CEBPA exerts a specific and biologically important proapoptotic role in pancreatic β cells through its downstream network targets

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ABSTRACT **Transcription factor CEBPA has been widely studied for its involvement in hematopoietic cell differentiation and causal role in hematological malignancies. We demonstrate here that it also performs a causal role in cytokine-induced apoptosis of pancreas β cells. Treatment of two mouse pancreatic α and β cell lines (αTC1-6 and βTC1) with proinflammatory cytokines IL-1β, IFN-γ, and TNF-α at doses that specifically induce apoptosis of βTC1 significantly increased the amount of mRNA and protein encoded by *Cebpa* and its proapoptotic targets, *Arl6ip5* and T*nfrsf10b*, in βTC1 but not in αTC1-6. *Cebpa* knockdown in βTC1 significantly decreased cytokine-induced apoptosis, together with the amount of *Arl6ip5* and *Tnfrsf10b*. Analysis of the network comprising CEBPA, its targets, their first interactants, and proteins encoded by genes known to regulate cytokine-induced apoptosis in pancreatic β cells (genes from the apoptotic machinery and from MAPK and NFkB pathways) revealed that CEBPA, ARL6IP5, TNFRSF10B, TRAF2, and UBC are the top five central nodes. In silico analysis further suggests TRAF2 as *trait d'union* node between CEBPA and the NFkB pathway. Our results strongly suggest that *Cebpa* is a key regulator within the apoptotic network activated in pancreatic β cells during insulitis, and *Arl6ip5*, *Tnfrsf10b*, *Traf2*, and *Ubc* are key executioners of this program.**

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INTRODUCTION

CEBPA was the first identified member out of six within the CEBP family of transcription factors (Johnson *et al.*, 1987; Tsukada *et al.*, 2011). It is involved in several important biological processes, including negative regulation of cell cycle progression, hematopoietic cell differentiation, and apoptosis; within them, it may act as either transcription activator or repressor, depending on cell context and target genes (Nerlov, 2007; Eyholzer *et al.*, 2010; Pulikkan *et al.*, 2010; Yoshida *et al.*, 2012; Zhang *et al.*, 2013). Several reports describe specific CEBPA mutations affecting its tumor-suppressive functions and associated with hematological malignancies such as acute myeloid leukemia (Gery *et al.*, 2005; Fuchs *et al.*, 2010). Insulitis is a pathological state of islets of Langerhans, which is triggered by the interplay between local hypersecretion of proinflammatory cytokines and ensuing infiltration of lymphocytes and macrophages (Donath *et al.*, 2008; Di Gialleonardo *et al.*, 2012). It activates complex genetic and epigenetic regulatory networks and frequently leads to apoptosis or dedifferentiation of pancreatic β cells (Darville and Eizirik, 2006; Eizirik *et al.*, 2009; Bramswig *et al.*, 2013; Schaffer *et al.*, 2013); these may be forerunner of diabetes mellitus (DM). To date, within the CEBP family, only CEBPD and DDIT3 have been associated with cytokine-induced apoptosis of pancreatic β cells (Allagnat *et al.*, 2012; Moore *et al.*, 2012). By analyzing mouse α and β pancreatic cells (αTC1-6 and βTC1) in a model of insulitis, we previously showed that cytokines significantly and specifically increased apoptotic levels of pancreatic β cells, in contrast to pancreatic α

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Abbreviations used: DE, differentially expressed; Dox, doxycycline; ECR, evolutionarily conserved region; HT, high-throughput; NC, untreated control; PT, post-treatment; RQ, relative quantity; TFBS, transcription factor–binding site; TLDA, TaqMan low-density array; TSS, transcription start site.

cells (Barbagallo *et al.*, 2013). In this article, we report our expression profiling of 92 genes belonging to the apoptotic machinery (AM; Di Pietro *et al.*, 2009) in αTC1-6 and βTC1, both at steady state and after treatment with proinflammatory cytokines interleukin 1β (IL-1β), interferon γ (IFN-γ), and tumor necrosis factor α (TNF-α). This allows us to identify and characterize the causal involvement of CEBPA and its downstream network targets in cytokine-induced apoptosis of β cells.

RESULTS

AM transcriptome profiling reveals marked differences between αTC1-6 and βTC1 in response to cytokines

By high-throughput (HT) real-time PCR, we demonstrated that cytokines significantly induced overexpression of 21 and 55 AM genes (out of 92 analyzed) in αTC1-6 and βTC1, respectively, during an experiment time course of 24 and 48 h as compared with matched, untreated controls (NCs; Limma test, Benjamini–Hochberg adjusted $p \leq 0.05$; Supplemental Tables S1 and S2). The number of differentially expressed (DE) genes in treated βTC1 was significantly higher than in αTC1-6 (Fisher's exact test, $p < 0.0001$). Only two genes (*Bmf* and *Gadd45b*) were specifically DE in αTC1-6, whereas 36 were specifically DE in βTC1 after treatment with respect to NCs (Table 1). Literature mining revealed that nine, four, and eight of 21 DE genes in αTC1-6 were proapoptotic, antiapoptotic, or potentially endowed with either proapoptotic or antiapoptotic functions, depending on cell phenotype, respectively; a similar analysis on DE genes in βTC1 led us to classify 27, 16, 12, of 55 genes as proapoptotic, antiapoptotic, or potentially endowed with either proapoptotic or antiapoptotic functions, depending on the cell phenotype, respectively (Supplemental Tables S1 and S2).

IL-1β, IFN-γ, and TNF-α up-regulate *Cebpa* mRNA and protein expression in βTC1

On the basis of our TaqMan low-density array (TLDA) data, we decided to focus on *Cebpa*: its steady-state mRNA levels were very low both in αTC1-6 and βTC1, but they significantly increased specifically in βTC1 after treatment with cytokines. We validated these data through specific single real-time PCR assays performed on three independent biological replicates. We observed a greater-than-twofold significant increase of *Cebpa* mRNA in βTC1 starting at 6 h after treatment (PT) with cytokines (Student's *t* test, $p < 0.001$; Figure 1A). Greater-than-ninefold increased expression of CEBPA protein was detected in βTC1 at 24 h PT as compared with NCs (Figure 1B). In contrast, no variation of CEBPA levels was detected in αTC1-6 at the same time points PT (Figure 1B).

