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Main Manuscript for

The T1D-associated lncRNA *Lnc13* modulates human pancreatic β cell inflammation by allele-specific stabilization of STAT1 mRNA.

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Author Contributions

ACR and IS contributed to the original idea, design and interpretation of experiments, researched data, contributed to discussion, and wrote the manuscript. IGM, AOG, TP, SK, MLC and DLE contributed to research data. NCV, LM, PM and BRG contributed with biological material. All authors have read and approved the manuscript. ACR and IS are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. IGM and

AOG have equally contributed to this work and thus, the first author has been determined alphabetically based on the last names.

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Abstract

The vast majority of type 1 diabetes (T1D) genetic association signals lie in non-coding regions of the human genome. Many have been predicted to affect the expression and secondary structure of long non-coding RNAs (lncRNAs), but the contribution of these lncRNAs to the pathogenesis of T1D remains to be clarified.

Here we performed a complete functional characterization of a lncRNA that harbors a SNP associated with T1D, namely *Lnc13*. Human pancreatic islets harboring the T1D-associated SNP risk genotype in *Lnc13* (rs917997*CC) showed higher *STAT1* expression than islets harboring the heterozygous genotype (rs917997*CT). Upregulation of *Lnc13* in pancreatic β cells increased activation of the pro-inflammatory STAT1 pathway, which correlated with increased production of chemokines in an allele-specific manner. In a mirror image, *Lnc13* gene disruption in β cells partially counteracts PIC-induced *STAT1* and pro-inflammatory chemokine expression. Furthermore, we observed that PIC, a viral mimetic, induces *Lnc13* translocation from the nucleus to the cytoplasm, promoting the interaction of *STAT1* mRNA with PCBP2. Interestingly, *Lnc13*-PCBP2 interaction regulates the stability of the *STAT1* mRNA, sustaining inflammation in β cells in an allele-specific manner.

Our results show that the T1D-associated *Lnc13* may contribute to the pathogenesis of T1D by increasing pancreatic β cell inflammation. These findings provide novel information on the molecular mechanisms by which disease-associated SNPs in lncRNAs influence disease pathogenesis and open the door to the development of novel diagnostic and therapeutic approaches based on lncRNA targeting.

Significance Statement

The mechanisms by which autoimmunity is triggered/amplified in type 1 diabetes (T1D) remain to be clarified and the lack of this basic understanding hampers efforts to prevent or arrest the disease. Testing the hypothesis that T1D-associated genetic variants in long non-coding RNAs (lncRNAs) affect important pathways that modify inflammation and pancreatic β cell survival will increase our understanding of T1D pathogenesis.

In the present work we describe a novel molecular mechanism by which the T1D-associated lncRNA *Lnc13* regulates pancreatic β cell inflammation. These findings provide novel information on the molecular mechanisms by which disease-associated SNPs in lncRNAs influence disease pathogenesis and open the door to the development of novel diagnostic and therapeutic approaches based on lncRNA targeting.

Main Text

Introduction

Type 1 diabetes (T1D) is a chronic autoimmune disease in which insulin-producing pancreatic β cells are specifically destroyed by the immune system. During the initial stages of the disease, pancreatic islets are infiltrated by immune cells leading to the generation of an inflammatory environment that promotes pancreatic β cell destruction (1). Local islet inflammation (insulitis) is partially driven by a "dialog" between β cells and the infiltrating immune cells, through the release of pro-inflammatory chemokines and cytokines by both β cells and immune cells. Increasing evidence suggests that the triggering of insulitis depends on the interaction between T1D candidate genes (2) and

environmental factors, such as viral infections (3, 4). The molecular mechanisms by which viruses induce an autoimmune response targeting β cells remain unclear, however genetically regulated host-immune response could play an important role in β cell demise through the stimulation of immune autoreactivity, first triggered and then maintained by potential persistent infections in the pancreas (3–5). Although virus-induced activation of specific transcription factors (IRF7, NFκB, STAT1 and STAT2, among others) is certainly a contributory factor to gene expression changes associated with β cell failure (3, 5), recent studies indicate that a new class of gene-regulatory molecules named long non-coding RNAs (lncRNAs) actively participate in the regulation of innate immune responses in different cell types (6, 7).

LncRNAs are non-coding RNAs that are 200 nucleotides or longer in transcript length and which structure is similar to the one of protein-coding genes (8). Several studies have implicated lncRNAs in a wide range of biological and cellular processes, including the modulation of the innate antiviral immune response through the activation of PRR-related signal transduction (9), the regulation of innate immune-associated chemokines (10) and other inflammatory genes (11). LncRNAs regulate a variety of normal immune responses but there is limited information on their implication in the development of autoimmunity and only few lncRNAs have been identified in this context (12).

Interestingly, the majority of immune disease-related genome-wide association signals map to non-coding regions of the genome, including lncRNAs. This suggests that risk variants located in lncRNAs could play regulatory roles, altering their function and leading

to dysregulated expression of genes or gene networks potentially important for the development of autoimmunity.

Lnc13 is a lncRNA located in chromosome 2q12 that harbors a polymorphism (rs917997) associated with several autoimmune diseases, including celiac disease (CeD) (13), inflammatory bowel disease (IBD) (14) and type 1 diabetes (T1D) (15). The expression level of this lncRNA is significantly decreased in small intestinal biopsy samples from patients with CeD and a recent study described that *Lnc13* regulates the expression of a subset of CeD-associated inflammatory genes through interaction with heterogeneous nuclear ribonucleoprotein D (16). Interestingly, while the risk signal in celiac disease corresponds to the rs917997*T allele, the opposite allele (rs917997*C) is the risk allele in T1D, suggesting that the SNP may alter the function of this lncRNA in a tissue or disease-specific mode.

In the current study, we describe a novel molecular mechanism by which T1D-associated *Lnc13* regulates virus-induced pancreatic β cell inflammation. We observed that *Lnc13* acts as a linker between the RNA binding protein PCBP2 and the 3'UTR of *STAT1* to stabilize the mRNA of *STAT1*. This stabilization leads to increased activation of the STAT1 pro-inflammatory pathway, contributing to local inflammation and eventual β cell destruction.

In conclusion, our results show that *Lnc13* participates in pancreatic β cell inflammation via regulation of the STAT1 signalling pathway. This pathway plays a crucial role in T1D-related β cell dysfunction and death, therefore the ability of *Lnc13* to influence STAT1 activation supports its role in the pathogenesis of the disease. These results serve as a proof

of concept of the role of T1D-associated lncRNAs in the progressive loss of pancreatic β cells in T1D and open a new avenue for the development of therapeutic approaches based on lncRNA expression modification.

Results

Lnc13 is expressed in human pancreatic β cells, is upregulated by viral infections and correlates with STAT1 expression in human pancreatic islets

We first evaluated the expression of *Lnc13* in the human pancreatic β cell line EndoC-βH1 compared with a set of human tissues. As previously described (16), *Lnc13* is ubiquitously expressed, albeit at various levels in several human tissues (Fig. 1A). The expression of *Lnc13* in the human EndoC-βH1 is around 20 times higher than the most *Lnc13*-expressing human tissues (heart, liver and muscle), likely due to the embryonic nature of the cell line. In contrast, expression of *Lnc13* in pancreatic human islets, in which the typical proportion of β cells is approximately 50-60% (17), is similar to that of thymus, colon and lung (Fig. 1A). Mining of publicly available RNA sequencing data in a set of human primary cells confirmed that *Lnc13* is expressed in most of the cell types, although its expression level is very low (less than 2 transcripts per million) (SI Appendix, Fig. S1A, upper graph). In terms of *Lnc13* copies per cell, EndoC-βH1 cells harbor more *Lnc13* molecules per cell (mean ± SEM; 2.78 ± 0.31 copies/cell) than other cell lines, such as HEK293 (kidney; mean ± SEM; 0.98 ± 0.49 copies/cell) and SHSY5Y (brain; mean ± SEM; 0.87 ± 0.29 copies/cell), among others (SI Appendix, Fig. S1B). These results are in concordance with previous studies showing that the majority of lncRNAs are of very low abundance, having a copy number even lower than one per cell (18–20).

