2019

Cyclophilin A as a molecular switch regulating prolactin receptor mediated signaling, mammary tumorigenesis and metastasis

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

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July 2019

Acknowledgements

I would first like to express my sincere gratitude to my thesis advisor, Dr. Charles Clevenger for the continuous support of my PhD study and related research, for his patience, motivation and immense knowledge. His passion for research is admirable and inspiring. In his lab, I have developed the independence and determination necessary to become an independent translational scientist. I feel confident and ready to pursue a career in translational/clinical cancer research. He created an amiable and compelling lab/work environment that made working in the lab both enjoyable and intellectually stimulating. His guidance helped me in all the time of research and writing of this thesis.

Besides my advisor, I would like to thank the rest of my thesis committee; Dr. Grant, Dr. Grossman, Dr. Gewirtz and Dr. Radhakrishnan, for their insightful comments and encouragement, but also for the hard questions which incented me to widen my research from various perspectives. I would like to thank the VCU CCTR program, particularly Dr. Joyce Lloyd and Dr. Devanand Sarkar. They have been extremely supportive and helpful throughout my graduate career at VCU. Having the support and resources offered by the CCTR was a significant aspect for my graduate career at VCU, and I am very grateful for the solid training I have received through the CAMM program. I would like to thank my fellow lab-mates for many stimulating discussions/ conversations and help over the last five years. The department of pathology and staffs deserve special mention for their research or administrative support. I thank my friends for their support, encouragements and company during my tenure at VCU.

Finally, I must express my profound gratitude to my parents and my brother for providing me with unfailing support and continuous encouragement throughout my years

of study and through the process of researching and writing this thesis. It was their love that raised me up again when I got weary. I greatly appreciate their continued love and support. Above all, I would like to thank my amazing wife for providing me with unconditional love, trust, timely encouragement, endless patience and keeping me sane over the past few months. Without her unconditional love, help and support, I would not be able to persist through this journey. I owe her everything. I would also like to extend my gratitude to my parents-in-law, brothers/sisters-in-law, uncle/aunt and cousins for believing in me, providing emotional support and heart-warming kindness. I thank my family and friends for everything and dedicate this work to them.

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List of Abbreviations

μg - microgram

μl - microliter

μm - micrometer

A - adenine

ANOVA - analysis of variance

ATCC - American Type Culture Collection

AQP3 - aquaporin 3

BSA - bovine serum albumin

°C - degrees Celsius

CCD - coiled-coil domain

CERM - conditional ERα in mammary epithelium

C-terminal - carboxyl-terminal

CCND1 - cyclin D1

cDNA - complementary DNA

CISH - cytokine inducible SH2 containing protein

CFP - Cyan fluorescent protein

CLD - cyclophilin-like domain

cm - centimeter

CsA - cyclosporine A

Ct - cycle threshold value

CTTN - cortactin

Cont - control

CypA - cyclophilin A

CypNK - cyclophilin natural killer

DBD - DNA binding domain

DMEM - Dulbecco’s modified eagle medium

DMSO - dimethyl sulfoxide

DNA - deoxyribonucleic acid

DNase - deoxyribonuclease

DUSP6 - dual-specificity phosphatase 6

EC - endothelial cells

ECD - extracellular domain

EDTA - ethylenediaminetetraacetic acid

EGFR - epidermal growth factor receptor

ER - estrogen receptor

EGR3 - early growth response 3

ERBB - erythroblastic leukemia viral oncogene homolog

ERK - extracellular signal-regulated kinase

FBS - fetal bovine serum

FFPE - formalin-fixed paraffin embedded

FLIM - Fluorescent Lifetime Imaging

FRET - Fluorescence Resonance Energy Transfer

FERM domain - protein four-point-one, ezrin, radixin, moesin

FDR - false discovery rate

G - guanine

GAPDH - glyceraldehyde 3-phosphate dehydrogenase

GBP1 - guanylate binding protein 1

GEO - gene enrichment ontology

GFP - green fluorescent protein

GHR - growth hormone receptor

GSKβ - Glycogen synthase kinase 3β

H - histone

HER2 - human epidermal growth factor receptor 2

hPRLr - human prolactin receptor

HRP - horseradish peroxidase

ICD - intracellular domain

IFN - interferon

IgG - immunoglobulin G

IL8 - Interleukin 8

IP - immunoprecipitation

JAK2 - janus kinase 2

JH - JAK homology

K - lysine

KLF4 - krupple-like factor 4

kb - kilobase

kDA - kilodalton

LD - linker domain

LPRK2 - leucine-rich repeat kinase 2

M - molar

MAPK - mitogen-activated protein kinase

MECs - mammary epithelial cells

mg - milligram

ml - milliliter

mM - millimolar

MMTV - mouse mammary tumor virus

mRNA - messenger RNA

N-terminal - amino-terminal

NBF - neutral buffered formalin

NFAT - nuclear factor of activated T cells

NF1B - nuclear factor 1/B

ng - nanogram

nm - nanometer

nM - nanomolar

NIM811-N-methyl-4-isoleucine cyclosporin

p - phosphorylation

P - proline

p53 - tumor protein 53

PBS - phosphate buffered saline

PCR - polymerase chain reaction

PDGF - platelet derived growth factor

PDX - patient-derived xenograft

PL - placental lactogen

PPI - Peptidyl prolyl isomerase

PR - progesterone receptor

PRL - prolactin

PRLr - prolactin receptor

p-Tail - Phosphotyrosyl Tail

PyMT - polyomavirus middle T-antigen

qRT-PCR - quantitative reverse transcription polymerase chain reaction

RIPA - radioimmunoprecipitation assay

RNA - ribonucleic acid

RNase - ribonuclease

S - serine

SEM - standard error of the mean

seq - sequencing

SFKs - src family of non-receptor tyrosine kinases

SFM - serum free media

SH2 - SRC homology 2

shRNA - short-hairpin RNA

siRNA - small interfering RNA

SOCS - suppressor of cytokine signaling

SRC - sarcoma proto-oncogene tyrosine kinase

SRF - serum response factor

STAT - signal transducer and activator of transcription 5

T - thymine

TAD - transactivation domain

TBS - tris-buffered saline

TBS-T - TBS containing 0.1% (v/v) Tween 20

TE - tris-EDTA

TNBC - triple negative breast cancer

Tris - tris(hydroxymethyl)aminomethane

V - volt

v/v - volume/volume percent

VAV - Vav guanine nucleotide exchange factor

VSMC - vascular smooth muscle cells

WAP - whey acidic protein

WT - wild-type

Y - tyrosine

YFP - yellow fluorescent protein

Abstract

CYCLOPHILIN A AS A MOLECULAR SWITCH REGULATING PROLACTIN RECEPTOR MEDIATED SIGNALING, MAMMARY TUMORIGENESIS AND METASTASIS

By Shawn Hakim, PhD

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2019.

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Current evidence supports a role for prolactin (PRL) and its cognate receptor (PRLr) in the development and progression of human breast cancer. Prolactin activates its receptor and induces rapid activation of proximal janus kinase 2 (Jak2). Jak2 associates with PRLr Box 1 motif via its FERM domain, phosphorylates the c-terminus of the PRLr, which leads to Stat5 recruitment and activation. Cyclophilin A (CypA) is a member of the immunophilin family of peptidyl-prolyl isomerases (PPIs), which is constitutively bound to the PRLr that catalyzes the cis-trans interconversion of proline imide bonds of peptides. The PPI activity of CypA is inhibited by the immunosuppressive drug cyclosporine A (CsA), and in turn the CypA-CsA complex inhibits calcineurin-mediated NFAT activation. Our lab has investigated CypA regulation of Jak2 and found a potential role for CypA**,** which binds to the PRLr at residue 334. Treatment with CsA inhibited CypA binding to the PRLr and blocked PRLr-driven activation of Jak2/Stat5. A recent study utilizing Fluorescence Resonance Energy Transfer (FRET) with transfectants expressing cyan- and yellow-fluorescent protein (CFP and YFP)-tagged forms of the growth hormone receptor (GHR,

an analog of the PRLr) showed that GHR activation induced a rotational movement in the C-terminus of the GHR, resulting in a loss of baseline FRET signal. Given this, we hypothesized that CypA isomerase activity on proline residues within the Box 1 motif of PRLr may facilitate conformational change in the intracellular domain of the PRLr. Like the GHR, PRL stimulation of transfectants expressing CFP-, YFP-tagged PRLr constructs results in a loss of FRET efficiency upon PRL stimulation. In contrast, treatment with NIM811 (a non-immunosuppressive form of CsA) or siRNA knockdown of CypA results in a return of FRET signal in response to PRL. These studies reveal that ligand stimulation of the PRLr results in a conformational change as measured by FRET signal to the receptor that is reversed by CypA inhibition or knockdown**,** implicating CypA as the mediator of this conformational change and ligand-induced signaling. To further assess the consequences of CypA inhibition on the PRLr/Jak2 mediated signaling/functions, analyses of phospho-tyrosine residues that are believed to be important for interactions/signaling were investigated in breast cancer cells. It was found that NIM811 inhibited prolactin-stimulated phosphorylation of Stat5-Y694, Jak2-Y1007/1008, PRLr-Y381/-Y587, and Src-Y416 in an ER/PR$^+$ T47D cell line in a time dependent manner. Like the drug inhibition, RNAi mediated knockdown of CypA also down-regulated PRLr/Jak2-associated phosphorylation following PRL stimulation. A microarray analysis of global gene expression in ER/PR$^+$ breast cancer cells revealed that NIM811 inhibited approximately 66% of the top 50 PRL induced genes. NIM811 inhibited ER+, ER-, and HER2+ breast cancer cell proliferation, survival, migration and anchorage-independent growth. Subsequent pre-clinical testing of NIM811 in relevant mouse mammary cancer models has found that NIM811 treatment of a TNBC (MDA231) xenograft inhibited

primary tumor growth and induced central tumor necrosis. Furthermore, loss of CypA (by constitutive genetic deletion) in the MMTV-PyMT mouse model demonstrated inhibition of tumorigenesis with significant reduction in lung and lymph node metastases. Overall, these results indicate that CypA modulates conformational change of the intracellular domain of the PRLr through its PPI activity, and alters PRLr/Jak2 complex signaling/functions in breast cancers and mammary epithelium, identifying this isomerase as a novel target for therapeutic intervention as a chemo-preventive and as an inhibitor of metastasis.

Chapter 1: Introduction

1.1 Breast and Mammary Cancer

1.1.1 Breast Cancer

In 2019, approximately 268,600 women are expected to be diagnosed with invasive breast cancer, and nearly 41,760 will die from the disease in the United States (American Cancer Society, 2019). Breast cancer is the second leading cause of cancer-related death and is the most common occurring cancer in women (American Cancer Society, 2019). One in eight women is affected by breast cancer in their lifetime (DeSantis, Fedewa et al. 2016). Breast cancer etiology is yet to be fully defined, but genetic, lifestyle and environmental factors are often associated with this multifactorial disease (Dignam and Mamounas 2004). It is a complex disease that exhibits a large degree of inter- and intra- tumoral heterogeneity (Perou, Sorlie et al. 2000). Breast cancer is mainly classified based on hormone receptors including estrogen (ER^+), progesterone (PR^+), and human epidermal growth factor receptor 2 (HER2) status. The five major molecular breast cancer subtypes consist of i) Luminal A, representing ER/PR^+ of low grade and low Ki-67 index (a proliferative marker); ii) Luminal B, ER/PR^+ of higher grade and proliferative index; iii) $HER2^+$ with or without ER; and iv) the “basal-like” or triple negative that do not express any of the three receptors (Perou, Sorlie et al. 2000, Brisken, Hess et al. 2015). In addition to these subtypes, the combined genomic/transcriptomic analyses of breast cancer have assisted in the identification of ten distinct breast cancer subtypes based on integrated clusters (Curtis, Shah et al. 2012). The hormone receptor positive cancers offer therapeutic targets for decreasing tumor growth by inhibiting signaling and malignant properties, however, treatment results in differential responses for patients due to inherent heterogeneity of the disease (Leong and Zhuang 2011).

Additional treatment options for breast cancer include radiation, chemotherapy and immunotherapy. A combination of two or more drugs is usually used to effectively treat breast cancer. However, for many patients even after greatly reducing the tumor, these regimens fail due to resistance/relapse and metastasis. A tailored or targeted approach to match precise subtypes is necessary to elicit the best responses in patients when administering treatment modalities (Kalluri and Weinberg 2009, Weis and Cheresh 2011).

1.1.2 Breast cancer progression and metastasis

Metastasis is responsible for around 90% of cancer related deaths (Spano, Heck et al. 2012). It is estimated that more than 25% of breast cancer patients will ultimately develop deadly metastasis, occurring even decades after time of diagnosis or primary tumor removal. The triple negative subtype of breast cancer is associated with a significantly high risk of progression and dissemination (Lorusso and Ruegg 2012). The prognosis for patients with metastatic disease is not favorable, only 25% of the patients survive an average of 5 years (Rabbani and Mazar 2007). Current therapies against metastatic breast cancer are still not effective, hence it is essential to develop targeted therapy against the metastatic cascade and understand mechanisms that drive the disease progression (Ding, Ellis et al. 2010).

The process of metastasis was originally explained by Stephen Paget's hypothesis of "seed and soil" in 1889 that organ-specific metastasis is due to the dependence of the seed (the cancer cell) on the soil (the secondary organ) (Paget 1989). It has been established that the formation of metastasis is a multi-step and multifunctional biological event that results from a sequential molecular cascade through which cancer cells spread from the primary tumors to distant sites, where they can proliferate and create secondary

neoplastic foci (Steeg 2006). During progression, a minor cell subpopulation acquires metastasis-driving mutations and capacities to effectively colonize distant organs when disseminated from primary tumors (Chaffer and Weinberg 2011). Next, tumor cells and stomal cells stimulate angiogenesis by production of soluble inflammatory and pro-angiogenic cytokines, which bind to endothelial cells. As a result, integrins and proteases are secreted, and the endothelial cells degrade the surrounding stroma with the aid of proteases (Avraamides, Garmy-Susini et al. 2008). Integrin interactions with stromal components are responsible for cancer cell migration through the area of degraded stroma (Avraamides, Garmy-Susini et al. 2008). In most cases, cells intravasating the primary tumor undergo epithelial to mesenchymal transition (EMT), mediated by molecules including TGFβ, MAP kinases, transcription regulators Twist and snail (SNA, SNAI1), Wnt, Notch and Hedgehog (Tse and Kalluri 2007). Ultimately, tumor cells alter inflammatory response, suppress the immune response, favor angiogenesis and modulate growth, survival and the release of motility factors (Hanahan and Weinberg 2011). Several genes and molecules mediating metastatic colonization of breast cancer to bone, lymph nodes, lung and brain have been identified, therefore, understanding the underlying mechanisms and regulation of these genes can generate novel targeted therapies against breast cancer metastasis.

1.1.3 Cytokines and hormone receptors in breast cancer

Cytokines employ both pro- and anti- cancer effects which determine the fate of a tumor (Borsig, Wolf et al. 2014). Tumor secreted cytokines assist in the recruitment of other immunosuppressive stromal cells for promoting metastasis (Hanahan and Coussens 2012). The Jak-Stat signaling pathway is crucial for exerting the effects of over fifty

cytokines, growth factors and hormones in mammals (Stark and Darnell 2012). Transcription factor Stat3 is important for cell proliferation, survival and maturation driven by IL-6 (Jones and Jenkins 2018). The interferon family of cytokines plays critical roles in innate immune response against infectious pathogens as well as neoplastic cells (Kalvakolanu 2017). Cytokine, hormone as well as their cognate receptors are important for breast cancer development and progression.

Breast cancer is a heterogeneous disease and hormone receptor status can differentiate between distinct subgroups, $Her2^{+/-}$, $ER^{+}PR^{+}$, $ER^{+}PR^{-}$, $ER^{-}PR^{+}$, and $ER^{-}PR^{-}$. A study of 155,175 women of at least 30 years old found that 63.5% were $ER^{+}PR^{+}$, 12.8% were $ER^{+}PR^{-}$, 3.1% were $ER^{-}PR^{+}$ and 20.6% were $ER^{-}PR^{-}$ (Dunnwald, Rossing et al. 2007). The discovery of estrogen and progesterone in breast tissue in the 1960s, provided the evidence for induction of a classical ligand-receptor pathway in breast cancer development (Jensen, Desombre et al. 1967). Certain hormones like prolactin (PRL) may regulate or increase progesterone receptor (PR) and estrogen receptor α (ERα) levels as well as estrogen (E2) sensitivity in breast cancer cells (Shafie and Brooks 1977, Gutzman, Miller et al. 2004). Such as, there is a 25% increase in ERα and 3-fold increase in PR in response to prolactin in murine mammary epithelial cells (Edery, Imagawa et al. 1985). However, endogenously produced prolactin is able to enhance ERα transcript levels, but not affect PR levels (Gutzman, Miller et al. 2004).

1.1.4 Mammary cancer and mouse models

Investigations of mouse knockout and transgenic models have been critical in determining the importance of hormone/receptor such as PRL/PRLr (prolactin receptor) or isomerase like Cyclophilin A (CypA) in regulating mammary cancer morphogenesis and metastasis.

Overexpression of PRL in the mammary epithelium results in the spontaneous development of $ER^{-/+}$ mammary tumors in transgenic mice, mimicking human breast cancer (Rose-Hellekant, Arendt et al. 2003). To investigate the action of PRL in pathogenesis, a transgenic mouse model called NRL-PRL was developed that mimics the high mammary PRL synthesis found in women (Arendt, Rugowski et al. 2011). In this model, expression of rat PRL transgene in mammary epithelia is driven by the PRL- and estrogen-insensitive NRL promoter, exposing the gland to locally elevated PRL (Rose-Hellekant, Arendt et al. 2003, Rose-Hellekant, Schroeder et al. 2007). Nonparous NRL-PRL mice develop mammary tumors that reveal many signatures of breast cancer including hyperplasias and intraepithelial neoplasias (ductal carcinoma in situ in women), and ultimately $ER\alpha^{+/-}$ carcinomas. These tumors found to be locally aggressive and often metastasize to the lymph nodes and lungs (Arendt, Rugowski et al. 2011).

ErbB2 is amplified in approximately 30% of all breast cancer (Slamon, Godolphin et al. 1989). A few rat erbB2 transgenic mouse models have been established including FVB-neuN mice carrying wild-type rat neu driven by the mouse mammary tumor virus (MMTV) promoter. Overexpression of ErbB2 in the mammary gland leads to mammary tumor growth around 40 week of age (Guy, Cardiff et al. 1992). BALB NeuT mice with activated rat neu oncogene develop spontaneous mammary tumors at around 20 weeks (Yamamoto, Ikawa et al. 1986). Several loss-of-function mouse models demonstrate delayed or disrupted tumorigenesis when crossed in the line expressing a transgenic mammary oncogene including Her2/ErbB2/neu. For example, cyclophilin A (CypA) (isomerase that regulates cytokine receptor signaling and metastasis) knockout ($CypA^{-/-}$) mice were crossed into MMTV-driven rat ErbB2 receptor containing mice (Muller, Sinn et

al. 1988) to assess its role in ErbB2 tumorigenesis. ErbB2 x $CypA^{-/-}$ mice demonstrate significantly delayed tumor latency/survival and reduced tumor multiplicity in comparison with the ErbB2 x $CypA^{+/+}$ cohort (Volker, Hedrick et al. 2018). Moreover, PRL deficient mice exhibit slower tumor growth/development induced by the polyoma middle T-antigen than PRL-expressing mice (Vomachka, Pratt et al. 2000). PyMT mammary gland-specific expression under the control of the MMTV promoter/enhance in transgenic mice (MMTV-PyMT) leads to extensive transformation of mammary epithelium. Specific expression of PyMT results in development of multifocal mammary adenocarcinomas and metastatic lesions in the lungs and lymph nodes (Guy, Cardiff et al. 1992). Mammary tumor formation and progression in these mice mimics distinct stages of human breast cancer such as hyperplasia, adenoma/mammary neoplasia, early and late carcinoma (Lin, Jones et al. 2003). Like human breast cancer, these mice demonstrate a gradual loss of steroid hormone receptors (estrogen and progesterone) and β-integrin, which relates to overexpression of ErbB2 and cyclin D1 in late-stage metastatic cancers (Brison 1993, Lapidus, Nass et al. 1998). The key features of the MMTV-PyMT mouse model of breast cancer are short latency, high penetrance, and high frequency of lung and lymph node metastasis with reproducible kinetics of progression regardless of pregnancy (Lin, Jones et al. 2003).

1.2 Prolactin, Prolactin Receptor Structure/Functions and Signaling

1.2.1 Prolactin

Prolactin (PRL) is a 23 kDA α-helix bundle polypeptide hormone first identified as a neuroendocrine hormone produced in lactotrophic cells of the anterior pituitary gland of vertebrates. PRL is also produced in other extra-pituitary sites, including the breast, T-

lymphocytes and the decidual cells of the endometrium (Clevenger, Chang et al. 1995, Harris, Stanford et al. 2004). Prolactin was discovered and purified from the anterior pituitary in 1933 and later named “pro-lactin” for its positive role in lactogenesis (Riddle, Bates et al. 1933, Hennighausen, Robinson et al. 1997). PRL has been implicated in many physiological and biological functions such as growth, immune regulation, metabolism, terminal differentiation of mammary gland, milk production, and reproduction (Ormandy, Binart et al. 1997, Freeman, Kanyicska et al. 2000). PRL ameliorates glucose homeostasis by elevating β-cell mass during pregnancy, where pituitary gland adenoma results in hyperprolactinemia that exacerbates insulin resistance (Sorenson and Brelje 1997, Park, Kim et al. 2011). PRL plays a critical role in human immune responses (Chavez-Rueda, Hernandez et al. 2005), where its effects on immunological systems may rely on concentration, ensuing in inhibition at high levels and immune-stimulation at modest levels (Zoli, Lizzio et al. 2002). PRL is part of the human lactogen family proteins including human growth hormone (GH) and human placental lactogen (PL) that share similar structure. Both GH and PL can bind to the prolactin receptor (PRLr), whereas PRL can only bind to the PRLr (Kelly, Djiane et al. 1991). Local synthesis of PRL has been found in both normal and malignant breast epithelium (Ginsburg and Vonderhaar 1995). It has been demonstrated that either estrogen or dopamine does not influence PRL transcription in extra-pituitary sites, but it is mediated by a different promoter (Gellersen, Kempf et al. 1994). PRL can regulate pro-proliferative and pro-differentiation functions through both endocrine and autocrine/paracrine mechanisms (Clevenger and Plank 1997, Ormandy, Binart et al. 1997). PRL targets and controls transcription of CCND1 (Cyclin D1) and increases its levels 2-fold in a cell culture model (Brockman and

Schuler 2005). Moreover, PRL also targets pro-proliferative CISH (Cytokine-Inducible SH2 Protein), which functions as a switch from differentiation to proliferation in epithelial cells (Raccurt, Tam et al. 2003). In contrast, PRL plays a pro-differentiation role by transcriptional activation of genes involved in milk production including whey acidic protein (WAP), β-casein and β-lactoglobulin (Andres, Schonenberger et al. 1987, Pittius, Sankaran et al. 1988).

1.2.2 Structure and functions of PRLr

The PRLr is a member of the cytokine class I receptor superfamily of proteins (Bazan 1990). The cytokine superfamily is composed of receptors such as interleukins, erythropoietin, thrombopoietin, granulocyte-colony stimulating factor and leptin (Bazan 1990). The first member of the hPRLr-family identified was the long PRLr isoform with a mass of 90 kDA, which is a Type 1 transmembrane receptor (Boutin, Edery et al. 1989, Kelly, Boutin et al. 1989). Subsequently, six different isoforms were identified, of which five of the isoforms are splice variants, with the shortest form being a product of proteolytic cleavage (Clevenger, Chang et al. 1995) (Table 1). Each splice variant has discrete signaling capabilities since each possesses different binding affinity for the ligand (PRL) and length of the cytoplasmic tail (Clevenger, Chang et al. 1995, Reynolds, Montone et al. 1997). The long form of the PRLr with the highest affinity for PRL and longest cytoplasmic tail is capable of propagating complete intracellular signaling.

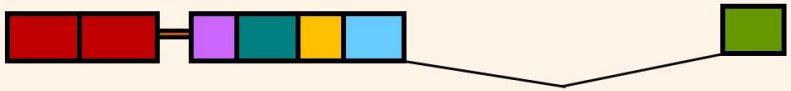
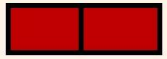
Isoform	Structures	Function	References
Long		Full length and full activation of downstream signaling	(Boutin, 1989; Kelly *et al.*, 1989)
Intermediate		Alternative splice variant and has reduced kinase activation	(Kline and Clevenger, 1999)
△S1		Reduced ligand binding and may only function under high PRL concentrations	(Kline *et al.*, 2002)
PRLRBP		Generated by cleavage event and antagonizes PRL action	(Kline and Clevenger, 2001)
TM-ICD		Generated by cleavage event and enhances signaling by unknown mechanisms	(Gadd and Clevenger, 2006)
S1a		Alternative splice variant and a dominant negative regulator of medium efficiency	(Hu *et al.*, 2001)
S1b		Alternative splice variant and a dominant negative regulator of high efficiency	(Hu *et al.*, 2001)

Table 1. Seven known human PRLr isoforms. Schematics showing extracellular domain (ECD) (red boxes) followed by transmembrane domain (red dash), which connects the intracellular domain (ICD) to ECD of PRLr isoform(s). The ICD composed of Box1 (purple), V-Box (teal), Box 2 (yellow) and X-Box (blue) (left to right). The green box after the X-Box represents the ICD tail consisting of tyrosine domains that are important for signal transduction. The line connecting regions within the ICD of intermediate, S1a and S1b depicts the truncated region.

The hPRLr consists of three major domains including the extracellular domain (ECD), the transmembrane domain and the intracellular domain (ICD) (Bazan 1990) (Fig. 1.1). The ECD has two Type-III fibronectin-like domains named S1 and S2 that are connected to the intracellular domain by a single transmembrane domain. The ECD of the PRLr has substantial homology to the other members of the cytokine receptor superfamily whereas the ICD shares little homology except the highly conserved Box 1 and Box 2 motifs (Liongue and Ward 2007). The ICDs have low sequence conservation, about 19% identity between the PRLr and growth hormone receptor (GHR), and there is a high degree of intrinsic disorder among all class I cytokine receptor ICDs (Sigalov and Uversky 2011).

