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The Role Of The Cell-Surface Protease Tmprss13 In Colorectal Cancer

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THE ROLE OF THE CELL-SURFACE PROTEASE TMPRSS13 IN COLORECTAL CANCER

by

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DISSERTATION

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DEDICATION

To my wife Veronica. I could not have made it this far without you. Together, I truly feel the sky is the limit. Thank you for your patience, input, and support, and I cannot express how excited I am to move together forward in our lives from this point.

To my girls Gwen and Sophie. I hope through my experience and example I can help you realize that you can accomplish all you want in life and more.

And in loving memory of my mother Jeanette Erazo. You have and always will be my greatest hero and role model, an exemplar of tenacity and perseverance. Whatever comes my way, I know I will overcome, with the strength you have helped instill in me. I hope to honor your memory in all I do.

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CHAPTER 1: INTRODUCTION

1.1 Normal colon structure and function

The colon and rectum form the final portion of the digestive tract. In humans, the colon can be anatomically sub-divided into three parts: the right, also known as the proximal or ascending, colon, found on the right side of the body receives digested food from the small intestine; the transverse colon, which travels across the abdomen from right to left; and the left, also known as the descending or distal colon, which is positioned on the left side of the body (**Figure 1.1**). Functionally, the colon serves to reabsorb water from indigestible food, leaving fecal matter to be stored in the rectum for later expulsion.

Colon tissue consists of three layers, with the innermost mucosa layer lining the lumen. Moving outward from the mucosa are the submucosal layer, muscularis propria, and serosa layers (**Figure 1.1A**). The mucosa contains the epithelial cell layer which is arranged in glandular structures known as the colon crypts; fingerlike invaginations lined by epithelial cells. including mucin-producing goblet cells (**Figure 1.1B**). The colon mucosal epithelium provides a cellular and molecular interface with the luminal contents to regulate reabsorption of water and salts. In addition to this function, the colon epithelium provides a physical barrier from environmental threats including toxins and infectious agents. The exclusionary properties of the gastric and intestinal mucosa are referred to as the "gastrointestinal (GI) barrier," and are reviewed in the following section (**Section 1.2**).

1.2 Intestinal barrier function – an overview.

The intestinal epithelium functions as the primary barrier to luminal contents in the GI tract. In the large intestine, maintenance of this barrier is crucial due to the presence of the most significant microbial population in the human body (Quigley, 2013). Mucus produced by the gut

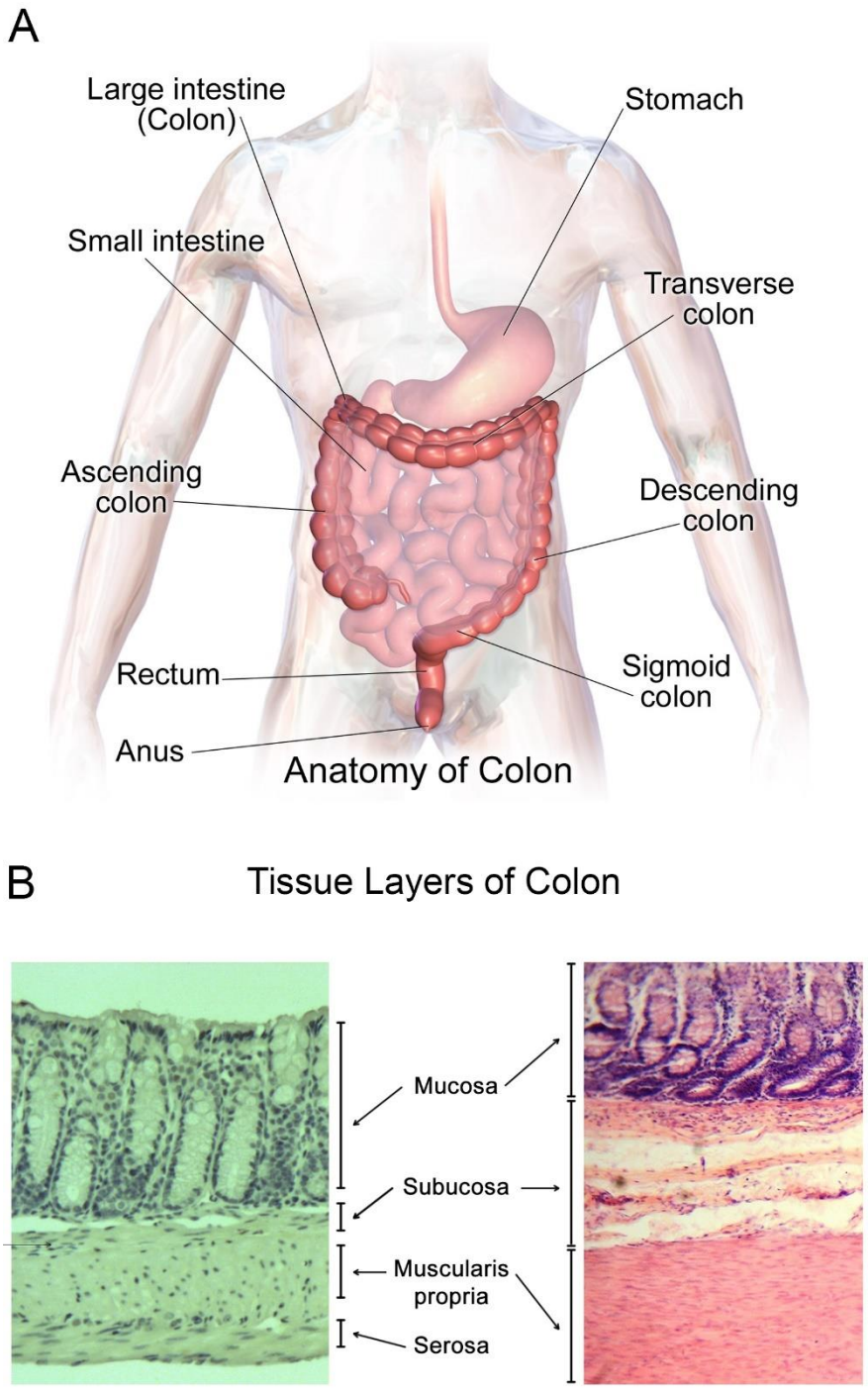

Figure 1.1: **Anatomical structure of the colon**
A) The colon is divided into four general regions; ascending, traverse, descending, and sigmoid colon. Colon position within a human body is shown, with major anatomical regions labelled. Figure from Blausen.com "Medical gallery of Blausen Medical 2014." *WikiJournal of Medicine* **1** (2) DOI:10.15347/wjm/2014.010. ISSN 2002-4436. **B)** Comparison of mouse (left panel) and human (right panel) colon to illustrate the homology of colon tissue. Sections are also labelled with the molecular layers of the colon. Human section images from Juan Carlos Fonseca Mata 2014, https://upload.wikimedia.org/wikipedia/commons/d/de/Large_intestine_histology.jpg.

functions as one of the first lines of defense from bacteria and from ingested contents, forming a protective layer throughout the GI tract (Okumura and Takeda, 2018). Due to microbial presence, the colon is coated with two distinct layers of mucus, with an outer layer which houses commensal flora and a dense inner layer, devoid of microbes, directly coating the epithelium, which produces the mucus (Johansson et al., 2008). The main barrier is provided by the epithelial cells of the mucosa, specifically through the junction complexes formed by adjacent cells **(Figure 1.2)**. The junction complexes are largely composed of tight junctions (TJs) and adherens junctions (AJs), which serve different functions (Groschwitz and Hogan, 2009). The TJs form between cells at the apical end of the intestinal epithelial cells, made up of claudins, occludin, zonula occludens (ZO)-1 and ZO-2 (Vancamelbeke and Vermeire, 2017). Tight junctions serve to restrict molecular passage to the intracellular space by providing barrier between apical and basal compartments of the cell. The AJs are composed of cadherin and nectin, transmembrane proteins which create extracellular adhesions between adjacent cells to maintain the epithelial sheet (Campbell et al., 2017). The intracellular domains of the cadherins and nectins also serve as modulators of intracellular signaling through intracellular domains.

Defects in intestinal barrier function are linked to GI disease, most notably inflammatory bowel disease (IBD) and colon cancer. Barrier defects are believed to underlie IBD, as immune responses to infiltrating gut flora can result in rampant inflammation (de Souza and Fiocchi, 2016). Disruption of the mucus barrier, such as through ablation of mucin-2 (Muc2) production, can lead to spontaneous inflammation and even colorectal cancer, likely due to compromised barrier function and bacterial infiltration (Van der Sluis et al., 2006). Inflammation and intestinal permeability function in a positive cyclic reinforcement, in which inflammation promotes permeability, and permeability promotes further inflammation. Single nucleotide polymorphisms

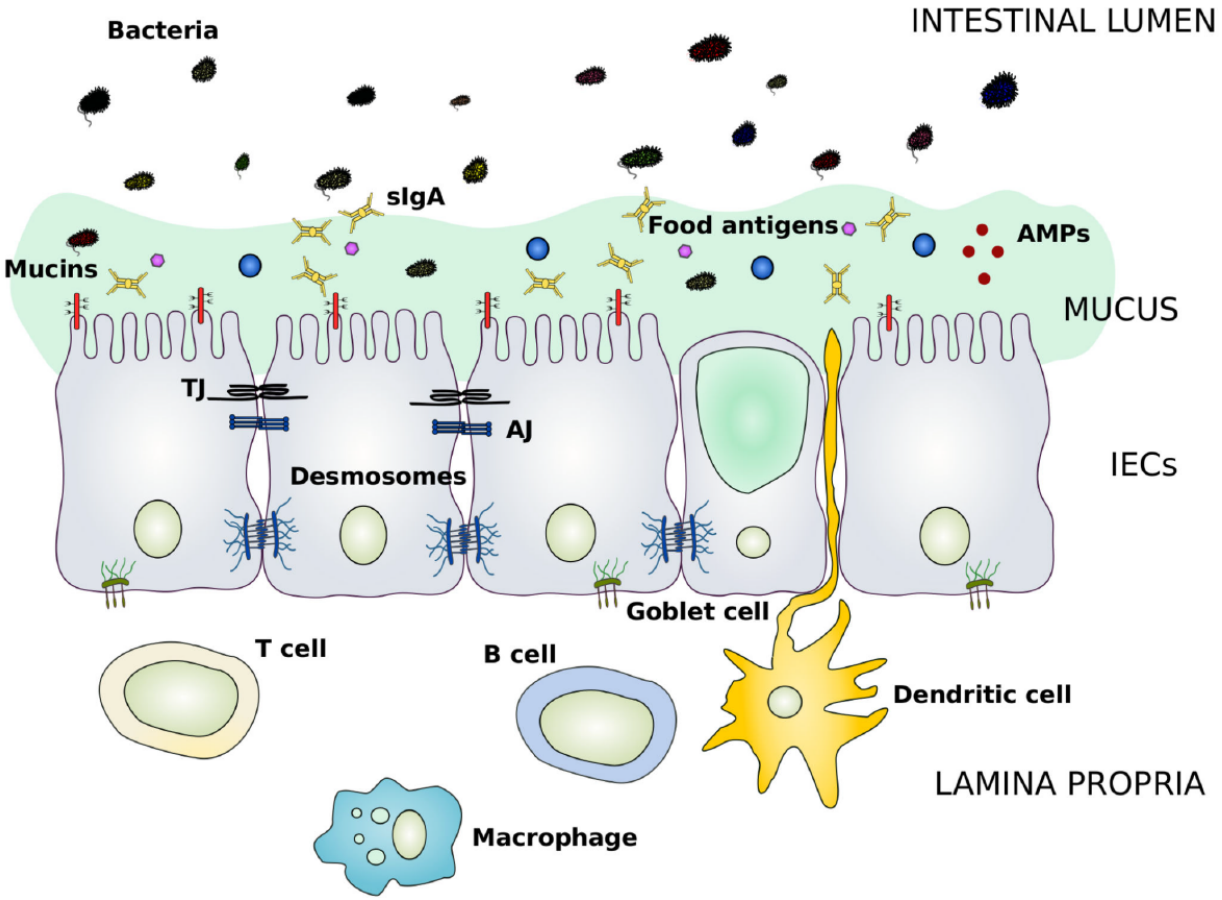

Figure 1.2 Illustration of intestinal barrier components
Cellular scale representation of intestinal barrier function by ntestinal epithelial cells (IECs). Tight junctions (TJs) and adherent junctions (AJs) confer both cellular adhesion to maintain the epithelial sheet and selective impermeability to restrict transport from colon luminal contents. Layers of mucus covering epithelial cells provide another layer of protection from the intestinal environment. The layer beneath the epithelium is made up of connective tissue and immune cells which make up the second line of immunological defense in the colon, following action of secreted antimicrobial peptides (AMPs) and secreted IgA (sIgA) antibodies. Figure used with permission from Vancamelbeke M & Vermeire S (2017) The intestinal barrier: a fundamental role in health and disease. Expert review of gastroenterology & hepatology 11:821-834.

in *CDH1*, which encodes the adherens junction protein E-cadherin, has been linked to ulcerative-colitis (Ventham et al., 2013; Grill et al., 2015). Variation in certain junction complex genes may predispose patients to increased intestinal permeability, increasing susceptibility inflammation. In Crohn's disease, patients in asymptomatic periods show evidence of intestinal permeability, which often precedes recurrence of inflammatory events (Vogelsang, 2008). Chronic inflammation is strongly linked to colorectal cancer development as well, making the impact of aberrant barrier

function far-reaching (Itzkowitz and Yio, 2004; Ullman and Itzkowitz, 2011; Grivennikov, 2013; Chen et al., 2017). Understanding the components contributing to maintaining intestinal barrier function and stable junction complexes is important to improve treatments for debilitating diseases like IBD and may also contribute to new preventative and treatment measures for GI malignancies.

1.3 Colorectal Cancer

Colorectal cancer (CRC) is one of the most common cancers in both men and women with an estimated 140,250 new cases in the United States and 50,630 CRC related deaths (ACS, 2018). This section provides a brief overview of what is known regarding CRC development, etiology, and treatment.

1.3.1 CRC development and subtypes

The vast majority of CRC cases are epithelial in origin. In most cases, morphological and pathological characterization of disease development follows a defined progression: localized hyperplasia in a single epithelial crypt of the colon will present histologically as an aberrant crypt focus. Aberrant crypt foci possess the potential to develop into an adenoma, or polyp, in the colon **(Figure 1.3)**. There are three histological classifications of colorectal polyps; tubular, villous, and an intermediate form known as tubulovillous. Tubular adenomas are identified by tubular crypt structures whereas villous adenomas exhibit very long thin villous structures growing within the (Myers and Arora, 2018). Whereas tubular adenomas tend to grow outward into the lumen, villous adenomas often have more flat growth patterns, making detection during colonoscopy difficult. Adenomas are thought to be the precursors to malignancy, and while it is not known what percentage of adenomas become malignant, identification and removal of adenomas through preventative colonoscopic screening is critical for risk management of disease (Rastogi and Wani, 2017).

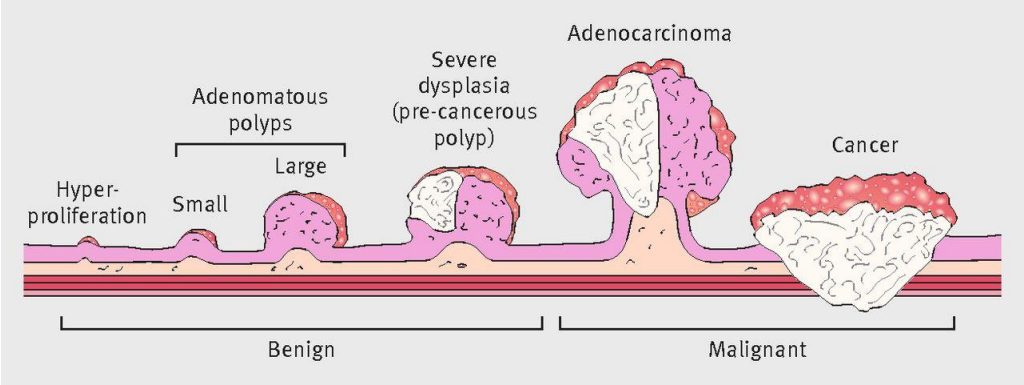

Figure 1.3: Colorectal cancer progression
Colorectal cancer follows a defined process over the course of years. Initial hyperproliferation in a single epithelial crypt can develop into an aberrant crypt focus, which has the potential to become an adenoma. Colorectal adenomas may undergo further genetic changes which promote development of invasive adenocarcinomas. Figure used with permission from Thrumurthy SG, Thrumurthy SSD, Gilbert CE, Ross P and Haji A (2016) Colorectal adenocarcinoma: risks, prevention and diagnosis. 354:i3590

A small percentage of adenomas will progress to adenocarcinomas, which account for roughly 95% of all cases of CRC (ACS, 2018). A rare subtype of colorectal adenocarcinoma, known as mucinous adenocarcinoma, makes up roughly 10-15% of CRC cases (Sundblad and Paz, 1982). Mucinous adenocarcinoma (MAC) typically presents with poorer prognosis and is characterized by increased production of mucin, making these tumors difficult to identify, resect, and treat. Even more infrequent are cases of colon signet ring cell carcinoma (SRCC), which account for less than 1% of CRC (Park et al., 2015). Early detection of SRCC has proven difficult and rare, making late stage diagnosis more likely with poor prognosis as a result. Both MAC and SRCC have higher recurrence rates, with MAC more likely to recur in the primary tumor site and SRCC more likely to recur at metastatic sites (Nitsche et al., 2013). The aggressive nature of these subtypes has made the need for improved screening techniques and identification of new treatments a high priority, as well as understanding the underlying events of CRC.

1.3.2 Molecular etiology of CRC

The established framework for solid tumor initiation implicates a series of multiple abnormal genetic changes that drive cancer development (Vogelstein et al., 1988). One of the most significant driver genes identified thus far has been the adenomatous polyposis coli (*APC*) gene, which is mutated in up to 80% of colon adenocarcinomas (Fearnhead et al., 2001). Mutations in the *APC* gene were first identified to be the causal factor in familial adenomatous polyposis (FAP), a rare inheritable form of colorectal cancer characterized by growth of hundreds of polyps in the colon and rectum (Groden et al., 1991; Joslyn et al., 1991; Kinzler et al., 1991; Nishisho et al., 1991; Bulow et al., 2006). The *APC* gene product has been found to regulate ß-catenin, with *APC* loss-of-function mutations leading to runaway activation of the Wnt signaling pathway (Kennell and Cadigan, 2009). Colorectal tumors also frequently contain mutations in the oncogene *KRAS*, which are present in roughly 30% of CRC cases (Schubbert et al., 2007). *KRAS* encodes a small GTPase (a hydrolase of guanosine triphosphate (GTP)) linked to downstream pathways important for cell growth, survival, and differentiation. Oncogenic mutations of *KRAS* leave the protein locked in the active GTP-bound conformation, leading to constitutive signaling of downstream effectors (Schubbert et al., 2007). The mutation status of *KRAS* in CRC has proven to be a strong prognostic marker, with wild-type *KRAS* tumors predicted to have better outcomes and be more responsive to treatment with the EGFR monoclonal antibody cetuximab (De Roock et al., 2008; Karapetis et al., 2008; Lee et al., 2015).

Defining the molecular events that promote mutations in oncogenes has been an intense field of study in cancer research. Mutations in several CRC driver genes are believed to result from chromosomal instability (CIN), in which large chromosomal portions are multiplied or deleted (Lengauer et al., 1998). For example, CIN has been implicated in loss of heterozygosity of *APC*

and *P53*, as well as amplification of mutant *KRAS* (Lengauer et al., 1998). CIN is typically the result of a defective mitotic machinery, such as multiple centrosomes, defective mitotic checkpoint signaling, and telomere dysfunction (Pino and Chung, 2010). Microsatellite instability (MSI), which results from deficiencies in the DNA mismatch repair pathway, has also been identified to promote mutations in CRC. MSI is prevalent in chromosomal stable tumors, making up roughly 10-15% of CRC (Ionov et al., 1993; Thibodeau et al., 1993). Presence of MSI in CRC tumors has become a prognostic marker and is believed to predict response to the standard of care treatment of adjuvant chemotherapy with 5-fluorouracil (Ribic et al., 2003; Carethers et al., 2004; Des Guetz et al., 2009).

1.3.3 Risk factors for CRC

About 75% of CRC cases have a sporadic origin, suggesting a strong contribution from environmental stimuli. Several factors have been found to contribute or associate with CRC incidence. Diet has long been recognized as a strong risk factor, with recent studies finding high intake of red or cured meats strongly associated with CRC (Chan et al., 2011; Mehta et al., 2017). The "Western diet," rich in red meat, dairy, and processed grains, is associated with elevated risk of CRC disease, with a historical disparity in CRC incidence between Asian and Western nations (Deng, 2017). While a directly causal link has yet to be identified, the Western diet has been found to promote low-grade inflammation, endotoxemia, hormonal imbalance related to obesity, and changes to gut flora (Pendyala et al., 2012; O'Keefe, 2016; Guerville et al., 2017). Increased production of carcinogenic bile acids, such as deoxycholic acid, resulting from high fat intake (as in the Western diet) has been demonstrated to induce spontaneous tumors in mouse models, further strengthening a link between diet and CRC (Bernstein *et al.*, 2011; Prasad *et al.*, 2014).

Inflammatory bowel disease (IBD), such as ulcerative colitis (UC) and Crohn's disease has also been found to strongly associate with CRC. For example, overall risk for patients of UC is estimated to be increased 2.4-fold above the rest of the population, with onset more likely at an earlier age (Jess et al., 2012). Aside from the elevated cancer risk, colitis-associated colorectal cancer, or CAC, also shows histological features characteristic of MAC and SRCC, the more aggressive forms of CRC (Itzkowitz and Yio, 2004). While sporadic CRC typically results from dysplasia originating in one or localized crypts, chronic inflammation often results in widespread dysplasia, creating numerous aberrant crypt foci and increasing the probability of CIN or MSI events that promote CRC (Ullman and Itzkowitz, 2011).

1.4 Targeting proteolysis in CRC: rationale, strategies, and outcomes

Treatment of CRC has been a major challenge in fighting advanced cancers. Standard of care treatment depends on when cancer is discovered. For localized tumors identified at early stages (stages 0-II), surgery is curative, however when lymph nodes positive for cancer are present (stage III), chemotherapy is often incorporated into treatment. At the heart of adjuvant chemotherapeutic regimens for CRC is 5-fluorouracil (5-FU). Cytotoxicity from 5-FU treatment is a result of miss-incorporation into nucleotides, resulting in DNA damage, and also through blockade of thymidylate synthase, preventing production of the nucleotide thymidine and promoting further miss-incorporation of 5-FU (Longley et al., 2003). Few advances have been made regarding chemotherapeutic treatment, with main changes being incorporation of the combination therapy FOLFOX (5-FU, the platinum-based drug oxaliplatin, and leuocovirin (folinic acid)), or FOLFIRI (5-FU, the topoisomerase inhibitor irinotecan, and leucovorin). These combination therapies have modest efficacy, with minimal benefit in advanced metastatic cancer (Van der Jeught et al., 2018). These drugs also have the downside of severe side effects; the effects

of FOLFOX and FOLFIRI drugs are not tumor selective and act on all proliferating cells, leading to hair loss, epithelial lesions, diarrhea, and increased susceptibility to infection.

