Abstract

SAG (Sensitive to Apoptosis Gene), also known as RBX2 or ROC2, is a RING component of CRL (Cullin-RING ligase), required for its activity. Our previous studies showed that Sag/Rbx2 co-operated with Kras or Pten loss to promote tumorigenesis in the lung and prostate, respectively, but antagonized Kras to inhibit skin tumorigenesis, suggesting a tissue/context dependent function of Sag. The role of SAG in KRAS-induced pancreatic tumorigenesis is unknown. In this study, we mined a cancer database and found that SAG is overexpressed in pancreatic cancer tissues and correlates with decreased patient survival. Whether Sag overexpression plays a causal role in pancreatic tumorigenesis is unknown. Here, we reported the generation of Sag transgenic mouse model alone (CS), or in combination with Kras\textsuperscript{G12D}, driven by p48-Cre (KCS mice) for pancreatic specific Sag expression. Sag transgenic expression alone has no phenotypical abnormality, but in combination with Kras\textsuperscript{G12D} promotes ADM (acinar-to-ductal metaplasia) conversion \textit{in vitro} and mPanIN1 formation \textit{in vivo} at the early stage, and impairs pancreatic functions at the late stage, as evidenced by poor glucose tolerance and significantly reduced $\alpha$-Amylase activity, and induction of cytogenesis and acinar cell loss, eventually leading to atrophic pancreata and shortened mouse life-span. Mechanistically, Sag transgenic expression altered several key signaling pathways, particularly inactivation of mTORC1 signaling due to Deptor accumulation, and activation of the antioxidant Nrf2-Nqo1 axis. Thus, Sag plays a stage dependent promotion (early) and fate-changing (late) role during Kras-pancreatic tumorigenesis, likely via regulating its key substrates, which control growth-related signal transduction pathways.

Keywords: ADM and mPanINs, Pancreatic tumorigenesis, Kras, SAG, Ubiquitin ligase

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the third leading cause of cancer death in the USA and one of the deadliest human malignancies with only 5 year survival rate of 9% [1]. Mechanistically, mutational activation of the KRAS oncogene occurs in 95% of cases, along with activation of the EGFR–MAPK and PI3K–AKT–mTORC signaling pathways [2,3]. However, Kras\textsuperscript{G12D} alone does not induce full-blown pancreatic cancer [4]. Therefore, identification of additional genetic alterations that interact with oncogenic Kras to accelerate the progression of mPanINs to invasive and metastatic PDAC is beneficial for establishing molecular targets for PDAC prevention and therapy.

SAG (Sensitive to Apoptosis Gene), also known as RBX2/ROC2, is the second member of RING family of SCF E3 ubiquitin ligases [5,6]. Although a large number of F-box proteins were found in the human genome [7] that selectively target various protein substrates, there are only two family members of RING proteins in human or mouse, RBX1/ROC1 and RBX2/ROC2/SAG that are required for the ligase activity by transferring ubiquitin from E2 to the substrate [5]. Our previous study using genetic
modified mouse models revealed that Rbx1 and Sag are not functionally redundant, since total KO of either gene causes embryonic lethality [8,9]. Our recent biochemical-based study showed that RBX1 exclusively binds to ubiquitin E2s CDC34 and UBCH5C to promote substrate polyubiquitylation via the K48 linkage, whereas SAG mainly binds to E2s UBCH10 and UBE2S to promotes substrate polyubiquitylation via the K11 linkage [10].

A potential role of SAG in human cancers was first implicated based on its overexpression in various carcinomas of lung, colon, stomach, cervix and liver, and an inverse correlation between SAG overexpression in lung cancer and patient survival [11–15]. To determine whether Sag plays a causal role in tumorigenesis, we have established several lines of mouse models. Sag transgenic expression caused early-stage suppression of tumor formation, but later-stage enhancement of tumor growth in skin tumorigenesis induced by DMBA–TPA [16]. In the UVB radiation model, Sag transgenic expression promoted skin hyperplasia, but had no significant effect on tumorigenesis [17]. More interestingly, Sag played a tissue- and context-dependent pro-oncogenic or pro-tumor suppressive role in tumorigenesis. Specifically, while Sag deletion in the lung and prostate significantly reduced tumorigenesis, triggered by KrasG12D or Pten loss, respectively [15,18], it accelerated skin tumorigenesis induced by KrasG12D [19]. Whether Sag plays a role in pancreas tumorigenesis, and, if so, the underlying mechanisms, is unknown.

