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[Continued on next page]

(54) Title: COMPOSITIONS AND METHODS FOR TREATING PANCREATIC CANCER

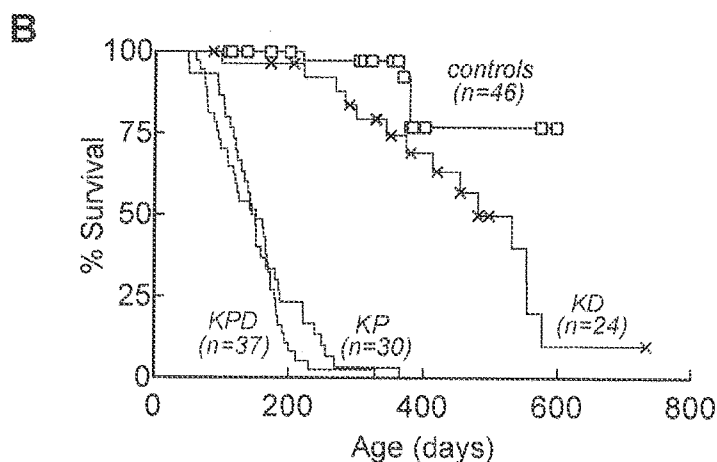


FIG. 1

(57) Abstract: The present disclosure provides compositions and methods for treating or reducing the risk of pancreatic cancer by administering compounds capable of inhibiting the expression or activity of RUNX3.



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COMPOSITIONS AND METHODS FOR TREATING PANCREATIC CANCER

CROSS REFERENCE TO RELATED APPLICATION

This application claims the benefit under 35 U.S.C. § 119(e) to U.S. Provisional Application 61/752,819 filed January 15, 2013, which application is incorporated
5 by reference herein in its entirety.

STATEMENT OF GOVERNMENT INTEREST

This invention was made with government support under Grant No. R01CA129357 awarded by the National Cancer Institute (NCI). The government *may have* certain rights in this invention.

10

STATEMENT REGARDING SEQUENCE LISTING

The Sequence Listing associated with this application is provided in text format in lieu of a paper copy, and is hereby incorporated by reference into the specification. The name of the text file containing the Sequence Listing
15 is 360056_413WO_SEQUENCE_LISTING.txt. The text file is 3.7 KB, was created on January 14, 2014, and is being submitted electronically via EFS-Web.

BACKGROUND

20 Technical Field

The present disclosure provides compositions and methods for treating or reducing the risk of pancreatic cancer and, more specifically, compounds capable of inhibiting the expression or activity of RUNX3 that are useful in minimizing or eliminating the metastasis of pancreatic cancer.

Description of the Related Art

Patients with carcinomas die of metastatic disease. Rarely do they succumb because of the primary tumor alone. This is especially true of pancreatic ductal adenocarcinoma or, more commonly, pancreas cancer.

- 5 Pancreas cancers appear to calibrate the often competing needs of local growth and distant spread to generate a profoundly lethal disease. While early metastasis characterizes the majority of clinical presentations, extension of locally invasive disease in others not only precludes surgical resection, but can also lead to demise.

- 10 Pancreatic ductal adenocarcinomas (PDAs) are thought to initiate most commonly in precursor lesions, termed pancreatic intraepithelial neoplasias (PanINs), which arise in the terminal ductules (Hruban *et al.*, *Am. J. Surg. Pathol.* 25:579, 2001). Activating mutations in the *KRAS* proto-oncogene occur early in preinvasive disease and are almost uniformly present (>90%) in
15 invasive PDA. Mutations in *CDKN2A/INK4A* are similarly abundant in invasive disease (>95%) and point mutations in *TP53* are also extremely common (>75%). Mutations in *DPC4/SMAD4*, the last of the cardinal genetic events associated with PDA, occur late in the PanIN-to-PDA progression scheme and are ultimately found in approximately 50% of invasive cancers (Hruban *et al.*,
20 *Clin. Cancer Res.* 6:2969, 2000).

- Ductal adenocarcinomas of the pancreas can also arise from two clinically, histologically, and genetically distinct classes of macroscopic cystic precursors, intraductal papillary mucinous neoplasms (IPMN) and mucinous cystic neoplasms (MCN) (Hruban *et al.*, *Gastroenterology Clinics of North*
25 *America* 36:831, 2007). Interestingly, although both MCNs and IPMNs can culminate in invasive PDA, the carcinomas that arise from these cystic routes portend significantly better prognoses for patients than the classical route (Hingorani, *Gastroenterology* 133:345, 2007; Hruban, 2007; Poultsides *et al.*, *Ann. Surg.* 251:470, 2010).

Pancreatic cancer is an unusually lethal disease with the highest 1-year and 5-year mortality rates of any cancer. Notoriously difficult to detect and resistant to all current therapeutic modalities, PDA has already advanced locally or frankly metastasized in most patients at the time of diagnosis; median survival in this setting is approximately 6 months (Hidalgo, *New England J. Med.* 362:1605, 2010). For the fortunate few for whom surgical resection is possible, median survival increases to 2 years but is not durable: survival at 5 years is only 20% and continues to decline to less than 2% at 10 years (Allison *et al.*, *J. Surg. Oncol.* 67:151, 1998). The majority of these resected patients also eventually die of metastatic disease, which indicates that clinical Stage I tumors are, in fact, already micrometastatic Stage IV. In this regard, PDA is unlike any other major epithelial malignancy, or solid tumor for that matter, in which chronologically distinct stages of disease permit meaningful surgical interventions that can prolong survival and even effect cures.

Hence, there remains a need in the art for alternative therapies and methods for reducing the risk of or treating pancreatic cancer and associated disorders. The present disclosure meets such needs, and further provides other related advantages.

BRIEF SUMMARY

In one aspect, the present disclosure provides methods for reducing the risk of or treating pancreatic cancer, metastasized pancreatic cancer, pancreatic cancer precursor lesions, or a metastatic niche associated with pancreatic cancer with a RUNX3 inhibitor, an agent capable of blocking the association of RUNX3 with a second transcription factor, an agent that enhances deacetylation of RUNX3, an agent that enhances ubiquitination of RUNX3, or any combination thereof.

These methods may be used as adjuvant or neoadjuvant therapies with existing therapies, such as surgery (e.g., Whipple operation), chemotherapy, radiation therapy, chemoradiation therapy, or any combination thereof. The

RUNX3 inhibitors may also be used in combination with other active agents, such as expression or activity inhibitors of Bmpr1a, Smad5, Tgfb3, Smad4, Bmp1, Itgb7, Tgfb1i1, Bmper, Ltbp1, Ltbp2, Itgb5, Id1, Tgfbi, Dlx2, Ctgf, Selp, Timp2, Col5a1, Ncam1, Thbs3, Mmp11, Sgce, Fn1, Vcam1, Ecm1, Adamts1, 5 Mmp2, Thbs1, Fbln1, Tgfbi, Cdh2, Mmp10, Timp3, Spp1, Vcan, Sparc, Col6a1, cyclin D, cyclin E, or any combination thereof, or in combination with conventional chemotherapies such as gemcitabine, nanoparticle albumin bound (nab)-paclitaxel, FOLFIRINOX combination (5-fluorouracil, leucovorin, irinotecan and oxaliplatin) or any combination thereof.

10 BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B show the effect of targeting endogenous *Kras*^{G12D} and *Trp53*^{R172H} expression and deletion of *Dpc4* to the murine pancreas. (A) Activation of *Kras*^{LSL-G12D/+} and *Trp53*^{LSL-R172H/+} and deletion of endogenous *Dpc4*^{flox/+} alleles upon pancreas-specific exposure to Cre recombinase (*p48*^{Cre/+}) 15 in quadruple mutant *Kras*^{LSL-G12D/+}::*Trp53*^{LSL-R172H/+}::*Dpc4*^{flox/+}::*p48*^{Cre/+} (*KPD*) mice. (B) Kaplan-Meier survival curves of *KP*, *KPD*, and control cohorts. Survival of *KPD* animals (151 days) is significantly less than control animals ($p < 0.001$) and *Kras*^{LSL-G12D/+}::*Dpc4*^{flox/+}::*p48*^{Cre/+} (*KD*) mice ($p < 0.001$), but not significantly different from *KP* mice (147 days, $p = 0.28$). A log rank test was 20 used for each pair wise combination.

Figure 2 shows the architectural and functional integrity of pancreata in *PD* and *KPD* animals. Immunohistochemical detection of digestive enzymes (amylase), hormones (insulin) and surface markers (CK-19) in distinct compartments of the pancreas from *Trp53*^{LSL-R172H/+}::*Dpc4*^{flox/+}::*p48*^{Cre/+} (*PD*) and 25 *Kras*^{LSL-G12D/+}::*Trp53*^{LSL-R172H/+}::*Dpc4*^{flox/+}::*p48*^{Cre/+} (*KPD*) and animals. (*is*, islets; *ac*, acini; arrow, ducts. Scale bars, 50 μ m.)

Figures 3A – 3N show the histological and molecular characterization of tumor progression in *KPD* mice. (A and B) Gross pathology of moribund *KPD* mice at necropsy. Dashed lines outline tumor. (C) Representative resected

KPD tumor. (D) Representative resected *KP* tumor. (E) Histology of pancreas in a young *KPD* animal. Arrow indicates PanIN-1A lesion. (F) CK-19 staining in *KPD* animal. Arrows, PanIN-2; arrowhead, PanIN-1. (G) Alcian blue staining (arrow) in a well-differentiated area of PDA from *KPD* animal. (H) Histology of pancreas in an older *KPD* animal. Arrow indicates PanIN-3 lesion. (I) Moderately well-differentiated histology in a *KPD* PDA. (J) Poorly differentiated PDA from a *KPD* animal. (K) Evidence of recombined *Kras*^{L-G12D} allele (1LoxP, upper band) and wild type (lower band) alleles in primary cells from *KPD* and *KP* tumors. (L) Evidence of recombined *Trp53*^{L-R172H} allele (1LoxP, upper band) and uniform loss of wild type *Trp53* (lower band) in primary carcinoma cells from *KPD* and *KP* animals. (M) Conditional (cond) *Dpc4* allele was recombined (rec) and the wild type (WT) allele retained in all *KPD* primary cells lines. WT, *D*^{flox/+} and *KDD* represent controls for various alleles. (N) Immunoblots of lysates from purified *KPD* and *KP* primary PDA cells for the indicated proteins. *d*, duodenum; *ac*, acinar cells. Scale bars, 50 μ m. See also Table S1.

Figures 4A – 4G show how heterozygous loss of *Dpc4* affects proliferation and cell cycle control. (A) Ki-67 expression in *KPD* PDA. (B) Ki-67 expression in *KP* PDA. (C) Cleaved caspase 3 (CC3) detection in *KPD* PDA. (D) Cleaved caspase 3 (CC3) detection in *KP* PDA. (E) Quantification of Ki-67 expression in *KPD* and *KP* tumor sections (mean $\pm$ SEM, *p=0.011). (F) Proliferation of purified primary PDA cells in the presence or absence of TGF β *in vitro*. Data points represent mean $\pm$ SEM. (G) Immunoblots of lysates from primary *KPD* and *KP* carcinoma cells for ppRB^{ser780}, Ccnd1 and p21 expression; Gapdh and Actin were used as loading controls for the respective blots. Normalized p21 expression values were 0.7 $\pm$ 0.02 in *KP* and 0.35 $\pm$ 0.27 in *KPD* carcinoma cells (mean $\pm$ SD). Scale bars, 50 μ m.

Figures 5A – 5H show how *Dpc4* status affects morphological and cellular behaviors associated with metastasis. (A and B) Immunofluorescence staining of actin stress fibers (A) and surface E-cadherin expression (B) in representative *KP* and *KPD* cells in the presence or absence of TGF β . Nuclei

are counterstained with DAPI. (C) Scratch wound migration assays of *KP* and *KPD* primary carcinoma cells in the presence or absence of TGF β . (D) Migration assays of scrambled (*Scr*) or *shDpc4*-transfected *KP* and *KPD* primary carcinoma cells in the presence or absence of TGF β . (E and F) Loss of

5 Dpc4 in metastatic PDA cells (arrowheads) in the liver, with retention of expression in adjacent hepatocytes (arrows). An isolated metastatic cell (white arrowhead) is also seen amidst hepatic parenchyma (*h*). (G) Focal loss of nuclear Dpc4 in invasive *KPD* PDA cells (arrowheads) with retention of expression in adjacent preinvasive (PanIN) lesions (arrow). (H) Focal loss of

10 nuclear Dpc4 in another example of invasive *KPD* PDA (arrowheads). Scale bars, 50 μ m.

Figures 6A – 6H show the epithelial to mesenchymal transition (EMT) and the metastatic potential of *KPD* and *KP* cells. (A) Immunofluorescence of actin stress fibers in representative *KP* and *KPD* cells in the presence or

15 absence of TGF β . Nuclei are counterstained with DAPI. (B) Immunofluorescence of E-cadherin expression in representative *KP* and *KPD* cells in the presence or absence of TGF β . Nuclei are counterstained with DAPI. (C) Scratch wound migration assays for *KP* and *KPD* cells in the presence or absence of TGF β . (D) Immunoblots after stable knockdown of

20 *Dpc4* in *KP* and *KPD* carcinoma cells. Gapdh was used as a loading control. (E and F) Immunofluorescence of actin stress fibers in scrambled (*Scr*) and *shDpc4*-transfected *KP* (E) and *KPD* (F) carcinoma cells in the presence or absence of TGF β . Nuclei are counterstained with DAPI. (G) Migration of scrambled (*Scr*) and *shDpc4*-transfected *KP* and *KPD* carcinoma cells in the

25 presence or absence of TGF β . (H) Representative H&E sections of lungs from mice injected by tail vein with murine *KP* or *KPD* PDA cells (*n* = 3 each). Scale bars, 50 μ m.

Figures 7A – 7H show the identification of Runx3 as metastasis-promoter and the post-translational regulation of Runx3 protein levels. (A) IPA

30 analysis of TGF β pathway array data. Red indicates upregulated genes and

green indicates downregulated genes in *KP* cells as compared to *KPD* cells. (B) Immunoblots for Runx3 in nuclear and cytoplasmic extracts from *KP* and *KPD* cells. Gapdh and Parp were used as cytoplasmic (C) and nuclear (N) markers, respectively. (C) Stable expression of murine *Flag-Runx3* in two
5 distinct *KPD* cell lines. Expression was confirmed by immunoblotting with both Flag and Runx3 specific antibodies. (D) The murine *Runx3* promoter contains five putative Runx3 binding sites (*Runx3*) at -1120, -1027, -962, -593 and -585 bp, respectively, from the transcription start site. The promoter also has two canonical SMAD binding elements (SBE). (E) Immunoprecipitation (IP) with
10 anti-Smad2 antibody followed by specific immunoblotting (IB) for indicated proteins on lysates prepared from *KP*#1 and *KPD*#1 PDA cells. Cells were incubated in the presence or absence of TGF β prior to lysis. IP with IgG was performed as control. (F) qRT-PCR analysis of *Hdac5* in *KPD* and *KP* cells (*p<0.035). Expression was normalized to preinvasive ductal cells. (G)
15 Immunoblots for Mdm2 in *KPD* and *KP* cells. Actin, loading control. (H) Immunoblots for Runx3 in *KPD* and *KP* cells treated with Trichostatin A (TSA) at the indicated concentrations. Gapdh, loading control.

Figures 8A – 8O shows how Runx3 promotes metastasis in murine PDA. (A) qRT-PCR analyses of relative transcript levels of *Runx3* in preinvasive (pre)
20 and invasive (PDA) ductal carcinoma cells (n = 3 each, *p=0.025, #p=0.045). (B) Immunoblots for Runx3 in preinvasive *K* cells and *KP* and *KPD* invasive PDA cells. Actin was used as loading control. (C) Runx3 IHC in wild type pancreas. Arrowheads, duct; ac, acinar cells; is, islet cells. (D) Intense nuclear Runx3 expression is seen in *KP* PDA (asterisk) with faint cytoplasmic
25 expression appreciable in adjacent PanINs (arrowhead). (E and F) Additional examples of nuclear Runx3 staining (arrows) in PDA from *KP* animals. (G and H) Representative sections from two different *KPD* PDAs show absence of nuclear Runx3 (arrowheads) and weak cytoplasmic staining. (I) Scratch wound migration assays of *Flag* and *Flag-Runx3* transfected *KPD* cells in the presence
30 or absence of TGF β . (J) Representative sections of lungs from mice injected

- by tail vein with *KPD#1* cells expressing *Flag* or *Flag-Runx3*. (K) Metastatic pulmonary tumor burden in mice injected by tail vein with *KPD#1* cells expressing *Flag* or *Flag-Runx3*. Two different levels from a total of three injected animals were assessed (means $\pm$ SEM; *p=0.0048). (L)
- 5 Electrophoretic Mobility Shift Assay (EMSA) of nuclear extracts incubated with labeled DNA probe containing *Runx3* promoter sequences. Lane 1, *KP* nuclear extract and labeled probe; lane 2, *KP* nuclear extract, labeled probe and excess unlabeled probe; arrow, mobility shift of biotinylated probe indicating specific binding; arrowhead, free probe. (M) Promoter activity of wild-type and
- 10 truncated (Δ) *Runx3* promoter in HEK293T cells with increasing concentrations of *Flag-Runx3* (*p=0.0001; **p=0.0002). (N) Immunoblots for Dpc4 and Runx3 in *KPD* primary carcinoma cells before (-) and after (+) exogenous overexpression of *Dpc4*. (O) Immunoblots for Dpc4 and Runx3 in *KPD* primary carcinoma cells before (-) and after (+) transfection with *shDpc4*.
- 15 Figures 9A – 9H show that RUNX3 promotes metastasis in human PDA. (A) qRT-PCR analyses of *RUNX3* expression in human PDA lines. Fold change normalized to HPDE cells. (B) Scratch wound migration assays of MiaPaCa-2, CFPAC-1 and Panc-1 human PDA cell lines in the presence or absence of transfected in the presence or absence of TGF β . (C) Scratch
- 20 wound migration assay of MiaPaca-2 cells after knockdown of *RUNX3* in the presence or absence of TGF β . (D) Scratch wound migration assay of CFPAC-1 cells after knockdown of *RUNX3* in the presence or absence of TGF β . (E) Scratch wound migration assay of Panc-1 cells after knockdown of *RUNX3* in the presence or absence of TGF β . (F) Representative sections of lungs from
- 25 NOD/SCID animals injected with *Flag* or *Flag-RUNX3* transfected MiaPaCa-2 cells. Arrows, metastases. (G) Representative sections of lungs from NOD/SCID animals injected with with *Flag* or *shRUNX3* transfected Panc-1 cells. Arrows, metastases. (G) Livers *in vivo* (left panels) and *ex vivo* (right panels) from NOD/SCID animals injected with *shRUNX3* or Scrambled (*Scr*)
- 30 transfected Panc-1 cells. Arrows, metastases.

Figures 10A – 10C show that RUNX3 controls metastasis in human pancreas cancer. (A) Expression of human *Flag-RUNX3* in MiaPaCa-2 cells. Expression of the construct in two separate clones (1 and 2) was confirmed by immunoblotting with both Flag- and RUNX3-specific antibodies. GAPDH was used as a loading control. C, control cells. (B) Efficiency of targeted depletion of *RUNX3* in CFPAC-1 cells with different RNAi constructs assessed by qRT-PCR. (C) Efficiency of targeted depletion of *RUNX3* in Panc-1 cells with different RNAi constructs assessed by qRT-PCR.

Figures 11A – 11D show that Runx3 promotes metastasis via modulation of ECM components. (A) IPA analysis of differentially regulated ECM pathway genes of in *KP* and *KPD* cells. Red indicates upregulated genes and green indicates downregulated genes in *KP* cells as compared to *KPD* cells. (B) The human *SPP1* promoter has two putative RUNX3 binding sites and two SMAD binding elements (SBE). (C) Expression of *SPP1* in *Flag-* and *Flag-RUNX3* transfected MiaPaca-2 cells assessed by qRT-PCR (*p = 0.0009). (D) The human *COL6A1* promoter has three putative RUNX3 binding sites (arrows).

