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THE ROLE OF RECEPTOR TYROSINE KINASE AXL IN PANCREATIC DUCTAL ADENOCARCINOMA AND ITS REGULATION BY HEMATOPOIETIC PROGENITOR KINASE 1

Xianzhou Song

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**The Role of Receptor Tyrosine Kinase Axl in Pancreatic Ductal Adenocarcinoma
and Its Regulation by Hematopoietic Progenitor Kinase 1**

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THE ROLE OF RECEPTOR TYROSINE KINASE AXL IN PANCREATIC DUCTAL
ADENOCARCINOMA AND ITS REGULATION BY HEMATOPOIETIC
PROGENITOR KINASE 1

A

DISSERTATION

Presented to the Faculty of

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of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

By

Song, Xianzhou, M.S.

Houston, Texas

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Dedication

I would like to dedicate this work to those who love and support me.

To my parents, Youlong Song (Dad) and Lijun Hu (Mom). To my sisters, Meiai Song and Meijuan Song. To all my intimate relatives and friends.

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First, I would like to express my sincere and utmost gratitude to my Ph.D. advisor, Dr. Huamin Wang, who gave me the chance to have Ph.D. training in his lab. His expertise, knowledge, experience benefits me greatly in my research. Moreover, my future research career will also be helped tremendously due to his committed and patient guiding.

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**The Role of Receptor Tyrosine Kinase Axl in Pancreatic Ductal Adenocarcinoma
and Its Regulation by Hematopoietic Progenitor Kinase 1**

Publication No. _____

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Supervisory Professor: Huamin Wang, M.D., Ph.D.

Pancreatic ductal adenocarcinoma (PDA) is one of the most aggressive malignancies with less than 5% of five year survival rate. New molecular markers and new therapeutic targets are urgently needed for patients with PDA. Oncogenic receptor tyrosine kinase Axl has been reported to be overexpressed in many types of human malignancies, including diffuse glioma, melanoma, osteosarcoma, and carcinomas of lung, colon, prostate, breast, ovary, esophagus, stomach, and kidney. However, the expression and functions of Axl in PDA are unclear. We hypothesized that Axl contributes to the development and progression of PDA. We examined Axl expression in 54 human PDA samples and their paired benign pancreatic tissue by immunohistochemistry, we found that Axl was overexpressed in 70% of stage II PDAs, but only 22% of benign ducts ($P=0.0001$). Axl overexpression was associated with higher frequencies of distant metastasis and was an independent prognostic factor for both poor overall and recurrence-free survivals in patients with stage II PDA ($p = 0.03$ and 0.04). Axl silencing by shRNA in pancreatic cancer cell lines, panc-28 and Panc-1, decreased tumor cell migration and invasion and sensitized PDA cells to apoptosis stimuli such as γ -irradiation

and serum starvation. In addition, we found that Axl-mediated Akt and NF- κ B activation and up regulation of MMP2 were involved in the invasion, migration and survival of PDA cells. Thus, we demonstrate that Axl plays an important role in the development and progression of PDA. Targeting Axl signaling pathway may represent a new approach for the treatment of PDA.

To understand the molecular mechanisms of Axl overexpression in PDA, we found that Axl expression was down-regulated by hematopoietic progenitor kinase 1 (HPK1), a newly identified tumor suppressor in PDA. HPK1 is lost in over 95% of PDAs.

Restoration of HPK1 in PDA cells down-regulated Axl expression. HPK1-mediated Axl degradation was inhibited by leupeptin, bafilomycin A1, and monensin, suggesting that HPK1-mediated Axl degradation was through endocytosis-lysosome pathway. HPK1 interacted with and phosphorylated dynamin, a critical component of endocytosis pathway. Overexpression of dominant negative form of dynamin blocked the HPK1-mediated Axl degradation. Therefore we concluded that HPK1-mediated Axl degradation was through endocytosis-lysosome pathway and loss of HPK1 expression may contribute to Axl overexpression in PDAs.

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CHAPTER 1: Backgrounds

Pancreatic cancer

Pathology of pancreatic cancer

Pancreatic cancer is one of the most malignant diseases. Pancreatic cancer is ranked as fourth leading cause for all cancer related death in United States¹⁻³. In 2010, about 43,140 new pancreatic cancer cases was expected; and about 36,800 pancreatic cancer patients was predicted to die from this disease¹. Only 24% of pancreatic patients can survive one year or more from the date of diagnosis. Overall 5 year survival rate is less than 5%¹⁻³. The absence of effective diagnostic markers for early stage pancreatic cancer partially counts for the high mortality. Most diagnosed pancreatic cancer patients have advanced diseases or distant metastasis^{4,5}. More desperate reality is that there are no effective treatments for pancreatic cancer^{4,5}. Traditional chemotherapy and radiation treatment have very minor impact on overall survival rate⁶⁻¹⁰. According to epidemiologic studies on pancreatic cancer has identified smoking, diabetes, alcohol, pancreatitis, obesity, age (more than 60 year), genetic predisposition, hereditary disorders, and family history as risk factors for pancreatic cancer¹¹⁻¹⁶.

Based on cellular origin, pancreatic cancer can be classified as exocrine or endocrine tumor. Exocrine tumors include pancreatic ductal adenocarcinomas (PDAs), cystic tumors, and cancer of the acinar cell origin. Endocrine tumors include gastrinomas, insulinomas, somatostatinomas, VIPoma, glucagonomas, PPomas, GNRHomas,

ACTHoma and other non-functional tumors¹⁷⁻²⁰. Pancreatic ductal adenocarcinoma (PDA) accounts for 90% of diagnosed pancreatic cancers¹¹,

Previous studies have shown that PDA arises from pancreatic intraepithelial neoplasia (PanIN), intraductal papillary mucinous neoplasm and mucinous cystic neoplasm. PanINs are microscopic lesions with neoplastic proliferation of epithelial cells, involving small pancreatic ducts²¹⁻²³. PanIN can be classified as PanIN-1(A/B), PanIN-2, and PanIN-3, according to the degree of cellular atypia and architecture atypia²¹⁻²³. PanIN-1 represents ductal lesions with minimal nuclear atypia; correspondingly, PanIN-3 represents severe dysplasia or carcinoma *in situ*²⁴.

Genetic alterations in pancreatic cancer

More than 90% of PDAs have KRas point mutation^{25,26}, and most KRas mutations are happened at codon 12²⁷. KRas is a GTPase. When bond to GTP, KRas is activated and functions as signaling molecular for many growth receptors²⁸. KRas-GTP activates down-stream signaling including mitogen-activated protein kinase (MAPK), phosphoinositide-3-kinase (PI3K), and serine/threonine protein kinase Akt pathways²⁹⁻³¹. Just like other members of Ras family, KRas has intrinsic GTP hydrolysis activity. When GTP is converted to GDP, the signaling function of KRas is switched off³². However, when KRas 12th amino acid Gly(G) is mutated to Valine(V) or Asp(D), the GTPase activity is inactivated. Mutated KRas constitutively binds to GTP, and is always at an activation status^{33,34}. Other frequently amplified oncogenes consist of Akt2, C-Myc,

MyB, and EGFR³⁵. The occurring rate of above gene amplification varied from 10% to 30% of all pancreatic patients³⁶⁻³⁸.

During the development and progression of PDA, a group of crucial tumor suppressor genes are frequently inactivated in pancreatic cancer, due to chromosomal deletion or mutation. CDKN2A/p16 is inactivated in almost all PDAs, by homozygous deletion, intragenic mutation or promoter hypermethylation³⁹⁻⁴¹. Protein p16 inhibits the cell cycle G1/S transition by binding to cyclin-dependent kinases Cdk4 and Cdk6⁴²⁻⁴⁵. Moreover, loss of p16 causes abnormal phosphorylation of retinoblastoma protein (Rb). Highly phosphorylated Rb will promote cell cycle progression^{43,46}. Another frequently inactivated tumor suppressor gene in pancreatic cancer is TP53. About 55–75% of PDAs have inactivated TP53^{47,48}. TP53 inactivation always starts from intragenic mutation of one allele, and ends with the loss of the second allele by chromosome deletion⁴⁸. TP53 is called as guardian of genome. When DNA is damaged, a DNA repair pathway can be triggered by TP53. TP53 induces cell cycle arrest and cell apoptosis, which insure the cells, with DNA damage, to repair the DNA or die^{47,49-51}. Inactivation of DPC4/SMAD4, by either homozygous deletion or mutation, is detected in about 55% of pancreatic cancer patients^{21,52,53}. SMAD4 is an important down-stream component of TGF β pathway, which has inhibitory function on normal cell growth^{54,55}. Other less often mutated tumor suppressor genes include BRCA2, MKK4, STK11, ALK1 and TGFBR⁵⁶⁻⁵⁸.

The development of PDAs is a process for accumulation of genetic alterations. Telomere shortening and KRas oncogenic mutation occur in early precursor lesion PanIN-1A⁵⁹.

The p16 inactivation was started to be observed on PanIN-1B lesions, with a steady increasing rate on higher grade lesions⁶⁰. SMAD4, TP53, and BRAC2 inactivation usually appears on high grade lesion PanIN-3 and late invasive adenocarcinoma^{21, 59, 61}. The pool of identified genetic alterations is steady increasing. The on-going function genome and whole cancer genome sequencing will help to reveal new altered genes,

Pancreatic cancer in mouse model

Pancreatic cancer development and progression has been recaptured in mouse models, which greatly extended our understandings about the molecular and cellular mechanisms of pancreatic cancer oncogenesis and development. Hingorani et al created a PDX1-Cre, LSL-KRasG12D mouse⁶². Pancreas/duodenum homeobox protein 1 (PDX1) is a pancreas specific transactivator, determining the pancreatic cell fate⁶³. PDX1-Cre construct ensures that Cre is specifically expressed in mouse pancreas. LSL-KRasG12D has an endogenous mouse KRas promoter, ensuring that the KRasG12D expression levels mimic the physiological endogenous KRas expression levels. PDX1-Cre, LSL-KRasG12D mice fully recaptured the full spectrum of development of human PDA⁶². PanIN lesions, from PanIN-1, PanIN-2 to PanIN-3, and spontaneous invasive PDA developed in PDX1-Cre, LSL-KRasG12D mice. This mouse model established the role of KRasG12D in initiating the PanIN lesions and PDAs.

The roles of tumor suppressor genes, like p16 (INK4a) and p53, have also been examined in mouse models. Deficiency of either p16 (INK4a), or p53 alone failed to induce pancreatic lesions in mouse. However, in the context of PDX1-Cre, LSL-KRasG12D

mouse, loss of p16 (INK4a), or p53 mutation caused rapid emergence of PanIN lesions, invasive and metastatic PDAs^{64 65}. These mouse models further confirmed that tumor suppressor functions of p16 and p53 in the KRasG12D initiated pancreatic cancer development. Ji et al developed an elastase-Cre, cLGL-KRasG12V transgenic mouse⁶⁶. Elastase is a pancreas acinar cell specific gene, driving acinar cell specific expression of Cre. cLGL-KRasG12V ensure overexpression of KRasG12V. Interestingly, elevated KRas activity quickly produced chronic pancreatitis, mouse PanINs, pancreatic ductal adenocarcinoma, and cystic papillary carcinoma. Based on above findings, a novel model of pancreatic cancer development was proposed. Elevated Ras activity will induce acinar-to-ductal metaplasia and inflammation. Inflammation will further cause genome instability, which will let loss of tumor suppressor genes occur. Above models suggest a key role of KRas in pancreatic diseases, including pancreatitis, PanIN lesions, and pancreatic cancer^{66, 67}. The established mouse models will provide us a good tool to explore the molecular mechanisms in pancreatic cancer development and progression, and to identify new diagnostic markers and therapeutic targets for PDA.

Axl

Molecular biology of Axl

According to human genome sequence, there are about 90 tyrosine kinase genes identified. Among them, 58 genes encode different receptor tyrosine kinase (RTK). Based on amino acid sequence similarity in the kinase domain and extracellular region, 58 RTKs were divided into 20 subfamilies⁶⁸. A typical RTK contains an extracellular

region and an intracellular tyrosine kinase domain. The unique feature of RTK makes it function as communicating bridge between extracellular environment and cell ⁶⁹⁻⁷¹. In general, RTK activity is triggered by secreted ligands. After binding of a RTK specific ligand, RTK will dimerize or oligomerize, and is activated by conformational change and trans-autophosphorylation in cytoplasmic kinase domain. The phospho-Tyr residue(s) on RTK cytoplasmic tail will provide docking sites for recruiting down-stream molecules, like adaptor proteins containing Src homology 2 (SH2) domain or phosphotyrosine binding (PTB) domain ⁷². Once adaptor proteins are recruited by RTK, the down-stream signaling(s) is initiated ⁶⁹⁻⁷¹. RTK was found to participate in cell growth, survival, differentiation, motility, and almost all other cellular processes, in either normal condition or disease status ⁶⁹⁻⁷¹. Deregulated RTKs play important roles in cancer development and treatment ^{73, 74}.

Receptor tyrosine kinase Axl is also called UFO, or Ark receptor ⁷⁵ {Janssen, 1991 #423, ⁷⁶. Axl, Tyro-3 together with Mer constitutes an Axl receptor family, or TAM family ^{77, 78-80}. Axl receptor family is featured with a unique KW(I/L)A(I/L)ES amino acid motif in cytoplasmic kinase domain, and 2 immunoglobulin-like (IgL) domains followed with 2 fibronectin type III (FNIII) domains in extracellular region, seeing figure 1 ^{75, 77, 78-81}. Axl gene encodes a protein with molecule weight of 97 KDa. However, Axl protein displays multiple bands with size between 120 and 140 KDa on SDS-PAGE, due to extensive post-translational modification ⁷⁵.

Axl receptor ligand, Gas6

Soon after identification of Axl, growth arrest specific 6 (Gas6) was identified as a ligand for Axl by an affinity matrix containing extracellular part of Axl⁸². Almost at the same time, Protein S, a Gas6 homologue, was identified as another ligand for Axl⁸³. Gas6 and Protein S were confirmed as ligands for all members of Axl receptor family^{84,85}. Protein S shares 43% identical amino sequence with Gas6⁸³. Moreover, Protein S has same domain structure as Gas6⁸³. Gas6 has a glutamic acid rich domain in N-terminal, four EGF-like repeats in middle region, and 2 globular laminin G-like (LG) domains in C-terminal⁸⁶. Whole molecular weight is about 75 KDa⁸⁶. Reactive Gas6 relies on carboxylation at N-terminal glutamic acid residues, through a vitamin K-dependent reaction^{85,87}. X-ray crystallography studies revealed that two Gas6 molecules can form a tetramer with two Axl molecules⁸⁸. Each Gas6 LG1 domain simultaneously contacts immunoglobulin-like (IgL) domain 1 of first Axl and immunoglobulin-like (IgL) domain 2 of second Axl. Two Axl molecules are bridged by two Gas6 molecules. There is no direct Axl/Axl, or Gas6/Gas6 contacts, seeing figure 1⁸⁸.

Axl activation and downstream signaling

Axl activation can follow a typical approach adopted by other RTKs. When ligand Gas6 binds to Axl, Axl tyrosine phosphorylation is detectable⁸². After mutational analysis of tyrosine residues on Axl C-terminal, tyrosine residues (Y-779, Y-821, and Y-866) were proposed as auto-phosphorylation sites. Mutation on above tyrosine sites blocked interaction with down-stream signaling molecules, like Grb2, phospholipase C γ (PLC γ), p85 subunits of PI3K, c-src and lck⁸⁹.

Axl activation may also be completed through a ligand-independent approach.

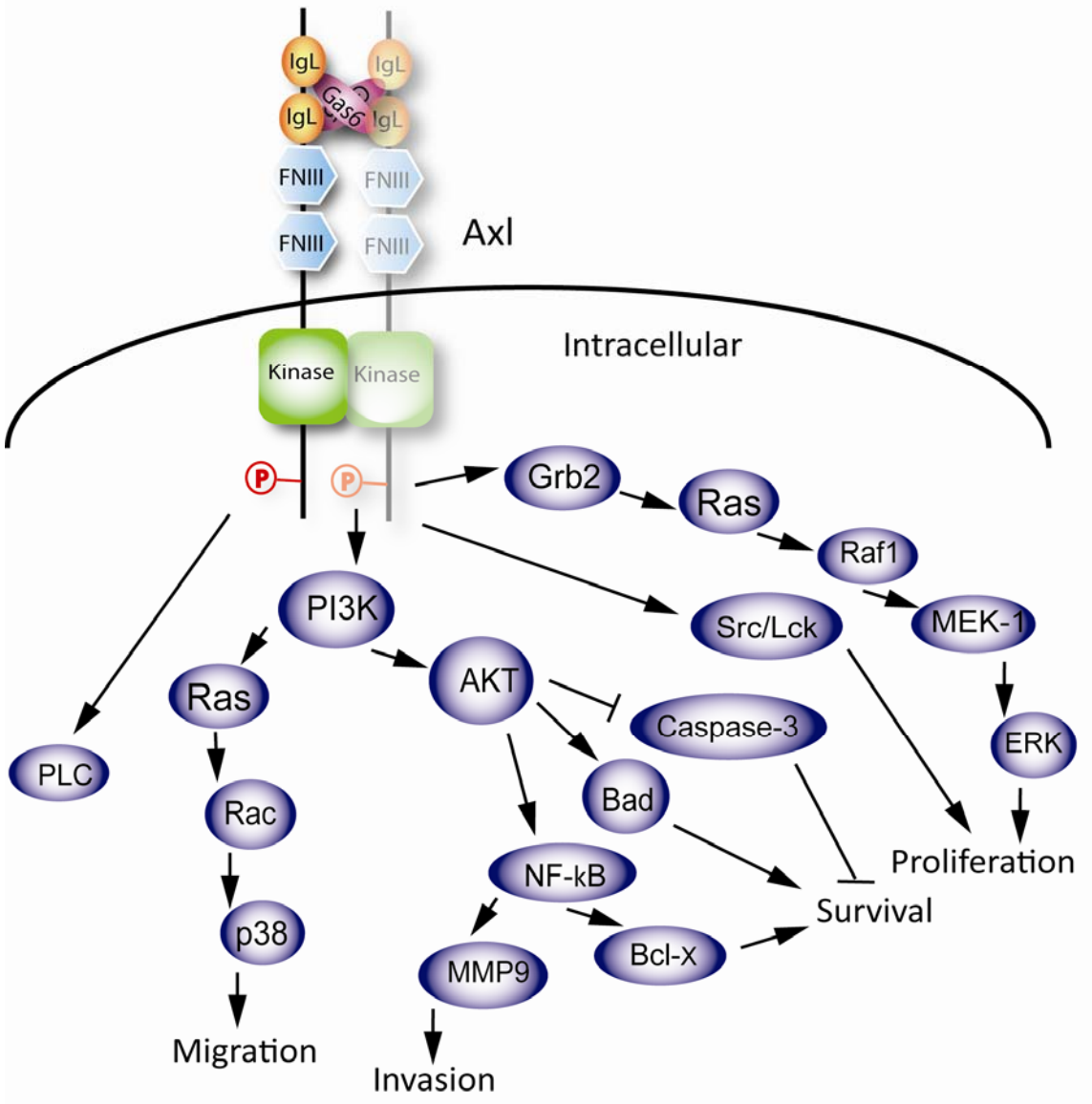
Overexpression of Axl in *Drosophila* S2 cells caused cell aggregation through Axl homophilic binding⁹⁰. Axl tyrosine phosphorylation was induced by Axl overexpression, even no additional Gas6 was added⁹⁰.

Activated Axl can stimulate several pathways which are involved in cell proliferation, survival and migration. Early studies by introducing chimeric EGFR/Axl receptor into leukemia 32D cells revealed that a MAPK/ERK pathway was activated to promote cell proliferation⁹¹. Adaptor proteins Grb2 and Shc bond to chimeric EGFR/Axl, when cells were stimulated with EGF protein. ERK activation by Axl require adaptor protein Grb2 and Shc⁹¹. Ectopic expression of dominant negative ERK in 32D cells abrogated Axl mediated cell proliferation⁹¹. Blocking Ras activity by antibody microinjection ablated Gas6 induced mitogenesis of NIH3T3 cells⁹². Phosphatidylinositol 3-kinase (PI3K)/Akt pathway was revealed to be a major survival pathway that is activated by Gas6/Axl signaling^{92, 93, 94}. Introducing dominant negative AKT (DN-AKT) into NIH3T3 cells blocked its Gas6 stimulated survival under serum starvation condition⁹². Gas6 stimulated JNK pathway through PI3/Akt pathway. Blocking Akt pathway by inhibitors also blocked JNK pathway⁹². Rac/Rho protein was also involved in Gas6 stimulated cell survival. Gas6 mediated PI3K activation may activate JNK through Rac/Rho protein⁹². Transcription factor NF- κ B was another signaling stimulated by Gas6 activated PI3/Akt survival pathway^{95, 96}. Gas6 stimulation caused I κ B degradation, which make NF- κ B accumulation in nuclear. Activated NF- κ B promoted the transcription of anti-apoptosis factor Bcl-x and Bcl-2^{95, 96}. In addition, P13K pathway activated by Gas6/Axl signaling

was also involved in cell migration. Gas6/Axl signaling can cause Gonadotropin-releasing hormone (GnRH) neuron migration through activating Rac → p38 MAPK → MAPKAP kinase 2 → HSP25 pathway, which is mediated by PI3K^{97,98}. Moreover, Other molecules activated by Gas6/Axl signaling includes Src, PLCγ, and Nck and RanBPM^{89,99}. The signaling pathways activated by Gas6/Axl signaling are summarized in figure 1.

Figure 1. The schematic presentation of Axl structure and summary of some Axl down-stream signaling

Axl has a typical tyrosine kinase domain in intracellular part, and two each of immunoglobulin-like (IgL) and fibronectin type III (FNIII) motifs in extracellular part. Ligand Gas6 specifically binds to IgL motifs and facilitates Axl dimerization. Dimerized Axl is activated by mutually trans-phosphorylating the Tyrosine on C-terminal. The phosphorylated Tyrosine sites provide docking sites for some down-stream signaling. Axl can activate Ras/MAPK and Src/Lck pathways which contribute to cell proliferation. Axl also can activate PI3K/Akt pathway to promote cell survival and invasion. Moreover, Axl is involved in Rac/p38 signaling which regulates the cell motility.



Axl functions in normal cells

So far many cellular functions of Gas6/Axl signaling have been explored. Regulating the immune response is one major function of Axl and the other two TAM family members. Triple knock-out mice of TAM receptors developed a spleen of 10 times bigger than a normal one¹⁰⁰. Aberrant lymphocyte proliferation was observed¹⁰⁰. TAM triple mutants eventually developed autoimmunity disease accompanied by hyperactivation of antigen presenting cells (APCs) like macrophage and dendritic cells¹⁰⁰. Symptoms related to rheumatoid arthritis, systemic lupus erythematosus, pemphigus vulgaris could be found in TAM triple mutants¹⁰⁰. Excessive TNF- α production was a driving factor for above autoimmune diseases^{100, 101, 102}. Moreover, TAM mutants displayed a defect in phagocytosis¹⁰³. Mer^{-/-} macrophage could not engulf apoptotic cells¹⁰³. During the process of clearing the apoptotic cells, Gas6 or Pro S binds to phosphatidylserine (PtdSer) which is specifically presented on the surface of apoptotic cell¹⁰⁴. After Gas6/Pro S binding to TAM family proteins on macrophages, a signaling cascade will be triggered to rearrange the actin cytoskeleton for phagocytosis¹⁰⁴⁻¹⁰⁸. Due to ineffective phagocytosis activity, TAM mutants eventually developed a degenerative phenotype in testis. The depletion of sperm cells turn mouse to be sterile¹⁰⁹. Axl, Tyro-3, and Mer are also required for normal function of natural killer (NK) cells^{110, 111}. NK cells with high Axl expression are more cytotoxic. Axl^{-/-} NK cells showed a 90% reduction in killing activity¹¹¹. After cytokine stimulation, NK cells with TAM triple mutants had less IFN- γ production. TAM are involved in NK cell differentiation¹¹¹.

The role of Gas6/Axl signaling in vascular smooth muscle cells (VSMCs) received a lot of investigation since Axl was cloned. Gas6 and Axl expression were increased at the sites of vascular injury. Gas6 attracted VSMCs but not the one with overexpressed Axl dominant negative form (Axl-DN) mutant migrated to injury sites ¹¹². Moreover, Gas6/Axl signaling protected VSMCs from serum starvation caused apoptosis ¹¹³. In VSMCs, a PI3K/AKT survival pathway could be activated by Gas6/Axl signaling ¹¹³.

Axl functions in cancer

Axl was initially identified as an oncogene from patients with chronic myelogenous leukemia (CML). Transient Axl overexpression caused NIH3T3 cells transformation ¹¹⁴. Later soon, more and more evidences of Axl as an oncogene were obtained from different types of cancer, including leukemia, colon, prostatic, breast, gastric, renal and lung cancers ¹¹⁵. In past years, more details about Axl functions on cancer progression have been elucidated. Axl functions in cancer are majorly focused on cell migration/invasion, metastasis, cell survival/proliferation/tumor growth, and angiogenesis.

Role of Gas6/Axl signaling in cell migration, invasion and metastases

Gas6/Axl signaling can induce migration in both normal and cancer cells. Extracellular Gas6 functioned as chemo-attractant and induced migration of vascular smooth muscle cells (VSMC). Overexpression of Axl in VSMC enhanced Gas6 induced migration; whereas, Dominant negative Axl (Axl-DN) reduced Gas6 induced migration ¹¹².

Gas6/Axl signaling was proposed to play a role in atherosclerosis and restenosis, where

VSMC migration was involved¹¹⁶. Migration of Gonadotropin-releasing hormone (GnRH) neuron, as well as dendritic cells are also regulated by Gas6/Axl signaling^{97, 117}.

In brain tumor, Axl dominant negative form (Axl-DN) inhibited glioma diffuse-invasive growth in a xenograft mouse model¹¹⁸. The migration and invasion rate was significantly lower in glioma cells harboring Axl-DN¹¹⁸. Inhibition of Gas6/Axl signaling decreased filopodia formation and make cells turn to a round shape. Similar effects caused by Axl inhibition were also found in mesotheliomas, hepatocarcinoma and cervical cancer¹¹⁹⁻¹²¹.

