Exploration into the Functional Impact of MUC1 on the Formation and Regulation of Transcriptional Complexes Containing AP-1 and p53

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Title
Exploration into the Functional Impact of MUC1 on the Formation and Regulation of Transcriptional Complexes Containing AP-1 and p53

By
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A DISSERTATION
Presented to the faculty of the Graduate College in the University of Nebraska
In Partial Fulfillment of Requirements
For the Degree of Doctor of Philosophy

Cancer Research Graduate Program
Under the Supervision of Dr. Michael A. Hollingsworth

University of Nebraska Medical Center
Omaha, Nebraska

July 18, 2016
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The transmembrane glycoprotein MUC1 is aberrantly expressed in the majority of pancreatic ductal adenocarcinoma cases and promotes tumor progression by engaging in morphogenetic signaling through its cytoplasmic tail. Furthermore, MUC1 can translocate to the nucleus and function as a transcriptional co-regulator in conjunction with transcriptional complexes containing activator protein-1 (AP-1) and p53. The specificity of these interactions are thought to rely on specific patterning of post-translational modifications within the cytoplasmic tail of MUC1.

Within this dissertation, we examined how MUC1 influences the formation and activity of these transcription factors and the resulting impact on tumor progression and metastasis. In our first set of studies, we evaluated the global pattern of post-translational modifications present within MUC1. While previous studies have shown phosphorylation of specific residues, we found that MUC1 exists in a hyperphosphorylated state containing potentially more than 10 phosphorylated residues. Furthermore, malignant tumors exhibit a higher degree of phosphorylation and changes in the environment also drive changes in this patterning. This is also true of several MUC1 splice variants, suggesting that MUC1 can readily integrate numerous signaling pathways to initiate the appropriate signaling compartment.

We further evaluated how MUC1 expression regulates the function of AP-1 and found that MUC1 promoted the formation of AP-1 dimers consisting of c-Jun and FRA-1. We further demonstrated that FRA-1 is a novel driver of pancreatic tumor migration, invasion, and overall progression both in conjunction, and independent of MUC1 expression. In vivo studies show increased expression of FRA-1 in pancreatic cancer and increased expression of FRA-1 target genes involved in epithelial-to-mesenchymal transition suggesting FRA-1 may be a potential therapeutic target in pancreatic cancer.
We explored this possibility utilizing inhibitors of bromodomain and extraterminal domain containing proteins, which had shown inhibitory effects on expression of FRA-1. We found we were unable to reproduce these effects, however, combining FRA-1 knockdown with BET inhibition resulted in additive effects in decreasing cellular growth. These effects are seemingly due to diminished expression of pro-growth and survival genes. Future studies targeting FRA-1 may identify better therapeutic partners.

Lastly, we examined how the mutational status of p53 influenced its interaction with MUC1. As mutant p53 has been shown to exhibit gain-of-function effects in cancer, we sought to evaluate whether different mutants would preferentially interact with MUC1 and any potential transcriptional changes that result. We found that MUC1 preferentially interacted with the hotspot mutant R273H, as compared to R175H and wildtype p53 in a dox-inducible model.
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Abbreviations

ADM: Acinar-to-Ductal Metaplasia
AP-1: Activator Protein 1
BET: Bromodomain and Extra-Terminal Motif Proteins
BETi: BET Inhibitor
CTGF: Connective Tissue Growth Factor
FOLFIRINOX: Leucovorin Calcium (Folinic Acid), Fluorouracil, Irinotecan Hydrochloride, and Oxiplatin
Gal: Galactose
GalNAc: N-acetyl-Galactosamine
GalNAc-Ts: GalNAc Transferases
GlcNAc: N-acetyl-Glucosamine
KPC: KrasLSL.G12D/+; p53R172H/+; PdxCretg/+ 
MMP1: Matrix Metalloprotease 1
MUC1.CT: MUC1 Carboxy-terminal Subunit
PanIN: Pancreatic Intraepithelial Neoplasia
PDAC: Pancreatic Ductal Adenocarcinoma
RTK: Receptor Tyrosine Kinase
TPA: 12-O-tetradecanoyl-phorbol-13-acetate
Acknowledgements

I would like to thank my advisor Michael Hollingsworth for his guidance over the past several years. It has been a great pleasure to work in his lab and develop my skills as a researcher. I thoroughly believe that working in his lab has given me the skills and independence necessary for a career in research. Throughout my graduate career I have been given the freedom to explore questions that have interested me, though Tony was always present to pull me back when I may be heading down a fruitless road. For this I am extremely grateful, as it allowed me to develop my ability to think critically and expanded my knowledge of many fields. I would also like to acknowledge my committee members: Dr. Angie Rizzino, Dr. Jennifer Black, and Dr. Surinder Batra, all of whom have provided me exceptional guidance over the years.

I am extremely grateful for my wife, Stephanie Hanson, who has put up with me over my years in graduate school. She has dealt with the long hours, the frustrating days of failure, as well as the joys of finally becoming a published scientist. She has always been there to keep me grounded when it would be easy to get caught up in the frustrations of failed experiments. She has had to put up with far more grumpy days than I should be allowed and has always been understanding and supportive of my career and for that I am extremely lucky.

We would like to thank the UNMC Advanced Microscopy Core for assistance with the confocal microscopy imaging. We acknowledge the UNMC Rapid Autopsy Program, which provided tissue microarrays for our Immunohistochemistry assays for FRA-1 and EMT gene targets. We also thank Dr. Fang Yu, who performed statistical analysis of the orthotopic studies and microarray data. We acknowledge the UNMC Flow Cytometry Core for performing the cell cycle analysis. We also thank the UNMC Sequencing core for preparing libraries and performing RNA-sequencing. We acknowledge the UNMC
Bioinformatics core facility for analyzing the RNA-seq data and assisting with QIAGEN Ingenuity® Pathway Analysis.

Lastly, I would like to thank my family and friends. Throughout the years everyone has been very supportive of my career path. It has been a long journey and I would like to thank everyone that has supported me along the way. Including the Eppley program as a whole, it has been a great few years.
CHAPTER I: INTRODUCTION
1. Pancreatic Cancer

1.1 Diagnosis, Prognosis and Treatment

Pancreatic cancer is currently the fourth-leading cause of cancer related deaths within the United States [1, 2]. This is projected to rise to the second-leading cause of cancer-related death by 2020 [3]. Despite extensive research focused on therapeutic treatment of cancer, the prognosis for patients diagnosed with pancreatic cancer remains poor, even as survival for other cancers have shown marked improvements. Furthermore, the majority of pancreatic cancer cases are the aggressive pancreatic ductal adenocarcinoma (PDAC), as opposed to the less malignant neuroendocrine tumors[4]. The current five-year survival rate for pancreatic cancer is approximately 6% and has remained largely unchanged for decades[2]. Surgical resection of the pancreas is the best option for patient survival, and patients that have undergone the Whipple procedure have a five-year survival rate near 20%[2, 5, 6]. However, due to the location of the tumor, non-specific symptoms at presentation, and propensity for distal metastases at diagnosis, only a fraction of patients are eligible for the procedure. Studies for early biomarkers of pancreatic cancer are currently ongoing, however, have not yet made a significant impact on the diagnosis of PDAC.

Over the past two decades the standard treatment of pancreatic cancer has largely consisted of the deoxycytidine analogue gemcitabine either alone, or in conjunction with other chemotherapeutic drugs, most recently Abraxane™ [7, 8]. The multidrug combination FOLFIRINOX has shown potential for extension of patient survival, however, the regimen is associated with significant toxicity [7, 9]. Ultimately none of the current standard therapies for pancreatic cancer provide curative benefit to patients, as the disease often resists treatment, either through intrinsic or adaptive mechanisms [10]. The molecular mechanisms involved in the formation and progression
of pancreatic cancer must be elucidated to identify novel therapeutic targets and improve
the outcome of patients.

1.2 Genetics and Development

PDAC develops through the accumulation of genetic alterations that confer a
selective advantage upon the cells. While the majority of pancreatic cancer cases are
ductal adenocarcinoma, the precise cell of origin remains debated. As the ductal cells
represent a minor fraction of the pancreas, it is hypothesized that acinar-to-ductal
metaplasia (ADM) plays a major role in the development of pancreatic cancer [11]. As
mutations accumulate, the normal epithelia of the pancreas undergo transition to
pancreatic intraepithelial neoplasias (PanINs), eventually developing into invasive
adenocarcinoma [12]. This transition is well defined, as cells progress from PanIN-1
through PanIN-3 with increasing dysplasia before ultimately becoming invasive
disease (Figure 1.1). The histology of pancreatic cancer is characterized by robust stromal
involvement and desmoplasia with the actual cancer cells representing a minor fraction
of the tumor (Figure 1.2). This stromal reaction has significant impact on the treatment
of PDAC as it creates a cellular, molecular, and physical barrier for drug delivery and
entry of effector lymphocytes and other immune cell types.

There are four major genetic alterations that commonly occur within pancreatic
cancer [13]. More than 90% of pancreatic cancers exhibit activating mutations within the
Kras oncogene and 50-75% of tumors exhibit alterations in the tumor suppressor p53
[14]. These two alterations are sufficient to induce spontaneous pancreatic tumor
development when engineered for pancreas cell specific expression in mice and provide
the basis for the KPC mouse model of spontaneous pancreatic cancer [15]. In addition
to these two drivers, loss of the CDKN2A locus and SMAD4 are also extremely common
Beyond the four common mutations, pancreatic cancer harbors patient specific mutations within a number of alternative pathways promoting oncogenic signaling through several distinct networks. As a result, pancreatic cancer represents a diverse malignancy that is proposed to consist of distinct molecular subtypes [16-18]. Most recently, it has been proposed that PDAC consists of four distinct subtypes based on combined genomic, epigenomic, and transcriptomic analyses, however, the conclusions from these studies remain controversial as tumors are often complicated by contamination by stromal cells [18]. Each of these subtypes appears to utilize different signaling pathways for growth and progression. Interestingly, expression of different mucin glycoproteins correlates with different subtypes of pancreatic cancer.
Figure 1.1: Development of Pancreatic Ductal Adenocarcinoma

The progression of events involved in the development of pancreatic ductal adenocarcinoma is well defined[12]. During development, lesions progress through a number of well-characterized stages (PanIN-1 through 3) before finally progressing to invasive carcinoma. Among the earliest events is the mutation of Kras, typically at hotspot residues 12 and 13. Other well-characterized events included loss of tumor suppressors such as p16, p53, and SMAD4.
Figure 1.1

Normal Epithelium, PanIN-1, PanIN-2, PanIN-3, PDAC

- KRAS Mutation
- Tp53 Loss
- SMAD4 Loss
- p16 Loss
**Figure 1.2: Histology of Pancreatic Ductal Adenocarcinoma**

Images representing either normal pancreas (A) or pancreatic ductal adenocarcinoma (B) are presented. Slides were counterstained with hematoxylin and scanned at 40X magnification using the Ventana Coreo slide scanner in the UNMC Tissue Sciences Core. Normal pancreas consists of both an exocrine and endocrine compartment. The exocrine function primarily consists of acinar cells, with a minor fraction of ductal cells, and cells within the Islet of Langerhans control the endocrine function. In comparison, pancreatic ductal adenocarcinoma exhibits an intense stromal reaction and in many tumors the proportion of stroma exceeds that of the tumor cells.
2. Mucins

2.1. Structure and Function

Mucins comprise a large family of glycoproteins expressed by a variety epithelia; including the respiratory, gastrointestinal, and reproductive tracts [19]. These high molecular weight glycoproteins are classified as either secretory mucins, which are secreted into the extracellular space, or membrane bound mucins that contain a transmembrane domain and are inserted into the apical surface of epithelial cells [19, 20]. Together these mucins make up a significant proportion of the proteins found in the mucosal layers that coat the epithelial surface at the air to cell interface [20-23]. The secretory family of mucins includes MUC2, MUC5AC, MUC5B, MUC6, and MUC7, whereas, MUC1, MUC3A, MUC3B, MUC4, MUC12, MUC13, MUC15, MUC16, MUC17, and MUC20 are all membrane bound [20]. Under normal physiological conditions, mucins play an essential role in lubrication, chemical sensing, and organization of the local microenvironment surrounding the cells [19]. Mucins also form a protective barrier to pathogens and are postulated to act as sensors of the surrounding environmental conditions [24-26]. Transmembrane mucins can propagate extracellular signals into the cells through phosphorylation of specific residues in the cytoplasmic tail, allowing cells to adapt to the surrounding environment [26].

A defining structural characteristic of mucin proteins is the presence of a tandem repeat domain or mucin domain [20]. The amino acid sequence and number of these repeats varies from mucin to mucin and are rich in serine, threonine, and proline residues resulting in the presence of many potential sites of O-linked glycosylation [27, 28]. The presence of O-glycosylation is critical for mucin function, as these oligosaccharides are involved in ligand-receptor interactions and mediate the gel-forming properties critical for organization of the extracellular environment [29, 30]. As the
number of these repeats is highly variable, individual mucins can contain a wide array of potential glycosylation patterns [20] (Figure 1.3).

The process by which mucin type O-linked glycosylation occurs is well characterized [31-34] (Figure 1.4). The initiating step involves the addition of N-acetyl-Galactosamine (GalNAc) to serine or threonine residues present in the mucin backbone to form the Tn-epitope and is catalyzed by a large family of enzymes called GalNAc-transferases (GalNAc-Ts) [31, 35]. These structures can then be further extended to form Core 1, 2, 3, or 4 structures based on the identity and linkage of the carbohydrate moiety [36]. Core 1 structures are formed by addition of Galactose (Gal) in a β1-3 linkage to GalNAc. In contrast to the diversity of GalNAc-Ts, a singular enzyme, Core 1 Gal-transferase, catalyzes this addition [37]. Core 1 structures can then be extended or Core 2 structures can be generated by addition of GlcNAc in a β1-6 linkage to the existing GalNAc of the Core 1 structure by C2GnTs [38-40]. As an alternative to Core 1, Core 3 structures can be generated through addition of GlcNAc in a β1-3 linkage to the Tn epitope [41]. Like Core 1 structures, Core 3 structures may be extended or act as the scaffold for Core 4 structure generation through addition of another GlcNAc in a β1-6 linkage [40]. While other core structures do exist, Core 1, 2, 3, and 4 structures comprise the primary glycan structures observed with the human body.

2.2 Deregulation of mucin expression and O-glycosylation in Cancer

Deregulated expression of mucins is observed in many malignancies, particularly within adenocarcinomas (Table 1.1). Elevated expression of MUC1 is common in pancreatic, breast, colon, lung, and prostate cancer [42-45]. Expression of MUC1 appears to be a major factor in the progression of pancreatic cancer, as knockout of MUC1 results in significantly prolonged survival in the KPC mouse model of pancreatic
cancer (Figure 1.5). This effect is presumably through the oncogenic signaling capacity of the MUC1 cytoplasmic tail. Similarly, knockout of MUC1 expression in a mouse model of mammary tumors also delayed tumor progression [46]. MUC4 expression is increased in colon adenocarcinoma samples and is a proposed marker of aggressive pancreatic cancer [42, 47]. Elevation of the MUC16 marker (CA125) is well studied in ovarian cancer, however, more recently expression of MUC16 has been implicated as a significant factor in the progression of pancreatic cancer [48-51].

Additionally, many tumors exhibit increased expression of truncated or aberrant O-glycans. These alterations in tumor glycobiology primarily occur through 2 mechanisms; Neo-synthesis or incomplete synthesis [52]. In particular, the expression of Core 1 based structures, such as T, Tn, or sialyl-Tn(STn), are observed in a majority human carcinomas, whereas in healthy tissues, these structures are typically absent [42, 53, 54]. Increased expression of other glycoepitopes, such as sialyl-Lewis^x/a, are also commonly observed [55]. In contrast, decreased expression of Core 3 and 4 structures are observed in gastric and colorectal cancers [56, 57]. In many instances, expression of these structures is driven by alterations in the expression of enzymes involved in the glycosylation process. For example, the extension of Core 1 structures relies on a single enzyme, C1GalT1. This enzyme requires a specific chaperone, COSMC, for proper folding and functional activity [58-60]. Cells lacking expression of COSMC have been shown to express increased levels of Tn and STn epitopes [61, 62]. Furthermore, a significant percentage of cancers exhibit hypermethylation of the COSMC gene, resulting in decreased expression of C1GalT1 and increased formation of Tn and STn epitopes [63]. Deregulation of other enzymes involved in O-linked glycosylation has been observed in a variety of cancers [55, 64-68].

Expression of these aberrant glycoforms is often used as a diagnostic or prognostic marker of tumor progression. CA19-9 detects the presence of sialyl lewis^x on
mucin core proteins[69, 70]. Other markers include DuPan-2, Span-1, CA50, CA242, CA195, CAM43, and SSEA[70-72]. All of these markers are mucin type oligosaccharides. While the levels of these markers are commonly monitored throughout tumor progression the sensitivity and specificity of these assays as diagnostic tools is relatively poor. As such, use of these assays has not proven useful for early diagnosis of pancreatic cancer.

Phenotypically, expression of aberrant O-glycans has been correlated with increased aggressiveness and metastatic behavior in a variety of cancers [53, 55, 63]. These effects are hypothesized to be the result of altered interactions between tumor cells and binding partners in the extracellular environment, such as selectins and integrins [55]. However, these alterations may result in perturbations to the signaling of mucin cytoplasmic tails leading to potentiation of oncogenic signals. Re-expression of enzymes involved in the extension of the carbohydrate chain, such as Core 3 synthase, results in a decrease in these aggressive properties in pancreatic cancer cells by influencing these interactions suggesting that targeting the glycosylation process may be a viable strategy in cancer [73].

2.3 MUC1: Signaling Through the Cytoplasmic Tail

MUC1 is the best-characterized transmembrane mucin for its signaling role within tumorigenesis; however, both MUC4 and MUC16 have also been shown to signal through their cytoplasmic tails [74, 75]. MUC1 is a type I transmembrane protein that exists as a heterodimer at the apical cell surface. The formation of the dimer is through an autoproteolytic cleavage event that occurs during translation of MUC1 at the SEA (sea urchin sperm protein, enterokinase, agrin) domain [19, 20, 76]. The resulting two subunits then associate in a strong, non-covalent interaction. The protein undergoes
extensive glycosylation, both O-linked and N-linked, before insertion into the plasma membrane [19, 20] (Figure 1.6). The N-terminus of MUC1 consists of a heavily O-glycosylated extracellular portion that extends from the cell surface and plays a role in organization of the glycocalyx [20]. The C-terminus of MUC1 (MUC1.CT) consists of a short extracellular domain, a transmembrane domain, and a 72 amino acid cytoplasmic tail.

In response to perturbations of the extracellular environment, binding of ligand molecules, or interaction with receptor tyrosine kinases (RTKs), MUC1 can become phosphorylated at specific residues within the cytoplasmic tail [26, 77-79]. Under normal physiological conditions, MUC1 is spatially separated from RTKs that are expressed on the basal surface. Loss of polarity, either through mechanical injury of the epithelia or as a result of cellular transformation, allows MUC1 to interact with these partners and drive downstream signaling (Figure 1.7) [26]. As the cytoplasmic tail of MUC1 contains 22 potential sites of phosphorylation, it is possible to integrate a wide range of signals through specific phosphorylation patterns. This combinatorial pattern of phosphorylation is likely critical for the specificity of interactions of the cytoplasmic tail with a host of effectors [77, 80-82] (Figure 1.8).

As a result of all these potential interaction partners, the propagation of signals through the cytoplasmic tail is extremely complex (Figure 1.9). Interaction of MUC1 with receptor tyrosine kinases can result in the activation of a number of downstream cascades. Phosphorylation of the MUC1 cytoplasmic tail has been shown to increase the interaction of MUC1 with Grb2 to drive downstream activation of ERK [81, 83]. MUC1 also promotes signaling through the PI3K/Akt signaling pathway to drive anti-apoptotic signaling [84, 85]. MUC1 also interacts with all four members of the ErbB family of receptor tyrosine kinases [86]. Other studies have shown that MUC1 is capable of initiating signaling through JNK to drive migration and invasion in
hepatocellular carcinoma[87, 88]. Induction of these signaling cascades results in eventual activation of transcriptional effectors, such as NF-κB and C/EBP, to alter the expression of genes critical in tumor progression and survival[81, 85, 89].

These interactions can also result in alterations to localization of signaling complexes. The interaction of MUC1 with the EGFR receptor results in not only phosphorylation of MUC1, but also in nuclear localization of EGFR [79]. How MUC1 translocates to the nucleus remains unknown, however, it may depend on endosomal internalization, similar to the mechanism of nuclear localization of ErbB2 [90]. Supporting this idea is the evidence that both MUC1 and EGFR can associate with CIN85, which regulates internalization of EGFR [91, 92]. Furthermore, the presence of specific tyrosine residues in the cytoplasmic tail is critical for clathrin-mediated endocytosis of MUC1, suggesting that phosphorylation of MUC1 may be a critical determinant in the localization of signaling complexes [93].

MUC1 expression also has significant impact on the surrounding tumor microenvironment through interactions with the surrounding stroma and tumor associated cells. Expression of select carbohydrate epitopes on MUC1 has been shown to promote the invasion of pancreatic cancer cells into the surrounding vascular [94]. As a result, MUC1 regulates the adhesive and anti-adhesive balance within the tumor. MUC1 has also been shown to play an immunomodulatory role through interactions with specific immune effector cells [95-97]. While these interactions are thought to be primarily immunosuppressive, the presence of anti-MUC1 autoantibodies within tumor patients suggests that MUC1 may be a viable target for immunotherapies [98, 99].

Specific studies have also demonstrated the capacity of MUC1.CT to interact with the transcription factors p53 and c-Jun [77, 80, 100]. The interaction of MUC1.CT with transcription factors is proposed to result in the translocation of MUC1 to the nucleus where it acts as a transcriptional co-regulator. The interaction of MUC1 and p53
has been shown to promote survival based signaling by promoting expression of p21, while inhibiting expression of the pro-apoptotic protein Bax [80]. This suggests that MUC1 may promote tumor cell survival by altering the normal function of p53 or by promoting gain-of-function effects with mutant p53. While MUC1 has no DNA binding capacity, it is found in complex with p53 at these promoter elements suggesting that MUC1 can modulate the formation of transcriptional complexes. Additional studies have shown that this interaction requires phosphorylation of specific motifs within the cytoplasmic tail [77]. These studies have also demonstrated that these complexes appear to impact the expression of connective tissue growth factor (CTGF) and matrix metalloprotease 1 (MMP1) through the binding of upstream promoter elements [77, 100]. This binding is accompanied by the apparent loss of the proto-oncogene, c-Jun, from the same promoter element. How the mutational status of p53 influences the interaction with MUC1.CT and the resulting impact on gene expression is unknown.

MUC1 has also been shown to associate with β-catenin through a serine rich motif present in the cytoplasmic tail [101, 102]. This motif is similar to the serine rich motif of APC that allows for interaction with β-catenin. Like the association with p53, this interaction has shown to promote translocation of MUC1 to the nucleus and alter the regulation of Wnt target genes [103]. This interaction is modulated by phosphorylation of specific residues in the cytoplasmic tail. Phosphorylation of a serine residue near the serine rich motif by GSK3β decreases the affinity of this interaction, whereas phosphorylation of tyrosine residues by c-Src appears to increase the affinity of MUC1 for β-catenin [104, 105]. These interactions may also influence the balance of adhesive properties by changing the localization of β-catenin from junctional complexes.

Translocation of MUC1 to the mitochondrial membrane has also been observed in select circumstances [106]. In these studies, MUC1 was shown to inhibit pore formation by interfering with Bax oligomerization. This binding requires the CQC motif
present within the cytoplasmic tail of MUC1. This association results in inhibition of cytochrome C release and blocking of the apoptotic pathway. The translocation of MUC1 to the mitochondria appears to rely on the association with the molecular chaperone Hsp90 [107]. Interestingly, expression of MUC1 has also been shown to promote FasL induced apoptosis suggesting MUC1 may balance several apoptotic pathways [108]. Supporting this idea is the evidence that MUC1 expression is often induced in response to cellular stressors, such as presence of reactive oxygen species (ROS) [109].

