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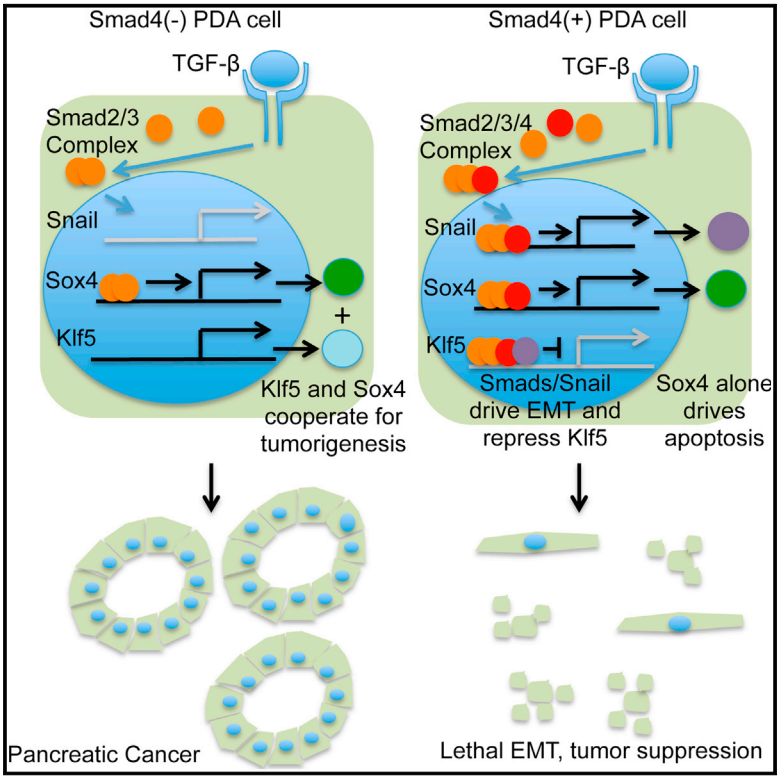
Cell

TGF-β Tumor Suppression through a Lethal EMT

Graphical Abstract



Highlights

- TGF-β-treated pancreatic cancer cells undergo lethal EMT, which is prevented by Smad4 loss
- TF Sox4 is converted from pro-tumor to pro-apoptotic during this process
- Altered Sox4 function due to EMT-linked repression of a key partner, lineage TF Klf5

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In Brief

TGF-β drives tumor suppression in pancreatic cancer cells by promoting EMT-linked remodeling of the transcription factor landscape, which converts TGF-β-induced Sox4 from an enforcer of tumorigenesis in the epithelial state into a promoter of apoptosis after EMT.

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TGF-β Tumor Suppression through a Lethal EMT

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SUMMARY

TGF-β signaling can be pro-tumorigenic or tumor suppressive. We investigated this duality in pancreatic ductal adenocarcinoma (PDA), which, with other gastrointestinal cancers, exhibits frequent inactivation of the TGF-β mediator Smad4. We show that TGF-β induces an epithelial-mesenchymal transition (EMT), generally considered a pro-tumorigenic event. However, in TGF-β-sensitive PDA cells, EMT becomes lethal by converting TGF-β-induced Sox4 from an enforcer of tumorigenesis into a promoter of apoptosis. This is the result of an EMT-linked remodeling of the cellular transcription factor landscape, including the repression of the gastrointestinal lineage-master regulator Klf5. Klf5 cooperates with Sox4 in oncogenesis and prevents Sox4-induced apoptosis. Smad4 is required for EMT but dispensable for Sox4 induction by TGF-β. TGF-β-induced Sox4 is thus geared to bolster progenitor identity, whereas simultaneous Smad4-dependent EMT strips Sox4 of an essential partner in oncogenesis. Our work demonstrates that TGF-β tumor suppression functions through an EMT-mediated disruption of a lineage-specific transcriptional network.

INTRODUCTION

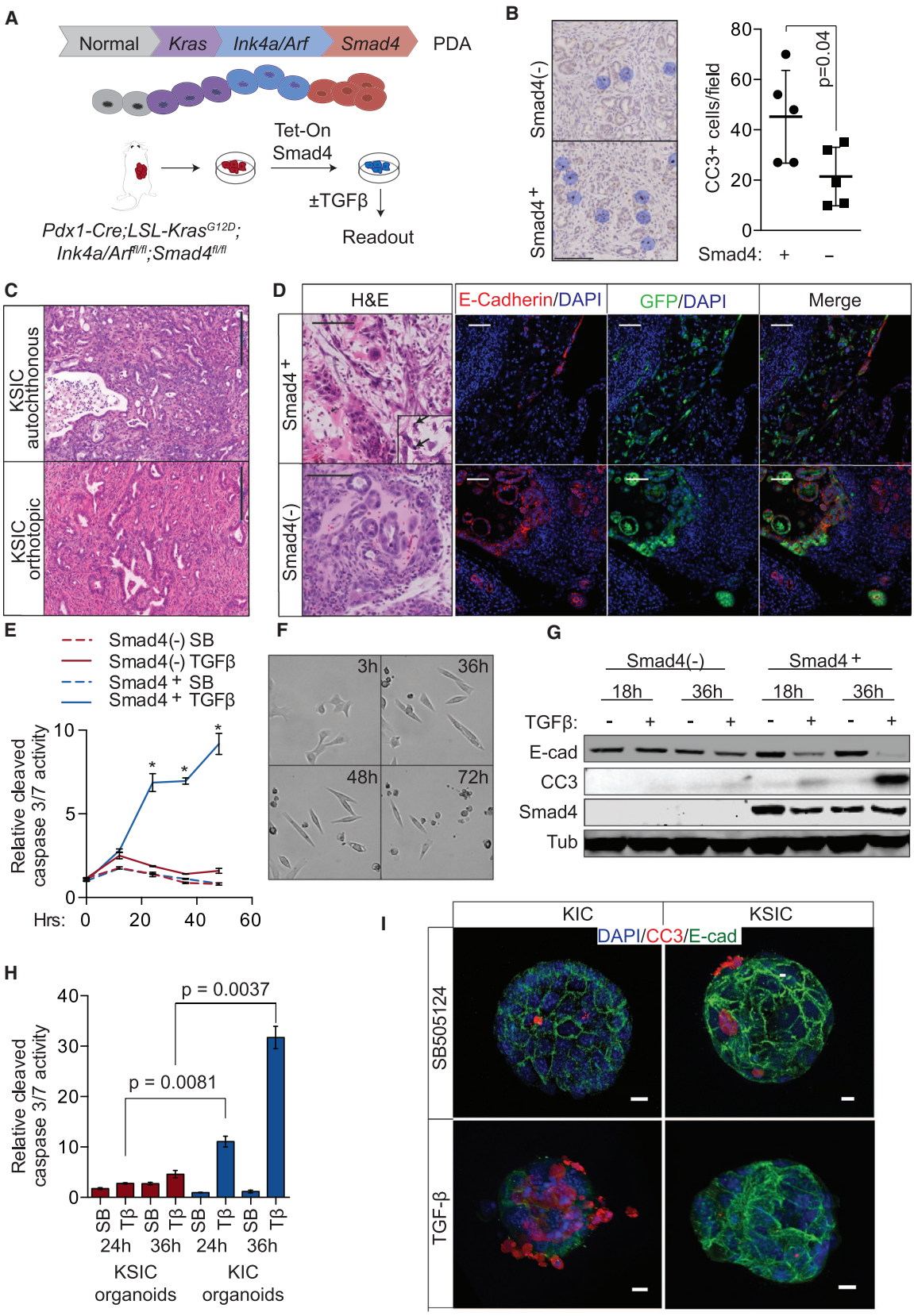
Cancer genes are typically classified as oncogenes or tumor-suppressor genes, but a growing number play a dual role and defy this classification (Stepanenko et al., 2013). TGF-β and its signaling pathway are a paradigm of duality in cancer (Massagué, 2008). This pathway is a key regulator of pluripotency, proliferation, and differentiation of metazoan cells (Gaarenstroom and Hill, 2014; Massagué, 2012; Oshimori and Fuchs, 2012). The effects of TGF-β depend on the cellular context, and this contextual nature is particularly manifest in tumors. TGF-β from the inflammatory tumor microenvironment may cause cancer cell apoptosis and tumor suppression (Guasch et al., 2007) or induce an epithelial-mesenchymal transition (EMT) that promotes cancer cell invasion and metastasis (Heldin et al., 2012) or promote cancer stem cell heterogeneity and drug resistance (Oshimori et al., 2015). The mechanistic basis for this duality is a long-unsolved question.

TGF-β is a major tumor-suppressive signal in the gastrointestinal tract and the pancreas (Goggins et al., 1998; Hahn et al., 1996). Acting through membrane receptor kinases, TGF-β activates Smad2 and Smad3 transcription factors (TFs), which bind Smad4 as an essential partner for many, though not all TGF-β responses (Massagué, 2012). Germline mutations in Smad4 result in familial juvenile polyposis syndrome (Howe et al., 1998), which predisposes to adenocarcinomas of the colon, stomach, and pancreas (Campos et al., 2015). Somatic inactivation of TGF-β receptors and Smad proteins are frequent in gastrointestinal carcinomas, with Smad4 inactivation in nearly half of pancreatic ductal adenocarcinomas (PDA) (Hahn et al., 1996).

Ras mutant premalignant cells are particularly susceptible to TGF-β-induced apoptosis (Guasch et al., 2007). In mouse models carrying a $Kras^{G12D}$ allele in the pancreatic epithelium, loss of *Smad4* accelerates progression to PDA (Bardeesy et al., 2006). Restoration of Smad4 expression in Smad4-defective cancer cells inhibits tumorigenic activity (Duda et al., 2003) and results in apoptosis (Bardeesy et al., 2006; Ramachandra et al., 2002). Notably, Smad4 is not required for normal development of the pancreatic epithelium (Bardeesy et al., 2006).

