Hematopoietic progenitor kinase 1 down-regulates the oncogenic receptor tyrosine kinase AXL in pancreatic cancer

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ABSTRACT

The oncogenic receptor tyrosine kinase AXL is overexpressed in cancer and plays an important role in carcinomas of multiple organs. However, the mechanisms of AXL overexpression in cancer remain unclear. In this study, using HEK293T, Panc-1, and Panc-28 cells and samples of human pancreatic intraepithelial neoplasia (PanIN), along with several biochemical approaches and immunofluorescence microscopy analyses, we sought to investigate the mechanisms that regulate AXL expression in pancreatic ductal adenocarcinoma (PDAC). We found that AXL interacts with hematopoietic progenitor kinase 1 (HPK1) and demonstrate that HPK1 down-regulates AXL and decreases its half-life. The HPK1-mediated AXL degradation was inhibited by the endocytic pathway inhibitors leupeptin, bafilomycin A1, and monensin. HPK1 accelerated the movement of AXL from the plasma membrane to endosomes in pancreatic cancer cells treated with the AXL ligand growth arrest–specific 6 (GAS6). Moreover, HPK1 increased the binding of AXL to Cbl proto-oncogene (c-Cbl); promoted AXL ubiquitination; decreased AXL-mediated signaling, including phospho-AKT and phospho-ERK signaling; and decreased the invasion capability of PDAC cells. Importantly, we show that AXL expression inversely correlates with HPK1 expression in human PanINs and that patients whose tumors have low HPK1 and high AXL expression levels have shorter survival than those with low AXL or high HPK1 expression (P < 0.001). Our results suggest that HPK1 is a tumor suppressor that targets AXL for degradation via the endocytic pathway. HPK1 loss of function may contribute to AXL overexpression and thereby enhance AXL-dependent downstream signaling and tumor invasion in PDAC.

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal tumors among all malignancies and ranks as the third leading cause of cancer related death in the United States. Pancreatic cancer is resistant to conventional chemotherapy and radiation therapies. The five-year survival rate for patients with pancreatic cancer is approximately 8.5% (1). The molecular mechanisms of pancreatic tumorigenesis remain unclear. Exploring the molecular mechanisms involved in the development, progression, and resistance to conventional therapies of pancreatic cancer may help to identify new therapeutic targets for this deadly disease.

Oncogenic receptor tyrosine kinase AXL play a major role in cancer cell survival, proliferation, migration, invasion, metastasis, and immunosuppressive tumor microenvironment (2-4). AXL and its ligand Gas6 are overexpressed in pancreatic cancer and carcinomas of other organs, including lung, colon, prostate, breast, ovary, esophagus, stomach, and kidney (5-18). Our previous study showed that AXL was overexpressed in 70% of human pancreatic cancer samples and that overexpression of AXL correlated significantly with higher rate of distant metastasis, shorter recurrence-free survival, and overall survival in patients with pancreatic cancer who underwent upfront pancreatectomy with curative intent (17). AXL silencing sensitizes pancreatic cancer cells to γ-irradiation and reduces their anchorage independent growth, migration, as well as invasion potential, which has been
attributed to the down-regulation of AKT signaling as well as transcription factors involved in epithelial mesenchymal transition (EMT) such as slug, snail and twist etc. (17,18). Subsequent studies targeting Gas6-AXL signaling pathways using either a small molecular inhibitor (BGB324), a high-affinity AXL decoy receptor, or neutralizing monoclonal antibodies mAbs) against AXL or Gas6 demonstrate that autocrine Gas6-AXL signaling is an important drivers for therapeutic resistance, disease progression and metastasis in pancreatic cancer and other human malignancies (2,19-26). Ludwig et al. showed that selective AXL kinase inhibitor, BGB324, not only inhibits the aggressiveness of pancreatic cancer and sensitizes pancreatic cancer cells to gemcitabine, but also induces an immune stimulatory microenvironment (2). Neutralizing mAbs against AXL or Gas6 inhibit AKT signaling in pancreatic cancer and in vivo tumor growth in xenograft tumor models (2,22). The results from these preclinical studies provide strong rationale for targeting Gas6-AXL autocrine pathway to improve the treatment efficacies and clinical outcomes in patients with pancreatic cancer and cancers of the other organs.

Previous studies by Leconet et al. showed that anti-AXL mAbs induced internalization and down-regulation of AXL in both pancreatic cancer and triple negative breast cancer cells (21,22). Their results suggest that endocytosis may be one of the major mechanisms regulating AXL expression in cancer cells. Consistent with this notion, Valverde reported that binding of Gas6 to AXL induces the phosphorylation, ubiquitination, and downregulation of AXL in human lens epithelial cells through endocytosis/lysosomal degradation, but not through proteasomal degradation (27). However, the molecular mechanisms regulating the expression of AXL in pancreatic cancer or other human malignancies are unclear.

