ARF6 and AMAP1 are major targets of KRAS and TP53 mutations to promote invasion, PD-L1 dynamics, and immune evasion of pancreatic cancer

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Although KRAS and TP53 mutations are major drivers of pancreatic ductal adenocarcinoma (PDAC), the incurable nature of this cancer still remains largely elusive. ARF6 and its effector AMAP1 are often overexpressed in different cancers and regulate the intracellular dynamics of integrins and E-cadherin, thus promoting tumor invasion and metastasis when ARF6 is activated. Here we show that the ARF6–AMAP1 pathway is a major target by which KRAS and TP53 cooperatively promote malignancy. KRAS was identified to promote eIF4E-dependent ARF6 mRNA translation, which contains a quadruplex structure at its 5'-untranslated region, by inducing TEAD3 and ETV4 to suppress PDCD4; and also eIF4E-dependent AMAP1 mRNA translation, which contains a 5'-terminal oligopyrimidine-like sequence, via up-regulating mTORC1. TP53 facilitated ARF6 activation by platelet-derived growth factor (PDGF), via its known function to promote the expression of PDGF receptor \( \beta \) (PDGFR\( \beta \)) and enzymes of the mevalonate pathway (MVP). The ARF6–AMAP1 pathway was moreover essential for PDGF-driven recycling of PD-L1, in which KRAS, TP53, eIF4A/4E-dependent translation, mTOR, and MVP were all integral. We moreover demonstrated that the mouse PDAC model KPC cells, bearing KRAS/TP53 mutations, express ARF6 and AMAP1 at high levels and that the ARF6-based pathway is closely associated with immune evasion of KPC cells. Expression of ARF6 pathway components is statistically correlated with poor patient outcomes. Thus, the cooperation among eIF4A/4E-dependent mRNA translation and MVP has emerged as a link by which pancreatic driver mutations may promote tumor cell motility, PD-L1 dynamics, and immune evasion, via empowering the ARF6-based pathway and its activation by external ligands.

ARF6 | mRNA translation | pancreatic driver oncopgenes | PD-L1 | mevalonate pathway

The 5-y overall survival rates of pancreatic ductal adenocarcinoma (PDAC) remain no more than 10% (1). Such poor outcomes of PDACs in most cases are a result of locally advanced malignancy (i.e., tumor invasion into surrounding tissues) already at the time of the initial diagnosis (2). Metastases are also frequently observed at the initial diagnosis (2). Consistently, a mathematical model suggested that metastatic subclones are generated late during the genetic evolution of PDACs, but long before tumors have grown to the size of clinical detection (3). A recent study also suggested that the acquisition of metastatic potential occurs at an early stage of pancreatic oncogenesis (4); however, the underlying molecular mechanisms remain largely unknown.

Four major driver mutations have been identified in PDAC, namely, mutations of KRAS, TP53, CDKN2A, and SMAD4/DPC4 (5–7). KRAS mutations occur in more than 90% to 95% of cases, often exert gain-of-oncogenic activities, and have been demonstrated to be an initiating event of PDAC oncogenesis (7–11). Oncogenic KRAS may moreover promote metabolic reprogramming to support tumor growth (12) and tumor signaling via stromal reciprocation (13). Mutations in TP53, which also often result in gain-of-oncogenic activities, occur in up to 70% of PDACs, potentially occurring at an early stage of pancreatic oncogenesis (4). To whom correspondence may be addressed. Email: sabeh@med.hokudai.ac.jp.

