An essential role for Argonaute 2 in EGFR-KRAS signaling in pancreatic cancer development

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Both KRAS and EGFR are essential mediators of pancreatic cancer development and interact with Argonaute 2 (AGO2) to perturb its function. Here, in a mouse model of mutant KRAS-driven pancreatic cancer, loss of AGO2 allows precursor lesion (PanIN) formation yet prevents progression to pancreatic ductal adenocarcinoma (PDAC). Precursor lesions with AGO2 ablation undergo oncogene-induced senescence with altered microRNA expression and EGFR/RAS signaling, bypassed by loss of pS3. In mouse and human pancreatic tissues, PDAC progression is associated with increased plasma membrane localization of RAS/AGO2. Furthermore, phosphorylation of AGO2Y393 disrupts both the wild-type and oncogenic KRAS-AGO2 interaction, albeit under different conditions. ARS-1620 (G12C-specific inhibitor) disrupts the KRASG12C-AGO2 interaction, suggesting that the interaction is targetable. Altogether, our study supports a biphasic model of pancreatic cancer development: an AGO2-independent early phase of PanIN formation reliant on EGFR-RAS signaling, and an AGO2-dependent phase wherein the mutant KRAS-AGO2 interaction is critical for PDAC progression.
KRAS mutations drive over 90% of pancreatic cancer, a disease with a dismal overall 5-year survival rate of only 9%. Like all RAS GTPases, KRAS is a molecular switch that transduces extracellular mitogenic signals by cycling between an active GTP-bound and an inactive GDP-bound state. Proteins that regulate the nucleotide loading of RAS, like GAPs or guanine exchange factors (GEFs), recruit RAS to the plasma membrane in response to activated growth factor receptors, such as EGFR. Recurrent oncogenic driver mutations in RAS result in the accumulation of its active GTP-bound form at the plasma membrane, leading to aberrant downstream signaling.

To investigate the role of KRAS cancer initiated by a conditionally activated allele of pancreatic cancer in vivo, we employed the GEMM of pancreatic AGO2 intervention to prevent pancreatic cancer progression. AGO2 association may, therefore, represent a point of therapeutic intervention to prevent pancreatic cancer progression.

Genetically engineered mouse models (GEMMs) of pancreatic cancer were developed by expression of a single oncogenic KRASG12D allele in the mouse exocrine pancreas. In this model, pre-invasive pancreatic intraepithelial (PanINs) lesions progress to pancreatic adenocarcinoma (PDAC) reflective of the human disease. Use of such GEMMs has been instrumental in defining the key events that characterize PanIN development and PDAC progression. Of particular relevance is the observation that EGFR is essential for KRASG12D-driven PanIN development. However, the requirement for EGFR at the early stage of PanIN development has not translated to successful treatment, while directly targeting KRAS also remains a challenge.

Earlier, we identified a direct interaction between KRAS and Argonaute 2 (AGO2), independent of KRAS mutation status, which was required for oncogenic KRAS-driven cellular transformation. Interestingly, Shen et al. had previously shown that EGFR phosphorylates AGO2 at tyrosine 393 under hypoxic stress. Here, we employed established mouse models of pancreatic cancer to determine the in vivo requirement of AGO2 in pancreatic cancer development. Our data show that oncogenic KRAS-initiated PanIN formation is reliant on EGFR and wild-type RAS signaling, independent of AGO2. Strikingly, however, we identify a critical dependence on AGO2 for PanIN progression to PDAC, bypassed by loss of p53. While defining an essential role for AGO2 in PDAC progression, we also further our understanding of how the KRAS-AGO2 interaction is regulated through EGFR activation. Disruption of the oncogenic KRAS-AGO2 association may, therefore, represent a point of therapeutic intervention to prevent pancreatic cancer progression.

Results

AG02 loss blocks PDAC progression and PanIN formation.

To investigate the role of AG02 in the development of pancreatic cancer in vivo, we employed the GEMM of pancreatic cancer initiated by a conditionally activated allele of KRAS, KRASLSL–G12D+ (KRASG12D, Fig. 1a). Crossing KRASG12D mice with animals harboring Cre recombinase knocked into the pancreas-specific promoter, p48 (p48Cre), yields KRASG12D; p48Cre mice that develop pancreatic intraepithelial neoplasia (PanINs) precursor lesions beginning around 8 weeks. Over time, these PanINs progress to pancreatic ductal adenocarcinoma (PDAC) and develop metastases. Next, we generated transgenic mice with both KRASG12D and conditionally deleted allele(s) of AG02 (ref. 13) (Fig. 1a). The resulting KRASG12D; p48Cre mice were either wild-type, heterozygous, or homozygous for the conditional allele of AG02 (hereafter referred to as AG02+/+; KRASG12D; p48Cre, AG020/0; KRASG12D; p48Cre, and AG020/0; KRASG12D; p48Cre, respectively). Genomic PCR confirmed Cre-driven excision and recombination of the oncogenic KRAS allele in pancreata from mice with KRASG12D; p48Cre alleles (Supplementary Fig. 1a). Further, qRT-PCR analysis showed significant reduction in AG02 expression in AG020/0; KRASG12D; p48Cre mice (Supplementary Fig. 1b).

Histology of pancreata from mice with Cre-mediated AGO2 ablation (AG020/0; p48Cre) showed normal morphology (Fig. 1b, left panels) with no differences in pancreatic weight compared with pancreata from AG02+/+; p48Cre mice (Supplementary Fig. 1c). This suggests that loss of AG02 does not grossly interfere with pancreas development. Immunohistochemistry (IHC) with a monoclonal antibody specific to AG02 (Supplementary Fig. 2) showed minimal expression of AG02 in the acinar cells of both AG02+/+; p48Cre and AG020/0; p48Cre pancreata (Fig. 1b, right panels). These data indicate a non-essential role for AG02 in the acinar cells during normal pancreatic development. However, expression of KRASG12D in the pancreatic acinar cells led to increased AG02 expression in the PanINs as well as the surrounding stroma in 12-week-old AG02+/+; KRASG12D; p48Cre mice (Fig. 1c, top panels). Notably, we observed PanIN lesions in AG020/0; KRASG12D; p48Cre mice lacking AG02 expression (Fig. 1c, lower panels) that were morphologically indistinguishable from those arising in AG02+/+; KRASG12D; p48Cre mice. Further, PanINs from both AG02+/+; KRASG12D; p48Cre and AG020/0; KRASG12D; p48Cre mice displayed high mucin content by Alcian blue staining and similar gross weights of the pancreas, indicating indistinct phenotypes at 12 weeks (Supplementary Fig. 3a–b).

