The Beta-Catenin/MUC1.CT Interaction in Pancreatic Cancer

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THE BETA-CATENIN/MUC1.CT INTERACTION IN PANCREATIC CANCER

by

Edwin Wiest

A DISSERTATION

Presented to the Faculty of
the University of Nebraska Graduate College
in Partial Fulfillment of the Requirements
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Biochemistry & Molecular Biology
Graduate Program

Under the Supervision of Professor Michael Hollingsworth

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MUC1 is overexpressed in over 90% of pancreatic cancer cases, and its interaction with beta-catenin promotes progression of the disease. Various in vitro and in vivo methods show that beta-catenin and MUC1 interact by way of the cytoplasmic tail of MUC1 (MUC1.CT). This interaction occurs in the membrane of pancreatic cancer cells but is found to a smaller extent in the nucleus as well. Biophysical methods suggest that MUC1 interacts with beta-catenin through a sequence of amino acids in the tail of MUC1 that sit very near the transmembrane domain of MUC1. In pancreatic ductal adenocarcinoma cells, it appears that EGF stimulation causes tyrosine residue phosphorylation of the cytoplasmic tail of MUC1 and a simultaneous reduction in the beta-catenin/MUC1.CT interaction in the membrane of the cells. The evidence presented here indicates that phosphorylation of the tail of MUC1 tends to decrease its interaction with beta-catenin.

While studying the beta-catenin/MUC1.CT interaction, it was inadvertently discovered that a Met receptor inhibitor, SU11274, fluoresces when excited by laser light of 488 nm. The inhibitor moves rapidly into cells and accumulates in discrete regions of the cell. Evidence suggests that SU11274 associates with the endoplasmic reticulum.
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<tr>
<th>Abbreviation</th>
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<tbody>
<tr>
<td>A.U.</td>
<td>Arbitrary unit</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CTGF</td>
<td>Connective tissue growth factor</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>eGFP</td>
<td>Enhanced green fluorescent protein</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERα</td>
<td>Estrogen receptor alpha</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FI</td>
<td>Fluorescence</td>
</tr>
<tr>
<td>FLIM</td>
<td>Fluorescence lifetime imaging</td>
</tr>
<tr>
<td>FRET</td>
<td>Förster resonance energy transfer</td>
</tr>
<tr>
<td>HDAC2</td>
<td>Histone deacetylase 2</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
</tr>
<tr>
<td>IP</td>
<td>Immunoprecipitation</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>ITC</td>
<td>Isothermal titration calorimetry</td>
</tr>
<tr>
<td>kcal</td>
<td>Kilocalorie</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>Lys</td>
<td>Lysate</td>
</tr>
<tr>
<td>MUC1.CT</td>
<td>Mucin 1 cytoplasmic tail</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PDAC</td>
<td>Pancreatic ductal adenocarcinoma</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PLA</td>
<td>Proximity ligation assay</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SPR</td>
<td>Surface plasmon resonance</td>
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<tr>
<td>Wnt</td>
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CHAPTER 1. INTRODUCTION

Pancreatic Cancer

The Pancreas

The pancreas is a mixed organ that produces enzymes as well as hormones. The endocrine cells are found in groups called the islets of Langerhans. These cells produce hormones such as insulin and glucagon. The acinar cells of the exocrine pancreas produce enzymes that aid in the digestion of food. Pancreatic ducts transport these enzymes to the duodenum [1].

Pancreatic Ductal Adenocarcinoma

Pancreatic cancer is associated with a dismal prognosis, which has been the case for decades. Pancreatic ductal adenocarcinoma (PDAC) is the most common form of pancreatic cancer, and it is the fourth leading cause of cancer death in developed countries [2]. The disease often goes undiagnosed for many years due to a lack of definite symptoms and specific tumor markers. By taking command of the local nervous system and vasculature the disease is able to metastasize rapidly. Pancreatic cancer is characterized by a complex and compact tumor microenvironment and is resistant to conventional treatments [2]. Barring improved treatment, the disease is predicted to be the second leading cause of cancer-related death by 2030 [3].

Pancreatic Cancer Microenvironment

The activating mutation in over 90% of pancreatic cancer cases is KRAS, and inactivation of tumor suppressors TP53, CDKN2A, and SMAD4 occur in 50-80% of cases [2]. In the context of heightened expression of oncogenic KRAS and loss of CDKN2A, signaling molecules such as transforming growth factor-alpha (TGFα), insulin-like growth factor 1 (IGF1), fibroblast growth factor (FGF), hepatocyte growth factor (HGF), and their respective receptors—epidermal growth factor receptor (EGFR),
receptor tyrosine-protein kinase erb-B2 (ERBB2), HER3, IGF1 receptor, FGF receptors, and hepatocyte growth factor receptor (MET)—trigger pancreatic cancer cell proliferation, migration, and invasion [4]. The overactive state of MET and EGFR is exacerbated by the formation of MET/EGFR heterodimers [4, 5]. In addition, aberrant transforming growth factor-beta (TGFβ) signaling leads to mitogen-activated protein kinase phosphorylation, proto-oncogene tyrosine-protein kinase Src (SRC) and AKT phosphorylation, and upregulation of the Wnt ligand \( WNT7B \) through a SMAD4-dependent mechanism [6].

**Pancreatic Cancer and Mucins**

Disease progression of pancreatic cancer involves increased expression, altered glycosylation, and aberrant localization of mucins [7]. Pancreatic lesions are characterized by a many-fold increase in MUC1 as well as de novo expression of MUC4, MUC5AC, MUC5B, MUC13, MUC15, MUC16 and MUC17 [7]. The loss of cell polarity that accompanies tumor formation involves a loss in asymmetric distribution of mucins, allowing (once apical) mucins to come in close proximity with (formerly basolaterally restricted) receptor tyrosine-protein kinases (RTKs) such as EGFR, ERBB2, ERBB3, and FGFR [7]. These RTKs regulate signaling cascades that give rise to the cancer phenotype: survival, growth, proliferation, and metastasis.

**Pancreatic Cancer and Wnt Signaling**

A body of evidence suggests that pancreatic ductal adenocarcinoma arises, at least in some cases, from ductal cells of the pancreas [8-10]. Wnt signaling is believed to encourage pancreatic cancer development [11, 12], and may even be required for pancreatic carcinogenesis in some cases [13]. It is known that Wnt target \( LGR5 \) is produced by a population of cells in the pancreas [14], that isolated pancreatic duct cells can self-renew [15], that following pancreatic damage, duct cells contribute to endocrine
and acinar cell regeneration [16], and that the stem cell marker Sox9 is expressed in duct cells (and not acinar or endocrine cells) that serve as progenitor cells for the exocrine pancreas during organ maintenance [17]. Given these facts, it is easy to imagine how dysregulated Wnt signaling may give rise to or aid in the progression of pancreatic ductal adenocarcinoma.

**Wnt Signaling**

Since the description of the first recognized member of the Wnt signaling family in 1982 the field has grown steadily. Wnt signaling is now understood to play a key role in growth and development as well as stem cell biology [14]. Wnt signaling involves a Wnt ligand (of which there are 19 known [18]) binding to a receptor complex comprised of the 7-transmembrane frizzled (FZD) protein and the low-density lipoprotein receptor-related protein 5/6 (LRP5/6) [14]. When a Wnt ligand binds to the FZD/LRP complex, the intracellular carrier of the Wnt signal, beta-catenin, accumulates in the cytoplasm and eventually moves into the nucleus where it forms a complex with DNA bound T-cell factor (TCF) or LEF (lymphoid enhancer-binding protein) to direct transcriptional programs involved in cell proliferation and differentiation [14, 19].

**Beta-Catenin**

The key driver of Wnt signaling within cells is beta-catenin, whose stability is tightly regulated by the beta-catenin destruction complex, which is composed of beta-catenin itself; two serine/threonine kinases, glycogen synthase kinase 3β (GSK-3β), and casein kinase 1 (CK1); a scaffolding protein, Axin; adenomatous polyposis coli (APC); and the E3-ubiquitin ligase, β-TrCP [20]. When FZD/LRP receptors are not bound to a Wnt ligand, CK1 and GSK-3β phosphorylate beta-catenin at serine residues on the N-terminus of beta-catenin. CK1 first phosphorylates serine residue 45, and then GSK-3β phosphorylates threonine residue 41, serine residue 37, and serine residue 33 [21]. This
phosphorylation pattern is a signal for the F-box-containing protein E3 ubiquitin ligase β-TrCP to ubiquitinate beta-catenin, which is then sent to the proteasome for degradation [14].

**Beta-Catenin in Cell-Cell Adhesion**

Cell-cell adhesion makes the production and maintenance of multicellular tissues possible. Adherens junctions and tight junctions enable epithelial cells to connect with one another to produce epithelial sheets. Epithelial-cadherin (E-cadherin), the transmembrane protein at the core of the adherens junction [22], is linked to the cytoskeleton by way of catenin molecules, including alpha-catenin and beta-catenin [23]. Because of its interaction with E-cadherin, beta-catenin is not only involved in intracellular signaling and gene transcription but also local control of the actin cytoskeleton [22]. The importance of proper maintenance of cell-cell adhesion is highlighted by the fact that loss of cell-cell adhesion is correlated with increased proliferation, and tumor invasiveness [22].

**Beta-Catenin Structure**

Beta-catenin has an N-terminal region of about 150 amino acids, an armadillo repeat domain (the “arm domain”), and a C-terminal tail that interacts with the general transcription apparatus. The arm domain contains 12 sets of three α helices (armadillo repeats), except repeat 7, which contains only two. Structural studies have shown that beta-catenin binding partners involved in adhesion (the classical cadherin cytoplasmic domain) and in Wnt signaling [T-cell factor transcription factors, ICAT, APC, and Axin] interact with the arm domain in a similar way [23]. Cell culture studies have shown that interactions between members of the adherens junction are regulated by phosphorylation. The tails of classical cadherins have a serine-rich region that is phosphorylated. Phosphorylation of this site within cadherins by GSK-3β and casein
kinase II (CKII) strengthens its affinity for beta-catenin by about 800-fold [24-26]. Mutation of these serine residues to alanine residues caused a reduction in cell-cell adhesion in NIH 3T3 cells [26]. Recently it was shown that three residues within this serine-rich region are required for high-affinity beta-catenin binding and are essential for proper cadherin–catenin complex formation and E-cadherin surface stability and function [27]. Src phosphorylation of beta-catenin Tyr654 reduces its affinity for cadherin [28], while CKII phosphorylation of beta-catenin increases its affinity for α-catenin [29]. Extracellular regions of cadherins on opposing cells bind to one another, and their cytoplasmic regions bind to the proteins p120 catenin and beta-catenin. Beta-catenin binds to α-catenin, which in turn binds to actin, but the association between these proteins is dynamic [30].

The N-terminal tail of beta-catenin has no intrinsic three-dimensional structure [31]. The arm domain mediates binding to TCF/LEF proteins, the beta-catenin destruction complex components Axin and APC, and the cytoplasmic domain of classical cadherins [31]. TCFs, APC, and cadherins share an amino acid sequence motif (DXθXφX_{2,7}E) where X is any amino acid, θ is an aliphatic hydrophobic residue, and φ is an aromatic residue. This motif binds to the beta-catenin superhelical groove that spans arm repeats 5-10 [24, 32].

*Wnt Signaling in Stem Cell Biology*

Wnt signaling is required for most stem cell types. In fact, Wnt proteins can be used to maintain embryonic stem cells in the pluripotent state [33], and Wnt signaling blockade cause elimination of hair follicles [34]. Also, two stem-cell specific Wnt target genes include Leucine-rich repeat-containing G-protein coupled receptor 5 (LGR5) and Axin2, which are involved in the maintenance of adult stem cells of the intestine, stomach, pancreas, liver, kidney, ovary, inner ear, taste buds, and mammary gland [14].
**Wnt Signaling in Cancer**

Because Wnt signals are involved in epithelial stem cell maintenance, it would be anticipated that mutations in Wnt pathway proteins are often found in carcinomas [14]. Aberrant Wnt signaling is well documented in colorectal cancer [35]. Loss of APC is the most common cause of colorectal cancer [12]. Wnt signaling abnormalities are also well studied in leukemia, melanoma, and breast cancer [12]. While it is well established that PDAC is mainly driven by oncogenic Ras signaling, the role of Wnt signaling in PDAC is not fully characterized [12]. Wnt mutations are not common in PDAC, but abnormal levels and nuclear localization of beta-catenin was observed in about 65% of PDAC (n=31) in one study [36].

**Noncanonical Wnt Signaling**

In addition to Wnt signaling through beta-catenin, Wnt ligands can also activate noncanonical Wnt signaling, which include the planar cell polarity (PCP) pathway and the Wnt/calcium pathway [37]. Noncanonical Wnt signaling, which is independent of beta-catenin, regulates cellular polarity and cell motility. Crosstalk occurs between the canonical and noncanonical Wnt signaling pathways. For example, in the PCP pathway Wnt activates RhoA and Rac leading to activation of JNK and AP1. AP1 is a heterodimeric transcription factor composed of any two of the following: cJun, JunB, JunD, cFos, Fra1, Fra2, ATF2, and CREB, and two of these (cJun and Fra1) are target genes of the canonical Wnt pathway [38]. Noncanonical Wnt signaling is implicated in many diseases including cancer. Studies in gastric cancer, melanoma, and glioblastoma indicate that malignant cells hijack noncanonical Wnt signaling to activate migration and metastasis [39-42].
MUCINS

*Mucins Defined*

Mucins are high molecular weight heavily glycosylated proteins. Mucins may be produced by secretory or polarized epithelial cells that line the luminal surfaces of respiratory, gastrointestinal, and reproductive tracts [43]. Mucins are also produced by hematopoietic tissues [44]. Mucins serve to protect and lubricate these surfaces. MUC1 also acts to influence adhesive properties of the cell by modifying gene expression upon structural and microenvironmental changes at the cell surface [45].

Mucins are classified as either membrane-bound or secretory mucins. Secretory mucins lack a transmembrane domain and are secreted into the extracellular space. These include MUC2, MUC5AC, MUC5B, MUC6, MUC7, MUC8, and MUC19. Membrane-bound mucins are type I membrane proteins with single transmembrane domains and C-terminal cytoplasmic tails. These include MUC1, MUC3A, MUC3B, MUC4, MUC12, MUC13, MUC15, MUC16, MUC17, and MUC20. Membrane-bound mucins may be released from cells through proteolytic cleavage. MUC1 secretory forms are likely splice variants lacking a transmembrane domain [46].

*Membrane-Bound Mucins*

Due to their presence at the luminal surface, transmembrane mucins also serve as sensors of the external environment. Outside-in signaling occurs upon ligand binding to the extracellular domain of the membrane-bound mucin or as a result of altered conformation caused by changes in pH, ionic composition, or other physical interactions at the luminal surface. Such signals are transmitted to the interior of the cell by way of post-translational modification of the cytoplasmic tail (e.g., phosphorylation, ubiquitination, proteolytic cleavage) [47].
**MUC1**

MUC1, a cell surface mucin, is 120 to 300 kDa. The actual molecular mass varies in proportion to the polymorphic variable number of tandem repeats (VNTR) domain. This mass can increase two-fold when MUC1 is fully glycosylated. MUC1 is actually composed of two subunits, a larger extracellular subunit that is heavily glycosylated, and a smaller fragment known as the MUC1 C-terminal subunit. The larger subunit is comprised of an N-terminal signal sequence and VNTR domain, which contains 20-100 repeats of the sequence GSTAPPAHGVTASAPDTRPA [31]. The smaller subunit is comprised of an extracellular stem of 58 residues, a 28 amino-acid transmembrane domain, and a 72 amino acid cytoplasmic tail. The three domains of the smaller subunit have a combined mass of 14 kDa, which is increased to about 25 or 30 kDa after glycosylation and phosphorylation. The CQC motif of the cytoplasmic tail has also been implicated as a self-association motif, enabling MUC1.CT to form dimers [48]. Also, the CQCRRK motif forms a turn, conferring some secondary structure to the otherwise simple random-coil structure of the cytoplasmic tail of MUC1 [49].

**MUC1.CT Signaling**

Concerning signaling, of the membrane-bound mucins MUC1 has been studied the most. MUC1.CT contains 72 residues, 23 of which may be phosphorylated (seven tyrosine residues, six threonine residues, and nine serine residues). Phosphorylation alters the binding affinity of MUC1.CT with various proteins, including transcription factors and kinases [47]. The interactions of MUC1.CT with kinases, transcription factors, and other proteins is summarized in Figure 1.
**Kinases** that phosphorylate MUC1.CT

- ZAP-70, PDGFR (targets MUC1.CT to nucleus with beta-catenin)
- PDGFR (targets MUC1.CT to nucleus with beta-catenin)
- PKCβ (decreases beta-catenin/MUC1 interaction)
- ERBB1, c-Src, Lyn, Lck, FGFR3

**Transcription factors** and other MUC1.CT interacting partners

- p53
- ER-α (protects MUC1.CT from proteasomal degradation and causes recruitment of p160)
- c-Jun (displaced by MUC1 at target genes, causing a rise in c-Jun levels)
- p120 catenin
- γ-catenin (targets MUC1.CT to nucleolus)
- APC
- Hsp70 (targets MUC1.CT to mitochondria)

**Figure 1.** MUC1.CT interacting partners
MUC1.CT and Heat Shock Proteins

MUC1 interacts with two heat shock proteins: Hsp70, and Hsp90 [50]. The association of MUC1.CT with Hsp70 and Hsp90 is involved in translocation of MUC1.CT to mitochondria [50]. MUC1.CT also interacts with the SH2 domain of Grb-2, purportedly when the tyrosine residue of the YTNP sequence of MUC1.CT is phosphorylated. SOS1 then binds the MUC1.CT/Grb-2 complex (by way of the SH3 domain of Grb-2), which enables signaling through Ras [51].

MUC1.CT and AP-2, Grb2

Studies in which the ectodomain of MUC1 was replaced with the ectodomain of Tac (to avoid the confounding issue of extracellular domain glycosylation) indicate that the tyrosine residues of the two MUC1.CT motifs YHPM and YTNP are required for efficient endocytosis of the MUC1 chimera. Mutation of the tyrosine residue of the YHPM motif essentially blocked coimmunoprecipitation of the chimera with AP-2, indicating that YHPM is recognized as a YXXφ motif by the μ2 subunit of AP-2. Mutation of the tyrosine residue of the YHPM motif blocked coimmunoprecipitation of the chimera with Grb2, which suggests a role for Grb2 in the endocytosis of MUC1 [52].

MUC1.CT and ErbB Kinase Family Members

Full-length MUC1 interacts with the four members of the ErbB family of RTKs [53, 54]. Whether or not EGF is present, MUC1 coimmunoprecipitates and colocalizes with ERBB1 in tumor cells. In addition, ligand bound ERBB1 phosphorylates MUC1.CT at the tyrosine residue of the YEKV motif [55]. This phosphorylation results in an increased affinity for the SH2 domain of c-Src, and beta-catenin and increases nuclear localization of MUC1.CT. Phosphorylation of the same residue is important for the interaction of MUC1.CT with Hsp90, which is necessary for the movement of MUC1.CT to the outer membrane of mitochondria [50].
It appears that MUC1 affects extracellular signal-regulated kinase (ERK) signaling independently of ErbB kinases. Stimulation of a CD8/MUC1.CT chimera caused tyrosine residue phosphorylation of MUC1.CT and activation of ERK1/2 [56]. SiRNA knockdown of MUC1 in two breast cancer cell lines caused a decrease in the transcription of MEK1 and led to a decrease in total and phosphorylated levels of MEK1/2 [57]. In addition, reduction of MUC1 in Jurkat lymphoma cells resulted in decreased T cell activation, including ERK1/2 phosphorylation and cell proliferation [58].

**MUC1.CT Phosphorylation by FGFR and PDGFR**

Activation of fibroblast growth factor receptor 3 (FGFR-3) causes MUC1.CT phosphorylation of the tyrosine residue within the YEKV motif with divergent results: MUC1.CT association with beta-catenin and nuclear targeting and Hsp90 association and targeting to the outer membrane of mitochondria [59]. In addition, platelet-derived growth factor receptor (PDGFR) activation causes MUC1.CT phosphorylation of the tyrosine residues within the YHPM and YVPP motifs with concomitant nuclear localization of MUC1.CT and beta-catenin [60].

**MUC1.CT Signaling through ICAM-1, MAP Kinase Pathways**

Studies indicate that intercellular adhesion molecule 1 (ICAM-1) serves as a ligand for MUC1 [61-63]. The binding of ICAM-1 to MUC1 activates calcium signaling that is independent of the mitogen-activated protein (MAP) kinase pathway [61]. Studies with signaling pathway inhibitors indicate that this calcium response involves Src, phosphatidylinositol 3-kinase (PI3K), and phospholipase C enzymes. The MUC1/ICAM-1 interaction increases migration of MUC1-expressing cells in vitro, which suggests that this interaction regulates cellular adhesion and motility [64]. The binding of *Pseudomonas aeruginosa* spurs phosphorylation of MUC1.CT resulting in the activation of the MAP kinase pathway [65].
**MUC1.CT and Src Family Non-RTKs**

Of the nine Src family non-RTKs, c-Src, Lyn, and Lck have been shown to bind and phosphorylate MUC1.CT at the tyrosine residue of the YEKV motif [58, 66-69]. The c-Src SH2 domain physically interacts with the YEKV motif. This interaction blocks the binding of GSK-3β to MUC1.CT. GSK-3β is also able to phosphorylate MUC1.CT. This leads to reduced interaction between MUC1.CT and beta-catenin. Therefore, c-Src phosphorylation of MUC1.CT at the tyrosine residue of the YEKV motif enhances the MUC1.CT/beta-catenin interaction and prevents the interaction of MUC1.CT with GSK-3β [67]. Other kinases known to phosphorylate MUC1.CT include the δ isoform of protein kinase C (PKCδ) and the ζ chain-associated protein kinase of 70 kDa (ZAP-70) [45, 70].

**MUC1.CT and TLR5**

A recent study shows that an interaction between MUC1.CT and toll-like receptor 5 (TLR5) interaction in A549 lung adenocarcinoma cells increases upon stimulation with TGF-α. This is apparently due to activation of EGFR and concomitant tyrosine residue phosphorylation of MUC1.CT [71]. In addition, stimulation of normal human bronchial epithelial cells with TNF-α increased MUC1 and EGFR protein levels, and stimulation of these cells with *Pseudomonas aeruginosa* caused an increase in MUC1.CT tyrosine residue phosphorylation and increased both the MUC1.CT/TLR5 and MUC1.CT/EGFR interactions [72].

**MUC1.CT and γ-Catenin**

The ERBB3 ligand heregulin is known to enhance interaction of MUC1.CT with γ-catenin. The MUC1.CT-γ-catenin complex is then targeted to the nucleolus. In fact, MUC1.CT can be coimmunoprecipitated with ERBB2, and stimulation with heregulin enhances this interaction [47].
**MUC1.CT and Transcription Factors**

MUC1.CT has been shown to interact with beta-catenin, p120 catenin, p53, and ER-α in the nucleus. Interaction between MUC1.CT and the inhibitor of κB (IκB) kinases (IKKs) have been reported as well [73]. Such interactions have been shown to influence transcription. For example, MUC1.CT has been shown to associate with transcription factors at the promoter of the connective tissue growth factor (CTGF) causing a change in CTGF expression [74]. It may be that MUC1.CT also enters the nucleus apart from any transcription factor. The CQC and the positively charged RRK MUC1.CT motifs are involved in its nuclear import and localization, respectively [53, 75].

Interaction of MUC1.CT with ER-α and members of the NF-κB signaling pathway is of immense biological consequence. It appears that MUC1.CT interaction with ER-α protects it from proteasomal degradation, and actually promotes the recruitment of p160 coactivator. Strikingly, the MUC1.CT/ER-α interaction is not ER ligand-dependent [76].

Concerning NF-κB, it appears that MUC1.CT activates IKKs, which trigger IκB degradation, enabling the transcriptional activity of NF-κB [73]. In addition, it has been shown that MUC1 displaces c-Jun from promoters of c-Jun target genes. This alteration in the AP-1 transcriptome relies on MUC1-mediated increase in steady state levels of c-Jun [77].

**MUC1 in cancer**

MUC1 overexpression occurs in many types of cancers including breast, ovarian, lung, colon, and pancreatic carcinomas [78], and MUC1 expression usually increases with progression of disease. In the case of breast cancer, MUC1 expression or levels of certain MUC1-associated glycans correlates with poor survival [79]. In the pancreas, in precursor lesions (pancreatic intraepithelial neoplasias or PanINs), MUC1 expression is low, but increases greatly in invasive carcinoma [80].
**MUC1 and Apoptosis**

MUC1 may aid in resistance to apoptosis and anti-tumor drug killing [81]. MUC1 can stimulate Akt and antiapoptotic protein Bcl-X to block genotoxin-induced apoptosis [82]. Additionally, chemotherapy-induced cell killing is attenuated in the presence of MUC1 while cell killing is increased upon loss of MUC1 expression [81-83]. MUC1 decreases the level of intracellular reactive oxygen species (ROS) and activates FOXO3a in response to oxidative stress [84, 85]. However, MUC1 expression can also induce Fas-mediated apoptosis [86], highlighting the complex nature of MUC1’s involvement in cell death.

**MUC1 Glycosylation in Cancer**

MUC1 expression on tumor cells differs from that of normal cells in terms of localization and glycosylation. MUC1 is normally found at the apical membrane of epithelial cells, but in tumor cells MUC1 expression is greatly increased, and MUC1 is found over the entire plasma membrane and in the cytoplasm [78]. Loss of apical MUC1 is associated with poor prognosis in lung cancer [87]. MUC1 is aberrantly glycosylated in tumors as well: the carbohydrate chains are typically truncated and include sugar moieties that are not normally present on MUC1 [88].

**MUC1.CT and Adhesion Properties of Cancer Cells**

The steric hindrance of the MUC1 extracellular domain provides an antiadhesive property to protect epithelial cells from pathogens, but this same feature allows tumor cells to evade immune cells [78, 89]. Also, MUC1 on tumor cells is associated with increased metastasis [90, 91]. This may result from interactions of MUC1 with adhesion molecules such as ICAM-1, which may assist these tumor cells in invading into the endothelium and attaching to cells in the metastatic niche [64].
**MUC1.CT and Cell Migration**

MUC1.CT colocalizes with beta-catenin and fascin and vinculin at sites of focal adhesion in the process of collagen matrix cell invasion [91]. Fascin supports cell protrusions in migrating cells. This suggests a role for MUC1.CT in cell migration.