Cebpa knockdown with specific small interfering RNAs decreases cytokine-induced apoptosis levels in βTC1

To investigate whether *Cebpa* overexpression was causally involved in apoptosis induction of cytokine-treated βTC1, we functionally knocked it down and then searched for alterations of apoptotic levels with respect to matched scramble-transfected, cytokine-treated βTC1 cells. Our data show a significant decrease of apoptosis (>2.5-fold) in βTC1 transfected with siRNAs targeting *Cebpa* and treated with cytokines for 24 h as compared with matched βTC1 transfected with scramble molecules and treated with cytokines (Tukey honestly significant difference [HAD] post hoc one-way analysis of variance [ANOVA] test, $p < 0.01$; Figure 2A). At the same time point, we observed a 5.5-fold decrease of CEBPA in βTC1 transfected with si*Cebpa* and treated with cytokines as compared with matched scramble-transfected cells (Figure 2B).

Cell type	DE genes
αTC1-6	*Bmf*, *Gadd45b*
βTC1	*Akt1*, *Apaf1*, *Bax*, *Bcl2l1*, *Birc2*, *Birc3*, *Cebpa*, *Chuk*, *Dapk1*, *Ddit3*, *Dedd2*, *Dffa*, *Dffb*, *Diablo*, *Ern1*, *Gadd45a*, *Htra2*, *Irs2*, *Jak2*, *Lrdd*, *Map3k14*, *Mapk14*, *Mapk4*, *Mdm2*, *Ptpn13*, *Rela*, *Rel*, *Stat5a*, *Stat5b*, *Tax1bp1*, *Tnfrsf10b*, *Tnfrsf21*, *Traf2*, *Traf3*, *Traf6*, *Trp53*

TABLE 1: Genes specifically DE in αTC1-6 and in βTC1 after treatment with cytokines.

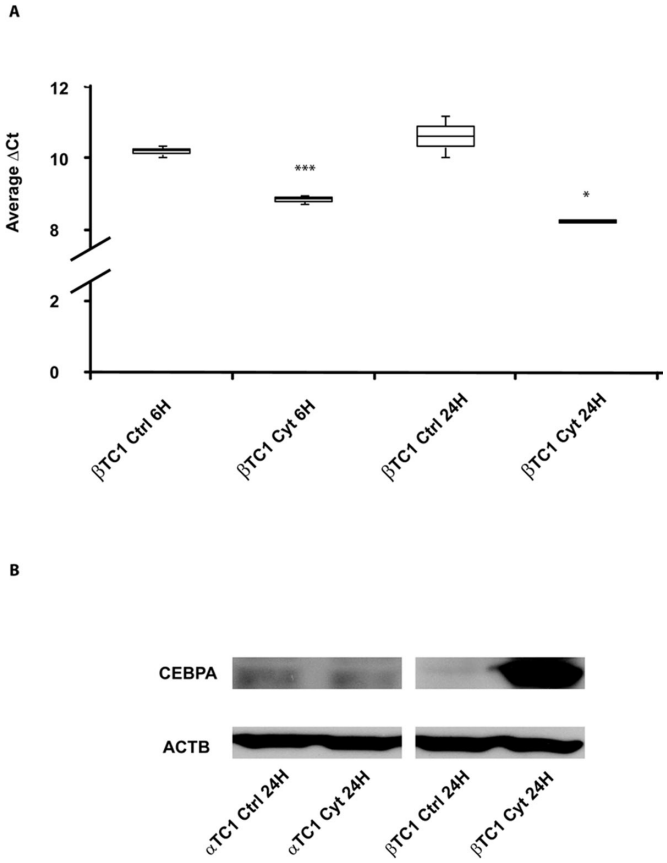

FIGURE 1: *Cebpa* mRNA and protein expression in pancreatic αTC1-6 and βTC1. (A) Levels of *Cebpa* mRNA in untreated (Ctrl, $n = 3$) and cytokine-treated (Cyt, $n = 3$) βTC1 at 6 and 24 h PT. Box plots with whiskers from minimum to maximum represent ΔCt values. *Two-tailed $p < 0.05$, ***two-tailed $p < 0.001$; paired Student's *t* test. (B) Western blot of CEBPA in αTC1-6 (left) and βTC1 (right) untreated (Ctrl) and treated (Cyt) with cytokines for 24 h. β-Actin (ACTB) was used as loading control.

Arl6ip5 and *Tnfrsf10b* expression positively correlates with that of *Cebpa* and increases in βTC1 after treatment with cytokines

Literature mining led us to identify a total of 16 genes as CEBPA targets (validated or whose expression had been demonstrated to be induced by CEBPA in other cell phenotypes; Supplemental Table S3). Owing to their known involvement in apoptosis induction, we focused on two genes, *Arl6ip5* and *Tnfrsf10b*. Single real-time PCR assays showed significant overexpression of both in βTC1 24 h PT as compared with matched, untreated controls (Figure 3, A and B). *Arl6ip5* mRNA expression was also positively correlated with that