Pro-inflammatory stimuli such as pro-inflammatory cytokines and viral infections have been shown to play a crucial role in pancreatic β cell demise through activation of several inflammation- and apoptosis-related pathways (21–23). The pro-inflammatory cytokines IL-1β+IFNγ did not modify *Lnc13* expression in human pancreatic β cells (SI Appendix, Fig. S2A). However, intracellular polyinosinic:polycytidylic acid (PIC), a synthetic mimic of viral double-stranded RNA (dsRNA), upregulated *Lnc13* expression by around 42% (Fig. 1B). Intracellular PIC-induced *Lnc13* upregulation was similar to that observed in other known PIC-inducible genes (S*TAT2* and *IRF7*) (Fig. 1B). In addition, we observed that infection of pancreatic human islets with the diabetogenic Coxsackie Virus B5 (CVB5; multiplicity of infection (MOI) of 5) for 24h led to a 2.5-fold increase in *Lnc13* expression (Fig. 1C). Previous studies have demonstrated that interaction of the mRNA decapping protein DCP2 with *Lnc13* under inflamed conditions contributes to the degradation of *Lnc13.* Indeed, in bone marrow derived macrophages from DCP2 knockout (KO) mice *Lnc13* transcript was upregulated after LPS stimulation, strongly suggesting that DCP2 recognized *Lnc13* and caused its degradation (16). In accordance with the increase in *Lnc13* expression, we observed that intracellular PIC exposure for 8 and 24h led to a significant decrease in the expression of *DCP2* (SI Appendix, Fig. S2B), suggesting that PIC-induced *DCP2* decay facilitates *Lnc13* upregulation as previously shown in other models (16). However, infection with CVB5 in human pancreatic islets did not alter *DCP2* expression (SI Appendix, Fig. S2C), probably due to the ability of some RNA viruses to sequester the cellular decay machinery to increase their infectivity, which will in turn reduce DCP2 function (24). In addition, we observed a very close correlation between PIC-induced *Lnc13* upregulation and the increased mRNA expression of the pro-inflammatory

transcription factor *STAT1* (r^2=0.99; p<0.05) (Fig. 1D). Moreover, CVB5-induced *Lnc13* upregulation in human islets was also accompanied with a 2.8-fold increase in *STAT1* mRNA expression when compared to non-infected islets (Fig. 1E). Interestingly, *STAT1* expression levels in primary human cells (SI Appendix, Fig. S1A, lower graph) were correlated with *Lnc13* expression (SI Appendix, Fig. S1C). In line with these results, determination of *Lnc13* and *STAT1* expression levels in human pancreatic islets from 43 individuals revealed that in general, islets expressing higher amounts of *Lnc13* also had higher expression levels of *STAT1* (Spearman's R=0.51 (0.24-0.71); p<0.001) (Fig. 1F).

The T1D-associated SNP risk genotype in Lnc13 gene correlates with increased STAT1 expression in human pancreatic islets

To determine whether the genotype of the T1D-associated SNP in *Lnc13* affects *STAT1* expression in human pancreatic islets, we next genotyped rs917997 in cDNA samples from human pancreatic islets. Out of these 43 samples, 15 were homozygous for the risk allele rs917997*C, 2 samples were homozygous for the protective allele rs917997*T and 26 samples were heterozygous (CT). As shown in Fig. 2A, human islets from individuals homozygous for the T1D risk allele rs917997*C showed higher *STAT1* mRNA expression levels as compared to individuals heterozygous (CT) or homozygous for the T1D protective allele rs917997*T.

Lnc13 overexpression activates the STAT1 signaling pathway and increases production of pro-inflammatory chemokines in an allele-specific manner

In order to clarify the role of *Lnc13* variants in *STAT1* upregulation, we overexpressed either the T1D risk allele rs917997*C (pLnc13-C) or the non-risk allele rs917997*T (pLnc13-T) in EndoC-βH1 cells. Both, the *Lnc13*-C and *Lnc13*-T, increased *Lnc13* mRNA expression by 783- and 2000-fold, respectively (Fig. 2B) which were paralleled by a drastic upregulation of *STAT1* mRNA expression when compared to non-transfected or pCMV6-transfected pancreatic β cells (Fig. 2C). The increase in transcript levels correlated with increased levels of total STAT1 and phosphorylated STAT1 (SI Appendix, Fig. S3), suggesting an increased activation of STAT1 signaling pathway even in the absence of pro-inflammatory stimuli.

The plasmid harboring the non-risk allele T (pLnc13-T) increased *Lnc13* mRNA expression more efficiently than the plasmid containing the risk allele C (pLnc13-C) (Fig. 2B). Thus, to control for differences in *Lnc13* overexpression levels, we next normalized *STAT1* expression by *Lnc13* expression values. Interestingly, this normalization revealed that *STAT1* mRNA expression was exacerbated in pLnc13-C-transfected β cells when compared to pLnc13-T-transfected ones (Fig. 2D). These results suggest that *Lnc13* harboring the risk allele for T1D (C allele) induces a more pronounced increase in *STAT1* expression than *Lnc13* containing the non-risk allele.

To evaluate the effect of *Lnc13* overexpression on STAT1-regulated pro-inflammatory chemokine expression, we next determined the expression of *CXCL10*, *CXCL9*, *CCL5* and

CXCL1 in *Lnc13*-overexpressing EndoC-βH1 cells. *Lnc13* overexpression (T and C) led to an increase in all pro-inflammatory chemokine transcripts (SI Appendix, Fig. S4A-D). Similar to *STAT1,* transcript levels for *CXCL10*, *CXCL9*, *CCL5* and *CXCL1* were higher in pLnc13-C-transfected cells compared to pLnc13-T-transfected ones (Fig. 3A-D), suggesting that *Lnc13* harboring the T1D-associated rs917997*C allele promotes the expression of pro-inflammatory chemokines more efficiently than the *Lnc13* containing the non-risk allele (rs917997*T).

The upregulation of *CXCL10* and *CCL5* mRNA expression in *Lnc13*-overexpressing β cells was confirmed at the protein level (SI Appendix, Fig. S4E-F). Similar to the results at the mRNA level, *Lnc13*-C-overexpressing cells secreted higher amounts of CXCL10 and CCL5 per *Lnc13* molecule than the *Lnc13*-T-overexpressing cells (Fig. 3E-F).

To determine whether *Lnc13* affects PIC-induced pro-inflammatory chemokine expression through modulation of the STAT signalling pathway, we exposed *Lnc13*-C-overexpressing EndoC-βH1 cells to PIC in the absence or presence of ruxolitinib, a JAK inhibitor. As shown in Fig. 3G, *Lnc13-C* overexpression enhanced PIC-induced *CXCL10* mRNA expression, and this effect was counteracted by the presence of ruxolitinib. In addition, we observed that in *Lnc13*-C-overexpressing β cells, binding of STAT1 transcription factor to the *CXCL10* promoter was increased when compared to pCMV6-transfected control cells (Fig. 3H), confirming that the upregulation of chemokine expression in *Lnc13*-overexpressing β cells is driven by increased activation of the STAT1 signalling pathway.

To determine the biological relevance of *Lnc13* upregulation-induced chemokine production, we performed a chemotactic migration assay using supernatants of pCMV6- or pLnc13-C-transfected β cells. As shown in Fig. 3I, supernatants of *Lnc13*-C-overexpressing β cells induce a higher migration of Jurkat T cells than the control supernatants.

We next disrupted *Lnc13* gene in the EndoC-βH1 cell line by generating a deletion of 1698 bp in the *Lnc13* gene using CRISPR-Cas9 technique (Fig. 4A). Due to the low proliferation rate and fragility of these cells, we did not obtain a homogenous *Lnc13*-disrupted β cell line. Nevertheless, the mix population of *Lnc13*-disrupted cells (CRISPR-Lnc13) showed a reduced expression of *Lnc13* of about 60% (Fig. 4B). In a mirror image of the overexpression experiments, *Lnc13* disruption partially counteracted the effect of intracellular PIC in *STAT1*, *CXCL10* and *CCL5* upregulation (Fig. 4C-E).

