ADAM8 as a drug target in pancreatic cancer

Uwe Schlomann1,2, Garrit Koller1, Catharina Conrad2, Taheera Ferdous1, Panagiota Golfi1, Adolfo Molejon Garcia1, Sabrina Höfling1, Maddy Parsons3, Patricia Costa4, Robin Soper4, Maud Bossard4, Thorsten Hagemann4, Rozita Roshani4, Norbert Sewald5, Randal R. Ketchum6, Marcia L. Moss7, Fred H. Rasmussen7, Miles A. Miller8, Douglas A. Lauffenburger8, David A. Tuveson9, Christopher Nimsky2 & Jörg W. Bartsch1,2

Pancreatic ductal adenocarcinoma (PDAC) has a grim prognosis with <5% survivors after 5 years. High expression levels of ADAM8, a metalloprotease disintegrin, are correlated with poor clinical outcome. We show that ADAM8 expression is associated with increased migration and invasiveness of PDAC cells caused by activation of ERK1/2 and higher MMP activities. For biological function, ADAM8 requires multimerization and associates with β1 integrin on the cell surface. A peptidomimetic ADAM8 inhibitor, BK-1361, designed by structural modelling of the disintegrin domain, prevents ADAM8 multimerization. In PDAC cells, BK-1361 affects ADAM8 function leading to reduced invasiveness, and less ERK1/2 and MMP activation. BK-1361 application in mice decreased tumour burden and metastasis of implanted pancreatic tumour cells and provides improved metrics of clinical symptoms and survival in a KrasG12D-driven mouse model of PDAC. Thus, our data integrate ADAM8 in pancreatic cancer signalling and validate ADAM8 as a target for PDAC therapy.
Pancreatic ductal adenocarcinoma (PDAC) has the highest mortality rate of solid organ cancers with a 5-year survival rate <5% (ref. 1). This cancer type is remarkably homogenous in that 95% of PDAC originate from oncogenic mutations in the KRAS gene. Hence, representative authentic mouse models of PDAC with pancreas-specific expression of Kras have been generated2. In mice and man, KRAS mutations cause early-stage pancreatic intraepithelial neoplasias (PanINs) with subsequent development of progressive PDAC. A hallmark of PDAC is the massive infiltration of tumour cells into the pancreas and surrounding tissues including lymphatic organs, spleen and peritoneum, and the concomitant metastasis to the liver and lungs3–5. Infiltration of pancreatic tumour cells depends critically on extracellular matrix (ECM) remodelling6. Given the importance of the ECM in PDAC, the proteolytic release of membrane proteins (shedding) as well as ECM (e.g., collagens and fibronectin) degradation has previously been postulated to have a pivotal role in shaping the tumour microenvironment7,8. Members of the Metzincin superfamily, matrix metalloproteases (MMPs) and/or ADAM (A disintegrin and metalloproteinase) proteases have been described in these processes1. In particular, the contribution of ADAMs to extracellular remodelling12 and tumour growth, infiltration, metastasis and angiogenesis by shedding of membrane-associated proteins may be important3,13,14. In PDAC patient samples, elevated expression levels of ADAM8 (CD156a, MS2) have been identified versus normal pancreatic tissues. In normal pancreas, ADAM8 expression is very low and restricted to the plasma membrane of ductal cells and, to a lesser extent, of islets and acinar cells. In PDAC tissues, ADAM8 is strongly expressed in tubular complexes and in cancer cells. Based on clinical data, high ADAM8 expression levels are associated with a poor patient prognosis, resulting in reduced survival and increased metastatic spread13.

ADAM8 is a proteolytically active member of the ADAM protease family originally described in inflammatory processes16–18 and subsequently in many systems of the body19. Increased expression of ADAM8 was observed in other neoplasias, such as high-grade glioma20, lung adenocarcinoma21, prostate cancer22 and, more recently, in squamous head and neck carcinoma23, medulloblastoma24, osteosarcoma25 and breast cancer26, suggesting that ADAM8 has an active role in tumour progression. Thus, understanding the functional role of ADAM8 in tumour biology is important.

ADAM8 is localized in a few distinct cell types and the analysis of ADAM8-deficient mice inferred dispensability for normal development and homeostasis27,28. ADAM8 is typically expressed at low levels, giving rise to the current hypothesis that it is functionally irrelevant for homeostasis unless induced by inflammatory stimuli17 or neoplasias. Once upregulated, ADAM8 can overlap with the substrate spectrum of ADAM10 and ADAM17, two major shedding enzymes, and cleave proteins with immune functions such as tumour necrosis factor receptor 1 (ref. 28), L-selectin29, CD23 (ref. 30) and CXCL1 (ref. 31), as well as cell adhesion proteins such as CHL1 (ref. 32), thereby potentially modulating immune response or cell adhesion. Cleavage of other ADAM8 substrates such as Tie-2, Flt-1, VE-cadherin, Flk-1, EphB4, KL-1, CD31 and E-selectin33 or by cleavage of fibronectin12 may control tumour angiogenesis. Moreover, a role for ADAM8 in metastases34 and in cell invasiveness15,20,22 has been postulated, although the mechanism underlying these processes is unknown.

ADAM8 is activated by autocatalysis in the trans-Golgi network35 and, unlike other ADAMs, not by furin-like convertases. For in vitro activity, ADAM8 requires homophilic multimerization of at least two ADAM8 monomers on the cell membrane. This specific interaction of ADAM8 monomers offers a potential strategy for blocking ADAM8 activity by preventing ADAM8 multimerization in vivo. We have demonstrated previously that ADAM8–ADAM8 interactions critically depend on the disintegrin (DI)/cytsteel (DC)-rich domain35.

In addition, ADAM family members can bind to integrins in vitro via their DC-rich domains36; as prototype, human ADAM15 contains a canonical RGD motif in the integrin-binding loop (IBL) of the DI domain37. However, even for non-RGD containing ADAMs such as ADAM9, integrin binding was demonstrated. ADAM9 binding to β1 integrin causes migration of melanoma cells38, and for ADAM8, binding to zyxin was shown in osteoclast turnover39, suggesting that these ADAM–integrin interaction have functional relevance.

Although ADAM8 has been associated with increased tumour cell migration, invasiveness and metastasis via a combination of catalytic, adhesion and cell signalling functions15,20, no mechanistic data on ADAM8 in tumour progression and specifically in PDAC are available. Here we provide evidence for an involvement of ADAM8 in cancer signalling and tumour progression. Furthermore, we validate ADAM8 as a target in PDAC by introducing a specific ADAM8 inhibitor.