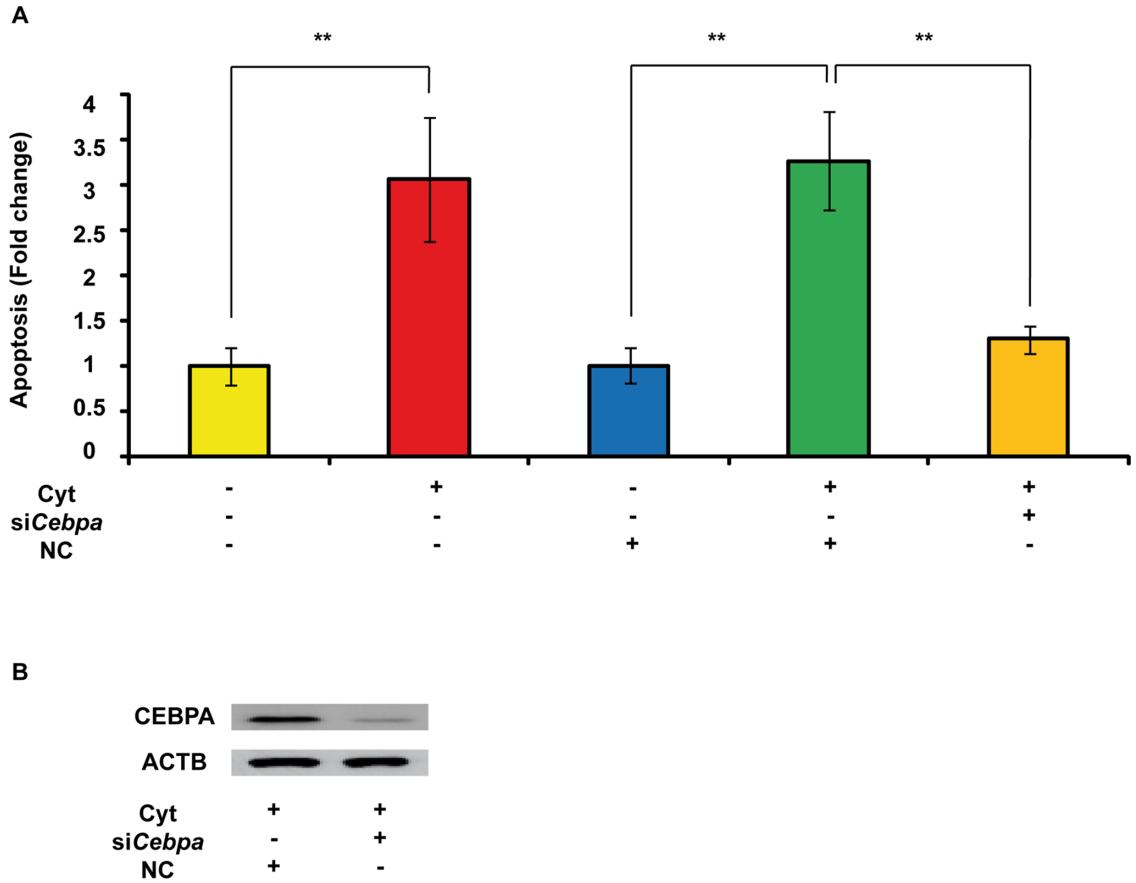

FIGURE 2: Apoptosis induced by cytokines in si*Cebpa*-transfected βTC1. (A) Apoptosis, determined by annexin V/PI method, is described as fold change with respect to controls. Data are presented as mean ± SD of three independent experiments ($n = 3$). **$p < 0.01$, Tukey honestly significant difference (HSD) post hoc one-way ANOVA test. (B) Western blot of CEBPA in si*Cebpa*-transfected βTC1 treated with cytokines and matched scramble-transfected controls. β-Actin (ACTB) was used as loading control.

of *Cebpa* ($r = 0.66$, $p = 0.019$; Pearson correlation test) during the whole time course of the experiment (6 and 24 h) in βTC1 (Supplemental Figure 1A); in the same biomolecular context, *Tnfrsf10b* mRNA expression was slightly but not significantly positively correlated with that of *Cebpa* ($r = 0.40$, $p = 0.19$; Pearson correlation test; Supplemental Figure 1B). We did not detect any significant variation of *Arl6ip5* mRNA expression in αTC1-6 at 24 h PT; instead, *Tnfrsf10b* mRNA levels significantly increased with respect to matched, untreated controls at the same time point, even if to a lower extent than in βTC1 (Figure 4A). However, and contrary to βTC1, the levels of TNFRSF10B protein did not vary in αTC1-6 after the same treatment with cytokines (Figure 4B). Expression of *Arl6ip5* and *Tnfrsf10b* mRNAs significantly decreased in βTC1 transfected with si*Cebpa* and treated with cytokines for 6 h as compared with scramble-transfected βTC1 exposed to the same cues (Figure 3, A and B). Similarly, ARL6IP5 and TNFRSF10B proteins decreased their expression (1.7- and 1.3-fold, respectively) in βTC1 transfected with si*Cebpa* and treated with cytokines for 24 h as compared with scramble-transfected βTC1 exposed to the same cues (Supplemental Figure S2).

Arl6ip5 and *Tnfrsf10b* knockdown decreases βTC1 apoptosis after treatment with cytokines

To evaluate the functional involvement of *Arl6ip5* and *Tnfsrf10b* in apoptosis induced by cytokines in βTC1, we transiently transfected βTC1 with siRNAs targeting both mRNAs, functionally knocking them down. In both cases, we detected a general decrease of apoptosis in cells transfected with siRNAs with respect to scramble-transfected cells. More specifically, functional knockdown of *Tnfrsf10b* determined a 1.5-fold decrease of apoptosis in βTC1 treated with cytokines as compared with matched controls (Figure 5A). The anti-apoptotic effect of *Arl6ip5* functional knockdown was less pronounced: βTC1 transfected with siRNAs targeting *Arl6ip5* showed 1.2-fold decrease of apoptosis when treated with cytokines as compared with matched scramble-transfected cells (Figure 6A). In the same experimental conditions, we observed 2.1- and 1.8-fold decrease of TNFRSF10B and ARL6IP5 proteins in βTC1 transfected with si*Tnfrsf10b* and si*Arl6ip5* and treated with cytokines as compared with matched scramble-transfected cells, respectively (Figures 5B and 6B).

Cebpa mRNA expression is significantly reduced in INS-1rαβ cells after *Pdx1* induction

To investigate the relationship between *Cebpa* expression and the differentiated phenotype of pancreatic β cells, we induced *Pdx1* expression in INS-1rαβ cells by treating them with doxycycline (dox) for 36 and 48 h. We detected a significant decrease of *Cebpa* mRNA in INS-1rαβ at 36 and 48 h after exposure to dox with respect to NCs. We also detected significantly decreased *Tnfrsf10b* mRNA levels in INS-1rαβ at 36 h after treatment with dox (Figure 7).

