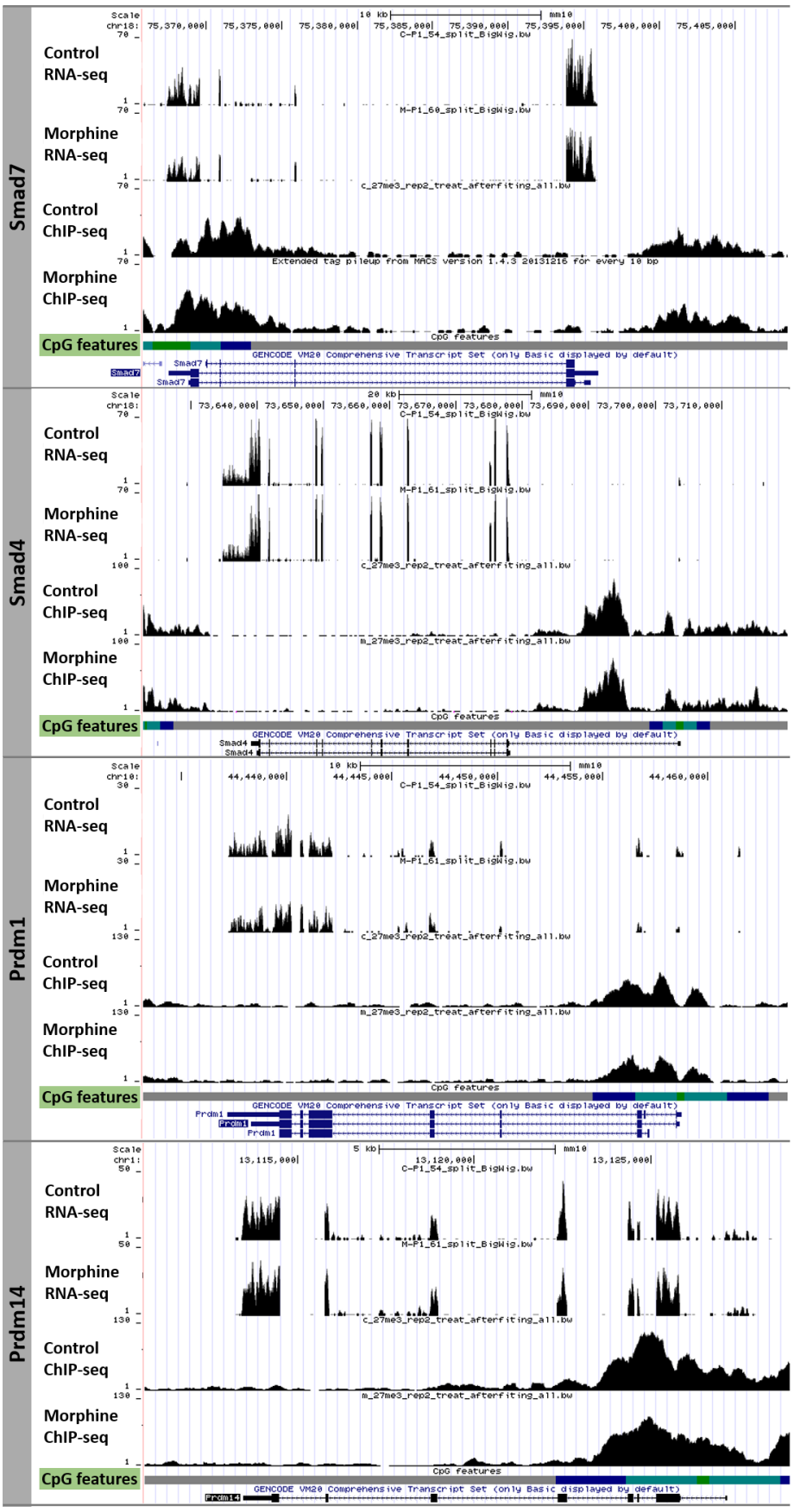

CpG features track was composed by CpG islands in green, Shores in light blue, Shelves in dark blue and Open sea in grey. Red boxes point out the enrichment and gene expression change location.

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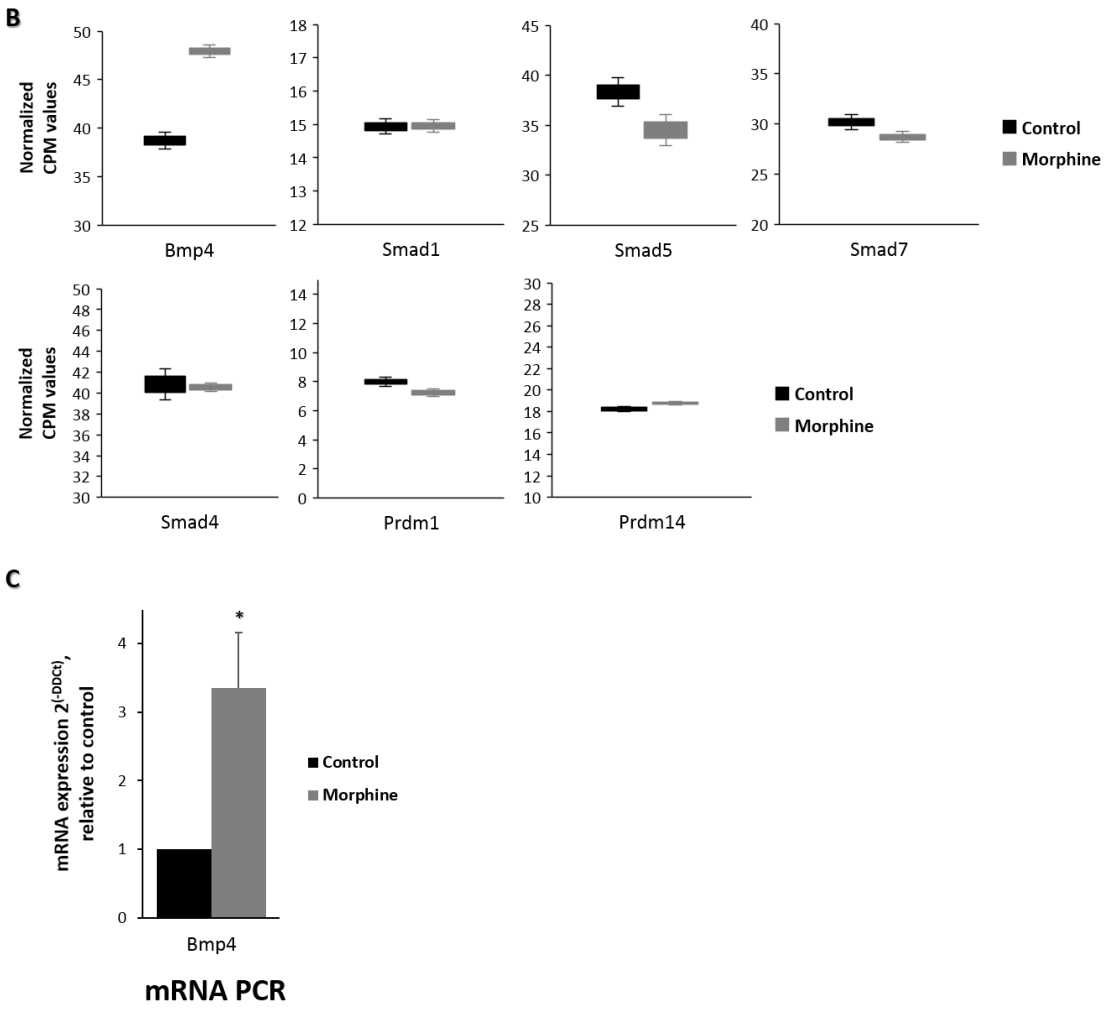

(B) Normalized CPM values of RNA-seq values summarized for *Bmp4* gene, and its signaling pathway components, *Smad1, Smad5, Smad7, Smad4, Prdm1* and *Prdm14*. (C) RT-qPCR analysis for the validation of *Bmp4* expression at mRNA level (left) and ChIP enrichment (right). *Gapdh* was used as housekeeping gene for mRNA level analysis and acquired Ct values were normalized respect to the control sample using 2^{ddCT}. ChIP-RT-qPCR amplification was normalized respect to the input sample. Statistical significance in B and C was determined by Student-T test (* p<0.05; **p<0.01; ***p<0.001. Sample size n=5.

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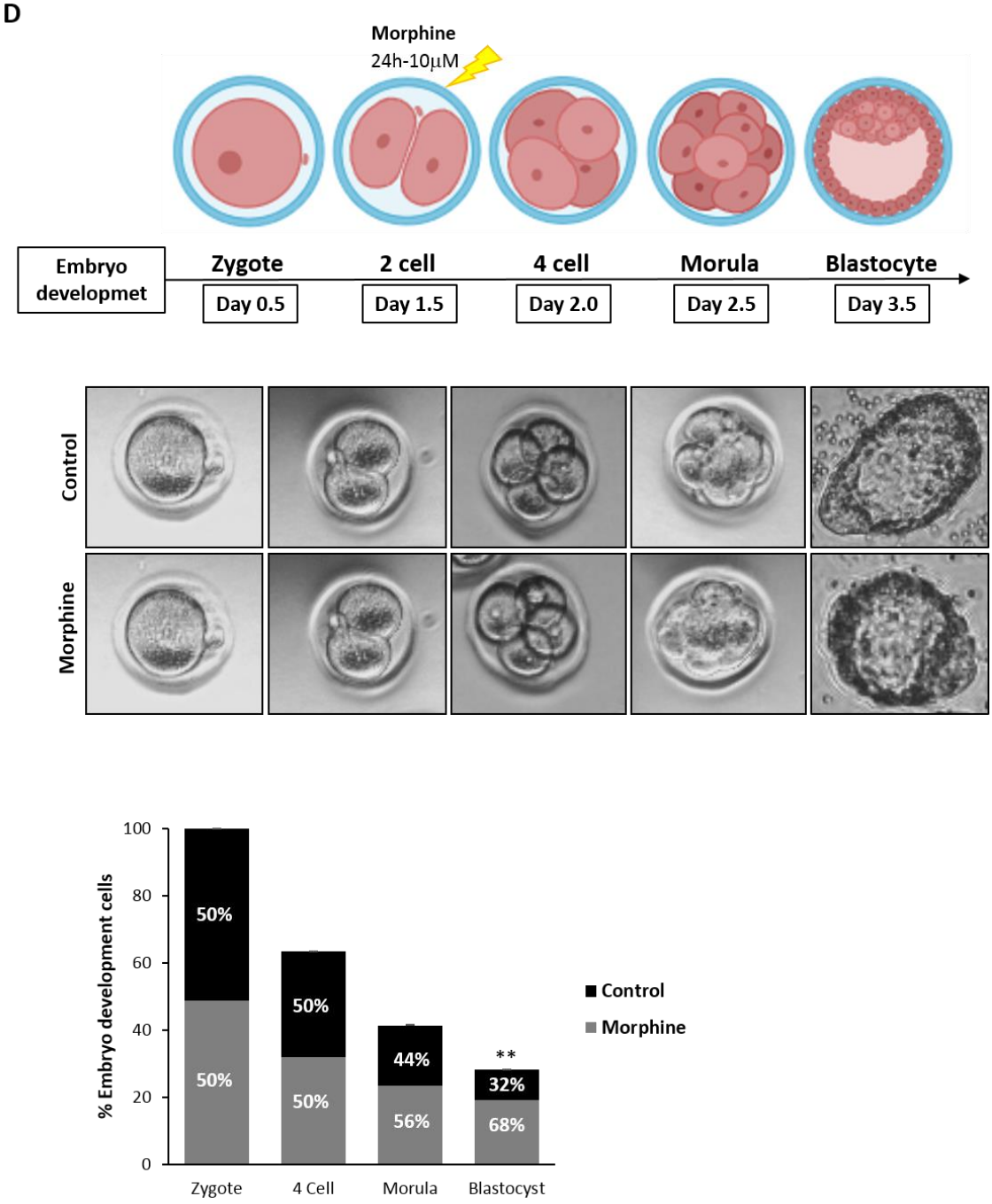

D) From the top to the bottom, embryo development study model; the results illustrated by culture pictures of zygote, 2cell embryos, 4 cell embryos, morula and blastocyst in control and morphine treated samples; and survival rate barr plot, summing up the total percentage of studied embryos and the respective percentage of control and morphine treated samples at each state. Statistical significance was determined by Student-T test (** $p<0.01$).

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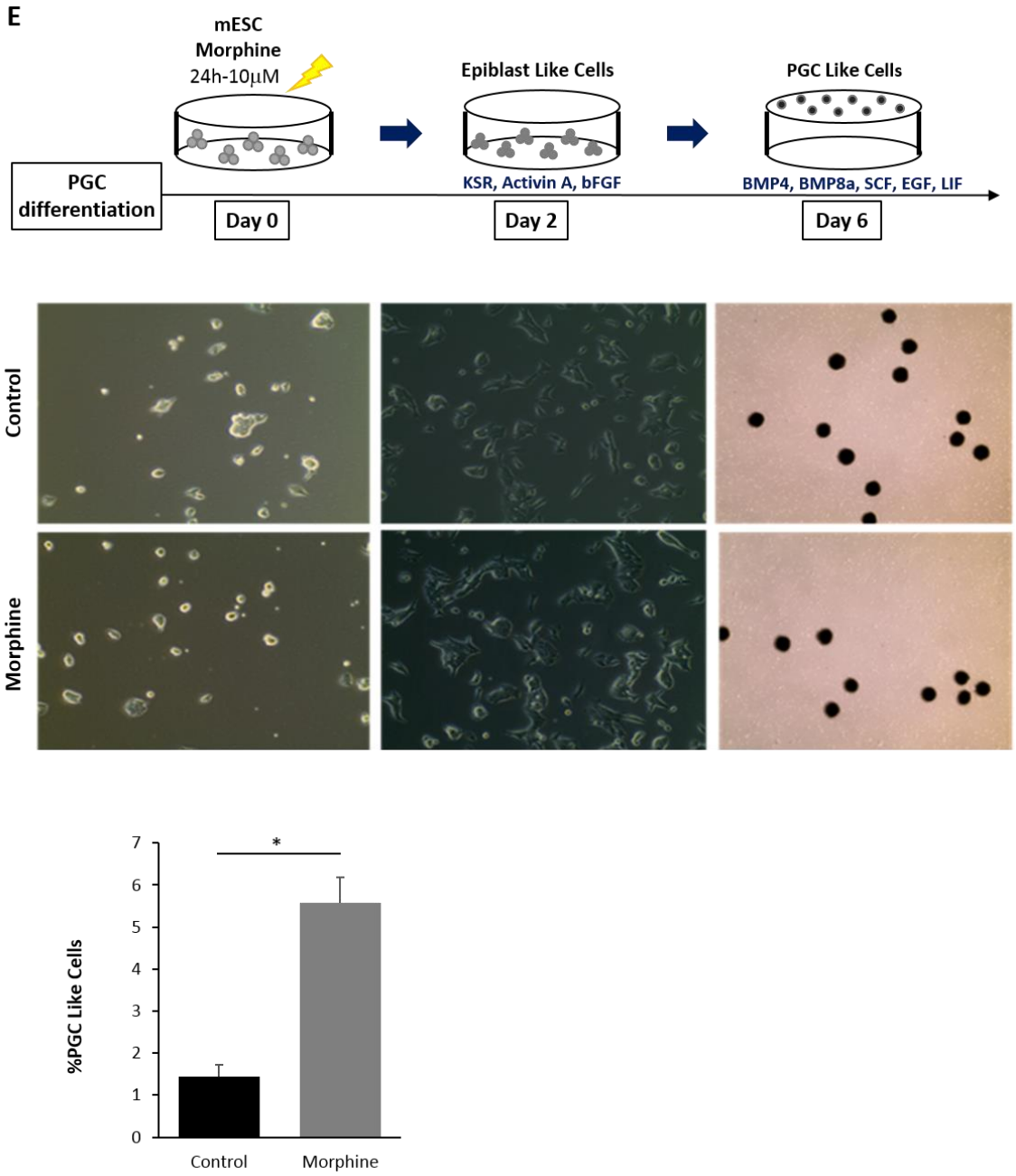

(E) From the top to the bottom, PGC differentiation study model; the results illustrated by culture pictures of mESCs, Epiblast like cells and PGC like cells; and plot summing up the percentage differences of obtained PGC Like Cells after differentiation. Statistical significance was determined by Student-T test (* $p<0.05$).

4.1.5.2. *In-vitro* epigenetic and genetic regulation of Polycom Complex 2 (PRC2) by chronic morphine treatment.

Integrative analysis revealed that morphine led to an increase on H3K27me3 enrichment at *Suz12* gene promoter that is consistent with its reduction on gene expression (Figure 4.18A). *Suz12* is a subunit belonging to polycomb repressive complex 2 (PRC2), the complex which primarily trimethylates H3K27, conferring a mark of transcriptionally silent chromatin. Chip-Seq and RNA-Seq approached confirmed that morphine was able to regulate PRC2 complex in mESC. Landscape in the UCSC genome browser showed an effect of chronic morphine treatment not only on *Suz12* but also on the other PRC2 components such as *Ezh2, Eed*, *Rbbp4 and Rbbp7* (Figure 4.18A). Morphine increased the H3K27me3 enrichment at promoters, which also correspond to CpG island region, in four members of the PRC2 components. Consistent to chromatin conformation by H3K27me3, RNA-seq traks showed a down regulation on all the mentioned PRC2 subunits, but specifically on *Suz12, Ezh2, Eed* gene expression pattern after morphine treatment, which was simplified with the CPM values boxplot (Figure 4.18B) and was validated previously by mRNA RT-qPCR (Figure 4.2C, repeated plots on Figure 4.18D). Our results, therefore, suggest that chronic morphine treatment lead to a global genome down regulation of H3K27me3 levels in mESCs by a self-regulation mechanism that involve PRC2 complex, in which morphine reduced the gene expression of main members of the complex by increasing H3K27me3 enrichment at the promoter levels.

In addition, other chromatin regulators, for instance, protein deacetylase *Sirt1* gene was identified after interactive analyses that is known to be an interactor of PRC2 complex (Montie HL et al. 2011), facilitating the binding of the PRC2 complex to the target promoters mostly linked to DNA damage response. Chronic morphine treatment also induced an increase on H3K27me3 enrichment at promoter level, corresponding to the highest enrichment peak at CpG island region (Figure 4.18A), and a decrease the gene expression pattern (Figure 4.18B), suggesting that another epigenetic mark such as acetylation could also be important on epigenetic mechanisms induced by morphine.

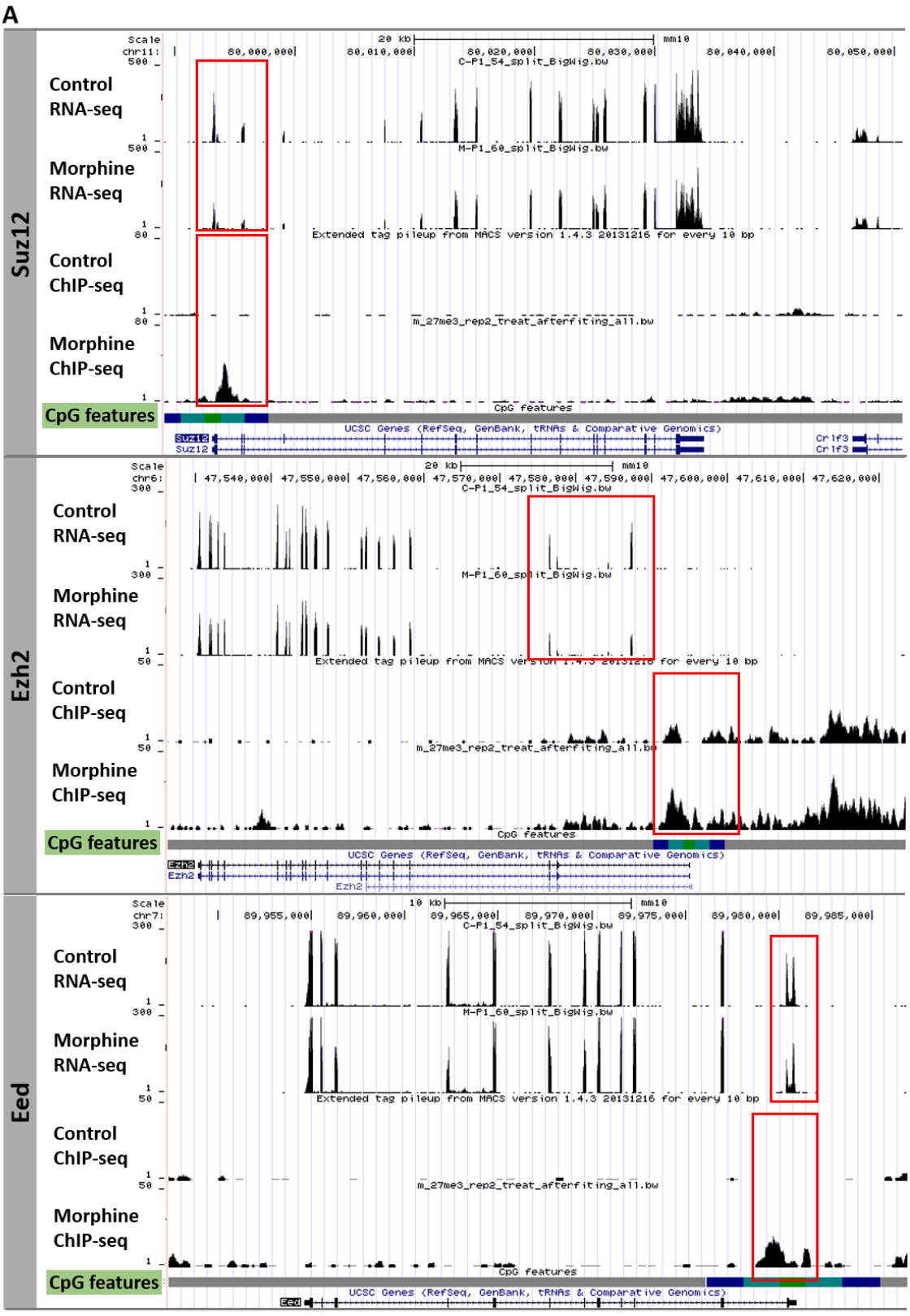

Figure 4.18. RNA-seq and H3K27me3 Chip-seq track integration around Suz12 and related PRC2 complex genes. (A) RNA-seq and H3K27me3 ChIP-seq track transitions enriched at promoter level, for *Suz12* and main components of PRC2 complex: *Ezh2, Eed, Rbbp4* and *Rbbp7. Sirt1* deacitylase is also included

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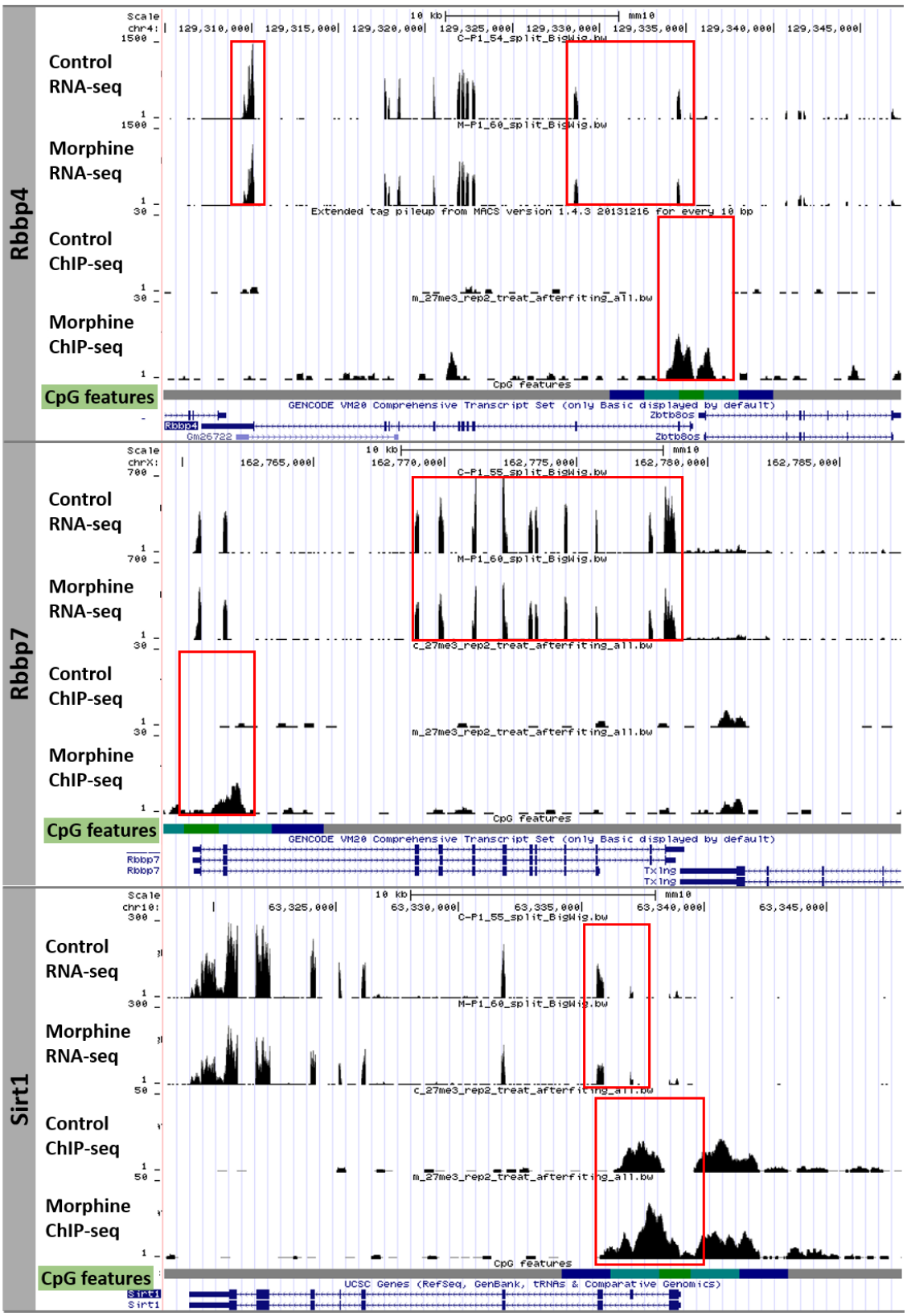

CpG features track was composed by CpG islands in green, Shores in light blue, Shelves in dark blue and Open sea in grey. Red boxes point out the enrichment and gene expression change location.

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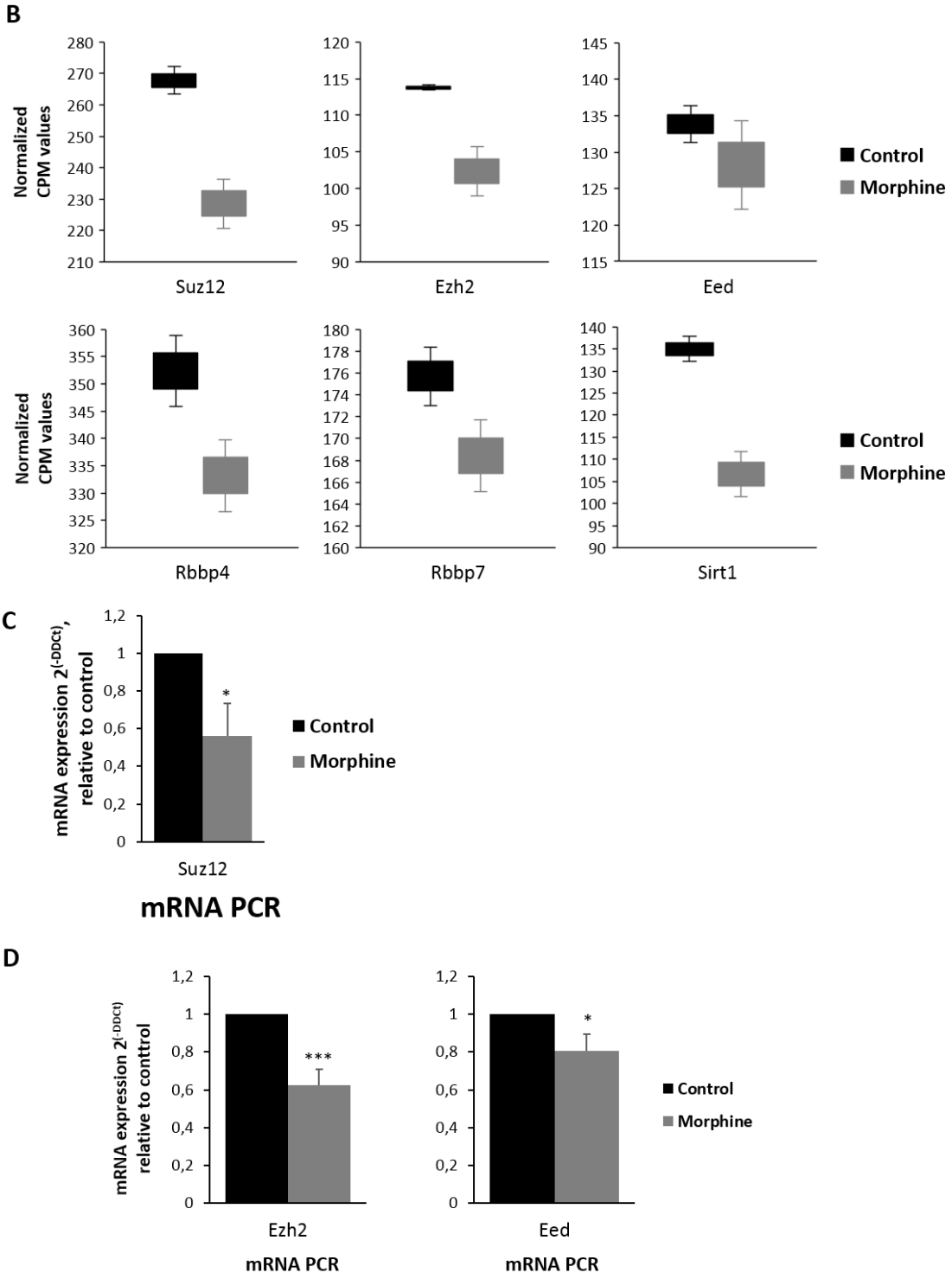

(B) Normalized CPM values of RNA-seq experiment summarized for *Suz12* gene, main PRC2 subunits *Ezh2, Eed, Rbbp4* and *Rbbp7*, together with *Sirt1* deacetylase. (C) RT-qPCR analysis for the validation of *Suz12* expression at mRNA level (left) and ChIP enrichment (right). (D) RT-qPCR for the validation of *Ezh2* and *Eed* expression at mRNA level. *Gapdh* was used as housekeeping gene for mRNA level analysis and acquired Ct values were normalized respect to the control sample using 2^{ddCT}. ChIP-RT-qPCR amplification was normalized respect to the input sample. Statistical significance in C and D was determined by Student-T test (* $p<0.05$; ** $p<0.01$; *** $p<0.001$. Sample size n=5.

4.1.5.3. Morphine induces a Smchd1 silencing through H3K27me3 enrichment conferring to the cell an active state of X chromosome

Another important epigenetic regulator identified by interactive analyses was the structural maintenance of chromosomes hinge domain containing 1 *(Smchd1*). SMCHD1 is involved in epigenetic repression throughout the genome, playing a critical role in silencing the inactive X chromosome in females (Wutz A 2011; Sakakibara Y et al. 2018) and in transcriptional repression of specific autosomal loci including some imprinting genes and protocadherins (Mould AW et al. 2013; Gendrel AV et al. 2013; Leong HS et al. 2013; Chen et al. 2015) and also Hox clusters (Chen K et al. 2015; Jansz N et al. 2018). X-chromosome inactivation (XCI) in mammals is a process that exemplifies the developmentally controlled formation of silent chromatin where one of the X chromosomes in females is inactivated for dosage compensation between the sexes. The process of XCI involves several steps that are orchestrated in developmental manner. The process implies the initiation of chromosome-wide silencing by XIST lncRNA, following the formation of a repressive compartment through DNA methylation and PRC mediated chromatin modifications. Finally, the last step is the maintenance of the stable repression of Xi in which SMCHD1 plays an important role. In this way, RNA-Seq and Chip-Seq approaches indicated that morphine induced a down-regulation of *Smchd1* gene expression by increasing the H3K27me3 enrichment at promoter (Figure 4.19A), suggesting that morphine may affect X chromosome inactivation, specifically in the stability and maintenance of chromosome silencing. This result was validated at mRNA level and ChIP enrichment level by RT-qPCR (Figure 4.19F).

To understand if morphine has also an effect on the initiation of chromosome-wide silencing, we evaluated the impact of morphine on *Xist* expression. Landscapes from RNA-Seq and Chip-Seq confirmed that morphine caused a down regulation of *Xist* gene expression mediated by an up-regulation of H3K27me3 along the gene body (Figure 4.19B). Acting as a mechanism in *cis* *XIST* is required for XCI, sequestering X chromosome from all other chromosomes within the same nucleus. However, this mechanism is not enough for complete silencing and subsequently, chromatin within *Xist* domain is repressed by recruiting non canonical PRC1 and PRC2 complexes leading to chromosome-wide histone modifications on the Xi. Our results indicated that morphine was also able to modify both complexes, non canonical PRC1 (Figure 4.19C) and PRC2 (Figure 4.18A), reducing the gene expression of all the components belonging to each complexes by modulating H3K27me3 chromatin conformation. Moreover, in this complex system around X chromosome inactivation, it seems that DNMT1 DNA methylatransferase is required for the methylation of the promoters on the Xi and as a maintenance mark for gene repression (Sado T et al. 2000; Wutz A 2011). Landscapes from RNA-Seq and Chip-Seq confirmed that morphine caused a down regulation of *Dnmt1* gene expression mediated by an up-regulation of H3K27me3 on promoter region (Figure 4.19D). To simplify the landscapes visualization, normalized CPM values of RNA-seq experiment recompiling all studied genes were also plotted (Figure 4.19E). ChIP-seq analyses also confirmed that morphine reduced the whole coverage of H3K27me3 enrichment in X chromosome after morphine treatment (Figure 4.19G), indicating that morphine was able to alter not only the initiation of chromosome-wide silencing but also the maintenance of XCI in mESC, providing to the cell an active state of transcription.

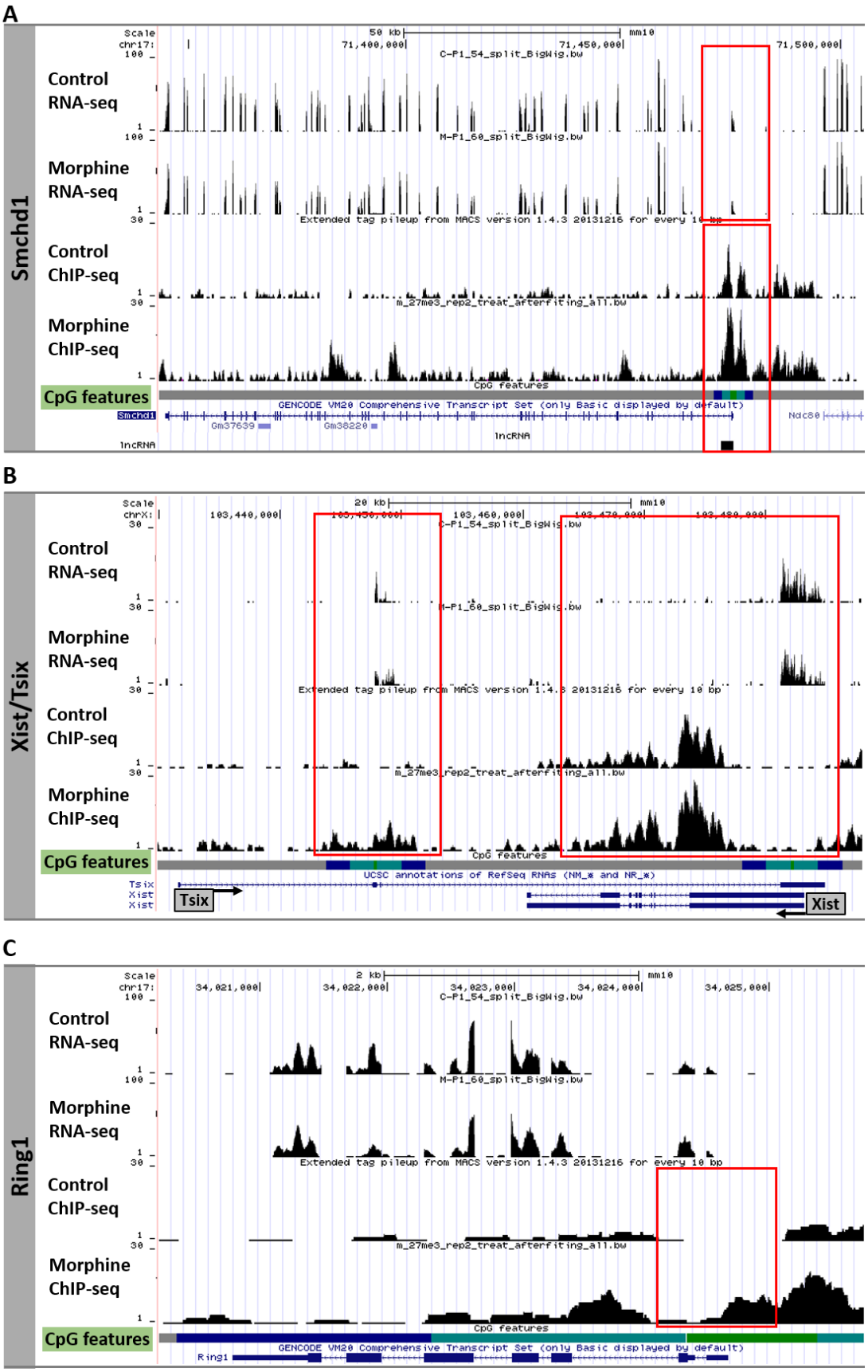

Figure 4.19. RNA-seq and H3K27me3 Chip-seq track integration around Smchd1 and around X chromosome inactivation process. RNA-seq and H3K27me3 ChIP-seq track transitions enriched at promoter level, for (A) *Smchd1*, (B) *Xist/Tsix* and (C) PRC1 subunits: *Ring1, Rnf2, Pcgf3, Pcfg5, Yaf2, Rybp, Cbx2* and *Cbx4*.