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and typically at a later time than KRAS mutations, and are frequently linked to invasive and metastatic phenotypes (1, 7), in which the induction of platelet-derived growth factor receptor β (PDGFRβ) was shown to be crucial (14). Oncogenic p53 may cooperate with oncogenic KRAS (26, 27). We found that highly invasive and metastatic PDAC cell lines express ARF6 and its signaling components at high levels and identified that KRAS and TP53 oncogenic mutations are causative for generating and help in activating the ARF6-based pathway. Intriguingly, ARF6 and AMAP1, as well as KRAS/TP53 oncogenic mutations, were moreover pivotal to the intracellular recycling of PD-L1, as well as its cell-surface expression. Consistently, mouse model experiments demonstrated a possible linkage of the ARF6-based pathway, as well as KRAS/TP53 oncogenes and associated events, with the immune evasion properties of PDACs. Our results demonstrate that the eIF4A/eIF4E/mRNA translational machinery, PDGFR, and MVP are excellent targets to block the invasive/metastatic phenotypes of PDACs and might also be useful for improving PD-1/PD-L1–based therapeutics, if cancer cells overexpress the ARF6-based pathway.

Results

High Expression Levels of Components of the ARF6–AMAP1 Pathway and Its Activation by PDGFR Are Crucial for PDAC Malignancy. MIAPaCa-2 cells (KRAS G12C and TP53 R248W/R273H) and Panc-1 cells (KRAS G12D and TP53 V272A/R273H) are typical pancreatic cancer cell lines with highly advanced malignancy. We first found that they both express ARF6, AMAP1, and GEP100 at high levels, similarly to highly invasive MDA-MB-231 breast cancer cells, as we showed previously (Fig. 1A) (17, 18, 25). The acquisition of mesenchymal phenotypes is associated with malignancy and the therapeutic resistance of different types of cancers, including PDACs (28). MIAPaCa-2 and Panc-1 cells show mesenchymal phenotypes (29) and express ZEB1, an EMT-associated transcriptional factor (29). ZEB1 is a core inducer of the mesenchymal component of the ARF6 pathway, EPB41L5 (23). Consistently, these cells also expressed EPB41L5 at high levels (Fig. 1A).

MIAPaCa-2 cells express PDGFRβ at high levels as a result of a TP53 mutation (14). We then found that PDGFRβ (i.e., stimulation with PDGFB-BB) activates ARF6 in these cells, and the silencing of GEP100 blocks the activation (Fig. 1B and SI Appendix, Fig. S1A). Moreover, the silencing of ARF6, GEP100, AMAP1, and EPB41L5 each substantially blocked PDGF-induced Matrigel invasion (Fig. 1C and SI Appendix, Fig. S1A). Silencing of these genes did not notably affect cell viability (SI Appendix, Fig. S1B).

To verify the general usage of the ARF6-based pathway in different PDACs, we then analyzed KPC cells, which were isolated from KPC (LSL-Kras(G12D/+); LSL-Tprp53(R172H); Pdx-1-Cre) mice, a well-established model of human PDAC (26). KPC cells are highly metastatic (26) and express ZEB1 (30) and PDGFRβ (14). We found that KPC cells express ARF6, GEP100, AMAP1, and EPB41L5 at high levels (Fig. 1D) and use these proteins for PDGF-induced ARF6 activation and Matrigel invasion (Fig. 1D and E and SI Appendix, Fig. S1C). We previously demonstrated in breast cancer cells and renal cancer cells that the ARF6-based pathway promotes metastasis in vivo, in which we either blocked the AMAP1 function by a small peptide or silenced the EPB41L5 expression by a short hairpin RNA (shRNA) (18, 20). Likewise, we here confirmed that silencing EPB41L5 in KPC cells blocked their metastasis into the lungs, in which we injected these KPC cells into tail veins of nude mice and measured their metastasis into the lungs (Fig. 1 F and G and SI Appendix, Fig. S1E). Again, silencing of ARF6 pathway components in KPC cells did not notably affect cell viability (SI Appendix, Fig. S1D).

We then performed immunohistochemical (IHC) analyses on the clinical specimens. Clinicopathological parameters of the patient cohort included in this study were summarized in Table S1. None of the patients received chemotherapy or radiation therapy before surgery. The high expression levels of AMAP1, EPB41L5, and PDGFRβ, each alone or in combination, statistically correlated with poor disease-free survival and poor overall survival of the patients (Fig. 1 H and J), whereas high expression of GEP100 did not statistically correlate with poor outcomes (SI Appendix, Fig. S1F and G). ARF6 antibodies that were clearly applicable for IHC were not available. Together with the above results, our results collectively indicate that the ARF6-based pathway is central to the malignancy of a significant population of PDACs.