Results

ADAM8 inhibition strategy. Cellular activation of ADAM8 occurs in two steps. The first is intracellular prodomain removal in vesicles, whereas the second is metalloprotease (MP) domain removal from membrane-bound-activated ADAM8 (Fig. 1a). Autocatalysis implies that ADAM8 multimerizes (Fig. 1a), and that the ADAM8 DC-rich domain is critical for multimerization as demonstrated previously by using an antibody directed against the DC domain35. To define the regions in the DC domain involved in ADAM8–ADAM8 interactions, homology modelling of the ADAM8 DC domain was performed based on the ADAM10 sequence derived from Janes et al.40 (Supplementary Figs 1 and 2). We hypothesized that an extended loop structure exposing the RGD-like positions of the ‘KDX’ motif in the IBL in human and mouse ADAM8 might be responsible for the observed interactions (Fig. 1b).

In mouse and human ADAM8, the amino-acid residues K and D are exposed towards the outer aspect of the DI domain, thereby forming a potential contact interface (Fig. 1b, labelled in magenta). To generate a peptidomimetic compound, a series of cyclic peptides (six amino acids) mimicking the motif ‘RLSDKD’ of mouse ADAM8 in the IBL were generated. Amino acids R, L or S were inserted in the peptide as α-amino acids to alter the conformational constraint of the KDK motif and to generate a potentially more stable peptide for in vivo work. The cyclic peptide sequence RLSKDK with ‘s’ as serine named BK-1361 was most effective in blocking ADAM8-dependent cell adhesion in mouse and human cells with similar efficiencies (Supplementary Fig. 3) and ADAM8–ADAM8 interactions as shown by reduced FRET ( Förster resonance energy transfer/ FLIM (fluorescence lifetime imaging) efficiency. In contrast, a control peptide (CP; RLsADK; Fig. 1c) had no effect.

Multimerization of ADAM8 was investigated by native gel electrophoresis (Fig. 1d). Under native conditions, 100 ng of pro-ADAM8 associates in dimeric (~120 kDa) and trimeric (~180 kDa) complexes of pro-ADAM8, while in the presence of BK-1361, only monomers (~60 kDa) were detected (Fig. 1d, left panel). Detection of dimers and trimers suggests that dimers associate by DI domain (homophilic) interactions, whereas trimers could be formed by a different mode of interaction. At higher concentrations of recombinant ADAM8, we detected even larger complexes as a result of greater order multimerization.
Supplementary Fig. 3e) in agreement with more than one interaction mode that results in ADAM8 complex formation. These interactions can be blocked by BK-1361, and we further analysed whether prevention of complex formation affects ADAM8 activity in vitro.

Activation of pro-ADAM8 was detected over a time course of 120 h (Fig. 1d, right panel) but blocked by BK-1361 (Fig. 1d, bar graph) with an IC50 of 120 ± 19 nM. In a cell-based assay, BK-1361 and other peptides (Table 1) were tested for their ability to inhibit ADAM8-dependent extracellular resulting in an active

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Activation of pro-ADAM8 was detected over a time course of 120 h (Fig. 1d, right panel) but blocked by BK-1361 (Fig. 1d, bar graph) with an IC50 of 120 ± 19 nM. In a cell-based assay, BK-1361 and other peptides (Table 1) were tested for their ability to inhibit ADAM8-dependent extracellular resulting in an active
soluble MP domain (Figs 1a and 3b). In cell lysates, presence of the remnant form indicates cellular processing of ADAM8 as seen with no or inactive CPs (Fig. 1e, upper panel). In cell supernatants (SNs), processing results in detectable activity of released ADAM8 MP. BK-1361, but not other BK peptide variants, decreased this activity (Fig. 1e, lower panel). BK-1361 blocks mouse and human ADAM8 in vitro with similar efficiencies, a prerequisite for testing BK-1361 in orthotopic PDAC models using human donor cells in mouse hosts.

We next tested BK-1361 and variants for their ability to inhibit shedding of CD23, a known substrate of ADAM8 (Fig. 3). BK-1361 concentration of up to 10 μM (Table 2).

ADAM8 inhibition by hydroxamates and cyclic peptides.

Table 2 | Specificity of BK-1361 against other ADAM proteases and MMPs.

| Protease | Peptide substrate           | IC50 (nM) |
|----------|-----------------------------|-----------|
| ADAM8    | Dabcyl-His-Gly-Asp-Ala-Lys-Ser-Lys(Fam)-NH2 | 120 ± 19  |
| ADAM9    | Dabcyl-Leu-Ala-Gln-Met-Lys-Ser-Lys(Fam)-NH2 | >10,000   |
| ADAM10   | Dabcyl-His-Gly-Asp-Ala-Lys-Ser-Lys(Fam)-NH2 | >10,000   |
| ADAM17   | Dabcyl-Leu-Ala-Gln-Met-Lys-Ser-Lys(Fam)-NH2 | >10,000   |
| MMP-2    | Mca-PLGL-Dpa-AR-NH2         | 10,000    |
| MMP-14   | Mca-KPLGL-Dpa-AR-NH2        | 10,000    |

ADAM8, a disintegrin and metalloproteinase 8. IC50 values of peptide variants tested for A8 inhibition in vitro and for inhibition of CD23 shedding. Recombinant A8 (100 ng total protein) was incubated for 24 h with the respective inhibitors/peptides in a concentration of 20-10,000 nM. All activity was monitored using the CD23 fluorescence assay described in the Methods section.

ADAM8 increases extracellular MMP activity. A proteolytic activity matrix assay (PrAMA)41 was used for simultaneous detection of multiple activities in Panc1_ctrl and Panc1_A8 cells that could account for the observed invasiveness. Briefly, PrAMA is based on the knowledge of individual FRET-substrate MMP/ADAM cleavage signatures using purified enzymes.41 Panels of FRET-substrate cleavage measurements can be used to infer a dynamic, quantitative and specific profile of MMP/ADAM proteolytic activities from complex enzyme mixtures such as SNs and solubilized membranes (see Methods and Supplementary Fig. 5). PrAMA inference revealed increased activities of MMP-2 in SNs and MMP-14 (MT1-MMP) in cell membranes of Panc1_A8 cells compared with Panc1_ctrl cells (Fig. 2g and Supplementary Fig. 5). Enhanced ADAM8 activities were detected in SNs and membranes of Panc1_A8 cells. Gelatinzymography and western blotting for MMP-2 and MMP-14 confirmed increased activity of MMP-2 and higher membrane concentration of MT1-MMP (Fig. 2h). However, elevated MMP activities in Panc1_A8 cells are not due to transcriptional activation of MMP-2 and MMP-14 (Supplementary Fig. 4).