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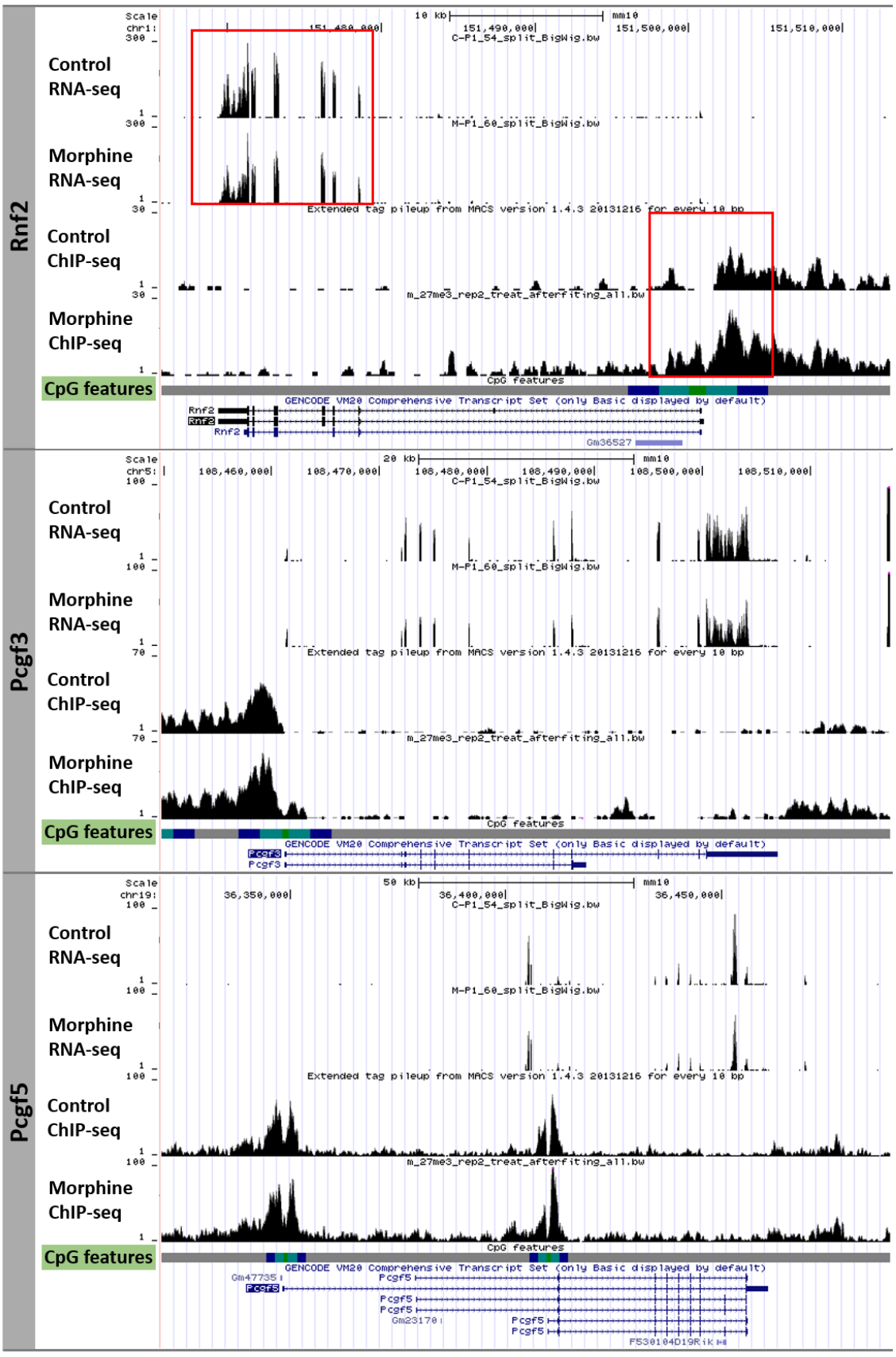
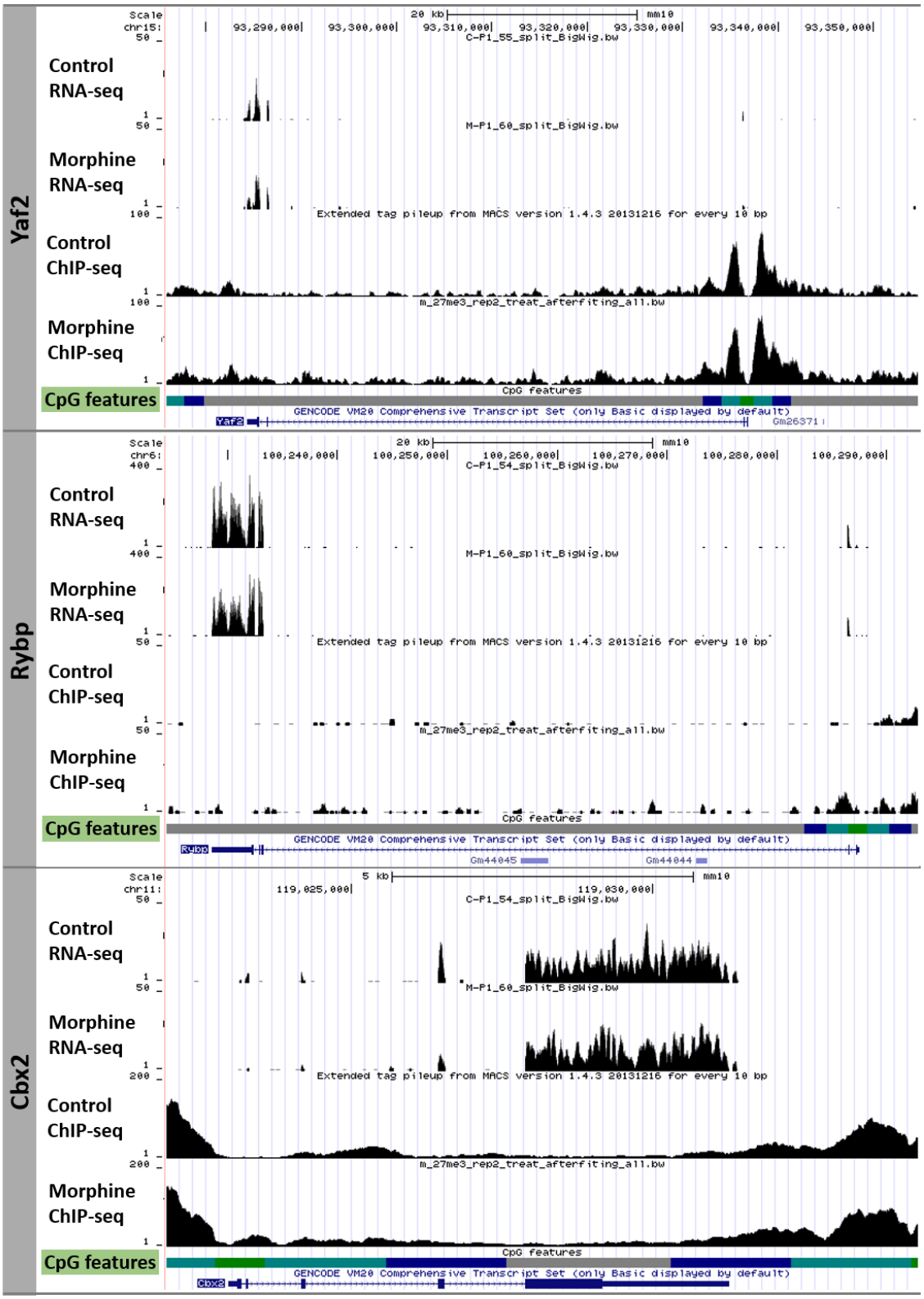
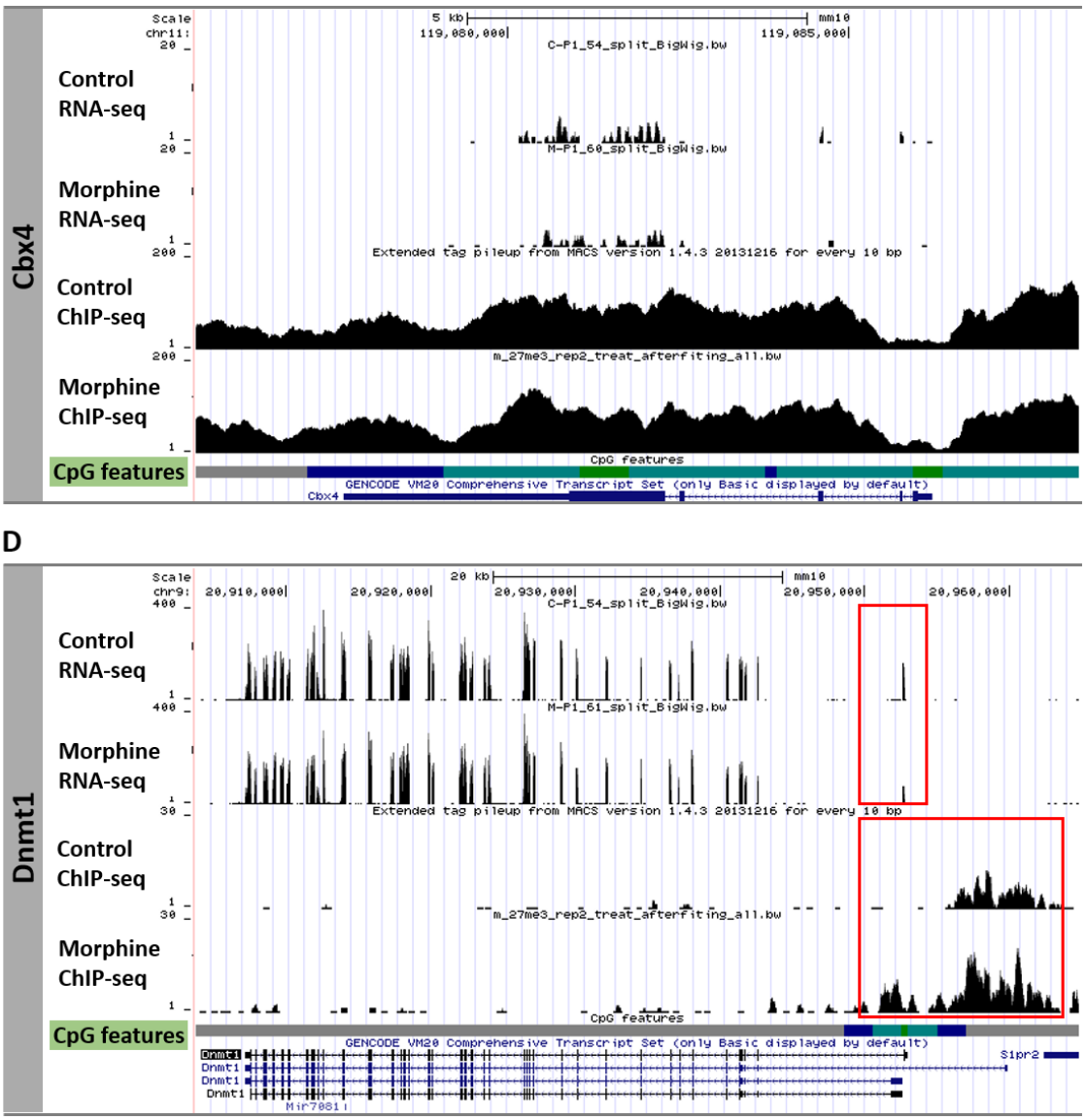

Rnf2
Control RNA-seq
Morphine RNA-seq
Control ChIP-seq
Morphine ChIP-seq
CpG features
Pcgf3
Control RNA-seq
Morphine RNA-seq
Control ChIP-seq
Morphine ChIP-seq
CpG features
Pcgf5
Control RNA-seq
Morphine RNA-seq
Control ChIP-seq
Morphine ChIP-seq
CpG features


Yaf2
Control RNA-seq
Morphine RNA-seq
Control ChIP-seq
Morphine ChIP-seq
CpG features
Rybp
Control RNA-seq
Morphine RNA-seq
Control ChIP-seq
Morphine ChIP-seq
CpG features
Cbx2
Control RNA-seq
Morphine RNA-seq
Control ChIP-seq
Morphine ChIP-seq
CpG features



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(D) RNA-seq and H3K27me3 ChIP-seq track transitions enriched at promoter level for *Dnmt1* gene. For all the landscapes shown in the figure, CpG features track was composed by CpG islands in green, Shores in light blue, Shelves in dark blue and Open sea in grey. Red boxes point out the enrichment and gene expression change location.

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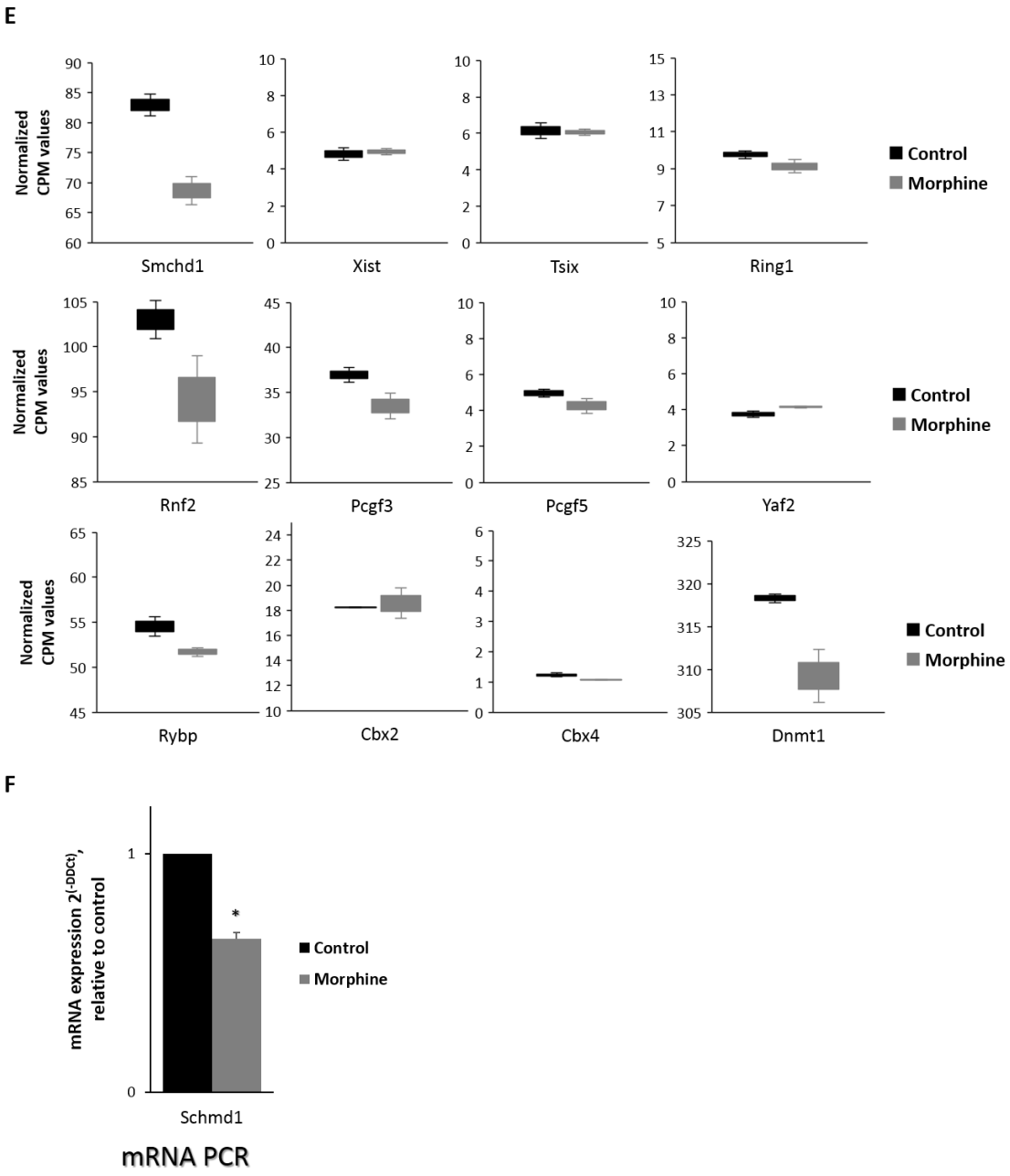

(E) Normalized CPM values of RNA-seq values summarized for *Smchd1* gene and X chromosome inactivation process participants*, Xits/Tsix*, PRC1 subunits *Ring1, Rnf2, Pcgf3, Pcgf5, Yaf2, Rybp, Cbx2* and *Cbx4*, and *Dnmt1* genes. (F) RT-qPCR analysis for the validation of *Smchd1* expression at mRNA level (left) and ChIP enrichment (right). *Gapdh* was used as housekeeping gene for mRNA level analysis and acquired Ct values were normalized respect to the control sample using 2^{ddCT}. ChIP-RT-qPCR amplification was normalized respect to the input sample. Statistical significance in B and C was determined by Student-T test (* p<0.05; **p<0.01; ***p<0.001. Sample size n=3.

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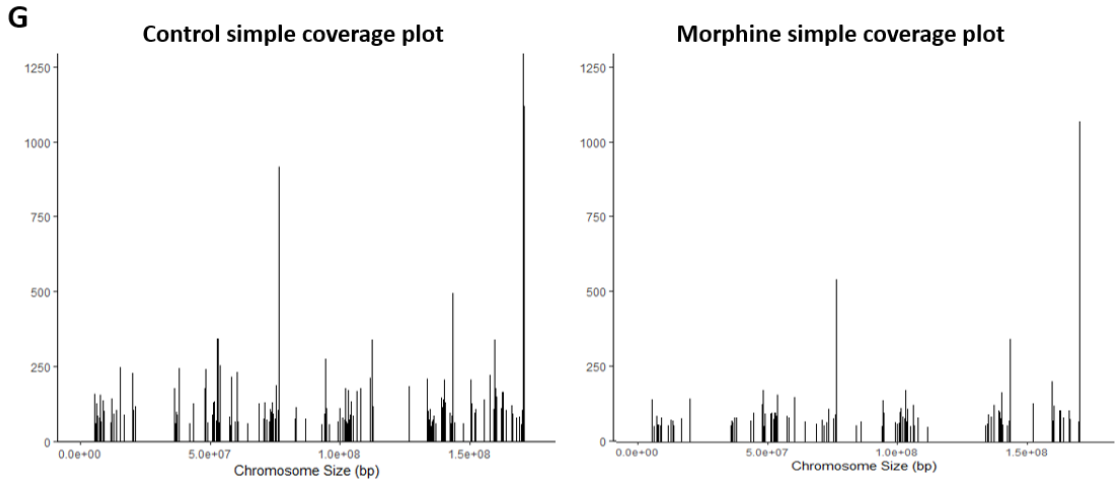


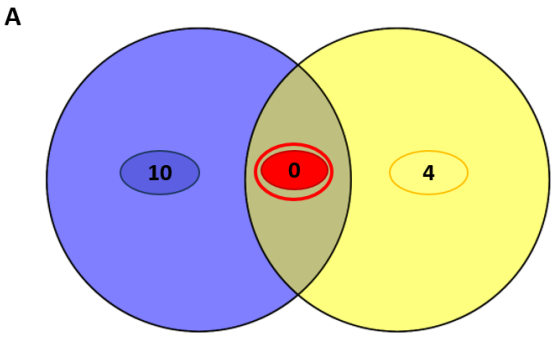
(G) Coverage plot of H3K27me3 enrichment, showing coverage of peak regions over X chromosome in control and morphine treated sample.

4.1.5.4. Morphine differentially affects specific imprinted genes and repetitive elements groups expression *in-vitro* in mESC

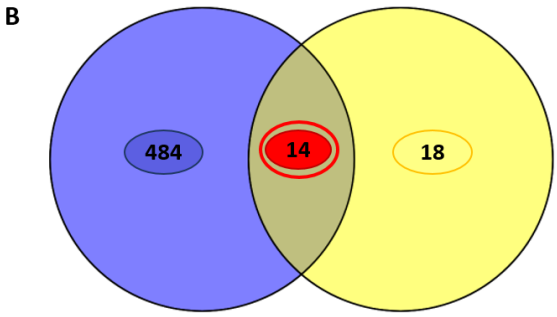
To understand how morphine can epigenetically modify the expression of imprinting genes and repetitive elements through changes in the distribution of H3K27me3, we next performed an interactive analysis between RNA-seq and ChIP-seq deregulated genomic features. For that purpose, we filtered out only those groups, which showed reversed patterns between feature expression and histone enrichment.

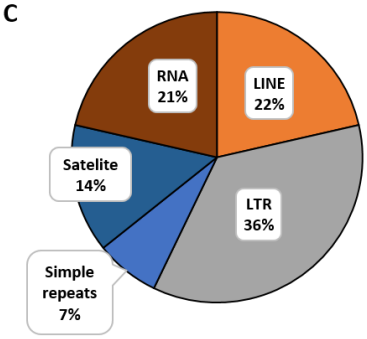
Regarding imprinting genes, unfortunately, we did not find any common gene between RNA-Seq and Chip-Seq data (Figure 4.20A). In spite of that we found some genes which belong to same imprinted cluster, that is the case of Ube3a (result from RNA-seq) and Snrpn, Snurf and Atp10a (results from ChIP-seq), all of them under control of SNRPN imprinted cluster, that was epigenetically regulated by morphine modifying the H3K27me3 chromatin distribution at ICR region. Moreover, we also identified Mirg (result from RNA-seq) and Begain (result from ChIP-seq) that belong to Dlk1 –Diol3 imprinted cluster. This coincidence open us a new field of studying these both imprinted regions more in deep, as morphine induced H3K27me3 histone conformational changes, could be affecting the general regulation of the imprinted cluster, and in that way changing the expression pattern of the components of the cluster.

Next, we focused on repetitive elements, and compared our results from H3K27me3 Chip-seq and RNA-seq after 24h morphine treatment. We obtained 14 matchings in different repetitive element groups (Figure 4.20B), belonging to LINE, LTR composed by ERVK subfamilies, Simple repeats, Satellite and RNA repeats composed by rRNA (Figure 4.20C). In (Figure 4.20D) a detailed table is added with the group and family name of each detected repetitive element.



Imprinted genes





D

Repetitive elements common in ChIP-seq and RNA-seq		
Repetitive Family	Repetitive Class	Repetitive element name
LINE	L1	Lx3A
LINE	L1	L1ME4c
LINE	L1	L1Md_F3
LTR	ERVK	MMERVK10C-int
LTR	ERVK	RMER17A2
LTR	ERVK	RLTR13A1
LTR	ERVK	ERVB7_2B-LTR_MM
LTR	ERVK	RLTR10E
Simple_repeat	Simple_repeat	_CTCGG_n
Satellite	Satellite	MMSAT4
Satellite	Satellite	MurSAT1
RNA repeats	tRNA	tRNA-Leu-TTA_m_
RNA repeats	rRNA	LSU-rRNA_Hsa
RNA repeats	rRNA	SSU-rRNA_Hsa

Repetitive elements

Figure 4.20. Interaction between H3K27me3 ChIP-seq and RNA-seq analysis in repetitive elements. (A) Venn diagram showing the overlap between H3K27me3 differentially enriched imprinted genes and RNA-seq, after chronic morphine treatment. (B) Venn diagram showing the overlap between H3K27me3 differentially enriched repetitive elements and RNA-seq, after chronic morphine treatment. (C) Common repetitive elements subclasses distribution plot. (D) Table resume of common repetitive elements in both techniques, specifying repetitive family, class and element names.

4.2 EPIGENETIC MEMORY INDUCED BY MORPHINE IN mESC

4.2.1. *In-vitro* epigenetic memory induced by morphine in mESC

To evaluate if morphine can induce a cellular memory, we next analyzed the dynamic changes over the time. After 24 hours of chronic treatment (P1), morphine was remove and mESC were maintain in culture for 48hours (P2) and 96 hours (P3) (Figure 4.21) in order to identify the histone marks that persist after morphine withdrawal.

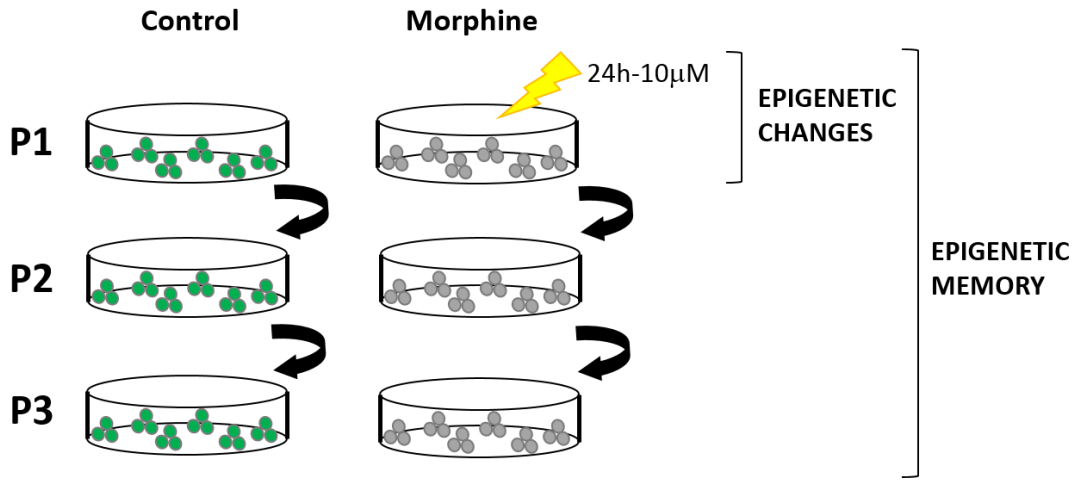


Figure 4.21. Scheme of mESC culture for *in-vitro* epigenetic memory determination. Three time points were analyzed: P1, cells collected just after 24h morphine treatment, P2 cells collected 24h after the treatment termination and P3, cells collected 48h after the treatment end.

Morphine induced a down-regulation of H3K27me3 levels in P1, which persisted in P2 and P3 (Figure 4.22A, B) even in the absence of morphine in the medium. In regard to H3K9me2 and H3K4me3, a similar dynamic was reported after morphine treatment, exhibiting an increase on histone levels in P1, followed by an acute decrease in P2 and a final recovery in P3. Finally, morphine could also induce an epigenetic memory in mESC by modifying the H3K4me2 levels. A decrease of H3K4me2 levels were measured after 24h of treatment that persisted in P2 but not in P3, in which an up-regulation of histone levels were measured.

To understand how morphine can produce an epigenetic memory in mESC by histone modifications, we evaluated the impact of morphine on their regulatory complexes over the three time points (Figure 4.22C) by RT-qPCR. In a concordance with H3K27me3 dynamic levels, we observed a significant down regulation in *Ezh2, and Eed* gene expression in P1, which persist over the time in P2 and P3 when morphine is absent.

In contrast, G9a/GLP complex genes, showed reversed expression patterns. On one hand, *G9a* presented a significant increase after morphine treatment, which was maintained at P2 and finished with a sharper upregulation at P3. On the other hand, *Glp* presented also a significant increase in gene expression after morphine chronic exposure, but then it was decrease during P2 and P3. Interestingly, this expression pattern after morphine also fit in with H3K9me2 levels dynamic after morphine treatment. Regarding the catalytic subunit of the Mll1 complex, it was

decreased in P1 and gradually increased between P2 and P3 time points, in contrast other components, as *Rbbp5* seem not to be substantially affected by morphine at P1, but followed a significant decrease at P2 and expression recovery at P3, consistent with H3K4me3 levels dynamics over time. Finally, in Setd1 complex genes, *Setd1a* presented a significant upregulation after morphine treatment, which decreased slightly (maintaining upregulated values) between P2 and P3. In contrast, *Setd1b* showed a pronounced decrease in gene expression between P1 and P2 with a final recovery at P3. Finally, *Cfp1* gene, displayed a pronounced increase at P1, then the expression value decreased to control line and finally in P3 the upregulation of the expression was recovered. Therefore, with the reverse pattern followed by both catalytic subunits of the complex the upside down gene expression pattern could be consistent with H3K4me2/me3 levels dynamic after morphine treatment.

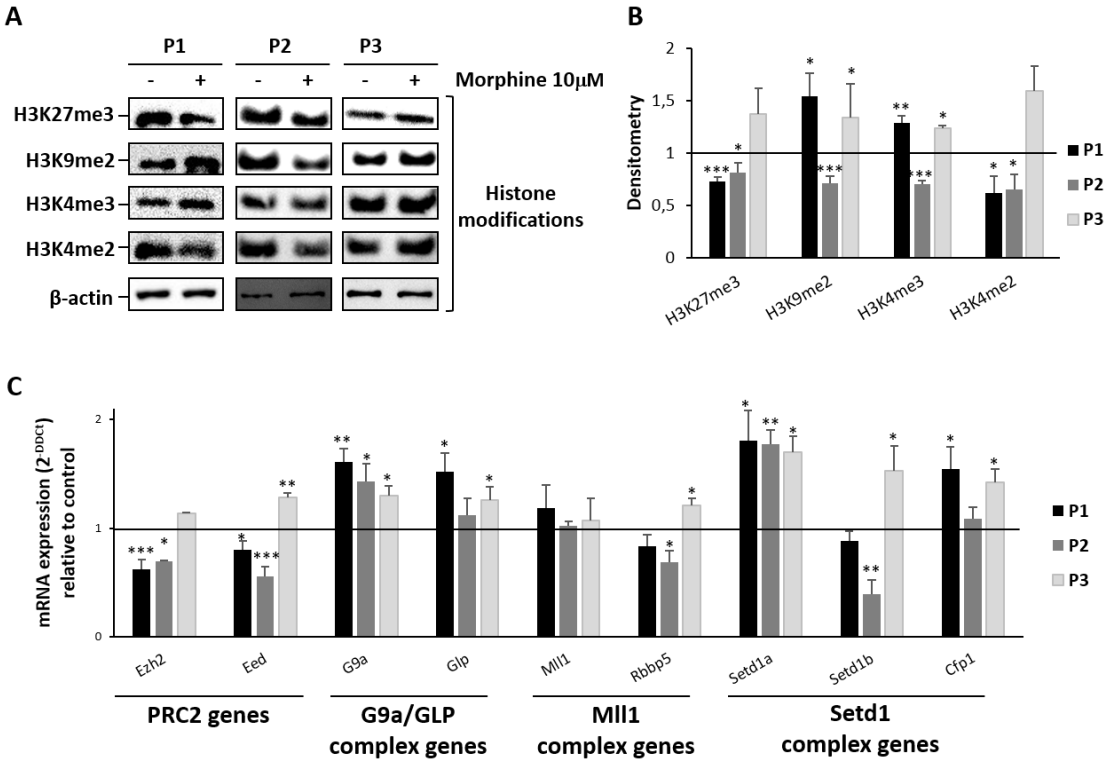


Figure 4.22. Dynamic changes of histone modification induced by morphine over time in mESC *in-vitro*. (A) Western blot analysis of H3K27me3, H3K9me2, H3K4me3, H3K4me2 after morphine treatment for 24h (P1), and 48h and 96h after morphine treatment withdrawal (P2 and P3 respectively). β-actin was used as loading control. (B) Quantification of H3K27me3, H3K9me2, H3K4me3, H3K4me2 levels measured by Image J software. (C) RT-qPCR analysis of genes belonging to each histone modification methylation complex. *Gapdh* was used as housekeeping gene. Acquired Ct values were normalized respect to the control sample using 2^{ddCT}. Statistical significance in B and C was determined by Student-T test (* p<0.05; **p<0.01; ***p<0.001). Sample size n=5.

This findings demonstrate that regardless the initial effect, morphine generates a global decrease at P2 time point which seem to be recovered at P3, both in histone modification levels and also in gene expression of specific methylation complexes, confirming our idea that they could be mediating this changes leaded by morphine.

Once epigenetic changes were described after 24h of morphine treatment, the next step was to identify which specific epigenetic changes could persist over time once the treatment was finished, contributing to the epigenetic memory.

4.2.2. Morphine induces global deregulation at transcriptomic level in mESC *in-vitro*, which could be maintained over time

For that purpose, we performed a systematic evaluation of transcriptomes at previously described experimental design of the three time points (P1, P2 and P3, Figure 4.23), through a RNA-Seq experiment.

First, we carried out the PCA plot, to demonstrate the distinct enrichment profiles between the three different time points (Figure 4.23A), and then performed a read count distribution plot, showing a correct normalization method (TMM normalization) (Figure 4.23B). Next, differential expression analysis was carried out with $p<0.05$ and $FDR<0.05$ criteria revealing a total of 932 DEGs at P1, 1196 DEGs at P2 and 2138 DEGs at P3 (Figure 4.23C, D). In these 932 DEGs at P1 after 24h morphine treatment, approximately 41% (n=386) were upregulated and 59% (n=546) were downregulated. On next pass, after morphine treatment withdrawal (P2), 37% of the DEGs (n=448) were upregulated and 63% (n=748) were downregulated. Finally on P3 (second pass after morphine treatment withdrawal) 49% of the identified DEGs (n=1039) were upregulated and 51% (n=1099) were downregulated. Partially overlapping gene groups were found between P1 and P2 (n=104), P2 and P3 (n=388) and P3 and P1 (n=336), and surprisingly 181n genes were detected to be DE in all three groups (Figure 4.23E). These results suggest that morphine induces a cell memory, as gene expression changes are observed even when morphine treatment is not present.

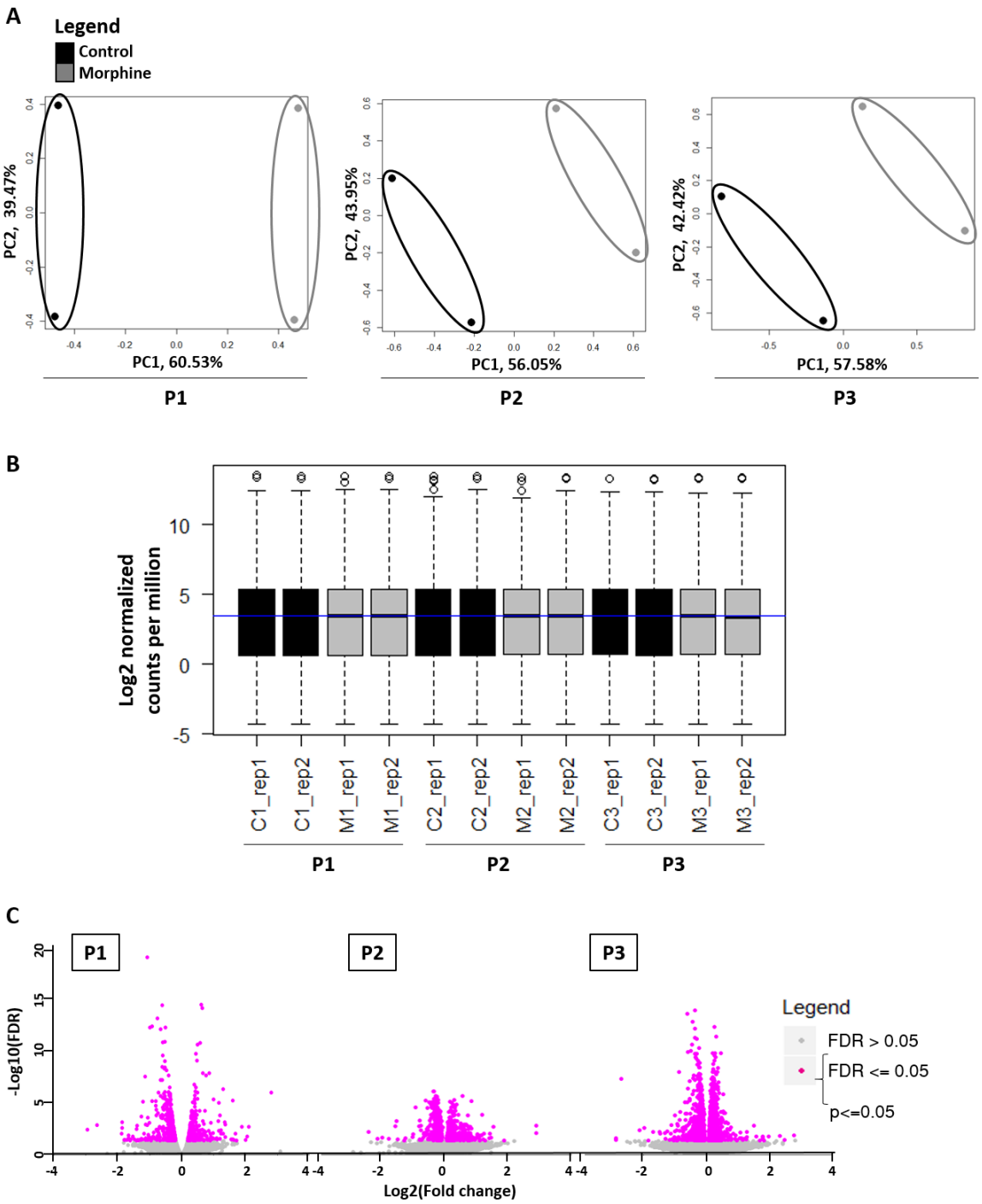


Figure 4.23. Differential gene expression analysis over time. (A) Principal component analysis plot of studied three time points. (B) Boxplot of read-count from three time points and respectively control and morphine treated samples of RNA-seq experiment. (C) Volcano plot of DEGs at P1, P2 and P3: in purple, significant genes (p<0.05 and FDR<0.05), in grey not significant genes.

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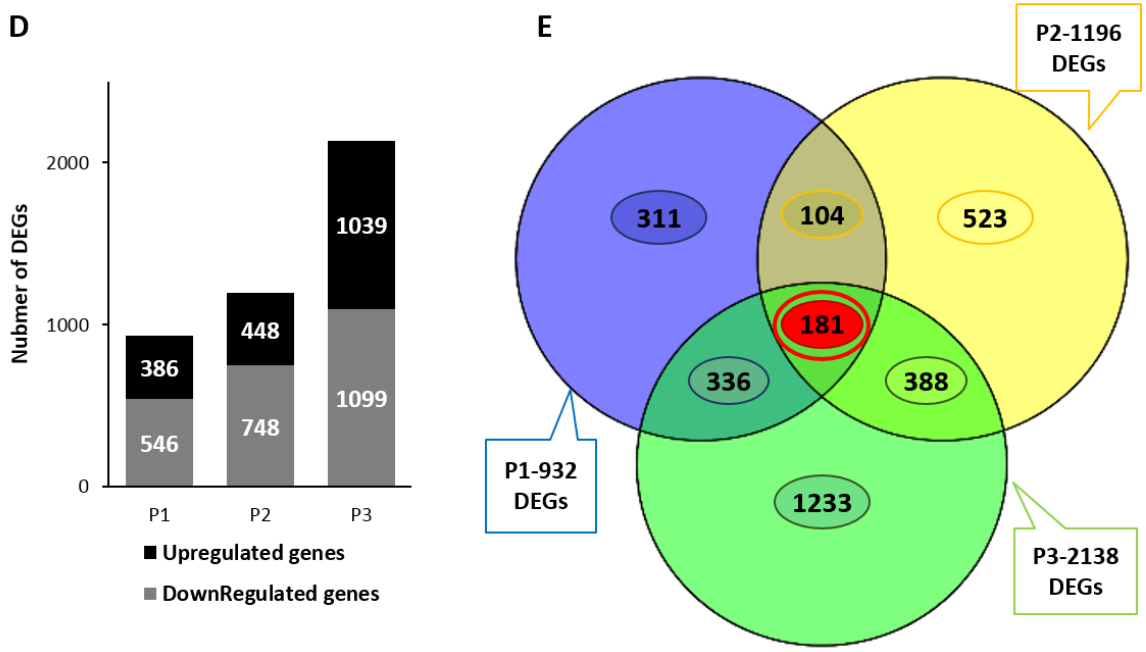


(D) Bar chart of the number of genes found to be upregulated or downregulated after 24h after treatment (P1) and next passes after treatment withdrawal (P2 and P3). (E) Venn diagram representing the number of DEGs between three time points.

Next, we explored the biological functions of genes that were deregulated between the three time points separately (Figure 4.24A). At P1, 24h after morphine treatment, DEGs were related with basic cellular functions, such as cell division processes mainly involved in mitosis. We also found DEGs related to basic nuclear functions as for example regulation of gene expression and DNA together with cellular functions such as metabolic and catabolic processes. DEGs corresponding to P2 were interestingly implicated in nuclear functions, such as, DNA repair, transcription process, regulation of gene expression, histone modification and chromatin remodeling functions, together with specific structural developmental processes, for instance, in utero embryonic development, heart, brain and reproductive structure development. This revealed that morphine treatment effect was persistent at P2 even after the withdrawal. In fact the described deregulation was permanent at P3 as DEGs were related to mainly nuclear functions such as, cell division process, specifically in meiosis, regulation of DNA replication process, together with transcription, regulation of gene expression and histone modification. Also more concrete processes related to structural developmental processes were deregulated at P3, for example, tissue development and regulation of neurogenesis process.

Furthermore, GO functional category analysis revealed several biological functions, which suffer dynamic changes over time (Figure 4.24B). It grabs attention, how DEGs related to developmental processes were found in all three time points. More specifically this was the pattern, which correspond to DEGS related to nervous system process. Besides, DEGs involved in cell cycle processes showed a minor increase between P1 and P2, which was maintained at P3. Surprisingly, DEGs involved in biological functions related to epigenetic mechanisms were the ones, which presented biggest variations, that is to say, DEGs related to chromosome organization and gene expression regulation, presented increased fold enrichment from P1 to P2, to further maintain around high values at P3, while protein modification processes seemed to present same values at three time points. Further, DEGs related to cellular response to DNA damage presented the highest enrichment point at P2, while DEGs involved in metabolic

processes, also presented a slight increase between P1 and P2 and it was maintained at P3. In contrast, DEGs related to cellular component organization or biogenesis processes showed the highest increase at P2, which was maintain in high values at P3. These findings concluded that morphine could be generating epigenetic memory in DEGs related to several biological processes since the observed fold enrichment pattern changed or maintained over different time points even after treatment withdrawal.

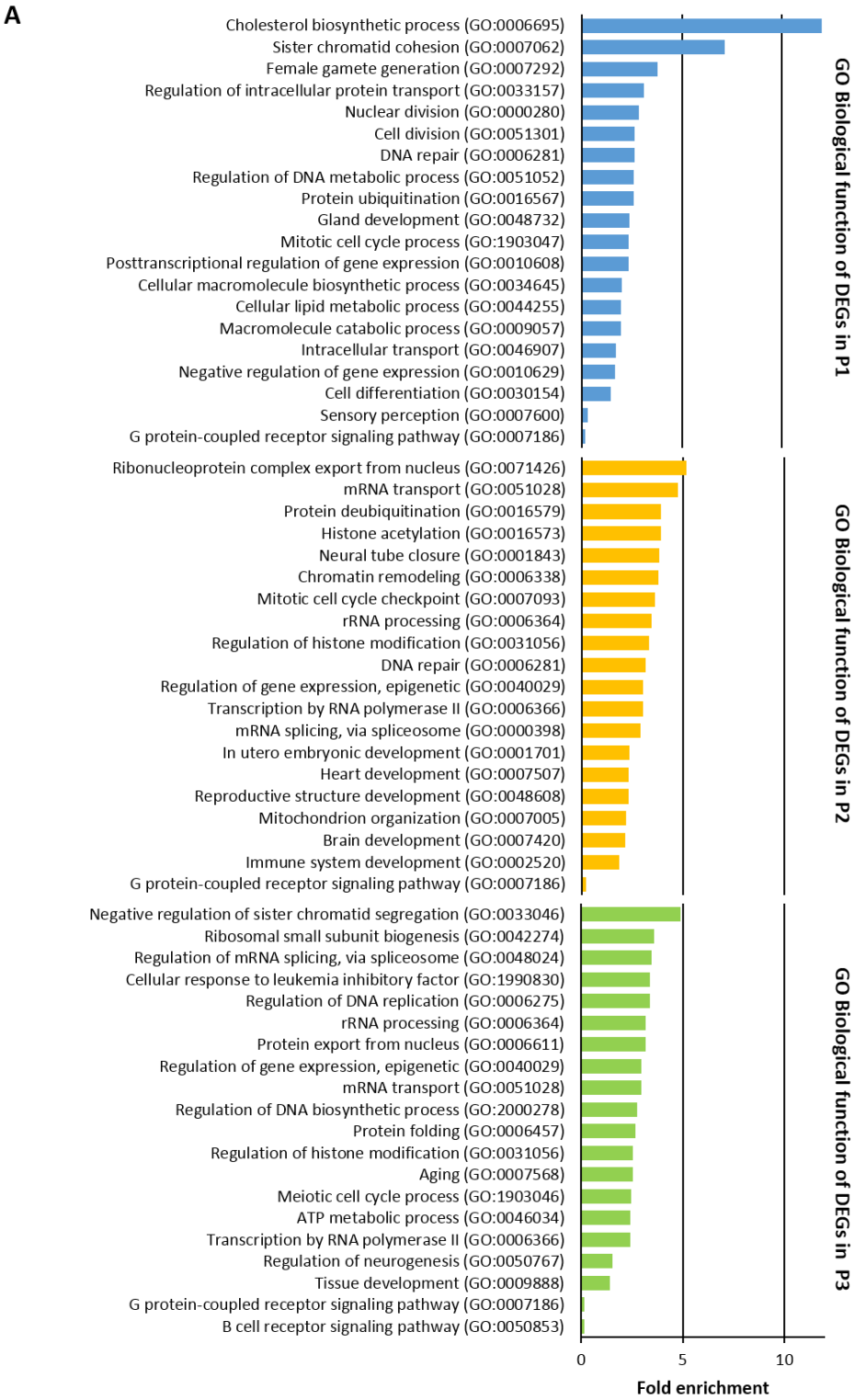
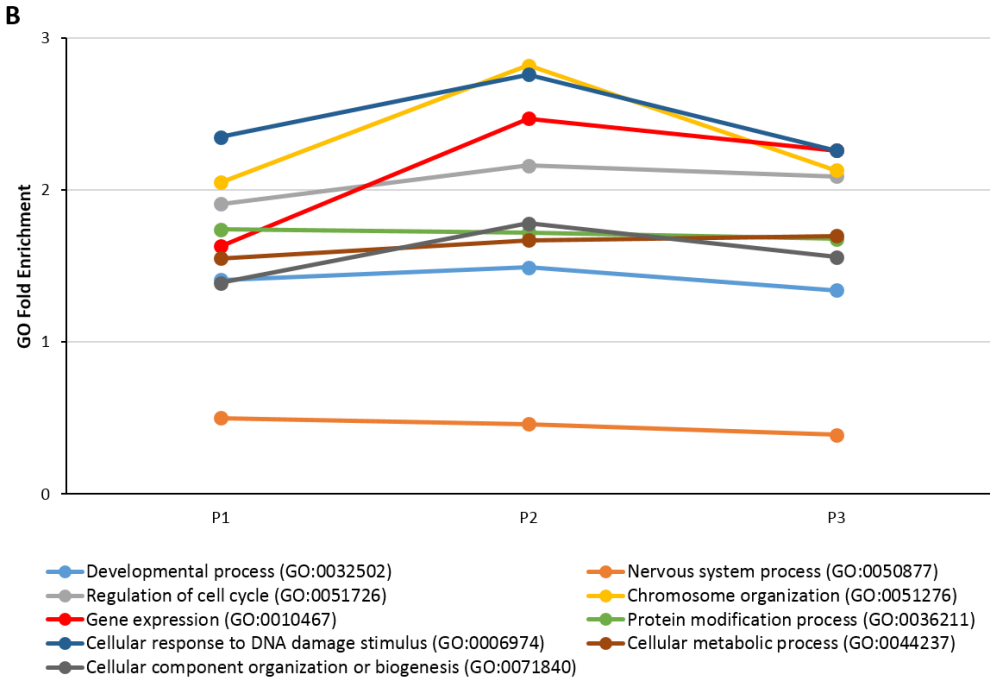


Figure 4.24. Analysis of GO biological functions for epigenetic memory description. (A) GO analysis for DEGs at each time point, showing the top biological functions. GO analysis was performed with the criteria of Bonferroni corrected for p<0.05. ***(Legend continued on next page)***



(B) Common GO biological functions between three time points.

