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 eIF4A-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 β (PDGFRβ) 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 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 oncogenes | 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,

Significance

Pancreatic ductal carcinomas (PDACs) have been extensively studied regarding their genomic alterations, microenvironmental intercommunication, and metabolic reprogramming. However, identification of the protein machinery of tumor cells that eventually drives malignancy as a result of driver mutations, and their associated events, is highly anticipated toward the development of precision medicine. The lack of such information regarding PDACs has hindered the elucidation of mechanisms driving malignancies, leaving them incurable. Here we demonstrated that the 2 well-known pancreatic driver mutations cooperatively activate a specific signaling pathway that promotes tumor invasion and immune evasion properties. Our results provide insights into the molecular basis by which malignancies often develop in parallel with oncogenesis and PDAC cell growth, as well as druggable targets for immunotherapies.

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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 moreover up-regulate mevalonate pathway (MVP) activity (15).

The small-GTPase ARF6 and its downstream effector AMAP1 (also called ASAP1 or DDEF1) are frequently overexpressed in different types of cancers and promote invasion and metastasis (16–21). In this pathway, AMAP1 binds to several different proteins, such as cortactin, paxillin, and protein kinase D2, to promote cortical actin remodeling and integrin recycling (22, 23). AMAP1 also binds to EPB41L5 (23), which is induced during epithelial–mesenchymal transition (EMT) to down-regulate E-cadherin and to up-regulate focal adhesion turnover (24). ARF6 can be activated by different types of receptor tyrosine kinases (RTKs), such as epidermal growth factor receptor (EGFR), via the guanine nucleotide exchanger GEP100 (also called BRAG2) (25) and also by G-protein–coupled receptors via EFA6 (20). Therefore, the ARF6–AMAP1 pathway appears to drive a cancer mesenchymal program in response to external stimuli, such as by growth factors and lysophosphatidic acid. Interestingly, MVP is essential for RTK-mediated ARF6 activation, in which geranylgeranyl transferase-II (GGT-II) prenylates RAB11b, and RAB11b then transports ARF6 to the plasma membrane to be activated by RTKs (21). Consequently, oncogenic-p53, via its activation of MVP activity (15), is crucial for the activation of ARF6 by RTKs in breast cancer cells (21). However, the mechanisms by which ARF6 and AMAP1 become overexpressed in cancer cells still remain unclear.

Previous studies using animal models have suggested that oncogenic p53 may cooperate with oncogenic KRAS to promote the invasion and metastasis of PDACs (26, 27). We here 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 demonstrated that the eIF4A/4E-dependent mRNA translation 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. 1*A*) (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. 1*A*).

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 PDGF-BB) activates ARF6 in these cells, and the silencing of *GEP100* blocks the activation (Fig. 1*B* and *SI Appendix*, Fig. S1*A*). Moreover, the silencing of *ARF6*, *GEP100*, *AMAP1*, and *EPB41L5* each substantially blocked PDGF-induced Matrigel invasion (Fig. 1*C* and *SI Appendix*, Fig. S1*A*). Silencing of these genes did not notably affect cell viability (*SI Appendix*, Fig. S1*B*).

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-Trp53(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. 1*A*) and use these proteins for PDGF-induced ARF6 activation and Matrigel invasion (Fig. 1 *D* and *E* and *SI Appendix*, Fig. S1*C*). 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 lung, 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. S1*E*). Again, silencing of ARF6 pathway components in KPC cells did not notably affect cell viability (*SI Appendix*, Fig. S1*D*).