We further investigated whether BK-1361 is able to affect ADAM8-dependent invasiveness and MMP secretion of Panc1_A8 cells (Fig. 2i). BK-1361 and peptide variants were tested for their ability to block invasiveness of Panc1_A8 cells (Fig. 2i). A dose-dependent effect of BK-1361 on invasion of
Figure 2 | Effect of ADAM8 expression in Panc1 cells. (a) Western blotting (WB) of ADAM8 in PDAC cell lines Panc1, Capan-1 and AsPC-1. (b) ADAM8 immunofluorescence (anti-ADAM8, green) in non-permeabilized Panc1_ctrl and Panc1_A8 cells. ADAM8 membrane staining in Panc1_ctrl (upper panel) and Panc1_A8 (lower panel) cells. Insert (lower panel), ADAM8 localization in membrane protrusions. Scale bar, 50 μm. (c) WB of whole-cell lysates (WCLs) and supernatants (SNs) from Panc1_ctrl and Panc1_A8 cells; proform (arrowhead), mature (circle) and remnant form (diamond) of ADAM8, respectively. In Panc1_A8 SN, an ~30-kDa soluble ADAM8 MP fragment is detectable (filled arrowhead). (d) ADAM8 activities in SNs of Panc1_ctrl and Panc1_A8 cells were measured as fluorescence units (FU; n = 5) by cleavage of CD23 substrate; ***P < 0.0001 (Student’s t-test); rel, relative. (e) Scratch assay of Panc1_ctrl versus Panc1_A8 cells treated either with BB-94 (100 nM ‘+’ and 1 μM ‘++’) or with BK-1361 (200 nM ‘+’ and 500 nM ‘++’). Cell counts after t = 0 and 12 h (three independent experiments in triplicates, n = 9); ANOVA was used as statistical test; *P < 0.01, **P < 0.001 (Student’s t-test). (f) Invasion of Panc1_ctrl and Panc1_A8 cells in collagen I (Coll I), collagen IV (Coll IV), fibronectin (FN) and Matrigel (MG) analysed in Boyden chambers (18 h). Data are presented as mean ± s.d. from five independent experiments. ANOVA test was used. *P < 0.01, ***P < 0.0001 (Student’s t-test). (g) PrAMA inference analysis of SNs and membranes (Mem) from Panc1_ctrl and Panc1_A8 cells using five fluorogenic peptides to detect MMP activities. Activities of MMP-2, MMP-14 and ADAM8 are shown as relative changes to Panc1_ctrl. (h) WB and gelatine zymography of Panc1_ctrl and Panc1_A8 SNs for MMP-2, MMP-2 activity and MMP-14. (i) Invasion of Panc1_A8 cells treated with BK-1361 (10, 100 and 1,000 nM) or 1,000 nM CPs for 6 h (Table 1). Invasion was determined after 18 h. ANOVA test was used. *P < 0.005, **P < 0.001, ***P < 0.0001. (j) PrAMA from Panc1_A8 cells treated with vehicle, BK-1361, BB-94 and U0126 (1 μM, each) for 24 h. Values are given relative to Panc1_A8 treated with vehicle only. ANOVA was used as statistical test.
Panc1_A8 cells was observed. From CPs, only BK-1364 had a slight effect on invasion. In parallel, we performed PrAMA assays in Panc1_A8 cells to evaluate MMP-2, MMP-14, and ADAM8 activities in the presence of BK-1361, BB-94 and the extracellular regulated kinase (ERK) inhibitor U0126, respectively (Fig. 2). BK-1361 and BB-94 reduced activities of MMP-2, MMP-14 and ADAM8. ERK inhibition had a slight effect on MMP-2 activity and a greater effect on MMP-14 activity, whereas ADAM8 activity was not affected. These findings argue for an ERK1/2-mediated effect on MMP-14, and, to a lesser extent, on MMP-2 activation. ADAM8 as regulator of ERK1/2 activation is not directly affected by U0126.

Our data suggest an effect of pharmacological ADAM8 inhibition on invasiveness of PDAC cells. To determine the effect of a genetic ADAM8 knockdown on cellular invasiveness, we selected AsPC-1 cells with high endogenous ADAM8 levels (Fig. 2a) and generated AsPC-1 cell clones carrying a stable knockdown of ADAM8 (sh_A8). Three representative cell clones from different sh_A8 constructs were analysed for ADAM8 knockdown (Fig. 3a–c), cell migration and invasion (Fig. 3d,e and Supplementary Movies 1, 2). Knockdown of ADAM8 expression (Fig. 3a–c), cell migration and invasion (Fig. 3d,e and Supplementary Movies 1, 2), and generated AsPC_1 cell clones carrying a stable knockdown of ADAM8 (sh_A8). Three representative cell clones were analysed for ADAM8 knockdown (Fig. 3a–c), cell migration and invasion (Fig. 3d,e and Supplementary Movies 1, 2). Knockdown of ADAM8 expression (Fig. 3a–c), cell migration and invasion (Fig. 3d,e and Supplementary Movies 1, 2). Knockdown of ADAM8 in AsPC-1 cells caused a significant drop in cell migration depending on the gene dosage of ADAM8. Invasion of AsPC-1 cells was similarly affected by ADAM8 dosage (Fig. 3e). BK-1361 treatment of wild-type (wt) AsPC-1 cells was similar to the genetic knockdown of ADAM8 with 87 ± 3.5% inhibition (Fig. 3f). PrAMA assays were performed with AsPC-1_shCtrl ± BK-1361 and AsPC-1_shA8 cell clones 1 and 2 (Fig. 3g). Reduction of MMP-2 and MMP-14 activities were observed in AsPC-1_shA8 clones. In BK-1361-treated AsPC-1_shCtrl cells, MMP-2 was similarly affected, however, the effect on MMP-14 was less pronounced (Fig. 3g).

**ADAM8 interacts with integrin β1 in pancreatic cancer cells.**

The membrane localization of ADAM8 in Panc1_A8 cells suggests that ADAM8 is complexed with cellular integrins, thereby enhancing cell migration and invasiveness. To investigate this, co-immunoprecipitation (co-IP) experiments were performed in Panc1 cells expressing either control or a tagged ADAM8 construct (ADAM8-BiPro). As a result, β1 integrin, present in comparable amounts in Panc1_ctrl and Panc1_A8 cells, was co-IP with ADAM8 (Fig. 4a–c). To analyse cellular ADAM8–β1

