The deubiquitinase USP9X suppresses pancreatic ductal adenocarcinoma

Pedro A. Pérez-Mancrea, Alistair G. Rust2, Louise van der Weyden2, Glen Kristiansen3, Allen Li4, Aaron L. Sarver5, Kevin A. T. Silverstein6, Robert Grützmann7, Daniela Aust7, Petra Rümmele8, Thomas Knösel9,10, Collin Herd11, Derek L. Stemple11, Ross Kettleborough11, Jacqueline A. Brosnan1, Ang Li1, Richard Morgan3, Spencer Knight4, Jun Yu6, Shane Stegeman9, Lara S. Collier13, Jelle J. ten Hoeve14,15, Jeroen de Ridder16, Alison P. Klein1, Michael Goggins4, Ralph H. Hruban1, David K. Chang16, Andrew V. Blankin16, Sean M. Grimmond19, *Australian Pancreatic Cancer Genome Initiative*16, David J. Adams2 & David A. Tuveson1

Pancreatic ductal adenocarcinoma (PDA) remains a lethal malignancy despite much progress concerning its molecular characterization. PDA tumours harbour four signature somatic mutations1–4 in addition to numerous lower frequency genetic events of uncertain significance. Here we use *Sleeping Beauty* (SB) transposon-mediated insertional mutagenesis5–8 in a mouse model of pancreatic ductal preneoplasia9 to identify genes that cooperate with oncogenic *Kras* to accelerate tumorigenesis and promote progression. Our screen revealed new candidate genes for PDA and confirmed the importance of many genes and pathways previously implicated in human PDA. The most commonly mutated gene was the X-linked deubiquitinase USP9x, which was inactivated in over 50% of the tumours. Although previous work had attributed a pro-survival role to USP9X in human neoplasia, we found instead that loss of *Usp9x* enhances transformation and protects pancreatic cancer cells from anoikis. Clinically, low USP9X protein and messenger RNA expression in PDA correlates with poor survival after surgery, and USP9X levels are inversely associated with metastatic burden in advanced disease. Furthermore, chromatin modulation with trichostatin A or 5-aza-2'-deoxycytidine elevates USP9X expression in human PDA cell lines, indicating a clinical approach for certain patients. The conditional deletion of *Usp9x* cooperated with *Kras* to accelerate pancreatic tumorigenesis in mice, validating their genetic interaction. We propose that USP9X is a major tumour suppressor gene with prognostic and therapeutic relevance in PDA.

The biological sequelae of PDA has been partially attributed to frequent and well characterized mutations in *KRAS* (>90%), *CDK2NA* (>90%), *TP53* (70%) and *SMAD4* (55%)1–4. Recent genome-wide analyses have uncovered numerous additional somatic genetic alterations, although the functional relevance of most remains uncertain. To explore the molecular genesis of PDA we previously generated a mouse model of pancreatic intraepithelial neoplasia (mPanIN) by conditionally expressing an endogenous *Kras* allele in the developing pancreas. Mice with mPanIN spontaneously progress to mPDA after a long and variable latency, providing an opportunity to characterize genes that cooperate with *Kras* to promote early mPDA. We hypothesized that such genes could be directly identified by applying insertional mutagenesis strategies5–8,10 in our mPanIN model, and that these candidates could represent ‘drivers’ of PDA development.

Accordingly, we interbred our mPanIN model with two distinct SB transposon systems and monitored mice for early disease progression. Our initial approach used the well characterized CAGGS-SB10 transgenic allele to promote transposition. Although CAGGS-SB10 promoted PDA, a variety of non-pancreatic neoplasms and a paucity of identified common insertion sites (CIS) in the recovered pancreatic neoplasms precluded a comprehensive analysis, potentially reflecting the variegated expression of *CAGGS-SB10* (ref. 12) (Supplementary Figs 1a and 2, and Supplementary Tables 1 and 3a). To increase the specificity and potency of SB mutagenesis, we generated a conditional SB13 mutant mouse by targeting the *Rosa26* locus in embryonic stem cells (Supplementary Fig. 3a, b). The pancreas-specific expression and function of the conditional SB13 allele was confirmed (Supplementary Fig. 3c), and we found that SB13-induced transposition by itself did not promote lethality or pancreatic tumorigenesis (Fig. 1a and Supplementary Fig. 4a). In contrast, *Kras*; *Pdx1-cre; T2/Onc; Rosa26-LSL-SB13 mice rapidly progressed and succumbed to invasive pancreatic neoplasms (Fig. 1a–c). A cohort of 117 *Kras*; *Pdx1-cre; T2/Onc; Rosa26-LSL-SB13 mice (Supplementary Fig. 1b) was monitored for tumour development, and 103 of these mice were available for full necropsy and tissue procurement. The majority of such mice harboured multi-focal pancreatic tumours, and 198 distinct primary tumours and metastases were subjected to histological and molecular analysis. Most mice had invasive carcinomas (66 of 103) that consisted of classical mPDA (78.8%) or invasive cystic neoplasms (21.2%); 34.8% of mice also contained metastases predominantly in their liver and lungs (Supplementary Fig. 4c). The remainder of the mice (37 of 103) had pre-invasive pancreatic tumours consisting of high-grade...
mPanIN and cyst-forming papillary neoplasms (Supplementary Fig. 4b).