As a result of all these interactions, MUC1 acts as a signaling hub for an extremely complex network of pathways. How MUC1 integrates all these pathways to initiate the correct downstream effect remains unknown. Furthermore, how MUC1 exerts its transcriptional co-regulatory role is still not fully understood. MUC1 appears to alter the formation of transcriptional machinery, however, whether these complexes are altered through direct association with MUC1 or through indirect alterations to upstream signaling cascades is unknown. Therefore further study of the molecular mechanisms involved in mucin signaling is needed.
Table 1.1: Deregulation of Mucin Expression in Cancer

Table list mucins for which expression is altered in different cancers. References are included for each cancer presented.
Table 1.1

<table>
<thead>
<tr>
<th>Mucin</th>
<th>Cancer</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MUC1, MUC4, MUC5AC, MUC6, MUC16</td>
<td>Pancreatic Ductal Adenocarcinoma</td>
<td>Remmers, et al[53]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hinoda, et al[110]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Huang, et al[111]</td>
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<tr>
<td></td>
<td></td>
<td>Haridas, et al[51]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Higashi, et al[50]</td>
</tr>
<tr>
<td>MUC1, MUC2, MUC3, MUC4, MUC5AC, MUC5B, MUC6</td>
<td>Breast Cancer</td>
<td>Ghosh, et al[112]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rakha, et al[113]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mukhopadhyay, et al[114]</td>
</tr>
<tr>
<td>MUC1, MUC2, MUC4, MUC5AC, MUC5B, MUC6, MUC17</td>
<td>Colon Cancer</td>
<td>Terada, et al[115]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Krishn, et al[42]</td>
</tr>
<tr>
<td>MUC1, MUC2, MUC4, MUC5AC, MUC6</td>
<td>Lung Cancer</td>
<td>Awaya, et al[44]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kwon, et al[116]</td>
</tr>
<tr>
<td>MUC1, MUC4, MUC16</td>
<td>Ovarian Cancer</td>
<td>Yin, et al[117]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chauhan, et al[118]</td>
</tr>
<tr>
<td>MUC1, MUC2, MUC4</td>
<td>Prostate Cancer</td>
<td>Singh, et al[45]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Osunkoya, et al[119]</td>
</tr>
<tr>
<td>MUC1, MUC2, MUC3, MUC4, MUC5AC, MUC5B, MUC6, MUC13</td>
<td>Gastric Cancer</td>
<td>Ho, et al[120]</td>
</tr>
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</table>
Figure 1.3: Structural Organization of MUC1, MUC4, and MUC16

Schematic representing the domain organization of the mucins: MUC1, MUC4, and MUC16. Structures are not drawn to scale. These mucins contain cleavage sites (highlighted by dashed line) and exist at the cell surface as a dimer. In addition to the common mucin repeat domain, each mucin contains regions of unique sequences conferring specific functions. The sequence of the cytoplasmic tails is presented with confirmed sites of phosphorylation (*) indicated.
Figure 1.3

**MUC1**

VNTR (25-125 repeats of 20 aa)

CT CQCRK NYGQL DIFPARDTYHPMSE YPTYTH GRYVPP SSTDRS PYEKVSAGNGSSLSYTE NPAVAATSANL

**MUC4**

VNTR (145-395 repeats of 16 aa)

CT LRFWGCSGARFSYFLNASEALP

**MUC16**

Tandem Repeats (60+ repeats of 156 aa)

CT VTRRR KKEGEYNVQQCQPG YQSHLDLQ

Imperfect repeats

Unique Sequence

NIDO

AMOP

Cys-rich

VWD

SEA domains (56)

SEA domains (56)
**Figure 1.4: Mucin Type O-Glycosylation**

Schematic representing the addition of carbohydrates involved in mucin type O-glycosylation. Initiation involves addition of GalNAc to serine or threonine residues present in the mucin backbone to form the Tn epitope. These structures can be further extended into Core 1, 2, 3, or 4 structures depending on the identity of the carbohydrate. In cancer, the formation of truncated glycans (highlighted in red box) is common due to disruption of normal enzymatic function.
Figure 1.4

Cancer Associated Epitopes

Core 1 Extension

Core 2

Core 2 Extension

Core 3

Core 3 Extension

Core 4

Core 4 Extension

T (Core 1)

STn

S/T

GalNAc

Gal

NeuAc

GlcNAc
Figure 1.5: Loss of MUC1 Extends KPC Survival

MUC1 wildtype and KO KPC mice were generated by crossing the standard MUC1 KO mouse into the KPC mouse model of pancreatic cancer. Survival was tracked over time and a Kaplan-Meier plot showing survival of KPC mice that are either wildtype for MUC1 expression or have MUC1 knocked-out was generated based upon the survival data. Death from cancer was confirmed by necropsy. Statistics were performed using 2-way ANOVA.
Figure 1.5

KPC Survival

![Graph showing survival rates for KPC with different MUC1 conditions]

- MUC1 WT
- MUC1 Knockout

p<0.001
Figure 1.6: Processing of MUC1

Following translation, MUC1 undergoes an autoproteolytic cleavage at its SEA domain. These two subunits are associated through a strong, non-covalent interaction. MUC1 then undergoes further processing in the Golgi with the attachment of carbohydrates in an O- (Serine/Threonine) and N-linked (Asparagine) fashion. MUC1 is then inserted into the plasma membrane and displayed at the cell surface.
auto-proteolytic cleavage of SEA domain
O- and N-linked glycosylation of extracellular domain
Insertion into plasma membrane
Figure 1.7: Model of MUC1 Activation in Response to Loss of Polarity

Figured based upon hypotheses presented in Singh, et al[26]. Under normal physiological conditions MUC1 and receptor tyrosine kinases (RTKs) are spatially separated. However, with loss of normal junctional complexes through mechanical injury or loss of polarity due to malignant transformation, MUC1 and RTKs can associate to drive downstream signaling cascades that determine cellular fate.
Figure 1.7

MUC1

RTK

Injury

Transformation
Figure 1.8: Known Interaction Partners of the MUC1 Cytoplasmic Tail

A schematic of the MUC1 cytoplasmic tail and known regions involved in the association with signaling partners. Phosphorylation sites with known kinases are highlighted in red text. Additional interaction partners are also shown below. These additional partners do not have known sites of interaction.
Figure 1.8

MUC1 cytoplasmic tail

CQCRRKNYGOLDIFPARDTVHPMSEYPTVTHGRYVPPSSTDRSPYEKVSAGNGGSSLNYTPAVAATSANL

Other Interaction Partners
γ-catenin KLF4
p120-catenin IKK
Caspase-8 c-Jun
Figure 1.9: Model of MUC1 Signaling in Cancer

MUC1 has been shown to initiate pro-oncogenic effects through a number of distinct mechanisms. 1) MUC1 can interact with RTKs (or other kinases) resulting in phosphorylation of the cytoplasmic tail. 2) MUC1 can as a chemical sensor of the surrounding environment and sequester various factors from the extracellular environment. 3) MUC1 can interact with and organize the surrounding microenvironment. 4) MUC1 propagates signals through a number of downstream effector pathways. 5) MUC1 can regulate the compartment of signaling in association with other factors. 6) MUC1 can translocate to the nucleus in association with transcription factors to alter gene expression. 7) Translocation of MUC1 to the mitochondrial membrane to inhibit the apoptotic response
Figure 1.9

The diagram illustrates a biological process involving signal transduction pathways. The steps are labeled as follows:

1. Activation of Grb2 and PI3K.
2. Generation of H+ ions.
3. Efflux of H+ ions.
4. Downregulation of ERK through PI3K-mediated AKT activation.
5. TF binding and transcriptional regulation.
6. TF activation and DNA binding.
7. Protein-DNA binding event.

The diagram shows the interaction between extracellular signaling molecules and intracellular pathways leading to transcriptional regulation.
3. AP-1

3.1 Structure and Function

Activator Protein-1 (AP-1) is a dimeric transcription factor canonically consisting of either a homodimer of Jun proteins or a heterodimer of Jun and Fos proteins [121]. The initial discovery and characterization of this factor demonstrated significant affinity for a common cis-element in the promoters of genes regulated by addition of phorbol esters, such as 12-O-tetradecanoyl-phorbol-13-acetate (TPA) [122, 123]. These promoter elements were termed TPA-response elements (TREs) and are among the best-defined sites of AP-1 binding [123]. The consensus sequence for the TRE is TGAC/GTCA. Interestingly, the individual components of AP-1 were discovered as viral oncogenes prior to the identification of the cellular homologs. V-Jun was discovered in the viral genome of avian sarcoma virus, whereas v-Fos was discovered within the genome of FBJ-murine osteosarcoma virus [124, 125]. Subsequent studies discovered the cellular homologs, c-Jun and c-Fos, which constitute mammalian AP-1.

Within humans there are 3 distinct JUN family members (c-Jun, JunB, and JunD) and 4 FOS family members (c-Fos, FosB, FRA-1, and FRA-2) [121, 126]. These proteins are part of a larger class of proteins called Bzip proteins that contain a basic region that facilitates the binding of DNA, and a leucine zipper for dimerization. A high degree of similarity exists across family members, particularly within the Bzip domain suggesting potential functional redundancy to a degree (Figure 1.10). Typically, functional dimerization of AP-1 proteins is dependent on phosphorylation of residues that stabilize the proteins. Phosphorylation of the delta domain in c-Jun is critical for regulation of its stability and is absent in the viral homolog v-Jun [127]. Phosphorylation of serines 63 and 73 within this domain, results in stabilization of the protein, and functional activity [128, 129]. However, it also creates binding sites for regulatory proteins that turn off the transcriptional signals by degrading c-Jun [130]. Similarly, the
stability of c-Fos and FRA-1 is regulated by the presence of a C-terminal degradation sequence [131, 132]. Like v-Jun, v-Fos lacks this domain and as a result is significantly more stable than its normal cellular counterpart [133]. Similarly, truncated ΔFosB that lacks the C-terminal degradation sequence also exhibits prolonged stability demonstrating that these sequences are critical for the regulation of Fos family proteins [134]. Phosphorylation of residues near the basic region has also been shown to regulate DNA binding of AP-1 proteins, presumably by altering the net positive charge required to associate with DNA [135].

3.2 Roles of Jun and Fos in Normal Development

The role of Jun and Fos proteins in normal developmental processes has been well studied through the generation of specific knockout mice (Table 1.2). Both knockouts of c-Jun and JunB are embryonic lethal at E12.5 and E10 respectively [136, 137]. Knockout of c-Jun results in significant abnormalities in the heart and liver [136, 138]. Defects in hepatocyte proliferation are maintained throughout adulthood, as perinatal liver specific deletion of c-Jun results in defects in liver regeneration following partial hepatectomy [139]. JunB knock-in can rescue the embryonic lethal phenotype of c-Jun knockout, however, mice still only survive a few days beyond birth [140]. Interestingly, these mice show normal induction of Jun/Fos target genes, however, are unable to induce genes regulated by Jun/ATF dimers, suggesting only partial redundancy between these proteins. Indeed, in other studies, c-Jun and JunB were shown to exert antagonistic effects [141, 142]. Overexpression of either c-Jun or JunB in transgenic mice shows no overt phenotype [143, 144]. Unlike c-Jun and JunB, JunD knockouts are viable; however, males exhibited impaired growth, hormone imbalances, and defects in reproduction due to faulty spermatogenesis [145].
FRA-1 is the only Fos protein whose knockout results in embryonic lethality. This occurs at approximately E9.5 [146]. The embryonic lethality of FRA-1 knockout is due to defects in placental development [147, 148]. Interestingly, these defects can be completely rescued by providing wild-type extra-embryonic tissues during development. These rescued mice display no overt phenotype suggesting that beyond placental development FRA-1 expression may be dispensable for normal developmental processes [146]. Interestingly, expression of JunB in place of FRA-1 can also partially rescue development, suggesting that JunB and FRA-1 may have similar functions in early development [146]. The similar phenotypes between the knockouts of these mice further support this. Overexpression of FRA-1 results in accelerated osteoblast differentiation and increased bone formation in mice [149].

Knockout of c-Fos results in viable mice, however, these mice lack osteoclasts [150, 151]. These mice also exhibit secondary alterations to the hematopoietic system, however; further studies have shown that c-Fos is dispensable for function of peripheral T-cells [152, 153]. Knock-in of FRA-1 in place of c-Fos is able to restore normal bone development demonstrating substantial redundancy between these proteins in normal development [154]. By comparison, overexpression of c-Fos further exacerbates the impact on bone development and promotes formation of osteosarcomas [155].

FosB null mice also develop normally, however, some studies have demonstrated that loss of FosB expression results in nurturing defects [156, 157]. Overexpression of FosB results in no discernable phenotype [144]. Unlike the other Fos proteins, FosB also exists as the variant ΔFosB. Overexpression of this splice variant results in disruption of normal cellular differentiation particularly within osteoblasts and T-cells [158, 159]. FRA-2 knockout results in disruptions to normal cartilaginous development and these mice die shortly after birth [160]. Overexpression of FRA-2 results in ocular abnormalities [161].
These knockout studies demonstrate the diverse range of function associated with AP-1. Despite the prediction that these proteins must functionally dimerize to exert functional effects, only JunB and FRA-1 knockouts exhibit similar phenotypes. Knock-in studies have demonstrated that these proteins do retain functional equivalency under some circumstances, however, in many instances these knock-ins only rescue part of the knockout phenotype. This suggests that these proteins also play unique roles within development. For example, in fibroblasts c-Jun is a positive regulator of cellular proliferation, whereas both JunB and JunD are negative regulators [138, 162-165]. These effects also appear to be context dependent as expression of c-Jun is critical for apoptosis in neuronal cells [166, 167].

3.3 AP-1 in Tumorigenesis

The formation of cancer involves deregulation of numerous cellular pathways [168]. These include disruptions to normal cellular proliferation, evasion of the immune system, resistance to cell death, the induction of angiogenesis, and the activation of pathways involved in invasion and metastasis. Interestingly, AP-1 has been shown to play a role in virtually every pathway associated with tumorigenesis [162, 164, 169-172](Figure 1.1).

The oncogenic potential of both c-Jun and c-Fos is well known as the cellular homologs of the viral oncogenes v-Jun and v-Fos [125, 173]. The oncogenic potential of c-Fos is dependent on the ability of c-Fos to functionally dimerize, as well as the maintenance of several structural motifs within the N and C-terminus [174-177]. Transformation of mammalian cells by c-Jun requires co-expression of oncogenic drivers, such as Ras, as well as the transactivation domain and the phospho-acceptor residues serine 63 and 73 [128, 178, 179]. Expression of c-Jun also results in decreased
expression of both p53 and p21 suggesting that c-Jun may interfere with normal tumor suppressor function [162]. Similar to c-Fos, FosB has also shown transformative potential in fibroblasts [180]. This potential also requires the presence of conserved motifs, particularly within the C-terminus [180-182].

The transformative potential of the other fos proteins (FRA-1 and FRA-2) are both weak by comparison. Overexpression of FRA-1 in fibroblasts does not result in significant morphological changes, but does promote anchorage-independent growth and tumor formation in nude mice [183, 184]. Despite the relatively weak transformation potential, expression of FRA-1 has also been shown to correlate with poor prognosis in breast cancer and drive an invasive phenotype [185-188]. Expression of FRA-1 in colorectal cancer also results in expression of genes involved in epithelial-to-mesenchymal transition (EMT) [170, 189]. FRA-2 is capable of transformation in chicken fibroblasts, but not rat fibroblasts [190, 191]. Interestingly, both JunB and JunD lack any known transforming activity [143, 192]. Furthermore, several studies have indicated that these proteins may actually act as tumor suppressors [163, 165].

3.4 Dimer Specific Functions of AP-1

While studies of human tumors have shown that expression of Jun and Fos proteins is altered in a wide range of cancers, the role that AP-1 plays in many of these tumors remains poorly understood; particularly with regard to the specific dimers working within these cells [170, 186, 193-195]. Due to the diverse phenotypes associated with different Jun and Fos proteins, it is likely that the composition of AP-1 is a critical determinant of the function. Based on the dimerization properties of Jun and Fos proteins, there are 18 potential dimer combinations; ignoring non-canonical dimers such as Jun:ATF and FRA1:Smad dimers (Figure 1.12). Several studies have demonstrated
that dimer composition can influence the DNA binding properties of AP-1 [196-199]. Studies using tether dimers of known composition have demonstrated that unique dimers regulate specific cellular effects, such as c-Jun:FRA-2 dimers inhibiting G0 arrest in 3T3 cells [198]. Jun and ATF dimers have been shown to specifically regulated growth factor independence [196]. FRA-1:Smad dimers have also shown to regulate invasive properties in response to TGFβ signaling [200]. As such, understanding the function of specific dimers and the hand-off from dimer to dimer remains of vital importance. It is feasible that the induction of unique dimers in response to different stimuli is critical in mediating the appropriate response to the microenvironmental changes encountered by the cell. Depending on the relative proportion of dimers, cells may proliferate, die, or migrate (Figure 1.13).
Table 1.2: Phenotypes of AP-1 Genetically Modified Mice

Table showing the associated phenotypes of published knockout and knock-in studies involving Jun and Fos proteins.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Phenotype</th>
<th>Tissues Affected</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-Jun</td>
<td>Embryonic Lethal (E12.5)[136]</td>
<td>Defects in heart and liver development</td>
</tr>
<tr>
<td>JunB</td>
<td>Embryonic Lethal (E10)[137]</td>
<td>Placental Tissue</td>
</tr>
<tr>
<td>JunD</td>
<td>Male Sterility [145]</td>
<td>Testis</td>
</tr>
<tr>
<td>c-Fos</td>
<td>Lack osteoclasts[150, 151]</td>
<td>Bone</td>
</tr>
<tr>
<td>FosB</td>
<td>Nurturing defects[156]</td>
<td>Brain</td>
</tr>
<tr>
<td>FRA-1</td>
<td>Embryonic Lethal (E9.5)[146]</td>
<td>Placental Tissue</td>
</tr>
<tr>
<td>FRA-2</td>
<td>Death at Birth[160]</td>
<td>Cartilage and Bone</td>
</tr>
</tbody>
</table>

**Phenotype of Knock-In Mice**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Phenotype</th>
<th>Tissues Affected</th>
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</thead>
<tbody>
<tr>
<td>JunB for c-Jun</td>
<td>Rescues embryonic lethality and some transcriptional targets. Mice die shortly after birth[140]</td>
<td>Heart</td>
</tr>
<tr>
<td>FRA-1 for c-Fos</td>
<td>Rescues phenotype[154]</td>
<td>None</td>
</tr>
<tr>
<td>JunB for FRA-1</td>
<td>Partially rescues phenotype[146]</td>
<td>None</td>
</tr>
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</table>

**Phenotype of Overexpression Mice**

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<tr>
<td>H2kb-cJun</td>
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</tr>
<tr>
<td>UbC-JunB</td>
<td>None[143]</td>
<td>None</td>
</tr>
<tr>
<td>H2kb-cFos</td>
<td>Osteosarcoma[144]</td>
<td>Bone</td>
</tr>
<tr>
<td>H2kb-FRA1</td>
<td>Increased Bone formation[149]</td>
<td>Bone</td>
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<tr>
<td>H2kb-FosB</td>
<td>None[144]</td>
<td>None</td>
</tr>
<tr>
<td>NSE-ΔFosB</td>
<td>Defects in Osteoclast and T-cell Differentiation[158, 159]</td>
<td>Bone and Thymus</td>
</tr>
<tr>
<td>CMV-FRA2</td>
<td>Ocular defects[161]</td>
<td>Eye</td>
</tr>
</tbody>
</table>
Figure 1.10: Structure of the JUN and FOS protein Family

Schematic diagrams representing conserved domains present within either the Jun or Fos family of proteins. The percentage of similarity and identity is presented for each of these conserved regions. Other critical regions are highlighted by name (Bzip domain, degrons, etc.). Known sites of phosphorylation are highlighted by lollipops (green are activating, red are inactivating, purple are localization, and white are unknown in function).
Figure 1.10

**JUN**

- **c-JUN**
  - 67.2% identical
  - 90.6% similar

- **JUNB**
  - 31.1% identical
  - 50.7% similar

- **JUND**
  - 16.7% identical
  - 83.3% similar
  - 42.4% identical
  - 64.3% similar
  - 44.4% identical
  - 88.9% similar
  - 48.1% identical
  - 77.7% similar
  - 76.6% identical
  - 95.3% similar

**FOS**

- **c-FOS**
  - 36% identical
  - 72.7% similar

- **FOSB**
  - 50% identical
  - 70% similar

- **FRA-1**
  - 29.6% identical
  - 77.7% similar
  - 67.2% identical
  - 90.6% similar

- **FRA-2**
  - 36% identical
  - 72.7% similar
  - 50% identical
  - 70% similar
Figure 1.11: Role of AP-1 in Tumorigenesis

The hallmarks of cancer are commonly used to highlight the deregulation of normal cellular processes in cancer. Interestingly, AP-1 has been shown to impact nearly every proposed hallmark of cancer through the regulation of specific genes. Other hallmarks have also been shown to induce AP-1 activity. Known AP-1 target genes and their role in these hallmarks are presented in adapted figure from Hanahan and Weinberg[168]. Effects in green indicate increased expression or activation of pathways whereas red indicates decreased expression or activity.
Figure 1.12: Combinatorial Possibilities of AP-1 Dimers

Based on the dimerization properties of Jun and Fos proteins there are 18 potential combinations for AP-1. 6 of these are “Jun:Jun” homodimers, whereas 12 are Jun:Fos heterodimers. The total number of AP-1 dimer combinations can further expand based on the inclusion of non-canonical dimers including ATF, MAF, CREB, and Smad proteins.
Figure 1.12

18 Potential Dimer Combinations
(Excluding “Non-Canonical” AP-1 combinations)
Figure 1.13: Model of AP-1 Response to Differing Stimuli

Hypothetical models of Jun and Fos protein expression in response to differing stimuli.

Studies have shown that depending on the stimulus the induction of Jun and Fos proteins differs. This suggests that the specific induction pattern of different Jun and Fos proteins may result in a different outcome in response to various stimuli. These induction patterns may also regulate the hand-off from one dimer combination to another, as the relative contribution of each dimer to the total of AP-1 shifts to mediate a lasting transcriptional response.
Figure 1.13
4. p53

4.1 Structure and Function

The TP53 gene located on chromosome 17 encodes the tumor suppressor p53. This gene locus contains 11 exons, the first of which is non-coding [201]. The canonical p53 (also called FLp53, p53α, or TAp53α) consists of 393 amino acids and contains 6 functional domains. These include 2 N-terminal transactivation domains (TAD1 and TAD2), a proline rich domain, DNA binding domain, tetramerization domain, and a C-terminal regulatory domain [201]. Depending on the use of internal start sites and alternative splice sites, the TP53 gene can encode at least 12 different variants of p53 (Figure 1.14) [202, 203]. These variants can dramatically alter function through changes in the normal domain structure of p53. For example, Δ40p53 utilizes an internal start site within the full-length p53 sequence and this isoform lacks the first transactivation domain, as well as the Mdm2 binding site that normally regulates stability. As a result, Δ40p53 exhibits different transcriptional activity and stability as compared to p53α [204-207].

Under normal cellular conditions, p53 is a critical regulator of progression through the cell cycle and is activated in response to deviations from normal homeostasis [208]. In the absence of stressors, p53 is maintained at very low levels, primarily through the regulatory effects of Mdm2 [209]. Mdm2 is an E3 ubiquitin ligase that regulates the turnover of p53 protein by promoting the ubiquitination and degradation of the protein [210]. This is accomplished through the binding of Mdm2 to p53 within TAD1. Induction of cell stress, such as through DNA damage, results in activation of stress related kinases ATR and ATM and promotes phosphorylation of p53 [211, 212]. Downstream activation of Chk1 and Chk2 can further phosphorylate p53 [213]. These stress kinases also phosphorylate Mdm2 interfering with degradation of p53 [214] (Figure 1.15). Once p53 is stabilized it will oligomerize to form tetramers and bind to the promoters of specific
targets, such as p21 or Puma, to promote either growth arrest and DNA repair or induce apoptosis [208]. Increased p53 activity also results in upregulation of Mdm2 to form a regulatory feedback loop to control p53 expression. As a result, the p53 response exhibits pulsatile dynamics with cyclical peaks and valleys of p53 expression allowing for multiple checks for lesions before re-entry into the cell cycle [215].

### 4.2 Loss of p53 function in Cancer

Loss of p53 function plays a major role in the development of most cancers and can occur through a number of mechanisms. Destabilization of the p53 protein, such as through E6 and E7 viral oncoproteins has been shown to play a major role in the development of cervical cancer following infection with the human papilloma virus (HPV) [216, 217]. Expression of these proteins is also commonly used in the process of immortalizing normal human cells for *in vitro* study [218, 219]. Expression of other viral proteins can also inactivate p53 including SV40 large T-antigen and adenovirus E1B protein [220]. Likewise, overexpression of Mdm2 has been observed in several cancers as a mechanism of decreasing p53 expression [221, 222]. Homologs of Mdm2, like MdmX, can also act as negative regulators of p53 and are also upregulated in neoplasms [223]. Methylation of the p53 promoter element can also drive loss of p53 expression [224-226]. Finally, loss of p53 function can occur through deletion, nonsense mutations, and missense mutations [227]. The vast majority of these mutations occur within the DNA binding domain of p53 and have long been thought to result in loss of DNA binding capacity (Figure 1.16). In particular there are approximately 6 hotspots that are consistently mutated including R175, G245, R248, R249, R273, and R282, all of which are present in the DNA binding domain.
Initially, it was postulated that tumor development required “two-hits” of tumor suppressors for complete loss of function and tumor progression [228, 229]. While this model holds true in some cases, as research has progressed exceptions to this rule have been found. These genes exhibit a haploinsufficient phenotype, suggesting that one functional allele fails to generate enough product to reach a normal threshold. Several studies have demonstrated that p53 exhibits a haploinsufficient phenotype [230, 231]. As a result, p53 germline mutations result in significantly increased risks for the development of cancer, both through haploinsufficiency and the increased likelihood of a secondary hit of the remaining functional allele [232].