Hematopoietic progenitor kinase 1 (HPK1), also named MAP4K1, is a mammalian Ste20-related serine/threonine kinase, which has been shown to regulate NFκB and JNK pathways in hematopoietic cells (28,29). We previously showed that HPK1 protein is expressed in normal pancreatic ductal cells, but is lost in pancreatic ductal adenocarcinomas (PDAC). Loss of HPK1 is strongly associated with the progression from early pancreatic intraepithelial neoplasia (PanIN) to PDAC. Restoring HPK1 expression in PDAC cells leads to cell cycle arrest and growth inhibition, which is due, in part, to the stabilization of p21 and p27(30). Therefore HPK1 may function as a novel tumor suppressor in pancreatic tumorigenesis. Our previous studies also demonstrated that loss of HPK1 in PDAC is mediated by CUL7/Fbxw8 ubiquitin ligase through 26S proteasome, which requires HPK1 kinase activity and autophosphorylation (31,32). To further explore the mechanisms of the tumor suppressor functions of HPK1, we identified AXL as one of the major HPK1 interacting proteins in PDAC cells using antibody array-based screening and examined the role of HPK1 in regulating AXL signaling. Our study not only reveals a novel mechanism by which HPK1 down-regulates oncogenic AXL through endocytic pathway, but also provides the new link between HPK1 and the oncogenic Gas6-AXL pathway in pancreatic cancer.

RESULTS

AXL physically associates with HPK1: To identify the binding partners of HPK1 in PDAC cells, we performed an antibody
array screening using Panc-1/HPK1 stable cells and identified AXL as one of the major binding partners of HPK1 in PDAC cells (Figure 1A). To confirm the interaction between HPK1 and AXL, we performed reciprocal co-immunoprecipitation using either anti-AXL, or anti-HPK1 and the lysates from HEK293T cells transfected with HPK1 and AXL plasmids. The AXL protein was effectively precipitated using an anti-HPK1 antibody (Figure 1B). Similarly, the HPK1 protein was precipitated using an anti-AXL antibody (Figure 1C). To demonstrate whether endogenous HPK1 interacts with endogenous AXL, reciprocal co-immunoprecipitation assays were formed using cell lysate of Jurket cells, which expressed both HPK1 and AXL. We found that endogenous HPK1 interacted with endogenous AXL in Jurket cells (Figure 1D and 1E). These data demonstrate a novel interaction between AXL and HPK1 proteins.

HPK1 possesses a kinase domain (KD, 1 to 274 amino acids) and C-terminal domain (CD, 275 to 833 amino acids) (33). The C-terminal domain includes 4 proline rich regions (PR1-PR4) and a citron homology domain (Figure 1F). To identify whether a discrete domain of HPK1 interacts with AXL, Flag-tagged HPK1, HPK1-KD, or HPK1-CD plasmid was transfected into 293T cells alone, or in combination with pCMV-AXL plasmid. Cells were collected 36 hours after transfection and co-immunoprecipitation were performed using M2 beads followed by immunoblotting using an anti-AXL antibody. The AXL protein was co-immunoprecipitated with full-length HPK1 and HPK1-CD, but not HPK1-KD (Figure 1G), indicating that AXL binds to the C-terminal domain of HPK1.

Oncogenic AXL is downregulated by HPK1 which requires HPK1 kinase activity: We noticed that AXL expression was consistently decreased when it was co-transfected with HPK1 (Figure 1G, lanes 4 and 5). To test whether AXL expression was downregulated by HPK1, fixed amount of pCMV-AXL was co-transfected with different amount pCI-Flag-HPK1 into 293T cells. AXL protein levels decreased in a dose-dependent manner with the increase of HPK1 expression (Figure 2A and 2B). To test the specificity of HPK1-mediated AXL down-regulation, we performed similar co-transfection experiments using increasing amount of pCI-Flag-HGK, which is also a member of MAP4K family but is overexpressed in PDAC (34). As shown in Figure 2C and 2D, increasing the expression levels of HGK protein did not affect AXL protein levels. To test whether HPK1-mediated AXL downregulation required HPK1 kinase activity, we performed co-transfection experiments using fixed amount of pCMV-AXL and increasing amount of pCI-Flag-HPK1-M46, a dominant-negative HPK1 construct that has a methionine substituted for Lys-46, which abrogates ATP binding and HPK1 kinase activity (35-37). Increasing amount of M46 did not show significant effect on the AXL protein levels (Figure 2E and 2F). These data suggest that HPK1 kinase activity is required for HPK1-mediated AXL downregulation.

