CALPAIN-1 AND CALPAIN-2 PROMOTE HER2/NEU TUMORIGENESIS AND INDEPENDENTLY PROMOTE METASTASIS IN TRIPLE NEGATIVE BREAST CANCER

by

James Alastair MacLeod

A thesis submitted to the Department of Pathology & Molecular Medicine

In conformity with the requirements for

the degree of Doctor of Philosophy

Queen's University

Kingston, Ontario, Canada

(August, 2018)

Abstract

Calpains are a family of calcium activated cysteine proteases. Calpain-1 and calpain-2 are obligate heterodimers composed of isoform specific catalytic subunits (encoded by *capn1* and *capn2*, respectively) coupled with the common, shared regulatory subunit calpain small subunit 1 (encoded by *capns1*). Here, we report a role for these calpains in promoting spontaneous tumor onset in a model of human HER2+ breast cancer, as well as a requirement for these calpains for tumor viability in an orthotopic engraftment model. Furthermore, we demonstrated that knockout of *capns1* correlated with enhanced sensitivity to the chemotherapeutic doxorubicin, the EGFR/HER2 inhibitor lapatinib, and sustained MAPK signaling in response to EGF.

In a model of triple-negative breast cancer we demonstrate that CRISPR/CAS9-mediated knockout of calpain-1 (*capn1*) or calpain-2 (*capn2*) independently attenuated metastatic colonization *in vivo*, and that knockout of either isoform was associated with attenuated production of MMP-9.

Collectively, our results suggest a pro-tumorigenic role for calpain-1/2 in both HER2+ and triple-negative breast cancer, which provides preclinical evidence in support of combining calpain inhibitors with commonly used therapeutic drugs.

Co-Authorship

The co-authors of this thesis are as follows: James A. MacLeod and Peter A. Greer.

Chapter 2: This manuscript has been accepted by *Oncotarget* pending minor revisions. The authors are James A. MacLeod, Yan Gao, Christine Hall, William J. Muller, Taran S. Gujral and Peter A. Greer. I performed all experiments in this study, with the exception of the reverse-phase protein array, which was performed by Taran S. Gujral. Technical assistance was provided by Yan Gao and Christine Hall, and the NIC transgenic mouse line was provided by William J. Muller. I drafted the manuscript, which was edited by Peter A. Greer.

Chapter 3: This manuscript is in preparation for submission. The authors are James A. MacLeod, Yan Gao, Christine Hall and Peter A. Greer. I performed all the experiments in this study, with technical assistance from Yan Gao and Christine Hall. I prepared the manuscript, with editing by Peter A. Greer.

Appendix 1: This review is pending submission. I prepared the review, with editing by Peter A. Greer.

Acknowledgements

I would like to first thank my supervisor Peter Greer for guiding throughout my studies, as well as members of my supervisory committee, Bruce Elliott and Waheed Sangrar, for their support and feedback over the years. I would also like to thank the Terry Fox Foundation Queen's Transdisciplinary Training Program in Cancer Research in partnership with the Canadian Institute of Health Research, the Robert Kisilevsky Fund for Research Education at Queen's, the Ontario Graduate Scholarship Program, and Queen's Graduate Award for funding.

I would also like to extend my thanks to Christine Hall, Yan Gao, Changnian Shi, Stacy Grieve, Victoria Hoskin, Christian McCartney, Stephanie Guy, Maximilian Niit, Kevin Ren, Matthew Gordon, and Jeff Mewburn.

Thank you to Joshua Powles for your humorous comments over the years. A special thank you to Theresa Cowan for your love and support, and the Cowan family for putting up with my extensive monopolization of their kitchen table.

Finally, and most importantly, I would like to thank my parents and family for their love and support. This would not have been possible without you.

Table of Contents

Abstract ii
Co-Authorship iii
Acknowledgements iv
List of Figures viii
List of Tables ix
List of Abbreviations x
Chapter One General Introduction 1
1.1 Breast Cancer Remains a Health Crisis 1
1.2 Classification and Treatment 1
1.3 The Calpain Family of Proteases 4
1.3.1 An Overview of Calpains 4
1.3.2 Expression Patterns in Cancer 8
1.4 Calpain in Cancer Pathogenesis 9
1.4.1 Calpain in Oncogenic Signaling and the Cell Cycle 9
1.4.2 Calpain in Apoptosis 11
1.4.3 Calpain in Invasion and Migration 13
1.5 Hypothesis and Aims 18
Chapter 2 Genetic disruption of calpain-1 and calpain-2 blocks tumorigenesis in mouse models of HER2+ breast cancer and sensitizes cancer cells to doxorubicin and lapatinib 20
2.1 Abstract 20
2.2 Introduction 21
2.3 Materials and Methods 22
2.3.1 Spontaneous Tumor Development and Reverse-Phase Protein Array Analysis 22
2.3.2 Cell Line Derivation 23
2.3.3 Migration, Invasion, Chemosensitivity, Hypoxic Response, and EGF Signaling Analysis 24
2.3.4 Matrix Metalloprotinease Assay 26
2.3.5 Orthotopic Tumor Growth and Experimental Metastasis Studies 26
2.3.6 Statistical Analysis 27
2.4 Results 27
2.4.1 Deletion of *capns1* in the mammary epithelium delays *her2/neu*-induced tumorigenesis 27
2.4.2 Establishment of a conditional *capns1* knockout *her2/neu*-driven mammary tumor epithelial cell line 32

2.4.3 *Capns1* deletion attenuates *in vitro* MTEC invasion but does not compromise migratory ability 34
2.4.4 *Capns1* KO sensitizes MTECs to doxorubicin and lapatinib challenge 36
2.4.5 Deletion of *capns1* blocks MTEC tumorigenesis in an orthotopic engraftment model 39
2.4.6 *Capns1* KO in MTECs correlates with enhanced EGFR expression and sustained EGF response 48
2.5 Discussion 50
Chapter 3 Calpain-1 and calpain-2 play non-redundant roles in tumor growth and metastasis in a model of triple-negative breast cancer 56
3.1 Abstract 56
3.2 Introduction 57
3.3 Methods 61
3.3.1 Generation of *capns1*, *capn1*, and *capn2* knockout cell lines 61
3.3.2 Immunoblotting Analysis 61
3.3.3 Zymography analysis of calpain and MMP activity 62
3.3.4 Orthotopic engraftment tumor growth and metastasis model 62
3.3.5 Statistical Analysis 63
3.4 Results 63
3.4.1 Knockout of *capns1*, *capn1*, and *capn2* in MDA-MB-231 cells 63
3.4.2 Deletion of *capns1*, *capn1*, or *capn2* delays primary tumor growth 65
3.4.3 *Capn1* and *capn2* provide non-redundant metastasis promoting roles 68
3.4.4 KO of *capns1*, *capn1*, and *capn2* attenuates MMP9 secretion 71
3.5 Discussion 73
Chapter 4 General Discussion 79
4.1 Summary 79
4.2 Reflections on roles for calpain in the *her2/neu* signaling network 80
4.3 Remarks on translational relevance in a *her2/neu* model 84
4.4 Calpain in triple-negative breast cancer: summary and future directions 86
References 88
Appendix One 104
Abstract 104
Contrasting roles for calpain in cancer cell survival and death 105
Calpain Modulates Autophagy and Apoptosis 105
Metabolic Coupling in Tumorigenesis 110

Calpain Modulates Metabolic Coupling 111
References 117

List of Figures

Figure 1.1 The crystal structure of calpain-2 bound to calpastatin. 7
Figure 2.1 CAPNS1 expression is ablated in mammary tumors from *NIC capns1*$^{flox/flox}$ mice. 29
Figure 2.2 Deletion of *capns1* in the mammary epithelium delays HER2/NEU-driven tumorigenesis. 30
Figure 2.3 Deletion of *capns1* in HER2/NEU-driven mouse mammary tumors correlates with a differential phosphoproteome. 31
Figure 2.4 CAPNS1 protein and calpain-1/2 activities are ablated by *capns1* deletion. 33
Figure 2.5 *capns1* KO attenuates MTEC *in vitro* invasion but does not affect migration. 35
Figure 2.6 *capns1* KO enhances sensitivity to lapatinib. 37
Figure 2.7 *capns1 KO* enhances sensitivity to doxorubicin. 38
Figure 2.8 *capns1 KO* reduces MTEC tumorigenic potential. 40
Figure 2.9 Late arising tumors in mice engrafted with *capns1 KO* MTECs display CAPNS1 expression. 41
Figure 2.10 *capns1 KO* is associated with reduced tumorigenic potential. 42
Figure 2.11 *capns1* KO does not affect hypoxia-induced stabilization of HIF1α. 44
Figure 2.12 *capns1* KO does not affect secretion or activation of matrix metalloproteinases 2 and 9. 46
Figure 2.13 *capns1* KO in MTECs does not compromise formation of lung metastatic lesions in an experimental metastasis model. 47
Figure 2.14 Knockout of calpain enhances EGFR expression and prolongs MAPK signaling. 49
Figure 3.1 CRISPR/CAS9 KO of *capns1, capn1,* or *capn2* in MDA-MB-231 cells. 64
Figure 3.2 *Capns1* and *capn2 KO* delays tumor growth at the orthotopic site. 66
Figure 3.3 CRISPR/CAS9 KO of calpain genes does not affect cell viability. 67
Figure 3.4 Tumors arising in orthotopically engrafted *Rag2*$^{-/-}$ *IL-2Rgc*$^{-/-}$ mice retain the calpain expression phenotype of the input MDA-MB-231 cells. 69
Figure 3.5 KO of *capns1, capn1, or capn2* is associated with reduced pulmonary metastasis. 70
Figure 3.6 KO of *capns1, capn1 or capn2* is associated with reduced secreted MMP9 activity. 72
Figure 3.7 Isoform-specific roles for calpain-1 and calpain-2 in tumorigenesis. 76
Figure 4.1 Integrating calpain into a signaling network in the *her2/neu* model highlighting proteins identified by RPPA analysis. 83

List of Tables

Table 3.1a Isoform-specific calpain substrates 59
Table 3.1b Isoform-specific calpain substrates 60

List of Abbreviations

17-AAG	Tanespimycin
ADAM	A disintegrin and metalloproteinase
AKT	Protein kinase B
ATG5	Autophagy related 5
BAK	BCL-2 homologous antagonist
BAX	BCL-2-associated X protein
BCL-2	B-cell lymphoma 2
BCL-$_{XL}$	B-cell lymphoma-extra large
BID	BH3 interacting-domain death agonist
BIM	B-cell like protein 11
BRCA	Breast cancer 1
CAF	Cancer-associated fibroblast
CAPN1	Calpain 1
CAPN2	Calpain 2
CAPN6	Calpain 6
CAPNS1	Calpain small subunit 1
CAPNS2	Calpain small subunit 2
CBSW	Calpain-type beta-sandwich domain
CCNB1	Cyclin B1
CDK	Cyclin dependent kinase
CRISPR	Clustered regularly interspaced short palindromic repeats
DCIS	Ductal carcinoma *in situ*
ECM	Extracellular matrix

EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EMT	Epithelial to mesenchymal transition
ER	Estrogen receptor
ERK	Extracellular signal-related kinase
FAK	Focal adhesion kinase
FISH	Fluorescent *in situ* hybridization
FOXO	Forkhead box O3
GRB2	Growth factor receptor-bound protein 2
GSK3β	Glycogen synthase kinase 3 beta
HER2	Human epidermal growth factor receptor 2
HER3	Human epidermal growth factor receptor 3
HGF	Hepatocyte growth factor
HIF	Hypoxia inducible factor
HSP90	Heat shock protein 90
IAP	Inhibitors of apoptosis
IFNγ	Interferon gamma
IGF	Insulin-like growth factor
IHC	Immunohistochemistry
IκBα	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha
JNK	c-Jun N-terminal kinase
Ki67	Marker of proliferation Ki-67
KO	Knockout
LCIS	Lobular carcinoma *in situ*
LVI	Lymphovascular invasion

MAPK	Mitogen activated protein kinase
MARCKS	Myristoylated alanine-rich C-kinase substrate
MDR	Multidrug resistance
MEF	Mouse embryonic fibroblast
MMP	Matrix metalloproteinase
MMTV	Mouse mammary tumor virus
MRP2	Multidrug resistance protein 2
MTEC	Mammary tumor epithelial cell line
MYBL2	Myb-like protein 2
MYC	Avian myelocytomatosis viral oncogene homolog
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PC	Protease core
PEF	Penta-EF-hand domain
Pgp	P-glycoprotein
PHLPP1	PH domain leucine-rich repeat-containing protein phosphatase 1
PI3K	Phosphatidylinositide 3-kinases
PKC	Protein kinase C
PLCγ	Phospholipase C
PP2A	Protein phosphatase 2
PR	Progesterone receptor
PTEN	Phosphatase and tensin homolog
PTP1B	Protein-tyrosine phosphatase 1B
RAC	Ras-related C3 botulinum toxin substrate 1
RAS	Transforming protein p21
Ras-GAP	Ras GTPase activating protein

RhoA	Ras homolog gene family member A
RIPA	Radioimmunoprecipitation assay
ROCK	Rho-associated, coiled-coil-containing protein kinase 1
RPPA	Reverse-phase protein array
RTK	Receptor tyrosine kinase
SHC	SH2-domain C1
SHP-2	Protein-tyrosine phosphatase 1D
Slug	Snail Family Transcriptional Repressor 2
SMAD	Small mothers against decapentaplegic homology 2
Snail	Zinc finger protein SNAI1
SOS	Son of sevenless
SRC	Proto-oncogene tyrosine-protein kinase Src
STAT1	Signal transducer and activator of transcription 1
TGF	Transforming growth factor
TKI	Tyrosine kinase inhibitor
TNBC	Triple-negative breast cancer
TNFα	Tumor necrosis factor alpha
TNM	Tumor/node/metastasis
TOB	Transducer of ERBB2
Twist	Class A basic helix-loop-helix protein 38
VEGF	Vascular endothelial growth factor
WT	Wildtype
Zeb1	Zinc finger E-box-binding homeobox

Chapter One General Introduction

1.1 Breast Cancer Remains a Health Crisis

Cancer is one of the leading health crises facing Canadians, with approximately 30% of Canadians expected to develop one form of cancer over their lifetime, and 25% of those afflicted individuals are expected to die from their disease(CCS, 2017). The economic ramifications of cancer treatment are substantial, with over $4.4 billion in economic costs, including $3.8 billion in direct healthcare costs (CCS, 2017). Breast cancer represents a substantial subset of this population, as 1 in 8 females are expected to develop the disease over the course of their lifetime. While not unheard of, the incidence of male breast cancer is substantially lower, as only 0.2% of newly diagnosed male cancers are those of the breast (CCS, 2017; Fentiman et al., 2006). In general, risk factors which contribute to breast cancer incidence include modifiable and non-modifiable factors such as, genetics (for example, BRCA1/2 gene variants), hormonal replacement therapy in post-menopausal woman, age at menarche, pregnancy, the use of oral contraceptives, alcohol consumption, exposure to carcinogens, and levels of physical activity (CCS, 2017; Chen et al., 2011; Kumle et al., 2002).

1.2 Classification and Treatment

Breast cancer may be broadly divided into two major types based on the ductal or lobular epithelial cell-of-origin. Carcinomas originating from both are also subclassified as either invasive or non-invasive. Non-invasive carcinomas are those confined to a local area that have not breached the basement membrane or started to invade into the surrounding tissue. These are termed either ductal carcinoma *in situ* (DCIS) or lobular carcinoma *in situ* (LCIS). On the other hand, those carcinomas that breach the basement membrane are deemed invasive ductal or invasive lobular carcinoma. The most common type

of breast cancer arises from ductal cells (Harvey, 2007), and while other types such as inflammatory breast cancer do exist, these are rather rare.

Classification of breast cancer is important as it informs prognosis. Classification schemes generally include histopathology, grade, and stage. Histopathology determines the cell-of-origin and informs whether the carcinoma is confined locally or has breached the basement membrane and become invasive. The grade of tumor refers to the differentiation state of the carcinoma. As tumors progress, they become less differentiated, and thus their similarity to normal adjacent tissue; and poorly differentiated tumors are associated with a worse prognosis. Tumor grade values can range from 1 to 3, with 3 representing those tumors which are poorly differentiated with a higher likelihood of metastasizing. Staging refers to the tumor/node/metastasis (TNM) system, where 'T' denotes to the primary tumor and reflects characteristics such as size and confinement of the carcinoma. 'N' refers to whether tumor cells are located in the regional lymph nodes, and 'M' indicates whether the tumor has metastasized beyond the regional lymph nodes (Edge and Compton, 2010).

Pathological assessment of breast cancer receptor status by immunohistochemistry (IHC) is also an important aspect of diagnosis, as this informs treatment. Pathologists stratify breast cancer by the expression of 3 major receptors - the estrogen receptor (ER), progesterone receptor (PR) and the human epidermal growth factor receptor (HER2). Pathologists classify breast cancer as either luminal A, luminal B, HER2 positive or triple-negative/basal-like based on the relative expression profiles of these receptors and other molecular and histological features.

The most frequent subtype of breast cancer is luminal A (50-60%), which is associated with low grade, low nuclear pleomorphism and low mitotic activity. Tumors falling into this category are ER/PR positive (>20% PR), and HER2 negative (Maisonneuve et al., 2014). The prognosis of patients with luminal A tumors is generally more favorable when compared to other subtypes and treatment involves the use of endocrine modulators such as tamoxifen and aromatase inhibitors (Guarneri and Conte, 2009; Yersal and Barutca, 2014).

Luminal B tumors are ER/PR positive, can be either HER2 positive or negative and are associated with a more aggressive phenotype and consequently a poorer prognosis (Cheang et al., 2015). Luminal B tumors may have lower expression of hormone receptors than luminal A tumors, yet higher expression of proliferation-related genes such as CCNB1 and MYBL2 (Ades et al., 2014), as well as greater than 14% expression of Ki67 (Maisonneuve et al., 2014); thus the key differences between luminal A and luminal B malignancies may not rest with overall receptor status, but rather with these unique molecular signatures (Ades et al., 2014; Reis-Filho et al., 2010). Approximately 15-20% of all diagnoses fall into the luminal B category. Treatment of luminal B tumors is similar to that of luminal A and relies on the use of endocrine modulators coupled with chemotherapy.

HER2 positive (HER2+) tumors are associated with high expression or amplification of the HER2 receptor and are generally high grade with an aggressive phenotype. HER2 positive tumors are diagnosed by demonstrating high levels of HER2 staining by IHC or identifying gene amplifications using fluorescent *in situ* hybridization (FISH). An IHC score of +3 (>30% of malignant cell membranes staining for HER2), or a FISH score of >6 copies/nucleus are recommended cutoffs for diagnosis (Wolff et al., 2007). Malignancies falling into this category are generally resistant to endocrine therapy, and thus are treated with anthracyclines (Gennari et al., 2008), taxanes (Burstein et al., 2001), antimetabolites (Geyer et al., 2006), and targeted inhibitors of the HER2 receptor (trastuzumab, lapatinib (EGFR/HER2)), or a trastuzumab-antimicrotubule conjugate, Kadcyla®). Approximately half of all patients treated with trastuzumab relapse with resistant disease within one year (Nahta and Esteva, 2006). Approximately 15-20% of all tumors can be classified as HER2 positive, and nearly half of all HER2 positive tumors also express the estrogen receptor, albeit at lower levels than their luminal counterparts (Yersal and Barutca, 2014).

The terms basal-like or triple-negative tumors have been used interchangeably in the literature, but generally refers to tumors which do not express the estrogen, progesterone, or HER2 receptor, and are highly aggressive with a propensity to metastasize to the brain and lung (Heitz et al., 2009). Since

pathological classification of clinical samples relies on expression of the three main receptors (ER/PR/HER2), clinical samples are usually deemed “triple-negative”, although from a research standpoint, the expression of basal myoepithelial markers such as the cytokeratins 5/14/17 can provide an additional level of classification (Yersal and Barutca, 2014). Basal-like/triple-negative tumors are the most challenging subtype to treat, as unlike their counterparts, no clinically approved generalized targeted therapeutics exist. However, molecular phenotyping is increasingly being used to design patient-tailored targeted therapeutics (Cleator et al., 2007). Often, these tumors are treated through surgery, radiotherapy, and general chemotherapy (Wahba and El-Hadaad, 2015).

Regardless of molecular subtype, approximately 70% of patients with local breast cancer receive adjuvant chemotherapy as part of their standard of care. However, only a subset of patients derive benefit from this, yet suffer side effects from their treatment and increase overall treatment-related costs (Blohmer et al., 2013). Multigene prognostic tools have since been developed (Prosigna, Oncotype DX, Mammaprint) that help to identify patients with a supraoptimal prognosis, and therefore not warranting adjuvant treatment with chemotherapy. Tools such as the PAM 50 can provide a 10-year predictive risk of distant recurrence. However, the majority of these prognostic tools all consider ER/PR negative breast cancer “high risk”, limiting their clinical utility for triple-negative/basal-like breast cancer (Yersal and Barutca, 2014).

1.3 The Calpain Family of Proteases

1.3.1 An Overview of Calpains

The calpain family consists of 16 mammalian calpain genes with various pathological associations, and are a family of calcium-dependent intracellular proteases (although CAPN6, CAPNS1, and CAPNS2 are not enzymatically active) (Campbell and Davies, 2012). Calpains were first purified from skeletal muscle tissue (Huston and Krebs, 1968) and rat brain (Guroff, 1964) in the 1960s. The two classical, or typical, calpains are ubiquitously expressed and were originally denoted μ-calpain (now

calpain-1) and m-calpain (now calpain-2), based on the micromolar and near millimolar calcium requirements, respectively, required for half-maximal *in vitro* activity (Goll et al., 2003). Physiological levels of calcium in the cell are considerably less, thus other regulatory mechanisms exist, discussed shortly. These two calpains are comprised of distinct 80 kDa catalytic subunits, encoded by *capn1* or *capn2*, respectively, coupled with a small 28 kDa regulatory subunit encoded by *capns1,* which is required for enzymatic stability and activity (Dutt et al., 1998; Elce et al., 1997; Graham-Siegenthaler et al., 1994; Tan et al., 2006c).

The structure of calpain-2 was determined by X-ray crystallography (Hosfield et al., 1999a). The catalytic subunit has four distinct domains (D I-IV), with the regulatory subunit having 2 domains (D V-VI). Updated nomenclature for the catalytic subunit refers to D-1 as protease-core (PC) domain 1, D-II as PC 2, D-III as calpain-type β-sandwich domain (CBSW), and D-IV as the PEF(L) domain. The updated nomenclature for the regulatory subunit refers to D-V as a glycine-rich domain, and D-VI as the penta-EF-hand (PEF) (S) domain (Calpain Research Portal 2018, calpain.net). Binding between the subunits is mediated by hydrophobic interactions between the C-terminal domains PEF(L) and PEF(S) of the large and small subunits, respectively, as well as contact between the anchor helix of the large subunit with the small subunit. The catalytic triad of calpain-2 is comprised of Cys105 in PC1, and His262 and Asn286 in PC2, which is improperly aligned in the Ca^{2+}-free structure. Calcium binding within the protease core of the enzyme induces a conformational reorientation of PC1 and PC2 to assemble a functional catalytic triad, involving ~5^0 rotation between PC1 and PC2 (Hosfield et al., 1999b). Although calpains are members of the Penta-EF hand family, having calcium-binding EF hands located in each of PEF(L) and PEF(S) domains, it is the binding by calcium at PC1 and PC2 that leads to activation of calpain (Moldoveanu et al., 2002). This occurs in a 2-stage mechanism involving the repulsion of the N-terminal anchor peptide from the large subunit, followed by the realignment of the active site to assemble the functional catalytic triad (Moldoveanu et al., 2002), although conceptual advances suggest that cleavage of the N-terminal anchor may not be necessary for activation (Chou et al., 2011a).

Calpain-1 shares approximately 55-65% sequence identity with calpain-2, and a chimeric crystal structure reveals similar structure to calpain-2, but with increased flexibility in the EF hands, and a tighter catalytic triad (Pal et al., 2003). Several mechanisms have been proposed which lower the calcium requirement for half-maximal activity, including phosphorylation by ERK (Glading et al., 2004) and protein kinase C (Xu and Deng, 2006), recruitment to the plasma membrane (Leloup et al., 2010) as well as activator proteins (Melloni et al., 2000; Melloni et al., 1998); although, the latter two studies require validation in cell-intrinsic systems. The notion that calpain may traffic to sites of localized calcium influx sufficiently high to achieve enzymatic activation – whether from intracellular stores such as the endoplasmic reticulum, or calcium channels at the plasma membrane – is the most plausible (Campbell and Davies, 2012). However, many studies suggest a role for calpain in localized signaling - for example, calpain-1 localizes to the intermembrane mitochondrial space (Garcia et al., 2005), and calpain-2 to the plasma membrane (Leloup et al., 2010) as well as caveolae (Kifor et al., 2003). To date, calpastatin is the only known endogenous inhibitor of calpains-1 and -2 (Ono et al., 2016). Calpastatin is a 70 kDa unstructured protein with four functional inhibitory domains, each consisting of three repeat subdomains (A, B, C), with the B subdomain exerting inhibitory function (Betts et al., 2003). This B subdomain inhibits calpain by binding in the active site, yet escaping cleavage itself by looping around the catalytic cysteine residue (Hanna et al., 2008; Moldoveanu et al., 2008). The repeated ABC domain suggests one molecule of calpastatin may inhibit up to four calpain molecules (Hanna et al., 2007) (Figure 1.1).

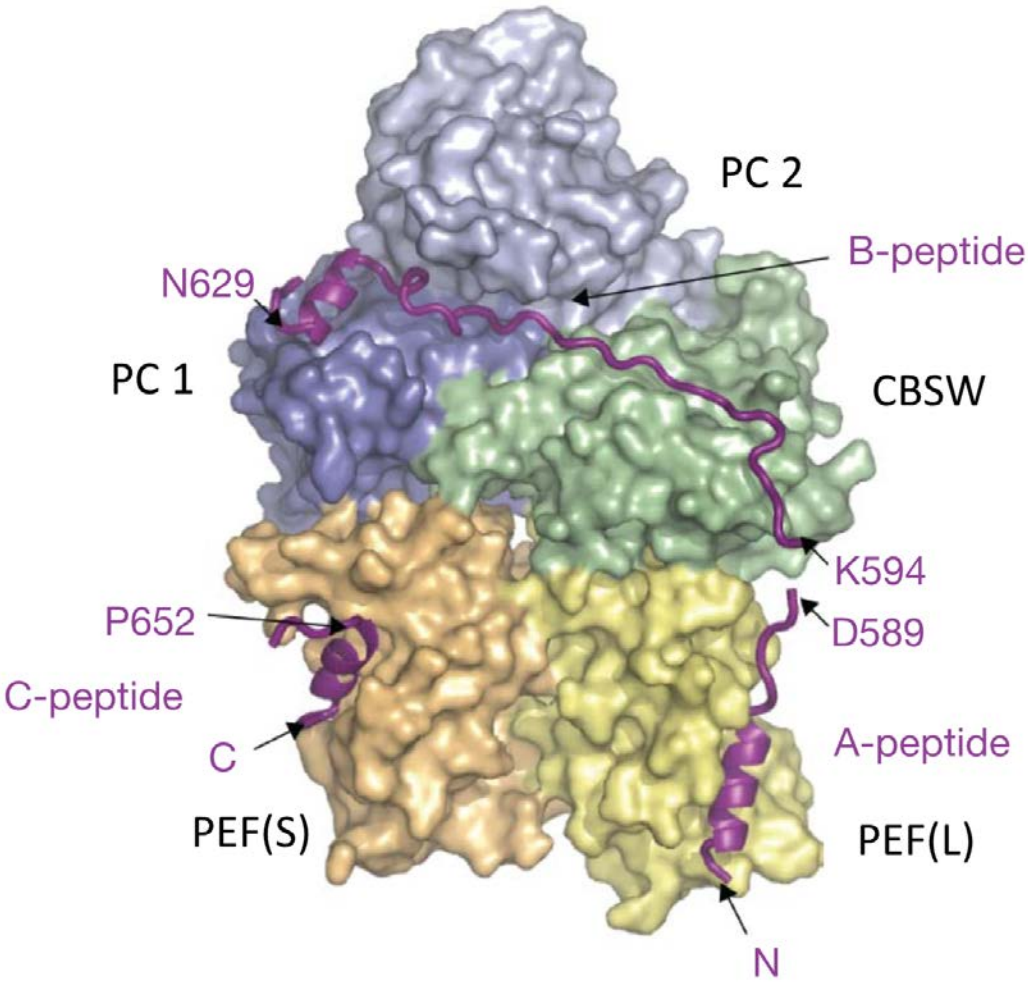

Figure 1.1 The crystal structure of calpain-2 bound to calpastatin.

When complexed with calpain, calpastatin forms helixes in subdomains A and C. Calpastatin evades proteolytic cleavage by calpain in the active sites between PC 1 and PC 2 by looping around the active cysteine residue. Modified from (Hanna et al., 2008).

Calpains-1 and -2 are implicated in a broad range of processes such as cell cycle regulation, migration, apoptosis, and autophagy (Cheng et al., 2018; Goll et al., 2003), as well as in a variety of disease states including Huntington's (Gafni and Ellerby, 2002), Alzheimer's (Saito et al., 1993), muscular dystrophy (Tidball and Spencer, 2000), and cancer (Storr et al., 2011a). The involvement of calpain in a range of cellular processes and diseases is perhaps unsurprising, as calpain-1 and calpain-2 lack a clearly defined consensus cleavage sequence, and thus have a vast substrate profile, with over 130 experimentally verified substrates (duVerle et al., 2010; Liu et al., 2011).

It is generally accepted that calpains participate in signaling pathways by making specific cuts to modulate, rather than abolish, the functions of their substrates. The context in which calpains are engaged is also critically important; for example, calpain-1 and calpain-2 have both pro- and anti-apoptotic roles. Challenge of calpain-1/2-deficient mouse embryonic fibroblasts (MEFs) with tumor necrosis factor α (TNFα) or staurosporine is associated with enhanced apoptosis relative to wild type MEFs, whereas serum starvation or challenge with etoposide is associated with enhanced survival (Tan et al., 2006c).

1.3.2 Expression Patterns in Cancer

Studies of calpain across various cancers have correlated elevated calpain expression with adverse cancer-specific survival. Expression of calpain-2 in basal-like and triple-negative breast cancer (TNBC) is associated with poor survival (Storr et al., 2012a), as well as reduced survival and resistance to platinum-based chemotherapy in ovarian cancer (Storr et al., 2012b). Calpain-1 expression has been correlated with adverse survival in HER2+ breast cancer (Storr et al., 2011c). The endogenous inhibitor calpastatin has also been negatively correlated with lymphovascular invasion (LVI) in breast cancer, implicating elevated calpain activity in LVI (Storr et al., 2011b). Calpain-2 is correlated with progression of prostate cancer, although epigenetic mechanisms may contribute to this observation (Mamoune et al., 2003). Calpain-1 is also associated with development and progression of renal cell carcinoma (Braun et al., 1999), and calpain-1 and calpain-2 with the development of meningiomas and schwannomas (Kimura

et al., 2000). However, not all calpains are associated with protumorigenic roles. Calpain-9 expression is reduced in gastric cancer (Yoshikawa et al., 2000), and calpain-3 is downregulated in aggressive melanoma (Moretti et al., 2009).

1.4 Calpain in Cancer Pathogenesis

The progression of a cancer from a local site to a distant organ is a multistep, complex process characterized by proliferation, invasion and migration at the local site leading to intravasation into the blood or lymphatic vasculature, and followed by extravasation and growth at a distant organ. Calpains have a diverse substrate profile, with over 130 known substrates and 360 different cleavage sites (Liu et al., 2011), and through these substrates are implicated in multiple stages throughout the metastatic process. Roles for calpain-1 and calapin-2 have been identified in biological processes ranging from cell migration (Dourdin et al., 2001; Franco et al., 2004b), invasion (Jang et al., 2010; Postovit et al., 2002b), survival and apoptotic signaling (Grieve et al., 2016; Ho et al., 2012; Tan et al., 2006b; Tan et al., 2006c), to cell cycle progression (Choi et al., 1997; Libertini et al., 2005). Calpain-1/2 knockdown in an orthotopic model of TNBC was recently associated with a significant reduction in metastatic potential (Grieve et al., 2016).

1.4.1 Calpain in Oncogenic Signaling and the Cell Cycle

The epidermal growth factor (EGF) family of growth factors regulates the activity of HER-family of receptor tyrosine kinases (RTKs), with ligand binding inducing the formation of receptor homo- or heterodimers (Yarden and Sliwkowski, 2001). There are four members of the HER-family (HER1-4, also known as ErbB1-4). HER1, the prototypic member of the epidermal EGFR family, and HER4 are ubiquitously expressed, while expression of HER2 and HER3 is more restricted. HER family members share a common structure, having an extracellular ligand binding domain, a single membrane-spanning region, and a cytoplasmic domain that includes a juxamembrane region, a tyrosine kinase domain and a

C-terminal tail, which contains multiple tyrosine phosphorylation sites (Yarden and Sliwkowski, 2001). HER3 is unique in that lacks intrinsic tyrosine kinase activity but can still participate in signaling by forming heterodimers with other members of the HER family (Yarden and Sliwkowski, 2001). HER signaling is mediated by phosphorylation of receptor C-terminal tails which provides docking sites for adapters such as SRC homology 2-domain containing (SHC), and growth factor receptor-bound protein 2 (GRB2), as well as enzymes including SHP-2, PI3K, Ras-GAP, SOS, and PLCγ. These effectors couple extracellular growth factors with intracellular downstream signaling pathways (Olayioye et al., 2000). Generally, HER-family RTK overactivation correlates with malignant phenotypes (Hynes and Lane, 2005).

Both calpain-1 and calpain-2 are implicated in promoting propagation of signaling from members of the HER family. A phosphorylation site on calpain-2 (S50) has been identified as a target of extracellular signal-related kinases (ERK), downstream of the EGFR (Glading et al., 2004) and represents a potential site of oncogenic signal propagation. HER2 is a key EGFR-family member and was first implicated as prognostic marker present in about 30% of breast cancers in 1987 (Slamon et al., 1987). Activation of EGFR family members, including HER2 is associated with activation of protein kinase B (AKT), a key player in regulating a multitude of cellular functions, which often plays a pro-survival role in response to cellular stress (Vivanco and Sawyers, 2002; Wen et al., 2000). Interestingly, activation of AKT by calpain-1 in trastuzumab-resistant SKBR3 cells is believed to be a critical survival mechanism (Kulkarni et al., 2010). AKT is upregulated in response to chemotherapeutic challenge (Belyanskaya et al., 2005; Liu et al., 2015) and knockout studies in MEFs implicate calpain-1 and calpain-2 in stress-mediated AKT activation (Tan et al., 2006c), likely through degradation of negative regulators such as protein phosphatase 2A (PP2A) (Bertoli et al., 2009) and PH domain leucine-rich repeat-containing protein phosphatase 1 (PHLPP1), which is an inhibitor of both AKT and ERK (Gao et al., 2005; Wang et al., 2013). Furthermore, calpain is implicated in regulating multidrug resistance (MDR) protein 1 stability (Grieve et al., 2016) implicating it in chemotherapeutic responses. Nuclear factor kappa-light chain

enhancer of activated B cells (NFκB) also promotes cancer survival and exhibits cross-talk with AKT (Hussain et al., 2012). Calpain has been implicated in the activation of the NFκB family through degradation of the inhibitor IκBα (Han et al., 1999; Lin et al., 1995; Shumway et al., 1999), which can suppress apoptosis by inducing expression of inhibitors of apoptosis (IAPs) and BCL-2 family members, as well as drive cell cycle progression (reviewed in (Dolcet et al., 2005)).

Calpains are also implicated in a number of phases of the cell cycle. Roughly, the cell cycle can be grouped into four phases, with the entry into phases governed by cyclins and cyclin dependent kinases (CDKs): (G1) where cells prepare to replicate their chromosomes; (S) where the chromosomes are replicated; (G2) where cells prepare to enter mitosis; and (M) mitosis where cells undergo division into two daughter cells. Using a flow cytometry, one group was able to demonstrate that inhibition of calpain was associated with a delay in passing the G1/S boundary, prolonged S phase progression, and a delay in G2/M progression (Janossy et al., 2004). Several calpain targets involved in regulating the cell cycle were identified including the cell cycle inhibitor p27 (Patel and Lane, 2000), which is influenced through an upstream calpain-AKT-FOXO3a-p27 pathway (Ho et al., 2012). Calpain also cleaves cyclin D1 (Choi et al., 1997), as well as cyclin E, which results in a hyperactive low-molecular weight cyclin E species that may further drive cell cycle progression (Wang et al., 2003). Calpain-mediated cleavage of p53 (Kubbutat and Vousden, 1997) may also subvert apoptosis. Cleavage of topoisomerases I (Chou et al., 2011b) and IIα (Jeon et al., 2018b) have also been described, which has implications both for cell cycle progression and sensitivity to chemotherapeutics (Jeon et al., 2018b).

1.4.2 Calpain in Apoptosis

BCL-2 family members include both pro-apoptotic members such as BIM and BID, and anti-apoptotic members such as BCL-2 and BCL-$_{XL}$, and apoptosis is regulated by a balancing act between these groups, referred to as the rheostat model. Pro-apoptotic members BAX and BAK induce apoptosis by permeabilizing the outer mitochondrial membrane and forming a proteolipid pore resulting in

cytochrome c release (Chipuk and Green, 2008; Green and Chipuk, 2008). Studies indicate that calpain can regulate members of this family to modulate apoptosis. Cleavage of BAX by calpain after etoposide or staurosporin challenge can generate a pro-apoptotic fragment which is resistant to inhibition by BCL-2 (Gao and Dou, 2000). Cleavage of BID by calpain in response to cisplatin challenge induces cytochrome-c release from the mitochondria (Mandic et al., 2002), and cleavage of ATG5 by calpain promotes its association with BLC-2 and subsequent release of cytochrome-c from the mitochondria. ATG5 has been implicated in autophagy, therefore calpain cleavage of ATG5 may represent a link between autophagy and apoptosis (Yousefi et al., 2006), as discussed in Appendix 1.

Calpain is also implicated in regulating apoptosis through cleavage of caspases. Caspases exist in latent forms and are activated to effect their functions. They are grouped roughly into long-domain and short-domain groups, with the long-domain caspases serving as initiator caspases (caspases-2/8/9/10), and the short-domain caspases (caspases-3/6/7) serving as effector caspases which cleave a range of cell targets to promote apoptosis. Caspases are produced as inactive zymogens which are activated by proteolysis (Riedl and Shi, 2004). Interestingly, cleavage of caspases by calpain can have opposing roles depending on the caspase in question. Cleavage of caspase-8 and caspase-9 by calpain attenuates their activities (Chua et al., 2000), whereas calpain cleavage of caspase-7 promotes apoptosis by increasing its activity (Gafni et al., 2009). Furthermore, calpain cleavage of the initiator caspase-12 in response to ER stress promotes apoptosis (Tan et al., 2006b). Calpain is also implicated in resistance to therapeutic challenge. Cleavage of the proto-oncogene c-MYC protein to MYC-Nick enhances the survival of colon cancer cells in response to chemotherapeutic challenge, in part by inducing autophagy (Conacci-Sorrell et al., 2014). Cleavage of topoisomerases I and IIα by calpain-2 in response to doxorubicin challenge also impairs therapeutic response in breast cancer cells (Jeon et al., 2018b).

