Molecular mechanisms governing CEACAM1-L-regulated colon cancer metastasis

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Abstract

The two major isoforms of CEACAM1 (CC1) have different effects on colorectal cancer (CRC) growth and metastasis. Izzi *et al.* have shown that CC1-L Tyrosine phosphorylation inhibits CRC growth and metastasis *in vivo* in xenograft assays, which opposes the minimal effect of CC1-S. It has also been shown in mouse colon carcinoma CT51 cells that the Ser503A residue is essential for Tyr488 phosphorylation, and that mutants of the three lysines (3K-3A) at the C-terminus of the protein display similar results. However, the impact on CRC metastasis of these last mutants is unknown. We introduced the Ser503 and 3K-3A mutants into the MC38 murine CRC cell line and selected and sorted cell populations for these mutants. Proliferation of these mutant cell lines was assessed *in vitro.* Then, the mutant CRC mouse cell lines (MC38) intrasplenically injected into mice (C57Bl/6) to investigate their metastatic ability *in vivo*. The metastatic burden was measured in the liver of these animals, and it was found that mutation of the Ser503, Ser516, and 3 terminal Lys abrogated the tumor inhibitory phenotype of CC1-L. Future studies include identifying the kinase responsible for Ser503 phosphorylation and identifying the pathways involved.

Résumé

Les deux isoformes principales de CEACAM1 (CC1) ont des effets différents sur la croissance et la métastase du cancer colorectal (CRC). Izzi *et al.* ont démontré par des analyses xénogreffes que la phosphorylation des tyrosines de CC1-L inhibe la croissance et la métastase du CRC *in vivo*, ce qui oppose l'effet minimal du CC1-S. Dans les cellules murines colorectales CT51, il a été démontré que le résidu Ser503 est essentiel pour la phosphorylation du Tyr488, et que les mutants des trois lysines (3K-3A) produisent des résultats similaires. Cependant, l'effet de ces mutants sur la métastase du CRC est inconnu. Nous avons donc généré des lignées mutantes dans la lignée colorectale métastatique MC38 par infections rétrovirales de ces mutants. Nous avons sélectionné des populations de cellules et isolé des populations uniformes par triage cellulaire. La prolifération de ces lignées cellulaires mutantes a été évaluée *in vitro.* Ensuite, les lignées cellulaires murines et mutantes du CRC (MC38) ont été injectées dans la rate des souris C57Bl/6 afin d'évaluer leur capacité métastatique *in vivo*. La charge métastatique du foie a été mesurée, et nous avons trouvé que la mutation des Ser503, Ser516, et des 3 Lys terminales diminue le phénotype de la lignée exprimant CC1-L, qui agit comme un inhibiteur des tumeurs. Les études ultérieures porteront sur l'identification de la kinase qui phosphoryle le Ser503 et des voies de signalisation qui sont impliquées.

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List of Abbreviations

Ab	Antibody
ACF	aberrant crypt foci
Akt	(also known as PKB - protein kinase B)
Ang-2	angiopoietin-2
BM	basement membrane
BMDC	bone marrow-derived cells
Bp	base pair
BSA	bovine serum albumin
CAFs	cancer-associated fibroblasts
CC1	CECAM1
CC1-L	CEACAM1 long isoform
CC1-S	CECAM1 short isoform
CDK	cyclin-dependent kinase
CEA	carcinoembryonic antigen
CEACAM1	Carcinoembryonic antigen cell adhesion molecule 1
CRC	colorectal cancer
ECM	extracellular matrix
EGF(R)	epidermal growth factor (receptor)
EMT	Epithelial Mesenchymal Transition
ERK	extracellular signal-regulated kinase
FAK	focal adhesion kinase
FBS	fetal bovine serum
FAP	familial adenomatous polyposis
h	hour(s)
HNPCC	hereditary non-polyposis CRC
hnRNP	heterogeneous nuclear ribonucleoprotein
Ig	immunoglobulin
IR	insulin receptor
ITIM	Immuno-receptor Tyrosine-based Inhibitory Motif

JPS	juvenile polyposis syndrome
LOH	loss of heterozygosity
Lys	Lysine
mAb	monocloncal antibody
MAP	mitogen-activated protein
MET	mesenchymal to epithelial transition
Min.	minute(s)
MFI	median fluorescence intensity
MMP	metalloprotease
MMR	mismatch repair
NK	natural killer
NO	nitric oxide
NOCs	N-nitroso compounds
pAb	polyclonal antibody
PDCD4	Programmed Cell Death 4
PDGFR	Platelet-derived growth factor receptor
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PSG	pregnancy-specific glycoproteins
PTEN	phosphatase and tensin homolog
ROS	reactive oxygen species
RTK	receptor tyrosine kinase
SD	standard deviation
Ser	Serine
TAMs	tumor-associated macrophages
TGF-β	transforming growth factor
TNFα	tumor necrosis factor alpha
Tyr	Tyrosine
uPAR	urokinase-type plasminogen activator receptor
VEGF(R)	vascular endothelial growth factor (receptor)

I. Introduction

1.1 Colorectal cancer

Colorectal cancer (CRC), cancer of the colon and rectum, is a very prevalent malignancy and remains a leading cause of death and morbidity worldwide, but particularly so in North America and Western Europe [1]. In fact, the Canadian Cancer Society predicted that in 2013 there would be 23, 800 new CRC cases and that 9, 200 deaths would ensue from CRC [2]. CRC arises due to environmental and genetic factors.

1.1.1 Environmental Factors and CRC

It is estimated that 30-40% of all tumors could be prevented by correcting diet and lifestyle [3]. The "Westernized" diet, high in red meat and fat, is related to increased CRC occurrence [4]. This is due to an increased level of N-nitroso compounds (NOCs) in the colon, which have been found to cause DNA damage [5]. At the same time, case-control studies showed that a diet high in fiber decreases CRC risk [6]. Furthermore, consistent alcohol consumption also increases risk for CRC via induction of cytochrome P4502E1 (CYP2E1), which converts many known pro-carcinogens to carcinogens [7]. Interestingly, tobacco use not only increases the risk of lung cancer but it also increases the risk of rectal cancer [8]. Obesity and lack of exercise also increase the risk of CRC, likely through inflammatory processes, adipokines, and estrogen [9]. Despite these well-defined environmental factors, CRC is mainly characterized by its genetic alterations.

1.1.2 Genetic Factors and CRC

On a genetic level, CRC arises from the accumulation of genetic changes in important oncogenes, tumor suppressor genes, and mismatch repair (MMR) genes [10]. Proto-oncogenes are genes that have the potential to induce cancer and, when activated, become oncogenes which promote pro-cancerous processes [11]. In contrast, a tumor suppressor gene is a gene that is normally expressed and acts to protect the cell from one or more pro-cancerous pathways [12]. When a tumor suppressor gene is inactivated by mutation, for example, the cell can become cancerous. Knudson first identified tumor suppressor genes during his investigation into childhood retinoblastoma [13, 14]. From these studies he also developed his "Two-hit"

hypothesis, which stated that, in the case of childhood retinoblastoma, the first genetic "hit" (mutation) is hereditary, and the second "hit" occurs during life (resulting in loss of heterozygosity (LOH) at that locus), and gives rise to cancer formation. MMR genes are responsible for repairing DNA damage as well as for recognizing and repairing DNA bases that have been inserted or deleted [15]. While there are many genes that are altered in colorectal cancer, as described by The Cancer Genome Atlas Network [16], there are six genes which are most frequently and characteristically altered in CRC: *APC* (Adenomatous polyposis coli), *KRAS* (Kirsten rat sarcoma viral oncogene homolog), *SMAD4* (Mothers against decapentaplegic homolog 4) also known as *Dpc4* (deleted in pancreatic carcinoma, locus 4), *TP53* (tumor protein 53), and the MMR genes *MLH1* (MutL homolog 1) and *MSH2* (MutS protein homolog 2) [17].

Tumor suppressor Genes

a) *APC*

APC is the predominant gene affected in colorectal cancer, as its inactivation is found in most sporadic colorectal cancers [18] and is also responsible for familial adenomatous polyposis (FAP) [19], a hereditary form of CRC. *APC* is a tumor suppressor gene located on the chromosome 5q21 locus [20], and encodes a scaffolding protein involved in the WNT signaling pathway [21]. The WNT pathway, which regulates signal transduction across the cell membrane, plays a large role in development and in carcinogenesis [22]. WNT signaling results in accumulation of β-catenin in the nucleus, which in turn activates transcription factors that promote cellular proliferation and migration [23, 24]. This protein is a relatively large 310 kDa protein and is multifunctional, with the central portion of the protein containing domains that interact with members of the WNT signaling pathway. There are three 15 amino acid repeats that bind to β-catenin, followed by seven 20 amino acid repeats that regulate β-catenin by phosphorylation [10]. There are also SAMP sequences that bind to axin. Glycogen synthase-3β kinase (GSK3β) interacts with APC as well. Both axin and GSK3β are members of the WNT signaling pathway. Mutations in *APC* result in increased nuclear β-catenin levels, which activates, amongst others, the *C-MYC* gene, and the Cdk4/cyclin D1/pRB/p16 pathway in CRC development [20].

Common mutations in *APC* result in a truncated protein, which lacks domains involved in down-regulating β-catenin via phosphorylation as well as its axin-binding domain [10].

Shortened APC proteins due to termination mutations are also quite unstable and degrade very quickly. These mutations are most often associated with FAP, whereas mutations in the mutation cluster region of *APC,* between codons 1286-1513, are mostly associated with spontaneous cases of CRC [25].

There are several *APC* mouse models that were created to better study the mutations found in human populations. An excellent review of these mouse lines is found by Fodde [26]. It should be noted that similar investigations in *APC*-mutant mice revealed that intestinal tumors did not exhibit somatic mutations in the *Trp53 (*mouse *TP53*) and *Kras* genes [26]. This difference is likely due to discrepancies in lifespan and genetics between humans and mice [10].

b) *SMAD4*

SMAD4 participates in the transforming growth factor (TGF-β) signaling pathway, which regulates many cancer-related cellular functions such as differentiation, apoptosis, and the cell cycle [27]. SMAD4 binds to receptor-related SMADs and is responsible for translocating the complex into the nucleus. SMAD4 mutations are commonly found in many types of cancers [28]. Found on chromosome 18, the tumor suppressor *SMAD4* is mutated in 50% of large colonic adenomas and 75% of CRCs [29-32]. Mutation of *SMAD4* is also associated with the hereditary disease juvenile polyposis syndrome (JPS), which predisposes children to polyps and cancer [33]. However it is not the only gene responsible for the disease. The SMAD proteins are members of a small family of proteins that are highly homologous, and contain two highly conserved domains joined by a linker domain: an N-terminal Mad homology domain-1 (MH1) and a C-terminal Mad homology domain-2 (MH2) [34]. In CRC, normally the MH2 domain is usually mutated in the region that permits hetero-oligomerization with other SMAD proteins [35, 36]. This blocks the TGF-β pathway and allows the tumor to proliferate autonomously [17] by preventing the transcription of the cyclin-dependent kinase (CDK) inhibitor p21 [37].

c) *TP53*

LOH of the tumor suppressor *TP53*, found on chromosome locus 17p13, is found in 75% of colorectal carcinomas [29, 38]. It encodes the p53 protein, the "guardian of the genome," which induces cell death by apoptosis upon sensing DNA damage and by up-regulating CDK inhibitor p21 [17]. p53 also regulates the cell cycle (G1/S transition), activates DNA repair

proteins, can induce senescence, and controls differentiation [39]. All of these processes are implicated in regulation of cancer, which is why p53 is deactivated in many cancers, including CRC. *TP53* is activated upon DNA damage, hypoxia, telomere erosion, deprivation of nutrients, and abnormal cellular proliferation driven by oncogene activation [40]. Approximately 85% of the mutations in *TP53* in cancer (especially CRC) are missense, and typically occur at codons 175, 245, 248, 273, and 282 [41-43]. In CRC, *TP53* mutations represent late events being most prominent in stage III adenocarcinomas [43].

Oncogenes

d) *KRAS*

The main oncogene associated with CRC is *KRAS,* which is mutated in roughly 50% of sporadic CRC carcinomas [29, 44, 45]. KRAS is a guanine nucleotide (GDP/GTP)-binding protein that is a self-inactivating signal transducer affecting the cancer-related cellular processes of proliferation, apoptosis, and senescence [46]. It is mutated in 95% of pancreatic cancers, 55% of thyroid cancers, and 35% of lung cancers [46]. It is located at chromosome locus 12p12.1, and encodes for a small GTPase that acts as a messenger between receptor activation and downstream pathways that affect such cellular processes as survival, proliferation, and differentiation [47, 48].

Mutations in *KRAS* are normally found at codons 12, 13, and 61, keeping it in a constitutively active state [49, 50]. Such mutated RAS proteins affect many pro-cancer downstream pathways. An excessive amount of RAS drives proliferation through the RAS-RAF-MEK-ERK pathway [51-53]. RAS is also part of the Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) pathway (which is antagonized by phosphatase and tensin homolog (PTEN)), and activates AKT to suppress apoptosis [17]. *MDM2* transcription is also affected by RAS activation, which explains the increased resistance to p53-dependent apoptosis [54]. In CRC, KRAS mutations are essential for the progression of stage I to stage II [55].

Mismatch repair genes

e) MMR genes, *MLH1* and *MSH2*

Mutations of *MLH1,* on chromosome3, and *MSH2*, on chromosome 2, give rise to microsatellite instability (MSI) [56], which is the state of genetic hypermutability. In fact,

mutation of these two genes is responsible for the hereditary non-polyposis CRC (HNPCC or Lynch syndrome). Normally, *MLH1* and *MSH2* help to detect incorrect bases in DNA and work to correct it. *MLH1* promoter hypermethylation (blocking transcription) and *MSH2* alteration inhibits normal base-pair correction, which gives rise to MSI and a pro-cancerous environment [57].

1.1.3 Development of CRC

A primary CRC tumor develops as a consequence of activating proto-oncogenes and by inactivating tumor suppressor genes, such as the ones mentioned above. The molecular pathology of CRC was well defined in 1990 by Fearon and Vogelstein [55] as a series of progressions from normal intestinal epithelia, to aberrant crypt foci (ACF), to polyps, to adenoma, to carcinoma **(Figure 1)** [10]. Each stage has a particular histological profile, and can be further defined by a number of genetic alterations.

a) ACF

ACF are tiny non-cancerous lesions that can only be seen by methylene blue staining or by microscopy [58], and are characterized by enlarged crypts and by thickened epithelium with reduced mucin, which behaves as a lubricant [1]. There are two main types of ACF: those that could develop into hyperplastic (increase of number of cells and not necessarily cancerous) polyps and normally have K*RAS* mutations, and those that could develop into dysplastic (change in cell phenotype, more likely to give rise to cancer) polyps and typically have *APC* mutations [1]. *KRAS* mutations typically occur between the ACF and adenoma stages of CRC progression.

The incidence of *APC* mutations in all stages of colorectal cancer and molecular analyses using relevant mouse models indicate that it is the earliest mutation in CRC development **(Figure 1)**, providing a selective advantage which allows the initiation of clonal evolution, which will be explained in section 1.2 [18]. Furthermore, starting with the *APC* mutation and continuing through to the adenoma and carcinoma stages, the levels of nuclear β-catenin continue to rise, leading to the activation of the pro-proliferation pathways mentioned above [59].

b) Adenoma

This stage is considered pre-cancerous and is marked by intra-epithelial neoplasia, dysplastic polyps, and general increased histological disorder [60]. Dysplasia normally begins towards the base of the crypt and continues toward the luminal surface, and the dysplastic cells have not extended beyond the lamina propria. The cells tend to be enlarged, to have hyperchromatism, and to have lost polarity [1]. Activating mutations of *KRAS* are considered to occur at this time, and are believed to act in synergy with *APC* mutations to permit growth and progression of the tumor. However, given the fact that 50% of CRC do not have *KRAS* mutations it is probable that either other oncogenes play a role or that epigenetic changes might replace the need for *KRAS* mutations.

c) Carcinoma

Carcinomas, characterized by an invasion into the muscularis mucosa, are marked by high histological disorder and by large and tall epithelial cells [1, 60]. Genetic alterations in *TP53* and *SMAD4* in this late stage of progression are required for further clonal expansion and malignant transformation [10].

The histopathological and genetic events that govern CRC progression from an ACF to carcinoma are, as explained above, relatively well understood. However, less is understood about the molecular and genetic changes that regulate the metastasis of CRC, as well as its timing. This is an important point considering that 40% of CRC patients develop hepatic metastasis and that, furthermore, most of these patients will succumb to their metastatic disease [61]. Given these facts, the goal of this Masters project is to contribute to the knowledge of the mechanisms controlling CRC hepatic metastasis.

1.2 Understanding Metastasis

Metastasis is defined as the "transfer of disease from one organ or part to another not directly connected to it" [62] and is one of the essential hallmarks of cancer as described by Hanahan and Weinberg [63]. There are currently six proposed models of the metastatic process, each with their own limitations, as described by Hunt [64]. The model that has been generally accepted to explain CRC metastasis is the linear progression model [65], first described by

Nowell as the clonal evolution model in the context of leukemia [66]. It proposes that the primary tumor accumulates somatic genetic alterations in a linear, clonal evolutionary fashion. During this process, a small number of cells in this primary tumor develop full metastatic potential [64, 67, 68]. The metastatic process is comprised of a number of interlinked sequential steps, all of which can be rate-limiting [69-71], and is often referred to as the metastatic cascade **(Figure 2)**.

1.2.1 The Metastatic Cascade

a) Migration and Invasion

The first step in metastasis is Epithelial Mesenchymal Transition (EMT). This describes the process of cells releasing contact with the basement membrane and with each other, and changing biochemically and physiologically into a mesenchymal cell, displaying increased migratory properties. This process is regulated by members of the SNAIL family (SNAI1 and SNAI2), by EMT-inducing transcription factors listed here [72], and by non-coding RNAs (members of the miR-200 family) [73]. Initiation of this process occurs via signaling from the stroma and from the tumor itself. Interestingly, an increased level of nuclear β-catenin has been functionally connected to EMT in CRC [74]. The β-catenin downstream target genes contribute to invasion and dissemination, and their roles are discussed below.

Invasion of CRC is regulated in part by prostaglandins, especially the E2 series (PGE2), which transactivates c-Met-R, increases tyrosine phosphorylation resulting in the accumulation of β-catenin in the nucleus, and up-regulates expression of the urokinase-type plasminogen activator receptor (uPAR) mRNA [75]. This is all contingent on the presence of functional EGFR, and this signaling has been shown to be essential to the invasive phenotype in CRC. In addition to this, the laminin γ chain [76], fibronectin [77], and the axon guiding factor L1 [78] have been found up-regulated at the invasive front of CRC, which implicates them in invasive regulation.

The ECM interacts with the tumor cell most commonly via cell adhesion receptors (integrins), and is comprised of five types of macromolecules: collagens, laminins, fibronectins, proteoglycans, and hyaluronans [65]. The basement membrane (BM) is a specialized type of ECM, and its degradation is an important step in EMT and in invasion of CRC cells. The presence of the BM contains tumor cells to that local environment and acts as a barrier to

invasion and metastatic spread. Once degraded, invasion and metastatic spread are facilitated [65]. The α6β4 integrin, which is normally involved in maintaining adhesion of epithelial cells to the BM, promotes cell migration on laminin-1 in CRC [79]. Furthermore, metastatic CRC cells are associated with the stromal cell expression/secretion of MMPs (collagenase IV), which degrade the ECM [80]. These processes and signaling pathways are all implicated in the increased invasive and migratory phenotype observed in cells undergoing this first step of metastasis.

The stroma secretes other pro-invasive and pro-migratory signals. Stromal cells express high levels of Platelet-derived growth factor receptor (PDGFR) in CRC. As shown by Kitadai *et al.* in their pharmacological inhibition studies, high PDGFR expression promotes CRC invasion [81]. Furthermore, it has been shown that cancer-associated fibroblasts (CAFs), which produce cytokines (TGF-β, TNFα, TFF, IGF, EGF, HGF), aid in tumor cell migration and invasion [82]. Genes located downstream of the TGF-β/SMAD2/SMAD3/SMAD4 axis activation, such as E-cadherin and Slug, have well-defined roles in EMT in CRC [83]. Loss of E-cadherin disrupts cell-cell adhesion, which in CRC allows for increased migration of the cancer cells as a result of an accumulation of Src and phospo-myosin, and of increased expression of guanine nucleotide exchange factor TIAM1 [84, 85]. Additionally, bone marrow-derived cells (BMDC), in particular tumor-associated macrophages (TAMs), secrete vascular endothelial growth factor (VEGF) in CRC, which promotes angiogenesis [86]. TAMs produce tumor necrosis factor alpha (TNFα), which increases TGF-β-induced EMT [87-89]. Finally, the cell surface glycoprotein CD44, which is normally involved in cell-cell adhesion and migration, was found to directly contribute to dissemination of tumor cells in CRC [90].

b) Angiogenesis

Mammalian cells need to be within 100 μm of a blood vessel in order to survive, which is the diffusion limit for oxygen as determined via pH and pO_2 measurements [91-93]. Oxygen and nutrients are obtained via the vasculature, and as such angiogenesis is an essential process in tumor progression. Angiogenesis is the process of neovascular growth in cancer, and this process normally occurs when the tumor around 2 cm^3 in size and is necessary for the tumor to grow any larger [94]. These vessels are typically different than healthy ones found elsewhere in the body in that they are leaky and tortuous in design, which usually results in hypoxic areas of a

tumor. Vessels in tumors are often leaky, meaning that there are larger-than-usual gaps in between endothelial cells and pericytes, thus facilitating metastasis. Angiognesis is an important step in the metastatic cascade because cells that have undergone EMT often use these vessels to access other sites in the body [65]. The common 'angiogenic switch' (though not the exclusive one [95, 96]) results from hypoxia, which promotes the transcription of *hypoxia-inducible factor* (*HIF-1*) [65]. Subsequently, HIF-1 assists in the expression of numerous angiogenic factors including basic fibroblast growth factor (bFGF), placental growth factor (PLGF), and vascular endothelial cell growth factor (VEGF) [97].

