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Altered Responses to Endoplasmic Reticulum Stress in Pancreatic Cancer

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ALTERED RESPONSES TO ENDOPLASMIC RETICULUM STRESS IN

PANCREATIC CANCER

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A

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by

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DEDICATION

I would first like to dedicate my dissertation to my husband and my children for the relentless support in helping me through these long years of finishing my Ph.D.

I would also like to dedicate my dissertation to all those pancreatic cancer patients who have passed waiting for the cure to come, those still waiting, and those who will fight. I hope that I will play some part in providing the path to treating this unforgiving disease.

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ALTERED RESPONSES TO ENDOPLASMIC RETICULUM STRESS IN PANCREATIC CANCER

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Pancreatic ductal adenocarcinoma (PDAC) represents the fourth most common cause of cancer-associated death in the United States. Little progress has been made in understanding how proteotoxic stress affects rapidly proliferating pancreatic tumor cells. Endoplasmic reticulum (ER) stress occurs when protein homeostasis in the ER lumen is perturbed. ER stress activates the unfolded protein response (UPR) to reduce the protein load in the ER. Under conditions of moderate ER stress, the UPR promotes cell cycle arrest which allows time for successful protein load reduction and enables cell survival. However, under conditions of high levels of ER stress the UPR induces cellular apoptosis. In this dissertation, I investigated the role of endoplasmic reticulum (ER) stress and its effects on the cell cycle in pancreatic cancer cells.

Activation of the unfolded protein response after ER stress induction was determined by comparing expression of key UPR mediators in non-tumorigenic pancreatic ductal cells to pancreatic cancer cells. Two arms of the UPR were

assessed: eIF2α/ATF4/CHOP and IRE1α/XBP1s. Pancreatic cancer cells exhibited altered UPR activation characterized by a delay in both phosphorylation of eIF2α and induction of spliced XBP1. Further evaluation of the UPR-mediated effects on cell cycle progression revealed that pancreatic cancer cells showed a compromised ability to inhibit G1 to S phase progression after ER stress. This reduced ability to arrest proliferation was found to be due to an impaired ability to downregulate cyclin D1, a key gatekeeper of the G1/S checkpoint. Abrogation of cyclin D1 repression was mediated through a slow induction of phosphorylation of eIF2α, a critical mediator of translational attenuation in response to ER stress.

In conclusion, pancreatic cancer cells demonstrate a globally compromised ability to regulate the unfolded protein response. This deficiency results in reduced cyclin D1 repression through an eIF2α-mediated mechanism. These findings indicate that pancreatic cancer cells have increased tolerance for elevated ER stress compared to normal cells, and this tolerance results in continued tumor cell proliferation under proteotoxic conditions.

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INTRODUCTION TO THE PANCREAS

The pancreas is a highly complicated secretory organ that serves as a critical component of the gastrointestinal system. Its main role in the digestive tract is to secrete digestive enzymes that promote breakdown of ingested food. Knowledge of the anatomy of the pancreas and its location relative to surrounding organ structures is important to the understanding of pancreatic physiology and its role in the digestive system.

Anatomy of the Pancreas

The pancreas is located in the retroperitoneum of the abdomen, just posterior to the stomach. In the adult, the pancreas is 12-20 cm in length and weighs between 70-110 grams. It is an obliquely positioned organ that extends from the C-loop of the duodenum to the splenic hilum. The pancreas is composed of mainly five segments: the head, uncinate process, body, neck, and tail. Each of these segments is in close proximity to important organ and vascular structures. The pancreatic head is surrounded by the second and third curvature of the duodenum. Anteriorly, the pylorus of the stomach, the first part of the duodenum, and the transverse colon lie adjacent to the pancreatic head. Posteriorly, the pancreatic head lies in close proximity to the right kidney and its renal vessels, the inferior vena cava, the right gonadal vein, and the right crus of the diaphragm. The uncinate process is variably present in the population. It projects inferiorly from the pancreatic head. Important surrounding structures include the aorta and inferior vena cava located posteriorly. The superior mesenteric vessels are located

superiorly to the uncinate process and emerge from below the neck of the pancreas.

Each of these regions of the pancreas has a distinct mechanism of development during embryologic growth that led to the subsequent arrangement of the pancreatic ductal system. During embryologic development of the foregut, the pancreas initially begins from the fusion of both the ventral and dorsal buds. The hepatic diverticulum gives rise to the ventral bud while the dorsal bud arises from the duodenum. The pancreatic segments are formed with rotation of the ventral bud around the duodenum and fusion with the dorsal bud. Both the inferior portion of the pancreatic head and uncinate process are derived from the ventral bud while the pancreatic body and tail originate from the dorsal bud. When these two buds fuse, the pancreatic ductal system is formed. Many normal anatomic variations exist, but the pancreatic ductal system is typically formed by the fusion of the ducts in the two buds. The pancreatic ductal system is most commonly composed of primarily the main pancreatic duct and the lesser pancreatic duct. The main pancreatic duct arises from fusion of the ducts in the ventral and dorsal buds into the Duct of Wirsung at the pancreatic head. It is formed from the many anastomosing ductules that drain the lobules of the pancreas. It combines with the common bile duct to form a shared segment, called the ampulla of Vater, which drains at the duodenal papilla into the duodenum. The lesser pancreatic duct arises from persistence of the duct from the dorsal bud as the Duct of Santorini and usually joins the main pancreatic duct. This accessory duct can drain into the duodenum at the minor papilla or in about 30% of cases, will end blindly without

draining (1). In about 10% of the population, fusion of the embryonic dorsal and ventral buds fails to occur and forms a normal variant called pancreas divisum (2). In this variant, the Duct of Santorini does not communicate with the Duct of Wirsung and the Duct of Santorini is mostly responsible for draining pancreatic secretions rather than the Duct of Wirsung.

Pancreatic Histology

The pancreas is a nodular gland that is composed of lobules loosely connected by connective tissue septa. The majority of the pancreas (85%) is comprised of exocrine cells while only 2% are endocrine (1). The remainder of the pancreas consists of extracellular matrix, blood vessels, and excretory ducts of the pancreas. The pancreas is composed of mainly three cell types: acinar cells, intercalated duct cells, and centroacinar cells. Acinar cells are arranged into a functional unit, called an acinus, with a central lumen. Centroacinar cells lie near the center of the acinus. The acinus then forms small intercalated ducts, which are lined by the intercalated duct cells. Multiple small intercalated ducts converge to form an interlobular duct. Each acinar cell contains zymogen granules that release digestive enzymes into the acinar lumen as pancreatic fluid. The zymogen granules contain 12-15 specific digestive enzymes, including amylase, proteases, and lipases, that aid in the processing of carbohydrates, protein, and fat (2). The composition of pancreatic enzymes in the pancreatic fluid is adjusted according to content of digested food. The centroacinar and intercalated duct cells contribute water, bicarbonate, and other electrolytes as the pancreatic juice flows from the

acini to the interlobular ducts to the main pancreatic duct before finally secretion into the duodenum. The resulting pancreatic juice is colorless, alkaline, and isosmotic. Each day, the pancreas secretes about 500 to 800 ml in order to process dietary intake.

The endocrine pancreas is composed of clusters of cells into structures called the islets of Langerhans. Each islet contains cells of five major types: alpha cells, β-cells, delta cells, epsilon cells, and PP cells. In contrast to the acinar cell which is responsible for the secretion of multiple enzymes from a single cell, each cell of the endocrine pancreas is mainly responsible for the secretion of one hormone. Alpha cells secrete glucagon, β-cells secrete insulin, delta cells secrete somatostatin, epsilon cells secrete ghrelin, and PP cells secrete pancreatic polypeptide. Each of these hormones is involved in the regulation of glucose or gastrointestinal function after a dietary challenge. Islet cell control is complicated through the interplay of multiple inputs from hormonal feedback loops, neural signals, and blood flow. Perfusion of the acinar cells comes from venous blood flow draining from the islets, thus allowing the endocrine pancreas to influence the secretions of the exocrine pancreas.

PANCREATIC DUCTAL ADENOCARCINOMA (PDAC)

Epidemiology

Pancreatic adenocarcinoma (PDAC) is a malignant tumor of the exocrine pancreas. This type of neoplasm represents 85% of all neoplasms that occur in the pancreas. Pancreatic cancer ranks as the fourth most common cause of cancer-associated deaths (3). While this malignancy only represents the tenth most common malignancy, the diagnosis of pancreatic adenocarcinoma carries the worst prognosis of all malignancies. An estimated 43,000 new cases of pancreatic cancer will be diagnosed with an estimated 36,800 deaths in 2010 (4). The rate of mortality closely follows the incidence rate with an abysmal 5-year survival rate of nearly 6% (4, 5).

Risk of developing PDAC is considered multifactorial from a combination of genetic and environmental factors. The overall lifetime risk of any individual is 1 in 71 (5). The most closely associated risk factor is increasing age. More than 68% of PDAC occur in individuals older than 65 years of age with a median age of 72 years (5). Incidence also predominates slightly in males over females (1.3:1) and is more common in blacks compared to other races (16.7 per 100,000 black men compared to 13.3 per 100,000 men of all races) (5). While several environmental risk factors have been implicated, the most consistently demonstrated preventable risk factor for PDAC is cigarette smoking with a 2.5 to 3.6 times the risk of a non-smoker (6). Other risk factors that have been identified include chronic pancreatitis and diabetes. Chronic cirrhosis, obesity, high fat or high cholesterol diets have been associated less conclusively with PDAC (7) Ref 9 in Hidalgo NEJM 2009).

Clinical Presentation, Diagnosis, and Staging of PDAC

A key factor in the poor prognosis of PDAC can be attributed to the late presentation and vague symptoms of the disease. The initial presentation of pancreatic cancer depends on the location of the tumor. Most pancreatic adenocarcinoma tumors arise in the pancreatic head and can result in obstructive cholestasis and jaundice. Common presenting symptoms include nausea, anorexia, vague abdominal discomfort, or deep dull upper abdominal pain that is poorly localized. Obstruction of the pancreatic duct by the mass may result in acute pancreatitis. In patients with newly diagnosed diabetes mellitus, pancreatic cancer should also be ruled out. Unfortunately in most cases, by the time symptoms become manifest, the cancer has already reached a level of progression that surgical resection is no longer an option.

Once a physician suspects a possible pancreatic tumor, the standard of care for the initial diagnosis and staging of PDAC is multiphase, multidetector helical computed tomography (CT) with intravenous contrast injection using a specific high-resolution protocol for the pancreas. This method of detection has been shown to have an accuracy of 80-90% in predicting surgical resectability (8). Positron-emission tomography has a supportive role in clarifying equivocal CT results. Additional studies, such as endoscopic retrograde ultrasonography (ERCP), can be used to obtain tissue samples for diagnosis and therapeutic stent placement for duct obstruction for tumors located in the pancreatic head. For patients with signs of advanced disease, such as weight loss, or in patients with large tumors or tumors of the body or tail of the pancreas, an exploratory laparotomy may be indicated to

determine level of metastases and vascular involvement (9). While many serum biomarkers have been investigated, the only currently clinically useful biomarker is CA 19-9 and can be used to monitor treatment and early detection of recurrence of pancreatic cancer (10-13). CA 19-9 is limited, however, since its utility has only been demonstrated in already diagnosed pancreatic cancer and cannot be used as a screening tool to detect previously undiagnosed pancreatic cancer.

Treatment of PDAC

Upon diagnosis of pancreatic adenocarcinoma, clinical management is determined by the 3 factors: tumor burden, invasion into local tissues, and metastasis to other sites. The extent of progression is determined at presentation through a staging process. The current gold standard for pre-operative tumor staging is through helical computed tomography (CT) to determine whether patients have surgically resectable disease. Staging of pancreatic adenocarcinoma is based on the American Joint Committee on Cancer classification system and is based on tumor grade, nodal status, and metastatic involvement (14). Tumor resectability as defined by the National Comprehensive Cancer Network (NCCN) Pancreatic Adenocarcinoma Panel is determined by the absence of distant metastases, absence of tumor thrombus and abutment or structural impingement on surrounding major vessels of the superior mesenteric vein and portal vein (15). The tumor must also show lack of involvement with the superior mesenteric artery, hepatic artery, and celiac axis as evidenced by clear fat planes around the structures. Only about 20% of patients present with potentially resectable disease at presentation (16).

Recent studies indicate a 5-year survival rate of 20% for surgical resection alone for pancreatic cancer with extension of the median survival time to 11-20 months (17-21). The European Study Group for Pancreatic Cancer Trial 1 (ESPAC-1) and Charité Onkologie 1 trial demonstrated that chemotherapy, either 5-fluorouracil (5-FU) or gemcitabine improved overall survival after surgical resection (20, 22, 23). Just recently, the ESPAC-3 trial demonstrated that gemcitabine was shown to be no more beneficial over 5-FU in completely resected pancreatic cancer to improve overall survival (24). Neoadjuvant therapy, or chemotherapy applied pre-operatively, has been shown to have equal benefit compared to adjuvant chemotherapy with an improved rate of local failures and decreased positive resection margins (25).

In 80% of cases, patients present with unresectable pancreatic cancer have disease that is either locally advanced or metastatic. Patients with metastatic disease are treated with systemic therapy for palliation of symptoms rather as an attempt at curative treatment. Currently, first-line therapy for locally advanced disease includes systemic therapy with gemcitabine or a gemcitabine-based combination therapy (26). Gemcitabine treatment alone was shown to provide a clinical benefit response in 23.8% of patients as well as a small extension in overall patient survival compared to 5-fluorouracil chemotherapy (27). Gemcitabine was shown to provide a modest improvement in survival to 5.65 months in comparison to 4.41 months with 5-fluorouracil (27). The only targeted therapeutic that shown to improve survival over gemcitabine alone was erlotinib, an epidermal growth factor inhibitor. Erlotinib provided a small but significant survival advantage of 6.24

months compared to 5.91 months for gemcitabine alone (28). The clinical significance of such a small survival extension is still being debated. Gemcitabine treatment in combination with a number of novel targeted therapies, including other agents aimed at the EGF receptor, have failed to provide benefit over gemcitabine alone in Phase III clinical trials (29-31). The combination of radiation with chemotherapy has yet to demonstrate convincing results of a survival advantage over chemotherapy (32).

Molecular Biology of Pancreatic Adenocarcinoma

Genetic predisposition is also a known component of development of PDAC. However, only about 5-10% of patients with PDAC have a family history of the disease (33). Strong evidence exists that the accumulation of both germline and somatic gene mutations leads to PDAC. The gradual accumulation of these gene mutations is believed to result in a sequential progression of normal pancreas to various stages of pre-malignant lesions, called pancreatic intraepithelial neoplasias (PanIns), to pancreatic adenocarcinoma. In a global genomic analysis of 24 pancreatic tumors, an average of 63 “likely relevant” genetic abnormalities in each tumor were identified (34). Each tumor possessed its own variation of genetic aberrations affecting signaling pathways that was unique from other tumors in the study. This heterogeneity of accumulated mutations points to the inherent difficulties in treating PDAC as a homogenous entity.

Four key genes have been identified as critical players in the carcinogenesis of pancreatic adenocarcinoma: KRAS, CDKN2A, TP53, and SMAD4/DPC4.

Presence of at least one of these gene mutations has been demonstrated in nearly all pancreatic cancers (35). Activating mutations in the KRAS oncogene has been identified in 90% of pancreatic adenocarcinomas (36). KRAS mutation, however, has demonstrated poor sensitivity (50%) owing to the presence of Ras pathway activation even when negative for KRAS mutation and does not accurately predict prognosis (37). KRAS has been demonstrated to be important to the initiation of carcinogenesis of pancreatic cancer (38). Mutation of this oncogene results in a constitutively active isoform of the Ras protein with consequent abnormal activation of proliferative and pro-survival signals. Accrual of additional genetic alterations, such as mutation or loss of CDKN2A or TP53, has been shown to be necessary to initiate carcinogenesis into PDAC. Mutations of CDKN2A have been demonstrated in 95% of PDACs. This mutation inactivates the p16 protein, a key regulator of the G1-S cell cycle transition, with a subsequent increase in proliferation. In 50-75% of cells, TP53 has been shown to be mutated. Mutation of this gene results in loss of control over DNA damage checkpoints and apoptosis with a resultant genomic instability that further promotes malignant transformation. A mutation in SMAD4/DPC4 mutation has been identified in about 50% of PDAC tumors and interferes with normal functioning of transforming growth factor β receptor (TGFβ). Mutation of SMAD4 has been associated with a worse prognosis in surgically resected pancreatic cancer (39).

ENDOPLASMIC RETICULUM STRESS AND THE UNFOLDED PROTEIN RESPONSE

Overview of ER Stress

Perturbations in the homeostasis of the cellular microenvironment may lead to changes in the organelle processing functions of the endoplasmic reticulum. The endoplasmic reticulum (ER) is a cellular organelle composed of a complex network of interconnecting tubules that is responsible for crucial control over protein biosynthesis, folding, quality control, and post-translational modifications in the secretory pathway. The ER is a necessary organelle for sequestration of incompletely folded and immature proteins requiring further modifications which would otherwise be unsuitable in the cytosolic environment.

Processes which challenge the ER's ability to maintain homeostasis result in a condition termed endoplasmic reticulum stress. These challenges interfere with ER protein folding function and cause an increase in misfolded and excess protein load in the ER. The accumulated protein load induces proteotoxic stress in the ER. ER stress signals for activation of the unfolded protein response (UPR). The UPR coordinates an integrated stress signaling pathway that balances survival against the damages created by excessive protein stress in the ER. This pathway's role is to re-establish protein homeostasis by reducing protein load within the ER. This pro-survival response is achieved through a complex system that attenuates protein translation, upregulates ER chaperone folding capacity, and increases degradation of aberrant protein through a process called ER-associated degradation (ERAD). If the protein load exceeds the UPR's capacity to decrease the proteotoxic stress, the

UPR converts from a pro-survival to a pro-apoptotic role with activation of the intrinsic apoptosis pathway.

The UPR is an integrated cellular response composed of the interplay among three ER transmembrane receptors which act as “sensors” to detect accumulated and misfolded proteins within the ER lumen. The three receptors that have been identified are called pancreatic ER kinase (PKR)-like ER kinase (PERK), inositol-requiring enzyme 1 (IRE1), and activating transcription factor 6 (ATF6). The mechanism of “detection” through these receptors is mediated through interactions with the ER molecular chaperone GRP78 (Binding Protein/Glucose-Regulated Protein 78kDa), and is also known as BiP (Binding Protein). GRP78 is constitutively bound to these ER receptors and maintains them in an inactive state. Currently, the most accepted theory is that the UPR is activated when misfolded proteins in the ER lumen compete with the UPR receptors for binding to GRP78 and cause its dissociation from the UPR receptors (41). GRP78 release subsequently relieves PERK, IRE1, and ATF6 of their inhibition. GRP78 thus exerts powerful control over the general regulation of the UPR (40).

PERK-eIF2α-ATF4 activation

Activation of PERK occurs through autophosphorylation and dimerization. PERK acts as a serine/threonine kinase with two known substrates: eukaryotic initiation factor 2α (eIF2α) and nuclear-factor E2-related factor 2 (Nrf2). For the purposes of this study, only eIF2α was studied. Upon ER stress induction, PERK phosphorylates the eIF2α subunit on Ser51, effectively inactivating the eIF2 ternary

complex. Subsequent global cap-dependent translation is inhibited (discussed further later). Paradoxically, not all protein synthesis is inhibited. A number of genes are selectively transcribed under stress conditions via cap-independent translation. In response to ER stress, one such selectively translated mRNA is activating transcription 4 (ATF4). The untranslated region of the ATF4 mRNA sequence contains three alternative untranslated open reading frames (uORFs) which are specifically translated in response to certain stresses. When active eIF2 ternary complexes are abundant, the primary open reading frame is translated, leading to a STOP codon and no protein is translated. When the availability of active eIF2 ternary complexes is limited, such as when eIF2α is phosphorylated, the alternative uORF is read and ATF4 is translated. General translational control by eIF2α phosphorylation will be discussed in more detail later.

IRE1α-XBP1s activation

The cytosolic domain of IRE1α possesses intrinsic endoribonuclease function which has only one known target: XBP1 precursor messenger RNA. GRP78 release results in disinhibition of IRE1α, causing its activation through dimerization and trans-autophosphorylation. Once activated, the endoribonuclease site splices an intron of 26 nucleotides from the XBP1 precursor mRNA. Upon splicing, a frameshift in the reading frame results in the translation of the spliced XBP1 mRNA into a spliced XBP1 (XBP1s) protein that acts as potent transcription factor for UPR gene expression (42, 43). XBP1s promotes transcription of genes

that upregulate ER chaperone expression, ER biogenesis in secretory cells such as the exocrine pancreas, and ER associated degradation (ERAD) (44-47).

ER Stress-Mediated Apoptosis

Under conditions of prolonged ER stress, cells undergo apoptosis induction. The PERK pathway promotes selective translation of ATF4. ATF4 subsequently induces CHOP (C/EBP homologous protein), which is also known as GADD153 (48). Although more closely studied in association with the PERK pathway, CHOP can additionally be induced by the IRE1α and ATF6 pathways (49). CHOP induction leads to derepression of protein synthesis via dephosphorylation of eIF2α by GADD34. Subsequent protein synthesis is increased and cell death is induced via continued accumulation of proteotoxic stress (50). CHOP has also been shown to promote expression of a number of pro-apoptotic genes and downregulation of anti-apoptotic genes, such as Bcl2 (51-53). Apoptosis via CHOP was demonstrated to be elicited via an ER stress-specific mechanism. CHOP-deficient cells demonstrated increased resistance to ER stress-mediated apoptosis (54). ER stress-mediated apoptosis is believed to be conducted via the proapoptotic Bcl2 proteins.

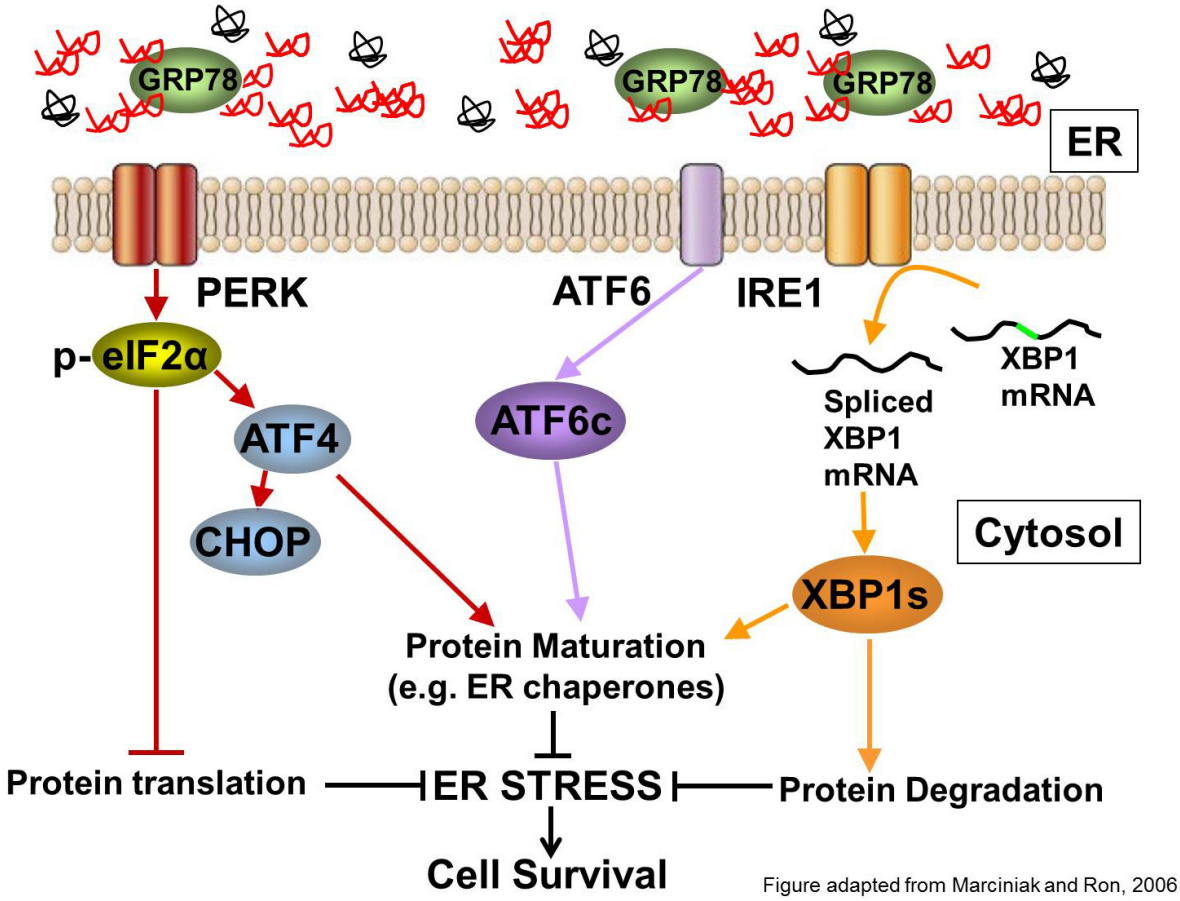


Figure 1. The unfolded protein response (UPR) pathway. The unfolded protein response pathway is initially activated by accumulation of misfolded proteins in the ER, resulting in release of GRP78 binding from the transmembrane receptors PERK, ATF6, and IRE1. GRP78 dissociation results in activation of PERK, ATF6, IRE1 cascades which promote inhibition of protein synthesis, upregulation of ER chaperones, and protein degradation. Initial activation of the UPR results in cell survival from decreased protein load and ER stress. Failure to reduce protein load results in apoptosis (not depicted). Adapted with permission from Physiological Reviews, Marciniak and Ron, 2006 (55). This is an unofficial adaptation of an article in a publication of the American Physiological Society. The American Physiological Society has not endorsed the content of this adaptation or translation, or the context of its use.

ER Stress and Cancer

Given the limited availability of a functional vascular supply in most pancreatic adenocarcinomas, the tumor microenvironment of pancreatic tumors would be expected to be deficient in important metabolites such as glucose, oxygen, and amino acids (56). This tumor microenvironment would provide for conditions predisposing tumors to an ER stress state. Tumor cells exhibit an increased dependency on glucose due to their reliance on glycolysis rather oxidative phosphorylation, otherwise known as the "Warburg effect" (57). Glucose deprivation results in decreased ATP availability and decreased efficiency of ATP-requiring protein folding enzymes. Additionally, inflammatory factors have also been implicated to be regulated by the UPR. The contribution of inflammation has been of key importance in the pathogenesis of cancer, particularly in pancreatic cancer. ER stress has been demonstrated to induce NFκB and may regulate it both negatively and positively and in a phase dependent fashion. A number of studies have established PERK and the UPR as an adaptive cellular mechanism to combat the protein stress created by hypoxic and metabolic stresses (58-61). This cytoprotective response is of particular importance in cancer since expression of UPR factors has been correlated to increased proliferative capacity, tumor growth, and metastasis (58, 61-63). Most recent evidence indicates that PERK may also help ameliorate proteotoxic stress in tumor cells, thus promoting their survival (62-64).

Particular attention has recently been paid to the UPR as new therapeutics targeting PERK are under investigation (59, 65). Multiple drugs have demonstrated

ER stress activation that promotes apoptosis, including proteasomal inhibitors and cisplatin (66-69). One barrier to chemoresistance in cancer with UPR targeting agents is perhaps the difficulty in identifying a therapeutic index between the cytoprotective versus apoptotic effects of ER stress induction. Although activation of ER stress can cause apoptosis in cancer cells, an obvious difficulty is the dual role that the UPR plays in mediating both apoptosis and survival. ER stress activation promotes cytoprotective processes, such as protein folding through ER chaperones like GRP78. GRP78 is frequently upregulated in cancers and has even been shown to be displayed on the cell surface of tumor cells (70, 71). A number of studies indicate that GRP78 plays a significant role in promoting tumorigenicity, angiogenesis, and chemoresistance. Ironically, despite evidence mentioned previously that the UPR is believed to play a mostly pro-tumorigenic role, overexpression of GRP78 inhibits the UPR through suppression of the UPR sensors. This dynamic that GRP78 and the UPR both promote tumorigenesis seem at odds with current evidence that one represses activation of the other. Whether this represents a difference in responses in various cell types or a modulation of ER stress based on the stress applied is yet to be clarified.

CYCLIN D1 REGULATION

In normal cells, D-type cyclins play a critical role in promoting cell cycle progression through the G1 restriction point. Cyclin D1 expression is dependent on cell cycle phase. Its expression rises early in G1 phase and declines rapidly upon entry into the G1/S phase boundary. Cyclin D1 forms a complex with cyclin-dependent kinase-4 (CDK4) or cyclin dependent kinase-6 (CDK6) that phosphorylates the retinoblastoma protein. Hyperphosphorylation of the retinoblastoma molecule results in its release of E2F transcription factors, thus allowing transcription of cell cycle genes promoting the G1 to S transition. Cyclin D1 thus acts as a rate-limiting step for progression through the G1 phase.

As expected, overexpression of cyclin D1 leads to more rapid progression through the G1 checkpoint and promotion of proliferation (72-74). These cyclins promote cell proliferation by transmitting mitogenic stimuli to the cell cycle pathways. Transcriptional regulation of cyclin D1 occurs through multiple inputs, including the Ras/Raf/MAPK and nuclear factor kappa B (NFκB) pathways. Ras/Raf/MAPK and NFκB pathways are two pathways known to contribute to the pathogenesis of pancreatic cancer (75).