1.4.3 Calpain in Invasion and Migration

Metastasis remains the leading cause of death for breast cancer patients. Tumor cells develop a propensity to metastasize when they begin to outgrow their microenvironment and seek a new site to colonize. One of the core features which underpins the ability of a tumor to metastasize is the intrinsic genomic instability of tumor cells which permits the acquisition of pro-metastatic traits as tumors evolve (Gupta and Massague, 2006). A review describing the hallmarks of cancer, including those that contribute to the metastatic cascade, was published by Hanahan and Weinberg (reviewed in (Hanahan and Weinberg, 2000; Hanahan and Weinberg, 2011). The six core hallmarks include self-sufficiency in growth signals, aversion to antiproliferative signals, subversion of apoptosis, unchecked proliferation, angiogenesis, and ultimately metastasis. Metastasis of cancer occurs through two key routes: the blood vasculature and the lymphatic vasculature.

Cancer cells have a strong propensity to metastasize when encountering stressors such as inflammation (Wu and Zhou, 2009) or hypoxia (Harris, 2002). Inflammation, acting through proto-oncogenes such as RAS, contributes to remodeling of the extracellular matrix (ECM) (Sparmann and Bar-Sagi, 2004). Fibroblasts are important players in the metastasis of cancer as they contribute to extracellular remodeling and angiogenesis that can promote tumor cell growth and dissemination (Orimo et al., 2005; Sappino et al., 1988). Fibroblasts are components of the connective tissue which synthesize and remodel fibronectin and collagen (Rodemann and Muller, 1991; Tomasek et al., 2002). They can promote the invasion of breast carcinoma (Hu et al., 2008), in part by forming direct E/N cadherin-mediated contacts with the cancer cells and assisting invasion through directed force, ECM remodeling, and secretion of chemotactic factors such as HGF (reviewed in (Ewald, 2017)). Fibroblast function can also be mediated through transforming growth factor (TGF)-α and TGFβ secreted from cancer cells, which activates SMAD, RAS, and PI3K. This leads to production of MMP9 and subsequent matrix remodeling (Stuelten et al., 2005). Calpain is implicated in promoting fibroblast motility through de-adhesion in response to EGF signaling (Glading et al., 2000). Calpain has also been noted to contribute to

the synthesis of TGFβ in fibroblasts and is also activated in response to TGFβ signaling, linking calpain to SMAD and AKT signaling (Li et al., 2015).

Hypoxia is also a strong driver promoting aggressive tumor growth and metastasis, and activates a transcriptional response promoting angiogenesis and stress response (Harris, 2002). Hypoxia-inducible factor 1α (HIF1α) is a ubiquitously expressed oxygen-labile subunit of the HIF1-α/β heterodimeric complex. HIF1α is normally degraded by an E3-ubiquitin ligase under normoxic conditions (Ohh et al., 2000), but is stabilized under hypoxic conditions to direct transcription of genes containing pro-survival hypoxic response elements including VEGF, which is needed to promote tumor-associated neoangiogenesis (Tug et al., 2009). Calpain modulates a hypoxic response through proteolysis of the actin crosslinker protein filamin-A, which results in enhanced nuclear localization of HIF1α (Zheng et al., 2014). While HIF1-α overexpression is often seen in primary breast cancer, it is strongly associated with distant metastasis (Zhong et al., 1999), and may contribute to immune escape.

In order for metastatic dissemination to occur, tumor cells generally undergo an epithelial-to-mesenchymal (EMT) phenotypic switch; which is characterized by altered cell-cell contacts and enhanced migratory and invasive properties. The EMT switch can be driven by hypoxia-related genomic instability (Yoo et al., 2011), TGF-β (Zavadil and Bottinger, 2005), hepatocyte growth factor (HGF) (Savagner et al., 1997), EGF (Lo et al., 2007), insulin-like growth factor (IGF) (Graham et al., 2008), and fibroblast growth factor (FGF) (Acevedo et al., 2007). These factors contribute to a phenotypic change characterized by loss of cell polarity, disruption of cell-cell and cell-substratum contacts, and cytoskeletal reorganization (Micalizzi et al., 2010). One of the hallmarks of EMT is the loss of E-cadherin, a key cell-cell adhesion molecule, as a result of transcriptional repression by Snail, Slug, and Twist. TGF-β, hypoxia, GSK-3β, NFκB, and MAPK signaling have been shown to induce the expression of Snail (De Craene et al., 2005), whereas hypoxia and TGF-β signaling regulate Slug and Twist expression (Cannito et al., 2008; Medici et al., 2008; Peinado et al., 2003; Yang and Wu, 2008). Zeb-1 is also an important transcription factor which contributes to EMT by repressing E-cadherin (Zhang et al., 2015), and is

correlated with NFκB activation (Chua et al., 2007). Calpain is implicated in this regulatory framework as it is activated downstream of ERK during MAPK signaling (Glading et al., 2004), contributes to NFκB signaling through proteolysis of IκBα (Lin et al., 1995), and mediates a hypoxic response through proteolysis of filamin-A (Zheng et al., 2014). Calpain also directly cleaves E-cadherin (Konze et al., 2014), which can further contribute to the loss of cell-cell contacts and contribute to motility.

While mesenchymal migration is well characterized, cancer cells have been noted to interconvert their phenotype between mesenchymal and amoeboid migration depending on the microenvironment (Taddei et al., 2013). Amoeboid-like migration is characterized by actomyosin contractility, and unlike mesenchymal modes of migration, amoeboid migration can occur in the absence of MMP-mediated proteolysis of the ECM. Of note, SRC-calpain-2-mediated integrin turnover is implicated in mesenchymal migration, and inhibition of calpain-2 in the presence of MMP inhibitors promotes amoeboid migration (Carragher et al., 2006). Amoeboid migration has a greater dependency on RhoA/ROCK, and Ezrin signaling (Sahai and Marshall, 2003). Protrusions from the leading edge of the cell make contact with collagen fibers in the extracellular matrix, and forcefully deform the collagen as cells migrate (Wilkinson et al., 2005; Wyckoff et al., 2006). Interestingly, tumor cells interconvert their migratory mechanism between mesenchymal and amoeboid in a Rac-dependent manner, whereby inactivation of Rac suppresses mesenchymal type movement (Sanz-Moreno et al., 2008). Therefore, tumor cells may switch migratory mechanisms to suit the microenvironment they encounter. Inhibiting key players of both mesenchymal and ameboid migration can synergize and attenuate overall tumor cell invasion (Sahai and Marshall, 2003). Clearly, the migratory process is complex and multifaceted.

In order to degrade the ECM, cancer cells make use of invadopodia, which are specialized, dynamic membrane-cytoskeletal structures that aid in the degradation of ECM as well as the vascular basement membrane – thus playing an important role in tumor cell intravasation (Seano and Primo, 2015). Invadopodia possess an F-actin rich core and are generally found on the ventral surface of tumor cells. Once formed, these structures can persist for hours and serve as sites for the secretion and activation

of matrix metalloproteinases (MMP), including MMP2, MM9, MMP14, as well as several proteins containing disintegrin and metalloproteinase domains (ADAMs). Calpain has been implicated in modulating the assembly of invadopodia through promoting SRC activity through cleavage-induced relocalization of PTP1B, as well as invadopodia disassembly through proteolysis of cortactin (Cortesio et al., 2008).

The *homo sapiens* MMP family is quite large, with 24 members, yet a large body of work suggests that MMP2 and MMP9 are key players as it relates to the invasion and metastasis of carcinomas; and they also have prognostic utility (Jones et al., 1999; Li et al., 2004; Somiari et al., 2006). While MMP expression is characteristic of normal tissue, upregulation and activation of MMP9 correlates with aggressive behavior of carcinomas (stage T2-T4) (Rha et al., 1997), and MMP9 is activated in a MMP2-dependent manner (Fridman et al., 1995). Of note, MMP2 is associated with calpain-2 expression in glioblastoma (Jang et al., 2010), and MMP9 expression can be driven downstream of AKT and ERK signaling (Lee et al., 2007). Furthermore, calpain-1 translocates to the mitochondria and drive oxidative-stress mediated MMP9 activation in endothelial cells (Moshal et al., 2006), consistent with a study indicating a role for the N-terminus of calpain-1 in mitochondrial targeting (Badugu et al., 2008). Calpain also activates the membrane-associated MMP14/MT1-MMP during angiogenesis (Kang et al., 2011; Kwak et al., 2012), and cancer cell invasion (Chen et al., 2013), suggesting that calpain activity in both the stromal compartment and cancer cells may also contribute to metastatic dissemination. Given calpain's role in the PI3K-AKT pathway (Ho et al., 2012; Tan et al., 2006c) and ERK-dependent regulatory phosphorylation (Glading et al., 2000; Zadran et al., 2010), calpain may also be implicated in the production and activation of MMPs in both cancer and stromal cells.

Cells are able to migrate through newly formed spaces in the matrix through the coordination of three key processes: extension of a leading edge and formation of focal adhesions, cell body contraction, and detachment of adhesions at the rear edge of the cell. Cell adhesion is mediated by the formation of early focal contacts characterized by integrin clustering and recruitment of focal adhesion kinase,

vinculin, and paxillin. Tyrosine phosphorylation promotes talin and paxillin incorporation into focal contacts and leads to tensin and zyxin recruitment (Lauffenburger and Horwitz, 1996; Raftopoulou and Hall, 2004). As focal contacts mature into focal complexes, the presence of FAK, tensin, and zyxin decreases (Carragher and Frame, 2004). Interestingly, calpain-2 localizes at focal adhesion structures (Beckerle et al., 1987). Consistent with calpain's purported involvement in focal complexes, treatment with pharmacological inhibitors of calpain reveals reduced retraction of trailing edge focal adhesions in addition to suppression of cell movement. Unsurprisingly, key players in cell adhesion, such as FAK, SRC, paxillin, talin, and β-integrins have been shown to be substrates of calpain (Cooray et al., 1996; Oda et al., 1993), and a critical step in the disassembly of focal adhesions during tail retraction involves calpain cleavage of talin (Franco et al., 2004b). A role for calpain in regulating both matrix degradation and cell migration substantiates its involvement in metastasis of breast cancer.

In the 1889 Stephen Paget first proposed the "soil and seed" hypothesis, postulating that there is a tendency for metastatic dissemination of certain cancers to target specific organs (the "soil") (Paget, 1889) , an observation which has gained support over time. Once cancer cells successfully intravasate into the blood or lymphatic system, they must not only survive transit through the circulation, but also successfully arrest and extravasate at an appropriate distant "soil" to enable subsequent proliferation as a metastatic lesion. Arresting cancer cell transit in the vasculature may be mediated by integrins – a family of transmembrane receptors which participate in signal transduction. There are over 24 functional heterodimeric integrin receptors, including a combination of 18 α-subunits and 8 β-subunits (Desgrosellier and Cheresh, 2010) which have affinity for different ECM components such as laminin, collagen, fibronectin, and vitronectin. This broad family serves as bi-directional messengers of signals, permitting the cell to sense and respond to the microenvironment (reviewed in (Seguin et al., 2015). Integrin activation is contingent upon a conformational change, adopting an "open" structure which occurs when it encounters an appropriate extracellular ligand, or when complexing with an intracellular adapter or scaffold protein (Shattil et al., 2010). For example, one study demonstrates a relationship

whereby active αvβ3 integrin permits vascular adhesion and metastatic colonization of MDA-MB-435 breast cancer cells, and is further supported by the finding that primary metastatic cells from a patient bound to platelets (likely through vitronectin (Trikha et al., 2002)) in an integrin-dependent manner and incorporated into a sheer-resistant thrombi (Felding-Habermann et al., 2001). Occlusion of tumor cells in capillaries may also provide an opportunity for tumor cells to extravasate.

Once tumor cells exit the vasculature, engagement of integrins may also promote survival and proliferative signals through encouraging the formation of focal adhesions. This results in the recruitment of FAK (Sieg et al., 1999), activation of MAPK (Campos et al., 2004) and PI3K-AKT pathways (Aoudjit and Vuori, 2001; Shaw et al., 1997), and transcription of BCL-2 (Matter and Ruoslahti, 2001), contributing to the overall survival and proliferation of metastatic cells. However, successfully transiting to, and invading the vasculature, extravasating, and proliferating at a metastatic site is a rare event, which explains why the vast majority of circulating tumor cells fail to form metastases (Luzzi et al., 1998).

1.5 Hypothesis and Aims

Advances in treatment have reduced mortality associated with breast cancer, yet some subtypes such as TNBC remain challenging to treat owing to lack of clinically approved generalized therapeutic targets. Calpain has multifaceted roles in tumorigenesis owing to its diverse substrate profile, implicating calpain in tumor cell survival, proliferation, and metastasis. Calpain modulates a number of key substrates which promote tumorigenesis, including NFκB (Shumway et al., 1999), HER2 (Kulkarni et al., 2010), and AKT (Ho et al., 2012; Tan et al., 2006c), as well as key players relating to chemotherapeutic response such as topoisomerase I and IIα (Chou et al., 2011b; Jeon et al., 2018a), and PGP (Grieve et al., 2016). Previous studies revealed that knockdown of *capns1* attenuates tumor growth and metastasis in a preclinical model of TNBC (Grieve et al., 2016). Here, I seek to explore the role of calpain-1 and calpain-2 in HER2+ breast cancer and elucidate an isoform-specific role for these calpains in TNBC.

I hypothesize that calpain-1 and calpain-2 foster the development of a tumor and provide pro-survival functions in response to cellular challenge by propagating oncogenic signaling and acting on key mitogenic and survival pathways, and that interfering with calpain functions will attenuate tumorigenesis and will cooperate with select chemotherapeutic and targeted agents. The aims of this thesis research were:

1) To explore the effects of conditional calpain-1/2 disruption in a transgenic mouse model of spontaneous *her2/neu*-driven mammary cancer.
2) To explore the impact of calpain-1/2 disruption on the tumorigenesis and susceptibility to therapeutic challenge of a mammary cancer cell line derived from a transgenic mouse model of *her2/neu*-driven mammary cancer.
3) To elucidate the effects of isoform-specific calpain-1 and calpain-2 knockout on primary tumor growth and metastasis in a preclinical model of TNBC.

Ultimately, the objectives of this work were to map the functional outcomes of calpain-1 and calpain-2 perturbation, with the goal of strengthening the rationale for the development and utilization of clinical calpain inhibitors.

Chapter 2

Genetic disruption of calpain-1 and calpain-2 blocks tumorigenesis in mouse models of HER2+ breast cancer and sensitizes cancer cells to doxorubicin and lapatinib

2.1 Abstract

Calpains are a family of calcium-activated cysteine proteases that participate in a wide range of cellular functions including migration, invasion, autophagy, programmed cell death and gene expression. Calpain-1 and calpain-2 isoforms are ubiquitously expressed heterodimers composed of isoform-specific catalytic subunits encoded by *capn1* or *capn2*, respectively, coupled with the obligate common regulatory subunit encoded by *capns1*. Here, we report that conditional deletion of *capns1* disrupts both calpain-1 and calpain-2 expression and activity, and this attenuates tumorigenesis in a spontaneous model of HER2+ breast cancer, and completely blocks tumorigenesis in an orthotopic engraftment model. Furthermore, we show that knockout of *capns1* correlates with enhanced sensitivity to the chemotherapeutic doxorubicin and the HER2/EGFR tyrosine kinase inhibitor lapatinib. Collectively, these results indicate pro-tumorigenic roles for calpains-1/2 in HER2+ breast cancer and provide compelling preclinical evidence that calpain-1/2 inhibitors would have significant anti-tumor effects if used alone or in combination with currently used chemotherapeutics and targeted agents.

2.2 Introduction

Calpains are a family of 16 calcium-dependent intracellular thiol proteases which are involved in a wide range of cellular and physiological functions (reviewed in (Campbell and Davies, 2012; Goll et al., 2003; Suzuki et al., 1995)). Aberrant expression or activity of calpains is implicated in the etiology of several diseases including diabetes, Alzheimer's, and cancer; this has stimulated considerable interest in the development and preclinical testing of calpain inhibitors (reviewed in (Ono et al., 2016)). Calpain-1 and calpain-2, the most widely studied members of this family, are ubiquitously expressed heterodimers composed of isoform-specific catalytic subunits encoded by *capn1* or *capn2*, respectively, coupled with an obligate common regulatory subunit encoded by *capns1*. Genetic disruption of *capns1* is associated with destabilization of the CAPN1 and CAPN2 catalytic subunits and loss of calpain-1 and calpain-2 activities *in vivo* (Arthur et al., 2000; Tan et al., 2006a). Approximately 130 calpain cleavage substrates have been described (Liu et al., 2011), and the effect of calpain cleavage on their functions varies from activation to inactivation to changes in subcellular localization. Since many of these substrates are components of key cell signaling pathways, calpains can have pleomorphic effects on cellular behavior, depending upon cell type, substrate expression, and the context in which calpain is activated. Many of the pathways calpain activity impinges upon are related to tumorigenesis (reviewed in (Storr et al., 2011a)), including key survival and apoptosis pathways such as the PI3K-AKT pathway (Ho et al., 2012; Tan et al., 2006b; Tan et al., 2006c), cell cycle checkpoints (Janossy et al., 2004; Libertini et al., 2005; Wang et al., 2003), migration and invasion (Dourdin et al., 2001; Franco et al., 2004a; Franco et al., 2004b; Postovit et al., 2002a), as well as the function of oncoproteins such as HER2 (Kulkarni et al., 2010) and MYC (Conacci-Sorrell et al., 2010). As calpain function is implicated in diverse signaling networks, perhaps unsurprisingly, there is evidence that calpain may engage in opposing roles; for example,

promoting apoptosis in response to challenge with etoposide or camptothecin, and protecting the cell from cytotoxic responses to tumor necrosis factor α or staurosporine (Tan et al., 2006c).

Translational studies have revealed that high calpain-2 expression correlates with adverse outcomes in basal-like or TNBC (Storr et al., 2012a), while high calpain-1 expression correlated with poor relapse-free survival in HER2+ breast cancer (Storr et al., 2011c). High calpain-2 levels were also associated with platinum resistance and poor overall survival in ovarian cancer patients (Storr et al., 2012b).

In vitro studies have linked calpain to trastuzumab resistance in HER2+ breast cancer cells through generation of a p95HER2 fragment (Kulkarni et al., 2010; Scaltriti et al., 2007), or efflux of chemotherapeutics like doxorubicin or other drugs by regulating multidrug resistance protein function (Grieve et al., 2016). Thus, a growing body of research suggests that clinical inhibition of calpain may cooperate or synergize with specific existing treatments to improve breast cancer patient outcomes.

In this study we use genetic manipulation of calpains-1/2 in HER2+ models of breast cancer to show that these calpains are involved in, but not required for, spontaneous tumor formation; however, calpain-1/2 knockout in established carcinoma cells abolished their tumor forming capability and enhanced *in vitro* sensitivity to doxorubicin and lapatinib.

2.3 Materials and Methods

2.3.1 Spontaneous Tumor Development and Reverse-Phase Protein Array Analysis

Transgenic mice expressing oncogenic rat HER2/NEU and Cre-recombinase in mammary epithelial cells from a bicistronic transcript under the control of the mouse mammary tumor virus promoter (*MMTV-NEU-IRES-CRE/NIC*) (Ursini-Siegel et al., 2008) were crossed with *capns1* floxed mice (Tan et al., 2006a) to produce compound *NIC capns1*$^{flox/flox}$ or *NIC capns1*$^{+/+}$ virgin females to explore the effect on HER2/NEU driven tumorigenesis of *capns1* deletion in the mammary epithelium.

Mammary glands were palpated weekly to assess spontaneous tumor formation. At endpoints, mice were euthanized and tumors resected, snap frozen in liquid N_2 and prepared for reverse-phase protein array (RPPA) analysis using recipe 10 and a panel of 128 antibodies as described elsewhere (Gujral et al., 2013; Luckert et al., 2012).

2.3.2 Cell Line Derivation

Mammary tumor epithelial cells (MTECs) were derived from *neu*^{NT} transgenic mice (strain TG.NK obtained from Jackson Labs) expressing oncogenic rat HER2/NEU in mammary epithelial cells under the control of the mouse mammary tumor virus promoter (Muller et al., 1988), crossed with *capns1*$^{flox/flox}$ mice (Tan et al., 2006a). A tumor from a *neu*^{NT} *capns1*$^{flox/flox}$ female mouse was excised and a cell suspension was developed by mechanical disruption and digestion with 0.25 % trypsin, and cultured in improved minimum essential medium supplemented with 1 % fetal bovine serum (FBS; Sigma), 10 μg/mL insulin (Sigma), 15 ng/mL EGF (Sigma), 1 μg/mL hydrocortisone (Sigma), and 20 nM estrogen (Sigma) as described by (Sangrar et al., 2015; Yu et al., 2006). The serum concentration for immortalized cells was increased to 5 % and supplemented with insulin, EGF, and hydrocortisone. Estrogen was discontinued after 10 passages, and immortalized cells were subsequently cultured in IMEM (Corning), supplemented with 5 % FBS (Sigma), and 1 % antibiotic-antimycotic (AA; Gibco) ("complete IMEM"). MTECs were maintained in a humidified incubator at 37.5 ^{o}C, 5 % CO_2.

To produce *capns1* knockout (KO) and vector control (WT) MTECs, $2.5x10^5$ cells were seeded on a 60 mm dish (Sarstedt) and transduced with retrovirus expressing Cre recombinase or an empty vector (Tan et al., 2006a) in a pMSCV-puro system (Clontech). Puromycin (Gibco) was used as a selectable marker, and disruption of the floxed *capns1* gene was validated by immunoblotting and HEPES-imidazole casein zymography. Cells surviving culture in 2 μg/mL puromycin were lysed into radio-immunoprecipitation assay buffer for immunoblotting (RIPA; 50mM Tris-HCl (Fisher), 150 mM NaCl (Fisher), 1 % NP-40 (Sigma), 0.1 % SDS (Sigma), and 0.5 % sodium deoxycholate (Sigma)

supplemented with protease and phosphatase inhibitors 1 mM sodium orthovanadate (Sigma), 1 mM phenylmethane sulfonyl fluoride (ICN Biomedicals), 5 μg/mL leupeptin (Sigma), and 2 μg/mL aprotinin (Sigma)); or lysed into HEPES/Triton X-100 lysis buffer for HEPES-imidazole casein zymography (as described by (Croall et al., 2002)). Lysates were clarified by centrifugation (12, 000 x g for 15 min at 4 ^{o}C), and protein quantification was performed using the Pierce bicinchoninic acid (BCA) kit (Thermo Scientific). *Capns1* KO was validated by immunoblotting using an in-house antibody generated against bacterially expressed rat calpain-2, which detects murine CAPN2 and CAPNS1 polypeptides (Samis et al., 1991), and by casein zymography to assess calpain-1 and calpain-2 enzymatic activity. Lysates from *capns1* KO or WT mouse embryonic fibroblasts (MEFs) (Tan et al., 2006a) were used as controls for immunoblotting and zymography.

2.3.3 Migration, Invasion, Chemosensitivity, Hypoxic Response, and EGF Signaling Analysis

To assess migration, 1.5 x 10^4 WT or *capns1* KO MTECs were seeded in sextuplicate on 96-well ImageLock plates (Essen Bioscience) and allowed to adhere overnight. The following day cell monolayers were scrape-wounded using the IncuCyte Wound Maker (Essen Bioscience) and washed twice with media. Adherent cells were then cultured in 100 μL of complete IMEM and placed in an IncuCyte system (Essen Bioscience) maintained at 37 ^{o}C, 5 % CO_2 and imaged with a 10x objective every 2 h until the wounds had closed.

For cell invasion assays, 8 μm-pore transwells (Corning #353097) were coated with 15 μg of growth-factor reduced matrigel (VWR) in 25 μL of serum-free IMEM and allowed to dry overnight. The following day, the matrigel was rehydrated with serum-free IMEM for 2 h in a humidified incubator at 37 ^{0}C, 5 % CO_2. One x 10^5 WT or *capns1* KO cells were added in triplicate wells to the top chambers in serum-free IMEM. Media in the lower chambers was replaced with complete IMEM. Cells were allowed to invade for 24 h, then non-invading cells were removed from the top chambers with cotton swabs, and

invading cells in the lower chambers were fixed in ice-cold methanol and stained with DAPI (Sigma) and imaged under a 10x objective.

To assess cytotoxicity, 2 x 10^4 WT or *capns1* KO MTECs were seeded in 96-well plates in triplicate, (Grenier Bio-One) and allowed to adhere overnight. Increasing concentrations of doxorubicin (Sigma) or lapatinib (Toronto Research Chemicals) were added to the wells and cells cultured for 72 h. At endpoint, the PrestoBlue reagent (ThermoFisher) was utilized to quantify cell viability by measuring fluorescence on a SpectraMax M2 plate reader (590 nm).

Hypoxic response was assessed by seeding 1.5 x10^6 WT or *capns1* KO MTECs in 10 cm dishes (Sarstedt) and allowing cells to adhere overnight in complete IMEM. The following morning cells were washed once with PBS and changed to serum-free IMEM. Positive controls were treated with 100 μM of cobalt chloride and half of the dishes were transferred to a hypoxic incubator (0.1 % O_2). After 24 h cells were placed on ice, rinsed once with PBS and lysed into RIPA (as described in 2.3.2) or nuclear extraction buffer (20 mM HEPES (Fisher) pH 7.4, 10 mM KCl (Fisher), 2 mM $MgCl_2$ (Fisher), 1 mM EDTA (Sigma), 1 mM EGTA (Sigma), 1 mM DTT (Fisher) supplemented with protease and phosphatase inhibitors as previously described. For nuclear extracts, cells were collected with a cell scraper and passed through a 27-gauge needle 10 times. Lysates were then incubated on ice for 20 min and centrifuged 720 x g for 5 min at 4 ^oC. The pellet was then washed once in 500 μL of nuclear extraction buffer and passed 10 times through a 25-gauge needle, followed by centrifugation for 10 min at 720 x g. The supernatant was discarded, and the pellet was resuspended in 2x SDS sample buffer (100 mM Tris-HCl (Fisher) pH 6.8, 0.2 % bromophenol blue (Sigma), 20 % glycerol (Fisher), 200 mM DTT (Fisher)) and boiled for 5 min. Protein quantification was performed using the DC Protein Assay (BioRad) according to the manufacturer's instructions. Immunoblotting was performed and samples probed for HIF1α (Cell Signaling CST# 36169), α-tubulin (Sigma Cat# T6199), and nuclear lamin B1 (Cell Signaling CST# 12586).

Response to EGF stimulation was assessed by seeding 7.5 x 10^4 WT or *capns1* KO MTECs in 60 mm dishes (Sarstedt) overnight in complete IMEM. MTECs were washed once in PBS and changed to serum-free IMEM overnight, then stimulated with 3 mL of 50 ng/mL EGF (Thermo Fisher) prepared in serum-free IMEM for the indicated times. Cells were placed on ice and washed once with PBS containing 1 mM sodium orthovanadate (Sigma) and lysed into RIPA (as described in 2.3.2). Immunoblotting was performed, and samples probed for EGFR (Cell Signaling CST# 2232), P-EGFR (Y845) (Cell Signaling CST# 2231), MEK (Cell Signaling CST# 9122), P-MEK (S217/221) (Cell Signaling CST# 9121), ERK (Cell Signaling CST# 9102), and P-ERK (T202/Y204) (Cell Signaling CST# 9101).

2.3.4 Matrix Metalloprotinease Assay

To assess the secretion of matrix metalloproteinase 2 and 9 (MMP2/9), 7 x 10^5 WT or *capns1* KO MTECs were seeded overnight on 60 mm tissue culture dishes (Sarstedt) in complete IMEM, and the following day plates were washed twice with PBS and overlaid with 3 mL of serum-free IMEM. Cells were allowed to condition the medium for 48 h and gelatin zymography was performed as described by (Toth et al., 2012).

2.3.5 Orthotopic Tumor Growth and Experimental Metastasis Studies

One x 10^6 WT or *capns1* KO MTECs were suspended in 50 μL of 50 % phosphate-buffered saline, and 50 % growth factor-reduced matrigel (VWR), and engrafted into the number four (abdominal) mammary gland of *BalbC-Rag2*$^{-/-}$*/IL2Rγc*$^{-/-}$ mice. After 7 d, staples were removed from surgical sites and tumor volumes assessed until endpoints. At endpoints, mice were euthanized, and tumors resected and lysed into RIPA buffer containing protease and phosphatase inhibitors (as described in 2.3.2) for calpain immunoblotting analysis. To assess metastatic potential, MTECs were transduced with GFP-expressing lentivirus (pWPXLd lentiviral vector was a gift from Didier Trono, Addgene plasmid # 12258), and 1 x 10^6 GFP-expressing WT or *capns1* KO MTECs were injected into the tail vein of *BalbC-Rag2*$^{-/-}$*/IL2Rγc*$^{-/-}$

mice. Mice were euthanized at 1, 48, and 168 h post-injection, and lungs were recovered for GPF imaging using a 20x objective. Mice were housed at the Queen's University Animal Care Facility, and all procedures were carried out in accordance with the guidelines specified by the Canadian Council on Animal Care, with approval from the University Animal Care Committee.

2.3.6 Statistical Analysis

All statistical analysis were performed using Prism Graphpad 5. A p-value of <0.05 was deemed significant for the student's t-test, and Log-Rank (Gehan-Breslow-Wilcoxon) test.

2.4 Results

2.4.1 Deletion of *capns1* in the mammary epithelium delays *her2/neu*-induced tumorigenesis

The stability and activity of calpain-1 and calpain-2 are contingent on the expression of the common shared regulatory subunit encoded by *capns1* (Graham-Siegenthaler et al., 1994; Meyer et al., 1996) and *capns1* knockout in transgenic mice abolishes both calpain-1 and calpain-2 (Arthur et al., 2000; Tan et al., 2006a). To test the potential involvement of calpain-1 and -2 in HER2-driven mammary tumorigenesis, we crossed the *NIC* transgenic mouse model (Ursini-Siegel et al., 2008) with conditionally targeted (*floxed*) *capns1* mice (Tan et al., 2006a). Co-expression of Cre recombinase with oncogenic Her2/Neu resulted in deletion of *floxed capns1* alleles and ablation of CAPNS1 expression in mammary tumors arising in *NIC capns1*$^{flox/flox}$ (*KO*) mice, while tumors from *NIC capns1*$^{+/+}$ (*WT*) mice retained CAPNS1 expression (Figure 2.1). Deletion of *capns1* in the mammary epithelium correlated with a significant delay in spontaneous tumor onset (median time *KO*=318 vs *WT*=300 days; p=0.0277*) (Figure 2.2). While these data show that calpain-1 and/or calpain-2 are not necessary for HER2/NEU-driven tumorigenesis, they indicate that these calpains contribute to carcinogenesis. To assess possible calpain-mediated signaling pathways that underpin this delay, an RPPA analysis was performed on tumor lysates

using 128 antibodies, predominantly directed against phosphopeptides in important signaling nodes (Gujral et al., 2013; Luckert et al., 2012). This analysis revealed five proteins (EGFR, JNK, STAT1, MARCKS and GSK3β) that were differentially phosphorylated in WT and KO tumors (Figure 2.3).

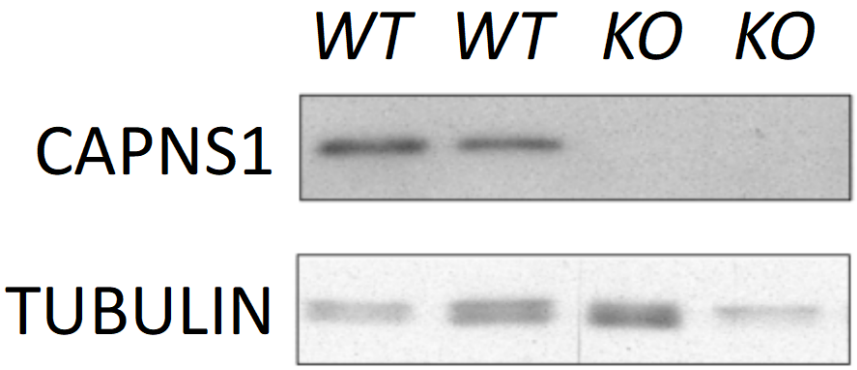

Figure 2.1 CAPNS1 expression is ablated in mammary tumors from *NIC capns1*$^{flox/flox}$ mice.

Tumor lysates from *NIC capns1*$^{flox/flox}$ (*KO*) or *NIC capns1*$^{+/+}$ (*WT*) female mice were assessed for CAPNS1 (28 kDa) or tubulin (50 kDa) expression by immunoblotting.

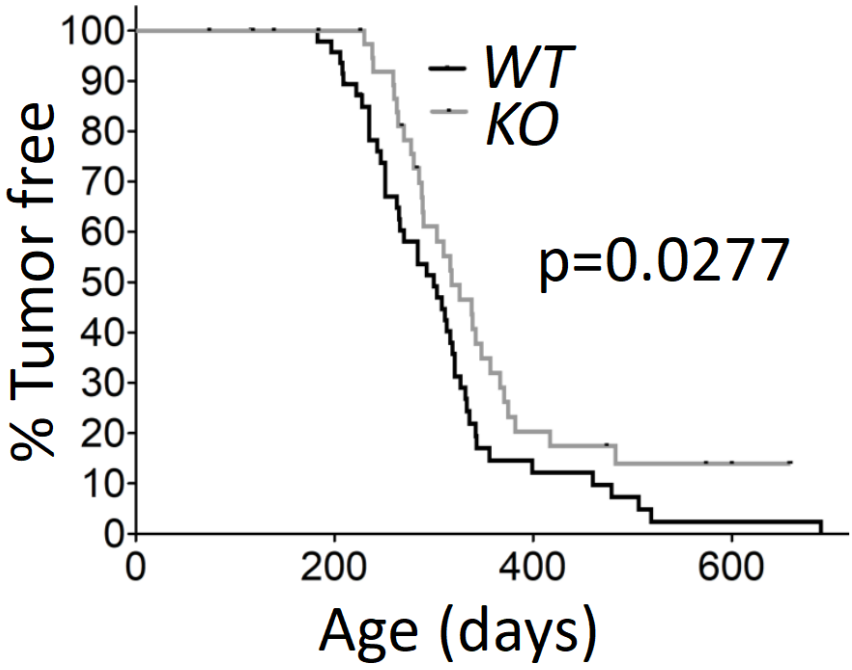

Figure 2.2 Deletion of *capns1* in the mammary epithelium delays HER2/NEU-driven tumorigenesis.

NIC capns1$^{flox/flox}$ (KO) or *NIC capns1*$^{+/+}$ (WT) female mice were assessed for tumor onset by weekly palpitation. Median tumor onset was 318 vs 300 days, (n = 43 vs 48, respectively, p=0.0277 Gehan-Breslow-Wilcoxon Test).

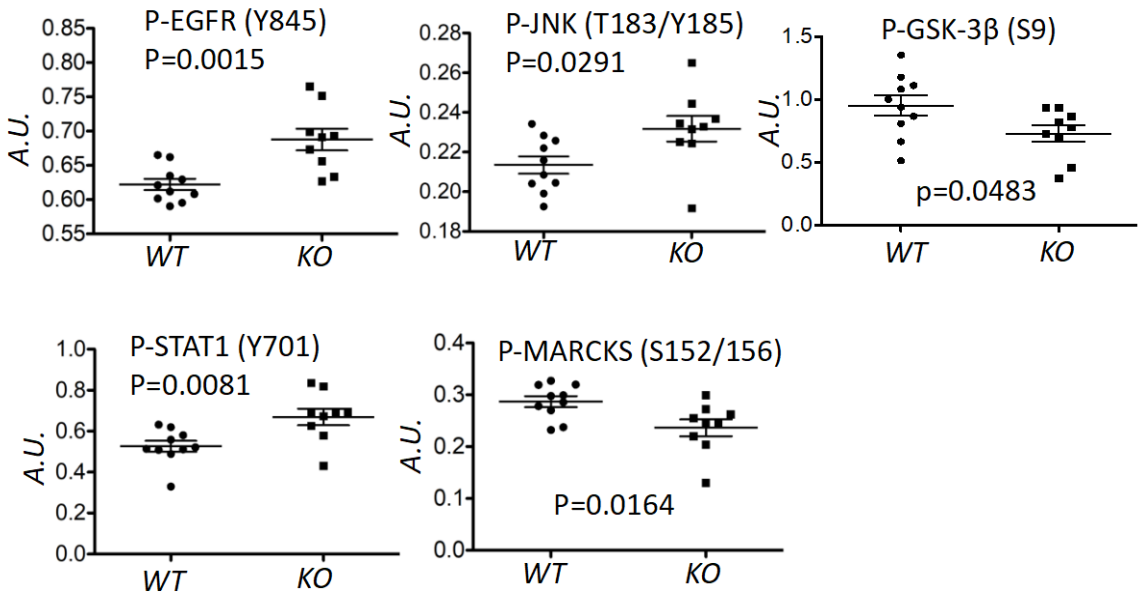

Figure 2.3 Deletion of *capns1* in HER2/NEU-driven mouse mammary tumors correlates with a differential phosphoproteome.

Spontaneous tumors arising in *NIC capns1*$^{flox/flox}$ (KO, n=9) or *NIC capns1*$^{+/+}$ (WT, n=10) female mice were subject to RPPA analysis with 128 antibodies. The indicated phosphoproteins displayed significantly different signal intensity (A.U.).

2.4.2 Establishment of a conditional *capns1* knockout *her2/neu*-driven mammary tumor epithelial cell line

In order to further study the involvement of calpains-1/2 in *her2/neu*-driven tumorigenesis we next established a conditional *capns1* knockout mammary carcinoma cell line model. *Floxed capns1* mice were crossed with Neu^{NT} transgenic mice (Muller et al., 1988), which express oncogenic HER2/NEU predominantly in the mammary epithelium under the control of the MMTV LTR. A tumor arising in a neu^{NT} $capns1^{flox/flox}$ female mouse was used to derive a mammary tumor epithelial cell (MTEC) line. To create isogenic MTECs without or with calpain-1/2 activity, we transduced these $capns1^{flox/flox}$ MTECs with retroviruses encoding either Cre-recombinase (KO) or the empty vector (WT). The retrovirus also encoded puromycin phosphotransferase, which enabled selection of transduced cell populations. Immunoblotting of the puromycin selected populations revealed the expected loss of CAPNS1 protein expression in Cre-transduced cells, and casein zymography showed the loss of both calpain-1/2 activities (Figure 2.4). Mouse embryonic fibroblasts (MEFs) generated from $capns1^{+/+}$ (WT) and $capns1^{-/-}$ (KO) embryos served as controls in this analysis (Tan et al, 2006). MTECs express predominantly calpain-2, with only trace amounts of calpain-1.

