MURINE CD248 AND ITS CYTOPLASMIC DOMAIN:
CHARACTERISING A NOVEL TUMOUR AND INFLAMMATION MARKER

by

MARGIT LAI WUN JUHÁSZ

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Abstract

CD248 is a member of a family of transmembrane glycoproteins containing an N-terminal C-type lectin-like domain. This family includes thrombomodulin and CD93, proteins known to modulate immunity, cell proliferation and homeostasis. CD248 is expressed in perivascular and stromal cells, during embryonic development and post-natally during inflammation and cancer. In the mesenchymal compartment of most normal adult tissues, CD248 expression is not detectable. It is believed that CD248 is involved in cross-talk between endothelial cells and pericytes, thereby playing a role in growth, metastasis and angiogenesis associated with the development of tumours and inflammatory lesions.

Intracellular signaling, mediated via the cytoplasmic domain of CD248, promotes tumour growth and inflammation. CD248 knock-out mice and mice expressing CD248 lacking the cytoplasmic domain were resistant to tumour growth and inflammatory arthritis. Although signaling pathways have not been delineated, examination of the cytoplasmic domain of CD248 reveals three highly conserved putative phosphorylation sites and a PDZ-binding motif. We hypothesised that these structural features are important for CD248 function.

We generated a variety of murine CD248 pcDNA constructs that encode CD248 with mutations in the cytoplasmic domain. We confirmed that all the
mutants were transcribed and translated. Mutant proteins were expressed on the cell surface, in a similar manner to wildtype CD248. Introduction of some mutant forms CD248 into cells caused CD248 to exhibit different intracellular localisation and induced changes in cellular morphology compared to wildtype. Limited functional studies demonstrated CD248-dependent alterations in cellular MMP-9 production. The findings underline an important role of CD248’s cytoplasmic domain in regulating cellular morphology and function that may impact its role in health and disease.

Extracellular interacting partners for CD248 have been previously described. Searches for intracellular partners interacting with the cytoplasmic domain have been less successful. Our results strongly suggest that these exist. Co-immunoprecipitation studies have revealed several putative interacting proteins that set the stage for future confirmatory and functional analyses.

The cytoplasmic domain of CD248 is important to study as it holds much promise as a therapeutic target for proliferative disorders. The information gathered in this project may be used to delineate clinically relevant CD248 signaling pathways.
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List of Abbreviations

A = adenine
Ala = alanine
ANG-2 = angiopoietin-2
APMA = 4-aminophenylmercuric acetate
Asp = aspartate
C = cytosine
CAIA = collagen-antibody induced arthritis
CCP = complement control protein
cDNA = complementary deoxyribonucleic acid
CHO = Chinese hamster ovary-K1
Cox-2 = cyclooxygenase-2
CTLD = C-type lectin-like domain
CyD = cytoplasmic deletion
C-terminus = carboxyl terminus
DAPI = 4',6-diamidino-2-phenylindole
DMEM = Dulbecco/Vogt modified Eagle's minimal essential medium
DMSO = dimethyl sulfoxide
DPC = days post conception
DPN = days post-natal
DTT = dithiothreitol
EGF = epidermal growth factor-like
EPO = erythropoietin
ERK1 = mitogen-activated protein kinase 3 (extracellular signal-regulated kinase 1)
ERK2 = mitogen-activated protein kinase 1 (extracellular signal-regulated kinase 2)
EBS = Ets transcription factor binding sites
Ets-1 = protein-C-ets-1
ER = endoplasmic reticulum
FACS = fluorescence-activated cell sorting
FAK = focal adhesion kinase
FasL = Fas ligand
FITC = fluorescein isothiocyanate
FGF-β = fibroblast growth factor beta
FLAG = FLAG octapeptide
G = guanine
G418 = geneticin
GAPDH = glyceraldehyde 3-phosphate dehydrogenase
GST = glutathione-S-transferase
HEK = human embryonic kidney
HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HER-2 = human epidermal growth factor receptor-2
HIF-1α = hypoxia inducible factor-1 alpha
HIF-2α = hypoxia inducible factor-2 alpha
HMVEC = human microvascular endothelial cells
HUVEC = human umbilical vascular endothelial cells
HSP 70 = heat shock 70 kDa protein 7
HSP 90 β2 = heat shock protein 90 beta-2
IAA = iodoacetamide
IFN-γ = interferon gamma
IGF-1 = insulin-like growth factor 1
IL-1β = interleukin 1-beta
IL-4 = interleukin 4
IL-6 = interleukin 6
IκBα = IkappaBalpha
IMAC = immobilised metal ion affinity chromatography
KO = knock-out
LLC = Lewis lung carcinoma
mAb = monoclonal antibody
Mac-2BP/90K = galectin-3 binding protein, 90 kDa
MAP2K1 = dual specificity mitogen-activated protein kinase kinase 1
MCF-7 = Michigan Cancer Foundation-7
MEF = murine embryonic fibroblast
MMP = matrix metalloproteinase
NOS = nitric oxide synthase
NP-CGG = 4-hydroxy-3-nitro-phenylacetyl chicken g-globulin
N-terminus = amino terminus
P1H12 = CD146
pAb = polyclonal antibody
PCR = polymerase chain reaction
PDGF-BB = platelet-derived growth factor-BB
PI-3K = phosphoinositol-3 kinase
PIGF = placental growth factor
PMA = phorbol 12-myristate 13-acetate
Q-RT-PCR = quantitative RT-PCR
RT-PCR = reverse transcription PCR
SAGE = serial analysis of gene expression
SCR = short consensus repeats
SDF-1 = stromal-cell derived factor 1
SDS = sodium dodecyl sulfate
SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ser = serine
SERCA-2 ATPase = sarco/endoplasmic reticulum Ca^{2+} ATPase
siRNA = small interfering ribonucleic acid (silencing RNA)
SM22α = transgelin
SMA = smooth muscle actin
SP-1 = specificity protein 1
SPR = surface plasmon resonance
Src = proto-oncogene tyrosine-protein kinase Src
T = thymine
TBS = tris-buffered saline
TBS-T = TBS with Tween-20
TEK/Tie2 = angiopoietin-1 receptor
TEM-1 = tumour endothelial marker 1
TEM-7 = tumour endothelial marker 7
TGF-α = tumour growth factor alpha
TGF-β = tumour growth factor beta
Thr = threonine
TIMP = tissue inhibitor of metalloproteinase
TNF-α = tumour necrosis factor alpha
TOLLIP = Toll-interacting protein
Val = valine
VEGF = vascular endothelial growth factor
VEGF-R = vascular endothelial growth factor receptor
WT = wildtype
Acknowledgements

“It was the best of times, it was the worst of times, it was the age of wisdom, it was the age of foolishness, it was the epoch of belief, it was the epoch of incredulity, it was the season of Light, it was the season of Darkness, it was the spring of hope, it was the winter of despair, we had everything before us, we had nothing before us . . . .”

Nothing quite describes the undertaking of a graduate degree as the first lines of Charles Dickens’ quintessential novel A Tale of Two Cities. At points, every graduate student feels foolish, despairing, “in-the-dark”, and with no sign of hope before them. However, it’s those moments of “Light”, accomplishment and belief that will eventually get us through.

On that note, I would like to thank all those who have helped me through the despairing moments and shaped my growing self, both as a young person as well as a researcher during my short time here.

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Without my friends, old and new (you know who you are), I would have not been provided with a perfect work-life balance. Thanks to you, I managed to finish my degree and have tons of fun in the “real world” at the same time (thereby maintaining what little sanity I have left).
And that, dear reader concludes all my acknowledgements except for one . . . thank you for taking your time to read my thesis, at least up to the Acknowledgements. But I promise, if you keep reading it really does get better!
Dedication

This thesis is dedicated to all the present graduate students and all those yet to come . . . remember Charles Dickens and persevere on.
1. Introduction

1.1 The importance of CD248

CD248, also known as tumour endothelial marker 1 (TEM 1) and endosialin, is a type I transmembrane glycoprotein belonging to a family of proteins containing an N-terminus C-type lectin-like domain that also includes thrombomodulin (CD141) and C1qRP (CD93). It was first discovered as a gene upregulated in tumour endothelium, however it is now believed that CD248 protein expression is associated with stromal fibroblasts and pericytes of cancer-associated or inflamed tissues. Although CD248 expression is barely detectable in healthy adult tissues, CD248 is highly upregulated during embryogenesis.

The field of CD248 research is rather nascent and therefore the function of this protein in health and disease still remains largely a mystery. It is believed that CD248 may be involved in cross-talk between the endothelial and stromal compartments of tumours and sites of inflammation, thereby playing a significant role in cellular migration and cell growth/proliferation, i.e. participating in tumour growth, tumour metastasis, expansion of inflammatory lesions, and the angiogenesis required for these processes. Due to CD248's apparent restriction to pathological conditions, researchers believe that CD248 has the potential to be an important therapeutic target.
1.2 Discovery

CD248 was first identified in tumour vessels by the FB5 monoclonal antibody, an antibody that was raised against human fetal fibroblasts fused with a myeloma cell line. The FB5 antibody was initially believed to react with vascular endothelial cells in malignant tumours and more weakly with tumour stromal fibroblasts and some tumour cells. Due to the putative expression in endothelial cells and its highly sialylated nature, this newly discovered protein, was ascribed the name “endosialin”\textsuperscript{1}.

In later studies, researchers performed Serial Analysis of Gene Expression (SAGE) on human colorectal tumour endothelium and found that a number of gene transcripts were upregulated. Of 46 transcripts, the most highly expressed was a gene that was referred to as “Tumour Endothelial Marker 1 (TEM1)”\textsuperscript{2}. One year later, Christian \textit{et al.} cloned and characterised the gene and cDNA encoding murine endosialin and determined that the cDNA for endosialin was identical to that which encodes TEM1\textsuperscript{3}. Further study revealed that expression of CD248/TEM1/endosialin (herein referred to as CD248) is limited to either the embryo or to tumour vasculature, but is essentially absent in adult tissues\textsuperscript{4; 5; 6}. These findings raised the exciting prospects of CD248 being a therapeutic target for cancer.
1.3 Expression pattern

In spite of its original detection with an antibody directed against fibroblasts, it was believed for several years that CD248 was endothelial-specific\(^1\);\(^2\);\(^4\);\(^7\). More detailed studies using approaches to distinguish endothelium from surrounding cells have unequivocally revealed that CD248 is a highly specific marker for activated perivascular cells and stromal fibroblasts, detectable in most cancers, as well as in some inflammatory lesions\(^8\);\(^9\);\(^10\);\(^11\);\(^12\);\(^13\).

1.3.1 Expression in vitro

In cell culture, CD248 expression is detectable in embryonic fibroblasts, smooth muscle cells, pericytes and mesenchymal stem cells\(^10\);\(^14\). It is important to note that human microvascular endothelial cells (HMVECs) and human umbilical vein endothelial cells (HUVECs) do not express CD248 under any circumstance, including following stimulation with a variety of factors including vascular endothelial growth factor (VEGF), insulin-like growth factor-1 (IGF-1), stromal cell-derived factor-1 (SDF-1), tumour necrosis factor-\(\alpha\) (TNF-\(\alpha\)), interleukin-1\(\beta\) (IL-1\(\beta\)), transforming growth factor-\(\beta\)1/2 (TGF-\(\beta\)1/2), phorbol myristate acetate (PMA), forskolin (coleonol), interferon-\(\gamma\) (IFN-\(\gamma\)), basic fibroblast growth factor (FGF-\(\beta\)), IL-4, or IL-6\(^1\);\(^9\);\(^15\);\(^16\).
It is interesting that CD248 is expressed by mesenchymal stem cells. These cells have the capacity to differentiate into osteoblasts, chondrocytes and adipocytes, and may play a role in tissue injury repair by homing to damaged sites. Mesenchymal stem cells also differentiate within tumour and inflammatory lesion microenvironments into activated synovial fibroblasts and/or tumour-associated fibroblasts, as well as pericytes. By differentiating to these specific cell types, mesenchymal stem cells play a role in fibrovascular expansion, thereby contributing to both tumour growth and progression of inflammatory lesions.

1.3.2 Expression during development

During fetal development, CD248 is temporally expressed. CD248 is most prominently detected in the embryo and rapidly progressively decreases immediately postnatally. The strongest CD248 expression in the embryo is found at epithelial-mesenchymal interfaces and in embryonic structures where an expression gradient may be observed, with greatest expression in fibroblasts located at the budding tips of these structures.

In mouse embryos, the CD248 transcript can already be detected at 8.5 days post conception (DPC). From then on, CD248 levels remain high throughout fetal development and can be detected in the dorsal aorta at 10 DPC, the perineural vascular plexus and vessels sprouting from the perineural
plexus into the neuroectoderm at 10.5-12 DPC \(^6\); \(^{19}\), mesenchymal stromal fibroblasts of the skin, head, lung, submandibular gland, optic cup, and the developing genitourinary system at 15 DPC \(^5\), the embryonic spleen (becoming more restricted to the interstitial fibroblasts of the red pulp with age) and the renal glomerulus at 16 DPC \(^{21}\), mesenchymal cells of the gastric cavity and dermis, as well as mesangial cells of the kidney from 17-17.5 DPC \(^6\); \(^{19}\). Throughout fetal development, CD248 expression increases in the developing vasculature of the heart, liver and neuroectoderm \(^5\). Furthermore, in the developing thymus and lymph nodes, CD248 can be found in mesenchymal cells and the vasculature of the epithelial medulla \(^{21}\) (Table 1.1).

Particularly interesting is the fact that although CD248 expression is highly expressed during embryonic development, deletion of the \textit{CD248} gene does not result in an apparent effect on the fetus and postnatally, the CD248 knockout mice develop normally under non-stress conditions \(^{12}\); \(^{22}\). These observations raise questions as to the role of CD248 in the embryo and suggest that there may be alternative pathways that are able to compensate for its loss.
Table 1.1: CD248-transcript detection throughout embryonic development

CD248 transcript is detected in a variety of tissues during embryonic development. The expression of CD248 is regulated in a temporal manner.

<table>
<thead>
<tr>
<th>Days Post Conception (DPC)</th>
<th>Tissues in which the Murine CD248 Transcript is Detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>Dorsal aorta</td>
</tr>
<tr>
<td>10.5-12</td>
<td>Perineural vascular plexus; vessels sprouting into the neuroectoderm</td>
</tr>
<tr>
<td>15</td>
<td>Mesenchymal stromal fibroblasts of skin, head, lung, submandibular gland, optic cup, genitourinary system</td>
</tr>
<tr>
<td>16</td>
<td>Embryonic spleen; renal glomerulus</td>
</tr>
<tr>
<td>17-17.5</td>
<td>Mesenchymal cells of the gastric cavity dermis; mesangial cells of the kidney</td>
</tr>
</tbody>
</table>

The expression of CD248 markedly and progressively decreases postnatally. At 1 day post-natal (DPN), CD248 is found in the dermis, stromal fibroblasts of the lung, vessels and red pulp of the spleen, as well as mesangial cells of the kidney. However, expression is almost undetectable in the vessels of the thymus and heart. By 9-10 DPN, CD248 expression in the dermis has decreased due to hair follicles undergoing apoptosis. However, CD248 expression can be found in the pericytes of the retina. At full maturity (70-80 DPN), CD248 expression has become restricted to perivascular cells of larger vessels, mesangial cells of the kidney, as well as the mesenchyme of organs with high proliferative capacity such as the uterus or ovaries (Table 1.2).
Table 1.2: Post-natal CD248-transcript detection in tissues

CD248 expression rapidly decreases postnatally. In most fully mature tissues, the CD248 transcript is not present.

<table>
<thead>
<tr>
<th>Days Post-Natal (DPN)</th>
<th>Tissues in which the Murine CD248 Transcript is Detected</th>
</tr>
</thead>
</table>
| 1                     | a) Found in dermis; lung stromal fibroblasts; splenic red pulp and vessels; mesangial cells of the kidney  
                        | b) Decreased expression in the vessels of the thymus and heart |
| 9-10                  | a) Found in pericytes of the retina  
                        | b) Decreased expression in the dermis due to cellular apoptosis |
| 70-80 (a.k.a. full maturity) | Expression restricted to pericytes of larger vessels, mesangial cells of the kidney, mesenchyme of organs with high-proliferative capacities |

1.3.3 Expression in the healthy adult

Postnatally, CD248 expression is retained in the dermis and perivascular cells of several organs. In adult mice, the CD248 transcript, as detected by Northern blot, has been shown to be present in almost all normal tissues. However, the CD248 protein has not uniformly been detected in most of these tissues, although it is readily found in tissues that are undergoing cellular proliferation.
Since 2005, researchers have uncovered CD248 expression in a number of human adult tissues under normal physiological conditions including: perivascular cells of the lung; stromal cells of the bladder, colon, endometrium, ovary, placenta and testis; bone marrow fibroblasts; red pulp of the spleen; mesangial cells of the renal glomeruli; lymphoid tissues such as the salivary gland, thyroid gland, thymus, pancreas and lymph nodes; smooth muscle cells; as well as mesenchymal cells stem cells. Most recently CD248 was detected in a subset of human naïve CD8+ T cells and CD8+ thymocytes, where it is believed that CD248 may be modulating T-cell proliferation through suppressive mechanisms.

1.3.4 Expression in cancer

As previously noted, CD248 was thought to be expressed on the tumour endothelium of almost all human carcinomas. However, subsequent studies determined that CD248 is found exclusively on tumour-associated mesenchymal cells, stromal fibroblasts and pericytes, and is not expressed by endothelial cells. In models of physiologic embryonic and tumour angiogenesis, CD248 co-localises with NG2, a pericyte marker, but does not co-localise with endothelial markers CD31 or isolectin B4. Cancer-associated CD248 expression in the perivascular and stromal cells varies in both cellular location and intensity and is dependent on the type of
cancer as well as the specific patient. Highest levels of CD248 expression are in SK-N-AS neuroblastomas, as well as subsets of sarcomas that include, for example, synovial sarcomas, osteosarcomas, liposarcomas, fibrosarcomas and malignant fibrous histiocytomas 8; 10; 11; 14; 15; 23; 24; 31; 32; 33; 34; 35.

This lack of homogeneity in CD248 expression impedes its utilisation as a therapeutic target. Nonetheless, the measurement of CD248 levels in tumours may have value in assessing prognosis. For example, increased CD248 expression in patients with breast cancer is associated with nodal involvement and poor patient outcome 32. In neuroblastomas, high CD248 levels are accompanied by high tumour grade and greater invasiveness 8. Furthermore, in studies of rectal cancer, the presence of CD248 in tumours correlated with an infiltrative growth pattern, the latter which also was directly related to increased expression of both p73 and Cox-2 (cyclo-oxygenase-2) 36.

1.3.5 Expression during inflammation

The role of CD248 in inflammation has not been as thoroughly examined as its role in cancer, although it has been well-documented that its expression increases with inflammatory diseases. In a salmonella-induced model of inflammation, CD248 expression increased in the spleen in a manner that correlated with the tissue remodeling that resulted from inflammatory damage 21. Also, patients with rheumatoid or psoriatic arthritis have increased numbers of
CD248-positive stromal cells in synovial tissues, whereas CD248 is not normally detected in adult joint \(^{12}\). Furthermore, in progressive kidney disease caused by IgA nephropathy, a subset of \(\alpha\text{-SMA}^+\) myofibroblasts and \(\alpha\text{-SMA}^-\) stromal cells exhibit increased CD248 expression while in a healthy kidney, expression is limited to mesangial cells \(^{13}\).

1.4 The origins of cells expressing CD248

Although CD248 is highly expressed during fetal development \(^{5,6,19}\), the origin of CD248-expressing cells remains a mystery. Somewhat surprising, given that it is not found in mature endothelial cells, CD248 expression has been detected in an endothelial progenitor cell population derived from CD133\(^+\)CD34\(^+\) human cells (VEGFR-2\(^+\)CD31\(^+\)CD45\(^-\)VE-Cadherin\(^+\)) \(^{27}\). Furthermore, CD248 is expressed by mesenchymal stem cells arising from bone marrow and adipose tissue \(^{10,28}\).