Figures 12A – 12H show how Runx3 orchestrates the metastasis niche via ECM components. (A) Immunoblots for Col6a1 and Spp1 in three different *KP* and *KPD* primary PDA cells. (B) *Spp1* transcript levels in *Runx3*-overexpressing *KPD* cells as determined by qRT-PCR. *p=0.0016. (C) Immunoblots for Col6a1 and Spp1 in control and *Runx3*-overexpressing *KPD* cells. (D) Relative transcript levels in *KP*, *KPD* and *KPD-Flag-Runx3* carcinoma cells measured by qRT-PCR. Results are normalized against transcript levels in preinvasive *K* cells (means $\pm$ SEM, n = 3 cell lines for each genotype, *p=0.045, **p=0.0059). (E) Human *COL6A1* promoter-luciferase assay in HEK293 cells transfected with *Flag* or *Flag-RUNX3* (*p=0.0024). (F) *COL6A1* expression as determined by qRT-PCR in scrambled (*Scr*) and *shRUNX3* (*sh#1* and *sh#2*) transfected Panc-1 cells (*p=0.02). (G) Scratch wound migration assay of control and *COL6A1*-transfected MiaPaCa-2 cells in the presence or

absence of TGF β . (H) Scratch wound migration assay of *KPD* cells after over expression of *Col6a1* in the presence or absence of TGF β .

DETAILED DESCRIPTION

The instant disclosure provides methods for treating pancreatic cancer or
5 for reducing the risk of pancreatic cancer or pancreatic cancer metastasis by
administering to a patient an inhibitor of RUNX3. A RUNX3 inhibitor may
function at the transcriptional level, translational level, or post-translational
level. For example, a RUNX3 inhibitor may reduce or block expression of a
nucleic acid molecule encoding RUNX3 (*e.g.*, alters transcription factor
10 function, alters promoter function), or the inhibitor may reduce the level of or
enhance the degradation of a RUNX3 RNA transcript (*e.g.*, antisense RNA,
small interfering RNA). In another example, a RUNX3 inhibitor may block the
translation of a RUNX3 mRNA. In a further example, a RUNX3 inhibitor may
enhance deacetylation, ubiquitination, or both of RUNX3 by activating, for
15 example, Hdac5, Mdm2, or both.

Also provided herein are inhibitors of RUNX3 for use in treating
pancreatic cancer, treating metastasized pancreatic cancer, reducing the risk of
pancreatic cancer, or reducing the risk of pancreatic cancer metastasis by
administering such compounds or compositions to a patient in need thereof.

20 Prior to setting forth this disclosure in more detail, it may be helpful to an
understanding thereof to provide definitions of certain terms to be used herein.
Additional definitions are set forth throughout this disclosure.

In the present description, any concentration range, percentage range,
ratio range, or integer range is to be understood to include the value of any
25 integer within the recited range and, when appropriate, fractions thereof (such
as one tenth and one hundredth of an integer), unless otherwise indicated.
Also, any number range recited herein relating to any physical feature, such as
polymer subunits, size or thickness, are to be understood to include any integer
within the recited range, unless otherwise indicated. As used herein, the terms

"about" and "consisting essentially of" mean $\pm 20\%$ of the indicated range, value, or structure, unless otherwise indicated. It should be understood that the terms "a" and "an" as used herein refer to "one or more" of the enumerated components. The use of the alternative (*e.g.*, "or") should be understood to
5 mean either one, both, or any combination thereof of the alternatives or enumerated components. As used herein, the terms "include," "have" and "comprise" are used synonymously, which terms and variants thereof are intended to be construed as non-limiting.

"Inhibit" as used herein refers to an alteration, reduction or abrogation,
10 directly or indirectly, in the expression of a target gene or in the activity of a target molecule (*e.g.*, RUNX3) relative to a control that is statistically, biologically, or clinically significant.

The "percent identity" or "sequence identity," as used herein, refers to the percentage of nucleic acid or amino acid residues in one sequence that are
15 identical with the nucleic acid or amino acid residues in a reference polynucleotide or polypeptide sequence, respectively, (*i.e.*, % identity = number of identical positions/total number of positions x 100) after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity of two or more sequences. For proteins, conservative
20 substitutions are not considered as part of the sequence identity. The comparison of sequences and determination of percent identity between two or more sequences is accomplished using a mathematical algorithm, such as BLAST and Gapped BLAST programs at their default parameters (*e.g.*, Altschul *et al.*, *J. Mol. Biol.* 215:403, 1990; Altschul *et al.*, *Nucleic Acids Res.* 25:3389, 1997; *see also* BLASTN or BLASTP at www.ncbi.nlm.nih.gov/BLAST).
25

A "conservative substitution" is recognized in the art as a substitution of one amino acid for another amino acid that has similar properties. Exemplary conservative substitutions are well known in the art (*see, e.g.*, WO 97/09433, page 10, published March 13, 1997; Lehninger, *Biochemistry*, Second Edition;

Worth Publishers, Inc. NY:NY (1975), pp.71-77; Lewin, Genes IV, Oxford University Press, NY and Cell Press, Cambridge, MA (1990), p. 8).

As used herein, "ribonucleic acid" or "RNA" means a nucleic acid molecule comprising at least one ribonucleotide molecule. It should be understood that "ribonucleotide" refers to a nucleotide with a hydroxyl group at the 2'-position of a β -D-ribofuranose moiety. The term RNA includes double-stranded (ds) RNA, single-stranded (ss) RNA, isolated RNA (such as partially purified RNA, essentially pure RNA, synthetic RNA, recombinantly produced RNA), altered RNA (which differs from naturally occurring RNA by the addition, deletion, substitution or alteration of one or more nucleotides), or any combination thereof. For example, such altered RNA can include addition of non-nucleotide material, such as at one or both ends of an RNA molecule, internally at one or more nucleotides of the RNA, or any combination thereof. Nucleotides in RNA molecules of the instant disclosure can also comprise non-standard nucleotides, such as naturally occurring nucleotides, non-naturally occurring nucleotides, chemically-modified nucleotides, deoxynucleotides, or any combination thereof. These altered RNAs may be referred to as derivatives or analogs of RNA containing standard nucleotides (*i.e.*, standard nucleotides, as used herein, are considered to be adenine, cytidine, guanine, thymidine, and uridine).

The term "dsRNA" as used herein refers to any nucleic acid molecule comprising at least one ribonucleotide and is capable of inhibiting or down regulating gene expression, for example, by promoting RNA interference ("RNAi") or gene silencing in a sequence-specific manner. The dsRNAs of the instant disclosure may be suitable substrates for Dicer or for association with RISC to mediate gene silencing by RNAi. One or both strands of the dsRNA can further comprise a terminal phosphate group, such as a 5'-phosphate or 5',3'-diphosphate. As used herein, dsRNA molecules, in addition to at least one ribonucleotide, can further include substitutions, chemically-modified

nucleotides, or non-nucleotides. In certain embodiments, dsRNA molecules comprise ribonucleotides up to about 100% of the nucleotide positions.

In addition, as used herein, the term dsRNA is meant to be equivalent to other terms used to describe nucleic acid molecules that are capable of mediating sequence specific RNAi, for example, nicked dsRNA (ndsRNA), gapped dsRNA (gdsRNA), short interfering nucleic acid (siNA), siRNA, micro-RNA (miRNA), short hairpin RNA (shRNA), short interfering oligonucleotide, short interfering substituted oligonucleotide, short interfering modified oligonucleotide, chemically-modified dsRNA, post-transcriptional gene silencing RNA (ptgsRNA), or the like. The term "large double-stranded (ds) RNA" refers to any double-stranded RNA longer than about 40 base pairs (bp) to about 100 bp or more, particularly up to about 300 bp to about 500 bp. The sequence of a large dsRNA may represent a segment of an mRNA or an entire mRNA. A double-stranded structure may be formed by self-complementary nucleic acid molecule or by annealing of two or more distinct complementary nucleic acid molecule strands.

In one aspect, a dsRNA comprises two separate oligonucleotides, comprising a first strand (antisense) and a second strand (sense), wherein the antisense and sense strands are self-complementary (*i.e.*, each strand comprises a nucleotide sequence that is complementary to a nucleotide sequence in the other strand and the two separate strands form a duplex or double-stranded structure, for example, wherein the double-stranded region is about 15 to about 24 or 25 base pairs or about 25 or 26 to about 40 base pairs); the antisense strand comprises a nucleotide sequence that is complementary to a nucleotide sequence of a target nucleic acid molecule or a portion thereof (*e.g.*, RUNX3); and the sense strand comprises a nucleotide sequence corresponding (*i.e.*, homologous) to the target nucleic acid sequence or a portion thereof (*e.g.*, a sense strand of about 15 to about 25 nucleotides or about 26 to about 40 nucleotides corresponds to the target nucleic acid or a portion thereof).

A dsRNA or large dsRNA may include a substitution or modification in which the substitution or modification may be in a phosphate backbone bond, a sugar, a base, or a nucleoside. Such nucleoside substitutions can include natural non-standard nucleosides (e.g., 5-methyluridine or 5-methylcytidine or a
5 2-thioribothymidine), and such backbone, sugar, or nucleoside modifications can include an alkyl or heteroatom substitution or addition, such as a methyl, alkoxyalkyl, halogen, nitrogen or sulfur, or other modifications known in the art.

As used herein, the term "RNAi" is meant to be equivalent to other terms used to describe sequence specific RNA interference, such as post
10 transcriptional gene silencing, translational inhibition, or epigenetics. For example, dsRNA and single stranded antisense molecules of this disclosure can be used to epigenetically silence genes at the post-transcriptional level, the pre-transcriptional level, or any combination thereof.

As used herein, "gene silencing" refers to a partial or complete loss-of-
15 function through targeted inhibition of gene expression in a cell, which may also be referred to as RNAi "knockdown," "inhibition," "down-regulation," or "reduction" of expression of a target gene. Depending on the circumstances and the biological problem to be addressed, it may be preferable to partially reduce gene expression. Alternatively, it might be desirable to reduce gene
20 expression as much as possible. The extent of silencing may be determined by methods described herein and known in the art, some of which are summarized in PCT Publication No. WO 99/32619. Depending on the assay, quantification of gene expression permits detection of various amounts of inhibition that may be desired in certain embodiments of this disclosure, including prophylactic and
25 therapeutic methods, which will be capable of knocking down target gene expression, in terms of mRNA level or protein level or activity, for example, by equal to or greater than 10%, 30%, 50%, 75% 90%, 95% or 99% of baseline (i.e., normal) or other control levels, including elevated expression levels as may be associated with particular disease states or other conditions targeted
30 for therapy.

As used herein, "target nucleic acid molecule" refers to any nucleic acid molecule whose expression or activity is to be altered (e.g., RUNX3). A target nucleic acid can be DNA, RNA, or derivatives or analogs thereof, and includes single, double, and multi-stranded forms. By "target site" or "target sequence" is meant a sequence within a target nucleic acid molecule (e.g., RUNX3 mRNA) that is "targeted" for cleavage by RNAi and mediated by a dsRNA construct of this disclosure containing a sequence within the antisense strand that is complementary to the target site or sequence.

By "sense region" or "sense strand" is meant one or more nucleotide sequences of a dsRNA molecule having complementarity to one or more antisense regions of the dsRNA molecule. In addition, the sense region of a dsRNA molecule comprises a nucleic acid sequence having homology or identity to a target sequence. By "antisense region" or "antisense strand" is meant a nucleotide sequence of a dsRNA molecule having complementarity to a target nucleic acid sequence. In addition, the antisense region of a dsRNA molecule can comprise a nucleic acid sequence regions having complementarity to one or more sense strands of a dsRNA molecule.

Furthermore, one or more dsRNA may be used to knockdown expression of a target mRNA or a related mRNA splice variant. In this regard, it is noted that a target gene may be transcribed into two or more mRNA splice variants. In certain embodiments, knockdown of one target mRNA splice variant without affecting one or more other target mRNA splice variants may be desired, or vice versa. Alternatively, knockdown of all transcription products of one or more target family genes is contemplated herein.

As used herein, "off-target effect" or "off-target profile" refers to the observed altered expression pattern of one or more genes in a cell or other biological sample not targeted, directly or indirectly, for gene silencing by a dsRNA. For example, an off-target effect can be quantified by using a DNA microarray to determine how many non-target genes have an expression level altered by at least 2-fold or more in the presence of a candidate dsRNA, or

derivative or analog thereof specific for a target sequence, such as one or more RUNX3 mRNA. A "minimal off-target effect" means that a dsRNA affects expression by about 2-fold or more of about 25% to about 1% of the non-target genes examined or it means that the off-target effect of substituted or modified dsRNA (e.g., having at least one uridine substituted with a 5-methyluridine or 2-thioribothymidine and optionally having at least one nucleotide modified at the 2'-position), is reduced by at least about 1% to about 80% or more as compared to the effect on non-target genes of an unsubstituted or unmodified dsRNA.

"Analog" as used herein refers to a compound that is structurally similar to a parent compound, but differs slightly in composition (e.g., one atom or functional group is different, added, or removed). An analog may or may not have different chemical or physical properties than the original compound and may or may not have improved biological or chemical activity. For example, an analog may be more hydrophilic or it may have altered activity as compared to a parent compound. An analog may mimic the chemical or biological activity of the parent compound (*i.e.*, it may have similar or identical activity), or, in some cases, may have increased or decreased activity. In other cases, the changes in an analog may impart certain desirable properties (e.g., improved stability, improved bioavailability, improved hydrophilicity, minimized off-target effects, minimized toxicity). An analog may be a naturally or non-naturally occurring (e.g., chemically-modified or recombinant) variant of the original compound. An example of an analog is a mutein (*i.e.*, a protein analog in which at least one amino acid is deleted, added, or substituted with another amino acid). An example of an RNA analog is an RNA molecule having a non-standard nucleotide, such as 5-methyluridine or 5-methylcytidine or 2-thioribothymidine. Other types of analogues include isomers (enantiomers, diastereomers, or the like) and other types of chiral variants of a compound, as well as structural isomers. An analog may be a branched or cyclic variant of a linear compound.

As used herein, the term "derivative" refers to a modification of a compound by chemical or biological means, with or without an enzyme, which

modified compound is structurally similar to a parent compound and (actually or theoretically) derivable from that parent compound. Generally, a "derivative" differs from an "analog" in that a parent compound may be the starting material to generate a "derivative," whereas the parent compound may not necessarily be used as the starting material to generate an "analog." A derivative may have different chemical, biological or physical properties of the parent compound, such as being more hydrophilic or having altered reactivity as compared to the parent compound. Derivatization (*i.e.*, modification) may involve substitution of one or more moieties within the molecule (*e.g.*, a change in functional group). For example, a hydrogen may be substituted with a halogen, such as fluorine or chlorine, or a hydroxyl group (-OH) may be replaced with a carboxylic acid moiety (-COOH). Other exemplary derivatizations include glycosylation, alkylation, acylation, acetylation, ubiquitination, esterification, and amidation.

The term "derivative" also refers to all solvates, for example hydrates or adducts (*e.g.*, adducts with alcohols), active metabolites, and salts of the parent compound. The type of salt that may be prepared depends on the nature of the moieties within the compound. For example, acidic groups such as carboxylic acid groups can form alkali metal salts or alkaline earth metal salts (*e.g.*, sodium salts, potassium salts, magnesium salts and calcium salts, and also salts with physiologically tolerable quaternary ammonium ions and acid addition salts with ammonia and physiologically tolerable organic amines such as, for example, triethylamine, ethanolamine or tris-(2-hydroxyethyl)amine). Basic groups can form acid addition salts, for example, with inorganic acids such as hydrochloric acid, sulfuric acid or phosphoric acid, or with organic carboxylic acids and sulfonic acids such as acetic acid, citric acid, lactic acid, benzoic acid, maleic acid, fumaric acid, tartaric acid, methanesulfonic acid or p-toluenesulfonic acid. Compounds that simultaneously contain a basic group and an acidic group, for example, a carboxyl group in addition to basic nitrogen atoms, can be present as zwitterions. Salts can be obtained by customary

methods known to those skilled in the art, for example, by combining a compound with an inorganic or organic acid or base in a solvent or diluent, or from other salts by cation exchange or anion exchange.

Other types of derivatives include conjugates and prodrugs of a parent compound (*i.e.*, chemically modified derivatives which can be converted into the original compound under physiological conditions). For example, the prodrug may be an inactive form of an active agent. Under physiological conditions, the prodrug may be converted into the active form of the compound. Prodrugs may be formed, for example, by replacing one or two hydrogen atoms on nitrogen atoms by an acyl group (acyl prodrugs) or a carbamate group (carbamate prodrugs). More detailed information relating to prodrugs may be found in, for example, Fleisher *et al.*, *Adv. Drug Del. Rev* 19:115, 1996; Design of Prodrugs, H. Bundgaard (ed.), Elsevier, 1985; or Bundgaard, *Drugs of the Future* 16:443, 1991.

The term "pyrimidine" as used herein refers to conventional pyrimidine bases, including standard pyrimidine bases uracil and cytosine. In addition, the term pyrimidine is contemplated to embrace natural non-standard pyrimidine bases or acids, such as 5-methyluracil, 2-thio-5-methyluracil, 4-thiouracil, pseudouracil, dihydrouracil, orotate, 5-methylcytosine, or the like, as well as a chemically-modified bases or "universal bases," which can be used to substitute for a standard pyrimidine within nucleic acid molecules of this disclosure.

The term "purine" as used herein refers to conventional purine bases, including standard purine bases adenine and guanine. In addition, the term purine is contemplated to embrace natural non-standard purine bases or acids, such as N2-methylguanine, inosine, or the like, as well as a chemically-modified bases or "universal bases," which can be used to substitute for a standard purine within nucleic acid molecules of this disclosure.

As used herein, the term "universal base" refers to nucleotide base analogs that form base pairs with each of the standard DNA/RNA bases with little discrimination between them, and is recognized by intracellular enzymes

(see, e.g., Loakes *et al.*, *J. Mol. Bio.* 270:426, 1997). Non-limiting examples of universal bases include C-phenyl, C-naphthyl and other aromatic derivatives, inosine, azole carboxamides, and nitroazole derivatives such as 3-nitropyrrole, 4-nitroindole, 5-nitroindole, and 6-nitroindole as known in the art (see, e.g.,
5 Loakes, *Nucleic Acids Res.* 29:2437, 2001).

As used herein, the term "isolated" means that the molecule referred to is removed from its original environment, such as being separated from some or all of the co-existing materials in a natural environment (e.g., a natural environment may be a cell).

10 The term "biological sample" includes a blood sample, biopsy specimen, tissue explant, organ culture, biological fluid (e.g., serum, urine, CSF) or any other tissue or cell or other preparation from a subject or a biological source. A subject or biological source may, for example, be a human or non-human animal, a primary cell culture or culture adapted cell line including genetically
15 engineered cell lines that may contain chromosomally integrated or episomal recombinant nucleic acid molecules, somatic cell hybrid cell lines, immortalized or immortalizable cell lines, differentiated or differentiable cell lines, transformed cell lines, or the like.

In further embodiments of this disclosure, a subject or biological source
20 may be suspected of having or being at risk for having a disease, disorder or condition, including a malignant disease, disorder or condition (e.g., pancreatic cancer). In certain embodiments, a subject or biological source may be suspected of having or being at risk for having a hyperproliferative disease (e.g., pancreatic cancer), and in certain other embodiments of this disclosure
25 the subject or biological source may be known to be free of a risk or presence of such disease, disorder, or condition.

By "subject" is meant an organism, which may be a donor or recipient of explanted cells, or the cells themselves. "Subject" also refers to an organism to which a small molecule, chemical entity, nucleic acid molecule, peptide or
30 polypeptide of this disclosure can be administered to inhibit RUNX3. In one

embodiment, a subject is a mammal or mammalian cell. In another embodiment, a subject is a human or human cell.

“Treatment,” “treating” or “ameliorating” refers to either a therapeutic treatment or prophylactic/preventative treatment. A treatment is therapeutic if
5 at least one symptom of disease (e.g., pancreatic cancer) in an individual receiving treatment improves or a treatment may delay worsening of a progressive disease in an individual, or prevent onset of additional associated diseases (e.g., metastases from pancreatic cancer).

A “therapeutically effective amount (or dose)” or “effective amount (or
10 dose)” of a RUNX3 inhibitor refers to that amount of compound sufficient to result in amelioration of one or more symptoms of the disease being treated (e.g., pancreatic cancer) in a statistically significant manner. When referring to an individual active ingredient, administered alone, a therapeutically effective dose refers to that ingredient alone. When referring to a combination, a
15 therapeutically effective dose refers to combined amounts of the active ingredients that result in the therapeutic effect, whether administered serially or simultaneously (in the same formulation or in separate formulations).