AG02 loss blocks PDAC progression and increases survival.

Surprisingly, mice aged over 400 days showed significantly increased pancreatic weights in both the AG02+/+; KRASG12D; p48Cre and AG020/0; KRASG12D; p48Cre cohort compared with AG020/0; KRASG12D; p48Cre mice, suggestive of a higher tumor burden in mice with at least one functional allele of AG02 (Fig. 1d). Histology of pancreata at the 400-day time point showed early late PanIN lesions and some PDAC development in AG02+/+; KRASG12D; p48Cre and AG020/0; KRASG12D; p48Cre mice with a distribution consistent with those previously reported. However, in the AG020/0; KRASG12D; p48Cre mice, mostly early stage PanIN lesions were observed, strikingly, with no evidence of PDAC (Fig. 1e). Occasionally, higher grade PanIN lesions were observed in AG020/0; KRASG12D; p48Cre pancreata, but these lesions invariably showed AG02 expression (Supplementary Fig. 4a), indicative of likely escape from Cre recombination, as has been previously noted in other contexts.

To examine the effect of AG02 loss on tumor-free survival, a cohort of transgenic mice was monitored over 500 days. Twelve of 12 AG02+/+; KRASG12D; p48Cre and 18 of 19 AG020/0; KRASG12D; p48Cre mice died over a median of 406 and 414 days, respectively, typical for a murine model expressing KRASG12D in the pancreas. Remarkably, however, all mice with homozygous AG02 deficiency (AG020/0; KRASG12D; p48Cre) had survived at the 500 day cut-off (Fig. 1f). PDAC was observed in pancreata of all mice that expressed AG02, but mice deficient for AG02 developed only early PanIN precursor lesions without progression to PDAC (Fig. 1g). Necropsies of experimental mice revealed frequent metastases and abnormal pathologies in the AG02+/+; KRASG12D; p48Cre and AG020/0; KRASG12D; p48Cre genotypes, but AG020/0; KRASG12D; p48Cre mice rarely showed abnormal pathologies and were without PDAC or metastases (Fig. 1g). Analyses of lungs with abnormal pathologies in two of the AG020/0; KRASG12D; p48Cre mice (marked as gray boxes) showed a single benign lesion each, associated with AG02 expression without indication of PDAC (Supplementary Table 2, Supplementary Fig. 4b). One mouse of the AG020/0; KRASG12D; p48Cre genotype developed a pancreatic cyst (without AG02 expression), histologically resembling the mucinous cystic neoplasm, and survived for 368 days (Supplementary Table 2, Supplementary Fig. 4a–b).
Supplementary Fig. 4b). Taken together, these data show that AGO2 is not essential for normal pancreatic development or KRASG12D-driven PanIN formation. Notably, however, AGO2 is indispensable for progression of PanINs to PDAC, despite the presence of other Argonaute proteins not deleted in this model with compensatory and overlapping RNAi functions.

PanINs with AGO2 loss undergo oncogene-induced senescence. Since precancerous lesions have been shown to undergo oncogene-induced senescence (OIS) in the pancreatic cancer mouse model, we performed OIS-associated β-galactosidase staining in pancreatic tissue sections of AGO2++/−;KRASG12D; p48Cre and AGO2−/−;KRASG12D;p48Cre mice. As shown in
p53 loss bypasses the OIS associated with AGO2 ablation. Since p53 loss leads to evasion of senescence and mutational inactivation of the TP53 gene has been observed in approximately 75% of PDAC patients, we determined the role of AGO2 in the context of p53 loss. For these studies, ablation of AGO2 expression was carried out in the KRASG12D;Trp53fl/fl (KPC) mouse model. In these mice, Cre activation simultaneously activates KRAS and reduces Trp53 levels. Tumor-free survival of chimeric mice with AGO2+/–;KRASG12D;Trp53fl/fl;+48Cre, AGO2fl/fl;KRASG12D;Trp53fl/fl;+48Cre, and AGO2fl/fl;KRASG12D;Trp53fl/fl;–48Cre genotypes was similar (Fig. 3a). PDAC and metastatic spread were also similar in all genotypes (Fig. 3b) despite efficient AGO2 ablation (Fig. 3c). Thus, a requirement for AGO2 in PDAC progression can be bypassed in a mouse model with TP53 aberrations, considered a late event in the development of pancreatic cancer.

Membrane association of RAS and AGO2 in PDAC development. Having identified an essential role for AGO2 in PDAC progression in mice, expression levels of AGO2 were next analyzed. Consistent with a role of AGO2 in KRAS-driven oncogenesis in AGO2+/–;KRASG12D;+48Cre mice, IHC analysis showed increased levels of AGO2 in PDAC and metastatic tissues as compared with early PanIN lesions (Fig. 4a). Transcript analysis in human pancreatic cancer suggested a significant increase in AGO2 expression in PDAC compared with normal pancreas (Supplementary Fig. 7a). To extend these observations at the protein level, we performed a systematic IHC analysis of a human pancreatic tissue microarray (TMA), comprising 44 duplicate pancreatic tissue cores, including PanIN, PDAC, and metastatic PDAC samples. AGO2 expression was remarkably higher in PDAC and metastatic PDAC cells compared with PanINs (Fig. 4b), and this increase was statistically significant (Fig. 4c). These data show that AGO2 protein levels are elevated with disease progression and suggest an important role for AGO2 in pancreatic cancer development in humans.