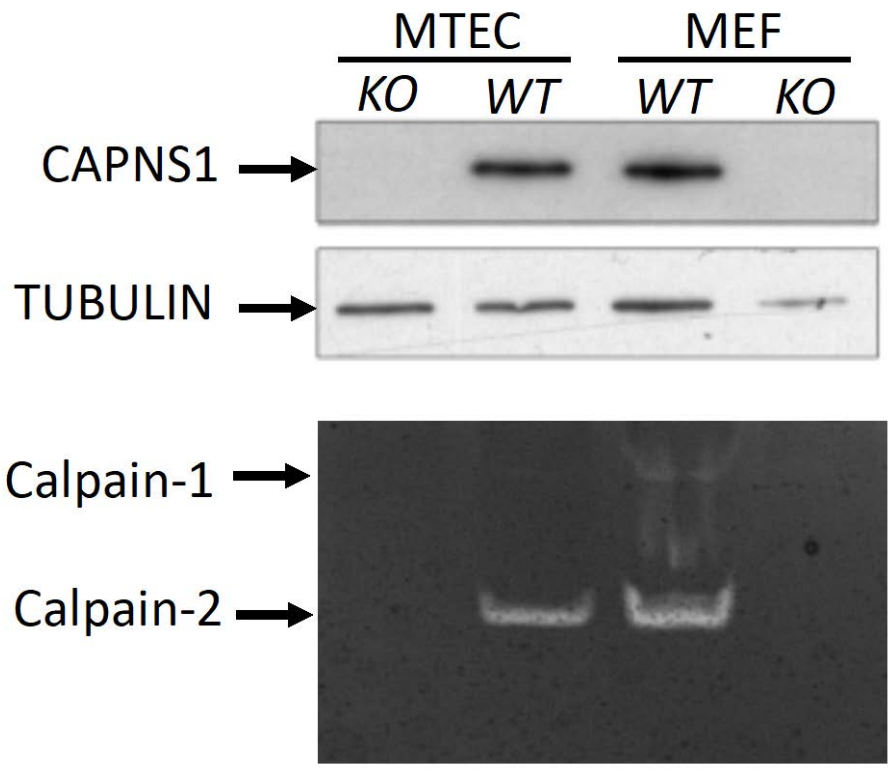

Figure 2.4 CAPNS1 protein and calpain-1/2 activities are ablated by *capns1* deletion.

(Upper) Lysates from *capns1*$^{flox/flox}$ MTECs transduced with Cre-expressing (KO) or control (WT) retroviruses; or *capns1*$^{+/+}$ (WT) or *capns1*$^{-/-}$ (KO) mouse embryonic fibroblasts (MEFs) were subjected to immunoblotting analysis with antibodies reactive against CAPNS1 (28 kDa) or tubulin (50 kDa). (Lower) Casein zymography assessment of calpain-1 and calpain-2 activities in lysates from the same panels of MTEC and MEF cells.

2.4.3 *Capns1* deletion attenuates *in vitro* MTEC invasion but does not compromise migratory ability

Calpains-1/2 have been implicated in membrane-cytoskeletal dynamics associated with cell migration and invasion (Franco et al., 2004b; Huttenlocher et al., 1997). *In vitro* migration and invasion were compromised by *capns1* KO in mouse embryonic fibroblasts (Dourdin et al., 2001; Postovit et al., 2002a) or *capns1* knockdown in MDA-MB-231 breast cancer cells (Grieve et al., 2016); and *capn2* knockdown was associated with dysregulated focal adhesion turnover (Franco et al., 2004b), lamellipodia dynamics (Franco et al., 2004a) in fibroblasts, as well as attenuated migration and invasion in mammary carcinoma cells (Ho et al., 2012). To determine whether calpains-1/2 were important for cell migration in the MTEC model, we performed wound healing assays with WT and KO cells. Surprisingly *capns1* KO MTECs did not display a migration defect (Figure 2.5 (A)). However, when the invasive ability of MTECs was assessed using matrigel-coated transwell Boyden chamber assays, *capns1* KO correlated with significantly attenuated cell invasion activity (Figure 2.5 (B), $p=0.006$).

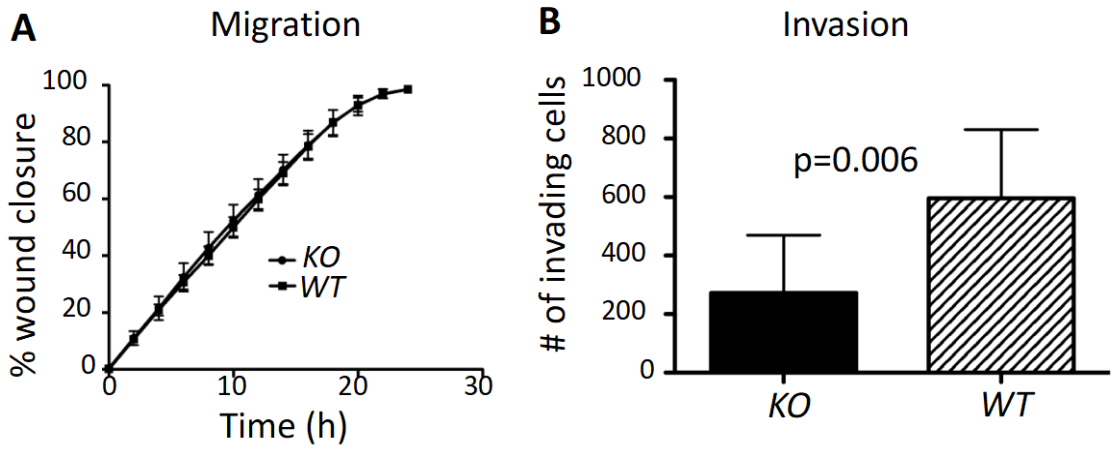

Figure 2.5 *capns1* KO attenuates MTEC *in vitro* invasion but does not affect migration.

(A) Temporal profiles of *capns1* KO and WT MTEC migration behavior in scratch-wound healing assays. Fifteen thousand MTECs were seeded on ImageLock 96-well plates and confluent monolayers were scratch-wounded after an overnight incubation. Migration was monitored in an IncuCyte system for 24 h. Data are the means ± SD of three independent experiments run in sextuplicate. (B) Boyden chamber assays measured *capns1* KO and WT MTEC invasion after 24 h through 8 mm pores of transwells coated with 15 mg of matrigel. Data are means ± SD of three independent experiments run in triplicate (p=0.006, student's t-test).

2.4.4 *Capns1* KO sensitizes MTECs to doxorubicin and lapatinib challenge

Lapatinib is a small molecule tyrosine kinase inhibitor (TKI) of both HER2 and EGFR. It has similar clinical efficacy to trastuzumab in a neoadjuvant setting (Robidoux et al., 2013), and may also be used as a second-line therapy when resistance to trastuzumab develops (Burstein et al., 2008; Geyer et al., 2006). Doxorubicin is an anthracycline which causes DNA damage by targeting topoisomerase II and is widely used as a cancer therapeutic. Doxorubicin resistance is mediated in part by members of the ABC family of transporters including P-glycoprotein (Pgp) and multidrug resistance protein 2 (MRP2) (Kartal-Yandim et al., 2016). We recently reported that expression of Pgp and MRP2, and sensitivity to doxorubicin correlates with calpain-1/2 expression (Grieve et al., 2016). Furthermore, cleavage of topoisomerase IIα by calpain-2 has been suggested as an additional mechanism of doxorubicin resistance (Jeon et al., 2018b). Thus, we sought to determine whether loss of calpain-1/2 in a HER2+ tumor model might cooperate with therapeutic challenge from these two commonly used treatments of breast cancer. Lapatinib exposure for 72 h was associated with a trend towards reduced viability in *capns1* KO MTECs, with significantly reduced viability at both 15 μM and 17 μM ($p=0.0033$ and $p=0.0066$, respectively) (Figure 2.6). Upon challenging *capns1* KO or WT MTECs with doxorubicin for 72 h, a similar trend towards increased cytotoxicity in KO MTECs was observed, with significantly reduced viability noted at 1 μM ($p=0.0271$) (Figure 2.7).

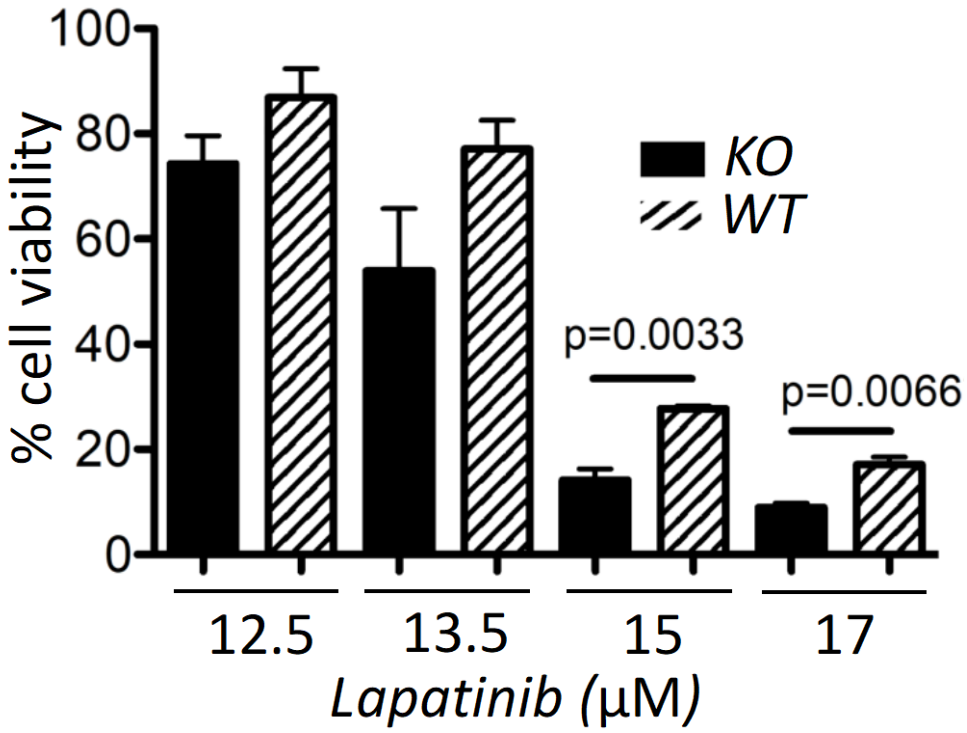

Figure 2.6 *capns1* KO enhances sensitivity to lapatinib.

Two x 10^4 *capns1* KO or WT MTECs were seeded on 96 well plates, then cultured overnight. The following day, cells were treated for 72 h with lapatinib at the indicated concentrations. Cell viability was assessed at endpoint using the PrestoBlue viability reagent. Data are means ± SD of three independent experiments with experimental triplicates ($p<0.05$, student's t-test).

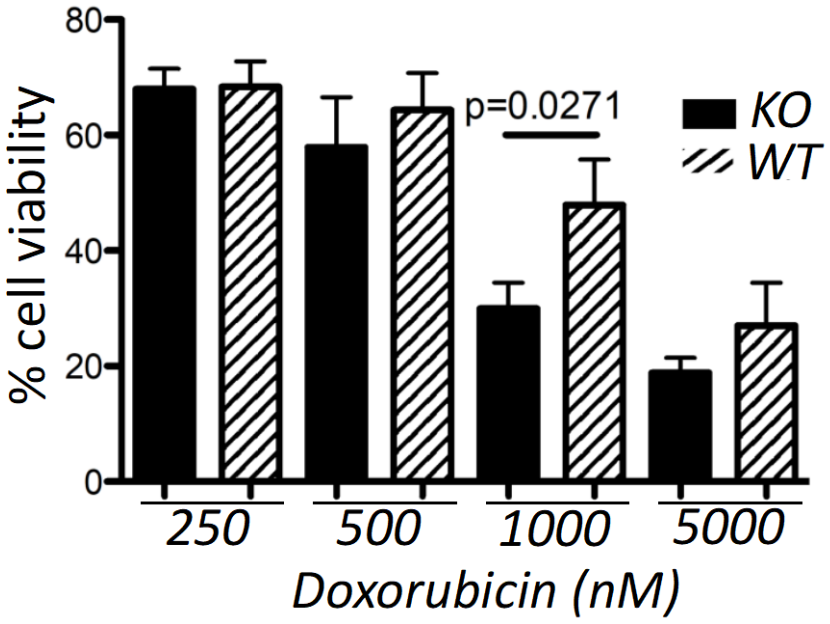

Figure 2.7 *capns1 KO* enhances sensitivity to doxorubicin.

Two x 10^4 WT or *capns1* KO MTECs were seeded on 96 well plates and cultured overnight. Doxorubicin was added the following day at the indicated concentrations and viability was assessed 72 h later using the PrestoBlue viability reagent. Data are means ± SD of three independent experiments with technical triplicates (p=0.0271, student's t-test).

2.4.5 Deletion of *capns1* blocks MTEC tumorigenesis in an orthotopic engraftment model

Previously, we showed that *shRNA*-mediated knockdown of *capn2* in an autocrine hepatocyte growth factor/Met receptor-driven murine mammary carcinoma engraftment model (Ho et al., 2012) or knockdown of *capns1* in a human TNBC xenograft model (Grieve et al., 2016) attenuated both tumor growth at the orthotopic site and metastatic progression. Here, using a *floxed capns1 her2/neu*-driven MTEC carcinoma model, we asked if complete ablation of calpain-1 and calpain-2 would result in a more effective block in tumorigenesis in an orthotopic engraftment model. WT MTECs produced rapidly growing tumors with a mean onset time of 29 days, while tumor onset was delayed to a mean of 79 days after engraftment with KO MTEC cells, at which point KO tumors grew at comparable rates to WT tumors (Figure 2.8). When resected and assessed by immunoblotting, comparable levels of CAPNS1 were observed in both WT and KO tumors (Figure 2.9). This indicates that KO tumors arose from rare "escaper" WT MTECs which had not undergone Cre-mediated deletion of the *floxed capns1* alleles and required several more weeks to emerge as palpable tumors. Delayed tumor growth behaviour was validated in an independent experiment with nearly identical results (Figure 2.10).

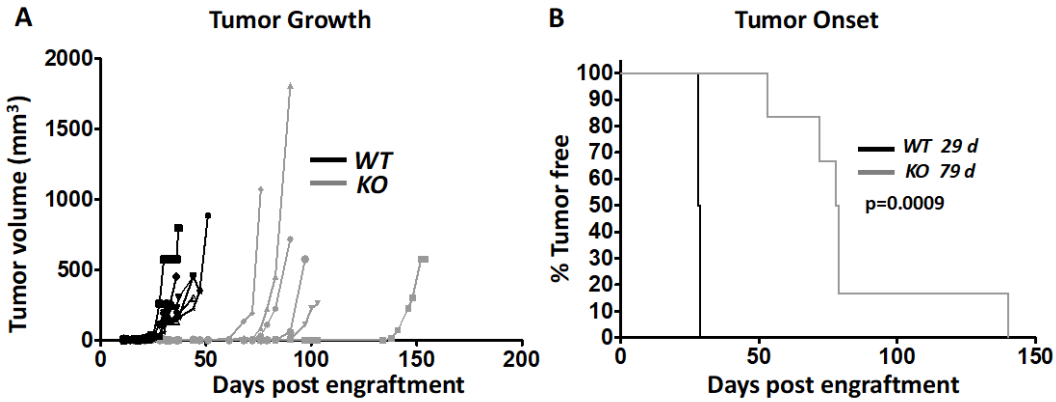

Figure 2.8 *capns1 KO* reduces MTEC tumorigenic potential.

One x 10^6 *capns1* KO or WT MTECs were engrafted into the number four mammary glands of female *Rag2*$^{-/-}$ *IL-2Rγc*$^{-/-}$ mice (n=6 for each cohort). (A) Caliper measurements of tumor volumes. (B) Median time to onset of palpable tumors at the engraftment site were 79 or 29 days for *KO* and *WT*, respectively (p=0.0009, Log-rank (Mantel-Cox) test).

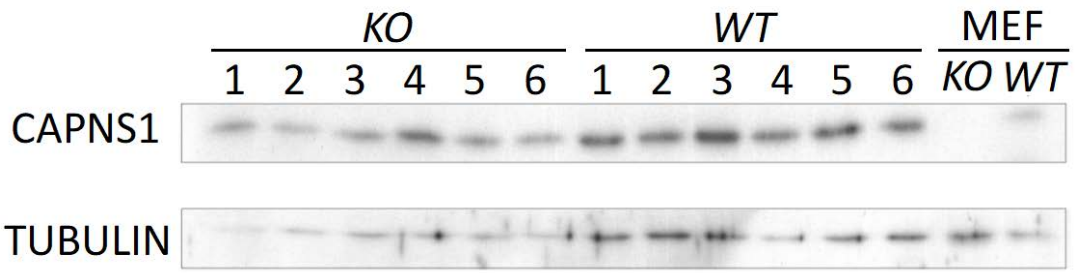

Figure 2.9 Late arising tumors in mice engrafted with *capns1 KO* MTECs display CAPNS1 expression.

Immunoblot analysis of tumor lysates reveals CAPNS1 expression (28 kDa) in tumors from both *capns1* WT and KO engrafted MTECs. Lysates from WT and KO MEF cells served as positive and negative controls for CAPNS1. Immunoblotting for tubulin (50 kDa) confirmed comparable sample loading.

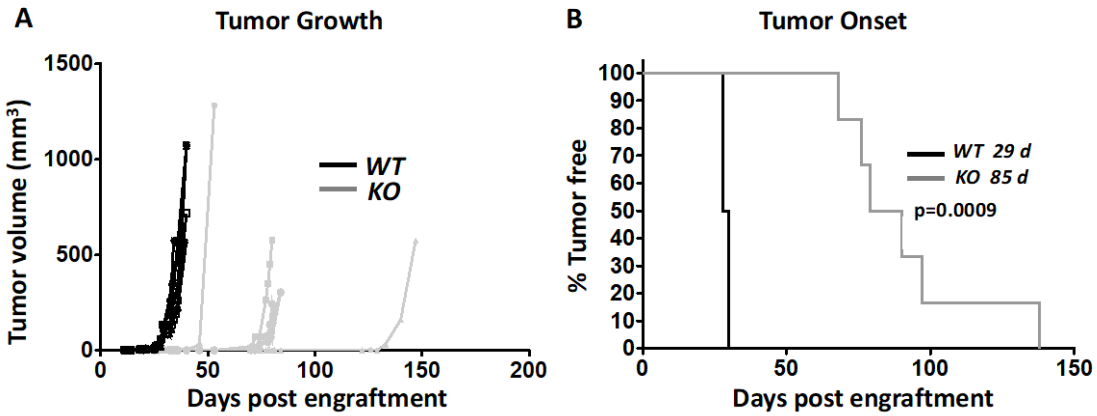

Figure 2.10 *capns1 KO* is associated with reduced tumorigenic potential.

One x 10^6 *capns1* KO or WT MTECs were engrafted into the number four mammary glands of female *Rag2*$^{-/-}$ *IL-2Rγc*$^{-/-}$ mice (n=6 for each cohort). (A) Caliper measurements of tumor volumes. (B) Medium time to onset of palpable tumors at the engraftment site were 85 or 29 days for KO and WT, respectively (p=0.0009, Log-rank (Mantel-Cox) test). All tumors arising in the KO group displayed expression of *capns1* (data not shown).

Tumor progression can be limited by failure to drive *de novo* angiogenesis in response to hypoxia due to an inability to trigger the angiogenic switch (Semenza, 2002). Given that calpain-deficient MTEC tumors failed to develop to a palpable size, we next explored the possibility that calpain-1/2 deficient cells might not be able to respond *in vivo* to hypoxia during tumor growth. Hypoxia-inducible factor 1α (HIF1α) is a transcription factor that is normally degraded by the proteasome via hydroxylation by prolyl hydroxylases under normoxic conditions, but is stabilized under hypoxic conditions to direct transcription of genes containing pro-survival hypoxic response elements including VEGF, which is needed to promote tumor-associated neoangiogenesis (Tug et al., 2009). Calpain has been shown to modulate a hypoxic response through generation of a filamin-A fragment which mediates nuclear localization of HIF1α (Zheng et al., 2014). To test the involvement of calpain in HIF1α regulation, we compared whole cell lysates and nuclear extracts of HIF1α levels in *capns1* KO and WT cells cultured at either 0.1 % (hypoxic) or ambient (normoxic) oxygen concentrations for 24 h in the absence of serum. Cobalt chloride was used as a positive control for HIF1α stabilization. Hypoxia or $CoCl_2$ stabilized comparable levels of HIF1α in WT and KO MTECs in both whole cell lysates and nuclear fractions (Figure 2.11), suggesting that calpain does not play a key role in the angiogenic switch in this model system.

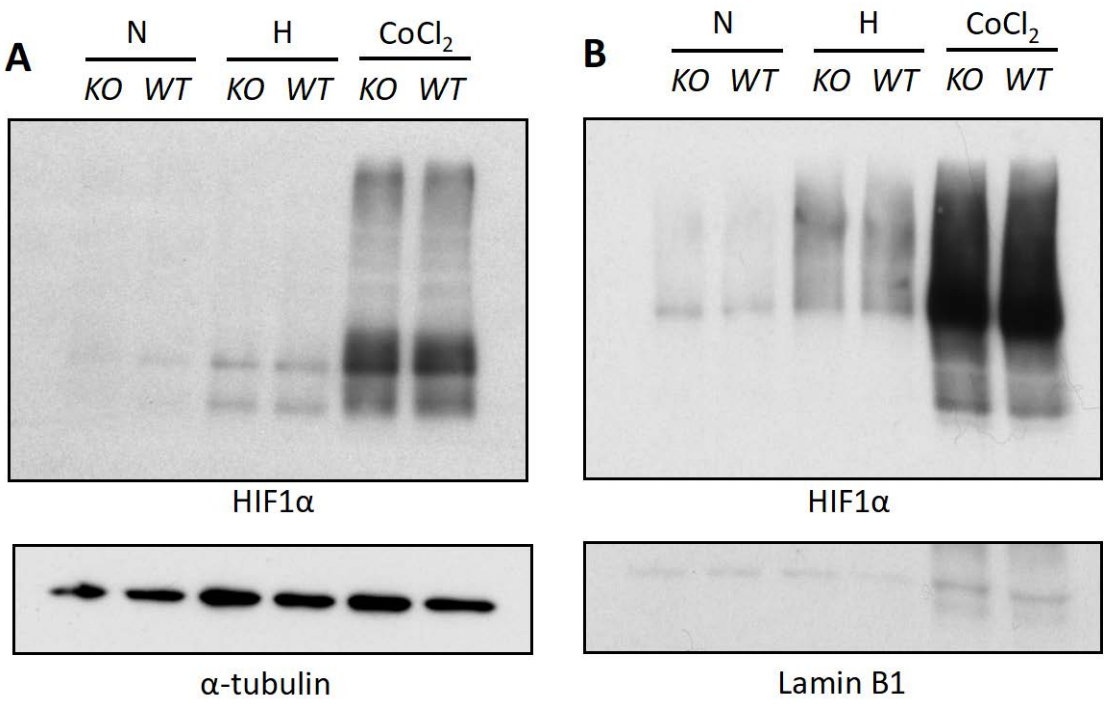

Figure 2.11 *capns1* KO does not affect hypoxia-induced stabilization of HIF1α.

One and a half x 10^6 *capns1* KO or WT MTECs were seeded for 24 h under conditions of hypoxia (H) (0. 1% O_2), normoxia (N) (21 % O_2) or challenge with $CoCl_2$ (100 mM). Lysates were collected and protein levels of HIF1α (120 kDa) were assessed by immunoblotting. Tubulin (50 kDa) and nuclear Lamin B1 (68 kDa) immunoblotting was performed to assess sample loading. (A) Whole cell lysate immunoblotting for HIF1α (B) Nuclear-enriched immunoblotting for HIF1α.

The production and activation of matrix metalloproteases (MMPs) has also been linked to tumor growth, invasion and metastasis (Jones et al., 1999; Li et al., 2004; Somiari et al., 2006). Gelatin zymography analysis of conditioned media from *capns1* KO and WT MTECs revealed abundant levels of MMP2 and lower levels of MMP9, but there were no differences between the WT and KO MTECs that might contribute to the observed differential tumor growth *in vivo* (Figure 2.12).

Finally, to determine if loss of *capns1* correlated with an overall inability to survive and proliferate *in vivo*, we injected *capns1* KO or WT MTECs into the tail veins of female *Rag2*$^{-/-}$ *IL-2Rγc*$^{-/-}$ mice and examined lung metastatic lesions at 1, 48, and 168 hours after injection (Figure 2.13). *Capns1* KO and WT MTECs both successfully seeded in the lungs and survived and proliferated into micro-metastatic lesions over the course of the next 2 to 7 days.

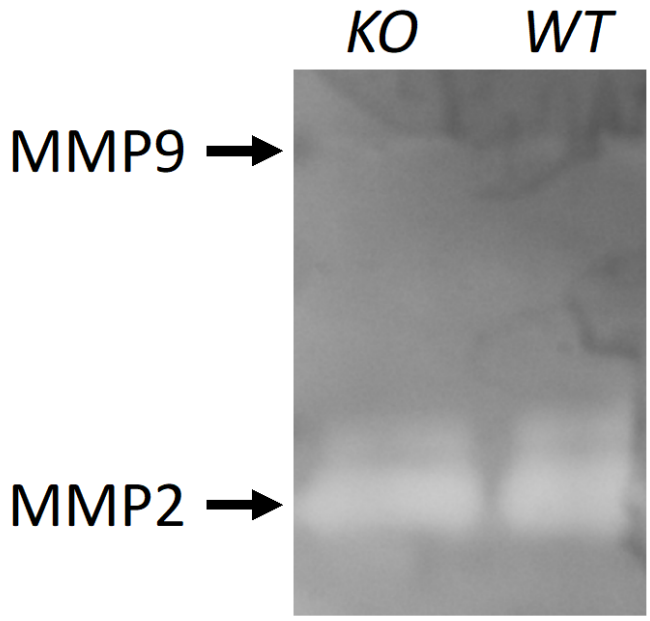

Figure 2.12 *capns1* KO does not affect secretion or activation of matrix metalloproteinases 2 and 9.

Seven x 10^5 *capns1* KO or WT MTECs were seeded and cultured overnight, then changed to serum-free medium the following day. Conditioned media was collected after 24 h and gelatin zymography performed to assess MMP activity.

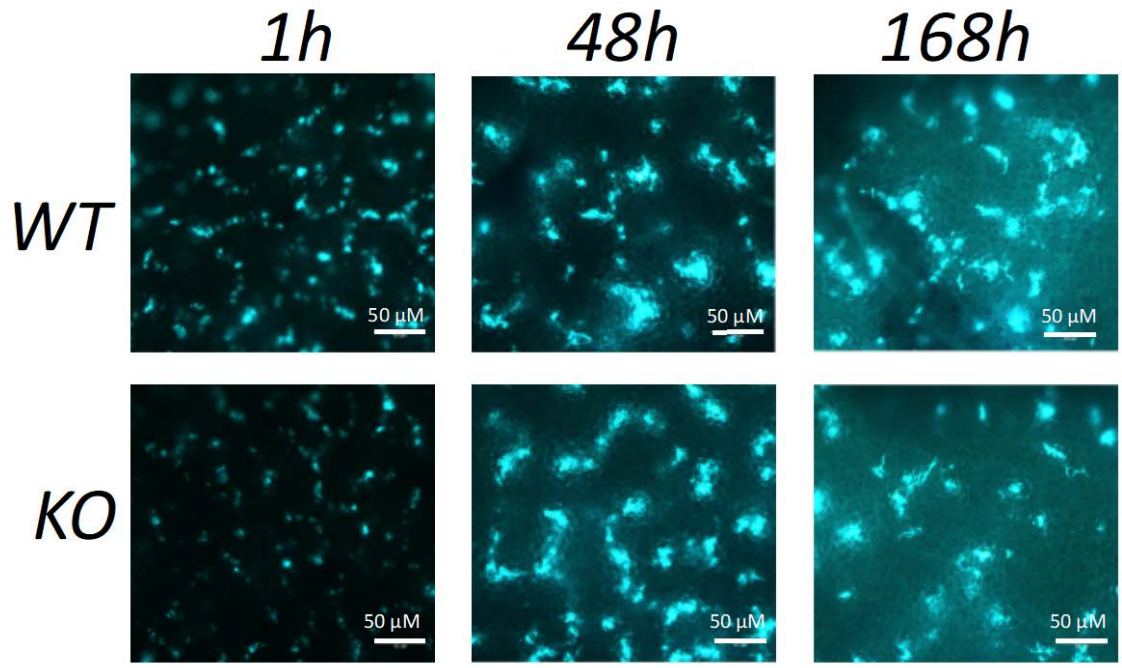

Figure 2.13 *capns1* KO in MTECs does not compromise formation of lung metastatic lesions in an experimental metastasis model.

One x 10^6 GFP transduced *capns1* KO or WT MTECs were injected into tail veins of *Rag2*$^{-/-}$*IL-2Rγc*$^{-/-}$ mice. Lungs were resected and imaged by fluorescence microscopy at the indicated post injection times.

2.4.6 *Capns1* KO in MTECs correlates with enhanced EGFR expression and sustained EGF response

EGFR is a key dimerization partner of HER2, and is frequently expressed in breast carcinomas (Walker and Dearing, 1999). Deletion of *capns1* was associated with enhanced phosphorylation of EGFR (Y845) in tumors from the *NIC* model (Figure 2.2); we therefore tested EGF-induced activation of EGFR *in vitro* in the MTEC system. Overnight serum starvation followed by EGF stimulation resulted in a rapid induction in pY845-EGFR levels in both *capns1* KO and WT MTECs (Figure 2.14). However, there is also a difference in kinetic behaviour, with a more sustained EGFR activation in the *capns1* KO MTECs; which correlates with more robust and sustained activation of MEK and ERK, as shown by their phosphorylation at activation loop sites (Figure 2.14 A,C,D). We also noted an increase in steady-state levels of EGFR in the *capns1* KO MTECs (Figure 2.14 B). These data are consistent with a role for calpains-1/2 in the regulation of EGFR signaling down the RAS-MAPK pathway.

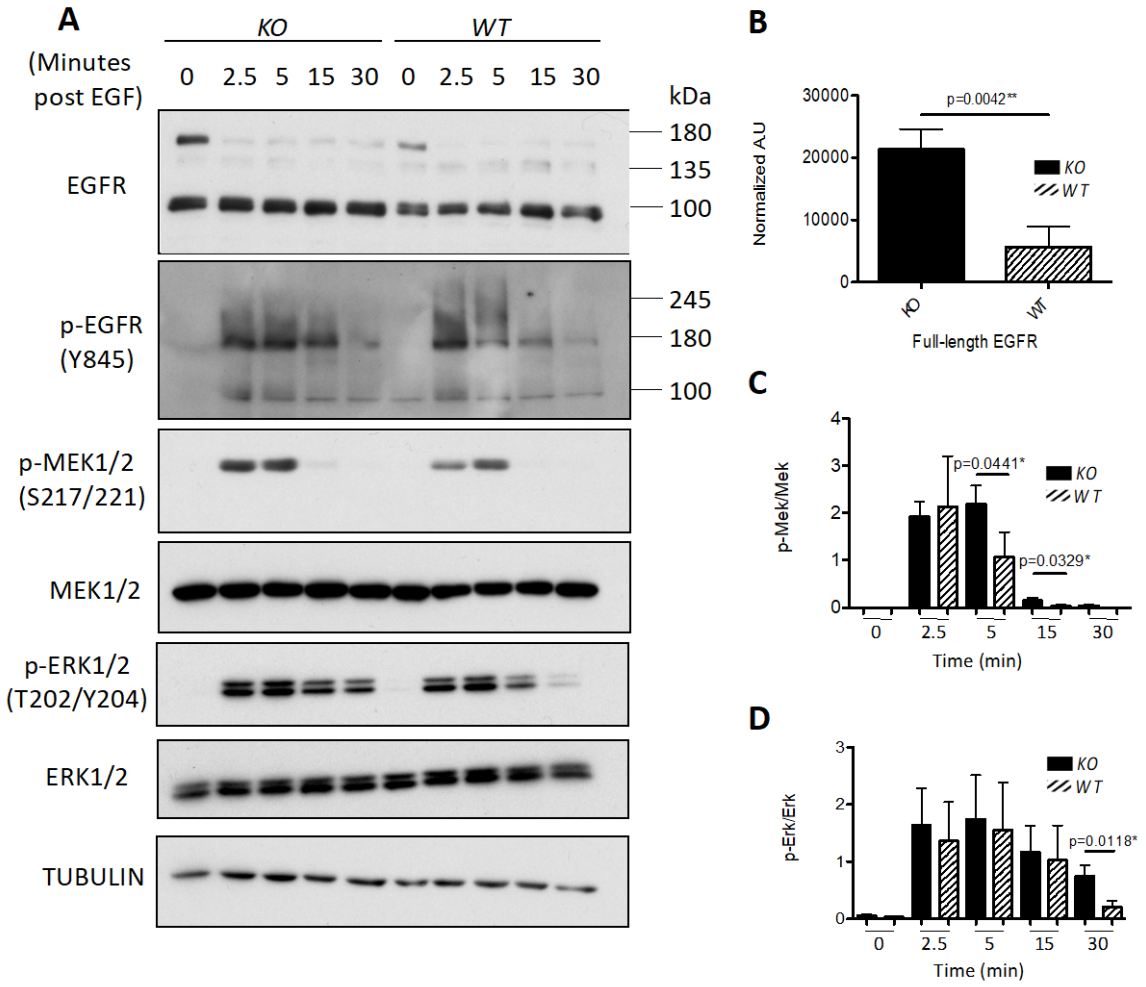

Figure 2.14 Knockout of calpain enhances EGFR expression and prolongs MAPK signaling.

7.5 x 10^4 WT or *capns1* KO MTECs were seeded overnight in 60 mm dishes. The following day cells were washed with PBS and changed to serum-free media. MTECs were stimulated with 50 ng/mL of EGF for the indicated times, and lysed into RIPA. (A) Assessment of *capns1* KO on MAPK signaling. (B) Immunoblot of full-length EGFR (175 kDa) at time 0. (C) Assessment of EGF-induced MEK (45 kDa) phosphorylation. (D) Assessment of EGF-induced ERK (42/44 kDa) phosphorylation.

2.5 Discussion

Approximately 15-20% of newly diagnosed breast cancers are the highly aggressive HER2+ subtype (Yersal and Barutca, 2014), and despite treatment with chemotherapy and targeted therapeutics such as trastuzumab and lapatinib, half of these patients relapse with resistant disease within one year (Gelmon et al., 2015). These clinical findings underscore the urgent need to develop more effective treatments for HER2+ breast cancer. This will require a better understanding of the biology of these tumors, including the signaling pathways that confer resistance to current treatments and identification of druggable components of these pathways that might represent novel combinatorial therapeutic targets.

Our previous work has shown that the ubiquitously expressed calpain-1 and -2 isoforms regulate cell survival in response to cytotoxic challenges (Tan et al., 2006b; Tan et al., 2006c) and promote breast cancer cell line resistance to chemotherapeutics including doxorubicin and cisplatin (Grieve et al., 2016). We also showed that *capn2* knockdown in a mouse mammary carcinoma cell line was associated with reduced tumor growth in a mouse orthotopic engraftment model (Ho et al., 2012), and *capns1* knockdown in the MDA-MB-231 triple negative breast cancer cell line correlated with reduced tumor growth and metastasis in an orthotopic xenograft model, and this effect was enhanced by combination with the HSP90 inhibitor, 17-AAG (Grieve et al., 2016).

The relationship between expression of calpains and clinical outcomes in various cancers are being explored (Storr et al., 2011a; Storr et al., 2015), and CAPN1 expression in HER2+ breast cancer has been positively correlated with shorter relapse-free survival and resistance to trastuzumab (Storr et al., 2011c). This is consistent with *in vitro* studies showing that *capns1* expression was associated with reduced trastuzumab sensitivity in the HER2+ human breast cancer cell lines SKBR3 and BT474 (Kulkarni et al., 2012).

Here we report the first study exploring the role of the ubiquitously expressed classical calpain-1/2 isoforms in a genetically engineered mouse model of spontaneous tumorigenesis driven by the *her2/neu* oncogene. By deleting *capns1* in the mammary epithelium of animals expressing oncogenic

HER2/NEU we show that calpains-1/2 are not required for tumorigenesis, but their absence is associated with a significant delay in tumor onset in this model (Figure 2.2). Phosphoproteomic analysis of these tumors was performed to explore the possibility that differences in the carcinogenic process in the presence or absence of calpains-1/2 might be revealed by distinct activation states of specific signaling nodes. Our analysis detected significant differences in 5 phosphoproteins (EGFR, GSK-3β, JNK, MARCKS and STAT-1) out of the 128 proteins queried (Figure 2.3), suggesting that the carcinogenic program pursued by these HER2/NEU-driven tumors was subtly different in the absence of calpains-1/2.

Glycogen synthesis kinase 3 β (GSK-3β) has complex roles in cell signaling, including a tumor suppressor function through inhibition of the β-catenin complex (Mancinelli et al., 2017). GSK-3β is inhibited by AKT-mediated S9 phosphorylation and activated by PP2A-mediated dephosphorylation. Reduced p-S9-GSK-3β levels in *capns1* KO tumors is consistent with previous observations of reduced AKT activation in *capns1* KO MEF cells (Tan et al., 2006c), and increased PP2A levels in *capn2* knockdown mammary carcinoma cells (Ho et al., 2012) and *capns1* KO MEFs (Bertoli et al., 2009).

The c-Jun N-terminal kinase (JNK) is activated in response to a number of ligands and stress stimuli and subsequently regulates transcription, migration and apoptosis (Huang et al., 2004). Increased pT193/Y185-JNK in *capns1* KO tumors was somewhat surprising since *capns1* KO MEF cells had previously been shown to display reduced activation in response to endoplasmic reticulum stress (Tan et al., 2006b). This difference may reflect distinct effects of calpain deficiency in cancer cells and immortalized fibroblasts, or distinct context-specific roles of calpain in endoplasmic reticulum stress and tumorigenesis.

Calpain-mediated cleavage of protein kinase C (PKC) is an evolutionally conserved mechanism for PKC activation (Hu et al., 2017), and PKCβ deletion was correlated with suppressed tumorigenesis in a transgenic mouse model of breast cancer (Wallace et al., 2014). Myristoylated alanine-rich protein kinase C substrate (MARCKS) displayed reduced pSer-152/S162 levels in *capns1* KO tumors. Elevated

p-Ser-152/156-MARCKS levels have been positively associated with more aggressive breast cancer and reduced sensitivity to chemotherapy in breast cancer cell lines (Chen et al., 2015).

STAT-1 behaves as a tumor suppressor through promotion of apoptosis as well as inhibition of angiogenesis, tumor growth, and metastasis (Stephanou and Latchman, 2003). A role for STAT-1 in IL-27 induced suppression of epithelial to mesenchymal transition has also been described (Kachroo et al., 2013). Increased pY701-STAT-1 observed in *capns1* KO tumors could reflect a role for calpain in regulating these tumor suppressor functions.