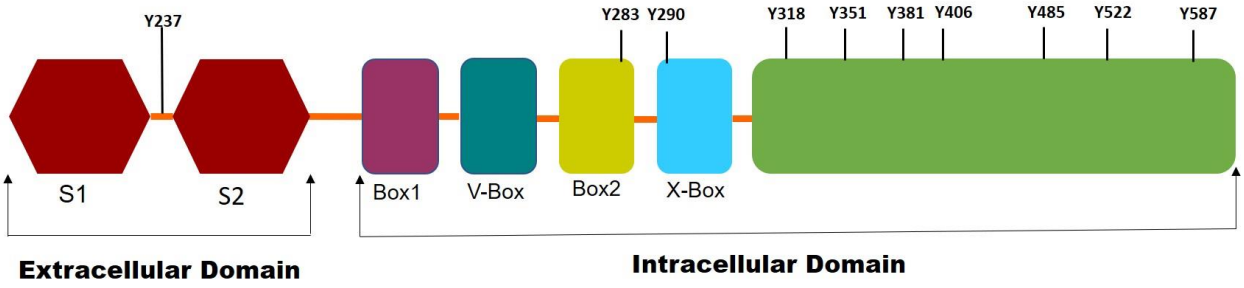


Figure 1.1 Full-length PRLr. The PRLr composed of an extracellular domain (ECD) including S1 and S2, required for ligand binding, transmembrane domain (TM), and an intracellular domain (ICD) consisting of a region of membrane-proximal homology to other cytokine receptors such as Box 1, V-Box, Box 2, X-Box and its unique cytoplasmic tail. The marking within the ICD represents the known tyrosine (Y) residues in the PRLr. Adapted from (Clevenger, Gadd et al. 2009).

The membrane proximal region of ICD consists of several motifs including Box 1, Variable Box (V box), Box 2, and X-box (Fig. 1.1). The proline rich Box 1 motif is necessary for Jak2 activation (DaSilva, Howard et al. 1994) while the signaling function of the hydrophobic and acidic Box 2 domain is mostly uncharacterized. The X-box is critical for

interaction of the PRLr with the prolyl isomerase Cyclophilin A (CypA). It is postulated that CypA induces a conformational change in PRLr-ICD upon PRL binding (Syed, Rycyzyn et al. 2003, Zheng, Koblinski et al. 2008).

The human intermediate PRLr isoform is an mRNA splice variant truncated in its ICD, but includes Box 1 and Box 2 motifs found in long hPRLr isoform. It is missing a section of its C-terminus due to an out-of-frame splice deletion resulting in a receptor containing a C-terminus of 13 amino acids with no homology to any other sequences of proteins in the database (Kline, Rycyzyn et al. 2002). The intermediate-form homodimer of the PRLr is capable of mediating PRL-induced survival but incapable of triggering PRL-activated proliferation. Like the long form of the PRLr, the intermediate form of PRLr can activate the Jak2 kinase, but is incapable of inducing Fyn tyrosine kinase activity (Kline, Rycyzyn et al. 2002).

1.2.3 Significance of conformational change of the PRLr in signal transduction

PRLr exists as pre-formed inactive homo- or hetero dimers at the cell surface, as dimerization is not sufficient for receptor activation (Gadd and Clevenger 2006). Hormone binding to the extracellular domains results in formation of active 1:2 hormone-receptor ternary complexes that lead to a conformational change in the receptor (Brown, Adams et al. 2005, Broutin, Jomain et al. 2010). The conformational change in the PRLr ICD provides a mechanism to answer the fundamental question of how the receptor is activated and modulates signal transduction to affect downstream gene expression and cell functions. The PRLr does not have intrinsic tyrosine kinase activity, unlike other cell surface receptors including epidermal growth factor receptor and fibroblast growth factor receptor. Hence, the presence of a tyrosine kinase, such as Jak2, is necessary to activate

PRLr-relevant signaling pathways. Jak2 is constitutively associated with the PRLr and PRL binding to the receptor, leads to the auto-phosphorylation of Jak2 (Leonard and O'Shea 1998). Once Jak2 is auto-phosphorylated, it then phosphorylates C-terminal PRLr residues such as tyrosine 587, thus becoming suitable for Stat5 (Signal Transducer and Activator of Transcription 5) recruitment and tyrosine phosphorylation by Jak2 (Pezet, Buteau et al. 1997, Levy and Darnell 2002). Tyrosine phosphorylated Stat5 then dimerizes, translocates to the nucleus and activates transcription of PRL responsive genes such as CyclinD1 and CISH (Schindler, Shuai et al. 1992, Brockman and Schuler 2005). PRL mediates its biological effects by binding to PRLr and activating signaling pathways including Jak2-STAT5, and Ras-MAPK in breast cancer (Clevenger and Kline 2001).

A negative feedback mechanism that restricts the degree of PRL-PRLr signal transduction is the ligand induced phosphorylation of PRLr S349 (a phosphodegron). The phosphorylation at S349 by Jak2 results in the interaction of the PRLr with the ubiquitin-protein E3 ligase β-TrCP, and thus enables the ubiquitination and subsequent degradation of the receptor (Li, Clevenger et al. 2006). Jak2 and its catalytic activity are thus necessary for not only mediating PRLr-induced signaling events, but also for regulating the magnitude and length of these signaling events.

1.2.4 Prolactin in mammary gland development

PRL is necessary for normal mammary gland development, as PRL induces both the proliferation and differentiation of the mammary gland, eventually resulting in milk synthesis and secretion (Clevenger, Chang et al. 1995). The primary driver of mammary gland development during puberty and pregnancy is the synchronization of pituitary and

ovarian hormones. Prior to puberty, the mammary gland remains an elementary structure with limited side branching and split. At the onset of puberty, the hypothalamic-pituitary-ovarian axis becomes activated, and the succeeding release of estrogen and progesterone stimulate further side branching and elongation throughout puberty (Lydon, DeMayo et al. 1995, Korach, Couse et al. 1996). During pregnancy, the hormones progesterone and PRL drive further development of the mammary gland. During this period, PRL is responsible for the proliferation of terminal differentiation of alveolar buds and milk protein production, while progesterone drives ductal side branching (Ormandy, Binart et al. 1997, Ismail, Amato et al. 2003).

The mammary gland is comprised of two types of tissue, i) the stroma that consists of adipocytes, fibroblasts and hematopoietic cells, and ii) luminal and basal cells that are supported by the stroma (Wiseman and Werb 2002, Sleeman, Kendrick et al. 2006). Luminal epithelium consist of ducts and the milk-secreting alveolar cells (Macias and Hinck 2012), whereas basal epithelium composed of myoepithelial cells, which give rise to the outer, protective layer of the gland (Gudjonsson, Adriance et al. 2005).

PRL and its cognate receptor have been implicated in mouse mammary gland development, based on investigation by hypophysectomy and genetic knockout mice. PRL and the PRLr are critical for regulating the proliferation and differentiation of normal mammary tissue. The mammary glands of PRL deficient mice show impaired lobular budding and ductal branching (Horseman, Zhao et al. 1997, Vomachka, Pratt et al. 2000). Moreover, PRLr-null mice develop small mammary glands with poorly branched ducts that results in lactation deficits (Ormandy, Binart et al. 1997, Ormandy, Camus et al. 1997). These mammary gland morphogenesis studies show that PRL/PRLr is not only

important for regulating expression of milk genes including β-casein (CSN2) and whey acidic protein (WAP), but also crucial for alveolar proliferation, terminal alveolar differentiation and milk secretion (Guyette, Matusik et al. 1979, Pittius, Sankaran et al. 1988).

1.2.5 Role of PRL/PRLr in mammary/breast cancer

Multiple studies revealed that PRL acts as an endocrine, autocrine and paracrine progression factor for mammary carcinoma in rodents and humans based on both *in vitro* and *in vivo* data (Clevenger, Furth et al. 2003, Graichen, Sandstedt et al. 2003). It was first reported more than 100 years ago that the development of mammary tumors is linked to a hormonal surge during pregnancy in mice (Lathrop and Loeb 1916). The introduction of pituitary isografts markedly increases spontaneous tumor formation in mice, and a large proportion of PRL is also found in these isografts (Boot and Muhlbock 1959). In contrast, a PRL suppressing agent, bromocriptine (dopamine agonist), significantly impedes mammary tumor formation in mice (Yanai and Nagasawa 1972). Later studies demonstrated that the elimination of an endocrine source of PRL resulted in tumor regression, whereas exogenous administration led to tumor re-occurrence in mice (Welsch 1985). Transgenic mice develop both estrogen receptor $(ER)^+$ and ER^- well differentiated mammary carcinoma within 12-18 months of life when PRL is overexpressed either at the endocrine or autocrine/paracrine levels (Wennbo, Gebre-Medhin et al. 1997, Clevenger, Furth et al. 2003). In contrast, PRL knockdown mice show increase latency in developing oncogene driven mammary tumors (Kelly, Binart et al. 2001). PRL has been strongly implicated in the pathogenesis of human breast cancer based on data at the cellular, epidemiologic and genetic levels (Maus, Reilly et al. 1999,

Clevenger 2003). The nurses' health study demonstrated a highly significant correlation between breast cancer risk and serum PRL levels (Hankinson, Willett et al. 1999). Postmenopausal women are at high risk of breast cancer development if their PRL is at "high normal" levels (Hankinson, Willett et al. 1999). Breast cancer cells produce PRL, which contributes to increased proliferation, survival, motility and invasion of breast cancer cells *in vitro* (Perks, Keith et al. 2004). Studies have found that PRLr is expressed in close to 98% of all human breast cancers based on immunohistochemistry, in situ hybridization and qRT-PCR analysis (Mertani, Garcia-Caballero et al. 1998). A significant increase in the ratio of PRLr/cell is also observed in breast cancer cells compared to normal mammary cells (Shiu 1979). PRL potentiates PRLr mRNA upregulation in MCF7 breast cancer cells and results in increased tumor cell growth potential (Ormandy, Hall et al. 1997). Similarly, a mouse xenograft study observed increased tumor formation when MB-MDA-435 breast cancer cells with high PRL and PRLr levels were implanted in nude mice. Also, these MDA-435 tumors expressed markedly more PRLr compared to controls (Liby, Neltner et al. 2003). PRLr knockdown impairs anchorage dependent and independent growth in breast cancer cells (Fiorillo, Medler et al. 2013). Based on a PRLr knockout transplants in mice, loss of mammary epithelial PRLr delays tumor formation by reducing cell proliferation in low-grade preinvasive lesions (Oakes, Robertson et al. 2007). Either gain or loss of PRL/PRLr in mammary epithelium demonstrates a role of this ligand/receptor in breast or mammary cancer. A ubiquitin mediated proteasomal degradation pathway strictly controls PRLr signaling for proper regulation of cellular processes such as proliferation, cell growth and survival (Li, Clevenger et al. 2006, Swaminathan, Varghese et al. 2008). However, an impaired degradation due to

decreased phosphorylation of S349 by Jak2 results in stabilization and accumulation of the PRLr and increased tumorigenesis (Li, Clevenger et al. 2006). A lack of S349 phosphorylation provides PRL with continuous accessibility to the receptor, and contributes to tumorigenic phenotypes by inducing signal transduction and proliferation/growth (Swaminathan, Varghese et al. 2008).

PRLr levels can be down-regulated by some cancer chemotherapeutics. A study demonstrated that post-menopausal women treated with tamoxifen for 7 days had significant decrease in PRLr mRNA levels in their tumors based on analysis of biopsy samples (de Castillo et al. 2004). Anti-estrogen therapy also leads to fast decrease in PRL-PRLr binding independent of ERα in both mouse mammary epithelial cells and human breast cancer cells (Biswas and Vonderhaar 1991). Based on another study, treatment with retinoid down-regulated PRLr mRNA, while treatment with retinoic acid reduced PRL-mediated Stat5a activation in breast cancer cells (Widschwendter, Widschwendter et al. 1999).

1.3 Jaks, Stats and Jak-Stat signaling pathway

1.3.1 Jak family of proteins and Jak2

The Jak family of proteins is composed of seven different Jak homology (JH) domains including a four-point-one, ezrin, radixin, moesin (FERM) domain (JH5, 6, 7) and a Src homology 2 (SH2) domain (JH3, and 4) (LaFave and Levine 2012) (Fig.1.2). Using the C-terminus SH2 domain, Jaks associate with the cytoplasmic tail of the cytokine receptors, then ligand induced conformational change of the receptor brings Jak dimers close enough to transphosphorylate the JH1 domain of each partner Jak (Ungureanu, Wu et al. 2011, Wallweber, Tam et al. 2014). Compared to the JH1 domain, the JH2 domain

has 10% catalytic activity and only been reported active in Jak2 (Ungureanu, Wu et al. 2011)(Fig. 1.2). Since the loss of the JH2 domain results in constant activity, it had been hypothesized to play an auto-inhibitory role (Saharinen, Takaluoma et al. 2000). The mechanisms by which JH2 negatively regulates JH1 is likely through an intramolecular interaction between JH2 and JH1 (Saharinen, Takaluoma et al. 2000). Deletion of JH2 in both Jak2 and Jak3 increases basal Jak activity, and JH1 (in trans) has been demonstrated to co-immunoprecipitate with the JH2 domain (Chen, Cheng et al. 2000, Saharinen and Silvennoinen 2002). Importantly, the JH2 domain is not a pseudo-kinase domain, as oppose to commonly accepted belief. Recently, it has been demonstrated that JH2 is an active protein kinase that phosphorylates two sites in Jak2, Serine 523 and Tyrosine 570 that allows to sustain a low basal level of Jak2 activity (Ungureanu, Wu et al. 2011).

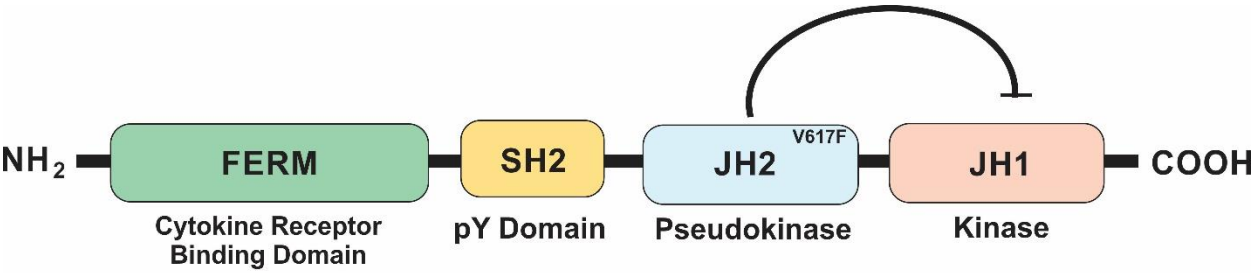


Figure 1.2 JAK2 Structure. JAK2 contains a tyrosine kinase domain (JAK homology 1 (JH1)), a pseudokinase domain (JH2), SH2-like domain, and a domain that resembles protein 4.1, ezrin, radixin and moesin (FERM). The latter domain is responsible for attachment to the cytosolic domains of cytokine receptors. The pseudokinase domain, JH2, functionally prevents the activation of the kinase domain, JH1 (curved arrow on the top). In contrast, mutations in the pseudokinase domain (light blue) lead to activation of JH1. Adapted from (Vainchenker and Constantinescu 2013).

Jak2 was originally named Tyk2 due to its well conserved protein tyrosine kinase domain next to a “kinase-like” domain (Sandberg, Wallace et al. 2004). The name Janus

originated from the two-faced Roman God of doorways, since the two Jak2 domains are involved in opposite activities (Stark and Darnell 2012). Jak2 acts as an important mediator of signaling of cytokines and hormones such as erythropoietin, interleukins, growth hormones, and prolactin (Imada and Leonard 2000).

Jak2 is pre-associated with the membrane proximal region of the PRLr (DaSilva, Howard et al. 1994, Rui, Kirken et al. 1994). Upon binding to the receptor, Jak2 auto-phosphorylates itself and then phosphorylates Stat5a and Stat5b on conserved tyrosine residues. Jak2 conditional knockout in epithelial cell lines has shown that Jak2 is necessary for the activation of Stat5 (Sakamoto, Creamer et al. 2007). Jak2 deficient mammary epithelial cells demonstrated a decreased proliferation index in estrogen and progesterone treated animals (Shillingford, Miyoshi et al. 2002). Moreover, Jak2 conditional knockout in mice leads to failed lactation due to impaired alveolar proliferation and differentiation, and a phenotype that closely mimics the PRLr knockout mouse model (Wagner, Krempler et al. 2004).

There are two well-known mutations of Jak2; the first mutation of E695K in the JH2 domain of Jak2 led to increased autophosphorylation and activation of Stat5 (Luo, Rose et al. 1997). Concurrently, several groups found a second mutation of valine to phenylalanine at position 617 in the JH2 domain of Jak2 results in a gain-of-function (Baxter, Scott et al. 2005, James, Ugo et al. 2005, Kralovics, Passamonti et al. 2005, Levine, Wadleigh et al. 2005). A majority of patients with Polycythemia Vera expresses this V617F mutation, but it is also frequent in other myeloproliferative neoplasms (Waterhouse, Follo et al. 2016)

1.3.2 Jak-Stat signaling pathway

The Jak/Stat signaling pathway is one of the highly conserved pathways found in a wide range of species (Harrison 2012). Ligands including growth factors, hormones, interferons or interleukins induce activation of transmembrane receptors that are associated with Jak/Stat signaling and activation. Since cytokine receptors do not have intrinsic kinase activity, Jak associates with the cytoplasmic domains of these receptors via Jak binding sites that are proximal to the membrane and form a complex mimicking the function of receptor tyrosine kinases (Behrmann, Smyczek et al. 2004, Babon, Lucet et al. 2014). These receptors activate the Jak/Stat signaling pathway through numerous combinations of Jak and Stat family members (Bousoik and Montazeri Aliabadi 2018).

The Suppressor of the Cytokine Signaling (SOCS) family of proteins negatively regulate the Jak2/Stat pathway (Starr and Hilton 1999). SOCS family members include CIS and SOCS1-7 that are structurally homologous (Naka, Fujimoto et al. 1999, Starr and Hilton 1999). SOCS expression is very low at resting state, but cytokine stimulation results in rapid induction of these proteins. SOCS1 and 3 block Jak and/or Stat phosphorylation to inhibit PRL signaling, whereas SOCS2 and CIS impede the inhibitory effects of SOCS1 and 3. Jak inhibition by SOCS proteins occurs through either a complex between Jak-SOCS or a complex made of PRLr-SOCS-Jak (Fujioka, Matozaki et al. 1996, Stofega, Wang et al. 1998, Timms, Swanson et al. 1999). CISH (cytokine-inducible SH2-containing protein) binds to the C-terminus of the PRLr to prevent Stat5a recruitment while not interfering with kinase activity of Jak2 (Ram and Waxman 2000). The proteasome-mediated degradation of the activated receptor complex is also accelerated by CISH. SOCS mediated inhibition results in a loss of sensitivity to PRL and other cytokines (Emanuelli, Peraldi et al. 2000).

1.3.3 Stats and their functions

Stats have roles both as transcription factors and signal transducers. Stats have an SH2 (Src homology 2) domain that mediates binding to phosphorylated tyrosine residues and a highly conserved C-terminal tyrosine residue that is required for activation of transcription (Decker and Kovarik 2000)(Fig 1.3). Activated Jaks phosphorylate this tyrosine residue and phosphorylated Stats form stable homo- or hetero- dimers with other Stats (Yu, Pardoll et al. 2009). Stats get dimerized and translocate to the nucleus after activation by tyrosine phosphorylation.

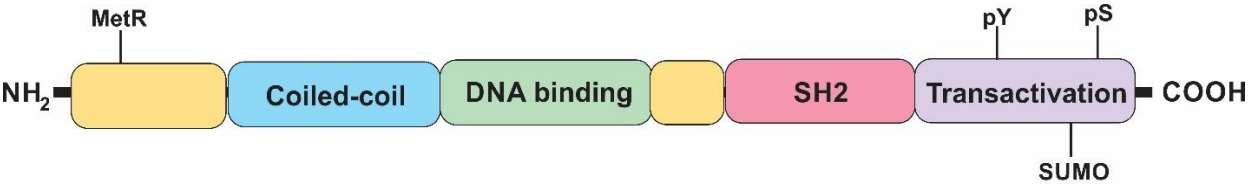


Figure 1.3 Structure of the STAT proteins. STAT structure is composed of a N-terminal domain (N-term), a Coiled-Coil domain (CCD), a DNA Binding domain (DBD), a Linker domain (LD), a SRC homology 2 domain (SH2), a Phosphotyrosyl Tail (p-Tail), and a C-terminus named the Trans-Activation Domain (TAD). Adapted from (Langenfeld, Guarracino et al. 2015).

Although Stats usually form homodimers, Stat1/2, Stat1/3 and Stat5a/b have been shown to form heterodimers (Subramaniam, Torres et al. 2001). Stat1 and Stat5 are transported into the nucleus by importin α5 and α3 transport receptors, respectively, whereas, importin α5 and α7 are involved in transporting Stat3 (Ma and Cao 2006). All three Stats (3,5,6) can be translocated to the nucleus in the un-phosphorylated state as well (Reich 2013).

Stat1 and Stat2 are known targets of interferon activation (Darnell, Kerr et al. 1994), whereas Stat1 is also activated by PDGF (platelet derived growth factor) (Vignais,

Sadowski et al. 1996). Stat1 primarily plays an inhibitory role in tumor growth by inducing cell death and vascularization while inhibiting cell growth (Sironi and Ouchi 2004). The outcome of Stat1 activation is the opposite of Stat3's role in cancer progression. However, Stat5 is involved in expression and regulation of proteins that are linked to Stat3 (Bousoik and Montazeri Aliabadi 2018). Stat3 is activated in a high fraction of breast tumors (Cotarla, Ren et al. 2004, Diaz, Minton et al. 2006), and both Stat3 and Stat5 have demonstrated important roles in mammary tumorigenesis (Barbieri, Pensa et al. 2010, Vafaizadeh, Klemmt et al. 2010). However, Stat1 activation is generally associated with better outcomes across all tumor types (Thomas, Snowden et al. 2015).

1.3.4 Stat5 and its role in mammary gland development and breast cancer

Stat5 is a transcription factor that is activated by PRL/PRLr through activation of Jak2 (Lebrun, Ali et al. 1994, Pezet, Ferrag et al. 1997). Activated Stat5 recognizes and binds to consensus elements in the DNA of various gene targets, resulting in their upregulation in cells (Schindler, Shuai et al. 1992). Stat5 plays an important role in mammary gland development, since its loss disrupts the development of luminal progenitor cells that results in impaired mammary alveologenesis. However, transgenic expression of Stat5A in the mammary epithelium rescues the luminal progenitor cell population and alveologenesis (Yamaji, Na et al. 2009). During pregnancy, Stat5A is critical for mammary gland differentiation and outgrowth, since Stat5A knockout mice are unbale to lactate (Liu, Robinson et al. 1997).

WAP-Tg transgenic mice develop mammary adenocarcinoma with a short latency. However, the heterozygous loss of Stat5a in WAP-Tag transgenic mice results in reduction in the number of tumor-bearing mice, decreased tumor size, delayed onset of

first tumor and enhanced apoptosis (Ren, Cai et al. 2002). Furthermore, loss of Stat5a markedly reduces ERα induced hyperplastic alveolar nodules in CERM (conditional ERα in mammary epithelium) mice (Miermont, Parrish et al. 2010). Stat5a transgenic mice have demonstrated increased lobuloalveolar outgrowth and delayed apoptosis, indicating that Stat5a is also a survival factor (Iavnilovitch, Cardiff et al. 2004).

In human breast cancer cell lines, Stat5 promotes cell survival and anchorage independent growth (Tang, Zuo et al. 2010). Stat5a regulates and directly enhances transcription of CISH and Cyclin D1, resulting in increased cell proliferation (Borges, Moudilou et al. 2008). Furthermore, increased expression of both Cyclin D1 and CISH have been found in primary breast tumors (Schindler, Shuai et al. 1992, Raccurt, Tam et al. 2003), leading to enhanced activation of the highly proliferative MAPK pathway and poorly differentiated tumors (Borges, Moudilou et al. 2008). In mammary tumorigenesis, Cyclin D1 is expressed in the excess of 50% and induces proliferation by promoting G1 to S phase transition of the cell cycle during tumorigenesis (Yu, Geng et al. 2001).

Stat5a is phosphorylated both at tyrosine (Y) and serine (S) residue(s) that are important for both mammary grand development and tumorigenesis. Stat5a has been shown to be phosphorylated at Y694 in a PRL-dependent manner where serine phosphorylation is constitutive in HC11 mammary epithelial cells (Wartmann, Cella et al. 1996). Stat5a S725 (Yamashita, Xu et al. 1998) as well as S779 located in the Stat5a SP motif (Beuvink, Hess et al. 2000) are identified as constitutively phosphorylated motifs in mammary glands from different developmental stages. It has been demonstrated that mutation of S779, S725 or both S779/725 into alanine residues has no substantial effects on PRL-induced transcriptional activation of a β-casein promoter reporter construct. In these conditions,

Stat5a S725A demonstrated continuous DNA binding activity compared to wild-type Stat5a because of upkeep of Stat5a Y694 phosphorylation. However, Stat5a S779A acted similar to the Stat5a wild-type (Beuvink, Hess et al. 2000).

1.3.5 Role of Jak2/Stat5 in mammary and breast cancer

The Jaks and Stats have been implicated in mammary/breast cancer. Jak2 and Stat5 are among the major proteins involved in inter-pathway crosstalk and regulate proliferation, differentiation, and survival of cancer cells (Vogelstein, Papadopoulos et al. 2013). The Jak/Stat pathway is activated upon binding of hormones (e.g. prolactin) and cytokines (e.g. interleukins) to their cognate receptors. In neoplasia, the ligands (e.g. PRL, PR, ER) are secreted by cells from the tumor microenvironment and/or cancer cells (Bissell and Radisky 2001). For example, following PRL stimulation, Jak2 is autophosphorylation/activated and then phosphorylate/activate Stat5. Phosphorylated Stat5 self-dimerize, and translocate to the nucleus to initiate oncogene mediated tumorigenesis (Wagner and Rui 2008). The mechanisms of Stat5 activation and translocation to the nucleus are not limited to Jak2 activation. There also have been links between Stat activation and the Src family of kinases to promote transcriptional regulation to affect cancer phenotypes (Kim, Song et al. 2009). The transcriptional activity of the Cyclin D1 locus that has been implicated in mammary tumorigenesis is stimulated by Jak2 activated Stat5 (Brockman, Schroeder et al. 2002). In mammary cancer, Jak2 and Stat5 may promote evasion from apoptosis and self-sufficiency in growth signals (Hanahan and Weinberg 2011). Gain of function mutations such as the Jak2 V617F mutation in myeloproliferative neoplasms can also activate Jak/Stat signaling pathway (Harrison, Kiladjian et al. 2012). Expression of a constitutively active Jak2 mutant, Jak2

V617F, in mammary epithelial cells (MECs) results in hyperactivation of Stat5. Furthermore, overexpression of Jak2 V617F leads to *in vitro* increased proliferation, resistance to cell death and promotes tumorigenesis in a xenograft model (Caffarel, Zaragoza et al. 2012). Jak2/Stat5 hyperactivation promotes resistance to PI3K/mTOR inhibitors by first restoring activation of Akt while upregulating pro-metastatic chemokine, IL-8 that induces positive feedback on Jak2/Stat5. Stat5 upregulates expression of IL-8, which binds to its receptor CXCR1 to induce invasion and metastasis (Britschgi, Andraos et al. 2012).

