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# ANALYSING THE CONTRIBUTION OF ATM/ATR PATHWAY ACTIVATION IN ESTABLISHING THE PREMATURE SENESCENCE OF E2F1/E2F2$^{-/-}$ BONE-MARROW-DERIVED MACROPHAGES

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# 1. Introduction

An aberrant cell cycle regulation can lead to pathological conditions such as tumorigenesis. For instance, it has been described a tight relationship between high levels of DNA replication that contribute to replicative stress and cancer development (Ekholm-Reed *et al.,* 2004; Hwang and Clurman, 2005). Both to avoid those potentially harmful processes and guarantee the correct physiology and function of a biological system, there is a homeostasic control during cell cycle. In this regard, under normal conditions when a cell is subjected to replicative stress, some alternatives pathways are induced. Senescence and apoptosis are some examples of these alternative mechanisms that regulate uncontrolled proliferation (Kastan and Bartek, 2004; Massagué, 2004).

An activation of those alternative pathways has been described in E2F1/E2F2$^{-/-}$ (double knockout or DKO from now onwards) mice. In this regard, Ana Zubiaga's research group has demonstrated that DKO bone-marrow-derived macrophages (BMDM) undergo an early rapid proliferation event related to DNA hyper-replication and have proved that this hyper-replication triggers a DNA damage response (DDR) pathway, shown by the accumulation of the phosphorylated form of the histone H2AX (γ-H2AX) among other markers in DKO cells (**Figure 1a**) (Iglesias *et al.,* 2010). In mammals, γ-H2AX protein is involved in the recruitment of repair or damage-signalling factors in response to DNA damage (Paull *et al.,* 2000). Moreover, this group described that the accumulation of γ-H2AX in DKO BMDM correlated with the deregulated accumulation of Mcm2 (**Figure 1b**), one of the six proteins that composes the catalytic core of the replicative helicase in eukaryotic cells. Apart from being a biological marker that indicates the active proliferative status of the cell (Chong *et al.*, 1996), Mcm2 is a direct target gene of E2F transcription factor. Therefore, it has being proposed that E2F1 and E2F2 act in this system as repressors of DNA hyper-replication or inhibitors of replicative stress (Iglesias *et al.*, 2010). Ultimately, the activation of the DDR pathway as a consequence of DNA hyper-replication culminates with DKO cells entering a premature senescent program, as it is shown by their senescent phenotype (**Figure 1c**) (Iglesias *et al.*, 2010). Senescence is defined as a permanent and irreversible state of cell cycle arrest in which cells are refractory to growth factor stimulation. Interestingly, the cell cycle arrest in DKO BMDM differs from the classical G1/S arrest, since the analysis of cell cycle distribution of DKO BMDM reveals that the arrest occurs in G2/M phase (**Figure 1d**) (Iglesias *et al.*, 2010). This type of arrest in G2/M phase has also been described in cells that undergo senescence upon oncogenic activation (Olsen *et al.*, 2002; Di Micco *et al.*, 2006). Taken together, these findings suggest that there is an activation of the G2/M checkpoint

in DKO BMDM which triggers G2/M cell cycle arrest and premature senescence as a consequence of the stimulation of the DDR pathway. However, the exact mechanism trough which DNA hyper-replication leads to DDR in absence of E2F1 and E2F2 remains undiscovered.

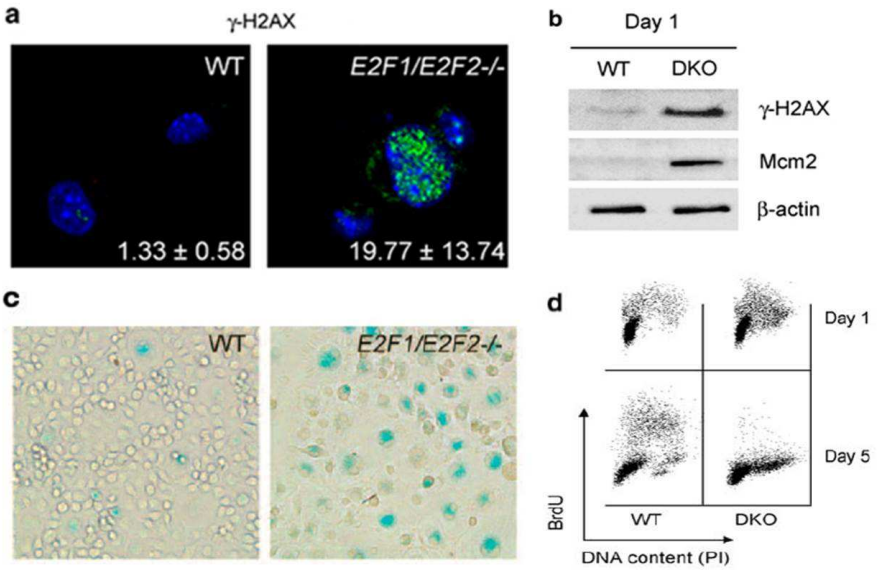


**Figure 1. DNA damage response activation, G2/M arrest and senescence in DKO cells (a)** Immunofluorescence assay showing DNA damage response activation in bone-marrow-derived macrophages (BMDM) by γ-H2AX accumulation in DKO cells. **(b)** The increased levels of γ-H2AX correlate with the accumulation of Mcm2 in DKO BMDM. **(c)** WT and DKO bone-marrow-derived cells were stimulated with MCSF for five days and then stained for SA-β-gal marker to establish the status of cellular senescence. **(d)** Cell cycle distribution analysis of representative WT and DKO bone-marrow-derived cells stimulated with MCSF for the indicated times. DNA content was assessed by staining with propidium iodide, and DNA synthesis was assessed by staining with an antibody to BrdU, and measured by flow cytometry. *Modified from Iglesias et al., 2010.*

