ABERRANT GENE EXPRESSION: DIAGNOSTIC MARKERS AND THERAPEUTIC TARGETS FOR PANCREATIC CANCER

Jeran Kent Stratford

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Approved by:

Jen Jen Yeh

Channing J. Der

Gary L. Johnson

Adrienne D. Cox

Timothy C. Elston

ABSTRACT

Jeran Kent Stratford: Aberrant gene expression: diagnostic markers and therapeutic targets for pancreatic cancer
(Under the direction of Jen Jen Yeh and Channing J. Der)

Pancreatic ductal adenocarcinoma (PDAC) is an aggressive cancer and the fourth leading cause of cancer-related death in the United States. The overall median survival for patients diagnosed with PDAC is five to eight months. The poor outcome is due, in part, to a lack of disease-specific symptoms that can be used for early detection, and as such, most patients present with locally advanced or metastatic disease at the time of diagnosis. Therefore, the need for diagnostic tools is both great and urgent. Furthermore, current chemotherapies have low response rates and high toxicity, limiting their use, and there are currently no effective targeted therapies for PDAC. Therefore, a greater understanding of the underlying biology of pancreatic cancer is needed to identify tumor-specific vulnerabilities that can be therapeutically exploited.

Pancreatic cancer development is driven by genomic changes that alter gene expression. Aberrant gene expression produces changes in protein expression, which in turn may confer growth advantages to the tumor; often the tumor then develops a dependency on continued aberrant gene and protein expression. Determining how aberrant genome-wide gene expression changes affect the biology of the tumor is of paramount importance in our continued efforts to improve patient therapy. By identifying and establishing a role for the overexpressed genes in PDAC we can discover new avenues for therapeutic intervention and

potentially predict the most effective therapy for each individual and avoid therapies that may have little clinical efficacy. My research aimed to identify aberrantly expressed genes in primary tumor samples from PDAC patients and characterize the diagnostic and therapeutic value of the identified genes. The work outlined in this dissertation focuses first on identifying a prognostic signature of genes with the ability to stratify patients into high and low risk groups, and second on assessing the biological importance of overexpression of the dual-specificity protein kinase TTK for pancreatic cancer oncogenesis. Together these two investigations provide a basis for translating molecular changes in tumor biology into improved therapy for pancreatic cancer patients.

To Mindy, my ever steady companion

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LIST OF ABBREVIATIONS

AA	Amino acid
AJCC	American joint committee on cancer
APC/C	Anaphase promoting complex/cyclosome
ATCC	American type culture collection
ATP4A	ATPase H+/K+ exchanging, alpha polypeptide
BRAF	v-Raf murine sarcoma viral oncogene homolog B
BRCA2	Breast cancer type 2, early onset
BUB1B	Budding uninhibited by benzimidazoles 1 mitotic checkpoint serine/threonine kinase B
BUB3	Budding uninhibited by benzimidazoles 3
CDC20	Cell division cycle 20
CDH1	Cell division cycle 20 related 1
CDK1	Cyclin-dependent kinase 1
CI	Confidence interval
CK2	Casein kinase 2
D-Box	Destruction box motif
DMSO	Dimethyl sulfoxide
DWD	Distance weighted discrimination
ECOG	Eastern cooperative oncology group
EGFR	Epidermal growth factor receptor
FDR	False discovery rate
FOSB	FBJ murine osteosarcoma viral oncogene homolog B

FU	Fluorouracil
G2/M	Gap 2 / mitosis
GAPS	GTPase activating proteins
GEO	Gene expression omnibus
GFP	Green fluorescent protein
GTPase	Guanine triphosphatase
GSG1	Germ cell associated 1
HRAS	Harvey rat sarcoma viral oncogene homolog
IPA	Ingenuity pathway analysis
IPMN	Intraductal papillary mucinous neoplasm
IRB	Institutional review board
JHMI	Johns Hopkins Medical Institutions
KEN	Lysine-glutamate-asparagine motif
KLF6	Kruppel-like factor 6
KNN	K-nearest neighbors imputation
KRAS	Kirsten rat sarcoma viral oncogene homolog
MAD1	Mitotic arrest deficient-like 1
MAD2	Mitotic arrest deficient-like 2
MAPK	Mitogen activated protein kinase
MCC	Mitotic checkpoint complex
MCN	Mucinous cystic neoplasm
MELK	Maternal embryonic leucine zipper kinase
miRNA	Micro ribonucleic acid

MTT	Thiazolyl blue tetrazolium bromide
NEB	University of Nebraska Medical Center Rapid Autopsy Pancreatic Program
NEK6	NIMA related kinase 6
NFKBIZ	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta
NLS	Nuclear localization signal
NOC	Nocodazole
NTE	N-Terminal extension
NRAS	Neuroblastoma RAS viral (v-ras) oncogene homolog
NSU	North Shore University Health System
NW	Northwestern Memorial Hospital
OS	Overall survival
$p16^{ink4a}$	Cyclin dependent kinase 4 inhibitor A
PanIN	Pancreatic intraepithelial neoplasia
PC	Principal component
PDAC	Pancreatic ductal adenocarcinoma
PIK3CA	Phosphatidylinositol-4,5-biophosphate 3-kinase, catalytic subunit alpha
PLCε	Phospholipase C epsilon
P/S	Penicillin and streptomycin
RalGDS	Ral guanine nucleotide dissociation stimulator
RalGEF	Ral guanine nucleotide exchange factor
RISC	RNA-induced silencing complex
RNA-Seq	Whole transcription ribonucleic sequencing

SAC	Spindle assembly checkpoint
SAM	Significance analysis of microarrays
SIGLEC 11	Sialic acid binding Ig-like lectin 11
siRNA	Small/short interfering RNA
SMAD4	Mothers against decapentaplegic homolog 4
SSP	Single sample predictor
TIAM1	T-cell lymphoma invasion and metastasis 1
TMA	Tissue microarray
TNM	Tumor, node, and metastasis
TP53	Tumor protein 53
TPR	Tetratricopeptide repeat
UNC	University of North Carolina
USP16	Ubiquitin specific peptidase 16

CHAPTER I

Introduction

Pancreatic cancer remains one of the most lethal cancers having an overall five-year survival rate of less than 5% (Cooper *et al.*, 2013; Wolfgang *et al.*, 2013; Heinemann *et al.*, 2013). The annual percent change in the number of people diagnosed with pancreatic cancer has increased by 0.7% and death rates have been rising by 0.4% each year (http://seer.cancer.gov). Pancreatic cancer is the fourth leading cause of cancer-related death in the United States and is projected to become the second leading cause of cancer-related death by 2030 (Burris *et al.*, 1997; Rahib *et al.*, 2014; Siegel *et al.*, 2014).

The poor prognosis of pancreatic cancer patients, in part, stems from the silent nature and lack of recognizable clinical symptoms, absence of early detection and diagnostic tools, inherent aggressiveness and high metastatic potential of the tumor, and resistance to standard chemotherapies. Surgical resection followed by adjuvant therapy remains the only potentially curative therapy. However, more than 80% of pancreatic cancer patients present with locally advanced or metastatic disease at diagnosis and are not considered candidates for surgery (Arnal and Wade, 1995; Sohn *et al.*, 2000).

Chemotherapy is the standard of care for metastatic or locally advanced pancreatic cancer. Gemcitabine, alone or in combination, has been the standard chemotherapeutic agent for PDAC for over 15 years (Hoff *et al.*, 2013; Werner *et al.*, 2013). Recently the gemcitabine-free FOLFIRINOX protocol has also been approved to treat pancreatic cancer (Conroy *et al.*, 2011). Although overall survival is increased for patients treated with chemotherapy, the low response

rate, high toxicity, and a lack of sustained efficacy pose significant hurdles to overcome (Oken *et al.*, 1982; Maginn *et al.*, 2014). Therefore there is a dire need to develop clinically effective strategies. I propose that a better understanding of the fundamental molecular biology of pancreatic cancer will allow for development of novel therapeutic approaches and diagnostic tools.

Pancreas anatomy and function

The pancreas is the main organ responsible for maintaining glucose homeostasis and regulating protein, lipid, and carbohydrate digestion. These functions are carried out by two physiologically distinct functional components: endocrine and exocrine pancreas. Endocrine pancreas is composed of four specialized cell types that are clustered into structures called the Islets of Langerhans (Bardeesy and DePinho, 2002). Islets are found throughout the pancreas and regulate metabolism and glucose homeostasis by secretion of hormones including glucagon, insulin, and somatostatin into the blood stream produced by alpha, beta, and delta cells respectively.

Exocrine pancreas accounts for the majority of the tissue mass of the pancreas and is responsible for the production and delivery of digestive zymogens into the gastrointestinal tract. Exocrine pancreas is composed of acinar and ductal cells that are organized into a branched network. The acinar cells synthesize and secrete the zymogens into the ductal lumen to be emptied into the small intestine to aid in digestion (Figure 1).

Pancreatic ductal adenocarcinoma development

Pancreatic cancer can arise from the endocrine or the exocrine pancreas components. Endocrine tumors arise from cells in the hormone-producing Islets of Langerhans of the pancreas and account for less than 5% of the total pancreatic cancer cases (Halfdanarson *et al.*, 2008). Exocrine tumors arise from pancreatic acinar or ductal cells and account for 95% of pancreatic

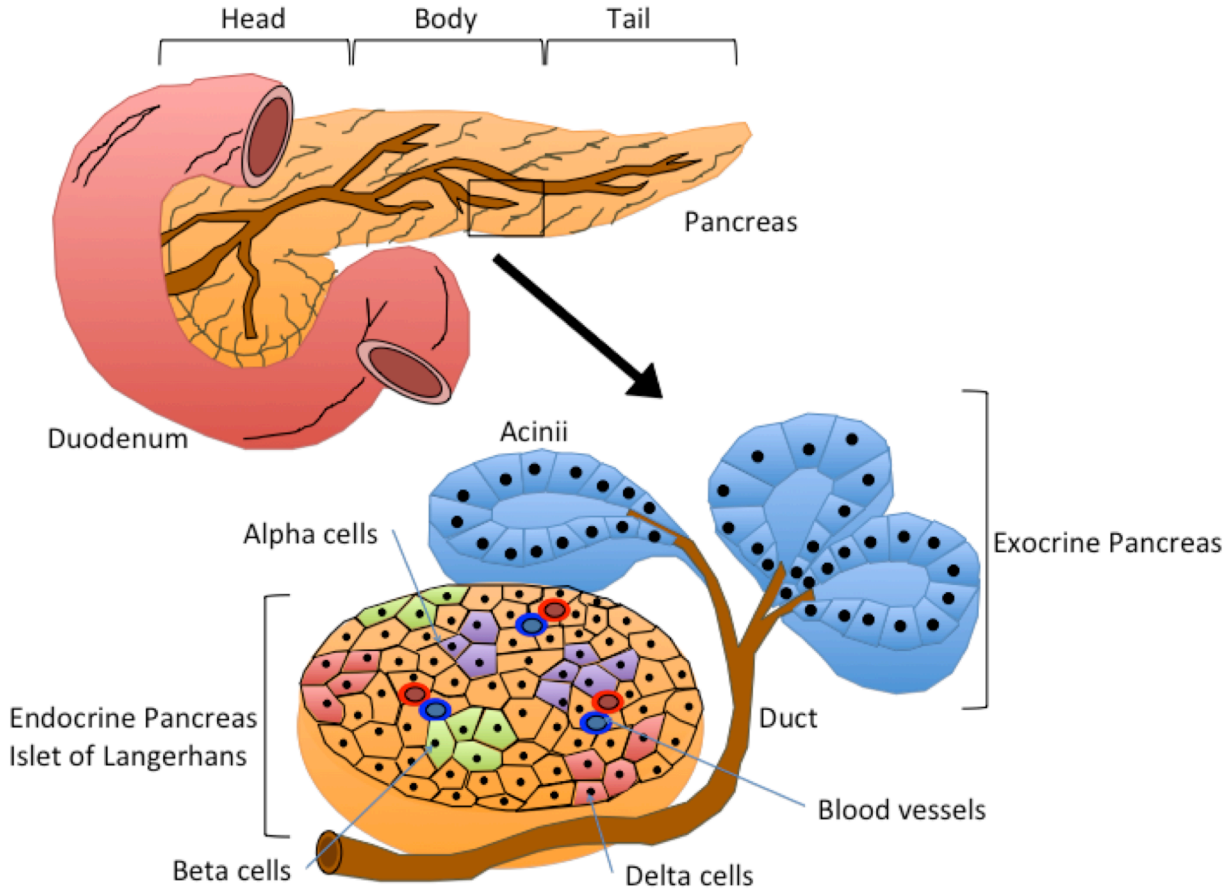

Figure 1. Anatomy of the pancreas. The pancreas is an organ situated behind the stomach and adjacent to the duodenum of the small intestine. The widest part of the pancreas is called the head, the middle section is referred to as the body, and the thinnest section is named the tail. The pancreas is composed of two functionally distinct compartments: endocrine and exocrine pancreas. Endocrine pancreas secretes hormones directly into a capillary network and is organized into Islets of Langerhans. Exocrine pancreas is composed of acinar cells that produce digestive enzymes that are secreted into ducts that feed into the small intestine to aide in digestion.

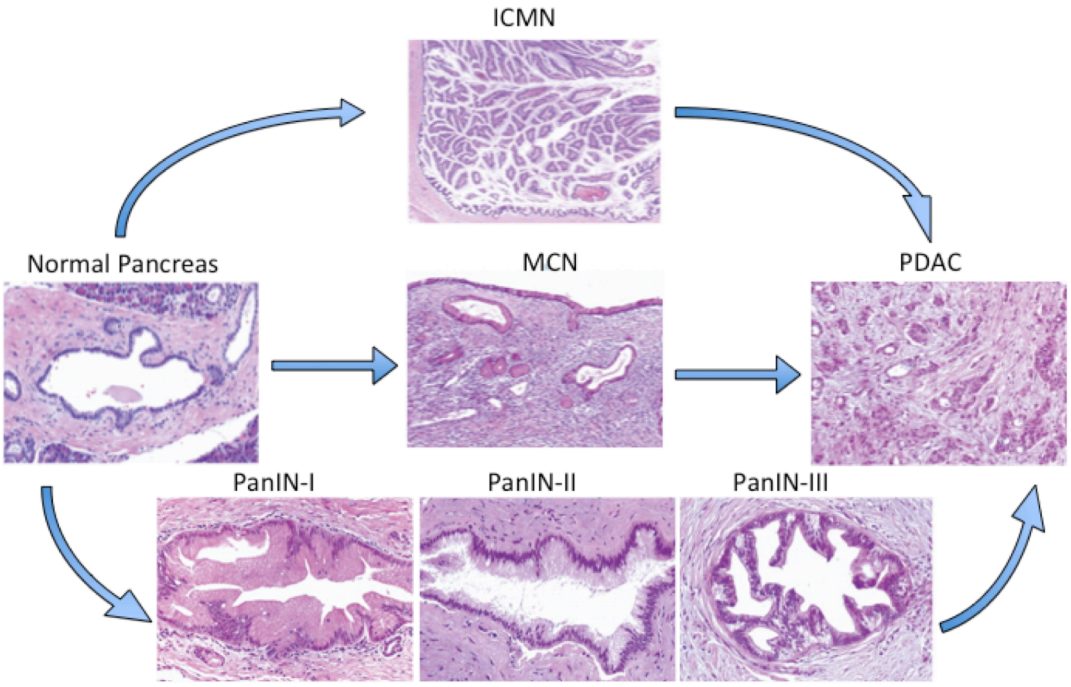

Figure 2. Precursor lesions of PDAC. Histopathological images of the normal pancreas and the three precursor lesions. Lesions progress towards pancreatic ductal adenocarcinoma in a temporal sequence. Increasing dysplasia can be observed during progression. Adapted from Hezel AF et al. (2006) Genes Dev. 20:1218-1249. PanIN: Pancreatic intraepithelial neoplasm precursor; PDAC: pancreatic ductal adenocarcinoma; MCN: mucinous cystic neoplasm; IPMN: intraductal papillary mucinous neoplasm.

tumors. Pancreatic ductal adenocarcinoma (PDAC) is the most common type of exocrine pancreatic cancer, accounting for more than 90% of total cases of exocrine pancreatic cancers (Antoniou *et al.*, 2013).

The progression from non-neoplastic cells to a malignant adenocarcinoma occurs through a series of precursor lesions characterized by increasing levels of dysplasia (Hezel *et al.*, 2006). Three premalignant, non-invasive lesions of PDAC have been identified: intraductal papillary mucinous neoplasm (IPMN), mucinous cystic neoplasm (MCN), and pancreatic intraepithelial neoplasia (PanIN) (Figure 2). Previous studies have identified increasing genomic instability in premalignant and malignant PDAC lesions.

Staging of pancreatic cancer

In 2009 the American Joint Committee on Cancer (AJCC) established an official guide for clinical staging of pancreatic cancer. The system is based on the TNM scale. The T variable describes the primary tumor size and invasion beyond the pancreas. The N variable describes the involvement of regional lymph nodes. The M variable describes the presence or absence of distant metastatic lesions (Table 1). Pancreatic cancers are staged from 0-IV; a stage 0 exhibits no evidence of a primary tumor and stage IV is any primary tumor with distant metastasis regardless of lymph node involvement (Table 2).

Pancreatic cancer has few symptoms and no early detection diagnostics. As such, over 50% of pancreatic cancers are classified as stage IV at the time of diagnosis (Seer cancer statistics review). The five-year survival rate of localized exocrine pancreas cancer patients is 26% whereas patients diagnosed with stage IV is 2%, (http://seer.cancer.gov/statfacts/html/pancreas.html, Figure 3).

Table 1. Pancreatic cancer TNM classification	
T Classification	**Primary Tumor**
T0	No evidence of primary tumor
Tis (Carcinoma *in situ*)	N/A
T1	Tumor limited to the pancreas, ≤2 cm in greatest dimension.
T2	Tumor limited to the pancreas, >2 cm in greatest dimension.
T3	Tumor extends beyond the pancreas but without involvement of the celiac axis or the superior mesenteric artery.
T4	Tumor involves the celiac axis or the superior mesenteric artery (unresectable primary tumor).
TX	Tumor cannot be assessed
N Classification	**Nodal Metastasis**
N0	No regional lymph node metastasis
N1	Regional lymph node metastasis
NX	Regional lymph nodes cannot be assessed
M Classifications	**Distant Metastasis**
M0	No distant metastasis
M1	Distant metastasis

Reproduced from Edge SB, Byrd DR, Compton CC, et al., eds.: AJCC Cancer Staging Manual. 7th ed. New York, NY: Springer, 2010, pp 241-9.

Table 2. AJCC stage groupings for pancreatic cancer				
Stage	**T Class**	**N Class**	**M Class**	**Treatment**
0	Tis	N0	M0	
IA	T1	N0	M0	Surgery Chemotherapy Chemoradiation
IB	T2	N0	M0	
IIA	T3	N0	M0	
IIB	T1	N1	M0	
	T2	N1	M0	
	T3	N1	M0	
III	T4	Any N	M0	Chemotherapy Palliative therapy
IV	Any T	Any N	M1	

Reproduced from Edge SB, Byrd DR, Compton CC, et al., eds.: AJCC Cancer Staging Manual. 7th ed. New York, NY: Springer, 2010, pp 241-9.

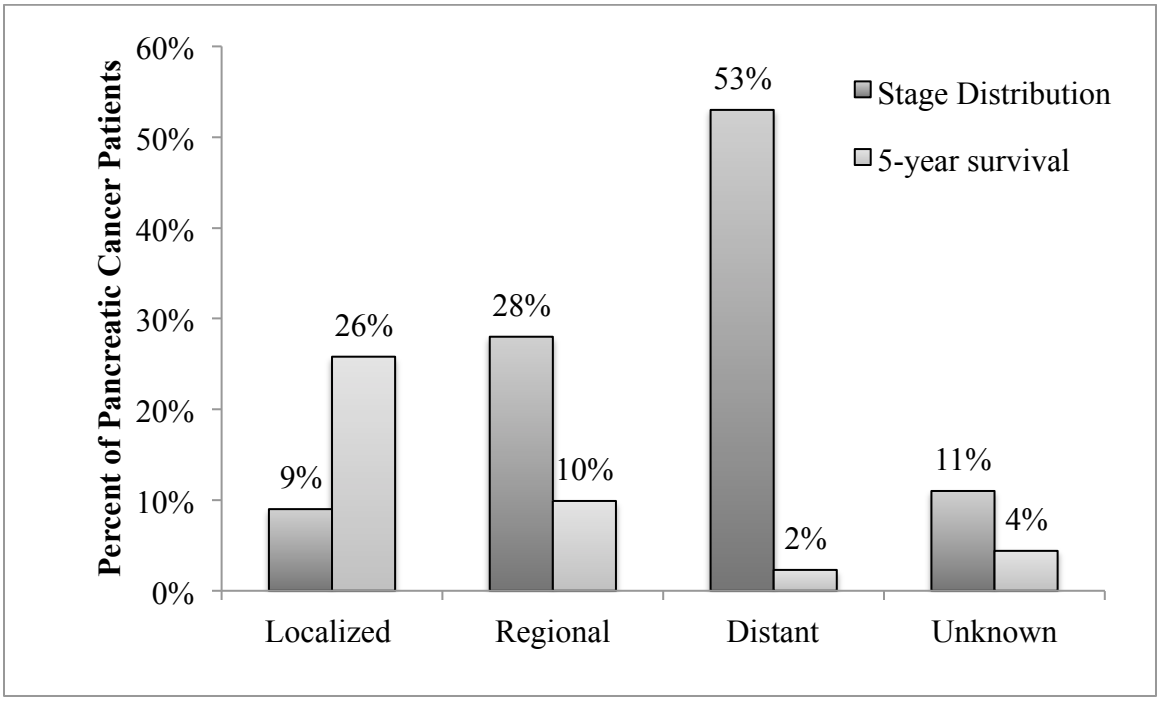

Figure 3. Early detection of pancreatic cancer associated with better 5-year survival. Patients with localized (confined to primary site) disease have the highest survival. Most patients are diagnosed with regional (regional lymph nodes involved) and distant (metastasis present) disease and have a worse prognosis. From SEER 18 2004-2010, all races, both sexes by SEER summary stage 2000.

Pancreatic ductal adenocarcinoma outcome and treatment

Pancreatic cancer is a lethal disease and is the fourth leading cause of cancer-related death in the United States (Siegel *et al.*, 2014). The overall median survival for patients diagnosed with PDAC is five to eight months. The five-year survival rate for PDAC is less than 5% (Bilimoria *et al.*, 2007). The poor outcome of PDAC patients stems from a strong tendency for tumors to invade and metastasize, resistance to chemotherapy, and a lack of disease-specific signs and symptoms that can be used for early detection. Because the symptoms of pancreatic cancer are absent through most of the development of the tumor, most patients are diagnosed at an advanced stage of PDAC and often present with tumors that have spread beyond the initial site. PDAC often invades through nerves and perineural spaces, lymphatic spaces, and small veins leading to metastasis beyond the pancreas (Cleary *et al.*, 2004).

Surgical resection followed by adjuvant chemotherapy is the only potentially curative therapy for PDAC. Patients with localized disease are considered as eligible candidates for surgical resection. The location of the tumor defines the surgical procedure. Of total cases, 20-25% of pancreatic tumors arise in the body or tail of the pancreas. To resect these tumors, a distal pancreatectomy is performed as a transection of the pancreas to the left of the superior mesenteric vein/portal vein trunk with possible resection of peripancreatic lymph nodes and potentially the spleen. This procedure carries up to a 5.9% mortality rate and a 45.3% major morbidity with the common causes of morbidity being pancreatic leak, splenic vein thrombosis, intra-abdominal abscess, and postoperative bleeding (Cuschieri *et al.*, 1996; Lillemoe *et al.*, 1999; Birkmeyer *et al.*, 2002; King *et al.*, 2008; Venkat *et al.*, 2012; Iacono *et al.*, 2013).

The majority of PDAC tumors, 60-70%, arise in the head of the pancreas. For PDAC located in the pancreas head, a pancreaticoduodenectomy, or Whipple procedure, is the procedure of choice. This procedure involves resection of the pancreatic head, duodenum, a portion of the jejunum common bile duct, gallbladder, and a partial gastrectomy but may be pylorus sparing (Figure 4). The Whipple procedure carries a 4% perioperative mortality rate and a 59% major morbidity with common causes of morbidity being infectious complications, dehydration, delayed gastric emptying, abscess formation, development of fistulas, and abdominal bleeding (Sohn *et al.*, 2000; DeOliveira, Winter, Schafer, Cunningham, Cameron, Yeo, and Clavien, 2006a; Yermilov *et al.*, 2008; SA Ahmad *et al.*, 2012).

