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Signaling by IL-1β + IFN-γ and ER stress converge on DP5/Hrk activation: a novel mechanism for pancreatic β-cell apoptosis

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Chronic inflammation and pro-inflammatory cytokines are important mediators of pancreatic β-cell destruction in type 1 diabetes (T1D). We presently show that the cytokines IL-1β + IFN-γ and different ER stressors activate the Bcl-2 homology 3 (BH3)-only member death protein 5 (DP5)/harakiri (Hrk) resulting in β-cell apoptosis. Chemical ER stress-induced DP5 upregulation is JNK/c-Jun-dependent. DP5 activation by cytokines also involves JNK/c-Jun phosphorylation and is antagonized by JunB. Interestingly, cytokine-inducted DP5 expression precedes ER stress: mitochondrial release of cytochrome *c* and ER stress are actually a consequence of enhanced DP5 activation by cytokine-mediated nitric oxide formation. Our findings show that DP5 is central for β-cell apoptosis after different stimuli, and that it can act up- and downstream of ER stress. These observations contribute to solve two important questions, namely the mechanism by which IL-1β + IFN-γ induce β-cell death and the nature of the downstream signals by which ER stress 'convinces' β-cells to trigger apoptosis.
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Diabetes is a major cause of morbidity and mortality worldwide, decreasing life quality and expectancy of millions of affected individuals.[1] The main forms of diabetes are type 1 diabetes (T1D) and type 2 diabetes (T2D). Both types are characterized by progressive pancreatic β-cell destruction, which is more marked in T1D.[2] In the early stages of T1D, macrophages and T cells infiltrate the islets of Langerhans and secrete pro-inflammatory cytokines such as IL-1β, IFN-γ and TNF-α contributing to β-cell apoptosis and the build up of insulitis.[3] Dying β-cells can act as 'danger signals' and, together with pro-inflammatory cytokines, induce the maturation of dendritic cells, activation of T cells and amplification of the immune reaction.[3,4] The final outcome is a loss of self-tolerance, induction of β-cell autoimmunity and progressive β-cell destruction, eventually rendering the affected individuals insulin dependent. Chronic inflammation (without autoimmune assault) has also been implicated in the development of T2D,[5] but the relevance of this inflammatory component for β-cell loss is a matter of debate.[2]

In vitro exposure of β-cells to IL-1β + IFN-γ, but not to either cytokine alone, causes functional changes similar to those observed in pre-T1D patients, namely elevated proinsulin/insulin ratio,[6] a preferential loss of first-phase insulin response to glucose[7] and β-cell death.[2,8] How pro-inflammatory cytokines destroy β-cells is a question that remains to be answered. Cytokines modulate the activity of several target genes and proteins in β-cells[2] inducing, among other effects, depletion of endoplasmic reticulum (ER) Ca^{2+}.[9] This hampers β-cell ER homeostasis, leading to ER stress and triggering of the unfolded protein response (UPR).[9,10] The consequence of the UPR is attenuation of global protein translation, upregulation of ER chaperones and degradation of irreversibly misfolded proteins. In case the UPR fails, apoptotic pathways are triggered.[11] As β-cells synthesize and secrete large amounts of insulin, they are particularly vulnerable to ER stress.[12] It is not clear, however, whether cytokine-induced ER stress is a direct cause of β-cell apoptosis or a parallel and/or downstream event.[12–14] In other cell types many pro-apoptotic stimuli, including severe ER stress, converge on the final activation of Bcl-2 homology 3 (BH3)-only family proteins.[15] These Bcl-2 members interact trough their BH3 domain and inactivate anti-apoptotic Bcl-2 proteins such as Bcl-2 and Bcl-XL, thereby liberating the pro-apoptotic Bcl-2 members Bax and Bak. Oligomerization of free Bax and Bak permeabilizes the outer mitochondrial membrane and triggers the release of apoptogenic molecules like apoptosis-inducing factor (AIF) and cytochrome *c*.[16] Cytoplasmic cytochrome *c* binds to APAF1 allowing the assembly of the apoptosome, which activates pro-caspases through a conformational change. This is commonly regarded as the 'point-of-no-return' in the cascade of events that delineate the intrinsic pathway of apoptosis.[15]

Death protein 5 (DP5)/harakiri (Hrk) belongs to the BH3-only protein family. It has been originally identified in rat sympathetic neurons[17] and in HeLa cells.[18] DP5 expression

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Abbreviations: T1D, type 1 diabetes; T2D, type 2 diabetes; IL-1β, interleukin-1β; IFN-γ, interferon-γ; TNF-α, tumor necrosis factor-α; DP5, death protein 5; Hrk, harakiri; BH3, Bcl-2 homology 3; AIF, apoptosis-inducing factor; OMM, outer mitochondrial membrane; UPR, unfolded protein response; ER, endoplasmic reticulum; siRNA, small interfering RNA; MAPK, mitogen-activated protein kinase; JNK, Jun N-terminal Kinase; NF-κB, nuclear factor-κB; IκB, inhibitory κB; NO, nitric oxide; iNOS, inducible form of NO synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CPA, cyclopiazonic acid; ChIP, chromatin immunoprecipitation
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was also detected in human pancreas[18] and earlier microarray analysis indicated that DP5 is activated in insulin-producing INS-1E cells after cytokine treatment.[19] In this study we clarified the mechanisms of DP5 induction and its role in β-cell apoptosis induced by chemical ER stressors and the cytokines IL-1β + IFN-γ. Our findings show that diverse apoptotic stimuli enhance DP5 expression through different pathways, resulting in cytochrome *c* release and apoptosis. Importantly, DP5 inactivation protects β-cells from IL-1β + IFN-γ- and chemical ER stress-mediated cell death. These findings point to novel avenues to prevent progressive β-cell loss in early T1D.

Results

The BH3-only protein DP5 is preferentially upregulated by cytokines and mediates β-cell death. Exposure of INS-1E or primary β-cells to IL-1β + IFN-γ induced cytochrome *c* release from the mitochondria but not nuclear AIF translocation (Figure 1a). Cytokine-mediated cytoplasmic cytochrome *c* localization was confirmed by western blot (Supplementary Figure S1A). Mitochondrial permeabilization is tightly regulated by proteins of the Bcl-2 family. Real-time RT-PCR and western blot showed that DP5 is the Bcl-2 protein preferentially upregulated in IL-1β + IFN-γ-treated INS-1E cells (Figure 1b and c). On the other hand, modulation of the anti-apoptotic Bcl-2 and Bcl-XL proteins by cytokines was not observed (Figure 1b; Supplementary Figure 1B). IL-1β or IFN-γ alone did not modify DP5 mRNA levels in INS-1E cells, but both cytokines together induced a 25-fold increase in DP5 expression (Supplementary Figure 1C). Cytokine-induced DP5 activation was also detected in FACS purified primary rat β-cells and in dispersed human islet cells (Figure 1d; Supplementary Figure 1D).

To study the role of DP5 in IL-1β + IFN-γ-induced β-cell apoptosis, we used small interfering RNA (siRNA) technology. Two different DP5 siRNAs inhibited DP5 mRNA expression in INS-1E cells by >60% as compared with an inactive control siRNA (Figure 2a). DP5 knockdown did not affect viability under basal conditions, but it decreased cytokine-induced apoptosis by 2-fold at 24 h (Figure 2b). The siRNA DP5-2, which induced a more marked knockdown effect, protected to a larger extent INS-1E cells from cytokine-induced apoptosis. In line with these findings, knockdown of DP5 diminished cytokine-induced cytochrome *c* release and subsequent caspase-3 activation and DNA fragmentation (Figure 2c–e; Supplementary Figure S2). Importantly, primary β-cells were also partially protected against IL-1β + IFN-γ-induced apoptosis by DP5 knockdown (Figure 2f).

ER stress activates JNK signaling in β-cells, which is required for DP5 induction. Earlier studies suggested that β-cells are particularly sensitive to ER stress and this may contribute for cytokine-induced apoptosis in early T1D.[9,12,14] To determine if ER stress results in DP5 activation we tested three different chemical ER stressors, namely cyclopiazonic acid (CPA), thapsigargin and tunicamycin. ER stress induction at different time points increased DP5 expression in INS-1E cells (Figure 3a; Supplementary Figure S3A and B) resulting in cytochrome *c* release (Supplementary Figure S4). The higher DP5 expression after CPA or thapsigargin addition as compared with tunicamycin correlated with the expression of ER stress markers induced by the different ER stressors (Supplementary Figure S5). Knockdown of DP5 by siRNA significantly attenuated CPA-, thapsigargin- and tunicamycin-mediated cell death (Figure 3b and c; Supplementary Figure S3C–F). Moreover, CPA treatment induced DP5 activation in primary rat β-cells and in dispersed human islet cells (Supplementary Figure S6A and B). Importantly, DP5 knockdown decreased CPA-mediated apoptosis in primary β-cells (Supplementary Figure S6C). Together, these results suggest that DP5 is a downstream mediator of ER stress-induced apoptosis in β-cells.

