A rapid in vivo screen for pancreatic ductal adenocarcinoma therapeutics

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ABSTRACT
Pancreatic ductal adenocarcinoma (PDA) is the fourth leading cause of cancer-related deaths in the United States, and is projected to be second by 2025. It has the worst survival rate among all major cancers. Two pressing needs for extending life expectancy of affected individuals are the development of new approaches to identify improved therapeutics, addressed herein, and the identification of early markers. PDA advances through a complex series of intercellular and physiological interactions that drive cancer progression in response to organ stress, organ failure, malnutrition, and infiltrating immune and stromal cells. Candidate drugs identified in organ culture or cell-based screens must be validated in preclinical models such as KIC (p48Cre;LSL-KrasG12D;Cdkn2a−/−) mice, a genetically engineered model of PDA in which large aggressive tumors develop by 4 weeks of age. We report a rapid, systematic and robust in vivo screen for effective drug combinations to treat Kras-dependent PDA. Kras mutations occur early in tumor progression in over 90% of human PDA cases. Protein kinase and G-protein coupled receptor (GPCR) signaling activates Kras. Regulators of G-protein signaling (RGS) proteins are coincidence detectors that can be induced by multiple inputs to feedback-regulate GPCR signaling. We crossed Rgs16::GFP bacterial artificial chromosome (BAC) transgenic mice with KIC mice and show that the Rgs16::GFP transgene is a KrasG12D-dependent marker of all stages of PDA, and increases proportionally to tumor burden in KIC mice. RNA sequencing (RNA-Seq) analysis of cultured primary PDA cells reveals characteristics of embryonic progenitors of pancreatic ducts and endocrine cells, and extraordinarily high expression of the receptor tyrosine kinase Axl, an emerging cancer drug target. In proof-of-principle drug screens, we find that weaning KIC mice with PDA treated for 2 weeks with gemcitabine (with or without Abraxane) plus inhibitors of Axl signaling (warfarin and BGB324) have fewer tumor initiation sites and reduced tumor size compared with the standard-of-care treatment.

INTRODUCTION
Pancreatic ductal adenocarcinoma (PDA) is the fourth leading cause of cancer-related deaths but is predicted to become more common owing to its association with smoking, diet, obesity and type 2 diabetes (Pannala et al., 2008; Rahib et al., 2014; Siegel et al., 2015). Three major classifications of pancreatic precancerous lesions are associated with progression to PDA: PanIN (pancreatic intraepithelial neoplasia), IPMN (intraductal papillary mucinous neoplasm) and MCN (mucinous cystic neoplasm) (Distler et al., 2014). Precancerous lesions can be common in the elderly or obese. For example, early PanINs were found in 65% of obese patients, and their presence was associated with intravisceral fat, and pancreatic intralobular fibrosis and fat (Rebours et al., 2015). IPMNs are the next most common pancreatic precancerous lesion associated with PDA (Maitra et al., 2005). They are found in the pancreatic main and branching ducts. MCNs occur predominantly in females, predominantly in the peripheral pancreas (Thompson et al., 1999).

Recent mathematical predictions attribute spontaneous mutations during cell division as initiators of PDA, making early detection and effective therapy the only two elements determining survival (Tomasetti and Vogelstein, 2015). Unfortunately, PDA symptoms present late in disease progression and, other than surgical resection, limited progress has been made in developing effective treatments after gemcitabine was introduced as a first-line therapy for advanced PDA (Burrus et al., 1997). Gemcitabine treatment alone or after resection is marginally effective in prolonging survival. One of the two predominant therapeutic regimens is gemcitabine combined with nab-paclitaxel (Abraxane), which was shown to increase survival to 8.5 months, compared with 6.7 months for patients who received gemcitabine alone (Von Hoff et al., 2013). In a follow-up study, 3% of patients in the gemcitabine plus nab-paclitaxel group were still alive after 42 months of treatment compared with none in the gemcitabine only group (Goldstein et al., 2015). The primary mechanism of function of paclitaxel is interference with microtubule depolymerization leading to mitotic failure (Schiff et al., 1979, 1980). Nab-paclitaxel has been shown to provide better tolerance and absorption than paclitaxel. In addition, nab-paclitaxel augments gemcitabine efficacy by reducing the level of its metabolizing enzyme, cytidine deaminase (Ibrahim et al., 2002; Frese et al., 2012). However, tumors are often resistant to this
TRANSLATIONAL IMPACT

Clinical issue
Pancreatic ductal adenocarcinoma (PDA) is the fourth leading cause of cancer-related US deaths, and is projected to be the second leading cause by 2025 because of its association with smoking, obesity and type 2 diabetes. PDA has the worst survival rate of any major cancer so far. The current standard-of-care provides only modest therapeutic gains. The two most desperately needed advances for extending life expectancy of individuals with PDA are improved therapeutics and the identification of early markers. PDA advances through a complex series of intercellular and physiological interactions that drive cancer proliferation in response to organ stress, and infiltrating immune and stromal cells, causing organ failure and subsequent malnutrition. Kras mutations occur early in tumor progression in over 90% of human PDA. However, Kras is refractory to direct inhibitors.