Although surgical resection of PDAC tumors is quite intensive, there are also substantial clinical benefits. Patients who are candidates for resection have an improved median overall survival of 23 months with a 15-30% five-year survival rates (Neuhaus *et al.*, 2008; Paulson *et al.*, 2013; Rahib *et al.*, 2014; Siegel *et al.*, 2014). Balancing the potential risks and benefits from surgical resection make the decision about which patients would benefit from the procedure quite complicated.

PDAC patients who undergo surgery have the best overall survival, but peripancreatic lymphatic involvement, peripancreatic nodal involvement, presence of distant metastasis, greater than 180-degrees encasement of the superior mesenteric artery or celiac axis, unreconstructable superior mesenteric vein/portal vein occlusion, and involvement of inferior vena cava or aorta are contraindications to surgical resection and are present in 80% of patients at the time of diagnosis (Sohn *et al.*, 2000; Seufferlein *et al.*, 2012). However, a classification of stage III borderline resectable disease has recently been defined as abutment

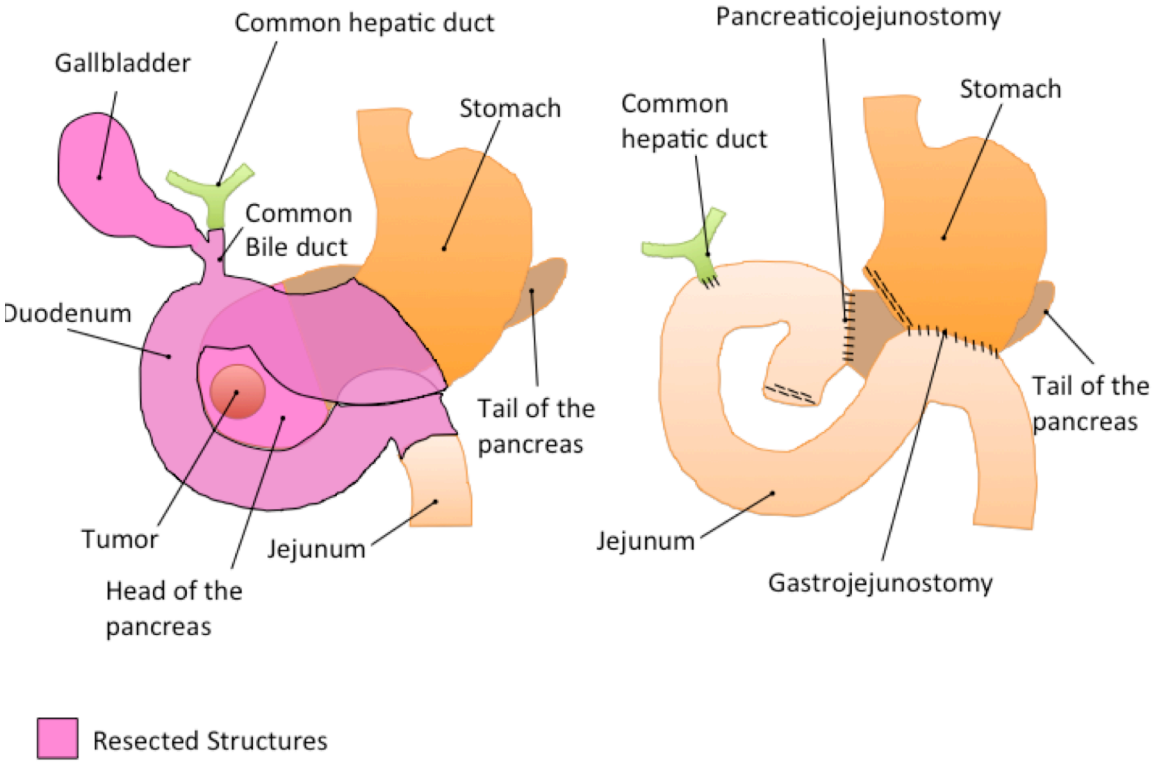

Figure 4. The Whipple procedure. Structure of the pancreas and other digestive organs before the procedure (left) and after (right) the procedure, with structures to be excised shown pink. Adapted from Freelove R and Walling AD. (2006) Am Fam Physician 73(3):485-492.

(less than 180-degree involvement) of the vasculature without any indication of metastatic disease (Callery *et al.*, 2009; Werner *et al.*, 2013; Wolfgang *et al.*, 2013). Although surgical resection of borderline resectable tumors is considered technically possible, the probability of a positive margin is high. Neoadjuvant therapy is recommended to patients with borderline resectable tumors in an effort to shrink the tumor in preparation for surgical resection. Patients whose tumors are converted from unresectable to resectable disease after neoadjuvant therapy and undergo tumor resection have comparable overall survival to primarily resectable patients (Conroy *et al.*, 2011; Cooper *et al.*, 2013; Heinemann *et al.*, 2013). Therefore, a pressing need exists to determine the patients who may respond to neoadjuvant therapy as a therapeutic strategy is established for patients with borderline resectable disease.

For the 80% of patients who are not eligible for surgery, chemotherapy is the standard of care. In 1997 gemcitabine (Gemzar) monotherapy was approved for first-line treatment of PDAC, having shown to improve overall survival compared to 5-fluorouracil (5-FU, 4.41 months for 5-FU vs. 5.4 months for gemcitabine) (Burris *et al.*, 1997; Maginn *et al.*, 2014). Gemcitabine is a nucleoside analog of cytidine and inhibits DNA synthesis. Many combination therapies of cytotoxic drugs with gemcitabine have been conducted but failed to provide increased clinical benefit. Recently a combination of gemcitabine and nanoparticle albumin-bound paclitaxel (nab-paclitaxel, Abraxane) was shown to have clinical efficacy over gemcitabine monotherapy. Paclitaxel is a stabilizing agent that prevents microtubule depolymerization to disrupt mitosis (Arnal and Wade, 1995; Bardeesy and DePinho, 2002). In a recent phase III clinical trial nab-paclitaxel in combination with gemcitabine improved response rate (7% for gemcitabine and 23% in combination), progression-free survival (3.7

months for gemcitabine alone vs. 5.5 months in combination), and overall survival (6.7 months for gemcitabine vs. 8.5 months in combination) (Halfdanarson *et al.*, 2008; Hoff *et al.*, 2013).

One of the best advancements in the treatment of unresectable PDAC is the combination therapy composed of the folic acid derivative leucovorin, 5-fluorouracil, topoisomerase I inhibitor irinotecan, and the DNA alkylating agent oxaliplatin, (FOLFIRINOX). In a phase III clinical trial FOLFIRINOX had a better response rate than gemcitabine alone (32% for FOLFIRINOX compared to 9% for gemcitabine). In addition FOLFIRINOX had a significantly higher time of progression-free survival (6.4 months for FOLFIRINOX vs. 3.3 months for gemcitabine) as well as overall survival (11.1 months for FOLFIRINOX compared to 6.8 months for gemcitabine). Although the clinical benefit of FOLFIRINOX is exciting, the use of FOLFIRINOX is limited due to greater toxicity. Patients given FOLFIRINOX experienced neutropenia, febrile neutropenia, thrombocytopenia, diarrhea, neuropathy, vomiting, and fatigue (Conroy *et al.*, 2011; Antoniou *et al.*, 2013). Therefore, FOLFIRINOX is reserved for the healthiest patients as defined as an Eastern Cooperative Oncology Group (ECOG) performance status less than 1 (Oken *et al.*, 1982; Hezel *et al.*, 2006) (Table 3). Studies are currently underway to modify the regimen to reduce toxicity and expand the population of patients who can receive this therapy. Given the complexity and the heterogeneity of PDAC, gaining a greater insight into the clinical characteristics, molecular biology, dependencies, and an understanding of the transformation and metastatic process will be required to drive future diagnostic and therapeutic developments.

Mitotic fidelity of PDAC

Genomic instability

Genomic instability and mutations are hallmarks of cancer (Hanahan and Weinberg, 2011; Siegel *et al.*, 2014). PDAC has been shown to exhibit characteristics of genomic instability including mutations (Bilimoria *et al.*, 2007; S Jones *et al.*, 2008; Biankin *et al.*, 2012), copy number variations (Harada *et al.*, 2008; Fu *et al.*, 2008), genomic rearrangements (PJ Campbell *et al.*, 2010), and microsatellite instability (Brentnall *et al.*, 1995). In addition to genetic instability, PDAC cells frequently missegregate their chromosomes (Hansel *et al.*, 2003), resulting in cellular aneuploidy, a state where cells have a chromosomal number other than 46 (Kops *et al.*, 2005). The process whereby cancer cells acquire and propagate chromosomal abnormalities is termed chromosomal instability (Gordon *et al.*, 2012). Three major mechanisms have been proposed whereby cells acquire chromosomal instability: mitotic checkpoint defects, centrosome over-duplication, or faulty sister chromatid cohesion (Duijf and Benezra, 2013).

Spindle assembly checkpoint

Mitotic checkpoints are important for regulating the proper progression through the cell cycle. Deregulation of mitotic entry and progression can lead to cellular transformation. Mitotic checkpoints exist throughout the cell cycle to halt mitotic progression until the current stage of the cell cycle is completed. Checkpoints remain active until error-free completion of the current mitotic process, thus ensuring proper timing and transition between stages and ultimately prevent the propagation of transformed cells.

Table 3. ECOG Performance Status	
Grade	**ECOG**
0	Fully active, able to carry on all pre-disease performance without restriction
1	Restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature, e.g., light house work, office work
2	Ambulatory and capable of all self-care but unable to carry out any work activities. Up and about more than 50% of waking hours
3	Capable of only limited self care, confined to bed or chair more than 50% of waking hours
4	Completely disabled, Cannot carry on any self-care. Totally confined to bed or chair
5	Dead

Reproduced from Oken MM et al., (1982) Am J Clin Oncol 5:649-655.

Of particular interest in regards to chromosomal instability is the spindle assembly checkpoint (SAC). Genetic screens in the budding yeast *Saccharomyces cerevisiae* originally identified mutants that failed to arrest in mitosis when the spindle was destroyed leading to the identification of the SAC. Later mammalian homologs were found with conserved function. The SAC regulates entry into anaphase during mitosis. SAC activation occurs in the presence of unattached kinetochores or syntelic kinetochore attachment, attachment of sister chromatids to microtubules emanating from the same spindle pole. Activation of the SAC is regulated by the activity of the T-cell tyrosine kinase (TTK, also known as Mps1 or CT96) (X Liu and Winey, 2012).

TTK

TTK is an evolutionarily-conserved protein kinase with homologs in yeast, xenopus, mice, rat, zebra fish, and drosophila (X Liu and Winey, 2012). The C-terminal kinase domain is highly conserved and kinase domain similarity is the main metric to identify homologs, yet the N-termini appear unrelated among species. In humans, TTK is an 857-amino acid (aa) 97-kDa protein. The C-terminus contains the conserved kinase domain (aa 515-794) followed by an unstructured tail (aa 794-857). Previous work revealed that the tail is required for exogenous substrate recruitment (Sun *et al.*, 2010). In addition, the extreme C-terminus contains a predicted nuclear localization signal (NLS) (aa 852-857) (X Zhang *et al.*, 2011).

Until recently the role of the much larger N-terminus of TTK has been poorly characterized. Expression of TTK N-terminus truncation mutants suggests that the N-terminus was important for subcellular localization and protein stability, but the functional domains were unknown. Newly published studies confirm the role of the N-terminus and identify tetratricopeptide repeat motifs (TPR, aa 61-192) preceded by an N-terminal extension (NTE, aa 1-60). These protein binding motifs are important for TTK kinetochore

localization (Thebault *et al.*, 2012; S Lee *et al.*, 2012; Nijenhuis *et al.*, 2013). Additionally, two LXXLL motifs (aa 65-69, 80-84), commonly referred to as nuclear receptor boxes, were found to be important for nuclear translocation at the G2/M boundary (X Zhang *et al.*, 2011).

TTK is also regulated by post-translational modifications. Ubiquitination is a process of covalently linking ubiquitin to a protein substrate. Whereas mono-ubiquitination is important for subcellular localization, poly-ubiquitination is generally a signal for protein degradation by the proteasome. TTK contains a single RXXL destruction box motif (D-Box, aa 256-259) that is recognized by the anaphase promoting complex, or cyclosome (APC/C) leading to ubiquitination of TTK and signaling for its degradation (Cui *et al.*, 2010).

Phosphorylation also regulates TTK function and activity. TTK is auto-phosphorylated as well as phosphorylated by other protein kinases. Mass spectrometry experiments have identified over 50 phosphorylation sites on TTK (J Kang *et al.*, 2007; Daub *et al.*, 2008; Guo *et al.*, 2008; Jelluma, Brenkman, McLeod, *et al.*, 2008; Oppermann *et al.*, 2009; Xu *et al.*, 2009; Tyler *et al.*, 2009; YH Yeh *et al.*, 2009; Mayya *et al.*, 2009; Kasbek *et al.*, 2010; Iliuk *et al.*, 2010; Olsen *et al.*, 2010; Rigbolt *et al.*, 2011; X Zhang *et al.*, 2011; Grosstessner-Hain *et al.*, 2011; Phanstiel *et al.*, 2011; Kettenbach *et al.*, 2011; Hegemann *et al.*, 2011; Stokes *et al.*, 2012; Franz-Wachtel *et al.*, 2012; L Zhang *et al.*, 2013; Zhou *et al.*, 2013; C-W Yeh *et al.*, 2014). Although most of these studies only identified the sites of phosphorylation, several of these sites have been functionally characterized. Phosphorylation of TTK regulates subcellular localization (T12, S15, T288, and S821), protein stability (S281, T288, and Y811) (YH Yeh *et al.*, 2009; Tyler *et al.*, 2009; J Liu *et al.*, 2013), and activation (T675, T676, and T686) (Mattison *et al.*, 2007; W Wang *et al.*, 2009). Characterizing the functional consequences of phosphorylation events on TTK and the

kinases responsible for these phosphorylation events will be required to understand the molecular role of TTK (Figure 5).

Originally identified in a screen using anti-phosphotyrosine antibodies, TTK mRNA and protein were observed in proliferating cells but not in resting cells. This study suggested that TTK may have a role in cell cycle regulation (Hogg *et al.*, 1994). Soon after, it was discovered that TTK had a conserved role regulating progression from metaphase to anaphase in the cell cycle. Since that time TTK has been shown to regulate DNA damage response through phosphorylation of CHK2 and BLM (Wei *et al.*, 2005; Leng *et al.*, 2006), chromosomal alignment by phosphorylating Borealin (Jelluma, Brenkman, McLeod, *et al.*, 2008), and post-mitotic arrest through phosphorylating p53(Huang *et al.*, 2009). TTK has also been shown to phosphorylate Smad2 and Smad3 but the downstream effects remain to be characterized (Zhu *et al.*, 2007). TTK has also been shown to localize to centrosomes (Bayliss *et al.*, 2012) and may play a similar role in centrosome duplication as observed in vertebrates (Fisk *et al.*, 2004) (Figure 6). Although the mechanism is not completely understood, TTK responds to a yet-unidentified signal produced by unattached kinetochores to prevent anaphase onset by activating the SAC. The way the SAC prevents early anaphase onset is by inhibiting APC/C.

APC/C

APC/C is a multi-subunit E3 ubiquitin ligase that ubiquitinates proteins containing a lysine-glutamate-asparagine (KEN) or D-BOX motifs. These proteins become polyubiquitinated and thereby targeted for proteosomal degradation by the 26S proteasome. APC/C requires association with cell division cycle 20 (Cdc20) or cell division cycle 20 related 1 (Cdh1) to be active (Hagting *et al.*, 2002). It is thought that Cdc20 and Cdh1 recruit

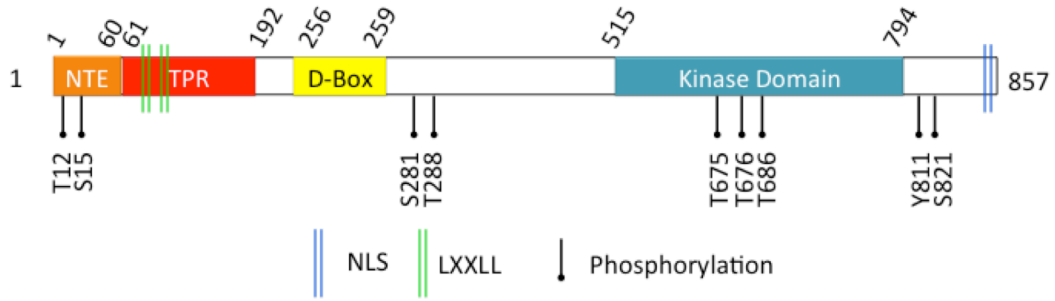

Figure 5. Domain structure of TTK. TTK protein contains a C-terminal kinase domain (blue), tandem tetratricopeptide repeats (TPR, red) preceded by an N-terminal extension (NTE, orange), and a single D-Box (yellow). TTK also contains a nuclear localization peptide sequence at the extreme C-terminus (blue lines) and tandem LXXLL motifs in the N-terminus (green lines) required for nuclear accumulation. Validated phosphorylation sites with known functions are also displayed (black lines).

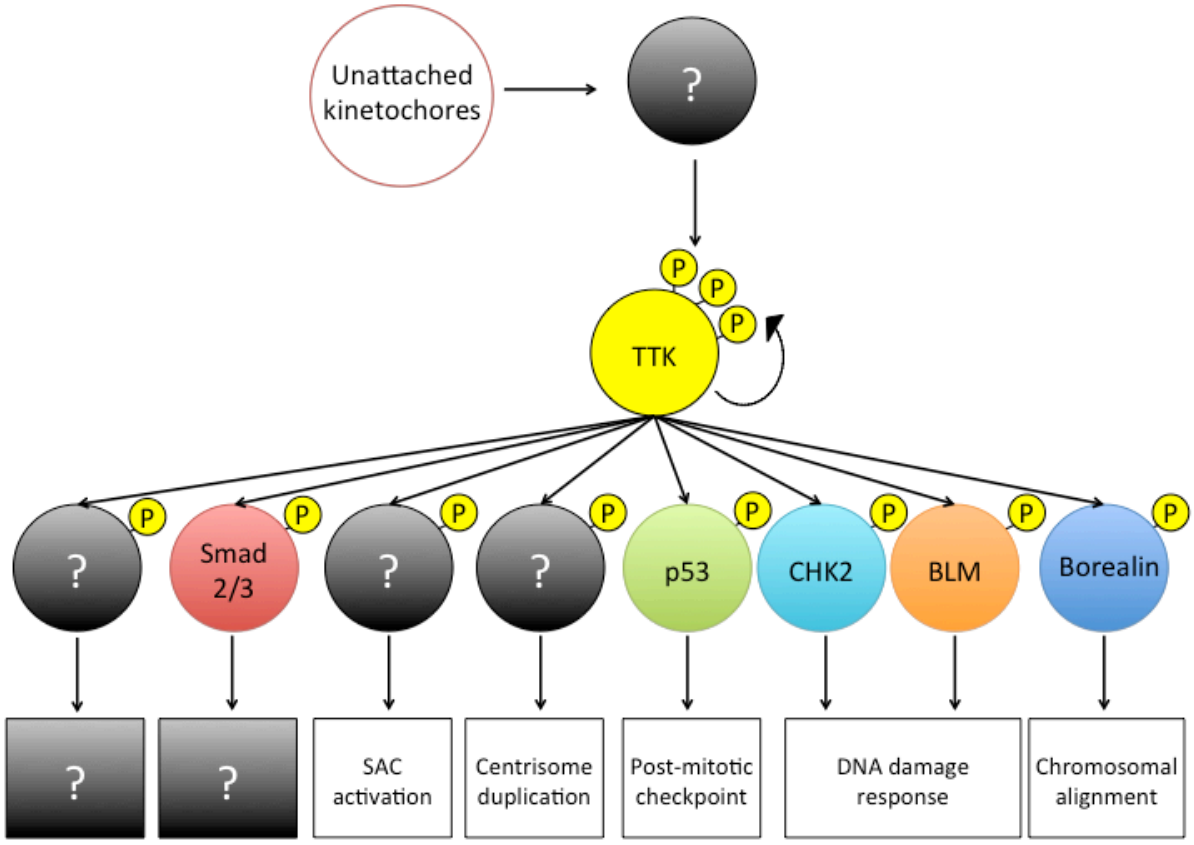

Figure 6. TTK signaling. TTK is activated downstream of unattached kinetochores by a yet-undefined mechanism. Trans- and auto-phosphorylation of T675, T676, T686 activate TTK and lead to downstream signaling. TTK phosphorylates multiple downstream substrates to mediate distinct cellular functions, yet gaps in the knowledge of the molecular signaling cascades remain unknown.

ubiquitination substrates to APC/C by binding to both partners. Cdc20 is important for regulating mitotic proteins in early mitosis, whereas APC/C binding to Cdh1 is important for the late stages of mitosis important for mitotic exit (Nasmyth, 2005; Lehman *et al.*, 2010).

APC/C^{Cdc20} has several well-characterized ubiquitination substrates among which are securin, and cyclin B. Securin is a chaperone protein responsible for inhibiting the protease activity of separase. Separase is a protease that cleaves cohesin, a multi-subunit protein that holds sister chromatids together, thus destroying the link between the sister chromatids in preparation for segregation during anaphase. Cyclin B is an activator of the cyclin-dependent kinase 1 (Cdk1), whose activities is responsible for progression through the cell cycle. Cyclin B/Cdk1 inhibits separase through direct phosphorylation. The simultaneous degradation of both securin and cyclin B by the APC/C relieve the inhibition of separase, allowing for separation of sister chromatids during anaphase (Nasmyth, 2005).

It is of vital importance that APC/C remain inactive until proper alignment and attachment of microtubules to each sister chromatid to ensure mitotic fidelity. The SAC accomplishes this function by sequestering the APC/C co-activator CDC20. Previous reports have shown that active mitotic arrest-deficient 2-like-1 (Mad2) can bind directly to CDC20, thereby preventing its association with APC/C. Unbound Mad2 exists in two conformations, an inactive "open" form (O-Mad2) and an active "closed" form (C-Mad2). As a monomer Mad2 is mostly found in the open, inactive state. C-Mad2 is found stably bound to a mitotic arrest-deficient 1-like-1 (Mad1) homodimer. Activation of O-Mad2 is thought to occur through a template model whereby O-Mad2 forms a homodimer with C-Mad2 that is bound to Mad1. Dimerization changes the conformation of O-Mad2 to C-Mad2. At this point the recently activated C-Mad2 can release the C-Mad2/Mad1 complex and bind to CDC20,

weakly capturing it. The binding of C-Mad2 and CDC20 is strengthened by the subsequent binding of budding uninhibited by benzimidazoles 1 mitotic checkpoint serine/threonine kinase B (BubR1) and budding uninhibited by benzimidazoles 3 (Bub3) to form the mitotic checkpoint complex (MCC). In the MCC, CDC20 is directly bound to C-Mad2 and BubR1, thus strengthening the sequestering effect. However, in the cytosol O-Mad2 cannot interact with the C-Mad2/Mad1 complex because the dimerization site is masked by $p31^{comet}$, which is a Mad2 binding protein that functions as a negative regulator of the SAC. $p31^{comet}$ outcompetes O-Mad2 for the C-Mad2 dimerization site due to its higher affinity. To overcome this negative regulation, the Mad1/C-Mad2/$p31^{comet}$ complex is recruited to unattached kinetochores. Although the mechanism is still under investigation, at unattached kinetochores $p31^{comet}$ disassociates from the C-Mad2/Mad1 dimer, allowing O-Mad2 to bind, convert to the closed conformation, and subsequently bind CDC20 (Figure 7).