Gas6 induced GnRH neuron migration was accompanied with cytoskeleton reorganization, and lamellipodia formation⁹⁷, which could be blocked by Axl-DN. Gas6/Axl signaling can activate Rac GTPase/p38 MAPK /MAPKAP kinase 2/HSP25 pathway. Blocking either Rac GTPase or p38 MAPK stopped the actin cytoskeletal remodeling, as well as GnRH neuron migration⁹⁷. In breast cancer cells, overexpression of Axl can activate MEK/ERK, NF-κB pathway. NF-κB cooperated with Brg-1, a subunit of SWI/SNF chromatin complex, to induce transcription of matrix metalloproteinase 9 (MMP-9), which was required for Axl caused cancer cell invasion. On other hand, Axl-DN inhibited the MMP-9 expression and cell invasion both *in vitro* and *in vivo*^{122, 123}.

Epithelial-to-mesenchymal transition (EMT) is considered as a critical beginning step for cancer progression and metastasis. During EMT process, cells lose polarity and adhesion, and gain mobility¹²⁴. Interestingly, Axl participates in EMT process through different ways. In breast cancer, vimentin induced EMT and migration can be blocked by Axl shRNA. Vimentin controlled Axl expression¹²⁵. In acute myeloid leukemia cells, ectopically overexpressing Axl increased expression of Twist, which is involved in EMT.

In pancreatic cancer cells, silencing Axl reduced the expression of Twist, Slug, and Snail, which can repress E-cadherin expression¹²⁶. Repression of E-cadherin is a crucial step for most EMT processes¹²⁴.

Role of Gas6/Axl signaling in cell survival, proliferation and tumor growth

Axl can contribute to tumor growth by promoting either cell survival, proliferation, or in combination. Axl was firstly recognized for its transformation activity in NIH3T3 cells. Gain of self-sustaining proliferation or extra cell survival signals is a typical process of cell transformation⁷⁵. Later on, Gas6 was observed to accelerate NIH3T3 cell entry into S phase, and cell recovery from serum starvation, suggesting Axl can contribute to both cell proliferation and survival⁹³. Gas6 activates Akt and Src kinases, whose inhibition blocked Gas6 induced cell survival and proliferation⁹³. In addition, NF-κB activation by Gas6 was required for Gas6 endowed NIH3T3 cell survival⁹⁵. Gas6/Axl signaling mediated cancer cell proliferation and survival are also observed on ocular melanoma, brain tumor, kaposi sarcoma, and mesothelioma^{118, 120, 127, 128}. Interestingly, in prostate carcinoma, Gas6/Axl signaling induced cell proliferation, but not protected cells from serum starvation caused apoptosis¹²⁹. Gas6 can activate both Akt and MEK in prostate carcinoma cells; however, only MEK activation is essential for Gas6 induced cell proliferation¹²⁹. Axl silencing had no effects on cutaneous squamous cell carcinoma (SCC) cell proliferation, but made cells sensitize to UV treatment. Axl silencing significantly altered the behavior of several apoptosis related molecular. Pro-apoptotic molecules Bad, Bax and Bak were more easily activated. Caspase activation and cytochrome C release were enhanced¹³⁰.

Gas6/Axl signaling was also found to be able to protect normal cells from apoptosis stimuli. Gas6 promoted GnRH neuronal cell survival during serum starvation by activating Akt and ERK pathways ¹³¹. Gas6 significantly reduced the amount of vascular smooth muscle cell (VSMC) in apoptosis, which was caused by serum deprivation. Gas6 activated Akt but not ERK was required for VSMC survival ¹¹³. Gas6/Axl signaling kept the hepatic stellate cells (HSC) survival during CCl₄ induced rat liver injury ¹³². Again, Gas6 activated PI3K/Akt pathway played a role in HSC survival ¹³².

Role of Gas6/Axl in angiogenesis

Accompanying the tumor growth, new blood vessels will be generated to provide nutrition and oxygen. The genesis of new blood vessel is called angiogenesis. The structure of freshly generated blood vessel can be described as vascular smooth muscle wall lined outside of endothelium cells. Gas6/Axl signaling can promote vascular smooth muscle cell (VSMC) survival and migration ^{112, 113}, which are required for angiogenesis. Moreover, Gas6/Axl signaling can also promote survival for umbilical vein endothelial cells (HUVECs) ¹³³. Gas6 prolonged survival of endothelial cells after depletion of growth factors. Gas6 also protected endothelial cells from TNF α induced death ¹³³. The role of Gas6/Axl signaling in angiogenesis was also observed in a breast cancer xenograft mouse model ¹³⁴. Axl silencing significantly reduced the amount of endothelial cells in breast cancer xenograft mouse model. Axl silencing blocked the endothelial tube formation in HUVEC culture plate. Furthermore, Axl inhibition blocked HUVEC proliferation ¹³⁴. The role of Axl in angiogenesis was further confirmed by using Axl

specific inhibitor R428, which can block vascular endothelial growth factor (VEGF)-induced corneal neovascularization in breast cancer xenograft mouse model¹³⁵.

Therapeutic significance of Axl

Since, in many types of cancer, Axl overexpression correlated well with cancer progression and metastasis, and inversely with overall patient survival rate, Axl can be used as a prognostic marker^{121, 123, 136-141}. Axl up-regulation is one of the major molecular events contributing to the cancer progression. Down regulation of Axl protein levels can be an approach to treat cancer. SiRNA or shRNA provided an efficient way to silence Axl expression. When breast cancer cell line MDA-MB-231 was introduced with Axl specific shRNA (shAxl), mouse xenograft growth of MDA-MB-231 was significant slower than vector alone control¹³⁴. ShAxl also significantly blocked ovarian cancer metastasis in a mouse xenograft model¹²³. Similar to the shAxl data, in both non-small cell lung cancer (NSCLC) and breast cancer, miR-34a and miR-199a/b was found to targeting Axl. Ectopic expression of above miRNA caused less migration, invasion and metastasis of cancer cells^{142, 143}. Since the tyrosine kinase activity is crucial for Axl oncogenic functions, developing Axl specific kinase inhibitor can be ideal strategy for cancer treatment. Amuvatinib (MP-470) binds to Axl very well (Kd=0.8 μ M). Treating gastrointestinal stromal tumor cells with MP-470 comprised its resistance to imatinib, which is a specific inhibitor for tyrosine kinases Abl, c-kit, and platelet-derived growth factor receptor (PDGFR)¹⁴⁴. Bosutinib (SKI-606) and R428 significantly reduced the breast cancer metastasis and prolonged model mouse survival^{135, 145}. R428 is specifically screened for inhibiting Axl kinase¹³⁵. The therapeutic effects of Bosutinib (SKI-606) and

R428 were also demonstrated in killing chronic lymphocytic leukemia (CLL) cells⁹⁹. Axl inhibitor DP-3975 greatly reduced the potential of migration and invasion in mesothelioma cells⁹⁰. Besides targeting the kinase enzymatic center, people have tried using anti-Axl monoclonal antibody to block Axl function. Axl mAbs 3G9 and 8B5 blocked its binding to ligand Gas6, and inhibited the NSCLC tumor growth in xenograft mouse model¹⁴⁶. One more option to antagonize Gas6/Pro S binding to Axl is to add soluble extracellular part of Axl (Axl-Fc) to cancer cells^{84, 147}.

Hematopoietic progenitor kinase 1 (HPK1)

Hematopoietic progenitor kinase 1 (HPK1) is a serine/threonine kinase, having a STE20-like kinase domain. HPK1 was firstly cloned from human fetal liver cDNA library^{35, 148}. Based on sequence homology, HPK1 is composed of a typical serine/threonine kinase domain in N-terminal region, four proline rich motifs in middle region, and a citron homology domain in C-terminal region³⁵. There are 28 serine/threonine kinases in mammalian share similar nuclear acid sequence with yeast kinase STE20¹⁴⁹. Since STE20 in yeast can activate MAP kinase pathway through activate Ste11p (MAP3K), STE20 is also named as MAP4K. In same way, HPK1 is called as MAP4K1³⁵. HPK1 is mainly expressed in hematopoietic cells³⁵, which was demonstrated by Northern Blotting assay.

The role of HPK1 in immune system

Since HPK1 was originally found in hematopoietic cells³⁵, the role of HPK1 in immune system received extensive investigation. HPK1 is involved in the development of several kinds of immune cells. HPK1 functions as negative regulator of dendritic cell activation¹⁵⁰. In HPK1 deficient mice, enhanced antigen presentation was observed. HPK1^{-/-} bone marrow dendritic cells (BMDCs) obtained superior antigen presentation capability, which was reflected by more T cells in response¹⁵⁰. In consistent with superior activity of BMDCs, the activation markers, IL-12, IL-1 β , TNF- α , and IL-6 were significantly increased in HPK1^{-/-} BMDCs, when cells were stimulated with Lipopolysaccharide (LPS). The major reason for increased activity of HPK1^{-/-} BMDCs is due to enhanced survival of BMDCs¹⁵⁰.

HPK1 is also a negative regulator of T cell mediated immune response¹⁵¹. HPK1^{-/-} T cells were hyperproliferative when they were stimulated with anti-CD3 antibody, which activated T cell receptor (TCR) signaling. HPK1^{-/-} T cells did not obtain extra survival potential when stimulated with anti-Fas or dexamethasone. HPK1^{-/-} mice showed superior T helper type 1 and 2 responses and expressed more IL-2, IL-4 and IF- γ cytokines. HPK1^{-/-} mice also showed aggravated autoimmunity, accompanied with hyper proliferation of autoreactive T cells¹⁵¹. HPK1 could down regulate T cell immune response through increasing T cell apoptosis after T cell activation. When CD4⁺ T cells were overexpressed with HPK1, a significant increased apoptosis rate was observed after TCR activation with anti-CD3 antibody. HPK1 also enhanced EL-4 thymoma apoptosis when cells were suffered with reactive oxygen species (ROS), which was introduced by adding H₂O₂¹⁵².

HPK1 plays an important role in TCR signaling. Four proline rich motifs (-P-x-x-P-) in middle region of HPK1 provide docking sites for many SH3 containing proteins. So far identified HPK1 associated SH3 containing adaptors includes growth factor receptor bound 2 (Grb2), Grb2-related adaptor protein (Grap), and Grb2-related adaptor downstream of Shc (Gads), HPK1-interacting protein of 55 kDa (HIP-55), B-cell associated molecule (Bam32), and Crk family¹⁵³. Since HPK1 activation involves the tyrosine phosphorylation, which serves as additional binding site for SH2 containing proteins, such as lymphocyte cytosolic protein 2 (LCP2 or SLP-76)¹⁵⁴. Moreover, HPK1 can activate some down-stream signaling molecules through its phosphorylation activity. In summary, HPK1 have diverse approaches to mediate signal transduction.

After activation of T cell receptor (TCR) by adding anti-TCR antibody to Jurkat cells, the HPK1 kinase activity was significantly activated. JNK (c-Jun N-terminal kinase) activation was triggered by overexpression of HPK1 in Jurkat cells and further enhanced by TCR engagement¹⁵⁵. However, HPK1 kinase dead form M46, where the ATP binding site was interrupted, could not activate JNK³⁵. HPK1 physically bond to and phosphorylated MAP3K1 (MEKK1), which is an up-stream activator of JNK^{35, 148}. HPK1 can activate JNK pathway through activating another MAP3K member, MLK-3. Interestingly, HPK1 selectively inhibit p38/ERK pathway^{35, 148}. As a down-stream target of MAP kinase, transcription factor AP-1 has its activation be suppressed by HPK1¹⁵⁵. HPK1 activation by TCR required the up-stream signaling components, lymphocyte-specific protein tyrosine kinase (LCK), Zeta-chain-associated protein kinase 70 (ZAP-70),

linker for activation of T cells (LAT) and SLP-76^{155, 156}. A proposed model for HPK1 activation is that, upon TCR activation, LCK and ZAP-70 is activated. Activated LCK and ZAP-70 then phosphorylated the adaptor LAT. Phosphorylated tyrosine on LAT provided docking sites for Grb2 or Gads protein, which contains both SH2 and SH3 domains. Through Grb2/Gads protein, HPK1 and SLP-76 are recruited to adaptor LAT. LCK and ZAP-70 phosphorylated some tyrosine sites on HPK1, which ensure HPK1 is further activated¹⁵⁴⁻¹⁵⁶. Complete activation of HPK1 involves phosphorylation of threonine 165 and serine 171. Serine 171 is phosphorylated by protein kinase D (PKD), while phosphorylation of threonine 165 is conducted by HPK1 auto-phosphorylation¹⁵⁷. Besides activating JNK pathway, HPK1 can promote T cell apoptosis by inhibiting NF- κ B activity¹⁵². Interestingly, HPK1 could be either a positive or negative regulator of NF- κ B depending on the T cell status¹⁵⁷. During T cell activation stage, HPK1 is a positive regulator of NF- κ B. However, in activation-induced cell death (AICD) stage which is after T cell expansion, HPK1 turns to a negative regulator of NF- κ B. Full length HPK1 physically associate with IKK complex component IKK β . HPK1 can phosphorylate IKK β and then activate IKK complex. Moreover, HPK1 can stimulate IKK complex by phosphorylating adaptor CARMA1¹⁵⁸, which is a member of CARD-containing membrane-associated guanylate kinase family. CARMA1 is crucial for TCR mediated NF- κ B activation¹⁵⁹. During T cell AICD stage, HPK1 is cleaved by caspase-3¹⁶⁰. HPK1-C terminal remains binding to IKK β . However, TCR induced NF- κ B activation is blocked^{157, 160}. HPK1-N terminal is still able to activate JNK pathway to promote T cell AICD^{152, 161}.

Role of HPK1 in cancer

So far there are very few reports on the function of HPK1 in cancer development. HPK1 may involve tumor progression through regulating immune system or directly suppressing cancer cell proliferation.

HPK1^{-/-} bone marrow dendritic cells (BMDCs) turned to be more potent antitumor vaccines¹⁵⁰. When tumor lysate-pulsed HPK1^{-/-} BMDCs was injected intratumorally to tumor-bearing mice, the tumor was completely eliminated. The control mice injected with lysate-pulsed WT BMDCs will reach maximal tumor size by day 50 after vaccination. However, mice injected lysate-pulsed HPK1^{-/-} BMDCs remained tumor-free for about 60 days. Lymphocytes collected from lymph nodes of mice injected with HPK1^{-/-} BMDCs obtained extra activity in killing Lewis Lung Carcinoma (LLC) cells¹⁵⁰. In consistent with the increased killing potential, T cells collected from HPK1^{-/-} BMDCs vaccinated mice had more IFN- γ expression.

As a negative regulator of T cell immune response, HPK1 can also regulate cancer progression by directly inhibiting T cell mediated anti-tumor response¹⁶². Non-small cell lung cancers (NSCLCs) have poor immunogenic response. One reason is that environmental prostaglandin E2 (PGE2) suppresses the anti-tumor immunity. Interestingly, PGE2 could not inhibit the IL-2 production on HPK1^{-/-} T cells. The proliferation rate of wild type T cells was significantly suppressed by PGE2 compared to HPK1^{-/-} T cells¹⁶². HPK1^{-/-} T cells were resistant to PGE2 induced apoptosis. In a xenograft mouse model for Lewis lung carcinoma, HPK1 knock-out mice barely

supported the tumor formation¹⁶². In summary, HPK1 could be a promoting factor of non-small cell lung cancer development by suppressing anti-tumor immune response.

On the contrary, HPK1 may function as a suppressor in cancer cells. For example, in Hodgkin's lymphoma B cells, HPK1 was required to mediate CD150 caused anti-proliferation and apoptosis¹⁶³. HPK1 was involved in CD150 activated JNK pathway¹⁶³. Previous research in our lab showed that HPK1 is expressed in normal pancreatic duct cells. However, HPK1 showed a progressive loss with the progression of PanIN lesions and not detectable in invasive PDAs¹⁶⁴. HPK1 was consistently lost in pancreatic cancer cell lines. The loss of HPK1 was due to the increased degradation. Proteasome inhibitor MG132 could bring back HPK1¹⁶⁴. The MG132 stabilized HPK1 could reduce pancreatic cancer cell proliferation rate and increase protein levels of cyclin-dependent kinase inhibitor 1/B (p21/p27), which are inhibitors of cell cycle progression^{165, 166}.

Endocytosis

One major form of protein trafficking is vesicle trafficking. Vesicle trafficking includes exocytosis and endocytosis, two processes which are reverse to each other. Endocytosis is a process that the cargo collected on the cell surface is compartmented by plasma membrane invagination, and vesicle fission from plasma membrane. Endocytosis plays important roles in the cell physiology. Endocytosis can allow large molecules that cannot permeate the plasma membrane to enter into cells. Endocytosis can also change the lipid and protein composition of plasma membrane. Moreover, endocytosis regulates the signal

transduction and response^{167, 168}. In a word, endocytosis provides an important approach for cells to communicate with environment. On physiological aspect, endocytosis is absolutely required for development, neuron-transmission, immune response, and cellular homeostasis¹⁶⁸.

Phagocytosis

Based on cargo character, endocytosis can be separated into phagocytosis and pinocytosis. Phagocytosis is usually triggered when large particles binds to corresponding receptors located on plasma membrane¹⁶⁸. The vesicle formed by phagocytosis process (phagosome) usually can reach 250 nm in diameter. In protozoon, phagocytosis is a common adopted way to obtain nutrition. While in metazoan and higher organism, phagocytosis is employed as a defensive trick to clean pathogens or cellular debris by some specialized cells like macrophages, and neutrophils¹⁶⁹. Phagocytosis is a strictly regulated process¹⁷⁰. When particle binds to the receptor, a signaling pathway is activated, and the actin goes to rearrangement until phagosome is formed¹⁷⁰. The destination of phagosome is usually ended by merging with lysosome, where the cargo is degraded¹⁷¹.

Pinocytosis

Pinocytosis, also called fluid-phase endocytosis, is a receptor independent and non-selectable endocytic process¹⁷². Pinocytosis starts with a clathrin coated pit, and end with a clathrin coated vesicle. During the pinocytosis, a bit of fluid from cell outside and its dissolved molecules is compartmented and get entry into cell¹⁶⁷. The diameter of

endosome formed through pinocytosis is usually less than 150 nm. Pinocytosis is a constitutively happened process in almost all cells, and extremely active in epithelial cell of intestinal, blood capillary, and kidney tubule¹⁷³.

Based on molecular machinery involved in the endocytosis, the endocytosis pathway can be divided into clathrin-mediated endocytosis, caveolin-mediated endocytosis, and actin-mediated endocytosis.

Clathrin-mediated endocytosis

Clathrin is a cytosolic protein that can bind to the outer surface of transport vesicles.

Formation of clathrin-coated vesicles (CCVs) requires Clathrin. Clathrin contains 1 heavy chain (180KD) and 2 light chain (33, 36KD). The basic assembly unit of clathrin is triskelion which is composed of 3 heavy chains and 3 light chains¹⁷⁴. Clathrin triskelion can self-assemble to a ball-shaped lattice. Clathrin alone can assemble a cage *in vitro* but not *in vivo*, suggesting the machinery for clathrin-mediated endocytosis requires other proteins¹⁷⁴.

A normal clathrin-coated vesicle structure needs adaptor proteins (AP) and accessory proteins¹⁷⁵. Plasma membrane-associated AP-2 complex is composed of 4 highly conserved subunits. AP-2 complex was located between the clathrin lattice and membrane, which provide docking sites for clathrin on vesicles. Clathrin alone can form coated vesicles *in vitro* with large variation in size. However, when adaptor protein was added, the size of clathrin coated vesicle was unified, indicating that AP-2 complex can

help to organize clathrin lattice¹⁷⁶. In another end, adaptor proteins can recognize the cytosolic part of cargo, like receptors. Adaptor proteins can facilitate cargo clustering¹⁷⁴.

One crucial step for clathrin-mediated endocytosis is to cut off the clathrin coated vesicles from plasma membrane. A large GTPase, dynamin, fulfill the above duty. Dynamin can be docked to plasma membrane by recognizing phosphatidylinositol 4,5-bisphosphate (PIP2). The dynamin, which is recruited and enriched by some accessory proteins, can polymerize around the neck of clathrin coated vesicles. Finally, through the GTP hydrolysis, Dynamin scissor the clathrin coated vesicles off the plasma membrane^{177, 178}. The clathrin coated vesicles, freed from plasma membrane, will be uncoated and finally fuse with early endosome¹⁷⁷.

Caveolin-mediated endocytosis

Almost all mammalian cells have flask-shaped invaginations, which are called caveolae. Caveolae are covered with caveolins and other coat proteins which are not found in clathrin coated vesicles¹⁷⁹. Usually, caveolae is formed in lipid raft which has enriched cholesterol and sphingolipid. The caveolae genesis sites on plasma membrane can be explained by its coat protein caveolins which can recognize cholesterol. Caveolins are acetylated membrane protein with molecular between 22-24 KD¹⁷⁹. Caveolins can self-assemble to a cage which holds the membrane invaginations. Knocking-out the caveolin-1 in mouse will damage the genesis of caveolin-mediated endocytosis^{180, 181}.

One of other important components of caveolae is Cavin protein. Cavins are small proteins with molecular weight between 10-15 KD. Cavin-1 silencing reduced the total amount of caveolae, and the size of caveolin 1 polymers^{179, 182}. Cavins can bind to Caveolin and physically locate the outer side of Caveolin cage¹⁷⁹.

Caveolin-mediated endocytosis is a strictly regulated process and involves a series of signaling cascade^{183, 184, 185}. For example, when SV40 virus binds to its receptor, a burst of tyrosine kinase activity was observed. Under signal stimulus, the actin around caveolae start to rearrange^{179, 185}. Finally the dynamin was recruited and the caveolae was scissor off from plasma membrane. The freed caveolae will fuse with other endosomes, such as early endosomes^{179, 185}. Caveolin-mediated endocytosis is expected to participate in many physiological behaviors, like signaling transduction, cargo transport, and adjust the plasma membrane lipid composition^{186, 187}.

Receptor-mediated endocytosis

The receptors on cell plasma membrane have an important role in endocytosis. Receptor mediated endocytosis can be clathrain, caveolin-dependent, or independent^{188, 189}.

Receptor mediated endocytosis allow cells to selectively internalize some nutrient components, like low-density lipoprotein (LDL), transferrin^{189, 190}. Accompanied with the clearance of receptor-bound ligands, the recycled receptor will be prepared to respond another around of stimuli^{188, 189}. Recently, the receptor mediated endocytosis had been explored for its potential in drug delivery. For example, therapeutic nanoparticles can be conjugated with ligands and its internalization by targeted cells will be increased¹⁹¹.

Though all receptors have some base level of intrinsic turnover through endocytosis pathway, the receptor internalization will be dramatically induced after ligand binding¹⁸⁹. Using receptor EGFR as an example, without EGF binding, the constitutive internalization is relatively slow ($t_{1/2} \approx 20\text{--}30$ min). However; the internalized the empty EGFR are recycled back to plasma membrane very quickly ($t_{1/2} \approx 5$ min)¹⁹². In contrast, after EGF binding, EGFR internalization rate is increased dramatically ($t_{1/2} \approx 4$ min), and only a partial of EGFR recycle back to the plasma membrane. Moreover, the recycling rate of EGF bound EGFR is expected to be slower than the empty EGFR^{192, 193}. A significant fraction of ligand bound EGFR will go through endosomal sorting¹⁹². The most frequently induced type of EGFR endocytosis by EGF is clathrin-mediated endocytosis. Nevertheless, in different cell type or physiological condition, EGFR may go through Caveolin-dependent, or other clathrin-independent endocytosis¹⁹³.

Receptor-mediated endocytosis is a process highly controlled by the receptor activated signaling. When EGF binds to EGFR, EGFR start to dimerize and the tyrosine activity is activated. The phosphor-Tyrosine sites on EGFR provide some docking sites for some adaptors, like Grb2, which can further recruit down-stream signaling molecular, or molecular regulating the endocytosis pathway¹⁹⁴. Ubiquitin E3 ligase Cbl, which is recruited by Grb2, will give EGFR a tag, which can be recognized by other endocytosis related adaptor, such as Hrs. and lead to endosomal sorting or degradation in lysosome^{194, 195}. In addition, some signaling molecular which is not directly activated by receptor itself will help to regulate receptor-mediated endocytosis. For example, protein kinase C (PKC)

can phosphorylate Thr⁶⁵⁴ site on EGFR, and cause decreased EGF endocytosis ¹⁹⁶.

Mitogen-activated protein kinase (MAPK)/p38 can even trigger the endocytosis of unoccupied EGFR, after phosphorylating serines in EGFR C-terminal domain ^{197, 198}.

Endocytosis organelles

On above sections, we described how endocytic vesicles are generated. In this section, we will introduce the common endocytosis organelles that participate in the endosomal sorting. Based on the biochemical character and functions, endocytosis organelles can be divided into early endosome (EE), multi-vesicular body (MVB)/late endosome (LE), and lysosome. The cargo in endocytic vesicle may selectively go through some, or all of above endocytic organelles.

Early endosome

Early endosome is irregular a membrane compartment containing some large vesicles (~400 nm diameter) and tubules (~60 nm diameter) ¹⁹⁹. The distinct morphological structures in early endosome are expected to be required for separating cargo in different space ¹⁹⁹. The cargo for returning back to plasma membrane is usually clustered in tubules, whereas, the cargo targeted to degradation will be collected in large vesicles ²⁰⁰. As a common sorting endosome for nascent endocytic vesicle to fuse with, early endosome is a very dynamic structure ^{199, 200}. The biogenesis of early endosome is still elusive. One possibility is that early endosome creates from the vesicles coming off the plasma membrane. Another possibility is that the vesicles budded from Golgi bodies give rise early endosome. On a giving early endosome, it may be a fusion product of several

vesicles from different sources. In matching with the sorting function, early endosome is featured with a mild low pH micro-environment (\sim pH6.3)²⁰¹. In this low pH solvent, ligand EGF is able to release from receptor EGFR²⁰¹. Early endosome specific GTPase Rab5 carries out crucial role in vesicle motility, budding and fusion. Except Rab5, the Rab5 effector, early endosomal antigen-1 (EEA1) can also serve as early endosome marker²⁰².