The G2/M checkpoint is controlled by the ATM/ATR axis, whose activation in response to DNA replication has been shown to promote senescence in different models (Herbig *et al.*, 2004; Di Micco *et al.*, 2006). ATM and ATR are two proteins that belong to the phosphatidylinositol 3-kinase (PI3K) family and catalyse the phosphorylation of proteins involved in DNA repair and proliferative control such as the histone H2AX (Burma *et al.,* 2001). Besides, the activation of ATM/ATR results in the phosphorylation of a plethora of downstream targets with the final effect of the inhibition of cell cycle progression, senescence or apoptosis. It has been shown a direct relationship between ATR/ATM activation in response to DNA damage with the establishment of premature senescence using caffeine as an inhibitor of both kinases. Caffeine (1,3,7-trimethylxantine), one of the most frequently ingested drugs in the world, at a concentration of 0.1–10 mmol/L is a potent inhibitor of ATM/ATR activity that can suppress cell cycle arrest and abolish DDR both *in vitro* an *in vivo* (Sarkaria *et al.,* 1999; Bache *et al.,* 2001). Recently, the research group of Bennet has used caffeine to block DDR *in vivo* using t-BHP, a hydrogen peroxide

analogue, as a DNA damage inductor in murine aortic vascular smooth muscle cells (Mercer *et al.,* 2012) (**Figure 2**).

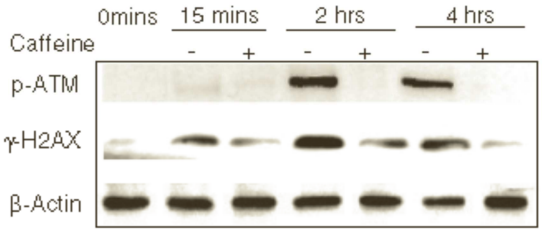


**Figure 2. Caffeine inhibits DDR**. Western blot analysis of phosphorylated ATM and γ-H2AX in murine aortic vascular smooth muscle cells after treatment with t-BHP in control cells or after 2.5 μM caffeine pre-treatment. *Modified from Mercer et al., 2012.*

Considering that the ATM/ATR pathway works as an alternative mechanism to control DNA damage and maintain cell cycle homeostasis in different animal and cellular models, it is reasonable to think that this same pathway acts as a surveillance mechanism to prevent uncontrolled proliferation by activating the G2/M arrest that triggers the premature senescent program in the DKO model.

## 1.1. Aim of the study

In this work we wanted to study the mechanism that lies beneath DDR activation in response to DNA hyper-replication in the DKO mouse model. Our hypothesis is that the uncontrolled replication of DNA that occurs in the absence of E2F1 and E2F2 leads to the activation of the ATR/ATM pathway which ultimately triggers a senescent program in DKO cells (**Figure 3**).

To determine whether the ATR/ATM pathway might be the surveillance mechanism in order to regulate uncontrolled proliferation in the DKO model, we used BMDM cultures in the presence of caffeine, the potent inhibitor of ATM/ATR activity.

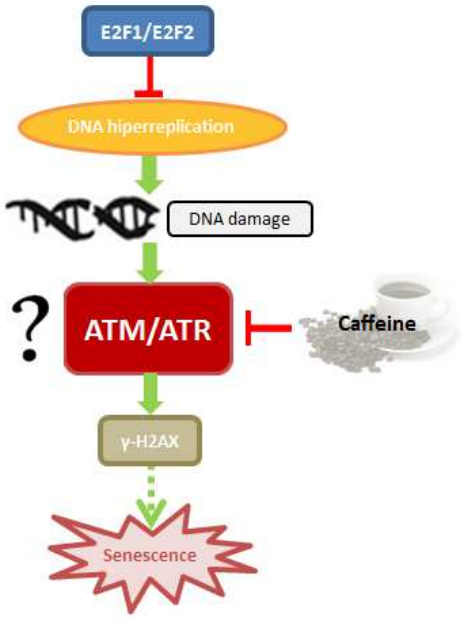


**Figure 3.** Proposed model for ATM/ATR control pathway in response to DNA hyper-replication.

The specific aims for this work were the following:

- To check whether caffeine inhibits DDR activation in DKO BMDM.
- To test whether caffeine inhibits premature senescence in DKO BMDM.

# 2. Materials and methods

## 2.1. Mouse strains and DKO genotyping

E2F1$^{-/-}$ and E2F2$^{-/-}$ mice belong to the 129/Sv x C57BL/6 mixed strain and were generated using homologous recombination (HR) in the laboratory of Dr Michael E. Greenberg. E2F1/E2F2$^{-/-}$ or DKO mice have been generated in Ana Zubiaga's lab by crossing E2F2$^{-/-}$ mice with E2F1 heterozygous mice. The breeding must be done in each generation, since DKO mice are sterile. The characteristics of this DKO model have been previously described (Li *et al.,* 2003; Iglesias *et al.,* 2004; Iglesias *et al.,* 2010). All procedures were approved by the University of the Basque Country Animal Care and Use Committee.

To confirm that newborn mice were knockout for E2F1 gene, thus confirming their DKO genotype, it was necessary to genotype them. Once the lab technician obtained a biopsy of the tail, I carried out a DNA extraction protocol based on the use of phenol and chloroform as described in literature (Laird *et al.*, 1991). After the DNA was extracted, a PCR was performed using three primers: 5´-GAGGGTTAGGGCTGATGGAT-3´, 5´-GAGTCCTCCGAAAGCAGTTG-3´ eta 5´-CCAGACGCCACTTGTGTAGC-´3. While the first two primers hybridize with the third exon of the E2F1 gene, the last one hybridizes with the neomycin sequence inserted by homologous recombination in the E2F1 locus, used to generate the knockout mice for E2F1. The design of the primers guarantees that a 207 bp or a 180 bp DNA fragment is obtained when WT or mutant alleles are amplified, respectively (**Figure 4**). In order to visualize those fragments, a DNA electrophoresis was carried out, migrating PCR results in a gel composed of 3% agarose and 0,01% ethidium bromide (EtBr). EtBr is a commonly used fluorescent aromatic tag that intercalates between DNA base pairs. When excited using ultraviolet light, the molecule emits orange light that permits the visualization of the amplicons.