It was shown earlier that *DP5* is a direct target gene of JNK/c-Jun.[20] ER stress activates JNK/c-Jun through the IRE1-TRAF2-ASK1 pathway.[21] In line with these data, the different ER-stressors tested induced phosphorylation of JNK and c-Jun in INS-1E cells (Supplementary Figure S7). We next analyzed the nucleotide sequence responsible for ER stress-induced DP5 transcription using *DP5* promoter reporter constructs. Figure 3d shows that the promoter sequence −125 to −85 is essential for CPA-induced *DP5* activation. Studies in neuronal cells have shown that this region harbors a conserved ATF site where c-Jun binds after phosphorylation by JNK.[20] Mutations introduced in the ATF site abolished CPA-induced *DP5* promoter activation in INS-1E cells (Figure 3e), indicating that this site is crucial for *DP5* gene transcription. To determine whether phosphorylated c-Jun is involved in DP5 expression after ER stress, we used the chemical JNK/c-Jun inhibitor SP600125.[22] This drug acts as a reversible ATP-competitive inhibitor of JNK in INS-1E cells[23] and has been shown to exhibit a selectivity of >20-fold relative to other tested kinases.[22] Decreasing CPA-induced c-Jun phosphorylation abolished DP5 mRNA upregulation and protected β-cells from apoptosis (Figure 3f–h). We therefore conclude that chemically induced ER stress activates DP5 in β-cells through JNK/c-Jun phosphorylation resulting in β-cell death.

JunB and c-Jun have antagonistic functions in the early induction of DP5 expression by cytokines. A key signaling mechanism induced by cytokines is the mitogen-activated protein kinase (MAPK) pathway leading to JNK and subsequently c-Jun phosphorylation in β-cells.[24] Consistently, we observed that IL-1β + IFN-γ treatment induced an early activation of JNK (15 min–1 h), followed by c-Jun phosphorylation at later time points (1–16 h) (Figure 4a). The JNK inhibitor SP600125 prevented cytokine-induced c-Jun phosphorylation and decreased DP5 activation (Figure 4b and c). Reporter analysis showed that the −85/−125 sequence in the *DP5* promoter is critical for its activation by cytokines (Figure 4d). Mutations of the ATF site for c-Jun binding, however, resulted only in a partial decrease of cytokine-induced DP5 expression (Figure 4e), suggesting that another mechanism is also involved in cytokine-mediated DP5 activation.

JunB has a protective function against ER stress and cytokine-induced apoptosis in β-cells[25] and, in some settings, JunB acts as a c-Jun antagonist.[26] We therefore examined whether modulation of JunB expression affects cytokine-

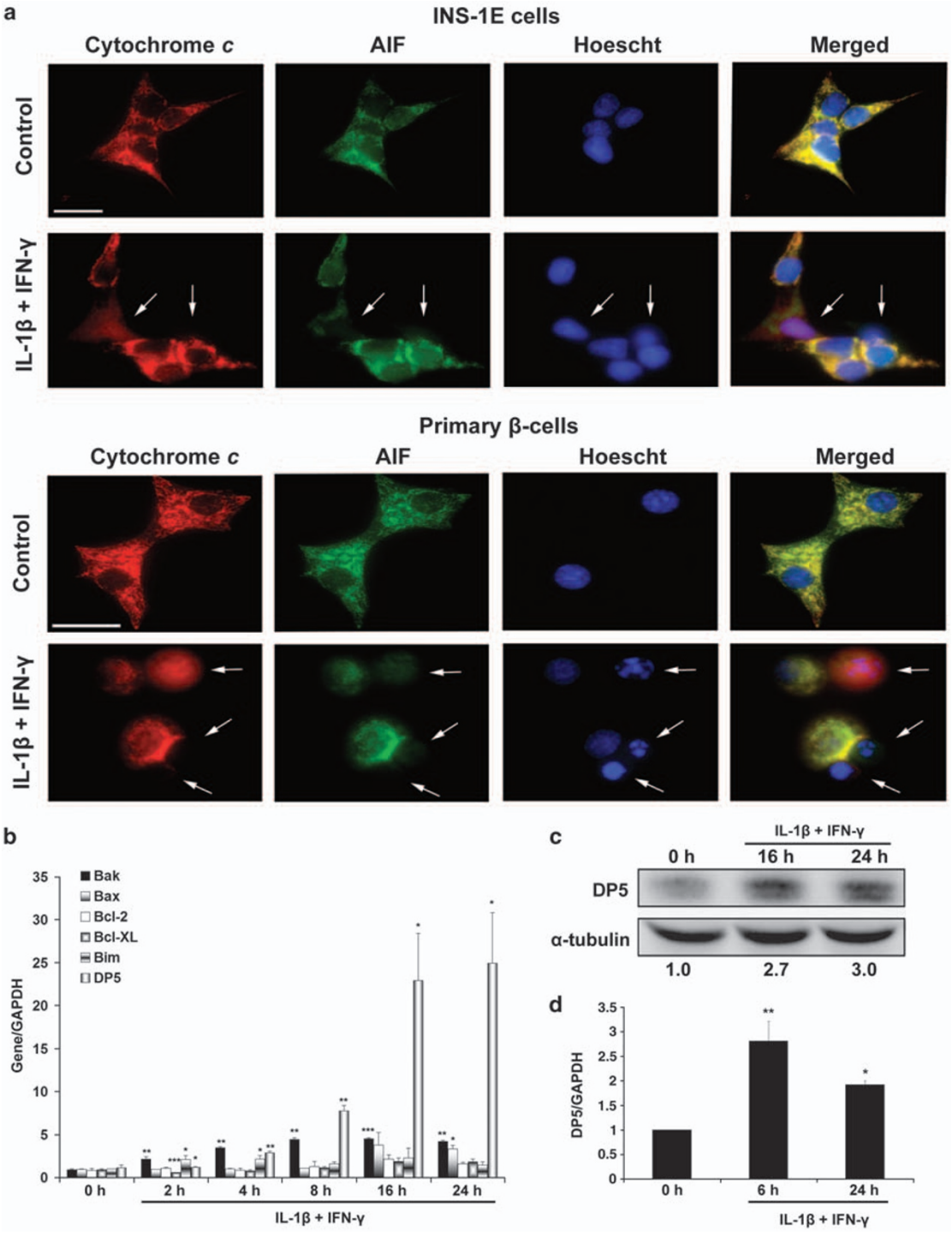


Figure 1 IL-1β + IFN-γ induce mitochondrial cytochrome *c* release and DP5 activation in β-cells. (**a**) Representative immunofluorescence images of cytochrome *c* (red) and AIF (green) localization in INS-1E and FACS purified primary β-cells treated for 24 h with IL-1β + IFN-γ. Nuclear morphology is shown by Hoescht staining (blue). Arrows indicate compaction of the nuclear chromatin and cytochrome *c* release from the mitochondria, bar 20 μm. (**b**) Expression of Bcl-2 family members as determined by real-time RT-PCR in INS-1E cells treated with IL-1β + IFN-γ. Values are represented as fold induction compared with control (non-cytokine-treated cells). $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *versus* control. (**c**) Western blot demonstrating increased expression of DP5 protein in cytokine-treated INS-1E cells. Quantification of DP5 band intensities is indicated at the bottom as a ratio to α-tubulin loading. (**d**) DP5 mRNA expression in purified rat β-cells treated with cytokines. Real-time RT-PCR for DP5 was performed at the indicated time points after cytokine addition. $^{*}P<0.05$, $^{**}P<0.01$

induced DP5 expression. Chromatin immunoprecipitation (ChIP) analysis using a JunB antibody confirmed protein binding to the ATF site in the *DP5* promoter (Figure 5a), whereas JunB overexpression decreased DP5 promoter activity; this effect was prevented by mutations in the ATF-binding site (Supplementary Figure S8A and B). In addition,