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**Figure 3 | ADAM8 inhibition reduces MMP-2 and MMP-14 activity and affects invasiveness.** (a) Stable ADAM8 knockdown (shA8) of AsPC-1 cell clones were generated and characterized for ADAM8 protein (b) and mRNA (c) expression; rel, relative. Values are given as mean values from three independent experiments performed in triplicates ± s.e.m. Using these cell clones, (d) scratch and (e) Matrigel invasion assays were performed. In both assays, an ADAM8 gene dosage-dependent effect was observed. (f) Matrigel invasion was determined after addition of BK-1361 (0–1,000 nM) or CPs (Table 1). For (d–f), values are given as mean values ± s.e.m. ANOVA was used as statistical test. *P<0.1, **P<0.01, ***P<0.005 (Student’s t-test). (g) PrAMA inference analysis of two AsPC-1 ADAM8 knockdown clones and of AsPC-1_shCtrl cells treated with BK-1361, relative to AsPC-1_shCtrl cells. Note that both genetic and pharmacological inhibition of ADAM8 show similar effects, that is, reduction of MMP-2 and MMP-14 activity with the exception of MMP-14 in AsPC-1 cells.
Figure 4 | ADAM8 multimerizes and interacts with integrin β1 in tumour cells. (a) Western blot (WB) of integrin β1 subunit (Int β1) in Panc1_ctrl (ctrl) and Panc1_A8 (A8) cells (~115 kDa). β1-Tubulin (β-tub) was used as loading control. (b) In Panc1 cells transiently expressing control vector (CV) or ADAM8-Bipro (A8BiPro), anti-Bipro or unrelated IgG was used for immunoprecipitation (IP). Blots were probed with polyclonal β1 integrin antibody. (c) Re-probing of blot (b) using polyclonal anti-ADAM8 antibody. (d) FRET/FLIM analysis of ADAM8-ADAM8 interactions. Panc1 cells were co-transfected with ADAM8-GFP and ADAM8-mCherry (A8-mCh) constructs; (upper panel) FLIM in the absence (control IgG) and (lower panel) in the presence of anti-β1 antibody (anti-β1), determined in nanoseconds (ns). (e) FRET efficiency (in %) for ADAM8–ADAM8 interaction in anti-β1 antibody-treated Panc1 cells (n = 10). As statistical test, ANOVA was used; **P < 0.001. (f–h) Localization of ADAM8 protein in MDA-MB-231 control (shCtrl, f) or in MDA-MB-231 cells with a stable β1 integrin knockdown. Staining of ADAM8 in red and β1 integrin in green. Boxed areas in f are a lamellipod structure zoomed in. In contrast to boxed area in f, the area in g shows diffuse staining of ADAM8 mainly in vesicles, the entire cell is flattened and cell area increases significantly. Scale bars (f, g), 10 μm. (h) Localization of ADAM8 in MDA-MB-231 cells treated with peptide BK-1362 (1 μM) or with BK-1361 (1 μM). Similar to g, cells flatten and localization of ADAM8 in lamellipodia is changed. (i) Quantification of cell areas of BK-1362- and BK-1361-treated cells, given as mean ± s.d. from 50 cell areas. **P < 0.001 (Student’s t-test). (j) Quantification of FRET efficiency between ADAM8-GFP and ADAM8-mCh in MDA-MB-231 control (shCtrl) and MDA-MB-231 with a stable β1 integrin knockdown (shβ1). In the right panel, antibody 12G10 was used to detect activated β1 integrin. (k) Interaction of ADAM8-GFP and ADAM8-mCh in MDA-MB-231 control (shCtrl) and MDA-MB-231 with a stable β1 integrin knockdown (shβ1). ANOVA was used; *P < 0.01 and **P < 0.001 (Student’s t-test).
intergrin and ADAM8--ADAM8 interactions, FRET/FLIM analyses were performed to detect FRET in cell lines expressing fusion proteins ADAM8-GFP and ADAM8-mCherry, respectively. ADAM8 multimerization was detected in Panc1 cells (Fig. 4d,e), indicated by FRET efficiency of 7.5 ± 0.93%. In cells, complex formation and membrane localization of ADAM8 in vivo involves β1 integrin, since treatment of Panc1 cells with a β1 integrin-blocking antibody (Fig. 4d,e) resulted in a significant drop to 3.1 ± 0.8% FRET efficiency. Moreover, MDA-MB-231 breast cancer cells lacking β1 integrin (Fig. 4f) show a significant change in cellular morphology while ADAM8 localization in lamellipodium structures is lost. Interestingly, administration of BK-1361 causes a similar change in cell morphology (Fig. 4h,i). We conclude that β1 integrin knockdown or specific ADAM8 inhibition have similar effects on ADAM8 membrane localization (Fig. 4j). In addition, areas of positive ADAM8-FRET in MDA-MB-231 cells were analysed for β1 integrin (Fig. 4k,l). In most areas, activated β1 integrin was detected by antibody 12G10, suggesting that ADAM8 interaction and β1 integrin activation are correlated.

To analyse whether ADAM8 interactions cause altered intracellular signalling, a mitogen-activated protein kinase (MAPK) array was used to screen for kinase phosphorylation in Panc1_ctrl and Panc1_A8 cells (Supplementary Fig. 6). As potential downstream effectors of the observed ADAM8-β1 integrin interaction, we investigated phosphorylation of focal adhesion kinase (FAK), ERK1/2, Akt and p38 in Panc1_A8 versus Panc1_ctrl cells. Focal adhesion kinase was described as β1 integrin interacting protein[2]. In western blots, increased phosphorylation of FAK at residue Tyr397 correlates with ADAM8 expression levels in Panc1 cells (Fig. 5a). In addition to pFAK, we detected increased phosphorylation of ERK1/2 (p44/p42) in Panc1_A8 cells by western blotting using corresponding phospho-specific antibodies (3.4-fold ± 0.2 Panc1_A8 versus Panc1_ctrl; Fig. 5b). The observed increase in pERK1/2 was reduced in Panc1_A8 cells treated with BK-1361 (Fig. 5b). To correlate ERK1/2 phosphorylation with the observed invasiveness of Panc1 cells, Matrigel invasion assays were performed in the presence of U0126 (Fig. 5c). U0126 blocked ERK1/2 phosphorylation in Panc1_ctrl and Panc1_A8 cells, and resulted in decreased invasion of Panc1_A8 cells. In addition, AsPC-1_shCtrl and three AsPC-1_shA8 cell clones with different ADAM8 levels were analysed for pERK1/2 levels (Fig. 5d). In cell clone AsPC-1_shA8/2, pERK1/2 levels were reduced by 2.9 ± 0.3 fold, suggesting that ADAM8 expression levels are correlated with pERK1/2. In AsPC-1 cells, ADAM8 levels affect MEK1/2, p-Akt and c-Raf activation (Fig. 5e). In addition, a β1 integrin antibody that blocks activation was able to reduce pERK1/2 levels in Panc1_A8 cells, demonstrating that β1 integrin is required for ADAM8-dependent ERK1/2 activation (Supplementary Fig. 7).