Lnc13 does not directly regulate transcription of STAT1 gene but participates in its mRNA stabilization in an allele-specific manner

To determine the molecular mechanisms by which *Lnc13* activates the STAT1 signaling pathway in pancreatic β cells, we first analyzed the potential effect of *Lnc13* on the transcriptional regulation of *STAT1*. Transcription of *STAT1* is regulated by several transcription factors, including homodimers of phosphorylated STAT1, heterodimers of phosphorylated STAT1/STAT2 or STAT1/STAT3, and thus, increased phosphorylated STAT1 may lead to increased *STAT1* transcription through an autoregulatory mechanism (25, 26). Taking into account that *Lnc13* overexpression led to increased pSTAT1 (SI

Appendix, Fig. S3), we first inhibited protein translation using cycloheximide to inhibit the synthesis of STAT1 protein, and determine whether *Lnc13* by itself was promoting *STAT1* expression upregulation. To this end, after overexpressing *Lnc13*-C, we exposed β cells to cycloheximide for 24h and determined the expression levels of *STAT1* total mRNA and *STAT1* primary transcript using specific primers. Cycloheximide treatment for 24h efficiently decreased STAT1 protein amounts (SI Appendix, Fig. S5A). As shown in SI Appendix, Fig. S5B, cycloheximide treatment led to a 5-fold decrease in *STAT1* primary transcript expression but did not affect the expression level of *STAT1* total mRNA. These data suggested that in the absence of *Lnc13*-induced pSTAT1 protein synthesis, *Lnc13* was not upregulating *STAT1* expression through a direct effect on its transcription and suggested an effect on the stabilization of already produced *STAT1* mRNA molecules.

To analyze whether *Lnc13* contributes to the stabilization of *STAT1* mRNA molecules, we next analyzed the amount of *STAT1* mRNA in pLnc13-C or pLnc13-T overexpressing β cells in the absence or presence of actinomycin D, an inhibitor of transcription. Actinomycin D treatment revealed that *Lnc13*-C was less stable than *Lnc13*-T, as exposure to actinomycin D for 6h downregulated *Lnc13* expression by 40% in pLnc13-C-transfected β cells, but did not have any effect in pLnc13-T-transfected cells (SI Appendix, Fig. S5C). Despite being less stable, *Lnc13*-C showed an increased capacity to stabilize *STAT1* mRNA when compared to *Lnc13*-T. Indeed, actinomycin D treatment did not affect *STAT1* mRNA levels in pLnc13-C-transfected β cells while pLnc13-T-transfected cells showed a 1.9-fold decrease in *Lnc13*-normalyzed *STAT1* mRNA expression after 6h of actinomycin D exposure (SI Appendix, Fig. S5D).

Lnc13 interacts with PCBP2 in the cytoplasm leading to enhanced STAT1 expression in response to viral dsRNA

Cellular location often determines the functional role of lncRNAs (27), so we next examined the subcellular localization of *Lnc13* in non-treated and PIC-transfected pancreatic β cells. Consistent with previous studies, we found that in untreated cells *Lnc13* is preferentially expressed in the nucleus, while PIC transfection induces its translocation to the cytoplasm (Fig. 5A).

Interestingly, previous data (16) showed that in mouse macrophages *Lnc13* can bind PCBP2 (*Poly(rC) binding protein 2*), a RNA binding protein that participates in antiviral cellular responses through 3'-UTR stabilization of the *STAT1* transcript (28). Exposure to intracellular PIC led to increased PCBP2 protein expression both in the nucleus and the cytoplasm of EndoC-βH1, mirroring *Lnc13* kinetics (Fig. 5B).

To test whether *Lnc13* interacts with PCBP2 in human pancreatic β cells, we performed a RNA immunoprecipitation (RIP) assay using cellular extracts of non-treated and PIC-transfected EndoC-βH1 cells. Using a specific antibody against PCBP2, we isolated PCBP2-bound RNA molecules and determined *Lnc13* levels by Q-PCR. As shown in Fig. 5C, *Lnc13* exhibited modest binding to PCBP2 in untreated cells and the amount of PCBP2-bound *Lnc13* RNA increased by 3-fold after PIC treatment. Similarly, we observed that *STAT1* mRNA was poorly bound to PCBP2 under basal conditions and this interaction was enhanced 17-fold after intracellular PIC exposure (Fig. 5C).

To examine whether *Lnc13* is needed to facilitate the binding between PCBP2 and *STAT1*, we next performed a RNA pulldown experiment. As shown in Fig. 5D, the interaction between PCBP2 and the 3'-UTR of *STAT1* was only detectable in the presence of *Lnc13*. Moreover, when the *Lnc13* harboring the risk allele for T1D (rs917997*C) was overexpressed, there was a stronger interaction between PCBP2 and the 3'-UTR-STAT1 than when the *Lnc13* harboring the protective allele for T1D (rs917997*T) was overexpressed (Fig. 5E). These results suggest that *Lnc13* acts as a linker between PCBP2 and *STAT1*, and that the presence of the risk allele in *Lnc13* promotes a stronger interaction between these two molecules, inducing a more efficient stabilization of *STAT1* mRNA.

To further confirm the interaction between *Lnc13* and the 3'-UTR of *STAT1*, we next performed a RNA antisense purification (RAP) experiment to purify *Lnc13*-bound RNA molecules in non-treated and PIC-treated nuclear and cytoplasmic fractions of pancreatic β cells. *Lnc13* was purified using biotinylated antisense complementary oligos (SI Appendix, Fig. S6A) and antisense oligos complementary to a similar lncRNA were used as control (SI Appendix, Fig. S6B). As shown in Fig. 5F, both in non-treated and PIC-treated nuclear fractions, the 3'-UTR of *STAT1* was not bound to purified *Lnc13* molecules. In contrast, in PIC-treated cytoplasmic fractions, the 3'-UTR of *STAT1* was significantly bound to purified *Lnc13* when compared to the control, suggesting that the interaction between *Lnc13* and the 3'-UTR of *STAT1* occurs in the cytoplasm after PIC exposure.

Finally, to clarify whether the region containing the T1D-associated SNP in *Lnc13* molecule wass implicated in the binding between PCBP2 and 3'-UTR-*STAT1* RNA molecule, we generated a mutant *Lnc13* (*Lnc13*-delSNP) that lacked a 507 bp region (1771-

2278 bp) harboring the SNP (rs917997; 2058 bp), and performed a RNA-protein interaction assay using a native agarose gel electrophoretic mobility shift assay. As shown in Fig. 5G, we observed changes in the migration pattern of *in vitro* transcribed 3'-UTR-*STAT1* RNA molecule upon mixing with protein lysates from PCBP2+*Lnc13*-C-, PCBP2+*Lnc13*-T- or PCBP2+*Lnc13*-delSNP-overexpressing cells. While migration pattern was similar between the PCBP2+*Lnc13*-C- and the PCBP2+*Lnc13*-T-overexpressing cells, cells expressing the mutant *Lnc13* (*Lnc13*-delSNP) presented a different migration profile, suggesting that the T1D-associated SNP region was implicated in the configuration of the *Lnc13*-*STAT1*-PCBP2 complex. Moreover, these results confirmed that in the absence of *Lnc13*, PCBP2 did not bind to the 3'-UTR of *STAT1*, as the 3'UTR-*STAT1* band was not detectable in cells in which only PCBP2 was overexpressed (Fig. 5G).

The implication of the T1D-associated SNP region in *Lnc13* was further confirmed by analyzing the expression of *STAT1*, *CXCL10* and *CCL5* in *Lnc13*-C- and *Lnc13*-delSNP-overexpressing EndoC-βH1 cells. As shown in Fig. 5H, cells overexpressing the mutant *Lnc13* expressed less *STAT1*, *CXCL10* and *CCL5* than cells overexpressing the wild type *Lnc13*-C. Taking together, these results indicate that *Lnc13* enhances *STAT1* expression levels by stabilizing its mRNA in the cytoplasm through interaction with the RNA-binding protein PCBP2. Moreover, these results suggest that the region harboring the T1D-associated SNP in *Lnc13* is important for the interaction between PCBP2 and 3'-UTR of *STAT1*, and thus, for the upregulation of *STAT1* and pro-inflammatory chemokine expression in pancreatic β cells.

Discussion

Most of the genetic variants associated with type 1 diabetes detected through genome-wide association studies are located in non-coding regions of the genome, making the characterization of their functional impact on the pathogenesis of the disease challenging (29). Advances in the annotation of the human genome have revealed that many disease-associated SNPs are located within lncRNAs, affecting their function through the disruption of their secondary structure (30). In the context of T1D, previous studies have pointed out the potential role of lncRNAs in pancreatic islet biology and T1D pathogenesis (31, 32), but the precise mechanisms involved remain to be characterized.

