Abstract
Pancreatic cancers driven by KRAS mutations require additional mutations for tumor progression. The tumor suppressor FBXW7 is altered in pancreatic cancers, but its contribution to pancreatic tumorigenesis is unknown. To determine potential cooperation between Kras mutation and Fbxw7 inactivation in pancreatic tumorigenesis, we generated P48-Cre;LSL-KrasG12D;Fbxw7fl/fl (KFCfl/fl) compound mice. We found that KFCfl/fl mice displayed accelerated tumorigenesis: all mice succumbed to pancreatic ductal adenocarcinoma (PDA) by 40 days of age, with PDA onset occurring by 2 weeks of age. PDA in KFCfl/fl mice was preceded by earlier onset of acinar-to-ductal metaplasia (ADM) and pancreatic intraepithelial neoplasia (PanIN) lesions, and associated with chromosomal instability and the accumulation of Fbxw7 substrates Yes-associated protein (Yap), c-Myc, and Notch. Using KFCfl/fl and FBXW7-deficient human pancreatic cancer cells, we found that Yap silencing attenuated growth promotion by Fbxw7 deletion. Our data demonstrate that Fbxw7 is a potent suppressor of KrasG12D-induced pancreatic tumorigenesis due, at least in part, to negative regulation of Yap.

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Introduction
Pancreatic cancer has an overall 5-year survival rate of 8% and is currently the fourth leading cause of cancer-related death in the United States. Nearly all human pancreatic cancers are characterized by mutations in KRAS followed in order of mutational frequency by P16, P53, and DPC4 [1]. Genetically engineered Kras mutant mouse models of pancreatic cancer recapitulate the human disease process beginning with acinar-to-ductal metaplasia (ADM), followed by pancreatic intraepithelial neoplasia (PanIN) formation, and ultimately pancreatic ductal adenocarcinoma (PDA) [2]. The introduction of other mutations such as p53, p16, or Dpc4 further accelerates the development of PDA. Using an inducible Kras mutant (G12D) mouse model, it was shown that Kras mutation is involved not only in initiation but also in the maintenance of PDA, as inactivation of mutant Kras in established tumors leads to tumor regression [3]. Furthermore, tumor regrowth following inactivation of mutant Kras involves expression of Yap which is also required for initiation of PDA in Kras mutant mice [4,5].

FBXW7, a component of the Skp1-Cullin1-Fbox E3 ubiquitin ligase complex, is a characterized tumor suppressor. For instance, conditional Fbxw7 deletion cooperates with APC mutation or P53

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1 One-sentence summary: This study for the first time identifies FBXW7 as a suppressor of mutant Kras-driven tumorigenesis.

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deletion to accelerate intestinal tumorigenesis in a haploinsufficient manner [6,7]. Although FBXW7 is most frequently mutated in colorectal, uterine, and bladder cancers, it is also a significantly mutated gene in pancreatic cancers [8,9]. Furthermore, in a Sleeping Beauty transposon insertional mutagenesis model of Kras \(^{G12D}\)-induced pancreatic tumorigenesis, Fbxw7 mutation cooperated with Kras \(^{G12D}\) to accelerate PDA formation with a high frequency (24%), suggesting that Fbxw7 may be an important tumor suppressor in Kras-driven pancreatic cancers [10]. In addition to mutation, there is also emerging evidence to suggest that FBXW7 is altered by other mechanisms in pancreatic cancers including by reduced gene expression and heightened protein degradation which correlate with reduced survival [11].

Although the tumor suppressor functions of FBXW7 have largely been attributed to its proteolytic regulation of oncogenic substrates such as Cyclin E, c-MYC, Notch, and c-JUN, FBXW7 regulates over 30 substrates and likely unknown substrates as well [12,13]. Loss of FBXW7 promotes genomic instability in part by increased Cyclin E expression [14] which may contribute to tumorigenesis. We have recently reported that FBXW7 regulates nonhomologous end-joining (NHEJ) via K63-linked polyubiquitination of XRCC4, promoting the interaction of XRCC4 with DNA double-strand breaks and other core NHEJ proteins [15]. This direct involvement of FBXW7 in NHEJ may also contribute to the genomic instability and tumorigenesis in FBXW7-deficient models.