Multivescicular Body (MVB)/late endosomes

A mature late endosome is also named multivescicular body (MVB). The morphology of MVB can be described as a spherical membrane compartment with multiple intraluminal vesicles (ILVs)^{203, 204}. MVB is generally considered as a product of early endosome maturation^{203, 204}. Right after early endosome formation, some part of cargo will recycle back to cellular plasma membrane, while left cargos will be sorted in late endosome and finally delivered to lysosome. Early endosome maturation process involves removal of recycling related proteins and integration of proteins directed to fusion with lysosome^{203, 204}. Small GTPase Rab protein replacement is one of symbolic events of endosome maturation. During maturation Rab5 is replaced by Rab7²⁰⁵. One key behavior of early endosome maturation is the formation of ILVs, which can be observed as small vesicles inside the endosome^{203, 204}. The mechanism of ILV formation is complicated and not univocal^{203, 204}. Plasma membrane inward budding on endosome may be an automatic behavior of membrane with given lipid composition²⁰⁶. Endosomal sorting complex required for transport (ESCRT) played roles in ILV formation and cargo sorting²⁰⁶⁻²⁰⁸. As the question of which proteins should be sorted in ILVs, ubiquitination on trans-

membrane proteins provides a recognition site for ESCRT machinery²⁰⁶⁻²⁰⁸. After MVB formation, the contained cargo will finally be delivered to lysosome by fusing together.

Lysosome

Lysosome is a digestive organelle in mammalian cells. In consistent with its protein degradation function, lysosome is filled with digestive enzymes, acid hydrolases²⁰⁹. There are about 50 acid hydrolases, which are specific to different protein substrates²⁰⁹. For the optimal activity of acid hydrolases, a favorable acidic pH(4.5~5.0) is maintained in lysosome by a group of lysosomal membrane proteins (LMPs)²⁰⁹. So far around 40 human disorders is associated with lysosomal enzyme deficiencies²¹⁰. Lysosome disorders have been found in cancer. For example, some of lysosomal cysteine proteinases (Cats) were found to be overexpressed in breast cancer²¹¹.

Aberrant endocytosis pathways in cancer

Accumulated evidences indicated that endocytosis pathways participate in cancer progression²¹². The first step of metastasis is cancer cell dissolve from cell-cell junctions, which are maintained by endocytic-exocytic cycles^{212,213}. In case of cancer, some oncogenic signaling will break this endocytic-exocytic balance. Overexpression of CDC42, a small GTPase, promoted the progression of testicular²¹⁴. CDC42-GTP can lead tight junction proteins to lysosome for degradation²¹⁵. Some oncogenic RTKs can phosphorylate E-cadherin, which is major component of adherent junctions. The phosphorylated E-cadherin recruits E3 ubiquitin ligase, Hakai. After ubiquitination, the internalization and lysosomal degradation of E-cadherin is dramatically increased²¹⁶. E3

ubiquitin ligase Cbl is crucial for down regulating receptor tyrosine kinase through endocytosis pathway. However, in cancer, the connection between Cbl and RTKs is somehow disrupted by mutations happened on either Cbl or RTKs^{217-219, 220}. The aberrations of components in endocytosis pathway were also frequently discovered in cancer²¹². Caveolin 1, an essential factor for caveolin-mediated endocytosis, is found to be involved in the progression of several types of cancer. Amplification of Caveolin 1 gene was found in aggressive breast cancer. Caveolin 1 is a marker for poor prognosis in gastrointestinal cancer²²¹⁻²²³. VPS37A, an ESRT-I protein, is down regulated in hepatocellular carcinoma (HCC). Knockdown of VPS37A stabilized EGFR expression and promoted HCC cell invasion²²⁴. More and more endocytosis aberrations have been reported in literatures. In summary, cancer progression needs the participation of endocytosis pathway. On other hand, the alteration of endocytosis pathway contributes to cancer progression.

Specific aims

Pancreatic cancer is one of the most malignant diseases. In USA, Pancreatic cancer causes approximately 36,800 deaths each year¹⁻³. Overall 5 year survival rate of pancreatic cancer patients is less than 5%¹⁻³. The absence of early diagnostic markers and effective treatments accounts for the high mortality^{4,5}. Exploring potential therapeutic targets is highly demanded. Though Ras mutation is considered as major driving factor of pancreatic cancer development^{26,62}, the molecular mechanisms leading to pancreatic cancer are unclear.

Oncogenic receptor tyrosine kinases (RTKs) play important roles in cancer development¹¹⁵. Axl was firstly identified by a DNA transfection-tumorigenesis assay with the DNA from chronic myelogenous leukemia (CML) patients. The sequence analysis revealed that it has one typical tyrosine kinase domain in the cytoplasmic part, and two immunoglobulin-like and two fibronectin like repeats juxtaposed in the extra-cellular domain which provides a bind site for ligand growth arrest specific factor 6 (Gas6), or Pro S. As a member of the receptor tyrosine kinase (RTK) family, Axl was tested to have cell transforming activities just like some other oncogenetic RTK members did. So far, the accumulating data of the Gas6-Axl pathway investigated with colon cancer, lung cancer, thyroid carcinomas, breast cancer, and glioma revealed that the elevated Axl closely associated with the tumor malignancy. Further experiments through manipulating Axl expression levels suggested that Axl can contribute to cell proliferation, anti-apoptosis migration, and invasion. However, the knowledge of Gas6/Axl pathway in pancreatic cancer is still a missing part.

Furthermore, how Axl protein levels are regulated in cancer is unknown. Hematopoietic progenitor kinase (HPK1) functions as tumor suppressor in pancreatic cancer.

Interestingly, HPK1 protein is lost in more than 95% of pancreatic ductal adenocarcinoma (PDA). The molecular mechanism of how HPK1 suppress cancer development is yet to be investigated. HIP-55 constitutively binds to HPK1. HIP-55 is involved in the receptor down regulation through endocytosis pathway^{225, 226}. HPK1 knockdown B- lymphoma cell displayed a significant less internalization rate of IgM which binds to B cell receptor (BCR)²²⁷. Our lab's research showed that Axl is a binding partner of HPK1. The physical connection between HPK1 and Axl provides a probability that Axl protein levels can be regulated by HPK1 through endocytosis pathway. For the purpose to address above questions, on the first step, I **hypothesized** that Axl has oncogenic functions on pancreatic ductal carcinoma (PDA) progression, and Axl protein levels is regulated by HPK1 through endocytosis pathway. To test above hypothesis, the following specific aims were developed.

- 1) To determine the oncogenic role of Axl in PDA progression

- 2) To investigate the role of HPK1 in Axl degradation through endocytosis pathway

CHAPTER 2: Materials and methods

Tissue Microarray

The pancreatic samples were collected from 54 patients who did not receive any cancer therapy. The tissue was fixed with formalin and embedded in paraffin. Two cores each of tumor and non-neoplastic pancreatic tissue were punched and aligned on a slide using a tissue microarrayer (Beecher Instruments, Sun Prairie, WI). More details about tissue array construct can refer to pervious paper published by our lab ²²⁸. The sample collection and following study was approved by the institutional review board of the M. D. Anderson Cancer Center ²²⁹.

Immunohistochemistry

The formalin-fixed, paraffin-embedded tissue was dried at 58°C for 1 hour in an oven, and deparaffinized by soaking slides in xylene, ethonal, and finally distilled water. Antigen was retrieved by steaming slides in target retrieval solution, 10 mM citrate buffer (Dako Inc. Cat# S1699) for 35 minutes. After slowly cooling down slides together with the target retrieval solution under room temperature, the slides was washed by PBS buffer for 3 times, 10 minutes per time, and endogenous peroxidase was blocked with 3% hydrogen peroxide solution in PBS buffer for 20 minutes. The leftover hydrogen peroxide was cleaned with PBS for 3 times as above. The tissue was blocked with 2.5% normal serum for 30 minutes to reduce nonspecific probing. Here, the second antibody was from goat. So, the normal serum should be normal goat serum. For probing Axl antigen, anti-Axl antibody (Rabbit source, Abcam Inc. Cat# 37861) was diluted 1:50

normal serum blocking solution, and covered the tissue for overnight at 4°C. After washing out the primary antibody with PBS buffer, the sections were incubated with biotinylated secondary anti-rabbit antibody (Vector Laboratories, Burlingame, CA) for 1 hour at room temperature, washed with PBS for 3 times as above. The section was labeled with avidin-biotin-peroxidase complex (ABC kit, Vector Laboratories, Burlingame, CA. Cat# PK-4001) for 30 minutes, and washed with PBS. To visualize the immunostaining, the sections were incubated with fresh DAB solution (Dako Inc. Cat# K3468) and monitored under microscope. After ideal signal was observed, the reaction was stopped by rinsing with water. Nuclear counterstain was performed by immersing slides in Mayer's Hematoxylin, and bluing reagent (Richard-Allan Scientific Co. Cat# 7301). Finally, slides were covered with coverslip using mount medium (Vector Laboratories, Burlingame, CA. Cat# H-5501)^{230, 231}. The completed Axl staining would be subjected to following analysis²²⁹.

Quantification of the Axl Expression in PDA samples

After immunohistochemistry staining of slide with pancreatic ductal adenocarcinoma tissue microarray, the slide was scanned and analyzed with Ariol 2.1 scanner and digital imaging instrument (Applied Imaging, San Jose, CA). The duct cells in both tumor and benign tissue were exclusively marked using a hand-draw tool. The intensity and percentage of Axl staining in marked cells was combined to give a score. Based on Axl staining score, the PDA specimens were categorized into 2 groups: Axl high (score ≥ 7.56), and Axl low (score < 7.56). Collected raw data were subjected to statistical analysis²²⁹.

Statistical analysis

Whether there is a significant difference between Axl high and low group was tested by Fisher exact test. Overall survival rate and recurrence-free survival time for each group were plotted using the Kaplan-Meier method²³². The statistical significance of difference between survival curves was evaluated by log-rank test. Overall survival time was counted from the date of diagnosis to date of death, or to the date of last follow-up. Recurrence-free survival time was counted from the surgery date to the date of first recurrence. All patients' data was obtained from MD Anderson institutional pancreatic cancer data base, which is under review of the US Social Security Index. Univariate Cox regression analysis was adopted to test prognostic significance of clinical and pathologic characteristics. The 2-sided significance level of $P < .05$ was set for all statistical analyses²²⁹.

Cell culture

Human pancreatic cancer cell lines, Panc-1, Capan-1, BxPC-3, and ASPC-1 were purchased from American Type Culture Collection (ATCC, Manassas, VA). The CFPAC-1, Panc-48, Panc-3, Capan-2, Panc-28, Hs766T, MIA PaCa-2, and L3.6pl pancreatic cancer cells were generously provided by Dr. Paul Chiao (The University of Texas M. D. Anderson Cancer Center). All cell lines were cultured in either Dulbecco's modified Eagle's medium (DMEM) or RPMI-1640 medium supplemented with 10% fetal bovine serum (Hyclone Inc. Cat#30014.03). The immortalized normal human pancreatic ductal epithelial (HPDE) cell line was kindly provided by Dr. Ming-Sound Tsao (Ontario

Cancer Institute, Toronto, ON, Canada). HPDE cells were cultured in Keratinocyte-SFM medium (GIBCO Inc. Cat#10724) supplemented with a kit (GIBCO Inc. Cat#37000) containing recombinant human EGF and bovine pituitary extract. All cells were maintained in a humidified incubator (Forma Scientific Co.) at 37 °C with 5% CO₂.

Western blotting assay and antibodies

The cell lysates were prepared with the lysis buffer containing 20 mM HEPES (pH7.4), 150 mM NaCl, 1% Triton X-100 (V/V), 10% glycerol (V/V), 50mM β-glycerophosphate, 2mM ethylenediaminetetraacetic acid (EDTA), 1mM phenylmethylsulfonyl fluoride (PMSF), 1mM Na₃VO₄, 10μg/mL leupetin, 1μg/mL pepstatin A, and 2.8μg/mL Aprotinin. After setting lysis on ice for 30 minutes, the lysates were clarified by centrifugation (2, 0817 x g for 15 minutes at 4°C). The protein concentration of cell lysates is measured by the Bradford assay (Bio-rad Co.). Protein were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride (PVDF) membrane. The blot was blocked with either 5% none fat milk in PBST (0.05% Tween-20) or 5% BSA in TBST (for phosphorylated protein). Then primary antibodies diluted in blocking buffer were used to probe target proteins on blot. Based on the animal source of primary antibody, proper secondary body, labeled with horseradish peroxidase, was chosen to hybridize with primary antibody. After detection with enhanced chemiluminescence (ECL, GE Health Co.), the PVDF membranes were exposed to x-ray film. The primary antibodies used here are anti-Axl (Goat, Cat# SC-1096, Santa Cruz Co.), anti-Gas6 (Goat, Cat# SC-1936, Santa Cruz Co.) anti-Actin (Goat, Cat# SC-1616, Santa Cruz Co.), anti-EGFR (Rabbit,

Cat# SC-03, Santa Cruz Co.), anti-PARP (Rabbit, Cat# SC-7150, Santa Cruz Co.), anti-p-Akt^{Ser473} (Rabbit, Cat#9271, Cell Signaling Co.), anti-Akt (Rabbit, Cat#9272, Cell Signaling Co.), anti-IGFBP6 (Rabbit, Cat# SC-13094, Santa Cruz Co.), and anti-MMP-2 (Mouse, Cat#IM33L, Oncogene Co.)²²⁹.

Stably silencing Axl expression in PDA cell lines

A set of lenti-virus-based Axl shRNA expression plasmids (5 constructs), pLKO.1-shAxl, were purchased from were purchased from Open Biosystem Co. (AL, U.S.). Each plasmid contains a unique hairpin sequence targeting to different region of Axl cDNA (Gene Bank entry, BC03229). The lenti-virus packaging plasmids psPAX2 and PMD2.G, and empty vector pLKO.1 were courtesy of Dr. Craig Logsdon lab (University of Texas, MD Anderson Cancer Center). The viral particles were obtained by co-transfecting above packaging plasmids and shRNA expression plasmid into HEK 293T cells. More details about lenti-virus packaging and clarifying could refer to previous published protocol²³³. After infecting Panc-1 and Panc-28 cells with viral particles for 24 hours, the cells were subjected to selecting culture with the conditioned medium containing puromycin at 2µg/mL for Panc-1 cell line and 1µg /mL for Panc-28 cell line. Then single colonies were picked for further selection and amplification. The Axl-silenced colonies were screened by Western blotting assay using parental cells as control. Here, Axl shRNAs with sequence 5'-gcggtctgcatgaaggaatttctcgagaaattccttcagcagaccgc-3' (Cat# TRCN0000001038), and 5'-cgaaatcctctatgtcaacatctcgagatggtgacatagaggatttcg-3' (Cat# TRCN0000001040) gave most efficient silencing. Multiple colonies of each stable cell

line made with above shRNAs were used in following experiments. Stable cell lines made with empty vector pLKO.1 served as vector alone control.

In vitro migration assay

The Costar 24-transwell (Becton Dickinson Labware Inc. Bedford, MA) was used in the migration assay. The lower compartment containing 0.6 mL of 2% FBS DMEM served as chemoattractant. 3×10^4 cells from each cell line were seeded with 0.6 mL of serum free medium in upper chambers. After 8 hours of migration assay. The filter was washed with PBS, cleansed with a cotton swap, and then fixed with methonal. To visualize the cells on filter, staining with hematoxylin was performed. Finally, the filter was cut off the chambers and mounted on a slide. Cells on each filter were counted under assistance of an Olympus BX40 microscope. The experiments were repeated 3 times. Differences between Panc-28, pLKO.1-Panc-28, and Sh-Axl-Panc-28 cell lines were compared and analyzed by student's t test.

In vitro chemoinvasion assay

24-well Biocat matrigel invasion chamber (Becton Dickinson Labware Inc. Bedford, MA) was used here for chemoinvasion assay²²⁹. 0.75mL of DMEM with 10% FBS located in the lower compartment served as chemoattractant. 2.5×10^4 cells from each cell line in experiment were seeded in the upper compartment, which contained 0.5mL of serum-free medium. Experimental samples were set in a triplicate manner. After 24 hours of incubation in a humidified incubator with 5% CO₂ at 37°C, the cells passed the membrane were fixed by methanol and stained with hematoxylin. The cell numbers in 5

predetermined fields on each membrane were counted under an Olympus BX40 microscope. The statistical differences in invasion rate between Panc-28, pLKO.1.1-Panc-28, and shAxl-Panc-28s cell lines were measured by students' t test.

Detection of secreted matrix metalloproteinase-2 (MMP-2)

PDA cells in test were grown to 80% confluence. The medium were changed to serum free medium and kept growing for another 24 hours. Then the serum free medium was collected. The secreted proteins were concentrated through Microcon Centrifugal Filter Device (Millipore Co. Cat#42410) as instructed by manual. Detection of secreted MMP-2 will follow a standard Western blotting procedure. Western blotting detection on another secreted protein, insulin-like growth factor binding protein 6 (IGFBP6)^{234, 235}, served as a loading control of total amount protein.

Treating cells with Gamma-irradiation

The effects of Axl silencing on cell response to Gamma-irradiation was measured by PARP cleavage assay. The number of cells in apoptosis was also reflected by counting the cells in sub-G1 stage. The irradiation source was provided by a cesium 137 (Model E-0103; US Nuclear Corp., Burbank, CA). One day before irradiation, 1×10^6 of cells was seeded into $\Phi 10$ cm culture dish, which will give about 50% confluence of cell density right before irradiation. The irradiation dose for Panc-28 and Panc-1 cell series are 20 and 30Gy respectively. After irradiation, the cells will keep grow in incubator for another 48 hours. Then the cells were collected for either Western blotting assay to detect PARP cleavage, or added with hypotonic solution (0.1% sodium citrate, 0.1% Triton X-100, 100

$\mu\text{g/mL}$ of RNase, and $50 \mu\text{g/mL}$ of propidium iodide). The propidium iodide stained cells were sorted through a flow-cytometer (Beckman Coulter Inc., Brea, CA) based on the amount of DNA in cells. The ratio of cells in sub-G1 were calculated, and compared between parental, vector alone, and Axl silenced cells.

Quantitative real time PCR (QRT-PCR)

The total RNA from HPDE, and pancreatic cancer cell lines were extracted by TRIzol (Invitrogen Inc.) method²³⁶. After cells were cultured in $\Phi 10\text{cm}$ plates to 80% confluence, the medium were removed; then 1mL of TRIzol reagent was added on cells. Setting plates under room temperature for 5 minutes, and pipetting lysates for several times, so the nucleo-protein could be disassociated from RNA completely. After collecting the lysate into a 1.5 mL eppendorf tube, $200 \mu\text{L}$ of chloroform was added. Samples were vortexed briefly and incubated at room temperature for 5 minutes. To separating aqueous phase, samples were centrifuged at maximal speed ($>12,000\text{g}$) with a laptop centrifuge for 15 minutes under 4°C . The upper aqueous phase, which contained RNA, was carefully transferred to a new eppendorf tube. To precipitate RNA, equal volume of isopropanol was added. After centrifuging tubes at maximal speed ($>12,000\text{g}$) for 30 minutes under 4°C , the supernatant was removed. RNA pellets were washed with 75% ethonal which was prepared with DEPC-water. Finally, RNA pellets were dissolved in certain amount of DEPC-water. RNA concentration was measured with Nano-dropper.

For cDNA synthesis, a reverse transcription system (Promega Co. Cat#A3500) was used²³⁷. Following the manual provided by the company, the random primer was chosen here.

The reaction condition was 42°C for half hour. Finally the reaction was stopped by heating samples at 95°C for 5 minutes. The final 20 µL of product were diluted by adding 180 µL of autoclaved water. In each PCR reaction with volume of 20uL, 5 µL of template was added. The products of quantitative real time-PCR were labeled with cyber green (Bio-Rad Co.). Axl primers used here were 5'-GGTGGCTGTGAAGACGATGA-3' (Forward), and 5'-CTCAGATACTCCATGCCACT-3' (Reverse). RT-PCR was performed using a thermal cycler (Bio-Rad Laboratories, Inc.) for 40 cycles consisting of 30s at 95°C (denaturation), 30s at 58°C (annealing), and 45s at 72°C (extension). Each sample test was triplicated. The mean values of cycle numbers at thresh holder were obtained. The RT-PCR performed with primers (forward, 5'-AAGGAGAGAAGGATATTCCTGGAC-3'; reverse, 5'-AGAGAGATTGAAAAGTTTGC GGAT-3') specific to Ribosomal Protein Small Subunit 6 (RPS6) served as internal control, reflecting the total amount of cDNA in reaction ¹⁶⁴.

Measuring NF-κB activity by luciferase reporter assay

In order to measure NF-κB activity, the NF-κB-Luc-reporter with a firefly luciferase reporter gene was transfected into PDA cells using FuGENE HD transfection reagent (Roche Co. Cat#04-709-705). Plasmids of NF-κB-Luc-reporter were kindly provided by Dr. Logsdon lab. Transfection procedure followed the standard protocol as described in product manual. In each well of 6-well plate, 2µg of NF-κB-Luc-reporter plasmids were transfected. After 48 hours of transfection, substrate D-luciferin was added to cells with

final concentration of 150 μ g/mL. The intensity of luminescence was recorded by IVIS bioluminescence system (Xenogen Co.)²³⁸.

Antibody array

An antibody array membrane, with 400 different immobilized antibodies, was purchased from Hypromatrix Company. The Flag-HPK1-Panc-1 cells were cultured in 2 of 15cm culture dish. Next day, after cell attaching the dish bottom, 1 μ M of proteasome inhibitor MG132 was added to the culture medium and cultured cells for another 36 hours. The cell lysate was prepared. The protein concentration was measured by bio-rad protein assay. The expression of Flag-HPK1 stabilized by MG132 was tested by Western blotting. At the beginning, the antibody membrane was blocked in 5% non-fat milk in TBST(150 mM NaCl, 25 mM Tris, 0.05% Tween-20, pH 7.5) at room temperature with gentle shaking for 1 hour. Then around 5 mg of whole cell lysate was incubated with antibody membrane at 4°C for overnight. After washing with TBST for 3 times, the membrane associated HPK1 was probed with anti-Flag antibody, which is conjugated with HRP complex.

Transfection

In 6-well culture plates, 6X10⁵ of HEK293T cells were seeded. After overnight growth, the cell density reached to around 80% confluence. 4 hours before transfection, the culture medium (DMEM+ 10%FBS) were changed to fresh medium. Transfection was completed with Calcium Phosphate transfection kit (Millipore Co. Cat#S-001)²³⁹. The designed plasmids were pipetted to 5 mL round-bottom tubes (Becton Dickinson Co.

Cat#352058). For scale of 10 transfections, solution A was prepared by mixing 200 μ L of 10XHBS, 1.75mL of sterilized ddH₂O, 30 μ L of NaOH, and 20 μ L of phosphate solution; Solution B was prepared by mixing 200 μ L of Calcium solution, and 1.8mL of sterilized ddH₂O. In each transfection, 100 μ L of solution A was added to mix with plasmid. Then 100 μ L of solution B was dropped into the mixture of solution A and plasmids, accompanied with vigorous vortex. After setting tubes in hood for 30 minutes, the mixture of plasmid and solution A/B was dropped into planned well of culture plates. After transfection of 12 to 16 hours, the culture medium was changed to fresh medium. Upon the experiment requirements, HEK293T with transfected plasmids continued growing for another 16 to 24 hours. At the end, cells were collected for following experiments.

Co-immunoprecipitation

Cell lysates used for co-immunoprecipitation were prepared as the same way used for Western blotting. In each co-immunoprecipitation, 200 to 500 μ g of total protein were mixed with 2 μ g of either antibody, or normal serum IgG. Additional lysis buffer was added to reach total volume of 300 to 500 μ L. After 3 hours of incubation at 4°C with gentle rotating. 20 μ L (before washing off slurry) of protein A or G beads (based on animal source or subtype of antibody) was added to precipitate the antibody-protein complex. The binding of beads with antibody-protein complex was completed by gently rotating tubes under 4°C for 1 to 2 hours. Beads were collected by spinning samples at 8,000 rpm for 1 min under 4°C. The non-specific associated proteins were washed with 500 μ L of lysis buffer for 2 times. The antibody-protein complex was eluted by adding

reducing protein loading buffer and boiling samples for 5 minutes. More technical details of co-immunoprecipitation could refer to previous published protocols^{240 241}. Co-immunoprecipitated proteins were detected by regular Western blotting. To immunoprecipitate the flag tagged protein, M2 beads (Sigma Co. Cat#2426), which has conjugated anti-flag antibody, was adopted. The antibodies used for co-immunoprecipitation were anti-Axl (Goat, Cat# SC-1096, Santa Cruz Co.), anti-HPK1 (Goat, Cat# SC-6231, Santa Cruz Co.), anti-EGFR (Rabbit, Cat# SC-03, Santa Cruz Co.), anti-Dynamin (Rabbit, Cat#SC-11362, Santa Cruz Co.), and anti-P-Thr (Mouse, Cat# SC-1096, Santa Cruz Co.).