As mentioned above, CD248\(^+\) cells are also found in populations of cells that participate in tumour and inflammation-associated angiogenesis. For example, a population of cells defined by their expression of both endothelial and dendritic markers, also express CD248. These CD45\(^+\)VE-Cadherin\(^+\)P1H12\(^+\)CD34\(^+\)CD31\(^+\)TEM7\(^+\)CD248\(^+\) cells, also known as vascular leukocytes, have the unique capability to create functional vessels \textit{in vivo} \(^{37}\).
1.5 Human CD248 gene structure and regulation

The human CD248 gene is located on chromosome 11. The gene is intron-less and encodes a single 2274 base pair transcript. The transcription initiation site is located 17 nucleotides upstream of the ATG start. CD248 transcription is upregulated in vitro when there is an increase in cell density or in response to hypoxia.

Hypoxia Inducible Factors (HIFs) are responsible for the transactivation of a large number of genes involved in environmental adaptation processes, including factors that contribute to angiogenesis such as VEGF, VEGFR1/2, Tie2/TEK, EPO, VE-cadherin, TGFα and ANG2. Angiogenesis is a critical process for normal growth and development, and is also key for tumour growth or inflammation, and embryonic development. Although multiple factors regulate angiogenesis, a key driving force is hypoxia, which stimulates adaptive responses to increase blood supply through the creation of new vessels and expansion of old vessels. HIF-2α activation requires only a slight lowering in oxygen tension thereby regulating pericellular hypoxia. Studies have shown that HIF-2α is more significant in upregulating the transcription of CD248 rather than HIF-1α due to the fact that the temporal induction of CD248 by hypoxia corresponds to the kinetic pattern observed with HIF-2α activation. Further supporting a likely relationship between CD248 and
HIF-2α is the fact that HIF-2α is prominently expressed in glial cells, blood vessels and fibroblasts, i.e. cells that also express CD248.

HIF-2α binds to the HRE (hypoxia-response element) in the distal part of the upstream regulatory region of CD248, a location adjacent to an Ets-binding site. There, HIF-2α directly interacts with Ets-1 through the N-terminus transactivation domain. In addition, HIF-2α interacts with Ets-1 and its two EBS (Ets transcription factor binding sites) elements in the proximal regulatory region in response to hypoxia. These two cooperative modes of action are also seen in other HIF-2 target genes. VEGFR2 is regulated in much the same way the distal promoter region of CD248 functions, whereas the proximal promoter region of CD248 performs like the promoter of VE-cadherin.

Upregulation of CD248 during high cell density is believed to be due in part to hypoxia. SP1 is a mediator of cell density effects and during times of sparse monolayer cell culture, this protein can induce CD248 expression. Furthermore, inhibition of SP1, in the setting of high cell density, will cause a decrease in CD248 expression. Knowing that SP1 is a transcriptional target of Ets-1 and Ets-1 expression is upregulated by HIF-1 protein, CD248 upregulation may also rely on HIF-1. Thus, hypoxia stabilises HIF-1, causing an increase in the production of Ets-1, thereby leading to upregulation of SP1 and further stimulating the transcription of CD248. In addition, an increase in Ets-1
might also yield Ets-1/HIF-2α interactions at the CD248 promoter that in turn may upregulate CD248 gene transcription 38.

1.6 Human CD248 domain architecture

CD248 is a member of the Group XIV family of C-type lectin-like domain (CTLD)-containing transmembrane proteins 53. Other proteins in this group include CD141 (thrombomodulin/BDCA-3) 54; 55; 56 and CD93 (C1qRp) 57; 58, both of which play important roles in inflammation, coagulation, complement regulation, and cellular adhesion. The most N-terminal 360 amino acid residues of CD248 show the most structural homology to thrombomodulin and CD93 3.

Human CD248 is a type I transmembrane protein consisting of 757 amino acids with a predicted molecular weight of 80.9 kDa. From the N-terminus, CD248 contains: a signal peptide that is cleaved co-translationally, a 157 amino acid residue globular CTLD, a 19 amino acid residue hydrophobic spacer, a 57 amino acid residue complement control protein (CCP) or Sushi domain, a 156 amino acid residue span containing three epidermal growth factor-like (EGF-like) repeats, a 269 amino acid sialomucin-like domain, a 48 amino acid residue single-pass transmembrane domain, and a 51 amino acid residue cytoplasmic tail. CD248 is heavily glycosylated, with an apparent molecular weight of approximately 165 kDa by SDS-PAGE. The CTLD and sialomucin-like regions of CD248 have predicted glycosylation sites for O-linked oligosaccharides.
(NetOGlyc 2.0) that can be removed with O-sialoglycoprotein endopeptidases (PNGase F, sialidase, O-glycosidase), reducing the molecular weight of human CD248 from an apparent 165 kDa to 95 kDa \(^3\).

### 1.7 Domain structure

C-type lectin-like domains (CTLD) are representative structurally similar motifs that are found in many species and that may or may not be calcium-binding. The domain is biologically important because of its ability to interact with multiple ligands, including proteins, lipids and especially carbohydrates. Proteins containing a CTLD participate in distinct cellular and physiological processes including apoptosis, cellular adhesion and immune regulation \(^59\). It is this domain that is similar in thrombomodulin, CD93 and CD248. The CTLD of thrombomodulin lacks a well-defined calcium-binding site \(^60\), and possesses potent anti-inflammatory properties \(^61\). The functions of the CTLDs of CD248 and CD93 have not yet been described.

Complement control protein (CCP) or Sushi domains (also known as short consensus repeats (SCR)) are commonly found in complement, coagulation, and adhesion proteins. The structure of the domain is highly conserved and is based on a \(\beta\)-sandwich \(^62\). Although the CCP of CD248 has not been characterised, mutations in complement proteins comprised of multiple CCPs may result in alterations in complement activation leading to serious disease \(^63; 64; 65\).
Figure 1.1: A schematic diagram depicting the domain architecture of CD248

From the N-terminus, CD248 contains a C-type lectin-like domain, a complement control protein domain, 3 epidermal growth factor-like repeats, a heavily glycosylated sialomucin-like region, a transmembrane domain, and a cytoplasmic tail that contains three putative phosphorylation sites and a PDZ-binding motif.
Epidermal Growth Factor-like (EGF-like) repeats maintain protein structure due to six cysteine amino acid residues that form three disulfide bridges \(^6\). Thrombomodulin contains six EGF-like repeats, four of which are essential for the molecule’s function in coagulation and fibrinolysis \(^5\). However, there is no evidence to date that CD248 plays a similar role in the hemostatic system.

Sialomucin-like domains are rich in serine, threonine, and proline residues accounting the potential for heavy glycosylation. Post-translational glycosylation is often seen in proteins that are important in cell-cell adhesion \(^3\). CD248 is predicted to contain 27 O-glycosylation sites (NetOGlycan 3.1) \(^1\) that may contribute to appropriate folding or proper biological interactions. These glycosylation sites likely account for the discrepancies seen between the predicted molecular weight as compared to the apparent molecular weight of CD248.

CD248 has a single hydrophobic transmembrane domain that anchors the protein to the cellular membrane. The transmembrane domain is followed by a highly conserved 51 amino acid residue cytoplasmic tail that contains three putative phosphorylation sites and a C-terminal PDZ-binding motif, the latter which was identified using the PROSCAN program \(^2\) (Figure 1.2). Previous studies using transgenic mouse models indicated that the cytoplasmic tail of CD248 is functionally important, probably by mediating intracellular signals \(^1\); \(^6\).
However, the precise molecular pathways have not been delineated, nor have the roles of the potential phosphorylation sites or the PDZ-binding motif within the cytoplasmic tail been elucidated.

PDZ-binding motifs are short consensus sequences of 3 or more amino acid residues, normally found at the most C-terminus of type I transmembrane proteins, which bind to intracellular proteins containing an 80-90 amino acid PDZ domain. PDZ motif-domain interactions are important in modulating many intracellular signaling pathways and are found in approximately 200 human proteins, including those of the Rap family, as well as other important kinases and phosphotases. No binding partners for the PDZ-binding motif of CD248 have yet been identified. However, the PDZ-binding motif of CD93 interacts with Gα adaptor protein, whereupon the protein mediates both leukocyte adhesion and phagocytosis.
Figure 1.2: Alignment of the amino acid sequence of cytoplasmic tail of CD248 from several different species

The cytoplasmic tail of CD248 is highly similar between species. The alignment shows that the three putative phosphorylation sites (red) as well as the C-terminal PDZ-binding motif (green) are conserved.
1.8 Murine CD248

Murine CD248 is an intronless single-copy gene located on chromosome 19. The gene is 2553 base pairs in length and the transcribed/translated product is a 765 amino acid protein that shares 77.5% overall sequence identity to that of human CD248 with highest conservation of the transmembrane and cytoplasmic domains. Murine CD248 has a predicted molecular weight of 92 kDa. The putative phosphorylation sites of the murine CD248 cytoplasmic tail are Thr731, Ser744 and Thr760.

1.9 The CD248\textsuperscript{KO/KO} mouse model and cancer

Mice lacking the CD248 gene (CD248\textsuperscript{KO/KO}) were generated in 2006 by homologous recombination in embryonic stem cells. The CD248\textsuperscript{KO/KO} mice developed normally and were fertile.

Upon further examination, the CD248\textsuperscript{KO/KO} mice responded to skin wounds with normal healing. However, as compared to wild-type mice, orthotopic xenografts of HCT116 colorectal tumour cells or heterotopic LLC (Lewis lung carcinoma) implanted in the liver and cecum of CD248\textsuperscript{KO/KO} mice grew more slowly, reached a lower tumour volume, were less invasive into neighbouring tissues, and yielded fewer metastases. Although the tumours of CD248\textsuperscript{KO/KO} mice were smaller in size, surprisingly, they had an increase in the
number of small vessels. A similar result was found using an intracranial glioblastoma model, i.e. tumours from CD248\textsuperscript{KO/KO} mice were more vascularised than the wild-type counterparts, although the tumours were not different in size.

Predicting that the cytoplasmic domain of CD248 may play a functional role, the Conway Lab generated mice that lacked this domain. As expected, the unstressed mice appeared normal. Using subcutaneously implanted T241 fibrosarcomas, they determined that the CD248 cytoplasmic deletion mice had the same phenotype as CD248\textsuperscript{KO/KO} mice, i.e. as compared to wild-type mice, the tumour volumes were decreased and the vessel density of tumours, the lumen area of vessels and the SMA\textsuperscript{+} pericyte coverage of vessels were similar to that of CD248 wildtype, suggesting an angiogenesis-independent decrease in tumour development.

The seemingly paradoxical decrease in tumour volume with an increase in vessel density may be due to CD248-deficient vessels failing to mature properly. This, however, has not been confirmed. Alternatively, stromal cell-derived tumour promoting factors that are CD248-dependent may regulate tumour cell survival and over-ride the contribution of angiogenesis. These hypotheses to explain uncoupling of tumour growth and angiogenesis require further study.
1.10 The CD248^{KO/KO} mouse model and inflammation

CD93 and thrombomodulin both play key roles in regulating complement activation, leukocyte adhesion, and inflammation \(^{57;70;71}\). It was therefore reasonable to consider that the third member of this family of CTLD-containing proteins, CD248, might also participate in inflammation. Indeed, CD248 expression was upregulated in rheumatoid fibroblast-like synoviocytes \(^9\) and lymphoid fibroblasts that take part in tissue remodeling after infection \(^{21}\).

In addition, CD248 is markedly upregulated in synovial tissue stromal cells of patients with rheumatoid and psoriatic arthritis as compared to normal healthy adult tissue that is without disease. Mice lacking CD248 or its cytoplasmic domain were tested in a model of collagen antibody-induced arthritis (CAIA). Compared to wild-type mice, these mice developed significantly less arthritis, measured by synovial hyperplasia, leukocyte infiltration and serum cytokine levels \(^{12}\). Thus, the findings were strongly supportive of a major contributory role for CD248 in facilitating an inflammatory response, mediated via its cytoplasmic domain. Although it seems reasonable to hypothesise that there are common CD248-dependent pathways that regulate both inflammation and tumour growth, this would have to be tested \(^{16}\).
1.11 CD248 and other roles in inflammation

In attenuated *Salmonella enterica* infection of mice, a model known to cause splenomegaly \(^72;73;74\), CD248 expression was increased in cells in the red pulp of the spleen, which temporally corresponded to the peak of splenic enlargement and a reduction in the size of B cell areas in the splenic white pulp. The findings suggested that CD248 expression by some stromal cell populations in lymphoid tissue contributes to tissue remodeling during development, infection and subsequent repair \(^21\).

To further understand the role CD248 plays in lymphoid tissue remodeling, studies were performed using CD248 deficient mice. After 4-hydroxy-3-nitro-phenylacetyl chicken g-globulin (NP-CGG) antigen immunization, lymph node expansion was greatly increased in wildtype mice versus CD248-deficient mice. Furthermore, lymph node expansion in the wildtype mice was dependent on the temporal expression pattern of CD248 \(^75\). The precise mechanism by which CD248 modulates lymph node expansion is not known, but might include CD248-dependent lymphoid tissue-associated stromal cell proliferation and migration \(^16\).
Several investigators have reported on possible signaling pathways regulated by CD248. Platelet-derived growth factor (PDGF) is a chemoattractant that participates in several cellular processes by triggering mitogenesis and promoting key events in angiogenesis. PDGF-dependent signaling events are noticeably modified by CD248 expression. In fibroblasts lacking CD248, there is a reduced rate of cell proliferation and also a decrease in PDGF-BB-directed migration through gelatin-coated transwells. When MG63 osteosarcoma cells are transfected with full-length CD248, they migrate more quickly than without CD248, and migration is further increased upon PDGF-BB addition. Further study revealed that pericyte proliferation and migration pathways induced by PDGF-BB were partly CD248-dependent. When PDGF-BB binds to PDGF-R, the receptor is phosphorylated, and the MAP kinase ERK-1/2 is activated, causing upregulation of the transcription factor c-Fos, a cellular proto-oncogene that participates in cellular proliferation and differentiation. Suppression of CD248 dampens this pathway, decreasing PDGF-R and ERK1/2 phosphorylation, and impairing c-Fos expression. CD248 therefore may play a role in pericyte proliferation by recruiting Src/PI-3 kinase and controlling the c-Fos pathway so the PDGF-BB signals through PDGF-R are amplified. Furthermore, the cytoplasmic domain of CD248 has been linked to regulating...
these PDGF-BB-derived signals. Mouse embryonic fibroblasts (MEFs) isolated from mice lacking the cytoplasmic domain of CD248 show less migratory response through transwells to PDGF-BB as compared to wildtype MEFs.

Other studies have focused attention on the cytoplasmic tail of CD248 and found that its deletion has implications on processes such as inflammation progression and tumour growth. In models of collagen-antibody induced arthritis, deletion of the cytoplasmic tail of CD248 causes a reduction in TGF-β induced angiogenic and pro-inflammatory events, decreased adhesion to monocytes, as well as lower levels of hypoxia-inducible factor 1α (HIF-1α) transcript, vascular endothelial growth factor (VEGF), placental growth factor (PIGF), and vascular endothelial growth factor receptor 1 (VEGFR-1). In addition to dampening leukocyte infiltration, these changes in cellular behavior and protein expression also reduce stromal cell activation, migration, and proliferation, leading to a decrease in pathologic angiogenesis.

In models of tumour growth, deletion of the cytoplasmic tail of CD248 resulted in a notable increase in transcript levels of SM22α (transgelin), Notch3, Jagged1, Hes, and Hey1. All these proteins, under specific contexts, may have tumour suppressor properties. Fibroblasts lacking the cytoplasmic tail of CD248 also showed a decrease in the release of active matrix metalloproteinase-9 (MMP-9), soluble TGF-β, and other yet-to-be identified soluble tumour cell proliferation factors. These alterations in cellular releasate
may explain the reduced tumour proliferation and migration evident in tumour models in mice lacking the cytoplasmic domain of CD248 \textsuperscript{12; 67}.

1.13 Interacting proteins

Several ligands for the extracellular domain of CD248 have been identified by immunoprecipitation and colocalisation, including collagens I and IV, fibronectin, and Mac-2BP/90K \textsuperscript{25; 84; 85}. Interactions of the CTLD of CD248 with extracellular matrix proteins such as collagen and fibronectin are important for cell attachment, as well as cellular migration during events such as tumour metastasis by increasing the release of extracellular matrix degrading proteins such as MMP-9 \textsuperscript{84}. Regardless of these findings, the overall effect and underlying mechanisms that increased CD248 expression has on different tumour types is uncertain. For instance, although CD248 co-localises with collagen IV and MMP-2 in developing brain capillaries \textsuperscript{25}, there is minimal co-localisation observed of CD248 and fibronectin in grade II-IV astrocytomas \textsuperscript{15}.

Mac-2BP/90K is a secreted glycoprotein that binds extracellular matrix proteins such as galectin-3, β1-integrins, collagens and fibronectin. This glycoprotein is highly expressed in malignant epithelial cells of the neoplastic compartment and may play roles in cell adhesion to neighbouring cells as well as the extracellular matrix \textsuperscript{86}. The domain of Mac-2BP/90K that interacts with extracellular matrix components is the same domain that binds CD248 \textsuperscript{85}. 
Studies show that a reduction of either Mac2-BP/90K in tumour epithelial cells and/or CD248 in fibroblasts results in increased cell adhesion between these primary fibroblasts and tumour epithelial cells. The hypothesis is that both proteins acting together regulate repulsive position signaling between epithelial and mesenchymal compartments of tumours causing tumour metastasis, i.e. Mac-2BP/90K binding to CD248 controls boundary formation by acting as a negative regulator of epithelial and mesenchymal cell adhesion. This repulsive position signaling is reminiscent of the Ephrin/Eph-receptor paradigm that facilitates angiogenesis, blood vessel remodeling and guidance of axons.

Interacting partners for the cytoplasmic domain of CD248 remain elusive; this is in spite of the important role that the cytoplasmic tail plays in promoting inflammation and tumour growth. Studies have not been performed to evaluate whether the extracellular and cytoplasmic domains of CD248 are involved in cross-talk pathways that might regulate cellular proliferation and immune responses.

1.14 Matrix metalloproteinases

Matrix metalloproteinases (MMPs) were first described during tadpole tail metamorphosis as collagen triple-helices were being degraded by enzymatic activity. Later studies described MMPs as a family of more than 20 neutral, endopeptidases containing a conserved zinc-binding motif. MMPs are secreted
as either pro-enzymes or zymogens that are consequently activated by the cleavage of a cysteine-containing NH₂ terminal peptide thereby causing the opening of the zinc-binding site. This activation mechanism is referred to as the “cysteine switch mechanism”. MMPs may be activated by a variety of biological and chemical molecules including proteases, other MMPs, organomercurials, urea, some detergents, reactive oxygen species, as well as intracellularly by furins. Once activated, MMP activity is controlled extracellularly by a class of inhibitors known as TIMPs (tissue inhibitors of metalloproteinases), and in the plasma by α₂-macroglobulin.

Initially, MMPs were regarded as only extracellular matrix modifiers, however with more recent evidence, the biological roles that MMPs perform have been expanded. Indeed, MMPs are now known to regulate a variety of reactions of cell-surface-associated proteins such as adhesion and structural molecules at intercellular junctions, mediators of apoptosis, cell-surface receptors, as well as a number of chemokines, cytokines, growth factors, and other proteases. Therefore, in addition to matrix remodeling, MMPs contribute to cellular behavior (migration, proliferation, differentiation, apoptosis), cell-to-cell communication, as well as intercellular adhesion. The actions of MMPs may thus participate in tumour progression and angiogenesis.

MMPs also play an important role during inflammation. By modifying or cleaving cell surface molecules, MMPs effectively activate pro-inflammatory
cytokines thereby recruiting leukocytes to the site of injury and further advancing the inflammatory reaction. MMP activation and inhibition in inflammatory situations is essential for proper wound healing and homeostasis. Dysregulation of these activities may contribute to the progression of some autoimmune disorders such as rheumatoid arthritis, where there may be a lack of intra-articular apoptotic cells and upregulated proliferation of the synovial membrane. The role that MMPs play in rheumatoid arthritis is of particular interest as CD248 has also been implicated in arthritis disease progression and severity.

1.15 CD248 and the MMPs

The expression of CD248 correlates with altered release of extracellular matrix degrading proteins such as MMP-9. When CHO cells overexpressed CD248, they release more MMP-9. However, even though CD248 has been shown to colocalise with MMP-2 in newly sprouting brain capillaries, there was no difference in MMP-2 release by CHO cells when CD248 expression was increased. Moreover, CD248 silencing in siRNA-transfected fibroblasts did not alter MMP-2 zymogen expression.