TTK is required to recruit Mad2 to unattached kinetochores (Abrieu *et al.*, 2001; Martin-Lluesma *et al.*, 2002; Santaguida *et al.*, 2010; Maciejowski *et al.*, 2010; Sliedrecht *et al.*, 2010; Hewitt *et al.*, 2010). Although TTK does not phosphorylate Mad2, TTK has been shown to phosphorylate Mad1 *in vitro* (Hardwick *et al.*, 1996). Furthermore, catalytic inhibition of TTK prevents hyper-phosphorylation of Mad1, maintenance of C-Mad2/Mad1 complex to the kinetochore, and continual recruitment of O-Mad2 to kinetochores (Hewitt *et al.*, 2010; Tipton *et al.*, 2013). These and other findings demonstrate the essential role of TTK for sustained SAC function. Inhibition of TTK with either siRNA depletion or pharmacologic inhibition of TTK catalytic activity causes SAC silencing, resulting in an accelerated mitotic transit time and aberrant chromosomal separation. Ultimately the result of TTK inhibition is massive cellular aneuploidy that is incompatible with cell viability

(Schmidt *et al.*, 2005; Tighe *et al.*, 2008; Jelluma, Brenkman, van den Broek, *et al.*, 2008; Kwiatkowski *et al.*, 2010).

The deregulation of the cell cycle by disrupting mitotic checkpoint machinery is an attractive approach for cancer therapy (Janssen and Medema, 2011; Aarts *et al.*, 2013). The requirement of TTK in proper SAC activation makes it an attractive target to inappropriately force cancer cells through the cell cycle. To explore this hypothesis, numerous pharmacologic inhibitors have been developed to selectively inhibit TTK, including AZ3146 (Hewitt *et al.*, 2010), Reversine (Santaguida *et al.*, 2010), MPS1-IN-1, MPS1-IN-2 (Kwiatkowski *et al.*, 2010), MPS-IN-3 (Tannous *et al.*, 2013), MPS-BAY1, MPS-Bay2a (Jemaà *et al.*, 2013), NMS-P715 (Colombo *et al.*, 2010), and MPI-0479605 (Tardif *et al.*, 2011). Pharmacologic inhibition of TTK has been shown to decrease cell proliferation of breast cancer cell lines *in vitro* and *in vivo* (Daniel *et al.*, 2011; Maire *et al.*, 2013). In addition, inhibition of TTK has been shown to decrease growth of other cancer cell lines including colorectal (Kwiatkowski *et al.*, 2010; Tardif *et al.*, 2011; Jemaà *et al.*, 2013), lung (Tardif *et al.*, 2011), glioblastoma (Tannous *et al.*, 2013), cervical (Tardif *et al.*, 2011; Jemaà *et al.*, 2013), pancreas (Slee *et al.*, 2013), and malignant melanoma (Colombo *et al.*, 2010).

These studies demonstrate the importance of TTK for continued growth of PDAC and to suppress chromosomal instability. Genomic instability, of which chromosomal instability is a component, is thought to drive cancer. Identification and characterization of the driving mutations and expression profiles in PDAC may identify vulnerabilities in PDAC to explore as therapies.

Genetics of pancreatic ductal adenocarcinoma

Progression from non-invasive precursor lesions towards PDAC is believed to occur through step-wise accumulation of genetic mutations (Figure 8)

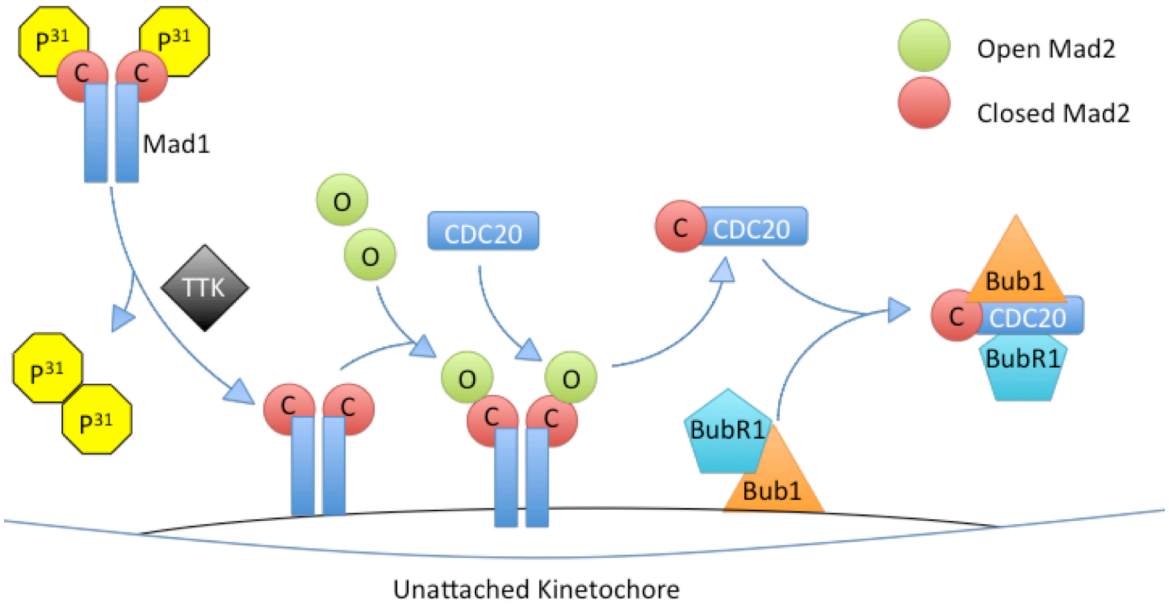

Figure 7. The spindle assembly checkpoint. TTK promotes kinetochore localization of a Mad1 homodimer bound to closed Mad2. Kinetochore binding causes the release of p31^{comet}. O-Mad2 (green circles labeled O) binds to closed Mad2 (red circles labeled C) and becomes activated and converts to closed Mad2 that can now bind and sequester CDC20. Further binding of BubR1 and Bub1 stabilizes the complex and continues sequesteration of CDC20 to prevent APC/C activation.

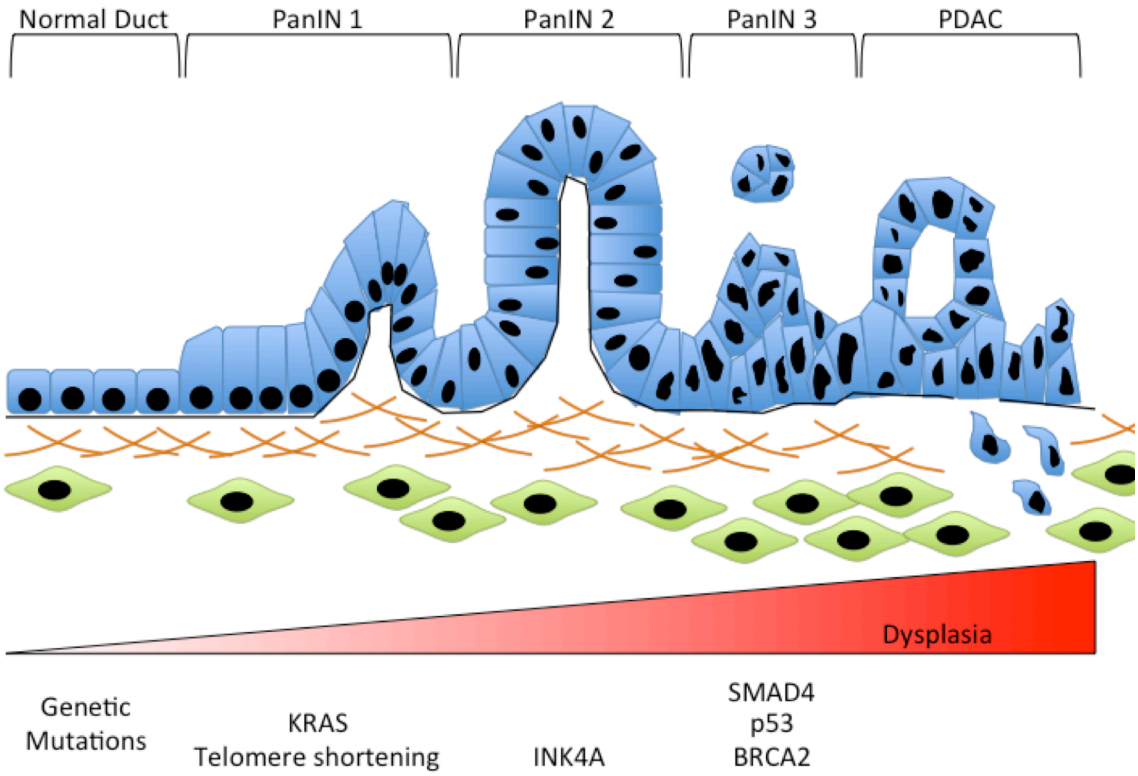

Figure 8. The morphological and genetic progression of PDAC. Normal duct epithelium progresses to invading carcinoma through a series of histologically defined precursor lesions. Accumulation of genetic mutations drive development from early stage PanIN1 lesions to advanced PanIN3 lesions that demonstrate increased dysplasia including elongation of the cell morphology, nuclear atypia and dysplastic growth. Development into invasive PDAC includes degradation of the extracellular matrix (orange) and infiltration through the stroma (green). Activating point mutations in KRAS occur early in the process and are followed by inactivating mutations in $p16^{ink4a}$, SMAD4, p53, and BRCA2 relatively later in the process. Adapted from Hruban RH et al., (2008) Int J Clin Exp Pathol 1:306-316.

(Hruban *et al.*, 2000; JJ Yeh and Der, 2007). One of the earliest mutations to arise in the development of PDAC, and most common, occurring in greater than 90% of tumors, is within the *KRAS* (Kirsten rat sarcoma) gene. *KRAS* is a member of the Ras family of small guanine triphosphatases (GTPases). Ras proteins act as a molecular switch to relay signals between the growth-promoting stimulation received at cell surface to the nucleus by initiating signal transduction cascades. In the nucleus, transcription factors convert the signals into gene expression changes that are more permissive of growth. Mutations in *KRAS* cause persistent signaling, even in absence of the growth-promoting signals, thereby driving PDAC development.

Work in mouse models of pancreatic cancer informs us that *Ras* mutations alone are sufficient to transform normal cells into carcinoma, however, it takes an extended period of time to form a tumor. The vast majority of pancreatic cancers required more than just *Ras* mutations. Whole genome sequencing of human primary PDAC tumors reveals that most tumors have an average of 48 genetic alterations. Some of these mutations cause activation of oncogenes, including *KRAS* and epidermal growth factor receptor (*EGFR*), as well as mutations that inactivate tumor suppressors, such as tumor protein 53 (*TP53*), breast cancer type-2 susceptibility protein (*BRCA2*), mothers against decapentaplegic homolog 4 (*SMAD4*, also known as *DPC4*) and cyclin dependent kinase 4 inhibitor A ($p16^{ink4a}$) (S Jones *et al.*, 2008). Although these mutations are more common in PDAC, each tumor is made up of unique set of acquired mutations.

Some mutations may be biologically inert and have little influence on tumor biology and are often referred to as passengers. Although the passenger genes may not provide a selective growth advantage for oncogenesis, they are of potential value as diagnostic

markers. Unlike traditional biomarkers, mutated protein and DNA are produced only by tumor cells (Q Wang *et al.*, 2011).

There are also mutations that directly provide cancer cells with a growth advantage; these mutations are referred to as driver mutations. Driver mutations often occur in key signal transduction nodes that regulate essential biological processes such as survival, proliferation, cellular invasion and motility, and apoptosis. Similarly to mutant *KRAS*, driver mutations often perturb cellular signaling resulting in altered transcription factor activity and gene expression changes. A genome-wide identification gene expression change, hereafter referred to as a gene signature, is one method to investigate the growth promoting consequences of driver mutations to tumor biology.

Gene expression profiling

It is now widely appreciated that cancer is a heterogeneous disease. Therefore, we need to improve our understanding of the molecular classification of patients to personalize the treatment plan. During tumor progression mutations will drive changes in gene expression to promote growth, and tumors may develop dependencies on key overexpressed genes. Often the tumor may become addicted to the overexpressed gene product to an extent where if the gene is removed the tumor is no longer viable. There is a great need to identify the products of overexpressed genes to which tumors become addicted. One way to accomplish this is by analysis of gene expression profiles of patient tumors. Statistical methods can be employed to determine a minimal number of genes that can stratify patients into high- and low-risk groups based on patient survival. Previously, gene signatures have been identified in multiple cancers with the ability to stratify patients into high- and low-risk groups associated with overall survival (Sørlie *et al.*, 2001; Beer *et al.*, 2002; van de Vijver *et al.*, 2002; Eschrich *et al.*, 2005). Not only does this method help identify biomarkers to help

dictate the treatment plan but it also generates a list of potential addictions of the tumor. In this way, genes that compose a gene signature can also be investigated for their potential use for therapeutic intervention.

Changes in gene expression occur through many molecular mechanisms including aneuploidy, gene amplification (Chou *et al.*, 2013), aberrant cell signaling (Singh *et al.*, 2009), aberrant micro RNA (miRNA) expression (Szafranska *et al.*, 2007), and epigenetic changes such as DNA methylation or histone modifications (McCleary-Wheeler *et al.*, 2013; Nones *et al.*, 2014) (Figure 9). Technological advances, such as complementary DNA (cDNA) microarrays and whole transcription RNA sequencing (RNA-seq), have provided a means to investigate gene expression on a genome-wide scale.

Several studies investigating aberrant gene expression are providing insight and understanding of the molecular biology that promotes malignant transformation, important to development of both diagnostic tools and therapeutic targets. Comparing the transcriptome of micro-dissected PanIN I-III precursor lesions with normal pancreas identified 1251 differentially regulated genes. Interestingly, only 47 differentially expressed genes were identified in PanIN-1B samples whereas PanIN2 had 438 differentially expressed genes when compared to normal pancreas. Of these genes, 30-60% of aberrantly expressed PanIN genes were also aberrantly regulated in PDAC, suggesting a functional role in disease progression (Buchholz *et al.*, 2005).

The utility of gene expression profiling as a diagnostic tool is an area of current investigation. Comparison of gene expression profiles of normal pancreas and chronic pancreatitis with PDAC cell lines and primary tumors identified differential expression of 158 genes in PDAC (Logsdon *et al.*, 2003). These genes not only provide new insights into

the molecular development of PDAC but could also be useful in differentiating between chronic pancreatitis and invasive PDAC, which has been a challenge for clinicians when the histology is inconclusive (Klöppel and Adsay, 2009).

In addition to diagnostic use, gene expression profiles may identify genes of biological significance for PDAC development. Comparison of the gene expression profiles of normal pancreas with pancreatic cancer cell lines and primary tumors have identified an enrichment of genes involved with specific molecular pathways in PDAC. One study identified 149 differentially up-regulated genes, 103 genes not previously identified in PDAC associated with cell-cell and cell-matrix interactions, cytoskeleton remodeling and calcium homeostasis (Iacobuzio-Donahue, Maitra, *et al.*, 2003). Further investigation into these pathways known to be involved with invasive properties of the cell may provide insight into the mechanism of metastasis and potential identify therapeutic targets.

Recent whole-genome profiling of PDAC performed by the International Cancer Genome Consortium (ICGC) and The Cancer Genome Atlas (TCGA, unpublished data from https://tcga-data.nci.nih.gov/tcga/) have unveiled vast heterogeneity in PDAC (Biankin *et al.*, 2012). Clustering of the gene expression of primary PDAC tumors demonstrates that similarities between tumors exist, perhaps subtypes of PDAC (Lowe *et al.*, 2007). Gene expression analysis in breast cancer led to the identification of disease subtypes associated with patient prognosis (Sørlie *et al.*, 2001). Assuming that subtypes exist, identification of subtypes of disease may help identify vulnerabilities and dependencies of PDAC that can be used to guide therapeutic choices as well as identify novel therapeutic targets.

Summary

Despite the low overall survival of PDAC patients, about 20% of patients with resectable PDAC will survive for five years following surgery. Although the reasons are not

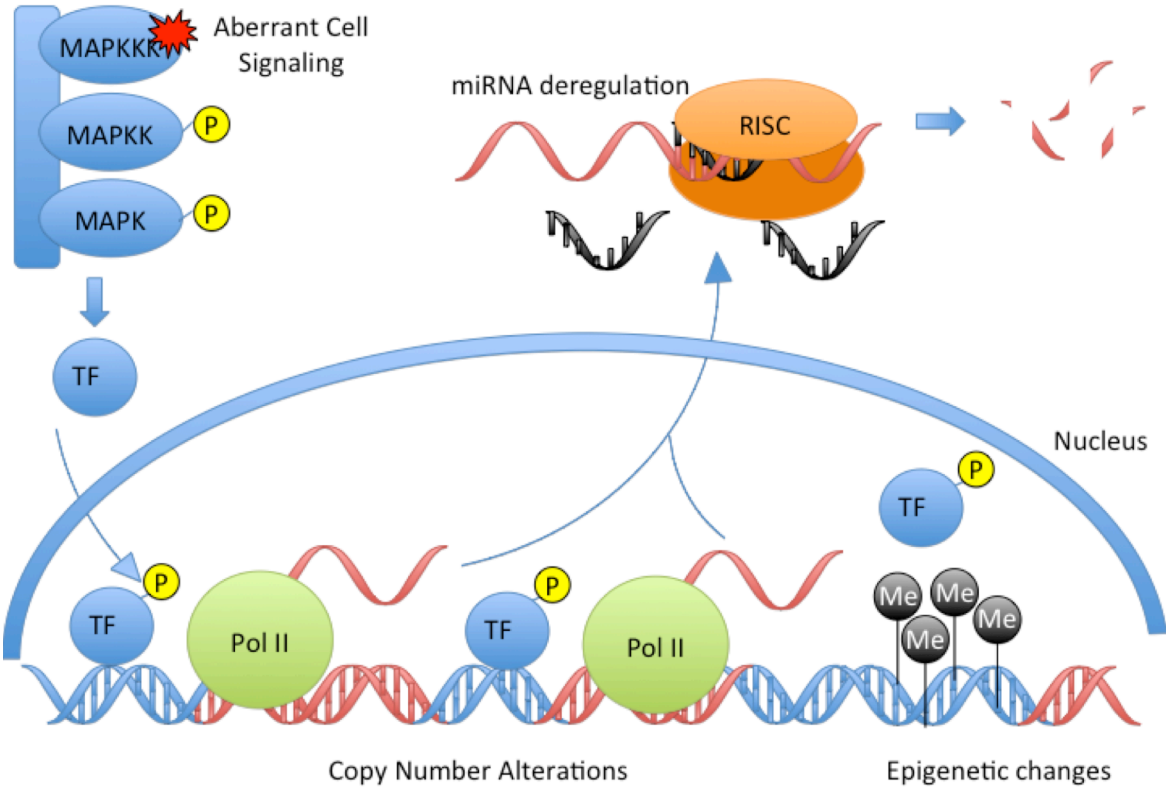

Figure 9. Mechanisms of aberrant gene expression. Aberrant gene expression occurs through many mechanisms in the cell. Mutation (red callout) can aberrantly phosphorylate (P) and activate MAPK signaling cascades resulting in enhanced transcription factor (TF) phosphorylation leading to nuclear translocation where it can enhanced transcription by RNA polymerase II (Pol II). Copy number alterations including gene duplication (red DNA) can also enhance gene expression. Aberrant epigenetic changes in DNA methylation (Me) or chromosomal structure can enhance/inhibit transcription factor accessibility to gene promoters and affect transcription. Enhanced expression of miRNA can increase RNA-induced silencing complex (RISC) and regulate mRNA expression.

completely known, it has been hypothesized that the long-term survivors may have a less aggressive form of the disease. Elucidating the molecular differences between more and less aggressive tumors could be helpful in determining a patient's therapy. In addition, by gaining a more complete understanding of tumor biology, including its addictions, dependencies, disposition for invasion and metastatic spread, we can better understand how to treat each newly diagnosed pancreatic cancer patient.

Here we have discussed the development, progression, characteristics, and therapy of PDAC. PDAC is a heterogeneous disease but there are mutations that are more common such as the mutational activation of the oncogene *KRAS*, and inactivation of the tumor suppressors *TP53*, $p16^{ink4a}$, and *SMAD4*. These driver mutations often result in changes in gene expression whose gene products may represent dependencies. By studying the changes in gene expression in *in vitro* and *in vivo* models of PDAC, we can identify novel diagnostic approaches, therapeutically useful targets, and further understand the molecular biology of PDAC to ultimately lead to advances in the way that we treat pancreatic cancer.

Chapter II.

A six-gene signature predicts survival of patients with localized pancreatic ductal adenocarcinoma.

Overview

Pancreatic ductal adenocarcinoma (PDAC) remains a lethal disease. For patients with localized PDAC, surgery is the best option, but with a median survival of less than 2 years and a difficult and prolonged postoperative course for most, there is an urgent need to better identify patients who have the most aggressive disease.

We analyzed the gene expression profiles of primary tumors from patients with localized compared to metastatic disease and identified a six-gene signature associated with metastatic disease. We evaluated the prognostic potential of this signature in a training set of 34 patients with localized and resected PDAC and selected a cut-point associated with outcome using X-tile. We then applied this cut-point to an independent test set of 67 patients with localized and resected PDAC and found that our signature was independently predictive of survival and superior to established clinical prognostic factors such as grade, tumor size, and nodal status, with a hazard ratio of 4.1 (95% confidence interval [CI] 1.7–10.0). Patients defined to be high-risk patients by the six-gene signature had a 1-year survival rate of 55% compared to 91% in the low-risk group.

Our six-gene signature may be used to better stage PDAC patients and assist in the difficult treatment decisions of surgery and to select patients whose tumor biology may benefit most from neoadjuvant therapy. The use of this six-gene signature should be investigated in prospective patient cohorts, and if confirmed, in future PDAC clinical trials,

its potential as a biomarker should be investigated. Genes in this signature, or the pathways that they fall into, may represent new therapeutic targets.

Introduction

Pancreatic ductal adenocarcinoma (PDAC), comprising over 90% of all pancreatic cancers, remains a lethal disease with an estimated 232,000 new cases, 227,000 deaths per year worldwide, and a less than 5% 5-year survival rate (Parkin *et al.*, 2005; Boyle *et al.*, 2008). Currently the standard of care for the 20% of patients with localized disease is surgery followed by chemotherapy, and in some cases radiation. Unfortunately, despite the use of adjuvant therapy, median survival remains at best 23 months (Neuhaus *et al.*, 2008). It is important to note, however, that up to 27% of patients with resected PDAC can survive for 5 year (Conlon *et al.*, 1996; NA Ahmad *et al.*, 2001; Cleary *et al.*, 2004; Han *et al.*, 2006; Winter *et al.*, 2006; Ferrone *et al.*, 2008; Schnelldorfer *et al.*, 2008). However, in studies examining actual long-term survivors (Conlon *et al.*, 1996; NA Ahmad *et al.*, 2001; Cleary *et al.*, 2004; Han *et al.*, 2006; Winter *et al.*, 2006; Ferrone *et al.*, 2008; Schnelldorfer *et al.*, 2008), only two have found that adjuvant therapy was associated with improved survival (NA Ahmad *et al.*, 2001; Winter *et al.*, 2006). In addition, randomized controlled trials of gemcitabine-based chemotherapy demonstrate an improvement in median survival of at best 3 months (Neuhaus *et al.*, 2008; Ueno *et al.*, 2009). One possible conclusion from these studies is that tumor biology dictates outcome and that our current adjuvant therapy has only a modest impact on altering a patient's course.

Hypothesizing that the dismal outcome of patients with localized disease is due to the presence of micrometastatic disease, current clinical investigation has focused on preoperative or neoadjuvant therapy (Evans *et al.*, 2008; Varadhachary *et al.*, 2008). This approach, in which patients who cannot tolerate the stress of therapy or who develop

metastatic disease during treatment are spared surgery, has demonstrated an overall survival of 34 months in this highly selected patient population (Evans *et al.*, 2008; Varadhachary *et al.*, 2008). Therefore the ability to select patients who would most benefit from a neoadjuvant approach may be important. One way to select these individuals is to define a prognostic gene signature that can identify patients with more aggressive tumor biology upfront.

Expression profiling of PDAC has lead to further studies of additional molecular diagnostic and prognostic markers (Grützmann, Saeger, *et al.*, 2004; Grützmann *et al.*, 2005; Goggins, 2007; Grote and Logsdon, 2007; Tonini *et al.*, 2007; Kolbert *et al.*, 2008). However, the search for genes of biological significance in these large datasets continues to be challenging. One approach to identify genes or pathways that are biologically relevant is to study those that are of prognostic significance (JJ Yeh, 2009). Lowe and colleagues found differential gene expression changes associated with nodal status in primary PDAC (Kim *et al.*, 2007), suggesting that molecular differences in primary PDAC do exist. We hypothesized that by comparing primary PDAC tumors at the extremes of disease, we would identify molecular changes reflective of differences in biology within primary PDAC tumors.