In vitro kinase assay of HPK1

10 μ g of pCI-Flag-HPK1, pCI-Flag-M46 (kinase dead form of HPK1), pCDNA-Flag-HPK1-KD, pCDNA-Flag-HPK1-CD, and pCDNA-Flag-Axl expression plasmid was transfected individually to HEK 293T cells, which was cultured in Φ 10cm plates, using Calcium Phosphate transfection kit. After 36 hours of transfection, cells were collected and lysised in lysis buffer (20mM of HEPES, pH7.4, 20mM of EGTA, 50mM of β -glycerophosphate, 1% Triton X-100, 10% Glycerol, 150mM of NaCl), which was added with fresh protease inhibitors. HPK1 complex, and its substrate Axl complex were immunoprecipitated by using M2-beads. Immunoprecipitated complexes were sequentially washed with 1mL of lysis buffer for 2 times, LiCl buffer (500mM of LiCl, 100mM of Tris, pH7.6, 0.1% of Triton X-100) for 2 times, and kinase buffer (20mM of Mops, pH7.2, 2mM of EGTA, 10mM of MgCl₂, 0.1% Triton X-100) for another 2 times. Immunoprecipitated HPK1 complex and substrate Axl complex were mixed together with

35 μ L of kinase buffer which contained 50 μ M of cold ATP and 10 μ Ci of [γ 32P]ATP. Kinase reaction was completed by incubation for 30 minutes at 30°C. Kinase reaction was stopped by adding Laemmli buffer and boiling for 5 minutes. After cooling down and briefly spinning the samples, boiled supernatants were loaded on a 10% SDS-polyacrylamide gel. When finished running, gel was exposed to an X-ray film. More details of in vitro HPK1 kinase assay can refer to previous published paper by Dr. Tan lab^{151, 242}. To test whether Dynamin was also a substrate of HPK1, endogenous Dynamin from HEK293T cells was immunoprecipitated by anti-dynamin II antibody (Rabbit, Cat#SC-6400, Santa Cruz Co.).

Measurement of protein half-life

Protein translation was inhibited by adding translation inhibitor cycloheximide (CHX) to final concentration of 100 μ g/mL. CHX was added at different time points from 0 to 6 hours. Protein Axl in measurement was detected by Western blotting. The intensity of Western blotting bands was quantified by software Image J. The ratio between Axl and loading control actin was calculated. The average value after 3 repeats was calculated and plotted in a 2 axis figure.

Construct of HPK1 inducible expression stable cell line

To establish HPK1 inducible expression system, a Lenti-X™ Tet-On® Advanced Inducible Expression System (Clontech Laboratories, Inc.) was adopted. First step, pLVX-Tet-On Advanced vector, which constitutively express a tetracycline-controlled transactivator, rtTA-Advanced, was introduced to Panc-1 cells. The lenti virus based

pLVX-Tet-On Advanced vector was packaged to lenti virus by co-transfecting with packaging plasmids, Lenti-X HTX Packaging Mix (Cat#632156), into 293T cells. The medium containing lenti-viral particles were collected to infect Panc-1 cells. Since pLVX-Tet-On Advanced vector has a Neo resistant gene, which can be used to select stable Panc-1 cell lines. Here, single colonies of pLVX-Tet-On-Panc-1 stable cell lines were selected under condition medium containing 400 μ g/mL of G418 (neomycin analogue). The expression levels of transactivator rtTA-Advanced in each stable colony were screened by Western blotting using antibody (Cat.Nos. 631108) specific to TetR protein. The transcriptional activity of rtTA-Advanced in Panc-1 cells was confirmed by temporary transfecting pLVX-Tight-Puro-Luc plasmid and following luciferase assay. Protein rtTA-Advanced has transcriptional activity only when tetracycline or doxycycline (tetracycline analogue) was present. Second step, lenti virus based pLVX-Tight-Puro was used to construct HPK1 expression plasmid. pLVX-Tight-Puro has a transactivator rtTA-Advanced response element in the PTight promoter region, and a puromycin resistant gene which can used for selecting stable cell line. The cDNAs of Flag-HPK1 and Flag-M46 (HPK1 kinase dead form) were cut from previous constructed pShuttle-CMV-Flag-HPK1/M46 (a construct for adenovirus based expression system) by restrict enzymes BglIII and EcoRV. The digested cDNAs was inserted into pLVX-Tight-Puro vector right after PTight promoter region through BamHI and EcoRI sites. Ligation was completed in 2 steps: first, ligating BglIII site with BamHI site through compatible stick ends; second, ligating EcorV site with EcoRI site through blunt-end ligation methods. The obtained pLVX-Tight-Puro-Flag-HPK1/M46 constructs were packaged to lenti virus using Lenti-X HTX Packaging Mix as described above. The collected lenti-viral particles

were used to infect pLVX-Tet-On-Panc-1 cell line. The stable colonies of pLVX-HPK1/M46-Panc-1 cells were selected under condition medium with 2 μ g/mL of puromycin. Doxycycline induced expression of HPK1 or M46 were checked by Western blotting using anti-HPK1 antibody.

CHAPTER 3:

To determine the oncogenic role of Axl in PDA progression

Introduction

Pancreatic cancer is the fourth leading cause of cancer death in the United States, preceded only by lung, colon, and breast cancers²⁴³. Despite the available treatment modalities for pancreatic ductal adenocarcinoma (PDA), including chemotherapy, radiotherapy, surgery, or a combination of these modalities, PDA has the worst prognosis of all major malignancies, with 5-year survival of less than 5%²⁴⁴. Even resectable PDA at an early stage frequently recurs either with metastatic disease in the liver, lung, or peritoneal cavity or with local recurrence in the pancreatic surgery bed. Therefore, functional studies of genetic alterations involved in its aggressive growth and metastasis are important to develop new treatment modalities for pancreatic cancer.

Increased expression of the epidermal growth factor (EGF) family of mitogenic peptides and their corresponding receptor tyrosine kinases (RTKs) related to the EGF receptor is a common feature of PDA²⁴⁵. The binding of growth factors to RTKs promotes dimerization and autophosphorylation in its cytoplasmic domain and the subsequent activation of downstream signal pathways that control a variety of cellular processes, such as proliferation, differentiation, migration, and survival^{246, 247}. Unlike other RTKs, which are activated by growth factors, Axl is a unique RTK in that it is activated by growth arrest specific factor 6 (Gas6), a member of the vitamin K–dependent proteins^{85, 88}. Axl protein has an extracellular domain resembling cell adhesion molecules, which

consists of 2 immunoglobulin-like domains and 2 fibronectin III-like motifs, and contains a typical intracellular kinase domain of an RTK^{85, 88}. Using an EGF-Axl chimeric receptor construct consisting of the extracellular and transmembrane domains of EGFR and the Axl kinase domain, Fridell et al showed that overexpression of this chimeric protein is sufficient to induce Axl's transforming activity and tumor formation in nude mice, suggesting that Gas6-independent mechanisms of Axl functions may also exist⁹¹.

Axl was originally identified as a transforming gene in chronic myelogenous leukemia and has been reported to be overexpressed in many types of human malignancies, including diffuse glioma, melanoma, osteosarcoma, and carcinomas of the lung, colon, prostate, breast, ovary, esophagus, stomach, and kidney^{75, 80, 118, 139, 145, 248}. Previous studies have shown that Axl signaling promotes tumor cell proliferation, migration, and invasion in these tumors. Both Axl and Gas6 were overexpressed in glioma cell lines and human glioma tissue samples and are associated with a poor prognosis in patients with glioblastoma multiforme¹³⁹. Inhibition of Axl signaling by a dominant-negative Axl mutant suppresses tumorigenesis, migration, and invasion and resulted in long-term survival of mice after intracerebral implantation of glioma cells compared with glioma cells transfected with wild-type Axl¹¹⁸. Axl was also overexpressed in metastatic prostatic carcinoma cell line compared with normal prostatic epithelial cells and other prostatic carcinoma cell lines²⁴⁹. In addition, Axl has been shown to be a key regulator involved in multiple steps of angiogenesis, including endothelial cell migration, proliferation, and tube formation in vitro¹³⁴. Knockdown of Axl expression not only impaired the formation of functional blood vessels in vivo but also reduced the growth of

MDA-MB-231 breast carcinoma cells in a xenograft mouse model¹³⁴. These findings indicate that Axl is critical for tumorigenesis, invasion, and angiogenesis. However, the expression and functions of Axl in PDA have not been well studied. In this study, we examined the expression of Gas6 and Axl proteins in 12 PDA cell lines, 54 pancreaticoduodenectomy specimens of stage II PDA, and their paired non-neoplastic pancreatic ductal epithelial cells. Using univariate and multivariate analysis, we correlated the expression of Axl with survival and other clinicopathologic features in patients with stage II PDA. To further examine the function of Axl in PDA, we used shRNA to knock down Axl expression in PDA cell lines and measured the effect of Axl knockdown on radiation-induced apoptosis and invasion ability. Our data showed that Axl and Gas6 are frequently overexpressed both in PDA cell lines and human PDA samples and play an important role in anti-apoptosis and invasion in pancreatic cancer. Therefore, targeting Axl signaling pathway may represent a new approach for the treatment of PDA.

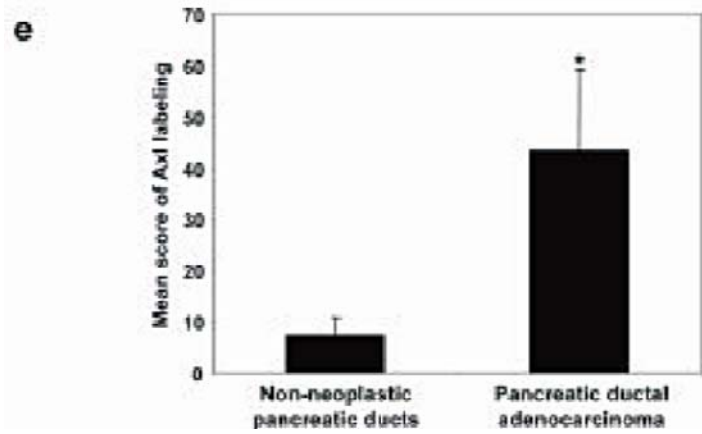
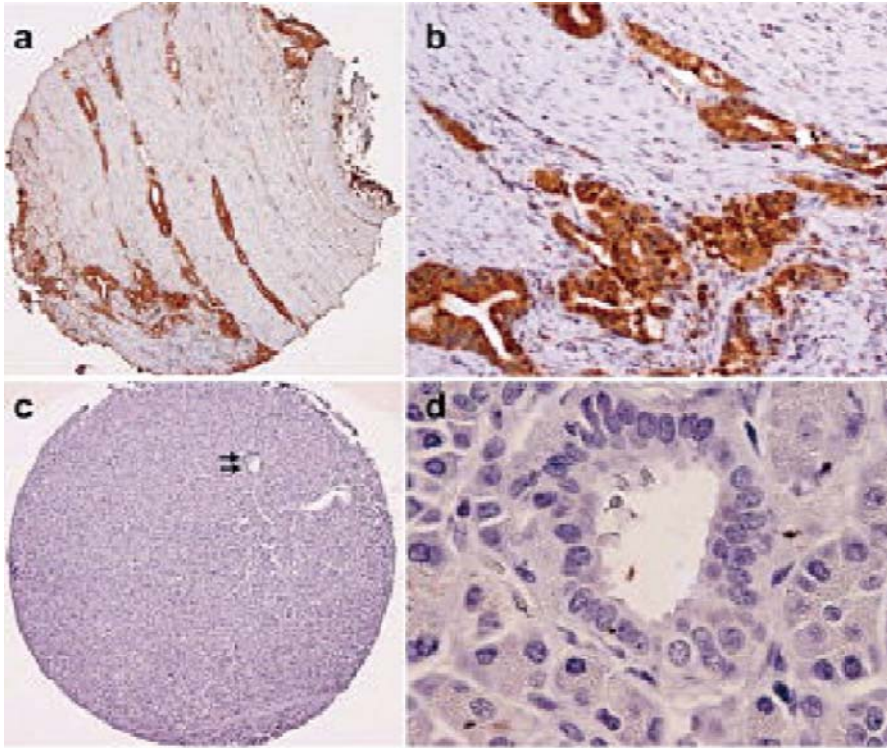
Results

Axl overexpression correlated with poor prognosis in stage II PDA patients.

To examine Axl expression in pancreatic cancer patients, a tissue microarray with specimens from 54 patients having stage II PDA was conducted, (See materials and methods section). Then the Axl expression levels were quantified and summarized through statistical approaches. As shown in figure 2a and b, Axl displayed a strong and diffuse cytoplasmic staining in pancreatic cancer duct cells, but not in normal ductal cells (Fig. 2 c and d). 38 of 54 stage II PDA specimens (70%) had high Axl expression. However; in 50 of paired non-neoplastic pancreatic ductal tissues, 11 of them (22%) had high Axl expression. The mean Axl expression scores of PDA samples and non-neoplastic pancreatic ductal tissues, which was 43.6 and 7.2 correspondingly (Fig. 2e), also showed a significant increase in the PDA samples ($p=0.0001$).

Figure 2. Immunohistochemical results showed Axl expression is increased in PDA samples.

Representative micrographs show strong cytoplasmic Axl staining in a moderately differentiated PDA (a and b) and no expression of Axl protein in normal pancreatic duct (original magnification, 20× for a and c, 200× for b, and 400× for d). (e) Axl expression in PDA samples and their paired non-neoplastic pancreatic ductal epithelial cells are quantified and summarized (*P < .01). The mean scores of Axl staining in PDA and non-neoplastic pancreatic samples are 43.6 and 7.2 respectively.



Clinicopathologic correlations between Axl expression and clinicopathologic pancreas in patients with stage II PDA progression was summarized in Table 1. Based on World Health Organization classification standards, 54 stage II PDA samples were classified as well-differentiated adenocarcinoma (8 cases, 15%), moderately differentiated adenocarcinoma (33 cases, 61%), and poorly differentiated adenocarcinoma (13 cases, 24%). 43 of 54 patients had R0 resection, in which surgical margins are microscopically negative. The remaining 11 patients had R1 resection²²⁹. As summarized in Table 1, the Axl expression levels did not correlate with tumor size ($p=0.71$), resection margin status ($p=1.0$), lymph node status ($p=0.55$), postoperative chemotherapy ($p=0.71$), and postoperative radiotherapy ($p=0.47$). However, the frequency of distant metastasis between Axl high and low patients showed a significant difference ($p=0.02$). 22 of 38 (58%) Axl-high PDA patients had distant metastasis, which was obviously higher than it in Axl-low PDA patients, in which only 4 of 16 (25%) patients had distant metastasis. The patients with Axl-high PDAs showed a worse prognosis. As demonstrated by Kaplan-Meier curves in figure 3, the median overall survival for Axl-high PDA patients were 25.0 ± 4.3 months, compared to 82.9 ± 35.3 months in Axl-low PDA patients ($p=0.02$; Fig. 3a). The median recurrence-free survival of Axl-high PDA patients was 12.6 ± 4.3 months, compared to 82.4 ± 55.7 months in patients with Axl-low PDAs ($p=0.008$; Fig. 3b). In multivariate analysis, high expression of Axl was associated with poor overall survival and recurrence-free survival ($P = .03$ and $P = .04$, respectively) independent of tumor size and lymph node status or stage, seeing Table (2)

Table 1. Clinicopathologic Correlation of Axl Expression in Stage II PDAs

Characteristics	N	Axl Low (n = 16)	Axl High (n = 38)	P
Age, y				0.14
<60	20	6	14	
60-70	22	9	13	
>70	12	1	11	
Sex				0.13
Female	18	3	15	
Male	36	13	23	
Tumor size, cm				0.71
≤2.0	11	4	7	
>2.0	43	12	31	
Tumor differentiation				0.24
Well	8	4	4	
Moderate	33	10	23	
Poor	13	2	11	
Margin status				1.0
Negative	43	13	30	
Positive	11	3	8	
Lymph node status (stage)				0.55
Negative (IIA)	20	7	13	
Positive (IIB)	34	9	25	
Postoperative chemotherapy				0.71
No	9	2	7	
Yes	45	14	31	
Postoperative radiotherapy				0.47
No	12	2	10	
Yes	42	14	28	
Recurrence/distant metastasis				0.02
No	14	8	6	
Local	14	4	10	
Distant	26	4	22	

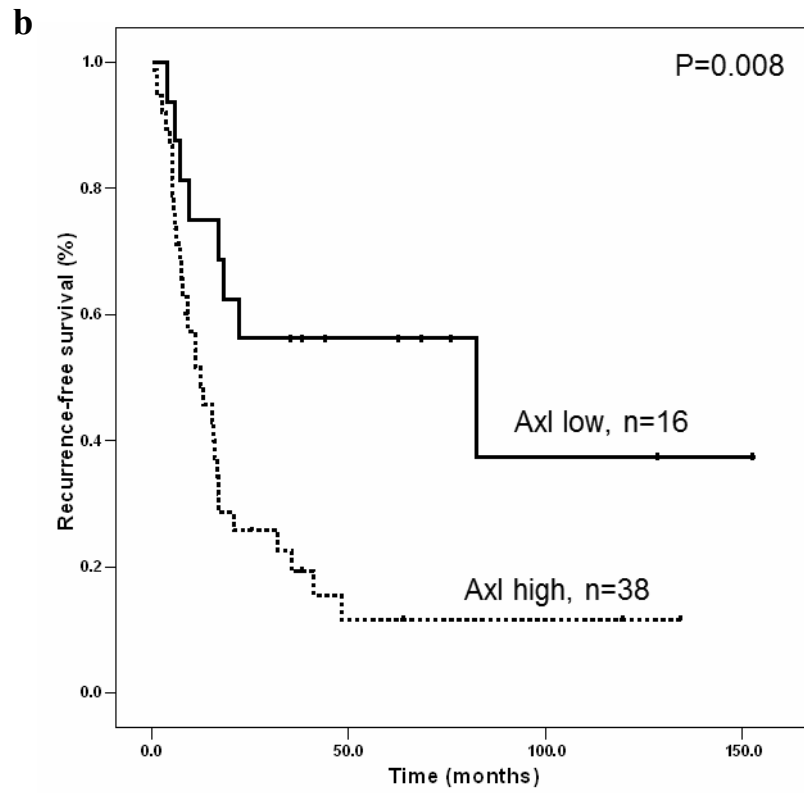
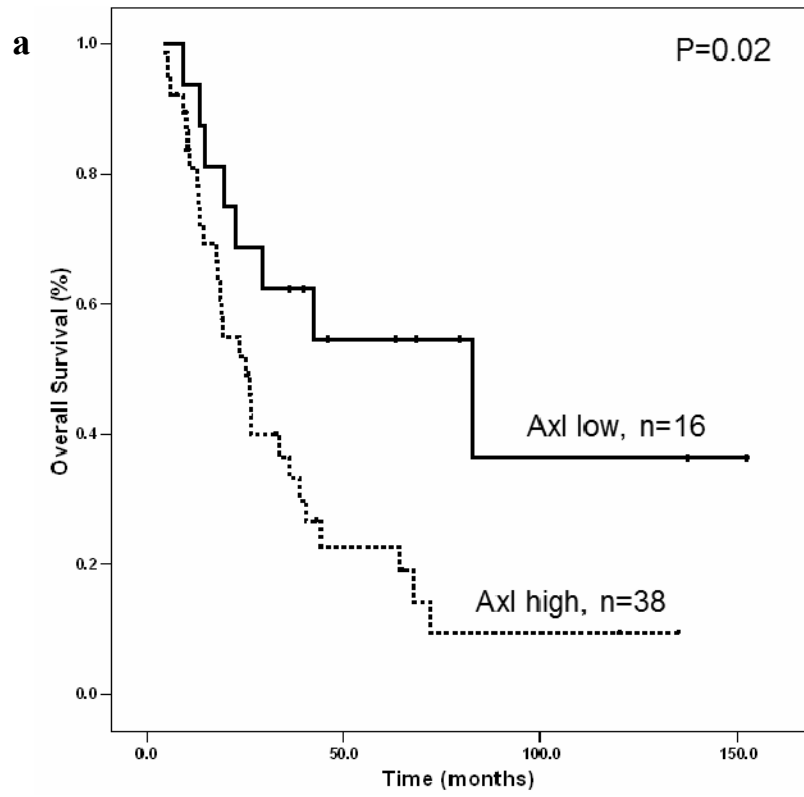
Table 2. Univariate and Multivariate Analysis of Overall and Recurrence-Free Survival in Patients With Stage II Pancreatic Ductal Adenocarcinomas

Parameters	N	Overall Survival		Recurrence-Free Survival	
		HR (95% CI)	P	HR (95% CI)	P
Univariate analysis					
Age, y					
<60 (reference)	20	1.00		1.00	
60-70	22	1.39 (0.66-2.91)	0.38	1.19 (0.59-2.41)	0.63
>70	12	1.46 (0.59-3.60)	0.42	1.40 (0.58-3.38)	0.45
Sex					
Female (reference)	18	1.00		1.00	0.36
Male	36	0.84 (0.43-1.66)	0.62	0.74 (0.38-1.42)	
Tumor size, cm					
≤2.0 (reference)	11	1.00		1.00	
>2.0	43	3.25 (1.15-9.24)	0.03	3.82 (1.35-10.82)	0.01
Tumor differentiation					
Well (reference)	8	1.00		1.00	
Moderate	33	1.80 (0.68-4.76)	0.23	1.92 (0.73-5.06)	0.19
Poor	13	1.72 (0.57-5.16)	0.34	1.81 (0.62-5.32)	0.28
Margin status					
Negative (reference)	43	1.00		1.00	
Positive	11	1.18 (0.51-2.72)	0.72	0.83 (0.36-1.88)	0.65
Lymph node status (stage)					
Negative (IIA)	20	1.00		1.00	
Positive (IIB)	34	2.20 (1.04-4.62)	0.04	3.31 (1.54-7.08)	0.002
Postoperative chemotherapy					
No (reference)	9	1.00		1.00	
Yes	45	1.92 (0.58-6.43)	0.29	1.50 (0.53-4.27)	0.45
Axl expression					
Low (reference)	18	1.00		1.00	
High	36	2.48 (1.13-5.46)	0.02	2.79 (1.27-6.13)	0.01
Multivariate analysis					
Tumor size (cm)					
≤2.0 (reference)	11	1.00		1.00	
>2.0	43	3.14 (1.10-8.93)	0.03	2.87 (0.96-8.54)	0.06
Lymph node status (stage)					
Negative (IIA)	20	1.00		1.00	
Positive (IIB)	34	1.22 (0.53-2.81)	0.65	2.12 (0.95-4.71)	0.07
Axl expression					
Axl low (reference)	16	1.00		1.00	
Axl high	38	2.40 (1.08-5.29)	0.03	2.36 (1.06-5.28)	0.04

Abbreviations: HR, hazard ratio; CI, confidence interval.

Figure 3. Kaplan-Meier curves for overall survival and recurrence-free survival by Axl expression in patients with stage II PDAs indicated that Axl high patients have worse prognosis.

(a) Median overall survival time for Axl-high and low PDA patients are 25.0 ± 4.3 months and 82.9 ± 35.3 months respectively ($P = .02$, log-rank method). (b) Median recurrence-free survival was 12.6 ± 3.5 months for Axl-high patients, and 82.4 ± 55.7 months for Axl-low patients ($P = .008$, log-rank method).

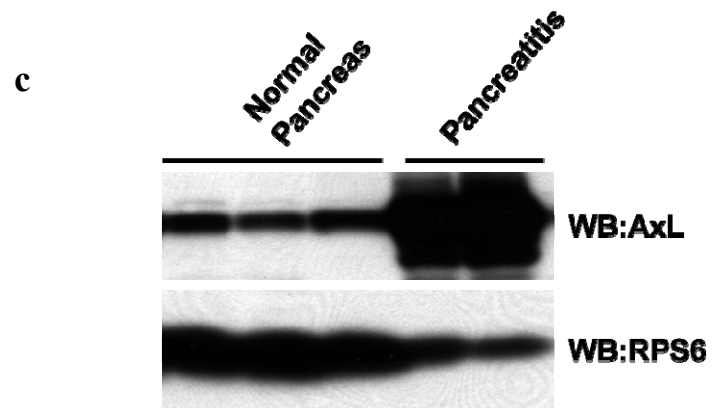
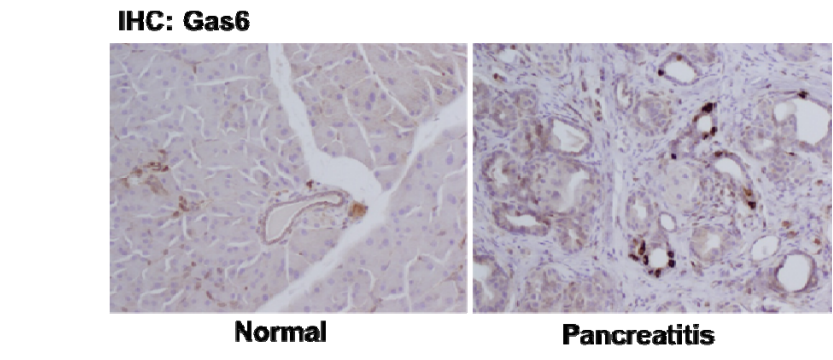
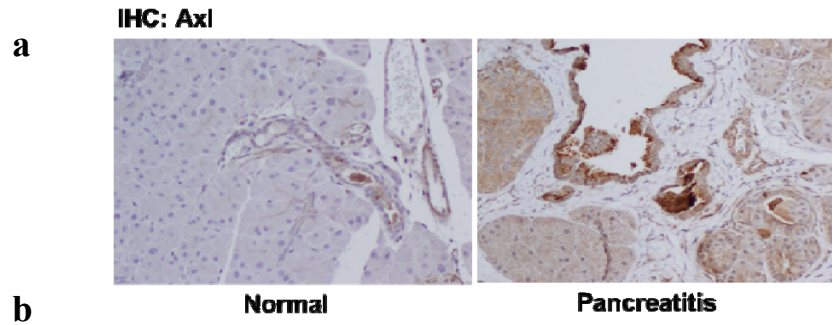


Axl and Gas6 were overexpressed in a transgenic mouse model of pancreatitis.