Much of the data point to the cytoplasmic domain of CD248 in regulating MMP-9 activation and release, rather than its ectodomain. TGF-β stimulated fibroblasts expressing CD248 lacking the cytoplasmic domain (CD248_CyD/CyD)
released less MMP-9 than wild-type (CD248<sup>WT/WT</sup>) fibroblasts<sup>67</sup>. Furthermore, transcript levels of SM22α, a small intracellular actin-binding protein known to repress TGF-β stimulated release of MMP-9<sup>96</sup>, were higher in CD248<sup>CyD/CyD</sup> fibroblasts as compared to CD248<sup>WT/WT</sup> fibroblasts<sup>67</sup>. SM22α is normally expressed by smooth muscle cells, mesenchymal cells, and tumour-associated fibroblasts<sup>97</sup>. In addition to suppressing MMP-9 release, SM22α reduces cell migration and may have tumour suppressor properties<sup>83</sup>. This finding suggests that signals mediated via the cytoplasmic domain of CD248 dampens SM22α expression, thereby increasing MMP-9 release from cells, allowing the degradation of the extracellular matrix around cells expressing CD248 (i.e. tumour-associated fibroblasts), allowing expansion and metastasis of tumours.

### 1.16 The future of CD248

There are many gaps in our understanding of the role of CD248 in health and disease. However, the fact that the little bit of information known about CD248 has large consequences on diseases such as cancer, arthritis, and kidney disease, gives the research community incentive to explore CD248 further and perhaps use this molecule as a potential diagnostic or therapeutic tool. For instance, in chronic renal disease, CD248 expression directly correlates with the expression of other established markers of kidney dysfunction<sup>13</sup>. CD248 expression is upregulated in a variety of human carcinomas<sup>2; 3; 4; 6; 7; 8; 10; 11; 14; 15; 22; 23; 26; 30; 31; 32; 36; 67</sup> and in the synovial tissues during inflammatory diseases.
such as arthritis\textsuperscript{12}. If the temporal expression pattern of CD248 during disease progression can be determined, it may prove to be a valuable biomarker, facilitating early therapeutic intervention to reduce disease.

Both CD93 and thrombomodulin are cleaved at the cell surface of endothelial cells by proteases\textsuperscript{56;57}. Furthermore, the soluble lectin-like domain of thrombomodulin is noted for its potent anti-inflammatory properties\textsuperscript{54;56;61;70;71}. Though a soluble form of CD248 has not been identified, it would not be surprising that such a form of CD248 also existed. Preliminary \textit{in vitro} studies done in our lab using a purified, un-glycosylated form of human CD248 suggest that some MMPs may cleave CD248 at several different sites. Since CD248 is upregulated during disease, a soluble form of CD248, if it does exist, may also be a valuable biomarker of disease progression.

Mice lacking CD248 or its cytoplasmic tail are resistant to tumour growth, tumour metastasis, and inflammation\textsuperscript{12;21;22;67}. Research has yet to find effective ways of suppressing CD248 or interfering with the signaling pathways associated with the cytoplasmic domain of CD248. Such approaches, however, could yield new therapeutic strategies. In 2008, investigators reported the use of an anti-CD248 antibody and saporin-conjugated anti-human secondary IgG to selectively inhibit the growth of both SK-N-AS neuroblastomas and A-673 Ewing’s sarcomas. However, this therapeutic approach had no effect on the growth of HT-1080 fibrosarcomas, a CD248 negative tumour\textsuperscript{31}. Similar to the
Herceptin/Her2-overexpression breast cancer model, an anti-CD248 antibody may be used to target CD248-positive tumours.

Indeed, a therapeutic antibody approach holds the most promise for researchers thus far. The structural domains of CD248 are critical for antibody production, as well as for creating targeted treatments for cancers and inflammation marked by the upregulation of CD248. Antibodies directed against the lectin-like domain of CD248 may be able to interfere with the CD248-positive cells' abilities to migrate and form tube formation. Most recently, anti-CD248 antibodies were used in imaging studies to detect the presence of ovarian cancer.

CD248 has known extracellular binding partners, and these interactions modulate cellular migration. However, intracellular binding partners for CD248 have not yet been definitively identified. The cytoplasmic domain of CD248 contains 3 highly conserved putative phosphorylation sites, as well as a C-terminal PDZ-binding motif consensus sequence. Finding proteins that interact with the cytoplasmic tail of CD248 will provide other points in the CD248 signaling pathways that may be targeted for therapeutic purposes. Perhaps it may even be possible that both the ecto-domain and cyto-domain of CD248 crosstalk through the interactions of binding proteins on a “CD248 scaffold”.


There are many questions that have yet to be answered about CD248's expression pattern and function, but that is what makes CD248 research such a thrilling field of study.

1.17 Hypothesis

We hypothesise that CD248, expressed by activated stromal fibroblasts, myofibroblasts, and perivascular cells, is important in promoting inflammation and tumour development via the cytoplasmic domain. We predict that disrupting CD248-dependent signaling via one or more putative phosphorylation sites or a PDZ-binding motif within the cytoplasmic domain will suppress cellular pathways associated with inflammatory responses and tumour growth.

1.18 Overall goal

The overall goal of this thesis project is to determine the role of the cytoplasmic tail of CD248 in health and disease.
1.19 Specific objectives

The goal will be achieved through four specific objectives.

1. An antibody detecting murine CD248 under several experimental conditions will be identified.

2. cDNAs encoding mutant forms of murine CD248 will be created and characterised.

3. Intracellular binding partners for murine CD248 will be identified.

4. The functional consequences of mutating the putative phosphorylation sites and the PDZ motif of the cytoplasmic domain of CD248 will be determined.
2. Materials and Methods

2.1 Cell culture

C57/B6 murine embryonic fibroblasts (MEFs) were harvested from 12.5-days post-coitum (DPC) mouse embryos. MEFs, human embryonic kidney (HEK) 293 T, 10T1, 3T3, and EAhy926 endothelial cell lines were cultured in DMEM media supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 2 mM sodium pyruvate, 50 U/mL penicillin, and 50 ug/mL streptomycin (Invitrogen, Frederick, MD).

Human umbilical vein endothelial cells (HUVEC) were cultured in EBM®-2 media supplemented with EGM-2 SingleQuots® (Lonza, Walkersville, MD).

Michigan Cancer Foundation-7 (MCF-7) and Chinese hamster ovary (CHO) cell lines were cultured in DMEM F12 media supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 2 mM sodium pyruvate, 50 U/mL penicillin, and 50 ug/mL streptomycin (Invitrogen, Frederick, MD).

Transient transfections of HEK 293 T and CHO with cDNAs were accomplished using FuGENE® 6 and FuGENE® HD transfection reagents (supplied by both Promega, Madison, WI and Roche Applied Science, Indianapolis, IN) according to the manufacturer’s instructions.
2.2 Immunoblotting of cell lysates (traditional methods)

Cells were lysed in RIPA buffer [30mM Tris-HCL (pH 7.4), 150 mM sodium chloride, 1% IGEPAL, 0.5% deoxycholate, 2 mM EDTA, 0.1% SDS in distilled water] or Triton X-100 buffer [1 mM EDTA, 2% Triton X-100 in 1x TBS]. Cell lysate concentrations were measured using the Pierce® BCA Protein Assay Kit (Thermo Scientific, Rockford, IL) and diluted in Laemmli sample loading buffer (then boiled). Equal amounts of protein per well were loaded under reducing conditions for separation by SDS-PAGE using 11% polyacrylamide gels. Gels were run in a Mini-PROTEAN gel system (Bio-Rad Laboratories, Hercules, CA) at 120 V for 20 minutes and 200 V for 40 minutes (or until dye front reached the bottom of the gel). Gels were then transferred using a Mini-PROTEAN wet transfer (Bio-Rad Laboratories, Hercules, CA) onto nitrocellulose membranes at either 100 V for one hour or 20 V overnight.

After transfer, the nitrocellulose membrane was washed in 1x TBS (Tris-Buffered Saline) for 10 minutes on a shaker. The wash was discarded and replaced with 15 mL 1:1 PBS:Odyssey® Blocking Buffer (v/v) (Li-COR Biosciences, Lincoln, NE) and shaken for one hour. Blocking solution was discarded and the membrane was washed with 1x TBS for 10 minutes with shaking. Wash was discarded and replaced with 5 mL 1:1 PBS:Odyssey® Blocking Buffer with the primary antibody (Table 2.1) at appropriate dilution as
suggested by the antibody data sheet. The membrane was left overnight at room
temperature, shaking in a parafilmed container.

The next morning, the primary antibody was removed and the membrane
was washed three times with 1x TBS with Tween-20 (Sigma, Saint Louis, MO)
(TBS-T), each wash for 10 minutes on the shaker. After the last wash, 5 mL 1:1
PBS:Odyssey® Blocking Buffer with the corresponding secondary antibody
(Table 2.2) (1:5,000 dilution, v/v) was placed on the membrane and incubated on
a shaker, in the dark, for one hour. The membrane was washed for 10 minutes
on the shaker with 1x TBS-T three more times.

A full-length un-glycosylated human CD248 protein with an N-terminal
GST (glutathione-S-transferase) tag (Abnova Corp., Taipei, Taiwan) acted as a
positive control on immunoblots.

Immunoblots were imaged with an Odyssey® Imager (LI-COR
Biosciences, Lincoln, NE) using Odyssey® Application Software Version 3.0.

Note: The preceding methods differ slightly for Anti-FLAG M2 mAb (Sigma, Saint
Louis, MO) in the following ways;
1. Blocking buffers comprised of 1x TBS, 5% skim milk powder (w/v) and 0.01%
sodium azide (w/v),
2. After primary antibody incubation, the membrane was washed once with 1x TBS for 10 minutes on the shaker,

3. After secondary antibody incubation, the membrane was washed with 1x TBS-T eight times for 5 minutes each on the shaker.
### Table 2.1: Primary antibodies used for immunoblot

<table>
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<th>Antibody Against</th>
<th>Species</th>
<th>Catalogue Number</th>
<th>Company</th>
</tr>
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<td>Chicken pAb</td>
<td>7-022008</td>
<td>Aves Lab Inc., Tigard, ON</td>
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<tr>
<td>CD248 N-term 2</td>
<td>Chicken pAb</td>
<td>8-022008</td>
<td>Aves Lab Inc., Tigard, ON</td>
</tr>
<tr>
<td>TEM1 N-term</td>
<td>Rabbit pAb</td>
<td>#4359</td>
<td>ProSci Inc., Poway, CA</td>
</tr>
<tr>
<td>TEM1 C-term</td>
<td>Rabbit pAb</td>
<td>#4357</td>
<td>ProSci Inc., Poway, CA</td>
</tr>
<tr>
<td>TEM1</td>
<td>Rabbit pAb</td>
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<td>Genesis Biotech Inc., Taipei, Taiwan</td>
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<td>sc-48098</td>
<td>Santa Cruz Biotechnology Inc., Santa Cruz, CA</td>
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<td>CD248</td>
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<td>p13</td>
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<tr>
<td>CD248</td>
<td>Rabbit pAb</td>
<td>p14</td>
<td>Gift from Dr. Clare Isacke</td>
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<td>TEM1</td>
<td>Rabbit pAb</td>
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<td>Abcam, Cambridge, MA</td>
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<td>*CD248</td>
<td>Rabbit pAb</td>
<td>18160-1-AP</td>
<td>ProteinTech Group, Chicago, IL</td>
</tr>
<tr>
<td>Antibody Against</td>
<td>Species</td>
<td>Catalogue Number</td>
<td>Company</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>------------</td>
<td>------------------</td>
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<tr>
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<td>60170-1-Ig</td>
<td>ProteinTech Group, Chicago, IL</td>
</tr>
<tr>
<td>CD248</td>
<td>Rabbit pAb</td>
<td>18594-1-AP</td>
<td>ProteinTech Group, Chicago, IL</td>
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<td>CD248</td>
<td>Rabbit pAb</td>
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<td>ProteinTech Group, Chicago, IL</td>
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<td>M2 FLAG</td>
<td>Mouse mAb</td>
<td>F3165</td>
<td>Sigma, Saint Louis, MO</td>
</tr>
<tr>
<td>Chicken IgG</td>
<td>Chicken pAb</td>
<td>10500C</td>
<td>Aves Lab Inc., Tigard, ON</td>
</tr>
<tr>
<td>Thrombomodulin (hTM)</td>
<td>Mouse mAb</td>
<td>CTM1009</td>
<td>Conway Lab 99</td>
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<tr>
<td>Nitric Oxide Synthase 1</td>
<td>Rabbit pAb</td>
<td>#06-528</td>
<td>Upstate® Biotechnology, Lake Placid, NY</td>
</tr>
<tr>
<td>Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)</td>
<td>Rabbit pAb</td>
<td>ab-9485-100</td>
<td>Abcam, Cambridge, MA</td>
</tr>
</tbody>
</table>

* The most successful antibody for murine CD248. This antibody was used for all subsequent experiments on immunoblot (at a dilution of 1:600) and for immunostaining (at a dilution of 1:100).
Table 2.2: Secondary antibodies used for immunoblot

<table>
<thead>
<tr>
<th>Antibody Against</th>
<th>Species</th>
<th>Colour</th>
<th>Catalogue Number</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chicken IgG (H+L)</td>
<td>Donkey pAb</td>
<td>IRDye® 800 CW</td>
<td>926-32218</td>
<td>LI-COR Biosciences, Lincoln, NE</td>
</tr>
<tr>
<td>Goat IgG (H+L)</td>
<td>Donkey pAb</td>
<td>IRDye® 800 CW</td>
<td>926-32214</td>
<td>LI-COR Biosciences, Lincoln, NE</td>
</tr>
<tr>
<td>Mouse IgG (H+L)</td>
<td>Donkey pAb</td>
<td>IRDye® 800 CW</td>
<td>926-32212</td>
<td>LI-COR Biosciences, Lincoln, NE</td>
</tr>
<tr>
<td>Mouse IgG (H+L)</td>
<td>Goat pAb</td>
<td>IRDye® 800 CW</td>
<td>926-32210</td>
<td>LI-COR Biosciences, Lincoln, NE</td>
</tr>
<tr>
<td>Rabbit IgG</td>
<td>Goat pAb</td>
<td>IRDye® 800 CW</td>
<td>926-32211</td>
<td>LI-COR Biosciences, Lincoln, NE</td>
</tr>
<tr>
<td>Mouse IgG (H+L)</td>
<td>Goat pAb</td>
<td>IRDye® 680 CW</td>
<td>926-32220</td>
<td>LI-COR Biosciences, Lincoln, NE</td>
</tr>
<tr>
<td>Rabbit IgG</td>
<td>Goat pAb</td>
<td>IRDye® 680 CW</td>
<td>926-32221</td>
<td>LI-COR Biosciences, Lincoln, NE</td>
</tr>
</tbody>
</table>
2.3 Immunoblotting of cell lysates (SNAP i.d. methods)

Nitrocellulose membranes were placed into specialised cassettes for use with the SNAP i.d.™ Protein Detection System (Millipore, Temecula, CA). Membranes were washed once with 1x TBS, followed by a five-minute incubation with a 15 mL blocking solution of 1:1 PBS:Odyssey Blocking Buffer (v/v) (LI-COR Biosciences, Lincoln, NE). The blocking solution was run through the system and the membrane was washed again with 1x TBS.

Primary antibody (Table 2.1) solution (consisting of 5 mL 1:1 PBS:Odyssey Blocking Buffer and antibody at a dilution twice that listed on the antibody data sheet) was incubated on the membrane for 10 minutes. The membrane was washed with 1x TBS-T three times before secondary antibody (Table 2.2) solution (consisting of 5 mL 1:1 PBS:Odyssey Blocking Buffer and appropriate antibody at a dilution of 1:5,000 (v/v)) was applied and incubated on the membrane, in the dark, for 10 minutes. The secondary antibody was removed and the blot washed three times with 1x TBS-T.

Immunoblots were imaged with an Odyssey® Imager (LI-COR Biosciences, Lincoln, NE) using Odyssey® Application Software Version 3.0.

Note: The preceding protocol differs slightly for Anti-FLAG M2 mAb (Sigma, Saint Louis, MO) in the following ways;
1. Blocking buffers comprised of 1x TBS, 0.3% skim milk powder (w/v),

2. After primary antibody incubation, the membrane was washed once with 1x TBS,

3. After secondary antibody incubation, the membrane was washed with 1x TBS-T eight times.

2.4 Site-directed mutagenesis

The cDNA encoding full-length murine CD248 had previously been subcloned into the expression vector pcDNA 3.1+. Site-directed mutagenesis was accomplished with this construct as a template using the QuikChange® XL II Site-Directed Mutagenesis Kit (for point mutations and insertions) and the QuikChange® Lightning Multi Site-Directed Mutagenesis Kit (for multiple point mutations) (both from Agilent Technologies, Santa Clara, CA) according to the manufacturer’s directions.

The following cycling parameters were used on a Mastercycler pro thermal cycler (Eppendorf, Hamburg, Germany):

QuikChange® XL II:

1 minute at 95 °C melting step followed by 18 cycles of:

50 seconds @ 95°C: melting step

60 seconds @ 60 °C: annealing step
7 minutes @ 68 °C: elongation step

Followed by a final elongation step @ 68 °C for 7 minutes.

QuikChange® Lightning Multi:

2 minutes at 95 °C melting step followed by 30 cycles of:

20 seconds @ 95 °C: melting step

30 seconds @ 55 °C: annealing step

5 minutes @ 65 °C: elongation step

Followed by a final elongation step @ 65 °C for 5 minutes.
Figure 2.1: Schematic diagram of site-directed mutagenesis

Site-directed mutagenesis uses a PCR-based method to introduce mutations into a parental cDNA template. The methylated and hemi-methylated parental DNA is subsequently digested and therefore only the mutated DNA is left.
Table 2.3: Site-Directed mutagenesis primers

<table>
<thead>
<tr>
<th>Mutation Completed</th>
<th>Forward Primer (5' → 3')</th>
<th>Reverse Primer (5' → 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thr731Ala</td>
<td>CCAACAAGCGGATCGCGG ACTGCTATCGC</td>
<td>GCGATACGAGTCCCGCGATCCG CTTGTTGG</td>
</tr>
<tr>
<td>Ser744Ala</td>
<td>CACATGCTGGGAACAAGG CCTCAACAGAACCATGC</td>
<td>GCATGGGTTCTGTGGAGGCCC TTCCCACTATGTG</td>
</tr>
<tr>
<td>Thr760Ala</td>
<td>CCTTACAGGGGTACAGGC CTGTAGAACCAGTG T</td>
<td>ACACTGGTTCTACAGGCCTGTA CCCCTGTAGG</td>
</tr>
<tr>
<td>Thr731Asp</td>
<td>CACCCACAAAGCGGATCG ACGACTGCTATCGCTGGG TS</td>
<td>ACCCAGCGATACGAGTCGTCG ATCCGCTTGGGTTGG</td>
</tr>
<tr>
<td>Ser744Asp</td>
<td>ACACATGCTGGGAACAAGG GACTCAACAGAACCATGCC</td>
<td>GCATGGGTTCTGTGGAGGCCC TTCCCACTATGTG</td>
</tr>
<tr>
<td>Thr760Asp</td>
<td>AGCCTTACAGGGGTACAG GACTGTAGCCAACGTG TG</td>
<td>CACACACTGGTTCTACAGTCCTG TACCCCTGTAGGCT</td>
</tr>
<tr>
<td>PDZ deletion</td>
<td>GTACAGACCTGTAGATGAT GGGGTGCAGATG</td>
<td>CATCTGCACCCCATCATCTACA GGTCTGTAC</td>
</tr>
<tr>
<td>N-terminus FLAG tag insertion Step 1</td>
<td>CAGGTCCCCTGGGACTAC ACGCCGGAGCCT</td>
<td>AGGCTCCGGCGTGTAGTCCCA GGGGACCTG</td>
</tr>
<tr>
<td>N-terminus FLAG tag insertion Step 2</td>
<td>CCCTGGGACTACAAGGAC GCACCTGGAGCCTC</td>
<td>GAGGCTCCGGCGGTCCTTTGT AGTCCCAGGG</td>
</tr>
<tr>
<td>N-terminus FLAG tag insertion Step 3</td>
<td>GACTACAAGGACGATGAC GACGCGCGGAGCCTC</td>
<td>CGAGGCTCCGGCGGTGCAGTCGCTTCAGC GTCCCTGTAGTC</td>
</tr>
<tr>
<td>Mutation Completed</td>
<td>Forward Primer ($5' \rightarrow 3'$)</td>
<td>Reverse Primer ($5' \rightarrow 3'$)</td>
</tr>
<tr>
<td>------------------------------------</td>
<td>------------------------------------------------------------------------</td>
<td>--------------------------------------------------------------------------</td>
</tr>
<tr>
<td>N-terminus FLAG tag insertion</td>
<td>GACGATGACGACAAGCAGGTCCCCTGGACGCCGGAGCC</td>
<td>GGCTCCGGCGTGCCCAGGGGACC TGCTTGTCGTCATCGTC</td>
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<td>Step 4</td>
<td></td>
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<tr>
<td>N-terminus FLAG tag Correction</td>
<td>GTCCCTGGACGCCGGAGCCTCGA</td>
<td>TCGAGGCTCCGGCGTCCAGGGGAC</td>
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</table>

Putative phosphorylation sites, shown in red, were either mutated to alanine to prevent phosphorylation of those sites (previously threonine or serine), or aspartate to mimic constitutive phosphorylation. These point mutations were introduced singly or in combination to create a series of mutants that may be used to determine the functional relevance of those putative phosphorylation sites with the cytoplasmic domain of CD248. Underlined in the above schematic is the PDZ-binding motif of CD248. This motif was also removed from CD248 in an attempt to determine if the PDZ-binding motif of CD248 is needed for interaction with potential cytoplasmic interacting protein partners.
Figure 2.2: The amino acid sequence of the cytoplasmic tail of murine CD248

The 51 amino acid cytoplasmic tail of murine CD248 has three putative phosphorylation sites (Thr731, Ser744, and Thr760) (red) and a PDZ-binding motif (green).

