

Differential activity of GSK-3 isoforms regulates NF- κ B and TRAIL- or TNF α induced apoptosis in pancreatic cancer cells

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While TRAIL is a promising anticancer agent due to its ability to selectively induce apoptosis in neoplastic cells, many tumors, including pancreatic ductal adenocarcinoma (PDA), display intrinsic resistance, highlighting the need for TRAIL-sensitizing agents. Here we report that TRAIL-induced apoptosis in PDA cell lines is enhanced by pharmacological inhibition of glycogen synthase kinase-3 (GSK-3) or by shRNA-mediated depletion of either GSK-3 α or GSK-3 β . In contrast, depletion of GSK-3 β , but not GSK-3 α , sensitized PDA cell lines to TNF α -induced cell death. Further experiments demonstrated that TNF α -stimulated I κ B α phosphorylation and degradation as well as p65 nuclear translocation were normal in GSK-3 β -deficient MEFs. Nonetheless, inhibition of GSK-3 β function in MEFs or PDA cell lines impaired the expression of the NF- κ B target genes Bcl-xL and cIAP2, but not I κ B α . Significantly, the expression of Bcl-xL and cIAP2 could be reestablished by expression of GSK-3 β targeted to the nucleus but not GSK-3 β targeted to the cytoplasm, suggesting that GSK-3 β regulates NF- κ B function within the nucleus. Consistent with this notion, chromatin immunoprecipitation demonstrated that GSK-3 inhibition resulted in either decreased p65 binding to the promoter of *BIR3*, which encodes cIAP2, or increased p50 binding as well as recruitment of SIRT1 and HDAC3 to the promoter of *BCL2L1*, which encodes Bcl-xL. Importantly, depletion of Bcl-xL but not cIAP2, mimicked the sensitizing effect of GSK-3 inhibition on TRAIL-induced apoptosis, whereas Bcl-xL overexpression ameliorated the sensitization by GSK-3 inhibition. These results not only suggest that GSK-3 β overexpression and nuclear localization contribute to TNF α and TRAIL resistance via anti-apoptotic NF- κ B genes such as Bcl-xL, but also provide a rationale for further exploration of GSK-3 inhibitors combined with TRAIL for the treatment of PDA.

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Subject Category: Cancer

Pancreatic ductal adenocarcinoma (PDA) is one of the most lethal human malignancies and is highly resistant to conventional chemotherapy due, in part, to its ability to resist induction of apoptosis.¹ There has been substantial interest in death receptor (DR)-initiated apoptosis as a potential means of circumventing chemotherapy resistance;^{2–4} and the death ligand TRAIL has undergone extensive preclinical and clinical testing due to its selective killing of cancer cells.^{3,5–8} Binding of TRAIL to DR4 and DR5 initiates assembly of the death-inducing signaling complex (DISC), which directly triggers caspase activation and, in some cells, activates the mitochondrial apoptotic pathway.^{5–9} Unfortunately, a potential

limitation of TRAIL-based therapy is that many cancer cells display TRAIL resistance, which can arise at any step of the apoptotic process from impairment of DISC formation to constitutive upregulation of anti-apoptotic proteins of both the extrinsic and mitochondrial pathways.^{1,7,10–12}

The outcome of TRAIL binding to cancer cells is highly context dependent. In addition to activating caspase-dependent cell death, DR4 and DR5 ligation can initiate signaling that results in increased survival, invasion and metastasis.^{12–15} In particular, TRAIL has also been reported to activate the transcription factor NF- κ B, which transactivates the genes encoding several key anti-apoptotic proteins,

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Abbreviations: ChIP, chromatin immunoprecipitation; cFLIP, cellular Flice-like inhibitory protein; cIAP2, cellular inhibitor of apoptosis protein 2; DISC, death-inducing signaling complex; DR, death receptor; EGFP, enhanced green fluorescent protein; FADD, Fas-associated protein with death domain; FITC, fluorescein isothiocyanate; GSK-3, glycogen synthase kinase-3; GSK-3i, glycogen synthase kinase-3 inhibitor; MEF, mouse embryonic fibroblast; NES, nuclear export signal; NLS, nuclear localization signal; NF- κ B, nuclear factor kappa-light-chain-enhancer of activated B cells; PDA, pancreatic ductal adenocarcinoma; PLA, proximity ligation assay; qRT-PCR, quantitative reverse-transcriptase polymerase chain reaction; shRNA, short hairpin RNA; TNF α , tumor necrosis factor alpha; TRAIL, tumor necrosis factor α -related apoptosis inducing ligand; XIAP, X-linked inhibitor of apoptosis protein.

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including cFlip, Bol-xL, Mcl-1 and IAPs, each of which has been implicated in TRAIL resistance.^{10,12,13,16,17} Novel strategies that overcome this survival signaling could potentially enhance the antitumor effects of TRAIL.

The two glycogen synthase kinase-3 (GSK-3) isoforms, GSK-3 α and GSK-3 β , were initially described as key enzymes involved in glycogen metabolism, but have subsequently been shown to regulate a diverse range of cellular functions, including apoptosis.^{18,19} In particular, GSK-3 β phosphorylates and regulates the stability of a number of proteins that are critical for proliferation and survival.²⁰ Importantly, GSK-3 β deficient mouse embryonic fibroblasts (MEFs) exhibit defective NF- κ B activation in response to TNF α , although the reason for this defect remains unclear.²¹ Conversely, constitutive NF- κ B activation in PDA contributes to cell proliferation and survival, as well as resistance to TNF α - and TRAIL-induced apoptosis.^{7,16,17,22}

We and others have shown that GSK-3 β is progressively overexpressed during progression from pancreatic intraepithelial neoplasia to advanced PDA and is localized to the nucleus in most moderately and poorly differentiated tumors.^{23–26} Additionally, it has been observed that GSK-3 β overexpression contributes to PDA cell proliferation and survival, whereas GSK-3 inhibition reduces pancreatic cancer cell viability *in vitro* and suppresses tumor xenograft growth *in vivo*, at least partly, *via* downregulation of NF- κ B activity.^{23–28} In view of the impact of NF- κ B target genes on TRAIL-induced apoptosis, we have assessed the impact of GSK-3 inhibition or downregulation on TRAIL-induced killing and have examined the mechanism of GSK-3 inhibitor (GSK-3i)-mediated TRAIL sensitization in PDA cells. Our results demonstrate for the first time that GSK-3 β , acting within the nucleus, regulates the action of NF- κ B at a subset of its target genes to modulate TRAIL-induced apoptosis.

Results

Isoform-specific GSK-3 depletion sensitizes PDA cells differentially to TNF α - and TRAIL-induced apoptosis. To provide a baseline for subsequent combination studies, we examined the effect of a potent and specific GSK-3i (LY2064827) on pancreatic cancer cell growth. We observed growth inhibition in all tested cell lines (Supplementary Figures S1a–e) with IC₅₀s ranging from 50 nM to 5 μ M after a 72-hour treatment (Supplementary Figure S1f). We next tested the effect of GSK-3i on TNF α and TRAIL-induced apoptosis (Figure 1). At concentrations that had little impact by themselves, GSK-3i enhanced TNF α -induced cell death as assessed by MTS assay, DNA fragmentation, annexin V binding and PARP cleavage (Figures 1a, c, e and g). Likewise, GSK-3i enhanced TRAIL-induced apoptosis in all PDA cell lines tested as demonstrated by DNA fragmentation, annexin V binding and PARP as well as CASP3 cleavage (Figures 1b, d, f, h and i and Supplementary Figure S2). This sensitization occurred with minimal effect on DR4 or DR5 mRNA expression and little if any change in cell surface levels of these receptors (Supplementary Figure S3).

Because GSK-3 inhibitors, including LY2064827, do not discriminate between the two GSK-3 isoforms, we knocked

down each GSK-3 isoform selectively using multiple lentiviral shRNA constructs (Figure 2a and Supplementary Figure S4a). Interestingly, GSK-3 β , but not GSK-3 α suppression, led to increased TNF α -induced apoptosis, as manifested by increased PARP and CASP3 cleavage as well as annexin V binding (Figures 2a, b and f and Supplementary Figure S4b). Consistent with this observation, more apoptosis was observed in GSK-3 β -null mouse embryonic fibroblasts (MEFs) than in wild-type (WT) MEFs after TNF α treatment (Figures 2c and f). In contrast, suppression of either GSK-3 α or GSK-3 β sensitized similarly to TRAIL-induced apoptosis in Panc04.03 and HupT3 cells (Figures 2d, e and g). Similar enhancement of TRAIL-induced apoptosis was observed using two additional GSK-3 α or GSK-3 β lentiviral shRNA construct (Supplementary Figures S4b and c). These results point to a unique role for GSK-3 β in suppressing TNF α -induced apoptosis, whereas both GSK-3 α and GSK-3 β contribute to TRAIL resistance in pancreatic cancer cells.