Considering that RAS is known to localize to the plasma membrane, if RAS and the RAS-AGO2 interaction could be localized at the plasma membrane in the mouse models and human tissues. Since most commercial KRAS-specific antibodies have been shown to be unsuitable for IHC or immunofluorescence (IF), we tested RAS10, a pan-RAS monoclonal antibody, and observed specific staining only in RAS expressing cells (Fig. 5a, Supplementary Fig. 7b–c). Surprisingly, relative to the surrounding normal tissue, IHC and IF analysis of mouse pancreatic tissues with this antibody detected high membranous RAS expression within the PanINs (Fig. 5b). Pre-incubation of the antibody with RAS peptides spanning the antibody epitope abrogated RAS staining (Fig. 5c). To corroborate the finding that RAS IHC and IF staining were primarily restricted to oncogenic KRAS-driven PanINs, we performed RNA in situ hybridization (RNA-ISH) using KRAS-targeted RNA probes (Supplementary Fig. 7d). As shown in Supplementary Fig. 7e, we observed KRAS transcripts restricted to the ducts of pancreatic lesions. The elevated KRAS transcript expression in PanINs is consistent with a recent study reporting increased oncogenic KRAS transcripts in engineered mouse models. We also validated the AGO2 monoclonal antibody using pancreatic tissue from experimental mice (Fig. 5d, Supplementary Fig. 2). Next, we assessed whether RAS and AGO2 co-localized in pancreatic tissues during PDAC progression. IF staining of normal acinar cells in the mouse pancreas displayed low and diffuse cytoplasmic staining of RAS and minimal expression of
AGO2, with a low measure of co-staining pattern (Pearson’s correlation for co-localization, PCC = 0.1) (Fig. 5e). Interestingly, as shown in Fig. 5e, RAS expression increased in PanINs, which further increased in PDAC (Supplementary Fig. 7f). In a parallel manner, AGO2 expression progressively increased in PanIN and PDAC tissues (Supplementary Fig. 7g), with a concomitant increase in plasma membrane localization and co-staining patterns with RAS (PanIN PCC = 0.5; PDAC PCC = 0.7). RAS staining could also be detected in the PanIN lesions of AGO2fl/fl; KrasG12D;p48Cre mice (Supplementary Fig. 8a). Localization of AGO2 to the plasma membrane was independently confirmed by analyzing co-localization with the membrane marker, E-cadherin (PCC = 0.43; Supplementary Fig. 8b). Furthermore, in pancreatic tissue obtained from normal mice treated with caerulein to
Fig. 2 AGO2 loss prevents PanIN to PDAC progression through OIS. a β-galactosidase staining of pancreatic sections from AGO2+/−;KRASG12D;p48Cre and AGO2+/−;KRASG12D;p48Cre mice at 70- and 500-day time points. Scale bar, 100 μm. b Scatter plot showing β-galactosidase staining in low grade PanINs. Data are from 47 PanINs from AGO2+/−;KRASG12D;p48Cre and 98 PanINs from AGO2+/−;KRASG12D;p48Cre from four individual mice at the 70-day time point and 30 PanINs from three individual mice at 500-day time points. Intensity of staining and percent cells within 30 low grade PanINs were used to determine the senescence score = intensity × percent positive cells. p values were determined using a two sided t-test. Data are presented as mean values +/− SEM. c Immunoblot analysis of RAS-driven MAPK (indicated by pERK) and PI3K (indicated by pAKT) signaling from individual pancreata obtained from mice of the indicated genotypes, aged to 400 days. Numbers on the left indicate protein molecular weights in kDa.

Fig. 3 p53 loss bypasses requirement for AGO2 during PDAC progression. a Kaplan–Meier tumor-free survival of AGO2+/−;KRASG12D;p53−/−;p48Cre, AGO2+/−;KRASG12D;Trp53fl/+;p48Cre, and AGO2+/−;KRASG12D;Trp53fl/+;p48Cre mice. b Chart showing PDAC (within the pancreas), the different metastatic lesions, and abnormal pathologies (black boxes) observed in each mouse of the indicated genotypes. c Representative H&E and AGO2 IHC in the indicated genotypes. Scale bar, 100 μm.

Induce pancreatitis, we observed wild-type RAS localization at the membrane without AGO2 co-staining (Supplementary Fig. 8c). This suggests specificity of the RAS-AGO2 co-staining observed during oncogenic KRAS-driven PDAC development. Importantly, extending the IF analysis to human pancreatic tissues, we observed a similar pattern of localization of RAS and AGO2 with increased RAS-AGO2 co-staining signals at the plasma membrane associated with pancreatic cancer progression (PCC, normal to PDAC increased from 0.1 to 0.5, respectively) (Fig. 5f).

For a direct assessment of the RAS-AGO2 interaction at single molecule resolution, we performed proximity ligation assays (PLA) using the RAS and AGO2 antibodies validated earlier (Fig. 5a, Supplementary Figs. 2, 8d). As shown in Fig. 5g, PLA signals indicative of RAS-AGO2 interaction were observed at the plasma membrane within PanINs arising in AGO2+/−;KRASG12D;p48Cre.
but not in AGO2^+/−;KRAS^G12D^/p48Cre mice. This further corroborates the IF analyses and provides evidence of membranous RAS-AGO2 interaction. Together, these data indicate that during pancreatic cancer development, AGO2 localizes at the plasma membrane, the site of RAS activity, and substantiates a role for AGO2 in the progression of PanINs to PDAC. Interestingly, in the KPC model described above (Fig. 3c, Supplementary Fig. 9a), AGO2 expression also increased during PDAC progression in AGO2-sufficient pancreata (Supplementary Fig. 9b). Significant overlapping membranous signals for RAS and AGO2 (PCC = 0.7) were observed in PDAC lesions from AGO2^+/−;KRAS^G12D^;Trp53^+/−:p48Cre mice (Supplementary Fig. 9c).

AGO2 regulates expression of microRNAs that control OIS. Considering a central role for AGO2 in the RNAi pathway, we compared the microRNA expression profiles of pancreata from AGO2^+/−;KRAS^G12D^;p48Cre and AGO2^+/−;KRAS^G12D^;p48Cre mice at the 500-day time point. Among the small number of microRNAs that showed differential expression between the two genotypes, the miR-29 and miR-30 families of microRNAs were significantly downregulated in pancreata expressing AGO2 (Fig. 6a, Supplementary Fig. 10a). Expression of this family of microRNAs was relatively upregulated within the PanINs lacking AGO2. These Rb-regulated microRNAs have been strongly associated with senescence, suggesting that their expression in the setting of AGO2 loss contributes to the OIS phenotype. A similar pattern of regulation was observed for members of the tumor suppressive let-7 family of microRNAs known to regulate cell proliferation and differentiation. On the other hand, oncogenic miR-21 was expressed to the same extent in the two genotypes. However, in the KPC model, downregulation of the miR-29, miR-30, and let-7 microRNA families was independent of AGO2 status (Supplementary Fig. 10b), allowing for progression to PDAC even in the absence of AGO2.

Transcriptomic profiles of pancreata obtained from the two genotypes showed that the block in PDAC progression in AGO2 loss was associated with reduced gene set enrichment scores for E2F targets, G2/M checkpoint controls, and canonical oncogenic KRAS signaling (Fig. 6b, Supplementary Fig. 11). Surprisingly, these transcriptional changes were accompanied with an increase in oxidative phosphorylation, considered conducive for PDAC.
development. These data suggest a temporal requirement for AGO2 for the biogenesis of select microRNAs to control PDAC progression.