Results
In this study, the authors report a rapid, systematic and robust in vivo screen for effective drug combinations to treat Kras-dependent PDA. Protein kinase and G-protein coupled receptor (GPCR) signaling activates Kras. Regulators of G-protein signaling (RGS) proteins are coincidence detectors that can be induced by multiple inputs to feedback-regulate GPCR signaling. The Rgs16::GFP transgene is a KrasG12D-dependent marker of all stages of PDA neoplasia and its expression increases proportionally to tumor burden in KIC mice – a genetically engineered mouse model of PDA. RNA sequencing analysis of cultured primary PDA cells shows characteristics of embryonic progenitors of pancreatic ducts and endocrine cells, and extraordinarily high expression of the receptor tyrosine kinase Axl, an emerging cancer drug target. In proof-of-principle drug screens, the authors find that PDA weanling mice treated for 2 weeks with gemcitabine plus Abraxane and inhibitors of Axl signaling (warfarin or BGB324) have fewer tumor initiation sites and reduced tumor size compared with KIC mice treated with standard-of-care treatments (either gemcitabine alone or gemcitabine plus Abraxane).

Implications and future directions
Candidate anti-cancer drugs identified in organ culture or cell-based screens must be validated for efficacy in preclinical models such as KIC mice. Rgs16::GFP is a robust and faithful in vivo reporter of PDA progression and sensitivity to new chemotherapeutic drug regimens, including Axl-targeted agents. This rapid in vivo screening strategy could potentially be applied to identify improved therapeutics for many other cancers.

combination (Neesse et al., 2014). The other common drug treatment, FOLFIRINOX, consisting of four different chemotherapy agents, is more effective but less well-tolerated (Becker et al., 2014; Mooncraft et al., 2014; Haeno et al., 2012). Therefore, there is a need for a systematic and robust in vivo screen that can accelerate the pace of discovery of improved PDA therapeutics.

PDA initiates as ductal neoplasia, derived from any of three pancreatic adult cell types – ductal progenitor cells, centroacinar cells, or acinar cells that have undergone acinar-to-ductal metaplasia (ADM) (Bonner-Weir et al., 2004; Rovira et al., 2010; von Figura et al., 2014). In humans, activated Kras and inactivated Cdkn2a are the earliest and most common genetic mutations identified in disease progression (Hezel et al., 2006; Iacobuzio-Donahue et al., 2012). Genetically engineered mouse models (GEMMs) based on these mutations have been developed to investigate PDA initiation and propagation. In the present study, we use KC (p48Cre;LSL-KrasG12D) and KIC (p48Cre;LSL-KrasG12D;Cdkn2a(−/−)) mice. Both lines form tumors because they express activated KrasG12D (KIC also has inactivation of the tumor suppressor Cdkn2a) in all three pancreatic lineages – ducts, acinar and endocrine cells – under control of the p48 (Ptf1a) promoter. By contrast, IC mice (p48Cre; KIC mice are an excellent GEMM for PDA therapeutic screens because neoplasia develops early, between 2 to 3 weeks of age, and large aggressive tumors develop in all mice by 4 weeks of age (Aguirre et al., 2003).

PDA is the most frequent major cancer harboring Ras mutations (e.g. KrasG12D) (Plyayeva-Gupta et al., 2011). Kras mutations are found in over 90% of human PDA cases (Iacobuzio-Donahue et al., 2012). KrasG12D expression is necessary but not sufficient to initiate neoplasia; GTP binding is required to activate KrasG12D (Huang et al., 2014). Ras guanine nucleotide exchange factors (Ras-GEFs) catalyze GDP dissociation, and subsequent GTP binding to Ras (Jeng et al., 2012). Protein kinase and G-protein coupled receptor (GPCR) signaling can stimulate Ras-GEFs to promote KrasG12D-dependent neoplasia (van Blesen et al., 1995; Kahn, 2014). Regulators of G-protein signaling (RGS) proteins are GTPase activating proteins (GAPs) for the Gi- and Gq-alpha subunits of heterotrimeric G proteins (Berman et al., 1996). Interestingly, RGS-resistant mutations in Goα and Goq were found in cells isolated from pancreatic cysts (Wu et al., 2011). RGS proteins are coincidence detectors that can be induced by and integrate multiple inputs to feedback-regulate the GPCR arm of the pathway, by virtue of their Gα-GAP activity (Ross and Wilkie, 2000; Huang et al., 2006; Villasenor et al., 2010; Pashkov et al., 2011). The induction of RGS proteins can therefore be monitored to report hyperactivated Ras signaling (Dohman et al., 1996; Dignard et al., 2008). Because Ras remains an elusive drug target (Stephen et al., 2014), we developed an in vivo screen for PDA therapeutics that is responsive to Kras signaling.

We previously described expression of an Rgs16::GFP bacterial artificial chromosome (BAC) transgene during embryonic and postnatal pancreas development in pancreatic progenitors, endocrine cells and duct cells (Villasenor et al., 2010). GFP was expressed in ducts and islet β-cells during neonatal pancreas development but was not detected in euglycemic adult mice. Rgs16::GFP was reactivated, first in ducts, then islet β-cells, under conditions of chronic insulin demand or hyperglycemia in mouse models of type 1 and type 2 diabetes, and during gestation. In humans, Rgs16 expression was observed in ducts of pancreatic cancer patients prior to detectable metastasis (Kim et al., 2010). Chronic stress might induce Rgs16 in progenitor cells within the pancreatic ductal epithelium (Bonner-Weir et al., 2004; Villasenor et al., 2010). To test whether Rgs16 is an early marker of PDA, we crossed the Rgs16::GFP transgene into KIC mice. Here, we show that the Rgs16::GFP transgene is a KrasG12D-dependent marker of all stages of neoplasia in KIC mice – IPMN, PanIN and PDA (Iacobuzio-Donahue et al., 2000; Maitra et al., 2005). The distribution and intensity of Rgs16::GFP expression is proportional to and coincident with tumor burden.