Because pancreatic cancer is characterized most frequently by Kras mutation (~100%) [1] as well as mutations or reduced expression of FBXW7 in a subset of human pancreatic cancers [8,9,11], in this study, we investigated whether FBXW7 acts as a tumor suppressor in Kras \(^{G12D}\)-driven pancreatic tumorigenesis. Using a pancreas conditional P48-Cre;LSL-Kras \(^{G12D}\};Flox/\text{n}\text{flox}\) mouse model, we found that homozygous deletion of Fbxw7 causes a dramatic acceleration of Kras \(^{G12D}\)-driven pancreatic tumorigenesis. Based on this finding, we went on to investigate the contribution of Fbxw7 substrates, such as Yap, to the mechanisms of tumorigenesis in both mouse and human pancreatic cancer cells.

Materials and Methods

Mouse Strains

The conditional LSL-Fbxw7\text{/flox}\text{/flox}\ mouse was a gift from Dr. Iannis Aifantis (New York University) [16]. Conditional LSL-Fbxw7\text{/flox}\text{/flox}; LSL-Kras \(^{G12D}\); and P48-Cre strains were interbred to obtain LSL-Kras \(^{G12D}\); LSL-Fbxw7\text{/flox}\text{/flox}; P48-Cre triple mutant animals (KFC). The mice were housed in specific pathogen-free facilities, and all the studies were conducted in compliance with the Institutional Committee on Use and Care of Animals guidelines. Genotyping was carried out by tail clipping from mice 2 weeks after birth. Tail specimens were incubated for 24 hours at 55°C in tail-lysis buffer containing proteinase K. NaCl was added, and cellular debris were pelleted by centrifugation. DNA was precipitated by the addition of isopropanol and washed with 70% ethanol. DNA pellets dissolved in water were used for polymerase chain reaction (PCR) analysis.

Cell Lines

Panc-1 cells were 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. The KFC cell line was derived from the tumor of a 3-week-old KFC mouse. Briefly, mouse pancreas was harvested and finely minced and incubated with a Collagenase V/HBSS (with Ca and Mg) solution at 37°C under constant shaking for 1.5 hours. Collagenase digestion of pancreas tissue was neutralized by RPMI-1640 medium containing 10% FBS. Digested tumor cells were filtered through a 40-μm filter and then cultured in RPMI-1640 medium containing 10% FBS, 2 mM glutamine, and antibiotics.

Acinar Cell Culture

The 3D culture of pancreatic acinar cells was prepared by digesting pancreata from 1-week-old mice with Collagenase P followed by culture in Matrigel as previously described [17]. Briefly, pancreata from KFC, KC, and control mice were cut into small pieces and digested with 2 mg/ml of Collagenase P (Roche Diagnostics) in HBSS for 15 minutes 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 ductlike structures was observed at days 1, 2, and 3.

Histopathologic Analysis

Histopathologic analysis was conducted by a pathologist (Y.Z.) 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 [2]. Pancreata were diagnosed according to the most severe phenotype observed, and data were expressed as the percentage of animals with each phenotype.

Immunohistochemistry and Immunoblotting

Immunohistochemistry was conducted as previously described [3]. Images were acquired with an Olympus BX-51 microscope, Olympus DP71 digital camera, and DP Controller software. Antibodies used for immunohistochemistry include those recognizing YAP (Cell Signaling, #4912, 1:500), c-MYC (Abcam, ab32072, 1:200), Notch (Cell Signaling, #3608, 1:250), Ki67 (BD Bioscience, Cat. 550609, 1:500), CK19 (Abcam, ab87000, 1:500), and Amylase (Sigma-Aldrich, A8273, 1:500).