**MUC1.CT and Wnt Signaling**

MUC1.CT interacts directly with three members of the Wnt signaling pathway: beta-catenin, APC, and GSK-3β [92-94]. MUC1.CT contains a motif, known as the serine-rich motif (SXXXXXSSL, where X is any amino acid) which interacts with beta-catenin. It has been reported that beta-catenin interacts with MUC1.CT through this motif [94, 95]. The affinity of interaction between beta-catenin and MUC1.CT depends on phosphorylation of MUC1.CT at specific residues, and this interaction increases levels of beta-catenin in the nucleus [60, 67]. MUC1.CT has also been shown to interact with p120 catenin [96].

It is important to note that not all researchers agree that MUC1 positively regulates beta-catenin activity. One group reported that in a nonmalignant cell line MUC1 decreased Wnt signal transduction through direct interaction with beta-catenin [97], and another group showed that in a breast cancer cell line and a pancreatic cancer cell line that MUC1 levels are inversely related to beta-catenin levels [98].

**MUC1/Wnt Pathway and Cancer**

Interactions of MUC1.CT with different components of the Wnt signaling cascade are related to cancer progression. Phosphorylation of the threonine residue within the TDRSPYEKV motif of MUC1.CT also increases its interaction with beta-catenin, while phosphorylation of the serine residue within this motif inhibits the same interaction [99]. The interaction between MUC1.CT and beta-catenin may sequester beta-catenin from its association with E-cadherin at the adherens junction. This would, in turn, stimulate anchorage-independent growth in vitro and might facilitate the metastasis of tumor cells.
In vivo [100, 101]. In fact, loss of MUC1 leads to a decrease in beta-catenin as a result of increased GSK3-β-mediated phosphorylation and degradation of beta-catenin [95].

In addition, it has been shown that MUC1.CT interacts with APC in breast cancer [92]. This interaction is enhanced by EGF stimulation. In fact, MUC1 knockout in the mouse mammary tumor virus (MMTV)-Wnt-1 transgenic background resulted in a delay in mammary tumorigenesis [91].

MUC1.CT peptides with wild-type GSK-3β and beta-catenin binding motifs increased the invasive potential of the MDA-MB-468 breast cancer cell line. This appears to be irrespective of the phosphorylation status of the MUC1.CT peptides. MUC1.CT peptides which lacked either the GSK-3β or beta-catenin binding motif no longer enhanced invasion [91]. This suggests a role for these interactions in invasion. Also, MUC1 is associated with increased beta-catenin levels in both the cytoplasm and nucleus of breast carcinoma cells. This is due to MUC1.CT blocking GSK-3β from phosphorylating beta-catenin [102]. It may be that MUC1.CT sequesters beta-catenin, keeping it from interaction with cadherins and the adherens junction. In some cell types, MUC1.CT promotes transcriptional activation of the beta-catenin-TCF-binding sites such as cyclin D1 [95].

Epidermal Growth Factor Receptor Signaling

EGFR Family

Discovered in 1959, Epidermal growth factor receptor (EGFR, also known as ERBB1/HER1) is the prototypical EGFR family member, which also includes ERBB2/HER2/Neu, ERBB3/HER3, and ERBB4/HER4 [103]. Though ERBB2 contains no ligand-binding domain, seven ligands are known to bind to the other three family members, including epidermal growth factor (EGF), transforming growth factor-α (TGFα), heparin-binding EGF-like growth factor (HB-EGF), betacellulin (BTC),
amphiregulin (AR), epiregulin (EPI), and epigen [104]. The classical EGFR ligand, EGF, is a 53 amino acid protein. The normal concentration of EGF in human fluids ranges from as much as 500 ng/mL in bile and milk to 1-2 ng/mL in plasma, serum, and saliva [103]. Upon ligand binding, these family members form homo- and heterodimers with each other, leading to transautophosphorylation (though ERBB3 contains no kinase domain). This results in the recruitment of signaling proteins and then rapid endosomal internalization of the receptors. Finally, the receptors are either recycled or destroyed in lysosomes [104].

**EGFR Structure**

EGFR is an 1186 amino acid transmembrane protein comprised of an extracellular ligand binding/dimerization arm domain, a transmembrane domain, and an intracellular tyrosine kinase/C-terminal tail domain. The kinase domain of the intracellular portion of EGFR contains an adenosine tri-phosphate (ATP) binding site. When a ligand docks on a member of the EGFR family, EGFR family receptors form dimers, which use the bound ATP to phosphorylate one another (transautophosphorylation), which leads to receptor activation. The kinase domain also contains lysine residues that are necessary for ubiquitination of the receptors for proper sorting or degradation of the receptors. The C-terminal tail is the site of many tyrosine residues, which may be phosphorylated to serve as sites for recruiting proteins involved in signal transduction [103].

**EGFR Signaling**

EGFR activates ERK/mitogen activated protein kinase (MAPK), phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/protein kinase B (AKT), proto-oncogene tyrosine-protein kinase Src (SRC), phospholipase C gamma 1 (PLC-γ1)/protein kinase C (PKC), c-Jun N-terminal kinase (JNK), and Janus kinase
(JAK)/signal transducer and activator of transcription (STAT) signaling pathways [103]. Following transphosphorylation of EGFR, the protein binds the Src homology 2 (SH2) domain of growth factor receptor binding protein 2 (GRB2). EGFR activation also leads to recruitment of Src homology and collagen (SHC). GRB2 then binds SOS1 (son of sevenless 1), which leads to activation of the RAS/rapidly accelerated fibrosarcoma (RAF)/mitogen-activated protein kinase kinase (MEK)/ERK signaling cascade, resulting in cell proliferation, differentiation, and survival [105, 106]. Evidence suggests that full-length EGFR is able to translocate into the nucleus. It has been hypothesized that EGFR may complex with importin-β by way of a nuclear localization signal, making interaction with nucleoporins of the nuclear pore complex and uptake into the nucleus possible [107].

**EGFR and Cancer**

This family of receptors plays a significant role in promoting cell proliferation, angiogenesis, cell migration, adhesion, metastasis, and the inhibition of apoptosis [103]. Because of the ability of ErbB family members to confer such oncogenic properties to cells, constitutive activation or overexpression of these receptors is often found in cancers, including carcinomas, sarcomas, non-small cell lung cancer, and malignant gliomas [103]. Mutation of EGFR can lead to constitutive activation, the majority of which stabilize ligand-independent dimerization with other ErbB family members [108, 109]. Other mutations allow endocytosis escape. One such mutation, EGFRvIII, occurs in a considerable portion of glioblastomas [110]. A point mutation, L858R, is a common EGFR tyrosine kinase domain mutation in non-small-cell lung cancer [111, 112]. This mutation leads to as much as fifty-fold increase in kinase activity in vitro [113]. Another kinase domain mutation, T790M, results in increased EGFR activity and confers resistance to tyrosine kinase inhibitors [114].
EGFR has been reported to be overexpressed in 30 to 95% of PDAC cases, depending on the study [115]. Once activated, EGFR induces cyclin D1, which complexes with CDK4/6 to initiate cell cycle progression [103]. This effect of activated EGFR is pivotal to its oncogenic capability, and aberrant levels of EGFR have been correlated with higher expression of cyclin D1 in non-small-cell lung cancer and breast cancer [116, 117].

**EGFR Inhibition as Cancer Therapy**

The two major therapies which target EGFR include humanized monoclonal antibodies against the extracellular domain of EGFR and tyrosine kinase inhibitors (TKIs). The antibodies block ligand binding and may also promote endocytosis and destruction of the receptor [118]. TKIs, such as Erlotinib, Gefitinib, and Lapatinib, are ATP mimetics that inhibit transautophosphorylation by competitively and reversibly binding to the ATP pocket of the tyrosine kinase domain [119]. Erlotinib, in combination with gemcitabine, is used as a first-line pancreatic cancer therapy [120].

**EGFR and Wnt Signaling**

Experimental evidence in keratinocytes indicates that EGFR phosphorylation of beta-catenin at tyrosine residue 654 decreases beta-catenin/E-cadherin binding, with loss of beta-catenin at the membrane and increased levels in the nucleus [121]. In an oral cancer study, again beta-catenin/E-cadherin interaction was found to decrease with EGF stimulation, and nuclear beta-catenin levels increased with concomitant increase in cyclin D1 levels [122]. This is in line with a triple-negative breast cancer study in which low membrane beta-catenin in the presence of EGFR correlated with unfavorable disease-free survival, but not in the absence of EGFR. In addition, the authors reported that decreased beta-catenin membrane staining correlated with increased nuclear beta-catenin staining [123].
The Interplay of EGFR, MUC1, and Wnt Signaling

Beta-catenin is stabilized by MUC1.CT [124], and nuclear beta-catenin levels rise with MUC1 overexpression [67, 124]. EGF is abundant in the microenvironment of pancreatic cancer, and active EGFR is highly expressed by pancreatic cancer cells [2, 4]. In addition, MUC1 has been reported to increase EGFR levels [7, 125]. Aberrant Wnt signaling [36, 126, 127] and EGFR activity [128-130] are well documented in pancreatic cancer. EGFR has been reported to directly phosphorylate the tyrosine residue of the YEKV motif of MUC1.CT, and this phosphorylation apparently increases the interaction between MUC1.CT and beta-catenin [55]. For these reasons, this dissertation is devoted primarily to understanding the interaction between beta-catenin and MUC1.CT in the context of EGF stimulation in the hope of demystifying this element of pancreatic cancer.
CHAPTER 2. MATERIALS AND METHODS

Reagents

Primary Antibodies

The beta-catenin antibody 15B8 [131] (53483, Santa Cruz) was a monoclonal antibody produced in mouse. This antibody recognizes the C-terminus of beta-catenin. The MUC1.CT antibody CT2 (80952, Abcam) was a monoclonal antibody produced in Armenian hamster. Mouse IgG isotype control antibody was 02-6100, Thermo Fisher Scientific. The Armenian hamster IgG isotype control antibody was 18479, Abcam. Actin antibody (A5441, Sigma-Aldrich) was used as a loading control for cytoplasm/membrane fractions. HDAC2 antibody was used (5113, Cell Signaling) as a loading control for nuclear fractions. An EGFR antibody (373746, Santa Cruz) was used as a membrane marker. An anti-phosphotyrosine antibody (7020, Santa Cruz) was used to determine MUC1.CT tyrosine residue phosphorylation.

Confocal microscopy primary antibodies for cell staining included beta-catenin antibody 15B8 (53483, Santa Cruz) and MUC1.CT antibody CT2 (80952, Abcam). For PDAC tissue staining MUC1.CT antibody CT2 and the rabbit monoclonal beta-catenin antibody D10A8 (8480, Cell Signaling) were employed.

For the SU11274 organelle localization study cell compartments were stained with the following antibodies: endoplasmic reticulum, anti-calreticulin Alexa Fluor 647 (1:100 v/v, 196159, Abcam); lysosomes, anti-Lamp1 (1:1,000 μg/µL, 24170, Abcam); early endosomes, anti-EEA1 Alexa Fluor 647 (1:50 v/v, 196186, Abcam); Golgi apparatus, anti-giantin (1:300 v/v, 80864, Abcam); late endosomes, anti-Rab9 (1:250 μg/µL, 179815, Abcam); recycling endosomes, anti-Rab11 (1:100 v/v, 700184, Thermo Fisher Scientific). Anti-rabbit Alexa Fluor 647 (1:3,000 μg/µL, A-21245, Thermo Fisher Scientific).
Scientific) was applied to the cells in the case that the primary antibody was not conjugated to Alexa Fluor 647.

**Secondary Antibodies**

The mouse secondary antibody used in western blotting was IRDye® 680RD anti-mouse (926-68072, LI-COR). The goat-anti Armenian hamster antibody was Affini Pure IgG (127-005-160, Jackson Immuno Research) to which IRDye 800CW NHS ester (929-70020) was conjugated per LI-COR protocol.

Confocal microscopy secondaries for fixed-cell experiments included goat-anti-Armenian hamster Alexa Fluor 488 (173003, Abcam), goat-anti mouse Alexa Fluor 568 (A11031, Life Technologies), and goat anti-rabbit Alexa Fluor 647 (A21245, Life Technologies). Goat anti-Armenian hamster Alexa Fluor 488 (173003, Abcam) and goat anti-rabbit Alexa Fluor 647 (A21245, Life Technologies) were used for staining PDAC tissue samples.

**Growth Factors**

Connective tissue growth factor (CTGF) (PHG0286, Invitrogen) was applied to cells at 100 ng/mL. Platelet-derived growth factor (PDGF) (PHG0045, Invitrogen) was applied to cells at 10 ng/mL. Hepatocyte growth factor (HGF) (H9661, Sigma Aldrich) was applied to cells at 10 ng/mL. Epidermal growth factor (EGF) (PHG0311, Thermo Fisher Scientific) was applied to cells at 10 ng/mL. EGF activity was confirmed by increased ERK phosphorylation (Thr 202/Tyr 204) in the cytoplasm/membrane and nuclear fractions of S2-013.MUC1F cells (data not shown).

**Buffers**

Unless indicated otherwise, cells were lysed in 250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM DTT at pH 7.4. Halt phosphatase/protease inhibitors (78440, Thermo Fisher Scientific) or Pierce
phosphatase and protease inhibitor (88667, Thermo Fisher Scientific) and 1 mM DTT were added to the lysis buffer immediately before use. The nuclear lysis buffer was 250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 10% glycerol, 0.1% SDS, pH 7.4.

The elution buffer for two-dimensional gel electrophoresis consisted of 7M urea (4.2g in 10 mL solution), 2M thiourea (1.5g in 10 mL solution), 3% CHAPS (0.3g in 10 mL solution), and 1% Triton-X 100 (1 mL 10% Triton-X 100 in 10 mL solution).

*MUC1.CT Peptides*

MUC1.CT peptides used in ITC experiments were purchased from Biomatik and Genscript. Peptides used in OpenSPR experiments were purchased from Biomatik, CSBio, Genscript, and RS Synthesis. Red font p followed by a red font S or Y indicates a phosphorylated serine or tyrosine residue. Red font E indicates a serine residue to glutamate residue phosphomimetic mutation. The Biomatik peptide was GRpYVPPSSTDRTSPpYEKVSAGNGGSSLSYTNPA (HPLC purity above 98%, identity confirmed by mass spectrometry). The CSBio peptides included the following: NYGQLDIFPARDEYHPMSEYPTHTHGRYVPPSSTDRTSPYEKVSAGNGGSSLSYTNPA VAATSAN and CQCRRKNYGQLDIFPARDEYHPMSEYPTHY (both with HPLC purity above 98% and identity confirmed by mass spectrometry). The Genscript peptides included the following: VSAGNGGSSLSYTNPA, GRYVPPSSTDRTSPYEKV, GRYVPPSSTDRTSPYEKVSAGNGGSSLSYTNPA, and GRYVPPSSTDRTSPYEKVSAGNGGEELEYTNPA (all with HPLC purity above 95% and identity confirmed by mass spectrometry and amino acid analysis). The RS synthesis peptides included the following: PYEKVSAGNGGSSLSYTNPA, PYEKVpSAGNGGSSLSYTNPA, PYEKVSAGNGGppSSLSYTNPA, PYEKVSAGNGGSpSLSYTNPA, PYEKVSAGNGGSSLpSYTNPA, KSVGSPNESTAYSGLNAGYP (scrambled peptide).
CQCRRKNYQLDIFPARDTPYHPMSEYPTYH, and

GRYVPPpSpSpTDRSPYEKVSAGNGGSSLTYTN (all with HPLC purity above 95%, TFA to chloride ion counter exchanged, and identity confirmed by mass spectrometry).

Recombinant Protein Constructs

M57 pPET28a-TEV-full-length human beta-catenin (Randall Moon, plasmid #17198, Addgene) was expressed in BL21 (DE3) E. coli [132] (kanamycin selection). When the bacteria reached an optical density (600 nm) of about 1.0, they were induced for 2 to 3 hours with 0.5 mM IPTG at 37°C (with 175 rpm rotation). For isothermal titration calorimetry (ITC) experiments, the protein was purified over a 5mL nickel column. Buffer A was 50 mM Tris, 150 mM NaCl, 10 mM imidazole, pH 8.0, and buffer B was 50 mM Tris, 150 mM NaCl, 600 mM imidazole, pH 8.0. A linear gradient (increasing percentage of imidazole) was used for elution of the protein. In ITC experiments the protein was purified further by size exclusion chromatography in 150 mM NaCl, 30 mM Tris, 2 mM beta-mercaptoethanol, pH 8.0. The same construct was used for OpenSPR experiments. In OpenSPR experiments the protein was purified with PBS, pH 8.0, using a linear gradient of imidazole to elute the protein from the column. The protein was dialyzed three times in PBS, pH 8.0 for at least two hours per dialysis step.

The E_{cyt} [24] construct was a gift from the lab of Dr. William Weis, Stanford School of Medicine. The protein was expressed in BL21 (DE3) E. coli (Ampicillin selection) to an optical density (600 nm) of about 1.0. The bacteria were induced for 4 hours with 0.5 mM IPTG at 37°C (with rotation at 175 rpm). The protein was purified with a GSTrap (28401748, GE Healthcare). The binding buffer was PBS, 2 mM DTT, pH 8.0. The elution buffer was PBS, pH 8.0, 2 mM DTT, 10 mM reduced glutathione. The protein was dialyzed three times in PBS, pH 8.0 for at least two hours per dialysis step.
His-tag MUC1.CT (pET-19b vector) was produced by Ryan Hanson in the Hollingsworth lab. Translated, it was of the sequence

MGHHHHHHHHHHSSGHIDDDKHMLECQCRRKNYQQLDIFPARDTYHPMSEYPTYHT HGRYVPPSTDSPYEKVSAGNGSLSYTNPAPAATSANL (MUC1.CT in italics).

The protein was expressed in BL21 (DE3) cells at 37°C, which were induced at an optical density (600 nm) of 0.5 to 1.0 for 4h at 37°C with 0.5 mM IPTG. The protein was purified with a cobalt affinity column. The binding buffer was 10 mM imidazole in PBS at pH 8.0. The protein was eluted in PBS, 1M imidazole, pH 8.0 (stepwise elution with 5 column volumes 30%, 3 column volumes 40%, 3 column volumes 50%, and 5 column volumes 100% elution buffer). The protein was concentrated, and the imidazole essentially removed by three rounds of buffer exchange with PBS, pH 8.0.

**Cell Line and Culture Conditions**

S2-013.MUC1F [133] is a pancreatic cancer cell line derived from a liver metastasis that was genetically engineered to express FLAG-tagged MUC1. CFPAC-1 is a pancreatic ductal adenocarcinoma cell line obtained from a patient with cystic fibrosis [134]. The cells were cultured in DMEM containing 5% FBS. Cells were incubated at 37°C and 5% CO₂. Cells were typically grown to 80% confluency prior to lysis for western blot or immunoprecipitation experiments and prior to imaging for confocal microscopy experiments.

**Constructs Used in Cells**

To study localization and trafficking of MUC1.CT and beta-catenin, cells labeled with fluorescent MUC1.CT and fluorescent beta-catenin were prepared. S2-013.MUC1 eGFP cells express eGFP at the C-terminus of MUC1.CT. These cells were transduced with mCherry murine beta-catenin (Addgene plasmid #55001, a gift from Michael Davidson). Murine beta-catenin differs by one amino acid residue in the C-terminus of
the protein (P706A). The mCherry beta-catenin was inserted into pCW57-MCS1-2A-MCS2, Addgene plasmid #71782, a gift from Adam Karpf. Stbl3 (Thermo Fisher Scientific) E. coli was used to prepare pCW57-MCS1-2A-MCS2/mCherry beta-catenin DNA to infect HEK 293T cells to package viruses, which were used to infect S2-013.MUC1 eGFP cells. The cells expressed mCherry beta-catenin upon 48 hours doxycycline treatment (16 µg/mL).

Methods

Subcellular Fractionation

To each 15-cm dish, 250 µL cytoplasm/membrane lysis buffer was applied. The lysate was passed through a 25G needle 10 times and then allowed to incubate on ice for 20 minutes. The lysate was centrifuged at 700g for 5 minutes at 4°C. The supernatant from this spin was centrifuged at 10,000g for 5 minutes. If not used directly, the cytoplasm/membrane fraction was stored at -80°C. The pellet produced from the first spin was washed once with 250 µL lysis buffer. The supernatant was then removed, and the pellet (mostly nuclei) was lysed with 100 µL of the nuclear lysis buffer. After sitting on ice for 10 minutes, the nuclear fraction was centrifuged at 10,000g for 5 minutes. Turbonuclease (1:1000 v/v) and DTT (1 mM) were added to the nuclear lysis buffer at the time of nuclear lysis. The nuclear fraction was stored at -80°C. Before freezing the lysates, 5 µL cytoplasm/membrane lysate and 10 µL nuclear lysate were placed in microcentrifuge tubes with NuPAGE (Thermo Fisher Scientific) sample buffer (5 µL per sample) in preparation for western blot. Membrane fractions were obtained with the Mem-PER™ Plus Membrane Protein Extraction Kit (Thermo Fisher Scientific).

Coimmunoprecipitation

For large scale immunoprecipitation experiments a beta-catenin affinity column was prepared by covalently binding 1 mg 15B8 antibody to 2 mL Pierce™ NHS-activated
agarose slurry (26200, Thermo Fisher Scientific). The isotype control column was made in an identical fashion with 1 mg mouse IgG1 antibody (3877, Santa Cruz). S2-013.MUC1F protein lysate (lysed in 1% NP40, 25 mM Tris-Cl, 150 mM NaCl, 1 mM EDTA, pH 7.4) was incubated on the column overnight at 4°C. Equal portions of lysate protein (same in volume and concentration) were applied to isotype control and beta-catenin affinity columns (typically 10 to 15 mg in 2 mL). Halt protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, 78440) was added to the lysis buffer prior to lysing the cells. The column was rinsed with 1 mL lysis buffer five to six times and six 1 mL 0.2M glycine (pH 3.0) elution fractions were collected.

For small scale immunoprecipitation experiments, 10 µg 15B8 or CT2 or IgG control antibody was applied to 50 µL Dynabeads (up to 200 µL Dynabeads for two-dimensional gel electrophoresis) protein G (10003D, Thermo Fisher Scientific) for 2 hours at 4°C or 10 minutes at room temperature for two-dimensional gel electrophoresis samples. Equal portions of lysate protein (same in volume and concentration) were applied to isotype control and specific antibody preparations. The beads were allowed to rotate overnight at 4°C or for 10 minutes at room temperature for two-dimensional gel electrophoresis samples. Fifty µL 2D-Xtract (G-Bioscience) was used to elute the beads for 30 minutes at 54°C. Two-hundred µL urea/thiourea buffer (described in Reagents section) was used to elute beads for 10 minutes at 70°C for two-dimensional gel electrophoresis samples.

Two-Dimensional Gel Electrophoresis

To each eluent (200 µL) from immunoprecipitation 1 µL strip ZOOM™ carrier ampholyte pH 3-10 (Thermo Fisher Scientific, ZM0021) and 1 µL bromophenol blue were added. The solution was added to a lane of the ZOOM™ IPG runner cassette (Thermo Fisher Scientific, ZM0003) holding a gel strip (Thermo Fisher Scientific, pH 3-10 linear, ZM0018). The gels were rehydrated overnight at room temperature.
For the first dimension the gel strips were electrophoresed at a constant 175V for 20 minutes, a gradient of 175 to 2000V for 50 minutes, and at a constant 2000V for 45 minutes. The gel strips were then equilibrated in 0.1 M DTT for 12 minutes and then 70 mM iodoacetamide solution for 12 minutes. For the second dimension the gel strips were placed horizontally over a 4-12\% Bis-Tris ZOOM\textsuperscript{TM} protein gel (NP0330BOX, Thermo Fisher Scientific). Agarose gel (0.5\%) was used to merge the gel strip with the protein gel. Then the proteins were electrophoresed for about 80 minutes at 150V.

\textit{Western Blot}

Bis-tris gels were run for 60 minutes at 150V. Gels were transferred to polyvinylidene fluoride (PVDF) for 80 minutes at 80V. The membranes were blocked in 5\% BSA for 1 hour. The membranes were incubated with primary antibody in 2.5\% BSA in PBS with 0.1\% Tween-20 overnight at 4°C with nutation. The beta-catenin antibody (15B8) was diluted at 1 μg/mL. The MUC1.CT antibody (CT2) was diluted at 0.25 μg/mL. Anti-mouse secondary (diluted at 1:10,000) and anti-Armenian hamster (diluted at 1:5,000) secondary antibodies were applied for 45 minutes at room temperature. The fluorescence of the secondary antibodies was detected with an Odyssey Imager (LI-COR, Bad Homburg, Germany) and quantified with Odyssey 3.0 software [135].

\textit{Confocal laser scanning microscopy}

A Zeiss laser scanning microscope 800 with Airyscan (Thornwood, NY) with a 40x/1.3 oil immersion objective lens was used to produce all images of cells unless otherwise specified. Unless otherwise indicated, the following excitation and emission parameters apply to confocal microscopy images. For DAPI and Hoechst 33342 dyes (detection of nuclei) the excitation wavelength was 405 nm and the emission spectrum detected was 411 to 488 nm. Alexa Fluor 488 and MUC1 eGFP were excited with a wavelength of 488 nm and the emission spectrum detected was 495 to 570 nm. The
excitation wavelength for excitation of Alexa Fluor 568 antibodies was 561 nm and the emission spectrum detected was 575 to 700 nm. The excitation wavelength for excitation of Alexa Fluor 647 was 640 nm and the emission spectrum detected was 646 to 700 nm. All images were acquired using sequential scanning of individual fluorophores with an image size of 1024x1024 pixels and a pixel scaling of 0.156 μm per pixel. Images were additionally averaged 2 to 8 times and collected using bidirectional scanning. All imaging parameters were universally applied across treatment groups of each experiment.