Experimental Procedures

Patients

PDAC samples from 15 patients with resected primary PDAC from the University of North Carolina at Chapel Hill (UNC) and 15 patients with metastatic PDAC from the University of Nebraska Medical Center Rapid Autopsy Pancreatic Program (NEB) were used to derive differentially expressed genes associated with metastatic disease. For the NEB samples, human pancreatic tumors from decedents who had previously been diagnosed with PDAC, and who generously consented to post mortem examinations, were obtained from the institutional review board (IRB)- approved NEB Tissue Bank. To ensure minimal

degradation of tissue, organs were harvested within 3 h post mortem and the specimens flash frozen in liquid nitrogen.

The training cohort included 34 patients with resected PDAC from Johns Hopkins Medical Institutions (JHMI). The testing or validation cohort included patients from two institutions: 48 from Northwestern Memorial Hospital (NW) and 19 from North Shore University Health System (NSU). All samples were collected between 1999 and 2007 at the time of operation and flash frozen in liquid nitrogen after approval by each individual IRB. The UNC IRB approved use of all de-identified samples for this study. All available samples were reviewed by a single pathologist (KAV). De-identified data including tumor, node, and metastasis (TNM), grade or differentiation, margin status, and survival were available for the majority of patients.

RNA isolation and Microarray Hybridization

All RNA isolation and hybridization was performed on Agilent (Agilent Technologies) human whole genome 4×44 K DNA microarrays and at UNC. RNA was extracted from macro-dissected snap-frozen tumor samples using Allprep Kits (Qiagen) and quantified using nanodrop spectrophotometry (Thermo Scientific). RNA quality was assessed with the use of the Bioanalyzer 2100 (Agilent Technologies). RNA was selected for hybridization using RNA integrity number and by inspection of the 18S and 28S ribosomal RNA. Similar RNA quality was selected across samples. One microgram of RNA was used as a template for DNA preparations and hybridized to Agilent 4×44 K whole human genome arrays (Agilent Technologies). cDNA was labeled with Cy5-dUTP and a reference control (Stratagene) was labeled with Cy3-dUTP using the Agilent (Agilent Technologies) low RNA input linear amplification kit and hybridized overnight at 65°C to Agilent 4×44 K whole

human genome arrays (Agilent Technologies). Arrays were washed and scanned using an Agilent scanner (Agilent Technologies). The data are publicly available in Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/ geo/query/acc.cgi?acc = GSE21501).

Microarray and Statistical Analysis

All array data were normalized using Lowess normalization. Data were excluded for genes with poor spot quality or genes that did not have mean intensity greater than 10 for one of the two channels (green and red) in at least 70% of the experiments. The log2 ratio of the mean red intensity over mean green intensity was calculated for each gene and went through LOWESS normalization (YH Yang *et al.*, 2002). Missing data were imputed using the k-nearest neighbors imputation (KNN) with k = 10 (Troyanskaya *et al.*, 2001). A distance weighted discrimination (DWD) was used to detect the systematic biases between the different datasets and then global adjustments made to remove these biases (Benito *et al.*, 2003). Genes that were significantly up- or down-regulated were identified using significance analysis of microarrays (SAM) (Tusher *et al.*, 2001). Two centroids were created using the mean gene expression profile of this significant gene list from the derivation set and used to develop a single sample predictor (SSP, nearest centroid algorithm) (Hu *et al.*, 2006) for an objective classifier. After DWD, the SSP was applied to a 34-patient training set where any new sample was compared to the resected centroid and assigned by the SSP distance function to the resected centroid using (1 2 Pearson correlation coefficient). The X-Tile software program, which assigns a two-population log-rank value to each sample and then determines the best cut-point, was used to determine the best threshold for classifying samples into high- and low-risk categories (Camp *et al.*, 2004). X-Tile predicted that the (12 Pearson correlation coefficient) distance of 1 would be the appropriate cut-point to stratify patients into a high-

and low-risk group (p = 0.006). A second independent validation cohort was then used as a test set using this predetermined cut-point to evaluate outcome.

Survival analysis was performed using the statistical software programs R, the R-package ‘‘survival,’’ and SPSS (SPSS, Inc.). Overall survival (OS) was analyzed using the Kaplan-Meier product-limit method and the significance of our variables was measured by the log-rank test. The Fisher exact test was used to analyze associations between two variables, the Pearson Chi-square test was used to analyze association between more than two variables. Multivariable analysis and analysis of continuous and ordinal variables was performed using the Cox proportional hazards regression method.

Tissue Microarrays

Tissue microarrays (TMAs; UNC2) were prepared from formalin-fixed paraffin-embedded tissue sections using a 2-mm punch. The arrays contained triplicate cores of matched normal and tumor tissue as well as chronic pancreatitis when available, from each patient. We prepared 5-μm sections from each TMA block. Hematoxylin and eosin stained slides from each TMA block were reviewed by a pathologist (KAV) to ensure that tissues were scored accurately.

Immunohistochemistry

Slides with 5-μM sections from the paraffin-embedded specimens were deparaffinized and rehydrated. The slides were then subjected to alkaline heat antigen-retrieval using 1% Tris EDTA for 20 min in a steamer. All slides were incubated with 3% H_2O_2 for 5 min and washed with TBS. The slides were further treated with protein block solution (bovine serum albumin) for 20 min. The sections were incubated with primary KLF6 1:150 antibody (sc-7158, Santa Cruz Biotechnology) for 60min at room temperature.

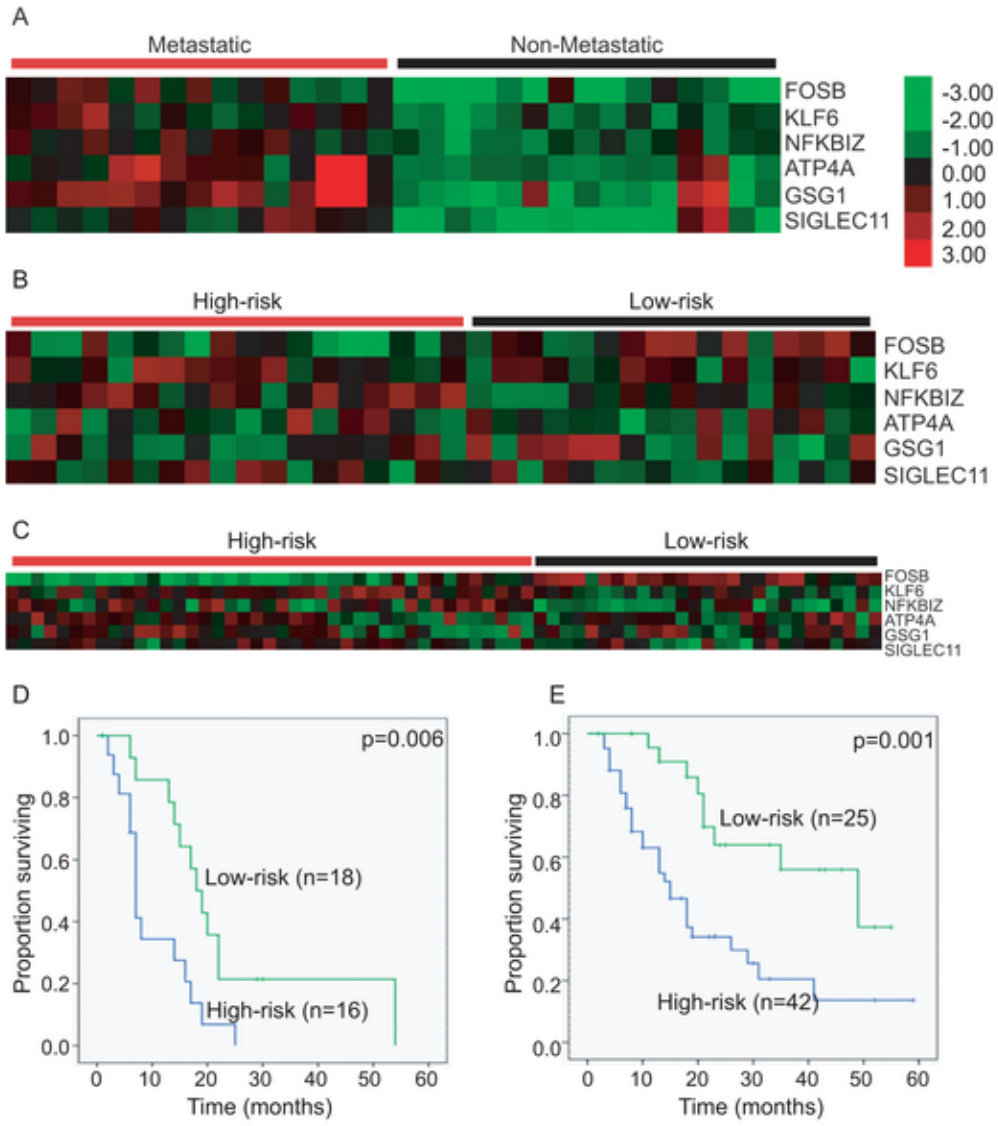

Figure 10. Identification, development, and application of a six-gene signature for PDAC. Clustering of (A) the six genes defined by SAM evaluation of the metastatic compared to nonmetastatic primary PDAC using a false discovery rate of 5%; (B) patient samples into high- and low-risk groups in a training set of 34 patients with localized and resected PDAC using the X-tile determined cut-point of a Pearson correlation coefficient of zero; (C) patient samples into high- and low-risk groups in an independent test set of 67 patients with localized and resected PDAC using the predetermined cut-point of zero. Kaplan-Meier overall survival of (D) the training set classified into high- and low-risk groups according to the X-tile determined cut-point of a Pearson correlation coefficient of zero; (E) and the independent test set classified into high- and low-risk groups according to the same predetermined cut-point. From: Stratford JK et al. (2010) PLoS Med 7(7): e1000307.

Following a TBS wash, the slides were incubated with secondary labeled Polymer-HRP anti-rabbit (Dako K4002) for 30 min. This was followed by a 5-min incubation with the substrate-chromogen, 3,39-diaminobenzidine (Vector SK-4100). The sections were counterstained with Harris hematoxylin. Positive KLF6 staining was defined as when more than 5% of cells expressed the marker and graded from 0 (no staining) to 4 (strong staining). The results of each protein marker were then expressed as intensity (I) and proportion (P) of positive epithelial cells and the score as the product of I and P (Hoos and Cordon-Cardo, 2001; JJ Yeh *et al.*, 2009). All stained slides were reviewed in a blinded fashion (JMA).

Results

Patient and Tumor Characteristics

In order to study the extremes of PDAC tumor biology, we collected a diverse set of resected PDAC specimens from patients with and without metastases. As the tumor microenvironment is increasingly recognized to play a critical role in tumorigenesis (Allinen *et al.*, 2004; Mueller and Fusenig, 2004; Comoglio and Trusolino, 2005; Troester *et al.*, 2009), tissues were macro-dissected in order to preserve the normal adjacent tissue and stroma of the tumors. The characteristics of the dataset used to derive the signature (derivation set) comprised 15 primary resected PDAC tumors (UNC1) and 15 primary tumors from patients with metastatic PDAC (NEB). The training set comprised 34 patients with primary PDAC and the independent validation test set comprised 67 patients with primary PDAC (Tables 4 and 5). There were no differences in RNA quality between the decedent and resected PDAC samples. Available treatment data of the patients in the training and test sets are also shown. One of 15 (7%) UNC1 patients received preoperative or neoadjuvant chemotherapy and 11/15 (73%) NEB patients received chemotherapy less than 6 months prior to death. No patient in the 34-patient training set received neoadjuvant

Table 4. Patient, tumor, and treatment characteristics in the derivation set.		
Demographics (Derivation Set)	NEB, n=15	UNC, n=15
Median follow-up (months)	NA	6 (1-35)
T stage		
1	NA	-
2	NA	2 (13%)
3	NA	12 (80%)
4	NA	1 (7%)
N stage		
0	NA	7 (47%)
1	NA	8 (53%)
M stage		
0	0	15 (100%)
1	15	0
Grade		
1	NA	2 (14%)
2	NA	8 (57%)
3	NA	4 (29%)
Margin		
Negative	NA	12 (80%)
Positive	NA	3 (20%)
Neoadjuvant therapy		
No	NA	14 (93%)
Yes	NA	1 (7%)
Adjuvant Therapy		
No	NA	11 (73%)
Yes	NA	4 (27%)
Chemotherapy		
No	3 (20%)	NA
Yes	12 (80%)	NA
Median Survival (months)	NA	9 (1-35)

NA, not available

From Stratford JK et al. (2010) PLoS Med 7(7): e1000307.

Table 5. Patient, tumor, and treatment characteristics in the training and testing sets.			
Demographics	**JHMI (Training Set), n=34**	**NW/NSU (Testing Set), n=67**	**UNC2 (TMA), n=50**
Median follow-up (months)	14 (2-54)	17 (2-59)	11 (0-51)
T stage			
1	-	2 (3%)	5 (10%)
2	6 (18%)	10 (16%)	8 (16%)
3	27 (79%)	51 (81%)	32 (66%)
4	1 (3%)	-	4 (8%)
N stage			
0	2 (6%)	25 (38%)	15 (31%)
1	32 (94%)	41 (62%)	34 (69%)
M stage			
0	34 (100%)	67 (100%)	47 (94%)
1	0	0	2 (4%)
Grade			
1	1 (3%)	2 (3%)	2 (4%)
2	13 (38%)	34 (54%)	26 (52%)
3	20 (59%)	27 (43%)	20 (40%)
Margin			
Negative	NA	51 (80%)	7 (14%)
Positive	NA	13 (20%)	2 (4%)
Neoadjuvant therapy			
No	34 (100%)	65 (97%)	7 (14%)
Yes	0	2 (3%)	1 (2%)
Adjuvant Therapy			
No	NA	30 (45%)	NA
Yes	NA	37 (55%)	NA
Median Survival (months)	13 (2-54)	21 (3-59)	12 (0-51)

NA, not available

From: Stratford JK et al. (2010) PLoS Med 7(7): e1000307.

chemotherapy. Only 3% (2/67) of patients in the test set received neoadjuvant chemotherapy and 45% (30/67) of patients received postoperative or adjuvant chemotherapy.

Gene Expression Differences in Nonmetastatic and Metastatic Primary Tumors

We hypothesized that we could enrich for molecular differences in primary PDAC, which may be clinically and biologically relevant, through examining primary tumors representing opposite spectrums of PDAC: early (localized) and late (metastatic) stage. To accomplish this, we compared nonmetastatic (UNC1) with metastatic (NEB) primary PDAC tumors. As the methods of procurement for these tumors differed, we used DWD to identify systematic biases between the two datasets (Benito *et al.*, 2003). This method has been used previously to successfully combine three breast cancer datasets across three microarray platforms (Hu *et al.*, 2006), across species (Herschkowitz *et al.*, 2007), and across multiple datasets (Oh *et al.*, 2006; Lu *et al.*, 2006). We therefore used DWD to adjust for the systematic biases between the UNC1 and NEB datasets by taking advantage of the fact that each dataset also had 15 normal pancreas samples assayed. In short, we used DWD to adjust these 15 tumor-normal pairs from both datasets to have similar distributions in principal component (PC) 16PC 2 space. After the DWD adjustment, we used SAM to identify differentially expressed genes (Tusher *et al.*, 2001; YH Yang *et al.*, 2002). Using a false discovery rate of 5%, we identified six genes that were differentially overexpressed between nonmetastatic and metastatic primary tumors: FBJ murine osteosarcoma viral oncogene homolog B (*FOSB*), Kruppel-like factor 6 (*KLF6*), nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta (*NFKBIZ*, *IKBZ*, *MAIL*), ATPase H+/K+ exchanging, alpha polypeptide (*ATP4A*), germ cell associated 1 (*GSG1*), and sialic acid binding Ig-like lectin 11 (*SIGLEC11*) (Figure 10A; Table S1, available online at

http://www.plosmedicine.org/).

Development of a Classifier Using the Six-Gene Signature

We examined the relationship of our six-gene signature to outcome using a training set of 34 patients with localized and resected PDAC. After identifying and adjusting for systematic bias using DWD (Benito *et al.*, 2003), a resected centroid-based predictor (Hu *et al.*, 2006) was created using the 30 samples in the derivation dataset. The centroid was then applied to the DWD-adjusted training set of primary PDAC patients to determine the performance of the six- gene signature. X-tile (Camp *et al.*, 2004) was used to determine the optimal distance function to the centroid cut-point for classifying this training set of patients into high-risk and low-risk groups on the basis of survival (Figure 10B and 10D). The optimal cut-point occurred at a Pearson correlation coefficient of zero ($p = 0.006$) with patients with Pearson correlation coefficients greater than zero in the low-risk and less than zero in the high-risk groups.

Application of the Six-Gene Signature to an independent Validation Cohort of 67 Patients

In order to evaluate the performance of the cut-point determined by X-tile (Camp *et al.*, 2004), we applied the cut-point to an independent validation test set of 67 patients with primary PDAC. Our predetermined Pearson correlation coefficient cut-point of zero distance to the centroid successfully stratified patients into high- (n=42) and low-risk groups (n=25) with a median overall survival (OS) of 15 versus 49 months ($p = 0.001$) (Figure 10C and 10E). Patients in the high-risk group had 1-, 2-, and 3-y estimated survival rates of 55%, 34%, and 21%, compared to 91%, 64%, and 56% in the low-risk group.

Previous studies in PDAC have found that nodal status is the most predictive of

outcome for patients with localized PDAC (Sohn *et al.*, 2000). We compared our prognostic signature to current clinical prognostic benchmarks. We found that tumors that were node positive (p=0.091) and grade 2 or 3 trended towards a shorter survival (p = 0.080). Neither T stage (p = 0.977) nor margin status (p = 0.223) were prognostic in this cohort. Treatment with adjuvant chemotherapy (p=0.699) or with neoadjuvant chemotherapy (p=0.409) was also not prognostic, although only two patients received neoadjuvant chemotherapy. We found no gene expression changes between the tumors of the two patients who received neoadjuvant chemotherapy and the tumors of patients who received no treatment prior to surgery.

An important feature of any prognostic signature is that it should be independent or additive to currently used clinicopathologic prognostic criteria. We therefore compared the prognostic importance of our molecular signature in the setting of grade (p = 0.417), nodal status (p = 0.381), T stage (p = 0.675), and margin status (p=0.295). We found that our six-gene signature was the only independent predictor of survival in the 57 patients with complete data, with a hazard ratio of 4.1 (95% confidence interval 1.7–10.0) (Table 6).

We also looked at whether our six-gene signature was confounded by available clinicopathological variables. We found no association between our molecular signature, and tumor size, grade, margin status, nodal status, and neoadjuvant or adjuvant chemotherapy in our independent test set (Table 7).

KLF6 Expression in Primary PDAC

In order to further validate the six-gene signature, we performed immunohistochemical analyses for KLF6, which showed a wide range of expression values between nonmetastatic versus metastatic samples (Figure 10A). To evaluate KLF6 protein

Table 6. Cox proportional hazards regression analysis of the six-gene signature.			
Variable	**Hazard Ratio**	**CI**	***p*-Value**
Six-gene signature	4.1	1.7-10.0	0.002
T stage	-	-	0.675
N stage	-	-	0.381
Grad	-	-	0.417
Margin status	-	-	0.295

CI, confidence interval.

From: Stratford JK et al. (2010) PLoS Med 7(7): e1000307.

Table 7. Relationship between the six-gene signature and clinicopathological variables.			
Variable	**Six-Gene Signature**		
	High Risk	**Low Risk**	***p*-Value**
T stage			
1	1 (50%)	1 (50%)	0.886
2	6 (60%)	4 (40%)	-
3	33 (65%)	18 (35%)	-
N stage			
0	13 (52%)	12 (48%)	0.203
1	28 (68%)	13 (32%)	-
Grade			
1	1 (50%)	1 (50%)	0.788
2	22 (65%)	12 (35%)	-
3	19 (70%)	8 (30%)	-
Margin			
Negative	31 (59%)	22 (41%)	0.344
Positive	9 (75%)	3 (25%)	-
Neoadjuvant therapy			
No	42 (65%)	23 (35%)	0.136
Yes	0 (0%)	2 (100%)	-
Adjuvant Therapy			
No	24 (65%)	13 (35%)	0.801
Yes	18 (60%)	12 (40%)	-

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Table 8. Comparison of individual genes in high- and low-risk groups Johns Hopkins Medical Institutions.					
	Group 1 (High Risk)		Group 2 (Low Risk)		
	Average	Stdev	Average	Stdev	2 Sided Type 2 T-Test
SIGLEC11	0.0604	0.8489	-0.3163	0.7000	0.2098
KLF6	0.3502	0.8063	0.1471	0.6985	0.2571
FOSB	-0.5505	.9013	0.4487	0.9527	0.0033
ATP4A	-0.1818	0.9529	-0.1826	0.7753	0.0010
NFKBIZ	0.5668	0.7767	-0.1826	0.7753	0.0010
GSG1	-0.2451	0.8350	0.1019	1.3196	0.1912

Stdev, standard deviation

From: Stratford JK et al. (2010) PLoS Med 7(7): e1000307.

Table 9. Comparison of individual genes in high- and low-risk groups Northwestern Memorial Hospital/NorthShore University Health System					
	Group 1 (High Risk)		Group 2 (Low Risk)		
	Average	Stdev	Average	Stdev	2 Sided Type 2 T-Test
SIGLEC11	0.1145	0.5742	-0.2116	0.7660	0.0492
KLF6	0.5304	0.6399	-0.2474	0.5880	0.0000
FOSB	-0.9593	0.8241	0.9310	0.7750	0.0000
ATP4A	0.0885	0.7960	0.1234	0.8217	0.8619
NFKBIZ	0.1985	0.9682	-0.7227	0.8165	0.0001
GSG1	0.0270	0.9123	0.1259	0.8499	0.6548

Stdev, standard deviation

From: Stratford JK et al. (2010) PLoS Med 7(7): e1000307.

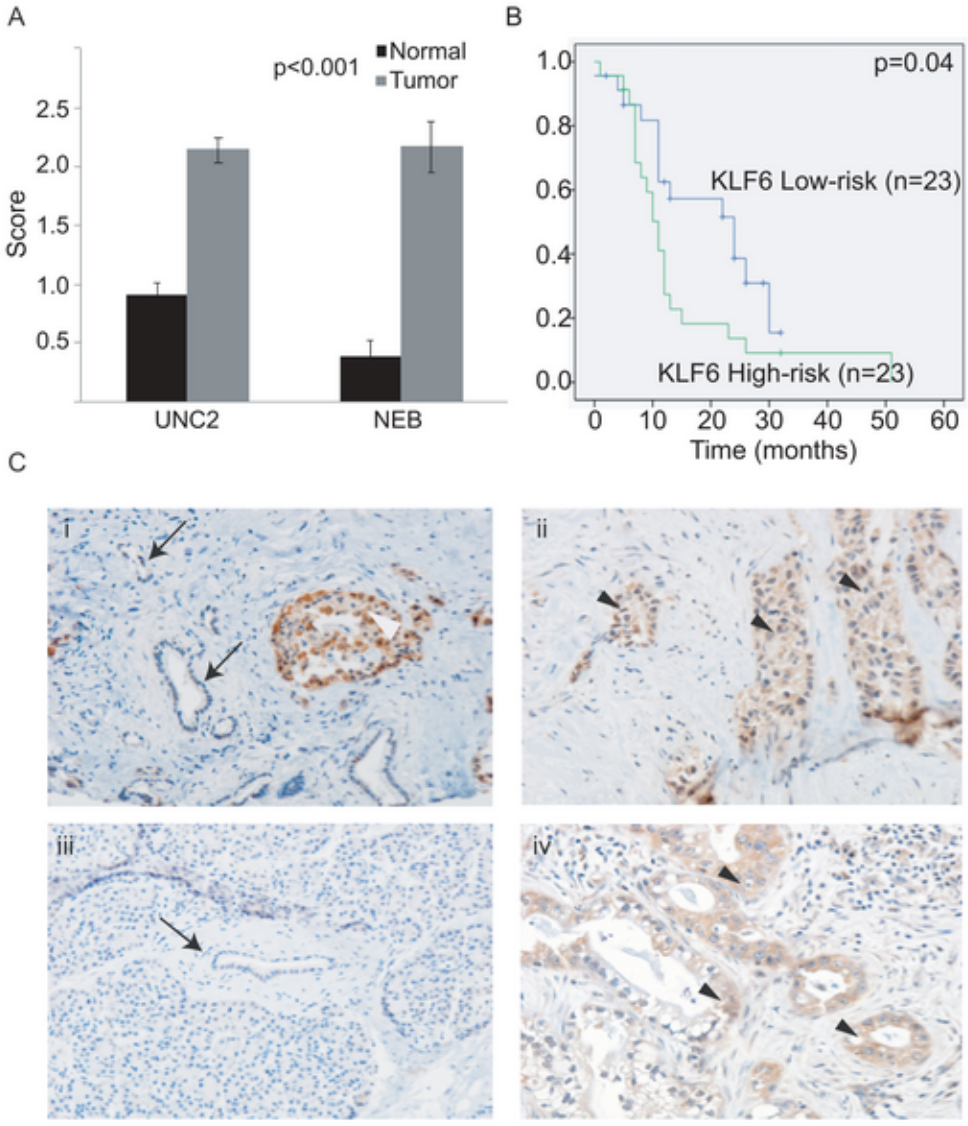

Figure 11. Significance of KLF6 and Fos B expression in primary PDAC. (A) KLF6 staining is significantly higher in PDAC compared to normal adjacent pancreas in an independent dataset of a 50-patient TMA (UNC2) as well as NEB samples used for the original analysis. (B) Kaplan-Meier overall survival of 50 patients classified by high and low KLF6 scores, using the median cutoff score of 1.5. (C) KLF6 immunostaining in the primary tumor of a patient who died of metastatic disease (ii) and in a resected primary tumor (iv). Minimal staining is seen in the matched normal adjacent tissue of both patients (i, iii). KLF6 immunostaining in islet cells (i, white arrowhead). Arrows illustrate normal ductal epithelium. Black arrowheads illustrate tumor. From: Stratford JK et al. (2010) PLoS Med 7(7): e1000307.

expression, we obtained another independent dataset of 50 patients represented on a TMA with matched normal, chronic pancreatitis, and PDAC (UNC2, Table 5). First, using the median score of 1.5 as the cutoff, we found that KLF6 expression was much higher in tumors compared to normal pancreas ($p<0.001$) (Figure 11A and 11C). KLF6 expression was strong in normal islet cells in agreement with a previous study (Figure 11Ci, white arrowhead) (Hartel *et al.*, 2008). Second, we found that KLF6 expression with a score greater than 1.5 (high) was associated with a shorter median survival of 11 months compared to 24 months for patients with KLF6 expression scores less than 1.5 (low) ($p = 0.04$) (Figure 11B).