Cancer cells avoid the tumor-suppressive action of TGF-β through inactivation of TGF-β receptors or Smad genes or through selective silencing of apoptotic TGF-β effects (Massagué, 2008). Carcinoma cells can undergo a TGF-β-induced EMT (Heldin et al., 2012). EMT is a developmental plasticity process involving a loss of epithelial features, such as expression of the cell-junction molecule E-cadherin, and a gain in mesenchymal features (Thiery et al., 2009). In cancer cells, EMT promotes invasiveness and stem cell-like features (Valastyan and Weinberg, 2011).

Apoptosis and EMT are generally viewed as separate fates for TGF-β-stimulated cancer cells and opposite poles of the duality of TGF-β in cancer. TGF-β triggers EMT through induction of Snail and Zeb1/2 (Thiery et al., 2009), but little is known about how the TGF-β pathway triggers apoptosis (Massagué, 2012). To address this question, we dissected the tumor-suppressive action of TGF-β in Ras mutant pancreatic cancer cells. We demonstrate that TGF-β induces PDA cells to undergo a Smad4-dependent EMT, and EMT then triggers apoptosis. The mechanism involves a conversion of the TF Sox4 from pro-tumorigenic to



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pro-apoptotic. This occurs as the result of Snail-mediated repression of Klf5, an essential master regulator of endodermal progenitors. Our results illustrate a paradigm in which TGF-β tumor-suppressive action revolves around an EMT-associated disruption of a pro-tumorigenic transcriptional network.

RESULTS

Smad4-Dependent EMT and Apoptosis in PDA Cells

Pdx1-Cre; lox-stop-lox (LSL)-*Kras*^{G12D} mice (KC mice) sustain selective activation of the Kras^{G12D} oncogene in the pancreatic epithelium and form pancreatic intraepithelial neoplasia (PanIN) lesions that fail to progress (Hingorani et al., 2003). When combined with *Cdkn2a*$^{fl/fl}$ and *Smad4*$^{fl/fl}$ alleles, the mice rapidly develop well-differentiated PDA with a median survival of under 8 weeks (Bardeesy et al., 2006) (Figure 1A). To investigate the effect of Smad4 signaling in premalignant lesions, we compared KC mice with KC mice bearing *Smad4* $^{fl/fl}$ alleles (KSC mice). We used caerulein treatment to induce acute pancreatitis, which results in widespread PanIN formation (Morris et al., 2010) and is accompanied by an inflammatory response that includes recruitment of TGF-β-secreting cells (Konturek et al., 1998). Three days after caerulein administration, we found a higher rate of apoptosis in the Smad4-wild-type pancreas than in the Smad4 mutant pancreas (Figure 1B), consistent with earlier evidence that the main outcome of tumor-suppressive TGF-β signaling is apoptosis (Guasch et al., 2007).

To examine the mechanism of apoptosis, we used mouse PDA cells isolated from KSC; *Cdkn2a*$^{fl/fl}$ (KSIC) mice. The cells, having been selected during tumorigenesis in the absence of Smad4 signaling, should be sensitive to the tumor-suppressive effect of restored Smad4 expression. We introduced a doxycycline (dox) inducible (TetOn) Smad4 construct into early-passage primary PDA cells and achieved a Smad4 expression level comparable to the endogenous level in Smad4 wild-type cells (Figure S1A). When subcutaneously injected into syngeneic mice, the Smad4-restored (Smad4$^+$) PDA cells had a lower tumor incidence than Smad4 mutant counterparts (Figure S1B). We induced pancreatitis with caerulein and orthotopically implanted PDA cells. Smad4 mutant PDA cells efficiently formed tumors (4/6 mice), whereas no tumors (0/7 mice) were detected after 2 months with Smad4$^+$ cells. The Smad4 mutant orthotopic tumors were histologically indistinguishable from autochthonous tumors formed in KSIC mice (Figure 1C) and presented with occasional liver metastasis, indicating that they retained the biological characteristics of their autochthonous counterparts.

Histologic analysis of the pancreata 3.5 days after injection of GFP-labeled PDA cells (see Figure S1A) revealed little evidence of cell death in injected Smad4 mutant cells but showed membrane blebbing and pyknotic nuclei in injected Smad4$^+$ cells (Figure 1D, see inset), suggesting activation of apoptosis. Of interest, the dead cells were intermingled with cancer cells displaying spindle morphology and loss of E-cadherin staining, consistent with an EMT, whereas the Smad4 mutant cells retained E-cadherin expression (Figures 1D and S1C). In cells constitutively expressing Smad4, dox-inducible expression of the anti-apoptotic protein Bcl-X$_L$ reduced cell death and cleaved caspase 3 (CC3) staining when orthotopically injected (Figures S1D and S1E). Knockdown of the TGF-β receptor subunit Tgfbr2 reversed the EMT phenotype in Smad4$^+$ cells (Figure S1F) and rescued tumor formation (Figure S1G), indicating that the phenotype is due to TGF-β action.

When cultured under low-mitogen cell suspension conditions, cancer cells form "oncospheres" that are enriched for tumor-initiating cells (Lonardo et al., 2011). Treatment of PDA oncospheres with TGF-β resulted in a Smad4-dependent reduction in growth and an increase in apoptosis (Figures S1H and S1I). TGF-β or the TGF-β receptor kinase inhibitor SB505124 (DaCosta Byfield et al., 2004) had no effect on the viability of Smad4$^+$ cells in monolayer culture with serum growth factors (Figure S1J). Serum starvation sensitizes PDA cells to TGF-β-induced apoptosis (Bardeesy et al., 2006), and growth factor-activated Akt mediates anti-apoptotic effects through Bcl-X$_L$ (Chen et al., 1998). Treatment of Smad4-expressing PDA cells with the Akt inhibitor MK2206 (Figure 1E) synergized with TGF-β to promote apoptosis, and this effect was inhibited by overexpression of Bcl-X$_L$ (Figure S1K). These results show that TGF-β/Smad4 signaling triggers Akt- and Bcl-X$_L$-sensitive apoptosis in Smad4$^+$ PDA cells.

Smad4-Dependent EMT Precedes Apoptosis in PDA Cells

To examine the possibility that EMT precedes apoptosis, we conducted time-lapse imaging of PDA cells for 3 days after the addition of TGF-β. Smad4 mutant cells exhibited little cell death throughout this period and retained epithelial morphology

Figure 1. TGF-β Induces EMT followed by Apoptosis in the Premalignant Pancreas

(A) Experimental scheme to model TGF-β-mediated tumor suppression by re-expression of Smad4 in Smad4 mutant PDA.

(B) Immunohistochemistry (IHC) for cleaved caspase 3 (CC3) in KC or KSC pancreata collected 2 days after caerulein treatment, quantification at right. Scale bars, 100 μm. Error bars represent ± SD.

(C) H&E staining of a representative KSIC autochthonous tumor (top) and orthotopic tumor (bottom). Scale bars, 200 μm.

(D) Representative H&E staining and GFP/E-cadherin immunofluorescence after orthotopic injections of the indicated cells, collected 3.5 days post-injection. Apoptotic cells are indicated with arrows (see high-magnification inset, Smad4$^+$ H&E section). Scale bars, 50 μm.

(E) Cells grown in monolayer in high-serum medium containing 2.5 μM MK2206 were treated with TGF-β (100 pM) or SB505124 (SB; 2.5 μM) and assayed for cleaved caspase 3/7 activity at the indicated times.

(F) Time-lapse microscopy of Smad4$^+$ cells at the indicated times following TGF-β treatment (see also Movie S1).

(G) Immunoblot of EMT marker E-cadherin (E-cad) and apoptosis marker cleaved caspase 3 (CC3) after TGF-β treatment.

(H) Pancreatic organoids cultured from KIC or KSIC mice were treated with MK2206 and TGF-β or SB505124 and were assayed for cleaved caspase 3/7 activity at the indicated times.

(I) Three-dimensional images of E-cadherin and CC3 immunofluorescence performed in KIC or KSIC PDA organoids treated as indicated for 24 hr. Scale bars, 10 μm. Values in (E) and (H) are results of three biological replicates. Bar graphs represent mean ± SD.