Herein we highlight a novel molecular mechanism by which the T1D-associated *Lnc13* modulates virus-induced inflammation in pancreatic β cells, namely by sustaining the activation of the STAT1 pro-inflammatory pathway in an allele-specific manner. We observed that viral dsRNA (a by-product of viral replication) and infection by Coxsackie virus B5 induces parallel up-regulation of *Lnc13* and *STAT1* expression in pancreatic β cells. In line with this, human pancreatic islets expressing higher amounts of *Lnc13* generally have increased mRNA expression of *STAT1*. Previous studies have demonstrated a link between activation of the STAT1 signaling pathway and T1D pathogenesis. Indeed, *STAT1* and its downstream transcription factor IRF1 are aberrantly expressed in pancreatic islets of T1D patients (33, 34), and deletion of *STAT1* in non-obese diabetic (NOD) mice prevents T1D development (35), suggesting a key role for this pro-inflammatory pathway in the pathogenesis of the disease.

Overexpression of *Lnc13* in pancreatic β cells led to increased *STAT1* activation and upregulated pro-inflammatory chemokine expression, suggesting that *Lnc13* plays a regulatory role in *STAT1*-driven inflammation at the pancreatic β cell level. Indeed, *Lnc13* disruption in pancreatic β cells partially prevented viral dsRNA-induced inflammation, as revealed by reduced *STAT1* and chemokine upregulation in *Lnc13*-disrupted β cells.

Interestingly, we observed that pancreatic islets harboring the risk T1D genotype in *Lnc13* (rs917997*CC) displayed higher *STAT1* expression levels than islets carrying the heterozygous (rs917997*CT) or protective genotype (rs917997*TT). These data suggest that individuals with the risk genotype in *Lnc13* express higher amounts of *STAT1* in their islets, and this may contribute to increased β cell inflammation and apoptosis observed in T1D pathogenesis (36). Indeed, *Lnc13*-C-overexpressing β cells showed higher activation of *STAT1* in comparison with *Lnc13*-T-overexpressing cells. In line with this increase in *STAT1* signaling, *Lnc13*-C-overexpressing cells expressed and released higher amount of pro-inflammatory chemokines. Expression of chemokines has been shown to be altered in several autoimmune diseases, including T1D (37, 38). Indeed, during early stages of T1D, pro-inflammatory chemokines released by pancreatic β cells play a crucial role in attracting immune cells to the islets and generating a local inflammatory environment that eventually leads to pancreatic β cell loss (1). In this context, the fact that *Lnc13* upregulation leads to increased STAT1 activation and chemokine production rising T cell migration, indicates its potential functional implication in the pathogenesis of the disease.

In contrast to T1D, in celiac disease (CeD), where the disease-associated risk allele in *Lnc13* is the T allele, downregulation of *Lnc13* in intestinal biopsies of active CeD patients correlated with increased expression of *STAT1* and other pro-inflammatory genes (16). The functional characterization of *Lnc13* in this model revealed that *Lnc13* represses the expression of CeD-related pro-inflammatory genes via its interaction with two chromatin-associated proteins (hnRNPD and HDAC1) that negatively regulate transcription. The CeD risk allele in *Lnc13* (allele T) decreased its affinity to bind hnRNPD and chromatin, resulting in higher expression of pro-inflammatory genes, and thereby increasing the predisposition to develop CeD (16).

The results presented here demonstrate that the function of *Lnc13* is cell- and stimuli-specific, but it is equally affected by the disease-associated SNP, impacting in the regulation of T1D-associated pathways at the pancreatic β cell level. Indeed, the molecular mechanism by which *Lnc13* regulates STAT1 signaling pathway and inflammation in pancreatic β cells is linked to a PCBP2-driven mRNA-stabilization mechanism in the cytoplasm. We found that in the presence of a viral infection (a potential environmental trigger in T1D) (3, 39, 40), *Lnc13* translocates from the nucleus to the cytoplasm acting as a linker between the 3'-UTR region of *STAT1* and PCBP2, forming a complex that stabilizes the *STAT1* mRNA molecule. It has been demonstrated that PCBP2 facilitates the antiviral activity of IFNα against the Hepatitis C virus by stabilizing the mRNA of *STAT1* and *STAT2* in hepatocyte cell lines (28). Stabilization of mRNA molecules usually implies the binding of PCBP2 to the 3'-UTRs (28, 41), but some studies have revealed that PCBP2 can also bind 5'-UTRs of mRNA molecules, inhibiting their translation (42).

We observed that *Lnc13* harboring the risk allele for T1D (rs917797*C) promoted a stronger interaction between PCBP2 and the 3'-UTR of *STAT1* mRNA molecule than *Lnc13* carrying the protective allele (rs917797*T). As previously described, this autoimmune disease-associated SNP (rs917997) induces a change in the secondary structure of *Lnc13* that most likely disrupts its function and capacity to bind specific proteins (16). Indeed, the function of lncRNAs relies in their ability to form different structures and induce molecular interactions with proteins and nucleic acids (43). In this sense, we have observed that deletion of the T1D-associated SNP region in *Lnc13* molecule alters the configuration of the *Lnc13*-*STAT1*-PCBP2 complex, affecting its capacity to promote *STAT1* activation and β cell inflammation.

As links between structure and function continue to emerge, it is increasingly apparent that polymorphisms or mutations located within lncRNAs contribute to disease pathogenesis through the dysregulation of disease-associated pathways (16, 44, 45). For example, lncRNA *CCAT2,* which harbors a cancer risk-associated SNP, regulates the metabolism of cancer cells through an interaction with the CFIm complex in an allele-specific manner (44). Furthermore, arteriosclerotic vascular disease-associated SNPs modifying the structure and splicing of the lncRNA *ANRIL* have been suggested to change its function and alter the expression of downstream inflammatory genes (45).

In conclusion, our results demonstrate that *Lnc13* is implicated in the regulation of virus-induced inflammation in pancreatic β cells through a molecular mechanism that implies allele-specific modulation of the STAT1 signaling pathway activation (Fig. 6). These findings provide novel information on the molecular mechanisms by which disease-

associated SNPs in lncRNAs influence disease pathogenesis and open the door to the development of novel diagnostic and therapeutic approaches based on lncRNA targeting.

Materials and Methods

For this study, EndoC-βH1 human pancreatic β cell line (Univercell Biosolutions, Paris, France), HEK-293 cell line (ATCC, CRL-1573) and JURKAT (ATCC, TIB-152) were used. cDNA samples from human pancreatic islets were obtained from two centers, namely Cisanello University Hospital, Pisa, Italy (directly or via the ULB Center for Diabetes Research, Brussels, Belgium) and Andalusian Center for Molecular Biology and Regenerative Medicine- CABIMER, Seville, Spain. Experiments using human islets were approved by the corresponding Ethical Committee.

The EndoC-βH1 human pancreatic β cell line was transfected using synthetic viral dsRNA mimic polyinosinic:polycytidylic acid (PIC) (InvivoGen, San Diego, CA, USA). *Lnc13* and coding gene expression levels were quantified by Q-PCR, starting from DNAseI (Qiagen) treated RNA using TaqMan expression assays (Applied Biosystems) or specific primers. Plasmids overexpressing *Lnc13* were generated by cDNA cloning in a modified pCMV6 vector. For confirmation of *Lnc13* binding to PCPB2 and/or 3'-UTR-STAT1, we used the RNA immunoprecipitation, RNA pulldown and RNA antisense purification (RAP) techniques. Mix mutant cell populations with disrupted *Lnc13* were generated using CRISPR-Cas9 system.

All the data, associated protocols and materials for this study are available in the SI Appendix.