To examine the mechanisms of HPK1-mediated AXL downregulation, we measured the half-life of AXL protein in presence or absence of HPK1. When co-transfected with HPK1, the estimated half-life of AXL protein was 2.4 hours, which was 2.3 hours shorter than that when AXL was transfected alone without HPK1 (Figure 2H and 2I). Overexpression of HPK1 had no significant effect on the expression levels of AXL mRNA (Figure 2G), suggesting that HPK1 downregulates AXL at the protein
level, rather than by affecting AXL mRNA expression.

**HPK1-mediated AXL downregulation is blocked by the inhibitors of endocytic pathway:** Activated receptor tyrosine kinases are often regulated by internalization and endocytic trafficking that can target the activated receptors for degradation in lysosomes (38,39). To examine whether HPK1-mediated AXL degradation requires lysosomal protease activity, AXL was transfected alone or with HPK1 into 293T cells. Eight hours after transfection, cells were treated with leupeptin for 16 hours, which inhibits serine, cysteine and threonine proteases in lysosome (40,41). HPK1-mediated AXL downregulation was blocked by leupeptin treatment (Figure 3A-3B). Lysosomal degradation is a consequence of cargo entering endosomal sorting (42,43). To determine whether HPK1-mediated AXL degradation requires that AXL proceeds through the endocytic pathway, we performed similar experiments using baflomycin A1 (BA1) and monensin, which specifically inhibits vacuolar-type H+ -ATPase and keeps a low pH environment in endosomal vesicles (44-46). Treatment with BA1 or monensin significantly inhibited HPK1-mediated AXL downregulation (Figure 3C-3F). These data indicate that HPK1 mediated AXL degradation occurs through endocytic trafficking and lysosomal degradation.

**HPK1 accelerates the movement of AXL protein from cytoplasmic membrane to late endosomes and promotes AXL degradation in pancreatic cancer cells:** To examine the role of HPK1 in endogenous AXL degradation in pancreatic cancer, the control Panc-28 cells and Panc-28/HPK1 stable cells were treated with Gas6 for different times and the cell lysates were subjected to immunoblotting using anti-AXL antibody. As shown in Figure 3G, Gas6 treatment accelerated endogenous AXL degradation in a time-dependent manner in Panc-28/HPK1 stable cells compared to the parental control cells. In Panc-28/HPK1 stable cells, AXL expression was markedly decreased at 4, 6 and 8 hours after Gas6 stimulation compared to control cells. These results suggest that HPK1 is involved in AXL protein degradation in pancreatic cancer.

To investigate whether HPK1-mediated AXL degradation in pancreatic cancer cell requires trafficking through the endocytic pathway, we examined the localization of AXL protein by immunofluorescence microscopy. In unstimulated Panc-28 control cells and Panc-28/HPK1 stable cells, AXL was located predominantly on the cell membrane (Green, dot staining pattern, 0°C, Figure 3H). When treated with Gas6 at 37°C for 40 minutes, we observed significant shift of membranous AXL expression to perinuclear vesicles that co-localized with the lysosome marker, LAMP1 (red) in Panc-28/HPK1 stable cells (yellow dots). However, no significant shift of membranous AXL expression was observed in Panc-28 control cells (Figure 3H, middle and lower panel). These results suggest that HPK1 accelerates AXL internalization and movement from cell membrane to late endosomes in Panc-28 cells when stimulated with Gas6.

**HPK1 enhances AXL and c-Cbl interaction and promotes AXL ubiquitination in vivo in pancreatic cancer cells:** Ubiquitin ligase c-Cbl has been shown to play a role in EGFR degradation through endocytosis and in GAS6-induced downregulation of AXL. To further examine the role of c-Cbl in HPK1-mediated AXL degradation, the Panc-1/Tet-HPK1 stable cells were treated with
doxycycline for 24 hours to induce HPK1 expression. The cell lysates were subjected to immunoprecipitation using anti-AXL followed by immunoblotting using anti-AXL, c-Cbl, HPK1 and GAPDH antibodies. As expected, Panc-1/Tet-HPK1 stable cells had markedly increased HPK1 protein expression after treated with doxycycline. HPK1 overexpression increased the binding of c-Cbl to endogenous AXL (Figure 4A). To examine whether HPK1 affects AXL ubiquitination, control and Panc-28/HPK1 stable cells, which have higher levels of AXL protein than Panc-1/HPK1 stable cells after GAS6 treatment, were treated with GAS6 for 30 min after serum starvation for 16 hours. Immunoprecipitations were performed using anti-AXL antibody followed by immunoblotting using anti-ubiquitin antibody. HPK1 enhanced the AXL ubiquitination in Panc-28/HPK1 stable cells after treated with GAS6 (Figure 4B).