If cells were to be treated with a growth factor, they were first serum starved for about 24 hours. When used, Erlotinib (Selleckchem) was applied at 1 μM (or an equal volume of DMSO as a vehicle control). Following fixation (4% PFA in PBS) and permeabilization (0.15% Triton-X 100 and 1% BSA in PBS) cells were typically blocked for 30 minutes with 5% BSA in PBS and then stained with primary antibody overnight at 4°C. Beta-catenin antibody 15B8 was applied at 1:200 μg/μL and MUC1.CT antibody CT2 was applied at 1:250 μg/μL. For the confocal microscopy experiments in which cells were probed for beta-catenin and MUC1.CT, the cells were blocked for 30 minutes in 5% BSA and 5% goat serum in PBS. Secondary antibodies were applied at 1:3,000 (μg/μL) for one hour at room temperature. Antibody diluent was typically 1% BSA in PBS. ProLong Gold Antifade mountant with DAPI (Thermo Fisher Scientific) was used to stain the nuclei of fixed cells. Coverslips were stored at 4°C if not imaged immediately.

For tissue staining of the PDAC sample formalin fixed paraffin embedded tissue was rehydrated followed by antigen retrieval with citrate buffer. After permeabilization (1.5% Triton X-100 in PBS) and blocking (5% BSA, 5% goat serum, 0.25% Triton X-100 in PBS) samples were stained with CT2 (1:100 v/v) and D10A8 (1:50 v/v) overnight at 4°C. Secondary antibodies Alexa Fluor 488 (1:100 v/v) and Alexa Fluor 647 (1:400 v/v)
were applied for 1 hour at room temperature. The antibody diluent was 5% BSA, 1% goat serum, and 0.10% Triton X-100 in PBS. Nuclei were stained with Duolink® In Situ Mounting Medium with DAPI (82040, Sigma-Aldrich).

Live Cell Imaging

Cells were cultured on glass bottom microwell dishes (P35G-1.5-14-C, MatTek). Cells were maintained at 37°C and 5% CO₂ during image acquisition.

SU11274 Spectral Properties and Imaging

SU11274 (at 32 µM) was excited using 488 nm and spectrally profiled with a Zeiss LSM 710 (Thornwood, NY). SU11274-associated fluorescence intensities were acquired in bins of approximately 10 nm in size ranging from 482 to 725 nm. Given the verified spectral properties of SU11274, SU11274-specific emission was collected using a 495-570 nm filter set.

SU11274 Cellular Uptake Assay

S2-013.MUC1F cells were treated with 2 µM SU11274 and imaged (under the aforementioned imaging parameters) for 15 minutes at 20 second intervals. To characterize inhibitor uptake in individual cells, mean fluorescence intensities from SU11274 were collected from discrete regions of interest (ROIs) representing individual cells (n=5 per time point). The averaged cell-specific SU11274 fluorescence intensities were normalized to the highest fluorescence intensity observed and plotted as a function of time. The rate of uptake was calculated by dividing the change in fluorescence intensity from consecutive intensity measurements by the increment of time elapsed between measurements (20 seconds).

Subcellular SU11274 localization

SU11274 (Selleckchem) was applied at 2 µM for 30 minutes prior to fixing, permeabilizing, and staining the cells with organelle-specific antibodies for 2 hours at
4°C. A rabbit isotype control antibody (NI01, MilliporeSigma) was applied at the same concentration as the specific primary antibodies. Anti-rabbit Alexa Fluor 647 was applied to the cells in the case that the primary antibody was not conjugated to Alexa Fluor 647.

Characterization of SU11274 concentration in the ER

SU11274 was applied to living cells at various concentrations (0, 0.1, 1, and 10 µM). ER colocalization was determined using ER-Tracker™ Red (10 nM, E34250, Thermo Fisher Scientific). ER-Tracker™ Red was excited using a 561 nm laser line and the resultant emission was collected using a 574-633 nm filter. Cells were imaged three minutes after the application of SU11274 and ER Tracker™ Red (added to cells simultaneously). Given that SU11274 emission could contaminate the ER-Tracker™ Red fluorescence collection, cells were sequentially imaged. Briefly, 488 nm excitation (used to excite SU11274) exhibits a 5% excitation efficiency for ER-Tracker™ Red, and the resultant ER-Tracker Red emission was completely independent of the SU11274 fluorescence detection filter. In cells treated with SU11274 only, SU11274 emission into the ER-Tracker™ Red specific channel was approximately 0.1% (0.10 ± 0.06%).

Areas of cells (n=9), cell medium (n=6), and the ER (n=9) were assigned as individual ROIs and the mean fluorescence intensity values for SU11274 in the cell medium, cells, and the ER were determined. Hoechst 33342 was used to stain the nuclei (blue). The Iso Data method (ZEN Blue) was used to optimize the image parameters to delimit the ER. SU11274-associated mean fluorescence intensities in individual cell ROIs and in acellular ROIs were analyzed to determine the relationship between SU11274 mean fluorescence intensity and SU11274 cell medium concentration. The SU11274 concentration was plotted as a function of the SU11274 mean fluorescence intensity measured in the cell medium. The average fluorescence intensity at 0 µM SU11274 of the cells and that of the ER was subtracted from cell and
ER data sets, respectively. The equation relating fluorescence intensity to SU11274 concentration ([SU11274] = 8.0749 x SU11274 Mean Fluorescence Intensity - 1.365) was used to estimate the SU11274 concentration in the cells and in the ER region of the cells at given cell medium SU11274 concentrations.

**Proximity Ligation Assay**

Duolink® In Situ Probemaker PLUS (Sigma-Aldrich) kit was used to make the CT2+ probe, which was applied to the cells at 1:100 v/v (1:100 µg/µL). The beta-catenin antibody (15B8) probe was added at 1:50 v/v (or 1:250 µg/µL). For the isotype control, the antibody was applied at 1:250 v/v (1:250 µg/µL). The total volume applied to each coverslip was 100 µL. The Duolink® (Sigma-Aldrich) proximity ligation assay (PLA) protocol was followed in order to quantify beta-catenin/MUC1.CT interactions in the cells.

**Mass Spectrometry**

Mass spectrometry was performed by Dragana Lagundzin, Ph.D., at the University of Nebraska Medical Center Mass Spectrometry and Proteomics Core Facility. After electrophoretic protein separation, gels were stained with Coomassie blue for 2h and left to destain overnight. Coomassie blue-stained gel pieces were manually cut using a sterile scalpel and kept in sterile microcentrifuge tubes. Gel pieces were washed with HPLC water and shrunk with acetonitrile (ACN) (100%). Proteins were reduced with 2 mM tris(2-carboxyethyl)phosphine (TCEP)/50 mM ammonium bicarbonate (AmBic) for 1h at 37°C. After incubation, ACN was added to TCEP to destain gel pieces. After gel pieces were dried by adding additional portion of ACN, thiol groups of proteins were alkylated with 55 mM iodoacetamide (IAA)/50 mM AmBic for 20 minutes in the dark, with rotation. Samples were dried with ACN, and 10 nM MS-grade trypsin (Promega) was added for protein digestion. Samples were incubated with trypsin
for 30 minutes on ice. After the excess trypsin was removed from the tubes, 25 mM AmBic was added to the gel pieces. Tryptic digestion continued overnight at 37°C. Digested peptides were then extracted from the gel with ACN (50%)/trifluoroacetic acid (0.1%) solution. Samples were dried in a Speedvac, dissolved in 15 µL formic acid (FA) (0.1%), and submitted for LC-MS/MS analysis.

In-gel digested peptide samples were analyzed using the high-resolution mass spectrometry LC-MS/MS system (LTQ Orbitrap Elite Velos Pro, Thermo Scientific, West Palm Beach, FL, USA), coupled with an Eksigent NanoLC-Ultra 1D plus (Eksigent, Dublin, CA, US) and nanoFlex chIPLC system (Eksigent), equipped with two alternating peptide traps. Ten microliters of each sample were loaded onto the peptide trap using 0.1% FA solvent. The samples were eluted using a 1h linear gradient of 0-60% ACN in 0.1% FA. Resolution of the full scan in the Orbitrap was set to 120,000 m/z with a range of 300 to 2000 Da. The collision energy was set at 35 kV.

The MS/MS spectra from the peptides were analyzed by assigning the fragments to the candidate sequence using the MASCOT search engine (Matrix Science, London, UK, version 2.5.1) with a Swissprot database (Taxonomy: Mammalia). Parameters on MASCOT were set as follows: enzyme, trypsin; max missed cleavage, 2; peptide charge, 1+, 2+, and 3+; peptide tolerance, ± 0.8 Da; fixed modifications, carbamidomethyl (C); variable modifications, oxidation (M), phospho (ST) and phospho (Y); MS/MS tolerance, ± 0.6 Da; instrument, ESI-TRAP. MASCOT results for different gel cuts of the same sample were combined and analyzed using Scaffold (Proteome Software, Inc., Portland, OR, version 4.4.5), which allows multiple search results to be condensed into a single result file. Peptide identifications were accepted if they were established at greater than 95.0% probability by the Peptide Prophet algorithm [136] with Scaffold delta-mass correction. Protein identifications were accepted if they were established at greater than 95.0%. Protein probabilities were assigned by the Protein
Prophet algorithm [137]. Proteins that contained similar peptides and could not be
differentiated based on MS/MS analysis alone were grouped to satisfy the principles of
parsimony. Proteins sharing significant peptide evidence were grouped into clusters.

*Fluorescence Lifetime Imaging/Förster Resonance Energy Transfer*

Fluorescence lifetime imaging (FLIM) of two-photon-excited eGFP was
performed using the 920-nm mode-locked femtosecond pulse train of a Spectra Physics
MaiTai Ti:S laser on a Leica TCS SP8 MP multiphoton laser scanning confocal
microscope (Leica Microsystems, Germany) using a Leica HC PL APO CS2 40X/1.3 NA
oil immersion objective at the Creighton University Integrated Biomedical Imaging
Facility (IBIF) with the assistance of Dr. Michael G. Nichols. Green and red non-
descanned fluorescence was separated using a 565-nm long pass dichroic mirror,
isolated with an ET 525/50M and HQ 645/75M band-pass filters (Chroma Technology,
Bellows Falls, VT, USA), respectively, and detected with high-sensitivity Super HyD
detectors and a time-correlated single photon counting module (830 SPC, Becker and
Hickl, Berlin, Germany). Individual imaging windows (128x128 pixels) acquired using a
zoom of 2.5 represented an area of approximately 110 µm (0.87 µm per pixel). Photons
were accumulated for 120s (total acquisition time).

*Isothermal Titration Calorimetry*

All reactions were measured with an ITC200 isothermal titration calorimeter from
Microcal (Northampton, MA). The reaction cell was identical to the reference cell, which
was filled with distilled water. In a typical isothermal titration calorimetry (ITC)
experiment the ligand solution was placed in the syringe and injected by a computer-
controlled step motor into the sample cell with the macromolecule solution. The heat
absorbed or released in each injection was measured by a thermoelectric device, which
increased or reduced the power input of the sample to maintain the same temperature between the sample and reference cells [138, 139].

A 40 μL syringe was used to inject the titrant, MUC1.CT peptide in this case. The solution was mixed by stirring the syringe at 1000 rpm. Typically, 2 μL of MUC1.CT peptide were injected into the sample cell containing beta-catenin. This was repeated four additional times with about 5 minutes between injections. The peptide and protein were both in 30 mM Tris and 150 mM NaCl at a pH of 8.0. The reaction heat of each injection was measured by integration of the area of the injection curve, corrected for the dilution heat of the titrant, and normalized by the moles of titrant added to yield the reaction change in enthalpy, \( \Delta H_{\text{ITC}} \) [138]. ITC experiments were designed to obtain the heat, \( \Delta H_{\text{ITC}} \), for each reaction by averaging the reaction heat of the five injections under unsaturated conditions. Typically, the solution of the MUC1.CT peptide in the titrating syringe had a ten-fold higher molarity than the recombinant beta-catenin solution to produce peaks under unsaturated conditions.

A PD Midi Trap G-10 column was used to remove excess TFA from MUC1.CT peptides in which TFA was present. The peptides were dissolved in the same buffer used to purify the beta-catenin in all ITC experiments.

**OpenSPR**

A Nicoya OpenSPR instrument and TraceDrawer software were used to determine kinetics of interaction between beta-catenin and MUC1.CT peptides. Freshly prepared His-tag beta-catenin (50 μg) was applied to a carboxyl sensor chip (SEN-AU-100-12-COOH, Nicoya LifeSciences). All experiments were conducted in PBS, pH 8.0. If TFA was present in the MUC1.CT peptide, it was removed by chloride replacement (at least two rounds of dissolving the peptide in 10 mM HCl followed by lyophilization to complete dryness). The peptides were dissolved in the same buffer used to purify the beta-catenin in all OpenSPR experiments.
Statistical analysis

Values were expressed as means ± standard error of the mean (SEM). Differences between groups were evaluated by a one-way ANOVA followed by non-parametric Mann Whitney tests (performed by GraphPad Prism 7.0d) for comparison between two groups. For Pearson correlation analysis in Chapter 4 the nonparametric Mann-Whitney test with Bonferroni method accounting for multiple comparisons was used to compare all non-control groups with the isotype antibody control. Differences were considered significant at p<0.05. Significant differences are indicated by asterisks only if confirmed with an experimental replicate unless otherwise noted in the text.
CHAPTER 3. THE BETA-CATENIN/MUC1.CT INTERACTION IN PANCREATIC CANCER

Introduction

The cytoplasmic tail of MUC1 and beta-catenin physically interact [95, 124]. Normally, MUC1 is restricted to the apical surface of epithelial cells. However, this apical polarity is lost in cancer cells, and MUC1 is expressed over the entire surface of the cell [47]. This expands the potential interactome of MUC1 to include proteins in the lateral surface as well as the basal surface of the cell. One such lateral protein is beta-catenin, which in cells normally associates with E-cadherin in the formation of adherens junctions. The beta-catenin/MUC1.CT interaction prevents degradation of beta-catenin in the cytoplasm, thereby permitting beta-catenin to translocate to the nucleus where it acts as a transcriptional coactivator (through association with TCF/LEF transcription factors) [95, 124]. Dysregulation of Wnt signaling correlates with increased expression of proto-oncogenes such as c-Myc and cyclin D1 in various human cancers [140, 141], including pancreatic cancer [142]. Additionally, some evidence suggests that aberrant beta-catenin signaling is necessary for pancreatic carcinogenesis [13].

Just as MUC1 is no longer restricted to the apical surface of cancer cells, receptor tyrosine kinases are no longer constrained to the basolateral surface [7]. This means that stimulation of RTKs by growth factors could lead to MUC1.CT phosphorylation, and, in fact, it has been reported that stimulation of certain RTKs not only causes phosphorylation of MUC1.CT, but also an increase in interaction between MUC1.CT and beta-catenin [67]. Serine/threonine kinases are also known to phosphorylate MUC1.CT [93, 99], and in some cases such phosphorylation increases interaction with beta-catenin [99].
The phosphorylation state or states of MUC1.CT that are important for interaction with beta-catenin are not fully characterized. Phosphorylation of tyrosine residues in MUC1.CT are believed to be important for this interaction [143]. A specific sequence in the C-terminus of MUC1, known as the serine-rich motif, has also been shown to be important for interaction with beta-catenin [94]. Because phosphorylation of serine residues in other beta-catenin binding serine-rich motif-containing proteins (i.e., E-cadherin and APC) increases binding affinity by two to three orders of magnitude [25, 144], one might easily conceive of a similar scenario between beta-catenin and MUC1.CT. However, phosphorylation of MUC1.CT does not always have a positive effect on its interaction with beta-catenin. GSK-3β has been shown to phosphorylate a serine residue of MUC1.CT in a span of residues just N-terminal to the serine-rich motif, and this phosphorylation actually has an inhibitory effect on its interaction with beta-catenin [93]. Thus, phosphorylation of MUC1.CT may in some cases promote and in others abolish interaction with beta-catenin. Because Wnt signaling results in cellular proliferation and the beta-catenin/MUC1.CT interaction is known to occur in and promote PDAC, understanding the nature of this interaction is of consequence.
Results

In order to understand the nature of the beta-catenin/MUC1.CT association at steady state in PDAC cells, S2-013.MUC1F cells were fractionated into cytoplasm/membrane (Figure 2A) and nuclear (Figure 2B) fractions and subjected to immunoprecipitation with a beta-catenin antibody as well as with a MUC1.CT antibody in a reciprocal experiment. The lysate and immunoprecipitation samples were subjected to western blot to determine the presence of beta-catenin and MUC1.CT.

A typical result of fractionation of S2-013.MUC1F cells into cytoplasm/membrane (Figure 2C) and nuclear fractions (Figure 2D) is also shown.
Figure 2. The beta-catenin/MUC1.CT association occurs both in the cytoplasm/membrane and nuclear fractions of S2-013.MUC1F cells.

(A) S2-013.MUC1F cells were lysed and fractionated. The cytoplasm/membrane fraction was subjected to immunoprecipitation with beta-catenin antibody 15B8 (and mouse IgG isotype control) and MUC1.CT antibody CT2 (and hamster IgG isotype control). Lys stands for lysate. (B) The nuclear fraction was also subjected to the same immunoprecipitation regime as in A. Notice the especially dark band at the top of the MUC1.CT band. This corresponds to the light chain of the mouse antibody used for immunoprecipitation. (C) This blot is a typical result of fractionation of S2-013.MUC1F cells into cytoplasm/membrane (presence of beta-actin and no H2B) and (D) nuclear (presence of H2B and no beta-actin) fractions prior to immunoprecipitation.
When we immunoprecipitated beta-catenin (15B8 antibody), we found that MUC1.CT coimmunoprecipitated with it from the cytoplasm/membrane fraction of the cells (Figure 2A). In the reciprocal experiment MUC1.CT was immunoprecipitated (CT2), and beta-catenin coimmunoprecipitated with it from the cytoplasm/membrane fraction. In Figure 2B we show that beta-catenin and MUC1.CT coimmunoprecipitated from the nuclear fraction as well. The fractionation method was confirmed (Figure 2C and D) by probing for β-actin (which was observed in the cytoplasm/membrane fraction and only minimally in the nuclear fraction) and H2B (observed in the nuclear fraction and not in the cytoplasm/membrane fraction). The results suggest that a subset of MUC1.CT isoforms between 20-25 kDa coimmunoprecipitate with beta-catenin.

In order to study the importance of phosphorylation of MUC1.CT on its association with beta-catenin, two-dimensional gel electrophoresis of beta-catenin immunoprecipitates were performed (Figure 3). Two-dimensional gel electrophoresis separates proteins by mass and charge, and any phosphorylation of MUC1.CT would alter the charge of the protein and its position within the gel. The immunoprecipitation samples are separated based on charge in the first dimension and then by molecular weight in the second dimension. The cytoplasm/membrane fraction of S2-013.MUC1F cells was used for this experiment. Because the beta-catenin antibody is a mouse antibody, and remnants of the antibody used in immunoprecipitation were anticipated in the gel, a mouse secondary antibody was used to manifest the presence of these antibody fragments in the isotype control (Figure 3A) and in the beta-catenin immunoprecipitate (Figure 3D). Then the membrane was probed for MUC1.CT (Figure 3B, isotype control; Figure 3E, beta-catenin immunoprecipitate) and finally for beta-catenin (Figure 3C, isotype control; Figure 3F, immunoprecipitate). Mouse antibody staining (Figure 3A and D) and MUC1.CT staining (Figure 3B and E) have been greyscaled.
Figure 3. MUC1.CT coimmunoprecipitates with beta-catenin from S2-013.MUC1F cells.

Two-dimensional gel electrophoresis was used to resolve MUC1.CT by molecular weight and isoelectric point from S2-013.MUC1F cell lysate. The cytoplasm/membrane fraction of S2-013.MUC1F cells was subjected to immunoprecipitation with an antibody that recognizes beta-catenin (15B8, mouse antibody) or an isotype control. Two-dimensional gel electrophoresis was used to resolve beta-catenin and MUC1.CT by molecular weight and isoelectric point. (A) The isotype control sample was electrophoresed in two dimensions. The membrane was then probed with secondary anti-mouse antibody to detect antibody fragments (immunoprecipitation remnants) prior to (B) probing the membrane for MUC1.CT. (C) The same membrane was then probed for beta-catenin. (D), (E), and (F) were probed in the same sequence as (A), (B), and (C). (F) This is a two-dimensional gel electrophoresis of the 15B8 immunoprecipitate from the cytoplasm/membrane fraction of S2-013.MUC1F cells. Beta-catenin is the red band between 75 and 100 kDa.
When we immunoprecipitated beta-catenin and then probed for MUC1.CT after two-dimensional gel electrophoresis (Figure 3E), we found beta-catenin appeared as a red band (Figure 3F) at about 75 kDa, predominantly with an isoelectric point of 5 to 8. This suggests that beta-catenin has been charge modified in S2-013.MUC1F cells. We also found that MUC1.CT coimmunoprecipitated with beta-catenin. MUC1.CT appears as a dark blotchy smear between 20 and 25 kDa with an isoelectric point of 4 to 7. The breadth of MUC1.CT forms at various isoelectric points suggests that MUC1.CT of various charge-related post translational modifications interacts with beta-catenin. Similar results were produced at least three times.

To study the beta-catenin/MUC1.CT association in a cell line with endogenous MUC1 expression, the PDAC cell line CFPAC-1 was selected. This cell line produces an easily detectable amount of both beta-catenin and MUC1.CT. The cytosol and membrane fractions of the cells were subjected to immunoprecipitation with a MUC1.CT antibody. Western blot of lysates and immunoprecipitation (IP) samples was performed. The membrane was probed for beta-catenin and MUC1.CT (Figure 4).
Figure 4. Beta-catenin associates with MUC1.CT in the membrane of CFPAC-1 cells.

CFPAC-1 cells were separated into cytosol and membrane fractions. Each fraction was divided in two and applied to either Dynabeads Protein G bound to isotype control hamster IgG antibody or Dynabeads Protein G bound to MUC1.CT (CT2) antibody. Lys stands for lysate.
We see in Figure 4 that the beta-catenin/MUC1.CT association occurs in a pancreatic cancer cell line with endogenous beta-catenin and MUC1. We immunoprecipitated MUC1.CT and probed for MUC1.CT (bands at 15 and 20 kDa) and beta-catenin (90 kDa) and found the association in the membrane fraction of the cells, but not in the cytosol fraction.

Proximity ligation assay (PLA) [145] was performed to detect the interaction between beta-catenin and MUC1.CT in S2-013.MUC1F cells (Figure 5). PLA enables one to detect molecular interactions that are no more than 40 nm apart [146]. A yellow spot in the image indicates an interaction between beta-catenin and MUC1.CT. MUC1.CT antibody only was applied to the negative control cells (132 cells in three fields) (Figure 5A), and beta-catenin and MUC1.CT antibodies were applied to the test samples (103 cells in three fields) (Figure 5B).
Figure 5. Beta-catenin and MUC1.CT interact in S2-013.MUC1F cells as determined by proximity ligation assay.

(A) The beta-catenin antibody was omitted for the negative control. (B) Each yellow spot indicates that a beta-catenin molecule is within 40 nm of a MUC1.CT molecule in the cell. (C) Three consecutive z stacks of the cells enable one to see that some of the interactions are occurring within the nuclei of the cells. The arrows point to nuclear interactions.
In the negative control cells (in which no beta-catenin antibody was applied), 0.05 ± 0.10 spots per cell were counted (Figure 5A). When beta-catenin and MUC1.CT antibodies were applied to the S2-013.MUC1F cells (Figure 5B), 17.0 ± 1.7 spots per cell were counted. The images in Figure 5C were produced by taking three sequential confocal microscopy images along the z-axis (z stacks) with 1 µm separation between images. The beta-catenin/MUC1.CT interactions can be found in the cytoplasm and/or membrane as well as within the DAPI stained nuclei (arrows). In a repeat experiment 1.3 ± 0.8 spots per cell were observed in the negative control samples and 10.7 ± 2.2 spots per cell were observed in the test samples.

PLA was performed to detect the interaction between beta-catenin and MUC1.CT in CFPAC-1 cells (Figure 6). Only MUC1.CT antibody was applied to the negative control cells (111 cells in three fields) (Figure 6A), and beta-catenin and MUC1.CT antibodies were applied to the test samples (70 cells in three fields) (Figure 6B).
Figure 6. Beta-catenin and MUC1.CT interact in CFPAC-1 cells as determined by proximity ligation assay.

(A) The beta-catenin antibody was omitted for the negative control. (B) Each yellow spot indicates that a beta-catenin molecule is within 40 nm of a MUC1.CT molecule in the cell. The nuclei are stained with DAPI (blue).
In the negative control cells (to which no beta-catenin antibody was applied), 1.5 ± 1.1 spots were detected per cell in 111 cells (Figure 6A). When beta-catenin and MUC1.CT antibodies were applied to the CFPAC-1 cells, a mean of 15.0 ± 2.2 interactions per cell were detected in 70 cells (Figure 6B). Thus, we show by PLA that beta-catenin/MUC1.CT interactions occur in the cytoplasm and/or membrane of CFPAC-1 cells. We have now observed that the beta-catenin/MUC1.CT association occurs in PDAC cells that overexpress MUC1 as well as those with endogenous expression of MUC1 by immunoprecipitation and PLA.

In order to study the nature of the association between beta-catenin and MUC1.CT in human tissue, a PDAC liver metastasis was stained for beta-catenin and MUC1.CT (Figure 7). The emission spectrum detected for DAPI (nuclei) was 400 to 491 nm. The emission spectrum detected for Alexa Fluor 488 (MUC1.CT) was 488 to 591. The emission spectrum detected for Alexa Fluor 647 (beta-catenin) was 645 to 700 nm.
Figure 7. Beta-catenin and MUC1.CT colocalize in pancreatic cancer.

(A) Secondary only control. (B) In a serial section beta-catenin is displayed in purple and MUC1.CT is displayed in green. DAPI was used to stain the nuclei (blue). Colocalization is shown in white.
In this PDAC liver metastasis sample beta-catenin staining is visible in the membrane of about half of the cells in the field (Figure 7). MUC1.CT (green) stained the lumen of ducts. Neither beta-catenin nor MUC1.CT was present in the nuclei of the cells. It appears that the association between beta-catenin and MUC1.CT in the pancreatic cancer sample occurs largely in the membrane of the cells, especially along the lumen of ducts. In some cases, large areas of beta-catenin/MUC1.CT colocalization occurred at points of contact between three or more cells.