These differences in the phosphorylation states of EGFR, GSK-3β, JNK, MARCKS and STAT-1 in *capns1* KO tumors represent important leads for future studies aimed at elucidating the molecular involvement of calpain in HER2+ breast cancers. EGFR in particular represents an important target since it is often dysregulated in human cancers. EGFR forms heterodimers with HER2 and contributes to survival and proliferative signaling in response to EGF (Hynes and Lane, 2005). Enhanced phosphorylation of the activation loop Y845 residue in *capns1* KO tumors indicated a higher level of activated EGFR (Figure 2.3). Both EGFR (Gregoriou et al., 1994) and HER2 (Kulkarni et al., 2010) have been identified as substrates of calpain, therefore this observation may simply be a reflection of an accumulation of more mature EGFR in the absence of calpain. Given the importance of this signaling node in HER2 tumorigenesis we focused further study on it using the *capns1 floxed* MTEC model system. There was significantly more of the presumptive mature ~150 kDa EGFR species in *capns1* KO MTEC cells (Figure 2.14 A,B), which supports a role for calpain-1/2 in regulating EGFR levels. While this presumptive mature EGFR is rapidly downregulated after EGF treatment in both *capns1* KO and WT MTECs, more of this 150 kDa band persists in KO cells, and more of a faster migrating 135kDa band was observed in the WT cells. It was tempting to predict that this smaller species arose by calpain cleavage of EGFR, since both EGFR (Gregoriou et al., 1994) and HER2 (Kulkarni et al., 2010) have been identified as a substrates of calpain. However, it is more likely that this 135 kDa band corresponds to immature EGFR in the ER/Golgi compartment. A prominent band at ~100 kDa was detected with antibodies to both

EGFR and pY845-EGFR (Figure 2.14 A). HER2 has been reported to give rise to a 95 kDa species which is associated with elevated PI3K/AKT signaling and trastuzumab resistance (Arribas et al., 2011; Clark et al., 2002; Nagata et al., 2004), and calpain is implicated the generation of intracellular fragments of HER2, as well as the activation of AKT (Kulkarni et al., 2010; Kulkarni et al., 2012; Tan et al., 2006c). EGFR is also a known calpain substrate, but the occurrence of this ~100 kDa species in the MTEC system was not correlated with *capns1* status, so it cannot be a uniquely calpain-generated fragment of EGFR. HER2 levels were very low in these MTEC cells, and even after forced ectopic over-expression of HER2, we were unable to correlate the presence of unique HER2 fragments with *capns1* status (data not shown).

Interestingly, EGF-induced pY845-EGFR levels persisted for longer in *capns1* KO MTECs (Figure 2.14 A), and this correlated with persistence of activated MEK1/2 and ERK1/2 levels (Figure 2.14 A,C,D). These observations are somewhat surprising given the delayed tumor onset in the *capns1* KO *NIC* transgenic model (Figure 2.2) and the complete block in tumorigenic potential in the *capns1* KO MTEC engraftment model (Figure 2.8). However, delayed tumor onset correlating with increased signaling through the ERK1/2 pathway has been observed previously in the Neu^{NT} transgenic mice model and MTEC lines derived from them (Sangrar et al., 2015). We speculate that this may be a reflection of compensatory signaling in the absence of calpain.

We also show that *capns1* KO in MTECs is associated with increased sensitivity to both doxorubicin and lapatinib (Figure 2.6 & 2.7). *Capns1* KO tumors arising *in vivo* in the *NIC* mice displayed enhanced activation of EGFR (Figure 2.3), and this was phenocopied *in vitro* in the MTEC model using EGF stimulation (Figure 2.14). Enhanced activation of EGFR may reflect a survival requirement in the absence of calpain. Given that lapatinib inhibits both HER2 and EGFR, we might attribute enhanced efficacy of lapatinib to an attenuation of compensatory EGFR signaling when calpain is ablated. In the case of doxorubicin, resistance is mediated in part by expression of Pgp, and we have shown that calpain correlates with Pgp expression (Grieve et al., 2016). Furthermore, calpain cleavage of topoisomerase IIα has been shown to promote doxorubicin resistance (Jeon et al., 2018b). Thus, survival

responses triggered by challenge with doxorubicin and lapatinib may engage calpain-dependent pathways, which bodes well for the potential of calpain inhibition to enhance the efficacy of these therapeutics.

The most compelling observation in this study was the complete ablation of the tumorigenic potential of MTECs upon *capns1* KO. Angiogenesis is known to be required for the growth of tumors beyond a size of approximately 2 mm^3. As calpain cleavage of filamin-A has been implicated in the nuclear localization of HIF1α (Zheng et al., 2014), we initially speculated that failure to trigger the angiogenic switch might account for these results. However, overall production as well as nuclear localization of HIF1α were unaffected by calpain status *in vitro* (Figure 2.11). Lung seeding after tail vein injection (Figure 2.13) and MMP-2 and MMP-9 production *in vitro* were also unaffected (Figure 2.12). This suggests that the block in tumorigenesis at the orthotopic site might relate to a role for calpain in stromal conditioning. For example, loss of calpain may impact the ability of MTECs to recruit pro-tumorigenic cancer-associated fibroblasts (CAFs) (Ewald, 2017); perhaps due to a failure to induce autophagy in the CAFs via a paracrine mechanism (Martinez-Outschoorn et al., 2010).

Capns1 KO in this MTEC model did not reduce cell migration in a scrape-wound assay (Figure 4A); however, *capns1* KO was associated with attenuated invasive behavior in matrigel-coated Boyden chamber assay (Figure 4B). This is interesting since *capns1* KO in MEFs has previously been associated with defects in cell migration (Dourdin et al., 2001) as well as invasion (Postovit et al., 2002a); and *capns1* knockdown in MDA-MB-231 cells correlated with reduced migration and invasion (Grieve et al., 2016). Calpain has also been linked with lamellipodia dynamics at the leading edge and focal adhesion turnover at the trailing edge of fibroblasts (Franco et al., 2004a; Franco et al., 2004b). We were surprised that loss of calpain-1/2 in the MTEC system did not attenuate their migration behavior, yet defective invasive behavior was conserved. MMPs are important players in cell invasion, and we have previously linked calpain to MMP production (Postovit et al., 2002a). EGFR and PI3K-AKT signaling has been implicated in the production of MMPs (Lee et al., 2007), and although we were unable to identify differential levels of active MMP-2 and MMP-9 by gelatin zymography (Supplemental Figure 6), this

assay is limited, as it dissociates tissue inhibitors of metalloproteineases (TIMPs), thus we cannot preclude the possibility that calpain participates elsewhere in the MMP activation cascade (Kwak et al., 2012).

In conclusion, our *in vivo* results demonstrate that loss of calpain-1/2 delays spontaneous tumor onset in a model of HER2+ tumorigenesis but does not prevent progression. Phosphoproteomic analysis suggests that tumors arising in the presence of calpain-1/2 engage a subtly different set of pro-tumorigenic signaling pathways from tumors arising in the absence of calpain-1/2. We also provide evidence that calpain is critically required for MTEC tumor progression in an orthotopic engraftment model. Finally, we show that KO of *capns1* enhances cytotoxicity mediated by doxorubicin and lapatinib. Taken together, these results suggest that inhibition of calpain in established malignancies may confer therapeutic benefits and may also enhance the efficacy of currently employed therapeutics like doxorubicin and lapatinib. This underscores the need to develop clinically effective pharmacological inhibitors of calpain-1/2.

Chapter 3

Calpain-1 and calpain-2 play non-redundant roles in tumor growth and metastasis in a model of triple-negative breast cancer

3.1 Abstract

Triple-negative breast cancer is challenging to treat owing to a lack of clinically approved targeted therapeutic agents. Previously, we demonstrated a pro-tumorigenic role for calpain-1 and/or calpain-2 in a MDA-MB-231 xenograft model by knocking down expression of the common obligate CAPNS1 regulatory subunit. Here we demonstrate that calpain-1 and calpain-2 play non-redundant essential roles in metastatic progression in an orthotopic MDA-MB-231 xenograft model by independently knocking out genes encoding the isoform-specific catalytic subunits CAPN1 and CAPN2, respectively. We also show that disruption of calpain-1 and calpain-2 is associated with suppression of tumor growth in this model. These observations of isoform-specific tumor growth and metastasis promoting roles for these two ubiquitously expressed calpains provide important preclinical evidence to justify and inform the future development of calpain inhibitors as cancer therapeutics.

3.2 Introduction

Triple-negative breast cancer (TNBC) is an aggressive subtype of breast cancer which is characterized by poor survival and increased likelihood of metastasis to the brain and lungs (Dent et al., 2007; Heitz et al., 2009). Unlike luminal or HER2 positive breast cancer, TNBC is characterized by a lack of overexpression of the estrogen, progesterone, or HER2 receptors; thus lacking generalizable targeted therapeutics and typically treated through surgery, radiotherapy, and chemotherapy. Agents targeting the epidermal growth factor receptor (EGFR) and downstream components of the RAS-MAPK pathway are being explored in preclinical studies and clinical trials (Baselga et al., 2013; Baselga et al., 2012b; Gelmon et al., 2015; Nagaria et al., 2017; Nagaria et al., 2013), however there is an urgent need for the identification of additional therapeutic targets in TNBC.

Calpain-1 and calpain-2 are calcium-dependent ubiquitously expressed intracellular proteases with pleotropic involvement in cellular behavior through their diverse protein substrates (Goll et al., 2003). They are heterodimers comprised of distinct isoform-specific catalytic subunits (encoded by *capn1* or *capn2*, respectively) coupled with a common regulatory subunit (encoded by *capns1*) that is required for stability and enzymatic activity of the catalytic subunits (Graham-Siegenthaler et al., 1994). Calpain-1 and calpain-2 lack clearly defined target cleavage sequences and typically cleave within disordered peptides, which often link structurally defined functional domains (duVerle et al., 2010; Liu et al., 2011). In this way, calpain cleavage typically modifies, rather than abolishes substrate functions through limited proteolysis. Many calpain substrates have been implicated in tumorigenesis (reviewed in (Storr et al., 2011a)) and in some cases calpain-1 and calpain-2 have been associated with specific cell functions, although there is limited evidence for isoform-specific cleavage of substrates. Compartmentalization of calpains may represent one mechanism by which substrate accessibility is regulated in an isoform-specific manner. For example, calpain-1 has been found localized at the mitochondria, which would contribute to its role in cleavage of apoptosis-inducing factor and BAX (Garcia et al., 2005). Perhaps the most well characterized isoform-specific role for calpain can be seen during cell migration. Calpain-1 activity is

implicated in forming adhesion complexes at the leading edge of a migrating cell through regulating Rho GTPase activity (Kulkarni et al., 1999), and calpain-2 is implicated in tail retraction through cleavage of substrates such as focal adhesion kinase, talin, paxillin, and spectrin (Franco et al., 2004a; Franco et al., 2004b). Germline deletion of *capns1* in mice is embryonic lethal (Arthur et al., 2000; Zimmerman et al., 2000); however, tissue-specific *capns1* deletion or regulated systemic deletion in adults is well tolerated (Amini et al., 2013; Li et al., 2011; Shimada et al., 2005; Tan et al., 2006a; Wernimont et al., 2010; Yang et al., 2015). Germline deletion of *capn1* in mice is associated with a mild hemostasis phenotype and cerebellar ataxia (Azam et al., 2001; Wang et al., 2016). In contrast, *capn2* deletion results in early embryonic death (Dutt et al., 2006; Takano et al., 2011). These observations argue that calpain-1 and calpain-2 have some unique non-redundant roles. Thus, while calpain-1 and calpain-2 have been associated with some specific roles (Table 3.1), our understanding of this is incomplete.

Tissue microarrays reveal that calpain-2 is associated with poor outcome in TNBC (Storr et al., 2012a), and knockdown of *capns1* in the TNBC cell line MDA-MB-231 attenuated tumor growth and metastasis in a preclinical orthotopic engraftment model (Grieve et al., 2016). In this study, we sought to determine an isoform-specific role for calpain-1 and calpain-2 in TNBC. Our findings implicate a set of non-overlapping substrates for calpain-1 and calpain-2 promoting the *in vivo* growth and metastatic colonization of MDA-MB-231 cells.

Process	Isoform	Substrate	Reference
Starvation & Oxidative Stress Response	Calpain-1	PHLPP1	(Wang et al., 2013; Wang et al., 2016)
mTOR-mediated protein synthesis	Calpain-2	PTEN	(Briz et al., 2013)
Platelet aggregation	Calpain-1 & Calpain-2	SRC	(Oda et al., 1993)
mTOR-mediated protein synthesis	Calpain-1 & Calpain-2	Hammartin, tuberin	(Briz et al., 2013)
Membrane localization	Calpain-1	Ezrin	(Hoskin et al., 2015)
Secretory process	Calpain-1	Ezrin	(Yao et al., 1993)
Cytosolic localization	Calpain-1 & Calpain-2	PTP1B	(Frangioni et al., 1993)
Invadopodia formation	Calpain-2	PTP1B, cortactin	(Cortesio et al., 2008)
Focal adhesion complex disassembly	Calpain-1 & Calpain-2	Paxillin, talin	(Carragher et al., 1999)
Integrin clustering, signaling, and focal adhesion disassembly	Calpain-2	Talin	(Franco et al., 2004b; Yan et al., 2001)
Cytoskeletal reorganization	Calpain-1	Talin	(Hayashi et al., 1999)

Table 3.1a Isoform-specific calpain substrates

Process	Isoform	Substrate	Reference
Integrin signaling and adhesion dynamics	Calpain-1 & Calpain-2	FAK	(Carragher et al., 1999; Chan et al., 2010; Cooray et al., 1996)
Synaptic plasticity	Calpain-1 & Calpain-2	PKC	(Bougie et al., 2009)
Tumor metastasis	Calpain-2	Cyclin E	(Libertini et al., 2005)
Platelet aggregation	Calpain-2	Integrin β1α, β2, β3, β7	(Pfaff et al., 1999)
Integrin clustering	Calpain-1	Integrin β3	(Bialkowska et al., 2000)
Adhesion dynamics	Calpain-2	Integrin β4	(Potts et al., 1994)
Adhesion dynamics	Calpain-2	Talin, spectrin, FAK, paxillin	(Franco et al., 2004a)
TNF-α signal transduction, inflammatory response	Calpain-1 & Calpain-2	IκBα	(Han et al., 1999; Shumway et al., 1999)

Table 3.1b Isoform-specific calpain substrates

3.3 Methods

3.3.1 Generation of *capns1*, *capn1*, and *capn2* knockout cell lines

The MDA-MB-231 human TNBC cell line was subjected to CRISPR/CAS9 genome editing using the LentiCRISPRv2 method described by (Sanjana et al., 2014; Shalem et al., 2014). sgRNAs were designed against the coding region of *capns1* (5′-ggcggctgcgcagtacaacc-3′), *capn1* (5′-cccaattcctccaagaccta-3′), and *capn2* (5′-cagccgaagggagcgagttc-3′). Twenty-five x 10^4 GFP-expressing (pWPXLD, Addgene #12258) MDA-MB-231 were seeded in Dulbecco's Modified Eagle Medium (Sigma) (10 % fetal bovine serum (FBS; Sigma), 1 % antibiotic-antimycotic (Gibco), and 1 % L-glutamine (Gibco)) ("complete DMEM") in 60 mm dishes (Sarstedt) and allowed to adhere overnight. The following morning, cells were transduced for with lentivirus targeting either *capns1*, *capn1*, or *capn2* in the presence of 10 μg/ml polybrene (Sigma). Fresh medium was added after 48 h and cells were expanded into 10 cm dishes (Sarstedt) in the presence of 2 μg/mL puromycin (Gibco) to select transduced cells. After preliminary immunoblotting analysis of polyclonal populations to assess loss of protein expression, serial-dilution on 96-well plates (Sarstedt) was used to isolate clones which were expanded for secondary immunoblotting analysis to validate gene-specific knockout (KO) clones.

3.3.2 Immunoblotting Analysis

Cell lysates for immunoblotting were prepared in radio-immunoprecipitation assay buffer (RIPA; 50 mM Tris-HCl (Fisher), 150 mM NaCl (Fisher), 1 % NP-40 (Sigma), 0.1 % SDS (Sigma), and 0.5 % sodium deoxycholate (Sigma) supplemented with protease and phosphatase inhibitors 1 mM sodium orthovanadate (Sigma), 1 mM phenylmethane sulfonyl fluoride (ICN Biomedicals), 5 μg/mL leupeptin (Sigma), and 2 μg/mL aprotinin (Sigma). Lysates were clarified by centrifugation for immunoblotting (12, 000 x g for 15 min at 4^{o}C), and protein quantification was performed using the Pierce bicinchoninic acid (BCA) kit (Thermo Scientific). *Capns1* KO was validated by immunoblotting using an in-house

antibody generated against bacterially expressed rat calpain-2, which detects CAPN2 (Arthur et al., 2000), and commercial antibodies from Santa Cruz were used to validate *capns1* (SC-32325) and *capn1* (SC-7531) KO.

3.3.3 Zymography analysis of calpain and MMP activity

Cells were seeded overnight in complete DMEM on 10 cm tissue culture dishes (Sarstedt) to achieve 70 % confluency the following day. Cells were washed once with PBS and lysed into HEPES/Triton X-100 lysis buffer for HEPES-imidazole casein zymography as described elsewhere (Croall et al., 2002). To assess the secretion of matrix metalloproteinases-2 and -9 (MMP-2/9), 1 x 10^6 WT, *capns1*, *capn1*, and *capn2* KO cells were seeded overnight on 60 mm tissue culture dishes (Sarstedt) in complete DMEM, and the following day plates were washed twice with PBS and overlaid with 3 mL of serum-free DMEM. Cells were allowed to condition the medium for 48 h; whereupon the supernatants was collected and clarified for 5 min at 180 x g. Gelatin zymography was then performed as described (Toth et al., 2012).

3.3.4 Orthotopic engraftment tumor growth and metastasis model

One x 10^6 WT, *capns1*, *capn1*, and *capn2* KO MDA-MB-231 cells were suspended in 50 μL of 50 % phosphate-buffered saline, and 50 % growth factor reduced matrigel (VWR), and engrafted into the number four (abdominal) mammary gland of *BalbC-Rag2*$^{-/-}$ *IL2Rγc*$^{-/-}$ mice (n=6 per cohort) using a Hamilton syringe (Sigma). Additional aliquots were retained to assess cell viability post-engraftment. To determine viability, cells were passed once through a Hamilton syringe and viable cells counted by Trypan blue exclusion assay (Sigma) with a hemocytometer. Staples were removed from surgical sites 7 days post-engraftment and tumor volumes were then assessed until tumors reached endpoint volumes of ~600 mm^3. At endpoints, tumors were resected via survival surgery and lysed into RIPA buffer containing protease and phosphatase inhibitors (as described previously) for immunoblot analysis. Metastatic growth

was allowed to develop for a further 14 days after tumor resection, whereupon mice were euthanized and lungs removed for biophotonic imagery. Lungs were imaged from each side and tumor burden was calculated as the percentage of GFP-positive pixels relative to the total area occupied by the lungs (sum of two sides). Quantification was performed using ImagePro Plus 6.0 and statistical analysis with Prism GraphPad.

3.3.5 Statistical Analysis

All statistical analysis were performed using Prism Graphpad 5. A p-value of <0.05 was deemed significant for the student's t-test, Log-Rank (Gehan-Breslow-Wilcoxon) test, and for an analysis of variance (ANOVA). Tukey's post-hoc test was used to determine significantly different sample means.

3.4 Results

3.4.1 Knockout of *capns1*, *capn1*, and *capn2* in MDA-MB-231 cells

Previously, we have shown that knockdown of *capns1* by RNAi in the triple-negative breast cancer line MDA-MB-231 is associated with attenuated primary tumor growth and metastasis (Grieve et al., 2016). We therefore sought to determine if there were isoform-specific roles for either calpain-1 or calpain-2 in tumorigenesis or if they provided essential yet redundant roles. CRISPR/CAS9 was used to generate MDA-MB-231 with null mutations in *capns1, capn1*, or *capn2*. After selection of clones, immunoblotting confirmed that CAPNS1, CAPN1, and CAPN2 protein expression was abolished (Figure 3.1A), and these results were corroborated by casein zymography (Figure 3.1B) which showed no detectable enzymatic activity.

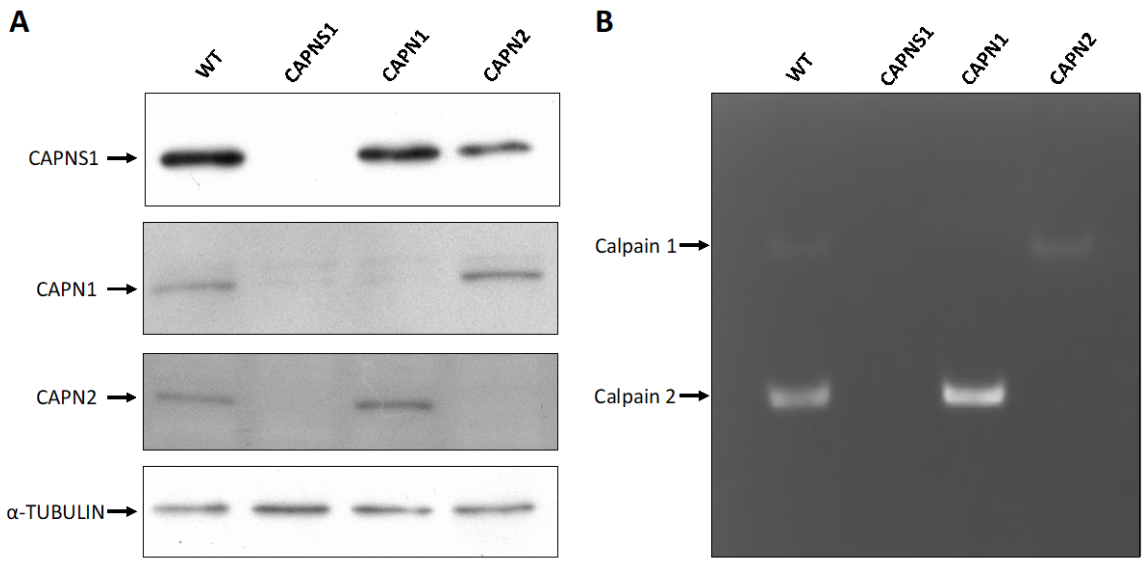

Figure 3.1 CRISPR/CAS9 KO of *capns1*, *capn1*, or *capn2* in MDA-MB-231 cells.

Twenty-five x 10^4 MDA-MB-231 cells were seeded in 60 mm^2 dishes and cultured overnight. The following day cells were transduced with a CRISPR/CAS9 lentiCRISPRv2 system expressing sgRNAs targeting *capns1*, *capn1*, and *capn2*. Puromycin-selected transduced cells were cloned in 96 well plates and gene KO was validated by (A) immunoblotting with antibodies for the indicated proteins (CAPNS1 (28 kDa), CAPN1 (80 kDa), CAPN2 (80 kDa), tubulin (50 kDa) and (B) casein zymography assessment of calpain-1 and calpain-2 activities.

3.4.2 Deletion of *capns1*, *capn1*, or *capn2* delays primary tumor growth

KO MDA-MB-231 cells of each genotype and control parental WT cells were engrafted into the number 4 abdominal mammary fat pads of *BalbC-Rag2*$^{-/-}$*IL2R*γ*c*$^{-/-}$ mice and tumor development were monitored. Latency in tumor growth was greatest in *capn2* KO (Figure 3.2A), however KO of *capns1*, *capn2*, and *capn1* were all associated with reduced doubling time compared to wildtype cells (Figure 3.2B). We confirmed that there were no differences in cell survival during the engraftment procedure by performing Typan blue exclusion assays on cells that were subjected to the same conditions as those that were engrafted (Figure 3.3).

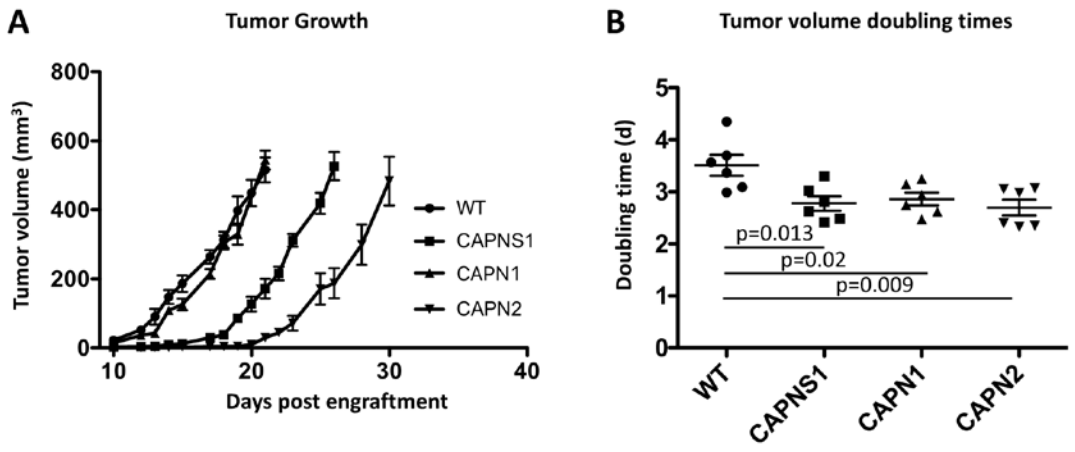

Figure 3.2 *Capns1* and *capn2 KO* delays tumor growth at the orthotopic site.

A. One x 10^6 GFP-expressing *capns1, capn1* or *capn2* KO or WT MDA-MB-231 cells were engrafted into the number four mammary gland of female *Rag2*$^{-/-}$ *IL-2Rγc*$^{-/-}$ mice (n=6 for each cohort). B. Doubling time of tumors shown in days. Measurements of tumor volumes reveal a delay in growth at the orthotopic site upon loss of CAPNS1, CAPN1, or CAPN2.

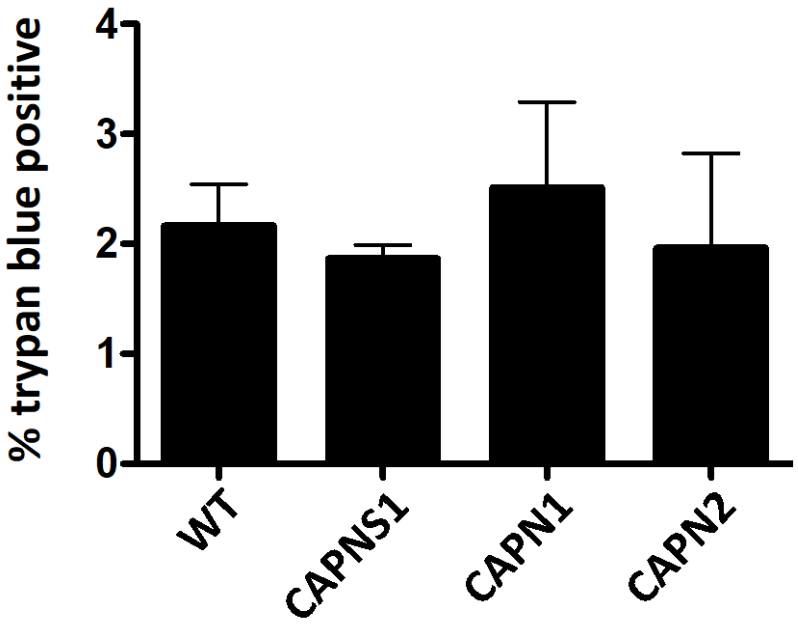

Figure 3.3 CRISPR/CAS9 KO of calpain genes does not affect cell viability.

Triplicate aliquots of *capns1*, *capn1*, *capn2* KO or WT cells suspended in matrigel were assessed for cell viability after sitting on ice for the duration of the engraftment procedure. Cells were passed through a Hamilton syringe once to mimic the engraftment procedure, mixed 1:1 with Trypan blue, and counted on a hemocytometer. Each line was counted in triplicate and represented as mean ± SD ($p>0.05$, ns, one-way analysis of variance (ANOVA)).

3.4.3 *Capn1* and *capn2* provide non-redundant metastasis promoting roles

Orthotopically engrafted tumors were resected by recovery surgery at volumes of 546.38 ± 76.76 mm^3 (mean ± SD) and immunoblotting analysis of these tumors confirmed that they did not arise from contaminating cells (Figure 3.4). After mice recovered from tumor resections, metastases were allowed to develop for 14 d post-resection; whereupon mice were euthanized and lungs were assessed for metastatic burden by biophotonic imaging. KO of *capns1*, *capn1*, and *capn2* all correlated with significant attenuation of lung metastasis (Figure 3.5).

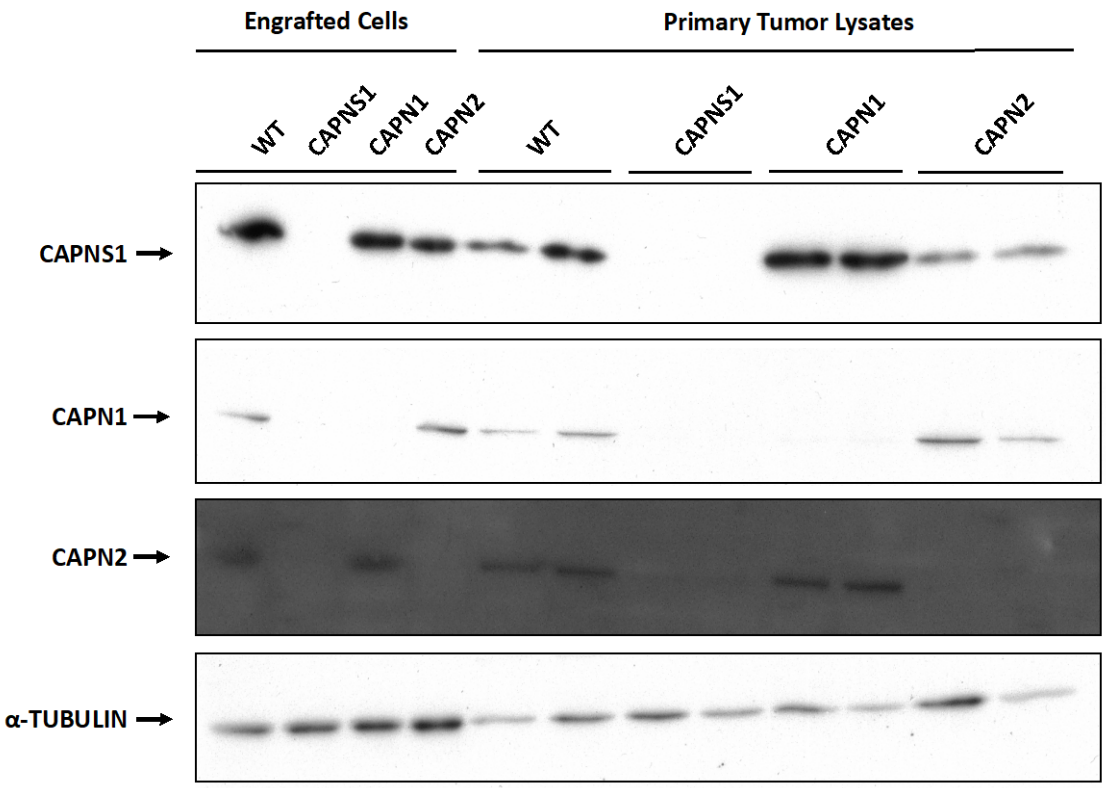

Figure 3.4 Tumors arising in orthotopically engrafted *Rag2*$^{-/-}$ *IL-2Rgc*$^{-/-}$ mice retain the calpain expression phenotype of the input MDA-MB-231 cells.

Tumors established with MDA-MB-231 cells of the indicated genotypes were resected by recovery surgery at volumes of 546.38 ± 76.76 mm^3 (mean ± SD) and subjected to immunoblotting analysis with antibodies for the indicated proteins (CAPNS1 (28 kDa), CAPN1 (80 kDa), CAPN2 (80 kDa), tubulin (50 kDa).

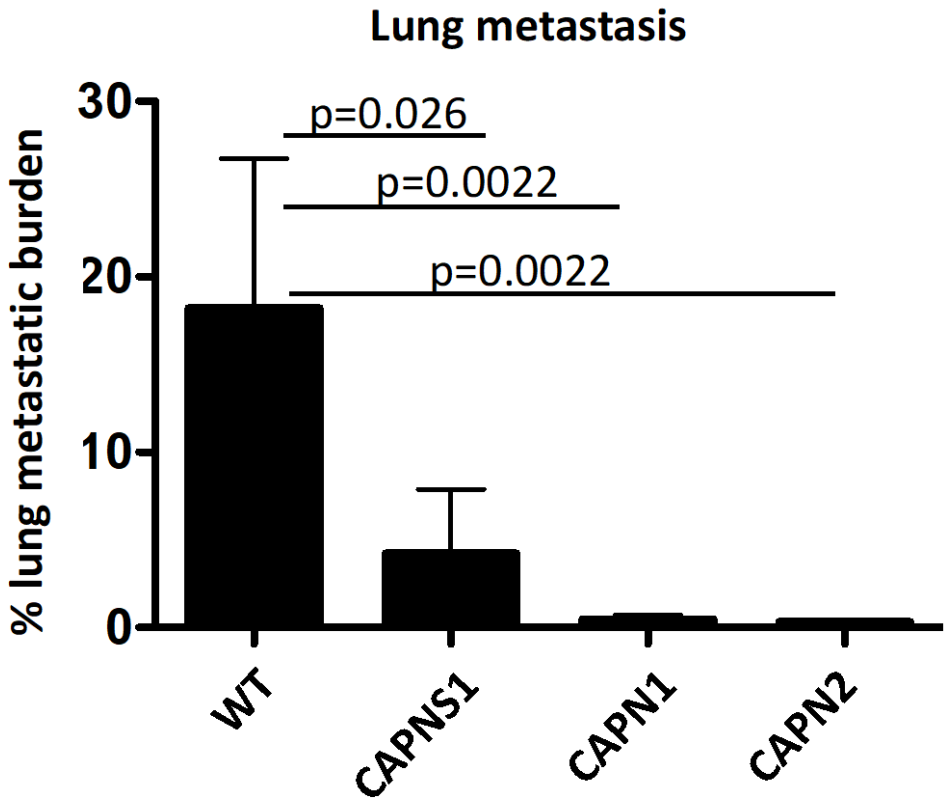

Figure 3.5 KO of *capns1, capn1, or capn2* is associated with reduced pulmonary metastasis.

At endpoints of orthotoptic engraftment studies, mice were euthanized and lungs removed for biophotonic imaging (n=6 per cohort). Lung metastatic burden was calculated as the percentage of GFP-positive pixels relative to the total area occupied by the lungs. Quantification was performed using ImagePro Plus 6.0 software, and statistical analysis with Prism GraphPad ($p<0.05$, student's t-test).

3.4.4 KO of *capns1*, *capn1*, and *capn2* attenuates MMP9 secretion

Given the important role of extracellular matrix degradation by matrix metalloproteinases (MMPs) in cancer cell invasion and metastasis, we sought to determine whether MMP production was affected by isoform-specific calpain-1/2 KO. We detected predominantly MMP9 in the supernatant of WT MDA-MB-231 cells, and determined that loss of either catalytic subunit (*capn1* and *capn2*) or the regulatory subunit (*capns1*) correlated with attenuated levels of secreted MMP9 (Figure 3.6).

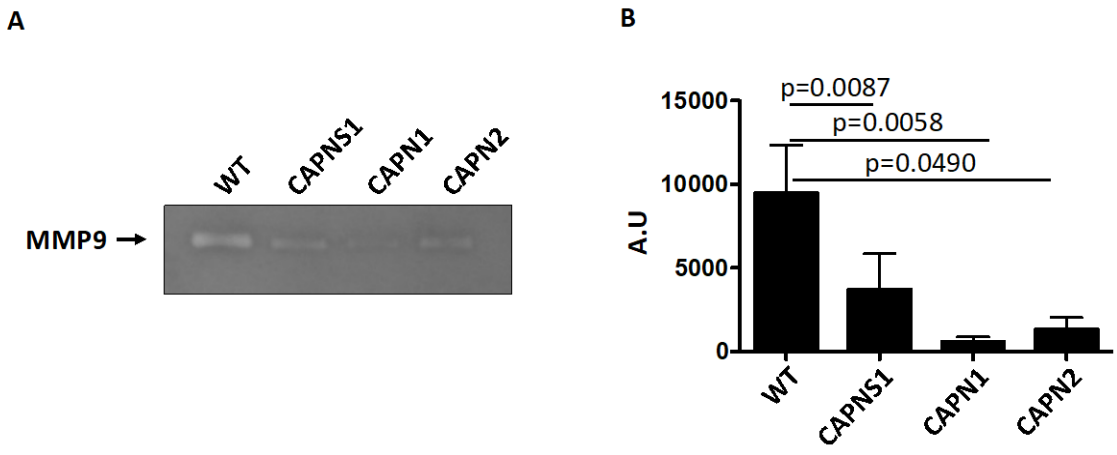

Figure 3.6 KO of *capns1, capn1 or capn2* is associated with reduced secreted MMP9 activity.

One x 10^6 *capns1, capn1, or capns1* KO, or WT MDA-MB-231 cells were seeded in 60 mm^2 dishes overnight. Cells were then washed with PBS and overlaid with 3 mL of serum-free DMEM and allowed to condition the medium for 48 h. At endpoint, supernates was collected and clarified by centrifugation. (A) Secreted MMP9 was assessed by gelatin zymography. (B) Semi-quantitative analysis of secreted MMP9 was performed by densitometry analysis of gelatin zymographs using ImageJ. Data are representative of three independent experiments ± SD ($p<0.05$, student's t-test).

3.5 Discussion

The progression of a tumor beyond the local site is a complex process involving invasion through the surrounding extracellular matrix, intravasation of blood or lymphatic vessels, survival through the circulation, and extravasation and growth at a distant metastatic site (reviewed in (Steeg, 2006)). Metastasis is one of the most challenging aspects of cancers to treat and is associated with poor prognosis compared to locally confined tumors (Steeg, 2016). Here, we show for the first time that calpain-1 and calpain-2 independently promote primary tumor progression, and play non-redundant, isoform-specific essential roles in metastatic colonization of triple-negative breast cancer.

Triple-negative breast cancer remains one of the most challenging to treat owing to a lack of generalizable targeted therapeutics. Furthermore, emerging preclinical data suggests that chemotherapeutic agents such as paclitaxel and doxorubicin, used to treat TNBC, can promote metastases when used in a neoadjuvant setting (Karagiannis et al., 2017), a paradigm similar to that seen with targeting of VEGF (Ebos et al., 2009; Paez-Ribes et al., 2009). Given that KO of both calpain-1 and calpain-2 independently attenuated metastatic colonization in TNBC, addition of calpain inhibitors to existing treatment regimens may prove relevant, especially in a neoadjuvant setting. Of note, familial deletion of calpain-1 in humans is associated with ataxia and spasticity (Wang et al., 2016), suggesting calpain-1 inhibition may be associated with neurologic toxicities.

Cancer cell invasion is aided by the production, secretion, and activation of MMPs at sites of integrin-containing structures called invadopodia (Albiges-Rizo et al., 2009; Brooks et al., 1996; Linder, 2007; Rolli et al., 2003). This aids in both interaction, with and degradation of, the extracellular matrix. Invadopodia are F-actin-rich structures which aid in degrading the extracellular matrix and facilitate invasion into the surrounding stromal compartment (Kelly et al., 1994); a critical step in tumor growth and metastatic dissemination. High levels of MMP9 are correlated with lymph node metastasis and reduced survival in breast cancer patients (Wu et al., 2008). KO of *capns1*, *capn1*, or *capn2* correlated with attenuated production of MMP9 (Figure 5). Both calpain-1 and calpain-2 are implicated in several

pathways leading to the production of MMP9; MMP9 is enhanced upon activation of the epidermal growth factor receptor (EGFR), the PI3K-AKT pathway (Lee et al., 2007), and NFκB (Huang et al., 2001). Loss of *capns1* is associated with impaired stress-mediated activation of AKT (Tan et al., 2006c), calpain-1 and calpain-2 degrade IκBα (Han et al., 1999; Shumway et al., 1999), and calpain-2 may separately be implicated in MMP production through MAPK signaling via phosphorylation by ERK at CAPN2 serine-50 (Glading et al., 2004).