Most research seems to support the role of cyclin D1 as a pro-tumorigenic factor in tumor cells. Cyclin D1 is known to be overexpressed in a number of cancers, including hepatocellular, breast, and esophageal (76-79). In pancreatic cancer, overexpression of cyclin D1 has been shown to be an indicator of poor prognosis and is correlated with poor postoperative survival (80, 81). Cyclin D1 has been heavily implicated in pancreatic cancer as a mechanism of chemoresistance

by promoting cell proliferation and inhibition of drug-induced apoptosis (82-84). Antisense knockdown of cyclin D1 allowed increased apoptosis in pancreatic, lung, and a number of squamous cell lines. Upregulation of endogenous cyclin D1 expression through enforced transcription also inhibited apoptosis.

In prior studies in mouse embryonic fibroblasts and NIH-3T3 cells, cell cycle arrest in the G1/S phase could be induced through activation of the of the PERK/phospho-eIF2α/ATF4 arm of the UPR (85, 86). Cell cycle exit was mediated by blocking cyclin D1 translation through phosphorylation of eIF2α (86). Another study by Tomida and Tsuruo observed that A2780 (ovarian carcinoma) and HT-29 (colon carcinoma) were also capable of downregulating cyclin D1 after being challenged with drugs that had been shown to induce ER stress (87-90).

CAP-DEPENDENT TRANSLATIONAL CONTROL OF GENE EXPRESSION

Gene expression is regulated at multiple levels. The final step in gene expression is translation of mRNA. In contrast to transcriptional regulation, changes in the translation of pre-existing mRNA's can elicit rapid modulation of protein levels to mediate the effects of the encoded proteins. Regulation of translation is reviewed in detail elsewhere (91, 92). For this study, mechanisms in a process called cap-dependent translation initiation will be examined. The regulatory mechanisms examined in this study involve the phosphorylation of eukaryotic initiation factor (eIF2α) and its control of translation during stress conditions.

During cap-dependent translation, eukaryotic translation initiation factor 4E (eIF4E) is maintained in an inactive state through inhibition by 4E-binding proteins (4E-BPs) (Figure 2). Mitogenic stimulation, such as through the phosphoinositol-3 kinase (PI3K) and mammalian target of rapamycin (mTOR) pathways, activate translation by phosphorylating the 4E-BPs. Phosphorylation of 4E-BPs causes eIF4E to be released and bind the mRNA 5' cap. Binding of the mRNA 5'cap recruits the 40S ribosomal subunit, which is responsible for scanning the mRNA for an AUG initiation codon.. Recognition of the AUG initiation codon is mediated through the ternary complex composed of the initiator Met-tRNA$_i^{Met}$, eIF2, and GTP. The assembled ribosomal complex scans the mRNA until encountering an AUG initiation codon with complementarity to the initiator Met-tRNA$_i^{Met}$. Recognition of the Met-tRNA$_i^{Met}$ to the AUG initiation codon results in irreversible hydrolysis of GTP, and eIF2-GDP is subsequently released. A 60S ribosomal subunit then joins the 40S subunit creating a larger 80S initiation complex which completes translation

of the mRNA. Activity of the ternary complex is renewed with exchange of the G-protein from eIF2-GDP to eIF2-GTP by the guanine exchange factor eIF2B. Phosphorylation of the α-subunit of the eIF2α results in inhibition of eIF2B, limiting the GDP to GTP exchange (Figure 3). This serves as a rate-limiting step since availability of the ternary complex regulates efficiency of translation efficiency. Small differences in levels of eIF2α phosphorylation have been shown to affect protein synthesis dramatically. In reticulocyte lysates, even a small increase in eIF2α phosphorylation as low as 5-10% is sufficient to cause a strong abrogation of translation (93). During conditions where eIF2 ternary complex is limited, ribosomal "global" control of translation initiation of mRNAs is inhibited while "selective" regulation allows translation of a particular group of mRNAs during stress conditions. This process will be described later.

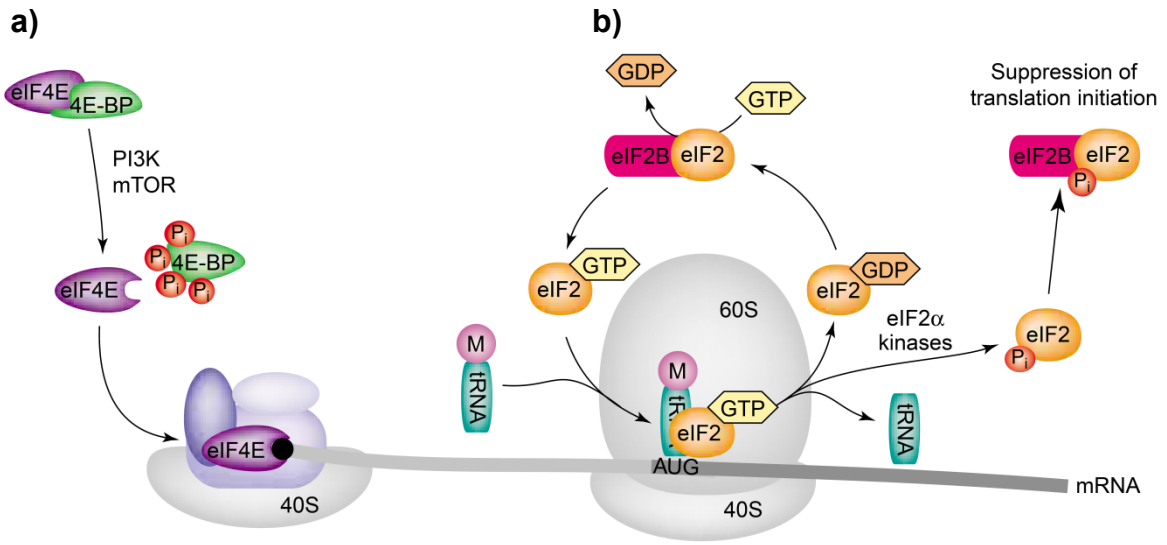


Figure 2. Initiation of cap-dependent translation. a) Translation initiation begins with formation of the cap-binding complex. Mitogenic stimulation through pathways such as PI3K or mTOR results in phosphorylation of 4E-BP proteins. eIF4E is released and binds the mRNA 5'cap (black circle). The 40S ribosomal subunit is recruited. **b)**, The ternary complex (eIF2, Met-tRNA$_i^{Met}$, GTP) participates in recognizing the AUG codon and initiating protein synthesis. Exchange of GDP for GTP by eIF2B recycles eIF2. eIF2α phosphorylation results in inhibition of eIF2B and inhibits further translation. (Reprinted with permission from *Trends in Molecular Medicine*, Calkhoven *et al*, December 2002) (91).

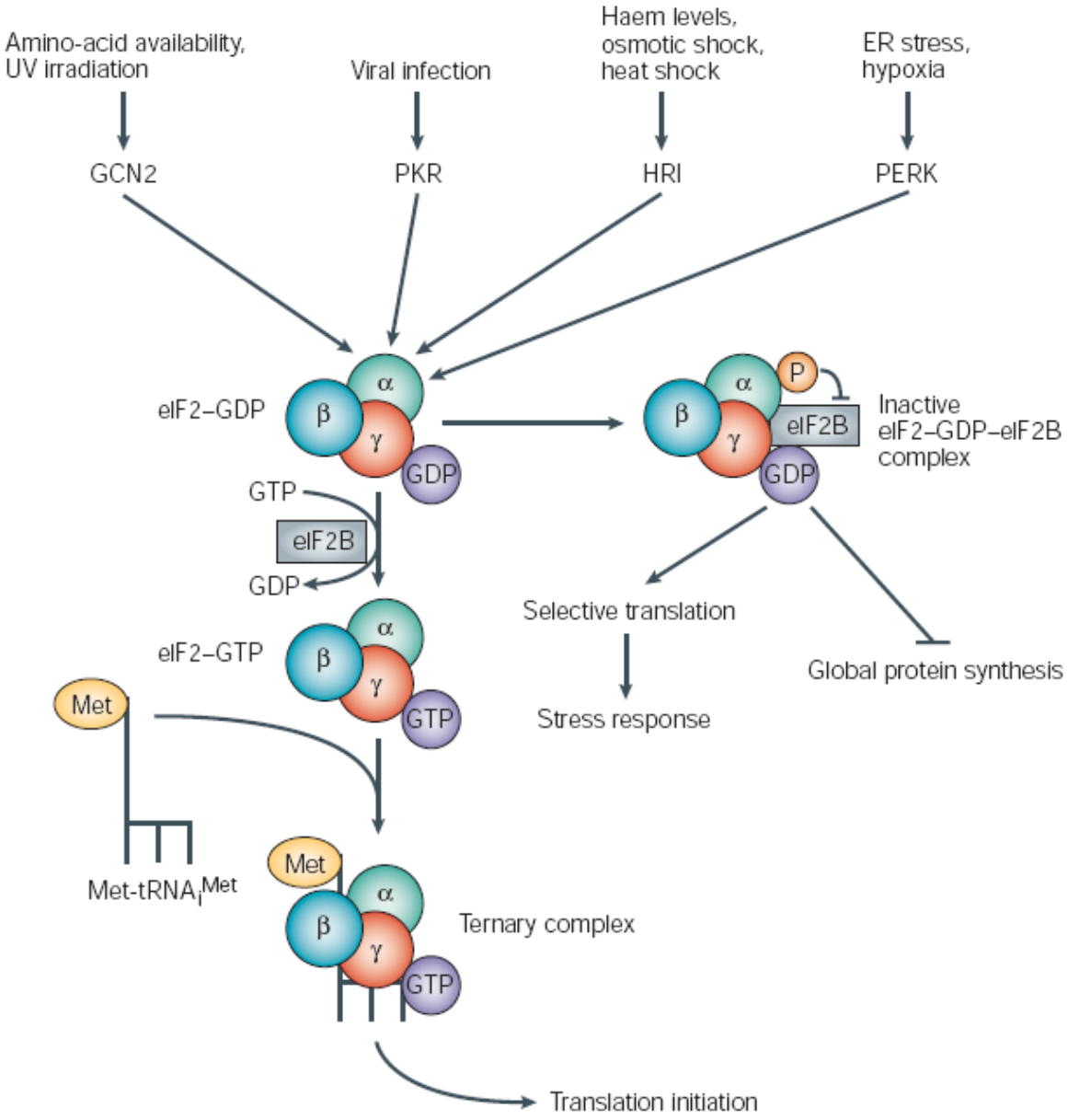


Figure 3. **Steps in translation initiation through eIF2α.** Multiple stress conditions converge at the eukaryotic initiation factor-2 complex. eIF2B acts as a guanine exchange factor that facilitates recycling of eIF2 through exchange of GDP for GTP. Translation is initiated when the ternary complex (eIF2, Met-tRNA$_i^{Met}$, GTP) is formed. Alternatively, phosphorylation of the eIF2α subunit prevents release of eIF2B and GDP exchange for GTP. Global protein synthesis is inhibited while simultaneously initiating translation of stress-specific proteins. (Reprinted with permission from *Nature Reviews: Molecular Cell Biology*, Holcik and Sonenberg, April 2005) (94).

EXPERIMENTAL RATIONALE AND HYPOTHESIS

Pancreatic cancer is one of the deadliest cancers with a poor survival rate owing to its aggressiveness and limited treatments available. One of the key obstacles facing treatment of pancreatic cancer is its chemoresistant nature. Understanding of how pancreatic cancer cells have adapted mechanisms to survive in the presence of stress is important to identifying mechanisms for sensitizing cells to treatment.

The main hypothesis of this dissertation is that **pancreatic cancer cells have an altered endoplasmic reticulum stress response**. The overall goal for this dissertation was to determine how pancreatic cancer cells respond to ER stress induction and how this may significantly affect chemoresistance. Since pancreatic cancer cells demonstrate features of hypovascularity and increased proliferation, availability of oxygen, nutrients, and glucose would be limited by vascular supply to a rapidly growing tumor. Hypoxia and lack of nutrient availability creates an conditions that promote induction of ER stress and activation of the unfolded protein response. In tumors, these same conditions also select for cells which are capable of adapting to a microenvironment of stress. Numerous studies have been published that support the pro-tumorigenic role of the UPR. However, very little has been published about how pancreatic cancer cells respond to induction of ER stress.

Chapter Two aims to determine whether pancreatic cancer cells respond similarly to ER stress induction as non-tumorigenic pancreatic ductal cells.

Activation of two branches of the UPR will be assessed: eIF2α/ATF4/CHOP and IRE1α/XBP1s.

Chapter Three aims to determine if altered responses to ER stress induction in pancreatic cancer cells have UPR-mediated effects on regulation of the cell cycle. Specifically, this aim explores translational control of cyclin D1 in pancreatic cancer cells.

CHAPTER TWO:

PANCREATIC CANCER CELLS HAVE AN ALTERED UNFOLDED PROTEIN RESPONSE TO ENDOPLASMIC RETICULUM STRESS INDUCTION

INTRODUCTION

A key feature of solid tumors is their adaptability to the environment created by the demands of a rapidly proliferating tumor. Tumor growth creates a vast need for oxygen and nutrients to maintain high proliferation rates. In pancreatic cancer, however, functional vascular supply is limited with a resultant decreased capability to deliver necessary oxygen, glucose, and nutrients to dividing tumor cells (56). This type of environment selects for cells that are able to cope with the hypoxic and metabolic stress created by these demands (95).

The tumor microenvironment creates conditions that predispose cells to disturbances in endoplasmic reticulum function. The endoplasmic reticulum (ER) is an organelle composed of an intracellular membrane network responsible for processing of secretory and transmembrane proteins. Accumulation of unfolded or misfolded proteins within the ER results in a perturbed homeostasis called ER stress. In the tumor microenvironment, conditions such as oxygen or glucose deprivation results in conditions that disrupt proper protein folding within the ER. ER stress is subsequently induced, which leads to activation of the unfolded protein response (UPR). The UPR is an integrated stress response pathway that is mediated by three ER transmembrane receptors PKR-like ER kinase, PERK/PEK/EIF2AK3, Inositol-Requiring Enzyme 1α (IRE1α), and activating transcription factor 6 (ATF6) (96-101). These transmembrane receptors are constitutively bound to ER chaperone GRP78 to maintain them in an inactive state. Accumulation of protein in the ER lumen results in GRP78 release and PERK, IRE1α, and ATF6 transduction of a stress response signal cascade to reduce

overall protein load in the ER. This process is achieved through 1) decreased protein translation, 2) increased expression of protein folding capacity, and 3) upregulated degradation of misfolded proteins.

Activation of the UPR has frequently been demonstrated to provide an adaptive mechanism for tumor cell survival in the presence of ER stress. Induction of both the PERK/eIF2α and IRE1α/XBP1s branches of the UPR have been implicated as playing dual roles in promoting apoptosis and survival in tumors (102). Inhibition of translation by the PERK/ eIF2α branch is thought to reduce protein stress and induce tumor dormancy. However, prolonged ER stress induces apoptotic mechanisms. IRE1α/XBP1 activation has been shown to promote tumor adaptation to hypoxia (103, 104).

Few studies have been performed to determine whether the UPR plays a role in promoting tumor cell survival or apoptosis in pancreatic cancer. This study aims to characterize the pancreatic cancer cell response to ER stress induction.

MATERIALS AND METHODS

Cell lines and culture

Non-tumorigenic pancreatic cell lines human pancreatic ductal epithelial (HPDE-E6/E7) cells were provided by Dr. M. Tsao (Ontario Cancer Institute, Toronto, Ontario, Canada). Non-tumorigenic pancreatic nestin-positive epithelial (hTERT-HPNE) cells and human pancreatic cancer cell lines AsPC-1, Su86.86, Hs766T, and HPAC were obtained from American Type Tissue Collection. Human pancreatic cancer cell lines Pa-Tu-8902, Colo357, and Pa-Tu-8988S were kind gifts from Drs. Eric Collisson and Joe Gray (University of California and Lawrence Livermore Laboratory, San Francisco, CA). HPDE cells were grown in keratinocyte media that was supplemented with epidermal growth factor and bovine pituitary extract (Invitrogen, Carlsbad, CA). For HPDE cell expansion, adherent cells were dissociated from plastic through trypsinization, and trypsin was neutralized with soybean trypsin inhibitor. HPNE cells were grown in Medium D as described previously (105). All human pancreatic cancer cell lines were grown in DMEM (Mediatech, Inc., Manassas, VA) supplemented with 10% fetal bovine serum. All pancreatic cancer cell lines were maintained in DMEM, supplemented with 10% fetal bovine serum. All cell lines were maintained in an incubator under conditions of 5% CO_2 at 37ºC. Cell lines were consistently maintained at confluencies of 80% or less to minimize stress. After cells were plated and allowed to adhere overnight, media was replaced 3-6 hours prior to start of each experiment to minimize metabolic stress from media consumption. All cell lines were maintained in antibiotic-free media and confirmed to be free of mycoplasma contamination by

testing with the Mycoalert Mycoplasma Detection Kit (Lonza BioWhittaker, Walkersville, MD).

Reagents and antibodies

Tunicamycin (TU) was purchased from Sigma-Aldrich (St. Louis, MO). The proteasome inhibitor bortezomib (PS-341, Velcade) was provided by Millenium Pharmaceuticals (Boston, MA). Primary antibodies specific to the proteins were obtained as follows: ATF4 and GRP78 (Santa Cruz Biotechnology, Santa Cruz, CA), $p27^{Kip1}$ (BD Biosciences Transduction Laboratories, San Diego, CA), total eIF2α, (Cell Signaling Technology, Beverly, MA), phosphorylated eIF2α (Ser52) (Invitrogen Biosource, Carlsbad, CA); β-actin (Sigma-Aldrich), CHOP (Thermo Scientific, Rockford, IL), and XBP1-s (Biolegend, San Diego, CA). Fluorescent secondary antibodies of IRDye CW800 goat anti-mouse, IRDye 680 goat anti-rabbit, andn IRDye 680 donkey anti-goat were purchased from Li-cor Biotechnology (Lincoln, NE).

Immunoblotting

Cells were lysed for 15 minutes on ice in Triton-X lysis buffer [1% Triton X-100, 150 mmol/L sodium chloride, 25 mmol/L Tris (pH 7.5), 1 mmol/L glycerol phosphate, 1 mmol/L sodium orthovanadate, 1 mmol/L sodium fluoride and protease inhibitor cocktail (Sigma)], collected by scraping, and incubated on ice an additional 10 minutes. Lysates were sonicated briefly and centrifuged at 14,000 x *g* at 4ºC for 15 minutes to clear cellular debris. 40 μg of total cellular protein of each

sample was loaded with 4X sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer to a final concentration of 50 mmol/L Tris-HCl, 2% SDS, 10% glycerol, 0.01% bromophenol blue, and 5% β-mercaptoethanol. Samples were heated to 95ºC for 5 minutes, resolved by SDS-PAGE, and subsequently transferred to nitrocellulose membranes. Membranes were blocked with either 5% nonfat milk in phosphate-buffered saline (PBS) or 3% bovine serum albumin (BSA) in tris-buffered saline (TBS) for antibodies detecting phosphorylated proteins for 1 hour. Blots were then incubated with primary antibodies overnight at 4ºC, washed, and probed with the appropriately targeted species of secondary antibody. Protein bands were visualized and quantified using the Odyssey Infrared Imaging System and manufacturer-supplied software (Li-Cor Biotechnology). For most membranes, more than one primary antibody was co-incubated and visualized using the Odyssey two-color detection system.

[6-^{3}H]-Thymidine Incorporation Assays

Selected cell lines were plated in 96-well plates so that the confluency of cells in the DMSO-treated control wells reached approximately 70-80% at the conclusion of the experiment. Cells were allowed to adhere over 24 hours. 3-6 hours prior to experiment start, media was replaced with fresh warmed media to minimize metabolic stress from media consumption. Cells were exposed to DMSO or 0.001, 0.01, 0.02, 0.04, 0.08, 0.16, 0.3, and 0.6 μg/ml tunicamycin for 48 hours. After treatment, cells were gently washed with PBS once and then pulse-labeled with 10 μCi/ml [6-^{3}H] thymidine (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) for 1 hour at 37ºC under normoxic conditions in an incubator. Non-

incorporated [6-^{3}H] thymidine was removed by gently washing cells with PBS once. Cells were then lysed in 100 μl of 0.1 M KOH and harvested onto fiberglass filters using a plate harvester. Incorporated tritiated thymidine was quantified using a scintillation counter.

Quantification of DNA Fragmentation

Analysis of DNA fragmentation was performed through propidium iodide (PI) staining. Selected cell lines were plated in 6-well plates so that the final confluency of cells in the control wells reached approximately 70-80%. Cells were allowed to adhere overnight. 3-6 hours prior to experiment start, media was replaced with fresh warmed media to minimize metabolic stress from media consumption. After exposure to various treatments, cells were harvested with 0.05% trypsin-EDTA, neutralized with serum-containing media and centrifuged at 500 x *g*. Cell pellets were washed twice with PBS, pelleted again by centrifugation, and fixed at least overnight in 70% ice-cold ethanol at 4ºC. Cells were then centrifuged at 1000 x *g* for 5 minutes and incubated in 50 μg/ml PI with 10 μg/ml RNase A for 30 minutes at room temperature while protected from light. Subsequent flow cytometric analysis was performed on a Beckman Coulter XL (Brea, CA) at the University of Texas M.D. Anderson Cancer Center's Flow Cytometry and Cellular Imaging Facility (Houston, TX). Cells in the sub-G0/G1 peak were considered apoptotic. The remaining cells were assessed for the percentage of cells in each of the cell cycle phases. Cell cycle analysis results were normalized to the DMSO-treated control

where proportion of cells in each phase was represented as a percentage of the DMSO-treated control unless otherwise specified.

RESULTS

Pancreatic cancer cells reduced inhibition of DNA synthesis after tunicamycin treatment compared to non-tumorigenic cells.

Evidence exists that the unfolded protein response pathways can play a cytoprotective role, particularly in adaptation to hypoxic stress (58, 104). The endoplasmic reticulum stress response in pancreatic cancer cells has not been previously assessed. To activate the UPR, the classic ER stressor tunicamycin was used as a tool to induce ER stress through its inhibition of dolichol-mediated synthesis of glycoproteins, which disrupts protein exit from the ER. To determine whether pancreatic cancer cells exhibit a different response to protein load induction, pancreatic cancer cells were exposed to a dose-dependent increase of tunicamycin for 48 hours. DNA synthesis was assessed through the incorporation of thymidine radioactively labeled with tritium and was used as an indirect measure of cell proliferation. At all doses ≥0.01 μg/ml tunicamycin, pancreatic cancer cell lines exhibited a statistically significant difference ($p<0.01$) in DNA synthesis rates compared to non-tumorigenic cells (Figure 4). Pancreatic cancer cell lines fail to reach comparable inhibition of DNA synthesis up to a dose of 0.6 μg/ml tunicamycin. A dose of 0.5 μg/ml tunicamycin was chosen for further experiments to evaluate the ER stress response in non-tumorigenic and pancreatic cancer cells.

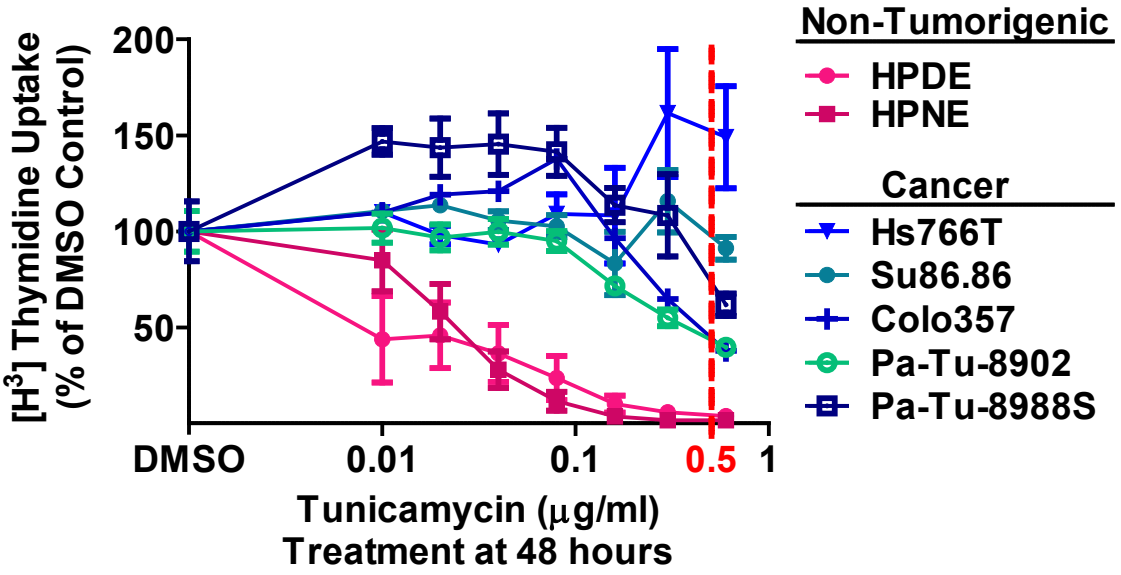


Figure 4. Pancreatic cancer cells have dose-dependent reduced inhibition of DNA synthesis with tunicamycin. Non-tumorigenic (pink curves, HPDE, HPNE) and pancreatic cancer cell lines (green curves, Hs766T, Su86.86, Colo357, Pa-Tu-8902, Pa-Tu-8988S) were exposed to increasing doses from 0.001 µg/ml to 0.6 µg/ml tunicamycin for 48 hours. Growth curves were measured through [6-3H]-thymidine uptake. $p<0.01$ with 2-way ANOVA and Bonferroni post-test comparing each non-tumorigenic cell line to each pancreatic cancer cell line for all concentrations ≥0.01 µg/ml tunicamycin. A tunicamycin dose of 0.5 µg/mL was chosen for further experiments. All experiments were repeated in at least 3 separate experiments, n=3. Error bars represent SEM.

Pancreatic cancer cells have delayed phosphorylation of eIF2α.

Tunicamycin treatment (0.5 μg/ml) in non-tumorigenic pancreatic ductal cells elicited a prompt phosphorylation of eIF2α within a minimal treatment duration of 2 hours. This rapid response was in contrast to pancreatic cancer cells requiring at least 8 hours of exposure before a clear increase in phosphorylation was induced. Non-tumorigenic cells had a greater magnitude of maximal phosphorylation with 2-fold (HPDE) or 4-fold (HPNE) induction by 4 hours. Of the three pancreatic cancer cell lines tested, HPAC reached the greatest magnitude of phosphorylation induction with a maximal induction of only 1.5-fold induction by 12 hours (HPAC).