VEGF, once expressed, interacts with its receptor (VEGFR2) to initiate several pro-angiogenic signaling cascades. For example, VEGF initiates the endothelial nitric oxide synthase (eNOS), PI3K, p38 and extracellular signal-regulated kinase (ERK) mitogen-activated protein (MAP) kinases, which results in vasodilation, migration, proliferation, and vascularization [98]. The mechanism behind VEGF activity in general is that VEGF binds surface receptor tyrosine kinases (the VEGFRs), which then dimerize and become phosphorylated [99], which in turn activates the pathways listed above. In addition to this, angiopoietin-2 (Ang-2), a member of the growth factor protein family, works in conjunction with VEGF to produce leaky vessels [100]. In human patients, the degree of angiogenesis is a predictor of survival [101] and was proven to be essential for metastasis to the liver by anti-VEGF antibody (bevacizumab) studies [102, 103].

Importantly, lymphangiogenesis may play a role in CRC liver metastasis as well. The lymphatic system plays an important role in draining fluid, macromolecules, and dead cells from tissues into lymph nodes as well as in the immune response via transportation of immune cells [65]. Lymphangiogenesis represents an alternative route to blood vessels for CRC cells to reach the liver from the site of the primary tumor. In fact, it has been proposed that it is easier for cancer cells to enter the lymphatic vessel due to the absence of inter-endothelial tight junctions, smooth muscle cells, pericytes, and intact BM [104]. Signaling between mainly VEGF-C and to a certain extent VEGF-D and their receptor (VEGFR3/Flt-4) regulates lymphangiogenesis. Activation of this receptor initiates up-regulation of the ERK and JNK MAP kinases, and the Pyk2, NF-κB and AKT signaling pathways, which regulate proliferation, survival, and sprouting [105, 106]. Further signaling from platelet-derived growth factor (PDGF)-BB and Ang-1 recruit mural cells to the site of lymphangiogenesis [94]. Interestingly, Ang-2 regulates

lymphangiogenesis by acting as an agonist to Tie2 receptor on lymphatic endothelial cells [107]. In addition to this, CRC cells express the chemokine receptor C-X-C chemokine receptor type 5 (CXCR5), and both lymphatic endothelial and hepatic cells express its ligand (BCA-1/CXCL13) [108]. This may partially explain the preference of metastatic spread to the liver in CRC.

c) Intravasation

EMT allows cells to migrate and, and coupled with angiogenesis the cells can invade the tissue surrounding the primary tumor. These cells encounter capillaries, venules, and/or lymphatic channels where they intravasate and enter the circulation. The molecular mechanisms behind intravasation are not yet well-defined, but there are currently two hypotheses.

The first hypothesis states that metastases develop due to direct blood vessel intravasation at the site of the primary tumor, while the second states that cancer cells first colonize lymph nodes and then continue to blood vessel intravasation [109]. In the first case, fragmentation upon entry into the blood vessel is a large problem that must be overcome by the tumor cells [110]. CRC in particular overcome this problem by overexpressing focal adhesion kinase (FAK), thereby resisting anoikis and surviving without ECM contact by circumventing integrin signaling [111]. FAK, which is the main driver of anoikis resistance in CRC, permits this by activating pathways such as ERK or Akt, which help the cell survive [112, 113]. *Ras* mutants, commonly found in CRC (see section 1.1.2), are upstream of ERK and serve to further promote the survival of the CRC cells. In the second hypothesis, metastatic cells first enter efferent lymph nodes and subsequently enter blood vessels via the thoracic duct and subsequently the *vena cava*, and this process is tightly linked to lymphangiogenesis (described in the context of liver metastasis in the Angiogenesis section) [65]. There are good arguments for both of these hypotheses, but the reality may be that both mechanisms occur in any given cancer.

Relatively little information regarding molecular mechanisms governing intravasation is available. This is due to the difficulty of creating experimental conditions that mimic the true cellular and molecular mechanisms, the fact that most metastatic experiments bypass the intravasation step [114], and the lack of animal models [115]. One study found that tumors with a high density of vascularization had an increased incidence of intravasation, and that increased levels of E-cadherin, α-catenin, β-catenin, metalloprotease (MMP)-2 and -9 did not influence intravasation in CRC [114]. Another study found that down-regulation of Programmed Cell

Death 4 (PDCD4) is associated with increased levels of invasion and intravasation in CRC [116]. Interestingly, preliminary studies show that PDCD4 is most likely regulated by topoisomerase inhibitors [117], COX-2 inhibitors [118], Myb [119], and Akt [120]. However, another study showed that PDCD4 expression is unequivocally negatively regulated by miR-21, which binds to nucleotides 228-249 in the 3' UTR [121]. PDCD4 binds the SP1/SP3 promoter motifs, which suppresses expression of the *uPAR* gene described in the Migration and Invasion section. This is important because, once uPar binds its receptor, the expression of proteases such as plasminogen and MMPs is activated. These proteases contribute to the degradation of the ECM, which favors intravasation [65]. The expression of *uPAR* is positively regulated by the AP-1 promoter, Src-mediated activation, as well as by Ser73 and Ser63 c-Jun phosphorylation. Furthermore, Src activity levels increase throughout the stages of CRC and are associated with an increased *in vitro* invasion of CRC cells [122].

d) Mechanical stress and evading apoptosis and immune cells in the circulation

Once entered into the blood stream, metastatic cells face an enormous number of challenges. Most solitary cancer cells in the circulation die via apoptosis [123]. These challenges include evading immune surveillance and apoptosis, and overcoming mechanical stress [65].

Cancer cells must evade Natural Killer (NK) cell-mediated destruction and T cell-mediated destruction. Either the NKG2/perforin or Trail pathways mediate tumor cell destruction in NK cells [124]. In cancer patients, the expression of IL-8 and IL-12, which stimulate these pathways in NK cells, is associated with lower burden of metastasis [125]. To overcome the immune system, cancer cells often form aggregates with platelets and use mechanisms associated with inflammation to protect against NK cell-mediated apoptosis and shear stress [65]. For example, cancer cells can induce up-regulation of glycoproteins which act as "non-specific blocking factors" and protect the cancer cells from immune attack [126]. Furthermore, integrins and adhesion molecules allow the metastatic cells to adhere to the endothelial cells that protect the cells from shear stress. Importantly, adhesion to the endothelial cells also aids metastatic cells to avoid anoikis that is induced by detachment from the ECM [70, 71].

Finally, the shear mechanical stress of being in the circulation is a challenge for cancer cells. This stress is greatest in the narrow microvasculature of the heart and with contracting skeletal muscle cells [127]. Cancer cells have overcome this challenge by overexpressing stress proteins like heat shock protein 70 (HSP70) and by associating with leukocytes and platelets in the blood stream [65], which helps to shield them from the stress. Cancer cells also survive the shear stress by regulating levels of nitric oxide (NO) and reactive oxygen species (ROS) [112], which are necessary for apoptosis of CRC cells in the liver [128]. In down-regulating NO and ROS, cancer cells evade apoptosis.

It is important to note that it is rare for a cell to survive the entire metastatic cascade. In a study by Fidler in 1970, it was shown that less than 0.1% of tumor cells were still viable after 24 hours of entering the circulation. Furthermore, less than 0.01% of these cells were able to form metastases [129]. This study supports the "metastatic inefficiency" model, which states that many cancer cells die upon entry to various anatomic sites [130]. This complements the now widely-accepted "seed and soil" hypothesis, first explained by Stephen Paget in 1889 [131].

Paget originally stated that the "seed" was the tumor cell and the "soil" was the target organ, and that for a metastasis to form the "seed" and the "soil" had to be compatible. Now, more than 120 years later, the "seed" is identified as the progenitor cell, initiating cell, or cancer stem cell, while the "'soil" is identified as the niche or organ microenvironment [132]. The cancer stem cell hypothesis states that cancers develop from a few cells that have the potential to grow infinitely, to resist apoptosis, and to divide asymmetrically [133]. In CRC it is assumed that cancer stem cells originate from mutations in normal colonic stem cells, located at the base of the crypt. Interestingly, the *Wnt* signaling target gene leucine-rich repeat containing G protein-coupled receptor 5 (Lgr5) is a marker of normal colonic stem cells [134]. Several markers of CRC stem cells have been identified in $Lgr5^+$ CRC cells, including CD133, CD44, CD166, and aldehyde dehydrogenase 1 (Aldh-1) [135]. The organ microenvironment refers to the appropriateness of the organ for the metastatic cell, and is comprised of local host cells (immune cells, fibroblasts, parenchyma), cytokines, chemokines, and ECM with associated proteins. There is now good evidence that the primary tumor in fact produces factors to create a premetastatic niche in the organ before metastasis [136].

e) Extravasation

Surviving metastatic cells (seeds) eventually find an environment (soil) that is appropriate for them, adhere to a vessel, and extravasate into an organ. Many cancers have preferred sites of metastasis, and CRC prefers to metastasize primarily to the liver, and sometimes the lungs [65]. To successfully extravasate, the ligands on the cancer cell and the receptors on the endothelial cell must engage using adhesive interactions, and there must be safe passage across the endothelium [69, 137].

A study using highly metastatic CRC (CX-1) cells showed that, upon entering the hepatic microcirculation, the host underwent a rapid inflammatory response resulting in TNFα production in the Kupffer cells. This in turn activates E-selectin expression, which facilitates binding to the blood vessel endothelial cells and extravasation [138]. In addition to this, CRC cell sialylated death-receptor-3 (DR3) is activated following binding to E-selectin [69, 139], implying that cells expressing DR3 will disseminate to the liver preferentially to bind E-selectin.

Initial contact between ligand and receptor is mediated by selectins, and results in the rolling of the cancer cell on the endothelium [65]. This rolling provokes the secretion of chemokines by the endothelium, which stimulates cancer cell and integrin activation. Integrin strengthens the adhesion via Ig-CAM members (ICAM).

Subsequent to adhesion, cancer cells start to migrate across the endothelium by extending invadopodia into the cell junctions. While the mechanism behind this is not clearly understood, Tremblay *et al.* found that the endothelial cell retraction in CRC cells was an ERK-dependent dissociation of the VE-cadherin/β-catenin complex, associated with the retraction of a p38-dependent actin filament [140, 141]. For the next step of extravasation, the cancer cells must adhere to the sub-endothelial BM/ECM to either grow and develop as a secondary neoplasm or they travel elsewhere [65]. Once across the membrane, the cells must colonize the new site.

The architecture of the liver also facilitates metastases formation via its unique microenvironment [142]. The hepatic microvasculature is dual by nature (two afferent arteries: portal and hepatic), tortuous, and slow [143]. In normal liver function, this aids in the filtration and recognition of circulating cells, bacteria, and molecules. However, this function can also aid in the arrest of circulating metastatic cells. In addition to this, the liver's macrophages (Kupffer cells in the endothelia) and the large quantity of surface molecules exposed via the fenestrated hepatic sinusoidal endothelial cells (HSEC's) facilitate efficient absorption of circulating

nutrients, toxins, bacteria and other pathogens, old cells, and toxins. Interestingly, this can also aid in the uptake of circulating metastatic cells.

The liver consists of a heterogeneous population of endothelial cells that aid in regeneration and renewal by repairing the hepatic microvasculature [144]. Metastatic cancer cells can take advantage of this process by secreting tumor-derived factors to use these same cells to contribute to stroma and blood vessel generation [142], creating a favorable microenvironment for metastatic development. Furthermore, activated myofibroblasts are a major source of ECM during liver injury and aid in hepatic regeneration. However, in response to tumor-derived factors these myofibroblasts contribute to a favorable microenvironment for metastatic development [142]. Finally, metastatic cells can also take advantage of the hepatic adaptive immune response to the prolonged up-regulation of immune factors and inflammation. This response is intended to save the liver parenchyma from damage, and consists of local immune suppression via up-regulation of IL-10, prostanoids, soluble ICAM-1, and TGF-β [145]. This microenvironment is favorable to infectious diseases, autoimmunity, and cancer metastasis, which likely explains the metastasis of CRC to this organ [146].

f) Colonization

This is the final step in the metastatic cascade, and is defined as the detectable growth of disseminated cancer cells [147]. This implies that the cancer cell has been able to avoid apoptosis in the new microenvironment, to divide, and to form new vascularization. An important and not well-understood mechanism that allows for colonization is mesenchymal to epithelial transition (MET), where the cell begins to re-express markers of the epithelium such as E-cadherin [100]. While MET is still not a very well-understood process, it has been shown that the proto-oncogene c-Met (also known as hepatocyte growth factor receptor) regulates MET in carcinogenesis [148]. In CRC, it is believed that CAFs contribute to this process by overexpressing cyclooxygenase-2 (COX2) and TGF-β [149], which creates a pro-proliferative environment.

It has also been found that CRC cells secrete CD44, which acts as a decoy receptor to inhibit interaction of CRC cells with their hyaluronate ligand in the ECM. This mechanism protects the CRC cells from apoptosis [150]. Furthermore, one study found that expression of β-Galactoside–Binding Protein Galectin 3 in the tumor cell is essential for colonization of

metastatic CRC in the liver [151]. A different study found that expression of the fibronectin receptor Integrin alphavbeta6 (αvβ6) was essential for liver colonization [152]. On the other hand, it was found that metastatic cells have to induce fibroblast stromal expression of periostin (POSTN), a component of the ECM, in order to colonize the liver [153]. Expression of growth factors (such as TGFα) from the ECM and of growth factor receptors in CRC cells (such as EGFR) has been shown to influence proliferation of metastatic cells in the liver [154]. In addition to this, expression of the chemokine receptor CXCR4 is essential for proliferation of hepatic CRC metastases [155].

Dr. Beauchemin's laboratory has defined CRC development very well in the context of CEACAM1 (Carcinoembryonic antigen cell adhesion molecule 1)-regulated tumors. CEACAM1 (CC1), an adhesion molecule that will be further introduced in the next section, which has reduced expression in the early phases of CRC development (tumor suppressor function) [156-158], but is up-regulated in later stages of the disease [159-161]. Furthermore, CC1 has several functions that implicate it in the metastatic process. Firstly, CC1 is an angiogenic factor that promotes VEGF activity. In fact, CC1 is involved in regulating the VEGFR2/eNOS/Akt pathway [162-164]. Considering the role that angiogenesis plays in the metastatic cascade, this is an important pro-metastatic function. CC1 can also engage in juxtacrine signaling while fulfilling its cellular adhesion function [165, 166]. This indicates that CC1 has a major role in CRC metastasis. Finally, the up-regulation of CC1 in the later stages of CRC disease combined with its proven expression at the invasive front of advanced human CRC tumors [160, 161, 167] suggests that CC1 does indeed regulate CRC metastasis. In addition to this, CC1 expression in both the colonic epithelium and the stroma contribute to cancer development [168-170]. Based on this evidence that CC1 plays an important role in metastasis, this project investigates epithelial CC1-regulated CRC metastasis.

1.3 The CEA Family

The carcinoembryonic antigen (CEA) was first discovered in 1965 by Gold and Freedman during studies investigating tumor-specific antigens of intestinal malignancies [171]. During the years that followed, other members of the CEA family were discovered. The CEA

family of proteins is divided into two main groups: the carcinoembryonic antigen cell adhesion molecules (CEACAM) found at the membrane, and the secreted pregnancy-specific glycoproteins (PSG) found in the trophoblast [166, 172-174]. In humans there are 12 genes that encode for the CEACAM proteins and 10 that encode for the PSG proteins, all of which are located in a cluster on human chromosome 19q13.1-13.2 (murine chromosome 7) [172]. These family members differ in function, cellular localization, expression pattern, and primary structure. The CEACAMs, which are part of the immunoglobulin (Ig) superfamily, are furthermore highly conserved across 28 mammalian species [175]. Interestingly, the most highly conserved member is CEACAM1 [172].

Members of the CEACAM family in mice are composed of either variable IgV-like N domains exclusively, or of one to eight N domains with one to three Ig constant (IgC)-like domains (of type A or B). However, in humans, CEACAMs have an IgV-like domain and zero to six IgC-like domains [172] **(Figure 3)**. Members of the CEA family are generally involved in adhesion interactions [173, 176-178], but have been shown to also have roles in neo-angiogenesis [179], innate and adaptive immune responses [166], insulin metabolism [180], tumor development [181], apoptosis [182], and certain members function as receptors for pathological bacteria and viruses [183, 184].

1.4 CEACAM1

This project focuses on CEACAM1 (CC1), formerly known as Bgp1, CD66a, and pp120 [172]. Identified in 1976 in the bile of patients with liver disease [185, 186], CC1 is the primordial CEACAM. CC1 is mostly a transmembrane glycoprotein [187], although some splicing isoforms are also secreted or anchored to the membrane. It is expressed in epithelia, vessel endothelia, and myeloid and lymphoid cells.

1.4.1 Gene, structure, and major isoforms

a) The Gene

There are nine exons in the *CEACAM1* gene, which can be alternatively spliced to generate twelve different isoforms, three of which are secreted instead of being membrane-bound [188] **(Figure 4)**. All members of the CEACAM family share the same intron and exon pattern. There is always an exon that encodes the 5′ untranslated region (UTR) and part of the leader peptide, followed by a different exon that encodes the rest of the membrane signal peptide and

that encodes the N-terminal IgV-like domain. Following this are other exons that each encode IgC-like domains of either type A or B, which are paired together in an A+B fashion. Finally, there are several exons that encode the C-terminal region and the 3′ UTR [189].

Transcription of the *CEACAM1* gene is induced by IFN-γ via an Interferon-Stimulated Response Element in its promoter region [190-192]. Alternative splicing heavily regulates *CEACAM1*, which is a post-transcriptional evolutionary strategy that allows one gene to code for several proteins [193, 194]. This process is mediated by *cis*-regulatory elements called exonic splicing enhancers and silencers, which either promote or inhibit the use of nearby splice-sites. The most common splicing effector proteins are part of the heterogeneous nuclear ribonucleoprotein (hnRNP) and serine/arginine-rich (SR) families [195, 196]. It was shown that alternative splicing controls the transcription of the CC1-long (-L) and CC1-short (-S) cytoplasmic tails [197]. hnRNP L and hnRNP A1 mediate transcription of CC1-S by interacting directly with exon 7, while hnRNP M is essential for transcription of CC1-L. In addition to this, interferon regulatory factor-1 (IRF-1) has also been shown to regulate *Cc1* alternative splicing in breast cancer cells (MDA-MB-468), favoring the CC1-L isoform [198]. Down-regulation of CC1 in prostate cancer was found to be regulated by Sp2 [199], a member of the Sp family of transcription factors that regulates gene expression by binding to GC boxes at their promoters [200].

b) The Structure

Consequently, all isoforms have the same three general structures: two, three, or four heavily glycosylated extracellular immunoglobulin domains, a transmembrane segment and either a long (71 amino acids, 73 in mice) or short (10 amino acids) cytoplasmic tail **(Figure 5)**. It is important to note that, while the human *CC1* gene encodes for only one protein, the murine *Cc1* gene encodes for two allelic variants: *Ceacam1a* and *Ceacam1b,* varying in the N-terminal region [201, 202]. However, most inbred mouse strains only express *Ceacam1a* [203]. Furthermore, mice have a homologous gene to *Cc1* called *Ceacam2*, which differs in protein structure, expression pattern, and function [173]. One difference between the twelve human splice isoforms is found via the addition of one, two, or three IgC domains. Another difference is the inclusion or exclusion of exon 7, which yields CC1-L and CC1- S cytoplasmic tails, respectively [166].

c) The Major Isoforms

The conventional way to distinguish between the isoforms is to refer to the number of extracellular immunoglobulin-like domains, the length of the cytoplasmic tail, and whether or not it is secreted. For example, the major murine isoforms are CC1-2L, CC1-2S, CC1-4L, and CC1-4S [172], although secreted forms have also been found [204]. In humans the major isoforms are CC1-4S, CC1-4L, CC1-3S, and CC1-3L [188]. For this reason, all work in this thesis involving CC1-L used CC1-4L. The CC1-L and CC1-S isoforms are generally both expressed in most cell types, but the balance between them is highly regulated and dependent on *cis*-acting regulatory elements and the down-regulation of hnRNP A1 and L, as well as the up-regulation of hnRNP M [197]. Interestingly, the ratio of long to short isoforms differs greatly dependent on cell type, phase of growth, and activation state [205, 206]. Our laboratory in fact showed that during CRC tumor growth, an optimal ratio of isoform expression is necessary to observe the tumor-inhibitory phenotype associated with overexpression of CC1, described in section 1.6 [207].