To this point, we have observed examples of association of beta-catenin and MUC1.CT in cells and tissue. It has been reported that the beta-catenin/MUC1.CT interaction is enhanced by phosphorylation of MUC1.CT [55], so we completed several experiments to elucidate the phosphorylation status of MUC1.CT that coimmunoprecipitates with beta-catenin. To this end, we prepared a beta-catenin affinity column. We incubated 13 mg of S2-013.MUC1F protein lysate (1% NP40 lysis) on a beta-catenin affinity column overnight at 4°C. The column was rinsed, and glycine elution fractions were collected. The lysate, a rinse fraction, and all elution fractions were analyzed by western blot (Figure 8A), and elution fraction 2 (E2) was analyzed by SDS-PAGE and Coomassie staining (Figure 8B).
Figure 8. Beta-catenin affinity column elution fractions for MUC1.CT analysis.

S2-013.MUC1F cytoplasm/membrane fraction lysate incubated on a beta-catenin antibody (15B8) agarose column. (A) Beta-catenin and MUC1.CT were detected in the lysate (Lys), flow through (FT), rinses (R), and elution fractions (E) by western blot. (B) Fraction E2 was analyzed by SDS-PAGE and Coomassie staining.
Because elution fraction 2 had the highest amount of beta-catenin (Figure 8A), we decided to pursue mass spectrometric analysis of this sample. The coimmunoprecipitating MUC1.CT is barely detectable in the western blot. Elution fraction 2 was subjected to SDS-PAGE and Coomassie staining (Figure 8B).

Following in-gel trypsin digestion, the sample was analyzed by tandem mass spectrometry. MUC1.CT peptide ions were identified by matching the mass and charge of the peptide ions detected to a Mascot database (Table 1). Ions score is -10log(P), where P is the calculated probability that the observed match between the experimental data and the Mascot database sequence is a random event. The expect score is the number of equal or better matches expected to occur by chance alone. The highest ions score and lowest expect score for each peptide are listed in Table 1.
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<td>5.6E-08</td>
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</table>

Table 1. S2-013.MUC1F cytoplasm/membrane beta-catenin coimmunoprecipitated MUC1.CT peptides identified by mass spectrometry, initial attempt.

Beta-catenin antibody immunoprecipitation elution fraction 2 (Figure 8) was subjected to in-gel trypsin digestion followed by tandem mass spectrometry. The peptides identified by mass spectrometry are listed here. Span indicates the region of MUC1 that the peptide spans (human MUC1, P15941, UniProtKB). Ion indicates the amino acid sequence of the ion identified. Ions Score is -10log(P), where P is the calculated probability that the observed match between the experimental data and the Mascot database sequence is a random event. The Expect Score is the number of equal or better matches expected to occur by chance alone.
The QGGFLGLSNIK peptide (Table 1) was identified eight times in the first replicate and two times in a second replicate. This stretch of amino acids of MUC1 is believed to be normally situated in the extracellular space. The high ions score and low expect score make it likely that MUC1 components that include this portion of MUC1 interact with beta-catenin. The NYGQLDIFPAR peptide was identified once in the first replicate and three times in the second technical replicate. This stretch of amino acids is within the cytoplasmic tail of MUC1. Again, the high ions score and low expect score indicate that this is a valid identification, suggesting that the forms of MUC1 that interact with beta-catenin include this sequence. In this experiment, no phosphorylated MUC1.CT was detected in the beta-catenin coimmunoprecipitate, suggesting that unphosphorylated MUC1.CT associates with beta-catenin.

Again, S2-013.MUC1F cells were lysed and the cytoplasm/membrane fraction collected. In this case, phosphatase inhibitors were used in every step of preparation prior to mass spectrometry to ensure no loss of phosphorylation of MUC1.CT peptides and to increase the odds of identifying phosphorylated peptides. The lysate was subjected to immunoprecipitation with beta-catenin antibody 15B8. The immunoprecipitates were isolated after SDS-PAGE and Coomassie staining. Following in-gel trypsin digestion, samples were analyzed by tandem mass spectrometry. MUC1.CT peptide ions were identified by matching the mass and charge of the peptide ions detected to a Mascot database (Table 2).
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Table 2. S2-013.MUC1F cytoplasm/membrane beta-catenin coimmunoprecipitated MUC1.CT peptides identified by mass spectrometry.

Beta-catenin antibody immunoprecipitates were analyzed by tandem mass spectrometry. The peptides identified are listed here. Span indicates the region of MUC1 that the peptide spans (human MUC1, P15941, UniProtKB). Ion indicates the amino acid sequence of the ion identified. Phosphorylated residues are indicated with a lowercase p followed by the amino acid residue symbol (both in red font). Ions Score is -10log(P), where P is the calculated probability that the observed match between the experimental data and the Mascot database sequence is a random event. The Expect Score is the number of equal or better matches expected to occur by chance alone.
Though we had used phosphatase inhibitors in the buffers of all stages of preparation of the mass spectrometry sample (lysis, rinse, and elution buffers) to maintain phosphorylation of the fraction of MUC1.CT that coimmunoprecipitates with beta-catenin, no phosphorylation was observed (Table 2). However, the NYGQLDIFPAR peptide of MUC1.CT was observed again. The high ions score (59) and low expect score (4.6E-5) indicate that this is a true identification. In addition, the MUC1.CT peptide EGTINVHDVETQFNQYK was observed. The high ions score (67) and low expect score (2.2E-6) signify the validity of this identification. This peptide is at the N-terminus of the 158 amino acid C-terminal subunit of MUC1.

As a control for nonspecific interactions of MUC1.CT with the Dynabeads used for immunoprecipitation, the following experiment was performed. S2-013.MUC1F cells were lysed and the cytoplasm membrane fraction collected. Again, phosphatase inhibitors were used in every step of preparation prior to mass spectrometry. The lysate was subjected to immunoprecipitation with an isotype antibody. The immunoprecipitates were isolated after SDS-PAGE and Coomassie staining. Following in-gel trypsin digestion, samples were analyzed by tandem mass spectrometry. MUC1.CT peptide ions were identified by matching the mass and charge of the peptide ions detected to a Mascot database (Table 3).
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**Table 3. S2-013.MUC1F cytoplasm/membrane fraction MUC1.CT peptides identified by mass spectrometry, isotype control.**

Isotype control antibody immunoprecipitates were analyzed by tandem mass spectrometry. The peptides identified are listed here. Span indicates the region of MUC1 that the peptide spans (human MUC1, P15941, UniProtKB). Ion indicates the amino acid sequence of the ion identified. Phosphorylated residues are indicated with a lowercase p followed by the amino acid residue symbol (both in red font). Ions Score is $-10\log(P)$, where $P$ is the calculated probability that the observed match between the experimental data and the Mascot database sequence is a random event. The Expect Score is the number of equal or better matches expected to occur by chance alone.
Neither unphosphorylated MUC1.CT peptide observed in the beta-catenin immunoprecipitation sample was observed in the isotype control experiment (Table 3), and only non-significant scores for the possible identifications indicate that no detectable MUC1.CT eluted from the isotype control column. To summarize mass spectrometry results to this point, MUC1.CT peptides were found in association with beta-catenin in the cytoplasm/membrane fraction of PDAC cells. However, none of these MUC1.CT peptides were phosphorylated. This result suggests that phosphorylation is not required for beta-catenin/MUC1.CT association.

We then looked for MUC1.CT peptides coimmunoprecipitating with beta-catenin from the nuclear fraction of cells. S2-013.MUC1F cells were lysed and the nuclear fraction collected. Phosphatase inhibitors were used in every step of preparation prior to mass spectrometry to ensure no loss of phosphorylation of MUC1.CT peptides and to increase the odds of identifying phosphorylated peptides. The lysate was subjected to immunoprecipitation with a beta-catenin antibody. The immunoprecipitates were isolated after SDS-PAGE and Coomassie staining. Following in-gel trypsin digestion, samples were analyzed by tandem mass spectrometry. MUC1.CT peptide ions were identified by matching the mass and charge of the peptide ions detected to a Mascot database (Table 4).
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Table 4. S2-013.MUC1F nuclear fraction beta-catenin coimmunoprecipitated MUC1.CT peptides identified by mass spectrometry.

Nuclear beta-catenin immunoprecipitates were analyzed by tandem mass spectrometry. The peptides identified are listed here. Span indicates the region of MUC1 that the peptide spans (human MUC1, P15941, UniProtKB). Ion indicates the amino acid sequence of the ion identified. Phosphorylated residues are indicated with a p followed by the amino acid residue symbol (both in red font). Ions Score is -10log(P), where P is the calculated probability that the observed match between the experimental data and the Mascot database sequence is a random event. The Expect Score is the number of equal or better matches expected to occur by chance alone.
The EGTINVHDTVQFNQYK and NYGQLDPAR MUC1.CT peptides were observed in the nuclear fraction for the beta-catenin coimmunoprecipitates analyzed by mass spectrometry (Table 4). The high ions score and low expect score for these peptides indicate the validity of these identifications. The low ions scores and high expect scores for the phosphorylated MUC1.CT peptides listed in Table 4 indicate that phosphorylated MUC1.CT did not coimmunoprecipitate with beta-catenin from the nuclear fraction of S2-013.MUC1F cells.

An experiment to control for nonspecific interactions of MUC1.CT with the Dynabeads was performed with the nuclear fraction as well. S2-013.MUC1F cells were lysed and the nuclear fraction collected. Phosphatase inhibitors were used in every step of preparation prior to mass spectrometry. The lysate was subjected to immunoprecipitation with an isotype antibody. The immunoprecipitates were isolated after SDS-PAGE and Coomassie staining. Following in-gel trypsin digestion, samples were analyzed by tandem mass spectrometry. MUC1.CT peptide ions were identified by matching the mass and charge of the peptide ions detected to a Mascot database (Table 5).
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Table 5. S2-013.MUC1F nuclear fraction MUC1.CT peptides identified by mass spectrometry, isotype control.

Nuclear isotype control immunoprecipitates were analyzed by tandem mass spectrometry. The peptides identified are listed here. Span indicates the region of MUC1 that the peptide spans (human MUC1, P15941, UniProtKB). Ion indicates the amino acid sequence of the ion identified. Phosphorylated residues are indicated with a lowercase p followed by the amino acid residue symbol (both in red font). Ions Score is -10log(P), where P is the calculated probability that the observed match between the experimental data and the Mascot database sequence is a random event. The Expect Score is the number of equal or better matches expected to occur by chance alone.
In the isotype control sample a few MUC1 peptides were observed by mass spectrometry in the nuclear fraction (Table 5), possibly due to non-specific sticking of low levels of MUC1 to either isotype control antibodies or the beads used in purification. The NYGQLDIFPAR peptide was detected with significant scores, but not the EGTINVHDVETQFNQYK peptide. The mass spectrometry results indicate that MUC1.CT associates with beta-catenin in both the cytoplasm/membrane and nuclear fractions of PDAC cells. It also appears that MUC1.CT phosphorylation is not required for this association.

We also wanted to probe the nature of the beta-catenin/MUC1.CT interaction through biophysical methods. In order to conduct in vitro studies with beta-catenin, the recombinant protein was produced in and purified from *E. coli*. The BL21 (DE3) [132] strain was selected for synthesis of the murine recombinant His-tag [147] protein beta-catenin (Figure 9). The human and murine proteins are both 781 amino acids in length. The human protein differs from the murine protein by a single amino acid in the C-terminus (proline to alanine at 706) [148, 149]. Protein samples were analyzed by SDS-PAGE and Coomassie Brilliant Blue [150] protein staining.
Figure 9. Full-length beta-catenin purification strategy.

This Coomassie stained gel shows the recombinant protein at various stages of purification. The protein is purified first by nickel affinity chromatography followed by size exclusion chromatography.
The protein is soluble (the pellet fraction has little beta-catenin), and nickel affinity chromatography [151] (imidazole elution) successfully purifies the protein (Figure 9). The protein has an apparent molecular weight between 75 and 100 kDa. After size exclusion chromatography [152] the protein was exceptionally pure.

It has been hypothesized that beta-catenin interacts with the serine-rich motif of MUC1.CT (SXXXXXSSL). Therefore, isothermal titration calorimetry (ITC) was performed to investigate this. Five different MUC1.CT serine-rich motif peptides were injected into a recombinant beta-catenin solution (Table 6). A representative example of an ITC trace (MUC1.CT peptide VSGNGGSSLSYTNPA) is shown in Figure 10. Dilution heat, the heat released when the buffer (without peptide) is injected into the beta-catenin protein solution, is subtracted from the heat released upon peptide injection in order to determine the enthalpy resulting from protein/peptide interaction.
Figure 10. Isothermal titration calorimetry trace of beta-catenin and a MUC1.CT peptide.

The peptide sequence is VSAGNGGSSLTYNPA. The enthalpy change is derived from the area under the curve of the trace.
Each peak corresponds to a point at which MUC1.CT peptide was injected into the beta-catenin protein solution (Figure 10). The area under the curve is used to determine the enthalpy change, which is plotted in the lower half of the figure. Four identical injections were used to estimate the enthalpy change for the interaction of beta-catenin with this specific MUC1.CT peptide. The enthalpy change determined was very low, suggesting a lack of interaction between beta-catenin and this serine-rich motif-containing MUC1.CT peptide.

The enthalpies of interaction of five MUC1.CT peptides with beta-catenin were determined by ITC. Two beta-catenin constructs were used to study the beta-catenin/MUC1.CT interaction: full-length beta catenin and the armadillo repeat region (residues 134-671) of beta-catenin (β59) [148]. In each case, at least four injections were made, and the average enthalpy change is displayed in Table 6. One phosphorylated MUC1.CT peptide and one phosphomimetic peptide (serine to glutamate residue mutations) were selected to study the impact of phosphorylation on interaction with beta-catenin.
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**Table 6. Beta-catenin and MUC1.CT peptide interaction enthalpy change as determined by isothermal titration calorimetry.**

The change in enthalpy was investigated with five MUC1.CT peptides, one of which was phosphorylated at two tyrosine residues (pY), and one of which was a serine residue to glutamate residue phosphomimetic (glutamate residues, E). The enthalpy change was determined with full-length beta-catenin as well as with β59, the armadillo repeat region (N- and C-termini omitted) of beta-catenin.
The enthalpy change upon interaction of beta-catenin and the MUC1.CT peptides tested was generally about -1 kcal/mol (Table 6). A hydrogen bond between two peptides has an enthalpy change of -0.5 to -1.5 kcal/mol [153]. When ITC was performed with beta-catenin and proteins that bind beta-catenin, the highest enthalpy change measured (phosphorylated cytoplasmic tail of E. cadherin) was -53.5 kcal/mol, and the weakest (third 20 amino-acid repeat of APC) was -4.5 kcal/mol [24]. The MUC1.CT peptide GRpYVPPSSTDRSpYEKVSAGNGSSLSYTNPA produced a similar result to the beta-catenin/APC interaction. However, this result was confounded by the fact that the enthalpy change was actually endothermic (2.2 kcal/mol) when the interaction between this MUC1.CT peptide and β59 (the armadillo repeat region of beta-catenin) was tested. The armadillo repeat region of beta-catenin is thought to be important in the beta-catenin/MUC1.CT interaction [95]. No ITC isotherms were produced between MUC1.CT peptides and beta-catenin, so the thermodynamic profiles of the reactions were not determined. It appears that none of these peptides, with or without the serine-rich motif and with or without phosphorylation, interact strongly with beta-catenin.

In order to study the interaction between beta-catenin and longer forms of MUC1.CT in vitro, His-tag MUC1.CT was purified by cobalt-affinity chromatography. After purification, the protein was concentrated. MUC1.CT is not stained by Coomassie or silver. Therefore, a Coomassie stained gel and western blot were used to confirm identity and to determine purity of the protein (Figure 11).
Figure 11. MUC1.CT purification strategy.

(A) The His-tag MUC1.CT protein fractions eluted from the cobalt affinity column were concentrated. A Coomassie stained gel indicates that the cobalt affinity purification removed many contaminating proteins. (B) A western blot confirms the identity of MUC1.CT.
This recombinant protein has a ten-residue histidine tag at the N-terminus and 72 amino acids of the cytoplasmic tail of MUC1. After the protein was eluted with imidazole, it was concentrated to about 0.2 mg/mL. The Coomassie staining was used to determine that impurities were removed, and the western blot confirmed the identity of the protein (Figure 11). With methods to produce and purify both proteins, we were now ready to conduct in vitro experiments with beta-catenin and MUC1.CT.

One such method that we employed was OpenSPR [154]. In an OpenSPR experiment, one of the proteins is covalently bound to a gold sensor chip by dimethylaminopropyl)-N′-ethylcarbodiimide (EDC) and N-hydroxy succinimide chemistry [155]. In this way we tested the interaction between full-length beta-catenin and MUC1.CT with beta-catenin bound to the sensor chip and MUC1.CT as the analyte. The reciprocal experiment was also performed (Figure 12).
Figure 12. OpenSPR traces of the interaction between recombinant beta-catenin and recombinant MUC1.CT.

(A) Beta-catenin was applied to the sensor chip and MUC1.CT served as the analyte. Duplicates of three concentrations of MUC1.CT (indicated to the right of each line) were used to determine the kinetics of interaction and the binding affinity. (B) In the reciprocal experiment MUC1.CT was applied to the sensor chip and beta-catenin served as the analyte. Three concentrations of beta-catenin (indicated to the right of each line) were tested to determine the kinetics of interaction and the binding affinity.
The concave down curve between approximately 0 and 250 seconds represents the association (binding) phase of interaction, and the concave up curve from 250 to 350 seconds represents the dissociation phase of the interaction (Figure 12). The computer-generated black line was used to determine the kinetics of interaction.

In addition to the experiments shown in Figure 12, OpenSPR was employed to test the interaction between full-length beta-catenin and ten MUC1.CT peptides (and a scrambled sequence control) (Table 7). Six of the MUC1.CT peptides tested were phosphopeptides. Ecyto (the full cytoplasmic tail of murine E-cadherin) [24] served as a positive control for interaction with beta-catenin. All experiments were performed in PBS (pH 8.0). The equilibrium dissociation constants (K_D), on rates (k_on), off rates (k_off), maximum SPR signal (B_max), and chi-squared (χ²) values are listed. Also, to control for the fact that covalently binding beta-catenin to the sensor chip might alter its ability to interact with MUC1.CT, the reciprocal experiment was performed, in which MUC1.CT was covalently bound to the sensor chip and beta-catenin served as the analyte.
<table>
<thead>
<tr>
<th>Analyte (beta-catenin bound to chip)</th>
<th>$K_D$</th>
<th>$k_{on}$ (1/Ms)</th>
<th>$k_{off}$ (1/s)</th>
<th>$B_{max}$</th>
<th>$\chi^2$</th>
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<tbody>
<tr>
<td>Ecyto</td>
<td>110 ± 20 nM</td>
<td>650 ± 20</td>
<td>7E-5 ± 1E-5</td>
<td>206</td>
<td>9.8</td>
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<tr>
<td>His-tag MUC1.CT (full-length)</td>
<td>13.8 ± 0.3 µM</td>
<td>174 ± 4</td>
<td>2.41E-3 ± 1E-5</td>
<td>345</td>
<td>16</td>
</tr>
<tr>
<td>NYGQLDIFPARDTDYHPSSEYPTYHTGRYVPPSSTDREPYPEKVSAGNGGSSLSYTNPAAATSANL</td>
<td>15 ± 0.2 µM</td>
<td>340 ± 50</td>
<td>5.2E-3 ± 1E-4</td>
<td>155</td>
<td>19</td>
</tr>
<tr>
<td>CQCRRKNYGQLDIFPARDTDYPYPMSEYPTYH</td>
<td>2.3 ± 0.1 µM</td>
<td>1,020 ± 40</td>
<td>2.32E-3 ± 1E-5</td>
<td>1,981</td>
<td>1,278</td>
</tr>
<tr>
<td>CQCRRKNYGQLDIFPARDTPYHPSSEYPTYH</td>
<td>2.3 ± 1 mM</td>
<td>10 ± 30</td>
<td>2.47E-3 ± 1E-5</td>
<td>2,118</td>
<td>3,868</td>
</tr>
<tr>
<td>KSVGSPNESTAYSGLNAGYP (Scrambled MUC1.CT sequence)</td>
<td>1.06 ± 0.01 mM</td>
<td>10 ± 5E3</td>
<td>1.09E-2 ± 1E-4</td>
<td>60</td>
<td>163</td>
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<tr>
<td>PYEKVSAGNGGSSLSYTNPA</td>
<td>270 ± 40 µM</td>
<td>30 ± 270</td>
<td>9.35E-3 ± 1E-5</td>
<td>176</td>
<td>129</td>
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<tr>
<td>PYEKVPAGNGGSSLYTNPA</td>
<td>45 ± 1 µM</td>
<td>90 ± 6E2</td>
<td>3.96E-3 ± 3E-5</td>
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<td>117</td>
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<tr>
<td>PYEKVSAGNGGpSSSLSYTNPA</td>
<td>160 ± 20 µM</td>
<td>50 ± 390</td>
<td>7.89E-3 ± 1E-5</td>
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<tr>
<td>PYEKVSAGNGGSpSLSYTNPA</td>
<td>210 ± 20 µM</td>
<td>50 ± 490</td>
<td>1.03E-2 ± 1E-4</td>
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<td>150</td>
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<tr>
<td>PYEKVSAGNGGSSpSYTNPA</td>
<td>130 ± 20 µM</td>
<td>80 ± 670</td>
<td>1.0E-3 ± 1E-4</td>
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<td>GRYVPPSSTDREPYEKVSAGNGGSSLSYTNPA</td>
<td>306 ± 9 µM</td>
<td>30 ± 100</td>
<td>9.18E-3 ± 1E-5</td>
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<td>GRYVPpSpSpTDREPYEKVSAGNGGSSLSYTNPA</td>
<td>6.49 ± 0.28 mM</td>
<td>10 ± 2E3</td>
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<table>
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<tr>
<th>Analyte (MUC1.CT bound to chip)</th>
<th>$K_D$</th>
<th>$k_{on}$ (1/Ms)</th>
<th>$k_{off}$ (1/s)</th>
<th>$B_{max}$</th>
<th>$\chi^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>His-tag beta-catenin</td>
<td>2.69 ± 0.08 µM</td>
<td>1490 ± 40</td>
<td>4.03E-3 ± 1E-5</td>
<td>510</td>
<td>211</td>
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</tbody>
</table>

**Table 7. Beta-catenin and MUC1.CT binding affinity as determined by OpenSPR.**

Beta-catenin/MUC1.CT binding affinities were determined by OpenSPR. The experimentally determined values are displayed. Phosphorylation is indicated with red font (e.g., pS: phosphorylated serine residue). The PYEKVSAGNGGSSLSYTNPA sequence was scrambled to serve as a negative control for that sequence only.
E_{cyt} served as a positive control for interaction with beta-catenin (Table 7). The beta-catenin/E_{cyt} interaction was reported to have a 40 nM binding affinity by ITC [24]. The OpenSPR result reported here is 110 nM. However, the binding affinity (as determined by ITC) was determined in a different buffer, so comparison is not direct. The binding affinity determined with His-tag MUC1.CT was confirmed with the (nearly) full-length MUC1.CT peptide. Notice also that in the reciprocal experiment (MUC1.CT bound to the sensor chip, beta-catenin as the analyte) the binding affinity was within an order of magnitude of that observed with beta-catenin bound to the sensor chip and MUC1.CT serving as the analyte. According to these results the N-terminal half of MUC1.CT binds about 20 times more tightly to beta-catenin than the C-terminal half of MUC1.CT (which contains the serine-rich motif). It appears that the beta-catenin/MUC1.CT interaction relies on the N-terminal portion of MUC1.CT rather than on the serine-rich motif of MUC1.CT. The highest binding affinity of any phosphorylated peptide was that of the sequence PYEKVpSAGGGSLSNTPA, though this binding affinity was about three times lower than that of full-length MUC1.CT. The OpenSPR results corroborated the ITC results: the beta-catenin/MUC1.CT interaction does not require MUC1.CT phosphorylation.

Several kinases are known to phosphorylate MUC1, and phosphorylation of MUC1.CT may affect its interaction with other proteins, including an increase in nuclear association of MUC1.CT with beta-catenin [47, 59]. Various growth factors were selected to study the impact of growth factor-induced phosphorylation of MUC1.CT on levels of beta-catenin and MUC1.CT in the cytoplasm/membrane and nuclear fractions of pancreatic cancer cells. The beta-catenin and MUC1.CT levels in the cytoplasm/membrane fraction and nuclear fraction were tested to determine whether or not MUC1.CT translocated into the nucleus upon growth factor stimulation.
S2-013.MUC1F cells were treated with 10 ng/mL EGF for 0, 5, 10, 30, and 60 minutes. Cells were then lysed and fractioned into cytoplasm/membrane and nuclear fractions. The following western blots show beta-catenin and MUC1.CT levels at the indicated time points (Figure 13). Actin was used to determine relative levels of beta-catenin and MUC1.CT in the cytoplasm/membrane fraction, and HDAC2 was used to determine relative levels of beta-catenin and MUC1.CT in the nuclear fraction. ImageJ software was used to quantify protein levels.
Figure 13. Impact of EGF on cytoplasm/membrane and nuclear beta-catenin and MUC1.CT in S2-013.MUC1F cells.

(A) A representative western blot shows levels of beta-catenin and MUC1.CT in the cytoplasm/membrane fraction of the cells at selected time points after 10 ng/mL EGF stimulation. The number below each lane is the level of each protein relative to the level of actin at each time point. The values are normalized to protein levels at time point 0. (B) This is a representative western blot showing levels of beta-catenin and MUC1.CT in the nuclear fraction of the cells at selected time points after 10 ng/mL EGF stimulation. The number below each lane represents the level of each protein relative to the level of HDAC2 at each time point. The values are then normalized to the protein level at time point 0.
This is a representative example of the experiments performed to study the impact of growth factors on beta-catenin and MUC1.CT levels (Figure 13). These experiments were performed at least three times, and bar graphs summarize the results (Figure 14-Figure 16).

S2-013.MUC1F cells were treated with 100 ng/mL CTGF (Figure 14), 10 ng/mL HGF (Figure 15), or 10 ng/mL PDGF (Figure 16) for 0, 5, 10, 30, and 60 minutes. Cells were then lysed and fractionated into cytoplasm/membrane (A) and nuclear (B) fractions. Each experiment was repeated in triplicate. Western blots were prepared for each trial. Actin was used to determine relative levels of beta-catenin and MUC1.CT in the cytoplasm/membrane fraction, and HDAC2 was used to determine relative levels of beta-catenin and MUC1.CT in the nuclear fraction. The level of each protein was expressed as a value normalized to the level of each protein at time point 0.
Figure 14. Impact of connective tissue growth factor on beta-catenin and MUC1.CT levels.