A)

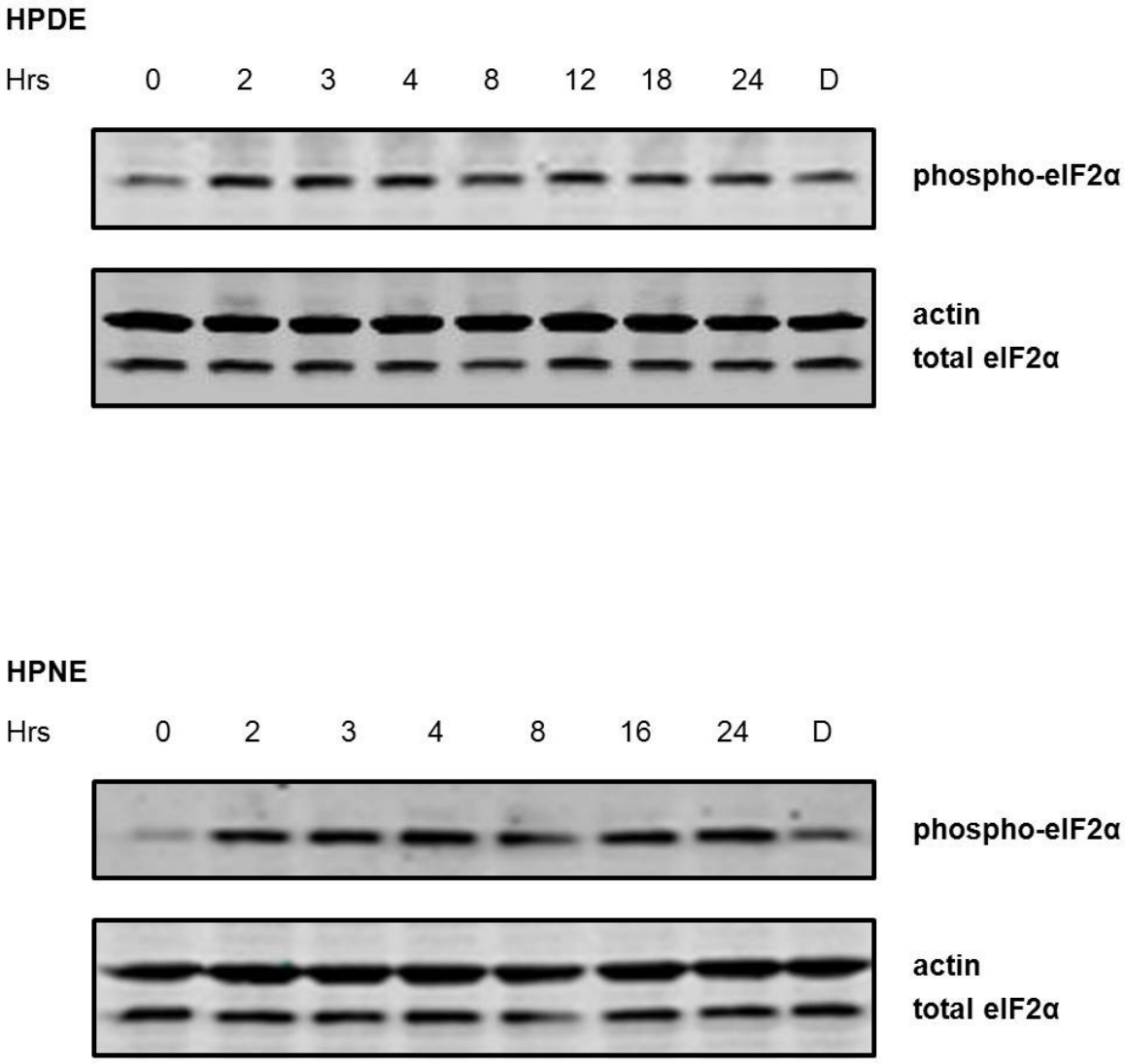
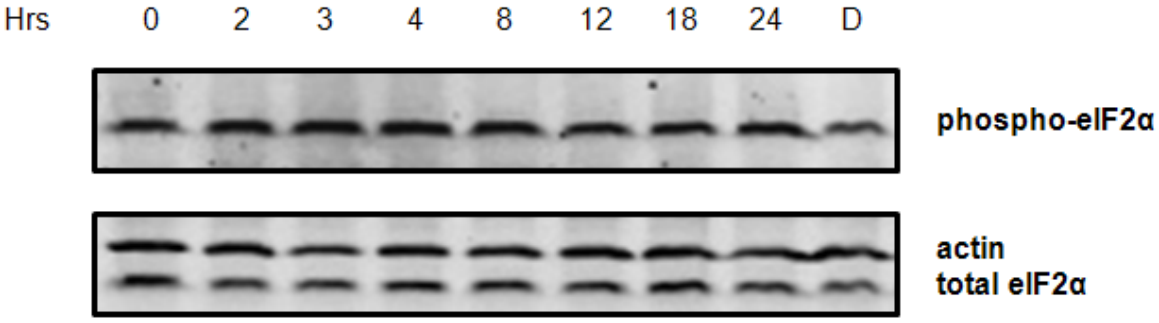


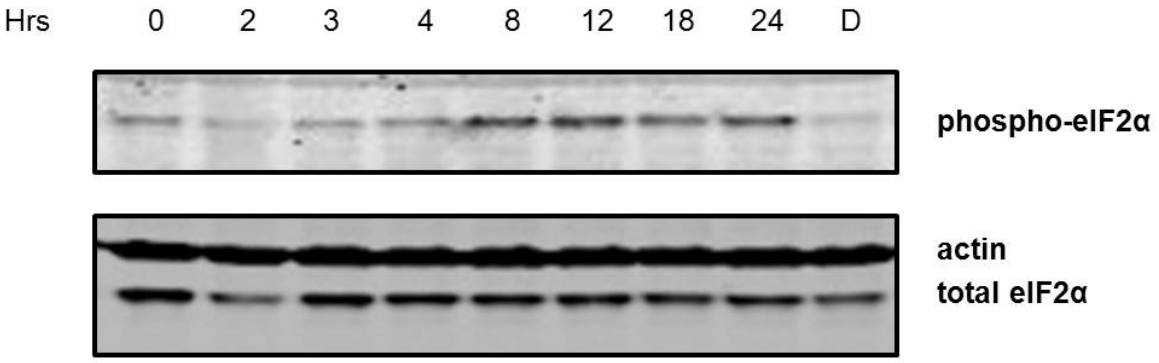
Figure 5. Phosphorylation of eIF2α is delayed in pancreatic cancer cells compared to non-tumorigenic pancreatic ductal cell lines. Treatment time courses over 24-hour incubation period of tunicamycin treatment. "D" represents treatment with DMSO vehicle over 24 hours. ***A***, non-tumorigenic pancreatic cell lines HPDE and HPNE rapidly phosphorylate eIF2α within 2 hours of tunicamycin treatment. ***B*** (next page), pancreatic cancer cell lines Pa-Tu-8902, HPAC, and AsPC-1 display delayed and markedly diminished phosphorylation of eIF2α. Each experiment was repeated in at least 2 separate experiments. Representative immunoblots are shown of similar data.

B)

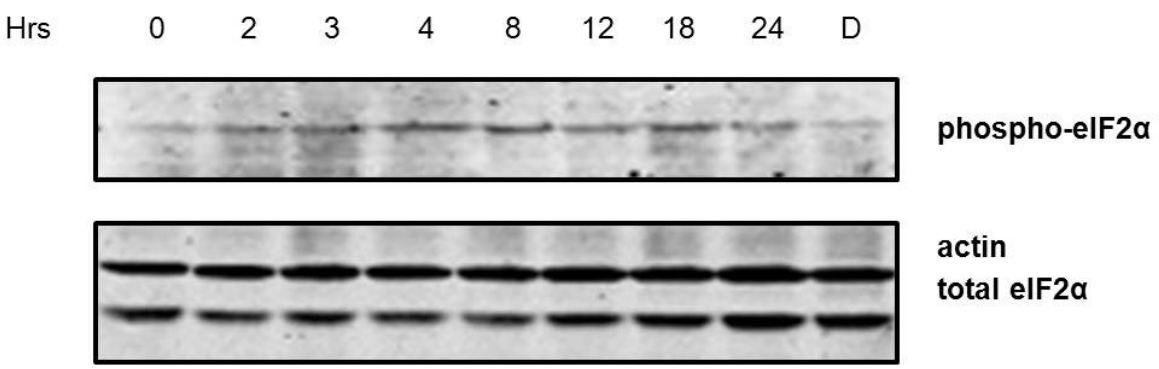
Pa-Tu-8902



HPAC



AsPC-1



Pancreatic cancer cells have delayed induction of ATF4.

Transmission of the delayed activation of the eIF2α arm of the UPR resulted in the delayed induction of the downstream effector ATF4. ATF4 is selectively translated while global protein synthesis is inhibited via eIF2α phosphorylation during ER stress. Consistent with phosphorylation of eIF2α, ATF4 was upregulated as early as 2 hours in non-tumorigenic cell lines HPDE and HPNE. In contrast, expression of ATF4 did not occur before 8 hours in pancreatic cancer cell lines. In addition to the delayed rate of ATF4 induction, pancreatic cancer cell lines also exhibited a diminished degree of induction. This altered response is most obvious when compared to the same positive control (Figure 6). Each immunoblot was loaded with the same lysate of HPNE that was treated with 100 nM bortezomib for 4 hours as a positive control for antibody effectiveness (106). ATF4 was robustly expressed in non-tumorigenic cell lines while pancreatic cancer cell lines had low induction, particularly in HPAC and AsPC-1 cells.

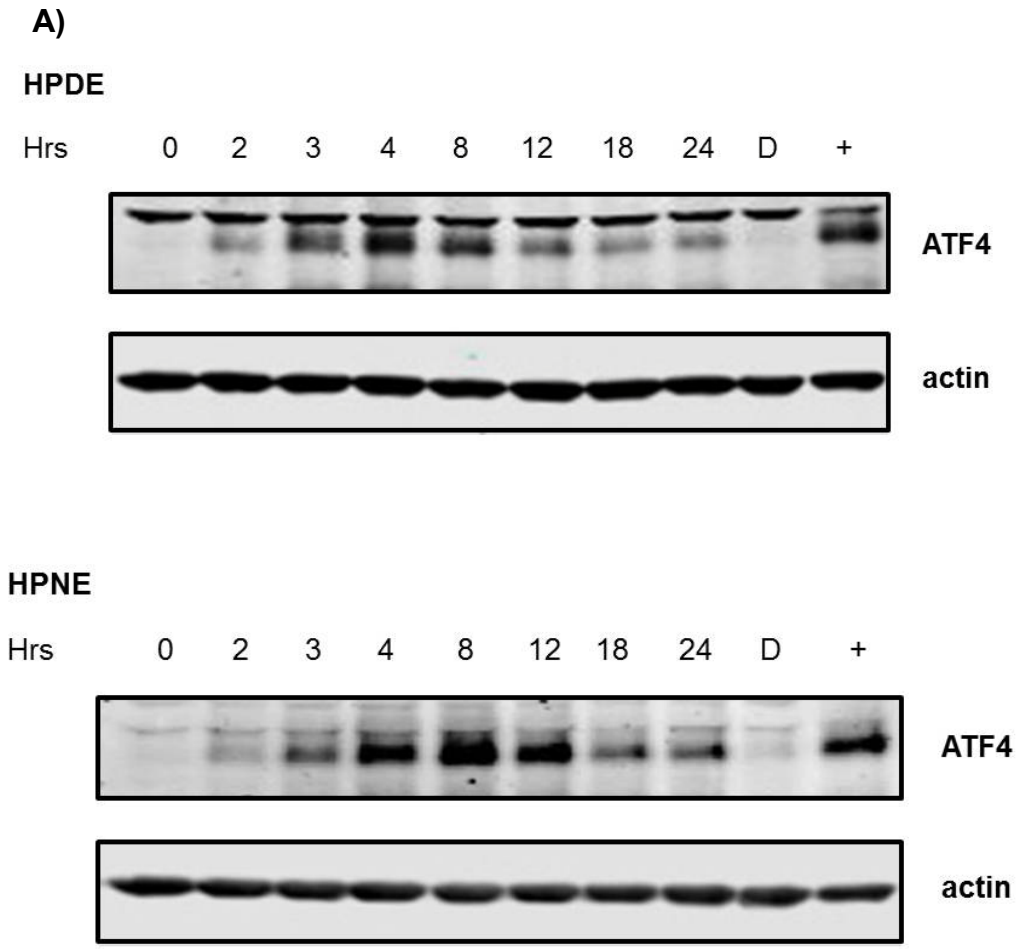
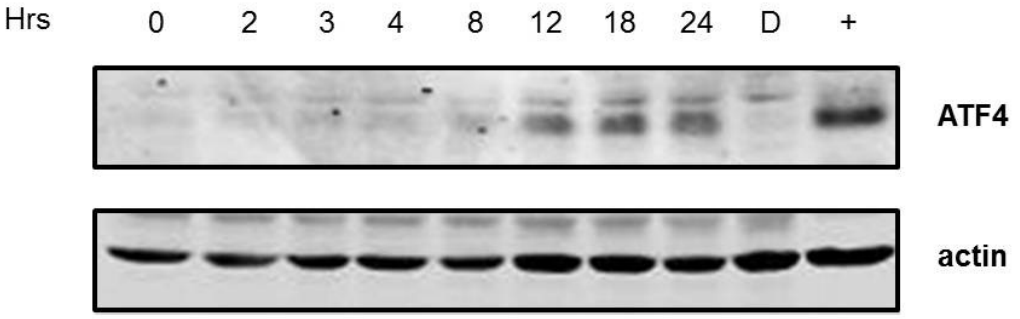


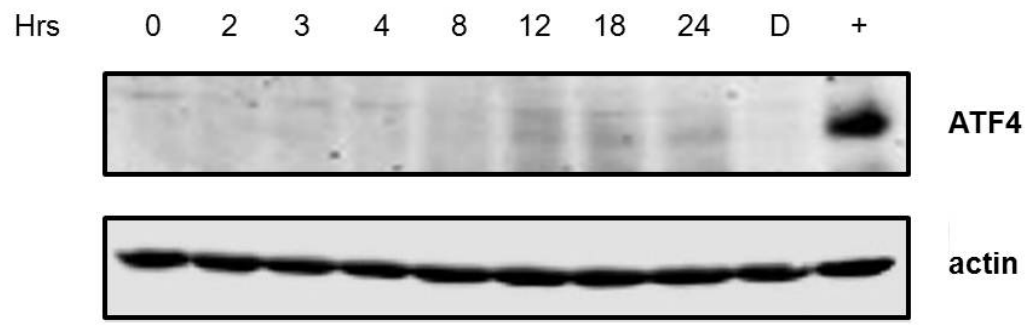
Figure 6. ATF4 induction is delayed in pancreatic cancer cells compared to non-tumorigenic pancreatic ductal cell lines. Treatment time courses over 24-hour incubation period of tunicamycin treatment. ***A***, non-tumorigenic pancreatic cell lines HPDE and HPNE exhibit early induction of ATF4 within 2 hours of tunicamycin treatment. ***B*** (next page), pancreatic cancer cell lines Pa-Tu-8902, HPAC, and AsPC-1 display delayed and markedly diminished induction of ATF4, particularly when compared to the same positive control. The DMSO-treated vehicle control, "D", for each cell line was treated for 24 hours. Positive control for antibody effectiveness: protein lysate from HPNE cells treated with 100 nM bortezomib for 4 hours. Each experiment was repeated in a minimum of 2 repetitions. Representative immunoblots are shown of similar data.

B)

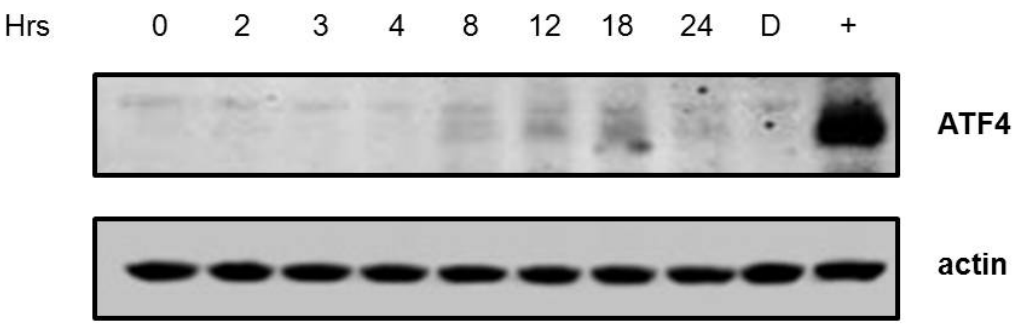
Pa-Tu-8902



HPAC



AsPC-1



Pancreatic cancer cells have delayed induction of CHOP.

Further assessment of events downstream of activation of the eIF2α arm of the UPR demonstrated that in addition to delayed activation of eIF2α and ATF4, CHOP was also delayed in its expression. Selective translation of ATF4 following ER stress induction resulted in transcription of its target gene, CHOP/GADD153. Consistent with a late activation of the eIF2α and ATF4 pathways, CHOP expression was also delayed in pancreatic cancer. CHOP expression occurred in non-tumorigenic pancreatic cells at a minimum of 3-4 hours compared to 8 hours in pancreatic cancer cells. The duration of CHOP expression was relatively similar in both non-tumorigenic and cancer cell lines with a prolonged induction to 24 hours of tunicamycin treatment.

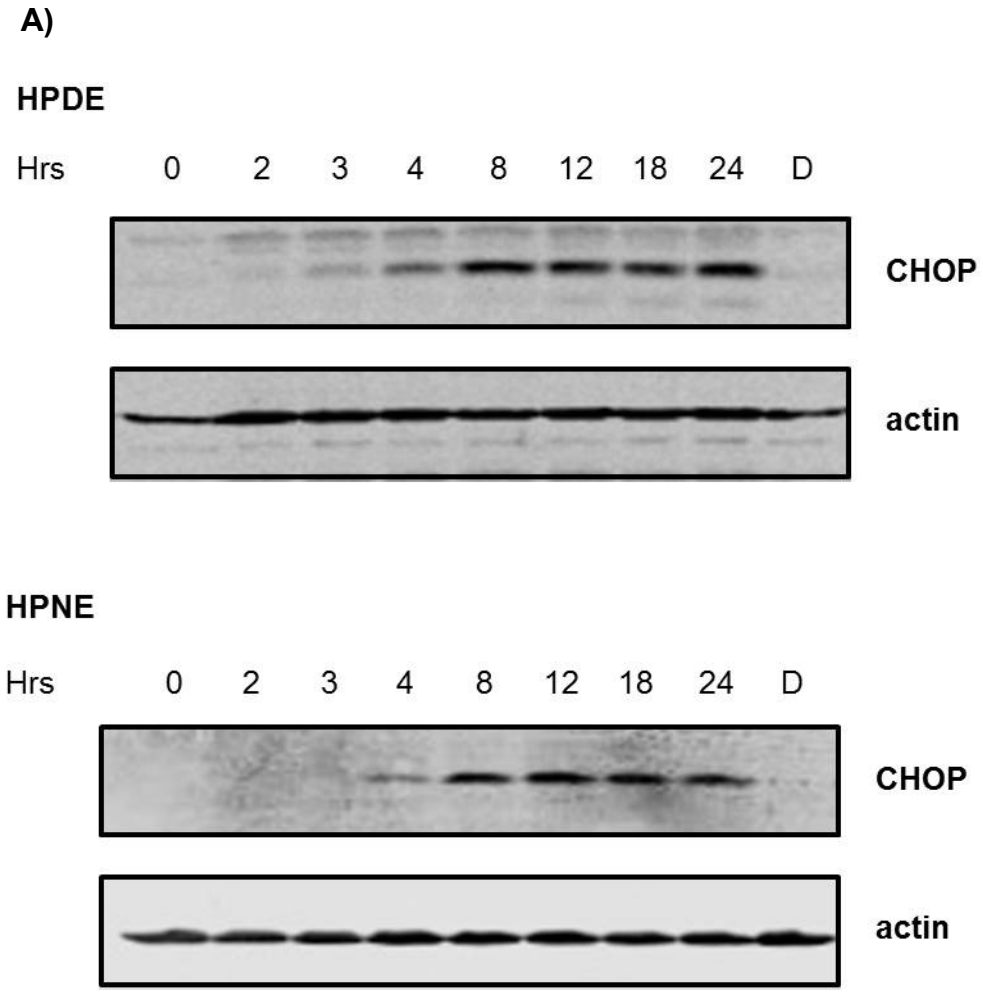
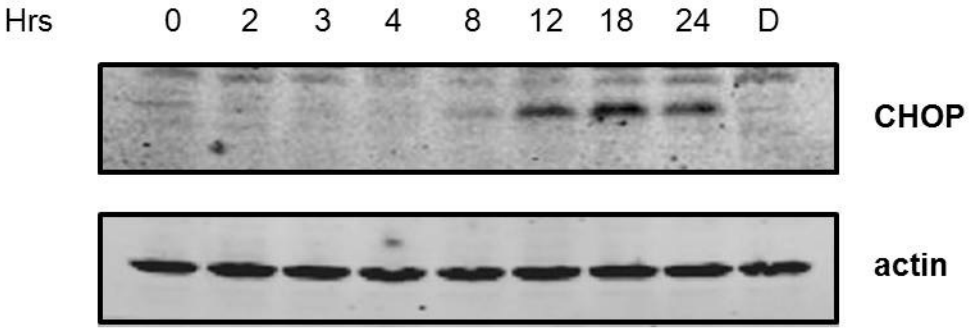


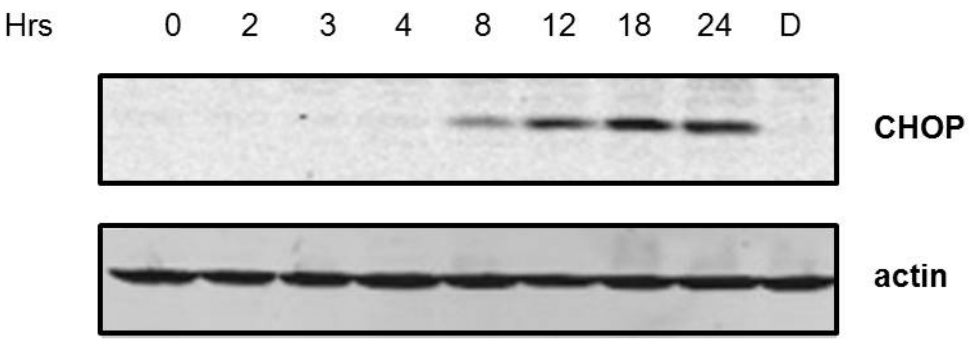
Figure 7. CHOP induction is delayed in pancreatic cancer cells compared to non-tumorigenic pancreatic ductal cell lines. Treatment time courses over 24-hour incubation period of tunicamycin treatment. ***A***, non-tumorigenic pancreatic cell lines HPDE and HPNE exhibit early induction of CHOP within 4 hours of tunicamycin treatment. ***B*** (next page), pancreatic cancer cell lines Pa-Tu-8902, HPAC, and AsPC-1 display delayed induction of CHOP within 8 hours of tunicamycin incubation. Each experiment was repeated in a minimum of 2 repetitions. Representative immunoblots are shown of similar data. The DMSO-treated vehicle control, "D", for each cell line was treated for 24 hours.

B)

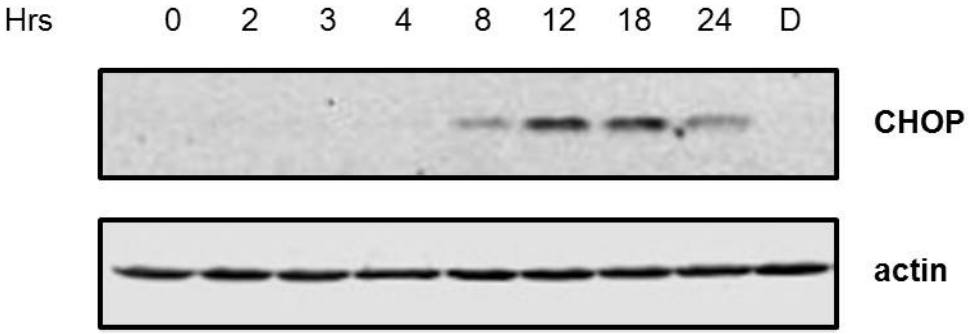
Pa-Tu-8902



HPAC



AsPC-1



Pancreatic cancer cell lines have reduced ability to activate the IRE1α/XBP1S arm of the UPR.

After establishing that the eIF2α/ATF4/CHOP arm of the UPR was late activated late in pancreatic cancer cells, I wanted to assess further whether impairment was isolated to the eIF2α arm of the UPR or generalized to the other branches of the UPR. Therefore, I next examined whether the IRE1α response to ER stress was also delayed. As a read-out for activation of IRE1α, I assessed whether expression of its downstream cleavage product, spliced XBP-1 (XBP1s), occurred late in pancreatic cancer cells compared to non-tumorigenic cells. IRE1α mediates splicing of the uncleaved precursor mRNA of XBP-1, the only identified cleavage product of the IRE1α endoribonuclease function. Clear visualization of XBP-1 splicing in pancreatic cancer cells did not occur until a minimum of 8 hours compared to as little as 3 hours for non-tumorigenic pancreatic ductal cells. Interestingly, non-tumorigenic cells reached a distinct peak of XBP1s expression at 8 hours, which rapidly diminished by 24 hours. In marked contrast, expression of XBP1s persisted from 8 hours until the end of the time course at 24 hours in pancreatic cancer cells.

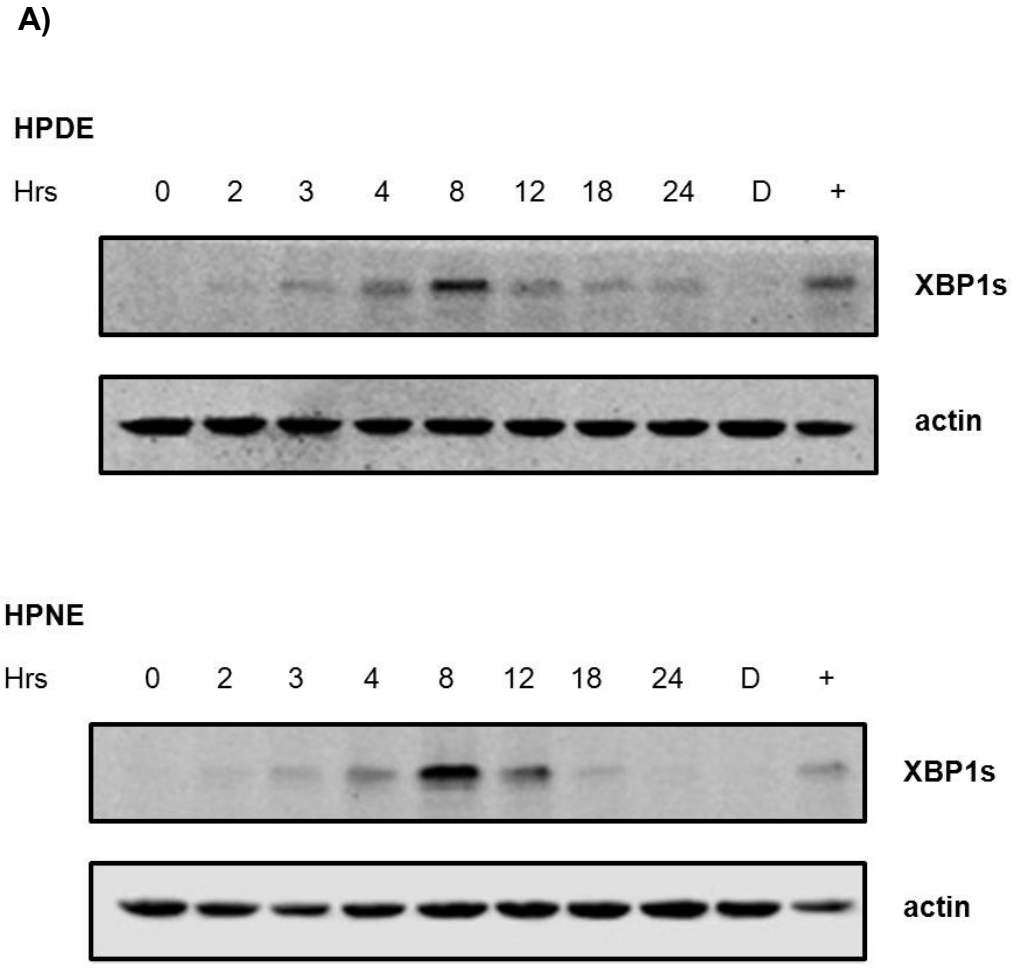
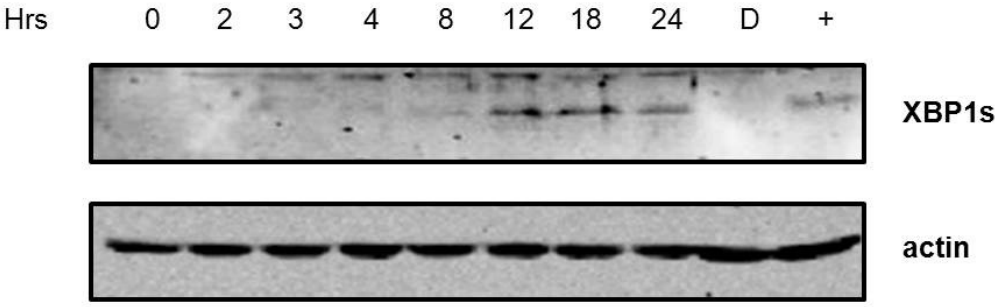


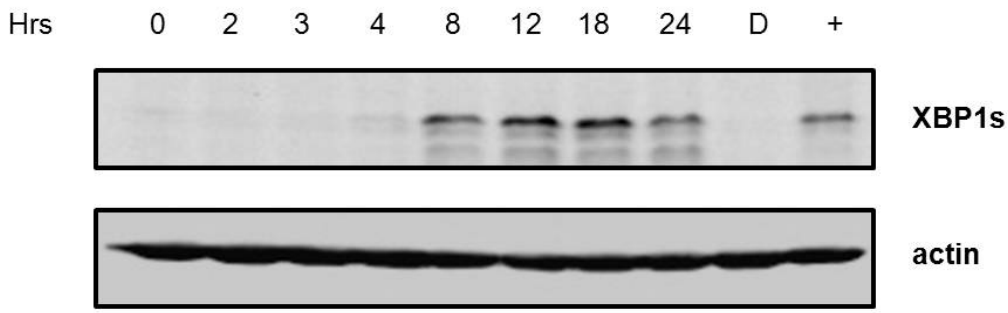
Figure 8. XBP1s expression is delayed in pancreatic cancer cells compared to non-tumorigenic pancreatic ductal cell lines. Treatment time courses over 24-hour incubation period of tunicamycin treatment. ***A***, non-tumorigenic pancreatic cell lines HPDE and HPNE exhibit early induction of XBP1s within 3 hours of tunicamycin treatment. Rise in XBP1s expression peaks at 8 hours and declines rapidly by 24 hours. ***B*** (next page), pancreatic cancer cell lines Pa-Tu-8902, HPAC, and AsPC-1 display delayed induction of XBP1s, requiring at least 8 hours of tunicamycin incubation. Activation of XBP1s remained elevated from 8 hours until 24 hours. Each experiment was repeated in a minimum of 2 separate experiments. Representative immunoblots are shown of similar data. The DMSO-treated vehicle control, "D", for each cell line was treated for 24 hours. Positive control for antibody effectiveness: protein lysate from HPDE cells treated with 10 µg/ml tunicamycin for 8 hours.