Previous studies show that pancreatic cancer development is that pancreatic cancer developed from precursor pancreatic intraepithelial neoplasia (PanIN)^{250 67 66}. Chronic pancreatitis will cause genomic instability and promote pancreatic development^{67, 251 66}. K-Ras was mutated in almost all pancreatic cancer. In a cLGL-KRas^{G12V} transgenic model, where KRas^{G12V} was overexpressed specifically in pancreas through Cre/loxP recombination system, extensive chronic pancreatitis (CP), PanIN, as well as PDA were observed^{66,67}. To further understand the role of Gas6/Axl signaling in the PDA development, The Gas6, Axl protein levels in mouse CP from cLGL-KRas^{G12V} mice, which is a courtesy of Dr. Logsdon Lab, were detected either by Western blotting, or immunohistochemistry. As shown in Fig. 4c, Axl protein levels in mouse pancreas with chronic pancreatitis was much higher than normal mouse pancreas. Axl immunohistochemistry results showed that duct cells in mouse chronic pancreatitis had more Axl staining (Fig. 4a). Gas6 immunohistochemistry results showed similar pattern as Axl. Gas6 staining in mouse CP tissue was stronger than it in normal mouse pancreatic ductal cells (Fig. 4b). In summary, Axl and Gas6 overexpression seems an early event in PDA development.

Figure 4. Axl and Gas6 protein levels were increased in Ras mouse model of pancreatitis and PDA.

(a) Immunohistochemical(IHC) staining of Axl in mouse chronic pancreatitis (CP) tissue showed that Axl was up-regulated. (b) IHC staining of Gas6 showed it is overexpressed in mouse CP tissue. (c) Axl Western blotting assay demonstrated that Axl was overexpressed in mouse CP tissues.



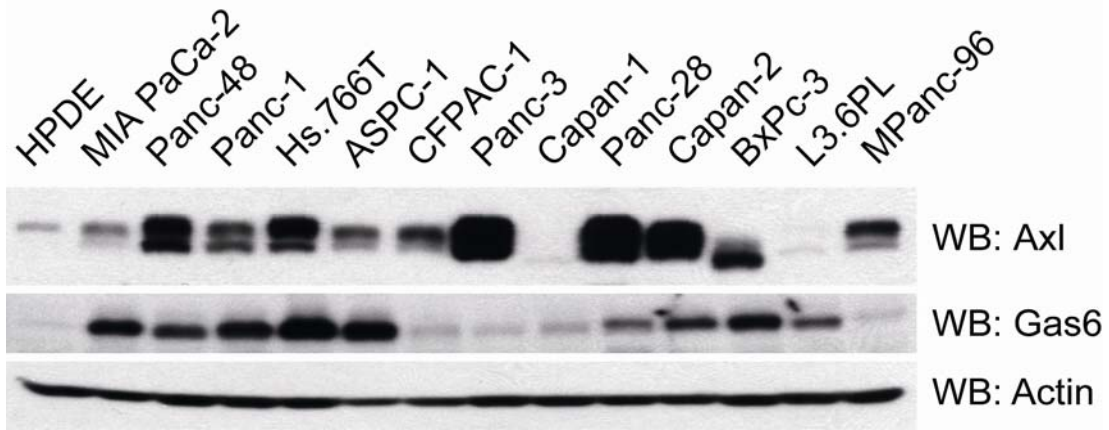
Most pancreatic cancer cell lines had overexpression of Axl and Gas6

Since most human PDA samples (70%) had overexpressed Axl. We expected that Axl expression was also high in pancreatic cancer cell lines. To test Axl, and Gas6 expression, 12 pancreatic cancer cell lines, as well as one immortalized normal pancreatic ductal epithelial cell line, HPDE, were cultured to 80% confluence, and then collected for West Blotting using either anti-Axl, or anti-Gas6 body, seeing materials and methods. In parallel, Cell samples were also collected for total mRNA extraction; and quantitative real time PCR was conducted to measure the mRNA levels of Axl. As shown in the figure 4a, comparing with HPDE cells, 9 of 12 (75%) PDA cell lines have Axl protein overexpression. They were Mia PaCa-2, Panc-48, Panc-1, Hs766T, ASPC-1, CFPAC-1, Panc-3, Panc-28, Capan-2, and BxPC3 cells. The ratio of PDA cell lines with high Axl expression was close to what we observed in PDA tissue samples (70%). Interestingly, Gas6 protein was also found for the first time to be overexpressed in most PDA cell lines (70%), which are MIA PaCa-2, Panc-48, Panc-1, Hs766T, Capan-1, Panc-28, Capan-2, BxPC3, and L3.6PL cells (Fig. 5a). Axl mRNA levels measured by quantitative RT-PCR also demonstrated that most PDA cell lines had overexpressed Axl (Fig. 5b).

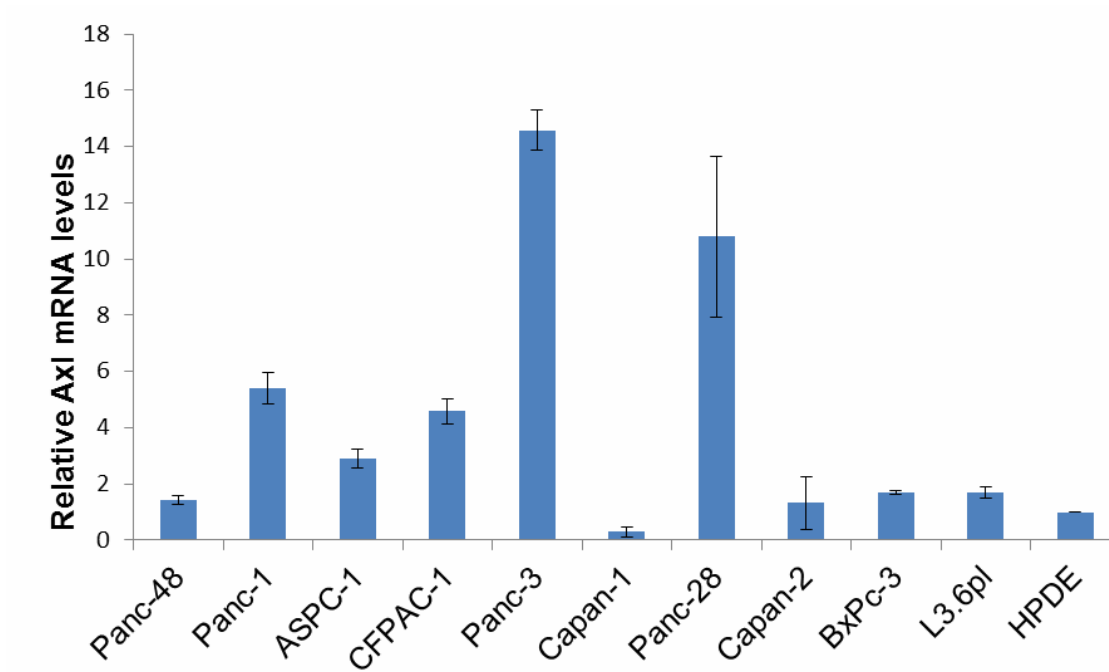
Figure 5. Overexpression of Axl and Gas6 in PDA cell lines

(a) Axl and Gas6 protein levels in PDA cell lines and immortalized normal pancreatic ductal epithelial cell HPDE (control) were measured by Western blotting assay. Most PDA cell lines have elevated Axl and Gas6 expression. (b) Relative Axl mRNA levels in PDA cell lines and HPDE cells. Quantitative RT-PCRs were performed using Axl and RPS6 (reference gene) specific primers. HPDE mRNA levels was set as 1. Majority of the PDA cell lines had elevated Axl mRNA levels.

a



b



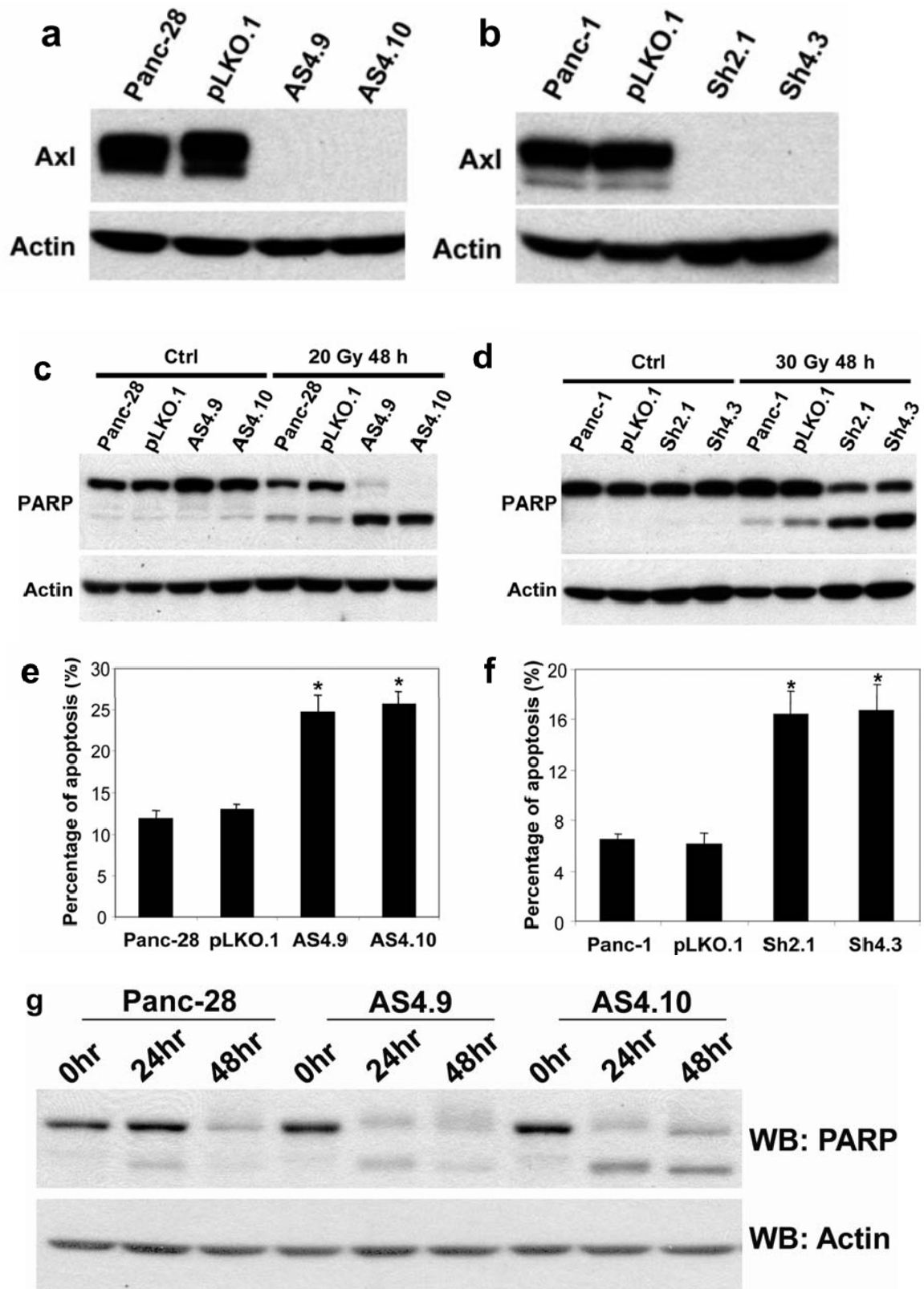
Axl silencing sensitized cells to apoptosis stimuli

In breast cancer, Axl can activate Akt and NF- κ B pathway to against apoptosis stimuli^{135 252 145}. To test whether Axl is involved in the survival of PDA cells, we silenced Axl expression by shRNA. Two shAxl stable colonies each generated from Panc-28 (AS4.9, AS4.10), Panc-1 (Sh2.1, Sh4.3) were selected for following experiments. As demonstrated by Axl Western blotting in Fig. 6a and b, each shAxl stable clone had no detectable Axl expression; whereas, the Axl protein levels in vector alone pLKO.1-Panc-28, and pLKO.1-Panc-1 stable clones were not changed, when compared with parental cell lines. To test whether Axl played a role in the cell apoptosis, above Axl silenced stable cell lines were treated with Gamma-irradiation with a dose of 20Gy on Panc-28 series, or 30Gy on Panc-1 series. 48 hours After irradiation treatment, cells were collected for either PARP cleavage assay, or counting sub-G(1) cells as described in materials and methods. Though PARP cleavage, the apoptosis marker, was detectable in all cells after irradiation, Axl silenced stable cell lines AS4.9, AS4.10, Sh2.1, and Sh4.3 had markedly increased PARP cleavage, compared to parental or vector controls (Fig. 6c and d. To quantify the amount of cells in apoptosis, the cells in Sub-G(1) were sorted and counted by FACS analysis. The average percentages of cells in apoptosis were 24.7% for AS4.9, 25.7% for AS4.10, which were significant higher than 11.8% for parental cell panc-28, and 13.0% for vector alone control pLKO.1-Panc-28 (Fig. 6e). Though Panc-1 was more resistant to radiation treatment, similar results were observed. The mean percentage of apoptotic cell of sh2.1, sh4.3, Panc-1 and pLKO.1 cells was 16.3%, 17.1%, 6.8% and 6.2% respectively (Fig. 6f). Axl silencing could also increase the apoptosis of PDA cells by serum starvation. The PARP cleavage was shown up in control pLKO.1-

Panc28 cells under serum starvation for 48 hours. However, in Axl silenced AS4.9, AS4.10 cells, the PARP cleavage was advanced to time point of 24 hours (Fig 5g), suggesting that Axl silencing could accelerate the cell apoptosis under serum starvation condition. In conclusion, Axl silenced PDA cells is more sensitive to apoptosis stimuli, gamma-irradiation and serum starvation. Our data showed that Axl play an important role in the survival of PDA cells.

Figure 6. Axl silenced PDA cells were more sensitive to apoptosis stimuli.

Compared to parental and vector control (pLKO.1) cells, Axl in Panc-28 (a) and Panc-1 (b) was efficiently silenced by shRNA (clone AS4.9, AS4.10, Sh2.1 and Sh4.3), which was demonstrated by Western blotting assay. (c and d) After treated with indicated dose of γ -irradiation, cells were collected at time point of 48 hours to detect PARP cleavage (a marker of cell in apoptosis) by Western blotting assay. As compared to parental and vector control cells, Axl silenced stable clones had significantly more PARP cleavage. (e and f) With the same γ -irradiation treatments, cells in sub-G(1) stage (a status reflects cell in apoptosis) were sorted and counted by PI-FACS analysis. Compared to controls, Axl silenced clones had more cells in sub-G(1) stage (* $p < 0.01$). (g) When treated with serum starvation for 24 or 48 hours, Panc-28 showed a significant PARP cleavage in a time point of 48 hours. However, Axl silenced clones AS4.9 and AS4.10 had shifted the PARP cleavage to an earlier time point of 24 hours.



Axl silencing decreases the invasion capability of PDA cells.

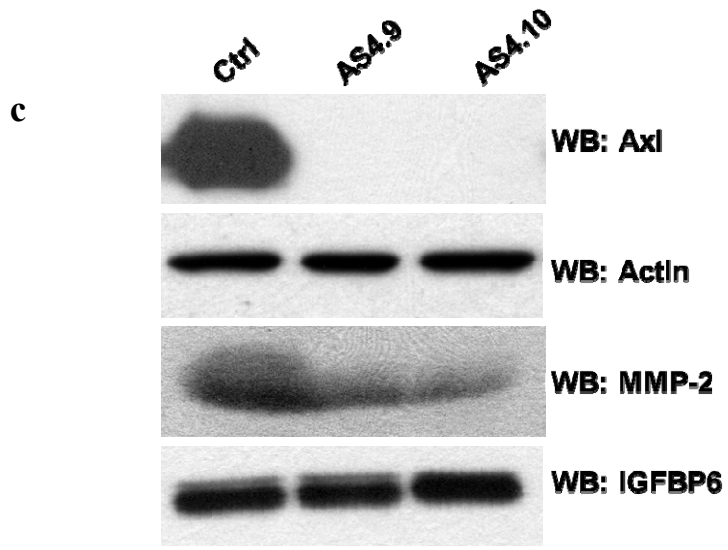
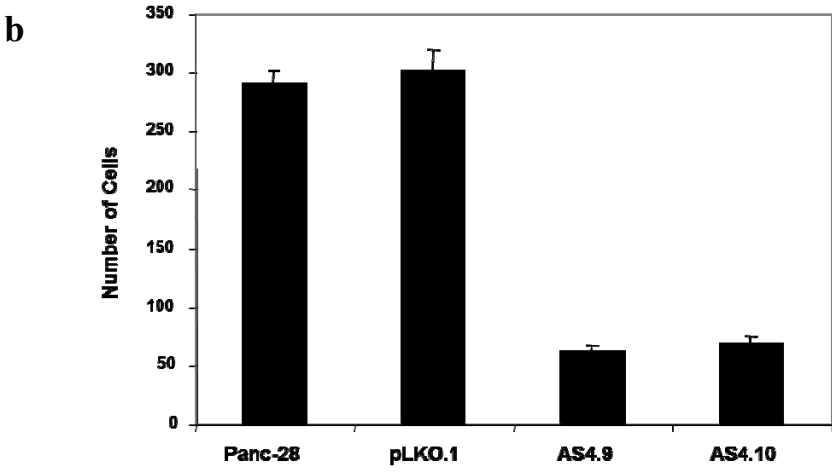
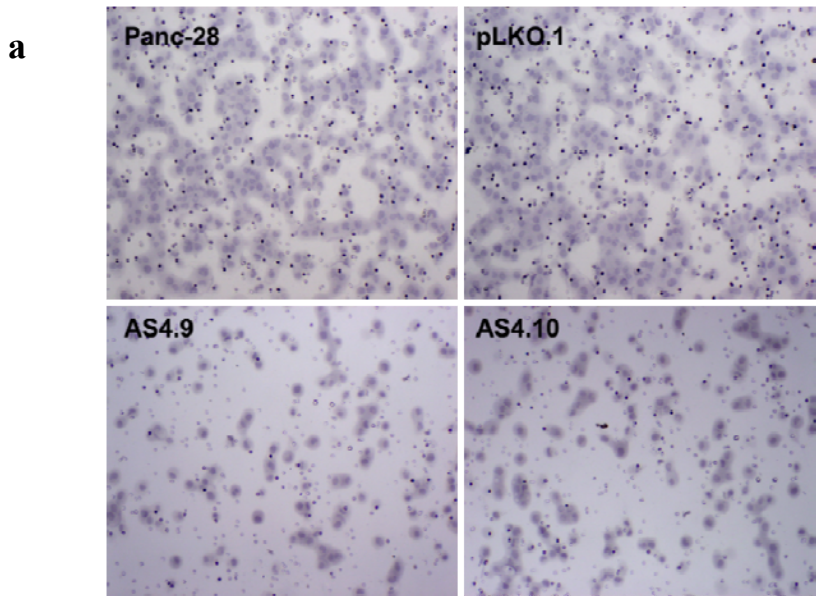
Previous works showed that dominant negative form of Axl can down regulate glioma cell invasion ability¹¹⁸. Axl could activate NF-kB pathway, and enhance expression of matrix metalloproteinase 9 (MMP-9), which are related to cell invasion capability¹²². Our tissue microarray data, which showed that Axl-high PDA patients had higher frequency of distant metastases than those patient with Axl-low tumors, we anticipated that overexpressed Axl may contribute to PDA progression by increasing the migration and invasion potential of pancreatic cancer cells. To test above hypothesis, a Matrigel in vitro invasion assay was performed. The number of cells invaded through themembrane was counted and compared between Axl silenced cell lines (AS4.9, AS4.10), and control cell lines (Panc-28, pLKO.1-Panc-28). As shown in Fig. 7b, the average number of invaded cells for AS4.9, and AS4.10 were 55, and 62 respectively per microscope field, which were significantly lower than 286 for Panc-28, or 301 for pLKO.1-Panc-28 (P<0.01).

Matrix metalloproteinase-2 (MMP-2) facilitates the cell invasion by digesting off the matrix collagen around cells²⁵³. Increased MMP-2 expression was often observed in cancer^{254 253}. To test whether Axl regulate the MMP-2 levels, the secreted proteins were collected and concentrated as described in materials and methods. Western blotting was performed using antibody specific to MMP2, and IGFBP6^{234, 235} which served as protein loading control. As shown in Fig. 7c, the proteins levels of MMP2, but not loading control IGFBP6 was lower in Axl silenced AS4.9 and AS4.10 cells, when compared to

control pLKO.1-Panc-28 cells. Above results indicated that Axl may enhance cancer cell invasion capability by increasing secreted MMP-2 levels.

Figure 7. Axl silencing reduced the invasion potential of PDA cells.

(a) Representative micrographs were selected from 4 cell lines used in in vitro Matrigel invasion assay. The number of cells invaded to trans-well membrane was lower in Axl silenced stable clones AS4.9 and AS4.10 than the controls Panc-28 and pLKO.1 clone. (b) The cells invading into trans-well membrane were counted under a microscope in 5 predetermined fields at $\times 200$ magnification. Each sample was assayed in triplicate, and assays were repeated at least twice. The average number of cells invaded through the membrane was plotted. A significant decrease of invaded cells in AS4.9 and AS4.10 clones was observed ($*P < 0.01$). (c) Axl silencing specifically down regulate MMP-2 expression. MMP-2 secreted into medium was concentrated by Millipore centrifugal filter device and detected by Western blotting assay. Comparing to panc-28, Axl silenced AS4.9 and AS4.10 has less MMP-2. Western blotting assay on IGFBP6 demonstrated that equal amount of total secreted protein was used in assay. Here, the Axl expression in cells used for collecting MMP-2 was also detected by Western blotting. Equal actin levels indicated that all cell pellets has equal amount of total protein.



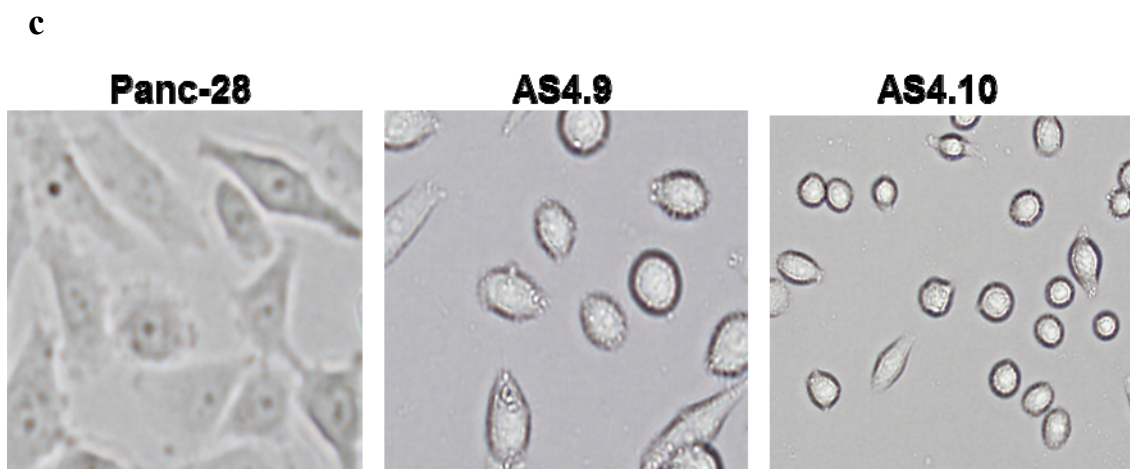
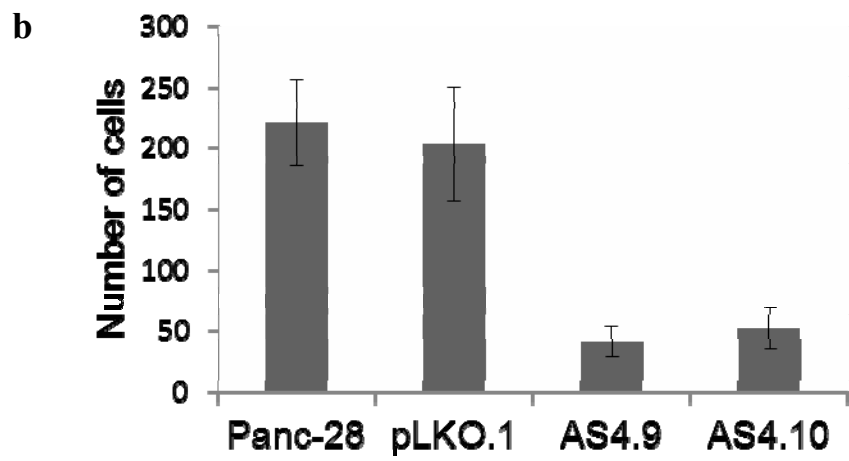
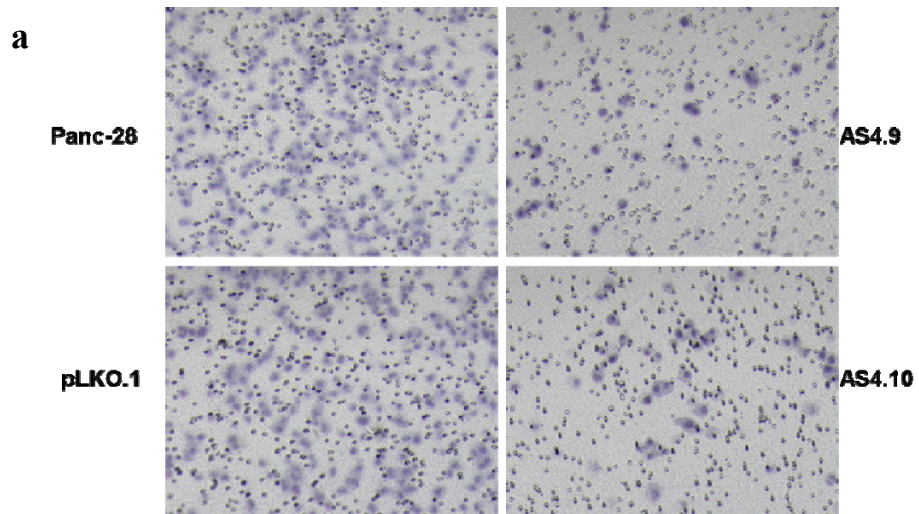
Axl silencing decreased the migration ability of PDA cells

Since Axl overexpression correlates with distant metastasis in patients with PDA and Axl increases invasion capability, we examined the probability that Axl can increase cancer cell migration ability. To test the effects of Axl on cancer cell migration, an in vitro migration assay were conducted. The cells migrated through membrane were photographed, and the number of migrated cells were counted. As shown in the representative microscope field of each cell line tested (Fig.7a), Axl silenced AS4.9, AS4.10 cell lines had much fewer cells migrated through membrane, when compared with control cell lines pLKO.1-Panc-28, and Panc-28. Migration assay was repeated three times. The average number of cells on each preselected microscope was summarized in Fig. 8b. They were 221 for Panc-28, 203 for pLKO.1-Panc-28, 42 for AS4.9, and 51 for AS4.10. The number of migrated cells in Axl silenced cell lines was lower than the control cells ($p < 0.01$).