(A) Levels of beta-catenin and MUC1.CT in the cytoplasm/membrane fraction of S2-013.MUC1F cells at selected time points after 100 ng/mL CTGF stimulation. (B) Levels of beta-catenin and MUC1.CT in the nuclear fraction of S2-013.MUC1F cells at selected time points after 100 ng/mL CTGF stimulation.
No significant changes in beta-catenin or MUC1.CT were observed upon treatment with CTGF (Figure 14). At times the substantial amount of variability made interpretation difficult. However, it appears that MUC1.CT may be slightly decreased in the nucleus at 10 minutes CTGF stimulation.
Figure 15. Impact of hepatocyte growth factor on beta-catenin and MUC1.CT levels.

(A) Levels of beta-catenin and MUC1.CT in the cytoplasm/membrane fraction of S2-013.MUC1F cells at selected time points after 10 ng/mL HGF stimulation. (B) Levels of beta-catenin and MUC1.CT in the nuclear fraction of S2-013.MUC1F cells at selected time points after 10 ng/mL HGF stimulation.
Again, no significant changes in beta-catenin or MUC1.CT levels were observed upon treatment with HGF (Figure 15). Again, variability made interpretation difficult. It may be that MUC1.CT and beta-catenin levels in the cytoplasm/membrane fraction are decreased with HGF stimulation.
Figure 16. Impact of platelet-derived growth factor on beta-catenin and MUC1.CT levels.

(A) Levels of beta-catenin and MUC1.CT in the cytoplasm/membrane fraction of S2-013.MUC1F cells at selected time points after 10 ng/mL PDGF stimulation.  (B) Levels of beta-catenin and MUC1.CT in the nuclear fraction of S2-013.MUC1F cells at selected time points after 10 ng/mL PDGF stimulation.
As with CTGF and HGF, no significant changes in beta-catenin or MUC1.CT levels were observed upon treatment with PDGF (Figure 16). It may be that beta-catenin in the nuclear fraction was decreased with PDGF stimulation.

S2-013.MUC1F cells were treated with 10 ng/mL EGF (Figure 17A and B) or 1 µM Erlotinib or DMSO vehicle control (Figure 17C and D) for the indicated amounts of time. Cells were then lysed and fractionated into cytoplasm/membrane (Figure 17A and C) and nuclear (Figure 17B and D) fractions. EGF experiments were repeated four times, and Erlotinib experiments were repeated in triplicate. Western blots were prepared for each trial. Actin was used to quantify levels of beta-catenin and MUC1.CT in the cytoplasm/membrane fraction, and HDAC2 was used for quantification of beta-catenin and MUC1.CT levels in the nuclear fraction. The level of each protein was expressed as a value normalized to the level of each protein at time point 0.
Figure 17. Impact of stimulating or inhibiting the epidermal growth factor receptor on beta-catenin and MUC1.CT levels.

(A) Levels of beta-catenin and MUC1.CT in the cytoplasm/membrane fraction of S2-013.MUC1F cells at selected time points after 10 ng/mL EGF stimulation. (B) Levels of beta-catenin and MUC1.CT in the nuclear fraction of S2-013.MUC1F cells at selected time points after 10 ng/mL EGF stimulation. (C) Levels of beta-catenin and MUC1.CT in the cytoplasm/membrane fraction of S2-013.MUC1F cells at selected time points after 1 µM Erlotinib treatment. (D) Levels of beta-catenin and MUC1.CT in the nuclear fraction of S2-013.MUC1F cells at selected time points after 1 µM Erlotinib treatment.

*p<0.05.
It appears that EGF affects MUC1.CT levels (Figure 17A and B). A decrease in MUC1.CT was observed in the cytoplasm/membrane fraction (at 10 and 60 minutes) and the nuclear fraction (at 5 and 10 minutes). Variability within the Erlotinib experiment made those results difficult to interpret (Figure 17C and D).

Because EGF in particular appears to alter MUC1.CT levels, two-dimensional gel electrophoresis was used to investigate the impact of EGF stimulation on MUC1.CT phosphorylation. To evaluate this within different cell compartments, cells were fractionated before two-dimensional gel electrophoresis.

CFPAC-1 cells (Figure 18A) and S2-013.MUC1F cells (Figure 18B) were untreated or treated for 5 minutes with 10 ng/mL EGF. Then cells were fractionated into cytosol, membrane, and nuclear fractions. Western blots indicate levels of beta-catenin and MUC1.CT following EGF stimulation. EGFR served as a membrane marker, and actin served as a cytosol marker. The blots were also probed for phosphotyrosine to determine whether or not EGF caused changes in phosphorylation of the cytoplasmic tail of MUC1.
Figure 18. Subcellular fractionation of CFPAC-1 and S2-013.MUC1F prior to two-dimensional gel electrophoresis.

The EGFR blot was used to verify membrane fraction isolation, and actin was used to verify cytosol fraction isolation. (A) CFPAC-1 cells were untreated (-) or treated (+) with 10 ng/mL EGF for 5 minutes and then fractionated into cytosol, membrane, and nuclear fractions. (B) S2-013.MUC1F cells were untreated (-) or treated (+) with 10 ng/mL EGF for 5 minutes and then fractionated in the same way.
We successfully separated the membrane fraction from other fractions as evidenced by the presence of EGFR and absence of beta-actin in the membrane fraction (Figure 18). We were therefore able to test the impact of EGF stimulation on MUC1.CT specifically in the membrane fraction.

CFPAC-1 cells were untreated or treated for 5 minutes with 10 ng/mL EGF. Following lysis, cells were fractionated into cytosol, membrane, and nuclear fractions. The membrane fraction was selected to investigate potential changes in phosphorylation of MUC1.CT. MUC1.CT was immunoprecipitated and then subjected to two-dimensional gel electrophoresis [156] (Figure 19). The blots were probed for MUC1.CT. The same blots were probed for phosphotyrosine also (Figure 19C and F). This experiment was repeated to ensure reproducibility.
Figure 19. EGF causes phosphorylation of MUC1.CT in the membrane of CFPAC-1 cells.

The membrane fraction of CFPAC-1 cells was subjected to immunoprecipitation with an antibody that recognizes the cytoplasmic tail of MUC1 (CT2, Armenian hamster antibody). Two-dimensional gel electrophoresis was used to resolve MUC1.CT by molecular weight and isoelectric point. (A) Secondary anti-hamster antibody was used to probe the membrane for antibody fragments prior to (B) blotting the membrane for MUC1.CT. (C) The same membrane was then probed for phosphotyrosine. (D), (E), and (F) are in the same sequence as (A), (B), and (C). (D), (E), and (F) are blots produced by two-dimensional gel electrophoresis of the CT2 immunoprecipitate from the membrane fraction of CFPAC-1 cells after 5 minutes of 10 ng/mL EGF stimulation. The arrows in the figure are described in the text below.
Four major MUC1.CT forms (black arrows, Figure 19B) exist in the membrane of CFPAC-1 cells spanning pH 5 to 7. After only 5 minutes of EGF stimulation phosphorylation of MUC1.CT occurred in all of the forms. A fifth MUC1.CT form (at about pH 6) appeared with EGF stimulation (yellow arrow, Figure 19E). The fraction of MUC1.CT phosphorylated in CFPAC-1 cells was visible at the top of the MUC1.CT band (25 kDa) (between red arrows, Figure 19F). This result supports the hypothesis that EGF stimulation causes MUC1.CT phosphorylation. We investigated the impact of EGF on the phosphorylation of membrane-fraction MUC1.CT in S2-013.MUC1F cells as well.

S2-013.MUC1F cells were untreated or treated for 5 minutes with 10 ng/mL EGF. Following lysis, cells were fractionated into cytosol, membrane, and nuclear fractions. The membrane fraction was selected to investigate potential changes in phosphorylation of MUC1.CT. MUC1.CT was immunoprecipitated and then subjected to two-dimensional gel electrophoresis (Figure 20). The blots were probed for MUC1.CT. The same blots were probed for phosphotyrosine also (Figure 20C and F). This experiment was repeated to ensure reproducibility.
Figure 20. EGF causes phosphorylation of MUC1.CT in the membrane of S2-013.MUC1F cells.

The membrane fraction of S2-013.MUC1F cells was subjected to immunoprecipitation with an antibody that recognizes the cytoplasmic tail of MUC1 (CT2, Armenian hamster antibody). Two-dimensional gel electrophoresis was used to resolve MUC1.CT by molecular weight and isoelectric point. (A) Secondary anti-hamster antibody was used to probe the membrane for antibody fragments prior to (B) blotting the membrane for MUC1.CT. (C) The same membrane was then probed for phosphotyrosine. (D), (E), and (F) are in the same sequence as (A), (B), and (C). (D), (E), and (F) are blots produced by two-dimensional gel electrophoresis of the CT2 immunoprecipitate from the membrane fraction of S2-013.MUC1F cells after 5 minutes of 10 ng/mL EGF stimulation. The arrows in the figure are described in the text below.
From this two-dimensional gel electrophoresis result, it appears that S2-013.MUC1F cells had seven major forms (black arrows, Figure 20B) of MUC1.CT. Notice also that some of these forms overlapped with those observed in Figure 3 (MUC1.CT coimmunoprecipitated with beta-catenin). The MUC1.CT form at pH 7 is most affected by EGF stimulation (red arrow, Figure 20F). Again, a 25 kDa MUC1.CT form becomes more visible with EGF stimulation, sitting just left of pH 7 (yellow arrow, Figure 20F). From the results of Figure 19 and Figure 20 it appears that EGF stimulation causes MUC1.CT phosphorylation of the membrane fraction of MUC1.CT.

EGF stimulation causes changes in levels of MUC1.CT (Figure 17) and in the phosphorylation of MUC1.CT (Figure 19 and Figure 20) in PDAC cells. To determine the impact of EGF stimulation on the association of beta-catenin and MUC1.CT, we performed immunoprecipitation experiments. S2-013.MUC1F cells were lysed after being untreated or treated with 10 ng/mL EGF for 5 minutes. The cytoplasm/membrane fraction was subjected to immunoprecipitation with a beta-catenin antibody (15B8). The lysates, isotype control immunoprecipitation samples, and beta-catenin specific immunoprecipitation samples were analyzed by western blot (Figure 21A). The experiment was repeated, except that in this case proteins extracted from the membrane were used in the immunoprecipitation, and MUC1.CT was immunoprecipitated (CT2) rather than beta-catenin (Figure 21B). Beta-catenin and MUC1.CT levels were normalized to no EGF treatment and quantified with ImageJ.
Figure 21. EGF causes loss of interaction between beta-catenin and MUC1.CT in the membrane fraction of S2-013.MUC1F cells according to coimmunoprecipitation data.

(A) When 10 ng/mL EGF was applied to S2-013.MUC1F cells for 5 minutes, the amount of beta-catenin that immunoprecipitated decreased as was the amount of coimmunoprecipitated MUC1.CT in the cytoplasm/membrane fraction. (B) In a similar experiment, upon 5 minutes 10 ng/mL EGF stimulation, the amount of beta-catenin that coimmunoprecipitated with MUC1.CT from the membrane fraction decreased. Numbers under the western blot lanes represent the relative amount of protein in the lane normalized to no EGF treatment.
EGF caused decreased MUC1.CT coimmunoprecipitation with beta-catenin in the cytoplasm/membrane fraction of S2-013.MUC1F cells (Figure 21). The reciprocal experiment (MUC1.CT immunoprecipitation, beta-catenin blot) conducted specifically in the membrane fraction of S2-013.MUC1F cells produced a corroborating result (Figure 21B).

To validate the result of diminished beta-catenin/MUC1.CT association observed by coimmunoprecipitation, confocal microscopy was employed. S2-013.MUC1F cells with mCherry beta-catenin and eGFP MUC1.CT were treated with 10 ng/mL EGF for 0, 20 minutes, or 4 hours and then fixed. Images were taken of cells expressing both fluorescent proteins with a confocal laser scanning microscope (Figure 22). Red indicates mCherry beta-catenin (Figure 22A-C), green indicates eGFP MUC1.CT (Figure 22D-F), and white indicates colocalization of the two fluorescent proteins (Figure 22G-I). The nuclei are stained with DAPI (blue). S2-013.MUC1F cells that produce no fluorescent proteins served as the negative control (Figure 22J-L). The thresholds for analyzing colocalization of MUC1.CT eGFP and mCherry beta-catenin were set based on the negative control S2-013.MUC1F cells that express neither fluorescent protein so that colocalization was virtually absent in the negative control cells. The emission spectrum detected for DAPI was 400 to 486 nm. The emission spectrum detected for MUC1.CT eGFP was 494 to 562 nm. The emission spectrum detected for mCherry beta-catenin was 574 to 637 nm.
Figure 22. EGF stimulation decreases colocalization of fluorescently labeled beta-catenin and MUC1.CT in S2-013 MUC1.CT eGFP/mCherry beta-catenin cells in a fixed-cell experiment.

Beta-catenin is labeled with mCherry (displayed in red, A-C), and MUC1.CT is labeled with eGFP (displayed in green, D-F). White indicates colocalization between the two fluorescent proteins (G-I). DAPI was used to stain the nuclei (blue). S2-013.MUC1F cells served as the negative control for both fluorescent protein level and colocalization (J-L). Cells are shown at three time points of EGF treatment. A, D, G, and J are time point 0. B, E, H, and K are time point 20 minutes. C, F, I, and L are time point 4 hours. Cells that expressed both mCherry beta-catenin and eGFP MUC1.CT were outlined in red for analysis.
It appears that in S2-013.MUC1 eGFP/mCherry beta-catenin cells the beta-catenin/MUC1.CT colocalization was most prevalent in the membrane of the cells (Figure 22). Upon EGF stimulation, we noticed loss in fluorescent beta-catenin and fluorescent MUC1.CT as well as an EGF-induced decrease in beta-catenin/MUC1.CT colocalization after twenty minutes of stimulation. In these cells this loss of colocalization appeared to persist for at least four hours.

To be certain that this observed loss of colocalization was not simply due to variability from one group of cells to another the experiment was conducted in living cells. S2-013.MUC1F cells with mCherry beta-catenin and eGFP MUC1.CT were treated with 10 ng/mL EGF for 0, 5, or 20 minutes. A group of living cells expressing both fluorescent proteins was imaged with a confocal laser scanning microscope at 0, 5, and 20 minutes post EGF stimulation. Red indicates mCherry beta-catenin (Figure 23A), green indicates eGFP MUC1.CT (Figure 23B). Colocalization of mCherry beta-catenin and eGFP MUC1.CT is indicated in white (Figure 23C). The emission spectrum detected for the Hoechst 33342 was 400 to 486 nm. The emission spectrum detected for MUC1.CT eGFP was 494 to 562 nm. The emission spectrum detected for mCherry beta-catenin was 574 to 637 nm.
Figure 23. EGF stimulation decreases colocalization of fluorescently labeled beta-catenin and MUC1.CT in S2-013 MUC1.CT eGFP/mCherry beta-catenin cells in a live-cell experiment.

A group of S2-013.MUC1 eGFP/mCherry beta-catenin cells were imaged at 0, 5, and 20 minutes after application of 10 ng/mL EGF (0, 5, and 20 minutes are arranged from left to right in each panel). Beta-catenin is labeled with mCherry (displayed in red, A), and MUC1.CT is labeled with eGFP (displayed in green, B). White indicates colocalization between the two fluorescent proteins (C). S2-013.MUC1F cells served as the negative control for both fluorescent protein levels and colocalization (D). Hoechst 33342 (1 µM) was used to stain the nuclei (blue).
As with the fixed-cell experiment it appears that EGF stimulation caused a loss in beta-catenin and MUC1.CT as well as diminished colocalization of the two proteins (Figure 23). This rapid EGF-induced loss in membrane colocalization was observed repeatedly in this cell line. Therefore, we quantified these results.

From the experiment illustrated in Figure 22, mean fluorescence intensities were collected from ROIs representing individual cells, and levels of mCherry beta-catenin (Figure 24A) and eGFP MUC1.CT (Figure 24B) were determined by averaging the mean fluorescence intensity of eGFP MUC1.CT and mCherry beta-catenin, respectively in cells expressing both fluorescent proteins. The extent of colocalization in the cells is expressed as relative area of colocalization, the percentage of pixels with co-occurrence of mCherry beta-catenin and eGFP MUC1.CT fluorescence intensities in which the fluorescence intensities of both channels exceed the thresholds established with the negative control (Figure 24C).
Figure 24. Effects of EGF on beta-catenin and MUC1.CT at the whole-cell level as determined by confocal microscopy.

(A) Levels of beta-catenin following 10 ng/mL EGF treatment. (B) Levels of MUC1.CT with EGF stimulation. (C) Beta-catenin/MUC1.CT colocalization with EGF stimulation at the whole-cell level.

**p<0.01
When ROIs representing individual cells were analyzed (Figure 24), it did not appear that EGF stimulation impacted beta-catenin or MUC1.CT levels with the exception of the rise in beta-catenin levels at 24h. Colocalization of beta-catenin and MUC1.CT was not significantly changed with EGF treatment at the whole-cell level. However, it may be that EGF prompts changes in the levels of beta-catenin and MUC1.CT and their colocalization in specific compartments within the cells. We therefore quantified changes within these compartments as well.

From the experiment illustrated in Figure 22 and Figure 24, ROIs representing individual cell membranes were analyzed. Levels of mCherry beta-catenin (Figure 25A) and eGFP MUC1.CT (Figure 25B) were determined by averaging the mean fluorescence intensity values for eGFP and mCherry beta-catenin, respectively, in the membrane ROIs of cells expressing both fluorescent proteins. The extent of membrane colocalization of mCherry beta-catenin and eGFP MUC1.CT is expressed as relative area of colocalization (Figure 25C).
Figure 25. Effects of EGF on beta-catenin and MUC1.CT in the membrane as determined by confocal microscopy.

(A) Levels of beta-catenin in the membrane with 10 ng/mL EGF stimulation. (B) MUC1.CT levels in the membrane with 10 ng/mL EGF stimulation. (C) The colocalization of beta-catenin and MUC1.CT in the membrane with 10 ng/mL EGF stimulation.

**p<0.01
When we quantified beta-catenin and MUC1.CT in the membrane of the cells, a significant decrease in MUC1.CT was observed at an early time point (20 minutes) and a later time point (4 hours) (Figure 25). We also noted a decline in beta-catenin/MUC1.CT colocalization in the cell membrane of these PDAC cells at both time points tested.

In the same experiment, we probed the levels and colocalization of these proteins in the cell nuclei. ROIs representing cell nuclei were analyzed. Levels of mCherry beta-catenin (Figure 26A) and eGFP MUC1.CT (Figure 26B) were determined by averaging the mean fluorescence intensities for MUC1.CT eGFP and mCherry beta-catenin, respectively, in the nuclear ROIs of cells expressing both fluorescent proteins. The extent of nuclear colocalization of mCherry beta-catenin and eGFP MUC1.CT is expressed as relative area of colocalization (Figure 26C). In a similar experiment, nuclear beta-catenin/MUC1.CT colocalization was determined at the 5-minute time point (Figure 26D).
Figure 26. EGF stimulation decreases colocalization of beta-catenin and MUC1.CT in the nuclei of S2-013.MUC1F cells as determined by confocal microscopy.

(A) Beta-catenin levels in the nucleus upon 10 ng/mL EGF stimulation.  (B) Levels of MUC1.CT in the nucleus following 10 ng/mL EGF stimulation.  (C) Colocalization of beta-catenin and MUC1.CT in S2-013.MUC1F (negative control) and S2-013.MUC1F cells with eGFP MUC1.CT and mCherry beta-catenin with 10 ng/mL EGF stimulation.  (D) In this graph, the effect of 10 ng/mL EGF stimulation on nuclear beta-catenin/MUC1.CT colocalization at the 5-minute time point is illustrated.

*p<0.01, ***p<0.001
EGF stimulation appears to decrease levels and colocalization of beta-catenin and MUC1.CT through experiments with fluorescently labeled proteins (Figure 22-Figure 26). However, the nuclear analysis of beta-catenin and MUC1.CT levels and colocalization was confounded by high 488 nm background signal (found in the negative control S2-013.MUC1F cells). To avoid this problem and to verify that EGF stimulation decreases beta-catenin/MUC1.CT colocalization, we used antibody labeling of beta-catenin and MUC1.CT in S2-013.MUC1F cells.

S2-013.MUC1F cells were treated with 10 ng/mL EGF for 0 minutes (Figure 27A), 5 minutes (Figure 27B), 20 minutes (Figure 27C), 4 hours (Figure 27D), and 24 hours (Figure 27E). The cells were fixed, permeabilized, blocked, and then stained for beta-catenin and MUC1.CT. The negative control cells received secondary antibody only (Figure 27F).
Figure 27. Effect of EGF on beta-catenin and MUC1.CT in S2-013.MUC1F cells, beta-catenin and MUC1.CT probed with antibodies.

S2-013.MUC1F cells were treated with 10 ng/mL EGF for 0 minutes (A), 5 minutes (B), 20 minutes (C), 4 hours (D), and 24 hours (E). The cells were stained for beta-catenin (red) and MUC1.CT (green). DAPI (blue) indicates position of nuclei. The negative control cells received secondary antibody only (F).
The cells appeared to change quite dramatically upon EGF stimulation by 20 minutes (Figure 27). Changes in levels and localization of beta-catenin and MUC1.CT were visible as well. Certain changes (such as an increase in nuclear beta-catenin) were persistent over time.

Images of the S2-013.MUC1F cells of Figure 27 were split into individual channels to evaluate the impact of EGF on beta-catenin (red) and MUC1.CT (green) separately (Figure 28).
Figure 28. Effect of EGF on beta-catenin and MUC1.CT in S2-013.MUC1F cells, split channels.

Images of cells (Figure 27) were split into individual channels to show beta-catenin (red), MUC1.CT (green), and nuclei (blue) at 0 minutes (A), 5 minutes (B), 20 minutes (C), 4 hours (D), and 24 hours (E) after application of 10 ng/mL EGF.
With the split-channel view it appears that EGF stimulation caused an increase in beta-catenin and MUC1.CT in the nuclei of the cells by 20 minutes (Figure 28). Notice also the punctate MUC1.CT structures in the nuclei at 20 minutes.

We next quantified the levels of beta-catenin and MUC1.CT in these cells (Figure 29). Mean fluorescence intensities were collected from ROIs representing individual cells (n=15), cell membranes (n=15), and nuclei (n=15) with ZEN Blue software to determine levels of beta-catenin and MUC1.CT.
Figure 29. Impact of EGF on levels of beta-catenin and MUC1.CT in S2-013.MUC1F cells, beta-catenin and MUC1.CT probed with antibodies.

Beta-catenin (A) and MUC1.CT (B) levels in whole cells upon 10 ng/mL EGF stimulation. Levels of beta-catenin (C) and MUC1.CT (D) in the cell membrane following EGF stimulation. Levels of beta-catenin (E) and MUC1.CT (F) in the nuclei following EGF stimulation.

*p<0.05, **p<0.01, ***p<0.001
At the whole-cell level beta-catenin levels have risen by 20 minutes and remain high (relative to the zero-minute time point) for four hours (Figure 29A and B). MUC1.CT is also higher at the four-hour time point at the whole-cell level. This is different from the result with fluorescently labeled proteins in which we observed no change in beta-catenin levels and a decrease in MUC1.CT by the four-hour time point (Figure 24). As opposed to what had been observed with the experiment with fluorescently labeled beta-catenin and MUC1.CT (Figure 25), in which beta-catenin levels were unchanged and MUC1.CT levels dropped by the four-hour time point, beta-catenin levels were apparently higher in the membrane fraction at the four-hour time point of this experiment (Figure 29C). The increase in beta-catenin and MUC1.CT in the nuclei of the cells after 20 minutes of EGF stimulation (Figure 29E and F) was striking. In fact, the MUC1.CT nuclear levels increased by a factor of seven. This increase was persistent throughout the time course. This stands in contrast to what was observed in the experiment with fluorescently labeled proteins in which no significant change was detected in beta-catenin levels and a decrease of MUC1.CT levels were observed in the nuclei of the cells (Figure 26).

The S2-013.MUC1F cells from the immediately preceding experiment (Figure 27-Figure 29) were analyzed in terms of colocalization of beta-catenin and MUC1.CT. Z stacks spaced 0.54 µm apart were produced for a field of cells at each time point. Consecutive z-stack images in sets of three (from the bottom towards the top of the cells) are shown for each time point (Figure 30).
Figure 30. Effect of EGF on colocalization of beta-catenin and MUC1.CT in S2-013.MUC1F cells, beta-catenin and MUC1.CT probed with antibodies.

The colocalization of beta-catenin and MUC1.CT is shown in white after 0 minutes (A), 5 minutes (B), 20 minutes (C), 4 hours (D), and 24 hours (E) EGF treatment (10 ng/mL). Beta-catenin is displayed in red, MUC1.CT is displayed in green, and nuclei are stained with DAPI (blue).
This view of the cells made it obvious that the location and amount of beta-catenin/MUC1.CT colocalization was changed by EGF stimulation (Figure 30). For example, the amount of colocalization diminished at the 5-minute time point, and by 20 minutes colocalization increased in the nuclei of the cells.

In the same experiment (Figure 27-Figure 30), colocalization of beta-catenin and MUC1.CT was quantified in ROIs representing the membrane (Figure 31A) and nuclei (Figure 31B) of the cells. The thresholds for MUC1.CT (Alexa Fluor 488) and beta-catenin (Alexa Fluor 568) fluorescence intensities were set so that the negative control cells (which received secondary antibody only) showed virtually no colocalization. Colocalization was expressed with the Pearson correlation coefficient where 1 indicates perfect linear correlation and -1 indicates the opposite. The Pearson correlation coefficient was measured in ROIs representing the membrane of fifteen cells and fifteen nuclei of S2-013.MUC1F cells (three from five independent fields).
Figure 31. Quantification of colocalization of beta-catenin and MUC1.CT in the membrane and nuclei of S2-013.MUC1F cells following EGF stimulation, beta-catenin and MUC1.CT probed with antibodies.