**HPK1-mediated AXL degradation decreases the invasion capability and downstream AKT and ERK signaling in pancreatic cancer:** In a previous study, we showed that AXL overexpression correlates with distant metastasis in PDAC patients and that AXL promotes invasion (17). To determine whether HPK1-mediated AXL degradation affects the invasion capacity of Panc-28 cells, we performed matrigel invasion assays. The number of cells invading through the membrane was compared between Panc-28/HPK1 stable cells and control cells. The average cell number invaded through the matrigel for Panc-28/HPK1 stable cell clones (HPK1 #1 and HPK1 #2) were significantly lower than that for Panc-28 parental and vector control (P<0.01, Figure 5A and 5B).

We next examined the effect of HPK1 on AXL downstream MAP kinase and AKT signaling pathways in response to Gas6 stimulation. Control or Panc-28/HPK1 stable cells were stimulated with Gas6 for 15 min to 120 min. We observed that HPK1 inhibited Gas6/AXL-mediated AKT activation throughout the entire stimulation period from 15 to 120 minutes and inhibited Gas6/AXL-mediated ERK activation at 30 min, 60 min and 120 min compared to the parental Panc-28 cells (Figure 5C, 5D). Similar results were observed in Panc-1/Tet-HPK1 cells. HPK1 down-regulated endogenous AXL and inhibited ERK activation in Panc-1/Tet-HPK1 cells when treated with Gas6 (Figure 6).

The expression of HPK1 inversely correlated with AXL expression in human pancreatic intraepithelial neoplasia (PanIN): To examine the significance of HPK1-mediated AXL degradation in pancreatic cancer, we examined the expression of HPK1 and AXL in 26 human PanIN lesions from 10 different patients by immunohistochemistry. Similar to our previous report, we found that HPK1 was expressed in normal pancreatic ductal cells in all patients and was expressed in 88.9% (8/9), 50% (5/10) and 28.6% (2/7) in PanIN1, PanIN2 and PanIN3 lesions respectively (Figure 7A-7D). In contrast, AXL was expressed in 20% (2/10), 22.2% (2/9), 70% (7/10) and 85.7% (6/7) in normal pancreatic ducts, PanIN1, PanIN2 and PanIN3 lesions respectively (Figure 7E-7H). Both the loss of HPK1 expression and AXL overexpression correlated with the progression from low-grade PanIN1 to high-grade PanIN3 (P<0.05). More importantly, we found a significant inverse correlation between HPK1 expression and AXL expression in human PanIN lesions. Among the 15 PanIN lesions that were positive for HPK1, AXL was expressed only in 33% (5/15) compared to 91% (10/11) AXL expression in the PanIN lesions that were
negative for HPK1 (P=0.005). These data provided strong support that HPK1 mediates AXL downregulation in pancreatic cancer.

To further examine the clinical significance of HPK1-mediated AXL degradation in pancreatic cancer, we perform survival analysis using the RNA sequencing data of HPK1 and AXL from 176 patients with pancreatic ductal adenocarcinoma in TCGA database. Patients, whose tumors were HPK1-low and high-AXL, had shorter survival (median survival: 481 days) compared to those with AXL-low or HPK1-high tumors (median survival: 1130 days, Figure 8, P<0.001).

Discussion

In previous studies, we and others showed that AXL is overexpressed in 70% of human PDAC samples and pancreatic cancer cell lines (17,18). Overexpression of AXL correlated with higher frequency of distance metastasis and poor prognosis in patients with PDAC who underwent surgical resection (17,18). AXL silencing decreases invasion/migration potential and increases apoptosis of PDAC cells in response to radiation (17). In addition, previous studies have shown that AXL is overexpressed in carcinomas of lung, breast, brain, colon, skin, prostate, ovary, liver and other types of malignancies, and contributes to the tumor progression in these malignancies (47-56). However, the mechanisms resulting in AXL overexpression in cancers are largely unknown. In this study, we showed for the first time that HPK1 physically associates with AXL using an antibody array assay and reciprocal co-immunoprecipitation assays. We observed that HPK1 down-regulated AXL protein expression and its downstream signaling pathways through endocytic trafficking coupled with lysosomal degradation. More importantly, our study demonstrated a strong inversely correlation between AXL protein expression and HPK1 expression in human PanIN lesions. Thus our results reveal a novel tumor suppressor function of HPK1 by targeting an important oncogenic receptor tyrosine kinase AXL for degradation. Consistent with this notion, we found that low expression of HPK1 and high expression of AXL correlated significantly with poor survival in patients with pancreatic ductal adenocarcinoma. Given the fact that HPK1 protein expression is lost in over 90% of human PDAC samples, our results suggest that loss of HPK1 expression may contribute to the overexpression of AXL in pancreatic cancer and thus reveal a new mechanism of AXL overexpression in cancer.