Both CC1-S and CC1-L are involved in homophilic binding [208] and in dimer formation at the cell surface [209]. CC1-L dimerization is a method of signal transduction [210]. Both the extracellular (N-terminal Ig domain) domain and the CC1-L cytoplasmic domain are involved in dimer formation, and CC1-L has a greater tendency to dimerize than CC1-S. It should be noted that the monomeric forms regulate intercellular adhesion [209, 211]. However, this project focuses on the cytoplasmic tail of CC1-L due to its important phosphorylatable Tyr and Ser residues, as well as its three terminal Lys **(Figure 6)**.

1.4.2 Important motifs and residues of CC1-L

The structure and function of the N-terminal region

Adhesion plays a major role in embryonic development as well as in the maintenance of the tissues of the body [212]. The N terminal IgV domain of CC1 is responsible for intercellular homophilic and heterophilic adhesion [213, 214]. In fact, CC1 was first found to mediate intercellular homophilic adhesion in Ca^{2+}-independent fashion [215]. It was found that N-domain residues Val39 and Asp40 are essential for homophilic adhesion [216], while retention of the N-domain is essential for heterophilic interactions with pathogens such as the Opa proteins of *Neisseria meningitidis* and *Neisseria gonorrhoeae* [217].

The function of the phospho-Tyr residues of the cytoplasmic tail

CC1-L has two unique phospho-Tyr residues (Tyr488 and Tyr515), both of which are located within Immuno-receptor Tyrosine-based Inhibitory Motifs (ITIM) **(Figure 6)**. CC1-L contains two ITIMs, which have a six amino acid binding motif (I/V/L/S)XYXX(L/V) and, upon tyrosine phosphorylation, serve as a docking site for kinases and phosphatases [218]. Several kinases phosphorylating CC1 have been identified to date, including members of the Src-family protein tyrosine kinases as well as receptor tyrosine kinases [219]. As shown in CRC epithelial HT29 cells, these kinases include c-Src, the insulin receptor (IR), and the epidermal growth factor receptor (EGFR) upon EGF treatment [220]. Other kinases include Btk kinase in B cells [221], Lyn in neutrophils [222, 223], and Lck and Fyn in T cells [166]. These kinases and their implication in CRC tumor development and metastasis will be explored further in section 1.5.

As shown in mouse CT51 CRC tumor cells and HEK293 cells, SHP-1 and SHP-2 (encoded by the *PTPN6* and *PTPN11* genes) are two well-characterized phosphatases that bind CC1-L [224, 225]. Interestingly, SHP-1 and -2 bind dimeric CC1-L preferentially, whereas c-Src binds both monomeric and dimeric CC1-L [226]. SHP-1 is mainly expressed in hematopoietic and epithelial cells, and is known to be a negative regulator of cell signaling. Conversely, SHP-2 is ubiquitously expressed and is widely accepted as a positive regulator of cell signaling [227, 228]. The two Src homology 2 (SH2) domains of SHP-1 and -2 bind CC1-L's Tyr488 and Tyr515, although Tyr488 binds with more affinity. For SHP-1 and -2 interaction, both of these Tyr residues must be present and at least one of them must be phosphorylated. Furthermore, the presence of the three terminal lysines (3 terminal Lys) was also essential for Tyr phosphorylation, and therefore for SHP-1 binding. Interestingly, the presence of phospho-Ser516, found within the second ITIM, was not essential for Tyr phosphorylation or for SHP-1 binding. The only difference in CC1 binding between SHP-1 and SHP-2 is the fact that both SH2 domains of SHP-1 can bind the phosphorylated Tyr488 and Tyr515, while only the N-terminal SH2 domain of SHP-2 can bind [225]. Dephosphorylation of CC1-L by SHP-1 affects insulin clearance in the liver, resulting in increased insulin signaling. The importance of insulin signaling in CC1-L-mediated CRC tumor development and metastasis will be further discussed in section 1.5.2.

The function of the phospho-Ser residues of the cytoplasmic tail

CC1 also contains important phospho-Ser residues. The CC1-L isoform contains the multifaceted Ser503 residue, which regulates CRC development [181], insulin clearance in the liver by mediating Tyr488 phosphorylation [229], and inhibition of Fas-mediated apoptosis in Jurkat T cells [230]. In search of the kinase responsible for phosphorylating Ser503, Fiset *et al.* found that cyclin-dependent kinase 2 (Cdk2) interacts with CC1-L, but that it did not phosphorylate Ser503 [231]. In fact, the kinase responsible for Ser503 phosphorylation in all of these instances has not yet been identified. Furthermore, there are 17 predicted phospho-Ser/Thr sites in CC1-L, 13 of which conform to the protein kinase C (PKC) consensus site. However, *in vitro* studies where CC1-L was phosphorylated with PKC β2 isoform revealed that only Ser449 is truly PKC-phosphorylated [232]. CC1-L also regulates several other signaling pathways that will be discussed in section 1.5.

1.5 Expression Pattern of CC1

CC1 is the most widely expressed CEA family member, and this breadth of expression is conserved between humans and mice. CC1 is found in epithelia, endothelia, and myeloid and lymphoid cells [187]. Interestingly, it is differentially regulated in a spatiotemporal fashion [189], and disruption of this regulation leads to tumor formation in many organs, as discussed in section 1.5.3.

1.5.1 Expression in Normal Tissues

Epithelial expression of CC1 is found in a wide array of tissues, including in the esophagus, stomach, epithelial cells of the duodenum, jejunum, and ileum, pancreas, gall bladder, kidney, bladder, prostate, cervix, endometrium, sweat and sebaceous glands, liver, and the columnar and caveolated cells of the colon [189]. In normal, healthy colon, CC1 is expressed in the mid-crypt of the colonic mucosa, and the glycocalyx (apical and lateral part of microvilli) [233]. Our laboratory has also previously found CC1-positive cells at the bottom of the colonic crypt in mice [168]. It is also localized at the apical surface of enterocytes (mature columnar epithelial cells), in microvesicles, filaments, and on highly differentiated epithelial cells at the crypt mouth [233, 234].

CC1 is also expressed during early stages of embryonic development in both humans and rodents. It is found in the placenta on the invasive extravillous trophoblasts [189]. In mice, it is

also expressed in the primitive gut, in the dermis, meninges, lung, kidney, salivary glands, and the pancreas (regions of epithelial-mesenchymal interaction). Finally, CC1 is also present during the processes of myogenesis and odontogenesis [235-237].

1.5.2 Expression in Immune Cells and Cell lines

CC1 is expressed by a number of haematopoietic cell types [238]. It is important to note that there is significant down-regulation of CC1 expression (both isoforms) in most immortalized and transformed epithelial cell lines used in the laboratory, which suggests a proliferative advantage for CC1-negative cells [158]. Primary mouse splenic T cells express CC1 [239], and resting T cells express low levels of CC1 *in vitro* [240] and *in vivo* [241]. However, within 30 minutes of mitogen stimulation, CC1 is localized at the surface of blast-like $CD4^+$ and $CD8^+$ T cells [239]. This means that, upon cellular activation, CC1 localizes quickly to the cell surface from an intracellular compartment. Resting human peripheral-blood $CD4^+$ T cells also express low levels of CC1, while $CD8^+$ T cells, γδ T cells [242, 243], and intestinal intraepithelial lymphocytes did not [244]. *In vivo*, T cells express CC1 in the lamina propria of celiac disease patients [244] and in the large intestines of patients suffering from inflammatory bowel disease (IBD) [245].

In contrast to T cells, CC1 is expressed constitutively in B cells [166]. Furthermore, upon activation with interleukin 2 (IL-2), $CD16^+$ and $CD56^+$ natural killer (NK) cells express CC1 at the cell surface [242, 243, 246]. In addition to this, neutrophils also express a variety of CEACAMs, including CC1. It is thought that CC1 is mostly involved in intercellular CC1-CC1 binding [247]. Finally, CC1 has been found in dendritic cells, where it is also thought to participate in intercellular binding [248]. Details of the role of CC1 in these immune cells are outlined in section 1.6.6.

The tumorigenic capacity of non-tumorigenic cell lines is increased when CC1 is down-regulated [249], while the overexpression of CC1-L in cancer cell lines inhibits tumorigenesis *in vitro* as well as *in vivo* [170, 249]. This is consistent with what is observed in terms of CC1 and immunosuppression. For example, a cell line expressing CC1 is protected from NK cell-mediated destruction, and this protection increases linearly with concentration of CC1 [250]. In summary, intercellular binding of CC1 inhibits cellular growth, while immune cell-mediated destruction is inhibited by CC1-dependent interactions with immune cells. This supports the fact that CC1-expressing tumors are aggressive due to their capacity to evade immune cell-mediated

destruction and that CC1-negative tumors are aggressive because they are able to escape proliferative regulation [251-253]. Further details on this dual role of CC1 are found in section 1.5.3.

CC1 is found in intercellular junctions and at the cell surface in unpolarized and stratified epithelial cells, whereas in simple, polarized epithelial cells CC1 is found in the apical microvilli [254]. In Madrin-Darby Canine Kidney (MDCK) epithelial cells, CC1-L polarized to both the apical and basolateral cell surfaces, while CC1-S only polarized to the apical surface [255]. In these same cells, it was found that PI3K-dependent shuttling to the endosome/lysosome compartment was dependent on Tyr phosphorylation. Furthermore, Tyr515 retention in the cytoplasmic tail was essential for lateral surface expression of CC1-L, but not Tyr488. This lateral surface CC1 was present in adherens junctions, and not in tight junctions or desmosomes [256]. Retention of CC1 at intercellular junctions is highly dependent on homophilic CC1-CC1 interactions as well as on interaction with actin [257]. Interestingly, in Swiss 3T3 fibroblast cells this localization is controlled by the Rho GTPases. Cdc42 and Rac1, as well as their downstream effector PAK1 can target CC1 to intercellular junctions via interactions with the transmembrane domain of CC1. However, activated Rho cannot and this results in an accumulation of CC1 at the cell surface [258].

1.5.3 Expression in Human Tumors

In terms of regulation of expression, CC1 isoforms are down-regulated in the early stages of several cancers including colon [158, 259], prostate [260], liver [261], and breast [262]. This down-regulation is most often found in hyperplastic lesions, which is an early phase in cancer progression [260, 263]. Furthermore, forcing re-expression of CC1 isoforms in colorectal and prostate cancer cells inhibited tumor development in syngeneic and immune-deficient mice [170, 249]. This implies that CC1-L expression was essential for this tumor-inhibitory phenotype, and indicates that CC1-L in this case behaved as a tumor suppressor. However, CC1 (especially CC1-L) is highly expressed at the invasive front of advanced human CRC [264-266] and it is re-expressed at the adenoma/high-grade dysplasia stage of CRC progression, which is associated with metastatic invasion [267].

CC1 is overexpressed in some aggressive forms of cancer such as melanoma [251], non-small cell lung [252], gastric [268], thyroid [269], and bladder [270]. Contrary to the above studies that associate CC1-L with tumor inhibition, these studies indicate that high CC1

expression is associated with metastasis [271]. This dual role is explained, at least in part, by the fact that CC1-L is involved in the angiogenic switch [272]. The down-regulation of CC1, associated with the early phases of many cancers as explained above, is concurrent with an up-regulation of CC1 in the endothelial cells of adjacent blood vessels. This down-regulation is also accompanied by an up-regulation of pro-angiogenic factors VEGF-A, -C, -D, and Ang-2. The adjacent blood vessels have an increased number of fenestrations, inter-endothelial and trans-endothelial gaps, and a higher degree of degradation of the basement membrane. [272] This increased vascular permeability, resulting in structural destabilization, is associated with the up-regulation of vascular endothelial growth factor (VEGF)-A, -C, and –D [273-276]. Furthermore, it was recently shown by our laboratory that down-regulation of CC1 increases vascular permeability *in vivo* [164]. Interestingly, pro-lymphangiogenic factors, VEGF-C and -D, were increased upon the down-regulation of CC1 [272, 276, 277]. In fact, CC1 plays an important role in tumor lymphangiogenesis and reprogramming of vascular endothelial cells to lymphatic endothelial cells. This is accomplished by CC1's interaction with lymphatic marker Prospero homeobox protein 1 (Prox1) and VEGF receptor 3 (VEGFR-3) [278].

Up until this point, I have discussed CC1-L expression in the "seed" (tumor cell). Our lab has recently shown, using CC1-deficient ($Cc1^{-/-}$) mice, that CC1 expression in the "soil" (liver parenchyma) increased hepatic metastatic burden in CRC [169]. Furthermore, our lab showed that colonocytes in $Cc1^{-/-}$ mice had an increased rate of proliferation and a decreased level of apoptosis [168], and that $Apc^{1638N/+}$:$Cc1^{-/-}$ mice had higher tumor burden than their wild-type (WT) counterparts [279]. It is clear that CC1 plays different roles in the soil and in the seed. Given these diverse functions of CC1, its function is tissue-specific and is regulated in a spatio-temporal manner [189]. My project focuses on the role of CC1-L in the tumor cell in CRC hepatic metastasis.

1.6 CC1 in CRC tumor progression and metastasis

As mentioned in section 1.5, CC1 is down-regulated in the early stages of CRC in humans, mice, and rats [156-158], which lead to the hypothesis that CC1 behaves as a tumor suppressor. In fact, CC1 expression is significantly decreased in human microadenomas and adenomas of CRC, indicating that CC1 down-regulation contributes to malignant transformation [157, 280]. Interestingly, a 1-bp deletion in the human *CC1* gene has been found in a microsatellite region of the 3' UTR, which is suspected to significantly decrease CC1 expression

[281] and thereby aid in tumor progression. CC1's role in CRC tumor inhibition has been thoroughly investigated *in vitro* as well as *in vivo*, and it has been found that Tyr488, Tyr515, Ser503, and the 3-terminal Lys all play a role in this phenotype [181, 225, 282, 283].

1.6.1 The cytoplasmic domain of CC1 inhibits tumor progression

Through cell culture work and xenograft models, the tumor inhibitory function of CC1 was investigated in prostate cancer and in CRC. It was found that expression of CC1 inhibited prostate cancer tumor progression [249, 284, 285] as well as CRC tumor progression [170]. Further investigation revealed that the presence of the cytoplasmic tail is necessary to maintain the tumor inhibitory phenotype [207, 282, 283]. Only CC1-L displays this phenotype, not CC1-S [207]. Given the knowledge that CC1-L classically mediates downstream signaling pathways via RTKs [188], the investigation into the essential phospho-residues for this phenotype began.

The Tyr Residues and the 3 Terminal Lys

Using CT51 CRC cells and allografts with Balb/c sygneneic mice, our laboratory showed that several residues were important for the tumor inhibitory phenotype [282]. Through deletions and mutational analysis, it was found that the presence of Tyr488, the 3 terminal Lys, and Val518 were all essential for CRC tumor inhibition. While Val518 is not a phospho-residue, it is important in the interaction between CC1-L and SHP-1 and -2 [225], and so it was investigated in the context of CRC progression. The importance of Val518 in both studies indicates that SHP-1 and -2 binding could also be involved in CRC development. Similarly, the 3 terminal Lys were investigated due to their influence on Tyr phosphorylation. Their presence was essential for Tyr phosphorylation, and therefore SHP-1 binding [225]. The Tyr515Phe mutation and the deletion of the N-terminal behaved like wild type (WT) CC1-L. However, when both Tyr488 and Tyr515 are deleted there is partial inhibition of tumor progression [282]. This indicates that, while the two Tyr residues have distinct signaling partners, they may be interdependent.

A study on prostate cancer progression also investigated some of these same residues, using DU145 human prostate cancer cells and xenografts in nu/nu mice [286]. It was found that CC1-L in these cells was phosphorylated in the C-terminal region (the last 65 amino acids). Furthermore, this study found that Tyr488 was not essential for the tumor-inhibitory effect of CC1-L, which is contradictory to the study by Izzi *et al.* discussed above [282]. This could be

due to the difference in cell transfection method, in cancer cell line, or in mouse model as nu/nu mice are immune-deficient.

The Ser503 Residue

Another study in our laboratory investigated the role of phospho-Ser503 in CRC tumor progression using CT51 CRC cells and Balb/c syngeneic mice as well [181]. It found that the tumor burden of mice injected with CT51 cells overexpressing the CC1-L Ser503Ala mutant was higher and similar to that of CC1-S. The search for the kinase responsible for Ser phosphorylation first investigated the already highly-suspected PKC and PKA [287, 288]. It was first found that phorbol ester phorbol-12-myristate-13-acetate (PMA), a known activator of PKC, increases basal phosphorylation and that CC1-L is sensitive to staurosporine, a general Ser kinase inhibitor. It was also found that the use of PKC-specific inhibitor calphostin C did not reduce the level of phosphorylation. Two other PKC-specific inhibitors, H7 and sphingosine inhibitors, also did not reduce phosphorylation levels. It was also determined that Ser503 is indeed a site of *in vivo* phosphorylation, but that it was likely not the sole residue responsible for serine phosphorylation by this PMA-inducible kinase. Interestingly, PMA-treated cells differed in morphology, CC1-L localization, and actin cytoskeleton organization as compared to untreated cells. This indicated a trafficking phenotype where CC1-L translocates from the cell membrane to the cytoplasm. In addition to this, the study mentioned above by Izzi *et al.* also revealed that Ser503 was essential for the CC1-L-mediated tumor inhibitory phenotype [282].

These studies revealed the recurring importance of these specific Tyr, Ser, and Lys residues in tumor progression. Interestingly, these residues also play essential roles in other CC1-L-mediated processes related to tumor progression and metastasis, which will now be described. In fact, most of CC1's functions participate in the hallmarks of cancer, including proliferation, migration, metastasis, apoptosis, and immune surveillance and evasion [63].

1.6.2 CC1-L, the IR and EGFR: regulator of proliferation

a) CC1-L and the insulin receptor

Insulin-induced internalization and recycling of the insulin receptor (IR) regulate cellular sensitivity to insulin. Once internalized, the IR is recycled while insulin is degraded via the endosome or lysosome [289]. CC1-L was identified as a substrate for the Tyr kinase activity of

the IR and EGFR decades ago [290, 291], and since then the details of these interactions have been investigated.

Upon insulin activation, the IR is autophosphorylated and CC1-L is endocytosed with the IR complex [292, 293]. Interestingly, this cellular process is dependent on Ser503 phosphorylation by a cAMP-dependent kinase that is not yet identified [293]. As for the IR, the presence of Tyr1316 of the β-chain is essential for CC1-L Tyr488 phosphorylation [294], which mediates interaction with an unknown adapter protein that facilitates the targeting of insulin for degradation [180]. CC1-L therefore down-regulates the mitogenic effects of insulin through insulin clearance [180]. However, CC1-L also down-regulates insulin through its interaction with Src homology 2 domain-containing (Shc) adaptor protein, which are associated with cellular proliferation [295].

Upon insulin activation, the IR phosphorylates CC1-L at Tyr488 which then binds to the SH2 domain of the Shc protein. Interestingly, in primary hepatocytes, the presence of both Tyr488 and Ser503 is essential for this interaction. In binding Shc, CC1-L inhibits its ability to couple the IR with Grb2 [295]. Upon binding to Shc, the Grb2/SOS and Grb2/GAB complexes up-regulate the mitogen-activated protein kinase (MAPK) and the PI3 kinase (PI3K)/Akt proliferative pathways [180]. When CC1 binds Shc upon insulin stimulation, the MAPK and PI3K/Akt proliferative pathways are thus down-regulated. It is this signaling that results in the phenotype of slower growth, decreased DNA synthesis, and slower progression through the cell cycle in CC1-L-expressing cells [295].

Sonia Najjar's laboratory has investigated *in vivo* CC1-L-mediated insulin clearance via the transgenic mouse model (L-SACC1) that expresses a Ser503Ala dominant negative mutant, consequently inhibiting phosphorylation at this residue [229]. The targeted expression of this mutant in the liver hepatocytes, driven by the ApoAI lipoprotein promoter, results in chronic hyperinsulinemia due to impaired insulin clearance. Furthermore, the transgenic mice develop visceral adiposity with fatty acid and triglyceride levels similar to the phenotype of type II diabetes. A similar phenotype was observed in *Ceacam1*$^{-/-}$ mice which were predisposed to hepatic steatosis by a high fat diet [296, 297]. The L-SACC1 mice and the *Ceacam1*$^{-/-}$ mice had increased fat due to the interaction between CC1-L and fatty acid synthase (FAS) upon IR activation. This resulted in insulin-mediated FAS inhibition. Importantly, phosphorylation of Tyr488 and Ser503 is essential for the CEACAM1-L-FAS interaction.