Next, we focused on analyzing the different expression patterns conformed by common DEGs found in three studied time points. We found two clear and reversed clusters (Figure 4.25): the first one composed by DEGs with upregulated expression after chronic morphine treatment, which gradually lose their expression until being downregulated at P3; and the second one, formed by DEGs with downregulated expression after chronic morphine exposure, but with gradual increase of the expression until being upregulated at P3.

To better understand those specific clusters, a gene ontology study was performed with the goal of identifying the specific biological functions related to genes affected by morphine after 24h treatment and which effect persisted on the next time points (Figure 4.25). First group of DEGs were related exclusively to biosynthetic biological functions, DEGs belonging to the second groups were related to nuclear functions, such as cell division and DNA replication processes, together with metabolic processes and biological functions involved in epigenetic processes, such as regulation of genetic expression, histone modification and chromatin organization.

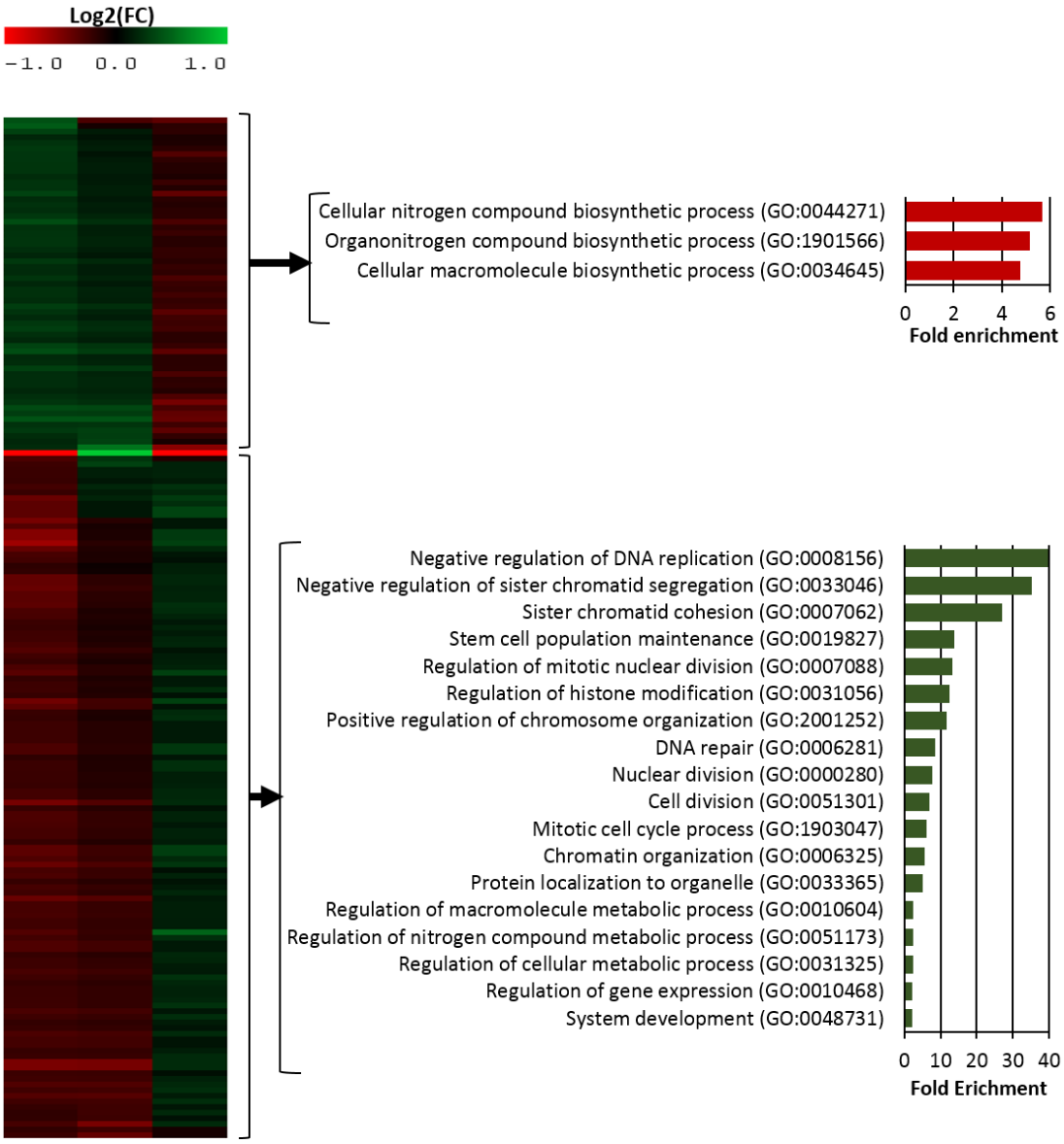


Figure 4.25. DEGs expression dynamics over time (P1, P2, P3). (A) Heatmap representation of DEGs over time. Heatmap representation of DEGs, identifying several clusters with different expression patterns over time. (B) GO biological functions for selected clusters. GO analysis for DEG at selected clusters, showing the top biological functions. GO analysis was performed with the criteria of Bonferroni corrected for $p<0.05$.

4.2.3 Integrative analyses between RNA-Sequencing and Chip-Sequencing data.

Going back to the previous table with the list of common genes between H3K27me3 enrichment at promoter level related with the expected change of gene expression, we also analyzed if all of them maintained the differential expression over time in studied three time points. Table 4.2 summarizes the list of common genes, composed by Smchd1, an epigenetic regulator; Tead1 and Mga, two DNA binding transcription factors; and Pgap1, Pikfyve and Psme4, three genes related to metabolism process. All of them presented same gene expression pattern over time (Figure 4.26), that is to say a decreased gene expression after 24h morphine treatment (P1), which was maintained up to P2, but it was increased at P3.

Table 4.2. Summary list of genes where H3K27me3 enrichment at promoter level was related to gene expression pattern, after 24h morphine treatment and which expression was DE over time.

ChIP-seq BSs and DBSs UP + RNA-seq DEGs Down in P1, and DE in P2 and P3		
Smchd1	Structural maintenance of chromosomes (SMC) hinge domain containing 1	Epigenetic silencing by regulating chromatic architecture
Tead1	TEA (DNA binding region) domain family member 1	DNA binding transcription factor activity (ERK signaling)
Mga	MAX gene associated	DNA binding transcription factor activity (MYC-MAX)
Pgap1	Post-GPI attachment to proteins 1	Metabolism
Pikfyve	Phosphoinositide kinase, FYVE type zinc finger containing	Metabolism
Psme4	Proteasome (prosome, macropain) activator subunit 4	Metabolism

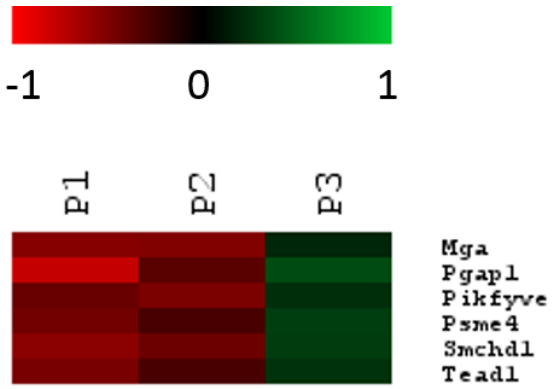


Figure4.26. Heatmap of the interaction between H3K27me3 ChIP-seq BSs and DBSs common with DEGs expression dynamics over time (P1, P2, P3).

4.2.3.1 Morphine induce a Smchd1 silencing through H3K27me3 enrichment conferring to the cell an active state of X chromosome

As it is shown in Table 4.2, Smchd1 gene has an important role on chromosome X inactivation, as it regulates chromatin organization through epigenetic repression of the genome. This fact together with its significant deregulation detected through ChIP-seq technique and maintained over time shown by RNA-seq technique, led as to think that it could be a good candidate to analyze more closely. We observed that after chronic morphine treatment, Smchd1 showed a higher H3K27me3 enrichment at promoter level (coinciding with a CpG island site) consistent with a decreased gene expression pattern validated by RT-qPCR (Figure 4.27B).

In mouse development X chromosome inactivation occurs in two waves (G. Pintacuda & A. Cerase 2015): the first one takes place from 2 to 4 cell embryonic stage and is known as imprinted X inactivation (iXCI) (Okamoto, I et al. 2004). Then, at blastocyst stage, the silencing is reverted and the active state of both Xs is re-established in the inner cell mass of the embryo. After implantation, during the epiblast formation, the second XCI wave occurs, where the X chromosome is randomly selected to be inactivated in a process known as random X inactivation (rXCI) (Mak, W et. Al 2004). As previously explained, the master regulator of the process is Xist lncRNA, and the subsequent maintenance is perform, on one hand though histone modification, manly related to H3K27me3 linked to PRC2 function (Wang et al 2001). On the other hand, DNA methylation is also essential for X inactivation process (Holliday and Pugh 1975). CpG islands found in gene promoter or enhancers are heavily methylated to repress the expression of every gene in the X inactivated chromosome and get the long term memory repressed state maintenance. Based on this information, the following step was to analyze the H3K27me3 enrichment and gene expression pattern of Xist gene and its antisense gene Tsix (Figure 4.27B). Both genes showed an increased enrichment of H3K27me3 after morphine chronic treatment, and consistently it was reflect in a slight decrease of gene expression level in both cases at P1, which was maintained at P2 but increased at P3. This pattern was the same as described for Smchd1, so this coincidence could be consistent with the idea that morphine induced the repression of the machinery responsible for the X chromosome inactivation, through H3K27me3 histone modification.

Based on our observations, morphine produced a global downregulation of H3K27me3 repressive mark on the whole X chromosome, possibly conferring to the cell an active state of transcription. In addition, the fact that morphine incremented the enrichment of H3K27me3 at promoter level of Smchd1, could be a mechanism of the cell to decrease its gene expression and repress its related gene silencing function. Furthermore, in its role of gene silencing, Smchd1 is related to some imprinted genes, clustered protocadherins (Chen, K. et al 2015; Gendrel AV et al. 2013; Leong HS et al. 2013; Moul AW et al. 2013) and clustered Hox genes (Natasha Jansz et al. 2018). It would be interesting to further have into consideration, the analysis of gene expression patterns of these Smchd1 related genes, to validate the hypothetical mechanism where morphine repressed Smchd1 expression through H3K27me3, and in turn reactivated the genes under its regulation.

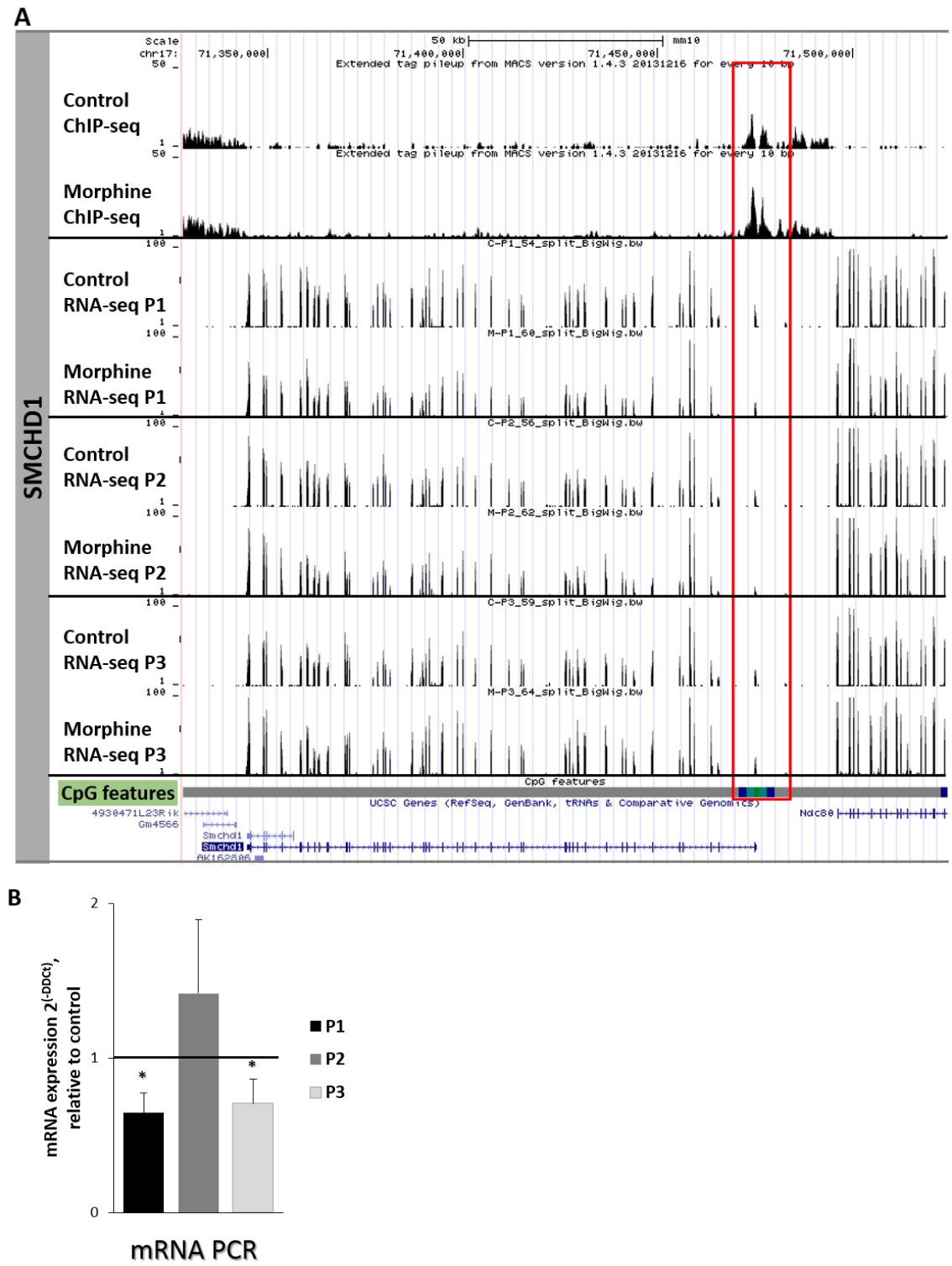


Figure 4.27. RNA-seq and H3K27me3 Chip-seq integration study around Smchd1 gene, and related involvement in X chromosome inactivation. (A) RNA-seq and H3K27me3 ChIP-seq track transitions enriched at promoter level, for Smchd1 gene. CpG features track was composed by CpG islands in green, Shores in light blue, Shelves in dark blue and Open sea in grey. Red boxes point out the enrichment and gene expression change location. (B) Coverage plot of H3K27me3 enrichment, showing coverage of peak regions over X chromosome in control and morphine treated sample.

4.2.3.2 Morphine alters imprinted gene expression in mESC *in-vitro*, at different time points

To follow with the study of observed coincidences between the three time points of the RNA-Seq analysis, we also focused on the DE imprinted genes that were in common. The results showed a total of 4 DE imprinted genes after 24h morphine treatment (P1) and 12 and 15 DE imprinted genes at P2 and P3 respectively, once the external stimulus was absent (Figure 4.28A). Furthermore imprinted type distribution was analyzed (Figure 4.28B, C, D), describing that at P1 mainly maternally expressed genes were DE, and also one with unknown expression. At P2 three imprinted genes classes were represented, being paternally expressed genes the prevalent DE group, but maternally expressed genes the one with the biggest FC. And at P3 three imprinted classes were represented with small FC values.

Analyzing the specific imprinted genes DE at each time point, unluckily we did not find any common genes between three time points (Figure 4.29A). However, going further and studying the clusters where those DE imprinted genes of each time point belong we found some coincidences, which are summarized at Figure 4.29B. For example, we found Mirg maternally expressed gene upregulated at P1 and Meg3 and Rian maternally expressed genes upregulated at P3 after morphine chronic exposure, all of them belonging to Dlk1/Dio3 imprinted cluster. Furthermore, the same happened with maternally expressed genes Dhcr7 (upregulated) at P1 and Tssc4 and Cd81 (both downregulated) at P3, which composed the Kcnq1 cluster. In addition, Ppp1r9a (decreased expression) at P2 and Peg10 (increased expression) at P3, both paternally expressed, formed part of the Sgce/Peg10 cluster. On the other hand, it is worthy to mention that there were some specific genes that even if were not DE at three time points, they were deregulated almost in two of the studied time points. One of them was Rhox5 unknowingly expressed gene, which suffered an increased expression at P1, which was maintained at P2. Zdbf2 (from Gpr1/Zdbf2 cluster) and Slc38a4 paternally expressed genes showed a downregulation at P2, followed by a slight recover at P3. Moreover, Igf2r maternally expressed gene which belonged to the Igf2r/Ain cluster displayed a downregulation at P2 which was maintained until P3. Interestingly, we detected genes, which are part of the Snurf/Snrpn cluster deregulated at three studied time-points. That is to say, at P1 Ube3a maternally expressed gene showed a downregulation after chronic morphine treatment; at P2 Snurf, Snrpn and Snhg14 paternally expressed genes were upregulated; and at P3 previously mentioned Ube3a and Snhg14 displayed increased gene expression.

As a conclusion, we have clear evidences that morphine induces changes in the gene expression of some imprinted genes through H3K27me3 histone modification. However, further studies are needed to better understand the mechanism of the specific regulation of H3K27me3 epigenetic mark in the detailed imprinted genes and clusters. Together with this and specifically in the case of Snrp/Snurf cluster and Igf2r cluster, it is worthy to mention that, previously we have described Smchd1 gene expression deregulation through H3K27me3 enrichment at promoter level after morphine chronic treatment, which is a specific regulator of Snrp and Igf2r imprinted cluster domain. (Gendrel AV et al. 2013; Leong HS et al. 2013; Moul AW et al. 2013; Arne W. Mould et al. 2013; Natasha Jansz et al. 2017; Kelan Chena et al. 2015). This is another field, which requires more experimentation before having a clear conclusion of the mechanism involved in Snrp/Snurf and Igf2r/Air epigenetic regulation.

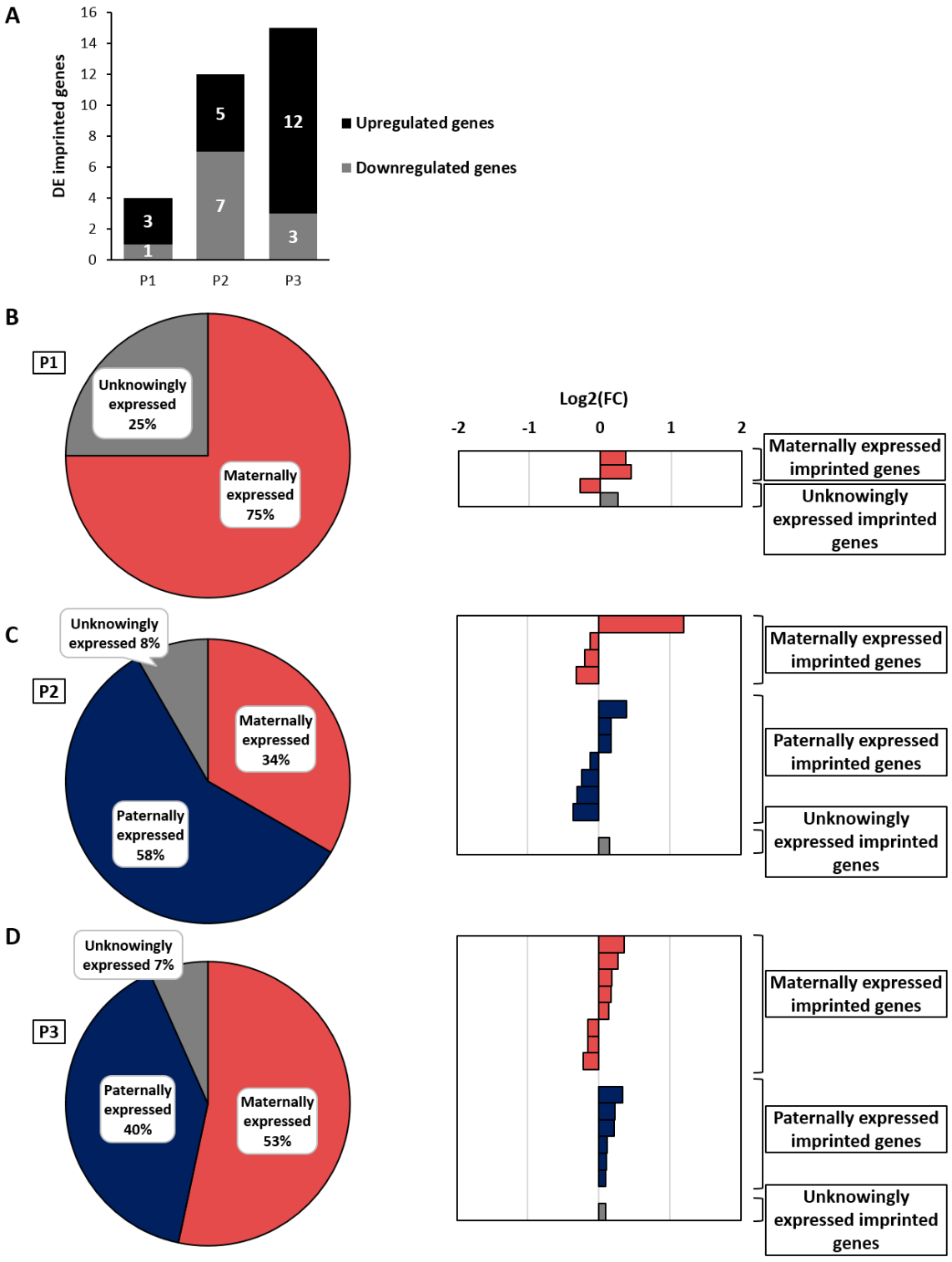
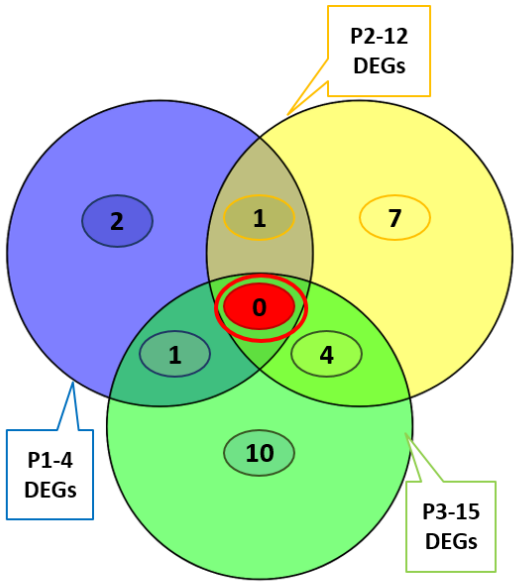


Figure 4.28. DE analysis in imprinted genes. (A) Bar chart of the number of imprinted genes found to be upregulated or downregulated over three time points. DE imprinted genes (B) in P1, (C) in P2 and (D) in P3. The number next to each class name corresponds to the number of DE subtype (p and FDR< 0.05). Each studied feature is presented next to the expression fold-change heatmap.

A



B

DE imprinted genes at P1		
Mirg	Maternally expressed	Dlk1/Dio3 cluster
Dhcr7	Maternally expressed	Kcnq1 cluster
Ube3a	Maternally expressed	Snurf/Snrpn cluster
Rhox5	Unknowingly expressed	--
DE imprinted genes at P2		
Snurf	Paternally expressed	Snurf/Snrpn cluster
Snrpn	Paternally expressed	Snurf/Snrpn cluster
Snhg14	Paternally expressed	Snurf/Snrpn cluster
Peg3	Paternally expressed	Peg3 cluster
Ppp1r9a	Paternally expressed	Sgce/Peg10 cluster
Zdbf2	Paternally expressed	Gpr1/Zdbf1 cluster
Mcts2	Paternally expressed	H13/Mcts2 cluster
H19	Maternally expressed	Igf2/H19 cluster
Igf2r	Maternally expressed	Igf2r/Air cluster
Cobl	Maternally expressed	--
Slc38a4	Paternally expressed	--
Rhox5	Unknowingly expressed	--

DE imprinted genes at P3		
Meg3	Maternally expressed	Dlk1/Dio3 cluster
Rian	Maternally expressed	Dlk1/Dio3 cluster
Tssc4	Maternally expressed	Kcnq1 cluster
Cd81	Maternally expressed	Kcnq1 cluster
Ube3a	Maternally expressed	Snurf/Snrpn cluster
Snhg14	Paternally expressed	Snurf/Snrpn cluster
Peg10	Paternally expressed	Sgce/Peg10 cluster
Zdbf2	Paternally expressed	Gpr1/Zdbf1 cluster
Igf2r	Maternally expressed	Igf2r/Air cluster
Copg2	Maternally expressed	Peg1/Copg2 cluster
Peg1	Paternally expressed	Peg1/Copg2 cluster
Tsix	Maternally expressed	Tsix/Zcchc13 cluster
Slc38a4	Paternally expressed	--
Impact	Paternally expressed	--
Lin28a	Unknowingly expressed	--

Figure 4.29. DE imprinted genes analysis and their expression dynamics over time, after morphine treatment (P1, P2 and P3), in mESC *in-vitro*. (A) Comparison of DE imprinted genes in three time points by a Venn diagram representation. (B) Table resume of DE imprinted genes common between P1-P2 overlapping, P2-P3 overlapping and P1-P3 overlapping, together with the cluster they belong to.

4.2.3.3 Morphine induces changes in repetitive elements expression in mESC *in-vitro*, which are maintained over time

Moreover, to deep into how morphine could change the expression pattern of each repetitive element class group after morphine treatment, the differential expression analysis was performed. This analysis revealed, a total of 32 DE repetitive elements after 24h morphine treatment (P1), 37 DE repetitive elements on next pass, after morphine treatment withdrawal (P2) and on P3 (second pass after morphine treatment withdrawal) 28 DE repetitive elements were identified (p and FDR<0.05) (Figure 4.30A). Surprisingly, most of the DE repetitive elements in P1 and P3 were downregulated and in contrast, at P3 they were mainly upregulated.

Deeping into those DE repetitive elements in each time point, class distribution was performed to understand what type of repetitive elements were altered after morphine treatment. DE repetitive elements were prevalently from the LINE, LTR, simple repeats, satellite and RNA repeats classes in the three time points (Figure 4.30B, C, D). Interestingly, none of SINE subfamilies were DE after morphine treatment. Most of the DE LTR subfamilies were endogenous retroviruses, with ERVK, ERVL and ERV1 being the most represented. For LINES, the majority of the subfamilies belonged to the L1 family of retrotransposons. Another highly represented group was RNA repeats, were mostly belonged to tRNA and rRNA subfamily at P1, snRNA subfamily at P2 and in a smaller level to tRNA subfamily at P3.

Next, we focused on finding common DE repetitive elements to establish a cellular memory after morphine treatment. Partially overlapping repetitive element groups were found between P1 and P2 (n=16), P2 and P3 (n=0) and P3 and P1(n=3), and interestingly 1 of them was present in the 3 studied time points as it is represented in Figure 4.31A. This DE repetitive element common in the 3 time points belonged to RNA repeats type and specifically to rRNA class: LSU-rRNA_Hsa. The table on Figure 4.31B also showed the DE repetitive elements that were overlapping between P1 and P2, which were mainly LTRs and repetitive elements overlapping between P1 and P3, which were composed by Satellite and DNA repeats. Following these findings, we focused on analyzing the different expression patterns conformed by DE repetitive elements common in three time points and also common in overlapping time points (Figure 4.31C). As expected from fold change values, we found a robust downregulation at P1 which was maintained at P2, which in contrast was recovered at P3 to values near to control except from DE repetitive elements commons at P1 and P3 which revealed a big upregulation at P3. Indeed, it is worthy to highlight that LSU-rRNA_Hsa common repetitive element between three time points is also DE deregulated at H3K27me3 enrichment level.

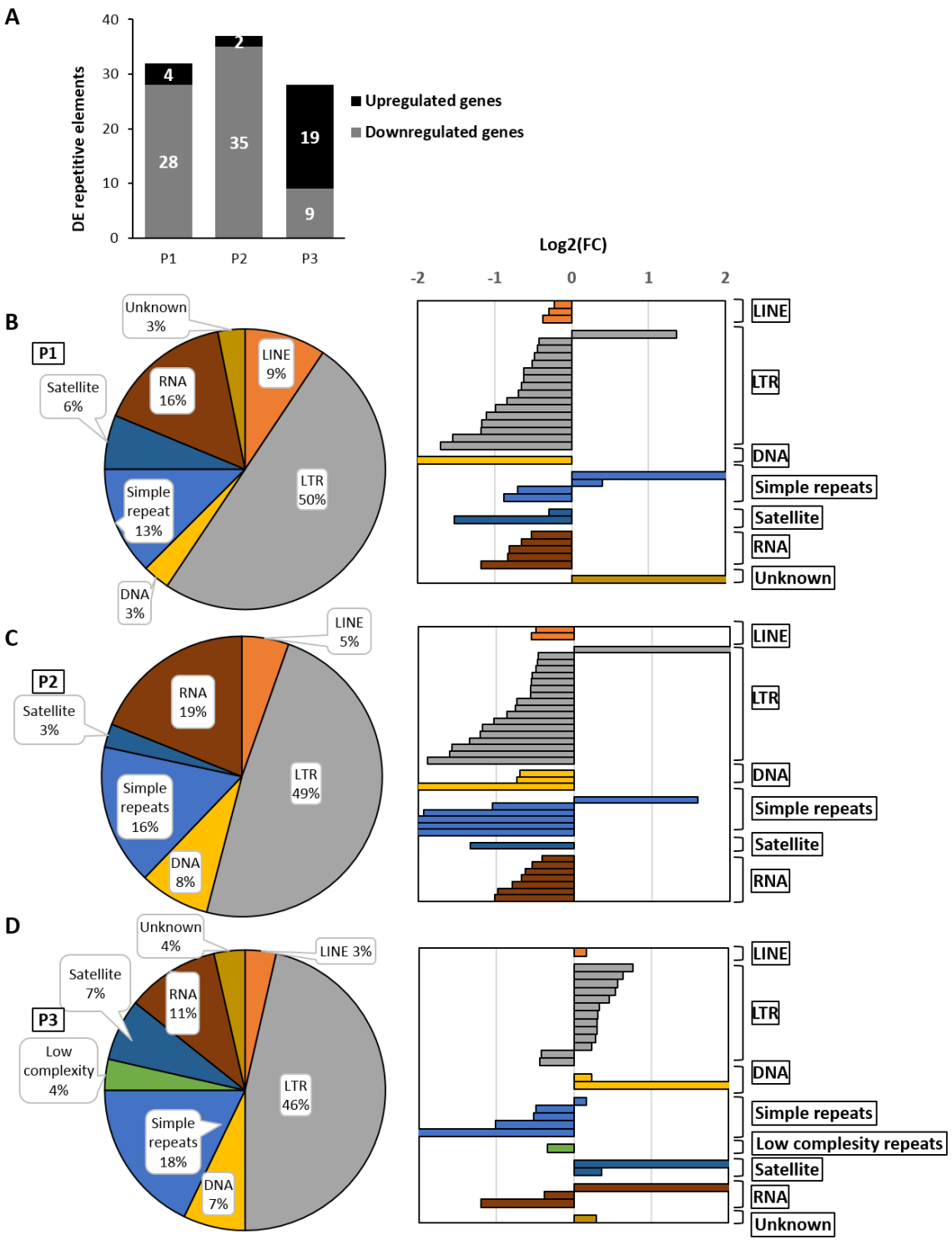
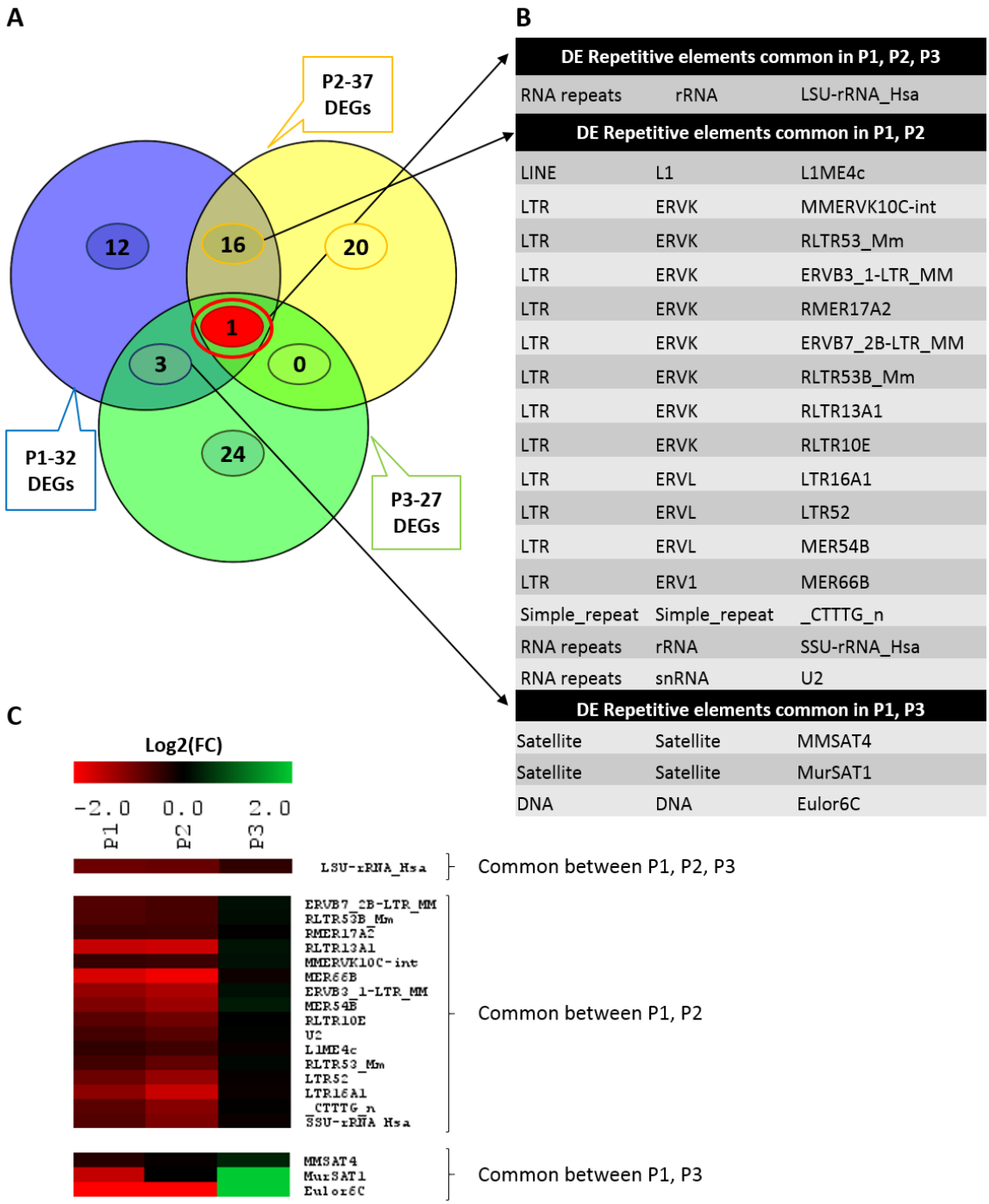


Figure 4.30. Differential expression analysis in repetitive elements over time (A) Bar chart of the number of repetitive elements found to be upregulated or downregulated over three time points. DE repetitive elements (B) in P1, (C) in P2 and (D) in P3. The number next to each class name corresponds to the number of DE subtype (p and FDR< 0.05). Each studied feature is presented next to the expression fold-change heatmap.



DE Repetitive elements common in P1, P2, P3		
RNA repeats	rRNA	LSU-rRNA_Hsa
DE Repetitive elements common in P1, P2		
LINE	L1	L1ME4c
LTR	ERVK	MMERVK10C-int
LTR	ERVK	RLTR53_Mm
LTR	ERVK	ERVB3_1-LTR_MM
LTR	ERVK	RMER17A2
LTR	ERVK	ERVB7_2B-LTR_MM
LTR	ERVK	RLTR53B_Mm
LTR	ERVK	RLTR13A1
LTR	ERVK	RLTR10E
LTR	ERVL	LTR16A1
LTR	ERVL	LTR52
LTR	ERVL	MER54B
LTR	ERV1	MER66B
Simple_repeat	Simple_repeat	_CTTTG_n
RNA repeats	rRNA	SSU-rRNA_Hsa
RNA repeats	snRNA	U2
DE Repetitive elements common in P1, P3		
Satellite	Satellite	MMSAT4
Satellite	Satellite	MurSAT1
DNA	DNA	Eulor6C

Figure 4.31. DE repetitive elements analysis and their expression dynamics over time, after morphine treatment (P1, P2 and P3), in mESC *in-vitro*. (A) Comparison of DE repetitive elements in three time points by a Venn diagram representation. (B) Table resume of DE repetitive elements common in three time points and between P1-P2 overlapping and P1-P3 overlapping. (C) Heatmap representation of expression dynamics of common DE repetitive elements over three time points. From top to bottom, DE repetitive element common between P1, P2 and P3; DE repetitive elements common between P1 and P2; and DE repetitive elements common between P1 and P3.

Therefore, it could be interesting to delve into the specific changes produced in repetitive elements specifically in groups such as LTRs, LINEs, DNAs, simple repeats but most importantly at RNA repeats and specifically at LSU-rRNA_Hsa, as they have shown to be potential candidates for epigenetic memory.

5. DISCUSSION

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5. DISCUSSION

During the last decades, it has been a remarkable increase related to research in epigenetic field, showing that environmental and lifestyle factors may influence different epigenetic mechanisms, such as DNA methylation, histone modifications and microRNA expression (Issa et al 2000; Romani et al. 2015; Abdul QA. et al. 2017). In fact, although some epigenetic changes are part of normal development and aging, there is a big concern in understanding how environmental factors can cause epigenetic changes that lead to health problems or disease, such as autoimmune (Quintero-Ronderos and Montoya. 2012) and neurodevelopmental disorder (Mastrototaro G et al. 2017), cardiovascular disease (Gonzalo-Calvo D et al. 2017; Ahuja YR et al. 2017; Muka T et al. 2016), and mainly cancer (Dawson MA and Kouzarides T. 2012; Toh TB. et al 2017).

Interestingly, to date, several studies support the idea that the epigenetic changes induced by the environment could be inherited. Environmental agents such as the diet (Whitelaw NC and Whitelaw E 2006), stress (Skinner, MK et al. 2014), endocrine disruptors (Skinner MK et al. 2014) and addictive drugs (Yohn, NL et al. 2015) have been described with the ability of modifying the phenotype of the offspring across generations. Much attention has been paid to determining the epigenetic mechanisms underlying drug abuse both within, as well as across generations (Nielsen, DA et al. 2012)

In this context, this Doctoral Thesis aims to describe and explore the epigenetic changes induced after chronic morphine exposure through histone modifications *in-vitro* in mESCs and *in-vivo* in mouse organism. Specifically this study is focused examining the relevance of changes produced through H3K27me3 repressive epigenetic mark in gene expression regulation, together with the implication in regulating imprinted genes, lncRNAs and repetitive elements in mESCs. Furthermore, all obtained outcomes were analyzed more in deep in terms of epigenetic memory. Therefore, the presented results disclosed for the first time novel and valuable information, shedding new light on to the understanding epigenetic mechanisms guided by H3K27me3 to further establish the epigenetic memory process.