Overexpression of wild-type and constitutively active Stat5 results in sporadic mammary cancer. Moreover, Stat5 variant without the C-terminal transactivation domain was able to transform mammary epithelial cells (Iavnilovitch, Cardiff et al. 2004). Pregnancy hormone (e.g. PRL) primed cells that co-express Cyclin D1 and nuclear Stat5 within alveolar units were primarily observed in multiparous mice that are also prone to develop mammary tumors (Wagner and Smith 2005). However, the incidence of mammary cancer was decreased in heterozygous Stat5a knockout mice that express the simian virus 40 large T-antigen (Ren, Cai et al. 2002).

A proportion of primary human breast cancer have nuclear localized, tyrosine phosphorylated Stat5a (Cotarla, Ren et al. 2004). Although phosphorylated Stat5a has been detected in luminal cells of primary tumors, it was significantly downregulated during metastatic progression of breast cancer, with a less than 20% of metastases demonstrating nuclear localized, tyrosine activated Stat5 (Nevalainen, Xie et al. 2004). Phosphorylated/activated Stat5a positively correlated with tumor differentiation and

favorable prognosis. The favorable association was more prominent in patients without lymph node metastasis (Wagner and Rui 2008).

1.4 Cyclophilin A

1.4.1 Cyclophilins and their functions

Cyclophilins are highly conserved proteins throughout evolution (Wang and Heitman 2005). They were originally discovered to be biological receptors for the immunosuppressive drug Cyclosporin (CsA), consequently, they are also called immunophilins. There are seven major Cyp isoforms in humans, namely: hCypA (also called hCyp18a), hCypB, hCypC, hCypD, hCypE, hCyp40, and hCypNK (first identified in human natural-killer cells) (Galat 2003). All Cyclophilins are composed of a common domain of around 109 amino acids, the cyclophilin-like domain (CLD), surrounded by regions exclusive to each member of the family that are associated with subcellular compartmentalization and functional specialization (Marks 1996). Cyclophilins are found in various subcellular compartments such as the cytosol, endoplasmic reticulum (ER), mitochondria and nucleus (Dornan, Taylor et al. 2003).

Cyclophilins act as molecular chaperones and catalyze the cis-trans isomerization of peptidyl-prolyl bonds using their specific enzymatic properties (Craig, Gambill et al. 1993). Cyclophilins also stabilize the cis-trans transition state and accelerate isomerization, a process that is considered crucial for both protein folding and during the assembly of multidomain proteins (Gothel and Marahiel 1999). In addition to protein folding and assembly, cyclophilins are also proficient in repairing damaged proteins under severe challenges, including oxidative stress, thermal stress, ultraviolet irradiation and changes in pH (Parsell and Lindquist 1993). Besides PPIase and chaperone activities,

cyclophilins have magnesium (Mg^{2+}) and calcium (Ca^{2+}) dependent nuclease activity like that of apoptotic endonucleases (Montague, Gaido et al. 1994), so they may be involved in apoptotic DNA degradation.

Cyclophilins are found in various cellular compartments of most tissues and have unique functions. Cyclophilin A (CypA) is primarily found in the cytosol and has both PPIase and chaperone activities (Lee 2010). Cyclophilin B (CypB) and Cyclophilin C (CypC) are primarily located in the ER and form protein complexes with various ER-stress related chaperones including glucose-regulated protein (Grp) 78 (GRp78/BiP), Grp94 and protein disulphide isomerase (Zhang and Herscovitz 2003, Kim, Choi et al. 2008). CypB ensures proper conformational changes of newly synthesized and secreted proteins (Zhang and Herscovitz 2003). Overexpressed CypB inhibits apoptosis linked to ROS and Ca^{2+} homeostasis after ER stress (Kim, Choi et al. 2008). CypB interacts with the PRLr and induces Stat5 mediated gene transcription (Rycyzyn and Clevenger 2002). CypC binds to osteopontin via CD147 and increases migration and invasion (Mi, Oliver et al. 2007). Cyclophilin D (CypD) is a component of the mitochondrial permeability transition pore (MPTP) and it's PPIase activity is necessary for binding CypD to the complex and its function (Lin and Lechleiter 2002). Cyclophilin E (CypE, also known as Cyp33) is found in the nucleus and contains a RNA-binding motif, but its cellular roles are not well characterized (Mi, Kops et al. 1996). Cyclophilin 20 (Cyp20) found in the mitochondria is involved in the folding, importing and processing of newly synthesized proteins and is functionally associated with mitochondrial Hsp70, Hsp60 and Hsp10 (Rassow, Mohrs et al. 1995). Cyclophilin 40 (Cyp40) associates with heat shock proteins including HsP70 and HsP90 and is induced by heat shock and hypoxia in cardiac cells (Zou, Guo et al.

1998, Mark, Ward et al. 2001). The association between Cyp40 and HsP exerts cytoprotective effects during ischaemia-reperfusion injury (Johnson and Craig 1997). Cyp40 has important functional implications for ERα and other steroid receptors in breast cancer (Ward, Mark et al. 1999). Cyclophilin natural killer (CypNK) is located in the cytosol with a large, hydrophilic and positively-charged carboxyl terminus, is also the largest known cyclophilin (Anderson, Gallinger et al. 1993). CypNK was identified as a component of the putative tumor recognition complexes where it may be a key tool in the molecular basis of tumor-cell immune recognition (Anderson, Gallinger et al. 1993, Rinfret, Collins et al. 1994).

1.4.2 Cyclophilin A and its regulation of the PRLr structure

CypA is part of the peptidyl-prolyl isomerase (PPI) family of enzymes that catalyzes the cis-trans interconversion of imide bonds of proline residues (Fig. 1.4) inducing protein backbone conformational change (Gothel and Marahiel 1999) and was initially identified

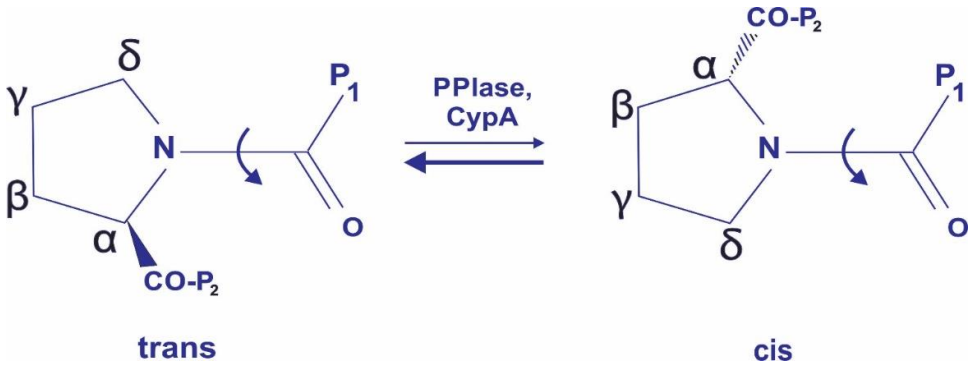


Figure 1.4 PPIase cis-trans isomerization. A schematic illustration of the *trans* and *cis* isomers of the peptide bond between proline (on the left of each structure shown) and another amino acid (P_1, on the right). The interconversion between the two forms is catalyzed by cyclophilins and other peptidyl-prolyl isomerases (PPIases). The carbon atoms of the proline are indicated by Greek letters; P_2 indicates a third amino

acid on the other side of the proline. The peptide bond has some double-bond character and is planar. Adapted from (Wang and Heitman 2005).

as a receptor of cyclosporine (CsA), an immunosuppressive drug (Handschumacher, Harding et al. 1984). CypA-CsA complex allosterically inhibits calcineurin, resulting in inhibition of T-cell activation (Roehrl, Kang et al. 2004). CypA is an 18 kDA isomerase, mostly cytosolic and found in all tissues in mammals, whereas, other cyclophilins are found in the ER, the mitochondria, or the nucleus (Galat 2003). Human CypA consists of an eight-stranded anti-parallel β-barrel structure, along with two α helices enclosing the barrel from either side. The area where CsA binds, is a compact hydrophobic core within the barrel consisting of seven aromatic and other hydrophobic residues (Kallen, Spitzfaden et al. 1991). CypA is post-translationally modified by acetylation and phosphorylation (Massignan, Casoni et al. 2007, Pan, Luo et al. 2008). CypA's acetylation at lysine residue 125 affects its enzymatic activity, its ability to bind CsA and calcineurin, and HIV-1 incorporation into a cell (Lammers, Neumann et al. 2010). CypA has a significant role in several cell functions such as protein folding, intracellular trafficking, signal transduction and transcriptional regulation (Satoh and Berk 2008).

CypA has been found to be a constitutively interacting protein with the prolactin receptor (PRLr), which is activated upon prolactin (PRL) binding to its cognate receptor (Clevenger, Furth et al. 2003, Zheng, Koblinski et al. 2008). CypA, through its PPI activity, induces the proximal activation of Jak2 and PRLr associated signaling upon binding of PRL to the PRLr (Zheng, Koblinski et al. 2008). PRL mediated signaling/gene expression is directly correlated with level or activity of CypA. It has been shown that overexpression

of an interaction-defective PRLr mutant (P334A), which impedes CypA association with the PRLr, downregulates PRLr/Jak2 mediated signaling (Zheng, Koblinski et al. 2008).

1.4.3 Functions of CypA in human diseases

CypA is a key regulator for cardiovascular diseases. It was demonstrated that extracellular CypA induces pro-inflammatory signals in endothelial cells (EC) and vascular smooth muscle cells (VSMC) (Jin, Melaragno et al. 2000, Suzuki, Jin et al. 2006). In CypA knockout mice, coronary artery intimal area, medial and adventitial thickening were markedly lower, whereas mice overexpressing CypA had enhanced thickening compared to WT mice (Satoh, Matoba et al. 2008). The anatomical severity of stable coronary artery disease was also correlated with plasma levels of CypA (Satoh, Matoba et al. 2008). In certain cases, CypA might be a useful marker for predicting the severity of acute coronary diseases (Yan, Zang et al. 2012). In patients with diabetes, the level of CypA in plasma samples are higher than healthy individuals (Ramachandran, Venugopal et al. 2012). CypA modulates the infection and replication of viruses affecting humans. CypA interacts with HIV accessory proteins including Vpr and Nef to mediate a step in the viral life cycle between penetration and reverse transcription (Zander, Sherman et al. 2003, Qi and Aiken 2008). CypA positively regulates replication of hepatitis C virus (HCV) (Patient, Hourioux et al. 2007). CypA is also important for hepatitis B virus (HBV), as higher serum CypA levels were found in chronic hepatitis B patients than in healthy volunteers (Tian, Zhao et al. 2010).

1.4.4 A role for CypA in cancer

CypA is overexpressed in cancer and regulates malignant transformation and metastasis. Based on several studies, overexpression of CypA drives cancer cell proliferation, prevention of apoptosis and oxidative-stress defense (Choi, Piao et al. 2007). CypA was first shown to be up-regulated in hepatocellular carcinoma (Corton, Moreno et al. 1998). Since then, a few other groups have demonstrated the overexpression of CypA in diverse cancers such as pancreatic cancer, lung cancer, squamous cell carcinoma and breast cancer using analytical methods such as protein profiling, immunohistochemistry and bioinformatics (Lee and Kim 2010).

In pancreatic cancer, CypA interacts with the proline-rich peptide in CD147's transmembrane domain to induce proliferation (Boulos, Meloni et al. 2007). Overexpression of CypA in small cell lung cancer stimulates cancer cell growth, while knockdown of CypA slows cancer cell growth (Howard, Zheng et al. 2004, Howard, Furumai et al. 2005). CypA was found to be up-regulated during malignant transformation of esophageal squamous carcinoma cells (Qi, He et al. 2008) and may regulate metastasis in these cells. In solid tumors, CypA overexpression renders resistance to hypoxia- and cisplatin-induced cell death in a p53 independent manner (Choi, Piao et al. 2007).

1.4.5 Cancer therapeutics targeting CypA

CypA inhibition by CsA blocks in vitro proliferation/growth in breast cancer cells while preventing mammary cancer metastasis (Zheng, Koblinski et al. 2008). A combination of two CypA inhibitors, CsA and Sanglifehrin A (SfA), increases the chemotherapeutic effect of cisplatin in glioblastoma multiforme (Han, Yoon et al. 2010). A clinical study found that women treated with CsA show a 50% reduction in incidence of breast cancer based on a

10-year follow-up (Stewart, Tsai et al. 1995), suggesting potential relevance of PPI inhibitors to target CypA in breast cancer. Treatment with anti-cancer drugs such as 5-aza-2'-deoxycytidine decreases CypA expression in pancreatic ductal carcinoma cancer cells (Cecconi, Astner et al. 2003) and sensitizes cells for enhanced chemotherapeutic effect of cisplatin in glioblastoma multiforme (Han, Yoon et al. 2010). However, liver cells overexpressing CypA demonstrate an increased resistance to doxorubicin and vincristine (Nigro, Pompilio et al. 2013). CypA has been known to regulate multiple malignant phenotypes that are critical for cancer progression, hence it is imperative to better understand its mechanisms in these processes and evaluate its potential as a target for cancer therapeutic intervention.

1.4.6 CsA and its non-immunosuppressive analogue, NIM811

An immunosuppressive drug, CsA, is a cyclic 11-amino-acid peptide and CypA is the major intracellular receptor for CsA (Handschumacher, Harding et al. 1984). The CsA-CypA complex binds to and inhibits a calcium-calmodulin activated serine/threonine phosphatase named calcineurin. Calcineurin inhibition blocks the translocation of NFAT (nuclear factor of activated T cells) from the cytosol to the nucleus, consequently blocking transcription of cytokines such as interleukin-2 (Liu, Farmer et al. 1991, O'Keefe, Tamura et al. 1992).

The non-immunosuppressive analog of cyclosporine is NIM811 (N-methyl-4 isoleucine cyclosporine). NIM811 is a mitochondrial permeability transition inhibitor. It is a four-substituted cyclosporine analogue that binds to cyclophilin, but cannot bind calcineurin, and hence lacks immunosuppressive activity (Ma, Boerner et al. 2006). Both cyclosporine and NIM811 inhibit PPIase activity of CypA with similar efficacy. NIM811

inhibits hepatitis C virus (HCV) activity and is a promising treatment for HCV patients (Ma, Boerner et al. 2006). NIM811 suppresses liver injury and stimulates liver regeneration after major hepatectomy (Rehman, Sun et al. 2011). It has been shown to have potential anti-inflammatory effects in acute lung injury and arthritis (Arora, Gwinn et al. 2005, Damsker, Okwumabua et al. 2009).

1.5 Src family kinase

1.5.1 Src family kinase and breast cancer

The Src family of non-receptor tyrosine kinases (SFKs) encompasses nine members, among them Src, Yes, Fyn, and Yrk are ubiquitously expressed in mammals (Thomas and Brugge 1997). The SFK family has a modular structure consisting of molecular hallmarks for these proteins. The N-terminal Unique domain (U or SH4) of SFK has 80 amino acid residues and is the most divergent among family members. The linear sequence is composed of a Src homology domain 3 (SH3), which has strong affinity for proline-rich sequences; and a SH2 domain that binds to the tyrosine phosphorylated residues of proteins (Fig. 1.5). These two domains are involved in vast interactions with networks of proteins/complexes to induce intracellular signaling (Pawson, Gish et al. 2001, Guarino 2010, Martin-Perez, Garcia-Martinez et al. 2015). SFKs generate signaling complexes using their SH2 and SH3 domain that diversify and amplify the initial receptor activation signaling (Fig. 1.5). Based on the physiological or pathological context, SFKs regulate cellular processes such as cell survival, proliferation, differentiation, motility, migration and invasion (Thomas and Brugge 1997, Guarino 2010). SFKs play key roles in the functional maintenance of various tissues and organs such as the bones, the mammary glands, the immune systems, the brains and are directly

involved in the development of numerous types of cancer (Guarino 2010, Zhang and Yu 2012).

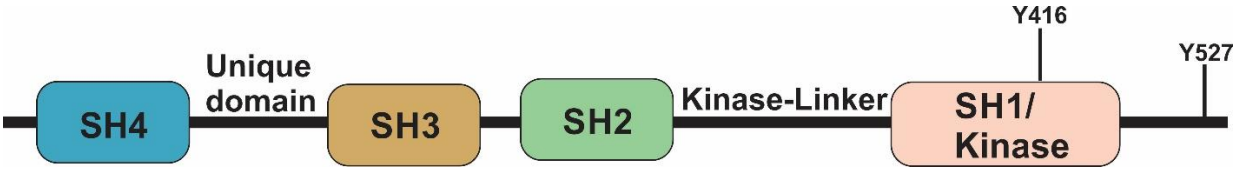


Figure 1.5 Structure of Src. Schematic of the linear domain arrangement of c-Src. The SH4 domain is important for myristoylation of Src and necessary for membrane binding and kinase activity *in vivo*. SH3 and SH2 are necessary for PxxP and pYEEI binding respectively. The Y416 residue in the kinase domain is important for phosphorylation-stimulation of kinase activity, whereas, the Y527 residue is necessary for phosphorylation-down-regulation of kinase activity. Adapted from (Reinecke and Caplan 2014).

1.5.2 SFK/Src relationships with PRL/PRLr and Jak2/Stat5 pathway

SFK has been implicated in the regulation of PRL signaling (Kelly, Ali et al. 1993). Increased production of PRL and overexpression of membrane PRLr in breast cancer (Shiu 1979), potentially results in amplified stimulation of catalytic activity of tyrosine kinases (Rui, Djeu et al. 1992). PRLr activation directly induces SFKs and Jak2 catalytic activity, consequently promoting proliferation, differentiation, or other cellular responses based on physiological context (Clevenger and Medaglia 1994, Berlanga, Fresno Vara et al. 1995).

Src kinases are also important signaling mediators of the PRLr by interacting with the Box1/2 domain (Acosta, Munoz et al. 2003). The regulation of Src kinase activity is dependent on phosphorylation of Y416 and Y295 residues of Src; and these events/phosphorylation have been linked to breast cancer progression (Kanomata,

Kurebayashi et al. 2011). Loss of Src expression significantly decreases PRL induced Jak2 activation, which is dependent on the SH2/SH3 domains of Src (Garcia-Martinez, Calcabrini et al. 2010), however, Src kinase activity is not dependent on Jak2 (Sakamoto, Creamer et al. 2007). *In vitro* overexpression of a kinase-inactive form of Src results in decreased PRL-induced proliferation, motility, and attachment of breast cancer cells (Acosta, Munoz et al. 2003, Gonzalez, Agullo-Ortuno et al. 2006). Moreover, treatment of ER^- breast cancer with the Src kinase inhibitor dasatinib has demonstrated some therapeutic efficacy for patients (Finn, Dering et al. 2007, Pichot, Hartig et al. 2009). A recent work demonstrated that the Src signaling signature is extensively upregulated in metastasis, and Src inhibitors including dasatinib are found to be cytotoxic to human breast cancer PDX spheroids (Alzubi, Turner et al. 2019). Overall, the Src kinase is relevant to both PRL-induced signaling, and breast cancer biology. Although the catalytic activity of SFKs is necessary for PRL induced phosphorylation of Sam68 (a SFKs mitotic substrate) (Fumagalli, Totty et al. 1994), phosphorylation/activation of PRLr, Jak2/Stat5, and ERK1/2 is insensitive to the inhibition of SFK catalytic activity. However, transient expression of dominant negative Src (SrcDM) significantly reduces PRL-induced cell proliferation and activation of Akt. Src inhibitor PP1/PP2 blocks PRL-induced activation of Shp2, Akt and p70S6k (Fresno Vara, Caceres et al. 2001).

1.6 Focus of this work and Hypothesis

1.6.1 Preliminary Findings

CypA is a constitutively interacting protein with the PRLr based on a yeast two-hybrid screen (Syed, Rycyzyn et al. 2003). Gain/loss of function approaches and inhibition of CypA activity with CsA in breast cancer cells revealed functional actions of CypA on the

PRLr/Jak2 complex (Zheng, Koblinski et al. 2008). Our lab investigated the action of CsA on ER^+ luminal breast cancer cells and found that it significantly reduced PRL induced activation of Jak2 and Stat5 (Zheng, Koblinski et al. 2008). Furthermore, CsA decreased both basal and PRL induced gene expression of CISH and Cyclin D1 (Zheng, Koblinski et al. 2008). To further assess the CsA mediated inhibition of CypA on the association of CypA with the PRLr and Jak2, immunoprecipitation (IP) of CsA-treated T47D cells showed reduced levels of CypA association with PRLr and Jak2 (Zheng, Koblinski et al. 2008). Together, these results revealed that the PPI activity of CypA contributes to the proximal signaling of the PRLr complex. It was hypothesized that CsA could also inhibit the development of human breast cancer *in vivo* as seen with the *in vitro* studies. To test this hypothesis, both ER^- MDA231 and ER^+ MCF7 human breast cancer cell lines were xenografted into nude mice (Zheng, Koblinski et al. 2008). It was found that CsA induced central primary tumor necrosis and decreased metastasis of both types of human breast cancer xenografts (Zheng, Koblinski et al. 2008). A site directed mutagenesis approach helped identify the CypA binding site at proline 334 on the prolactin receptor (Zheng, Koblinski et al. 2008). When cells expressed the P334A PRLr mutant, a significantly decreased CypA-PRLr association, Jak2/Stat5 phosphorylation and PRL induced gene expression was observed (Zheng, Koblinski et al. 2008). These data demonstrated that CypA binds to the PRLr and affect Jak2/Stat5 signaling, but the fundamental question remained as to how CypA modulates the structure/function relationships of the PRLr. CsA is a potent inhibitor of CypA and capable of modulating breast cancer cell functions, but it is still immunosuppressive with toxic side effects. The immunosuppressive properties of CsA, through its engagement of calcineurin, precludes the use of this agent

in breast cancer patients with anything else other than end stage disease. Therefore, the action of a non-immunosuppressive and non-toxic analog of CsA called NIM811 (N-methyl-4-isoleucine cyclosporine) with higher affinity for CypA compared to cyclosporine (Ma, Boerner et al. 2006) is explored, to evaluate its efficacy in breast cancer cell functions and mammary tumorigenesis/metastasis. The inhibition of PRLr/Jak2/Src phosphorylation by NIM811 is evaluated because certain tyrosine phosphorylation(s) are believed to be important for PRL signaling and breast cancer pathogenesis. Using the non-immunosuppressive NIM811 or loss of function approach, we sought to extensively investigate the role of CypA in modulating breast/mammary cancer cell functions both *in vitro* and *in vivo*. Importantly, we ask the fundamental question of how CypA regulates the structure/function relationships of PRLr to modulate signaling, global gene expression, breast cancer cell functions and mammary cancer progression.

1.6.2 Hypothesis

CypA as a molecular switch inducing a conformational change of the PRLr to regulate PRLr/Jak2 complex mediated signaling, mammary tumorigenesis and metastasis.

1.6.3 Significance

This work elucidates a fundamental mechanism of CypA prolyl isomerase action on the PRLr, leading to activation of proximal kinases, downstream gene expression, breast cancer cell functions and mammary cancer progression. These studies illuminate the basis of mechanisms of CypA prolyl isomerase action in human breast cancer. The experimental results from both *in vitro* and *in vivo* studies delineated here are highly relevant and can translate to the patient.

Chapter 2: Methods and Materials

2.1 Cell culture and reagents

T47D and MCF7 human breast cancer cell lines were obtained from ATCC (Manassas, VA) and cultured in Dulbecco's modified Eagles's medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin (Life Technologies). All cells were incubated in a humidified 5% CO_2/95% air atmosphere at 37°C. Human recombinant PRL was a gift from Dr. Anthony Kossiakoff (University of Chicago, Chicago, IL). PRL was added to cells to yield a final concentration of 250 ng/ml. N-methyl-4-isoleucine-cyclosporin (NIM811) was obtained from Novartis and pre-incubated for 2-4 hours prior to stimulation with PRL.

2.2 Western blotting and analysis

Cell lysates were analyzed by western blot analysis as previously described in (Zheng, Koblinski et al. 2008). Cells were grown in 100 cm^2 dishes until 70%-80% confluence followed by starvation for 16-24 hrs before PRL treatment (100 ng/ml) in conditioned media (DMEM, Life Technologies). Cells were lysed in RIPA buffer and cell lysates were blotted by specific antibodies. Antibodies utilized for western blot analysis were obtained from the following sources and used as described: anti-pPRLr 381 (Custom antibody from New England Biolabs, 1:500), anti-pPRLr 587 (Custom antibody from New England Biolabs, 1:500), anti-PRLr (Life Technologies, 1:1000), anti-p-Jak2 1007/1008 (Cell Signaling Technology, 1:500), anti-Jak2 (Cell Signaling Technology, 1:1000), anti-pStat5 (Cell Signaling Technology, 1:1000), anti-Stat5 (Santa Cruz Biotechnology, 1:1500), anti-pSrc416 (Cell Signaling Technology, 1:500), anti-Src (Cell Signaling Technology, 1:1000), anti-CISH (Santa Cruz Biotechnology, 1:1000), anti-CyclinD1 (Santa Cruz Biotechnology, 1:1000), anti-Cyclophilin A (Santa Cruz Biotechnology, 1:1000), and anti-

GAPDH (Cell Signaling Technology, 1:2000) diluted in TBS-T with 3% milk or BSA as suggested by the specific manufacturer. Target proteins were visualized by enhanced chemiluminescence (GE Healthcare), and images were captured using Fujifilm LAS-3000 system. The band intensities were quantified by LAS-3000 analysis tools and normalized to those of their respective loading control bands. Data were expressed as fold changes compared with an appropriate control.

2.3 Immunoprecipitation (IP)

Transfected T47D cells were lysed in immunoprecipitation buffer (20 mM Tris-HCL (pH 7.5), 150 mM NaCl, 1% (v/v) Nonidet P-40, 100 mM NaF, 5 mM $MgCl_2$, 0.5 mM Na_3VO_4, 1 mM PMSF, 1X protease inhibitor mixture, and 1X phosphatase inhibitor mixture. Lysates were pre-cleared with 50 ul of recombinant protein G-agarose beads with rotation at 4° C for 1 hour. Next, an immunoprecipitation protocol using Dynabeads was performed (IP kit/protocol from Novex, Life Technologies). Immunoprecipitates were eluted in 30 ul of 2X Laemmli sample buffer (BioRad) containing 5% (v/v) β-mercaptoethanol and analyzed by western blotting.