GSK-3 β is not essential for TNF α -induced p65 nuclear translocation. To determine how GSK-3 modulation affects death ligand signaling, we started by evaluating the effect of GSK-3 β deletion on key steps of NF- κ B activation. TNF α -treatment of either WT or GSK-3 β -null MEFs resulted in robust I κ B α phosphorylation by 5 min and loss of total I κ B α protein by 15 min (Figure 3a), with robust accumulation of p65 in the nucleus by 20 min in both cell lines (Figure 3b). The pattern and extent of nuclear p65 oscillations were also similar at extended periods following TNF α treatment (Figures 3c and d). Taken together, these results suggest that GSK-3 β is not essential for either the initial wave of I κ B α degradation leading to p65 nuclear entry or the subsequent I κ B α gene expression that drives the kinetics of the p65 nuclear oscillations.

Because the predominant form of NF- κ B in the cytosol in resting cells and in the nucleus upon NF- κ B activation is a p50/p65 heterodimer, we further investigated the effect of GSK-3 β on p50/p65 interactions using a proximity ligation assay (PLA), a highly sensitive technique to detect protein–protein interactions *in situ*. As predicted, the p50/p65 interactions were observed almost exclusively in the cytosolic compartment in resting cells, mainly in the nucleus 20 min after TNF α exposure, and largely in the cytosol again at 60 min (Figures 3e and f). There was no significant difference in the number or the sites of p50/p65 interactions following TNF α exposure in WT versus GSK-3 β null MEFs (Figures 3e and f). Accordingly, GSK-3 β deficiency does not appear to impact TNF α -induced canonical NF- κ B activation up to the point of p50/p65 nuclear entry.

Isoform-specific regulation of BCL2L1 and BIRC3 by GSK-3 in pancreatic cancer cells. In further experiments, we examined the effects of GSK-3i and isoform-specific shRNA on NF- κ B target genes that affect the DR pathway. Dose-response experiments demonstrated that concentrations of 200–1000 nM GSK-3i decreased phosphorylation of GSK-3 α / β Y276/Y216 and glycogen synthase S641 (Figure 4a), increased nuclear accumulation of β -catenin (Figure 4b), and increased activity of pLenti-TopFlash Luciferase reporter (Figure 4c), which reflects β -catenin