5.1. CHRONIC MORPHINE TREATMENT INDUCES GLOBAL EPIGENETIC CHANGES IN HISTONE MODIFICATIONS

Opioids modify the expression profile of certain mRNAs in many tissues, including central nervous system (CNS), the main target of drugs of abuse (Przewlocki, R. et al. 2004). Up to now, extensive studies have been focused on identifying morphine-induced changes in gene expression (Loguinov AV et al. 2001; Ammon-Treiber S et al. 2010; Anghel A et al. 2005; McClung CA et al. 2005; Rhodes JS et al. 2005; Ziolkowska B et al. 2012), but none of them has identified a mechanism describing how morphine induces the expression change. Trying to find some clues, researchers have studied epigenetic marks such as DNA methylation changes or histone modifications related to morphine exposure.

DNA methylation is the most stable epigenetic modification, regulating the transcriptional plasticity of mammalian genome, as the DNA hypermethylation in gene promoters represses gene expression (Razin and Cedar 1991; Jaenisch and Bird 2003; Wong et al. 2011). In terms of DNA methylation, information of its regulation in addiction models remains limited. Even if a small number of studies have investigated DNA methylation changes at particular genes of interest, such as the Oprm1 gene, LINE1 retrotransposon, and other genes implicated in brain functions, after acute or chronic morphine exposure (Doehring A et al. 2013; Cecil CAM et al. 2015), information on the regulation of DNA methylation in morphine addiction models remains limited. Furthermore, those studies are focused only in specific cells or tissues, such as blood or sperm (Xu J et al. 2018; Chorbov V et al. 2011), and mainly related to the brain, but there is not a global trend of methylation pattern, as it changes depending on the analyzed region (Barrow et al. 2018). It is worthy to mention, the presence of a contribution that seeds some light in the subjacent mechanism of DNA methylation induced by morphine intake, as it relates the process to redox based changes in global DNA methylation pattern (Trivedi M et al. 2014), producing a global demethylation similar to what it is described for cocaine (Novikova et al. 2008; Tian et al. 2012). However, there has not yet been a genome-wide mapping of such regulation, as it has been done with other drugs as cannabinoids (Watson CT et al. 2015), or other toxics and addiction models (Suzuki T et al. 2013; Yoon A et al. 2017; Yang AY et al. 2014).

In terms of histone modifications, few studies have implicated a role for histone acetylation or methylation in opiate action (Sanchis-Segura et al. 2009; Wang et al. 2010; Jing et al. 2011; Sheng et al. 2011; Rehni et al. 2012; Day and Sweatt, 2011; Maze and Nestler, 2011; Peter and Akbarian, 2011). Nevertheless, only one studies the specific effect of chronic morphine exposure at H3K9me2 histone modification in the nucleus accumbens (Sun HS et al. 2012). Therefore, in this manuscript we describe for the first time chronic morphine exposure induced changes in different active and repressive histone modifications in mESC.

Very little is known about morphine deregulation at H3K27me3 epigenetic repressive mark. In fact only a study carried out in brain ventral tegmental area, describes a specific H3K27me3 enrichment to Bnf gene induced by morphine (Koo JW et al. 2015). Our study showed a downregulation of H3K27me3 after chronic morphine treatment, which is confirmed also in other study models considering gestational alcohol exposure (Veazey et al. 2013) or alcohol treatment on mice (Chater-Diehl EJ et al. 2016).

Moreover, we identified a global upregulation of H3K9me2 in mESC after chronic morphine exposure, opposite to what Sun et al. found at the nucleus accumbens (Sun HS et al. 2012), and to what it is described for repeated cocaine exposure at the same region (Maze I et al. 2010; Covington HE et al. 2011). However, our results were similar to what other researchers described for prenatal alcohol exposure (Subbanna et al. 2013, 2014, 2015; Veazey et al. 2013, 2015; Bekdash et al, 2013; Govorko et al. 2012), or THC exposure in different areas of the brain. Furthermore, it is worthy to mention, that every time a deregulation of H3K9me2 is described in previous researches, it is also related to the same deregulation for G9a, highlighting its key role in the regulation machinery of H3K9me2. To this extend, we also identified this positive correlation between G9a expression and H3K9me2 protein levels, providing G9a as a direct contribution of H3K9me2 regulating machinery.

Regarding H3K4me2/me3 we described a global downregulation and upregulation respectively after morphine exposure. No information was found concerning H3K4me2 changes related to drug of abuse. But interestingly, our described H3K4me3 upregulation has been identified in other studies performed with other drug or toxic exposures, such as cannabinoid receptor type 2 agonist JWH133 in male germ cells (Giacomo DD et al. 2019) or ethanol exposed mice (Chater-Diehl EJ et al. 2016).

Because posttranslational regulation at the protein level affects RNA transcription, histone modifications may play important roles not only in previously explained immediate response but also in long term response dynamically. Our study seeds light in histone modification levels dynamics trying to describe changes, which maintain over time, even when the stimuli is absent, as possible candidates for cell memory. We found a global downregulation at H3K27me3 and H3K4me2 levels after chronic morphine treatment, which were maintained during 24h and upregulated 48h after treatment end. Mainly H3K9me2 and H3K4me3 in a lower level, presented an increased histone pattern after morphine treatment, which was downregulated after 24h and again upregulated after 48h. Finding trends in time points over published data has not been possible, given the limited number of histone studies to date. We only found punctual researches studying histone modifications while a continuous treatment of morphine administration on mouse nucleus accumbens (Sun HS et al. 2012), or of continuous treatment of retinoic acid in hESCs during 6 days (Shahhoseini M et al. 2013). On the other hand, at 2, 24 and 48h after a single injection of drugs such as THC in adolescent and adult female mice (Prini P et al. 2018). None of them is comparable with our study model as they don't have into account a chronic drug exposure and an evaluation of the effect once the stimulus is absent. Further studies are needed with arranged time windows, important to establish cell memory regarding to histone modifications.

The collective results of this study suggest that histone modifications are each affected by morphine exposure in mESCs in distinct genomic and temporal patterns.

5.2. CHRONIC MORPHINE TREATMENT LED TO A GLOBAL GENOME DOWN REGULATION OF H3K27me3 LEVELS IN mESCs BY PRC2 MODULATION

Because of the limited information about H3K27me3 changes after morphine exposure and taking into account its implication in processes such as embryonic development, genomic imprinting, X chromosome inactivation and cellular memory (Kouzarides T. 2007; Vallot C et al. 2016, Inoue A et al. 2017; Matoba et al. 2018; Zheng H et al. 2016), we decided to focused our investigation specifically in changes related to H3K27me3. The global downregulation induced by morphine at H3K27me3 histone level was confirmed by ChIP-seq experiment, as we identified less enrichment points in morphine treated samples compared to control sample. These results were similar to those described previously by ChIP-seq and ChIP-microarray for H3K27me3 in alcohol exposure models (Veazey KJ et al. 2013; Chater-Diehl EJ et al. 2016), or by ChIP-seq in THC exposure models in lymph nodes (Yang X et al 2014). Furthermore, as H3K27me3 is an epigenetic repressive mark (Wang Z et al. 2008), we expected a global upregulation of gene expression together with the described downregulation of the histone after morphine exposure. However, we observed more downregulated genes in RNA-seq experiment after 24h morphine treatment. This could be explained by the wide increase of H3K9me2 we observed after the treatment, which could be repressing the gene expression resulting in the downregulation we observed at gene level.

Interestingly, we described a big increment in H3K27me3 enrichment at promoter level, fact that has been reported by diverse studies focused in specific genes (Keifer J et al. 2017; Koo JW et al. 2015). Moreover, when analyzing the TSS centered plot on control sample we could identify the typical feature for H3K27me3, which is a sharp dip around the TSS region, corresponding to the position of the nucleosome-depleted zone (Jiang C et al. 2009; Young MD 2011), related to gene body enrichment in other drugs exposure (Yang X et al. 2014). Surprisingly, morphine treated sample showed a broad peak around the TSS region, more likely to the pattern related to H3K4me3 (Pan G et al. 2007), highlighting the increased promoter enrichment after the treatment.

Regarding CpG islands, we have described a big H3K27me3 enrichment mainly at promoter of genes with CpG islands. Deeping into this result, we have found several studies describing the presence of bivalent promoter domains as regulation machinery of developmentally important genes in ES cells, as compared to other cell types (Azuara V et al. 2006; Mikkelsen TS et al. 2007). These bivalent promoters are composed of coexistence of active and repressive histone modifications such as H3K4me3 and H3K27me3. It has been reported that bivalent promoters are CpG rich while H3K27me3 only promoters are CpG poor (Mantsoki A et al. 2015), fact that led as to think that morphine induced increase of H3K27me3 enrichment at gene promoters rich in CpG islands, could be a specific change produced at bivalent promoters.

Concerning the biological functions related to genes enriched to H3K27me3 histone mainly at promoter level after chronic morphine exposure, as we expected because of the epigenetic repressive characteristic of the histone modification, we found the regulation of transcription function. Other predictable biological functions were many brain related functions such as neuron generation, differentiation or projection, which were deregulated. In fact, morphine treatment is highly related with apoptosis process in neurons (Hu S et al. 2002) and reduced

neurogenesis (Eisch AJ et al. 2000; Zhang Y et al. 2016). Together with that H3K27me3 is described as an important participant in neural development and as a regulator of neurogenic gene expression (Hirabayashi et al. 2009; Hwang et al. 2014; Pereira et al. 2010), even if the process nowadays is not well understood. In this concern, we would like to further complete the present results, with a study focused on the behavior of mice after chronic morphine exposure and the analysis of specific regions of the brain related to H3K27me3. Furthermore, we have identified biological functions related to other specific organs, in genes enriched to H3K27me3 after morphine treatment. One of them is heart morphogenesis, together with the vasculature development, results supported by the fact that endogenous opioids modulate blood pressure and cardiac function by stimulating cardiac synthesis of atrial natriuretic factor (Ernest S et al. 1997). This factor is described as responsible for body fluid homeostasis and blood pressure control, one more biological function described in GO from H3K27me3 enriched genes after the treatment. We could appreciate that H3K27me3 enrichment in heart, at the *in-vivo* model in mice, as we found a big increase in global H3K27me3 in both, male and female mice after chronic morphine treatment. It is worthy to mention that all of studied histone modification appear to be increased in male and female hearth organs after a chronic morphine treatment, this could be explain because of the key role of the histone modifications during hearth development (Bruneau BG 2010; Zhang QJ and Liu ZP, 2015), process that becomes altered after the treatment. The other mentioned organ in biological function aspect is kidney development. Numerous publications suggest that chronic morphine treatment cause structural abnormalities and renal dysfunction, renal injuries, chronic renal failure or cell degeneration (Arerangaiah R et al. 2007; Perneger TV et al. 2001; Sumathi T et al. 2009; Senturk M et al. 2009). Furthermore, we have found that morphine treatment could be related to acute kidney injury in critically ill children as a risk factor (Glanzmann C et al. 2016). We have analyzed histone modification patterns in kidney of male and female mice, and have found mainly an upregulation of all histone modifications in females, while males presented a decreased pattern. This involvement of histone modifications in kidney development (Xiang X et al. 2018; Mimura I 2015).

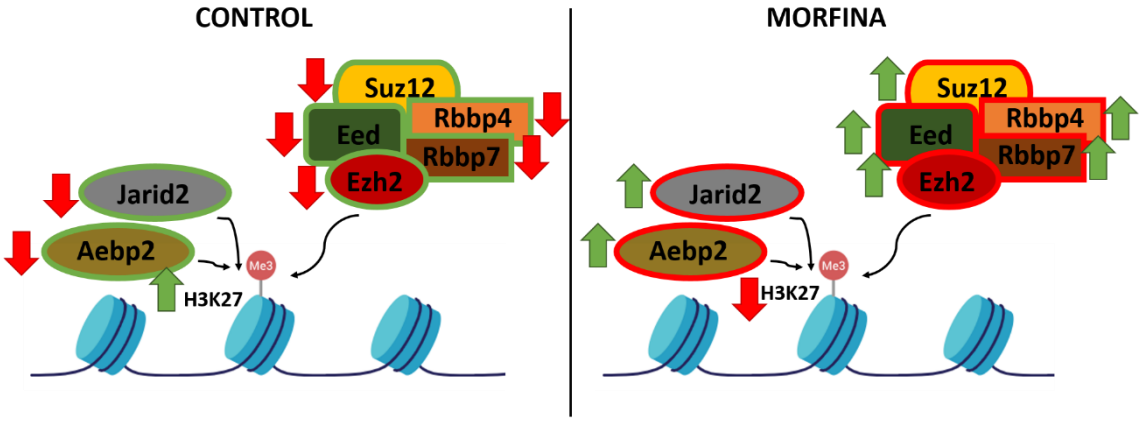

Figure 5.1. Model of H3K27me3 downregulation by PRC2 modulation, composed from our results. Green arrows mean H3K27me3 enrichment upregulation and red arrows mean H3K27me3 down regulation. Red circles around PRC2 subunits mean gene expression downregulation and green circles mean gene expression upregulation.

Indeed the opioid receptors are widely distributed throughout the body and opioid treatment can alter or modulate many different physiological and psychological processes (Kibaly C et al. 2019), tightly related with the regulation we observed in anatomical structure morphogenesis biological function, though the H3K27me3 enrichment. That is why we observed such increase and decreases of different histone modifications in almost all the studied organs in male or female morphine treated mice, except from lungs. Specifically in H3K27me3 we observed mainly an upregulated pattern in all organs except from male kidney, but more importantly in both reproductive organs, ovaries and testis, where H3K27me3 level decreases greatly. In fact, the chronic morphine treatment is related to reduction in the initiation of follicular growth (Lintern-Moore S et al. 1979), but there is no published relationship between morphine effect through H3K27me3, even if H3K27me3 decrease at ovaries is related to cancer (Wei Y et al. 2008; Chapman-Rothe et al. 2013; He WP 2015). In contrast, in male reproductive organs, the effect of morphine is more related to the modulation of testosterone effect, rather than changing structural features (Yilmaz B et al. 1999), and also with altered sperm production and sertoli cell population (Mafra F et al. 2011), but little is known about the H3K27me3 in testis in general. Other mentioned biological functions are involved in regulation of metabolic processes and cell secretion, tightly related to liver and pancreas function. Evidence has accumulated indicating that opiates and opioids may influence carbohydrate metabolism (Matsuma et al. 1984) and endocrine pancreatic secretion (Giugliano D et al. 1988) in a direct way. After morphine treatment we perceived increased of all studied histone modification levels in male and female liver and pancreas, except from H3K4me3 enrichment in female liver which appeared to be decreased. In fact, histone modifications are described as key machinery involved in regulating gene expression, which leads to an organic injury, caused by drug's side effects (Xu WTY 2015). Furthermore, there is a widely described implication of studied histone modifications in the development of all analyzed organs, mostly related with cancer, when an alteration occurs in the normal pattern (Kanwal R & Gupta S 2011; Kanwal R & Gupta S 2012).

Following with the biological functions described from H3K27me3 enriched genes, we have found few of them related to differentiation of diverse cell types, cell development and proliferation. Indeed, there is a contradiction between publications, where some researchers have found that morphine has an inhibitory effect in cell proliferation and others defend that morphine promotes the process (Gach K et al. 2011; Singhal PC et al. 1992; Feizy N et al. 2015; Wu G et al. 2018; Willner D et al. 2014). Moreover, the opioid peptidic system activates lot of intracellular signaling pathways, that is way we found functions related to regulation of signal transduction, cell communication and specific enzymatic activities such as protein quinase activity (Narita M et al. 1994; Al-Hasani R et al. 2011; Antolak A et al. 2017) or protein phosphorylation (Chakrabarti S et al 2001).

From all the identified DEGs with decreased expression, after chronic morphine exposure in mESCs, one of the most interesting genes, which correlated with H3K27me3 enrichment, was Suz12. This gene is a component of the polycomb group protein, which together with Ezh2, Eed and Aebp2, regulates H3K27me3. All of them showed a decrease expression pattern in RNA-seq experiment and also in RT-qPCR analysis supported by the specific enrichment of H3K27me3 to CpG enriched promoters, significantly in Ezh2. Taking into account these results, our study provides novel insight into the regulation of PRC2/H3K27me3 in mESC by chronic morphine, as we described a global downregulation of the histone mark after morphine exposure, but also a repression guided by H3K27me3 enrichment of the PRC2 components. In the case of Bmi1, a subunit of PRC1, we observe a significant decrease both in RNA-seq expression patterns and the results of RT-PCR analysis, but H3K27me3 enrichment was not affected by morphine exposure.

This supports our idea of morphine induced specific regulation of PRC2/H3K27me3. To add robustness to our presented results it would be a good choice to perform Ezh2 overexpression or silencing experiments to prove that the mechanism starts by the reduction in PRC2 which in turns reduces H3K27me3 methylation, similar what they do with G9a and H3K9me2 in nucleus accumbes (Sun et al. 2012). In fact, it is shown that morphine and cocaine reduce H3K9me2 levels in nucleus accumbens of chronic treated mice and that the regulation of the histone modification is performed by G9a/GLP enzyme (Maze I et al. 2010; Maze I et al. 2011; Sun H et al. 2012). As previously mention, we observed opposite results after chronic exposure in mESC, but we also perceived that G9a could be the regulator of H3K9me2, as both expression patterns change in the same direction. Following with our observed Mll1 expression changes in mESC, we could see that it was reversely related to H3K4me3, results that must be studied more in deep as both have been described to have a direct relationship of expression. In contrast, it showed the same expression pattern as H3K4me2 global histone level did. In fact, it is shown that other addictive drugs as methamphetamine, increased Mll1 expression depending on the number of dose, together with H3K4 mehtylation level (Aguilar-Valles A et al. 2014).

Turning again to H3K27me3, it is widely described that PRC2 is the regulation mechanism of H3K27me3 (Hosogane M et al. 2016), but little is known about external stimuli induced changes in its mechanism. The global downregulation of this repressive machinery following chronic exposure to morphine, or other external stimuli, could lead to aberrant transcriptional control that contributes to abnormal adaptations or cause diseases. Overexpression of PRC2 components is related with various cancers such as melanoma, lymphoma, and breast and prostate cancer (Varambally S et al. 2002; Kleer CG et al. 2003; Karanikolas BD et al. 2009; Li X et al. 2009). In contrast, linked with some of the effects of morphine explained previously, deletion of PRC2 components in somatic cells led to a marked reduction in cell proliferation (Bracken AP et al. 2003; Varambally S et al. 2002) or developmental defects during embryogenesis or in tumorgenesis, indicating the physiological relevance of H3K27me3 (Kim and Roberts 2016; Surface et al. 2010). This described physiological effects, support our results even if the fact that the lack or decrease of PRC2 in our case is caused by morphine. In contrast, there is no reported evidence of H3K27me3 enrichment in PRC2 subunits as a self-regulatory mechanism, to produce morphine induced H3K27me3 global downregulation.

In terms of histone modifications and their regulatory subunits, we did not find any robust consensus in the *in-vivo* experimentation results, as most of the changes observed in mESC did not maintained when analyzing the animal model and different tissues. Possibly, being a higher organism a more complex mechanism is involved in the regulation of histone modification in each of the organs or specific cell types composing each organ. Therefore, more experimentation is required to shed light in this aspect.

5.3. MORPHINE PROMOTES PGC AND EARLY EMBRYO DEVELOPMENT BY Bmp4 GROWTH FACTOR.

From all the identified DEGs after chronic morphine exposure in mESCs, we only found one of them with increased expression that correlated with a decreased enrichment of H3K27me3. This result could be highlighting a regulation mechanism where the decreased H3K27me3 enrichment at Bmp4 gene promoter region, stimulates Bmp4 gene expression upregulation induced by morphine.

Bmp4 is a growth factor and it is tightly related to embryonic development in mammals. Specifically it acts, as a direct responsible of germ cell specification (Yasuhide Ohinata et al. 2009). In fact, it plays an essential role in generation of primordial germ cells (PGCs), the origins for both the oocytes and the spermatozoa, as it is widely used in protocols of differentiation cells from the epiblast to PGCs (K.A.Lawson et al 1999). In addition, Bmp4 is also described as a regulator of blastocyst cavitation and swelling together with trophoblast epithelial morphogenesis (Nicolas C. Rivron et al 2018; R. Michael Roberts et al. 2018).

In the manuscript both embryonic stages have been studied, starting from a chronic morphine treatment and reaching the differentiation or development process once the treatment was absent. In both cases, we found higher cell amount at the end of the processes that is to say, at the end of PGC differentiation and embryonic development until blastocyst stage, even if there was no detectable morphological changes. This could be related with previously mentioned biological functions from H3K27me3 enriched genes, such as, cell population proliferation and cell development regulation. Previously with have talk about the contradictory findings in this topic, as some researchers have described that morphine in low dose acts as a cell proliferation promoter in different cell types, such as tumors (Gach K et al. 2011) and mesenchymal cells (Singhal PC et al. 1992), among others. However, there are other studies which support the inhibitory action of morphine in cell proliferation (Feizy N et al. 2015; Wu G et al. 2018; Willner D et al. 2014), but importantly that the inhibition of proliferation is followed by an increased differentiation rate in neural stem cells populations. Analyzing more in deep the reported morphine effectin PGC differentiation, this is what could be happening after morphine treatment and the observed Bmp4 increase. However, none publication has been found related specifically to morphine, opioid or drug treatments in PGC differentiation. In the case of Bmp4 overexpression significance in PGC differentiation, we have found a recent publication, which relates the Bmp4 exposure time with higher or lower PGC differentiation frequency (Talaei-Khozani et al. 2014). As they explain time and concentration of Bmp4 addiction to promote PGC differentiation is a controversial topic. We found an internal upregulation at the beginning of the differentiation process, thus we do not know if the results could be extrapolated to an addiction of Bmp4 in a typical differentiation protocol. Furthermore, H3K27me3 is involved in the repression of somatic and meiotic genes in PGCs (Mu et al, 2014), thus, we found important to follow analyzing H3K27me3 involvement in PGC differentiation process after chronic morphine treatment.

On the other hand, related to embryo development, we have found very little publications on the field. Most of them are focused on studying the effect of opioids on uterus and the implantation process of the blastocyst (Chen Y et al. 2014; Tang X et al. 2015; Wu W et al. 2016). Only one of them analyzed morphine exposure *in-vitro*, in two-cell stage of the embryonic

development process and triggered to blastocyst, as we did in our manuscript. They concluded that morphine exerts an inhibitory effect on blastocyst formation when treating 2-cell embryos (Chen Y et a. 2014), totally opposite of what we described with our finding. It is worthy to mention, that their experimentation was focused on inducing an opioid signaling effect on the embryo to analyze the underlying mechanism, and that is why the use an "excessive morphine exposure", which specifically is a treatment of 50uM concentration. That value is five times more abundant than the treatment we use for our experimentation (Zadina JE et al. 1992; Prenus RV et al. 2012) and possibly toxic for the embryo, as for example at neural stem cells a dose of 100uM morphine is established as cytotoxic, incrementing the cell apoptotic level (Feizy N et al. 2015). Another important detail is that they do not specify the treatment timing, that is to say, if the treatment is for 24h or it lasts all the development process until blastocyst stage. Similar blastocyst stage studies have been reported to analyze the implantation on the uterus by cannabinoid receptor signaling pathway, concluding that low concentrations prepare blastocysts to be competent for the implantation process, however high concentrations fail to induce blastocyst competency, resulting in pregnancy termination (Wang H et al. 2003).

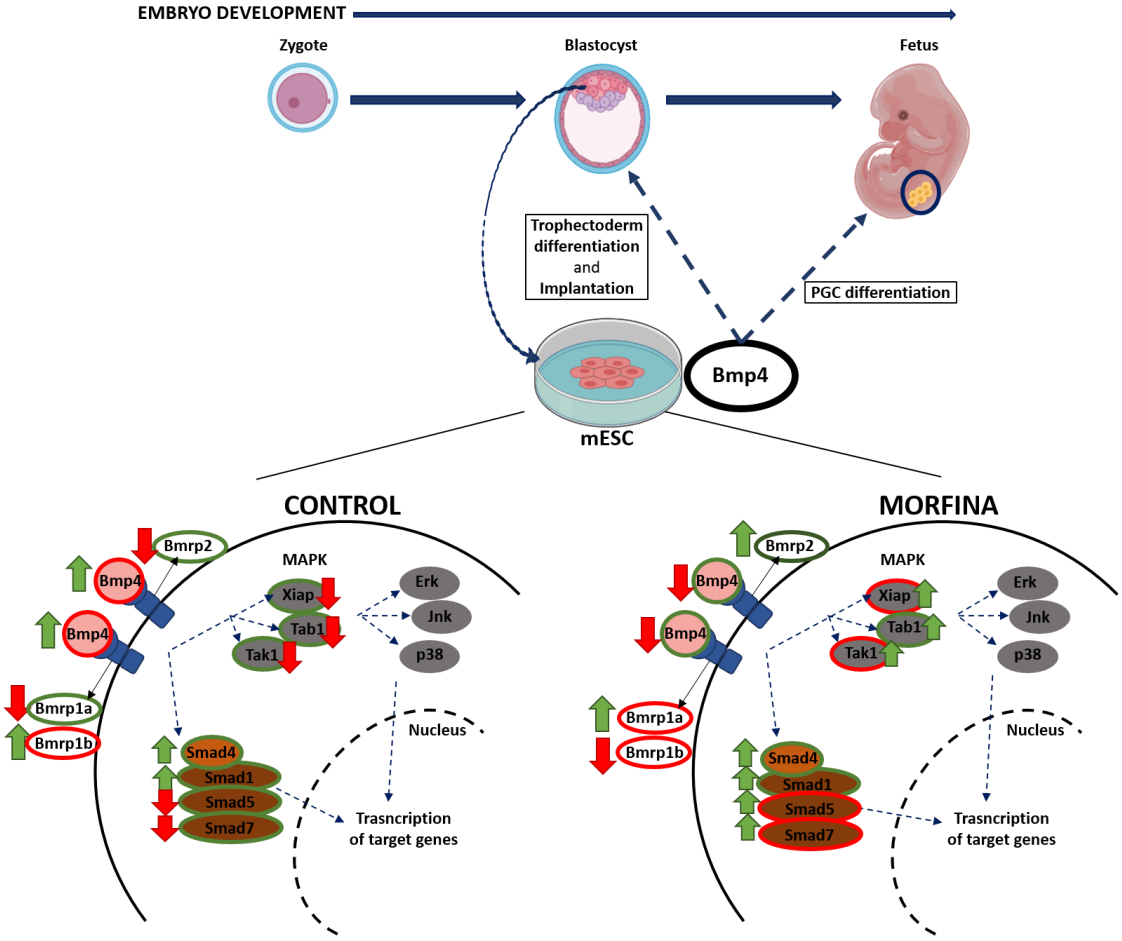

Figure 5.2. Model of Bmp4 promoting early embryo development and PGC differentiation, through changes in H3K27me3, composed from our results. Green arrows mean H3K27me3 enrichment upregulation and red arrows mean H3K27me3 down regulation. Red circles around Bmp4 signaling pathway genes, mean gene expression downregulation and green circles mean gene expression upregulation.

Furthermore, returning to the fact that Bmp4 has an important role in signaling during early embryonic differentiation, some recent studies (C. De Paepe et al 2018), have related the presence of supplemented levels of Bmp4 with impaired blastocyst formation and final apoptosis. To explain the observed role of Bmp4 in blastocyst apoptosis, this study present a mechanism involving a downregulation of Sirt1 deacetylase, thus producing a higher acetylated p53, with apoptotic function. This specific mechanism could be consistent with our findings as we also detect a downregulation of Sirt1 expression related to increased enrichment of H3K27me3 at promoter level.

From those results, we concluded that morphine treatment induced increased PGC like cells and blastocyst formation. This last result could logically hide an underlying mechanism regulated by morphine, which nowadays is unknown. As Bmp4 acts in both studied time points, our next step would be to validate its gene expression after morphine treatment in PGC like cells and blastocyst stage, to see if it is consistent with the pattern observed at mESCs study model. If it appear to be increased, with the capability to maintain morphine induced changes across embryonic development through germline differentiation process and early embryonic development, therefore could have a strong candidate for epigenetic memory. Afterwards, it could be interesting to validate these observed changes when performing a chronic treatment of mouse female and males and carrying out the embryonic development *in-vivo*.

5.4. MORPHINE AND IMPRINTING

5.4.1. Different genomic distribution of epigenetic mark H3K27me3 at ICR/DMRs of imprinted genes induced by morphine in mESCs

As it is widely described, epigenetic mechanisms play an important role in the control of gene expression, to allow correct gene expression patterns at appropriate time points. An additional mechanism of gene regulation is the genomic imprinting, an epigenetic process affecting less than 1% of genes in the mammalian genome. It consists in genes which expression occurs from only maternal or paternal allele, and the other allele is silenced (Ferguson-Smith AC, Surani MA 2001). In order to achieve this functional haploidy at selected genes, epigenetic mechanisms are utilized to differentiate between the genetically identical sequences and confer monoallelic activity.

As mechanism of this process, both DNA methylation and post-translational histone modifications have been described to be enriched more on one chromosome compared to its homologue one at the majority of imprinted loci in the mouse and human genome (Edwards CA, Ferguson-Smith AC 2007; Delaval K, Feil R 2004). Mainly, both DNA methylation and histone modification act over differentially methylated regions (DMRs), which are found at imprinting control regions (ICRs), the fundamental elements in imprinting regulation (Edwards CA, Ferguson-Smith AC 2007). Many publications in this field, have described differential enrichment of specific histone modifications at some ICRs and imprinted gene promoters or transcription start sites (TSSs) (Lee J et al. 2002; Lane N et al. 2003; Borgel J et al. 2010; Reik W et al. 2001). These marks include all histone modifications that we have studied, H3K4me2 and H3K4me3 on unmethylated chromosomes or active alleles and H3K27me3 and H3K9me2 enriched on the methylated chromosomes or inactive alleles, among others (Carr MS et al. 2007; Delaval K et al.

2007; Regha K et al. 2007; Umlauf D et al. 2004; Vu TH et al. 2004; Yamasaki-Ishizaki Y et al. 2007; Yang Y et al. 2003; Kim JM et al. 2009). Importantly, individual genes and individual ICRs show different combinations of enriched histone modifications, with cell-type specificity. Furthermore, it has been described that the dysregulation of this epigenetic mechanisms, mainly considering changes in methylation of DMR/ICR regions, are associated with loss of imprinting of the linked genes in the clusters and as a conclusion with different imprinting disorders and diseases, such as placental, fetal and postnatal growth restriction (Kalish JM et al. 2014; Plasschaert et al. 2014). In our results we have observe a big increase of H3K27me3 enrichment at both DMR and ICR regions of the studied imprinted genes, after morphine chronic exposure, the opposite of what is described for ordinary enrichment of H3K27me3 in imprinted genes (McEwen KR and Ferguson-Smith AC 2010). This fact could be indicating a repressive status of imprinted genes after the present of an external stimulus. However, despite many studies having previously assessed epigenetic modifications at particular imprinted genes, the functional role that histone modifications play in imprinting establishment and maintenance, is difficult to consider and is currently unknown. Some of them, relate H3K27me3 activity with H3K4me3, as bivalent regulators of imprinted genes promoters, identifying a gene expression even when H3K27me3 is enriched at the ICR region of some imprinted genes, caused because of the H3K4me3 enrichment too (Maupetit-Mehouas S et al. 2016). However, most of the times H3K27me3 histone mark is found at developmentally repressed imprinted genes, with the role of repression of the normally active alleles in a similar manner to non-imprinted genes (McEwen KR and Ferguson-Smith AC 2010).

Looking to the results of the interaction between H3K27me3 enrichment and transcriptomic analysis after chronic morphine exposure, we did not find any candidate imprinted gene, which seemed to be regulating the gene expression through H3K27me3 enrichment. Nevertheless, interestingly we found genes belonging to the same cluster between both techniques. That is the case of Mirg (result from RNA-seq) and Begain (result from ChIP-seq) that belong to Dlk1 – Diol3 imprinted cluster; and the case of Ube3a (result from RNA-seq) and Snrpn, Snurf and Atp10a (results from ChIP-seq), both belonging to Snrpn imprinted cluster. Both clusters are widely studied in different tissues and cell types, but there is a lack of information about histone modification enrichment studies after exposure of an environmental treatment. There are some studies about prenatal exposure to opioids, but none of them is focused on analyzing the changes at imprinted genes (Schrott LM et al 2014; Byrnes EM and Vasoler FM 2017). These clusters have been studied in other drug or addition models, such as parental alcoholic exposure, through DNA methylation changes (Knezovich JG and Ramsay M 2012; Marijonen H et al. 2018), highlighting none significant differences in the methylation patterns of the ICR/DMR of mainly Snrpn clusters. On the other hand, a hypomethylation pattern has been described in the genes belonging to Dlk1-Dio3 genes in current and former smoker patients with lung cancer (Molina-Pinelo S et al. 2018). This information together with our results, open us a new field of studying these both imprinted regions more in deep, in terms of altered chromatin remodeling as a regulatory mechanism of imprinting process. Nevertheless, future studies are needed to clarify and complete the underlying mechanism, in terms of specific expression and methylation patterns in our study model.

5.4.2. Morphine induce a Smchd1 silencing through H3K27me3 enrichment conferring to the cell an active state of X chromosome

Among the genes, which correlated between ChIP-seq and RNA-seq results over time, we found Smchd1, a gene with a key role on chromosome X inactivation, as it regulates chromatin organization through epigenetic repression of the genome. We observed that after chronic morphine treatment, Smchd1 showed a higher H3K27me3 enrichment at promoter level (coinciding with a CpG island site) consistent with a decreased gene expression pattern. This decreased expression was maintained until 24h after morphine exposure was finished and then started to recovered and became upregulated.

As it is related to X chromosome inactivation, we focused on its function in this process. In mouse development X chromosome inactivation occurs in two waves (G. Pintacuda & A. Cerase 2015): the first one takes place from 2 to 4 cell embryonic stage and is known as imprinted X inactivation (iXCI) (Okamoto, I et al. 2004). Then, at blastocyst stage, the silencing is reverted and the active state of both Xs is re-established in the inner cell mass of the embryo. After implantation, during the epiblast formation, the second X chromosome inactivation wave occurs, where the X chromosome is randomly selected to be inactivated in a process known as random X inactivation (rXCI) (Mak, W et. Al 2004). As previously explained, the master regulator of the process is Xist lncRNA, and the subsequent maintenance is perform, on one hand though histone modification, manly related to H3K27me3 linked to PRC2 function (Wang et al 2001). On the other hand, DNA methylation is also essential for X inactivation process (Holliday and Pugh 1975). CpG islands found in gene promoter or enhancers are heavily methylated to repress the expression of every gene in the X inactivated chromosome and get the long term memory repressed state maintenance. Based on this information, we also described the H3K27me3 enrichment pattern and the expression level of Xist gene and its antisense gene Tsix. Both genes showed an increased enrichment of H3K27me3 after morphine chronic treatment, and consistently it was reflect in a slight decrease of gene expression level in both cases at P1, which was maintained at P2 but increased at P3. This pattern was the same as described for Smchd1, so this coincidence could be consistent with the idea that morphine induced the repression of the machinery responsible for the X chromosome inactivation, through H3K27me3 histone modification.

Going deeply in morphine effect during X inactivation process, we have found a publication where control and opioid addicted females X chromosome inactivation ratio was compared (Vousooghy N et al. 2015). But, unlike us, they did not find an increase in X chromosome inactivation skewness between treated and untreated samples or unbalanced expression of genes associated with X chromosome because of the opioid effect. Considering this, we focused on the function of Smchd1. As we have previously explained, data published describes that its main role consists in gene expression silencing of X-linked genes in X chromosome inactivation (XCI) maintenance, once the process of inactivation itself is started with Xist expression (Michal R. Gdula et al. 2019; Yuki Sakakibara et al 2018; Kesley et al. 2015; Ryu-Suke Nozawa et al. 2013). This fact, led as think about how morphine treatment affects H3K27me3 enrichment through X chromosome to link it with a more active state of the X chromosome. For that purpose, we analyzed specifically the coverage of H3K27me3 enrichment through the whole X chromosome after morphine treatment and observed that the general pattern of enrichment is decrease on

morphine treated sample. H3K27me3 has been extensively studied linked to X inactivation. It is describatedthat the PRC2 is the responsible of the deposition of the repressive mark, through Ezh2 enzymatic component in the early X inactivation, in a Xist-dependent fashion (Plath et al. 2003; Silva et al. 2003; de la Cruz et al. 2005; Zhao et al. 2008). Based on this, PRC2-dependent H3K27me3 deposition has been proposed to act as a recruitment signal for PRC1, which further starts the process of chromosome X inactivation (de Napoles et al. 2004). Based on our observations, morphine produced a global downregulation of H3K27me3 repressive mark on the whole X chromosome, just the opposite of what happens in the ordinary process. Thus, this could possibly confer to the cell an active state of transcription. In addition, the fact that morphine incremented the enrichment of H3K27me3 at promoter level of Smchd1, could be a mechanism of the cell to decrease its gene expression and repress its related gene silencing function. It could be an interesting option to analyze the distribution of the rest of histone modifications in the X chromosome, to further complete or understand the underlying mechanism of the effect of morphine in the X chromosome inactivation. Also it would be worthy to study the distribution in Tsix, Xist and Smchd1 genes, to evaluate the changes induced by morphine respect the described ordinary pattern.

Furthermore, in its role of gene silencing, Smchd1 is related to some clustered protocadherins (Chen, K. et al 2015; Gendrel AV et al. 2013; Leong HS et al. 2013; Mould AW et al. 2013) and clustered Hox genes (Natasha Jansz et al. 2018), together with some imprinted genes such as Snrpn and Igf2r clusters. Snrpn imprinted cluster genes show an epigenetic signature consisting in a loss of imprinting in the absence of Smchd1 (Mould AW et al. 2013). As previously mentioned, from our results Smchd1 expression seem to be decreased after 24h morphine exposure and possibly mediated by H3K27me3 repression, and this decreased expression is maintained during the consequent 24h. This disruption induced by morphine, could affect the regulation of the entire Snrpn imprinted cluster. Thus, it would be interesting to further have into consideration, the analysis of gene expression patterns of these Smchd1 related genes, to validate the hypothetical mechanism where morphine repressed Smchd1 expression through H3K27me3, and in turn reactivated the genes under its regulation.

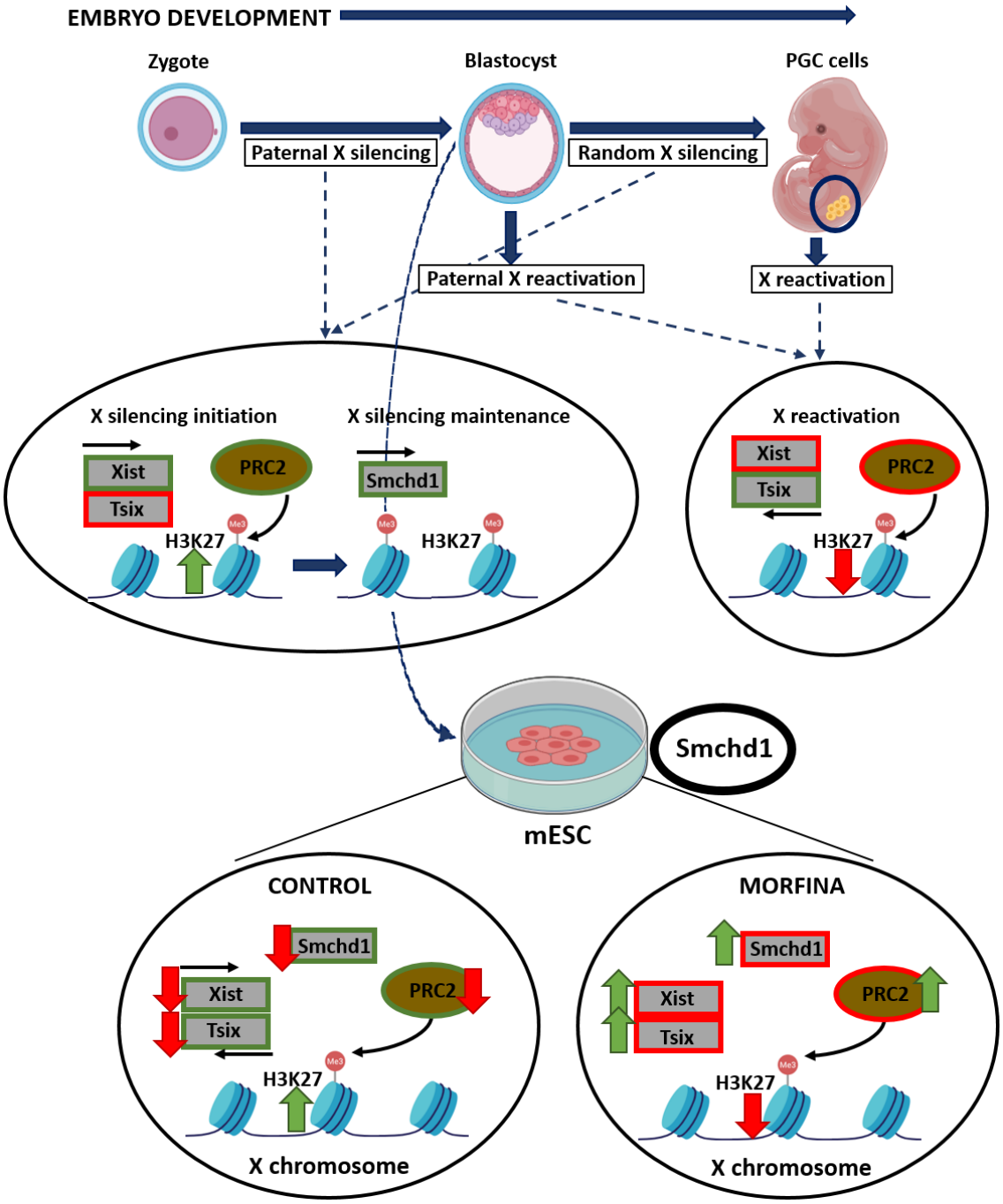

Figure 5.3. Model of Smchd1 silencing through H3K27me3 and reactivation of X chromosome, composed from our results. Green arrows mean H3K27me3 enrichment upregulation and red arrows mean H3K27me3 down regulation. Red boxes and circles around genes, mean gene expression downregulation and green boxes and circles mean gene expression upregulation.