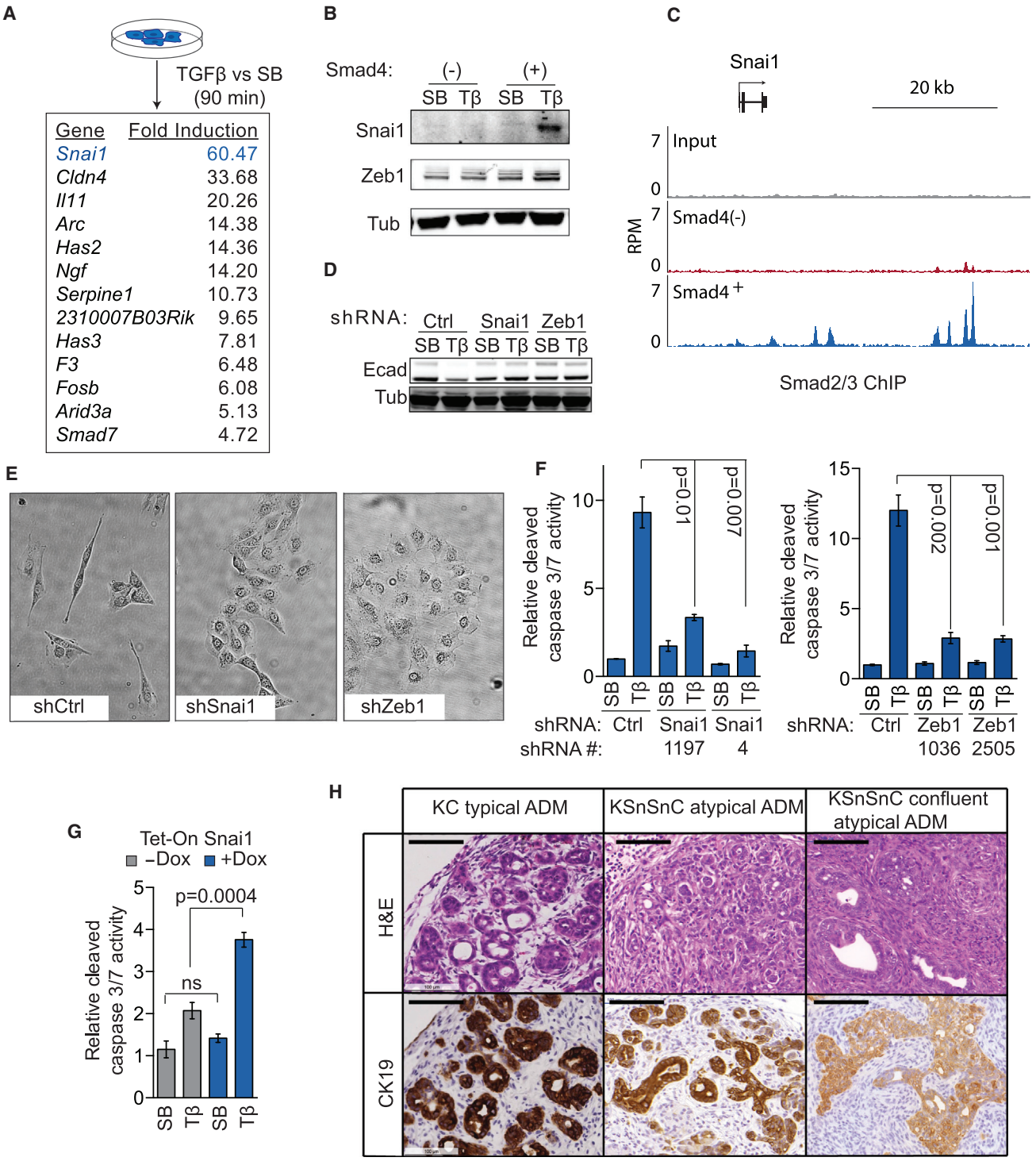

Figure 2. Snail and Zeb1 Promote EMT and Apoptosis

(A) List of most strongly induced genes after 90 min of TGF-β treatment in Smad4^{+} cells.

(B) Immunoblot for Snail and Zeb1 in Smad4 mutant and Smad4^{+} cells after 24 hr of TGF-β.

(C) Smad2/3 binding to the *Snai1* locus after 1 hr of TGF-β treatment in Smad4 mutant or Smad4^{+} cells.

(D) Immunoblot for E-cadherin in Smad4^{+} cells expressing the indicated shRNAs 24 hr post-TGFβ/SB treatment.

(E) Images of Smad4^{+} cells treated with TGF-β for 72 hr and expressing control shRNAs, or shRNAs targeting *Snail* or *Zeb1*.

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(Movie S1). In contrast, Smad4$^+$ cells started with epithelial morphology but within 24 hr lost cellular polarity and cell-cell contacts and assumed a spindle-like appearance. From 24–72 hr, most spindle-like cells began to shrink, formed membrane blebs, and dissociated into apoptotic bodies (Movie S1; Figure 1F). Consistent with this sequence of events, TGF-β treatment in Smad4$^+$ cells decreased E-cadherin levels at 18 hr, followed by CC3 accumulation (Figure 1G).

We generated organoids from mouse PDA tissues (Boj et al., 2015). Organoids from KSIC tumors were little affected by TGF-β addition. In contrast, KIC organoids underwent extensive apoptosis after TGF-β addition (Figure 1H), accompanied by a loss of structural integrity and increased CC3 staining (Figure 1I). Peak apoptosis was preceded by a strong increase in the expression of the EMT transcription factor (EMT-TF) Snail (Figure S1L) (Thiery et al., 2009) and by a loss of E-cadherin staining (Figure 1I). CC3-positive cells were invariably E-cadherin negative (Figure S1M).

In tumor cell isolates from mice of multiple genotypes, Smad4 mutant cells were uniformly resistant to TGF-β-induced EMT, and Smad4 restoration resulted in EMT followed by apoptosis (Figures S1N and S1O). Smad4-wild-type PDA tumors developing in the KIC model give rise to epithelial cells that undergo apoptosis in response to TGF-β (Bardeesy et al., 2006). As with KIC organoids, these lines underwent EMT preceding apoptosis in response to TGF-β (Figures S1M and S1N). The human Smad4 mutant pancreatic cancer cell line BxPC3 was resistant to TGF-β-induced EMT and apoptosis, and Smad4 re-expression sensitized these cells to undergo EMT and apoptosis (Figures S1P and S1Q).

EMT TFs Prime Cells for TGF-β-Induced Apoptosis

We performed RNA-seq after 90 min and 24 hr of TGF-β treatment. Snail was prominently induced at the mRNA and protein levels (Figures 2A and 2B). Smad2/3 ChIP-seq analysis at 90 min post-TGF-β treatment showed binding of Smad2/3 to the *Snai1* locus in Smad4$^+$ cells but not Smad4 mutant cells (Figure 2C). Zeb1 and Zeb2 are EMT-TFs that respond to TGF-β in other cell types (Heldin et al., 2012). Zeb1 was highly expressed in PDA cells regardless of the Smad4 status and was only marginally increased by TGF-β in Smad4$^+$ cells (Figure 2B). Zeb2 was not detected under any of these conditions (data not shown).

shRNAs targeting *Snail* and *Zeb1* transcripts (Figures S2A and S2B) blocked TGF-β/Smad4-induced E-cadherin downregulation (Figure 2D). Moreover, Snail and Zeb1 depletion in Smad4$^+$ PDA cells suppressed the induction of EMT-like morphological changes (Figure 2E) and apoptosis by TGF-β (Figure 2F). When we introduced a TetOn-Snail construct into Smad4$^+$ cells, Snail overexpression did not induce apoptosis in the absence of TGF-β but synergized with TGF-β in the induction of apoptosis (Figure 2G). These results indicate that Snail induction by TGF-β, together with basal Zeb1 expression, is necessary but not sufficient to cause apoptosis in PDA cells, and induction of apoptosis must involve an additional TGF-β-dependent input.

Snail Inhibits Pancreatic Tumorigenesis in Mice

The requirement of Snail for TGF-β/Smad4-induced apoptosis suggested that Snail might inhibit pancreatic tumorigenesis. We crossed mice bearing a conditional allele of *Snai1* with KC mice to generate *Pdx1-Cre*; *LSL-Kras^{G12D}*; *Snai1$^{+/flox}$* (KSnC) and *Pdx1-Cre*; *LSL-Kras^{G12D}*; *Snai1$^{flox/flox}$* (KSnSnC) mice. At 8 weeks, KSnC and KSnSnC pancreata were indistinguishable from their KC littermates (data not shown).

Spontaneous progression to invasive PDA in KC mice is rare and when encountered occurs after 12 months of age (Hingorani et al., 2003). Typically, the development of PDA requires the inactivation of tumor suppressors (Aguirre et al., 2003; Hingorani et al., 2005) and develops through progression of increasingly atypical PanIN lesions. Consistent with prior reports, cohorts of KC mice sacrificed at 4–5 months showed various grades of acinar-to-ductal metaplasia (ADM) and PanIN, but no invasive PDAC was seen. PanINs were seen at approximately the same frequency in KSnC and KSnSnC mice. By contrast, 7/9 KSnSnC mice had developed multifocal regions of atypical ADM, with confluent and highly disorganized CK19$^+$ cells displaying nuclear atypia, consistent with carcinoma in situ (Figures 2H and S2C). These results suggest that carcinoma in situ arose directly from ADM without progression through a PanIN intermediary. Such regions were not seen in KC mice and occasionally seen in KSnC mice (Table S1). One of the KSnSnC mice, aged 4.75 months, progressed to invasive PDAC, with liver metastases (Figure S2C). These data suggest that Snail plays a tumor-suppressive role at an early stage in PDA carcinogenesis.

Sox4 Is Required for EMT-Associated Apoptosis

Given that apoptosis enhancement by Snail overexpression in Smad4-expressing PDA cells required an additional TGF-β input, we searched for additional TGF-β-induced factors that cooperate with Snail to induce cell death. Based on our RNA-seq data, we constructed miR-30-based shRNA libraries (Zuber et al., 2011) targeting the mRNAs induced by TGF-β over 90 min in Smad4$^+$ PDA cells (Table S2). These pools were transduced into Smad4$^+$ PDA cells, which were then treated with SB505124 or TGF-β for 5 days. shRNA representation was then determined by deep sequencing (Figure 3A). shRNAs targeting the TGF-β receptor subunits *Tgfbr1* and *Tgfbr2*, included as positive controls, were enriched following cell treatment with TGF-β (Figure 3B). Snai1.1197, which caused a potent knockdown of *Snai1* and rescue of cell viability (refer to Figure 2F), was among the top 10% of enriched shRNAs in each of two replicates in the screen.

Sox4 scored as a candidate factor required for TGF-β-induced apoptosis (Figure 3B). Sox4 (SRY-related HMG box 4) is widely

(F) Smad4$^+$ cells expressing *Snai1*-targeting (left panel) or *Zeb1*-targeting shRNAs (right panel) were treated with TGF-β or SB for 36 hr then assayed for caspase 3/7 activity.

(G) Smad4$^+$ cells transduced with Tet-On Snail were treated with or without dox for 12 hr followed by TGF-β or SB, then assayed for caspase activity after 18 hr.

(H) H&E and CK19 IHC for representative ADM or atypical ADM lesions in KC or KSnSnC mice. Scale bars, 100 μm.