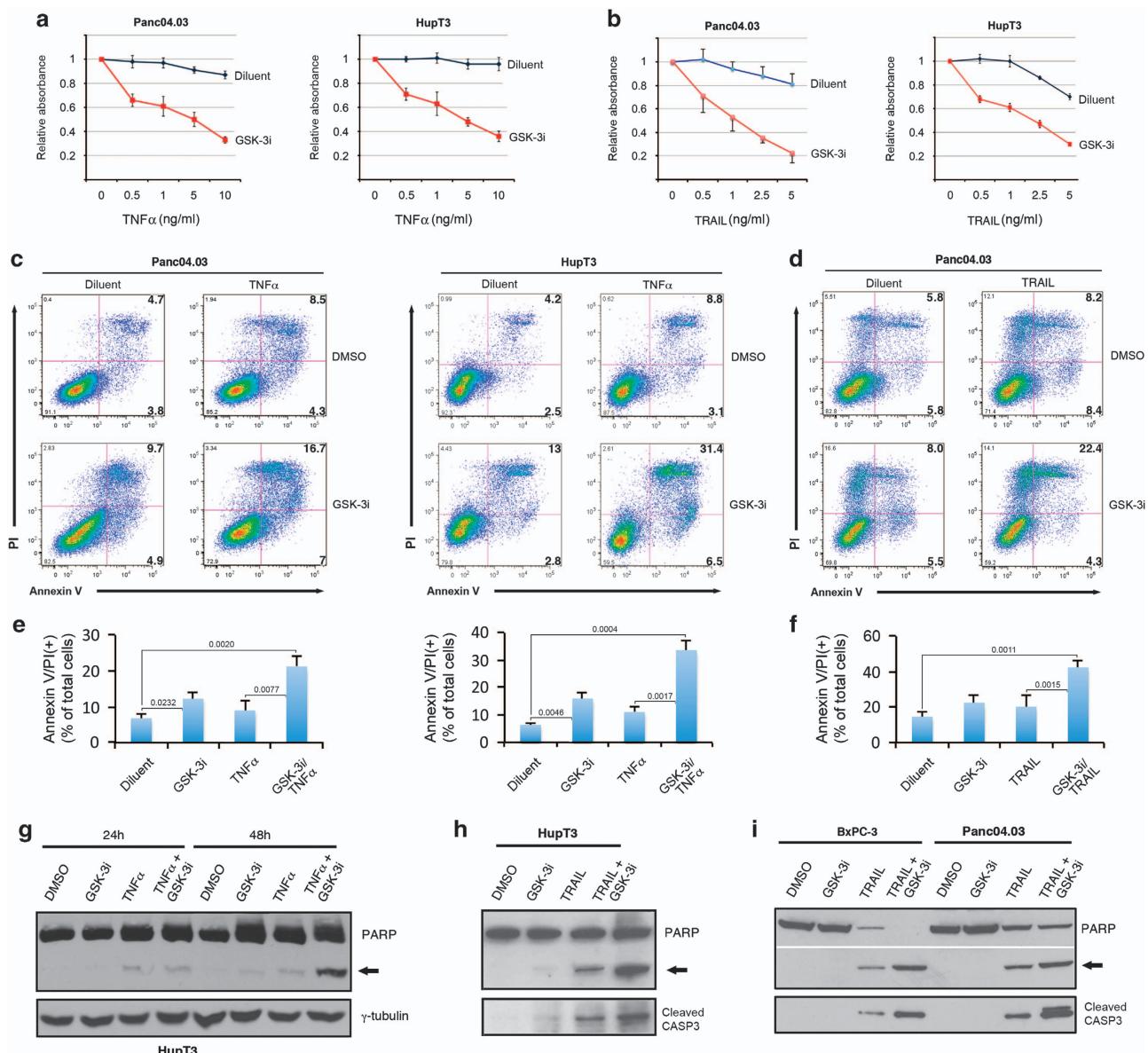


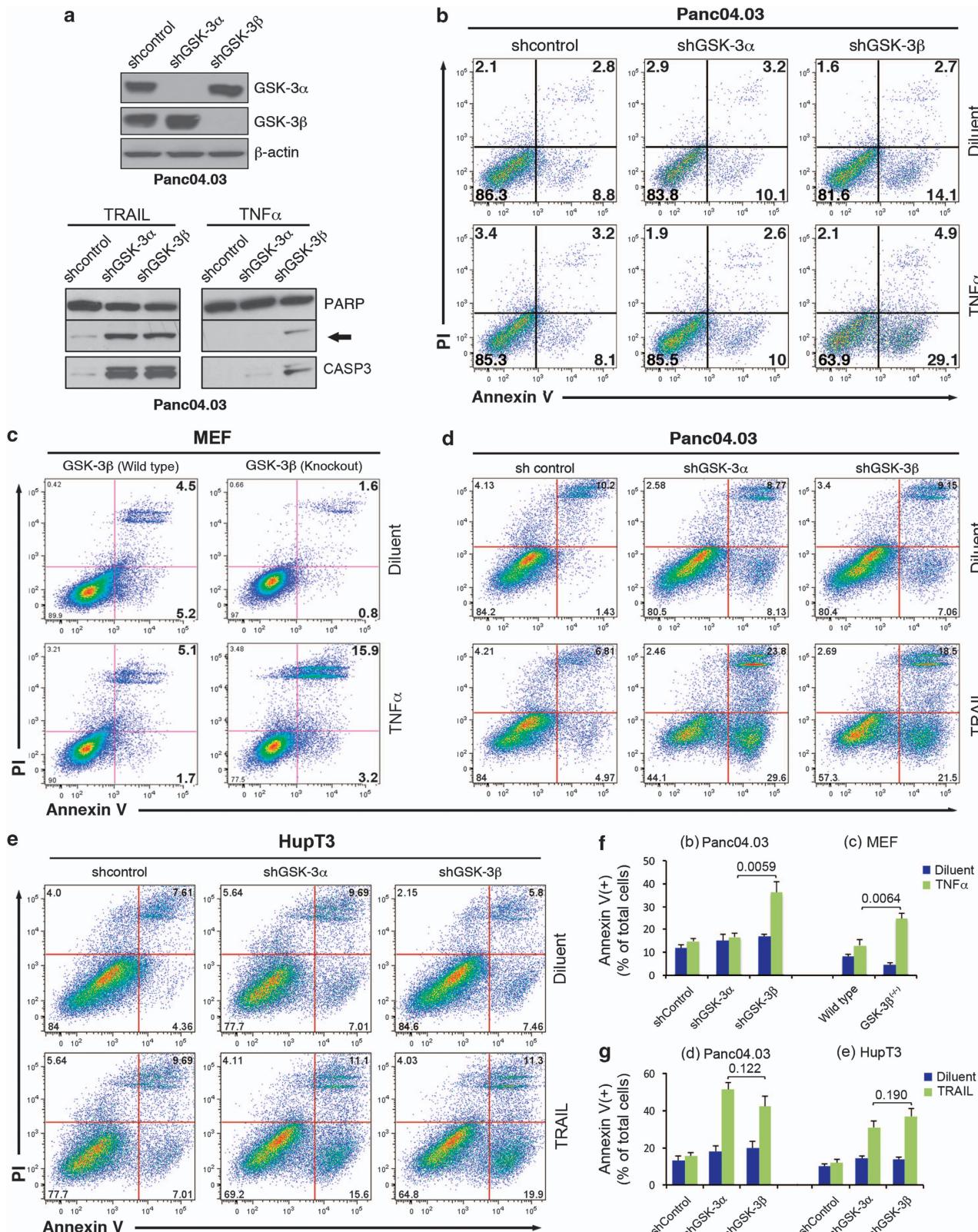
Figure 1 GSK-3 inhibition enhances both TNF α - and TRAIL-induced apoptosis in pancreatic cancer cells. (a, b) Cell proliferation determined by MTS assay. Pancreatic cancer cells were pretreated for 30 min with 0.2 μ M of GSK-3i (LY2064827) or diluent (0.1% DMSO) followed by co-treatment with the indicated concentrations of TNF α or TRAIL for 24 h. (c–f) Cells were treated with diluent, GSK-3i and TNF α or TRAIL for 24 h as in (a) and dually stained with annexin V-FITC and PI for flow cytometry. (e, f) Results from three independent experiments were quantified and shown as mean \pm S.D. with indicated P-values (Student's t-test). (g) HupT3 cells were treated with GSK-3i and TNF α alone or in combination for 24 and 48 h, respectively. Whole-cell extracts were prepared for immunoblot analysis for PARP1. (h, i) Indicated pancreatic cancer cells were treated with GSK-3i (0.2 μ M) and TRAIL (2 ng/ml) alone or in combination for 24 h. Whole-cell extracts were immunoblotted with antibodies specific for PARP1 and cleaved CASP3. Arrows indicate cleaved PARP1 (g–i).

nuclear accumulation. The same 500 nM GSK-3i concentration that produced near-maximal pLenti-TopFlash Luciferase activation (Figure 4c) decreased expression of Bcl-xL, cIAP2 and cFlip (both long and short isoforms), while I κ B α and Bcl-2, and XIAP were not affected (Figure 4d). Interestingly, in cells expressing GSK-3 isoform-specific shRNA, Bcl-xL and cIAP2 were decreased with GSK-3 β , but not with GSK-3 α suppression (Figure 4e and data not shown). cFlip, however, was not reduced by either GSK-3 α or GSK-3 β shRNA, suggesting that both kinases might need to be inhibited to affect this protein.

GSK-3 β modulates NF- κ B binding and cofactor recruitment at the *BCL2L1* and *BIRC3* promoters. The preceding results suggest that GSK-3 β , but not GSK-3 α modulates a subset of NF- κ B target genes. This possibility was further evaluated by qRT-PCR and chromatin immunoprecipitation (Figure 5). qRT-PCR revealed that I κ B α mRNA increased more than 20-fold following a 45-min TNF α treatment in both WT and GSK-3 β null MEF cells (Figure 5a). In contrast, Bcl-xL and cIAP2 mRNA levels increased in WT MEFs but not GSK-3 β -null MEFs (Figure 5a). These alterations in transcript levels

largely correlated to protein expression changes as determined by immunoblotting (Supplementary Figure S5). Depletion of GSK-3 β in Panc04.03 cells similarly impaired

TNF α -induced expression of Bcl-xL and cIAP2 but spared I κ B α (Figure 5b). These results indicate that GSK-3 β loss does not globally impact NF- κ B transcriptional activity, but



instead impairs the expression of a specific set of NF- κ B target genes.

As we found no gross defect in GSK-3 β -null cells up to p65 nuclear entry, we next examined whether GSK-3 may affect binding of p65 and p50 to the promoters. For the I κ B α promoter, we found no significant change in either p65 or p50 loading or histone 4 lysine 16 acetylation (a marker of gene activation) following GSK-3i treatment (Figure 5c). In contrast, at the promoter of the *BIRC3* gene, which encodes cIAP2, p65

binding and H4K16ac modification were significantly reduced, with only a marginal decrease in p50 binding (Figure 5c). Unexpectedly, we detected increased p50 binding at the promoter of the *BCL2L1* gene, which encodes *Bcl-xL* (Figure 5d), while p65 binding was only marginally decreased (Figure 5e). Further analysis of the *BCL2L1* promoter indicated that GSK-3i treatment was also associated with increased SIRT1 and HDAC3 loading along with reduced binding of lysine 310-acetylated p65 and RNA pol II, indicating