We then performed immunohistochemical (IHC) analyses on the clinical specimens. Clinicopathological parameters of the patients at the time of pancreatectomy are summarized in *SI Appendix*, 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 *I*), whereas high expression of GEP100 did not statistically correlate with poor outcomes (*SI Appendix*, Fig. S1 *F* 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. 2*A*). *siKRAS* also reduced ARF6 and AMAP1 protein levels in Panc-1 cells bearing mutant *KRAS*, but not in BxPC3 cells bearing intact *KRAS* (*SI Appendix*, Fig. S2*A*). *KRAS* silencing in MIAPaCa-2 cells also reduced *ARF6* mRNA levels by about one-half (*SI Appendix*, Fig. S2*B*), which was consistent with a previous report (31), whereas *AMAP1* mRNA was not notably affected (*SI Appendix*, Fig. S2*B*). We have previously shown that ARF6 and AMAP1 proteins are overexpressed at levels severalfold 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. 2*B*) (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. 2*B*), 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. 2*C*). eIF4A acts as a subunit of the eIF4F complex, which initiates mRNA

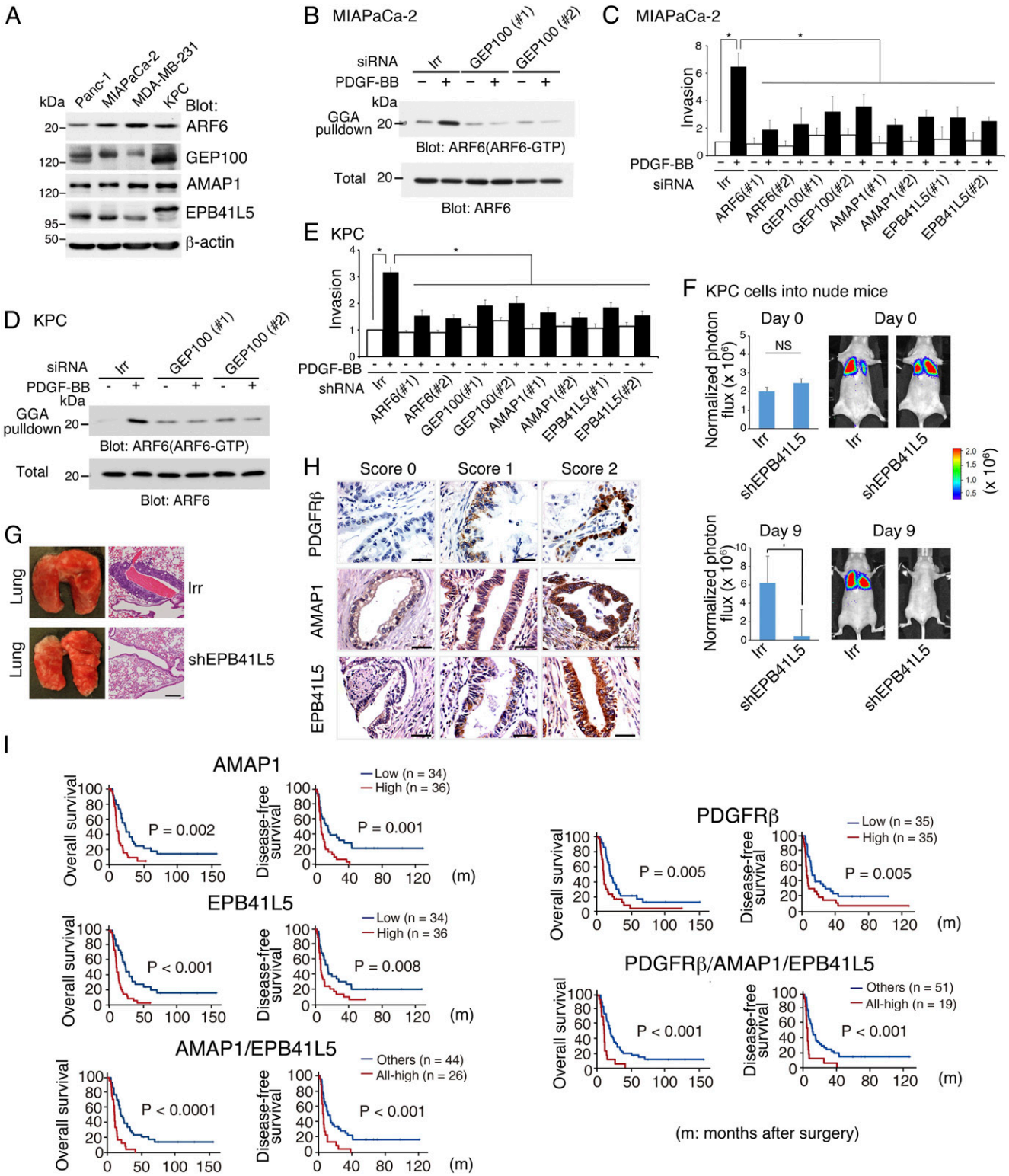

Fig. 1. The ARF6–AMAP1 pathway is central to PDAC malignancy. (*A*) Expression of ARF6 pathway components in PDAC cells, analyzed by Western blotting. β-Actin was used as a loading control. (*B–E*) PDGF activates ARF6 via GEP100 (*B* and *D*) and promotes cell invasion via ARF6, GEP100, AMAP1, and EPB41L5 (*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*).