The search for novel CRC treatments has focused on identification of tumor-promoting genes or pathways that can be pharmacologically targeted. Inhibitors used in treatment today target angiogenesis, as with the VEGF monoclonal antibody bevacizumab, and EGFR signaling, with the EGFR monoclonal antibody cetuximab. The effectiveness of bevacizumab has been modest, extending progression free survival by a few months (Saltz et al., 2008). Treatment efficacy with cetuximab depends on the mutation status of *KRAS*, with antibody treatment most effective in patients with wild-type *KRAS* (De Roock et al., 2008; Karapetis et al., 2008). With the limited treatment options in advanced CRC, the identification of novel targets becomes a more salient goal in CRC research.

The diversity among proteases provides rich opportunity in developing cancer treatments. Many proteases have highly defined expression patterns in human tissues, enabling greater target selectivity in comparison to classical cytotoxic chemotherapeutics. An important goal to improve quality of life for patients is minimizing the impact of non-specific activity of therapeutic drugs. With proteases often exhibiting *de novo*/ectopic expression or overexpression in cancer, minimizing side effects may become possible with selective therapeutics. Currently, proteolytic inhibition remains a novel and experimental treatment in cancer, but several key pathways and molecules have been identified as targets.

One of the major pro-oncogenic roles for proteolysis in cancer is the activation of hepatocyte growth factor, or HGF. HGF is a growth factor synthesized by fibroblast and inflammatory stromal cells as the propeptide pro-HGF and secreted into the extracellular space. In the extracellular environment or the pericellular space bound to its tyrosine kinase receptor Met,

pro-HGF is proteolytically processed to active HGF, which initiates the Met-signaling pathway to promote proliferation and invasion in cancer (Cooper et al., 1984; Bottaro et al., 1991). Several proteases have been identified to activate HGF, but the most efficient are the secreted serine protease HGF activator (HGFA) and membrane-anchored serine matriptase (Owen et al., 2010; Kawaguchi and Kataoka, 2014). Increased Met signaling has been identified in breast, colon, and ovarian cancers, and associated with poor outcomes and advanced disease (Camp et al., 1999; Bardelli et al., 2013; Kim et al., 2018). Due to the high degree of homology among proteases competent in pro-HGF cleavage, developing selective inhibitors poses a significant challenge, however multiple approaches to targeting proteolysis become available once the critical protease in a defined cancer type is known (for further review of matriptase and activation of the HGF/Met pathway in breast cancer, see section "**1.6.1: Type II transmembrane proteases**"). This can be addressed by inhibitory antibodies, which can block enzymatic activity through zymogen activation inhibition, allosteric or orthosteric inhibition, or inhibition of co-factors (Kirchhofer et al., 2018). Preclinical testing of antibodies developed against matriptase has been used to image patient-derived xenografts in mice with a high degree of specificity; however, the therapeutic benefits are yet to be determined (Darragh et al., 2010).

The development of small molecule inhibitors is another means of proteolytic targeting. Rational drug design is a common strategy for drug development. For targeting proteolysis, the structural properties of protease substrates can reveal the peptide sequences that confer protease specificity, which can be modified for inhibiting catalysis. Key to success with this approach is understanding the roles of the proteases being targeted and the impact of selective inhibition. Some of the first synthetic protease inhibitors were developed for matrix metalloproteinases (MMPs), which were viewed as drivers of metastasis due to their tissue remodeling activity. Inhibitors that

showed great promise in preclinical models failed to exert the same impact in human trials as treatment for advanced metastatic cancers, and often resulted in significant side effects (Coussens et al., 2002; Turk, 2006). Limited knowledge about physiological function of the various MMPs was available when these inhibitors were developed, and our understanding of MMP group has grown significantly since. Past inhibitors are now understood to have had broad specificity, and it has also been demonstrated that MMPs can also play protective roles in cancer, making MMP inhibitors potentially carcinogenic (Maquoi et al., 2002). Timing of treatment may have also played a role in poor outcome of human trials; MMPs are now believed to contribute to events preceding metastasis, making pre-metastatic treatment more likely to be effective (Fingleton et al., 1999). Targeting of specific MMPs with more selective inhibitors may prove to be a more effective strategy as the specific roles played by these proteases are better understood.

1.5 An overview of major classes of human proteases

Proteases are one of the most important classes of enzymatic proteins, with over 580 proteases recognized to date (**Figure 1.4**). In general, these enzymes function by catalyzing hydrolysis of peptide bonds for the purpose of degrading extracellular matrix proteins or processing, or shedding of cell adhesion molecules, growth factors, and cytokines. As a mechanism to regulate enzymatic activity, proteases are generally synthesized as zymogens, or pro-enzymes, which require proteolytic processing to allow for catalytic activity. Proteolytic networks in which zymogen activation of different proteases are linked, factor heavily into several key cellular processes, such as apoptosis, cell-cycle regulation, migration, immune responses neuronal outgrowth, and regulate development of tissues (Mason and Joyce, 2011; Bonnans et al., 2014; Sevenich and Joyce, 2014). In this section, the characteristics, key enzymes, and function of the three largest protease groups are briefly reviewed.

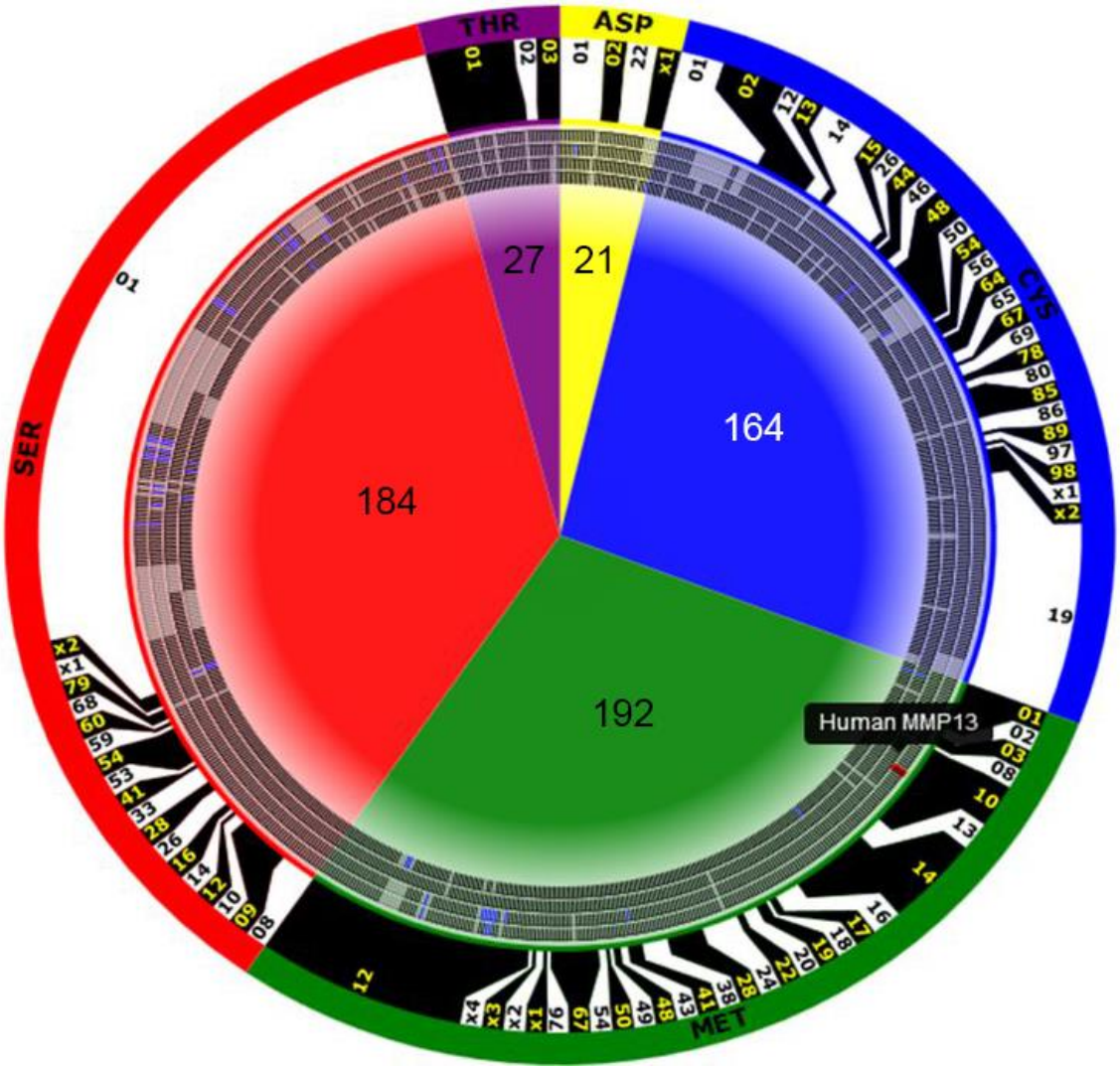

Figure 1.4: The major classes of proteases
The human genome encodes five major classes of proteases, grouped by catalytic mechanism. Serine (SER), metalloproteinase (MET), cysteine (CYS), threonine (THR), and asparartic (ASP) proteases are represented, with number of currently known proteases indicated within the pie chart. Black and white labels on outer ring represent subgroupings within each protease class. Figure used with permission from Pérez-Silva JG, Español Y, Velasco G and Quesada V (2016) The Degradome database: Expanding roles of mammalian proteases in life and disease. Nucleic Acids Research 44:D351-D355.

1.5.1 Metalloproteinases

Metalloproteinases make up the largest class of proteases, with 194 identified members in the human genome. This class is so-called due to the utilization of metal ion for enzymatic activity. One of the most well-known classes of metalloproteinases is the metzincin family, which is characterized by the use of zinc ion for catalysis and a conserved methionine residue in the catalytic active site. Metzincins encompasses the matrix metalloproteinases (MMPs), a disintegrin and metalloproteinases (ADAMs), and ADAM with thrombospondin motif proteases (ADAMTSs) (Kraniak et al., 2018). Like many other classes of proteases types, metalloproteinases are synthesized as zymogens requiring proteolytic cleavage for enzymatic activation, allowing for regulation of catalytic activity (Ra and Parks, 2007).

Of these enzymes, the MMPs are the most well-studied in cancer as they play important roles in remodeling tumor microenvironments through degradation of extracellular matrix components and many are subject to dysregulated expression in disease (Kessenbrock et al., 2010). Structurally, the MMPs share three domains: The N-terminal hydrophobic signal sequence, prodomain, and catalytic domain. However, there exists a significant amount of diversity in the structure and roles these proteases play (Nagase, 1997; Visse and Nagase, 2003). To date, 28 MMPs have been identified (Brinckerhoff and Matrisian, 2002; Pérez-Silva et al., 2016). The MMPs can be classified into six subgroups; collagenases (MMP-1, -8, -13, and -14), gelatinases (MMP-2, -9), stromelysins (MMP-3, -7, and -10), membrane-type MMPs (MT-MMP1 - 6), and matrilysin, and MMPs that do not fit previous classification (Overall and Lopez-Otin, 2002; Kraniak et al., 2018). The MMPs are important for several physiological processes. MMPs have been shown to promote angiogenesis (MMP-2, 3, 9), induce pro-inflammatory signaling (MMP-2, -8, -9), and cellular migration (MT-MMP1) among them (Kajita et al., 2001; Xu et al., 2001; Jin

et al., 2006; Thirkettle et al., 2013). In the case of MMP-8, increased expression is associated with anti-metastatic outcomes in breast cancer (Gutierrez-Fernandez et al., 2008). This effect appears to be tissue-specific, with increased expression of MMP-8 in ovarian and colorectal cancer being linked to poor survival (Gutierrez-Fernandez et al., 2008). It is therefore important to consider different roles for proteases depending on contextual environment. It is perhaps too simplistic to relate an effect seen with a protease in one tissue to all others. As more is discovered about both pro- and anti-tumorigenic effects of MMP expression, more informed targeting strategies can be employed to ensure successful outcomes.

1.5.2 Cysteine proteases

Cysteine proteases are so named for the cysteine, histidine, and asparagine catalytic triad shared among them. The cathepsins, caspases, and calpain protease families are all categorized as cysteine proteases, encompassing enzymes involved in a myriad of physiological processes (Alnemri et al., 1996; Grzonka et al., 2001). As is typical for proteases, they are synthesized as zymogens requiring proteolytic cleavage for activation. Activation of caspases is intricately linked in a proteolytic cascade, allowing for tightly regulated control of apoptotic responses. In the apoptotic proteolytic cascade initiator enzymes are activated in response to death receptor signaling (caspase-8) or induction of the mitochondrial pathway of apoptosis (caspase-9) (Nuñez et al., 1999). Initiator caspases can then activate effector caspases, such as caspase-3, which promotes chromatin condensation and DNA fragmentation (Nuñez et al., 1999; Porter and Janicke, 1999).

Unlike the caspases, which function within the cytosol, the cysteine cathepsins function extracellularly and within intracellular compartments, such as lysosomes. Cathepsin, including cathepsin B and H, found within lysosomes play important roles in protein degradation (Turk et

al., 2000). Cathepsins also play an important role in bone remodeling; expression of cathepsin K is highly localized to osteoclasts, where protease secretion promotes the degradation of type I collagen (Troen, 2004). Cathepsin K knockout mice display a hypermineralization phenotype that is replicated in a rare human disease known as pycnodysostosis, caused by a mutation in the gene encoding cathepsin K (Boskey et al., 2009; Clarke and Hollak, 2015). These studies have helped inform the therapeutic potential of cathepsin K inhibition, which has been proposed as putative target option for osteoporosis, but expression of cathepsin K in other tissues, such as brain, makes inhibition risky until the consequences of cathepsin K blockade are better understood (Drake et al., 2017).

1.5.3 Serine proteases

The serine protease family comprises the second largest protease family in the human genome. These proteases are important for numerous physiological processes, such as thrombin in blood coagulation, enteropeptidase in digestion, granzymes in immune response, matriptase and prostasin in epithelial homeostasis, and plasmin in fibrinolysis (Szabo and Bugge, 2008; Di Cera, 2009; Szabo and Bugge, 2011; Miller and List, 2013). Enzymatically, this family is characterized by an amino acid catalytic triad consisting of histidine, aspartic acid, and serine, the latter amino acid functioning as a nucleophile towards the electrophilic carbonyl of a peptide bond. One of the most studied subfamilies is the S1 group of serine proteases, which are structurally characterized by an active site containing a chymotrypsin-like fold. This group includes the digestive proteases trypsin and chymotrypsin, as well as the thrombin enzyme involved in blood coagulation. Serine proteases include both secreted and membrane-bound family members.

One of the best studied physiological processes regulated by serine proteases is blood coagulation. In blood coagulation, soluble fibrinogen proteins in the bloodstream are cleaved to

create fibrin, which polymerizes to create insoluble aggregates that bind platelets to form blood clots (Palta et al., 2014). A major regulator of this process is the secreted serine protease thrombin. Thrombin is an S1 protease that is synthesized as the zymogen prothrombin which undergoes activation by the blood clotting protease Factor Xa in response to injury to promote formation of a blood clot. A G20210A mutation in the prothrombin gene has been identified to be linked to increased risk of developing blood clots (Shemesh et al., 2017). Mutant prothrombin produces clots that have a distinct structure, however pharmacological compounds that can exploit this difference have not been identified (Janion-Sadowska et al., 2017).

On the flip-side of blood coagulation is the removal of clots to maintain hemostasis through a process known as fibrinolysis. Fibrinolysis controls the size of blood clots and serves to degrade clots once they are formed. This process is controlled by a proteolytic cascade involving secreted serine proteases. Fibrinolysis is largely performed by the serine protease plasmin (Palta et al., 2014). Plasminogen, the zymogen form of plasmin, is released by the liver to the circulatory system where it is activated to plasmin by tissue plasminogen activator (tPA), a serine protease secreted from the endothelium, enabling degradation of fibrin by plasmin. The serine protease urokinase plasminogen activator (uPA) is also capable of activating plasminogen. uPA has also been strongly implicated in the metastasis of several malignancies, such as breast, lung, and gastrointestinal cancers (Gandolfo et al., 1996; Johnsen et al., 1998; Duffy, 2004; Dano et al., 2005; Dass et al., 2008). The proteases involved in coagulation and fibrinolysis have widespread expression patterns throughout the vascular system, making modulation of their activity for therapeutic purposes challenging. Other serine protease groups with more restricted expression and subcellular localization patterns may prove more useful in that regard.

1.6 Membrane-anchored serine proteases

Membrane-anchored serine proteases represent spatially controlled cell-surface localized proteolysis. While secreted serine proteases play important roles in systemic processes such as coagulation and fibrinolysis, the membrane-bound forms ensure cell-surface/pericellular activity, providing a level of spatial regulation of activity. The membrane-anchored serine proteases are classified into three general groups based on the way they are attached to the cell membrane; type I transmembrane, type II transmembrane, and glycosylphosphatidylinositol (GPI)-anchored serine proteases (Antalis et al., 2010) **(Figure 1.5)**.

Type I transmembrane serine proteases are classified by a carboxy-terminal transmembrane domain and extracellular amino-terminal domain. Tryptase γ1 exists as the sole example of a type I transmembrane serine protease expressed in humans and belongs to the tryptase group of largely secreted proteases associated with mast cell granules (Wong et al., 1999; Caughey et al., 2000). The function of this protease remains unknown, but it may be involved in mounting immune responses. For example, administering a recombinant form of tryptase γ1 to the trachea of wild-type mice induces interleukin (IL)-13 expression, but fails to elicit the same effect in STAT6- and IL-4Rα-null mice (which both lack IgE expression), indicating that this protease may play a role in allergy responses (Wong et al., 2002). While the physiological substrates of tryptase γ1 remain to be discovered, the restricted expression of this protease to mast cells as well as the ability of recombinant tryptase γ1 to induce IL-13 express do support a role for this enzyme in immunity.

The GPI-anchored serine proteases are represented by prostasin and testisin (**Figure 1.5**). Prostasin, also known as channel-activating protease 1 (CAP-1), is widely expressed in epithelial tissues such as prostate, colon, lung, kidney, pancreas, salivary gland, liver, and bronchial tubes

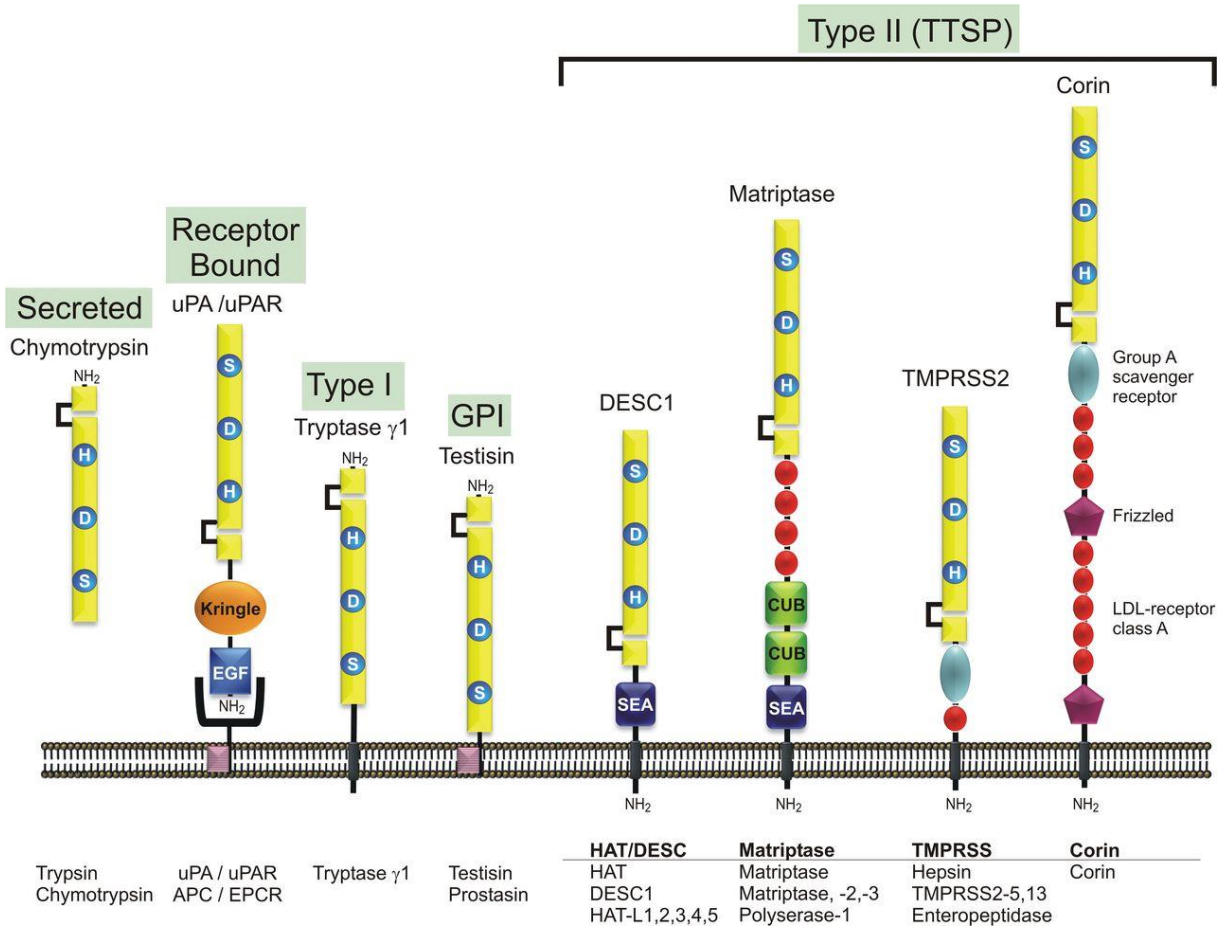

Figure 1.5: Summary of the serine protease diversity of structure
The structure and mode of membrane attachment among serine protease are summarized above, along with the four subgroups of type II transmembrane serine proteases (TTSP). The membrane-anchored serine proteases exist as one of three types; type I, type II, and glycophosphatidylinositol (GPI)-anchored. Both type I and II are single-pass transmembrane proteases, differing in the orientation of their carboxyl- and amino-terminal ends. The GPI-anchored proteases are tethered to the cellular membrane through a GPI-anchor. Secreted and receptor-bound examples of proteases are also shown, with representative types displayed. Figure used with permission from Antalis TM, Buzza MS, Hodge KM, Hooper JD and Netzel-Arnett S (2010) The cutting edge: membrane-anchored serine protease activities in the pericellular microenvironment. The Biochemical journal 428:325-346. http://www.biochemj.org/content/428/3/325.article-info

(Yu et al., 1994; List et al., 2007b). Prostasin plays important roles in prenatal development, as evidenced by the embryonic lethality of prostasin knockout mice caused by impaired placental development and function (Hummler et al., 2013). Prostasin also plays important roles postnatally in the development and maintenance of epidermal and intestinal barrier function (Leyvraz et al., 2005; Netzel-Arnett et al., 2006; Buzza et al., 2013; Hummler et al., 2013; Friis et al., 2016;

Keppner et al., 2016). Loss of prostasin expression is a common trend in cancer and has been observed in prostate, breast, bladder, gastric, and colorectal cancers (Chen and Chai, 2002; Takahashi et al., 2003; Sakashita et al., 2008; Chen et al., 2009; Bao et al., 2016). Overexpression of prostasin in human CRC cell xenografts grown in nude mice resulted in significantly attenuated tumor growth, suggesting a tumor suppressive function for prostasin (Bao et al., 2016).