Acknowledgments

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Figures

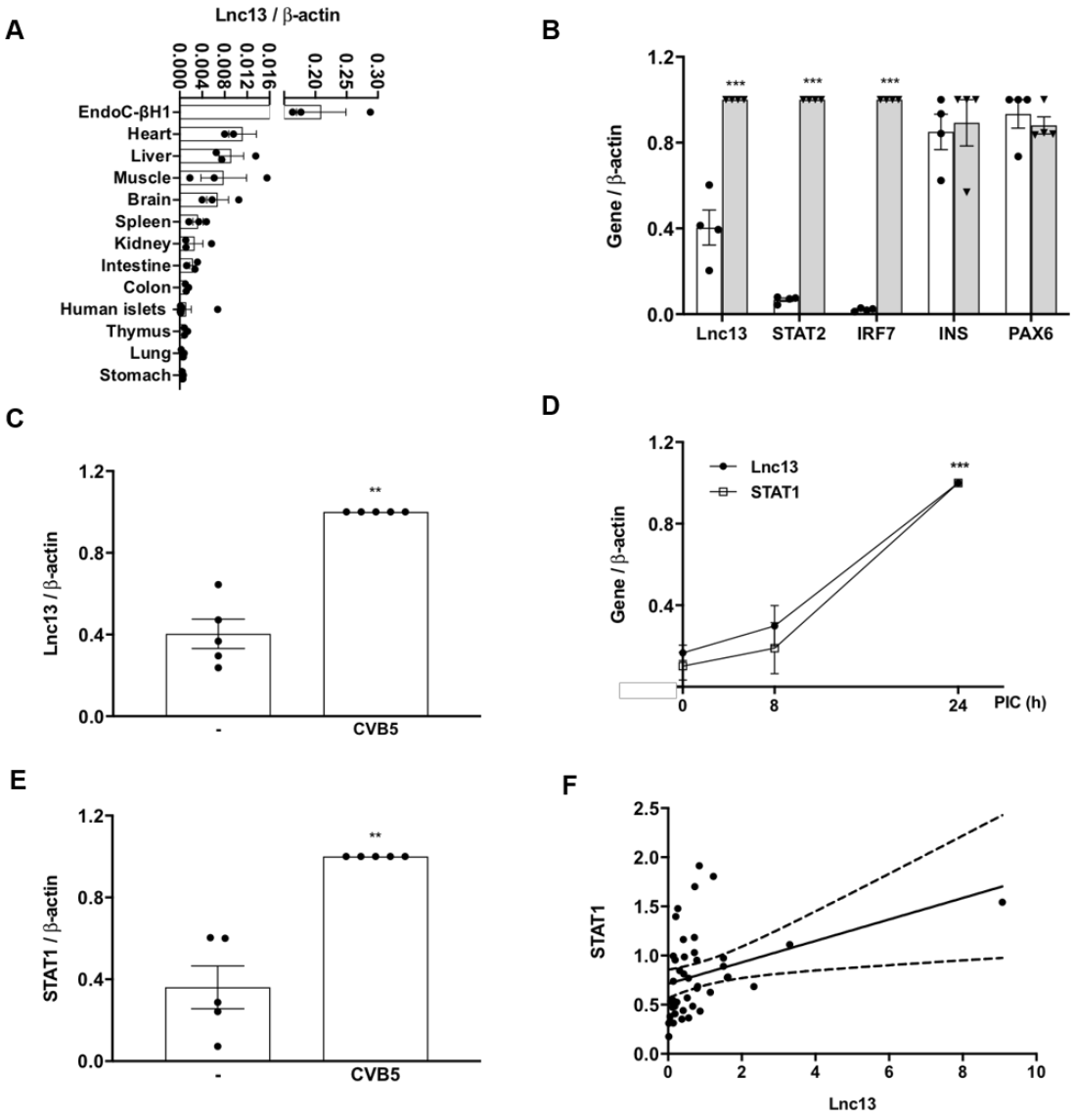

Figure 1. *Lnc13* is expressed in pancreatic β cells, upregulated by viral infections and correlates with *STAT1* expression in human pancreatic islets. (A) *Lnc13* expression was analyzed in the human β cell line EndoC-βH1, in human pancreatic islets and in a set of human tissues (heart, liver, muscle, brain, spleen, kidney, intestine, colon, thymus, lung and stomach). *Lnc13* expression was determined by Q-PCR and normalized by the

housekeeping gene *β-actin*. Results are means ± SEM of 3 experimental replicates. (B) Human EndoC-βH1 cells were left untransfected (-; white bars) or transfected with PIC (1µg/ml; grey bars) for 24h. Expression of *Lnc13*, *STAT2* and *IRF7* (positive controls), *INS* and *PAX6* (negative controls) was assayed by Q-PCR and normalized by the housekeeping gene *β-actin*. Results are means ± SEM of 4 independent experiments; ***p<0.001 vs. untreated cells; Student's *t* test. (C) Human EndoC-βH1 cells were left uninfected (-) or infected with the Coxsackie virus B5 (CVB5; MOI 5) for 24h. *Lnc13* expression was determined by Q-PCR and normalized by the housekeeping gene *β-actin*. Results are means ± SEM of 5 independent experiments; **p<0.01; Student's *t* test. (D) EndoC-βH1 were kept untreated (i.e. 0h) or treated with PIC for 8 or 24h. Relative *Lnc13* and *STAT1* expression was determined by Q-PCR and normalized by the housekeeping gene *β-actin*. Results are means ± SEM of 6 independent experiments; ***p<0.001 *vs.* time 0h; Student's *t* test. (E) Human EndoC-βH1 cells were left uninfected (-) or infected with the Coxsackie virus B5 (CVB5) for 24h. *STAT1* expression was determined by Q-PCR and normalized by the housekeeping gene *β-actin*. Results are means ± SEM of 5 independent experiments. **p<0.01; Student's *t* test. (F) *Lnc13* and *STAT1* expression was determined in 43 human pancreatic islets of non-diabetic individuals. Expression values were normalized by the housekeeping gene *β-actin*. Spearman's correlation analysis was performed to check for correlation between *Lnc13* and *STAT1* expression values; Spearman's R = 0.51 (0.24-.0.71); p<0.001.

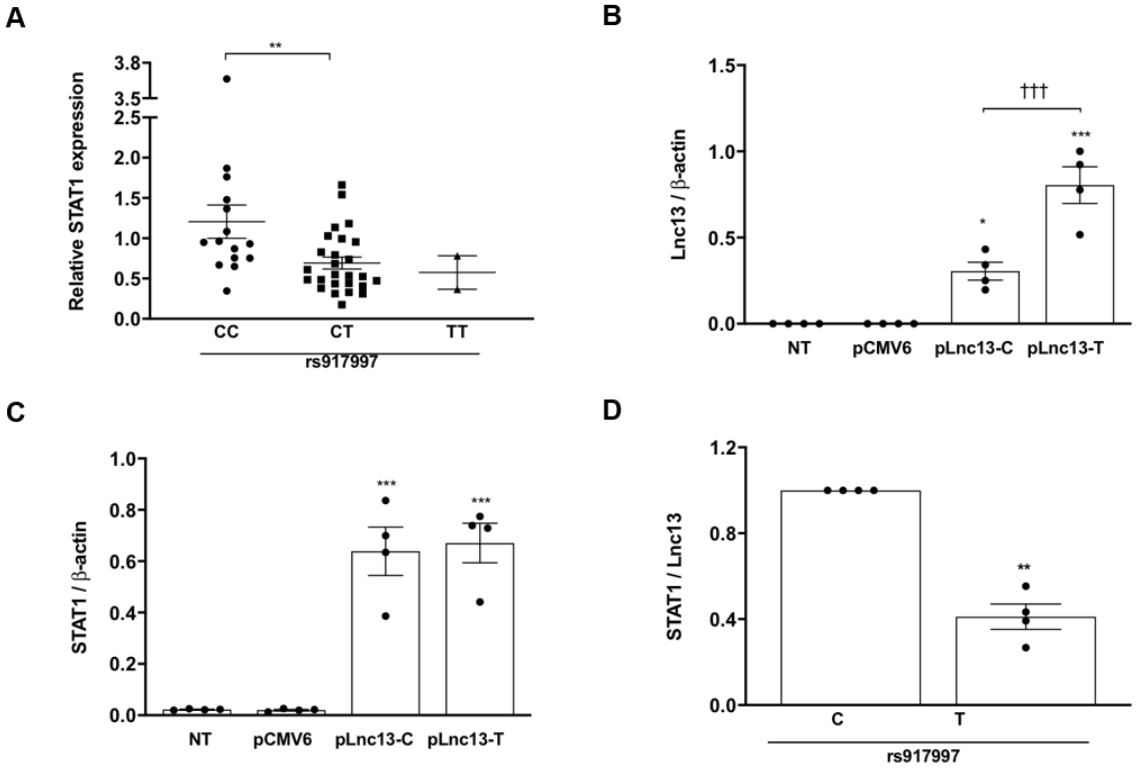

Figure 2. The T1D-associated SNP genotype in *Lnc13* gene correlates with *STAT1* expression in human pancreatic islets and overexpression of *Lnc13* in β cells leads to an upregulation of *STAT1* mRNA expression in an allele-specific manner. (A) Expression of *STAT1* in human pancreatic islets stratified by the genotype of the T1D-associated SNP rs917997 in *Lnc13* gene. Results are means ± SEM of 15 samples with the homozygous risk genotype (CC), 26 samples with the heterozygous genotype (CT) and 2 samples with the homozygous protective genotype (TT) genotype. **p<0.01; Student's *t* test. (B-C) EndoC-βH1 cells were left untransfected (NT) or transfected with pCMV6, pLnc13-C or pLnc13-T. After 48h, expression levels of *Lnc13* (B) and *STAT1* (C) were