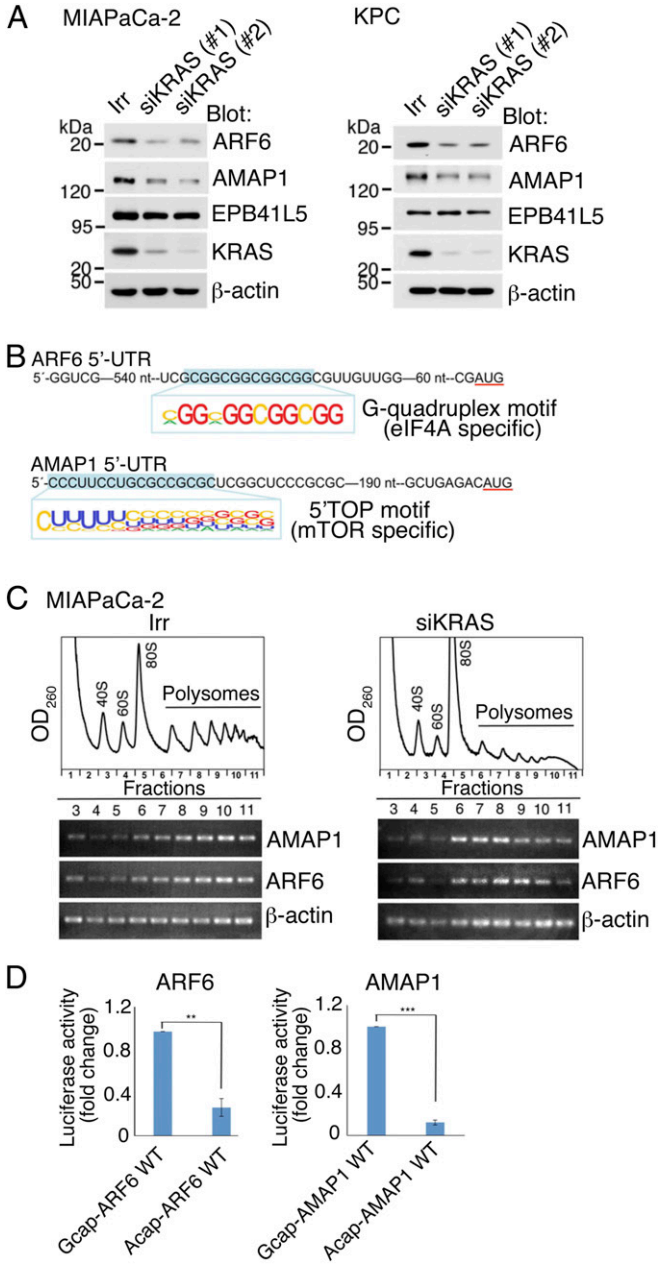

Fig. 2. *KRAS* promotes ARF6 and AMAP1 expression via their mRNA translation. (*A*) Representative immunoblots of ARF6, AMAP1, and EPB41L5 in cells treated with *KRAS* siRNAs or with an Irr control. Two different siRNAs (#1 and #2) were used. β-Actin was used as a control. Irr, an irrelevant siRNA. (*B*) 5′-UTRs of *ARF6* mRNA (the G-quadruplex motif) and *AMAP1* mRNA (the TOP motif). (*C*) Polysome profiles, as detected by absorbance at 254 nm after 15% to 60% sucrose gradient ultracentrifugation of RNAs from MIAPaCa-2 cells treated with si*KRAS* or an irrelevant siRNA (Irr). Ribosomal peaks (40S, 60S, and 80S) and polysomal peaks are shown. *B*, *Bottom* shows detection of *AMAP1*, *ARF6*, and *β-actin* mRNAs by PCR. A representative result from 3 independent experiments is shown. Actin was used as a control. (*D*) Translational activity of the 5′-UTRs of *ARF6* and *AMAP1* mRNAs, bearing either a 5′-Gcap or 5′-Acap and constructed into the polyadenylated firefly luciferase reporter, assessed in vitro using micrococcal nuclease-treated MIAPaCa-2 extracts. Results are shown as ratios by normalizing values obtained from each of the Gcap constructs as 1 ($n = 3$). Error bars, mean ± SEM; $^{**}P < 0.01$, $^{***}P < 0.001$ by ANOVA.