KRAS Oncogenic Mutation Promotes ARF6 and AMAP1 Protein Expression via mRNA Translation. We then found that ARF6 and AMAP1 protein expression is under the control of KRAS. Silencing of the KRAS in MIAPaCa-2 cells and KPC cells significantly decreased the protein levels of ARF6 and AMAP1, but not EPB41L5 or β-actin (Fig. 2A). siKRAS also reduced ARF6 and AMAP1 protein levels in Panc-1 cells bearing mutant KRAS, but not in BscPC3 cells bearing intact KRAS (SI Appendix, Fig. S2A). KRAS silencing in MIAPaCa-2 cells also reduced ARF6 mRNA levels by about one-half (SI Appendix, Fig. S2B), which was consistent with a previous report (31), whereas AMAP1 mRNA was not notably affected (SI Appendix, Fig. S2B). We have previously shown that ARF6 and AMAP1 proteins are overexpressed at levels several-fold higher in highly invasive breast cancer cells than in normal mammary epithelial cells (17, 18). Therefore, the 2-fold augmentation of ARF6 mRNA expression by KRAS does not appear to be the entire mechanism by which KRAS overexpresses ARF6 in PDACs.

We then found that ARF6 and AMAP1 mRNAs are both under translation control by KRAS. ARF6 and AMAP1 mRNAs are both rich in G/C content in their 5′-untranslated regions (UTRs) (74% and 88%, respectively) (19). Moreover, ARF6 mRNA contains a G-quadruplex structure at the 5′-UTR (Fig. 2B) (32), which is indicative of its translational control by the RNA helicase eIF4A (33). The 5′-UTR of AMAP1 mRNA contains a 5′-terminal oligopyrimidine (TOP)-like sequence (Fig. 2B), which is indicative of its control by mTOR and eIF4E (34, 35). Polysomal profiling of mRNAs prepared from MIAPaCa-2 cells and their siKRAS–treated derivative then demonstrated that KRAS silencing shifted ARF6 and AMAP1 mRNAs to be localized to less dense polysome fractions (Fig. 2C). eIF4A acts as a subunit of the eIF4F complex, which initiates mRNA
Fig. 1. The ARF6–AMAP1 pathway is central to PDAC malignancy. (A) Expression of ARF6 pathway components in PDAC cells, analyzed by Western blotting. β-Actin was used as a loading control. (B–E) PDGF activates ARF6 via GEP100 (B and D) and promotes cell invasion via ARF6, GEP100, AMAP1, and EPB41L5 (C and E). Two different siRNAs (#1 and #2) were used. Irr, a control siRNA with an irrelevant sequence. In C and E, results are shown as ratios by normalizing values obtained for Irr-treated cells as 1 (n = 3). Error bars, mean ± SEM; *P < 0.001. (F and G) Lung metastases of KPC cells, expressing a luciferase reporter gene and transfected with an EPB41L5 shRNA plasmid (shEPB41L5, sequence #2) or a control empty vector (Irr), in nude mice. In F, bioluminescence intensities from the chests were measured on days 0 and 9. Results are shown as the mean ± SEM; *P < 0.05 (n = 5 for each group); NS, not significant. In G, representative images of the lungs are shown. (H) Representative IHC images of PDGFRβ, AMAP1, and EPB41L5 in human primary PDACs. (I) Kaplan–Meier plots with regard to the different levels of PDGFRβ, AMAP1, EPB41L5, and their combinations (high, score of 1 or 2; low, score 0). P values were obtained by ANOVA (C, E, and F) and by the log-rank test (I).
translation in a manner dependent on the 7-methylguanylate cap (Gcap) of the 5′-end of mRNAs to sustain protein synthesis (36). The function of eIF4E also depends on the 5′-end Gcap, but not the 5′-end adenylate cap (Acap)-dependent translation of ARF6 and AMAP1 mRNAs was confirmed (Fig. 2D). These results suggested that KRAS acts to promote the translation of ARF6 and AMAP1 mRNAs in a 5′-cap-dependent manner.