4.3 p53 as a gain-of-function oncogene

Missense mutations of p53 were long considered loss-of-function mutations that promoted tumor progression by removing normal cellular checkpoints regulated by p53 through abrogation of p53’s DNA binding capacity. However, unlike most tumor suppressors, p53 mutations are primarily single amino acid substitutions as opposed to nonsense mutations that yield either a truncated protein or no product at all. Explorations into the activity of p53 mutants have suggested that mutant p53 may function as an oncogene in a gain-of-function manner (Figure 1.17) [233-238].

Early studies in mice found that the presence of a mutant p53 allele was not equivalent to a p53 null allele [239]. Similarly, in the KPC mouse model of pancreatic cancer, expression of mutant p53 results in a widely metastatic disease whereas p53 knockout mice exhibit a more localized disease [15]. This phenotype is also observed in other mouse models of cancer [240, 241] While missense mutations alter the structure and residues involved in DNA binding, several studies have demonstrated that mutant p53 retains some DNA binding to strong p53 promoters like the p21 promoter[242].
Interestingly, these mutants often lose the capacity to bind the promoter of the pro-apoptotic protein Bax, suggesting that mutant p53 preferentially promotes survival signaling while impairing apoptosis. These mutations also result in gain-of-function binding to new DNA elements in a structure dependent manner[237, 243].

Beyond alterations to the DNA binding properties, mutant p53 also exhibits enhanced stability and increased expression is often observed within tumors. This appears to be due in part to impaired regulatory feedback between Mdm2 and p53 [244]. Mutation of p53 also results in novel interactions with new partners to drive pro-tumorigenic effects [245-247]. A subset of p53 mutants gain the ability to associate with related family members p63 and p73 to inhibit growth suppression [248]. A number of other mutants have also shown the capacity to interact with the transcription factor SP-1 to drive expression of SP-1 genes [249, 250]. Other studies have implicated mutant p53 in alterations to DNA synthesis and proliferation, chemoresistance, acquisition of stem cell characteristics, and induction of angiogenesis [251-254]. As a result, reactivating wildtype p53 function through the refolding of mutant p53 is a widely explored therapeutic option [255-258].
**Figure 1.14: Structure of Full-Length p53 and its Variants**

A schematic diagram presenting the domain structure of full-length p53 and its variants from internal start sites or alternative splicing. Δ variants arise through the use of internal ATG start sites within the amino acid sequence, whereas β and γ variants arise from alternative splicing of C-terminal exons. As a result, the TP53 gene can give rise to 12 unique forms of p53.
Figure 1.14

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<th>Protein</th>
<th>TAD1</th>
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<th>Transactivation Domain</th>
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DQTSFKENC
MLLDLRWCYFLINSS
Figure 1.15: p53 Response to DNA Damage
In response to DNA damage, ATM is activated and phosphorylates Chk1. Both Chk1 and ATM can phosphorylate p53 resulting in stabilization of the protein. ATM also phosphorylates Mdm2 to inhibit its activity and further stabilize p53. P53 then can induce transcription of either survival/growth arrest genes like p21 or induce cell death pathways through induction of pro-apoptotic proteins. Increased p53 activity also drives increased expression of Mdm2 in an autoregulatory manner to turn off p53 signaling.
Figure 1.15

DNA damage

ATM

Chk1

Mdm2

p53

p21

PUMA

Survival/Growth arrest

Cell Death
Figure 1.16: Mapping of p53 Mutations

Schematic representation of known mutations in p53 in pancreatic ductal adenocarcinoma. Length of each lollipop indicates the relative proportion of mutation. Hotspot mutations typically occur within the DNA binding domain of p53, whereas mutations in other domains are uncommon. Data were collected from cBioportal and from the TCGA.
Figure 1.16

Pancreas TP53 TCGA mutations

DNA Binding Domain

- Missense mutation
- Truncating mutation
- Inframe Indel
- Multiple mutation classes

0 100 200 300 393 aa
Mutant p53 has been proposed to gain oncogenic potential through a number of different mechanisms. A) Binding to p63 and p73 and inhibiting transcription of downstream targets. B) Association with novel transcription factor partners to alter expression of their targets. C) Binding of novel DNA elements in a structure specific fashion. D) Association with novel non-transcriptional partners.
Figure 1.17

- Inhibition of p63/73 gene targets
- Gain of novel interaction partners
- Co-regulation of other transcription factor targets
- Structure specific DNA binding
5. Statement of Research

Based on the known role of MUC1 as an integrator of cellular signals and known associations with both AP-1 and p53, we sought to explore how MUC1 alters the formation and regulation of these transcriptional complexes. We focused upon how the MUC1 cytoplasmic tail is capable of integrating multiple signals to initiate appropriate downstream signaling. Furthermore, we investigated how expression of MUC1 impacts AP-1 with special focus on roles in migration, invasion, and metastasis in pancreatic cancer. Lastly, we examined how the mutational status of p53 can influences its association with the cytoplasmic tail of MUC1 and the potential influence of gain-of-function effects.
Chapter II: Materials and Methods
Antibodies

The anti-MUC1 antibody CT2 was generously provided by Dr. Sandra J Gendler or ordered from Abcam (ab80952). Phospho-specific antibodies against the MUC1 cytoplasmic tail were previously generated within the lab. Antibodies against c-Jun and phosphoSerine73 c-Jun were obtained from Abcam (ab31419, ab32447). Antibodies against phosphoFRA-1, phospho-c-Fos, ERK, and phosphoERK were purchased from Cell Signaling (#5841, #5348, #9107, and #4377 respectively). Antibodies measuring the DNA damage response (pChk1, pChk2, phospho-p53, PUMA, and p21) were also obtained from Cell Signaling (#9947S, #12450S, and #2947S respectively). Antibodies against Cyclin D1 and Cyclin A were purchased from Cell Signaling (#2926 and #4656 respectively). FRA-1, c-Fos, and H2B were obtained from Santa Cruz (sc-28310, sc-8047, sc-8650) and ATF2 from Novus Biologicals (H00001386-M02). β-actin, p53 (DO-1), and Flag (M2) antibodies were obtained from Sigma Aldrich.

BET Inhibitor Treatments

BET inhibitors JQ-1 and OTX015 were purchased from SelleckChem (Cat.No S7110 and S7360). Inhibitors were suspended in DMSO and stored as aliquots until use. Cells received either inhibitor treatment or DMSO control at the indicated doses. Cells were only treated once per experiment.

Cell Culture

Panc1, Colo357, and AsPC-1 cells were obtained from American Type Culture Collection and S2013 cells were obtained from the originator of the line [69]. S2013.Neo and MIF were generated as previously described [102]. Panc1.MUC1 and Neo were generated from stable transfection of pSIN-ires-neo using lentiviral transduction. Panc1.MUC1-FRA1, Panc1.Neo-FRA1, S2013.Neo-FRA1, and
S2013.MIF-FRA1 lines were generated by stable transfection of pLVX.puro using lentiviral transduction. FRA-1 knockdown was performed using the previously characterized shRNA TRCN0000019539 [259] or scrambled control purchased from OpenBiosystems. Cells were selected in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 4 µg/ml Puromycin, 10% fetal bovine serum (FBS), and 1X HyClone penicillin/streptomycin mix (100 U/ml penicillin and 100 µg/ml).

Colo357, Panc1, and HPDE inducible p53 cell lines were generated by sequential lentiviral transduction of pLVX.Tet-ON, and pLVX.Tight-puro generously provided by Dr. Angie Rizzino, followed by sequential selection in DMEM supplemented with 1000 µg/ml G418 or 4 µg/ml Puromycin. Once selection had occurred cells were maintained in 10% DMEM supplemented with HyClone pen/strep mix. Cells were maintained at 37°C in a humidified environment with 5% CO₂.

**Cell Cycle Analysis**

Cell cycle analysis was performed using the propidium iodide flow cytometry kit purchased from Abcam (ab139418). Briefly, supernatant was removed and adherent cells were trypsinized and removed from plate by rinsing with original supernatant. Total cells were counted and 1 million cells were pelleted and fixed with 70% EtOH. After all time points were collected, cells were prepared for flow cytometry. Fixed cells were pelleted and washed 2X in 1X PBS. Cells were then resuspended in 400 µl 1X Propidium Iodide + RNase Staining Solution. Cells were incubated in the dark for 30 minutes at 37 °C before being taking to the UNMC flow cytometry core for analysis. Average percentages of cells in each phase were then plotted for each treatment group.

**Chromatin Shearing and Chromatin Immunoprecipitation (ChIP)**
A 15 cm dish of cells was fixed in 1% formaldehyde at room temperature for 10 minutes according to the MagnaChIP protocol (Millipore). After fixation cells were quenched with 1X glycine from kit. Cells were washed and collected in 1X PBS containing protease inhibitors. Cells were then pelleted and supernatant removed. 500 µl of cell lysis buffer was added for each 15 cm dish. Cells were incubated on ice for 15 minutes before being pelleted again. 500 µl of nuclear lysis buffer was added and lysate was aliquoted into Covaris AFA sonication tubes. DNA shearing was done for 6 minutes on the Covaris S2 sonicator with 2% duty cycle, 3 intensity, and 200 cycles per burst. Shearing was assessed using agarose gel and chromatin stored at -80°C until use.

Chromatin immunoprecipitation was performed according to MagnaChIP (Millipore) protocol using 5 µg FRA-1 (sc-605) antibody or IgG control per 50 µl chromatin. Eluted DNA was analyzed by qRT-PCR and compared to both input and IgG controls.

Co-immunoprecipitation

Cells were grown to 80-90% confluence and lysed in co-immunoprecipitation buffer (50 mM Tris-Cl, 150 mM NaCl, 0.5% NP40, pH 8.0) in the presence of protease and phosphatase inhibitors (ThermoFisher 78440). Buffer was adjusted to 300 mM NaCl to promote extraction of nuclear proteins. Lysates were incubated on ice for 30 minutes and insoluble debris removed by centrifugation at 16,000g. 300 µl of lysate was incubated with Protein G beads (ThermoFisher 1003D) and antibodies against the protein of interest or an IgG control at 4°C for 2 hours to form complexes. For p53-Flag pulldown, M2 agarose beads were used instead of Protein G. Beads were washed 3X with Co-IP wash buffer (50 mM Tris-Cl, 150 mM NaCl, 0.1% NP40) and proteins eluted by boiling in SDS sample buffer. Each co-immunoprecipitation study was performed multiple times to ensure reproducibility.
Construct Generation

FRA-1 constructs were designed by PCR amplification of FRA-1 cDNA purchased from OpenBiosystems. Primers used were designed for placement of a HA-epitope tag on the C-terminus of FRA-1. Amplified fragments were then restriction digested and ligated into pLVX.puro vector for lentiviral transduction. DNA sequencing of the plasmid confirmed appropriate sequence.

Inducible p53 constructs were designed by PCR amplification of cDNA from OpenBiosystems and a C-terminal 3X-Flag tag added through PCR. Amplified products were cloned into the pLVX.Tight-puro construct from Clontech. Specific p53 mutants were generated using the site-directed mutagenesis kit from Agilent and all mutants were confirmed by DNA sequencing.

Homologous arm constructs for CRISPR mediated fusion protein knock-ins were designed using primers specific for regions upstream and downstream of the predicted CRISPR cut site. Amplicons were approximately 1.5 kb. PCR amplification of these regions was performed following isolation of genomic DNA from cell lines. Amplicons were then cloned into original AAV vector provided by Dr. John Albeck.

DNA Damage Studies

Cells were exposed to either UV or X-ray irradiation. X-ray irradiation was performed using RS-2000 irradiator in the Biological Irradiation Core and cells were treated with the indicated doses. UV damage was induced by exposure to DNA gel box for 5 minutes. Following DNA damage cells were returned to the incubator for 1 hour before lysis and examination of DNA damage response by western blot analysis.

Doxycycline Treatment of Inducible Cell Lines
Fresh doxycycline was prepared in autoclaved water and stored as aliquots until use. Cells were primarily treated with 1 µg/ml doxycycline unless otherwise indicated. Cells were treated for 24 hours prior to analysis of p53 expression and downstream effects except for long-term studies. For studies longer than 24 hours, media was replaced with fresh doxycycline containing media every 24 hours to retain expression of p53 as doxycycline degraded.

**Immunoblotting**

Proteins were transferred from gels to Immobilon-FL PVDF membrane using the Bio-Rad transfer system at 100V, 0.3 A, for 70 minutes. Membranes were rinsed in 1X PBS then blocked for 1 hour in a 1:1 mixture of 1X PBS and Licor Blocking buffer. Primary antibodies were incubated for 1 hour in 0.1% PBST:Licor buffer. All primary antibodies were used at a concentration of 1 µg/ml. Membranes were washed three times with 0.1% PBST. Secondary antibodies were conjugated to IrDyes (Licor) and incubated for 1 hour in the dark in 0.1% PBST/Licor buffer with 0.01% SDS to reduce background. Blots were washed three more times, rinsed with 1X PBS, and visualized using the Odyssey Imaging System.

**Immunohistochemistry**

Slides containing primary pancreatic tumor, liver metastases, or uninvolved pancreas were obtained from the UNMC Rapid Autopsy Program. Staining was performed using the Dako Envision+ kit (K4006) with a hematoxylin counter-stain. Anti-FRA-1 (sc-28310), Anti-ZEB1 (ab180905), Anti-slug (ab27568) and IgG control were used at a concentration of 10 µg/ml. Following staining, slides were mounted and imaged.
**Invasion and migration assays**

Assays were carried out using matrigel invasion plates or control migration plates from BD Biosciences (#354480 and #354578 respectively). Cells were grown to approximately 60% confluence and serum starved 24 hours prior to assay. Treated cells received a 2-hour pretreatment of 10µM U0126 (Sigma). Prior to plating, matrigel matrix in invasion plates was rehydrated for 2-4 hours with serum free media at 37°C, migration plates required no pre-treatment. 100,000 cells per well were plated for invasion assays and 25,000 cells per well for migration assays. Chemoattractant in the lower well was 10% FBS containing DMEM. Cells were incubated at 37°C for 24 hours, then inserts were washed and stained using the DiffQuick staining kit. Membranes were dried and mounted on slides for quantification. Each experimental group was examined in two independent experiments with each group plated in triplicate. Statistical analysis was performed using 2-tailed student’s t-test.

**KPC MUC1 knockout mice and cell lines**

KPC mice were bred at UNMC to carry the PDX-1-Cre transgene [260], the LSL-KRAS<sup>G12D</sup> knock-in mutation [261] and the LSL-Trp53<sup>R172H</sup> knock-in mutation [262]. In the context of the KPC background, mice were bred to the Muc1 knockout mouse [263] to generate KPC mice that express Mucin-1 (KPC WT) or are deficient in Mucin-1 expression (KPC KO). Cell lines were derived from primary tumors of each genotype and utilized for further analysis. Cell lines were collected by collagenase digestion of primary tumors and fibroblasts removed through selective trypsinization of the cells over multiple passages.

**Lentiviral Generation**
Lentivirus was generated through transfection of 293A cells with 3:1:4 ratio of packing vectors psPAX2 and pMD2.G along with the appropriate lentiviral vector containing our sequence of interest. Transfection was performed using the X-fect protocol from Clontech. Lentivirus was collected at 24, 48, and 72 hours post-transfection. Virus containing media was passed through a 0.45 µm SFCA filter and polybrene (6 µg/ml) added to the filtered media. Viral media was then applied to transduced cells.

**Methylene Blue Proliferation Assay[264]**

2,000 cells were plated in 6 wells of a 96-well plate for each cell line. One plate was used for each time point measured (0, 24, 48, 72, and 96 hours [also 120 and 144 hours in Colo357 studies]). After each time point cells were fixed with formalin and kept at 4° until all time points were taken. To generate the 0 hour time point the cells were allowed to adhere for two hours and then immediately fixed. Following fixation cells were stained for 30 minutes with 1% methylene blue in 0.01M borate buffer (pH8.5). Plates were rinsed 4 times in 0.01M borate buffer and the dye eluted with 100 µl of 1:1 ethanol and 0.1M HCl. The absorbance at 650 nm was measured with a plate reader and results normalized to background. 2-way ANOVA was used to assess differences in proliferation between multiple cell types and treatment groups.

**Orthotopic Mouse Studies**

All mouse studies were performed according to UNMC IACUC specifications. 150,000 tumor cells were injected orthotopically into the pancreas of nude mice. S2013.Neo and MIF cell lines and their FRA-1 knockdown counterparts were utilized for the study. Groups consisted of 12-13 mice for a total of 50 mice overall, all mice were female. Tumors were allowed to develop for 30 days, at which time mice were sacrificed.
and tumors measured. Presence of ascites and metastasis was initially assessed based on gross observation during necropsy. Tissues for each metastatic site and primary tumor were formalin fixed. The UNMC tissue sciences core facility cut and stained H&E slides for each sample in the experiment. Metastases were confirmed by microscopy before final scoring. Statistical analysis of differences in tumor growth was assessed using ANOVA and Bonferroni method for multiple comparisons.

**Phosphatase Assay:**

MUC1 wild type mouse stomach was lysed into T-Cell Protein Tyrosine Phosphatase buffer (50 mM Tris-HCl, 100 mM NaCl, 2 mM Na₂EDTA, 5 mM DTT, 0.01% Brij35, pH 7.5) supplemented with protease inhibitor cocktail tablet (Roche) using a 27 gauge needle for full lysis. Lysates were divided evenly into 2 microcentrifuge tubes with 1 receiving 20 µl (200 U) T-Cell Protein Tyrosine Phosphatase (New England Biolabs) and the other receiving a phosphatase inhibitor to reduce active phosphatase activity in the whole lysate. Samples were incubated at 30°C in a water bath for 3 hours to yield maximal dephosphorylation. Samples were then buffer exchanged into 2D-Xtract buffer using Zeba desalting columns and analyzed with 2D gel electrophoresis.

**Proximity Ligation Assay (PLA)**

All PLA reagents used were from Duolink PLA kit. Cells were grown in 12-well plates on poly-lysine coated slides. Cells were fixed with 4% PFA supplemented with 120 mM sucrose. The reaction was quenched by removal of PFA and addition of 0.1 M glycine for 5 minutes followed washes with 1X PBS. Cells were permeabilized using 500 µl of 0.15% Triton-X-100 with 1% BSA in 1X PBS for 15 minutes then blocked with 1 drop of blocking solution for 30 minutes at 37°C. Blocking solution was removed and coverslips incubated with the primary antibodies in antibody diluent (1:200) overnight at
4°C. After incubation, coverslips were washed three times with PLA wash buffer A for 5 minutes. PLA secondary probes were added and incubated at 37°C for 1 hour in the dark. Coverslips were washed twice more with PLA wash buffer A and ligation reaction mix was added to the coverslips for 30 minutes at 37°C. After two more washes in buffer A the amplification-polymerase solution was added for 100 minutes at 37°C. Coverslips were washed twice in 1X PLA wash buffer B then once in 0.1X buffer B. Coverslips were mounted with Fluoromount G with DAPI. Results were visualized with confocal microscopy and the interactions were quantified using Blobfinder. All experimental groups were performed in quadruplicate and multiple independent fields per slide were used for quantification. Statistical analysis was performed by 2-tailed student’s t-test.

**Purification of Recombinant MUC1**

Recombinant expression of MUC1 was induced in C41 or C43 E.coli through transformation with the pGEX4T-1 plasmid. The full-length cDNA sequence of the MUC1 C-terminus was optimized for E.coli expression before being cloned into this vector to generate a C-terminally tagged GST-MUC1 construct. GST-purification kit was ordered from Clontech (Cat# 635619) and purification of recombinant protein was performed according to the provided protocol. Pulldown of recombinant MUC1 was confirmed by western blot analysis of elution fractions.

**R programming**

All applications involving R programming language were performed using RStudio. Heatmaps were generated in R for all Immunohistochemistry experiments. Hierarchical clustering was also performed using R programming language to generate the distance scores and cluster the individual tumors utilized in Bailey, et al[18].
Individual box plots for each gene were also designed using R programming to cluster each tumor into its identified subtype and plot the normalized RNA-seq expression values.

**RNA Isolation, RT-PCR, and RNA-seq**

Cells were grown to 80-90% confluence on 15 cm dishes, rinsed with 1X PBS and RNA isolated using the Qiagen RNeasy kit. For inducible p53 studies, cells were treated with indicated doses of doxycycline for 24 hours prior to RNA isolation. Isolated RNA was aliquoted and stored at -80 until use. RNA was converted to cDNA using the Verso cDNA kit (ThermoFisher) and cDNA was stored at -20 until use. All RT-PCR primers were obtained from PrimerBank. RT-PCR for each primer set was performed in triplicate and Sybr Green was used for signal detection. Fold change was calculated using the $\Delta\Delta Ct$ method and converted to log$_2$ fold change or normalized to 18s rRNA for plotting multiple genes on the same graph. For single comparisons statistical significance was assessed by student’s t-test. For multiple comparisons 2-way ANOVA was used. For RNA-seq assays, RNA was collected from 3 biological replicates using the Qiagen RNeasy kit and submitted to the UNMC sequencing core for library preparation and sequencing using the Illumina HiSeq2500. Results were analyzed using tophat/cutdiff and edgeR analysis programs by the UNMC bioinformatics core. Differentially expressed genes were then run through Qiagen’s Ingenuity Pathway Analysis to identify cellular pathways that were altered between cell lines[265].

**Subcellular Fractionation**

Cytoplasmic and Nuclear fractions were obtained using the nuclear fractionation protocol from Abcam. Cells were grown to 80-90% confluence and lysed into Buffer A (10mM HEPES, 1.5 mM MgCl$_2$, 10mM KCl, 0.5mM DTT, and 0.5% NP40) and then
incubated on ice for 30 minutes. Lysates were spun down at 3000 rpm for 10 minutes at 4°C to pellet nuclei. The supernatant was removed as the cytoplasmic fraction and the nuclear pellet was washed 3 times in Buffer A to remove potential contaminants. The nuclear pellet was lysed in Buffer B (5mM HEPES, 1.5mM MgCl$_2$, 0.2mM EDTA, 0.5mM DTT, 26% glycerol, supplemented with 300mM NaCl added fresh). To ensure lysis, the nuclear pellet was passed through a 25-gauge needle and the lysates incubated on ice for 15 minutes. Lysates were then spun down at 16,000g for 15 minutes to pellet insoluble debris. The supernatant was collected as the nuclear fraction. Fraction purity was assessed by western blotting with an antibody to Histone 2B.