2.4 Plasmids

The plasmid vectors that encode cyan FP (pE-CFP) and yellow FP (pE-YFP) were originally obtained from Clonetech (Clonetech Laboratories Inc., Mountain View, CA). Four separate variants of either CFP or YFP of long-form and intermediate-form of the PRLr were generated by inserting/cloning into pECFP-N1 and pEYFP-N1 vectors using standard techniques as per instructions from Clonetech. Sequencing primers used are as follows: forward primer 5'-CGCAAATGGGCGGTAGGCGTG-3' and reverse primer 5'-CGTCGCCGTCCAGCTCGACCAG-3'. All mutants or corrected constructs were

generated using a Phusion Mutagenesis Site Directed kit (Life Technologies) according to the manufacturer's instructions.

2.5 Plasmid Transfection

HEK293 cells were obtained from ATCC (Manassas, VA) and maintained as a monolayer culture in Dulbecco's Modified Eagle's Medium (DMEM; Life Technologies, Inc; Grand Island, NY) containing 10% fetal bovine serum (FBS; Atlanta Biologicals; Flowery Branch, GA) and grown in a humidified 37°C incubator containing 5% CO_2. For PRL treatment, cells were synchronized in serum-free medium containing 0.1% bovine serum albumin (BSA) for overnight or 24 hours prior to treatment. PRL was added to yield a final concentration of 250 ng/ml for the indicated timepoints. For CypA inhibition, cells were treated with 10 μg/ml of the non-immunosuppressive, NIM811 (Novartis Institute) or DMSO as the vehicle control for 2 hours prior to PRL treatment.

2.6 siRNA transfection

On-target plus siRNA targeting PPIA (Cat # LQ-004979-04-0002) and non-targeting control (Cat # D-001810-10-05) were purchased from Dharmacon. T47D cells were transfected with siRNA at a final concentration of 25 nM. Transfections were performed using RNAiMAX (Life Technologies, Inc.) following the manufacturer's protocol. Transfection complexes were prepared in OptiMEM medium (Thermo Fisher). For a 6-well plate, transfections were carried out using 7.5 μl RNAiMAX and 3×10^5 cells per well. Other conditions were scaled accordingly. Twenty-four hours post-transfection, the complexes were removed and replaced with fresh growth medium. After another 24 hours, the cells were arrested. Cell harvesting for membrane extracts analysis or live cell imaging was carried out after 24 hours of starvation/arrest and PRL treatments.

2.7 Membrane extracts FRET analysis

FRET analysis of membrane extracts from transfected HEK293 cells were performed on a fluorescence plate reader and as described in (Brooks, Dai et al. 2014). 293 Cells were transfected with PRLr-CFP and PRLr-YFP or single-color controls or negative control or FRET positive control. Transfection was performed according to the manufacturer's protocol for the Lipofectamine 3000 transfection kit from Life Technologies. Forty-eight hours post transfection and subsequent starvation for 16-24 hours, cells were stimulated with prolactin for 30 minutes. After stimulations, cells were washed twice with PBS and protease inhibitor. Then, cells were incubated for 15 minutes in Buffer A (25 mM sodium phosphate and 5 mM $MgCl_2$, and 2 mM Na_2VO) at 4°C. Cells were scraped off the plates and homogenized using a tissue homogenizer. Homogenized cells were subjected to three rounds of freezing and thawing using a liquid nitrogen bath and 37°C water bath with mixing between each thaw. Subsequently, lysed cells were centrifuged at 17000g for 20 minutes, re-suspended in Buffer A and centrifuged again for 20 minutes followed by re-suspension in Buffer A. Each suspension was thawed and placed in a well of a black bottom 96 well plate and fluorescence readouts were performed on a plate reader with CFP-Ex/Em 420/486 and YFP-Ex/Em 492/550 nm filters.

2.8 Live cell FRET analysis

Transfections and treatments were performed as described for membrane extracts experiments. Cells were imaged live at 37°C by confocal microscopy and images acquired on a LSM710 Zeiss inverted confocal microscope equipped with a 20x objective. CFP was excited at 430 nm and detected from 460 to 490 nm, and YFP was excited at 514 nm, and detected from 528 to 603 nm. Excited power was approximately 100-400

uW. The regions of interest (ROIs) were designed to correspond with cell expressing CFP or YFP or both. The sensitized emission image was ratioed to the excitation intensity-corrected $M_{directAcceptor}$ or F_{donor} image to obtain the apparent FRET efficiency picture.

2.9 Live cell FLIM-FRET analysis

HEK293 cells were cultured in glass-bottomed Petri dishes with glass bottom (MaTek) to reach approximately 70% confluence. Cell culture and microscopy imaging was performed in Optimem at 37^{o}C. The imaging was executed using a Zeiss 710 confocal system, equipped with an Axiovert Z1 inverted microscope, 63x oil immersion objective (NA 1.4), 440 nm pulsed (80 MHz) diode laser (50 mW nominal power), and a multiline argon ion laser (15 mW nominal power of the 488 nm line). The system contained two hybrid detectors (on external port), controlled with two SPC 150 time-correlated single photon counting (TCSPC) modules (Becker & Hickl). Fluorescence of CFP was excited using the diode laser and registered from single confocal sections (pinhole set to 1.2 Airy unit) in s in 450 – 485 nm (CFP, donor) and 510 – 560 nm (YFP, acceptor) range. Images of fluorescence (512x512 pixels) were collected in TCSPC mode (the hybrid detectors), with pixel size of 0.18 µm, 6.3 µs pixel dwell time and the results were integrated for 300s. Fluorescence lifetime of the donor was calculated from the integrated TCSPC images on a pixel-by-pixel basis using monoxponential fit (least squares method, intensity weighted), implemented in SPC Image (Becker & Hickl). Empirical impulse response function (IRF), measured with quenched rhodamine was used in the fit.

2.10 FRET/FLIM to assess conformational change of the PRLr

Forster resonance energy transfer (FRET) is used to study protein interactions or conformational changes within a protein/receptor (e.g. growth hormone receptor). FRET is based on a biomolecular fluorescence quenching process that the excited state energy of a donor fluorophore is non-radiatively transferred to a ground state acceptor molecule (Suhling, Hirvonen et al. 2015). The process relies on a dipole-dipole coupling process and was quantitatively described by Forster in 1946. For FRET to occur, the emission spectrum of the donor (e.g. cyan fluorescence protein/CFP) and the absorption spectrum of the acceptor (e.g. yellow fluorescence protein/YFP) must overlap, be within <10 nm and not perpendicular to each other (Suhling, Hirvonen et al. 2015).

The widely used FLIM (Fluorescence Life-time Imaging) is the identification/measurement of FRET in live cells based on fluorescence life-time decay of the donor molecule. FRET/FLIM is often used to study conformational changes within a protein (Hunt, Keeble et al. 2012). Truncated growth hormone receptor (GHR) with FRET reporters (CFP, YFP pair) demonstrated growth hormone (GH) mediated activation of the receptor and Jak2. GH induced conformational change in the GHR intracellular (ICD) domain is assessed by loss of FRET ratio as opposed to uninduced condition (Brooks, Dai et al. 2014). Likewise, we fluorescently tagged the carboxyl terminal of the PRLr with either CFP or YFP and transfected the pair in HEK 293 cells to assess FRET/FLIM utilizing plate reader and confocal live cell imaging. The FLIM analysis provided a method for acquiring FRET measurements that is independent of the intensity of the fluorescent molecules, since it depends on measurement of the donor fluorescence lifetime in the absence or presence of an acceptor (Wallrabe and Periasamy 2005).

2.11 shRNA Constructs

TripZ shRNA with mature antisense 5'-TAGGATGAAGTTCTCATCT-3' (V3THS_304403) and 5'-TCTGCTGTCTTTGGGACCT-3' (V3THS_304404) target sequences against PPIA (CypA) as well as non-targeting control shRNA controls were purchased from Dharmacon as glycerol stocks. Bacterial cultures were grown with Carbenicillin (100 µg/ml, Fisher Scientific) and subsequently plasmids were purified using the EndoFree^{R} Plasmid Maxi Kit (Qiagen) according to the manufacturer's instructions. Purified plasmid DNA were transfected in HEK 293T cells using Lipofectamine 3000 (Life Technologies) according to the manufacturer's instructions.

2.12 Lentivirus production and transduction

Transfection of TripZ shRNA or control shRNA was performed with necessary components to produce/collect virus 48 hours post-transfection according to Dharmacon protocol. MCF7 and T47D cells were infected with filtered virus and puromycin selected 48 hours post infection according to Dharmacon instructions. Following antibiotic selection, Doxycycline at a concentration of 1 µg/ml was used for induction of TurboRFP/shRNA expression.

2.13 Cell viability

Trypan blue exclusion method as described (Strober 2001) was used to assess cell viability. 1×10^5 cells were plated and cultured for 24 hours, and then serum starved for overnight. Cells were treated with PRL and/or NIM811 in indicated concentrations for the indicated timepoints. DMEM, along with trypsinized cells were centrifuged at 200 x g, and then resuspended in PBS and Trypan blue. Cells were counted on a Countess

Automated Cell Counter (Invitrogen) and the percentage of live cells was calculated. Cell viability was measured in the same manner for the duration of the experiment.

2.14 Wound healing assay

MDA231 and T47D confluent cell monolayers were wounded with a p200 pipette tip and cultured in serum-free medium in the presence of various concentrations of NIM811. Representative images of the wound closure assay were acquired with a phase-contrast microscope at indicated times. The wound areas were measured using Image J and the percentage of the wound closed was calculated.

2.15 Boyden chamber migration assay

MDA231 and T47D cells were placed in the top of a trans-well chamber with 8 µm pore positron emission tomography membranes in the presence of various doses of NIM811. Serum free media (SFM) with 2% FBS medium was placed in the bottom of the chamber. After 24 hours, the number of cells migrating to the lower surface of the membrane was quantified by CyQuant^{TM} (Invitrogen).

2.16 Soft agar colony formation assay

A bottom agar was prepared by solidifying 1 mL of 0.8% SeaPlaque agarose (BioWhitaker) in 10 % FBS-containing growth media in each well of a 6-well plate. The bottom agar was overlaid with 800 µl of a 0.45% top agar mixture containing 10,000 cells per well in the presence of various concentrations of NIM811. The plates were incubated at 37°C for 14 (MDA231 cells) to 21 (T47D cells) days, colonies were counted using a light microscope with an ocular grid. Only colonies (≥50 µm) were counted with Image J software. Five random fields were counted for each well and the average number of colonies per well is shown.

2.17 RNA extraction, cDNA synthesis, and qRT-PCR

T47D or MCF7 cells were plated in 10 cm plates as described in the later section and treated with DMSO (0.1%) control, PRL (250 ng/mL in 0.1% DMSO), and/or NIM811 (10 ug/ml in 0.1% DMSO). After treatments, cells were washed with PBS and mRNA was isolated with a PureLink^{TM} RNA Mini Kit (Invitrogen/Fisher Scientific) according to manufacturer's instructions. cDNA was synthesized using 1 µg of total mRNA with iScript^{TM} Reverse Transcription Supermix (BioRad) according to the manufacturer's protocol. qPCR was performed using iTaq^{TM} Universal SYBR^{R} Green Supermix (Biorad), 20 ng of DNA, and 1 nmol/L primers listed in Table 2 using a BioRad CFX96 Real-Time PCR thermocycler. Data were normalized to GAPDH and fold changes were represented as $2^{-\Delta\Delta Ct}$ ($2^{-(CtTarget-CtGAPDH)PRL-(CtTarget-CtGAPDH)Control}$) using untreated DMSO control as a baseline.

Gene Name	Primer	Sequence (5' to 3' direction)
CISH	Forward	5'- AGAGGAGGATCTGCTGTGCAT-3'
	Reverse	5'- GGAACCCCAATACCAGCCAG-3'
CCND1	Forward	5'-CCGTCCATGCGGAAGATC-3'
	Reverse	5'- GAAGACCTCCTCCTCGCACTT-3'
GAPDH	Forward	5'- CATGAGAAGTATGACAACAGCCT-3'
	Reverse	5'- AGTCCTTCCACGATACCAAAGT-3'

Table 2. qRT-PCR primers. These primers were used to measure mRNA levels of CISH, CCND1, and GAPDH in T47D cells using qRT-PCR methods as described in this chapter.

2.18 Cell culture and RNA Isolation for microarray

Differential gene expression was assessed in T47D cells treated with DMSO (0.1%) control, PRL (250 ng/mL in 0.1% DMSO), and/or NIM811 (10 ug/ml in 0.1% DMSO). Prior to RNA isolation, T47D cells were plated at 60% confluency in 10-cm plates and incubated for 24 hours in complete media followed by an additional 24 hours of serum starvation in DMEM (Life Technologies) and 1X ITS Liquid Media Supplement (Sigma Aldrich). Cells were then treated for 4 hours with either DMSO or NIM811 before stimulation with PRL. After 2-hour PRL stimulation, cells were washed with PBS and RNA was extracted using the MagMAX-96 for Microarrays Total RNA Isolation Kit (Invitrogen, Life Technologies) in an automated fashion using the magnetic particle processors MagMAX Express. RNA purity was judged by spectrophotometry at 260, 270, and 280 nm. RNA integrity was assessed by running 1 µl of every sample in RNA 6000 Nano LabChips on the 2100 Bioanalyzer (Agilent Technologies).

2.19 Microarray hybridization and Data Acquisition

Each of the four cell treatment conditions (PRL^-/DMSO, PRL^+/DMSO, PRL^-/NIM811, PRL^+/NIM811) were assessed in three independently grown biological replicates. Each of the 12 RNA samples were hybridized in duplicate to two Human Genome U133A 2.0 Arrays (Affymetrix, Santa Barbara, CA) according to the Affymetrix protocol as previously described (Dumur, Nasim et al. 2004) with modifications : Starting with 500 ng of total RNA, we performed a single-strand cDNA synthesis primed with a T7-(dT24) oligonucleotide. Second strand cDNA synthesis was performed with *E. coli* DNA Polymerase I, and biotinylation of the cRNA was achieved by *in vitro* transcription (IVT) reaction using the GeneChip 3' IVT Express Kit (Affymetrix, Santa Clara, CA). After a

37°C-incubation for 16 hours, the labeled cRNA was purified using the cRNA cleanup reagents from the GeneChip Sample Cleanup Module. As per the Affymetrix protocol, 10 µg of fragmented cRNA were hybridized on the GeneChip HG U133A 2.0 Arrays (Affymetrix Inc., Santa Clara, CA) for 16 hours at 60 rpm in a 45 °C hybridization oven. The arrays were washed and stained using the GeneChip Hybridization, Wash, and Stain Kit in the Affymetrix fluidics workstation. Every chip was scanned at a high resolution, on the Affymetrix GeneChip Scanner 3000 7G according to the GeneChip Expression Analysis Technical Manual procedures (Affymetrix, Santa Barbara, CA). After scanning, the raw intensities for every probe were stored in electronic files (in *.DAT* and *CEL* formats) by the GeneChip Operating Software v1.4 (GCOS) (Affymetrix, Santa Barbara, CA).

2.20 Differential Expression Analysis

The 12 resultant *.CEL* files were analyzed using the R statistical computing language and environment (RDevelopmentCoreTeam 2011). The data quality of each microarray was assessed by examining the average background, percent of probe sets called present by the MAS5 detection call algorithm (Gautier, Cope et al. 2004), and the 3':5' ratio for GAPDH and ACTIN. Additionally, to detect potential spatial artifacts resulting from sub-optimal hybridization conditions, probe level linear models were fit using the R Bioconductor package, "affyPLM", and plots of the residuals were examined for each microarray (Bolstad, Collin et al. 2004, Gentleman, Carey et al. 2004). Differential expression was assessed using the "affy" and "limma" Bioconductor packages (Gautier, Cope et al. 2004, Smyth 2004). Briefly, probesets were quantile normalized and processed by the Robust Multi-Array Average (RMA) algorithm (Irizarry, Bolstad et al.

2003) before the manufacturer's control probesets and probesets considered "absent" in 20 or more arrays by the MAS5 algorithm were filtered from all arrays (Archer and Reese 2010). Differential expression between treatment conditions was then assessed via moderated t-test adjusted for multiple hypotheses by the Benjamini & Hochberg method (Benjamini and Hochberg 1995). The false discovery rate (FDR) was controlled so that only those probesets with $q < 0.01$ were deemed significant.

Hierarchical clustering was performed on the top 100 prolactin induced and top 100 prolactin inhibited genes (as defined by the expression fold change between the PRL^+ / DMSO and PRL^- / DMSO microarrays) utilizing the GenePattern public server (Eisen, Spellman et al. 1998, de Hoon, Imoto et al. 2004, Reich, Liefeld et al. 2006). Microarrays were clustered using a pairwise average-linkage method and Pearson correlation as the similarity metric; genes were not subjected to clustering and are presented as originally ordered in the *.GCT* file supplied to the module.

STAT5 target genes were defined as previously reported (Kang, Yamaji et al. 2014) and include genes flanked by the classic STAT5 palindromic repeat binding motif as well as those identified by Kang et al. utilizing STAT5 ChIP-seq on murine mammary tissues at parturition. After converting *Mus musculus* gene annotations to human gene symbols, 847 unique genes comprised the STAT5 target gene list used in this study.

2.21 Mouse MMTV-PyMT model

For tumorigenesis studies, the well-recognized mouse model of transgene-driven tumorigenesis (Muller, Sinn et al. 1988) utilizing the MMTV-driven expression of polyomavirus middle T in mouse mammary glands was employed. PyMT mice were

purchased from The Jackson Laboratory (Jax; Stock # 002374, FVB/N-Tg(MMTV-PyVT)634Mul/J). Hemizygous males were cross-bred with our transgenic CypA$^{-/-}$ female mice (Volker, Hedrick et al. 2018) until a PyMT^{+}/CypA$^{+/-}$ male mouse was obtained. This sire was then bred with the Cyp A$^{-/-}$ dams until PyMT^{+}/Cyp A$^{+/-}$ and PyMT^{+}/CypA$^{-/-}$ females were obtained for use in this study. Tumors were monitored beginning at weaning until time of dissection via palpating and calipering. Mice were kept in accordance to an approved IACUC protocol which allowed the humane endpoint of when any single tumor reached 17 mm in diameter. Dissection timepoints included 5 weeks (hyperplasia phase in PyMT model), 8 weeks (adenoma phase), 11 weeks (invasive carcinoma phase), and 13 weeks (distal metastasis phase). Mice were weighed and whole mammary glands containing tumors were calipered upon dissection. Lungs and lymph nodes were weighed and collected as well. Following fixation with 10% buffered neutral-buffered formalin, tumors were paraffin-embedded and processed for histologic and histochemical analyses.

2.22 Mouse xenograft model

Four to six weeks old female nude mice were used for *in vivo* xenograft study. To assess the effects of NIM811 on the xenograft growth, MDA231 cells (0.5×10^{6}) were suspended in Matrigel and injected into the teat of the fourth abdominal mammary gland of nude mice as described (Harrell, Dye et al. 2006). When tumor volume reached 80-100 mm^3, animals were randomized into three groups and were treated with vehicle control (olive oil) and NIM811 (20 mg/kg/day, 50 mg/kg/day) for 4 weeks by twice-daily gavages. Tumor growth was measured by weekly caliper measurement using the formula length × breadth^2/2. At the end of experiment, mice were sacrificed. The entire primary mammary

tumors were removed and weighed. In addition, all visceral organs, bones, brain, and superficial lymph nodes were harvested for microscopic examination for metastasis. Metastases were detected by the presence of mCherry fluorescent protein using a Zeiss SteroDiscovery. V12 fluorescence dissecting microscope with an AxioCam MRm digital camera. One half of each tumor and other organs were fixed, embedded, sectioned, and stained with H&E. The necrotic area of the primary tumor was quantified by morphometric determination of the proportion of total tumor area that was necrotic in H&E staining section.

2.23 Histology

All mammary glands, lymph nodes, and lungs were fixed in 10% neutral buffered formalin (NBF) for 24-48 hours, depending on the size of the tumor, and then stored in 70% ethanol at 4 degrees. All tissues were processed together to form FFPE blocks and sectioned into 5 µm thick sections by the VCU Cancer Mouse Models Core facility (CMMC) as well as Anatomic Pathology Research Services (APRS) at VCU Health for histological analysis by hematoxylin, Gill no. 3 (cat. # GHS332, Sigma Aldrich, St. Louis, MO) and eosin Y (cat. # 318906, Sigma Aldrich, St. Louis, MO) staining as described (Volker, Hedrick et al. 2018). Positivity of lymph nodes was determined as lymph nodes containing at least 1 macrometastasis. FFPE hematoxylin and eosin stained axillary and accessory axillary lymph nodes were assessed for the presence of metastases by CVC, a board-certified pathologist, under brightfield microscopy. Slides were then scanned by the NanoZoomer RS Digital Slide Scanner (Hamamatsu Photonics, Bridgewater, NJ) to analyze scanned images using the NDPview2 software (Hamamatsu Photonics). Lung metastases were counted, and borders of metastases drawn as free ROIs in this

software. The software calculated a scaled area from the scanned image metadata. Areas of each metastasis as well as each lung lobe were totaled for each mouse. The metastasis area was normalized to total lung area and expressed as a percent to account for the anatomical size difference of the organs between the WT and KO mice (Volker, Hedrick et al. 2018).

2.24 Statistical Analysis

Statistical analysis was performed using appropriate statistical methods including the Student's t-test, one- and two-way ANOVA and parametric tests using GraphPad Prism V7.Og (GraphPad Software, Inc.) and JMP version 12.0. The data are shown as the mean with error bars showing ± SEM. Statistical significance indicated as *P < 0.05, **P < 0.01, *** P < 0.005 and ****P < 0.001.

Chapter 3: Results

3.1 Overexpression of PRLr-CFP and PRLr-YFP in HEK 293 cells to assess FRET/FLIM signal

PPIs can function to modulate signal transduction of proteins through their isomerase activity to switch proteins into either an active or inactive conformer (Dunyak and Gestwicki 2016). It has been shown that CypA binds to the P334 residue of the PRLr to mediate its isomerase activity upon PRL binding to the receptor (Zheng, Koblinski et al. 2008). A recent study utilizing FRET strategies with transfectants expressing CFP- and YFP- tagged forms of the growth hormone receptor (GHR, an analog of the PRLr) showed that GHR activation induced a rotational movement in C-terminus of the GHR, resulting in a loss of baseline FRET and activation of Jak2 kinase (Brooks, Dai et al. 2014).

To investigate the mechanisms of CypA regulation of PRL-induced conformational change of the PRLr-ICD, we have generated and successfully expressed cyan- and yellow-fluorescent protein (CFP and YFP) tagged forms of the PRLr-CFP, -YFP pair in HEK 293 (null for PRLr) cells. The PRLr-CFP, -YFP pair was chosen based on their spectral overlap and as this FRET pair was used to study GHR activation (Brown, Adams et al. 2005, Brooks, Dai et al. 2014). Like the growth hormone receptor work, we have used the relatively shorter intermediate-form of the PRLr to measure FRET since the long cytoplasmic tail of the long-form of the PRLr produced low FRET efficiency in our experimental model. A positive-control CV5 (CFP-YFP construct that is attached with a linker), negative-control (vector alone) and PRLr-CFP were used to set FRET signal parameters.

As shown in Fig 3.1A, cell lysates with indicated transfectants were analyzed using a plate reader and FRET ratios were reported as relative fluorescence units. Expression of the

PRLr-CFP, -YFP pair demonstrated highly significant FRET signal compared to the PRLr-CFP alone (Fig 3.1A). To demonstrate the dynamic conformational change in live cells, FLIM techniques/methods were used to measure the percent FRET efficiency. In Fig 3.1B, percent FRET efficiency of live cells with PRLr-CFP only or PRLr-CFP, -YFP pair were assessed based on FLIM. Compared to the PRLr-CFP only condition, expression of the PRLr-CFP, -YFP pair demonstrated significantly higher FRET efficiency (Fig. 3.1B). These data demonstrate that the PRLr-CFP, -YFP pair generates significantly higher FRET ratios/efficiency compared to the control (PRLr-CFP only), indicating the close proximity (< 10 nm) of the intermediate cytoplasmic tails of the prolactin receptor hetero-dimers. Furthermore, FRET signal is only detected in the presence of the PRLr-CFP, -YFP pair as opposed to PRLr-CFP or -YFP transfectant alone.

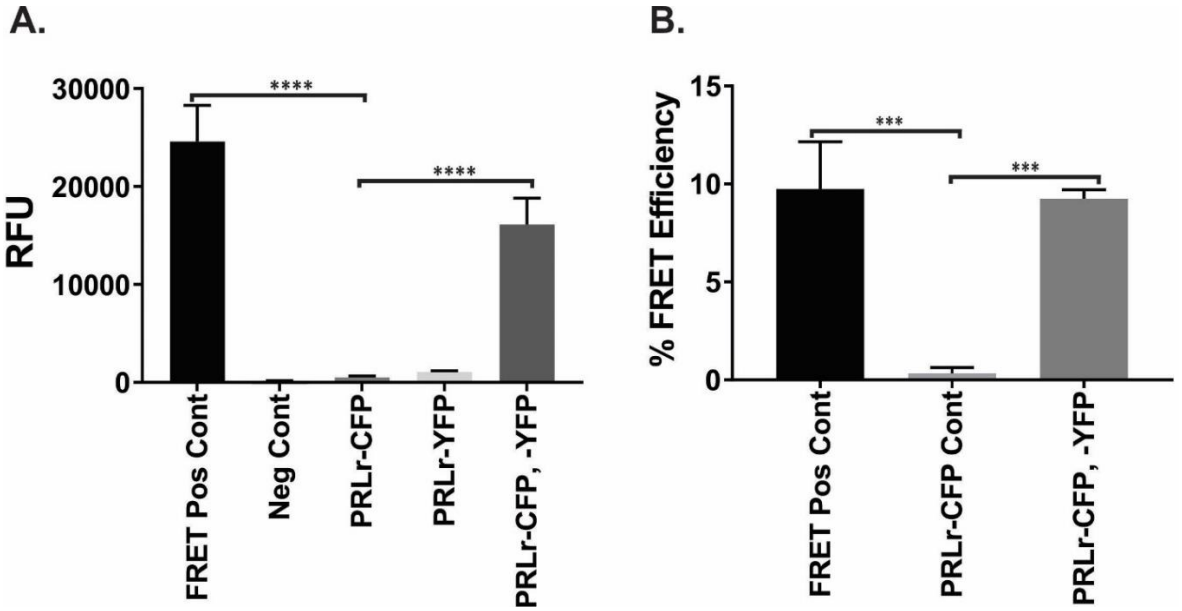


Figure 3.1 FRET/FLIM signals of PRLr-CFP, -YFP pair compared to PRLr-CFP alone. (A) Negative (neg) Control (Cont) or positive (pos) cont or PRLr-CFP or PRLr-YFP or both (PRLr-CFP, -YFP) were transfected in HEK293 cells. Cell lysates were analyzed as described in Methods to assess FRET signal. FRET ratio was calculated for each condition and reported as relative fluorescence units (RFU). *Columns,* mean of three independent experiments; error *bars,* SEM. ****P < 0.001. **(B)** The indicated constructs were transfected as in panel A. Percent efficiency of live cells FRET signal was assessed using FRET/FLIM as described in Methods. *Columns,* mean of three independent experiments; *error bars*, SEM. ***P < 0.005.