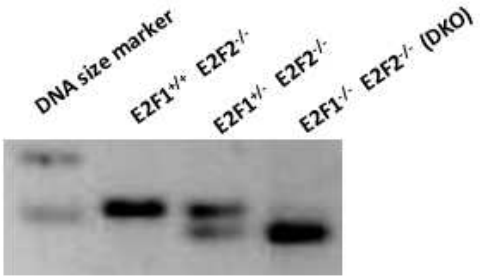


**Figure 4.** Representative figure of mice genotyping using PCR for E2F1 to confirm DKO genotype in the offspring of E2F1$^{+/-}$ and E2F2$^{-/-}$ bred mice.

## 2.2. Primary cell culture conditions and treatment with caffeine

Cell cultures were performed in an incubator with saturated humidity, at 5% $CO_2$ concentration and 37ºC. Once the lab technician had extracted the femurs of WT and DKO mice, I continued with subsequent injections of Dubelcco's modified Eagle medium (DMEM, Gibco-BRL) in the bone-marrow canal. Obtained cells were washed in phosphate buffered saline (PBS) and

contaminant erythrocytes were eliminated by hypotonic lysis. Cells were finally washed in PBS and resuspended in complete medium containing DMEM medium with 10% Fetal Bovine Serum (FBS, Gibco-BRL) and 1/100 penicillin/streptomycin. Before cell culture, cell count was done using a Neubauer chamber or haemocytometer and trypan blue (Invitrogen) exclusion assay was performed to discriminate between viable and unviable cells.

Bone-marrow progenitor cells are induced into a proliferation concomitant to macrophage differentiation using DMEM medium with 10% FBS and 1/100 p/s in presence of 20% of L-cell conditioned medium as a source of MCSF (Celada *et al.,* 1996). I further referred to "20% L-cell conditioned medium" as MCSF, which is equivalent to 6.7 ng/ml of recombinant MCSF. MCSF, macrophage colony-stimulating factor, is a cytokine that induces cell proliferation and differentiation into macrophages within 6 days from the primary culture of monocyte progenitors (Liu *et al.,* 1999) (**Figure 5**).

Besides, macrophage differentiation is accompanied by the early induction of the differentiation marker CD11b (Brackman *et al.,* 1995). CD11b is a specific integrin expressed in macrophage cell surface that can be used to monitor the correct differentiation into this cell type. Once the differentiation process ends, WT cells are able to continue proliferating during approximately 10 days before the nutrient depletion induces cells to enter senescence. The growing and differentiation of the cells was monitored visually using an inverted phase contrast microscope (Leica microsystems DM IL LED) coupled to a camera device.

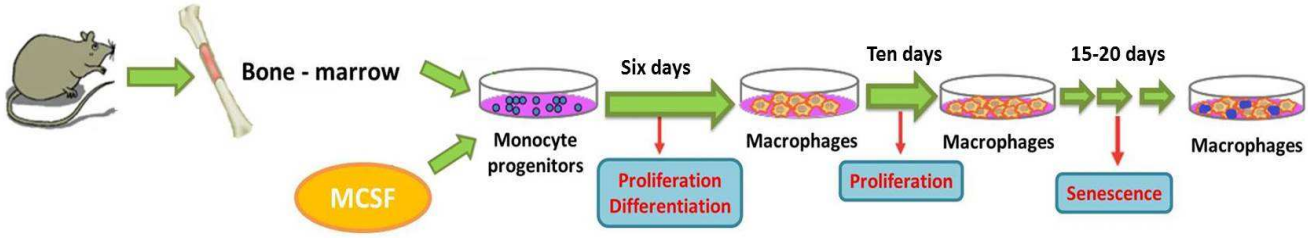


**Figure 5.** Schematic representation of the experimental design used to culture bone-marrow-derived progenitor stimulated with MCSF to induce proliferation and differentiation of this cells into macrophages. *Modified from Iglesias thesis, 2005.*

Where indicated, cells were treated with 0.5 or 4 mM of caffeine (Sigma, St Louis, MO, USA). Those concentrations were chosen based on the results of Sarkaria *et al.,* who described the biochemical properties of the *in vitro* inhibition that caffeine exerts over ATR and ATM proteins in hematopoietic cells (Sarkaria *et al.*, 1999). As it can be seen in the inhibition curves for ATM and ATR kinase activity both proteins show similar sensitivities to caffeine, with a 50% inhibition of kinase activity at drug concentrations of 0.2 and 1.1 mM (**Figure 6a and 6b**). In this regard, a treatment of 0.5 mM of caffeine was chosen since it is included in this range, whereas 4 mM of caffeine is representative of a nearly total inhibition of the kinase activity.

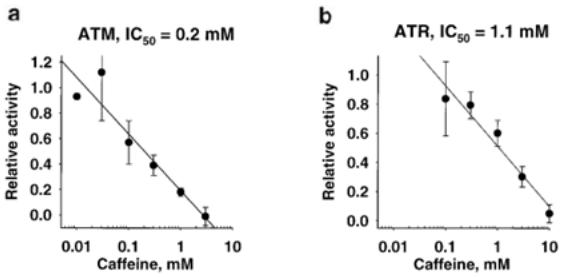


**Figure 6.** Inhibition of the kinase activity of **(a)** ATM and **(b)** ATR at graded concentrations of caffeine. *Modified from Sarkaria et al., 1999.*

## 2.3. Flow cytometry analysis using CFSE and anti-CD11b staining

In order to monitor proliferation and differentiation of WT BMDM simultaneously, I decided to perform a flow cytometry analysis using CellTrace™ CFSE Proliferation Kit (Life Technologies). The molecular probe carboxyflluorescein diacetate succinimidyl ester, abbreviated as CFSE, passively diffuses into cells. It is colourless and non-fluorescent until the acetate groups are cleaved by intracellular amines, forming fluorescent conjugates that are well retained. These complexes are retained by the cells throughout development and meiosis, as it is inherited by daughter cells after cell division occurs, decreasing its fluorescent intensity with each division approximately twofold. Those particular features make CFSE staining an excellent method for monitoring the proliferation of WT BMDM.