Immunoblotting was conducted as previously described [15]. FBXW7 was detected by FBXW7 immunoprecipitation followed by immunoblotting. Antibodies used for immunoblotting in this study include those recognizing FBXW7 (Bethyl, A301-720A, 1:1000), c-MYC (Abcam, ab32072, 1:200), Notch (Cell Signaling, #3608, 1:250), Ki67 (BD Bioscience, Cat. 550609, 1:500), CK19 (Abcam, ab87000, 1:500), and Amylase (Sigma-Aldrich, A8273, 1:500).

Lentivirus-Based shRNA Infection and siRNA Transfection

Cells were infected with lentivirus (control 5′-GCAAGCT GAACCTGAAATTTCAT-3′; FBXW7: 5′-ACAGGACAGTTTCA AAA-3′) in the presence of 8 μg/ml of polybrene. Silencing efficiency was detected 72 hours after infection by immunoblot. For Yap RNAi experiments, cells were transfected with Oligofectamine (Invitrogen) transfection reagent. The two independent siYap oligonucleotide sequences used were 5′-CCACACACCUAGAAAGA-3′ (siYap-2) and 5′-GACCGUACACUCUGAGA-3′ (siYap-4). Nonspecific siRNA was used as a control.
**Results**

**Acceleration of Kras<sup>G12D</sup>-Pancreatic Tumorigenesis by Fbxw7 Deletion**

To investigate the involvement of Fbxw7 in mutant KRAS-driven pancreatic tumorigenesis, we crossed P48-Cre;LSL-Kras<sup>G12D</sup> mice [2] with Fbxw7<sup>F/F</sup> mice [6] to generate mice with pancreas specific activation of Kras<sup>G12D</sup> and heterozygous or homozygous deletion of Fbxw7 (designated KFC<sup>F/F</sup> or KFC<sup>F/F</sup>, respectively; Figure 1A). We first confirmed deletion of Fbxw7 by examining Fbxw7 protein levels in pancreata from 1-week-old mice and found Fbxw7 protein to be substantially reduced in pancreata from KFC<sup>F/F</sup> mice compared with those from KC control mice or KFC<sup>F+/</sup> mice (Figure 1B). To begin to establish a phenotype for these mice, survival was monitored in KC, KFC<sup>F+/</sup>, KFC<sup>F/F</sup> as well as in F<sup>F/F</sup> mice. We observed a profound effect of homozygous Fbxw7 deletion on survival of KFC<sup>F/F</sup> mice (Figure 1C) with a median survival time of 28.5 days. Most KFC<sup>F/F</sup> displayed early symptoms of morbidity and were euthanized; 100% of KFC<sup>F/F</sup> mice succumbed to PDA by 40 days compared with only 7% of KFC<sup>F+/</sup> and none of the KC or F<sup>F+/</sup> mice during the observation period. Gross examination of KFC<sup>F/F</sup> mice revealed smaller body size, enlarged pancreata, ascites, and duodenum adhesions in most mice with pancreas main duct obstruction and liver metastases occurring in a subset of mice (Supplementary Figure 1, A–B). Histological examination of pancreas sections from 1-month-old KFC<sup>F/F</sup> mice revealed moderately to poorly differentiated PDA accompanied by PanIN3 lesions (Figure 1D). In contrast, pancreata from KC or F<sup>F+/</sup> mice appeared largely normal, although early-stage PanIN lesions were occasionally observed in KFC<sup>F+/</sup> pancreata (Supplementary Figure 1F). To further explore the effects of heterozygous Fbxw7 deletion, we also examined pancreata from 6-month-old mice. Whereas KC mice showed some PanIN lesions, KFC<sup>F+/</sup> contained more PanIN lesions, ranging from PanIN1A to PanIN3 in both genotypes, but no PDA (Figure 1D). Pancreata from F<sup>F+/</sup> mice were overall normal with the exception of occasional enlarged ductal cells, consistent with a prior study [18] (Supplementary Figure 1G).