The Pearson correlation coefficient for beta-catenin/MUC1.CT colocalization was determined in the membrane (A) and the nuclei (B) of fifteen cells at each time point following EGF treatment. Colocalization in cells to which only secondary antibody was applied is displayed alongside beta-catenin/MUC1.CT colocalization values. ***p<0.001
The loss in membrane beta-catenin/MUC1.CT colocalization was confirmed; however, it was observed at the five-minute time point in one experiment and at the twenty-minute time point in a repeat experiment (Figure 31A). In either case EGF induced a decline in membrane beta-catenin/MUC1.CT interaction in S2-013.MUC1F cells after a short amount of time. This loss in colocalization at the membrane with 20 minutes EGF stimulation was also observed in the experiment with fluorescently labeled beta-catenin and MUC1.CT (Figure 25). It is important to note that the decrease in colocalization in the membrane of the S2-013.MUC1F cells with EGF stimulation did not coincide with changes in levels of beta-catenin and MUC1.CT. Beta-catenin and MUC1.CT levels in the membrane were unchanged by EGF stimulation at the 20-minute time point (Figure 29C and D), while a statistically significant drop in beta-catenin/MUC1.CT membrane colocalization occurred at that point (Figure 31A). While we observed a loss in nuclear colocalization of beta-catenin and MUC1.CT in the experiment with fluorescently labeled proteins (Figure 26), no statistically significant change occurred in nuclear colocalization in this experiment (Figure 31B).

Because EGF induced changes in levels and colocalization of beta-catenin and MUC1.CT, we next tested the impact of an EGFR inhibitor on the levels and colocalization of these proteins. S2-013.MUC1F cells were treated with 1 µM Erlotinib for 0 minutes (Figure 32A), 20 minutes (Figure 32B), or 4 hours (Figure 32C). An equal volume of DMSO (0.01% v/v) was applied to a set of cells as a vehicle control. The cells were fixed, permeabilized, blocked, and then stained for beta-catenin and MUC1.CT.
Figure 32. Effect of Erlotinib on levels of beta-catenin and MUC1.CT in S2-013.MUC1F cells.

S2-013.MUC1F cells were treated with 1 μM Erlotinib for 0 minutes (A), 20 minutes (B), or 4 hours (C). The cells were stained for beta-catenin (red) and MUC1.CT (green). DAPI (blue) indicates position of nuclei. The negative control cells received secondary antibody only (D).
It appears from these images that Erlotinib increased levels of beta-catenin and MUC1.CT, especially by the four-hour time point (Figure 32). These increases were most obvious in the membrane of the cells.

Images of the S2-013.MUC1F cells of Figure 32 were split into individual channels to evaluate the impact of Erlotinib on beta-catenin (red) and MUC1.CT (green) separately (Figure 33).
Figure 33. Effect of Erlotinib on beta-catenin and MUC1.CT in S2-013.MUC1F cells, split channels.

Images of cells (Figure 32) were split into individual channels to show beta-catenin (red), MUC1.CT (green), and nuclei (blue) at 0 minutes (A), 20 minutes (B), and 4 hours (C) after application of 1 µM Erlotinib.
Beta-catenin levels in the nuclei of the cells was higher by 4 hours Erlotinib treatment (Figure 33). Like EGF treatment (Figure 29F), Erlotinib affected nuclear MUC1.CT levels in S2-013.MUC1F cells, but at a later time point (four hours rather than 20 minutes).

With cells from this Erlotinib-treatment experiment, ROIs representing cell membranes (n=15) and nuclei (n=15) were analyzed with ZEN Blue software to determine levels of beta-catenin and MUC1.CT (Figure 34).
Figure 34. Impact of Erlotinib on levels of beta-catenin and MUC1.CT in S2-013.MUC1F cells.

Beta-catenin (A) and MUC1.CT (B) levels in whole cells upon 1 µM Erlotinib (or DMSO) treatment. Levels of beta-catenin (C) and MUC1.CT (D) in the cell membrane following Erlotinib (or DMSO) treatment. Levels of beta-catenin (E) and MUC1.CT (F) in the nuclei following Erlotinib (or DMSO) treatment.

***p<0.001
Quantification of the images confirmed that significant increases in nuclear beta-catenin and nuclear MUC1.CT occurred with 4 hours Erlotinib treatment in S2-013.MUC1F cells (Figure 34). Significant increases in the level of both proteins were observed at the four-hour time point at the whole-cell level, in the membrane, and in the nuclei of the cells. EGF and Erlotinib both caused an increase in beta-catenin and MUC1.CT levels in the nuclei of the cells. However, while EGF stimulation caused significant changes in both MUC1.CT and beta-catenin levels in the nuclei of the cells at 20 minutes (Figure 29E and F), Erlotinib required a longer period of action before causing significant changes in levels of beta-catenin and MUC1.CT.

S2-013.MUC1F cells from the preceding experiment (Figure 32-Figure 34) were analyzed in terms of beta-catenin/MUC1.CT colocalization. Consecutive z-stack images in sets of three (from the bottom towards the top of the cells) are shown for each time point (Figure 35).
Figure 35. Effect of Erlotinib on colocalization of beta-catenin and MUC1.CT in S2-013.MUC1F cells.

The colocalization of beta-catenin and MUC1.CT is shown in white after 0 minutes (A), 20 minutes (B), and 4 hours Erlotinib treatment (1 µM). Beta-catenin is displayed in red, MUC1.CT is displayed in green, and nuclei are stained with DAPI (blue).
The Erlotinib-induced increase in colocalization of beta-catenin and MUC1.CT is clear by the four-hour time point (Figure 35). This involved both the membrane and nuclei of the cells.

In the same Erlotinib experiment (Figure 32-Figure 35), colocalization of beta-catenin and MUC1.CT was quantified in ROIs representing individual cells (Figure 36A), the membrane (Figure 36B), and nuclei (Figure 36C) of the cells. The Pearson correlation coefficient was measured in fifteen membrane and fifteen nuclear regions of the S2-013.MUC1F cells (three of each from five independent fields).
Figure 36. Quantification of colocalization of beta-catenin and MUC1.CT in whole cells, in the membrane, and in the nuclei of S2-013.MUC1F cells following Erlotinib treatment.

The Pearson correlation coefficient for beta-catenin/MUC1.CT colocalization was determined in whole cells (A), the membrane (B), and the nuclei (C) of cells at each time point following Erlotinib treatment. Beta-catenin/MUC1.CT colocalization in cells to which only secondary antibody was applied and cells treated with DMSO (vehicle control) are displayed alongside beta-catenin/MUC1.CT colocalization values.

***p<0.001
The increase in colocalization of beta-catenin and MUC1.CT was confirmed at the whole-cell level and in the membrane of the Erlotinib-treated S2-013.MUC1F cells (Figure 36). Thus, as one might expect due to their opposite impact on EGFR, EGF stimulation decreased membrane beta-catenin/MUC1.CT colocalization (Figure 31A), while Erlotinib increased it (Figure 36B). As with EGF stimulation (Figure 31B), Erlotinib did not induce changes in nuclear beta-catenin/MUC1.CT colocalization (Figure 36C).

We also investigated the impact of EGF and Erlotinib on a PDAC cell line with endogenous beta-catenin and MUC1.CT. CFPAC-1 cells were treated with 10 ng/mL EGF for 0 minutes (Figure 37A), 5 minutes (Figure 37B), 20 minutes (Figure 37C), 4 hours (Figure 37D), and 24 hours (Figure 37E). The cells were fixed, permeabilized, blocked, and then stained for beta-catenin and MUC1.CT.
Figure 37. Effect of EGF on beta-catenin and MUC1.CT in CFPAC-1 cells, beta-catenin and MUC1.CT probed with antibodies.

CFPAC-1 cells were treated with 10 ng/mL EGF for 0 minutes (A), 5 minutes (B), 20 minutes (C), 4 hours (D), and 24 hours (E). The cells were stained for beta-catenin (red) and MUC1.CT (green). DAPI (blue) indicates the nuclei. The negative control cells received secondary antibody only (F).
The changes induced by EGF in CFPAC-1 cells were clearly different from the changes induced in S2-013.MUC1F cells (compare Figure 27 to Figure 37). For example, membrane beta-catenin levels rose dramatically after 20 minutes of EGF stimulation in CFPAC-1 cells, whereas a rise in membrane beta-catenin was observed at the four-hour time point in S2-013.MUC1F cells (Figure 29C).

Images of the CFPAC-1 cells of Figure 37 were split into individual channels to evaluate the impact of EGF on beta-catenin (red) and MUC1.CT (green) separately (Figure 38).
Figure 38. Effect of EGF on beta-catenin and MUC1.CT in CFPAC-1 cells, split channels.

Images of cells (Figure 37) were split into individual channels to show beta-catenin (red), MUC1.CT (green), and nuclei (blue) at 0 minutes (A), 5 minutes (B), 20 minutes (C), 4 hours (D), and 24 hours (E) after application of 10 ng/mL EGF.
As with S2-013.MUC1F cells, EGF stimulation caused nuclear accumulation of MUC1.CT in CFPAC-1 cells at the twenty-minute time point (compare Figure 28C to Figure 38C). However, the dramatic increase in membrane beta-catenin at 20 minutes in the CFPAC-1 cells was not observed in the S2-013.MUC1F cells (compare Figure 28C to Figure 38C).

In the same CFPAC-1 experiment (EGF stimulation), the mean fluorescence intensities of ROIs representing individual cells (n=15), cell membrane regions (n=10), and nuclei (n=15) were analyzed with ZEN Blue software to determine levels of beta-catenin and MUC1.CT (Figure 39).
Figure 39. Impact of EGF on levels of beta-catenin and MUC1.CT in CFPAC-1 cells, beta-catenin and MUC1.CT probed with antibodies.

Beta-catenin (A) and MUC1.CT (B) levels in whole cells upon 10 ng/mL EGF stimulation. Levels of beta-catenin (C) and MUC1.CT (D) in the cell membrane following EGF stimulation. Levels of beta-catenin (E) and MUC1.CT (F) in the nuclei following EGF stimulation.

**p<0.01, ***p<0.001
The increase in levels of beta-catenin and MUC1.CT was significant at the whole-cell level, in the membrane, and in the nuclei of cells at 20 minutes EGF stimulation (Figure 39). However, while this increase persisted in the nuclei of S2-013.MUC1F cells (Figure 29E and F), the EGF-induced nuclear increase of beta-catenin and MUC1.CT was not seen at the four-hour time point in CFPAC-1 cells.

CFPAC-1 cells from the preceding experiment (Figure 37-Figure 39) were analyzed in terms of beta-catenin/MUC1.CT colocalization. Z stacks (serial cross-section images) spaced 0.54 µm apart were produced for a field of cells at each time point (Figure 40). Consecutive z-stack images in sets of three (from the bottom towards the top of the cells) are shown for each time point. Beta-catenin is displayed in red, MUC1.CT in green, nuclei (DAPI) in blue, and colocalization of beta-catenin and MUC1.CT is shown in white.
Figure 40. Effect of EGF on colocalization of beta-catenin and MUC1.CT in CFPAC-1 cells, beta-catenin and MUC1.CT probed with antibodies.

The colocalization of beta-catenin and MUC1.CT is shown in white after 0 minutes (A), 5 minutes (B), 20 minutes (C), 4 hours (D), and 24 hours (E) EGF treatment (10 ng/mL). Beta-catenin is displayed in red, MUC1.CT is displayed in green, and nuclei are stained with DAPI (blue).
Because the EGF-induced increase in levels of beta-catenin and MUC1.CT occurred at the 20-minute time point, it is not surprising that the colocalization of the proteins was so striking at that time (Figure 40C). Colocalization of beta-catenin and MUC1.CT returned to the original level by 24 hours EGF stimulation.

In the same EGF-treated CFPAC-1 cell experiment, colocalization of beta-catenin and MUC1.CT was analyzed in ROIs representing the membrane (Figure 41A) and nuclei (Figure 41B) of the cells. The Pearson correlation coefficient was measured in ten membrane and fifteen nuclear regions of CFPAC-1 cells (two membrane regions and three nuclei from five independent fields).
Figure 41. Quantification of colocalization of beta-catenin and MUC1.CT in the membrane and nuclei of CFPAC-1 cells following EGF stimulation, beta-catenin and MUC1.CT probed with antibodies.

The Pearson correlation coefficient for beta-catenin/MUC1.CT colocalization was determined in the cell membrane (A) and the nuclei (B) at each time point following EGF treatment. Colocalization in cells to which only secondary antibody was applied is displayed alongside beta-catenin/MUC1.CT colocalization values.

***p<0.001
Analysis of the CFPAC-1 cells confirmed what appeared to be an increase in beta-catenin/MUC1.CT colocalization at 20 minutes EGF stimulation (Figure 41A and B). Concerning membrane colocalization of the proteins, this result was opposite to that observed in S2-013.MUC1F cells (Figure 31A). Also, while no statistically significant change occurred in nuclear beta-catenin/MUC1.CT colocalization in S2-013.MUC1F cells (Figure 31B), a rise in beta-catenin/MUC1.CT colocalization was observed in CFPAC-1 nuclei at 20 minutes EGF stimulation (Figure 41B).

Because EGF caused dramatic changes in beta-catenin and MUC1.CT levels and colocalization in CFPAC-1 cells, we next investigated their response to EGFR inhibition. CFPAC-1 cells were treated with 1 µM Erlotinib for 0 minutes (Figure 42 A), 20 minutes (Figure 42 B), or 4 hours (Figure 42 C). The cells were fixed, permeabilized, blocked, and then stained for beta-catenin and MUC1.CT.
Figure 42. Effect of Erlotinib on levels of beta-catenin and MUC1.CT in CFPAC-1 cells.

CFPAC-1 cells were treated with 1 µM Erlotinib for 0 minutes (A), 20 minutes (B), or 4 hours (C). The cells were stained for beta-catenin (red) and MUC1.CT (green). DAPI (blue) indicates the nuclei of the cells. The negative control cells received secondary antibody only (D).
Erlotinib impacts neither beta-catenin nor MUC1.CT in CFPAC-1 cells at the 20-minute time point. However, the cells were visibly perturbed by the drug by four hours (Figure 42). This clumped-cell phenotype occurred in both replicates of this experiment. MUC1.CT was universally increased in the cells by four hours.

Images of the CFPAC-1 cells of Figure 42 were split into individual channels to evaluate the impact of Erlotinib on beta-catenin (red) and MUC1.CT (green) separately (Figure 43).
Figure 43. Effect of Erlotinib on beta-catenin and MUC1.CT in CFPAC-1 cells, split channels.

Images of cells (Figure 42) were split into individual channels to show beta-catenin (red), MUC1.CT (green), and nuclei (blue) at 0 minutes (A), 20 minutes (B), and 4 hours (C) after application of 1 µM Erlotinib.
The level of beta-catenin in the membrane of CFPAC-1 cells has dramatically increased by four hours of Erlotinib treatment (Figure 43C). This increase was actually visible at the 20-minute time point (Figure 43B).

In the same CFPAC-1 experiment (Figure 42), ROIs representing the cell membrane (n=10) and nuclei (n=15) were analyzed with ZEN Blue software to determine levels of beta-catenin and MUC1.CT (Figure 44).
Figure 44. Impact of Erlotinib on levels of beta-catenin and MUC1.CT in CFPAC-1 cells as determined by confocal microscopy.

Beta-catenin (A) and MUC1.CT (B) levels in whole cells upon 1 µM Erlotinib (or DMSO) treatment. Levels of beta-catenin (C) and MUC1.CT (D) in the cell membrane following Erlotinib (or DMSO) treatment. Levels of beta-catenin (E) and MUC1.CT (F) in the nuclei following Erlotinib (or DMSO) treatment.

**p<0.01, ***p<0.001
After quantifying levels of both beta-catenin and MUC1.CT in the CFPAC-1 cells, it was clear that Erlotinib caused increases in both proteins at the whole-cell level, in the membrane, and in the nuclei of the cells (Figure 44). This change started at the 20-minute time point, and beta-catenin and MUC1.CT levels were two to four times higher by the 4-hour time point. A similar result was observed with Erlotinib-treated S2-013.MUC1F cells, except in that case the rise in levels of beta-catenin and MUC1.CT required a longer period of treatment (Figure 34).

The CFPAC-1 cells from the preceding experiment (Figure 42-Figure 44) were analyzed in terms of beta-catenin/MUC1.CT colocalization. Z stacks (serial cross-section images) spaced 0.54 µm apart were produced for a field of cells at each time point (Figure 45). Consecutive z-stack images in sets of three (from the bottom towards the top of the cells) are shown for each time point.
Figure 45. Effect of Erlotinib on colocalization of beta-catenin and MUC1.CT in CFPAC-1 cells.

The colocalization of beta-catenin and MUC1.CT is shown in white after 0 minutes (A), 20 minutes (B), and 4 hours Erlotinib treatment (1 µM). Beta-catenin is displayed in red, MUC1.CT is displayed in green, and nuclei are stained with DAPI (blue).
As with S2-013.MUC1F cells (Figure 35) it appears that Erlotinib causes increased beta-catenin/MUC1.CT colocalization in the membrane of CFPAC-1 cells (Figure 45). This increase continued until at least four hours after treatment.

With cells from the CFPAC-1 Erlotinib experiment, the Pearson correlation coefficient was measured in ten ROIs representing the membrane compartment (Figure 46A) and in fifteen nuclei (Figure 46B) (two membrane regions and three nuclei from five independent fields).
Figure 46. Quantification of colocalization of beta-catenin and MUC1.CT in the membrane and nuclei of CFPAC-1 cells following Erlotinib treatment.

The Pearson correlation coefficient for beta-catenin/MUC1.CT colocalization was determined in the membrane (A), and the nuclei (B) of fifteen cells at each time point following Erlotinib treatment. Colocalization in cells to which only secondary antibody was applied and cells treated with DMSO (vehicle control) are displayed alongside beta-catenin/MUC1.CT colocalization averages.

**p<0.01, ***p<0.001
Quantification of the colocalization confirmed that an increase in membrane beta-catenin/MUC1.CT colocalization occurred in Erlotinib-treated CFPAC-1 cells by 20 minutes (Figure 46A). In the membrane compartment colocalization tripled by four hours. A similar trend was also observed in the nuclei of the cells (Figure 46B). With S2-013.MUC1F cells, increased beta-catenin/MUC1.CT colocalization was observed in the membrane of the cells also, but this required four hours of Erlotinib treatment (Figure 36B). However, as opposed to what was observed in CFPAC-1 cells, no beta-catenin/MUC1.CT colocalization change was measured in the nuclei of S2-013.MUC1F cells with Erlotinib treatment (Figure 36C).

We also employed PLA to determine how EGF changes the interaction between beta-catenin and MUC1.CT in S2-013.MUC1F cells (Figure 47). Cells were untreated or treated with 10 ng/mL EGF for 5 minutes. Cells were fixed and then analyzed by PLA. A yellow spot in the image indicates that a beta-catenin molecule was within 40 nm of a MUC1.CT molecule. Images of cells were taken with a confocal laser scanning microscope. A representative image of EGF-untreated cells (Figure 47A) and a representative image of EGF-treated cells (Figure 47B) are shown.
Figure 47. EGF stimulation decreases the interaction between beta-catenin and MUC1.CT as determined by proximity ligation assay.

(A) Each yellow spot indicates that a beta-catenin molecule is within 40 nm of a MUC1.CT molecule. (B) A representative PLA image of the cells 5 minutes after 10 ng/mL EGF stimulation. (C) The negative control sample for the 0-minute time point and (D) for the 5-minute time point.
A drop in the average number of beta-catenin/MUC1.CT interactions occurred with only 5 minutes EGF stimulation (Figure 47). This supports what was observed by coimmunoprecipitation and confocal microscopy experiments with S2-013.MUC1F cells.

PLA was also performed with EGF-untreated and EGF-treated CFPAC-1 cells to determine how EGF impacted the interaction between beta-catenin and MUC1.CT in this PDAC cell line. The results of experiments with both S2-013.MUC1F (Figure 48A) and CFPAC-1 (Figure 48B) cells were quantified and graphed. Cells were untreated or treated with 10 ng/mL EGF for the indicated periods of time. Cells were fixed and then analyzed by PLA. Negative control cells were probed with a MUC1.CT antibody and an isotype control antibody, and the test samples were probed with a MUC1.CT antibody and a beta-catenin antibody. Images of cells were produced by confocal scanning laser microscopy and analyzed with ImageJ software. Three fields of cells were analyzed at all time points for both cell lines, and the experiment was conducted at least twice with both cell lines.
Figure 48. EGF stimulation decreases the interaction of beta-catenin with MUC1.CT as determined by proximity ligation assay.

(A) The number of beta-catenin/MUC1.CT interactions per cell was quantified in S2-013.MUC1F cells treated with EGF for the indicated periods of time. The negative control samples were treated with an isotype control antibody and a MUC1.CT antibody, while the test samples were treated with a beta-catenin and a MUC1.CT antibody. (B) CFPAC-1 cells were tested in the same way.
The number of interactions between beta-catenin and MUC1.CT tended to decrease (mean of 2.4 vs. 0.8 interactions per cell) upon stimulation of cells with EGF, though no statistically significant difference was determined (Figure 48A). Though CFPAC-1 cells did not follow the same trend as S2-013.MUC1F cells, the interaction between beta-catenin and MUC1.CT was similar to that which was observed in the CFPAC-1 cells in the confocal microscopy experiment (initial rise followed by a return to previous levels). As with the S2-013.MUC1F cells, the CFPAC-1 PLA results were not statistically significant (Figure 48B).

To summarize the confocal microscopy experimental results to this point, we saw that both EGF and Erlotinib affected the beta-catenin/MUC1.CT association in PDAC cells, suggesting that EGFR regulates the beta-catenin/MUC1.CT association. Typically, the responses of S2-013.MUC1F and CFPAC-1 cells to the treatments were incongruous. However, with both S2-013.MUC1F and CFPAC-1 cell lines, an increase in beta-catenin/MUC1.CT colocalization occurred in the membrane compartment with Erlotinib treatment. One should also note that changes in beta-catenin/MUC1.CT colocalization in S2-013.MUC1F cells (Figure 31, Figure 36) did not correspond to changes in beta-catenin and MUC1.CT levels (Figure 29, Figure 34), while changes in beta-catenin/MUC1CT colocalization in CFPAC-1 cells (Figure 41, Figure 46) always followed the trend in levels of beta-catenin and MUC1.CT (Figure 39, Figure 44). It appears that the mechanism by which the beta-catenin/MUC1.CT association is regulated differs in these cell lines.

While coimmunoprecipitation does not require that proteins physically interact (proteins can coimmunoprecipitate if they are merely in associating protein complexes), and confocal microscopy can only resolve distances of about 250 nm [157], Förster resonance energy transfer (FRET) only occurs if interacting proteins are within 10 nm of one another [158], which is on the order of the size of an individual protein molecule.
This allows one to study the physical interaction of proteins. In addition, when FRET is coupled with fluorescence lifetime imaging (FLIM), one can detect protein-protein interaction events that occur within cells even at low levels because FLIM is independent of the concentrations of the donor and acceptor fluorophore molecules [159]. For these reasons, we employed FRET FLIM to assess the nature of the beta-catenin/MUC1.CT interaction. To that end, we conducted FRET FLIM studies with nonfluorescent S2-013.MUC1F cells, S2-013 cells with eGFP MUC1.CT, and S2-013 cells with eGFP MUC1.CT and mCherry beta-catenin [159-161]. Fixed cells were excited with 517 nm light, and the lifetimes of emitted light were collected. All observed lifetimes are shown for each cell type in Figure 49.
Figure 49. The interaction between beta-catenin and MUC1.CT as determined by FRET FLIM.

All fluorescence lifetimes of light emitted by cells excited by 488 nm laser light in S2-013 cells with no fluorescent protein expression, in S2-013 cells expressing eGFP labeled MUC1.CT, and in S2-013 cells expressing both eGFP labeled MUC1.CT and mCherry beta-catenin are displayed in this histogram.
After taking scores of images of S2-013.MUC1F, S2-013.MUC1 eGFP, and S2-013.MUC1 eGFP/mCherry beta-catenin cells and making hundreds of measurements of lifetimes of photons we produced a histogram to summarize all lifetimes observed (Figure 49). The lifetimes fell into four major groups: one at 0.3 ns, one at 0.4 ns, one at 2.25 ns, and a fourth at 2.4 ns. The nonfluorescent cells produced autofluorescent lifetimes at about 0.4 ns and at 2.6 ns. The 0.4 ns lifetimes of the nonfluorescent cells aligns with a lifetime commonly observed in S2-013.MUC1 eGFP/mCherry beta-catenin cells. However, the number of counts for the nonfluorescent cells at 0.4 ns was much lower than that observed in the fluorescent cells (consider the area under the curve). The 0.3 ns lifetime was only observed in the S2-013.MUC1 eGFP cells. Though it is not certain, it is possible that this lifetime was due to homo-FRET (FRET between two MUC1.CT eGFP molecules). Homo-FRET is not typically detected with FRET FLIM [162], though it has been reported [163]. The second shortest lifetime (about 0.4 ns) was not observed in S2-013.MUC eGFP cells, but it occurred when mCherry beta-catenin was present. Perhaps this lifetime relates to an interaction between an mCherry beta-catenin molecule and a MUC1.CT eGFP dimer (or multimer). Because the 2.25 ns lifetime pool only occurred in the S2-013.MUC1 eGFP/mCherry beta-catenin cells, it likely resulted from low-efficiency FRET between individual molecules of MUC1.CT eGFP and mCherry beta-catenin. The highest lifetime (2.4 ns) most likely corresponds to unbound (free) MUC1.CT eGFP [164].

The impact of EGF stimulation on beta-catenin/MUC1.CT interaction was studied by FRET FLIM. Nonfluorescent S2-013.MUC1F cells, S2-013 cells with eGFP MUC1.CT, and S2-013.MUC1 eGFP and mCherry beta-catenin cells were untreated or treated with 10 ng/mL EGF for 5 minutes. Fixed cells were excited with 488 nm light. ROIs representing individual cells and cell compartments were analyzed to determine the impact of mCherry beta-catenin on MUC1.CT eGFP fluorescence lifetimes as well as
to determine the impact of EGF on the beta-catenin/MUC1.CT interaction. An example of compartmental analysis of a cell is shown in Figure 50.
Figure 50. FRET FLIM compartmental analysis of an S2-013.MUC1 eGFP/mCherry beta-catenin cell.