5.5. MORPHINE AND LONG NON-CODING RNA

In recent years, non-coding RNAs (ncRNAs) such as microRNAs (miRNAs) and long non-coding RNAs (lncRNAs) have gained attention for their remarkable role in cellular function and disease development related to drug addiction (Bali KK et al. 2014; Briana AB et al. 2014). As general functions, non-coding RNAs participate in DNA synthesis, maintain genome stability, and promote epigenetic modifications (Cech TR and Steitz JA 2014; Frias-Lasserre D et al. 2017), and specifically miRNAs can regulate opioid functions (Toyanna K et al. 2017; Zheng H et al. 2012), and play a critical role in the signal cascade from receptor to systemic responses in the modulation of adult neurogenesis and *in-vivo* memory (Zheng H et al. 2012). However, researches focused on lncRNAs are very limited. It is described that lncRNAs can interact with mRNA, bind to transcription factors, modulate chromatin remodeling and directly regulate target proteins. With our results, we described morphine induced interaction of H3K27me3 with different lncRNAs groups. We perceived a big increase of differentially enriched lncRNAs in our results and the most characterized groups were intergenic, antisense, intronic and processed transcripts lncRNAs. In fact, other publications have confirm the role of H3K27me3 regulating different lncRNAs expression in normal conditions, as an epigenetic machinery similar to that of protein coding genes (Wu SC et al. 2010). These H3K27me3 enriched lncRNAs related to adjacent coding genes, which belonged mainly to biological functions described in previous sections of the discussion proper to those related to opioid use. Specifically, the top biological functions were, cell differentiation processes; intracellular signal transduction functions related to hippo signaling, which controls the organ size in animals through the regulation of cell differentiation or apoptosis; regulation of metabolic process; cell adhesion; axon development; gonad development; respiratory system development etc. All of them are tightly related to opioid abuse side effects (Hirabayashi et al. 2009; Hwang et al. 2014; Pereira et al. 2010; Gach K et al. 2011; Singhal PC et al. 1992; Feizy N et al. 2015; Wu G et al. 2018; Willner D et al. 2014; Narita M et al. 1994; Al-Hasani R et al. 2011; Antolak A et al. 2017; Matsuma et al. 1984).

To identify the specific lncRNAs, which could be regulated by H3K27me3 epigenetic mark, we also performed a transcriptomic analysis of morphine induced gene expression changes at lncRNA level. Different from we described until this point, we found more or less the same quantity of upregulated and downregulated lncRNAs. Surprisingly the adjacent coding genes of these differentially expressed lncRNAs, were not related to the same biological functions as that we found through the H3K27me3 enrichment. This led us to think about another epigenetic mechanism regulating lncRNAs expression. GO results revealed high deregulation in signal transduction involved in mitotic G1 DNA damage checkpoint, cholesterol biosynthesis and transport, negative regulation of striated and cardiac muscle cell apoptotic process, telomere maintenance, etc. In fact, morphine is described to participate in most of the mentioned biological functions. For example, Mandyam CD et al. identified the regulation of the cell cycle mitosis phase by chronic morphine in mouse subgranular zone of the brain (Mandyam CD et al. 2004). Supporting our results, other researchers have published results confirming cholesterol synthesis increase (Chankandi M et al. 2015), or specifically functions of cardioprotection by ncRNAs (Melo Z et al. 2018). Furthermore, we found similarities in the results of experimentation carried out in human heroin addicts in nucleus accumbens. Among other lncRNAs they described Neat1 (Nuclear enriched abundant transcript 1) as one of the differentially expressed lncRNA, similar to what we found in our experimentation in mESCs

model after chronic exposure. Neat1 is associated with nuclear paraspeckles, ribonucleoprotein structures found in the nuclei of differentiated mammalian cells that appear to regulate the nuclear retention of select mRNAs, allowing for rapid post-transcriptional regulation of expression of certain genes (Wilusz JE et al. 2009; Bond CS and Fox AH 2009).

When mixing both results to identify if H3K27me3 epigenetic mark could be regulating lncRNAs expression, as expected an in line of observed with the despaired deregulated biological functions, we found a small number of reversely related lncRNAs. From those that have an adjacent coding gene, we could identify lncRNAs related to previously mentioned Bmp4 and Smchd1 genes, and also Ttc14, 1700001L05Rik, Gm13227, Gm13230, Gm26573 and AK035187 genes.

We performed the transcriptome analysis in different time points to identify morphine induced lncRNA expression deregulations, maintained over time to establish cell memory results. We found that even when the stimulus is absent the deregulated lncRNA amount gets bigger. Furthermore, 227 lncRNA were described to maintain the deregulation pattern through studied three time points, with adjacent coding genes related to negative regulation of DNA metabolic process and stem cell population maintenance. These results are in line with our previous results regarding morphine induced biological functions. From all of that lncRNAs, we only identify one which reversely correlated with H3K27me3 enrichment values and in turn could be regulated by H3K27me3 repressive mark after morphine chronic exposure, and during posterior periods once the treatment is finished. This lncRNA is an intron overlapping lncRNA related to Smchd1 gene. Together with previous results, this intron overlapping lncRNA could be involved somehow in the machinery of chromosome X silencing. However, additional functional experiments are needed to clarify and robustly construct a mechanism network with these results.

5.6. MORPHINE AND REPETITIVE ELEMENTS.

The majority of the mouse genome is comprised of interspersed and tandem repetitive sequences (at least 35%), most of which are represented by endogenous retroviruses, long interspersed nuclear elements (LINEs), short interspersed nuclear elements (SINEs), Long terminal repeats (LTRs) transposable element superfamilies, as well as direct transposition of genomic DNA and satellite sequences. In mouse, the major SINEs are the B1 and B2 elements (Deininger P 2011; Krayev AS et al. 1980; Haynes SR et al. 1981). B1 repeats share some homology with human Alu repeats, whereas B2 repeats have no human homoleg and resemble tRNAs. The mouse LINE elements, has homologs in other mammalian species and also polymorphic dispersed minisatellite and microsatellite repeats have been described mouse and other species Toth G et al. 2000). Last years have seen increased interest in understanding their regulation because of the important roles in genome evolution, development and disease (Bourque G et al. 2008; Erwin JA et al. 2015; Bollati V et al. 2011; Bodega B and Orlando V 2014).

To date little is known about epigenetic changes induced by opioid exposure in culture cells of treated animals focused on repetitive elements. Most of the researches have been performed through DNA methylation changes, as it is described the capacity of morphine to modulate cellular oxidative stress and methyl group donation (Trivedi et al. 2014). For example, LINE-1 retrotransposons have been identified as hypomethylated in leukocytes of chronic heroin users and in neuronal cell lines, increasing their mRNA expression (Doehring et al. 2013; Trivedi et al. 2014). Others have analyzed repetitive elements through histone methylation changes after direct injection of morphine in the nucleus accumbens (Sun et al. 2012). In this sense, from our ChIP-seq experiment we have identify specific repetitive elements related to H3K27me3 enrichment changes after chronic exposure of morphine.

We have observed a global upregulation of H3K27me3 enriched repetitive elements the opposite of what we expected after the global downregulation pattern of H3K27me3 after chronic morphine exposure, and also comparing with other types of drugs such as alcohol exposure models (Veazey KJ et al. 2014). This repetitive elements with increased H3K27me3 enrichment belonged mainly to LTRs, RNA repeats and simple repeats, being both groups the most enriched ones to H3K27me3 also in normal conditions (Day DS et al. 2010). Studies of mESCs mutants deficient for the Suv39h histone methylatransferase have described a decreased H3K9me3 levels in the repetitive elements, together with an increase in H3K27me3 enrichment (Peters AH et al. 2003), suggesting a potential compensation mechanism between both histone modifications. It could be an interesting step forward to analyze the repetitive elements distribution after morphine chronic exposure in other histone modifications such as H3K9me3 to form a complete mechanism.

Furthermore, to keep on a potential mechanism basis for the observed epigenetic alterations we analyzed the mRNA levels of transcripts identifying a global downregulation of repetitive elements transcripts, in line with the described H3K27me3 repressive mark increased enrichment (Leeb M et al. 2010). This repetitive elements with decreased gene expression belonged mainly to LTRs and also to RNA repeats in a lower degree, according to H3K27me3 enrichment results. Therefore, our results demonstrate a potential regulation of H3K27me3 in repetitive elements induced by morphine chronic exposure. Some publications have described H3K27me3 mainly to act as a repressor of ERLV and MaLR retrotransposon family group and

H3K9me3 o repress directly ERVK retrotransposon family group, with the help of Trim28 in mESC and early embryos (Rowe HM et al. 2010; Chrichton JH et al. 2013). In our case, we observed an increased H3K27me3 enrichment mainly to ERVK retrotransposon family, suggesting a possible combined mechanism of repetitive elements regulation induced by morphine.

Other striking result of our analysis proceeded from cell memory experimental results. In fact, we found a substantial group of LTRs composed by previously mentioned ERVKs and some ERVLs which maintained a significant downregulated pattern induced by morphine just after the treatment and until 24h later, when the stimuli was absent. Surprisingly, only one of the repetitive elements preserved a significat decreased expression pattern in the studied three time points, consisting on a rRNA repeat: LSU-rRNA-HAS. This large subunit ribosomal ribonucleic acid is the largest of the two major RNA components of the ribosome, and after morphine exposure it appears to be downregulated during P1, P2 and P3. Furthermore, its gene expression downregulation did not match with the downregulation of H3K27me3 enrichment also observed. Therefore, it must be another epigenetic mechanism regulating its long lasting repression started with morphine exposure and maintained once the treatment was end, worthy to analyze in future studies. Moreover, even if this repeat element has been mentioned in very small quantity of publications related to DNA methylation, none of them clarifies its specific function in mESC or other organism, thus this is an important field to follow studying and characterizing this repetitive family in the future.

6. ONDORIOAK
CONCLUSIONS

6. ONDORIOAK

1- Morfinaren tratamendu kronikoak, *in-vitro* H3K27me3-ren, H3K9me2-ren, H3K4me2-ren eta H3K4me3-ren mailan eta histonen aldaketen erregulazio konplexuetan desdoitze orokorra sortzen du, denboran zehar irauten dutenak naiz eta tratamendua presente ez egon.

2- Morfinaren tratamendu kronikoak, *in-vivo* H3K27m3-ren, H3K9me2-ren, H3K4me2-ren eta H3K4me3-ren mailan eta histonen aldaketen erregulazio konplexuetan desdoitze orokorra sortzen du organismoko sistemetako organo garrantzitsuenetan.

3- Kromatinaren azterketaren bitartez, morfinaren tratamenduak sortutako H3K27me3 marka epigenetiko errepresiboaren jaitsiera globala berretsi da, CpG irletan ugariak diren geneen sustatzaile eremuetan aldiz aberastasuna identifikatuz. Hauek, batez ere nerbio sisteman, zelularen funtzio basikoetan, zelularen desberdintze prozesuan, mugikortasunean, transkripzioaren erregulazioan eta organoen garapenean parte hartzen dute.

4- Transkriptomaren analisian, geneen adierazpenaren erregulazioan aldaketak identifikatu dira, morfinaren tratamendu kronikoaren ostean eta baita azterturiko hurrengo denbora puntuetan, nahiz eta tratamendua presente ez egon. Denboran zeharreko eragin hau, prozesu metabolikoekin, erreplikazio eta zatiketa prozesuekin eta geneen adierazpenaren erregulazioarekin erlazioa duten geneetan identifikatu da, memoria epigenetikoaren sorrera baieztatuz.

5- Morfinaren eraginez H3K27me3-k inpronta genomikodun gene klusterren erregulazioan parte hartu lezake, bere distribuzio aldaketek batez ere geneen DMR/ICR eremuetan eragiten dutelako. Ez da denboran zehar mantentzen den eta transkripzioan eragiten duen H3K27me3-ren distribuzioaren aldaketarik aurkitu, baina denboran puntu ezberdinetan eragina erakusten duten inpronta genomikodun geneen klusterrak identifikatu dira.

6- lncRNA-etan izugarrizko desdoitzea sortzen du morfinak H3K27me3-aren distribuzioari eta transkripzioari dagokionean. Morfinak sortutako lncRNA-en transkripzio aldaketek denboran zehar dute eragina, batez ere DNA-ren prozesu metabolikoetan eta zelula amen populazioaren mantenuan parte hartzearekin erlazionatuta. Horietatik, bi lncRNA introniko identifikatu dira H3K27me3-ren distribuzio aldaketen ondorioz sortuak izan daitezkeenak.

7- Era berean elementu errepikakorretan ere, H3K27me3-ren distribuzioaren eta traskripzioaren denboran zeharreko aldaketak hauteman dira, hainbat azpifamiliatan. Horietatik rRNA azpifamiliako elementu errepikakor bat identifikatu da H3K27me3-ren aberastearen ondorioz, denboran zehar adierazpena erreprimitua mantendu dezakeena.

8- Morfinaren tratamendu kronikoak H3K27me3-ren distribuzioaren aldaketen bitartez, Bmp4 hazkuntza faktorearen adierazpenean eragin lezake. Era horretan, Bmp4-ren aldaketek, enbrioi goiztiarren eta jatorrizko hozi zelulen desberdintze prozesuaren garapena azkartuz.

9- Morfinaren ondorioz hautemandako H3K27me3 mailaren jaitsiera orokorra, PRC2 erregulazio konplexuaren azpiunitateen adierazpenaren jaitsieraren ondorioz gertatu daiteke. PRC2-aren jaitsiera, H3K27me3-k azpi unitate horietan sortzen duen auto-erregulazio prozesuaren eraginez gerta daiteke, eta prozesu horren eredua proposatzen da.

10- Morfinak, H3K27me3-ren distribuzio aldaketen bitartez X kromosomaren aktibazioa azkartu lezake, Xist/Tsix eta Smchd1 X kromosomaren inaktibazio eta mantentzeaz arduratzen diren geneak erreprimituz, eta X kromosomarekiko aberastasuna gutxituz.

6. CONCLUSIONS

1- Morphine chronic exposure, leads to global H3K27me3, H3K9me2, H3K4me2 and H3K4me3 levels changes and histone modifying regulatory complexes expression deregulation *in-vitro*, which are maintain over time, even when the treatment is absent.

2- Morphine chronic exposure generates global H3K27me3, H3K9me2, H3K4me2 and H3K4me3 levels changes and histone modifying regulatory complexes expression deregulation *in-vivo,* in the main organs of organism systems.

3- Global downregulation of H3K27me3 epigenetic repressive mark is determined, through chromatin analysis approaches. However, an upregulation is identified mainly in promoters rich in CpG islands. These genes are related to nervous system, cell basic functions, cell differentiation processes, locomotion, transcription regulation and organ development.

4- Trancriptomic analysis reveals changes in gene expression after chronic morphine treatment, together with other evaluated time points when the treatement is absent. This “over time” effect is identified in genes related to metabolic processes, cell replication and division and gene expression regulation, suggesting epigenetic memory formation.

5- H3K27me3 regulates different imprinting clusters through morphine produced distribution changes, mostly at DMRs/ICRs. There is not a “over time” transcriptomic change in any specific gene, but different genes from a same cluster present expression changes in the studied time points.

6- Morphine produces a wide deregulation at H3K27me3 enrichment in lncRNA and their transcriptome. Transcriptomic changes are maintain over time in lncRNAs originated from genes related to DNA metabolic processes and stem cell population maintenance. From those, two intronic overlapping lncRNAs are identified, which could be generated by H3K27me3 distribution changes.

7- H3K27me3 distribution and transcriptomic changes are observed in repetitive elements subfamilies over time. From those, a repetitive element which belongs to rRNA subfamily is identified to present a H3K27me3 enrichment and repressed expression maintained over time.

8- Morphine chronic exposure, affects Bmp4 growth factor expression through H3K27me3 distribution changes. This accelerates early embryonic development and primordial germ cells differentiation processes.

9- Global H3K27me3 level downregulation generated by morphine chronic treatment is produced by the downregulation of PRC2 subunits. A self-regulatory model is suggested, where H3K27me3 distribution regulates PRC2 expression.

10- Morphine leads to H3K27me3 distribution changes accelerating the silenced X chromosome reactivation. H3K27me3 represses X chromosome silencing initiator Xist/Tsix genes and silencing maintenance Smchd1 gene and produces a global downregulation of H3K27me3 at X chromosome enrichment.

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ERANSKINAK

APPENDIXES

I. ERANSKINA

Analisi bioinformatikoaren datuen formatua

Atal honetan, analisi bioinformatikoan zehar erabili ziren fitxategien formatuen ezaugarriak deskribatzen dira.

- Datu gordinak – fastq

Sekuentziazio prozesuaren ondoren sortutako datuak, **FASTQ** formatuan jaso genituen, hau da, FASTA formatuaren bertsio aldatu batean, .fq edo .fastq luzapena zutenak. Datu gordin hauek, sekuentziatutako laginen nukleotidoen eta hauen izendapenaren kalitatearen informazioa zeramaten. Orokorrean artxibo formatu honek sekuentziazio prozesuak izendatutako "read" bakoitzeko 4 lerroko informazioa ematen du: goiburuko lerroa, "@" ikurrarekin hasi eta "read"-aren izena eta deskribapena duena; nukleotido sekuentzia; bigarren goiburua: "+" ikurrarekin hasi eta lehenengo goiburuko informazioa errepikatzen duena; azkenik, kalitatearen informazioaren puntuazioa (kalifikazioa zenbat eta hobeagoa, orduan eta fidagarriagoa izango da sortutako informazioa eta kalifikazioa baxua denean nukleotidoen izendapena zalantzan jartzen dute). .fastq formatuan dagoen fitxategi bat, honelako da orokorrean:

```
@SRR566546.970 HWUSI-EAS1673_11067_FC7070M:4:1:2299:1109 length=50
TTGCCTGCCTATCATTTTAGTGCCTGTGAGGTGGAGATGTGAGGATCAGT
+SRR566546.970 HWUSI-EAS1673_11067_FC7070M:4:1:2299:1109 length=50
hhhhhhhhhhghhghhhhhhfhhhhhfffffe`ee[`X]b[d[ed`[Y[^Y
@SRR566546.971 HWUSI-EAS1673_11067_FC7070M:4:1:2374:1108 length=50
GATTTGTATGAAAGTATACAACTAAAACTGCAGGTGGATCAGAGTAAGTC
+SRR566546.971 HWUSI-EAS1673_11067_FC7070M:4:1:2374:1108 length=50
hhhhgfhhcghghggfcffdhfehhhhcehdchhdhahehffffde`bVd
...
```

- Lerrokatzearen datuak – sam, bam, bai

.fastq fitxategiak erreferentziazko genomari lerrokatu ondoren, emaitza duten fitxategiak orohar, .**SAM** edo .**BAM** formatuan jasotzen dira. Biek informazio bera biltzen dute, baina formatu ezberdinetan adierazten dute. .sam fitxategiak ingeleseko "Sequence Alignment/Map"-en dute jatorria eta sekuentzien lerrokatzearen informazioa biltzen dute, tabulazio bidez mugaturiko testu batean, hau da, gizakientzat irakurgarria den formatuan. .bam fitxategiak ordea, .sam fitxategien bertsio bitarra dira, konprimatuagoak eta beraz, lan egiteko errazagoak. Sekuentziazioaren munduan, fitxategi formatu hauek, lerrokatze informazioa biltzeko formatu estandarrean bilakatu dira.

.sam fitxategiek, goiburuak dituzte @ zeinuaz hasten direnak eta bi letrazko kodigo batek jarraituaz (@HD, @SQ, @RG, @PG, @CO). Ilara hauek, lerrokatzea gertatu den kromosoma bakoitzeko informazioa biltzen dute:

```
@HD     VN:1.0  SO:coordinate
@SQ     SN:chr1 LN:249250621
@SQ     SN:chr10        LN:135534747
@SQ     SN:chr11        LN:135006516
...
```

Goiburuaren ostean, lerrokatutako read-en informazioa aurkezten da, ilara bakoitzeko lerrokatze bat ageriz, hainbat zutabetan banatua. Zutabe horietako bakoitzean, honako datu hauek laburbiltzen dira: read-aren izena, .sam fitxategiaren seinalea, kromosoma zenbakia (read-ak ez badu lerrokatzerik "*" agertuko da kromosoma zenbakiaren ordez), read-aren posizioa, MAPQ (lerrokatze kalitatea), CIGAR balioa, binakako muturren bikotearen informazioa, binakako muturren bikotearen posizioa, ereduaren neurria, read-aren sekuentzia, read-aren kalitatea, lerrokatze programaren seinale espezifikoak. .sam formatuan dagoen fitxategi bat, honelako da orokorrean:

```
HWI-ST1001:137:C12FPACXX:7:2313:17391:30032  272  chr1  13494  1 51M *  0  0
ACTGCCTGGCGCTGTGCCCTTCCTTTGCTCTGCCCGCTGGAGACAGTGTTT
CFFFFHHJJJJIJJJJIJJJJJJJJJJJJJJJJJJJJJJHHHHFA+FFFC@B                    AS:i:-3
XN:i:0  XM:i:1  XO:i:0  XG:i:0NM:i:1  MD:Z:44G6  YT:Z:UU  XS:A:+  NH:i:3
CC:Z:chr15  CP:i:102517626  HI:i:0
```

Gainera, analisi bioinformatikoan zehar lan-tresna batzuek .bam fitxategia, era berezi batean antolatua egotea behar izaten dute. Horrela fitxategiko posizio datuak antolaketa lexikografikoa erabiliz ordenatzen dira, hau da, kromosoma zenbakia kontuan hartuz (1, 10, 11, 12, 13, 14, …, 2, 3, 4…) eta ondoren posizio koordenatuak. **SORTED.BAM** fitxategiak beraz, read-en azterketa modu eraginkorragoan egitea ahalbidetuko du.

Bestalde, analisiko beste pausu batzuetan, .bam fitxategiaren "aurkibide" bat behar izaten da, read-en informazioa bizkorragoan antzeman ahal izateko. Aurkibide fitxategi hauek **BAI** formatuan egongo dira, eta .bam fitxategiaren izen berdina edukiko dute. Fitxategi honek .bam fitxategiko informazioaren eduki taula bezala funtzionatzen du eta analisi bioinformatikoen programak .bam fitxategiko atal konkretuetara edo intereseko ataletara bideratzen ditu, fitxategi osoa aztertu ordez.

SAM/BAM/BAI formatuei buruzko informazio gehiago, The SAM Format Specification dokumentuan aurkitu daiteke (v1.4-r985; http://samtools.sourceforge.net/SAM1.pdf).

- Irudikatze prozesuaren datuak – bed, bedGraph, GTF, bigWig

Erreferentziazko genomari lerrokatutako sekuentzien datuak irudikatzeko "genomako nabigatzaileak" (ingelesezko Genome Browser) erabiltzen dira. Bertan sekuentziak ikusarazteak, sortu ditugun datuak hurbilagotik aztertzea eta analisiaren nondik norakoa ezartzea ahalbidetzen du. Genomako nabigatzaileen artean erabilienak bi dira:

- **UCSC Genome Browser**, Kaliforniako Unibertsitateak (Santa Cruz) sortutako genoma nabigatzailea da (https://genome.ucsc.edu/) (Kent WJ eta lank. 2002). "On line" erabiltzen da eta askotariko ornodun eta ornogabe espezieen erreferentziazko lerrokatze datuak ditu erabilgarri. Sekuentzien irudikapena errazteko hainbat tresna ditu eta baita datuen fitxategi eta dokumentazioak deskargatzeko aukera ere.
- **IGV Integrative Genome Viewer**, era lokalago batean erabili daiteke bakoitzaren ordenagailuan (http://software.broadinstitute.org/software/igv/) (Robinson JT eta lank. 2011, Thorvaldsdottir H eta lank. 2013). UCSC nabigatzaileak baino datu gutxiago ditu aukeran baina, askoz ere azkarragoa da fitxategi potoloak irudikatzerako orduan

Bi genoma nabigatzaileetan, era askotariko sekuentziazio fitxategi formatuak kargatu daitezke. Orokorrean ChIP-Seq esperimentuko emaitzak irudikatzeko fitxategiak .**BED/.BEDGRAPH** izango

dira eta RNA-Seq esperimentuko emaitzak irudikatzeko fitxategiak aldiz .**GTF/.BEDGRAPH** izango dira.

.bed formatuan dauden fitxategiek genomako leku konkretuak deskribatzen dituzten koordenatuen datuak dira. Koordenatu horiek orokorrean ChIP-Seq sekuentzien tontorrak deskribatzen dituzte beste ezaugarri batzuen artean. Fitxategi hauetan lehenengo hiru zutabeak beharrezkoak dira, kromosoma zenbakia, ezaugarriaren hasiera adierazten duen posizio base zenbakia eta ezaugarriaren bukaera adierazten duen posizio base zenbakia. Ondorengo zutabeek informazio osagarria azaltzen dute, adibidez, ezaugarriaren izena, balioa, haria etab. Fitxategi hauen adibidea jarraian adierazten da:

```
chr7 127471196 127472363 Pos1 0 + 127471196 127472363 255,0,0
chr7 127472363 127473530 Pos2 0 + 127472363 127473530 255,0,0
```

.bedGraph fitxategi formatuak, sekuentzien datu kuantitatiboak irudikatzeko erabiltzen dira, genomako leku konkretuetan. Datuak biltzeko tamaina aldakorreko tarteak erabiltzen ditu eta ChIP-Seq eta RNA-Seq esperimentuetako emaitzak irudikatzeko fitxategirik erabilienak dira. Goiburu bezala, fitxategiaren deskribapena azaltzen duen lerroa dago eta ondoren, .bed fitxategien antzera lehenengo hiru zutabeek, kromosoma zenbakia, ezaugarriaren hasierako posizio basea eta ezaugarriaren bukaerako posizio basea azaltzen dute eta laugarren zutabean deskribatzen den koordenatuaren balioa azaltzen da. bedGraph fitxategien adibide bat honakoa da:

```
track type=bedGraph name="BedGraph Format" description="BedGraph format"
visibility=full color=200,100,0 altColor=0,100,200 priority=20
chr19 49302000 49302300 -1.0
chr19 49302300 49302600 -0.75
chr19 49302600 49302900 -0.50
chr19 49302900 49303200 -0.25
chr19 49303200 49303500 0.0
chr19 49303500 49303800 0.25
```

.gff/.gtf fitxategiak, gene eta transkritoak biltzeko formatu konkretuak dira. Batez ere, RNA-Seq esperimentuko emaitzen analisi bioinformatikoa burutzeko tresnen fitxategiak izan ohi dira. Fitxategi honen zutabeetan, kromosomaren zenbakia, deskribatzen den genomaren ezaugarriaren izena, ezaugarriaren hasierako posizio base zenbakia, ezaugarriaren amaierako posizio base zenbakia, balioa, haria… agertzen dira besteak beste.

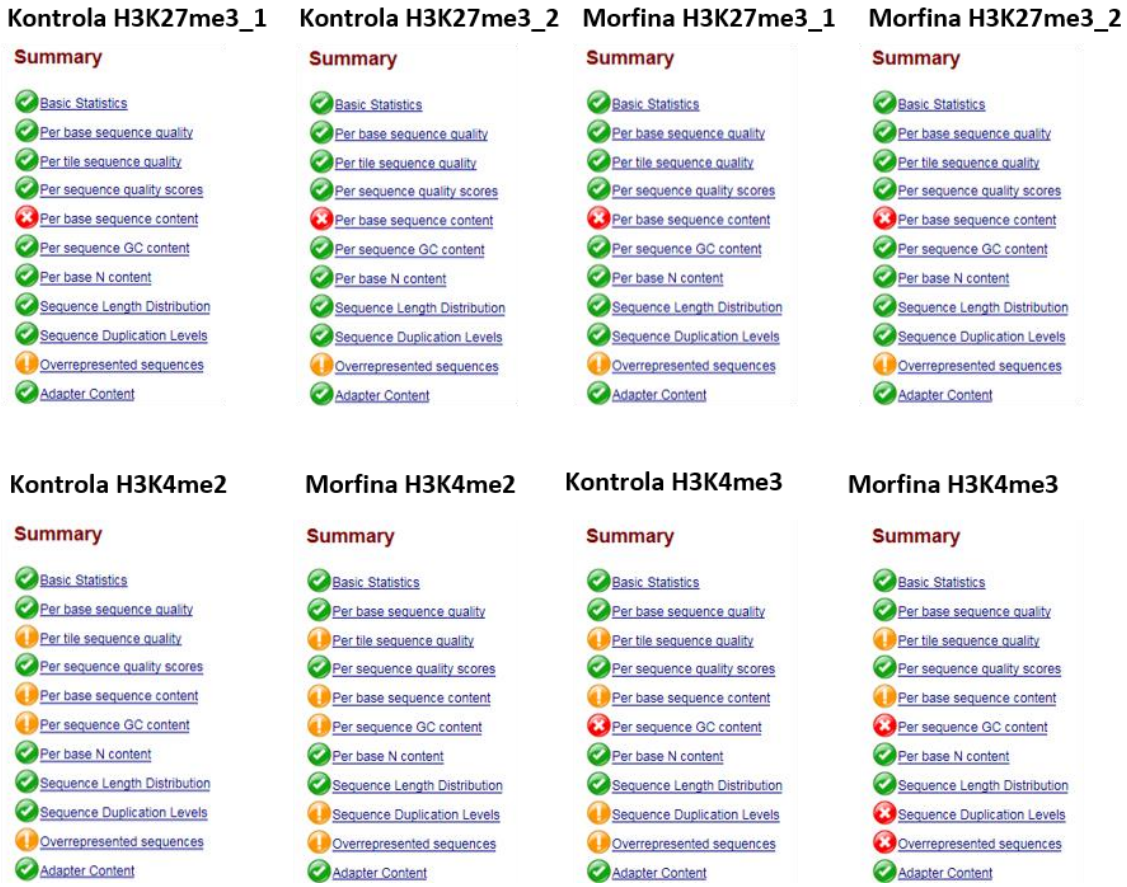
```
20 gene exon 9873504 9874841 gene_id "ENSBTAG00000020601"
20 gene CDS  9873504 9874841 gene_id "ENSBTAG00000020601"
20 gene exon 9877488 9877679 gene_id "ENSBTAG00000020601”
20 gene CDS  9877488 9877679 gene_id "ENSBTAG00000020601"
```

bigWig fitxategiak datu trinko eta jarraiak genomako nabigatzaileetan grafiko eran ikusarazteko erabiltzen dira eta askotan .bedGraph fitxategietatik eratorriak izaten dira. Fitxategi hauek indexatutako binario formatuan daude ta hauen erabileraren abantailetako bat ikusarazi nahi diren koordenatuen datuak bakarrik bidaltzen dituela da genoma nabigatzaileko zerbitzarira, beraz, bigWig fitxategien erabilerak ikusarazteko prozesua asko azkartzen du.

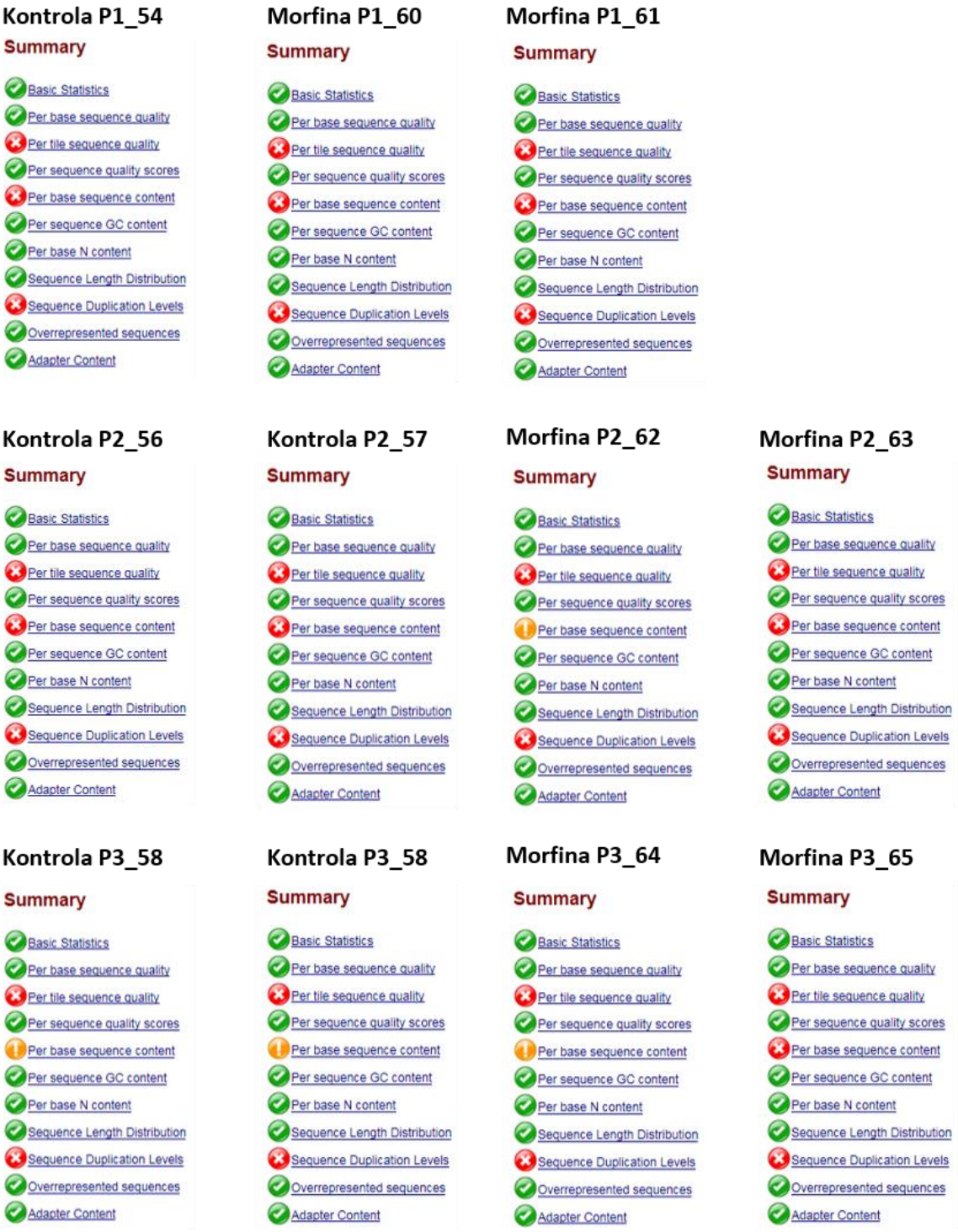
II. ERANSKINA

FASTQC analisiaren emaitzen irudiak

- Laburpena atalari dagozkien irudiak.



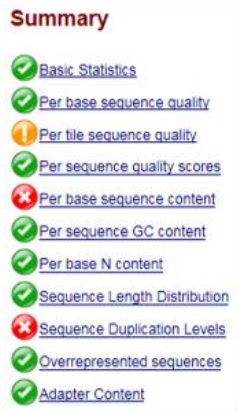
2.1.Irudia. FastQC "laburpena" moduluaren emaitzak. Irudiak FastQC analisiko laburpen moduluaren emaitzak erakusten ditu ChIP-seq esperimentuko lagin guztietan zehar.



2.2.Irudia. FastQC "laburpena" moduluaren emaitzak. Irudiak FastQC analisiko laburpen moduluaren emaitzak erakusten ditu (A) RNA-seq esperimentuko L4-an sekuentziatu ziren laginetan zehar eta,

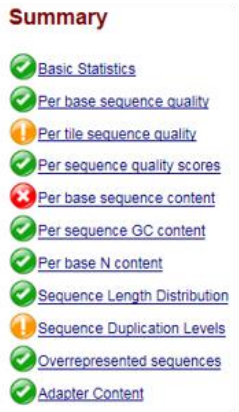
Orri oinaren jarraipena hurrengo orrialdean

Kontrola P1_54

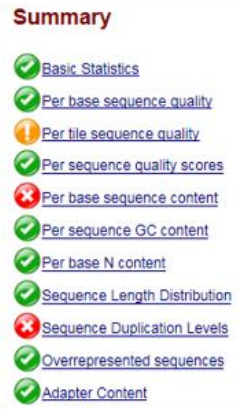


Kontrola P1_55

Morfina P1_60



Morfina P1_61



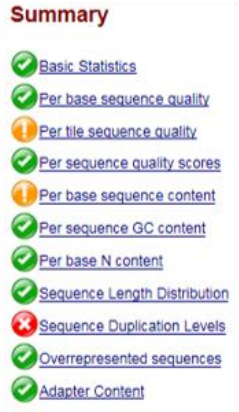
Kontrola P2_56

Kontrola P2_57

Morfina P2_62

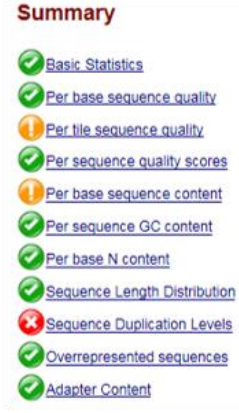
Morfina P2_63

Kontrola P3_58

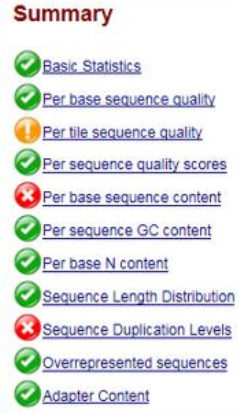


Kontrola P3_58

Morfina P3_64



Morfina P3_65



(B) RNA-seq esperimentuko L5-an sekuentziatu ziren laginetan zehar.