When cells in migrating, filopodial extensions were formed in front ends. So the morphology of migrating cells displayed a polar spindled shape²⁵⁵⁻²⁵⁷. We expected that Axl silencing may change cell morphology. As shown in Fig.7c, control cells pLKO.1-Panc-28 had polarized spindle shape. However, Axl silenced AS4.9, AS4.10 displayed a typical depolarized round shape. The morphological change in Axl silenced cells may imply less formation of filopodial extensions. In conclusion, Axl silencing can make pancreatic cancer cells lose migration ability.

Figure 8. Axl silencing in PDA cells caused less migration potential.

(a) Representative micrographs of *in vitro* migration assay showed that Axl silenced cells AS4.9 and AS4.10 had less migrated cells than controls Panc-28 and vector alone, pLKO.1 cells. Here 2% FBS was served as chemo-attractant for cell migration. (b) Migrated cells in 5 predetermined fields of each assay were counted under microscope. The average number of cells in each field were calculated. As summarized in this figure, Axl silenced cells AS4.9 and AS4.10 had significantly lower number of migrated cells ($p < 0.01$). (c) Panc-28 cells displayed a spindle shape; whereas, AS4.9 and AS4.10 cells displayed a spherical shape, indicating Axl silenced cells has less filopodial extensions.

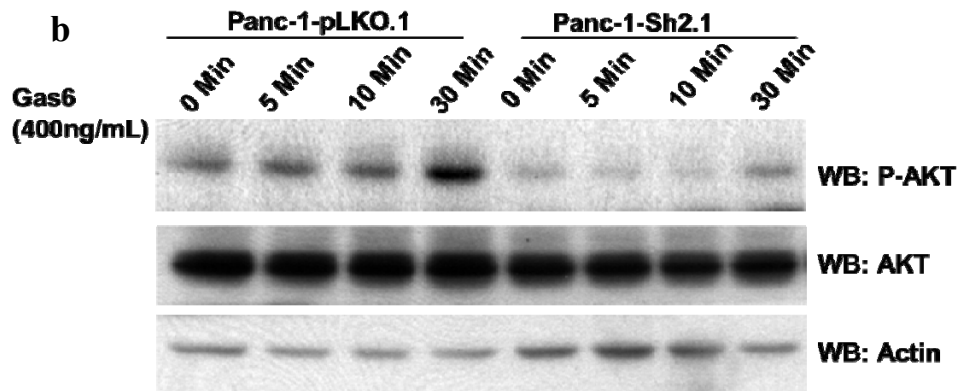
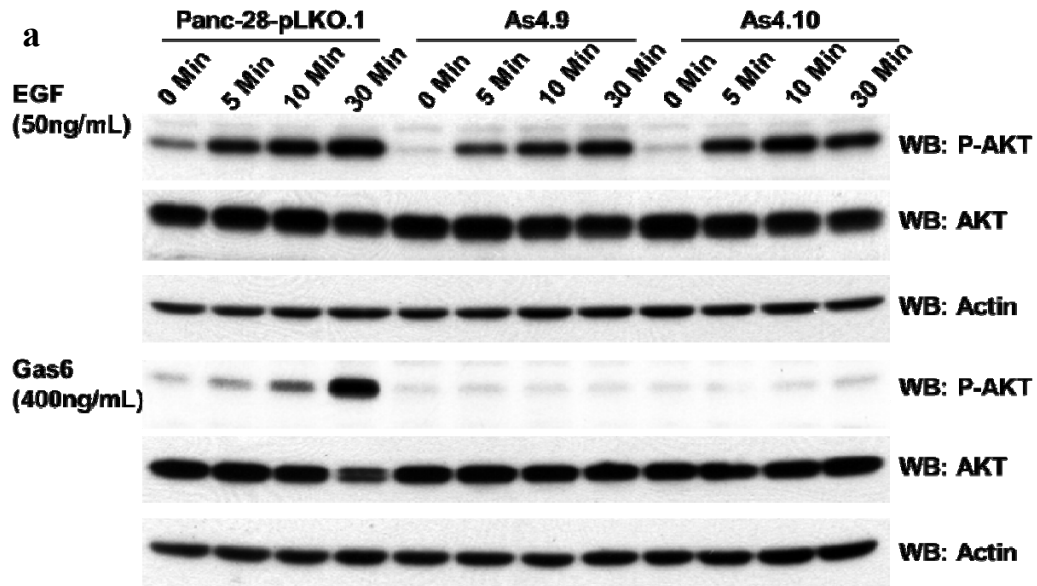


Axl silencing abolished basal and Gas6 activated Akt activation in PDA Cells.

Akt signaling pathway played important roles in cell survival and cell transformation. Activated receptor tyrosine kinase, like EGFR can recruit and activate phosphoinositide 3-kinase (PI3-K), which further phosphorylate and activate a serine/threonine protein kinase Akt^{31, 258-262}. As a receptor tyrosine kinase, Axl may also activate Akt. To test whether Axl silencing can abolish Gas6 stimulated Akt activation. Axl silenced AS4.9, AS4.10 as well as controls pLKO.1-Panc-28 were grown to 80% confluence, and then changed to serum free medium for overnight. Finally, cells were treated with 400 ng/mL of human recombinant Gas6 (R&D systems Co. Cat#885-GS) for 5, 10, or 30 minutes. In parallel, another set of samples treated with 50ng/mL EGF served as positive control. As shown in Fig.9a, after Gas6 stimuli, the levels of phospho-Akt increased dramatically in control pLKO.1-Panc-28 cells, but not in Axl silenced AS4.9, and AS4.10 cells. However, as positive control, EGF could stimulate Akt phosphorylation in all cells. Comparing the phospho-Akt levels in samples (lanes labeled as 0 min) without Gas6, or EGF stimuli, the basal levels of phospho-Akt in Axl silenced AS4.9, AS4.10 cells were lower than control pLKO.1-Panc-28 cells. Similar results were observed in another PDA cell line in experiment (Fig. 9b). Under same condition of Gas6 stimuli, Panc-1 cells had more basal and stimulated phospho-Akt than Axl silenced sh2.1 cells. The Akt activation mediated by Gas6/Axl signaling further supported that Axl can promote cell survival and invasion which in part may be due to activation of Akt.

Figure 9. Axl silencing abolished Gas6 mediated Akt activation.

(a) After serum starvation for overnight, vector alone cell Panc-28-pLKO.1, and Axl silenced Panc-28 cells AS4.9, AS4.10 were treated with either EGF (50ng/mL) or Gas6 (400 ng/mL) for the indicated time. After stimulation, phospho-Akt and total Akt in cells were detected by Western blotting assay. EGF activated Akt in both Axl silenced Panc-28 cells and Panc-28-pLKO.1 cell; However, Gas6 stimulated Akt only control Panc-28-pLKO.1 cell, but not in Axl silenced Panc-28 AS4.9 and AS4.10 cells. The basal levels of phospho-Akt in AS4.9 and AS4.10 cells were also lower than Panc-28-pLKO.1 cell (lanes with 0 min of treatment). The total Akt levels are similar in all cells, either treated with EGF, or Gas6. (b) Similar phospho-Akt pattern was observed in panc-1 cells, where Axl silencing (cell Sh2.1) also abolished Gas6 stimulated Akt activation.

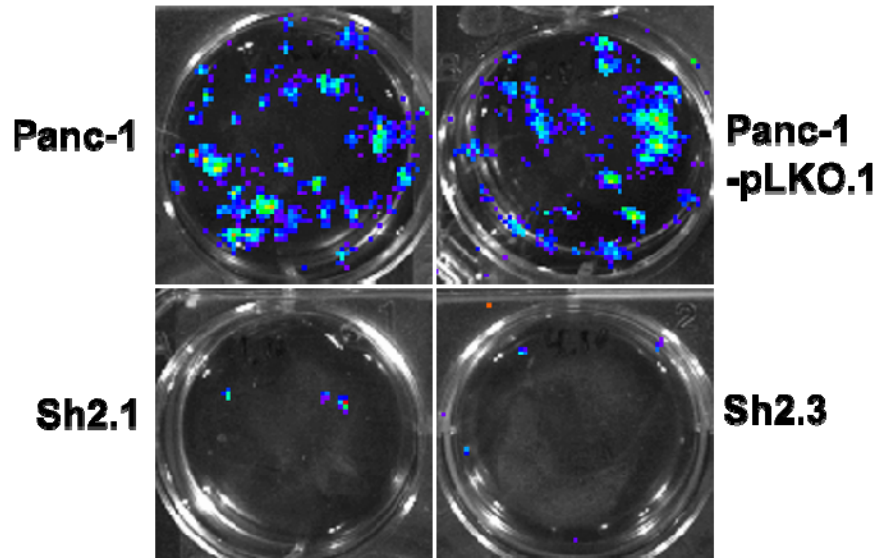


Axl silencing diminished basal NF-κB activity

Transcription factor, nuclear factor of κB light chain (NF-κB), played important roles in cell transformation, survival, migration, invasion and tumor metastasis^{263 264 265}. NF-κB is constitutively activated in many cancers, including pancreatic cancer²⁶⁶. NF-κB could be activated by Akt signaling²⁶⁵. Axl silencing down regulated Akt signaling, so NF-κB activity was expected to be down regulated as well. To test whether Axl affect NF-κB activity, a luciferase assay was performed. The intensity of luminescence recorded by IVIS bioluminescence system showed that luciferase activity was hardly detectable in Axl silenced Sh2.1, and Sh4.3 cells. However, the corresponding control cells, Panc-1 and Panc-1-pLKO.1, had relative strong luciferase activity (Fig. 10). Above results demonstrated that Axl could regulate the NF-κB activity. Axl silencing reduced the constitutive NF-κB activity in PDA cells.

Figure 10. Axl silencing in PDA cells reduced basal NF- κ B activity.

NF- κ B-Luc-reporter plasmids were transfected to Panc-1, Panc-1-pLKO.1(vector alone control), and Axl silenced Sh2.1, Sh2.3 cells. After adding substrate D-luciferin, bioluminescence coming from luciferase activity was recorded by IVIS bioluminescence system in same set-up. The representative photographs here showed that Panc-1, Panc-1-pLKO.1, Sh2.1, and Sh4.3 had correspondingly $3.74e+05$, $3.57e+05$, $3.78e+04$, and $8.49e+04$ photons.



Discussion

Pancreatic cancer was one of most malignant cancer³. The five-year survival rate for pancreatic cancer patients was less than 5%³. However, the molecular mechanisms of pancreatic cancer development and progression were not well known. The therapeutic options for pancreatic cancer are quite limited, and not effective²⁶⁷. Exploring new therapeutic approach or finding new therapeutic targets is urgently demanded.

Deregulated receptor tyrosine kinases received many interests for their prominent oncogenic functions and their applications in cancer therapy⁷³. Treatment with EGFR inhibitor erlotinib slightly improved clinical outcomes of pancreatic cancer patients^{268, 269}. Receptor tyrosine kinase Axl was found to has oncogenic functions on many cancers, including lung, breast, brain, colon, skin, prostate, ovary, and other cancers with solid tumors^{118, 127, 139, 248, 252, 270-274}. However, the role of Axl in pancreatic cancer is unknown.

Our tissue microarray data demonstrated that Axl was overexpressed in about 70% of patients with PDAs. Statistical analysis of Axl expression with clinicopathologic correlation revealed that Axl-high stage II PDA patients had significant shorter overall and recurrence-free survival (P=0.03 and 0.04 respectively). Axl-high stage II PDA patients also displayed a significant higher rate to have long distance metastasis (p=0.02). Our findings about the role of Axl in pancreatic cancer was corroborated by Koorstra group's research, which demonstrated that Axl overexpression was detected in 55% of pancreatic cancer patients¹²⁶. The differential overexpression rate of Axl revealed by two groups could be explained by criteria variation. Koorstra's research also demonstrated

that Axl-high patients had significant less survival rate and more lymph node metastasis¹²⁶, which was consistent with our findings.

We also checked the Axl and Gas6 protein expression in PDA cell lines and immortalized normal pancreatic ductal epithelial cell line (HPDE) by Western blotting assay. Both Axl and Gas6 overexpression were detected in 75% of cancer cell lines. Quantitative real time-PCR results also demonstrated that Axl mRNA levels were elevated in most pancreatic cancer cell lines. However, the elevated Axl mRNA levels in some PDA cell lines, like Panc-48 and Capan-2, are not consistent with the Axl protein levels. Both Panc-48 and Capan-2 have highly elevated Axl protein levels, but only slightly increased Axl mRNA levels. Such inconsistency suggests that Axl overexpression in PDA cell lines could be regulated in both mRNA and protein levels. Another inconsistency is that not all PDA cell lines with Axl overexpression have Gas6 overexpression, like CFPAC-1, Panc-3 and Mpanc-96 cell lines. In those Gas6 absent PDA cell lines, we still believe significant levels of Axl tyrosine kinase activity are maintained, because overexpressed Axl can dimerize without the assistance of Gas6⁸⁰.

To investigate the functions of Gas6/Axl signaling in pancreatic cancer, we silenced the Axl expression using a lenti-virus based shRNA expression system. After treating cells with γ -irradiation, we found that Axl silenced Panc-28 and Panc-1 cells had more Poly ADP ribose polymerase (PARP) cleavage (an apoptosis marker)^{275, 276}, and more cells in Sub-G0/G1 phase (cells in apoptosis)^{277 278}. Serum starvation also caused more PARP cleavage in Axl silenced Panc-28 cells. All together, we concluded that Axl silenced cells

are more sensitive to apoptosis stimuli, like γ -irradiation and serum starvation. In other word, Axl overexpression may contribute to pancreatic cancer cell survival.

Since Axl-high pancreatic cancer patients had higher frequency of distant metastasis, we expected that Axl may contribute to pancreatic cancer cell invasion and migration. We performed matrigel invasion and migration assay to test the invasion and migration potential of Axl silenced Panc-28 cells. As we expected, Axl silenced cells were revealed to have significant less cell invasion and migration capability. Furthermore, we demonstrated that Axl silenced Panc-28 cells turns to round shape morphology, indicating there are less filopodial extensions which is required for cell in migration²⁵⁵⁻²⁵⁷. Consistent with the lower invasion rate in Axl silenced cells, Axl silencing reduced the amount of secreted Matrix metalloproteinase-2 (MMP-2), which is involved in the cell invasion by digesting the matrix collagen surrounding cells^{253 254 279 280}.

Akt signaling was often activated by receptor tyrosine kinases like EGFR, insulin growth factor receptor (IGFR), Platelet-derived growth factor receptors (PDGFR), c-Met and other oncogenic receptors^{281 282 283 284, 285}. Activated Akt, phospho-Akt, contributes cancer cell survival, proliferation, and invasion^{286 259 31 287 288}. We checked phospho-Akt levels in Axl silenced Panc-28 and Panc-1 cells after Gas6 stimulation. As we anticipated, Axl silencing abolished Akt activation in both cell lines after Gas6 stimulation. Interestingly, the basal levels of phsopho-Akt in Axl silenced cells were also perceivably lower. One probable explanation is that constitutively expressed endogenous Gas6 activates the Axl signaling. We also checked the NF- κ B activity in Axl silenced Panc-28

cells by luciferase reporter assay. NF- κ B can be activated by Akt. After Akt phosphorylating I κ B kinase (IKK), NF- κ B will be released from inhibitory I κ B proteins and translocated to nucleus. As a transcription activator, NF- κ B will transcribe many genes that contribute to cell transformation, survival, migration, invasion and tumor metastasis^{263, 264, 265, 289}. NF- κ B is frequently activated in many solid tumors, including pancreatic cancer^{266, 290}. As we expected, Axl silenced Panc-28 cells has significant less luciferase activity, suggesting Axl silencing reduced the NF- κ B activity. However, we cannot conclude that down-regulated Akt activity is the only reason for reduced NF- κ B activity, because Axl can also activate mitogen-activated protein (MAP) kinase pathway which is able to activate NF- κ B as well^{30, 92, 291, 292}. To consolidate the conclusion that Axl silenced Panc-1 cells has less NF- κ B activity; we still need co-transfect a renilla luciferase report as an internal control. Moreover, an electrophoretic mobility shift assay (EMSA) for measuring the NF- κ B binding activity is necessary. Since MMP-2 transcription can be activated by NF- κ B²⁹³, the reduced NF- κ B activity in Axl silenced PDA cells could account for the MMP-2 down regulation.

Our research further confirmed that Axl overexpression was a common phenomenon among different type of cancers. Before our research in pancreatic cancer, Axl overexpression was observed in lung, breast, brain, colon, skin, prostate, ovary, and other cancers with solid tumors^{118, 127, 139, 248, 252, 270-274}. About the question how Axl overexpression changed the pancreatic cancer cellular behaviors, we found that Axl silencing can cause cells more sensitive to apoptosis stimuli and less cell invasion and migration capability. More than that, Koorstra's research found that Axl can contribute to

pancreatic cell transformation and anchorage independent growth. Though dominant negative form of Axl will inhibit brain tumor growth in a xenograft mouse model, Axl silencing did not significantly affect pancreatic cancer cell growth rate (data not shown)¹¹⁸. One probable reason is that the Panc-28 and Panc-1 cells in tests have a bundle of proliferation signaling. Redundancy of proliferation signaling compromises the Axl silencing. The weak effects on cancer cell proliferation may be not rare for Gas6/Axl signaling. Overexpressing Gas6 in NIH3T3 did not cause increased DNA synthesis²⁹⁴.

So far there are still many remaining questions about how Axl contribute to pancreatic cancer progression, especially the molecular mechanisms. Akt and NF- κ B activation by Gas6/Axl signaling definitely provided some potential mechanisms for Axl contributing to pancreatic cancer cell survival^{95, 295, 296}. However, we cannot ignore that Axl can also activate MAP kinase pathway, which is involved in cell survival⁹². About how Axl contribute to pancreatic cancer metastasis, one probable explanation is that Axl can regulate Epithelial-Mesenchymal Transition (EMT) by up-regulating transcription of transcriptional factors Twist, Slug, and Snail, which are required for EMT^{126, 297}. During EMT process, cancer cells will lose cell-cell adhesion and cell polarity, and convert cells to an invasion/migration status^{298, 299, 300}. To systematically elucidate the molecular mechanism for how Axl contributes to pancreatic cancer progression, microarray data for discovering genes altered by Axl will be necessary.

The most interest issue raised by our research is that Axl can be used as therapeutic target. Down-regulating Gas6/Axl signaling in pancreatic cancer will be a direction that worth

putting extensive efforts on. We are not alone in developing drugs against Axl signaling. An anti-Axl monoclonal antibody developed by Li et al was tested to be able to inhibit breast cancer and non-small cell lung cancer (NSCLC) growth in xenograft mouse model^{145, 146}. Tyrosine kinase small molecular inhibitor, R248, developed by Holland SJ et al also displayed an obvious inhibitory effect on breast tumor growth, cell invasion and tumor metastasis¹³⁵. Some small molecules originally screened for inhibiting other oncogenic tyrosine kinases, like c-Ret, c-Kit, Met, Rad51, PDGFR, Src, and Abl, were also identified as Axl inhibitors, such as Amuvatinib, Bosutinib, Foretinib and BMS-777607^{144, 270, 301-304}. To best characterize effects brought by Axl inhibitors, we will test them in a pancreatic cancer xenograft mouse model. We expect Axl specific inhibitors will prevent PDA growth in xenograft mouse model.

Pancreatic cancer is extremely resistant to chemotherapy, which can be reflected by the extremely low survival rate. Only 5% of diagnosed pancreatic cancer patients can survive more than 5 years³. Epithelial-Mesenchymal Transition (EMT) is recognized as one reason of chemotherapy resistance^{305, 306 307 308}. Reversing EMT will be a strategy to overcome chemotherapy resistance. For example, reversing EMT of non-small cell lung cancer (NSCLC) cells by overexpressing cadherin-1 obviously reduced the resistance to erlotinib, an EGFR kinase inhibitor³⁰⁹. Silencing of EMT trigger gene ZEB-1 in pancreatic cancer cells increased the cell sensitivity to a common chemotherapy drug, gemcitabine³⁰⁷. Interestingly, Axl silencing in pancreatic cancer cells significantly downregulated mRNA levels of Twist, Slug, and Snail, who can also trigger EMT process. All above analysis rationalized that inhibiting Gas6/Axl signaling would be a

good way to against chemotherapy resistance in pancreatic cancer. So Axl inhibitors could be used as an adjuvant therapy of traditional chemical or irradiation therapy for PDA patients.

In a word, our research for the first time demonstrated that Axl overexpression correlated with poor survival time and distant metastasis in PDA patients. Axl promotes cell survival under irradiation, and cell migration/invasion. Our research firstly demonstrated that Axl could be adopted for prognostic marker for PDA patients. Moreover, Axl can be used as potential therapeutic target for PDA. Developing Axl specific inhibitors will be a topic that worth large investment.

CHAPTER 4:

To investigate the role of HPK1 in Axl degradation through endocytosis pathway

Introduction

In previous charter, we showed that Axl protein levels are dramatically increased in PDA tissues and PDA cell lines. Axl overexpression contributes to PDA progression. Down-regulating Axl expression is one potential approach for PDA treatment^{115, 145}. Therefore, understanding the mechanism of regulating Axl expression is critical for developing probable therapeutic method against Axl. Receptor tyrosine kinase undergoes endosomal sorting and lysosomal degradation^{188, 193}. However, the deregulated endocytosis pathway, or mutations leading to insufficient connection with endocytosis pathway can stabilize the receptor tyrosine kinase and prolong the down-stream signaling. For example, VPS37A, a component of endosomal sorting complex required for transport 1 (ESCRT-1) is frequently deleted or down regulated in heptohepatocellular carcinoma (HCC) and ovarian cancer. VPS37A down regulation causes EGFR cytoplasmic retention and exacerbate cancer progress^{224, 310}. So far, the molecular regulations of Axl expression in both mRNA and protein levels are unclear. Little is known about Axl endosomal sorting in both normal cells and transformed cells. Since Axl plays an important role in PDA progression, it would be critical to understand the molecular mechanisms involved in the regulation of Axl expression in PDA.

Recently our lab's research found that HPK1 functions as tumor suppressor in PDA. HPK1 is expressed in normal pancreatic duct but is lost over 95% of PDAs. Restore

HPK1 expression in PDA cancer cell lines inhibits cell proliferation. HPK1 constitutively binds to HPK1-interacting protein of 55 kDa (HIP-55) and Grb2 proteins, both of which are involved in endocytosis^{193, 311-313}, suggesting HPK1 may be also involved in endocytosis pathway. Consistent with this notion that HPK1 silenced B lymphoma cells has slower IgM internalization rate²²⁷. In immune system, HPK1 functions as negative regulator of T- cell receptor (TCR) and B-Cell receptor (BCR) induced signaling, HPK1-HIP-55 complex co-localize with TCR. HIP-55 was detected to localize in early endosome²²⁶. HIP-55 promoted TCR down-modulation through endocytosis in both basal and ligand-dependent ways²²⁶. All those data suggested that HPK1 may participate in receptor mediated endocytosis. Here I expected that HPK1 plays an important role in regulating Axl protein levels through endocytosis pathway. Moreover, the molecular mechanism of how HPK1 function as tumor suppressor is unknown. Down-modulating Axl signaling could be an approach adopted by HPK1.

Results

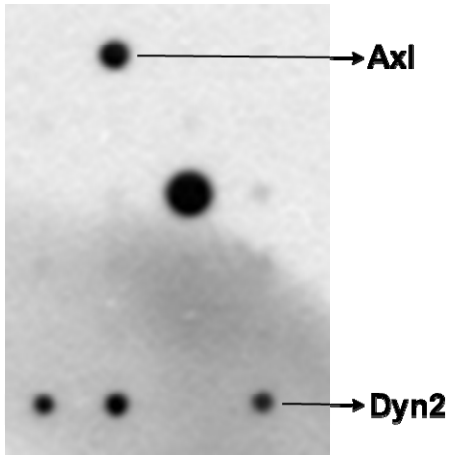
Axl physically associated with HPK1.

In order to identify the binding partners of HPK1 in PDA cells, an antibody array was performed. Flag-HPK1 in Flag-HPK1-Panc-1 #18 cell line was stabilized by MG132. Cell lysates were collected to incubate with an antibody membrane. Complexes containing antibody, antibody targeted proteins, and its associated HPK1 protein were formed on membrane. Anti-flag antibody was used to probe associated HPK1. As shown in Fig. 11a, HPK1 interacted with Axl in Panc-1 cells. In addition, our antibody array data also showed that HPK1 interacted with dynamin 2. To further confirm that Axl physically interacted with HPK1, a reciprocal co-immunoprecipitation assay was performed. 3 μ g of pCMV-Axl, 1 μ g of pCI-Flag-HPK1, or in combination were co-transfected by calcium phosphate transfection into 293T cells which were grown in Φ 6cm culture dish. The pcDNA3.1(+) empty vector were served as complement DNA, and made total amount of DNA in each transfection equal to 4 μ g. 36 hours after transfection, cell pellets were collected; and whole cell lysates were prepared. The co-immunoprecipitation with either anti-Axl, or anti-HPK1 were performed. Experimental results demonstrated that anti-Axl can pull down HPK1 protein. In reciprocal assay, anti-HPK1 can pull down Axl protein (Fig. 11b). Above experimental results demonstrated that Axl physically associated with HPK1. To test whether HPK1 interact with endogenous Axl protein, Jurkat cells were collected for co-immunoprecipitation with either anti-Axl or anti-HPK1 antibody. Jurkat cells express both Axl and HPK1. As shown in Fig. 11c, endogenous HPK1 interacted with endogenous Axl in Jurkat cells.