As mentioned in section 1.4.2, dephosphorylation of CC1-L by SHP-1 and -2 affects insulin clearance in the liver, resulting in increased insulin signaling to the IRS-PI3K-Akt pathway in liver and muscle. Mice deficient in SHP-1 (*Ptpn6* $^{me\text{-}v/me\text{-}v}$ also known as viable motheaten) have hyperphosphorylated CC1-L, which effectively blocks insulin stimulation. The lack of SHP-1, resulting in blocked CC1-L insulin signaling, caused increased glucose tolerance and sensitivity in these mice [298]. The link is therefore clear between CC1-L and insulin signaling, confirming the role that CC1-L plays in regulating cellular proliferation.

b) CC1-L and the EGFR

CC1-L is linked to proliferation via EGFR signaling as well [299]. In fact, CC1-L is phosphorylated by both the IR and the EGFR at the same site: Tyr488, with prior phosphorylation of Ser503. Similarly, Shc recruitment by CC1-L results in the uncoupling of EGFR signaling from the Ras/MAPK pathway. Using the MCF-7 (breast cancer) and COS-7 (fibroblast-like) cell lines, it was shown that there is a decrease in cell growth after transfection of CC1-L after EGF stimulation [299]. EGFR is activated in epithelial cells by adipose tissue that secretes heparin binding (HB)-EGF into the portal vein [300]. HB-EGF increases proliferation of hepatocytes in the L-SACC1 mice, which is in line with its role in mitogenesis during liver regeneration [301]. Due to the increased hepatocyte proliferation and metabolic derangement that accompanies the inactivation of CC1-L in the liver, this study showed that CC1-L regulates obesity and insulin resistance with EGFR activation and abnormal epithelial cell growth [299].

1.6.3 CC1 and the cytoskeleton: regulator of migration

CC1 also has a role to play in migration due to its interactions with several cytoskeletal proteins. CC1-S binds to calcium-modulated protein (calmodulin) in a calcium-dependent reaction [302]. Calmodulin is the Ca^{++} regulatory subunit of the myosin light-chain kinase that is highly involved in cytoskeletal functions and enzyme activities in eukaryotic cells [303-305]. Furthermore, increased levels of Ca^{++} are associated with the blocking of CC1 dimerization, which is most likely regulated by calmodulin that binds the CC1-L cytoplasmic tail [209]. This interaction would inhibit these dimers from preferentially binding to Src kinases to conduct downstream signaling [210]. CC1 can also bind tropomyosin and globular actin [306], which reaffirms CC1's role with the cytoskeleton. The Phe449 and Lys451 residues bind actin during

cytoskeletal re-organization [307]. Interaction between CC1-S and fibrillar actin (F-actin) occurs, but is thought to be indirect [257, 306]. It is important to note that CC1-L is found at the cell-cell contacts (apical and lateral surface) of epithelial cells [255, 257]. The Shively laboratory proposed that CC1-S serves as a potential G-actin polymerization site, and that polymerized actin filaments anchor on CC1-L [306].

In addition to this, interaction between Filamin A (FLNa) and CC1 was discovered via the Yeast Two-Hybrid system [308]. FLNa is important in modulating cell shape and in motility by cross-linking filamentous actin to orthogonal networks, by binding to β-integrin subunits thereby anchoring actin filaments to the extracellular matrix, and by providing a scaffold for guanosine-triphosphatases (GTPases) of the Ras and Rho families [309, 310]. It was also found that Rho GTPase activity was essential for CC1-L-targeting to the cell-cell contacts in epithelial cells. Cdc42 and Rac1 induced this targeting, and the transmembrane domain of CC1-L was essential [258]. Furthermore, migration studies revealed that CC1-L binding to FLNa reduced migration and cell scattering [311], indicating that CC1-L regulates migration. The migratory role of CC1-L in melanoma cells was defined when it was found to increase migratory and invasive activities, which were blocked upon introduction of anti-CC1 antibodies [312].

1.6.4 CC1-L: regulator of invasion and metastasis

a) CC1 and invasion and metastasis

Up-regulation of CC1 is associated with increased invasiveness, metastatic spread, and unfavorable prognosis in non-small cell lung cancer [313], thyroid cancer [269], gastric carcinoma [268], pancreatic tumors [314], malignant melanoma [312], and metastatic CRC cells [161, 266]. CC1 expression is correlated with the development of metastatic disease, and there is a correlation between CC1 expression and poor prognosis in the above-mentioned cancers [252]. In fact, in a human thyroid microarray CC1 expression matched metastatic incidence. Upon CC1 transfection into WRO cells (human thyroid tumor), there was decreased cell cycle progression, up-regulation of p21, decreased phosphorylation of Rb, and increased cell matrix adhesion, migration and invasion [269]. Furthermore, CC1 binds integrin $\alpha_v\beta_3$ at the invasive front of melanomas and at the apical surface of glandular cells of endometrium [165]. The localization of the CC1/integrin β_3 complex at the tumor-stroma interface in these two settings implicates CC1 in cellular invasion.

Re-expression of CC1 in the advanced stages of CRC, with a predominance of CC1-L over CC1-S, is also indicative of a correlation between CC1 expression and increased hematogenous metastasis, lymph node involvement, and decreased survival [265]. Ieda *et al.* further showed that CC1-L is up-regulated at the invasive front of CRC tumors, indicating a role for CC1-L in CRC invasion. This study also emphasizes the importance of a balance of CC1-S and CC1-L for maintenance of a non-cancerous phenotype. Although this study did not elucidate the mechanism behind this regulation, it hypothesized that CC1 and the β_3 integrin may be functionally connected, as they are in neutrophils [315] and melanoma [312].

Our laboratory recently investigated the contribution of stromal-derived CC1 on hepatic metastasis of CRC [169]. Arabzadeh *et al.* showed that there was a significant reduction in *Cc1*$^{-/-}$ liver metastatic burden, after intrasplenic injection of metastatic CRC MC38 cells and after tail vein injection of B16F10 melanoma cells. Upon evaluation, it was found that there was decreased early survival and proliferation of MC38 cells within the *Cc1*$^{-/-}$ liver. There was also down-regulation of CCL2, CCL3, and CCL5 chemokines, which are essential for myeloid-derived bone marrow-derived cell (BMDC) emigration to distant sites [316, 317]. Indeed, there was a decrease in $CD11b^{+}Gr1^{+}$ myeloid-derived BMDC and immune cell recruitment to the metastatic site. CC1's role in this was confirmed by adoptive transfer experiments and metastatic assays. This study demonstrated that $CD11b^{+}Gr1^{+}$ myeloid-derived BMDCs are key metastatic contributors in *Cc1*$^{-/-}$ mice, but that other cells present in the liver likely also contribute to metastasis formation. Interestingly, the metastatic nodules in the *Cc1*$^{-/-}$ mice displayed increased vascular density, but had less mature vessels. This increase in angiogenesis was not attributed to VEGF levels, but rather to the granulocyte colony-stimulating factor (G-CSF) [318]. Lu *et al.* demonstrated that G-CSF induces the expression of prokineticin 2 (Bv8) from $CD11b^{+}Gr1^{+}$ myeloid-derived suppressor cells (MDSCs). Finally, decreased levels of activated STAT1 and STAT3, along with increased levels of activated Akt were found in *Cc1*$^{-/-}$ metastatic liver. The elevated Akt levels are in line with CC1's role as a regulator of apoptosis [263, 319]. Overall, the role of CC1 in regulating invasion and metastasis is dependent on stage of disease and type of tumor.

b) CC1 and angiogenesis

CC1 was first implicated in angiogenesis by the demonstration of its expression on microvessels of proliferating tissue, in wound healing edges in granuloma tissue [320], and in immature blood vessels of various tumors [321]. It was then identified as an angiogenic factor via angiogenesis assays [162], and it was identified as a key mediator of vascular integrity and formation of the *in vivo* vascular network, particularly during normal neo-vascularization [322]. Furthermore, endothelial CC1-L affects cytoskeletal architecture and integrin-mediated signaling, implicating it in angiogenic activation [323]. Volpert *et al.* showed that prostate carcinoma cells overexpressing CC1-L inhibited endothelial cell migration and corneal neovascularization *in vivo*. They also showed that this increased the number of endothelial cells [324]. Interestingly, they found that the Ser503 residue was essential for this phenotype. In prostate intraepithelial neoplasia (PIN), down-regulation of CC1-L was accompanied by its up-regulation in adjacent vessels [272]. This was associated with increased vascularization and decreased vessel stabilization. Moreover, endothelial CC1 promotes lymphatic reprogramming of vascular endothelial cells, which increases tumor lymphangiogenesis [278]. This increased tumoral access to lymphatic vessels implicates CC1 in metastasis.

A study from our laboratory found that CC1 is an important regulator of vascular permeability [164]. This study links CC1-L and the VEGFR2/Akt/eNOS-mediated vascular permeability pathway. CC1-null primary endothelial cells had increased vascular permeability because of increased NO production, which is a known angiogenesis promoter that regulates vascular remodeling, permeability, and integrity [325, 326]. The increased NO levels were a consequence of increased Akt and endothelial nitric oxide synthase (eNOS) activation [326]. Indeed, eNOS produces NO, but can also be stimulated to induce VEGF expression, which produces NO in turn via PI3K/Akt-dependent eNOS activation [326]. Treatment with VEGF in this study resulted in elevated VEGFR2 activity as well as increased Src- and SHP-1-dependent CC1-L Tyr phosphorylation. Appropriately, this study found that the CC1-L Tyr488Phe mutant and CC1-S yielded lower NO production, due to the lack of SHP-1 signaling [225]. Interestingly, this study also found that the CC1-L Ser516Ala mutant had no effect on the phenotype.

CC1 also plays an important role in metastatic development in myeloid cells via promotion of angiogenesis. $CD11b^{+}$ myeloid cells endogenously express high levels of CC1,

and this is associated with VEGF-dependent angiogenesis [327]. CC1 also regulates angiogenesis in a Bv8 (prokinecticin-2)-dependent manner [318]. CC1-null mice with B16F10 melanoma allografts had increased MDSC presence in the tumors, which themselves had increased growth and angiogenesis. MDSCs, in fact, produce Bv8 via the G-CSFR-mediated pathway.

In sum, CC1 regulates metastatic angiogenesis via at least two pathways. CC1 mediates the levels of activated STAT3 in liver metastatic nodules as shown with intrasplenic injection of metastatic CRC MC38 cells and with tail vein injection of B16F10 melanoma cells [169]. In addition to this, CC1 regulates angiogenesis via the G-CSFR-Bv8 signaling pathway, as shown in myeloid cells [318].

1.6.5 CC1-L: regulator of apoptosis

The role of CC1 in apoptosis has been defined in breast cancer. CC1-S mediates apoptosis during mammary morphogenesis, and in the case of breast cancer, the malignant phenotype can be reverted with re-expression of CC1-S in 3D culture matrices [182]. In this study it was found that CC1-S regulates apoptosis by translocation of Bax from the cytosol to the mitochondria and release of cytochrome *c* into the cytoplasm, which is part of a mitochondrial signaling pathway. CC1 also has a pro-apoptotic role in human pulmonary artery endothelial cells [324]. Interestingly, this study found that the CC1-L Ser503Ala mutant reverted the phenotype and resulted in less apoptosis. Furthermore, the hyperplastic growth of colonic epithelium is due to reduced apoptosis (as opposed to increased proliferation) [328]. Increased proliferation and decreased apoptosis was also observed in colonocytes of *Cc1*$^{-/-}$ mice [168].

Nittka *et al.* demonstrated in HT29 human CRC cells and human T lymphocyte Jurkat cells that pro-apoptotic signaling likely occurs via CC1 signal transduction. In this case, the pro-apoptic signaling is carried out via CC1 by homo- and heterophilic adhesion to other CEACAMs at the cell surface [263]. CC1 crosslinking was also necessary for ERK1/2 signaling in human leukocytes [329]. The results of these studies indicate that CC1 regulates morphogenesis by apoptosis.

1.6.6 CC1-L: regulator of immune evasion

The long and short isoforms of CC1 also play pivotal roles in immune cell functions, often times participating in different signaling pathways. This section will examine CC1's role in T cells, B cells, Natural killer cells, neutrophils, and dendritic cells.

a) T cells

Upon IL-2, IL-7, IL-15 cytokine treatment, CC1 at the cell surface is up-regulated *in vitro* [243, 245, 330, 331]. Details of T cell CC1 expression patterns are found in section 1.5.2. It is important to note that CC1 is heavily glycosylated, and that different glycoforms have different functions [166]. Given this information, it is interesting that CC1 expressed in T cells do not have the sialyl-Lewis X moieties that are found in granulocytes [330, 332].

CC1 regulates immune responses and immune-mediated diseases in T cells, in addition to adhesion [166]. CC1 also regulates T cell adhesion to other lymphocytes and to tumor cells, and it regulates cytolytic activity [244]. This regulation is accomplished via CC1-L Tyr phosphorylation by the kinases Fyn or Lck [333], which results in the shuttling of CC1-L to lipid rafts where it binds the CD3ε chain [334] and in the shifting of dimers to monomers, favored for calmodulin binding. CC1-L is then dephosphorylated by SHP-1, and associates with the T cell receptor (TCR)-associated CD3ζ chain and ZAP70 Tyr kinase. This interaction inhibits TCR signaling, decreasing signaling [220, 334, 335] through the MAPK pathway (decreased activation of JNK and ERK) and decreasing levels of IFN-γ, IL-4, and IL-2 [334, 335]. CC1-L thus regulates inflammatory pathways in T cells as an inhibitory co-receptor, in a SHP-1-dependent fashion [220].

The ratio of CC1-L/CC1-S is important for this inhibitory phenotype. CC1-3S transfection into Jurkat T cells displayed the opposite phenotype of CC1-3L transfection, resulting in increased MAPK signaling and cytokine production [335]. Interestingly, transfection of both isoforms into these same cells displayed a phenotype in between that of CC1-S and CC1-L alone [226]. One possible explanation for this phenotype is that the incidence of CC1-L homodimers decreases in the presence of CC1-S, which would prevent SHP-1 association.

b) B cells

Similarly to T cells, CC1-L plays a co-inhibitory role in B cell FcγRIIB (receptor to the Fc domain of IgG) signaling. The cytoplasmic Tyr residues are also essential for the SHP-1 and -2 - mediated interaction [336]. However, CC1 stimulates B cell activity when it homodimerizes in *trans* [205]. This contradictory role likely depends on the functional state of the cell, which has been proven to be similar in epithelial cells [337]. Another study using a Burkitt's lymphoma B cell line found that, upon CC1-L Tyr phosphorylation and subsequent interaction with SHP-1, PI3K signaling and CD19 LFA-1-induced B cell aggregation are reduced. This leads to CC1-L-mediated apoptosis [338].

c) Natural killer cells

Resting natural killer (NK) cells do not express CC1, but upon activation with IL-2 $CD16^-$ and $CD56^+$ NK cells up-regulate its expression at the cell surface [243, 246, 330]. CC1 inhibits MHC-class-I-independent cytotoxic activity via homophilic *trans*-ligation in NK cells [339]. Indeed, as is the case in other immune cells, the presence of the long cytoplasmic tail is essential for this inhibitory phenotype [250]. CC1 is also implicated in suppression of tumor immunosurveillance in cancers that up-regulate CC1 expression, such as melanoma. The abundance of CC1 at the cell surface allows for homophilic binding on NK cells, preventing NK-mediated tumor cell destruction [250]. In particular, CC1 binds the NK gene 2 member D (NKG2D) receptor, and consequently SHP-1 dephosphorylates Vav1, blocking cytolytic signaling [340]. Given this information and the fact that increased invasiveness accompanies CC1 up-regulation, CC1 expression is thought to be responsible for the poor prognosis for survival for melanoma [251]. CC1 further aids tumor cells in escaping immune surveillance by down-regulating NKG2D ligands, rendering tumor cells less sensitive to NK cell-mediated lysis [341]. Interestingly, soluble CC1-Fc chimeric proteins have been shown to block the CC1 *trans* homophilic interaction between tumor cells and NK cells, effectively blocking the inhibition of NK cell-mediated tumor cell destruction [342].

d) Neutrophils

CC1 is expressed on activated neutrophils, stimulated by various activating signals such as the chemotactic formylated peptide N-formyl-methionine-leucine-phenylalanine (fMLP) [343,

344], the calcium ionophore A23187, tissue plasminogen activator (tPA) [345], and retinoic acid (RA) [346]. In rat granulocytes (which only express CC1), CC1 is an activation and differentiation marker [347], and upon stimulation CC1-L is Tyr phosphorylated by kinases such as pp60c-Src, Lyn, and Hck [219, 222]. Furthermore, Singer *et al.* discovered that CC1 regulates the delay of spontaneous apoptosis via treatment of polymorphonuclear granulocytes (PMN) with a CC1-specific mAb, which resulted in delayed spontaneous apoptosis in a dose- and time-dependent manner [347]. The Erk1/2 pathway, recognized as anti-apoptotic, mediates this pro-survival effect of CC1 in granulocytes. Upstream of this is the dephosphorylation of CC1 by the phosphatase SHP-1, which as previously stated binds the cytoplasmic domain of CC1-L upon Tyr phosphorylation [224]. Singer *et al.* also investigated the role of CC1 in stress-induced apoptosis, and found that CC1 mediates delayed FasL-induced apoptosis via caspase-3. In monocytes in general, CC1-L also increases survival via the PI3K and Akt signaling pathways [348].

e) Dendritic Cells

An anti-CC1 antibody (AgB10) stimulates the maturation of dendritic cells (DC) [248]. This antibody also resulted in the release of chemokines such as CC-chemokine ligand 3 (MIP1α) and CXC-chemokine ligand 2 (MIP2), IL-6, and IL-12. Interestingly, these effects were also seen with the treatment with an anti-FcγRIIB co-inhibitory antibody [349]. CC1 Lewis X epitopes also regulate DC maturation by binding to Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN), a C-type lectin receptor that typically binds to blood vessel endothelial cells and activates $CD4^+$ T cells [350-352]. These studies show that CC1 negatively regulates DC maturation.

The expression of CC1 in various immune cells is essential for escape from immune surveillance, and for immune cell progression. CC1's regulation of these processes is often SHP-1 or -2-dependent, which implicates CC1-L as an essential isoform. This is an interesting fact considering that most immune cells that express CC1-L also express CC1-S, indicating that the role of CC1 is dependent on a balance between the two isoforms. Importantly, Chen *et al.* discovered that, in Jurkat T cells, CC1-L and CC1-S have distinct inhibitory and stimulatory functions, respectively [335].

1.7 CEA and CC6 in CRC tumor progression and metastasis

CEA (also called CC5) and CC6 represent two other prominent members of the CEA protein family. They are also heavily implicated in CRC progression and metastasis [188]. They are both expressed in columnar epithelial and goblet cells in the colon [353, 354] and, although not studied in this project, their roles in CRC tumor progression and metastasis are important for the discussion of future direction.

1.7.1 CEA

CEA has been used as a CRC tumor marker for the past 50 years. However, its suitability as a biomarker has been questioned due to comparative studies using new imaging techniques and targeting methods [188]. One study found that CC6 was a more suitable biomarker and that tumor-associated CEA levels had no prognostic value in CRC patients [355]. However, another study that compared CEA to other CRC biomarkers such as EGFR, tumor-associated glycoprotein-72 (TAG-72), and folate receptor-α (FRα) found that CEA was by far the superior biomarker in terms of sensitivity [356]. Moreover, CEA serum levels were found to be indicative of overall survival with CRC by a study that used the Surveillance Epidemiology and End Results (SEER) database in the United States [357]. CEA has also been investigated as a marker for CRC metastasis.

It has been shown that CEA contributes to CRC liver metastasis [358], and that soluble CEA plays a role in enhanced liver metastasis [359]. It is the interaction between the CEA receptor (CEAR) and the hnRNP M, expressed at the surface of liver Kupffer cells. This interaction brings about pro-inflammatory signaling (IL-1α and β, IL-6, TNF-α) which results in increased expression of cell adhesion molecules (ICAM-1, VCAM-1, E-selectin), and increased adherence of metastatic cells in the vasculature [360]. CEA also signals through the DR5 receptor (TRAIL-R2), which leads to increased metastasis through decreased anoikis (a form of programmed cell death, induced by detachment from the ECM) [361].

Experiments using an $APC^{Min/+}$ (C57Bl/6 background) mouse model showed that CEA expression does not influence tumorigenesis [362]. However, another experiment using the CEABAC mouse model (FVB background), which expressed CEA, CC6, and CC7, showed increased incidence of CRC [363]. Of course, these differences might be due to different genetic background and/or different levels of expression of the CEA family in the CEABAC mouse.

Finally, CEA has been proven to play a role in tumor development as shown in *in vitro* studies as well. Its function as an intercellular adhesion molecule indicates that it can form CEA-CEA bridges between tumor cells or between tumor and stromal cells [176]. In addition to this, CEA has been found to activate integrin signaling in lipid raft subdomains, which in turn activates integrin-linked kinase (ILK), PI3K, and AKT pathways [364]. It is via this signaling that CEA plays a role in inhibiting differentiation programs [365, 366], inhibiting anoikis and apoptosis [367], and interrupting cell polarization [366]. CEA also inhibits NK cell cytolytic activity via the N domain [178].

Potential antitumor vaccines are in fact exploring CEA's interactions with immune cells, as reviewed in [368]. Furthermore, an elevated CEA serum level indicates metastasis and poor prognosis [369]. In fact, CEA expression contributes to liver metastasis in CRC by inhibiting TGF-β signaling [370].

1.7.2 CC6

There is unfortunately no CC6 transgenic mouse model available yet to fully investigate the role of CC6 *in vivo* [188]. However, it has been shown that CC6 overexpression in pancreatic cells inhibits CRC differentiation and anoikis, and that this inhibits hepatic metastasis of these cells [366, 371]. In fact, overexpression of CC6 in Capan2 pancreatic cancer cells was shown to provide resistance to gemcitabine (chemotherapetuic drug for pancreatic cancer), while the silencing of CC6 in BxPC3 cells resulted in chemosensitization to the drug [372]. In this instance, Src-dependent AKT signaling was responsible for the difference in phenotype. Furthermore, CC6 expression is associated with increased invasiveness [372], which matches the aggressiveness of pancreatic tumors seen in patients: absence of CC6 expression is associated with lack of lymph node involvement, higher survival, and lower stage of disease [373]. In fact, anti-CC6 antibody drug conjugate therapeutic investigations have yielded very promising results, and clinical trials are the next step for this field [188].