**KRAS Oncogene Promotes ARF6 mRNA Translation by Suppressing PDCD4.** We next found that ARF6 expression is highly sensitive to silvestrol, an eIF4A inhibitor (37). Silvestrol (10 nM) readily blocked ARF6 expression almost completely in MIAPaCa-2 cells and KPC cells, in which AMAP1 and β-actin levels were not notably affected (Fig. 3A). PDCD4 acts as a negative regulator of eIF4A via direct binding (38). Silencing of the KRAS promoter PDCD4 expression at both the protein and the mRNA levels (Fig. 3B and SI Appendix, Fig. S2B), and consequently, forced expression of PDCD4 by cDNA transfection reduced ARF6 levels (Fig. 3C). Thus, ARF6 mRNA appeared to be under the translational control of eIF4A, in which the KRAS oncogene is likely to up-regulate eIF4A activity via suppressing PDCD4 expression. Consistently, PDCD4 mRNA levels were significantly lower in the presence of mutant (MT) KRAS than in the presence of wild-type (WT) KRAS in The Cancer Genome Atlas (TCGA) RNASeq dataset of PDACs (https://cancergenome.nih.gov/) (Fig. 3D). Our results were also consistent with a previous report showing the decreased expression of PDCD4 mRNA in highly invasive PDAC cells (39).

**KRAS Oncogene Induces TEAD3 and ETV4 to Suppress PDCD4.** We then sought to understand the possible mechanism by which KRAS suppresses PDCD4 (for this, we analyzed only human cells, as factors involved in transcriptional regulation may be different between humans and mice). We isolated DNA fragments 5′ upstream of the transcription start site (TSS) of PDCD4 and measured their transcriptional activities in response to KRAS in MIAPaCa-2 cells and Panc-1 cells, by ligating them into the 5′ end of the firefly luciferase gene. Analysis of different length fragments (#1 to #6; Fig. 3E and SI Appendix, Fig. S3A) demonstrated that a region encompassing 1.0 kb and 0.7 kb 5′ upstream of the TSS responds well to KRAS (i.e., its transcriptional activity was augmented upon KRAS silencing) (Fig. 3E and SI Appendix, Fig. S3A and B). Genome-wide RNASeq analysis using MIAPaCa-2 cells then demonstrated that different transcriptional factors (TFs) each possessing putative binding sites at the PDCD4 promoter region become suppressed upon KRAS silencing (to less than 1.5-fold; SI Appendix, Fig. S3C). Among them, the expression of TEAD3, FOXL1, ETV4, and FOSSL1 mRNAs showed statistically significant negative correlations with PDCD4 mRNA levels in the TCGA RNASeq dataset; and moreover, the expression of these 4 mRNAs was higher in the presence of the MT KRAS gene than that in the presence of the WT KRAS gene (SI Appendix, Fig. S3D). We then focused on these 4 TFs, shRNA-mediated silencing of TEAD3 and ETV4 in MIAPaCa-2 cells promoted transcription of the PDCD4 #4 fragment, but not the #5 fragment, compared with Irr-treated controls in the luciferase assay (Fig. 3F and SI Appendix, Fig. S3E). Silencing of FOXL1 and FOSSL1 also up-regulated PDCD4 #4 transcription, but to a lesser extent than TEAD3 and ETV4 silencing (Fig. 3F and SI Appendix, Fig. S3E). Up-regulation of PDCD4 protein levels by shTEAD3 and shETV4, and also by shFOXL1 and shFOSSL1 to lesser extents, was also confirmed (Fig. 3G). We also detected the binding of TEAD3 and ETV4 to the PDCD4 locus in these cells (Fig. 3H and SI Appendix, Fig. S3 F and G). Therefore, it is likely that the KRAS oncogene induces several TFs, such as TEAD3 and ETV4, to suppress PDCD4, whereas we have yet to identify the mechanisms by which KRAS induces these TFs.
KRAS Oncogene Promotes AMAP1 mRNA Translation via mTORC1.