The term “pharmaceutically acceptable” refers to molecular entities and compositions that do not produce allergic or other serious adverse reactions
20 when administered using routes well known in the art.

A “patient in need” or “subject in need” refers to a patient or subject at risk of, or suffering from, a disease, disorder or condition (e.g., pancreatic cancer) that is amenable to treatment or amelioration with a RUNX3 inhibitor or a composition thereof, as provided herein.

25 In certain embodiments, provided are compounds for reducing the risk of or treating pancreatic cancer or metastatic pancreatic cancer include inhibitors of RUNX3 expression, such as small molecules, chemical entities, nucleic acid molecules, peptides or polypeptides. In particular embodiments, the RUNX3 inhibitor is a dsRNA (interfering RNA) or antisense RNA. In certain further

embodiments, the RUNX3 inhibitor comprises an isolated nucleic acid molecule with the sequence set forth in SEQ ID NO.:4.

In certain further embodiments, provided are methods for reducing the risk of or treating pancreatic cancer, comprising administering to a subject in
5 need thereof an inhibitor of RUNX3 expression or activity, such as a small molecule, chemical entity, nucleic acid molecule, peptide or polypeptide. In particular embodiments, the RUNX3 inhibitor is a dsRNA (interfering RNA) or antisense RNA. In certain further embodiments, the RUNX3 inhibitor comprises an isolated nucleic acid molecule with the sequence set forth in SEQ ID NO.:4.

10 By way of background, *Runx3* belongs to a family of transcription factors involved in development and differentiation (Ito, *Adv. Cancer Res.* 99:33, 2008; Speck *et al.*, *Cancer Res.* 59:1789s, 1999) and whose best characterized members are *Runx1* and *Runx2*. The specificity of the upregulation in *Runx3* in *KP* cells was underscored by the lack of significant changes in these close
15 family members (-1.2-fold for *Runx1* and 1.5-fold for *Runx2*; not shown). *RUNX1* and *RUNX2* have been identified as tumor suppressor genes (TSGs) in hematopoietic malignancies (Blyth *et al.*, *Nat. Rev. Cancer* 5:376, 2005). Its alternate designation of *AML2* notwithstanding, what little is known about the role of *RUNX3* in malignancy comes primarily from studies of solid tumors,
20 where it has been implicated on both sides of the cancer divide, and sometimes with contradicting conclusions in the same cancer.

Several studies point toward a tumor suppressive role for RUNX3 (e.g., Bae and Choi, *Oncogene* 23:4336, 2004), but a small number of reports indicate that it has oncogenic potential and that this potential might extend
25 beyond murine lymphomas (Debernardi *et al.*, *Genes Chromosomes Cancer* 37:149, 2003; Lacayo *et al.*, *Blood* 104:2646, 2004). For instance, *RUNX3* overexpression has been reported in basal cell (Salto-Tellez *et al.*, *Oncogene* 25:7646, 2006), skin (Lee *et al.*, *Clin. Exp. Dermatol.* 36:769, 2011b), head and neck (Tsunematsu *et al.*, *PLoS One* 4:e5892, 2009) and ovarian cancers (Lee
30 *et al.*, *Gynecol. Oncol.* 122:410, 2011a). Finally, in gastric carcinoma, *RUNX3*

has been separately identified as either a TSG by some groups (e.g., Li *et al.*, 2002) or an oncogene by others (e.g., Levanon *et al.*, 2011). Indeed, its specific role in tumorigenesis remains the subject of considerable controversy (see, also, Ito *et al.*, *Oncogene* 28, 1379, 2009; Levanon *et al.*, *EMBO Mol.*

5 *Med.* 3:593, 2011; Levanon *et al.*, *Mech. Dev.* 109:413, 2001; Li *et al.*, *Cell* 109:113, 2002 and see below).

Pancreatic ductal adenocarcinoma (PDA) is notorious for its extreme penchant for metastatic spread. The chronological sequence in which cardinal mutations in *KRAS*, *P53* and *SMAD4/DPC4* occur can define the

10 histopathology and metastatic potential of the resultant PDAs. For the first, the present disclosure teaches that heterozygous mutation of *Smad4/Dpc4* early in the progression of precursor lesions expressing *Kras*^{G12D} and *Trp53*^{R172H} shifts the disease burden toward increased primary tumor growth while almost completely abolishing distant metastases. Conversely, data provided herein
15 shows that heterozygous *Dpc4* mutation late in progression further augments inherently prolific metastasis. Unexpectedly, it was found that this switch governing the balance between proliferation and dissemination is regulated by the transcription factor, *RUNX3*, which orchestrates a coherent metastatic program that both drives cell migration and remodels the nascent metastatic
20 niche.

While not wishing to be bound by theory, *RUNX3* might orchestrate a coordinated program that shifts the pancreatic cancer cell's priorities from proliferation to dissemination and metastatic colonization of distant sites.

Overcoming the barriers to successful metastasis requires achieving a
25 migratory capability, as well as the ability to refashion the extra cellular matrix (ECM) and microenvironment to support growth in a foreign organ. In *KPD* cells, resting levels of Runx3 are kept tightly in check via coordinated Mdm2- and Hdac5-mediated protein degradation, together with relatively low levels of transcriptional initiation because of decreased Runx3/Smad4 complex
30 formation. In *KP* cells, however, Mdm2 and Hdac5 are both relatively low,

allowing basal levels of Runx3 protein to reach a threshold at which point transcription is further rapidly increased by positive feedback of Runx3 on its own promoter. Elevated levels of Runx3 activate the “metastatic switch,” increasing cell cycle checkpoint gene expression and expression, along with
5 secretion of proteins that support and promote migration and colonization. In this manner, *RUNX3* helps regulate a cell fate decision node between the competing demands of local growth and invasion on the one hand, and dissemination and metastatic colonization on the other. In shifting cellular resources from proliferation to colonization, *RUNX3* drives cells to refashion
10 their microenvironment to overcome the biologic barriers to establishing a metastatic foothold.

In further embodiments, provided are methods for reducing the risk of or treating pancreatic cancer, comprising administering to a subject in need thereof a compound capable of blocking the association of RUNX3 with a
15 second transcription factor, such as SMAD4 or a complex of SMAD4 with SMAD2. In further embodiments, provided are methods for reducing the risk of or treating pancreatic cancer, comprising administering to a subject in need thereof an agent that enhances deacetylation of RUNX3, such as an activator of *HDAC5* activity (preferably a pancreas-specific activator of *HDAC5*, such as
20 increasing expression via gene therapy). In still further embodiments, provided are methods for reducing the risk of or treating pancreatic cancer, comprising administering to a subject in need thereof an agent that enhances ubiquitination of RUNX3, such as an activator of *HDAC5* activity (preferably a pancreas-specific activator of *HDAC5*), an activator of *MDM2* activity (preferably a
25 pancreas-specific activator of *MDM2*), or a combination thereof. In further embodiments, the methods further comprise administering an expression or activity inhibitor of a cyclin D (such as cyclin D1), a cyclin E, or a combination thereof.

In yet other embodiments, provided are methods for reducing the risk of
30 or treating metastasized pancreatic cancer, comprising administering to a

subject in need thereof an inhibitor of RUNX3 expression or activity, such as a small molecule, chemical entity, nucleic acid molecule, peptide or polypeptide. In particular embodiments, the RUNX3 inhibitor is a dsRNA (interfering RNA) or antisense RNA. In certain further embodiments, the RUNX3 inhibitor comprises
5 an isolated nucleic acid molecule with the sequence set forth in SEQ ID NO.:4.

In still further embodiments, provided are methods for reducing the risk of or treating a pancreatic cancer precursor lesion, comprising administering to a subject in need thereof an inhibitor of RUNX3 expression or activity, such as a small molecule, chemical entity, nucleic acid molecule, peptide or
10 polypeptide. In particular embodiments, the RUNX3 inhibitor is a dsRNA (interfering RNA) or antisense RNA. In certain further embodiments, the RUNX3 inhibitor comprises an isolated nucleic acid molecule with the sequence set forth in SEQ ID NO.:4.

In certain further embodiments, provided are methods for reducing the
15 risk of or treating a metastatic niche associated with pancreatic cancer, comprising administering to a subject in need thereof an inhibitor of RUNX3 expression or activity, such as a small molecule, chemical entity, nucleic acid molecule, peptide or polypeptide. In particular embodiments, the RUNX3 inhibitor is a dsRNA (interfering RNA) or antisense RNA. In certain further
20 embodiments, the RUNX3 inhibitor comprises an isolated nucleic acid molecule with the sequence set forth in SEQ ID NO.:4.

Within additional aspects of this disclosure, combination formulations and methods are provided comprising an effective amount of RUNX3 inhibitor of the present disclosure in combination with one or more secondary or
25 adjunctive therapies. Such therapies may be additional active agents that are formulated together or administered coordinately with the RUNX3 inhibitors of this disclosure to control pancreatic cancer or associated condition, as described herein. Useful adjunctive or neoadjuvant therapies for combinatorial formulation or coordinate treatment methods include, for
30 example, enzymatic nucleic acid molecules, allosteric nucleic acid molecules,

antisense, decoy, or aptamer nucleic acid molecules, antibodies such as monoclonal antibodies, enzymes (*e.g.*, hyaluronidases), small molecules and other organic or inorganic compounds including metals, salts and ions, steroids, non-steroidal anti-inflammatory drugs (NSAIDs), and other drugs or active
5 agents or procedures indicated for treating pancreatic cancer, including surgery, chemotherapy, radiation therapy, chemoradiation therapy, or the like.

In certain embodiments, any of the aforementioned methods further comprise a second method of treatment, such as surgery, chemotherapy, radiation therapy, or any combination thereof. For pancreatic cancer or a
10 pancreatic cancer precursor lesion, several therapeutic regimens are known in the art. For example, the Whipple procedure, or pancreaticoduodenectomy, is the most commonly performed surgery to remove pancreatic tumors. Pancreatic cancer is considered resectable if the tumor appears to be localized to the pancreas without invasion into important surrounding structures, such as
15 the mesenteric blood vessels (that supply blood to the intestines) located adjacent to the head portion of the pancreas. Furthermore there should be no evidence of metastatic spread to the liver or the intestinal lining. In a standard Whipple operation, a surgeon will remove the head of the pancreas, the gallbladder, part of the duodenum (*i.e.*, the uppermost portion of the small
20 intestine), a small portion of the stomach called the pylorus, and the lymph nodes near the head of the pancreas. Then the remaining pancreas and digestive organs are reconnected so that pancreatic digestive enzymes, bile, and stomach contents will flow into the small intestine during digestion. In another type of Whipple procedure, known as pylorus preserving Whipple, the
25 bottom portion of the stomach, or pylorus, is not removed. In either case, such a surgery can last from about 6 hours to about 10 hours.

When pancreatic cancer has grown beyond the confines of the pancreas to invade surrounding vital structures, such a locally advanced pancreatic cancer is not treated by surgery. Treatment of locally advanced pancreatic
30 cancer includes chemotherapy and radiation therapy. Exemplary

chemotherapeutic drugs used for the treatment of pancreatic cancer include 5-fluorouracil, leukovirin, gemcitabine, cisplatin, irinotecan, paclitaxel, nanoparticle albumin bound (nab)-paclitaxel, docetaxel, capecitabine, oxaliplatin, and the FOLFIRINOX combination (5-fluorouracil, leucovorin, irinotecan and oxaliplatin). Exemplary radiation therapy is delivered in daily fractions over a six week period to a total dose of approximately 5,000 rads, which may be external (e.g. high energy X-rays) or internal (e.g., radiation contained in needles, seeds, wires, or catheters, which are placed directly into or near a tumor). In certain embodiments, chemotherapy may be administered together or sequentially with the radiation therapy.

Exemplary chemotherapeutic agents include alkylating agents (e.g., cisplatin, oxaliplatin, carboplatin, busulfan, nitrosoureas, nitrogen mustards, uramustine, temozolomide), antimetabolites (e.g., aminopterin, methotrexate, mercaptopurine, fluorouracil, cytarabine, gemcitabine), taxanes (e.g., paclitaxel, nab-paclitaxel, docetaxel), anthracyclines (e.g., doxorubicin, daunorubicin, epirubicin, idarubicin, mitoxantrone, valrubicin), bleomycin, mytomyacin, actinomycin, hydroxyurea, topoisomerase inhibitors (e.g., camptothecin, topotecan, irinotecan, etoposide, teniposide), monoclonal antibodies (e.g., alemtuzumab, bevacizumab, cetuximab, gemtuzumab, panitumumab, rituximab, tositumomab, trastuzumab), vinca alkaloids (e.g., vincristine, vinblastine, vindesine, vinorelbine), cyclophosphamide, prednisone, leucovorin, oxaliplatin, hyalurodinases.

Other exemplary adjunctive or neoadjuvant therapies for use with a RUNX3 inhibitor include expression or activity inhibitors of Bmpr1a, Smad5, Tgfb3, Smad4, Bmp1, Itgb7, Tgfb1i1, Bmper, Ltbp1, Ltbp2, Itgb5, Id1, Tgfb1, Dlx2, or any combination thereof. Still further exemplary adjunctive or neoadjuvant therapies for use with a RUNX3 inhibitor include expression or activity inhibitors of Ctgf, Selp, Timp2, Col5a1, Ncam1, Thbs3, Mmp11, Sgce, Fn1, Vcam1, Ecm1, Adamts1, Mmp2, Thbs1, Fbln1, Tgfb1, Cdh2, Mmp10, Timp3, Spp1, Vcan, Sparc, Col6a1, or any combination thereof. In yet further

exemplary adjunctive or neoadjuvant therapies for use with a RUNX3 inhibitor include expression or activity inhibitors of a cyclin D, a cyclin E, or a combination thereof, or in combination with conventional chemotherapies such as gemcitabine, nab-paclitaxel, FOLFIRINOX combination, or any combination thereof. In yet other embodiments, an adjunctive or neoadjuvant therapy for use with a RUNX3 inhibitor includes an agent capable of degrade hyaluronic acid (HA), such as hyaluronidases, a CD44 antagonist, or a combination thereof.

To practice the coordinate administration methods of this disclosure, a RUNX3 inhibitor is administered simultaneously or sequentially in a coordinated treatment protocol with one or more secondary or adjunctive therapies, such as surgery, chemotherapy, radiation therapy, chemoradiation therapy, or the like. The coordinate administration may be done in either order, and there may be a time period while only one or both (or all) therapies, individually or collectively, exert their biological activities. A distinguishing aspect of all such coordinate treatment methods is that a RUNX3 inhibitor present in a composition elicits some favorable clinical response, which may or may not be in conjunction with a secondary clinical response provided by the secondary therapeutic agent. For example, the coordinate administration of a RUNX3 inhibitor with a secondary therapeutic agent as contemplated herein can yield an enhanced (e.g., synergistic) therapeutic response beyond the therapeutic response elicited by either or both a RUNX3 inhibitor or secondary therapeutic agent or procedure alone.

In further embodiments, provided are compositions for reducing the risk of or treating pancreatic cancer or metastatic pancreatic cancer that include an inhibitor of RUNX3 expression (such as small molecules, chemical entities, nucleic acid molecules, peptides or polypeptides) and a pharmaceutically acceptable carrier, diluent or excipient. In certain further embodiments, the composition contains a RUNX3 inhibitor that is a dsRNA (interfering RNA) or antisense RNA. In still further embodiments, the composition contains a

RUNX3 inhibitor that is an isolated nucleic acid molecule with the sequence set forth in SEQ ID NO.:4.

For the purposes of administration, the compounds of the present disclosure may be administered as a raw chemical or may be formulated as pharmaceutical compositions. Pharmaceutical compositions of the present disclosure may comprise a small molecule, chemical entity, nucleic acid molecule, peptide or polypeptide, and a pharmaceutically acceptable carrier, diluent or excipient. The small molecule, chemical entity, nucleic acid molecule, peptide or polypeptide composition will be in an amount that is effective to treat a particular disease or condition of interest - that is, in an amount sufficient for reducing the risk of or treating pancreatic cancer, metastases arising from the pancreatic cancer, a pancreatic cancer precursor lesion, a metastatic niche associated with pancreatic cancer or any of the other associated indication described herein, and preferably with acceptable toxicity to a patient. Compounds for use in the methods described herein can be determined by one skilled in the art, for example, as described in the Examples below. Appropriate concentrations and dosages can be readily determined by one skilled in the art.

Administration of the compounds of this disclosure, or their pharmaceutically acceptable salts, in pure form or in an appropriate pharmaceutical composition, can be carried out using any mode of administration for agents serving similar utilities. The pharmaceutical compositions of this disclosure can be prepared by combining a compound of this disclosure with an appropriate pharmaceutically acceptable carrier, diluent or excipient, and may be formulated into preparations in solid, semi-solid, liquid or gaseous forms, such as tablets, capsules, powders, granules, ointments, solutions, suppositories, injections, inhalants, gels, microspheres, and aerosols. Exemplary routes of administering such pharmaceutical compositions include oral, topical, transdermal, inhalation, parenteral, sublingual, buccal, rectal, vaginal, and intranasal.

The term "parenteral" as used herein includes subcutaneous injections, intravenous, intramuscular, intrasternal injection or infusion techniques. Pharmaceutical compositions of this disclosure are formulated to allow the active ingredients contained therein to be bioavailable upon administration of the composition to a patient. Compositions that will be administered to a subject or patient take the form of one or more dosage units, where for example, a tablet may be a single dosage unit, and a container of a compound of this disclosure in aerosol form may hold a plurality of dosage units. Actual methods of preparing such dosage forms are known, or will be apparent, to those skilled in this art (see, *e.g.*, *Remington: The Science and Practice of Pharmacy*, 22nd Edition (Pharmaceutical Press, 2012). The composition to be administered will, in any event, contain a therapeutically effective amount of a compound of this disclosure, or a pharmaceutically acceptable salt thereof, for reducing the risk of or treating pancreatic cancer, metastases arising from the pancreatic cancer, a pancreatic cancer precursor lesion, a metastatic niche associated with pancreatic cancer or other condition of interest in accordance with the teachings of this disclosure.

As a solid composition for oral administration, the pharmaceutical composition may be formulated into a powder, granule, compressed tablet, pill, capsule, chewing gum, wafer or the like form. Exemplary solid compositions can contain one or more inert diluents or edible carriers. In addition, one or more additives may be present, including binders such as carboxymethylcellulose, ethyl cellulose, microcrystalline cellulose, gum tragacanth or gelatin; excipients such as starch, lactose or dextrins, disintegrating agents such as alginic acid, sodium alginate, Primogel, corn starch and the like; lubricants such as magnesium stearate or Sterotex; glidants such as colloidal silicon dioxide; sweetening agents such as sucrose or saccharin; a flavoring agent such as peppermint, methyl salicylate or orange flavoring; or a coloring agent. When a pharmaceutical composition is in the form of a capsule, such as a gelatin capsule, it may contain, in addition to

materials of the above type, a liquid carrier such as polyethylene glycol or oil or combinations thereof.

The pharmaceutical composition may be in the form of a liquid, such as an elixir, syrup, solution, emulsion, or suspension. In certain embodiments, a liquid composition may be formulated for oral administration or for delivery by injection, as two examples. When intended for oral administration, exemplary compositions may further contain, in addition to one or more compounds of this disclosure, a sweetening agent, preservative, dye/colorant, flavor enhancer, or any combination thereof. Exemplary compositions intended for administration by injection may further contain a surfactant, preservative, wetting agent, dispersing agent, suspending agent, buffer, stabilizer, isotonic agent, or any combination thereof.

Liquid pharmaceutical compositions of this disclosure, whether they are solutions, suspensions or other like forms, may further comprise adjuvants, including sterile diluents such as water for injection, saline solution, preferably physiological saline, Ringer's solution, isotonic sodium chloride, fixed oils such as synthetic mono or diglycerides which may serve as the solvent or suspending medium, polyethylene glycols, glycerin, propylene glycol or other solvents; antibacterial agents such as benzyl alcohol or methyl paraben; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid (EDTA); buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic. Physiological saline is a preferred adjuvant. An injectable pharmaceutical composition is preferably sterile.