2.4.1 Inserting an N-terminal FLAG Tag

An N-terminal FLAG tag was inserted into the existing murine CD248 construct using four steps of site-directed mutagenesis. The tag was inserted immediately after (3’ of) the signal peptide (shown underlined) to ensure that the recombinant protein would be correctly directed to the plasma membrane in transfected cells. The first four amino acids of CD248 were inserted along with the FLAG tag (shown in red and bold) in attempts to ensure that the protein would traffick normally in the transfected cells.


Figure 2.3: The amino acid sequence of the N-terminal of murine CD248 with an inserted FLAG-tag

The N-terminal FLAG-tag (red/bold) was inserted after the signal peptide (underlined) along with first four amino acids of CD248 (red).
2.5 Sequencing

After site-directed mutagenesis, the mutated pcDNA3.1+ murine CD248 cDNA constructs were fully sequenced using the DNA Sequencing Laboratory (UBC, Vancouver, BC) and Eurofins MWG Operon (Huntsville, AL).

All mutated and wild-type cDNAs were fully sequenced using the ApE – A plasmid Editor (Universal) (http://biologylabs.utah.edu/jorgensen/wayned/ape/) to confirm that only desired changes were introduced.
<table>
<thead>
<tr>
<th>CD248 Primer Number Name</th>
<th>5' → 3'</th>
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<tr>
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<td>AAGTCGTTGAGCTCAGAATGC</td>
</tr>
<tr>
<td>Forward 2</td>
<td>CTAGCAGGGGAGCTGGCAGCG</td>
</tr>
<tr>
<td>Forward 3</td>
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</tr>
<tr>
<td>Forward 4</td>
<td>GCACACTGGCTGTCGATGGCT</td>
</tr>
<tr>
<td>Forward 5</td>
<td>TGTGTGGAAGAGGTGGACGGGT</td>
</tr>
<tr>
<td>Forward 6</td>
<td>GCTTTGAGTGTACTGCAGCG</td>
</tr>
<tr>
<td>Forward 7</td>
<td>CCACCTTAGGCCCCCAGGGGC</td>
</tr>
<tr>
<td>Forward 8</td>
<td>AAGTCTTCCCTCCCCCACCAGGC</td>
</tr>
<tr>
<td>Forward 9</td>
<td>GAGCCCCTCTAGTCCCAAGGG</td>
</tr>
<tr>
<td>Forward 10</td>
<td>CCAGAGGCAGCCTTACAGGGG</td>
</tr>
<tr>
<td>Reverse 1</td>
<td>AACCTCCAGGCCACTCAGCCT</td>
</tr>
<tr>
<td>Reverse 2</td>
<td>TGCTGGGCTCAAAGAACAGAA</td>
</tr>
<tr>
<td>Reverse 3</td>
<td>GCAAGCAGCACCACCAAGAAG</td>
</tr>
<tr>
<td>Reverse 4</td>
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<tr>
<td>Reverse 5</td>
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<tr>
<td>Reverse 6</td>
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<td>Reverse 7</td>
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<tr>
<td>Reverse 8</td>
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<tr>
<td>Reverse 9</td>
<td>AAGGCGGTGTCTCTGCTCTCCC</td>
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<tr>
<td>Reverse 10</td>
<td>CAGCAGCAGGCGACGAGCAT</td>
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</table>
2.6 Production and purification of murine CD248 cDNAs

For each pcDNA3.1+ murine CD248 construct, 1 uL of cDNA (of varying concentrations) was transformed into 50 uL JM109 competent cells (Promega, Madison, WI). Bacteria was incubated on ice for 30 minutes, heat shocked at 42 °C for 45 seconds, and placed on ice for 2 minutes before being plated on LB Agar plates supplemented with ampicillin (Sigma, Saint Louis, MO).

Minipreps of cDNA were completed using the QIAprep® Spin Miniprep Kit (Qiagen Sciences, Maryland, USA) according to the manufacturer’s directions.

Maxipreps of cDNA were completed using the PureLink™ HiPure Plasmid Filter Maxiprep Kit (Invitrogen, Löhne, Germany) according to the manufacturer’s directions.

2.7 Immunofluorescence

2.7.1 Cell surface staining for murine CD248

HEK 293 T cells and CHO (either transiently transfected, or expressing stable WT or mutant murine CD248) cells were grown on poly-L-Lysine (Sigma, Saint Louis, MO) glass coverslips. Transient transfections of cells, with WT or mutant murine CD248 cDNA constructs, were performed using FuGENE® 6 and
FuGENE® HD transfection reagents (Promega, Madison, WI and Roche Applied Science, Indianapolis, IN) according to the manufacturer’s instructions. Coverslips were placed in 24 well tissue culture plates and washed three times with PBS. The coverslips were placed on ice and 1% BSA in PBS block (w/v) (BSA from Fisher BioReagents®, Fair Lawn, NJ; PBS from Sigma, Saint Louis, MO) was added for 10 minutes. Block was removed and 200 uL primary antibody at a dilution of 1:100 in 1% BSA was added and incubated on ice for 40 minutes. The primary antibody was removed and coverslips were washed three times with PBS, then 200 uL of secondary antibody at a dilution of 1:400 in 1% BSA was added and incubated for 20 minutes, in the dark, on ice. After the secondary antibody was removed, coverslips were washed three times with PBS. Cells were fixed on the coverslips using 4 °C 3.7% paraformaldehyde in PBS (Sigma, Saint Louis, MO) for 20 minutes, in the dark, on ice. The coverslips were then mounted on glass slides using ProLong® Gold antifade reagent with DAPI (Invitrogen Molecular Probes®, Eugene, OR). Slides were kept in the dark overnight to dry and then sealed with clear nail polish (Sally Hansen, Barrie, ON).

2.7.2 Intracellular staining for murine CD248

Cells were grown on coverslips as above. The cells were fixed by immersing the coverslips in -20 °C acetone (Sigma, Saint Louis, MO) for 2 minutes. The coverslips were then immersed into PBS (Sigma, Saint Louis, MO), placed into
24 well tissue culture dishes, and washed twice more with generous amounts of PBS.

After fixation, cells were permeabilised with a buffer containing 0.1% Triton X-100 in PBS (v/v) (Sigma, Saint Louis, MO) for 10 minutes. All subsequent blocking solutions contained 1% BSA in PBS (w/v) with 0.1% Triton X-100 (v/v) (Sigma, Saint Louis, MO). All remaining steps of immunostaining were done as previously described.

2.7.3 Intracellular staining for actin and focal adhesions

Cells were grown on coverslips as above and coverslips were placed into 24-well tissue culture plates. The cells were fixed by immersing the coverslips in 3% paraformaldehyde (PFA) in PBS (v/v) (Sigma, Saint Louis, MO) for 15 minutes. The coverslips were then washed with generous amounts of PBS (Sigma, Saint Louis, MO) three times.

After fixation, cells were permeabilised with a buffer containing 0.1% Triton X-100 in PBS (v/v) (Sigma, Saint Louis, MO) for 10 minutes. All subsequent blocking solutions contained 1% BSA in PBS (w/v) with 0.1% Triton X-100 (v/v) (Sigma, Saint Louis, MO). The focal adhesion kinase (FAK) antibody was used at a dilution of 1:150 and phalloidin-488 was used at a dilution of
1:500. Subsequent immunostaining methodology remained the same as previously described.

2.7.4 Intracellular staining for SERCA 2 ATPase and murine CD248

Cells were grown on coverslips as above. The cells were fixed by immersing the coverslips in -20 °C acetone (Sigma, Saint Louis, MO) for 2 minutes. The coverslips were then immersed into PBS (Sigma, Saint Louis, MO), placed into 24 well tissue culture dishes, and washed twice more with generous amounts of PBS.

After fixation, cells were permeabilised with a buffer containing 0.1% Triton X-100 in PBS (v/v) (Sigma, Saint Louis, MO) for 10 minutes. All subsequent blocking solutions contained 1% BSA in PBS (w/v) with 0.1% Triton X-100 (v/v) (Sigma, Saint Louis, MO). Anti-SERCA 2 ATPase (a marker for the endoplasmic reticulum) was used at a dilution of 1:200 and stained alongside anti-CD248 at a dilution of 1:100 to show possible localisation of mutant murine CD248 to the endoplasmic reticulum. All remaining steps of immunostaining were done as previously described.
2.7.5 Imaging

Slides were imaged with a BX61 Motorised Fluorescence Microscope (Olympus, Center Valley, PA) at the Life Science Institutes Tissue Processing Facility (UBC, Vancouver, BC).

Table 2.5: Primary antibodies used for immunofluorescence

<table>
<thead>
<tr>
<th>Antibody Against</th>
<th>Species</th>
<th>Catalogue Number</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD248</td>
<td>Rabbit pAb</td>
<td>18160-1-AP</td>
<td>ProteinTech Group, Chicago, IL</td>
</tr>
<tr>
<td>Rabbit IgG</td>
<td>Rabbit IgG (dilute 1:10 before use)</td>
<td>10500C</td>
<td>Invitrogen, Frederick, MD</td>
</tr>
<tr>
<td>Thrombomodulin</td>
<td>Mouse mAb (dilute 1:10 before use)</td>
<td>CTM1009</td>
<td>Conway Lab 99</td>
</tr>
<tr>
<td>Focal Adhesion Kinase (FAK)*</td>
<td>Rabbit pAb</td>
<td>sc-557</td>
<td>Santa Cruz Biotechnology Inc., Santa Cruz, CA</td>
</tr>
<tr>
<td>SERCA-2 ATPase*</td>
<td>Mouse mAb</td>
<td>MA3-910</td>
<td>Affinity BioReagents, Rockford, IL</td>
</tr>
</tbody>
</table>

*Antibodies kindly provided by the lab of Dr. Ivan Robert Nabi.
Table 2.6: Secondary antibodies used for immunofluorescence

<table>
<thead>
<tr>
<th>Antibody Against</th>
<th>Species</th>
<th>Colour</th>
<th>Catalogue Number</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit IgG (H+L)</td>
<td>Goat pAb</td>
<td>Alexa-Flour® 488</td>
<td>A11008</td>
<td>Invitrogen Molecular Probes®, Eugene, OR</td>
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<tr>
<td>Mouse IgG, IgM (H+L)</td>
<td>Goat pAb</td>
<td>Alexa-Flour® 488</td>
<td>A10680</td>
<td>Invitrogen Molecular Probes®, Eugene, OR</td>
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<tr>
<td>Rabbit IgG (H+L)</td>
<td>Goat pAb</td>
<td>Alexa-Flour® 568</td>
<td>A11011</td>
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<tr>
<td>Phalloidin</td>
<td>Not applicable</td>
<td>Alexa-Flour® 488</td>
<td>A12379</td>
<td>Invitrogen Molecular Probes®, Eugene, OR</td>
</tr>
</tbody>
</table>

2.8 Confocal microscopy

Confocal imaging of immunofluorescence-stained cells was accomplished with a Fluoview FV1000 Laser Scanning Confocal Microscope (Olympus, Center Valley, PA) at the Life Science Institutes Imaging Facility (UBC, Vancouver, BC), using the imaging program Fluoview FV1000 Software Version 3.0a (Olympus, Center Valley, PA).
2.9 Creating CHO cell lines that stably express mCD248

We first determined that the minimum concentration of geneticin (G418) (Invitrogen, Frederick, MD) required to induce CHO cell death was 400 ug/mL.

CHO cells were transiently transfected with either pcDNA3.1\(^+\) or murine CD248 pcDNA3.1\(^+\) cDNA constructs (wildtype, all Alanine, PDZ del., all Alanine + PDZ del., all Aspartate) using FuGENE HD® (Promega, Madison, WI) according to the manufacturer's directions. After allowing cells to recover for 48 hours, selection was completed using DMEM F12 media (Invitrogen, Frederick, MD) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 2 mM sodium pyruvate, 50 U/mL penicillin, 50 ug/mL streptomycin, and 400 ug/mL of G418. Media was changed every two to three days and after two weeks of selection, cells expressing murine CD248 cDNA constructs were selected using fluorescence-activated cell sorting (FACS) at the UBC Multi-Used Flow Cytometry (FACS) Facility (Life Sciences Institute, UBC, Vancouver).

Once selected for and sorted, cells were maintained and grown in DMEM F12 media (as described above) containing 200 ug/mL of G418.
2.10 Fluorescence-activated cell sorting of live CHO cell lines

CHO cells expressing either pcDNA3.1+ or murine CD248 pcDNA3.1+ cDNA constructs (listed above) were lifted from a 10 cm tissue culture plate using 1 mM PBS/EDTA (pH 8.0) and gently centrifuged to pellet the cells for 5 minutes. One million cells were resuspended in 500 uL FACS buffer (2% FBS, 0.002 M EDTA in PBS). Cells were then stained with 250 uL primary antibody at a dilution of 1:100 in FACS buffer and incubated on ice for 40 minutes. Gently spinning for 5 minutes isolated the cells and 3 washes with PBS were completed. Cells were then stained with 250 uL secondary fluorescent antibody at a dilution of 1:400 in FACS buffer and incubated on ice, in the dark for 20 minutes. The cells were further washed 3 times with PBS and resuspended in 500 uL of FACS buffer. FACS was performed using a FACSARia IIu (BD Biosciences, San Jose, CA).

Cells were sorted as bulk or singly into a 96 well tissue culture plate. Single cell colonies that survived in the 96 well plate were harvested and grown. The bulk sorted cells were serially diluted to a point that single cell colonies could be picked and grown.
Table 2.7: Primary antibodies used for FACS

<table>
<thead>
<tr>
<th>Antibody Against</th>
<th>Species</th>
<th>Catalogue Number</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD248</td>
<td>Rabbit pAb</td>
<td>18160-1-AP</td>
<td>ProteinTech Group, Chicago, IL</td>
</tr>
<tr>
<td>Rabbit IgG</td>
<td>Rabbit IgG (dilute 1:10 before use)</td>
<td>10500C</td>
<td>Invitrogen, Frederick, MD</td>
</tr>
</tbody>
</table>

Table 2.8: Secondary antibodies used for FACS

<table>
<thead>
<tr>
<th>Antibody Against</th>
<th>Species</th>
<th>Colour</th>
<th>Catalogue Number</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit IgG (H+L)</td>
<td>Goat pAb</td>
<td>Alexa-Flour® 488</td>
<td>A11008</td>
<td>Invitrogen Molecular Probes®, Eugene, OR</td>
</tr>
</tbody>
</table>

2.11 Flow cytometry of CHO cells, stable mCD248 (WT or mutant) CHO cell clones and B6 murine embryonic fibroblasts (MEFs)

CHO cells expressing either pcDNA3.1+ or murine CD248 pcDNA3.1+ cDNA constructs (listed above), B6 MEFs (CD248-positive control), and CHO cells (CD248-negative control) were lifted from a 10 cm tissue culture plate using 1 mM PBS/EDTA (pH 8.0) and gently centrifuged to pellet the cells for 5 minutes.
One million cells were resuspended in 100 uL FACS buffer (2% FBS, 0.002 M EDTA in PBS) and placed into a 96-well tissue culture plate. FACS buffer was removed with gentle spinning for two minutes. Cells were then stained with 100 uL primary antibody at a dilution of 1:100 in FACS buffer and incubated on ice for 40 minutes. Gently spinning for two minutes isolated the cells and three washes with PBS were completed. Cells were then stained with 100 uL secondary fluorescent antibody at a dilution of 1:400 in FACS buffer and incubated on ice, in the dark for 20 minutes. The cells were further washed 3 times with PBS and resuspended in 500 uL of FACS buffer containing 3.7% paraformaldehyde (Sigma, Saint Louis, MO) (v/v).

Flow cytometry was performed at the UBC Multi-User Flow Cytometry (FACS) Facility (Biomedical Research Centre, UBC, Vancouver) using a FACSCalibur (BD Biosciences, San Jose, CA). Data obtained from the instrument was analysed using FlowJo (Tree Star, Inc., Ashland, OR).

**Table 2.9: Primary antibodies used for flow cytometry**

<table>
<thead>
<tr>
<th>Antibody Against</th>
<th>Species</th>
<th>Catalogue Number</th>
<th>Company</th>
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<tr>
<td>CD248</td>
<td>Rabbit pAb</td>
<td>18160-1-AP</td>
<td>ProteinTech Group, Chicago, IL</td>
</tr>
<tr>
<td>Rabbit IgG</td>
<td>Rabbit IgG (dilute 1:10 before use)</td>
<td>10500C</td>
<td>Invitrogen, Frederick, MD</td>
</tr>
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</table>
Table 2.10: Secondary antibodies used for flow cytometry

<table>
<thead>
<tr>
<th>Antibody Against</th>
<th>Species</th>
<th>Colour</th>
<th>Catalogue Number</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit IgG (H+L)</td>
<td>Goat Ab</td>
<td>Alexa-Flour® 488</td>
<td>A11008</td>
<td>Invitrogen Molecular Probes®, Eugene, OR</td>
</tr>
</tbody>
</table>

2.12 RT-PCR of stable mCD248 (WT or mutant) CHO cell clones

CHO single cell colonies were grown in 6 well tissue culture plate. RNA was extracted from the cells using the RNeasy® Mini Kit (Qiagen Sciences, Maryland, USA) according to the manufacturer’s directions.

The 500 ng of RNA from the cells was reverse transcribed into single-stranded cDNA using the Quantitect® Reverse Transcription Kit (Qiagen Sciences, Maryland, USA) according to the manufacturer’s directions.

PCR was performed on the cDNA using the GoTaq® Green Master Mix 2x (Promega, Madison, WI) according to the manufacturer’s directions.

The following cycling parameters were used on a Mastercycler pro thermal cycler (Eppendorf, Hamburg, Germany):
2 minute at 94 °C melting step followed by 30 cycles of:

- 30 seconds @ 94°C: melting step
- 60 seconds @ 60 °C: annealing step
- 1 minutes @ 72 °C: elongation step

Followed by one last elongation step @ 72 °C for 5 minutes.

The PCR products were visualised on a 2% agarose gel and imaged with an Odyssey® Imager (LI-COR Biosciences, Lincoln, NE) on the 700 channel using Odyssey® Application Software Version 3.0.