KRAS signaling can activate mTOR (40), and consistently, mTOR is known to be highly activated in the majority of PDACs (41). mTOR, when complexed with Raptor (i.e., mTORC1), phosphorylates 4EBP1, which is a negative regulator of eIF4E, and releases eIF4E (42). We found that siRNA-mediated silencing of MTOR and RPTOR, but not RICTOR, significantly reduces AMAP1 protein levels in MIAPaCa-2 cells and KPC cells, to be accompanied with the reduced phosphorylation of 4EBP1 (Fig. 3I and SI Appendix, Fig. S3I). mTOR inhibitors, such as rapamycin and Torin 1 (35), were also successful in reducing AMAP1 levels, without notably affecting ARF6 levels (Fig. 3J and SI Appendix, Fig. S3J). Therefore, AMAP1 mRNA appeared to be under the translational control of mTORC1. EIF4EBP1 (encoding 4EBP1) expression is impaired in more than 50% of primary PDACs (43), and the AMAP1 gene is frequently

Fig. 3. (A–J) Mechanisms by which KRAS up-regulates mRNA translation of ARF6 (A–H) and AMAP1 (I and J) in PDACs. (A) Suppression of ARF6 levels by silvestrol. (B) Induction of PDCD4, coupled with reduction of ARF6 levels, upon KRAS silencing. (C) Suppression of ARF6 levels by the forced expression of PDCD4. (D) Box-and-whisker plots of PDCD4 mRNA levels in wild-type KRAS-expressing (WT) and mutant KRAS-expressing (MT) PDACs of the TCGA RNASeq dataset (n = 151). P < 0.01 by the Welch t test. (E) Schematic drawing of the 5′-upstream fragments of the PDCD4 TSS (#4 to #6) and their transcriptional activities, as assessed by the luciferase reporter assay, in response to siKRAS in MIAPaCa-2 cells (for regions #1 to #3; SI Appendix, Fig. S3A). **P < 0.01 by ANOVA. (F and G) Luciferase reporter assay results of the PDCD4 promoter region (F) and protein levels of ARF6 and PDCD4 (G) in MIAPaCa-2 cells, infected with lentiviruses bearing shRNAs, as indicated. (H) Binding of TEAD3 and ETV4 to the PDCD4 promoter region in MIAPaCa-2 cells. Input, 5% of total lysate used for the immunoprecipitation. IgG, an irrelevant IgG. For positions a–f, see SI Appendix, Fig. S3F. (I and J) AMAP1 levels in MIAPaCa-2 cells, infected with lentiviruses bearing the indicated shRNAs (I) or treated with vehicle (DMSO) or mTOR inhibitors, as indicated (J). Levels of ARF6, 4EBP1, and phosphorylated 4EBP1 are also shown. In B, F, G, and I, 2 different shRNAs (#1 and #2) were used, and levels of the target proteins are shown. Irr, an irrelevant shRNA. Representative results are shown in each immunoblot from at least 3 independent experiments. β-Actin was used as a control. Error bars indicate the mean ± SEM; **P < 0.01, ***P < 0.001 by ANOVA.
amplified in PDACs (44). These alterations might also promote AMAP1 protein levels in PDACs, in cooperation with the KRAS oncogene.