A pharmaceutical composition of this disclosure may be intended for topical administration, in which case the carrier may comprise a suitable solution, emulsion, ointment, gel base, or any combination thereof. The base, for example, may comprise petrolatum, lanolin, polyethylene glycols, bee wax,

mineral oil, diluents such as water and alcohol, emulsifiers, stabilizers, or any combination thereof. Thickening agents may be present in a pharmaceutical composition of this disclosure for topical administration. If intended for transdermal administration, the composition may include a transdermal patch or
5 iontophoresis device.

A pharmaceutical composition of this disclosure may be intended for rectal administration, in the form, for example, of a suppository, which will melt in the rectum and release the active compound(s). A composition for rectal administration may contain an oleaginous base as a suitable nonirritating
10 excipient. Exemplary bases include lanolin, cocoa butter, polyethylene glycol, or any combination thereof.

A pharmaceutical composition of this disclosure may include various materials that modify the physical form of a solid or liquid dosage unit. For example, a composition may include materials that form a coating shell around
15 the active ingredient(s). Exemplary materials for forming a coating shell may be inert, such as sugar, shellac, or other enteric coating agents. Alternatively, active ingredient(s) may be encased in a gelatin capsule.

In certain embodiments, compounds and compositions of this disclosure may be in the form of a solid or liquid. Exemplary solid or liquid formulations
20 include semi-solid, semi-liquid, suspension, and gel forms. A pharmaceutical composition of this disclosure in solid or liquid form may further include an agent that binds to the compound of this disclosure and thereby assists in the delivery of the compound. Suitable agents that may act in this capacity include a monoclonal or polyclonal antibody, a protein, or a liposome.

25 A pharmaceutical composition of this disclosure may consist of dosage units that can be administered as an aerosol. The term aerosol is used to denote a variety of systems ranging from those of colloidal nature to systems consisting of pressurized packages. Delivery may be by a liquefied or compressed gas or by a suitable pump system that dispenses the active
30 ingredients. Aerosols of compounds of this disclosure may be delivered in

single phase, bi-phasic, or tri-phasic systems in order to deliver the active ingredient(s). Delivery of the aerosol includes the necessary container, activators, valves, subcontainers, and the like, which together may form a kit.

Pharmaceutical compositions of this disclosure may be prepared by methodology well known in the pharmaceutical art. For example, a pharmaceutical composition intended to be administered by injection can be prepared by combining a compound of this disclosure with sterile, distilled water to form a solution. A surfactant may be added to facilitate the formation of a homogeneous solution or suspension. Surfactants are compounds that non-covalently interact with the compound of this disclosure to facilitate dissolution or homogeneous suspension of a compound in an aqueous delivery system.

Compounds of this disclosure, or their pharmaceutically acceptable salts, are administered in a therapeutically effective amount, which will vary depending upon a variety of factors including the activity of the specific compound employed; the metabolic stability and length of action of the compound; the age, body weight, general health, sex, and diet of the patient; the mode and time of administration; the rate of excretion; the drug combination; the severity of the particular disorder or condition; and the subject undergoing therapy. Following administration of RUNX3 inhibitors according to the formulations and methods of this disclosure, test subjects will exhibit about a 10% up to about a 99% reduction in one or more symptoms associated with the disease or disorder being treated (e.g., pancreas cancer), as compared to placebo-treated or other suitable control subjects.

Compounds of this disclosure, or pharmaceutically acceptable derivatives thereof, may also be administered simultaneously with, prior to, or after administration of one or more other therapeutic agents. Such combination therapy includes administration of a single pharmaceutical dosage formulation which contains a compound of this disclosure and one or more additional active agents, as well as administration of the compound of this disclosure and each

active agent in its own separate pharmaceutical dosage formulation. For example, a compound of this disclosure and the other active agent can be administered to the patient together in a single oral dosage composition such as a tablet or capsule, or each agent administered in separate oral dosage formulations. Where separate dosage formulations are used, the compounds of this disclosure and one or more additional active agents can be administered at essentially the same time, *i.e.*, concurrently, or at separately staggered times, *i.e.*, sequentially; combination therapy is understood to include all these regimens.

It will also be appreciated by those skilled in the art that in the process described herein the functional groups of intermediate compounds may need to be protected by suitable protecting groups. Such functional groups include hydroxy, amino, mercapto, and carboxylic acid. Suitable protecting groups for hydroxy include trialkylsilyl or diarylalkylsilyl (for example, *t*-butyldimethylsilyl, *t*-butyldiphenylsilyl or trimethylsilyl), tetrahydropyranyl, benzyl, and the like. Suitable protecting groups for amino, amidino and guanidino include *t*-butoxycarbonyl, benzyloxycarbonyl, or the like. Suitable protecting groups for mercapto include -C(O)-R" (where R" is alkyl, aryl or arylalkyl), *p*-methoxybenzyl, trityl or the like. Suitable protecting groups for carboxylic acid include alkyl, aryl or arylalkyl esters. Protecting groups may be added or removed in accordance with standard techniques, which are known to one skilled in the art and as described herein. The use of protecting groups is described in detail in Green, T.W. and P.G.M. Wutz, *Protective Groups in Organic Synthesis* (1999), 3rd Ed., Wiley. As one of skill in the art would appreciate, the protecting group may also be a polymer resin such as a Wang resin, Rink resin or a 2-chlorotrityl-chloride resin.

It will also be appreciated by those of skill in the art, although such protected derivatives of compounds of this disclosure may not possess pharmacological activity as such, they may be administered to a mammal and thereafter metabolized in the body to form compounds of this disclosure which

are pharmacologically active. Such derivatives may, therefore, be described as “prodrugs”. In certain embodiments, compounds of this disclosure are in the form of a prodrug.

Furthermore, all compounds of this disclosure that exist in free base or
5 acid form can be converted to their pharmaceutically acceptable salts by treatment with the appropriate inorganic or organic base or acid by methods known to those skilled in the art. Salts of the compounds of this disclosure can be converted to their free base or acid form by standard techniques.

EXAMPLES

EXAMPLE 1

TARGETING CONDITIONAL ALLELES TO THE MURINE PANCREAS

Conditional *Trp53*^{LSL-R172H/+} (Olive *et al.*, *Cell* 119:847, 2004; Hingorani *et al.*, 2005), *Dpc4*^{flox/+} (Yang *et al.*, *Genesis* 32:80, 2002), *Kras*^{LSL-G12D/+} (Hingorani *et al.*, *Cancer Cell* 4:437, 2003; Hingorani *et al.*, 2005; Izeradjene *et al.*, *Cancer Cell* 11:229, 2007), and *p48*^{Cre/+} (Kawaguchi *et al.*, *Nat. Gen.* 32:128, 2002; Izeradjene *et al.*, 2007) strains were interbred to obtain various multiple mutant animals (Figure 1A). In particular, the following transgenic mice, along with various littermate control animals, on a mixed BISwiss/129/SvJae/C57Bl/6 background were generated: (1) *Kras*^{LSL-G12D/+}::*Trp53*^{LSL-R172H/+}::*p48*^{Cre/+} (KP); (2) *Kras*^{LSL-G12D/+}::*Trp53*^{LSL-R172H/+}::*Dpc4*^{flox/+}::*p48*^{Cre/+} (KPD); (3) *Trp53*^{LSL-R172H/+}::*Dpc4*^{flox/+}::*p48*^{Cre/+} (PD); (4) *Kras*^{LSL-G12D/+}::*Dpc4*^{flox/+}::*p48*^{Cre/+} (KD), and (5) *Kras*^{LSL-G12D/+}::*Dpc4*^{flox/flox}::*p48*^{Cre/+} (KDD). Nude mice (Athymic NCr-nu/nu, NCI Frederick USA) and NOD.SCID/NCr mice (NOD.SCID Core, FHCRC) were used in pulmonary metastasis assays.

When Cre recombinase is expressed, exon 8 of the floxed *Smad4/Dpc4* allele results in a frame-shift, rapid degradation of the transcript, and no evidence of SMAD4/DPC4 protein expression (Izeradjene *et al.*, 2007). For *Trp53*^{R172H} and *Kras*^{G12D}, the Lox-stop-Lox (LSL) transcription stop sequence is deleted in the presence of Cre recombinase, which leads to expression of the mutant genes.

Histology and Immunohistochemistry

Tissues were fixed in 10% neutral buffered formalin, paraffin embedded and 5 µm sections were prepared. Routine H&E and immunostaining was done as previously described (Hingorani *et al.*, 2003). Biotinylated secondary antibody and the Elite Vectastain® kit (Vector Lab, CA, USA) were used for

signal detection, while DAB was used as a chromogen substrate. Primary antibodies used were insulin (1:200, Dako, CA, USA), amylase (1:800, Sigma, Chicago, IL, USA), and cytokeratin-19 (CK-19) (Phenopath Laboratories, WA, USA).

- 5 Activation of point-mutant *p53* expression and concomitant heterozygous deletion of *Smad4/Dpc4* in the absence of oncogenic *Kras*^{G12D} (*i.e.*, PD mice) did not affect normal pancreatic development or function. PD animals developed and aged normally, while parenchymal architecture, as well as the synthetic functions of the exocrine and endocrine compartments of the gland,
10 were unperturbed by the genetic manipulations (Figure 2).

EXAMPLE 2

EFFECT OF TRP53 MUTANT WITH DPC4 DELETION ON PANIN-TO-PDA PROGRESSION

- The effect of concomitant *p53* and *Dpc4* mutation on pancreatic neoplasias initiated by oncogenic *Kras* was investigated to determine whether
15 the histological progression reverted back from the cystic MCN-to-PDA route to the classical PanIN-to-PDA route.

- KPD quadruple mutant animals were aged until moribund, and the resulting cancers subjected to comprehensive analyses of gross pathology, histopathology, cellular behaviors and molecular properties. The significance of
20 the data was determined by the Student's *t*-test (two-tailed) for all *in vitro* studies. A value of $p < 0.05$ was deemed significant. Fisher's exact *t*-test was performed for comparison of metastatic disease burdens in *KP* vs. *KPD* cohorts. All statistical analyses were done using GraphPad Prism 5.0 software.

- Belying its role as a tumor suppressor gene (TSG), concomitant
25 heterozygous mutation of *Dpc4* did not hasten demise when compared with *KP* mice; both *KPD* and *KP* mice had a median survival of approximately 5 months (Figure 1B). Also, in contradistinction to the macroscopic cystic lesions of the pancreatic body and tail that predominate in *KD* and *KDD* animals (Izeradjene *et al.*, 2007), *KPD* animals develop solid, multinodular tumors of the gland

similar to those that develop in *KP* mice (see Figures 3A and 3B).

Unexpectedly, the primary tumors in *KPD* animals were larger, in both size and mass, and occupied more of the organ than *KP* PDAs (Figures 3C and 3D).

The histologic progression in the ducts followed the classical PanIN-to-PDA sequence and not the cystic MCN-to-PDA route. Early grade PanIN lesions were seen in young animals (Figures 3E – 3G), which subsequently progressed to higher grade PanINs (Figure 3H) and then to invasive PDA (Figures 3I and 3J). Strong expression of CK-19 (Figure 3F), a epithelial cell marker, confirmed the ductal epithelial nature of these neoplasms, and alcian blue staining revealed abundant cytoplasmic mucin (Figure 3G). The histologic morphology was predominantly glandular (see Figure 3I and Table 1), although other less common histologies also seen in human PDAs, including anaplastic and sarcomatoid, could also be found and were similarly rare in the murine tumors (Figure 3J).

Overall, concomitant mutation of *p53* prevents heterozygous deleted *Dpc4* from shifting the differentiation state of PanIN lesions initiated by oncogenic *Kras* toward an MCN phenotype.

KPD animals revealed a further dramatic and unexpected finding – an almost complete absence of metastatic disease. *KP* animals, by comparison, develop highly metastatic disease to organ sites and with frequencies that faithfully mimic the patterns seen in the majority of patients. Indeed, the heavy macroscopic tumor burden in *KP* mice, principally to the liver and lungs, contributes significantly to their demise. Liver metastases occur in 63% of *KP* animals and lung metastases in 44%; overall, fully 74% of *KP* animals develop metastatic disease (Hingorani *et al.*, 2005). In stark contrast, only 3 of 37 (8.1%) *KPD* animals developed macroscopically evident metastases and an additional 5 (13.5%) had microscopic metastases only (Table 1; $p=0.0001$ for the difference between the two genotypes). In the few cases of disseminated disease in *KPD* animals, metastases were seen only in the liver and never in

more distant organs (Table 1), and they did not contribute substantively to overall disease burden.

Table 1. Clinical Spectrum of Disease in *KPD* Mice

ID	Age	PDA	Histology		Metastases		Ascites	Cachexia
			1°	2°	Liver	Lung		
1	151	Y	G		N	N	Y	*
2	169	Y	G		N	N	Y	Y
3	165	Y	G		N	N	N	Y
4	329	Y	G	U	Y ^M	N	N	N
5	120	Y	G	U	N	N	*	*
6	125	Y	G		N	N	Y	Y
7	183	*	*	*	*	*	*	*
8	74	*	*	*	*	*	*	*
9	172	Y	G	U	Y ^M	N	N	Y
10	193	Y	G	C ^m	N	N	Y	Y
11	109	Y	G		N	N	N	N
12	180	Y	G	U	Y ^m	N	Y	Y
13	95	Y	G	U	Y ^m	N	N	Y
14	76	Y	G		N	N	Y	N
15	66	Y	G		N	N	N	Y
16	102	Y	N		N	N	N	N
17	190	Y	G		Y ^m	N	Y	N
18	211	Y	G	U	N	N	N	N
19	141	*	*	*	*	*	*	*
20	98	Y	G		N	N	N	Y
21	73	Y	G	C ^m	Y ^m	N	N	Y
22	60	Y	G		N	N	N	Y
23	77	*	*	*	*	*	*	*
24	88	Y	U		N	N	N	N
25	172	Y	G	C ^m ; U	Y ^m	N	Y	Y
26	108	Y ¹	G		N	N	N	N

ID	Age	PDA	Histology		Metastases		Ascites	Cachexia
			1°	2°	Liver	Lung		
27	178	Y	G		N	N	N	N
28	92	Y	G	U	Y ^M	N	Y	N
29	78	Y	G	U	N	N	N	N
30	200	Y	G		N	N	N	N
31	181	Y	G		N	N	N	N
32	162	Y	G		N	N	N	N
33	123	Y	G		N	N	N	N
34	167	Y	G		N	N	N	N
35	230	Y	G		N	N	N	N
36	160	*	*		*	*	*	*
37	165	*	*		*	*	*	*

Y, disease present; N, no disease detected; G, glandular; U, undifferentiated; ^M, macroscopic; ^m, microscopic; *, tissue not evaluable secondary to necrosis; ¹, focally invasive PDA but animal succumbed to thymoma; C, cystic

Thus, the evolution of pancreas cancer in *KPD* mice is characterized by a shift toward a significantly increased primary tumor burden, together with an almost complete absence of metastatic disease, in contradistinction to the *KP* mice, but with the overall result of essentially identical survival in these two models of pancreas cancer.

EXAMPLE 3

GENETIC PROGRESSION OF *KPD* PANCREAS CANCER

To characterize the molecular basis for the very distinct *in vivo* behavior of *KPD* PDAs, primary ductal carcinoma cells were purified from the tumors using previously described procedures (Hingorani *et al.*, 2005; Schreiber *et al.*, *Gastroenterology* 127:250, 2004). Pure populations of PDA cells were recovered after 3-4 passages on defined substrates in serum-free culture media. To ensure fidelity to the autochthonous cancers, all subsequent

molecular and cellular studies were performed on low passage number cells (less than 15 times for all assays) grown in DMEM/F12 supplemented with 10% FBS, 0.5% Glucose, and 1XPen/Strep.

To examine recombination events, high-quality total RNA was extracted
5 from cells (about 80% confluency) using TRIzol reagent (Invitrogen, CA, USA), treated with DNase (Applied Biosystems, CA, USA), and further purified using the RNeasy kit (Qiagen, Germany). RNA was quantified using the NanoDrop. For cDNA synthesis, 2 µg total RNA was reverse transcribed using RT² first strand kit (SA Biosciences).

10 The isolated PDA cells were examined for recombination and activation of the conditional *Kras*^{LSL-G12D} and *Trp53*^{LSL-R172H} alleles, as well as deletion of the *Dpc4* allele (Figures 3K–3M). Recombination of *Trp53*^{LSL-R172H} was invariably accompanied by spontaneous loss of the wild-type allele (LOH) (Figure 3L), as typically occurs in human PDAs with point mutations in one
15 *TP53* allele (Hingorani *et al.*, 2005). Interestingly, invasive *KPD* PDA cells did not undergo LOH at the *Dpc4* locus and instead retained the remaining wild-type allele.

For immunoblot analysis, whole cell lysates were prepared using 1X RIPA buffer (Millipore, CA, USA) supplemented with 1X protease inhibitor
20 (Sigma, MO, USA) and phosphatase inhibitor cocktails I and II (Sigma, MO, USA). For immunoblot experiments, 20 µg (murine) or 30 µg (human) whole cell protein lysate were separated on a 4-12% Bis-tris gel system (Invitrogen, CA, USA) and transferred to PVDF membrane (Millipore, CA, USA). Primary antibodies used were Dpc4, p16, p19, Actin (all at 1:2000, Santa Cruz, CA,
25 USA), and p53 (1:1000, Vector Lab, CA, USA).

These immunoblot analyses confirmed detectable levels of *Trp53* consistent with expression of the point-mutant allele as well as persistent, albeit decreased, expression of *Dpc4* (as compared with cells with both *Dpc4* alleles intact) (Figure 3N). Interestingly, *Cdkn2a/Ink4a* (p16) expression was
30 frequently lost, while expression of the contiguous *p19* allele was retained,

indicating specific promoter methylation rather than genomic deletion as the mechanism for silencing *p16*. The loss of *p16* is notable as prior studies of both *KP* (Hingorani *et al.*, 2005) and *Kras*^{LSL-G12D/+}::*p16/p19*^{-/-}::*Cre* (Bardeesy *et al.*, *Proc. Nat'l. Acad. Sci. U.S.A.* 103, 5947, 2006) animals found no additional loss of other major TSG during the course of disease progression, identifying non-overlapping mutational spectra in those two model systems. The increased selection pressure to lose *p16* in the setting of heterozygous *Dpc4* mutation further underscores the latter's behavior as a tumor "promoter" in this particular context.

- 10 The spontaneous progression of PDAs with *Kras*^{G12D}, *Trp53*^{R172H}, *Dpc4*^{+/-} and loss of *p16* also represents the first autochthonous model of pancreas cancer encompassing all four cardinal mutations seen in human PDA.

EXAMPLE 4

PROLIFERATION AND CELL CYCLE CONTROL IN PANCREAS TUMOR CELLS OF KPD MICE

- 15 The increased primary tumor size at the expense of metastatic disease seen in *KPD* compared with *KP* PDAs could potentially be due to increased proliferation, decreased apoptosis, or a combination of both. These different properties were examined in both *in vivo* resected tumors and *in vitro* purified primary ductal carcinoma cells.

- 20 For immunocytochemistry, the primary antibody used was Ki-67 (1:25, Dako, CA, USA), which is a marker of proliferating cells. For cell proliferation assays, equal numbers of murine or human cells (30,000) were plated in triplicate for each cell line and incubated in the presence or absence of TGFβ (5 ng/ml, R&D Systems, USA). Cells were counted manually at 24 hour intervals. Data points represent the mean ±SEM of triplicate determinations from at least 3 independent experiments. All experiments were conducted in triplicate at least 3 independent times on at least 2 different cell lines from each genetic background. Representative photographs were taken from a minimum of 6-8

separate high power fields. For all assays, media was replaced every 24 hours with or without TGF β and photographed every 24 hours.

Ki-67 expression was significantly increased in *KPD* versus *KP* tumors (40% vs. 20%, $p=0.0114$; Figures 4A, 4B and 4E). Apoptosis, however, was
5 similarly negligible in PDAs in both genetic contexts (Figures 4C and 4D). Primary *KPD* PDA cells in culture also had a higher proliferative rate than *KP* cells (Figure 4F), and once again very similar and low apoptotic rates (not shown). *KPD* cells were only partially growth-arrested by TGF β (Figure 4F).