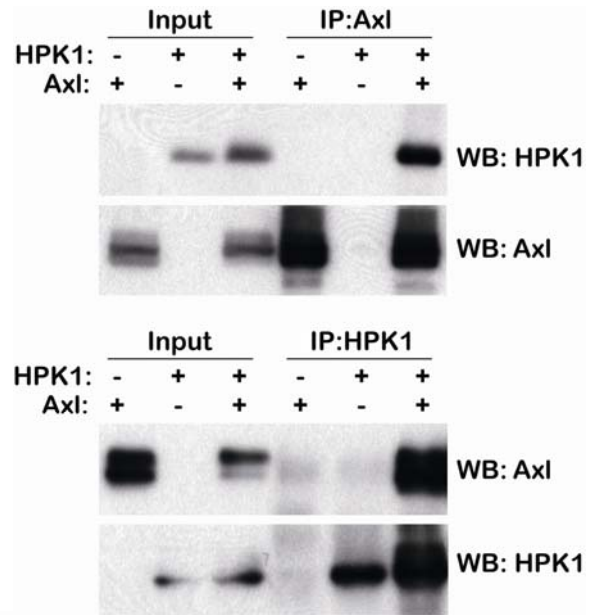
Figure 11. HPK1 physically interacted with Axl.

(a) Antibody array results showed that Axl and dynamin 2 were two binding partners of HPK1. Flag-HPK1-Panc-1 stable cell line was treated with MG132 to stabilize Flag-HPK1 protein. Then the cell lysates were collected and incubated with antibody array membrane (Hypromatrix Co.). HPK1 interacting proteins was probed and detected by anti-Flag-HRP. The dots for Axl and dynamin 2 were indicated by arrow. (b) Interaction between HPK1 and Axl was detected by reciprocal co-immunoprecipitation. In 293T cells, Axl, HPK1, or in combination, were transiently overexpressed. Cell lysates were collected for co-immunoprecipitation with either anti-Axl (upper panel) or anti-HPK1 (below panel) antibody. The immunoprecipitated proteins were detected by Western blotting using either anti-HPK1, or anti-Axl antibody. (c) Endogenous interaction between HPK1 and Axl was detected in Jurkat cells. 500 μ g of cell lysates were incubated with either anti-Axl or anti-HPK1 antibody. The co-immunoprecipitated proteins were detected by Western blotting using anti-HPK1 and anti-Axl antibodies. The amount of input was 10% of lysates used in co-immunoprecipitation assay.

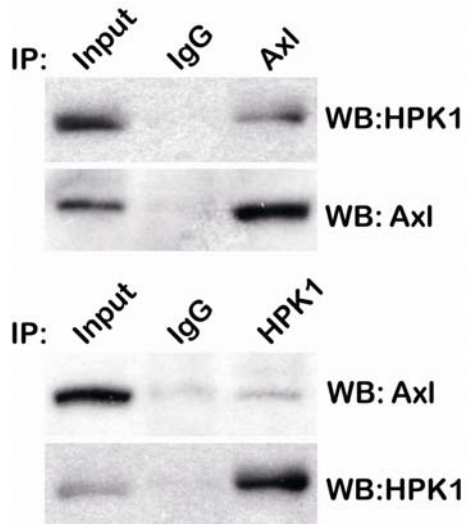
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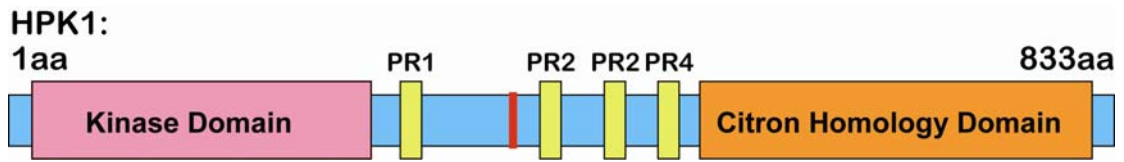
Axl mainly interacted with the C-terminal domain of HPK1

HPK1 is composed of kinase domain (KD, 1 to 274 amino acids) and C-terminal domain (CD, 275 to 833 amino acids)³¹⁴. C-terminal domain includes 4 Proline rich region and citron homology domain. The domain structure of HPK1 is shown in Fig. 12a. To identify which domain of HPK1 interact with Axl, 1 μ g each of pCI-Flag-HPK1, pcDNA-Flag-HPK1-KD, pcDNA-Flag-HPK1-CD was transfected alone, or in combination with 3 μ g of pCMV-Axl to 293T cells grown on Φ 6cm culture dish. Empty vector pcDNA3.1(+) was used as complement DNA just like above. 36 hours after transfection, cell pellets were collected for followed co-immunoprecipitation. Flag-HPK1, Flag-HPK1-KD, and Flag-HPK1-CD were immunoprecipitated by M2 beads. The co-immunoprecipitated Axl protein was detected by Western blotting using antibody specific to Axl. As shown in Fig. 12b, Axl protein was co-immunoprecipitated with Flag-HPK1-CD; a detectable amount of Axl protein was co-immunoprecipitated with Flag-HPK1; however, the Axl co-immunoprecipitated with Flag-HPK1-KD was not detectable. Above experiment indicated that Axl interacted with HPK1 mainly through C-terminal domain of HPK1.

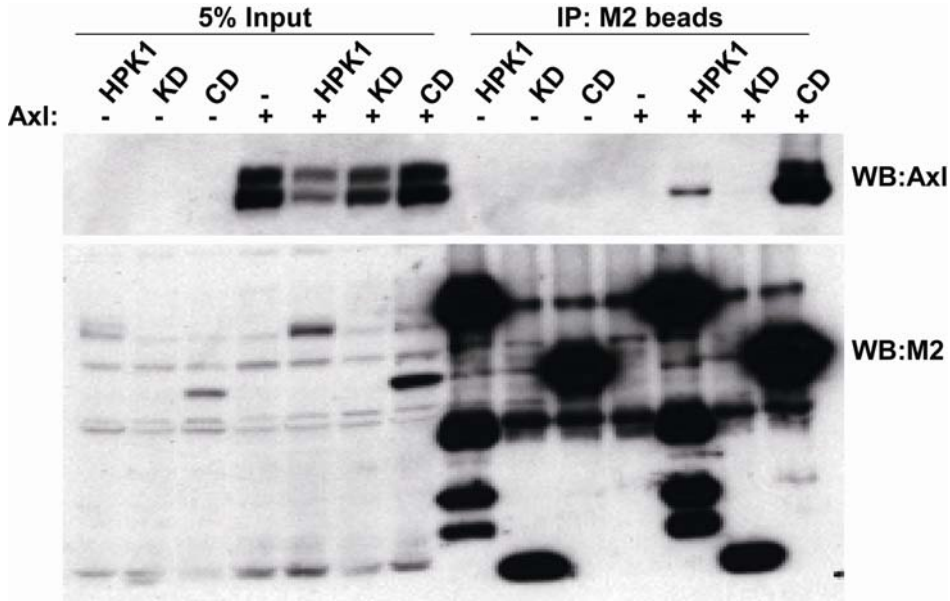
Figure 12. HPK1 C-terminal domain interacted with Axl.

(a) Schematic presentation of HPK1 structure. C-terminal domain includes four Proline rich regions and one Citron homology domain. (b) HPK1 interacted with Axl mainly through the C-terminal domain. In 293T cells, Axl expression plasmid was transfected alone, or co-transfected with Flag-HPK1, kinase domain (Flag-HPK1-KD), or C-terminal domain (Flag-HPK1-CD) expression plasmids. Cell lysates were collected for co-immunoprecipitation with M2-beads (have specific affinity for Flag peptide). Immunoprecipitated Axl was detected by Western blotting assay. HPK1-CD but not HPK1-KD pulled down a significant amount of Axl

a



b

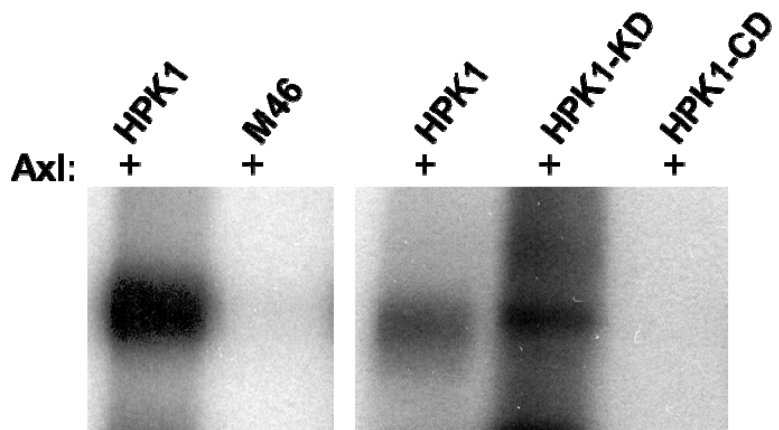


Axl can be phosphorylated by HPK1 in vitro.

Since HPK1 is a serine/ threonine kinase³¹⁴, and HPK1 physically associate with Axl. It would be interesting to examine whether HPK1 can phosphorylate Axl. A HPK1 in vitro kinase assay was performed as described by materials and methods. HPK1, M46 (kinase dead form of HPK1), HPK1-KD, HPK1-CD, as well as Axl were prepared by transfecting expression plasmids, pCI-Flag-HPK1, pcDNA-Flag-HPK1-KD, pcDNA-Flag-HPK1-CD, and pcDNA-Flag-Axl, individually into 293T cells. Protein expression levels were checked by Western blotting to ensure similar amount of HPK1, M46, HPK1-KD, and HPK1-CD were obtained for *in vitro* kinase assay. All above flag tagged exogenous protein were immunoprecipitated by M2 bead pull-down. The *in vitro* kinase assay showed that HPK1 and HPK1-KD, but not kinase dead forms, M46 and HPK1-CD, can phosphorylate Axl (Fig. 13). So we could conclude that Axl may be a substrate of HPK1.

Figure 13. Axl was *in vitro* phosphorylated by HPK1.

Flag tagged Axl, HPK1, M46 (kinase dead form of HPK1), HPK1-KD, HPK1-CD were individually expressed in 293T cells by transient transfecting corresponding expression plasmid. Each protein was precipitated by M2 beads. *In vitro* kinase was conducted by mixing Axl with one of HPK1 protein. The incorporated radioisotope labeled ATP was visualized after exposing SDS-PAGE gel to X-ray film. HPK1 and HPK1-KD but not M46 and HPK1-CD can phosphorylate Axl.



Axl expression is down regulated by HPK1 which requires HPK1 kinase activity.

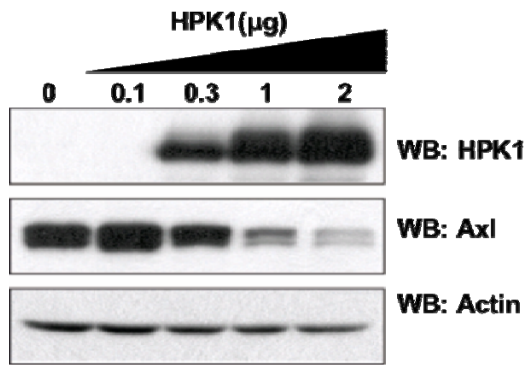
Since HPK1 interacts with and phosphorylates Axl, we hypothesized that Axl expression can be down regulated by HPK1. To test this hypothesis systematically, fixed amount of pCMV-Axl (0.3 μ g) was co-transfected with different amount pCI-Flag-HPK1, from 0, 0.1, 0.3, 1 to 2 μ g, into 293T cells, using plasmid pcDNA3.1(+) served as complement DNA. 36 hours after transfection, the 293T cells were collected. Axl and HPK1 protein levels in each sample were checked by Western blotting using anti-Axl anti-HPK1 respectively. As displayed in Fig. 14a, the protein levels of HPK1 sequentially increased as more pCI-Flag-HPK1 was transfected; correspondingly Axl protein levels gradually decreased and became hardly detectable when 2 μ g of pCI-Flag-HPK1 was co-transfected. Dose dependent decrease in Axl protein levels suggested that Axl expression was down regulated by HPK1. Similarly, treatment of Flag-HPK1-Panc-1 cells with MG132 which stabilized HPK1 protein led to marked decrease of Axl (Fig. 14b). In addition, we showed that endocytosis/lysosome inhibitor NH₄Cl can inhibit HPK1 mediated Axl degradation (Fig. 14b).

To test whether Axl down regulation relied on HPK1 kinase activity, HPK1 kinase dead form M46 was co-transfected with Axl using the same condition as HPK1 above. As shown in Fig. 14c, increasing amount of M46 did not change the Axl protein levels, suggesting kinase activity was required for Axl down regulation. In summary, Axl expression was down regulated by HPK1, and the HPK1 kinase activity was required for Axl down regulation.

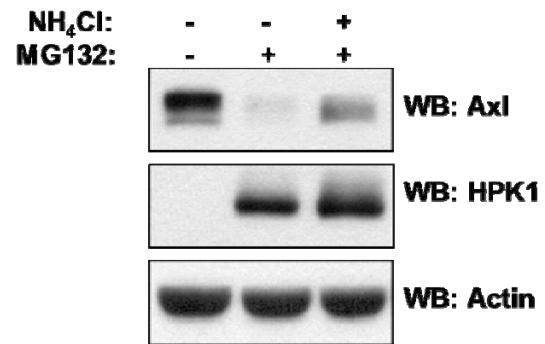
Figure 14. Axl was down regulated by HPK1 and its kinase activity was required.

(a) Overexpressed HPK1 down regulated Axl expression. In 293T cells, different amount of pCI-HPK1 (from 0, 0.1, 0.3, 1 to 2 μ g) expression plasmid was co-transfected with 0.3 μ g of pCMV-Axl expression plasmid. The protein levels of Axl and HPK1 were detected by Western blotting using anti-Axl and anti-HPK1 antibodies. HPK1 down-regulating Axl showed a dose dependent manner. (b) Axl was down regulated in Panc-1 cells. Once HPK1 protein in Flag-HPK1-Panc-1 stable clone was stabilized by M132 (1 μ M for 24 hours), Axl was not detectable any more. However, when endocytosis/lysosome inhibitor NH_4Cl was present, significant amount of Axl expression was restored. (c) Overexpression of M46, a kinase dead form of HPK1, did not down regulate Axl expression. The same experiment procedure was used as (a), except transfecting pCI-M46 here instead of pCI-HPK1.

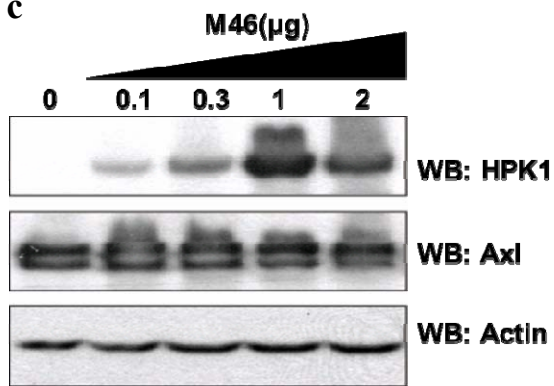
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Axl protein half-life is shortened by HPK1

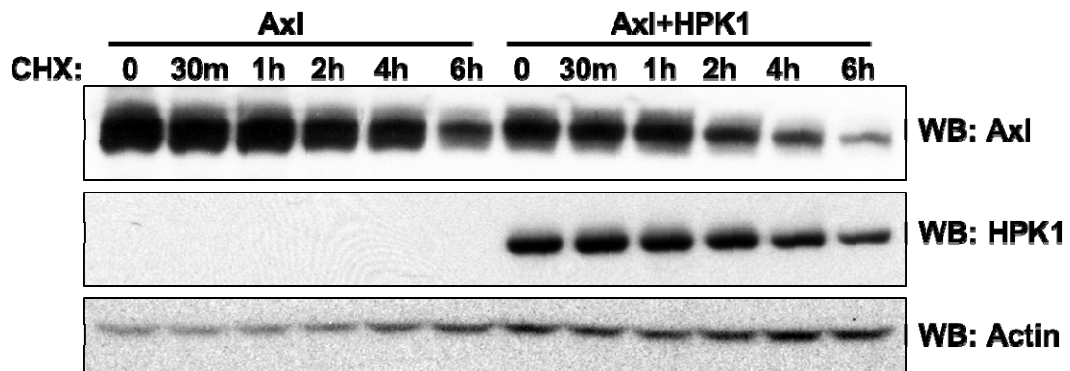
There are several ways to determine the protein expression levels, including mRNA levels, translational activity, and protein stability. Since HPK1 associated physically with and phosphorylated Axl, HPK1 down regulating Axl expression was most likely a posttranslational behavior. To test the stability of Axl protein when HPK1 was present or not, 1 μ g of pCMV-Axl was co-transfected with either 3 μ g of pcDNA-Flag-HPK1, or 3 μ g of pcDNA3.1 (complement DNA) into 293T cells in Φ 6cm culture plate. 16 hours after transfection, the cells were equally split onto 6 wells. Three hours later, CHX was added for different time period as indicated on Fig. 15a. Axl protein levels detected by Western blotting demonstrated that, when HPK1 was present, Axl protein degraded much faster (Fig. 15a). After repeating multiple times of Axl half-life assay, the relative Axl protein levels were quantified and plotted. The estimated half-life of Axl, when HPK1 was present, was about 2.4 hours which was about 2.3 hours shorter than it without the presence of HPK1 (Fig. 15b).

The mRNA levels of Axl were also measured by quantitative RT-PCR. The results showed that the presence of HPK1 did not change the Axl mRNA levels (Fig. 15c). So it could be concluded that Axl protein degradation rate was accelerated by HPK1, or in another word, Axl lost stability when HPK1 was present.

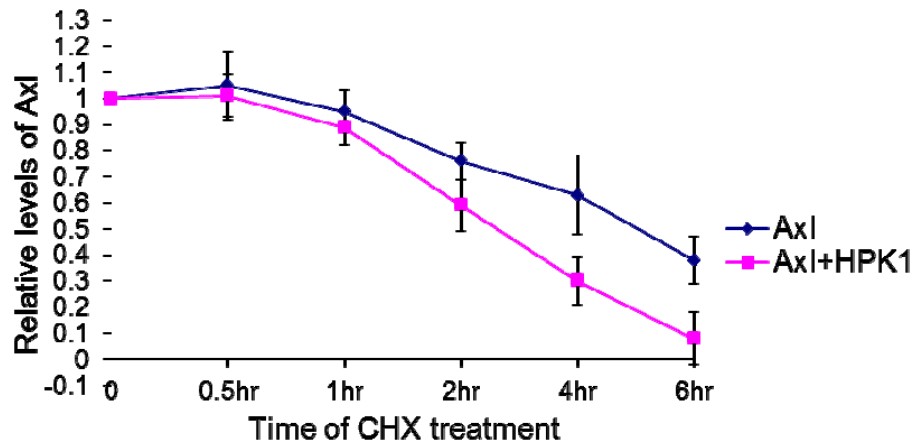
Figure 15. Axl protein half-life was shortened by HPK1.

(a) pCMV-Axl alone, or together with pCI-HPK1 was transfected into 293T cells. Then the cells were treated with 100 μ g/ mL of Cycloheximide (CHX) to inhibit protein translation. After inhibition for different time points, from 0, 0.5, 1, 2, 4 and 6 hours , cell lysates were collected for Western blotting to measure Axl protein levels. When HPK1 was present, the Axl protein levels decreased more dramatically. (b) The relative protein levels of Axl in each sample were quantified with Image J software. The average value from multiple experiments were plotted. The half-life of Axl protein was about 4.7 hours; however, when HPK1 was present, the Axl protein half-life was decreased to about 2.4 hours. (c) The relative Axl mRNA levels were not changed by HPK1. 0.3 μ g of pCMV-Axl expression plasmid was transfected alone into 293T cells or in combination with 2 μ g of pCI-HPK1. 36 hours after transfection, cells were collected for quantitative RT-PCR on Axl and RPS6. RPS6 was served as reference gene. NH₄Cl (10mM) was added 16 hours before harvesting cells.

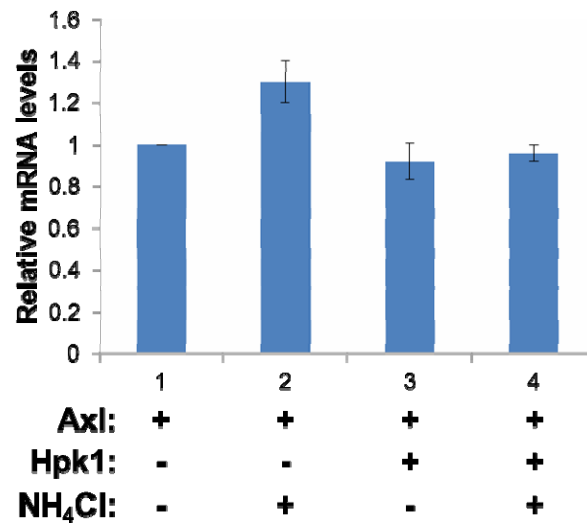
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Axl degradation caused by HPK1 is blocked by lysosome and endocytosis inhibitors.

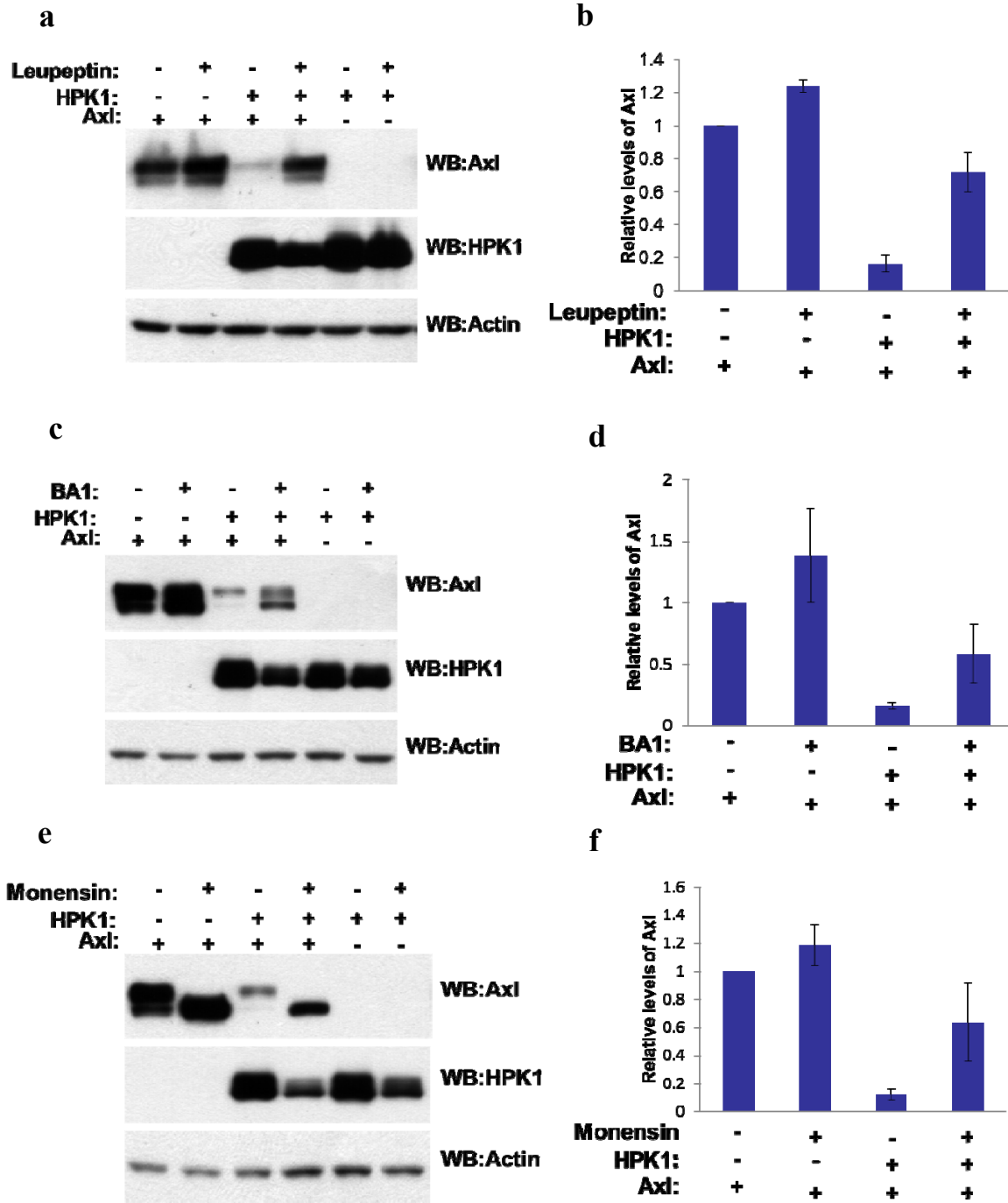
One common pathway for receptor degradation was endocytosis coupled lysosomal degradation^{315, 316}. To test whether Axl degradation by HPK1 was going through lysosome, lysosome function was disrupted by leupeptin, which can inhibit serine, cysteine and threonine proteases located in lysosome^{317, 318}. In 293T cells which were grown in 6 well plates, 0.3µg of pCMV-Axl was transfected alone or in combination with 1µg of pCI-Flag-HPK1. 8 hours after transfection, cells were treated with leupeptin to a final concentration of 100ug/mL for 16 hours. Axl, HPK1 and actin (loading control) protein levels were detected by Western blotting. Experiment was repeated for more than 3 times. The intensity of Axl and actin Western blotting signal was measured by ImageJ software (NIH). The relative levels of Axl was calculated as a ratio between Axl and actin expression levels, and the mean value was plotted on a bar figure. As shown in Fig. 16a, transient overexpression of HPK1 can down regulate Axl expression dramatically, which was consistent with the previous findings in Fig. 14a. However, when leupeptin was present, HPK1 mediated Axl degradation was inhibited and Axl protein levels were comparable to control which was transfected with pCMV-Axl alone and no leupeptin was added. When HPK1 was not overexpressed, leupeptin alone could slightly increase Axl protein levels (125% in Fig. 16b).