Discussion

We profiled and compared nonmetastatic and metastatic primary PDAC tumors and identified a six-gene signature. Although this signature was not derived on the basis of outcomes, we show that it was prognostic in a true test set of resectable PDAC patients. Importantly, our six-gene signature was independently predictive of survival, stratifying patients with median survivals of 15 compared to 49 months, outperforming current pathological staging criteria, suggesting that our signature will be a powerful prognostic tool for patients with localized PDAC.

PDAC continues to be a devastating disease with few long-term survivors. Surgery remains the standard therapy for patients diagnosed with resectable PDAC (Yeo *et al.*, 1997). Yet with a median survival only of less than 2 y after surgery, the attendant postoperative mortality rate of 2%–6% (Yermilov *et al.*, 2008; Eppsteiner *et al.*, 2009), and postoperative complication and hospital readmission rates of 59% (DeOliveira, Winter, Schafer, Cunningham, Cameron, Yeo, and Clavien, 2006b; Yermilov *et al.*, 2008), the decision for surgery should be made cautiously. Therefore, improved patient selection for therapy is necessary. For the majority of patients who cannot undergo surgery, gemcitabine

chemotherapy remains the best option, yet only 5%–10% of patients respond to the treatment (Abou-Alfa *et al.*, 2006; Van Cutsem *et al.*, 2009). Given the current therapeutic limitations, additional prognostic tools are needed to help a patient decide whether to have surgery, and/or neoadjuvant chemotherapy, or when to consider participation in a clinical trial.

Our analysis identified a surprisingly small number of genes with differential expression between early compared to late stage primary PDAC (Table S1, available online at http://www.plosmedicine.org/). This finding suggests that primary PDAC may be largely homogenous from a global gene expression standpoint. Nonetheless, the differences that we identified appear to be clinically and therefore biologically important. Our findings of molecular differences in resected primary PDAC tumors suggest that there is subtle biological variation in these tumors that influences outcome. A review of previous published studies did not identify differential expression of our six genes (Crnogorac-Jurcevic *et al.*, 2001; 2002; Iacobuzio-Donahue *et al.*, 2002; Logsdon *et al.*, 2003; Iacobuzio-Donahue, Ashfaq, *et al.*, 2003; Iacobuzio-Donahue, Maitra, *et al.*, 2003; Crnogorac-Jurcevic *et al.*, 2003; Grützmann, Pilarsky, *et al.*, 2004; Grützmann *et al.*, 2005; Ishikawa *et al.*, 2005; Segara *et al.*, 2005; Kim *et al.*, 2007; Lowe *et al.*, 2007; Karanjawala *et al.*, 2008). This finding is not surprising, as previous studies examined differential gene expression changes between either normal pancreas or chronic pancreatitis and PDAC (Crnogorac-Jurcevic *et al.*, 2001; 2002; Iacobuzio-Donahue *et al.*, 2002; Logsdon *et al.*, 2003; Iacobuzio-Donahue, Ashfaq, *et al.*, 2003; Iacobuzio-Donahue, Maitra, *et al.*, 2003; Crnogorac-Jurcevic *et al.*, 2003; Grützmann, Pilarsky, *et al.*, 2004; Grützmann *et al.*, 2005; Ishikawa *et al.*, 2005; Segara *et al.*, 2005; Lowe *et al.*, 2007; Karanjawala *et al.*, 2008). Only one study has looked at gene expression changes between PDAC of different stages (Kim *et al.*, 2007). Ours was

the first, to our knowledge, to study molecular differences between nonmetastatic versus metastatic primary tumors and identify and validate a prognostic signature for PDAC.

Of the six genes identified in this study, most do not have an obvious role in carcinogenesis. Three of the six genes demonstrated significantly higher expression in the poor prognostic groups (SIGLEC11, KLF6, NFKBIZ; Tables 8-9). ATP4A, GSG1, and SIGLEC-11 have not been studied in cancer. SIGLEC-11 is thought to be expressed by tissue macrophages and also the brain microglia (Angata *et al.*, 2002). Interestingly, a missense mutation of SIGLEC-11 (S465A) was identified in the mutation discovery screen of the recent genome-wide sequencing of PDAC (S Jones *et al.*, 2008). NFKBIZ, also called IkappaB zeta, binds to the p50 subunit of nuclear factor (NF)-kappaB and is important for interleukin-6 (IL-6) induction and may be induced by IL-1 receptor and Toll-like receptors (Angata *et al.*, 2002). Given the prevalence of chronic pancreatitis and high degree of stromal fibrosis, it is possible that NFKBIZ may play a role in PDAC and inflammation.

KLF6 is a transcription factor and its full-length transcript is thought to be a tumor suppressor gene involved in prostate, lung, and ovarian carcinogenesis (DiFeo *et al.*, 2009). However a splice variant KLF6- SV1 has been shown to have oncogenic properties. The oligonucleotide probes used in the Agilent whole human genome array and the antibody against KLF6 did not differentiate between the full-length and splice variant. In agreement with a previous study (Hartel *et al.*, 2008), we found that KLF6 protein expression was higher in tumors than normal pancreas. In addition we found that higher KLF6 expression was associated with worse survival. Hartel et al. further investigated KLF6-SV1 expression in their study using real-time PCR and demonstrated that the higher KLF6 expression seen in tissues was associated with a higher ratio of KLF6-SV1 compared to full-length KLF6.

Therefore our findings that KLF6 expression is higher in tumors and is prognostic is likely in agreement with this study.

Only one patient in the UNC1 cohort was treated with neoadjuvant chemotherapy compared to 80% of NEB patients who were treated with palliative chemotherapy. Although there is a possibility that our signature may be reflective of gemcitabine treatment or perhaps resistance, as NEB patients died of metastatic disease despite gemcitabine treatment, the successful application of our six-gene signature on an independent test set of patients where only 3% of patients with localized PDAC were treated with neoadjuvant therapy suggests that it is a rigorous predictor of prognosis in previously untreated patients. We found no association between our six-gene signature and whether a patient received adjuvant chemotherapy. In addition, chemotherapy treatment in this cohort, either pre- or postoperative, did not demonstrate a survival advantage.

Another concern is the validity of our hypothesis that gene expression changes at different stages of primary PDAC development may occur and be important for prognosis. Our study is in agreement with Lowe and colleagues' findings that differential gene expression changes can be identified within primary PDAC (Kim *et al.*, 2007). However, they did not address the prognostic value of their findings. Several studies have also suggested that gene expression changes in metastasis may be found in primary tumors. In a study of molecular differences between primary tumors and metastases, Golub and colleagues identified a gene expression signature of metastasis present that could be identified in primary tumors (Ramaswamy *et al.*, 2003). In addition, studies in melanoma have suggested that metastatic cells may be found in the parent primary tumor (Fidler and Kripke, 1977). Finally studies in breast cancer have demonstrated that gene expression

changes found in breast cancer cells with metastatic potential may be prognostic and predictive of patients who will develop metastasis (Minn, Y Kang, *et al.*, 2005; Minn, Gupta, *et al.*, 2005; Minn *et al.*, 2007). Our study is the first to demonstrate that molecular differences in metastatic PDAC can be identified at earlier stages, and that these differences are predictive of future behavior. Whether these molecular changes are biologically associated with metastatic potential will require further investigation.

We have applied our six-gene signature to an independent dataset of 67 patients, and have validated its prognostic value. In addition, we have validated the protein expression of KLF6 in a 50-patient TMA. Although not nearly as powerful a predictor of prognosis as our six-gene signature, we found that KLF6 expression was prognostic in our 50-patient TMA. Further validation studies will be needed to see if KLF6 alone may be a useful prognostic marker as others have shown (Hartel *et al.*, 2008). Our findings suggest that the prognostic value of KLF6 is strengthened in evaluating the six genes in their entirety.

Studies of patients with resectable PDAC demonstrate median survivals of up to 22 months, equivalent to the median survival of patients in our training and testing cohorts (Neoptolemos *et al.*, 2001; Neuhaus *et al.*, 2008; Ueno *et al.*, 2009). Our finding that our six-gene signature is able to stratify patients, with startling differences in survival, suggests that it may be used to select patients for therapies. For example, for patients who are at high operative risk, knowledge of a median survival of 49 compared to 15 months, may be helpful in the operative decision-making process. Similarly, patients who have a poor prognosis based on the six- gene signature may be considered for neoadjuvant therapy. Currently, the minority of centers use neoadjuvant therapy as a standard of care, most instead reserve this for patients with locally advanced unresectable or borderline resectable tumors. Therefore the

current decision-making process is based on anatomical considerations. Our prognostic signature may refine this paradigm such that neoadjuvant therapy is offered to patients on the basis of biological considerations, regardless of resectability, and may allow us to further study and maximize the benefits of neoadjuvant treatment. In addition, as new therapies are developed, it may help to determine whether patients may require more or less aggressive treatment. Finally, our findings that there are molecular differences associated with late-stage primary tumors, which translate into differences in prognosis, suggest that the six genes in this signature should be further studied for their potential as biomarkers, and some of these genes, or the pathways that they fall into, may represent new therapeutic targets.

Chapter III

Genetic and pharmacological inhibition of TTK impairs pancreatic cancer cell line growth by inducing lethal chromosomal instability

Overview

Pancreatic ductal adenocarcinoma (PDAC), which accounts for the majority of pancreatic cancers, is a lethal disease with few therapeutic options. Genomic profiling of PDAC has identified a complex and heterogeneous landscape. Further understanding of PDAC molecular biology will facilitate the identification of potential therapeutic strategies. We analyzed the gene expression profiles of primary tumors from patients compared to normal pancreas and identified high co-overexpression of core components of the spindle assembly checkpoint (SAC), including the protein kinase TTK. We found overexpression of TTK protein in a subset of PDAC primary tumors and cell lines. siRNA-mediated depletion or catalytic inhibition of TTK resulted in an aberrant cell cycle profile, multi- and micro-nucleation, induction of apoptosis, and decreased cell proliferation and transformed growth. Selective catalytic inhibition of TTK caused override of the SAC-induced cell cycle arrest. Together these results suggest the importance of the role of TTK-dependent SAC activation to prevent excessive chromosomal instability and support growth. Unexpectedly, we identified ubiquitin specific peptidase 16 (Usp16), a ubiquitin hydrolase, as a phosphorylation substrate of TTK. Usp16 regulates chromosomal condensation and G2/M progression by deubiquitinating histone H2A. Phosphomimetic mutants of Usp16 show enhanced proteosomal degradation and may prolong the G2/M transition allowing for

correction of replication errors. Taken together, our results suggest a critical role for TTK in preventing aneuploidy-induced cell death in pancreatic cancer.

Introduction

PDAC represents 95% of all pancreatic cancers and is the fourth leading cause of cancer related deaths in the United States. Median survival of pancreatic cancer patients is five to eight months with fewer than 5% of patients surviving longer than five years after diagnosis. The poor prognosis stems from the frequent presence of metastatic disease at the time of or shortly after diagnosis. The current standard of care for metastatic pancreatic cancer is chemotherapy. Although chemotherapeutic approaches including gemcitabine, nab-paclitaxel, and FOLFIRINOX have improved patient survival (Conroy *et al.*, 2011; Gourgou-Bourgade *et al.*, 2013; Hoff *et al.*, 2013), the discovery of new and better drugs targets remains essential for the continued improvement of therapies for PDAC.

Genomic and mouse model studies have advanced our understanding of PDAC tumor biology and have identified a high degree of chromosomal instability in PDAC (Moskovitz *et al.*, 2003; Hingorani *et al.*, 2005; Gotoh *et al.*, 2011). One aspect of chromosomal instability is the unequal segregation of chromosomes during mitosis, resulting in aberrant chromosomal numbers and cellular aneuploidy of both daughter cells (Thompson and Compton, 2008). It has long been postulated that chromosomal instability is an important mechanism for tumor adaptation (Nowell, 1976; Lengauer *et al.*, 1998). However, recent studies have hypothesized that the adaptive capacity of cancer cells to aneuploidy is limited (Birkbak *et al.*, 2011; Tang *et al.*, 2011). Aneuploid cancer cells must maintain a delicate balance between sustaining an altered genome that enhances proliferation yet confines continued chromosomal instability within survivable limits (Kops *et al.*, 2004; Janssen *et al.*, 2009). As such, there is a pressing need to characterize the adaptive mechanisms that control

this balance to potentially identify therapeutic targets that can shift this balance from sustainable to non-viable chromosomal instability.

Chromosomal segregation during mitosis is a multi-step process and errors during this often result in aneuploidy (Gordon *et al.*, 2012). Therefore, the timing of each step is tightly regulated in order to maintain mitotic fidelity. In normal cells, mitotic checkpoints ensure that each step is completed prior to continuing through the next phase of the cell cycle. The importance of these checkpoints in cancer is an area of current interest as inactivation of mitotic checkpoints has been shown to enhance chromosomal instability (Duijf and Benezra, 2013), which may result in aneuploidy and decreased cell fitness.

The spindle assembly checkpoint (SAC) is a conserved mitotic checkpoint found from yeast to mammals that ensures accurate segregation of chromosomes during mitosis. During metaphase, sister chromatids congregate at the metaphase plate prior to separation during anaphase. The SAC is activated to prevent the premature onset of anaphase until bi-oriented microtubule attachment at each kinetochore. Failure to activate the SAC has previously have been shown to promote chromosomal instability (Kops *et al.*, 2004; Thompson and Compton, 2008; Rao *et al.*, 2009; Janssen *et al.*, 2009).

SAC activation requires the expression and activity of the protein kinase TTK, also known as Mps1/Pyt/CT96, (X Liu and Winey, 2012). Overexpression of TTK has been proposed to be an adaptive mechanism whereby cells cope with aneuploidy. In agreement with this hypothesis, high levels of TTK mRNA have been observed in multiple cancer types and been shown to be protective against aneuploidy (Thykjaer *et al.*, 2001; Yuan *et al.*, 2006; Salvatore *et al.*, 2007; Landi *et al.*, 2008; Mizukami *et al.*, 2008; Daniel *et al.*, 2011; Maire *et al.*, 2013). Previous studies have investigated the role of TTK in cancer using pharmacologic

inhibitors in cancer cell lines. Pharmacologic inhibition of TTK in colorectal and glioblastoma cancer cell lines reduced cell viability, caused aberrant cell cycle progression, increased aneuploidy, and increased apoptosis (Kwiatkowski *et al.*, 2010; Tardif *et al.*, 2011; Jemaà *et al.*, 2013; Tannous *et al.*, 2013). Similarly, a recent study provides a tantalizing rationale to look at the role of TTK overexpression in PDAC. This study found that pharmacologic inhibition of TTK in PDAC caused aberrant override of SAC-dependent cell cycle arrest, increased chromosomal instability, increased apoptosis and decreased clonogenic survival (Slee *et al.*, 2014). While this study demonstrated that pharmacologic inhibition of TTK might prevent PDAC growth, several questions remain unanswered about the biological role of TTK in PDAC. First, although the pharmacologic inhibitor used was selective for TTK, it also inhibits casein kinase 2 (CK2), maternal embryonic leucine zipper kinase (MELK), and NIMA-related kinase 6 (NEK6) (Colombo *et al.*, 2010). Second, TTK has a large N-terminal domain with protein binding motifs that may participate in disparate pathways and processes that support PDAC growth that cannot be directly studied using catalytic site ATP-competitive pharmacologic inhibitors. Third, Slee et al. focused on the effects of TTK inhibition of PDAC cell lines after several cell divisions and the acute role of TTK for PDAC growth remains undetermined. Finally, the requirement of TTK for cellular transformation and *in vivo* tumorigenesis in PDAC were not evaluated.

Although this study clearly implicates the importance of TTK catalytic activity for continued PDAC cell line growth, how TTK promotes growth remains unclear, mainly because only few TTK phosphorylation substrates have been identified (Zhu *et al.*, 2007; Jelluma, Brenkman, van den Broek, *et al.*, 2008; Huang *et al.*, 2009; Sun *et al.*, 2010).

The overall goal of this study was to investigate the role of TTK in mitotic progression, proliferation and transformation of PDAC and identify the molecular mechanism whereby TTK limits chromosomal instability. We found increased mRNA expression of core SAC components and elevated mRNA and protein expression of TTK in a subset of PDAC cell lines and human tumors, suggesting that TTK might protect cells from excessive chromosomal instability through SAC activation. We also found that TTK phosphorylates and regulates the protein stability of Usp16, an enzyme required to promote chromosomal condensation, suggesting that TTK functions at multiple stages of the cell cycle to maintain genome stability. Together these findings establish the importance of TTK to adapt to and maintain viable levels of aneuploidy in PDAC.

Experimental procedures:

Tissue Collection

Human tumor lysates from flash-frozen samples were homogenized in NP40 lysis buffer, resolved by SDS–PAGE, and evaluated by western blot analysis.

RNA Microarray analysis

Gene expression microarray data of 184 normal colon and primary pancreatic tumor samples from patients was obtained from the Gene Expression Omnibus (GEO) database (accession number GSE21501). Normalization, quality control, and imputation of array data was performed as previously described (Stratford *et al.*, 2010). Expression data from multiple probes were collapsed by the mean for each sample. Statistical significance was assessed using a two-tailed unpaired t-test comparing SAC component expression in normal versus tumor tissue. Differentially expressed genes were identified using statistical analysis of microarrays (SAM) using a 5% false discovery rate (FDR) (Tusher *et al.*, 2001). Gene

networks were were investigated for enrichment of differentially expressed kinases using Ingenuity Pathway Analysis (IPA, Ingenuity Systems, Mountain View, CA).

Cell culture and reagents

All cell lines except HPDE, HPNE, 293FT, HuPT3 were obtained from American Type Culture Collection (ATCC, LGC Promochem). The HPDE cell line was obtained from Ming-Sound Tsao (University of Toronto, Toronto). The HuPT3 cell line was obtained from Dan Billadeau (Mayo Clinic, Rochester, MN). 293FT cells were purchased from Invitrogen (Grand Island, NY). The HPNE cell line was obtained from Michel Ouellete (UNMC Eppley Cancer Center) and has been described previously (KM Lee *et al.*, 2003; PM Campbell *et al.*, 2007). All cell lines were maintained at 37°C and 5% CO_2. HPAC, PANC-1, MIA PaCa-2, T3M4, HPAF-II, HPNE, HPDE, 293T, 293FT, and HeLa cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM, Corning). BxPC-3, Panc 02.03, Panc 10.05, AsPC-1, SW-1990, HuPT3, CFPAC-1, Capan-1 and Capan-2 cell lines were maintained in RPMI-1640 with 4.5g/L glucose. All media was supplemented with 10% (vol/vol) fetal bovine serum (FBS, Hyclone), and 100 U/ml penicillin and 100 U/ml streptomycin (P/S, Gibco). Capan-1 cells which were supplemented with 15% (vol/vol) FBS.

Antibodies

Primary antibodies used for immunoblot or immunoprecipitation include: Mouse anti-β-actin (IB, 1:5000, AC-15, Sigma Aldrich) mouse anti-TTK (IB, 1:2000, 4-112-3 Millipore) rabbit anti-Cyclin B1 (IB, 1:1000, 4138 Cell Signaling), rabbit anti-Histone H3 phospho Ser 10 (IB, 1:1000, 9701 Cell Signaling), mouse anti-FLAG (IB, 1:1000, M2 Sigma Aldrich), mouse anti-phospho tyrosine (IB, 1:1000, 9411 Cell Signaling), mouse anti-phospho serine (IB, 1:1000, 4A4 Millipore), mouse anti-phospho threonine (IB, 1:1000, Cell Signaling 3986)

rabbit anti-Usp16 (IB, 1:2000, Bethyl A301-615A) mouse anti-HA (1:1000, Covance 16B12), and mouse anti-GFP (1:5000 Clontech JL8). HRP conjugated secondary antibodies include goat anti-mouse (1:10000, Thermo) or goat anti-rabbit (1:10000, Thermo).

Immunoprecipitation and immunoblotting

Two to five micrograms of specified antibodies were bound to 20-50 µl of Protein G Dynabeads (Invitrogen) overnight at 4°C. Cells were collected following treatments as specified in the figure legends. Cells were harvested and then lysed in NP-40 lysis buffer (50 mM Tris pH 7.5, 10 mM $MgCl_2$, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, and 10% glycerol) supplemented with phosphatase (Roche) and protease (Sigma) inhibitor cocktails. Cell lysates were incubated with antibody bound Dynabeads overnight at 4°C. Beads were washed three times with lysis buffer and resuspended in protein sample buffer containing β-mercaptoethanol and examined by immunoblotting.

Immunoblots were performed by running protein samples on either 15% (for proteins under 40 kDa) or 8% SDS-PAGE followed by transfer of resolved proteins to PVDF membranes for 90 min at 4°C. All immunoblots were blocked in 5% milk/TBST solution for 1 h at room temp with gentle agitation. Primary antibodies were diluted in 5% (weight/vol) milk or 5% BSA. Primary antibodies were incubated overnight at 4°C. Following incubation with primary antibody blots were washed 3x with TBST and then incubated with secondary HRP-conjugated antibodies for 1 h at room temp with gentle agitation. Blots were again washed 3x with TBST and developed by ECL/chemiluminescence and auto-radiograph film (Kodak) or the Biorad XRS+ imaging system.

FLAG pull-down were performed on cell lysates using the pull-down buffer (50 mM Tris pH 7.6, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1 mM EDTA, 1 mM

EGTA) and supplemented with protease inhibitors (Roche). Lysates were clarified by centrifugation. 10 mg of protein were used in each pull down. Anti-FLAG M2 magnetic beads (Sigma) were used to immunoprecipitate FLAG-tagged Usp16. Following extensive washing with TBS, FLAG-Usp16 was eluted with FLAG peptide (Sigma) in TBS.

cDNA expression

Expression vectors for firefly luciferase (plasmid #17477), FLAG-HA-Usp16 (Plasmid #22595) were obtained from Addgene. GFP-Usp16 was prepared by amplifying Usp16 cDNA from FLAG-HA-Usp16 followed by subcloning into pEGFP-C3 using the XhoI and BamHI sites. GFP-Usp16 3xA and 3xE were prepared by site-directed mutagenesis and confirmed by sequencing.