In a proof-of-principle for drug screens, we find that weaning KIC mice with PDA treated with the standard-of-care combination of gemcitabine and nab-paclitaxel (Abraxane) (Masellis et al., 2009; Frese et al., 2012; Von Hoff et al., 2013; Neesse et al., 2014) for 2 weeks have significantly lower Rgs16::GFP expression, and reduced tumor size and occurrence. The Axl tyrosine kinase receptor is associated with aggressive cancer and poor patient outcome in breast and pancreatic cancer (Gjerdrum et al., 2010; Song et al., 2011; Reichl et al., 2015). Axl, and its γ-carboxylated ligand, Gas6, are associated with drug-resistant tumor relapse (Linger et al., 2008; Song et al., 2011; Schmidt et al., 2012; Kirane et al., 2015). We therefore evaluated novel combinations of standard-of-care PDA chemotherapeutics with Gas6/Axl signaling inhibitors in our rapid in vivo PDA therapeutic assay. We show that warfarin or an Axl kinase inhibitor (BGB324), in combination with gemcitabine and nab-paclitaxel, significantly reduce tumor
initiation and growth. Thus, the in vivo PDA model harboring the Rgs16::GFP reporter is an efficient system for identifying effective drug combinations, and for identifying novel or repurposed drugs to treat PDA.

RESULTS
Rgs16::GFP is a Kras$^{G12D}$-dependent reporter of PDA initiation and growth
We introduced the Rgs16::GFP reporter into KC and KIC mice because PDA initiates in pancreatic duct-like cells, either following ADM or neoplastic growth of progenitor cells, anywhere from the head to the tail of the pancreas but not in the hepatopancreatic duct (supplementary material Fig. S1) (Aguirre et al., 2003). The affected cell types are consistent with the expression domain of p48 (Ptf1a) during pancreas development (Kawaguchi et al., 2002). Rgs16::GFP is expressed in embryonic and neonatal pancreatic ducts (Villasenor et al., 2010) and, in adults, in ducts early in the response to chronic high insulin demand and in mid-late gestation in pregnant females (Villasenor et al., 2010). Finally, endogenous Rgs16 is expressed in human PDA [The Cancer Genome Atlas (TCGA), http://cancergenome.nih.gov/; Kim et al., 2010].

Analysis of pancreata harvested from postnatal day 29 (P29) mice demonstrated that Rgs16::GFP expression in pancreatic tumors is dependent on the (heterozygous) Kras$^{G12D}$ allele; KIC and KC mice had high Rgs16::GFP expression (Fig. 1). Pancreata in KIC mice at P29 had widespread ductal neoplasia. No GFP expression was observed in IC mice (Fig. 1), which do not harbor the Kras$^{G12D}$ allele and do not develop tumors. Background fluorescence was minimal in KIC mice lacking the GFP transgene (data not shown).

An important advantage of the Rgs16::GFP;KIC reporter mice is that the entire pancreas can be rapidly evaluated under a fluorescence dissection microscope to quantitate non-overlapping regions of GFP expression. In hundreds of pancreata dissected at multiple ages, every neoplastic lesion detected in bright-field microscopy expressed Rgs16::GFP (e.g. supplementary material Fig. S1A,B). A three-dimensional rotational movie shows Rgs16::GFP expression in a PDA tumor, several small areas of neoplasia and normal acinar cells in lobes at the head of the pancreas (supplementary material Movie 1; Fig. S2).

Rgs16::GFP intensity increases with PDA initiation and growth
In KIC mice, Cre-recombinase is first expressed by the p48 (Ptf1a) promoter in the embryonic progenitor cells that give rise to the three epithelial cell lineages in the adult – ducts, and exocrine and endocrine pancreas (Kawaguchi et al., 2002). Therefore, all cells in these pancreatic lineages express oncogenic Kras$^{G12D}$ and the tumor suppressor gene Cdkn2a is deleted (see supplementary material Fig. S3: the entire pancreas is marked by TdTomato in p48Cre;LSL-TdT reporter mice). Despite expression of Kras$^{G12D}$ and deletion of Cdkn2a throughout the pancreas, Rgs16::GFP is only expressed in a few early PanINs sparsely scattered throughout the pancreas 2 weeks after birth (P15; Fig. 2A). At P15, the pancreas appears morphologically and functionally normal, with the exception of these early PanINs. Thus, Rgs16::GFP reports the precise region of activated Kras$^{G12D}$ signaling at tumor initiation and throughout progression.

Fig. 1. Rgs16::GFP is a Kras$^{G12D}$-dependent reporter of pancreatic neoplasia. Rgs16::GFP is expressed in early pancreatic ductal neoplasia (IPMN, PanIN and PDA) but not in functional acinar cells of Rgs16::GFP;KIC (GFP-KIC) and Rgs16::GFP; KC (GFP-KC) mice (age=P29). No fluorescence background was detected in PDA tumors in mice lacking the Rgs16::GFP transgene (KIC or KC without GFP; not shown) nor in GFP-IC or normal glycemic Rgs16::GFP (GFP-WT; not shown) pancreata. Live fluorescence microscopy is shown. Scale bars: 1 mm.