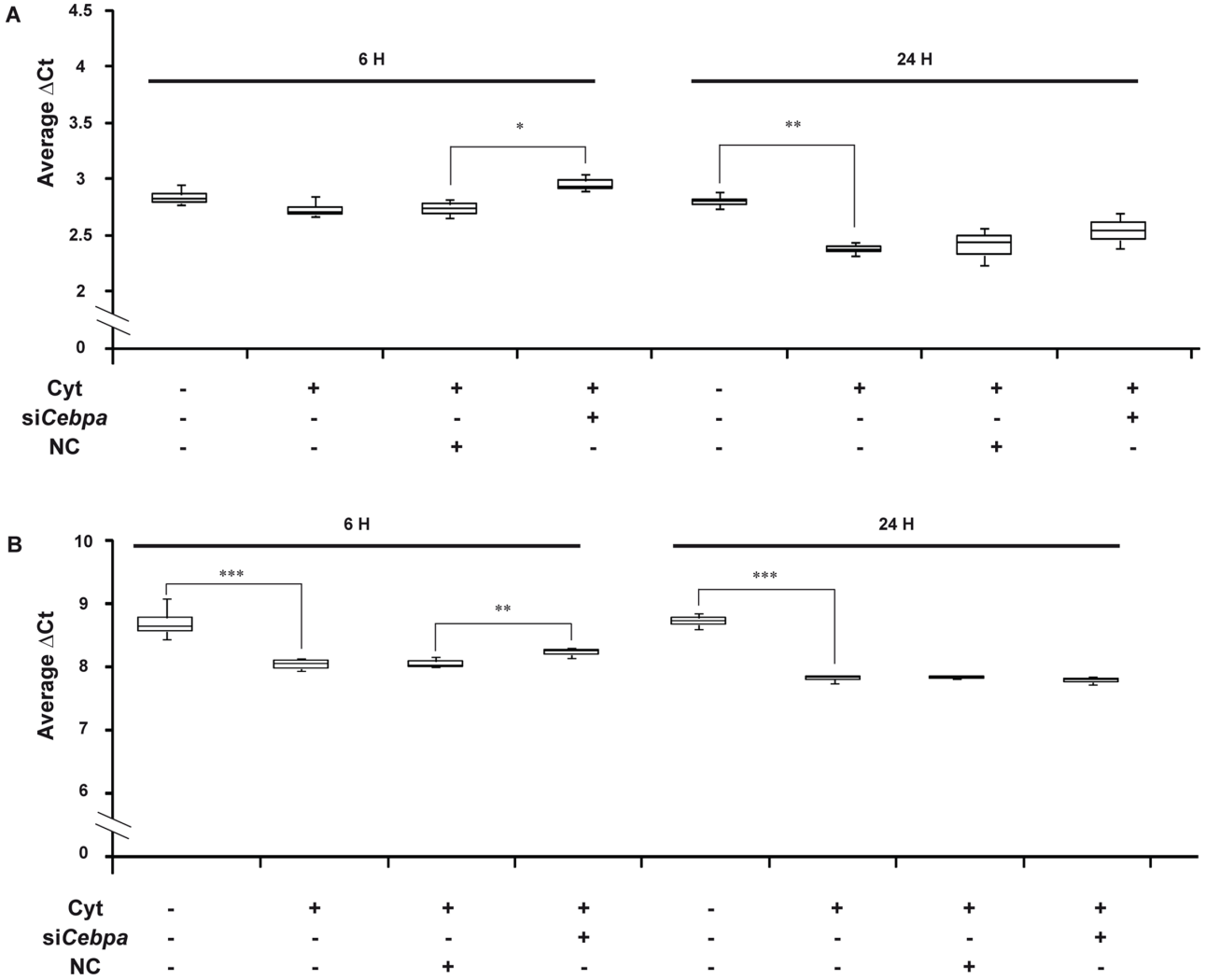

FIGURE 3: *Arl6ip5* and *Tnfrsf10b* mRNA expression at steady state and after treatment with cytokines for 6 and 24 h in si*Cebpa*-transfected βTC1. (A) Levels of *Arl6ip5* mRNA at 6 and 24 h PT. (B) Levels of *Tnfrsf10b* mRNA at 6 and 24 h PT. Box plots with whiskers from minimum to maximum represent Δ*Ct* values. *Two-tailed $p < 0.05$, **two-tailed $p < 0.01$, ***two-tailed $p < 0.001$; paired Student's *t* test ($n = 3$).

Reconstruction of *Cebpa* downstream transcriptional network

In silico analysis through the LASAGNA algorithm revealed four and six CEBPA transcription factor–binding sites (TFBSs) within a 5-kb genomic region upstream of the *Arl6ip5* and *Tnfrsf10b* transcription start site (TSS), respectively (Supplemental Figure 3, A and B). Of interest, by using the ECR Browser we identified a TFBS for NFKB1 (localized within an evolutionarily conserved region [ECR] in both humans and mice) near the CEBPA TFBS centered 2251 base pairs upstream of the *Arl6ip5* TSS (Supplemental Figure 3A). The ECR Browser also identified a TFBS for DDIT3:CEBPA centered 3070 base pairs upstream of the *Tnfrsf10b* TSS and conserved between humans and mice (Supplemental Figure 3B). On the basis of these data, we generated a network (made of physical and functional interactions, mapped within the whole mouse interactome) of 182 nodes and 336 edges arising from the union of the following subnetworks: 1) ARL6IP5, CEBPA, NFKB1, PDX1, TNFRSF10B, and their first-neighbors interactants; 2) proteins encoded by AM genes, analyzed in TLDA (this article); and 3) proteins encoded by genes belonging to mitogen-activated protein kinase (MAPK) and NFkB pathways (Ragusa *et al.*, 2012). The latter pathways were considered because of their known critical involvement in triggering apoptosis of cytokine-treated pancreatic β cells. Intersecting results from the analysis of several centrality parameters (see *Materials and Methods*) showed CEBPA, ARL6IP5, TNFRSF10B, TRAF2, and UBC as the top five central nodes (Figure 8). In silico analyses of biological processes and pathways of this network showed an overrepresentation of genes involved in cell death and degenerative (DM and Alzheimer's disease) and neoplastic diseases (Supplemental Table S4).

In silico analysis of promoter regions of *Cebpa, Arl6ip5, Tnfrsf10b, Traf2*, and *Ubc* reveals the presence of several cytokine-responsive elements evolutionarily conserved between humans and mice

To find a direct link between the top central nodes of this network (ARL6IP5, CEBPA, TNFRSF10B, TRAF2, UBC) and apoptosis induction by cytokines of pancreatic β cells, we searched for evolutionarily conserved TFBSs for CEBP family members and for cytokines-responsive elements within their putative promoter regions. The ECR

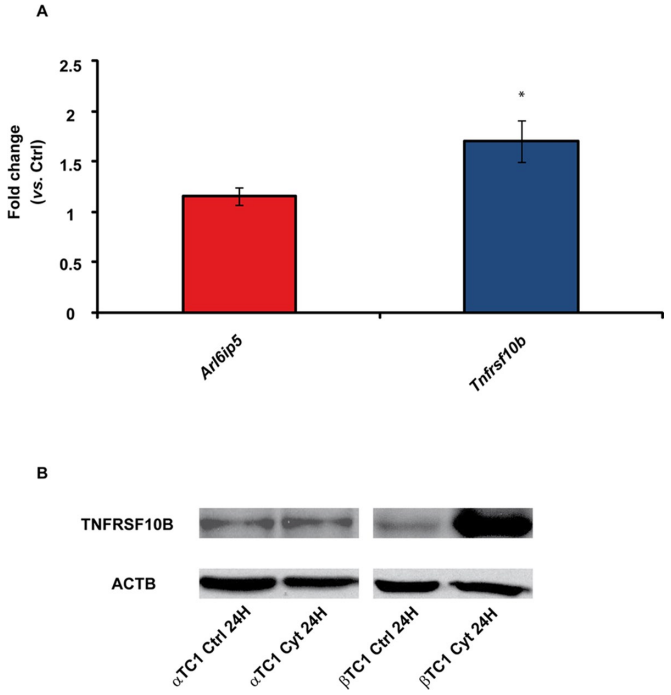

FIGURE 4: *Arl6ip5* and *Tnfrsf10b* mRNA expression in αTC1-6 and βTC1. (A) *Arl6ip5* (red bar) and *Tnfrsf10b* (blue bar) mRNA expression in αTC1-6 after 24 h of treatment with cytokines. Values are reported as fold changes relative to matched untreated controls. *Two-tailed $p < 0.05$; paired Student's *t* test ($n = 3$). (B) Western blot of TNFRSF10B in αTC1-6 (left) and βTC1 (right) untreated (Ctrl) and treated (Cyt) with cytokines for 24 h. β-Actin (ACTB) was used as loading control.