The candidate genes identified from the SB13 screen represented unanticipated candidates as well as many genes and pathways previously implicated in human PDA (Table 1 and Supplementary Tables 2, 3a and 4). Indeed, various members of the TGF-β pathway, including Smad3, Smad4, Tgfbr1 and Tgfbr2, were collectively mutated in 32% of the tumours. CISs were ranked by tumour frequency where the spatial distribution of insertion sites was analysed using the narrowest 15K kernel scale. Chr, chromosome; CIS height, estimate of the number of insertions within a CIS; n, number of tumours from which the CIS was found.

Table 1 | Top 20 candidate CIS genes that cooperate with KrasL12D to promote mPDA in KrasL12D; Pdx1-cre; T2/Onc; Rosa26-LSL-SB13 mice

| Gene    | Chr | CIS peak location | CIS height | n | Mutation in humans |
|---------|-----|-------------------|------------|---|-------------------|
| Usp9x   | X   | 126911773         | 158.1256   | 101.341 | –                 |
| Pten    | 9   | 32872602          | 64.5204    | 61.96  | –                 |
| Fndc3b  | 12  | 27562591          | 13.7096    | 55.67  | –                 |
| Setd5   | 6   | 113057997         | 35.6175    | 52.71  | –                 |
| Arhgap5 | X   | 87567353          | 21.6666    | 48.80  | Yes (ref. 5)      |
| Fam193a | 5   | 34705809          | 24.3555    | 45.78  | –                 |
| Ctnna1  | 3   | 33524886          | 20.2017    | 43.50  | –                 |
| Pum1    | 4   | 13028487          | 12.7948    | 41.66  | Yes (ref. 5)      |
| Far1    | 14  | 123587858         | 9.407      | 39.47  | –                 |
| Foxp1   | 6   | 98921646          | 19.5831    | 38.60  | –                 |
| Arid1a  | 4   | 13326839          | 32.1628    | 38.47  | Yes (ref. 5)      |
| Acvr1b  | 15  | 101024934         | 31.1752    | 38.47  | Yes (ref. 15)     |
| Map4k3  | 17  | 81109860          | 13.2385    | 38.45  | Yes (ref. 5)      |
| Stag2   | X   | 39535994          | 16.8613    | 37.48  | –                 |
| Mll3    | 3   | 22982314          | 16.0001    | 37.43  | Yes (ref. 5)      |
| Abn2/Snhb | 2  | 122267680         | 12.3174    | 37.41  | –                 |
| Ahrgap5 | 12  | 53644560          | 37.416     | 35.61  | –                 |
| Gsk3b   | 16  | 38106972          | 21.79      | 35.43  | –                 |

100 µm. d, Usp9x is the major CIS in KrasL12D; Pdx1-cre; T2/Onc; Rosa26-LSL-SB13 PDA tumours (x axis denotes genome, y axis –log P value), with bidirectional insertions. (+) indicates parallel to Usp9x expression; (–) indicates antiparallel. e, Usp9x exon 2-2/T2/Onc chimaeric mRNA in SB13 tumours. f, g, Usp9x protein expression in normal pancreatic ducts (arrow (4)), but not in neoplastic cells (arrows (g)), in SB13 PDA harbouring Usp9x insertions. Scale bar: 100 µm.

The most frequent CIS observed was the X-linked deubiquitinase Usp9x, a gene that had not been previously associated with PDA or other types of carcinoma in humans or mouse models. Indeed, the COSMIC data base revealed only one USP9X mutation in a case of ovarian cancer, although the functional relevance of this mutation has not been characterized (COSMIC mutation ID 73237). Usp9x was disrupted in over 50% of all tumours, with 341 insertions noted in the 101 tumours harbouring this CIS (Fig. 1d and Table 1). Furthermore, Usp9x was also identified as a CIS in four samples from the initial SB10 screen (Supplementary Table 1), supporting its candidacy as a PDA genetic determinant. We confirmed that Usp9x was disrupted in tumours by isolating chimaeric fusion mRNAs that spliced the Usp9x transcript to the T2/Onc transposon (Fig. 1e). In addition, the Usp9x protein was specifically absent in neoplastic cells in pancreatic tumours bearing intragenic insertions (Fig. 1f, g).