Values in (F) and (G) are the average of three biological replicates. Bar graphs represent mean ± SD.

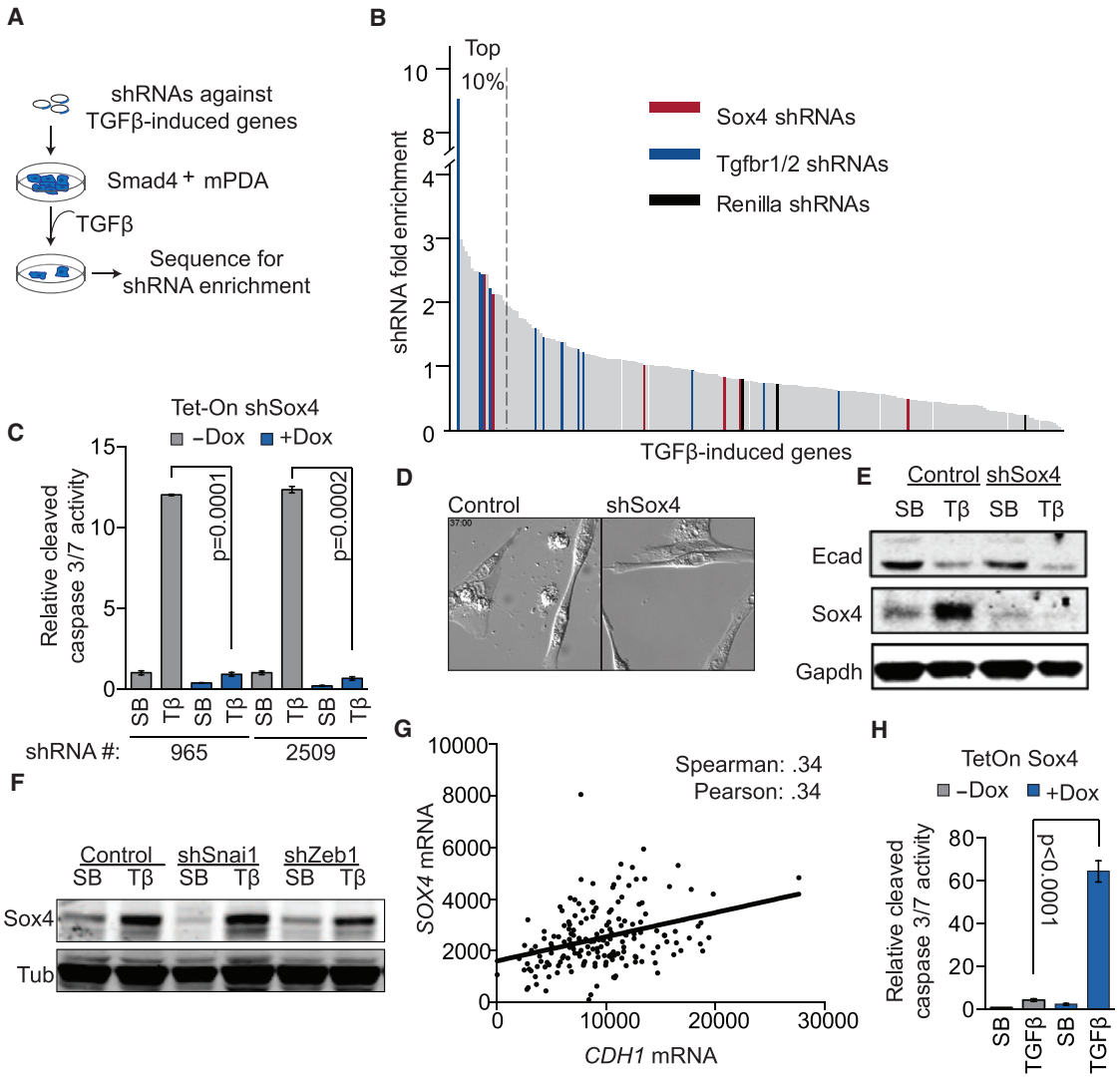

Figure 3. Sox4 Is Required for TGF-β-Driven Apoptosis

(A) Experimental scheme to screen for factors required for TGF-β-induced cell death.

(B) Plot depicting shRNA enrichment/depletion in TGF-β– versus SB-treated cells. Positive control shRNAs targeting *Tgfbr1* and *Tgfbr2* and negative control shRNAs targeting Renilla luciferase are indicated. See also Table S2.

(C) Indicated TetOn Sox4 shRNAs were transduced into Smad4^{+} cells; cells were pretreated with or without dox for 24 hr, then treated as indicated. Caspase activity was assayed after 36 hr.

(D) Images of control or Sox4 KD cells treated with TGF-β for 37 hr (see also Movie S2).

(E) Immunoblot for the indicated factors after Sox4 depletion and 24 hr of SB/TGF-β treatment.

(F) Sox4 immunoblot after 24 hr of TGF-β treatment in cells bearing control shRNAs or shRNAs targeting the *Snai1* or *Zeb1* transcripts. Samples and load control same as in Figure 2D.

(G) Publicly available RNA-seq expression data from The Cancer Genome Atlas (TCGA) derived from 179 PDA tumors were used to plot *SOX4* and *CDH1* levels. Spearman and Pearson correlation coefficients are indicated.

(H) TetOn Sox4 Smad4^{+} PDA cells were treated as indicated and assayed for apoptosis after 18 hr.

Values in (C) and (H) are averages of two and three biological replicates, respectively. Bar graphs represent mean ± SD.

expressed during mouse embryogenesis and functions in the development of many tissues (Vervoort et al., 2013). After birth, Sox4 expression is restricted to progenitor cells in pancreatic, intestinal, uterine, and mammary epithelia and maintains epithelial progenitor identity in a range of tissues (Doulatov et al., 2013; Ikushima et al., 2009; Zhang et al., 2013b), including pancreatic epithelium (Wilson et al., 2005). Sox4 is upregulated in various types of carcinoma and leukemia (Vervoort et al., 2013).

RNAi-mediated knockdown of Sox4 in Smad4^{+} PDA cells abrogated TGF-β-mediated apoptosis (Figure 3C) but had little effect on EMT morphology (Figure 3D; Movie S2) or E-cadherin expression (Figure 3E). Conversely, shRNA-mediated depletion

of Snail or Zeb1 had no effect on Sox4 expression (Figure 3F). Sox4, through its regulation of Ezh2, was implicated in EMT in mammary epithelial cells (Tiwari et al., 2013b). However, Sox4 knockdown in PDA cells had little effect on Ezh2 mRNA levels (data not shown). Moreover, *SOX4* mRNA levels show a positive correlation with those of *CDH1* (E-cadherin) in human PDA (Figure 3G), indicating that Sox4 is not a mediator of EMT in this context. In PDA cells, TetOn-driven Sox4 expression triggered extensive apoptosis only after TGF-β addition (Figure 3H). These results show that neither Snail nor Sox4 are pro-apoptotic separately, but the simultaneous induction of EMT and Sox4 by TGF-β is lethal to PDA cells.

The Smad4-Independent TGF-β Program and Sox4

The involvement of Sox4 in EMT-associated apoptosis was surprising given its role as a progenitor identity factor in epithelial tissues, including the pancreas. An additional parallel between Sox4 and pancreatic development emerged when we examined the requirement of Smad4 for Sox4 expression. Although Smads 2 and 3 play a key role in the development and patterning of the foregut and pancreas (Wiater and Vale, 2012), Smad4 is dispensable for normal pancreas development (Bardeesy et al., 2006). Notably, Sox4 induction by TGF-β was Smad4 independent in all Smad4 mutant mouse lines that we tested (Figures 4A and 4B and data not shown). Consistent with the Smad4-independent nature of Sox4 induction, IHC staining for Sox4 showed strong nuclear positivity in both Smad4 null and Smad4-restored PDA cells in vivo (Figure 4C).

RNA-seq transcriptomic analysis revealed 165 genes that were differentially expressed >2-fold after addition of TGF-β for 90 min to Smad4^{+} PDA cells. Of these responses, 145 were Smad4 dependent (Smad4 WT:mutant fold-change ratio > 1.5), including well-known Smad4-dependent genes such as *Smad7*, *Interleukin-11*, and *Snai1* (Figure 4D). An additional set, including *Sox4, Junb, Sgk1*, and *Fn1*, were equally induced by TGF-β regardless of Smad4 status, with induction occurring over a longer timescale (Figure 4D). Smad2/3 bound similarly to the *Sox4* locus in the presence and the absence of Smad4 by ChIP-seq (Figure 4E). Thus, Sox4 is in a small group of genes whose response to TGF-β involves Smad2/3 but is independent of Smad4 (Figure 4F).

Role of Sox4 in PDA Tumor-Initiating Activity

These observations raised the possibility that Sox4, although pro-apoptotic to PDA cells that undergo a TGF-β-induced EMT, may be important for the tumor-initiating capacity of these cells. For an unbiased assessment of this possibility, we screened PDA TFs for their requirement in oncosphere growth. To identify putative master regulators (Lee and Young, 2013), we ranked PDA TFs by RNA abundance and created pooled miR-E shRNA libraries targeting the top 93 TFs (Table S3) (Fellmann et al., 2013; Zuber et al., 2011). We transduced these libraries into Smad4 mutant PDA cells, grew the cells as oncospheres for 18 days, and determined the relative enrichment or depletion of individual shRNAs (Figure 5A). shRNAs targeting *Myc*, a TF required for PDA formation (Saborowski et al., 2014), were strongly depleted from the oncospheres, validating our approach (Figure 5B). *Sox4* shRNAs were also strongly depleted. PDA cells required Sox4 for growth as oncospheres (Figures 5C and 5D) but not growth in monolayer (Figure 5E). Importantly, Sox4 depletion in PDA cells inhibited tumor formation in both the subcutaneous (Figure 5F) and orthotopic settings (Figure 5G). Cells grown as oncospheres exhibited higher Sox4 levels than cells grown in adherent conditions (Figure 5H). Pretreatment of Smad4 mutant oncospheres with TGF-β resulted in increased capacity for secondary oncosphere formation (Figure 5I).