**Tumorigenesis in Kras<sup>G12D</sup>;Fbxw7-Deleted Pancreata Is Preceded by ADM and PanIN and Associated with Chromosomal Instability**

We then sought to characterize the progression of precursor lesions leading to PDA in KFC<sup>F/F</sup> mice. To this end, pancreata from 1-, 2-, and 3-week-old KC, KFC<sup>F+/</sup>, and KFC<sup>F/F</sup> mice were examined histologically and scored for the presence of ADM, PanINs, and PDA. In KC and KFC<sup>F+/</sup> mice at 1 to 2 weeks of age, pancreata were composed mainly of acinar cells with no observable ADM or PanIN lesions (Figure 2, A and B). By 3 weeks of age, pancreata from KC and KFC<sup>F+/</sup> mice began to develop ADM with progression toward PanIN2 in the KFC<sup>F+/</sup> mice. In striking contrast, KFC<sup>F/F</sup> pancreata contained PanIN1 and PanIN2 lesions as well as stroma accumulation at 1 week, which advanced in combination with PanIN3 and PDA at 2 weeks. By 3 weeks of age, 100% of KFC<sup>F/F</sup> pancreata contained PDA. To confirm the acceleration of ADM in KFC<sup>F/F</sup> pancreata, we used 3D cultures of acinar clusters and observed the formation of ductlike structures by as early as 1 day in KFC<sup>F/F</sup> acini (Figure 2, C and D). Further characterization of KFC<sup>F/F</sup> pancreata showed increased CK19, decreased amylase, and increased Alcian Blue staining, confirming ADM and progression toward PanIN lesions, respectively (Supplementary Figure 2).

**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. P values of <.05 were considered statistically significant.

**Accumulation of Fbxw7 Substrates in Fbxw7-Deleted Pancreata**

To begin to determine the mechanisms leading to accelerated pancreatic tumorigenesis in the KFC<sup>F/F</sup> mice, pancreata from 1-week-old mice were analyzed for expression of oncogenic substrates of Fbxw7 [12,13]. We hypothesized that Fbxw7 substrates involved in Kras<sup>G12D</sup>-driven tumorigenesis would be accumulated in response to homozygous Fbxw7 deletion. Of the substrates investigated, we found that the levels of c-Myc, Yap, and Notch, but not c-Jun, Cyclin E, and Mcl-1, were elevated in KFC<sup>F/F</sup> pancreata as well as in the KFC<sup>F+/</sup> pancreata, although to a lesser extent (Figure 3A). Evaluation of these proteins immunohistochemically confirmed their increased expression within aberrant ductal structures in KFC<sup>F/F</sup> pancreata (Figure 3B). In addition, we also assessed the proliferative marker Ki67 and found significantly higher levels of Ki67 in KFC<sup>F/F</sup> pancreata (Figure 3B, Supplementary Figure 4A). These changes in KFC<sup>F/F</sup> pancreata appeared to be independent of Kras activity, as downstream effectors of Kras (pErk1/2 and pAkt) were not elevated in KFC<sup>F/F</sup> pancreata (Supplementary Figure 4B). Taken together, these data demonstrate an association between increased c-Myc, Yap, and Notch expression and Kras<sup>G12D</sup>-induced tumorigenesis in Fbxw7 homozygous deleted mice.