In this study, we found that Sag is overexpressed in human pancreatic cancer, which is associated with poor patient survival. To pursue whether Sag overexpression plays a causal role in PDAC, we generated the CS (p48-Cre;Sag-Tg) or KCS (KrasG12D; p48-Cre;Sag-Tg) mouse models in which Sag transgenic expression alone, or in combination with Kras activation in the pancreas, driven by p48-Cre recombinase. We found that while Sag transgenic expression alone has no phenotype, it promotes ADM conversion and mPanIN formation at the early stage, but impairs pancreatic function by inducing cystogenesis and acinar cell loss at the late stage, leading to much shortened life-span. Mechanistically, Sag transgenic expression altered multiple signaling pathways, including inactivation of mTORC1 signaling and activation of the Nrf2-Nqo1 signaling axis. Thus, the role of Sag in pancreatic tumorigenesis induced by Kras is stage dependent and likely controlled by its key substrates, which affect several signaling pathways.

Materials and methods

Generation of Sag transgenic mice

Transgenic mouse with pancreas-specific overexpression of Sag were generated by using 5.2 kb of the mouse p48 promoter to drive expression of Sag under control of LoxP-EGFP-STOP-LoxP (p48-LGSL-FLAG-Sag) sequence (Fig. 2A). Note that the SV40 poly A is used as the transcriptional stop signal for EGFP, while BGH poly A is the stop signal for Sag overexpression plays a causal role in PDAC, we generated the CS mice (referred to hereinafter as KC mice) to create mice with Sag overexpression and Kras activation in pancreas, driven by p48-Cre recombinase (designated as KCS mice). A potential role of SAG in human cancers was first implicated based on its overexpression in various carcinomas of lung, colon, stomach, cervix and liver, and an inverse correlation between SAG overexpression in lung cancer and patient survival [11–15].

For glucose tolerance tests, 6-month KC and KCS mice were fasted overnight (14 h) then injected i.p. with glucose (2 g/kg). Tail vein blood glucose was measured using a blood glucose meter (Roche Diagnostic) at 0, 15, 30, 60, and 120 min after injection. For Amylase activity assay, pancreata from 6-month KC and KCS mice were prepared and the amylase activity was determined by using an amylase activity assay kit (Sigma-Aldrich), which results in a colorimetric (405 nm) product. The amylase activity is proportional to the amount of substrate (ethylidene-pNP-G7) cleaved by the amylase.

Cell line and characterization of p48-LGSL-FLAG-Sag in vitro

Mouse pancreatic acinar cell line 266-6 was obtained from the American Type Culture Collection. Cells were cultured in Dulbecco’s modified Eagle medium supplemented with 10% FBS, 2 mM glutamine, and antibiotics. Cells were transfected with p48-LGSL-FLAG-Sag by Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocols. Transfected 266-6 cells were further infected with Ad-Cre (adenovirus encoding Cre recombinase) to delete the EGFP-STOP segment DNA flanked by LoxP and get FLAG-Sag expression.

Fig. 1. Sag is overexpressed in human PDAC and correlates with poor patient survival. (A) The scatter plot displays the differential expression levels of SAG transcripts in paired PDAC and non-cancer pancreas tissues for the GSE15471 dataset. (B) Kaplan–Meier curve to show the association between the levels of SAG expression and overall survival of patients with PDAC.

In this study, we found that Sag is overexpressed in human pancreatic cancer, which is associated with poor patient survival. To pursue whether Sag overexpression plays a causal role in PDAC, we generated the CS (p48-Cre;Sag-Tg) or KCS (KrasG12D; p48-Cre;Sag-Tg) mouse models in which Sag transgenic expression alone, or in combination with Kras activation in the pancreas, driven by p48-Cre recombinase. We found that while Sag transgenic expression alone has no phenotype, it promotes ADM conversion and mPanIN formation at the early stage, but impairs pancreatic function by inducing cystogenesis and acinar cell loss at the late stage, leading to much shortened life-span. Mechanistically, Sag transgenic expression altered multiple signaling pathways, including inactivation of mTORC1 signaling and activation of the Nrf2-Nqo1 signaling axis. Thus, the role of Sag in pancreatic tumorigenesis induced by Kras is stage dependent and likely controlled by its key substrates, which affect several signaling pathways.
Immunohistochemistry and immunoblotting