To characterize the cellular and molecular pathways affected by Usp9x in PDA, we used RNA interference to deplete Usp9x levels in the tumours. Additionally, the Rb–p16Ink4a pathway was disrupted in 21% of the tumours. CISs representing the orthologues of additional human PDA genes included Fbxw7 (24.2%), Arid1a (19.1%), Acvr1b (19.1%), Skil1 (also symbolized as Lkb1) (6.5%), Mll3 (6%), Smarcad1 (6%) and Pbrm1 (4.5%)13–15. Trp53 was the only commonly mutated PDA gene conspicuously absent, although the p53 regulatory deubiquitinase Usp7 was a CIS (6.5%)16. Several CISs previously noted in insertional mutagenesis screens for hepatocellular carcinoma or gastrointestinal tract adenocarcinoma11. This indicates that many tumour progression pathways may be common to pancreatic, liver and gastrointestinal/colorectal tumours.
mPDA cell lines (Supplementary Fig. 5a). Although Usp9x depletion did not affect the proliferation of monolayer cultures (Supplementary Fig. 5b), it significantly increased colony formation in soft agar (Fig. 2a, Supplementary Fig. 5c) compared to cells transfected with scrambled short hairpin RNAs. Furthermore, knock down of Usp9x potently suppressed anoikis in mPDA cells (Fig. 2b). These properties of Usp9x were predominantly dependent on its intrinsic deubiquitinating activity (Supplementary Fig. 6a, b).

Because USP9X was previously reported to positively regulate SMAD4 transcriptional activity and SMAD4 is commonly mutated in PDA, we hypothesized that Usp9x loss would attenuate Smad4 function or TGF-β responsiveness in PDA cell lines. However, irrespective of Usp9x expression level, mPDA cell lines expressed Smad4 and were equally sensitive to p21 induction, growth inhibition and morphological alterations after exposure to TGF-β1 (Supplementary Fig. 7). Therefore, we were unable to assign a specific role to Usp9x in the regulation of the Smad4–TGF-β pathway in mPDA cells or tumours.

We next investigated several additional proteins reported to be regulated by Usp9x and involved in pathways relevant to cellular transformation. Although USP9X has been shown to bind to and regulate two proteins involved in cell survival, ASK1 (ref. 18) and MCL1 (refs 9, 19), we could not detect obvious changes in Ask1 or Mcl1 protein levels upon Usp9x loss (Fig. 2c). Usp9x has also been reported to deubiquitinate and thereby stabilize the E3 ligase Itch30; decreased protein levels of Itch were observed in mouse and human PDA cells upon the depletion of Usp9x (Fig. 2c and Supplementary Fig. 8a). Notably, ectopic Itch expression was sufficient to promote anoikis in mPDA cells (Fig. 2d), and Itch was partially responsible for the ability of Usp9x to promote anoikis and suppress colony formation (Supplementary Fig. 6c, d). Because Itch is known to promote the degradation of several proteins relevant to cell proliferation and survival31, we evaluated the protein expression of likely candidates including c-Jun, p63 and c-Flip but observed no alterations (Supplementary Fig. 8b). Furthermore, the Itch gene was identified as a CIS in 13% of cases (Supplementary Table 2). Therefore, Usp9x mutation may promote tumorigenesis in part by disabling Itch function, and the Usp9x–Itch pathway may work to constrain pancreatic tumorigenesis.

To determine whether USP9X expression is aberrant in human PDA, three distinct patient cohorts were assessed. First, we analysed a cohort of 100 Australian patients who underwent surgery for localized PDA and had detailed information available concerning clinical-pathological characteristics and outcome (Supplementary Fig. 9 and Supplementary Tables 5 and 6). Tumour DNA from 88 patients in this cohort failed to yield somatic mutations in USP9X, consistent with previous reports (data not shown). Notably, the low expression of USP9X mRNA correlated with poor survival after surgery (P = 0.0076) (Fig. 3a), and multivariate analysis revealed that USP9X expression was an independent poor prognostic factor after surgery (Supplementary Table 7). We next analysed autopsy specimens from a separate cohort of 42 American patients to determine that USP9X protein expression inversely correlated with a widespread metastatic pattern (P = 0.0212) (Fig. 3b), and bore no relation to SMAD4 expression (Supplementary Table 8). A third collection of PDA specimens obtained from resected German patients (n = 404) was used to determine that USP9X and ITCH protein levels were decreased (Supplementary Fig. 10) and expressed in a similar manner (Spearman–Rho correlation = 0.47; P < 0.01; Supplementary Table 9a) in tumours compared to normal pancreatic tissue. Furthermore, the proportion of tumours that had undetectable USP9X (13.6%) or ITCH (30.5%) protein correlated with a worse outcome (Supplementary Fig. 11, Supplementary Table 9b, c), particularly regarding USP9X in the subset of high-grade tumours (Fig. 3c and Supplementary Tables 10 and 11). Collectively, these findings implicate the loss of USP9X expression as a relevant event in human pancreatic cancer progression.