Collectively, the results show that TGF-β induces Sox4 expression in PDA cells independently of Smad4, and Sox4 supports tumor-initiating properties of PDA cells, consistent with the role of Sox4 in the epithelial progenitor state. Yet, Sox4 triggers apoptosis in cells that undergo a TGF-β/Smad4-induced EMT, implying that EMT switches Sox4 function from pro-tumorigenic in epithelial PDA cells to pro-apoptotic if these cells undergo an EMT (Figure 4F).

Klf5 as a Lineage-Survival Gene that Cooperates with Sox4 in PDA

To define the Sox4 transcriptional program in epithelial PDA cells, we performed Sox4 ChIP-seq in these cells and identified approximately 3,000 Sox4-bound peaks, including in the established Sox4 target *Nestin* (Figure S3A). Analysis of sequences within the Sox4 peaks revealed enrichment of consensus binding motifs for other TFs (Figure S3B), including the related but often functionally opposed TFs Klf4 and Klf5 (Ghaleb et al., 2005). Interestingly, Klf5 had scored in our PDA oncosphere formation screen as a mediator, whereas Klf4 scored as an inhibitor (Figure 5B). Klf5 enforces epithelial identity (Zhang et al., 2013a), is required for the integrity and oncogenicity of intestinal stem cells (Nakaya et al., 2014), and is a lineage-survival oncogene in gastric cancer (Chia et al., 2015). We used RNAi to confirm the requirement of Klf5 for oncosphere formation by PDA cells (Figure 6A). Knockdown of Klf5 in oncospheres, which express high levels of Sox4 (refer to Figure 5H), resulted in activation of apoptosis (Figure S3C). As with Sox4, Klf5 knockdown strongly inhibited orthotopic tumor formation (Figure 6B).

Nuclear Klf5 staining was absent in the normal pancreas, present in rare cells in ADM lesions, and nearly uniform in neoplastic lesions (Figure S3D), suggesting that pancreatic tumorigenesis involves the expansion of a Klf5^{+} progenitor pool. Consistent with its high expression the *Klf5* locus is flanked by a super enhancer in PDA cells, as determined by Med1 ChIP-seq (Figure S3E) (Whyte et al., 2013). High, lineage-specific expression is a hallmark of lineage-survival oncogenes such as MITF in melanoma (Figure 6C). Similarly, *KLF5* expression in human tumors is lineage restricted, with *KLF5* ranking among the most highly expressed TFs in GI and squamous carcinomas (Figure 6C), tumors that exhibit high rates of loss of TGF-β signaling. Lineage-survival oncogenes often exhibit genomic amplification (Garraway and Sellers, 2006), and TCGA copy-number data show amplifications of *KLF5* in colorectal, esophageal, stomach, and pancreatic carcinomas (data not shown). Taken together, these data suggest that Klf5 functions as a lineage-survival gene in pancreatic and other GI cancers.

Genome-wide Sox4 ChIP-seq revealed that approximately 80% of Sox4 peaks were associated with an overlapping Klf5 peak (Figure 6C). These data indicate that Klf5 may be an

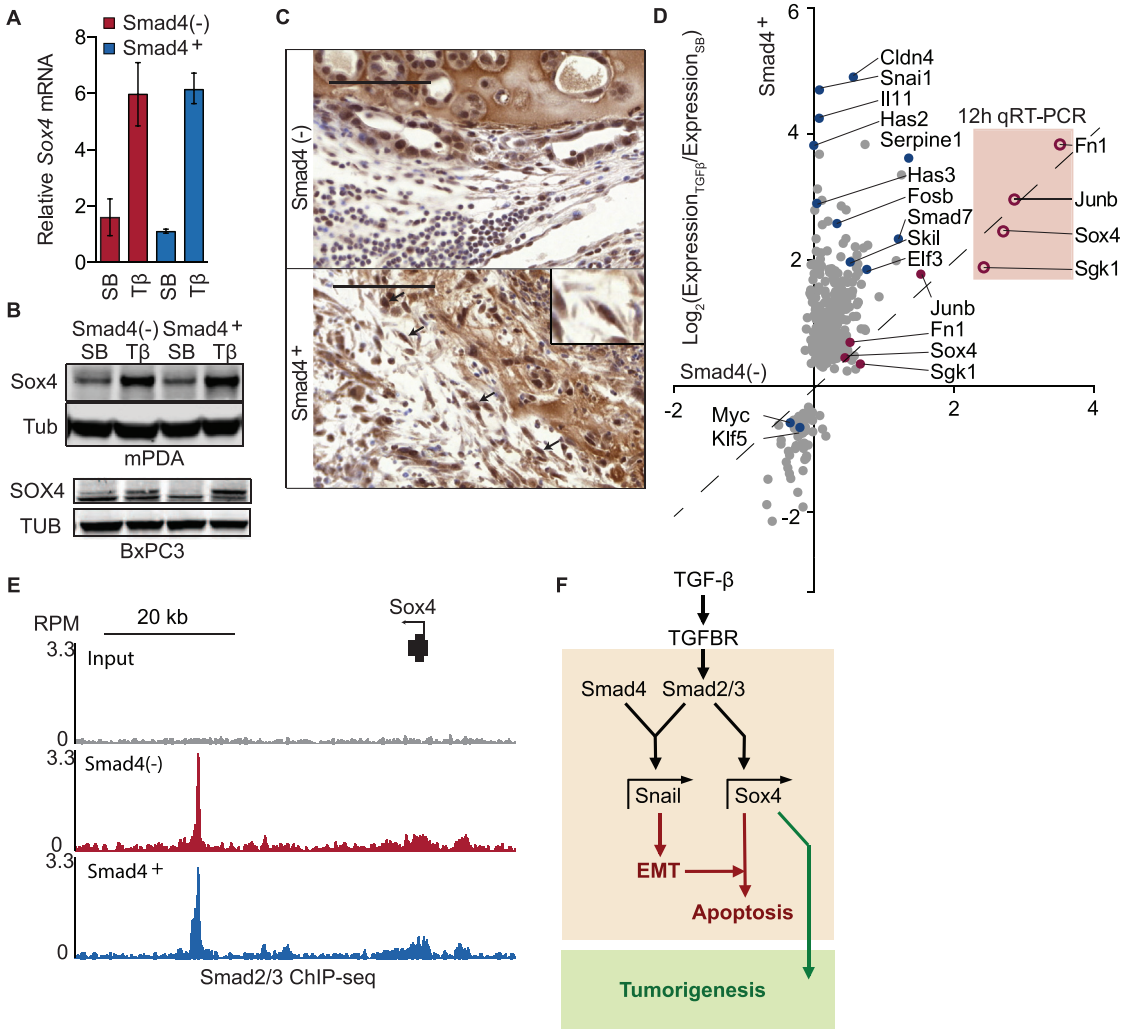

Figure 4. Sox4 Is Part of a Smad4-Independent Group of TGF-β-Induced Genes

(A) Sox4 mRNA levels in the indicated cells after 24 hr of the indicated treatment. Values are the mean and range of four technical replicates and representative of at least three separate experiments.

(B) Immunblot for Sox4 after 24 hr of the indicated treatment in mPDA (top) and BxPC3 cells (bottom).

(C) Immunohistochemistry for Sox4 performed 72 hr after Smad4^{-} or Smad4^{+} PDA cells were implanted in the pancreata of syngeneic mice. Inset shows higher-magnification image of Sox4^{+} EMT cancer cell.

(D) Smad4 dependence of 90 min TGF-β genes. Fold induction in Smad4 mutant and Smad4^{+} cells is plotted on the x and y axes, respectively. Select Smad4-independent genes were assayed by qRT-PCR after 12 hr induction and plotted (red-shaded region).

(E) Smad2/3 binding to the Sox4 locus as determined by ChIP-seq performed in Smad4 mutant and Smad4^{+} cells.

(F) Summary diagram showing cooperation between Smad4-dependent and Smad4-independent branches to induce apoptosis.

important determinant of Sox4 function in PDA. Consistent with this, Klf5 knockdown in Smad4 mutant PDA cells was accompanied by increased apoptosis (Figures 6E and S3F), which was further accentuated by TGF-β addition. Knockdown of Foxa2, another highly expressed TF, did not promote TGF-β-induced apoptosis (Figure 6E). In a reciprocal experiment, enforced expression of Klf5 in Smad4^{+} cells prevented TGF-β-induced apoptosis (Figure 6F) and E-cadherin downregulation (Figure 6G) and increased the tumorigenic activity of Smad4^{+} PDA cells (Figures 6H and S3G). These results suggested that a low Klf5:Sox4 ratio might trigger PDA cell death. We used tandem expression of Sox4 shRNAs with a Klf5 shRNA to confirm that Sox4 promotes apoptosis upon Klf5 depletion (Figure 6I), thus linking apoptosis in PDA to an imbalance between Sox4 and Klf5.