B)

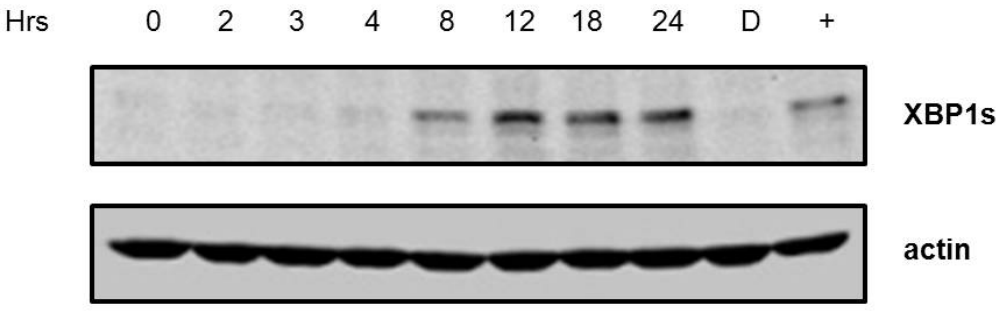
Pa-Tu-8902



HPAC



AsPC-1



GRP78 response is similar in pancreatic cancer cells and non-tumorigenic pancreatic ductal cells

We next investigated GRP78 expression as a potential explanation for the impairment of both for eIF2α and IRE1α/XBP1s arms of the UPR. Since both UPR arms were affected, the cause of this impairment was likely upstream of both pathways. Since GRP78 constitutively binds the UPR transmembrane receptors to maintain them in an inactive state, upregulation of GRP78 could possibly require an increased threshold of protein load in the ER before PERK and IRE1α could be activated. GRP78 could also act as an adaptive mechanism once ER stress was induced to increase capacity for protein load. Consequently, we would expect either an increased basal expression of GRP78 prior to ER stress or an increased GRP78 induction once ER stress occurred to counter the accumulation of ER proteins.

However, we observed that in contrast to delayed responses in both eIF2α/ATF4/CHOP and IRE1α/XBP1s branches, no clear differences in upregulation of GRP78 were observed between non-tumorigenic and pancreatic cancer cells. In previously performed studies (negative data not shown), no consistent differences between non-tumorigenic and pancreatic cancer cell lines were observed. Similar responses to activation of GRP78 occurred in both pancreatic cancer cells and non-tumorigenic cells. Both HPDE and HPNE cells had a similar rate of GRP78 induction to pancreatic cancer cells with clear upregulation occurring at 12 hours. Upregulation of GRP78 in pancreatic cancer cells occurred at variable intervals, ranging from a minimum of 8 hours (HPAC) up to 18 hours

(AsPC-1). The maximum reached induction level from baseline for GRP78 expression was also similar among non-tumorigenic pancreatic ductal cells and pancreatic cancer cells. Both types of cell lines upregulated GRP78 by 2-4 times the baseline value over 24 hours.

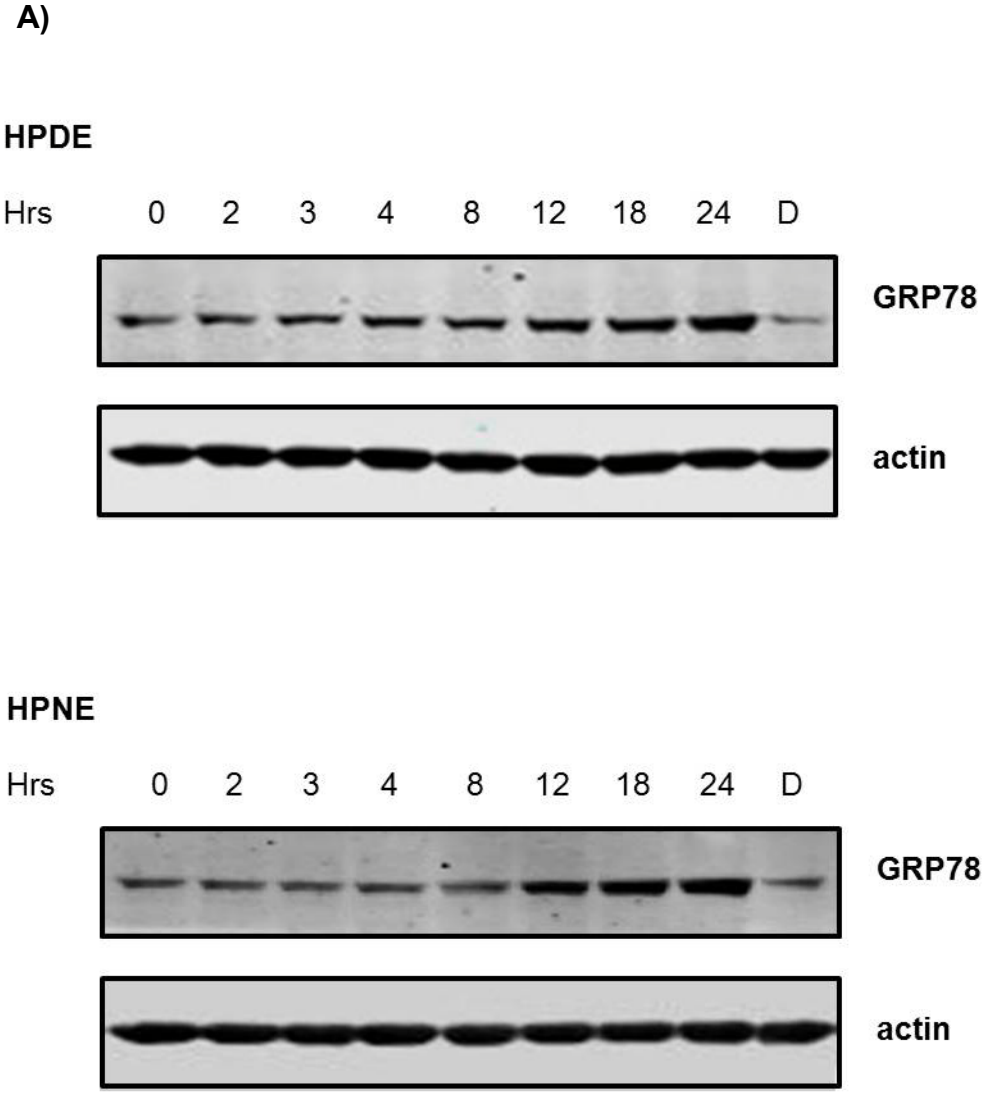
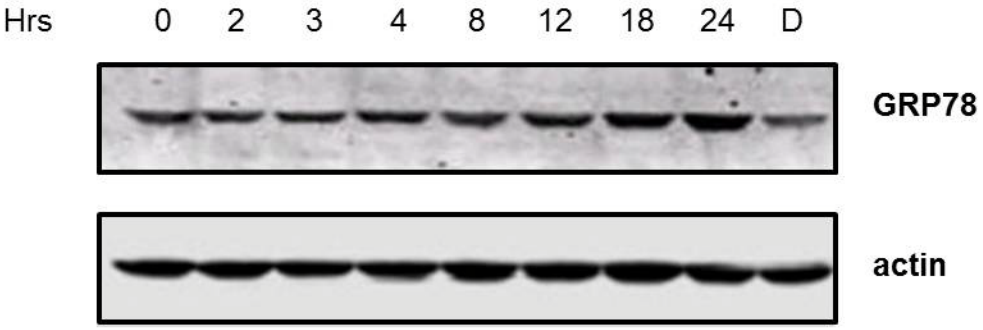


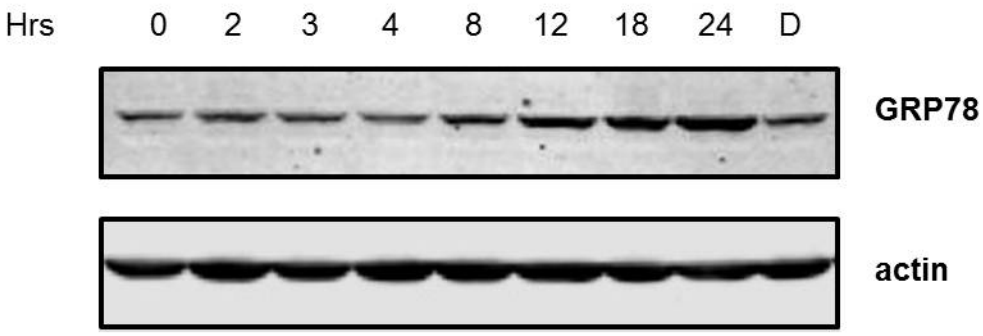
Figure 9. GRP78 expression is similar in both pancreatic cancer cells and non-tumorigenic pancreatic ductal cell lines. Treatment time courses over 24-hour incubation period of tunicamycin treatment. ***A***, non-tumorigenic pancreatic cell lines HPDE and HPNE exhibit induction of GRP78 at 8 hours of tunicamycin treatment. ***B*** (next page), pancreatic cancer cell lines Pa-Tu-8902, HPAC, and AsPC-1 display induction of GRP78 between 8 and 18 hours of tunicamycin incubation. Each experiment was repeated in a minimum of 2 repetitions. Representative immunoblots are shown of similar data. The DMSO-treated vehicle control, "D", for each cell line was treated for 24 hours.

B)

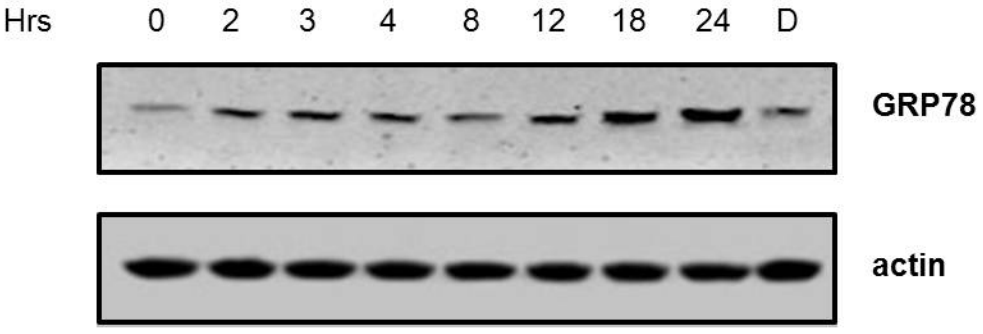
Pa-Tu-8902



HPAC



AsPC-1



Treatment with tunicamycin does not induce apoptosis in most cell lines.

To assess whether cells were being lost to apoptosis during the duration of tunicamycin treatment, rates of apoptosis were determined at 24 hours. ER stress normally activates a pro-survival response initially in cells by activating mechanisms that mitigate protein stress within the ER. However, apoptosis is induced if the cell is unable to reduce the protein load. The pro-apoptotic switch of the UPR is thought to occur through induction of CHOP and subsequent activation of caspases. As demonstrated by Figure 10, neither non-tumorigenic nor pancreatic cancer cells underwent any statiscally significant apoptosis greater than their respective DMSO-treated controls after treatment with tunicamycin. The exception to this observation was pancreatic cancer cell line HPAC. Longer durations up to 24 hours of tunicamycin exposure induced 16.3 +/- 5.2% apoptosis. Preliminary time course studies indicate that apoptosis of more than 10% did not occur until after 16 hours of tunicamycin exposure (data not shown).

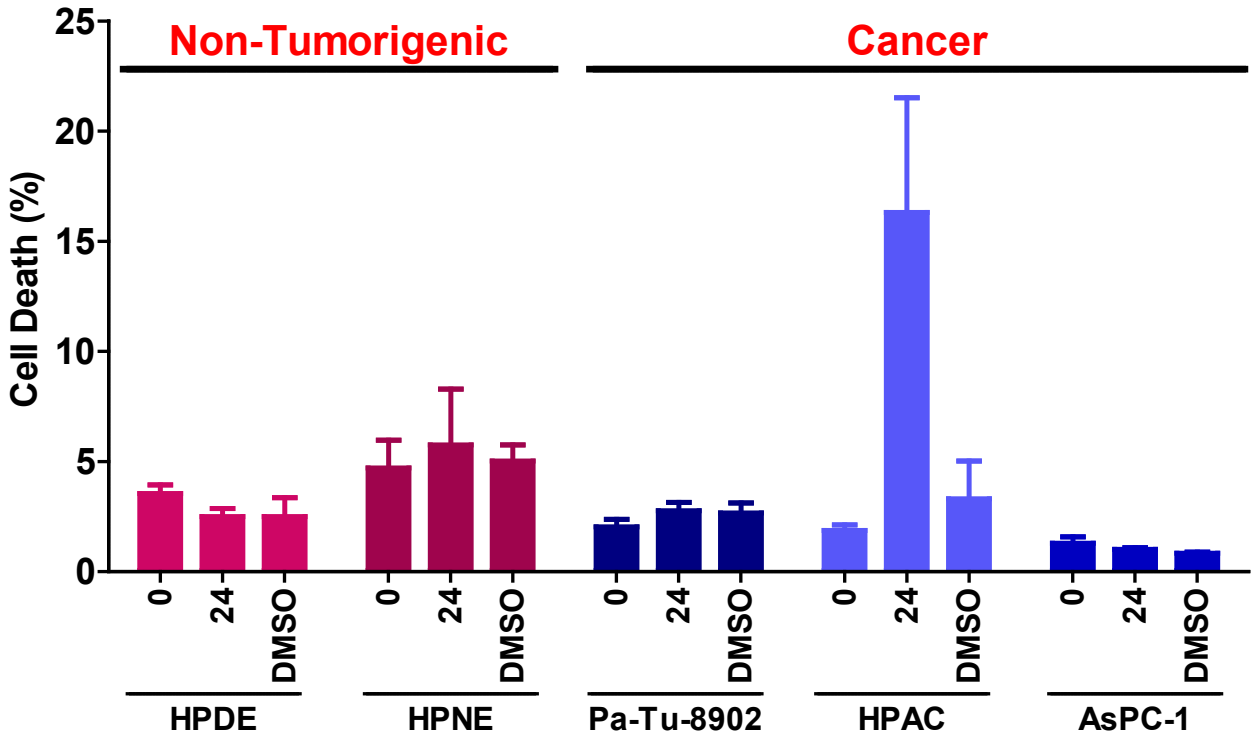


Figure 10. Most non-tumorigenic pancreatic ductal and pancreatic cancer cell lines do not undergo apoptosis following tunicamycin treatment. Cell death measured via propidium iodide staining and quantified by flow cytometry. Cells in the sub-G0/G1 were considered apoptotic. Non-tumorigenic pancreatic ductal cells (in pink hues, HPDE, HPNE) and pancreatic cancer cells (in blue hues, Pa-Tu-8902, HPAC, AsPC-1) were treated with DMSO vehicle or tunicamycin (0.5 µg/ml) for 24 hours. Columns, *n*=3; bars represent SEM.

DISCUSSION

This study aimed to determine whether the pancreatic cancer cell lines regulated the unfolded protein response differently from non-tumorigenic cells. Our research demonstrates that pancreatic cancer cell lines possess a deficient ability to activate either the eIF2α/ATF4/CHOP or IRE1α/XBP1s arms of the UPR. The identification of two branches that fail to be activated by ER stress induction indicates that a global response to UPR activation is impaired. This finding implicates a defect upstream of the UPR branches.

The failure to activate the UPR could represent a number of problems within the cell. The two most salient explanations for a deficient UPR response would be either an overall failure to detect ER stress or a pre-existing compensatory mechanism enabling the cells to handle a higher protein capacity at basal conditions. The current prevailing theory of how the UPR is activated is through disinhibition of the UPR transmembrane "sensors" through GRP78 release. Conceivably, the presence of inactivating mutations or the presence of one or more inhibitory proteins could exist for either PERK or IRE1α in pancreatic cancer cells. Such inactivating mutations or interacting proteins preventing their oligomerization or autophosphorylation could make the cells less responsive to ER stress because the UPR sensors would be inhibited. Consequently, cells would be less sensitive to detection of protein stress within the ER. However, the presence of mutations in both PERK and IRE1α would seem to be highly improbable. While PERK mutations do exist in humans (Wolcott-Rallison Syndrome), this seems to be an unlikely explanation since PERK mutation has not been specifically linked to the

development of cancer (107). Studies in mice with established germline mutations in PERK have demonstrated diabetes along with a number of systemic abnormalities, such as osteoporosis and growth retardation (108). Conceivably, the observance of pancreatic β-cell deficiency with PERK mutation could be an explanation for the high incidence of diabetes in pancreatic cancer patients, but this was beyond the scope of this study. The alternative possibility of a binding protein that modulates PERK and IRE1α activity is most logically filled by the role of GRP78. However, my studies indicate that induction rate and the degree of induction of GRP78 is similar among non-tumorigenic and pancreatic cancer cell lines. Additionally, I previously performed studies which indicated no significant difference in basal GRP78 protein expression between non-tumorigenic and pancreatic cancer cells (negative data not shown). Other proteins that may interact with both molecules have not been identified.

The second possible explanation for defective eIF2α activation is a mechanism enabling a higher protein load before the UPR is activated. Past research has indicated that a decreased ability to phosphorylate eIF2α promotes cell transformation (109-112). Seemingly contrary to this finding, most recent evidence indicates that the PERK/eIF2α pathway acts as a cytoprotective mechanism by mitigating the increased protein stress placed on proliferating tumor cells (58, 61-64). Deficiency of PERK activity has been repeatedly shown to induce apoptosis cells because of the failure to abrogate protein synthesis, thus creating an environment of toxic protein stress. While our study may appear inconsistent with the most recent studies, we propose that pancreatic cancer cells possess an

adaptive mechanism upstream of the UPR that pre-empts the need for its protective effects in pancreatic cancer cells. We propose that a pre-existing over-abundance protein folding mechanisms, such as ER chaperones, would prevent protein aggregation. An increase in such mechanisms may essentially act as a protein "buffer" system that allows a higher protein capacity within the cell before stress is induced. Upregulation of a number of ER chaperones, such as GRP78 or GRP94, have indeed been documented to be upregulated in multiple cancers (113). This concept is supported by a previous study by Yamaguchi *et al*, which established a link between ER chaperone levels with delayed responses in the UPR (114). They demonstrated that HEK293 cells with a kinase-dead PERK demonstrated an over-abundance of calreticulin, a chaperone involved in maintaining ER protein quality control, at both basal and after ER stress induction. Deficiency in PERK function resulted in complete block of ATF4 expression and delayed induction of CHOP (114). These findings are remarkably similar to my results where a deficient eIF2α response also resulted in delayed and diminished activation of ATF4 and slower induction of CHOP in pancreatic cancer cell lines.

To determine whether the altered response to ER stress induction was isolated to the eIF2α arm of the UPR, I further examined the IRE1α/XBP1s signaling. I identified that in addition to delayed activation of the eIF2α arm of the UPR, activation of the IRE1α branch was also delayed. XBP1 splicing was utilized as a readout for IRE1α activation. We found that XBP1s expression persisted in pancreatic cancer cells even up to 24 hours of treatment. This is in comparison to non-tumorigenic cell lines where XBP1s expression quickly increased up to 8 hours

with a rapid decline by 12 hours. Once activated, the IRE1-XBP1s pathway appears to elicit a full and sustained response. This increase in XBP1s expression may represent a compensatory increase in activation of an alternative UPR pathway given the continued deficiency in phosphorylation of the eIF2α pathway. Despite a delay in the initial activation of this response, the persistent expression of XBP1s in pancreatic cancer cells may be a pro-tumorigenic activation of the UPR that indicates more than a shift in the protein load capacity of these cells. XBP1s activation may represent a true deficiency in the phosphorylation of eIF2α. These findings are supported with previous findings where Perk-deficient embryonic stem cells demonstrated persistent IRE1α activation up to 16 hours while subsiding within 4-6 hours in their wild-type counterparts (60). Interestingly, the XBP1s pathway has been frequently attributed to promoting mostly pro-tumorigenic functions. Expression of XBP1s was shown to be correlated with hypoxic regions tumor and was demonstrated to be critical to tumor growth under hypoxic conditions (104). XBP1s has also been shown to regulate survival-promoting pathways that upregulate ER chaperones and ER biogenesis. These findings suggest that possibly two mechanisms of adaptability to ER stress induction exist: increased tolerance to UPR activation and compensatory XBP1s activation once the threshold for its activation is reached.

One mechanism that warrants further investigation is the possibility that autophagy may play a role in protecting pancreatic cancer cells from ER stress. Autophagy entails another process with dual cytoprotective and cytotoxic functions. The cytoprotective component of autophagy attempts to counter proteotoxic stress

in cells by sequestration of proteins away from the cellular environment into autophagosomes. Autophagy has been consistently shown to be activated in response to ER stress as a complementary mechanism to the UPR to alleviate protein stress (61). Inhibition of this process has been shown to enhance cell death by eliminating a pathway for preventing protein accumulation, thus creating a toxic cellular environment. Although propidium iodide staining revealed that in most non-tumorigenic and pancreatic cancer cell lines did not undergo a significant amount of cell death, autophagy may play a role in ameliorating protein stress by storage of proteins into autophagosomes. However, the delayed activation of the eIF2α pathway would appear to argue against the presence of accumulated proteins and autophagy unless a defect in ER stress detection existed. Additionally, ATF4 has been implicated as a potential mechanistic link from the UPR to autophagy that helps mediate adaptation of tumor cells to hypoxic stress (58, 115). However, in my studies, the late and reduced activation of ATF4 would suggest that autophagy were not being induced through ATF4 in pancreatic cancer cells.

Tunicamycin treatment did not induce significant apoptosis compared to their DMSO-treated controls in non-tumorigenic cell lines and 2 out of 3 pancreatic cancer cell lines. Ironically, protection from apoptosis by two different mechanisms is likely occurring in non-tumorigenic compared to pancreatic cancer cell lines. Non-tumorigenic cells were capable of phosphorylating eIF2α and effectively reducing protein load. This mechanism of translational attenuation has been repeatedly shown to promote cell survival by preventing continued protein accumulation through translational inhibition (60, 116). One pancreatic cancer cell

line, HPAC, was the only cell line to exhibit sensitivity to apoptosis induction by tunicamycin treatment (Figure 10). However, preliminary evaluation of apoptosis induction through a time course over 24 hours revealed that significant induction of apoptosis to more than 10% required at least 20 hours of tunicamycin treatment (data not shown). The limited apoptosis observed up to that time point would suggest that HPAC cells were able to accommodate increased protein load. An explanation for this observation could possibly be attributed to defects in autophagy activation. If defects in the autophagy pathway existed, especially in conjunction with a defective activation of translational repression via the eIF2α pathway, toxic proteins could accumulate in the cellular environment. HPAC cells would likely be less capable of handling the excess protein. In the presence of ineffective eIF2α-mediated translational repression, more proteins would continue to be synthesized and further contribute to protein accumulation. Consequently, excess and malfolded proteins would be free to aggregate within the cell creating a toxic microenvironment, resulting in increased cell death.

The differing response in HPAC may suggest a potential susceptibility of a subset of pancreatic cancer cell lines to ER stress that is not encountered in non-tumorigenic cells. While HPAC cells exhibited similar delayed activation of the UPR, these cells appeared to be more prone to apoptosis. This finding is more consistent with published studies indicating that a deficient phosphorylation of eIF2α resulted in continued protein synthesis and overwhelming protein load (60, 117). These results would suggest that HPAC cells may have a truly deficient eIF2α activation once the threshold for ER stress is achieved. Conjecture would suggest

that while HPAC cells still possess a compensatory mechanism for accommodating increased protein load, saturation of this system results in overwhelming protein stress that cannot be relieved by translational attenuation through a deficient eIF2α response.

While this study did not find a primary cause for the deficient activation of the UPR, identification of the explanation for this finding could lead to potential new targets that would enable pancreatic cancer cells to be sensitized to ER stress-inducing agents. Admittedly, a key problem with targeting ER stress in cancer therapy is the lack of specificity in differentiating normal from cancer cells. Proteasomal inhibitors, such as bortezomib, appear to have partially circumvented this problem in some aspects by selectively inducing ER stress-mediated apoptosis in tumor cells because of their high protein turnover rate. In pancreatic cancer cells, bortezomib has been shown to induce ER stress-mediated apoptosis (66, 67, 118). The narrow therapeutic index between inducing apoptosis in normal versus cancer cells impedes an effective cancer-specific strategy that does not also create toxicity. This obstacle is no more evident than in pancreatic cancer. A critical problem frequently encountered in the treatment of pancreatic cancer is balancing the toxicity induced by chemotherapies compared to the therapeutic benefit obtained. I suspect that a component of the chemoresistance encountered along with the poor toxicity profile of chemotherapeutics in pancreatic cancer is due to an elevated tolerance for protein stress, as supported by my study. I hypothesize that pancreatic cancer cells are able to withstand such a high level of protein stress before the induction of the ER stress response that it far exceeds the protein stress

capacity of normal cells. My previous studies have yet to identify any level of basal ER stress in pancreatic cancer cells despite a hypovascular tumor microenvironment that should predispose cells to ER stress induction (negative data not shown). I suspect that ER stress is not induced at basal levels in part because an adaptive mechanism to higher protein load exists in pancreatic cancer cells. Identification of a cancer-specific target that "shifts the curve" toward a threshold level of protein stress in pancreatic cancer cells that would essentially sensitize the cells to earlier induction of ER stress. I propose that rather than identifying agents that act as single chemotherapeutic therapies, ER stress-targeting agents should be used in combination therapy to "prime" cells for sensitization ER stress-mediated apoptosis by other tumor selective agents.

CHAPTER THREE:

CYCLIN D1 REGULATION IN PANCREATIC CANCER CELLS IS IMPAIRED IN RESPONSE TO ER STRESS

INTRODUCTION

Loss of cell cycle control is thought to be an important component of tumorigenesis in cancer. Cyclin D1 has been shown to be overexpressed in multiple cancers, including in pancreatic cancer (77-80, 119). Its overexpression in pancreatic cancer has been associated with poor prognosis (80). In up to 90% of pancreatic tumors, KRAS is mutated to an oncogenic isoform and results in a constitutively activated RAS-RAF-MEK pathway. The tumorigenic effects of this activating mutation has been well-established as a promoter of cyclin D1 transcription, cyclin D1 protein translation and stability, and assembly of cyclin D1 holoenzyme complexes with CDK4 and CDK6 (120-124).

In malignancy, the dysregulation of cyclin D1 and subsequent uncontrolled tumor cell proliferation is driven at multiple regulatory levels, including transcriptional, translational, and post-translational regulation steps. The uncontrolled growth of these transformed cells places strenuous requirements on solid tumors to supply adequate oxygen and nutrients to rapidly growing tumors. In poorly vascularized tumors such as pancreatic adenocarcinoma, hypoxic stress, nutrient and glucose deficiency, as well as altered calcium homeostasis are thought to promote signaling pathways required to sustain continued tumor growth. One such pathway known to respond to these increased metabolic and hypoxic stresses is endoplasmic reticulum stress. As a cytoprotective mechanism in normal cells, ER stress induction activates a UPR-mediated translational attenuation, which has been previously shown to reduce translation of cyclin D1 and lead to subsequent cell cycle arrest (85, 86). An expected response to increased metabolic demand in

a pancreatic tumor would be reduction of cyclin D1 through the UPR. Contradictory to this, however, previous studies have established that pancreatic cancer cells demonstrate increased cyclin D1 expression.

I hypothesized that pancreatic tumors are unable to downregulate cyclin D1 translation in response to endoplasmic reticulum stress and that this impaired repression was mediated through an eIF2α-dependent mechanism. Previous findings from Chapter Two identified an altered unfolded protein response in pancreatic cancer cells to ER stress induction. In this study, I challenged pancreatic cancer cells with two classical ER-inducing stress agents, tunicamycin and thapsigargin, to investigate the response of pancreatic cancer cells to ER stress. I found that in comparison to non-tumorigenic pancreatic cells, a subset of pancreatic cancer cells were consistently unable to downregulate cyclin D1 and exit the cell cycle after ER stress induction. Further analyses demonstrated that this delayed inhibition of cyclin D1 levels was due to impaired translational repression mediated by the eIF2α pathway of the UPR. These findings suggest that a subset of pancreatic cancer cells have increased tolerance to ER stress-mediated cell cycle arrest.

MATERIALS AND METHODS

Cell lines and culture

Non-tumorigenic cell line human pancreatic ductal epithelial (HPDE-E6/E7) cells was provided by Dr. M. Tsao (Ontario Cancer Institute, Toronto, Ontario, Canada). The human pancreatic nestin-positive epithelial (hTERT-HPNE) cell line was obtained from the American Type Tissue Collection. 22 pancreatic cancer cell lines were initially screened in this study: human pancreatic cancer cell lines AsPC-1, Hs766T, BxPC3, MiaPaca-2, Su86.86, HPAC (American Type Tissue Collection); MPanc96 (Dr. Timothy J. Eberlein, Washington University, St. Louis, MO); L3.6pl (Dr. Isaiah J. Fidler, MD Anderson Cancer Center, Houston); SW1990, SUIT-2, T3M4, DAN-G, COLO357, Pa-Tu-8902, Pa-Tu-8988T, HCG-25, 3.27, 2.03, HPAFII, CAPAN2, and CFPAC1 (Drs. Joe Gray, Eric Collison, and Martin McMahon, University of California and Lawrence Livermore Laboratory, San Francisco, CA). HPDE cells were grown in keratinocyte media and supplemented with epidermal growth factor and bovine pituitary extract (Invitrogen, Carlsbad, CA). For HPDE cell expansion, adherent cells were dissociated from plastic through trypsinization, and trypsin was neutralized with soybean trypsin inhibitor. HPNE cells were grown in Medium D as described previously (105). All human pancreatic cancer cell lines were maintained in DMEM (Mediatech, Inc., Manassas, VA) supplemented with 10% fetal bovine serum. Wild-type eIF2α mouse embryonic fibroblasts (MEFs) and mutant eIF2α (S51A) MEFs with a non-phosphorylatable eIF2α at serine 51 were generously provided by David Ron (New York University, New York, NY). S51A and WT MEFs were maintained in DMEM (Mediatech, Inc., Manassas, VA),

supplemented with 10% fetal bovine serum, 10 μmol/L non-essential amino acids (Lonza BioWhittaker, Walkersville, MD), 0.55 μmol/L β-mercaptoethanol (Sigma, St. Louis, MO), and L-glutamine (Lonza BioWhittaker, Walkersville, MD). All cell lines were maintained in an incubator under conditions of 5% CO_2 at 37ºC. Cell lines were consistently maintained at confluencies of 80% or less to minimize stress. After cells were plated and allowed to adhere overnight, media was replaced 3-6 hours prior to start of each experiment to minimize metabolic stress from media consumption. All cell lines were maintained in antibiotic-free media and confirmed to be free of mycoplasma contamination by testing with the Mycoalert Mycoplasma Detection Kit (Lonza BioWhittaker, Walkersville, MD).