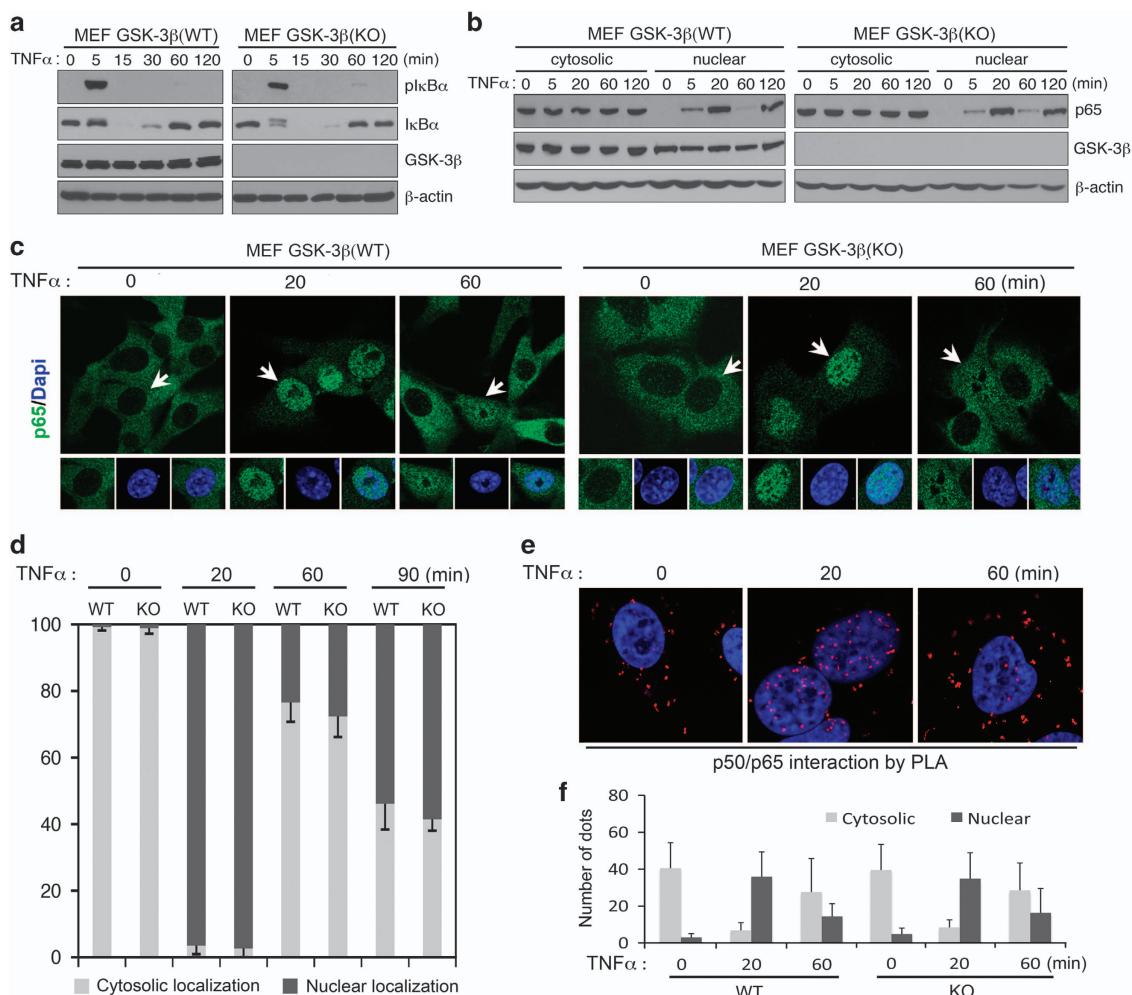


Figure 3 GSK-3 β is not essential for TNF α -induced I κ B α degradation or p65 nuclear translocation. (a, b) Western blot analysis of whole-cell extracts (a) or cytosolic and nuclear extracts (b) from MEFs treated with 10 ng/ml TNF α for the indicated periods of time. Note that there is no discernible difference in TNF α -induced I κ B α phosphorylation/degradation (a) or p65 nuclear translocation (b) between WT and GSK-3 β -null MEF cells. (c) Immunofluorescence staining and confocal microscopic imaging showing p65 expression/localization in MEF cells following TNF α stimulation (10 ng/ml) for the indicated periods of time. (d) Quantification of images (c) for percentage of p65 with predominant cytoplasmic or nuclear localization in wild-type (WT) and Gsk-3 β ^{-/-} (KO) MEF cells at the indicated time points. Arrows point to the cells shown in inset with p65 predominantly localized to the cytoplasm (0 min) or nucleus (20 and 60 min). (e) Confocal microscopic imaging showing *in situ* p50 and p65 interaction detected by PLA following TNF α stimulation. (f) Quantification of dots corresponding to sites of interaction as shown in (e) as mean \pm S.D. as described in Materials and Methods

Figure 2 Isoform-specific role of GSK-3 in TRAIL- and TNF α -induced apoptosis. (a) Whole-cell extracts prepared from cells stably transduced with isoform-specific lentiviral shRNAs or scrambled control shRNA were subjected to immunoblotting to demonstrate specific and efficient knockdown of endogenous GSK-3 proteins (upper panel). Lentiviral shRNA transduced cells were treated with TRAIL or TNF α for 24 h. Whole-cell extracts were subjected to immunoblot against PARP1 and cleaved CASP3 (lower panel). Arrow indicates cleaved PARP1. (b, c, f) Panc04.03 stably transduced with lentiviral shRNA (b) or MEFs (c) were treated with TNF α (20 and 2 ng/ml, respectively) for 18 h and stained with annexin V-FITC and PI for flow cytometry. (d, e, g) Cells stably transduced with the indicated shRNA were treated with TRAIL (5 ng/ml) or diluent for 18 h, stained with annexin V-FITC/PI and examined by flow cytometry. (f, g) Results from three independent experiments were quantified and shown as mean \pm S.D. with indicated P-values (Student's t-test). Note that only GSK-3 β knockdown or deletion enhanced TNF α -induced apoptosis in Panc04.03 or MEF cells (f), whereas knockdown of either GSK-3 α or GSK-3 β sensitized the cells equally to TRAIL (g)

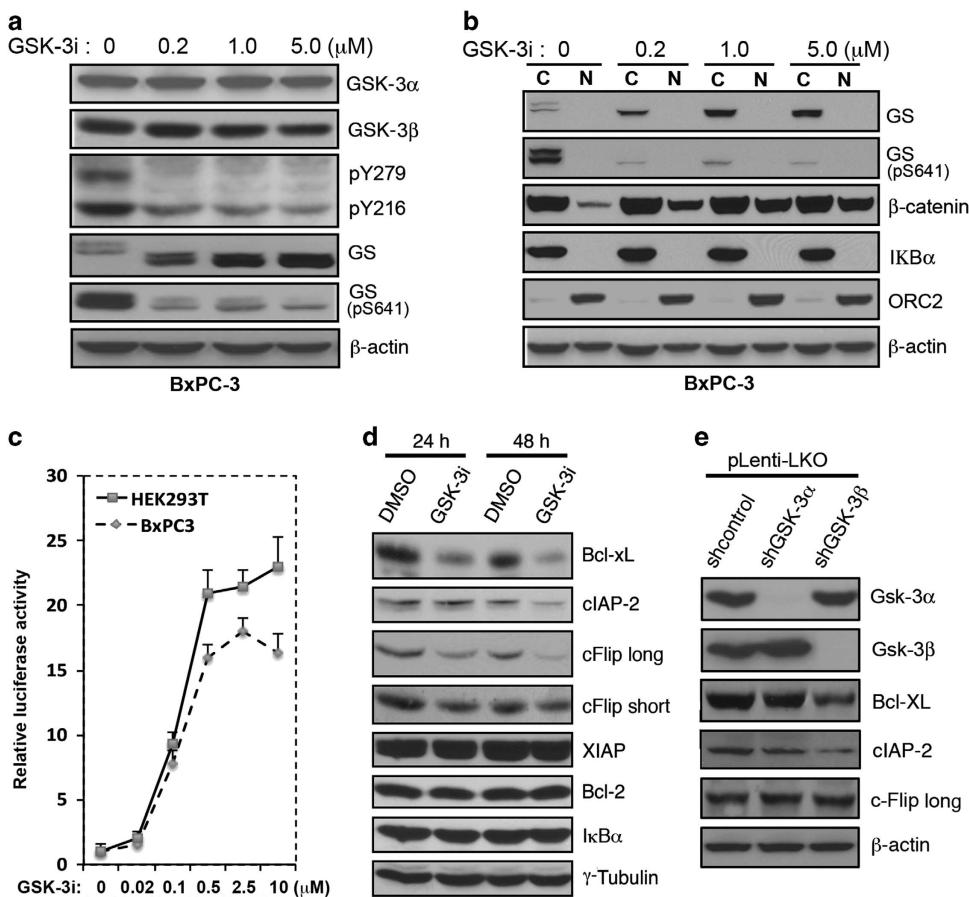


Figure 4 GSK-3 inhibition and GSK-3 β suppression regulate a subset of anti-apoptotic NF- κ B target genes. (a, b) Western blot analysis of whole-cell extracts (a) or cytosolic and nuclear fractions (b) with indicated antibodies. BxPC-3 cells were treated for 24 h with the indicated concentrations of GSK-3i before preparation of protein extracts. (c) Luciferase reporter assay in BxPC-3 and HEK293T cells stably transduced with TopFlash and treated with the indicated concentration of GSK-3i for 6 h. The relative firefly luciferase activities were normalized to renilla luciferase activity and plotted as a histogram. (d) After Panc04.03 cells were treated with 0.5 μ M of GSK-3i for 24 and 48 h, respectively, whole-cell extracts were prepared and immunoblotted with the indicated antibodies. (e) Whole-cell extracts from Panc04.03 cells stably transduced with GSK-3 α or GSK-3 β lentiviral shRNA were probed with the indicated antibodies

that the GSK-3i induced the formation of repressive chromatin. These results suggest that GSK-3i may differentially impact p65 and/or p50 binding and unloading from chromatin depending on the target gene promoter.

Nuclear GSK-3 β contributes to Bcl-xL and cIAP2 expression. The preceding results not only show that GSK-3 β modulates the effects of NF- κ B on a specific subset of its target genes, but also suggest that these effects occur after NF- κ B enters the nucleus. Consistent with these results, we detected abundant nuclear GSK-3 β in all pancreatic cancer cell lines examined (Figure 6a). Moreover, nuclear GSK-3 β exhibited minimal S9 phosphorylation, an auto-inhibitory modification, suggesting that the nuclear pool of GSK-3 β is highly active in these cells (Figure 6a). These results prompted us to determine the role of nuclear GSK-3 β in the regulation of these NF- κ B target genes. For this purpose, we generated a set of mammalian suppression/re-expression plasmids that knocked down endogenous GSK-3 β and expressed modified GSK-3 β that contained either a nuclear export signal (NES) for cytoplasmic targeting or a nuclear localization signal (NLS) for nuclear targeting.

Immunofluorescence and cellular fractionation followed by immunoblotting confirmed that GSK-3 β (S9A).NES.F and GSK-3 β (S9A).NLS.F localized mainly to the cytoplasm and nucleus, respectively (Figures 6b and c). Using this reconstituted system, we investigated the effect of GSK-3 β subcellular localization on the expression of I κ B α , Bcl-xL and cIAP2. Consistent with our previous data, neither depletion of GSK-3 β nor re-expression of a cytosolic or nuclear form of GSK-3 β affected I κ B α gene expression (Figure 6d). Interestingly, expression of cytosolic GSK-3 β resulted in reduced expression of both Bcl-xL and cIAP2 similar to that of GSK-3 β depletion (Figure 6d). In contrast, expression of either the S9A mutant or the nuclear-localized GSK-3 β resulted in increased expression of both Bcl-xL and cIAP2 (Figure 6d). Taken together, these data indicate that active nuclear GSK-3 β has the potential to regulate NF- κ B target genes involved in cell survival.