One of the major mechanisms that controls the signaling of receptor tyrosine kinases is through internalization, early and late endosomes and then delivery to lysosome for degradation (39,57,58). In this study, we showed that HPK1-mediated AXL degradation can be completely blocked by the lysosomal proteinase inhibitor, leupeptin. HPK1-mediated AXL degradation was also significantly blocked by inhibitors of endocytosis, bafilomycin A1 and monensin. Thus, we concluded that HPK1-mediated AXL degradation occurs by targeting AXL for endocytic trafficking and lysosomal degradation. Our results are consistent with previous report that binding of Gas6 to AXL induces the phosphorylation and down-regulation of AXL in human lens epithelial cells through endocytosis/lysosomal degradation (27).

AXL trafficking and down-regulation have been proposed to significantly influence downstream signaling. Treatment using monoclonal
antibodies (mABs) against AXL induced down-regulation of AXL by internalization and inhibits its downstream AKT activation, which leads to inhibition of proliferation and migration of pancreatic cancer cells in vitro and leads to growth inhibition in vivo in pancreatic cancer xenograft models (22). Similar findings have also been reported in triple negative breast cancer cells, in which AXL mAB induces internalization and degradation of AXL, and inhibited AXL/Gas6 signaling, EMT, and cell migration/invasion (21). Consistent with these reports, we found that HPK1-mediated AXL down-regulation through endocytosis coupled lysosomal degradation led to decreased activation of AKT and ERK and inhibited the invasion potential of pancreatic cancer cells. Our data suggest that targeting the HPK1-Gas6/AXL pathway may represent a new therapeutic strategy for pancreatic cancer.

In this study, we found that HPK1 interacted with AXL preferentially through its C-terminal domain and resulted in down-regulation of AXL protein. It is interesting that HPK1-mediated AXL degradation is dependent on HPK1 kinase activity. In addition, we found that HPK1 increased the binding of AXL to c-Cbl and increased AXL protein ubiquitination. It is possible that HPK1 phosphorylates AXL at unidentified serine/threonine residue(s), which subsequently led to the binding to AXL to c-Cbl, which promotes AXL ubiquitination and accelerates AXL internalization, and trafficking through endosomes to lysosomes for degradation.

HPK1 has been shown to be a negative regulator in the activation of T lymphocytes (59). A recent study using HPK1 M46 transgenic mice showed that HPK1 kinase activity is required for the immunosuppressive functions of HPK1. Inactivation of kinase domain enhanced the anti-tumor immune responses and anti-PD-L1 efficacy (37). These findings suggested that targeting HPK1 kinase activity in immune cells using selective small molecular inhibitors in combination with the immune checkpoint inhibitor therapy may be an alternative strategy for effective cancer treatment (37). However our study demonstrated that HPK1 kinase activity is required in the HPK1-mediated degradation of oncogenic tyrosine receptor AXL and inhibition of its downstream signaling pathways in pancreatic cancer. The function of HPK1 in the interplay between tumor cells and activation of immune cells in tumor microenvironment needs to be investigated.

MATERIALS AND METHODS

Cell lines and transfection: HEK293T, Panc-1 and Panc-28 cells were grown in DMEM supplemented with 10% fetal bovine serum and 100 units/ml streptomycin/penicillin. HEK293T cells were transfected with different amounts of plasmids expressing AXL, HPK1, and its mutants, as indicated in figure legends. To establish HPK1 stable cell lines using Panc-28 cells, retrovirus based expression system pBABE-Flag–tagged HPK1 was used. To establish inducible HPK1 expression system in Panc-1 cells (Panc-1/Tet-HPK1), a lentivirus based expression system pBABE-Flag–tagged HPK1 was used. To establish inducible HPK1 expression system in Panc-1 cells (Panc-1/Tet-HPK1), a lentivirus based expression system pBABE-Flag–tagged HPK1 was used. To establish inducible HPK1 expression system in Panc-1 cells (Panc-1/Tet-HPK1), a lentivirus based expression system (Clontech Laboratories, Inc.) was used. The cells were subsequently selected in the presence of puromycin (1-2 µg/ml). The stable clones expressing HPK1 were screened by RT-PCR and immunoblotting. Panc-1 and Panc-28 were chosen for this study because both cell lines expressed high levels of AXL and Gas6 proteins.
Antibody array-based screening for HPK1 interacting proteins in pancreatic cancer cells. The cell lysates from Panc-1/HPK1 stable cells were incubated with a membrane filter arrayed with 400 antibodies (Hypromatrix, Worcester, MA), which was pre-blocked in a buffer containing 5% nonfat milk. After overnight incubation at 4°C, the antibody-antigen-HPK1 complex was detected by horseradish peroxidase-conjugated anti-Flag antibody, followed by chemiluminescence according to the manufacturer’s protocol.