Expression of testisin is much more restricted in comparison to prostasin, with expression under normal physiological conditions restricted to the testes. Testisin has been demonstrated to be required for spermatozoa maturation, with testisin ablation resulting in immobile spermatozoa (Netzel-Arnett et al., 2009). Loss of testisin expression as a result of hypermethylation of the 5' CpG island in the gene encoding testisin and has been shown to promote testicular cancer (Hooper et al., 1999; Manton et al., 2005; Kempkensteffen et al., 2006). In contrast to what is observed in the testes, testisin is strongly expressed in human invasive epithelial ovarian cancers and cervical cancers, while being undetectable in normal ovarian or cervical tissue (Shigemasa et al., 2000; Manton et al., 2005; Netzel-Arnett et al., 2009). Because of the restricted expression of testisin to testicular tissue under normal physiological conditions, expression is viewed as *de novo*/ectopic in ovarian and cervical cancer, making a selective inhibitor of testisin promising in terms of limiting side effects in female patients. Targeting of testisin has been accomplished using an anthrax toxin engineered to be cleaved by testisin. Testisin activation of anthrax protective antigen bound to anthrax lethal factor then induced cytotoxicity where the protease is overexpressed. This modified toxin has been shown to effectively induce cell death in a cervical cancer cell xenograft mouse model of testisin-expressing cancer (Martin et al., 2015).

1.6.1 Type II transmembrane proteases

The type II transmembrane serine proteases (TTSPs) make up the largest family of membrane-anchored serine proteases. The TTSPs are distinguished from type I membrane proteases by an extracellular carboxyl-terminal end containing the catalytic domain, and intracellular amino-terminal domain. They are single-pass transmembrane proteins and are synthesized as single-chain zymogens requiring proteolytic cleavage for activation. This group is further divided into four subfamilies: HAT/DESC, corin, matriptasae, and hepsin/TMPRSS subfamilies (**Figure 1.6**). TTSPs play several key roles in tissue development, epithelial homeostasis, and dysregulated expression of several TTSP examples has been demonstrated to have disease-promoting consequences, however several family members remain to be studied (List et al., 2006a; Szabo and Bugge, 2008; Choi et al., 2009; List, 2009; Antalis et al., 2011; Szabo and Bugge, 2011; Webb et al., 2011).

The human airway trypsin-like (HAT)/differentially expressed in squamous cell carcinoma (DESC) subfamily of TTSPs is composed of eight proteases, of which only four have been characterized (Szabo and Bugge, 2008). This subfamily is characterized among TTSPs by their simple structure of the extracellular stem domain, which contains a single sea urchin sperm protein, enteropeptidase, agrin (SEA) domain. As the name suggests, this class of proteases play important roles in the human airways, with expression of HAT and HATL5 largely localized to the human trachea and bronchial epithelia (Yasuoka et al., 1997; Yamaoka et al., 1998; Takahashi et al., 2001). Outside of human airways, expression of HAT, HAT-like 4 (HATL4), and HATL5 has been identified in the epithelia of skin, tongue, esophagus and cervix, and expression of HATL3 discovered in the spinal cord and brain (Stallmach and Gloor, 2008; Sales et al., 2011; Miller et al., 2014; Duhaime et al., 2016; Zhang et al., 2017). Characterization of DESC-4 has identified

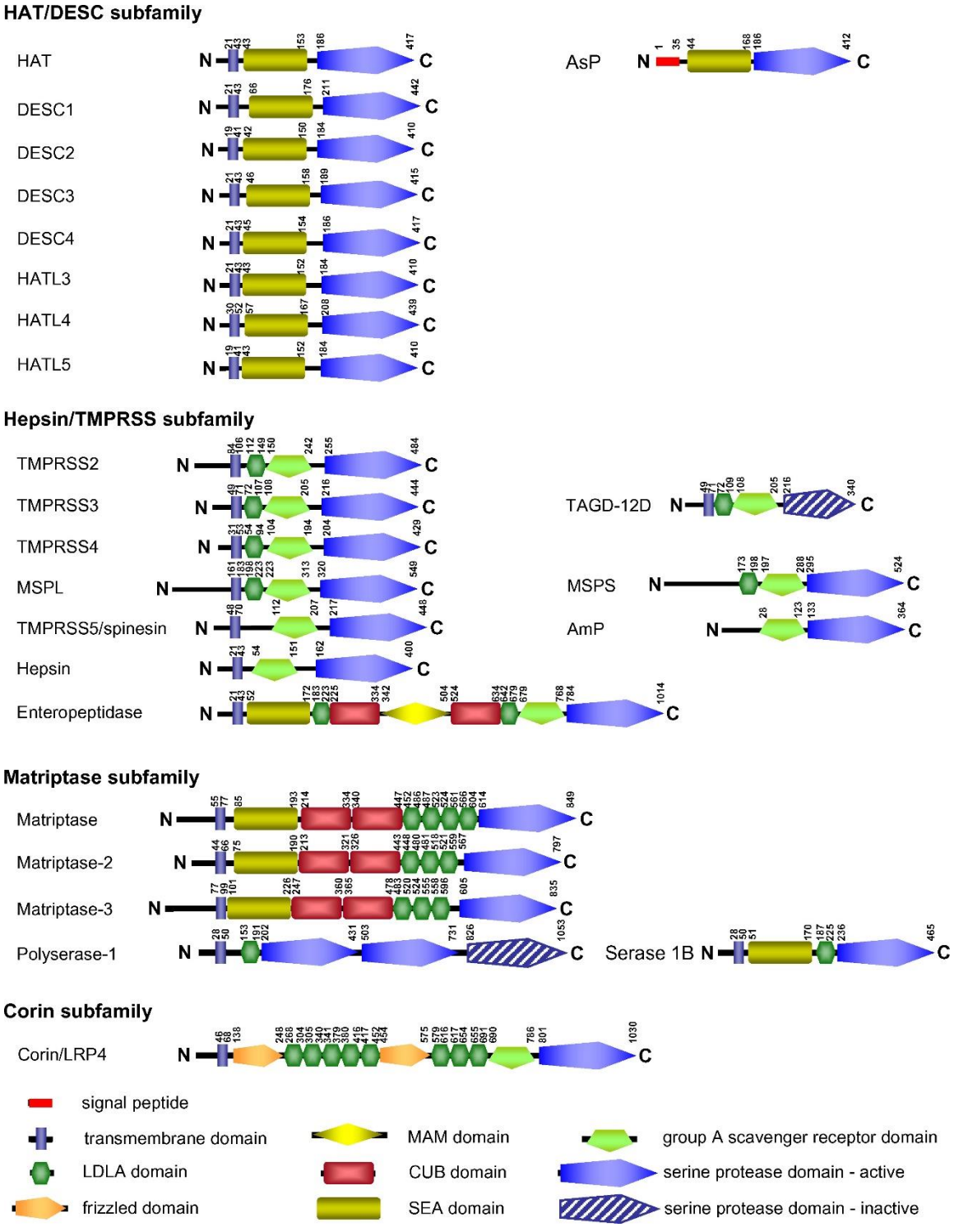

Figure 1.6: Predicted domain structures of the known human TTSPs
Numbers indicate the position of each domain in the amino acid sequence of native, pro-enzyme molecules. Figure reprinted with permissions from Szabo R and Bugge TH (2008) Type II transmembrane serine proteases in development and disease. International Journal of Biochemistry and Cell Biology 40:1297-1316.

expression in the brain, heart, liver, colon, lung, and tongue (Behrens et al., 2004).Much remains to be discovered regarding the functional properties of this TTSP subfamily. HAT was first discovered in the sputum of patients with chronic airway diseases, and has since been demonstrated to promote mucus production and hypersecretion, which has made it a potential target for respiratory disease (Yasuoka et al., 1997; Chokki et al., 2004; Liu et al., 2013). Perhaps as a form of parallel evolution, the activity of these proteases contributes to viral infection through the cleavage of viral proteins, as observed with HAT cleavage of influenza A and B hemagglutinin (Bottcher-Friebertshauser et al., 2012; Baron et al., 2013). HATL4, another HAT/DESC member, has been identified as important for non-airway functions. Knockout HATL4 mouse pups exhibited increased transdermal water loss compared to wild-type mice, despite the lack of a morphological skin defect (Zhang et al., 2017). With skin remaining visibly intact, it is remains unclear the how HATL4 affects barrier function. Expression profiles of HAT/DESC proteases have also been found to be altered in cancer. Study of HAT and HATL5 expression in cervical and esophageal squamous cell carcinomas reveal loss of protease expression with cancer (Miller et al., 2014; Duhaime et al., 2016). Differentially expressed in squamous cell carcinoma (DESC)-1 is expressed in skin, prostate, testes, and oral epithelia (Lang and Schuller, 2001; Hobson et al., 2004). It remains unclear whether loss of TTSP expression in these cancers plays a role in tumor progression.

Corin makes up the sole member of the corin subfamily. Among TTSPs, corin possesses a unique structure with a stem region containing two frizzled, eight low density lipoprotein receptor class A (LDLa), and one group A scavenger receptor (SCR) domain. Expression of this protease is highest in heart tissue, but is also found in kidney, skin, and pregnant uterus (Enshell-Seijffers et al., 2008; Polzin et al., 2010; Ichiki et al., 2011; Fang et al., 2013). This protease was found to

be an activator of pro-atrial natriuretic peptide (pro-ANP) to ANP, which is required for management of blood pressure (Wu et al., 2009). Deficiencies in corin expression have been associated with hypertension as well as chronic kidney disease, highlighting the importance of this protease as a convertor of natriuretic peptides (Chan et al., 2005; Fang et al., 2013).

The matriptase family is the most extensively studied subfamily of TTSPs, made up of four members; matriptase, matriptase-2, matriptase-3, and polyserase-1. The matriptase proteases share a high degree of homology, containing a single SEA, two Cls/Clr, urchin embryonic growth factor and bone morphogenic protein-1 (CUB) domains in the stem region between transmembrane/intracellular domains and the serine protease domain. Matriptase-2 and matriptase-3 are distinguished by three LDLa domains, whereas matriptase contains four. Polyserase-1 stands out with three catalytic serine protease domains, one of which is inactive (Cal et al., 2003). Matriptase, along with hepsin, is the most studied TTSP and is widely expressed in epithelial tissues (Oberst et al., 2003; List et al., 2006b; Affara et al., 2009). Mouse model studies have uncovered roles for matriptase in epidermal and gastrointestinal differentiation and barrier function, hair follicle development, and thymic maturation (List et al., 2002; List et al., 2003; List et al., 2006b; Netzel-Arnett et al., 2006; Buzza et al., 2010). Mice with targeted deletion of the gene encoding matriptase die 1-2 days after birth due to dehydration due to severely compromised epidermal barrier function (List et al., 2002; List et al., 2009). In addition to the important physiological roles, matriptase has also been identified as a driver of cancer. Deregulation of matriptase has been observed in several forms of cancer, including breast and colon (Vogel et al., 2006; List, 2009; Zoratti et al., 2015; Zoratti et al., 2016). Matriptase in breast cancer has been demonstrated to be functionally important for driving tumor proliferation and invasiveness through conversion of pro-hepatocyte growth factor (pro-HGF) to HGF, resulting in activation of the Met

signaling pathway (Bergum et al., 2012; Zoratti et al., 2015; Zoratti et al., 2016). Thus, mice with reduced levels of matriptase display a significant delay in oncogene-induced mammary tumor formation and blunted tumor growth. Importantly, inhibition of matriptase catalytic activity using a selective small-molecule inhibitor abrogates the activation of Met and its downstream targets in cultured human breast cancer cells and primary murine mammary carcinoma cells in response to pro-HGF (Zoratti et al., 2015; Zoratti et al., 2016). Studies designed to determine the efficacy of matriptase inhibitors in breast cancer treatment *in vivo* are currently ongoing. The strong link between matriptase and mammary tumor growth provides promising new avenues to target proteolysis for therapeutic purposes.

The fourth TTSP subfamily is the Hepsin/TMPRSS group, which encompasses hepsin, enteropeptidase, and TMPRSS2, 3, 4, and 13. Members of this subfamily all contain a single SCR domain within the extracellular stem region. Enteropeptidase, differs most from the other Hepsin/TMPRSS proteases with one SEA domain, one meprin, A5 antigen, and receptor protein phosphatase μ (MAM) domain, two CUB domains, and two LDLa domains in addition to the single SCR domain. This protease was one of the first discovered TTSPs, discovered in the digestive system of Ivan Pavlov's dogs (Zheng et al., 2009). Enteropeptidase is chiefly expressed in the duodenum where it functions to convert trypsinogen to trypsin, a serine protease secreted from pancreatic glands. This active trypsin acts with the other two principal digestive proteinases—pepsin and chymotrypsin—to break down dietary protein into peptides and amino acids.

Along with matriptase, the namesake protease of this subfamily, hepsin, is one of the most well-studied TTSPs. Originally found to be expressed in hepatocytes, hepsin has been found to also be highly expressed in kidney, and ubiquitously expressed in most other major tissues at low

levels (Leytus et al., 1988; Tsuji et al., 1991; Su et al., 2004). A number of physiological functions have been proposed for hepsin. A role for hepsin in receptor tyrosine kinase signaling is also proposed based on the ability of hepsin to efficiently activate HGF and macrophage-stimulating protein (MSP) (Herter et al., 2005; Ganesan et al., 2011). Hepsin is also believed to be a part of proteolytic activation cascades, having been shown to activate uPA (Chen et al., 2010; Smith and Marshall, 2010; Tervonen et al., 2016). Activation of pro-HGF by hepsin may be restricted to particular tissues such as the liver, as hepsin knockout mice display increased levels of hepatic pro-HGF and decreased Met activation (Hsu et al., 2012). Interestingly, hepsin knockout mice were found to be viable and fertile, indicating that hepsin is not essential for embryonic development and postnatal survival (Wu et al., 1998; Yu et al., 2000). However, it was later discovered that hepsin null mice exhibit hearing loss as a result of an aberrant tectorial membrane in the cochlea, indicating hepsin is required for proper cochlear develop, albeit through a yet unknown mechanism (Guipponi et al., 2007).

Hepsin has been extensively studied in cancer following the discovery of upregulated hepsin in prostate cancer (Luo et al., 2001; Magee et al., 2001; Stamey et al., 2001; Chen et al., 2003; Stephan et al., 2004). Upregulation of hepsin is also observed in gastric, ovarian, renal and breast cancer (Tanimoto et al., 1997; Klezovitch et al., 2004; Betsunoh et al., 2007; Miao et al., 2008; Xing et al., 2011; Zhang et al., 2016). Conversion of pro-HGF and pro-MSP to active growth factors by hepsin has been proposed to contribute to its pro-oncogenic properties (Herter et al., 2005; Kirchhofer et al., 2005; Ganesan et al., 2011). For example, overexpression of hepsin in mammary organoids resulted in downregulation of the hepsin inhibitor hepatocyte growth factor activator inhibitor (HAI)-1 and promotion of HGF signaling (Tervonen et al., 2016). Hepsin has also been demonstrated to promote cellular motility, and degradation of basement membrane in

prostate cancer is also attributed to hepsin activity, further implicating this protease as a key player in cancer invasion and metastasis (Xuan et al., 2006; Tripathi et al., 2008; Xing et al., 2011). This role was further cemented *in vivo* through induction of transgenic hepsin expression in the probasin-Large T antigen mouse model of prostate cancer, which led to development of invasive tumors with metastases to liver, lung, and bone (Klezovitch et al., 2004). Use of monoclonal antibodies to target hepsin was demonstrated to have anti-invasive properties, preventing degradation of extracellular matrix proteins and activation of the pro-forms of HGF, MSP, and uPA, highlighting the anti-cancer potential of hepsin targeting (Xuan et al., 2006; Ganesan et al., 2011; Koschubs et al., 2012).

The remaining TMPRSS proteases (TMPRSS2-4, 13) remain relatively uncharacterized. TMPRSS2 has been linked to prostate cancer due to the discovery of TMPRSS2-ETC-related gene (ERG) fusion protein (Clark and Cooper, 2009; Shah and Small, 2010). The catalytic function of TMPRSS2 is not linked to the precancerous properties of this fusion protein, however it highlights the unique regulation of TMPRSS2 expression. Thus, like many genes expressed in reproductive organs, TMPRSS2 is under the control of androgenic signaling, which is not known to be the case for other TTSPs (Clark and Cooper, 2009). TMPRSS3 mutations that abrogate catalytic activity have been linked to various forms of deafness in human populations, with TMPRSS3-mediated activation of epithelial sodium channel (ENac) implicated in this phenotype (Guipponi and Vuagniaux, 2002). The properties and physiological roles of TMPRSS13 are discussed in **Section 1.8**.

1.6.2 Regulation of TTSP proteolytic activity by endogenous inhibitors

Proper function of TTSP activity on the cellular surface requires tight regulation of enzymatic activity to prevent the negative consequences of excess proteolysis. Endogenous

regulation of TTSP catalysis occurs largely though two kunitz-type inhibitors: hepatocyte growth factor activator inhibitor-1 and -2 (HAI-1 and -2). Both HAI-1 and HAI-2 are transmembrane proteins possessing two extracellular kunitz domains, with expression profiles largely in epithelial tissues, mirroring several TTSPs including matriptase (Szabo et al., 2008). First discovered in conditioned media of the gastric cancer cell line MKN45, HAI-1 was initially believed to be a secreted inhibitor (Shimomura et al., 1997). HAI-1 was found to potently inhibit the activity of the secreted serine protease hepatocyte growth factor activator (HGFA). Further study revealed that HAI-1 exists as a type I transmembrane protein, which can be shed from the cell surface after forming a complex HGFA (Kataoka et al., 2000). HAI-2 was similarly discovered in MKN45 conditioned media, however also found to be membrane bound (Kawaguchi et al., 1997). Both HAI-1 and HAI-2 regulate the activity of many proteases, including membrane-anchored proteases such as matriptase and hepsin, and are expressed in many epithelial tissues, indicating an important role for proteolytic regulation at the cellular surface (Benaud et al., 2001; Oberst et al., 2001; Kirchhofer et al., 2005; Kojima et al., 2008; Szabo et al., 2008). Despite the apparent overlap in expression of HAI-1 and -2, genetic ablation of either inhibitor alone leads to embryonic lethality, suggesting highly defined roles for each inhibitor which cannot be compensated for by the other (Tanaka et al., 2005; Szabo et al., 2009). Through the use of spatially restricted and lox-Cre inducible loss of expression, both HAI-1 and HAI-2 have been found to be crucial to development, with neural tube defects and aberrant placental development resulting from inhibitor loss (Tanaka et al., 2005; Szabo et al., 2007; Szabo et al., 2009). The defects resulting from HAI-1 and HAI-2 ablation can be rescued by co-deletion of matriptase, implicating matriptase as a major target for these inhibitors and as the key protease responsible for the embryonic defects, however matriptase is part of proteolytic cascades involving prostasin as well as uPA, kallikreins and MMPs,

indicating these proteases may act as master regulators (Jin et al., 2006; Uhland, 2006; Szabo et al., 2007; Szabo et al., 2009; Sales et al., 2010).

1.7 Role of TTSPs and their inhibitors in intestinal development and function

Proteolysis in epithelial tissues is a highy regulated process with significant consequences when the balance is tipped. This can occur either through changes in protease or cognate inhibitor expression. In this section the physiological importance of TTSPs and their inhibitors in intestinal epithelium is discussed.

1.7.1 Matriptase in the gastrointestinal tract

(The following adapted section was originally published in Varela et al. 2018 Physiological functions and role of matriptase in cancer. *In* Extracellular Targeting of Cell Signaling in Cancer. J.W. Janetka and R.M. Benson, eds. Pp. 91-124. Hoboken, NJ: John Wiley & Sons.)

Inducible matriptase ablation or expression techniques have been extremely useful in identifying the various roles matriptase plays beyond epidermis, allowing circumvention of the lethality of global matriptase deletion in mice. Matriptase ablation restricted to intestinal epithelia in mice via lox-Cre recombination results in severe defects in colon function, with animals surviving a maximum of two months postnatally due to severe diarrhea (Kosa et al., 2012). Matriptase is crucial for proper development of the colon mucosal layer, as these mice exhibited hyperproliferation of their epithelia, chronic inflammation, and lack of mucin production. Interestingly, small intestine function and morphology/histology remained unaffected, suggesting that matriptase is critical mainly for large intestine development and function (Kosa et al., 2012). Matriptase not only has a key function in colon development, but also in the maintenance of homeostasis. Inducible ablation of matriptase in adult mice leads to a severe decline in health, with significant weight loss leading to moribund states in as few as 9 days (List et al., 2009). This severe

weight loss is a consequence of losing intestinal epithelial barrier function (List et al., 2009) (**Figure 1.7**). This was further demonstrated by increased diffusion of a tracer from the colorectal lumen, through the epithelial and into the bloodstream (List et al., 2009). This functional defect was caused by loss of tight junction (TJ) proteins in intestinal epithelial cells. Additionally, matriptase loss in the intestines results in hyperproliferation of epithelial cells, a consequence of inflammation resulting from increased intestinal permeability (List et al., 2009). Matriptase hypomorphic mice with decreased global expression of matriptase also exhibit impairments in gut impermeability, with crypt epithelia expressing selectively high levels of one particular claudin, claudin-2, a TJ protein that in contrast to other claudins promotes epithelial permeability (Buzza et al., 2010) (**Figure 1.7**). An increase in intestinal permeability has serious implications for inflammation, permitting greater exposure to foreign antigens and bacterial infiltration. Patients with inflammatory bowel disease (IBD) such as Crohn's disease (CD) and ulcerative colitis (UC) suffer from chronic diarrhea, and persistent intestinal inflammation, and display leaky gut barrier. Quantitative PCR analysis of matriptase transcripts in CD and UC patient intestines showed downregulation of matriptase in the colon compared to normal colon, but not in the small intestine (Netzel-Arnett et al., 2012). While this does not answer the question whether low levels of matriptase contribute to IBD or *vice versa*, a laboratory model of colitis, induced by administration of dextran sulfate sodium (DSS), produces severe inflammation in matriptase hypomorphic mice, compared to controls, indicating a protective role for matriptase in gut inflammation (Netzel-Arnett et al., 2012). The roles matriptase plays in epithelial function are numerous, making complete understanding of enzymatic activity crucial to understanding the impact of modulation of matriptase levels or activity as a therapeutic option.