Repression of Klf5 Turns Sox4 into a Pro-apoptotic TF

EMT-TFs repress the expression of TFs that determine the epithelial state (Cicchini et al., 2006). Our RNA-seq data revealed a loss in the expression of 20 abundant PDA TFs, including Klf5, during EMT (Figure S4A). We confirmed the Smad4-dependent downregulation of Klf5 and other endodermal TFs (Figure 7A). In vivo, nuclear Klf5 was expressed in Smad4^{-} PDA cells but was undetectable in Smad4^{+} cells with EMT morphology (Figures 7B and S4B). Knockdown of Snail and Zeb1 blunted the

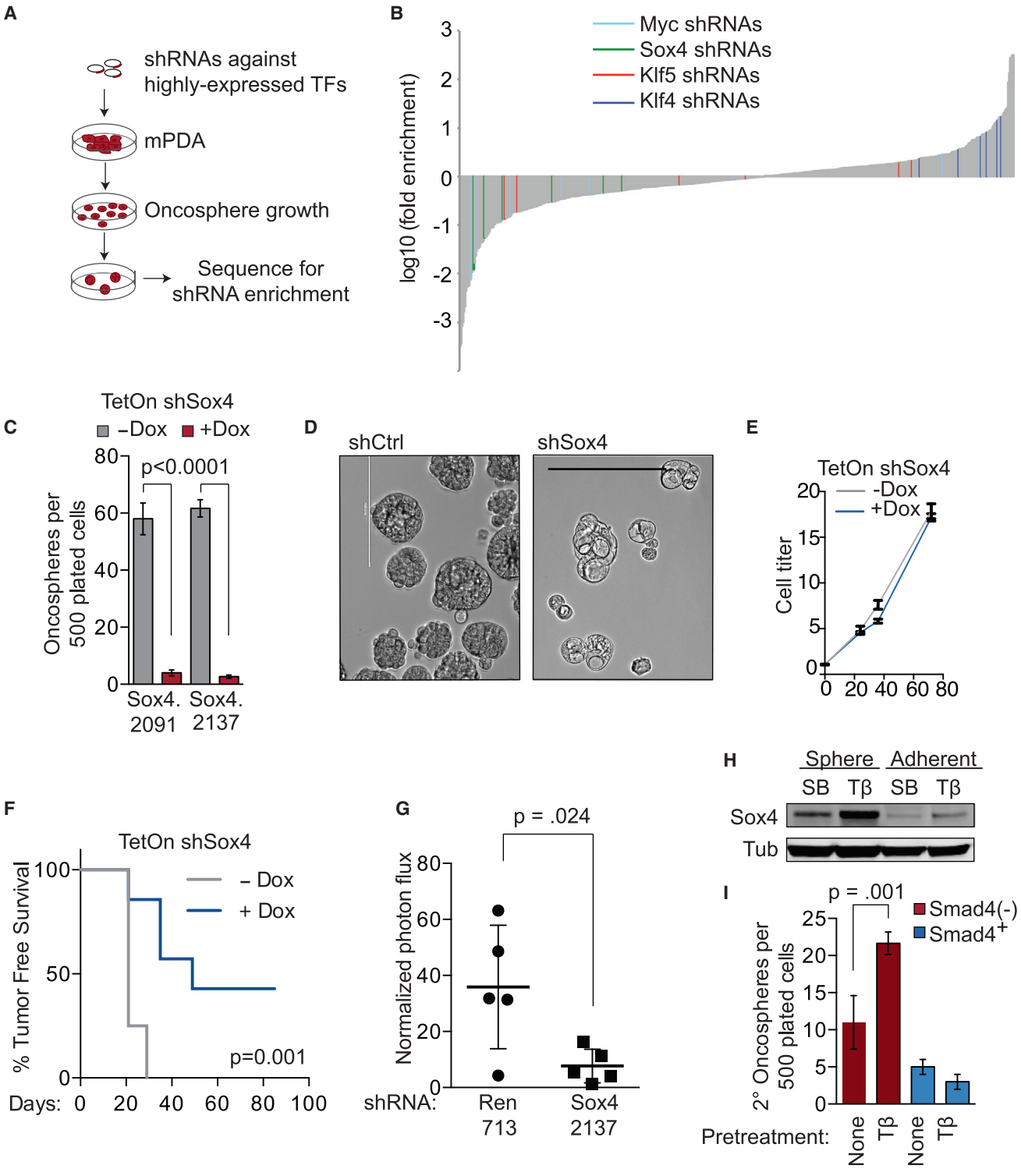

Figure 5. Sox4 Plays a Pro-tumorigenic Role in PDA Cells

(A) Experimental scheme for identification of tumorigenic TFs in PDA.

(B) Waterfall plot depicting results of an shRNA screen of the 93 most highly expressed TFs in PDA cells. Cells expressing the library were grown as oncospheres for 18 days with one passage. Data presented are the merged results of two separate pools. See also Table S3.

(C) PDA cells expressing the indicated shRNAs were plated as oncospheres and counted after 1 week.

(D) Bright-field images of cells expressing a control or Sox4 shRNA grown as oncospheres for 1 week. Scale bar, 200 μm.

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downregulation of Klf5 by TGF-β (Figure S4C). *KLF5* mRNA levels showed a strong positive correlation with *CDH1* expression in human PDA (Spearman's $\rho = 0.54$; Figure S4D).

The *Klf5* locus showed Smad4-dependent binding of Smad2/3 (Figure S4E), suggesting that TGF-β-activated Smads play a direct role in *Klf5* repression in the context of an EMT. A major Smad-binding site immediately downstream of Klf5 is centered around two E boxes (CACCTG), known to serve as binding sites for both Zeb1 and Snail (data not shown) (Burk et al., 2008). An identical region exhibits Zeb1 binding in an ENCODE ChIP-seq dataset from human hepatocellular carcinoma HepG2 cells (Figure S4F) (ENCODE Project Consortium, 2012). Knockdown of either Snail or Zeb1 did not prevent Smad binding to universal TGF-β-responsive genes *Smad7* and *Serpine1* (Figure 7C) but eliminated Smad binding at the Klf5-downtream site and at the E-cadherin locus (Figure 7C), a known site of Smad/EMT-TF cooperation (Vincent et al., 2009). These data indicate that Smads induce, then cooperate with EMT-TFs to repress Klf5 and E-cadherin.

To identify Sox4-dependent mediators of apoptosis in the post-EMT context, we performed RNA-seq on Smad4$^+$ cells with and without Sox4 knockdown. After 24 hr of TGF-β treatment, 1,169 genes were induced by TGF-β. Of these, 290 showed a >2-fold difference in their TGF-β responses in Sox4-depleted cells compared to controls. The set of TGF-β gene responses that were lost in Sox4-depleted PDA cells included Bim (encoded by *Bcl2l11*) and Bmf (Figure 7D). Bim and Bmf are BH3-only pro-apoptotic proteins, which are sequestered by Bcl-X_L (Piñon et al., 2008). These observations were consistent with the ability of Bcl-X_L to blunt TGF-β-induced apoptosis in this context (refer to Figures S1C and S1F). We confirmed that the increased expression of Bim and Bmf in response to TGF-β in PDA cells requires Sox4 (Figure 7E). Thus, repression of Klf5 by TGF-β-induced EMT-TFs switches Sox4 from a pro-tumorigenic enforcer in malignant endodermal progenitors to an inducer of tumor-suppressive, pro-apoptotic genes (Figure 7F).

DISCUSSION

Our results provide a mechanistic basis for the duality of TGF-β in pancreatic adenocarcinoma. The mechanism involves a Smad4-dependent EMT that perturbs a pro-tumorigenic transcriptional network, resulting in apoptosis. This perturbation involves the simultaneous activation of conflicting programs: one, involving Sox4, appears geared to reinforce an endodermal progenitor identity of PDA cells, whereas another, EMT, represses a set of endodermal TFs that would normally partner with Sox4 to establish this progenitor state. Repression of these endodermal factors, most notably Klf5, switches the activity of Sox4 from pro-tumorigenic in the presence of cooperating endodermal TFs to pro-apoptotic in their absence (Figure 7G). These observations reveal a driving force for the accumulation of *SMAD4* mutations in pancreatic cancer and possibly other endoderm-derived carcinomas.

TGF-β Control of Cellular Identity Drives Lineage-Specific Tumor Suppression

TGF-β superfamily members are powerful orchestrators of cell-identity changes throughout development, and our results implicate this property in tumor suppression. Pre-neoplastic lesions are dependent on TFs that maintain progenitor populations endemic to the tissue of origin (Garraway and Sellers, 2006). In tumor types most sensitive to TGF-β, Klf5 appears central to the identity of these progenitors. Klf5 marks proliferative progenitors in the developing GI tract (Ohnishi et al., 2000). Klf5 deletion in mouse intestinal stem cells results in apoptosis and depletion of Lgr5$^+$ cells (Nakaya et al., 2014; Nandan et al., 2015) and abrogates tumor formation (Nakaya et al., 2014). *KLF5* displays amplifications or stabilizing mutations (Bialkowska et al., 2014; Chia et al., 2015) and high-level expression restricted to a set of GI and squamous tumor types most prone to genetic inactivation of the TGF-β pathway.

Sox4 is associated with stem/progenitor cell properties in many contexts (Doulatov et al., 2013; Ikushima et al., 2009; Zhang et al., 2013b). We show that in PDA cells, Sox4 co-binds the genome with Klf5 and cooperates with Klf5 to establish this progenitor-like state. By repressing endodermal TFs, TGF-β drives the lethal collapse of the Sox4-Klf5 network, imposing a cell-identity-specific bottleneck. Cells could emerge from this TGF-β-imposed bottleneck either by (a) losing TGF-β signaling components, (b) attaining a self-renewing epigenetic state compatible with TGF-β signaling, or (c) activating signaling pathways that blunt the effects of TGF-β. Smad4-wild-type mouse models often give rise to tumors with loss of E-cadherin and sarcomatoid differentiation (Bardeesy et al., 2006; Rhim et al., 2012); cells derived from these tumors resist death when treated with TGF-β (Bardeesy et al., 2006), a likely example of (b). Such tumors are rare in human pancreas cancer (Kane et al., 2014), indicating that different mechanisms of escape from TGF-β suppression predominate in human tumors.