To investigate whether the observed FAK and ERK1/2 activation depends on membrane-bound ADAM8, Panc1 cells were transfected with wt ADAM8 (A8 wt), an ADAM8 construct lacking the cytoplasmic domain (A8ΔCD), or a soluble ADAM8 (A8ecto) construct (Fig. 5f). First, we confirmed that all constructs are catallytically active, as all three ADAM8 proteins shed CD23 (sCD23) from the cell membrane. Interestingly, neither A8ΔCD nor the ectodomain of ADAM8 were able to activate FAK and ERK1/2 (Fig. 5f), suggesting that intracellular signalling mediated by ADAM8 requires membrane localization of ADAM8 and the presence of the cytoplasmic domain. As potential substrates for ERK activation, that is, the epidermal growth factor receptor (EGFR) ligand family such as Heparin-binding epidermal growth factor (HB-EGF), EGFR or amphiregulin, were screened (Supplementary Fig. 8). We have not identified significant EGFR ligand release, so that ADAM8-β1 integrin interactions might act independent from EGFR signalling.

**Role of ADAM8 in PDAC and effect of BK-1361 in vivo.** Based on our findings that ADAM8 inhibition blocks invasiveness and ERK1/2 signalling in PDAC cells, we used BK-1361 to validate ADAM8 as a therapeutic target in PDAC in vivo. Initially, acute and chronic toxicity analyses were performed in C57BL/6j mice. In single and repeated dose applications, doses of up to 10 μg g⁻¹ body weight were well tolerated as mice showed no abnormalities, weight loss or motor performance over 4 weeks. After necropsy, organs investigated showed no abnormalities at histological level (Supplementary Fig. 9). Owing to the lack of acute and chronic toxicity, a dose of 10 μg g⁻¹ body weight was applied daily for subsequent in vivo applications.

Initially, orthotopic injections of Panc1 cells into mouse pancreas were performed (Fig. 6a–g) in three cohorts (n = 12 each); cohort 1 received Panc1_A8 cells, cohort 2 received Panc1_ctrl cells and cohort 3 received Panc1_A8 cells followed by daily intraperitoneal injection of 10 μg g⁻¹ BK-1361. Mice were monitored for 12 days, by which time most of the mice injected with Panc1_A8 cells were moribund and reached end point criteria. In contrast, mice injected with Panc1_ctrl cells or Panc1_A8/BK-1361 treatment showed improved clinical parameters. At end point, pancreatic tumours formed from Panc1_ctrl cells were significantly smaller than from Panc1_A8 cells (Fig. 6a,b). Moreover, tumours obtained from mice that received Panc1_A8 cells and daily injections of BK-1361 were significantly smaller. These data indicate that inhibition of ADAM8 reduced tumour load to almost the value of Panc1_ctrl-derived tumours (0.42 g for Panc1_ctrl versus 0.62 g for Panc1_A8/BK-1361). By histology, a significant invasion of Panc1_A8 cells into the pancreatic tissue was detected, whereas in tumours derived from Panc1_A8/BK-1361 cells, tumour masses embedded in Matrigel were primarily localized to the implantation site even after 12 days, as the boundaries of pancreas and implanted tumour mass were still distinct (Fig. 6c). In addition, there were signs of necrosis inside the implanted tumour treated with BK-1361 (Fig. 6c), inferring that non-invasive Panc1 cells undergo necrotic changes. Moreover, ADAM8 levels in Panc1_ctrl cells located in the tumour were increased under hypoxic conditions. Co-staining for pERK1/2 was observed in infiltrative ADAM8-positive tumour cells (Fig. 6d, upper panel).

Metastasis and infiltration is the major cause for the observed morbidity in PDAC. Since ADAM8 was discussed in the context of infiltration and metastasis[26,34], we investigated orthotopic mice for infiltration of close structures such as peritoneum, diaphragm and spleen, and liver metastasis. From mice injected with Panc1_A8 cells, we found significant infiltrates in adjacent organs (Fig. 6e,f). Macroscopic inspection and haematoxylin/eosin stain of tissue sections revealed higher invasion into spleen and diaphragm of Panc1_A8-injected mice (Fig. 6e). Analysis of infiltration areas showed enhanced invasive behaviour of Panc1_A8 cells versus Panc1_ctrl and Panc1_A8/BK-1361 cells (Fig. 6f) in peritoneum, diaphragm and spleen. ADAM8 staining of liver sections revealed occurrence of micrometastases with higher frequencies in Panc1_A8 implanted mice compared with Panc1_ctrl and Panc1_A8/BK-1361 (Fig. 6g). Metastases frequencies were markedly different between Panc1_A8, Panc1_ctrl and Panc1_A8/BK-1361. Furthermore, the implantation of AsPC-1_shCtrl and AsPC-1_shA8 cells was analysed (Fig. 6h). AsPC-1_shCtrl cells caused large streams of tumour cells invading the pancreatic tissue with an infiltration area of 21 ± 2.8%. In contrast, AsPC-1_shA8 cells were located close to the injection site and showed less invasive behaviour with infiltration areas of 3.4 ± 1.2% (P > 0.01). Thus, data derived from genetic ADAM8 knockdown support the results obtained with ADAM8 inhibition using BK-1361.
The therapeutic effect of ADAM8 inhibition in vivo was analysed in mice with genotype KrasLSL-G12D, Trp53R172H/+, Pdcd4Cre+ (KPC)2, a genetically engineered PDAC mouse model. Injections of BK-1361 were started around the onset of PanINs. KPC control groups received injections of either saline (as in a clinical setting) or CP (BK-1362). Control groups showed progression to PDAC with a median survival of 15.5 weeks for saline-treated mice and 16 weeks for BK-1362. In contrast, BK-1361-treated KPC mice have extended median survival times of 24.2 weeks and 16 weeks for BK-1362. In BK-1361-treated KPC mice, ADAM8 and pERK1/2 staining is restricted to acinar structures, suggesting that despite occurrence of neoplasias, tumour infiltration was low. In BK-1361-treated KPC mice, ADAM8 and pERK1/2 staining is restricted to acinar structures, suggesting that despite occurrence of neoplasias, tumour infiltration was reduced while the acinar architecture was more conserved.

