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 Tnfrsf10b, 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 NFκB 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 NFκB 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.

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 (Alagnrat 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
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 < 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 siCebpa and treated with cytokines as compared with matched scramble-transfected cells (Figure 2B).

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

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

| Cell type | DE genes                                      |
|-----------|----------------------------------------------|
| αTC1-6    | Akt1, Apaf1, Bax, Bcl2/11, Birc2, Birc3, Cebpa, Chuk, Dapk1, Ddit3, Dedd2, Dffa, Dffb, Diablo, Ern1, Gadd45a, Htra2, Irs2, Jak2, Lrdd, Mapk3/14, Mapk14, Mapk4, Mdm2, Ptp13, Rola, Rel, Stat5a, Stat5b, Tax1bp1, Tnfrsf10b, Tnfrsf21, Traf2, Traf3, Traf6, Trp53 |
| βTC1      | Bmf, Gadd45b                                  |

![Figure 1: Cebpa mRNA and protein expression in pancreatic βTC1.](image-url)
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 (Supplementary 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; Supplementary 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 siCebpa 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 siCebpa 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 Tnfrsf10b 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 siTnfrsf10b and siArl6ip5 and treated with cytokines as compared with matched scramble-transfected cells, respectively (Figures 5B and 6B).

Cebpa mRNA expression is significantly reduced in INS-1αβ 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-1αβ cells by treating them with doxycycline (dox) for 36 and 48 h. We detected a significant decrease of Cebpa mRNA in INS-1αβ at 36 and 48 h after exposure to dox with respect to NCs. We also detected significantly decreased Tnfrsf10b mRNA levels in INS-1αβ at 36 h after treatment with dox (Figure 7).
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, TNRFRSF10B, 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, TNRFRSF10B, 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 cytokine-responsive elements within their putative promoter regions. The ECR

![Figure 3](https://example.com/figure3.png)

**Figure 3:** Arl6ip5 and Tnfrsf10b mRNA expression at steady state and after treatment with cytokines for 6 and 24 h in siCebpa-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 ΔCt values. *Two-tailed p < 0.05, **two-tailed p < 0.01, ***two-tailed p < 0.001; paired Student’s t test (n = 3).
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DISCUSSION
we found that the frequency of NFkB-binding sites was significantly
ing this gene list with all the other AM genes analyzed in the TLDA
further revealed that
α
promoter did not contain any TFBS that could be linked
Turtox-induced apoptosis that we observed in si
α
TC1 strongly suggests that up-regulation of
significantly contributes to the induction of proapoptotic pathways in
its proapoptotic transcriptional targets ARL6IP5 and
Arl6ip5
and
Tnfrsf10b
mRNA expression positively correlated
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fold change (vs. Ctrl)
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FIGURE 4: Arl6ip5 and Tnfrsf10b mRNA expression in αTC1-6 and
beta TC1. (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 β TC1 (right) untreated (Ctrl) and
treated (Cyt) with cytokines for 24 h. 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. Fi-
nally, 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 (Pear-
son 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 physio-
logical differentiation of myeloid cells, as in their neoplastic transfor-
mation (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 proinflamma-
tory 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 pro-
grams 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. Not-
withstanding 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 signifi-
cantly and specifically up-regulated in βTC1 after treatment with
cytokines (Figure 1, A and B). The significant protection from cy
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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 NFκB1 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 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 TLD data for Traf2 and Ortiz 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).

**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...
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-αβ 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 TLDA, 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−ΔΔCt 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
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 Cebpα 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 TNfsf10b mRNA (FlexiTube siRNAs #2, #3, #4; Qiagen) or with scrambled molecules (AllStars Negative Control siRNA; Qiagen) at a final concentration of 100 nM (siCebpα and siTnfsf10b) or 50 nM (siArl6ip5), 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 βTC1-6 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 algorith (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, interactome data were automatically retrieved from the Biogrid, interacting proteins 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 over-representation 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/mghome/nomen/gene.shtml).

ACKNOWLEDGMENTS

We thank F. Magherini, A. Modesti, F. Piva, G. Pellegrini, and G. Principato for discussions in the early phases of this project. We thank F. Ortis for sharing data with us. We also acknowledge the technical collaboration of A. Vasta and thank A. Scaccianente (CO.Di.SAN SPA, Belpasso, Catania, Italy), S. Fanti (BioGenerica s.r.l., Mascalucia, Catania, Italy), and F. Montanaro (Biotecnica, Acciacastello, Catania, Italy) for their kind cooperation. This work was supported by funds from Ministero dell’Università e della Ricerca Scientifica e Tecnologica to M.P. and F.P.

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