2D gel electrophoresis

Cells were grown in 150 mm dishes until approximately 90% confluent. Cells were washed with 1X PBS and lysed with 8M urea/thiourea containing 2D-Xtract buffer (G-Biosciences) supplemented with complete protease and phosphatase inhibitor (ThermoScientific) and removed from the dish by scraping. Mouse tissues were homogenized using dounce homogenizers directly into 2D-Xtract buffer supplemented with protease and phosphatase inhibitors. The resulting lysates, cells and tissue, were then alkylated with 5 µl/ml N, N-dimethylacrylamide and placed on a rotator for 20 minutes at room temperature. Alkylated samples were quenched with DTT to a final concentration of 200 mM, and centrifuged at 13,000 rpm for 30 minutes to pellet remaining debris. Protein concentration was determined by Bradford analysis and diluted to a loading concentration of 175 µg/ 155 µl, with 1 µl of the appropriate pH ampholytes (Invitrogen 3-10 or 4-7) and 1 µl 0.25% Bromophenol blue. 155 µl of sample was loaded into each lane with the IPG Zoom strip and allowed to rehydrate the gel strip for a minimum of 2 hours. Following rehydration, loading wells were removed and filter paper saturated with 600 µl DI water was affixed to the designated location. Zoom tray
was loaded into the running apparatus and the outer well filled with 600 ml DI water. Isoelectric focusing was performed per manufacturers specifications (Zoom IPG runner, Invitrogen). Following first dimension electrophoresis, IPG strips were equilibrated in 10 ml 1X SDS sample buffer with 100 mM DTT for 12 minutes then with a 12 minute equilibration in 10 ml 1X SDS sample buffer with 232 µg iodoacetamide. IPG strips were loaded into 4-12% Bis-Tris Zoom Gels, IPG well (Invitrogen) and sealed with 0.5% MES or MOPS agarose, depending on second dimension running buffer. Gels were loaded onto the Invitrogen running apparatus with MES or MOPS running buffer depending on the molecular weight of proteins of interest and run at 175 V for 80 minutes.
Chapter III: Post-translational Modification of the MUC1 Cytoplasmic Tail and Its Splice Variants
1. **Key Findings**

- MUC1 exists in an extensively phosphorylated state in normal mouse pancreas.
- Alterations to the surrounding environment or transformation alters the post-translational modification of MUC1.
- MUC1.CT is N-glycosylated and this form appears to translocate to the nucleus.
- Processing of MUC1 does not appear to involve TACE, however, another self-cleaving module may be present within the MUC1.CT.
- MUC1 splice variants exhibit extensive phosphorylation suggesting a functional role in cell signaling.
2. Introduction

The cytoplasmic tail of MUC1 is postulated to act as a sensor of the extracellular environment; integrating information from the surroundings and propagating intracellular signaling events to drive cellular responses [26]. While numerous studies have demonstrated specific phosphorylation events within the cytoplasmic tail and the corresponding impact on cell signaling, no studies have evaluated the global phosphorylation status of MUC1 [77-79, 82, 100, 104, 266]. The cytoplasmic tail of MUC1 contains 21 potentially phosphorylated residues. This allows for a potential of more than 2 million combinatorial patterns of phosphorylation. This patterning may explain the capacity of MUC1 to alter many distinct pathways, in many instances involving phosphorylation of the same residue [20, 26, 77, 100].

Phosphorylation of MUC1 is thought to be critical for it to translocate to the nucleus where it functions as a transcriptional co-regulator in association with transcription factors, such as p53 and β-catenin [26]. Interestingly, the precise fragment of MUC1 that translocates within the nucleus remains unknown. It has been proposed that MUC1 may potentially undergo a proteolytic cleavage event, similar to Notch signaling, and only the cytoplasmic tail may go to the nucleus [267]. Another possibility is the endosomal transport of MUC1 into the nucleus, similar to the mechanism of ErbB2 translocation [90].

Further adding to the complexity of signaling through MUC1 is the presence of several splice variants [268]. In many instances, these splice variants retain at least a portion of the cytoplasmic tail, potentially allowing for distinct signaling mechanisms. The C-terminal subunit of MUC1 also contains a consensus sequence for N-linked glycosylation. These glycosylation sites may be critical mediators of interactions with various signaling partners in the extracellular compartment.
Given the role of MUC1 in oncogenic signaling, we sought to explore the range of post-translational modifications on MUC1. Presumably, modification of these sites allows for MUC1 to relay a wide array of signals into the cell. To assess these modifications, we utilized 2D gel electrophoresis, as well as phosphorylation specific antibodies in an attempt to map the patterns observed on MUC1. We performed additional studies evaluating the potential for N-glycosylation of the MUC1.CT, as well as the possibility of proteolytic cleavage of the cytoplasmic tail. We found that MUC1 exists in an extensively phosphorylated state, and that this phosphorylation can be impacted by cellular transformation or stimulation with different factors. Furthermore, MUC1 appears to translocate to the nucleus as 3 distinct forms, one of which is N-glycosylated. These forms are apparently independent of proteolytic cleavage by ADAM17; however, in vitro studies suggest that self-cleavage effects may occur within the cytoplasmic tail as a result of chemical reactions involving the cysteine residues of the cytoplasmic tail. Furthermore, MUC1 splice variants are also extensively phosphorylated suggesting potential signaling roles for these variants in addition to full-length MUC1.

3. Results

MUC1.CT is Extensively Phosphorylated in Response to Environmental Factors

The C-terminal subunit of MUC1 (MUC1.CT) contains a wide array of potential post-translational modification sites. In particular, the cytoplasmic tail contains several serine, threonine, and tyrosine residues (Figure 3.1A). To assess the post-translational status of MUC1, we performed 2D gel electrophoresis to identify changes in predicted size or isoelectric point in normal mouse pancreas and in KPC tumors. We observed the presence of 3 distinct bands of approximately 25, 20 and 15 kDa deviating from the predicted molecular weight of 17 kDa of the 158 amino acid, MUC1.CT. This was true
for both normal mouse pancreas and KPC pancreatic tumors. We also observed significant deviations from the predicted isoelectric point of 6.4. Phosphorylation is predicted to cause a decrease in the observed isoelectric point [269]. To test whether these deviations were the result of phosphorylation, we treated mouse pancreas extracts with 200 U T-cell tyrosine phosphatase for 3 hours at 30°C and repeated the 2D gel analysis. Treatment resulted in a shift of isoforms from an apparent PI of 4.0-4.6 back towards a range of 4.9-6.0, approaching the predicted isoelectric point of 6.4, though this shift was incomplete, suggesting the presence of additional modifications of serine and threonine residues on the MUC1.CT (Figure 3.1B). Interestingly, KPC tumors exhibited higher levels of the lower isoelectric point forms than normal mouse pancreas, suggesting hyperphosphorylation in these tumors.

To further assess potential phosphorylation events of MUC1.CT, we performed western blots of normal mouse pancreas utilizing phosphospecific antibodies raised against unique MUC1 phosphoepitopes. We observed positive results with antibodies against a number of sites, suggesting that MUC1.CT is indeed extensively phosphorylated at a variety of sites (Figure 3.1C). In addition, the different gel mobilities shown in 3.1.C suggest that the rather broad bands observed with antibodies against the MUC1CT (e.g. Figure 3.1.B) are comprised of isoforms of MUC1CT that are differentially phosphorylated at multiple sites in different combinations. To confirm that receptor tyrosine kinases and other stimuli signal through MUC1CT, we evaluated the effect of stimulating MUC1 expressing cells with EGF. Stimulation of cells with either FBS or EGF resulted in differential patterning of MUC1 phosphorylation events, suggesting that different stimuli promote different phosphorylation patterns of MUC1.CT (Figure 3.2).

MUC1.CT is N-glycosylated and Present in the Nucleus
The juxtamembrane portion of the extracellular domain of the MUC1.CT contains a consensus site for N-glycosylation (NXS/T). Previous studies have suggested that MUC1.CT is N-glycosylated at this site [270]. We sought to confirm the presence of N-linked glycosylation at this site. Furthermore, we wanted to identify whether this form was present within the nucleus, given the previously reported finding that the extracellular part of β-dystroglycan can traffic to the nucleus [271]. We performed western blot analysis of normal mouse pancreas extract following treatment with the glycosidase PNGase F (Figure 3.3A). PNGase F treatment resulted in a decrease in the apparent molecular weight of the 25 kDa band to the 20 kDa band, indicating that MUC1.CT is N-glycosylated. To test whether this form is present within the nucleus, we performed subcellular fractionation of S2013.Neo and MIF cells (Figure 3.3B) and treated the nuclear fraction of S2013.MIF cells with PNGase F, followed by western blot analysis. PNGase treatment induced a shift of MUC1.CT from 25 to 20 kDa indicating loss of N-glycosylation and suggesting that this form is present within the nucleus (Figure 3.3C). This supports the hypothesis that the 25 kDa band of MUC1 seen in these experiments contains the C-terminus with N-glycosylation, whereas the 20 kDa band contains non-glycosylated forms of MUC1.CT.

MUC1.CT Processing is Unaffected by a TACE Inhibitor

There have been reports of putative cleavage sites within the C-terminal portion of MUC1 for the ADAM17 protease, also called TACE [272]. This cleavage results in truncation of the predicted 58 amino acid extracellular domain of MUC1CT. As we had previously identified the 25 and 20 kDa bands of MUC1.CT, we sought to examine whether cleavage by TACE was responsible for the formation of smaller bands, including the 15 kDa band. We treated S2013.MIF cells with the TACE inhibitor, TAPI-2, at increasing doses for 12 or 24 hours and monitored the processing of MUC1 by western
blot analysis (Figure 3.4). We observed no significant differences in any of the MUC1 bands detected by CT2. This suggests that processing by TACE is not responsible for the 15 kDa form of MUC1.CT.

The cysteine residues of MUC1.CT have been suggested to critically regulate a number of processes, including dimerization. Interestingly, several studies have shown nucleophilic chemical reactions within proteins resulting in self-cleavage events (Figure 3.5A). These excised segments are termed inteins and the cleavage events caused by this chemical reaction can be catalyzed by the addition of reducing agents [273]. To examine the potential dimerization of MUC1 in vitro we have purified recombinant MUC1 from E.coli cells and examined the properties under reducing or non-reducing conditions. Under non-reducing conditions, we observe an approximately 40 kDa band consistent with a dimer of MUC1. Interestingly, we have observed in initial studies with recombinant MUC1.CT that the presence of DTT results in formation of a cleavage fragment of approximately 15 kDa and this is absent in non-reduced conditions, suggesting the possibility of a self-cleavage event involving the cysteine residues of MUC1 (Figure 3.5B). Future studies using cysteine mutants may help to confirm this possibility, though serine residues have also been shown to be involved in this type of cleavage.

**MUC1 Splice Variants are Extensively Phosphorylated**

The MUC1 gene contains 7 exons and can be alternatively spliced into a wide variety of alternative products [268]. Many of these variants retain the cytoplasmic tail suggesting the possibility for differential signaling depending on the splice variant. While the signaling capacity of MUC1 has been well studied, the potential for signaling through the tail of variants has not been explored in depth. To assess the potential phosphorylation of MUC1 splice variants we performed 2D gel analysis of recombinant
X, Y, and SV3 splice variants expressed in HPNE cells (Figure 3.6A). Western blots of the different isoforms show distinct changes in molecular weight for each isoform. HPNE-mock transfected cells showed no specific bands. Similar to the standard form of MUC1, we observed significant deviations to the left of the predicted isoelectric (Figure 3.6B), suggesting that these MUC1 splice variants can be extensively phosphorylated and suggesting that they may play a role in cellular signaling.

4. Discussion

The transmembrane mucin MUC1 plays a critical role in tumorigenesis through the integration of microenvironmental signals and induction of downstream signaling cascades to promote cellular responses and adaptation to changes in the extracellular microenvironment [26, 43, 81, 83]. We propose that MUC1CT serves as a node to integrate morphogenetic signals from a number of diverse networks, in part through its capacity to receive multiple differential phosphorylation events. A number of studies have demonstrated that phosphorylation of specific residues in the MUC1 cytoplasmic tail are critical to drive its association with signaling factors and transcription factors; however, until this report, no studies have evaluated the global phosphorylation status of MUC1 [77, 100, 105, 274]. The studies reported here demonstrate that MUC1 exhibits a wide array of modifications, including potentially 10 or more phosphorylation events within the cytoplasmic tail. The fact that many of these modifications occur within the normal pancreas support the hypothesis that phosphorylation of MUC1 contributes to normal signaling in cells, and the finding that this pattern is significantly altered in cancer supports the hypothesis that this biological process is co-opted and modified by malignant processes in tumor cells.
Most studies have focused on tyrosine phosphorylation of the MUC1CT as a major player in MUC1 signaling. Our results show that tyrosine phosphorylation is prominent in normal and malignant pancreatic cells; however, treatment with tyrosine phosphatase does not remove all modifications, suggesting that a number of serine and threonine residues are also phosphorylated. Preliminary western blot analysis of MUC1 using phosphospecific antibodies support this hypothesis, though the absolute specificity of these antibodies has not been extensively validated. Interestingly, we found significant alterations in the global patterns of phosphorylation by stimulating cells with different growth factors, supporting the concept that MUC1 contributes to the integration and relay of information about the cytokine status and other factors from surrounding environment. Malignant transformation also results in hyperphosphorylation of the cytoplasmic tail, suggesting alterations in signaling pathways during the progression to malignancy. Based on these results, it is likely that the combinatorial pattern of the phosphorylated residues is critical for initiating or regulating distinct downstream responses. With the 22 potential sites of phosphorylation, there are more than 2,000,000 potential phosphorylation patterns, allowing for specific integration of multiple signals.

We also explored potential modifications other than phosphorylation of the cytoplasmic tail. Previous studies have shown that MUC1 contains a consensus site for N-linked glycosylation within the C-terminal subunit, which appears to be N-glycosylated [270]. We confirmed this finding in our studies and provide evidence that this form of MUC1 can traffic to the nucleus. Similar studies have shown this effect with β-dystroglycan, which has a general structure similar to MUC1 [271]. While these studies are preliminary and perhaps controversial, the N-glycosylation of MUC1 has been reported to regulate its interaction with EGFR [270, 275]. Association of MUC1 and EGFR has also been shown to promote its nuclear translocation, suggesting the
possibility that these signaling complexes are transported via an endosomal mechanism that would allow translocation of both extracellular and intracellular portions of MUC1.CT. Further studies using additional methods of distinguishing protein trafficking and improved methods of cellular fractionation will help to address these questions.

We and others have found that the C-terminal portion of MUC1 exists as at least 3 distinct forms. While we have postulated that two of these forms are full-length MUC1.CT either with or without N-glycosylation, to date the smallest form seen commonly by gel electrophoresis has not been identified. We tested the hypothesis that these forms were the result of proteolytic cleavage by the TACE protease, but found no effect using a specific inhibitor, suggesting that this smaller form may result from cleavage from an unidentified protease or may represent a distinct variant. Preliminary biochemical studies of recombinant MUC1.CT raise the possibility that this smaller form may be the result of a self-cleavage event involving nucleophilic reactions of cysteine residues; however, validation of this hypothesis requires further study. These in vitro studies also suggest that MUC1 can readily exist as a dimer, whether this occurs in vivo remains controversial. Use of cysteine mutants may help to validate these studies, though serine residues can engage in intein cleavages as well. Mass-spectrometry identification of the fragment may assist in the identification of critical residues and determination of whether any excision of internal residues actually occurs.

Interestingly, 2D gel analysis of known MUC1 splice variants showed extensive phosphorylation similar to canonical MUC1. As these variants exhibit alterations in the extracellular domains, they may propagate signals differently than full-length MUC1. This may also account for the diverse phenotypes associated with MUC1 expression. These findings also require additional study.

The studies reported in this chapter suggest that the C-terminal subunit of MUC1 is a dynamic structure, driving downstream signaling in part by conducting and
integrating signals from growth factor receptors and other factors in the surrounding environment. Future studies examining the specific residues that are phosphorylated under given conditions should provide further insight into the specific nature and integration of these signals. For example, some phosphorylation events may require previous phosphorylation to create docking sites for other kinases, which may contribute to our observation of widely different patterns of phosphorylation. Better understanding of how these processes occur will allow for potential targeting of specific partner interaction sites within the cytoplasmic tail to preferentially impede certain signaling networks that may be activated in pancreatic cancer.
Figure 3.1: MUC1.CT is Extensively Phosphorylated

A) Sequence of the 158 MUC1.CT. Extracellular, transmembrane, and cytoplasmic tail domains are highlighted in each box. Consensus N-glycosylation site and potential phosphorylation sites(*) are also highlighted. B) 2D gel western blot analysis of MUC1.CT in normal mouse pancreas and KPC tumor. For each experiment 175 µg of protein lysate were used. Isoelectric point is mapped below, as well as a prediction of isoelectric point for each phosphorylation event. Treatment of samples with tyrosine phosphatase results in shift towards predicted isoelectric point. These studies were performed on multiple, independent samples. C) Western blot analysis of MUC1.CT using phosphospecific antibodies against the cytoplasmic tail. Each epitope is highlighted below the blot with the Red residue indicating the site of phosphorylation. Western blots were repeated multiple times to optimize antibody concentration and confirm reproducibility.
Figure 3.1

A. MUC1.CT

SEA cleavage

Putative TACE cleavage

N-glycosylation

MUC1.CT

* Potential Phosphorylation Site

B. MUC1.CT Increased Phosphorylation in KPC PDAC Tumors

Normal Mouse Pancreas
MUC1.CT

KPC Pancreatic Tumor
MUC1.CT Phosphorylation

Normal Mouse Pancreas
TC-PTP (Tyr Phosphatase) Treatment
MUC1.CT

C. pH

# Phosphates

IB: EYPT YGQL SPYE SAGN YHTH SSLS TYHP SEYP SSLS SSLS TYHT YHPM YVPP YTNP
Figure 3.2: Different Stimuli Induce Different Phosphorylation Patterns on MUC1

Panc1.MUC1-HA cells were serum starved for 24 hours prior to stimulation with either 100 ng/ml EGF or 10% FBS containing media for 30 minutes. Cells were lysed and analyzed by 2D gel electrophoresis followed by western blot analysis for MUC1 expression. Each was sample was prepared identically with 175 µg loaded on the gel. Blots were scanned at the same time to control for exposure. EGF stimulation caused a specific hyperphosphorylated shift to very low pH range, whereas FBS stimulation resulted in a wide range of phosphorylation patterns spread out over the entire 3-10 pH range. The images are representative images of multiple experiments.
Figure 3.2

Panc1.MUC1-HA

IB: MUC1(CT2)

+EGF

25 20 15
3 10

IB: MUC1(CT2)

+FBS

25 20 15
3 10
Figure 3.3: N-Glycosylated MUC1 is Present in the Nucleus

A) Western blot analysis of MUC1.CT in normal mouse pancreas with or without PNGase F treatment. Samples were prepared identically and split prior to treatment to yield two equal concentrations for the gel. With treatment a shift in molecular weight is observed corresponding to loss of N-glycosylation. B) Western blot of S2013.Neo and MIF cytoplasmic and nuclear fractions for expression of MUC1. MUC1 is present in both the cytoplasmic and nuclear fractions and exists as all 3 species we commonly observe. GAPDH was used as a loading control for the cytoplasmic fraction. C) Western blot analysis of nuclear MUC1 following treatment with PNGase F. The shift in molecular weight indicates a loss of N-glycosylation. H2B serves as the loading control. All western blots were repeated multiple times to ensure reproducibility. For all western blots 30 µg of lysate was used.
Figure 3.3

A. Mouse Pancreas

PNGaseF + –

IB: MUC1 (CT2)

B. S2013.Neo S2013.MIF

Cytoplasmic Nuclear

IB: MUC1
IB: GAPDH
IB: H2B

C. S2013.MIF

PNGaseF – +

IB: MUC1
IB: H2B
Figure 3.4: MUC1.CT Processing is not Impacted by TACE Inhibition

Western blot analysis of MUC1 expression in S2013.MIF cells following treatment with TAPI-2 over 12 or 24 hours. 30 µg of protein lysate was loaded for each sample. No substantial changes were observed in any of the products detected by CT2. β-actin serves as a loading control.
Figure 3.4

<table>
<thead>
<tr>
<th>TAPI-2 (μM)</th>
<th>S2013.MIF</th>
<th>S2013.MIF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ctl 1</td>
<td>Ctl 2</td>
</tr>
<tr>
<td>12 hours</td>
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<tr>
<td>IB: MUC1(CT2)</td>
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<tr>
<td>24 hours</td>
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<tr>
<td>IB: β-actin</td>
<td>![Image]</td>
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Figure 3.5: Putative In Vitro Self-Cleavage of MUC1.CT

A) General schematic of the chemical processes involved in intein self-cleavage. Initial steps involve nucleophilic attack typically involving cysteine and serine residues. These steps are followed by rearrangements that result in excision of the intein and joining of the two-extein portions. These reactions can be catalyzed in vitro through addition of reducing agents. B) Multiple preparations of recombinant His-tagged MUC1.CT (158 amino acids) were isolated from C41 or C43 E.coli using Nickel affinity column and analyzed by western blot analysis under non-reducing or reducing conditions. Non-reducing conditions show potential dimerization of monomeric MUC1, reduction causes loss of these high molecular weight forms and formation of smaller molecular weight forms, suggesting a self-cleavage event.
Figure 3.5

A. N-terminus of N-extein interacts with the Intein, leading to nucleophilic attack and cyclization. The resulting structure can then undergo N-acyl shift to form a dimer or undergo MUC1 autocleavage?

B. Non-reducing and reducing conditions show differences in MUC1:MUC1 dimer and MUC1 autocleavage? IB: MUC1(CT2)
**Figure 3.6: MUC1 Splice Variants are Extensively Phosphorylated**

**A)** Exon map showing the alternative splicing of MUC1/X, MUC1/SV3, and MUC1/Y. All three of these variants retain the cytoplasmic tail and are detectable using the CT2 antibody. MUC1/Y does not undergo SEA cleavage and thus is larger than MUC1/X. MUC1/SV3 lacks all of exon 4 and is the smaller than both X and Y.

**B)** 2D gel analysis was performed using HPNE cells transfected with specific MUC1 splice variants. All gels contained 175 µg of protein lysate. The MUC1 antibody CT2 was used for detection. The variants did exhibit slightly higher than predicted molecular weight and the smear of higher weight forms suggests that these variants may be glycosylated. However, each form did run according to its relative size as compared to the other variants. These variants all showed significant deviations to the left off the predicted isoelectric point (*), indicating extensive phosphorylation.
Figure 3.6

A.

MUC1

1 2 3 4 5 6 7

MUC1/X

1 2 3 4 5 6 7

MUC1/SV3

1 2 3 4 5 6 7

MUC1/Y

1 2 3 4 5 6 7

SEA cleavage

Cytoplasmic tail

Ex.3 skip

F.S

Ex.4 skip

F.S

F.S

B.

HPNE-Mock

HPNE-MUC1/SV3

HPNE-MUC1/Y

HPNE-MUC1/X

20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95

20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95

20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95

20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95

*
Chapter IV: Identification of FRA-1 as a Novel Player in Pancreatic Cancer in Cooperation with a MUC1:ERK Signaling Axis

This work has published in Oncotarget[169]

"Identification of FRA-1 as a Novel Player in Pancreatic Cancer in Cooperation with a MUC1:ERK Signaling Axis"

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Identification of FRA-1 as a Novel Player in Pancreatic Cancer in Cooperation with a MUC1: ERK Signaling Axis

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Keywords: FRA-1, MUC1, ERK, pancreatic cancer, invasion
1. Key Findings
   - Expression of MUC1 drives increased expression and activity of c-Jun
   - MUC1 promotes association of c-Jun and FRA-1, likely in an ERK dependent manner
   - FRA-1 promotes migration, invasion, and overall tumor growth in pancreatic cancer cells
   - Expression of FRA-1 is increased in clinical specimens of pancreatic ductal adenocarcinoma
   - Expression of FRA-1 correlates with expression of pro-EMT genes in a subset of clinical specimens
   - MUC1 modulates expression of FRA-1:EMT genes
2. Introduction

Pancreatic ductal adenocarcinoma (PDAC) is a prominent cause of cancer related deaths worldwide. Despite recent advances in therapeutic treatment, the prognosis for patients remains relatively unchanged, with a median survival of about 6 months and a 5-year survival of only 6% [1]. Several factors contribute to the poor outcome of pancreatic cancer, including difficulties in early diagnosis and the propensity of the cancer to metastasize to distant sites early in progression [1, 276]. As such, there is a vital need for improved understanding of the mechanisms by which pancreatic cancer cells disseminate throughout the body and potential ways to specifically target these metastatic cells. MUC1, a member of the mucin family of glycoproteins that is commonly overexpressed and aberrantly glycosylated in pancreatic cancer [277] is known to modulate the invasive and metastatic potential of cancer cells. MUC1 acts by influencing the balance of adhesive and anti-adhesive properties, and by engaging in morphogenetic signaling that communicates information about structural and microenvironmental conditions at the cell surface to the nucleus in a manner that alters gene expression [20]. MUC1 exists at the cell surface as a heterodimer comprised of a large N-terminal extracellular mucin domain that is non-covalently associated with a C-terminal domain containing a short extracellular domain, transmembrane region, and a cytoplasmic tail [19]. The cytoplasmic tail is differentially phosphorylated by different receptor tyrosine kinases and serine and threonine kinases in response to cytokine stimulation, physical interactions with counter-receptors, or other factors, and acts as a relay of signals from the cell surface to the nucleus [19, 20, 26]. In cancer, MUC1 potentiates oncogenic signaling through downstream effectors [26] and acts as a transcriptional co-regulator in conjunction with transcription factors such as p53, β-catenin, and c-Jun [26, 77, 102]. This wide range of interaction partners allows MUC1 to
act as a signaling hub, integrating signals from cytokine receptor status, cellular structure, and other microenvironmental conditions to alter cellular behavior. Among these, are the capacity to influence migration and invasion potential [278-280].