- Oinarrizko estatistikak atalari dagozkien irudiak

Kontrola H3K27me3_1

Measure	Value
Filename	ESC_9212_TTAGGC.fastq
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequences	44101637
Sequences flagged as poor quality	0
Sequence length	50
%GC	42

Kontrola H3K27me3_2

Measure	Value
Filename	9212_ESC_30442_TTAGGC.fastq.gz
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequences	59753562
Sequences flagged as poor quality	0
Sequence length	50
%GC	41

Morfina H3K27me3_1

Measure	Value
Filename	ESM_9213_GATCAG.fastq
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequences	43447815
Sequences flagged as poor quality	0
Sequence length	50
%GC	42

Morfina H3K27me3_2

Measure	Value
Filename	9213_ESM_30443_GATCAG.fastq.gz
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequences	63495840
Sequences flagged as poor quality	0
Sequence length	50
%GC	42

Kontrola H3K4me2

Measure	Value
Filename	c_me2_1.fastq
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequences	100633093
Sequences flagged as poor quality	0
Sequence length	51
%GC	42

Kontrola H3K4me3

Measure	Value
Filename	c_me3_1.fastq
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequences	60474336
Sequences flagged as poor quality	0
Sequence length	51
%GC	42

Morfina H3K4me2

Measure	Value
Filename	m_me2_1.fastq
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequences	64309345
Sequences flagged as poor quality	0
Sequence length	51
%GC	40

Morfina H3K4me3

Measure	Value
Filename	m_me3_1.fastq
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequences	59802586
Sequences flagged as poor quality	0
Sequence length	51
%GC	44

2.3.Irudia. FastQC "oinarrizko estatistikak" moduluaren emaitzak. Irudiak FastQC analisiko oinarrizko estatistikak moduluaren emaitzak erakusten ditu (A) ChIP-seq esperimentuan sekuentziatu ziren laginetan zehar,

Kontrola P1_54

Measure	Value
Filename	28454_ACAGTG_L004_R1_001.fastq.gz
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequences	18087614
Sequences flagged as poor quality	0
Sequence length	50
%GC	49

Kontrola P2_56

Measure	Value
Filename	28456_CTTGTA_L004_R1_001.fastq.gz
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequences	21029954
Sequences flagged as poor quality	0
Sequence length	50
%GC	50

Kontrola P2_57

Measure	Value
Filename	28457_AGTTCC_L004_R1_001.fastq.gz
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequences	18349484
Sequences flagged as poor quality	0
Sequence length	50
%GC	49

Kontrola P3_58

Measure	Value
Filename	28458_GTGAAA_L004_R1_001.fastq.gz
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequences	26576548
Sequences flagged as poor quality	0
Sequence length	50
%GC	49

Kontrola P3_59

Measure	Value
Filename	28459_GAGTGG_L004_R1_001.fastq.gz
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequences	26985487
Sequences flagged as poor quality	0
Sequence length	50
%GC	50

Morfina P1_60

Measure	Value
Filename	28460_ACTGAT_L004_R1_001.fastq.gz
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequences	24249350
Sequences flagged as poor quality	0
Sequence length	50
%GC	50

Morfina P1_61

Measure	Value
Filename	28461_CGATGT_L004_R1_001.fastq.gz
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequences	13492328
Sequences flagged as poor quality	0
Sequence length	50
%GC	50

Morfina P2_62

Measure	Value
Filename	28462_TGACCA_L004_R1_001.fastq.gz
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequences	23871820
Sequences flagged as poor quality	0
Sequence length	50
%GC	50

Morfina P2_63

Measure	Value
Filename	28463_CAGATC_L004_R1_001.fastq.gz
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequences	26554998
Sequences flagged as poor quality	0
Sequence length	50
%GC	49

Morfina P3_64

Measure	Value
Filename	28464_ATGTCA_L004_R1_001.fastq.gz
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequences	20609037
Sequences flagged as poor quality	0
Sequence length	50
%GC	49

Morfina P3_65

Measure	Value
Filename	28465_CCGTCC_L004_R1_001.fastq.gz
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequences	18816073
Sequences flagged as poor quality	0
Sequence length	50
%GC	49

2.4. Irudia. FastQC "oinarrizko estatistikak" moduluaren emaitzak. Irudiak FastQC analisiko oinarrizko estatistikak moduluaren emaitzak erakusten ditu (A) RNA-seq esperimentuko L4-an sekuentziatu ziren laginetan zehar eta,

Orri oinaren jarraipena hurrengo orrialdean

Kontrola P1_54

Measure	Value
Filename	28454_ACAGTG_L005_R1_001.fastq.gz
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequences	20352544
Sequences flagged as poor quality	0
Sequence length	50
%GC	49

Kontrola P1_55

Measure	Value
Filename	28455_GCCAAT_L005_R1_001.fastq.gz
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequences	40174883
Sequences flagged as poor quality	0
Sequence length	50
%GC	49

Kontrola P2_56

Measure	Value
Filename	28456_CTTGTA_L005_R1_001.fastq.gz
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequences	16480100
Sequences flagged as poor quality	0
Sequence length	50
%GC	50

Kontrola P2_57

Measure	Value
Filename	28457_AGTTCC_L005_R1_001.fastq.gz
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequences	18756151
Sequences flagged as poor quality	0
Sequence length	50
%GC	49

Kontrola P3_58

Measure	Value
Filename	28458_GTGAAA_L005_R1_001.fastq.gz
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequences	11682198
Sequences flagged as poor quality	0
Sequence length	50
%GC	49

Kontrola P3_59

Measure	Value
Filename	28459_GAGTGG_L005_R1_001.fastq.gz
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequences	8987682
Sequences flagged as poor quality	0
Sequence length	50
%GC	50

Morfina P1_60

Measure	Value
Filename	28460_ACTGAT_L005_R1_001.fastq.gz
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequences	9879080
Sequences flagged as poor quality	0
Sequence length	50
%GC	50

Morfina P1_61

Measure	Value
Filename	28461_CGATGT_L005_R1_001.fastq.gz
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequences	24731812
Sequences flagged as poor quality	0
Sequence length	50
%GC	50

Morfina P2_62

Measure	Value
Filename	28462_TGACCA_L005_R1_001.fastq.gz
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequences	13489117
Sequences flagged as poor quality	0
Sequence length	50
%GC	50

Morfina P2_63

Measure	Value
Filename	28463_CAGATC_L005_R1_001.fastq.gz
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequences	11203542
Sequences flagged as poor quality	0
Sequence length	50
%GC	49

Morfina P3_64

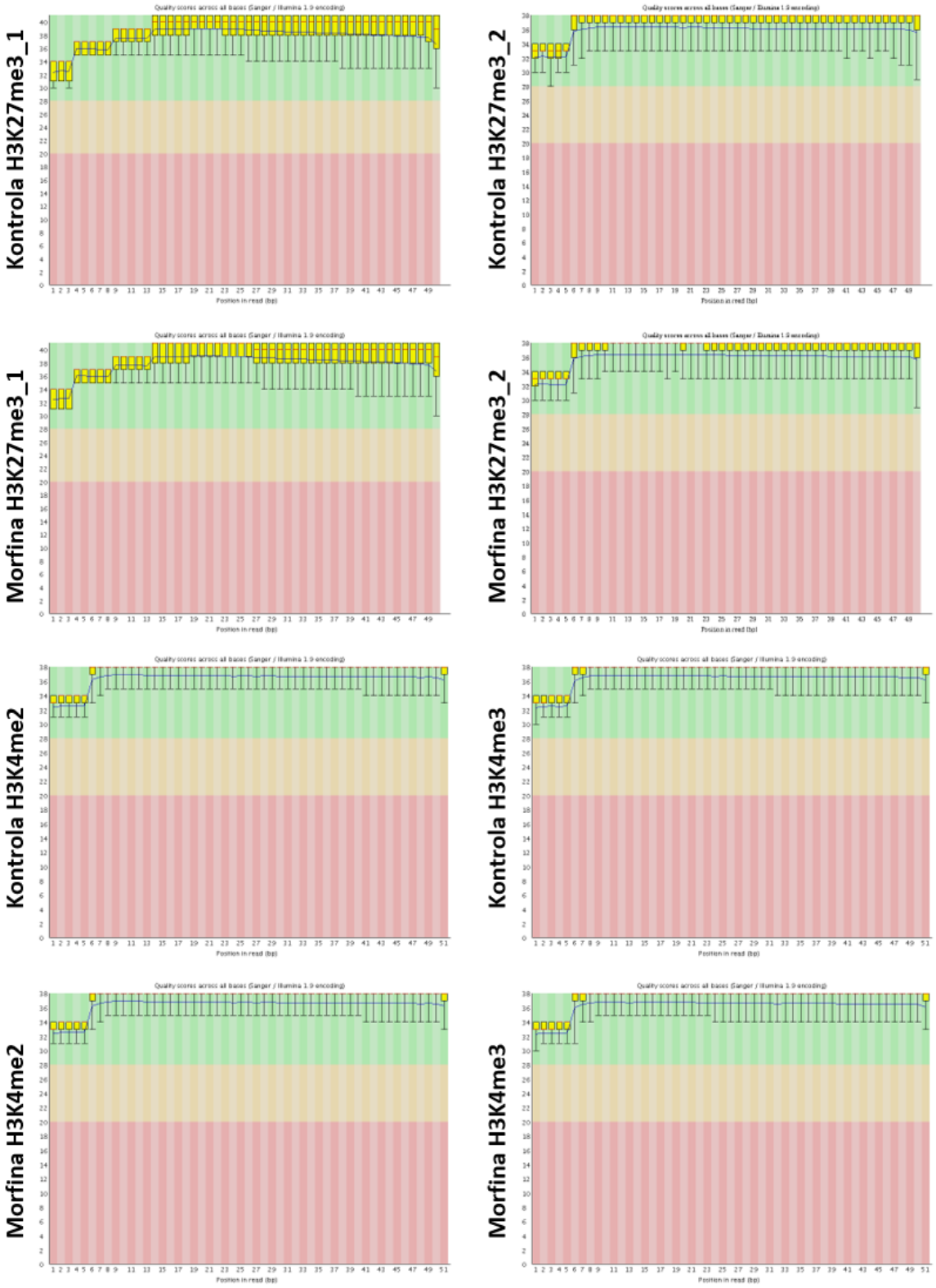
Measure	Value
Filename	28464_ATGTCA_L005_R1_001.fastq.gz
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequences	17872149
Sequences flagged as poor quality	0
Sequence length	50
%GC	49

Morfina P3_65

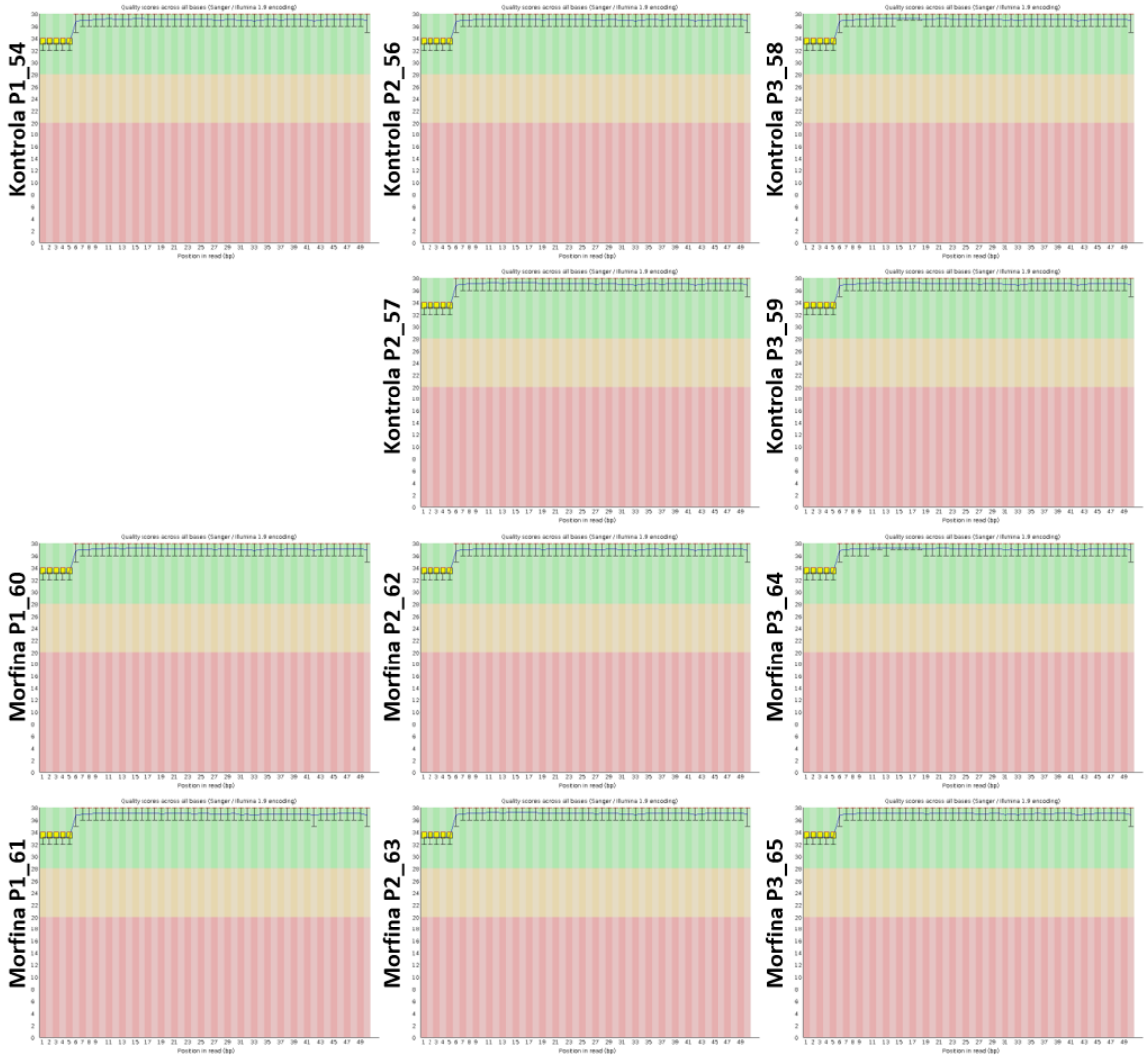
Measure	Value
Filename	28465_CCGTCC_L005_R1_001.fastq.gz
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequences	19918967
Sequences flagged as poor quality	0
Sequence length	50
%GC	49

(B) RNA-seq esperimentuko L5-an sekuentziatu ziren laginetan zehar.

- Sekuentzien base bakoitzeko kalitatea atalari dagozkien irudiak

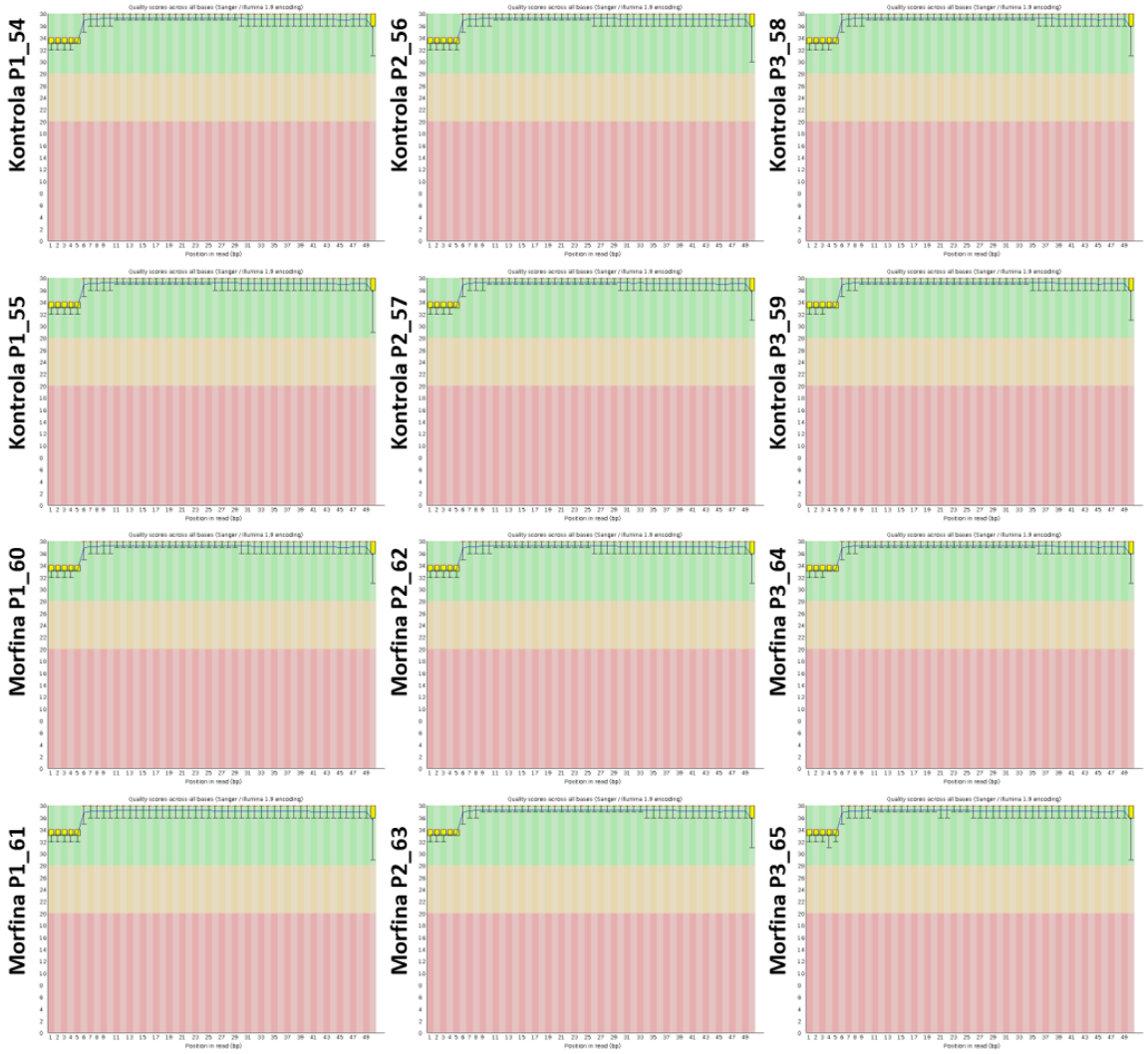


2.5. Irudia. FastQC "sekuentzien base bakoitzeko kalitatea" moduluaren emaitzak. Irudiak FastQC analisiko sekuentzien base bakoitzeko kalitatearen emaitzak erakusten ditu ChIP-seq esperimentuko laginetan.



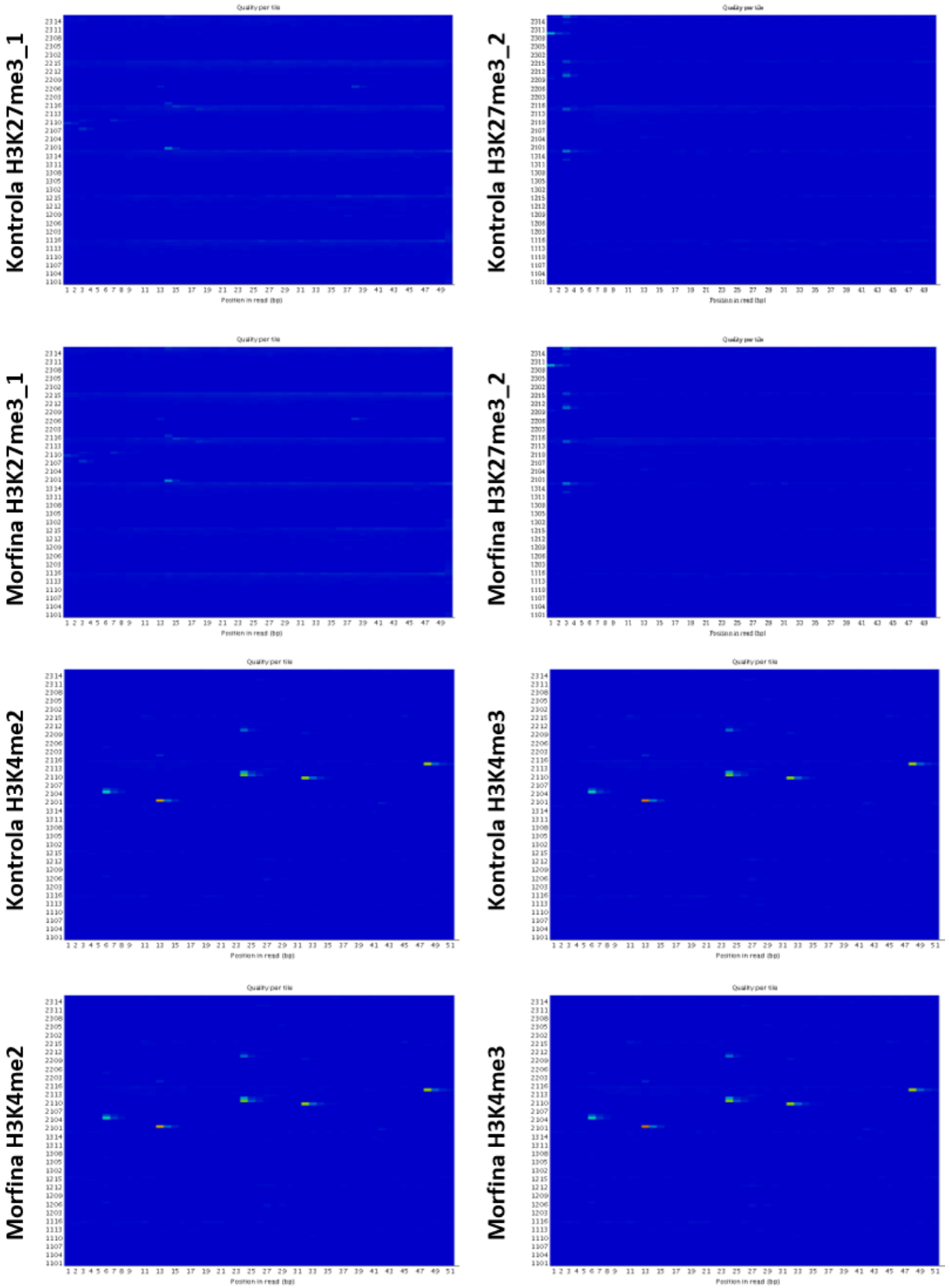
2.6. Irudia. FastQC "sekuentzien base bakoitzeko kalitatea" moduluaren emaitzak. Irudiak FastQC analisiko sekuentzien base bakoitzeko kalitatearen emaitzak erakusten ditu (A) RNA-seq esperimentuko L4-an sekuentziatu ziren laginetan eta,

Orri oinaren jarraipena hurrengo orrialdean

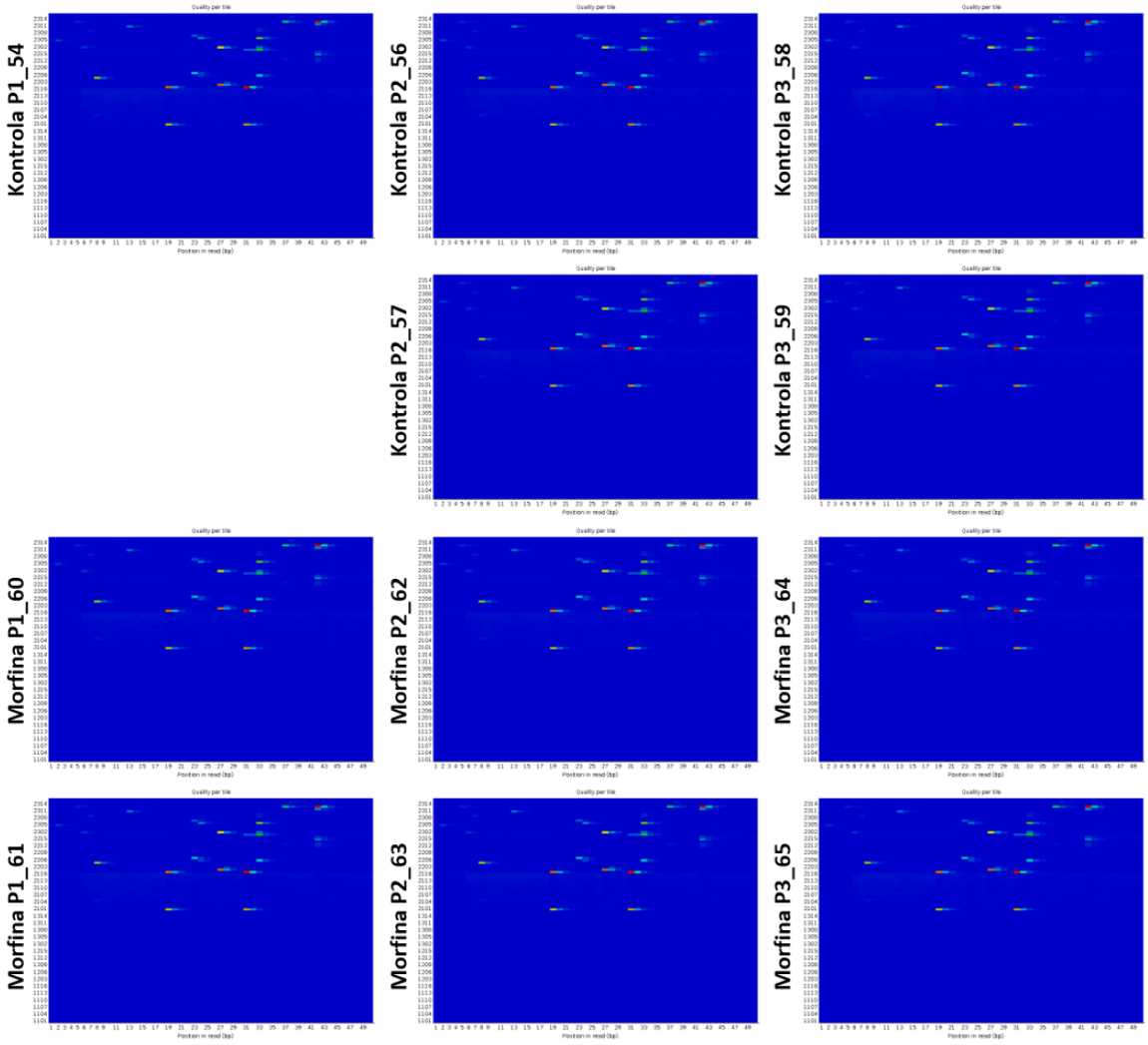


(B) RNA-seq esperimentuko L5-an sekuentziatu ziren laginetan zehar.

- Sekuentzien lauza bakoitzeko kalitatea atalari dagozkien irudiak

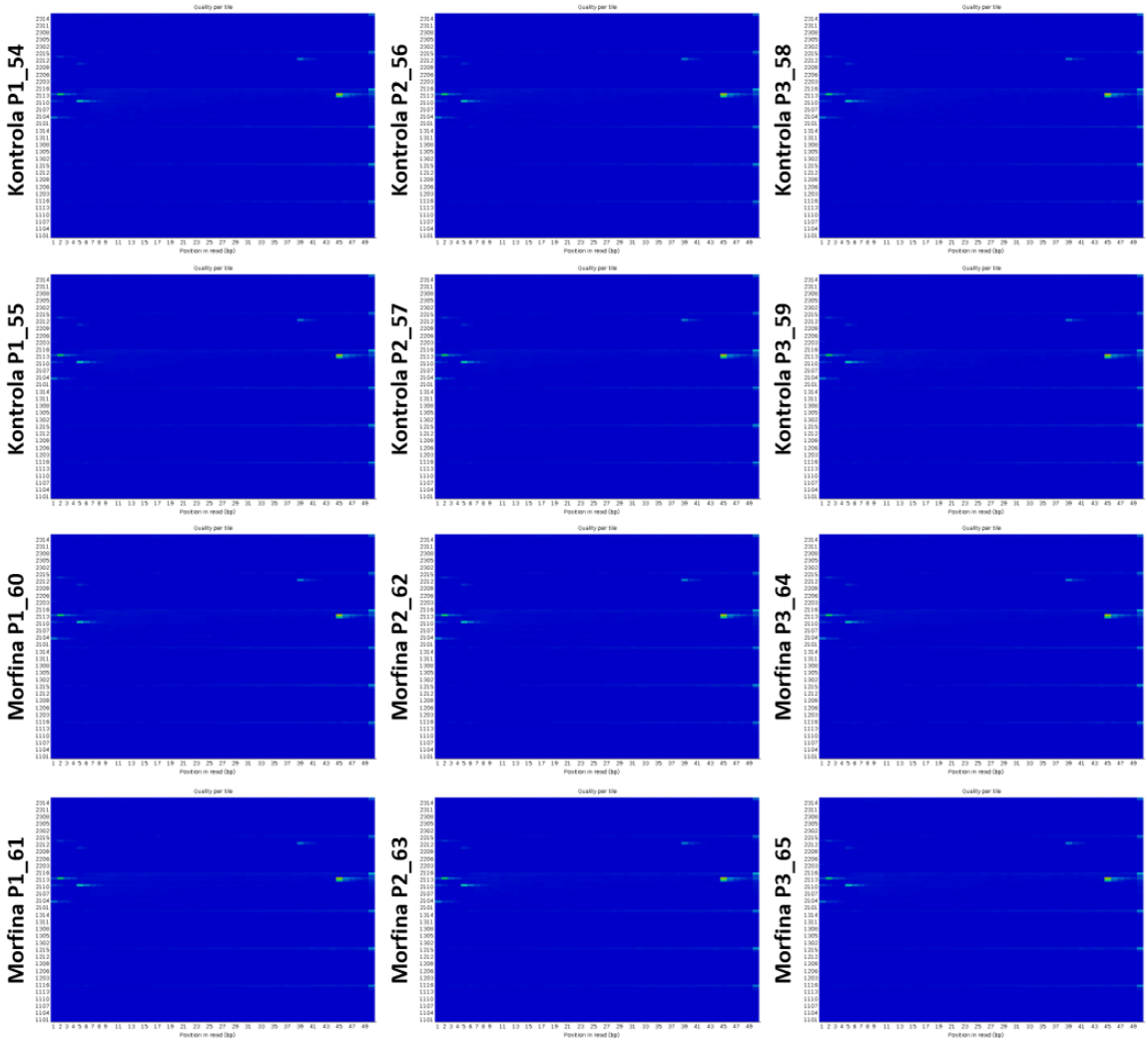


2.7. Irudia. FastQC "Sekuentzien lauza bakoitzeko kalitatea" moduluaren emaitzak. Irudiak FastQC analisiko sekuentzien lauza bakoitzeko kalitatearen emaitzak erakusten ditu ChIP-seq esperimentuko laginetan.



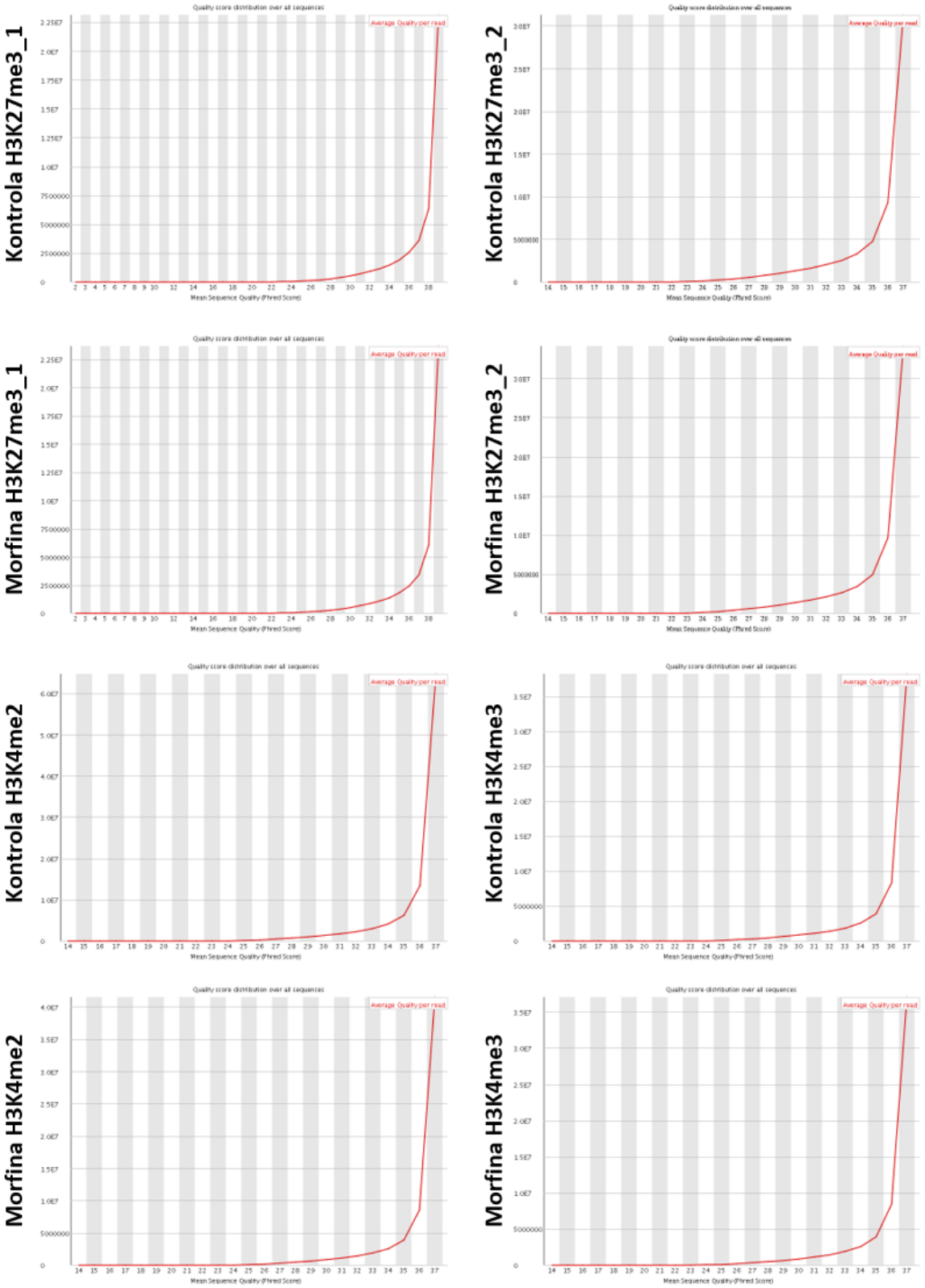
2.8. Irudia. FastQC "sekuentzien lauza bakoitzeko kalitatea" moduluaren emaitzak. Irudiak FastQC analisiko sekuentzien lauza bakoitzeko kalitatearen emaitzak erakusten ditu (A) RNA-seq esperimentuko L4-an sekuentziatu ziren laginetan eta,

Orri oinaren jarraipena hurrengo orrialdean



(B) RNA-seq esperimentuko L5-an sekuentziatu ziren laginetan zehar.

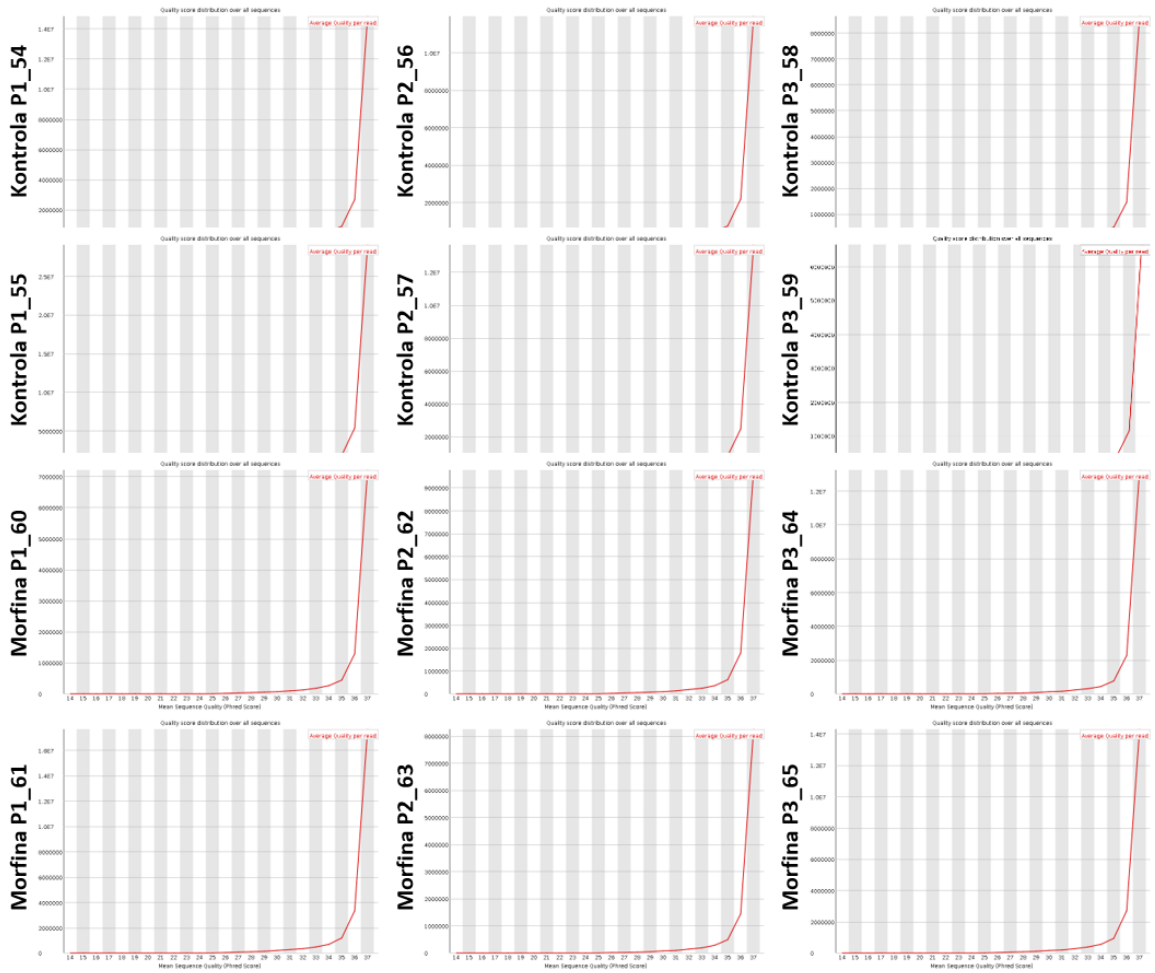
- Sekuentzien bakoitzeko kalitatezko balioak atalari dagozkien irudiak



2.9. Irudia. FastQC "sekuentzia bakoitzeko kalitatezko balioak" moduluaren emaitzak. Irudiak FastQC analisiko sekuentzia bakoitzeko kalitatezko balioak erakusten ditu ChIP-seq esperimentuko laginetan.

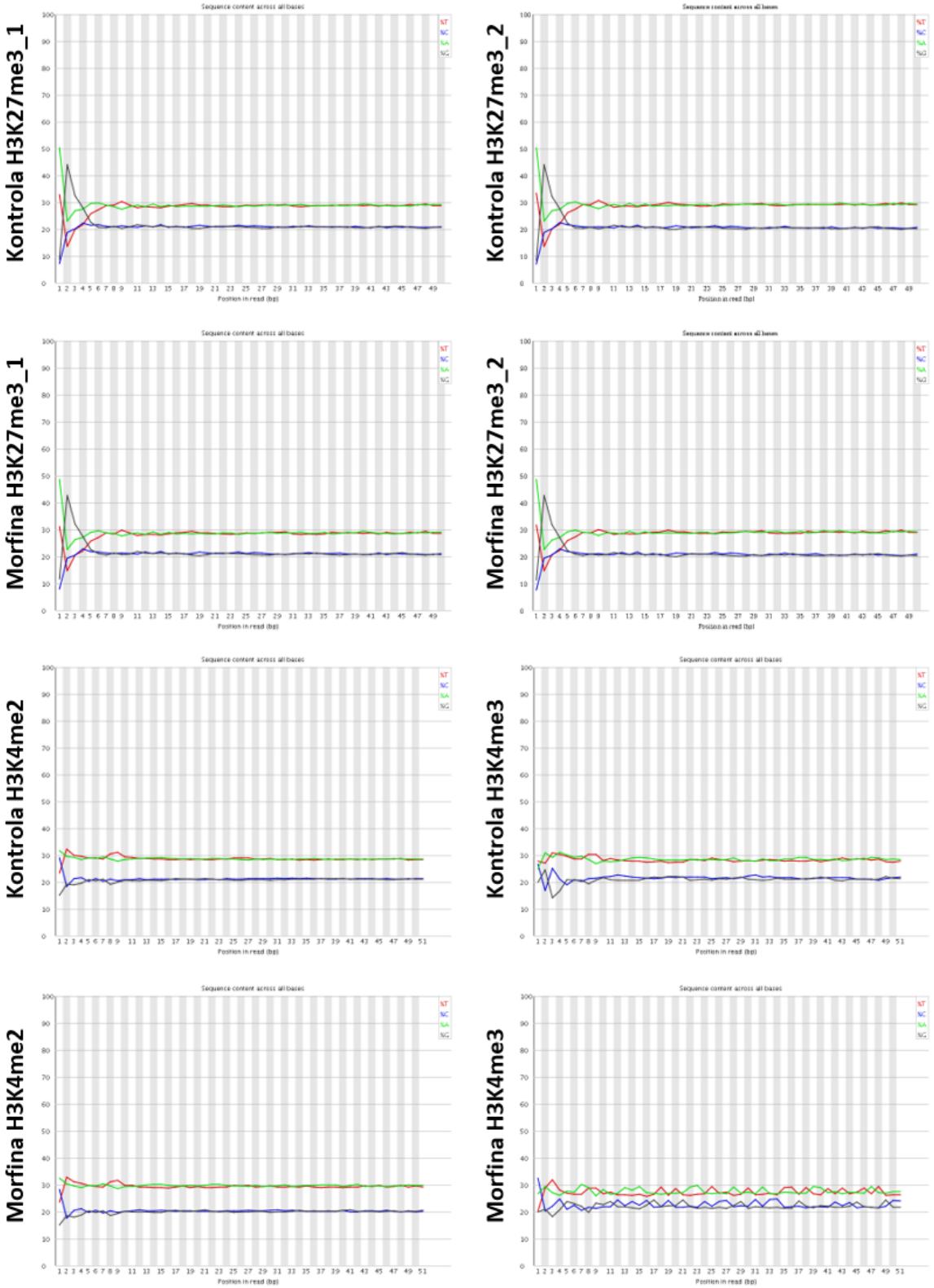
2.10. Irudia. FastQC "sekuentzia bakoitzeko kalitatezko balioak" moduluaren emaitzak. Irudiak FastQC analisiko sekuentzia bakoitzeko kalitatezko balioen emaitzak erakusten ditu (A) RNA-seq esperimentuko L4-an sekuentziatu ziren laginetan eta,

Orri oinaren jarraipena hurrengo orrialdean

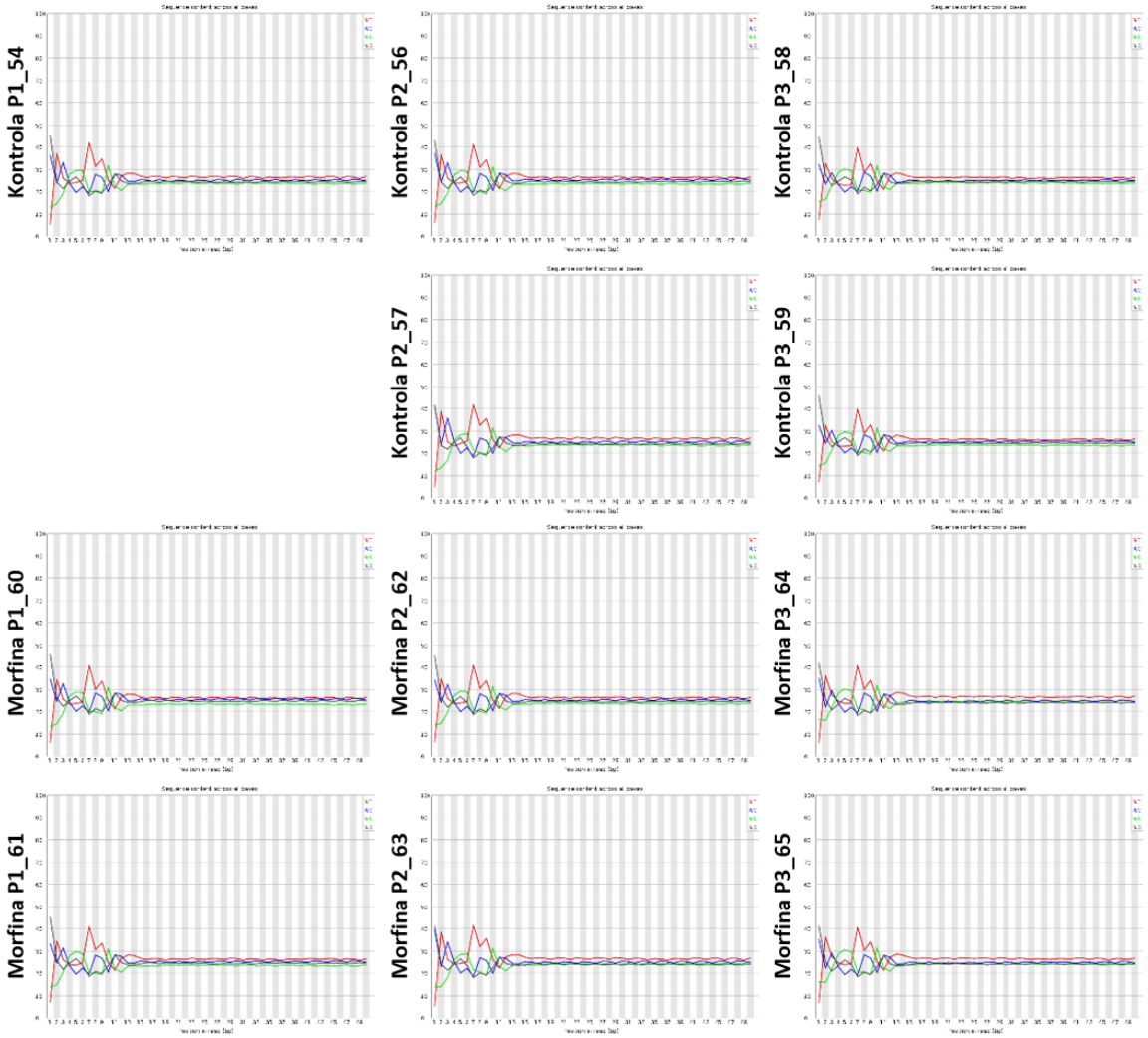


(B) RNA-seq esperimentuko L5-an sekuentziatu ziren laginetan zehar.