Understanding key calpain substrates involved in driving tumorigenesis remains a work in progress. Studies implicate calpain isoforms as having dominant functions in certain cancers; For example, calpain-2 is associated with reduced relapse-free survival in triple-negative breast cancer (Storr et al., 2012a), focal adhesion turnover (Franco et al., 2004b), and the formation and turnover of invadopodia (Cortesio et al., 2008). Calpain-1 has been reported to play a dominant role in HER2+ breast cancer, and is associated with resistance to trastuzumab (Storr et al., 2011c), and focal adhesion dynamics during cell spreading (Croce et al., 1999; Glading et al., 2002; Satish et al., 2005). KO of *capns1, capn1,* or *capn2* were associated with a reduction in tumor doubling time, suggesting that these calpain isoforms play a role in cell cycle progression. A previous study supports the notion that calpains are implicated in driving cell cycle progression (Janossy et al., 2004), and others have shed some light on candidate isoform-dominant functions in regulating the cell cycle, such as calpain-2 in attenuating $p27^{kip1}$ expression (Ho et al., 2012), and promoting cyclin E activity (Libertini et al., 2005). Here, we provide evidence that calpain-1 and calpain-2 independently promote orthotopic tumor growth, as well as pulmonary metastasis in TNBC. Calpain-2 KO was associated with delayed tumor growth, suggesting that calpain-2 substrates may be critically involved in driving cell cycle progression, or associated with a rate limiting step in primary tumor growth, such as coopting the vasculature. Both calpain-1 and calpain-2 are associated with MMP production *in vitro*, suggesting that they both contribute to cell invasion and metastasis. Metastases are targeted in large part through chemotherapy, therefore it will also be important to understand whether there is an isoform-specific contribution to chemotherapeutic resistance.

Knockdown of *capns1* in MDA-MB-231 cell was associated with greater sensitivity to doxorubicin (Grieve et al., 2016), likely through attenuating ABC transporter function, and calpain-2 has been implicated in resistance to the clinical agent doxorubicin through cleavage of topoisomerase IIα (Jeon et al., 2018b). Unraveling an isoform-specific role for calpain in resistance to clinically approved TNBC therapeutics is an important next step. Here, we posit a model highlighting differential roles for calpain-1 and calpain-2 in primary tumor growth and metastasis based on studies suggesting dominant functions for these calpains (Figure 3.7).

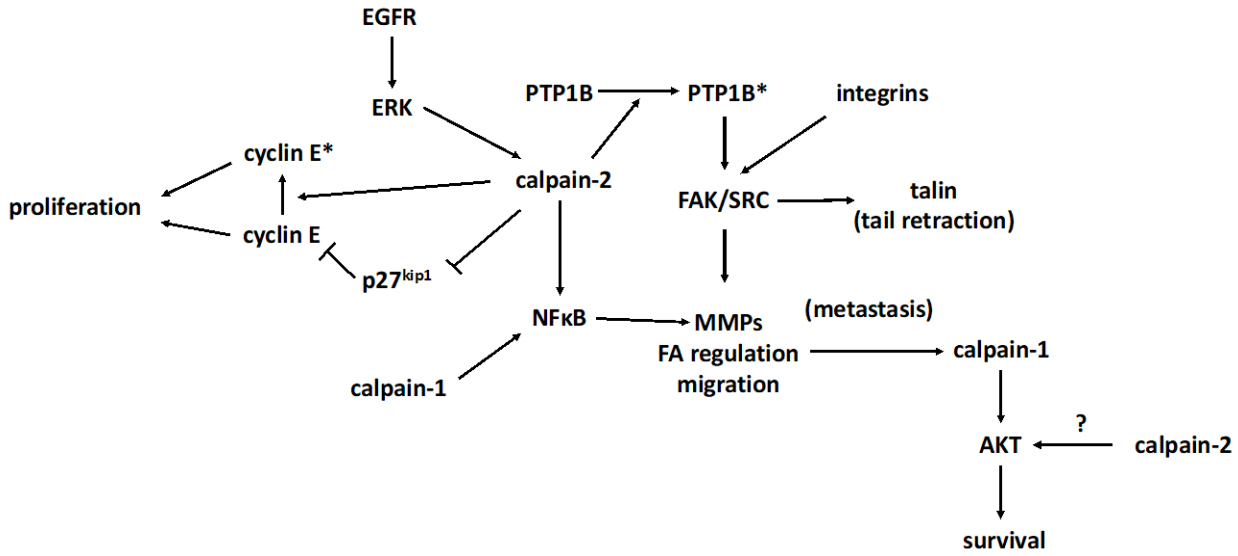

Figure 3.7 Isoform-specific roles for calpain-1 and calpain-2 in tumorigenesis.

Calpain-2 has multifaceted roles in tumorigenesis by promoting proliferation through cleavage of cyclin E and attenuation of $p27^{KIP1}$, as well as promoting cell migration and invasion by enhancing talin-mediated talin retraction, MMP production, and regulating focal adhesion dynamics through NFκB and SRC. Calpain-1 may also contribute to migration through NFκB-mediated MMP production, although it is unlikely to be involved in tail retraction. Calpain-1 may also play an important role in promoting the survival of disseminated cells through activation of AKT.

Calpain-2 has been implicated in the activation of SRC through cleavage of PTP1B, promoting FAK activation and integrin turnover (Carragher et al., 2006; Cortesio et al., 2008; Zhang et al., 2006), as well as focal adhesion disassembly during tail retraction (Franco et al., 2004b). Both calpain-1 and calpain-2 are implicated in the activation of NFκB, which can contribute to MMP production and invasion (Han et al., 1999; Huang et al., 2001; Shumway et al., 1999). Furthermore, we have found that RNAi knockdown of calpain-2 is associated with increased expression of the cell cycle inhibitor $p27^{Kip1}$ (Ho et al., 2012). Calpain-2 is also responsible for increasing cyclin E activity by generating a low molecular weight form that is resistant to inhibition by $p27^{kip1}$ (Libertini et al., 2005).

Data supporting isoform-specific roles for calpain-1 are less prominent in the literature. Tissue microarray studies suggest that increased calapin-1 expression is correlated with tumor metastasis in renal cell carcinoma (Braun et al., 1999). It has also been suggested that calpain-1 cleaves the negative regulator of AKT, PH domain and leucine-rich repeat protein phosphatase 1 (PHLPP1) in neuronal cells (Wang et al., 2013; Wang et al., 2016), and calpain- 1 has also been implicated in the activation of AKT in trastuzumab-resistant HER2+ breast cancer (Kulkarni et al., 2010). This raises speculation as to whether calpain-1 may predominantly promote survival of disseminated cells, whereas calpain-2 may contribute to primary tumor growth and metastatic dissemination from the orthotopic site.

To explore this model in greater depth, focal adhesion turnover will need to be assessed, which may allow attribution of a dominant role for either isoform in cell migration. Assessing levels of regulators of cell cycle such as $p27^{kip1}$ in tumors at endpoint would help to elucidate a dominant role for a calpain isoform in primary tumor growth. Quantitative analysis of circulating tumor cells from the blood may be informative as this might reveal an isoform-specific role for calpain in metastatic shedding as well as overall survival of cancer cells in the circulation. Importantly, *in vivo* studies should be completed with rescue and kinase-dead cell lines to address the issue of clonal variation. Finally, determining whether KO of either isoform sensitizes cancer cells to clinically approved therapeutics such as doxorubicin is also an important consideration which can be rapidly validated *in vivo*. Future exploration of the aforementioned

molecular pathways (Figure 3.7) will serve as a springboard to determining the relative contribution each calpain isoform makes to tumor progression.

Chapter 4

General Discussion

4.1 Summary

Knockout of *capns1* in a transgenic model of spontaneous mammary tumor development driven by the *her2/neu* oncogene delayed tumor onset but did not block tumor development. Differential phosphoproteins identified by a reverse-phase protein array (RPPA) suggest that calpain functions impinge on several key signaling pathways involved in tumorigenesis and provides a basis for identifying key calpain substrates that regulate tumor progression. If deletion of *capns1* results in tumors engaging an alternative set of pathways to drive carcinogenesis, it is possible that calpain inhibition will enhance the efficacy of chemotherapies or targeted agents aimed at these pathways. Interestingly, *ex vivo* deletion of *capns1* in a carcinoma cell line derived from a *her2/neu*-driven tumor blocked the tumorigenic potential of these cells in an orthotopic engraftment model. The delayed development of tumors comprised of calpain positive escaper clones suggests that calpain expression is required to sustain the tumorigenic potential of these *her2/neu* carcinoma cells and raises the important question: would effective therapeutic inhibition of calpain halt progression or cause regression of established tumors?

Deletion of *capns1*, *capn1* or *capn2* in a mouse xenograft model of TNBC revealed that calpain-1 and calpain-2 play non-redundant roles in tumorigenesis, including major roles in the promotion of metastasis. Although deletion of *capns1*, *capn1* or *capn2* attenuated primary tumor growth in this TNBC orthotopic model, the magnitude of this effect was small with only a slight reduction in the rate of tumor growth, suggesting that therapeutic targeting of calpain may confer maximal benefits in suppression of metastasis. This is especially relevant as emerging preclinical evidence suggests that neoadjuvant treatment with chemotherapeutics such as paclitaxel and doxorubicin may promote metastasis

(Karagiannis et al., 2017). Thus, combination of calpain inhibition with these chemotherapeutics might provide a more effective treatment, including a reduced risk of metastasis.

4.2 Reflections on roles for calpain in the *her2/neu* signaling network

Several proteins exhibited differential phosphorylation states in the presence or absence of calpain-1 and calpain-2 in the transgenic model of spontaneous tumor development driven by *her2/neu*, including some that are implicated in response to therapeutic challenges and oncogenesis. Validation of the RPPA results by IHC is an important next step. Myristoylated alanine-rich C kinase substrate (MARCKS) is an actin-binding protein involved in cytoskeletal reorganization and cell migration and is a substrate of both protein kinase C (PKC) and calpain (Dedieu et al., 2004). Phosphorylation of MARCKS was decreased in *capns1* KO *her2/neu* tumors. PKC-mediated phosphorylation of MARCKS confers protection from degradation, and involves translocation from the membrane to the cytosol (Hu et al., 2017; Spizz and Blackshear, 1996). Activation of PKC by calpain may represent one mechanism by which MARCKS phosphorylation is regulated (Fong et al., 2017). Phosphorylated MARCKS is associated with increased tumor angiogenesis and resistance to paclitaxel (Chen et al., 2015) and erlotinib (Chen et al., 2014) in breast and lung cancer, respectively. Furthermore, phosphorylation of MARCKS by PKC has been noted to enhance HER2 signaling by displacing the antiproliferative protein TOB (Jin Cho et al., 2001). Therapeutic strategies targeting PKC have been met with limited success (reviewed in (Roffey et al., 2009)), thus targeting calpain may be a viable alternative to attenuate oncogenic signaling from HER2, as well as improve chemotherapeutic response.

Signal transducer and activator of transcription 1 (STAT-1) is a transcription factor responsible for mediating downstream signaling in response to interferon and cytokine signaling (Stephanou and Latchman, 2003). Data from knockout mice suggest that STAT-1 behaves as a tumor suppressor (Kaplan et al., 1998), and pY701-STAT-1 has been identified as a favorable prognostic marker in breast cancer patients (Widschwendter et al., 2002). STAT-1 has been found complexed with EGFR and is

phosphorylated in response to EGF in a SRC-dependent manner (Olayioye et al., 1999), thus EGFR signaling contributes to the phosphorylation of STAT-1 (Quelle et al., 1995; Raven et al., 2011). Given that *capns1* knockout tumors displayed elevated pY845-EGFR, this may represent one mechanism by which STAT-1 phosphorylation was also elevated. Interestingly, STAT-1 expression appears to be critically important for mediating an *in vivo* antitumor response to lapatinib and doxorubicin in *neu*-driven tumors (Hannesdottir et al., 2013), although the authors did not directly address the phosphorylation state. Elevated STAT-1 signaling in response to IFNγ may also attenuate SMAD-3 response to TGFβ through inducing expression of inhibitory SMAD-7 (Ulloa et al., 1999). Collectively, these observations suggest that elevated levels of p-Y701-STAT-1 in *capns1* knockout tumors may reflect a role for dysregulated STAT-1 in delayed *her2/neu*-driven carcinogenesis.

GSK-3β is a serine/threonine kinase which is implicated in glycogen synthesis, autophagy, and tumorigenesis (reviewed in ((Mancinelli et al., 2017)). *capns1* KO *her2/neu* tumors displayed attenuated phosphorylation of the inhibitory reside S9 of GSK-3β, suggesting elevated GSK-3β activity. GSK-3β is inhibited by a number of players including AKT (Cross et al., 1995), MAPK signaling (Saito et al., 1994) and PKA (Li et al., 2000). Attenuated p-GSK-3β (S9) is somewhat paradoxical, as *capns1* KO *her2/neu* tumors displayed elevated p-Y845-EGFR; thus we might exepct elevated inhibitory phosphorylation (S9) of GSK-3β stemming from MAPK signaling. GSK-3β can regulate the cell cycle through modulating cyclin E (Welcker et al., 2003) and cyclin D1 (Diehl et al., 1998). GSK-3β activity is also associated with resistance to chemotherapy in p53 mutant colorectal cancers (Grassilli et al., 2013). However, expression of inactive GSK-3β can prevent apoptosis (Pap and Cooper, 1998), suggesting that GSK-3β can serve multifaceted roles depending on the nature of the cellular challenge. Importantly, active GSK-3β can serve as a tumor suppressor through promoting degradation of β-catenin (Patel et al., 2004).

The c-Jun N-terminal kinase (JNK) is a member of the mitogen activate protein kinase (MAPK) pathway and is phosphorylated and activated in response to a number of extracellular stimuli such as EGF, TGFβ, and TNFα to effect cellular migration and apoptosis (Huang et al., 2004; Park et al., 1997;

Tournier et al., 2000). *capns1* KO *her2/neu* tumors displayed an elevation in JNK phosphorylation. In breast cancer, activation of JNK downstream of EGF signaling can promote cell motility through upregulation of IRS-2 (Cui et al., 2006), implicating JNK in invasion and metastasis. JNK inhibition has been noted to activate AKT and downregulate GSK-3β (Fornoni et al., 2008). RPPA analysis of mouse *her2/neu* tumors revealed elevated JNK activity in the absence of calpain, however, we have previously shown that *capns1* deficient mouse embryonic fibroblasts displayed reduced JNK activity in response to ER stress (Tan et al., 2006b). These differences may be a reflection of the different cell types and metabolic conditions.

Elevated p-Y845-EGFR was prominent in *capns1* knockout *her2/neu* mouse tumors, and represents an interesting candidate for crosstalk between GSK-3β, MARCKS, JNK, and STAT-1, outlined in Figure 4.1. As EGFR has been identified as a substrate of calpain (Gregoriou et al., 1994), it is plausible that enhanced signaling is a result of reduced calpain-mediated cleavage of EGFR, or downstream changes in signaling to compensate for the absence of calpain expression. EGFR has been shown to elevate JNK activity (Cui et al., 2006), which would have effects on cell motility and modulate cellular response to apoptotic stimuli. Activation of ERK downstream of EGFR can promote calpain activity (Glading et al., 2004) that is involved in AKT activation (Tan et al., 2006a); and both ERK and AKT can suppress GSK-3β (Choi et al., 2012). EGFR signaling may also drive STAT-1 phosphorylation (Olayioye et al., 1999), and calpain could activate PKC (Hu et al., 2017) resulting in MARCKS phosphorylation.

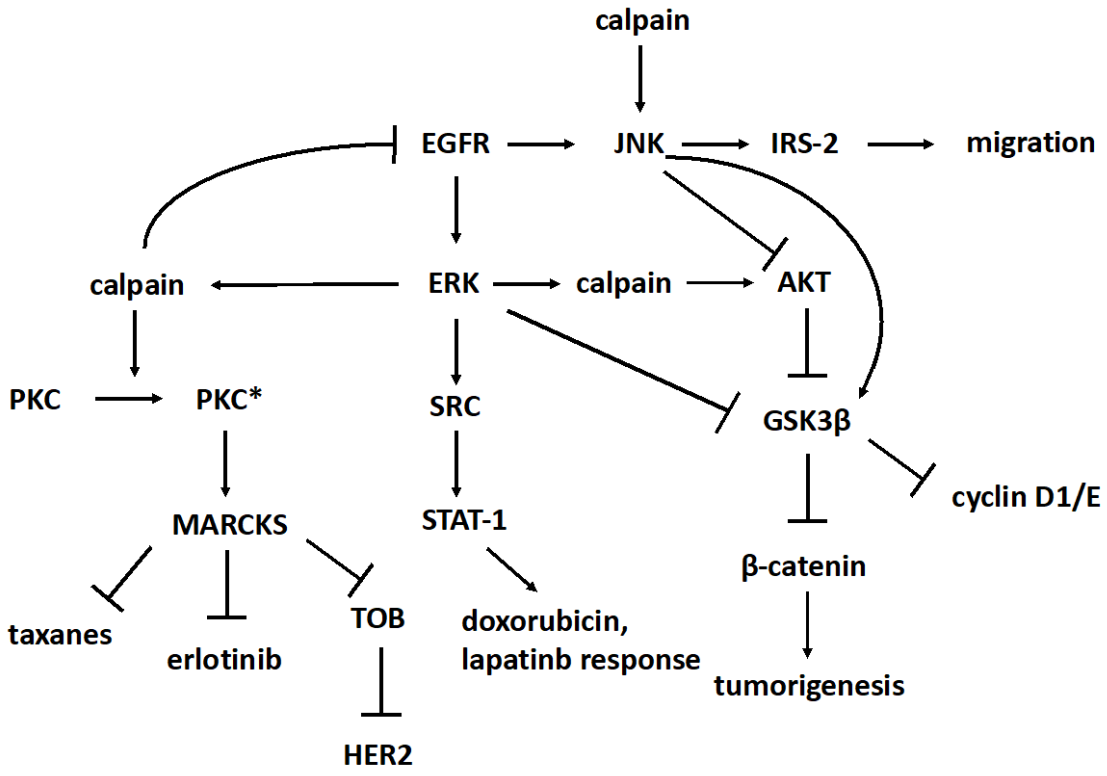

Figure 4.1 Integrating calpain into a signaling network in the *her2/neu* model highlighting proteins identified by RPPA analysis.

Calpain may contribute to the therapeutic resistance and invasive potential of cancer cells by activating protein kinase C, and driving MARCKS activity, resulting in increased HER2 signaling and resistance to taxanes and erlotinib. Calpain-mediated activation of JNK may also contribute to cancer cell migration through the IRS-2 adaptor, yet serve some anti-tumor roles through promoting GSK3β activity, which may attenuate pro-tumorigenic roles of β-catenin, as well as inhibit the cell cycle. Activation of ERK downstream of EGFR is associated with inhibition of GSK3β; and calpain may negatively regulate this pathway through cleavage of EGFR. Activation of STAT-1 downstream of ERK may mediate an anti-tumor response to doxorubicin and lapatinib.

4.3 Remarks on translational relevance in a *her2/neu* model

The absence of calpain appears to have resulted in the engagement of a differential set of signaling pathways during the carcinogenic process in a spontaneous *her2/neu*-driven model of mammary tumorigenesis. The RPPA data suggest at least five key proteins were differentially regulated; however, these require validation in a cancer-cell intrinsic manner. It is plausible that the observed phosphoprotein signals arose from stromal cells, thus it will be important to also assess whether deletion of calpain affects the composition and function of the surrounding stroma.

In the orthotopic engraftment model, *capns1* KO MTECs did not form tumors; although rare escaper clones did eventually give rise to *capns1* WT tumors. Nuclear localization of HIF1α was not affected by the absence of calpain in this MTEC model, suggesting that this aspect of the angiogenic switch was not compromising tumor development. However, this does not preclude the possibility that calpain is required for tumor angiogenesis *in vivo*, or in a HIF1α-independent manner (Folkman, 2002). Assessing the secretion of cytokines by WT and *capns1* KO MTECs under hypoxic conditions might provide clues about the proangiogenic potential of these cells, in addition to highlighting differential paracrine signaling which may impact other stromal cells including fibroblasts and immune cells.

Assessing the effect of calpain deletion after the tumor has formed will also be important to explore using the orthotopic *capns1 floxed* MTECs model. A tamoxifen-inducible Cre-ER transgene in these cells or tumor injection with Cre-expressing adenovirus could be used recapitulate a treatment model with a calpain inhibitor. Given that cultured *capns1* KO MTECs phenocopied the elevated p-Y845-EGFR levels seen in the *capns1* KO transgenic spontaneous tumor model, and the *capns* KO MTECs were more sensitive to lapatinib and doxorubicin than *capns1* WT MTECs, this provides rationale to test calpain inhibition/deletion in these preclinical mouse models in combination with erlotinib, lapatinib, trastuzumab, and doxorubicin.

Erlotinib is a tyrosine kinase inhibitor (TKI) targeting both EGFR and HER2 (Schaefer et al., 2007). The use of erlotinib in breast cancer has been met with limited success thus far, although selection

of patients by p-EGFR status was not employed (Dickler et al., 2008). The combination of trastuzumab and erlotinib has shown some promise in a phase I trial (NCT00033514). If p-Y845-EGFR represents an important survival mechanism in *capns1* KO *her2/neu* tumors, then calpain inhibition may enhance the efficacy of erlotinib.

Lapatinib is another inhibitor of both EGFR and HER2, which is used to treat both early and advanced breast cancer, often in combination with trastuzumab, a neutralizing antibody inhibitor of HER2 (Baselga et al., 2012a; Blackwell et al., 2010). Given that *capns1* KO MTECs challenged with lapatinib *in vitro* displayed enhanced cytotoxicity, it will be important to validate this observation in an *in vivo* tumor model. Additionally, since elevated p-Y701-STAT1 was observed in the RPPA analysis, this provides rationale to test both lapatinib and doxorubicin *in vivo*, as this site mediates an antitumor response to these agents (Hannesdottir et al., 2013). It would be important to consider the use of a syngeneic model system for these experiments, as some STAT-1 tumor suppressor functions are immune-mediated (Hannesdottir et al., 2013). Doxorubicin is a widely used anthracycline agent, and resistance to doxorubicin may be mediated in part by cleavage of topoisomerase II by calpain (Jeon et al., 2018b). Importantly, calpain is also implicated in generating p95HER2, a fragment associated with resistance to trastuzumab (Kulkarni et al., 2010; Scaltriti et al., 2007). Thus, combining calpain inhibitors with trastuzumab, lapatinib and doxorubicin could confer considerable benefit.

The development of specific and effective calpain inhibitors remains a work in progress. Many of the current calpain inhibitors also impact cysteine cathepsins, and although strategies utilizing calpastatin peptidomimetics show some promise, they do not inhibit the entire calpain family (reviewed in (Ono et al., 2016)). However, many inhibitors which have moved into clinical trials have been discontinued (Olesoxime (NCT00868166), cyclosporine A (NCT02496975)), highlighting the challenges associated with developing an effective clinical calpain inhibitor. The use of a calpain inhibitor to validate experimental findings - such as attenuated metastasis upon calpain knockout in the model of TNBC - is important as there may be a role for calpain in the stromal compartment, and systemic inhibition of

calpain may be disadvantageous. Our observations using the NIC transgenic mouse model suggests that deletion of *capns1* in the mammary epithelium is well tolerated, and is associated with delayed tumor onset, however inhibition of calpain in innate and adaptive immune cells may interfere with their ability to appropriately interact with the developing tumor. For example, engagement of the T-cell receptor/CD3 complex is an important step in the activation of $CD8^+$ and $CD4^+$ T-cells, that is rapidly followed by calpain-mediated cytoskeletal rearrangement and pseudopodia formation (Selliah et al., 1996), suggesting that systemic inhibition of calpain may have broad ranging impacts on tumor development.

In summary, conditional deletion of *capns1* in a model of spontaneous *her2/neu* tumor development delays primary tumor onset, but does not prevent tumors from forming, suggesting that calpain contributes to the oncogenic process. Given that *ex vivo* deletion of *capns1* in the MTEC line is associated with a complete block in orthotopic tumor development, with tumors arising only from rare *capns1* WT escaper clones, this suggests that calpain is required to sustain the tumorigenic potential of carcinoma cells that developed in the presence of calpain. Furthermore, the RPPA analysis revealed a number of proteins which are differentially regulated in the absence of calpain; and some of these, such as EGFR, provide rationale to test clinically approved therapeutics such as erlotinib in combination with calpain inhibitors in preclinical model systems. Success in these systems would form the basis for development and utilization of clinical calpain inhibitors.

4.4 Calpain in triple-negative breast cancer: summary and future directions

Knockout of *capns1*, *capn1* or *capn2* in a model of triple-negative breast cancer revealed that ablation of both calpain-1 and calpain-2 modestly attenuate primary tumor doubling time, and revealed that these two calpains were associated with non-redundant essential roles in metastatic progression. These observations inform rationale for the development of isoform-specific calpain inhibitors, and highlight the need to expand our understanding of dominant functions for calpain isoforms in specific cancers subtypes, as well as specific processes associated with tumorigenesis. Considering some recent

preclinical data that suggests chemotherapeutic agents used to treat TNBC may actually promote metastases (Karagiannis et al., 2017), it would be important to understand whether inhibition of either calpain-1 or calpain-2 can interfere with the associated underlying pathways. While these exciting and novel observations with calpain isoform-specific KO TNBC xenograft models are encouraging, it will be important to validate these findings with additional clones, as well as rescue calpain-1 and calpain-2 expression with CRISPR/CAS9-resistant *capn1* and *capn2* vectors to rule out off-target effects. If the metastatic phenotype is restored in rescue experiments, it would validate the conclusion that *capn1* and *capn2* function independently as metastasis promoting genes. The use of proteomic techniques such as terminal amine isotopic labeling of substrates may be a useful step in determining calpain-1 and calpain-2 isoform-specific degradomes. Identifying key calpain substrates involved in the metastatic cascade may also highlight novel candidates for therapeutic targeting.

References

Acevedo, V.D., R.D. Gangula, K.W. Freeman, R. Li, Y. Zhang, F. Wang, G.E. Ayala, L.E. Peterson, M. Ittmann, and D.M. Spencer. 2007. Inducible FGFR-1 activation leads to irreversible prostate adenocarcinoma and an epithelial-to-mesenchymal transition. *Cancer Cell*. 12:559-571.

Ades, F., D. Zardavas, I. Bozovic-Spasojevic, L. Pugliano, D. Fumagalli, E. de Azambuja, G. Viale, C. Sotiriou, and M. Piccart. 2014. Luminal B breast cancer: molecular characterization, clinical management, and future perspectives. *J Clin Oncol*. 32:2794-2803.

Albiges-Rizo, C., O. Destaing, B. Fourcade, E. Planus, and M.R. Block. 2009. Actin machinery and mechanosensitivity in invadopodia, podosomes and focal adhesions. *J Cell Sci*. 122:3037-3049.

Amini, M., C.L. Ma, R. Farazifard, G. Zhu, Y. Zhang, J. Vanderluit, J.S. Zoltewicz, F. Hage, J.M. Savitt, D.C. Lagace, R.S. Slack, J.C. Beique, M. Baudry, P.A. Greer, R. Bergeron, and D.S. Park. 2013. Conditional disruption of calpain in the CNS alters dendrite morphology, impairs LTP, and promotes neuronal survival following injury. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 33:5773-5784.

Aoudjit, F., and K. Vuori. 2001. Integrin signaling inhibits paclitaxel-induced apoptosis in breast cancer cells. *Oncogene*. 20:4995-5004.

Arribas, J., J. Baselga, K. Pedersen, and J.L. Parra-Palau. 2011. p95HER2 and breast cancer. *Cancer Res*. 71:1515-1519.

Arthur, J.S., J.S. Elce, C. Hegadorn, K. Williams, and P.A. Greer. 2000. Disruption of the murine calpain small subunit gene, Capn4: calpain is essential for embryonic development but not for cell growth and division. *Mol Cell Biol*. 20:4474-4481.

Azam, M., S.S. Andrabi, K.E. Sahr, L. Kamath, A. Kuliopulos, and A.H. Chishti. 2001. Disruption of the mouse mu-calpain gene reveals an essential role in platelet function. *Mol Cell Biol.* 21:2213-2220.

Badugu, R., M. Garcia, V. Bondada, A. Joshi, and J.W. Geddes. 2008. N terminus of calpain 1 is a mitochondrial targeting sequence. *J Biol Chem*. 283:3409-3417.

Baselga, J., I. Bradbury, H. Eidtmann, S. Di Cosimo, E. de Azambuja, C. Aura, H. Gomez, P. Dinh, K. Fauria, V. Van Dooren, G. Aktan, A. Goldhirsch, T.W. Chang, Z. Horvath, M. Coccia-Portugal, J. Domont, L.M. Tseng, G. Kunz, J.H. Sohn, V. Semiglazov, G. Lerzo, M. Palacova, V. Probachai, L. Pusztai, M. Untch, R.D. Gelber, M. Piccart-Gebhart, and A.S.T. Neo. 2012a. Lapatinib with trastuzumab for HER2-positive early breast cancer (NeoALTTO): a randomised, open-label, multicentre, phase 3 trial. *Lancet*. 379:633-640.

Baselga, J., P. Gomez, R. Greil, S. Braga, M.A. Climent, A.M. Wardley, B. Kaufman, S.M. Stemmer, A. Pego, A. Chan, J.C. Goeminne, M.P. Graas, M.J. Kennedy, E.M. Ciruelos Gil, A. Schneeweiss, A. Zubel, J. Groos, H. Melezinkova, and A. Awada. 2013. Randomized phase II study of the anti-epidermal growth factor receptor monoclonal antibody cetuximab with cisplatin versus cisplatin alone in patients with metastatic triple-negative breast cancer. *J Clin Oncol*. 31:2586-2592.

Baselga, J., J.G. Segalla, H. Roche, A. Del Giglio, H. Pinczowski, E.M. Ciruelos, S.C. Filho, P. Gomez, B. Van Eyll, B. Bermejo, A. Llombart, B. Garicochea, M.A. Duran, P.M. Hoff, M. Espie, A.A. de Moraes, R.A. Ribeiro, C. Mathias, M. Gil Gil, B. Ojeda, J. Morales, S. Kwon Ro, S. Li, and F. Costa. 2012b. Sorafenib in combination with capecitabine: an oral regimen for patients with HER2-negative locally advanced or metastatic breast cancer. *J Clin Oncol*. 30:1484-1491.

Beckerle, M.C., K. Burridge, G.N. DeMartino, and D.E. Croall. 1987. Colocalization of calcium-dependent protease II and one of its substrates at sites of cell adhesion. *Cell*. 51:569-577.

Belyanskaya, L.L., S. Hopkins-Donaldson, S. Kurtz, A.P. Simoes-Wust, S. Yousefi, H.U. Simon, R. Stahel, and U. Zangemeister-Wittke. 2005. Cisplatin activates Akt in small cell lung cancer cells and attenuates apoptosis by survivin upregulation. *Int J Cancer*. 117:755-763.

Bertoli, C., T. Copetti, E.W. Lam, F. Demarchi, and C. Schneider. 2009. Calpain small-1 modulates Akt/FoxO3A signaling and apoptosis through PP2A. *Oncogene*. 28:721-733.

Betts, R., S. Weinsheimer, G.E. Blouse, and J. Anagli. 2003. Structural determinants of the calpain inhibitory activity of calpastatin peptide B27-WT. *J Biol Chem*. 278:7800-7809.

Bialkowska, K., S. Kulkarni, X. Du, D.E. Goll, T.C. Saido, and J.E. Fox. 2000. Evidence that beta3 integrin-induced Rac activation involves the calpain-dependent formation of integrin clusters that are distinct from the focal complexes and focal adhesions that form as Rac and RhoA become active. *J Cell Biol*. 151:685-696.

Blackwell, K.L., H.J. Burstein, A.M. Storniolo, H. Rugo, G. Sledge, M. Koehler, C. Ellis, M. Casey, S. Vukelja, J. Bischoff, J. Baselga, and J. O'Shaughnessy. 2010. Randomized study of Lapatinib alone or in combination with trastuzumab in women with ErbB2-positive, trastuzumab-refractory metastatic breast cancer. *J Clin Oncol*. 28:1124-1130.

Blohmer, J.U., M. Rezai, S. Kümmel, T. Kühn, M. Warm, K. Friedrichs, A. Benkow, W.J. Valentine, and W. Eiermann. 2013. Using the 21-gene assay to guide adjuvant chemotherapy decision-making in early-stage breast cancer: a cost-effectiveness evaluation in the German setting. *Journal of Medical Economics*. 16:30-40.

Bougie, J.K., T. Lim, C.A. Farah, V. Manjunath, I. Nagakura, G.B. Ferraro, and W.S. Sossin. 2009. The atypical protein kinase C in Aplysia can form a protein kinase M by cleavage. *J Neurochem*. 109:1129-1143.

Braun, C., M. Engel, M. Seifert, B. Theisinger, G. Seitz, K.D. Zang, and C. Welter. 1999. Expression of calpain I messenger RNA in human renal cell carcinoma: correlation with lymph node metastasis and histological type. *Int J Cancer*. 84:6-9.

Briz, V., Y.T. Hsu, Y. Li, E. Lee, X. Bi, and M. Baudry. 2013. Calpain-2-mediated PTEN degradation contributes to BDNF-induced stimulation of dendritic protein synthesis. *J Neurosci*. 33:4317-4328.

Brooks, P.C., S. Stromblad, L.C. Sanders, T.L. von Schalscha, R.T. Aimes, W.G. Stetler-Stevenson, J.P. Quigley, and D.A. Cheresh. 1996. Localization of matrix metalloproteinase MMP-2 to the surface of invasive cells by interaction with integrin alpha v beta 3. *Cell*. 85:683-693.

Burstein, H.J., I. Kuter, S.M. Campos, R.S. Gelman, L. Tribou, L.M. Parker, J. Manola, J. Younger, U. Matulonis, C.A. Bunnell, A.H. Partridge, P.G. Richardson, K. Clarke, L.N. Shulman, and E.P. Winer. 2001. Clinical activity of trastuzumab and vinorelbine in women with HER2-overexpressing metastatic breast cancer. *J Clin Oncol*. 19:2722-2730.

Burstein, H.J., A.M. Storniolo, S. Franco, J. Forster, S. Stein, S. Rubin, V.M. Salazar, and K.L. Blackwell. 2008. A phase II study of lapatinib monotherapy in chemotherapy-refractory HER2-positive and HER2-negative advanced or metastatic breast cancer. *Ann Oncol*. 19:1068-1074.

Campbell, R.L., and P.L. Davies. 2012. Structure-function relationships in calpains. *Biochem J*. 447:335-351.

Campos, L.S., D.P. Leone, J.B. Relvas, C. Brakebusch, R. Fassler, U. Suter, and C. ffrench-Constant. 2004. Beta1 integrins activate a MAPK signalling pathway in neural stem cells that contributes to their maintenance. *Development*. 131:3433-3444.

Cannito, S., E. Novo, A. Compagnone, L. Valfre di Bonzo, C. Busletta, E. Zamara, C. Paternostro, D. Povero, A. Bandino, F. Bozzo, C. Cravanzola, V. Bravoco, S. Colombatto, and M. Parola. 2008. Redox mechanisms switch on hypoxia-dependent epithelial-mesenchymal transition in cancer cells. *Carcinogenesis*. 29:2267-2278.

Carragher, N.O., and M.C. Frame. 2004. Focal adhesion and actin dynamics: a place where kinases and proteases meet to promote invasion. *Trends Cell Biol*. 14:241-249.

Carragher, N.O., B. Levkau, R. Ross, and E.W. Raines. 1999. Degraded collagen fragments promote rapid disassembly of smooth muscle focal adhesions that correlates with cleavage of pp125(FAK), paxillin, and talin. *J Cell Biol*. 147:619-630.

Carragher, N.O., S.M. Walker, L.A. Scott Carragher, F. Harris, T.K. Sawyer, V.G. Brunton, B.W. Ozanne, and M.C. Frame. 2006. Calpain 2 and Src dependence distinguishes mesenchymal and amoeboid modes of tumour cell invasion: a link to integrin function. *Oncogene*. 25:5726-5740.

CCS. 2017. Canadian Cancer Statistics 2017.0-142.

Chan, K.T., D.A. Bennin, and A. Huttenlocher. 2010. Regulation of adhesion dynamics by calpain-mediated proteolysis of focal adhesion kinase (FAK). *J Biol Chem*. 285:11418-11426.

Cheang, M.C., M. Martin, T.O. Nielsen, A. Prat, D. Voduc, A. Rodriguez-Lescure, A. Ruiz, S. Chia, L. Shepherd, M. Ruiz-Borrego, L. Calvo, E. Alba, E. Carrasco, R. Caballero, D. Tu, K.I. Pritchard, M.N. Levine, V.H. Bramwell, J. Parker, P.S. Bernard, M.J. Ellis, C.M. Perou, A. Di Leo, and L.A. Carey. 2015. Defining breast cancer intrinsic subtypes by quantitative receptor expression. *The oncologist*. 20:474-482.

Chen, B., J. Tang, Y.S. Guo, Y. Li, Z.N. Chen, and J.L. Jiang. 2013. Calpains are required for invasive and metastatic potentials of human HCC cells. *Cell Biol Int*. 37:643-652.

Chen, C.H., C.T. Cheng, Y. Yuan, J. Zhai, M. Arif, L.W. Fong, R. Wu, and D.K. Ann. 2015. Elevated MARCKS phosphorylation contributes to unresponsiveness of breast cancer to paclitaxel treatment. *Oncotarget*. 6:15194-15208.

Chen, C.H., S. Statt, C.L. Chiu, P. Thai, M. Arif, K.B. Adler, and R. Wu. 2014. Targeting myristoylated alanine-rich C kinase substrate phosphorylation site domain in lung cancer. Mechanisms and therapeutic implications. *Am J Respir Crit Care Med*. 190:1127-1138.

Chen, W.Y., B. Rosner, S.E. Hankinson, G.A. Colditz, and W.C. Willett. 2011. Moderate alcohol consumption during adult life, drinking patterns, and breast cancer risk. *JAMA*. 306:1884-1890.

Cheng, S.Y., S.C. Wang, M. Lei, Z. Wang, and K. Xiong. 2018. Regulatory role of calpain in neuronal death. *Neural Regen Res*. 13:556-562.

Chipuk, J.E., and D.R. Green. 2008. How do BCL-2 proteins induce mitochondrial outer membrane permeabilization? *Trends Cell Biol*. 18:157-164.

Choi, C.H., B.H. Lee, S.G. Ahn, and S.H. Oh. 2012. Proteasome inhibition-induced p38 MAPK/ERK signaling regulates autophagy and apoptosis through the dual phosphorylation of glycogen synthase kinase 3beta. *Biochem Biophys Res Commun*. 418:759-764.