The KRAS-AGO2 interaction limits RAS activation. Next, we sought to explore how AGO2 alters the EGFR-RAS signaling axis for two reasons: (1) EGFR has been shown to be essential for PanIN formation in the KRAS\textsuperscript{G12D}-driven pancreatic mouse model, and (2) EGFR activation has been shown to directly inhibit AGO2 function through phosphorylation of its tyrosine 393 residue. Immunoblot analysis of pancreatic tissues from 12-week-old mice with AGO2\textsuperscript{+/+};KRAS\textsuperscript{G12D};p48Cre, AGO2\textsuperscript{fl/+};KRAS\textsuperscript{G12D};p48Cre, and AGO2\textsuperscript{fl/fl};KRAS\textsuperscript{G12D};p48Cre genotypes showed a marked increase in AGO2 levels relative to normal pancreata (Supplementary Fig. 12a) concordant with IF analysis (Fig. 5e). Consistent with published studies, total EGFR levels were also elevated in KRAS\textsuperscript{G12D} mice irrespective of the AGO2 genotype (Supplementary Fig. 12a). However, in early PanINs initiated by oncogenic KRAS, significantly higher levels of
phospho-EGFR (Y1068) were observed in pancreatic tissues of AGO2+/−;KRASG12D;p48Cre mice (Fig. 6c, Supplementary Fig. 12a–b), indicating activated EGFR signaling in the absence of AGO2 expression. IHC analysis confirmed the elevated phospho-EGFR levels observed in tissue lysates were restricted to PanIN lesions of AGO2+/−;KRASG12D;p48Cre mice (Fig. 6d). IHC of total EGFR showed no significant difference in expression in pancreatic tissues between genotypes (Fig. 6d, Supplementary Fig. 13a–b). As previously noted, irrespective of AGO2 genotype, lesions from later time points showed a marked reduction in total EGFR levels7,8 in mice (and with disease progression in human tissue), further supporting the significance of EGFR signaling in the early stages of disease (Supplementary Fig. 13a,c). Importantly, immunoblot analysis showed that EGFR activation was accompanied with a remarkable increase in total RAS levels but not oncogenic KRASG12D levels (Fig. 6c, Supplementary Fig. 12), raising an intriguing possibility that signaling in early stage PanINs is along the EGFR-wild-type RAS axis.

To investigate this further, we isolated pancreatic ducts from 12-week-old AGO2+/−;KRASG12D;p48Cre and AGO2+/−;KRASG12D;p48Cre mice and cultured them as organoids39 in the absence of EGF (Supplementary Fig. 14). Immunoblot analysis showed increased levels of phospho-EGFR and total RAS in the organoids with AGO2 loss, while KRASG12D expression showed no change (Fig. 6e), mirroring the observations from pancreatic tissue lysates. Given that AGO2 is a direct phosphorylation substrate of the EGFR kinase12, our experiments define a reverse feedback upregulation of phospho-EGFR via AGO240–43.

To estimate the levels of activated wild-type RAS and oncogenic KRAS due to EGFR activation in AGO2 loss, we performed the RAF-binding domain (RBD) assay using isomorph specific antibodies. As shown in Fig. 6e, significant increases in activated RAS were readily detected using pan-RAS and KRAS-specific antibodies. A modest increase in KRASG12D-GTP levels was also observed. These observations reveal that AGO2 ablation activates EGFR signaling and results in increased GTP loading of both the wild-type and oncogenic KRAS.

Next, we monitored the extent of wild-type KRAS-GTP and KRASG12D-GTP levels upon treatment of the organoids with erlotinib. Remarkably, erlotinib treatment of organoids expressing AGO2 showed an increase in KRASG12D-GTP levels. On the other hand, EGFR inhibition in AGO2-deficient organoids had no effect on the increased KRASG12D-GTP levels (Fig. 6f). Conversely, in the absence of AGO2, EGFR inhibition dramatically reduced wild-type KRAS-GTP levels. The total KRAS-GTP levels in AGO2-sufficient organoids tracked with those of KRASG12D-GTP levels upon erlotinib treatment. These experiments clarify the role of AGO2 as a regulator of KRAS activity. In its absence, both oncogenic and wild-type RAS forms are activated, yet unlike oncogenic KRAS, activated wild-type KRAS is sensitive to EGFR inhibition. Further, the activation of EGFR, wild-type KRAS, and downstream ERK, all remained sensitive to erlotinib treatment (Fig. 6f).

To probe if AGO2 loss activates wild-type RAS even in the absence of mutant KRAS, we performed immunoblot analysis and RAS activation assays using AGO2−/− MEFs that do not harbor any form of oncogenic RAS. As shown in Fig. 6g, AGO2−/− MEFs also exhibit increased phospho-EGFR and wild-type RAS levels along with elevated wild-type RAS-GTP levels, which were significantly reduced when rescued with AGO2. We then made stable lines expressing either vector, full-length wild-type AGO2, or RAS binding-deficient AGO2 (K112A/E114A) to interrogate the specific effects of the KRAS-AGO2 interaction on RAS signaling. As shown in Fig. 6h, both wild-type AGO2 and AGO2K112A/E114A decreased phospho-EGFR activation. While the wild-type form of AGO2 limited RAS activation, the AGO2 mutant lacking RAS-binding residues sustained RAS activation. Further, increased pERK activation observed in the AGO2−/− MEFs could be rescued with AGO2 but not the RAS binding-deficient mutant (Fig. 6h). Using diverse models, these data define AGO2 as a key regulator that limits KRAS activation and downstream effector engagement through its direct interaction with KRAS.

To further explore how AGO2 could regulate KRAS-GTP levels, we performed biochemical assays that monitor the levels of KRAS-GTP in the presence of known RAS regulators. Specifically, using purified catalytic domains of Neurofibromin 1-GAP and SOS1-GEF, we measured KRAS-GTP levels in the presence or absence of AGO2. As shown in Fig. 6i, AGO2 had no effect on the intrinsic GTPase activity of KRAS and did not alter NFI1-GAP activity on KRAS. However, SOS1-mediated nucleotide exchange on wild-type RAS was significantly reduced in the presence of AGO2 (Fig. 6i) in a dose-dependent manner (Fig. 6k). In similar assays, oncogenic forms of KRAS were resistant to both GAP and GEF activity. Since both SOS and AGO2 compete for binding to the KRAS Switch II domain through Y64, our data predicts that the KRAS-AGO2 interaction limits wild-type RAS activation by competitively blocking SOS association (Fig. 6). Thus, in addition to the microRNA and transcriptomic changes observed with AGO2 ablation, loss of the KRAS-AGO2 interaction likely leads to increased ERK activation associated with the OIS phenotype.