For CFSE staining, $10^7$ bone-marrow-derived WT cells were incubated with 2.16 µM of CFSE as previously described (Infante *et al.,* 2008). Stained cells were cultured in 12-well plates in presence of 20% MCSF and complete medium (detailed above) for five days. Each of the five days, early differentiating macrophages were detected with cell surface staining, performed using anti-CD11b antibody conjugated to phycoerythrin (Becton & Dickinson, San Jose, CA, USA). Finally, fluorescence was detected using a FACSCalibur (Becton & Dickinson) flow cytometer and results were analysed using ModFit 3.0 software. For each day of culture, I analysed the number of generations and the proliferative index in the CD11b-positive population.

## 2.4. Staining for senescence-associated-β-galactosidase activity

To establish the status of cellular senescence, cells were stained to detect β-galactosidase activity, as previously described (Dimri *et al.,* 1995). The origin or function of senescence-associated-β-galactosidase activity (SA-β-gal) is not known yet. However, there are some hypotheses that relate the increase in SA-β-gal activity with an elevated lysosomal activity in senescent cells.

For SA-β-gal staining, $0{,}8x10^6$ bone-marrow-derived progenitors from both WT and DKO were obtained and cultured, as described previously, in 6-well plates. At day six, when a senescent phenotype was clearly observed in DKO cells, cells were washed is PBS and fixed for 3-5 minutes (at room temperature) in 2% formaldehyde/0.2% glutaraldehyde. After washing again, cells were incubated at 37º with fresh senescence associated staining solution: 1 mg of 5-bromo-4-chloro-3-indolyl P3-D-galactoside (X-Gal) per ml in a buffer containing 40 mM citric acid/sodium phosphate, pH 6.0; 5 mM potassium ferrocyanide; 5 mM potassium ferricyanide; 150 mM NaCl; 2 mM MgCl2. Senescent cells exhibited β-gal activity, that is detected in single cells by X-gal, which forms a local blue precipitate when processed after 2-4 hours. Staining was maximal in 7-10 hours, as observed using an inverted phase contrast microscope (Leica microsystems DM IL LED) coupled to a camera device.

## 2.5. Protein analysis by western blotting

To study the protein expression of both Mcm2 and γ-H2AX, western blot analyses were performed. $15x10^6$ bone-marrow derived cells from each genotype (WT and DKO) were primarily cultured in 20 cm diameter plates as described above. After 24 hours of culture, floating and adherent cell were harvested and cellular protein was extracted as previously described (Infante *et al.,* 2008) using a buffer containing 500 mM $NaPO_4H$, pH 7.2; 500 mM EDTA; 500 mM EGTA; 500 mM NaCl; 500 mM NP-40; 100 mM β-glucophosphate; 100 mM PMSF; 200 mM $Na_3VO_4$ and a cocktail of protease and phosphatase inhibitors (Roche). Protein concentration from each sample was determined using DC Protein Assay (Bio-Rad) with a BSA standard curve. 20 µg of protein were loaded on each lane of the sodium dodecyl sulphate-polyacrylamide gels (SDS-PAGE) for protein separation, using 10% of acrylamide for Mcm2 and 15% for γ-H2AX. The migration was carried out at 100 V and then acrylamide gels where transferred onto a nitrocellulose membrane at the same voltage (Bio-Rad). Proper transference of the protein to the membrane was confirmed after Ponceau staining. Membranes were then washed with TTBS (Tris 50 mM, NaCl 150 mM, 5% tween 20) and blocked with 5% of skimmed milk in TTBS for one hour. Membranes were incubated with primary antibodies goat anti-Mcm2 (diluted 1:400; Santa Cruz), rabbit anti-γ-H2AX (phosphorylated in serine 139; diluted 1:1000; Millipore) and mouse anti-β-actin, as an endogenous control (diluted 1:3000; Sigma) overnight in 10 ml of 5% skimmed milk (anti-Mcm2 and anti-β-actin) or 3% BSA (anti γ-H2AX) in TTBS. Inmunocomplexes were finally visualized with horseradish peroxidase-conjugated anti-goat, anti-rabbit or anti-mouse IgG antibodies (Santa Cruz) using chemiluminiscence detection with ECL (Santa Cruz) with a ChemiDoc camera (Bio-

Rad). Semi-quantification was performed using the image analysis software provided by the manufacturer, normalising the results for Mcm2 and γ-H2AX with β-actin expression.

# 3. Results

## 3.1. Wild type bone-marrow-derived macrophage (BMDM) culture monitoring

Before starting with the caffeine treatment, I wanted to assure that I was able to recapitulate the method used to differentiate and stimulate macrophage growing detailed in previous studies. For this purpose, a cell culture of WT bone-marrow progenitor cells was performed as described above.

The growing and differentiation towards macrophage morphology was monitored during six days (**Figure 7a**). In the presence of 20% of MCSF monocyte progenitors undergo a notorious morphological change that correlates with their differentiation into macrophages (Liu *et al*, 1999). Whereas cells at day 0 displayed a circular shape and were floating in the medium, it is clearly observable on phase-contrast microscopy that, as the monocyte precursors difference over time, the cells enlarged in size and became adherent to the plate surface. After six days, cultured cells adopted a fusiform shape with lamelipods characteristic of macrophages, presented an increased number of cytoplasmic granules and had spread on the surface of the plate, thus indicating positive proliferation concomitant to differentiation.