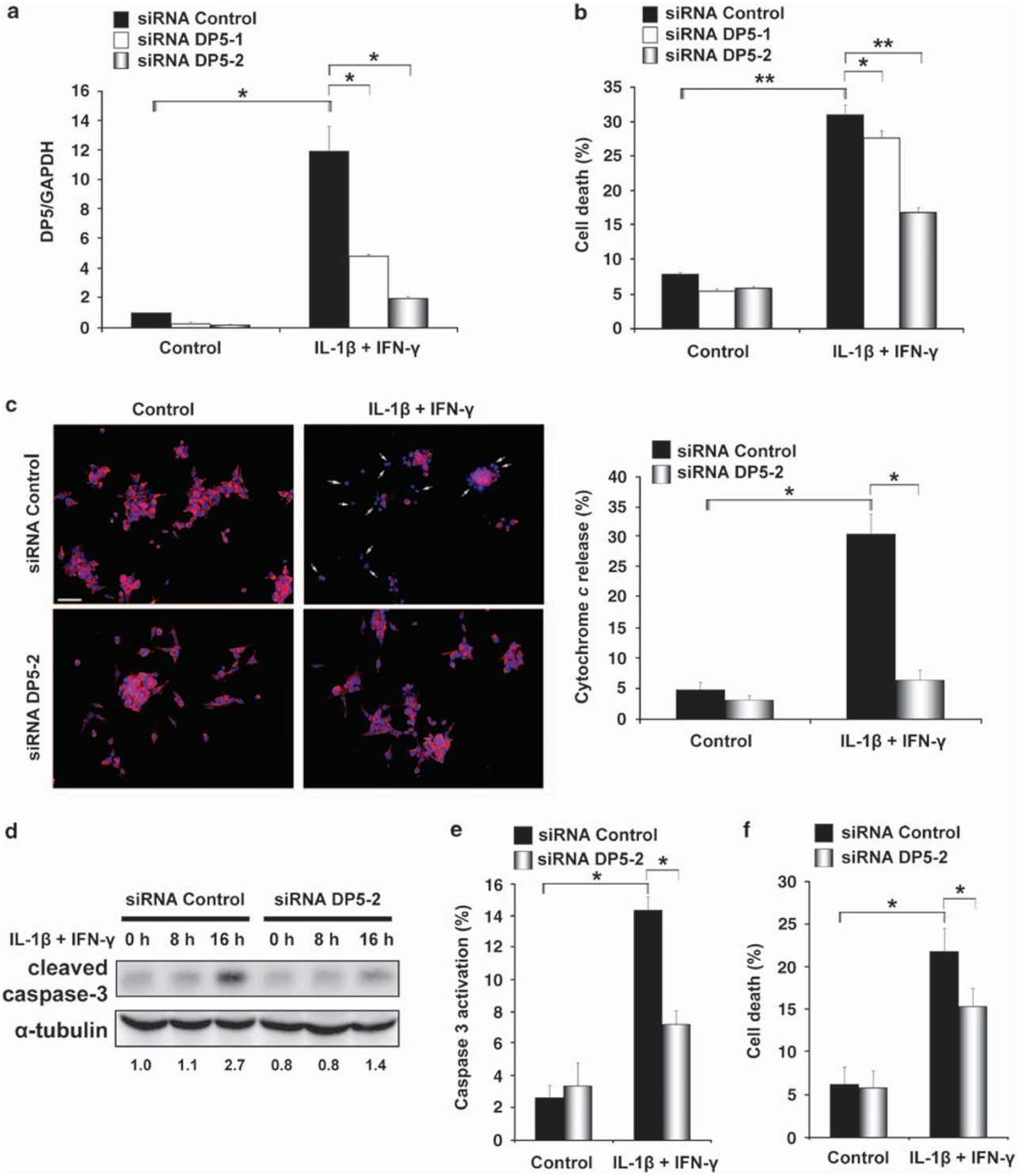


Figure 2 DP5 knockdown protects β-cells against IL-1β + IFN-γ-induced apoptosis. (**a**) INS-1E cells were transfected with inactive siRNA (control) or two different DP5 siRNAs (siRNA DP5-1 and siRNA DP5-2). Real-time RT-PCR for DP5 and GAPDH expression was performed 16 h after cytokine exposure. $^{*}P<0.05$. (**b**) Cytokines and/or DP5 or control siRNAs were added for 24 h to INS-1E cells and cell death was measured by HO/PI. $^{*}P<0.05$, $^{**}P<0.01$. (**c**) Untreated control or cytokine-treated INS-1E cells (either siRNA control- or siRNA DP5-transfected) were stained with antibodies specific for cytochrome *c* and Hoescht for nuclear morphology evaluation, followed by immunofluorescence analysis. The percentage of cells exhibiting diffuse or no cytochrome *c* staining was determined among the entire population of control or cytokine-treated cells, bar 40 μm. $^{*}P<0.05$. (**d**) INS-1E cells were transfected with control or with DP5-2 siRNAs, and western blot for cleaved caspase-3 was performed 8 and 16 h after cytokine addition. (**e**) INS-1E cells were transfected with control or with DP5-2 siRNAs, treated with cytokines for 24 h and caspase-3 activation was then determined and expressed as % of the total cell population. $^{*}P<0.05$. (**f**) DP5 knockdown decreases primary β-cell death 48 h after cytokine treatment. $^{*}P<0.05$

fibroblasts constitutively lacking JunB showed increased DP5 mRNA and protein expression compared with wild-type cells (Supplementary Figure S8C and D). We have shown earlier that adenovirus-mediated JunB overexpression protects INS-1E cells and primary β-cells against cytokine-induced apoptosis.[25] Importantly, we have now found that adenovirus overexpressing JunB significantly reduces DP5 induction (Figure 5b). Conversely, knockdown of JunB using two previously described siRNAs increased cytokine-mediated cell death[25] and DP5 expression (Figure 5c). Knockdown of DP5-attenuated apoptosis induced by JunB inactivation and cytokine treatment, indicating that higher DP5 expression is indeed responsible for JunB knockdown-increased cell death in INS-1E cells and primary β-cells exposed to cytokines (Figure 5d–g). As a whole, these results suggest that JunB acts as a DP5 repressor and support the hypothesis that c-Jun and JunB have antagonistic functions in IL-1β + IFN-γ-mediated DP5 activation.

Cytokine-induced nitric oxide formation synergizes with JNK/c-Jun to induce DP5 expression. The transcription factor nuclear factor-κB (NF-κB) is an important mediator of cytokine-induced β-cell apoptosis.[27] NF-κB is formed by homo- or heterodimers of five NF-κB family members. These dimers are usually present in an inactive form in the cytoplasm, in which they remain bound to a group of related inhibitory κB (IκB) proteins.[28] IL-1β + IFN-γ induce degradation of the IκB-α protein, p65 nuclear translocation and NF-κB activation (Figure 6a; Supplementary Figure S9A and B). As NF-κB is induced early after cytokine exposure preceding ER stress, we evaluated whether this transcription factor modulates DP5 upregulation in INS-1E cells. Infection with the adenovirus AdIκB$^{(SA)2}$, expressing an IκB 'super-repressor', prevented cytokine-induced NF-κB nuclear translocation (Supplementary Figure S9C) and decreased by >70% DP5 induction (Figure 6b). However, we did not find NF-κB-binding sites in the regulatory sequences of the *DP5* promoter (data not shown), suggesting an indirect regulation by this transcription factor. Nitric oxide (NO), generated by the inducible form of NO synthase (iNOS), contributes for cytokine-induced gene expression[19] and apoptosis.[29] We observed that adenovirus-mediated inactivation of NF-κB prevented iNOS expression (Figure 6c) and NO formation (Supplementary Figure S9D) as reported earlier.[30] To test whether NF-κB-induced NO formation is responsible for cytokine-driven increase in DP5 mRNA expression we next used the NO blocker L-NMA (Supplementary Figure S9E). Blocking NO production partially prevented the late (after 8 h) but not the early expression of DP5 after cytokine treatment (Figure 6d). In line with these results, L-NMA diminished cytokine-induced cytochrome *c* release (Supplementary Figure S1A). Reporter analyses indicated that inactivation of the c-Jun-binding site and L-NMA addition fully prevents cytokine-induced *DP5* promoter activity (Figure 6e). Moreover, combined inactivation of JNK/c-Jun by SP600125 or a cell-permeable JNK inhibitor peptide[31] and NO formation by L-NMA abolished cytokine-induced DP5 mRNA upregulation (Figure 6f) and protected β-cells from apoptosis (Figure 6g; Supplementary Figure S9F). These data indicate that MAPK and iNOS/NO pathways synergise in *DP5* activation after IL-1β + IFN-γ exposure resulting in β-cell death.