One critical transcription complex impacted by MUC1 is activator protein 1 (AP-1), a transcription factor comprised of Jun and Fos proteins that were among the first oncogenic proteins discovered [125, 173]. The Jun family of proteins includes c-Jun, JunB, and JunD, and the Fos family consists of c-Fos, FosB, FRA-1, and FRA-2 [126]. Jun and Fos proteins exhibit varying degrees of transforming effects on cells, and the function of these proteins is dependent on the formation of specific dimers. Jun proteins can homodimerize or form Jun:Fos heterodimers. Dimers that comprise AP-1 transcription factors can bind to TPA response elements (TRE) within DNA to regulate transcription, though the DNA elements bound depend in part on the composition of the dimer [123, 126]. The AP-1 regulated targets, matrix metalloproteases 1 (MMP1) and connective tissue growth factor (CTGF), are known targets of MUC1’s co-regulatory activity [77, 100], and we have previously shown that MUC1 over-expression decreases the apparent binding of c-Jun to the CTGF promoter element [100]. However, the impact of MUC1 expression on other targets of AP-1 has not been studied and how MUC1 mediates the displacement of AP-1 from specific promoters remains unknown. Based on the capacity of different AP-1 dimers to bind unique promoter elements, we hypothesized that MUC1 may alter the composition of the AP-1 dimer as a result of integrating oncogenic signaling events to regulate expression of genes associated with migration and invasion.

In this chapter, we examined how MUC1 modulated AP-1 (c-Jun and FRA-1) activity and thereby affected the migratory and invasive properties of pancreatic cancer cells. Our results provide the first evidence that in concert with ERK activation, MUC1 modifies the formation of AP-1 dimers to preferentially favor c-Jun:FRA-1, which in turn
enhances the migration and invasive potential of pancreatic cancer cells *in vitro*. We show that MUC1 acts as a dominant regulator of FRA-1 function at the CTGF promoter and promotes expression of FRA-1 targets involved in migration and invasion. Increased expression of FRA-1 mRNA and protein was also observed in clinical PDAC samples, and a subset of clinical samples exhibited a FRA-1: EMT gene expression signature. Knockdown of FRA-1 significantly impacted tumor growth *in vivo*, further supporting the hypothesis that a novel FRA-1/MUC1 axis contributes to the aggressiveness of PDAC.

3. Results

**MUC1 increases levels of active c-Jun in tumor cells**

Previous studies demonstrated that MUC1 affects AP-1 regulation of target genes in pancreatic cancer cell lines [77, 100]. We initially evaluated the possibility that this was due in part to the influence of MUC1 on levels of c-Jun in two MUC1-overexpressing human pancreatic tumor cell lines, S2013.MIF and Panc1.MUC1, as compared to their low-expressing counterparts. Analysis of total c-Jun in cytoplasmic and nuclear fractions showed an increase in total c-Jun within the nucleus of MUC1 overexpressing cells (Fig. 4.1A & B). MUC1 expression also increased c-Jun activation through phosphorylation at serine 73. Similarly, examination of pancreatic tumor cell lines derived from tumors of KPC mice that expressed or were null for Muc1 showed a modest increase in total c-Jun within the nucleus when MUC1 was expressed (Fig. 4.1C). These observations were confirmed *in vivo* by analysis of normal mouse pancreas and primary pancreatic tumors derived from Muc1 expressing and Muc1-null KPC mice, which showed undetectable levels of c-Jun in normal pancreas as compared to tumor samples, and that c-Jun expression was further enhanced in tumors expressing
Muc1 (Figure 4.1D). Unlike human tissues, normal mouse pancreas expresses high levels of MUC1.

**MUC1 promotes the formation of c-Jun:FRA-1 dimers**

We next investigated the hypothesis that MUC1-mediated increases in c-Jun levels were due to alterations in dimerization partnerships that are known to stabilize c-Jun. Previous studies have shown that MUC1 expression leads to displacement of c-Jun from promoters [77, 100]. The composition of c-Jun heterodimers is known to impact the DNA binding affinity and specificity [197, 198]. We therefore evaluated dimer composition by proximity ligation assays to assess the effect of MUC1 on interactions between c-Jun and a subset of known dimerization partners (c-Fos, FRA-1, and ATF2), which were chosen based on published roles in DNA binding, transformation, or metastatic phenotype. Representative images of PLA experiments are shown in Figure 4.2A. Quantification was performed using the Blobfinder program and results are presented as a representation of mean interactions per cell [281], which were further subdivided into cytoplasmic and nuclear localization (Figure 4.2B). MUC1 overexpression did not significantly affect nuclear interactions between c-Jun and ATF2 or c-Fos; however, c-Jun:FRA-1 interactions were significantly increased (Fig. 4.2C). As a secondary validation that MUC1 promoted the association of c-Jun and FRA-1, we performed co-immunoprecipitation/western blotting assays to detect stable interactions between FRA-1 and c-Jun. The results showed increased amounts of c-Jun associated with FRA-1 in cells over-expressing MUC1 (Figure 4.2D) confirming that MUC1 promoted the association of c-Jun and FRA-1.

**MUC1, ERK, and FRA-1 regulate the migratory and invasive potential of pancreatic cancer cells**
Upregulation of FRA-1 is commonly observed in metastatic breast cancer [186-188, 282], where it is hypothesized that FRA-1 acts as a driver of invasion and metastatic spread of cancer cells. We sought to determine if FRA-1 played a similar role in pancreatic cancer. Given our evidence that FRA-1:c-Jun interactions increased with MUC1 overexpression, that FRA-1 can be phosphorylated via ERK, and that MUC1 is known to promote signaling through the ERK pathway [81, 126], we investigated the effects of MUC1 over-expression on ERK activation. We determined the levels of total ERK1/2 and phosphorylated ERK1/2 by western blot analysis on subcellular fractions of S2013.Neo and MIF cells. The results showed increased levels of phosphoERK2 in the nucleus of MUC1-overexpressing cells (Figure 4.3A). Panc1.MUC1 cells also exhibit increased ERK2 phosphorylation (Figure 4.4). We confirmed that ERK activation was responsible for activation of FRA-1 by treating S2013.Neo and MIF cells with the MEK inhibitor U0126. Western blot analysis indicated that U0126 reduced phosphorylation of both ERK and FRA-1 (Figure 4.3B).

The results of migration and invasion assays using Boyden chamber inserts showed that inhibition of ERK signaling by U0126 treatment resulted in approximately 40% reduction of the number of migrating MUC1 expressing cells, while no effect was observed in S2013.Neo cells (Figure 4.3C), supporting the hypothesis of activation of FRA-1 enhanced motility in pancreatic cancer cells in concert with MUC1. Similar results were observed for the invasive potential of S2013.MIF cells, indicating increased sensitivity to loss of FRA-1 activity (Figure 4.3D). We confirmed the role of FRA-1 in modulating motility by shRNA knock down studies [confirmed by RT-PCR and western blot analysis (Figure 4.5A)]. Decreased FRA-1 expression resulted in decreased migration and invasion. Similar to the effect of ERK inhibition, knockdown of FRA-1 decreased migration and invasion to the greatest degree in cells overexpressing MUC1. Conversely, overexpression of FRA-1 (Figure 4.5B) greatly increased the migratory and
invasive properties of S2013 cells, whether or not MUC1 was expressed, though cells overexpressing MUC1 and FRA-1 showed the highest migratory and invasive activities (Figure 4.3C and 4.3D). Similarly, overexpression of FRA-1 in Panc1.Neo or Panc1.MUC1 cells significantly increased both migration and invasion (Figure 4.3E and 4.3F), whereas knockdown decreased these properties. Notably, as for S2013 cells, these effects were higher in cells expressing higher levels of MUC1. Overexpression of FRA-1 also altered the morphology of the cells. Increased numbers of elongated cells with filipodia-like projections were observed in culture (Figure 4.6A-B). Evaluation of these cells for proliferation revealed that at 48 hours, FRA-1 overexpression did not impact cellular growth; however, over longer time frames FRA-1 slightly enhanced proliferation as assessed by methylene blue growth assay [264] (data not shown).

Loss of FRA-1 expression decreases tumor growth and metastases

To evaluate the role of FRA-1 in the development and progression of pancreatic ductal adenocarcinoma, we performed orthotopic studies evaluating tumor growth of S2013.Neo and MIF cells with or without knockdown of FRA-1 expression. 150,000 cells were injected into the pancreas of nude mice and tumors allowed to progress for 30 days before mice were sacrificed. Knockdown of FRA-1 expression resulted in significant reduction of primary site growth as assessed by both tumor weight and tumor volume in S2013.Neo (Figure 4.7A-B). This effect was enhanced in S2013.MIF cells (Figure 4.7D-E). Interestingly, one mouse in the S2013.MIF-FRA1 kd group failed to develop a palpable tumor, though a small tumor was detected by microscopy. Knockdown of FRA-1 also resulted in a reduction in the development of ascites.

Metastatic spread was assessed by gross analysis of tissues during necropsy and confirmed by microscopic examination of collected tissues. Knockdown of FRA-1 resulted in a 10-30% reduction in metastases in S2013.Neo, though some sites showed
no differences, such as liver metastasis. The effects were less pronounced with high levels of MUC1 expression in S2013.MIF (Figure 4.7C & F).

**FRA-1 is upregulated in pancreatic cancer**

The role of FRA-1 in pancreatic cancer remains relatively unexplored, though it is expressed in numerous pancreatic cancer cell lines [194]. We investigated the possibility that FRA-1 contributes in vivo to pancreatic cancer progression by evaluating gene expression of FOSL1, which encodes FRA-1, in PDAC samples. Our initial analysis included evaluation of the GEO database for microarray expression data of pancreatic ductal adenocarcinoma samples that were compared to normal pancreatic tissues. Using the data series of GSE16515, consisting of 52 samples (36 tumors and 16 normal samples), we evaluated the gene expression levels of FRA-1 [283, 284]. Analysis of relative expression levels of FOSL1 using the Generalized Estimating Equation (GEE) [285] revealed significant upregulation (p<0.001) in tumors as compared to normal samples (Figure 4.8A). To confirm the results were not skewed by a few highly expressing tumors, we compared the 16 tumors that were matched to samples of uninvolved pancreas included in this data set. Log2Fold Change was utilized to compare overexpression between tumor and normal samples. We observed that 15 of 16 samples showed upregulation of FOSL1, and 8 exhibited a change of greater than 2 fold (Figure 4.8B).

Our in vitro studies suggest FRA-1 expression is important for invasive potential. To assess whether FRA-1 expression changed during the progression of pancreatic cancer, we evaluated the data series GSE42952, which includes tumor stage and some matched primary and metastatic tumors. FOSL1 expression was plotted for each tumor stage identified within the data set, ignoring absent calls (Figure 4.8C) [284, 286]. For metastatic sites we differentiated between the identified liver or peritoneal metastatic
site. Late stage tumors showed a slightly higher trend of FOSL1 expression, particularly within liver metastases, but the low number of samples prevented us from making reliable conclusions based solely on these data. As a second evaluation, we performed immunohistochemistry using tissue microarrays of matched sets of primary and metastatic tumors derived from the UNMC Rapid Autopsy Program. FRA-1 expression was examined in primary site tumor, metastatic sites, and normal pancreas from multiple patients (Figure 4.9). A heatmap was generated based upon the intensity of staining observed within tumor cells with representative images for scoring presented (Figure 4.8D). Most cancer cells exhibited robust nuclear staining, whereas FRA-1 was absent in normal pancreas samples; however there were no consistent trends of higher expression in metastatic samples in this limited analysis. Expression of FRA-1 also varied in different tumor samples. Thus we conclude that FRA1 is upregulated in some but not all pancreatic cancers.

**FRA-1 overexpressing tumors exhibit a FRA-1:EMT signature**

Recently a FRA-1:EMT signature has been proposed for colorectal cancer cells overexpressing a flagged FRA-1 construct [170]. Eight genes identified as regulated by FRA-1 were found to represent part of an Epithelial to Mesenchymal Transition (EMT) associated signature: VIM, FN1, FOSL1, ZEB1, SNAI2, AXL, TGFB2, and SMAD3. We chose to examine gene expression of 6 of these genes (FN1, ZEB1, SNAI2, AXL, TGFB2, and SMAD3) in PDAC samples. The GSE16515 PDAC data set was analyzed for a similar signature. We evaluated paired samples that overexpressed FOSL1 at least two-fold, which were predicted to exhibit a FRA-1 associated phenotype [283]. Calculation of Log2 Fold Change for each paired set of tumor and normal samples for these targets (Figure 4.10A-F) showed a substantial correlation between FRA-1 expression and upregulation of these EMT signature mRNAs. 5 of the 6 targets (FN1,
ZEB1, SNAI2, AXL, and SMAD3) were upregulated in at least 60% of the tumors with high FRA-1 expression; however TGFB2 showed no consistent trend. Tumor samples 5, 8, 11, 13, and 15 were most consistent with the predicted FRA-1 signature, mirroring the predicted trend 100% of the time for genes other than TGFB2. These results support the hypothesis that pancreatic cancer exhibits a FRA-1 driven EMT signature, though only within a subset of cases.

To examine whether protein expression of these genes correlated with FRA-1 in patient samples, we performed IHC for Slug (SNAI2) and ZEB1 on matched sets from our rapid autopsy program (Figures 4.11 & 4.12). Slug was observed in most samples, though it was absent in a FRA-1 negative tumor. ZEB1 was absent in most samples, but present in a few tumors highly expressing FRA-1. A heatmap representing the IHC pattern was generated using R (Figure 4.13). These results suggested that even though there were effects on mRNA levels, there was not a direct correlation between expression of FRA-1 and the protein products of its target genes Slug and ZEB1 in clinical samples, demonstrating that factors other than mRNA levels influence steady state protein levels of these proteins.

**MUC1 regulates expression of FRA-1:EMT gene targets**

As previous studies have demonstrated the capacity of MUC1 to regulate expression of MMP1 and CTGF, we sought to examine how the interplay of MUC1 and FRA-1 impacted expression of these genes[77, 287]. We performed RT-PCR analyses of our S2013.Neo, S2013.MIF, Panc1.Neo, and Panc1.MUC1 cell lines in conjunction with FRA-1 overexpression or knockdown to examine expression of MMP1 and CTGF. Expression of FOSL1 was used as a positive control to confirm overexpression or knockdown of FRA-1. Results indicated that increased MUC1 expression dramatically impacts expression of MMP1 and CTGF in S2013 cells. Overexpression or knockdown
of FRA-1 did not reverse these effects, indicating a dominance of MUC1 effects at these sites, whereas loss of FRA-1 in S2013.Neo resulted in CTGF expression comparable to that observed in S2013.MIF (Figure 4.14A). These results were more modest in Panc1 cells and reflect a less robust impact of MUC1 on expression of these targets in this cell line (Figure 4.14B).

To examine whether MUC1 impacted expression of FRA-1:EMT genes, we performed additional RT-PCR studies examining expression of SNAI2, ZEB1, TGFB2, AXL, SMAD3, and FN1. Expression of MUC1 in S2013 cells caused significant upregulation of many of the FRA-1:EMT genes, including SNAI2, ZEB1, AXL, and FN1. Loss of FRA-1 caused significant reduction of these genes to levels comparable to the S2013.Neo cell line. The impact of FRA-1 knockdown in the Neo cell line was relatively modest on most FRA-1:EMT genes (Figure 4.14C). Once again these effects were less pronounced in the Panc1 cell lines, though AXL was impacted by MUC1 expression (Figure 4.14D).

4. Discussion

That MUC1 affects gene expression is well established [80, 89, 103, 288]; however, the mechanism by which MUC1 regulates transcription and affects tumor progression is not fully understood. MUC1.CT is known to interact with a wide range of transcription factors including p53, β-catenin, c-Jun, and others [77, 100, 102]. Previous studies have shown that MUC1 displaces c-Jun from promoters of known target genes; the data presented here demonstrates this effect is not the result of decreasing the levels of c-Jun within the cell. Rather, our results indicate that high levels of MUC1 alter the AP-1 transcriptome in part by increasing steady state levels of c-Jun protein. We went on to demonstrate that this stabilization of c-Jun results in enhanced association
with FRA-1 in cells expressing high levels of MUC1, suggesting that MUC1 alters the stoichiometry of AP1 protein complexes, which in turn modifies transcriptional activity (Figure 4.15).

We found that MUC1 acts as a dominant regulator of FRA-1 activity that in turn modulates expression of CTGF and MMP1. Additionally, MUC1 upregulated expression of several genes associated with FRA-1 mediated EMT. Steady state mRNA levels for these genes were reduced upon FRA-1 knockdown. Together, these results suggest that MUC1 serves as a co-activator for FRA-1 at many FRA1-EMT sites, whereas it may function as a de-repressor of FRA-1 at the CTGF and MMP1 sites. The results were less apparent in Panc1 cells, which may be attributed to differences in context dependent constitutive signaling between the S2013 and Panc1 cell lines. Indeed, the impact of MUC1 on the migration and invasion of Panc1 cells was more modest than the effects on S2013 cells. Thus, it is not surprising that expression of genes involved in migration and invasion are not substantially altered in the Panc1 cells.

Consistent with the findings reported here, FRA-1 has been shown to impact migration, invasion, and metastasis in a number of different cancers [126, 170, 187, 188]. In particular, the role of FRA-1 is well characterized in breast cancer, which also commonly exhibits MUC1 over-expression and consequent effects on signaling. Similar to breast cancer, FRA-1 enhances the migratory and invasive capacity of pancreatic cancer cells. The finding of a cooperative effect between MUC1 and FRA-1 that resulted in substantial increases in migration and invasion addresses in part the finding that these factors show differential effects on transcription of different genes. It is likely that one function of MUC1 is to integrate morphogenetic and oncogenic signals that arise from cell surface structural conditions, cytokine and growth factor stimulation and steady state signaling within the cell [19] to help enact programs of transcriptional response to these composite sets of stimuli from the cellular microenvironment and internal signaling.
apparatus. Programs of cellular activity (e.g. EMT, cell migration, cell division, other cellular functions) require differential transcriptional responses (up-regulation and down-regulation of different sets of genes), and so it is not surprising that an integrator of signaling such as MUC1 would act as both a co-activator and a co-repressor. For results examined here, inhibiting ERK activation or knocking down expression of FRA-1 produced similar effects in MUC1 expressing cells, which supports the hypothesis that MUC1 integrates ERK signaling with morphometric signals related to motility and invasion. Furthermore, we demonstrated that MUC1 enhanced steady state ERK activation in pancreatic cancer cells, further supporting the link between ERK activation and the functional activity of MUC1 and FRA-1. Previous studies have also demonstrated the capacity for MUC1 to promote ERK mediated signaling, however, these studies did not evaluate the impact on downstream transcriptional machinery[81, 86]. These results serve as the first reported evidence of cooperative signaling between MUC1 and FRA-1. This finding could have important implications not only in pancreatic cancer, but also in other cancers with aberrant expression of cell surface mucin proteins that engage in signal transduction [19].

Despite the known synergy between AP-1 and oncogenic Ras, few studies have examined the expression of AP-1 proteins in pancreatic cancer, which contains K-Ras mutations in a vast majority of cases [128, 287]. Our analysis of data from the GEO database suggested that FRA-1 is transcriptionally upregulated during the progression from normal to cancerous pancreatic tissue and FRA-1 mRNA may also be upregulated as the tumor progresses to metastasis. Immunohistochemistry supported these analyses in part, as pancreatic tumors exhibited robust nuclear staining for FRA-1 and expression was retained in liver metastases. FRA-1 staining was absent in samples of normal pancreas. A set of FRA-1 target genes identified in studies of colorectal cancer studies was confirmed here for pancreatic cancer. Recently, it has been proposed that
pancreatic cancer consists of 4 distinct subtypes based on genomic analyses [18]. Interestingly, the squamous subtype of pancreatic cancer proposed exhibits high expression of FOSL1, TGFB2, SNAI2, and FN1; all genes we’ve studied within the FRA1:EMT phenotype. This suggests that our proposed FRA1:EMT subset and the squamous subtype of pancreatic cancer may be the same group. Immunohistochemistry analysis of clinical samples obtained at autopsy provided additional support for the hypothesis that FRA-1 was associated with expression of these genes; however, it was also apparent that the levels of expression of proteins encoded by the target genes are influenced by other factors. Future studies in which the tumors can be stratified according to the recently identified subtypes may allow for easier correlations to be drawn. Additionally, the dependence of FRA-1 activity on ERK activation suggests that tumors (such as pancreatic cancer) bearing activating mutations with the Ras-Raf-MEK-ERK cascade are likely to exhibit FRA-1 based effects.

Our results support the hypothesis that FRA-1 contributes significantly to metastasis of pancreatic cancer, at least within a subset of cases and also plays an important role in overall tumor progression. Reduction of FRA-1 expression by as little as 2-3 fold produced significant reductions in primary site tumor growth in an orthotopic model of pancreatic cancer. Metastases were also reduced, though they were not completely inhibited. Recent studies have highlighted a potential role for FRA-1 in anchorage independent growth [289]. Other studies have highlighted the importance of FRA-1 in promoting YAP driven oncogenesis [290], which is important in the progression of pancreatic cancer. These results suggest FRA-1 may be a viable target to inhibit the growth and dissemination of pancreatic cancer cells. To date no specific inhibitors to FRA-1 have been characterized, though various inhibitors such as bromodomain inhibitors impact FRA-1 expression [289, 291]. As FRA-1 exhibited a number of effects
independent of MUC1, future studies focused on FRA-1 alone may provide further insight into the possibility of targeting FRA-1.

In conclusion, our work presents the first evidence that MUC1 can function by altering the composition of AP-1 protein complexes involved in transcriptional regulation. This function explains some of the effects of MUC1 on the expression of genes involved in migration and invasion, particularly those that are known targets of FRA-1. We further highlight the functional role of these changes as drivers of metastatic and invasive potential in pancreatic cancer cells. Given that 90% of pancreatic cancer patients exhibit metastatic spread at diagnosis, the mechanisms behind the early dissemination of pancreatic cancer cells need further study [1]. Whereas the mechanism identified in this manuscript identifies aspects of the biology of MUC1 in modulating transcriptional effects, our in vivo and in vitro studies suggest FRA-1 can independently contribute to effects on tumor progression. Additional study of FRA-1 in pancreatic tumor specimens is warranted, as is further study of the specific and redundant functions of c-Jun heterodimers in pancreatic cancer. Future studies should also be undertaken to identify potential therapeutic targets of specific AP-1 heterodimers.
**Figure 4.1: MUC1 Increases Expression of c-Jun Protein**

A-B) Cytoplasmic and nuclear fractions of S2013.Neo and MIF cells were western blotted for c-Jun, phosphoJun, and MUC1; H2B blotting was evaluated for normalization and purity assessment. C) Cytoplasmic and nuclear fractions of cell lines established from the tumors of KPC mice that either expressed (MUC1 WT) or lacked MUC1 (MUC1 KO) were blotted for c-Jun and MUC1 expression with H2B used for normalization and purity assessment. D) Whole cell lysates prepared from normal mouse pancreas and tumors derived from KPC mice (either MUC1 WT or KO) and were blotted for expression of c-Jun and MUC1 with β-actin as a loading control.
Figure 4.1

   
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<td>-</td>
<td>1.0</td>
<td>2.8</td>
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B. Panc.Neo  Panc1,MUC1  Panc.Neo  Panc1,MUC1
   
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<td>-</td>
<td>1.0</td>
<td>1.7</td>
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</table>

C. KPC KO cell line  KPC WT cell line  KPC KO cell line  KPC WT cell line
   
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<tr>
<th>Cytoplasmic</th>
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<th>IB: c-Jun</th>
<th>IB: MUC1</th>
<th>IB: H2B</th>
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<td>-</td>
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<td>1.0</td>
<td>1.3</td>
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</table>

D. MUC1 KO Pancreas  MUC1 WT Pancreas  KPC KO  KPC WT
   
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<tr>
<th>Normal</th>
<th>Tumor</th>
<th>IB: c-Jun</th>
<th>IB: MUC1</th>
<th>IB: β-actin</th>
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<tr>
<td>-</td>
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<td>1.0</td>
<td>1.4</td>
<td></td>
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</table>
Figure 4.2: MUC1 Enhances the Interaction of c-Jun and FRA-1 in Pancreatic Cancer Cells

A) Proximity ligation assay examining interactions of c-Jun with the proteins ATF2, c-Fos, and FRA-1 in S2013.Neo and MIF cells. Experiments were performed in four independent assays; with multiple fields were quantified for each experiment. Representative fields are shown and red dots indicate protein-protein interaction. B) Quantification of interactions between c-Jun and associated partners. Quantification was performed using the Blobfinder program and results presented as the average number of interactions per cell±SEM. Significance was assessed using Student’s t-test p<0.05 was considered significant. C) Comparison of the nuclear interactions of c-Jun and the associated proteins in S2013.Neo and MIF cells. Results represent the percentage of nuclear interactions/total interactions±SEM. Significance was assessed with Student’s t-test D) FRA-1 was immunoprecipitated from Neo and MUC1 overexpressing cell lines. The association of FRA-1 and c-Jun was then assessed by western blot analysis of co-immunoprecipitated c-Jun. Levels of c-Jun were normalized based upon amount of FRA-1 pulled down and compared between Neo and MUC1 cell lines. Western blot analysis of input confirms MUC1 expression.
Figure 4.2

A. 