**MVP Is Pivotal to ARF6 Activation by PDGFR.** MVP activity, as well as that of GGT-II and RAB11b, is essential for EGFR-mediated ARF6 activation in breast cancer cells; and hence constitutive MVP up-regulation by mutant p53 facilitates ARF6 activation by EGFR, as already mentioned. We then analyzed the involvement of TP53, as well as MVP and associated factors, in the PDGF-mediated ARF6 activation in PDACs. As expected, RAB11b and GGT-II were found to be essential for PDGF-mediated ARF6 activation and Matrigel invasion of MIAPaCa-2 cells (Fig. 4 A and B). Likewise, silencing of TP53, as well as simvastatin, an inhibitor of hydroxymethylglutaryl-CoA reductase, significantly blocked these activities (Fig. 4 C–F). On the other hand, PDGF did not notably activate ARF6 in Capan-2 cells, which possessed KRAS mutation but their TP53 is intact (45). Likewise, Capan-2 cells were weakly invasive even in the presence of PDGF, and silencing of ARF6 and AMAP1 did not notably affect their invasiveness (SI Appendix, Fig. S4 F and G). Cell viability was not notably affected by these siRNAs or by simvastatin (SI Appendix, Fig. S4 A–E and H). Therefore, as in the case of breast cancer cells, the TP53 mutation may act to promote RTK-mediated ARF6 activation in PDACs, in which MVP, GGT-II, and RAB11b also appear to be integral.

**KRAS and TP53 Oncogenes Promote PD-L1 Recycling and Cell Surface Expression via ARF6 and AMAP1.** PD-L1 and PD-L2 are ligands for PD-1 that activate immune checkpoints. High expression levels of PD-L1, but not PD-L2, statistically correlate with the poor outcome of patients with PDAC (46). Not only high expression levels, but also the dynamic nature of PD-L1, being actively recycling between the cell surface and endosomal compartments, might also be important for the efficient formation of PD-L1/PD-L1–based immune synapses, as shown with T cell antigen receptors in immune synapse assembly (47, 48). ARF6 primarily acts to promote recycling, particularly the outward flow of plasma membrane components, such as integrins (22, 49). Thus, we were finally interested in investigating whether the ARF6–AMAP1 pathway is involved in PD-L1 dynamics.

We first found that PD-L1, induced by IFNγ (50), is mostly localized at the cell periphery to be well colocalized with cortical actins in MIAPaCa-2 cells (Fig. 5A). Silencing of ARF6 and AMAP1, as well as the silencing of KRAS and TP53, each significantly affects the colocalization of PD-L1 with cortical actins and causes a diffuse distribution of PD-L1 throughout the cell surface (Fig. 5 A and B). Silvestrol and simvastatin as well as the silencing of GGT-II, mTOR, and RAPTOR also demonstrated similar effects (Fig. 5 A, C, and D), whereas shRICTOR did not (Fig. 5D). EPB41L5 links AMAP1 with E-cadherin dynamics (23, 24), and we found that EPB41L5 is not involved in PD-L1 dynamics (Fig. 5A). The induction of...
Fig. 5. ARF6 and AMAP1, as well as KRAS and TP53, are pivotal to PD-L1 dynamics and cell surface expression. (A–D) Representative images of immunofluorescence staining for PD-L1 (red) and F-actin (green) in IFNγ-treated MIAPaCa-2 cells, pretreated with siRNAs/shRNAs (A, B, and D) and simvastatin or silvestrol (C), as indicated. Nuclei were visualized by DAPI (blue). (Scale bars, 10 μm.) (E) Recycling of PD-L1 to the cell surface in the presence or absence of PDGF in IFNγ-treated MIAPaCa-2 cells, pretreated with siRNAs, as indicated. (F) PD-L1 cell surface expression in IFNγ-treated or nontreated MIAPaCa-2 cells, pretreated with siRNAs. MFI, median fluorescence intensity. (G) s.c. tumor growth of AMAP1-silenced KPCs (shAMAP1 #1 and #2) or control KPCs (Irr) in immunodeficient BALB/c nude mice and immunocompetent C57BL/6 mice. Tumors were measured every 2 to 4 d starting on day 5. Data are representative of 3 independent experiments with at least 6 mice per group. In A, D, E, and F, sequence #1 was used for each siRNA/shRNA. In A, B, and D–G Irr indicates an irrelevant siRNA/shRNA. Error bars indicate the mean ± SEM; *P < 0.05, **P < 0.01, ***P < 0.001 by ANOVA (F and G).
PD-L1 by IFNγ, as determined by protein immunoblotting, was not notably affected by the above gene silencing, except a slight decrease upon TP53 silencing (SI Appendix, Fig. S5 A–E).