Transfections for all cDNA were accomplished using a HBS/$CaCl_2$ or TransIT-LT1 (Mirius Bio) protocol and cells were examined for expression 48-72 h post transfection

siRNA transfection

All siRNA described were obtained from Thermo and are part of their ON-TARGETplus SMARTpools of siRNA. siRNAs were transfected using RNAiMax (Invitrogen).

Cell proliferation assays

For the MTT proliferation assay, 300 x 10^3 cells were transfected with siRNA using RNAiMax transfection reagent. Forty-eight hours post transfection 1 x 10^3 cells were plated into 6 identical wells of a 96-well plate. Proliferation was assessed by addition of the MTT reagent for 4 h. Measurements were taken at an A_{560} on the Synergy 2 (Biotek).

For the soft agar assay, 10^4 siRNA transfected cells were suspended between layers of 0.6% (bottom) and 0.3% (top) bacto-agar (BD Biosciences) in three wells of a 6-well dish.

Cells were supplied with growth medium twice a week and allowed to form colonies for 7-28 days. Colonies were visualized by staining with MTT (2 mg/ml). Colonies were imaged and quantitated using ImageJ (National Institute of Health).

Cell cycle analysis by flow cytometry

DNA content was assayed using propidium iodide. Cells were harvested, washed and fixed in 70% ethanol. Cells were permeabolized with 0.5% Triton X-100 and stained with propidium iodide. Stained cells were quantitated on the CyAN flow cytometer (Beckman Coulter). Distribution of cells in each stage of the cell cycle was performed using ModFit (Verity Software House).

Immunofluorescence

PANC-1 and HPAC cells were plated on poly-L-lysine (Sigma) coated coverslips. Cells were fixed in 4% paraformaldehyde, permeabolized with 0.2% Triton X-100. Cells were then blocked in 5% BSA in PBS. Cells were allowed to stain for 2 h at room temperature, washed three times with PBS. Alexa Fluor-conjugated secondary antibodies (Invitrogen) and DAPI were allowed to bind for 1 h. Stained cells were washed three times with PBS, followed by one wash with distilled water. Coverslips were then mounted with Fluorsave (Calbiochem).

All confocal images were obtained on a Zeiss 710 spectral confocal laser scanning confocal microscope equipped with a 405, 458, 488, 514, 543, 594, 633 nm excitation lines. Images were obtained using a 40X or 63X oil plan/Apo objective. Multicolor images were acquired using sequential scanning. All images were visualized using ImageJ and cropped using Photoshop.

Apoptosis Evaluation

Apoptosis was evaluated using an Annexin V assay (Roche) following manufacturer's protocol. Live cells were harvested in Tryp-LE (Gibco), washed in PBS, incubated in binding buffer with FITC conjugated Annexin V. Necrotic cells were discriminated by using a propidium iodide counterstain. Stained cells were quantitated on the CyAN flow cytometer (Beckman Coulter). At least 10,000 events were analyzed for each sample.

P^{32} incorporation assay

Kinase assays were performed using recombinant active GST-Tagged TTK (SignalChem). FLAG-Usp16 was purified from 293FT cells stably expressing FLAG-Usp16. Kinase assay was performed at 30°C for 15 and 30 min in 50 µl assay buffer (5 mM MOPS pH 7.2, 2.5 mM beta glycerol phosphate, 5 mM $MgCl_2$, 1 mM EGTA, 0.4 mM EDTA, 200 µM ATP, 0.4 mCi/ml [ϒ-^{32}P]ATP, 50 µM DTT, 50 ng/µl BSA). Five microliters of the reaction was spotted onto Whatman P81 cellulose phosphate filter paper. Reactions were quenched by washing the filter paper with 10% phosphoric acid three times for 5-10 min each. Filter paper was washed once with ethanol and dried. Filter papers were placed into scintillation vials, scintillation fluid was added to each vial, and P^{32} incorporation was assessed on the Beckman LS6500.

Results:

High expression of spindle assembly checkpoint kinases identified in PDAC

Protein kinases are the most frequently mutated family of genes that promote cancer and are therefore an attractive class of highly druggable molecular targets (Lahiry *et al.*, 2010). However, mutations in protein kinases are relatively uncommon in PDAC (17.6%)

compared to breast (75%), lung (86%), and colon (93%) cancers (S Jones *et al.*, 2008; Biankin *et al.*, 2012; Cancer Genome Atlas Network, 2012; Koboldt *et al.*, 2012) (https://tcga-data.nci.nih.gov/). Gene amplifications and overexpression of protein kinases has also been shown to promote oncogenesis (Chou *et al.*, 2013). Thus we examined what protein kinases were overexpressed in PDAC by analyzing gene expression profiles of previously published cDNA microarrays of primary PDAC tumors (n=30) and normal pancreas (n=20) (Stratford *et al.*, 2010). We identified differentially expressed kinases using the Statistical Analysis of Microarrays (SAM) software (Tusher *et al.*, 2001; YH Yang *et al.*, 2002). Of 3899 differentially expressed genes between primary patient tumors and normal pancreas, 106 probes were representing 91 kinases were identified (Table 10).

We identified biological networks regulated by the differentially expressed kinases using IPA. We found that the cell cycle was one of the most enriched biological pathways regulated by our list of kinases in PDAC (Table 11, Figure 12A, 13), with 7 of the 10 most upregulated kinases (Table 12). Interestingly, three of these seven kinases are involved with the spindle assembly checkpoint (SAC): BUB1, BUB1B, and TTK. Further interrogation of the gene expression profiles found statistically significant co-overexpression of both kinase and non-kinase components of the SAC in PDAC tumors compared to normal pancreas (Figure 12B). Together these data suggests that the SAC may be important for mitotic fidelity in PDAC.

TTK kinase activity is essential to activate the SAC and prevent early anaphase onset (Hardwick *et al.*, 1996; Bettencourt-Dias *et al.*, 2004; MH Jones *et al.*, 2005; May and Hardwick, 2006). In order to evaluate the role of TTK we evaluated the expression of TTK at the protein level and confirmed that a subset of primary PDAC patient samples had elevated

TTK protein expression compared to normal pancreas tissue (Figure 12C). In addition, we evaluated TTK protein expression in a panel of 14 PDAC cell lines by immunoblot analysis (Figure 12D). We selected two cell lines with moderate TTK expression, HPAC and PANC-1, to conduct our studies.

TTK is necessary for PDAC growth

To determine the functional consequence of TTK expression on PDAC growth we depleted TTK in PANC-1 and HPAC cell lines using a pool of 5 individually targeted siRNAs (Figure 14). TTK protein knockdown was confirmed by immunoblot analysis (Figure 15A). HPAC and PANC-1 cells depleted of TTK showed a significant decrease in proliferation as determined by the thiazolyl blue tetrazolium bromide (MTT) assay (Figure 15B). Catalytic inhibition of TTK with AZ3146 (AstraZeneca) phenocopied the decrease in proliferation seen with siRNA in HPAC and PANC-1 cells (Figure 15C). We next examined the requirement for TTK to support PDAC transformed growth by measuring anchorage-independent colony formation in soft agar, a standard assay for cellular transformation. Genetic depletion or catalytic inhibition of TTK in PANC-1 and HPAC cell lines significantly decreased transformed growth of the cells (Figure 15 D-F).

Override of the spindle assembly checkpoint and aberrant cell cycle progression and mitotic aberrancies and apoptosis

It is well established that TTK is required for proper SAC activation and function (X Liu and Winey, 2012). To understand the molecular mechanism whereby TTK supports PDAC growth we investigated the effects of TTK inhibition on SAC activation. HPAC and PANC-1 cells were arrested in mitosis with 100 ng/ml nocodazole and then challenged with

Table 10. Overexpressed protein kinases in primary PDAC compared to normal pancreas, with their respective fold-change values and false discovery rates.

Gene ID	Gene Name	Gene Product	Fold Change	q-value(%)
AGI_HUM1_OLIGO_A_23_P201988	*MASTL*	microtubule associated serine/threonine kinase-like	1.75	0.53
AGI_HUM1_OLIGO_A_23_P20248	*MAP2K1*	mitogen-activated protein kinase kinase 1	1.42	2.49
AGI_HUM1_OLIGO_A_23_P207896	*CSNK1D*	casein kinase 1, delta	1.38	2.02
AGI_HUM1_OLIGO_A_23_P213114	*TEC*	tec protein tyrosine kinase	1.33	4.20
AGI_HUM1_OLIGO_A_23_P215461	*LIMK1*	LIM domain kinase 1	1.54	1.43
AGI_HUM1_OLIGO_A_23_P216920	*NEK6*	NIMA (never in mitosis gene a)-related kinase 6	1.31	5.07
AGI_HUM1_OLIGO_A_23_P24997	*CDK4*	cyclin-dependent kinase 4	1.33	2.93
AGI_HUM1_OLIGO_A_23_P251342	*GSK3B*	glycogen synthase kinase 3 beta	1.32	0.39
AGI_HUM1_OLIGO_A_23_P252106	*RIPK2*	receptor-interacting serine-threonine kinase 2	1.35	2.93
AGI_HUM1_OLIGO_A_23_P256312	*MST1R*	macrophage stimulating 1 receptor (c-met-related tyrosine kinase)	2.00	0.13
AGI_HUM1_OLIGO_A_23_P259586	*TTK*	TTK protein kinase	2.97	0.00
AGI_HUM1_OLIGO_A_23_P3204	*MAPK6*	mitogen-activated protein kinase 6	1.41	2.93
AGI_HUM1_OLIGO_A_23_P342067	*UHMK1*	U2AF homology motif (UHM) kinase 1	1.36	2.02
AGI_HUM1_OLIGO_A_23_P35219	*NEK2*	NIMA (never in mitosis gene a)-related kinase 2	3.42	0.00
AGI_HUM1_OLIGO_A_24_P166663	*CDK6*	cyclin-dependent kinase 6	1.66	1.43

Table 10 (continued). Overexpressed protein kinases in primary PDAC compared to normal pancreas, with their respective fold-change values and false discovery rates.

Gene ID	Gene Name	Gene Product	Fold Change	q-value(%)
AGI_HUM1_OLIGO_A_23_P359245	*MET*	met proto-oncogene (hepatocyte growth factor receptor)	2.38	0.00
AGI_HUM1_OLIGO_A_23_P39684	*TLK1*	tousled-like kinase 1	1.29	2.49
AGI_HUM1_OLIGO_A_23_P397341	*PKMYT1*	progestin and adipoQ receptor family member IV	1.79	0.00
AGI_HUM1_OLIGO_A_23_P418413	*OXSR1*	oxidative-stress responsive 1	1.33	1.12
AGI_HUM1_OLIGO_A_23_P42784	*STK31*	serine/threonine kinase 31	1.91	2.93
AGI_HUM1_OLIGO_A_23_P51646	*PLK3*	polo-like kinase 3 (Drosophila)|Tctex2 beta	1.57	5.07
AGI_HUM1_OLIGO_A_23_P55578	*RIOK3*	RIO kinase 3 (yeast)	1.48	2.49
AGI_HUM1_OLIGO_A_23_P55584	*RIOK3*	RIO kinase 3 (yeast)	1.53	1.77
AGI_HUM1_OLIGO_A_23_P56978	*PTK6*	PTK6 protein tyrosine kinase 6	3.21	0.00
AGI_HUM1_OLIGO_A_23_P57667	*PLXNA1*	plexin A1	1.71	0.00
AGI_HUM1_OLIGO_A_23_P66732	*GSG2*	germ cell associated 2 (haspin)	1.44	2.49
AGI_HUM1_OLIGO_A_23_P75989	*PAK1*	p21/Cdc42/Rac1-activated kinase 1 (STE20 homolog, yeast)	1.49	2.02
AGI_HUM1_OLIGO_A_23_P76731	*RAGE*	renal tumor antigen	1.52	2.02
AGI_HUM1_OLIGO_A_23_P94422	*MELK*	maternal embryonic leucine zipper kinase	1.80	0.53

Table 10 (continued). Overexpressed protein kinases in primary PDAC compared to normal pancreas, with their respective fold-change values and false discovery rates.

Gene ID	Gene Name	Gene Product	Fold Change	q-value(%)
AGI_HUM1_OLIGO_A_24_P251899	*CSNK1A1*	casein kinase 1, alpha 1	1.30	3.47
AGI_HUM1_OLIGO_A_24_P313504	*PLK1*	endoplasmic reticulum to nucleus signaling 2|polo-like kinase 1 (Drosophila)	1.52	2.02
AGI_HUM1_OLIGO_A_24_P319613	*NEK2*	NIMA (never in mitosis gene a)-related kinase 2	2.14	0.00
AGI_HUM1_OLIGO_A_24_P333663	*MAPK6*	mitogen-activated protein kinase 6	1.45	3.47
AGI_HUM1_OLIGO_A_24_P37441	*PDK1*	pyruvate dehydrogenase kinase, isozyme 1	1.76	0.53
AGI_HUM1_OLIGO_A_24_P42603	*TRIO*	triple functional domain (PTPRF interacting)	1.43	5.07
AGI_HUM1_OLIGO_A_24_P830690	*PDPK1*	3-phosphoinositide dependent protein kinase-1	1.36	4.20
AGI_HUM1_OLIGO_A_24_P94054	*STK4*	serine/threonine kinase 4	1.51	4.20
AGI_HUM1_OLIGO_A_32_P140501	*AXL*	AXL receptor tyrosine kinase	1.49	5.07
AGI_HUM1_OLIGO_A_32_P25204	*PRKDC*	similar to protein kinase, DNA-activated, catalytic polypeptide	1.35	3.47
AGI_HUM1_OLIGO_A_32_P62997	*PBK*	PDZ binding kinase	2.76	0.00
AGI_HUM1_OLIGO_A_32_P119174	*IPPK*	inositol 1,3,4,5,6-pentakisphosphate 2-kinase|centromere protein P	1.37	5.07
AGI_HUM1_OLIGO_A_24_P245646	*TP53RK*	TP53 regulating kinase	1.30	3.47

Table 10 (continued). Overexpressed protein kinases in primary PDAC compared to normal pancreas, with their respective fold-change values and false discovery rates.

Gene ID	Gene Name	Gene Product	Fold Change	q-value(%)
AGI_HUM1_OLIGO_A_24_P76319	*LOC642609*	similar to tau tubulin kinase 2	1.44	5.07
AGI_HUM1_OLIGO_A_23_P10559	*AATK*	apoptosis-associated tyrosine kinase	-1.98	1.77
AGI_HUM1_OLIGO_A_23_P110791	*CSF1R*	colony stimulating factor 1 receptor, formerly McDonough feline sarcoma viral (v-fms) oncogene homolog	-1.89	4.20
AGI_HUM1_OLIGO_A_23_P125596	*RPS6KA6*	ribosomal protein S6 kinase, 90kDa, polypeptide 6	-1.28	4.20
AGI_HUM1_OLIGO_A_23_P126416	*TIE1*	tyrosine kinase with immunoglobulin-like and EGF-like domains 1	-1.73	1.43
AGI_HUM1_OLIGO_A_23_P128447	*LRRK2*	leucine-rich repeat kinase 2	-1.64	0.22
AGI_HUM1_OLIGO_A_23_P134125	*MAP3K5*	mitogen-activated protein kinase kinase kinase 5	-1.41	3.47
AGI_HUM1_OLIGO_A_23_P142304	*MKNK2*	MAP kinase interacting serine/threonine kinase 2	-1.74	2.93
AGI_HUM1_OLIGO_A_23_P142310	*MKNK2*	MAP kinase interacting serine/threonine kinase 2	-1.61	2.02
AGI_HUM1_OLIGO_A_23_P147711	*NPR1*	natriuretic peptide receptor A/guanylate cyclase A (atrionatriuretic peptide receptor A)	-1.75	0.14
AGI_HUM1_OLIGO_A_23_P159169	*AATK*	apoptosis-associated tyrosine kinase	-1.29	3.47
AGI_HUM1_OLIGO_A_23_P164057	*MAPK7*	microfibrillar-associated protein 4|mitogen-activated protein kinase 7	-2.40	0.53
AGI_HUM1_OLIGO_A_23_P16817	CLK1	CDC-like kinase 1	-1.67	1.12

Table 10 (continued). Overexpressed protein kinases in primary PDAC compared to normal pancreas, with their respective fold-change values and false discovery rates.

Gene ID	Gene Name	Gene Product	Fold Change	q-value(%)
AGI_HUM1_OLIGO_A_23_P202245	*RET*	ret proto-oncogene	-1.46	5.07
AGI_HUM1_OLIGO_A_23_P169819	*EPHA3*	EPH receptor A3	-1.87	2.02
AGI_HUM1_OLIGO_A_23_P205900	*NTRK3*	neurotrophic tyrosine kinase, receptor, type 3	-1.91	0.27
AGI_HUM1_OLIGO_A_23_P207517	*PDK2*	pyruvate dehydrogenase kinase, isozyme 2	-1.36	1.77
AGI_HUM1_OLIGO_A_23_P211985	*SNRK*	SNF related kinase	-1.60	1.77
AGI_HUM1_OLIGO_A_23_P219105	*FGFR1*	fibroblast growth factor receptor 1 (fms-related tyrosine kinase 2, Pfeiffer syndrome)	-1.46	3.47
AGI_HUM1_OLIGO_A_23_P253602	*BMX*	BMX non-receptor tyrosine kinase	-1.81	1.12
AGI_HUM1_OLIGO_A_23_P300033	*PDGFRA*	platelet-derived growth factor receptor, alpha polypeptide	-2.04	2.02
AGI_HUM1_OLIGO_A_23_P301304	*FGFR1*	fibroblast growth factor receptor 1 (fms-related tyrosine kinase 2, Pfeiffer syndrome)	-1.75	1.43
AGI_HUM1_OLIGO_A_23_P34804	*NTRK1*	neurotrophic tyrosine kinase, receptor, type 1	-1.55	4.20
AGI_HUM1_OLIGO_A_23_P372923	*FGFR1*	fibroblast growth factor receptor 1 (fms-related tyrosine kinase 2, Pfeiffer syndrome)	-1.47	5.07
AGI_HUM1_OLIGO_A_23_P374695	*TEK*	TEK tyrosine kinase, endothelial (venous malformations, multiple cutaneous and mucosal)	-2.89	0.00
AGI_HUM1_OLIGO_A_23_P397455	*ACVR1C*	activin A receptor, type IC	-1.67	5.07

Table 10 (continued). Overexpressed protein kinases in primary PDAC compared to normal pancreas, with their respective fold-change values and false discovery rates.

Gene ID	Gene Name	Gene Product	Fold Change	q-value(%)
AGI_HUM1_OLIGO_A_23_P46618	*PLXNA2*	plexin A2	-1.94	3.47
AGI_HUM1_OLIGO_A_23_P55107	*ULK2*	unc-51-like kinase 2 (C. elegans)	-1.82	0.27
AGI_HUM1_OLIGO_A_23_P424	*MARK1*	MAP/microtubule affinity-regulating kinase 1	-1.64	2.02
AGI_HUM1_OLIGO_A_23_P61674	*CLK4*	CDC-like kinase 4	-1.56	2.02
AGI_HUM1_OLIGO_A_23_P84974	*NRK*	Nik related kinase	-2.27	3.47
AGI_HUM1_OLIGO_A_24_P106112	*PKD2*	polycystic kidney disease 2 (autosomal dominant)	-1.41	4.20
AGI_HUM1_OLIGO_A_24_P179585	*MARK1*	MAP/microtubule affinity-regulating kinase 1	-1.47	4.20
AGI_HUM1_OLIGO_A_24_P243749	*PDK4*	pyruvate dehydrogenase kinase, isozyme 4	-3.69	0.00
AGI_HUM1_OLIGO_A_24_P263144	*BMX*	BMX non-receptor tyrosine kinase	-1.98	0.22
AGI_HUM1_OLIGO_A_24_P319923	*MYLK*	myosin, light chain kinase	-2.25	4.20
AGI_HUM1_OLIGO_A_24_P4171	*FGFR1*	fibroblast growth factor receptor 1 (fms-related tyrosine kinase 2, Pfeiffer syndrome)	-1.39	4.20
AGI_HUM1_OLIGO_A_24_P71973	*KDR*	kinase insert domain receptor (a type III receptor tyrosine kinase)	-1.68	1.77
AGI_HUM1_OLIGO_A_32_P100379	*PDGFRA*	platelet-derived growth factor receptor, alpha polypeptide	-1.79	0.39

Table 10 (continued). Overexpressed protein kinases in primary PDAC compared to normal pancreas, with their respective fold-change values and false discovery rates.

Gene ID	Gene Name	Gene Product	Fold Change	q-value(%)
AGI_HUM1_OLIGO_A_32_P105865	*ROCK1*	Rho-associated, coiled-coil containing protein kinase 1	-1.60	5.07
AGI_HUM1_OLIGO_A_32_P183765	*ERBB4*	v-erb-a erythroblastic leukemia viral oncogene homolog 4 (avian)	-2.13	3.47
AGI_HUM1_OLIGO_A_32_P200586	*CLK1*	CDC-like kinase 1	-1.37	3.47
AGI_HUM1_OLIGO_A_32_P94160	*PRKAA2*	protein kinase, AMP-activated, alpha 2 catalytic subunit	-1.60	2.49

Table 11. Biological pathways enriched with differentially expressed kinases.		
Network name	***p*-Value**	**# of nodes**
Post-translational modification	1.14E-67 – 3.85E-04	66
Cell cycle	6.76E-24 – 4.16E-04	43
Amino acid metabolism	1.46E-19 -1.45E-08	18
Small molecule biochemistry	1.46E-19 – 2.42E-06	25
Cell death and survival	5.53E-19 – 4.16E-01	58

Table 12. Function of the 10 most overexpressed kinases			
Kinase	**Fold Change**	**FDR (Q-Value)**	**Function**
BUB1	3.887	0	Cell cycle
NEK2	3.424	0	Cell cycle
CHEK1	3.362	0	Cell cycle
PTK6	3.213	0	Differentiation and apoptosis
TTK	2.972	0	Cell cycle
PBK	2.756	0	Cell cycle
STYK1	2.65	0.125	Proliferation and survival
MET	2.381	0	Growth factor sensing
CDK1	2.343	0	Cell cycle
BUB1B	2.114	0	Cell cycle

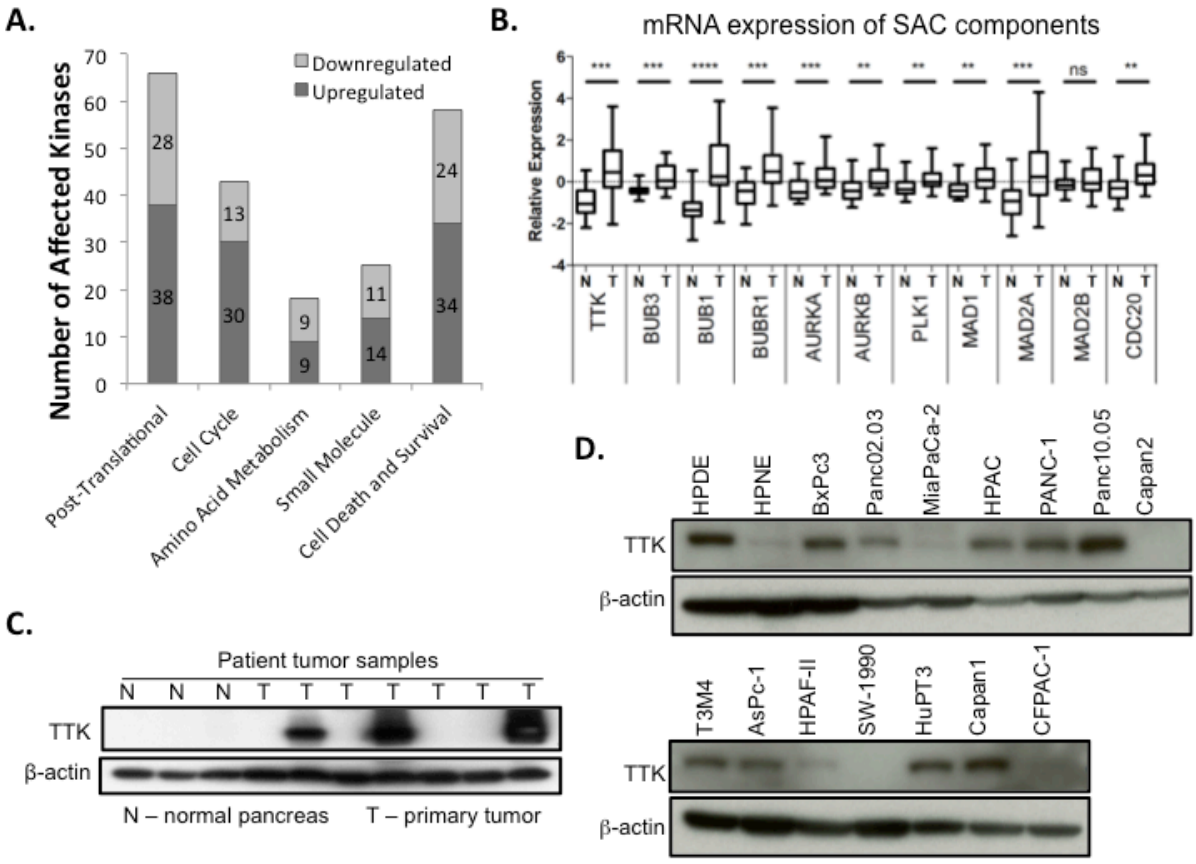

Figure 12. Increased expression spindle assembly checkpoint components and its regulator TTK in PDAC. (A) Ingenuity Pathway Analysis software identified prominent cellular functions that were significantly affected by differentially expressed kinases between normal and primary PDAC. (B) Box and whisker plot of median, upper, and lower quartiles of mRNA expression of core components and regulators of the spindle assembly checkpoint. Asterisk represent the p-value of the Mann-Whitney test (ns: $p \geq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$). (C) Expression of TTK in a panel of patient samples. N = normal pancreas and T = primary tumor. (D) Expression of TTK in immortalized pancreas epithelium (HPNE and HPDE) and PDAC cell lines.