Table 2.11: PCR primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5' → 3')</th>
<th>Reverse (5' → 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Murine CD248 (cytoplasmic domain)</td>
<td>ACATGTGTCTTTCTTGTTGTTGCTGC</td>
<td>GACCCAGCGATAGCAGTCCGT</td>
</tr>
<tr>
<td>Murine CD248 (mucin domain)</td>
<td>GGGCCCCTACCACCTCCTCA GT</td>
<td>AGGTGGGTGGACAGGGCTCAG</td>
</tr>
<tr>
<td>Murine GAPDH</td>
<td>ACCCACCAGCAAGGACACT</td>
<td>GCTCCCTAGGCCCTCTCTGA</td>
</tr>
<tr>
<td>Universal GAPDH100</td>
<td>GACCACAGTCCATGCCATCCTG</td>
<td>ATGACCTTGCCCACAGCTTGG</td>
</tr>
</tbody>
</table>
2.13 Quantitative PCR of stable mCD248 (WT or mutant) expressing CHO cell clones

The cDNA generated from CHO stably expressing mCD248 clones (as described above) was used to quantitate the amount of murine CD248 transcript contained within each clone. Q-PCR was also done on cDNA from HUVEC cells and CHO cells stably transfected with pcDNA3.1\(^+\) for negative controls for mCD248, as well as B6 wildtype MEFs for a positive control for mCD248. The original pcDNA3.1\(^+\) construct containing murine CD248 (used to create all the cytoplasmic mutations) was also used as a positive control, to ensure the mCD248 primers worked correctly. The mCD248 primers were targeted to the extracellular sialomucin-like region of the DNA sequence in order to ensure the cytoplasmic mutations did not hinder the binding of the primers to the mCD248 cDNA.

Using 5 uL of Fast SYBER® Green Master Mix (Invitrogen, Frederick, MD), 0.5 uL of forward primer, 0.5 uL of reverse primers, 1 uL of cDNA of interest, and 3 uL of double-distilled RNase/DNase free water, Q-PCR was performed on a 790HT Real-Time PCR System (Applied Biosystems, Carlsbad, CA) at the Genotyping Facility (Biomedical Research Centre, UBC, Vancouver).

Analyses of the samples were carried out by normalising the mCD248 transcript expression against the amount of GAPDH transcript expressed. The
mCD248:GAPDH transcript value from cDNA isolated from CHO cells stably expressing pcDNA 3.1+ was normalized arbitrarily to “1”, and all other mCD248:GAPDH transcript values obtained from the other cDNA samples were calculated with this normalisation in effect.

Table 2.12: Quantitative PCR primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5' → 3')</th>
<th>Reverse (5' → 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Murine CD248 (mucin domain)</td>
<td>GACCACAGTCCATGCCATC ACTGC</td>
<td>AGGTGGGTGGACAGGGCTC AG</td>
</tr>
<tr>
<td>Universal GAPDH(^{100})</td>
<td>GACCACAGTCCATGCCATC ACTGC</td>
<td>GACCACAGTCCATGCCATC ACTGC</td>
</tr>
</tbody>
</table>

2.14 Gelatin zymography for MMP-9

Serum-free conditioned media was collected from cells grown in a 6 well tissue culture plate and kept on ice. Equal volumes of media under non-reducing conditions in Laemmli sample loading buffer (not boiled) were separated by 10% SDS-PAGE polyacrylamide gel that was prepared with 0.1% gelatin. The gel was then run at 150V for 90 minutes (or until the 37 kDa marker reached the bottom of the gel) in a Mini-PROTEAN gel running system (BioRad Laboratories, Hercules, CA). The gel was placed in a Triton X-100 Exchange Buffer [20 mM Tris-HCl (pH 8.0), 150 mM sodium chloride, 5 mM calcium chloride, 2.5% Triton
X-100] on a shaker for 60 minutes. The gel was then washed three times, for 10 minutes each, on a shaker with Incubation Buffer [20 mM Tris-HCl (pH 8.0), 150 mM sodium chloride, 5 mM calcium chloride]. The gel was then placed in Incubation Buffer in the 37 °C incubator overnight. The next morning, the buffer was removed and the gel was stained with Coomassie Brilliant Blue Solution [0.025% Brilliant Blue R (Sigma, Saint Louis, MO), 40% methanol, 7% acetic acid] for one hour on a shaker. Then, the gel was destained with a solution consisting of 40% methanol and 7.5% acetic acid for two hours on a shaker before being transferred to distilled water.

Gels were imaged on the Odyssey® Imager (LI-COR Biosciences, Lincoln, NE) on the 700 channel using Odyssey® Application Software Version 3.0.

2.14.1 Activating MMP 9 and MMP 2 proteins for positive controls

MMP 9 and MMP 2 zymogens were kindly provided by the group of Dr. Christopher Overall (UBC, Vancouver, B.C.). 1200 ng of MMP zymogen was added to 6 ul of 10 mM 4-aminophenylmercuric acetate (APMA) and 53 uL of MMP Activation Buffer [25 mM Tris-HCl pH 7.4, 150 mM sodium chloride, 10 mM calcium chloride, 5 uM zinc chloride]. The solution was vortexed to mix and incubated for one hour at 37 °C. In parallel, as a non-activation control, another
sample of MMP zymogen was placed in 59 uL of MMP Activation Buffer and incubated.

Detectable amounts of the purified activated and non-activated MMPs were loaded onto the MMP zymographs as controls.

2.14.2 MCF-7 Cells transiently transfected with WT Murine CD248

MCF-7 cells were grown in 6 well tissue culture plates and transiently transfected using FuGENE® HD transfection reagents (Promega, Madison, WI) according to the manufacturer’s instructions. After 48 hours, the media was exchanged for serum-free media. After two hours, the cells were stimulated with 0 nM (DMSO-alone control), 1 nM, 10 nM and 100 nM PMA in DMSO (Sigma, Saint Louis, MO) for 1 hour. Media was collected for gelatin zymography.

2.14.3 CHO transiently transfected cells

CHO cells were grown in 6 well tissue culture plates and transiently transfected using FuGENE® 6 and FuGENE® HD transfection reagents (Promega, Madison, WI and Roche Applied Science, Indianapolis, IN) according in serum-free media. After 48 hours, the conditioned media was collected for gelatin zymography.
2.14.4 CHO stably expressing murine CD248 (WT or Mutant) cDNA constructs

CHO cells were grown in 6 well tissue culture plates. Once confluent, the media was exchanged for serum-free media. After 48 hours, conditioned media was collected for gelatin zymography.

2.15 Phospho-immunoprecipitations

Anti-phosphoserine rabbit polyclonal antibody (Abcam, Cambridge, MA) was coupled to Dynabead® epoxy using the Dynabeads® Antibody Coupling Kit (Invitrogen, Oslo, Norway) at a concentration of 7 ug of antibody per mg of Dynabead® epoxy.

B6 wild-type murine embryonic fibroblasts (MEFs) and CHO cells were grown in 10 cm tissue culture plates. The CHO cells were transiently transfected with either wildtype or “all A” pcDNA3.1+ murine CD248 cDNA using FuGENE® HD transfection reagent (Promega, Madison, WI and Roche Applied Science, Indianapolis, IN) according to the manufacturer’s instructions. After 24 hours, cells were starved overnight and the next morning they were stimulated with 1 mM PMA for one hour. Cells were then lysed according to the Dynabeads® Co-Immunoprecipitation Kit (Invitrogen, Oslo, Norway) manufacturer’s instructions.
Co-immunoprecipitation of cell lysates was performed using the Dynabeads® Co-Immunoprecipitation Kit with the DynaMag™-2 Magnetic Particle Concentrator (Invitrogen, Oslo, Norway) according to manufacturer’s directions.

The proteins eluted directly from the bead epoxy and were resuspended in 1x Laemmli reducing buffer and separated by 11% SDS-PAGE polyacrylamide gel in a Mini-PROTEAN gel running system (Bio-Rad Laboratories, Hercules, CA). Transfer and immunoblotting of eluents using anti-CD248 rabbit polyclonal antibody was completed to detect if phosphorylated murine CD248 was pulled out of the cell lysates using the covalently-bound anti-phosphoserine-Dynabead® epoxy.
2.16 FLAG-tag immunoprecipitation

Figure 2.4: Diagrammatic illustration of the FLAG-tag immunoprecipitation method

M2 anti-FLAG monoclonal antibody is covalently bound to a Dynabead® epoxy. Cell lysates are mixed with the beads and FLAG-tagged proteins (namely murine CD248) are pulled down, along with putative binding partners. The samples are separated by SDS-PAGE gel, protein bands are cut out and subsequently subjected to in-gel tryptic digestion. Isolated proteins are then identified by mass spectrometry.

Anti-FLAG M2 mAb F3165 (Sigma, Saint Louis, MO) was coupled to Dynabead® epoxy using the Dynabeads® Antibody Coupling Kit (Invitrogen, Oslo, Norway) at a concentration of 7 ug of antibody per mg of Dynabead® epoxy.
HEK 293 T cells were grown in 10 cm tissue culture plates. The cells were transiently transfected with either wildtype or FLAG-tagged wildtype pcDNA3.1+ murine CD248 cDNA using FuGENE® HD transfection reagent (Promega, Madison, WI and Roche Applied Science, Indianapolis, IN) according to the manufacturer’s instructions. Cells were then lysed according to the Dynabeads® Co-Immunoprecipitation Kit (Invitrogen, Oslo, Norway) manufacturer’s instructions.

Co-immunoprecipitation of cell lysates was performed using the Dynabeads® Co-Immunoprecipitation Kit with the DynaMag™-2 Magnetic Particle Concentrator (Invitrogen, Oslo, Norway) according to manufacturer’s directions.

The protein pellet left after elution from the bead epoxy was resuspended in 1x Laemmli reducing buffer and separated by 11% SDS-PAGE polyacrylamide gel in a Mini-PROTEAN gel running system (Bio-Rad Laboratories, Hercules, CA). Gels were either stained using EZBlue™ Gel Staining Reagent (Sigma, Saint Louis, MO), or immunoblotted using both the anti-CD248 (ProteinTech Group, Chicago, IL) and anti-FLAG (Sigma, Saint Louis, MO) antibodies to ensure the efficient pull-down of the FLAG WT mCD248 protein from the HEK cell lysates.
Coomassie-blue stained gels were imaged with the Odyssey® Imager (LI-COR Biosciences, Lincoln, NE) on the 700 channel using Odyssey® Application Software Version 3.0.

2.17 In-gel tryptic digestion

Coomassie stained gels obtained from the immunoprecipitation experiments explained above were processed using in-gel tryptic digestion, obtaining peptides that could then be identified by mass spectrometry. Each lane on the SDS-PAGE gel was cut into 10 bands of differing sizes reflecting the approximate amount on protein in each band, as seen by Coomassie stain. The bands were chopped into 1 mm x 1 mm pieces that were suspended in 10 separate 1.7 mL tube (Axygen, Inc., Union City, CA) with 100 uL of acetonitrile (Sigma, Saint Louis, MO) or enough acetonitrile to cover all the gel pieces. The gel pieces were incubated at room temperature in a thermomixer (Eppendorf, Hamburg, Germany) at 600 rpms for 15 minutes, allowing the gel to shrink and turn opaque. The acetonitrile was removed and discarded.

The gel pieces were then incubated in 100 uL of 10 mM DTT (dithiothreitol) (Sigma, Saint Louis, MO) in 100 mM ammonium bicarbonate (NH₄HCO₃) for 30 minutes at 56 °C in a thermomixer at 600 rpms. After incubation the DTT was removed and discarded. The gel pieces were shrunk in 100 uL of acetonitrile as before. The acetonitrile was removed and discarded.
The gel was then washed with 150 uL 100 mM ammonium bicarbonate and the pieces were allowed to swell. Again, the gel pieces were shrunk in 100 uL of acetonitrile as before, and the acetonitrile was removed then discarded.

To the gel pieces, 100 uL of 55 mM iodoacetamide in 100 mM ammonium bicarbonate was added and the pieces were incubated at room temperature for 45 minutes, in the dark, in a thermomixer at 600 rpms. The iodoacetamide solution was then removed and discarded. The gel pieces were shrunk in 100 uL of acetonitrile and washed in 150 uL of 100 mM ammonium bicarbonate as above. The gel pieces were shrunk one last time in 100 uL of acetonitrile, as before, then the acetonitrile was removed and discarded.

Trypsin (Promega, Madison, WI) was made at a concentration of 1 ug/uL in acidic re-suspension buffer as per manufacturer’s directions, and kept on ice until needed. The gel pieces were rehydrated in 100 uL 50 mM ammonium bicarbonate, 5 mM calcium chloride, with 0.15 ug of trypsin per sample and incubated at room temperature. (At 15 minutes, the gel pieces were checked to ensure buffer still covered the tops of the gel. If the buffer evaporated, more was added as necessary.) The samples were incubated at 37 °C in a thermomixer at 600 rpms overnight.

After incubation, 15 uL of 25 mM ammonium bicarbonate was added to the gel pieces and trypsin solution. The mixture was then incubated at 37 °C for 15 minutes in a thermomixer at 600 rpms. Another 100 uL of acetonitrile was
added and the mixture was incubated again at 37 °C for 15 minutes in a thermomixer at 600 rpms. The supernatant containing the peptides was removed and placed into separate labeled 1.7 mL tubes. Afterwards, 50 uL of 5% formic acid (Sigma, Saint Louis, MO) was added to the gel pieces and incubated at 37 °C for 15 minutes in a thermomixer at 600 rpms. Again, 100 uL of acetonitrile was added and the mixture was incubated as before. The supernatant was removed and added to the previous supernatant samples. The samples were taken to a vacuum centrifuge (Thermo Scientific, Holbrook, AZ) and spun until dry. The dried proteins were reconstituted in 15 uL 5% formic acid.

2.18 In-solution tryptic digestion

In-solution tryptic digestion was carried out directly on samples after elution off the Dynabead® epoxy. The lyophilised protein samples were resuspended in 100 uL 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) pH 8.0 buffer (Sigma, Saint Louis, MO), vortexed to mix, and put into a 99 °C thermomixer (Eppendorf, Hamburg, Germany) at 600 rpms for 5 minutes. 1 uL of 2% SDS (sodium dodecyl sulfate) (w/v) (Sigma, Saint Louis, MO) and 4 uL of DTT solution (5 mg DTT: 10 mL 100 mM ammonium bicarbonate) was added to the protein solution. After vortexing, protein samples were incubated at room temperature in a thermomixer at 600 rpms for 30 minutes. Then, 20 uL of iodoacetamide solution (5 mg iodoacetamide: 10 mL 100 mM
ammonium bicarbonate) was added to protein samples. After vortexing, the samples were incubated at room temperature for 20 minutes, in the dark, in a thermodixer at 600 rpms.

Following incubation, 2 uL of endoproteinase LysC solution (1.0 mg Lys C: 1 mL digestion buffer) (Promega, Madison, WI) was added to protein samples at incubated for three hours at room temperature in a thermodixer at 600 rpms. To the samples, 1 ug of trypsin was added per 50 ug of sample protein and incubated overnight at 37 °C in a thermodixer at 600 rpms. The digested peptides were lyophilised in a vacuum centrifuge (Thermo Scientific, Holbrook, AZ) until dry. The dried protein samples were reconstituted in 15 uL 5% formic acid.

2.19 Mass spectrometry

Mass spectrometry was completed at the Centre for Blood Research Mass Spectrometry Suite at the Life Sciences Institute (UBC, Vancouver, BC). Samples were separated via nano-flow liquid chromatography (C18, 50 um I.D., 20 cm length, 200 nL/min) using a water/acetonitrile/acetic acid gradient with an LC Packings Ultimate HPLC pump. Column eluent was sprayed into a Q-Star mass spectrometer (AB/Sciex, Concord, ON) using 10 um nanospray emitters (New Objective, Inc., Woburn, MA). Raw data were converted to mascot generic format using Analyst Control software and searched against the SwissProt Homo
sapiens protein database, using the search algorithm on Mascot Distiller (v2.3) (Matric Science, Boston, MA).
3. Results

3.1 Identifying an antibody to detect murine CD248

To identify antibodies that specifically detected murine CD248, 12 commercially available antibodies, 4 other antibodies generated in the Conway lab (from Aves Lab Inc.) and in a lab in England were tested using Western blots under non-reducing and reducing conditions (Figures 3.1 And 3.2). Antibodies from Aves Lab Inc. did not specifically detect CD248 (Figure 3.1 a); nor did a commercially available antibody from Sigma (Figure 3.1 b). These antibodies exhibited non-specific binding in cell lysates that contained CD248 (HEK 293 T cells transfected with WT murine CD248, B6 murine embryonic fibroblasts, and 3T3 murine fibroblasts) and lysates from cells that reportedly do not express CD248 (EAhy endothelial hybrid cells, HEK 293 T cells without transfection, and B6 MEFs obtained from three different CD248 knock-out mice).

An immunoblot of cell lysates (10T1 murine fibroblasts, 3T3 murine fibroblasts, B6 murine embryonic fibroblasts, and CD1 murine embryonic fibroblasts), revealed the appearance of a murine CD248 band at an apparent molecular weight of 75 kDa when using a Rabbit anti-CD248 polyclonal antibody 18160-1-AP under reducing conditions. HUVEC (human umbilical vein endothelial cells) cell lysate was used as a negative control since it is a cell-line known to not express CD248. As seen in Figure 3.2 the 75 kDa band is indeed
absent in the HUVEC lysate. A full-length, un-glycosylated, human CD248 construct with an N-terminal GST tag was used as a positive control.

HEK (human embryonic kidney) 293 T cells were transfected with human and murine CD248 cDNAs. Immunoblots revealed that higher molecular CD248 species (presumably glycosylated forms of the protein) are visible, appearing at the apparent molecular weights of 100 and 150 kDa, as seen in Figure 3.3.
Figure 3.1: Immunoblots of cell lysates detected with antibodies raised against CD248 antigens

Non-specific signals are observed with two chicken anti-CD248 antibodies (Aves Lab Inc.) raised against N-terminal peptides from CD248 (a) and a rabbit anti-CD248 polyclonal antibody from Sigma (b). Signals are evident in lanes where lysates are from cells that do not express CD248 (EAhy926, HEK 293 T without transfection, and B6 MEF lysates obtained from three different CD248-knockout mice).
Figure 3.2: Immunoblot of murine fibroblast cell lysates using ProteinTech rabbit anti-CD248 polyclonal antibody 18160-1-AP under reducing conditions

Cells were grown in 10 cm tissue culture plates and lysed in RIPA buffer. Protein content of cell lysates was quantified with the BCA assay and equal amounts of total protein were loaded into each well. The 75 kDa band present in the murine fibroblast lysates is now identified as murine CD248.
Figure 3.3: Immunoblot of transfected HEK (human embryonic kidney) cell lysates using ProteinTech rabbit anti-CD248 polyclonal antibody 18160-1-AP under reducing conditions

Cells were grown in 10 cm tissue culture plates and transfected with human and murine CD248 cDNA constructs using FuGENE® HD reagent. After 48 hours, cells were lysed with RIPA lysis buffer. Protein content of cell lysates was quantified by BCA assay and equal amounts of total protein were loaded into each well. Different forms of CD248 are present in the cell lysates, presumably representing variable glycosylation.
3.2 Site-directed mutagenesis of murine CD248's cytoplasmic tail

In 2000, Opavsky et al. identified three putative phosphorylation sites (Thr731, Ser744, and Thr760) and a putative PDZ-binding motif (amino acids 763-765)\textsuperscript{20}, shown in Figure 3.4. The putative phosphorylation sites were mutated using site-directed mutagenesis to alanine (to knock-out phosphorylation potential) and aspartates (to mimic constitutive phosphorylation, \emph{i.e.} “always phosphorylated”). The putative PDZ-binding motif was also deleted using site-directed mutagenesis. Site-directed mutagenesis was performed on a pcDNA3.1\textsuperscript{+} murine CD248-containing construct previously prepared in our lab. Table 3.1 summarises the cytoplasmic murine CD248 mutants created.
Figure 3.4: The cytoplasmic tail of murine CD248 identifying putative phosphorylation sites and PDZ-binding domain

Putative phosphorylation sites were mutated to alanine to knock-out phosphorylation potential, and aspartate to mimic constitutive phosphorylation. These mutations were performed in combination with and without deletion of the PDZ-binding motif, as well as in combination with each other.
Table 3.1: Site-directed mutagenesis of murine CD248 cytoplasmic tail creating murine CD248 mutant cDNA

Site-directed mutagenesis of murine CD248’s cytoplasmic tail was performed on an existing pcDNA3.1 murine CD248-containing cDNA construct. Mutagenesis led to the creation of 22 different cytoplasmic murine CD248 mutants.