We found that USP9X was expressed throughout murine and human tumour development and lost focally in PDA (Supplementary Figs 12 and 13). Additionally, human PDA cell lines expressed lower levels of USP9X compared to non-PDA cancer cell lines (Supplementary Fig. 14). To investigate additional potential mechanisms of USP9X regulation in PDA, human cell lines were treated with the DNA methylase inhibitor 5-aza-2′-deoxycytidine and the HDAC inhibitor trichostatin A. Both inhibitors modestly increased the USP9X mRNA and protein levels in most cell lines, indicating that USP9X may be epigenetically silenced in vivo (Fig. 3d and Supplementary Fig. 15). Furthermore, although the promoter region of USP9X was not heavily methylated in tumour samples or PDA cells harbouring low protein expression (data not shown), treatment with 5-aza-2′-deoxycytidine did decrease colony formation of human PDA cells and this was partially reversed by concomitantly knocking down USP9X (Supplementary Fig. 16).

To confirm that Kras
\(^{G12D}\) cooperated with Usp9x loss to promote pancreatic cancer, a conditional Usp9x
\(^{f/f}\) allele was generated (Supplementary Fig. 17a) and interbred with Kras
\(^{LSL-G12D}\), Pdx1-cre mice to evaluate the impact on mPanIN progression. The mosaic expression of Usp9x in pancreas from Pdx1-cre; Usp9x
\(^{f/f}\) mice was confirmed by immunohistochemistry (Supplementary Fig. 17b). We found that all hemizygous male mice and heterozygous female mice carriers of the Usp9x
\(^{f/f}\) allele in the background of Kras
\(^{LSL-G12D}\), Pdx1-cre rapidly developed advanced mPanIN and microinvasive neoplasms within 3 months of age (Fig. 3e, f and Supplementary Fig. 18). Immunohistochemical analysis of mPanINs from heterozygous female mice demonstrated absence of Usp9x expression in the pre-neoplastic and neoplastic cells (Supplementary Fig. 18), indicative of additional events such as X inactivation of the other locus in female mice32,33. mPanINs in Kras
\(^{LSL-G12D}\), Pdx1-cre; Usp9x
\(^{f/f}\) mice expressed intranuclear Smad4, similar to Kras
\(^{LSL-G12D}\), Pdx1-cre mice (Supplementary Fig. 19a). Additionally, early passage pancreatic cell cultures
Figure 3 | USP9X loss promotes PDA. a–c, Decreased USP9X expression correlates with shortened survival in an Australian post-surgical cohort (a) (8.7 versus 18.4 months, \( P = 0.0076 \); log-rank test), increased metastatic burden in an American autopsy series (b) (54% versus 19%, \( P = 0.0212 \); Fisher’s exact test), and diminished survival in a German post-surgical cohort (c) (11.1 versus 16.1 months, \( P = 0.037 \); log-rank test). d, Trichostatin A (TSA, red) and 5-aza-2′-deoxycytidine (AZA, blue) modestly increase USP9X mRNA expression in a panel of eight human PDA cell lines. The mean and s.e.m. of one representative experiment performed in triplicate are shown.

Prepared from these mice confirmed the absence of the Usp9x protein and altered regulation of Itch (Supplementary Fig. 19b). Although some mice died of local or metastatic pancreatic cancer, aggressive oral papillomas often required the culling of young mice and demonstrated that \( Kras^{G12D} \) and Usp9x loss also cooperated to transform keratinocytes (Supplementary Fig. 19c).

Although a recent report implicated USP9X as a pro-survival gene by stabilizing MCL1 (ref. 9), potential inhibitors of USP9X should be prepared with caution as we find that Usp9x has tissue-specific effects including a tumour suppressor role in oncogenic \( Kras \)-initiated pancreatic carcinoma. USP9X is probably epigenetically silenced in a subset of PDA, thus explaining why previous DNA sequencing efforts have failed to identify this as a participant in carcinogenesis, and indicating that clinically available epigenome modulators may be beneficial agents in such patients. ITCH is a likely mediator of pancreatic tumour suppression by USP9X, and continued investigation of the USP9X–ITCH pathway is warranted. More generally, the pan-pancreatic tumour suppression by USP9X, and continued investigation of beneficial agents in such patients. ITCH is a likely mediator of indicating that clinically available epigenome modulators may be subset of PDA, thus explaining why previous DNA sequencing efforts assessed previously20–22. Reads from sequenced tumours were mapped to the mouse genome assembly NCBI m37 and merged together to identify SB insertion sites, as previously described10. Redundant sequences, as well as insertions in the \( En2 \) gene and in the T2/Onc donor concatemer resident chromosome (chromosome 1), were removed. Mouse survival curves and cell culture experiments were analysed with the GraphPad prism program. The IHC histoscoring from the TMA samples and Kaplan–Meier survival curves were analysed with SPSS18, and the spearman–rho correlation coefficient (two-sided) between USP9X and ITCH was calculated. The IHC USP9X histocore and analysis was conducted using Fisher’s exact test on post-mortem samples.