A Tumor-Suppressive EMT

A remarkable finding here is that tumor suppression is coupled to an EMT, a developmental program thought to promote the acquisition of malignant traits in advanced carcinomas (Valastyan and Weinberg, 2011). This coupling occurs through the function of the EMT-TFs Snail and Zeb1, which are induced by

(E) PDA cells transduced with a TetOn Sox4 shRNA were cultured in full serum medium in two-dimensional culture and were assayed for cell viability at the indicated times (in hr).
(F) Tumor-free survival after KSIC mPDA cells tranduced with TetOn Sox4 shRNAs were injected subcutaneously into mice maintained on or off dox diet. Plot reflects results of pooled experiments using Sox4.965 and Sox4.2509 shRNAs. For each condition (on or off dox), n = 10 mice; survival compared by log-rank test.
(G) Luciferase-labeled KSIC cells transduced with the indicated shRNAs were injected orthotopically in mice and imaged after 4 weeks. Luminescence was normalized to day 0 values. Statistical significance was calculated by a two-tailed unpaired t test. Error bars represent ± SD.
(H) Sox4 protein expression after SB/TGF-β treatment of cells grown in adherent conditions (right) or after 3 days in oncosphere conditions (left).
(I) Secondary oncosphere formation of Smad4 mutant and Smad4$^+$ PDA cells. Primary oncospheres were cultured for 2 weeks in the presence or absence of 20 pM TGF-β. Spheres were dissociated by trypsinization and replated for 1 week prior to counting.
Values in (C) and (I) are the average of three biological replicates. Bar graphs represent mean ± SD.

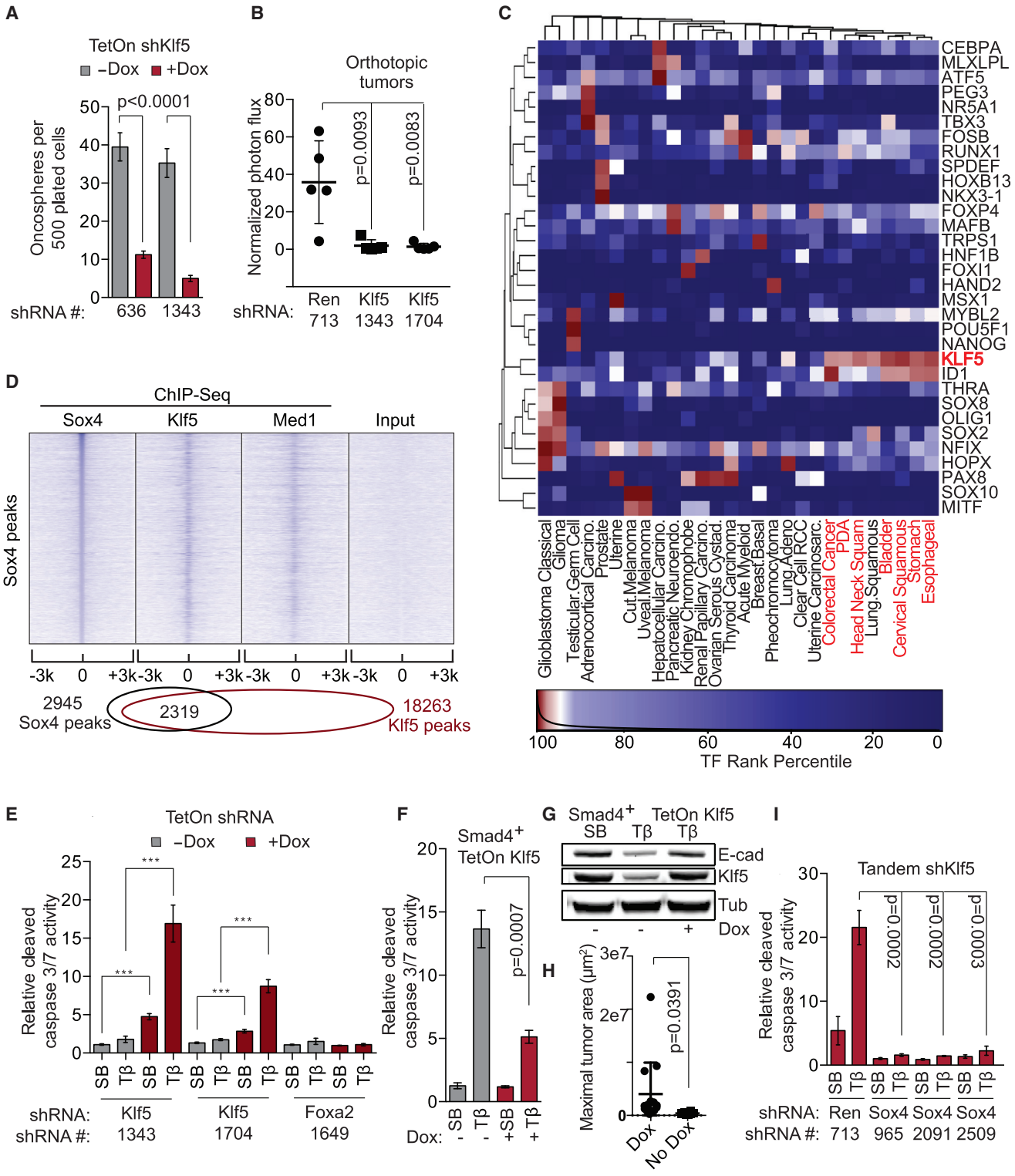

Figure 6. Klf5 Is a Lineage-Survival Gene that Determines Sox4 Function

(A) KSIC mPDA cells transduced with TetOn Klf5 shRNAs were grown as oncospheres for 2 weeks as indicated then quantified.

(B) KSIC cells were transduced with the indicated shRNAs and implanted orthotopically in mice maintained on dox feed. Mice were imaged after 4 weeks. Control group same as in Figure 5G. Error bars represent ± SD.

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and cooperate with Smad4 to bring about the repression of Klf5. Kras mutations sensitize PDA cells to TGF-β-induced EMT (Horiguchi et al., 2009) and sensitize keratinocytes to TGF-β-induced apoptosis (Guasch et al., 2007). Taken together, these observations suggest that Kras mutations sensitize incipient carcinoma cells to lethal EMT. Human PDA shows frequent repression-associated methylation of the *ZEB2* promoter, whereas miR-200a/200b, which suppress ZEB1 and ZEB2, are overexpressed (Li et al., 2010).

Our work highlights a role of EMT in tumor suppression and provides a mechanism for EMT-triggered apoptosis. An association of EMT with apoptosis is not unprecedented. Serum-starved canine kidney epithelial cells undergo EMT followed by cell death in response to TGF-β (Peinado et al., 2003). EMT can also precede apoptosis in response to TGF-β in murine mammary epithelial cells (Gal et al., 2008; Tiwari et al., 2013a). In the presence of sustained TGF-β treatment, the emergence of proliferative cells that survive EMT requires 2–3 weeks (Gal et al., 2008), which is consistent with our findings that lethal EMT imposes a narrow bottleneck on naive epithelial cells. Although the bulk of naive PDA cells are susceptible to EMT-linked death, EMT-permissive cells can emerge from the TGF-β-imposed bottleneck (Bardeesy et al., 2006; Gal et al., 2008; Rhim et al., 2012). Moreover our results indicate that tumorigenicity linked to an EMT is not broadly endowed on incipient carcinoma cells but is rather a trait that must be selected during carcinoma progression.

The Dual Nature of TGF-β and Core TF Regulation

Our results clarify the long-unexplained dual role of TGF-β in cancer. Despite its clear tumor-suppressive properties, a high level of TGF-β signaling in PDA tumors is associated with poor prognosis (Friess et al., 1993; Wagner et al., 1999). Our observation that Sox4 is induced in a Smad4-independent manner provides one potential mechanism by which TGF-β may promote aggressiveness in Smad4 null PDA and could account for the predominance of Smad4 loss over TGF-β receptor inactivation in human PDA. Approximately half of PDA harbor *SMAD4* mutations, but few harbor *TGBFR* mutations (Goggins et al., 1998), underscoring the requirement to bypass Smad4-dependent lethal EMT but not TGF-β-mediated tumor-promoting effects. PDA tumors with wild-type SMAD4 status tend to have fewer metastases (Iacobuzio-Donahue et al., 2009) perhaps reflecting an increased susceptibility of these disseminated cells to suppression by stromal TGF-β. Our results that conditional *SNAI1* knockout PDA retains metastatic competency are consistent with a recent report that EMT is not always connected with effective metastatic dissemination in PDA (Zheng et al., 2015). Smad4-independent induction of Sox4, a known component of pancreatic progenitors (Wilson et al., 2005), also provides a potential resolution to the apparently paradoxical observation that activin receptor mutant mice develop a severely hypoplastic pancreas, whereas Smad4 is dispensable for normal pancreas development (Bardeesy et al., 2006; Kim et al., 2000).

Sox4 both mirrors and contributes to the context-dependent dual nature of TGF-β. Sox4 is an established stem/progenitor factor that functions as a driver of apoptosis in some contexts (Vervoort et al., 2013). We show that the major determinant of context is the cellular repertoire of transcriptional master regulators. Why would a stemness factor be endowed with the ability to induce apoptosis in the absence of appropriate transcriptional partners? We speculate that this property evolved as a mechanism to eliminate stem cells with developmentally untenable TF configurations.