Reagents and antibodies

Tunicamycin (TU) and cycloheximide (CHX) was purchased from Sigma-Aldrich (St. Louis, MO). Thapsigargin (TG) was obtained from EMD Chemicals (Gibbstown, NJ). The proteasome inhibitor bortezomib (PS-341, Velcade) was provided by Millenium Pharmaceuticals (Boston, MA). Primary antibodies specific to the proteins were obtained as follows: cyclin D1 (Santa Cruz Biotechnology, Santa Cruz, CA), $p27^{Kip1}$ (BD Biosciences Transduction Laboratories, San Diego, CA), phospho-Rb, total Rb, phospho-GSK3α/β, total GSK3β (Cell Signaling Technology, Beverly, MA). Fluorescent secondary antibodies of IRDye CW800 goat anti-mouse and IRDye 680 goat anti-rabbit were purchased from Licor Biotechnology (Lincoln, NE).

Cell Cycle Analysis

Analysis of cell cycle phases and DNA fragmentation were performed through propidium iodide (PI) staining. Selected cell lines were plated in 6-well plates so that the final confluency of cells in the control wells reached approximately 60-70%. Cells were allowed to adhere overnight. 3-6 hours prior to experiment start, media was replaced with fresh warmed media to minimize metabolic stress from media consumption. After exposure to various treatments, cells were harvested with 0.05% trypsin-EDTA, neutralized with serum-containing media and centrifuged at 500 x *g*. Cell pellets were washed twice with PBS, pelleted again by centrifugation, and fixed at least overnight in 70% ice-cold ethanol at 4ºC. Cells were then centrifuged at 1000 x *g* for 5 minutes and incubated in 50 μg/ml PI with 10 μg/ml RNase A for 30 minutes at room temperature while protected from light. Subsequent flow cytometric analysis was performed on a Beckman Coulter XL (Brea, CA) at the University of Texas M.D. Anderson Cancer Center's Flow Cytometry and Cellular Imaging Facility (Houston, TX). Cells in the sub-G0/G1 peak were considered apoptotic. The remaining cells were assessed for the percentage of cells in each of the cell cycle phases. Cell cycle analysis results were normalized to the DMSO vehicle control where the proportion of cells in each phase was represented as a percentage of the DMSO-treated control unless otherwise specified.

Immunoblotting

Cells were lysed for 15 minutes on ice in Triton-X lysis buffer [1% Triton X-100, 150 mmol/L sodium chloride, 25 mmol/L Tris (pH 7.5), 1 mmol/L glycerol phosphate, 1 mmol/L sodium orthovanadate, 1 mmol/L sodium fluoride and protease inhibitor cocktail (Sigma)], collected by scraping, and incubated on ice an additional 10 minutes. Lysates were sonicated briefly and centrifuged at 14,000 x *g* at 4ºC for 15 minutes to clear cellular debris. 40 μg of total cellular protein of each sample was loaded with 4X sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer to a final concentration of 50 mmol/L Tris-HCl, 2% SDS, 10% glycerol, 0.01% bromophenol blue, and 5% β-mercaptoethanol. Samples were heated to 95ºC for 5 minutes, resolved by SDS-PAGE, and subsequently transferred to nitrocellulose membranes. Membranes were blocked with either 3% bovine serum albumin (BSA) in tris-buffered saline (TBS) for detection of phosphorylated proteins or 5% nonfat milk in phosphate-buffered saline (PBS) for 1 hour. Blots were then incubated with primary antibodies overnight at 4ºC, washed, and probed with the appropriately targeted species of secondary antibody. Protein bands were visualized and quantified using the Odyssey Infrared Imaging System and manufacturer-supplied software (Li-Cor Biotechnology). For most membranes, more than one primary antibody was co-incubated and was visualized using the two-color detection system.

Real-Time Quantitative Polymerase Chain Reaction

Total RNA was collected and isolated in Trizol reagent (Invitrogen, Carlsbad, CA) per manufacturer's protocol. 1 μg of total mRNA was subjected to genomic DNA elimination treatment and reverse transcribed for 30 minutes using the QuantiTect Reverse Transcription Kit by Qiagen (Hilden, Germany) following manufacturer's instructions. The resulting cDNA was diluted 1 volume to 10 volumes. Subsequently, quantitative PCR was performed with reaction mixtures consisting of 1 μl cDNA, 12.5 μl 2X PCR master mix (Promega, Madison, WI), 10 μM forward and reverse primers, 0.25 μl 100X SYBR Green I (Invitrogen, Carlsbad, CA). Primer pairs targeting genes of interest were designed using Lasergene software (DNAstar, Madison, WI). Primer pairs were designed for target gene cyclin D1 and reference gene 18S for mRNA amplification. Product amplification was quantified using the iCycler iQ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). To determine the linear range of amplification and to account for primer efficiency of each primer set, quantification values were calculated based on a standard curve constructed from a dilution series (125). Each experimental condition was tested in triplicate. The mean of the triplicate samples target gene were normalized to the mean of triplicate 18S values from the same sample set. Results were expressed as a ratio of the relative expression of treated samples to control samples +/- SEM. Specificity of each primer set for one product was verified by the formation a single melting peak and by agarose gel electrophoresis.

Quantification of Protein Synthesis

Selected cell lines were plated in 96-well plates so that the confluency of cells in the control wells reached approximately 60-70% at the conclusion of the experiment. Cells were allowed to adhere over 24 hours. 3-6 hours prior to experiment start, media was replaced with fresh warmed media to minimize metabolic stress from media consumption. Cells were then incubated for 4 hours with DMSO, 0.5 μg/ml tunicamycin, or 10 nM thapsigargin. After treatment, cells were gently washed with PBS once and then pulse-labeled in leucine-free media (MP Biomedicals, Solon, OH) with 2 μCi/ml L-[4,5-^{3}H] leucine (PerkinElmer, Waltham, MA) for 2 hours in an incubator at 37ºC and 5% CO_2. Cycloheximide (50 μM) was included as a positive control for protein synthesis inhibition and was co-incubated with tritiated leucine during the 2 hour incubation. Non-incorporated L-[4,5-^{3}H] leucine was removed by gently washing cells in ice-cold phosphate-buffered saline once. Cells were then lysed in 100 μl of 0.1 M KOH and harvested onto fiberglass filters using a plate harvester. Incorporated tritiated leucine into proteins was quantified using a scintillation counter.

Measurement of Cyclin D1 Degradation

Degradation of cyclin D1 was assessed in non-tumorigenic cell line HPDE and human pancreatic cancer cell line Hs766T. Cell lines were incubated in tunicamycin (0.5 μg/ml), cycloheximide (50 μM), or tunicamycin (0.5 μg/ml) with cycloheximide (50 μM) and collected at 10-minute intervals up to 60 minutes. Cyclin D1 protein was immunoblotted and bands quantified as described above.

Cyclin D1 levels were normalized to β-actin levels. The slopes of the time courses were calculated and compared using the GraphPad Prism 5.03 statistical software (GraphPad Software, San Diego, CA). The equations for the slopes were used to calculate the half-lives, $t_{1/2}$, of cyclin D1.

[6-^{3}H]-Thymidine Incorporation Assays

Wild-type and S51A mouse embryonic fibroblasts (MEFs) were plated in 96-well plates so that the confluency of cells in the DMSO-treated control wells reached approximately 70-80% at the conclusion of the experiment. Cells were allowed to adhere for at least 24 hours. 3-6 hours prior to experiment start, media was replaced with fresh warmed media to minimize metabolic stress from media consumption. Cells were exposed to DMSO or 0.5 μg/ml tunicamycin for 4 hours. After treatment, cells were gently washed with PBS once and then pulse-labeled with 10 μCi/ml [6-^{3}H] thymidine (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) for 1 hour at 37ºC under normoxic conditions in an incubator. Non-incorporated [6-^{3}H] thymidine was removed by gently washing cells with PBS once. Cells were then lysed in 100 μl of 0.1 M KOH and harvested onto fiberglass filters using a plate harvester. Incorporated tritiated thymidine was quantified using a scintillation counter.

Statistical Analysis

Unless otherwise specified, experiments represented in figures represent of at least three independent experiments. Statistical analyses were conducted with the GraphPad Prism 5.03 statistical software (GraphPad Software, San Diego, CA). Statistically significant differences between two groups were determined using the two-tailed unpaired Student's *t* test. For three or more groups, comparison by two-way ANOVA was performed followed by a Bonferroni's post-test analysis. Statistical significance was determined as $p < 0.05$ in all cases.

RESULTS

PANCREATIC CANCER CELLS HAVE IMPAIRED CELL CYCLE INHIBITION AFTER TUNICAMYCIN TREATMENT

In Figure 4 from Chapter Two, I established that on-tumorigenic pancreatic ductal cells were more sensitive to inhibition of DNA synthesis after ER stress than pancreatic cancer cell lines. This response was in compared to the impaired ability of pancreatic cancer cells to inhibit DNA synthesis at the same respective doses. Since DNA synthesis measures proliferation of viable cells, we also established in Chapter Two that the inhibition of DNA synthesis in non-tumorigenic cells was not due to a loss of cells to apoptosis. Since differences in DNA synthesis and non-tumorigenic and pancreatic cancer cells could not be attributed to apoptosis, we next assessed whether this difference could be the result of differences in cell cycle status.

Non-tumorigenic pancreatic ductal cells undergo G1 phase arrest.

I examined whether the decrease in DNA synthesis correlated with induction of cell cycle arrest. I performed cell cycle analysis using propidium iodide staining followed by flow cytometry to determine whether non-tumorigenic cells underwent cell cycle arrest after ER stress. In non-tumorigenic pancreatic ductal cells, treatment with tunicamycin resulted in a time-dependent reduction of cells in S-phase coinciding with an increased percentage of cells entering G0/G1 phase arrest (Figure 11 and Figure 12).

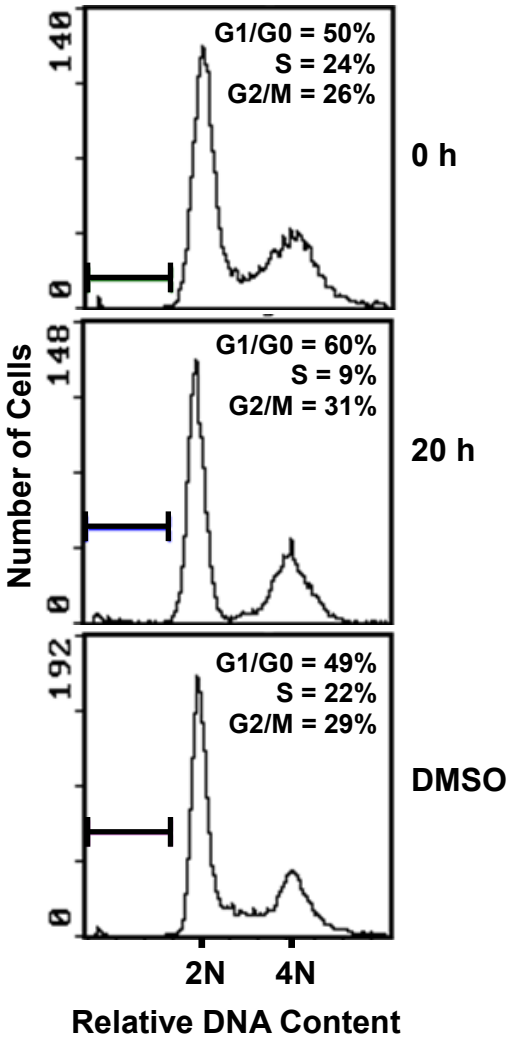


Figure 11. Treatment with tunicamycin induced a G1 phase arrest in non-tumorigenic cell line HPDE. HPDE cells were treated for 0 or 20 hours with DMSO or tunicamycin (0.5 µg/ml). Cell cycle analysis demonstrated an accumulation of cells in G0/G1 and a decreased percentage of cells in S-phase after incubation with tunicamycin. Similar results were obtained in at least 3 separate experiments.

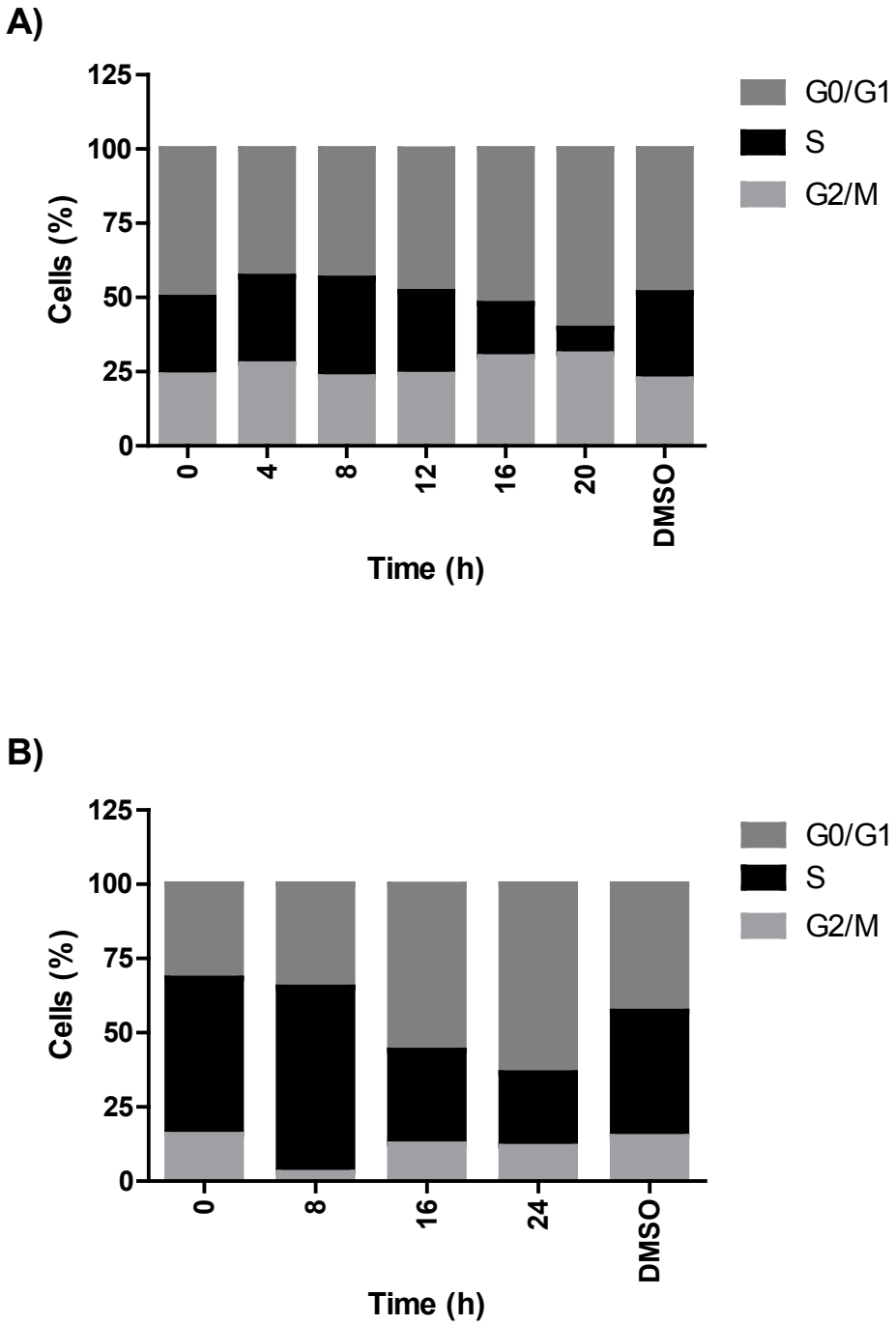


Figure 12. Non-tumorigenic cell lines demonstrate a time-dependent decrease of cells in S-phase with a proportionate increase of cells in G0/G1 phase. Representative data from cell cycle analysis by flow cytometry using propidium iodide staining is shown. Non-tumorigenic pancreatic ductal cells HPDE, ***A***, and HPNE, ***B***, were treated with tunicamycin (0.5 µg/ml) over a 24-hour time course. The DMSO-treated control was treated for 24 hours. Experiments were performed in two independent experiments with similar results.

Inhibition of cell cycle progression is reduced in pancreatic cancer cells.

To determine whether the differences between in DNA synthesis observed in Figure 4 between non-tumorigenic and pancreatic cancer cells, we next performed cycle analysis on pancreatic cancer cell lines. In comparison to non-tumorigenic cells, pancreatic cancer cells demonstrated an increased percentage of cells in S-phase following tunicamycin treatment (Figure 13). For the purposes of comparing across cell lines with different starting fractions of cells in S-phase, the percentages of cells in S-phase in the tunicamycin-treated samples were normalized to those of their respective DMSO-treated controls. Pancreatic cancer cell lines exhibited a statistically significant ($p<0.05$) inability to inhibit progression to S-phase compared to non-tumorigenic cells.

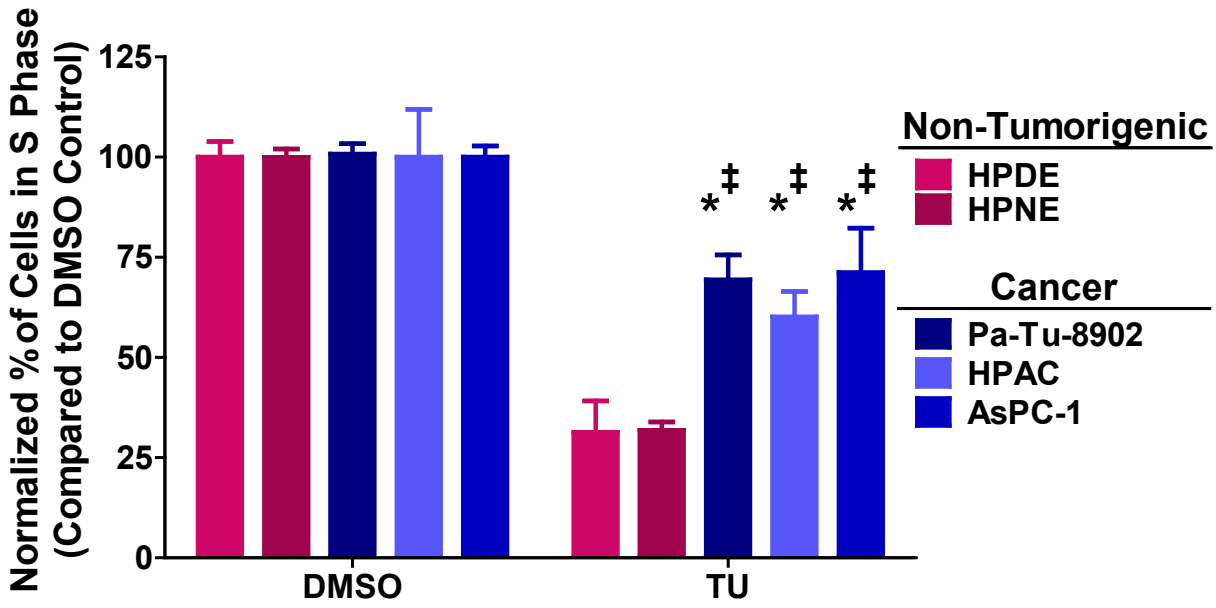


Figure 13. Pancreatic cancer cells have an increased proportion of cells in S-phase after tunicamycin treatment. S-phase results from cell cycle analysis by flow cytometry using propidium iodide staining. Non-tumorigenic pancreatic ductal cells (in pink hues, HPDE, HPNE) and pancreatic cancer cells (in blue hues, Pa-Tu-8902, HPAC, AsPC-1) were treated with vehicle DMSO or tunicamycin (TU, 0.5 µg/ml) for 24 hours. Human pancreatic cancer cells displayed a decreased ability to inhibit progression to S-phase compared to non-tumorigenic cell lines. Percentages of cells in S-phase were normalized to their respective DMSO-treated controls. Statistical analysis performed by 2-way ANOVA. Columns, n=3; error bars represent SEM. *, $p<0.05$ as compared to HPDE with respective treatment, ‡, $p<0.05$, as compared to HPNE, with respective treatment.

Tunicamycin causes cyclin D1 loss in non-tumorigenic cell lines

Since we observed that non-tumorigenic pancreatic cells successfully entered G1 phase arrest, we next examined cyclin D1. We considered cyclin D1 the most reasonable target given its critical role as a key regulator of the G1 to S-phase transition. Additionally, previous studies by the Diehl laboratory have established that the UPR plays a regulatory role in cyclin D1 translation (85, 86).

Treatment with tunicamycin (0.5 μg/ml) for 4 hours with comparison to DMSO-treated controls demonstrated that non-tumorigenic cells effectively downregulated cyclin D1 levels to 34 +/- 3% and 41 +/- 2% of remaining protein in HPDE and HPNE cells, respectively. These results indicate non-tumorigenic cells repressed cyclin D1 expression by >50% compared to their DMSO-treated controls. These results were consistent with the involvement of cyclin D1 in mediating G0/G1 phase arrest in non-tumorigenic cells.

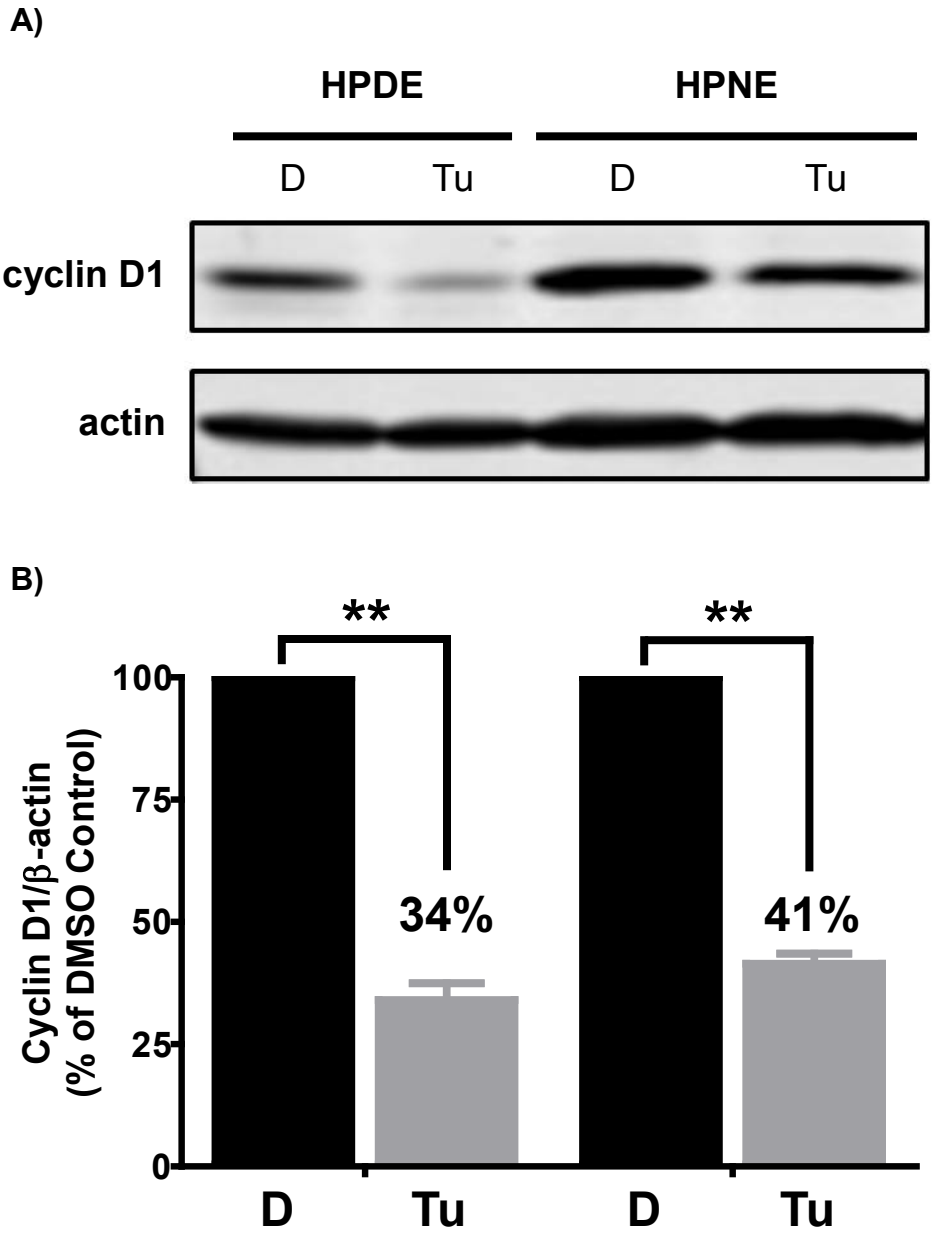


Figure 14. Tunicamycin inhibits cyclin D1 protein levels in non-tumorigenic pancreatic ductal cell lines. HPDE and HPNE cells were exposed to DMSO (D) or 0.5 µg/mL tunicamycin (Tu) for 4 hours. ***A***, Representative immunoblots of similar data are shown. ***B***, Cyclin D1 protein levels were measured by immunoblotting and quantified using Odyssey software. Columns represent *n* = 3, error bars represent standard error of the mean (SEM).

Tunicamycin fails to downregulate cyclin D1 protein levels in a screen of pancreatic cancer cell lines

Given that previous experiments showed non-tumorigenic pancreatic cells were capable of inducing G0/G1 phase arrest that correlated with a decrease in cyclin D1 levels following tunicamycin treatment, we next examined whether pancreatic cancer cells were capable of downregulating cyclin D1 protein levels. Non-tumorigenic pancreatic cell lines HPDE and HPNE were used as comparison cell lines that most closely resemble normal pancreatic ductal cells. In these non-tumorigenic pancreatic cell lines, cyclin D1 expression was consistently downregulated levels to <50% of their DMSO-treated controls (Figure 14). This was consistent with experiments in wild-type mouse embryonic fibroblasts (MEFs), which also decreased cyclin D1 to <50% of baseline values (discussed later).

In an initial screen of 22 pancreatic cancer cell lines demonstrated that 15 out of 22 (68%) cell lines were unable to inhibit cyclin D1 levels by >50% of their DMSO-treated controls (Figure 16). Nearly half (10 of 22) of the screened cancer cell lines had further difficulty inhibiting cyclin D1, repressing expression by <25% of their DMSO-treated controls. For the purposes of this study, we defined ER stress-responsive cells as cell lines which downregulated cyclin D1 by >50% of their baseline values. Cell lines that downregulated cyclin D1 by <50% were designated as “resistant” to ER stress-mediated downregulation. Although a majority of cell lines demonstrated an impaired ability to downregulate cyclin D1 in our initial screen of 22 pancreatic cancer cells lines, the degree of impairment of cyclin D1 was highly

variable. This variability is not an unexpected finding given the marked heterogeneity among pancreatic tumors (34).