AGO2K112A/E114A phosphorylation disrupts the KRAS-AGO2 interaction. Considering that AGO2 loss leads to increased signaling via EGFR and wild-type RAS through increased access of SOS to KRAS (Fig. 6–k), we posited that AGO2 binding to KRAS may also represent a rate limiting step in the activation of wild-type KRAS during growth factor stimulation. To explore this premise, we assayed for KRAS-AGO2 interaction across a panel of cell...
Interestingly, EGF stimulation resulted in a dramatic decline in endogenous KRAS and AGO2 despite activation of the EGFR/ERK and MAPK/AKT pathway (Fig. 7b, Supplementary Fig. 15c). Disruption of the wild-type RAS-AGO2 interaction was also observed when HEK293 (wild-type KRAS) cells expressing FLAG-tagged AGO2 were stimulated with EGF; the interaction was rescued by treatment with cells erlotinib (Fig. 7c). This strongly suggests that EGFR kinase activity was critical for the disruption of the wild-type KRAS-AGO2 interaction. In contrast, DLD-1 cells harboring mutant KRAS<sup>G13D</sup> showed no loss of
KRAS and AGO2 association either by EGF or erlotinib treatment (Fig. 7d).

To test if the previously identified site of EGFR-mediated phosphorylation12 on AGO2 at tyrosine 393 has a role in binding to KRAS, we analyzed the ability of a phosphorylation-deficient AGO2Y393F mutant to bind RAS under different conditions. In HEK293 (wild-type KRAS) cells, EGFR stimulation led to dissociation of wild-type AGO2 from RAS, but the AGO2Y393F mutant continued to bind RAS with or without EGFR activation (Fig. 7e), indicating that phosphorylation of this residue is critical for dissociation. Expression of these AGO2 constructs in Mia PaCa-2 (KRASG12C) cells showed no discernible change in RAS binding upon EGFR activation (Fig. 7f).

Next, we tested oncogenic KRAS-expressing cells with H2O2, known to inactivate tyrosine phosphatases by oxidation and, thus, activate EGFR45. In both H358 and Mia PaCa-2 cells expressing oncogenic KRAS, the KRAS-AGO2 interaction could be readily disrupted following H2O2 treatment (Fig. 7g–h). This disruption was also found to be dependent on the Y393 phosphorylation site of AGO2, since mutant AGO2Y393F remained recalcitrant to H2O2 treatment in Mia PaCa-2 cells (Fig. 7i).

To track the localization of the RAS-AGO2 interaction upon growth factor activation, we performed PLA on cells expressing wild-type or mutant KRAS. The use of either RAS or AGO2 antibodies alone did not show signals for the RAS-AGO2 PLA (Supplementary Fig. 16a). Interestingly, serum-starved PC3 cells showed increased membrane localization of both RAS and AGO2 proteins contributing to the increase in membrane-localized RAS-AGO2 PLA signals under these conditions (Fig. 7j, upper panels). RAS-AGO2 interaction PLA signals were significantly reduced upon EGFR stimulation and restored to levels observed under serum-sufficient conditions. IF analyses also showed a similar pattern of RAS-AGO2 co-staining under these different culture conditions (Supplementary Fig. 16b). A similar pattern of RAS-AGO2 interaction PLA signals was observed in wild-type RAS expressing MCF-7 cells (Fig. 7j, panel I). In contrast, both HCT116 and H358 cells (Fig. 7j, panels II and III), expressing oncogenic forms of KRAS, showed higher basal levels of RAS-AGO2 PLA signals compared with wild-type RAS expressing cells that remained consistent under different cell culture conditions. Combined, these data suggest that the wild-type KRAS-AGO2 interaction at the membrane is sensitive to EGFR-stimulated phosphorylation of AGO2Y393, while the oncogenic KRAS-AGO2 interaction is unaffected by ligand-activated EGFR. These data suggest that the Y393 phosphorylation site in AGO2 determines binding of both wild-type and oncogenic forms of KRAS; however, while AGO2 phosphorylation in wild-type RAS expressing cells can be achieved by EGFR stimulation, mutant RAS-expressing cells require sustained EGFR activation through inhibition of tyrosine phosphatases.

ARS-1620 disrupts the oncogenic KRAS-AGO2 interaction. Finally, we tested if direct targeting of oncogenic KRAS could affect the endogenous mutant KRAS-AGO2 interaction. Interestingly, the mutant KRAS-AGO2 interaction was disrupted when H358 (Fig. 8a) and Mia-PaCa-2 cells (Fig. 8b), harboring KRASG12C mutant alleles, were treated with ARS-162046, a covalent G12C inhibitor. The disruption of endogenous KRASG12C-AGO2 interaction in these cells was concentration-dependent and reflects the differential sensitivities of the two cell lines to ARS-162047. In a similar assay, ARS-1620 treatment had no effect on the KRASG12D-AGO2 interaction in Panc 05.04 (Fig. 8c) or Panc 10.05 cells (Fig. 8d). Given that ARS-1620 binds an allosteric Switch II pocket (SW-IIIP)47 on GDP-loaded KRASG12C, the disruption of KRASG12C-AGO2 binding provides orthogonal evidence that AGO2 makes contact with the Switch II region in KRAS. This data also proves that besides SOS, the easily detectable, endogenous membrane-bound KRASG12C-AGO2 interaction is an additional target of G12C inhibitors.

Discussion

GEMMs mirror the stepwise progression of human pancreatic cancer, starting with benign precursor lesions (PanINs) driven by mutant KRAS18,48,49. Here, a GEMM of AGO2 loss delineates pancreatic cancer development into two distinct phases (Fig. 9). The first phase of PanIN development is triggered by oncogenic KRAS and depends on EGFR-RAS mediated proliferation; this is followed by a second phase of PDAC progression that requires AGO2 expression to overcome OIS. While low-grade PanINs have been known to undergo senescence20, our model represents the first instance where loss of a direct interactor of KRAS induces OIS to abrogate PDAC progression. Despite the increased presence of NK cells30, the senescent cells are not cleared, allowing increased lifespan of mice lacking AGO2. Interestingly, the OIS phenotype is characterized by both the limited RNAi activity of AGO2 (to downregulate miRNAs that control cell proliferation/senescence, such as the let-7 and miR-29/30 families31–34) and a concomitant increase in oncogenic and wild-type KRAS activity and downstream effector signaling. While the microRNA regulation may be a result of the complete lack of AGO2 expression, the effects on RAS signaling can be attributed to the KRAS-AGO2 interaction. Notably, other members of the Argonaute family fail to compensate for these functions,
reiterating the specific role of AGO2 in KRAS-driven pancreatic cancer. Since the microRNAs identified here also regulate pancreatic autophagic process and OIS, it will be interesting to determine the role of AGO2 in autophagy.