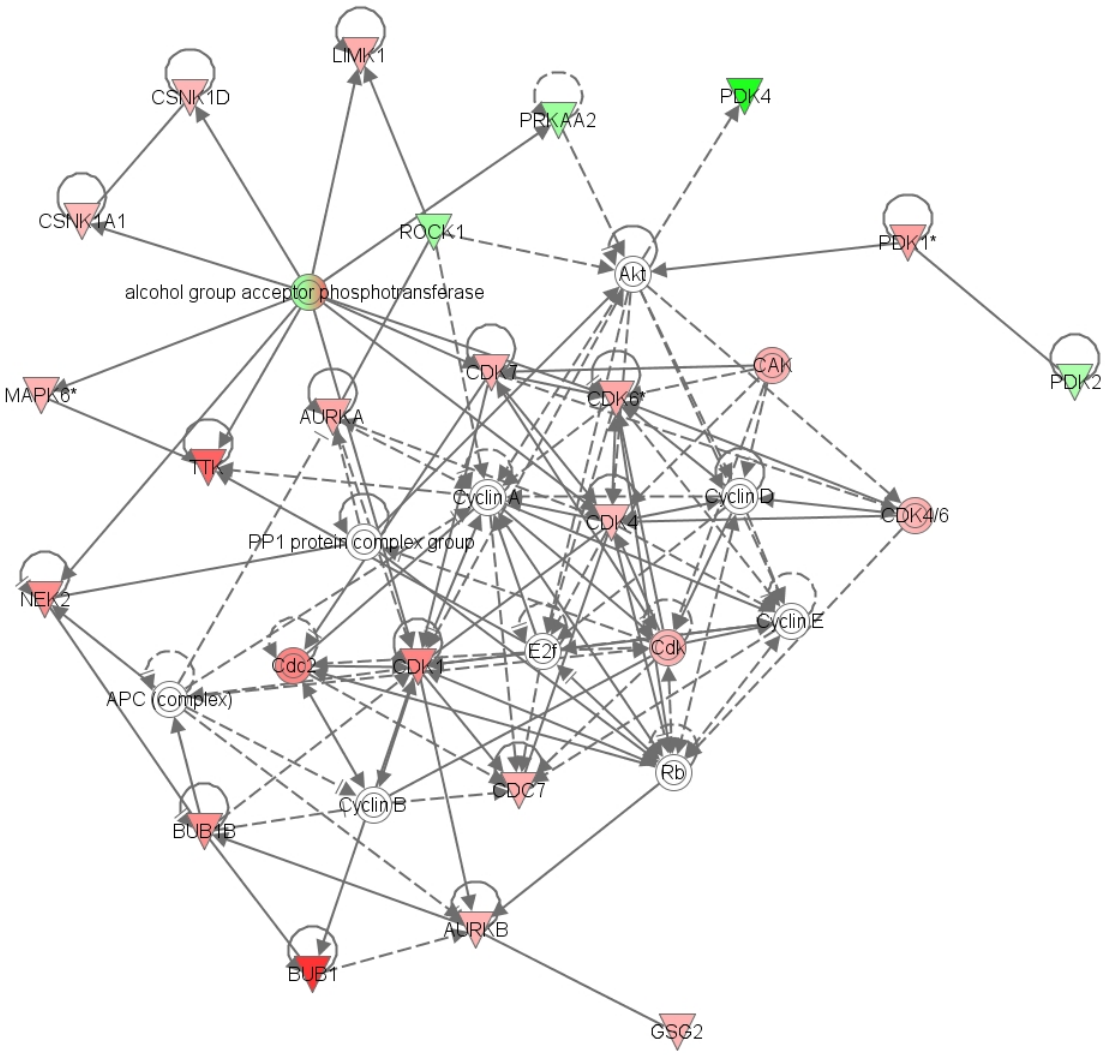

Figure 13. Ingenuity pathway analysis of differentially expressed kinases in primary PDAC compared to normal pancreas identified by unbiased methods. All 106 differentially regulated kinases were analyzed by integrated pathway analysis using Ingenuity. The posttranslational modification cell cycle, cellular assembly and organization, one of the most dominant networks, is depicted here. Signaling pathways are colored according to expression, green representing downregulation and red representing upregulation with the expression fold change represented by more intense colors.

2 μM AZ3146. We monitored the stability of the mitotic marker cyclin B to assess silencing of the SAC. Catalytic inhibition of TTK caused a drop in the levels of cyclin B (Figure 16A), indicating that the catalytic inhibition of TTK caused an escape from checkpoint mediated mitotic arrest and accelerated mitotic progression. We next investigated the effect of checkpoint silencing on cell cycle progression. We determined the cell cycle phase distribution of HPAC and PANC-1 cells treated with siRNA or AZ3146 after 72 h by propidium iodide staining and flow cytometry analysis. Aberrant distribution of cells in each cell cycle phase occurred, specifically an increase post G2 cells, indicative of multi-nucleation (Figure 16B-C).

TTK is required to prevent aneuploidy and apoptosis

Studies in other tumors have found that TTK depletion causes premature mitotic progression and often results in aberrant chromosomal segregation and aneuploidy (Fisk *et al.*, 2003; Tighe *et al.*, 2008; Tannous *et al.*, 2013). In PDAC, TTK inhibition induced gains in the X chromosome and chromosome 17. However, the effect of TTK inhibition on the nuclear architecture is unknown. We examined the nuclear architecture of PANC-1 cells stably transduced with a lenti-viral vector encoding H2B-GFP followed by depletion of TTK using siRNA. Fluorescent microscopy revealed gross multi- and micro-nucleation in TTK depleted cell lines compared to mismatch siRNA controls, which reflected widespread chromosomal segregation defects (Figure 16D-E). These results demonstrate the requirement of TTK for mitotic fidelity and that depletion of TTK results in an increase of chromosomal instability and aneuploidy.

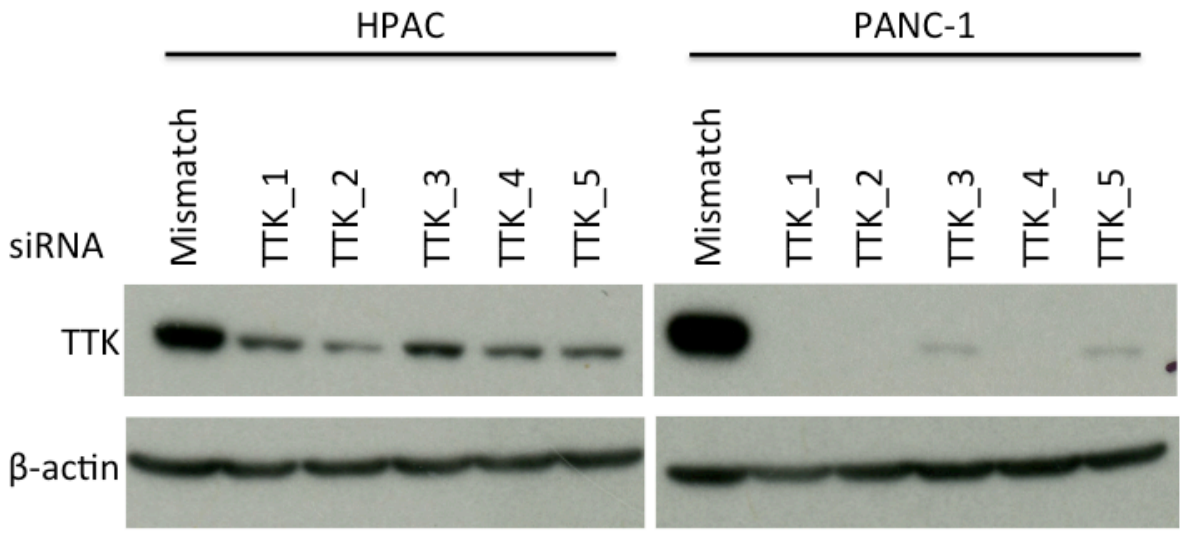

Figure 14. Individual targeting of TTK of each siRNA used in the pool. Immunoblot analysis of HPAC and PANC-1 cell lines 48 h after transfection with 10 nM siRNA targeting TTK.

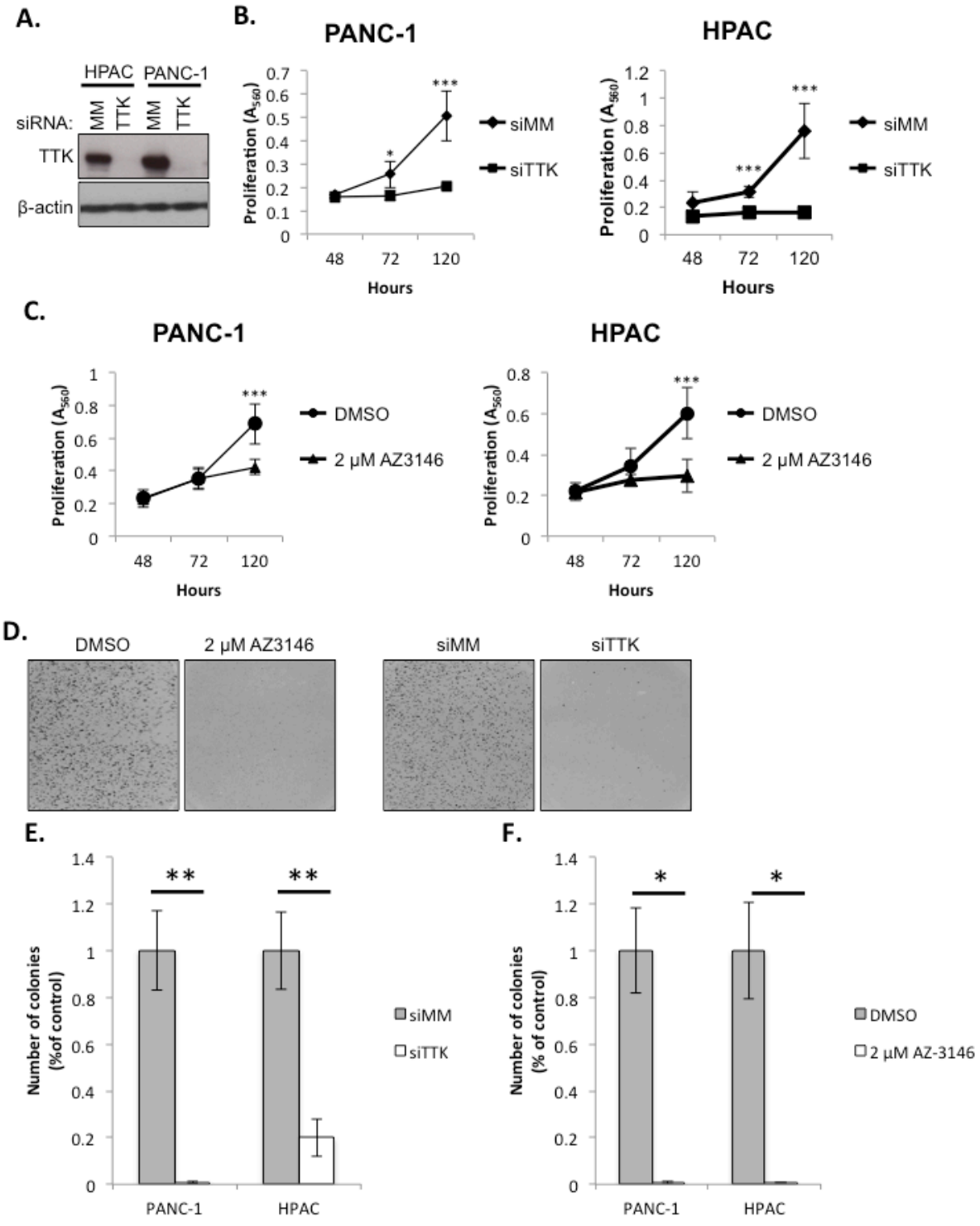
A.
HPAC
PANC-1
siRNA:
MM
TTK
MM
TTK
TTK
β-actin
B.
PANC-1
HPAC
Proliferation (A560)
Hours
48
72
120
siMM
siTTK
*
***
C.
PANC-1
HPAC
DMSO
2 μM AZ3146
D.
DMSO
2 μM AZ3146
siMM
siTTK
E.
F.
Number of colonies (%of control)
Number of colonies (% of control)
PANC-1
HPAC
**
*
2 μM AZ-3146

Figure 15. Genetic and pharmacologic inhibition of TTK decrease growth of PDAC cell lines. (A) Immunoblot analysis of HPAC and PANC-1 cell extracts showing protein level of TTK in control mismatch siRNA (siMM) and a TTK siRNA (siTTK) pool 48 h after transfection. (B) Growth of HPAC and PANC-1 PDAC cell lines transfected with control siMM and siTTK show reduced proliferation with TTK depletion. Cells were measured for proliferation at 48, 72, and 120 h as indicated. (C) Growth of HPAC and PANC-1 PDAC cell lines treated with DMSO control or 2 μM AZ3146. Cells were measured for proliferation at 48, 72, and 120 h as indicated. (D) Representative images of colony formation of the PANC-1 cell line in soft agar. (E) Quantitation of colony formation in soft agar of the HPAC and PANC-1 cell lines after transfection of either control or TTK targeted siRNA. Samples normalized to control. (F) Quantitation of colony formation in soft agar of the HPAC and PANC-1 cell lines after with continuous treatment with vehicle (DMSO) or AZ3146. Normalized to DMSO control. Asterisk represent the P-value of the two sided T-test (ns: $P \geq 0.05$,*:≤ 0.05, P **: $P \leq 0.01$, ***: $P \leq 0.001$, ****: $P \leq 0.0001$). Results representative of at least 2 experiments.

Unstable aneuploidy is often associated with cell death from mitotic catastrophe. To assess whether TTK is important to preventing apoptosis in PDAC cell lines, we monitored apoptotic induction by flow cytometry. siRNA mediated TTK depletion in PANC-1 cells resulted in increased Annexin V staining. However, at the same time point, catalytic inhibition of TTK did not promote apoptosis (Figure 16F-G).

Usp16 is a direct substrate of TTK

TTK has been shown to regulate mitotic progression by phosphorylation of mitotic regulators (Leng *et al.*, 2006; Jelluma, Brenkman, van den Broek, *et al.*, 2008). However, substrates of TTK that mediate mitotic progression require further characterization. To further delineate the molecular mechanism whereby TTK regulates proper mitotic progression necessary for growth we sought to identify phosphorylation substrates of TTK. To identify putative substrates of TTK we used the Scansite3 (http://scansite.mit.edu/) prediction software with an input phosphorylation consensus motif recently identified by Hennrich et al (Hennrich *et al.*, 2013). This motif consists of a threonine residue with acidic amino acids in the −2, and/or −3 positions and hydrophobic branched-chain amino acids (leucine, valine and isoleucine) in the +2 and +3 position (Hennrich *et al.*, 2013). Using this approach we identified 410 putative TTK phosphorylation substrates in humans. We focused on phosphorylation substrates with known roles in mitotic networks (Table 12) and identifiedthe ubiquitin specific peptidase 16 (Usp16, also known as Ubp-M) as a putative phosphorylation substrate of TTK.

To examine if Usp16 is a direct phosphorylation substrate of TTK we performed an *in vitro* kinase assay with ADP-P^{32} and measured substrate incorporation of P^{32}. Incorporation of P^{32} on purified Usp16 was enhanced when incubated with active TTK (SignalChem) and

inhibited upon addition of 2 μM AZ3146 (Figure 17A). These results indicate that Usp16 is directly phosphorylated by TTK *in vitro*. We next sought to identify the TTK dependent phosphorylation sites on Usp16 by performing mass spectrometry. TTK activity was stimulated with nocodazole in 293FT cells exogenously expressing a flag-tagged Usp16. Cells were then concurrently challenged with DMSO control or 2 μM AZ3146 for 4 h. FLAG-Usp16 from the cell lysate was purified by immunoprecipitation, digested with trypsin, and enriched for phosphopeptides. Amino acid composition of phosphopeptides was then identified by orbi-trap mass spectrometry. Analysis of the resulting spectra identified three TTK-dependent phosphorylation sites within Usp16: S415, S552, T554 (Figure 17B).

TTK dependent phosphorylation of Usp16 causes protein degradation

To investigate the importance of phosphorylation of these three residues on Usp16 function we created a green fluorescent protein (GFP) tagged phospho-mimetic mutant of Usp16 where glutamic acid residues were substituted for the three identified phosphorylation sites (GFP-Usp16 3xE), and a phospho-deficient mutant of Usp16 where alanine residues were substituted for the three identified phosphorylation sites (GFP-Usp16-3xA). Immunoblot analysis of 293FT cells transfected with cDNA for wild type and mutant Usp16 revealed decreased expression of the phospho-mimetic mutant of Usp16. RT-PCR with Usp16 specific primers confirmed equivalent mRNA expression, suggesting that Usp16 phosphorylation promotes protein degradation. Inhibition of the proteasome with 10 μM MG-132 restored expression of the phosphomimetic Usp16 mutant (Figure 17C-E). Taken together, these data suggest that Usp16 is a phosphorylation substrate of TTK and that Usp16 phosphorylation on S415, S552, or S554 leads to proteasome degradation of Usp16. **Discussion:**

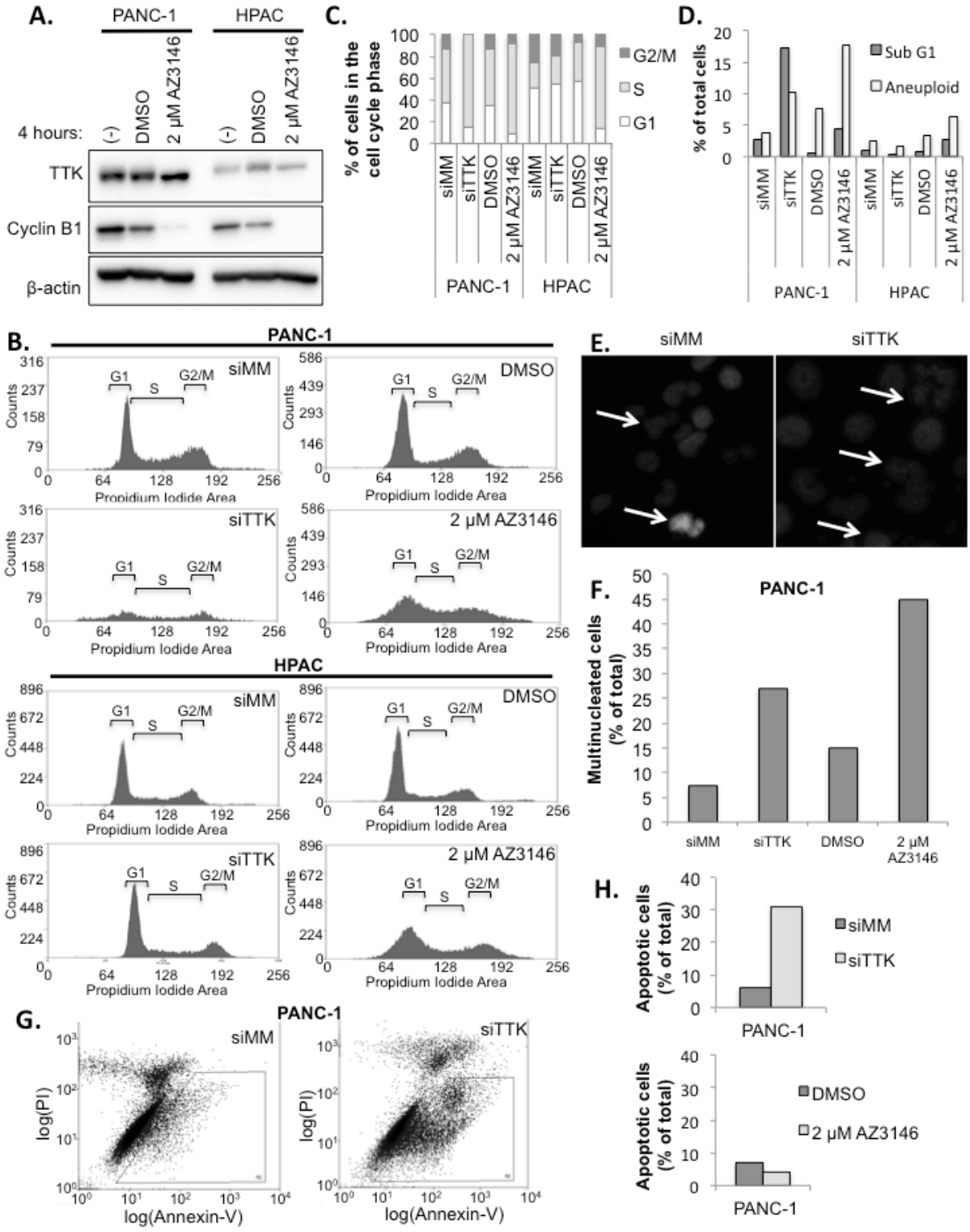
A.
PANC-1
HPAC
4 hours:
(-)
DMSO
2 μM AZ3146
TTK
Cyclin B1
β-actin
C.
% of cells in the cell cycle phase
G2/M
S
G1
siMM
siTTK
DMSO
2 μM AZ3146
PANC-1
HPAC
D.
% of total cells
Sub G1
Aneuploid
B.
PANC-1
siMM
DMSO
siTTK
2 μM AZ3146
Counts
Propidium Iodide Area
HPAC
E.
siMM
siTTK
F.
PANC-1
Multinucleated cells (% of total)
G.
PANC-1
log(PI)
log(Annexin-V)
H.
Apoptotic cells (% of total)
siMM
siTTK
DMSO
2 μM AZ3146

Figure 16. TTK inhibition overrides the SAC mediated cell cycle arrest and leads to aberrant cell cycle progression, multi-nucleation and apoptosis. (A) Immunoblot of HPAC and PANC-1 PDAC cell lines arrested in mitosis by treatment with nocodazole. Cells were then treated with 2 μM AZ3146 for 4 h and probed for expression of cyclin B1. (B) Representative flow cytometry plots of the cell cycle of HPAC and PANC-1 cell lines of 2 experiments. Cells were transfected with control or TTK targeted siRNA. 72 h post transfection cells were fixed and stained with propidium iodide. DNA content was assessed by flow cytometry. (C) Quantitation of B showing distribution of cells in each phase of the cell cycle. (D) Confocal microscopy of the PANC-1 cell line stably expressing a GFP-Histone 2B construct to visualize DNA. Chromosomal instability is visible in cells depleted of TTK in the form of multi- and micro-nucleation. (E) Quantitation of cells with multi- or micro-nucleated phenotypes. (F) Scatterplots showing induction of apoptosis with depletion of TTK. PANC-1 cells were transfected with control or a TTK targeted siRNA pool. 72 h post transfection cells were harvested and stained with the apoptotic marker Annexin V and the counterstained with propidium iodide to visualize necrotic cells. (G) quantitation of the apoptotic induction of cells used in F.