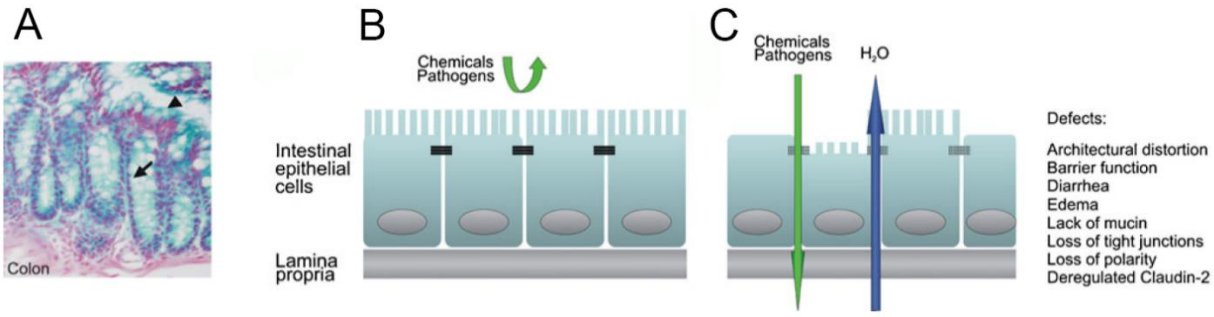

Figure 1.7: Impact of matriptase loss in intestinal barrier function
(A) In the intestine, matriptase is expressed in goblet cells (arrow) and in surface mucosal cells (arrowhead). (B) Continual matriptase expression is required for tissue maintenance and results in defects in tight junctions (black horizontal bars) and cell polarity. (C) Ablation of matriptase in the large intestine causes architectural distortion, loss of tight junctions, and compromised barrier function resulting in edema and diarrhea and causing premature death. Figure modified with permission from Miller GS and List K (2013) The matriptase-prostasin proteolytic cascade in epithelial development and pathology. Cell and Tissue Research 351:245-253.

1.7.2 The role of HAI-2 in intestinal homeostasis

HAI-2 has, in addition to its role in proteolytic regulation during embryonic development described above (**section 1.6.2**), recently emerged as an important regulator of proteolysis in postnatal epithelial tissues. One of the first clues for this role was identifying a mutation in *SPINT2*, the gene encoding HAI-2, underlying a form of congenital tufting enteropathy (CTE) (Slae et al., 2013). This disease is characterized by severe diarrhea and loss of sodium through the bowels, with disorganized epithelial organization in the small intestine with epithelial structures resembling tufts throughout the mucosa. One potential mechanism has been identified using cell culture models implicating aberrant tight junction formation as a result of dysregulated matriptase activity (Kawaguchi et al., 2019). CTE-associated *SPINT2* mutations were found to render the HAI-2 protein products inefficient inhibitors of matriptase, with the resulting increase in matriptase activity contributing to impaired tight junctions (Wu et al., 2017). Studies using mice expressing mutant forms of *SPINT2* result in intestinal defects similar to CTE, with similar changes to tight junctions as seen in tissue culture studies (Szabo and Bugge, 2018). Interestingly, this defect does not develop in mice also expressing a zymogen-locked form of prostasin (Szabo and

Bugge, 2018). Prostasin and matriptase are linked through a reciprocal zymogen activation loop, in which matriptase promotes prostasin zymogen activation and prostasin, in a catalytically-independent manner, promotes matriptase zymogen activation (Friis et al., 2013). Conceivably, loss of HAI-2 promotes prostasin activation through decreased regulation of matriptase, however several serine proteases are efficiently inhibited by HAI-2, including TMPRSS13, TMPRSS2, and hepsin (Hashimoto et al., 2010; Faller et al., 2014; Murray et al., 2017). Thus, a comprehensive understanding of which proteases are critically contributing to CTE awaits further studies.

1.8 TMPRSS13

This dissertation focuses on the function of TMRPSS13 in CRC. To date a relatively uncharacterized protease, this section reviews what is known about the properties of TMPRSS13 in human tissue function and disease.

1.8.1 TMPRSS13 discovery and biochemistry

TMPRSS13, also known as mosaic serine protease large-form (MSPL) remains one of the least characterized TTSPs to date. TMPRSS13 was first identified to be expressed in several epithelial tissues, such as lung, placenta, pancreas, prostate (Kim et al., 2001; Kido and Okumura, 2008). Two human forms of TMPRSS13 were initially sequenced, with four identified isoforms currently identified (Hashimoto et al., 2010; Faller et al., 2014). Physiologically relevant substrates have yet to be identified but TMPRSS13 has been shown to be an efficient activator of epithelial sodium channel (ENaC) in a cellular model and cell-free experiments have demonstrated cleavage of pro-HGF to HGF (Hashimoto et al., 2010; Faller et al., 2014). Like other proteases, TMPRSS13 is synthesized as a zymogen and has been shown to be capable of autoactivation (Murray et al., 2017). Catalytic activity of TMPRSS13 has been shown to be efficiently inhibited by HAI-2 and HAI-1 (Hashimoto et al., 2010; Faller et al., 2014). Inhibition of activity appears to be required for

proper trafficking of TMPRSS13 to the cell surface (Murray et al., 2017). Interestingly, this protease contains the longest intracellular domain of the TTSP group which is subject to phosphorylation (Murray et al., 2017) Furthermore, TMPRSS13 is glycosylated in its extracellular domains (Murray et al., 2017). It remains unclear how these posttranslational modifications affect the physiological function of TMPRSS13.

1.8.2 TMPRSS13 roles in physiology/pathology

Much remains to be uncovered regarding the physiological roles of TMPRSS13. Initial examination of the physiological and pathological significance of TMPRSS13 identified a role for proteolytic activity in viral infection. TMPRSS13 in the bronchial tubes was found to efficient cleave viral hemagglutinin, thereby promoting viral infection (Kido et al., 2008; Okumura et al., 2010). While no inhibitors have been tested yet, this does suggest targeting of proteolytic activity of TMPRSS13, and other hemagglutinin-competent proteases may have anti-viral benefit.

Information about the physiological role of TMPRSS13 comes from characterization of a TMPRSS13-decicient mouse model. The TMPRSS13-deficient mouse was generated through homologous recombination using a promoterless LacZ-Neo fusion gene, resulting in deletion of exon 10 and partial deletion of exon 9 (Madsen et al., 2014). Characterization of mice homozygous for the targeted allele demonstrated impaired development of the stratum corneum, and increased epidermal fluid loss in neonates compared to wild-type mice, uncovering a role for TMPRSS13 in epidermal differentiation and barrier function (Madsen et al., 2014). Observations from our laboratory show flaky skin and delayed pelage hair growth in young homozygous mice, however the mice become indistinguishable when they reached weaning age (day 21-22) **(Figure 1.8)**. A similar defect in the skin of matriptase hypomorphic mice has also been observed, hinting towards

a shared pathway or proteolytic cascade between TMPRSS13 and matriptase (List et al., 2002; List et al., 2007a).

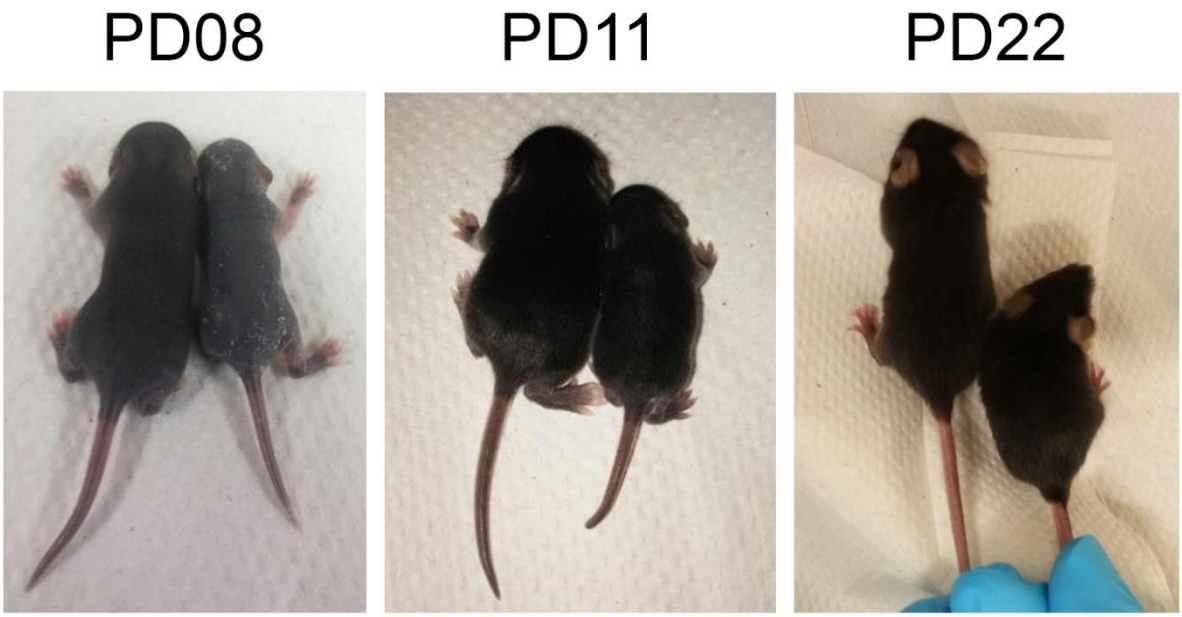

Figure 1.8: Flaky skin and delayed hair growth in TMPRSS13-deficient mice
Mice were photographed on postnatal day (PD) 8, 11, and 22 days postnatally to track duration of a flaky skin and sparse hair phenotype observed in TMPRSS13-deficient mice (rightmost mouse in each panel). Interestingly, while wild-type (left) and TMPRSS13-deficient mice were easily distinguished at PD08 days of age, by PD22 mice were visually indistinguishable, indicating a transient impact of TMPRSS13 loss for skin and hair development.

1.9 Dissertation outline, hypothesis and aims.

Studies investigating the basic biochemical properties of TMPRSS13 and its role in epidermal function are nascent, and to date no studies been published on the functional role this protease may play in malignancy. The entry point of this dissertation work was the discovery of TMPRSS13 transcript upregulation in CRC compared to normal colon (**Chapter 2**). In trying to understand the pathophysiological role of TMPRSS13, we formulated three aims to study TMPRSS13 protein expression in CRC and to determine the functional role of this protease using both CRC cell culture models and a genetic mouse loss-of-function model. *My central hypothesis is that TMPRSS13 expression is increased as normal cells undergo neoplastic transformation and is a critical player in pro-oncogenic processes in CRC.*

Aim 1: Determine the TMPRSS13 protein expression levels and localization in normal colon versus colorectal cancer.

We performed a comprehensive characterization of TMPRSS13 protein expression in human tissue arrays by immunohistochemistry (described in **Chapter 2**).

Aim 2: Determine the consequences of TMPRSS13 loss-of-function and gain-of-function for key pro-oncogenic cellular processes in human CRC cells

RNAi mediated knock-down and transient plasmid-mediated overexpression of TMPRSS13 in multiple human colon cancer cell lines were used to study the role of this protease in proliferation, cell survival, apoptosis, and invasion (described in **Chapter 2**).

Aim 3: Determine the contribution of TMPRSS13 to normal intestinal barrier function and to colon cancer progression in vivo

The role of TMPRSS13 in gastrointestinal barrier function was assessed using a TMPRSS13-deficient mouse model. We utilized a chemical colon carcinogenesis model to compare tumor multiplicity and growth in TMPRSS13-deficient mice compared to TMPRSS13-sufficient mice (described in **Chapter 3**).

CHAPTER 2: TMPRSS13 PROMOTES CELL SURVIVAL, INVASION, AND RESISTANCE TO DRUG-INDUCED APOPTOSIS IN COLORECTAL CANCER

2.1 Introduction

According to the American Cancer Society, colorectal cancer (CRC) is the third most common cancer in men and in women and the second leading cause of cancer-related deaths in men and women in the United States (ACS, 2018). Early diagnosis is critical to successful treatment for this disease when surgical intervention is a viable option. The prognosis for advanced and metastatic CRC is poor due to limited medical treatment options. Fluorouracil (5-FU) and its pro-drug form capecitabine are currently the most routinely used agents, alone or in combination with drugs such as oxaliplatin and irinotecan (1999; Mamounas et al., 1999; Gill et al., 2004). Although targeted therapies have been successful in the treatment of some types of cancers including breast cancer, they have limited efficacy in adjuvant treatment of colorectal cancer (i.e., cetuximab, panitumumab, bevacizumab, ramucirumab, ziv-aflibercept, and regorafenib) and add relatively small survival benefits for those with advanced disease (De Roock et al., 2008; Brenner et al., 2014; Mody et al., 2018). Therefore, there is an urgent need to develop novel drug regimens for patients suffering from advanced CRC. To this end, understanding the molecular mechanisms driving CRC represents a critical step toward the development of novel targeted therapeutics for this particularly deadly type of cancer.

Proteolysis is a tightly regulated process under normal physiological conditions and proteolytic dysregulation constitutes a hallmark and contributing factor to cancer (Johnsen et al., 1998; Affara et al., 2009; Mason and Joyce, 2011; Bonnans et al., 2014; Sevenich and Joyce, 2014). The type II transmembrane serine protease (TTSP) subfamily is a relatively new classification of membrane-anchored serine proteases, many of which play key roles in processes

exploited in cancer, such as tissue remodeling, cellular migration and invasion, colonization, and metastasis (Hooper et al., 2001; Netzel-Arnett et al., 2003; Szabo and Bugge, 2008; Bugge et al., 2009; Antalis et al., 2011; Sevenich and Joyce, 2014). Thus, studies of TTSPs in cancer using cell culture and animal models have identified pro-oncogenic properties of several of these proteases (Szabo and Bugge, 2011; Webb et al., 2011; Murray et al., 2016; Tanabe and List, 2017). Work from our laboratory recently demonstrated that the TTSP, matriptase promotes tumor growth and invasion *in vitro* and *in vivo* through activation of the hepatocyte growth factor (HGF)/c-Met signaling cascade in breast cancer (Zoratti et al., 2015; Zoratti et al., 2016).

TMPRSS13 (transmembrane protease, serine 13; also known as mosaic serine protease large-form, MSPL) is expressed in several epithelial tissues including epithelia of the oral cavity, esophagus, bladder, stomach, and skin (Kido and Okumura, 2008; Madsen et al., 2014). TMPRSS13-deficient mice display abnormal skin development in newborn mice, leading to a mildly compromised barrier function, as measured by the transepidermal fluid loss rate of neonates (Madsen et al., 2014). Long-term studies (up to 12 months of age) showed no detectable histological tissue abnormalities in the skin or any other tissues and no differences in longevity, reproduction, and rearing of pups were observed (Varela, Murray, List, unpublished studies). TMPRSS13 is also expressed in mouse and human respiratory epithelium and several studies have demonstrated a role for TMPRSS13 in influenza infection due to its ability to proteolytically modify the viral protein hemagglutinin, thereby mediating fusion between viral and endosomal membranes during virus entry into the cell (Kido et al., 2008; Choi et al., 2009; Okumura et al., 2010; Zmora et al., 2014).

In this study, we identify TMPRSS13 as a differentially expressed TTSP in human CRC. Mechanistically, we demonstrate a role for TMPRSS13 in CRC cell survival, invasiveness and resistance to apoptosis-inducing agents.

2.2 Materials and Methods

In silico Analysis

The Oncomine™ online platform (http://www.oncomine.org) was used to perform a meta-analysis of TMPRSS13 across multiple transcriptome-wide studies of colorectal cancer samples in comparison to normal colon. Relevant datasets were identified utilizing the differential analysis; cancer vs. normal analysis; and TMPRSS13 gene filters.

Tissue samples, immunohistochemistry, and evaluation of staining

Colorectal (CO1921) tissue arrays containing both cancer and normal samples were obtained from US Biomax, Inc (Rockville, MD). Paraffin-embedded arrays were cleared in xylene (Fisher Scientific) and rehydrated in a graded series of ethanol solutions. For antigen retrieval, tissue arrays were boiled in reduced pH citrate antigen retrieval buffer for 10 minutes. Endogenous peroxidase activity was quenched by incubating tissue arrays in 3% H_2O_2 for 15 minutes. Arrays were blocked with 2.5% bovine serum albumin (Sigma) in PBS for 1 hr at room temperature and incubated overnight in anti-TMPRSS13 antibody (PA5-30935, Thermo Fisher Scientific) at 4°C in a humidified chamber. All washing steps were performed with PBS. Non-immune rabbit IgG (Neomarkers, Fremont, CA) was used as a negative control. Visualization of bound primary antibody was performed using a biotinylated anti-rabbit secondary antibody and conjugated horseradish peroxidase H contained in the VECTASTAIN ABC kit (Vector Laboratories). Enzymatic reactions were carried out with 3,3-diaminobenzidine substrate (Sigma-Aldrich) and arrays were subsequently counterstained with hematoxylin. Stained slides were washed and

dehydrated in a series of graded ethanol solutions followed by xylene and mounted with glass coverslips using Permount (Fisher Scientific). Microscopic images were acquired on a Zeiss Axio Scope A.1 using digital imaging.

To evaluate staining intensity in colon tissue arrays, samples were manually assessed microscopically. Epithelial staining of TMPRSS13 was rated on the basis of intensity in 20x microscopic fields on a scale of 0 to 3, where: 0 = no epithelial staining; 1 = majority weakly stained epithelial cells OR few moderately stained epithelial cells among a majority of non-stained cells; 2 = majority moderately stained epithelial cells OR few strongly stained among a majority of weakly or non-stained cells; 3 = majority strongly stained epithelial cells.

Cell lines and culture conditions

HCT116 (ATCC, Manassas, VA) cells were cultured with minimal essential media (MEM) supplemented with 10% fetal bovine serum (FBS)(Atlanta Biologicals, Flowery Branch, GA), 10 units/ml Penicillin and 10 µg/ml Streptomycin (Gibco), and 1x non-essential amino acids. DLD-1 cells (ATCC, Manassas, VA) were cultured with RPMI-1640 media supplemented with 10% FBS and 10 units/ml Penicillin and 10 µg/ml Streptomycin (Gibco). Cells were maintained in a humidified incubator at 37°C with an atmosphere of 5% CO_2.

RNAi-mediated gene silencing

For TMPRSS13 knockdown in DLD-1 and HCT116 colon cancer cells, two independent Stealth RNAi siRNA duplexes (Invitrogen/ThermoFisher Scientific) targeting TMPRSS13 (siRNA 1 = HSS130531, siRNA 2 = HSS130532) were used. A matched %GC negative scramble control (12935300) was included in all experiments. Reverse transfections, in which siRNA-lipid complexes were added to wells prior to seeding, were performed using Lipofectamine RNAiMAX (Invitrogen/ThermoFisher Scientific) following manufacturer instructions. Cells were transfected

in 6-well plates for all experiments except for cell-counting experiments and flow cytometric analyses, in which 12-well plates were used and reagent levels adjusted according to transfection reagent manufacturer guidelines. Media was replaced every 48 hrs during siRNA treatment for all experiments. Cellular lysates were collected three-, five-, and seven-days post-transfection for proliferation experiments, four-days post-transfection for drug treatment experiments, and four- and five-days post-transfection for flow cytometry experiments.

Cell counting

DLD-1 and HCT116 cells were reverse transfected for siRNAs targeting of TMPRSS13 and seeded onto 12-well plates at 50,000 cell/well for DLD-1 and 250,000 cells/well for HCT116. Live cells were trypsinized three-, five-, and seven-days post-transfection, pelleted by centrifugation at 200 x g for 5 min, and resuspended in sufficient media to approximate an appropriate cell/mL concentration for counting. Samples were mixed 1:1 with 0.4% trypan blue stain (Gibco/ThermoFisher Scientific) to distinguish viable and dead cells. Counting was performed using a hemocytometer.

Western blot analysis

Cultured DLD-1 or HCT116 cells were washed three times with ice-cold PBS and lysed in well using ice cold RIPA buffer—150 mM NaCl; 50 mM Tris/HCl, hP 7.4; 0.1% SDS; 1% NP-40; with protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO); and phosphatase inhibitor cocktail (Sigma Aldrich) and cleared by centrifugation at 12,000 x g at 4°C. Quantification of cell lysate protein concentrations was performed using the Pierce BCA Protein Assay Kit (Thermo Scientific, Waltham, MA). Lysate samples were prepared with SDS lysis buffer (50 mM Tris-HCl, pH 6.8, 0.25% bromphenol blue, 5% glycerol, 1.5% SDS, and 100 mM dithiothreitol) and boiled for 5 min prior to loading for gel electrophoresis. Protein separation was performed through SDS-

PAGE using 4-15% Bio-rad precast TGX gels followed by blotting onto 0.2 µm polyvinylidene difluoride membranes. Membranes were blocked with 2.5% dry milk powder in TBS-T (Tris-buffered saline, 0.1% Tween 20) for 1 hr at room temperature and subsequently incubated overnight at 4°C in primary antibodies diluted in 2.5% dry milk powder in TBS-T. Primary antibodies used for western blotting were rabbit anti-TMPRSS13 (1:2000, ab59862, Abcam), rabbit anti-cleaved caspase-3 (1:500, 9661, Cell Signaling Technology), rabbit anti-PARP (1:1000, 9532, Cell Signaling Technology), and mouse anti-β-actin (1:10000, Sigma Aldrich). Secondary antibodies were goat anti-rabbit and goat anti-mouse (Millipore, Billerica, MA) HRP-conjugated antibodies. Detection of antibodies was performed using ECL Western Blotting substrate or Super-Signal West Femto Chemiluminescent Substrate (Pierce, Rockford, IL).

Transient transfections with TMPRSS13 expression vector

DLD-1 and HCT116 cells grown in 6-well plates were reverse transfected using Lipofectamine LTX (Invitrogen) according to manufacturer instructions using 500 ng of plasmid. The vectors used for transfections were empty vector pcDNA3.1 (Invitrogen/ThermoFisher Scientific) and pcDNA3.1-TMPRSS13 (human full length), previously generated in our laboratory (Murray et al., 2017). Media was replaced 24 hrs following transfection and cells were subjected to drug treatment as described below, with lysates collected three-days hrs post-transfection in drug treatment experiments.

Drugs and drug treatments

For drug treatment experiments, stock solutions of paclitaxel (Sigma Aldrich), 5-FU (Sigma Aldrich), HA14-1 (Cayman Chemical), and staurosporine (STS) (Cell Signaling Technology) were diluted in DMSO and stock solution of carboplatin was diluted in water. In siRNA-treated DLD-1 cells, a 48 hr treatment with paclitaxel at a final concentration of 10 µM

was performed two-days post-transfection with sample collection occurring four-days post-transfection. siRNA-treated DLD-1 and HCT116 cells were subjected to 1 hr treatment of HA14-1 four-days post-transfection at final concentrations of 30 and 60 µM, respectively. For transient mammalian expression vector transfections, DLD-1 cells transiently transfected with vectors were subjected to 48 hr treatments with paclitaxel (10 µM), carboplatin (50 µM), and 5-FU (100 µM) 24 hrs post-transfection, after which lysate samples were harvested. In transiently transfected DLD-1 and HCT116 cells, treatments with HA14-1 or STS were performed four-days post-transfection. Concentration and duration of treatment for DLD-1 cells was 1.5 hr at 10 µM for HA14-1 treatment and 1 µM for 4 hours for STS. In HCT116 cells, treatment with HA14-1 lasted 1 hr at 60 µM.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from cultured cells using the RNeasy Plus Kit (Qiagen) according to manufacturer instructions. Reverse transcription of RNA isolates was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The qPCR reactions were performed with probes for TMPRSS13 (Hs00361060_m1, TaqMan®, Applied Biosystems) and expression levels were analyzed using the $-2^{\Delta\Delta Ct}$ method and normalized to HPRT1 or GAPDH housekeeping genes.