Through these studies, we show that AGO2 is localized to the plasma membrane, a known site for KRAS activity but not RNA silencing activity. Importantly, we show that AGO2 expression at the membrane increased during PDAC progression, both in mouse and human PDAC, highlighting membrane redistribution of AGO2 in clinical disease progression. Given that posttranscriptional regulation can involve dynamic polarization of mRNA targets, it will be interesting to understand the effects of AGO2 membrane localization on its RNAi function to control translation. Functionally, we find that p53 loss precludes a requirement for both AGO2 and its RNAi activity, yet, like in human tissues, RAS/AGO2 membrane localization is retained in this model. Since aberrations in KRAS and p53 do not occur simultaneously, the mouse model with p53 loss represents a
Fig. 7 Phosphorylation of AGO2[Y393] disrupts its interaction with KRAS. a Immunoprecipitation (IP) of endogenous AGO2 upon EGF stimulation (5′) in the indicated cancer cells expressing wild-type RAS followed by immunoblot analysis of KRAS. For MCF7 cells, endogenous co-IP analysis was performed using both AGO2 and KRAS-specific antibodies. For each cell line and panel in this figure, MAPK activation and levels of various proteins are shown as input blots. b IP of endogenous AGO2 upon EGF stimulation (5′), in the indicated cancer cells harboring different KRAS mutations, followed by immunoblot analysis of KRAS. c Co-IP and immunoblot analysis of RAS and AGO2 upon EGF stimulation of HEK293 (wild-type KRAS) cells expressing FLAG-AGO2 or (d) DLD-1 (KRASG12D) cells in the presence or absence of erlotinib. e EGF stimulation and RAS co-IP analysis in HEK293 (wild-type KRAS) and f MIA PaCa-2 (KRASG12C) cells expressing FLAG-tagged AGO2 (wild-type or Y393F). IP of endogenous AGO2 upon H2O2 treatment (4′), in H358 (g) and MIA PaCa-2 (h) cells harboring KRAS mutations, followed by immunoblot analysis of KRAS. i H2O2 treatment and KRAS-AGO2 co-IP analysis in MIA PaCa-2 (KRASG12C) cells expressing FLAG-tagged AGO2 (wild-type or Y393F). Numbers on the left of the immunoblots in this panel indicate protein molecular weights in kDa. j Left panels, Representative images of single target (RAS or AGO2) and RAS-AGO2 interaction PLA in wild-type RAS expressing PC3 cells across the indicated cell culture conditions. PLA signals appear as red dots around DAPI stained nuclei in blue. Scale bar, 50 μm.

different etiology for PDAC progression and limits our understanding of the requirement of AGO2 in advanced disease states.

This study also reveals how EGFR activation allows fine-tuning of RAS signaling by disrupting the membrane RAS-AGO2 association under conditions of stress (starvation or presence of oncogenic KRAS). Interestingly, phosphorylation of AGO2 by EGFR simultaneously inhibits the last step of microRNA biogenesis12 and activates RAS at the plasma membrane62. We observe that EGF stimulation is sufficient to disrupt the wild-type KRAS-AGO2 interaction, but disruption of the oncogenic KRAS-AGO2 interaction requires activation of the growth receptor through inhibition of cytosolic tyrosine phosphatases. However, under both of these conditions, interaction with KRAS is dependent on the Y393 phosphorylation status of AGO2. In fact, inhibition of tyrosine phosphatase PTP1B through oxidative stress has been shown to lead to accumulation of AGO2[Y393] in oncogenic RAS-expressing cells, leading to senescence63. It is also intriguing that EGFR-mediated phosphorylation of AGO2[Y393] disrupts KRAS binding in a manner reminiscent of AGO2-Dicer binding and renders AGO2 incapable of RNAi activity12,64. While both KRAS and EGFR aberrations are mutually exclusive, both alter AGO2 RNAi function through distinct mechanisms11.

Extending these observations to our mouse model, we predict that AGO2 loss phenotypically mimics AGO2[Y393] phosphorylation. During EGFR-dependent PanIN development (Fig. 9), neither phosphorylated AGO2[Y393] nor the setting of AGO2 loss permits KRAS association, and this stage is, therefore, independent of AGO2. On the other hand, during progression to PDAC that is less reliant on EGFR, accumulation of AGO2[Y393] in its non-phosphorylated form promotes KRAS binding, which is essential for PDAC progression. Mouse models expressing only the RAS binding-deficient form of AGO2 will validate the biological role of the KRAS-AGO2 interaction.

Finally, we find that the G12C covalent inhibitor disrupts the mutant KRASG12C-AGO2 interaction. The inhibitor is known to covalently attach to cysteine residues and make contacts at the Switch II pocket. This binding has been shown to interfere with nucleotide exchange because of reduced SOS1-KRASG12C interaction47. Given that SOS and AGO2 compete for binding to the Switch II domain to regulate RAS-GTP levels, it is not surprising that AGO2 also fails to bind inhibitor-bound KRAS. Therefore, our data suggests that abrogation of the oncogenic KRAS-AGO2 association at the plasma membrane may represent a therapeutic opportunity for pancreatic cancer that warrants further investigation.
Oncogene-induced senescence. OIS due to ablation results in increased expression of microRNAs that regulate cell proliferation and senescence and also activates KRAS to promote oncogene-AGO2 interaction and is, therefore, AGO2-independent. PDAC progression is associated with increased expression of KRAS and AGO2 at the membrane. PanINs which progress to higher grade PanINs, PDAC, and metastases. PanIN formation requires EGFR that can phosphorylate AGO2 to disrupt the KRAS-AGO2 interaction and is, therefore, AGO2-independent. PDAC progression is associated with increased expression of KRAS and AGO2 at the membrane.