Pancreatic tissues were fixed in 10% formalin and embedded in paraffin. Five-μm-thick sections were cut for H&E and immunohistochemistry staining. Immunohistochemistry was conducted as previously described [20]. Images were acquired with an Olympus BX-51 microscope, Olympus DP71 digital camera, and DP Controller software. Antibodies used for immunohistochemistry include those recognizing Ki67 (BD Bioscience, Cat. 550609, 1:500), CK19 (Abcam, ab87000, 1:500), Amylase (Sigma-Aldrich, A8273, 1:500), Muc5A (Abcam, ab3649, 1:1000), p-S6K (Novus, NB600-1049, 1:200), p-4EBP1 (Cell Signaling, #2855, 1:200), p-mTOR (Cell Signaling, #2976, 1:100), c-JUN (Cell Signaling, #9165, 1:200), Erbin (Abcam, ab124653, 1:100), Nqo1 (Santa Cruz, sc-32793, 1:100). The immunoblotting analysis was performed as described [21]. Antibodies used include those recognizing EGFP (Abcam, ab87000, 1:500), SAG (clone: Sag-10) [13,21], β-TrCP (Cell Signaling, #4394, 1:1000), DEPTOR (Cell Signaling, #11816, 1:1000), p-Erk1/2 (Cell Signaling, #9101, 1:1000), Erk1/2 (Cell Signaling, #9102, 1:1000), NRF2 (Cell Signaling, #12721, 1:1000), FLAG (Sigma, A2220, 1:2000), and β-actin (Sigma Aldrich, Clone AC-40, 1:5000).

Acinar cell culture

The 3D culture of pancreatic acinar cells was prepared by digesting pancreata from 2-month-old mice with Collagenase P and then culturing in Matrigel as previously described [20]. Briefly, pancreata from KC, KCS, and control mice were cut into small pieces and digested with 2 mg/ml of Collagenase P (Roche Diagnostics) in HBSS for 15 min at 37 °C. Cells were then washed three times with HBSS with 5% FBS and filtered through 100-μm nylon meshes. After centrifugation, the cell suspension was mixed 1:1 with Matrigel and plated onto the collagen layer. The acinar cell/Matrigel mix was allowed to solidify for 1 hour at 37 °C before adding medium. The formation of duct-like structures was observed at days 1, 2, and 3.
Histopathologic analysis

Histopathologic analysis was conducted by a pathologist at the University of Michigan (Dr. Ingrid Bergin) on all deidentified hematoxylin and eosin (H&E)-stained slides. Pancreata sections were evaluated for ADM, PanIN1, PanIN2, PanIN3, and PDA lesions based on a previously reported classification system [4]. Pancreata were diagnosed according to the most severe phenotype observed, and data were expressed as the percentage of animals with each phenotype.

Statistical analysis

Survival curves were calculated according to the Kaplan–Meier method, and statistical differences were analyzed by the log-rank and Gehan–Breslow–Wilcoxon tests using GraphPad Prism. A two-sided, unpaired Student’s t test was used for other statistical analyses. The p values of <0.05 were considered statistically significant.

Results

SAG is overexpressed in human PDAC, which correlates with poor patient survival

Previous studies from our laboratory and others have shown that SAG is overexpressed in a panel of human tumor tissues, including lung, colon, stomach, and liver, as compared to paired normal tissues, and SAG overexpression in lung cancer is positively correlated with poor survival of patients [11–15]. To determine potential alterations of SAG expression in human PDAC samples, we performed a datamining analysis in the Gene Expression Omnibus (GEO) database. In a study containing 36 paired human PDAC samples and their non-cancer pancreas tissues (GSE15471), SAG expression is significantly higher in the PDAC samples in comparison to the normal pancreas [22] (Fig. 1A). To associate SAG overexpression with patient survival, we performed the survival analyses of PDAC patients in TCGA based on SAG expression levels (High vs. Low/medium). According to the log-rank test from Kaplan–Meier survival analysis, high expression of SAG is associated with poor overall survival of the PDAC patients (http://ualcan.path.uab.edu/cgi-bin/TCGA-survival1.pl?genename=RNF7&dataset=PAAD) (Fig. 1B). Thus, SAG is indeed overexpressed in human PDAC tissues and SAG overexpression may serve as a novel prognostic biomarker for PDAC patients.