Analysis of apoptosis via Annexin V/propidium staining and flow cytometry

Flow cytometry analysis was performed in the Wayne State University Flow Cytometry core. Labelling of Live HCT116 cells was performed using the Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit (Molecular Probes) according to manufacturer instructions. Specifically, siRNA-treated HCT116 cells were cultured in 12-well plates, with media replaced every 48 hours. Following four- and five-days post-transfection, cells were trypsinized with 0.25% Trypsin-EDTA

(Gibco), centrifuged at 200 x g for 5 min, and cell pellets washed twice with ice-cold PBS. Following washes, 100 ul of cellular suspension from each biological sample were combined with 400 ul of 5x annexin binding buffer and gently mixed. Cells were analyzed within 30 minutes of staining with an LSR II (Becton Dickinson) cytometer. Cytometric data was plotted and analyzed with FlowJo software.

Invasion assay

Following two-day post-TMPRSS13 silencing period, DLD-1 cells were serum-starved for 5 hrs, then seeded with serum-free media onto permeable support inserts (8.0 µm pore size, Falcon) coated with 1 mg/mL Cultrex® in 24-well plates. Cells were cultured on coated inserts placed into wells containing serum-containing media as a chemoattractant for 24 hrs, after which invading cells were fixed using Z-fix (Anatech, Battle Creek, MI) and stained using Diff-Quik (Seimens, Newark, DE). Images of inserts were collected using an EZ4D Stereo Zoom microscope with integrated digital camera (Leica Microsystems, Buffalo Grove, IL), and invading cells quantified from images using ImageJ software.

Statistical Analysis

All statistical analyses were performed using Prism software (GraphPad, San Diego, CA). For immunohistochemical staining, differences in staining scores between cancer grade groups were analyzed using the non-parametric Kruskal-Wallis ANOVA test, with post-hoc comparisons performed with Dunn's test. For proliferation experiments, differences between siRNA treatment groups were analyzed using the two-way ANOVA test, with siRNA treatment and timepoint as independent factors. Post-hoc comparisons were performed between siRNA treatments for each timepoint using Tukey's multiple comparisons test. One-way ANOVA tests with Tukey multiple

comparisons post-hoc tests were used for comparison of siRNA treatment groups in flow cytometry, invasion, and qPCR experiments.

2.3 Results

2.3.1 TMPRSS13 is upregulated in human colorectal cancer

As part of an ongoing effort to determine the expression and function of the TTSP family in healthy colon and CRC, we performed a systematic expression analysis of TTSPs in cancer through *in silico* datamining, using the Oncomine™ microarray database. TMPRSS13 transcripts were found to be significantly upregulated in human colon adenocarcinomas compared to normal human colon (TGCA, 2012)(**Figure 2.1A**). TMPRSS13 expression in the normal colon has previously been reported to be low in both human and mouse tissue (Kido et al., 2008; Faller et al., 2014; Madsen et al., 2014). To confirm the elevated levels of TMPRSS13 at the protein level in human CRC, we performed immunohistochemical (IHC) staining of human colon tissue arrays. Low protein expression levels of TMPRSS13 was detected in some normal colon crypt epithelial cells with no or minimal staining in the lamina propria (**Figure 2.1B**, lower right panel); however, the majority of normal colon samples displayed no detectable expression. TMPRSS13 protein was localized on the cell surface of the epithelial cells and no nuclear staining was observed. This cell surface localization is in agreement with the expected distribution of the membrane anchored topology of TMPRSS13 (Murray et al., 2017) . Strong expression of TMPRSS13 in epithelial-derived colon adenocarcinoma was detected (**Figure 2.1B**, lower left panel). When primary antibodies were substituted with non-immune rabbit IgG in serial sections of all samples no significant staining was observed (**Figure 2.1B**, upper panels).

Evaluation of differential expression of TMPRSS13 in CRC was completed using normal and cancerous tissue samples with grades ranging from I to III (Normal colon; N=14, Grade I;

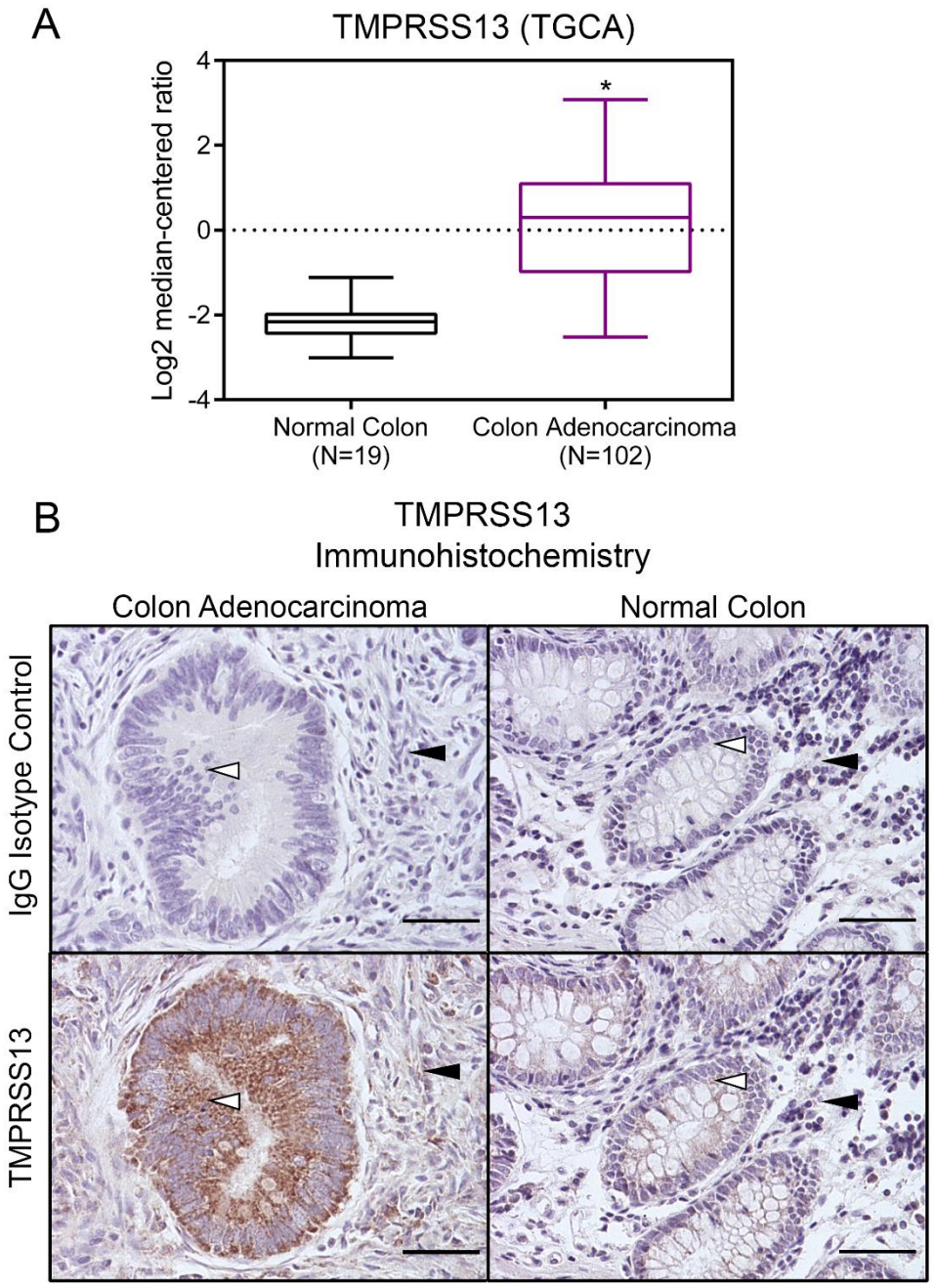

Figure 2.1: TMRSS13 transcript and protein is upregulated in human colorectal cancer
(A) Box and whisker plot representing TMPRSS13 mRNA expression data in normal and colon adenocarcinoma tissue samples. TMPRSS13 gene expression values in normal colon (N=19, black bar) and colon adenocarcinoma tissue (N=102, purple bars) are shown. Box and whiskers indicate interquartile ranges with median indicated as horizontal line in box (* = p = 2.96E-26, fold change = 4.918). **(B)** Representative samples from tissue array IHC analysis of TMPRSS13 protein expression in normal human colon (right panels) and colon adenocarcinoma (left panels) samples. Primary antibody was substituted with non-immune IgG in serial section of all samples and no significant staining was observed (upper panels). Black arrowheads indicate epithelial cells with undetectable or weak TMPRSS13 staining in normal colon (upper left panel) compared to strong TMPRSS13 staining observed in epithelial cells of a grade II colon adenocarcinoma (lower left panel). No or minimal staining was detected in the lamina propria of the colon (white arrowheads). Scale bars = 50 µm.

N=16, Grade II; N=65, and Grade III; N=23 colorectal adenocarcinoma). The CRC tissue arrays were incubated with anti-TMPRSS13 rabbit antibody (Figure 2A, representative samples shown) and non-immune rabbit IgG as a negative control (Figure 2A, upper right panel). Staining intensity was scored on a scale from 0 to 3 (see "Materials and Methods"). In terms of TMPRSS13 expression, the majority of normal colon samples (11/14) displayed no detectable expression while three samples showed low expression (**Figure 2.2B**). In contrast, all CRC samples were scored positive for TMPRSS13 staining (**Figure 2.2B**). Well-differentiated, low-grade carcinomas (Grade I) showed low to moderate cell surface staining (**Figure 2.2A**, upper right panel), while the majority of moderately differentiated carcinoma (Grade II) displayed moderate to strong cell surface staining (**Figure 2.2A**, lower left panel). In poorly differentiated carcinomas (grade III), TMPRSS13 expression was mainly cell surface localized with some areas displaying dispersed staining (**Figure 2.2A**, lower right panel), and the majority of samples showed low to moderate staining. Statistical analysis showed a significant increase in staining intensity in CRC in comparison to normal tissue ($p < 0.01$) (**Figure 2.2B**). Together these findings demonstrate differential TMPRSS13 expression in CRC, validating that increased levels of transcripts are accompanied by increased protein levels, and indicates a proteolytic imbalance in the colorectal tumor microenvironment.

2.3.2 Loss of TMPRSS13 impairs survival in CRC cells

Based on our observations that TMPRSS13 is upregulated in human CRC on the transcript and protein level, we set out to determine the role of TMPRSS13 in pro-oncogenic cellular processes, using two different human CRC cell lines, DLD-1 (high TMPRSS13 expression) and HCT116 (low TMPRSS13 expression) colorectal cancer cell lines both derived from colorectal adenocarcinoma. DLD1 cells harbors mutations in the *KRAS, PIK3C, APC* and *TP53* genes.

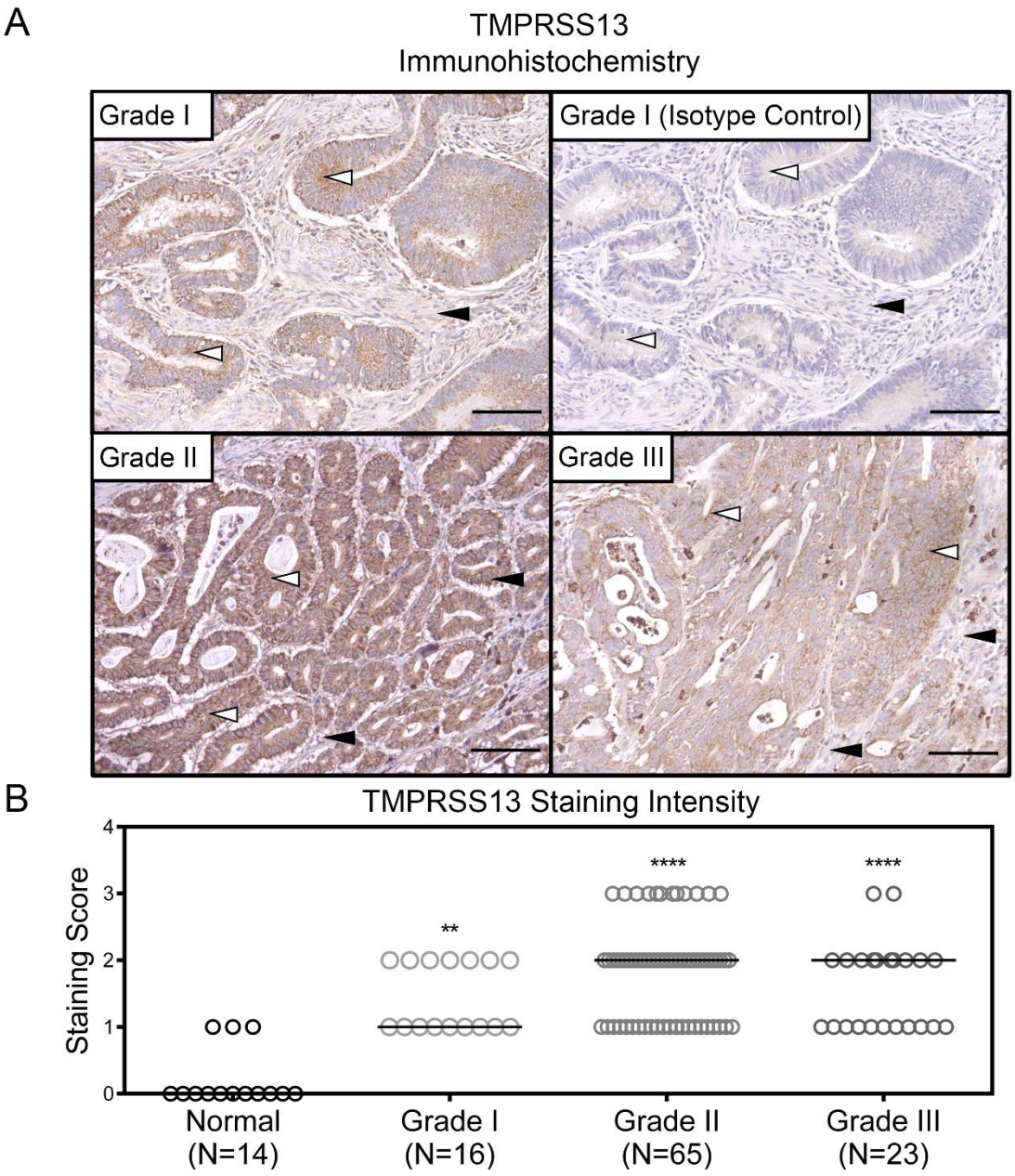

Figure 2.2: TMPRSS13 expression is consistently upregulated across human colorectal cancer of different grades

(A) Representative samples from tissue array IHC analysis of TMPRSS13 protein expression in Grade I (upper left panel), Grade II (lower left panel), and Grade III (lower right panel) colorectal adenocarcinomas. Primary antibody was substituted with non-immune IgG in serial section of all samples and no significant staining was observed (upper right panel). Black arrowheads indicate epithelial cells with undetectable or weak TMPRSS13 staining in normal colon (upper left panel) compared to strong TMPRSS13 staining observed in epithelial cells of a grade II colon adenocarcinoma (lower left panel). No or minimal staining was detected in the lamina propria of the colon (white arrowheads). **(B)** Staining intensities were determined as described in "Materials and Methods" and presented in a scatterplot categorized by cancer grade. Each circle represents one individual patient. Normal colon (N=14), Grade I (N=16), Grade II (N=65), and Grade III (N=23) colorectal adenocarcinoma. (** = $p < 0.01$; **** = $p < 0.0001$; determined by Dunn's test post-hoc, following Kruskal-Wallis ANOVA). Scale bars = 100 µm.

HCT116 cells harbors *KRAS, PIK3C,* and wildtype *APC* and *TP53 genes* (Ahmed et al., 2013). Both cell lines grow primary tumors upon orthotopic microinjection in nude mice with dissemination of cancer cells to local and distant sites (Cespedes et al., 2007). To assess the consequence of TMPRSS13 loss-of-function for cell survival, two non-overlapping siRNAs targeting TMPRSS13 were used and cells were counted at different time points. A significant decrease in the numbers of viable TMPRSS13-silenced cells in both cell lines compared to scramble controls was observed (**Figure 2.3A**). TMPRSS13-silencing was confirmed in DLD-1 cells by Western blotting (**Figure 2.3B**) whereas qPCR analysis was used to verify silencing of TMPRSS13 in HCT116, due to lower baseline expression levels in this cell line (**Figure 2.3C**). The multiple bands observed by Western blot analysis most likely represent different isoforms of TMPRSS13 (four isoforms produced by alternative splicing have been reported (Kido and Okumura, 2008; Murray et al., 2017)). To further characterize the cellular effects of TMPRSS13 knock-down, we assessed the level of cleaved Poly (ADP-ribose) polymerase (PARP) as a marker for cells undergoing apoptosis in cell lysates by Western blotting. Robust increases in cleaved PARP levels upon TMPRSS13 silencing in both DLD-1 cells (**Figure 2.3B**) and HCT116 cells (**Figure 2.3D**) was observed for all three time points (days 3, 5, and 7). For a detailed comparative analysis of TMPRSS13-deficient cell populations versus TMPRSS13-sufficient cell populations, staining with Annexin V-AlexaFluor™ 488 conjugate (AV488) in conjunction with the vital dye propidium iodide (PI) followed by Flow Cytometry analysis was used for identification of early and late stage apoptotic cells. The Flow cytometric AV488/PI analysis data demonstrates that a significantly higher proportion of TMPRSS13-silenced HCT116 cells were undergoing apoptosis compared to control cells (**Figure 2.4A** and **2.4B**, left panels). The largest relative difference was observed in early apoptotic cells (AV488-positive/PI-negative) following TMPRSS13-silencing

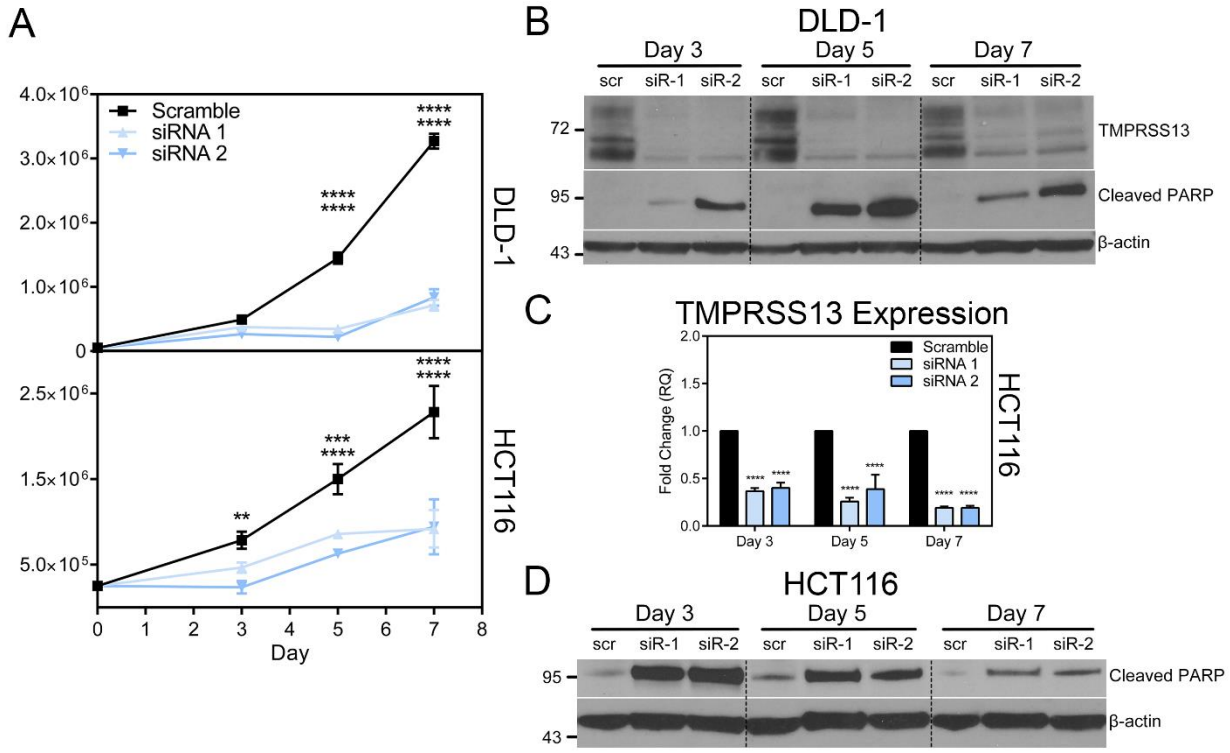

Figure 2.3. Silencing of TMPRSS13 impairs proliferation and leads to increased PARP cleavage in colorectal carcinoma cells

(A) TMPRSS13 was silenced using two non-overlapping synthetic RNA duplexes (siRNA 1 and siRNA 2) in the human colorectal carcinoma cell lines DLD-1 (top panel) and HCT116 (bottom panel) and cells were counted on day 3, day 5, and day 7 following siRNA treatment. A %GC matched non-targeting RNA duplex was used as negative control (scr). The number of viable cells counted was plotted for each time point. Error bars indicate SD (*** = $p < 0.001$; **** = $p < 0.0001$, determined by Tukey's post-hoc test, following two-way ANOVA). **(B)** Verification of TMPRSS13 knockdown in DLD-1 cells was performed by Western blot analysis. TMPRSS13 was efficiently silenced by both siRNA 1 and siRNA 2 (siR-1 and siR-2, upper panel). Apoptosis was assessed by probing for the presence of cleaved PARP (middle panel) and anti-ß-actin was used as control for equal loading (bottom panel). Dashed lines indicate cropped lanes. **(C)** Verification of TMPRSS13 knockdown in HCT116 cells was performed by qPCR analysis using fold-change analysis normalized to HPRT1. Error bars indicate SEM (**** = $p < 0.0001$, determined using a one-way ANOVA with Tukey post-hoc tests). **(D)** Assessment of apoptosis following TMPRSS13 knockdown was performed by detection of cleaved PARP in HCT116 cells by Western blot analysis (top panel). Anti-ß-actin was used as control for equal loading (bottom panel). Dashed lines indicate cropped lanes.