Immunoprecipitation and immunoblotting. Immunoprecipitation and immunoblotting were performed as previously described (30). For each co-immunoprecipitation, 500 µg total proteins were mixed with 2 µg of either antibody, or normal serum IgG. The precipitated proteins were analyzed by 10% SDS-PAGE, which was then electroblotted onto polyvinylidene difluoride membranes (Novex, Grand Island, NY), blocked in 5% skim milk in 1× TBST, and probed with the primary antibodies indicated in the figure legends. The following antibodies were used for immunoprecipitation or immunoblotting: anti-β-actin and anti-Flag (M2) were purchased from Sigma-Aldrich (St. Louis, MO); Anti-HPK1 (N-19) and anti-AXL were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX), anti-p-AKT 

Ubiquitination of AXL in pancreatic cancer cells. Control and Panc-28/HPK1 stable cells were treated with 100 ng/ml GAS6 for 30 min after serum starvation for 16 hours. Immunoprecipitations were performed at 4°C overnight using anti-AXL antibody (Santa Cruz Biotech sc-1097). Immunoprecipitated and input samples were run on SDS-PAGE, transferred to nitrocellulose membranes, and blotted using anti-ubiquitin (P4D1, sc-1097) and anti-AXL (sc-1097) antibodies.

Immunofluorescence microscopy. Panc-28 control and Panc-28/HPK1 stable cells were plated onto glass coverslips in 24-well plates to about 70% confluence. Prior to fixation, the cells were serum-starved for 16 hours and then incubated with Gas6 (400 ng/mL) for 30 min on ice. Plates either remained on ice (0 °C) or were transferred to 37 °C for 40 min to allow AXL internalization. The cells were then fixed with methanol for 5 min at -20°C and incubated at 4 °C overnight in 1% BSA containing antibodies directed against AXL (1:200). Following

and the loading control, actin, was calculated. The average value after 3 repeats was calculated and plotted.

Quantitative RT-PCR analysis of AXL mRNA: Total RNA from 293T cells was extracted using TRIzol (Invitrogen Inc.). The cDNA was synthesized using a reverse transcription system (Promega Co.). RT-PCR was performed using a thermal cycler (Bio-Rad Laboratories, Inc) with the primers (AXL forward: 5’-GGTGGCTGTGAAGACGATGA-3’ and reverse: 5’-CTCAGATAC TCCATGCCACT-3’; ribosomal protein small subunit 6 (RPS6) forward: AAGGAGAGAGGATATTGGAC-3’ and reverse: 5’-AGAGAGATGAAAAAGTTTGCGGAT-3’).

Half-life of AXL protein. The half-life of AXL protein was measured in HEK293T cells co-transfected with AXL and HPK1 constructs compared with those transfected with AXL alone. The cells were treated with 100 µg of cycloheximide (CHX) for 0, 30 min, 1 hour, 2 hours, 4 hours, and 6 hours. The AXL protein levels were measured by immunoblotting and quantified using the ImageJ software. The ratio between AXL and the loading control, actin, was calculated. The average value after 3 repeats was calculated and plotted.
PBS washes, cells were incubated with secondary antibodies at room temperature for 45 min. Subsequently, cells were fixed with 4% PFA for 5 min and permeabilized with methanol for 2 min at -20°C. Following PBS washes, the cells were incubated in 1% BSA containing antibodies directed against LAMP1 (1:100) for 1 hour at room temperature. After PBS washes, the cells were incubated with secondary antibodies at room temperature for 45 min, then washed with PBS, and coverslips were mounted with VECTASHIELD Antifade Mounting Medium with DAPI (Vector Laboratories, Inc., Burlingame, CA). The cell images were obtained using Olympus IX73 inverted microscope.

**Treatment of pancreatic cancer cells with Gas6.** Cells were subjected to serum starvation for 16 hours, and then treated with recombinant human Gas6 (400 ng/mL, R&D Systems Inc., Minneapolis, MN) for the time length indicated in the figures. The cells were harvested, and the expression of phospho-AKT, phospho-ERK and total ERK were detected by immunoblotting.

**In vitro chemoinvasion assay.** Chemoinvasion assays were performed using 24-well BioCoat Matrigel invasion chambers as described previously (17). Briefly, the lower compartment contained 0.6 mL of DMEM medium with 5.0% fetal bovine serum as chemoattractants or serum-free DMEM medium as a control. In the upper compartment, 2.5 × 10⁴ cells/well were placed in triplicate wells and incubated for 24 hours at 37°C in a humidified incubator with 5% CO₂. Then the cells that passed through the filter into the lower wells were stained with Giemsa (Fisher Scientific, Orangeburg, NY) and counted under a microscope in 5 predetermined fields. All assays were repeated at least 3 times. The differences in the invasion rates between the parental PDAC cells, vector control cells, and the PDAC cells with stable HPK1 expression were analyzed by one-way ANOVA tests.