Full Methods and any associated references are available in the online version of the paper.

Received 30 May 2011; accepted 5 April 2012.
Published online 29 April 2012.

1. Almoguera, C. et al. Most human carcinomas of the exocrine pancreas contain mutant c-K-ras genes. Cell 53, 549–554 (1988).
2. Caldas, C. et al. Frequent somatic mutations and homoygous deleions of the \( p16 \) (MTS1) gene in pancreatic adenocarcinoma. Nature Genet. 8, 27–32 (1994).
3. Redston, M. S. et al. p53 mutations in pancreatic carcinoma and evidence of common involvement of homocopolymer tracts in DNA microdeletions. Cancer Res. 54, 3025–3033 (1994).
4. Hahn, S. A. et al. PDC4, a candidate tumor suppressor gene at human chromosome 18q21.1. Science 271, 350–353 (1996).

5. Jones, S. et al. Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. Science 321, 1801–1806 (2008).

6. Coller, L. S., Carlson, C. M., Ravmohor, S., Dupuy, A. J. & Largaespada, D. A. Cancer gene discovery in solid tumors using transposon-based somatic mutagenesis in the mouse. Nature 436, 272–276 (2005).

7. Dupuy, A. J., Largaespada, D. A., Copeland, N. G. & Jenkins, N. A. Mammalian mutagenesis using a highly mobile somatic Sleeping Beauty transposon system. Nature 436, 221–226 (2005).

8. Hingorani, S. R. et al. Preinvasive and invasive ductal pancreatic cancer and its detection in the mouse. Cancer Cell 4, 437–450 (2003).

9. Schwickart, M. et al. Deubiquitinating USP9X stabilizes MCL1 and promotes tumour cell survival. Nature 463, 103–107 (2010).

10. Keng, V. W. et al. A conditional transposon-based insertional mutagenesis screen for genes associated with mouse hepatocellular carcinoma. Nature Biotechnol. 27, 264–274 (2009).

11. SPORE grant to D.A.T., D.A.L. and C.A.I.-D.; grants CA62924, CA128920 and CA106610 from the National Institutes of Health; the American Pancreatic Cancer Genome Initiative; and the Mouse Genome Interoperability Initiative.

12. Australian Pancreatic Cancer Genome Initiative

13. Garvan Institute of Medical Research

14.van der Kooy, K. et al. LGALS1 is a colorectal cancer susceptibility gene. Nature 463, 133–139 (2010).

15. Nagai, H. et al. Ubiquitin-like sequence in ASK1 plays critical roles in the recognition and stabilization by USP9X and oxidative stress-induced cell death. Mol. Cell 36, 805–818 (2009).

16. Sun, H. et al. Boror ubiquitination and Usp9x inhibition block kinase signalling and promote CML cell apoptosis. Blood 117, 3151–3162 (2011).

17. Mouchantaf, R. et al. The ubiquitin ligase Itch is auto-ubiquitylated in vivo and in vitro but is protected from degradation by interacting with the deubiquitylating enzyme USP9X. J. Biol. Chem. 282, 38738–38747 (2007).

18. Ferraioli, M. et al. Deubiquitinating UKP39 stabilizes MCL1 and promotes tumour cell survival. Nature 436, 221–226 (2005).

19. Deubiquitination of p53 by HAUSP is an important pathway for p53 stabilization. Nature 416, 548–563 (2002).

20. Hingorani, S. R. et al. Insertional mutagenesis identifies multiple networks of cooperating genes driving intestinal tumorigenesis. Nature 463, 133–139 (2010).

21. Hingorani, S. R., Schwickart, M., Wang, X., Skalnikova, K. & Hingorani, S. R. Deubiquitination of p53 by HAUSP is an important pathway for p53 stabilization. Nature 416, 548–563 (2002).

22. Wang, X., Soloway, P. D. & Clark, A. G. Paternally biased X inactivation in mouse neural tissue. Genome Biol. 11, R79 (2010).

23. Jackson, E. L. et al. Analysis of lung tumour initiation and progression using conditional expression of oncogenic Kras. Genes Dev. 15, 3243–3248 (2001).

24. March, H. N. et al. Insetional mutagenesis identifies multiple networks of cooperating genes driving intestinal tumorigenesis. Nature Genet. 43, 1202–1209 (2011).

25. Uren, A. G. et al. A high-throughput splinkerette-PCR method for the isolation and sequencing of retroviral insertion sites. Nature Protocols 4, 789–798 (2009).

Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We thank P. Labosky for assistance in generating the Rosa26-LSL-SB13 mouse; B. Bhagavan for pathology consultation; M. Tsa for providing the HPDE cell line; and N. Copeland and K. Mann for sharing pre-published information. We thank A. Gopinathan, H. Tiriac, D. Engle, D. Chan, F. Connor, S. Derkits and R.79 (2010).