3.2 NIM811 inhibits Jak2 phosphorylation and conformational change of the PRLr based on FRET

Based on our previous and current data (Fig. 3.1), we hypothesized that CypA isomerase activity on the proline residue of the PRLr leads to conformational/rotational change of the intracellular domain of the PRLr. To assess expression and functions of the PRLr-CFP, -YFP heterodimer, we transiently transfected the PRLr-CFP, -YFP pairs and then treated with PRL and/or NIM811. As demonstrated in Fig. 3.2A, PRLr-CFP, -YFP pairs expressed similarly in all three conditions. Inhibition of CypA PPIase activity by NIM811 decreased phosphorylation of Jak2 in a PRL dependent manner (Fig 3.2A), demonstrating functions of these PRLr FRET pairs. To demonstrate the significance of CypA PPI activity in the conformational change of the PRLr-ICD, receptor heterodimers were pre-treated with NIM811 and stimulated with PRL. In Figure 3.2B, PRL stimulation of CFP-, YFP-tagged PRLr pairs resulted in a significant loss of FRET ratio/signal around 70%. In contrast, treatment with NIM811 resulted in a return of FRET signal at approximately 50% in the presence of PRL. These data indicate that NIM811 blocks ligand-induced conformational change of the PRLr-ICD, through its inhibition of CypA PPI activity.

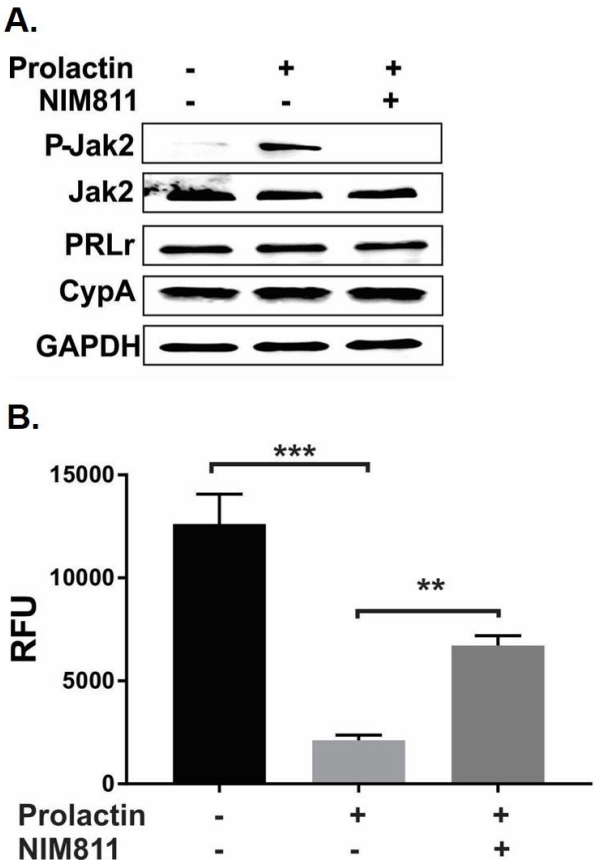


Figure 3.2 NIM811 inhibits PRL induced phosphorylation of Jak2 and conformational change of the PRLr-intracellular domain. (A) Both PRLr-CFP and PRLr-YFP transfected and treated with -/+ PRL in the presence or absence of NIM811. Cell lysates were probed with indicated antibodies. **(B)** Constructs were transfected and treated as indicated in panel A. Cell lysates were analyzed, and FRET ratios were calculated and reported as RFU as described in methods. *Columns,* mean of three independent experiments; *error bars,* SEM. ** $P < 0.01$, *** $P < 0.005$.

3.3 CypA inhibition blocks PRL induced conformational change of the PRLr assessed by FRET/FLIM

To further investigate the dynamic conformational change of the PRLr induced by PRL over time, we have used live cell FRET/FLIM strategies as described in the Methods. As performed earlier, PRLr-CFP, -YFP pairs were transiently transfected and pre-treated with NIM811 and/or stimulated with PRL. As shown in Fig. 3.3A, PRL-induced PRLr-CFP, -YFP expressing cells generated markedly reduced FRET signal compared to the unstimulated condition, whereas, NIM811 treatment in the presence of PRL returned FRET signal closer to the unstimulated state. Quantification of the FRET images using FLIM methods further demonstrate that PRL induced activation of PRLr-CFP, -YFP heterodimers resulted in a significant loss of percent FRET efficiency at approximately 70% (separation of cytoplasmic tails) compared to the uninduced condition, whereas treatment with NIM811 returned the FRET efficiency to around 50% in a PRL dependent manner (Fig. 3.3B). The partial return of FRET efficiency in Figs. 3.2B and 3.3B may be due to a compensatory role of other PPIAse(s) like PIN1(Shen, Esnault et al. 2009) in inducing conformational change of the PRLr. These data demonstrate that NIM811 inhibits PRL-induced conformational change of the PRLr based on FRET/FLIM efficiency.

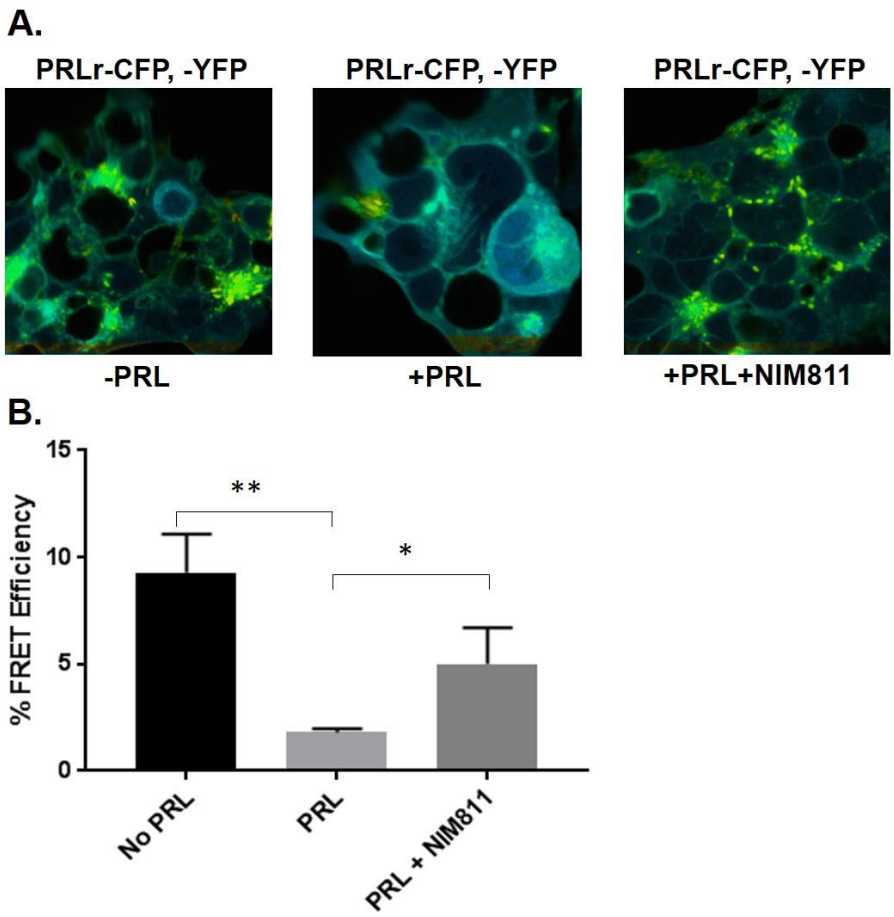


Figure 3.3 CypA inhibition blocks PRL induced conformational change of the PRLr-intracellular domain in live cells. Fluorescence resonance energy transfer (FRET) of HEK 293 Cells. **(A)** Representative confocal images showing FRET signals of HEK 293 cells with both PRLr-CFP and PRLr-YFP transfectants pre-treated with NIM811 and/or PRL. **(B)** Quantification of FRET efficiency was determined using live cell FRET/FLIM as described in the Methods and presented as percent FRET efficiency. *Columns,* mean of three independent experiments; *error bars,* SEM. *, P< 0.05 **, P< 0.01.

3.4 Knockdown of CypA increases FRET signals of the PRLr receptor pairs in a ligand dependent manner

In addition to the pharmacological inhibition of CypA as seen in Figs. 3.2 and 3.3, we assessed whether CypA knockdown would modulate FRET signal, which is a measure of the conformational change of the PRLr as an independent control. We used CypA siRNA to knockdown CypA expression in HEK 293 cells (Fig. 3.4A). As demonstrated in Fig. 3.4A, CypA siRNA effectively knocked down CypA protein expression compared to control siRNA. As observed with NIM811 inhibition of CypA, we hypothesized that CypA knockdown would also block prolactin induced conformational change of the PRLr. Indeed, PRL significantly induced conformational change in the PRLr-ICD, which led to a significant decrease in FRET ratio/signal compared to the unstimulated condition (Fig 3.4B). However, knockdown of CypA resulted in a return of FRET signal at approximately 95% in a PRL dependent manner (Fig. 3.4B). Compared to the NIM811-inhibited condition (Fig. 3.2), the return of FRET is more robust in the knockdown condition (Fig. 3.4), indicating that CypA may have other functions in addition to PPIase activity for regulating conformational change of the PRLr. Together, these data demonstrate that loss of CypA blocks PRL-induced conformational change of the PRLr.

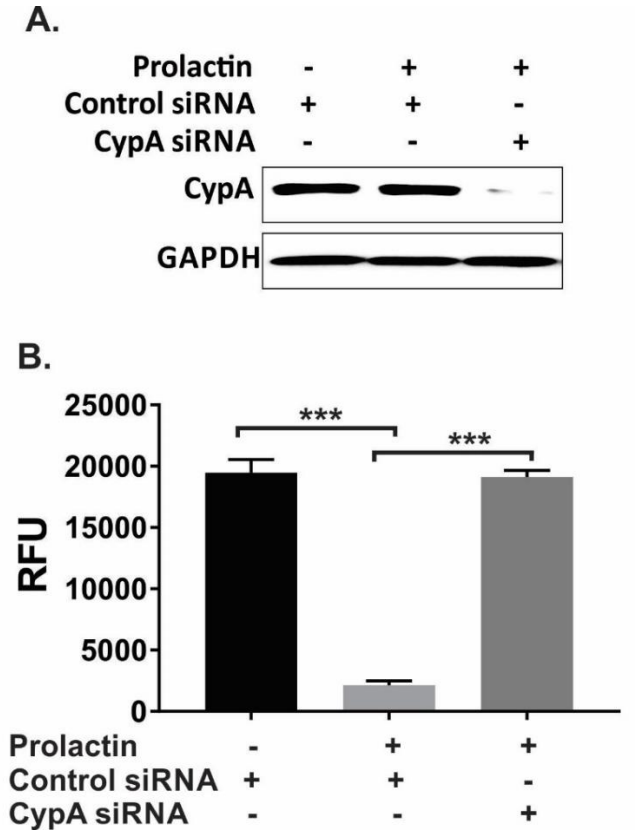


Figure 3.4 siRNA mediated knockdown of CypA significantly blocks PRL-induced conformational change in the PRLr-intracellular domain. (A) Control siRNA or CypA siRNA was transfected, and concurrently both PRLr-CFP and PRLr-YFP were also transfected in HEK 293 cells as per protocols described in the Methods. Following transfection, cells were pre-treated with NIM811 and/or PRL. Cell lysates were probed with anti-CypA or anti-GAPDH. **(B)** Constructs were transfected and treated as indicated in panel A. Cell lysates were analyzed using a plate reader, and FRET ratios were calculated and reported as RFU as described in the Methods. *Columns,* mean of three independent experiments; *error bars,* SEM. (*** $P < 0.005$).

3.5 Loss of CypA impedes PRL induced conformational change of the PRLr assessed by FRET/FLIM

To further investigate CypA knockdown effects in the dynamic conformational change of the PRLr induced by PRL over time, we utilized live cell FRET/FLIM strategies as previously described. PRLr-CFP, -YFP pairs were transiently transfected in cells having control siRNA or CypA siRNA in -/+ PRL conditions. As shown in Fig. 3.5A, PRL stimulation of PRLr-CFP, -YFP expressing cells generated markedly reduced FRET signal/intensity compared to the unstimulated control siRNA condition. However, siRNA knockdown of CypA returned FRET signal close to the levels of the unstimulated state (Fig 3.5A). In Fig. 3.5B, quantification of the FRET images using FLIM methods further demonstrated that PRL induced activation of the PRLr-CFP, -YFP pairs resulted in a significant loss of percent FRET efficiency (separation of cytoplasmic tails), whereas CypA knockdown returned the FRET efficiency to a similar level of the uninduced condition (Fig. 3.5B). Taken together, these results demonstrate that CypA knockdown significantly impedes PRL-induced conformational change of the PRLr assessed by FRET/FLIM.

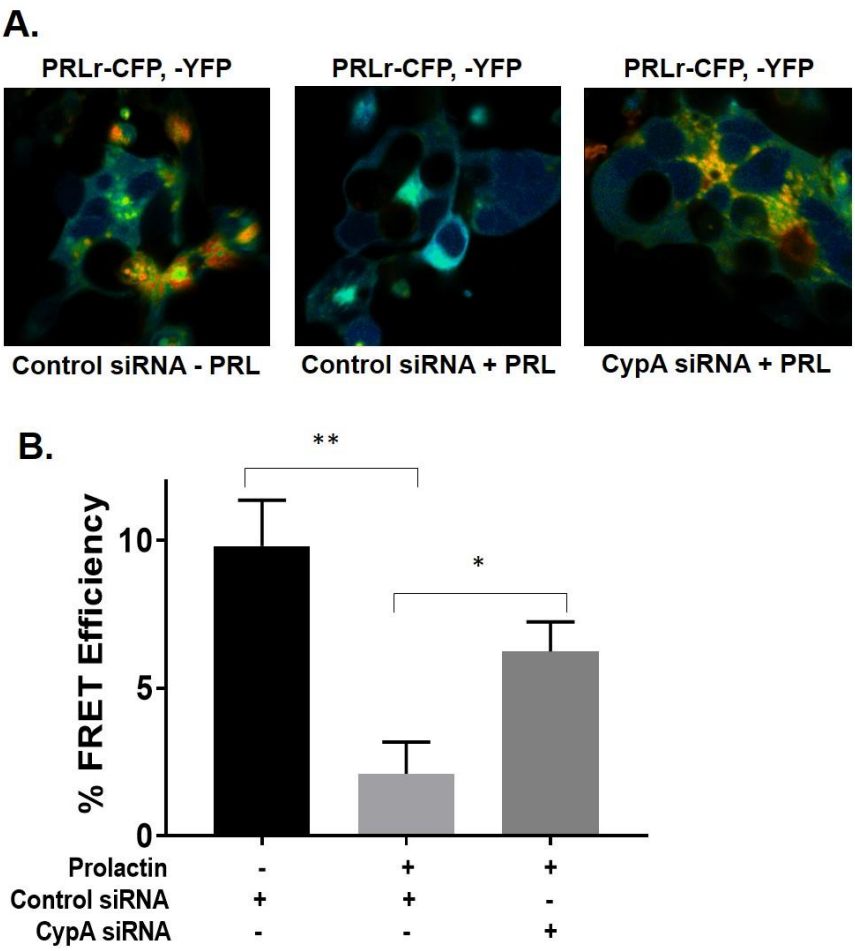


Figure 3.5: CypA knockdown blocks PRL induced conformational change of the PRLr-intracellular domain. (A) Fluorescence resonance energy transfer (FRET) of HEK 293 Cells. Representative confocal images showing percent FRET efficiency of HEK 293 cells with both of the PRLr-CFP, PRLr-YFP transfectants in either control siRNA or CypA siRNA condition(s) as treated with -/+ PRL and/or NIM811. **(B)** Quantification of FRET efficiency was determined using live cell FRET/FLIM as described in the Methods and presented as percent FRET efficiency. *Columns,* mean of three independent experiments; *error bars,* SEM. *, P< 0.05 **, P< 0.01.

3.6 NIM811 inhibits phosphorylation of PRLr-Stat5 signaling intermediates

Based on recent studies, CypA has been found to be a constitutively interacting protein with PRLr and Jak2 (Leonard and O'Shea 1998, Zheng, Koblinski et al. 2008). Inhibition of CypA activity with the immunosuppressive PPI inhibitor CsA in breast cancer cells blocked Jak2 and Stat5 phosphorylation (Zheng, Koblinski et al. 2008). Herein, we assessed the consequences of the CypA mediated conformational change of the PRLr on phosphorylation/activation of the receptor proximal signaling intermediates. Phospho-specific anti-PRLr, Jak2, and Src antibodies were utilized to test the effects of NIM811 on the PRL-induced phosphorylation given their significance to PRLr-associated signaling. To that end, ER/PR$^+$ T47D breast cancer cells were pre-treated with NIM811 or DMSO and then stimulated as a function of time with PRL. The effects of NIM811 on tyrosine-phosphorylation of PRLr, Jak2, Src and Stat5 were significant (Fig. 3.6A). In Fig. 3.6B, the quantitative assessment of western blots (band intensity) in Fig. 3.6A, revealed a significant decrease in tyrosine-phosphorylation of the PRLr and the PRLr-associated signaling machinery in NIM811 pre-treated, PRL stimulated breast cancer cells. In Fig. 3.6, there were marked increases in pJak2-Y1007/1008 within 7.5-15 minutes following PRL stimulation, however a 3-5-fold inhibition of p-YJak2 was observed with NIM811 treatment as a function of time (Fig. 3.6B), confirming the importance of CypA-PPI activity regulating Jak2 activity in breast cancer cells. Following PRL stimulation, there were also significant increases in phosphorylation of PRLr itself, at residues 381 and 587 which are thought to play roles in the PRLr engagement of Src and Stat5, respectively (Pezet, Ferrag et al. 1997, Brooks 2012) within 7.5-15 minutes, while a concomitant 2-5-fold decrease in phosphorylation with the treatment of NIM811 was observed as a function of

time (Fig. 3.6B). Src is an associated kinase of the PRLr/Jak2 complex and Src-Y416 residue is involved in Src activation. In Fig. 3.6, a marked increase in Src phosphorylation within 7.5-15 minutes of PRL stimulation with a concomitant >3-fold decrease in phosphorylation were observed when treated with NIM811. Stat5 tyrosine phosphorylation is mediated by the Jak2 tyrosine kinase at tyrosine residue 694 (Clevenger 2004). Following PRL stimulation, Stat5 was significantly Y-phosphorylated within 7.5-30 minutes while demonstrating an associated 5-7-fold decrease in the levels of p-Y694 with NIM811 treatment as a function of time (Fig 3.6). Taken together, these data indicate that CypA inhibition decreases phosphorylation/activation of PRLr-Stat5 signaling intermediates that contribute to downstream gene expression and breast cancer cell functions.

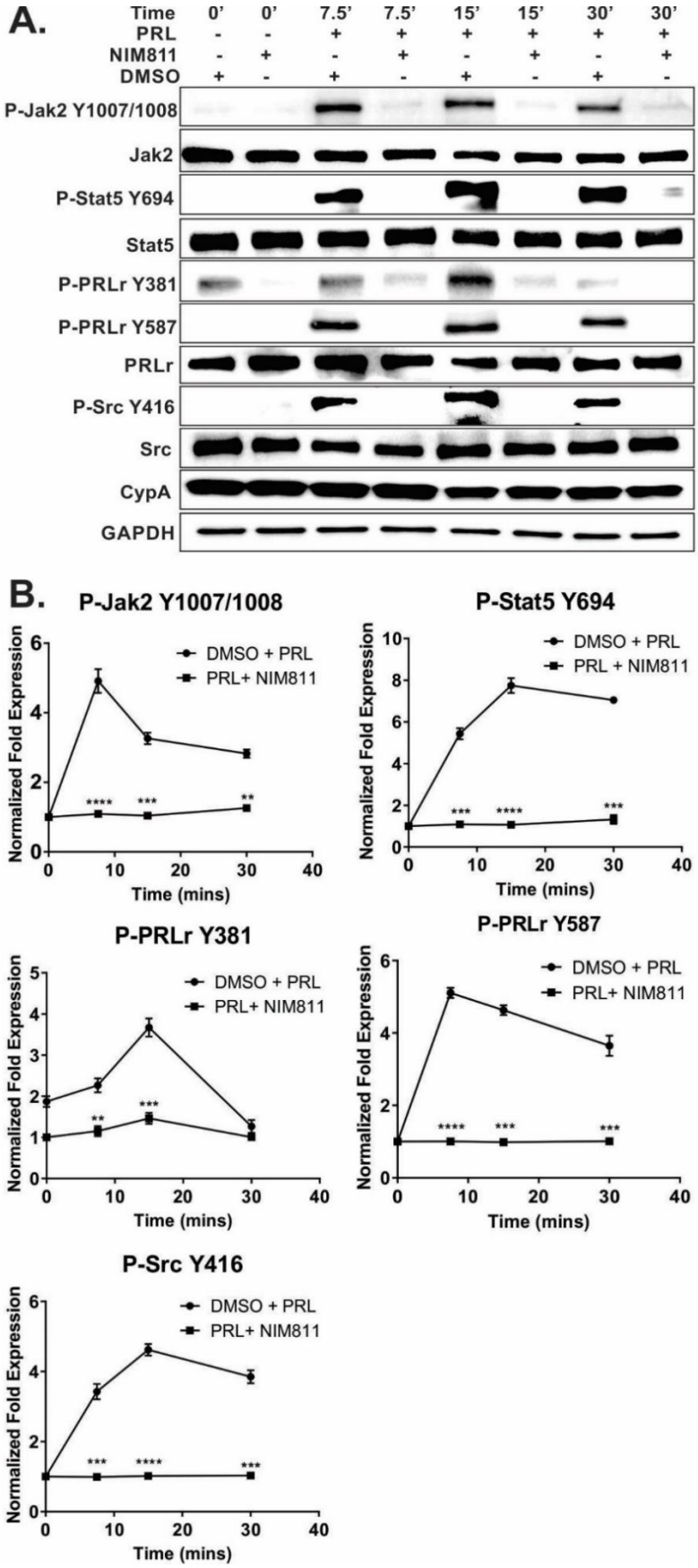

A.
Time 0' 0' 7.5' 7.5' 15' 15' 30' 30'
PRL - - + + + + + +
NIM811 - + - + - + - +
DMSO + - + - + - + -
P-Jak2 Y1007/1008
Jak2
P-Stat5 Y694
Stat5
P-PRLr Y381
P-PRLr Y587
PRLr
P-Src Y416
Src
CypA
GAPDH
B.
P-Jak2 Y1007/1008
P-Stat5 Y694
P-PRLr Y381
P-PRLr Y587
P-Src Y416
DMSO + PRL
PRL+ NIM811
PRL + NIM811
Normalized Fold Expression
Time (mins)

Figure 3.6: CypA inhibition modulates phosphorylation of PRLr-Stat5 signaling intermediates. (**A)** T47D cells were serum starved for 16-24 hours, pre-treated with NIM811 (10 µg/ml) in DMSO or DMSO only for 4-6 hours and/or stimulated with human PRL (250 ng/ml) for the indicated times. Blots were probed with the indicated antibodies. (**B)** Quantification of fold expression of phospho- proteins normalized to total proteins in panel A as indicated. *Lines,* mean of three independent experiments; *error bars* in SEM. **, P< 0.01 ***, P< 0.005 ****, P< 0.001.

3.7 NIM811 inhibits phosphorylation of AKT and MAPK

We found that CypA inhibition by NIM811 significantly reduced phosphorylation/activation of Jak2-Stat5 signaling intermediates (Fig. 3.6). To determine whether CypA inhibition can affect the phosphorylation/activation of the parallel/downstream molecules of the Jak2-Stat5 pathway, we assessed phosphorylation of Akt and MAPK in response to PRL and/or NIM811 as a function of time. In Fig. 3.7A-B, there was a marked decrease of around 5-fold in phosphorylation of Akt at 30 mins of PRL stimulation when pre-treated with NIM811. Furthermore, there were also a significant reduction of 6-7-fold in phosphorylation of MAPK both at 15 and 30 mins of PRL stimulation when pre-treated with NIM811 (Fig. 3.7A & C). These data indicate that CypA regulates the PI3K-MAPK signaling pathway in a PRL dependent manner, since inhibition of CypA by NIM811 significantly down-regulated phosphorylation/activation of both Akt and MAPK.