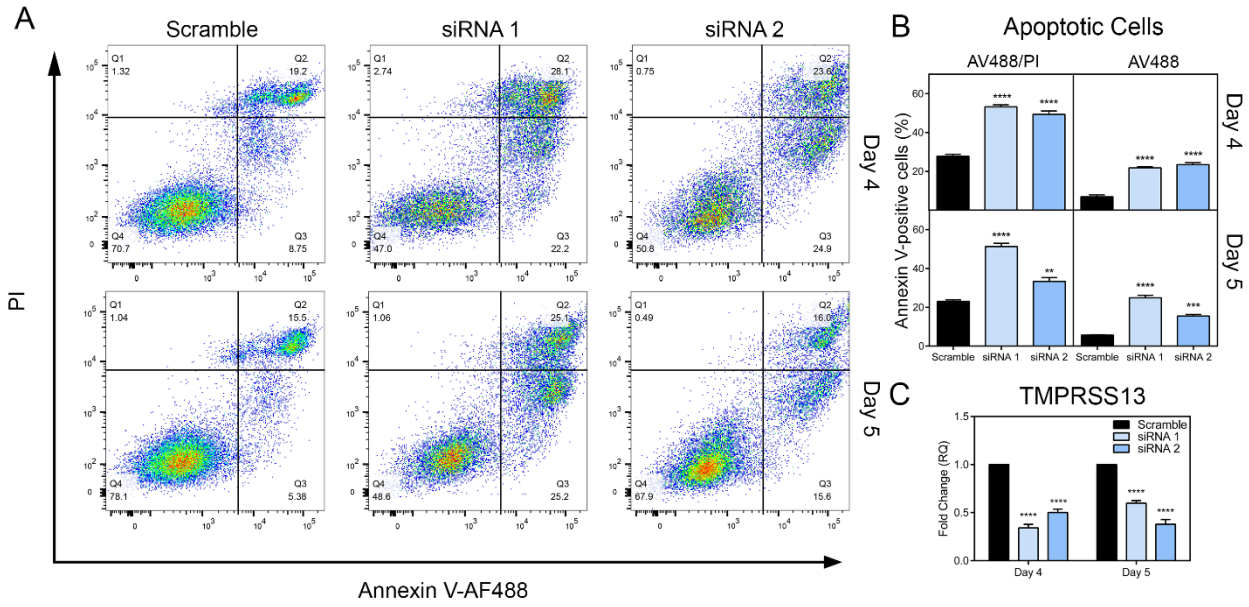

Figure 2.4. Silencing of TMPRSS13 causes apoptosis in colorectal carcinoma cells
(A) Apoptosis in HCT116 cells was quantified by flow cytometry using an Annexin V-Alexa Fluor-488 conjugate (AV488) and propidium iodide (PI) at 4 days and 5 days post-siRNA treatment. Dot plots summarizing populations stained with AV488 and PI staining are shown for two non-overlapping synthetic RNA duplexes (siRNA 1 and siRNA 2). A %GC matched non-targeting RNA duplex was used as negative control (Scramble). Unstained cells represented by Q4, with early-apoptotic AV488-positive stained cells represented in Q3, late-apoptotic AV488/PI-positive stained cells in Q2, and necrotic PI-stained cells in Q1. **(B)** Analysis of AV488-positive populations, encompassing both early- and late-apoptotic cells (AV488/PI, left panels) and early apoptosis (AV488, right panels), shows a significant increase in apoptotic cells among TMPRSS13 siRNA-treated cells. Error bars indicate SEM. (** = $p < 0.01$; *** = $p < 0.001$; **** = $p < 0.0001$, compared to scramble control. Determined by Tukey post-hoc test, following one-way ANOVA). **(C)** qPCR analysis of TMPRSS13 expression normalized to HPRT1 in HCT116 cells was used to verify gene silencing. Error bars indicate SEM (**** = $p < 0.0001$).

(**Figure 2.4B**, right panels). The relative fractions of early apoptotic cells were: 22% for siRNA1, 23% for siRNA1 versus 9% for control (day 4) and 25% for siRNA1, 16% for siRNA1 versus 5% for control (day 5)(**Figure 2.4B**, right panels). Silencing of TMPRSS13 in HCT116 was verified by qPCR (**Figure 2.4C**). DLD-1 were not amenable to the AV488/PI assay due to poor annexin-V staining, which is in line with previous reports (Dicker et al., 2005). Together, the decrease in viable cells, increased PARP-cleavage, and higher relative apoptotic cell populations in flow cytometry analysis observed following TMPRSS13-silencing cells demonstrate an important role for TMPRSS13 in CRC cancer cell survival and apoptosis.

2.3.3 Increased TMPRSS13 expression promotes resistance to drug-induced apoptosis in CRC cells

Spurred by the data presented above showing that TMPRSS13 expression is elevated in CRC and plays a critical role for CRC cell survival/apoptosis, we performed gain-of-function experiments to determine whether overexpression of the protease is sufficient for cells to acquire protective properties against apoptotic stimuli (**Figure 2.5**). For this purpose, we utilized a panel of drugs including the chemotherapeutic drugs, paclitaxel, carboplatin, and 5-fluorouracil; the broad-spectrum kinase inhibitor staurosporine; and the small-molecule Bcl-2 inhibitor HA14-1. These drugs have shown to cause cytotoxicity in a variety of cells through different mechanisms, with apoptosis as the ultimate outcome (Couldwell et al., 1994; Long and Fairchild, 1994; Murray et al., 1997; Longley et al., 2003; Kessel and Reiners, 2007; Hassan et al., 2014). Full-length human TMPRSS13 was overexpressed in DLD-1 and HCT116 cells using transient plasmid transfection and confirmed by Western blot (**Figure 2.5A, B**, upper panels). The responses to drug treatment were assessed by Western blot detection of caspase-3 cleavage (**Figure 2.5A, B**, middle panels). Interestingly, overexpression TMPRSS13 led to a profound decrease in detected cleaved

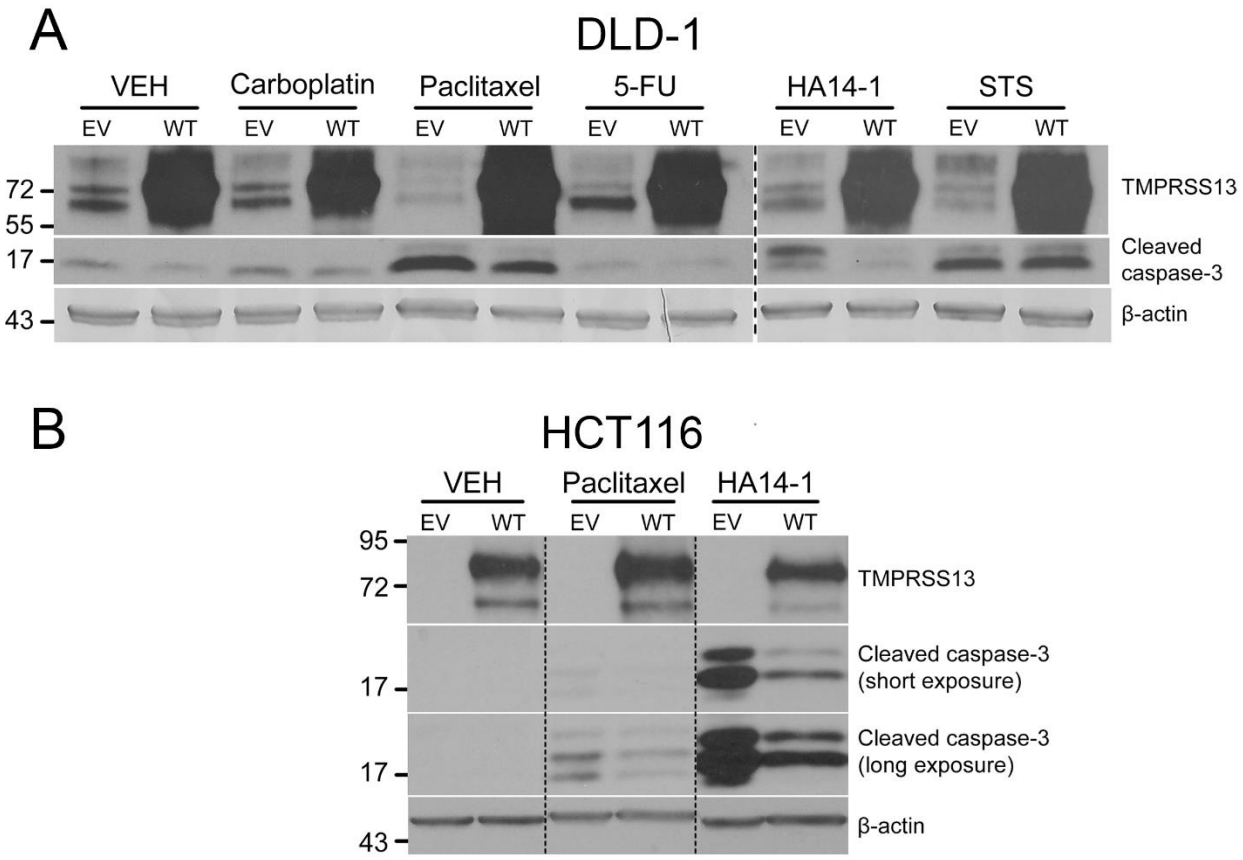

Figure 2.5. Overexpression of TMPRSS13 in colorectal cancer cells in confers resistance to drug-induced apoptosis

(A) DLD-1 cells were transfected with a mammalian expression vector containing full-length wildtype human TMPRSS13 cDNA. Controls include same expression vector without cDNA insert (empty vector = EV) and one where no vector was included with the transfection agent (mock). Cells were treated for 48 hrs with 50 µM carboplatin, 10 µM paclitaxel, or 100 µM 5-fluorouracil (5-FU); 10 µM HA14-1 for 1.5 hrs; or 1 µM staurosporine (STS) for 4 hrs. Cell lysates were collected 72 hrs post-transfection. TMPRSS13 overexpression was verified by Western blot analysis (top panel). Apoptosis was determined by the detection of cleaved caspase-3 (middle panel) and anti-ß-actin was used as control for equal loading (bottom panel). Heavy dashed line indicates separate membranes. **(B)** HCT116 cells were transfected as described for DLD-1 cells to express TMPRSS13 and treated with 10 µM paclitaxel for 48 hrs or 60 µM HA14-1 for 1 hr. Cellular lysates were collected 72 hrs post-transfection and analyzed for TMPRSS13 expression and for the presence of cleaved caspase-3 by Western blot analysis. Anti-ß-actin was used as control for equal loading (bottom panels).

caspase-3 in CRC cells in response to HA14-1. Thus, both in DLD-1 cells, which have a relatively high level of endogenous TMPRSS13, and HCT116 cells with relatively low endogenous TMPRSS13 level (**Figures 2.5A** and **B**) were protected from HA14-1-induced apoptosis upon increased TMPRSS13 expression. Both cells lines also displayed TMPRSS13-mediated protection from apoptosis upon paclitaxel treatment. TMPRSS13 overexpression had no detectable effect on caspase-3 cleavage upon treatment with carboplatin, 5-FU, or staurosporine. The observed lack of response to 5-FU, which is widely used in advanced CRC, emphasizes the major clinical obstacle posed by acquired resistance to this and other chemotherapy, which occurs in 90% of patients with metastatic cancer (Hassan et al., 2014; Hammond et al., 2016).

2.3.4 Loss of TMPRSS13 renders CRC cells more sensitive to drug-induced apoptosis

To further investigate the role of TMPRSS13 in drug induced apoptosis, we investigated whether silencing of TMPRSS13 would increase sensitivity to drug-induced apoptosis, thereby exploring the potential of a novel combination treatment strategy using TMPRSS13 inhibitors together with chemotherapy to improve treatment efficacy (**Figure 2.6**). HA14-1 and paclitaxel were selected based on the protective effect TMPRSS13 overexpression had against these two apoptosis-inducing agents.

HA14-1 or paclitaxel was added to TMPRSS13-silenced cells and levels of cleaved PARP and caspase-3 were detected by Western blotting (**Figure 2.6A-C**, middle panels). TMPRSS13 knock down was confirmed by Western blotting for DLD-1 (**Figures 2.6A** and **B**, top panels) and by qPCR for HCT116 cells (**Figure 2.6D**). As expected, silencing of TMPRSS13 alone induced apoptosis in DLD-1 cells (**Figure 2.6A**) and HCT116 cells (**Figure 2.6C**). Importantly, HA14-1 treatment in combination with TMPRSS13 silencing further increased the level of apoptotic markers indicating that the combination elicits a stronger apoptotic response than targeting either

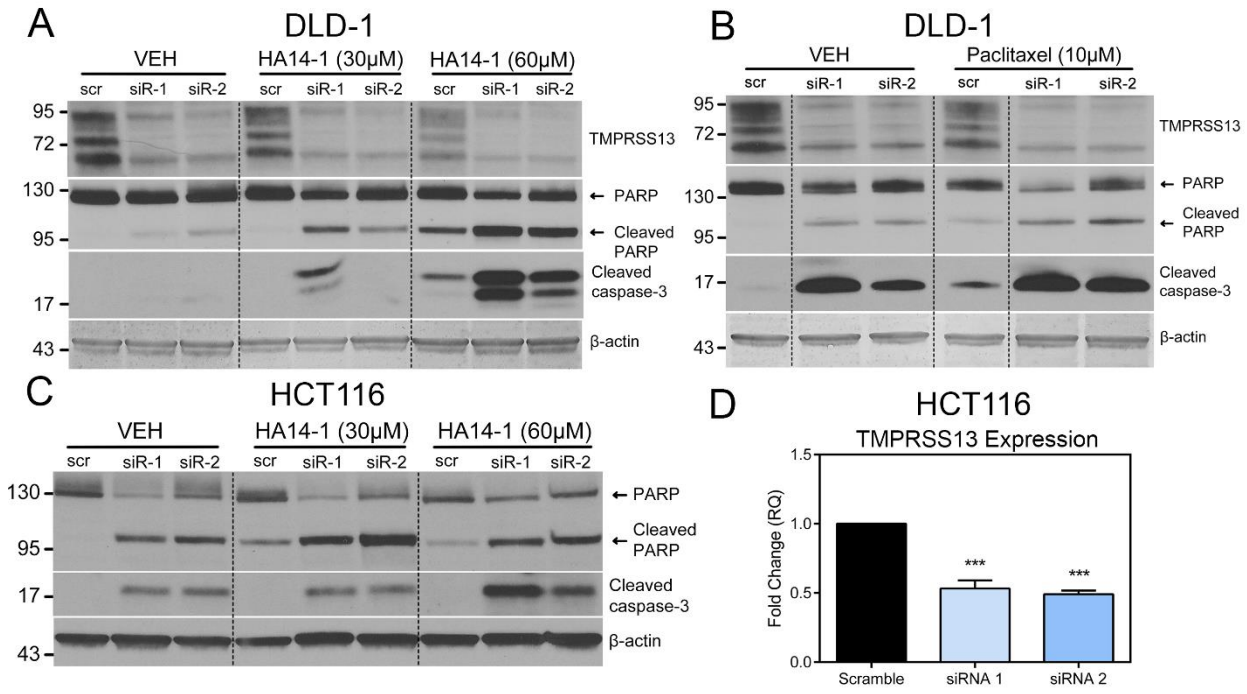

Figure 2.6. TMPRSS13 silencing sensitizes colorectal cancer cells to drug-induced apoptosis
Whole cell lysates from DLD-1 **(A, B)** and HCT116 cells **(C)** were collected 96 hrs following siRNA treatment using two non-overlapping synthetic RNA duplexes (siRNA 1 and siRNA 2= siR-1 and siR-2). A %GC matched RNA duplex was used as negative control (scr). A control where no RNA duplex was included with the transfection agent was also included (mock). Prior to lysis, cells were subject to treatment with HA14-1 for 1 hr (30 or 60 μM) or paclitaxel for 48 hrs (10 μM) as indicated. **(A, B)** Western blot analysis was used to verify TMPRSS13 knockdown in DLD-1 cells (top panels) and the presence of cleaved PARP or cleaved caspase-3 was used as markers of apoptosis in DLD-1. Dashed lines indicate cropped lanes. **(A,B)** and HCT116 cells **(C)**, respectively (middle panels). Anti-ß-actin was used as control for equal loading (bottom panels). Dashed lines indicate cropped lanes. **(D)** qPCR analysis of TMPRSS13 expression normalized to HPRT1was used to verify gene silencing in HCT116 cells. Error bars indicate SEM (**** = $p < 0.0001$, determined using a one-way ANOVA with Tukey post-hoc tests).

Bcl-2 (by HA14-1) or TMPRSS13 individually. The apoptotic response in DLD-1 cells to paclitaxel treatment was also enhanced in TMPRSS13-siRNA treated cells, though the potentiation by combination targeting compared to single targeting was less pronounced (**Figure 2.6B**).

2.3.5 Loss of TMPRSS13 reduces invasive potential of CRC cells

In order for progression from localized to advanced and metastatic CRC to occur, cancer cells acquire properties consistent with a propensity to invade into surrounding tissues and distal organs. The role of TMPRSS13 for the invasion potential of CRC cells was studied using a transwell assay in which cells were seeded on top of an extracellular matrix hydrogel in reduced serum media and allowed to invade overnight towards full serum media in the bottom chamber (**Figure 2.7**). Upon silencing of TMPRSS13, a significant decrease in invasive potential was observed in DLD-1 cells (**Figure 2.7**). Importantly, the experiment was carried out at day 2 after siRNA transfection when no difference in cell number is observed between control and TMPRSS13 silenced DLD-1 cells (**Figure 2.2A**). This minimizes interference from differences in cell proliferation/survival. These data suggest that TMPRSS13, in addition to anti-apoptotic functions, also promotes cellular invasion; two key pro-oncogenic functional properties involved both in primary tumor, growth, invasion, and metastasis.

2.4 Discussion

In this study we sought to identify uncharacterized TTSPs dysregulated in cancers. We discovered that TMPRSS13 transcript is upregulated in CRC. Using immunohistochemistry, increased TMPRSS13 protein levels were verified in both well-differentiated and poorly differentiated cancers compared to normal colon, suggesting that this protease is a potential player in promoting CRC progression. Through further characterization of TMPRSS13 in tissue culture models, we found that TMPRSS13 expression promoted cellular survival and invasive potential in

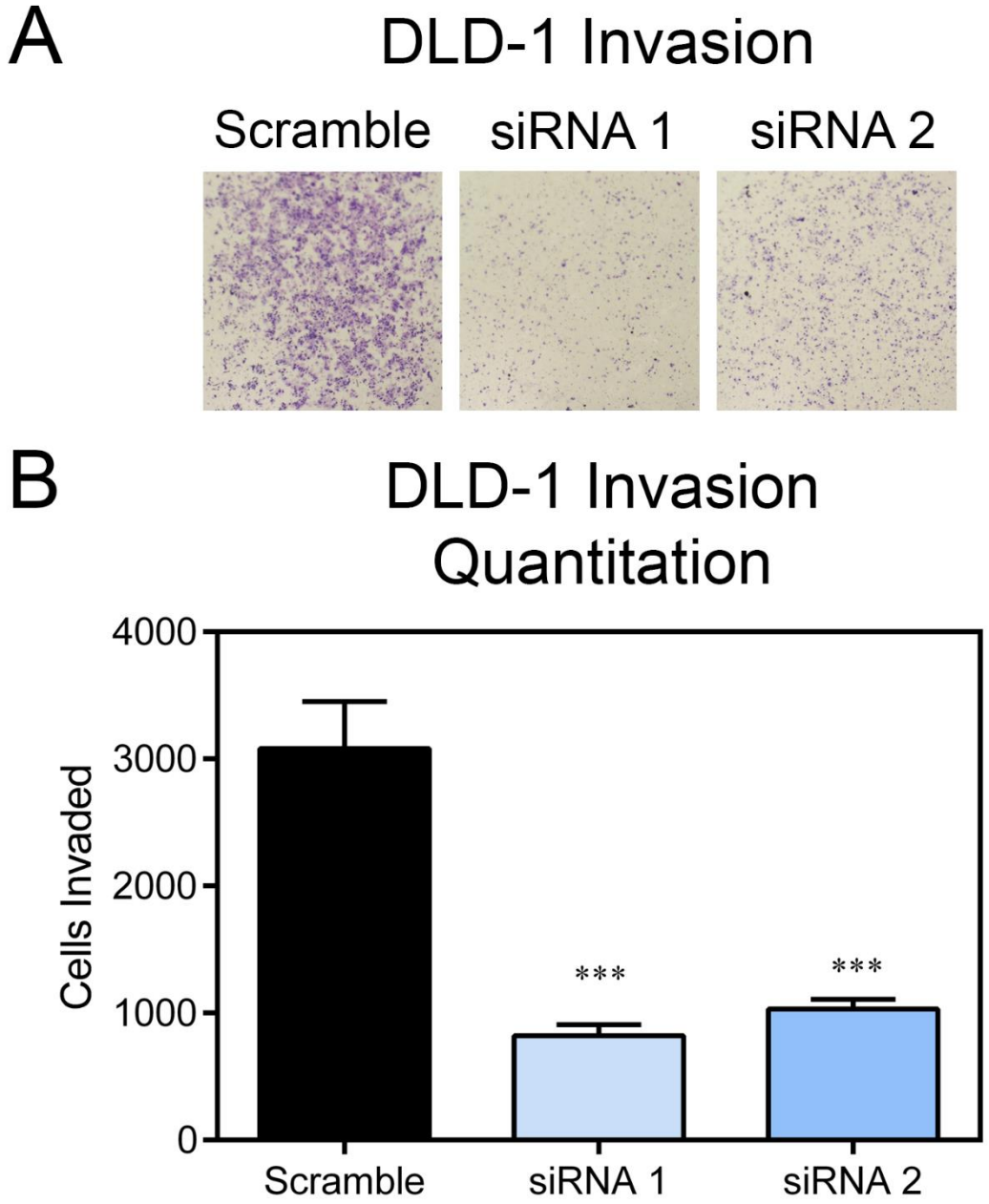

Figure 2.7. TMPRSS13 silencing reduces the invasive potential of colorectal cancer cells
(A) Invasion assay was performed in siRNA-treated DLD-1 cells to assess impact of TMPRSS13 on invasive potential. Two non-overlapping synthetic RNA duplexes (siRNA 1 and siRNA 2) were used and a %GC matched RNA duplex was used as negative control (Scramble). 48 hrs following siRNA treatment, DLD-1 cells were seeded in serum-free media onto transwell inserts coated with 1 mg/ml Cultrex basement membrane gel inserted into 24-well plates with serum-containing media. Cells were incubated for 16 hrs and invading cells were fixed and stained with crystal violet. Representative images of Cultrex-coated transwell membranes containing invading cells are shown. **(B)** Invading cells were counted and numbers analyzed by ANOVA, with Tukey's post-hoc test for multiple comparisons. Error bars indicate SEM (n = 3, *** = $p < 0.001$).

CRC cell lines. Flow cytometric analysis of HCT116 cells revealed a significant increase in Annexin V-positive/PI-negative TMPRSS13-silenced cells, indicative of cells undergoing early stages of apoptosis. In colon epithelia, apoptosis is a tightly regulated process crucial to maintenance of epithelial integrity (Delgado et al., 2016). Suppression of apoptosis in cancer is critical for tumor progression, and various pathways are altered to facilitate escape from cell death (Mohammad et al., 2015). Our findings suggest TMPRSS13 that is a key player in promoting resistance to apoptosis in CRC cells. These properties of TMPRSS13 have not previously been described in any cancer type. A recent study demonstrated that expression of another TTSP, TMPRSS4 correlates with colorectal cancer pathological stage (Huang et al., 2014). Futhermore, RNA-mediated silencing experiments in HCT116 cells revealed that TMPRSS4 is involved in the regulation of cell proliferation, apoptosis, and invasion. It has also been shown that knockdown of TMPRSS3 inhibits gastric cancer cell proliferation, invasion and EMT via regulation of the ERK1/2 and PI3K/Akt pathways (Li et al., 2018).