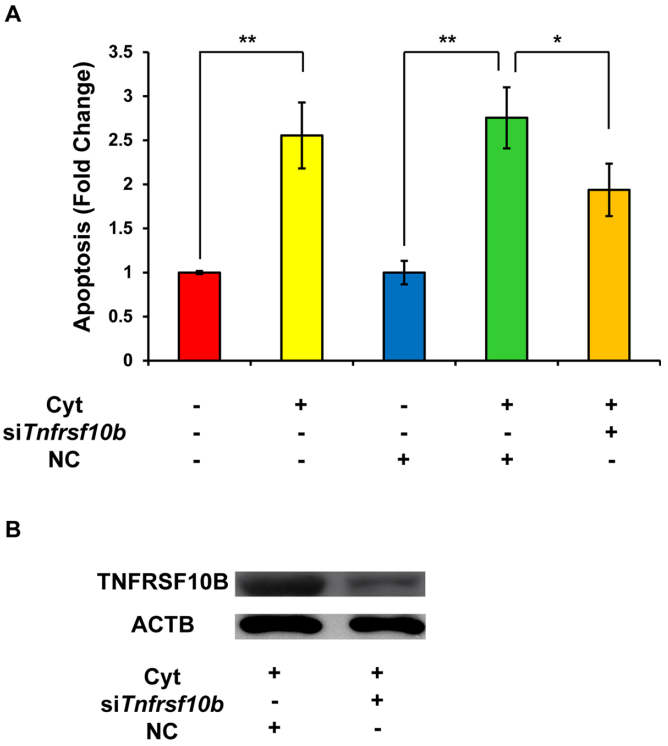

FIGURE 5: Apoptosis induced by cytokines in βTC1 transfected with si*Tnfrsf10b*. (A) Apoptosis determined by annexin V/PI method, described as fold change with respect to controls. Data are presented as mean ± SD of three independent experiments ($n = 3$). *$p < 0.05$, **$p < 0.01$, Tukey HSD post hoc one-way ANOVA test. (B) Western blot of TNFRSF10B in si*Tnfrsf10b*-transfected βTC1 treated with cytokines and matched scramble-transfected controls. β-Actin (ACTB) was used as loading control.

Browser revealed TFBSs conserved between humans and mice for several transcription factors within the putative promoter regions of *Arl6ip5*, *Cebpa*, and *Traf2*; among these transcription factors, we detected AP1, STAT, and NFkB, which are known to be key mediators of the immune response. *Tnfrsf10b* promoter showed a TFBS for DDIT3 evolutionarily conserved between humans and mice. Finally, *Ubc* promoter did not contain any TFBS that could be linked either to cytokine-responsive elements or to TFs belonging to CEBP family (Supplemental Table S5). Of interest, our TLDA and single real-time PCR assays data demonstrate that *Rela* and *Jun* mRNAs (which encode members of NFkB and AP1 complexes, respectively) significantly increased in amount specifically in βTC1 after treatment with cytokines: expression of both did not significantly change in αTC1-6 under the same cues (Figure 9). TLDA data on AM genes further revealed that *Cebpa* mRNA expression positively correlated with that of 24 other genes significantly up-regulated in βTC1 (Pearson correlation test, $p < 0.01$; Supplemental Table S6). By comparing this gene list with all the other AM genes analyzed in the TLDA that were also up-regulated in βTC1 after treatment with cytokines, we found that the frequency of NFkB-binding sites was significantly higher in the first group than in the second ($p = 0.0451$, two-sample *t* test).

DISCUSSION

Several reports confirmed the critical role of CEBPA in the physiological differentiation of myeloid cells, as in their neoplastic transformation (Friedman *et al.*, 2003; Paz-Priel *et al.*, 2011). In this article, we report for the first time that expression of both its encoded mRNA and protein is significantly up-regulated in mammalian pancreatic β cells undergoing apoptosis induced by proinflammatory cytokines. By specifically integrating our molecular data in the context of the AM network, we identified a set of genes that were prioritized as new effectors of apoptotic and dedifferentiation programs within pancreatic β cells after treatment with cytokines (Table 1 and Supplemental Tables S1 and S2). These results confirm 1) the high molecular complexity of cell regulatory networks (Sarkar *et al.*, 2009) and 2) the ensuing need to analyze final biological outcomes of complex phenomena rather than calculate ratios of up- or down-regulated proapoptotic or antiapoptotic genes, respectively. Notwithstanding this complexity, our approach allowed us to pinpoint AM genes that were specifically DE after treatment with cytokines in both α and β cells. This led us to focus attention on transcription factor CEBPA: it was expressed at very low levels in steady-state αTC1-6 and βTC1 (both as mRNA and protein), but it was significantly and specifically up-regulated in βTC1 after treatment with cytokines (Figure 1, A and B). The significant protection from cytokine-induced apoptosis that we observed in si*Cebpa*-transfected pancreatic βTC1 strongly suggests that up-regulation of *Cebpa* significantly contributes to the induction of proapoptotic pathways in these cells. Its proapoptotic transcriptional targets ARL6IP5 and TNFRSF10B are important in carrying out this function within the involved cell network. Indeed, expression of these genes followed the same trend as CEBPA, and their functional knockdown significantly reduced βTC1 apoptosis induced by cytokines (Figures 5 and 6 and Supplemental Figure 1, A and B). The greater antiapoptotic effect of TNFRSF10B knockdown on βTC1 treated with cytokines as