A group of cells was excited by 488 nm laser light. The emitted photons were counted and their lifetimes determined. Regions of interest (green border) were drawn around whole cells (A), the membrane (B), the cytoplasm (C), the synthetic machinery (D), or the nucleus (E) of each cell analyzed.
After imaging cells, mean fluorescence intensities were collected from ROIs representing individual cells and various cell compartments (membranes, cytoplasm, synthetic machinery, and nuclei) in order to analyze the impact of EGF stimulation at a subcellular level. The synthetic machinery of the cell is the perinuclear area of the cell with visibly high levels of eGFP. This region appears white in Figure 50.

Membrane, cytoplasm, synthetic machinery, and nuclear compartments were individually analyzed in nonfluorescent S2-013.MUC1F cells, S2-013 cells with eGFP MUC1.CT, and S2-013.MUC1 eGFP and mCherry beta-catenin cells with or without EGF stimulation. The measured fluorescence lifetimes were pooled into five groups: 0 to 0.35 ns, 0.35 to 0.70 ns, 0.70 to 2.00 ns, 2.00 to 2.30 ns, and 2.30 to 3.00 ns. The following pie graphs (Figure 51 to Figure 55) summarize the results of this eGFP lifetime pool analysis. Approximately fifty measurements were made for each group analyzed.
Figure 51. The effect of EGF on MUC1.CT and the impact of EGF on the MUC1.CT/beta-catenin interaction in whole S2-013.MUC1 eGFP and S2
013.MUC1 eGFP/mCherry beta-catenin cells as determined by FRET FLIM.

The pie charts display the lifetime distributions for photons emitted by 488 nm laser-excited S2-013.MUC1 eGFP cells untreated (A) and treated with 10 ng/mL EGF (B), and S2-013.MUC1 eGFP/mCherry beta-catenin cells untreated (C) and treated with 10 ng/mL EGF (D).
At the whole-cell level (Figure 51) EGF stimulation caused the percentage of lifetimes in pool 1 to decrease by about 3.4% in S2-013.MUC1 eGFP cells. This means that homo-FRET (MUC1 eGFP dimerization) has decreased. At the same time, an increase of about 2.7% in the percentage of lifetimes in pool 5 occurred. This means that a higher percentage of MUC1.CT eGFP molecules have entered an unbound state with EGF treatment. Concerning the S2-013.MUC1 eGFP/Cherry beta-catenin cells a decrease of 0.9% in pool 4 and an increase of 1.2% in pool 5 indicate that a lower percentage of interactions is taking place between mCherry beta-catenin and MUC1.CT eGFP molecules, and a higher percentage of MUC1.CT eGFP molecules are in the free state.
Figure 52. The effect of EGF on MUC1.CT and the impact of EGF on the MUC1.CT/beta-catenin interaction in the membrane of S2-013.MUC1 eGFP and S2-013.MUC1 eGFP/mCherry beta-catenin cells as determined by FRET FLIM.

The pie charts display the lifetime distributions for photons emitted by the membranes of 488 nm laser-excited S2-013.MUC1 eGFP cells untreated (A) and treated with 10 ng/mL EGF (B), and S2-013.MUC1 eGFP/mCherry beta-catenin cells untreated (C) and treated with 10 ng/mL EGF (D).
In the membrane of cells (Figure 52) EGF stimulation caused the percentage of lifetimes in pool 1 to decrease by about 2.5% in S2-013.MUC1 eGFP cells. This means that homo-FRET (MUC1 eGFP dimerization) has decreased. At the same time, an increase of about 5.8% in the percentage of lifetimes in pool 5 occurred. This means that a higher percentage of MUC1.CT eGFP molecules have entered an unbound state with EGF treatment. Concerning the membrane compartment of S2-013.MUC1 eGFP/Cherry beta-catenin cells, an increase of 4.6% in pool 2 indicates that a higher percentage of MUC1.CT eGFP dimers are interacting with mCherry beta-catenin molecules. A decrease of 7.3% in pool 4 indicates that a lower percentage of MUC1.CT eGFP/mCherry beta-catenin heterodimer interactions are taking place. An increase of 4% in pool 5 indicates that a higher percentage of MUC1.CT eGFP molecules are in the free state. This corresponds well with coimmunoprecipitation and confocal microscopy data for S2-013.MUC1F cells.
Figure 53. The effect of EGF on MUC1.CT and the impact of EGF on the MUC1.CT/beta-catenin interaction in the cytoplasm of S2-013.MUC1 eGFP and S2-013.MUC1 eGFP/mCherry beta-catenin cells as determined by FRET FLIM.

The pie charts display the lifetime distributions for photons emitted by the cytoplasm of 488 nm laser-excited S2-013.MUC1 eGFP cells untreated (A) and treated with 10 ng/mL EGF (B), and S2-013.MUC1 eGFP/mCherry beta-catenin cells untreated (C) and treated with 10 ng/mL EGF (D).
In the cytoplasm of cells (Figure 53) EGF stimulation caused the percentage of lifetimes in pool 1 to decrease by about 1% in S2-013.MUC1 eGFP cells. This means that homo-FRET (MUC1 eGFP dimerization) has slightly decreased. No change has occurred in lifetime pool 5, or the MUC1.CT eGFP unbound state. Concerning the cytoplasm compartment of S2-013.MUC1 eGFP/Cherry beta-catenin cells, a decrease in pool 2 of about 2.4% indicates that a lower percentage of MUC1.CT eGFP dimers are interacting with mCherry beta-catenin molecules. A decrease of 7.6% in pool 4 indicates that a lower percentage of MUC1.CT eGFP/mCherry beta-catenin heterodimer interactions are taking place. An increase of 9.8% in pool 5 indicates that a higher percentage of MUC1.CT eGFP molecules are in the free state. This interpretation corresponds well with immunoprecipitation and confocal microscopy data for S2-013.MUC1F cells.
Figure 54. The effect of EGF on MUC1.CT and the impact of EGF on the MUC1.CT/beta-catenin interaction in the synthetic machinery of S2-013.MUC1 eGFP and S2-013.MUC1 eGFP/mCherry beta-catenin cells as determined by FRET FLIM.

The pie charts display the lifetime distributions for photons emitted by the synthetic machinery (perinuclear region of particularly high MUC1.CT eGFP concentration) of 488 nm laser-excited S2-013.MUC1 eGFP cells untreated (A) and treated with 10 ng/mL EGF (B), and S2-013.MUC1 eGFP/mCherry beta-catenin cells untreated (C) and treated with 10 ng/mL EGF (D).
In the synthetic machinery (Figure 54) a different trend was observed. EGF stimulation caused the percentage of lifetimes in pool 1 to decrease by about 9.8% in S2-013.MUC1 eGFP cells. This means that homo-FRET (MUC1 eGFP dimerization) has decreased. A decrease in lifetime pool 5 of about 1.3% indicates that a higher percentage of MUC1.CT eGFP molecules are in a bound state. Concerning the synthetic machinery compartment of S2-013.MUC1 eGFP/Cherry beta-catenin cells, an increase in pool 1 of about 1.5% indicates the presence of a higher percentage of MUC1.CT eGFP homo-FRET pairs. A decrease of 5.4% in pool 2 indicates that less MUC1.CT eGFP dimers are interacting with mCherry beta-catenin molecules. An increase of 16.3% in pool 4 indicates that a higher percentage of MUC1.CT eGFP/mCherry beta-catenin heterodimer interactions are taking place. A decrease of 12.4% in pool 5 indicates that a lower percentage of MUC1.CT eGFP molecules are in the free state. This result was unlike that of the membrane and cytoplasm compartments where an increase in MUC1.CT eGFP in the free state was observed with EGF stimulation (Figure 52 and Figure 53).
Figure 55. The effect of EGF on MUC1.CT and the impact of EGF on the MUC1.CT/beta-catenin interaction in the nuclei of S2-013.MUC1 eGFP and S2-013.MUC1 eGFP/mCherry beta-catenin cells as determined by FRET FLIM.

The pie charts display the lifetime distributions for photons emitted by the nuclear region of 488 nm laser-excited S2-013.MUC1 eGFP cells untreated (A) and treated with 10 ng/mL EGF (B), and S2-013.MUC1 eGFP/mCherry beta-catenin cells untreated (C) and treated with 10 ng/mL EGF (D).
Concerning the nuclei (Figure 55) EGF stimulation caused very little change to occur in either cell type. The percentage of lifetime pools in S2-013.MUC1 eGFP cells remained virtually unchanged. Concerning the nuclear compartment of S2-013.MUC1 eGFP/Cherry beta-catenin cells, a decrease in pool 1 of about 1.5% indicates the presence of a lower percentage of MUC1.CT eGFP homo-FRET pairs. An increase of 1.3% in pool 4 indicates that a higher percentage of MUC1.CT eGFP/mCherry beta-catenin heterodimer interactions are taking place.
**Discussion**

The steady state coimmunoprecipitation data show that beta-catenin and MUC1.CT interact in the cytoplasm and/or membrane as well as in the nucleus of PDAC cells (Figure 2–Figure 6). The Rapid Autopsy sample stained for beta-catenin and MUC1.CT (Figure 7) indicates that the beta-catenin/MUC1.CT interaction occurs in pancreas cancer tissue as well, particularly in the membrane of the cells. The mass spectrometry data provide support that the beta-catenin/MUC1 interaction occurs both in the cytoplasm/membrane and nuclear fractions (Table 1). According to mass spectrometry data this interaction includes MUC1 forms with a portion of MUC1 that normally sits just above the plasma membrane in the extracellular space. The N- and C-terminal subunits of MUC1 interact noncovalently and the C-terminal subunit (that crosses the cell membrane and includes the cytoplasmic tail) is only 158 amino acids in size [78]. It is easy to imagine that the entire C-terminal subunit of MUC1 enters the cell and interacts with beta-catenin.

In the first attempt to identify beta-catenin-coimmunoprecipitating MUC1.CT peptides by mass spectrometry, two MUC1 peptides were observed in the cytoplasm/membrane fraction (Table 1–Table 5). For example, the QGGFLGLSNIK peptide was identified eight times in the first sample and two times in a technical replicate. This peptide is just five residues N-terminal to the C-terminal subunit of MUC1. The ions score and expect score make it likely that forms of MUC1 including this portion interacted with beta-catenin for those experiments. However, this peptide was not again seen in biological replicates. The NYGQLDIFPAR peptide was identified once in the first sample and three times in a technical replicate. The ions score of 72.5 and expect score of 2.6E-4 suggest that this is not a random match nor a false positive. On the second attempt to observe MUC1.CT peptides in a beta-catenin coimmunoprecipitate, special attention was paid to maintaining phosphorylation. Again,
the NYGQLDIFPAR peptide was observed in the cytoplasm/membrane fraction. The ions score (59) and expect score (4.6E-5) indicate that this is a true identification. In addition, the MUC1.CT peptide EGTINVHDVETQFNQYK was observed. This peptide is at the N-terminus of the 158 amino acid C-terminal subunit of MUC1. The ions score (67) and expect score (2.2E-6) are again indicative that this is a valid identification (Table 2). This raises the possibility that the entire small MUC1 subunit associates with beta-catenin. Neither of these peptides were observed in the isotype control sample, lending credibility to the possibility that beta-catenin and MUC1 interact through this region of MUC1 (Table 3).

The EGTINVHDVETQFNQYK and NYGQLDIFPAR peptides were also observed in the nuclear fraction for the beta-catenin coimmunoprecipitates analyzed by mass spectrometry (Table 4). The ions score and expect score for the EGTINVHDVETQFNQYK peptide were 77 and 6.9E-7, respectively. The same peptide was observed in the nuclear isotype control sample, but in that case the ions score and expect score were very low, only 16 and 0.27, respectively. The identification of EGTINVHDVETQFNQYK in nuclear isotype control immunoprecipitation is much more likely to be a random match occurring by chance alone than that of the nuclear beta-catenin coimmunoprecipitate sample.

Concerning the OpenSPR results, large chi-squared ($\chi^2$) values of some of the OpenSPR results mean that the experimental curves did not fit well to the mathematically modelled curves in those cases. Also, in some cases the $k_{on}$ value error is larger than the value itself, making these results difficult to interpret. The OpenSPR results still provide valuable information about the beta-catenin/MUC1.CT interaction.

Interestingly, when the CQCRRKNYGQLDIFPARDTYHPMSEYPTYH peptide (which contains the MUC1.CT peptide detected by mass spectrometry) was analyzed by OpenSPR for interaction with beta-catenin, the highest binding affinity was observed (2.3
± 0.1 µM, Table 7). However, the chi-squared was high, at 1,278. Fortunately, another longer peptide encompassing nearly the entire stretch of MUC1.CT (NYGQLDIFPARDTYHPMSEYPTYHTHGRYVPPSTDRSPYEKVSAGNGGSLSYTNPA) had a much lower chi-squared value of 19, though its binding affinity, 15 ± 0.2 µM, was six times lower than that of the CQCRRKNYGQLDIFPARDTYHPMSEYPTYH peptide. The beta-catenin binding affinity for full-length recombinant His-tag MUC1.CT was 13.8 ± 0.3 µM, with a fairly low chi-squared value of 16. Interestingly, ITC and SPR data indicate that beta-catenin does not interact strongly with the latter C-terminal half of MUC1.CT (GRYVPPSTDRSPYEKVSAGNGGSLSYTNPA). This peptide had a very low exothermic enthalpy change with beta-catenin as determined by ITC (-1.1 kcal/mol) and a binding affinity of 306 ± 9 µM by OpenSPR (Table 7), though the chi-squared value is fairly high, at 185. The binding affinity of the phosphorylated version of this peptide (pYHPM) was over seven times lower (2.3 ± 1 mM) with an even higher chi-squared value of 3,868. The highest binding affinity observed with a phosphorylated MUC1.CT peptide was that of PYEKVpSAGNGGSLSYTNPA, with a binding affinity of 45 ± 1 µM (six times higher binding affinity than the unphosphorylated form). Still, the highest beta-catenin/MUC1.CT binding affinities observed involved unphosphorylated MUC1.CT.

The ITC data (Table 1) provide evidence that the interaction between beta-catenin and MUC1.CT does not involve the C-terminal half of MUC1.CT (GRYVPPSTDRSPYEKVSAGNGGSLSYTNPA), which includes the previously hypothesized SAGNGSSL binding site. This is in contrast to the report that this serine-rich motif of MUC1.CT is responsible for beta-catenin binding [94]. OpenSPR data confirmed the lack of interaction of beta-catenin with this stretch of amino acids in
the cytoplasmic tail of MUC1. In addition, the phosphomimetic form of the serine-rich motif peptide GRYVPPSSTDSPYEKVSAGNGGEELEYTNPA actually produced an endothermic enthalpy change, which means that the interaction was energetically unfavorable.

Mass spectrometry, ITC, and OpenSPR data taken together paint a picture that beta-catenin and MUC1.CT interact in the low micromolar range, and this interaction is through the N-terminal half of the cytoplasmic tail, the portion of the molecule that would be closest to the cell membrane.

Several kinases are known to phosphorylate MUC1, and phosphorylation of various MUC1.CT residues have been implicated in its interaction with various transcription factors [47]. However, it is not clear from the results presented here how CTGF, PDGF, or HGF impact MUC1.CT and beta-catenin levels and colocalization. Only trends were observed. These trends include decreased MUC1.CT in the nucleus at 10 minutes CTGF stimulation (Figure 14), decreased MUC1.CT and beta-catenin in the cytoplasm/membrane fraction after 60 minutes of HGF stimulation (Figure 15), and decreased MUC1.CT in the nucleus of PDGF-treated cells at 60 minutes (Figure 16).

EGF seems to have an effect on both MUC1 and beta-catenin. This is not a surprise given that EGFR has been reported to regulate both proteins [55, 165]. What had not been previously explored was the impact of EGF stimulation on the interaction between beta-catenin and MUC1. Western blot data show that EGF stimulation reduces MUC1.CT in the cytoplasm/membrane fraction within 5 minutes of stimulation and in the nucleus after 10 minutes but does not significantly change beta-catenin levels (Figure 17). The impact of Erlotinib was determined at the one-hour and four-hour time point to produce this western blot data (Figure 17). According to the western blot data, Erlotinib does not seem to significantly impact cytoplasm/membrane or nuclear beta-catenin or MUC1.CT at these longer time points.
Subcellular fractionation successfully separated the membrane fraction from other fractions as evidenced by the presence of EGFR and absence of beta-actin in the membrane fraction (Figure 18). This set the stage for determining the impact of EGF stimulation on MUC1.CT specifically in the membrane fraction. It appears that four major MUC1.CT forms exist in the membrane of CFPAC-1 cells, and that EGF stimulation, even at five minutes, causes phosphorylation of MUC1.CT (Figure 19). It appears that the fraction of MUC1.CT phosphorylated in CFPAC-1 cells situates itself at the top of the MUC1.CT band (at about 25 kDa). In addition, it appears that a fifth MUC1.CT form becomes visible by two-dimensional gel electrophoresis upon EGF stimulation. This band is at a pH of about 6.

S2-013.MUC1F cells have seven major forms of MUC1.CT (Figure 20). The MUC1.CT form farthest to the right in Figure 20 seems to be the most affected by EGF stimulation, as it is very pronounced in the phosphotyrosine blot. As in the case of CFPAC-1 cells, a MUC1.CT form becomes more visible with EGF stimulation. This band sits just left of pH 7 at about 25 kDa. It is unknown if in this case EGFR is directly phosphorylating MUC1.CT or a kinase downstream of EGFR signaling. Both possibilities have been reported to occur at the YEKV motif of MUC1.CT [55, 67].

Based on coimmunoprecipitation data, EGF stimulation appears to decrease the interaction of beta-catenin and MUC1.CT (Figure 21). Beta-catenin immunoprecipitates from the cytoplasm/membrane fraction of S2-013.MUC1F cells had diminished levels of coimmunoprecipitating MUC1.CT. The reciprocal experiment conducted in the membrane fraction of S2-013.MUC1F cells produced a similar result in which immunoprecipitated MUC1.CT had decreased coimmunoprecipitating beta-catenin.

When this phenomenon was studied in S2-013.MUC1 eGFP/mCherry beta-catenin cells, the decrease in beta-catenin/MUC1.CT colocalization was observed specifically in the membrane in both a fixed-cell (Figure 22) and in a live-cell (Figure 23)
experiment. This loss in colocalization is visible at only five minutes of stimulation. At the whole-cell level (Figure 24) EGF stimulation does not appear to greatly impact beta-catenin or MUC1.CT levels, except that beta-catenin levels have significantly risen at the 24-hour time point.

In the membrane of the cells treated with EGF one can observe a trend of decreased beta-catenin and MUC1.CT (Figure 25). At the four-hour time point, membrane MUC1.CT is significantly diminished. The result of EGF stimulation is a seven-fold drop in membrane colocalization at the 20-minute mark that persists for at least four hours.

The impact of EGF stimulation on nuclear beta-catenin/MUC1.CT colocalization is not clear based on the confocal microscopy data in Figure 26. The background 488 nm signal is high in the nucleus, making it difficult to tell whether or not the apparent drop in MUC1.CT levels in the nucleus is accurate or not. The impact of the EGFR axis on beta-catenin and MUC1.CT in cancer cells is made clearer in Figure 27 through Figure 46. It appears that EGF causes a slight increase in beta-catenin at the whole-cell level that persists for four hours as well as a moderate rise in MUC1.CT by the four-hour time point. In the membrane it appears that beta-catenin levels rise by the four-hour time point, but no significant change in MUC1.CT were observed in the membrane of S2-013.MUC1F cells over the course of the 24-hour experiment. However, a significant decrease in colocalization of beta-catenin and MUC1.CT occurred in the membrane at 5 minutes in one experiment and 20 minutes in a replicate. In the nuclei a nearly two-fold increase in beta-catenin was observed with EGF treatment that persisted throughout the time course. An even greater increase in MUC1.CT was observed in the nuclei of these cells, also starting at the 20-minute time point and enduring throughout the 24-hour time course. However, no significant increase in beta-catenin/MUC1.CT interaction in the nucleus accompanied this rise in protein levels. It seems that the nuclear beta-
catenin/MUC1.CT interaction remains totally unperturbed by EGF stimulation in these cells. When EGFR was inhibited in S2-013.MUC1F cells, an increase in beta-catenin/MUC1.CT interaction was observed at the whole-cell level and in the membrane. Again, the beta-catenin/MUC1.CT interaction in the nucleus was unchanged by the treatment (Figure 36).

In CFPAC-1 cells, EGF treatment also caused a rise in beta-catenin at the whole-cell level at 20 minutes, but this returned to pre-treatment levels by four hours as opposed to that of S2-013.MUC1F cells, which persisted for at least four hours. In addition, MUC1.CT levels rose by the twenty-minute time point, which did not occur until the later four-hour time point in the case of S2-013.MUC1F cells. However, the MUC1.CT level dropped by the four-hour time point. While beta-catenin sluggishly increased after four hours of EGF stimulation in S2-013.MUC1F cells, beta-catenin levels rose nearly two-fold by the twenty-minute mark in CFPAC-1 cells but did not persist. Membrane and nuclear beta-catenin and MUC1.CT followed the same trend of a transient increase at the twenty-minute time point in CFPAC-1 cells, as did the beta-catenin/MUC1.CT interaction in both the membrane and nuclei of these cells (Figure 41). Whereas EGF treatment affected the CFPAC-1 cells and S2-013.MUC1F cells differently, EGFR inhibition had a similar effect in that it caused levels of beta-catenin and MUC1.CT to rise and persist in the cells, as did beta-catenin/MUC1.CT colocalization, particularly in the membrane of the cells. However, unlike S2-013.MUC1F cells, CFPAC-1 cells showed a significant increase in beta-catenin/MUC1.CT colocalization in the nuclei (Figure 46).

The loss in beta-catenin/MUC1.CT interaction in S2-013.MUC1F cells is visible at the five-minute time point by PLA also (Figure 47). It does appear that upon EGF stimulation, the beta-catenin/MUC1.CT interaction is reduced in S2-013.MUC1F cells at five minutes. However, in neither S2-013.MUC1F nor CFPAC-1 cells was the EGF-
induced change in beta-catenin/MUC1.CT interaction determined to be significant by PLA (Figure 48).

The FRET FLIM data (Figure 49) shows two short (0.3 and 0.4 ns) and two long lifetimes (2.25 and 2.4 ns). The published lifetime of eGFP in living cells is 2.4 ns [164], however this is very much dependent on the cellular environment of the fluorophore [166]. In the case of the S2-013.MUC1 eGFP and S2-013.MUC1 eGFP/mCherry beta-catenin cells the unbound lifetime of eGFP was concordant with the published value of 2.4 ns. The 2.25 ns lifetime pool is found only in the S2-013.MUC1 eGFP/mCherry beta-catenin cells. Though 2.25 ns is not very different from 2.4 ns, the two lifetimes are distinct. Thus, it appears that low efficiency FRET is occurring between mCherry beta-catenin and MUC1.CT eGFP. For this to occur, the molecules must be within 10 nm of one another [158], providing more evidence that beta-catenin and MUC1.CT physically interact.

The FRET FLIM data brings another interesting aspect of MUC1.CT biology to light. The very short 0.30 ns lifetime in MUC1 eGFP cells may indicate that MUC1 eGFP molecules are quenching energy from one another, a phenomenon called homo-FRET [167]. This could result from MUC1.CT associating with itself, or it could be an artefact due to the presence of eGFP at the C-terminus of the molecule. However, it has been reported that MUC1.CT does dimerize through its CQC motif [48]. In accordance with the idea that MUC1.CT and beta-catenin interact, mCherry beta-catenin increases the very short lifetime (0.3 ns) observed in MUC1 eGFP cells to a slightly longer lifetime (0.4 ns). This may be an occurrence of more efficient FRET between mCherry beta-catenin and MUC1 eGFP, or it may be that an mCherry beta-catenin molecule is interacting with a dimer of eGFP MUC1 molecules, modulating the homo-FRET lifetime.

Concerning the impact of EGF stimulation on the beta-catenin/MUC1.CT interaction in S2-013.MUC1 eGFP/mCherry beta-catenin cells, the FRET FLIM data
corresponds well with what has been shown by coimmunoprecipitation, PLA, and confocal microscopy data. The percentage of lifetimes above 2.3 ns at the whole-cell level (Figure 51), in the membrane (Figure 52), and in the cytoplasm (Figure 53) fractions increased with EGF treatment. The longer lifetime indicates that eGFP molecules are released into an unbound state upon EGF stimulation. In addition, the 2.0 to 2.3 ns pool decreases by 7 to 8% in the membrane and cytoplasm fractions of S2-013.MUC1 eGFP/mCherry beta-catenin cells, suggesting that interactions between mCherry-beta-catenin and MUC1 eGFP are lost upon EGF stimulation. In addition, it appears that homo-FRET (the very short lifetime pool) is decreased upon EGF stimulation in S2-013.MUC1 eGFP cells by about 3% at the whole-cell level and in the membrane fraction of S2-013.MUC1 eGFP cells. It seems that MUC1.CT loses association with itself and beta-catenin with EGF stimulation. It is interesting that the opposite trend was observed in the synthetic machinery of the cells, where about 16% more MUC1.CT enters a beta-catenin bound state upon EGF stimulation (Figure 54). Perhaps this is related to EGF-induced endocytosis of MUC1 [168] and trafficking through the perinuclear region of the cell. Also, the FRET FLIM data indicate that no change occurs in the nuclei of the cells with EGF stimulation in five minutes. Perhaps a longer period of EGF stimulation is required to see changes there.