Choi, Y.H., S.J. Lee, P. Nguyen, J.S. Jang, J. Lee, M.L. Wu, E. Takano, M. Maki, P.A. Henkart, and J.B. Trepel. 1997. Regulation of cyclin D1 by calpain protease. *J Biol Chem*. 272:28479-28484.

Chou, J.S., F. Impens, K. Gevaert, and P.L. Davies. 2011a. m-Calpain activation in vitro does not require autolysis or subunit dissociation. *Biochim Biophys Acta*. 1814:864-872.

Chou, S.M., T.H. Huang, H.C. Chen, and T.K. Li. 2011b. Calcium-induced cleavage of DNA topoisomerase I involves the cytoplasmic-nuclear shuttling of calpain 2. *Cellular and molecular life sciences : CMLS*. 68:2769-2784.

Chua, B.T., K. Guo, and P. Li. 2000. Direct cleavage by the calcium-activated protease calpain can lead to inactivation of caspases. *J Biol Chem*. 275:5131-5135.

Chua, H.L., P. Bhat-Nakshatri, S.E. Clare, A. Morimiya, S. Badve, and H. Nakshatri. 2007. NF-kappaB represses E-cadherin expression and enhances epithelial to mesenchymal transition of mammary epithelial cells: potential involvement of ZEB-1 and ZEB-2. *Oncogene*. 26:711-724.

Clark, A.S., K. West, S. Streicher, and P.A. Dennis. 2002. Constitutive and inducible Akt activity promotes resistance to chemotherapy, trastuzumab, or tamoxifen in breast cancer cells. *Molecular cancer therapeutics*. 1:707-717.

Cleator, S., W. Heller, and R.C. Coombes. 2007. Triple-negative breast cancer: therapeutic options. *The Lancet. Oncology*. 8:235-244.

Conacci-Sorrell, M., C. Ngouenet, S. Anderson, T. Brabletz, and R.N. Eisenman. 2014. Stress-induced cleavage of Myc promotes cancer cell survival. *Genes & development*. 28:689-707.

Conacci-Sorrell, M., C. Ngouenet, and R.N. Eisenman. 2010. Myc-nick: a cytoplasmic cleavage product of Myc that promotes alpha-tubulin acetylation and cell differentiation. *Cell*. 142:480-493.

Cooray, P., Y. Yuan, S.M. Schoenwaelder, C.A. Mitchell, H.H. Salem, and S.P. Jackson. 1996. Focal adhesion kinase (pp125FAK) cleavage and regulation by calpain. *Biochem J*. 318 (Pt 1):41-47.
Cortesio, C.L., K.T. Chan, B.J. Perrin, N.O. Burton, S. Zhang, Z.Y. Zhang, and A. Huttenlocher. 2008. Calpain 2 and PTP1B function in a novel pathway with Src to regulate invadopodia dynamics and breast cancer cell invasion. *J Cell Biol*. 180:957-971.
Croall, D.E., K. Moffett, and H. Hatch. 2002. Casein zymography of calpains using a 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-imidazole buffer. *Analytical biochemistry*. 304:129-132.
Croce, K., R. Flaumenhaft, M. Rivers, B. Furie, B.C. Furie, I.M. Herman, and D.A. Potter. 1999. Inhibition of calpain blocks platelet secretion, aggregation, and spreading. *J Biol Chem*. 274:36321-36327.
Cross, D.A., D.R. Alessi, P. Cohen, M. Andjelkovich, and B.A. Hemmings. 1995. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature*. 378:785-789.
Cui, X., H.J. Kim, I. Kuiatse, H. Kim, P.H. Brown, and A.V. Lee. 2006. Epidermal growth factor induces insulin receptor substrate-2 in breast cancer cells via c-Jun NH(2)-terminal kinase/activator protein-1 signaling to regulate cell migration. *Cancer Res*. 66:5304-5313.
De Craene, B., F. van Roy, and G. Berx. 2005. Unraveling signalling cascades for the Snail family of transcription factors. *Cell Signal*. 17:535-547.
Dedieu, S., S. Poussard, G. Mazeres, F. Grise, E. Dargelos, P. Cottin, and J.J. Brustis. 2004. Myoblast migration is regulated by calpain through its involvement in cell attachment and cytoskeletal organization. *Exp Cell Res*. 292:187-200.
Dent, R., M. Trudeau, K.I. Pritchard, W.M. Hanna, H.K. Kahn, C.A. Sawka, L.A. Lickley, E. Rawlinson, P. Sun, and S.A. Narod. 2007. Triple-negative breast cancer: clinical features and patterns of recurrence. *Clin Cancer Res*. 13:4429-4434.
Desgrosellier, J.S., and D.A. Cheresh. 2010. Integrins in cancer: biological implications and therapeutic opportunities. *Nat Rev Cancer*. 10:9-22.
Dickler, M.N., H.S. Rugo, C.A. Eberle, E. Brogi, J.F. Caravelli, K.S. Panageas, J. Boyd, B. Yeh, D.E. Lake, C.T. Dang, T.A. Gilewski, J.F. Bromberg, A.D. Seidman, G.M. D'Andrea, M.M. Moasser, M. Melisko, J.W. Park, J. Dancey, L. Norton, and C.A. Hudis. 2008. A phase II trial of erlotinib in combination with bevacizumab in patients with metastatic breast cancer. *Clin Cancer Res*. 14:7878-7883.
Diehl, J.A., M. Cheng, M.F. Roussel, and C.J. Sherr. 1998. Glycogen synthase kinase-3beta regulates cyclin D1 proteolysis and subcellular localization. *Genes & development*. 12:3499-3511.
Dolcet, X., D. Llobet, J. Pallares, and X. Matias-Guiu. 2005. NF-kB in development and progression of human cancer. *Virchows Arch*. 446:475-482.
Dourdin, N., A.K. Bhatt, P. Dutt, P.A. Greer, J.S. Arthur, J.S. Elce, and A. Huttenlocher. 2001. Reduced cell migration and disruption of the actin cytoskeleton in calpain-deficient embryonic fibroblasts. *J Biol Chem*. 276:48382-48388.
Dutt, P., J.S. Arthur, D.E. Croall, and J.S. Elce. 1998. m-Calpain subunits remain associated in the presence of calcium. *FEBS letters*. 436:367-371.
Dutt, P., D.E. Croall, J.S. Arthur, T.D. Veyra, K. Williams, J.S. Elce, and P.A. Greer. 2006. m-Calpain is required for preimplantation embryonic development in mice. *BMC Dev Biol*. 6:3.
duVerle, D., I. Takigawa, Y. Ono, H. Sorimachi, and H. Mamitsuka. 2010. CaMPDB: a resource for calpain and modulatory proteolysis. *Genome informatics. International Conference on Genome Informatics*. 22:202-213.
Ebos, J.M., C.R. Lee, W. Cruz-Munoz, G.A. Bjarnason, J.G. Christensen, and R.S. Kerbel. 2009. Accelerated metastasis after short-term treatment with a potent inhibitor of tumor angiogenesis. *Cancer Cell*. 15:232-239.
Edge, S.B., and C.C. Compton. 2010. The American Joint Committee on Cancer: the 7th edition of the AJCC cancer staging manual and the future of TNM. *Ann Surg Oncol*. 17:1471-1474.

Elce, J.S., P.L. Davies, C. Hegadorn, D.H. Maurice, and J.S. Arthur. 1997. The effects of truncations of the small subunit on m-calpain activity and heterodimer formation. *Biochem J*. 326 (Pt 1):31-38.
Ewald, A.J. 2017. Pulling cells out of tumours. *Nat Cell Biol*. 19:147-149.
Felding-Habermann, B., T.E. O'Toole, J.W. Smith, E. Fransvea, Z.M. Ruggeri, M.H. Ginsberg, P.E. Hughes, N. Pampori, S.J. Shattil, A. Saven, and B.M. Mueller. 2001. Integrin activation controls metastasis in human breast cancer. *Proc Natl Acad Sci U S A*. 98:1853-1858.
Fentiman, I.S., A. Fourquet, and G.N. Hortobagyi. 2006. Male breast cancer. *Lancet*. 367:595-604.
Folkman, J. 2002. Role of angiogenesis in tumor growth and metastasis. *Seminars in oncology*. 29:15-18.
Fong, L.W.R., D.C. Yang, and C.H. Chen. 2017. Myristoylated alanine-rich C kinase substrate (MARCKS): a multirole signaling protein in cancers. *Cancer Metastasis Rev*. 36:737-747.
Fornoni, A., A. Pileggi, R.D. Molano, N.Y. Sanabria, T. Tejada, J. Gonzalez-Quintana, H. Ichii, L. Inverardi, C. Ricordi, and R.L. Pastori. 2008. Inhibition of c-jun N terminal kinase (JNK) improves functional beta cell mass in human islets and leads to AKT and glycogen synthase kinase-3 (GSK-3) phosphorylation. *Diabetologia*. 51:298-308.
Franco, S., B. Perrin, and A. Huttenlocher. 2004a. Isoform specific function of calpain 2 in regulating membrane protrusion. *Exp Cell Res*. 299:179-187.
Franco, S.J., M.A. Rodgers, B.J. Perrin, J. Han, D.A. Bennin, D.R. Critchley, and A. Huttenlocher. 2004b. Calpain-mediated proteolysis of talin regulates adhesion dynamics. *Nat Cell Biol*. 6:977-983.
Frangioni, J.V., A. Oda, M. Smith, E.W. Salzman, and B.G. Neel. 1993. Calpain-catalyzed cleavage and subcellular relocation of protein phosphotyrosine phosphatase 1B (PTP-1B) in human platelets. *EMBO J*. 12:4843-4856.
Fridman, R., M. Toth, D. Pena, and S. Mobashery. 1995. Activation of progelatinase B (MMP-9) by gelatinase A (MMP-2). *Cancer Res*. 55:2548-2555.
Gafni, J., X. Cong, S.F. Chen, B.W. Gibson, and L.M. Ellerby. 2009. Calpain-1 cleaves and activates caspase-7. *J Biol Chem*. 284:25441-25449.
Gafni, J., and L.M. Ellerby. 2002. Calpain activation in Huntington's disease. *J Neurosci*. 22:4842-4849.
Gao, G., and Q.P. Dou. 2000. N-terminal cleavage of bax by calpain generates a potent proapoptotic 18-kDa fragment that promotes bcl-2-independent cytochrome C release and apoptotic cell death. *J Cell Biochem*. 80:53-72.
Gao, T., F. Furnari, and A.C. Newton. 2005. PHLPP: a phosphatase that directly dephosphorylates Akt, promotes apoptosis, and suppresses tumor growth. *Molecular cell*. 18:13-24.
Garcia, M., V. Bondada, and J.W. Geddes. 2005. Mitochondrial localization of mu-calpain. *Biochem Biophys Res Commun*. 338:1241-1247.
Gelmon, K.A., F.M. Boyle, B. Kaufman, D.G. Huntsman, A. Manikhas, A. Di Leo, M. Martin, L.S. Schwartzberg, J. Lemieux, S. Aparicio, L.E. Shepherd, S. Dent, S.L. Ellard, K. Tonkin, K.I. Pritchard, T.J. Whelan, D. Nomikos, A. Nusch, R.E. Coleman, H. Mukai, S. Tjulandin, R. Khasanov, S. Rizel, A.P. Connor, S.L. Santillana, J.A. Chapman, and W.R. Parulekar. 2015. Lapatinib or Trastuzumab Plus Taxane Therapy for Human Epidermal Growth Factor Receptor 2-Positive Advanced Breast Cancer: Final Results of NCIC CTG MA.31. *J Clin Oncol*. 33:1574-1583.
Gennari, A., M.P. Sormani, P. Pronzato, M. Puntoni, M. Colozza, U. Pfeffer, and P. Bruzzi. 2008. HER2 status and efficacy of adjuvant anthracyclines in early breast cancer: a pooled analysis of randomized trials. *J Natl Cancer Inst*. 100:14-20.
Geyer, C.E., J. Forster, D. Lindquist, S. Chan, C.G. Romieu, T. Pienkowski, A. Jagiello-Gruszfeld, J. Crown, A. Chan, B. Kaufman, D. Skarlos, M. Campone, N. Davidson, M. Berger, C. Oliva, S.D. Rubin, S. Stein, and D. Cameron. 2006. Lapatinib plus capecitabine for HER2-positive advanced breast cancer. *N Engl J Med*. 355:2733-2743.

Glading, A., R.J. Bodnar, I.J. Reynolds, H. Shiraha, L. Satish, D.A. Potter, H.C. Blair, and A. Wells. 2004. Epidermal growth factor activates m-calpain (calpain II), at least in part, by extracellular signal-regulated kinase-mediated phosphorylation. *Mol Cell Biol*. 24:2499-2512.

Glading, A., P. Chang, D.A. Lauffenburger, and A. Wells. 2000. Epidermal growth factor receptor activation of calpain is required for fibroblast motility and occurs via an ERK/MAP kinase signaling pathway. *J Biol Chem*. 275:2390-2398.

Glading, A., D.A. Lauffenburger, and A. Wells. 2002. Cutting to the chase: calpain proteases in cell motility. *Trends Cell Biol*. 12:46-54.

Goll, D.E., V.F. Thompson, H. Li, W. Wei, and J. Cong. 2003. The calpain system. *Physiol Rev*. 83:731-801.

Graham, T.R., H.E. Zhau, V.A. Odero-Marah, A.O. Osunkoya, K.S. Kimbro, M. Tighiouart, T. Liu, J.W. Simons, and R.M. O'Regan. 2008. Insulin-like growth factor-I-dependent up-regulation of ZEB1 drives epithelial-to-mesenchymal transition in human prostate cancer cells. *Cancer Res*. 68:2479-2488.

Graham-Siegenthaler, K., S. Gauthier, P.L. Davies, and J.S. Elce. 1994. Active recombinant rat calpain II. Bacterially produced large and small subunits associate both in vivo and in vitro. *J Biol Chem*. 269:30457-30460.

Grassilli, E., R. Narloch, E. Federzoni, L. Ianzano, F. Pisano, R. Giovannoni, G. Romano, L. Masiero, B.E. Leone, S. Bonin, M. Donada, G. Stanta, K. Helin, and M. Lavitrano. 2013. Inhibition of GSK3B bypass drug resistance of p53-null colon carcinomas by enabling necroptosis in response to chemotherapy. *Clin Cancer Res*. 19:3820-3831.

Green, D.R., and J.E. Chipuk. 2008. Apoptosis: Stabbed in the BAX. *Nature*. 455:1047-1049.

Gregoriou, M., A.C. Willis, M.A. Pearson, and C. Crawford. 1994. The calpain cleavage sites in the epidermal growth factor receptor kinase domain. *Eur J Biochem*. 223:455-464.

Grieve, S., Y. Gao, C. Hall, J. Hu, and P.A. Greer. 2016. Calpain Genetic Disruption and HSP90 Inhibition Combine To Attenuate Mammary Tumorigenesis. *Mol Cell Biol*. 36:2078-2088.

Guarneri, V., and P. Conte. 2009. Metastatic breast cancer: therapeutic options according to molecular subtypes and prior adjuvant therapy. *The oncologist*. 14:645-656.

Gujral, T.S., R.L. Karp, A. Finski, M. Chan, P.E. Schwartz, G. MacBeath, and P. Sorger. 2013. Profiling phospho-signaling networks in breast cancer using reverse-phase protein arrays. *Oncogene*. 32:3470-3476.

Gupta, G.P., and J. Massague. 2006. Cancer metastasis: building a framework. *Cell*. 127:679-695.

Guroff, G. 1964. A Neutral, Calcium-Activated Proteinase from the Soluble Fraction of Rat Brain. *J Biol Chem*. 239:149-155.

Han, Y., S. Weinman, I. Boldogh, R.K. Walker, and A.R. Brasier. 1999. Tumor necrosis factor-alpha-inducible IkappaBalpha proteolysis mediated by cytosolic m-calpain. A mechanism parallel to the ubiquitin-proteasome pathway for nuclear factor-kappab activation. *J Biol Chem*. 274:787-794.

Hanahan, D., and R.A. Weinberg. 2000. The hallmarks of cancer. *Cell*. 100:57-70.

Hanahan, D., and R.A. Weinberg. 2011. Hallmarks of cancer: the next generation. *Cell*. 144:646-674.

Hanna, R.A., R.L. Campbell, and P.L. Davies. 2008. Calcium-bound structure of calpain and its mechanism of inhibition by calpastatin. *Nature*. 456:409-412.

Hanna, R.A., B.E. Garcia-Diaz, and P.L. Davies. 2007. Calpastatin simultaneously binds four calpains with different kinetic constants. *FEBS Lett*. 581:2894-2898.

Hannesdottir, L., P. Tymoszuk, N. Parajuli, M.H. Wasmer, S. Philipp, N. Daschil, S. Datta, J.B. Koller, C.H. Tripp, P. Stoitzner, E. Muller-Holzner, G.J. Wiegers, V. Sexl, A. Villunger, and W. Doppler. 2013. Lapatinib and doxorubicin enhance the Stat1-dependent antitumor immune response. *Eur J Immunol*. 43:2718-2729.

Harris, A.L. 2002. Hypoxia--a key regulatory factor in tumour growth. *Nat Rev Cancer*. 2:38-47.

Harvey, J.A. 2007. Unusual breast cancers: useful clues to expanding the differential diagnosis. *Radiology*. 242:683-694.

Hayashi, M., H. Suzuki, S. Kawashima, T.C. Saido, and M. Inomata. 1999. The behavior of calpain-generated N- and C-terminal fragments of talin in integrin-mediated signaling pathways. *Arch Biochem Biophys*. 371:133-141.

Heitz, F., P. Harter, H.J. Lueck, A. Fissler-Eckhoff, F. Lorenz-Salehi, S. Scheil-Bertram, A. Traut, and A. du Bois. 2009. Triple-negative and HER2-overexpressing breast cancers exhibit an elevated risk and an earlier occurrence of cerebral metastases. *Eur J Cancer*. 45:2792-2798.

Ho, W.C., L. Pikor, Y. Gao, B.E. Elliott, and P.A. Greer. 2012. Calpain 2 regulates Akt-FoxO-p27(Kip1) protein signaling pathway in mammary carcinoma. *J Biol Chem*. 287:15458-15465.

Hosfield, C.M., J.S. Elce, P.L. Davies, and Z. Jia. 1999a. Crystal structure of calpain reveals the structural basis for Ca(2+)-dependent protease activity and a novel mode of enzyme activation. *EMBO J*. 18:6880-6889.

Hosfield, C.M., Q. Ye, J.S. Arthur, C. Hegadorn, D.E. Croall, J.S. Elce, and Z. Jia. 1999b. Crystallization and X-ray crystallographic analysis of m-calpain, a Ca2+-dependent protease. *Acta crystallographica. Section D, Biological crystallography*. 55:1484-1486.

Hoskin, V., A. Szeto, A. Ghaffari, P.A. Greer, G.P. Cote, and B.E. Elliott. 2015. Ezrin regulates focal adhesion and invadopodia dynamics by altering calpain activity to promote breast cancer cell invasion. *Mol Biol Cell*. 26:3464-3479.

Hu, J., K. Adler, C.A. Farah, M.H. Hastings, W.S. Sossin, and S. Schacher. 2017. Cell-Specific PKM Isoforms Contribute to the Maintenance of Different Forms of Persistent Long-Term Synaptic Plasticity. *J Neurosci*. 37:2746-2763.

Hu, M., J. Yao, D.K. Carroll, S. Weremowicz, H. Chen, D. Carrasco, A. Richardson, S. Violette, T. Nikolskaya, Y. Nikolsky, E.L. Bauerlein, W.C. Hahn, R.S. Gelman, C. Allred, M.J. Bissell, S. Schnitt, and K. Polyak. 2008. Regulation of in situ to invasive breast carcinoma transition. *Cancer Cell*. 13:394-406.

Huang, C., K. Jacobson, and M.D. Schaller. 2004. MAP kinases and cell migration. *J Cell Sci*. 117:4619-4628.

Huang, S., C.A. Pettaway, H. Uehara, C.D. Bucana, and I.J. Fidler. 2001. Blockade of NF-kappaB activity in human prostate cancer cells is associated with suppression of angiogenesis, invasion, and metastasis. *Oncogene*. 20:4188-4197.

Hussain, A.R., S.O. Ahmed, M. Ahmed, O.S. Khan, S. Al Abdulmohsen, L.C. Platanias, K.S. Al-Kuraya, and S. Uddin. 2012. Cross-talk between NFkB and the PI3-kinase/AKT pathway can be targeted in primary effusion lymphoma (PEL) cell lines for efficient apoptosis. *PLoS One*. 7:e39945.

Huston, R.B., and E.G. Krebs. 1968. Activation of skeletal muscle phosphorylase kinase by Ca2+. II. Identification of the kinase activating factor as a proteolytic enzyme. *Biochemistry*. 7:2116-2122.

Huttenlocher, A., S.P. Palecek, Q. Lu, W. Zhang, R.L. Mellgren, D.A. Lauffenburger, M.H. Ginsberg, and A.F. Horwitz. 1997. Regulation of cell migration by the calcium-dependent protease calpain. *J Biol Chem*. 272:32719-32722.

Hynes, N.E., and H.A. Lane. 2005. ERBB receptors and cancer: the complexity of targeted inhibitors. *Nat Rev Cancer*. 5:341-354.

Jang, H.S., S. Lal, and J.A. Greenwood. 2010. Calpain 2 is required for glioblastoma cell invasion: regulation of matrix metalloproteinase 2. *Neurochem Res*. 35:1796-1804.

Janossy, J., P. Ubezio, A. Apati, M. Magocsi, P. Tompa, and P. Friedrich. 2004. Calpain as a multi-site regulator of cell cycle. *Biochemical pharmacology*. 67:1513-1521.

Jeon, K.H., H.V. Yu, and Y. Kwon. 2018a. Hyperactivated m-calpain affects acquisition of doxorubicin resistance in breast cancer cells. *Biochim Biophys Acta*. 1862:1126-1133.

Jeon, K.H., H.V. Yu, and Y. Kwon. 2018b. Hyperactivated m-calpain affects acquisition of doxorubicin resistance in breast cancer cells. *Biochim Biophys Acta*.

Jin Cho, S., M. La, J.K. Ahn, G.G. Meadows, and C.O. Joe. 2001. Tob-mediated cross-talk between MARCKS phosphorylation and ErbB-2 activation. *Biochem Biophys Res Commun*. 283:273-277.

Jones, J.L., P. Glynn, and R.A. Walker. 1999. Expression of MMP-2 and MMP-9, their inhibitors, and the activator MT1-MMP in primary breast carcinomas. *J Pathol*. 189:161-168.

Kachroo, P., M.H. Lee, L. Zhang, F. Baratelli, G. Lee, M.K. Srivastava, G. Wang, T.C. Walser, K. Krysan, S. Sharma, S.M. Dubinett, and J.M. Lee. 2013. IL-27 inhibits epithelial-mesenchymal transition and angiogenic factor production in a STAT1-dominant pathway in human non-small cell lung cancer. *J Exp Clin Cancer Res*. 32:97.

Kang, H., H.I. Kwak, R. Kaunas, and K.J. Bayless. 2011. Fluid shear stress and sphingosine 1-phosphate activate calpain to promote membrane type 1 matrix metalloproteinase (MT1-MMP) membrane translocation and endothelial invasion into three-dimensional collagen matrices. *J Biol Chem*. 286:42017-42026.

Kaplan, D.H., V. Shankaran, A.S. Dighe, E. Stockert, M. Aguet, L.J. Old, and R.D. Schreiber. 1998. Demonstration of an interferon gamma-dependent tumor surveillance system in immunocompetent mice. *Proc Natl Acad Sci U S A*. 95:7556-7561.

Karagiannis, G.S., J.M. Pastoriza, Y. Wang, A.S. Harney, D. Entenberg, J. Pignatelli, V.P. Sharma, E.A. Xue, E. Cheng, T.M. D'Alfonso, J.G. Jones, J. Anampa, T.E. Rohan, J.A. Sparano, J.S. Condeelis, and M.H. Oktay. 2017. Neoadjuvant chemotherapy induces breast cancer metastasis through a TMEM-mediated mechanism. *Sci Transl Med*. 9.

Kartal-Yandim, M., A. Adan-Gokbulut, and Y. Baran. 2016. Molecular mechanisms of drug resistance and its reversal in cancer. *Crit Rev Biotechnol*. 36:716-726.

Kelly, T., S.C. Mueller, Y. Yeh, and W.T. Chen. 1994. Invadopodia promote proteolysis of a wide variety of extracellular matrix proteins. *J Cell Physiol*. 158:299-308.

Kifor, O., I. Kifor, F.D. Moore, Jr., R.R. Butters, Jr., and E.M. Brown. 2003. m-Calpain colocalizes with the calcium-sensing receptor (CaR) in caveolae in parathyroid cells and participates in degradation of the CaR. *J Biol Chem*. 278:31167-31176.

Kimura, Y., H. Saya, and M. Nakao. 2000. Calpain-dependent proteolysis of NF2 protein: involvement in schwannomas and meningiomas. *Neuropathology*. 20:153-160.

Konze, S.A., L. van Diepen, A. Schroder, R. Olmer, H. Moller, A. Pich, R. Weissmann, A.W. Kuss, R. Zweigerdt, and F.F. Buettner. 2014. Cleavage of E-cadherin and beta-catenin by calpain affects Wnt signaling and spheroid formation in suspension cultures of human pluripotent stem cells. *Mol Cell Proteomics*. 13:990-1007.

Kubbutat, M.H., and K.H. Vousden. 1997. Proteolytic cleavage of human p53 by calpain: a potential regulator of protein stability. *Mol Cell Biol*. 17:460-468.

Kulkarni, S., K.B. Reddy, F.J. Esteva, H.C. Moore, G.T. Budd, and R.R. Tubbs. 2010. Calpain regulates sensitivity to trastuzumab and survival in HER2-positive breast cancer. *Oncogene*. 29:1339-1350.

Kulkarni, S., T.C. Saido, K. Suzuki, and J.E. Fox. 1999. Calpain mediates integrin-induced signaling at a point upstream of Rho family members. *J Biol Chem*. 274:21265-21275.

Kulkarni, S., L. Saju, C. Farver, and R. Tubbs. 2012. Calpain4 is required for activation of HER2 in breast cancer cells exposed to trastuzumab and its suppression decreases survival and enhances response. *Int J Cancer*. 131:2420-2432.

Kumle, M., E. Weiderpass, T. Braaten, I. Persson, H.O. Adami, and E. Lund. 2002. Use of oral contraceptives and breast cancer risk: The Norwegian-Swedish Women's Lifestyle and Health Cohort Study. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology*. 11:1375-1381.

Kwak, H.I., H. Kang, J.M. Dave, E.A. Mendoza, S.C. Su, S.A. Maxwell, and K.J. Bayless. 2012. Calpain-mediated vimentin cleavage occurs upstream of MT1-MMP membrane translocation to facilitate endothelial sprout initiation. *Angiogenesis*. 15:287-303.

Lauffenburger, D.A., and A.F. Horwitz. 1996. Cell migration: a physically integrated molecular process. *Cell*. 84:359-369.

Lee, C.W., C.C. Lin, W.N. Lin, K.C. Liang, S.F. Luo, C.B. Wu, S.W. Wang, and C.M. Yang. 2007. TNF-alpha induces MMP-9 expression via activation of Src/EGFR, PDGFR/PI3K/Akt cascade and promotion of NF-kappaB/p300 binding in human tracheal smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol*. 292:L799-812.

Leloup, L., H. Shao, Y.H. Bae, B. Deasy, D. Stolz, P. Roy, and A. Wells. 2010. m-Calpain activation is regulated by its membrane localization and by its binding to phosphatidylinositol 4,5-bisphosphate. *J Biol Chem*. 285:33549-33566.

Li, F.Z., P.C. Cai, L.J. Song, L.L. Zhou, Q. Zhang, S.S. Rao, Y. Xia, F. Xiang, J.B. Xin, P.A. Greer, H.Z. Shi, Y. Su, W.L. Ma, and H. Ye. 2015. Crosstalk between calpain activation and TGF-beta1 augments collagen-I synthesis in pulmonary fibrosis. *Biochim Biophys Acta*. 1852:1796-1804.

Li, H.C., D.C. Cao, Y. Liu, Y.F. Hou, J. Wu, J.S. Lu, G.H. Di, G. Liu, F.M. Li, Z.L. Ou, C. Jie, Z.Z. Shen, and Z.M. Shao. 2004. Prognostic value of matrix metalloproteinases (MMP-2 and MMP-9) in patients with lymph node-negative breast carcinoma. *Breast Cancer Res Treat*. 88:75-85.

Li, M., X. Wang, M.K. Meintzer, T. Laessig, M.J. Birnbaum, and K.A. Heidenreich. 2000. Cyclic AMP promotes neuronal survival by phosphorylation of glycogen synthase kinase 3beta. *Mol Cell Biol*. 20:9356-9363.

Li, Y., J. Ma, H. Zhu, M. Singh, D. Hill, P.A. Greer, J.M. Arnold, E.D. Abel, and T. Peng. 2011. Targeted inhibition of calpain reduces myocardial hypertrophy and fibrosis in mouse models of type 1 diabetes. *Diabetes*. 60:2985-2994.

Libertini, S.J., B.S. Robinson, N.K. Dhillon, D. Glick, M. George, S. Dandekar, J.P. Gregg, E. Sawai, and M. Mudryj. 2005. Cyclin E both regulates and is regulated by calpain 2, a protease associated with metastatic breast cancer phenotype. *Cancer Res*. 65:10700-10708.

Lin, Y.C., K. Brown, and U. Siebenlist. 1995. Activation of NF-kappa B requires proteolysis of the inhibitor I kappa B-alpha: signal-induced phosphorylation of I kappa B-alpha alone does not release active NF-kappa B. *Proc Natl Acad Sci U S A*. 92:552-556.

Linder, S. 2007. The matrix corroded: podosomes and invadopodia in extracellular matrix degradation. *Trends Cell Biol*. 17:107-117.

Liu, Z., J. Cao, X. Gao, Q. Ma, J. Ren, and Y. Xue. 2011. GPS-CCD: a novel computational program for the prediction of calpain cleavage sites. *PloS one*. 6:e19001.

Liu, Z., G. Zhu, R.H. Getzenberg, and R.W. Veltri. 2015. The Upregulation of PI3K/Akt and MAP Kinase Pathways is Associated with Resistance of Microtubule-Targeting Drugs in Prostate Cancer. *J Cell Biochem*. 116:1341-1349.

Lo, H.W., S.C. Hsu, W. Xia, X. Cao, J.Y. Shih, Y. Wei, J.L. Abbruzzese, G.N. Hortobagyi, and M.C. Hung. 2007. Epidermal growth factor receptor cooperates with signal transducer and activator of transcription 3 to induce epithelial-mesenchymal transition in cancer cells via up-regulation of TWIST gene expression. *Cancer Res*. 67:9066-9076.

Luckert, K., T.S. Gujral, M. Chan, M. Sevecka, T.O. Joos, P.K. Sorger, G. Macbeath, and O. Potz. 2012. A dual array-based approach to assess the abundance and posttranslational modification state of signaling proteins. *Sci Signal*. 5:pl1.

Luzzi, K.J., I.C. MacDonald, E.E. Schmidt, N. Kerkvliet, V.L. Morris, A.F. Chambers, and A.C. Groom. 1998. Multistep nature of metastatic inefficiency: dormancy of solitary cells after successful extravasation and limited survival of early micrometastases. *The American journal of pathology*. 153:865-873.

Maisonneuve, P., D. Disalvatore, N. Rotmensz, G. Curigliano, M. Colleoni, S. Dellapasqua, G. Pruneri, M.G. Mastropasqua, A. Luini, F. Bassi, G. Pagani, G. Viale, and A. Goldhirsch. 2014. Proposed new clinicopathological surrogate definitions of luminal A and luminal B (HER2-negative) intrinsic breast cancer subtypes. *Breast Cancer Res*. 16:R65.

Mamoune, A., J.H. Luo, D.A. Lauffenburger, and A. Wells. 2003. Calpain-2 as a target for limiting prostate cancer invasion. *Cancer Res*. 63:4632-4640.

Mancinelli, R., G. Carpino, S. Petrungaro, C.L. Mammola, L. Tomaipitinca, A. Filippini, A. Facchiano, E. Ziparo, and C. Giampietri. 2017. Multifaceted Roles of GSK-3 in Cancer and Autophagy-Related Diseases. *Oxid Med Cell Longev*. 2017:4629495.

Mandic, A., K. Viktorsson, L. Strandberg, T. Heiden, J. Hansson, S. Linder, and M.C. Shoshan. 2002. Calpain-mediated Bid cleavage and calpain-independent Bak modulation: two separate pathways in cisplatin-induced apoptosis. *Mol Cell Biol*. 22:3003-3013.

Martinez-Outschoorn, U.E., C. Trimmer, Z. Lin, D. Whitaker-Menezes, B. Chiavarina, J. Zhou, C. Wang, S. Pavlides, M.P. Martinez-Cantarin, F. Capozza, A.K. Witkiewicz, N. Flomenberg, A. Howell, R.G. Pestell, J. Caro, M.P. Lisanti, and F. Sotgia. 2010. Autophagy in cancer associated fibroblasts promotes tumor cell survival: Role of hypoxia, HIF1 induction and NFkappaB activation in the tumor stromal microenvironment. *Cell Cycle*. 9:3515-3533.

Matter, M.L., and E. Ruoslahti. 2001. A signaling pathway from the alpha5beta1 and alpha(v)beta3 integrins that elevates bcl-2 transcription. *J Biol Chem*. 276:27757-27763.

Medici, D., E.D. Hay, and B.R. Olsen. 2008. Snail and Slug promote epithelial-mesenchymal transition through beta-catenin-T-cell factor-4-dependent expression of transforming growth factor-beta3. *Mol Biol Cell*. 19:4875-4887.

Melloni, E., M. Averna, F. Salamino, B. Sparatore, R. Minafra, and S. Pontremoli. 2000. Acyl-CoA-binding protein is a potent m-calpain activator. *J Biol Chem*. 275:82-86.

Melloni, E., M. Michetti, F. Salamino, and S. Pontremoli. 1998. Molecular and functional properties of a calpain activator protein specific for mu-isoforms. *J Biol Chem*. 273:12827-12831.

Meyer, S.L., D. Bozyczko-Coyne, S.K. Mallya, C.M. Spais, R. Bihovsky, J.K. Kaywooya, D.M. Lang, R.W. Scott, and R. Siman. 1996. Biologically active monomeric and heterodimeric recombinant human calpain I produced using the baculovirus expression system. *Biochem J*. 314 (Pt 2):511-519.

Micalizzi, D.S., S.M. Farabaugh, and H.L. Ford. 2010. Epithelial-mesenchymal transition in cancer: parallels between normal development and tumor progression. *J Mammary Gland Biol Neoplasia*. 15:117-134.

Moldoveanu, T., K. Gehring, and D.R. Green. 2008. Concerted multi-pronged attack by calpastatin to occlude the catalytic cleft of heterodimeric calpains. *Nature*. 456:404-408.

Moldoveanu, T., C.M. Hosfield, D. Lim, J.S. Elce, Z. Jia, and P.L. Davies. 2002. A Ca(2+) switch aligns the active site of calpain. *Cell*. 108:649-660.

Moretti, D., B. Del Bello, E. Cosci, M. Biagioli, C. Miracco, and E. Maellaro. 2009. Novel variants of muscle calpain 3 identified in human melanoma cells: cisplatin-induced changes in vitro and differential expression in melanocytic lesions. *Carcinogenesis*. 30:960-967.

Moshal, K.S., M. Singh, U. Sen, D.S. Rosenberger, B. Henderson, N. Tyagi, H. Zhang, and S.C. Tyagi. 2006. Homocysteine-mediated activation and mitochondrial translocation of calpain regulates MMP-9 in MVEC. *Am J Physiol Heart Circ Physiol*. 291:H2825-2835.

Muller, W.J., E. Sinn, P.K. Pattengale, R. Wallace, and P. Leder. 1988. Single-step induction of mammary adenocarcinoma in transgenic mice bearing the activated c-neu oncogene. *Cell*. 54:105-115.

Nagaria, T.S., C. Shi, C. Leduc, V. Hoskin, S. Sikdar, W. Sangrar, and P.A. Greer. 2017. Combined targeting of Raf and Mek synergistically inhibits tumorigenesis in triple negative breast cancer model systems. *Oncotarget*. 8:80804-80819.

Nagaria, T.S., J.L. Williams, C. Leduc, J.A. Squire, P.A. Greer, and W. Sangrar. 2013. Flavopiridol synergizes with sorafenib to induce cytotoxicity and potentiate antitumorigenic activity in EGFR/HER-2 and mutant RAS/RAF breast cancer model systems. *Neoplasia*. 15:939-951.

Nagata, Y., K.H. Lan, X. Zhou, M. Tan, F.J. Esteva, A.A. Sahin, K.S. Klos, P. Li, B.P. Monia, N.T. Nguyen, G.N. Hortobagyi, M.C. Hung, and D. Yu. 2004. PTEN activation contributes to tumor inhibition by trastuzumab, and loss of PTEN predicts trastuzumab resistance in patients. *Cancer Cell*. 6:117-127.

Nahta, R., and F.J. Esteva. 2006. HER2 therapy: molecular mechanisms of trastuzumab resistance. *Breast Cancer Res*. 8:215.

Oda, A., B.J. Druker, H. Ariyoshi, M. Smith, and E.W. Salzman. 1993. pp60src is an endogenous substrate for calpain in human blood platelets. *J Biol Chem*. 268:12603-12608.

Ohh, M., C.W. Park, M. Ivan, M.A. Hoffman, T.Y. Kim, L.E. Huang, N. Pavletich, V. Chau, and W.G. Kaelin. 2000. Ubiquitination of hypoxia-inducible factor requires direct binding to the beta-domain of the von Hippel-Lindau protein. *Nat Cell Biol*. 2:423-427.

Olayioye, M.A., I. Beuvink, K. Horsch, J.M. Daly, and N.E. Hynes. 1999. ErbB receptor-induced activation of stat transcription factors is mediated by Src tyrosine kinases. *J Biol Chem*. 274:17209-17218.

Olayioye, M.A., R.M. Neve, H.A. Lane, and N.E. Hynes. 2000. The ErbB signaling network: receptor heterodimerization in development and cancer. *EMBO J*. 19:3159-3167.

Ono, Y., T.C. Saido, and H. Sorimachi. 2016. Calpain research for drug discovery: challenges and potential. *Nature reviews. Drug discovery*. 15:854-876.

Orimo, A., P.B. Gupta, D.C. Sgroi, F. Arenzana-Seisdedos, T. Delaunay, R. Naeem, V.J. Carey, A.L. Richardson, and R.A. Weinberg. 2005. Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. *Cell*. 121:335-348.

Paez-Ribes, M., E. Allen, J. Hudock, T. Takeda, H. Okuyama, F. Vinals, M. Inoue, G. Bergers, D. Hanahan, and O. Casanovas. 2009. Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis. *Cancer cell*. 15:220-231.

Paget, S. 1889. The distribution of secondary growths in cancer of the breast. 1889. *Cancer Metastasis Rev*. 8:98-101.

Pal, G.P., T. De Veyra, J.S. Elce, and Z. Jia. 2003. Crystal structure of a micro-like calpain reveals a partially activated conformation with low Ca2+ requirement. *Structure*. 11:1521-1526.