EXPERIMENTAL PROCEDURES

Animal Studies

All animal experiments were done in accordance with a protocol approved by the MSKCC Institutional Animal Care and Use Committee. All implantations were carried out in FVB mice purchased from the Jackson Laboratory or Harlan Laboratories, aged 4–6 weeks, except orthotopic implantation of Sox4 and Klf5 knockdown cells, which were performed in 6-week-old athymic mice. Mice bearing a Snail flox allele (B6;129S-Snai1tm2Grid/J) were purchased from the Jackson Laboratory and were crossed with *Pdx1-Cre; LSL-Kras^{G12D}* mice in FVB background. Caerulein treatment was performed as described (Morris et al., 2010). Orthotopic injections were performed as described (Rhim et al., 2012), except cells were suspended in 25 μl matrigel prior to injection. For Smad4$^{+/-}$ tumor formation, 3,000 cells were injected; for tracking cell-fate post-injection, 100,000 cells were injected. For TetOn Smad4 in vivo studies, cells were pretreated with dox for at least 12 hr prior to injection, and mice were maintained on dox for the duration of the study. Sox4 and Klf5 KD orthotopic injections were performed with 5,000 cells each, without prior caerulein treatment. shRNAs were induced for 2 days with dox prior to injection. Three-week luciferase signal was normalized to the day 0 value measured in the exposed post-injection pancreas.

In Vitro Cell-Based Assays

For in vitro cleaved caspase activity measurements, cells were plated in 96-well format at 500 cells/well for 12–24 hr prior to treatment with MK2206

(C) Heatmap showing highly expressed TFs in cancer exhibiting lineage specificity. TCGA RNA-seq data were used to rank TFs by average expression levels in the indicated tumor types. To identify lineage-specific TFs, we included any TF with expression in the top 0.5% in at least one tumor type, excluding TFs that exhibited expression in the top 10% in more than half of the tumor types analyzed. The latter step excluded 22 TFs. Tumor types exhibiting >10% of loss of TGF-β signaling are indicated in red.

(D) Heatmap showing overlap between Sox4, Klf5, and Med1 ChIP-seq peaks. Below is a venn diagram depicting overlap between Sox4 and Klf5 ChIP-seq peaks.

(E) Smad4 mutant PDA cells transduced with the indicated TetOn shRNAs were treated with dox for 48 hr to deplete the target protein, followed by treatment with SB/TGF-β. Cells were assayed for caspase 3/7 activity after 36 hr SB/TGF-β treatment.

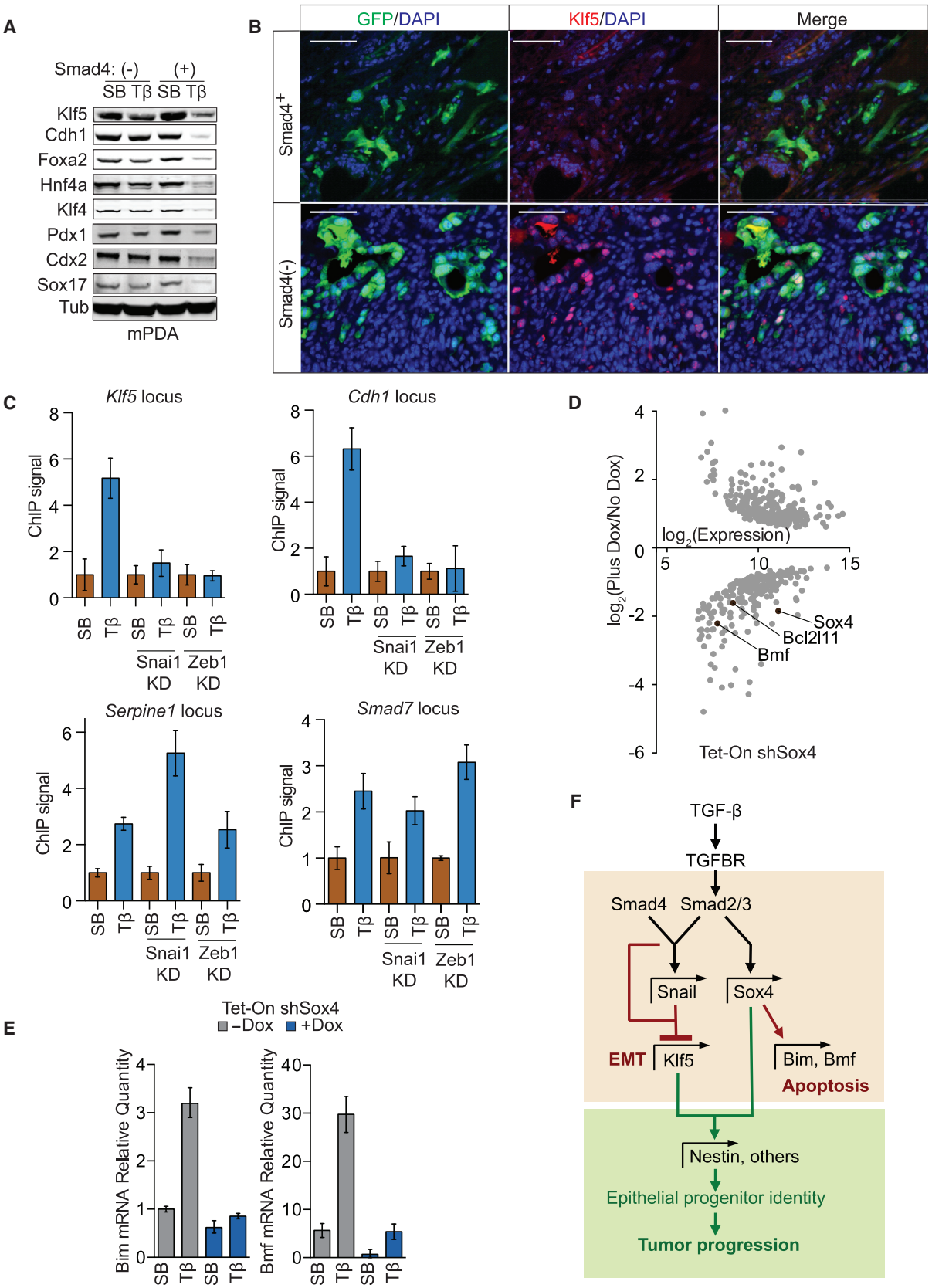
(F) A TetOn Klf5 construct was introduced into constitutive Smad4^{+} cells. Klf5 was induced with dox for 12 hr prior to the addition of MK2206. Cells were then treated with SB/TGF-β for 36 hr and assayed for caspase 3/7 activity.

(G) TetOn Klf5 cells were treated as in (F), then immunoblotted after 24 hr of the indicated treatment.

(H) 3,000 Smad4^{+} TetOn Klf5 cells were injected orthotopically in caerulein-treated mouse pancreata. Mice were sacrificed after 2 weeks, and pancreata subjected to serial sectioning and quantification of maximal tumor surface area, plotted as indicated (On dox n = 14, Off dox n = 10; unpaired two-tailed t test).

(I) Klf5.1343 shRNA was cloned into a tandem TetOn shRNA vector together with shRNAs targeting Renilla luciferase or Sox4. Cells were incubated with dox for 48 hr, then treated as indicated and assayed for caspase 3/7 activity after 36 hr.

Values in (A), (E), (F), and (I) are the average of three biological replicates. Graphs show mean ± SD; p values were calculated by a two-tailed t test.



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(Tocris, 2.5 μM) and SB505124 (Sigma, 2.5 μM) or TGF-β (R&D, 100 pM). Cells were assayed for cleaved caspase 3/7 activity using CaspaseGlo (Promega) and normalized to cell titer using Cell TiterGlo (Promega). Experiments were performed in triplicate and analyzed with Student's unpaired t test. For oncosphere formation assays, 500 cells/well were plated in ultra-low attachment 96-well plates (Corning) in DMEM/F-12 medium supplemented with B-27 serum replacement and 5 μg/ml Heparin. Organoids were cultured as described (Boj et al., 2015).

shRNA Screening

Pooled miR-30- and miR-E-based shRNA libraries were ordered from custom arrays and cloned into pTRIPZ or SGEP, respectively. Libraries were transduced into cells at transduction efficiencies of ~10% to ensure single-copy representation of shRNAs. For TGF-β screening, shRNAs were expressed for 3 days prior to addition of SB505124 or TGF-β. For TF screening, cells were expanded for two passages prior to plating in oncosphere conditions. To ensure adequate library representation, in all experiments libraries were represented by a cell number ≥ (1000 × library size) prior to relevant treatment. Additional procedures were performed as described (Zuber et al., 2011).

ACCESSION NUMBERS

The raw RNA-seq and ChIP-seq data for this manuscript are available at GEO under the accession number GSE72069.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, three tables, and two movies and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2016.01.009.

AUTHOR CONTRIBUTIONS

C.J.D. and Y.-H.H. performed most experiments. M.C. performed ChIP-seq experiments, and J.S. performed experiments in organoids. Y.Z. and Y.-H.H. performed bioinformatic analysis. N.B. provided mouse strains. C.A.I.-D. performed pathology analysis. J.M. conceived and oversaw the project, and C.J.D., Y.-H.H., and J.M. designed the study and wrote the paper.

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Figure 7. EMT-Linked Klf5 Repression Promotes an Apoptotic Sox4 Program

(A) Immunoblot for highly expressed endoderm-associated TFs after 24 hr of the indicated treatment.

(B) The indicated cells were injected as in Figure 1D. Klf5 and GFP were then detected by immunofluorescence. Scale bars, 50 μm.

(C) Smad2/3 ChIP-qRT-PCR performed at the indicated loci in the presence of control or Snail/Zeb1 shRNAs. Error bars represent the mean and range of four technical replicates, representative of two separate experiments. Cells were treated with SB/TGF-β for 12 hr prior to crosslinking.

(D) Sox4-dependent gene expression in TGF-β-treated Smad4^{+} cells. Cells bearing TetOn Sox4 shRNAs were cultured ± dox for 24 hr prior to treatment with TGF-β. After another 24 hr, RNA was extracted and RNA-seq was performed. Sox4-dependent differentially expressed genes were then plotted.

(E) qRT-PCR validation of Bim and Bmf as Sox4-dependent gene responses in TGF-β-treated Smad4^{+} cells. Values are the result of four technical replicates.

(F) Diagram summarizing effects of TGF-β signaling in naive PDA cells.

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