Despite advances in systemic CRC therapy, the 5-year survival rate is still only 12.5% and the primary reason for treatment failure is believed to be acquired resistance to therapy which occurs in 90% of patients with metastatic cancer (Siegel et al., 2014). Genetic analysis of tumors has been an increasingly useful tool in decision-making regarding adjuvant chemotherapeutic regimens in CRC, with advances identifying the presence of microsatellite instability and CpG island methylated phenotype as predictors of 5-FU response and survival benefit (Van Rijnsoever et al., 2003; Carethers et al., 2004). Yet, the majority of advanced CRCs eventually develop resistance to 5-FU and other cytotoxic drugs. This prompted us test whether TMPRSS13 contributes to a drug resistant phenotype in CRC cells. Indeed, upon overexpression of TMPRSS13, CRC cells exhibited resistance to treatment with the apoptosis-inducing drugs HA14-

1 and paclitaxel. Conversely, a profoundly increased sensitivity to HA14-1-induced and, to a lesser extent, paclitaxel-induced cellular death in TMPRSS13-silenced cells was observed. Taxanes, including paclitaxel, suppress microtubule polymerization dynamics, which results in the slowing or blocking of mitosis at the metaphase-anaphase boundary and subsequently cells die by apoptosis (Xu et al., 2009). Taxanes have failed to demonstrate significant clinical benefit in phase II trials in colorectal cancer (CRC) and are not used as standard of care (Einzig et al., 1996; Swanton et al., 2006). In cell culture experiments it was demonstrated that paclitaxel-induced apoptosis was enhanced by simultaneous inhibition of the mitogen-activated protein kinase pathway in CRC. Thus, the treatment of the colon cancer cell lines SW480 and DLD-1 with paclitaxel resulted in increased activation of the MAPK pathway, which was blocked by PD98059, a MEK inhibitor. In both cell lines, MAPK inhibition by the MEK inhibitor PD98059 led to an enhancement of paclitaxel-induced apoptosis (Xu et al., 2009). Synergistic inhibition of colon cancer cell growth with paclitaxel and the PI3K/mTOR dual inhibitor BEZ235 through apoptosis in HCT116 and HT-29 colon cancer cells was also reported (Zou et al., 2016)**.** Further studies are needed to determine whether paclitaxel in combination with other inhibitors could be viable therapeutic strategy in CRC.

In our studies we observed the most profound effect when combining TMPRSS13 targeting with HA14-1, which represent a rational starting point to further explore this combination option. Based on the selective action of HA14-1 on Bcl-2 (Wang et al., 2000; Kessel and Reiners, 2007), it is plausible that TMPRSS13 modulates the molecular pathway that drives intrinsic apoptotic pathway as a result of exposure to stressors, such as drug treatment. We frequently observed decreases Bcl-2 levels upon TMPRSS13 silencing (data not shown); however, since it was not consistently detected under different cell culture conditions, a sound conclusion could not

be substantiated. Bcl-2 is often upregulated in cancer, including CRC (Violette et al., 2002). Bcl-2 signaling has also been shown to promote Akt signaling independent of mitochondrial involvement (Mortenson et al., 2007). Further investigation is needed to determine how TMPRSS13 promotes the response to Bcl-2 inhibition. It is noteworthy that inhibitors targeting the Bcl-2 protein have gained increased attention in recent years. In 2016 the FDA approved venetoclax, a selective small-molecule inhibitor of BCL-2, for treatment of chronic lymphocytic leukemia (CLL). Particularly in hematological malignancies, multiple clinical trials are assessing the possible use of venetoclax alone or in combination with other chemotherapies (Montero and Letai, 2018). It remains to be seen whether the positive results observed in diseases like CLL using anti-apoptotic inhibition will be mirrored in solid tumors, including CRC, that are commonly more difficult to treat in the clinic. Promising results have recently been reported on venetoclax combined with tamoxifen in ER- and BCL-2-positive metastatic breast cancer with an overall response rate of 61% and a 72% clinical benefit rate (Lindeman et al., 2018). In the primary clinical setting, about 80% of ER-positive tumors are BCL-2–positive. Importantly, no patients have had to discontinue due to toxicity (Lindeman et al., 2018).

Identifying whether TMPRSS13 is frequently co-expressed with Bcl-2 would be important for identifying patient populations with the most favorable response rate to TMPRSS13/Bcl-2 combination treatment. Collectively, our studies have demonstrated a strong role for TMPRSS13 in conferring cells with invasive potential and protecting cells from apoptosis. These phenotypes have not been reported in any disease context to date and highlight the potential of TMPRSS13 as a potential target in CRC. One factor to consider is the potential side effects of TMPRSS13 inhibition *in vivo*. What makes TMPRSS13 an attractive target in CRC is the low baseline levels of colon expression, potentially limiting the adverse effects of TMPRSS13 inhibition.

Furthermore, based on previous studies in mouse genetic loss-of-function models, TMPRSS13 deficiency has no discernible consequences for adult mouse (Madsen et al., 2014). However, as the function of TMPRSS13 under challenged conditions is still uncharacterized, particularly pertaining to cytotoxic stress, it remains to be evaluated what the consequences of TMPRSS13 inhibition on function and homeostasis of the colon and other tissues are.

In summary, our current study represents a comprehensive expression characterization of TMPRSS13 in healthy and CRC tissues that demonstrated differential expression of this TTSP, mechanistic studies on the cellular level demonstrating that TMPRSS13 plays a critical role for CRC cell survival and protection from drug-induced apoptosis. These novel findings lay the foundation for continuing studies to further decipher the molecular mechanisms by which TMPRSS13 exerts its pro-oncogenic functions and to promote the development of TMPRSS13 inhibitors and their potential as targeted therapeutic drugs in CRC.

CHAPTER 3: IMPACT OF TMPRSS13 DEFICIENCY IN A MOUSE MODEL OF COLORECTAL CANCER

3.1 Introduction

Colorectal cancer (CRC) remains one of the most common cancers in both men and women worldwide (ACS, 2018). One of the most at-risk populations are those with inflammatory bowel disease (IBD), such as Crohn's disease (CD) and ulcerative colitis (UC). The risk of developing CRC in IBD patients rises with increased duration of inflammation and occurrence of cancer results in a unique form of CRC known as colitis-associated colorectal cancer (CAC)(Ekbom et al., 1990b; Ekbom et al., 1990a; Jess et al., 2012). The etiology of CAC shares much in common with sporadic CRC development, such as mutations in key oncogenes (such as *APC* and *P53*) and chromosomal instability, but the sequence in which genetic changes occur in CAC differs as a result of chronic inflammation (Ullman and Itzkowitz, 2011). Chronic inflammation is known to increase oxidative stress through production of reactive oxygen species (ROS) by innate immune cells (Kundu and Surh, 2008). Accumulation of ROS in epithelial cells increases the probability of cancer-driving genetic changes, including mutations in *APC*, *P53*, and *BCL2* (Roessner et al., 2008).

The colon has several characteristics to mitigate risk of inflammation. Despite being exposed to a large commensal microbial population, the colon mucosa features the barrier function of the intestinal epithelium (Clayburgh et al., 2004; Watson et al., 2005). This barrier is maintained by apical junction complexes. In IBD, these junctions can become impaired, contributing to increased intestinal permeability (Clayburgh et al., 2004). While the epithelial barrier possesses a degree of permeability to permit the transport of water and soluble nutrients, it restricts access of pathogenic antigens and toxicants. Aberrant permeability allows for unregulated exposure to the

luminal contents, which can induce inflammation and result in further damage the epithelia and barrier loss. Changes in the gut microbiome can also contribute to colon inflammation. Dysregulated populations of bacteria and increased bacterial metabolites from bacterial fermentation can influence the mucosal immune system, tipping the scale towards an inflammatory phenotype (Sun et al., 2016).

The link between inflammation and CRC has been well established in the azoxymethane/dextran sulfate sodium (AOM/DSS) model of colorectal cancer. This model has helped inform several of the immune responses underlying intestinal inflammation. Tumorigenesis with this model mirrors human CRC progression, making it suitable for examining the impact of genetic factors on early stages of tumor development (De Robertis et al., 2011).

The role of proteases in maintaining barrier function in epithelial tissues has been well described with the type II transmembrane serine protease (TTSP) matriptase in epidermis and intestine (List et al., 2002; List et al., 2009; Kosa et al., 2012). Deregulated proteolysis as a consequence of loss of hepatocyte growth factor activator inhibitor-2 (HAI-2) in the intestine also has been shown to disrupt epithelial homeostasis (Szabo and Bugge, 2018). Several TTSPs have yet to be characterized in intestinal physiology or disease, including TMPRSS13. TMPRSS13 is a expressed in a number of epithelial tissues, such as skin, intestine, tongue, and kidney (Madsen et al., 2014). We have identified this protease to be upregulated in human CRC and important for cancer cell survival, with loss resulting in increased sensitivity to drug-induced stress (See **Chapter 2**). TMPRSS13 thus makes a potential target candidate in treating CRC. This study marks the first investigation of the role of TMPRSS13 in intestinal function and cancer. To that end, we hypothesized that TMPRSS13 promotes CRC. We utilized a colitis-associated model of CRC to test whether TMPRSS13-deficient mice would be resistant to development of tumors.

3.2 Materials and Methods

Animals

All procedures involving live animals were performed in an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited vivarium following institutional guidelines and standard operating procedures. TMPRSS13-deficient mice (*T13*$^{-/-}$) were generated by Deltagen Inc. and acquired from Jackson Laboratories (Bar Harbor, ME). The mice were generated by homologous gene recombination using a gene-targeting vector containing a promoterless LacZ-Neo fusion gene (β-galactosidase reporter) in 129P2 OlaHsd-derived E14 embryonic stem cells. Recombination resulted in an in-frame insertion and deletion of exon 10 and 92 bp of exon 9 encoding the Asp residue of the catalytic triad. Expression results in a fusion protein consisting of the first 377 amino acids of TMPRSS13 fused to β-galactosidase (Madsen et al., 2014). Genotyping was performed using the following primer sets for Tmprss13: moIMR0012 (5'-GGGTGGGATTAGATAAATGCCTGCTCT-3'), oIMR6316 (5'-AAATGACCCACCTAATTAGCTGTAG-3'), and oIMR6318 (5'-GCCTCAATGAGACCTGTTGGATCAC-3'). To generate cohorts for study, male mice heterozygous for the TMPRSS13-deficiency allele (*T13*$^{+/-}$) were crossed to female *T13*$^{+/-}$ mice to generate wild-type (*T13*^{wt}), *T13*$^{+/-}$, and *T13*$^{-/-}$.

Azoxymethane/DSS model of CRC

For the azoxymethane (AOM)/dextran sulfate sodium (DSS) CRC model, 8-week old mice were subject to intraperitoneal injections with 12.5 mg/kg AOM in sterile phosphate buffered saline. Seven days following AOM treatment, mice were given DSS (1.75% w/v) in drinking water for five days, after which mice were switched to regular drinking water. Mouse body weight was recorded throughout course of DSS treatment and mice were monitored for signs of severe colitis,

such as weight loss, dehydration, bloody stools, and rectal prolapse throughout course of treatment. Mice were euthanized 110 days following AOM treatment.

Tissue Preparation

Colon tissue was dissected whole from euthanized mice for macroscopic and histological examination of tumors, with colons washed with PBS and opened longitudinally. Tumors were counted, and colon mass was recorded. Dissected colons were fixed in 10% neutral-buffered zinc formalin (Z-fix) (Anatech, Battle Creek, MI), embedded in paraffin and cut into 5 µm sections. Sections were stained with hematoxylin and eosin.

3.3 Results

3.3.1 Impact of TMPRSS13 on AOM/DSS-induced tumor development

The role of TMPRSS13 in the development and progression of CRC was examined using a colitis-associated cancer model in TMPRSS13-deficient mice. The AOM/DSS model is an established model of chemically-induced tumorigenesis, involving administration of a genotoxic agent, AOM, followed by repeated exposure to DSS, a gastrointestinal irritant. Tumor progression with this model closely mimics that of human CRC, with development of aberrant crypt foci which progress to become adenocarcinomas (Tanaka et al., 2003; De Robertis et al., 2011). The advantage of using this two-hit model is two-fold: a) AOM/DSS in combination produces tumors in a shorter time interval than by using a carcinogen alone, and b) it functions as an inflammatory model for CRC, which is translationally relevant considering that patients suffering from IBDs, such as Crohn's disease and ulcerative colitis, are at a markedly higher risk for developing CRC (Ekbom et al., 1990b; Ekbom et al., 1990a; Jess et al., 2012). We utilized this model with a mouse with disrupted TMPRSS13 expression resulting from a lacZ insertion spanning exons 9 and 10 of the TMPRSS13 gene. Reverse transcription analysis of TMPRSS13 mRNA using multiple probes

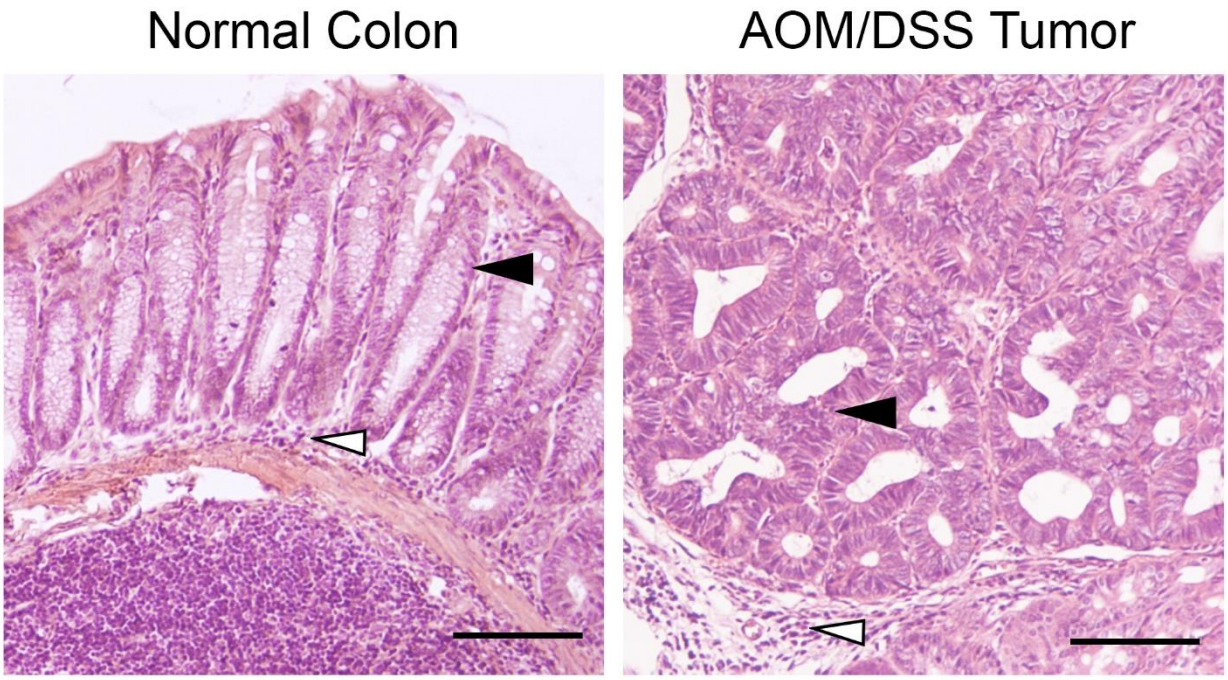

Figure 3.1: AOM/DSS-induced tumor histology
To model the role of TMPRSS13 in development of CRC, mice were subject to AOM treatment followed by a single cycle of DSS treatment lasting 5 days. At 110 days, mice were euthanized and colons dissection for analysis. Images are representative of tumor tissue collected from mice at study endpoint. H&E-stained sections of paraffin-embedded mouse tissue are shown, with normal colon on the left panel and a representative adenoma on the right panel. Black triangles point to epithelial cells of the colon crypts (left panel) and of tumor epithelia (right panel). Lamina propria is indicated by white triangles. Scale bar = 200 µm.

spanning exons 8 through 13 reveal a lack of TMRPRSS13 expression in *T13*$^{-/-}$ mice (Madsen et al., 2014). Previous characterization of this mouse demonstrated that mice heterozygous for the deficient allele (*T13*$^{-/-}$) were indistinguishable from wild-type controls and only exhibited a mild skin phenotype in neonates (Madsen et al., 2014). Our own observations (**Figure 1.8**) show this difference to be observed transiently during the early post-natal period (21 days).

The AOM/DSS treatment schedule proved to be successful with just one cycle of DSS treatment (**Figure 3.1**). The majority of treated animals developed tumors (63% of *T13*^{wt} (n = 16), 83% of *T13*$^{+/-}$ (n = 29), and 75% of *T13*$^{-/-}$ (n = 20)) (**Figure 3.2A**). Despite variation in tumor incidence, no statistically significant differences between the three genotypes were observed.

As a measure of tumor burden we also weighed whole colon from rectum to cecum. We found that *T13*$^{-/-}$ mice exhibited increased colon weight compared to wild-type controls, likely as a result of larger tumor mass (**Figure 3.2B**). No significant difference was observed between *T13*$^{+/-}$ and *T13*^{wt} or *T13*$^{-/-}$. In humans, tumors that develop in different anatomical regions of the colon will also exhibit different molecular aberrations. By splitting the colon into three general regions—proximal colon, mid colon, and distal colon/rectum—we assessed the frequency of tumor formation by region (**Figure 3.3A**). We observed a non-significant trend suggesting that *T13*$^{-/-}$ mice develop tumors more distal colon/rectal tumors, whereas *T13*$^{+/+}$ mice developed more mid colon tumors than other genotypes (**Figure 3.3B**). Mice heterozygous for the disrupted TMPRSS13 allele displayed an intermediate phenotype, with tumors observed in both mid and distal regions.

3.3.2 Impact of TMPRSS13 deficiency on intestinal barrier function

Throughout the duration of DSS treatment, mice were monitored and weighed for signs of severe colitis. Other reported dosages for DSS treatment proved to be too high, causing severe

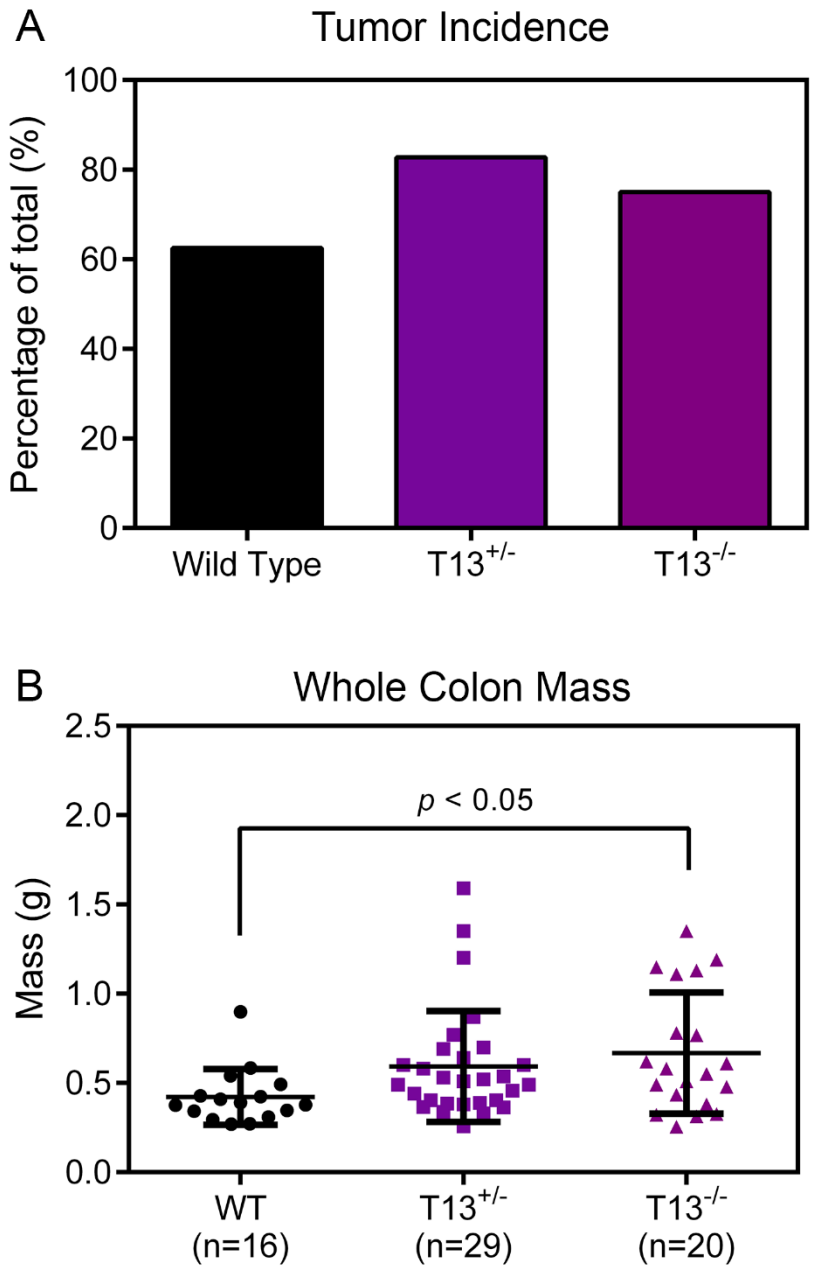

Figure 3.2: Summary of tumor incidence and colon mass in AOM/DSS-treated mice
Dissected colon tissue was analyzed for tumors visually upon collection and microscopically after tissue fixation and paraffin embedding. **(A)** Bar graph representing the percentage of AOM/DSS-treated mice exhibiting tumors. Wild-type mice (n = 16), $T13^{+/-}$ (n = 29), $T13^{-/-}$ (n = 20). Differences were analyzed using Chi-square test, p = 0.3194. **(B)** Scatterplot of whole mouse colon weights following dissection as a measure of tumor burden *in situ*. Mean values are indicated with error bars representing SEM. Tukey post-hoc test used to analyze differences following a one-way ANOVA. T13 = TMPRSS13.