**Immunohistochemical analysis for HPK1 and AXL expression in human pancreatic intraepithelial neoplasia.** The use of human tissue in this study was approved by the Institutional Review Board of MD Anderson Cancer Center. Whole tissue sections, which contained histologically verified pancreatic intraepithelial neoplasia (PanIN) of different grades, from 10 patients were used for immunohistochemistry. Immunohistochemical staining for HPK1 and AXL was performed on 4-µm unstained sections using the antibodies and conditions as described previously (17,30). The expression of AXL and HPK1 in PanIN lesions and adjacent normal pancreas were evaluated by a pathologist who specializes in pancreatic cancer.

**Survival analysis:** The RNA sequencing data of HPK1 and AXL expression and survival data of 176 pancreatic cancer patients in the Cancer Genome Atlas (TCGA) database were downloaded from the Human Protein Atlas (https://www.proteinatlas.org). The expression of HPK1 and AXL was categorized as low or high using the cutoff values set by TCGA (3.42 for HPK1 and 14.09 for AXL). Survival curves were constructed using the Kaplan-Meier method, and the log-rank test was used to evaluate the statistical significance of differences.

**Conflict of interest:** The authors declare that they have no conflicts of interest with the contents of this article.
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Abbreviations used are: HPK1, hematopoietic progenitor kinase 1; PDAC, pancreatic ductal adenocarcinoma; mABs, monoclonal antibodies; PanIN, pancreatic intraepithelial neoplasia; EMT, epithelial-to-mesenchymal transition.
FIGURE LEGENDS

Figure 1. HPK1 physically interacts with AXL. A, Antibody array results and map showed that HPK1 interacts with AXL (marked by arrow). B and C, Interaction between HPK1 and AXL was detected by reciprocal co-immunoprecipitation. HEK293T cells were transfected with pCMV-AXL alone or co-transfected with pCMV-AXL and Flag-HPK1 expression plasmids and co-immunoprecipitation was performed as described in the Materials and Methods. D and E, endogenous HPK1 interacted with endogenous AXL in Jurket cells. F. A schematic drawing of the domain structure of HPK1 protein. G. AXL binds to the C-terminal domain of HPK1. HEK293T cells were transfected with AXL alone or co-transfected with AXL and Flag-HPK1, Flag-HPK1 kinase domain (KD), or Flag-HPK1 C-terminal domain (CD) expression plasmids. Cell lysates were collected for co-immunoprecipitation with M2-beads. Immunoprecipitated AXL was detected by immunoblotting.

Figure 2. AXL is downregulated by HPK1 and HPK1 kinase activity is required for HPK1 mediated AXL downregulation. A and B, Overexpressed HPK1 downregulates AXL expression in a dose-dependent manner when co-transfected with pCMV-AXL expression plasmid into HEK293T cells. C-F. Co-transfection of HGK or HPK1-M46, a kinase dead form of HPK1 with pCMV-AXL expression plasmid into HEK293T cells does not affect the AXL expression. The expression levels of AXL protein and actin were quantified using Image J software. Mean values of relative AXL protein expression from three independent experiments were calculated and plotted on bar graphs (B, D, and F). G. AXL mRNA expression levels measured by quantitative RT-PCR analysis. RPS6 was used as an internal control. The data are shown as bar graph from three independent experiments. H and I. HPK1 decreases the stability of AXL protein. HEK293T cells were transfected with pCMV-AXL alone or co-transfected with pCMV-AXL and pCI-Flag-HPK1 and then treated with 100 µg/mL of Cycloheximide (CHX) to inhibit protein translation for 0, 0.5, 1, 2, 4 and 6 hours. The cell lysates were collected for immunoblotting to measure expression levels of AXL proteins. The expression levels of AXL protein and actin in each sample were quantified using ImageJ software. The average expression levels of AXL protein after adjusted to actin levels from multiple experiments were plotted. The estimated half-life of AXL protein was 4.7 hours in the absence of HPK1 and 2.4 hours in the presence of HPK1.

Figure 3. HPK1 downregulates AXL through endocytic/lysosomal pathway. A-F, lysosome inhibitors: Leupeptin, Bafilomycin A1 and Monensin blocked HPK1-mediated AXL degradation. HEK293T cells were transfected with pCMV-AXL alone or co-transfected with pCI-HPK1. The transfected cells were treated with 100µg/mL of leupeptin, 0.1µM Bafilomycin A1 or 100 µM Monensin for overnight. AXL and HPK1 protein levels were detected by immunoblotting using AXL and HPK1 antibodies (A, C, and E). The expression levels of AXL protein and actin were quantified using Image J software. Mean values of relative AXL protein expression from three independent experiments were calculated and plotted on bar graphs (B, D, and F). G, HPK1
accelerates endogenous AXL degradation in Panc28/HPK1 stable cells. Panc28 control and Panc28/HPK1 stable cells were serum starved for 16 hours, then treated with 400 ng/mL Gas6 for the time lengths as indicated. The expression levels of AXL and HPK1 were detected by immunoblotting. H. HPK1 accelerates the movement of AXL protein from cytoplasmic membrane to late endosomes in pancreatic cancer cells as detected by immunofluorescence. Panc-28 control and HPK1 stable cells were treated with Gas6 (400 ng/mL) at 37°C to observe AXL internalization or at 0°C after serum-starvation. The cells were fixed with methanol, and incubated with antibody targeting AXL (green, Alexa Fluor 488) and LAMP1 (red, Alexa Fluor 564). Co-localization of AXL and LAMP1 (yellow) was visualized with mounting medium with DAPI (blue) using Olympus IX73 inverted microscope.