Construction and characterization of Kras\textsuperscript{G12D}, p48-Cre;FLAG-Sag (KCS) mouse model

KRAS mutations occur in almost all PDAC tissues, and mutated KRAS initiates pancreatic tumorigenesis and drives PDAC development [24,25]. To investigate if SAG overexpression seen in PDAC tissues indeed plays a causal role in pancreatic tumorigenesis triggered by KRAS mutation, we generated a Sag transgenic mouse model using 5.2 kb of the mouse pancreas-specific transcription factor 1 (Ptf1a)/p48 promoter to drive expression of FLAG-tagged Sag under control of LoxP-EGFP-STOP-LoxP (p48-LGSL-FLAG-Sag) sequence with the SV40 poly A being used as the transcriptional stop signal for EGFP, whereas the BGH poly A as the stop signal for FLAG-Sag (Fig. 2A). To validate that the p48-LGSL-FLAG-Sag transgene was working properly, we first characterized the transgene by transfecting it into a mouse pancreatic acinar cell line 266-6. Indeed, the cells transfected with p48-LGSL-FLAG-Sag construct exhibited high EGFP expression, whereas EGFP expression was completely abrogated in the same cells infected with Ad-Cre (adenovirus encoding Cre recombinase) which resulted in removal of the EGFP encoding sequence (Fig. 1B). FLAG-Sag expression and EGFP depletion was further confirmed by immunoblotting in 266-6 cells transfected with p48-LGSL-FLAG-Sag upon Ad-Cre infection (Fig. 2C), suggesting that transgenic construct is working, as expected.

To exclude the possibility of an off-target effect, we selected 2 independent transgenic lines (#659 and #694) for further studies. Compared to a control line (#630, without p48-LGSL-FLAG-Sag transgene), both transgenic lines expressed EGFP protein in the pancreas (Fig. 2D). We then crossed the p48-LGSL-FLAG-Sag mice with the KrasLSL\textsuperscript{G12D}/p48\textsuperscript{Cre/} mice (designated as KC) to create pancreas-specific Sag transgenic expression in the KC background (designated as KCS) as well as the p48-Cre; p48-LGSL-FLAG-Sag (designated as CS) mice. We determined FLAG-Sag expression in both lines upon introduction of p48-Cre allele in the pancreas and found that exogenous FLAG-Sag is indeed expressed, and to the levels similar to the endogenous Sag (Fig. 2E). Taken together, these results clearly show that p48-Cre drives FLAG-Sag expression in the pancreatic tissues of compound mice.

Sag transgenic expression cooperates with Kras mutation to trigger pancreatic atrophy and shorten the life-span

Both lines of KCS mice were born with expected Mendelian frequency. While CS mice with Sag transgenic expression without Kras\textsuperscript{G12D} did not show any abnormality up to adulthood, the KCS mice started to show the signs of growth retardation with a hunched-posture after 4-5 months of age. We, therefore, dynamically observed the body weight of CS, KC, and KCS mice in both genders and found that KCS mice started to lose body weight at around 15 weeks with significant weight loss at week of 30, which is sex-independent (Fig. 3A). Indeed, KCS mice have smaller body size, and the pancreata from the KCS mice were much smaller and atrophic (Fig. 3B). The pancreas/body (w/w) ratio was significantly reduced in KCS mice as compared to the KC mice, while the other organs in KCS mice were proportional to mouse weight, except for the spleen which also had a smaller ratio (Fig. 3C and D). Survival of CS, KC and KCS (two independent lines) mice was monitored for up to 600 days. Remarkably, the KCS mice (both lines) had significantly shortened life-span, compared to the KC mice (Fig. 3E). In line with these growth-retarded and pancreas-atrophic phenotypes, most KCS mice became ill and had to be euthanized between 8 and 10 months. The biochemical analyses of KCS mice revealed signs of diabetes with much higher values in the glucose tolerant test (GTT) (Fig. 3F), and reduced α-Amylase levels in the sera (Fig. 3G). Thus, it is likely that KCS mice may die of defective pancreatic functions before fully developing PDAC. Taken together, these results clearly showed that Sag transgenic expression in combination with Kras\textsuperscript{G12D} activation significantly retards pancreatic development, and impairs pancreatic functions, which certainly contributes to the shortened life-span.