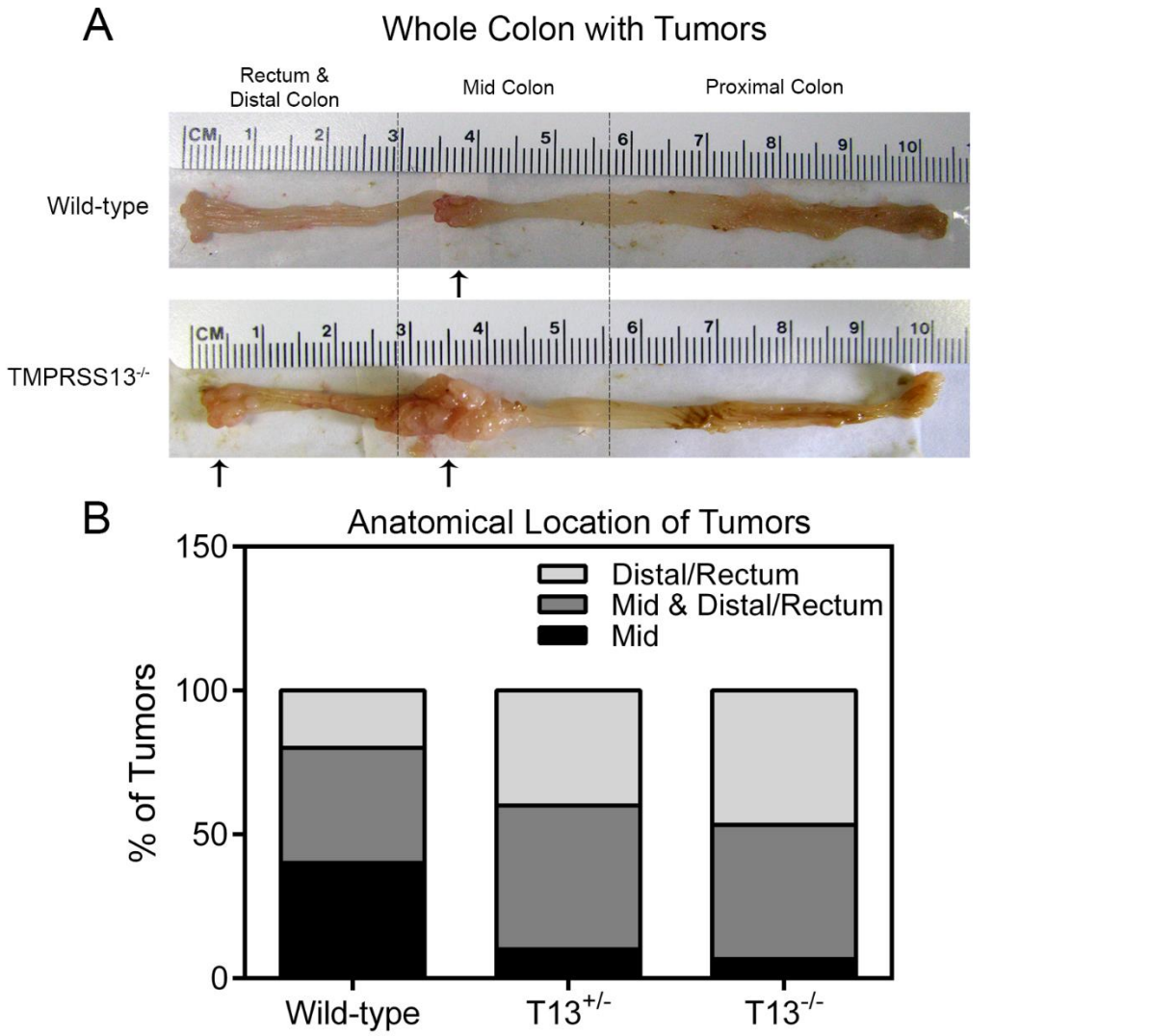

Figure 3.2: The impact of TMPRSS13 on location of tumor development in the colon
Tumor location was found to be vary non-significantly between wild-type and TMPRSS13-deficient mice ($T13^{-/-}$). **(A)** Images of representative colon with tumors. Genotypes for each panel are shown. Colon samples are oriented rectum on the left to proximal colon on the right. The anatomical regions used to sort tumor location are indicated by labels and separated by dotted line. Tumors are labelled using arrows. **(B)** Graphical representation of tumor location. Black bar represents mice exhibiting tumors only in the mid colon; dark gray represents mice exhibiting tumors in both mid colon and distal colon/rectal region; light gray represents mice exhibiting tumors exclusively in the distal colon/rectum. No statistically significant difference was observed following chi-square analysis ($p = 0.1765$).

diarrhea and weight loss for our strain of mice (Tanaka et al., 2003; Netzel-Arnett et al., 2012). We found a five-day regimen of 1.75% (w/v) DSS in water to induce a level of colitis from which mice could recover upon cessation of DSS exposure. Signs of colitis were not observed until roughly four days after DSS exposure. Weight loss began to occur on day four as well, with most severe signs of colitis and weight loss occurring following cessation of DSS exposure. Upon plotting weight loss as a percentage of initial weight, we found that *T13*$^{-/-}$ mice experienced increased weight loss as a result of DSS treatment compared to controls (*T13*$^{+/-}$) (**Figure 3.4**). This difference proved to be statistically significant on days 7-11, where *T13*$^{-/-}$ mouse experienced nearly 20% loss in weight, compared to roughly 12% loss in control mice. Weight loss was indicative of an increased sensitivity to DSS-induced colitis experienced by TMPRSS13 deficient mice, suggesting an important role for this protease in intestinal epithelial function.

3.4 Discussion

To identify the role of TMPRSS13 in CRC, we utilized the AOM/DSS model in TMPRSS13-deficient mice. Tumor development was observed in mice after exposure to a single cycle of DSS, with the majority of mice presenting with tumors. Analysis of differences between *T13*^{wt}, *T13*$^{+/-}$, and *T13*$^{-/-}$ deficient mice did not reveal differences with respect to tumor incidence, however we observed a significant increase in colon mass in TMPRSS13-deficient mice compared to wildtype mice. We also identified a genotype-dependent trend in tumor location within the colon, where TMPRSS13 mice exhibited more distal colon and rectal tumor, whereas wild-type mice developed more mid colon tumors. In our model, we also found *T13*$^{-/-}$ mice to be more susceptible to DSS treatment, suggesting both increased sensitivity to cellular stress and increased intestinal permeability. Taken together, these findings signal a role for TMPRSS13 in intestinal homeostasis, a role not yet identified for this protease, and in the development of CRC.

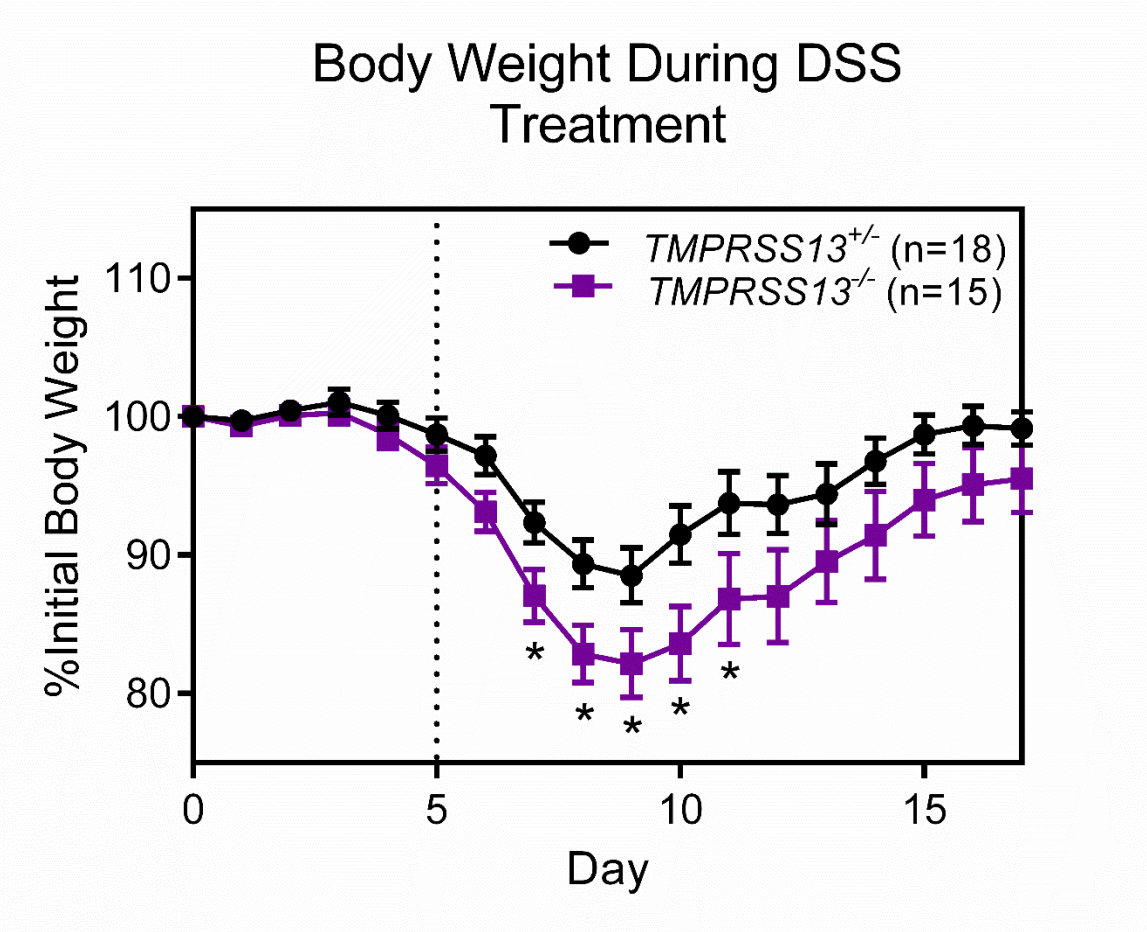

Figure 3.4: TMPRSS13-deficient mice exhibit increased sensitivity to DSS-treatment
Line graph of mouse weights throughout course of DSS treatment. Mice were exposed to DSS (1.75% w/v) in drinking water for five days, after which regular drinking water was restored. Dotted line indicated cessation of DSS treatment. TMPRSS13-deficient mice exhibited greater weight loss in response to DSS treatment in comparison to control (*TMPRSS13*+/-) mice. Determined through two-way ANOVA with Tukey post-hoc test. * = $p < 0.05$.

Previous characterization of the TMPRSS13-deficient mouse line utilized for these studies did not identify histological defects in gastrointestinal tissue, consistent with our own observations (Madsen et al., 2014). The increased severity of colitis in TMPRSS13-deficient mice following DSS treatment therefore represents a functional impairment in colon function that has yet to be reported. One possible explanation for this increased DSS sensitivity is that TMPRSS13 deficiency contributes to impaired intestinal barrier function. The importance of regulated proteolysis in intestinal epithelial function is well demonstrated by studies of matriptase in mice. Hypomorphic mice expressing highly reduced levels of matriptase generally develop normally expect for mild epidermal defects, but exhibit increased colitis and weight loss in response to DSS treatment, similarly to our observations with TMPRSS13-deficient mice (Netzel-Arnett et al., 2012). In loss of function studies, mice with intestinal-specific genetic matriptase ablation exhibit severe colitis resulting from a compromised epithelial barrier function (Kosa et al., 2012). Conversely, unregulated proteolysis may also have a negative impact. Analysis of mRNA transcripts of human CRC tissue reveals attenuated inhibition of matriptase by HAI-1 (Vogel et al., 2006). In mice, loss of HAI-2, the endogenous inhibitor of TMPRSS13, matriptase, and prostasin, results in prenatal lethality as a result of unregulated proteolysis (Szabo and Bugge, 2018; Kawaguchi et al., 2019). Loss of HAI- also leads to degradation of junctional proteins such as epithelial cell adhesion molecule (EpCam) and claudin-7, which could be rescued *in vivo* when HAI-2 loss occurs in mice expressing a zymogen locked form of prostasin, or by co-deletion of prostasin in intestinal organoid culture (Szabo and Bugge, 2018; Kawaguchi et al., 2019). These studies demonstrate how 1) proteolytic regulation of intestinal homeostasis is important for maintaining barrier function, such that either increased and decreased proteolysis is detrimental and 2) how the matripase-prostasin proteolytic network contribute to intestinal homeostasis. In our own

preliminary observations, we have found TMPRSS13 to potentially participate in the matriptase/prostasin network, with siRNA-mediated knockdown of TMPRSS13 resulting in upregulation of prostasin and matriptase (data not shown). While more work needs to be done to fully elucidate these interactions, it remains possible that loss of TMPRSS13 contributes to deregulation of the activity of other proteases important for normal epithelial function.

The impairment of normal intestinal function in TMPRSS13-deficient mice has a potentially far-reaching impact in our studies, as the contribution of colitis in the AOM/DSS model is key to tumor development. Based on previous findings implicating a role for TMPRSS13 in CRC, we hypothesized a TMPRSS13-deficient mouse would be more resistant to tumorigenesis. Unexpectedly, in the AOM/DSS colitis-associated cancer model, we observed that TMPRSS13-deficient mice displayed and increased colon mass, suggesting an increased propensity of TMPRSS13-deficient mice to develop tumors compared to wild-type mice. We speculate that increased DSS sensitivity resulting from TMRPSS13-deficiency can be attributed to increased intestinal permeability, a key factor in colitis. Severe and chronic inflammation in the intestines is a strong risk factor CRC development (Ullman and Itzkowitz, 2011; Monteleone et al., 2012). Inflammation resulting from gut permeability has been modeled in mice lacking matriptase expression in the intestinal tract, which spontaneously development colon adenocarcinomas as a result of loss of barrier function and rampant inflammation (Kosa et al., 2012). In the context of how inflammation contributes to carcinogenesis, it is vital to understand the role of TMPRSS13-deficiency in normal colon function to be able to completely gauge the role of TMPRSS13 on cancer. Any oncogenic properties conferred by TMPRSS13 expression can be masked by a barrier defect resulting from loss of function. Thus, the AOM/DSS model complicates the interpretation pertaining to the contribution of TMPRSS13 in CRC.

Use of a CRC mouse model lacking the inflammatory element of AOM/DSS could potentially provide a clearer picture of TMPRSS13 as a tumor promoter. One of the most commonly used genetic models is the ApcMin mouse expressing mutant *APC*, a well-characterized tumor suppressor gene in CRC (Moser et al., 1995). This model recapitulates the inheritable form of CRC, familial adenomatous polyposis (FAP), also caused by mutations in the *Apc* gene (Groden et al., 1991; Kinzler et al., 1991). Unfortunately, the ApcMin mouse, is a poor model for CRC due to the infrequent number of adenomas that develop in the colon, with the majority of lesions found in the small intestine, however this model could potentially inform how TMPRSS13 may interact with the changes caused by mutant *APC*. To best model the impact of TMPRSS13 in CRC would require eliminating the impact of a defective barrier, Orthotopic xenografts circumvent this challenge by allowing for the growth of eells implanted in a normal intestinal background (Hoffman, 1999). This model also allows for easy incorporation of genetic modifications, such as mutations affecting TMRPSS13 catalytic activity or zymogen activation, Orthotopic grafts would permit the study of the impact of TMPRSS13 on tumor growth but would not allow for modeling the contribution of TMPRSS13 in CRC initiation, as grafts require implantation of human tumor tissue or cancer cell lines. Thus, care must be taken in choice of model when studying an organ system as tightly regulated as the colon. It is worth noting that the TMPRSS13-deficient mouse utilized in this study produces a TMPRSS13 protein fusion product with β-galactosidase, however the LacZ insertion replaces the exon encoding the catalytic triad aspartic acid (Madsen et al., 2014). Due to the lack of phenotype in mice heterozygous for this *TMPRSS13* allele, we consider it unlikely that the protein product has any dominant negative properties or contribution to development of CRC.

A non-significant trend in the location of AOM/DSS-induced tumors was found to vary between genotypes. Namely, *T13*$^{-/-}$ exhibited more distal colon/rectal tumors than wild-type animals, which had more mid-colon tumors. Interestingly, expression of TMPRSS13 in the mouse colon is highest in the distal colon, whereas expression is minimal in the proximal colon (Faller et al., 2014). We did not observe the development of tumors in the proximal colon of any animals. The variation observed in tumor location may reflect subregions within the colon where TMPRSS13 plays functional roles. More detailed analyses of the molecular differences across the colon are necessary to better understand how the colorectal subregions differ. In humans, certain CRC molecular profiles are associated with the location where tumors are found. Importantly, mutation status of *KRAS* and *BRAF* is strongly associated with tumor location. Specifically, distal colon tumors of the distal colon are more likely to lack mutations in *KRAS/BRAF*, which, along with microsatellite instability, are more common in proximal tumors. (Missiaglia et al., 2014; Arnold et al., 2017; Loree et al., 2018). Mutations status of *KRAS/BRAF* is a strong predictor of response to treatment with the anti-EGFR monoclonal antibody cetuximab, making tumor location a prognostic marker (Arnold et al., 2017; Ghidini et al., 2018). Understanding how the expression of TMPRSS13 varies among proximal and distal tumors in humans and how TMPRSS13 interacts with both proximal and distal molecular profiles can help inform the potential benefit of therapeutically targeting this protease,

These findings suggest a role for TMPRSS13 in maintenance of barrier function, a function that past studies have identified requires tight regulation of the matriptase/prostasin proteolytic network. TMPRSS13 may contribute to this proteolytic chain to promote epithelial homeostasis, but more work is required to determine this possibility. The impact of TMPRSS13 on CRC

development remains inconclusive, however the use of alternative models may aid in interrogating the oncogenic potential of this protease.

CHAPTER 4: CONCLUSIONS AND FUTURE DIRECTIONS

The body of work presented here represent one of the first characterizations of the type II transmembrane serine protease TMPRSS13 in a cancer of any type. Following up on the identification of upregulated TMPRSS13 transcript in human colorectal cancer (CRC), we found that TMPRSS13 protein was also increased in human CRC. We sought to determine whether TMPRSS13 functioned to drive cancer development in growth. Through the use of tissue culture, we discovered that TMPRSS13 played a role in cellular survival, evidenced by the increase in apoptosis following attenuation of TMPRSS13 through siRNA-mediated silencing of gene expression. Silencing of TMRSS13 expression also resulted in reduced invasiveness of human CRC lines and increased susceptibility to drug-induced apoptosis. Additionally, TMPRSS13 overexpression was found to promote resistance to Bcl-2 inhibition and paclitaxel treatment. Taken together, these findings suggest that increased TMRPSS13 expression plays a vital role in the survival of CRC cells, and may confer resistance to drug treatments. Baseline expression levels of TMPRSS13 in normal colon are low, minimizing the potential impact of TMPRSS13 inhibition and maximizing tumor selectivity (Faller et al., 2014; Madsen et al., 2014). These factors make TMPRSS13 an attractive putative target in CRC.

To better understand the potential of TMPRSS13 as a therapeutic option, it is important to better understand the pathways underlying the phenotypes we observed. For example, loss of TMPRSS13 resulted in both increased cell death and increased sensitivity to certain cytotoxic agents. In particular, HA14-1, a selective Bcl-2 inhibitor, was especially effective at inducing apoptosis following TMPRSS13 knockdown, an effect that was dramatically reduced by TMPRSS13 overexpression. Understanding what pathways may synergize or the interaction between the apoptotic pathways regulated by Bcl-2 expression and TMPRSS13 is key to exploiting

the pro-survival role TMRPSS13 plays. Following changes to intrinsic pathway proteins following induction of apoptosis in a TMPRSS13-deficient or -sufficient genetic background would help inform of differences influenced by protease activity

In addition to our tissue culture studies, we also utilized a TMPRSS13-deficient mouse in conjunction with a colitis-associated model of CRC, hypothesizing that reduced TMPRSS13 would have a protective effect against cancer. We found that TMRSS13-deficient mice exhibited greater tumor burden compared to wild-type controls, suggesting that TMPRSS13 loss had a mal-adaptive role rather than a protective one. Induction of tumors in mice was performed using the azoxymethane (AOM)/dextran sulfate sodium (DSS) model of CRC, which involves induction of colitis through DSS treatment. TMPRSS13-deficient mice displayed increase sensitivity to DSS-treatment, suggesting an impairment in epithelial function. Considering the strong link between colitis severity and duration with CRC, it is difficult to determine the contribution of TMPRSS13 to CRC, however this finding does highlight a role for TMPRSS13 in normal intestinal function. Proteolysis in the colon is tightly regulated, with overactive protease activity or loss of proteolysis having negative impact (Kosa et al., 2012; Szabo and Bugge, 2018). It is not yet known what role TMPRSS13 plays in maintaining normal intestinal epithelial function, but an interaction with the matriptase-prostasin axis may be implicated, with yet unpublished from our lab demonstrating loss of TMPRSS13 impacts both matriptase and prostasin levels in tissue culture models. It would be important to characterize how loss of TMPRSS13-competency in our mouse model affects matriptase and prostasin activity, which may inform both how TMPRSS13 can impact epithelial homeostasis, as well as identify a proteolytic network utilizing TMPRSS13.

Our mouse model also revealed a difference between TMPRSS13-deficient mice and wild-type mice with respect to where tumors developed along the colon, such that TMPRSS13-

deficiency resulted in greater frequency of distal colon and rectal tumors, which were infrequent in wild-type mice. Much remains to be understood about the differential molecular profiles along the span of the colon, but considerable differences are found in the expression patterns of proximal colon tumors compared to distal colon tumors (Loree et al., 2018). A comprehensive examination of TMPRSS13 expression along both human and mouse colon would help inform the types of tumors expression of this protease is associated with. This has the biggest impact on treatment. One of the major issues in CRC treatment is resistance to drug therapy. Key molecular changes have been identified to strongly associate with poor response to treatment. For example, mutations in *KRAS*/*BRAF* are associated with poor response to cetuximab and poor outcome (De Roock et al., 2008; Karapetis et al., 2008; Arnold et al., 2017). MSI is highly associated with poor response to 5-fluorouracil treatment, the standard of care chemotherapeutic agent (Ribic et al., 2003; Carethers et al., 2004; Des Guetz et al., 2009). Identification of overexpression of TMPRSS13 in proximal tumors, which are more difficult to treat, would greatly increase the value of TMPRSS13 by providing a target in refractory cancer. The study outlined in this dissertation thus provides a jumping off point for further study of this multi-faceted protease in CRC.

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ABSTRACT

THE ROLE OF THE CELL-SURFACE PROTEASE TMPRSS13 IN COLORECTAL CANCER

by

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Colorectal cancer (CRC) is one of the most common and deadly cancers in both men and women in the United States. Extracellular proteolysis is often dysregulated in cancer including (CRC), resulting in degradation of extracellular matrix, as well as cleavage, processing, or shedding of cell adhesion molecules, growth factors, and cytokines. Several members of the type II transmembrane serine protease (TTSP) family have been shown to play critical roles in cancer progression; however, many family members have not yet been characterized in malignancy. We identified TMPRSS13 transcript to be upregulated in CRC compared to normal colon. This increase was confirmed at the protein level by immunohistochemical analysis of CRC tumor tissue arrays. Mechanistic studies revealed increased apoptosis an impaired invasive potential following TMPRSS13 silencing in human CRC cells. Importantly, TMPRSS13 gain-of-function promoted resistance to the apoptosis-inducing agents paclitaxel and HA14-1 Reversely, TMPRSS13 loss-of-function caused increased sensitivity of CRC cells to drug-induced apoptosis. Use of a TMPRSS13-deficient mouse line in the azoxymethane/dextran sulfate sodium (DSS) model of CRC revealed increased sensitivity to DSS treatment. This sensitivity manifested as increased colitis and severe weight loss indicative of a defect in intestinal barrier function. TMPRSS13-

deficient mice also exhibited greater tumor burden compared to wild-type mice, likely as a result of increased severity of colitis. Together these findings demonstrate a critical role for TMPRSS13 in pro-oncogenic processes as well as intestinal homeostasis and identify TMPRSS13 as potential novel target for CRC cancer therapy.

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Publications

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