Bcl-xL and XIAP, but not cIAP2, contribute to GSK-3i-mediated sensitization to TRAIL- and TNF α -induced apoptosis. Bcl-xL, cIAP2 and XIAP are all important apoptotic regulators. To assess their roles in TRAIL- and

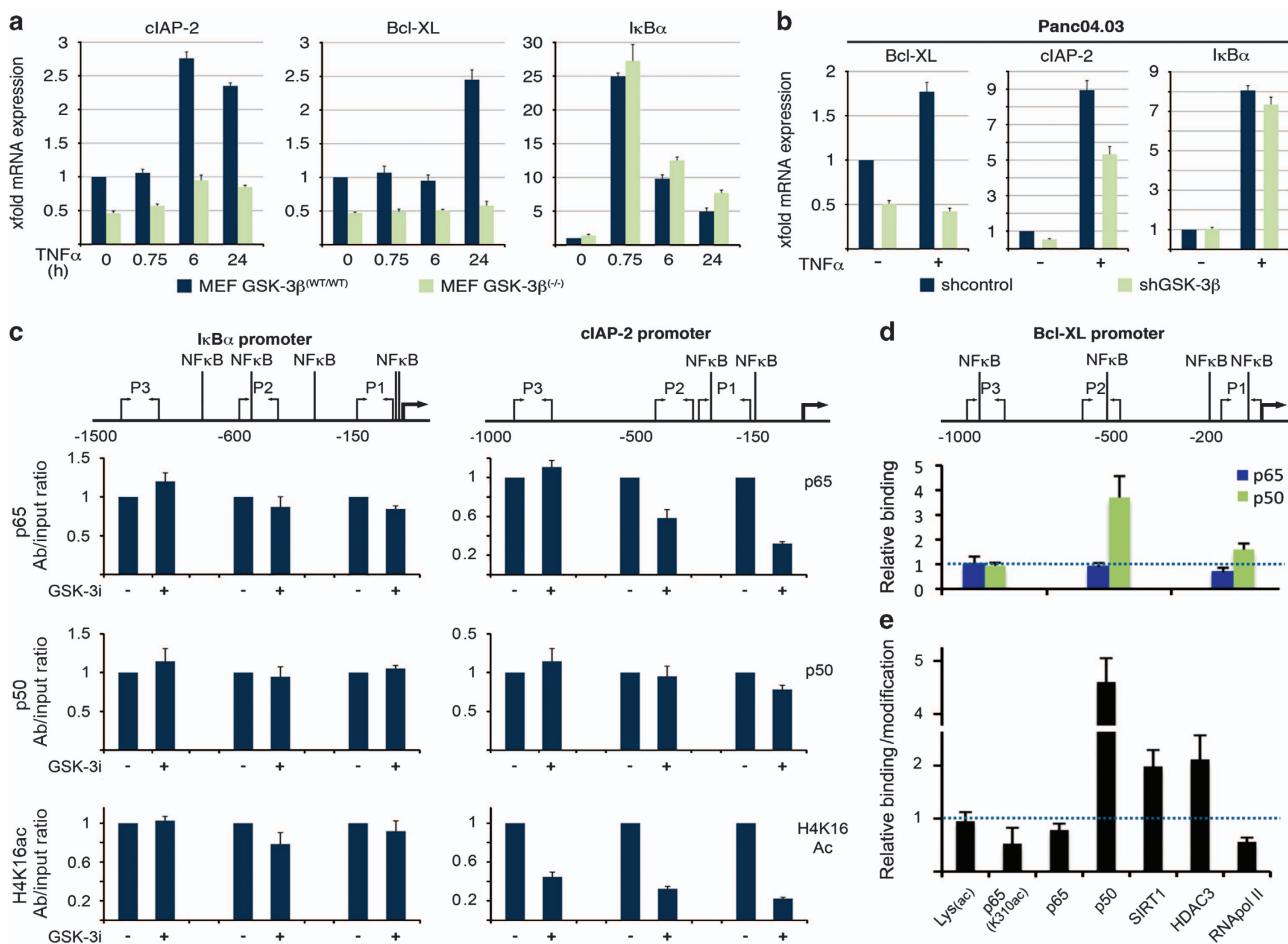


Figure 5 GSK-3 β differentially affects the binding of NF- κ B p65 and p50 to the *BCL2L1* and *BIRC3* promoters. **(a, b)** qRT-PCR analysis of MEF and Panc04.03 cells following TNF α -stimulation. Note that Bcl-xL and cIAP2 transcripts, but not I κ B α , were reduced in Gsk-3 β -null MEFs compared with WT control (a). Similarly, Bcl-xL and cIAP2, but not I κ B α mRNA expression was reduced in TNF α -treated Panc04.03 cells transduced with lentiviral GSK-3 β shRNA (b). **(c–e)** ChIP analysis of Panc04.03 cells treated with GSK-3i (0.5 μ M LY2064827) or diluent (0.1% DMSO) for 16 h. The relative amount of DNA precipitated with the specified antibodies was accessed by qPCR using three sets of primers targeting promoter regions containing the depicted NF- κ B sites. The qPCR was performed in triplicate; and the result is normalized to diluent control.

TNF α -induced apoptosis in PDA cells, we used lentiviral shRNA to generate HupT3 cells with stable knockdown of each gene and determined their response to TRAIL and TNF α (Figures 7a–c). Suppression of XIAP as well as cIAP2 increased basal levels of apoptosis (shXIAP = 14.9% \pm 1.27 and shcIAP2 = 10.9% \pm 0.29 versus shcontrol = 7.6% \pm 0.59). At the concentrations studied, TRAIL induced more apoptosis than TNF α in scramble control. Importantly, suppression of either Bcl-xL or XIAP markedly enhanced TRAIL-induced apoptosis (25.7% \pm 1.0 and 28.5% \pm 2.9, respectively, versus 16.2% \pm 1.9 in scrambled control). However, only shBcl-xL cells displayed significantly increased sensitivity to TNF α (16.5% \pm 0.6 versus 8.2% \pm 0.4 in diluent control), whereas shXIAP cells exhibited higher spontaneous apoptosis, but no significant enhancement of TNF α activity (15.2% \pm 1.3 versus 14.9% \pm 1.3 in diluent control). On the other hand, cIAP2 knockdown did not affect either TRAIL (16.6% \pm 0.7 versus 16.2% \pm 1.9 in shcontrol) or TNF α -induced apoptosis (11.4% \pm 1.2 versus 10.7% \pm 1.0 in shcontrol). These results highlight potentially distinct roles for these anti-apoptotic

proteins when apoptosis is initiated by different stimuli and also indicate that only Bcl-xL is both regulated by GSK-3 β suppression or GSK-3i and contributes to TRAIL resistance. To further examine if Bcl-xL actively participates in GSK-3i-mediated TRAIL sensitization, we expressed Bcl-xL off an NF- κ B- and GSK-3i-independent promoter in BxPC-3 cells (Figure 7d) and measured apoptosis following treatment with either GSK-3i or TRAIL (Figure 7e). Similar to HupT3 cells, Bcl-xL knockdown BxPC-3 cells transduced with empty vector showed markedly increased apoptotic induction following GSK-3i or TRAIL exposure (7.1 and 9.0% increase relative to diluent control (Figure 7e upper panel). Importantly, Bcl-xL knockdown cells reconstituted with pLenti-Flag.Bcl-xL, although yielding more spontaneous apoptosis (5.7% versus flag vector 1.1%), partially reversed GSK-3i and TRAIL-induced apoptosis (net increase of apoptosis down from 10 to 4.7%; and 16.1 to 3.3%, respectively) under the same treatment conditions (Figure 7e, lower panel). These results confirm that Bcl-xL downregulation contributes to GSK-3i-induced sensitization to TRAIL.