S2013.Neo  S2013.MIF

c-Jun:ATF2

c-Jun:c-Fos

c-Jun:FRA-1

B. 

c-Jun:ATF2 Interactions

S2013.Neo  S2013.MIF

C-Jun:ATF2 Interactions

S2013.Neo  S2013.MIF

** p<0.01

C-Jun:c-Fos Interactions

S2013.Neo  S2013.MIF

** p<0.01

C-Jun:FRA-1 Interactions

S2013.Neo  S2013.MIF

** p<0.01

C. 

Percentage of Nuclear Interactions

S2013.Neo  S2013.MIF

*** p<0.001

D. 

IP: FRA-1  IgG  Panc1.Neo  Panc1.MUC1

IB: FRA-1  IB: c-Jun

Input

1.0  1.4

IB: MUC1


IB: FRA-1  IB: c-Jun

Input

1.0  2.1

IB: MUC1
Figure 4.3: ERK Activity and FRA-1 Drive Invasion and Migration of Pancreatic Cancer Cells

A) Cytoplasmic and nuclear fractions were isolated from S2013.Neo and MIF cells and western blot analysis performed for total ERK1/2, phosphoERK1/2, and MUC1. H2B was used for normalization and purity assessment. Densitometry values are shown below the figures. B) S2013.Neo and MIF cells were serum starved for 24 hours, treated with the MEK inhibitor U0126 or DMSO control for 2 hours, then stimulated with 10% FBS containing media to induce ERK activation. Western blots for phosphoERK1/2 and phosphoFRA-1. C-F) Effects of inhibiting ERK Signaling (U0126), knocking down FRA1 mRNA, or overexpressing FRA1 mRNA on Migration (C & E) and Invasion (D & F) in the context of low or high expression of MUC1.
Figure 4.3

A. 

<table>
<thead>
<tr>
<th></th>
<th>Cytoplasmic</th>
<th>Nuclear</th>
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<tbody>
<tr>
<td>S2013.Neo</td>
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</tr>
<tr>
<td>S2013.MIF</td>
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<td>1.0</td>
</tr>
<tr>
<td>S2013.Neo</td>
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</tr>
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<td>S2013.MIF</td>
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</table>

IB: ERK1/2
IB: phosphoERK1/2
IB: MUC1
IB: H2B

B.

FBS Stimulation U0126 (10 μM)

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<thead>
<tr>
<th></th>
<th>+</th>
<th>+</th>
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<td>1.0</td>
<td>1.7</td>
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<td>S2013.MIF</td>
<td>1.0</td>
<td>0.4</td>
<td>1.0</td>
<td>0.5</td>
</tr>
</tbody>
</table>

IB: pERK1/2
IB: pFRA1
IB: MUC1
IB: b-actin

C.

- Parental
- U0126 Treated
- FRA1 knockdown
- FRA1 overexpression

D.

- Untreated
- U0126 Treated
- FRA1 knockdown
- FRA1 overexpression

E.

- Parental
- FRA1 knockdown
- FRA1 overexpression

F.

- Parental
- FRA1 knockdown
- FRA1 overexpression

*p<0.05  **p<0.01  ***p<0.001
Figure 4.4: ERK Activity is Increased in Panc1.MUC1 Cells

A) Western blot analysis evaluating the expression of total ERK1/2 and phosphoERK1/2 in Panc1 cells. Relative amounts were determined by densitometry using H2B as a loading control. Calculations were performed for each localization and are for ERK2 only. Results show increased levels of phosphoERK with MUC1 expression.
Figure 4.4

<table>
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<tr>
<th></th>
<th>Panc1,Neo</th>
<th>Panc1,MUC1</th>
<th>Panc1,Neo</th>
<th>Panc1,MUC1</th>
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<tbody>
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<td>0.9</td>
<td>1.0</td>
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<td>IB:phosphoERK1/2</td>
<td>1.0</td>
<td>1.3</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>IB:MUC1</td>
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<tr>
<td></td>
<td>IB:H2B</td>
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**Figure 4.5: Validation of FRA-1 Knockdown and Overexpression Cell Lines**

**A)** Characterization of FRA-1 knockdown cell lines. FRA-1 expression was knocked down by shRNA and expression of FRA-1 was assessed by both western blot analysis and RT-PCR. Reduction in FRA-1 expression was similar between the Neo and MUC1 expressing counterparts.  

**B)** Characterization of FRA-1 levels following stable overexpression of HA-tagged FRA-1 construct. Lysates were probed for FRA-1 expression using an anti-HA antibody and MUC1. Actin served as a loading control. Both the Panc1 and S2013 cell lines express similar levels of FRA-1 independent of MUC1 expression allowing for comparisons across cell lines.
Figure 4.5

A.

<table>
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<tr>
<th>S2013Neo</th>
<th>S2013Neo-FRAkd</th>
<th>S2013MIF-FRAkd</th>
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<tbody>
<tr>
<td>IB: FRA-1</td>
<td>IB: MUC1</td>
<td>IB: β-actin</td>
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</table>

IB: FOSL1 expression

Log2 Fold Change (KO/parental)

Neo KO/parental vs. MIF KO/parental

B.

<table>
<thead>
<tr>
<th>Panc. Neo</th>
<th>Panc. Neo-FRA1</th>
<th>Panc. MUC1</th>
<th>Panc. MUC1-FRA1</th>
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<tbody>
<tr>
<td>IB: HA</td>
<td>IB: MUC1</td>
<td>IB: β-Actin</td>
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</table>

IB: FOSL1 expression

Log2 Fold Change (KO/parental)

Panc. Neo KO/parental vs. Panc. MUC1 KO/parental
Figure 4.6: FRA-1 Expression Alters Cellular Morphology

Images of cancer cells with and without FRA-1 overexpression were taken using an EVOS digital microscope. Cells were then examined for changes in cellular morphology. Both Panc1 (A) and S2013 cells (B) exhibit increased numbers of cells with cellular projections when FRA-1 was overexpressed (indicated by arrows).
Figure 4.6

A.

<table>
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<tr>
<th>- FRA-1</th>
<th>+ FRA-1</th>
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<tbody>
<tr>
<td>Panc1.Neo</td>
<td>Panc1.MUC1</td>
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B.

<table>
<thead>
<tr>
<th>- FRA-1</th>
<th>+ FRA-1</th>
</tr>
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<tbody>
<tr>
<td>S2013.Neo</td>
<td>S2013.MIF</td>
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</table>
Figure 4.7: Knockdown of FRA-1 Decreases Tumor Growth and Metastasis

A-B) Tumor weight and Tumor volume were plotted for mice injected with S2013.Neo (n=12) or S2013.Neo-FRA1 kd (n=12) cells. The mean was calculated ± SD. Knockdown of FRA-1 resulted in significant reduction of both weight and volume (Bonferroni adjusted p-values following ANOVA and Bonferroni method for multiple comparisons).

C) Presence of ascites or metastasis was assessed for each individual mouse and the fraction of total mice for each condition was calculated.

D-E) Tumor weight and volume plotted for mice injected with S2013.MIF (n=13) or S2013.MIF-FRA1 kd (n=13) cells. The mean was calculated ± SD (Bonferroni adjusted p-values following ANOVA and Bonferroni method for multiple comparisons).

F) Presence of ascites or metastasis was assessed and presented for each individual mouse.
Figure 4.7

A. Tumor weight (g)

B. Tumor Volume (mm³)

C. Fraction of Mice

D. Tumor Weight (g)

E. Tumor Volume (mm³)

F. Fraction of Mice
Figure 4.8: FOSL1 is Overexpressed in Pancreatic Ductal Adenocarcinoma Samples

A) Gene expression data from the NCBI GEO dataset GSE16515 was analyzed for expression of FOSL1 (FRA1) using microarray expression values. Expression values from tumor and normal samples are presented. Statistical comparison of the groups was performed using the Generalized Estimating Equation (GEE) to account for the paired tumor and normal samples. Analysis revealed a significant increase in FOSL1 expression in the tumor group (p<0.001) as compared to normal. B) To confirm tumor samples upregulated FOSL1 16 paired tumor and normal samples were compared. The log$_2$Fold Change in gene expression for tumor/normal was calculated and plotted. 15 of 16 samples show upregulation in the tumor. 8 of 16 showed an upregulation greater then 2 fold (log$_2$Fold Change >1) C) Gene expression data for staged pancreatic tumor specimens was mined using the NCBI GEO dataset GSE42952. The expression of FOSL1 was plotted for each specified staging with absent calls ignored. D) Immunohistochemistry was performed to evaluate the protein expression of FRA-1 in pancreatic cancer. A heatmap representing relative staining for FRA-1 was generated using R. Scoring was based upon intensity of stain observed only within tumor cells. Representative images for the scoring are presented below the heat map.
Figure 4.8

A. 

**relative FOSL1 expression**

B. 

**FOSL1**

Paired sets of Cancer and Normal Tissue

C. 

**Log2 FOSL1 expression**

** *** p<0.001

D. 

Primary Metastasis

Normal Pancreas 1 Normal Pancreas 2

RAP46 RAP59 RAP61 RAP62 RAP63 RAP64 RAP65 RAP66 RAP67 RAP68 RAP69 RAP70 RAP71 RAP72 RAP73 RAP74
Figure 4.9: Analysis of FRA-1 Expression in PDAC Samples

Tissue microarrays were stained for expression of FRA-1. Samples were collected from the UNMC Rapid Autopsy Program. Each number represents an individual patient. Primary and metastatic sites were stained and compared to normal pancreas. Metastatic sites were collected from the liver unless otherwise indicated.
Figure 4.10: Pancreatic Cancer Samples Exhibit Similar FRA1:EMT Signature as Colorectal Cancer Cells

A-F) Gene expression data from the NCBI GEO dataset GSE16515 for 6 FRA1:EMT signature genes (FN1, ZEB1, SNAI2, AXL, TGFB2, SMAD3) were plotted. For genes with multiple probes the average of all probes was used. Only tumors showing >2-fold upregulation of FRA-1 were used for this analysis.
Figure 4.10

A. FN1

B. ZEB1

C. SNAI2

D. AXL

E. TGFB2

F. SMAD3
Figure 4.11: Analysis of Slug Expression in PDAC

Tissue microarrays were stained for expression of Slug. Samples were collected from the UNMC Rapid Autopsy Program. Each number represents an individual patient. Primary and metastatic sites were stained and compared to normal pancreas. Metastatic sites were collected from the liver unless otherwise indicated.
**Figure 4.12: Analysis of ZEB1 Expression in PDAC Samples**

Tissue microarrays were stained for expression of ZEB1. Samples were collected from the UNMC Rapid Autopsy Program. Each number represents an individual patient.

Primary and metastatic sites were stained and compared to normal pancreas.

Metastatic sites were collected from the liver unless otherwise indicated.
Figure 4.13: Heatmap of Immunohistochemistry Studies

Heatmaps were generated based upon staining of FRA-1, Slug, and ZEB1 in pancreatic cancer samples. Scoring was based upon relative intensity of staining and each TMA was scored independently to avoid bias based upon antibody differences. Scoring was restricted to tumor cells.
Figure 4.13

Primary Site

Metastatic Site

FRA-1
Slug
ZEB1
Figure 4.14: MUC1 Regulates Specific AP-1 and FRA-1 Targets

A-B) RT-PCR studies were performed to examine the impact of FRA-1 on the MUC1 regulated genes MMP1 and CTGF. FOSL1 expression was measured to confirm overexpression or knockdown of FRA-1. Results were analyzed by 2-way ANOVA and p-values represent comparison of FRA-1 overexpression and knockdown lines to parental unless otherwise indicated by lines. MUC1 expression caused significant alterations to expression of MMP1 and CTGF. Alteration of FRA-1 expression had no impact on expression in S2013.MIF, but significantly altered expression of these genes in S2013.Neo.

C-D) Additional RT-PCR studies were performed to examine the impact of MUC1 and FRA-1 on expression of putative FRA1:EMT genes. Results were analyzed by 2-way ANOVA for multiple comparisons and p-values indicate significant differences between parental and FRA-1 knockdown cell lines unless otherwise indicated by lines. In S2013 cells (C) MUC1 caused significant increases in expression of these genes. Loss of FRA-1 abrogated this effect and restored expression to levels similar to S2013.Neo cells. Similar effects were observed for the regulation of AXL in Panc1 cells (D).
Figure 4.14

A. S2013

B. Panc1

C. S2013

D. Panc1

* p<0.05, ** p<0.01, *** p<0.001
**Figure 4.15: MUC1 and ERK Cooperate to Drive Association of c-Jun and FRA-1**

Schematic representation of our proposed mechanism in MUC1 expressing pancreatic cancer cells. Phosphorylation of the MUC1 cytoplasmic tail drives downstream activation of ERK, likely involving the association of MUC1 with receptor tyrosine kinases. Increased ERK activity results in phosphorylation of FRA-1 and promotes dimerization with c-Jun. The transcriptional complex is then stabilized and allows for expression of genes involved in migration, invasion, and overall tumor progression.
Figure 4.15

Pro-invasive genes
Pro-migratory genes
Tumor progression
Chapter V: FRA-1 Knockdown Confers Additive Benefits to BET Inhibition
1. Key Findings
   - The impact of BET inhibition on the expression of FRA-1 is variable and likely depends on multiple factors
   - Overexpression of FRA-1 does not protect cells from growth inhibition with BETi
   - Knockdown of FRA-1 confers additive effects on growth inhibition with BETi
   - Knockdown of FRA-1 does not synergize with G1/G0 arrest induced by BETi
   - Knockdown of FRA-1 results in decreased expression of pro-growth genes and increased expression of cell death/cytostatic genes
2. Introduction

Fos-related antigen 1 (FRA-1) is a member of the Fos family of proteins [126], which together with the Jun family of proteins, comprise the dimeric transcription factor AP-1 [121]. Initial discovery of the viral oncoproteins v-Jun and v-Fos highlighted the activity of AP-1 in cancer and has led to extensive exploration of this pathway for targeted therapy in cancer [121, 292]. The combinatorial diversity of AP-1 that arises from its capacity to form 18 potential dimeric combinations has led to the demonstration that AP-1 imparts a wide range of effects, including promotion of cellular growth, apoptosis, and invasive behavior [121]. These effects are dependent on the composition of the dimer and are often cell type dependent [121, 165, 198]. FRA-1 has been identified as a significant driver of invasion and metastasis in breast cancer and colorectal cancer, however, the impact of FRA-1 on pancreatic cancer remains relatively unexplored [170, 186, 188, 189].

Pancreatic cancer remains one of the most difficult cancers to treat, with a 5-year survival rate hovering around 6-7% [1]. Current therapies are largely ineffective and as a result, there is a vital need to identify novel targets. Recently, we found that FRA-1 expression is increased in a significant fraction of pancreatic ductal adenocarcinoma (PDAC) samples [169]. Enhanced expression of FRA-1 in pancreatic cancer cells increased migratory and invasive properties, similar to effects observed in breast cancer cells. Knockdown of FRA-1 diminished these properties and resulted in diminished tumor growth and metastasis in vivo. Additional studies have supported a role for FRA-1 in the three-dimensional growth of pancreatic cancer cells in collagen [289]. These studies suggest that FRA-1 may be a potential therapeutic target in pancreatic cancer.

While there are no current therapies directed against FRA-1, treatment of cells with inhibitors of bromodomain and extracellular terminal (BET) proteins has shown a
propensity to decrease expression of FRA-1 and c-Myc [289, 291]. BET expression is deregulated in a host of cancers and there are currently several clinical trials in progress examining the efficacy of BET inhibition (BETi). In pancreatic cancer, BETi has shown the ability to inhibit growth both in vitro and in patient-derived xenografts [293]. Despite potential effects on FRA-1, most studies of BETi have focused on its effects with c-Myc [291, 294-296]. One study in lung cancer showed that overexpression of FRA-1 failed to protect cells from BETi [297]. Our previous results implicating FRA1 in pancreatic cancer [169] led us to examine how expression of FRA-1 impacted the efficacy of BETi on pancreatic cancer, and whether dual inhibition of FRA-1 and BET proteins would result in synergistic decreases in tumor growth. Furthermore, we attempted to confirm that BET inhibition caused decreased expression of FRA-1.

We found that treatment of pancreatic cancer cell lines with the BET inhibitors JQ1 and OTX015 failed to consistently decrease expression of FRA-1 in 2-dimensional growth. Similar to studies in lung cancer, overexpression of FRA-1 did not protect cells from BETi. However, knockdown of FRA-1 in conjunction with BETi resulted in an additive decrease in cellular growth, apparently through independent molecular mechanisms of action. We observed no cooperative effects on cell cycle arrest in cells treated with BETi and FRA-1 knockdown; however, RNA-seq analysis of FRA-1 knockdown cells revealed significant alterations in cell growth and survival pathways, suggesting that loss of FRA-1 decreases overall cellular viability. Our results suggest that targeting of FRA-1 is useful and may increase the therapeutic effect of BETi.

3. Results

BET Inhibition Exhibits Variable Impact on FRA-1 Expression
Previous studies have shown the treatment of cells with BET inhibitors commonly results in decreased expression of FRA-1 [289, 291, 298]. To determine if JQ1 or OTX015 treatment downregulated FRA-1 in pancreatic cancer cell lines, we treated Colo357, AsPC1, Panc1, and S2013 cell lines with increasing concentrations of either JQ-1 or OTX015 (50nM to 500nM). Cells were grown as a monolayer and treated for 48 hours before lysis. Western blot analysis of FRA-1 expression showed mixed responses to BET inhibition. Both Colo357 (Figure 5.1A) and AsPC1 (Figure 5.1C) exhibited no decrease in expression of FRA-1 in response to either JQ1 or OTX015. Interestingly, AsPC1 showed increased expression of FRA-1 as the dose of BET inhibitor was increased. Panc1 (Figure 5.1B) and S2013 (Figure 5.1D) cells showed modest decreases in the expression of FRA-1 with higher doses of OTX015, though no decrease of expression was observed with JQ-1. Thus, we conclude that BETi alone does not consistently modulate expression of FRA-1 in pancreatic cancer cells.

To evaluate the role of FRA-1 expression on the efficacy of BET inhibitors, we generated both FRA-1 knockdown and FRA-1 overexpression cell lines using the S2013 and Panc1 pancreatic cancer cell lines (Figure 5.1E and 5.1F). Expression of FRA-1 was assessed by both western blot analysis and RT-PCR analyses to confirm that FRA-1 was appropriately knocked-down or overexpressed.

**Knockdown of FRA-1 Expression Confers Additive Impacts on Cell Growth with BET Inhibition**

Previous studies examining the mechanisms of BET inhibition have focused primarily on the role of c-Myc on growth. Given that recent studies by our lab and others have demonstrated that FRA-1 influences pancreatic cancer cell growth both *in vitro* and *in vivo*, we sought to examine whether knockdown of FRA-1 expression would cooperate with, or inhibit effects of BET inhibition. Parental, FRA-1 overexpressing, and FRA-1
knockdown cell lines were treated with increasing doses of JQ1, OTX015, or a DMSO vehicle control. Methylene Blue assays were performed to assess cellular proliferation over time.

We found that effects of BET inhibition on cell proliferation were not affected synergistically by FRA-1 overexpression or knock-down in both S2013 (Figure 5.2A-C and Figure 5.3A-C) and Panc1 (Figure 5.4A-C and Figure 5.5A-C) cells. This shows that FRA-1 does not protect cells from BET inhibition. Nonetheless, significant inhibition of growth was observed in most cell lines at 250nM of either JQ1 or OTX015 after 96 hours, and loss of FRA-1 expression significantly decreased growth independent of BET inhibition for both cell lines in an additive manner. As a result, the combination of FRA-1 knockdown and BET inhibition was significantly better than BET inhibition alone (Figures 5.2-5.5). This was true for both S2013 and Panc1 cells at virtually every dose of BET inhibitor, though 1 µM OTX015 showed no significant difference in Panc1 cells with knockdown of FRA-1 (Figure 5H).

**Loss of FRA-1 Does Not Synergize with BETi mediated G1 Arrest**

As loss of FRA-1 alone is sufficient to decrease cellular growth, we sought to examine whether the mechanism was independent or synergistic with BETi. Previous studies of FRA-1 have shown that loss of FRA-1 can result in alterations in cyclin expression [299-301]. This in turn leads to slower progression through the cell cycle and decreased cellular proliferation. To test whether FRA-1 caused aberrations in cell cycle progression, we performed cell cycle analysis on parental and FRA-1 knockdown lines in the presence or absence of BET inhibition. For these studies, the 500 nM concentration was chosen based on results showing significant differences in growth between the parental and FRA-1 knockdown cell lines. BET inhibition caused accumulation of cells in G1, consistent with the literature [294, 302]. Loss of FRA-1 resulted in accumulation in
G2/M in Panc1 cells and accumulation in S phase in S2013 cells, suggesting an effect on cell cycle progression; however, we did not observe synergistic accumulation of cells in G1/G0 (Figure 5.6A-B).

To test whether FRA-1 expression influenced expression of cyclins, we performed western blot analysis of lysates from 96-hour treatment time points. Western blots for Cyclin A and Cyclin D showed that FRA-1 loss promoted decreased expression in response to BET inhibition in Panc1 cells (Figure 5.6A). However, these effects were not observed in S2013 cells, suggesting that other factors are involved in decreasing cellular growth (Figure 5.6B).

**Loss of FRA-1 Results in Decreased Cellular Fitness**

We performed RNA-seq analysis of the S2013.Neo and S2013.piKO FRA1 cell lines to identify mechanisms that may explain the decreased cellular proliferation observed in FRA-1 knockdown cells. Differences in gene expression between the cell lines were analyzed using QIAGEN’s Ingenuity® Pathway Analysis to identify cellular pathways that were altered by decreased FRA-1 expression [265]. Heatmaps generated using QIAGEN’s Ingenuity® Pathway Analysis demonstrate the increased expression of cell death genes (Figure 5.6C) and decreased expression of proliferative genes (Figure 5.6D), suggesting that loss of FRA-1 may diminish cellular viability. Additionally, pathways associated with cellular movement and motility were significantly impacted, which is consistent with the known role for FRA-1 in the migration and invasion of cells (Figure 5.6E).
4. Discussion

Bromodomain inhibitors have been studied recently as potential therapeutics for a wide range of cancers [303-305]. Many of the currently available inhibitors elicit cytostatic effects on tumor growth, rather than cytotoxic effects [306, 307]. As such, a priority is identifying potential targets that can synergize with BET inhibitors, or in combination enhance cytotoxicity. Previous studies have identified FRA-1 as a factor commonly impacted by BET inhibition [289, 291, 298]. In the studies reported here, we sought to examine whether targeting FRA-1 would synergize with the BET inhibitors JQ1 and OTX015. Whereas previous studies in breast cancer cell lines found that JQ1 treatment resulted in decreased expression of FRA-1, we found no consistent impact of BET inhibition on FRA-1 expression in pancreatic cancer. BET inhibition is known to have variable effects with known targets such as Myc, the most characterized target [293]. Interestingly, FRA-1 has been shown to control expression of several known Myc targets, and in some instances FRA-1 affects expression patterns for genes that may compensate for loss or lack of Myc expression [299]. This may explain in part the context dependent effects of BET inhibitors.

Cells with reduced FRA-1 activity through knockdown or treatment with BET inhibitors exhibited a significant reduction in growth as compared to parental lines. However, this effect appeared to be additive, as loss of FRA-1 expression alone reduced cellular growth, albeit to a lesser extent. Our finding that overexpression of FRA-1 failed to protect pancreatic cancer cells from the growth inhibitory effects of BET inhibitors provided additional evidence that BET inhibitors function independently of FRA-1. This is consistent with results reported in lung cancer [297]. BET inhibition is reported to induce G1/G0 arrest, which we also observed [306, 307]. Knockdown of FRA-1 failed to enhance the arrest induced by either JQ1 or OTX015, further suggesting that oncogenic
mechanisms of FRA-1 activity can be independent of BETi. As well, western blots examining cyclin levels failed to identify a consistent mechanism of growth arrest. For Panc1 cells, loss of FRA-1 induced a cooperative reduction of Cyclin A and Cyclin D1 expression in response to BET inhibition, however, these effects were not observed in S2013 cells, suggesting that the growth inhibitory effects of knocking down FRA-1 are not due to effects on these cyclins.