Methods

Mouse strains. LSL-KRAS<sup>G12D</sup> (ref. 14) (Kras<sup>LSL-G12D</sup> and p48Cre mice<sup>15</sup>) were obtained from Marina Pasca di Magliano, University of Michigan. Conditionally floxed AGO2 (ref. 13) (AGO2<sup>fl/fl</sup>) mice and p53<sup>fl/fl</sup> mice were purchased from Jackson labs (Bar Harbor, Maine). PCR genotyping for KRAS<sup>LSL-G12D;p48Cre</sup>, p53<sup>fl/fl</sup>-<sup>1/2</sup>, and AGO2 alleles, from DNA isolated from mouse tails, was performed using standard methodology. To generate experimental and control mice, AGO2<sup>fl/fl</sup>p48Cre and KRAS<sup>G12D</sup>p48Cre lines were intercrossed to generate AGO2<sup>fl/fl;KRAS<sup>G12D;</sup>p48Cre</sup> mice. These two lines were then intercrossed to generate the experimental mice. Given that mice were maintained on a mixed background, littermate controls were systematically used in all experiments (sex ratio per cohort was balanced). All animals were housed in a pathogen-free environment, and all procedures were performed in accordance with requirements of the University of Michigan IACUC. Cre activation in acinar cells of pancreata of mice with mutant KRAS alleles was validated by genotyping using the KRAS<sup>LSL-G12D</sup> conditional PCR detailed in Supplementary Table 3.

Histology, immunohistochemistry, and IF. Paraffin-embedded tissues from mice were processed using standard methodology. Details of the primary antibodies used for IHC are provided in Supplementary Table 1. Immunohistochemistry and IF staining were performed using standard techniques. For IF, slides were viewed using a Nikon 1A-B confocal microscope. To estimate co-localization of proteins, the Coloc2 program (ImageJ) was used to determine Pearson’s coefficient. Cells within the PanIN/PDAC or metastatic regions (from mouse and human tissues), excluding the stromal compartment, were used to determine the extent of overlap.

In panels with normal tissue shown in Fig. 5, acinar cells were used for co-localization analyses. Average values over three different areas are shown.

Proximity ligation assay (PLA). Cell lines were cultured in 8-well chamber slides. After the indicated treatment/stimulation, cells were fixed with 4% paraformaldehyde and then permeabilized using 0.1% Tween. Subsequent PLA staining was performed as per the protocol provided by the manufacturer (DUOlink kit, Millipore/Sigma). Mouse RAS10 and rabbit AGO2 antibodies, validated in this study, were used at 1:250 dilution to detect signals either alone or in combination. Negative controls were performed using either single antibody (Supplementary Fig. 8d), or tissue lacking AGO2 (Fig. 5d). Images were obtained using the Nikon A1B inverted confocal microscope. For mouse tissue PLA, the paraffin-embedded sections were processed as for IF analysis. PLA was then performed using RAS10 or AGO2 antibodies, either alone or in combination, and imaged using the Nikon A1B confocal microscope.

Human TMA analysis. Pancreatic TMAs and frozen human tissue repositories were established by a pathologist (L.S.) and developed at the Tissue and Molecular Pathology Core in the Department of Pathology, University of Michigan, after IRB approval as described<sup>16</sup>. The Institutional Review Board at the University of Michigan approved the study (protocol number: HUM0098128). Patients with pancreas resections for pancreatitis, cystic neoplasms, or PDA from 2002 to 2015 at the University of Michigan Health System were included in the study. The electronic medical record was examined for clinical and demographic patient information. Date of surgery and date of last patient contact were recorded from the
RNA in situ hybridization (RNA-ISH). RNA-ISH was performed to detect Kras mRNA on formalin-fixed paraffin-embedded (FFPE) tissue sections using the RNAScope 2.5 HD Brown kit (Advanced Cell Diagnostics, Newark, CA) and target probes (molecular beacon oligonucleotides) (412491). mRNA levels were normalized to the expression of the housekeeping gene, S100A4

Quantitative RT–PCR. Pancreatic total RNA was isolated using the AllPrep DNA/RNA/miRNA Universal Kit (Qagen). For quantification of mRNA transcripts, RNA was extracted from the indicated samples, and cDNA was synthesized using the SuperScript III System according to the manufacturer’s instructions (Invitrogen). Quantitative RT–PCR was conducted using primers detailed in Supplementary Table 3 with SYBR Green Master Mix (Applied Biosystems) on the StepOne Real-Time PCR System (Applied Biosystems). Relative mRNA levels of the transcripts were normalized to the expression of the housekeeping gene GAPDH.

MiRNA expression profiles by qPCR of mouse miRNA panels. Pancreatic total RNA was isolated using AllPrep DNA/RNA/miRNA Universal Kit (Qiagen). Five nanograms of total RNA from each sample was converted into cDNA using miRCURY™ LNA Universal RT microRNA PCR Universal cDNA Synthesis Kit II. Quantitative micro RT–PCR was performed using exiLENT SYBR Green Master mix with microRNA ready to use PCR mix, MouseKid rat panel I, V4,M (Exiqon, Cat #203713) on ABI 7900HT Fast Real-time PCR system (Applied Biosystems).

Data were analyzed using GenEX vers 6 software.

Transcriptome analysis. mRNA was quantitated on the Illumina platform using the Riboseq library preparation protocol. Transcriptome data processing and quality control were performed using RSeQC package. Sequencing alignment was performed using splice aware aligner STAR with two pass alignment option using mm10 reference build. featureCounts from Rsubread package was used to get the count matrix for expression quantification. R-package edgeR DGEList object was used to import, organize, filter, and normalize the data by the method of trimmed mean of M-values (TMM) using the calcNormFactors. This was followed by limma and voom analyses where default settings for the limma were used. Deseq2 was set up according to a standard protocol. HIC scoring was performed on each TMA block using standard protocol, and unstained slides were prepared for immunohistochemical (IHC) staining and IHC scoring was performed by a pathologist (I.S.).

In vitro assay to measure KRAS-GTP levels. Purified catalytic domains of SOS1 (Cytoskeleton) and N1F (Creative Biomart) were used to carry out nucleotide exchange and GTPase activity. Full-length KRAS and KRAS V12 (Biorime) were purified using Ni-NTA beads expression at the University of Michigan Proteomics Core. AGO2 was purchased from Sino Biologicals. Halo tag protein was obtained from Promega.

Plasmids. Full-length FH-AGO2 constructs were obtained from Addgene (pIR-Eneo-FLAG/HA-AGO2 10822, PETHomas Tuschl). AGO2 V5HIS mutant construct was generated using the QuikChange II XI Site-Directed Mutagenesis Kit (Agilent) from the FH-AGO2 plasmid described above using the primers detailed in Supplementary Table 3. DNA sequencing was confirmed using Sanger sequencing at the University of Michigan Sequencing Core.