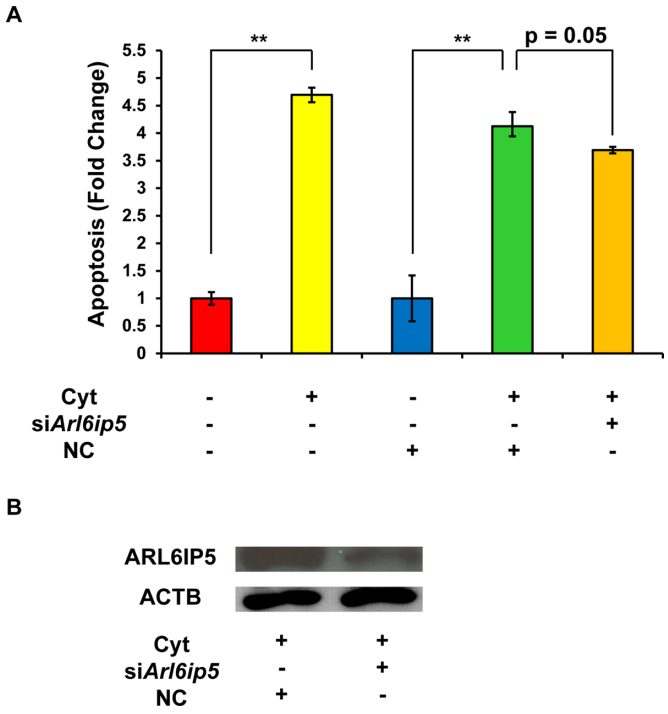

FIGURE 6: Apoptosis induced by cytokines in βTC1 transfected with si*Arl6ip5*. (A) Apoptosis determined by annexin V/PI method, described as fold change with respect to controls. Data are presented as mean ± SD of three independent experiments ($n = 3$). **$p < 0.01$, Tukey HSD post hoc one-way ANOVA test. (B) Western blot of ARL6IP5 in si*Arl6ip5*-transfected βTC1 treated with cytokines and matched scramble-transfected controls. β-Actin (ACTB) was used as loading control.

compared with ARL6IP5 is likely due to the relative importance of their biological roles within this specific biological system. TNFRSF10B was previously shown to be part of the apoptotic cascade triggered by CEBPA in a rat liver stellate cell line (Wang X *et al.*, 2009); it also was demonstrated to positively control TRAIL-mediated apoptosis of pancreatic β cells (Ou *et al.*, 2002). ARL6IP5 has been shown to be a key mediator of CEBPA-induced cell death in several cell types (Wang *et al.*, 2008; Vento *et al.*, 2010), but it has never been investigated in pancreatic cells. The identification of two adjacent TFBSs for NFKB1 and CEBPA within the putative promoter region of *Arl6ip5* further suggests that transcriptional control of this gene could be mediated by cooperation of the two transcription factors (Supplemental Figure 3A). Their functional association has been shown to mediate the transcription of several genes involved in inflammatory cytokine–induced neutrophil production (Wang D *et al.*, 2009). Furthermore, CEBPA binding to *Arl6ip5* promoter has been reported in a murine fibroblast cell line and in human adipocytes (Wang *et al.*, 2008; Lo *et al.*, 2011; Supplemental Table 3). Our data suggest that induction of *Pdx1* negatively controls the expression of *Cebpa* mRNA: this is consistent with previous studies demonstrating that Polycomb-mediated repression of *Cebpa* is crucial to maintain the differentiated phenotype of β cells upon commitment of pancreatic endocrine precursor cells (Davis and Eddy, 2013). The relevant biological role performed by *Cebpa* and its target genes within the apoptotic network of pancreatic β cells after their exposure to cytokines is also confirmed by their network centrality. The same analysis allowed us also to identify *Traf2* and *Ubc* as two other central genes within the network: of interest, both genes are up-regulated in pancreatic β cells exposed to cytokines (see our TLDA data for *Traf2* and Ortis *et al.* (2010) for *Ubc*). Similar to the expression of *Rela* and *Jun*, that of *Traf2* mRNA also significantly increased specifically in βTC1 after treatment with cytokines as compared with matched, untreated controls (Table 1 and Supplemental Tables S1 and S2). These data, together with the extended network that we generated starting from CEBPA and its targets, spotlighted a set of genes whose expression was specifically altered in βTC1 as compared with αTC1-6. We suggest that all of them may variously contribute to β cell demise after treatment with cytokines. Taken together, our data strongly suggest that the increased expression of *Cebpa* is causally involved in apoptosis of pancreatic β cells exposed to proinflammatory cytokines IL1β, IFNγ, and TNFα. The proapoptotic role of *Cebpa* within this system is very likely exerted through its proapoptotic targets *Arl6ip5*, *Tnfrsf10b*, and *Traf2*. Accordingly, *Cebpa* and its targets may be prioritized as new candidates for cytokine-induced apoptosis of pancreatic β cells and DM (Lee *et al.*, 2013). Understanding the complex cross-talk mechanisms between pancreatic α or β cell differentiation programs and antiapoptotic or proapoptotic pathways activated in response to proapoptotic stimuli (such as cytokines) in the two cell phenotypes will allow us to identify and prioritize new candidate genes involved in the onset of DM and also potentially reveal new therapeutic strategies (Talchai *et al.*, 2012). Owing to the central role played by *Cebpa* in the apoptotic network of pancreatic β cells, it is also important to further increase our knowledge of its upstream and downstream regulatory transcription networks (Eyholzer *et al.*, 2010; Pulikkan *et al.*, 2010).

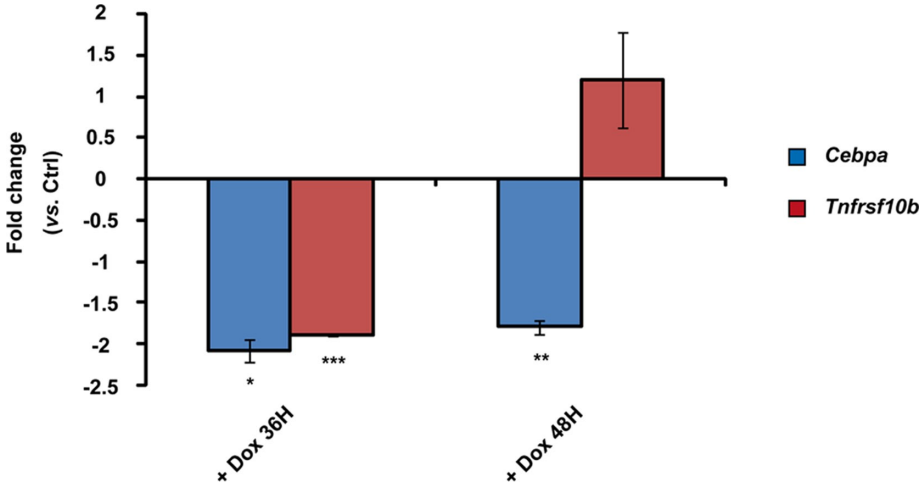

FIGURE 7: *Cebpa* and *Tnfrsf10b* mRNA expression in INS-1rαβ after induction of *Pdx-1* mRNA expression by treatment with doxycycline for 36 and 48 h. Values are reported as fold changes relative to matched untreated controls. *Two-tailed $p < 0.05$; **two-tailed $p < 0.01$; ***two-tailed $p < 0.001$; paired Student's *t* test ($n = 3$).