Address correspondence and requests for materials to D.J.A. (da1@sanger.ac.uk) or D.A.T. (david.tuveson@cancer.org.uk). www.nature.com/reprints. The authors declare no competing financial interests.

©2013 Macmillan Publishers Limited. All rights reserved
METHODS

Mouse strains. Kras<sup>LSL-G12D</sup> (ref. 24), Pdx1-cre (ref. 8), T2/Onc (ref. 6), CAGGS-SR10 (ref. 6) and Rosa26-SL-BSI13 strains were interbred to generate Kras<sup>LSL-G12D</sup>, Pdx1-cre, T2/Onc; CAGGS-SB10 (KCTSB10) and Kras<sup>LSL-G12D</sup>, Pdx1-cre, T2/Onc; Rosa26-SL-BSI13 (KCTSB13) compound mutant mice. Non-quadruple mutant mice represented the comparison cohorts. Genomic DNA from tumours developed in KCTSB10 and KCTSB13 mice was obtained using the Puregene Core Kit A (Qiagen) and splinkerette PCRs were performed as described previously<sup>25,26</sup>. For the KCU cohort, Kras<sup>LSL-G12D</sup>, Pdx1-cre and Usp9x<sup>y</sup> mice were interbred with Usp9x<sup>y</sup> mice to generate the Kras<sup>LSL-G12D</sup>, Pdx1-cre; Usp9x<sup>y</sup>/y (KC) compound mutant mice, as well as the two control cohorts Pdx1-cre; Usp9x<sup>y</sup> (CU) and Kras<sup>LSL-G12D</sup>, Pdx1-cre (KC).

Generation of Rosa26-SL-BSI13 knock-in mice. TLI ES cells<sup>27</sup> were electroporated with linearized pRosa26-SL-SA-BSI13-BGHpolyA targeting construct and correctly targeted puromycin-resistant clones were identified by Southern blot. Germline transmission of the targeted allele was confirmed by Southern blot analysis of tail DNA from the agouti mice by microinjection into C57BL/6 blastocysts. Germline transmission of the correctly targeted puromycin-resistant clones were identified by Southern blot.

Plasmids. Plasmids, shRNAs and transfections. The plasmid pEF/GW-51/LacZ (Invitrogen) was used as control. The plasmid pBabe-neo (Oligoengine) expressed a short hairpin against mouse and human Usp9x (R960-25, Invitrogen), anti-c-Flip (ALX-804-127, Enzo Life Sciences), anti-c-Itch (sc-6266, Santa Cruz), anti-Myc tag (2276, Cell Signaling), anti V5 (5453, Cell Signaling), anti-Usp9x (A301-351A, Bethyl), anti-CC3 (a kind gift from R. reviewed by the present study). Cells were treated with 1 μM trichostatin A (Sigma) for 24 h or with 5 μM 5-aza-2’-deoxycytidine (Sigma) for 96 h where indicated to obtain RNA and protein lysates to assess USP9X expression. For anchorage-independent growth assay, cells were treated with 5 μM 5-aza-2’-deoxycytidine (Sigma).

Retroviruses. Phoenix cells were plated 24 h before transfection using the Profection Mammalian Transfection System Calcium Phosphate (Promega). Target cells were infected with retroviruses produced in the Phoenix packaging cell lines (24 and 48 h after transfection) in the presence of 8 μg ml<sup>-1</sup> polybrene (Sigma) and were selected with 2 μg ml<sup>-1</sup> puromycin (Sigma) or 1 mg ml<sup>-1</sup> G418 (Clontech). Experiments were performed using at least two independent cell line infected pools. Human PDA cells lines Panc1, SUIT2 and PATU2 infected with retroviral vectors expressed the ectropic receptor (eCoR).

Transformation, aneikia and EMT assays. Cells (1.5 × 10<sup>5</sup>) cells were plated in triplicate in 12-well plates and counted as indicated using a Z2 Coulter (Beckman). Cells were fed every other day. Anchorage-independent growth was assessed by colony formation in soft agar. Briefly, 150 cells were plated in duplicate in DMEM with 15% serum and 0.34% low-melting-point agarose (LMP, BioGene) onto 6-cm dishes coated with 0.5% LMP. Cells were fed twice a week and grown for 2 weeks. Colonies were counted in nine different ×20 fields. For the aneikia assay, 10<sup>4</sup> cells per 0.5 ml were plated in 24-well ultra-low cluster plates (Costar) to allow them to grow in suspension for 4 days. Cells were collected, washed with cold PBS and protein lysates were obtained. Cell line T4878 was cultured in matrigel as previously described<sup>27</sup>, plating 1,000 cells per well. Cells were fed every 2 days and grown for 4 days. Epithelial-to-mesenchymal transition (EMT) was determined by plating 10<sup>4</sup> cells per 6-well plates for 4 days to allow attachment, followed by treatment with human TGF-β1 (5 ng ml<sup>-1</sup>) (RD Systems) for 24 h. p21 induction was assessed after treatment with human TGF-β1 (5 ng ml<sup>-1</sup>) (RD Systems) for 24 h.