Cell culture, transfection, and EGFR stimulation. All cell lines (detailed in Supplementary Table 4) were obtained from the American Type Culture Collection (ATCC) or as indicated. Cells were cultured following ATCC culture methods in media supplemented with the corresponding serum and antibiotics. Additionally, cells were routinely genotyped and tested bi-weekly for mycoplasma contamination. Only cells with the correct genotype and that were mycoplasma free were used for the experiments. For EGFR stimulation, cells were grown to approximately 80% confluence and washed with PBS three times. Cells were incubated overnight (16 h) in serum free media. EGFR stimulation was performed for 5 min with 100 ng/mL of EGFR (Gibco) at 37 °C. After stimulation, cells were washed and protein lysates were prepared in K Buffer lysis buffer. For tyrosine kinase inhibition, cells were pretreated with 15 µM of erlotinib for 1 h prior to EGF stimulation, as described above. HEK293 or MIA PaCa-2 cells were transfected with different AGO2 constructs using Fugene HD (Promega) or Lipofectamine 3000 (Invitrogen) according to the manufacturer’s protocols. For EGFR stimulation with transient AGO2 construct overexpression, cells were transfected ~16 h prior to overnight serum starvation and EGF stimulation.

RAS-less MEFs were a kind gift from the RAS initiative. Details of how these cells were developed and their growth characteristics can be found at https://www. cancer.gov/research/key-initiatives/ras/ras-central/blog/2017/rasless-mefs- drug-screens.

Immunoprecipitation (IP) analysis. For immunoprecipitation analysis, protein lysates were prepared in K Buffer (20 mM Tris pH 7.0, 5 mM EDTA, 150 mM NaCl, 1% Triton X100, 1 mM DTT, phosphatase inhibitors, and protease inhibitors). Typically, 150–200 µg of protein lysates (RAS10 IP: 150 µg; AGO2 IP: 200 µg) were pre-cleared with 10 µl of Protein A/G agarose beads (Santa Cruz) for 1 h. Pre-cleared lysates were incubated with 5–10 µg of the indicated primary antibodies targeting the protein of interest or with corresponding isotype controls overnight at 4 °C. Thirty microliters of Protein A/G beads were then added to immune complexes and incubated for 1–3 h at 4 °C, spun, and washed in 150–300 mM NaCl containing K-buffer prior to separation of immunoprecipitates by SDS-PAGE. To determine the varying levels of KRAS expressed in different cell lines (with and without EGF stimulation), shown in Fig. 7, pan RAS10 antibody was used for immunoprecipitation followed by immunoblot analysis using KRAS-specific SC-30 antibody.

β-galactosidase assay. β-galactosidase staining was performed using the Senesence β-Galactosidase Staining Kit #9860 (Cell Signaling) on 10 µM-thick frozen sections of mouse pancreas, as per the manufacturer’s protocol.

Statistics and reproducibility. Reproducibility of results were ensured by (1) involving multiple members of the team to collect data, (2) analyzing pathologies with two independent pathologists, and (3) repeating cell line-based experiments at least twice. Many experiments were repeated by multiple members of the group.
The data are representative of multiple biological replicates, and the number of times each of the individual data was repeated with similar results is indicated below.

Main Figures
Two: 5c, 5f, 6d, 6e, 6f, 7g, 7h, 7i, 8c, 8d
Three: 1b, 2c, 2d, 5a, 5g, 6g, 6h, 7a, 7b, 7d, 7i, 8a, 8b
Four: 2f, 7e, 7f
Six: 6l, 6j, 6k
Eight: 3c
Ten: 4a, 1c, 5b, 4d, 5e

Also note that for Fig. 2a, the image is representative of data collected in Fig. 2b, and representative images for Fig. 4b were obtained by analysis of six PanINs, six PDAC, and two metastatic tissues.

**Data availability**

All data generated or analyzed during this study are included in this article. This includes raw data for the immunoblot (Main Figs. 2c, 6c, e, f, g, h, 7a, b, c, d, e, f, g, h, i, 9a, b, c, d; and Supplementary Figs. 2b, c, 12a, b, c) and microRNA analyses (Fig. 6a, Supplementary Fig. 10a, b). A reporting summary for this article is available as a Supplementary Information file. The RNA sequencing data (for Fig. 4b and Supplementary Fig. 11) have been deposited in GEO under the accession number GSE147781.

Received: 26 July 2019; Accepted: 20 April 2020; Published online: 04 June 2020

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Acknowledgements

We thank Kristin M. Jackette, Fengyun Su, Grace Tsaioff, Lisha Wang, and Javed Siddiqui for their assistance with experimental work. We thank Mandy Davis and Marta Hernadi-Muller for their help with processing paraffin-embedded slides. We also thank Saravana Dhanasekaran, Miriam Gandham, and Markus Eberl for technical assistance. We thank members of the RAS Initiative, Dhirendra Simanshu (for discussions) and Jim Hartley (for providing RASless MEFs). We are also thankful for RAS/AGO2-related discussions with Phillip Sharp, Eric Fearon, and David Bartel. We acknowledge Sisi Gao for help in manuscript preparation. S.S. is a Genentech Fellow. J.S. is supported by NCI K08CA24222. H.C.C. is supported by NIH Grants U01 CA224145 and DOD CA170568. A.M.C. is a National Cancer Institute Outstanding Investigator (R35CA231996), Howard Hughes Medical Institute Investigator, A. Alfred Taubman Scholar, and American Cancer Society Professor.

Author contributions

Mouse experimental data were generated by J.C.T., S.C., A.G., X.L., and S.S. Contributions to other experimental data were made by S.S., R.F.S., V.L.D., S.E., S.E., X.C., M.M., S.P., J.I.A., and C.K.-S. R.M. and J. S. coordinated the pathology assessment. J.S. provided the human TMA and performed IHC scoring. X.W. performed RNA ISH. J.W. and J.T. supported work on AGO2 phosphorylation. P.V. and Y.Z. provided bioinformatics support. S.J.E. and X.C. helped with project management. H.C.C. supervised the pancreatitis experiments. S.S. and A.M.C. jointly conceived the study. S.S., C.R.-S., and A.M.C. wrote the manuscript. Funding and overall supervision of the study was provided by A.M.C.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information is available for this paper at https://doi.org/10.1038/s41467-020-16309-2.

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Peer review information Nature Communications thanks Lorenzo Galluzzi and the other, anonymous, reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

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