**Rescue of Growth Promotion Caused by Fbxw7 Deletion by Depletion of Yap**

Based on recent studies implicating Yap in Kras<sup>G12D</sup>-induced pancreatic tumorigenesis [4,5], we hypothesized that Fbxw7 may promote tumorigenesis through a Yap-dependent mechanism. To test this hypothesis, we first evaluated the ability of Fbxw7 to regulate Yap protein levels in a cell line established from KFC<sup>F/F</sup> pancreatic
tumors. Consistent with the low levels of Yap observed in pancreata from KC mice (relative to KFC), exogenously expressed FBXW7 reduced Yap, c-Myc, and Notch protein levels in primary KFC-derived cells (Figure 4A) without substantial changes in other Fbxw7 substrates (Cyclin E, Mcl-1, mTOR; Supplementary Figure 5A). We then depleted Yap from KFC cells to determine its contribution to the increased growth of KFC cells and found that Yap depletion caused a significant decrease in the growth of KFC cells which was similar to the effect observed by exogenous FBXW7 expression (Figure 4B). Interestingly, Yap depletion was also associated with reduced c-Myc protein levels (Figure 4A). To further define the role of YAP in KFC fl/fl tumorigenesis, we utilized human pancreatic cancer cells that were deleted of FBXW7 by shRNA and then further silenced for YAP by siRNA. FBXW7 shRNA-treated Panc-1 and MiaPaCa-2 cells demonstrated increased levels of YAP and c-MYC levels without change in other FBXW7 substrates (Figure 4C; Supplementary Figure 5A). Silencing of YAP by either of two independent siRNAs caused a reduction in c-MYC protein levels and, more importantly, significantly inhibited the growth of FBXW7-deficient human pancreatic cancer cells (Figure 4D, Supplementary Figure 5D). These results are consistent with the accelerated tumorigenesis and increased Yap levels observed in KFC fl/fl pancreata. Taken together, these data demonstrate that Yap accumulation is causally related, at least in part, to the accelerated growth observed in Fbxw7-deficient, Kras mutant pancreatic cancer cells.

Figure 1. Deletion of mouse Fbxw7 accelerates KrasG12D-driven pancreatic tumorigenesis. (A) Genetic makeup of the KFC model. (B) Western blot analysis of Fbxw7 protein levels in KC, KFC fl/+, and KFC fl/fl pancreata specimens from 1-week-old mice. (C) The survival of KC, FC, KFC fl/+, and KFC fl/fl mice is expressed using the Kaplan-Meier method, and statistically significant differences from KC mice are shown (P < .0001 ***). (D) H&E staining of pancreata at 1 and 6 months. Scale bar, 100 μm. Each image is representative of at least three independent animals.
In this study, we show for the first time that Fbxw7 is a suppressor of Kras mutation-driven pancreatic tumorigenesis. The dramatically accelerated tumorigenesis observed in the KFCfl/fl mice was associated with accumulation of oncogenic Fbxw7 substrates such as c-Myc, Notch, and Yap. Importantly, we found that Yap expression was a driving mechanism of the accelerated tumorigenesis in response to Fbxw7 deletion given that Yap depletion attenuated growth in both KFC primary cells as well as FBXW7-deficient human pancreatic cancer cells. These findings suggest that alterations in FBXW7 may be an important contributor to human pancreatic tumorigenesis. Furthermore, our data suggest that Fbxw7 is a relatively potent suppressor of pancreatic tumorigenesis given the development of tumors by less than 1 month in response to Fbxw7 deletion relative to

**Figure 2.** Pancreatic tumor progression in KFCfl/fl mice. (A) H&E staining of KC, KFCfl/+, and KFCfl/fl pancreata at 1, 2, and 3 weeks. Scale bar, 100 μm. (B) Pathologic analysis of the lesions of KC, KFCfl/+, and KFCfl/fl pancreata at 1, 2, and 3 weeks. Data represent at least three independent mice for each genotype and time point. (C) Deletion of Fbxw7 accelerates ADM. Transmitted light images of control, KC, and KFCfl/fl pancreatic cell clusters in 3D culture from day 0 to 3 are shown. Scale bar, 100 μM. (D) Quantification of ductlike structures. Statistically significant differences were determined by a two-sided, unpaired Student’s t test and are indicated (*P < .01**).