Immunoblots were used to characterize the expression of several
10 additional critical cell cycle components and the primary antibodies used were CyclinD1, Actin, and GAPDH (all at 1:2000, Santa Cruz, CA, USA); p53 (1:1000, Vector Lab, CA, USA), ppRb⁷⁸⁰ (1:1000, Cell Signaling, MA, USA); and p21 (1:1000, AbCam, MA, USA). These data show that the higher proliferative index of *KPD* cells could be attributed to higher basal levels of cyclins D1
15 (Figure 4G) and E1 (data not shown), which bind to and activate Cdk4 and Cdk2, respectively; lower levels of p21 (Figure 4G); and correspondingly increased levels of phosphorylated Rb (Figure 4G), liberating E2F to promote cell cycle entry.

Overall, these data indicate that increased primary tumor size seen in
20 *KPD* compared with *KP* PDAs appears to be due to increased proliferation with no alteration in apoptosis levels.

EXAMPLE 5

EFFECT OF DPC4 MUTATION ON EMT AND MIGRATION OF KPD CELLS *IN VITRO*

A series of functional assays were performed to further explore the
25 almost complete absence of metastatic disease in *KPD* pancreas cancers. For all experiments, a single retained wild-type *Dpc4* allele and correspondingly reduced protein expression (*i.e.*, about 50% of WT levels) were confirmed in *KPD* cells prior to assay (Figure 3N and data not shown). The *in vitro* assays, while reflective of only some of the capabilities necessary for *in vivo* metastasis,

nevertheless underscore the profoundly distinct intracellular wiring and responses to external stimuli in *KP* and *KPD* cells.

First, *KPD* pancreas cancer cells were probed by immunofluorescence for their ability to undergo epithelial-to-mesenchymal transition (EMT), a
5 program of morphological and molecular changes implicated in the metastatic phenotype. Briefly, cells were grown on glass cover slips and treated for 72 hours in the presence or absence of TGF β (5 ng/ml); media was replaced every 24 hours. For E-cadherin immunofluorescence, cells were fixed in ice cold methanol:acetone (1:1) for 15 minutes. For F-actin IF, cells were fixed in 4%
10 paraformaldehyde for 30 minutes at RT followed by brief rinse in 0.15M glycine to quench the PFA. After fixation, the cells were washed with PBS and blocked for 1 hour at RT with 2% fatty acid-free BSA/2.5% goat serum. Cells were incubated with E-cadherin primary antibody (1:200, BD Biosciences) or Alexafluor488 F-actin (1:1000) for 1 hour at RT. Alexafluor 488 secondary
15 antibody was used to visualize E-cadherin antibody. Cells were counterstained with DAPI and the slips mounted with Prolong Gold Anti-fade (Invitrogen, CA, USA). Images were acquired using a Nikon Eclipse 80i microscope with NIS Elements Software (Nikon, v3.1).

KPD carcinoma cells did develop, if perhaps less abundantly, actin
20 stress fibers in response to TGF β (Figures 5A and 6A), while also down-regulating surface expression of E-cadherin, a member of the superfamily of Ca²⁺-dependent adhesion proteins that, in part, defines the epithelial phenotype (Figures 5B and 6B). Also, *KP* PDA cells readily underwent TGF β -induced EMT (Figures 5A, 5B, 6A and 6B). Hence, these data show that EMT is
25 essentially intact in *KPD* PDA cells.

In addition to examining EMT, carcinoma cells were examined for their ability to migrate. For migration assays, cells were first incubated in the presence or absence of TGF β (5 ng/ml) for 60 hours to achieve growth arrest (in order to separate out effects of proliferation rather than migration; see
30 Izeradjene *et al.*, 2007), then serum starved for 8 hours. Confluent cultures

were scratched with a 20 μ l pipette tip to generate a wound in three horizontal stripes per well; each experimental condition was performed in triplicate wells. All experiments were conducted in triplicate at least 3 independent times on at least 2 different cell lines from each genetic background. A minimum of six
5 points across each wound were assessed independently by two investigators at the indicated time intervals for the presence of cells within the expanse. Representative photographs were taken from a minimum of 6-8 separate high power fields. For all assays, media was replaced every 24 hours with or without TGF β and photographed every 24 hours.

10 Despite the qualitative ability to undergo EMT, the basal migration of *KPD* cells across an introduced wound in monolayer culture was significantly decreased compared with *KP* cells; moreover, the migration of *KP* cells was stimulated by TGF β , while *KPD* cells did not respond in kind (Figures 5C and 6C).

15 Again, both the baseline properties as well as the response to TGF β were very different in the presence or absence of heterozygous *Dpc4* mutation.

EXAMPLE 6

EFFECT OF DPC4 MUTATION ON EMT AND MIGRATION OF KPD CELLS *IN VIVO*

Purified *KP* and *KPD* carcinoma cells were also injected into the
20 circulation of immunocompromised mice and scored for their ability to form pulmonary metastases. Briefly, murine cells were studied in a classical tail vein metastasis assay in nude mice (Athymic NCr-nu/nu). At least 3 animals were injected (1×10^5 or 1×10^6 cells/injection, depending on the experiment) for each independently derived cell line and all experiments were performed at least
25 twice. Animal health was monitored daily and all study animals in a given cohort were sacrificed when any of the animals in that particular group showed signs of cachexia, lethargy, or distress. Metastatic disease burdens were assessed as a ratio of cross-sectional area occupied by tumor to whole lung area using ImageJ software (NIH).

KP cells formed numerous, large, pleural and parenchymal metastases leading to demise from respiratory failure. *KPD* cells, on the other hand, formed far fewer and smaller metastatic deposits in the lungs of animals that appeared clinically robust at comparable time points (Figure 6H, top panels).

- 5 These results parallel what was observed in the *in vitro* immunofluorescence assay of Example 5.

EXAMPLE 7

EFFECT OF LATE *DPC4* MUTATION ON METASTATIC CAPABILITY OF INVASIVE *KP* CARCINOMA CELLS

- 10 These experiments examine whether late mutation of *Dpc4* (*i.e.*, after the acquisition of an already invasive phenotype) would not inhibit the ability of PDA cells to metastasize by assessing the functional consequences of knockdown of *Dpc4* expression in *KP* invasive carcinoma cells (Figure 6D).

- Dpc4* expression was knocked down using a short hairpin RNA (shRNA) sequence (GATTAACACTGCAGAGTAA, SEQ ID NO.:1) targeting murine *DPC4* procured in a pGIPZ vector (Open Biosystems). A control sequence (scrambled, scr) was used as a negative control. High-titer lentivirus was made and PDA cells were infected and selected for stable expression of the *DPC4*-specific shRNA. Knockdown was confirmed by qRT-PCR. Briefly, total RNA
- 15 was reverse transcribed using the high capacity cDNA synthesis kit (Applied Biosystems, CA, USA) and qRT-PCR performed using SYBER green supermix (Bio-Rad, VA, USA) on a CFX-96 thermal cycler. PCR reactions included initial denaturation at 95°C for 5 minutes, followed by 40 cycles of incubation at 95°C for 30 seconds and 60°C for 1 minute. Melting curves were generated for each
- 20 reaction to exclude false positive signal from primer dimers. Murine CphA was used as an internal control (forward primer SEQ ID NO.:11, GAGCTGTTTGCAGACAAAGTTC; and reverse primer SEQ ID NO.:12, CCCTGGCACATGAATCCTGG). *DPC4* transcript levels in murine cell lines were analyzed by qRT-PCR using the Taq-man assay per the manufacturer's

recommendations (Applied Biosystems, CA, USA) with GAPHD as an internal control.

Tellingly, late ablation of *Dpc4* expression in *KP* PDA cells did not completely prevent EMT in response to TGF β . Some of the morphological changes associated with EMT (e.g., actin stress fiber formation) were still
5 evident (Figure 6E), although the cells did exhibit less of a tendency to separate from each other. Even after complete loss of *Dpc4* expression in *KPD* carcinoma cells some morphological features of EMT in response to TGF β also persisted (Figure 6F). More startlingly, not only did depletion of *Dpc4* in *KP*
10 cells not decrease their migration, but instead migration increased slightly, and TGF β still promoted this motility (Figures 5D and 6G). Complete ablation of *Dpc4* expression in *KPD* cells also increased their basal migratory behavior modestly, which was again further potentiated by TGF β (Figures 5D and 6G), perhaps awakening a previously dormant metastatic phenotype.

15 Finally, results from *in vivo* pulmonary metastasis assays were consistent with the *in vitro* migration data, revealing that complete knockdown of *Dpc4* in *KPD* cells enhanced their metastatic potential, and completely extinguishing *Dpc4* in *KP* cells did not diminish their already considerable metastatic potential (Figure 6H).

20 In view of the results of these *in vitro* migration and *in vivo* metastasis assays, the status of *Dpc4/Smad4* in paired primary and metastatic tumors in the very few *KPD* animals that had developed metastases was analyzed. Liver metastases of *KPD* animals showed loss of nuclear Dpc4 expression (Figures 5E and 5F), and examination of the respective primary tumors revealed focal
25 loss of Dpc4 (Figures 5G and 5H), indicating a potential source for the disseminated cells.

Thus, late loss of *Dpc4* in invasive *KP* carcinoma cells further unleashes its invasive and metastatic behavior, while completing the loss of *Dpc4* in cells that underwent heterozygous *Dpc4* deletion prior to the development of an

invasive phenotype (*i.e.*, as in *KPD* cells) bestows a newly acquired metastatic potential.

EXAMPLE 8

ROLE OF *RUNX3* IN PANCREAS CANCER METASTASIS

To further examine the dramatically different behaviors of *KP* and *KPD* carcinomas in otherwise nearly identical genetic contexts, comparative profiling was performed. Initially, the profiling was focused on the TGF β -Smad signaling pathway genes. Expression array analyses on purified *KP* and *KPD* primary carcinoma cells ($n = 3$ each) using a customized TGF β -signaling specific quantitative real time-PCR platform was performed. Array analyses with SYBR Green PCR Master Mix (SA Biosciences) were carried out according to the manufacturer's protocol on a Bio-Rad CFX-96 PCR System (Bio-Rad, VA, USA). Gene expression differences were determined using the $2^{-\Delta\Delta C_t}$ method following standard protocols (SA Biosciences). Human CPHA was used as an internal control (forward primer SEQ ID NO.:13, CCCACCGTGTTCCTTCGACATT; and reverse primer SEQ ID NO.:14, GGACCCGTATGCTTTAGGATGA).

There were 15 genes identified as being up-regulated at least 2-fold in *KP* cells as compared to *KPD* cells, which might represent metastasis-promoting genes (Table 2). As expected, *Smad4* expression was approximately 2-fold higher in *KP* than *KPD* cells. Completely distinguishing itself, however, was *Runx3*, which was up-regulated 36-fold in *KP* cells (Table 2 and Figure 7A).

Table 2. TGF β Pathway Array

**Analysis of *KP* vs. *KPD* PDA
Cells***

Gene	Fold Change
Runx3	36.0541

Gene	Fold Change
Dlx2	7.4322
Tgfb1	4.6685
Id1	3.1935
Itgb5	3.1581
Ltbp2	3.1563
Ltbp1	3.0789
Bmper	2.997
Tgfb1i1	2.9623
Itgb7	2.6516
Bmp1	2.4641
Smad4	2.3829
Tgfb3	2.3613
Smad5	2.1156
Bmpr1a	2.0592

* Genes upregulated at least 2-fold in *KP* compared to *KPD* carcinoma cells. Values are expressed as fold change ($n = 3$ each).

- 5 To further define the role of murine *Runx3* in the biological behavior of PDA, qRT-PCR (forward primer SEQ ID NO.:17, AGAGTTTCACGCTCACAATC; and reverse primer SEQ ID NO.:18, GGAGAAGGGGTTTCAGGTC) was used established that transcript levels correlated with metastatic potential in a larger series of primary cell lines. *KP*
- 10 PDA cells had approximately 20-fold higher *Runx3* expression than *KPD* cells by quantitative PCR (200-fold and 10-fold respectively, relative to WT ductal cells; $n = 3$ each, Figure 8A). Transcript levels corresponded to protein levels (Figure 8B).

- As a transcription factor, the subcellular localization of *Runx3* is also of
- 15 functional consequence, so isolated cytoplasmic and nuclear fractions from *KP* and *KPD* cells were examined. Nuclear and cytoplasmic fractions of murine PDA cell lines were prepared using the nuclear cytoplasmic kit from Active Motif

(CA, USA). Approximately 20 µg of nuclear and cytoplasmic cell fractions were loaded on 4-12% Bis-Tris gel and probed with mouse Runx3 (1:500, GenScript, NJ, USA). Also used were Parp (1:1000) and Gapdh (1:2000, both from Santa Cruz Biotechnology, CA, USA, which were used as nuclear and cytoplasmic
5 markers, respectively.

The data show that both types of carcinoma cells express Runx3 in the nuclear compartment (Figure 7B), albeit at different levels, consistent with the qRT-PCR and immunoblot data. Specific immunohistochemistry for Runx3 revealed no discernible expression in normal ducts and acini, while islets
10 showed predominately cytoplasmic Runx3 staining (Figure 8C). *KP* tumors (Figures 8D – 8F) had moderate to strong nuclear Runx3 staining, whereas *KPD* tumors (Figures 8G and 8H) showed very weak expression at best. Preinvasive lesions adjacent to *KP* carcinomas were also negative for Runx3 expression (Figure 8D). Both *KP* and *KPD* tumors showed strong nuclear
15 staining in infiltrating lymphocytes, a common site for elevated Runx3 and a useful internal control (Yarmus *et al.*, *Proc. Nat'l. Acad. Sci. U.S.A.* 103:7384, 2006).

To further investigate its effects on metastatic potential, *Runx3* was stably over-expressed in two different primary *KPD* carcinoma cell lines (Figure
20 7C). Briefly, full length mouse *Runx3* was obtained from Open Biosystems (Huntsville, AL, USA) and subcloned into a pMSCV Puro IRES GFP vector (Addgene, MA, USA) to generate a *Flag-Runx3* construct. Fidelity of the cloned genes was confirmed by sequencing. High-titer retrovirus was made in HEK293T cells using a standard protocol (Addgene, MA, USA). Cells were
25 selected with the appropriate antibiotic for at least 5 days or until all uninfected cells were dead. Transgene expression was confirmed by immunoblot using Flag tag-specific and Runx3-specific antibodies.

Runx3 overexpression greatly increased the basal migration of *KPD* cells (Figure 8I), which was also less sensitive to attenuation by TGFβ, behaviors
30 more reminiscent of *KP* cells than basal *KPD* cells. *KPD-Runx3* cells also

formed significantly increased numbers of pulmonary metastases in immunocompromised mice, a property virtually absent from control transfected *KPD* cells and again more like *KP* cells (Figures 8J and 8K).

EXAMPLE 9

5

REGULATION OF *RUNX3* LEVELS

The following experiments were performed to investigate the potential mechanisms that regulate basal levels of *Runx3*. Analysis of the promoter region of the murine *Runx3* locus revealed five consensus-binding sites for *Runx3* itself clustered into two groups (Figure 7D). An electrophoretic mobility shift assay (EMSA) was performed using nuclear and cytoplasmic protein fractions extracted according to manufacturer's protocol (Active Motif, CA, USA) and protein concentrations determined by Bradford assay (Bio-Rad, VA, USA). Complimentary oligonucleotide sequences were designed to span two putative consensus *Runx3* binding sites – (1) mRunx3EMSAF1:

15 GGTCCCTCAACCACAGAACCACAAGGCCAGGCCCT (SEQ ID NO.:2) and (2) mRunx3EMSA complement1: CCAGGGAGTTGGTGTCTTGGTGTTCGGTCCGGGA (SEQ ID NO.:3). The oligonucleotides were end-labeled with Biotin 3' end-labeling kit (Thermo Scientific, IL, USA). Nuclear protein extracts (10 µg) were incubated with

20 labeled probe +/- 4-fold excess unlabeled probe for 20 minutes at room temperature and separated on 7.5% nondenaturing polyacrylamide gels. Resolved protein-DNA complexes were transferred to nylon membrane and processed for detection using Light Chemiluminescent EMSA kit (Thermo Scientific, IL, USA).

25 The EMSA showed that nuclear extracts from *KP* carcinoma cells bound the probes specifically (Figure 8L). To determine if these *Runx3* sites were functionally responsive to *Runx3*, a full length murine *Runx3* promoter (containing 5 putative *Runx3* binding sites) and a truncated version lacking *Runx3* binding sites were each cloned into a pGL2-luciferase expression vector

(Promega, WI, USA) to perform a luciferase reporter assay. Full length or truncated *Runx3* promoter (0.1 µg) constructs were co-transfected with increasing concentrations of *Flag-Runx3* along with 0.05 µg of the β-gal plasmid into HEK293T cells. Cell lysates were prepared at 48 hours in Reporter Lysis Buffer (Promega WI, USA), substrate added and luciferase activity recorded on a luminometer (Veritas, WI, USA). β-gal activity was used to normalize the transfection efficiency. Results represent means ± SEM of triplicate wells from three independent experiments.

The luciferase assay showed that increased levels of Runx3 led to increased expression of the luciferase reporter (Figure 8M). These data indicate that Runx3 binds to its promoter and increases its own transcription through a positive feedback mechanism.

EXAMPLE 10

REGULATION OF RUNX3 LEVELS AT THE TRANSCRIPTIONAL LEVEL

As transducers of TGFβ signaling, the Runx family of transcription factors is known to interact with R-Smads. Interestingly, the *Runx3* promoter region also contains two canonical Smad binding elements (Figure 7D), indicating potential transcriptional control by R-Smads as well. *In silico* analyses of promoter regions of various genes for putative Runx3 binding sites and SBE (SMAD binding elements) were performed with publically available software (see, e.g., www.cbrc.jp/research/db/TFSEARCH.html) and regions up to -1200 bp upstream of the transcription start site (+1TSS) were examined.

To examine whether the transcriptional activity of Runx3 was exercised in complex with Smad4/Dpc4, immunoprecipitation assays were performed. Briefly, cells were lysed by sonication in 20 mM HEPES (pH 7.5), 400 mM KCl, 5% Glycerol, 5 mM EDTA, 0.4% Np40, supplemented with 1X protease inhibitor (Sigma, MO, USA), phosphatase inhibitor cocktails I and II (Sigma, MO, USA), and the lysates cleared by centrifugation. Extracts were diluted to 20 mM HEPES (pH 7.8), 50 mM KCl, 5% Glycerol, 2.5 mM MgCl₂, 0.05% NP40 and

incubated with Smad2 antibody (2.5 µg/ml from AbCam, Cambridge MA, USA) and Protein A-Sepharose beads for 4 hours at 4°C. Beads were washed and co-purified proteins liberated in boiling Laemmli buffer and analyzed by immunoblot with the appropriate primary antibody. ExactaCruz secondary
5 antibodies (Santa Cruz biotechnology) were used to reduce background from IgG.

The immunoprecipitation data show that Runx3 was co-immunoprecipitated in complexes with Smad2 and Smad4 and, furthermore, more complex formation occurred in *KP* cells as compared to *KPD* cells (Figure
10 7E). Moreover, TGFβ appeared to favor complex formation in *KP* cells and inhibit it in *KPD* cells, echoing its opposing effects on their respective cell behaviors. In addition, exogenous over-expression of *Smad4* increased Runx3 levels in *KPD* cells (Figure 8N), while targeted depletion of *Smad4* (using SEQ ID NO.:1) had the converse effect (Figure 8O).

15 In sum, Runx3 appears to control its own transcription in complexes with Smad4.

EXAMPLE 11

REGULATION OF RUNX3 LEVELS AT THE POST-TRANSLATIONAL LEVEL

A positive feedback loop may mean that a certain threshold of activity
20 would be needed for Runx3 transcriptional activation to result in rapid amplification of the system; thus, the resting levels of the *Runx3* protein might be subject to multiple levels of control. Indeed, both histone deacetylases (Hdac) and ubiquitin-coupled degradation have been implicated in a concerted mechanism for regulation of the Runx family of proteins – Hdacs remove acetyl
25 groups from critical lysine residues on Runx proteins exposing them to Mdm2-mediated ubiquitination and targeted degradation (Bae and Lee, *Gene* 366:58, 2006; Chi *et al.*, *Cancer Res.* 69:8111, 2009). The class II Hdacs (Hdac-4 and -5), specifically, have been shown in other contexts to be most important for regulating Runx3 (Jin *et al.*, *J. Biol. Chem.* 279:29409, 2004).