**Figure 5** | ADAM8 intracellular kinase signalling. (a) Antibody detection of FAK phosphorylation (Y397) in Panc1_A8 and Panc1_ctrl cells 24 h after plating cells on plastic. Total FAK antibody was used to control equal loading and anti-ADAM8 antibody confirms ADAM8 expression. A β-tubulin antibody was used as loading control. Note a 2.8-fold increase in pFAK in Panc1_AB cells compared with Panc1_ctrl cells. (b) Western blotting (WB) of relative (rel.) phosphorylation of ERK1/2, in Panc1_ctrl and Panc1_AB cells as determined by antibodies directed against phosphorylated forms of respective kinases. Lower panel, ERK1/2 phosphorylation in Panc1_ctrl and Panc1_AB cells in the presence of ERK1/2 inhibitor U0126 (10 μM) after 18 h incubation, indicating a strong correlation between ERK1/2 phosphorylation and invasion in Panc1 cells. ANOVA statistical test was used; *P<0.01, **P<0.001 (Student’s t-test). (c) Correlation of ADAM8 and pERK1/2 levels in AsPC-1 control (shCtrl) and knockdown (shA8) cells. In clone AsPC-1_shA8/2, levels of pERK1/2 are reduced by threefold. (d) Relative invasiveness of Panc1_ctrl and Panc1_AB cells (in duplicates) in the presence of ERK1/2 inhibitor U0126 (10 μM) in protein lysates of AsPC-1_shCtrl and AsPC-1_shA8 cells. Kinases and their respective phosphorylation sites are indicated on the right. (e) Relative kinase phosphorylation of the ERK and AKT signalling pathways after 18 h incubation with (+) or without (−) U0126 (10 μM) in protein lysates of AsPC-1_shCtrl and AsPC-1_shA8 cells. Kinases and their respective phosphorylation sites are indicated on the right. (f) Membrane-bound ADAM8 with cytoplasmic domain is required for FAK and ERK1/2 activation. Panc1 cells were transiently transfected with full-length ADAM8 construct (A8 wt), ADAM8 lacking the cytoplasmic domain (A8ΔCD), or with a construct encoding the soluble ectodomain (A8ecto) of ADAM8, respectively. To control for cellular ADAM8 activity, the amount of soluble CD23 was determined in SNs by using an HA-tag antibody. The presence of ADAM8 protein variants, relative levels of pFAK and pERK1/2 were detected by WB, using β-tubulin as loading control. Levels of FAK and ERK phosphorylation were quantified from three independent experiments, given as mean ± s.e.m. WCL, whole-cell lysates.
Figure 6 | Effect of ADAM8 inhibition in an orthotopic pancreatic cancer model. (a) Pancreas morphology after orthotopic injection of Panc1_A8, Panc1_ctrl or Panc1_A8 cells followed by BK-1361 injection (10 μg·g⁻¹ daily, n = 12 per group). (b) Tumour load (g) 12 days after implantation (n = 12); Tukey’s linear contrast test was used. **P < 0.001, ***P < 0.0001 (Student’s t-test) (c) Representative ADAM8 IHC/Periodic Acid Schiffs (PAS) staining of pancreas tumours from mice injected with Panc1_A8, Panc1_ctrl compared with implanted Panc1_A8/BK-1361 treated. Significant invasion of Panc1_A8 cells (arrows, upper panel left); across the implantation border (asterisk, upper left panel); Panc1_ctrl and Panc1_A8/BK-1361 injected tumours show less invasion from implantation site. Lower panel, ADAM8 staining; infiltration and loss of ductal architecture, increase in ADAM8 in Panc1_ctrl-implanted tumours and delineated between implantation site and surrounding pancreas tissue in Panc1_A8/BK-1361-treated mice. Lower left panel, enlarged image shows invasion of ADAM8-positive tumour cells; enlarged images (boxed in upper panel); box1: upregulation of ADAM8 in cell mass of Panc1_ctrl cells; box 2: enlarged view of the border between implantation site and surrounding ductal tissue; box 3: signs of intratumoral necrosis in tumours treated with BK-1361 (scale bars, 100 μm). (d) pERK1/2 staining of tumours. Only for Panc1_A8 (top), tumour cells stain positive for pERK1/2 (scale bar, 100 μm). (e) Morphology of spleen and diaphragm from mice injected with either Panc1_A8, Panc1_ctrl, or Panc1_A8/BK-1361. In PAS stains (lower panel), infiltration of tumour cells in the diaphragm is only seen in Panc1_A8. Scale bars, 100 μm. (f) Quantification of infiltration areas in peritoneum, diaphragm and spleen. Values are in % of total tissue area ± s.e.m. (n = 12). (g) ADAM8⁺ micrometastases in livers from Panc1_A8-injected mice; in contrast, metastases (mets) were mostly absent in livers from Panc1_ctrl and Panc1_A8/BK-1361-treated mice (scale bar, 200 μm); relative frequency (Freq.) of liver metastases in mice injected with Panc1_A8, Panc1_ctrl and Panc1_A8/BK-1361, respectively (n = 12). (h) Pancreas histology (PAS, ADAM8 IHC) after orthotopic implantation of AsPC-1_shCtrl and AsPC-1_shA8/2 cells. Scale bar, left upper panel, 100 μm. ADAM8⁺ cells in pancreas injected with AsPC-1_shCtrl cells, weaker staining with AsPC-1_shA8 cells. Infiltration areas in pancreata from six mice per group were quantified. Mean ± s.e.m. **P < 0.01 (Student’s t-test).
(Fig. 7g). Staining intensities of pERK1/2 and ADAM8 is reduced in BK-1361-treated KPC mice (Fig. 7h), suggesting that, in vivo, ADAM8 inhibition leads to reduced activation of pERK1/2.

**Discussion**

Our study links the available clinical data on ADAM8 expression in pancreatic cancer cell lines and in PDAC to mechanistic data on ADAM8 in PDAC tumour progression. Using a novel proof-of-concept ADAM8 inhibitor, we demonstrate that ADAM8 inhibition in PDAC leads to reduction of tumour load, infiltration and metastasis in vivo by affecting downstream signalling of ADAM8, thus further supporting the important role of ADAM8 in PDAC.

ERK1/2 signalling is considered a major pathway in PDAC, and the EGF/EGFR pathway has been established as an...
upstream effector of ERK1/2, so that PDAC development and progression is associated with overexpression of EGFR ligands and ADAM17 (refs 43,44). Aberrant EGF signalling is associated with ERK1/2 signalling in tumour proliferation and migration of pancreatic cancer cells45. Since ADAM8 is an active shedding enzyme, evidence of a function of ADAM8 in EGF/EGFR signalling was investigated. No ADAM8-dependent shedding of EGFR ligands such as EGF, epiregulin, amphiregulin and HB-EGF was detected in substrate screens (Supplementary Fig. 8). This suggests that ADAM8 could act independent from the EGF/EGFR pathway, as demonstrated for ADAM10 and ADAM17 (ref. 14). Thus, it is likely that ADAM8 stimulates non-EGFR pathways for PDAC progression, which is in agreement with recent experimental observations that progression of PDAC is beyond sole EGF function46. For this hypothesis, we provide experimental evidence by demonstrating a link between ADAM8 and ERK1/2 signalling.

Our data suggest that ADAM8 interacts with β1 integrin, an essential signalling module in PDAC47. The β1-associated signalling pathways involving kinases such as FAK, p38, Akt and ERK1/2 are altered by expression of ADAM8. In particular, ERK1/2 activation is correlated with ADAM8 levels, as shown for cells either overexpressing ADAM8 or AsPC-1 cells bearing a genetic ADAM8 knockdown. In accordance with potent effects of ERK1/2 inhibitors PD98059 and U0126 on reducing Panc1 cell invasion, we propose an ADAM8-induced ERK1/2 activation via the ADAM8 cytoplasmic domain, thereby regulating MMP activities more effectively than inhibitors for Akt and p38.