**Discussion**

In this study, we show for the first time that Fbxw7 is a suppressor of Kras mutation-driven pancreatic tumorigenesis. The dramatically accelerated tumorigenesis observed in the KFCfl/fl mice was associated with accumulation of oncogenic Fbxw7 substrates such as c-Myc, Notch, and Yap. Importantly, we found that Yap expression was a driving mechanism of the accelerated tumorigenesis in response to Fbxw7 deletion given that Yap depletion attenuated growth in both KFC primary cells as well as FBXW7-deficient human pancreatic cancer cells. These findings suggest that alterations in FBXW7 may be an important contributor to human pancreatic tumorigenesis. Furthermore, our data suggest that Fbxw7 is a relatively potent suppressor of pancreatic tumorigenesis given the development of tumors by less than 1 month in response to Fbxw7 deletion relative to
latency periods of 2 to 9 months in other KnTS\(^{G_{12D}}\) models (e.g., \(p16^{ink4a}\), \(P53\), and \(Dpc4\)) [21–23]. Although \(FBXW7\) is most frequently mutated in colorectal, uterine, and bladder cancers, it is also a significantly mutated gene in pancreatic cancers [8,9]. \(FBXW7\) mutations are generally heterozygous, missense, loss-of-function mutations occurring in the WD domain that function in a dominant negative manner [13,24]. There are however emerging evidence also to suggest that \(FBXW7\) is altered by other mechanisms in pancreatic cancers including by reduced gene expression and heightened protein degradation [11]. It is important for future studies to define the spectrum of genetic and posttranslational alterations of \(FBXW7\) in both primary and

![Figure 3. Analysis of Fbxw7 substrates in pancreata.](image)

(A) Western blot analysis of the indicated proteins in lysates obtained from pancreata of 1-week-old mice for each of the indicated genotypes. (B) Immunohistochemical assessment of Yap, c-Myc, Notch, and Ki67 in KC, KFC\(^{fl/+}\), and KFC\(^{fl/fl}\) pancreata at 1 week. Scale bar, 100 \(\mu m\).
metastatic human pancreatic cancers. In addition, given the frequency of KRAS and FBXW7 mutations in colorectal cancers, studies to investigate their effects on intestinal tumorigenesis are warranted.

Prior studies have shown that Fbxw7 is a haploinsufficient tumor suppressor [7,25]. In mice with P53 deletion, heterozygous Fbxw7 deletion resulted in the development of multiple tumor types [25]. Similarly, intestinal tumorigenesis in APC mice was accelerated by heterozygous deletion of Fbxw7 [7]. In the current study, we found that homozygous deletion of Fbxw7 produced a dramatic acceleration of pancreatic tumorigenesis that was not observed under heterozygous conditions. However, consistent with a haploinsufficient function of Fbxw7, KFC B- mice did have an increased frequency of PanIN lesions relative to KC mice (Figure 2B), leading to the development of PDA in a subset of these mice (7%). Taken together, these results are consistent with a haploinsufficient effect of Fbxw7 on PanIN development but clearly demonstrate that the most profound effects of Fbxw7 on KratG12D-induced tumorigenesis require homozygous deletion.

Our findings implicate Yap accumulation as being causally related, at least in part, to the accelerated growth observed in Fbxw7-deficient, Krat mutant pancreatic cancer cells. Furthermore, it is also possible that YAP accumulation is associated with the observed genomic instability, as other studies have shown that YAP can promote genomic instability [26]. Interestingly, we also found c-Myc, another established Fbxw7 substrate, consistently elevated in KFC fl/fl pancreata and FBXW7-deficient human pancreatic cancer cells. Although it is conceivable that direct regulation of c-Myc by Fbxw7 could be a mechanism of tumorigenesis, Yap depletion led to reduced expression of c-Myc (Figure 4, A and C, and Supplementary Figure 5 C). This finding, together with a recent report demonstrating YAP-dependent c-MYC expression [27], suggests that c-Myc may function downstream of Yap in KratG12D-induced pancreatic tumorigenesis. Future studies are required to fully define the mechanisms of Fbxw7-mediated regulation of Yap and c-Myc in pancreatic tumorigenesis.

In conclusion, this study demonstrates that Fbxw7 suppresses KratG12D-induced pancreatic tumorigenesis via a Yap-dependent mechanism. These findings suggest that YAP and the Hippo pathway should be explored as a therapeutic strategy in pancreatic cancers with...
FBXW7 alterations. Furthermore, based on the genomic instability associated with FBXW7 deletion [14,15], investigation of therapeutic strategies exploiting DNA damage repair pathways is warranted for cancers with FBXW7 alterations.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.neo.2016.08.009.

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