Pap, M., and G.M. Cooper. 1998. Role of glycogen synthase kinase-3 in the phosphatidylinositol 3-Kinase/Akt cell survival pathway. *J Biol Chem*. 273:19929-19932.

Park, J., I. Kim, Y.J. Oh, K. Lee, P.L. Han, and E.J. Choi. 1997. Activation of c-Jun N-terminal kinase antagonizes an anti-apoptotic action of Bcl-2. *J Biol Chem*. 272:16725-16728.

Patel, S., B. Doble, and J.R. Woodgett. 2004. Glycogen synthase kinase-3 in insulin and Wnt signalling: a double-edged sword? *Biochemical Society transactions*. 32:803-808.

Patel, Y.M., and M.D. Lane. 2000. Mitotic clonal expansion during preadipocyte differentiation: calpain-mediated turnover of p27. *J Biol Chem*. 275:17653-17660.

Peinado, H., M. Quintanilla, and A. Cano. 2003. Transforming growth factor beta-1 induces snail transcription factor in epithelial cell lines: mechanisms for epithelial mesenchymal transitions. *J Biol Chem*. 278:21113-21123.

Pfaff, M., X. Du, and M.H. Ginsberg. 1999. Calpain cleavage of integrin beta cytoplasmic domains. *FEBS Lett*. 460:17-22.

Postovit, L.-M., P. Dutt, N. Dourdin, M. Park, P.A. Greer, C.H. Graham, and J.S. Elce. 2002a. Expression of MMP-2 and u-PA is dependent on calpain in SV40 T-antigen transformed fibroblasts. *Biochem. Biophys. Res. Comm.* (In press).

Postovit, L.M., P. Dutt, N. Dourdin, M. Park, P.A. Greer, C.H. Graham, and J.S. Elce. 2002b. Calpain is required for MMP-2 and u-PA expression in SV40 large T-antigen-immortalized cells. *Biochem Biophys Res Commun*. 297:294-301.

Potts, A.J., D.E. Croall, and M.E. Hemler. 1994. Proteolytic cleavage of the integrin beta 4 subunit. *Exp Cell Res*. 212:2-9.

Quelle, F.W., W. Thierfelder, B.A. Witthuhn, B. Tang, S. Cohen, and J.N. Ihle. 1995. Phosphorylation and activation of the DNA binding activity of purified Stat1 by the Janus protein-tyrosine kinases and the epidermal growth factor receptor. *J Biol Chem*. 270:20775-20780.

Raftopoulou, M., and A. Hall. 2004. Cell migration: Rho GTPases lead the way. *Dev Biol*. 265:23-32.

Raven, J.F., V. Williams, S. Wang, M.L. Tremblay, W.J. Muller, J.E. Durbin, and A.E. Koromilas. 2011. Stat1 is a suppressor of ErbB2/Neu-mediated cellular transformation and mouse mammary gland tumor formation. *Cell Cycle*. 10:794-804.

Reis-Filho, J.S., B. Weigelt, D. Fumagalli, and C. Sotiriou. 2010. Molecular profiling: moving away from tumor philately. *Sci Transl Med*. 2:47ps43.

Rha, S.Y., J.H. Kim, J.K. Roh, K.S. Lee, J.S. Min, B.S. Kim, and H.C. Chung. 1997. Sequential production and activation of matrix-metalloproteinase-9 (MMP-9) with breast cancer progression. *Breast Cancer Res Treat*. 43:175-181.

Riedl, S.J., and Y. Shi. 2004. Molecular mechanisms of caspase regulation during apoptosis. *Nat Rev Mol Cell Biol*. 5:897-907.

Robidoux, A., G. Tang, P. Rastogi, C.E. Geyer, Jr., C.A. Azar, J.N. Atkins, L. Fehrenbacher, H.D. Bear, L. Baez-Diaz, S. Sarwar, R.G. Margolese, W.B. Farrar, A.M. Brufsky, H.R. Shibata, H. Bandos, S. Paik, J.P. Costantino, S.M. Swain, E.P. Mamounas, and N. Wolmark. 2013. Lapatinib as a component of neoadjuvant therapy for HER2-positive operable breast cancer (NSABP protocol B-41): an open-label, randomised phase 3 trial. *The Lancet. Oncology*. 14:1183-1192.

Rodemann, H.P., and G.A. Muller. 1991. Characterization of human renal fibroblasts in health and disease: II. In vitro growth, differentiation, and collagen synthesis of fibroblasts from kidneys with interstitial fibrosis. *Am J Kidney Dis*. 17:684-686.

Roffey, J., C. Rosse, M. Linch, A. Hibbert, N.Q. McDonald, and P.J. Parker. 2009. Protein kinase C intervention: the state of play. *Current opinion in cell biology*. 21:268-279.

Rolli, M., E. Fransvea, J. Pilch, A. Saven, and B. Felding-Habermann. 2003. Activated integrin alphavbeta3 cooperates with metalloproteinase MMP-9 in regulating migration of metastatic breast cancer cells. *Proc Natl Acad Sci U S A*. 100:9482-9487.

Sahai, E., and C.J. Marshall. 2003. Differing modes of tumour cell invasion have distinct requirements for Rho/ROCK signalling and extracellular proteolysis. *Nat Cell Biol*. 5:711-719.

Saito, K., J.S. Elce, J.E. Hamos, and R.A. Nixon. 1993. Widespread activation of calcium-activated neutral proteinase (calpain) in the brain in Alzheimer disease: a potential molecular basis for neuronal degeneration. *Proc Natl Acad Sci U S A*. 90:2628-2632.

Saito, Y., J.R. Vandenheede, and P. Cohen. 1994. The mechanism by which epidermal growth factor inhibits glycogen synthase kinase 3 in A431 cells. *Biochem J*. 303 (Pt 1):27-31.

Samis, J.A., D.W. Back, E.J. Graham, C.I. DeLuca, and J.S. Elce. 1991. Constitutive expression of calpain II in the rat uterus during pregnancy and involution. *Biochem J*. 276:293-299.

Sangrar, W., C. Shi, G. Mullins, D. LeBrun, B. Ingalls, and P.A. Greer. 2015. Amplified Ras-MAPK signal states correlate with accelerated EGFR internalization, cytostasis and delayed HER2 tumor onset in Fer-deficient model systems. *Oncogene*. 34:4109-4117.

Sanjana, N.E., O. Shalem, and F. Zhang. 2014. Improved vectors and genome-wide libraries for CRISPR screening. *Nat Methods*. 11:783-784.

Sanz-Moreno, V., G. Gadea, J. Ahn, H. Paterson, P. Marra, S. Pinner, E. Sahai, and C.J. Marshall. 2008. Rac activation and inactivation control plasticity of tumor cell movement. *Cell*. 135:510-523.

Sappino, A.P., O. Skalli, B. Jackson, W. Schurch, and G. Gabbiani. 1988. Smooth-muscle differentiation in stromal cells of malignant and non-malignant breast tissues. *Int J Cancer*. 41:707-712.

Satish, L., H.C. Blair, A. Glading, and A. Wells. 2005. Interferon-inducible protein 9 (CXCL11)-induced cell motility in keratinocytes requires calcium flux-dependent activation of mu-calpain. *Mol Cell Biol*. 25:1922-1941.

Savagner, P., K.M. Yamada, and J.P. Thiery. 1997. The zinc-finger protein slug causes desmosome dissociation, an initial and necessary step for growth factor-induced epithelial-mesenchymal transition. *J Cell Biol*. 137:1403-1419.

Scaltriti, M., F. Rojo, A. Ocana, J. Anido, M. Guzman, J. Cortes, S. Di Cosimo, X. Matias-Guiu, S. Ramon y Cajal, J. Arribas, and J. Baselga. 2007. Expression of p95HER2, a truncated form of the

HER2 receptor, and response to anti-HER2 therapies in breast cancer. *J Natl Cancer Inst*. 99:628-638.
Schaefer, G., L. Shao, K. Totpal, and R.W. Akita. 2007. Erlotinib directly inhibits HER2 kinase activation and downstream signaling events in intact cells lacking epidermal growth factor receptor expression. *Cancer Res*. 67:1228-1238.
Seano, G., and L. Primo. 2015. Podosomes and invadopodia: tools to breach vascular basement membrane. *Cell Cycle*. 14:1370-1374.
Seguin, L., J.S. Desgrosellier, S.M. Weis, and D.A. Cheresh. 2015. Integrins and cancer: regulators of cancer stemness, metastasis, and drug resistance. *Trends Cell Biol*. 25:234-240.
Selliah, N., W.H. Brooks, and T.L. Roszman. 1996. Proteolytic cleavage of alpha-actinin by calpain in T cells stimulated with anti-CD3 monoclonal antibody. *J Immunol*. 156:3215-3221.
Semenza, G.L. 2002. HIF-1 and tumor progression: pathophysiology and therapeutics. *Trends Mol Med*. 8:S62-67.
Shalem, O., N.E. Sanjana, E. Hartenian, X. Shi, D.A. Scott, T. Mikkelson, D. Heckl, B.L. Ebert, D.E. Root, J.G. Doench, and F. Zhang. 2014. Genome-scale CRISPR-Cas9 knockout screening in human cells. *Science*. 343:84-87.
Shattil, S.J., C. Kim, and M.H. Ginsberg. 2010. The final steps of integrin activation: the end game. *Nat Rev Mol Cell Biol*. 11:288-300.
Shaw, L.M., I. Rabinovitz, H.H. Wang, A. Toker, and A.M. Mercurio. 1997. Activation of phosphoinositide 3-OH kinase by the alpha6beta4 integrin promotes carcinoma invasion. *Cell*. 91:949-960.
Shimada, M., M.J. Mahon, P.A. Greer, and G.V. Segre. 2005. The receptor for parathyroid hormone and parathyroid hormone-related peptide is hydrolyzed and its signaling properties are altered by directly binding the calpain small subunit. *Endocrinology*. 146:2336-2344.
Shumway, S.D., M. Maki, and S. Miyamoto. 1999. The PEST domain of IkappaBalpha is necessary and sufficient for in vitro degradation by mu-calpain. *J Biol Chem*. 274:30874-30881.
Sieg, D.J., C.R. Hauck, and D.D. Schlaepfer. 1999. Required role of focal adhesion kinase (FAK) for integrin-stimulated cell migration. *J Cell Sci*. 112 (Pt 16):2677-2691.
Slamon, D.J., G.M. Clark, S.G. Wong, W.J. Levin, A. Ullrich, and W.L. McGuire. 1987. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science*. 235:177-182.
Somiari, S.B., R.I. Somiari, C.M. Heckman, C.H. Olsen, R.M. Jordan, S.J. Russell, and C.D. Shriver. 2006. Circulating MMP2 and MMP9 in breast cancer -- potential role in classification of patients into low risk, high risk, benign disease and breast cancer categories. *Int J Cancer*. 119:1403-1411.
Sparmann, A., and D. Bar-Sagi. 2004. Ras-induced interleukin-8 expression plays a critical role in tumor growth and angiogenesis. *Cancer Cell*. 6:447-458.
Spizz, G., and P.J. Blackshear. 1996. Protein kinase C-mediated phosphorylation of the myristoylated alanine-rich C-kinase substrate protects it from specific proteolytic cleavage. *J Biol Chem*. 271:553-562.
Steeg, P.S. 2006. Tumor metastasis: mechanistic insights and clinical challenges. *Nat Med*. 12:895-904.
Steeg, P.S. 2016. Targeting metastasis. *Nature reviews. Cancer*. 16:201-218.
Stephanou, A., and D.S. Latchman. 2003. STAT-1: a novel regulator of apoptosis. *Int J Exp Pathol*. 84:239-244.
Storr, S.J., N.O. Carragher, M.C. Frame, T. Parr, and S.G. Martin. 2011a. The calpain system and cancer. *Nat Rev Cancer*. 11:364-374.
Storr, S.J., K.W. Lee, C.M. Woolston, S. Safuan, A.R. Green, R.D. Macmillan, A. Benhasouna, T. Parr, I.O. Ellis, and S.G. Martin. 2012a. Calpain system protein expression in basal-like and triple-negative invasive breast cancer. *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO*. 23:2289-2296.

Storr, S.J., R.A. Mohammed, C.M. Woolston, A.R. Green, T. Parr, I. Spiteri, C. Caldas, G.R. Ball, I.O. Ellis, and S.G. Martin. 2011b. Calpastatin is associated with lymphovascular invasion in breast cancer. *Breast*. 20:413-418.

Storr, S.J., S. Safuan, C.M. Woolston, T. Abdel-Fatah, S. Deen, S.Y. Chan, and S.G. Martin. 2012b. Calpain-2 expression is associated with response to platinum based chemotherapy, progression-free and overall survival in ovarian cancer. *Journal of cellular and molecular medicine*. 16:2422-2428.

Storr, S.J., N. Thompson, X. Pu, Y. Zhang, and S.G. Martin. 2015. Calpain in Breast Cancer: Role in Disease Progression and Treatment Response. *Pathobiology*. 82:133-141.

Storr, S.J., C.M. Woolston, F.F. Barros, A.R. Green, M. Shehata, S.Y. Chan, I.O. Ellis, and S.G. Martin. 2011c. Calpain-1 expression is associated with relapse-free survival in breast cancer patients treated with trastuzumab following adjuvant chemotherapy. *International journal of cancer. Journal international du cancer*. 129:1773-1780.

Stuelten, C.H., S. DaCosta Byfield, P.R. Arany, T.S. Karpova, W.G. Stetler-Stevenson, and A.B. Roberts. 2005. Breast cancer cells induce stromal fibroblasts to express MMP-9 via secretion of TNF-alpha and TGF-beta. *J Cell Sci*. 118:2143-2153.

Suzuki, K., H. Sorimachi, T. Yoshizawa, K. Kinbara, and S. Ishiura. 1995. Calpain: novel family members, activation, and physiologic function. *Biol Chem Hoppe Seyler*. 376:523-529.

Taddei, M.L., E. Giannoni, G. Comito, and P. Chiarugi. 2013. Microenvironment and tumor cell plasticity: an easy way out. *Cancer Lett*. 341:80-96.

Takano, J., N. Mihira, R. Fujioka, E. Hosoki, A.H. Chishti, and T.C. Saido. 2011. Vital role of the calpain-calpastatin system for placental-integrity-dependent embryonic survival. *Mol Cell Biol*. 31:4097-4106.

Tan, Y., N. Dourdin, C. Wu, T. De Veyra, J.S. Elce, and P.A. Greer. 2006a. Conditional disruption of ubiquitous calpains in the mouse. *Genesis*. 44:297-303.

Tan, Y., N. Dourdin, C. Wu, T. De Veyra, J.S. Elce, and P.A. Greer. 2006b. Ubiquitous calpains promote caspase-12 and JNK activation during endoplasmic reticulum stress-induced apoptosis. *J Biol Chem*. 281:16016-16024.

Tan, Y., C. Wu, T. De Veyra, and P.A. Greer. 2006c. Ubiquitous calpains promote both apoptosis and survival signals in response to different cell death stimuli. *J Biol Chem*. 281:17689-17698.

Tidball, J.G., and M.J. Spencer. 2000. Calpains and muscular dystrophies. *Int J Biochem Cell Biol*. 32:1-5.

Tomasek, J.J., G. Gabbiani, B. Hinz, C. Chaponnier, and R.A. Brown. 2002. Myofibroblasts and mechano-regulation of connective tissue remodelling. *Nat Rev Mol Cell Biol*. 3:349-363.

Toth, M., A. Sohail, and R. Fridman. 2012. Assessment of gelatinases (MMP-2 and MMP-9) by gelatin zymography. *Methods Mol Biol*. 878:121-135.

Tournier, C., P. Hess, D.D. Yang, J. Xu, T.K. Turner, A. Nimnual, D. Bar-Sagi, S.N. Jones, R.A. Flavell, and R.J. Davis. 2000. Requirement of JNK for stress-induced activation of the cytochrome c-mediated death pathway. *Science*. 288:870-874.

Trikha, M., Z. Zhou, J. Timar, E. Raso, M. Kennel, E. Emmell, and M.T. Nakada. 2002. Multiple roles for platelet GPIIb/IIIa and alphavbeta3 integrins in tumor growth, angiogenesis, and metastasis. *Cancer Res*. 62:2824-2833.

Tug, S., B. Delos Reyes, J. Fandrey, and U. Berchner-Pfannschmidt. 2009. Non-hypoxic activation of the negative regulatory feedback loop of prolyl-hydroxylase oxygen sensors. *Biochem Biophys Res Commun*. 384:519-523.

Ulloa, L., J. Doody, and J. Massague. 1999. Inhibition of transforming growth factor-beta/SMAD signalling by the interferon-gamma/STAT pathway. *Nature*. 397:710-713.

Ursini-Siegel, J., W.R. Hardy, D. Zuo, S.H. Lam, V. Sanguin-Gendreau, R.D. Cardiff, T. Pawson, and W.J. Muller. 2008. ShcA signalling is essential for tumour progression in mouse models of human breast cancer. *EMBO J*. 27:910-920.

Vivanco, I., and C.L. Sawyers. 2002. The phosphatidylinositol 3-Kinase AKT pathway in human cancer. *Nat Rev Cancer*. 2:489-501.
Wahba, H.A., and H.A. El-Hadaad. 2015. Current approaches in treatment of triple-negative breast cancer. *Cancer Biol Med*. 12:106-116.
Walker, R.A., and S.J. Dearing. 1999. Expression of epidermal growth factor receptor mRNA and protein in primary breast carcinomas. *Breast Cancer Res Treat*. 53:167-176.
Wallace, J.A., J.R. Pitarresi, N. Sharma, M. Palettas, M.C. Cuitino, S.T. Sizemore, L. Yu, A. Sanderlin, T.J. Rosol, K.D. Mehta, G.M. Sizemore, and M.C. Ostrowski. 2014. Protein kinase C Beta in the tumor microenvironment promotes mammary tumorigenesis. *Frontiers in oncology*. 4:87.
Wang, X.D., J.L. Rosales, A. Magliocco, R. Gnanakumar, and K.Y. Lee. 2003. Cyclin E in breast tumors is cleaved into its low molecular weight forms by calpain. *Oncogene*. 22:769-774.
Wang, Y., V. Briz, A. Chishti, X. Bi, and M. Baudry. 2013. Distinct roles for mu-calpain and m-calpain in synaptic NMDAR-mediated neuroprotection and extrasynaptic NMDAR-mediated neurodegeneration. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 33:18880-18892.
Wang, Y., J. Hersheson, D. Lopez, M. Hammer, Y. Liu, K.H. Lee, V. Pinto, J. Seinfeld, S. Wiethoff, J. Sun, R. Amouri, F. Hentati, N. Baudry, J. Tran, A.B. Singleton, M. Coutelier, A. Brice, G. Stevanin, A. Durr, X. Bi, H. Houlden, and M. Baudry. 2016. Defects in the CAPN1 Gene Result in Alterations in Cerebellar Development and Cerebellar Ataxia in Mice and Humans. *Cell Rep*. 16:79-91.
Welcker, M., J. Singer, K.R. Loeb, J. Grim, A. Bloecher, M. Gurien-West, B.E. Clurman, and J.M. Roberts. 2003. Multisite phosphorylation by Cdk2 and GSK3 controls cyclin E degradation. *Molecular cell*. 12:381-392.
Wen, Y., M.C. Hu, K. Makino, B. Spohn, G. Bartholomeusz, D.H. Yan, and M.C. Hung. 2000. HER-2/neu promotes androgen-independent survival and growth of prostate cancer cells through the Akt pathway. *Cancer Res*. 60:6841-6845.
Wernimont, S.A., W.T. Simonson, P.A. Greer, C.M. Seroogy, and A. Huttenlocher. 2010. Calpain 4 is not necessary for LFA-1-mediated function in CD4+ T cells. *PloS one*. 5:e10513.
Widschwendter, A., S. Tonko-Geymayer, T. Welte, G. Daxenbichler, C. Marth, and W. Doppler. 2002. Prognostic significance of signal transducer and activator of transcription 1 activation in breast cancer. *Clin Cancer Res*. 8:3065-3074.
Wilkinson, S., H.F. Paterson, and C.J. Marshall. 2005. Cdc42-MRCK and Rho-ROCK signalling cooperate in myosin phosphorylation and cell invasion. *Nat Cell Biol*. 7:255-261.
Wolff, A.C., M.E. Hammond, J.N. Schwartz, K.L. Hagerty, D.C. Allred, R.J. Cote, M. Dowsett, P.L. Fitzgibbons, W.M. Hanna, A. Langer, L.M. McShane, S. Paik, M.D. Pegram, E.A. Perez, M.F. Press, A. Rhodes, C. Sturgeon, S.E. Taube, R. Tubbs, G.H. Vance, M. van de Vijver, T.M. Wheeler, D.F. Hayes, O. American Society of Clinical, and P. College of American. 2007. American Society of Clinical Oncology/College of American Pathologists guideline recommendations for human epidermal growth factor receptor 2 testing in breast cancer. *J Clin Oncol*. 25:118-145.
Wu, Y., and B.P. Zhou. 2009. Inflammation: a driving force speeds cancer metastasis. *Cell Cycle*. 8:3267-3273.
Wu, Z.S., Q. Wu, J.H. Yang, H.Q. Wang, X.D. Ding, F. Yang, and X.C. Xu. 2008. Prognostic significance of MMP-9 and TIMP-1 serum and tissue expression in breast cancer. *Int J Cancer*. 122:2050-2056.
Wyckoff, J.B., S.E. Pinner, S. Gschmeissner, J.S. Condeelis, and E. Sahai. 2006. ROCK- and myosin-dependent matrix deformation enables protease-independent tumor-cell invasion in vivo. *Curr Biol*. 16:1515-1523.
Xu, L., and X. Deng. 2006. Protein kinase Ciota promotes nicotine-induced migration and invasion of cancer cells via phosphorylation of micro- and m-calpains. *J Biol Chem*. 281:4457-4466.

Yan, B., D.A. Calderwood, B. Yaspan, and M.H. Ginsberg. 2001. Calpain cleavage promotes talin binding to the beta 3 integrin cytoplasmic domain. *J Biol Chem*. 276:28164-28170.
Yang, J., Z. Wu, N. Renier, D.J. Simon, K. Uryu, D.S. Park, P.A. Greer, C. Tournier, R.J. Davis, and M. Tessier-Lavigne. 2015. Pathological Axonal Death through a MAPK Cascade that Triggers a Local Energy Deficit. *Cell*. 160:161-176.
Yang, M.H., and K.J. Wu. 2008. TWIST activation by hypoxia inducible factor-1 (HIF-1): implications in metastasis and development. *Cell Cycle*. 7:2090-2096.
Yao, X., A. Thibodeau, and J.G. Forte. 1993. Ezrin-calpain I interactions in gastric parietal cells. *The American journal of physiology*. 265:C36-46.
Yarden, Y., and M.X. Sliwkowski. 2001. Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol*. 2:127-137.
Yersal, O., and S. Barutca. 2014. Biological subtypes of breast cancer: Prognostic and therapeutic implications. *World J Clin Oncol*. 5:412-424.
Yoo, Y.G., J. Christensen, J. Gu, and L.E. Huang. 2011. HIF-1alpha mediates tumor hypoxia to confer a perpetual mesenchymal phenotype for malignant progression. *Sci Signal*. 4:pt4.
Yoshikawa, Y., H. Mukai, F. Hino, K. Asada, and I. Kato. 2000. Isolation of two novel genes, down-regulated in gastric cancer. *Jpn J Cancer Res*. 91:459-463.
Yousefi, S., R. Perozzo, I. Schmid, A. Ziemiecki, T. Schaffner, L. Scapozza, T. Brunner, and H.U. Simon. 2006. Calpain-mediated cleavage of Atg5 switches autophagy to apoptosis. *Nat Cell Biol*. 8:1124-1132.
Yu, Q., E. Sicinska, Y. Geng, M. Ahnstrom, A. Zagozdzon, Y. Kong, H. Gardner, H. Kiyokawa, L.N. Harris, O. Stal, and P. Sicinski. 2006. Requirement for CDK4 kinase function in breast cancer. *Cancer Cell*. 9:23-32.
Zadran, S., H. Jourdi, K. Rostamiani, Q. Qin, X. Bi, and M. Baudry. 2010. Brain-derived neurotrophic factor and epidermal growth factor activate neuronal m-calpain via mitogen-activated protein kinase-dependent phosphorylation. *J Neurosci*. 30:1086-1095.
Zavadil, J., and E.P. Bottinger. 2005. TGF-beta and epithelial-to-mesenchymal transitions. *Oncogene*. 24:5764-5774.
Zhang, P., Y. Sun, and L. Ma. 2015. ZEB1: at the crossroads of epithelial-mesenchymal transition, metastasis and therapy resistance. *Cell Cycle*. 14:481-487.
Zhang, Z., S.Y. Lin, B.G. Neel, and B. Haimovich. 2006. Phosphorylated alpha-actinin and protein-tyrosine phosphatase 1B coregulate the disassembly of the focal adhesion kinase x Src complex and promote cell migration. *J Biol Chem*. 281:1746-1754.
Zheng, X., A.X. Zhou, P. Rouhi, H. Uramoto, J. Boren, Y. Cao, T. Pereira, L.M. Akyurek, and L. Poellinger. 2014. Hypoxia-induced and calpain-dependent cleavage of filamin A regulates the hypoxic response. *Proc Natl Acad Sci U S A*. 111:2560-2565.
Zhong, H., A.M. De Marzo, E. Laughner, M. Lim, D.A. Hilton, D. Zagzag, P. Buechler, W.B. Isaacs, G.L. Semenza, and J.W. Simons. 1999. Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. *Cancer Res*. 59:5830-5835.
Zimmerman, U.J., L. Boring, J.H. Pak, N. Mukerjee, and K.K. Wang. 2000. The calpain small subunit gene is essential: its inactivation results in embryonic lethality. *IUBMB life*. 50:63-68.

Appendix One

A Calpain Perspective on Autophagy and Metabolism in Cancer

James A. MacLeod[1,2] and Peter A. Greer[1,2,]

[1] Department of Pathology and Molecular Medicine, Queen's University, Kingston, Ontario, Canada

[2] Division of Cancer Biology and Genetics, Queen's Cancer Research Institute, Kingston, Ontario, Canada

Abstract

Autophagy and metabolism are two emerging hallmarks of cancer. Autophagy is a complex process that has been implicated in protecting cancer cells from therapeutic challenge in a cell-intrinsic manner, as well as supporting tumorigenesis. The calpain protease system contributes to the regulation of autophagy through acting on upstream mediators such as hypoxia-inducible factor alpha (HIF1-α), as well as a key effector of autophagy, beclin-1.

Metabolic coupling of cancer cells and cancer-associated fibroblasts (CAFs) has also been recognized for its role in enhancing tumor progression through optimizing response to locally available metabolites. Calpain modulates glucose receptor (GLUT) and monocarboxylate transporter (MCT) expression, implicating calpain in these processes.

A growing body of research suggests that disruption of autophagy may enhance the efficacy of specific cancer therapeutics, and that interfering with metabolic coupling within a tumor may also enhance anticancer strategies. This review highlights key areas of calpain function in autophagy and metabolic coupling and provides rationale for the therapeutic use of calpain inhibitors in cancer treatment.

Contrasting roles for calpain in cancer cell survival and death

The calpain system is comprised of a family of calcium-dependent intracellular proteases encoded by 15 mammalian genes. The most widely studied calpains, calpain-1 and calpain-2 are heterodimers comprised of CAPN1 and CAPNS1 (calpain-1) or CAPN2 and CAPNS1 (calpain-2), where CAPN1 and CAPN2 are the catalytic subunits and CAPNS1 is an obligate regulatory subunit (Goll et al., 2003). These two calpain isoforms lack defined peptide target cleavage sequences and have diverse substrate profiles (Liu et al., 2011). Tissue microarrays (TMAs) have revealed discordant results when correlating calpain expression with patient prognosis across a variety of cancers. High expression of calpain-2 was associated with reduced survival in triple-negative and basal-like breast cancer (Storr et al., 2012a), as well as resistance to platinum-based chemotherapy in ovarian cancer (Storr et al., 2012b). However, low expression of both calpain-1 and calpain-2 were associated with lower survival rates in gastroesophageal adenocarcinomas (Storr et al., 2013). It is important to note that TMAs represent correlative observations, and additionally do not capture the activity level of these calpains, which is a current challenge in the calpain research field. These discordant results may also be a reflection of the context-specific nature of calpain activation and effects of calpain cleavage on the function and regulation of its substrates, which may vary depending upon the specific cancer type, as well as therapeutic challenge utilized. Studies of mouse embryonic fibroblasts revealed that calpain promotes apoptosis upon challenge with the topoisomerase inhibitors etoposide or camptothecin yet protects cells from apoptosis induced by the pro-inflammatory mediator tumor necrosis factor alpha or the broad-spectrum protein kinase inhibitor staurosporine (Tan et al., 2006b).

Calpain Modulates Autophagy and Apoptosis

Autophagy is a physiological response that cells undergo in response to cell stress. It is characterized by the formation of a double-membrane autophagosome structure around cytosolic proteins and organelles and the subsequent fusion with lysosomes where the contents are catabolized and recycled

to sustain cell viability (reviewed in (Kang et al., 2011)). Unlike apoptosis, which results in cell death, autophagy serves to promote survival, but if left unchecked can lead to cell death.

The role of autophagy in tumorigenesis is complex. Some studies note that autophagy can attenuate breast cancer progression under conditions of metabolic stress through protecting genome integrity (Karantza-Wadsworth et al., 2007). Other studies show that autophagy can protect cancer cells from specific therapeutic challenges. For example, knockdown of the pro-autophagy protein 5 (ATG5) in radioresistant MDA-MB-231 breast cancer cells sensitized them to gamma radiation (Apel et al., 2008). In the breast cancer lines MCF-7, MCF-7-HER2 (tamoxifen-resistant), and T-47D, inhibiting autophagy sensitized cells to tamoxifen and camptothecin (Abedin et al., 2007; Qadir et al., 2008). Upregulated autophagy has also been linked to the development of trastuzumab resistance in the HER2 positive breast cancer cell line SKBR3 (Vazquez-Martin et al., 2009); and the authors extended this work to show that inhibition of autophagy in a trastuzumab-resistant patient derived cell line markedly suppressed tumor growth in a xenograft model (Cufi et al., 2013). Related research on calpain and response to trastuzumab suggests that calpain may contribute to the survival of resistant cell populations, as well as the generation of a cytoplasmic HER2-derived fragment (p95Her2), which is a biomarker of trastuzumab resistance (Kulkarni et al., 2010; Scaltriti et al., 2007). Several clinical trials have been launched exploring autophagy inhibition in breast cancer. One assessing a microtubule inhibitor (ixabepione) in combination with the autophagy inhibitor hydroxychloroquine in metastatic breast cancer was terminated due to slow patient accrual (NCT00765765), and results are pending for a 2017 study investigating neoadjuvant chloroquine administration in ductal carcinoma *in situ* prior to surgical resection (NCT01023477).

Inhibiting autophagy does not enhance the efficacy of all types of therapeutic challenges. For example, in a preclinical colorectal cancer model, inhibition of autophagy attenuated tumor growth after radiotherapy in immune-deficient mice but had the opposite effect in immune-competent mice in which radiotherapy exerts a significant anticancer effect through stimulating immune engagement in a process involving autophagy-mediated ATP release from dying cells (Ko et al., 2014). Thus, the type of cancer,

immune status, and the nature of therapeutic challenge are relevant when targeting autophagy in a clinical context.

Given that autophagy appears to play an important role in protecting tumors from certain therapeutic challenges, there has been a great deal of interest in autophagy inhibition as a potential cancer therapeutic strategy (Chude and Amaravadi, 2017). Calpain was first implicated in autophagosome formation in mouse embryonic fibroblasts, where it was revealed that loss of calpain correlated with impaired autophagy and increased apoptosis (Demarchi et al., 2007; Demarchi et al., 2006). One of the key drivers of autophagy is the stabilization of HIF1-α by hypoxia, a common feature of cancer (Bellot et al., 2009; Zhang et al., 2008). HIF1-α promotes autophagy by translocating to the nucleus where it binds hypoxia response elements (HREs) and activates transcription of genes including B-cell lymphoma 2/protein 3 like (BNIP3/BNIP3L) which disrupts Bcl-2 sequestration of the pro-autophagy protein beclin-1 (Mazure and Pouyssegur, 2009; Sinha and Levine, 2008) (Figure 1). While calpain has previously been implicated in both pro- and anti-apoptotic responses to therapeutic challenge (Cao et al., 2003; Tan et al., 2006b; Wood et al., 1998), under hypoxic challenge, calpain can facilitate the nuclear translocation of HIF1-α, consistent with a pro-survival function. Mechanistically, calpain cleavage of the cytoplasmic actin-binding protein filamin-A (FLNA) generates a C-terminal FLNA fragment which binds HIF1-α and promotes its nuclear translocation and subsequent transcriptional activity (Zheng et al., 2014). In another example, hypoxia and starvation challenge in colon cancer cells leads to calpain cleavage of the nuclear oncoprotein MYC, resulting in a cytoplasmic localized MYC-nick fragment that promotes autophagy and resistance to chemotherapeutic drugs through a mechanism involving recruitment of acetyltransferases that modify tubulin and ATG3 (Conacci-Sorrell et al., 2014).

Calpain has also been implicated in regulating a switch between autophagy and apoptosis through cleavage of ATG5 and Beclin-1 (Figure 2), with apoptosis initiated by truncated ATG5 triggering cytochrome-c release from the mitochondria (Yousefi et al., 2006; Zhu et al., 2015). However, the high-mobility group box 1 (HMGB1) protein protects both ATG5 and Beclin-1 from calpain cleavage (Figure

1), preventing the generation of pro-apoptotic fragments (Zhu et al., 2015). Contextual differences for pro-survival functions of calpain in autophagy are exemplified by a study showing that calpain cleavage of beclin-1 in RAS-activated intestinal epithelial cells promotes anchorage-independent survival (Yoo et al., 2010), consistent with TMA data suggesting that higher levels of beclin-1 are associated with favorable prognosis in locally advanced colon cancer (Li et al., 2009a). The authors note several possible explanations for this, including the possibility that autophagy protects genomic integrity under metabolic stress. However, preclinical data support the notion that inhibition of autophagy in colorectal cancer enhances the efficacy of first-line chemotherapeutics including 5-fluorouracil (5-FU) (Li et al., 2009b; Selvakumaran et al., 2013), and a phase I/II clinical trial exploring the standard of care plus the autophagy inhibitor hydroxychloroquine is underway (NCT01206530).

The activities of both AKT and mTOR may also inhibit autophagy (Nazio et al., 2013; Russell et al., 2013; Wang et al., 2012) through regulating a core initiator of autophagosome formation, unc-51 like autophagy activating kinase 1 (ULK1). Although calpain has previously been implicated in the activation of AKT (Ho et al., 2012; Tan et al., 2006a), these studies supported a predominantly pro-tumorigenic role. Given that hypoxic tumor cells may rely on autophagy for survival, acting through a key regulator such as HIF1-α (Zhang et al., 2008), calpain may preferentially promote autophagy under these conditions. As illustrated in Figure 2, calpain may play a dominant role under hypoxic conditions where calpain functions may promote autophagy by enhancing nuclear localization and activity of HIF1-α, leading to BNIP3/BNIP3L expression and liberation of beclin-1 from the BCL-2/beclin-1 complex. While calpain can contribute to apoptotic pathways through the cleavage of endogenous ATG5, it could be speculated that this is not a dominant function, as only enforced expression of ATG5 appears to enhance the role of calpain-cleaved ATG5 in driving apoptosis (Yousefi et al., 2006). Figure 1 highlights key areas where calpain interacts in pathways related to autophagy and apoptosis. A dominant role for calpain may be seen under conditions of hypoxia, where transcription of genes containing hypoxia response elements (HREs) by HIF1-α occurs, or in cancers expressing activating mutations in RAS or MYC.

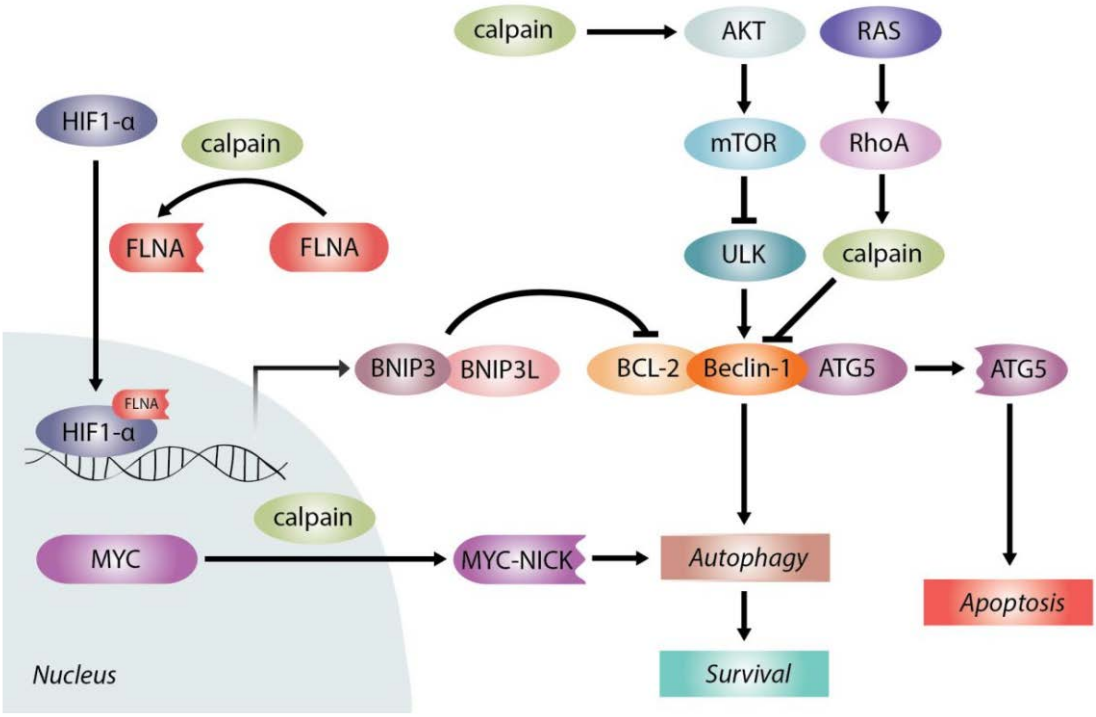

Figure 1. Calpain roles in autophagy and apoptosis.

The schematic illustrates proposed roles of calpain in regulation of autophagy and apoptosis. HIF1-α is stabilized under hypoxic conditions and can initiate signals supporting cell viability through autophagy. Calpain cleaved FLNA promotes nuclear translocation of HIF1-α to drive transcription of HRE-containing genes (Zheng et al., 2014) including BNIP3 and BNIP3L, BH3-domain containing members of the BCL-2 family. BNIP3/BNIP3L displace BCL-2 from Beclin-1, releasing Beclin-1 to promote autophagy (Mazure and Pouyssegur, 2009), which can contribute to cell survival under conditions of stress. Calpain-mediated activation of AKT may enhance mTOR function, allowing mTOR to phosphorylate and inhibit ULK1, a key driver of Beclin-1/ATG5-mediated autophagosome formation (Nazio et al., 2013; Russell et al., 2013). However, under conditions of stress, a reduction in AKT signaling may favor apoptosis. Oncogenic RAS may contribute to the activation of calpain via RhoA, which may attenuate autophagy through cleavage of Beclin-1 (Yoo et al., 2010). Generation of MYC-Nick by calpain may also enhance autophagy (Conacci-Sorrell et al., 2014). Calpain may also regulate a switch from autophagy to apoptosis in the absence of HMBG1, by cleavage of ATG5 and Beclin-1, yielding (ATG5*), which may contribute to apoptosis (Yousefi et al., 2006; Zhu et al., 2015).