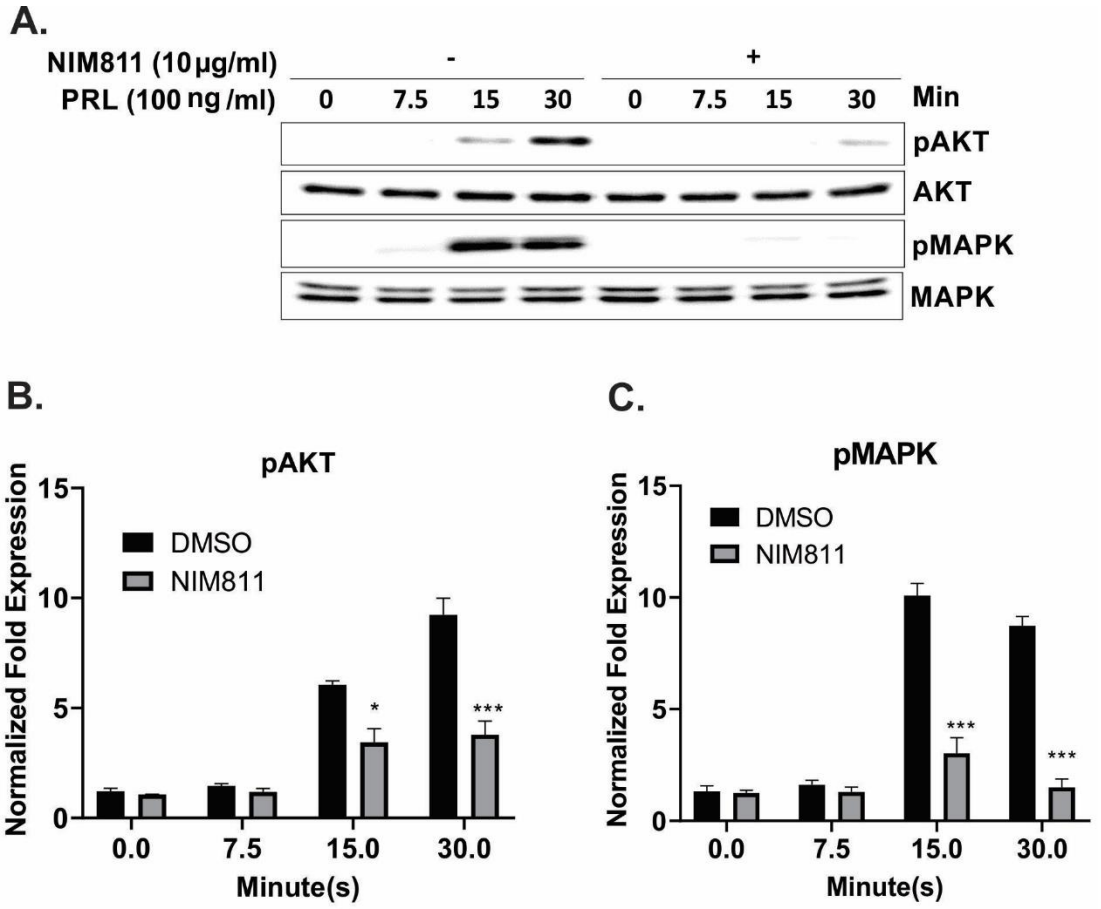


Figure 3.7: NIM811 inhibits phosphorylation of AKT and MAPK. (A) T47D Cells were serum starved for 16-24 hours, pre-treated with NIM811 (10 µg/ml) in DMSO or DMSO only for 4-6 hours and/or stimulated with PRL (250 ng/ml) at the indicated times. Blots were probed with the indicated antibodies. **(B, C)** Quantification of fold expression of phospho-proteins (pAKT and pMAP) normalized to their respective total proteins in panel A as indicated. Quantification of blots in panel A. *Column,* mean of three independent experiments; *error bars* in SEM. *, P< 0.05 ***, P< 0.005.

3.8 Stable knockdown of CypA modulates phosphorylation of PRLr-Stat5 signaling intermediates

Given the effects of NIM811 treatment on breast cancer cell signaling (Fig. 3.6A-B) and PRLr mutant action on Jak2/Stat5 phosphorylation (Zheng, Koblinski et al. 2008), it was reasoned that loss-of-function of CypA should alter phosphorylation/activation of PRLr-Stat5 signaling intermediates as well as verify on target activity of NIM811. Therefore, doxycycline (1 ug/ml) inducible shRNA-mediated molecular knockdown of CypA was performed in ER/PR$^+$ T47D breast cancer cells. Like the pharmacological inhibition of CypA PPI activity by NIM811, loss of its expression in breast cancer cells had significant effects in phosphorylation/activation of PRLr, Jak2, Stat5 and Src in a PRL-dependent manner (Fig. 3.8A). Following PRL stimulation for 15 minutes, pPRLr-Y381 and -Y587 were induced markedly in the shRNA control condition, while a significant decrease of >4-fold in phosphorylation at these sites in the CypA knockdown condition were observed in T47D cells (Fig. 3.8B). As expected, pYJak2 was significantly induced with prolactin stimulation in control cells while >5-fold reduction in pYJak2 levels was observed with the loss of CypA (Fig. 3.8B). Similar to the pSrc-Y416 levels in Fig. 3.6A-B, pSrc-Y416 was significantly induced within 7.5-15 minutes of PRL stimulation in control cells, while demonstrating a >2-fold reduction in phosphorylation in CypA knockdown cells (Fig. 3.8B). Lastly, Jak2-mediated phosphorylation of Stat5 was significantly induced by PRL stimulation, while a >7-fold reduction in phosphorylation was observed in the absence of CypA (Fig. 3.8B). Taken together, loss of CypA significantly decreases phosphorylation/activation of the PRLr-Stat5 signaling intermediates as seen with NIM811 inhibition.

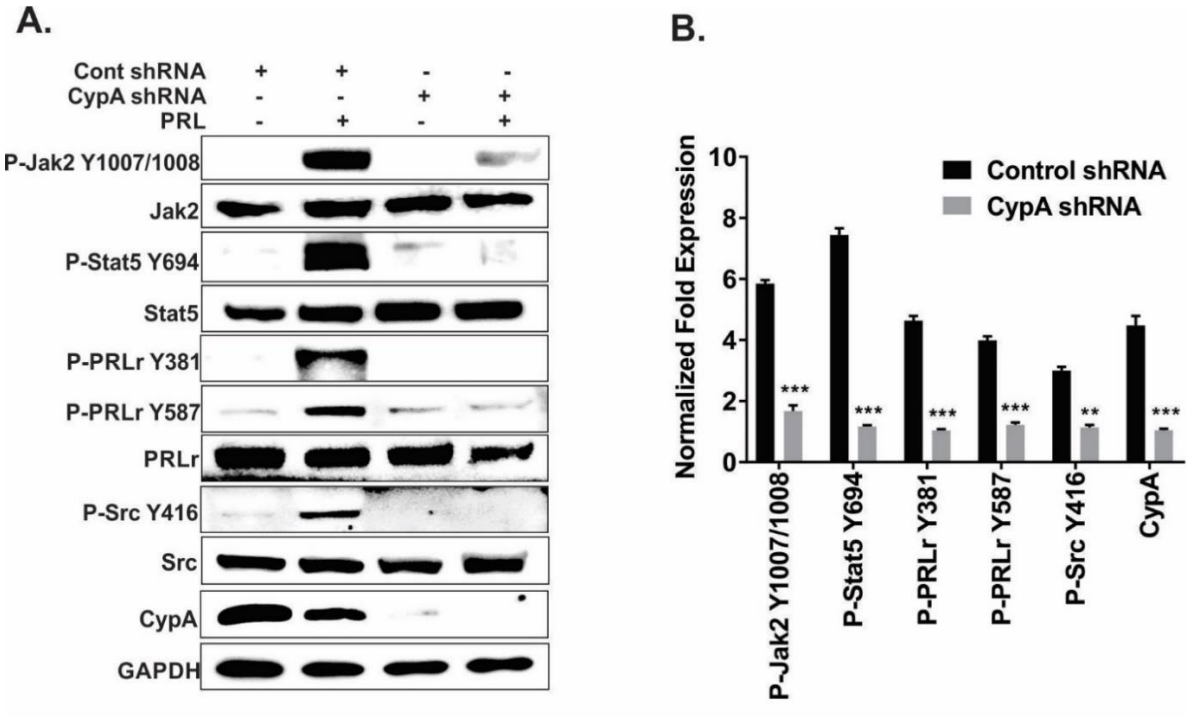


Figure 3.8 Knockdown of CypA modulates phosphorylation of PRLr-Stat5 signaling intermediates. (**A)** T47D cells stably expressing non-silencing control or CypA shRNA was induced by treatment of doxycycline (1 ug/ml) for 48 hours. Cells were serum starved for 16-24 hours, and then stimulated with PRL (250 ng/ml) for 15 minutes. Blots were probed with the indicated antibodies. (**B)** Quantification of fold expression of PRL treated phospho-proteins normalized to their respective total proteins in panel A (column 2 and 4 left to right) as indicated. *Columns,* mean of at least three independent experiments; *error bars,* SEM. **, P< 0.01 ***, P< 0.005.

3.9 Rescue overexpression of CypA modulates phosphorylation of PRLr-Stat5 signaling intermediates

To determine whether the decrease in phosphorylation of PRLr-Stat5 intermediates was directly due to CypA knockdown and not an off-target effect, a rescue overexpression was performed. T47D cells with CypA knockdown were transfected with CypA-WT plasmid (Zheng, Koblinski et al. 2008), and subsequently cell lysates were analyzed as previously described. The rescue overexpression of CypA in the CypA-knockdown cells was adequate to restore p-YPRLr, pYJak2, pYStat5 and pYSrc levels to PRL-induced endogenous levels (Fig. 3.9A-B). The PRL induced activation of PRLr, Jak2, Stat5 and Src were directly correlated to CypA levels by both knockdown and rescue overexpression approaches. Taken together, these data indicate that loss of CypA had profound effects on tyrosine phosphorylation of PRLr-Stat5 intermediates, which was restored by overexpression of a rescue construct.

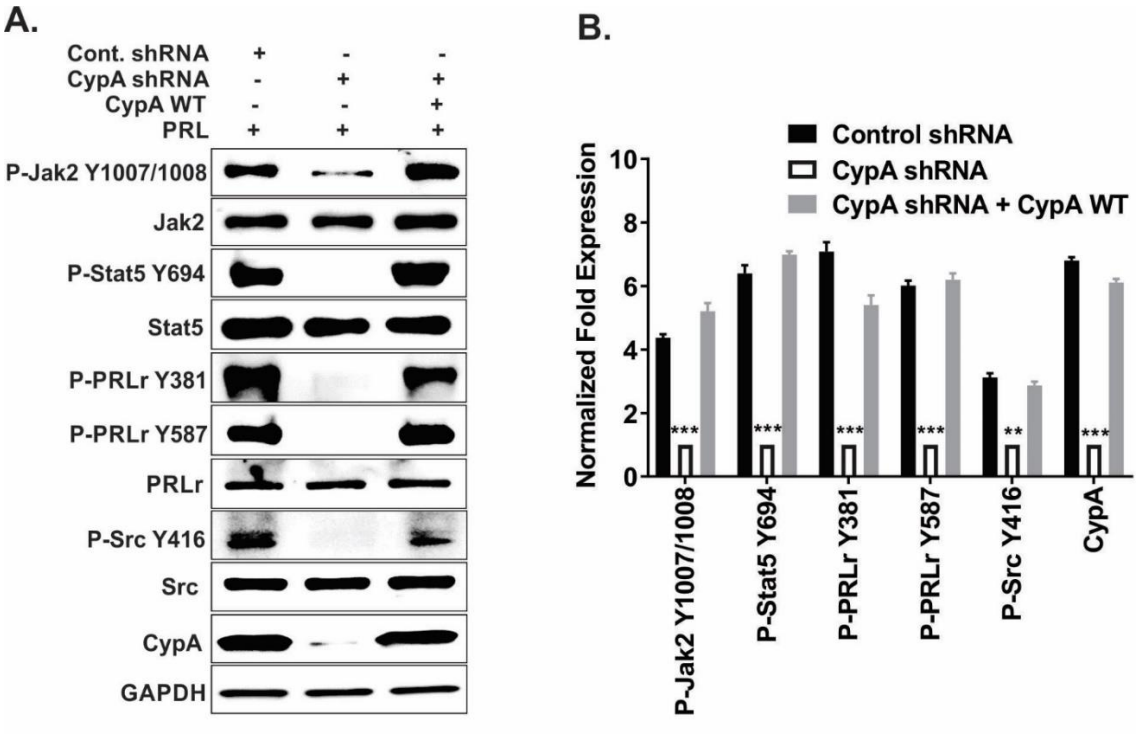


Figure 3.9 Rescue overexpression of CypA restores phosphorylation of PRLr-Stat5 signaling intermediates. (A) Stable expression of non-silencing control or CypA shRNA and/or CypA-WT (overexpression) were transfected in T47D cells. Cells stably expressing non-silencing shRNA control or CypA shRNA was induced by treatment of doxycycline (1 ug/ml) for 48 hours, followed by serum starved for 16-24 hours, then stimulated with PRL (250 ng/ml) for 15 minutes. Blots were probed with the indicated antibodies. **(B)** Quantification of fold expression of phospho-proteins normalized to total proteins in panel A as indicated. *Columns,* mean of three independent experiments: *error bars,* SEM. **, P< 0.01 ***, P< 0.005.

3.10 CypA knockdown impedes association of the proximal proteins to the PRLr and phosphorylation/activation

Considering the inhibition of PRL-induced phosphorylation/activation of Jak2, Src and Stat5 with NIM811 treatment (Fig. 3.6) or CypA knockdown (Fig. 3.8) in breast cancer cells, and PRLr mutant (unable to bind CypA) action on Jak2/Stat5 phosphorylation (Zheng, Koblinski et al. 2008), we sought to investigate whether the knockdown of CypA alters association of Jak2, Src and Stat5 to the PRLr and subsequent phosphorylation/activation of these signaling intermediates. Immunoprecipitation of the PRLr was performed and probed with indicated antibodies in Fig 3.10A. Loss of CypA expression in breast cancer cells had profound effects in PRL-induced phosphorylation/activation of signaling intermediates associated with the PRLr (Fig. 3.10A). Not only phosphorylation, but CypA knockdown blocked PRLr association with Jak2, Src and Stat5 (Fig. 3.8A), but not global expression of these proteins (Fig 3.6, 3.8). As demonstrated, in Fig. 3.8B, quantification of the phosphorylation normalized to the total protein indicated a significant decrease in phosphorylation of PRLr, Jak2, Stat5 and Src associated with the PRLr in CypA knockdown cells compared to the control. Moreover, CypA knockdown also significantly minimized association of Jak2 around 50%, and both Src and Stat5 at around 25% with the PRLr (Fig. 3.10B). These indicate that the function of CypA is important primarily for Jak2 but also for Src and Stat5 association with the PRLr. Overall, loss of CypA impedes association of the proximal molecules to the PRLr and their subsequent phosphorylation.

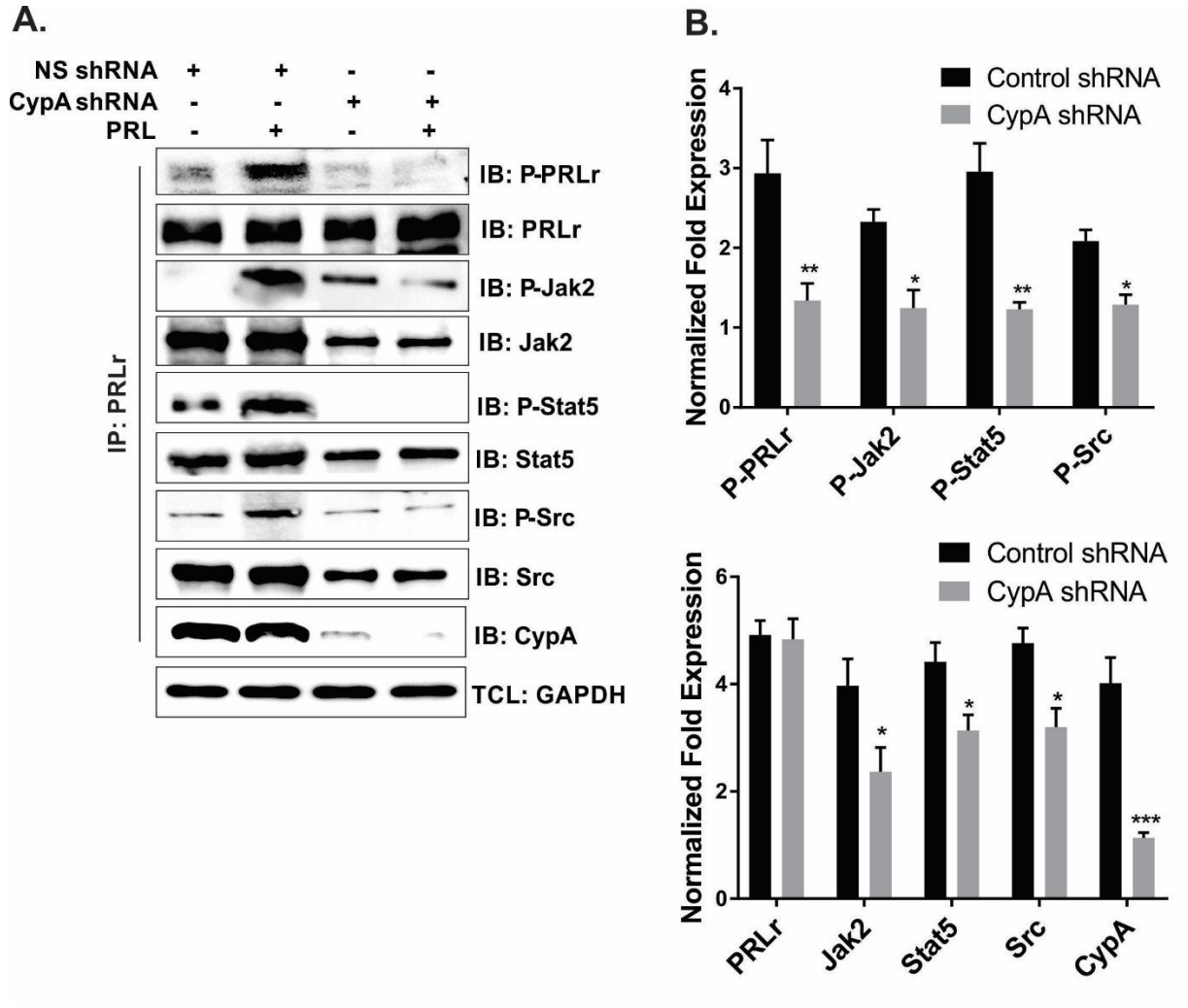


Figure 3.10 Knockdown of CypA modulates phosphorylation of PRLr-Stat5 signaling intermediates and their association with the PRLr. (A) Immunoprecipitation of PRLr in indicated conditions including stable expression of non-silencing control or CypA shRNA in T47D cells and serum starved for 16-24 hours, then stimulated with PRL (250 ng/ml) for 15 minutes. Immunoblots were probed with the indicated antibodies. **(B)** Quantification of fold expression of phospho-proteins normalized to the respective total protein and total proteins normalized to loading control in panel A as indicated. *Columns,* mean of at least three independent experiments; *error bars,* SEM. *, P< 0.05 **, P< 0.01 ***, P< 0.005.

3.11 CypA inhibition decreases mRNA and protein levels of CISH and Cyclin D1

Prolactin and its cognate receptor regulate downstream genes through the Jak2-Stat5 signaling pathway. PRL signaling in breast cancer cells induces several breast cancer relevant genes including; the estrogen receptor (ER), cyclin D1, and CISH (Fang, Rycyzyn et al. 2009). These genes have been implicated in the pathogenesis of breast cancer (Sherr 1996, Raccurt, Tam et al. 2003). Herein, we sought to determine whether CypA inhibition by NIM811, could alter expression of these PRL responsive downstream genes. mRNA and protein expression of CISH and CyclinD1 were assessed in NIM811 pre-treated ER/PR^{+} T47D breast cancer cells utilizing qRT-PCR and western blot analysis respectively. As demonstrated in Fig 3.11A, CISH and Cyclin D1 mRNA levels were significantly down-regulated with NIM811 treatment in a PRL-dependent manner. Furthermore, NIM811 treatment resulted in a highly significant decrease in the excess of 50% in PRL-induced expression of both CISH and Cyclin D1 at the protein level (Fig. 3.11B) based on quantification of their normalized expression (Fig. 3.11C-D). Overall, these findings demonstrate that inhibition of CypA prolyl-isomerase activity directly correlated with PRL-induced gene expression of CISH and Cyclin D1.

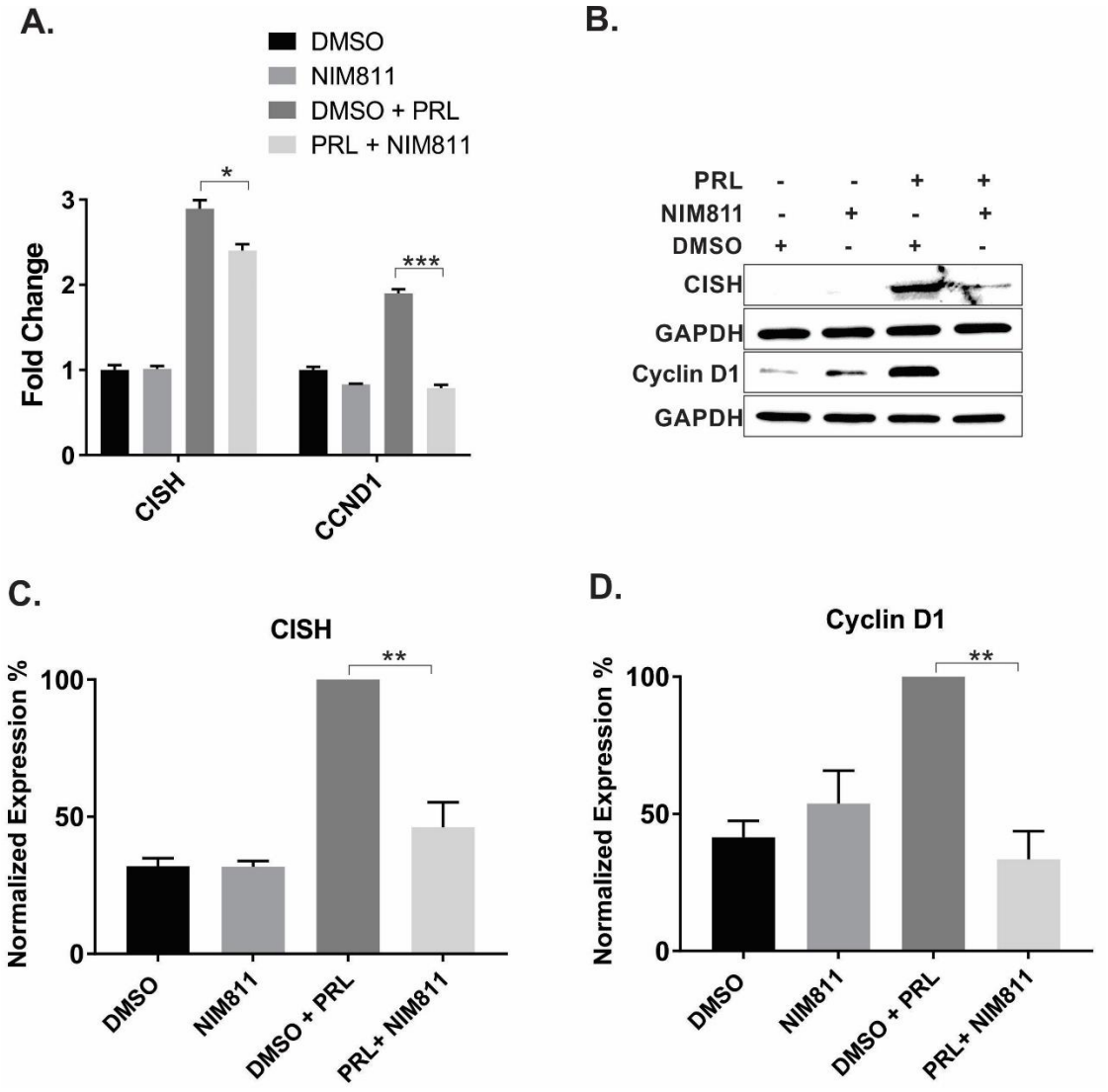


Figure 3.11 mRNA and protein expression of prolactin responsive genes, CISH and Cyclin D1. (A) mRNA expression of CISH and Cyclin D1. T47D cells pre-treated with NIM811 (10 µg/ml) for 4 hours followed by PRL (250 ng/ml) stimulation for 2 hours. **(B)** Protein expression of CISH and Cyclin D1 in T47D cells treated the same as in Panel A**.** Quantification of % expression of CISH **(C)** and Cyclin D1 **(D)** normalized to respective GAPDH in panel B. *Columns,* mean of three independent experiments; *error bars,* SEM. *, P < 0.05 **, P< 0.01 ***, P< 0.005.

3.12 CypA inhibition alters global gene expression in breast cancer cells

In a precedent study, microarray analysis revealed 120 prolactin-induced genes up-regulated by wild-type, but not mutant (mutation in the trans-activation domain) PRLr in T47D cells (Fiorillo, Medler et al. 2013). We reasoned that inhibition of the PPI activity of CypA may alter PRL-induced global gene expression based on its role in structure/function relationships of the PRLr and associated proximal/downstream signaling. To determine the extent to which prolactin and NIM811 altered global gene expression in human breast cancer cells, we analyzed expression microarrays and RNA derived from T47D cells treated with prolactin and/or NIM811. Analysis of the resultant data with a fold change cutoff of 1.2 revealed that PRL induced the expression of 535 genes and inhibited the expression of 372 genes, while NIM811 induced 1,725 genes and inhibited 1,737 genes. NIM811 inhibited expression of the PRLr target genes *CCND1* and *CEBPB* and opposed the expression changes stimulated by PRL on a global scale (Fig. 3.12). NIM811 significantly (FDR<0.01) inhibited 57% of the top 100 prolactin induced genes (Fig. 3.12A) and significantly (FDR<0.01) induced 62% of the top 100 prolactin inhibited genes (Fig. 3.12B). Of the 535 and 372 genes induced and inhibited, respectively, by prolactin, NIM811 significantly (FDR<0.01) inhibited 50% (268/535) and induced 50% (185/372). Using a candidate gene approach with a fold change cutoff of 2.3, we selected/identified top NIM811 inhibited PRL-induced genes including EGR3, AQP3, DUSP6, LIN28A, CCND1, NF1B, KLF4, CTTN, GBP1 and SRF that are implicated in promoting malignant phenotypes including proliferation, growth, migration, invasion, stemness of breast and other cancers (Table 3).