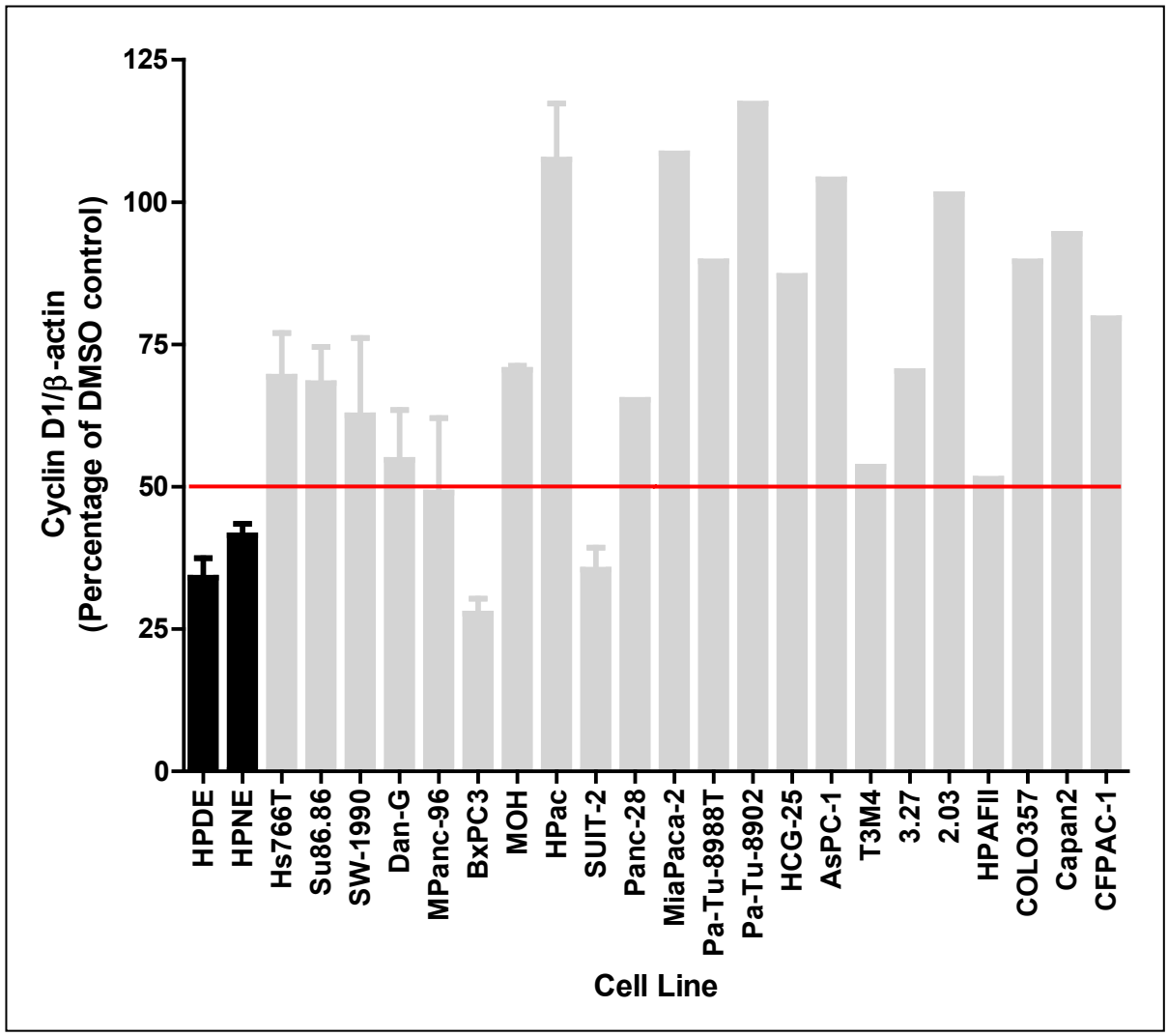


Figure 15. Screen of pancreatic cancer cell lines suggests impaired inhibition of cyclin D1 protein expression. Pancreatic cell lines were treated with DMSO or 0.5 μg/ml tunicamycin for 4 hours. Quantification results from immunoblots for cyclin D1 normalized to β-actin. All cell lines have been represented as a percentage of their cyclin D1/β-actin expression compared to their respective DMSO-treated controls. Black columns represent non-tumorigenic pancreatic ductal cell lines; gray columns represent pancreatic cancer cell lines. Red line indicates distinction at 50% marker indicating "resistance" to ER stress-mediated downregulation of cyclin D1. Columns with error bars represent mean (n=3); bars represent standard error of the mean (SEM). Columns with no error bars represent screened cell lines where immunoblot was performed only once.

Tunicamycin has impaired repression of cyclin D1 levels in pancreatic cancer cell lines

To confirm the results of the initial screen, three of the pancreatic cancer cell lines that were least able to downregulate cyclin D1 were selected for further studies: Pa-Tu-8902, HPAC, and AsPC-1. Time courses over 24 hours demonstrated an impaired cyclin D1 repression in pancreatic cancer cell lines compared to non-tumorigenic cell lines after exposure to tunicamycin (Figure 16). In non-tumorigenic cells, cyclin D1 reduction by >50% occurred within 4 hours of tunicamycin exposure. Over the same exposure times of tunicamycin treatment, the proportion of cyclin D1 lost from baseline in cancer cell lines was dramatically deficient compared to non-tumorigenic cell lines. While non-tumorigenic cells were capable of downregulating cyclin D1 levels to <30% of baseline in HPDE cells and <10% of baseline in HPNE cells by the end of the 24-hour tunicamycin exposure, pancreatic cancer cells were unable to reduce cyclin D1 levels to below 50% over the entire 24 hours Figure 16). In comparison, non-tumorigenic pancreatic ductal cells reduce cyclin D1 levels to <50% in less than 4 hours. All experiments were performed at least twice with similar trends in expression.

A)

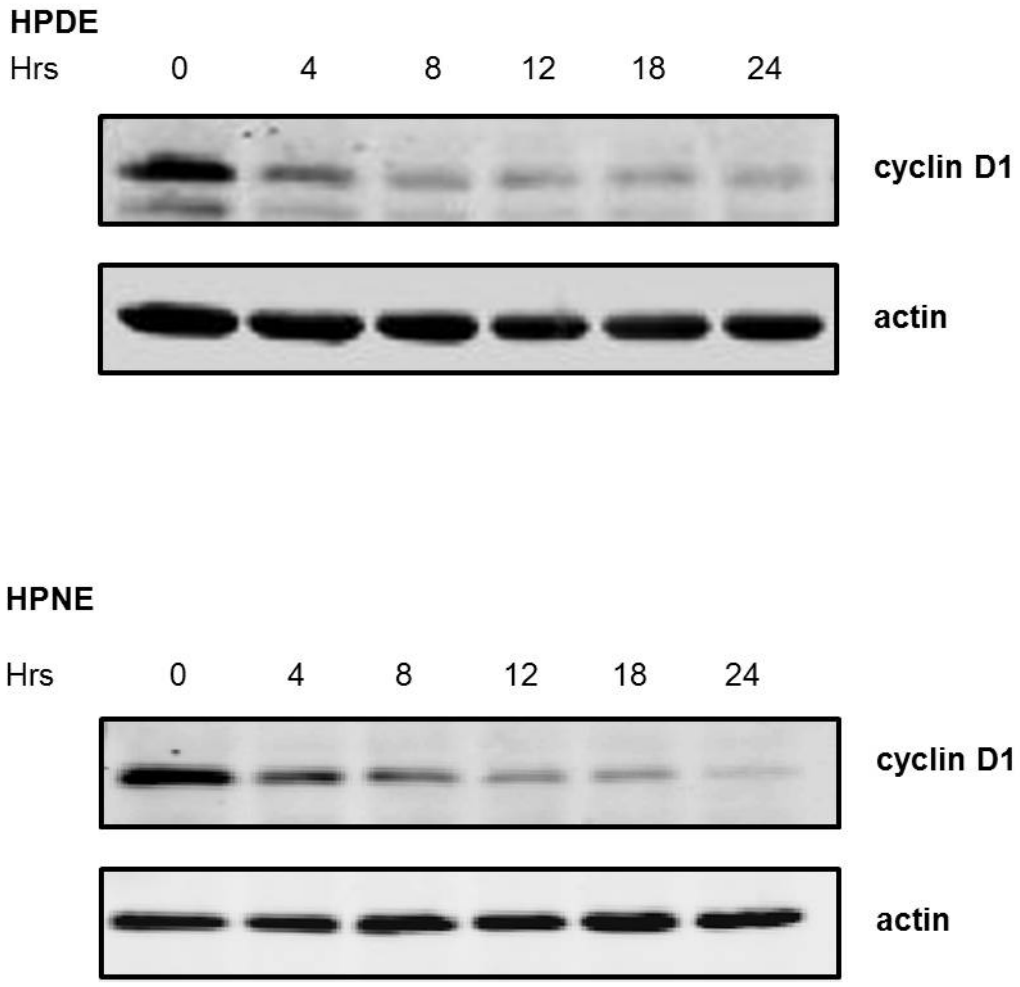
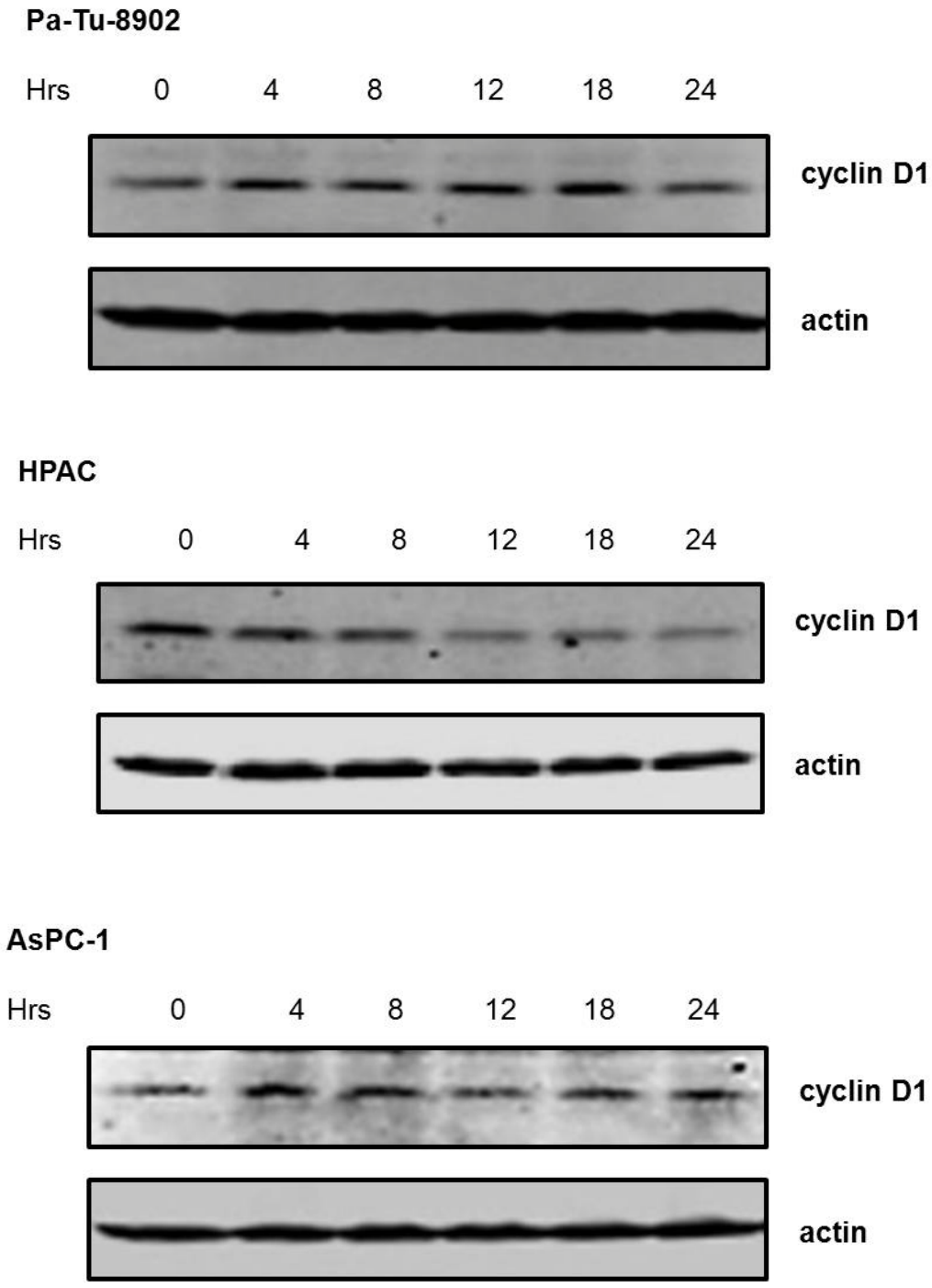


Figure 16. Time-dependent effects of tunicamycin on cyclin D1 protein. Non-tumorigenic pancreatic cell ductal cell lines (***A***, HPDE and HPNE) and pancreatic cancer cell lines (***B***, next page, Pa-Tu-8902, AsPC-1, and HPAC) were exposed to tunicamycin (0.5 µg/ml) for durations of 0, 4, 8, 12, 18, and 24 hours. The DMSO-treated vehicle control, "D", for each cell line was treated for 24 hours. Cyclin D1 levels were measured by immunoblotting. All experiments were repeated at least twice with similar results obtained.

B)


Pa-Tu-8902
Hrs 0 4 8 12 18 24
cyclin D1
actin
HPAC
Hrs 0 4 8 12 18 24
cyclin D1
actin
AsPC-1
Hrs 0 4 8 12 18 24
cyclin D1
actin

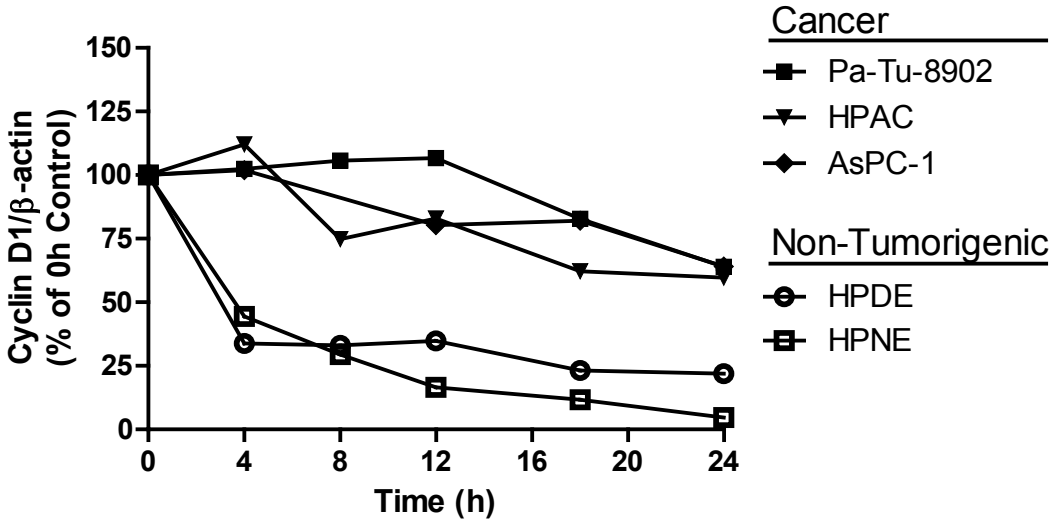


Figure 17. Pancreatic tumor cells have diminished cyclin D1 repression after tunicamycin. Graphical comparison of cyclin D1 levels after time-dependent tunicamycin treatment in pancreatic cancer cell and non-tumorigenic pancreatic ductal cells. Pancreatic cancer cell lines (AsPC-1, Pa-Tu-8902, and HPAC) and non-tumorigenic cells were exposed to tunicamycin (0.5 μg/ml) for 4 hours. 40 μg of whole cell lysate was loaded onto SDS-PAGE gels, transferred, and immunoblots quantified by Odyssey software. Values were normalized to β-actin as an internal loading control and represented as a percentage of the 0-hr time point. All time courses were repeated in at least two separate experiments with similar results. Curves are the representative results of data quantified from immunoblots of time courses for each cell line.

Pancreatic cancer cells do not decrease phosphorylation status of retinoblastoma protein as effectively in response to tunicamycin

To further confirm that non-tumorigenic pancreatic cells were undergoing cell cycle arrest via cyclin D1 downregulation, the phosphorylation status of retinoblastoma (Rb) protein was evaluated. Cyclin D1 is a key regulator of the G1-S phase checkpoint through assembly of a complex with cyclin-dependent kinase-4 (CDK4) and cyclin-dependent kinase-6 (CDK6). Cyclin D1 subsequently causes phosphorylation of Rb protein. Hyperphosphorylation of Rb protein results in its release of E2F transcription factors, which results in the transcription of genes responsible for promoting the G1 to S-phase transition and continued cell cycle progression. Hypophosphorylation of Rb protein, therefore, results in continued binding to E2F with cell cycle arrest at the G1 to S-phase transition.

Since non-tumorigenic cell lines entered G0/G1 phase arrest with an associated decrease in cyclin D1, we expected a decrease in Rb phosphorylation status of our non-tumorigenic cell lines. Conversely, we predicted pancreatic cancer cell lines should have decreased loss of Rb phosphorylation. As expected, in our non-tumorigenic cell line HPNE, tunicamycin caused complete loss of Rb phosphorylation by 18 hours of treatment. In contrast, phosphorylation of Rb persisted even until 24 hours in pancreatic cancer cell lines Pa-Tu-8902 and HPAC. Loss of phosphorylation occurred earlier and to a greater degree in non-tumorigenic cell line HPNE than pancreatic cancer cell lines. These results are consistent with prior cell cycle analysis experiments demonstrating induction of G1 cell cycle arrest in non-tumorigenic cells but block or delayed arrest in pancreatic cancer cell lines.

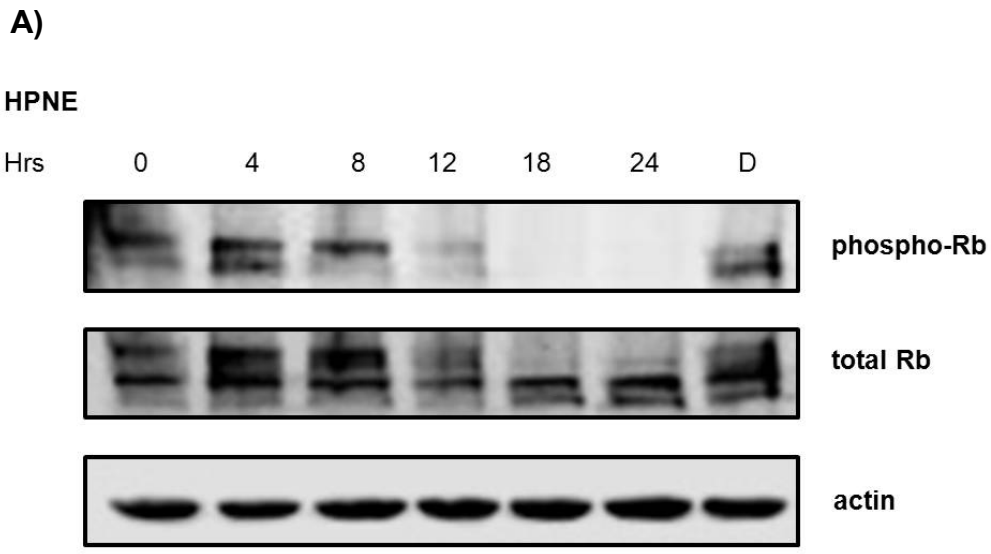
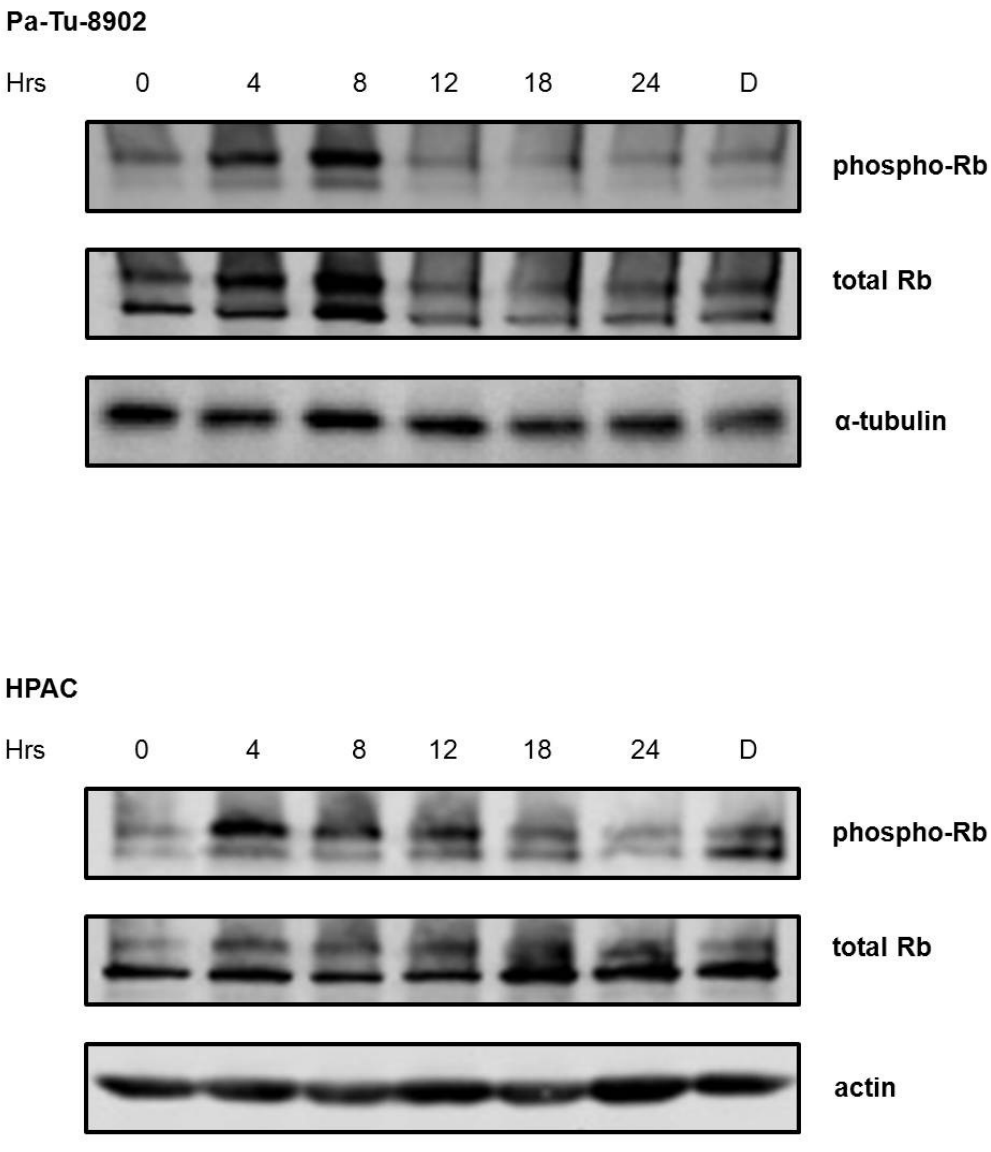


Figure 18. Phosphorylation status of retinoblastoma protein correlates with cell cycle arrest. Immunoblots of phospho-Rb following tunicamycin exposure (0.5 µg/ml) over 24 hour time course. ***A***, Non-tumorigenic pancreatic cell ductal cell line HPNE exhibits complete loss of phosphorylation of retinoblastoma protein by 18 hours of tunicamycin exposure. ***B*** (next page), pancreatic cancer cell lines (Pa-Tu-8902, AsPC-1, and HPAC) display phosphorylation of retinoblastoma protein through the entire 24 hour time course. The DMSO-treated vehicle control, "D", for each cell line was treated for 24 hours. All experiments were repeated at least twice with similar results obtained. Representative immunoblots are shown of similar data.

B)


Pa-Tu-8902
Hrs 0 4 8 12 18 24 D
phospho-Rb
total Rb
α-tubulin
HPAC
Hrs 0 4 8 12 18 24 D
phospho-Rb
total Rb
actin

DECREASED CYCLIN D1 LOSS IN PANCREATIC CANCER CELLS IS DUE TO IMPAIRED INHIBITION OF TRANSLATION

Regulation of cyclin D1 occurs at multiple levels including transcription, translation, and degradation. To determine the cause for differences in cyclin D1 protein expression between non-tumorigenic and pancreatic cancer cell lines, I examined the various levels cyclin D1 protein regulation to identify the principal mechanism for differences in cyclin D1 levels.

Early loss of cyclin D1 protein is not attributed to loss of cyclin D1 mRNA.

Since a reduction in cyclin d1 levels in non-tumorigenic cells could also be a reflection of decreased messenger RNA levels, I determined through quantitative polymerase chain reaction (Q-PCR) whether cyclin D1 mRNA levels were affected by treatment with tunicamycin.

I previously showed that cyclin D1 loss to <50% of the initial protein level in non-tumorigenic cells occurs within 4 hours of tunicamycin exposure (Figure 16). Q-PCR results demonstrate that reduction in cyclin D1 mRNA levels in all cell lines did not occur until after the initial loss of cyclin D1 protein observed at 4 hours. While cyclin D1 mRNA levels were shown to diminish over the entire 24-hour time course in non-tumorigenic pancreatic ductal cells, a significant reduction of its message did not occur until at least 8 hours of treatment in HPDE cells and 12 hours in HPNE cells. These results were consistent across repetitions. These observations were also noted previously in NIH-3T3 cells where a cyclin D1 mRNA loss was also not observed until 8 hours (86). Interestingly, pancreatic cancer cells

demonstrated a reduced ability to reduce cyclin D1 mRNA by 24 hours. Parallel treatment of cell lines with DMSO-treated control for 24 hours indicated that the reduction in cyclin D1 mRNA levels in HPNE cells was not due to a growth inhibition from increased confluency. In pancreatic cancer cell lines, the DMSO-treated control never demonstrated more inhibition of cyclin D1 mRNA than the 24-hour tunicamycin-treated sample. This indicates that any inhibition of cyclin D1 mRNA levels reflected an effect of tunicamycin treatment rather than vehicle or confluency. In the representative experiment for AsPC-1 cells in Figure 19, the DMSO-treated control showed cyclin D1 mRNA levels (61 +/- SD 6%) that were statistically equivalent to the 24-hour tunicamycin-treated sample (61% +/- SD 9%). This indicates that the downregulated cyclin D1 mRNA effect observed in AsPC-1 was a result of confluency and not of treatment. Repetitions of the time course showed that AsPC-1 cyclin D1 mRNA levels at 24 hours were always either statistically equivalent or above the DMSO-treated control. The results are consistent with an impaired ability of AsPC-1 to downregulate cyclin D1 mRNA. Of note, Q-PCR experiments with HPDE cells did not include a DMSO-treated control, but cell cycle analysis of HPDE cells consistently demonstrated that cell cycle was unaffected by treatment with DMSO while tunicamycin treatment caused cell cycle arrest (Figure 11 and Figure 12).

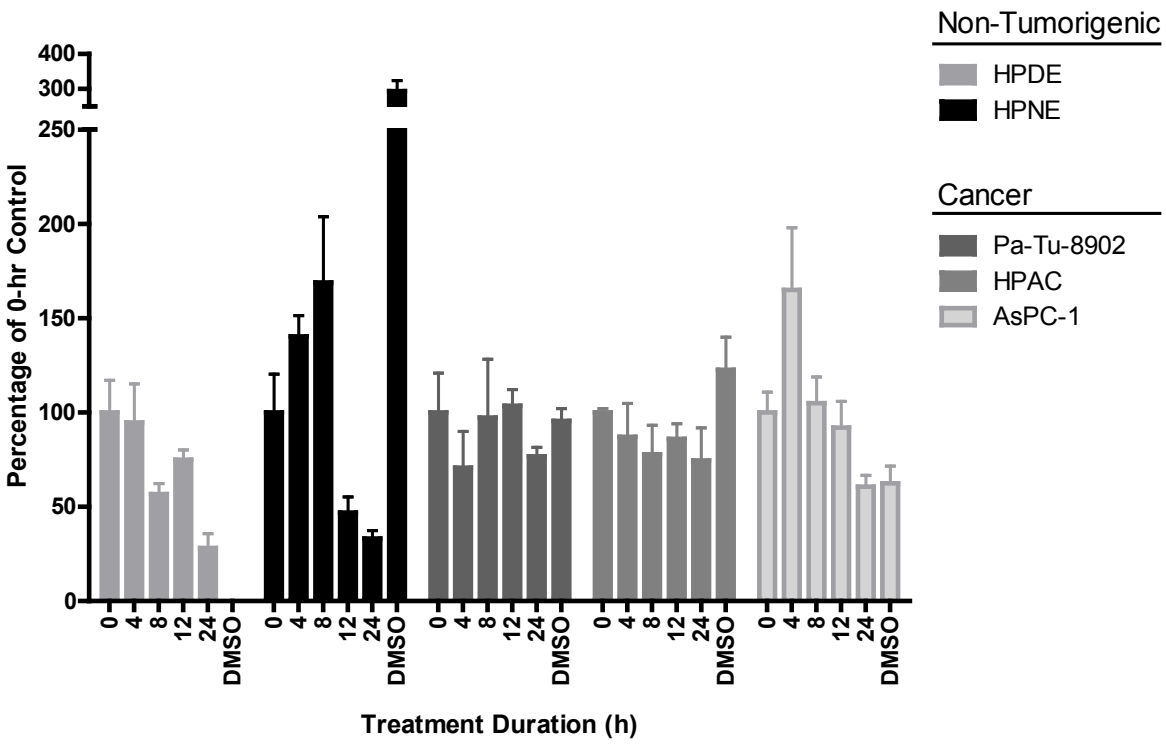


Figure 19. Loss of cyclin D1 mRNA occurs after cyclin D1 protein levels decline. Quantitative polymerase chain reaction was used to measure levels of cyclin D1 mRNA in respective cell lines at 0, 4, 8, 12, and 24 hours of treatment with tunicamycin (0.5 µg/ml). Cyclin D1 loss does not begin to decline until at least 8 hours in both non-tumorigenic or pancreatic cancer cell lines. Representative results are shown. Similar results were obtained from at least 2 different repetitions. Error bars represent standard deviation (SD).

Pancreatic cancer cells have diminished ability to attenuate translation in pancreatic cancer cells.