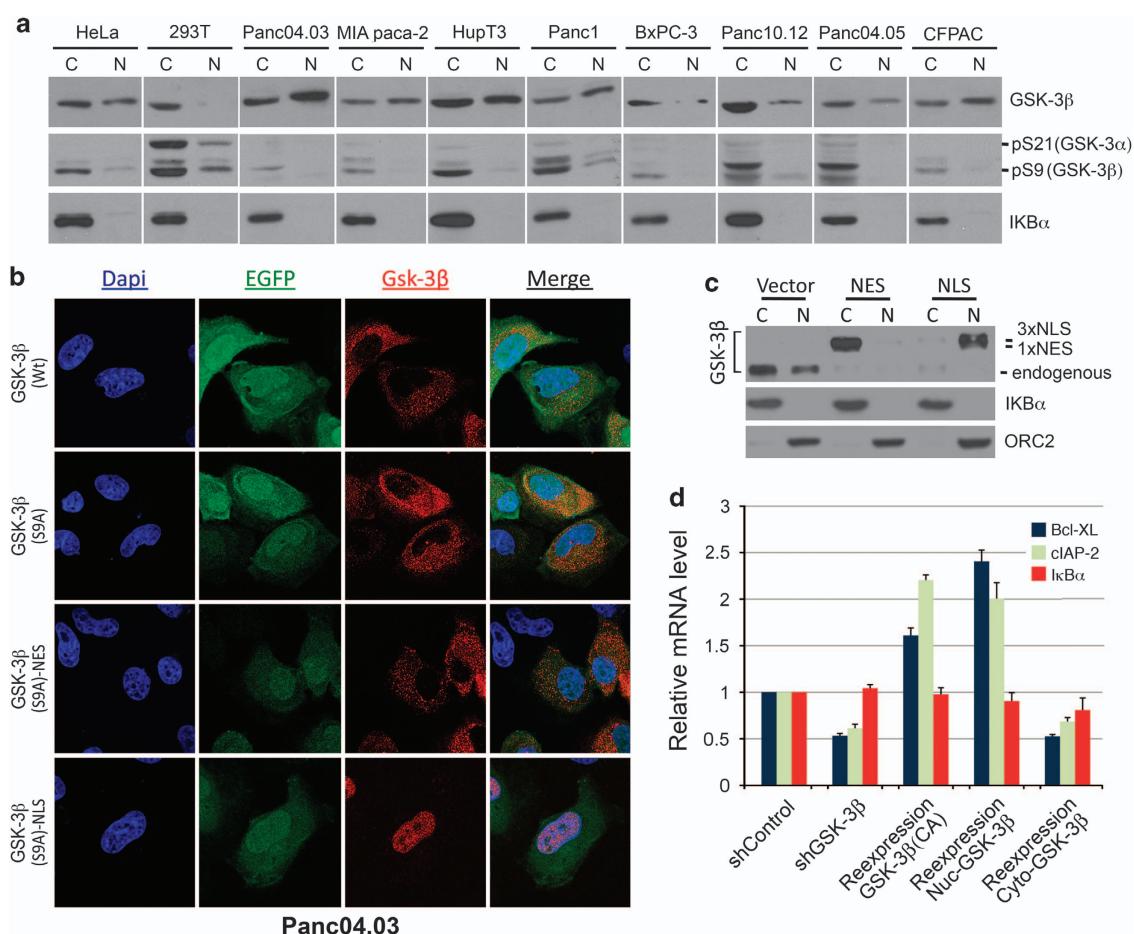


Figure 6 Nuclear GSK-3 β is active and contributes to regulation of Bcl-xL and cIAP2. (a) Western blot analysis of cytosolic and nuclear fractions from the indicated cell lines. Note the abundant presence of nuclear GSK-3 β and minimal S9 phosphorylation in all pancreatic cancer cells. (b) Immunofluorescence staining and confocal microscopic imaging showing the expression/localization of GSK-3 β in Panc04.03. Cells were transfected for 48 h with pCMV-eGFP-H1P suppression/re-expression vectors for the expression of C-terminal Flag-tagged GSK-3 β including WT, S9A mutant, and S9A with NLS and NES signals. The plasmid also expresses EGFP driven by an independent promoter, making it feasible to identify individual transfected cells. (c) Cytosolic and nuclear protein samples from above transfected cells were subjected to immunoblotting with indicated antibodies. ORC2 is used as a marker for nuclear protein. (d) qRT-PCR showing effect of reconstituted GSK-3 β expression on Bcl-xL, cIAP2 and I κ B α expression. Results of one representative experiment are shown as mean of triplicates \pm S.D. and normalized to RPLP0 expression

Discussion

In this work, we examined the hypothesis that GSK-3 inhibition can overcome key anti-apoptotic mechanism(s) associated with TRAIL resistance. The rationale behind this combination was based on the premise that GSK-3 treatment downregulates the expression of key anti-apoptotic proteins that mediate TRAIL resistance. A connection between GSK-3 β and TRAIL-induced apoptosis was first described in prostate cancer cells, in which GSK-3 inhibition or GSK-3 β suppression eliminated TRAIL resistance.²⁹ A more recent study reported a similar caspase-dependent TRAIL sensitization involving the mitochondrial apoptotic pathway in both prostate and pancreatic cancer cells.³⁰ The present work not only confirmed that GSK-3 inhibition enhanced TRAIL-induced apoptosis in various pancreatic cell lines, but also uncovered differential effects of GSK-3 isoforms on TRAIL- and TNF α -induced cell death. Furthermore, our results demonstrated a unique mechanism by which nuclear GSK-3 regulates the NF- κ B target genes *BCL2L1* and *BIRC3*.

Most published work on GSK-3 has focused on GSK-3 β , while much less is known regarding the relative contribution of GSK-3 α despite its widespread tissue distribution.^{31,32} Research using genetically engineered mouse models revealed that GSK-3 α and GSK-3 β largely compensate for each other during embryogenesis, as demonstrated for β -catenin via the Wnt pathway.³³ Nevertheless, isoform-specific phenotypes have been reported, including the observation that GSK-3 β loss causes a defect in NF- κ B activation and craniofacial anomalies,^{21,34} while GSK-3 α loss results in premature death and acceleration of age-related pathologies due to activation of mTORC1 and associated suppression of autophagy markers.³⁵ A recent study has identified a distinct role for GSK-3 α in noncanonical NF- κ B signaling via stabilization of TAK1-TAB downstream of oncogenic K-Ras.^{27,32} These reports point to GSK-3 isoform-specific effects on the regulation of NF- κ B activity.

Because GSK-3 α and GSK-3 β share a highly homologous kinase domain, the available GSK-3 kinase inhibitors, including LY2064827, do not distinguish between the two isoforms.³⁶