To further confirm that monocyte precursors were differentiating to macrophages, I performed a flow cytometry analysis using the fluorescent probe CFSE to monitor proliferation during 5 days. As CFSE is diluted approximately twofold with each cell division, the number of cells present in each generation and, consequently, the proliferative index (PI) of the culture could be estimated. Besides, cells were counterstained with a fluorescent antibody of CD11b, an integrin described as a specific biomarker for macrophage maturation (Brackman *et al.,* 1995). As the population of CD11b cells increased over time after MCSF stimulation, these cells gradually lost their CFSE fluorescence (**Figure 7b**). The proliferation analysis was then performed choosing the population positive for anti-CD11b staining (depicted as a red box in **Figure 7b**). As it can be seen in **Figure 7b and 7c**, WT cells undergo a rapid proliferation from the third day of culture onwards and until the final days of culture. Moreover, whereas at day 3 the proliferation index of the chosen population had an estimated value of 3.85, this parameter was increased nearly threefold at day 5 (PI=9.6), in correlation with the observations made using phase-contrast microscope. Taken together, these results confirm that the population undergoing proliferation in presence of MCSF consisted of differentiating macrophages.

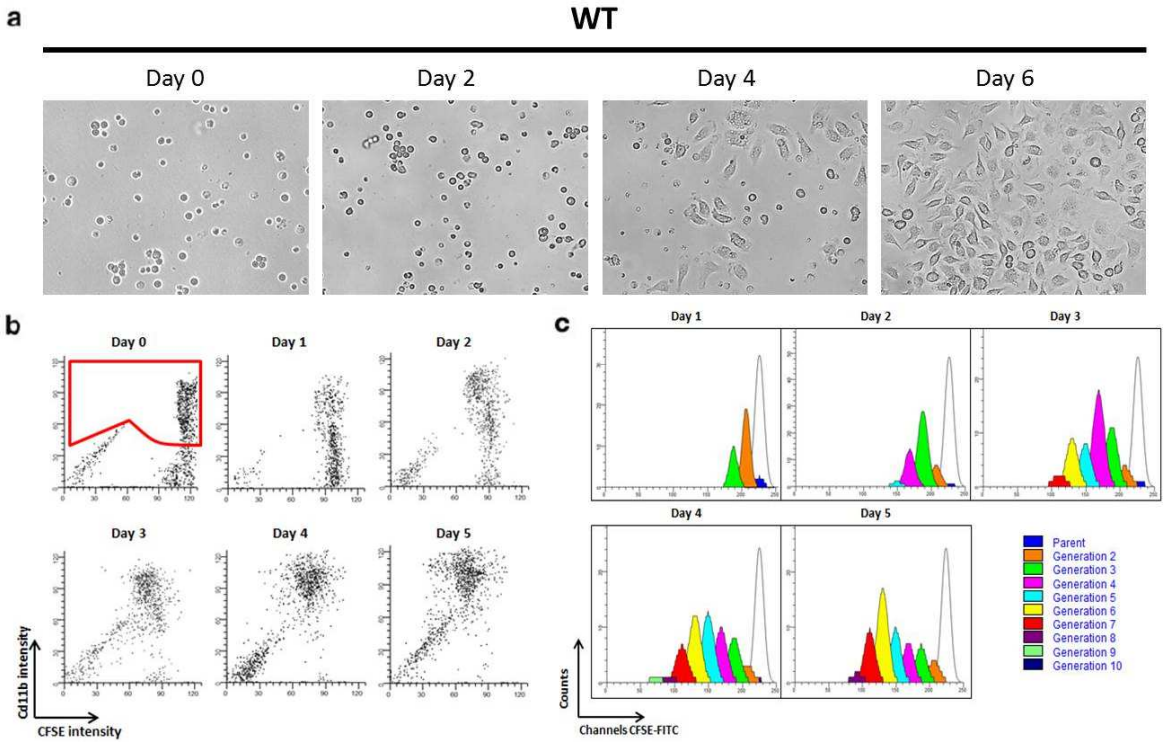


**Figure 7. Proliferation and differentiation of wild type bone-marrow progenitors into macrophages in the presence of MCSF**. **(a)** Monitoring of WT bone-marrow-derived cell culture in days 0, 2, 4 and 6 in presence of MCSF using a phase-contrast microscope. **(b)** Analysis of proliferation from WT bone-marrow-derived cells stained with CFSE, cultured with MCSF for five days and then stained with anti-CD11b. The population of cells positive for CD11b staining is marked with a red box. **(c)** Proliferation Wizard software was used to identify CD11b positive cells in different generations as indicated. Note that the parental population (unshaded) is the brightest population, with subsequent cell divisions showing reduced CFSE signal and increased CD11b signal.

## 3.2. DKO BMDM cells undergo a rapid proliferation event, show DDR activation and enter premature senescence

After demonstrating that bone-marrow-derived cells differentiated into macrophages in the presence of MCSF, I proceeded to analyse the phenotype of DKO BMDM in the same conditions. For this purpose, WT and DKO cells were cultured in parallel and visual, molecular and biochemical analysis were performed.

Previous studies have shown that DKO BMDM undergo a rapid proliferation followed by a premature senescence (Iglesias *et al.,* 2010). Using a phase-contrast microscope, I analysed the phenotypic differences between WT and DKO BMDM cells in culture (**Figure 8a**). Consistent with the previous findings of Iglesias *et al.,* at the fourth day of culture DKO cells proliferated faster in comparison to WT cells. In correlation with this rapid proliferation, DKO cells acquired a more differenced phenotype earlier too, as it was clearly shown by their fusiform shape at days 4 and 6. Moreover, the amount of cells was higher in the case of DKO cells, confirming that a hyperproliferative event had occurred in the initial stages of the culture. At the sixth day and,

more dramatically, at the eighth day of culture, several circular flattened cells with a large cytoplasm could be observed in the DKO culture (pointed out using red arrows in **Figure 8a**), suggesting that those DKO BMDM cells had entered premature senescence. Although several senescent cells could also be observed in WT at day eight, the number of senescent cells is visually higher in the DKO culture.