Sag transgenic expression promotes early mPanIN formation, followed by pancreatic cystogenesis with the loss of acinar structures

To determine the potential effect of Sag-transgenic expression on Kras-induced pancreatic tumorigenesis, we histologically assessed the pancreata from KCS mice at 2, 4, and 6 months of age. At 2 months of age, increased numbers of mPanIN1a/1b structures were evident in KCS mice, but not in KC mice. At 4 months of age, more mPanIN1a/1b structures were found, along with the appearance of cystic structures in the KCS mice, whereas only some scattered mPanIN1 structures started to show in the KC mice. Remarkably, at the age of 6 months, although the extent of mPanINs and cysts in the pancreata of KCS mice varies, most of KCS mice showed profound cystic structures with limited mPanIN1 structures and an overall loss of normal acinar cells and islets, while the KC mice developed some of mPanIN2-3 structures and maintained normal acinar cells.
structures (Fig. 4A). Consistent with the H&E staining and serum α-Amylase activity assay, the immunohistochemistry staining revealed that the acinar cell marker α-Amylase was almost completely lost in the pancrease of KCS mice at 6 months, compared to their KC littermates (Fig. 4B, left). While the expression of cytokeratin 19 (CK19), a biomarker for duct-like lesions in PDAC [23], showed comparable levels (Fig. 4B, middle), the expression of Muc5AC, a marker for invasive progression of PDAC [24], was remarkably reduced in the KCS mice, compared to the
KC littermates (Fig. 4B, right). We further assessed the proliferative marker Ki67 and found significantly decreased levels of Ki67 in KCS pancreata (Fig. 4C), which is in agreement with acinar cell loss and pancreas atrophy. Taken together, these results demonstrate that Sag transgenic expression promotes the mPanIN formation at the early stage, followed by cystogenesis which severely damages the acinar structures and impairs the proliferation and normal function of the pancreas, leading to an atrophic and diabetic phenotypes and shortened mouse life-span.

**Fig. 4.** KC-Sag transgenic mice show accelerated PanIN formation at early stage, and cystic structures in the later stage. (A) H&E staining of pancreata of KC and KCS mice at 2, 4, and 6 months. Scale bar, 100 μm. Each image is representative of at least three independent animals. (B) Immunohistochemical assessment of Amylase, CK19, and Muc5A in KC and KCS pancreata at 6 months. Scale bar, 100 μm. (C) Immunohistochemical assessment of Ki67 in KC and KCS pancreata at 6 months. Scale bar, 100 μm (Left). Quantitation of Ki67-positive cells in each of the indicated genotypes (Right). Five randomly selected, nonoverlapping high-power images (20× objective) were taken from each slide of each group. Nuclei positive for Ki67 were counted as actively proliferating cells. Data are the mean percentage of total counted nuclei for each of 5 randomly selected fields ± SE (p < 0.01**).
Sag transgenic expression accelerates acinar-to-ductal metaplasia conversion with enlarged duct-like structures

The early appearance of the mPanIN1 structure could imply a rapid and profound earlier onset of ADM in KCS pancreata. To confirm the ADM acceleration by Sag transgenic expression, we used the 3D cultures of acinar clusters and found the formation of duct-like structures as early as 1 day in KCS mouse acini. A higher percentage was also found in acinar cells from KCS, as compared to those from KC (Fig. 5A and B). Remarkably, the duct-like structures from KCS acini have a much larger size at day 2 and 3, which is consistent with the observed increase in PanIN lesions in vivo (Fig. 5A and C). These data suggest that Sag transgenic expression cooperates with mutant Kras to promote ADM conversion at the early stage. The larger size of duct-like structure may be associated with later stage of cystogenesis seen in the pancreata of KCS mice.

Sag transgenic expression dysregulates multiple signaling pathways

SAG/RBX2, is the RING component of the Cullin-RING ligases (CRLs), and specifically for SCF/CRL1 and CRL5 which promote the ubiquitylation and degradation of many key signaling molecules to regulate a number of cellular processes [5]. We previously showed that Sag promotes the ubiquitylation and degradation of c-Jun, IκBα, p27, and Erbin to regulate skin tumorigenesis [16,17,19,21], and apoptosis [25], HIF-1α for hypoxia regulation [26], and β-TrCP to regulate its substrates [10]. We next sought to examine the protein levels of some key substrates in the pancreata from two independent KCS and KC mice, respectively. Immunoblotting analysis revealed that Sag transgenic expression significantly reduced the levels of Erbin, an inhibitor of Ras activation of Raf [19], HIF-1α, and β-TrCP1, moderately reduced the levels of c-Jun, but had no effect on IκBα and p27 (Fig. 6A, top panels), suggesting a context dependent degradation of known Sag substrates. Decreased levels of
Erbin and c-Jun were also confirmed by immunohistochemistry staining (Fig. 6B).