Real-time PCR. Total RNA from human PDA cell lines was extracted using the RNeasy Mini Kit (Qiagen), and total RNA (1 μg) was reverse transcribed into cDNA using the High Capacity RNA-to-cDNA kit (Applied Biosystems). Human USP9X expression was analysed by quantitative PCR (qPCR) using TaqMan gene expression assays Hs00245009_m1 (Applied Biosystems) on a 7900HT Real-Time PCR system (Applied Biosystems). Gene expression was normalized to human ACTB and GAPDH expression, assessed with the gene expression assays Hs99999903_m1 (Applied Biosystems), and showed relative to control samples. Western blot analysis. Cells were washed three times in cold PBS and lysed with boiling lysis buffer (1% SDS, 10 mM, pH 7.5 Tris, 50 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>). Lysates were boiled for 5 min, passed through a 20 μm needle to shear genomic DNA and centrifuged for 10 min at 14,000 r.p.m. Equivalent amounts of protein were resolved in 4–12% gradient SDS–PAGE gels (Invitrogen), transferred to Immobilon–P transfer membranes (Millipore), and incubated with the corresponding antibodies including anti-Ask1 (NB110-55482, Novus Biologicals), anti-Mcl1 (5453, Cell Signaling), anti-Usp9x (A301-351A, Bethyl), anti-CC3 (a kind gift from R. reviewed by the present study). Cells were treated with 1 μM trichostatin A (Sigma) for 24 h or with 5 μM 5-aza-2’-deoxycytidine (Sigma) for 96 h where indicated to obtain RNA and protein lysates to assess USP9X expression. For anchorage-independent growth assay, cells were treated with 5 μM 5-aza-2’-deoxycytidine (Sigma).

Immunohistochemistry. Formalin-fixed paraffin-embedded (FFPE) mouse tissues were cut into 3-μm tissue sections, and antigen retrieval was performed in 10 mM, pH 6.0 citric acid (for Usp9x and E-cadherin) or 10 mM, pH 8.0 EDTA (for Smo and pSm) for 20 min. BD) and 90°C for 20 min. BD) and signal detection for immunohistochemistry was achieved with biotinylated secondary antibodies (Vector Laboratories) using the Elite Vectastain ABC kit and peroxidase substrate DAB kit (Vector Laboratories). Primary antibodies used were anti-Usp9x, 1:200 (A301-351A, Bethyl); E-cadherin, 1:60182, BD) and anti-CC3 (a kind gift from R. reviewed by the present study).

Cell culture. Tumour pancreatic cancer cell lines were established from Kras<sup>LSL-G12D</sup>; Pdx1-cre (T4878 and T9394), and Kras<sup>LSL-G12D</sup>, Pdx1-cre; Usp9x<sup>y</sup> (KCU1 and KCU2) mice as described previously<sup>24</sup>. Cells were subsequently cultured in DMEM (Invitrogen), supplemented with 10% FCS (HyClone). The normal human pancreatic ductal cell line HPDE was provided by M. Tsao and cultured as described previously<sup>30,31</sup>. The human pancreatic cancer cell lines AsPC1 (CRL-1682) and BxPC3 (CRL-1687) were acquired from ATCC and cultured according to instructions. The other cell lines were obtained from Clare Hall Laboratories (CRUK). The human cell lines Panc1, MiaPaCa2, 81R.84, Hs766T, PATU2, SUIT2, A48 and MDA-Panc3 (PDA); CaCO2 and SW1116 (colorectal cancer); SKBR3 (breast cancer) and A549 (lung cancer) were cultured in DMEM supplemented with 10% FCS. The human cell lines U937 (histiocytic lymphoma), Ramos (Burkitt’s lymphoma), NCI-H2179 (lung cancer) and ZR75-1 (breast cancer) were cultured in RPMI (Invitrogen) supplemented with 10% FCS. Cells were treated with 1 μM trichostatin A (Sigma) for 24 h or with 5 μM 5-aza-2’-deoxycytidine (Sigma) for 96 h where indicated to obtain RNA and protein lysates to assess USP9X expression. For anchorage-independent growth assay, cells were treated with 5 μM 5-aza-2’-deoxycytidine (Sigma).