Quantitative RT-PCR showed that expression of murine *Hdac5* (forward primer SEQ ID NO.:15, AGCCCACCACGTTCTTTG; and reverse primer SEQ ID NO.:16, GTCATCACGGCTGTCATAGGG) was approximately 3-fold higher in *KPD* carcinoma cells ($n=3$) than *KP* cells ($n=3$) (Figure 7F). In addition,
5 resting levels of Mdm2 protein (detected with primary antibody at 1:2000, Santa Cruz, CA, USA) were significantly higher in *KPD* than in *KP* cells (Figure 7G).

To directly test of this potential involvement of Hdac5 in the post-translational control mechanism of Runx3, a histone deacetylase inhibitor was used to determine whether Runx3 levels increased when deacetylation of
10 Runx3 by Hdac5 was minimized or abrogated (*i.e.*, whether the acetylation level of Runx3 does in fact affect physiologic degradation). Briefly, cells (4×10^5) were plated in 60cc dishes and trichostatin A (TSA) (Tocris Biosciences, MO, USA), an Hdac5 inhibitor, was added at 0, 10, or 40 μ M at 24 hours. Total RNA or whole cell lysates were prepared as described herein after 48 hours of
15 treatment, and the latter resolved on 4-12% Bis-Tris gels, transferred to PVDF and probed with Runx3 antibody.

Treatment of *KPD* carcinoma cells with TSA did indeed raise levels of Runx3 to approximately those found in *KP* cells (Figure 7H).

Thus, a plausible mechanism for setting Runx3 levels in epithelial cells
20 may involve autologous transcriptional control, as well as post-translational modifications and degradation. The elevated basal levels of Hdac5 and Mdm2 in *KPD* cells, together with decreased levels of Smad4 available to form activating transcriptional complexes, probably helps maintain Runx3 below a critical set-point, but decreasing the levels or activities of either enzyme
25 (ubiquitin ligase or deacetylase) would probably allow Runx3 levels to rise above this threshold where it could rapidly amplify its effects by increasing its own transcription.

EXAMPLE 12

ROLE OF *RUNX3* IN HUMAN PANCREAS CANCER CELLS

To investigate the role of *RUNX3* in human pancreas cancer cells, *RUNX3* protein levels were assessed in a panel of well-characterized human pancreatic cancer cell lines, which were compared to non-transformed human pancreatic ductal epithelial (HPDE) cells in a qRT-PCR assay (Figure 9A). From this series, the lowest (MiaPaCa-2) and two of the highest (CFPAC1 and Panc-1) *RUNX3*-expressing lines were chosen for further study. The primary antibody used to detect human *RUNX3* was R35G4 (1:500, AbCam, MA, USA).

As an initial analysis, the human pancreas cancer cell lines were tested in the scratch wound migration assay described herein. The basal levels of *RUNX3* in these human PDA cell lines correlated with their migratory potential, as MiaPaCa-2 cells migrated far less well than CFPAC-1 and Panc-1 cells (Figure 9B). Moreover, the respective responses of these human cell lines to TGF β also followed the pattern seen with murine PDA cells. That is, TGF β modestly inhibited the migration of MiaPaCa-2 cells, as it similarly did for low *Runx3*-expressing *KPD* cells, whereas it promoted or left unaffected the high basal migration of CFPAC1 and Panc-1 cells (Figure 9B), similar to high-*Runx3* expressing *KP* cells.

Human *RUNX3* was cloned into pBABE hygromycin vector and stable *RUNX3* expressing HEK293T cells were prepared using a similar protocol to that used to make the murine version. Also, shRNA sequences targeting human *RUNX3* to knock down expression were procured in pGIPZ vectors (Open Biosystems). High-titer lentivirus was made and PDA cells were infected and selected for stable expression of the *RUNX3*-specific shRNA (SEQ ID NO.:4, CGGCAGAAGCTGGAGGACC). Knockdown was confirmed by qRT-PCR.

Overexpression of human *RUNX3* in MiaPaCa-2 cells greatly increased their migration (Figures 9C and 10A). Conversely, depletion of *RUNX3* in

CFPAC1 and Panc-1 cells inhibited their migration (Figures 9D, 9E, 10B and 10C).

To determine whether overexpression of *RUNX3* in human PDA cells also promotes metastasis, pulmonary metastasis assays were performed using NOD/SCID mice. MiaPaCa-2 cells overexpressing *RUNX3* showed a significantly greater metastatic burden compared to cells transfected with control vector (Figures 9F). Similarly, Panc-1 cells, with and without targeted depletion of *RUNX3*, were found to have their overall metastatic potential decreased considerably as compared to control cells. Interestingly, both control and *RUNX3*-knockdown Panc-1 cells produced a comparable number of lung metastases (Figure 9G); however, the ability of Panc-1 cells to seed and support secondary metastases to the liver was considerably decreased by *RUNX3* depletion (Figure 9H), which was reflected in the overtly distinct gross pathology and significantly different liver weights between the two groups: 4.3 +/- 0.6g ($n = 4$) vs. 0.53 +/- 0.06g ($n = 3$), for control and knockdown cells, respectively.

Thus, the *RUNX3*-dependent metastatic drive and responses to TGF β observed in human PDA cells parallels the phenotypes and behaviors seen in the murine counterpart PDA cells.

20

EXAMPLE 13

ROLE OF *RUNX3* IN ESTABLISHMENT OF A METASTATIC NICHE

The inability of *KPD* cells to efficiently seed metastases even after direct introduction into the bloodstream indicated that they may differ from *KP* cells in more than just the ability to migrate. Of interest was how the tumor epithelium refashions its microenvironment, including the nascent metastatic niche, not only to resist systemic therapy (Provenzano *et al.*, *Cancer Cell* 21:418, 2012) but to also support growth, invasion, and disease dissemination. Accordingly, focused array profiling for ECM genes comparing the highly metastatic *KP* and non-metastatic *KPD* carcinoma cells was performed.

A survey of the top differentially expressed genes revealed a number of genes previously associated with critical ECM functions involved in local invasiveness and modeling of the metastatic niche (Table 3), including matrix metalloproteinases such as MMP2, 10 and 11 (Krantz *et al.*, *Mol. Cancer Res.* 9:1294, 2011); Timps (Schelter *et al.*, *Clin. Exp. Metastasis* 28:793, 2011); Versican (Kim *et al.*, *Nature* 457:102, 2009; Said *et al.*, *J. Clin. Invest.* 122:1503, 2012); Spp1 (Ding *et al.*, *Nature* 470:269, 2011; Dudley *et al.*, *Cancer Cell* 14:201, 2008); and Sparc (Bradshaw, *J. Cell. Commun. Signal.* 3:239, 2009).

10

**Table 3. ECM Focused PCR
Array in KP vs. KPD PDA**

Cells*	
Gene	Fold Change
Col6a1	12.5194
Sparc	8.3402
Vcan	7.6945
Spp1	7.1535
Timp3	6.9182
Mmp10	6.4396
Cdh2	4.3222
Tgfbi	4.2408
Fbln1	3.557
Thbs1	3.3539
Mmp2	3.1125
Adamts1	3.0894
Ecm1	2.9625
Vcam1	2.8629
Fn1	2.7477
Sgce	2.7383
Mmp11	2.7008
Thbs3	2.5626

Gene	Fold Change
Ncam1	2.4502
Col5a1	2.0393
Timp2	2.0159
Selp	2.013
Ctgf	1.9957

* Genes upregulated at least 2-fold in *KP* compared to *KPD* carcinoma cells. Values are expressed as fold change ($n = 3$ each).

5 One of the most highly upregulated genes in *KP* compared to *KPD* carcinoma cells was osteopontin (*SPP1*), which was previously found to be upregulated in human PDA and shown to increase the invasion of human PDA cell lines *in vitro* (Kolb *et al.*, *Cancer Biol. Therap.* 4:740, 2005). But, some genes showing upregulation, including the top-ranked one, *Col6a1*, were less
10 familiar (Table 3 and Figure 11A).

 With regard to *COL6A1* gene, little is known about its specific function(s) in cancer and, in particular, the pathogenesis of PDA. But, *COL6A1* has been shown to associate in specific and structural binding interactions with HA (Kielty *et al.*, *J. Cell Biol.* 118:979, 1992; McDevitt *et al.*, *FEBS Letters* 294:167, 1991)
15 and also to bind to fibrillary type I collagen (Bonaldo *et al.*, *Biochemistry* 29:1245, 1999), which is itself complicit in the aggressiveness and metastatic dissemination of PDA (Armstrong *et al.*, *Clin. Cancer Res.* 10:7427, 2004; Shields *et al.*, *Biochem. J.* 441:541, 2012; Shintani *et al.*, *Cancer Res.* 66:11745, 2006). In addition, *COL6A1* has been identified in the serum
20 proteome of PDA patients (Pan *et al.*, *PLoS One* 6:e27574, 2011; Yu *et al.*, *J. Proteome Res.* 8:1565, 2009).

 Another interesting finding was that CD44, the surface receptor for hyaluronic acid (HA), was also upregulated in *KP* cells as compared to *KPD* cells, albeit to a slightly lesser extent (1.7-fold, data not shown). Thus, an
25 additional benefit of degrading HA in PDA may derive from disrupting this well-

known signaling pathway (Toole, *Nat. Rev. Cancer* 4:528, 2004). Thus, in certain embodiments, PDA may be treated with a RUNX3 inhibitor in combination with degrading HA, disrupting the CD44 signaling pathway (Toole, *Nat. Rev. Cancer* 4:528, 2004), or a combination thereof.

5

EXAMPLE 14

ROLE OF *SPP1* AND *COL6A1* IN PANCREAS CANCER CELL BEHAVIOR

To examine the potential role of SPP1 and COL6A1 in metastatic niche formation in PDA, the promoter regions and expression patterns of these genes, as well as their effect on cell behavior, were analyzed.

10 Immunoblots were performed as described herein, wherein the primary antibodies used were Actin (control) (1:2000, Santa Cruz, CA, USA); Col6a1 (1:1000 Santa Cruz, CA, USA); and Spp1 (1:200 Santa Cruz, CA, USA).

Human *COL6A1* was cloned into pBABE hygromycin vectors and transgenic cells made as described herein.

15 Transcript levels in Runx3-overexpressing carcinoma cells were determined using qRT-PCR as described herein. The Spp1 mouse forward and reverse primers used were SEQ ID NO.:5 (TTTCACTCCAATCGTCCCTACA) and SEQ ID NO.:6 (TCAGTCCATAAGCCAAGCTATCA), respectively; the Col6A1 mouse forward and reverse primers were SEQ ID NO.:7

20 (CTGCTGCTACAAGCCTGCT) and SEQ ID NO.:8 (CTGCTGCTACAAGCCTGCT), respectively; and the *COL6A1* human forward and reverse primers were SEQ ID NO.:9 (ACACCGACTGCGCTATCAAG) and SEQ ID NO.:10 (CGGTCACCACAATCAGGTACTT), respectively.

For luciferase assays, the human *COL6A1* promoter region was
25 subcloned into a pGL2 vector from a Bac clone (ID RP11-892E8) obtained from Children's Hospital Oakland Research Institute, USA. For *SPP1* and *COL6A1* assays, 0.1 - 0.3 µg of promoter DNA, together with human *Flag-RUNX3* or *Flag* vector, were transfected into HEK293T cells. Cell lysates were prepared at 48 hours in Reporter Lysis Buffer (Promega WI, USA), substrate added and

luciferase activity recorded on a luminometer (Veritas, WI, USA). β -gal activity was used to normalize the transfection efficiency. Results represent means $\pm$ SEM of triplicate wells from three independent experiments.

The *SPP1* promoter regions were found to have two consensus RUNX3
5 binding sites and two SMAD binding elements (SBE) in the human (Figure 11B) and murine genes (data not shown). Similarly, the *COL6A1* promoter region was also found to have two *RUNX3* consensus-binding sites in the human (Figure 11D) and murine (data not shown).

Spp1 was shown to be specifically upregulated in *KP* as compared to
10 *KPD* PDA cells (Figure 12A) and its expression in both murine (Figures 12B and 12C) and human PDA cells (Figure 11C) is sensitive to *Runx3*. Similarly, Col6a1 was found to be differentially expressed at the protein level in *KP* and *KPD* cells (Figure 12A). Furthermore, luciferase assays confirmed that *RUNX3* directly stimulates *COL6A1* transcription in both species (Figures 12D and
15 12E). Overexpression of *Runx3* correspondingly increases Col6a1 protein levels (shown for murine cells in Figure 12C), whereas silencing of *RUNX3* reduces *COL6A1* transcript levels (shown for human cells in Figure 12F).

Having firmly established the specificity of *RUNX3*-regulation of *COL6A1* levels in both species, *COL6A1* was examined for the ability to directly
20 influence behavior in a cell autonomous manner or, in other words, to act as an autocrine enabler of dissemination. Overexpression of *COL6A1* in MiaPaca-2 PDA cells (Figure 12G), and of *Col6a1* in *KPD* carcinoma cells (Figure 12H), significantly increased their respective migratory abilities in scratch wound migratory assays, and also freed them from a dependence on TGF β . Thus, in
25 addition to providing a potentially critical component of the three dimensional architecture of a developing tumor, Col6a1 also appears to directly stimulate cell motility.

Overall, these results indicate that *RUNX3* serves to both expel the “seed” and prepare the “soil” by directly stimulating cell migration and

expressing a host of proteins to remodel the extracellular matrix to favor metastatic colonization.

The various embodiments described above can be combined to provide further embodiments. All of the U.S. patents, U.S. patent application
5 publications, U.S. patent applications, foreign patents, foreign patent applications and non-patent publications referred to in this specification and/or listed in the Application Data Sheet are incorporated herein by reference, in their entirety. Aspects of the embodiments can be modified, if necessary to employ concepts of the various patents, applications and publications to
10 provide yet further embodiments.

These and other changes can be made to the embodiments in light of the above-detailed description. In general, in the following claims, the terms used should not be construed to limit the claims to the specific
embodiments disclosed in the specification and the claims, but should be
15 construed to include all possible embodiments along with the full scope of equivalents to which such claims are entitled. Accordingly, the claims are not limited by the disclosure.

CLAIMS

What is claimed is:

1. A method for reducing the risk of or treating pancreatic cancer, comprising administering to a subject in need thereof an inhibitor of RUNX3 expression or activity.
2. A method for reducing the risk of or treating pancreatic cancer, comprising administering to a subject in need thereof an agent capable of blocking the association of RUNX3 with a second transcription factor.
3. A method for reducing the risk of or treating pancreatic cancer, comprising administering to a subject in need thereof an agent that enhances deacetylation of RUNX3.
4. A method for reducing the risk of or treating pancreatic cancer, comprising administering to a subject in need thereof an agent that enhances ubiquitination of RUNX3.
5. A method for reducing the risk of or treating metastasized pancreatic cancer, comprising administering to a subject in need thereof an inhibitor of RUNX3 expression or activity.
6. A method for reducing the risk of or treating a pancreatic cancer precursor lesion, comprising administering to a subject in need thereof an inhibitor of RUNX3 expression or activity.

7. A method for reducing the risk of or treating a metastatic niche associated with pancreatic cancer, comprising administering to a subject in need thereof an inhibitor of RUNX3 expression or activity.
8. The method according to any of the preceding claims wherein the RUNX3 inhibitor is a small molecule, chemical entity, nucleic acid molecule, peptide or polypeptide.
9. The method according to any of the preceding claims wherein the RUNX3 inhibitor disrupts or inhibits association of RUNX3 with Smad4 or a Smad2/Smad4 complex.
10. The method according to any of the preceding claims further comprising administering an expression or activity inhibitor of Bmpr1a, Smad5, Tgfb3, Smad4, Bmp1, Itgb7, Tgfb1i1, Bmper, Ltbp1, Ltbp2, Itgb5, Id1, Tgfb1, Dlx2, or any combination thereof.
11. The method according to any of the preceding claims further comprising administering an expression or activity inhibitor of Ctgf, Selp, Timp2, Col5a1, Ncam1, Thbs3, Mmp11, Sgce, Fn1, Vcam1, Ecm1, Adamts1, Mmp2, Thbs1, Fbln1, Tgfb1, Cdh2, Mmp10, Timp3, Spp1, Vcan, Sparc, Col6a1, or any combination thereof.
12. The method according to any of the preceding claims further comprising administering a pancreas-specific activator of *Hdac5* activity.
13. The method according to claim 3 wherein the agent that enhances deacetylation of RUNX3 is a pancreas-specific activator of *Hdac5* activity.

14. The method according to claim 4 wherein the agent that enhances ubiquitination of RUNX3 is a pancreas-specific activator of *Hdac5* activity or Mdm2 activity.

15. The method according to any of the preceding claims further comprising administering an expression or activity inhibitor of a cyclin D, a cyclin E, or a combination thereof.

16. The method according to any of the preceding claims further comprising a second method of treatment selected from surgery, chemotherapy, radiation therapy, or any combination thereof.

17. The method according to claim 16 wherein each treatment is performed simultaneously, successively, or concomitantly.

18. A compound for reducing the risk of or treating pancreatic cancer, comprising an inhibitor of RUNX3 expression.

19. A compound for reducing the risk of or treating metastatic pancreatic cancer, comprising an inhibitor of RUNX3 activity.

20. The compound according to claim 18 or 19 wherein the RUNX3 inhibitor is a small molecule, chemical entity, nucleic acid molecule, peptide or polypeptide.

21. The compound according to claim 18 wherein the RUNX3 inhibitor is an interfering RNA or antisense RNA.

22. The compound according to claim 18 wherein the RUNX3 inhibitor is an interfering RNA having the sequence as set forth in SEQ ID NO.:4.

23. A composition for reducing the risk of or treating pancreatic cancer, comprising an inhibitor of RUNX3 expression and a pharmaceutically acceptable carrier, diluent or excipient.

24. A composition for reducing the risk of or treating metastatic pancreatic cancer, comprising an inhibitor of RUNX3 activity and a pharmaceutically acceptable carrier, diluent or excipient.

25. A composition according to claim 23 or 24 wherein the composition further comprises a chemotherapeutic selected from gemcitabine, nanoparticle albumin bound (nab)-paclitaxel, FOLFIRINOX combination (5-fluorouracil, leucovorin, irinotecan and oxaliplatin), or any combination thereof.

26. The method according to any one of claims 1-17 wherein the subject is further administered a chemotherapeutic selected from gemcitabine, nanoparticle albumin bound (nab)-paclitaxel, FOLFIRINOX combination (5-fluorouracil, leucovorin, irinotecan and oxaliplatin), or any combination thereof.