Metabolic Coupling in Tumorigenesis

The tumor microenvironment can profoundly affect cancer progression, as cancer cells engage in metabolic coupling between adjacent cancer cells and stromal cells to provide a supportive microenvironment for a developing neoplasm. This metabolic coupling may represent a strategy to adapt to changes in oxygen perfusion as solid tumors develop. The Warburg Effect was first described in 1956 (Warburg, 1956a; Warburg, 1956b) and refers to the propensity for cancer cells to employ glycolysis to metabolize glucose to lactate in the cytosol, rather than shunting pyruvate into the aerobic mitochondrial oxidative phosphorylation system. Several theories exist to explain why cancer cells preferentially utilize glycolysis, despite being less efficient than aerobic respiration in the production of ATP. The separation of pre-malignant lesions from the vascular network may favor the development of anaerobic glycolysis in a nascent tumor (Gatenby and Gillies, 2004), and lactate may be utilized by cancer cells or tumor-associated stroma to support carcinogenesis (Koukourakis et al., 2006; Sonveaux et al., 2008; Vander Heiden et al., 2009). Furthermore, an acidic microenvironment, which is driven by glycolysis, may promote a hypermutable state (Yuan et al., 2000), and facilitate release of VEGF from extracellular vesicles (Taraboletti et al., 2006), aiding tumor progression and metastatic dissemination.

Lactate is a key product of glycolysis and is notable as it can promote fibroblast activity (Trabold et al., 2003). Fibroblasts aid in remodeling the extracellular matrix and can also provide metabolites to a developing tumor as a result of metabolic reprogramming; representing an interesting area of crosstalk between autophagy and metabolism (Chiavarina et al., 2010; Martinez-Outschoorn et al., 2011; Martinez-Outschoorn et al., 2010). Fibroblasts deposit fibronectin, which contributes to the induction of epithelial-to-mesenchymal transition in MCF-7 cells in a calpain-dependent manner (Li et al., 2017), and cancer-associated fibroblasts (CAFs) can aid metastatic dissemination by force-directed migration (Ewald, 2017). In one model, MCF-7 cells were able to induce paracrine autophagy in CAFs via oxidative stress mediated stabilization of HIF1-α, prompting CAFs to undergo aerobic glycolysis and provide nutrients to adjacent cancer cells (Martinez-Outschoorn et al., 2010). Increased cell intrinsic mitochondrial autophagy

under hypoxic conditions can also protect cancer cells from generation of reactive oxygen species (Zhang et al., 2008). Another study reports on HIF1-α and lactate mediated metabolic coupling of CAFs and cancer cells whereby CAFs enter into what is described as a "biunivocal relationship" and may extrude lactate for use by adjacent cancer cells as a result of oxidative stress mediated stabilization of HIF1-α (Fiaschi et al., 2012). Lactate itself has also been reported to stabilize HIF1α (Sonveaux et al., 2012), and this may represent one mechanism by which cancer cells recruit CAFs and associated stroma to drive tumor progression, as well as respond to local fluctuations in oxygen by transferring lactate from hypoxic cells to oxygenated cells (Fiaschi et al., 2012; Sonveaux et al., 2008).

Calpain Modulates Metabolic Coupling

Metabolic coupling between cancer cells and stroma is facilitated by transporters for glucose and lactate. Glucose is imported into mammalian cells by a family of glucose transporters (GLUT), encoded by 14 genes. Tissue expression varies, however GLUT1 is frequently upregulated in tumors (Macheda et al., 2005; Younes et al., 1996). In particular, tumors with high expression of GLUT1 are associated with poor prognosis (Szablewski, 2013), fewer infiltrating $CD8^+$ effector cells (Singer et al., 2011), and drug resistance (Cao et al., 2007). Inhibition of GLUT1 has been associated with improved sensitivity to chemotherapeutic agents (Cao et al., 2007; Rastogi et al., 2007). Expression and localization of GLUT1 is driven by AKT (Barthel et al., 1999), GSK3β-mTOR signaling (Buller et al., 2008), HIF1-α (Robey et al., 2005), and lactate (Jantsch et al., 2011).

Lactate is transported into or out of cells by the family of monocarboxylic acid symporters (MCT1-4) (reviewed in (Halestrap, 2012)). Within cells, lactate is interconverted to pyruvate by lactate dehydrogenase-A (LDH-A), which allows pyruvate to be fed into the tricarboxylic acid cycle. Elevated expression of MCT1 and MCT4 are associated with a variety of cancers (Gallagher et al., 2007; Miranda-Goncalves et al., 2013; Pinheiro et al., 2012; Pinheiro et al., 2008), and expression of MCT4 is associated with poor prognosis. MCT4 is a transcriptional target of HIF1-α, and MCT1 is induced by MYC, and

repressed by p53 (Boidot et al., 2012; Doherty et al., 2014; Ullah et al., 2006). MYC has also been implicated in driving LDH-A expression, which is associated with cellular transformation and aerobic glycolysis (Lewis et al., 2000; Shim et al., 1997). Interestingly, expression of the lactate transporter MCT1 is sufficient to drive lactate-mediated angiogenesis and stabilization of HIF1-α in both cancer and endothelial cells, as lactate has been shown to stabilize HIF1-α (Sonveaux et al., 2012; Vegran et al., 2011), and disruption of MCT1 *in vivo* has antitumor effects (Sonveaux et al., 2008). These antitumor effects may result from changes to intracellular pH, impeded import of lactate, and attenuated angiogenesis (Doherty et al., 2014).

As illustrated in Figure 2, metabolic coupling can be characterized at a macro level by the efflux of lactate from hypoxic cancer cells, for use by a neighboring population of oxygenated cells. Hypoxic cells distant from the vasculature may induce autophagy in CAFs, providing metabolites to hypoxic cancer cells as well as employing CAFs in metastatic dissemination. Lactate and hypoxia also co-opt the vasculature and induce angiogenesis, contributing to overall tumorigenesis. Calpain can be incorporated into this model whereby promotion of AKT signaling (Tan et al., 2006b) enhances mTOR activity (Hay and Sonenberg, 2004) and subsequent transcription of GLUT1 (Barthel et al., 1999; Buller et al., 2008), facilitating glucose uptake by hypoxic cells. This effect can be compounded by the presence of intracellular lactate which can further stabilize HIF1-α, and calpain can aid translocation of HIF1-α to the nucleus through cleavage of FLNA (De Saedeleer et al., 2012; Sonveaux et al., 2012; Zheng et al., 2014). Both lactate and calpain can cause degradation of IκBα (Pianetti et al., 2001; Shumway et al., 1999; Sonveaux et al., 2012; Vegran et al., 2011), allowing nuclear translocation and transcription of NFκB target genes, which can contribute to angiogenesis (Vegran et al., 2011), as well as induction of MCT1 (Boidot et al., 2012). Induction of MCT1 may be one mechanism by which hypoxic cells efflux lactate for use by oxygenated cancer cells and fibroblasts. This metabolic coupling can be further enhanced through calpain-mediated degradation of p53 (Gonen et al., 1997; Kubbutat and Vousden, 1997) which can lead to upregulated GLUT1 and GLUT4, facilitating glucose uptake in hypoxic cells, and the efflux or import of

lactate via MCT1 expression (Boidot et al., 2012; Schwartzenberg-Bar-Yoseph et al., 2004). LDH-A facilitates the interconversion of lactate and pyruvate; therefore its function may be relevant, especially for oxygenated cells which uptake lactate. The proto-oncogene MYC is activated in many cancers and can drive expression of both LDH-A and MCT1 (Doherty et al., 2014; Shim et al., 1997). Importantly, MYC transcriptional activity is suppressed by calpain (Conacci-Sorrell et al., 2014), therefore an anti-cancer function for calpain may exist, and therapeutic strategies targeting calpain in cancers with oncogenic MYC may be more effective in disrupting autophagy (Figure 2) than metabolic coupling.

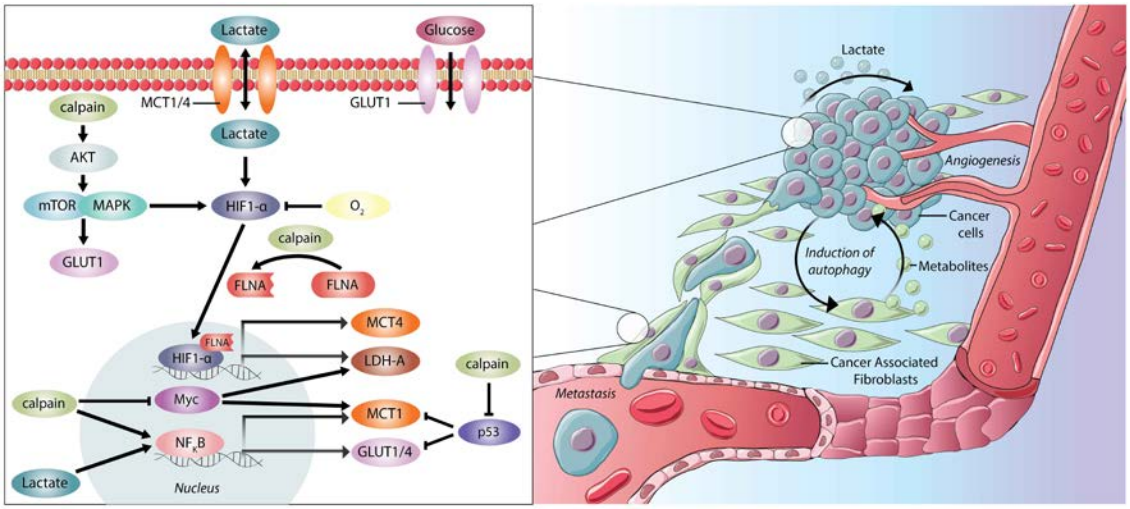

Figure 2. Calpain roles in dynamic metabolic coupling.

GLUTs and MCTs are key players in metabolism and tumor-stroma coupling by regulating glucose uptake and lactate uptake or efflux. Lactate is a key metabolite of both cancer and stromal cells and can profoundly affect tumorigenesis through incorporation into metabolic pathways of oxygenated cancer cells (Sonveaux et al., 2008). Lactate is produced by hypoxic cancer cells and enhances HIF1-α stability and promotes angiogenesis (De Saedeleer et al., 2012), as well as enhancing activation of NFκB in stromal cells (Sonveaux et al., 2012; Vegran et al., 2011), thereby modulating a hypoxic response. Lactate may be effluxed and interconverted to pyruvate in oxygenated cells by LDH-A. Efflux of lactate is facilitated by MCTs under the regulation of NFκB, p53 (Boidot et al., 2012), HIF1-α (Ullah et al., 2006), and MYC (Doherty et al., 2014). Calpain can promote MCT expression through degradation of IκB (Shumway et al., 1999), allowing NFκB to translocate to the nucleus, as well as promote nuclear accumulation of HIF1-α (Zheng et al., 2014). However, calpain may attenuate MCT expression through cleavage of MYC (Conacci-Sorrell et al., 2014). Expression of GLUTs facilitates glucose uptake and is regulated by many of the same signaling members, in addition to p53 (Schwartzenberg-Bar-Yoseph et al., 2004), which is a calpain substrate (Kubbutat and Vousden, 1997). Collectively, these signaling pathways can drive dynamic GLUT and MCT expression to facilitate tumor progression in response to changes in oxygen availability. Furthermore, the recruitment of CAFs can support the growth of cancer cells by providing metabolites as a result of induced autophagy (Martinez-Outschoorn et al., 2011), as well as directly aiding metastatic dissemination (Ewald, 2017).

Concluding remarks

There is increasing evidence for calpain promoting tumor progression and metastasis, yet to date the mechanisms underpinning these effects remain elusive. Here, we highlight how autophagy can protect cancer cells from therapeutic challenge, and how metabolic coupling contributes to tumor progression through a key metabolite, lactate. Understanding which calpain substrates are in involved in established pathways which regulate autophagy and metabolic coupling sets the stage for incorporation of calpain inhibitors into existing treatment strategies. Work remains to validate a dominant role for calpain in these processes, especially under hypoxia, during which some of the paradoxical roles for calpain may be resolved. For example, as seen in Figure 2, calpain cleavage of ATG5 may contribute to apoptosis (Yousefi et al., 2006), yet understanding which, if any, therapeutic challenges exert their function through this pathway is required. Furthermore, beclin-1 has been described as a haploinsufficient tumor suppressor, which is expressed at low levels in some breast and ovarian cancers (Yue et al., 2003). Although beclin-1 possesses tumor suppressor functions, evidence suggests that inhibition of autophagy in combination with therapeutic challenge can improve tumor response. Therapeutic suppression of autophagy through inhibition of calpain may be more effective in tumors with high levels of CAFs, or homozygous expression of beclin-1 in the cancer cells, as autophagy would likely be regulated in a canonical manner. Tissue microarrays may be an effective tool to address this question by looking at the prevalence of beclin-1 loss specifically in cancer cells of different origin, as well as correlating the level of autophagy occurring in either the cancer cells or the CAFs with clinical outcome. Another approach may involve assessing response to therapeutic challenge using xenografts of fibroblast and cancer cells with CRISPR/Cas9 knockout of beclin-1 in the cancer cells.

Calpain cleavage of filamin-A under hypoxia is also postulated to be especially relevant in promoting both autophagy and metabolic coupling. The extent to which co-occurrence of cleaved filamin-A and nuclear HIF1-α is found in clinical samples would shed light on the strength of this relationship. This could also be explored *in vitro* through hypoxic culture of cancer cells with or without

CRISPR/CAS9 knockout of calpain and subsequent immunoblotting for HIF1α in the nuclear fraction. Conceptually, disrupting metabolic coupling appears to be a bona fide therapeutic strategy, and a first in class phase I human trial of AZD3965 targeting the MCT1 metabolic symporter has recently launched (NCT01791595).

Consideration should also be given to whether calpain participates in regulating immune escape, since lactate has been linked to modulating immune engagement (Mendler et al., 2012). Therefore calpain-mediated disruption of MCT expression in immunotherapy may represent another therapeutic avenue. Furthermore, autophagy has been implicated in antigen presentation and adaptive immunity (Ma et al., 2013), suggesting broad clinical implications for calpain inhibitors. Linking the mechanism of action of different therapeutic modalities with calpain engagement will also need to be considered in a cancer-specific manner. Thus, the use of systemic calpain inhibitors may be advantageous only under certain circumstances, such as tumors with a high degree of hypoxia, and strong recruitment of cancer associated fibroblasts.

In conclusion, calpain substrates are intricately involved in processes such as tumor cell survival and metastasis. Emerging evidence shows that calpains are dysregulated in cancer, yet functional characterization of calpain substrate involvement in carcinogenesis and development of effective calpain inhibitors remain as works in progress (reviewed in (Ono et al., 2016)). Understanding calpain engagement in response to therapeutic challenges will shed light on the utility of calpain inhibitors in treatment strategies aimed at disrupting autophagy and tumor-stroma coupling in cancer.

References

Abedin, M.J., D. Wang, M.A. McDonnell, U. Lehmann, and A. Kelekar. 2007. Autophagy delays apoptotic death in breast cancer cells following DNA damage. *Cell Death Differ*. 14:500-510.

Apel, A., I. Herr, H. Schwarz, H.P. Rodemann, and A. Mayer. 2008. Blocked autophagy sensitizes resistant carcinoma cells to radiation therapy. *Cancer Res*. 68:1485-1494.

Barthel, A., S.T. Okino, J. Liao, K. Nakatani, J. Li, J.P. Whitlock, Jr., and R.A. Roth. 1999. Regulation of GLUT1 gene transcription by the serine/threonine kinase Akt1. *J Biol Chem*. 274:20281-20286.

Bellot, G., R. Garcia-Medina, P. Gounon, J. Chiche, D. Roux, J. Pouyssegur, and N.M. Mazure. 2009. Hypoxia-induced autophagy is mediated through hypoxia-inducible factor induction of BNIP3 and BNIP3L via their BH3 domains. *Mol Cell Biol*. 29:2570-2581.

Boidot, R., F. Vegran, A. Meulle, A. Le Breton, C. Dessy, P. Sonveaux, S. Lizard-Nacol, and O. Feron. 2012. Regulation of monocarboxylate transporter MCT1 expression by p53 mediates inward and outward lactate fluxes in tumors. *Cancer Res*. 72:939-948.

Buller, C.L., R.D. Loberg, M.H. Fan, Q. Zhu, J.L. Park, E. Vesely, K. Inoki, K.L. Guan, and F.C. Brosius, 3rd. 2008. A GSK-3/TSC2/mTOR pathway regulates glucose uptake and GLUT1 glucose transporter expression. *American journal of physiology. Cell physiology*. 295:C836-843.

Cao, X., X. Deng, and W.S. May. 2003. Cleavage of Bax to p18 Bax accelerates stress-induced apoptosis, and a cathepsin-like protease may rapidly degrade p18 Bax. *Blood*. 102:2605-2614.

Cao, X., L. Fang, S. Gibbs, Y. Huang, Z. Dai, P. Wen, X. Zheng, W. Sadee, and D. Sun. 2007. Glucose uptake inhibitor sensitizes cancer cells to daunorubicin and overcomes drug resistance in hypoxia. *Cancer Chemother Pharmacol*. 59:495-505.

Chiavarina, B., D. Whitaker-Menezes, G. Migneco, U.E. Martinez-Outschoorn, S. Pavlides, A. Howell, H.B. Tanowitz, M.C. Casimiro, C. Wang, R.G. Pestell, P. Grieshaber, J. Caro, F. Sotgia, and M.P. Lisanti. 2010. HIF1-alpha functions as a tumor promoter in cancer associated fibroblasts, and as a tumor suppressor in breast cancer cells: Autophagy drives compartment-specific oncogenesis. *Cell Cycle*. 9:3534-3551.

Chude, C.I., and R.K. Amaravadi. 2017. Targeting Autophagy in Cancer: Update on Clinical Trials and Novel Inhibitors. *Int J Mol Sci*. 18.

Conacci-Sorrell, M., C. Ngouenet, S. Anderson, T. Brabletz, and R.N. Eisenman. 2014. Stress-induced cleavage of Myc promotes cancer cell survival. *Genes & development*. 28:689-707.

Cufi, S., A. Vazquez-Martin, C. Oliveras-Ferraros, B. Corominas-Faja, E. Cuyas, E. Lopez-Bonet, B. Martin-Castillo, J. Joven, and J.A. Menendez. 2013. The anti-malarial chloroquine overcomes primary resistance and restores sensitivity to trastuzumab in HER2-positive breast cancer. *Sci Rep*. 3:2469.

De Saedeleer, C.J., T. Copetti, P.E. Porporato, J. Verrax, O. Feron, and P. Sonveaux. 2012. Lactate activates HIF-1 in oxidative but not in Warburg-phenotype human tumor cells. *PLoS One*. 7:e46571.

Demarchi, F., C. Bertoli, T. Copetti, E.L. Eskelinen, and C. Schneider. 2007. Calpain as a novel regulator of autophagosome formation. *Autophagy*. 3:235-237.

Demarchi, F., C. Bertoli, T. Copetti, I. Tanida, C. Brancolini, E.L. Eskelinen, and C. Schneider. 2006. Calpain is required for macroautophagy in mammalian cells. *J Cell Biol*. 175:595-605.

Doherty, J.R., C. Yang, K.E. Scott, M.D. Cameron, M. Fallahi, W. Li, M.A. Hall, A.L. Amelio, J.K. Mishra, F. Li, M. Tortosa, H.M. Genau, R.J. Rounbehler, Y. Lu, C.V. Dang, K.G. Kumar, A.A. Butler, T.D. Bannister, A.T. Hooper, K. Unsal-Kacmaz, W.R. Roush, and J.L. Cleveland. 2014. Blocking lactate export by inhibiting the Myc target MCT1 Disables glycolysis and glutathione synthesis. *Cancer Res*. 74:908-920.

Ewald, A.J. 2017. Pulling cells out of tumours. *Nat Cell Biol*. 19:147-149.

Fiaschi, T., A. Marini, E. Giannoni, M.L. Taddei, P. Gandellini, A. De Donatis, M. Lanciotti, S. Serni, P. Cirri, and P. Chiarugi. 2012. Reciprocal metabolic reprogramming through lactate shuttle coordinately influences tumor-stroma interplay. *Cancer Res*. 72:5130-5140.

Gallagher, S.M., J.J. Castorino, D. Wang, and N.J. Philp. 2007. Monocarboxylate transporter 4 regulates maturation and trafficking of CD147 to the plasma membrane in the metastatic breast cancer cell line MDA-MB-231. *Cancer Res*. 67:4182-4189.

Gatenby, R.A., and R.J. Gillies. 2004. Why do cancers have high aerobic glycolysis? *Nat Rev Cancer*. 4:891-899.

Goll, D.E., V.F. Thompson, H. Li, W. Wei, and J. Cong. 2003. The calpain system. *Physiol Rev*. 83:731-801.

Gonen, H., D. Shkedy, S. Barnoy, N.S. Kosower, and A. Ciechanover. 1997. On the involvement of calpains in the degradation of the tumor suppressor protein p53. *FEBS Lett*. 406:17-22.

Halestrap, A.P. 2012. The monocarboxylate transporter family--Structure and functional characterization. *IUBMB life*. 64:1-9.

Hay, N., and N. Sonenberg. 2004. Upstream and downstream of mTOR. *Genes & development*. 18:1926-1945.

Ho, W.C., L. Pikor, Y. Gao, B.E. Elliott, and P.A. Greer. 2012. Calpain 2 regulates Akt-FoxO-p27(Kip1) protein signaling pathway in mammary carcinoma. *J Biol Chem*. 287:15458-15465.

Jantsch, J., M. Wiese, J. Schodel, K. Castiglione, J. Glasner, S. Kolbe, D. Mole, U. Schleicher, K.U. Eckardt, M. Hensel, R. Lang, C. Bogdan, M. Schnare, and C. Willam. 2011. Toll-like receptor activation and hypoxia use distinct signaling pathways to stabilize hypoxia-inducible factor 1alpha (HIF1A) and result in differential HIF1A-dependent gene expression. *J Leukoc Biol*. 90:551-562.

Kang, R., H.J. Zeh, M.T. Lotze, and D. Tang. 2011. The Beclin 1 network regulates autophagy and apoptosis. *Cell Death Differ*. 18:571-580.

Karantza-Wadsworth, V., S. Patel, O. Kravchuk, G. Chen, R. Mathew, S. Jin, and E. White. 2007. Autophagy mitigates metabolic stress and genome damage in mammary tumorigenesis. *Genes & development*. 21:1621-1635.

Ko, A., A. Kanehisa, I. Martins, L. Senovilla, C. Chargari, D. Dugue, G. Marino, O. Kepp, M. Michaud, J.L. Perfettini, G. Kroemer, and E. Deutsch. 2014. Autophagy inhibition radiosensitizes in vitro, yet reduces radioresponses in vivo due to deficient immunogenic signalling. *Cell Death Differ*. 21:92-99.

Koukourakis, M.I., A. Giatromanolaki, A.L. Harris, and E. Sivridis. 2006. Comparison of metabolic pathways between cancer cells and stromal cells in colorectal carcinomas: a metabolic survival role for tumor-associated stroma. *Cancer Res*. 66:632-637.

Kubbutat, M.H., and K.H. Vousden. 1997. Proteolytic cleavage of human p53 by calpain: a potential regulator of protein stability. *Mol Cell Biol*. 17:460-468.

Kulkarni, S., K.B. Reddy, F.J. Esteva, H.C. Moore, G.T. Budd, and R.R. Tubbs. 2010. Calpain regulates sensitivity to trastuzumab and survival in HER2-positive breast cancer. *Oncogene*. 29:1339-1350.

Lewis, B.C., J.E. Prescott, S.E. Campbell, H. Shim, R.Z. Orlowski, and C.V. Dang. 2000. Tumor induction by the c-Myc target genes rcl and lactate dehydrogenase A. *Cancer Res*. 60:6178-6183.

Li, B.X., C.Y. Li, R.Q. Peng, X.J. Wu, H.Y. Wang, D.S. Wan, X.F. Zhu, and X.S. Zhang. 2009a. The expression of beclin 1 is associated with favorable prognosis in stage IIIB colon cancers. *Autophagy*. 5:303-306.

Li, C.L., D. Yang, X. Cao, F. Wang, D.Y. Hong, J. Wang, X.C. Shen, and Y. Chen. 2017. Fibronectin induces epithelial-mesenchymal transition in human breast cancer MCF-7 cells via activation of calpain. *Oncol Lett*. 13:3889-3895.

Li, J., N. Hou, A. Faried, S. Tsutsumi, T. Takeuchi, and H. Kuwano. 2009b. Inhibition of autophagy by 3-MA enhances the effect of 5-FU-induced apoptosis in colon cancer cells. *Ann Surg Oncol*. 16:761-771.

Liu, Z., J. Cao, X. Gao, Q. Ma, J. Ren, and Y. Xue. 2011. GPS-CCD: a novel computational program for the prediction of calpain cleavage sites. *PloS one*. 6:e19001.

Ma, Y., L. Galluzzi, L. Zitvogel, and G. Kroemer. 2013. Autophagy and cellular immune responses. *Immunity*. 39:211-227.
Macheda, M.L., S. Rogers, and J.D. Best. 2005. Molecular and cellular regulation of glucose transporter (GLUT) proteins in cancer. *J Cell Physiol*. 202:654-662.
Martinez-Outschoorn, U.E., S. Pavlides, A. Howell, R.G. Pestell, H.B. Tanowitz, F. Sotgia, and M.P. Lisanti. 2011. Stromal-epithelial metabolic coupling in cancer: integrating autophagy and metabolism in the tumor microenvironment. *Int J Biochem Cell Biol*. 43:1045-1051.
Martinez-Outschoorn, U.E., C. Trimmer, Z. Lin, D. Whitaker-Menezes, B. Chiavarina, J. Zhou, C. Wang, S. Pavlides, M.P. Martinez-Cantarin, F. Capozza, A.K. Witkiewicz, N. Flomenberg, A. Howell, R.G. Pestell, J. Caro, M.P. Lisanti, and F. Sotgia. 2010. Autophagy in cancer associated fibroblasts promotes tumor cell survival: Role of hypoxia, HIF1 induction and NFkappaB activation in the tumor stromal microenvironment. *Cell Cycle*. 9:3515-3533.
Mazure, N.M., and J. Pouyssegur. 2009. Atypical BH3-domains of BNIP3 and BNIP3L lead to autophagy in hypoxia. *Autophagy*. 5:868-869.
Mendler, A.N., B. Hu, P.U. Prinz, M. Kreutz, E. Gottfried, and E. Noessner. 2012. Tumor lactic acidosis suppresses CTL function by inhibition of p38 and JNK/c-Jun activation. *Int J Cancer*. 131:633-640.
Miranda-Goncalves, V., M. Honavar, C. Pinheiro, O. Martinho, M.M. Pires, C. Pinheiro, M. Cordeiro, G. Bebiano, P. Costa, I. Palmeirim, R.M. Reis, and F. Baltazar. 2013. Monocarboxylate transporters (MCTs) in gliomas: expression and exploitation as therapeutic targets. *Neuro Oncol*. 15:172-188.
Nazio, F., F. Strappazzon, M. Antonioli, P. Bielli, V. Cianfanelli, M. Bordi, C. Gretzmeier, J. Dengjel, M. Piacentini, G.M. Fimia, and F. Cecconi. 2013. mTOR inhibits autophagy by controlling ULK1 ubiquitylation, self-association and function through AMBRA1 and TRAF6. *Nat Cell Biol*. 15:406-416.
Ono, Y., T.C. Saido, and H. Sorimachi. 2016. Calpain research for drug discovery: challenges and potential. *Nature reviews. Drug discovery*. 15:854-876.
Pianetti, S., M. Arsura, R. Romieu-Mourez, R.J. Coffey, and G.E. Sonenshein. 2001. Her-2/neu overexpression induces NF-kappaB via a PI3-kinase/Akt pathway involving calpain-mediated degradation of IkappaB-alpha that can be inhibited by the tumor suppressor PTEN. *Oncogene*. 20:1287-1299.
Pinheiro, C., A. Longatto-Filho, J. Azevedo-Silva, M. Casal, F.C. Schmitt, and F. Baltazar. 2012. Role of monocarboxylate transporters in human cancers: state of the art. *J Bioenerg Biomembr*. 44:127-139.
Pinheiro, C., A. Longatto-Filho, L. Ferreira, S.M. Pereira, D. Etlinger, M.A. Moreira, L.F. Jube, G.S. Queiroz, F. Schmitt, and F. Baltazar. 2008. Increasing expression of monocarboxylate transporters 1 and 4 along progression to invasive cervical carcinoma. *Int J Gynecol Pathol*. 27:568-574.
Qadir, M.A., B. Kwok, W.H. Dragowska, K.H. To, D. Le, M.B. Bally, and S.M. Gorski. 2008. Macroautophagy inhibition sensitizes tamoxifen-resistant breast cancer cells and enhances mitochondrial depolarization. *Breast Cancer Res Treat*. 112:389-403.
Rastogi, S., S. Banerjee, S. Chellappan, and G.R. Simon. 2007. Glut-1 antibodies induce growth arrest and apoptosis in human cancer cell lines. *Cancer Lett*. 257:244-251.
Robey, I.F., A.D. Lien, S.J. Welsh, B.K. Baggett, and R.J. Gillies. 2005. Hypoxia-inducible factor-1alpha and the glycolytic phenotype in tumors. *Neoplasia*. 7:324-330.
Russell, R.C., Y. Tian, H. Yuan, H.W. Park, Y.Y. Chang, J. Kim, H. Kim, T.P. Neufeld, A. Dillin, and K.L. Guan. 2013. ULK1 induces autophagy by phosphorylating Beclin-1 and activating VPS34 lipid kinase. *Nat Cell Biol*. 15:741-750.
Scaltriti, M., F. Rojo, A. Ocana, J. Anido, M. Guzman, J. Cortes, S. Di Cosimo, X. Matias-Guiu, S. Ramon y Cajal, J. Arribas, and J. Baselga. 2007. Expression of p95HER2, a truncated form of the

HER2 receptor, and response to anti-HER2 therapies in breast cancer. *J Natl Cancer Inst*. 99:628-638.
Schwartzenberg-Bar-Yoseph, F., M. Armoni, and E. Karnieli. 2004. The tumor suppressor p53 down-regulates glucose transporters GLUT1 and GLUT4 gene expression. *Cancer Res*. 64:2627-2633.
Selvakumaran, M., R.K. Amaravadi, I.A. Vasilevskaya, and P.J. O'Dwyer. 2013. Autophagy inhibition sensitizes colon cancer cells to antiangiogenic and cytotoxic therapy. *Clin Cancer Res*. 19:2995-3007.
Shim, H., C. Dolde, B.C. Lewis, C.S. Wu, G. Dang, R.A. Jungmann, R. Dalla-Favera, and C.V. Dang. 1997. c-Myc transactivation of LDH-A: implications for tumor metabolism and growth. *Proc Natl Acad Sci U S A*. 94:6658-6663.
Shumway, S.D., M. Maki, and S. Miyamoto. 1999. The PEST domain of IkappaBalpha is necessary and sufficient for in vitro degradation by mu-calpain. *J Biol Chem*. 274:30874-30881.
Singer, K., M. Kastenberger, E. Gottfried, C.G. Hammerschmied, M. Buttner, M. Aigner, B. Seliger, B. Walter, H. Schlosser, A. Hartmann, R. Andreesen, A. Mackensen, and M. Kreutz. 2011. Warburg phenotype in renal cell carcinoma: high expression of glucose-transporter 1 (GLUT-1) correlates with low CD8(+) T-cell infiltration in the tumor. *Int J Cancer*. 128:2085-2095.
Sinha, S., and B. Levine. 2008. The autophagy effector Beclin 1: a novel BH3-only protein. *Oncogene*. 27 Suppl 1:S137-148.
Sonveaux, P., T. Copetti, C.J. De Saedeleer, F. Vegran, J. Verrax, K.M. Kennedy, E.J. Moon, S. Dhup, P. Danhier, F. Frerart, B. Gallez, A. Ribeiro, C. Michiels, M.W. Dewhirst, and O. Feron. 2012. Targeting the lactate transporter MCT1 in endothelial cells inhibits lactate-induced HIF-1 activation and tumor angiogenesis. *PLoS One*. 7:e33418.
Sonveaux, P., F. Vegran, T. Schroeder, M.C. Wergin, J. Verrax, Z.N. Rabbani, C.J. De Saedeleer, K.M. Kennedy, C. Diepart, B.F. Jordan, M.J. Kelley, B. Gallez, M.L. Wahl, O. Feron, and M.W. Dewhirst. 2008. Targeting lactate-fueled respiration selectively kills hypoxic tumor cells in mice. *J Clin Invest*. 118:3930-3942.
Storr, S.J., K.W. Lee, C.M. Woolston, S. Safuan, A.R. Green, R.D. Macmillan, A. Benhasouna, T. Parr, I.O. Ellis, and S.G. Martin. 2012a. Calpain system protein expression in basal-like and triple-negative invasive breast cancer. *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO*. 23:2289-2296.
Storr, S.J., X. Pu, J. Davis, D. Lobo, A.M. Reece-Smith, S.L. Parsons, S. Madhusudan, and S.G. Martin. 2013. Expression of the calpain system is associated with poor clinical outcome in gastro-oesophageal adenocarcinomas. *J Gastroenterol*.
Storr, S.J., S. Safuan, C.M. Woolston, T. Abdel-Fatah, S. Deen, S.Y. Chan, and S.G. Martin. 2012b. Calpain-2 expression is associated with response to platinum based chemotherapy, progression-free and overall survival in ovarian cancer. *Journal of cellular and molecular medicine*. 16:2422-2428.
Szablewski, L. 2013. Expression of glucose transporters in cancers. *Biochim Biophys Acta*. 1835:164-169.
Tan, Y., N. Dourdin, C. Wu, T. De Veyra, J.S. Elce, and P.A. Greer. 2006a. Conditional disruption of ubiquitous calpains in the mouse. *Genesis*. 44:297-303.
Tan, Y., C. Wu, T. De Veyra, and P.A. Greer. 2006b. Ubiquitous calpains promote both apoptosis and survival signals in response to different cell death stimuli. *J Biol Chem*. 281:17689-17698.
Taraboletti, G., S. D'Ascenzo, I. Giusti, D. Marchetti, P. Borsotti, D. Millimaggi, R. Giavazzi, A. Pavan, and V. Dolo. 2006. Bioavailability of VEGF in tumor-shed vesicles depends on vesicle burst induced by acidic pH. *Neoplasia*. 8:96-103.
Trabold, O., S. Wagner, C. Wicke, H. Scheuenstuhl, M.Z. Hussain, N. Rosen, A. Seremetiev, H.D. Becker, and T.K. Hunt. 2003. Lactate and oxygen constitute a fundamental regulatory mechanism in wound healing. *Wound Repair Regen*. 11:504-509.

Ullah, M.S., A.J. Davies, and A.P. Halestrap. 2006. The plasma membrane lactate transporter MCT4, but not MCT1, is up-regulated by hypoxia through a HIF-1alpha-dependent mechanism. *J Biol Chem*. 281:9030-9037.

Vander Heiden, M.G., L.C. Cantley, and C.B. Thompson. 2009. Understanding the Warburg effect: the metabolic requirements of cell proliferation. *Science*. 324:1029-1033.

Vazquez-Martin, A., C. Oliveras-Ferraros, and J.A. Menendez. 2009. Autophagy facilitates the development of breast cancer resistance to the anti-HER2 monoclonal antibody trastuzumab. *PLoS One*. 4:e6251.

Vegran, F., R. Boidot, C. Michiels, P. Sonveaux, and O. Feron. 2011. Lactate influx through the endothelial cell monocarboxylate transporter MCT1 supports an NF-kappaB/IL-8 pathway that drives tumor angiogenesis. *Cancer Res*. 71:2550-2560.

Wang, R.C., Y. Wei, Z. An, Z. Zou, G. Xiao, G. Bhagat, M. White, J. Reichelt, and B. Levine. 2012. Akt-mediated regulation of autophagy and tumorigenesis through Beclin 1 phosphorylation. *Science*. 338:956-959.

Warburg, O. 1956a. On respiratory impairment in cancer cells. *Science*. 124:269-270.

Warburg, O. 1956b. On the origin of cancer cells. *Science*. 123:309-314.

Wood, D.E., A. Thomas, L.A. Devi, Y. Berman, R.C. Beavis, J.C. Reed, and E.W. Newcomb. 1998. Bax cleavage is mediated by calpain during drug-induced apoptosis. *Oncogene*. 17:1069-1078.

Yoo, B.H., X. Wu, Y. Li, M. Haniff, T. Sasazuki, S. Shirasawa, E.L. Eskelinen, and K.V. Rosen. 2010. Oncogenic ras-induced down-regulation of autophagy mediator Beclin-1 is required for malignant transformation of intestinal epithelial cells. *J Biol Chem*. 285:5438-5449.

Younes, M., L.V. Lechago, J.R. Somoano, M. Mosharaf, and J. Lechago. 1996. Wide expression of the human erythrocyte glucose transporter Glut1 in human cancers. *Cancer Res*. 56:1164-1167.

Yousefi, S., R. Perozzo, I. Schmid, A. Ziemiecki, T. Schaffner, L. Scapozza, T. Brunner, and H.U. Simon. 2006. Calpain-mediated cleavage of Atg5 switches autophagy to apoptosis. *Nat Cell Biol*. 8:1124-1132.

Yuan, J., L. Narayanan, S. Rockwell, and P.M. Glazer. 2000. Diminished DNA repair and elevated mutagenesis in mammalian cells exposed to hypoxia and low pH. *Cancer Res*. 60:4372-4376.

Yue, Z., S. Jin, C. Yang, A.J. Levine, and N. Heintz. 2003. Beclin 1, an autophagy gene essential for early embryonic development, is a haploinsufficient tumor suppressor. *Proc Natl Acad Sci U S A*. 100:15077-15082.

Zhang, H., M. Bosch-Marce, L.A. Shimoda, Y.S. Tan, J.H. Baek, J.B. Wesley, F.J. Gonzalez, and G.L. Semenza. 2008. Mitochondrial autophagy is an HIF-1-dependent adaptive metabolic response to hypoxia. *J Biol Chem*. 283:10892-10903.

Zheng, X., A.X. Zhou, P. Rouhi, H. Uramoto, J. Boren, Y. Cao, T. Pereira, L.M. Akyurek, and L. Poellinger. 2014. Hypoxia-induced and calpain-dependent cleavage of filamin A regulates the hypoxic response. *Proc Natl Acad Sci U S A*. 111:2560-2565.

Zhu, X., J.S. Messer, Y. Wang, F. Lin, C.M. Cham, J. Chang, T.R. Billiar, M.T. Lotze, D.L. Boone, and E.B. Chang. 2015. Cytosolic HMGB1 controls the cellular autophagy/apoptosis checkpoint during inflammation. *The Journal of clinical investigation*. 125:1098-1110.