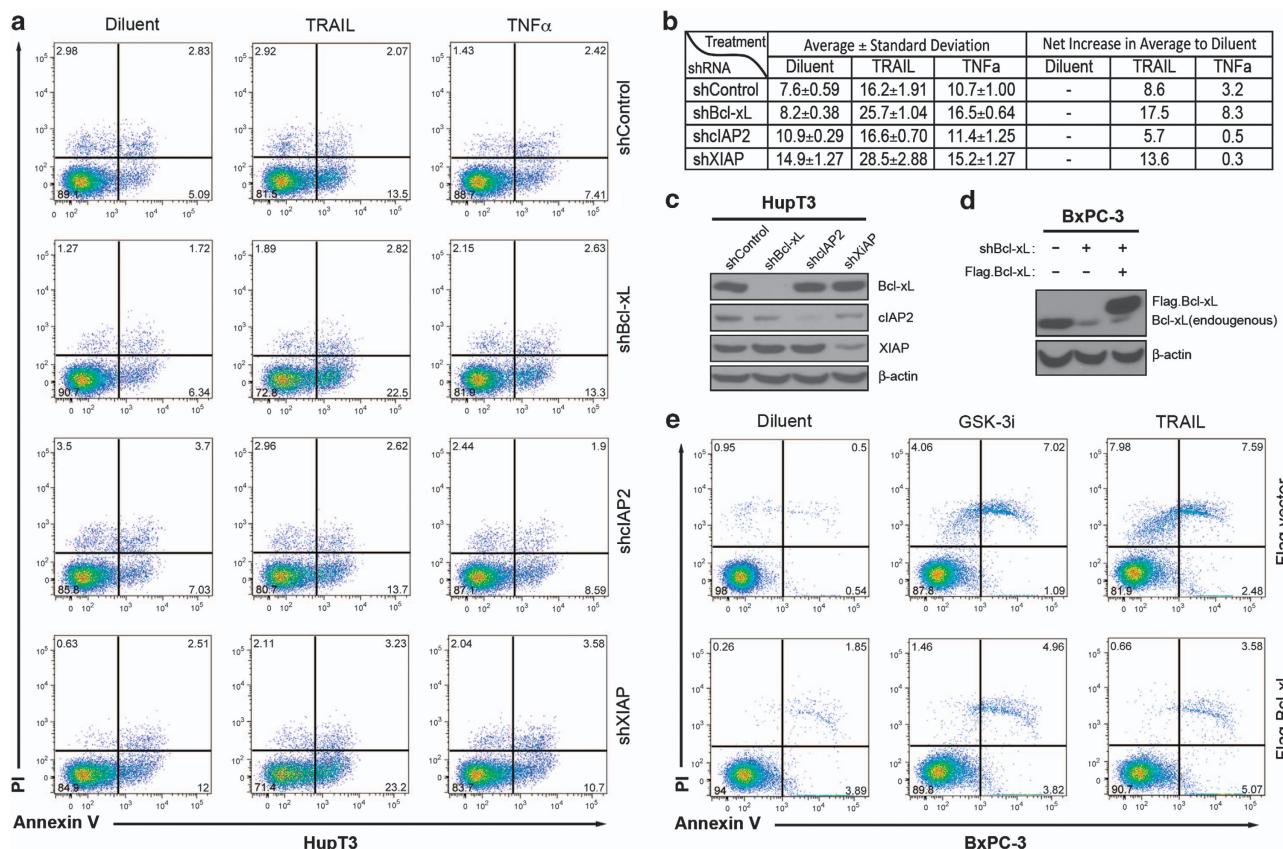


Figure 7 Bcl-xL and XIAP participate in GSK-3i-induced sensitization to TRAIL/ and TNF α . (a) Representative flow cytometric profile of annexin V-FITC and PI stained HupT3 cells to evaluate apoptosis induction. HupT3 cells with stable expression of lentiviral shRNA for Bcl-xL, clAP2 or XIAP (or scrambled control) were treated with diluent, TNF α or TRAIL as indicated for 18 h before harvesting and staining. (b) Summarized results from three independent analyses as percentage of annexin V-FITC and/or PI cells to total gated cells were shown as average \pm S.D. (c, d) Whole-cell extracts from above HupT3 cells (c) and BxPC-3 cells stably re-expressing Flag.Bcl-xL (d) were subjected to immunoblotting to confirm efficient suppression of endogenous gene expression and rescued Bcl-xL expression. (e) BxPC-3 cells with stable lentiviral-mediated expression of Flag.Bcl-xL were treated and stained as in (a) for apoptosis analysis

We used isoform-specific lentiviral shRNA vectors to evaluate the role of each GSK-3 isoform in TRAIL-induced apoptosis. Contrary to the reported minor effect of GSK-3 α ,³⁰ we showed that both isoforms similarly contributed to TRAIL resistance in PDA cells. This discrepancy likely arises from the efficiency of GSK-3 α suppression. Our analysis utilized multiple shRNAs, each of which achieved nearly complete suppression of GSK-3 α at both mRNA and protein levels and caused similar TRAIL sensitization. Our data are also consistent with a previous report by Sun *et al.*³⁷, which identified GSK-3 (both GSK-3 α and 3 β) as a component of an anti-apoptotic protein complex with DDX3 and clAP1 associated with DR5 in breast cancer cells.³⁷ Although the precise mechanism and substrate(s) of GSK-3 action within this membrane complex remain to be defined, GSK-3 inhibition was found to overcome TRAIL resistance by promoting the initial step of DISC formation leading to caspase-8 and caspase-3 activation.³⁷ Our results, which focused on another aspect of TRAIL killing, also point to an effect of both GSK-3 β isoforms in modulating TRAIL sensitivity.

In contrast to TRAIL, only GSK-3 β suppression led to sensitization of TNF α -induced cell death in PDA, an effect recapitulated in GSK-3 β -null MEFs. As the first report on

genetic ablation of the murine GSK-3 β gene leading to a defect in NF- κ B activation,²¹ the involvement of GSK-3 β in NF- κ B signaling has been documented in many cell types. However, conflicting results have been reported regarding the role of GSK-3 β loss on NF- κ B activation, ranging from major defects in TNF α -induced I κ B α phosphorylation/degradation, to minimal or no effect on the cytosolic signaling and p65 nuclear translocation.^{28,38–40} Our group previously proposed that GSK-3 β exerts its effect on NF- κ B at a point distal to activation of the IKK complex in PDA cells, whereas a role of GSK-3 β in maintaining constitutive NF- κ B signaling via IKK was also reported.^{25,28} Here we provide evidence that GSK-3 β is basically dispensable during TNF α -induced canonical NF- κ B activation up to p65/p50 nuclear entry, and is only involved in the regulation of a subset of genes such as *BCL2L1* and *BIRC3*, but not *I κ B α* and several other NF- κ B target genes (Figures 4 and 5 and data not shown).

Our observation that GSK-3 β affects only a subset of NF- κ B target genes, raised the possibility that GSK-3 β contributes to NF- κ B target gene expression in a promoter- or chromatin context-dependent manner. In fact, GSK-3 inhibition in Panc04.03 cells affected NF- κ B binding to the *BCL2L1* and *BIRC3* promoters, but not the *I κ B α* promoter. Where present,

the effects of GSK-3 inhibition were characterized by either decreased p65 or increased p50 binding along with increased HDAC3 and SIRT1 recruitment. The underlying mechanism is not presently clear, but may involve posttranslational modification of NF- κ B proteins, as GSK-3 β has been shown to phosphorylate both p65 and p50.^{24,41,42} GSK-3 β has also been reported to phosphorylate and regulate the processing/degradation of NF- κ B1 (p105), the precursor of p50 and, therefore, could alter levels of p50 in cells.⁴³ Although detailed mechanism(s) underlying GSK-3 β regulation of NF- κ B hetero- and homodimer formation and chromatin binding remain to be further characterized, the difference in DNA binding specificity/affinity as well as in transcriptional activity of NF- κ B dimer forms may constitute a mechanism for selective activation/repression of a subset of NF- κ B target genes.^{44–46} In light of our direct experimental evidence that nuclear-localized GSK-3 β can efficiently drive the expression of the NF- κ B target genes *BCL2L1* and *BIRC3* in pancreatic cancer cells, it is of interest that many GSK-3 β substrates are nuclear transcription factors.²⁰ As GSK-3 β is overexpressed and localized to nuclei in the majority of moderately and poorly differentiated PDAs, it is tempting to speculate that, in addition to driving the expression of select NF- κ B target genes such as *BCL2L1* and *BIRC3*, nuclear GSK-3 β might affect the action of other transcription factors as well.

Among the key NF- κ B-target anti-apoptotic proteins linked to TRAIL resistance, cFLIP functions at the apex of death receptor initiated signaling by preventing the recruitment and activation of caspase-8.⁴⁷ A recent report has identified cFLIP (both long and short isoforms) as a crucial negative regulator of death receptor-induced apoptosis in pancreatic carcinoma cells.⁴⁸ We initially found that the long isoform of cFLIP is reduced in GSK-3i treated cells, but this was not observed in either GSK-3 α or GSK-3 β knockdown cells, suggesting a potential redundant role of GSK-3 α or GSK-3 β in its regulation. Interestingly, suppression of Bcl-xL or XIAP, but not cFLIP, sensitized PDA cells to TRAIL-induced apoptosis. Moreover, expression of Bcl-xL off a GSK-3i-independent promoter could partially reverse the GSK-3i-induced TRAIL sensitization. Our data, which are consistent with previous work showing that both Bcl-xL and XIAP are important in mediating TRAIL resistance,^{7,11,49–52} demonstrate that down-regulation of Bcl-xL by GSK-3i or GSK-3 β suppression accounts for part of the GSK-3i-induced death ligand sensitization in PDA cells.

The signaling cascades activating NF- κ B pathways are attractive targets for cancer therapy.^{7,10,22} However, the complex mechanisms underlying constitutive NF- κ B activation, including both canonical and noncanonical pathways in PDA, require a combination of inhibitors in order to widely block NF- κ B activities to achieve therapeutic benefit. Results presented here demonstrate that combining GSK-3i with TRAIL dramatically enhances TRAIL cytotoxicity in pancreatic cancer cells. Our further studies show that GSK-3 β in the nucleus of pancreatic cancer cells contributes to TRAIL resistance by impacting binding of NF- κ B dimers to a subset of NF- κ B-responsive promoters, ultimately modulating the expression of specific anti-apoptotic NF- κ B target genes such as *BCL2L1*. The present findings, together with published work, identify GSK-3-mediated mechanisms of TRAIL resistance

at the plasma membrane and in the nucleus. Taken together, these observations provide a strong rationale as well as a molecular basis for further study of TRAIL in combination with GSK-3i in order to potentially improve PDA therapy.