Generally, the intracellular recycling of cell surface molecules is crucial for their colocalization with cortical actin structures (51). We then found that PDGF promotes the recycling back of PD-L1 to the plasma membrane and that the silencing of ARF6 and AMAP1, but not EPB41L5, almost completely abolished the PDGF-induced recycling of PD-L1 (Fig. 5E). Cell surface levels of PD-L1, as measured using a fluorescence-activated cell sorter, were also significantly reduced upon the silencing of ARF6 and AMAP1 (Fig. 5F and SI Appendix, Fig. S5F). On the other hand, PD-L1 internalization was not notably affected by these gene silencing (SI Appendix, Fig. S5G), to be consistent with the basic function of ARF6 (22, 49). We also confirmed that silencing of KRAS and TP53 each affects the PDGF-mediated PD-L1 recycling (SI Appendix, Fig. S5H). Taken together, our data collectively indicated that the ARF6–AMAP1 pathway promotes PD-L1 recycling and cell surface expression in response to PDGF and that the cooperation of KRAS and TP53 oncogenes may thereby be causative to promote PD-L1 dynamics. However, we still do not know what protein(s) links AMAP1 with PD-L1.

Prompted by the above findings, we finally tested whether the ARF6–AMAP1 pathway is involved in the immune evasion of cancers. We noticed that constitutive silencing of ARF6 for a long time somewhat affects cell viability and proliferation, whereas silencing of AMAP1 did not (SI Appendix, Fig. S5 I and J) (20). We therefore prepared KPC cells in which AMAP1 was silenced by the shRNA method (#1 and #2) and s.c. injected them into immunodeficient BALB/c mice or into immunocompetent C57BL/6 mice, the latter of which are syngeneic to KPC cells. Although the silencing of AMAP1 did not affect the growth of KPC cells in BALB/c mice, as observed in culture, this silencing remarkably impaired their growth in C57BL/6 mice (Fig. 5G). Thus, our data supported a notion that the ARF6–AMAP1 pathway is crucially involved in the immune evasion of PDAC cells, whereas the precise mechanisms therein involved still remain unclear.

**Discussion**

In this study, we show that cooperation between KRAS and TP53 oncogenic mutations of PDACs activates the ARF6–AMAP1 pathway to promote tumor malignancies including the immune evasion properties. In this process, KRAS primarily acts to promote eIF4A/4E-dependent mRNA translation to up-regulate ARF6 and AMAP1 protein expression, whereas on the other hand, TP53 acts to facilitate ARF6 activation by PDGFR, in which the up-regulation of MVP by this oncogene is also crucial. We moreover demonstrated that the ARF6–AMAP1 pathway promotes PD-L1 recycling and cell surface expression in response to RTK activation, in which the KRAS/TP53 oncogenic mutations, as well as eIF4A/4E, mTOR, GGT-II, and MVP, are crucial (Fig. 6). Furthermore, a close association between the ARF6–AMAP1 pathway and the tumor immune evasive phenotype was observed in vivo. Our results thus highlight PDAC as a disease of enhanced mRNA translation and protein geranylgeranylation, in which these events are likely to be the primary targets of the driver oncogenes in promoting tumor malignancies.

Our results confirmed a prevailing notion that the onset of mesenchymal programs is crucial for cancer malignancy, by showing a tight correlation between high EPB41L5 expression levels and the poor prognosis of patients with PDAC. We moreover demonstrated that EPB41L5 promotes metastasis of
PDAC cells. We were interested in understanding the mechanisms involved in the induction of EBP41L5, as well as in the induction of ZEB1, which then induces EBP41L5 (23). In this regard, although TP53 mutations appeared to be crucially involved in the induction of the ZEB1-EBP41L5 axis in MiaPaCa-2 cells and KPC cells, as well as in a significant population of primary PDACs, as we have observed previously with breast cancer cells (23), we found that the link between TP53 and induction of the mesenchymal axis is not a linear process nor simply mediated by particular miRNAs (52). Likewise, the induction of ZEB1 in KPC tumors was reported to be noncell autonomous and to occur sporadically, even within the same tumor mass (30). Thus, we still do not know the precise mechanisms by which EBP41L5 and its associated mesenchymal properties emerge in PDACs. Whether EBP41L5 functions only in association with AMAP1, as well as with the ARF6 pathway, in PDAC cells also needs to be determined.