Fig. 2. Rgs16::GFP is a Kras$^{G12D}$-dependent reporter of PDA expansion. (A) Early lesions appear by postnatal day 15 (P15) in Rgs16::GFP;KIC (GFP-KIC) mice, marked by Rgs16::GFP expression in ducts. As tumors grow, GFP expression increases. (A; lower panels) Pancreata of normal glycemic, age-matched Rgs16::GFP (Ctrl) mice. Live fluorescence microscopy is shown. (B) Quantification of GFP expression based on the averages of the highest intensity pictures of each pancreas of GFP-KIC (G-KIC) and control (G-Ctrl) mice between P15 to P29. Rgs16::GFP expression increases with age and PDA expansion. GFP fluorescence of PDA tumors at P29 (arrows) is about 10,000-fold greater than non-tumorigenic, euglycemic Rgs16::GFP mice. Age and number of mice in Rgs16::GFP;KIC [n(G-K)] and Rgs16::GFP-Control [n(G-C)] groups are noted.
Between the ages P15 to P29, average GFP fluorescence increased more than 100-fold as early PanINs appeared throughout the pancreas and PDA tumors grew (Fig. 2B). P29 is therefore an optimal time to assess tumor growth in weanlings because individual tumors achieve near-maximal brightness, more than 10,000-fold above background fluorescence.

**Rgs16::GFP is expressed in ADMs, PanINs and PDAs**

Pancreatic neoplasia secrete mucins and other polysaccharide-decorated proteins detected by Alcian Blue/Periodic Acid-Schiff (AB/PAS) staining. PDAs in KIC mice have intense Rgs16::GFP expression (supplementary material Fig. S4) but little or no AB/PAS staining, whereas Rgs16::GFP is significantly lower in regions of neoplasia that are AB/PAS-positive (Fig. 3, inserts). High-resolution confocal microscopy revealed that Rgs16::GFP is not expressed in normal acinar cells, consistent with fluorescence microscopy of dissected pancreata (Figs 1,2). KrasG12D-evoked ADM induced Sox9 expression, as previously reported (Kopp et al., 2012; Krah et al., 2015), and low Rgs16::GFP expression was observed in most cells (Fig. 4). By contrast, Rgs16::GFP expression was significantly higher and co-expressed with Sox9 in duct-like PanIN lesions, consistent with the findings shown in Fig. 3. The marker of proliferation-competent cells, Ki67, was co-expressed with Rgs16::GFP in many cells within PDAs and PanINs but almost never in regions of ADM (supplementary material Fig. S5). This pattern of high Rgs16::GFP expression in PanINs and PDAs was confirmed by immunofluorescence with Muc1, Ecad and endogenous Rgs16 (Fig. 4, Fig. 5A). Exceptional cases induced intense Rgs16::GFP expression in acinar-like cells (supplementary material Fig. S6) co-expressing high carboxypeptidase A1 (CPA1; supplementary material Fig. S7). This pattern was observed in peripheral lobes with edema that sit beyond (proximal to) tumor nodules in KIC pancreata. These seem to be specialized responses in which Rgs16 and CPA1 expression is secondary to PDA tumor growth.

**Rgs16::GFP is expressed in pancreatic ductal progenitor and PDA cells**

KIC mice (6-8 weeks) and PDA primary cells in culture co-expressed Rgs16::GFP with markers of pancreatic progenitor cells and epithelial-mesenchymal transition (EMT), such as Sox9, Muc1 and Ecad (Fig. 4, Fig. 5C; β-catenin and TGF-β supplementary material Fig. S8). Orthotopic transplantation of Rgs16::GFP+ primary cells derived from PDA tumors at 6 weeks rapidly regenerated GFP-positive pancreatic cancer in duct-like structures in close proximity with vasculature in recipient NOD-SCID mice (Fig. 5B). By contrast, stroma of the non-transgenic host did not express GFP.

To gain more information about the gene expression profile of the KIC PDA cells, we performed RNA sequencing (RNA-Seq) analysis of the transcriptome, and found that PDA cells in primary culture are closely related to embryonic (E13.5) pancreas (Fig. 5D,E). PDA cells and E13.5 progenitor cells of the ductal and endocrine lineages expressed Pdx-1 and Sox9 (Fig. 5F). Similarities in the transcriptomes diverged noticeably by E17.5 and E18.5, as pancreata begin to express more genes in maturing endocrine and exocrine cells, and fewer mesenchymal genes. The transcriptomes of PDA primary cells and normal adult pancreas are only distantly related; PDA cells did not express appreciable levels of markers of mature acinar or endocrine cell types.

In a survey of cancer-associated receptors and ligands in primary PDA cells in culture, we noted that the receptor tyrosine kinase Axl...
was highly expressed, with modest expression of its endogenous ligand Gas6 (Fig. 5F, Table 1). Axl and Gas6 are highly expressed in many human primary PDA tumors (TCGA data), containing both cancer and stromal cells. Axl was the most highly enriched receptor kinase expressed in PDA cells for which we had inhibitors of active ligand maturation and receptor antagonists to test PDA initiation and progression. Furthermore, Axl is associated with EMT and drug resistance in carcinomas (Zhang et al., 2012; Byers et al., 2013). Therefore, we sought to establish a rapid in vivo assay to assess the effect of clinical Axl inhibitors on PDA initiation and progression.