RNA-seq analysis of the S2013 cell lines suggested that the additive effects of FRA-1 knockdown and BET inhibition resulted from reduced expression of pro-growth genes and consequent decreases in cellular proliferation. Previous studies in triple negative breast cancer demonstrated that FRA-1 enhanced cellular growth and expression of pro-growth genes [185]; however, this has not been previously reported for pancreatic cancer. Interestingly, expression of FRA-1 has been shown to occur in a recently identified putative subtype of pancreatic cancer [18]. Taken together, these results suggest that FRA-1 may be a viable target in a range of cancers and that inhibiting AP-1 complexes may be additive to effects of BET inhibitors. Unfortunately, targeting other AP-1 complexes has proven extremely difficult in the clinical setting [308]. Recently a selective AP-1 inhibitor (T-5224) demonstrated efficacy in a few preclinical models of cancer [309]. Future studies examining the efficacy of this drug in pancreatic cancer may provide insight into the utility of broadly targeting AP-1.
Figure 5.1: FRA-1 Exhibits Variable Responses to BET Inhibition

A) Colo357, B) Panc1, C) AsPC1, and D) S2013 cells were treated with increasing doses of the BET inhibitors OTX015 or JQ1 and cell lysates were evaluated for the impact on expression of FRA-1. β-actin was used as a loading control. Western blots were repeated three times to ensure reproducibility. E) FRA-1 knockdown cell lines were evaluated for FRA-1 expression by western blot analysis and RT-PCR analysis. Overall gene expression was normalized to 18s rRNA for RT-PCR analysis. Both Panc1 and S2013 cell lines showed knockdown of FRA-1 expression. F) FRA-1 overexpression cell lines were generated in Panc1 and S2013 cells. Expression of HA tagged FRA-1 was confirmed by western blot analysis for the HA-tag. Increased expression of FRA-1 was also confirmed by RT-PCR analysis after normalizing to 18s rRNA.
Figure 5.1

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IB: β-actin

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IB: FRA-1
IB: β-actin
Figure 5.2: JQ1 Treatment Results in Additive Decreases in Cell Growth with FRA-1 Knockdown in S2013 Cells

A) S2013 parental cells, B) HA-FRA1, and C) FRA-1 knockdown cells were treated with different concentrations of JQ1 or DMSO control. Cellular growth was measured by absorbance at 650 nm with methylene blue assay. Absorbance was plotted over time to generate growth curves and statistical analyses were performed using 2-way ANOVA comparing to the control (*p<.05, **p<.01, ***p<0.001). Absorbance values represent average of 6 wells for each cell line to control for variability in cell plating and growth due to plate position. D-H) The growth curves for the parental, HA-FRA1, and FRA-1 knockdown lines were compared at each individual concentration. Differences between the treated parental lines and the FRA-1 knockdown line were assessed using 2-way ANOVA for each time point (*p<0.05, **p<0.01, ***p<0.001). Each assay was performed multiple times to ensure reproducibility.
Figure 5.3: OTX015 Treatment Results in Additive Decreases in Cell Growth with FRA-1 Knockdown in S2013 Cells

A) S2013 parental cells, B) HA-FRA1, and C) FRA-1 knockdown cells were treated with different concentrations of OTX015 or DMSO control. Cellular growth was measured by absorbance at 650 nm with methylene blue assay. Absorbance was plotted over time to generate growth curves and statistical analyses were performed using 2-way ANOVA comparing to the control (*p<.05, **p<.01, ***p<0.001). Absorbance values represent average of 6 wells for each cell line to control for variability in cell plating and growth due to plate position. D-H) The growth curves for the parental, HA-FRA1, and FRA-1 knockdown lines were compared at each individual concentration. Differences between the treated parental lines and the FRA-1 knockdown line were assessed using 2-way ANOVA for each time point (*p<.05, **p<.01, ***p<0.001). Each assay was performed multiple times to ensure reproducibility.
Figure 5.3

A. S2013.Neo OTX015Tx

B. S2013 HA-FRA1 OTX015Tx

C. S2013 pKO FRA1 OTX015Tx

D. 50 nM OTX015

E. 100 nM OTX015

F. 250 nM OTX015

G. 500 nM OTX015

H. 1 uM OTX015

Neo.Tx vs. pKO.Tx

* p<0.05  ** p<0.01  *** p<0.001
Figure 5.4: JQ1 Treatment Results in Additive Decreases in Cell Growth with FRA-1 Knockdown in Panc1 Cells

A) Panc1 parental cells, B) HA-FRA1, and C) FRA-1 knockdown cells were treated with different concentrations of JQ1 or DMSO control. Cellular growth was measured by absorbance at 650 nm with methylene blue assay. Absorbance was plotted over time to generate growth curves and statistical analyses were performed using 2-way ANOVA comparing to the control (\(p<.05\), \(**p<.01\), \(***p<0.001\)). Absorbance values represent average of 6 wells for each cell line to control for variability in cell plating and growth due to plate position. D-H) The growth curves for parental, HA-FRA1, and FRA-1 knockdown lines were compared at each individual concentration. Differences between the treated parental lines and the FRA-1 knockdown line were assessed using 2-way ANOVA for each time point (*\(p<.05\), **\(p<.01\), ***\(p<0.001\)). Each assay was performed multiple times to ensure reproducibility.
Figure 5.5: OTX015 Treatment Results in Additive Decreases in Cell Growth with FRA-1 Knockdown in Panc1 Cells

A) Panc1 parental cells, B) HA-FRA1, and C) FRA-1 knockdown cells were treated with different concentrations of OTX015 or DMSO control. Cellular growth was measured by absorbance at 650 nm with methylene blue assay. Absorbance was plotted over time to generate growth curves and statistical analyses were performed using 2-way ANOVA comparing to the control (*p<.05, **p<.01, ***p<0.001). Absorbance values represent average of 6 wells for each cell line to control for variability in cell plating and growth due to plate position. D-H) The growth curves for the parental, HA-FRA1, and FRA-1 knockdown lines were compared at each individual concentration. Differences between the treated parental lines and the FRA-1 knockdown line were assessed using 2-way ANOVA for each time point (*p<.05, **p<.01, ***p<0.001). Each assay was performed multiple times to ensure reproducibility.
Figure 5.5

A. Panc1.Neo OTX015

B. Panc1.HA-FRA1 OTX015

C. Panc1.piKO FRA1 OTX015

D. 50 nM OTX015

E. 100 nM OTX015

F. 250 nM OTX015

G. 500 nM OTX015

H. 1 uM OTX015

Legend:
- Panc1.Neo ctl
- Panc1.Neo +50 nM OTX
- Panc1.HA-FRA1 ctl
- Panc1.HA-FRA1 +50 nM OTX
- Panc1.piKO FRA1 ctl
- Panc1.piKO FRA1 +50 nM OTX

Comparisons:
- Neo.Tx vs. piKO.Tx

Significance levels:
- *** p < 0.001
- ** p < 0.01
- * p < 0.05
- ns = not significant
Figure 5.6: Loss of FRA-1 Decreases Cellular Fitness but Fails to Synergize with BET Inhibition

A) Cell cycle analysis of Panc1 or B) S2013 cells with or without FRA-1 knockdown at selected time points following treatment with BET inhibitors. The percentage of cells observed within each phase is presented for each time point. Additionally, western blot analysis was performed to examine the impact of FRA-1 expression and BET inhibition on the expression of Cyclin A2 and Cyclin D1. β-actin served as a loading control. C) Heatmap generated from Ingenuity® Pathway analysis demonstrating increased expression of genes involved in cell death or D) cellular proliferation. Shading is based upon differential Z-score. E) List of significantly altered pathways between S2013.Neo and S2013.plKO FRA-1 cell lines identified using QIAGEN's Ingenuity® Pathway Analysis. P-values are calculated based on the odds of finding x number of genes differentially regulated within the same network.
Figure 5.6

A. Panc1

B. S2013

C. Cell Death and Survival

D. Cellular Growth and Proliferation

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Chapter VI: Impact of p53 Mutation Status on the Interaction with MUC1 and Potential Gain of Function Effects
1. Key Findings

- MUC1 preferentially interacts with the R273H hotspot mutant of p53, as compared to wildtype or R175H p53
- Expression of R273H results in increased expression of CTGF, but wildtype and R175H p53 are unable to induce CTGF
- Expression of p53 under steady state conditions has no impact on the cellular growth of Colo357 cells
- Irradiation of wildtype p53 expressing cells causes growth arrest quicker than mutant p53 expressing cells
- Irradiation induces phosphorylation of wildtype p53 and induction of PUMA, but does not result in phosphorylation of mutant p53
- UV damage induces phosphorylation of wildtype and mutant p53, but this effect is variable depending on the mutation
2. Introduction

The tumor suppressor p53 is mutated in approximately 50-75% of pancreatic ductal adenocarcinoma cases [310]. The majority of p53 mutations occur within the DNA binding domain of p53 resulting in disruptions to normal DNA binding [311]. As a result, therapeutic intervention to restore wildtype p53 function has been extensively studied [312]. While it was initially postulated that these mutations resulted in loss of p53 function, recent studies have indicated that p53 mutants retain variable DNA binding capacity and may exhibit gain-of-function effects [243, 313-317]. Indeed, in the KPC pancreatic cancer mouse model, loss of p53 tumors exhibit a distinct phenotype from tumors arising from mutant p53 [15]. Other studies have also demonstrated that a p53 null allele is not equivalent to a mutant allele, further supporting the potential for gain-of-function effects [239].

The transmembrane glycoprotein MUC1 has been shown to interact with p53 resulting in alterations to downstream transcriptional targets and changes in promoter occupancy [77, 80, 100]. Preliminary data from our laboratory suggest that these interactions are determined in part by phosphorylation of specific residues within the cytoplasmic tail of MUC1. How the mutational status of p53 influences these interactions has not been explored. To study this, we designed a doxycycline-inducible system to express specific p53 isoforms in pancreatic cancer cells. We found that the hotspot mutant R273H appears to preferentially interact with MUC1, however, both wildtype and R175H mutant p53 also interacted with MUC1. Furthermore, when we examined expression of CTGF, a gene previously suggested to be regulated by the interaction of p53 and MUC1 at upstream promoter elements [100], we found that expression of R273H caused the most robust increase in expression of CTGF as compared to wildtype or R175H p53. We also examined expression of MMP1, which is
thought to be downregulated in response to this interaction [77]. We observed that both wildtype and R175H caused a modest decrease in expression at low doses; however, R273H was unable to decrease MMP1 expression. These results suggest that the mutational status of p53 may influence particular gain-of-function effects on gene targets. Furthermore, some of these effects may be dose dependent on the overall levels of p53.

Interestingly, p53 expression did not alter the growth kinetics of Colo357 cells as assessed by methylene blue assay. This suggests that under steady state conditions these cells have become unresponsive to p53. However, when we irradiated cells we found that wildtype p53 expression caused cells to cease growth sooner than either R175H or R273H. When we examined the response of these cells to irradiation we found that wildtype p53 was phosphorylated in response to DNA damage, whereas neither mutant was phosphorylated under these conditions. Furthermore, wildtype p53 showed induction of the pro-apoptotic protein PUMA. These results suggest that specific mutants of p53 may lose responsiveness to DNA damage stressors.

3. Results

Colo357 Cells Express High Levels of Endogenous MUC1 and no p53

To identify a suitable cell line for studies involving re-expression of different isoforms of p53, we performed western blot analysis of several pancreatic cancer cell lines. In order to minimize complications from variables in MUC1 and p53 expression we sought a cell line that expressed endogenous MUC1 and either wildtype or no p53. S2013 and Panc1 cells were not usable for these assays due to lack of endogenous MUC1 expression and natural expression of mutant p53. We found that Colo357 expressed high levels of endogenous MUC1 (Figure 6.1). Furthermore, while Colo357
Designing a Dox-Inducible System for p53 Expression

As a starting point, we designed 3 distinct p53 constructs; wildtype, R175H, and R273H (Figure 6.2A). The R175H and R273H mutations are both hotspot mutations in the DNA binding domain and commonly occur in pancreatic cancer cell lines [319]. Furthermore, the R175H mutation is orthologous to the R172H mutation that was used in generation of the KPC mouse model of pancreatic cancer. These mutations also represent distinct classes of mutants, as R175H disrupts DNA binding by altering the structure of p53, whereas the R273H mutation disrupts a residue involved in direct DNA binding [311]. We cloned the human wildtype p53 cDNA sequence into the plvx.Tight-puro vector from Clontech (graciously provided by Dr. Angie Rizzino) and added a triple flag sequence to the C-terminus to facilitate detection and isolation. Site-directed mutagenesis was performed to generate mutants and sequencing was used to confirm mutations (Figure 6.2B). To validate the induction of recombinant p53 in transduced cells, increasing doses of doxycycline were applied in culture and p53 expression assessed by western blot analysis by both a p53 antibody and an antibody against the flag sequence (Figure 6.2C). Dose dependent induction of p53 was observed for all 3 constructs. Each cell line exhibited similar levels of p53 expression upon induction.

MUC1 Preferentially Interacts with the R273H Hotspot Mutant

To assess how the mutational status of p53 impacts interaction with MUC1, we performed reciprocal co-immunoprecipitation assays for both proteins. Lysates were prepared from each cell line and levels of p53 and MUC1 were similar in the sample inputs for immunoprecipitation (Figure 6.3A). Immunoprecipitation of p53 was performed...
using M2 agarose beads and western blot analysis was performed for both p53 and co-associated MUC1. We observed a modest increase in associated MUC1 with the R273H mutant; however, this result was confounded by the background signal from antibody light chain used in the immunoprecipitation (Figure 6.3B). The reciprocal experiment also showed increased association between MUC1 and R273H, supporting the hypothesis that MUC1 shows enhanced interaction with this mutant as compared to wild-type and R175H (Figure 6.3C), though there were interactions between MUC1 and all 3 forms of p53.

**Expression of R273H Causes Induction of CTGF Expression**

Previous studies have shown that the interactions between MUC1 and p53 modulate the expression of MMP1 and CTGF by binding upstream promoter elements [77, 100]. To examine whether the mutational status of p53 influenced the expression of these genes, we treated Colo357.ip53 cells with no doxycycline, 300 ng/ml, or 1000 ng/ml to induce expression of wildtype, R175H, or 273H p53 respectively in each cell line. RNA was isolated and expression of MMP1 and CTGF was evaluated by qRT-PCR (Figure 6.4). Expression of p53 was also confirmed by two independent primer sets. Induction of p53 was dose dependent and similar for each cell line as compared to their untreated parental counterpart. MMP1 did not show substantial changes in expression, though at 300 ng/ml both wildtype and R175H expression caused a modest reduction in expression. This was abrogated at 1000 ng/ml suggesting potential dose dependent effects. By comparison, CTGF expression was significantly induced by R273H expression in a dose dependent manner. Wildtype and R175H p53 also showed modest increases in CTGF, however, this was significantly less than R273H and only high dose wildtype p53 reached statistical significance (p=0.048), suggesting R273H preferentially induces CTGF expression.
Mutant p53 is Less Responsive to DNA Damage

We performed methylene blue based proliferation assays to determine if different p53 mutants influenced the growth of pancreatic tumor cells. Cells received either no treatment or 1 µg/ml doxycycline refreshed daily for 1 week. Interestingly, under steady state conditions we observed no significant impact of p53 expression on cellular growth. This was true even for expression of wildtype p53 (Figure 6.5A-D). Under normal cellular conditions, p53 often does not exert any effect on cellular growth until the cell is stressed [208]. To test whether cells expressing p53 would respond differently in response to stress conditions, we performed cell proliferation assays with 10 Gray irradiation of the cells at 48 hours, and compared these results to our non-irradiated controls. We found that irradiation decreased growth in all 3 cell lines as compared to non-irradiated controls; however, both R175H and R273H cells maintained growth near control levels until the 144 hour timepoint. In contrast, cells expressing wildtype p53 showed significantly decreased cellular growth at 120 hours (Figure 6.6A-C). These results suggest that mutant p53 may be less responsive to induction of stress responses in these cells.

To further investigate this hypothesis, we performed western blot analysis to examine the induction of the stress response following DNA damage. Cells either received no doxycycline or 1 µg/ml doxycycline with increasing doses of radiation. All 3 cell lines showed similar response profiles for phosphorylation of Chk1 and Chk2. However, western blot analysis for phosphorylation of p53 at Serine 15, a residue involved in stabilization and transcriptional activity, showed that only wildtype p53 was phosphorylated at this residue (Figure 6.7). Cells that did not receive doxycycline showed no induction of p53, confirming that the phosphorylated p53 we observed was derived from the inducible recombinant construct. Additionally, at 20 Gray irradiation,
wildtype p53 cells showed induction of the pro-apoptotic protein PUMA. This induction was not observed in mutant p53 expressing cells. This difference appears to be specific to irradiation, as UV damage was capable of inducing phosphorylation of all 3 p53 forms, though R175H showed less phosphorylation than wildtype or R273H p53 (Figure 6.8).

4. Discussion

The interaction between MUC1 and p53 has been confirmed by a number of independent studies [77, 80]. Interestingly, these studies show that MUC1 and p53 co-occupy promoters that are not regulated by p53 under normal circumstances. While previous studies have shown that phosphorylation of the MUC1 cytoplasmic tail is an important regulatory step of this interaction, no studies have evaluated the impact of p53 mutational status. As p53 is mutated in a significant fraction of pancreatic tumors, mutation of p53 could significantly impact the outcome of these interactions [310]. We evaluated how mutations impacted this interaction using wildtype, R175H, and R273H mutants of p53. We found that the R273H mutant preferentially interacted with MUC1; however, MUC1 showed interaction with all 3 p53 forms. Several studies have shown that different p53 mutants can exhibit different gain-of-function effects within cancer [235, 316]. The differential interactions suggest that MUC1 may play a role in the regulation of these gain-of-function effects in pancreatic cancer. Indeed, previous studies showing that MUC1 and p53 can alter the expression of CTGF and MMP1 were performed using S2013 cells that carry the R273H mutation [77, 100]. Our studies support a role for the R273H mutation in regulation of CTGF expression, as only our inducible R273H cell line was capable of significantly inducing expression of CTGF. Interestingly both wildtype and R175H p53 affected MMP1 expression with low dose induction; however, this effect was abrogated with higher dose expression. This
suggests that p53 mutants may also exhibit dose dependent effects on certain promoters. As mutant p53 is significantly more stable than wildtype p53, some gain-of-function effects may be a result of changes of overall protein concentration in these dose dependent experiments.

The fact that MUC1 is also capable of interacting with wildtype p53 suggests that this interaction contributes to normal cellular responses. In normal tissues MUC1 is spatially segregated from many receptor tyrosine kinases that can phosphorylate the cytoplasmic tail [26]. Similarly, p53 expression is normally tightly regulated and expressed at extremely low levels under unstressed conditions [208, 223]. However, during mechanical injury, the barriers that normally separate MUC1 and RTKs can be removed, allowing the two molecules to interact and engage in signaling. Cellular stress also causes induction and stabilization of p53. As such, it is possible that the interaction of MUC1 and p53 may represent a critical mediator of the response to cellular injury, helping the cell to make a decision about whether to live or die. Cancer cells may co-opt this mechanism and use it to survive stresses such as loss of polarity through constitutive signaling through the MUC1 cytoplasmic tail.

Interestingly, under steady state conditions expression of p53 had no impact on the growth of Colo357 cells. The fact that Colo357 also lacks detectable expression of endogenous p53 suggests that these cells may have become largely insensitive to p53 expression. Irradiating cells resulted in cellular death and appropriate phosphorylation of wildtype p53, suggesting that under stressed conditions p53 may still exert functional effects in these cells. Furthermore, cells expressing wildtype p53 exhibited an earlier decrease in proliferation in response to irradiation and induction of the pro-apoptotic protein PUMA. These effects were diminished in both mutants suggesting that these variants confer resistance to irradiation induced cell death by delaying or abrogating induction of apoptotic proteins. Several studies have shown that mutants of p53 often
fail to induce expression of the apoptotic protein Bax [242]. The interaction of MUC1 with p53 also preferentially blocks induction of Bax suggesting another means by which this signaling axis delays or inhibits apoptosis [80].

Future studies examining gain-of-function effects of these mutants at promoter elements will provide further insight into how MUC1 regulates the transcriptional machinery of tumor cells. While we have observed differential interaction between MUC1 and a few hotspot mutants of p53, design of additional mutants using the hotspots at G245, R248, R249, and R282 could provide further insight into the molecular mechanisms and effects of these interactions. Interestingly, preliminary data in our lab suggest that MUC1 interacts with p53 through its regulatory domain, whereas the hotspot mutations all reside within the DNA binding domain. This may explain why MUC1 is capable of interacting with all 3 of our p53 species; however, mutation may result in structural changes that alter the affinity of this interaction. In vitro biochemical assays of interaction may allow us to better understand these dynamics and remains an important area of future investigation.
Figure 6.1: Expression of MUC1 and p53 in Pancreatic Cancer Cell Lines

Western blot analysis screening expression of MUC1 and p53 in the pancreatic cell lines Panc1, S2013, and Colo357. Both Panc1 and S2013 cells lack endogenous MUC1 expression, requiring exogenous transfection to study MUC1. Furthermore, these cell lines express the R273H mutant of p53 at detectable levels making them unsuitable for our system. Colo357 cells represented the best combination of high endogenous MUC1 and no co-expression of mutant p53 and thus were chosen for our studies.
Figure 6.1

IB: p53

IB: MUC1(CT2)

IB: β-actin
Figure 6.2: Characterization of a Dox-Inducible p53 Expression System

We chose to study 2 hotspot mutants (R175H and R273H), as well as wildtype p53. A general schematic (A) of the expressed protein is provided highlighting functional domains, the site of mutation (star), and the attachment of a 3X Flag sequence for detection. **B)** Generation of specific site mutations was confirmed by sequencing and a chromatogram of the results is presented. The R175H and R273H mutant codons are highlighted in red. **C)** Inducible expression of p53 was confirmed by western blot analysis for both p53 (DO-1) and the Flag tag (M2). Increasing concentrations of doxycycline resulted in a dose dependent induction of p53 in all 3 cell lines. β-actin was used as a loading control. Consistent induction was confirmed by multiple replications of the dose curve.
**Figure 6.2**

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**Transactivation**

**Proline-rich Domain**

**DNA-binding Domain**

**Tetramerization Domain**

**Regulatory**

**3X Flag**

**WT p53**

**R175H p53**

**R273H p53**

**V173**

**R174**

**R175**

**C176**

**P177**

**E271**

**V272**

**R273**

**V274**

**C275**

**TAD1**

**TAD2**

**N-term**

**C-term**
**Figure 6.3: MUC1 Preferentially Interacts with the R273H Mutant**

Evaluation of the effects of mutation of p53 on interactions with MUC1: reciprocal co-immunoprecipitation assays.  **A)** Western blot analysis of input samples confirming equal concentrations of proteins were used for immunoprecipitation in all 3 cell lines.  **B)** Immunoprecipitation of p53 using M2 (anti-FLAG) agarose beads and western blot analysis of p53 and MUC1 shows slightly increased levels of MUC1 pulldown in the R273H lane; however, this interpretation is complicated by light chain reactivity detected by the secondary antibody, as seen in the IgG control lane.  **C)** Immunoprecipitation of MUC1 shows increased co-immunoprecipitated R273H p53, as compared to wildtype and R175H p53. IgG lanes confirm that the interaction is specific. Co-immunoprecipitation studies were repeated 3 independent times to ensure reproducibility.
Figure 6.3

A. Colo357

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Input

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Figure 6.4: R273H Preferentially Induces CTGF Expression

qRT-PCR analysis of p53, MMP1, and CTGF mRNA expression in Colo357.WTp53, R175H, and R273H cell lines. Expression of each gene was normalized to the untreated parental control. Induction of p53 was shown to be dose dependent and similar induction is observed for all 3 cell lines. MMP1 expression showed modest decrease with low dose expression of wildtype or R175H p53. This was abrogated with high dose expression. All 3 p53 forms promoted some induction of CTGF, however only R273H promoted a significant increase in CTGF expression in a dose dependent manner. At high doses of Dox, expression of wildtype p53 induced CTGF above statistical threshold (p=0.048). Statistics were performed using two-tailed student’s t-test comparing the doxycycline treated lines to the untreated parental control. Each sample was performed in triplicate in two independent experiments.
Figure 6.4

![Graph showing relative expression levels for various conditions.](image)

- **Colo357.WTp53 -Dox**
- **Colo357.WTp53 +300 ng/ml Dox**
- **Colo357.WTp53 +1000 ng/ml Dox**
- **Colo357.R175H -Dox**
- **Colo357.R175H +300 ng/ml Dox**
- **Colo357.R175H +1000 ng/ml Dox**
- **Colo357.R273H -Dox**
- **Colo357.R273H +300 ng/ml Dox**
- **Colo357.R273H +1000 ng/ml Dox**

* p<0.05  ** p<0.01  *** p<0.001
**Figure 6.5: Expression of p53 Does Not Impact Steady State Growth**

Methylene blue assays measuring proliferation of all 3 p53 cell lines in the presence or absence of 1 µg/ml doxycycline. Results are presented as the average absorbance at 650 nm. Expression of wildtype (A), R175H (B), or R273H (C) showed no significant impact on growth as compared to the non-dox control cells. A comparison of all 3 lines (D) shows that the form of p53 has no impact on steady state growth. Statistical analysis was performed using 2-way ANOVA. Absorbance represents an average of 6 independent wells to control for variability in plating and growth due to plate position.
Figure 6.5

A.  