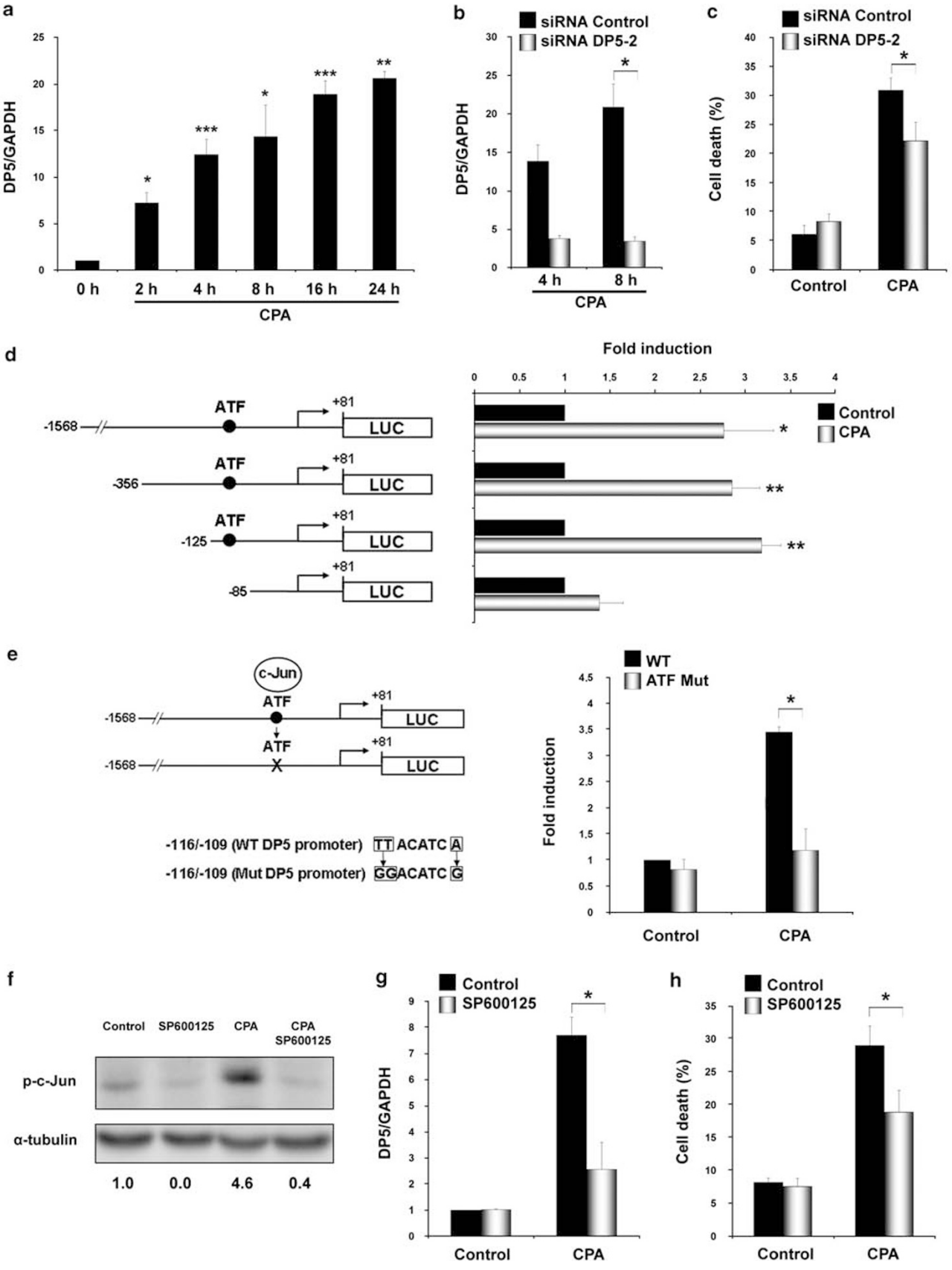
DP5 expression is upstream and modulates the ER stress response in cytokine-treated β-cells. The mitochondria and ER can coordinately trigger apoptosis.[32] Bcl-2 proteins are located at both the mitochondria and ER membranes, regulating induction of autophagy and apoptosis.[33] Interestingly, DP5 knockdown significantly decreased cytokine-induced mRNAs for the ER stress markers XBP-1spliced (XBP-1s), BiP, ATF4, ATF3 and Chop (Figure 7a). Reduced eIF2 phosphorylation and Chop protein expression were also detected in DP5 knockdown cells after cytokine exposure (Figure 7b). In addition, cytokine-induced DP5 expression preceded Chop upregulation (Supplementary Figure S10A), and Chop knockdown using previously described siRNAs[23] did not prevent cytokine- or CPA-mediated DP5 activation (Supplementary Figure S10B and C). These results suggest that DP5 modulates ER homeostasis and that DP5 overexpression precedes and contributes for cytokine-induced ER stress.

Discussion

In this study we show that activation of the BH3-only protein DP5, also known as Hrk, is a critical step for IL-1β + IFN-γ- and chemical ER stressors-induced β-cell death. These observations contribute to answer two important questions, namely the mechanism by which IL-1β + IFN-γ induce β-cell demise and the nature of the downstream signals responsible for apoptosis triggering after severe ER stress.

The main findings of the present work are summarized in Figure 7c. Inactivation of the SERCA2 pump leading to ER Ca^{2+} depletion (e.g. CPA and thapsigargin) or inhibition of N-linked protein glycosylation in the ER (e.g. tunicamycin) activates JNK and c-Jun phosphorylation through ER stress induction. Analysis of DP5 promoter regulation showed that c-Jun phosphorylation is required for ER stress-mediated DP5 expression. Activated DP5 is crucial for β-cell apoptosis, as suggested by the observation that DP5 knockdown prevents cell death induced by the three different ER stressors. Of special relevance, DP5 is activated in insulin-producing INS-1E, primary β-cells and dispersed human islets after ER stress. Pancreatic β-cells are the prototype of differentiated secretory cells and synthesis of insulin increases by more than 10-fold under physiological stimulatory conditions.[34] These cells share with neurons the expression of a large number of genes and proteins[35] and DP5 was found to modulate neuronal cell death.[36] It will be therefore of interest to determine whether DP5 has a similar role as a mediator of ER stress-induced apoptosis in other secretory cells or in neurons.

DP5 is also an important effector of IL-1β + IFN-γ-induced β-cell death as evidenced by the fact that blocking DP5 with specific siRNAs prevents cytokine-induced cytochrome *c* release, caspase-3 activation and apoptosis in β-cells (Figure 2). In this context, the activation and role of DP5 is more complex than in the case of pure ER stressors (Figure 7c). IL-1β + IFN-γ induce both pro- and anti-apoptotic signals, with the pro-apoptotic ones eventually prevailing.[2,37] In line with earlier findings,[37] we observed that cytokines induce an early and parallel activation of JNK/c-Jun and NF-κB. These upstream phenomena lead to a complex downstream cross talk, which was unveiled to a large extent in this study (Figure 7c). Thus, NF-κB upregulates JunB and this is eventually overruled by the inhibitory


a
DP5/GAPDH
0 h
2 h
4 h
8 h
16 h
24 h
CPA
b
siRNA Control
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DP5/GAPDH
4 h
8 h
CPA
c
siRNA Control
siRNA DP5-2
Cell death (%)
Control
CPA
d
Fold induction
ATF
+81
LUC
-1568
-356
-125
-85
Control
CPA
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-116/-109 (WT DP5 promoter) TT ACATC A
-116/-109 (Mut DP5 promoter) GG ACATC G
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effects of JNK.[25] Early c-Jun activation mediates DP5 upregulation after cytokine exposure whereas its antagonist partner JunB inhibits cytokine-induced DP5 expression. The antagonistic role of c-Jun and JunB has been described in other cell types as both proteins participate in AP-1 transactivation.[38] We have shown earlier that overexpression of JunB prevents cytokine-mediated β-cell death,[25] a phenomenon now explained by the inhibitory effect of JunB on DP5. Under pathological conditions cytokine/JNK-induced c-Jun activation eventually prevails over JunB and upregulates DP5. Indeed, analysis of *DP5* promoter constructs indicates that c-Jun has an important function for cytokine-induced DP5 expression. Inhibition of c-Jun phosphorylation, however, partially abolished cytokine-induced DP5 activation, suggesting the involvement of an alternative regulatory mechanism.