determined by Q-PCR and normalized by the housekeeping gene *β-actin*. Results are means ± SEM of 4 independent experiments; ***p<0.001 and *p<0.05 *vs.* pCMV6-transfected cells; †††p<0.001 *vs.* pLnc13-C-transfected cells; Student's *t* test. (D) *STAT1* mRNA expression in pLnc13-C- and pLnc13-T-transfected β cells corrected by *Lnc13* expression values to control for differences in *Lnc13* allele stability. Results are means ± SEM of 4 independent experiments; **p<0.01; Student's *t* test.

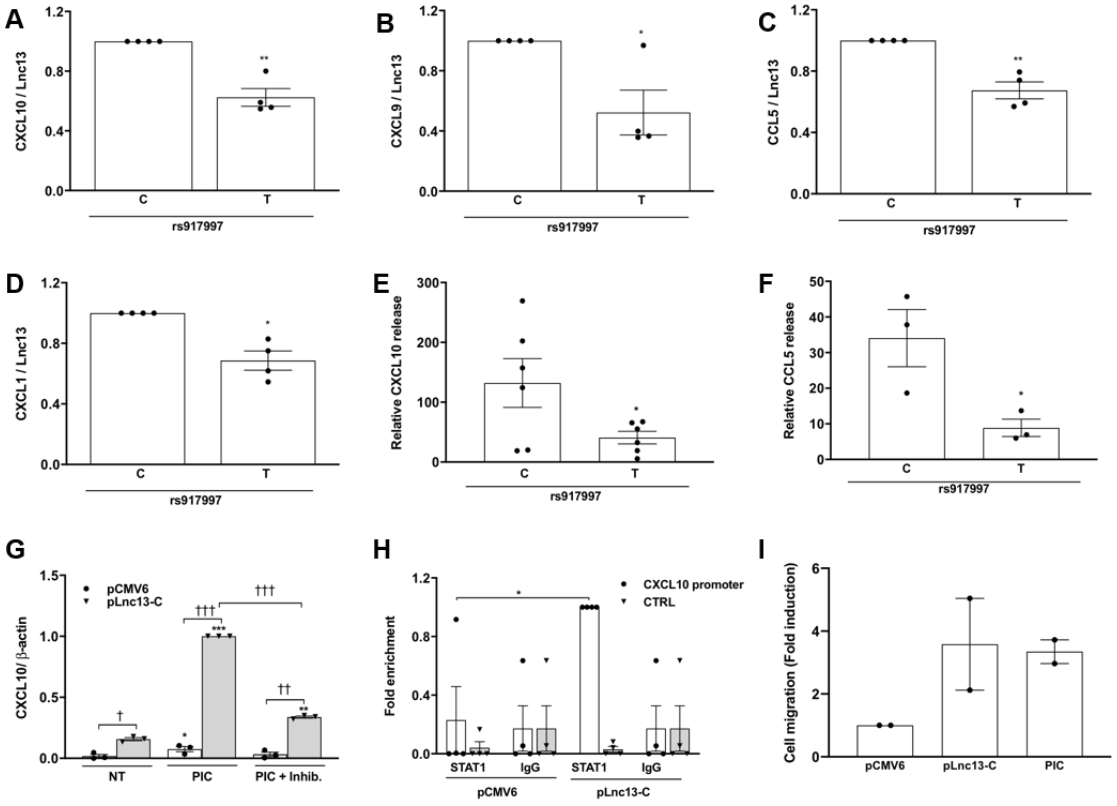

Figure 3. Overexpression of *Lnc13* upregulates pro-inflammatory chemokine production in an allele-specific manner in pancreatic β cells through STAT1 signaling activation. (A-D) EndoC-βH1 cells were transfected with pLnc13-C or pLnc13-T and mRNA levels of *CXCL10*, *CXCL9*, *CCL5* and *CXCL1* were determined by Q-PCR and normalized by the housekeeping gene *β-actin* and corrected by *Lnc13* expression values to control for differences in *Lnc13* allele stability. Results are means ± SEM of 4 independent experiments; **p<0.01 and *p<0.05 *vs.* pLnc13-C-transfected cells; Student's t test. (E-F) EndoC-βH1 cells were transfected with pLnc13-C or pLnc13-T and CXCL10 (E) and CCL5 (F) protein release to the medium was determined by ELISA in cell supernatants.

CXCL10 and CCL5 values (pg/ml) were corrected by *Lnc13* expression values to control for differences in *Lnc13* allele stability. Results are means ± SEM of 6 and 3 independent experiments, respectively. *p<0.05 *vs.* pLnc13-C-transfected cells; Student's *t* test. (G) Human EndoC-βH1 cells were transfected with pCMV6 or with pLnc13-C and subsequently left untreated (NT), treated with intracellular PIC (1 µg/ml) for 24 h (PIC) or treated with PIC and ruxolitinib for 24 h (PIC + Inhib). *CXCL10* mRNA expression was measured by Q-PCR and normalized by the housekeeping gene *β-actin*. The results are means ± SEM of 3 independent experiments; **p<0.01 and *p<0.05 *versus* NT and transfected with the same plasmid; $^{\dagger\dagger\dagger}p$<0.001, $^{\dagger\dagger}p$<0.01 and $^{\dagger}p$<0.05 as indicated; ANOVA followed by Student's *t* test followed with Bonferroni correction. (H) EndoC-βH1 were transfected with pCMV6 or pLnc13-C, chromatin was fragmented and precipitated with anti-STAT1 or anti-IgG (as negative control), CXCL10 promoter or a control region (Oct4 gene body) was amplified by Q-PCR. Results are means ± SEM of 4 independent experiments; *p<0.05 as indicated; ANOVA followed by Student's *t* test followed with Bonferroni correction. (I) Supernatants of pCMV6- or pLnc13-C-transfected EndoC-βH1 cells were used to determine chemotactic migration of Jurkat cells using a transwell system and a fluorescence-based assay. Supernatant of PIC-transfected cells was used as positive control. The results are means ± SEM of 2 independent experiments.