Immunoprecipitation, PLA, confocal microscopy, and FRET FLIM data suggest that EGF stimulation decreases MUC1.CT interaction with beta-catenin in S2-013.MUC1F cells. Because EGF stimulation causes tyrosine residue phosphorylation of MUC1.CT in the membrane fraction of S2-013.MUC1F, and this stimulation decreases membrane beta-catenin/MUC1.CT colocalization in S2-013.MUC1F cells in 5 minutes, it seems reasonable to hypothesize that tyrosine residue phosphorylation of MUC1.CT diminishes the beta-catenin/MUC1.CT interaction. This is certainly in line with mass spectrometry analysis of beta-catenin coimmunoprecipitates in which MUC1.CT
phosphorylation was not observed, and with ITC and OpenSPR data, in which phosphorylation (of either tyrosine or serine residues) tended to decrease the beta-catenin/MUC1.CT binding affinity.
CHAPTER 4. MET INHIBITOR SU11274 LOCALIZES AT THE ENDOPLASMIC RETICULUM

Introduction

In the course of examining activities of Met, a receptor tyrosine kinase that transduces signals from the extracellular space to the cell interior when its ligand, hepatocyte growth factor (HGF) binds to its extracellular domain [169], we noted that one small molecule inhibitor SU11274, a pyrrole indolinone compound, first described in 2003 [170] showed unexpected properties of fluorescence and cellular uptake into specific organelles. This compound is reported to inhibit the Met receptor activation with an IC50 of 10 nM as determined in a cell-free assay [171], due to the ability of SU11274 to bind tightly to the ATP pocket of the Met receptor [170].
Results

SU11274 fluoresces when excited by a laser at 488 nm. Figure 56A is the emission spectrum of SU11274. In order to produce the emission spectrum SU11274 was applied to S2-013.MUC1F cells at 32 µM.

SU11274 is a pyrrole indolinone compound that also contains a sulfonamide group. Its structure is shown in (B). The compound readily enters cells. (C) S2-013.MUC1F cells were treated with 2 µM SU11274 and imaged every 20 seconds for 10 minutes with a 488 nm laser. ROIs were drawn around five individual cells. The five SU11274 mean fluorescence intensity measurements were averaged, and the emitted light produced upon excitation was measured and plotted as a function of time. All values were normalized to the highest fluorescence intensity measurement observed. In the same experiment the rate of absorption of SU11274 by S2-013.MUC1F cells was determined by dividing the change in mean fluorescence intensity between successive measurements by the increment of time elapsed between measurements (20 seconds) (D). All values were normalized to the highest rate measurement observed.
After applying 32 µM SU11274 to S2-013.MUC1F cells, the cells were excited with 488 nm laser light. The emission spectrum (A) was produced by taking SU11274 mean fluorescence intensity measurements in approximately 10 nm bandpass increments from 482 to 725 nm. The structure of SU11274 (chemical formula: C_{28}H_{30}CIN_{5}O_{4}S) is shown in (B). (C) S2-013.MUC1F cells were treated with 2 µM SU11274 and imaged every 20 seconds for 10 minutes. Regions of interest were drawn around five individual cells. The five intensity measurements were averaged, and the emitted light produced upon excitation was measured and plotted as a function of time. The highest intensity measurement was set at 1, and all other values are relative to it. (D) The rate of absorption of SU11274 by S2-013.MUC1F cells was determined by dividing the change in SU11274 mean fluorescence intensity from consecutive intensity measurements by the increment of time elapsed between measurements. The largest rate measurement was set at 1, and all other values are relative to it.
We were able to determine the emission spectrum of SU11274, which peaks at 515 nm (Figure 56A). Ostensibly, the structure of the compound facilitates both its fluorescence properties as well as its ability to concentrate in cells (Figure 56B). The rapid rate of cellular uptake of SU11274 slowly levels off after about 2 minutes (Figure 56C and D), probably because the cells reach a point of saturation.

SU11274 was applied at 2 µM for 30 minutes prior to fixing, permeabilizing, and staining the cells with antibodies for specific subcellular compartments. A confocal image split into individual channels shows SU11274 localization in green (Figure 57A) and the ER (calreticulin staining) of the cells in purple (Figure 57B). The combined image is shown in Figure 57C. Relative area colocalization of SU11274 with subcellular compartments is shown in Figure 57D. The isotype antibody control was used to establish the threshold for the SU11274 fluorescence intensity and the Alexa Fluor 647 fluorescence intensity in order to make colocalization measurements. Relative area colocalization is the percentage of pixels with co-occurrence of SU11274 and Alexa Fluor 647 fluorescence intensities in which the fluorescence intensities of both channels exceed the threshold established with the isotype antibody negative control. Colocalization measurements were made with ten cells (five cells in two fields). The Pearson correlation coefficient is a measure of the linear correlation between the SU11274 fluorescence intensity and the Alexa Fluor 647 fluorescence intensity (Figure 57E).
**Figure 57.** SU11274 localization.

S2-013.MUC1F cells were treated with 2 µM SU11274 for 30 minutes. The cells were stained with antibodies for various cell compartments. A confocal image split into individual channels shows SU11274 localization (A) and the ER (calreticulin staining) of the cells (B). The combined image is shown in (C). DAPI (blue) indicates the location of the nuclei, and differential interference contrast is shown in white. Relative area colocalization for the negative control and the cell compartments is shown in (D). The Pearson correlation coefficient between SU11274 fluorescence intensity and Alexa Fluor 647 (cell compartment) fluorescence intensity is shown in (E).

***p<0.001 (endoplasmic reticulum compared to all other groups), **p<0.01.
It is clear that SU11274 colocalizes with the ER in terms of the area of overlap of SU11274 and the ER marker calreticulin (Figure 57A-D). In addition, the linear correlation of SU11274 and the ER marker calreticulin or that of the early endosome marker EEA1 significantly exceed that of the isotype control and all other cell compartment markers tested (Figure 57E). This indicates that SU11274 preferentially localizes in the ER region of cells as well as early endosomes. Because the ER volume is so much larger than that of the early endosomes, it appears to predominantly localize in the ER region of the cells.

In an effort to quantify the concentration of SU11274 in cells and in the ER, we performed the following experiment. SU11274 was applied to living cells at 0, 0.1, 1, and 10 µM. At the same time ER-Tracker™ Red was applied to cells at 10 nM in order to define the ER. Cells were imaged three minutes after SU11274 and ER-Tracker™ Red were applied (Figure 58). The SU11274 mean fluorescence intensity in ROIs representing cells, acellular areas, and ER regions was measured. Figure 58A and B contain representative images from cells treated with 10 µM SU11274. The SU11274 mean fluorescence intensity of ROIs representing acellular areas at various cell medium concentrations of SU11274 was quantified to determine the relationship between SU11274 mean fluorescence intensity and SU11274 concentration. The SU11274 concentration in the cell medium is plotted as a function of the SU11274 mean fluorescence intensity measured in the ROIs representing acellular areas (Figure 58C). The equation relating SU11274 mean fluorescence intensity to SU11274 concentration was used to determine the SU11274 concentration in the cells and in the ER region of the cells at given cell medium SU11274 concentrations (Figure 58D).
Figure 58. SU11274 concentration in cells and ER.

SU11274 was applied to living cells at 0, 0.1, 1, and 10 µM. ER-Tracker™ Red was applied to cells at 10 nM to define the ER. The SU11274 mean fluorescence intensity of ROIs representing individual cells (circled in red, n=9), acellular areas (circled in white, n=6), and the ER (circled in yellow, n=9) was measured. The Iso Data method (ZEN Blue) was used to set the threshold for the ER-Tracker™ Red fluorescence intensity to delimit the ER. Hoechst 33342 was used to stain the nuclei (blue). (A) Cells to which 10 µM SU11274 was applied, and (B) the ER region of the same cells in yellow. The SU11274 concentration in the cell medium is plotted as a function of the 488 nm SU11274 mean fluorescence intensity measured in the ROIs representing acellular areas (C). The SU11274 mean fluorescence intensity at 0 µM SU11274 of the cells and that of the ER were subtracted from cell and ER data sets, respectively. The equation relating SU11274 mean fluorescence intensity to SU11274 concentration was used to determine the SU11274 concentration in the cells and in the ER of the cells at given cell medium SU11274 concentrations (D). *p<0.05, ***p<0.001.
We found that the relationship between the applied SU11274 cell medium concentration and the SU11274 mean fluorescence intensity (of the areas without cells, circled in white, Figure 58A) was linear (Figure 58C). The equation that defines the relationship is SU11274 concentration = 8.0749 x SU11274 mean fluorescence intensity - 1.365. With this equation we determined the concentration of SU11274 in S2-013.MUC1F cells (Figure 58D) and in the ER region (shown in yellow in Figure 58B) of the cells. For example, the concentration of SU11274 in the cells is about 300 µM, and in the ER it is nearly 600 µM when that of the cell medium is only 10 µM. SU11274 accumulates rapidly in the ER of cells. This property may be of therapeutic utility.

SU11274 (Figure 59A) has a certain moiety that is similar to the phenyl sulfonylurea group found in the compound glibenclamide (Figure 59B) and glimepiride (Figure 59C). Figure 59D shows a cryo-electron microscopy derived-structure of a sulfonylurea receptor of ATP sensitive-potassium channel composed of four SUR1 subunits and four Kir6.2 subunits [172]. Glibenclamide (which is the sulfonylurea portion of ER-Tracker™ Red) and glimepiride are known to bind sulfonylurea receptors of ATP sensitive-potassium channels (Figure 59D) [173, 174]. The cryo-electron microscopy image of the sulfonylurea receptor of ATP sensitive-potassium channel has been used with the authors’ permission. Because sulfonylurea receptors of ATP sensitive-potassium channels are known to populate the ER [175], and because SU11274 shares structural similarity with two compounds that bind to such channels, perhaps SU11274 rapidly accumulates in the ER by way of a sulfonylurea receptor of ATP sensitive-potassium channel.
Figure 59. Sulfonylurea receptors of ATP-sensitive potassium channels and drugs that bind them.

(A) Structure of SU11274, glibenclamide (B), and glimepiride (C) with the common motif boxed. (D) Cryo-electron microscopy derived-structure of a sulfonylurea receptor of ATP sensitive-potassium channel composed of four SUR1 subunits and four Kir6.2 subunits. Four molecules of ATP bound to the channel are shown in green bound to Kir6.2 subunits. Four glibenclamide molecules shown in red are bound to SUR1 subunits. Cryo-electron microscopy structure used with permission.
Discussion

The endoplasmic reticulum is involved in biosynthesis of lipids, folding and assembly of proteins, and regulation of calcium signaling [176]. Loss of function of the endoplasmic reticulum is related to several diseases, including cystic fibrosis, diabetes mellitus, Alzheimer’s, and Parkinson’s [176]. SU11274 accumulates rapidly in cells (Figure 56), specifically in the ER region (Figure 57). In fact, after treating cells with 100 nM SU11274 for only three minutes, we found that the cellular SU11274 concentration was nearly 100 times that of the cell medium, and the concentration in the ER region was over 350 times that of the cell medium (Figure 58). It is noteworthy that the Pearson correlation coefficients indicate that SU11274 preferentially localizes with both the ER and early endosomes (Figure 57E).

SU11274 shares a structural feature, a phenyl sulfonamide group, with glibenclamide (the ER-targeting moiety of ER-Tracker™ Red) and glimepiride. These drugs are known to bind sulfonylurea receptors of ATP sensitive-potassium channels (Figure 59) [173, 174]. It may be that SU11274 interacts with the ER by way of this channel.

Discovered in 1942, sulfonylureas are the most common drug used in the treatment of diabetes mellitus [177]. Glibenclamide and glimepiride, second generation sulfonylureas, act on β-pancreatic cells by binding and inhibiting the sulfonylurea receptor of ATP sensitive-potassium channel, which stops the inflow of potassium ions into the cells. This causes depolarization of the cell membrane and results in an influx of calcium ions, which leads to a release of insulin [177]. Interestingly, SU11274 has been shown to directly impact β-cells. SU11274 caused β-cell death and loss of β-cell mass in rats after two weeks of treatment [178]. However, this stands in contrast to the impact of glimepiride on β-cells. In a six-month clinical study, glimepiride was found to enhance β-cell secretory capacity after six months of treatment [179].
Though sulfonylureas and SU11274 affect cells differently, the moiety that they share may be a useful tool to target drugs specifically to the ER. Cancer patients would possibly benefit from ER-targeted therapy. In most types of cancer, the tumor microenvironment is limited in oxygen and glucose, so oxidative ER protein folding and glycosylation-assisted ER protein folding are impaired. This leads to activation of the unfolded protein response (UPR). In fact, cancer cells often rely on UPR for growth and survival [176]. Targeting the endoplasmic reticulum in B cell malignancies may be especially advantageous. B-chronic lymphocytic leukemia (B-CLL) and multiple myeloma (MM) cells have more developed ER networks than normal lymphocytes [180]. In addition, these cells rely on the unfolded protein response (UPR) for plasma cell differentiation [180].

Some proteins crucial to UPR activity, such as X-box binding protein 1 (XBP1), glucose-regulator protein 78 kDa (GRP78), and inositol-requiring enzyme 1 (IRE1) have been targeted for the treatment of breast cancer, myeloma, melanoma, and atherosclerosis [181-183]. It may be that modifying such a drug with a phenyl sulfonamide group would cause it to accumulate in the ER region resulting in greater efficacy. Such targeted therapy would allow decreased dosage and decreased toxicity. Perhaps locked within the fluorescent molecule SU11274 is a key to ER-targeting drugs of the future.
CHAPTER 5. DISCUSSION AND FUTURE STUDIES

Concerning future SU11274 studies, the fact that SU11274 is fluorescent is of itself perhaps something that may be clinically useful. Finding a way to chemically target a drug to the ER, perhaps with a phenyl sulfonamide group, may also prove to be useful. Inducing ER stress seems to be a particularly effective way to treat certain types of cancers, such as leukemia and lymphoma. In the case that targeting a drug to the endoplasmic reticulum would be helpful, causing such a drug to concentrate at the endoplasmic reticulum would mean that less of the drug could be used, reducing toxicity.

Presently, it is not clear if SU11274 accumulates inside of the ER or at the surface of the ER. Does SU11274 bind to sulfonylurea receptors of ATP sensitive-potassium channels or pass into the lumen of the ER? It will be important to answer such questions to know how useful this phenyl sulfonylurea group will be in targeting the ER of cells.

MUC1 and beta-catenin are important players in many cellular processes. MUC1 plays a role in the housekeeping function of lubricating and protecting luminal surfaces of respiratory, gastrointestinal, and reproductive tracts. However, it also plays an important role in signal transduction, that is communicating the current state of the cell exterior to the nucleus so that the cell can reprogram transcription to adapt to various stresses. Beta-catenin does not communicate information about the extracellular environment with the nucleus. Rather, it provides cues to the nucleus that it is time for division, the accumulative effect of which is the proliferation of cells.

Cancer is something like a wound that does not heal, which sets the stage for an inflammatory microenvironment [184] swarming with growth factors and cytokines [185]. Such signaling molecules act by binding to specific receptors. However, the signal must then be transduced into the cell interior in order to have the proper effect on the cell. In
the case of cells that overexpress MUC1, many have hypothesized that phosphorylation of the cytoplasmic tail of MUC1 would allow for interaction with various protein partners, such as beta-catenin, which would allow the signal to reach the nucleus.

The purpose of this project was to understand how extracellular cues impact signaling through the cytoplasmic tail of MUC1 by way of beta-catenin. Because cancer cells have lost polarity, the normally apical MUC1 and basolateral receptor tyrosine kinases are able to interact [7]. It is thought that in cancer cells growth factor stimulation of receptor tyrosine kinases, such as EGFR, causes phosphorylation of MUC1.CT resulting in its association with various transcription factors, such as beta-catenin [55]. The hypothesis is that increased interaction between beta-catenin and MUC1.CT stabilizes beta-catenin and leads to nuclear localization of beta-catenin [124], which results in increased expression of proto-oncogenes such as cyclin D1, c-Myc, and matrix metalloproteinase-7 (MMP7) [96, 142].

The fact that EGF stimulation decreases colocalization and Erlotinib treatment tends toward increased colocalization between the molecules supports the idea that EGFR-mediated phosphorylation of MUC1.CT regulates the interaction between beta-catenin and MUC1.CT, but the nature of this regulation may be different than previously thought. Two-dimensional gel electrophoresis of MUC1.CT immunoprecipitates indicates that EGF stimulation causes increased phosphorylation of tyrosine residues of MUC1.CT. However, counter to a previous report [55], it seems that EGF stimulation results in a decreased association between beta-catenin and MUC1.CT.

With EGF stimulation a trend of decreased beta-catenin in the membrane was observed in S2-013.MUC1 eGFP/mCherry beta-catenin cells, as others have reported [121, 165, 186]. A significant decrease was also observed in the levels of MUC1.CT in the membrane of these cells. In the case of S2-013.MUC1F cells stained with beta-catenin and MUC1.CT antibodies, this loss in membrane beta-catenin and MUC1.CT
was not recapitulated. However, in both cases, a loss in membrane colocalization of the two proteins was observed.

It is not clear if this loss in colocalization is due entirely to decreased levels of the interacting partners or loss of beta-catenin/MUC1.CT binding affinity upon MUC1.CT phosphorylation. However, beta-catenin and MUC1.CT levels appear to remain steady or only moderately decrease in the minutes that follow EGF stimulation. The rapid drop in colocalization at the membrane suggests that the loss in association is not simply due to a loss in beta-catenin and MUC1.CT concentration. EGF-induced phosphorylation seems to physically deter the interaction between beta-catenin and MUC1.CT.

The physiological implications of the loss of the beta-catenin/MUC1.CT association with EGF stimulation are not known, and conflicting reports make speculation difficult. Some have proposed that MUC1.CT binding to beta-catenin stabilizes beta-catenin allowing for its increased presence in the nucleus [124]. Beyond simply changing the quantity of Wnt target gene expression, one group reported that MUC1.CT binding to beta-catenin stabilizes beta-catenin and also modifies the genes that beta-catenin targets [74]. Contrarily, one group found that MUC1.CT actually decreased Wnt signaling [97], and a second group showed that MUC1 levels are inversely related to beta-catenin levels [98].

It seems clear how the beta-catenin/MUC1.CT interaction provides a survival advantage to cancer cells. As discussed previously, beta-catenin is a transcription coactivator. In cancer the transcription program beta-catenin activates is not only one of proliferation but also of invasion and metastasis [187, 188]. Epithelial to mesenchymal transition is important for cancer progression [189]. Because all three proteins associate with the membrane, perhaps MUC1 competes with E-cadherin for beta-catenin binding, and this beta-catenin/MUC1 interaction at the membrane helps cancer cells maintain their mesenchymal phenotype by reducing the pool of beta-catenin at adherens
junctions. In addition, as has been proposed, the beta-catenin/MUC1.CT interaction stabilizes beta-catenin in the cytoplasm and allows for beta-catenin nuclear accumulation and its cancer-driving transcriptional activity [95, 124].

So what survival benefit does EGF-stimulated abolition of the beta-catenin/MUC1.CT interaction provide the cancer cell? In cancer cells MUC1 and beta-catenin are able to interact due to loss in cell polarity [47]. When EGFR is activated by EGF, membrane-bound MUC1.CT and beta-catenin [186, 190] have both been reported to be phosphorylated. EGFR activation results in EGFR endocytosis [191]. Perhaps when EGFR endocytosis occurs, the membrane-bound MUC1 is taken inside the cell along with EGFR. It may be that MUC1 maintains a pool of E-cadherin-free beta-catenin that can be internalized along with EGFR and MUC1. No longer bound to MUC1 but now in an endosome, beta-catenin escapes the degradation complex. EGFR is known to enter the nucleus. Conceivably this pool of beta-catenin enters the nucleus with EGFR by way of nuclear envelope-associated endosomes [192]. Here it is then able to activate transcription of genes involved in driving proliferation and invasion. In fact, MUC1 has been proposed to play a role in nuclear accumulation of EGFR, and MUC1/EGFR nuclear association has been shown to be important in upregulation of cyclin D1, a transcriptional target of Wnt signaling [193]. Could it be that EGF stimulation provides a pathway for beta-catenin to enter the nuclei of MUC1 overexpressing cancer cells?

It is surprising that EGF stimulation of pancreatic cancer cells actually reduces interaction between beta-catenin and MUC1.CT. It is unclear if this is a transient effect or is maintained chronically. In the context of cancer, the cells are bathed in growth factors constantly [184, 185]. It will be important to more fully elucidate the long-term aspects of EGF-stimulated changes in the interaction between beta-catenin and
MUC1.CT in order to better understand cancer, especially that which involves high levels and/or aberrantly localized MUC1 and beta-catenin.

Fluorescent MUC1.CT and fluorescent beta-catenin molecules have made it possible to see inside of the cells in order to understand the interaction between the two molecules. However, the particular genetics of the S2-013 cell line align with only an undefined subgroup of pancreatic ductal adenocarcinomas. It seems advisable to repeat these experiments in other pancreatic cancer cell lines in order to look for overarching themes so that a broader understanding of the interaction between these molecules in pancreatic cancer can be obtained.

EGF stimulation causes a rise in beta-catenin levels in the membrane of CFPAC-1 cells, whereas it tends to cause nuclear beta-catenin to rise in S2-013.MUC1F cells. Confocal microscopy and PLA data of CFPAC-1 cells indicate that a transient rise in beta-catenin/MUC1.CT interaction occurs with EGF stimulation, whereas S2-013.MUC1 cells tend to lose beta-catenin/MUC1.CT interaction with EGF stimulation. CFPAC-1 cell behavior stands in contrast to reports that EGF diminishes membrane beta-catenin [121, 165, 186]. It may be of use to determine the underlying cause for this discrepancy.

The OpenSPR data support the idea that beta-catenin and MUC1.CT interact, at least in vitro. The ITC data coupled with the OpenSPR data suggest that the beta-catenin/MUC1.CT interaction involves the N-terminal half of the cytoplasmic tail of MUC1.CT. This would mean that the serine-rich motif of MUC1.CT is not involved in the interaction as has been postulated [94]. Limited data in this dissertation suggest that the interaction relies on the residues of MUC1.CT that lie closest to the membrane (of membrane-bound MUC1). Interestingly, the immunoprecipitation data, confocal microscopy data, and PDAC tissue sample point to the fact that the highest proportion of beta-catenin/MUC1.CT interaction is taking place in the membrane of the cells. This would not be surprising, however, given that MUC1 is a transmembrane protein and that
beta-catenin is an important player in adherens junctions at the membrane of cells. In order to more fully characterize the beta-catenin/MUC1.CT interaction, it will be necessary to define the portion of MUC1.CT that physically associates with beta-catenin.

The clinical relevance of this dissertation would be enhanced by studies conducted in human tissue samples comparing normal pancreas to pancreatic ductal adenocarcinoma. This would show whether or not increased interaction of beta-catenin and MUC1.CT occurs with cancer progression and whether or not this interaction is occurring in the nuclei of cells, to determine the extent to which MUC1.CT impinges on Wnt signaling in PDAC. Studies conducted with the University of Nebraska’s Rapid Autopsy Program (RAP) suggest, as in the cell culture data, that the interaction between beta-catenin and MUC1.CT occurs largely in the membrane of PDAC cells. To date, no colocalization of beta-catenin and MUC1.CT has been observed in the nuclei of these RAP samples. However, it is not difficult to imagine that only a very small subset of MUC1.CT would interact with beta-catenin, and even a small amount of MUC1.CT interacting with beta-catenin could be amplified to produce a large impact on the transcriptional program of the cells. It may be that this small pool of beta-catenin/MUC1.CT complexes is simply below the current means of detection. Others have reported cytoplasm/membrane compartment beta-catenin/MUC1 colocalization in gastric cancer [194] and colorectal cancer [195]. Both noted a trend of strong nuclear beta-catenin staining in the invasive front of the tumor. Interestingly, in colorectal cancer the areas of strongest beta-catenin/MUC1 colocalization are near the invasive front of the tumor. Neither group reported nuclear MUC1 localization, though the groups probed for full-length MUC1 rather than the cytoplasmic tail of MUC1.

It remains to be determined how other growth factors alter the interaction between MUC1 and beta-catenin. CTGF, which binds to the receptor tyrosine kinase Tropomyosin receptor kinase A (TrkA) [196], was studied (Figure 14) because this
matricellular protein promotes fibrosis and cancer progression in multiple types of cancer [197], including pancreatic cancer [198]. In addition, MUC1.CT has been observed at the promoter of the gene [74]. PDGF has been reported to impact the beta-catenin/MUC1.CT interaction. In this study, it was reported that PDGFR catalyzed the phosphorylation of the tyrosine residues in the HGRYVPP and RDTYHPM motifs of MUC1.CT resulting in greater nuclear colocalization of MUC1.CT and beta-catenin. This in turn increased cancer cell invasion and metastasis in an animal model [60].

The impact of HGF on colocalization of MUC1 with various protein partners may be of interest as Met is able to bind MUC1.CT [199], and one report indicates that Met transactivates Wnt signaling in glioblastoma [200]. Furthermore, in a rat hepatoma cells line HGF stimulation disrupted beta-catenin/E-cadherin association and caused transactivation of beta-catenin through phosphorylation of tyrosine residues 654 and 670 [201]. Perhaps Met phosphorylation of MUC1.CT changes its cadre of interacting partners and the downstream results.

It may be of use to determine how growth factors and growth factor receptor inhibitors impact the interaction between MUC1 and other transcription factors such as p53 and ERα. The impact of Met-mediated MUC1.CT phosphorylation on its interaction with p53 has already been partially characterized [199].

In addition to questions about interactions of MUC1.CT with signaling molecules, many questions about MUC1 biology remain unanswered. For example, how does the plasma-membrane-bound MUC1 move into the cytosol? And how does it re-enter membranes, such as the outer membrane of mitochondria [50, 81]? What is the metabolic fate of MUC1.CT? Does MUC1.CT play any role in signaling in normal cell physiology? Answers to such questions will help to unravel the mystery of pancreatic cancer.
In conclusion, the beta-catenin/MUC1.CT interaction has been reported by multiple groups [91, 94, 124, 202]. The work presented here shows that a beta-catenin/MUC1.CT interaction occurs in cells and in vitro. However, only a certain fraction of MUC1.CT coimmunoprecipitates with beta-catenin and vice versa. This may be the result of a weak or transient interaction. Yet compared to other noncovalent interactions in biology such as the millimolar ubiquitin/ubiquitin binding domain interaction and the femtomolar biotin-streptavidin interaction [203, 204], the beta-catenin/MUC1.CT interaction is of moderate strength. Perhaps the interaction requires that one or both partners fit specific post-translational modification parameters. Indeed, if EGF-stimulated tyrosine residue phosphorylation of MUC1.CT decreases its interaction with beta-catenin and phosphorylation generally weakens the beta-catenin/MUC1.CT interaction, it seems that the beta-catenin/MUC1.CT interaction requires that the proteins have a specific phosphorylation pattern. Perhaps the specific population of MUC1.CT molecules that interacts most with beta-catenin is that which is least phosphorylated.
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