Adenovirus-mediated inactivation of NF-κB decreases DP5 mRNA induction (Figure 6). As no binding site for NF-κB was found in the *DP5* promoter we reasoned that the transcription factor was signaling through a downstream effector. An interesting candidate in this respect is the NO radical: this molecule contributes for cytokine induction of β-cell death by modulating the expression of nearly 50% of the modified genes,[19,29] and iNOS, the enzyme responsible for NO formation, is directly regulated by NF-κB in β-cells.[2,29] Inhibition of iNOS activity and NO production by L-NMA prevented the late upregulation of DP5 after cytokine treatment, whereas blocking both c-Jun and iNOS fully abolished cytokine-induced *DP5* promoter activity and mRNA induction, leading to a nearly complete protection against apoptosis (Figure 6g). The mechanism(s) of NO-induced gene modulation in β-cells remain unclear, but it may be mediated through activation/repression of transcription factor(s) by *S*-nitrosation, intracellular zinc quellation, modulation of reactive oxygen species or changes in cellular redox balance as shown in other cell types.[39] An important effect of cytokine-induced NO in β-cells is inhibition of the SERCA2 pump, depleting ER Ca^{2+} stores and triggering ER stress.[9] Considering that NO also contributes to DP5 activation, it is a logical assumption that NO-dependent DP5 induction may occur through ER stress, as observed with the chemical ER stressors (Figure 7c). In line with this possibility, it has been shown in other cell types that the transcription factor Chop, a downstream component of the ER stress response, regulates the BH3-only protein Bim.[40] In our model, cytokine-induced DP5 activation precedes ER stress and Chop induction, and siRNA-mediated Chop knockdown failed to prevent DP5 upregulation (present data). Conversely, DP5 inhibition decreased cytokine-induced activation of ER stress markers, suggesting the novel concept that DP5 is, under some conditions (i.e. exposure to the pro-inflammatory cytokines IL-1β + IFN-γ), an upstream regulator of ER stress, whereas under others (i.e. chemical ER stressors) it acts mainly as a downstream effector of ER stress-induced mitochondrial permeabilization, cytochrome *c* release and cell death.

How DP5 activation induces ER stress remains to be determined, but it does not seem to depend on Ca^{2+} traffic from the mitochondria to the ER (unpublished observation). A crucial effect of DP5 is to hamper Bcl-XL and, to a less extent, Bcl-2 activity.[18,41] Earlier studies have shown that overexpression of these anti-apoptotic Bcl-2 members protects mouse and human β-cells against cytokine-induced apoptosis.[42–44] Bcl-XL and Bcl-2 are located at both the mitochondrial and ER membranes.[32] In the ER, these proteins interact with Ca^{2+} channels regulating Ca^{2+} content.[45] It is conceivable therefore that DP5 inhibits Bcl-XL and Bcl-2 function at the ER level, contributing to Ca^{2+} depletion and ER stress induction. Together our results suggest that combined impairment of the mitochondria and ER organelles by DP5 is a critical step in IL-1β + IFN-γ-mediated β-cell apoptosis.

In conclusion, we have shown that DP5 has a central role for pancreatic β-cell death in the context of severe ER stress and exposure to the pro-inflammatory cytokines IL-1β + IFN-γ, acting as a regulatory/effector protein at different levels of the apoptotic pathways. DP5 may be thus an interesting target for the prevention of β-cell demise in T1D.

Materials and Methods

Cell culture and treatments. Pancreatic islets were isolated from adult Wistar rats (Charles River Laboratories Belgium, Brussels, Belgium), housed and used according to the guidelines of the Belgian Regulations for Animal Care. All the experimental protocols presently used were approved by the Ethical Committee for Animal Experiments of the ULB. Islets were isolated by collagenase digestion and β-cells were purified by autofluorescence-activated cell sorting (FACS, FACStar, Becton-Dickinson and Co.; Sunnyvale, CA, USA) and pre-cultured for 48 h in HAM's F-10 medium before subsequent experimental procedures.[9,23] Insulin-producing INS-1E cells were cultured in RPMI-1640 medium (Invitrogen, Paisley, UK) supplemented with 5% FCS.[19] Recombinant rat or mouse IFN-γ (R&D Systems, Abingdon, UK) and human recombinant IL-1β (a kind gift from Dr. CW Reinolds, National Cancer Institute, Bethesda, MD, USA) were used at the following concentrations: IFN-γ, 100 and 500 U/ml for, respectively, INS-1E cells and primary β-cells; IL-1-β, 10 and 50 U/ml for, respectively, INS-1E cells and primary β-cells. The NO blocker L-NMA (NG-methyl-L-arginine, Sigma, Steinheim, Germany) was used at 2.5 mM. Culture medium was collected for nitrite determination (nitrite is a stable product of NO oxidation) by the Griess method.[19] The peptide JNK inhibitor L-TAT-JNKi (a kind gift of C Bonny and M Mathieu; XigenPharma, Lausanne, Switzerland) was dissolved in culture medium at 10 μM concentration. SP600125 (10 μM), CPA (20 μM), Thapsigargin (1 μM) and Tunicamycin (5 μM) were

Figure 3 ER stress-induced β-cell apoptosis is mediated through DP5 upregulation. (**a**) Time course analysis of CPA-induced DP5 mRNA in INS-1E cells, as evaluated by real-time RT-PCR. Data shown are represented as means ± S.E.M. of DP5 values corrected for GAPDH. *$P<0.05$, **$P<0.01$, ***$P<0.001$ *versus* 0 h. (**b**) INS-1E cells were transfected with control or DP5 siRNAs and DP5 mRNA expression was evaluated by real-time RT-PCR 4 and 8 h after CPA addition. *$P<0.05$. (**c**) siRNA-mediated DP5 knockdown decreased INS-1E cell death 24 h after ER stress induction by CPA. *$P<0.05$. (**d**) The structure of DP5 promoter fragments of different lengths is shown in the left side of the figure. The ATF sites are represented by filled black circles. INS-1E cells were co-transfected with reporter plasmids and pCMV-RL for 6 h and then treated for 24 h with CPA. Luciferase activity was normalized to *Renilla* luciferase activity and expressed as fold induction compared with respective controls, considered as 1 (right side of the figure; the bars correspond to the promoter fragments shown in the left side). *$P<0.05$, **$P<0.01$ *versus* control. (**e**) Mutation of the ATF site abolished activation of the *DP5* promoter. The wild-type ATF site was mutated as it is represented (left). INS-1E cells were transfected with *DP5* luciferase reporter construct containing the wild type (WT) or ATF mutated (ATF Mut) site and treated with CPA as described in Figure 3d. Luciferase activity is represented as in (**d**). *$P<0.05$. (**f**) Western blot analysis of phospho-c-Jun (p-c-Jun) expression in INS-1E cells after 8 h treatment with CPA and/or the JNK-inhibitor SP600125. (**g**) Inhibition of JNK-attenuated upregulation of DP5. INS-1E cells were treated with CPA in the presence or absence of SP600125; 16 h after treatment expression of DP5 was determined by real-time RT-PCR. *$P<0.05$. (**h**) Quantification of INS-1E cell death by HO/PI after 24 h CPA and/or SP600125 treatment. *$P<0.05$