MATERIALS AND METHODS

Cell culture and treatment with cytokines

Mouse glucagonoma cell line αTC1-6 was obtained from the American Type Culture Collection (ATCC; Manassas, VA); it was

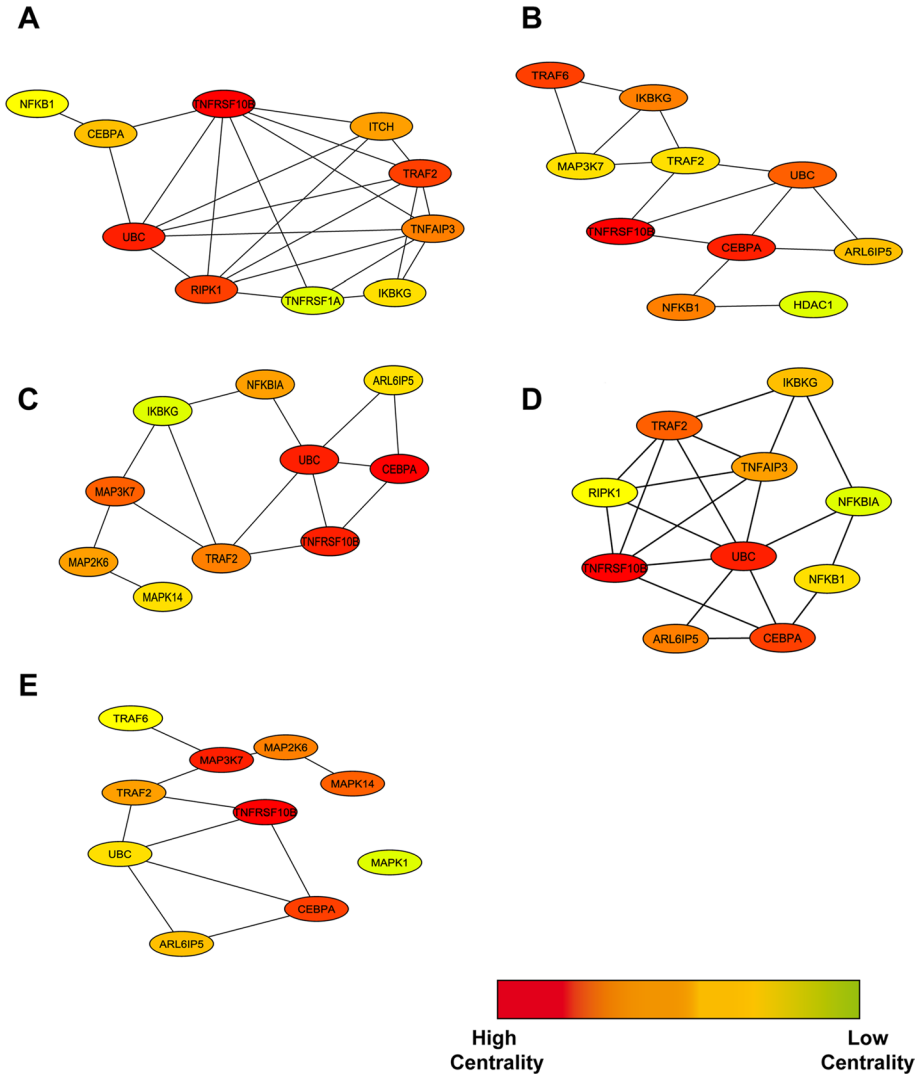

FIGURE 8: Network centralities calculated through cytoHubba plug-in. (A) MCC; (B) Degree; (C) Bottleneck; (D) Closeness; (E) Betweenness.

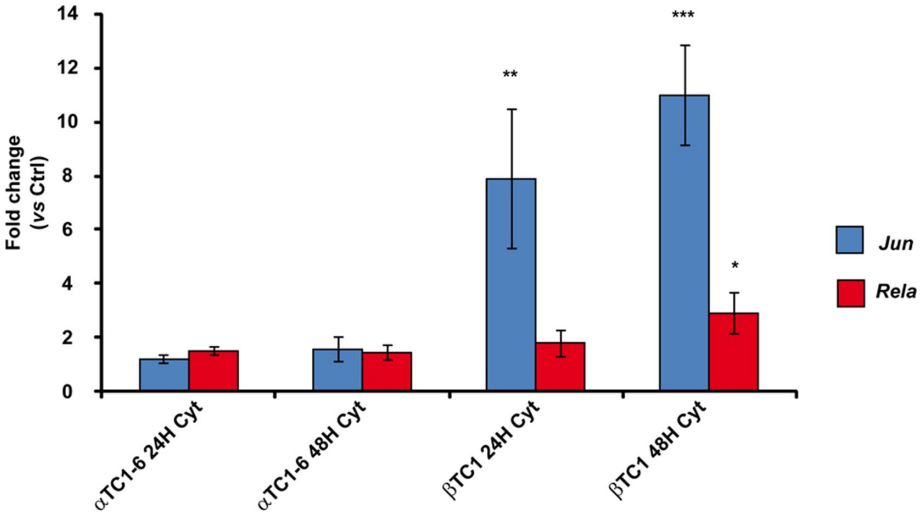

FIGURE 9: *Jun* (blue bar) and *Rela* (red bar) mRNA expression in αTC1-6 and βTC1 after treatment with cytokines for 24 and 48 h. Values are reported as fold changes relative to matched untreated controls. *Two-tailed $p < 0.05$; **two-tailed $p < 0.01$; ***two-tailed $p < 0.001$; paired Student's t test ($n = 3$).

cultured in complete DMEM (Sigma-Aldrich, Saint Louis, MO) as previously described (Barbagallo *et al.*, 2013). Mouse insulinoma cell line βTC1 was also from ATCC and was cultured in DMEM (Barbagallo *et al.*, 2013). Cells were passaged once a week after trypsinization and replaced with new medium twice weekly. Treatment with cytokines was performed as previously described (Barbagallo *et al.*, 2013). INS1-rαβ was cultured and treated as described (Hansen *et al.*, 2012).