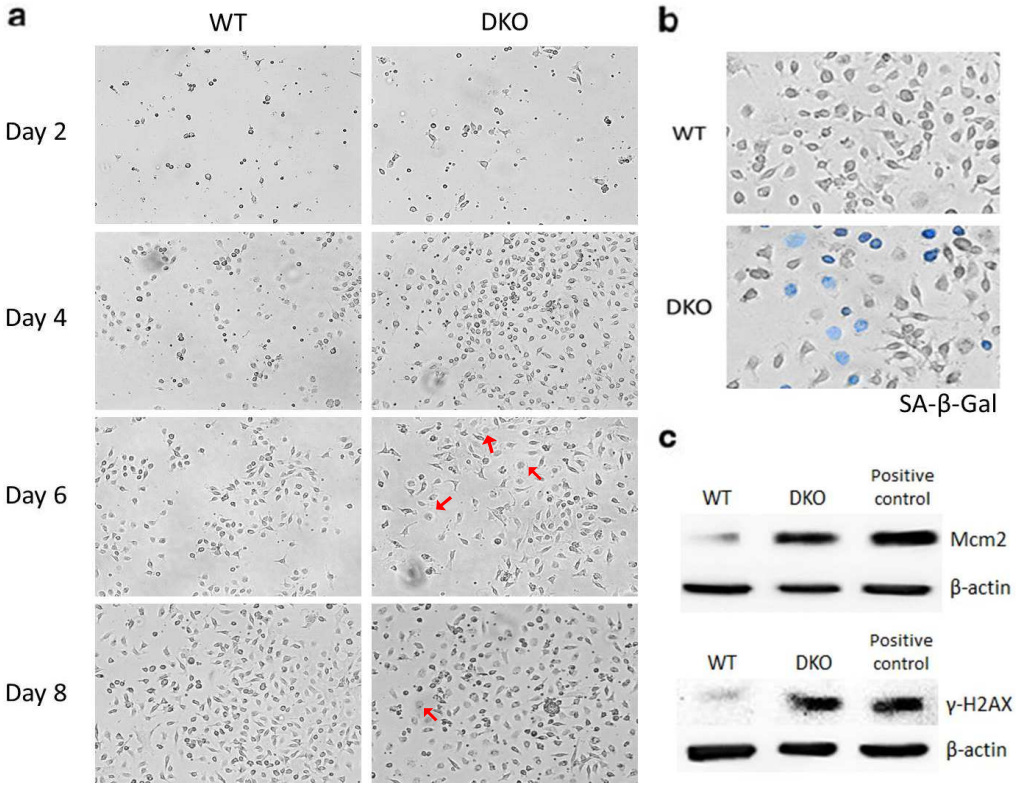


**Figure 8. Rapid hyper-proliferation, DDR activation and premature senescence in DKO cells**. **(a)** Monitoring of WT and DKO BMDM in presence of MCSF at days 2, 4, 6 and 8. Senescent cells at the DKO culture are signalled by red arrows. **(b)** BMDM WT and DKO cells were stimulated with M-CSF for 6 days and then stained for SA-β-gal marker to establish the status of cellular senescence. **(c)** Western blot analysis of Mcm2 and γ-H2AX. WT and DKO cells were stimulated with MCSF and harvested 1 day after. Proteins were analysed by immunoblotting with the indicated antibodies. Expression of β-actin was used as loading control and protein was extracted from HEK293 cell culture was used as a positive control in both cases.

To confirm that flattened cells in DKO were senescent, I stained both WT and DKO cells at the sixth day of culture for senescence-associated-β-galactosidase (SA-β-gal) activity as described above. DKO BMDM, but not WT cells, stained positive for SA-β-gal (**Figure 8b**). These results confirmed that DKO cells proliferate rapidly during the initial days of culture compared with WT cultures, reach a plateau on days 4 or 5 and enter premature senescence.

Following the observation of the altered features exhibited by proliferating DKO cells, I examined the expression of Mcm2 protein, which is a S-phase marker directly related to the replication of DNA, 24 hours after the stimulation with MCSF. As it has been previously described (Iglesias *et al.,* 2010), a western blot analysis of the protein content of DKO cells showed a

remarkably higher amount of Mcm2 in comparison to wild type cells (**Figure 8c**). This fact suggests that the hyperproliferation event might be a consequence of the deregulated accumulation of Mcm2. Additionally, the western blot analysis of γ-H2AX (the phosphorylated form of the histone H2AX, marker of DDR), 24 hours after the stimulation with MCSF, also showed a singular accumulation in stimulated DKO cells (**Figure 8c**). Taken together, these results indicate that I was able to recapitulate the method used to differentiate bone-marrow progenitors into macrophages and check the characteristic phenotype of DKO cells as it has been previously described (Iglesias *et al*., 2010).

### 3.3. Optimization of the concentration of caffeine

Once I assured that the phenotype of DKO BMDM cells I had observed was consistent with the one previously described in the literature, I decided to treat those cells with caffeine in order to test my initial hypothesis. To start, an optimization of the concentration of the drug was performed treating WT BMDM with two representative concentrations of caffeine: 0.5mM and 4mM.

WT BMDM cell cultures treated with 0.5 or 4 mM of caffeine in presence of MCSF were monitored and visually analysed using a phase-contrast microscope as described above (**Figure 9**). After four days, WT cells treated with the vehicle showed a macrophage morphology characterised by fusiform shape and granulocytes in their cytoplasm. At the same time, cells treated with 0.5 mM of caffeine presented a similar phenotype, although it seemed that differentiation and proliferation were slowed down. This observation was confirmed when observing cells treated with 4 mM caffeine, where no differentiated macrophages could be observed. Therefore, the results suggest that caffeine might be toxic at 4 mM under these culture conditions for WT BMDM. Moreover, the morphological change that BMDM undergo in the presence of 0.5 mM caffeine argues for a direct link between the addition of the drug and its effect in the proliferation/differentiation process of monocyte progenitors towards macrophages, something already described in other cellular models at the same concentration of caffeine (Hashimoto *et al.*, 2004).