**A 2-week in vivo assay: PanIN and PDA tumor growth is suppressed at P29 by a combination of Axl inhibitors, gemcitabine and Abraxane**

The therapeutic screening strategy was to treat KIC pups between P15-P28 (when PanINs and PDAs are in rapid expansion) with Table 1.

| Therapeutic  | Primary target in PDA | RNA-Seq (FPKM) | Pancreas |
|--------------|-----------------------|----------------|---------|
|              | Cell type              | Gene | PDA culture | E13.5 | E18.5 | Adult |
| BGB324       | Cancer cell            | Axl  | 66.70       | 6.47  | 8.25  | 4.10  |
| Warfarin     | Cancer cell and stroma | Gas6 | 1.39        | 3.43  | 17.42 | 14.10 |

BGB324 inhibits Axl kinase; warfarin inhibits γ-carboxylation of Gas6.

RNA-Seq samples: PDA culture, n=2; E13.5, n=1; E18.5, n=5; adult, n=5.

FPKM, fragments per kilobase of exon per million fragments mapped.
anticancer drugs, sacrifice mice at P29, and capture Rgs16::GFP intensity with a dissection fluorescence microscope. Images of the five brightest non-overlapping fields of Rgs16::GFP expression were then collected, representing the regions of greatest tumor burden (supplementary material Fig. S9). This quantitative protocol represents a much more rapid approach (about 5% of the time) than traditional histology for analysis of the entire pancreas from each mouse in a cohort of 20 adults.

To validate this in vivo screening approach, we treated KIC mice with gemcitabine+Abraxane (GA), a current standard-of-care for PDA therapy (Von Hoff et al., 2013). GA therapy significantly reduced PanIN lesions throughout the pancreas (supplementary material Fig. S9B; note the fields of low GFP expression in treated animals compared to untreated animals), and reduced average and median GFP expression (Fig. 6B). GA was more effective than gemcitabine alone at inhibiting PanIN initiation, relative to untreated controls (supplementary material Figs S9, S10, statistical analysis in supplementary material Tables S1, S2). Thus, this method detected that a standard-of-care drug treatment was able to impede tumor growth. However, toxic side effects inhibited growth of weanling mice (supplementary material Fig. S11). Furthermore, although GA treatment reduced PanIN lesions, large PDA tumors emerged at the same frequency in untreated and GA-treated mice (three trials), consistent with its modest effects in humans (Becker et al., 2014). As in humans, tumor heterogeneity exists between mice, reflected by differences in Rgs16::GFP expression levels, histology, immunofluorescence and response to treatment in KIC mice. Heterogeneity also exists within each pancreas – some regions were apparently unaffected at P29 whereas other areas had ADM, PanIN, and/or small or large PDA tumors.

To address whether blocking Axl signaling would improve GA effectiveness, we tested two clinical Axl inhibitors, BGB324 (Ben-Batalla et al., 2013) and warfarin, in combination with GA in PDA primary cell culture and KIC Rgs16::GFP reporter mice. In PDA primary cell culture, high concentrations of gemcitabine killed cells, and BGB324 was cytostatic, but warfarin had no effect (supplementary material Fig. S12). In KIC mice, warfarin had no effect by itself but, when combined with gemcitabine+Abraxane (WGA), PanIN lesions were further reduced and, importantly, WGA significantly reduced the growth of large PDA tumors observed in untreated and GA-treated mice (Fig. 6). Similar inhibitory effects were seen when GA was combined with BGB324 (BGA) or warfarin plus BGB324 (WBGA) (Fig. 6). Together, these data demonstrate that this screening strategy in KIC Rgs16::GFP mice is a relatively rapid means for identifying novel
or repurposed drugs, as well as new drug combinations, for treatment of Kras-dependent PDA.

**DISCUSSION**

The dismal survival rate of patients diagnosed with pancreatic cancer justifies an intense preclinical effort to identify novel PDA therapeutics. GEMMs have substantial benefits for drug screening, including recapitulation of all major stages of PDA and the complex interplay between precancerous neoplasia, adenocarcinoma, immune components and stromal elements (Cook et al., 2012). We used the KIC model because it is currently the most aggressive GEMM of PDA available (Aguirre et al., 2003). KIC mice harbor the two earliest genetic mutations common to most individuals with PDA: oncogenic mutations in Kras, which confer GAP-resistance (e.g. KrasG12D), and deletions of the tumor suppressor Cdkn2a. In the KIC GEMM, neoplasia initiate around 2 weeks of age and tumors (1-2 mm) develop in all untreated mice by P29. Furthermore, toxic and off-target drug effects inhibit growth of weanling mice and are easily measured as reduced body weight (supplementary material Fig. S11). Although weanlings might be hypersensitive to toxic drugs, the screen allows a rapid assessment of in vivo drug efficacy and specificity in weanling pups at a relatively modest cost.