Although ADAM8 can cleave fibronectin12, the effect of ADAM8 on cell migration into different ECM substrates could better be explained by ERK1/2 activation in Panc1 cells that regulates the extracellular activities of MMPs. In accordance, increased extracellular activities of MMP-2 and MMP-14 were detected by PrAMA assays. MMP-2 was shown to promote increased extracellular activities of MMP-2 and MMP-14 were better be explained by ERK1/2 activation in Panc1 cells that

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in phase III trials owing to unwanted side effects. Even a short in vivo half-life of ~34 min for BK-1361 (Supplementary Fig. 10), comparable to the half-life of cilengitide, should not limit the therapeutic use of BK-1361, and thereby provides a platform for further preclinical studies including alternative delivery routes and structure-activity relationship.

Methods

Mice. Kras\(^{LOH}\) – G12D; ptp53\(^{R172H}\) – Pdx2\(^{-/-}\) (KPC) were described earlier.2 After weaning, all mice were genotyped. Equal numbers of male and female mice were taken for analysis. All animal experiments were conducted in accordance with Home Office regulations under a relevant project (T.H.) and with the German Law on the protection of animals, and were approved by the Local Government (Regierungspärismus Giessen, I.W.B.).

Cell lines. Panc1 and AsPC-1 cells were obtained from Sigma (UK); HEK-293, COS7 and MDA-MB-231 cells were purchased from American Type Culture Collection (ATCC). AsPC-1 cells were grown in RPMI medium; all other cell lines COS7 and MDA-MB-231 cells were purchased from American Type Culture Collection (ATCC). Purities were comparable to the half-life of cilengitide, should not limit the therapeutic use of BK-1361, and thereby provides a platform for further preclinical studies including alternative delivery routes and structure-activity relationship.

Quantitative PCR analysis. After reverse transcription using 1 ng total mRNA, quantitative PCR was performed using SYBR Green kits (Bio-Rad, USA, 1:2000). Amplification was performed in a 96-well black-coated plate using a MyCycler (Bio-Rad Laboratories, Hercules, CA). After each cycle, the fluorescence was measured using the Typhoon laser scanning system. Relative quantification was performed by the Pfaffl algorithm, and expressed as the fold change of gene expression in the experimental samples compared to the control samples.

Native gel electrophoresis. To assess complex formation of recombinant human ADAM8 (Met1-Pro497) was obtained from R&D Systems. ADAM8 autocaltotic activation assays were performed in 50 mM Tris, 10 mM CaCl\(_2\), and 150 mM NaCl, pH 7.5 (TCN) buffer for the indicated times at 37°C. For inhibition assays using the cyclic peptide inhibitor, concentrations varied from 10 nM up to 5 \(\mu\)M.

Activity assays with ADAM8. Activity of recombinant human ADAM8 catalytic/DI domain was monitored at 2 min intervals using the fluorescent substrate Dabcyl\((\text{4-((4-(dimethylamino)phenyl)azo)benzoic acid})\) as a quencher.3 Activity assays with ADAM8 were performed in a 96-well black-coated plate using a MyCycler (Bio-Rad Laboratories, Hercules, CA). After each cycle, the fluorescence was measured using the Typhoon laser scanning system. Relative quantification was performed by the Pfaffl algorithm, and expressed as the fold change of protein expression in the experimental samples compared to the control samples.

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Determination of MMP/ADAM activities in PDAC cell lines. PRAME activity was measured by processing Panc1, AsPC-1, and MKN-28 cells through a seven-plate well process for 24 h in serum-free DMEM medium without phenol red. Medium was removed, spun to remove cell fragments and set aside for assaying. Cells were washed with PBS and then scraped from the plate and resuspended in a 1.5 ml tube in a cold solution of 0.25 M sucrose, 50 mM Tris, pH 8, and 4 % Brij-35. Reactions were run in a 96-well black-coated plate. The concentration of PRAME enzyme was typically 10 ng per reaction. For inhibition assays using the cyclic peptide inhibitor, concentrations varied from 10 nM up to 5 \(\mu\)M.

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ADAM8, ADAM10, ADAM17, MMP-2 and MMP-14 that were determined using protease activities were inferred with PrAMA by comparing the pattern of substrate fluorescence units versus time were monitored with a Fluostar BMG Optima using incubated with either 20 mM substrate concentrations in 60 μl of assay buffer (see above) were incubated with either 20 μl of medium or 10 μl of resuspended membranes. Fluorescence units versus time were monitored with a Fluostar BMG Optima using excitation and emission wavelengths of 485 and 530 nm, respectively. Specific protease activities were inferred with PrAMA by comparing the pattern of substrate cleavage rates for each sample to a matrix of known substrate specificities for ADAM8, ADAM10, ADAM17, MMP-2 and MMP-14 that were determined using purified enzymes (Miller et al.35). Before performing PrAMA, substrate cleavage rates were first converted to fold change over control measurements as indicated. Each PrAMA experiment was repeated three times and values for activities are given as relative to defined control measurements (a.u.).

Immunofluorescence. MDA-MB-231, Panc1 cells and stable derivatives were grown on coverslips, and were fixed with 3.7% paraformaldehyde. ADAM8 was detected in unpermeabilized cells by goat polyclonal antibody AF1031 (R&D Systems) as primary antibody. As secondary antibody, we used goat-anti-rabbit-Cy3 (1:500, Sigma). Images were acquired with confocal microscopy imaging using a Nikon A1R microscope equipped with CFI Plan Fluor ×40 oil objective. Images were captured and exported with NIS Elements software (Nikon) and presented as TIF files.

Immunoblotting and IP experiment. Equal amounts of protein were loaded onto 10% reducing polyacrylamide (PAA) gels. The proteins were transferred on nitrocellulose under non-denaturing conditions at 120 V for 1 h followed by Coomassie destaining solution to visualize bands of active enzyme. The Matrigel was allowed to set (10 s), the needle was gently removed from the pancreas and the area was swabbed with iodine to devitalize any stray cells in the injection site. The pancreas and spleen were replaced in the abdomen and the incision site closed with 3–4 interrupted 9-0 Prolene sutures (Ethilon 5–0 PS-3, Ethicon, Piscataway, NJ, USA). The Matrigel method of orthotopic tumour implantation has resulted in 100% tumour uptake with little evidence of extra-pancreatic leakage. Panc1 and AsPC-1 cell-derived tumours were analysed 12 days after implantation.