CC6 is also a good biomarker and predictor of overall and disease-free survival in CRC patients [355]. It has been shown that CC6 plays an important role in regulating metastasis, due to the increased E-cadherin promoter activity that accompanies CC6 suppression [374]. In addition to this, another study showed that increasing CC6 expression in HCT116 CRC cells increased invasion through the ECM, while suppressing CC6 expression in LoVo CRC cells decreased invasion [375]. Both CEA and CC6 were found to contribute to CRC metastasis in an

experiment using GW-39 CRC cells pre-treated with Fab' fragments (region of the Ab that binds to the antigen) of mAbs against CEA and CC6 [374]. It showed that cells pretreated with the mAbs resulted in decreased metastatic burden *in vivo*, affirming CEA and CC6's roles in CRC metastasis.

Given the importance of both CEA and CC6 is CRC development and metastasis, future experiments for this project will investigate their roles in the context of liver metastasis of MC38 CRC cells in C57Bl/6 mice. This will be further discussed in the Future Directions section.

1.8 Objectives

The objective of this thesis work is to understand the role that CC1-L plays in CRC metastasis to the liver. Previous works mentioned above implicate key residues in the cytoplasmic tail in CRC tumor development: Tyr488, Tyr515, Ser503, and the 3 terminal Lys. A CC1-negative population of metastatic CRC MC38 cells overexpressing CC1-L, CC1-S, and Tyr488,515Phe had previously been characterized *in vitro* and *in vivo* in this laboratory (Arabzadeh *et al.*, manuscript in preparation for *Gastroenterology* 2014). These studies showed that CC1-L yields a lower metastatic burden than both CC1-S and Tyr488,515Phe. Within this thesis project, I overexpressed mutations of the remaining residues (Ser503Ala, 3Lys3Ala) in addition to one control mutation (Ser516Ala) in the MC38 CRC cell line. In order to remain syngeneic to the previous work in this laboratory, C57Bl/6 *CC1*$^{+/+}$ mice were used. This project first aimed to characterize the phenotype resulting from overexpression of these three mutants *in vitro* and *in vivo.* The main residue of interest was Ser503, due to its regulation of CRC development [181, 282], insulin clearance in the liver via Tyr488 phosphorylation [229], inhibition of Fas-mediated apoptosis in Jurkat T cells [230], and its regulation of angiogenesis in prostate carcinoma cells [324]. From the results of this thesis, we expect to clarify the mechanisms whereby CC1-L regulates metastasis in CRC.

Figure 1: Histopathology of colorectal cancer.

The histopathology of colorectal cancer (CRC) is defined by a series of morphological changes, which are accompanied by mutations in specific oncogenes and tumor suppressors. The epithelium of the colon has many invaginations called crypts, which serve to increase the surface area. The lower third of the crypt contains the dividing cells, while the upper two thirds contain the differentiated cells. The earliest stage of colorectal neoplasia is an aberrant crypt focus (ACF), which can consist of either dysplastic or non-dysplastic cells. Dysplastic cells are more likely to become a polyp, which is a benign tumor that protrudes into the intestinal lumen. Polyps can be hyperplastic or adenomatous, the latter being more likely to progress to a malignant carcinoma. Each step along this path is associated with the loss of function of a tumor suppressor or with the gain of function of an oncogene, as depicted in the figure. In addition to this, higher nuclear levels of β-catenin are associated with higher malignancy.

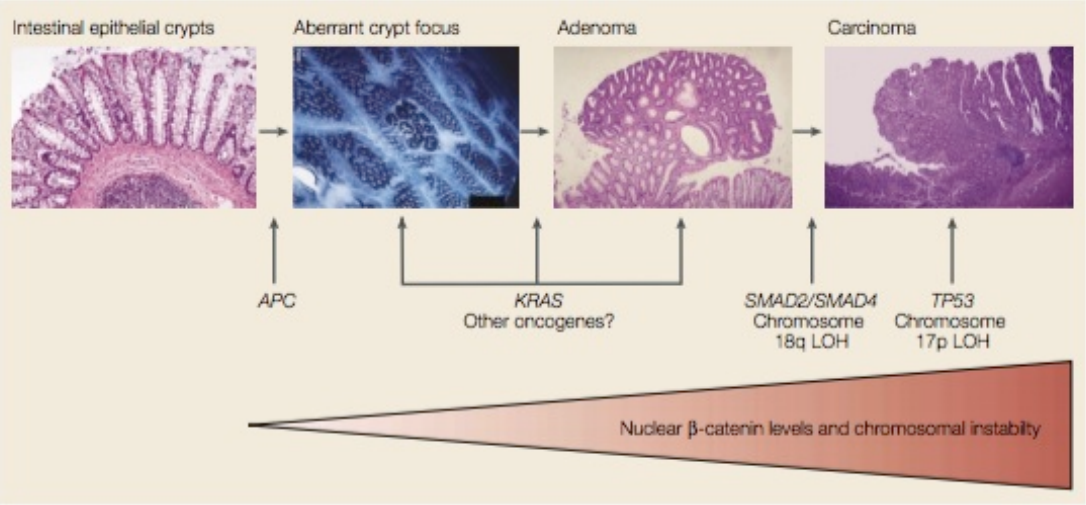

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Figure 2: The Metastatic Cascade.

Metastasis is a series of steps that are often referred to as the metastatic cascade, and each step can be rate-limiting. **A)** The primary tumor proliferates. **B)** Certain cells acquire the ability to invade the local tumor environment. **C)** These cells intravasate into the vasculature where they must evade destruction by immune cells, survive of the mechanical stress of the vessel, and evade anoikis. **D)** Attachment to the vessel endothelial cell and extravasation. **E)** Establishment of secondary neoplasm at a distant site and metastatic growth.

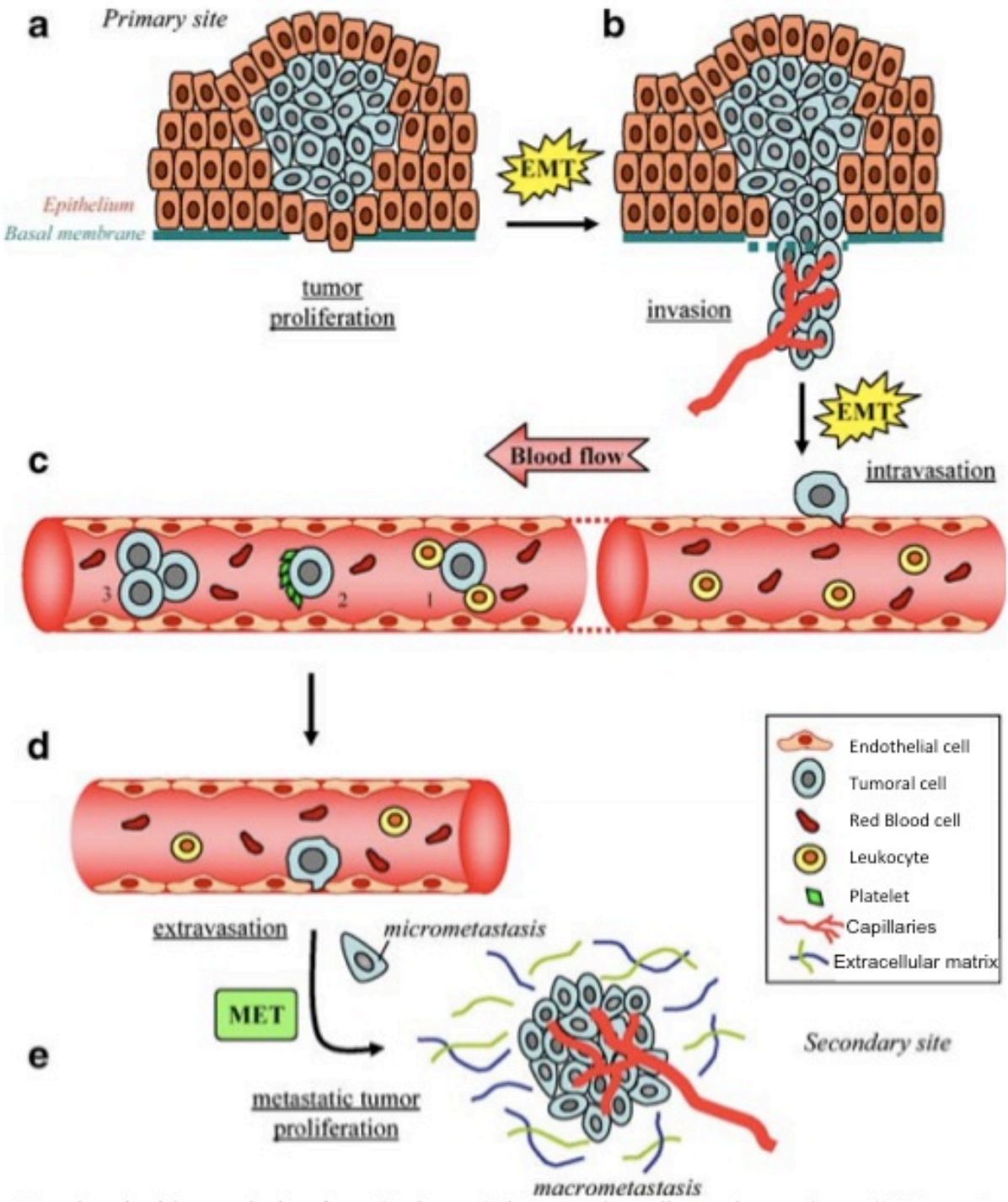

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Figure 3: The human CEACAM isoforms.

All CEACAM1 isoforms have one variable-like domain (N domain). Following this, there are a variable number of C2-like immunoglobulin domains (A or B), leading to the transmembrane domain. This is followed by the intracellular domain, which consists of either a long or short cytoplasmic tail. It is important to note that there are secreted forms of CEACAM1 that lack the transmembrane and intracellular domains. CC1 also has many N-linked glycosylation sites, represented here by balls and sticks.

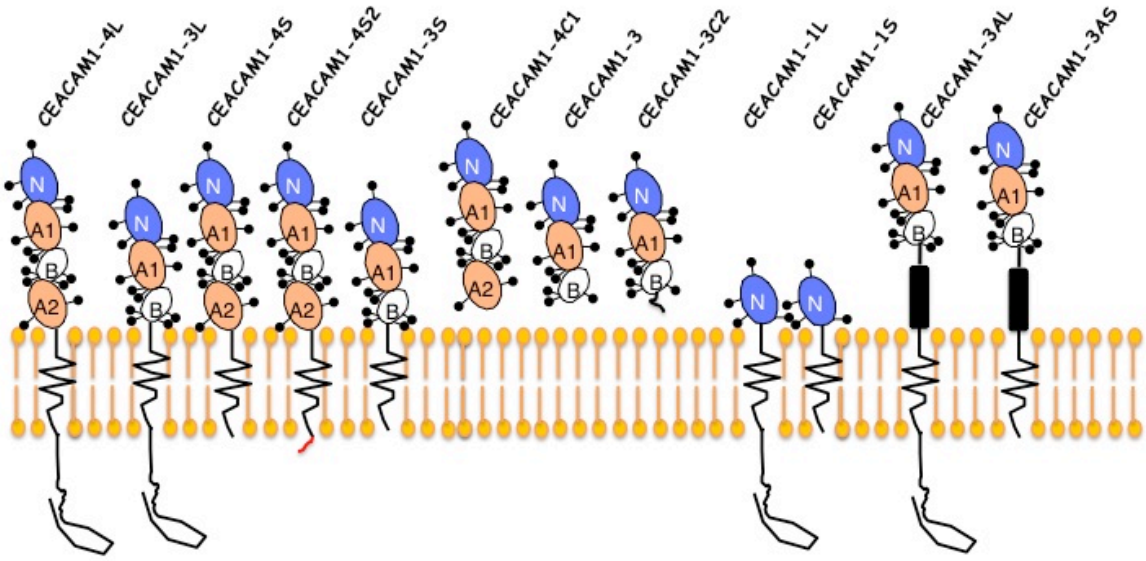

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Figure 4: Alternative splicing and the long and short isoforms of CC1.

Alternative splicing produces the two major isoforms of CC1: CC1-L which is characterized by a long cytoplasmic tail, and CC1-S which is characterized by a short cytoplasmic tail. The difference between the two isoforms is the inclusion or exclusion of exon 7.

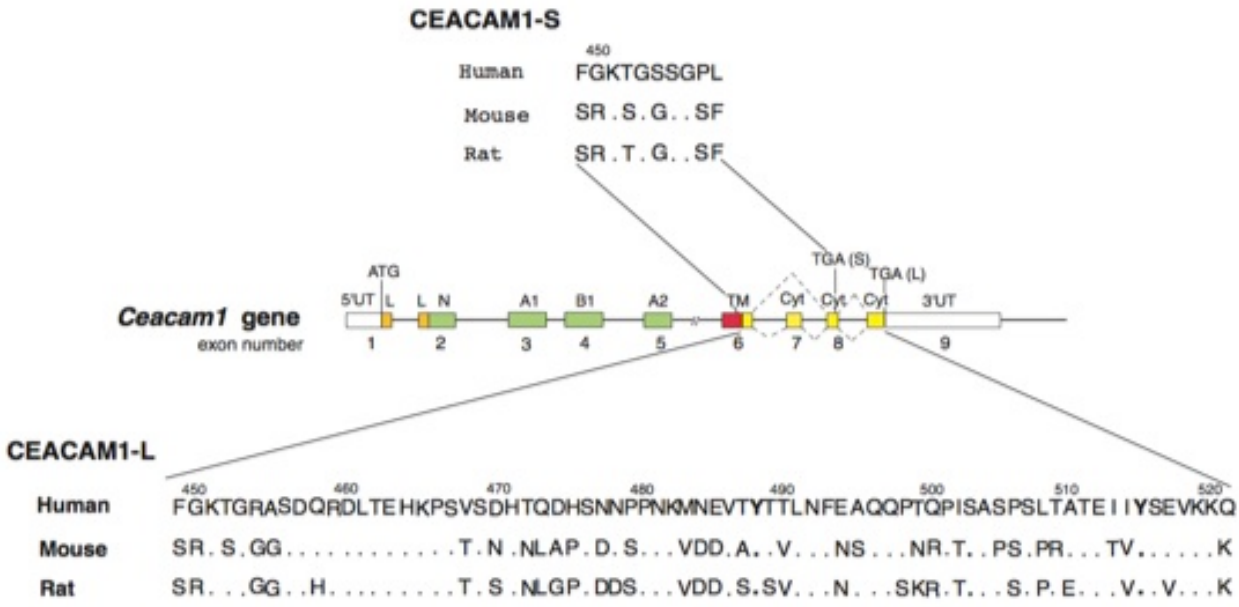

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Figure 5: The short and long cytoplasmic tail of CC1-L.

Both isoforms (CC1-L and CC1-S) have the same three general structures: three, or four (shown here) heavily glycosylated (depicted by the sticks and balls) extracellular immunoglobulin domains (termed A or B), a transmembrane segment and either a long (71 amino acids, 73 in mice) or short (10 amino acids) cytoplasmic tail.

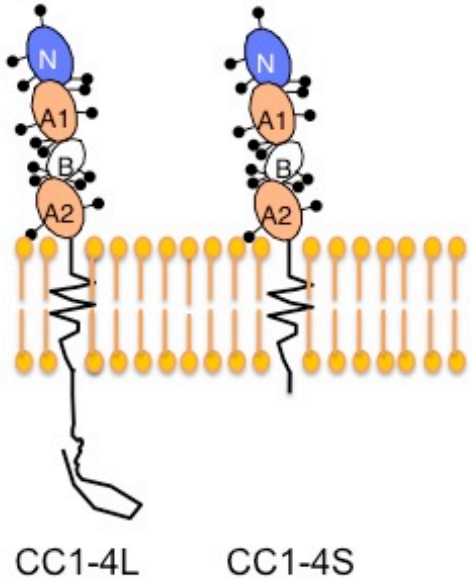

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Figure 6: The important residues and motifs of CC1-L.

CC1-L contains several residues that are essential for its tumor inhibitory phenotype: two phospho-Tyr residues (Tyr488 and Tyr515), one phospho-Ser residue (Ser503), and the 3 terminal Lys (3K). The two phospho-Tyr residues are located within ITIM motifs, which serve as a docking site for kinases and phosphatases.


Y488
S503
Y515 3K
YFLYSRKSGGGSDQRDLTEHKPSTSNHNLAPSDNSPNKVDDVAYTVLNFNSQQPNRPTSAPSSPRATETVYSEVKKK
445
ITIM
ITIM
521

II. Materials and Methods

2.1 ΨII Cell Transfection by Calcium Phosphate Co-Precipitation

The ΨII packaging cell line was seeded to achieve 70% confluency on the day of the experiment, and were grown in α-MEM medium (Multicell), supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco), 100U/ml penicillin, 10mg/ml streptomycin (Multicell), and 2μM L-glutamine (Gibco). Four different calcium phosphate co-precipitation transfections [376] were carried out using 5 μg of the retroviral vector plasmid DNA pLXSN including either: no insert (neo), or including the CEACAM1-L-S503A, 3K-3A, and S516A mutants. Transfected ΨII cells were selected for two weeks with G418 (geneticin, Multicell) at a concentration of 1 mg/mL.

2.2 Retroviral-mediated Metastatic MC38 cell infection

The stably transfected ΨII cells were seeded to achieve 70% confluency the day of the experiment, while the metastatic MC38 cells provided by Dr. Pnina Brodt (McGill University) and Dr. Shoshana Yakar [377], were seeded to ahieve 50% confluency the day of the experiment and grown in D-MEM medium (Gibco), supplemented with 10% heat-inactivated FBS (Gibco), 100U/ml penicillin, and 10mg/ml streptomycin (Multicell). The supernatant from the ΨII cells was passed through a 0.45μm filter and mixed with 80 μL of polybrene (1 mg/mL). The MC38 cells were incubated overnight with the virion-containing medium from untrasnfected and transfected ΨII. The untransfected cells served as a negative control. The next day, this was repeated to achieve a superinfection. G418 was added on the third day at a concentration of 750 μg/mL and cells were selected for two weeks. The cells were analyzed on the FACScan (BD Biosciences) after selection using an in-house rabbit anti-CC1 pAb (3759 1:50) [169] and the goat anti-rabbit FITC-conjugated secondary antibody (Multicell, 1:100). The cells were subsequently sorted by the McGill Flow Cytometry Facility on the FACSAria Cell Sorter/Analyzer (BD Biosciences), using the same antibodies, to obtain uniform populations expressing high levels of the S503A, 3K-3A, and S516A CC1-L mutants. The highest-expressing 40% of cells were selected out of the population via FACS sorting and this population

was subsequently expanded. Each experimented is started with a fresh vial of frozen cells from this sort.

The MC38 empty vector control (MC38 CT) and the MC38 CC1-L control cell lines were generously provided by Dr. Azadeh Arabzadeh, research associate in the laboratory. They were prepared using the same protocol described above (Arabzadeh *et al.*, manuscript in preparation).

2.3 Animals

All in-house 11H11 *Ceacam1*$^{+/+}$ wild type mice (C57BL/6 backcross 16) were bred through homozygote matings by Claire Turbide, and all other *Ceacam1*$^{+/+}$ wild type mice mice on a C57BL/6 background were purchased from Harlan. The 2D2 and 11H11 *Ceacam1*$^{-/-}$ knockout mice (C57BL/6 backcross 21) were also bred in house. All mice are fed the Charles River diet and kept on a bedding of wood shavings. Mice brought in from outside our facility were given two weeks to acclimatize to the diet and bedding.

The mice were housed in pathogen-free conditions at the McGill Animal Resources Centre. All experiments were reviewed and approved by the McGill University Animal Care committee, and were done in compliance with the Canadian Council on Animal Care.

2.4 Mouse Experiments

All experimental mice were of the same age (between 8-12 weeks old) and sex (males only). The intrasplenic injection liver metastasis assay was performed as described previously [378, 379]. To summarize, the mice were anesthetized with a cocktail consisting of ketamine (50 mg/mL), xylazine (5 mg/mL), acepromazine (1 mg/mL), and saline (0.9%). Viable MC38-derived cells ($2x10^5$) were suspended in 50 µl of phosphate-buffered saline containing 2% FBS and were injected intrasplenically into the mice. All cells used in these experiments were analyzed via flow cytometry to ensure that the expression levels of CC1-L were comparable. The spleen was removed three min. after injection of the cells, following ligation of the vascular supply and venous drainage. The wound was then sutured. The *Ceacam1*$^{+/+}$ wild type mice were sacrificed 14 days post-injection, while the *Ceacam1*$^{-/-}$ knock out mice were sacrificed 14-17 days post-injection. Liver metastases were retrieved and processed for analyses (described below).