Figure 4. HPK1 enhances AXL and c-Cbl interaction and promotes AXL ubiquitination in pancreatic cancer cells. A, the Panc-1/Tet-HPK1 stable cells were treated with 100 ng/ml doxycycline for 24 hours. The cell lysates were subjected to immunoprecipitation using anti-AXL followed by immunoblotting using anti-AXL, Cbl, HPK1 and GAPDH antibodies. B, Control and Panc-28/HPK1 stable cells were treated with 100 ng/ml GAS6 for 30 min after serum starvation for 16 hours. Immunoprecipitations were performed using anti-AXL antibody followed by immunoblotting using anti-ubiquitin antibody.

Figure 5. Overexpression of HPK1 reduces the invasion potential and inhibits Gas6-mediated AKT and ERK activation in pancreatic cancer cells. A. Representative micrographs showing the number of cells invaded through the membrane for Panc28 control cells and two Panc28/HPK1 stable clones (HPK1 #1 and HPK1 #2) in in vitro Matrigel invasion assays. B. The cells invading through membrane were counted under a microscope in 5 predetermined fields at ×200 magnification. The control and HPK1 stable cells were assayed in triplicate and assays were repeated three times. The average number of cells invaded through the membrane for the control and HPK1 stable cells was plotted. A significant decrease in the number of invaded cells was observed in both HPK1 stable clones compared to the control cells (*P <0.001). C and D. HPK1 inhibits Gas6-mediated AKT and ERK activation in pancreatic cancer cells. Panc28 control and Panc28/HPK1 stable cells were serum starved for 16 hours, then treated with 400 ng/mL Gas6 for the time lengths as indicated. The expression levels of AXL, p-AKT (ser473), AKT, p-ERK, total ERK, HPK1 and tubulin control were detected by immunoblotting and were quantified using Image J software. Mean values of relative AXL protein expression (AXL/tubulin ratio), p-AKT/AKT ratio and p-ERK/ERK ratio from three independent experiments were calculated and plotted on bar graph (D).

Figure 6. HPK1 inhibits Gas6-mediated AXL degradation and ERK activation in Panc-1/Tet-HPK1 stable cells. Panc-1/Tet-HPK1 stable cells, untreated or treated with doxycycline, were serum starved for 16 hours, then treated with 400 ng/mL Gas6 for the time lengths as indicated. The expression levels of AXL, HPK1, p-ERK, total ERK, and GAPDH control were detected by immunoblotting.
Figure 7. AXL protein expression inversely correlates with HPK1 expression in human pancreatic intraepithelial neoplasia (PanIN). A-D, representative micrographs show the expression of HPK1 in normal pancreas (A), PanIN1 (B), PanIN2 (C) and PanIN3 (D). E-H, representative micrographs show the expression of AXL in normal pancreas (E), PanIN1 (F), PanIN2 (G) and PanIN3 (H). HPK1 is expressed in normal pancreatic ducts and PanIN1, but not expressed in PanIN2 and PanIN3. In contrast, AXL is not expressed in normal pancreatic ducts and PanIN1, but expressed in PanIN2 and PanIN3.

Figure 8. Kaplan-Meier survival curves stratified by the expression levels of HPK1 and AXL in 176 patients with pancreatic ductal adenocarcinoma. The expression data of HPK1 and AXL and survival data were downloaded from TCGA database (www.proteinatlas.org). Patients, whose tumors were HPK1-low and AXL-high had the shorter survival (median survival: 481 days) compared to those with AXL-low or HPK1-high tumors (median survival: 1130 days, P<0.001).
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
| GAS6 (h) | 0  | 0.5 | 1  | 2  | 4  |
|---------|----|-----|----|----|----|
| Axl     | 150| 100 |    |    |    |
| HPK1    | 97 |     |    |    |    |
| p-Erk   | 44 |     |    |    |    |
| Erk     | 44 |     |    |    |    |
| GAPDH   | 36 |     |    |    |    |

Figure 6
Figure 7
Figure 8
Hematopoietic progenitor kinase 1 down-regulates the oncogenic receptor tyrosine kinase AXL in pancreatic cancer
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