RNA preparation and HT real-time reverse transcription-PCR

Total RNA was extracted with TRIzol (Life Technologies, Foster City, CA), according to the manufacturer's instructions. RNA quantification was performed by Qubit Fluorometer (Life Technologies). DNA contamination was removed using deoxyribonuclease 1 (DNase I Amplification Grade; Life Technologies). DNase-treated RNA was reverse transcribed by using High Capacity RNA-to-cDNA Kit (Life Technologies) according to manufacturer's instructions. Resulting cDNAs (200 ng per sample-loading port) were loaded into custom TLDAs, format 96a (Life Technologies), and amplified through a standard thermal cycling profile on an ABI PRISM 7900HT Fast Real-Time PCR System (Life Technologies). Single-gene specific assays were performed through real-time PCR by using Fast SYBR Green Master Mix (Life Technologies) according to manufacturer's instruction. To allow statistical analysis, PCRs were performed in three independent biological replicates. Primer sequences are available upon request.

TLDA design and data analysis

Custom TLDA was designed to analyze the expression of 92 transcripts of genes known to be involved in apoptosis (AM genes) plus four candidate reference genes (*Actb*, *Hprt*, *Tuba1a*, and *18S RNA*). AM genes were selected on the basis of our previous characterization of the AM (Di Pietro *et al.*, 2009) and from the literature. The complete list of AM genes analyzed is given in Supplemental Table S7. *Actb* and *Hprt* were chosen as the best housekeeping genes in our experimental model according to geNorm analysis (Vandesompele *et al.*, 2002). Relative quantities (RQs) of each mRNA were calculated according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Values are reported as average fold changes of three independent biological replicates; RQ values of <1 were converted into negative fold changes using –1/RQ. Limma test was carried out by Real-Time Statminer software: mRNAs with

Benjamini–Hochberg adjusted $p \leq 0.05$ were considered DE (Smyth, 2004).

Western analysis

Protein lysates and their quantification were obtained as previously described (Anello *et al.*, 2004; Barbagallo *et al.*, 2013). A 50-μg amount of total protein extract was loaded into 10% SDS polyacrylamide gel (Hoefer miniVE; GE Healthcare, Little Chalfont, United Kingdom) and blotted to nitrocellulose membranes by iBlot Dry Blotting System (Life Technologies). Membranes were probed by rabbit monoclonal antibodies to CEBPA (Abcam, Cambridge, United Kingdom), TNFRSF10B (Santa Cruz Biotechnology, Dallas, TX), rabbit polyclonal antibodies to ARL6IP5 (Abcam), and mouse monoclonal antibodies to ACTB (β-actin; Sigma-Aldrich). ACTB (β-actin) was used as loading control. Protein detection and densitometric analysis were performed as previously described (Barbagallo *et al.*, 2013).

Transfection and siRNA reagents

βTC1 cells were transfected with a mixture of two different siRNAs targeting *Cebpa* mRNA (FlexiTube siRNAs #1 and #2; Qiagen, Venlo, Netherlands), three different siRNAs targeting *Arl6ip5* mRNA (FlexiTube siRNAs #2, #5, #6; Qiagen), three different siRNAs targeting *Tnfrsf10b* mRNA (FlexiTube siRNAs #2, #3, #4; Qiagen) or with scrambled molecules (AllStars Negative Control siRNA; Qiagen) at a final concentration of 100 nM (si*Cebpa* and si*Tnfrsf10b*) or 50 nM (si*Arl6ip5*), using HiPerFect Transfection Reagent (Qiagen) according to manufacturer's instruction. Briefly, 120,000 cells/well were seeded in 12-well plates (SPL Life Sciences, Pocheon, South Korea) and reverse transfected at different time points. Annexin V/PI assay and RNA extraction were performed after transfection to evaluate alterations of 1) apoptosis levels of αTC1-6 and βTC1 and 2) amount of CEBPA transcriptional targets. Percentage of apoptotic or necrotic β cells was assessed through flow cytometry, as previously described (Barbagallo *et al.*, 2013).

In silico characterization of CEBPA transcriptional network

Candidate transcriptional targets of CEBPA were retrieved from both the AnimalTFDB database (Zhang *et al.*, 2012) and the literature (Supplemental Table S3). In silico identification of CEBPA-binding sites in a 5-kb region upstream from the TSS of predicted transcriptional targets was performed through the LASAGNA algorithm (Lee and Huang, 2013). We submitted the sequence of the region of interest such as FASTA and selected TRANSFAC TFBSs as matrix to search for CEBPA-binding sites. The rVista 2.0 tool (http://rvista.dcode.org/) integrated into the ECR Browser has been used to find conserved TFBSs within each ECR within putative promoters (Ovcharenko *et al.*, 2004). The parameters used to define an ECR were minimum length 100 base pairs and at least 70% sequence identity. We defined a putative promoter region as a genomic window spanning from 5 kbp upstream to 111 base pairs (the median value of the 5′ untranslated region length) downstream from the TSS of each gene. The TELiS database (Cole *et al.*, 2005) was queried to compare the average number of TFBSs between two gene lists; frequency analysis was performed with a high scanning stringency (90) and a promoter size of 1200 base pairs. Mouse interactome data were automatically retrieved from the Biogrid, version 3.2.107, database through Cytoscape software, version 3.0.1 (Shannon *et al.*, 2003). Interactions within MAPK and NFkB pathways were retrieved from InnateDB (Lynn *et al.*, 2008). Network centrality parameters (MCCs; Degree, Bottleneck, Closeness, Betweenness) were calculated through the Cytoscape plug-in CytoHUBBA (Lin *et al.*, 2008). Gene ontologies and pathway overrepresentation analyses were performed through the DAVID tool (Huang *et al.*, 2009).

Statistical analysis

The Limma test, Student's *t* test, the ANOVA test, Pearson's correlation test, and Fisher's exact test were used as statistical tests for the comparisons described. Adjusted *p* value ≤ 0.05 was considered significant. The statistical tests and correction methods used to calculate *p* values are specified throughout the text and figure legends.

Nomenclature of genes and proteins

Rules for official gene and protein symbols of the International Committee on Standardized Genetic Nomenclature for Mice were followed throughout the text (Eppig *et al.*, 2012; www.informatics.jax.org/mgihome/nomen/gene.shtml).

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