To determine the mechanism for a reduced capacity to reduce cyclin D1 protein levels, I next tested whether pancreatic cancer cells had diminished capacity to reduce cyclin D1 levels due to differences in translation. Since the PERK/eIF2α pathway is a well-established regulator of global protein synthesis, differences in cyclin D1 protein levels seemed most likely to involve this pathway. Additionally, ER stress has previously been established as a potent inhibitor of cyclin D1 translation through the UPR's PERK/eIF2α pathway (86). Since tunicamycin and thapsigargin are classic inducers of ER stress, I examined the inhibitory effects of tunicamycin and thapsigargin on the synthesis of new proteins through biosynthetic labeling with L-[4,5-3H]-leucine. Pancreatic cancer cells exhibited a marked ineffectiveness in inhibiting protein synthesis (Figure 20). Pancreatic cancer cells could reduce L-[4,5-3H]-leucine incorporation by no greater than 12% of their DMSO-treated controls. In contrast, non-tumorigenic pancreatic ductal cells were able to reduce protein synthesis at least by 39% of their respective DMSO-treated controls ($p<0.001$). These findings indicate that pancreatic cancer cells possess a clear dysregulation in inhibiting translation in response to ER stress. The decreased ability to suppress translation was consistent across two ER stressors with different mechanisms of disturbing protein folding provides support that this deficiency in regulation of translation applies to ER stress in general, not specific drugs.

The finding that translation is not inhibited in pancreatic cancer cells is consistent with our phospho-eIF2α data in Chapter Two. I showed previously that pancreatic cancer cells had an increased time of onset as well as a decreased magnitude of eIF2α phosphorylation. My data demonstrating that pancreatic cancer cells possess a delayed ability to phosphorylate eIF2α correlates well with this chapter's results delineating impaired cyclin D1 downregulation and a deficient translational inhibition in response to ER stress.

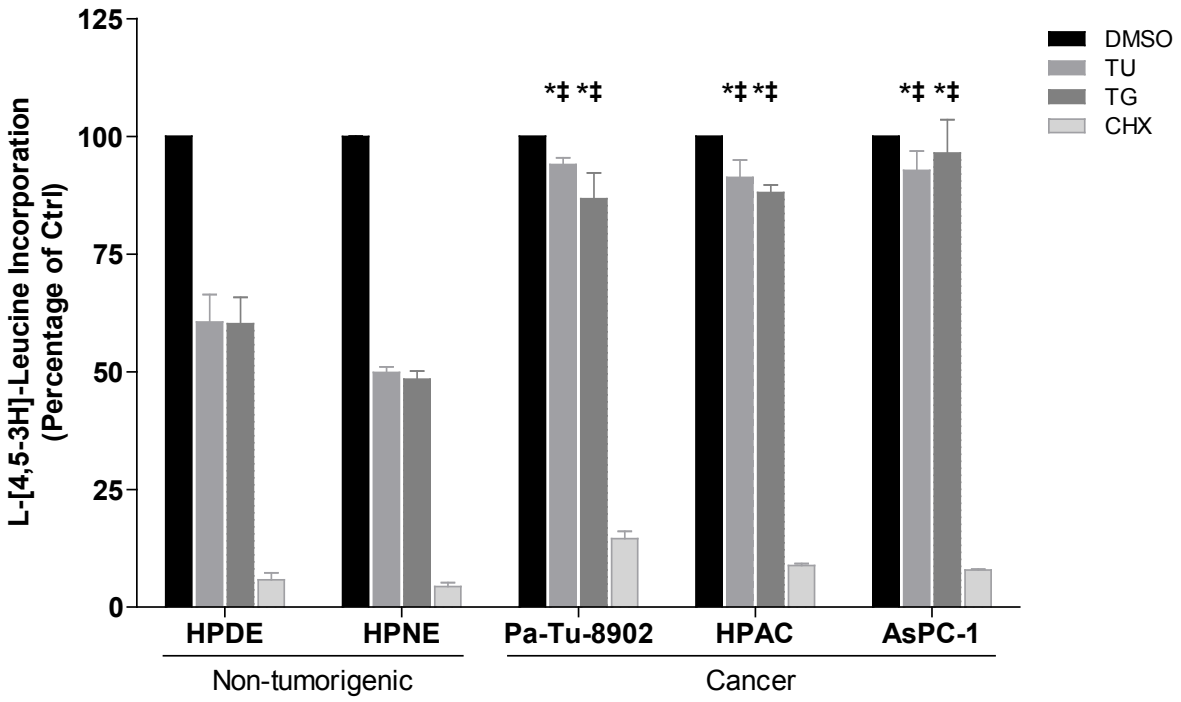


Figure 20. Pancreatic cancer cells demonstrate diminished ability to attenuate translation. Non-tumorigenic pancreatic ductal cell lines and pancreatic cancer cell lines were treated for 4 hours with DMSO, tunicamycin (TU, 0.5 µg/ml), or thapsigargin (TG, 10 nM) in ideal media. Cells were then pulsed with L-[4,5-3H]-leucine for 2 hours in leucine-free media. Cycloheximide (CHX, 50 µM) was included as a positive control for protein synthesis inhibition. Values represent the percentage of L-[4,5-3H]-leucine incorporated compared to the DMSO-treated control. *, $p<0.001$, as compared to HPDE; ‡, $p<0.001$, as compared to HPNE. Comparisons are made between treatments of tunicamycin or thapsigargin. All experiments were performed in at least three independent experiments.

Phosphorylation of cyclin D1 degradation regulator GSK3β does not correlate with expression of cyclin D1.

The third possible explanation for a reduced ability to downregulate cyclin D1 in pancreatic cancer cells is an increased accumulation of cyclin D1 protein due to decreased degradation. To determine whether delayed repression of cyclin D1 was caused by decreased degradation, phosphorylation status of glycogen synthase kinase-3β (GSK3β) was evaluated. Phosphorylation of cyclin D1 at threonine 286 by the GSK3β facilitates degradation of cyclin D1 and its redistribution from the nucleus to the cytoplasm. Cyclin D1 proteolysis occurs through ubiquitination and degradation by the 26S subunit of the proteasome.

GSK3β is regulated through phosphorylation which results in its inactivation. Subsequently, upregulated GSK3β phosphorylation results in inhibited cyclin D1 degradation and an increase in cyclin D1 protein. Since pancreatic cancer cells have decreased repression of cyclin D1 levels, an increase in the phosphorylation of GSK3β would be expected. However, no change in phosphorylation status of GSK3β occurred through the time course in either pancreatic cancer cells or non-tumorigenic cells (Figure 21). These data indicate that GSK3β is not involved in cyclin D1 protein levels.

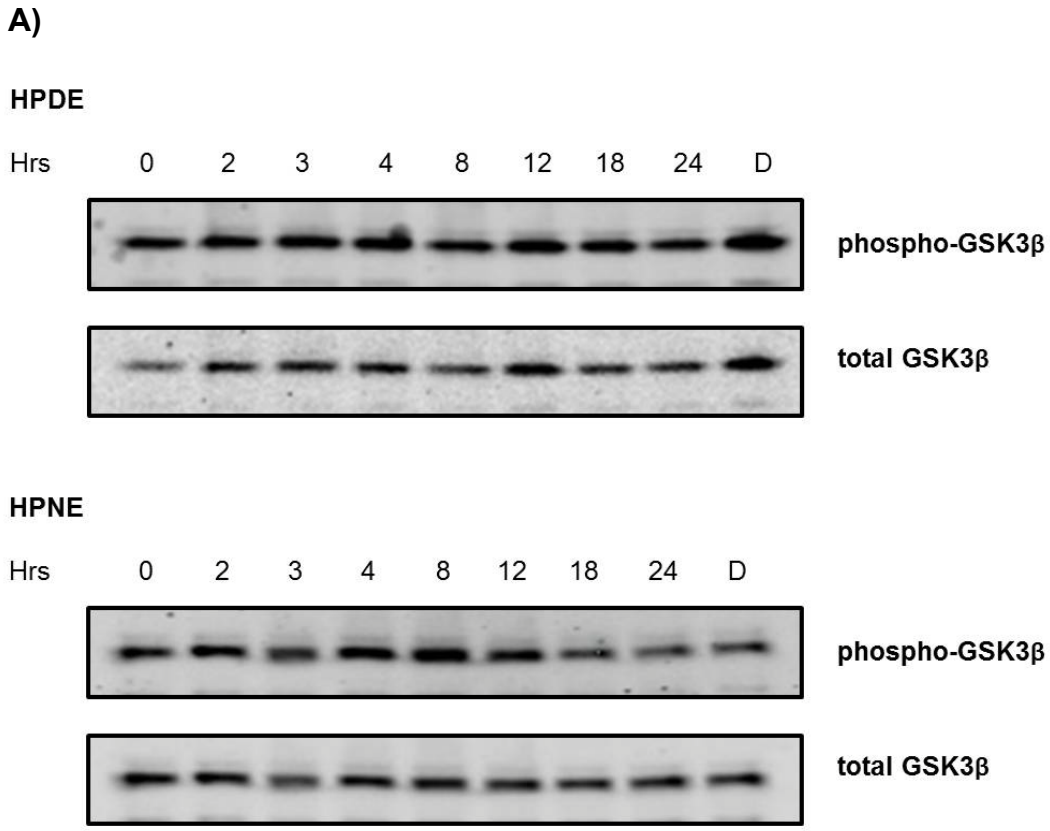
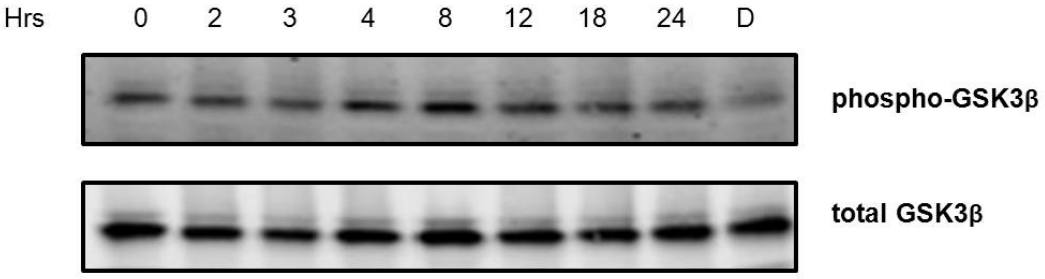


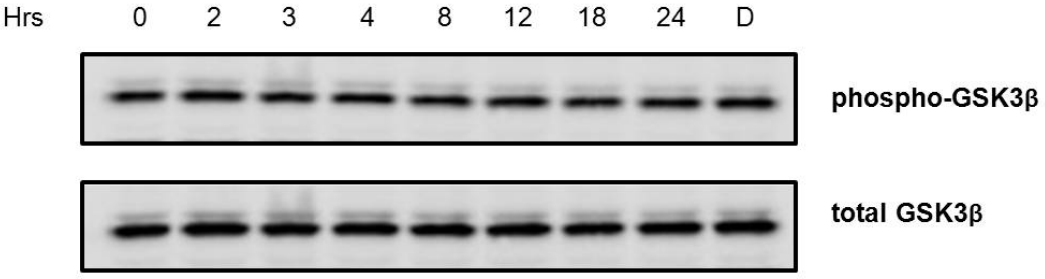
Figure 21. Phosphorylation of glycogen synthase kinase-3β does not correlate with cyclin D1 loss. Immunoblot of phospho-GSK3β following tunicamycin exposure (0.5 µg/ml) over 24 hour time course. Non-tumorigenic pancreatic cell ductal cell lines (***A***, HPDE and HPNE) and pancreatic cancer cells lines (***B****,* next page, Pa-Tu-8902, AsPC-1, and HPAC) do not change phosphorylation status of GSK3β in response to tunicamycin. The DMSO-treated vehicle control, "D", for each cell line was treated for 24 hours. All experiments were repeated at least twice with similar results obtained. Representative blot of similar data is shown.

B)

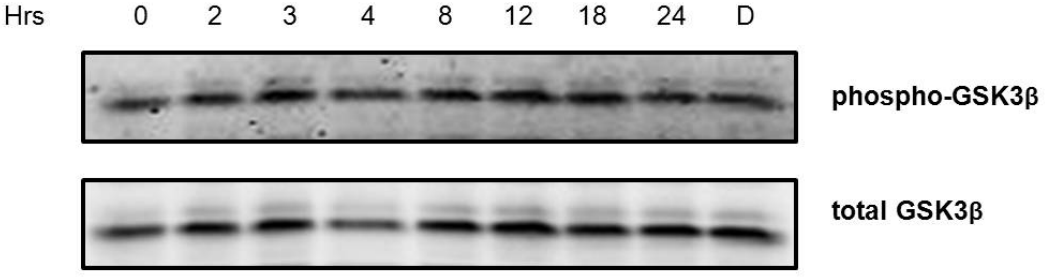
Pa-Tu-8902



HPAC



AsPC-1



Cyclin D1 degradation is not responsible for decreased downregulation of cyclin D1 in pancreatic cancer cells.

Since cyclin D1 degradation has also been shown to be regulated by molecules other than GSK3β, such as p38SAPK2 and Skp- Cullin- F-box (SCF) complexes, I also analyzed general cyclin D1 degradation rates. The protein synthesis inhibitor cycloheximide (CHX) was utilized as a tool to examine the half-life, $t_{1/2}$, of cyclin D1. The average $t_{1/2}$ for cyclin D1 was previously reported to be only ~25 minutes in NIH-3T3 cells (86).

If impaired loss of cyclin D1 in pancreatic cancer cell lines were due to an accumulation of cyclin D1, I would expect to see decreased degradation of cyclin D1 and an increased $t_{1/2}$. Examination of degradation rates of cyclin D1 over 60 minutes demonstrated a comparable $t_{1/2}$ of cyclin D1 at ~26 minutes with CHX alone and ~28 minutes with combined tunicamycin and cycloheximide treatment. This was not substantially different compared to a $t_{1/2}$ of ~24 minutes when treating pancreatic cancer cell line Hs766T with combined tunicamycin and cycloheximide treatment. Unexpectedly, Hs766T actually consistently trended toward a higher $t_{1/2}$ of cyclin D1 at ~31 minutes when treated with CHX alone. These results suggest that the initial turnover rate of cyclin D1 is slightly slower at a $t_{1/2}$ of 31 minutes, tunicamycin actually promotes rather than inhibits degradation of cyclin D1 to a $t_{1/2}$ of 24 minutes in the pancreatic cancer cell line. My findings provide strong evidence that impaired cyclin D1 downregulation in this pancreatic cancer cell line was not due to increased accumulation of cyclin D1.

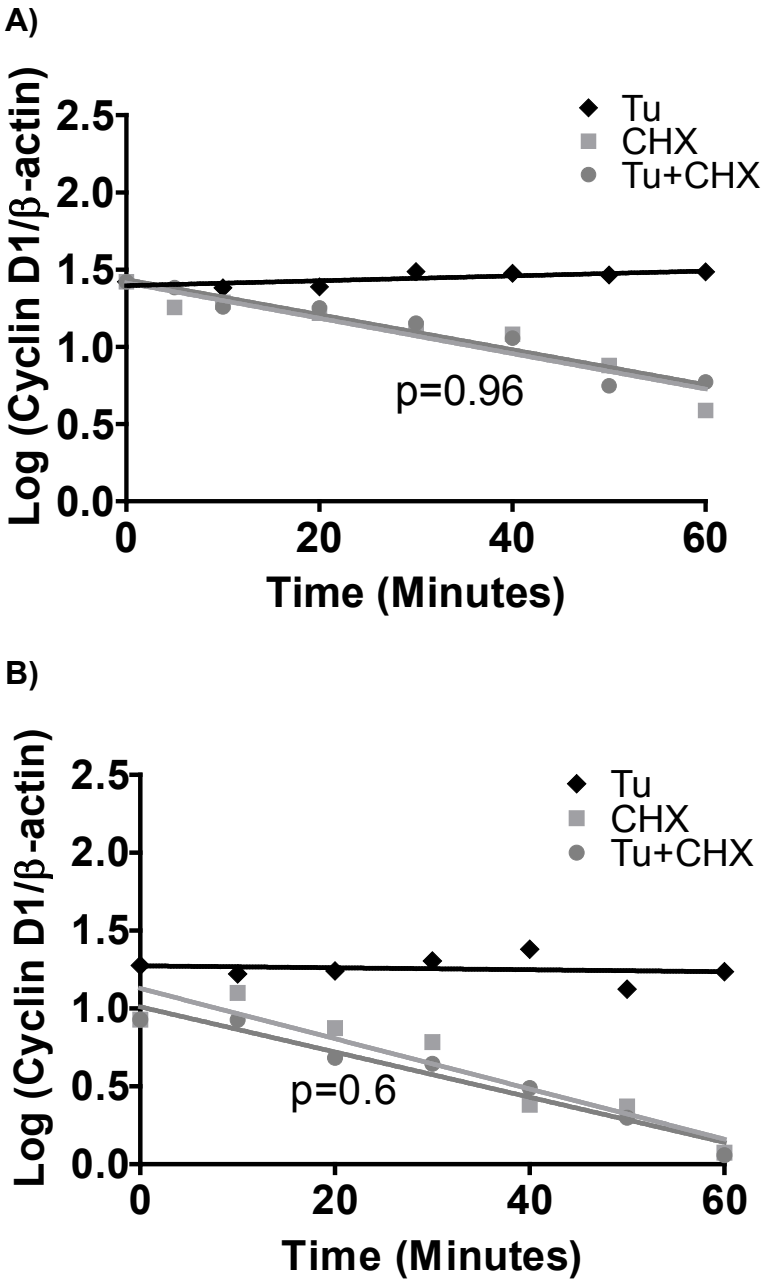


Figure 22. Cyclin D1 degradation is not inhibited in pancreatic cancer cell lines. Cyclin D1 protein levels were quantified on immunoblots of tumorigenic cell line HPDE, ***A***, and human pancreatic cancer cell line, Hs766T **B**. Cell lines were treated with tunicamycin (Tu, 0.5 µg/ml), cycloheximide (CHX, 50 µM), or combination tunicamycin (0.5 µg/ml) with cycloheximide (50 µM) in a time course of increments of 10 minutes up to 60 minutes. Slopes were not significantly different between combination Tu+CHX or CHX alone in either cell line.

Expression of $p27^{Kip1}$ does not correlate with cell cycle arrest

Expression of cyclin-dependent kinase inhibitor $p27^{Kip1}$ in non-tumorigenic cell line HPNE demonstrates brief downregulation until 12 hours of tunicamycin treatment before a rapid increase in $p27^{Kip1}$. Overexpression of $p27^{Kip1}$ should induce cell senescence. However, $p27^{Kip1}$ expression is also induced in pancreatic cancer cell lines within 2 hours of treatment without any induction of cell cycle arrest. This indicates that expression of $p27^{Kip1}$ does not correlate with induction in non-tumorigenic cells or failed induction of cell cycle arrest in pancreatic cancer cells.

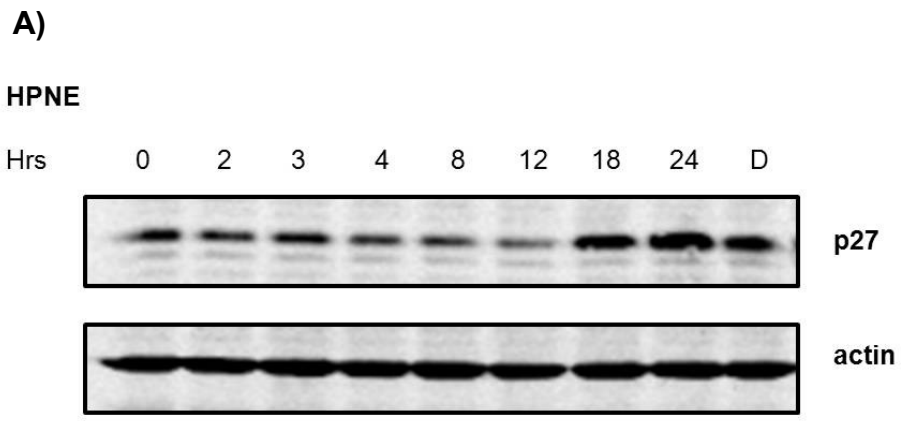
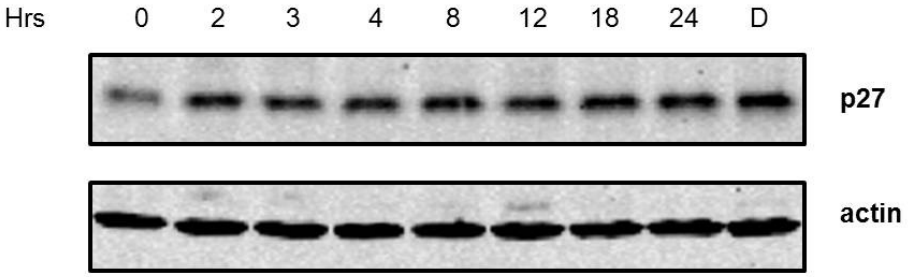


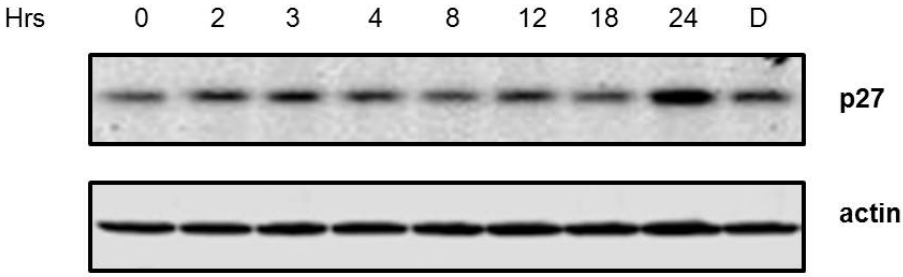
Figure 23. $p27^{Kip1}$ expression does not correlate with cell cycle arrest. Immunoblot of $p27^{Kip1}$ following tunicamycin exposure (0.5 µg/ml) over 24 hour time course. ***A***, non-tumorigenic pancreatic cell ductal cell line HPNE exhibits initial decline of $p27^{Kip1}$ until 18 hours before rapidly increasing until 24 hours. ***B*** (next page), pancreatic cancer cell lines (Pa-Tu-8902, AsPC-1, and HPAC) display an initial increase of $p27^{Kip1}$ expression as early as 2 hours. The DMSO-treated vehicle control, "D", for each cell line was treated for 24 hours. All experiments were repeated at least twice with similar results obtained.

B)

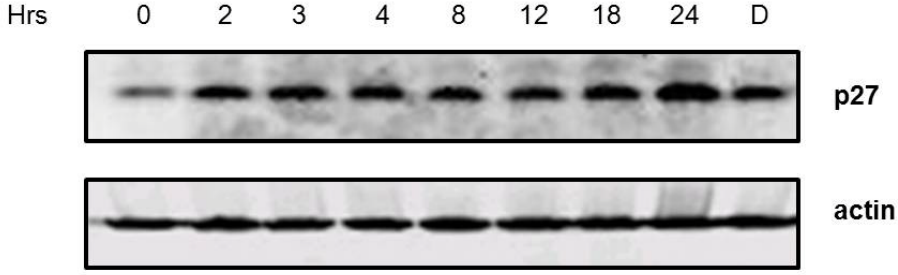
Pa-Tu-8902



HPAC



AsPC-1



CONTROL OF CELL CYCLE ARREST DEPENDS ON EIF2α PHOSPHORYLATION IN MUTANT MOUSE EMBRYONIC FIBROBLAST SYSTEM

To determine whether the eIF2α pathway could be a possible mechanism for the defective translational attenuation, I examined whether eIF2α phosphorylation was required to inhibit translation by utilizing a mutant mouse embryonic fibroblast (MEF) model system. In this mutant model MEF system, the phosphorylation site of eIF2α at Ser51 was mutated to an Ala51 (S51A MEFs). Mutation of this site prevents the phosphorylation of eIF2α and subsequently inhibits GDP to GTP exchange by eIF2B, effectively preventing global inhibition of protein synthesis.

Failure to phosphorylate eIF2α leads to an inability to attenuate translation in mouse embryonic fibroblasts (MEFs).

To illustrate that translational inhibition was dependent on eIF2α phosphorylation, I compared S51A and WT MEFs ability to incorporate L-[4,5-3H]-leucine as a measure of protein synthesis. WT MEFs inhibited incorporation of L-[4,5-3H]-leucine to 59.0% +/- 5.3% with tunicamycin and 51.8% +/- 2.5% with thapsigargin from their baseline values. In contrast, S51A MEFs demonstrated a complete inability of S51A MEFs to inhibit translation. These findings recapitulated previously published experiments using these phosphorylation-deficient MEFs (86). This findings are comparable to the deficient translational inhibition observed in pancreatic cancer cells (Figure 20) and provide evidence that abrogated translational inhibition was mediated through eIF2α phosphorylation.

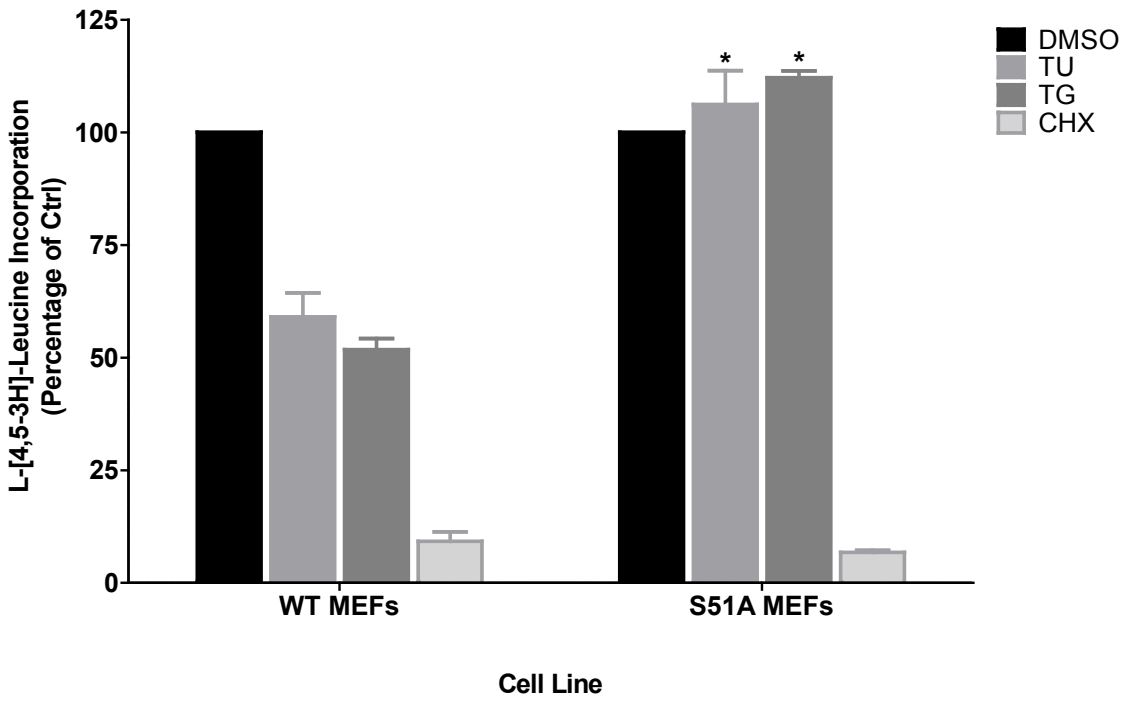


Figure 24. Phosphorylation-deficient MEFs demonstrate diminished ability to attenuate translation. WT and S51A MEFs were treated for 4 hours with DMSO, tunicamycin (TM), or thapsigargin (TG) in ideal media. Cells were then pulsed with L-[4,5-3H]-leucine for 2 hours in leucine-free media. Phosphorylation-deficient S51A MEFs were completely unable to abrogate protein synthesis compared to WT MEFs, which showed strong translational inhibition after ER stress induction. Cycloheximide (CHX) was included as a positive control for protein synthesis inhibition. Values represent the percentage of L-[4,5-^{3}H]-leucine incorporated compared to the DMSO-treated control. *, $p<0.001$, as compared to WT MEFs according to respective treatments of tunicamycin or thapsigargin. Columns, *n*=3, error bars represent SEM.

Cyclin D1 loss is dependent on eIF2α in a S51A mutant mouse embryonic fibroblast model system.

Since our MEF model system demonstrated that translational attenuation was shown to be controlled through eIF2α, we next examined whether cyclin D1 repression was also deficient in our S51A MEFs. If our hypothesis were correct that cyclin D1 loss were dependent on eIF2α-mediated translational inhibition, we predicted that S51A MEFs would be unable to downregulate cyclin D1, similarly to pancreatic cancer cell lines. Expression of cyclin D1 protein after tunicamycin (0.5 μg/ml) treatment in S51A MEF cells was compared to wild-type (WT) MEF cells. WT MEFs were able to cause cyclin D1 loss to 52 +/- 5% of DMSO-treated levels. S51A MEFs, in contrast, were completely unable to repress cyclin D1 levels ($p<0.05$), which was consistent with our expectations and previous studies that demonstrated cyclin D1 loss was mediated through eIF2α phosphorylation. These findings provide further support that the reduced ability to inhibit cyclin D1 in pancreatic cancer cells is dependent on eIF2α phosphorylation.