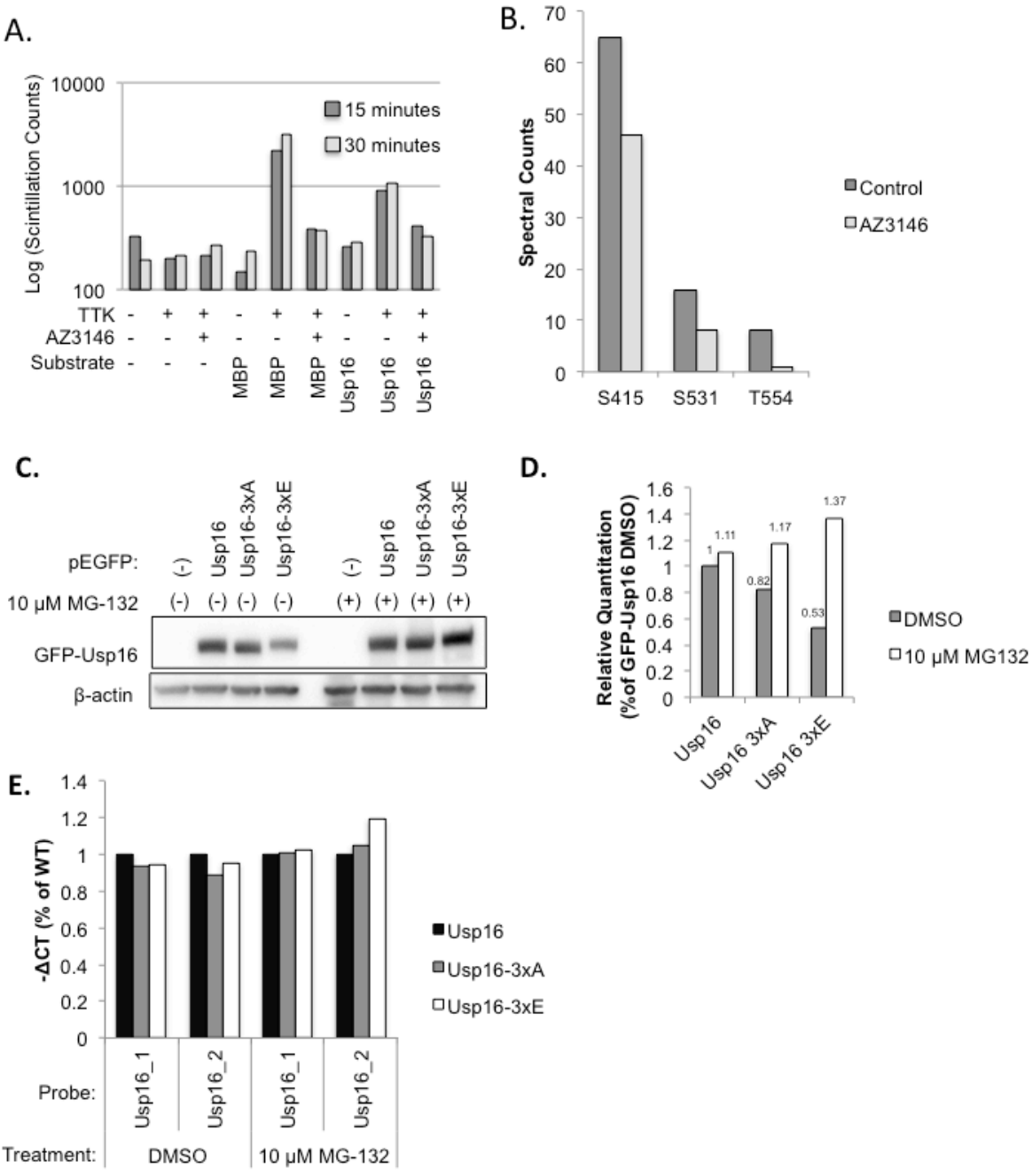
A.
Log (Scintillation Counts)
10000
1000
100
15 minutes
30 minutes
TTK
AZ3146
Substrate
MBP
MBP
MBP
Usp16
Usp16
Usp16
B.
Spectral Counts
70
60
50
40
30
20
10
0
S415
S531
T554
Control
AZ3146
C.
pEGFP:
(-)
Usp16
Usp16-3xA
Usp16-3xE
10 μM MG-132
GFP-Usp16
β-actin
D.
Relative Quantitation (%of GFP-Usp16 DMSO)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1
1.11
0.82
1.17
0.53
1.37
Usp16
Usp16 3xA
Usp16 3xE
DMSO
10 μM MG132
E.
-ΔCT (% of WT)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Probe:
Usp16_1
Usp16_2
Usp16_1
Usp16_2
Treatment:
DMSO
10 μM MG-132
Usp16
Usp16-3xA
Usp16-3xE

Figure 17. Usp16 is a TTK phosphorylation substrate. (A) *In vitro* kinase assay measuring TTK dependent phosphorylation by p32 incorporation measured by liquid scintillation counts. Representative of 2 independent experiments. (B) Exogenously expressed FLAG-Usp16 was immunoprecipitated from DMSO and AZ3146 treated mitotic 293FT cells, digested with trypsin and enriched for phosphopeptides. Phosphorylated residues of Usp16 were identified by mass spectrometry. Spectral counts of representative of individual experiments are shown. (C) Immunoblot analysis of 293FT cells transiently transfected with control GFP, GFP-Usp16, GFP-Usp16 3xA (phosphodeficient mutant) or GFP-Usp16 3xE (phosphomimetic mutant) and treated with control DMSO or MG-132. (D) Densitometry of (C). (E) RT-PCR of Usp16 using 2 independent taqman probes from cells used in from (C) normalized to β-actin and represented as percent of WT-Usp16.

Patients with PDAC have limited therapeutic options. Discovery of new and better drug targets is essential for the continued advancement of better therapies. Chromosomal instability and aneuploidy are characteristics of PDAC (Schreiner *et al.*, 2003; Aguirre *et al.*, 2004; Heidenblad, 2004; Hingorani *et al.*, 2005; Hezel *et al.*, 2006; Bardeesy *et al.*, 2006). The SAC limits chromosomal instability by ensuring faithful segregation of sister chromatids. Here we show RNA overexpression of the core components of the SAC in primary PDAC tumors compared to normal pancreas. We hypothesize that targeting the SAC function may alter the ability of cancer cells to adapt to aneuploidy and may be a possible therapy for PDAC.

TTK is a protein kinase required for SAC activation and was overexpressed in our dataset (X Liu and Winey, 2012). TTK was a gene previously identified in a 25-gene signature associated with chromosomal instability and aneuploidy in cancer (Carter *et al.*, 2006). Thus, overexpression of TTK may represent an adaptive mechanism to sustain the growth of chromosomally unstable tumors. In support of this hypothesis, overexpression of TTK has been previously observed in multiple tumor types including PDAC (Slee *et al.*, 2014), breast (Yuan *et al.*, 2006; Daniel *et al.*, 2011; Maire *et al.*, 2013; Al-Ejeh *et al.*, 2014), bladder (Thykjaer *et al.*, 2001), esophagus (Mizukami *et al.*, 2008), lung (Landi *et al.*, 2008), anaplastic thyroid (Salvatore *et al.*, 2007), and glioblastoma (Tannous *et al.*, 2013).

To investigate the role of TTK as an adaptive response to aneuploidy in PDAC we investigated the effect of both catalytic and genetic depletion of TTK on mitotic progression. In agreement with a previous study we show that catalytic inhibition of TTK caused an aberrant override of the SAC mediated cell cycle arrest in PDAC (Slee *et al.*, 2014). We anticipated that SAC override would result in aneuploidy as previously observed in other

cancer cell lines. While, we found that both knockdown and catalytic inhibition of TTK in the PANC-1 cell line increased aneuploidy, the HPAC cell line did not. Although this result was unexpected, previous reports have demonstrated heterogeneous response to TTK inhibition (Jemaà *et al.*, 2013). The difference in response could perhaps be explained by the hypothesis that cells with extra chromosomes may have a greater requirement for SAC function (Storchová *et al.*, 2006; Z Yang *et al.*, 2008). However, previous reported karyotypes show a modal number of 63 and 61 for the PANC-1 and HPAC cell lines respectively, suggesting that the level of aneuploidy does not account for this discrepancy (Lieber *et al.*, 1975; Gower *et al.*, 1994). Alternatively, the discrepancy could be attributed to differences in the genetic background of the two cell lines. Previous studies have shown that aneuploidy can stimulate a p53-dependent senescence-like growth arrest (Thompson and Compton, 2010; Li *et al.*, 2010). PANC-1 cells have inactivating mutations in both alleles of p53, whereas HPAC cells have two wild-type p53 alleles (Deer *et al.*, 2010). Mitotic errors resulting from TTK inhibition in HPAC cells may trigger p53 mediated growth arrest and prevent aneuploidy whereas PANC-1 lacking functional p53 continue to aberrantly divide. Further investigation in more PDAC cell lines with a wild-type p53 background will be required to determine whether TTK inhibition induces senescence-like phenotypes.

Importantly, we found that both pharmacologic inhibition and knockdown of TTK severely decreased PDAC cell line proliferation and transformed growth. Our results are in agreement with prior reports in other tumor types where either pharmacologic inhibition or genetic knockdown of TTK resulted in a similar decrease in the growth of multiple cancer cell lines (Schmidt *et al.*, 2005; Colombo *et al.*, 2010; Kwiatkowski *et al.*, 2010; Sliedrecht *et al.*, 2010; Tardif *et al.*, 2011; Daniel *et al.*, 2011; Tannous *et al.*, 2013; Jemaà *et al.*, 2013;

Maire *et al.*, 2013; Slee *et al.*, 2013). However, a key observation from our study is that we observed greater impairment of proliferation with genetic knockdown compared to pharmacologic inhibition. These data suggest that non-kinase domains of TTK are important to support proliferation of aneuploid cells. Although poorly characterized, the much larger N-terminus of TTK contains tandem tetratricopeptide repeats known to be important for protein binding (Thebault *et al.*, 2012; S Lee *et al.*, 2012; Nijenhuis *et al.*, 2013). However, the binding partners are currently undetermined and future studies will be required to identify TTK binding partners and characterize how they contribute to adapting to aneuploidy.

We also observed that 72 h of catalytic inhibition of TTK with AZ3146 did not induce apoptosis in the PANC-1 cell line whereas genetic knockdown of TTK did. In contrast to our results, Slee et al. found that pharmacologic inhibition of TTK with NMS-P715 in PANC-1 cells did induce apoptosis (Slee *et al.*, 2014). Differences in apoptotic induction may be due to non-TTK effects suggesting the need to further characterize these inhibitors.

In agreement with previous studies, we clearly demonstrate that the catalytic activity of TTK is required for cancer cell line mitotic regulation and growth. However, the phosphorylation substrate(s) of TTK are less clear. In an attempt to characterize the molecular pathways downstream of TTK, we identified Usp16 as a novel phosphorylation substrate of TTK. Usp16 dependent deubiquitination of histone H2A is a prerequisite for chromosomal condensation (Kouzarides, 2007; Joo *et al.*, 2007). We identified 3 TTK-dependent phosphorylation sites on Usp16: S415, S552, and T554. Point mutations in Usp16 (S415E, S552E, T554E) exhibited enhanced degradation compared to wild-type Usp16. We propose that phosphorylation and subsequent degradation of Usp16 may represent another

mechanism whereby TTK regulates genome stability by preventing chromosomal condensation and thus allowing more time to correct for errors that accumulated during DNA replication.

The high levels of chromosomal instability that exists in PDAC present a window that may be exploited for therapy. Our results demonstrate that SAC inactivation through inhibition of the upstream activator TTK decreases the ability of PDAC to adapt and support the growth of aneuploid cells. TTK inhibition has previously been shown to enhance chromosomal instability and sensitivity of cancer cell lines when combined with the microtubule targeting drugs vincristine or taxol (Janssen *et al.*, 2009; Tannous *et al.*, 2013; Jemaà *et al.*, 2013). Consistent with this idea, the microtubule targeting nab-paclitaxel in combination with gemcitabine was approved for PDAC therapy (Hoff *et al.*, 2014). Future studies will be required to determine whether TTK inhibition sensitizes PDAC cells to nab-paclitaxel. Altogether our results support continued study of the molecular mechanism that allow PDAC to adapt to chromosomal instability.

CHAPTER IV

Conclusions and Future Directions

In this dissertation I investigated the role of aberrant gene expression as a diagnostic marker and the therapeutic value of the gene products of an aberrantly-expressed gene in PDAC. Through the analysis of genome-wide gene expression profiles from PDAC patients, I have been able to identify a gene signature that can stratify patients into a high- and low-risk groups associated with overall survival. In addition, I identified and characterized the functional role of overexpression of the protein kinase TTK in PDAC oncogenesis.

Three main observations arise from my studies: 1) The impact of gene expression signatures for diagnostic and prognostic evaluation of PDAC. 2) The therapeutic value of TTK in PDAC. 3) The identification of Usp16 as a phosphorylation substrate of TTK. Future studies are required to further understand the clinical relevance of these findings.

Diagnostic gene expression for PDAC

One reason behind the high mortality rate of pancreatic cancer is the lack of selective and sensitive diagnostic tools for early detection. It is now accepted that there are three main precursor lesions: IPMN, MCN, and PanIN. While differences in morphological features and clinical outcome exist, each of these three precursor lesions can, through a multistep process, develop into invasive PDAC. Early detection of these lesions remains a challenge, yet development of diagnostic tools to detect tumors before the disease becomes invasive is necessary to allow for potentially curative surgical resection. The genetic progression of each of these lesions is also unique, yet some common genetic events, such as activating mutations

in *KRAS* and inactivating mutations of *p53*, and loss of $p16^{ink4a}$, have been observed in each of the precursor lesions (Hezel *et al.*, 2006; Smith *et al.*, 2012). This pattern of common mutations suggests that, in part, similar signaling pathways are deregulated and may contribute to oncogenesis. Comparison of gene expression profiles of PanIN, MCN, and IPMN precursor lesions may help identify these pathways and provide novel insight into the development of PDAC. Identification of gene expression changes of secreted proteins may help identify potential early biomarkers of disease. Although overexpression or loss of mRNA that encodes for secreted proteins may be identified, extensive confirmation would be required before hits could be classified as biomarkers.

Gene expression signatures and subtypes of disease

Whole-exome and genome sequencing have demonstrated that PDAC is a heterogeneous disease with diverse molecular subtypes (MH Jones *et al.*, 2005; Biankin *et al.*, 2012; Cowley *et al.*, 2013). As previously seen with breast cancer (Parker *et al.*, 2009), identification of the intrinsic molecular subtypes would impact clinical practices by helping asses risk for surgical procedures, therapy choice based on chemoresistance profiles, and identification of novel therapeutic targets. Therefore, it is vital that the molecular determinants of PDAC be defined. In this dissertation I present the first study to validate a prognostic gene signature for PDAC. It is important to note that this signature was developed by studying the molecular differences between nonmetastatic and metastatic primary tumors, suggesting that subtypes can be identified based on intrinsic tumor biology. Collisson et al. have since used consensus clustering of gene expression data to identify three molecular subtypes of PDAC: classical, quasimesenchymal, and exocrine-like. However, analysis of human and mouse PDAC cell lines failed to identify all three subtypes, suggesting that future work is needed to validate and potentially expand the subtypes of PDAC. It would be

interesting to perform consensus clustering on the TCGA and ICGC pancreatic cancer gene expression data set and compare any subtypes identified.

As subtypes of PDAC are identified, it will be necessary to perform extensive characterization of each subtype. Understanding the subtype-specific morphology, pathology, and clinical associations will be required to identify subtype-specific dependencies that can be targeted for therapy. Jones et al. identified 12 pathways and process whose component genes were genetically altered in PDAC (S Jones *et al.*, 2008). A similar analysis of gene expression data would complement the mutational analysis, identifying potential nodes for therapeutic intervention.

Therapeutic value of TTK for PDAC

Another observation that arose from my studies is the importance of TTK in PDAC growth. Based on the *in vitro* work we hypothesize that TTK is important for *in vivo* PDAC tumorigenesis. To evaluate this hypothesis it would be necessary to study the effect of TTK inhibition in genetically engineered mouse model. The KrasLSL.G12D/+; p53R172H/+; PdxCretg/+ mouse model (KPC) faithfully recapitulates many of the characteristics of human PDAC including morphology, stromal desmoplasia, and metastasis (Westphalen and Olive, 2012). To study the affect of TTK expression I propose to create a mouse model in the KPC mouse that also has a knockout of TTK. In zebra fish, inactivating mutations in the TTK orthologous gene *nightcap*, was embryonic lethal (Poss *et al.*, 2002). Additionally, homozygous deletions of SAC components in mice are also embryonic lethal (Foijer *et al.*, 2008). Furthermore, complete checkpoint inhibition has been shown to have adverse effects on non-transformed immortalized breast cancer cell lines (Kwiatkowski *et al.*, 2010; Maire *et al.*, 2013), suggesting that in addition to pancreas specific depletion, knockdown may also need to be inducible to allow for proper organ development. Doxycycline-inducible tissue

specific knockdown has previously been demonstrated in the pancreas (Collins *et al.*, 2012; Ying *et al.*, 2012). A key benefit of this model is that protein knockdown can be depleted in an established tumor, allowing characterization of TTK expression for tumor maintenance. Based on my cell culture studies, I anticipate that TTK depletion in this setting will exhibit an increase in cell cycle defects resulting in impaired tumor growth.

Standard therapeutic intervention of protein kinases is accomplished by pharmacologic inhibition of the kinase activity. Since TTK is a larger protein (~97 kDa) with previously determined non-kinase domain sequences, it is possible that non-kinase functions of TTK also contribute to PDAC growth. To determine whether abolishing catalytic function of TTK alone decreases tumor growth I would create a conditional knock-in mouse model. In the KPC background I would replace the wild-type TTK gene with a mutant TTK with a catalytic domain mutation (D664A) previously shown to abolish TTK kinase activity (L Zhang *et al.*, 2013).

Clinical efficacy of signal transduction inhibitors, in particular protein kinase inhibitors, has been hampered by emergence of cells resistant to the inhibitor. Resistance may arise as a consequence of pharmacologic inhibition. Cells can become resistant by activation of alternative pathways to reactivate a node downstream of inhibitor's target, and is an example of acquired resistance. In contrast, some tumors display intrinsic resistance. Intrinsic resistance occurs when cells within the tumor are inherently resistant to therapy before treatment begins. Initial clinical efficacy is observed with inhibitor treatment, as the sensitive tumor cells are eliminated and tumor size is reduced. However, the intrinsically resistant cells are unaffected by the inhibitor and eventually leading to tumor recurrence.

Resistance to a TTK pharmacologic inhibitor is a likely scenario. Therefore future

studies are needed to anticipate possible mechanisms of resistance with the goal to identify molecular targets that can be used to make a more effective combination therapy. To identify intrinsic and acquired resistance mechanisms I would isolate PDAC cell line variants after prolonged treatment with a high dose of the TTK inhibitor AZ3146. Comparison of exome sequencing of sensitive (pre-treatment) and resistant variants would identify mutations associated with resistance. Functional analysis would be required to determine the mechanism of resistance.

Aberrant activation of compensatory signaling cascades is another resistance mechanism that occurs in cancer, often referred to as feedback or reprogramming. To investigate compensatory reprogramming of the kinome that results from TTK inhibition I would determine global chances in kinase activation using multiplexed inhibitor beads and mass spectrometry (MIB/MS) (Duncan *et al.*, 2012). Comparison of kinase activity profiles of PDAC cell lines pre- and post-treatment with a TTK inhibitor may identify signal transduction pathways that become activated to compensate for loss of TTK catalytic activity. Once these pathways are identified, combination of kinase inhibitors that target TTK as well as the compensatory pathways will need to be evaluated *in vitro* and *in vivo*. Anticipating the mechanisms of resistance will hopefully decrease the rates of recurrence in PDAC patients.

Previous studies have also suggested that combination therapies of anti-mitotic drugs with TTK inhibitors enhance sensitivity. A study of glioblastoma cell lines found that inhibition of TTK enhanced sensitivity to the microtubule targeting drug vincristine (Tannous *et al.*, 2013). In addition, treatment of colorectal cancer cell lines with sub-lethal does of a TTK inhibitor (Mps-Bay1 or Mps-Bay2a) with paclitaxel demonstrated a

synergistic effect. siRNA mediated knockdown of TTK demonstrated similar synergy when combined with paclitaxel (Jemaà *et al.*, 2013). Recently a microtubule-targeted drug nab-paclitaxel in combination with gemcitabine was approved for PDAC therapy (Hoff *et al.*, 2014). Future studies will be required to determine if TTK inhibition could synergize with nab-paclitaxel to not only improve patient response, but also allow a lower dose of paclitaxel and reduce adverse side effects.

TTK phosphorylation substrates

Previous studies have found the importance of TTK for multiple functions in the cell including SAC activation, genotoxic stress response, centrosome duplication, cytokinesis, and meiosis, yet for the most part the molecular mechanism and phosphorylation substrates of TTK remain unclear (X Liu and Winey, 2012). Additional phosphorylation events may occur specifically in cancer due to aberrant TTK overexpression and mislocalization. Only recently have TTK substrates been identified, and the list is continually growing.

A more comprehensive portrait of TTK substrates is needed for elucidating the molecular roles of TTK in the cell as well as identifing markers of response to anti-TTK therapy. The canonical function of TTK in regulation of the SAC is conserved in many eukaryotes, suggesting that there may be conserved substrates. In this work we identified 410 putative TTK phosphorylation substrates in humans through bioinformatics analysis. To identify conserved phosphorylation substrates of TTK that may function to maintain genome stability I would perform a similar bioinformatics interrogation of conserved TTK orthologs in mice (*Esk*), zebra fish (*nightcap*), drosophila (*ALD*) and xenopus (*TTK*) to determine putative substrates. Hits could be determined by finding the homologous substrates that overlap between species. Validation and functional analysis of these hits would then be required *in vitro* and *in vivo*.

In addition to a bioinformatics approach, TTK phosphorylation substrates can also be identified by detailed proteomic approaches. There are nine available pharmacologic inhibitors of TTK, with varying selectivity for TTK. To identify phosphorylation substrates of TTK I would compare phosphorylation event changes identified by mass spectrometry of cells treated with TTK inhibitors. By identifying the proteins with decreased phosphorylation following treatment with multiple inhibitors I will limit identification of false positive substrates due to off-target inhibitor activity. These unbiased approaches will provide a strong basis for the continued studies and TTK biology and identify markers of response to anti-TTK therapy.

Usp16 substrates and regulation

Proper chromosome dynamics during mitosis require TTK expression and function and inhibition of TTK promotes genome instability. My identification of Usp16 as a TTK phosphorylation substrate adds new dimensions to TTK signaling. Usp16 is a deubiquitinating enzymes whose best known function is deubiquitinating histone H2A to allow phosphorylation of serine 10 on histone H3 and trigger chromosomal condensation (Joo *et al.*, 2007). Based on my studies here, inhibition of TTK causes stabilization of Usp16 protein. Overexpression of Usp16 has previously been identified in Down syndrome where Usp16 is amplified and reduces self-renewal of hematopoietic stem cells, reduces the expansion of mammary epithelial cells, and accelerated senescence (Adorno *et al.*, 2013), yet the molecular biology that mediate these phenotypes are unknown. To better understand how increased Usp16 mediates these functions it is necessary to conduct future studies to identify the deubiquitination substrates of Usp16.

In addition to the three TTK-dependent sites of phosphorylation on Usp16 our mass spectrometry approach also identified sites of phosphorylation that were not altered with

TTK inhibition in PDAC cell lines. In order to gain a better understanding of Usp16 biology in PDAC it will be necessary to identify the kinases that mediate these functions but also elucidate the effect of the remaining phosphorylation events on activity, localization, and substrate specificity.

Summary

PDAC remains a lethal disease and only incremental improvement in therapy has been made. Through analysis of aberrant gene expression in PDAC we sought to translate the molecular biology of PDAC into clinically relevant diagnostic and therapeutic tools. In aggregate, our studies found two roles of aberrant gene expression in PDAC: 1) as a diagnostic tool to aid in making decisions about patient therapy and 2) as a tool to identify therapeutic targets.

Future studies will be required to identify molecular subtypes associated with drug resistance and patient survival. In addition, future studies are required to validate the role of TTK in PDAC therapy, anticipate mechanisms of resistance, and improve our understanding of TTK biology by identifying and characterizing TTK phosphorylation and Usp16 ubiquitination substrates.

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