- Sekuentzia bakoitzeko base edukia atalari dagozkien irudiak

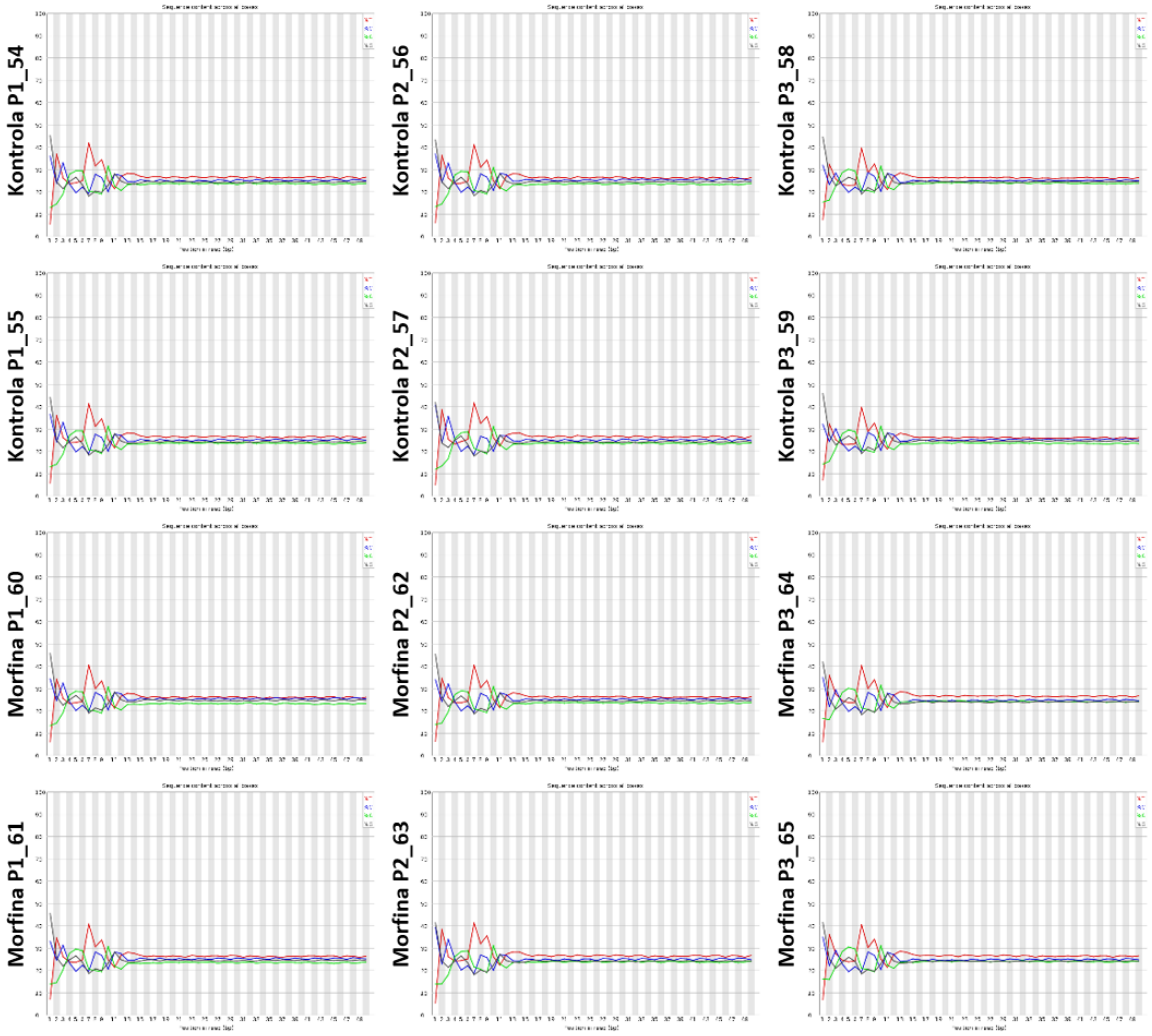


2.11. Irudia. FastQC "sekuentzia bakoitzeko base edukia" moduluaren emaitza. Irudiak FastQC analisiko sekuentzia bakoitzeko base edukia erakusten du ChIP-seq esperimentuko laginetan.



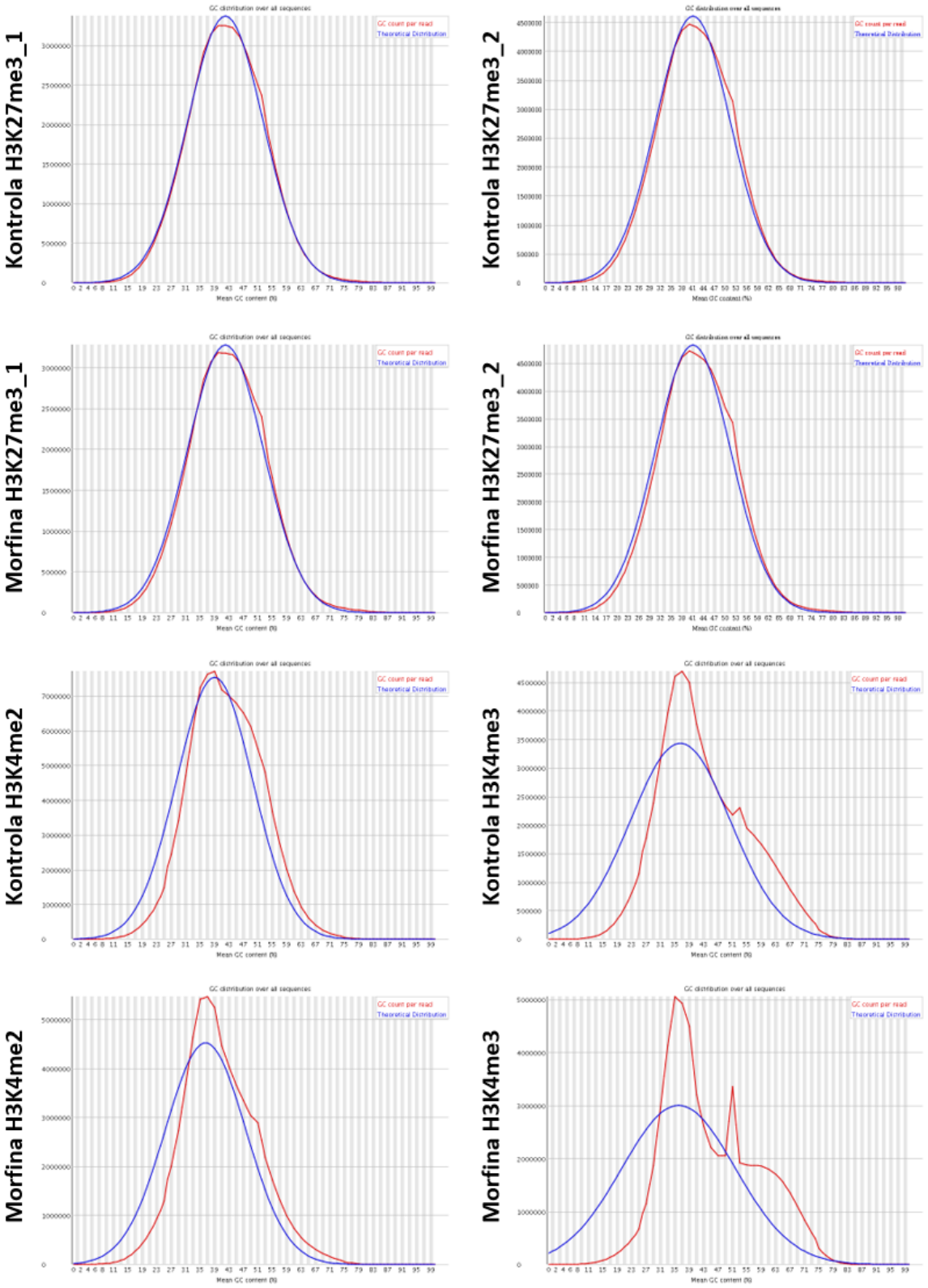
2.12. Irudia. FastQC "sekuentzia bakoitzeko base edukia" moduluaren emaitzak. Irudiak FastQC analisiko sekuentzia bakoitzeko base edukiaren emaitzak erakusten ditu (A) RNA-seq esperimentuko L4-an sekuentziatu ziren laginetan eta,

Orri oinaren jarraipena hurrengo orrialdean

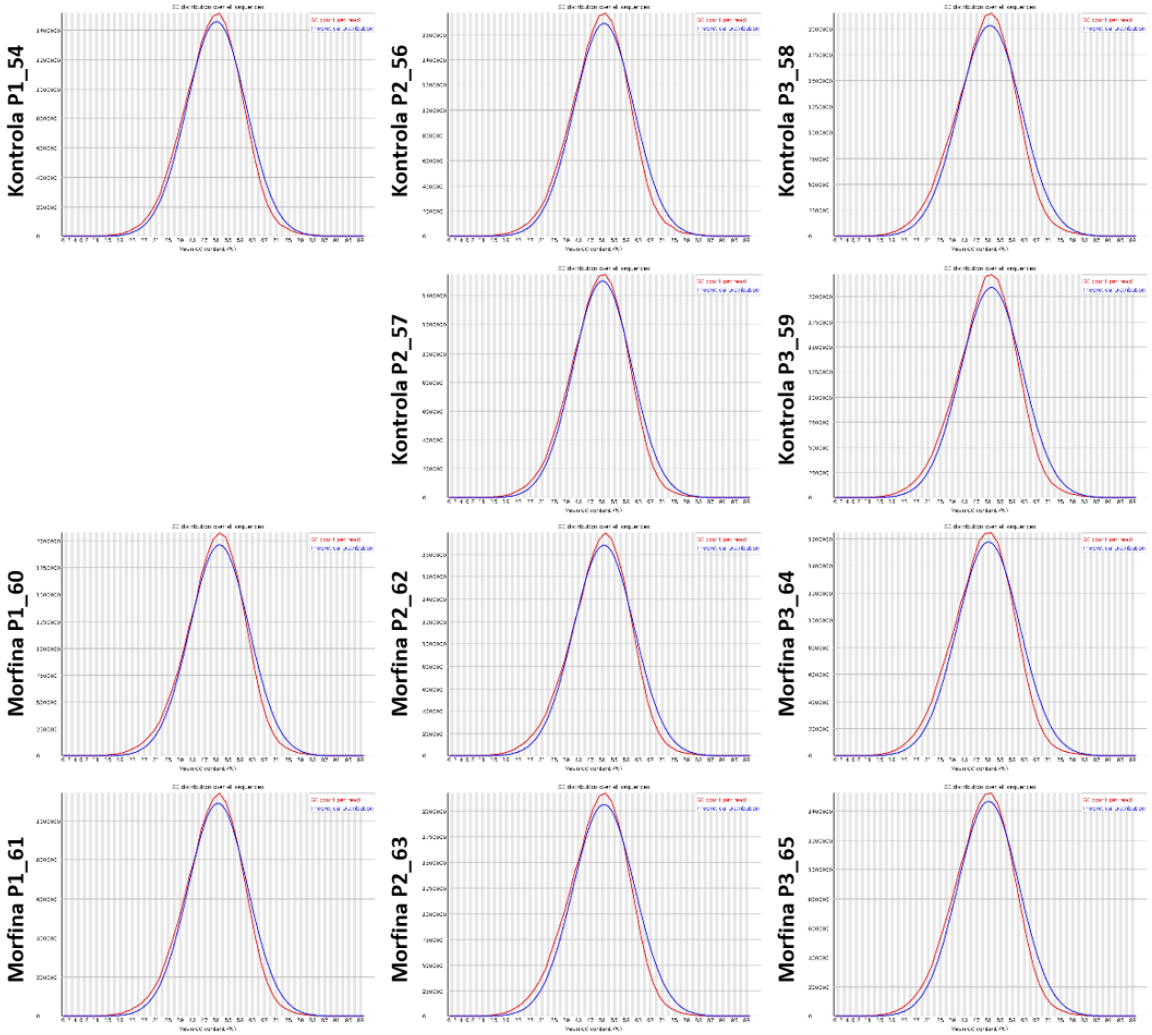


(B) RNA-seq esperimentuko L5-an sekuentziatu ziren laginetan zehar.

- Sekuentzia bakoitzeko GC edukia atalari dagozkien irudiak

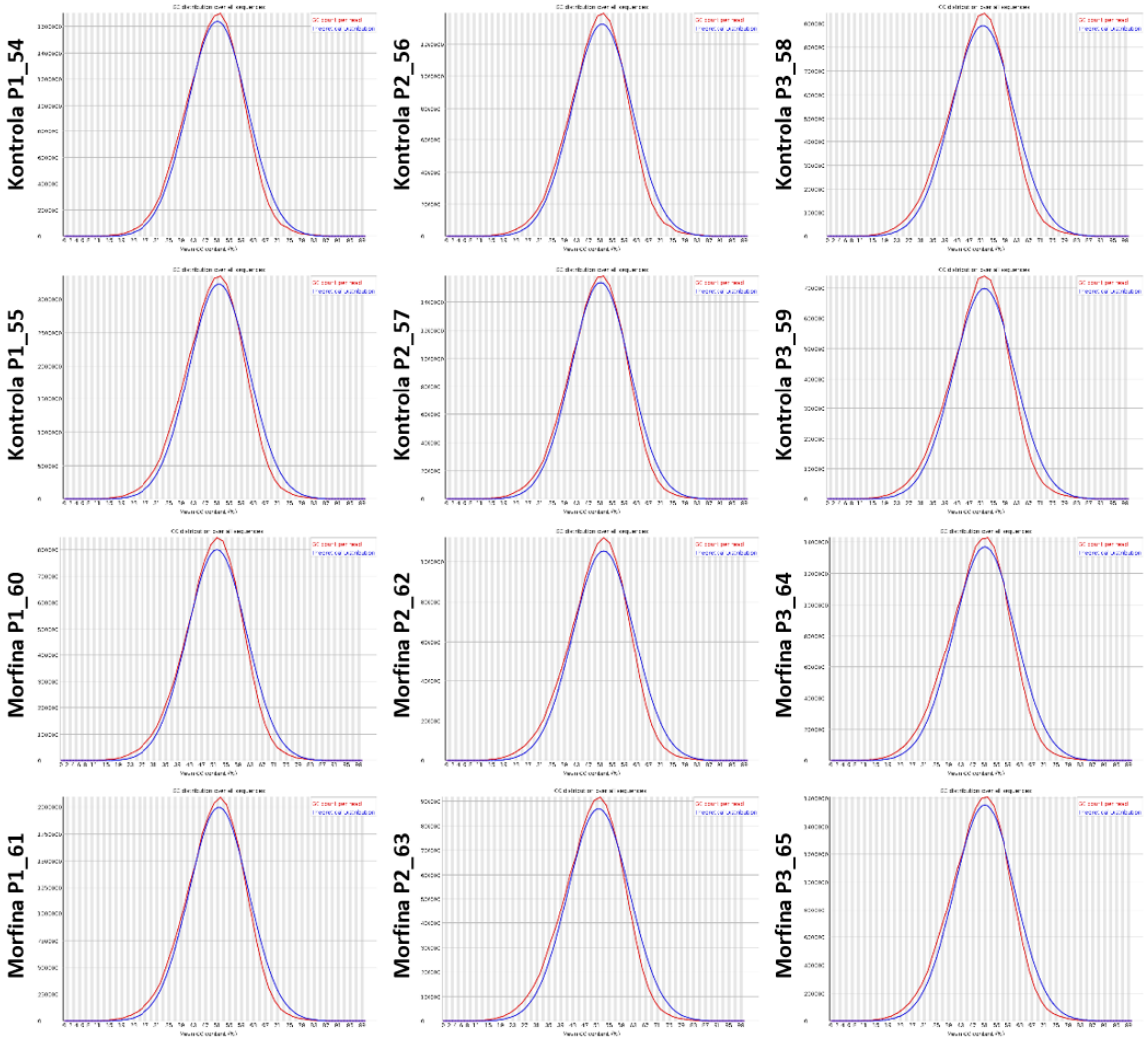


2.13. Irudia. FastQC "sekuentzia bakoitzeko GC edukia" moduluaren emaitza. Irudiak FastQC analisiko sekuentzia bakoitzeko GC edukia erakusten du ChIP-seq esperimentuko laginetan.



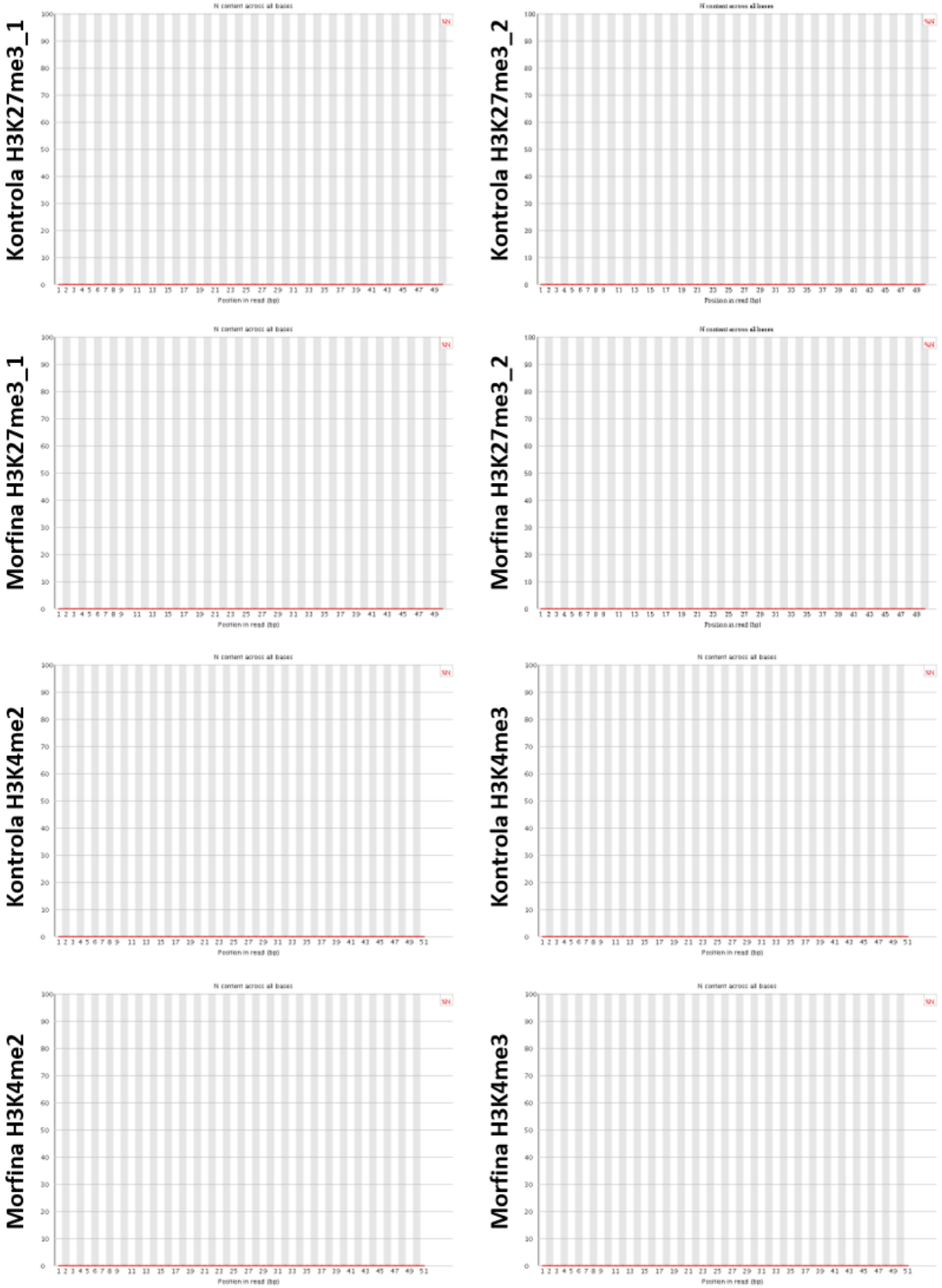
2.14. Irudia. FastQC "sekuentzia bakoitzeko GC edukia" moduluaren emaitzak. Irudiak FastQC analisiko sekuentzia bakoitzeko GC edukiaren emaitzak erakusten ditu (A) RNA-seq esperimentuko L4-an sekuentziatu ziren laginetan eta,

Orri oinaren jarraipena hurrengo orrialdean



(B) RNA-seq esperimentuko L5-an sekuentziatu ziren laginetan zehar.

- Base bakoitzeko N edukia atalari dagozkien irudiak



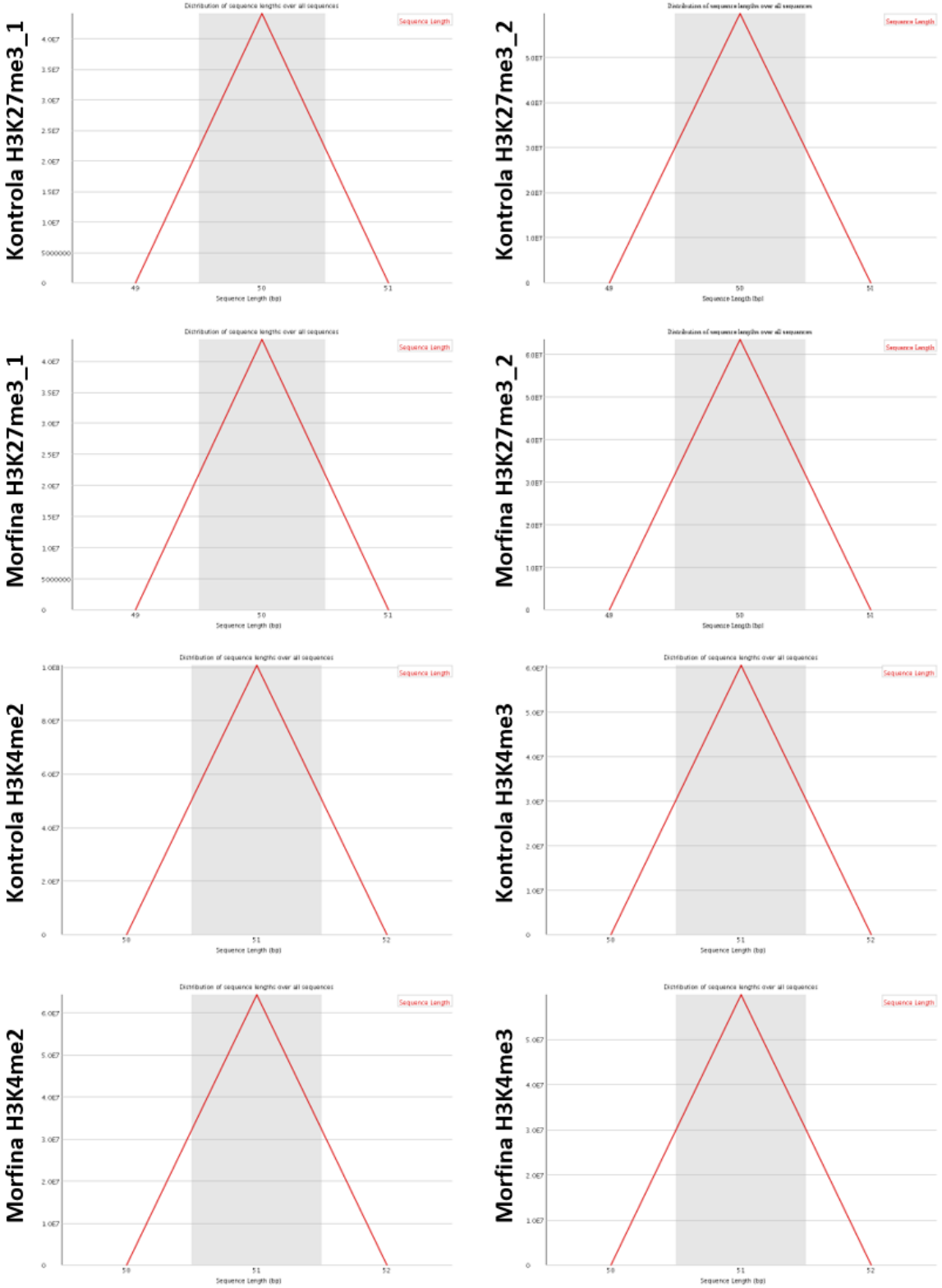
2.15. Irudia. FastQC "base bakoitzeko N edukia" moduluaren emaitzak. Irudiak FastQC analisiko base bakoitzeko N edukiaren emaitzak erakusten ditu ChIP-seq esperimentuan sekuentziatu ziren laginetan zehar,

2.16. Irudia. FastQC "base bakoitzeko N edukia" moduluaren emaitzak. Irudiak FastQC analisiko base bakoitzeko N edukiaren emaitzak erakusten ditu (A) RNA-seq esperimentuko L4-an sekuentziatu ziren laginetan eta,

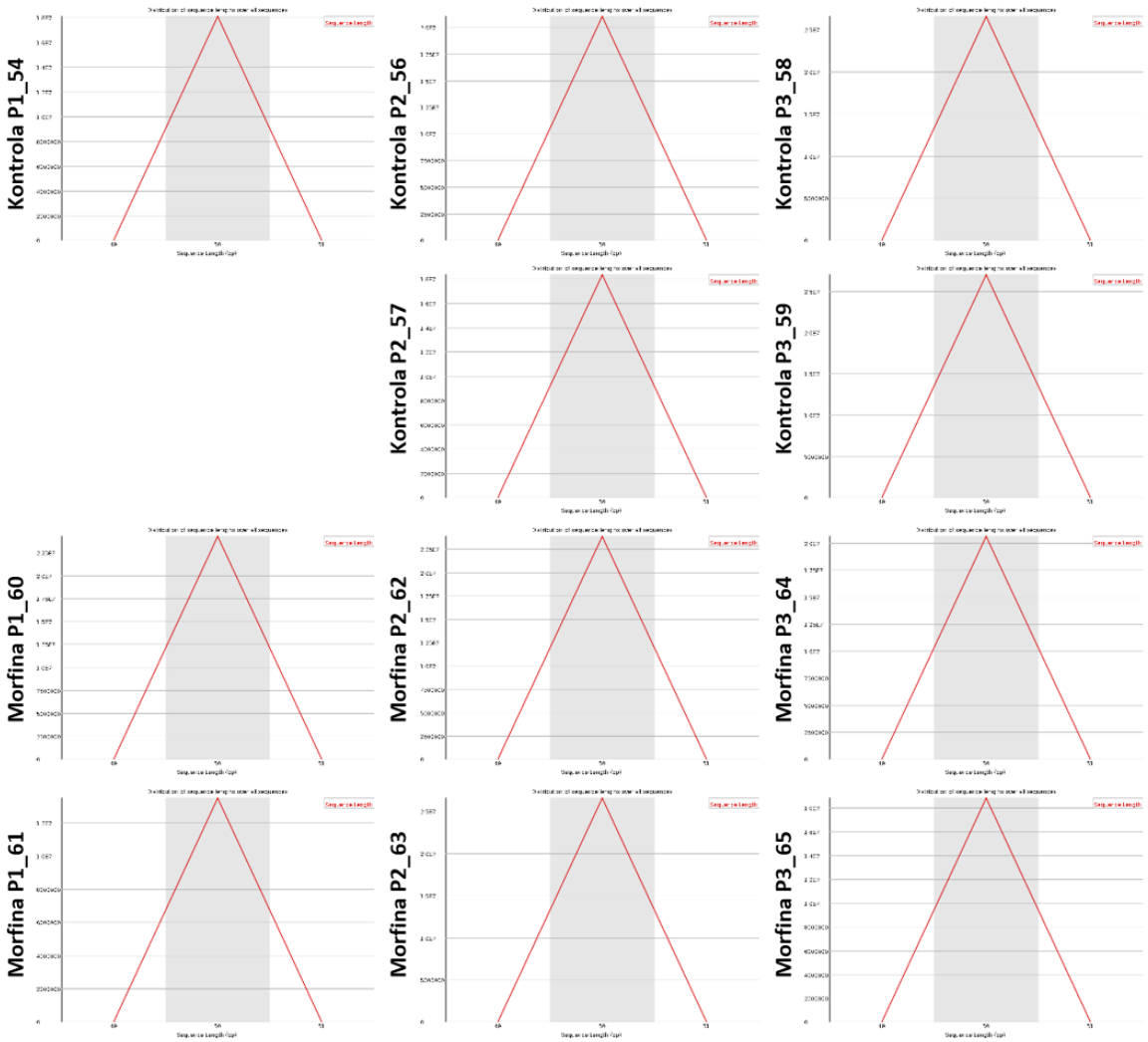
Orri oinaren jarraipena hurrengo orrialdean

(B) RNA-seq esperimentuko L5-an sekuentziatu ziren laginetan zehar.

- Sekuentzia luzeeraren distribuzioa atalari dagozkien irudiak

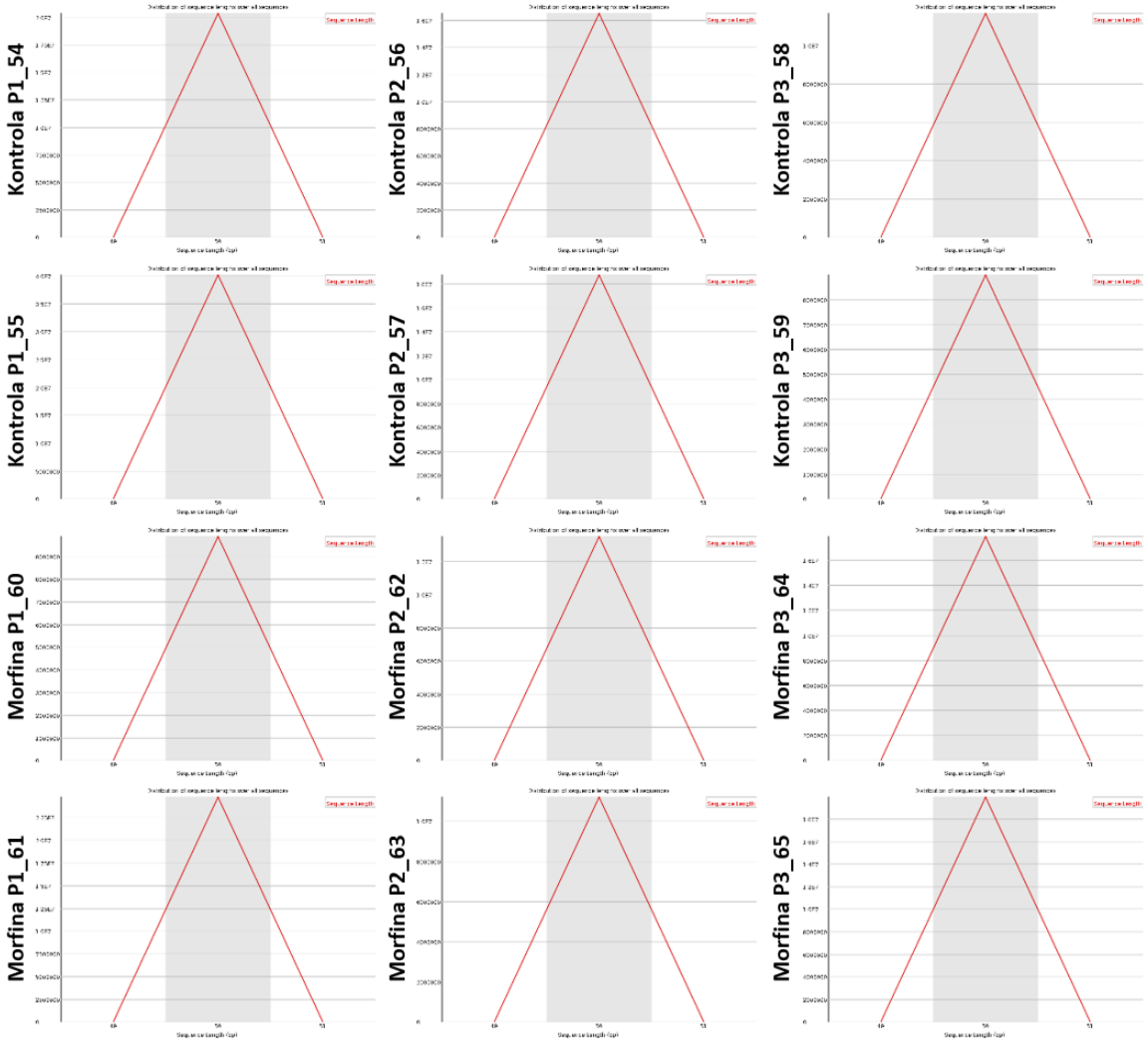


2.17. Irudia. FastQC "sekuentzien luzeraren distribuzioa" moduluaren emaitzak. Irudiak FastQC analisiko sekuentzien luzeraren distribuzioaren emaitzak erakusten ditu ChIP-seq esperimentuan sekuentziatu ziren laginetan zehar,



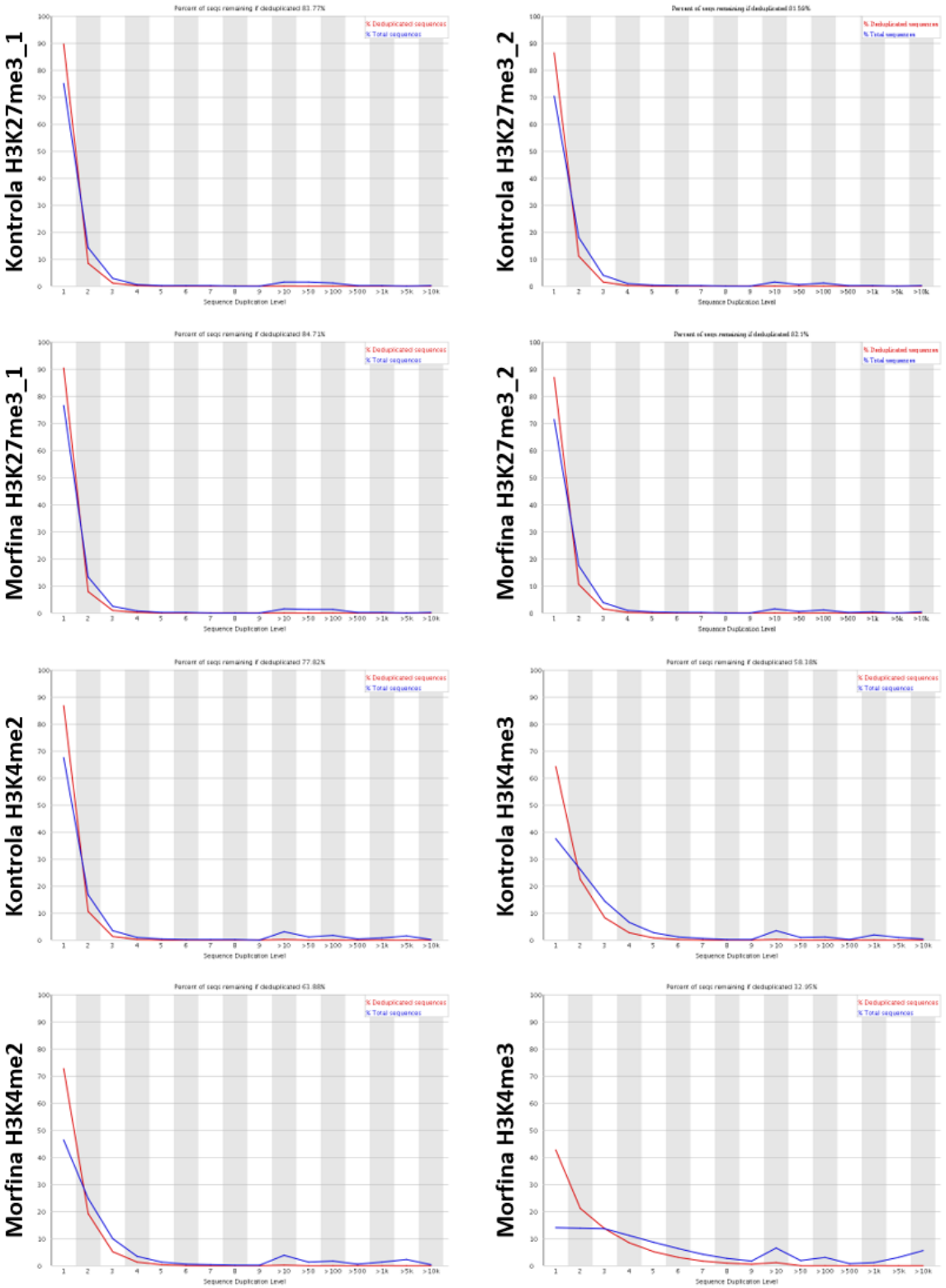
2.18. Irudia. FastQC "sekuentzien luzeraren distribuzioa" moduluaren emaitzak. Irudiak FastQC analisiko sekuentzien luzeraren distribuzioaren emaitzak erakusten ditu (A) RNA-seq esperimentuko L4-an sekuentziatu ziren laginetan eta,

Orri oinaren jarraipena hurrengo orrialdean

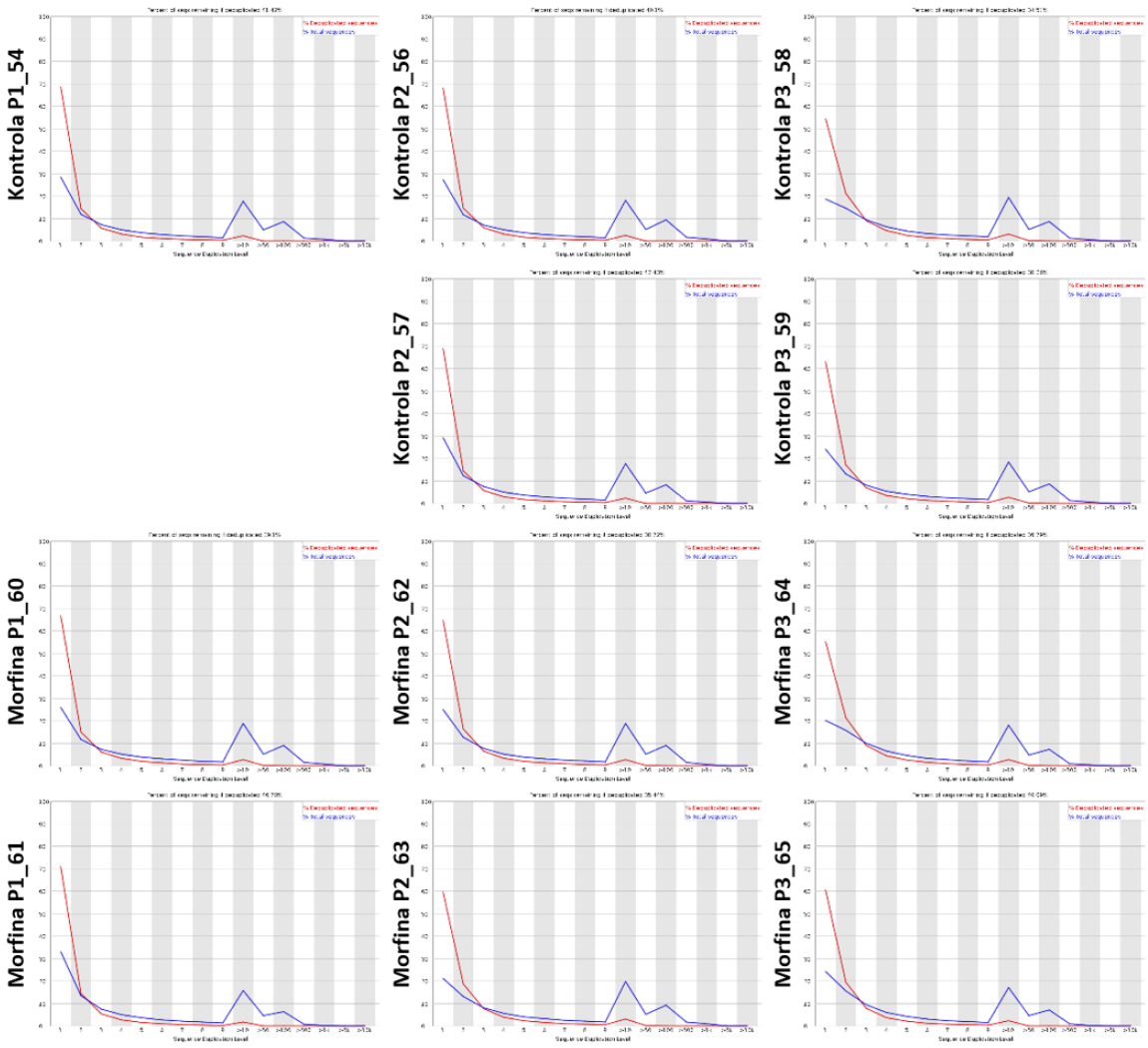


(B) RNA-seq esperimentuko L5-an sekuentziatu ziren laginetan zehar.