ACKNOWLEDGMENTS. This work has been supported by grant 6012781008 of the National Institutes of Health, Bethesda, USA. This work was supported by grant 6012781008 of the National Institutes of Health, Bethesda, USA.
Immunohistochemistry was performed on 5 times of patients after surgery from each centre were indistinguishable. In the subgroup there was no significant increase in patient survival. The median survival (35%) were chiefly treated with 5FU or gemcitabine-based regimens, but in this cohort, as few parameters were significant in univariate analysis, all were initially considered for Cox Proportional Hazard multivariate analysis in a backward elimination model, and assessed with the SPSS18 Software (IBM) with overall survival used as the primary endpoint. For the ICGC/APGI cohort, clincopathological variables analysed with a P value of less than 0.25 on log-rank test were entered into Cox proportional hazard multivariate analysis and the model was resolved using backward elimination. Statistical analysis was performed using StatView 5.0 Software (Abacus Systems). Disease-specific survival was used as the primary endpoint.

27. Tompers, D. M. & Labosky, P. A. Electroporation of murine embryonic stem cells: a step-by-step guide. Stem Cells 22, 243–249 (2004).
28. de Ridder, J., Uren, A., Kool, J., Reinders, M. & Wessels, L. Detecting statistically significant common insertion sites in retrovirial insertion mutagenesis screens. PLOS Comput. Biol. 2, e166 (2006).
29. Grützmann, R. et al. Gene expression profiling of microdissected pancreatic ductal carcinomas using high-density DNA microarrays. Neoplasia 6, 611–622 (2004).
30. Pilarsky, C. et al. Activation of Wnt signalling in stroma from pancreatic cancer identified by gene expression profiling. J. Cell. Mol. Med. 12, 2823–2835 (2008).
31. Oberdoerffer, P. et al. Efficiency of RNA interference in the mouse hematopoietic system varies between cell types and developmental stages. Mol. Cell. Biol. 25, 3896–3905 (2005).
32. Nathan, J. A. et al. The ubiquitin E3 ligase MARCH7 is differentially regulated by the deubiquitylating enzymes USP7 and USP9X. Traffic 9, 1130–1145 (2008).
33. Murray, R. Z., Jolly, L. A. & Wood, S. A. The FAM deubiquitylating enzyme localizes to multiple points of protein trafficking in epithelia, where it associates with E-cadherin and beta-catenin. Mol. Biol. Cell 15, 1591–1599 (2004).
34. Olive, K. P. et al. Inhibition of Hedgehog signaling enhances delivery of chemotherapy in a mouse model of pancreatic cancer. Science 324, 1457–1461 (2009).
35. Ouyang, H. et al. Immortal human pancreatic duct epithelial cell lines with near normal genotype and phenotype. Am. J. Pathol. 157, 1623–1631 (2000).
36. Furuwaka, T. et al. Long-term culture and immortalization of epithelial cells from normal adult human pancreatic ducts transfected by the E6E7 gene of human papilloma virus 16. Am. J. Pathol. 148, 1763–1770 (1996).
37. Debnath, J., Muthuswamy, S. K. & Brugge, J. S. Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in three-dimensional basement membrane cultures. Methods 30, 256–268 (2003).
38. Iacobuzio-Donahue, C. A. et al. DPC4 gene status of the primary carcinoma correlates with patterns of failure in patients with pancreatic cancer. J. Clin. Oncol. 27, 1806–1813 (2009).
Corrigendum: The deubiquitinase USP9X suppresses pancreatic ductal adenocarcinoma

Pedro A. Pérez-Mancera, Alistair G. Rust, Louise van der Weyden, Glen Kristiansen, Allen Li, Aaron L. Sarver, Kevin A. T. Silverstein, Robert Grützmann, Daniela Aust, Petra Rümmele, Thomas Knösel, Colin Herd, Derek L. Stemple, Ross Kettleborough, Jacqueline A. Brosnan, Ang Li, Richard Morgan, Spencer Knight, Jun Yu, Shane Stegeman, Lara S. Collier, Jelle J. ten Hoeve, Jeroen de Ridder, Alison P. Klein, Michael Goggins, Ralph H. Hruban, David K. Chang, Andrew V. Blankin, Sean M. Grimmond, Australian Pancreatic Cancer Genome Initiative, Lodewyk F. A. Wessels, Stephen A. Wood, Christine A. Iacobuzio-Donahue, Christian Pilarsky, David A. Largaespada, David J. Adams & David A. Tuveson

In this Letter, several authors from the Australian Pancreatic Cancer Genome Initiative were inadvertently omitted: Christopher J. Scarlett and Warren Kaplan from the The Kinghorn Cancer Centre; and Aldo Scarpa from the ARC-NET Center for Applied Research on Cancer. Amitabha Das was incorrectly listed as Amithabad Das. Furthermore, the correct address for the ARC-NET Center for Applied Research on Cancer, University of Verona, is: Policlinico GB Rossi, Piazzale LA Scuro 10, 37134 Verona, Italy. In the acknowledgements section, S.A.W. was supported by the National Health and Medical Research Council of Australia (NHMRC); and the APGI investigators by the University of Verona and Italian Ministry of University and Research (FIRB RBAP10AHJBJ). These errors have been corrected in the HTML and PDF of the original paper.