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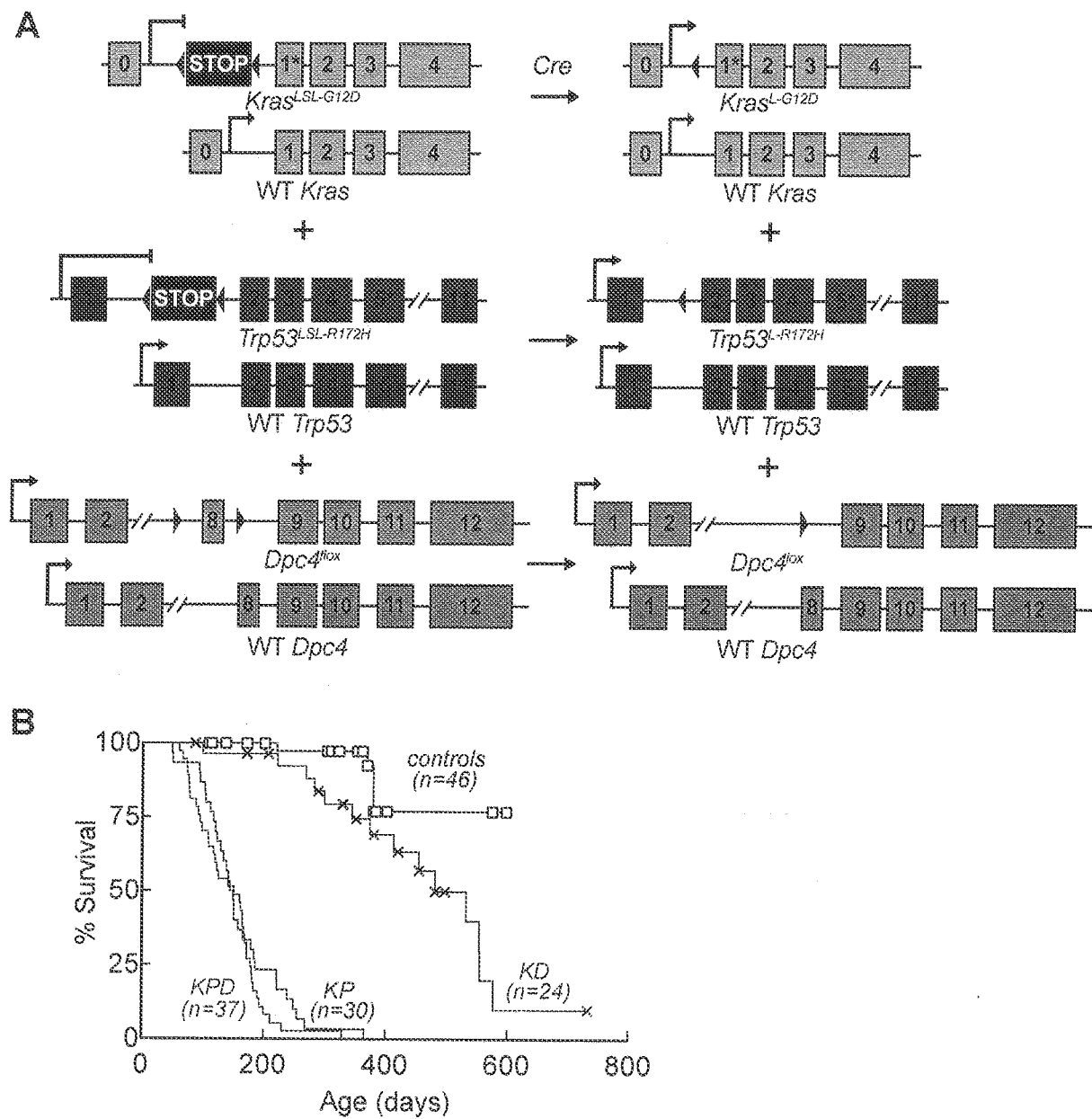


FIG. 1

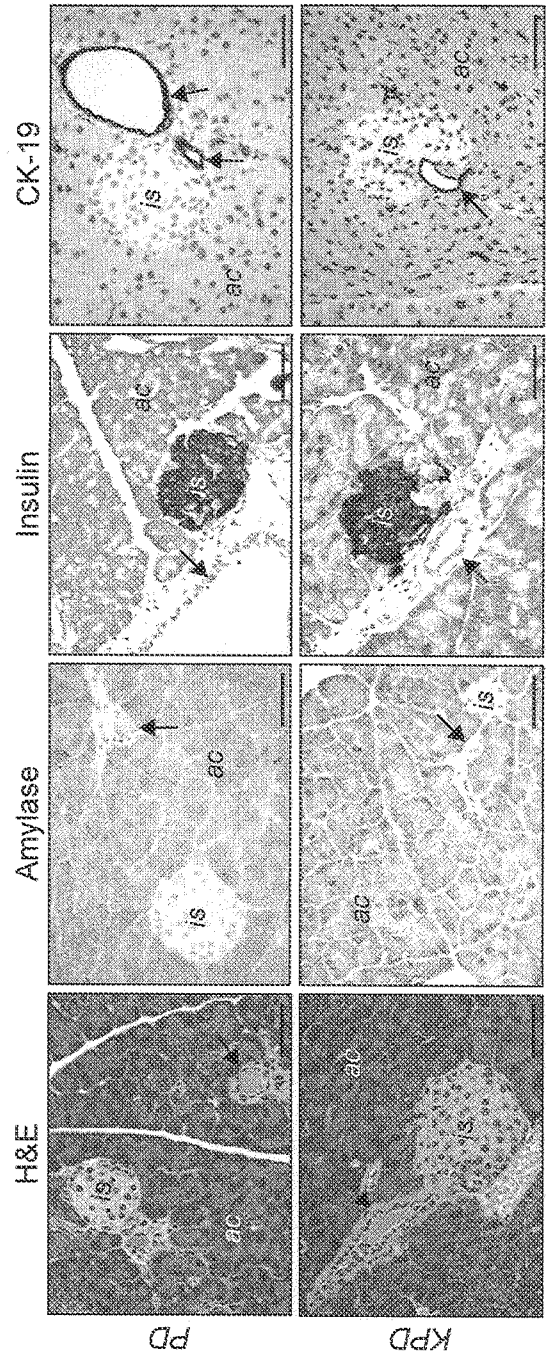


FIG. 2

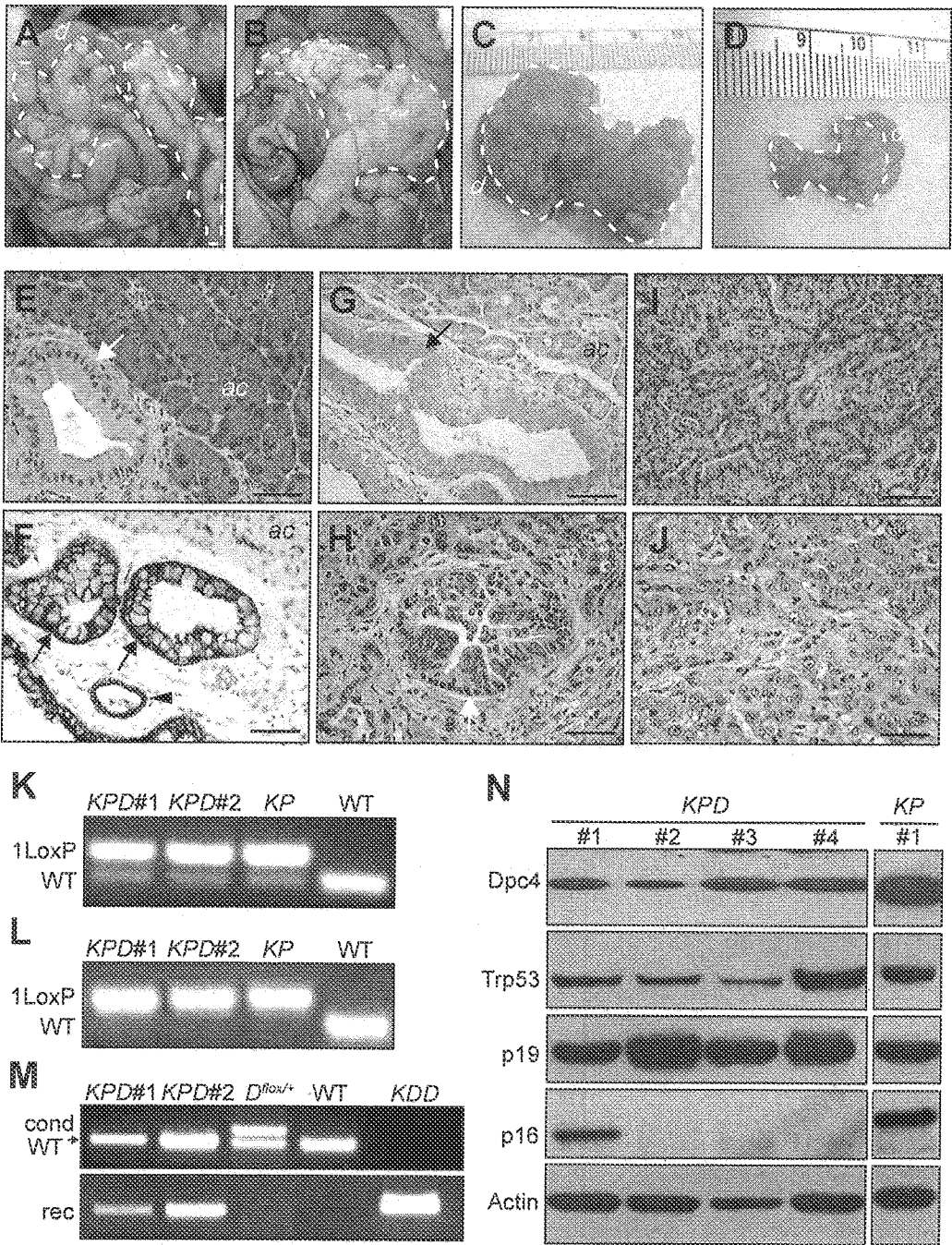


FIG. 3

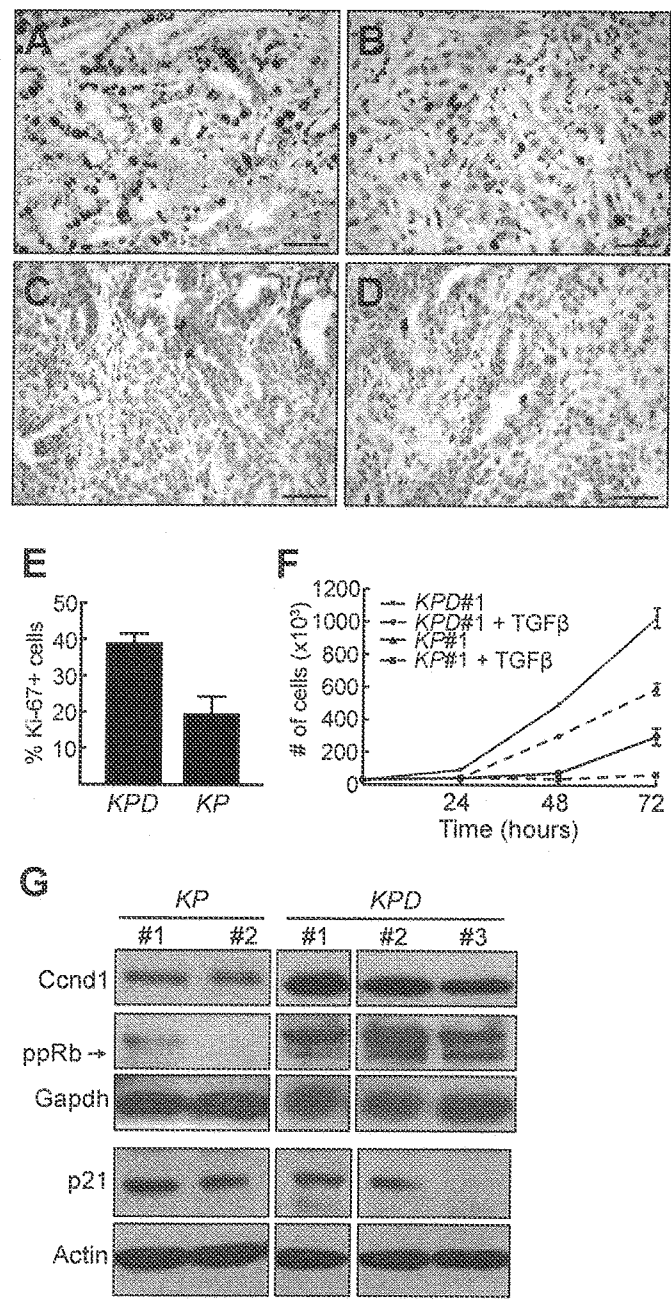


FIG. 4

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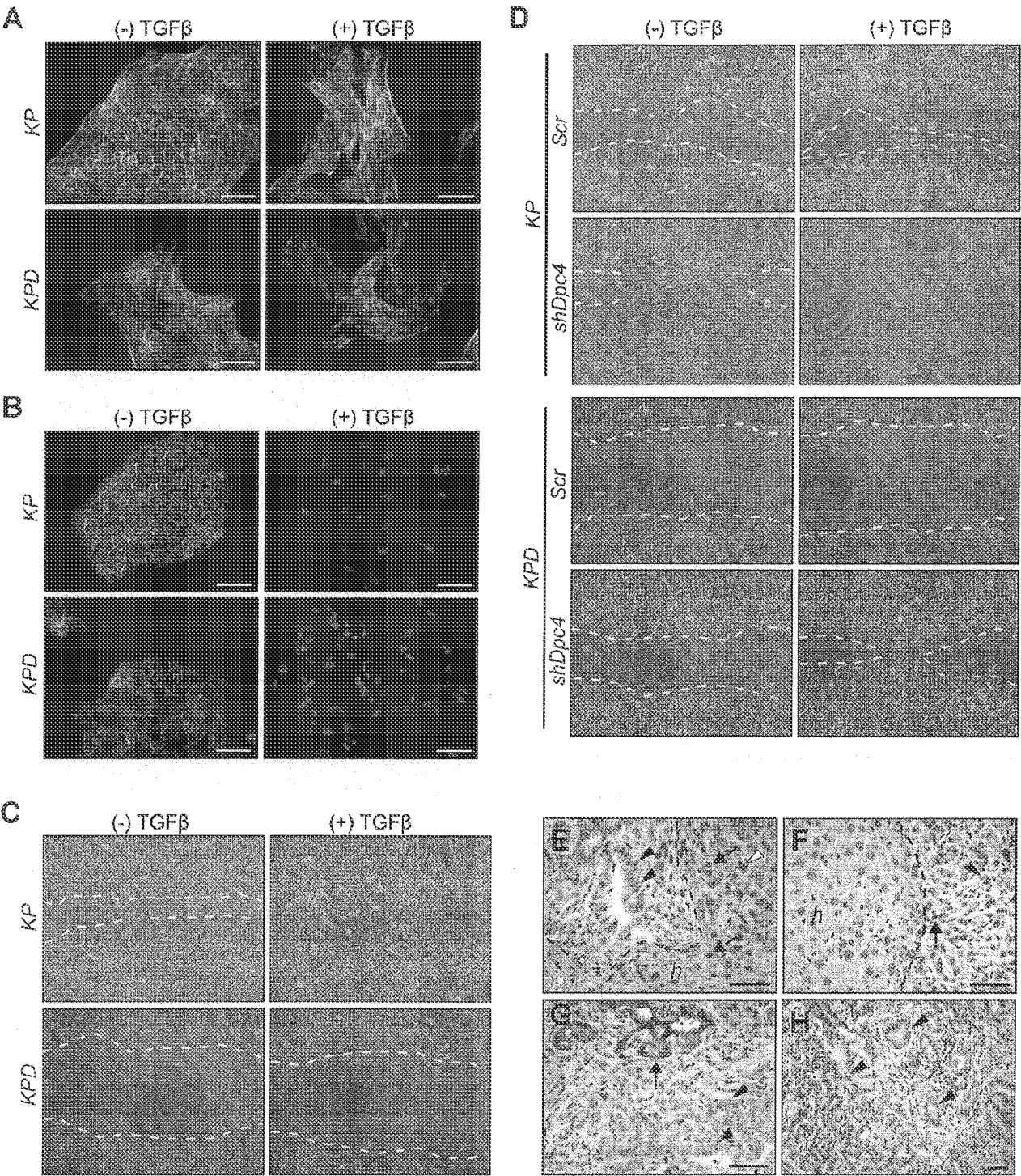