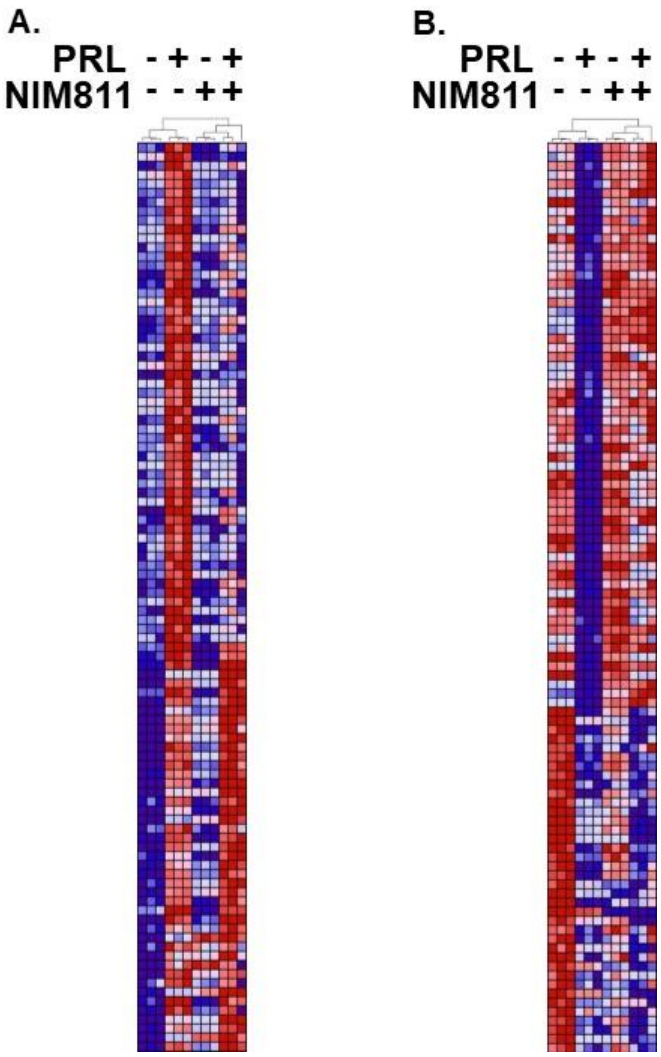


Figure 3.12 NIM811 opposes prolactin induced gene expression globally. (A, B) Heat maps depict hierarchical clustering of the top 100 prolactin induced **(A)** and prolactin inhibited **(B)** genes (represented by rows) identified by microarray analysis. Samples are represented by columns and cluster with biological replicates according to the treatment conditions indicated above each dendrogram (PRL; prolactin). Red and blue represent high and low gene expression values, respectively. Differential expression between treatment conditions was assessed via moderated t-test adjusted for multiple hypotheses by the Benjamini and Hochberg method. The false discovery rate (FDR) was controlled so that only those probesets with $q<0.01$ were deemed significant.

PRL vs. PRL + NIM811				
Gene Symbol	**Fold Change**	**Gene Name**	**Type of Gene**	**Functions**
EGR3	7.5374	Early Growth Response-3	Transcriptional regulator belongs to EGR family	Regulates cellular metabolism and survival in breast cancer
AQP3	6.4463	Aquaporin 3	Membrane transporter protein	Required for CXCL12-induced breast cancer cell signaling and migration
DUSP6	6.4123	Dual-Specificity phosphatase 6	Serine/ Threonine specific protein phosphatase	Promotes growth, migration/invasion of TNBC *in vitro*
LIN28A	4.2193	RNA binding protein	RNA-binding protein and post-translational regulator	Induces EMT and stemness in breast cancer
CCND1	4.0426	Cyclin D1	Cell cycle regulator of G1 to S phase progression	High amplification correlated with poor prognosis for ER+ breast cancer patients
NF1B	3.1323	Nuclear factor I/B	Transcription factor	Overexpressed in human metastatic high grade neuroendocrine lung tumors
KLF4	2.7320	Kruppel-like factor 4	Zinc finger transcription factor	Required for maintenance of breast cancer stem cells, promotes cell migration/invasion
CTTN	2.6310	Cortactin	Monomeric protein promotes polymerization	Cortactin promotes colorectal cancer cell proliferation by activating the EGFP-MAPK
GBP1	2.4208	Guanylate Binding Protein 1	GTPase belong to the dynamin superfamily	GBP1 promotes growth of TNBC and its expression is controlled by EGFR
SRF	2.3793	Serum Response Factor	Transcription factor binds to Serum Response Element	Promotes breast cancer stemness through YAP-IL6 axis

Table 3. NIM811 opposes PRL-induced expression of cancer-promoting genes. List of top hits of NIM811 opposed PRL-induced genes using candidate gene approach with a fold change cutoff of 2.3, extracted from the gene array (Fig. 3.12). The above listed genes including EGR3 (Shajahan-Haq, Boca et al. 2017), AQP3 (Satooka and Hara-Chikuma 2016), DUSP6 (Song, Wu et al. 2015), LIN28A (Liu, Li et al. 2013), CCND1 (Ahlin, Lundgren et al. 2017), NF1B (Semenova, Kwon et al. 2016), KLF4 (Yu, Li et al. 2011), CTTN (Zhang, Liu et al. 2017), GBP1 (Quintero, Adamoski et al. 2017) and SRF (Kim and Lim 2016) are significantly inhibited by NIM811 and have been implicated in promoting malignant phenotypes including proliferation/growth, migration, invasion, and stemness of breast and other cancers.

3.13 CypA inhibition alters Stat5 target genes in breast cancer cells

Our data demonstrated that NIM811 impedes Stat5 phosphorylation and its downstream target including CISH and Cyclin D1 (Figs. 3.6, 3.11). We sought to determine if, like its global effects, NIM811 opposed the actions of prolactin at Stat5 target genes. Indeed, utilizing a previously reported set of Stat5 target genes (Kang, Yamaji et al. 2014), we found that of the 46 Stat5 target genes significantly induced by prolactin with a fold change greater than 1.2, 52% (24/46) were also significantly inhibited by NIM811 (Fig. 3.13A). Of the 26 Stat5 target genes significantly inhibited by prolactin with a fold change greater than 1.2, 62% (16/26) were significantly induced by NIM811 (Fig. 3.13B). These data indicate that NIM811 alters Stat5 target genes in breast cancer cells.

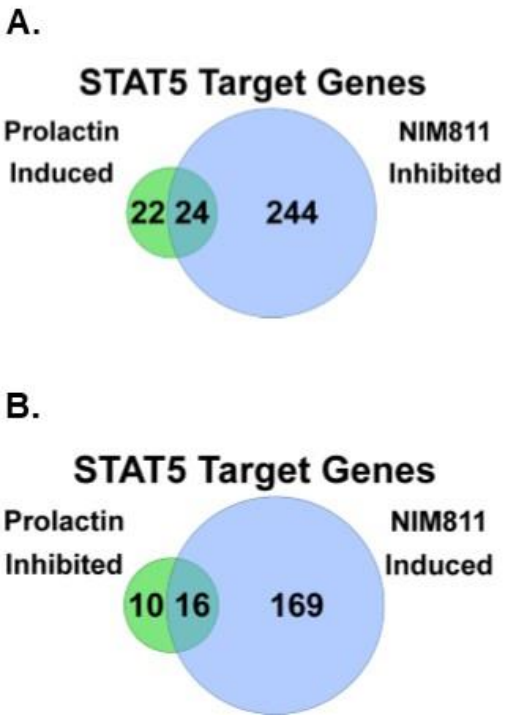


Figure 3.13 NIM811 opposes prolactin induced Stat5 target genes (Kang, Yamaji et al. 2014) (A, B) from the microarray analysis that are significantly prolactin induced **(A)** or prolactin inhibited **(B)** (fold change>1.2) and that are also significantly inhibited **(A)** or induced **(B)** by NIM811. Differential expression between treatment conditions was assessed via moderated t-test adjusted for multiple hypotheses by the Benjamini and Hochberg method. The false discovery rate (FDR) was controlled so that only those probesets with $q<0.01$ were deemed significant.

3.14 Concordance of NIM811 inhibited gene set with the Kinase perturbation experiments deposited in the Gene Expression Omnibus (GEO)

Consistent with CypA's regulation of the prolactin receptor in respect to Jak2 in the PRLr-Stat5 signaling cascade, gene set enrichment analysis comparing NIM811 inhibited genes with GEO (Gene Expression Omnibus) kinase perturbation experiments revealed highly significant similarity with Jak2 knockdown, LRRK2 mutant, GSK3β knockdown, SYK Inhibition and IGF1 inhibition and Jak1 inhibition cohorts (Fig. 3.14). In Fig 3.14, the arrays listed based on the degree of similarity with the NIM811-inhibited gene set in descending order. These arrays demonstrate that mutant form, inhibition or loss of expression of any of these genes resulted in differential gene signature that is very similar to our NIM811-inhibited gene set.

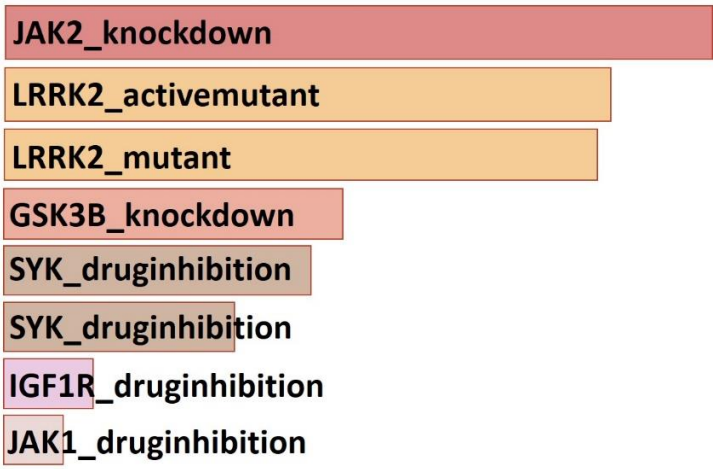


Figure 3.14 Kinase perturbation experiments deposited in the Gene Expression Omnibus (GEO). Bar graph depicts the highest scoring gene set enrichments when NIM811 inhibited genes (N=1737) with a fold change >1.2 are compared with gene signatures from kinase perturbation experiments deposited in the Gene Expression Omnibus (GEO). The gene signatures with the highest concordance with NIM811 inhibited gene set are listed in descending order from top to bottom (#1-8) based on p-value ranking. 1) Jak2_knockdown_192_GSES4645, p-value < 3.18E-12 2) LRRK2_activemutant_159_GSE36321, p-value < 3.50E-12 3) LRRK2_mutant_GDS4401, p-value < 5.24E-12 4) GSK3β_knockdown_206_GDS4305, p-value < 2.27E-09 5) SYK_druginhibition_153_GSE34176, p-value < 5.22E-09 6) SYK_druginhibition_154_GSE34176, p-value < 1.23E-08 7) IGF1R_druginhibition_46_GSE14024, p-value < 1.62E-07 and 8) JAK1_druginhibition_166_GSE38335, p-value < 3.79E-07.

3.15 CypA inhibition or knockdown inhibits breast cancer cell proliferation

CypA inhibition significantly downregulated CISH and Cyclin D1 both at mRNA and protein levels (Fig. 3.11). Furthermore, NIM811 markedly inhibited global gene expression of pro-proliferative and pro-growth genes based on the microarray analysis (Fig. 3.12). To assess whether the non-immunosuppressive, NIM811 could alter cell proliferation, ER/PR$^+$ (T47D) cells were treated with NIM811. NIM811 treatment markedly decreased cell proliferation in a PRL dependent manner (Fig. 3.15A). Furthermore, we addressed any off-target effects of NIM811 by performing shRNA knockdown of CypA in ER/PR$^+$ (T47D) cells and assessed proliferation. We found that loss of CypA also significantly reduced ER/PR+ breast cancer cell proliferation (Fig. 3.15B). These data indicate that CypA plays a role in altering breast cancer cell proliferation.

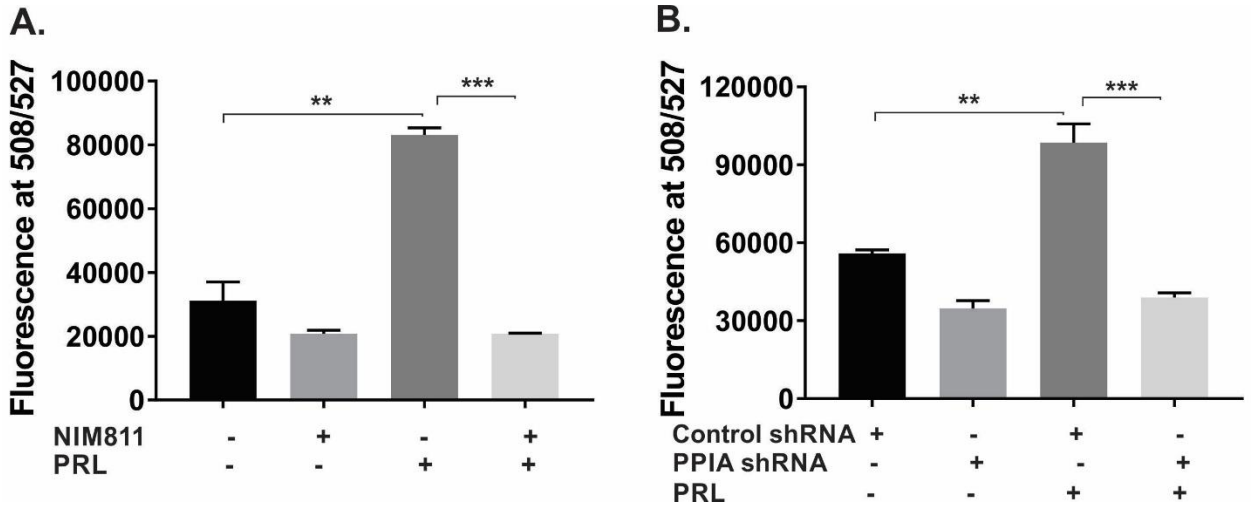


Figure 3.15 Inhibition or knockdown of CypA significantly decreases T47D cell proliferation. Cell proliferation was measured by the CyQuant (Thermo Fisher) proliferation assay. **(A)** Cells were pre-treated with NIM811 (10 µg/ml) and/or PRL (250 ng/ml). **(B)** Non-targeting control or CypA shRNA transfected in T47D cells and/or PRL (250 ng/ml). *Columns,* mean of three independent experiments; *error bars,* SEM. **, P < 0.01 ***, P<0.005.

3.16 NIM811 inhibits breast cancer cell viability and anchorage-independent growth

Breast cancer cell growth, particularly anchorage-independent growth is a key hallmark of breast cancer development and progression (Paoli, Giannoni et al. 2013). Given the function of CypA, not only at the level of the receptor but at other signaling loci that contribute to progression of breast cancer (Rycyzyn, Reilly et al. 2000) as well, we reasoned that the utilization of a non-immunosuppressive PPI inhibitor such as NIM811 might inhibit malignant phenotypes of breast cancer. NIM811 inhibited PRL/Jak2 complex-mediated signaling (Fig. 3.6), and downstream gene expression (Fig. 3.11), therefore, the effects of NIM811 treatment on breast cancer cell viability and anchorage independent growth were examined. Trypan blue exclusion methods were employed to investigate the effects of NIM811 in ER/PR+ (T47D) and TNBC (MDA 231) breast cancer cell viability. When cultured in monolayer in the presence of NIM811, both T47D (Fig. 3.16A) and MDA231 (Fig. 3.16B) demonstrated a dose-dependent inhibition of cell viability.

To determine the effects of NIM811 on anchorage independent growth, soft-agar colony formation assays were performed. As demonstrated in Fig. 3.17, NIM811 treatment markedly reduced anchorage independent growth of T47D (Fig. 3.17A) and MDA 231 (Fig. 3.17B) breast cancer cells in a dose dependent manner. These data indicate that PPIAse activity of CypA regulates *in vitro* malignant phenotypes of breast cancer cells based on the inhibitory effects of NIM811 observed in both breast cancer cell viability and anchorage independent growth.

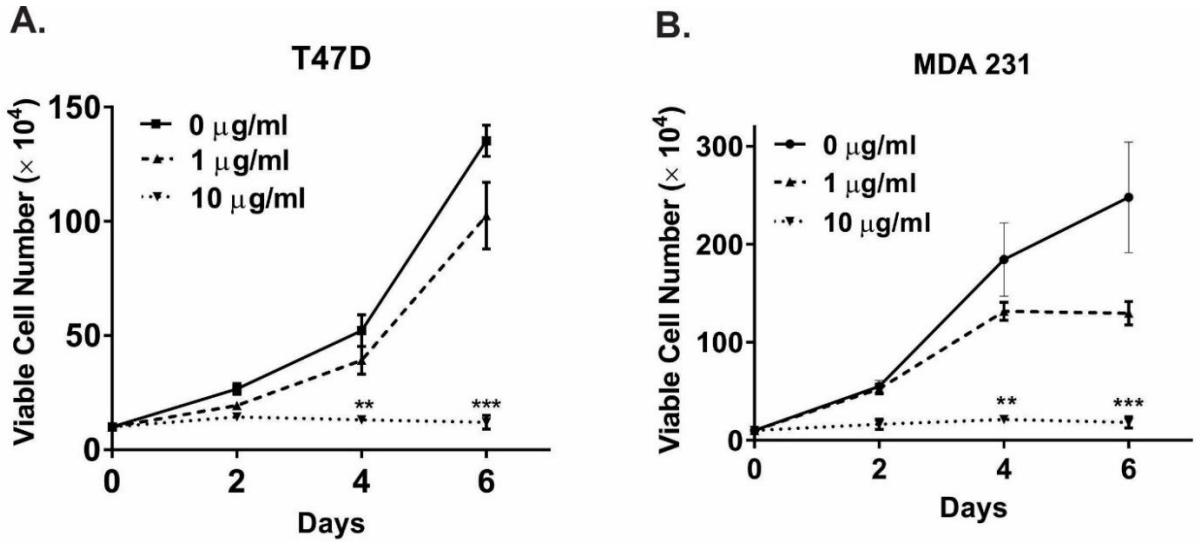


Figure 3.16 NIM811 treatment markedly decreases cell viability of breast cancer cells. (**A)** T47D and **(B)** MDA-MB-231 cells were incubated with various doses of NIM811. Viable cells were quantified every other day by trypan blue exclusion methods. *Lines,* mean of three independent experiments; *error bars,* SEM. **, P< 0.01 ***, P< 0.005.

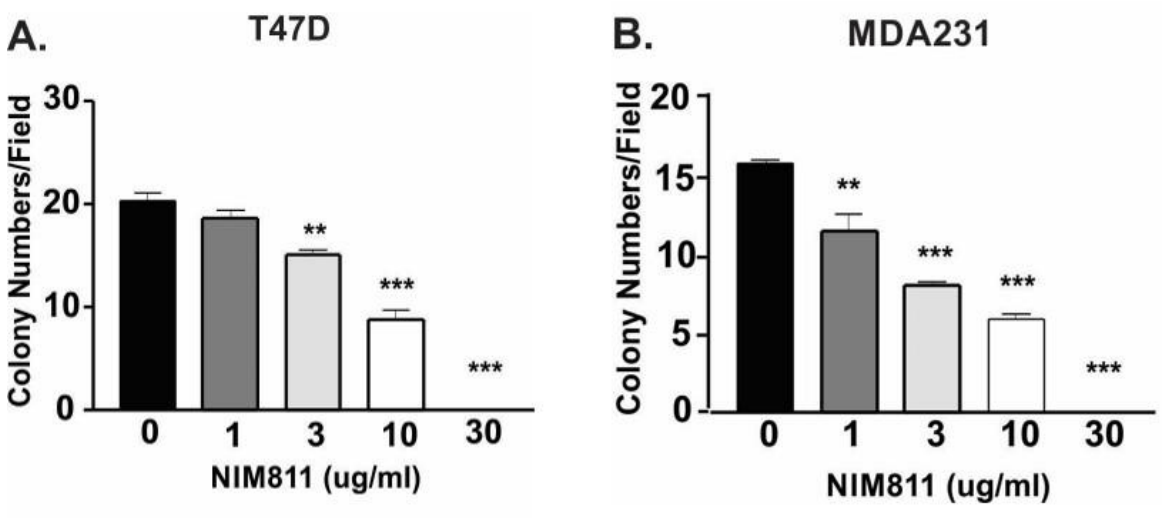


Figure 3.17 NIM811 treatment reduces anchorage-independent growth of breast cancer cells. (A) T47D and (**B)** MDA-MB-231 cells incubated with or without various doses of NIM811 were mixed with 0.45% agarose and overlaid over a 0.8% bottom agar in 6-well plates. Cells were incubated for 14-21 days. Five randomly selected fields were counted for each well to assess anchorage independent growth. Each treatment was done in triplicate. *Column,* mean of three independent experiments; *error bars,* SEM. **, P< 0.01 ***, P< 0.005.

3.17 High dose of NIM811 treatment induces apoptosis

Pharmacological inhibition of CypA by NIM811 or CypA knockdown markedly reduced ER/PR$^+$ breast cancer cell proliferation in a PRL dependent manner (Fig. 3.15). Furthermore, NIM811 inhibited viability of both ER/PR$^+$ and TNBC breast cancer cells in a dose dependent manner (Fig. 3.16). To determine the dose of NIM811 required to induce apoptosis, we treated ER/PR$^+$ breast cancer cells with various doses of NIM811 (Fig. 3.18). We used known apoptosis markers such as cleaved PARP and cleaved caspase 3 to measure/assess cell death. As demonstrated in Fig 3.18, there was a marked increase in cleaved-caspase 3 and cleaved-PARP induction at both 30 and 100 ug/ml doses of NIM811 treatment for 96 hours. These data indicate that NIM811 induces apoptosis of breast cancer cells, only at higher doses.

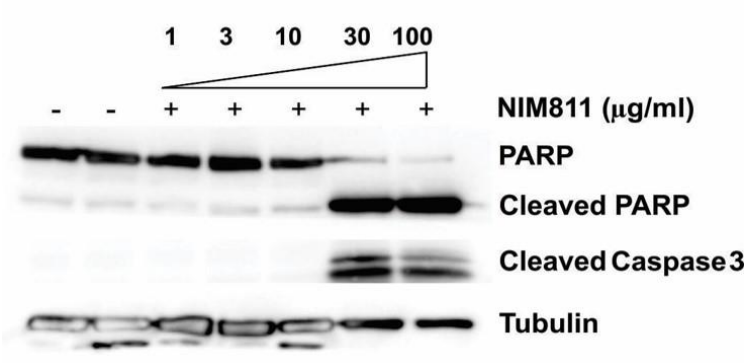


Figure 3.18 Treatment of high dose of NIM811 induces apoptosis. T47D cells are treated with various doses of NIM811 for 96 hours. Cell lysates are harvested and blotted with PARP or cleaved caspase 3 antibody as indicated. α-Tubulin is used as a loading control.

3.18 NIM811 inhibits motility and migration of breast cancer cells

Prolactin stimulates the cytoskeletal re-organization and motility of breast cancer cells through activating Nek3 and Vav2 pathways (Miller, DeMaria et al. 2005). Given this, we assessed motility and migration of ER/PR+ (T47D) and TNBC (MDA 231) cells utilizing wound-healing and trans-well migration assays. The wound healing assay was utilized to measure motility by percent of wound closure (Maus, Reilly et al. 1999), as determined morphometrically. MDA 231 cells were treated with NIM811 for 24- and 48-hour time-periods. The highly metastatic MDA231 cells exhibited markedly reduced motility compared to the un-treated control based on quantification of percent wound closure, while MDA231 cells had moderate effects in wound closure (Fig. 3.19A-B).

Next, trans-well assays were performed as described in (Schmitt, Andrews et al. 2016) to determine breast cancer cell migration. Both T47D and MDA 231 cells were treated with the same range of doses of NIM811 as used previously. As shown in Fig. 3.20, NIM811 significantly reduced migration of both ER/PR^{+} (Fig. 3.20A) and ER/PR^{-} (Fig. 3.20B) breast cancer cells. These data demonstrated that NIM811 treatment significantly inhibits motility and migration of breast cancer cells derived from different subtypes of breast cancers *in vitro*, suggesting a possible *in vivo* role for CypA-PPI inhibition in the treatment of breast cancer malignancies.

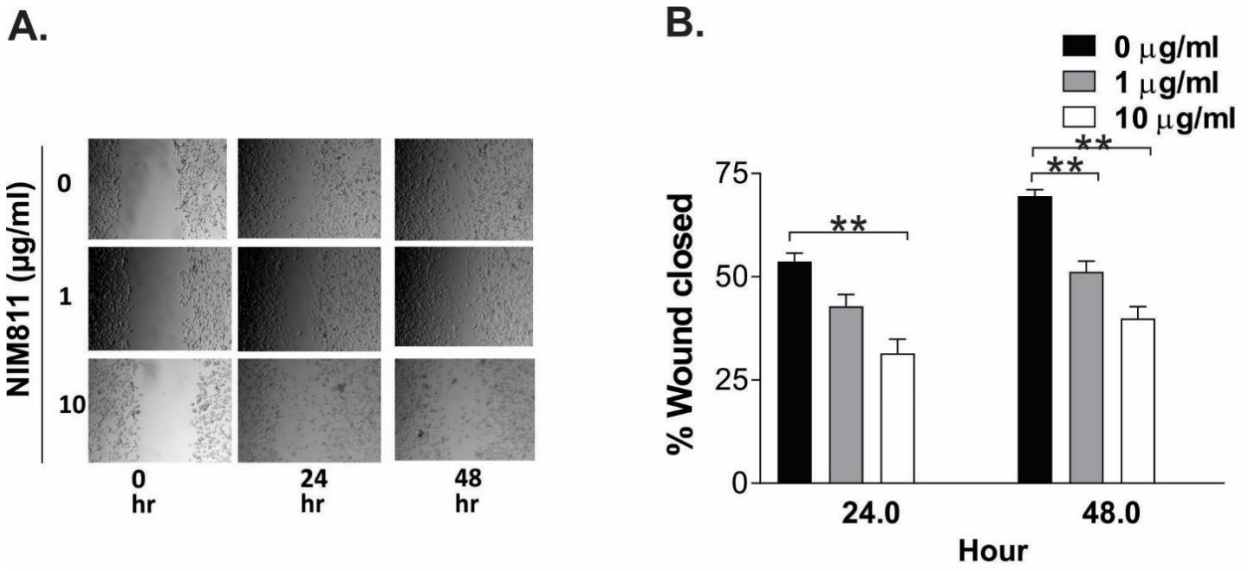


Figure 3.19 CypA inhibition decreases TNBC cell motility. (A) Confluent MDA-MB-231 cells were wounded with a pipette tip and cultured in serum-free medium in the presence of various doses of NIM811. Representative images of the wound closure assay were acquired with a phase-contrast microscope and **(B)** the percentage of the wound closures were quantified. *Columns,* mean of three independent experiments; error *bars,* SEM. ** P<0.01.