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. The 5′-end Gcap-dependent, but not the 5′-end adenylate cap (Acap)-dependent translation of *ARF6* and *AMAP1* mRNAs was confirmed (Fig. 2*D*). 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. 3*A*). PDCD4 acts as a negative regulator of eIF4A via direct binding (38). Silencing of the *KRAS* promoted PDCD4 expression at both the protein and the mRNA levels (Fig. 3*B* and *SI Appendix*, Fig. S2*B*), and consistently, forced expression of PDCD4 by cDNA transfection reduced ARF6 levels (Fig. 3*C*). 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. 3*D*). 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. 3*E* and *SI Appendix*, Fig. S3*A*) 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. 3*E* and *SI Appendix*, Fig. S3 *A* 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. S3*C*). Among them, the expression of *TEAD3*, *FOXL1*, *ETV4*, and *FOSL1* 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. S3*D*). 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. 3*F* and *SI Appendix*, Fig. S3*E*). Silencing of *FOXL1* and *FOSL1* also up-regulated *PDCD4* #4 transcription, but to a lesser extent than *TEAD3* and *ETV4* silencing (Fig. 3*F* and *SI Appendix*, Fig. S3*E*). Up-regulation of PDCD4 protein levels by *shTEAD3* and *shETV4*, and also by *shFOXL1* and *shFOSL1* to lesser extents, was also confirmed (Fig. 3*G*). We also detected the binding of TEAD3 and ETV4 to the *PDCD4* locus in these cells (Fig. 3*H* 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.