Since lysosomal degradation is a consequence of cargo entering endosomal sorting^{319, 320}, we expected that Axl lysosomal degradation was blocked when endocytosis pathway was interrupted. Baflomycin A1 can specifically inhibit vacuolar-type H⁺-ATPase which keeps a low pH environment in endosomal vesicles³²¹⁻³²³. In order to test whether HPK1

caused Axl degradation went through endocytosis pathway, an identical experiment was performed using Baflomycin A1. The dose of Baflomycin A1 used here was 0.1 μ M at final concentration. As shown in Fig. 16c and d, significantly amount of Axl come back when cells were treated with Baflomycin A1, even high levels of HPK1 was present. We also tested another frequently used endocytosis inhibitor Monensin. Monensin is a Na⁺/H⁺-exchanging ionophore, and can make endosome lose H⁺ gradients³²⁴. The effect of Monensin on Axl down regulation are shown in Fig. 16e and f. Here the final concentration of Monensin was 100 μ M. When Monensin was added, HPK1 could not efficiently down regulate Axl expression. In addition, Monensin also inhibited Axl glycosylation. In conclusion, HPK1 caused Axl protein degradation was blocked by either lysosome or endocytosis inhibitors, implying Axl degradation was going through endocytosis coupled lysosome.

Figure 16. Lysosome and endocytosis inhibitors blocked HPK1 caused Axl down regulation.

(a) Lysosome inhibitor Leupeptin blocked HPK1 caused Axl degradation. In 293T cells, pCMV-Axl was transfected alone or together with pCI-HPK1. Then the lysosome function was inhibited by 100 μ g/mL of leupeptin for overnight. Axl and HPK1 protein levels were detected by Western blotting using antibody specific to Axl and HPK1 respectively. (b) Relative protein levels of Axl in each sample were quantified by Image J software. Mean values of multiple experiments were calculated and plotted on a bar histogram. When leupeptin was not present, HPK1 down regulated Axl levels to 10.1% of control. However, when leupeptin was added, the Axl protein levels came back to 58% of the control. (c and d) Endocytosis inhibitor Baflomycin A1 blocked HPK1 caused Axl degradation. Same experimental procedure as described in (b and c) was adopted here. Final concentration of Baflomycin A1 was 0.1 μ M. (e and f). (e and f) Endocytosis inhibitor Monensin (100 μ M) blocked HPK1 caused Axl degradation.



HPK1 interacts with dynamin.

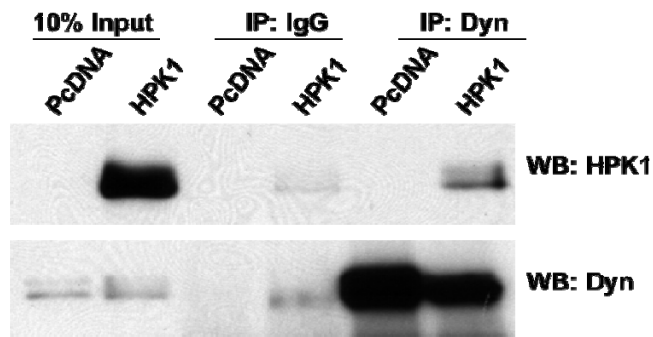
Dynamin is a member of GTPase family. The major function of dynamin is to polymerize around the neck of invaginated plasma vesicles. Through GTPase activity, Dynamin cuts off vesicles from plasma membrane^{325, 326}. Besides GTPase domain, dynamin has middle domain (MD), GTPase effect domain (GED), pleckstrin homology (PH), proline-rich domain (PRD), and SH3-like domain^{325, 326}. All those domains not only are required for normal dynamin function, but also provide docking sites for some binding partners^{325 326}. Dynamin includes 3 conserved homologous called dynamin 1, 2 and 3^{325, 326, 327}. Dynamin 1 is exclusively expressed in neuron cells. Dynamin 3 is highly expressed in testis tissue. Dynamin 2 is commonly expressed in each type of cells^{328, 329, 327}.

Previous research found that dynamin directly interacted with HIP-55, which is involved in receptor endocytosis^{225, 311, 312}. HIP-55 was firstly identified as a HPK1 interacting protein³¹³, implying that HPK1, HIP-55, and dynamin may form a protein complex.

Interestingly, our antibody array data demonstrated that dynamin 2 was a binding partner of HPK1 (Fig.11a). To further confirm that HPK1 associated with dynamin. 10 μ g of pCI-Flag-HPK1 was transfected into 293T cells which were grown in Φ 10cm culture plates. Cells transfected with 10 μ g of pcDNA3.1(+) plasmids served as negative control. 36 hours after transfection, 293T cells were collected for co-immunoprecipitation assay with either anti-dynamin antibody, or normal rabbit IgG. The immunoprecipitated HPK1 was detected by Western blotting. As shown in Fig. 17, significant amount of HPK1 was co-immunoprecipitated with anti-dynamin antibody but not normal rabbit IgG.

Figure 17. HPK1 physically bound to dynamin.

HPK1 expression plasmid pCI-HPK1 or vector control pCDNA3.1(+) were transfected into 293T cells respectively. Cell lysates were collected for co-immunoprecipitation with anti-dynamin (Dyn) antibody, using normal rabbit IgG as control. Immunoprecipitated protein was detected by Western blotting using antibody specific to HPK1 or dynamin. Anti-Dyn but not normal IgG pulled down a significant amount of HPK1.



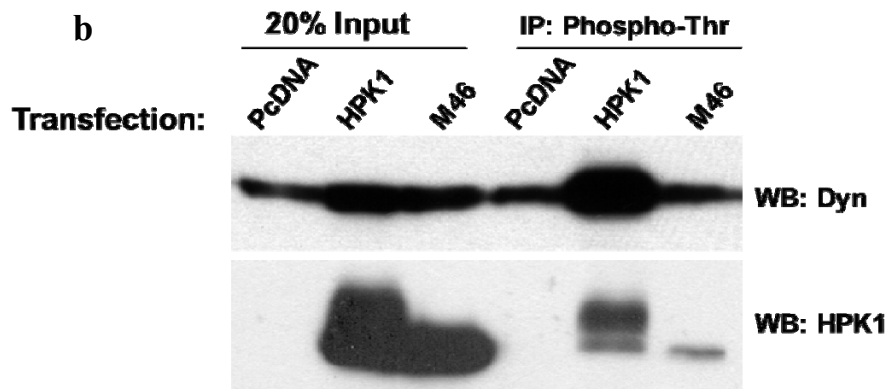
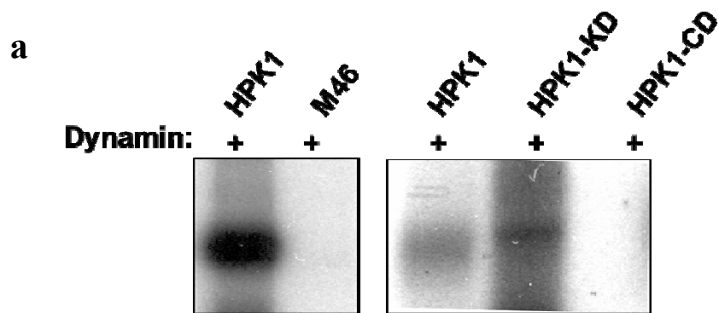
Dynamin is phosphorylated by HPK1.

In order to test whether HPK1 can phosphorylate dynamin, Protein of HPK1, kinase dead form M46, HPK1-KD, HPK1-CD was obtained as described above for *in vitro* HPK1 kinase assay with Axl as a substrate. Endogenous dynamin was collected from 293T cells by immunoprecipitation with anti-dynamin antibody. HPK1 *in vitro* kinase assay demonstrated that HPK1 and HPK1-KD, but not M46 and HPK1-CD could phosphorylate dynamin (Fig. 18a).

To test whether HPK1 can phosphorylate dynamin *in vivo*, 10 μ g of pCI-Flag-HPK1, pCI-flag-M46, or pcDNA3.1(+) (transfection control) was transiently transfected individually into 293T cells. Proteins having phospho-Thr residue(s) were immunoprecipitated by anti-phospho-Thr antibody. The amount of dynamin in immunoprecipitated protein pool was detected by Western blotting. Experiment results demonstrated that the amount of phospho-Thr-dynamin was significantly higher in 293T cells transfected with pCI-Flag-HPK1 than cells transfected with either pCI-Flag-M46, or pcDNA3.1(+) (Fig. 18b). Therefore, our data showed that dynamin was phosphorylated on Thr residue(s) by HPK1.

Figure 18. HPK1 phosphorylated dynamin (Dyn).

(a) HPK1 phosphorylated Dyn *in vitro*. An *in vitro* kinase assay was performed by mixing HPK1, M46, Kinase domain (HPK1-KD), C-terminal domain (HPK1-CD) with dynamin respectively when $^{32}\gamma$ -ATP was added. All HPK1 proteins were immunoprecipitated by M2 Beads from 293T cells with corresponding transiently overexpressed protein. Endogenous dynamin was immunoprecipitated from 293T cell lysates by anti-Dyn antibody. The results demonstrated that HPK1 and HPK1-KD can phosphorylate dynamin. (b) HPK1 phosphorylated dynamin *in vivo*. 293T cells were transfected with either pCI-HPK1, pCI-M46, or pCDNA vector control. Proteins with phosphorylation on threonine were immunoprecipitated with anti-phospho-Thr antibody. The immunoprecipitated dynamin were detected by Western blotting. When HPK1 was present, significantly more phosphorylated dynamin was co-immunoprecipitated.



Dynamin is required for Axl degradation

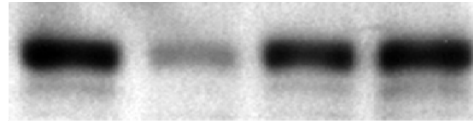
As described above, dynamin was required for receptor internalization which is the initial step for receptor entering endosomal sorting^{326, 327, 330}. Disrupting of dynamin function would be expected to block Axl internalization and further lysosomal degradation.

Dyanmin-K44A, a dominant negative form, loses affinity to GTP, and inhibits receptor internalization^{331, 332}. To test whether HPK1 caused Axl degradation is through receptor internalization or endocytosis pathway, 0.3 μ g of pCMV-Axl was co-transfected with pCI-Flag-HPK1 (0.5 μ g), dynamin-K44A (0.5, or 1 μ g), or both. 24 hours after transfection, Axl levels in cells were tested by Western blotting. As shown in Fig. 19, When dynamin-K44A was overexpressed, HPK1 mediated Axl protein degradation was completely blocked, suggesting dynamin is required for Axl internalization, and further confirmed that endocytosis pathway was involved in HPK1 mediated Axl degradation.

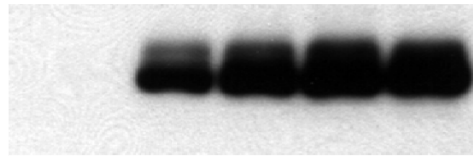
Figure 19. HPK1 mediated Axl degradation was blocked by dominant negative dynamin K44A .

In 293T cells, HPK1, Axl, or in combination with dynamin K44A was transiently overexpressed. Axl protein levels were checked with Western blotting assay. When dynamin K44A was present, HPK1 mediated Axl degradation could not be observed.

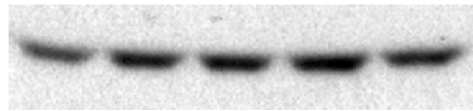
Dyn K44A(μg):	1	-	0.5	1
HPK1(0.5μg):	-	+	+	+
Axl(0.3μg):	+	+	+	+



WB: Axl



WB: HPK1



WB: Actin

Discussion

We found Axl is overexpressed in PDA tissues and cell lines. However, the molecular mechanism of Axl up-regulation is unclear. HPK1 served as tumor suppressor in pancreatic cancer¹⁶⁴. The molecular mechanism how HPK1 suppress pancreatic cancer development was unknown either. Our research demonstrated that HPK1 physically associated with Axl, which was demonstrated by an antibody array assay (Fig. 11a), and a reciprocal co-immunoprecipitation assay (Fig. 11b). Physically association between Axl and HPK1 was further confirmed by identifying that C-terminal domain of HPK1 bond to Axl. HPK1 phosphorylated Axl by an *in vitro* kinase assay. Whether HPK1 directly interact Axl and whether HPK1 itself phosphorylate Axl need to be determined with purified proteins.

Axl protein levels were found to be down-regulated by overexpressed HPK1 but not M46 in a dose dependent manner (Fig. 14). Other than to attenuate T cell receptor (TCR) signaling by phosphorylating SLP-76¹⁵¹, we are the first to demonstrate that HPK1 may attenuate Axl receptor signaling. Axl was further found to be down regulated by HPK1 through decreasing Axl protein half-life (Fig. 15a and b). However, Axl mRNA levels, reflected by quantitative real time PCR, were not altered by HPK1 (Fig. 15c). Such findings implied that phosphorylation of Axl by HPK1 may lead Axl to protein degradation system.

Here we investigated the mechanisms of Axl degradation caused by HPK1. One major way to degrade receptors, which are located on plasma membrane, is going through

endocytosis/lysosome pathway. Receptors are first internalized, and then delivered to lysosome through early and late endosomes^{333, 193, 316}. In order to test the roles of endocytosis/lysosome pathway in HPK1 induced Axl degradation, we treated cells with proteinase inhibitor leupeptin. Leupeptin inhibited HPK1 mediated Axl degradation, suggesting lysosome was involved in HPK1 mediated Axl degradation. Since receptors are delivered to lysosome through endosomal sorting, we expect that inhibiting endosome functions will also block HPK1 mediated Axl degradation. Consistent with this notion, endocytosis inhibitors bafilomycin A1, and monensin inhibited HPK1 mediated Axl degradation. The roles of endocytosis in HPK1 mediated Axl degradation was further confirmed by the fact that dominant negative dynamin-K44A completely abolished HPK1 mediated Axl degradation. Therefore, HPK1 down regulates Axl protein expression in PDA through endocytosis/lysosome pathway.

Interestingly, we found that HPK1 also physically associated with dynamin 2 in Panc-1 cells by an antibody array assay (Figure 11a). Dynamin 2 has same function as dynamin 1. The difference is that dynamin 2 is expressed in all types of cells, whereas dynamin 1 is exclusively expressed in neuron cells^{329 328}. The absence of dynamin 1 in Panc-1 cells can explain why dynamin 1 was not detected to associate with HPK1 in same antibody array assay. A co-immunoprecipitation assay with anti-dynamin 2 antibody further confirmed that HPK1 physically associate with dynamin 2. Previous research showed that dynamin directly bond to HIP-55, a molecule involved in endocytosis²²⁵. HIP-55 has been identified as a direct binding partner of HPK1^{226, 313}. So HPK1 may form a complex with HIP-55 and dynamin. HPK1 may also go through Grb2 to bind to dynamin.

Grb2 has also been identified to bind directly with either HPK1, or dynamin^{334, 335, 336}.

Both HIP-55 and Grb2 are adaptor proteins containing SH3 domains. HPK1 may directly interact with dynamin or interact with dynamin through HIP-55, or Grb2. Our *in vitro* kinase assay with immunoprecipitated HPK1 complex and dynamin 2 complex demonstrated that HPK1 can phosphorylate dynamin (Fig. 18). Moreover, we found that HPK1 can phosphorylate threonine amino acid on dynamin *in vivo* (Fig 18). Because HPK1 kinase dead form M46 could not increase the amount of phospho-Thr-dynamin, combining with the phenomenon that HPK1 physically associated with dynamin, it further suggested that dynamin may be a substrate of HPK1.

In summary, we found that HPK1 could cause Axl degradation. An endocytosis coupled lysosome pathway was required for HPK1 caused Axl degradation. In future direction, we will put more efforts on determining whether HPK1 caused Axl degradation contributes to pancreatic cancer progression. In order to keep a close mimic of physiological condition, Gas6 will be added to pLVX-HPK1-Panc-1 cells with or without Dox which induced HPK1 expression. Then the cell proliferation, migration/invasion, or resistance to apoptosis stimuli will be measured and compared. When HPK1 is present, Gas6 induced cell proliferation, survival, migration, or invasion is supposed to be less, compared to when HPK1 was absence. This part of experiment will answer the physiological significance of HPK1 mediated Axl degradation.

Though we found that HPK1 could phosphorylate Axl, and dynamin, there are still many remaining questions about molecular mechanisms of Axl degradation. So far we don't

know which site(s) of Axl is phosphorylated by HPK1. To identify the phosphorylation sites, we will purify recombinant protein of Axl. After *in vitro* kinase assay with HPK1, the phosphorylation sites on Axl will be analyzed by mass spectrometry³³⁷. The identified phosphorylation site(s) will be mutated to check whether phosphorylation is required for HPK1 caused Axl degradation. The mutants which could not be phosphorylated by HPK1 also can be used to address whether HPK1 caused Axl degradation contributes to pancreatic progression.

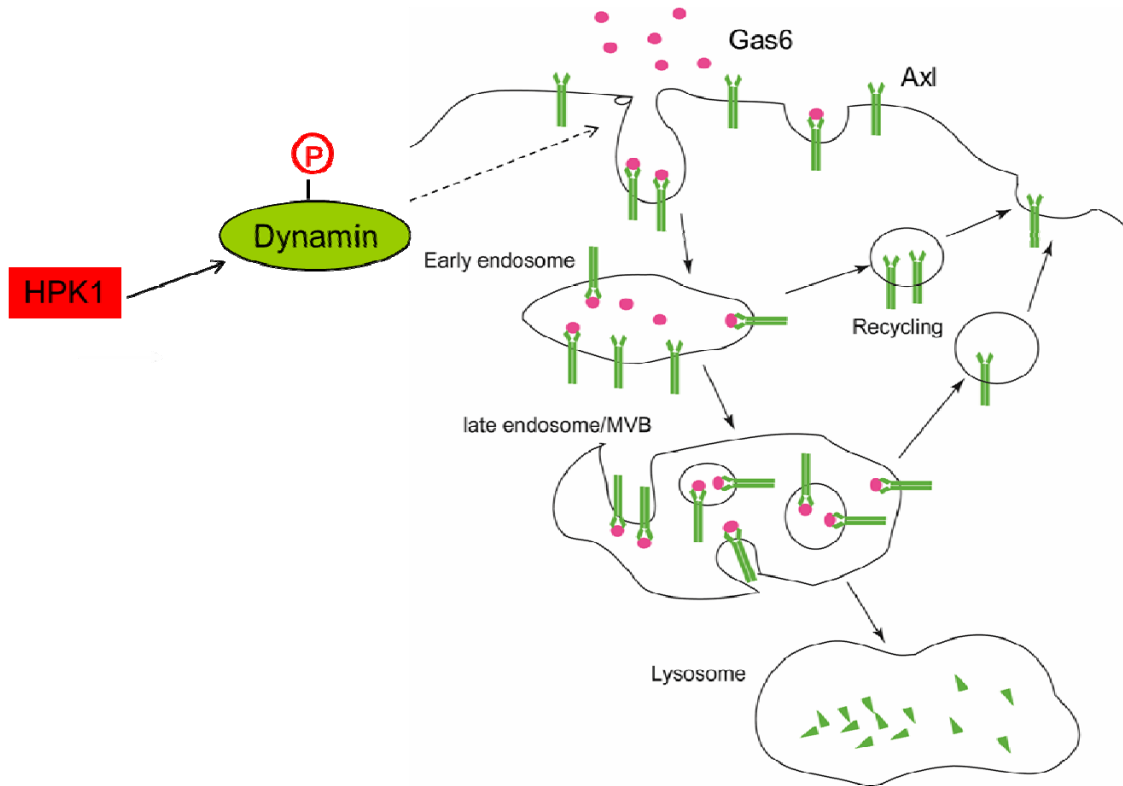
Another big question is how Axl phosphorylation by HPK1 leads to lysosomal degradation. It has been reported that Ser/Thr phosphorylation in receptor cytoplasmic tail regulates receptor internalization. Phosphorylation on Ser^{1046/7} sites is required for EGFR internalization³³⁸. Phosphorylation by p38 cause accelerated EGFR internalization^{198, 339}. Whether HPK1 regulate Axl internalization is an immediate question needing answers. For address this question, Gas6 will be labeled by rado-isotope, the Gas6 internalization rate will be measured when HPK1 is present or not. In case of EGFR degradation, ubiquitination of EGFR by Cbl E3 ligase play an important role. After EGF binding to EGFR, EGFR become dimerized and activated through trans-phosphorylation on tyrosine sites. Then Cbl is recruited through adaptor protein Grb2. Ubiquitin on EGFR provides a recognition site for ubiquitin-interacting motifs contained proteins, like Eps15, Eps15R and Epsins, which are components of endosytosis machineries^{193, 340-343}. In Human lens epithelial cells (HLEC), Gas6 treatment caused Axl degradation, accompanied with Gas6 induced Axl-Cbl interaction, and Axl ubiquitination. Gas6 caused Axl degradation was blocked by endocytosis inhibitors³⁴⁴. Whether Axl

phosphorylation by HPK1 can increase Cbl recruitment and Axl ubiquitination is yet to be determined.

Due to its unique role in vesicle scission, dynamin occupied a critical position in endocytosis³⁴⁵. Accumulated evidences revealed that dynmain polymerization is a strictly regulated process^{346, 347, 348}. Dynamin itself is a highly phosphorylated protein³⁴⁹. Phosphorylation of dynamin by PKC dramatically increases dynamin GTPase activity³⁵⁰³⁵¹. Re-phosphorylation by cdk5 assures that dynmain can be reused in next round of internalization^{352, 353}. However, the role of many other identified phosphorylation sites on dynamin and the kinase involved in dynamin phosphorylation are unknown. Here, we found dynamin can be phosphorylated by HPK1 both *in vitro* and *in vivo*. Whether and how dynamin phosphorylation by HPK1 contributes to Axl internalization will be another interesting aspect to pursue. Identification of phosphorylation sites on dynamin by HPK1 will help to address above issue. An immediate question that we can answer is whether phosphorylation by HPK1 affects dynamin GTPase activity and dynamin polymerization capability. One more interesting thing is that when cells are stimulated with EGF, dynamin was phosphorylated on some tyrosine sites³⁵⁴, which is required for EGFR internalization. Gas6 is expected to have same effects as EGF on dynamin phosphorylation. Whether HPK1 down regulate Gas6 caused dynamin phosphorylation on tyrosine sites is another interesting question to answer. In a word, those experiments will answer how HPK1 affects dynamin function to degrade Axl protein. A model proposed for HPK1 mediated Axl degradation is displayed in Fig. 20.

Figure 20. Model of HPK1 mediated Axl degradation through endocytosis/lysosome pathway.

HPK1 interacts with and phosphorylates dynamin. Phosphorylated dynamin promotes Axl internalization rate. Internalized Axl will be sequentially delivered to early endosome, late endosome, and then lysosome, where Axl is finally degraded.



Toward a more comprehensive understanding of the influence on pancreatic cancer progression by HPK1 mediated Axl degradation, we will check Axl down-stream signalings potentially affected by HPK1. Since the highly conserved activation mechanism of Akt adopted by Axl and EGFR ^{120, 229}, we expect HPK1 can also attenuate Gas6 stimulated Akt activity. Other Axl down-stream effectors, like ERK, MAPK, SRC, and PKC, will also be considered as test objects ⁸⁰. However, we may not anticipate HPK1 will bring similar impacts on all Axl down-stream effectors ^{355, 356, 357}. Those works will further answer the biological significance of HPK1 mediated Axl degradation in PDA cells.

Since stabilizing HPK1 expression in Panc-1 cells caused Axl degradation (Fig. 14b), down-regulating Gas6/Axl signaling could be one approach adopted by HPK1 to function as tumor suppressor in PDA ¹⁶⁴. The loss of HPK1 in PDA is going through proteasome pathway ¹⁶⁴. Inhibiting proteasome pathway in PDA is one potential way to down regulate Axl expression and further improve clinic outcomes of PDA patients.

In a word, our research for the first time demonstrated that Axl protein degradation could be through endocytosis/lysosome pathway. HPK1 is firstly found to down regulate Axl expression through endocytosis/lysosome pathway. Since HPK1 was found to interact with and phosphorylate dynmain, HPK1 can be a component of endocytosis pathway. Our research enriched the knowledge that HPK1 can function as a negative regulator of receptor more than activating the JNK pathway ³¹⁴, but can directly modulate receptor expression. Our research demonstrated that, except inhibiting the Axl kinase activity,

accelerating Axl degradation through endocytosis/lysosome pathway will be an interesting direction to consider for its application in PDA therapy. Moreover, how HPK1 participate in endocytosis/lysosome pathway is needed to be investigated more in the future.

Summary

Our general goal is to understand the molecular mechanism of pancreatic ductal adenocarcinoma (PDA) development, and identify potential therapeutic molecular targets. Here, we found receptor tyrosine kinase Axl is overexpressed in 70% of PDA patients and 75% of PDA cell lines. Axl overexpression correlates with shorter overall survival time of PDA patients, and severer distant metastasis. In PDA mouse model, Axl overexpression is early event happened in chronic pancreatitis. Silencing Axl by shRNA sensitizes PDA cells to apoptosis stimuli, and decreases the cell migration/invasion potential. Axl silencing abolishes ligand Gas6 stimulated Akt activation and decreases secreted MMP-2 levels and NF- κ B activity, all of which have oncogenic function in PDA tumorigenesis. Our research successfully demonstrated that Axl can be a useful prognostic marker of PDA patients and Axl can be developed as therapeutic target.

During the process of investigating the molecular mechanism of Axl up-regulation in PDA, we found hemopoietic progenitor kinase 1 (HPK1) interacts with and phosphorylates Axl. HPK1 down regulates Axl protein levels through decreasing Axl protein stability. Restoring HPK1 expression in PDA cell reduces Axl expression. Endocytosis/lysosome pathway is involved in HPK1 mediated Axl degradation. HPK1 binds to and phosphorylates dynamin, a crucial component of endocytosis pathway. Dominant negative form of dynamin completely blocks HPK1 mediated Axl degradation. Since HPK1 is degraded by proteasome pathway in PDA, besides directly inhibiting Axl kinase activity, inhibiting proteasome pathway provides one more option to decrease Axl protein levels and further to slow down PDA progression.

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Vita

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