FIG. 5

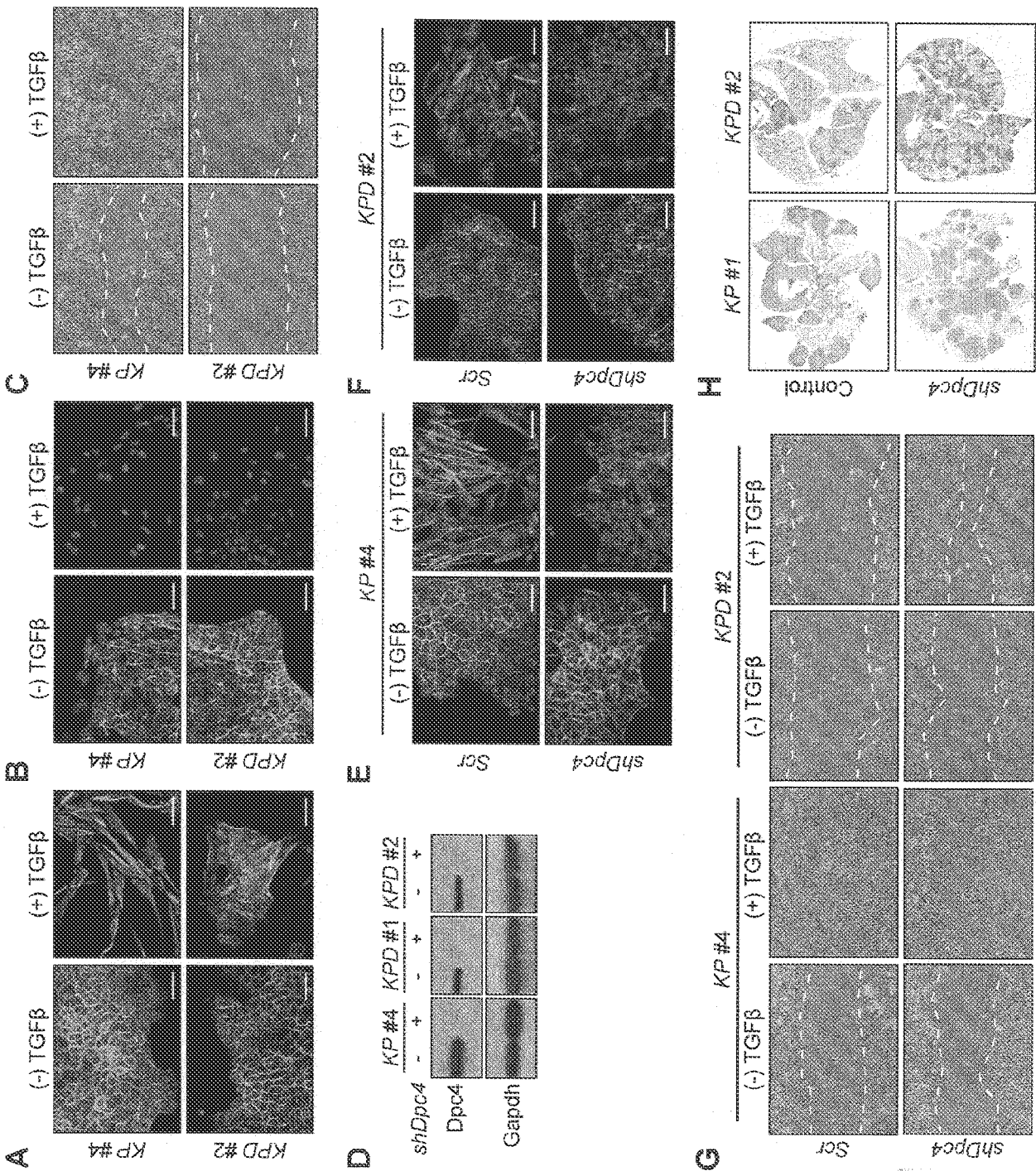


FIG. 6

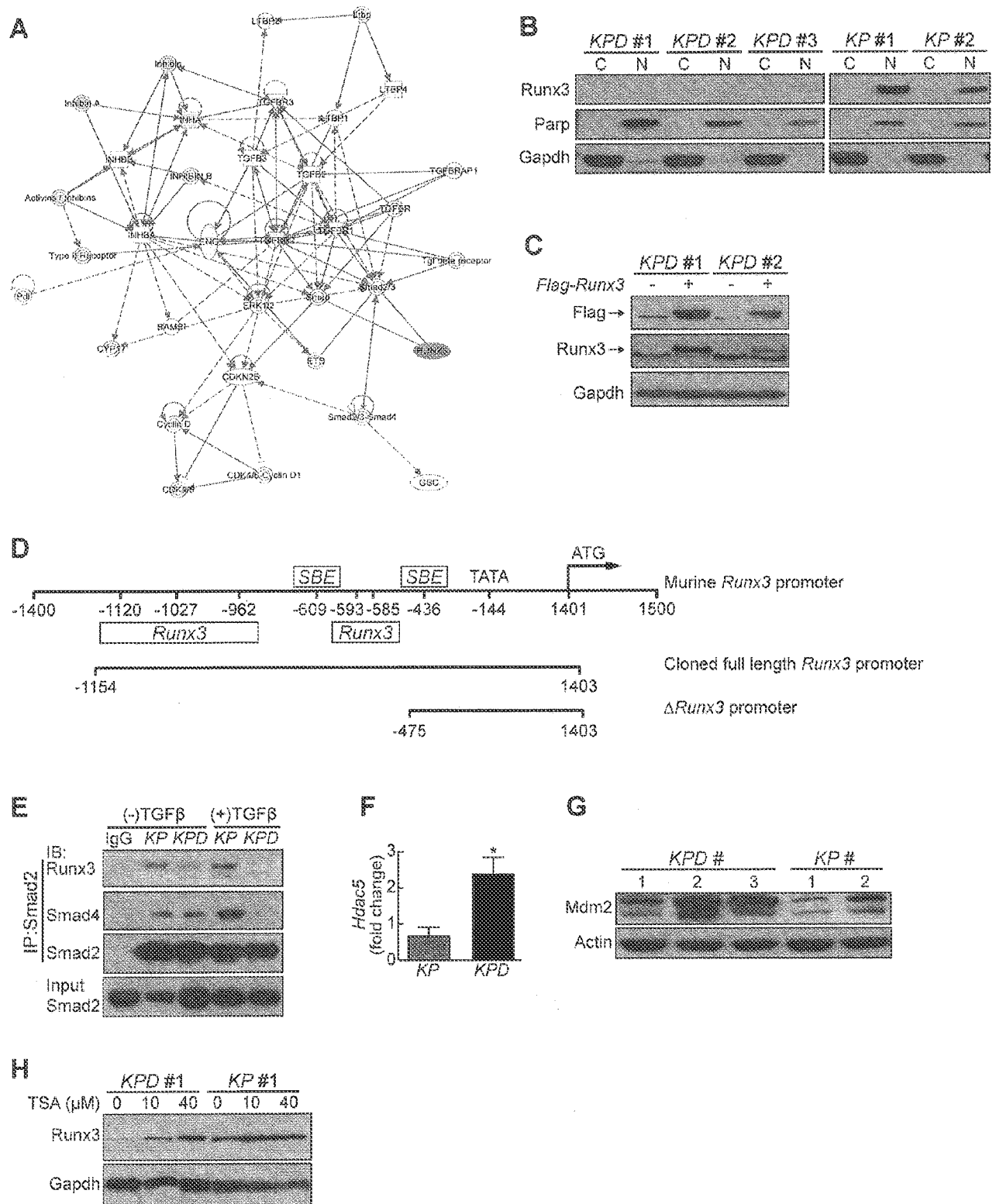


FIG. 7

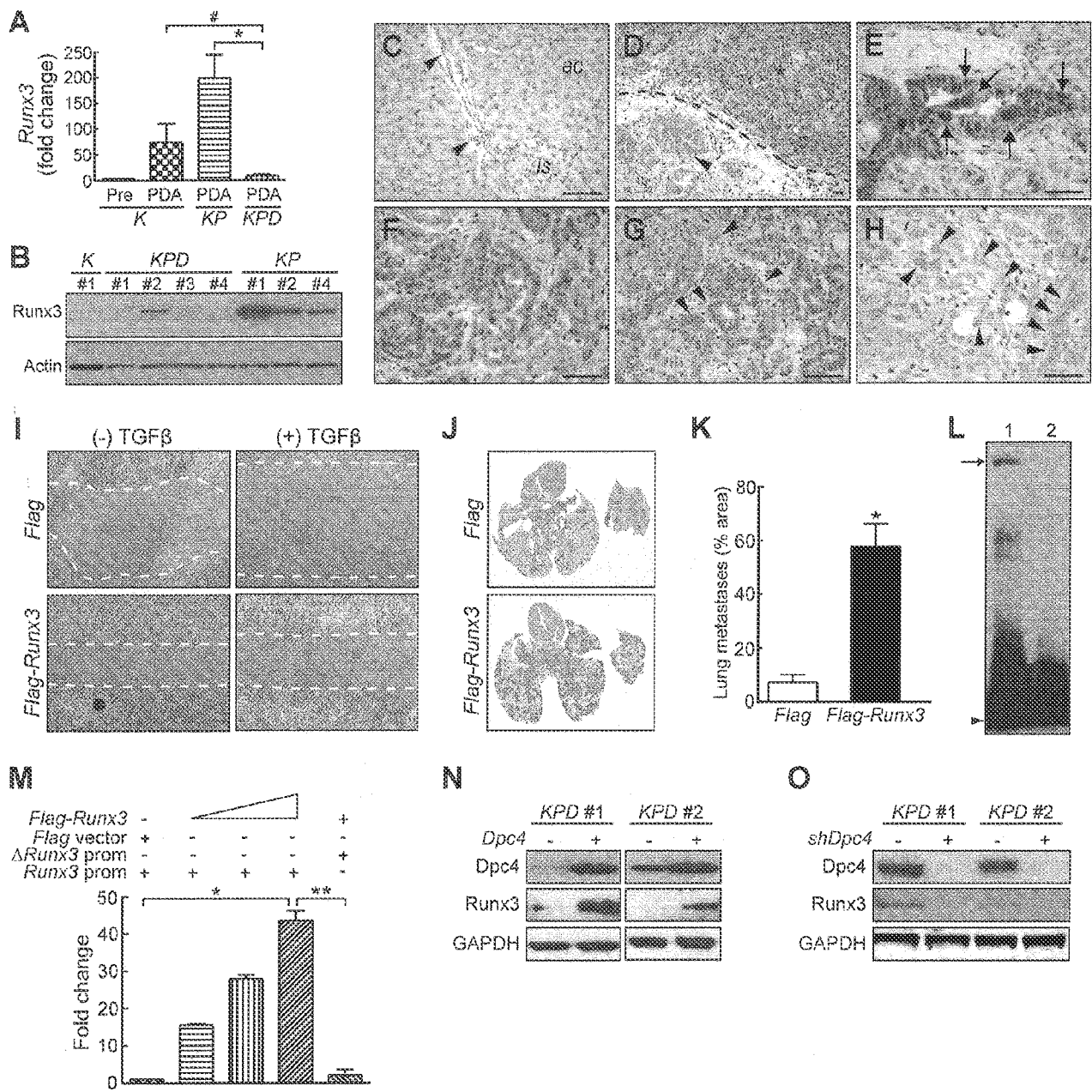
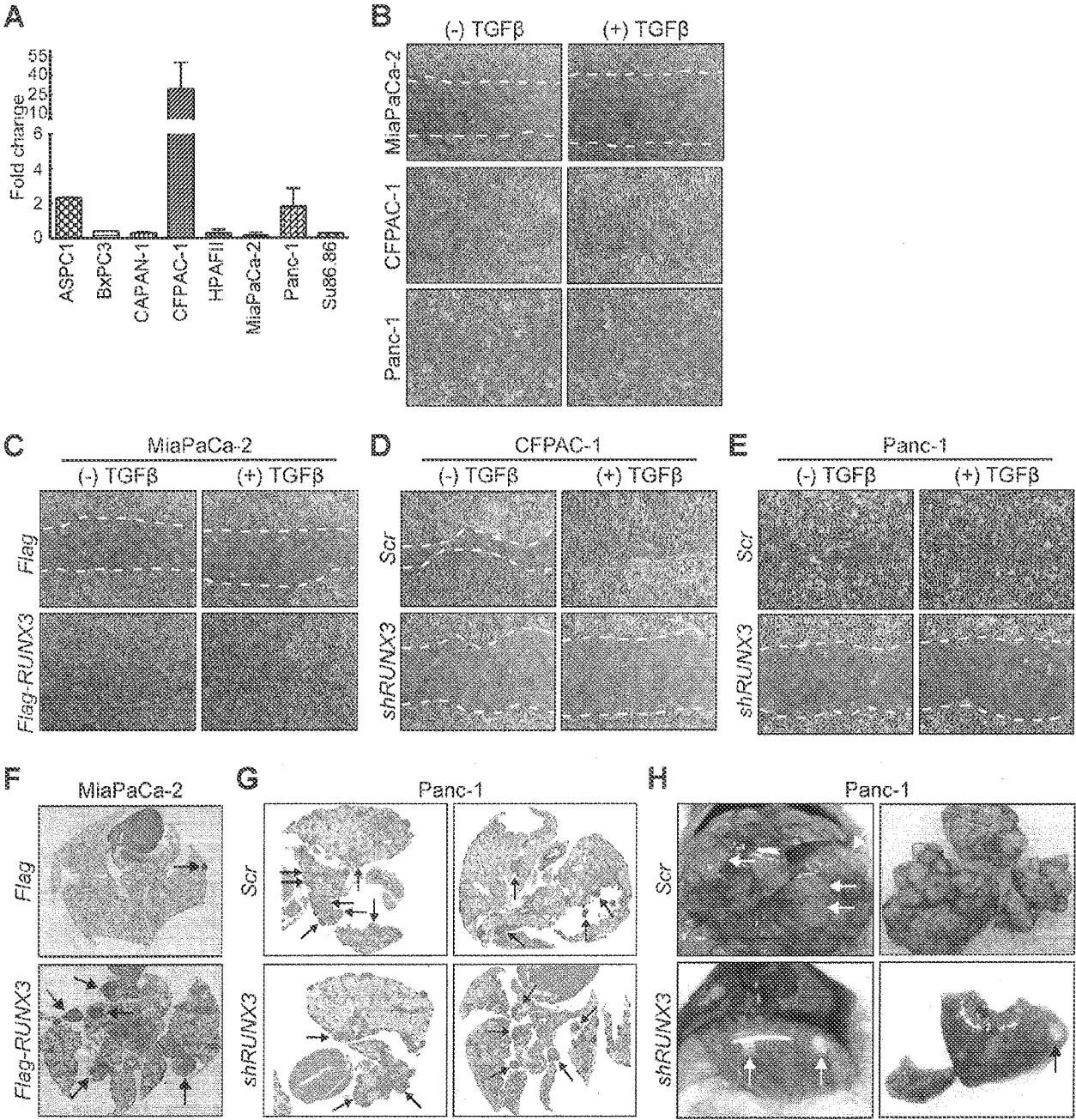


FIG. 8

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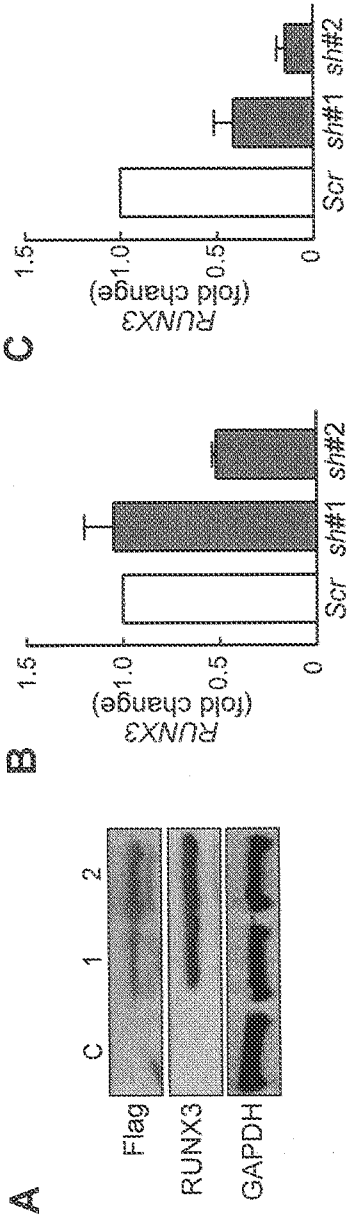
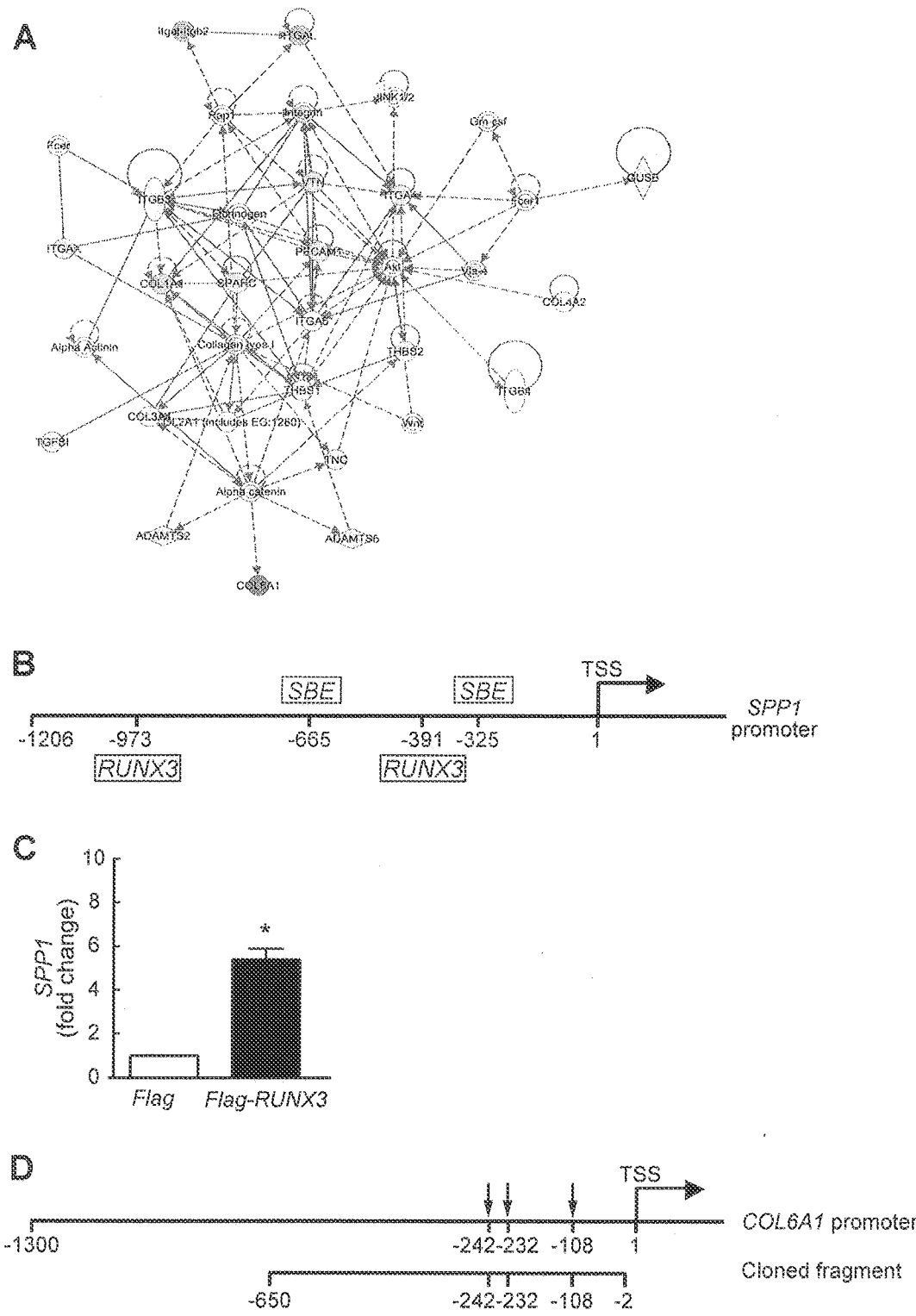


FIG. 10

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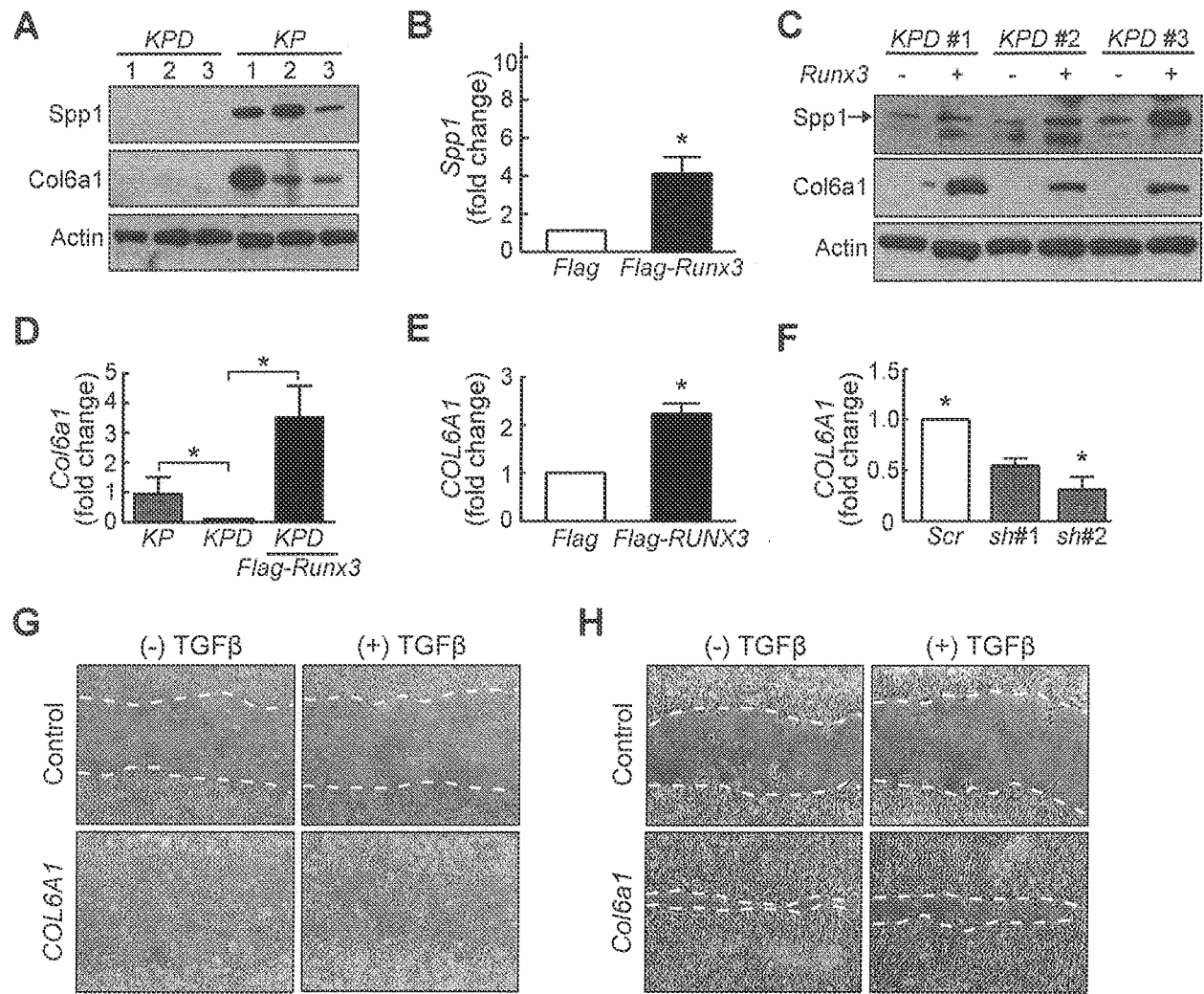


FIG. 12

INTERNATIONAL SEARCH REPORTInternational application No.
PCT/US2014/011536**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 1-17, 26
because they relate to subject matter not required to be searched by this Authority, namely:
Claims 1-17, 26 pertain to a method for treatment of the human by therapy, and thus relate to a subject matter which this International Searching Authority is not required, under Article 17(2)(a)(i) of the PCT and Rule 39.1(iv) of the Regulations under the PCT, to search.
2. ☒ Claims Nos.: 17
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
Claim 17 is unclear, since it refers to claim 16 which is not drafted in accordance with PCT Rule 6.4(a) (PCT Article 6).
3. ☒ Claims Nos.: 9-12, 15, 16, 26
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of any additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2014/011536**A. CLASSIFICATION OF SUBJECT MATTER****A61K 48/00(2006.01)i, A61K 31/337(2006.01)i, A61P 35/00(2006.01)i**

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K 48/00; C07K 14/525; C12P 21/02; A61K 31/337; A61P 35/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean utility models and applications for utility models

Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

eKOMPASS(KIPO internal) & Keywords: RUNX3, pancreatic cancer

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Li, J. et al., "RUNX3 expression in primary and metastatic pancreatic cancer", J. Clin. Pathol., 2004, Vol. 57, pp. 294-299 See abstract, page 298, right-column, lines 15-25.	18-20, 23, 24
A		21, 22, 25
A	US 2004-0146986 A1 (BAE, S. C. et al.) 29 July 2004 See abstract and claims 1-18.	18-25
A	WO 2006-057623 A1 (NATIONAL UNIVERSITY OF SINGAPORE) 1 June 2006 See abstract; pages 1-6; and claims 1-28.	18-25

☐ Further documents are listed in the continuation of Box C.☒ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

30 April 2014 (30.04.2014)

Date of mailing of the international search report

01 May 2014 (01.05.2014)

Name and mailing address of the ISA/KR


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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US2014/011536

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2004-0146986 A1	29/07/2004	AT 402256 T	15/08/2008
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		DK 1356030 T3	03/11/2008
		EP 1356030 A1	29/10/2003
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		US 2008-0261883 A1	23/10/2008
		US 7368255 B2	06/05/2008
		WO 02-061069 A1	08/08/2002
WO 2006-057623 A1	01/06/2006	None	