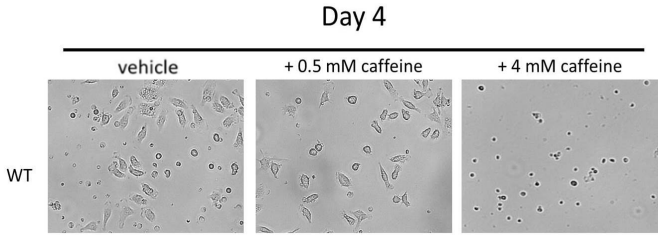


**Figure 9.** Caffeine treatment affects the differentiation and proliferation of WT BMDM. BMDM were stimulated with MCSF in the presence of 0.5 and 4 mM of caffeine and analysed 4 days later using a phase-contrast microscope.

### 3.4. Treatment of DKO cells with caffeine prevents premature senescent phenotype, reduces Mcm2 accumulation and stimulates phosphorylation of H2AX

Once observed the results obtained in the optimization assay, I decided to check the effect that caffeine has over the senescence and the activation of DDR in DKO BMDM. As a consequence of the toxicity of caffeine at 4 mM, I used 0.5 mM caffeine or control treatment.

To assess whether caffeine rescued premature senescence in the DKO BMDM cells, a SA-β-gal assay was performed at the sixth day of culture as described above. Interestingly, while DKO cells stained positive for β-gal activity, neither WT control nor caffeine treated WT and DKO cells showed positive staining (**Figure 10a**). These results indicate that caffeine prevents DKO cells from entering premature senescence.

Simultaneously, in order to address if the absence of senescence correlated with DDR inhibition as a result of the inhibitory activity of caffeine over ATM/ATR, the expression of γ-H2AX was analysed. For that purpose, I made a western blot analysis of the cellular protein extracted 24 hours after WT and DKO BMDM cells were cultured in the presence or absence of 0.5 mM caffeine (**Figure 10b**). Under these conditions, caffeine treatment did not affect γ-H2AX expression in WT BMDM relative to control WT cells. Surprisingly, there was a brusque increase of γ-H2AX accumulation in DKO treated cells. Therefore, the absence of senescence and the unexpected increase in H2AX histone phosphorylation may indicate that caffeine might be abrogating G2/M arrest while at the same time DNA damage is accumulating.

Provided that G2/M arrest is bypassed in the presence of caffeine, I might expect an increase in the accumulation of S-phase proteins such as Mcm2. Since caffeine seems to inhibit the block in G2/M, DKO BMDM would continue hyper-proliferating in an uncontrolled fashion. To check this hypothesis, a western blot analysis for Mcm2 was performed under the same conditions (**Figure 10b**). Similarly to what was seen in γ-H2AX, there were no observable changes in Mcm2 expression in WT cells. Interestingly, DKO cells showed an abrupt reduction of Mcm2 expression after being treated with caffeine. The reduced accumulation of Mcm2 might indicate that DNA replication is reduced in the presence of caffeine in DKO BMDM.

To clearly compare the differences in the expression of both γ-H2AX and Mcm2 in the presence or absence of caffeine a semi-quantification was performed, normalising the results using β-actin as an endogenous control (**Figure 10c**). Altogether, it seems that caffeine has no effect on the expression of neither Mcm2 nor γ-H2AX in WT BMDM cells. However, there is more than a twofold decrease in Mcm2 expression and an observable increase of γ-H2AX accumulation in DKO BMDM cultures treated with caffeine 0.5mM. These results suggest that the absence of

senescence observed in the presence of caffeine could be caused by a combination of reduced proliferation of the DKO cells and a G2/M arrest abrogation, but yet without inhibition of DDR, as it is indicated by the increase in the accumulation of γ-H2AX.

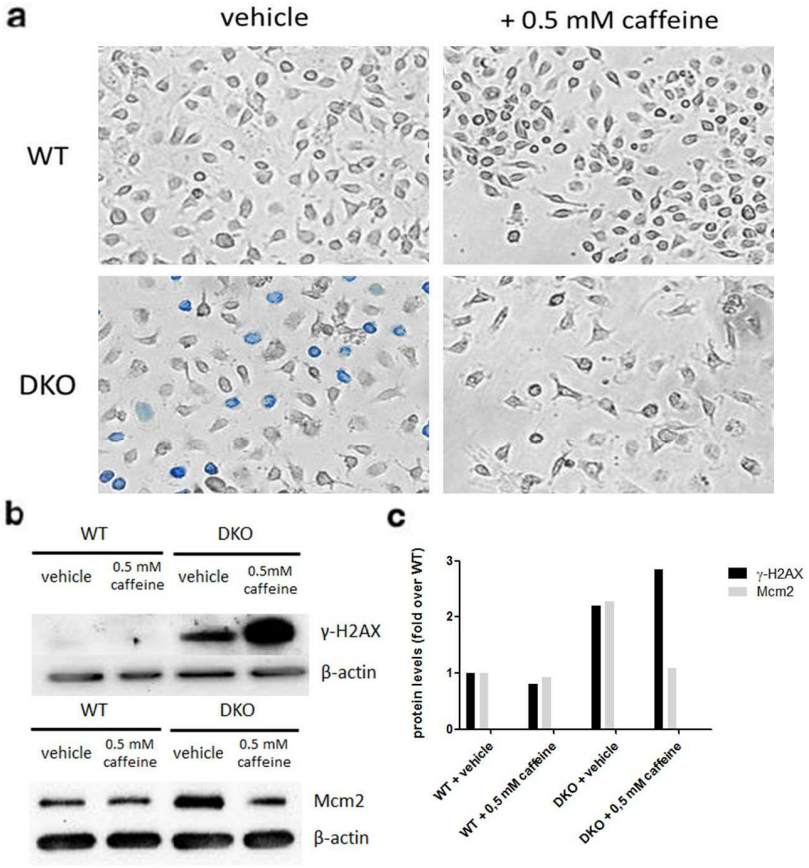


**Figure 10. Caffeine treatment prevents cellular senescence and results in inhibition of Mcm2 expression and induction of γ-H2AX accumulation**. **(a)** BMDM WT and DKO cells were stimulated with M-CSF for 6 days in presence or absence of 0.5 mM caffeine and then stained for SA-β-gal marker to establish the status of cellular senescence. **(b)** Western blot analysis of γ-H2AX and Mcm2. WT and DKO cells were stimulated with MCSF in presence or absence of 0.5 mM caffeine and harvested 1 day after. Proteins were analysed by immunoblotting with the indicated antibodies. Expression of β-actin was used as loading control. **(c)** Semi-quantification of western blot results expressed as fold over WT using β-actin expression as a housekeeping control to normalize the results.