2.5 Preservation of Tissue

After the mice were sacrificed, blood was collected and frozen at -80°C, and the livers were removed, weighed, and prepared for preservation. For immunohistochemistry/immunofluorescence, livers were fixed in 10% phosphate-buffered formalin overnight, dehydrated in 70% ethanol, and then embedded in paraffin by the Histology Core (Goodman Cancer Research Centre). For immunofluorescence analyses, liver tissue was flash-frozen in O.C.T. Compound (Tissue Tek) for cryo-sectioning. Normal areas of fresh liver and metastatic nodule tissue were also immediately frozen on dry ice and preserved at -80°C.

2.6 Determination of Metastatic Burden

Paraffin-embedded liver sections (4 μm thick, four step sections/ mouse, separated by 200 μm) were stained with hematoxylin and eosin to differentiate between normal liver parenchyma and metastatic nodules. These slides were first scanned using a ScanScope XT digital scanner (Aperio Technologies, Vista, CA, USA). The average metastatic burden was computed using a pixel contrast detection algorithm (Pixel Contrast Analysis) designed by our colleague Shawn McGuirk (PhD candidate, McGill University) and reported as an area fraction (ratio of surface nodule area/total surface area). To use the Pixel Contrast Analysis, the user first defines the pixel color(s) representative of the metasatic lesions. The program then analyzes all pixels and determines the area covered by metastatic lesions as well as the total area. The metastatic burden, measured as an area fraction, is computed from these two values.

2.7 Immunohistochemistry

Immunohistochemistry performed to detect CEACAM1 was performed on formalin-fixed, paraffin-embedded liver tissue. Antigen retrieval was performed on de-paraffinized sections incubated with 10 mM sodium citrate buffer (pH 6.0) at 95°C for 20 minutes. The samples were blocked with a 10% solution of bovine serum albumin (BSA) containing 2% goat serum in tris-buffered saline with 0.05% Tween and 0.025% Triton-X-100 (TBST) for 30 minutes at 20°C. The sections were incubated for 18 h at 4°C with an in-house mouse anti-CC1 monocolonal Ab (1:750 and labelled with Alexa Fluor 594 secondary antibody (1:600) for one hour at room temperature. They were counterstained with 4',6-diamidino-2-phenylindole (DAPI) at 1 μg/mL (DAKO) for 20 minutes. Sections were mounted in DAKO Cytomation

mounting medium and analyzed on a Zeiss LSM Pascal on Axiovert 200 microscope. The Ki67 antigen was retrieved following the protocol described above. The anti-Ki67 (Abcam, 1:1000) primary and the Alexa Fluor 488 secondary Ab (1:800) were used.

2.8 xCELLigence Proliferation Assay

To assess the proliferative properties of the MC38-derived cell lines, a proliferation assay was performed on the Roche xCELLigence machine following the protocol provided by Roche with the machine. All cells used in these experiments were analyzed via flow cytometry to ensure that the expression levels of CC1-L were comparable. Viable MC38-derived cells were suspended in 1 mL of 10% FBS RPMI media, as described above, and $2x10^5$ cells (100 μL) were plated into each well of the E-plate. Each cell line was run in duplicate or triplicate, and at least two wells were loaded with media only in order to assess background levels. The E-plate has gold microelectrodes at the bottom of each well, which detect electrical impedence and reports a value called cell index. The cell index is an arbitrary value that represents the cellular biological status, which is comprised of cell number, viability, strength of binding, and morphology. Once attached to the docking station, the cell index at 30 minute intervals is recorded live on the software on the computer for 48 h. In this experiment, doubling time was caclulated between 3 and 45 h using the software provided by Roche with the machine. The doubling time is calculated using the cell index values, by first converting the data into its logarithmic form and then doing a linear regression analysis to determine both the slope and the standard deviation of the slope. The doubling time is calculated from this slope.

2.9 Pervanadate and MG132 Treatments

In order to determine Tyr phosphorylation status of the cell lines, the general Tyr phosphorylation inhibitor pervanadate was used. All cells used in these experiments were analyzed via flow cytometry to ensure that CC1-L expression levels were comparable. Cells were grown to ~ 70% confluency in 15 cm plates. The cells that received no pervanadate treatment (negative controls) were scraped off of the plate and lysed first. The pervanadate was then prepared (0.5 mL of 50 mM vanadate, 1.9 mL 30% H_2O_2, 7.6 mL H_2O) and was incubated at room temperature for 15 min. Following this, the cells were treated with 300 μL of pervanadate for 7.5 min. at 37°C, scraped off of the plate, and lysed. All cells were lysed within 10 min. of treatment. To assess possible CC1-L degradation, certain cells were also treated with

the proteasome inhibitor MG132 (N-(benzyloxycarbonyl)leucinylleucinylleucinal) (10 μM) for 12 h.

Following protein quantification (bicinchoninic acid assay, Pierce), an immunoprecipitation was performed using the CC1-specific in-house rabbit pAb and 200 μg of total lysate. The total lysate was first precleared with 10 μg of IgG (rabbit) for 1 h at 4°C on a rotary shaker. The volume was equalized to 500 μL among samples using lysis buffer. Then the lysate was precleared with 30 μL of the slurry of Protein A beads for 1 h at 4°C on a rotary shaker. The samples were centrifuged at 4000 rpm for 2 min. at 4°C three times, each time being washed with lysis buffer. The lysate was then incubated with 2 μg of the CC1-specific antibody mentioned above for 2 hrs at 4°C on rotary shaker. Next, the lysate was incubated with 30 μL of the slurry of Protein A beads for 1 h at 4°C on a rotary shaker. Finally, the samples were centrifuged at 4000 rpm for 2 min. at 4°C three times, each time being washed with lysis buffer. The beads were resuspended in 30 μL of 2x loading dye and heated at 95°C for 10 min. Visualization of Tyr phosphorylation was carried out via Western blotting with the 4G10 anti-phospho-Tyr antibody. The blots were then stripped and incubated with the CC1-specific mouse mAb. Total cell lysate was also analyzed via Western blotting as an indicator of the efficiency of the immunoprecipitation.

2.10 Statistical Analysis

All results are presented as mean ± SD unless otherwise stated. Statistical significance between samples was assessed by analysis of variance (ANOVA). A P value of <0.05 was considered statistically significant.

III. Results

3.1 Expression of CC1 in metastatic MC38 CRC cell line

To evaluate the importance of specific residues in the metastasis-inhibitory phenotype of CC1-L, metastatic CRC MC38 C57Bl/6 cells were infected with wild-type CC1-L, empty vector control, and the CC1-L mutants (Ser503Ala, 3 terminal Lys3Ala, and Ser516Ala). After antibiotic selection was completed, cell populations of each mutant cell line were sorted by FACS sorting and stock of cells were frozen down for further use. A representative immunoblot of total CC1-L expression was comparable among CC1-L wild-type and variant cell lines (Figure 7A, B, n=3). As expected, the empty vector control (CT) cell line did not express CC1-L, and was statistically different than all other cell lines ($p<0.05$). There is also significance between both the CC1-L ($p<0.005$) and 3K-3A ($p<0.05$) cell lines and the Ser503Ala (S503A) cell line. These differences are likely due to the large variation in the values of S516A.

Surface expression of CC1 was evaluated using flow cytometry (Figure 7C). This was first evaluated as a percentage of total cells expressing CC1-L (Figure 7D). There was a very significant difference in percentage of total cells expressing CC1-L between the CT cell line and every other cell line ($p<0.0005$). Surface CC1-L expression was next evaluated as a median fluorescence intensity (MFI) of the cell lines (Figure 7E). There was no significant difference in MFI between the CC1-L-expressing cell lines. Overall, these data confirm that the CT cell line does not express any CC1-L, and that the other cell lines express a comparable amount of total and surface CC1-L.

3.2 Proliferation rate of empty vector control, CC1-L wild-type, and CC1-L-variant cell lines

In order to characterize the phenotypic differences *in vitro* between these cell lines, a proliferation assay was performed using the Roche xCELLigence machine. This method was chosen due to its accuracy, consistency, and ease of use [380]. The metastatic CRC MC38 cell lines described above (CT, CC1-L, S503A, 3K-3A, S516A) were evaluated in triplicate in three separate experiments. The log phase of growth was used in the calculation of doubling time (~3 to 45 h). A representative graph of three independent experiments shows a doubling time of ~ 17

h for CT, ~ 27 h for CC1-L, ~21 h for S503A, ~19 h for 3K-3A, and ~19 h for S516A ± SD (Figure 8). The doubling times of all cell lines were significantly different from that of CT (p<0.005). Similarly, the doubling times of all cell lines were significantly different from that of both CC1-L (p<0.0005) and S503A (p<0.0005). Overall, the CC1-L cell line proliferated the slowest (highest doubling time), and the CT cell line proliferated the quickest (lowest doubling time). All variants (S503A, 3K-3A, and S516A) proliferated at rates more similar to that of CT than CC1-L. In summary, mutation of these residues in the MC38 background partially restored the proliferative capacity of the cells relative to CC1-L wild-type.

3.3 Metastatic burden of wild-type mice injected intrasplenically with empty vector control, CC1-L wild-type, and CC1-L variant cell lines

Intrasplenic injections were performed to evaluate the metastatic ability of the MC38 cell lines (CT, CC1-L, S503A, 3K-3A, and S516A) on 40 male C57Bl/6 mice in total (8 mice/cell line). This method of experimental metastasis bypasses the metastatic intravasation step, but has been used as a model since 1986 [378]. Each mouse was injected with 2 x 10^5 cells (in 50 μL), the expression of which was verified via flow cytometry the day prior to the surgery. All injections were performed within 4 h of cell trypsanization to avoid cell death. Two weeks post-surgery, the mice were sacrificed and their livers were examined for metastatic burden by H & E staining of liver sections separated by 50 microns (Figure 9A). Analysis of the livers using the Pixel Contrast Analysis (described in Materials and Methods) showed that the CT cell line resulted in the highest metastatic burden (significantly different from that of the 3K-3A cell line, p<0.005), the CC1-L cell line displayed the lowest metastatic burden (significantly different from that of all other cell lines, p<0.0005), and the variant cell lines (S503A, 3K-3A, and S516A) yielded metastatic burdens more similar to that of the CT cell line (Figure 9B). The results of this experiment show that the CC1-L wild-type-expressing cells maintain the metastasis-inhibitory phenotype as previously described by our laboratory in the context of CRC development [170]. This experiment also showed that the CT cell line resulted in the highest metastatic burden, and that all three variants (S503A, 3K-3A, S516A) displayed metastatic burdens more similar to that of the CT than the CC1-L cell line. In summary, as reported for CT51 tumorigenic cell line, modifications of these residues in CC1-L lead to phenotypic differences in metastasis.

3.4 CC1 degradation and phosphorylation status not statistically different between CC1-L wild-type and variant cell lines

In order to elucidate signaling pathways that are differentially affected in the CC1-L variant cell lines compared to the CC1-L wild-type and the empty vector control cell lines, phosphorylation status of the wild-type and Ser503Ala cell lines was evaluated. The current literature demonstrates that, at least in the context of insulin clearance, Ser503 phosphorylation is essential for Tyr488 phosphorylation, which in turn permits insulin-induced phosphorylation, leading to insulin clearance in 3T3 fibroblast cells [293]. To investigate phosphorylation status, we decided to use pervanadate, which is a known insulin mimetic and irreversible inhibitor of protein-Tyr phosphatases [381]. Vanadium compounds are known to behave as insulin, in that they lower glucose levels in insulin-resistant forms of diabetes via a PI3K- and PKB-independent pathway [382]. Treating cells with this Tyr phosphatase inhibitor would permit the assessment of the physiological level of CC1 Tyr phosphorylation. In keeping with the work mentioned by Najjar *et al.*, it was expected that CC1-L would be phosphorylated to a significantly lesser degree in MC38 cells expressing the Ser503Ala mutant as compared to the wild-type. We noticed after the first pervanadate experiment that there was differential expression of CC1 itself in the wild-type and Ser503Ala cell lines (Figure 10A). To investigate the possibility of proteasomal CC1 degradation following cell lysis, cells were treated with the proteasome inhibitor MG132 in conjunction with pervanadate inhibition of phosphorylation. Following an immunoprecipitation with an in-house anti-CC1 pAb [169], phosphorylation status and CC1 expression was examined (Figure 10A).

Using the ImageJ (NIH) analysis software, Western blots were analyzed to determine if there was a difference in CC1 expression between untreated and treated cells (Figure 10B). Actin from the whole lysate control for this experiment was used as a loading control for this analysis. ANOVA analysis revealed that there was no significant difference in CC1 expression between the treated and untreated cells, indicating that CC1 expression is stable in both cell lines.

To investigate the phosphorylation status of the CC1-L wild-type and Ser503Ala cell lines, cells were treated with pervanadate. Upon immunoprecipitation using the in-house anti-CC1 pAb and 50 μg of lysate, Western blots were incubated with the 4G10 anti-phospho-Tyr and an anti-CC1 antibody. A representative experiment of three is shown (Figure 10C). While

the same general trend is followed in all three experiments (greater fold increase of Tyr phosphorylation in the wild-type cell line as opposed to the Ser503Ala cell line), ImageJ (NIH) analysis determined that there was no significant difference in phosphorylation status between these two cell lines (Figure 10D). Furthermore, the large error values associated with these data indicate that either there is no pronounced difference in phosphorylation status or that there is much technical variability that needs to be addressed. Given the significant error values of this data, this experiment needs to be repeated in order to reach a solid conclusion.

3.5 Continued CC1 expression in metastatic nodules of *Cc1*$^{-/-}$ mice injected intrasplenically with empty vector control, CC1-L wild-type, and CC1-L variant Ser503Ala cell line

One important question to answer when performing these assays is to verify that CC1 expression has been maintained in the tumors developing *in vivo* for 14-17 days. We encountered many technical difficulties in the assessment of CC1 expression in the wild-type mouse background due to the presence of CC1 expressed endogenously in many tissue types. To obviate this difficulty, an *in vivo* experiment was repeated in *Cc1*$^{-/-}$ mice. A Western blot performed on tumor lysates from 9 of these mice displayed that CC1-L expression was maintained in all mice after 15-17 days and that mice injected with the empty vector control (CT) did not express CC1-L (Figure 11). Other mice were excluded from the data because of surgical complications or because lung metastasis was observed in *Cc1*$^{-/-}$ mice, which displayed zero hepatic metastatic burden (Arabzadeh, A., personal communication). Three of the four mice injected with the Ser503Ala cell line had reduced CC1-L expression as compared to the mice injected with the CC1-L wild-type cell line. The blot was also probed with an actin-specific antibody, which served as a loading control. All mice injected with the CT cell line were sacrificed 15 days post-injection and all mice injected with the CC1-L and Ser503Ala cell lines were sacrificed 17 days post-injection. In summary, the expression of CC1-L appears to be maintained throughout the development of the metastatic nodules whereas that of the Ser503Ala mutant is, in general, significantly reduced over the same period. It is presently unclear why this mutant's expression is significantly decreased. Repeating this experiment with more mice per cell line and calculating metastatic burden of their livers would provide insight as to whether or not the reduced CC1 expression in the Ser503Ala cell line results in decreased metastatic burden.

If this were the case, it would suggest that the *Cc1*$^{-/-}$ environment negatively selects against the Ser503Ala mutant, indicating that this residue is particularly important in the knockout context.

Figure 7: CC1 expression in metastatic CRC MC38 C57Bl/6 cell lines

A) Western blot on whole cell lysates showing the total expression of CC1 in the infected MC38 cell lines (empty vector control (CT), wild-type protein (CC1-L), 3Lys3Ala (3K-3A), Ser503Ala (S503A), Ser516Ala (S516A)), using an in-house monoclonal anti-CC1 antibody. Anti-actin immunoblotting confirmed equal loading of proteins. **B)** Quantification of CC1 expression in the cell lines relative to CC1-L using ImageJ (NIH) on 3 replicate gels ± SD. *p<0.005, **p<0.005, paired one-tailed T-test. **C)** Flow cytometry profiles displaying CC1 surface expression for all 4 cell line (wild-type CC1-L, 3Lys3Ala mutant, Ser503Ala mutant, and Ser516Ala compared to the empty vector control cell line (CT)**). D)** Quantification of the flow cytometry profiles. The percent of CC1-positive cells were calculated from 5 replicate experiments ± SD. ***p<0.0005, Student's T-test. **E)** Quantification of the flow cytometry profiles. The median fluorescent intensity was calculated from 5 replicate experiments ± SD. *p<0.05, **p<0.005, Student's T-test.

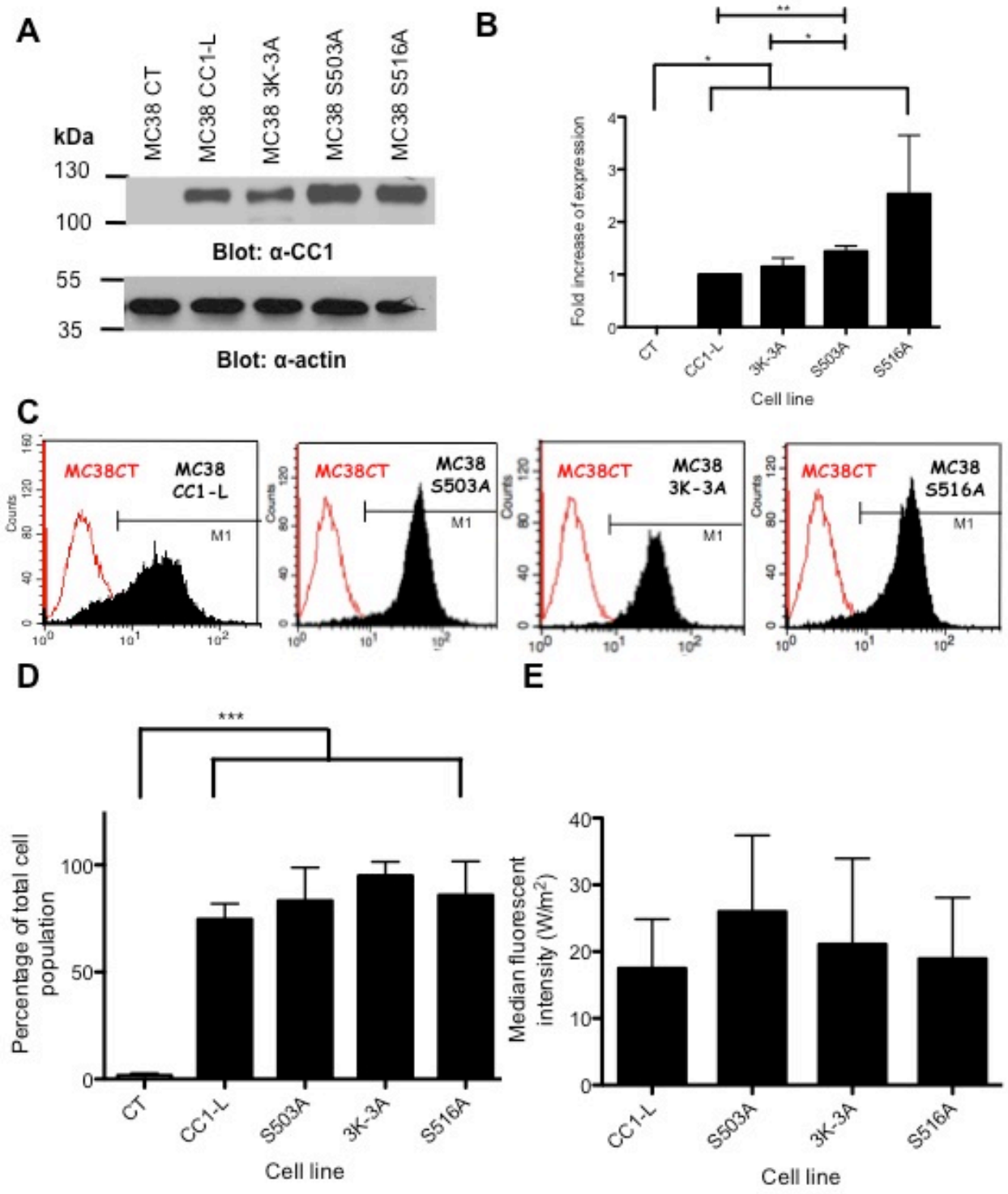

A
MC38 CT
MC38 CC1-L
MC38 3K-3A
MC38 S503A
MC38 S516A
kDa
130
100
Blot: α-CC1
55
35
Blot: α-actin
B
Fold increase of expression
CT
CC1-L
3K-3A
S503A
S516A
Cell line
C
MC38CT
MC38 CC1-L
MC38 S503A
MC38 3K-3A
MC38 S516A
Counts
M1
D
Percentage of total cell population
Cell line
E
Median fluorescent intensity (W/m²)
Cell line

Figure 8: Doubling time of the metastatic CRC MC38 C57Bl/6 cell lines.

The Roche xCELLigence system was used to evaluate the proliferative capacity of MC38 cell lines overexpressing the empty vector control (CT), the wild-type protein (CC1-L), the Ser503Ala mutation (S503A), the 3Lys3Ala mutation (3K-3A), and the Ser516Ala (S516A) mutation. Over a period of 60 hours, the system recorded the impedance value as a Cell Index value, which is a quantitative value of cell number. From this real-time xCELLigence cell index data, doubling time was generated using the RTCA software, provided by Roche with the instrument. Cell lines were analyzed in triplicate, and this is a representative trial of 3. Doubling time calculated for each cell line ± SD is displayed; $**p<0.005$, $***p<0.0005$, ANOVA.