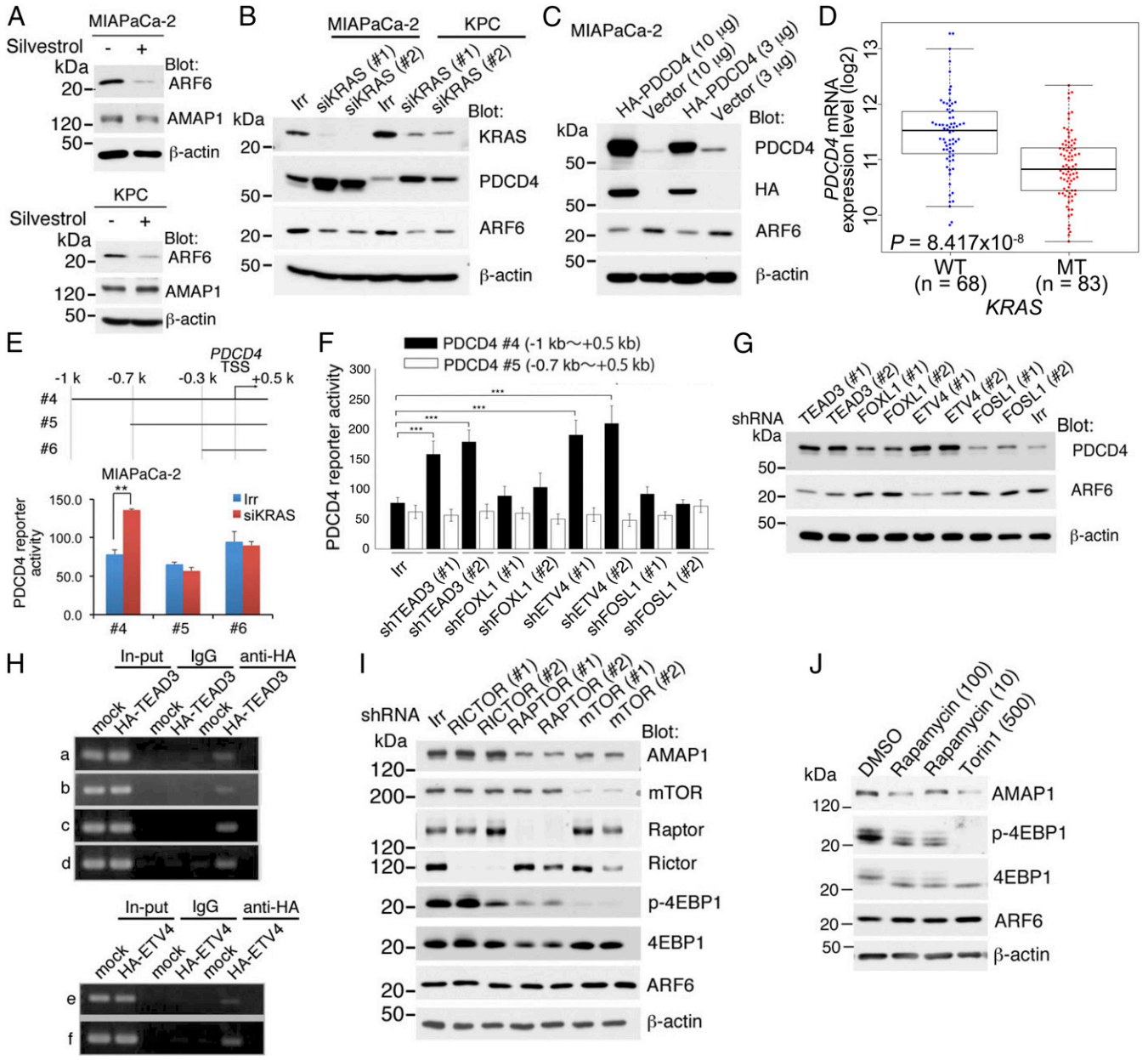

Fig. 3. (*A–J*) Mechanisms by which *KRAS* up-regulates mRNA translation of *ARF6* (*A–H*) and *AMAP1* (*I* and *J*) in PDACs. (*A*) Suppression of ARF6 levels by silvestrol. (*B*) Induction of PDCD4, coupled with reduction of ARF6 levels, upon *KRAS* silencing. (*C*) Suppression of ARF6 levels by the 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. S3*A*). $^{**}P < 0.01$ 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. S3*F*. (*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.