## 4. Discussion and further work

The aim of this work was to check whether the activation of the ATR/ATM pathway was responsible for triggering a premature senescent program in DKO cells as a consequence of an uncontrolled replication of DNA in absence of E2F1 and E2F2.

In summary, our results show that the addition of caffeine, an ATR/ATM activity inhibitor, abolishes premature senescence in DKO BMDM, as it is confirmed by the negative results obtained in the SA-β-gal staining of those cells (**Figure 10a**). Surprisingly, the expression of both Mcm2 and γ-H2AX is altered in the presence of caffeine (**Figure 10b and 10c**). There is a higher

accumulation of γ-H2AX, a direct target of ATM/ATR, when cells are treated with caffeine. If caffeine acts to inhibit ATM/ATR activity, I would expect the phosphorylation of the histone H2AX to be decreased by the addition of the drug. Moreover, Mcm2 is a direct target of E2F that has been described as a marker for DNA replication which gives insight into the proliferative status of the cell. Since caffeine acts as an inhibitor of ATM/ATR activity, proteins that are involved in the DDR that is activated as a consequence of DNA hyper-replication in the DKO model, I would not expect any modifications in Mcm2 expression when caffeine is added.

The simplest explanation of this data is that ATM and ATR are actually superactivated in the DKO model. The absence of both E2F1 and E2F2 leads to an uncontrolled cell proliferation. In this cellular context, it is reasonable to think that the addition of caffeine could provoke an unexpected and abnormal effect. Similar experiments carried out by Cortez argue for this hypothesis, where the treatment of HCT116 cells with caffeine does not prevent ATM/ATR-dependent phosphorylation of CHK1 and CHK2, two proteins that act downstream of ATR/ATM to induce cell-cycle arrest in G2/M in response to DNA damage (Cortez, 2003). However, caffeine yet still inhibited checkpoint response initiated by hydroxyurea as a DNA damaging agent in those cells. This could support the idea that caffeine, in some cellular contexts, prevents cellular senescence without directly inhibiting ATM/ATR activity. Moreover, if G2/M arrest is abrogated by caffeine even though the damaged DNA is not repaired, the cell would continue proliferating without entering senescence while this damage is increasingly accumulated. Although the increased accumulation of γ-H2AX and the restoration of senescence support this hypothesis in the DKO model, the mitigation of Mcm2 is not explained.

A second model that might explain the results is that caffeine, since it is a purine analogue, can intercalate into DNA and interfere with DNA repair activities by preventing the binding of repair enzymes and proteins such as the histone H2AX. (Tornaletti *et al.*, 1989). Consequently, caffeine may be preventing the repair of the damaged DNA that results from hyper-replication in the DKO model and thereby promoting hyper-activation of ATM/ATR and their targets. However, this hypothesis does not explain either the decrease in Mcm2 protein or the absence of senescence in DKO treated cells.

Apart from preventing G2/M phase arrest induced by DNA damage, caffeine at a concentration of 5 mM, has been reported to induce G1/S phase arrest in human lung adenocarcinoma cell line (Qi *et al.*, 2002). Thus, caffeine has been described as a pleiotropic agent that shows various effects on the cell cycle and may cause cell growth arrest and inhibition of proliferation, resulting in anticarcenogenic effects. Moreover, Hashimoto *et al.*, demonstrated

that pretreatment with caffeine resulted in an indirect inhibition of cyclin D1-cdk4 complex activation in mouse epidermal JB6 C1 41 cells (Hashimoto *et al.*, 2004). This inhibition causes arrest in G1 phase accompanied by inhibition of cell proliferation in response to cell growth stimulation. The arrest in G1 was a consequence of the direct inhibition of PI3K, an effector of the growth signalling pathway Akt-GSK-3, which provokes several effects on the physiological functions of cells, including cellular growing. It might be possible that this same mechanism is occurring in DKO BMDM, where the inhibition of growth signalling cascades by caffeine provokes a decrease in proliferation, thus inhibiting Mcm2 accumulation. According to this model, caffeine would be behaving as an inhibitor of DNA hyper-replication, ultimately preventing senescence. Nevertheless, this hypothesis does not explain why there is an accumulation of γ-H2AX.

On account of the data presented in this study, it could be concluded that caffeine does not act individually upon ATM/ATR activity in the DKO model. Therefore, the results of the experiment can only be explained if caffeine is inhibiting various targets simultaneously. While the inhibition of the Akt-GSK-3 signalling pathway explains the inhibition of proliferation confirmed by the decrease in Mcm2 expression, the abrogation of G2/M arrest could explain the increase in γ-H2AX and the absence of senescence. However, as a consequence of the mixed results, the implications of the ATM/ATR pathway in DKO BMDM are not fully eviscerated and the initial hypothesis has not been fully tested.

An interesting experiment for the future would be to repeat the experiments illustrated in this study using specific inhibitors of the ATM/ATR DDR pathway (Pitts *et al.*, 2014). New generation small molecule inhibitors (SMIs) KU-55933 or KU-60019 could be used to specifically target ATM activity; VE-821 to inhibit ATR kinase activity; AZD-7762 to inhibit CHK1 and CHK2, direct targets of ATM/ATR. Moreover, siRNAs could be developed against those proteins to see whether the phenotype is rescued and elucidate the mechanism through which DNA hyper-replication leads to DDR in the DKO model. Gaining insight into the alternative mechanisms that control cell cycle in response to replicative stress or other DNA insults would allow the development and design of more efficient drugs for treating pathologies such as cancer, where cell cycle related biochemical pathways are deregulated.

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