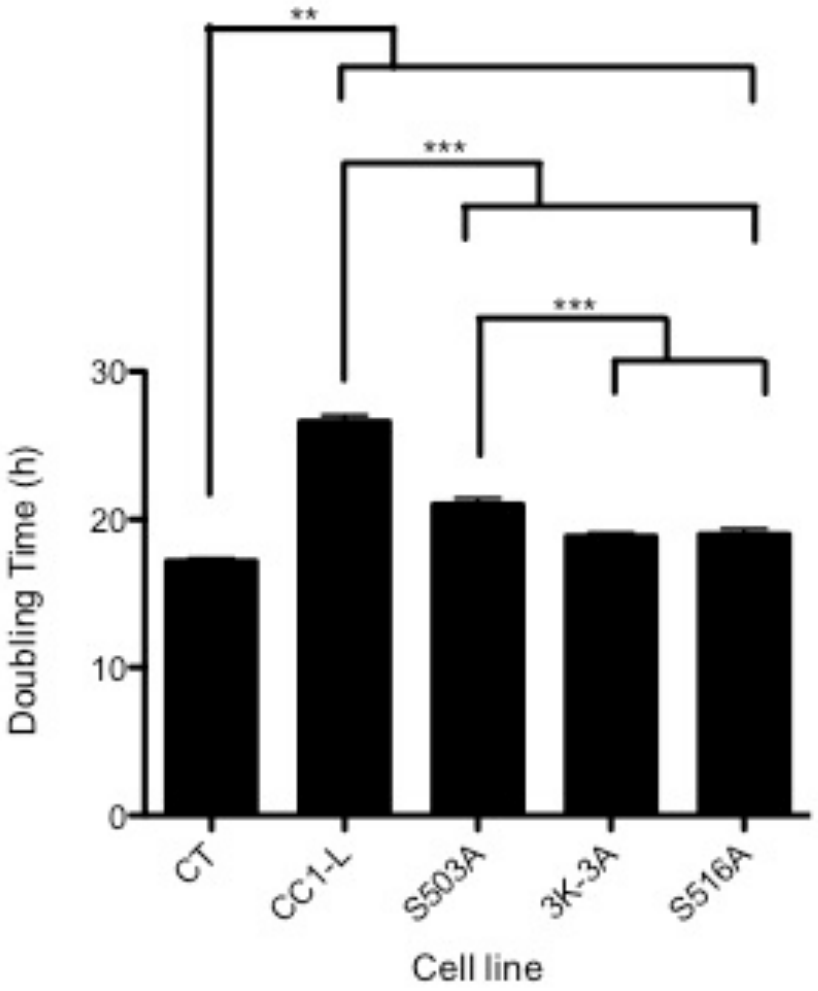
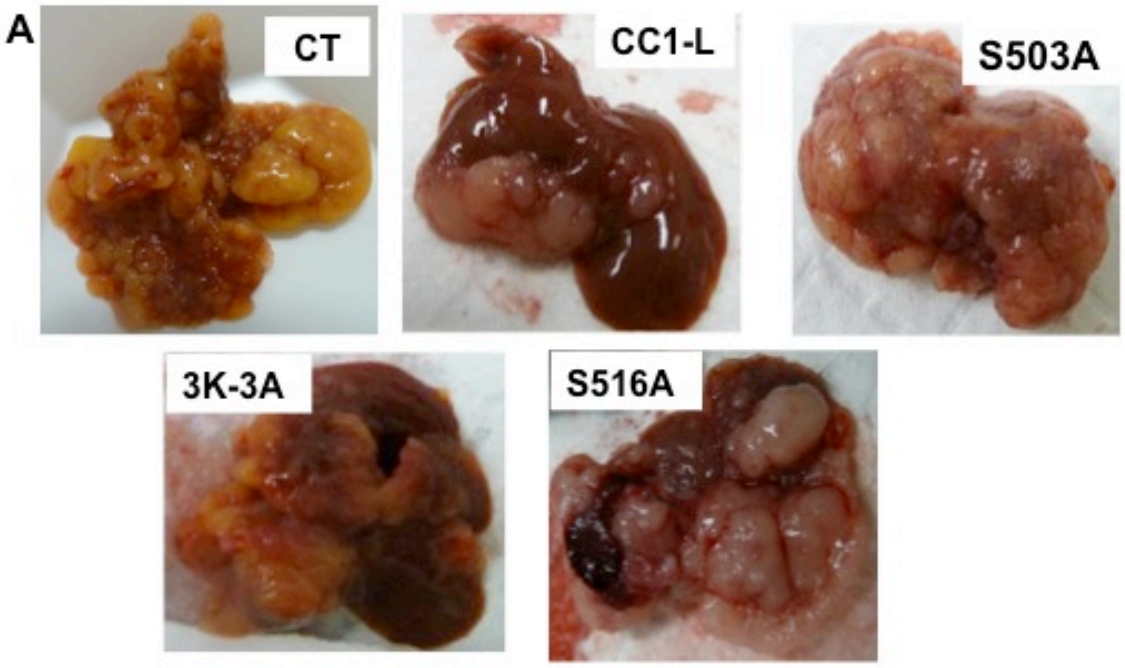

**
***
***
30
20
10
0
Doubling Time (h)
CT
CC1-L
S503A
3K-3A
S516A
Cell line

Figure 9: Metastatic tumor burden in the liver of wild-type C57Bl/6 mice post-intrasplenic injection of metastatic CRC MC38 C57Bl/6 cell lines.

MC38 cells overexpressing the empty vector (CT), the wild-type CC1-L protein (CC1-L), the Ser503Ala mutation (S503A), the 3Lys3Ala mutation (3K-3A) and the Ser516Ala (S516A) mutation were intrasplenically injected into 40 male C57Bl/6 mice (8 per cell line) to evaluate the metastatic capacity of each cell line. Mice were sacrificed two weeks post-surgery and livers were examined for metastatic burden, as described in Materials and Methods. **A)** Representative metastatic tumor burden of C57Bl/6 wild-type mice 2 weeks following intrasplenic injection of metastatic CRC MC38 cells expressing wild-type CC1-L and variants. The specimens were then paraffin-embedded and stained with hematoxylin and eosin, with 4 step sections per liver/mouse. **B)** Quantification of metastatic burden as a fraction of total tissue area, using the Pixel Contrast Analysis that compares color contrast in pixels. The results were obtained from 39 mice (one excluded from CT group due to surgical complications); $^{**}p<0.005$, $^{***}p<0.0005$, ANOVA.


A
CT
CC1-L
S503A
3K-3A
S516A

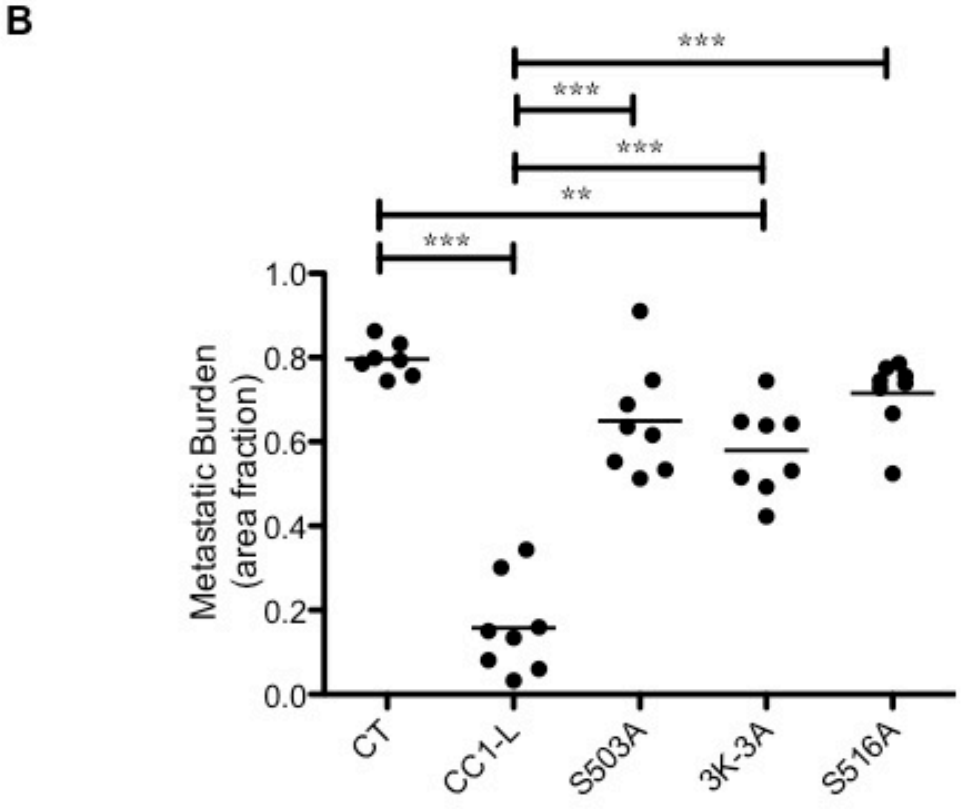

B
***
***
***
**
***
Metastatic Burden
(area fraction)
1.0
0.8
0.6
0.4
0.2
0.0
CT
CC1-L
S503A
3K-3A
S516A

Figure 10: Phosphorylation status and CC1-L degradation.

A) Western blot on immunoprecipitated lysate (with an in-house anti-CC1 pAb [169]) showing the results of treatment with the MG132 proteasome inhibitor to evaluate protein degradation (see **B**) in conjunction with the phosphatase inhibitor, pervanadate, to evaluate Tyr phosphorylation status (see **C, D**) in metastatic CRC MC38 cells overexpressing the empty vector control (CT), the wild- type CC1-L protein, and the Ser503Ala (S503A) mutant. Blots were incubated with the 4G10 anti-phospho-Tyrosine antibody to evaluate phosphorylation status, stripped and then blotted with an in-house anti-CC1 antibody. Also included as a control for MG132-treated cells were cells treated with DMSO (last three lanes). As a further control, total cell lysate from treated and untreated cells was also incubated with the 4G10 anti-phospho-Tyrosine and CC1 pAb (not shown), then stripped and incubated with an antibody against actin. **B)** Quantification of MG132 experiment (see **A**), displayed as fold difference in total CC1 expression. CC1 expression of the CC1-L and Ser503A cell lines without MG132, with MG132, and with DMSO are displayed. There was no significant difference between cell lines (ANOVA). ImageJ (NIH) analysis software was used to quantify the Western blot. **C)** Western blot displaying the results of treatment with the phosphatase inhibitor, pervanadate, on metastatic CRC MC38 cells overexpressing wild-type CC1-L, an empty vector control (CT), and the Ser503Ala variant. Cells were treated with pervanadate for 7.5 minutes and then lysed and quantified (BCA assay, run in triplicate). 50 μg of whole cell lysate was used to immunoprecipitate CC1 (in-house antibody). This is a representative experiment of 3. Blots were revealed with either anti-pTyr or anti-CC1 antibodies. **D)** Quantification of pervanadate experiment (see **B**), displayed as a fold increase in phosphorylation between treated and untreated cells. ImageJ (NIH) analysis software was used to quantify the Western blot, and no significant difference between cell lines was found using ANOVA.

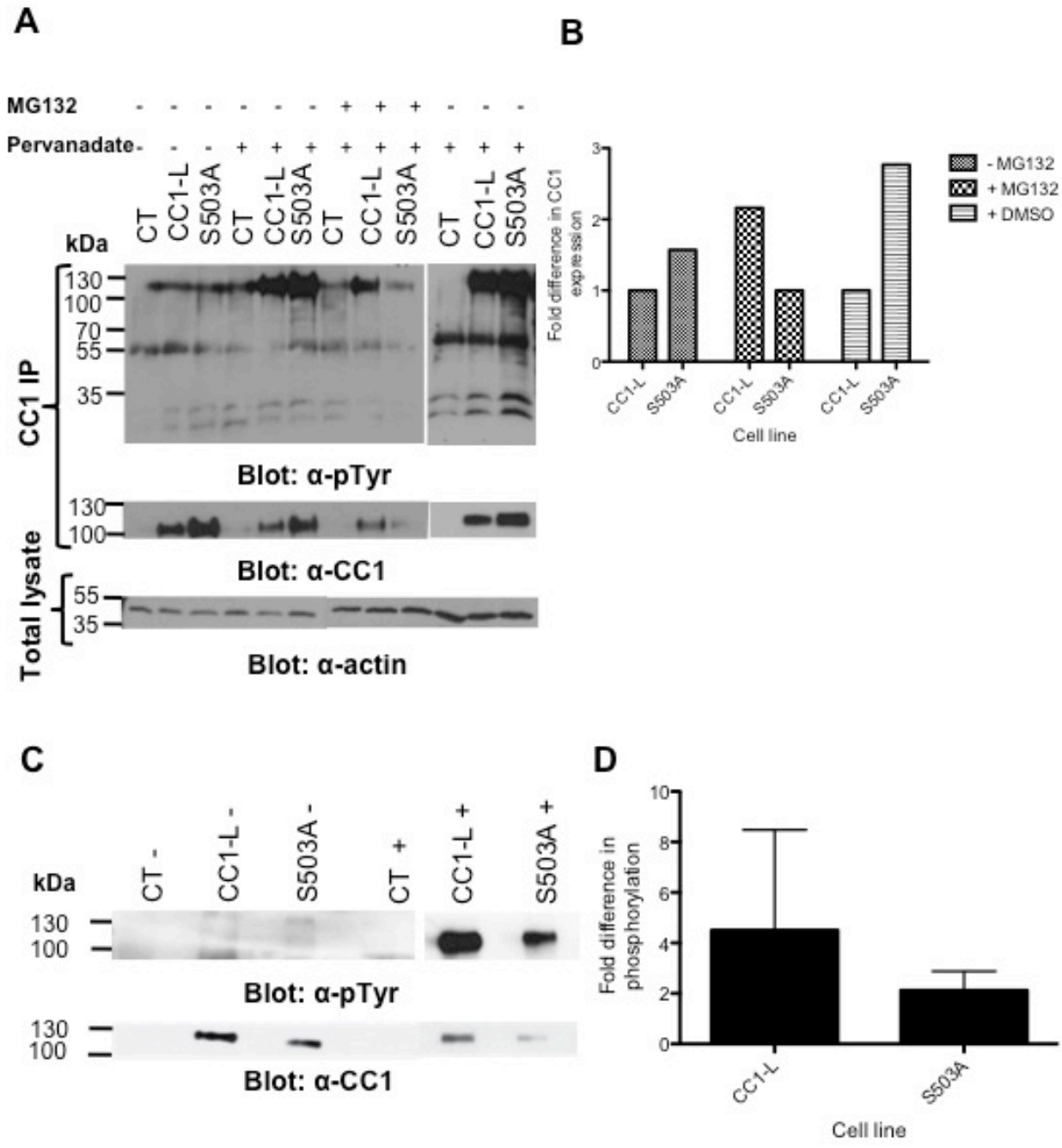
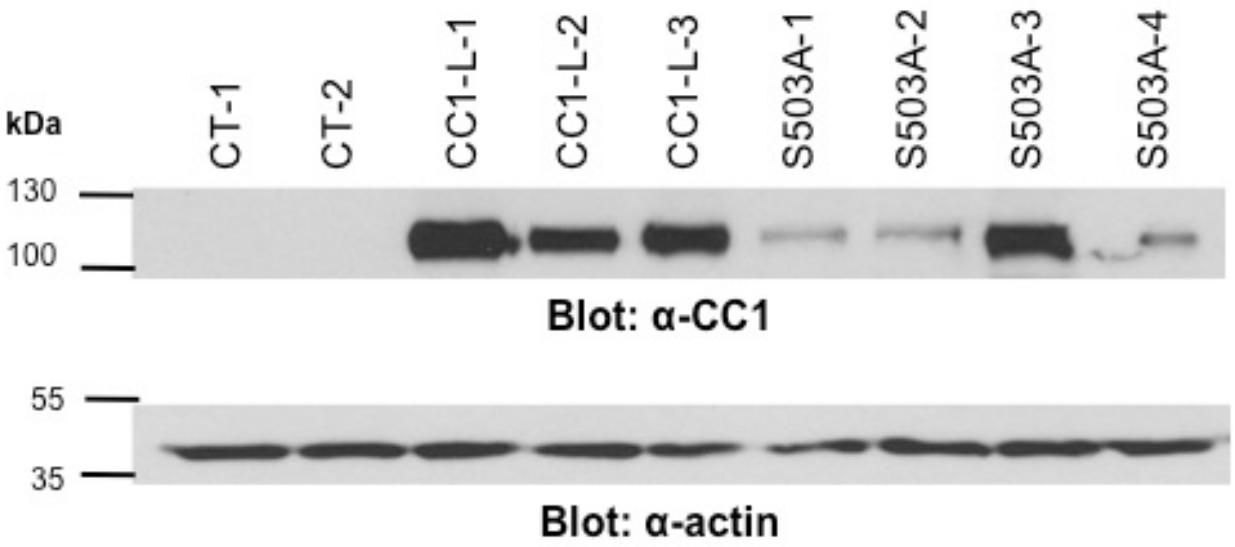

A
MG132
Pervanadate
CT
CC1-L
S503A
kDa
130
100
70
55
35
CC1 IP
Blot: α-pTyr
Blot: α-CC1
Total lysate
Blot: α-actin
B
Fold difference in CC1 expression
- MG132
+ MG132
+ DMSO
Cell line
C
Blot: α-pTyr
Blot: α-CC1
D
Fold difference in phosphorylation
Cell line

Figure 11: Expression of CC1 in the metastatic nodules of CC1$^{-/-}$ mice post-intrasplenic injection of metastatic CRC MC38 C57Bl/6 cell lines.

MC38 cells overexpressing the empty vector (CT), the wild-type CC1-L protein (CC1-L), the 3Lys3Ala mutation (3K-3A), and the Ser503Ala mutation (S503A) mutation were intrasplenically injected into 14 male *Cc1*$^{-/-}$ C57Bl/6 mice to evaluate the metastatic capacity of each cell line in the *Cc1*$^{-/-}$ background. Mice were sacrificed 15-17 days post-surgery and livers were examined for metastases formation. A Western blot using metastatic nodule total lysates of C57Bl/6 *Cc1*$^{-/-}$ mice following intrasplenic injection of metastatic CRC MC38 cells expressing wild-type CC1-L and variants. Blots were incubated with an in-house CC1-specific mAb. Anti-actin immunoblotting confirmed equal loading of proteins.


kDa
CT-1
CT-2
CC1-L-1
CC1-L-2
CC1-L-3
S503A-1
S503A-2
S503A-3
S503A-4
130
100
Blot: α-CC1
55
35
Blot: α-actin

IV. Discussion

In this work, the importance of the CC1-L Ser503, 3 terminal Lys, and Ser516 residues in CRC metastasis was evaluated. The metastatic CRC MC38 cell lines were used to define an *in vitro* proliferative phenotype of mutants of these residues. They were also used to define an *in vivo* phenotype with regard to the incidence of hepatic metastasis in an experimental metastasis model.

4.1 *In vitro* characterization of MC38 CC1-L wild-type and variant cell lines

Increased cell cycle progression is a common change that promotes tumor progression and metastatic growth [212]. Given the tumor-inhibitory property of CC1-L, we hypothesized that the cells expressing CC1-L wild-type would have the highest doubling time in comparison to the empty vector control (CT) and the CC1-L mutants. We further hypothesized that the CT cells would display the lowest doubling time, while the mutants of the residues believed to be important for CC1-L function (Ser503, 3 terminal Lys, Ser516) would have doubling times closer to that of the CT than the wild-type cells. In an effort to elucidate the *in vitro* phenotype of the MC38 CT, CC1-L wild-type, and CC1-L variant cell lines, doubling times were evaluated. These results were compared against previous proliferation experiments conducted in our laboratory using the CT and CC1-L wild-type cell lines (Arabzadeh *et al.,* manuscript in preparation), and the doubling times were dissimilar. This thesis found that the CT cell line had a doubling time of ~ 17 h and the CC1-L cell line had a doubling time of ~ 27 h, while the unpublished manuscript found doubling times of ~ 5 h and ~ 13 h, respectively. While a trend is followed in these experiments, there is a discrepancy between the values of doubling times. This could be explained by a slightly different number of cells seeded and by a slightly different time window of data points. With respect to the doubling time of the MC38 CT cell line, 17 h is comparable to the doubling times found for the MC38 cell line by Hu *et al.* (24 h) [383]. This discrepancy will be investigated via further proliferation trials using the xCELLigence system. These doubling times can further be compared to the cell cycle of normal eukaryotic cells, which is around 24 hours [384].

The Ser516Ala CC1-L variant cell line displayed an unexpected phenotype. It was originally supposed to serve as a control mutation for this project, based on the paper by Huber *et*

al., which showed that the Ser516Ala mutation did not affect CC1-L's binding to SHP-2 or CC1-L phosphorylation [225]. This is the first evidence of the importance of the Ser516Ala mutation in the context of metastatic CRC MC38 cell proliferation. This phenotype is likely either associated with the location of Ser516 within the second ITIM of CC1-L or with the disruption of any interaction with binding partners of Tyr515 (previously shown to play a partial role in CRC development [282]), blocking downstream signaling. As mentioned in section 1.4.2, ITIMs are docking sites for kinases and phosphatases [218]. To test the importance of this ITIM for the functioning of the Ser516 residue, another mutant with the ITIM deleted could be generated. If these cells behave the same way as the Ser516Ala mutant, then this would indicate that the Ser516Ala mutant obstructs ITIM binding or favors binding of a different partner.