To accelerate the discovery of effective drugs that inhibit PDA in vivo, we developed Rgs16::GFP as a sensitive reporter of PanIN and PDA initiation, progression and tumor size by 4 weeks of age (P29) in KIC mice. Rgs16::GFP expression is KrasG12D-dependent in KC and KIC mice. GFP is not expressed in IC mice (which do not develop neoplasia), and PDAs that develop in the GEMMs have no background fluorescence in the absence of the GFP transgene. Endogenous Rgs16 is not abundant in normal adult exocrine pancreas, nor is it usually induced in dedifferentiating (injured) acinar cells early in the process. For example, Rgs16 is not induced in the pancreas (primarily acinar cells) of adult Ptf1a knockout (KO) mice, although Cpa1 (an acinar cell marker) declines precipitously during the 2-week progression of acinar cell dedifferentiation (Krah et al., 2015). KrasG12D opposes Ptf1a maintenance of terminally differentiated pancreas and promotes acinar dedifferentiation in P29 KC and KIC mice (Krah et al., 2015). Widespread, high Rgs16::GFP expression in acinar-like cells (co-expressing high CPA1; supplementary material Fig. S7) was sometimes observed in peripheral lobes with edema that sit beyond (proximal to) tumor nodules in KIC pancreata (supplementary material Fig. S6). These are specialized responses in which Rgs16 and Cpa1 expression is secondary to PDA tumor growth. Tumors and/or calcified ductal stones might block the duct [analogous to pancreatic ductal ligation (Xu et al., 2008)], and promote edema and signaling that stimulates this unusually intense co-expression of Cpa1 and Rgs16. Whereas Rgs16 expression persisted, co-expression with Cpa1 in these dedifferentiating pancreatic lobes was presumably transient, because these structures (and CPA1) were absent in PanIN and in older KIC mice with solid tumors occupying the entire transformed pancreas.

An important point is that, although all pancreatic cells in KIC mice express KrasG12D and have inactivated Cdkn2a (indeed, p48::Cre drove TdTomato expression throughout the pancreas; supplementary material Fig. S3), Rgs16::GFP is absent at P15 except for expression in the earliest lesions, and then throughout tumor progression. Huang et al. (2014) showed that oncogenic Kras requires GTP loading for enhanced activity. Presumably, Rgs16::GFP is marking the precise location of, and perhaps the cells directly engaged in, receptor-dependent activation of KrasG12D signaling. Thus, these in vivo reporter mice could help to identify drugs that directly or indirectly inhibit KrasG12D activation during ADM, PanIN formation and PDA progression.

We used Rgs16::GFP to evaluate novel PDA therapeutic combinations in a rapid (2 week) in vivo screen. GFP intensity increased as a function of tumor burden in the pancreas (Fig. 2). Therefore, drugs and novel small molecules that inhibit PDA progression in vivo can be readily identified by their ability to reduce Rgs16::GFP fluorescence intensity in dissected pancreata (Fig. 6, supplementary material Figs S9, S10). We showed that a standard-of-care combination of gemcitabine+Abraxane (GA) reduced initiation of neoplasia in KIC;Rgs16::GFP reporter mice. However, GA did not significantly reduce growth of the largest pancreatic tumors, consistent with modest effects in prolonging survival in humans (Becker et al., 2014).

To identify additional genes that might be involved in PDA initiation and tumor growth, and therefore be potential targets for drug treatment, we characterized the gene expression profile of primary PDA cells in culture by RNA-Seq. The transcription profile of PDA cells was most closely related to embryonic progenitors of ducts and islets, the same embryonic cell types that express Rgs16 (Villasenor et al., 2010). Rgs16::GFP is a marker of embryonic pancreatic stem cells at E9.0 in the pancreatic bud, and continues to be expressed in Sox9-positive duct cells and insulin-positive endocrine cells at E15.5 (Villasenor et al., 2010). Thus, monitoring Rgs16 expression might lead to the identification of receptors and ligands important in stem cell function and cancer initiation. We found that expression of the tyrosine kinase receptor Axl was tenfold higher in primary PDA cells compared to normal embryonic (E13.5) pancreas and 16-fold higher than normal adult pancreas (Fig. 5F; Table 1), consistent with observations by others (Song et al., 2011). It was found that Axl and Rgs16 tend to be coordinately expressed in human PDA primary tumors (from analysis of co-expression of the human primary PDA samples characterized in supplementary material Figs S13 and S14). High levels of Axl expression in advanced cancers from diverse cellular origins suggest that tumor-cell-associated Axl might be a fundamental contributor to malignant progression (Holland et al., 2010). Indeed, our recent results support the notion that Axl signal transduction is required to maintain epithelial plasticity traits of aggressive pancreatic tumors, including tumorigenicity, invasiveness, survival, drug sensitivity and metastasis (Kirane et al., 2015). Additionally, Axl inhibition has been shown to block cell migration and reduce metastasis in breast cancer models (Gjerdrum et al., 2010; Paccez et al., 2013; Dunne et al., 2014; Paolino et al., 2014).

We found that Gas6, Axl ligand, was expressed in normal adult pancreas (and primary human PDA tumors; TCGA) but its expression was tenfold lower in PDA primary culture cells (Table 1). Warfarin inhibits post-translational γ-carboxylation of Gas6, which is necessary for its ability to activate Axl signaling (Lew et al., 2014). Whereas warfarin by itself had no effect on PDA, warfarin combined with gemcitabine+Abraxane (WGA) had three important effects in KIC;Rgs16::GFP reporter mice. Compared to GA therapy alone, WGA further reduced the initiation of neoplasia, lowered median tumor size, and significantly reduced growth of the largest tumors. We used low doses of WGA that have, individually, proven safe in humans. WGA retarded growth in weanling mice but this is attributable to gemcitabine and Abraxane. We have recently shown that warfarin exerts its anti-cancer effects by inhibiting Gas6-mediated Axl activation in PDA tumor cells (Kirane et al., 2015). A selective Axl
kinase inhibitor, BGB324, had similar effects on PDA initiation and growth when combined with GA (manuscript in preparation). Systemic Axl inhibition might also exert anti-tumor effects through host-response-dependent mechanisms (Paolino et al., 2014; Kirane et al., 2015). We screened a total of 53 mice with GA plus warfarin or BGB324. All mice survived treatment from P15 to P29, and about 40% had lower rates of neoplasia compared to the control mice that we analyzed.