***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 shRNA-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. 3*I* and *SI Appendix*, Fig. S3*H*). mTOR inhibitors, such as rapamycin and Torin 1 (35), were also successful in reducing AMAP1 levels, without notably affecting ARF6 levels (Fig. 3*J* and *SI Appendix*, Fig. S3*I*). 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

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-1/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. 5*A*). We then found that the 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. 5*D*). EPB41L5 links AMAP1 with E-cadherin dynamics (23, 24), and we found that EPB41L5 is not involved in PD-L1 dynamics (Fig. 5*A*). The induction of

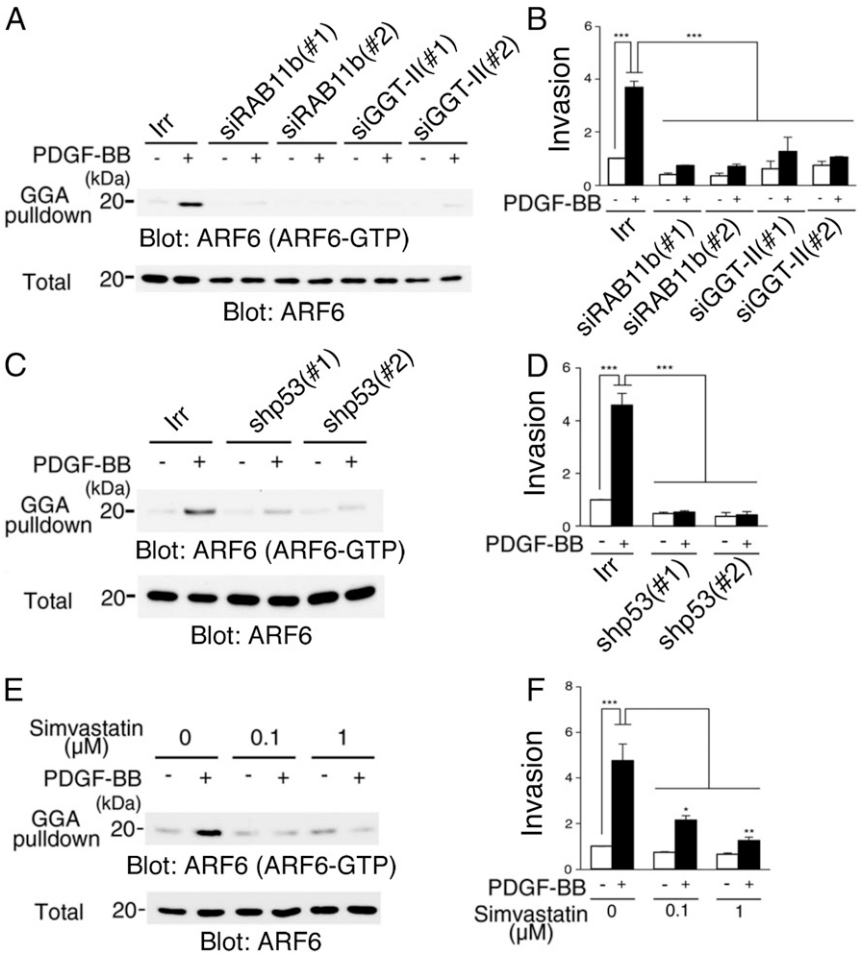

Fig. 4. Requirement for *TP53* and MVP in PDGF-induced ARF6 activation and cell invasion in MIAPaCa-2 cells. (*A*–*F*) Blockade of PDGF-induced ARF6 activation (*A*, *C*, and *E*) and cell invasion (*B*, *D*, and *F*) by silencing of *RAB11b* or *GGT-II* (*A* and *B*), by silencing of *TP53* (*C* and *D*), and by simvastatin (*E* and *F*). Error bars indicate the mean ± SEM; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ by ANOVA (*B*, *D*, and *F*).