- Sekuentzia bikoizketa maila atalari dagozkien irudiak

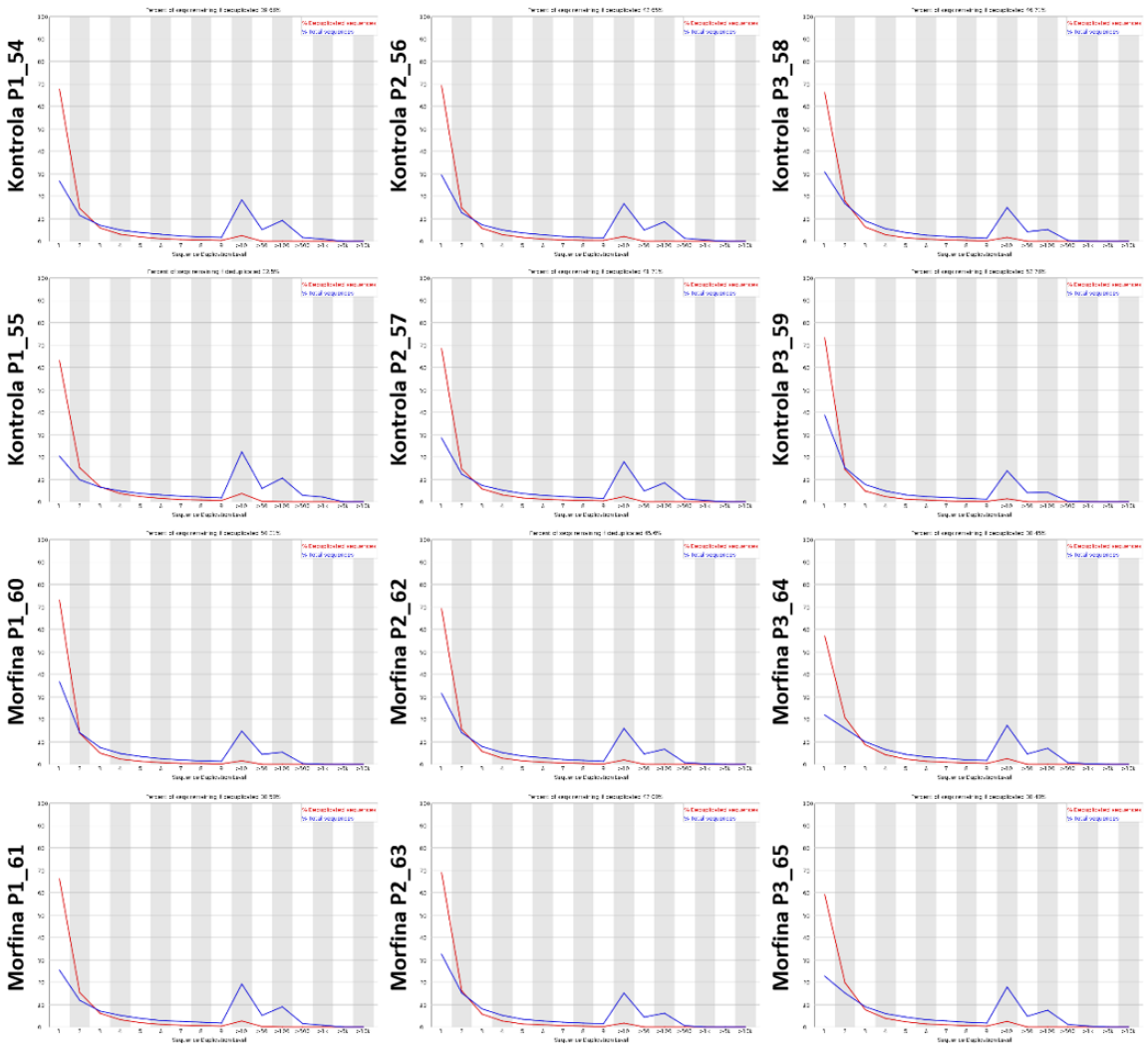


2.19. Irudia. FastQC "Sekuentzien bikoizketa maila" moduluaren emaitzak. Irudiak FastQC analisiko sekuentzien bikoizketa maila erakusten du ChIP-seq esperimentuko laginetan.



2.20. Irudia. FastQC "Sekuentzien bikoizketa maila" moduluaren emaitzak. Irudiak FastQC analisiko sekuentzien bikoizketa mailaren emaitzak erakusten ditu (A) RNA-seq esperimentuko L4-an sekuentziatu ziren laginetan zehar.

Oinaren jarraipena hurrengo orrialdean



(B) RNA-seq esperimentuko L5-an sekuentziatu ziren laginetan zehar.

- Gain-irudikatutako sekuentziak atalari dagozkien irudiak

Kontrola H3K27me3_1

Overrepresented sequences

Sequence	Count	Percentage	Possible Source
GATCGGAAGAGCACACGTCTGAACTCCAGTCACTTAGGCATCTCGTATGC	137185	0.31106555069599795	TruSeq Adapter, Index 3 (100% over 50bp)

Kontrola H3K27me3_2

Overrepresented sequences

Sequence	Count	Percentage	Possible Source
GATCGGAAGAGCACACGTCTGAACTCCAGTCACTTAGGCATCTCGTATGC	226061	0.37832221617181583	TruSeq Adapter, Index 3 (100% over 50bp)

Morfina H3K27me3_1

Overrepresented sequences

Sequence	Count	Percentage	Possible Source
GATCGGAAGAGCACACGTCTGAACTCCAGTCACGATCAGATCTCGTATGC	163504	0.3763227218676014	TruSeq Adapter, Index 9 (100% over 50bp)

Morfina H3K27me3_2

Overrepresented sequences

Sequence	Count	Percentage	Possible Source
GATCGGAAGAGCACACGTCTGAACTCCAGTCACGATCAGATCTCGTATGC	282120	0.44431257228820026	TruSeq Adapter, Index 9 (100% over 50bp)

Kontrola H3K4me2

Overrepresented sequences

Sequence	Count	Percentage	Possible Source
GATCGGAAGAGCACACGTCTGAACTCCAGTCACACAGTGATCTCGTATGCC	179100	0.17797326372548242	TruSeq Adapter, Index 5 (100% over 51bp)

Morfina H3K4me2

Overrepresented sequences

Sequence	Count	Percentage	Possible Source
GATCGGAAGAGCACACGTCTGAACTCCAGTCACGTGAAACGATCTCGTATG	223782	0.34797742069989984	TruSeq Adapter, Index 19 (97% over 40bp)

Kontrola H3K4me3

Overrepresented sequences

Sequence	Count	Percentage	Possible Source
GATCGGAAGAGCACACGTCTGAACTCCAGTCACGCCAATATCTCGTATGCC	239582	0.39617136102164063	TruSeq Adapter, Index 6 (100% over 51bp)

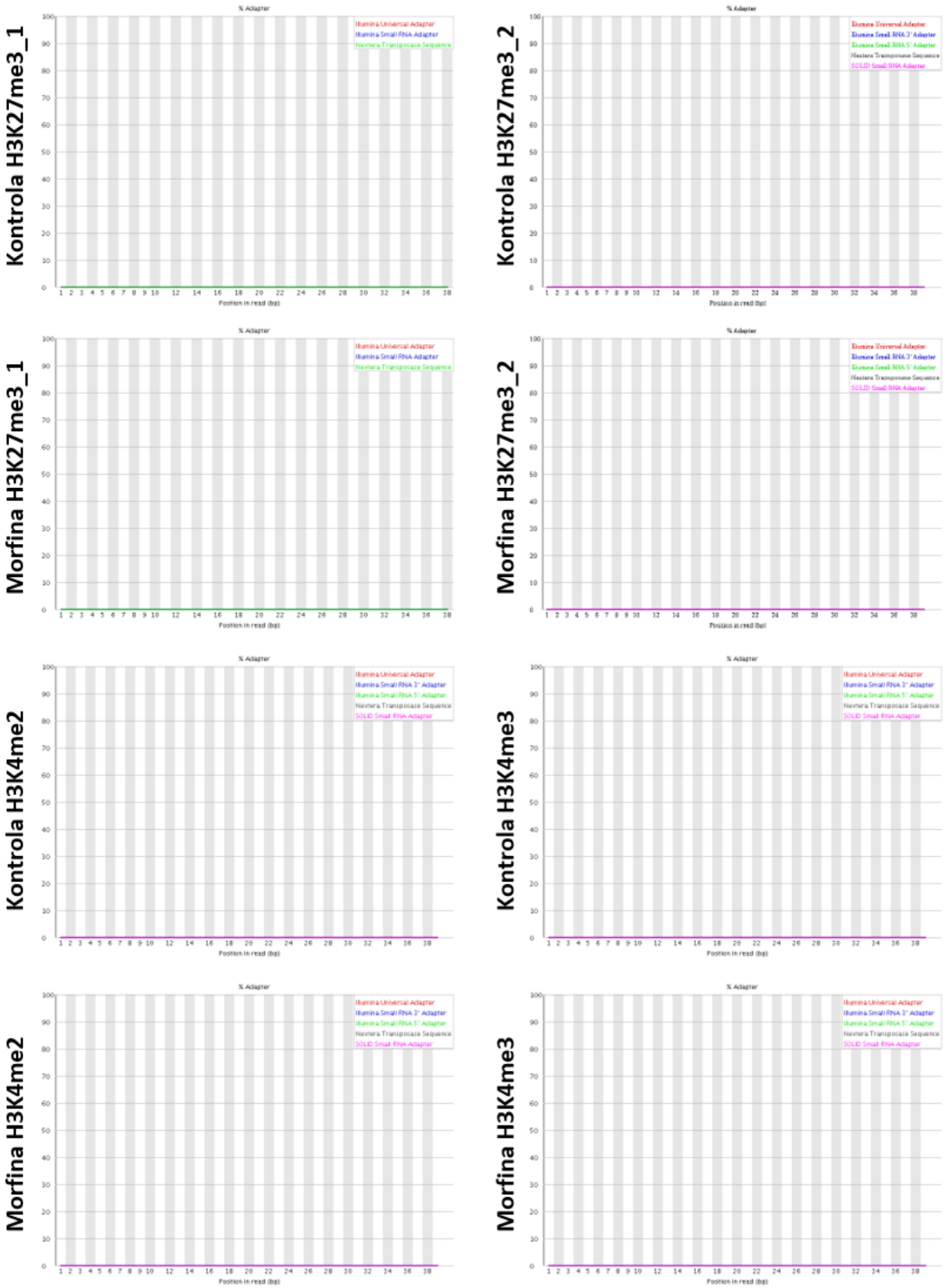
Morfina H3K4me3

Overrepresented sequences

Sequence	Count	Percentage	Possible Source
GATCGGAAGAGCACACGTCTGAACTCCAGTCACCTTGTAATCTCGTATGCC	1347789	2.2537302985526413	TruSeq Adapter, Index 12 (100% over 51bp

2.21. Irudia. FastQC "gain irudikatutako sekuentziak" moduluaren emaitzak. Taulan ageri diren nukleotidoz osaturiko zatiak, FastQC analisiak ChIP-seq esperimentuko laginetan detektaturiko gain-irudikatutako sekuentziak dira.

- Egokitzaileen edukia atalari dagozkien irudiak



2.22. Irudia. FastQC "egokitzaileen edukia" moduluaren emaitzak. Irudiak FastQC analisiko sekuentzien egokitzaileen edukia erakusten du ChIP-seq esperimentuko laginetan.

2.23. Irudia. FastQC "egokitzaileen edukia" moduluaren emaitzak. Irudiak FastQC analisiko sekuentzien egokitzaileen edukiaren emaitzak erakusten ditu (A) RNA-seq esperimentuko L4-an sekuentziatu ziren laginetan zehar.

Oinaren jarraipena hurrengo orrialdean

(B) RNA-seq esperimentuko L5-an sekuentziatu ziren laginetan zehar.

III. ERANSKINA

Egokitzaileen mozketaren emaitzak

- Behin egokitzaileak moztuta, prozesuaren txostenak (3.1. Taula) honako informazioa errazten du: laginen read kopurua, egokitzaileak zituzten read kopurua eta behin egokitzaileak moztuta, luzeera urria erakutsi zuten read-en kopurua.

3.1.Taula. Trim_Galore! tresnaren emaitzen laburpena. Egokitzailedun read kopurua adierazten da eta baita behin egokitzaileak moztuta, 50bp baino tamaina gutxiagoko read kopurua ere.

Fitxategia	Prozesaturiko Read kopurua	Egokitzailedun Read kopurua	Ezabatutako sekuentzia motzak
c_27me3_rep1	44101637	150685 (0.3%)	1451315 (3.3%)
m_27me3_rep1	43447815	179020 (0.4%)	1399063 (3.2%)
c_27me3_rep2	59753562	248740 (0.4%)	652232 (1.1%)
m_27me3_rep2	63495840	310679 (0.5%)	731609 (1.2%)
c_4me2_rep1	100633093	277941 (0.3%)	823238 (0.8%)
m_4me2_rep1	64309345	313288 (0.5%)	670817 (1.0%)
c_4me3_rep1	60474336	362085 (0.6%)	801100 (1.3%)
C1_rep1_L4	18087614	24679 (0.1%)	94517 (0.5%)
C2_rep1_L4	21029954	17520 (0.1%)	103210 (0.5%)
C2_rep2_L4	18349484	10135 (0.1%)	78399 (0.4%)
C3_rep1_L4	26576548	39666 (0.1%)	139753 (0.5%)
C3_rep2_L4	26985487	49616 (0.2%)	154559 (0.6%)
M1_rep1_L4	24249,350	17549 (0.1%)	113387 (0.5%)
M1_rep2_L4	13492328	14517 (0.1%)	71273 (0.5%)
M2_rep1_L4	23871820	21358 (0.1%)	116674 (0.5%)
M2_rep2_L4	26554998	15826 (0.1%)	115755 (0.4%)
M3_rep1_L4	20609037	37023 (0.2%)	111855 (0.5%)
M3_rep2_L4	18816073	19597 (0.1%)	95386 (0.5%)
C1_rep1_L5	20352544	26678 (0.1%)	213287 (1.0%)
C1_rep2_L5	40174883	23130 (0.1%)	389480 (1.0%)
C2_rep1_L5	16480100	13074 (0.1%)	171377 (1.0%)
C2_rep2_L5	18756151	10172 (0.1%)	174124 (0.9%)
C3_rep1_L5	11682198	16589 (0.1%)	121493 (1.0%)
C3_rep2_L5	8987682	15829 (0.2%)	98659 (1.1%)
M1_rep1_L5	9879080	6736 (0.1%)	99804 (1.0%)
M1_rep2_L5	24731812	24360 (0.1%)	264494 (1.1%)
M2_rep1_L5	13489117	11531 (0.1%)	137218 (1.0%)
M2_rep2_L5	11203542	6376 (0.1%)	103111 (0.9%)
M3_rep1_L5	17872149	30963 (0.2%)	182534 (1.0%)
M3_rep2_L5	19918967	19059 (0.1%)	202080 (1.0%)

- Honenbestez, egokitzaileak moztu ondoren egin zen FastQC analisiaren laburpen irudia hasierako FastQC irudiarekin alderatuz gero (3.1.Irudia, bakarrik H3K27me3-ren laginak azaltzen dira), ikus dezakegu nola, behin egokitzaileak moztuta “gain-irudikatutako sekuentziak” atalean genuen zeinu gorria desagertu eta modulu horren ebaluazioa emaitza arrunt bihurtu zen.

3.1.Irudia. Egokitzaileak moztu ondorengo FastQC analisia. Irudiak FastQC analisiko laburpen moduluaren emaitzen adibide bat erakusten da H3K27me3 laginetan, behin egokitzaileak moztu ondoren. Gain-irudikatutako sekuentziak atala emaitza arrunt bezala ageri da.

IV.ERANSKINA

ChIP-seq laginen analisi espezifikoaren datuak

- Behin lerrokatzearen prozesua bukatuta, programak laburpen txostena sortu zuen (4.1. Taula) honako informazioa erraztuz: lerrokatzeak erabili dituen read kopuruak, 0 aldiz, behin eta behin baino gehiagotan lerrokatu ziren read-ak eta lerrokatze proportzio orokorra azaltzen dira zehaztuta, lagin bakoitzeko.

4.1.Taula. Bowtie2 tresnaren lerrokatze emaitzak laburtzen dira. Lerrokatzea burutu duten read kopurua, 0 aldiz, behin eta behin baino gehiagotan lerrokatu diren read-ak eta lerrokatze proportzio orokorrak adierazten dira.

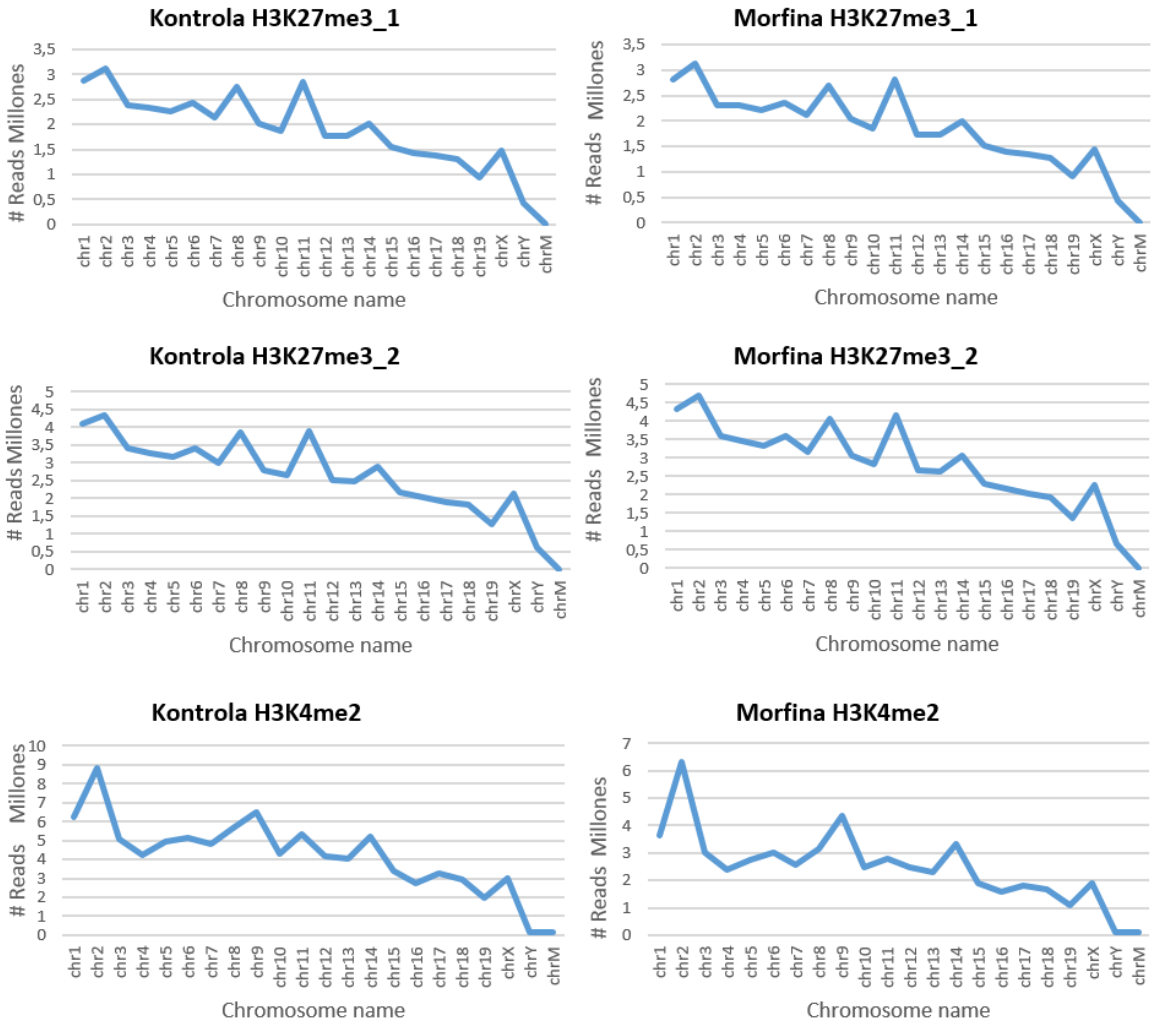
Fitxategia	Read kopurua	0 aldiz lerrokatuak	1 aldiz lerrokatuak	>1 aldiz lerrokatuak	Lerrokatze maila osoa
c_27me3_rep1	42650322	1322882 (3.10%)	32707145 (76.69%)	8620295 (20.21%)	96.90%
m_27me3_rep1	42048752	1316566 (3.13%)	31948253 (75.98%)	8783933 (20.89%)	96.87%
c_27me3_rep2	59101330	975671 (1.65%)	45549025 (77.07%)	12576634 (21.28%)	98.35%
m_27me3_rep2	62764231	1027650 (1.64%)	47930017 (76.37%	13806564 (22.00%)	98.36%
c_4me2_rep1	99809855	5261824 (5.27%)	66156365 (66.28%)	28391666 (28.45%)	94.73%
m_4me2_rep1	63638528	6835723 (10.74%)	37290022 (58.60%)	19512783 (30.66%)	89.26%
c_4me3_rep1	59673236	46554230 (78.02%)	4874749 (8.17%)	8244257 (13.82%)	21.98%
m_4me3_rep1	57665533	32027613 (55.54%)	8414855 (14.59%)	17223065 (29.87%)	44.46%

- .sorted.bam fitxategitik eratorritako datu estatistikoak ondorengo taulan azaltzen dira (4.2.Taula). Aurreko datuekin alderatu daitezke, lerrokatze mailak %96-97 balioetan mantentzen direla ikusteko.

4.2.Taula. .sorted.bam fitxategitik eratorritako lerrokatzearen emaitza estatistikoak. Lerrokatzea burutu duten read kopurua, lerrokatutako read-ak eta lerrokatze maila orokorra adierazten dira.

FITXATEGIA	READ KOPURUA	LERROKATUTAKO READ-AK	LERROKATZE MAILA
c_27me3_rep1	42650322	41327440	96,90%
m_27me3_rep1	42048752	40732186	96,87%
c_27me3_rep2	59101330	58125659	98,35%
m_27me3_rep2	62764231	61736581	98,36%
c_4me2_rep1	99809855	94548031	94,73%
m_4me2_rep1	63638528	56802805	89,26%
c_4me3_rep1	59673236	13119006	21,98%
m_4me3_rep1	57665533	25637920	44,46%

- .sorted.bai fitxategitik eratorritako .txt fitxategiko emaitzekin kromosoma bakoitzeko read kopurua irudikatzen zuen grafikoa egin genuen, kromosoma guztietan zehar hainbat milioiko read-en aberastea genuela baieztatuz (4.1. Irudia).



4.1.Irudia. .sorted.bai fitxategitik eratorritako lerrokatzearen emaitzak. Kromosoma bakoitzean lerrokatzea burutu duten read kopurua adierazten da.

- Picard programaren lerrokatzearen inguruko datu estatistikoek (4.3. Taula), read kopurua, lerrokatze read-ak, lerrokatze maila, desdoitze maila (erreferentziazko genomarekin bat ez datorren base kopuruaren tasa), errore maila (lerrokaturiko read-etatik erreferentziazko genomarekin bat ez datozen base kopurua) eta INDEL maila (txertatze eta ezabatze gertaeren kopurua lerrokatutako 100 baseko). Guzti hauek maila oso baxuetan aurkitu genituen, beraz, ondorioztatu genuen gure sekuentziak era egokian lerrokatu zirela erreferentziazko genomara.

4.3. Taula. Picard tresnaren bidez lortutako lerrokatzearen emaitza estatistikoak. Lerrokatzea burutu duten read kopurua, lerrokatutako read-ak, lerrokatze maila orokorra , desdoitze maila, errore maila eta indel maila adierazten dira.

FITXATEGIA	READ KOPURUA	LERROKATZE READ-AK	LERROKATZE MAILA	DESDOITZE MAILA	ERRORE MAILA	INDEL MAILA
c_27me3_rep1	42650322	41327440	0.968983	0.002962	0.00211	0.000256
m_27me3_rep1	42048752	40732186	0.96869	0.003032	0.002095	0.000258
c_27me3_rep2	59101330	58125659	0.983492	0.003348	0.002498	0.000269
m_27me3_rep2	62764231	61736581	0.983627	0.003413	0.002473	0.000271
c_4me2_rep1	99809855	94548031	0.947282	0.005394	0.002754	0.000314
m_4me2_rep1	63638528	56802805	0.892585	0.006425	0.002649	0.000358

V. ERANSKINA

RNA-seq laginen analisi espezifikoaren datuak

- Behin lerrokatzearen prozesua bukatuta, programak laburpen txostena sortu zuen (5.1. Taula) honako informazioa erraztuz: lerrokatzeak erabili dituen read kopuruak, 0 aldiz, behin eta behin baino gehiagotan lerrokatu ziren read-ak eta lerrokatze proportzio orokorra azaltzen dira zehaztuta, lagin bakoitzeko.

5.1. Taula. hisat2 tresnaren lerrokatze emaitzak laburtzen dira. Lerrokatzea burutu duten read kopurua, 0 aldiz, behin eta behin baino gehiagotan lerrokatu diren read-ak eta lerrokatze proportzio orokorrak adierazten dira.

Fitxategia	Read kopurua	0 aldiz lerrokatuak	1 aldiz lerrokatuak	>1 aldiz lerrokatuak	Lerrokatze maila osoa
C1_rep1_L4	17993097	1170052 (6.50%)	13541823 (75.26%)	3281222 (18.24%)	93.50%
C2_rep1_L4	20926744	1429856 (6.83%)	15635077 (74.71%)	3861811 (18.45%)	93.17%
C2_ rep2_L4	18271085	1200198 (6.5%)	13778635 (75.41%)	3292252 (18.02%)	93.43%
C3_ rep1_L4	26436795	1635668 (6.19%)	20111152 (76.07%)	4689975 (17.74%)	93.81%
C3_ rep2_L4	26830928	1641811 (6.12%)	20426707 (76.13%)	4762410 (17.75%)	93.88%
M1_ rep1_L4	24135963	1456011 (6.03%)	18362222 (76.08%)	4317730 (17.89%)	93.97%
M1_ rep2_L4	13421055	845504 (6.30%)	10172029 (75.79%)	2403522 (17.91%)	93.70%
M2_ rep1_L4	23755146	1530284 (6.44%)	17836552 (75.09%)	4388310 (18.47%)	93.56%
M2_ rep2_L4	26439243	1706849 (6.46%)	19850860 (75.08%)	4881534 (18.46%)	93.54%
M3_ rep1_L4	20497182	1290302 (6.30%)	15573012 (75.98%)	3633868 (17.73%)	93.70%
M3_ rep2_L4	18720687	1178874 (6.30%)	14215448 (75.93%)	326365 (17.77%)	93.70%
C1_rep1_L5	20139257	1163309 (5.78%)	15270928 (75.83%)	3705020 (18.40%)	94.22%
C1_ rep2_L5	39785403	2118175 (5.32%)	30461529 (76.56%)	7205699 (18.11%)	94.68%
C2_rep1_L5	16308723	1030383 (6.32%)	12249078 (75.11%)	3029262 (18.57%)	93.68%
C2_ rep2_L5	18582027	1097895 (5.91%)	14103911 (75.90%)	3380221 (18.19%)	94.09%
C3_ rep1_L5	11560705	692188 (5.99%)	8799841 (76.12%)	2068676 (17.89%)	94.01%
C3_ rep2_L5	8889023	539517 (6.07%)	6758928 (76.04%)	1590578 (17.89%)	93.93%
M1_ rep1_L5	9779276	569643 (5.83%)	7442862 (76.11%)	1766771 (18.07%)	94.17%
M1_ rep2_L5	24467318	1308015 (5.35%)	18749139 (76.63%)	4410164 (18.02%)	94.65%

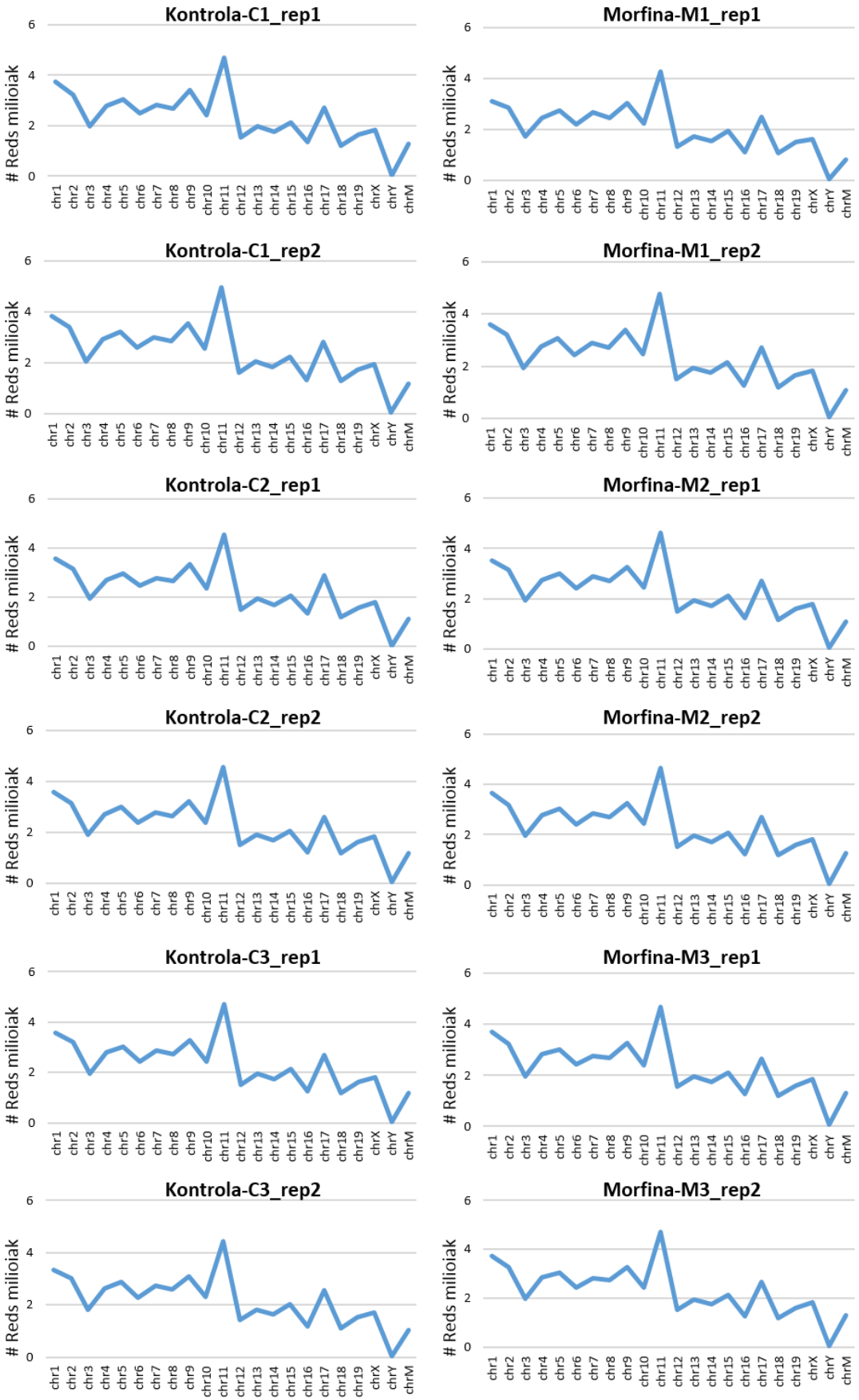
M2_ rep1_L5	13351899	811297 (6.08%)	10057002 (75.32%)	2483600 (18.60%)	93.92%
M2_ rep2_L5	11100431	691075 (6.23%)	8340628 (75.14%)	2068728 (18.64%)	93.77%
M3_ rep1_L5	17689615	1014160 (5.73%)	13515150 (76.40%)	3160305 (17.87%)	94.27%
M3_ rep2_L5	19716887	1112341 (5.64%)	15075804 (76.46%)	3528742 (17.90%)	94.36%

- Erreplika bakoitzean nahastutako bi lane-etako datuekin eratutako .sorted.bam fitxategien lerrokatzetik eratorritako datu estatistikoak ondorengo taulan (5.2.Taula) adierazten dira, bi lane-tako informazioa nahasteak lerrokatze maila altua mantentzen dela azalduz.

5.2. Taula. .sorted.bam fitxategitik eratorritako lerrokatzearen emaitza estatistikoak. Lerrokatzea burutu duten read kopurua, lerrokatutako read-ak eta lerrokatze maila orokorra adierazten dira.

FITXATEGIA	READ KOPURUA	LERROKATUTAKO READ-AK	LERROKATZE MAILA
C-P1_54	53074566	50741205	%95.60
C-P1_55	55241486	53123311	%96.16
C-P2_56	52079818	49619579	%95.27
C-P2_57	51216827	48918734	%95.51
C-P3_58	52454700	50126844	%95.56
C-P3_59	49373444	47192116	%95.58
M-P1_60	47133115	45107461	%95.70
M-P1_61	52542578	50389059	%95.90
M-P2_62	51981412	49639831	%95.49
M-P2_63	52430078	50032154	%95.43
M-P3_64	52609002	50304540	%95.62
M-P3_65	52998770	50707555	%95.68

- .sorted.bai fitxategitik eratorritako .txt fitxategiko emaitzekin kromosoma bakoitzeko read kopurua irudikatzen zuen grafikoa egin genuen, kromosoma guztietan zehar hainbat milioiko read-en estaldura genuela baieztatuz (5.1. Irudia).



5.1. Irudia. .sorted.bai fitxategietatik eratorritako lerrokatzearen emaitzak. Kromosoma bakoitzean lerrokatzea burutu duten read kopurua adierazten da.

- Picard programaren lerrokatzearen inguruko datu estatistikoek (5.3. Taula), read kopurua, lerrokatze read-ak, lerrokatze maila, desdoitze maila (erreferentziazko genomarekin bat ez datorren base kopuruaren tasa), errore maila (lerrokaturiko read-etatik erreferentziazko genomarekin bat ez datozen base kopurua) eta INDEL maila (txertatze eta ezabatze gertaeren kopurua lerrokatutako 100 baseko). Guzti hauek maila oso baxuetan aurkitu genituen, beraz, ondorioztatu genuen gure sekuentziak era egokian lerrokatu zirela erreferentziazko genomara.

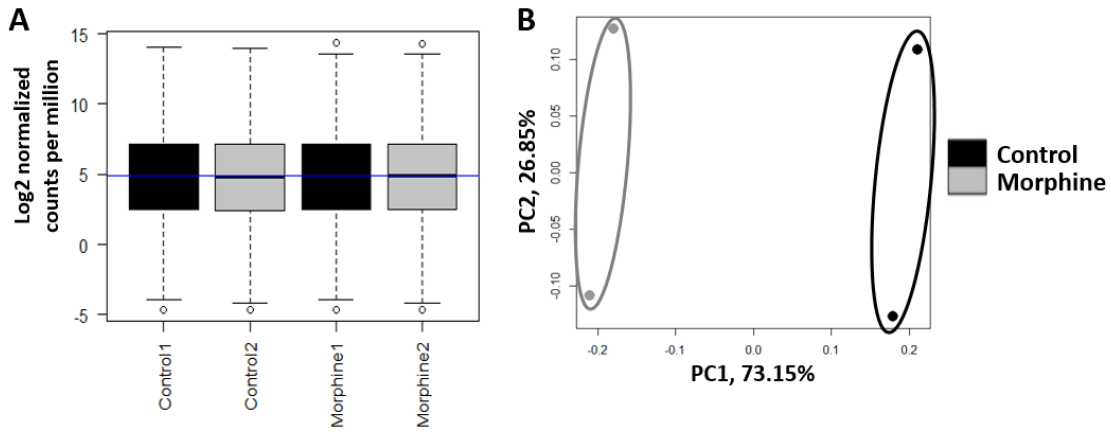
5.3.Taula. Picard tresnaren bidez lortutako lerrokatzearen emaitza estatistikoak. Lerrokatzea burutu duten read kopurua, lerrokatutako read-ak, lerrokatze maila orokorra , desdoitze maila, errore maila eta indel maila adierazten dira.

FITXATEGIA	READ KOPURUA	LERROKATZE READ-AK	LERROKATZE MAILA	DESDOITZE MAILA	ERRORE MAILA	INDEL MAILA
C-P1_54	38132354	35798993	0.938809	0.001228	0.001262	0.000016
C-P1_55	39785403	37667228	0.94676	0.000997	0.001027	0.000015
C-P2_56	37235467	34775228	0.933928	0.001318	0.001348	0.000019
C-P2_57	36853112	34555019	0.937642	0.001264	0.001299	0.000015
C-P3_58	37997500	35669644	0.938737	0.00134	0.001372	0.000018
C-P3_59	35719951	33538623	0.938933	0.00139	0.001421	0.000016
M-P1_60	33915239	31889585	0.940273	0.001369	0.001401	0.000015
M-P1_61	37888373	35734854	0.943161	0.001183	0.001215	0.000015
M-P2_62	37107045	34765464	0.936897	0.001341	0.001374	0.000016
M-P2_63	37539674	35141750	0.936123	0.00138	0.001414	0.000016
M-P3_64	38186797	35882335	0.939653	0.001267	0.001298	0.000018
M-P3_65	38437574	36146359	0.940391	0.001245	0.001277	0.000017

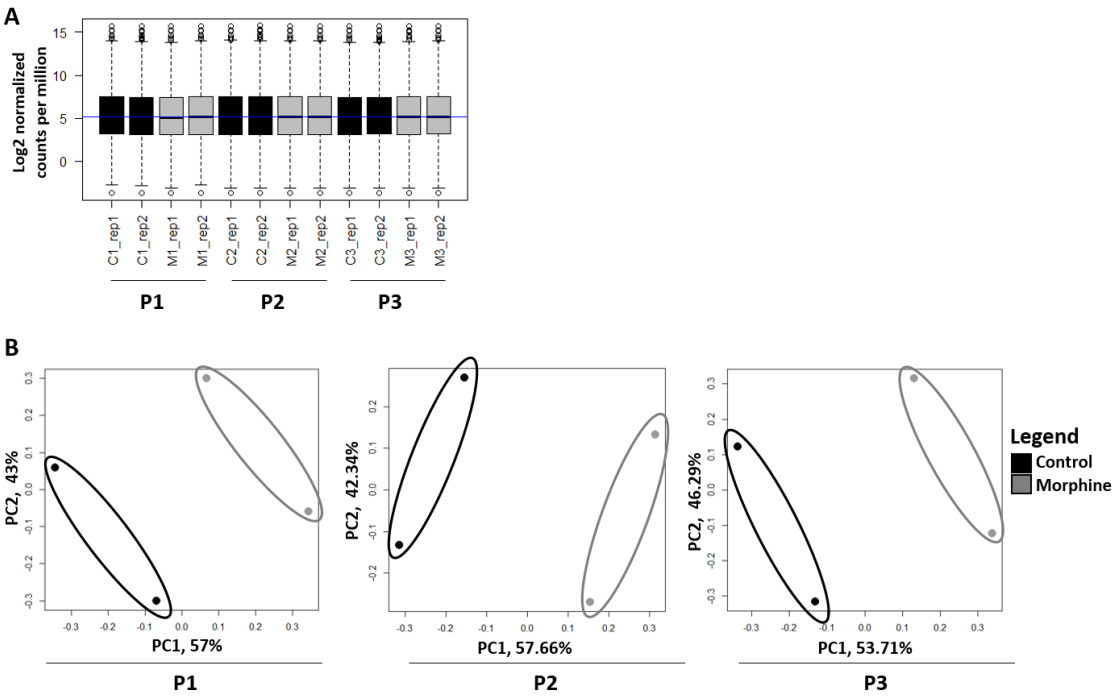
VI. ERANSKINA

Ezaugarri genomikoen izendatzeen irudiak

- ChIP-seq eta RNA-seq laginetan elementu errepikakorren analisiaren TMM normalizazioaren ondoren datuen distribuzioa normala zela (6.1.A. eta 6.2A Irudiak, ChIP-seq eta RNA-seq laginetan hurrenez hurren) eta PCA-ren bitartez konparaketa guztietan kontrol eta morfina laginen bereizketa garbia zela baieztatu zen (6.1.B eta 6.2.B Irudiak, ChIP-seq eta RNA-seq laginetan hurrenez hurren).



6.1. Irudia. ChIP-Seq-aren edgeR analisi estatistikoaren emaitzak elementu errepikakorretan. (A) Normalizatu gabeko eta (B) normalizatutako datuen distribuzioa, kutxa grafikoaren bidez. (C) Normalizatu abeko eta (D) normalizatutako PCA grafikoa.



6.2.Irudia. ChIP-Seq-aren edgeR analisi estatistikoaren emaitzak elementu errepikakorretan. (A) Normalizatu gabeko eta (B) normalizatutako datuen distribuzioa, kutxa grafikoaren bidez. (C) Normalizatu gabeko eta (D) normalizatutako PCA grafikoa.