<table>
<thead>
<tr>
<th>DNA Mutation</th>
<th>Resulting Amino Acid Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>A2191G</td>
<td>Thr731Ala</td>
</tr>
<tr>
<td>A2230G, G2231C</td>
<td>Ser744Ala</td>
</tr>
<tr>
<td>A2278G</td>
<td>Thr760Ala</td>
</tr>
<tr>
<td>A2191G, A2278G</td>
<td>Thr731Ala, Thr760Ala</td>
</tr>
<tr>
<td>A2191G; A2230G, G2231C; A2278G</td>
<td>Thr731Ala, Ser744Ala, Thr760Ala a.k.a. “all A”</td>
</tr>
<tr>
<td>A2191G, C2192A, G2193C</td>
<td>Thr731Asp</td>
</tr>
<tr>
<td>A2230G, G2231A</td>
<td>Ser744Asp</td>
</tr>
<tr>
<td>A2278G, C2279A</td>
<td>Thr760Asp</td>
</tr>
<tr>
<td>A2191G, C2192A, G2193C; A2230G, G2231A; A2278G, C2279A</td>
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</tr>
<tr>
<td>G2201 to T2209 deletion</td>
<td>Thr767-Ser764-Val765 deletion a.k.a. “PDZ deletion”</td>
</tr>
<tr>
<td>A2191G; A2230G, G2231C; A2278G; G2201 to T2209 deletion</td>
<td>Thr731Ala, Ser744Ala, Thr760Ala; Thr767-Ser764-Val765 deletion a.k.a. “all A + PDZ deletion”</td>
</tr>
<tr>
<td>A2191G, C2192A, G2193C; A2230G, G2231A; A2278G, C2279A; G2201 to T2209 deletion</td>
<td>Thr731Asp, Ser744Asp, Thr760Asp; Thr767-Ser764-Val765 deletion a.k.a. “all D + PDZ deletion”</td>
</tr>
</tbody>
</table>
3.3 Detecting murine CD248 wildtype and mutant protein

We confirmed that the cytoplasmic mutants of murine CD248 were detectable by immunoblot and immunofluorescence using the anti-CD248 polyclonal antibody. The cytoplasmic mutant mCD248 cDNA, when transfected into HEK 293 T cells and CHO cells, yields a protein that is detectable by immunoblot under reducing conditions. It is also detectable on the cell surface by immunofluorescence (Figure 3.5), in a pattern similar to that observed with wildtype murine CD248. These two findings are important as further experiments to determine differences in expression levels or function of these mutant proteins relies on these detection approaches.
Figure 3.5: Immunofluorescence of WT or mutant mCD248 transfected HEK 293 T cells

HEK cells were transfected with wildtype or mutant mCD248 cDNAs and grown on 10% poly-L-lysine coverslips. Live cells were stained with anti-CD248 antibody (green) and DAPI (blue) at 4°C, without fixation or permeabilisation, to visualise cell surface staining. Unstained, isotype-match, secondary antibody-alone and untransfected HEK cells were used as controls for murine CD248 as no signals appear.

3.4 Addition of an N-terminal FLAG-tag to wildtype and mutant forms of murine CD248

In the event that a murine CD248-detecting antibody might not be found, we inserted a cDNA encoding a FLAG tag into all of the murine CD248 cDNA constructs (described above). This was performed to allow us to specifically
detect expression of CD248 on immunoblots and in cells using anti-FLAG tag antibodies. In addition, the FLAG tag provided a mechanism to perform CD248 co-immunoprecipitation studies to pull-down interacting proteins. Furthermore, the FLAG tag was a potentially useful tool for transfecting cell lines that already naturally expressed CD248.

Using site-directed mutagenesis, as described in Materials and Methods, a FLAG tag was inserted at the N-terminus of CD248, as seen in Figure 3.6. It was hoped that the N-terminal location would not disturb the production and cellular localisation of CD248 the least. The FLAG tag was inserted after CD248’s signal peptide to ensure that the protein would be directed to the plasma membrane of the transfected cell, and after the first four amino acids of CD248 to ensure the protein would have a higher chance of being properly expressed. In addition, the first four amino acids were repeated once after the FLAG tag, again to ensure proper expression of the CD248 protein.

Though the addition of the FLAG tag could potentially change the function and the folding patterns of murine CD248, the results gathered to date show no such alterations in CD248’s protein production or trafficking. The CD248 forms produced in HEK 293 T and CHO cells are both detectable by immunoblot and by immunofluorescence on the cell surface.
The N-terminus FLAG-tag was inserted using site-directed mutagenesis (see Materials and Methods).

3.5 Detecting murine CD248 FLAG-tagged wildtype and mutant protein by immunoblot and immunofluorescence

We tested whether the FLAG-tagged CD248 could be detected by immunoblot using both the CD248 polyclonal antibody mentioned earlier and an M2 FLAG-tag antibody. As shown in Figure 3.7, the FLAG-tagged proteins, expressed following transfection of the corresponding cDNA into HEK 293 T cells using FuGENE® HD, were detectable by immunoblot under reducing conditions, using both FLAG and CD248 antibodies, at the apparent molecular weights of 80, 100 and 150 kDa.
We also tested whether the recombinant CD248 forms with or without the FLAG tag similarly localised to the cell surface. Immunofluorescence utilising dual staining for both CD248 and FLAG, and individual staining, showed the FLAG-tagged wildtype and mutant murine CD248 proteins were indeed functional in the sense that they trafficked to the cell surface once produced and could be detected by both antibodies (Figure 3.8).
Figure 3.7: Immunoblot of transfected HEK cell lysates using anti-CD248 polyclonal antibody and anti-FLAG monoclonal antibody under reducing conditions

Cells were grown in 10 cm tissue culture plates and transfected with cDNAs encoding human and wildtype/"all A" murine CD248 with and without the FLAG tag. After 48 hours, cells were lysed with RIPA lysis buffer. Cell lysates were quantified for protein content by BCA assay and equal amounts of total protein were loaded into each well. Variously glycosylated forms of CD248 protein are present in the cell lysate.
Figure 3.8: Detection of FLAG WT murine CD248 on CHO cells using both anti-CD248 polyclonal antibody and anti-FLAG monoclonal antibody

Cells were grown on 10% poly-L-lysine coverslips and stained using anti-CD248 polyclonal antibody (red), anti-FLAG monoclonal antibody (green), and DAPI (blue). On CHO cells transfected with the FLAG WT mCD248 cDNA construct, dual-staining occurred (yellow). Isotype and secondary antibody controls showed no staining for CD248. FLAG mutant murine CD248 protein also localised to the cell surface in much the same manner as the wildtype variant (data not shown).

3.6 Creating CHO wildtype murine CD248 and mutant murine CD248 stably-expressing cell lines

To perform further analyses of murine CD248’s function, we sought to establish cells that stably express the different mutant forms of murine CD248 at equivalent levels. CHO cells were transfected using FuGENE® HD with the WT mCD248, with pcDNA3.1+ (as a negative control for experiments), and with
cDNA constructs encoding mCD248 “all A”, mCD248 “all D”, mCD248 “PDZ del.”, and mCD248 “all +PDZ del.” Transfected cells were selected with geneticin (G418) at a dose designed to kill cells that were not expressing the neomycin resistant gene. As shown in Figure 3.9, fluorescence-activated cell sorting (FACS) was used to identify those cells that expressed the highest amounts of murine CD248, and these were sorted in both bulk and then singly. Single cells were plated into 96-well dishes and grown into colonies that could be expanded and used for further analyses.

Five WT mCD248 clones (2N, 3N, 8N, 9N, and 12N), ten mCD248 “all A” clones (1-10), five “PDZ del.” clones (1-5), ten “all A + PDZ del.” clones (1-10), and 11 “all D” clones survived.
Figure 3.9: Fluorescence-activated cell sorting of CHO cells expressing WT murine CD248

After selection, CHO cells were stained with anti-CD248 polyclonal antibody and FACS was performed to identify cells expressing murine CD248 on their surface. Using unstained, isotype matched and secondary antibody-alone controls, as well as CHO cells stably transfected with the empty vector pcDNA3.1+, gating voltages were set to only allow those cells expressing murine CD248. The gating for mCD248 “all A”, “all D”, “PDZ del.”, and “all A + PDZ del.” was completed with the same gating parameters as for WT mCD248. Cells were bulk-sorted and singly sorted, for mCD248 (WT or mutant) expression, into 96-well tissue culture plates. Those single-cell clones that survived in the 96-well plates were grown in selection media with G418 until they were confluent in a 10 cm tissue culture plate. The clones were frozen in liquid nitrogen for further experiments.
3.7 Stably expressing mCD248 CHO cell lines express wildtype and mutant 
murine CD248 protein on their cell surface

We confirmed that the stably expressing mCD248 (WT or mutant) CHO cell lines 
retain expression on the cell surface by flow cytometry. Untransfected CHO cells 
and CHO cells stably expressing pcDNA3.1+ were used as CD248-negative 
controls; wildtype B6 MEFs were used as a CD248-positive control.

Two clones of each mCD248 mutant stably expressing CHO cell line were 
used to show the cell surface expression of wildtype, “all A”, “all A + PDZ del.”, 
“PDZ del.” and “all D” murine CD248 protein. It is clear that the stable CHO cell 
lines express CD248 on their cell surface as shown in the representative 
histograms and cell scatter plots (Figure 3.10).

In spite of claims in the literature to the contrary, it is apparent that there is 
minimal cell-surface expression of CD248 on CHO cells. However, the CHO 
cells that are stably expressing mCD248 (WT or mutant) have a much higher 
level of expression of CD248 protein than CHO cells that are non-transfected.
Figure 3.10: Representative histograms and cell scatter plots for flow cytometry of CHO mCD248 (WT or mutant) stable expressing cells lines

To show that the single-cell clones retained expression of murine CD248 (WT or mutant) on their cell surface, flow cytometry was performed using an anti-CD248 polyclonal antibody (FITC channel, labeled as FL1-H). Using unstained, isotype-matched, anti-CD248 only, and secondary antibody-alone controls, as well as CHO cells expressing pcDNA 3.1+, gating voltages were set. Untransfected CHO cells were used as a negative control for CD248 expression (a). B6 MEFs were used as a positive control for murine-CD248 expression (b). CHO cells expressing WT murine CD248 (complete with the aforementioned controls) show cell surface expression of CD248 (c). In addition, CHO cells expressing “all A” (d), “PDZ del.”, “all A + PDZ del.” and “all D” forms of murine CD248 also showed cell surface expression of the protein by flow cytometry. By cell scatter plot, the gating for live cells (e) and FITC (f) was made using unstained CHO cells. Untransfected CHO cells show minimal cell surface expression of CD248 (g), but cells stably transfected with “all D” murine CD248 show much greater cell surface expression (h).

3.8 Relative quantification of murine CD248 protein in all stably expressing mCD248 CHO cell lines

The amount of murine CD248 expressed by the different stable CHO cell lines was compared by using serial immunoblots of cell lysates in which a range of total protein amounts (2.28 ug, 5 ug, 10ug and 15 ug) were separated by SDS-PAGE. The subsequent blots were probed with both anti-CD248 polyclonal antibody (and anti-GAPDH polyclonal antibody as a loading control) to determine the relative amounts of murine CD248 expressed by each clone. The blots
revealed the ranking of murine CD248 protein expression levels in each set of clones (Figure 3.11).

For WT mCD248 expression CHO clones, it was determined that the CD248 protein expression was greatest in clone 9N, followed (in descending order) by 12N, 3N, and 2N. For “all A” mCD248 expressing CHO clones it was determined that the CD248 protein expression was greatest in clone 3, followed (in descending order) by clones 2, 8, 4, 5, 9, 7, and 1. For “PDZ del.” mCD248 clones, CD248 expression was greatest in clone 4, followed (in descending order) by clones 5, 1, 2, and 3. For “all A + PDZ del.” mCD248 clones, CD248 expression was greatest in clone 8, followed (in descending order) by 2, 10, 1, and 9. For “all D” mCD248 clones, CD248 expression was greatest in clone 9, followed (in descending order) by 6, 10 and 7.

The preceding represents only those clones that expressed sufficient CD248 to be detectable when 15 ug of total protein lysate was loaded for SDS-PAGE and immunoblotting.
Figure 3.11: Serial immunoblots of CHO mCD248 “all A” clone lysates at differing total protein amounts

CHO cells, CHO stably transfected with pcDNA3.1", and ten CHO clones from single cell colonies stably expressing murine CD248 “all A” protein, not transfected were grown in 10 cm tissue culture plates. Once confluent, cells were lysed with Triton X-100 lysis buffer. Protein lysates at 2.28 µg (a), 5 µg (b), 10 µg (c), and 15 µg (d) of protein were separated by SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted with anti-CD248 polyclonal antibody. Murine CD248 bands appear at ~100 kDa and ~150 kDa. Lanes marked with an “X”
are blank as the protein concentration of the lysate did not allow the loading of that amount of protein. GAPDH was used as a loading control (data not shown).

This process was repeated for the 5 WT mCD248 clones, 10 mCD248 “all A + PDZ del.” clones, 5 mCD248 “PDZ del.” clones, and 11 mCD248 “all D” clones (data not shown).

### 3.9 Relative quantification of murine CD248 mRNA in mCD248 (WT or murine) stably expressing CHO clones

To confirm that transcript levels of CD248 match the protein levels for each of the stably transfected CHO cell lines, quantitative real-time PCR (Q-RT-PCR) was performed, B6 wildtype MEF cDNA was isolated as a positive control for mCD248 transcript. Negative controls were derived from RNA prepared from CHO cells stably transfected with the empty vector pcDNA3.1+ and from HUVECs. Double-distilled water was also run alongside all the samples to ensure no contamination of the primers occurred (Figure 3.12).

Primers were designed to amplify the sialomucin-like region of mCD248, as it was a concern that the DNA mutations in the cytoplasmic tail may hinder the primers’ abilities to bind. Universal GAPDH primers were used for the control for the relative quantification of mCD248. The Ct values of mCD248 transcript remained within a small range in all CD248 positive samples. However, the GAPDH Ct values varied considerably from one set of clones to another. The thousands of fold increases of mCD248 transcript seen in the “all A” and “all A +
PDZ del." may be partly due to the fact that these samples had significantly less GAPDH transcript, and therefore the huge increase in CD248 transcript may be an artifact of the calculation process.

From the Q-PCR results, it is possible to rank the CHO cell clones by the amount of mCD248 transcript. For wildtype mCD248 stably expressing CHO cells, clone 3N makes the most mCD248 transcript followed by clones (in descending order) 8N, 2N, 9N and 12N. For the “PDZ del.” mutant mCD248 stably expressing CHO cells, clone 4 makes the most mCD248 transcript followed by (in descending order) clones 2, 5, 1 and 3. For “all A” mutant mCD248 stably expressing CHO cells, clone 3 (shown previously at the protein level) makes the most mCD248 transcript followed by (in descending order) clones 2, 8, 4, 9, 5, 6, 10, 7 and 1. For “all A + PDZ del.” mutant mCD248 stably expressing CHO cells, clone 10 makes the most mCD248 transcript followed by (in descending order) clones 5, 1, 6, 7, 9, 2, 4 and 3. For “all D” mutant mCD248 stably expressing CHO cells, clone 7 makes the most mCD248 transcript followed by (in descending order) clones 3, 6, 1, 11, 8, 9, 10, 2, 5 and 4. These findings in general do match the rankings made for murine CD248 protein expression by stably transfected CHO cell clones, with notable exceptions in the wildtype mCD248 and “all D” mutants.
Figure 3.12: Relative quantification of mCD248 transcript in CHO cells stably transfected with WT or mutant mCD248 cDNA

Quantitative RT-PCR was performed on cDNA isolated from all the wildtype and mutant murine CD248 stably expressing CHO cell clones to determine the amount of mCD248 transcript in comparison to GAPDH transcript. B6 wildtype MEF cDNA was used as a positive control for CD248 transcript expression; HUVEC and CHO cells stably transfected with the empty pcDNA3.1+ vector acted as negative controls for CD248 transcript expression. The ratio of mCD248 transcript to GAPDH transcript (in the form of Ct values) was calculated and the mCD248:GAPDH of cDNA from CHO pcDNA3.1+ stably transfected cells was set to have the value of 1; all other cDNA ratios were then normalized to the ratio obtained from CHO pcDNA3.1+ stably transfected cells. The ratios for B6 MEFs, wildtype mCD248, “PDZ del.” and “all D” mCD248 remain within a small range. However, the ratios for “all A” and “all A + PDZ del.” are much greater than the other samples.
### 3.10 Murine CD248 as detected by immunofluorescence and confocal microscopy

#### 3.10.1 Surface staining of murine CD248

HEK 293 T and CHO cells were transiently transfected with cDNAs encoding wildtype and the different mutant forms of CD248 (Thr731Ala, Ser744Ala, Thr760Ala, Thr731Asp, Ser744Asp, Thr760Asp, PDZ del., “all A”, “all D”, “all A + PDZ del.”) with and without the FLAG-tag. The cells produced murine CD248 protein that was detectable using either the anti-CD248 antibody or anti-FLAG antibody or both antibodies (for the FLAG-tagged proteins), on the surface of the cell (data shown previously).

#### 3.10.2 Intracellular staining of murine CD248 in CHO cells stably expressing wildtype or mutant mCD248 cDNA

Wildtype murine CD248 trafficked to the cellular surface under normal conditions. If cells were not permeabilised during the staining process, only cell surface expression of CD248 was detected. However, by permeabilising cell membranes, it was possible to detect CD248 within the cell (Figure 3.13).

Under permeabilised conditions, wildtype murine CD248 is present throughout the cytoplasm of the cell and can also be found on the cell surface.
CHO cells stably expressing the wildtype form of mCD248 show a cellular morphology that is similar to non-transfected CHO cells or CHO cells expressing the empty vector pcDNA3.1+. Cells expressing the constitutively phosphorylated form of mCD248, “all D”, also exhibited mCD248 expression throughout the cytoplasm with similar cell morphology to the cells stably expressing the wildtype protein, although the “all D” cells appeared larger in size or more spread out.

Conversely, CHO cells stably expressing the “all A” and “PDZ del.” mutant forms of mCD248 exhibit different cell morphology. Firstly, the nuclei and the cells themselves are larger. The cell membranes are distorted and have many finger-like protrusions. Secondly, the pattern of murine CD248 staining is markedly different from that of the wildtype mCD248 stably expressing cells. The mutant forms of murine CD248 appear to accumulate within perinuclear organelles of the cytoplasm and there is not as strong staining at the cell surface. Separately, expression of the “all A” and “PDZ del.” cytoplasmic mutant forms in cells also results in morphologic changes in the cells and distorted patterns of localisation of the CD248.

Somewhat surprisingly, CHO cells stably expressing mCD248 “all A + PDZ del.” do not exhibit major alterations. Rather, they are more similar to the “wildtype” phenotype, with almost normal-sized cells and mCD248 expression that is equally spread through the cytoplasm, with distinct cell surface localisation. These findings suggest that there may be a double-negative effect
occurring via the cytoplasmic domain of CD248 in which one mutation may compensate for the other to achieve a more “normal” phenotype.

Figure 3.13: Immunofluorescence detection of CD248 in CHO cells stably expressing wildtype and mutant forms of murine CD248 protein

CHO cells stably expressing empty vector pcDNA3.1+, wildtype mCD248 or mutant mCD248 cDNA were grown on 10% poly-L-lysine coverslips. Cells were stained for murine CD248 (red) and DAPI (blue). Cells expressing mutant forms of mCD248 exhibit different cell morphology and cellular localisation of murine CD248 as compared to cells expressing wildtype mCD248. The “all A” and “PDZ del.” mCD248 mutants produced the most radical changes, followed by the “all D” mutants which are larger than cells expressing wildtype mCD248 (but otherwise phenotypically similar). The “all A + PDZ del.” mCD248 mutant is the least different. The white scale bar represents 10 um.
A side view of CHO cells stably expressing WT or mutant mCD248 protein shows that the cells expressing mutant forms of the protein exhibit increased spreading. This observation may account for the seemingly "larger" cells obtained when cells are expressing mutant CD248 proteins. Subsequent studies will need to be done to determine if the actual volume of cells expressing mutant CD248 is bigger or not.

3.10.3 Mutant murine CD248 localises to the endoplasmic reticulum

The pattern of expression of the mutant forms of CD248 was reminiscent of that for endoplasmic reticulum (ER). We attempted to confirm this by assessing whether CD248 colocalises to that organelle. Double-staining with SERCA2 ATPase, a marker for the ER, showed that the mutant murine CD248 proteins were indeed trapped in the ER (Figure 3.14), unlike wildtype murine CD248.
Figure 3.15: Permeabilised CHO cells stably expressing murine CD248 show localisation of mutant murine CD248 to the endoplasmic reticulum

Confocal images of CHO cells stably expressing mCD248 “all A” show abnormal cellular trafficking of murine CD248 (a). DAPI (blue) shows the nuclei of the cells. The white scale bar represents 10 μm. Colocalisation of murine CD248 (red, Ch3) and SERCA2 ATPase (green, Ch2) is unusual in that most of the mutant murine CD248 appears to be in the endoplasmic reticulum (b). Compared to WT murine CD248, in which CD248 antigen colocalisation occurs only to a small extent with SERCA2 ATPase (c), the differential localisation of mutant murine CD248 is interesting.