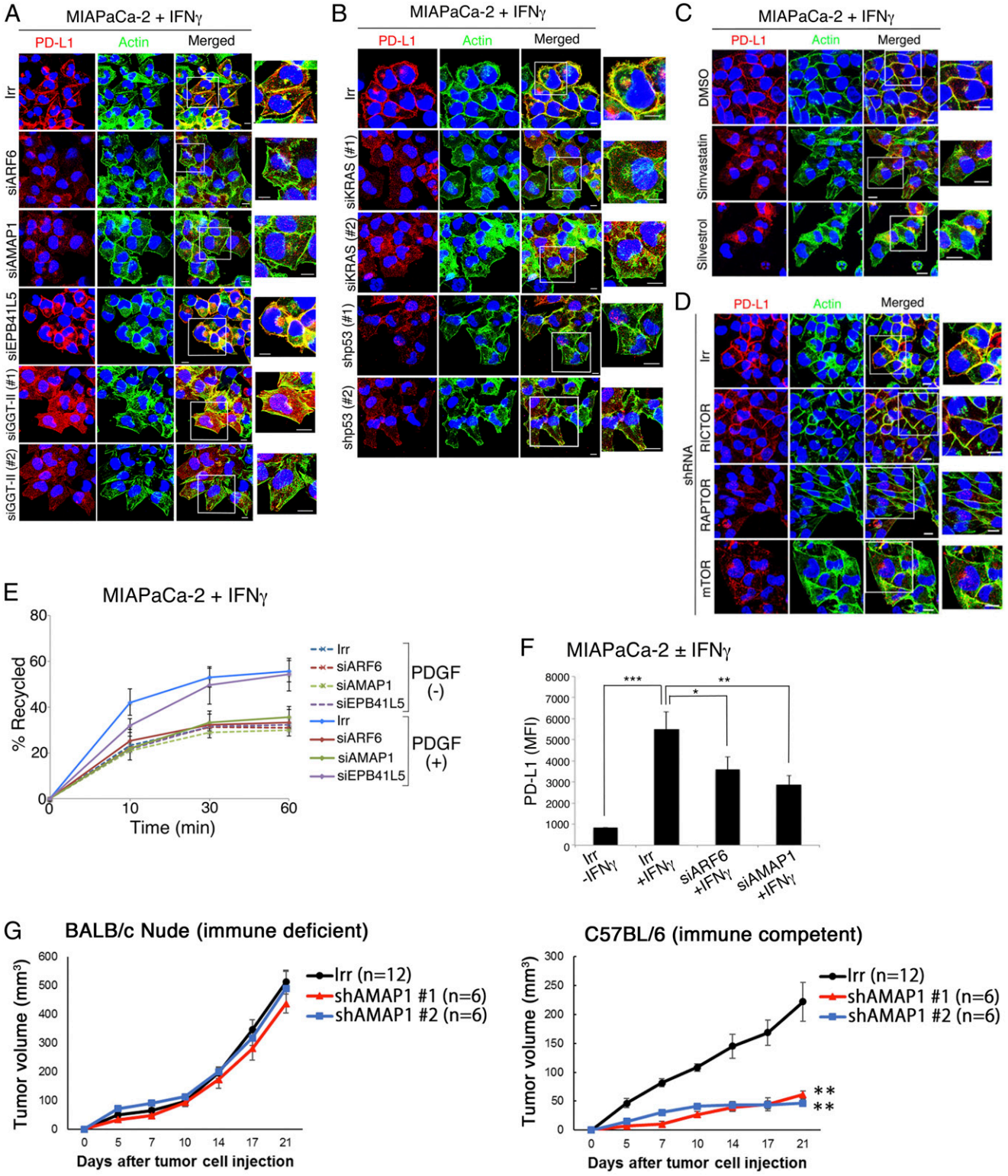

Fig. 5. ARF6 and AMAP1, as well as *KRAS* and *TP53*, are pivotal to PD-L1 dynamics and cell surface expression. (*A*–*D*) Representative images of immunofluorescence staining for PD-L1 (red) and F-actin (green) in IFNγ-treated MIAPaCa-2 cells, pretreated with siRNAs/shRNAs (*A*, *B*, and *D*) and simvastatin 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. 5*E*). 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. 5*F* and *SI Appendix*, Fig. S5*F*). On the other hand, PD-L1 internalization was not notably affected by these gene silencings (*SI Appendix*, Fig. S5*G*), 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. S5*H*). 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. 5*G*). 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