B.  

C.  

D.  

Absorbance 650 nm

Time (hrs)

0  24  48  72  96  120  144

ns  ns  ns  ns  ns  ns  ns

Absorbance 650 nm

Time (hrs)

0  24  48  72  96  120  144

ns  ns  ns  ns  *** ns

Absorbance 650 nm

Time (hrs)

0  24  48  72  96  120  144

ns  ns  ns  ns  ns  ns

Absorbance 650 nm

Time (hrs)

0  24  48  72  96  120  144

ns  ns  ns  ns  *** ns

A.  WT+Dox  WT

B.  R175H+Dox  R175H

C.  R273H+Dox  R273H

D.  R273H+Dox  R175H+Dox  WT+Dox
Figure 6.6: Mutant p53 Delays the Onset of Radiation Induced Growth Inhibition

Methylene blue proliferation assays measuring cellular growth of p53 cell lines with or without 10 Gray irradiation at 48 hours. Results are presented as the average absorbance at 650 nm. With irradiation all cell lines showed slowed growth and showed loss of proliferation at 144 hours. However, both R175H (B) and R273H (C) maintained near control level growth until 144 hours, whereas wildtype p53 growth decreased 24 hours earlier (A). Statistical analysis was performed using 2-way ANOVA. Absorbance represents an average of 6 independent wells to control for variability in plating, growth due to plate position, and irradiation exposure.
Figure 6.6
Figure 6.7: Mutation of p53 Alters Activation of DNA Damage Response

Colo357. WT p53 (A), R175H p53 (B), or R273H p53 (C) were treated with increasing doses of radiation in the presence or absence of 1 µg/ml doxycycline. 1 hour post-irradiation cells were lysed and examined by western blotting for induction of stress related signals phosphoChk1, phosphoChk2, phosphoSer15-p53, as well as expression of p21 and PUMA. All 3 lines showed similar profiles for phosphorylation of Chk1 and Chk2. However, only wildtype p53 showed phosphorylation at Serine 15 and induction of PUMA expression. Western blots are representative images of multiple experiments.
**Figure 6.7**

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Figure 6.8: R175H p53 Exhibits Altered Response to UV Damage

Colo357. WT, R175H, R273H, and control cells were exposed to UV irradiation to induce DNA damage. 1 hour post UV exposure, cells were lysed and examined for phosphorylation of p53 and p21 expression by western blotting. UV damage induced phosphorylation of all 3 mutants, however, R175H showed diminished activity. Loss of p21 expression indicates cells had initiated the apoptotic response. Western blots are representative images of multiple experiments.
Figure 6.8

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<tr>
<td>UV</td>
<td>-</td>
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IB: p53 (DO-1)

IB: pSer15-p53

IB: p21

IB: β-actin
Chapter VII: Summary and Future Directions
7. Summary

The role of transmembrane mucins in the progression of cancer is well established and the knowledge of their signaling capacity continues to grow [75, 81, 86, 87, 102, 268, 320, 321]. MUC1 remains the most studied of these mucins. The capacity of MUC1 to alter the activity of different signaling cascades under different contexts has been demonstrated; however, the manner and extent to which MUC1 integrates multiple signals has not been well-characterized. We have demonstrated that the C-terminal subunit of MUC1 exists in a wide array of modified states, including numerous distinct phosphorylated forms, suggesting that MUC1 integrates several signals concurrently to affect multiple signaling and biological pathways within the cell. Furthermore, changes in biological conditions of the cell result in broad alterations in signaling and transcriptional regulation, lending support to the hypothesis that MUC1 acts as a sensor of the surrounding environmental conditions and commensurate modifier of cell activity [19, 26]. Interestingly, specific MUC1 splice variants also appear to exist in extensively modified states, suggesting additional means of regulating signaling pathways by altering the N-terminal portion of MUC1 exposed to the extracellular space. Expression of truncated glycans has also been shown to modulate the tumorigenic properties of mucins, suggesting that while the C-terminus is engaged in signaling, the N-terminal extracellular domain may play a significant role in the detection of the surroundings to initiate or modify those signals [42, 73, 322, 323].

While several studies have shown the impact of MUC1 on downstream signaling cascades such as ERK, few studies have evaluated the corresponding impact on transcriptional activity downstream of these effectors [81, 86]. We found that expression of MUC1 promoted shifts in the composition of the dimeric transcription factor AP-1 to favor c-Jun:FRA-1 dimers, seemingly due to induction of upstream ERK activity. As the
composition of AP-1 has been shown to influence the DNA binding properties of the transcription factor, this suggests that MUC1 can reprogram gene expression by altering the formation of specific transcriptional complexes [196-198]. As many transcription factors are rapidly stabilized or degraded in response to post-translational modification, the integration of extracellular signals through MUC1 may represent a critical regulatory step of gene expression in response to changes in the environment. In our studies, MUC1 expression resulted in increased expression of known targets of FRA-1 involved in epithelial-to-mesenchymal transition. Preliminary studies also suggest that MUC1 expression increases occupancy of FRA-1 at these sites. Different stimuli may induce differential downstream impact on transcriptional complexes, altering the gene expression profiles accordingly. This may explain in part how MUC1 impacts numerous pathways under different cellular contexts [87, 169, 274, 321, 324].

Interestingly, expression of FRA-1 itself also appears to play a role in pancreatic tumor progression. Orthotopic tumor models demonstrated that knockdown of FRA-1 decreases tumor growth. These studies represent the first evidence for FRA-1 playing an in vivo role in the progression of pancreatic cancer, which provides support for previous in vitro evidence [289]. Gene and protein expression analysis of human PDAC samples further suggests elevation of FRA-1 expression in PDAC. While we did not observe significant correlations between FRA-1 and protein levels of EMT targets, future studies utilizing more quantitative measures may allow for better models to study the correlations between these proteins. Stratification of pancreatic cancer samples into distinct subtypes may also provide clarity for these comparisons. Furthermore, in vitro studies suggest that targeting FRA-1 may represent a viable option to decrease tumor growth. While our studies did not demonstrate synergy between FRA-1 knockdown and BETi, several other methods of targeting AP-1 dimers exist including DNA aptamers and
specific AP-1 inhibitors and future studies may provide better insight into the targeting of FRA-1 [309].

While the impact of MUC1 on the formation of AP-1 appears to involve upstream signaling cascades, MUC1 has been shown to regulate the function of p53 through direct binding [77, 80]. Interestingly, this results in changes to promoter occupancy of complexes containing both p53 and MUC1. Mutation of p53 is extremely common in pancreatic cancer and is often accompanied by gain-of-function effects, including alterations to DNA binding capacity [235, 237, 314]. Our use of inducible models of p53 expression showed that MUC1 preferentially associates with the R273H p53 mutant as opposed to wildtype or R175H p53. This p53 mutant appears to exert specific gain-of-function control at the CTGF locus, as expression of R273H in Colo357 cells can induce expression of CTGF, whereas wildtype and R175H show relatively little induction. Studies showing that MUC1 can regulate expression of CTGF have primarily been performed using cell lines expressing the R273H p53 mutant, supporting the hypothesis that MUC1 may preferentially exert gain of function effects with this mutant [100]. Interestingly, mutant p53 often binds DNA in a structure specific manner, suggesting that mapping regions of predicted alternative DNA structures may provide insight into both p53 and MUC1 binding in cancer. Studies correlating specific p53 to patient outcomes may also help to identify p53 mutants that drive distinct gain-of-function effects within pancreatic cancer.

While these studies have provided significant insight into biological roles of MUC1 in gene regulation, induction of signaling cascades, regulation of transcriptional complexes, and overall tumor progression, they remain limited through our lack of quantitative understanding of the mechanism. Indeed, while MUC1 is known to drive downstream signaling cascades, the rate at which this occurs and the magnitude of the effect is not known. This limits the predictive power of our model systems and hampers
efforts to effectively target MUC1 and downstream effectors. Future studies using more quantitative models, such as the CRISPR based systems, single cell imaging, super resolution microscopy, and real-time tracking will allow us to better define the molecular circuits involved in MUC1 signaling. The expansion of human samples available for study through the UNMC Rapid Autopsy Program and Next Gen Sequencing datasets of these samples will further allow for deep quantitative analyses allowing for better understanding of the human condition and correlation of these models with in vitro models. Expansion of studies from the biological systems into the computational systems will allow for greater understanding of the complexities of pancreatic cancer and offer many tools for the future study of pancreatic cancer.

7.1 Evaluate the Impact of MUC1 on the Global Promoter Occupancy of p53, c-Jun, and FRA-1

We have shown that expression of MUC1 influences the formation of AP-1 to favor c-Jun:FRA-1 dimers. Previous studies have shown that MUC1 displaces c-Jun from both the MMP1 and CTGF promoters [77, 100]. No studies have evaluated how MUC1 impacts the occupancy of these factors on a global scale. To address this question, we have initiated experiments to isolate chromatin from MUC1 overexpressing cells and their parental counterparts to perform ChIP-seq for c-Jun and FRA-1. Co-occupancy of c-Jun and FRA-1 at promoter elements will be confirmed using ChIP-reChIP assays. Preliminary studies performing ChIP followed by qRT-PCR of select sites showed changes in promoter occupancy of several FRA-1:EMT genes; however, these preliminary studies need to be repeated and further optimized (Figure 7.1). These studies will be paired with RNA-seq analysis of the cell lines to identify genes that are differentially regulated due to changes in promoter occupancy. To date we have
performed RNA-seq analysis of S2013.Neo and MIF cells, as well as FRA-1 overexpression and knockdown lines (Figure 7.2A). The differences in gene regulation by FRA-1 between the S2013.Neo and MIF cells further support differences in global DNA binding (Figure 7.2B).

The previous studies showing displacement of c-Jun from promoter elements also highlighted increased occupancy of MUC1 and p53 at these sites [77, 100]. We wish to examine how the mutational status of p53 influences its binding capacity at these sites and others within the genome. Recent studies of p53 have shown that different mutants of p53 retain different DNA binding properties [235, 236, 313, 314]. We have shown that MUC1 preferentially interacts with the R273H hotspot mutant. Our qRT-PCR data also suggests that different p53 mutants exhibit differential regulation of these gene targets. We hypothesize that MUC1 may regulate certain gain-of-function effects by regulating DNA binding depending on the mutant of p53 expressed. As such, we intend to extend these studies to evaluate changes in p53 binding in conjunction with MUC1 expression using our dox inducible models of p53. Using ChIP-seq we can assess whether different p53 mutants exhibit differential DNA binding with MUC1 expression.

7.2 Assess the role of MUC1 in the temporal regulation of AP-1 induction

Jun and Fos proteins are both immediate early genes and are rapidly induced in response to various stimuli [121]. While we demonstrated that MUC1 could readily alter the composition of the AP-1 dimer, we still don’t understand the stoichiometry and temporal dynamics of this process. As different Jun and Fos proteins exhibit different kinetics, the rise and fall of these proteins likely regulates the relative composition of AP-1 over time. The expression of particular dimers may also be critical for the appropriate
regulation of downstream gene targets over time. Furthermore, as MUC1 relays information from the surrounding microenvironment, any changes in the surroundings may result in a different temporal response if MUC1 is present or absent.

To study the levels of AP-1 proteins in real time, we are currently designing a CRISPR based system that will allow for the generation of knock-in fluorescent Jun or Fos proteins whose expression and interactions can be followed by fluorescence assays and by Fluorescence Resonance Energy Transfer (FRET). This system is being modeled on previous work by Dr. John Albeck, and he has generously provided initial constructs that are being used as a framework for our studies [325]. These constructs consist of homologous arms to c-Jun or FRA-1 as well as an in-frame cDNA sequence for a fluorescent protein (Figure 7.3). These constructs are paired with a CRISPR construct containing a specific sgRNA against genomic sequences within FRA-1 or c-Jun. After transfection, cells are sorted for fluorescence that should only be present in cells that have undergone appropriate genomic editing. Initial pilot experiments have shown modest efficacy (Figure 7.4). However, the number of positive cells has been insufficient for experimentation to date. In order to increase efficiency of targeting, we have moved to use lentiviral transduction of CRISPR constructs and mutation of specific PAM sequences to eliminate non-specific cutting.

As a secondary approach, we are generating fusion protein constructs for AP-1 proteins should the CRISPR approach prove to be too difficult with the available facilities. These constructs will allow us to study stabilization of AP-1 proteins in response to post-translational modification, however, we will not be able to evaluate responses that lead to increased AP-1 gene expression as these constructs won’t have the endogenous genomic promoter elements and enhancers.

If our experimental system is successful, the response of AP-1 proteins will be measured in real-time using live fluorescent microscopy of cells. How different stimuli
influence responsiveness will be assessed by multi-well assays in which cells are treated with various growth factors, drugs, or other stimuli in the presence or absence of MUC1 expression. Fluorescence intensity will be tracked over time and used as readout of AP-1 protein levels and confirmed using time-course studies based on the real-time imaging results.

7.3 Stratification of Rapid Autopsy Specimens by Molecular Subtype

In recent years, several studies have explored the diversity of pancreatic cancer and categorized tumors on the basis of genomic, transcriptomic, and epigenomic features [16-18]. Due to the robust stromal involvement in pancreatic cancer, these studies remain controversial, as the molecular characteristics are often complicated through the inclusion of non-tumor cells in the analysis. Within UNMC, we have access to a substantial number (~100) of pancreatic tumor specimens through the UNMC Rapid Autopsy Program (RAP). We will attempt to validate these subtypes through clustering analysis of gene expression in these tumors. While previous studies have primarily used RNA-seq to measure gene expression, this is a costly approach. In collaboration with Dr. Fang Yu of the UNMC Biostatistics Department, we are incorporating transcriptome data from the recent manuscripts to identify the most differentially regulated genes and generate a smaller subset of genes to evaluate as biomarkers of biological subsets of pancreatic cancer (Figure 7.5).

To test this gene set, we will perform hierarchical clustering based on Euclidean distance using previously published work from other groups to assess how well this signature can recapitulate the subtypes previously identified (Figure 7.6). As these subtypes remain controversial, we will attempt to incorporate findings from multiple studies to avoid overfitting the data or biasing our results to one paradigm or another.
Once we have chosen a biomarker gene profile, we will perform digital PCR on RAP samples using Nanostring technology to generate our expression profile. This technology has the benefit of being more cost-effective than RNA-seq and requiring lower RNA quality, which is easier to achieve with stored RAP specimens. We will then perform unsupervised clustering of our gene expression and identify the number of subtypes observed and compare them to previously published subtypes. These stratified groups can then be used for further analyses examining survival, treatment response, metastatic spread, and correlation with protein expression.


The interaction of MUC1 and p53 is postulated to involve the regulatory domain of p53, however, this does not explain why we observe differences in the interaction with mutations occurring within the DNA binding domain of p53 [80]. In order to effectively function as a transcription factor, p53 must oligomerize to form a tetramer. Previous studies in the lab have suggested that MUC1 may modulate the ability to form these oligomeric complexes, however, these studies remain unconfirmed. In collaboration with Edwin Wiest, we have focused on purifying recombinant MUC1 and p53 (Figure 7.7). The interactions between these two proteins will then be assessed by biochemical assays including Isothermal Titration Calorimetry (ITC). We will further explore the impact of mutation through the generation of specific p53 mutants, including the R175H and R273H mutants previously studied.

We would also like to further expand our studies to include additional hotspot mutants at G245, R248, R249, and R282. These mutants will be generated in our Dox inducible system and validated using the same approaches used for R175H and R273H.
As p53 can also utilize internal start sites and splice sites, we would like to additionally generate inducible constructs of these variants. We have already generated the Δ40p53 variant (Figure 7.8). As these proteins exhibit different transcriptional and regulatory dynamics, they may further influence the gain-of-function effects observed within cancer. There has also been no exploration of whether these variants are capable of interacting with MUC1. To evaluate the putative roles mediated by association of MUC1 and p53, and those driven by p53 alone, we intend to expand these studies to include shRNA-mediated knockdown or CRISPR knockout of MUC1.

7.5 Evaluation of FRA-1 Expression and its Role in Early Pancreatic Cancer Development

In cancer, deregulation of immediate early gene products, such as c-Myc, is extremely common [326]. Expression of these genes is often rapidly induced in response to oncogene expression, typically as a result of post-translational stabilization due to constitutive signaling. As a result, many of these genes play a critical role in the development and progression of early tumors. In pancreatic cancer, mutation of Kras is the most common driver of tumor development [13]. Several studies have shown that the pro-oncogenic activity of AP-1 proteins, like c-Jun and FRA-1 relies on co-expression of an oncogenic driver like Kras [287, 301, 327]. We found that expression of FRA-1 promoted increased invasive behavior and knockdown of FRA-1 decreases tumor growth. Furthermore, while we found that tumors typically exhibited increased expression of FRA-1, the stage at which this occurs remains unknown.

To evaluate expression of FRA-1 in early tumor development, we will perform immunohistochemistry for FRA-1 using PanIN confirmed slides. In preliminary studies, we found that FRA-1 expression does not substantially increase until PanIN-3 (Figure...
This is despite the presence of Ras mutations in earlier PanIN lesions, suggesting that additional regulatory changes must occur before FRA-1 is stabilized. Interestingly, expression of miR-34 is lost in later stages of development (PanIN-3 to PDAC) [328]. Expression of miR-34 has been shown to inhibit expression of FRA-1 and is normally regulated by p53, suggesting that loss of p53 and miR-34 may be critical events for induction of FRA-1 [329, 330]. Further immunohistochemistry with expanded samples will help to confirm the stage at which FRA-1 expression increases. Pairing these studies with expression of p53 and miR-34 will further elucidate the genomic alterations critical for FRA-1 induction.

In long-term studies, it would be of interest to cross FOSL1 floxed mice into the KC and KPC mouse models of pancreatic cancer. These studies would tease out the importance of FRA-1 expression in the formation and progression of pancreatic cancer. Furthermore, comparing the results from KC and KPC mice may allow for the determination of the importance of p53 in these effects. Unfortunately, the FOSL1 floxed mouse is not publicly available at this time.
Figure 7.1: MUC1 Expression Alters Occupancy of FRA-1 at EMT Gene Promoters

FRA-1 bound chromatin was immunoprecipitated from S2013.Neo and MIF cells and analyzed for promoter occupancy by qRT-PCR. Known sites of promoter binding were used for AXL, FN1, SNAI2, and TGFB2, and normalized to IgG and Input controls. MUC1 expression resulted in increased occupancy at genes involved in EMT (AXL, FN1, SNAI2), however, TGFB2 showed no substantial enrichment.
Figure 7.1
**Figure 7.2: Differential Expression of Select Genes in S2013.Neo and MIF Lines**

**A)** Table of differentially regulated genes between S2013.Neo and MIF cells. Genes on the left are upregulated (green arrow) in MIF cells. Genes on right (red arrow) are downregulated in MIF cells. **B)** Venn diagrams highlighting the differential regulation of FRA-1 targets in S2013.Neo and MIF cells. MIF cells show higher numbers of unique targets as compared to Neo cells suggesting changes to FRA-1 function between the lines.
Figure 7.2

A. Genes Differentially Regulated MIF/Neo

<table>
<thead>
<tr>
<th>MUC1</th>
<th>SOX2</th>
<th>SNAI2</th>
<th>CTGF</th>
<th>ADAM11</th>
<th>MMP1</th>
<th>S100A4</th>
<th>ID1</th>
<th>JAG1</th>
<th>HES1</th>
</tr>
</thead>
</table>

B. Genes Negatively Regulated by FRA-1

Genes Positively Regulated by FRA-1
Figure 7.3: Vector Maps of Homologous Constructs for AP-1 CRISPR

Vector map showing the design of homologous arm constructs for the generation of FRA-1 and c-Jun fluorescent protein fusions using CRISPR. Homologous arms are approximately 1.5kb sequences up or downstream of CRISPR cut site. Fluorescent protein sequence lacks ATG start site requiring genomic incorporation to yield a functional product.
Figure 7.3
Figure 7.4: Validation of CRISPR Constructs

CRISPR constructs targeting either FRA-1 or c-Jun were transfected into S2013 or 293A cells with homologous donor constructs. Cells were assessed for expression of fluorescent fusion products by confocal microscopy. A small number of red (FRA1) and yellow (c-Jun) cells are observed in each study confirming that the CRISPR constructs are functioning.
Figure 7.4
Figure 7.5: Differential Expression of Genes Between PDAC Subtypes

Using previously published expression data from the PDAC subtype manuscript by Bailey, et al [18], and box plots for each of the approximately 18,000 genes examined were generated using R-programming language. Statistical analysis of differential gene expression was performed using both SAM and LIMMA methods. Genes that were differentially regulated between specific subtypes are presented as validation of these analyses.
Figure 7.5

**Unc-51 Like Kinase 3**

- ADEX
- Immunogenic
- Progenitor
- Squamous

**Tenascin C**

- ADEX
- Immunogenic
- Progenitor
- Squamous

**CD38**

- ADEX
- Immunogenic
- Progenitor
- Squamous

**PRSS3P1**

- ADEX
- Immunogenic
- Progenitor
- Squamous
Figure 7.6: Hierarchical Clustering of Tumors by Gene Expression Profile

Gene expression profiles were identified from Bailey, et. al [18] manuscript of pancreatic cancer subtypes. These profiles were than used to perform hierarchical clustering based upon Euclidean distance to determine how many genes were required to recapitulate the original findings.
Figure 7.6

A. 32 Gene Expression Profile

B. 102 Gene Expression Profile
Figure 7.7: Purification of Recombinant MUC1

E. Coli optimized cDNA sequence for MUC1 was cloned into the pGEX.4T1 expression vector to generate recombinant GST-tagged MUC1. These constructs were transfected into C41 or C43 bacterial cells and recombinant MUC1 isolated using a GST column. Purification was confirmed by western blot analysis of elution fractions (E1-E6).
Figure 7.7

Input FT E1 E2 E3 E4 E5 E6 Input FT E1 E2 E3 E4 E5 E6

C41 cells C43 cells
**Figure 7.8: Expression of Dox-Inducible p53 Truncated Mutants**

**A)** Schematic of additional p53 constructs designed. Tagless p53 lacks the 3X Flag sequence. FL-p53 should only express full-length p53, whereas Δ40p53 should express N-terminally truncated p53 lacking the first 40 amino acids. **B)** Mutations of methionine to valine were introduced into the p53 sequence to generate FL p53 and Δ40p53. DO-1 epitope is highlighted to show that Δ40p53 is not detectable with this antibody. **C)** Inducible expression of these constructs was measured with 1 µg/ml doxycycline and western blot analysis for both p53 (DO-1) and the Flag tag. Overlay of these two signals highlights FL-p53 in yellow, whereas tagless is absent by Flag and Δ40p53 is absent by DO-1. Interestingly, FL p53 induces a predicted full-length size as well as another truncated form smaller than Δ40p53, suggesting utilization of another internal start site at amino acid 133 or 160. These studies suggest that for full design of the system, these methionine residues will all have to be mutated.
Figure 7.8

A. Tagless p53

N-term

C-term

FL p53

Mini p53

B.

C.

Dox (1ug/ml)

Colo-357 Tagless FL Δ40

IB: p53(DO-1)

IB: Flag

2 channel merge
Figure 7.9: Expression of FRA-1 in PanIN Lesions

Immunohistochemistry was performed on PanIN lesion slides to assess expression of FRA-1. 3 separate slides were used for each lesion (PanIN-1 through 3). Representative images for each stage are shown (PanIN-1 through PDAC). Heatmaps showing the relative expression are presented showing that FRA-1 is only modestly expressed until the PanIN-3-PDAC transition.
Figure 7.9

PanIN-1

PanIN-2

PanIN-3

PDAC

Normal Pancreas 1

Normal Pancreas 2

PanIN-1

PanIN-2

PanIN-3

RAP46

RAP49

RAP89

RAP67

RAP81

RAP82

RAP57

RAP63

RAP64

RAP48

RAP49

RAP89

RAP67

RAP82

RAP57

RAP63

RAP64

RAP46

RAP49

RAP89

RAP67

RAP82

RAP57

RAP63

RAP64

RAP46

RAP49

RAP89

RAP67

RAP82

RAP57

RAP63

RAP64

RAP46

RAP49

RAP89

RAP67

RAP82

RAP57

RAP63

RAP64

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1

2

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