Materials and Methods

Regents and antibodies. GSK-3i (LY2064827) was obtained from Eli Lilly (Indianapolis, IN, USA). Recombinant human TRAIL, human and mouse TNF α were purchased from R&D Systems (Minneapolis, MN, USA). Fluorescein isothiocyanate (FITC)-conjugated annexin V was from Invitrogen (Carlsbad, CA, USA). Blasticidin and puromycin were obtained from InvivoGen (San Diego, CA, USA). Antibodies from Cell Signaling Technologies (Beverly, MA, USA) include rabbit monoclonal anti-GSK-3 α / β (D75D3), Bcl-xL (54H6), phospho-IKB α Ser32/36 (5A5), p65 (D14E12), cFLIP and Cleaved Caspase-3 (Asp175). Antibodies from Epitomics (Burlingame, CA, USA) include rabbit monoclonal anti-GS, phospho-GS (pS641) and rabbit polyclonal anti-cFLIP2. Antibodies from BD PharMingen (San Diego, CA, USA) include mouse monoclonal anti-Bcl-2, XIAP, GSK-3 β , β -catenin, ORC2 and PARP. Antibodies from Santa Cruz Biotechnology (Santa Cruz, CA, USA) include rabbit polyclonal anti-p65, p50, I κ B α and mouse monoclonal anti-p50. Mouse monoclonal β -actin, γ -tubulin, anti-FLAG M2 and M2 agarose were from Sigma-Aldrich (St. Louis, MO, USA).

Plasmid construction. For knockdown of endogenous gene expression, including GSK-3 α , GSK-3 β , Bcl-xL, cFLIP and XIAP, we used lentivirus-mediated short hairpin RNA (shRNA) expression system (Sigma, St. Louis, MO, USA).⁵³ The plasmids were either constructed in pLKO.1 vector or obtained from the Mayo Clinic RNA Interference Shared Resource (RISR). At least two distinct shRNA target sequences for each gene with confirmed suppression (over 70% decrease at mRNA level relative to scrambled control) were used (Supplementary Table S1). For stable reconstituted expression of Bcl-xL, we cloned its full-length coding region with an N-terminal Flag-tag in pLenti6.3 vector (Invitrogen). To generate GSK-3 β mammalian suppression/re-expression constructs, cDNA sequence (5'-CACTGGTCACGTTG GAAAGA-3') derived from human GSK-3 β 3' UTR region was first cloned as a hairpin into pCMS4-eGFP-H1P vector.⁵³ Human GSK-3 β protein coding region (both WT and constitutively active S9A mutant) were subcloned into this pCMS4-eGFP-H1P-shGSK-3 β vector at MluI/NotI sites. For suppression/re-expression of cytosolic and nuclear-localized GSK-3 β , the stop codon was mutated and replaced with a coding sequence for either NES from the human protein kinase inhibitor α or 3 tandem copies of the NLS sequence from SV40 large T-antigen, which is followed by a C-terminal Flag-tag. All cDNA and shRNA expression plasmids were verified by direct sequencing at the Mayo Molecular Biology Core Facility.

Lentiviral packaging, transduction and selection of stable cells. Lentivirus packaging, cell infection and selection of pLKO-shRNA stable pancreatic cancer cells with puromycin were performed as previously described following institutional biosafety regulations.⁵³ For stable overexpression of BCL-XL in BxPC-3 cells, pLenti6.3-Flag.BCL-XL or pLenti6.3-Flag.vector viral infected cells were selected in culture media supplemented with blasticidin (5 μ g/ml) for two passages (about 6 days) and pooled blasticidin-resistant cells were used as stable overexpression cells. To generate pLenti-TopFlash luciferase vector, we used pLenti6.3-Topo vector (Invitrogen) as a backbone and replaced the ClaI and MluI fragment (containing the CMV promoter, Topo cloning site the V5 tag coding sequence) with seven tandem copies of TCF/LEF binding site followed by the firefly luciferase ORF amplified from pGL3E vector (Promega, Madison, WI, USA). The resulting pLenti-7xTopFlash luciferase vector was packaged into lentiviral particles and used for infection of 293T and BxPC-3 cells, respectively. Blasticidin-selected stable cell clones were used in dual luciferase reporter assays.

Cell culture/transfection, protein extraction and immunoblotting. GSK-3 β -null mouse embryonic fibroblasts (MEFs) and matching WT MEFs were a kind gift from Dr. Jim Woodgett (Ontario Cancer Institute, Toronto, ON, Canada).²¹ HEK293T, HeLa and all pancreatic cancer cell lines were obtained from ATCC, maintained under recommended culture conditions and transfected as previously described.^{53–55} Whole cell or nuclear/cytosolic fractionated protein extracts were prepared, quantified and subject to SDS-PAGE and immunoblotting.

Cell proliferation and apoptosis analysis. Proliferation of MEFs and pancreatic cancer cells was measured by MTS assay (Promega) as described.⁵³

To assess DNA fragmentation, pancreatic cancer cells were incubated in buffer containing 0.1% (w/v) Triton X-100, 50 mg/ml PI and 0.1% (w/v) sodium citrate overnight at 4 °C and analyzed by flow cytometry for subdiploid events. For annexin V and PI staining, MEFs or pancreatic cancer cells were detached by trypsinization and stained with annexin V labeled with FITC (eBioscience, San Diego, CA, USA) and propidium iodide (PI, 1 mg/ml, Sigma) for 20 min. Cells (20 000 per condition) were then analyzed on a Becton Dickinson FACS Caliber flow cytometer (Franklin Lakes, NJ, USA) as described.⁵⁶ The fraction of cells positive for annexin V and/or PI was calculated using FlowJo software (Tree Star, Ashland, OR, USA).⁵⁴

Immunofluorescent staining, proximity ligation assay (PLA) and confocal microscopy. For localization of endogenous p65 in MEFs, WT and GSK-3 β -null MEFs were grown on coverslips for 24 h and stimulated with 2 μ g/ml of TNF α for the indicated periods of time. Cells were fixed, permeabilized, stained with rabbit monoclonal anti-p65 (1 : 300 dilution) and analyzed via confocal microscopy as previously described.⁵³ To detect subcellular distribution of GSK-3 β , Panc04.03 cells were transfected with pCMCS4-H1P-eGFP- GSK-3 β vectors by electroporation and grown on coverslips for 48 h before staining with mouse monoclonal anti-flag M2 antibody (1 : 800). For PLA detection of p50/p65 interaction *in situ*, we used the Duolink *in situ* PLA kit from Olink Bioscience according to the supplier's instructions. Mouse mAb against p50 (SC8414, Santa Cruz Biotechnology) and rabbit mAb against p65 (#8242, Cell Signaling Technology) were used as primary antibodies at dilutions of 1 : 50 and 1 : 300, respectively. The anti-rabbit plus and anti-mouse minus secondary antibodies were used as PLA probes and Texas red as detection reagent (Supplementary information).

Luciferase reporter assay. Luciferase reporter assays were performed as previously described.⁵⁷ Firefly luciferase activity was normalized to Renilla luciferase. The results were expressed as mean 'fold induction'. Mean values of at least three independent experiments are displayed \pm S.D.

RNA extraction and qRT-PCR. RNA extraction using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA), generation of cDNA with Superscript III reverse transcription Kit (Invitrogen) and PCR using the comparative CT method with the SYBR Green PCR Master Mix (Applied Biosystems, Grand Island, NY, USA) and the ABI Prism 7900TM Sequence Detection System have previously been described.⁵³ Experiments were performed in triplicate using three independent cDNAs. Primer sequences are in Supplementary Table S2.

Chromatin immunoprecipitation assays. Panc04.03 cells were treated with the GSK-3i LY2064827 (0.5 μ M) or diluent (DMSO) for 16 h. Treated cells were cross-linked and subjected to chromatin immunoprecipitation as described.⁵⁵ The following antibodies were used for ChIP: rabbit polyclonal antibodies to p65 and p50 (Santa Cruz Biotechnologies), monoclonal H3K14ac, RNA polymerase II and rabbit polyclonal HDAC3 and SIRT1 (Millipore, Billerica, MA, USA), rabbit polyclonal anti-p65 (acetylated K310), and anti-acetylated lysine (Abcam, Cambridge, MA, USA). Three sets of qPCR primers were designed for I κ B α , Bcl-xL and cJAP2 within promoter/enhancer or intergenic regions based on the location of NF- κ B binding sites. The result was first normalized to input or control IgG, then to diluent control. Experiments were performed in triplicate. Primer sequences are presented in Supplementary Table S3.

Conflict of Interest

The authors declare no conflict of interest.

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