Rgs16:GFP expression is essentially extinguished in pancreas of normal, euglycemic mice by P15 and completely absent by P28 (Villasenor et al., 2010). By contrast, Rgs16:GFP expression in the largest PDA tumors was about 1-million times brighter than control pancreas, or regions of KIC pancreas not yet affected by KrasG12D expression. Equally important for identification of the most effective PDA therapeutics, the median GFP expression in untreated mice was 1000-fold higher than non-GFP KIC mice, or Rgs16:GFP control mice lacking PDA. Warfarin+GA significantly retarded PDA initiation and progression. Although this in vivo assay is sensitive and rapid, it is primarily a chemopreventive screen in young animals, whereas PDA typically initiates in middle age and is diagnosed late in life. Therefore, the best drug candidates identified in this rapid in vivo assay should be validated in survival and tumor regression studies in adults. An early phase clinical trial is under consideration for low-dose warfarin based on the combination of findings we report here, other preclinical studies (Brown, 1973; McCulloch and George, 1987; Schulman and Lindmarker, 2000; Kirane et al., 2015) and anecdotal observations in patients (Brown, 1973; McCulloch and George, 1987; Schulman and Lindmarker, 2000; Kirane et al., 2015). Although we still found significant tumor progression in WGA-treated KIC mice, this might be caused by other receptors activating KrasG12D. Further inhibition might be achieved by adding another inhibitor to the combination therapy.

In summary, the screening method described here reveals sensitivity to new drug regimens that inhibit KrasG12D-mediated oncogenesis. These findings suggest that patients with successful resection of PDA and clear margins of resection might benefit most from repurposed low-dose warfarin treatment in combination with gemcitabine chemotherapy. Future studies will test new drugs as they become available to help identify the most effective and targeted PDA therapeutics.

**MATERIALS AND METHODS**

**Mouse lines and genotyping**

The mouse lines used were KIC (p48Cre+/ KrasG12D+/; Cdkn2d−/−), KC (p48Cre+/ KrasG12D−/−; Cdkn2d−/−), Rgs16::GFP; KIC [KIC mice crossed with Rgs16::GFP BAC transgenic mice to generate Rgs16::GFP; p48Cre+/ KrasG12D+/; Cdkn2d−/− (Rgs16::GFP; KIC reporter mice). Genotyping was done using clipped tails before sacrifice and confirmed with spleen DNA after dissection. Mice were maintained at a 12-h day, 12-h light cycle on normal chow ad libitum according to the rules and standards of UT Southwestern Institutional Animal Care and Use Committee. Rgs16 mice were identified by blue light excitation of GFP in the brain of newborn pups or in the retina of adult mice. Genotyping of KIC mice was done with the following primers: p48Cre-FW (For: 5′-CTCTGAAATATGCTTGGCAG-3′; Rev: 5′-CAGGGTTGATACATCGACTCC-3′; product: 392 bp), LSL-KrasG12D (For: 5′-CTACGACACCATGTCTTGGG-3′; Rev: 5′-TCTGAGTTGTTGAAAATGCT-3′; product: 327 bp), and Cdkn2d−/− (For: 5′-TTTGGCCAGAGATGCACATC-3′; Rev: 5′-CCAAGTGTGGCAA-ACCAGGCTC-3′; product: 145 bp for wild type, 179 bp for loxp inserted allele). All PCR conditions started with genomic denaturation at 94°C for 10 min followed by 33 cycles of 94°C denaturation for 30 s, 60°C annealing for 1 min, and 72°C elongation for 1 min. PCR products were run in a 1% agarose gel.

**Fluorescent microscopy and GFP quantification**

Pancreatic expression of Rgs16::GFP in Rgs16::GFP; KIC mice was captured under a Zeiss Lunar tissue dissection microscope (eye piece =10×) with Filter Set Lunar 13 (excitation: BP 470/20, emission: BP 505-530) illuminated by Osram HBO 103 W/2 mercury short-arc (without reflector) fluorescent lamp. The microscope objective was NeoLunar S (0.8×, FWD 80 mm) and the total image magnification was selected to be 25×. Images were captured via a single-channel camera (Hamamatsu 60-C, 1′, 1′) in 1344×1024 resolution with 1× exposure and 1× binning, analog gain=10, and analog offset=2 settings. Pancreatic fields representing the tumor burden (three to four fields for P15 pups and five or more for pups from P23 onwards) of the pancreases were imaged, covering up to 50% of the organ surface area. All images were saved in gray-scale 16-bit tiff format. Images were quantified using NIH ImageJ software with background subtraction with a radius of 50 pixels. A variable and tight threshold was set to eliminate residual background. Intensities of all particles with size ≥5 pixels were summed to obtain the total light intensity per image.

**Tissue clearing and microscopy**

Pancrea and a small section of duodenum containing the ampulla of Vater were dissected, fixed overnight in PFA, and washed in PBS (4°C). Tissues were stored in PBS (4°C). Tissue clearing and microscopy was done as described by Soderblom et al. (2015), based on previous studies (Becker et al., 2012; Kopp et al., 2012; Krah et al., 2015). Production of the three-dimensional (3D) rotational movie was done as described by Soderblom et al. (2015) using IMARIS.