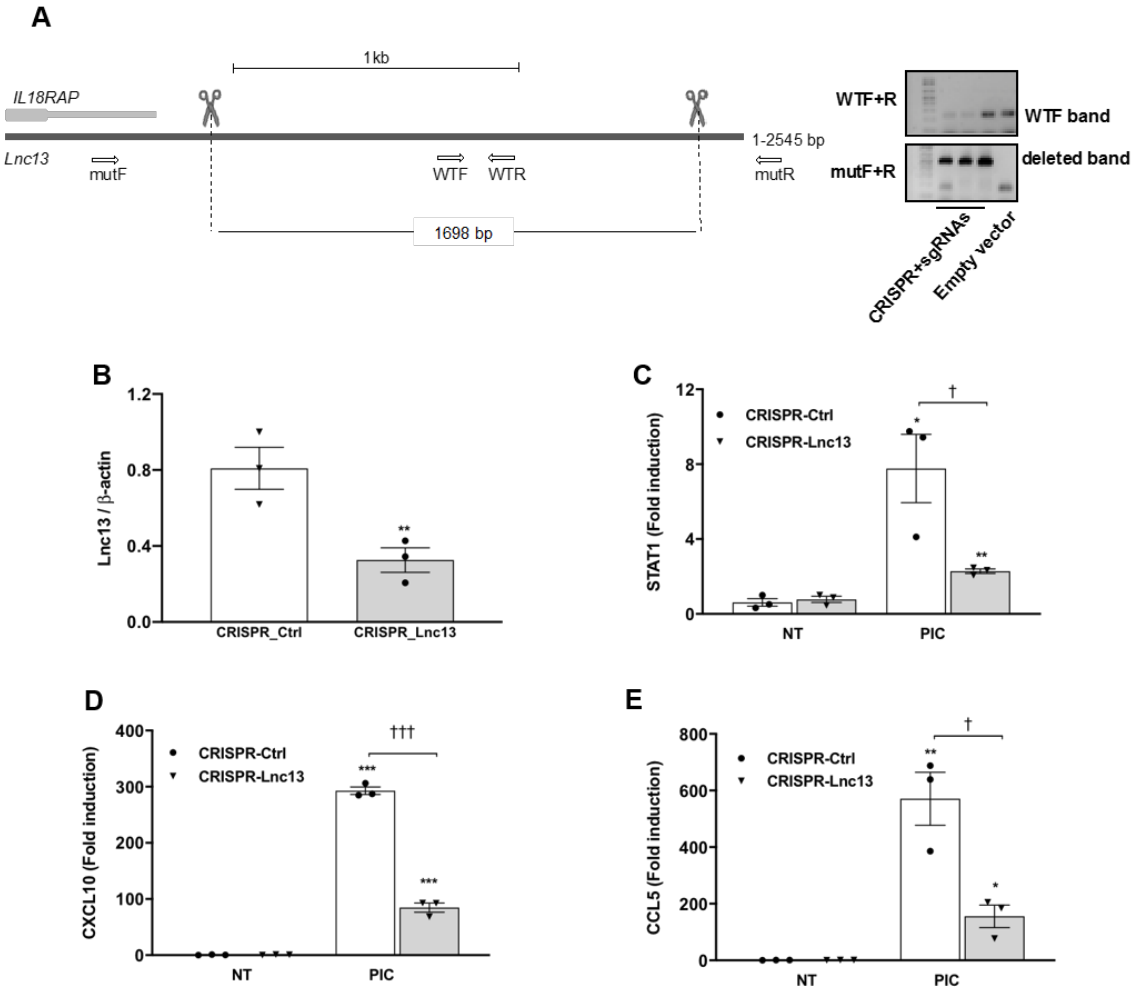

Figure 4. *Lnc13* gene disruption in pancreatic β cells partially counteracts the effect of PIC in *STAT1* and pro-inflammatory chemokine expression upregulation. (A) *Lnc13* disruption was performed by generating a deletion of 1698 bp using CRISPR-Cas9 technique and sgRNAs targeting *Lnc13* gene. Presence of the deletion was confirmed by PCR using a pair of primers located inside the deleted region (for detection of unedited cells; WTF and WTR) and a pair of primers located outside the deleted region (for detection

of edited cells; mutF and mutR). (B) EndoC-βH1 cells transfected with an empty px459 vector (CRISPR-Ctrl) or with a vector harboring the *Lnc13* targeting sgRNAs (CRISPR-*Lnc13*). *Lnc13* expression was determined by Q-PCR and normalized by the housekeeping gene *β-actin*. The results are means ± SEM of 3 independent cell populations. **p<0.01; Student's *t* test. (C-E) Control and *Lnc13*-disrupted mixed cell populations were exposed to intracellular PIC for 24h, *STAT1* (C), *CXCL10* (D) and *CCL5* (E) expression was determined by Q-PCR and normalized by the housekeeping gene *β-actin*. The results are represented as fold induction and are means ± SEM of 3 independent cell populations. ***p<0.001, **p<0.01 and *p<0.05 vs. non-treated cells; †††p<0.0001, ††p<0.01 and †p<0.05 as indicated.

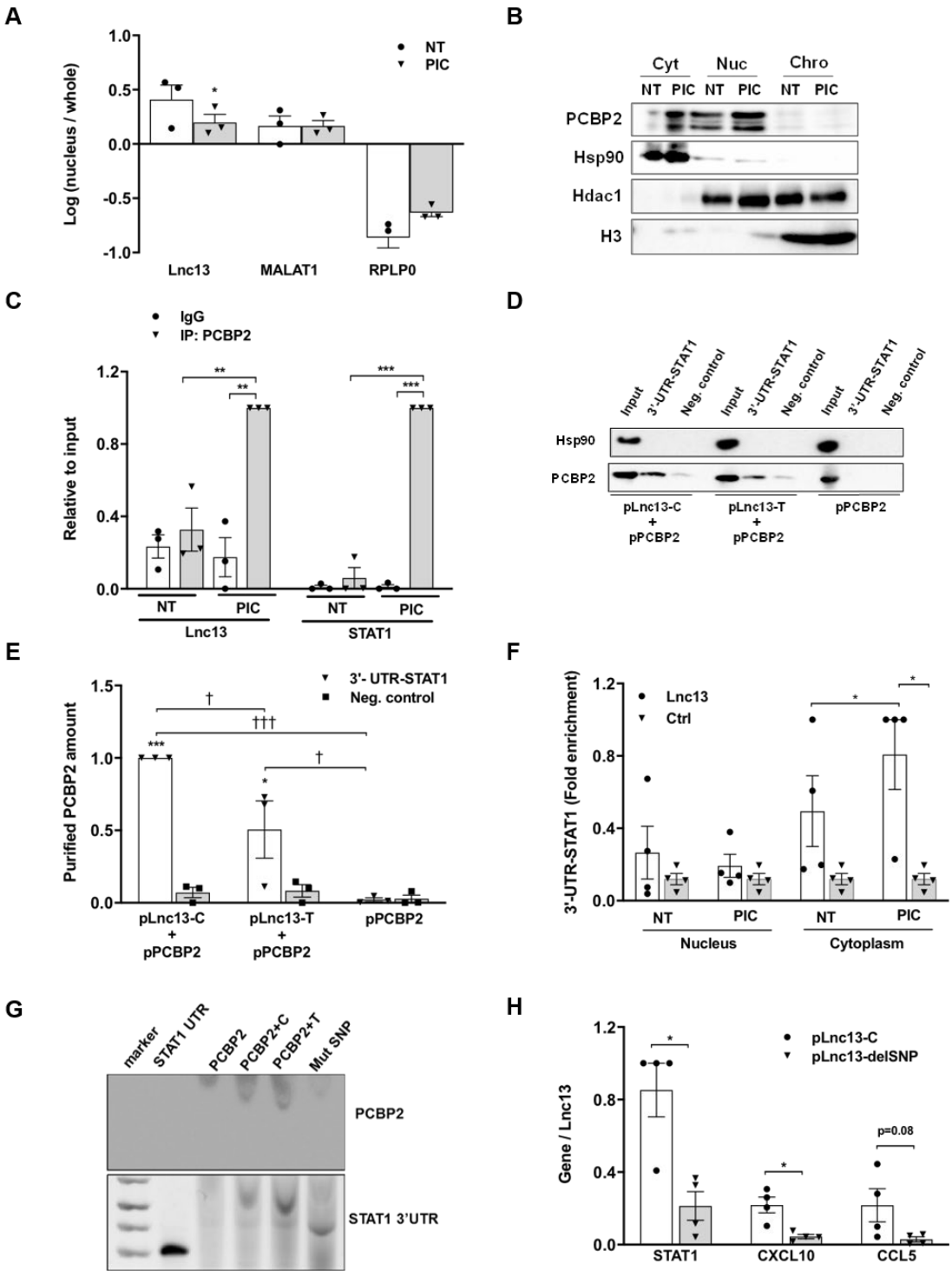
A
Log (nucleus / whole)
NT
PIC
Lnc13
MALAT1
RPLP0
B
Cyt
Nuc
Chro
NT PIC NT PIC NT PIC
PCBP2
Hsp90
Hdac1
H3
C
IgG
IP: PCBP2
Relative to input
NT
PIC
Lnc13
STAT1
D
Input
3'-UTR-STAT1
Neg. control
Hsp90
PCBP2
pLnc13-C + pPCBP2
pLnc13-T + pPCBP2
pPCBP2
E
3'- UTR-STAT1
Neg. control
Purified PCBP2 amount
pLnc13-C + pPCBP2
pLnc13-T + pPCBP2
pPCBP2
F
Lnc13
Ctrl
3'-UTR-STAT1 (Fold enrichment)
NT
PIC
Nucleus
Cytoplasm
G
marker
STAT1 UTR
PCBP2
PCBP2+C
PCBP2+T
Mut SNP
PCBP2
STAT1 3'UTR
H
pLnc13-C
pLnc13-delSNP
Gene / Lnc13
p=0.08
STAT1
CXCL10
CCL5