This *in vitro* experiment also demonstrated that all CC1-L variant cell lines have a doubling time closer to that of the CT cell line, indicating that Ser503, the 3 terminal Lys, and Ser516 are all essential residues for the tumor inhibitory effect of CC1-L. To confirm these phenotypes, migration and invasion assays using the xCELLigence system or boyden chamber assays should also be performed. This experiment therefore proved our hypothesis and displayed an increase in cell cycle, which as stated earlier is an important change for metastatic growth. In fact, it has previously been shown that CC1-L amino acids 454-518 participate in the increased motility of epithelial cells that overexpress CC1-L [256]. This was found to be due to an increased abundance of desmosomes and a disorganization of cytokeratin filament. To investigate whether Ser503, the 3 terminal Lys, and Ser516 are important in motility assays, we evaluated these same cells for their metastatic ability *in vivo*.

4.2 *In vivo* characterization of wild-type and variant MC38 cell lines in wild-type mice

In order to evaluate the metastatic capacity of MC38 CT, CC1-L wild-type, and CC1-L variant cell lines in the context of CRC hepatic metastasis, *in vivo* intrasplenic injections were performed on 40 male *Cc1* wild-type C57Bl/6 mice. Based on previous results in our laboratory , we hypothesized that the CT cell line would have the highest metastatic burden, while the CC1-L wild-type cell line would have the lowest. We further hypothesized that the CC1-L mutants would have a metastatic burden closer to the CT than the CC1-L wild-type cell line. Results of the CT and CC1-L cell lines were compared against those obtained previously in the laboratory (Arabazdeh *et al.*, manuscript in preparation). A CC1-L variant cell line, Tyr515,488Phe, resulted in a metastatic burden more similar to that of the CT cell line than the CC1-L wild-type,

indicating that these residues are essential for the tumor-inhibitory phenotype observed with CC1-L. The results of this experiment therefore support these previous findings.

Similarly to the proliferation experiments, the Ser516Ala mutant cell line displayed a phenotype more similar to the CT than the CC1-L cell line. The consistent phenotype between the proliferation and *in vivo* experiments indicates that Ser516 is indeed an important residue in the context of CRC hepatic metastasis. This experiment shows the first evidence of the importance of all CC1-L mutations (Ser503Ala, 3Lys3Ala, Ser516Ala) in CRC hepatic metastasis, and re-affirms the tumor inhibitory role of CC1-L in CRC progression. This experiment further proved our hypothesis, showing that the CC1-L residues are in fact important in the context of CRC hepatic metastasis.

4.3 Signaling

After evaluating the phenotypes of the CC1-L variant cell lines *in vitro* and *in vivo* and determining the significance of these residues in CRC hepatic metastasis, we decided to investigate possible signaling pathways that might be affected by the mutations. To do this, possible differences in Tyr phosphorylation status between the cell lines were investigated using the Tyr phosphatase inhibitor, pervanadate (Figure 10A). As a first trial, only the mutation that is best defined in the literature was included in further experiments (Ser503Ala). After the first experiment, a difference in total CC1 expression between the CC1-L and Ser503Ala cell lines was noticed. To determine if this was due to pervanadate-induced CC1 degradation or rather due to a difference in CC1 expression in the cell lines themselves, cells were also treated with MG132, a proteasome inhibitor.

The MG132 experiment showed that there was no difference in CC1 expression between treated and untreated cells, which is consistent with what was observed by Sundberg *et al.* in their pervanadate experiment using MDCK cells expressing CC1-L and Ser503Ala [256]. Examining Figure 7C, D, and E there is also a difference in surface CC1-L expression levels between these two cell lines. Although this difference is insignificant and only representative of surface CC1-L, it might be responsible for the difference in CC1 expression seen in Figure 10A. Given these data, it was determined that the difference in CC1 expression between the cell lines witnessed via this Western blot is likely due to different expression levels in the cell lines themselves. However, the inconsistency in the data indicates that further replicate experiments are required to draw any final conclusions.

Based on previous results [293], we hypothesized that, upon pervanadate treatment of the MC38 cells, the level of phosphorylation would be greater in the CC1-L cell line than the Ser503Ala cell line. This hypothesis was based on the study by Najjar *et al.*, which showed that phosphorylation of the Ser503 residue was essential for Tyr488 insulin-induced phosphorylation, leading to insulin clearance in 3T3 fibroblast cells [293]. This means that the total level of Tyr phosphorylation would decrease in the absence of Ser503 phosphorylation. We evaluated whether or not similar molecular mechanisms in this paper were responsible for CRC metastasis (Figure 10C). Although the level of phosphorylation was generally higher in the CC1-L wild-type cell line than Ser503Ala, there was no significant difference in phosphorylation status between them (Figure 10D). This indicates that Ser503 phosphorylation might not be essential for Tyr488 phosphorylation in MC38 cells, but that it could play a small role either directly or indirectly. Similar findings have been observed in the CT51 cell line, a tumorigenic non-metastatic CRC cell line [225]. However, the data presented herein is inconclusive and could also indicate that further replicates with less total cell lysate to increase sensitivity are needed to see a significant difference in Tyr phosphorylation. Overall, further studies are needed to uncover the mechanism governing this.

These data oppose the results of another experiment examining CC1-L lateral localization, which used pervanadate to evaluate the Tyr phosphorylation status of CC1-L wild-type and Ser503Ala and 3Lys3Ala mutants in MDCK kidney cells [256]. Sundberg *et al.* found that the level of phosphorylation compared to CC1-L wild-type was greatly increased in the cells expressing the S503A (15x) and 3K-3A (272x) mutants, indicating that mutation of Ser503 and 3Lys facilitated CC1-L Tyr phosphorylation. There are a few likely reasons why this study yielded different results, including different cell lines, different cellular processes (implying different binding partners such as kinases), and different methods of transfection (resulting in different expression levels of CC1). It is possible that, in MC38 cells, Ser503 phosphorylation does not regulate Tyr488 phosphorylation. This experiment did not prove our hypothesis and instead suggested that there is very likely a different mechanism governing CC1-L Tyr phosphorylation in this metastatic CRC cell line. However, the data from this experiment is inconclusive and further replicates of this experiment are needed. As well, the genetic background of the MC38 cells might affect these results and these mutants should be tested in a different genetic background, namely the BALB/c-derived CT26 metastatic CRC cells.

4.4 Intrasplenic injections in *Cc1*$^{-/-}$ mice

Due to difficulty in showing retention of CC1 expression over time *in vivo* via IHC and immunofluorescence in the metastatic nodules in the first *in vivo* experiment, we decided to repeat the experiment in *Cc1*$^{-/-}$ mice. We hypothesized that this would eliminate background CC1 expression in the liver parenchyma and it would be simpler to show CC1 expression in the metastatic nodules. Unfortunately, our CC1-specific antibody either bound to what is likely a CC1-like molecule in the liver parenchyma or the blocking was insufficient, and I was unable to show CC1 expression in the metastatic nodules. However, CC1 expression 15-17 days post-injection was confirmed via a Western blot using lysate from the metastatic nodules of these mice (Figure 11A). Given that experimental conditions for both *in vivo* experiments were consistent and that the time between injection and sacrifice was similar (14 days vs 15-17 days), it is inferred that CC1 expression was also likely maintained in the *Cc1* wild-type mice. The decreased CC1 expression level in three of the four mice injected with the Ser503Ala cell line should be noted, and further studies will look to uncover the mechanism governing this. Looking to see if there is a correlation with metastatic burden, for example, would be informative. Overall this experiment showed that CC1-L expression is maintained 15-17 days post-injection in *Cc1*$^{-/-}$ mice, and that a mutation of Ser503Ala appears to confer instability in this cell line. Furthermore, it was the first investigation of the Ser503Ala mutation in the context of a CC1-null background, and in this sense was informative.

4.5 Future Directions

This project represents the basis for further studies regarding the mechanisms by which the Ser503, 3 terminal Lys, and Ser516 residues govern CC1-L-mediated CRC hepatic metastasis. The *in vitro* and *in vivo* phenotypes defined during this thesis provide evidence that these residues are essential for the tumor-inhibitory effect of CC1-L, and the investigation into the possible signaling pathways demonstrates that Ser503 phosphorylation may act independently of Tyr488 and Tyr515, which also play a role in CRC hepatic metastasis (Arabzadeh *et al.*, manuscript in preparation).

4.5.1 Immediate investigations

Immediate future work includes demonstrating the expression of CC1 in the metastatic nodules of the *Cc1* wild-type and knock-out mice. While the Western blot of tumor lysate from

the *Cc1*$^{-/-}$ mice strongly suggests that expression was also maintained in the experiment using *Cc1* wild-type mice, immunofluorescence experiments demonstrating metastatic nodule-specific CC1 expression is necessary. This would provide evidence that the phenotype observed is linked to CC1 expression of the injected cells. It would also enable co-staining in future studies. If the monoclonal CC1-specific antibody also recognizes the CC1-like protein during immunofluorescence staining on frozen and paraffin-embedded tissue, then CC1 expression could be confirmed via RNA extraction, cDNA conversion, followed by PCR amplification of CC1. Once evidence of this is shown, the following markers will be evaluated via immunofluorescence: Ki67 (proliferation), CD3 (T cells), CD8 (suppressor/cytotoxic T cells), F4/80 (macrophages), CD31 (angiogenesis), α-smooth muscle actin (SMA) (vessel maturity), CD11b (dendritic and NK T myeloid-derived leukocytes), and Gr-1 (granulocytes). Conditions for all these antibodies have already been defined in our laboratory. The results of these markers would be compared against the data from intrasplenic surgeries on *Cc1*$^{-/-}$ mice using MC38 parental cells [169], which found that reduced liver metastasis was controlled, at least in part, by CD11b+ Gr1+ myeloid-derived suppressor cells (MDSCs).

In addition to this, the xCELLigence proliferation trials will be repeated to confirm the doubling times observed. MTT proliferation assays were also performed, and the doubling times observed differed from those shown by the xCELLigence data (MTT showed ~ 10.2 h for CT, ~ 13.9 h for CC1-L, and ~ 10.9 h for Ser503Ala). While the values of these doubling times are different from those obtained via the xCELLigence experiments, the trend remains the same. Once a consistent doubling time is obtained, migration and invasion assays can be performed either using the xCELLigence system or Boyden chambers to assess the migratory capacity of the MC38 cell lines. This would provide further evidence for the importance of the Ser503, 3 terminal Lys, and Ser516 residues of CC1-L for the tumor inhibitory phenotype. Given the interesting phenotypes observed in the CC1-L mutants in terms of CC1-L cellular localization [256], cellular localization will be investigated. Differential localization of CC1-L in the mutants used in this thesis could be functionally informative. Conditions for this experiment have been defined.

Evaluation of the differences in cytokines and chemokines between the various cell lines should also be performed in an effort to elucidate signaling pathways affected by the various mutants. The results of this assay would be very interesting to compare to the results published

previously in our laboratory [169], which found that CCL2, CCL3, and CCL5 (known for regulating macrophage and monocyte recruitment) were all down-regulated in the *Cc1*$^{-/-}$ mice upon intrasplenic injection of MC38 cells. In conjunction with this, a Western blot using tumor and liver lysate from the mice of the *Cc1* wild-type experiment would be helpful for identifying affected signaling pathways. These lysates would be examined for phospho-proteins and their total protein counterparts, such as STAT1 (pro-apoptosis, anti-proliferation), STAT3 (increased malignancy, pro-survival), STAT6 (anti-proliferation), NF-κB (pro-survival), Akt (pro-survival), and Erk (pro-proliferation). Analysis of this blot could reveal differential levels of certain phospho-proteins, indicating activation or down-regulation of certain pathways and cellular processes.

4.5.2 Mechanistic investigations

Following the above experiments, the signaling pathways and mechanisms involved in the observed phenotypes would be examined. The type of experiment would depend on the residue being investigated.

A) Ser503Ala, Ser516Ala

The Ser503 residue is the most well-defined in the literature of the residues investigated in this project, as is described in section 1.6. We believe it is an important residue in the context of CC1-L-regulated CRC hepatic metastasis due to its importance in CRC tumor progression [181]. One possible mechanism is that Ser503 interacts with the nearby ITIM containing Tyr488, enabling CC1-L's interaction with kinases/phosphatases, affecting metastatic pathways (described in section 1.2). One way to test this possibility would be to investigate the importance of this ITIM on the ability of Ser503 to regulate CRC metastasis. To do this, an additional mutant which lacks the ITIM could be compared via co-immunoprecipitations to the Ser503Ala mutant and the CC1-L wild-type cell lines. CC1-L in the cells lacking the ITIM should not be able to interact with typical binding partners such as AP1 [188]. If CC1-L in the cells with the Ser503Ala mutation is unable to be co-immunoprecipitated with AP1, this could indicate that Ser503 influences the ITIM signaling. If this were the case, the obligatory presence of Ser503 for Shc's interaction with CC1-L [295] would be explained. This would be because Ser503 would act in tandem with the ITIM containing Tyr488. Furthermore, this would implicate Ser503 in many other signaling pathways, described in section 1.6. Ser503 is also essential for

IR signaling [180], where CC1-L is a target of the IR and affects Akt and PI3K signaling. It is therefore possible that Ser503 acts independently of Tyr488 and affects downstream members of the Akt and PI3K pathways, promoting vascular permeability (angiogenesis) as outlined in section 1.6.4.

Identification of potential Ser kinases and mechanisms related to Ser503 phosphorylation are likely to provide novel insights as to how CC1-L regulates tumor cell migration and invasion *in vivo*. To identify the signaling pathway and mechanisms involved in the phenotype observed with the Ser503Ala and Ser516Ala mutations, identification of the Ser/Thr kinase that phosphorylates these residues or of the phosphatases that dephosphorylate them in the context of CRC hepatic metastasis is necessary. Ideally, a kinase assay would be performed (such as the Omnia Kinase Assay available by Life Technologies). The assay by Life Technologies, for example, involves mixing using cell/tissue lysate with the chelation-enhanced fluorophore, which is incorporated into CC1-L. Once phosphorylated, Mg^{2+} is chelated and forms a bridge between CC1-L and the kinase. This reaction increases fluorescence, indicating that CC1-L is a targe of the kinase used. If a potential kinase for Ser503 or Ser516 is identified via a kinase assay, an inhibitor for this kinase ("kinase X") could be used. Treatment of CC1-L wild-type and mutant cells with this inhibitor, followed by Western blot with phospho-kinase X and total kinase X antibodies would confirm the involvement of this kinase. Unfortunately, this method of investigation is the most costly. A more economic method of investigation and the first to be considered involves searching for consensus sequences in order to identify potential kinases.

A brief overview of consensus sequences for the Ser503 residue, among 13 other Ser/Thr in CC1-L, has identified the consensus sequence for protein kinase C (PKC). However, only Ser449 was shown to be phosphorylated by PKC [232]. In addition to this, Ser503 was found to interact with but not be phosphorylated by Cdk2 [231]. Examination of consensus sequences involving the Ser503 residue revealed one promising candidate kinase, mitogen- and stress-activated kinase (MSK) 1/2. The consensus sequence of this kinase is not specific, but is defined as R-X-X-pSpT, which fits Ser503 and its surrounding residues [385]. Interestingly, the Ser516 of CC1-L also fits this sequence. MSK1 and MSK2 are isoforms that share 64% homology [386]. MSK1 is activated downstream of the ERK1/2 and MAPK signaling pathways, while the activation of MSK2 is not yet well-defined but believed to be similar to that of MSK1 [386]. MSK1/2 are expressed in the majority of cell types, and are localized to the nucleus [386, 387].

However, CC1 is either membrane-bound or in endocytic vesicles or endosomes, and therefore does not overlap with the currently defined location of MSK1/2. That being said, Phan *et al.* found that the cytoplasmic tail localized to the nucleus if delivered by adenovirus [199]. It would be informative to define the cellular localization of MSK1/2 in metastatic MC38 CRC cells to confirm if it could be the kinase. The role of MSK1/2 in cancer is not yet well-defined, but its expression has been implicated in the formation of breast cancer [388]. Furthermore, MSK1 has been shown to play a pro-proliferative role in HaCat cells and A431 human epidermoid carcinoma cells [389], and cell transformation was reduced upon treatment with the MSK1 inhibitor, H89 [386]. Moreover, MSK1 was essential for EGF- or TPA-induced cell transformation via histone phosphorylation [390]. Finally, *Msk1/2* knockout mice displayed reduced melanoma development as compared to wild-type mice [386]. Given these ties to tumor development and to the ERK1/2 and MAPK signaling pathways, MSK1/2 could be the kinase that phosphorylates Ser503. Use of the H89 inhibitor on the MC38 CT, CC1-L, Ser503Ala, and Ser516Ala cells would provide insight into this possibility.

B) 3 terminal Lys3Ala

The investigation of the 3 terminal Lys of CC1-L requires a different method of investigation to identify potential signaling pathways involved. Classically, Lys residues are often polyubiquitinated, leading to proteasomal degradation of the protein and recycling of the ubiquitins [391]. During preliminary pervanadate experiments using the 3Lys3Ala cell line (not shown), no signs of increased CC1 stability due to the conversion of Lys to Ala were observed. We then searched for proteasome-independent functions of ubiquitin that could be affected by the 3Lys3Ala mutant. A review of the literature revealed that monoubiquitination of three consecutive Lys leads to endocytosis of the protein [392]. In fact, many plasma membrane proteins are ubiquitinated and then endocytosed [393]. Given that CC1-L has previously been shown to endocytose in conjunction with the IR and the TCR in the context of insulin clearance and cellular proliferation [220, 292], monoubiquitination-mediated endocytosis of CC1-L could be an important mechanism. In the paper by Choice *et al.* it was shown that the MAPK and PI3K/Akt pathways are down-regulated upon CC1 internalization. In terms of a metastatic context, the MAPK and PI3K/Akt signaling pathways are up-regulated during angiogenic, proliferative, and migratory processes [98]. One potential mechanism for the 3 terminal Lys is

insulin-induced monoubiquitination-mediated CC1-L endocytosis, followed by down-regulation of the MAPK and PI3K/Akt pathways. This anti-angiogenic and anti-proliferative process would be disrupted by mutation of the 3 terminal Lys to 3Ala, resulting in increased pro-tumorigenic pathways. To study the possibility of increased endocytosis in the MC38 cells expressing CC1-L wild-type versus the 3Lys3Ala mutant, *in vitro* localization experiments of CC1-L could be performed. While it is true that CC1-L is defined as being localized to the membrane in CT51 CRC cells [181], perhaps insulin treatment in metastatic MC38 CRC cells would result in relocalization.

The mutation of the 3 terminal Lys to 3 Ala may have also impacted the ability of calmodulin to bind. As mentioned in section 1.6.3, there is a calmodulin binding site at the end of the CC1-L cytoplasmic domain, which impacts cytoskeletal function and downstream signaling mediated by CC1-L dimerization [302]. To investigate the impact of Lys mutation on calmodulin binding, GST pull-downs could be performed.

4.5.3 Long-term investigations

All future studies based on the findings of this thesis should consider the mutational background of the cell line being used. Mutation of oncogenes and tumor suppressors can greatly influence observed phenotypes. For example, there are two major differences in the mutational background between the CT26 mouse CRC cell line (derived from the same tumor as the CT51 CRC cell line used previously in this laboratory to define the role of CC1-L in CRC development) and the metastatic MC38 cell line. The CT26 cell line has a K-Ras mutation (G12D) and is positive for Smad4, while the MC38 cell line expresses the wild-type K-Ras mutation and has no Smad4 activity [394, 395]. Any comparison between these two cell lines would have to consider the different signaling environments. It would be beneficial to conduct future studies with a wide array of cell lines in order to account for such differences in background mutations and to better understand their importance. Furthermore, considering the importance of CEA and CC6 in CRC metastasis as mentioned in section 1.7, it would be informative to further investigate the molecular mechanisms involved. The murine cell line examined provided the advantage of working in an immunocompetent syngeneic mouse background and to avoid the complications of CEA and CC6 expression, as there are no murine equivalents to CEA and CC6. Investigations into human CRC metastatic cell lines will need to take these two factors.

This thesis shows that the Ser503, 3 terminal Lys, and Ser516 all play an important role in CC1-L-mediated CRC hepatic metastasis. The experiments regarding potential mechanisms indicate that the molecular mechanisms may be novel in comparison to mechanisms defined for CRC development. Elucidation of the signaling governing CC1-L-mediated CRC metastasis may uncover novel therapeutic avenues targeting CC1-L. Furthermore, the use of anti-CC1 immunotherapy in the treatment of melanoma indicates the great potential benefit of refining treatment by understanding the mechanisms involved [396]. In fact, there are promising studies using CC1-specific antibodies in the context of CRC. The WL5 anti-human CC1-specific antibody, for example, prevented the formation of lung metastases in mice with human CRC xenografts [397]. Further evidence for the potential of treatment with anti-CC1 antibodies comes from the study by Lu *et al.* who, using MC38 cells, showed that mice treated with an anti-CC1 antibody displayed lower tumor burden [318]. The CC1 field is poised for clinical trials targeting CC1 in cancer.

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