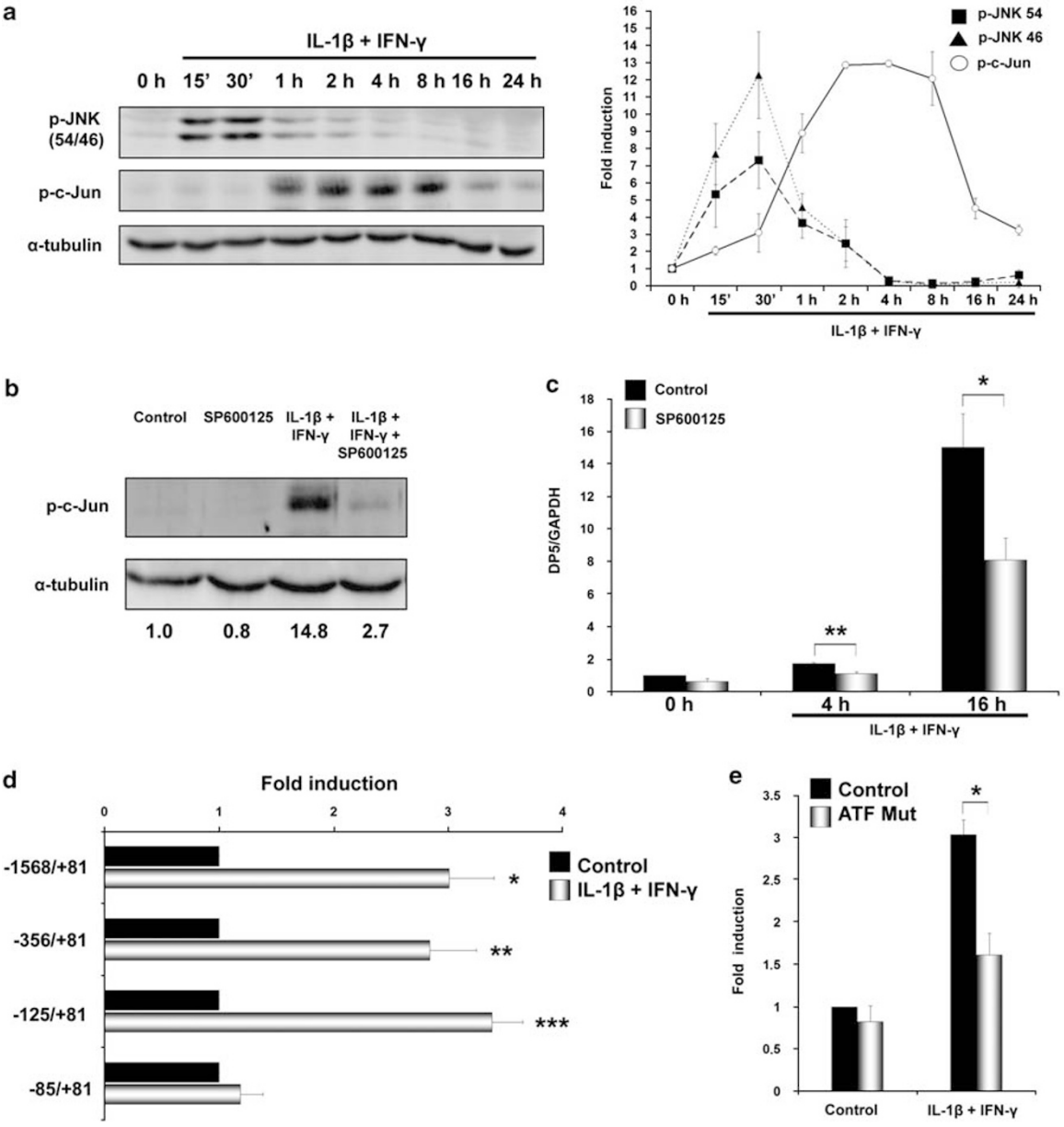

Figure 4 IL-1β + IFN-γ activate the JNK/c-Jun pathway leading to an early DP5 upregulation. (**a**) Time course analysis of IL-1β + IFN-γ-induced JNK activation in INS-1E cells. Cell lysates were subjected to western blotting with antibodies against phospho-JNK (p-*JNK*), phospho-c-Jun (p-c-Jun) or α-tubulin as loading control (left side of the figure). Quantification of densitometry bands is shown in the right side of the figure as fold induction compared with control after correction for α-tubulin. (**b**) Western blot analysis of p-c-Jun activation in INS-1E cells after 8 h of cytokines and/or SP600125 addition. Quantification of the p-c-Jun expression, corrected for α-tubulin, is indicated in the bottom of the figure. (**c**) JNK inactivation by SP600125 decreased cytokine-mediated DP5 upregulation in INS-1E cells. $^{*}P<0.05$, $^{**}P<0.01$. (**d**) INS-1E cells were co-transfected with the *DP5* promoter plasmids described in Figure 3d and pCMV-RL for 6 h and then treated for 36 h with cytokines. Luciferase activity is represented as in Figure 3d. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *versus* control. (**e**) Mutation of the ATF site in the *DP5* promoter diminished activation of cytokine-mediated reporter expression in INS-1E cells. $^{*}P<0.05$

purchased from Sigma and dissolved in DMSO. Cytokines, L-NMA, CPA, Thapsigargin and Tunicamycin concentrations were selected based on our earlier time course and dose–response studies.[10,19,29]

Infection with recombinant adenoviruses. After 48 h pre-culture, cells were infected either with AdLUC (control luciferase expressing virus), Ad$I\kappa B^{(SA)2}$ (encoding a previously described NF-κB super-repressor protein)[30] or AdJunB (expressing the rat JunB protein, purchased from RIKEN Bio Resource Center, Tsukuba, Japan).[25] INS-1E cells were infected for 2 h at 37°C with an MOI of 10 for Ad$I\kappa B^{(SA)2}$ or 20 for AdJunB. AdLUC was used at an MOI of 10 or 20 as control for, respectively, Ad$I\kappa B^{(SA)2}$ or AdJunB.

Immunofluorescence. Cells were plated in poly-lysine-coated coverslip plates, treated with cytokines for the indicated time points, fixed with 4% paraformaldehyde and permeabilized with 0.3% Triton. The cells were then incubated overnight with the primary antibodies. The correspondent secondary antibody FITC or Rhodamine conjugated (Jackson ImmunoResearch, Westgrove, PA, USA; diluted 1/200) was used for visualization by inverted fluorescence microscopy (Zeiss Axiovert 200, Oberkochen, Germany). Primary antibodies used