Gelatination and coagulation. Protein samples were prepared in non-reducing sample buffer without boiling before the experiment. Gelatin (0.1%) was added in a separating gel to co-polymerize with PAA. During electrophoresis, proteins are separated in the PAA gel, while SDS present in the gel preserves MMPs in an inactive state. After the run, gels were washed with renaturing buffer (2.5% Triton X-100; 2 mM Tris pH 8.0, 150 mM NaCl2, 1% NP40, 0.5% sodium deoxycholate, 1.5 mM MgCl2 and cOmplete). The sonicated lysates were centrifuged two times at full speed to remove cell debris. Cleared lysates were subjected to IP using specific antibodies in concentrations of 0.2 μg ml−1 (for anti-BiPro) and 2.5 μg ml−1 (for anti-ADAM8) overnight at 4°C. As unspecific binding controls, IgG was used instead of specific antibodies. After binding of precipitated material to Protein-G-sepharose and washing steps, the precipitated proteins were eluted by boiling in 2 × SDS-loading buffer and were analysed by western blotting.

FRET/FLIM. FLIM was performed and data were analysed as described previously39. FLIM capability was provided by time-correlated single-photon counting electronics (Becker & Hickl, SPC 700). Widefield acceptor monomeric Red Fluorescent protein (mRFP) images were acquired using a CCD camera (Hamamatsu) at exposure times of <100 ms. Data were analysed using TR2 software (developed by Dr Paul Barber). All histogram data are plotted as mean FRET efficiency from >12 cells per sample. Lifetime images of exemplary cells are presented using a pseudocolour scale whereby blue depicts normal GFP lifetime (C0). As unspecific binding controls, IgG was used instead of specific antibodies. After binding of precipitated material to Protein-G-sepharose and washing steps, the precipitated proteins were eluted by boiling in 2 × SDS-loading buffer and were analysed by western blotting.

Activated MAPK assay. The human phospho-MAPK Array Kit (ARY002B; R&D Systems) was used to analyse the influence of ADAM8 on the activation state of altogether 26 MAPKs and other serine/threonine kinases. The assay was used according to the manufacturer's instructions. In brief, cells of the stable control PANC1 clone and the ADAM8 overexpressing clone were lyzed in lysis buffer 6, sonicated and a Bradford assay was used to determine the protein concentrations. Each array membrane was incubated in 2 ml array buffer 5 for 1 h at room temperature. After removal of array buffer 5 from the membranes, the lystate Detection Antibody Cocktail was added and the mixture was incubated for 1 h at room temperature. After removal of array buffer 5 from the membranes, the lystate Detection Antibody mixture was added to the membrane. The membranes were incubated at 4°C on a rocker overnight. Each membrane was washed three times with 1 × wash buffer (20 ml, 10 min each) and incubated with 2 ml streptavidin-HRP (1:20,000 in array buffer 5) for 30 min at room temperature on a rocker. After three repeating washing steps with 1 × wash buffer (20 ml, 10 min each), an ECL substrate (Thermo Scientific) for HRP enzyme and an ECL camera (Intas, Gottingen, Germany) were used to detect the signals. The signals were quantified with the National Institutes of Health (NIH) ImageJ software package (freeeware).

ADAM8 DI structure modelling. A BLAST (a) search of the Protein Data Bank (b) using the ADAM8 sequence revealed the top structure hits as Russell's viper venom metalloproteinase 26 (X2a) (c) and vascular smoothmuscle-inhibiting protein-2 (Zero) (d). ZEx3 and ZERO align structurally at 2.33 Å using the alignment in Supplementary Fig. 1. The structure of ADAM10 (ZA07) (e) was also aligned even though it was eighteenth in the BLAST results. The per cent identity matrix is shown in Supplementary Fig. 2.

Administration of cyclic peptide BK-1361 in mice. Lyophilized cyclic peptide was dissolved in sterile PBS to a concentration of 1 μg ml−1. Since neither an acute (single injection) nor a chronic toxicity (repeated weekly injections over a total of 4 weeks) using a dosage of 10 μg ml−1 body weight via intraperitoneal route was observed based on organ histology after necropsy (Supplementary Fig. 9). this dosage regimen was administered throughout the experiments presented in this study. Equal volumes of sterile PBS were injected as control. For KPC mice, BK-1361 injections were started at 4 weeks of age and continued for over 8 weeks. Mice were constantly monitored and were killed when they reached end point criteria.

Orthotopic injections. Orthotopic pancreatic cancer tumours were implanted into 6- week-old female CD1 nu/nu mice. Ketamine/xylazine (100/10 mg kg−1) was used for anaesthesia. The left side of the mouse was shaved and the fur was completely removed. The surgical area was sterilized with an iodine solution (Povidone-iodine, Novaplug, Irving, TX, USA) and a small incision (<1 cm) was made through the skin and abdominal wall at the base of the spleen. The spleen was sharply pulled through the incision, exposing the tail of the pancreas. The cell- Matrigel solution (1 × 106 cells in 50 μl) was injected into the tail of the pancreas. The Matrigel was allowed to set (∼10 s), the needle was gently removed from the pancreas and the area was swabbed with iodine to devitalize any stray cells in the injection site. The pancreas and spleen were replaced in the abdomen and the incision site closed with 3–4 interrupted 9-0 Prolene sutures (Ethilon 5–0 PS-3, Ethicon, Piscataway, NJ, USA). The Matrigel method of orthotopic tumour implantation has resulted in 100% tumour uptake with little evidence of extra-pancreatic leakage. Panc1 and AsPC-1 cell-derived tumours were analysed 12 days after implantation.

Statistical analysis. Invasion assays and western blot data were analysed by one- way ANOVA. For in vivo experiments, two-way ANOVA using Shapiro–Wilks normality was used. For pairwise comparisons of tumour weights/volumes and tissue pathologies (acinar tissue and infiltration rates), including Image analyses, Tukey-type linear contrast tests were used. Survival was estimated as a Kaplan–Meier survival curve, and the statistical analysis was carried out using a log-rank test for the censored data. Based on the obtained results, the data were considered not significant (P>0.05), significant * (P<0.05), highly significant ** (P<0.01) or very highly significant *** (P<0.001).

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32. Author contributions

This study was conceived by J.W.B., D.A.T., C.N., D.A.L., T.H., M.P., M.L.M. and N.S.; experiments were performed by J.W.B., G.K. and M.L.M.; acquisition of all the data...
was done by U.S., G.K., C.C., T.F., P.G., A.M.G., S.H., P.C., R.S., M.B., R.R., R.R.K., F.H.R. and M.A.M.; the analysis and interpretation of data was performed by U.S., G.K., C.C., J.W.B., M.L.M., D.A.L. and M.A.M.; the final manuscript was prepared by U.S., G.K., C.C., M.L.M. and J.W.B.; and the whole study was supervised by J.W.B.

Additional information
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