**Drug dosages**

Rgs16::GFP; KIC mice were injected intraperitoneally with gemcitabine (Eli Lilly; Indianapolis, IN, USA) (12.5 mg/kg body weight/day given 3 days/week, dissolved in PBS), Abraxane (Cellgene; Summit, NJ, USA) (5 mg/kg body weight/day given 2 days/week, diluted in 2% saline solution), BGB324 (BerGenBio, Bergen, Norway) (5 mg/kg body weight/day given 5 days/week, dissolved in a mix of DMSO:ethanol:Kolliphor EL/water with 4:4:8.84 v/v ratio), and warfarin (0.2 mg/kg body weight/day given 5 days/week, dissolved in PBS) according to the injection schedules specified in supplementary material Fig. S9. These doses were validated in previous mouse studies (Dineen et al., 2010; Kutluk Cekin et al., 2013; O斯塔poff et al., 2013, 2014; Aguijera et al., 2014). Note that 30% cyclodextrin was discontinued as a vehicle for BGB324 (v2B, v2G) because it was not active. GA was not affected (supplementary material Fig. S9; GA, v2B, GA, v2G). To verify that tumor progression remains constant over the duration of these experiments, assays of untreated mice were interspersed with drug-treated cohorts, and warfarin-treated pups were the final cohort we tested. Gemcitabine, Abraxane and warfarin were purchased from the UT Southwestern clinical pharmacy; BGB324 was a gift from BerGenBio.

Rgs16::GFP PDA primary cell culture

Primary Rgs16::GFP PDA cells were harvested from 6-week-old KIC mice. Cells were grown in 25 mM Glucose DMEM (HyClone) with 10% FBS (Serum Source International), penicillin and streptomycin (Life Technologies) on rat tail collagen type 1 (BD Biosciences) coated plates (0.5 µg/cm2) in a humidified incubator at 37°C and 5% CO2. Cells reaching 80% confluence were washed twice with PBS (HyClone) and lifted with 0.05% Trypsin-EDTA (HyClone) treatment up to 10 min in the incubator. For drug tests in supplementary material Fig. S12, primary PDA cells were incubated with gemcitabine (10 µM dissolved in PBS), warfarin (1 µM or 10 µM dissolved in water), BGB324 (1 µM or 10 µM dissolved in DMSO) or warfarin and BGB324 together for 24 h in a 37°C incubator. PDA live cell numbers were obtained via counts using a hemocytometer following 2% trypan blue staining to distinguish dead cells under an inverted microscope.

**PDA primary cell RNA-Seq**

Cultured Rgs16::GFP PDA cells were stimulated with 40% FBS containing growth medium, incubated overnight and subjected to FACs to separate the GFP-positive and -negative cell populations. After isolating RNA via TRIzol (Life Technologies) treatment, the transcriptional profile of each
GFP-PDA population was revealed via RNA-Seq performed on poly-A selected mRNA. Mouse sequence reads were aligned to the mm9 genome assembly using TopHat v2.0.9 (Trapnell et al., 2009). All default settings were used except: -G option and -no-novel-juncs. The Cuffdiff module available in Cufflinks software v2.1.1 was used to quantify the expression by the FPKM method (Trapnell et al., 2010, 2012). The geometric method (median of the geometric means of fragment counts across all libraries) was used to normalize and scale FPKM values.

**Dendrogram – mouse PDA culture and tissues**

We calculated pair-wise distances between all sample expression data using the ‘euclidean’ method in dist () R function to check the similarities between samples. This method calculates the distance between the two vectors. We performed hierarchical clustering on this distance matrix using the ‘ward’ method in hclust () R function.

**Multidimensional scaling**

We used multidimensional scaling (MDS) to assess the differences between samples. For this, we used normalized expression matrix for all genes in the genome across all samples and computed distances between each sample pair using the euclidean method; cmdscale () in stats R package (R Development Core Team, 2014) was used to represent these distances between each pair of samples in 3D space.

**Heatmap**

Z-score was computed for a selected set of markers in each category across all samples and plotted using heatmap.2 () function available in gplots R package (Warnes et al., 2015).

**Statistical analysis of GBP expression**

GBP values were converted to log_{10} scale prior to statistical analysis. Graphs and their statistical comparisons were done using GraphPad Prism software with unpaired and two-tailed Student’s t-test. Significance between groups was indicated as ns (not significant); *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001. Error bars in all the graphs are standard error of the mean (s.e.m.). See supplementary material Tables S1 and S2 for additional statistical analysis.

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**Competing interests**

R.A.B. and T.M.W. are co-founders of Tuelvol Therapeutics. J.B.L. is a co-founder of BerGenBio. R.A.B. receives research funding from BerGenBio.

**Author contributions**

Conception and design: T.M.W. and R.A.B. Development of methodology: T.M.W., O.O., R.A.B., A.B.A., P.T. and J.B.L. Acquisition of data: O.O., T.M.W., R.B.H., A.B.A., G.H.S., V.P., Y.Z., V.H.C.M. and P.T. Analysis and interpretation of data (e.g. statistical analysis, biostatistics, etc.): O.O., A.L.C., Y.Z., V.B., M.A.H., G.H.S., R.K.K. and T.M.W. Writing, review and/or revision of the manuscript: T.M.W., O.O., R.A.B. and J.B.L. Study supervision: T.M.W.

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**Supplementary material**

Supplementary material available online at http://dmm.biologists.org/lookup/suppl/doi:10.1242/dmm.020933/-/DC1

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