In line with reduction of β-TrCP1, we observed accumulation of Deptor, a well-known substrate of β-TrCP1 [27–29], and a naturally occurring inhibitor of both mTORC1 and mTORC2 [30]. Indeed, consistent with Deptor accumulation, mTorc1 signaling was significantly inactivated in KCS pancreata tissues, as evidenced by decreased levels of p-mTOR, p4EBP1, pS6K (Fig. 6A, middle panels), which was further confirmed by reduced levels of pS6K, p4EBP1 and p-mTOR assessed by immunohistochemistry staining (Fig. 6B). Finally, we found that while it had minor, if any, effect on the Ras-MAPK pathway, Sag transgenic expression caused significant accumulation of Nrf2, an antioxidant transcription factor [31] with a corresponding increase in its downstream target, Nqo1 (NAD(P)H:quinone oxidoreductase 1) by both immunoblotting and immunohistochemistry staining (Fig. 6A and B, bottom panels). Taken together, Sag transgenic expression altered multiple signaling pathways mainly via promoting ubiquitylation and degradation of selective sets of substrates, leading to inactivation of the mTORC1 pathway, which likely contributes to reduced proliferation and pancreas atrophy in KCS mice.

Discussion

SAG/RBX2 is a dual E3 for neddylation and ubiquitylation: when coupled with UBE2F neddylation E2, SAG acts as a neddylation E3 to promote cullin-5 neddylation; when coupled with ubiquitylation E2s, UBE2C/UBE2S or UBCH5C, SAG formed complex with cullin-5 or cullin-1, respectively and acts as ubiquitylation E3 to promote ubiquitylation and degradation of many substrates [5,10,32]. Our previous study showed that SAG is overexpressed in various human cancers, and SAG silencing inhibits pancreatic cancer cell growth both in vitro and in an

![Fig. 6](image-url).

Fig. 6. SAG transgenic expression altered multiple signal pathways. (A) Western blot analysis of the indicated proteins in lysates of pancreatic tissues from two independent 6-month-old KC and KCS mice. (B) Immunohistochemical assessment of c-Jun, Erbin, p-S6K, p-4EBP1, and p-mTOR in KC and KCS pancreata at 6 months of age. Scale bar, 100 μm.
orthotopic in vivo mouse model [5,13]. In line with these observations, here we show with cancer database profiling that SAG is overexpressed in a cohort of human PDAC samples, and higher SAG expression levels are associated with poor prognosis in PDAC patients. However, it is previously unknown whether this overexpressed SAG indeed plays a causal role in pancreatic tumorigenesis.

To this end, we generated two SAG transgenic mouse lines with pancreas-selective expression driven by a 5.2 kb p48 promoter fragment upon Cre-mediated recombination. While mice with SAG transgenic expression in the pancreas alone develop normally without any visible abnormal phenotypes, SAG transgenic expression under the background of KrasG12D (KCS) demonstrated many abnormal phenotypes, including atrophic pancreata with impaired pancreatic functions, smaller body size, and much shorter life-span. While SAG-transgenic expression indeed promotes the development of low-grade mPanIN structure (PanIN1a and 1b) at the early stage, no further development to high-grade mPanINs or PDAC was found. Instead, KCS mice at 6 months had profound pancreatic atrophy with various sized cystic structures. Consistent with these abnormal pancreas features, the in vitro 3D-culture assay showed that SAG-transgenic expression accelerated ADM conversion with much enlarged duct-like structures. These results suggested that SAG transgenic expression accelerates the process of Kras-induced precursor lesions at the early stage, but altered the cell fate from neoplastic progression to cystogenesis, leading to the massive loss of acinar cells, reduced proliferation, atrophic pancreas with impaired pancreatic function, and eventually early death. The dual roles of SAG overexpression in Kras-mutated mouse pancreata implies that SAG might target different sets of substrates in a cell-context (acinar cells vs. intraepithelial neoplastic cells) dependent and time dependent manner. This is reminiscent of the role of SAG in skin carcinogenesis induced by DMBA/TPA, where SAG-transgenic expression leads to a reduced skin hyperplasia by targeting c-Jun/AP-1 at the early stage, but enhanced tumor growth via activating NF-kB by targeting IxB to inhibit apoptosis at the later stage [16].