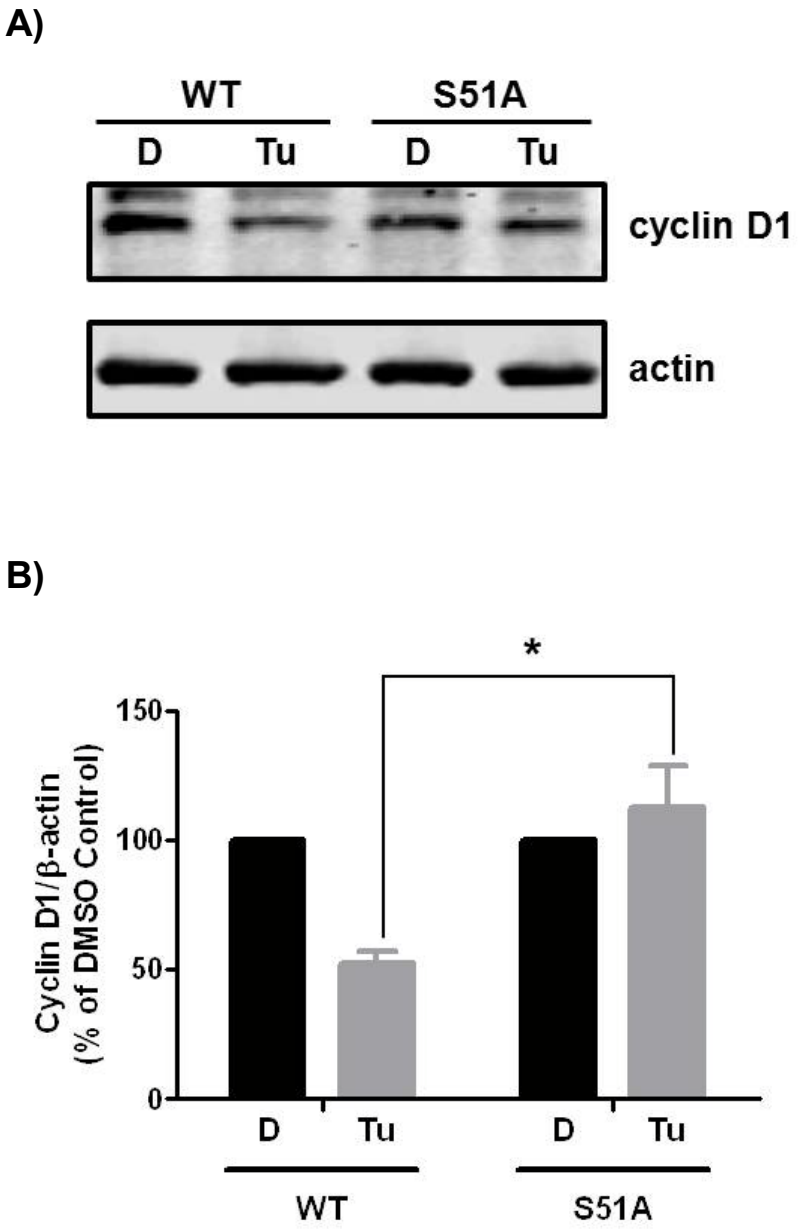


Figure 25. Phosphorylation-deficient MEFs are unable to downregulate cyclin D1 levels. Wild-type and phosphorylation-deficient S51A MEFs were exposed to DMSO (D) or 0.5 μg/ml tunicamycin (Tu) for 4 hours. ***A***, Representative immunoblot of cyclin D1 levels in wild-type (WT) and phosphorylation-deficient MEFs (S51A). ***B***, Cyclin D1 levels were measured by immunoblotting and normalized to β-actin levels. The results of three independent experiments were quantified, *, $p<0.05$.

Inhibition of DNA synthesis is dependent on eIF2α in S51A mutant mouse embryonic fibroblast (MEF) model system.

Since cyclin D1 repression was demonstrated to be deficient in S51A MEFs, we next determined whether DNA synthesis was also dependent on eIF2α phosphorylation. Inhibition of DNA synthesis was considered an indirect measure of reduced proliferation and therefore, cell cycle arrest. Since we previously demonstrated that cyclin D1 loss was dependent on eIF2α phosphorylation, we predicted that DNA synthesis would be fail to be inhibited. As predicted, S51A MEFs were unable to inhibit DNA synthesis in a dose-dependent manner. While S51A MEFs exhibited a slightly positive slope, WT MEFs demonstrated a steeply negative slope indicating prominent inhibition of DNA synthesis with increasing tunicamycin concentrations. These results are consistent with my studies in pancreatic cancer where both DNA synthesis and G0/G1 cell cycle arrest had reduced inhibition.

Through these experiments in the mutant model MEF system examining translation, cyclin D1 loss, and DNA synthesis inhibition, I provide strong support that the reduced ability of pancreatic cancer cells to repress cyclin D1 and undergo cell cycle arrest is mediated through reduced eIF2α phosphorylation.

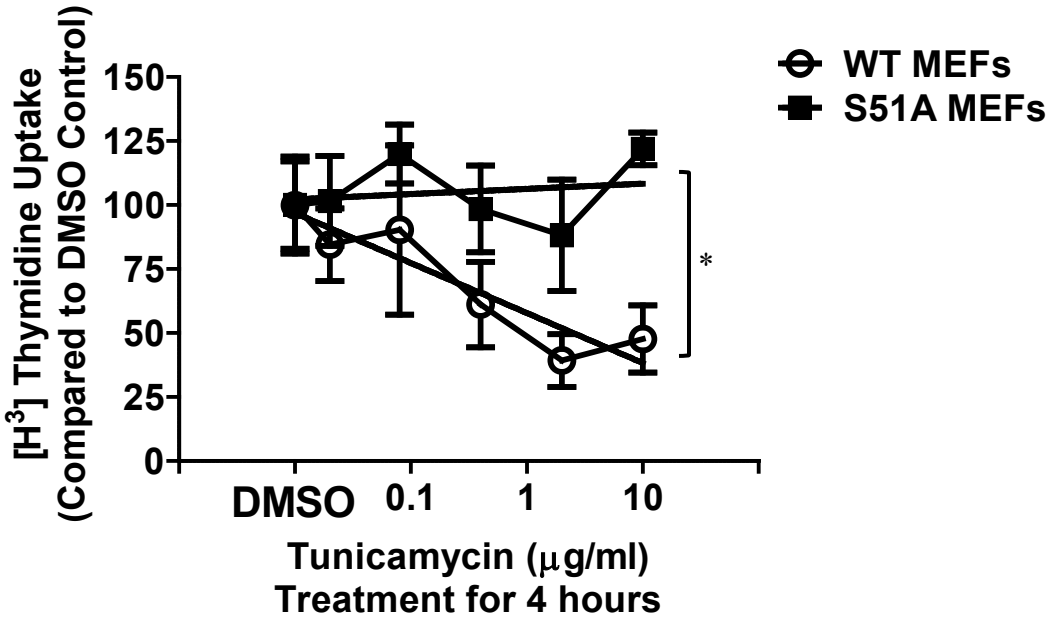


Figure 26. Tunicamycin is less effective in inhibiting DNA synthesis in phosphorylation-deficient S51A MEFs compared to wildtype MEFs. Wildtype and S51A MEFs were exposed to increasing concentrations of tunicamycin (DMSO-treated control, 0.016, 0.08, 0.4, 2, and 10 μg/ml. S51A MEFs clearly demonstrate an inability to inhibit DNA synthesis with increasing concentrations of tunicamycin. This is in contrast to WT MEFs which exhibit a dose-dependent inhibition of DNA synthesis. Slopes were calculated for each dose-dependent curve, *, $p<0.05$. Results were obtained from two independent experiments.

DISCUSSION

In Chapter Two, we discovered that pancreatic cancer cells possessed an altered unfolded protein response to ER stress induction. Phosphorylation of the eIF2α and activation of the IRE1α/XBP1s pathways were delayed in comparison to non-tumorigenic cells. We also made the observation that pancreatic cancer cells also required higher concentrations of tunicamycin to inhibit DNA synthesis (). We aimed to determine whether the altered UPR observed in Chapter Two also impacted cell cycle regulation. This study makes a novel discovery demonstrating that a subset of pancreatic cancer cells has a diminished ability to downregulate cyclin D1 in response to ER stress induction. This dysregulation was dependent on translational control of cyclin D1 through delayed activation of the PERK/eIF2α arm of the UPR.

Cell cycle analysis comparing non-tumorigenic pancreatic ductal cells to pancreatic cancer cell lines indicated a decreased ability in pancreatic cancer cells to enter cell cycle arrest after treatment with tunicamycin. Cell cycle exit occurred in the G1 to S phase transition in non-tumorigenic cells while pancreatic cancer cells demonstrated a statistically significant increased percentage of cells entering S phase. Consistent with cell cycle arrest in G1 phase, HPNE cells exhibited loss of phosphorylation of retinoblastoma protein while continued cycle progression correlated with retention of Rb phosphorylation in pancreatic cancer cells. Examination of cyclin D1 as a possible regulatory target of the UPR was chosen given its critical role in mediating the G1 to S phase transition. Additionally, in NIH-3T3 and mouse embryonic fibroblasts, the PERK arm of the UPR has been

documented to regulate cyclin D1 translation and cause G1 cell cycle arrest (85, 86, 126).

Cyclin D1 is a well-known driver of cell cycle progression critical to the pathogenesis of many types of cancer. It is frequently overexpressed in different types of cancer and has also been heavily implicated as a mechanism of chemoresistance, particularly in pancreatic cancer (78, 79, 82-84, 127, 128). Cyclin D1 levels are closely regulated through mitogenic signaling inputs, such as through the Ras/Raf/MAPK pathway. Persistent mitogenic signaling is required for active cyclin D1/CDK4 complexes. In normal cells, withdrawal of growth factors results in decreased Ras signaling and rapid turnover of cyclin D1. Regulation of this process enables cells to quickly adjust to growth requirements to changes in the extracellular environment. In pancreatic adenocarcinomas, oncogenic KRAS is mutated in >90% of cases and likely makes a significant contribution to driving cyclin D1 expression (129, 130). The increased proliferative demand created by KRAS, cyclin D1, and other growth-promoting factors leads to increased tumor growth which outstrips the vascular supply. The limited availability of hypoxia, nutrients, and glucose creates a tumor microenvironment predisposed to induction of ER stress. The conundrum remains that if conditions in the tumor should promote ER stress, activation of the UPR should lead to an inhibition of cyclin D1 translation. Hypothetically, if a low level of ER stress were present, cells should enter a state of tumor dormancy with the reduction of cyclin D1. However, given the hypovascularity, increased proliferative demand, and chemoresistant character of

pancreatic adenocarcinomas, these tumors likely possess an adaptive stress response to counter the increased hypoxic and metabolic demands.

Our studies indicate that a subset of pancreatic cancer cells exhibit a decreased ability to downregulate cyclin D1 compared to non-tumorigenic cell lines. Both non-tumorigenic pancreatic ductal cell lines, HPDE and HPNE, and WT MEFs exhibited abrogation of cyclin D1 levels to <50% of baseline values by 4 hours. I demonstrate that a majority (15/22) of pancreatic cancer cells exhibited an altered cyclin D1 downregulation in response to tunicamycin treatment. In the 24-hour time course evaluated, pancreatic cancer cells failed to achieve a level of cyclin D1 repression. This finding demonstrated that pancreatic cancer cells clearly possessed a deficient cyclin D1 response to tunicamycin inhibition. Previous studies have also indicated that the ER stress mediates cell cycle arrest via decreased cyclin D1 expression in A2780 (ovarian) and HT-29 (colon) cancer cell lines (87). However, no studies have been published indicating a resistance to ER stress-induced arrest in cancer via UPR regulation of cyclin D1.

Cyclin D1 is regulated at multiple levels, including transcription, translation, and degradation. While gene amplification has been shown to play a role in mediating cyclin D1 overexpression in a number of cancers, this is not a prevalent finding in PDAC (131). To determine the mechanism of persistence of cyclin D1 in pancreatic cancer, we examined mRNA levels, translation, and degradation of cyclin D1. Our results indicate that regulation of cyclin D1 is dependent on translational inhibition rather than at the mRNA level or protein degradation.

Transcriptional regulation of cyclin D1 could also represent another explanation for the reduced loss of cyclin D1 in pancreatic cancer cell lines. The regulation of cyclin D1 transcription by mitogenic pathways, particularly the RAS/RAF/MAPK pathway through Myc or upregulation of NFκB, is especially important given their roles in the pathogenesis in PDAC. Our study determined that while cyclin D1 mRNA loss eventually occurred in non-tumorigenic pancreatic cells, this loss occurred after cyclin D1 protein reduction was observed. This finding indicated that the early regulation of cyclin D1 protein levels in response to tunicamycin was not through reduced mRNA levels. However, our findings that cyclin D1 protein loss occurred prior to mRNA reduction indicated another mechanism dominated early cyclin D1 protein repression. This finding is consistent with a prior study in NIH-3T3 cells that showed that cyclin D1 mRNA levels remained unchanged after 8 hours of tunicamycin exposure while cyclin D1 protein levels were lost after just 4 hour of treatment (86). Importantly, these results do not discount the possibility that transcription of cyclin D1 mRNA may play a significant role after more extended durations of tunicamycin treatment. Pancreatic cancer cells possessed an impaired response in downregulating both mRNA and protein levels over a prolonged treatment duration. The RAS/RAF/MAPK pathway may indeed play a contributory role in the persistence of cyclin D1 mRNA levels over the entire 24-hour time course. Another explanation for the differences in the responsiveness at the mRNA level of non-tumorigenic cells could potentially be explained by the initial regulation of cyclin D1 at the protein level. I hypothesize that the early attenuation of cyclin D1 protein translation mitigates the E2F feed-

forward response to further stimulate cyclin D1 transcription. This would result in a compounded effect of further reducing cyclin D1 protein in non-tumorigenic cells through transcriptional and subsequent translational processes. In contrast, a persistent cyclin D1 level in pancreatic cancer cells promotes further cell cycle progression.

We also determined through degradation experiments that slower degradation of cyclin D1 was not occurring. On the contrary, it appeared that cyclin D1 degradation was actually being promoted in the pancreatic cancer cell line Hs766T compared to the non-tumorigenic cell line HPDE. The significance of this finding has not been elucidated and is beyond the scope of this thesis. GSK-3β phosphorylation, measured as an indirect measure of cyclin D1 degradation also indicated that cyclin D1 was not accumulating in pancreatic cancer cell lines due to decreased degradation.

Further analysis through [H^3]-leucine incorporation revealed that impaired repression of cyclin D1 was occurring through a statistically significant inability to downregulate translation in pancreatic cancer cells ($p<0.001$) as compared to non-tumorigenic pancreatic ductal cells. Since inhibition of translation was impaired in pancreatic cancer cell lines, results from eIF2α phosphorylation from Chapter Two were compared to cyclin D1 protein levels. Time-dependent expression of cyclin D1 closely correlated with phosphorylation of eIF2α and downstream effectors ATF4 and CHOP in both non-tumorigenic and pancreatic cancer cells. The relationship between eIF2α phosphorylation and translational repression of cyclin D1 were also confirmed with my own experiments in MEFs with eIF2α mutated at its Ser51

phosphorylation site. Data in the MEFs recapitulated previously published work establishing translational repression of cyclin D1 through eIF2α phosphorylation (85, 86).

Most experiments in this study focused on tunicamycin to induce ER stress. Admittedly, the effects observed in this study could be limited to tunicamycin's function as an N-linked glycosylation. Specific usage of this drug is notable since many growth factors, such as EGFR, require N-linked glycosylation for maturation (132, 133). However, to circumvent the possibility that the observed cell cycle regulatory effects are specific to tunicamycin or N-linked glycosylation, cells were also treated with thapsigargin, a calcium ATPase inhibitor in Figure 24. The impaired ability to reduce [H^3]-leucine incorporation was similar across non-tumorigenic cells and pancreatic cancer cells after treatment with tunicamycin or thapsigargin. Since thapsigargin functions through a mechanism distinct from thapsigargin, the cell cycle effects are unlikely solely due to the inhibition of glycosylation.

Cyclin D1 loss in non-tumorigenic cells correlated with previous studies (Chapter Two) where eIF2α phosphorylation occurred prior to cyclin D1 loss. Phosphorylation of eIF2α in pancreatic cancer cells was consistently delayed and correlated with persistence of cyclin D1 levels as compared to non-tumorigenic pancreatic ductal cells. The mechanism linking eIF2α phosphorylation was further corroborated by experiments in phosphorylation-deficient mutant S51A eIF2α MEFs. In non-tumorigenic pancreatic cells, pancreatic cancer cells, and our model

MEF system, cyclin D1 repression consistently correlated with translational inhibition, phosphorylation status, and stage of cell cycle progression.

The findings in this chapter underline the key observations that pancreatic cancer cells are unable to downregulate cyclin D1 due to an impaired activation of the eIF2α arm of the UPR. A subsequent impaired G0/G1 phase arrest occurs, which allows continued proliferation. As mentioned in Chapter Two, UPR activation has not been observed in pancreatic cancer with a moderate induction of ER stress at 0.5 μg/ml tunicamycin. Pancreatic cancer cells instead exhibit an increased tolerance to ER stress that enables continued proliferation under conditions of increased protein load.

These results have important clinical implications given the problem that some chemotherapies have been noted to induce ER stress-mediated growth arrest and apoptosis in some cancer types. However, a heightened ability to withstand the increased protein load created by chemotherapies would allow pancreatic tumors to be resistant to the ER stress-mediated effects. While many studies suggest that perhaps cyclin D1 should be targeted, I suggest that cyclin D1 only represents a readout for an underlying problem that impedes activation of the UPR in pancreatic cancer. Future studies identifying the mechanism that prevents UPR activation would provide a novel target for the treatment of pancreatic cancer.

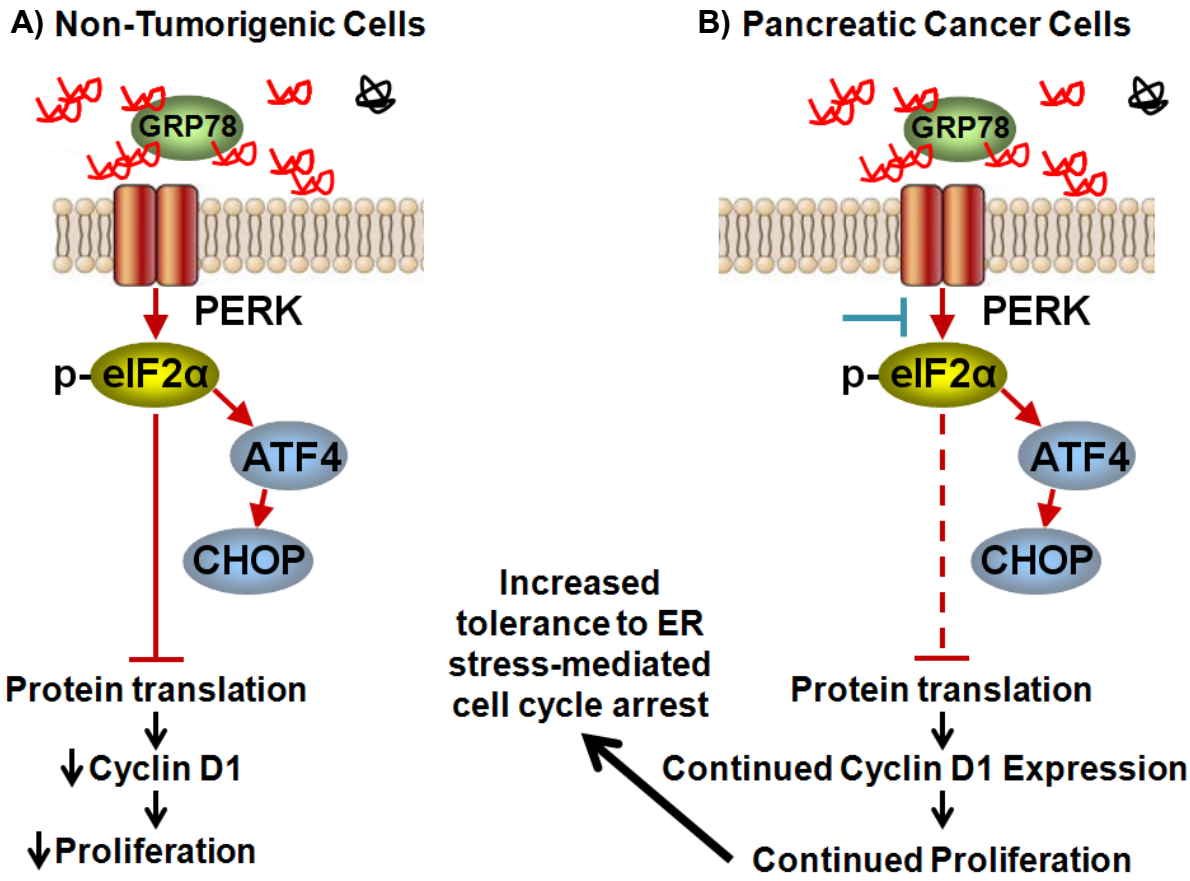


Figure 27. Proposed mechanism of pancreatic cancer cell tolerance to ER stress-mediated cell cycle arrest. In non-tumorigenic cells, ***A***, ER stress activates PERK. PERK phosphorylates eIF2α which inhibits protein translation and also selectively translates ATF4 and induces CHOP expression. Inhibition of protein translation results in decreased cyclin D1 protein levels and cell cycle arrest/reduced proliferation. In pancreatic cancer cells, **B**, we propose that an unknown mechanism prevents activation of PERK. Therefore, phosphorylation of eIF2α is inhibited with subsequent decrease in attenuation of protein translation, as well as delayed ATF4 and CHOP induction. Since translation is not inhibited effectively, cyclin D1 continues to be expressed and cell cycle progression/proliferation continues. The overall result is increased tolerance to the growth inhibitory effects of ER stress in pancreatic cancer cells.

CHAPTER FOUR:

SUMMARY AND FUTURE DIRECTIONS

SUMMARY

Pancreatic adenocarcinoma is one of the deadliest cancers, ranking as the fourth most common cause of death associated with cancer. The disease is extraordinarily deadly, with a an overall 5-year survival rate of only 5.6% and a median survival of less than 6 months (4, 5). Major obstacles face advancement of treatment options for pancreatic cancer. Current medicine lacks the ability to detect pancreatic cancer early and the therapeutic options available provide only brief extensions of survival. Given the poor responsiveness of pancreatic cancer to chemotherapy, new targets and pathways need to be investigated for potential therapies. A key impedance to progress in treatment of pancreatic cancer is the chemoresistance it exhibits to most chemotherapies. Identifying methods that sensitize chemoresistant pancreatic cancer cells is a critical component in advancing treatment for this deadly disease.

Because of the innate ability of pancreatic adenocarcinoma to survive in the context of hypoxia, metabolic stresses, as well as applied stresses such as through chemotherapy, the overall goal of this dissertation was to determine whether pancreatic cancer cells utilize adaptive mechanisms to survive in these stresses. *The hypothesis for this project was that pancreatic cancer cells have an altered unfolded protein response to endoplasmic reticulum stress*.

To test this hypothesis, I first examined whether pancreatic cancer cells exhibited differences from non-tumorigenic cell lines in response to the classic ER stressor tunicamycin. Pancreatic cancer cells exhibited a clear difference in their UPR activation that permeates the PERK/eIF2α/ATF4 and IRE1α/XBP1s branches.

Delayed induction of both of these arms of the UPR indicates that a global mechanism upstream of ER stress activation exists. The mechanism of how pancreatic cancer cells avoid early activation of the UPR warrants further investigation. Potential explanations for this finding includes upregulated ER chaperones that provide an increased capacity for protein load before stress induction, increased autophagy sequestration of aberrant proteins, or factor(s) that have yet to be identified that inhibit activation of the ER transmembrane UPR receptors.

Additionally, this study determined that the altered unfolded response resulted in an important impairment of cyclin D1 translational repression and cell cycle arrest. I determined that while mRNA regulation may influence cyclin D1 protein levels over longer periods of tunicamycin treatment, the early loss of cyclin D1 in non-tumorigenic but not pancreatic cancer cells was primarily caused by an impaired eIF2α pathway. Delayed activation of this branch of the UPR resulted in an impaired attenuation of protein translation. Over the same 24 hour exposure to tunicamycin, pancreatic cancer cells were incapable of achieving the same proportion of cyclin D1 loss as non-tumorigenic pancreatic cells.

Of note, although a screen of pancreatic cancer cell lines revealed that a majority (15/22) of pancreatic cancer cell lines exhibited a reduced ability to downregulate cyclin D1, a large minority (7/22) still demonstrated an intact response to ER stress induction. This variability in response illustrates the heterogeneity encountered in pancreatic cancer cells and the hindrance facing treatment of pancreatic cancers with single agents.

In conclusion, this dissertation provides evidence of an altered unfolded protein response in pancreatic cancer cells that results in continued proliferation under conditions that should induce ER stress. These findings demonstrate that pancreatic cancer cells possess an adaptive mechanism that enables continued proliferation in the presence of ER stress-inducing conditions.

FUTURE DIRECTIONS

What is the mechanism for global resistance to UPR activation in pancreatic cancer cells?

My current study identified that a mechanism upstream of the UPR exists that enables late activation of the PERK/eIF2α/ATF4/CHOP and IRE1α/XBP1s arms of the UPR. However, further investigation of potential mechanisms for this global resistance to UPR activation is required if a potential therapeutic target is to be identified for further evaluation. Some suggested causes of this impaired UPR activation include increased ER chaperone expression or autophagy. Increased ER chaperone expression may provide increased protein load capacity that requires increased protein entry into the secretory ER pathway in order to induce ER stress. Autophagy may provide increased autophagosomal capacity for sequestering of aggregated proteins away from the ER, thus limiting proteotoxic stress. Determination of a causative factor for the increased tolerance for ER stress in pancreatic cancer would provide a therapeutic target that could sensitize pancreatic cancer cells to ER stress-mediated apoptosis. Additionally, more extensive study of UPR activation after treatment with other ER stressors needs to be included to

determine whether this global impairment of the UPR extends to ER stress conditions beyond tunicamycin's inhibition of N-linked glycosylation. Other ER stress conditions, such as hypoxia, have also been established as inducing translational attenuation by the eIF2α arm of the UPR. Additional studies are required to examine whether the global impairment of the UPR in pancreatic cancer cells extends to other types of ER stress.

How does the pancreatic tumor microenvironment influence ER stress activation?

This study investigated the role of the UPR activation in pancreatic cancer cells. However, the contribution of the tumor microenvironment was not directly tested in this experiment. We do not underestimate the importance of the pancreatic tumor microenvironment, particularly given that our hypothesis assumes a hypovascular tumor microenvironment imparts the need for adaptability to ER stress. Pancreatic adenocarcinomas also contain extensive stroma that has been shown to influence vascular supply to pancreatic cancer cells in mouse transgenic models (134). We can further examine the tumor microenvironment by stably transfecting pancreatic cancer center lines with luciferase reporters for XBP1 splicing and ATF4 activation.

Does persistent XBP1s activation in pancreatic cancer cells enable continued tolerance to ER stress?

XBP1s has been demonstrated to play a critical role in tumor cell survival during hypoxia. XBP1-deficient cells displayed an increased susceptibility to apoptosis during severe hypoxic stress and impaired tumor growth (104). Additionally, XBP1 expression has been shown to be highly expressed in hypoxic tumor regions (103). Our studies indicate that once activated, XBP1s is robustly expressed for an extended duration in pancreatic cancer cells compared to a rapid decline in non-tumorigenic cell lines. I hypothesize that this response is a compensatory mechanism for deficient eIF2α phosphorylation. Further investigation of the importance of XBP1s in pancreatic cancer cell survival could be achieved through knockdown of XBP1s.

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VITA

Jennifer Hsing Choe was born on February 5, 1980 in Columbus, Ohio to her parents, Su-Yun Hsing and Hsu-Hui Hsing. She moved to Nederland, Texas in 3rd grade and eventually graduated from Nederland High School in 1998. In the fall of 1998, she entered the University of Texas at Austin where she received a B.A. in the liberal arts honors program Plan II and biochemistry with a minor in English. Jennifer was also awarded the distinction of admission to the Dean's Scholars Natural Sciences Honors Program. At the University of Texas at Austin, she was mentored for 3 years by Dr. Angela Belcher in the Department of Chemistry and Biochemistry studying biological nanoparticle self-assembly systems. Besides research, she was also involved in a wide breadth of student activities including the Natural Sciences Representative-Elect to the University of Texas at Austin Student Government, representative-elect to the Dean's Scholars Honors Program Council, editor-in-chief of a student-interest magazine, along with a number of student mentoring programs. In 2002, Jennifer matriculated into the M.D./Ph.D. program at the University of Texas-Houston Medical School and Graduate School of Biomedical Sciences in Houston, Texas. During her first 3 years in medical school, Jennifer was actively involved in student activities such as chairing the Cultural and Religious Awareness Committee and acting as a member of the both of the M.D/Ph.D. program's Admissions Committee and as the M.D./Ph.D. representative on the medical school's Curriculum Committee. In 2005, she joined Craig Logsdon's lab at the University of Texas at M.D. Anderson Cancer Center to begin her Ph.D. thesis work. She has since presented posters at the American Association of Cancer Research and the American Pancreatic Association annual meetings. She was also awarded an NIH-funded T32 fellowship from the Center for Clinical and Translational Science program from 2007-2009 and two travel awards. After completion of her Ph.D. thesis, Jennifer will soon graduate from medical school and enter her residency in internal medicine at the Baylor College of Medicine in Houston, Texas. Jennifer is married to Jonathan Choe, has two young daughters, Eva and Natalie, and unabashedly admits to having 5 cats.