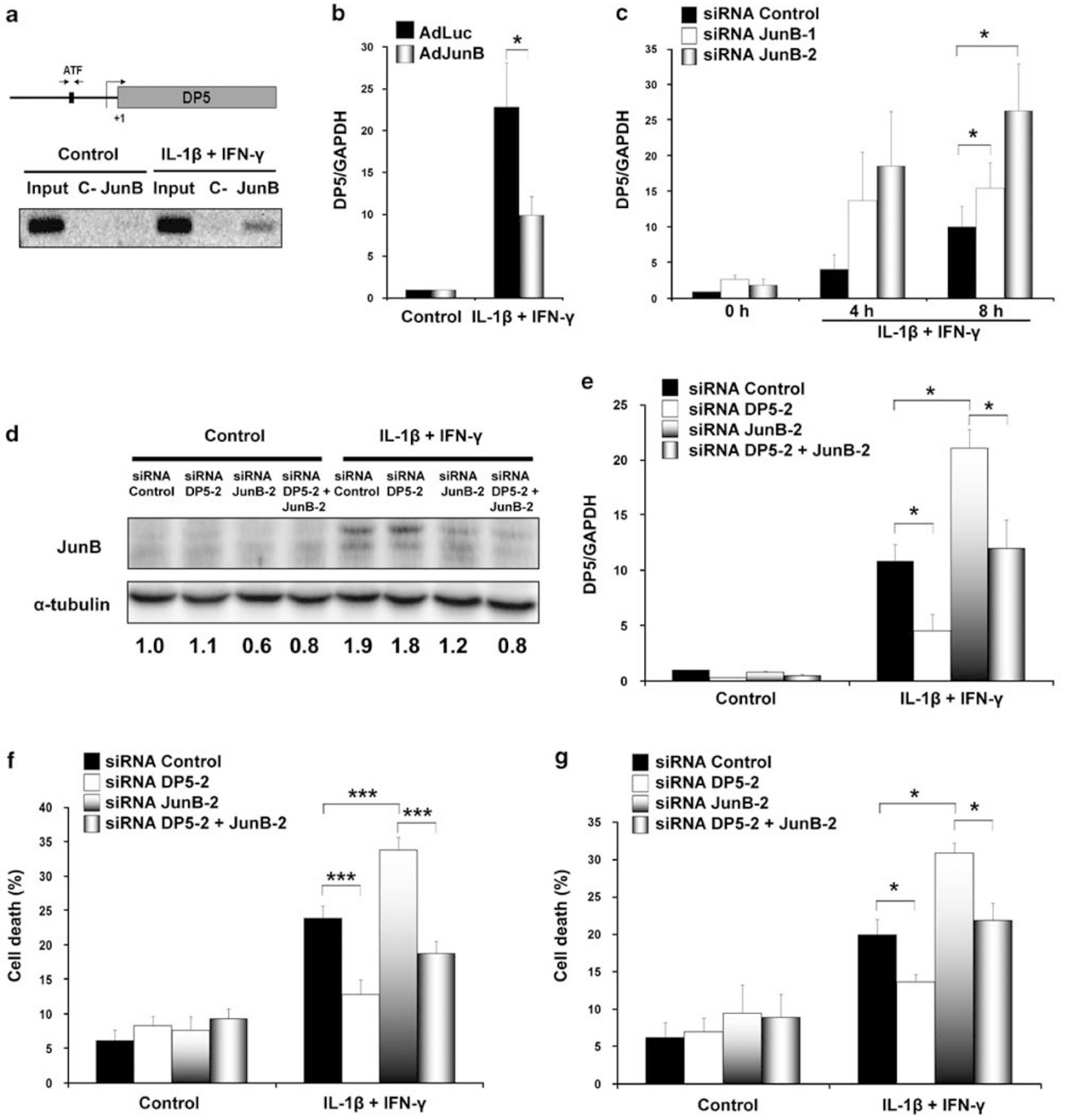

Figure 5 JunB antagonizes c-Jun and prevents cytokine-mediated early DP5 activation. (**a**) Schematic representation of the *DP5* promoter with primers used flanking the ATF site from −116 to −109 relative to the transcriptional start site. ChIP showed binding of JunB to the ATF site in the *DP5* promoter 4 h after cytokine treatment as assessed by PCR. (**b**) AdJunB, but not AdLuc (control adenoviral vector), diminishes cytokine-induced DP5 mRNA expression. INS-1E cells were infected with AdJunB or AdLuc and then exposed for 16 h to cytokines. DP5 expression was analyzed by real-time RT-PCR. $^{*}P<0.05$. (**c**) Knockdown of JunB augments cytokine-induced DP5 mRNA expression. INS-1E cells were transfected with control or JunB siRNAs and treated with cytokines. DP5 expression was analyzed by real-time RT-PCR at the indicated time points. $^{*}P<0.05$. (**d**) Western blotting for JunB expression in INS-1E cells transfected with siRNAs targeting DP5 (DP5-2), JunB (JunB-2) or control and then treated or not for 8 h with cytokines. (**e**) DP5 mRNA expression was measured by real-time RT-PCR in INS-1E cells after transfection with siRNAs targeting DP5 and/or JunB (as in **d**) followed by cytokine treatment for 8 h. $^{*}P<0.05$. (**f**) Knockdown of DP5 prevents the increase in cell death observed in INS-1E cells exposed to cytokines in the presence of siRNAs targeting JunB. Cytokines were added for 24 h to INS-1E cells transfected earlier with siRNAs as in **d**), and cell death was measured by HO/PI. $^{***}P<0.001$. (**g**) Knockdown of DP5 prevents the increase in cell death observed in primary β-cells exposed to cytokines in the presence of an siRNA targeting JunB. Cytokines were added for 48 h to FACS-purified β-cells cells transfected earlier with siRNAs as in **d**), and cell death was measured by HO/PI. $^{*}P<0.05$

were anti-cytochrome *c* (1 : 200, BD Biosciences, San Jose, CA, USA) and anti-AIF (1 : 200, Cell Signaling, Danvers, MA, USA). All fluorescence images shown are representative for 2–4 independent experiments.

Western blotting. Equal amounts of proteins were resolved by 10% SDS-PAGE. Immunoblot analysis was performed with antibodies against JunB, IκB-α, eIF2, Chop (Santa Cruz Biotechnology, CA, USA) Santa Cruz Biotechnology,

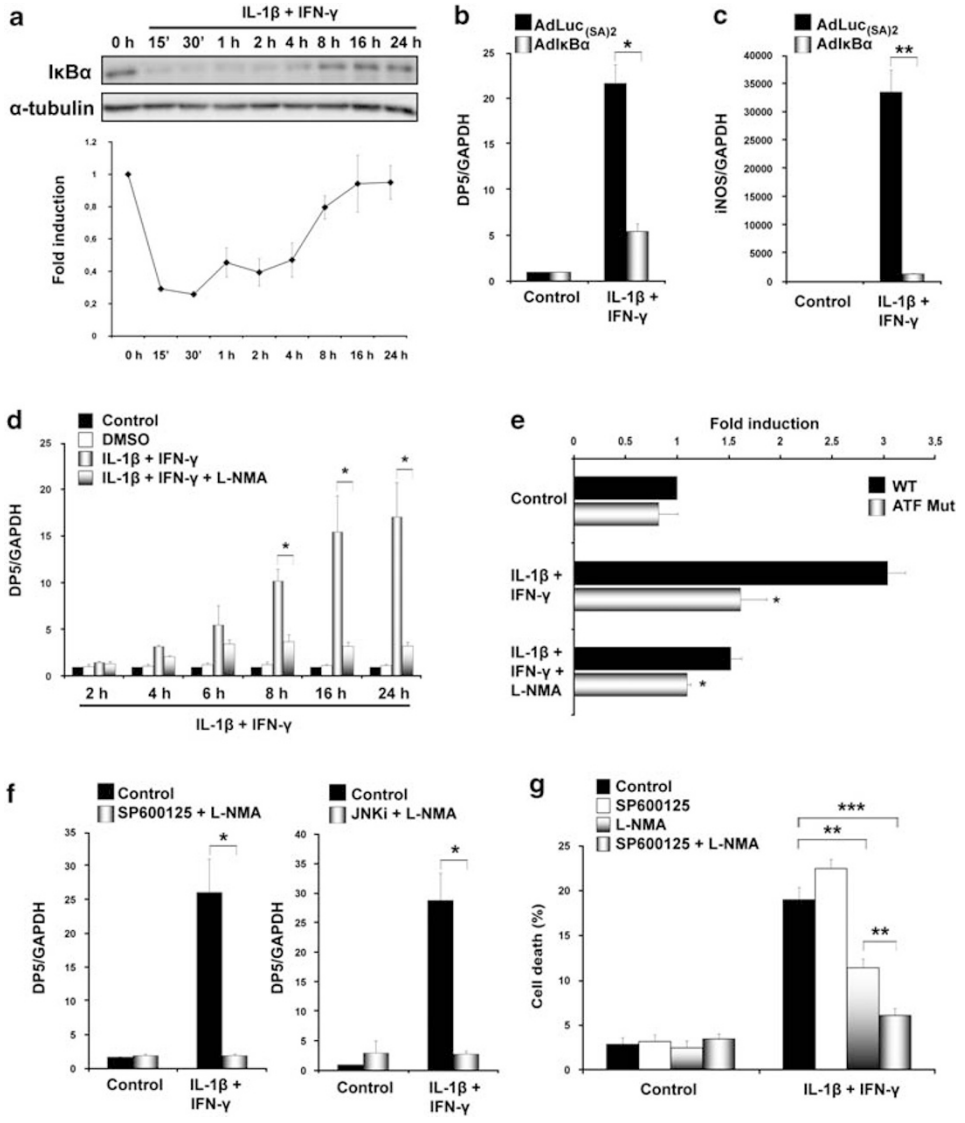

Figure 6 Cytokine-induced iNOS expression and NO formation synergize with JNK/c-Jun to activate DP5. (**a**) Time course analysis of IL-1β + IFN-γ-induced IκB-α degradation in INS-1E cells. Quantification of densitometry bands is shown at the bottom of the figure as fold induction compared with control (non-cytokine treated) after correction for α-tubulin. (**b**, **c**) AdIκB$^{(SA)2}$ prevents cytokine-induced DP5 and iNOS expression. INS-1E cells were infected with AdLUC or AdIκB$^{(SA)2}$, as in **a**), and then exposed to IL-1β + IFN-γ for 16 h. Expression of DP5 and iNOS were determined by real-time RT-PCR. $^{*}P<0.05$, $^{**}P<0.01$. (**d**) The iNOS blocker L-NMA decreases the late, but not the early, cytokine-induced DP5 expression. Time course of DP5 mRNA expression after cytokines and/or L-NMA exposure. $^{*}P<0.05$. (**e**) Reporter analysis of WT or ATF Mut DP5 promoter after cytokine and/or L-NMA treatment. Luciferase activity was measured and expressed as in Figure 3e. $^{*}P<0.05$ *versus* WT. (**f**) INS-1E cells were treated for 16 h with IL-1β + IFN-γ, SP600125 or JNK inhibitor peptide (JNKi), L-NMA or combination as indicated. DP5 expression was measured by real-time RT-PCR. $^{*}P<0.05$. (**g**) Percentage of cell death (HO/PI) in INS-1E cells after exposure for 24 h to cytokines, SP600125, L-NMA or combination as indicated. $^{**}P<0.01$, $^{***}P<0.001$

Santa Cruz, CA, USA, p-c-Jun, p-JNK, p-eIF2, cleaved caspase-3, β-actin (Cell Signaling), DP5 (AbD Serotec, Oxford, UK) and α-tubulin (Sigma). The proteins were detected using the corresponding horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology), and chemiluminescence Supersignal (Pierce, Rockford, IL, USA). Protein loading was normalized for the β-actin or α-tubulin signal. Band intensity was quantified *versus* a control sample (considered as 1), using Aida1D analysis software (Fujifilm, London, UK). All blots shown are representative for 2–4 independent experiments.