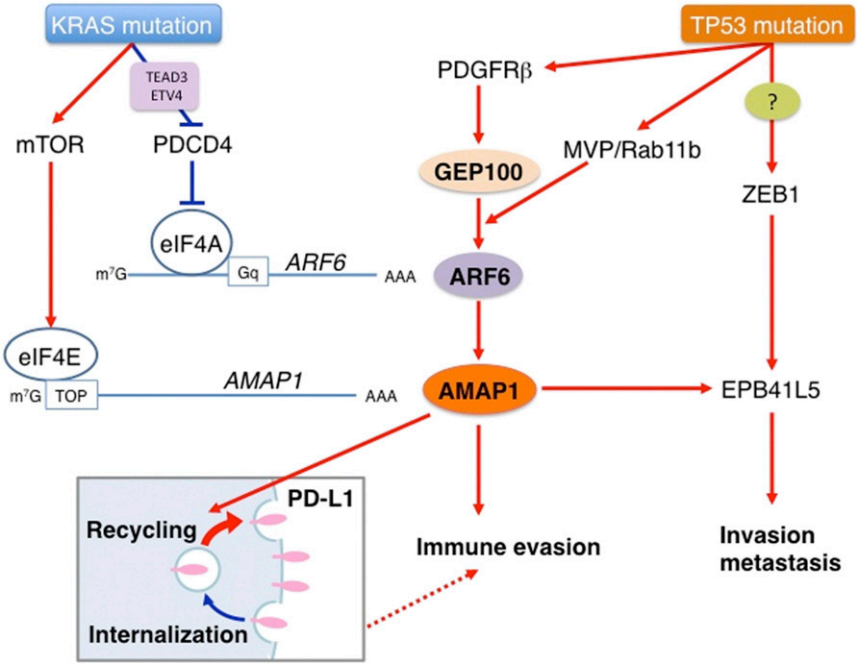

Fig. 6. Our proposed model of *KRAS* and *TP53* oncogenes driving PDAC malignancy via the ARF6–AMAP1 pathway. *KRAS* promotes the 5′-cap–dependent translation of *ARF6* and *AMAP1* mRNAs, primarily via enhancing the activities of eIF4A and eIF4E, respectively. *TP53* facilitates ARF6 activation by RTKs, via enhancing the expression of PDGFR (14) and MVP (15), in which MVP activity is essential to geranylgeranylate RAB11b to transport ARF6 to the plasma membrane for its activation by RTKs (21). EPB41L5 is induced during EMT by ZEB1 (23). Although *TP53* mutations can induce ZEB1 and hence EPB41L5, the molecular basis of this link appears to be complicated in PDACs and is not simply mediated by miRNAs. The ARF6–AMAP1 pathway drives tumor cell motility, in which the interaction of AMAP1 with EPB41L5, PRKD2, and other proteins is necessary to promote intracellular dynamics of β1 integrins and E-cadherin, as well as the cortical actin remodeling (main text). The ARF6–AMAP1 pathway also promotes PD-L1 dynamics and is closely associated with the immune evasion of PDACs, whereas factors linking AMAP1 with PD-L1 and immune evasion are unknown.

PDAC cells. We were interested in understanding the mechanisms involved in the induction of *EPB41L5*, as well as in the induction of *ZEB1*, which then induces *EPB41L5* (23). In this regard, although *TP53* mutations appeared to be crucially involved in the induction of the *ZEB1-EPB41L5* 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 EPB41L5 and its associated mesenchymal properties emerge in PDACs. Whether EPB41L5 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 interpretation 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, as well as the high production of ATP within tumor cells, appear to 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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