3.10.4 Actin stress fibers are present in CHO cells stably transfected with mutant mCD248

CHO cells stably transfected with the mutant forms of mCD248 (“all A”, “PDZ del.”, “all A + PDZ del.” and “all D”) are morphologically dissimilar from cells stably transfected with either the empty vector pcDNA3.1+ or wildtype mCD248 cDNA. In addition to being larger, CHO cells expressing mutant mCD248 have increased numbers of actin stress fibers and an increased evidence of focal adhesions (Figure 3.15).

Wildtype B6 MEFs were also stained in an effort to study the organisation of actin fibers and focal adhesions under conditions in which wildtype murine CD248 is constitutively expressed. As seen in Figure 3.15, there are no actin stress fibers and few focal adhesions as compared to cells expressing mutant forms of murine CD248.
Figure 3.16: Increased numbers of actin stress fibers and focal adhesions are present in CHO cells stably transfected with mutant mCD248

Stably transfected CHO cells were grown on 10% poly-L-lysine coverslips. The cells were stained for actin and focal adhesions using phalloidin (green) and anti-focal adhesion kinase (FAK) (red); DAPI (blue) identifies the nuclei. CHO cells stably transfected with mCD248 “all A”, “PDZ del.”, “all A + PDZ del.” and “all D” cDNA have a different cellular morphology from CHO cells stably transfected with empty vector pcDNA3.1+ and wildtype mCD248. Wildtype B6 MEFs were used to visualise the “native” state of actin and focal adhesions with constitutive CD248 expression. CHO cells expressing mutant mCD248 are larger, have increased numbers of actin stress fibers, and more focal adhesions. The white scale bar represents 10 um.
3.11 Activating MMP-9 and MMP-2 zymogens to use as standards in further MMP zymography

As described earlier, CD248 is known to regulate MMP expression. To evaluate the effects of the cytoplasmic domain and the specific mutant forms, we established a functional assay to measure MMP expression and activation. MMP-9 and MMP-2 zymogens were obtained from the lab of Dr. Christopher Overall. The zymogens were activated using 4-aminophenylmercuric acid (APMA). 10 ng of zymogen and activated protein were assessed by zymography (an SDS-PAGE acrylamide gel infused with 0.1% gelatin) (Figure 3.16). Activation of the MMP zymogens was evident by signal shifts from 71 kDa to 62 kDa for MMP-2, and 76.4 kDa to 68.1 kDa for MMP-9. The lowest faint band appearing in the active MMP-2 lane is likely due to a loss of the C-terminal hemopexin-like domain resulting in a shift of ~20 kDa.

Some MMPs such as MMP-9 and MMP-2 are auto-catalytic \(^{102}\). This explains the gelatin clearings observed in the lanes containing the zymogen forms MMP-9 and MMP-2. During the overnight incubation at 37 °C, a small amount of MMP is activated which in turn cleaves other MMPs. Since the gel has already been run, the proteins are confined to their position on the gel after electrophoresis. However, the now-active forms degrade the gelatin, causing the clearing seen at the supposed zymogen positions.
Figure 3.17: Activating MMPs 2 and 9 using APMA

MMP-2 and MMP-9 zymogens were activated with APMA. MMP-2 zymogen (71 kDa) and MMP-9 zymogen (76.4 kDa) are converted to activated MMP-2 (62 kDa) and activated MMP-9 (68.1 kDa). The lowest band in the activated MMP-2 lane (~45 kDa) is a result of the loss of the hemopexin-like C-terminal domain.
3.12 Effect of transient expression of WT mCD248 or mCD248 “all A + PDZ del.” by MCF-7 cells on MMP-9 production

MCF-7, a breast cancer cell line known to secrete MMP-9 upon stimulation \(^{103}\), was transiently transfected with cDNA encoding either WT mCD248 or mCD248 “all A + PDZ del.”. After transfection, the cells were allowed to recover for 24 hours and then were starved overnight. The next morning the cells were stimulated in 100 nM PMA in DMSO and allowed to rest again overnight. The final morning, the cells were counted to ensure equal numbers and the conditioned media was removed. Equal volumes of media were loaded for analysis by gelatin zymography.

We first determined that the addition of DMSO alone at maximal concentration used with PMA does not induce secretion of MMP-9 by MCF-7 cells. Previous work in the lab also established that under the conditions used, transfection of the cDNAs yielded similar amounts of total WT mCD248 and the mCD248 “all A + PDZ del.” protein in CHO cells. Therefore, any difference in MMP-9 secretion (Figure 3.17) between the mutant forms would likely be attributable to the mutation of the cytoplasmic domain of the protein.

MMP-9 zymogen secretion is markedly increased in MCF-7 cells transiently expressing WT mCD248 cDNA as compared to the cells after sham transfection. Not surprisingly, MMP-9 secretion is decreased in cells expressing
mCD248 “all A + PDZ del.”. However, MMP-9 secretion was still more than from the control sham-transfected MCF-7 cells. This finding is in accordance with previous data obtained by the Conway laboratory, in which the deletion of the cytoplasmic domain of murine CD248 causes a decrease in MMP-9 secretion by murine embryonic fibroblasts.

Interestingly, in spite of changes in total zymogen, generation of active MMP-9 does not change with any of the CD248 forms.
Figure 3.18: MCF-7 cells transiently expressing WT mCD248 cDNA secrete more pro-MMP-9 than with control sham-transfected cells or cells expressing mCD248 “all A + PDZ del.”

MCF-7 cells were seeded in equal numbers into 6-well tissue culture plates. The cells were then either sham transfected (lanes 3,4), or transfected with WT mCD248 (lanes 1,2) or mCD248 “all A + PDZ del.” (lanes 5,6). After treatment with 100 nm PMA was described in the text, cells were counted, and equal volumes of conditioned media were assessed for MMP-9 presence and activity by gelatin zymography. As compared to sham-transfected cells, WT mCD248 causes a greater increase in total MMP-9 than mCD248 “all A + PDZ del.”.

3.13 Effect of WT mCD248 and FLAG WT mCD248 expression in CHO cells on MMP-9 production

The dependence of CD248 on MMP-9 production prompted us to evaluate whether the FLAG-tag linked to CD248 had any effect. For this reason, we used CHO cells to compare the effects of transiently expressed WT mCD248 with FLAG WT mCD248 on MMP-9 secretion from CHO cells. Studies were
performed in duplicate as above, except that cells were not treated with PMA. Conditioned media was analysed by gelatin zymography, and cell lysates were evaluated by Western immunoblot for expression of CD248 and GAPDH (Figure 3.18).

We first confirmed that the quantity of CD248 is not affected by the presence of the FLAG-tag (Figure 3.18 a). Since CHO cells were not stimulated with PMA, MMP-9 is present only in its zymogen form. The amount of MMP-9 produced by cells transiently expressing either WT mCD248 or FLAG WT mCD248 was increased compared to that of the sham transfected CHO cells. Densitometry analyses indicated that MMP-9 increased by a factor 1.2 ± 0.1 in cells expressing WT mCD248 and by a factor of 1.4 ± 0.1 in cells expressing FLAG WT mCD248 (p=NS).

Overall, the lack of significant difference in MMP-9 activation using cells expressing either WT mCD248 or FLAG WT mCD248 supports the notion that the N-terminal FLAG-tag does not significantly affect the function of murine CD248, at least in this assay.
**Figure 3.19:** CHO cells expressing WT mCD248 and FLAG WT mCD248 induce similar increases in total MMP-9 secretion after 48 hours

CHO cells were grown in 6-well plates and transfected in duplicate with cDNAs encoding either WT mCD248 or FLAG WT mCD248 cDNA. Cells were starved for 48 hours prior to media collection and cell lysis. (a) Equal amounts of cell lysate protein were separated by SDS-PAGE and immunoblotting with antibodies against CD248, FLAG-tag, and GAPDH (loading control). (b)
30 μL of media was analysed by gelatin-zymography (left panel) and densitometry was performed using 3 independent experiments to generate the data shown in the right panel.

### 3.14 MMP-9 production in CHO cell lines stably expressing wildtype and cytoplasmic mutant forms of murine CD248

Based on immunoblots used to semi-quantify the amount of murine CD248 expressed by the CHO cell clones stably expressing wildtype and mutant forms of CD248, it was determined that the wildtype clone 12N, “all A” clone 9, “PDZ del.” clone 5 and “all A + PDZ del.” clone 1 expressed similar amounts of murine CD248. Following a 48 hour starvation period and confirmation that the numbers of cells were equal at this endpoint, these cell lines were assessed for baseline secretion of MMP-9 using gelatin zymography.

We recognised that stable transfection of the empty vector pcDNA3.1+ also causes an increase in MMP-9 production by CHO cells, compared to non-transfected CHO cells. This finding highlights the importance of appropriate controls, and must be accounted for in all studies. Nonetheless, in these pilot studies, MMP-9 secretion by CHO cells stably expressing mCD248 “all A + PDZ del.” is further decreased as compared to CHO cells stably expressing the empty vector, and wildtype CD248 causes an increase in secretion. Stable expression of mCD248 mutants “all A” and “PDZ del.” does not appear to cause a notable change in MMP-9 production, as compared to WT mCD248. Further studies are required to confirm these findings.
Figure 3.20: Stably expressed wildtype and mutant mCD248 cDNA causes a change in MMP-9 production by CHO cells

Equal volumes of conditioned media from CHO cells were assessed by gelatin zymography as described in the text. MMP-9 zymogen signals were subjected to densitometry. The results shown in this figure were gathered from three technical repeats, each in duplicate. The expression of wildtype murine CD248 causes a non-significant increase in MMP-9 secretion. Stable expression of mCD248 "all A + PDZ del." also causes a non-significant decrease in MMP-9 secretion compared to WT mCD248.
3.15 Creating an MMP-9 standard curve for absolute quantification

In order to quantitatively assess the amount of MMP-9 protein released by cells into conditioned media, it was necessary to determine at what point MMP-9 signals are linear on an MMP zymogram. MMP-9 zymogen was activated using APMA. A standard zymogram curve was generated by using active and pro-MMP-9 from 0.156 ng to 10 ng. From densitometry analyses, linearity was achieved in the range from 0.156 ng to 0.625 ng (Figure 3.20). Lanes containing more than 0.625 ng of protein plateaued in densitometry, i.e. the signal became saturated.

For future MMP-9 studies it will now be possible to perform absolute quantitation to determine the amount of MMP-9 released. Time did not allow us to apply this technology to the preceding studies in which we compared MMP-9 secretion from the different CD248-expressing cells. However, once repeated, we believe that our findings will be confirmed, as the observed MMP-9 signals by zymography appeared subjectively to be within the linear range.
Figure 3.21: Standard curves generated by zymography for pro-MMP-9 and active MMP-9

MMP-9 was activated using APMA and known amounts of the pro-MMP-9 and active MMP-9 protein were separated and visualised by gelatin zymography. Densitometry of signal revealed linearity in the range of 0.156 ng and 0.625 ng. This curve, when run on the same zymogram as conditioned cell media, may be used to quantify the amount of pro- or active MMP-9.
3.16 Finding binding partners for murine CD248

Preliminary studies performed in Dr. Conway’s lab using the MAPPIT (mammalian protein-protein interaction trap) technology had putatively identified TOLLIP (Toll-interacting protein) as a possible partner for the cytoplasmic domain of CD248. TOLLIP is a negative regulator of NFκB and Toll signaling during the inflammatory response \(^{104}\). As a loss of CD248 or CD248’s cytoplasmic domain leads to a decrease in inflammation \(^{12}\), it was postulated that CD248 sequesters negative regulators of inflammatory pathways thereby facilitating inflammation.

We sought to identify intracellular interacting proteins for CD248. Several approaches were considered. For this thesis, we focused on using the entire CD248 as bait to pull out interacting proteins from cell lysate. Co-immunoprecipitation of FLAG-tagged wildtype murine CD248 was followed by mass spectrometry to identify the putative CD248 interacting proteins. To achieve this end, M2 anti-FLAG monoclonal antibody was covalently bound to Dynabead(® epoxy. HEK cell lysates expressing either WT mCD248 or FLAG WT mCD248 were incubated with the beads. Only the FLAG WT mCD248 protein was expected to bind to the immobilised antibodies, and thus the pulled-down CD248 binding proteins would be restricted to this immunoprecipitate. The signal obtained using lysates from cells expressing WT mCD248 would provide a background control for the immunoprecipitation.
Pulled down proteins were eluted and the samples were separated by SDS-PAGE. Gels were either further processed for immunoblotting with anti-CD248 polyclonal antibodies or M2 anti-FLAG antibodies (Figure 3.21) or stained with Coomassie to visualise all the proteins contained in the eluents (Figure 3.22 a). As expected, FLAG WT murine CD248 protein is pulled-down by the M2 anti-FLAG-Dynabead® epoxy (Figure 3.23), whereas the WT murine CD248 protein is not pulled down, *i.e.* it is not detectable by either immunoblot or mass spectrometry. The Coomassie stained gels were subjected to in-gel tryptic digestion and cut into bands as shown in Figure 3.22 b. The proteins obtained from the gel pieces were analysed by mass spectrometry, which identified several proteins that may be potential interacting partners with murine CD248 (Table 3.2).
Figure 3.22: Immunoblots of immunoprecipitations using M2 anti-FLAG-Dynabead® epoxy and HEK cell lysates

HEK cells were grown in 10 cm plates and transfected with cDNAs encoding either WT mCD248 or FLAG WT mCD248. After 48 hours, the cells were lysed and immunoprecipitated as described in the text. Pulled-down proteins were immunoblotted to assess for the presence of FLAG WT murine CD248 and for WT murine CD248. Murine CD248 is pulled-down only when the HEK cells express FLAG WT mCD248.
Figure 3.23: Coomassie-stained gel of immunoprecipitation reactions using M2 anti-FLAG-Dynabead® epoxy and HEK cell lysates

a) Pulled-down proteins after immunoprecipitation reactions with HEK cell lysates containing either WT murine CD248 or FLAG WT murine CD248 were separated by SDS-PAGE and then Coomassie-stained. Murine CD248 is “pulled-down” when the HEK cell lysate contains FLAG WT mCD248, however the protein is not present when the HEK cell lysate contained WT mCD248.

b) Coomassie-stained pull-downs after immunoprecipitation were cut into 10 matching gel bands that were subjected to in-gel tryptic digestion. Due to the stringent nature of the elution buffer, some covalently bound antibody was also removed from the beads. Sample 8 contained the FLAG WT mCD248; sample 6 contained the 50 kDa M2 anti-FLAG heavy chains; and sample 2 contained the 25 kDa anti-FLAG light chains.
Figure 3.24: Sample mass spectra of murine CD248 collected from the in-gel tryptic digestion samples

Murine CD248 was identified by mass spectrometry with a number of peptides, including E-L-G-G-N-L-A-T-P-R, in the HEK cell immunoprecipitation samples from lysates transfected with FLAG WT mCD248 cDNA. Murine CD248 peptides were not present in HEK cell immunoprecipitation samples from lysates transfected with WT mCD248 cDNA.
Table 3.2: Proteins identified to interact with murine CD248

Mass spectrometry identified several potential binding partners for murine CD248. These partners will be further studied to confirm their binding and to assess the pathophysiologic relevance. Only those with the highest scores and that were found in both of 2 independent experiments are shown.

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<td>Plakophilin-3</td>
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4. Discussion

4.1 Identification and characterisation of anti-CD248 antibodies

In attempting to find sensitive and specific anti-CD248 antibodies, we net several challenges. First, most of the commercial anti-CD248 antibodies (ProSci Inc., Genesis Biotech Inc., Santa Cruz Biotechnology, Sigma, Novus Biologicals, Abcam, Millipore, and those from ProteinTech Group with the exception of 18160-1-AP) and anti-CD248 peptide antibodies generated under license by Dr. Conway and others developed by laboratories (Aves Lab Inc., the Macfadyen Laboratory, and the Isacke Laboratory), were, on testing with immunoblot, non-specific.

CD248 is a heavily glycosylated protein due to its sialomucin-like extracellular domain. Glycosylation often proves to be problematic when performing immunoblot after SDS-PAGE, as the apparent molecular weight(s) often appears “smeared” on gel, and/or different from the native molecular weight based on the amino acid composition. Moreover, depending on the cellular source, CD248 may have different glycosylation patterns and thus different apparent molecular weights. Previous reports have shown CD248 with apparent molecular weights ranging from ~50 kDa to ~250 kDa. In our studies with the ProteinTech Group rabbit polyclonal anti-CD248 (18160-1-AP), the most prominent band for murine CD248 under reducing conditions appears at 75 kDa,
but also with signals at 100 kDa, 150 kDa and 50 kDa when the cell lysate concentration is increased.

As multiple bands are immuno-detected on Western blot by the CD248 antibody, we were concerned that this antibody was also non-specifically binding to other proteins. Using CD248 antibody that was “blocked” with its antigen, we showed that we confirmed the specificity of the antibody, i.e. that the immuno-detected bands all represent murine CD248.

The band that appears at the apparent molecular weight of 75 kDa is likely the unglycosylated form of murine CD248, with the higher molecular weight bands corresponding to differentially glycosylated forms of the protein. We hypothesise the unglycosylated form of murine CD248 is detected by the antibody more readily because the antibody was raised against an unglycosylated ectodomain of CD248. We cannot comment on the difference in function based on glycosylation, but undoubtedly this is important.

This antibody was fortunately also capable of specifically identifying CD248 in immunofluorescence studies of cells, allowing detection on the cell surface and intracellularly. The 18160-1-AP antibody, in conjunction with Dynabead® Protein G epoxy (Invitrogen, Oslo, Norway), was also used successfully to immunoprecipitate murine CD248 in both transfected HEK 293 T
cell lysates as well as cell lysates that normally express CD248 (B6 MEFs and
10T1) (data not shown).

The 18160-1-AP anti-CD248 antibody was generated using the ectodomain of human CD248 from amino acids 21 to 401. The antibody cross-reacts with murine CD248. It was important for this project that the cytoplasmic mutants of murine CD248 protein could be immuno-detected in some manner, either directly with a CD248 antibody or via a FLAG-tag approach. For this reason, and prior to completing the validation of the 18160-1-AP anti-CD248 antibody, we created cDNA constructs encoding wildtype and mutant forms of CD248 with an N-terminal FLAG tag. Fortunately all of the recombinant cytoplasmic mutant forms of CD248, with and without the N-terminal FLAG-tag, could be immuno-detected with the anti-CD248 antibody. Nonetheless, the FLAG-tag has proven to be a valuable tool for identification of interacting proteins (see below).

To expand our capabilities, the lab has undertaken the production of a soluble, full-length, extracellular domain of human CD248, tagged with 6x His using both mammalian cells and *Pischia pastoris* expression systems. The recombinant protein will be used for various purposes, such as producing our own antibodies (monoclonal and polyclonal) that may better detect the different glycosylated forms of CD248, or for crystallization of the ectodomain, or perhaps to create our own ELISA for quantifying CD248.
4.2 Characterisation of the putative signaling properties of the cytoplasmic domain of CD248

The cytoplasmic mutant mCD248 constructs were generated from an existing cDNA vector, pcDNA3.1+ encoding wildtype murine CD248. As the putative phosphorylation sites and PDZ-binding motif of CD248’s cytoplasmic domain are highly conserved across species, these sites were mutated. The putative phosphorylation sites of Thr731, Ser744 and Thr760 were mutated to alanine to prevent phosphorylation and to aspartate to mimic constitutive phosphorylation. The model of aspartate mimicking a state of constant phosphorylation has been used with success to determine the phosphorylation states and activation in a number of proteins including the c-kit receptor tyrosine kinase, dual specificity mitogen-activated protein kinase kinase 1 (MAP2K1), and IκBα (IκBα). The PDZ-binding motif was also deleted in hopes of abolishing CD248’s ability to bind some cytoplasmic proteins of potential importance. These mutations were performed in several combinations including an all alanine, an all aspartate, an all alanine without the PDZ-binding motif, an all aspartate without the PDZ-binding motif, and both threonines to alanines variants.