In an attempt to elucidate the underlying mechanisms of SAG action, we used immunoblotting and immunohistochemistry staining methods to measure the protein levels of several SAG substrates known to regulate cell growth or be responsive to stress in pancreatic tissues from KCS vs. KC mice. We found that while SAG transgenic expression did not affect the levels of p27 or IxB, it did reduce the levels of Erbin, HIF-1x, c-Jun and β-TrCP1. Furthermore, SAG transgenic expression increased the levels of Nrf2 and its transcriptional target, Nqo1. While reduced Erbin, c-Jun and the levels of p27 or IκB would be expected to reduce cell growth or be responsive to stress in pancreatic tissues from KCS vs. KC mice. However, it is previously unknown whether this overexpressed SAG indeed plays a causal role in pancreatic tumorigenesis.

In addition, we also observed increased protein levels of the antioxidant protein Nrf2 and its transcription target Nqo1 in KCS pancreata. The abnormal expression and activation of Nrf2 has been observed at different stages of pancreatic cancer and correlated with its initiation, progression, metastasis, and chemoresistance [39,40]. Interestingly, Nrf2 also plays dual roles in pancreatic carcinogenesis. At the early stage, Nrf2 exerts a tumor-suppressive role through binding to antioxidant response elements (AREs) and promoting its downstream target gene expression, which regulates the cellular antioxidant/detoxification response [41]. At the late stage, however, Cullin3-Keap1 mutation and silencing are frequently observed, which cause Nrf2 accumulation, leading to pancreatic cancer cell metastasis and chemoresistance [39,42]. Thus, it is also possible that elevated Nrf2 resulting from SAG transgenic expression contributes to blockage of disease progression from the early precursor lesions.

How SAG transgenic expression causes Nrf2 accumulation is unclear. Our previous study using a KrasG12D skin cancer model showed that SAG deletion triggered Nrf2 accumulation; whereas in a prostate cancer model triggered by Pten loss, SAG deletion caused Deportor accumulation [18]. These opposite results in other organs from this KCS-PDAC study further demonstrate that SAG indeed plays a tissue/context-dependent role. By tissue-specific targeting of a subset of its substrates, SAG could play an oncogene- or tumor suppressor-cooperating role in regulation of tumorigenesis. Indeed, SAG is pro-oncogenic in the lung [15] and tumor-suppressive in the skin [19] during KrasG12D-induced tumorigenesis, and in the prostate, SAG is pro-oncogenic during tumorigenesis induced by Pten loss [18]. In this pancreatic KCS model, SAG appears to be a Kras-cooperating gene at the early stage, but tumor suppressive by inactivating mTORC1 signaling at the later stage.

Finally, it is worth noting that two-fold increase of SAG levels via transgenic expression (at the level comparable to endogenous, Fig. 2E) did cause biochemical changes in the levels of several key proteins, likely due to increased ligase activity of SAG-associated CRL E3s, which contributes to observed phenotypical changes, as shown in this study. This observation is consistent with our previous reports that SAG is stress inducible protein [5,43], and induced SAG in response to stressed conditions, such as mitogen stimulation, hypoxia exposure or ROS, did cause enhanced degradation of several substrates, such as c-JUN and IκB [16,21,25,26]. On the other hand, SAG deletion caused accumulation of its substrates in a context dependent manner, again leading to phenotypic changes [15,18,19,44].

In summary, our study demonstrates that pancreatic specific SAG transgenic expression in combination with KrasG12D promotes ADM and early mPanIN formation, but without preceding to later stage mPanINs, nor adenocarcinoma. Instead, SAG transgenic expression at the later stage caused acinar cell loss, pancreatic atrophy, and pancreatic cytogenesis, which could be attributable to alterations in multiple signaling pathways including the β-TrCP1/Depotr/mTOR axis and Nrf2 accumulation. The future study is directed toward delineating the detailed molecular mechanisms of SAG in different stages of pancreatic tumorigenesis and progression using rescuing approaches.

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Authors’ contributions

Q.Z. and Y.S. conceived and designed the experiments, Q.Z., D.W., and M.T. performed the experiments. All the authors analyzed the data. M.M. and Y.S. supervised the study. Q.Z. and Y.S. wrote the paper. All authors reviewed and approved the manuscript before submission.
Competing interests

The authors declare that they have no competing interests.

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