Figure 5. Upon exposure to PIC *Lnc13* translocate from the nucleus and facilitates the interaction between PCBP2 and the 3'-UTR of *STAT1* mRNA. (A) EndoC-βH1 cells were exposed to synthetic dsRNA PIC (1μg/ml) for 24h and relative *Lnc13* expression was determined in nuclear and whole extracts. Expression of *MALAT1* and *RPLPO* was used as a control for nuclear and whole cell fractions, respectively. Amounts of specific nuclear RNA was measured by Q-PCR and compared to the total amount of RNA in the whole cell. Results are represented as log (nucleus /whole) and are means ± SEM of 3 independent experiments; *p<0.05; Student's *t* test. (B) EndoC-βH1 were exposed to PIC (1μg/ml) for 24h and cytoplasm (Cyt), nuclear (Nuc) and chromatin (Chro) fractions were purified. PCBP2 protein expression was determined in all cellular compartments by Western blot and protein expression of Hsp90, HDAC1 and H3 was used as a control for cytoplasmic, nuclear and chromatin fractions, respectively. The results are representative of 3 independent experiments. (C) EndoC-βH1 cells were left non-transfected (NT) or transfected with PIC for 24h. RNA immunoprecipitation was performed using a specific antibody for PCBP2 and *Lnc13* and *STAT1* expression was determined in PCBP2-bound RNA by Q-PCR. Results are means ± SEM of 3 independent experiments and the amount of *Lnc13* and *STAT1* are expressed as relative to the input. IgG was used as negative control; ***p<0.001 and **p<0.01 as indicated; ANOVA followed by Student's *t* test with Bonferroni correction. (D) *In vitro* transcribed biotinylated 3'-UTR region of *STAT1* was incubated with cellular extracts overexpressing *Lnc13*-C+PCBP2, *Lnc13*-T+PCBP2 or PCBP2. Afterwards, 3'-UTR-STAT1-bound proteins were purified using streptavidin beads and PCBP2 and Hsp90 (as negative control) were detected by Western blot. Incubation with streptavidin beads alone was used as negative control. The results are

representative of 3 independent experiments. (E) Densitometry results for purified PCBP2 amounts in RNA pull down experiments are represented as means ± SEM of 3 independent experiments. ANOVA followed by Student's *t* test with Bonferroni correction; ***p<0.001 and *p<0.01 vs. negative control; †††p<0.001 and †p<0.05 as indicated. **(**F) *Lnc13* antisense purification was performed in non-treated (NT) and PIC-treated nuclear and cytoplasmic fractions of EndoC-βH1 cells. *Lnc13*-bound 3'-UTR-*STAT1* amounts were determined by Q-PCR using specific primers. A non-related similar lncRNA was used as negative control (Ctrl). Results are represented as fold enrichment and are means ± SEM of 4 independent experiments. *p<0.05 as indicated; ANOVA followed by Student's *t* test with Bonferroni correction. (G) RNA-protein interaction assay. Cells were transfected with pPCBP2 alone, pPCBP2+pLnc13-C, pPCBP2+pLnc13-T or pPCBP2+pLnc13-delSNP. Cell lysates were incubated with *in vitro* transcribed 3'-UTR-*STAT1* molecules and a native agarose gel electrophoretic mobility shift assay was performed (lower panel). After electrophoresis, proteins were transferred to a nitrocellulose membrane for PCBP2 visualization (upper panel). The 3'-UTR-*STAT1* RNA molecule alone was loaded as control. The results are representative of three independent experiments. (H) EndoC-βH1 cells were transfected with a plasmid encoding *Lnc13*-C or a mutant *Lnc13* in which the region containing the T1D-associated SNP was deleted (pLnc13-delSNP). *STAT1*, *CXCL10* and *CCL5* expression was determined by Q-PCR and normalized by the housekeeping gene *β-actin* and corrected by *Lnc13* expression values. Results are means ± SEM of 4 independent experiments. p<0.05 as indicated; Student's *t* test.

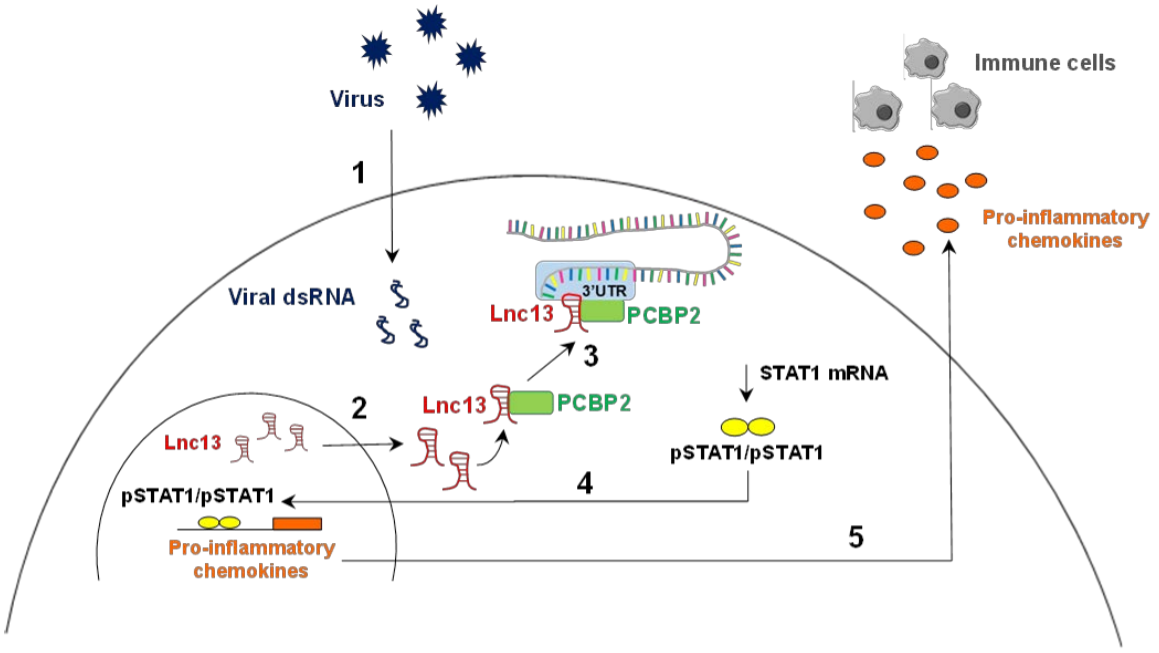

Figure 6. *Lnc13* participates in virus-induced pancreatic β cell inflammation by stabilizing STAT1 mRNA through interaction with PCBP2 protein. Viral infections in pancreatic β cells lead to the generation of viral dsRNA that is recognized by viral dsRNA cytoplasmic receptors (1). Viral dsRNA induces a decrease in DCP2 expression that leads to an increase in *Lnc13* RNA levels. In addition, viral dsRNA provokes the translocation of *Lnc13* from the nucleus to the cytoplasm (2). Once in the cytoplasm, *Lnc13* interacts with PCBP2 to bind the 3'UTR of *STAT1* mRNA and induce its stabilization (3). Increased *STAT1* mRNA levels lead to increased protein and provides the substrate for generation of activated STAT1 (phosphorylated STAT1; pSTAT1). pSTAT1 forms homodimers and translocate to the nucleus, promoting the expression of several pro-inflammatory chemokines (4). Chemokines are then released by pancreatic β cells, attracting immune cells into the islets (5). In genetically susceptible individuals harbouring the T1D risk

genotype in *Lnc13* (rs917997*CC), the function of *Lnc13* is affected, leading to an excessive antiviral inflammatory response that contributes to β cell destruction in type 1 diabetes.