RTKs are known to activate RAS. It is well documented that the overexpression of RTKs, such as EGFR and Her2, is a major risk factor for breast cancer (53), and consistently, RAS mutations are very rare in breast cancers (54). Thus, it was enigmatic as to why PDGFR overexpression was a risk factor of PDACs (14), although most PDACs already have the KRAS oncogene. RTKs directly activate ARF6 via GEP100 (25). Likewise, we showed here that PDGFR activates ARF6 via GEP100 in PDACs. Therefore, our results provide a possible interplay for PDGFR overexpression being a risk factor of PDACs. On the other hand, GEP100 binds to different RTKs. Thus, the overexpression of RTKs other than PDGFR might also be a risk factor of PDACs, if cells have the KRAS oncogene as well as show enhanced MVP activity.

eIF4A-dependent translation requires large amounts of ATP (36). Likewise, mTOR activation requires high levels of cellular ATP (i.e., a high ATP/AMP ratio) to suppress the inhibition by AMPK (42). Moreover, MVP starts from acetyl-CoA and needs its precursors, such as glucose, fatty acids, and amino acids. On the other hand, the KRAS oncogene may also promote glucose uptake and anabolic metabolism in PDACs (12). Likewise, TP53 mutations (i.e., loss of normal p53) can be associated with the enhanced glucose uptake and metabolism of tumor cells (55–57). Taken together, nutrition-rich conditions within the tumor environment may lead to high production of ATP within tumor cells, which may be prerequisites that predispose the KRAS/TP53 oncogenes to fuel the ARF6–AMAP1 pathway to promote malignancy.

Clinical therapies targeting immune checkpoints still remain largely ineffective in PDACs. The active recycling of PD-L1 might be favorable for the efficient formation of PD-1/PD-L1–based immune synapses, as discussed earlier, and may also weaken the effect of antibody-based therapies via enhanced endocytosis of antibodies bound to PD-L1 (58). Enhanced cell-surface expression of PD-L1 on its own might also help tumor cells to outcompete microenvironmental immune cells through enhanced glucose uptake and consumption (59). Although we demonstrated that the ARF6–AMAP1 pathway is closely associated with the immune evasive properties of PDACs, whether enhanced PD-L1 dynamics are at the core of driving the immune evasive phenotype needs to be clarified. Furthermore, whether pharmacological inhibition of this pathway, such as by statins, or silvestrol and mTOR inhibitors, abates immune evasive properties of PDACs also awaits to be tested.

**Materials and Methods**

**Patients and Tissue Samples.** All clinical specimens were selected from patients who underwent pancreatectomy at Hokkaido University Hospital between January 1999 and December 2005 and were analyzed retrospectively. None of the patients received chemotherapy or radiation therapy before surgery. Clinicopathological parameters of the patients at the time of pancreatectomy are summarized in SI Appendix, Table S1. This study was approved by the Institutional Review Board of Hokkaido University Hospital (study approval no. 014-0084). Comprehensive agreement regarding specimen storage was obtained in writing from all patients at the time. PDAC is one of the most aggressive types of solid malignancies. In particular, the 5-y survival rate remains low at ~5% to 7%. Therefore, at the beginning of our study, these patients, who underwent pancreatectomy between January 1999 and December 2005, had passed away or were unable to give informed consent. However, the Institutional Review Board of Hokkaido University Hospital recognized the importance of analyzing these clinical specimens in our study and approved this study without requiring written consent from individual patients.

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