An assumption made during these steps of the project was that CD248, at least under some conditions, is indeed phosphorylated, and that this occurs at one or more of the three putative sites in the cytoplasmic domain. In spite of attempting to do so, we have yet to confirm that this is the case. Identification of
the phosphorylation potential of CD248 and what extracellular stimuli are responsible is key in characterising relevant intracellular signaling pathways. The creation of the above mutant mCD248 constructs was an important step to determine CD248’s phosphorylation potential. Co-immunoprecipitation reactions have been performed using phosphoserine/threonine antibodies covalently bound to Dynabead® epoxy in hopes of pulling-down phosphorylated murine CD248 from PMA-stimulated WT mCD248 transfected cell lysates. However, the results from these experiments have not yet been definitive in demonstrating phosphorylation of CD248. Future studies will include a series of further co-immunoprecipitations using cell lysates that natively express murine CD248 such as wildtype B6 MEFs or 10T1 fibroblasts that would be compared to lysates that do not express the murine CD248 such as HUVECs or B6 MEFs isolated from CD248 knockout mice. Similarly, B6 MEFs obtained from mice that lack the cytoplasmic domain (available in our lab) may also be used as a negative control.

Another approach to determine the phosphorylation potential of murine CD248 would use the technology of phosphoproteomics. Following stimulation with factors considered likely to induce phosphorylation, the cells are lysed, the proteins are separated using ion exchange chromatography or SDS-PAGE, and the separated proteins are digested into peptides. Titanium oxide beads (TiO₂) or immobilised metal ion affinity chromatography (IMAC) is used to enrich for
phosphopeptides which are then run on a mass spectrometer for identification 108; 109; 110.

Imbalances in protein phosphorylation, *i.e.* hypo- or hyper-phosphorylation of proteins, may result in functional changes that may manifest as changes in structure or function of a cell. However, as we have not yet confirmed that the cytoplasmic domain of CD248 undergoes phosphorylation under any conditions, it is possible that some or all of our observations made during this thesis may be due to non-phosphorylation-dependent structural changes in the cytoplasmic domain of CD248. For instance, as noted below, stable expression of mCD248 “all A” and “all D” mutant proteins yielded similar phenotypes. The phosphorylation states of these mutant CD248s should be completely “on” or “off” respectively, and one would hypothesise that dissimilar phenotypes would be observed. These findings suggest that other cytoplasmic motifs contained in CD248 may be important.

**4.3 The cytoplasmic domain of CD248: a role in cellular senescence?**

Transient expression systems are more likely to be confounded by inter-experimental variability due to, for example, differences in transfection-translation efficiency and quality of cDNA. We therefore generated CHO cell lines that stably express the wildtype and mutant forms of murine CD248. Rather than express all of the > 20 mutant forms at this early stage of study, stable lines
were made that express wildtype, “all A”, “PDZ del.”, “all A + PDZ del.”, and “all D” mCD248.

In considering which cells to use for stably expressing the mutant forms of CD248, we would have preferred to use those that are physiologically relevant, such as mesenchymal cells, stromal cells, fibroblasts or pericytes/smooth muscle cells. Ideally they would not express CD248, i.e. be derived from the CD248\(^{\text{KO/KO}}\) mice, or be knocked down with siRNA. At the time of these studies, the CD248\(^{\text{KO/KO}}\) mice for deriving MEFs were not available, and attempts to knock-down CD248 in wildtype MEFS were not reliably working. Therefore, CHO cells were used as they are easily transfected and express almost undetectable amounts of CD248. Moreover, they express high amounts of MMP-9, which was considered valuable for functionally assessing the role of the mutant forms of CD248 when introduced into these cells.

The most striking feature of the wildtype and mutant murine CD248 stably transfected CHO cells was their vastly different morphology. CHO cells that were stably transfected with the empty vector, and those that expressed wildtype mCD248 and mCD248 “all D” exhibited similar morphologies to non-transfected CHO cells. On the other hand, CHO cells stably expressing mCD248 “all A”, mCD248 “PDZ del.”, and somewhat less so, mCD248 “all A + PDZ del.” exhibited dramatic changes in morphology. These cells appeared larger or more spread out, and were spindle-shaped with a distorted cellular membrane with
many protrusions. At this moment, it is unclear if the cells expressing mutant mCD248 are truly larger in physical size (bigger volume), or if the apparent change in cell size is due to increased spreading of the cells (same volume but increased surface area). If CD248 is involved in cell migration during diseases such as cancer and inflammation, perhaps mutations in CD248 cause alterations in expression or function of proteins involved in cell adhesion, thereby causing phenotypic changes such as enhanced spreading. This notion that mutations in CD248’s cytoplasmic tail modulate cellular spreading, is supported by the number of important cytoskeletal proteins, found to interact with CD248, that were identified in the immunoprecipitation experiments (discussed in the next section). Further studies, using advanced confocal analysis and/or flow cytometry, will be necessary to assess changes in the volume of cells expressing the different mutant forms of CD248.

CD248 appeared to become trapped in the cytoplasm of the “all A” and “PDZ del.” CHO cells, although it still trafficked to the cell surface. The synthetic pathways of proteins are complex and delicately regulated. After transcription, mRNA is transported from the nucleus to the cytoplasm where translation occurs either by free ribosomes or ribosomes attached to the rough endoplasmic reticulum. At the endoplasmic reticulum, newly synthesized proteins are properly folded and undergo post-translational modifications including the formation of disulfide bonds, addition of oligosaccharide chains (glycosylation), and insertion into the intracellular membrane (important for transmembrane proteins). The
synthesized proteins are then transported to the Golgi apparatus where they undergo further post-translational modifications including glycosylation and phosphorylation. Once the protein is fully mature, it is trafficked to its proper place within the cell, i.e. secreted, transported to proper organelle, left in the cytoplasm or inserted into the cell membrane. It is reasonable to expect that mutations that alter the native structure of a protein may result in alterations at any step in the synthetic and trafficking pathway.\textsuperscript{111, 112}

In the case of murine CD248, preliminary evidence suggests that “all A” and “PDZ del.” mutant forms of CD248 localised primarily in the ER, with reduced trafficking to the cell surface as compared to WT CD248. This observation may indeed be due to defects in the protein synthesis pathway in which mutant, “malformed” murine CD248 protein becomes trapped and accumulates within the endoplasmic reticulum. The mutant proteins likely do not undergo normal post-translational changes (folding, glycosylation, phosphorylation \textit{etc.}) and are therefore not normally trafficked to the cell surface.

Further study revealed in particular that the cells expressing mCD248 “all A”, “PDZ del.”, and “all A + PDZ del.” exhibited a striking increase in actin stress fibers. Although further studies are required, altogether, these changes are suggestive of cellular senescence. Cellular senescence is defined as a stage in which healthy diploid cells lose their ability to undergo mitosis and divide. It is thought that cellular senescence evolved as a way for cells to evade and prevent
the onset of cancer, *i.e.* cells that accumulate too much DNA damage stop undergoing mitosis \cite{113; 114; 115}. This model of cellular senescence in which cells are “protected” from undergoing transformation to tumour phenotype is in line with what was observed with the CD248KO/KO mice and the mice lacking the cytoplasmic domain of CD248. These mice were resistant to the development of cancer, suggesting that an intact cytoplasmic domain of CD248 is necessary for tumour growth and that abrogating its function may promote senescence \cite{22; 67}. That the putative sites for phosphorylation and the PDZ-binding motif might play a role in cellular senescence is intriguing and may be used as a site for future therapeutic intervention.

More studies will be required, however, to determine if CD248 via the cytoplasmic domain does in fact regulate cellular senescence. The actin stress fibers observed in each mutant cell line will have to be quantified, cell size should be measured, colocalisation studies must be repeated and optimised using a better marker, and cell cycle analyses must be performed. If the finding are confirmed, we would try to identify which of the putative phosphorylation sites is/are responsible by using cells that express CD248 with individual mutations, rather than mCD248 “all A” that has so far been used. We have in fact, generate, selected and sorted “Thr731Ala”, “Ser744Ala” and “Thr760Ala” mCD248 expressing CHO cells as single clones into 96 well plates for stable expression.
4.4 Identification of CD248-interacting proteins

The preceding findings underline the importance of the integrity of the cytoplasmic domain of CD248 and strongly suggest that it interacts with intracellular partners that regulate cellular function. To identify interacting partners of murine CD248, we couple co-immunoprecipitation experiments with mass spectrometry analyses. For the immunoprecipitation experiments, both CHO cells and HEK 293 T cells were initially used. However, we ultimately chose HEK cells, as the co-immunoprecipitations yielded less background and non-specific signal.

Subsequent to immunoprecipitation, the protein samples for mass spectrometry must be prepared either by in-gel digestion or by in-solution digestion. In-solution digestion is generally restricted for samples that are known to contain adequate amounts of detectable protein. Once we were able to reduce the non-specific signal in the eluent, and the protein concentration was high enough in the co-immunoprecipitation experiments, we tried in-solution tryptic digestion once. By this approach, however, we could not readily confirm that we could readily pull-down CD248, which was the natural in-sample positive control. We believe that this is due to the fact that CD248 is a transmembrane glycoprotein that is relatively insoluble for the in-solution digestion approach. For this reason, subsequent experiments were performed using the in-gel tryptic digestion technique, which was always successful at revealing the expected in-sample CD248 control.
Two separate experiments analysed by mass spectrometry revealed 12 putative binding-partners to murine CD248. Interestingly, several of the protein hits lay important roles in cytoskeletal organisation. For example, ensconsin (microtubule-associated protein 7), the second highest scoring protein, is involved in the stabilisation of the mitotic spindle during mitosis \(1^{16}; 1^{17}\). Other cytoskeletal proteins found include plakophilin-3 (a protein that plays a role in desmosome adhesion and links cadherins to intermediate filaments) \(1^{18}; 1^{19}\), cadherin-2 (a protein thought to play a role in transendothelial migration of cancerous cells) \(1^{20}; 1^{21}\), and flotillin-2 (a highly conserved protein found in lipid raft domains where it mediates signaling between growth factor receptors and intracellular transduction pathways) \(1^{22}; 1^{23}\). Interactions with these cytoskeletal proteins may prove to be important for the function of CD248. For example, perhaps the mCD248 “all A” does not bind properly to ensconsin, thereby causing a defect in the mitotic spindle, resulting in cells that cannot divide properly and yielding a cellular senescence phenotype.

The protein identified with the highest score as an interacting partner for murine CD248 was heat shock 70 kDa protein 7 (HSP 70). Heat shock proteins are a family of proteins \(1^{24}; 1^{25}; 1^{26}\) involved in the cellular stress response \(1^{27}\), act as intracellular chaperones, prevent protein aggregation, transport proteins across cell membranes, perform housekeeping duties by transporting old/damaged proteins to the proteasome \(1^{28}; 1^{29}\), and play roles in cardiovascular disease progression \(1^{30}\). HSP 70 is especially interesting as it may bind antigens,
present these peptides to the immune system and thereby increase antigen-specific T cell activation. HSP 70 is being tested as an immunologic adjuvant that could increase the potency of vaccines for certain types of cancer. Perhaps HSP 70 is a potential chaperone for murine CD248 formation.

Another heat shock protein, heat shock protein 90 β2, one of the most abundant proteins in eukaryotic cells, was also pulled down by CD248. HSP 90 β2 is known for its role in vascular relaxation as well as its ability to stabilise a number of proteins involved in tumour growth, such as phosphatidylinositol-3 kinases (PI3K), protein kinase B (AKT), proto-oncogene tyrosine-protein kinase Src (v-Src), Bcr/Abl, and mutant forms of p53; HSP 90 β2 also participates in angiogenesis by inducing vascular endothelial growth factor (VEGF) and nitric oxide synthase (NOS) pathways, and by promoting metastasis by aiding matrix metalloproteinases such as MMP-2. If the expression of CD248 does enhance tumourigenesis and metastasis, perhaps it is working in conjunction with HSP 90 β2.

In addition to interactions with cytoskeletal proteins and heat shock proteins, murine CD248 was also found to interact with a G-protein, specifically guanine nucleotide-binding protein G(t) subunit α-3. It is known that the class II PDZ-motif on the C-terminus of CD248’s family member CD93 (amino acid sequence GTDC), binds to GIPC1, a PDZ-domain containing adaptor protein that allows further interactions with other G proteins to help modulate leukocyte
migration and phagocytosis. Murine CD248 contains a class I PDZ-motif (amino acid sequence TSV), which is different from CD93. The PDZ-domains of interacting proteins bind specifically to their complementary C-terminus PDZ-motifs on transmembrane proteins. However, this specificity does not preclude the possibility that CD248 and CD93 may have similar intracellular functions and therefore interact with similar classes of proteins through their respective C-terminus PDZ-motifs.

Further studies are required to confirm these interactions and to validate their relevance in cell biological systems. As a start, one could use a “reverse immunoprecipitation” approach, in which the putative binding partner is used to pull-down CD248. Immunofluorescence studies could be used to determine whether the putative binding partners colocalises with CD248. If they do interact, we would then determine the relevant structure of CD248, i.e. the cytoplasmic domain, and the contribution of the sites for phosphorylation and/or the PDZ-binding motif. Subsequent siRNA knockdowns of the putative interacting proteins could be performed to assess the phenotype and to compare it with the findings seen with the mutant forms of CD248, as described in this thesis.

If the preceding studies are confirmatory, addition physical-biochemical approaches could be used to assess the binding properties of the CD248-interactin proteins. Surface plasmon resonance (SPR), which depends on the availability of sufficient purified proteins, relies on the binding of two proteins one
immobilised to a resin and the other flowing in solution on top of the resin \(^{140}\). SPR would allow binding constants to be determined, and sets the stage for analyzing the effects of mutation and other interacting proteins.

4.5 Functional consequences of CD248 mutations: effects on MMP-9 secretion

As mentioned, several studies could be performed to assess the functional effects of the CD248 cytoplasmic domain mutations. We focused on evaluating MMP-9 secretion. MMPs are known for their abilities to degrade and remodel the extracellular matrix, allowing processes such as the growth and metastasis of tumours, as well as the further development of inflammatory lesions \(^{102; 141; 142}\). Previous studies \(^{67; 84}\), and present studies performed in the Conway laboratory, indicate that MMP-9 secretion, by a variety of cells, is moderately increased upon the introduction of wildtype CD248. However, in cells expressing CD248 lacking the cytoplasmic domain, MMP-9 secretion is decreased compared to cells expressing wildtype CD248 \(^{67}\).

CHO cells stably expressing mCD248 “all A + PDZ del.” and MCF-7 cells transiently expressing mCD248 “all A + PDZ del.” showed a decrease in total MMP-9 secretion, as compared to cells expressing wildtype mCD248. These findings, albeit only preliminary and not statistically significant, support a role for
the cytoplasmic domain in regulating key intracellular functions that play a role in inflammation and cancer.

![Diagram of cell size and morphology changes](image)

**Figure 4.1: Summary of the cellular changes observed with the stable expression of WT or mutant mCD248 protein**

Stable expression of WT or mutant mCD248 proteins in CHO cells resulted in changes in cell size, cell morphology, antigen localisation and non-significant changes in MMP-9 secretion in CHO cells. Cell size was determined based on the diameter the cells at their widest point.

CD248 via its cytoplasmic domain has also been reported to have other effects, such as modulating platelet-derived growth factor-BB (PDGF-BB) mediated cell migration, secretion of vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) \(^{10; 75; 84}\). Specifically, B6 MEFs lacking the
cytoplasmic domain of CD248 do not migrate as efficiently as wildtype B6 MEFs.

It will be interesting to see whether CHO cells expressing mCD248 “all A + PDZ del.” have a similar defect. Matrigel migration assays, as used for this sort of study, have been used with great success to map the transformation of human mammary epithelial cells into cells exhibiting properties of secretory breast cancers. These functional assays may help elucidate the role of CD248 in cancer progression and inflammation.
5. **Conclusion**

CD248 plays an important physiologic role in disease progression as it pertains to cancer and a range of inflammatory diseases. Increasing evidence supports the notion that the integrity of the cytoplasmic domain of CD248 is central to its overall function, and that this domain mediates critical intracellular signals by interacting with cytoplasmic protein partners. By focusing on the putative phosphorylation sites and PDZ-binding motif, which are conserved within the cytoplasmic domain of CD248, we have established that specific mutations therein do induce dramatic morphologic and functional cellular changes. These changes are reminiscent of those that may occur during cellular senescence, a process that is increasingly recognised as contributing to cancer, inflammation, aging and many other diseases. Our findings also strongly indicate that structures within the cytoplasmic domain of CD248 likely directly interact with intracellular proteins that play key roles in cell division, motility, and migration.

It is important that investigators continue to study CD248-dependent intracellular signaling pathways, as they are likely to reveal novel targets for therapeutic intervention for a range of inflammatory and malignant diseases.
References


**Appendix I – Experimental methods used with cells transiently and stably expressing WT or mutant mCD248**

<table>
<thead>
<tr>
<th>Clones</th>
<th>Transient Trans.</th>
<th>Stable Trans.</th>
<th>mCD248 Immunofluorescence (Cell Surface)</th>
<th>mCD248 Immunofluorescence (ER)</th>
<th>mCD248 Flow Cytometry (Cell Surface)</th>
<th>Immuno-blot for mCD248</th>
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Note: Bracketed numbers indicate the number of clones used for each experiment.
**Appendix II – Summary of changes observed using cells transiently or stably expressing WT or mutant mCD248**

<table>
<thead>
<tr>
<th>Clones</th>
<th>Transient Trans.</th>
<th>Stable Trans.</th>
<th>Cell Morphology</th>
<th>Cell Size</th>
<th>Increased Actin Stress Fibers and Focal Adhesions</th>
<th>MMP-9 Secretion</th>
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<td>Normal CHO-like (1)</td>
<td>~ 10 um (1)</td>
<td>No</td>
<td>Baseline (1)</td>
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<tr>
<td>WT</td>
<td>X (5)</td>
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<td>Normal CHO-like (3)</td>
<td>~ 10 um (3)</td>
<td>No (2)</td>
<td>Increased compared to pcDNA3.1* (not sig.) (Clone 12N)</td>
</tr>
<tr>
<td>Thr731 Ala</td>
<td>X</td>
<td></td>
<td>Normal CHO-like</td>
<td>~ 10 um</td>
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</tr>
<tr>
<td>Ser744 Ala</td>
<td>X</td>
<td></td>
<td>Normal CHO-like</td>
<td>~ 10 um</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thr760 Ala</td>
<td>X</td>
<td></td>
<td>Normal CHO-like</td>
<td>~ 10 um</td>
<td></td>
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</tr>
<tr>
<td>Thr731 Asp</td>
<td>X</td>
<td></td>
<td>Normal CHO-like</td>
<td>~ 10 um</td>
<td></td>
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</tr>
<tr>
<td>Ser744 Asp</td>
<td>X</td>
<td></td>
<td>Normal CHO-like</td>
<td>~ 10 um</td>
<td></td>
<td></td>
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<tr>
<td>Thr760 Asp</td>
<td>X</td>
<td></td>
<td>Normal CHO-like</td>
<td>~ 10 um</td>
<td></td>
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<tr>
<td>“all A”</td>
<td>X (10)</td>
<td></td>
<td>Spreading (5)</td>
<td>&gt; 10 um (5)</td>
<td>Yes (2)</td>
<td>Increased compared to pcDNA3.1* (not sig.) (Clone 9)</td>
</tr>
<tr>
<td>Clones</td>
<td>Transient Trans.</td>
<td>Stable Trans.</td>
<td>Cell Morphology</td>
<td>Cell Size</td>
<td>Increased Actin Stress Fibers and Focal Adhesions</td>
<td>MMP-9 Secretion</td>
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<tr>
<td>“PDZ del.”</td>
<td>X (5)</td>
<td></td>
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<td>Increased compared to pcDNA3.1+ (not sig.) (Clone 5)</td>
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<td>~ 10 um</td>
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</tr>
<tr>
<td>FLAG Thr760 Ala</td>
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<td>~ 10 um</td>
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<td>FLAG Thr731 Asp</td>
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<td>Normal CHO-like</td>
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<td>X</td>
<td></td>
<td>Normal CHO-like</td>
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<td>Clones</td>
<td>Transient Trans.</td>
<td>Stable Trans.</td>
<td>Cell Morphology</td>
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<td>MMP-9 Secretion</td>
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<tr>
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<td>X</td>
<td></td>
<td>Normal CHO-like</td>
<td>~ 10 um</td>
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<td>Normal CHO-like</td>
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<tr>
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<td>Normal CHO-like</td>
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Note: Bracketed numbers indicate the number of clones used for each observation.