Cell viability and caspase-3 activity assay. The percentage cell death was determined in at least 600 cells in each experimental condition by inverted fluorescence microscopy after addition of the DNA dyes Hoechst 342 (20 μg/ml) and propidium iodide (10 μg/ml) (HO/PI).[9,19,23] Viability was evaluated by at least two observers, one of whom was unaware of sample identity. NunViewTM 488 Caspase-3 Kit for Live Cells (Biotium Inc., Hayward, CA, USA) was used for determination of caspase-3 activation after cytokine treatment. Cells were nuclear-stained with the DNA dye Hoechst 342 (20 μg/ml) and then incubated with caspase-3 substrate for at least 15 min. Caspase-3 substrate cleavage stains the nucleus of positive cells green (ultraviolet excitation at 450–490 nm). The percentage of caspase-3-positive cells was calculated against the total number of Hoechst-stained cells (ultraviolet excitation at 365–380 nm).

siRNA treatment. Cells were transfected overnight with 30 nM of siRNAs against DP5 (siRNA DP5-1: GATGTGAACTCTGAGACTTTGTGAT; siRNA DP5-2: TCACAGTTTCTTGGTGCTAAGTGTA) or medium GC content inactive control siRNA (Invitrogen, Carlsbad, CA, USA) using 1 μl of DharmaFECT lipid reagent

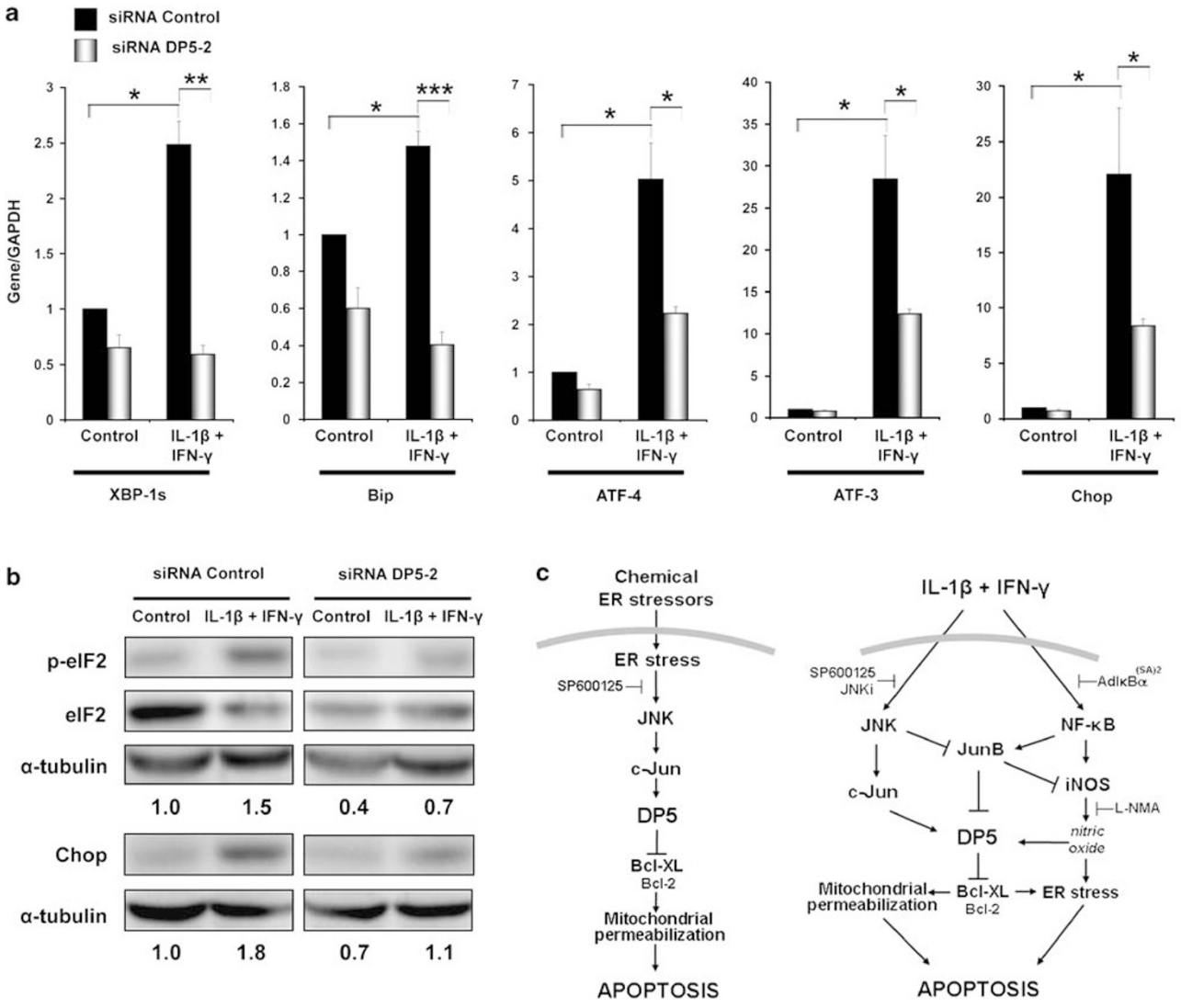

Figure 7 DP5 can act up- or downstream of ER stress to mediate β-cell demise. (**a**) INS-1E cells were transfected with inactive (control) or siRNA DP5-2 and then exposed for 16 h to IL-1β + IFN-γ. Expression of mRNAs encoding for different ER stress markers was measure by real-time RT-PCR. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. (**b**) Western blot demonstrating decreased p-eIF2α and Chop protein levels in cytokine-treated (24 h) DP5 knockdown cells. Quantification of protein band intensities is indicated at the bottom as a ratio to α-tubulin loading. (**c**) Proposed model for the role of DP5 in β-cell apoptosis after exposure to ER stress (left) or cytokines (right). Severe ER stress activates JNK/c-Jun in β-cells leading to DP5 expression, cytochrome *c* release and apoptosis. Cytokines induce an early JNK activation, leading to c-Jun phosphorylation and DP5 expression. In parallel, NF-κB activation results in iNOS expression and transient JunB upregulation. JunB has an inhibitory effect on iNOS and DP5 expression, but its expression is progressively inhibited by JNK. iNOS-mediated NO formation is responsible for the late DP5 increased expression. Activation of DP5 induces cytochrome *c* release and contributes to NO-induced ER stress. The combination of these signals eventually results in cytokine-induced β-cell apoptosis

(Dharmacon, Chicago, IL, USA) to 150 nM of siRNA in Opti-mem (Invitrogen), with an efficiency of transfection $>90\%$.[23,25] After overnight incubation, transfection medium was replaced by regular culture medium for cell recovery.

Real-time RT-PCR. mRNA expression was determined by real-time RT-PCR using SYBR Green fluorescence on a LightCycler instrument (Roche, Manheim, Germany). Primer sequences for mouse and rat DP5, Bak, Bax, Bcl-2, Bcl-XL, Bim, Chop and iNOS are provided in Supplementary Table S1. Expression of the gene of interest was divided by the house keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and expressed as fold induction compared with control. GAPDH expression is not modified under the present experimental conditions (data not shown). All results shown are the means ± S.E.M. of 3–5 independent experiments.

ChIP assay. ChIP assays were performed by standard techniques as described before.[25] Extracts were pre-cleared by 1 h incubation with protein A/Herring sperm DNA, and immunoprecipitation was performed by incubating the samples overnight at 4°C with the JunB antibody (Santa Cruz Biotechnology), using preimmune serum as negative controls, and then 1 h with protein A/Herring sperm DNA. Protein–DNA complexes were washed as per standard ChIP techniques. After elution, proteinase K treatment and reversal of crosslinks, DNA fragments were analyzed by standard PCR. Input DNA was analyzed simultaneously and used as normalization.

DP5 promoter reporter assay. Previously described rat *DP5* promoter constructs tethered to luciferase reporter gene were used.[20] INS-1E cells were transfected using lipofectamine reagent (Invitrogen) with 250 ng of different luciferase reporter construct and the pRL-CMV plasmid (50 ng, with *Renilla* used as internal control for transfection efficiency). Twenty-four hours after transfection, the cells were exposed for 24 h to IL-1β + IFN-γ or CPA (same concentration as above). Luciferase activities of cell lysates were then determined and expressed as Firefly/*Renilla* (relative luciferase activity).

Statistical analysis. Data are shown as means ± S.E. of 3–5 independent experiments, and comparisons between groups are made by paired *t*-test or by ANOVA followed by *t*-test with the Bonferroni correction, as indicated.

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Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on *Cell Death and Differentiation* website (http://www.nature.com/cdd)