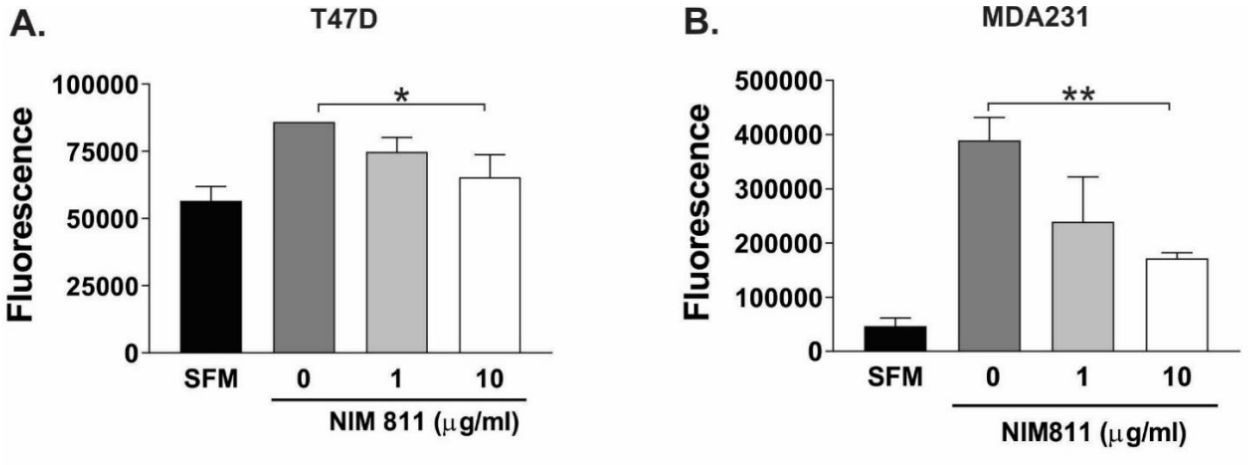


Figure 3.20 CypA inhibition significantly reduced migration of breast cancer cells. Migration was measured by a Boyden chamber assay. Prolactin stimulated migration of T47D **(A)** and MDA-231 **(B)** breast cancer cells were inhibited by NIM811. SFM (serum-free media) was the negative control. *Columns,* mean of three independent experiments; *error bars*, SEM. *, $P < 0.05$, ** $P<0.01$.

3.19 CypA inhibition induces tumor necrosis and inhibits lymph node metastasis of TNBC xenograft

As NIM811 had significant effects on monolayer cell growth, anchorage independent growth, migration and associated signaling in breast cancer cells, it was reasoned to determine the effects of NIM811 therapy in a mouse xenograft model of breast cancer. It was hypothesized that NIM811 would inhibit the outgrowth of human breast cancer *in vivo*. To test this hypothesis, triple negative MDA-231 breast cancer cells were injected into the fourth lactiferous ducts of nude mice, using a novel approach that dramatically facilitates the metastatic spread of breast cancer xenografts (Harrell, Dye et al. 2006). When xenograft tumors were formed, mice were randomized into three groups and treated with vehicle control (olive oil), or NIM811 at both 20 mg/kg/day and 50 mg/kg/day. Tumors were allowed to establish for 2 weeks prior to twice daily gavage of NIM811 in olive oil administered for a total of 4 weeks. While all NIM811 treated tumors revealed a trend toward a reduction in weight and size over the period, these parameters did not achieve statistical significance (data not shown). However, two parameters were found to be markedly altered as functions of NIM811 treatment, including central tumor necrosis and lymph node metastasis (Fig. 3.21). As demonstrated in Fig. 3.21A, central tumor necrosis of TNBC was significantly increased compared to vehicle control. NIM811 effectively induced central tumor death at both the 20 and 50 mg/kg/day doses based on quantification of necrotic area (Fig. 3.21A). As shown in Fig. 3.21B, NIM811 markedly decreased lymph node metastasis of TNBC xenografts. Notably, the 20 mg/kg/day dose of NIM811 was sufficient to significantly inhibit the lymph node metastasis (Fig. 3.21B).

Taken together, NIM811 therapy had significant effects on inhibition of both lymph node metastasis and the induction of central tumor necrosis in TNBC xenografts.

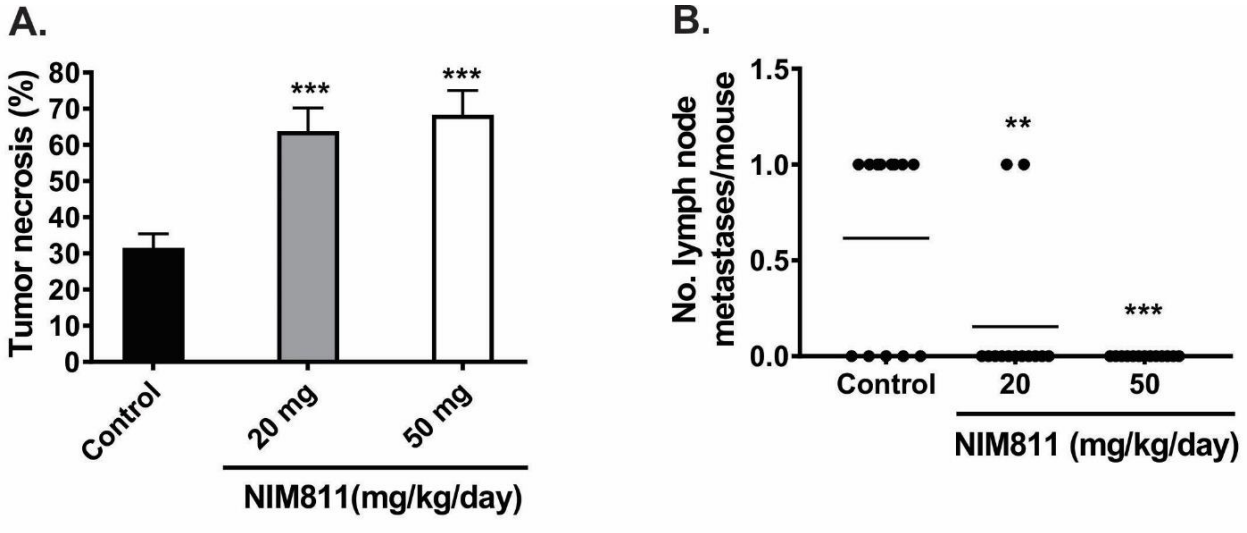


Figure 3.21 NIM811 induces tumor necrosis and inhibits lymph node metastasis in TNBC xenograft. **(A)** Quantification of the necrotic area of primary tumor based on histologic examination of H&E-stained sections. ***P<0.001. **(B)** Lymph node metastases were detected by histologic examination of H&E-stained sections of lymph nodes harvested from mice treated for 12 weeks with either NIM811 or vehicle control. **P<0.01, ***P<0.005.

3.20 Loss of CypA reduces tumor burden of PyMT mice

Several mouse loss-of-function models demonstrate delayed or disrupted tumorigenesis when crossed into the appropriate line expressing a transgenic mammary oncogene (Ren, Cai et al. 2002, Shen and Brown 2005, Sakamoto, Triplett et al. 2010). We postulated that mammary tumorigenesis driven by a mammary oncogene, such as MMTV-PyMT, would be significantly reduced by its introduction into the $CypA^{-/-}$ background. This was based on the following observations: i) Significant reduction in tumor latency in ErbB2 x $CypA^{-/-}$ compared to wild-type control (Volker, Hedrick et al. 2018), ii) CypA regulates PRLr-Stat5 signaling intermediates in breast cancer cells (Fig. 3.6 & 3.8); iii) CypA inhibition decreases anchorage independent growth (Fig. 3.17) and migration (Fig. 3.20); and iv) NIM811 treatment induces necrosis and blocks metastasis in human breast cancer xenografts (Fig. 3.21). To determine a role for CypA in the PyMT mouse model, $CypA^{+/-}$ x FVB and PyMT x FVB mice were mated and after remating of select progeny, $CypA^{+/+}$ and $CypA^{-/-}$ (xFVB) female littermates were followed for mammary tumor development for 5-14 weeks.

To assess mammary cancer progression through different cancer stages of $PyMT^{+}$ x $CypA^{-/-}$, H&E staining of 4^{th} mouse mammary gland was performed. As shown in Fig. 3.22A, each age correlated to a particular cancer stage: 5 weeks with hyperplasia, 8 weeks with adenoma, 11 weeks with invasive carcinoma, and 13 weeks with metastasis (Fig. 3.22A). $PyMT^{+}$ x $CypA^{+/+}$/$CypA^{+/-}$ mice had markedly higher invasive carcinoma at 11 weeks and metastasis at 13 weeks compared to the $PyMT^{+}$ x $CypA^{-/-}$ mice (Fig. 3.22A). Each gland had regions of each stage that were scored in addition to “% normal”. This scoring was quantified to compare significance between cancer phenotypes and

genotype. Since primary tumor burden is a key parameter to determine initial cancer growth/development, mammary glands of $CypA^{-/-}$ were calipered/excised, with these weights normalized to total mouse weight and compared to control ($CypA^{+/+}$, $CypA^{-/+}$) mice. The primary tumor burden for $CypA^{-/-}$ mice significantly decreased compared to $CypA^{+/+}$ mice whereas only moderate decrease was observed for $CypA^{-/+}$ mice (Fig. 3.22B). The data demonstrate that loss of CypA significantly reduces mammary tumor burden of PyMT mice.

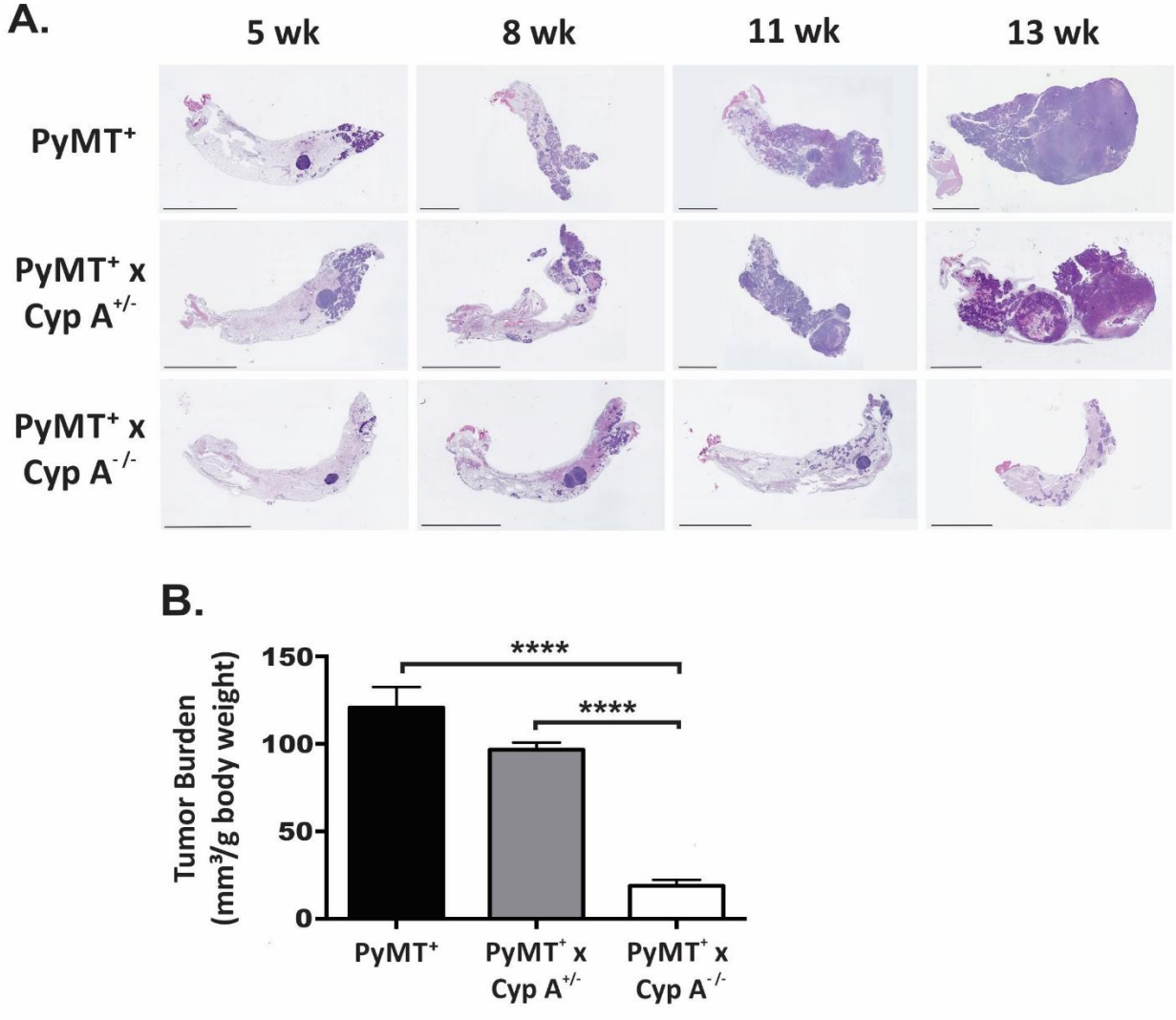


Figure 3.22 Loss of CypA decreases tumor burden. **(A)** Representative images of hematoxylin and eosin stained mouse mammary gland containing tumor as a result of cross-breeding the WT ($PyMT^+$) mouse to the Cyp $A^{-/-}$ mouse (n = 12). Different ages are representative of distinct cancer stages in the WT ($PyMT^+$) model: 5 weeks – hyperplasia; 8 weeks – adenoma; 11 weeks – invasive carcinoma; 13 weeks – metastasis. **(B)** Tumor burden was assessed as the sum of all primary mammary tumor volumes normalized to body weight in grams. For B, data reported as means with SEM. **** = $p < 0.001$.

3.21 CypA knockout markedly reduces lung and lymph node metastasis

To elucidate a role for CypA in mammary cancer metastasis, we assessed both lymph nodes and lung metastasis. Quantification of percent positive lymph nodes of $CypA^{+/+}$, $CypA^{-/+}$ and $CypA^{-/-}$ mice demonstrated that $CypA^{-/-}$ mice had a marked reduction in lymph node metastasis compared to $CypA^{+/+}$ (Fig. 3.23A). To determine lung metastasis, both the metastases area as well as number of metastases were measured. $CypA^{-/-}$ mice demonstrated significant decreases in both the number of metastases (Fig. 3.23B) and metastases area (Fig. 3.23C). Even after correction for primary tumor burden (Fig. 3.23D), $CypA^{-/-}$ mice demonstrated reduced levels of metastases compared to $CypA^{+/+}$ mice. These results demonstrated that loss of CypA and its corresponding function has significant impacts on the reduction of metastasis of lymph nodes and lung in mammary cancer. These data demonstrate that CypA significantly contributes to multistage mammary cancer progression.

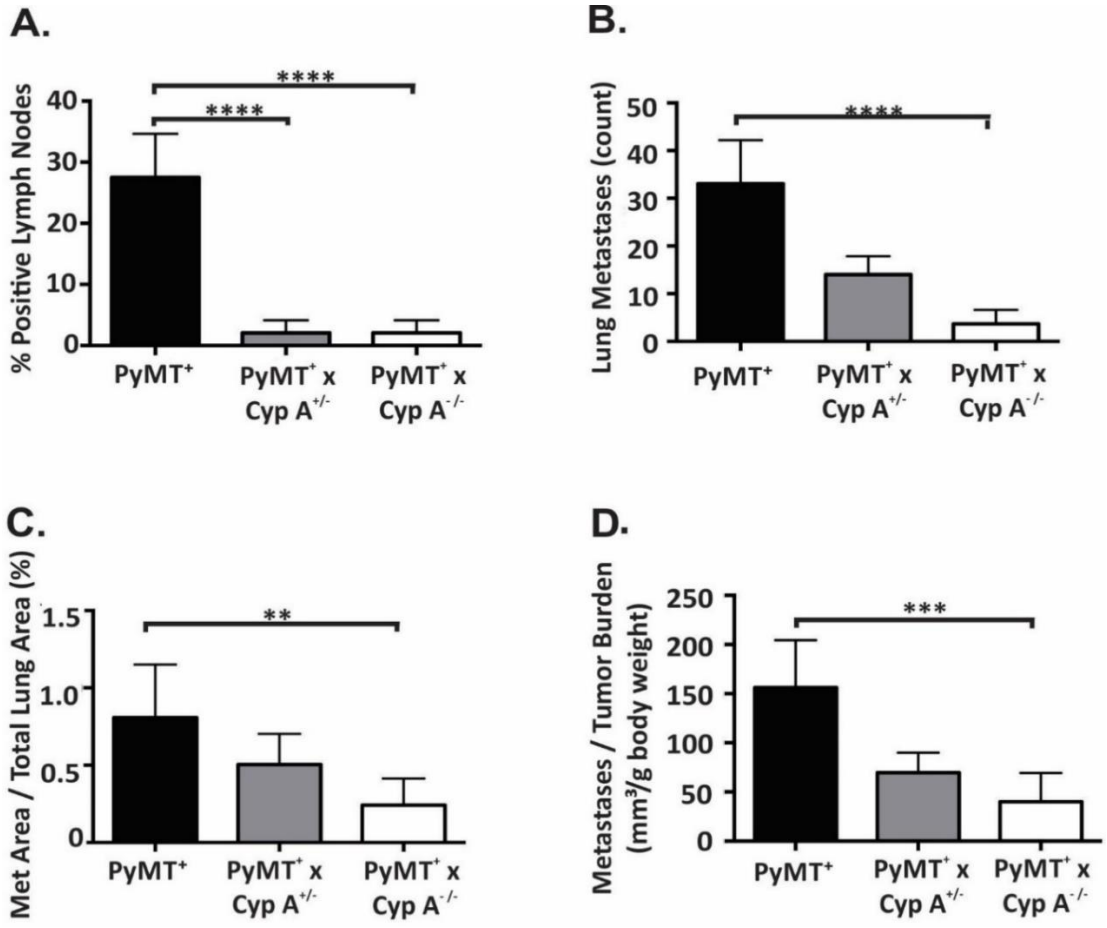


Figure 3.23. Loss of CypA reduces lung and lymph node metastasis. **(A)** Positivity of lymph nodes was determined as lymph nodes containing at least 1 macrometastasis from FFPE H&E-stained axillary and accessory axillary lymph nodes, assessed by CVC, a board-certified pathologist, under brightfield microscopy. **(B)** Lung metastases from H&E-stained FFPE lung sections were counted. **(C)** Borders were drawn of metastases identified in B and a scaled area was calculated for each metastasis as well as each lung lobe for each mouse. Metastases total area was normalized to total lung area. **(D)** Lung metastases were counted and normalized to per tumor burden. All experiments contained n = 12 mice per cohort. For A, data reported as means with SEM. For B, C, and D medians with range were reported due to the lack of a normal distribution of the metastases data. ** = $p < 0.01$, **** = $p < 0.001$.

3.22 CypA deletion enhances tumor-free survival of PyMT mice

Our previous data demonstrated that erbB2 x CypA$^{-/-}$ significantly enhanced tumor latency and tumor-free survival in mice compared to erbB2 x CypA$^{+/+}$ (Volker, Hedrick et al. 2018). To assess the role of CypA in oncogene driven tumor-free survival, we assessed tumor latency and tumor-free survival in PyMT^{+} x CypA$^{+/+}$ vs PyMT^{+} x CypA$^{-/-}$ mice. As presented in a scattered plot in Fig. 3.24A, a significant delay in tumor latency was noted in the CypA$^{-/-}$ mice (CypA$^{+/+}$ mean latency = 36 days vs. CypA$^{-/-}$ = 51 days). In parallel, survival of the CypA$^{-/-}$ females was significantly enhanced (CypA$^{+/+}$ = 129 days vs. CypA$^{-/-}$ = 90 days) (Fig. 3.24B). However, CypA$^{-/+}$ x PyMT females demonstrated no increase in tumor latency or survival, paralleling the curves of the CypA$^{+/+}$ mice (Fig. 3.24A-B). These data indicate that loss of CypA significantly enhances both tumor latency and tumor-free survival in PyMT^{+} x CypA$^{-/-}$ mice.

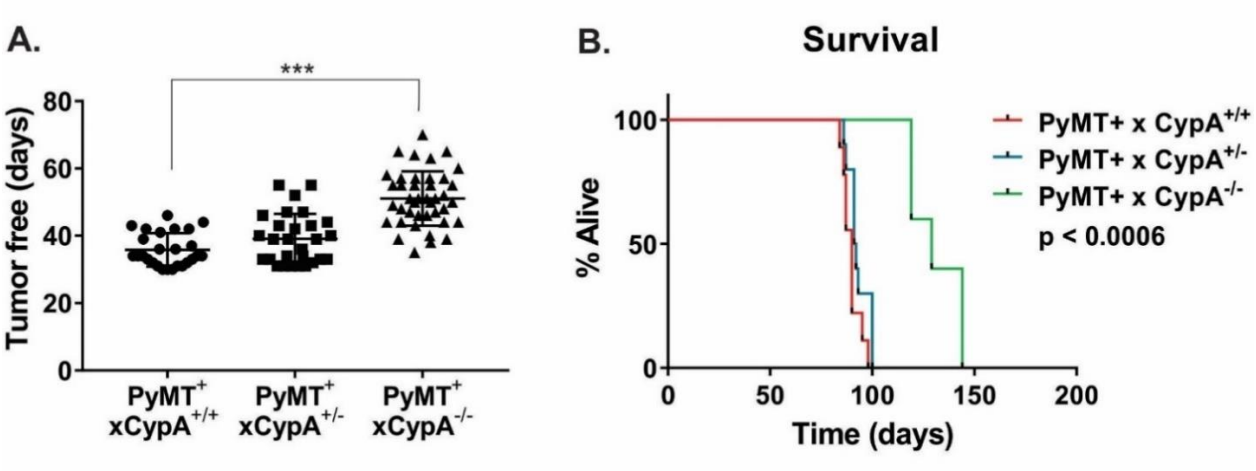


Figure 3.24 CypA deletion enhances tumor-free survival of PyMT mice. **(A)** $CypA^{-/-}$ x $PyMT^{+}$ mice demonstrate significantly delayed tumor latency ($P < 0.005$). **(B)** Kaplan-Meier plots of overall survival ($P < 0.0006$) reveal highly significant differences between Cyp $A^{+/+}$ and Cyp $A^{-/-}$ mice when bred into the PyMT mouse model of mammary tumorigenesis. Statistics based on n = 12 for each cohort based upon comparison to WT ($PyMT^{+}$) (**** $p < 0.001$).

3.23 CypA deletion inhibits tumor multiplicity and invasive carcinoma

Analysis of mammary tumor multiplicity in the mouse cohorts at the time of euthanasia/death; $CypA^{+/+}$ mice (average of 9.85 tumors/mouse) demonstrated significantly more discrete carcinomatous foci than did $CypA^{-/-}$ mice (average of 4.93 tumors/mouse) (Fig. 3.25A). As demonstrated in Fig. 3.25B, $PyMT^{+}$ x $CypA^{-/-}$ cohort had a highly significant proportion of tumors in hyperplasia stage compared to either $CypA^{+/+}$ or $CypA^{-/+}$ cohort. In contrast, $PyMT^{+}$ x $CypA^{-/-}$ mice had significantly lower percentage of invasive carcinomas compared to $CypA^{+/+}$ or $CypA^{-/+}$ mice (Fig. 3.25B). These results demonstrate that loss of CypA significantly reduces number of primary tumors and invasive carcinoma in $PyMT^{+}$ x $CypA^{-/-}$ mice.

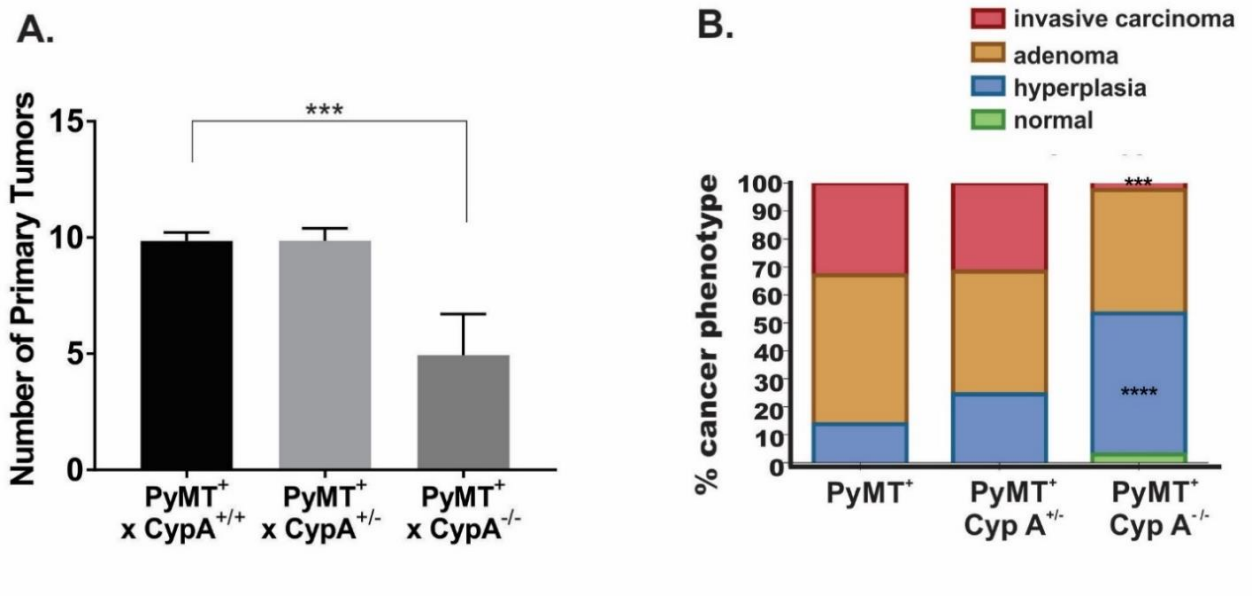


Figure 3.25 CypA deletion inhibits PyMT primary tumors and invasive carcinoma. **(A)** Compared to $CypA^{+/+}$ x $PyMT^{+}$, $CypA^{-/-}$ x $PyMT^{+}$ mice demonstrate significantly reduced number of primary tumors ($P < 0.005$). **(B)** Percent cancer phenotypes based on histological analysis performed by CVC, a board-certified pathologist, under brightfield microscopy. Percent of total denotes the portion of FFPE mouse mammary gland hematoxylin and eosin stained tissue sections exhibiting features of (1) invasive carcinoma, (2) adenoma, (3) hyperplasia, (4) normal mammary tissue. Statistics based upon an n = 12 for each cohort based upon comparison to WT ($PyMT^{+}$) (**** $p < 0.001$).

Chapter 4: Discussion

4.1 Summary and significance of novel findings

While significant progress in delineating the signaling networks that are activated after ligand binding to prolactin receptor has been made, the precise mechanisms exploited to activate proximal receptor-associated kinases remain enigmatic. Given the dependence of various disease processes on such pathways, the need to ameliorate our understanding of these events is crucial from both a mechanistic and therapeutic vantage point. Herein, we elucidated fundamental mechanisms of PRL-induced conformational change of the PRLr that mediates signal transduction to regulate mammary cancer progression. Furthermore, we revealed a PRL dependent activation profile of receptor proximal signaling molecules and global gene expression signatures; and how their inhibition by a non-immunosuppressive blocker of CypA, NIM811, significantly alters breast cancer cell functions, mammary tumorigenesis and metastasis.

Our comprehensive analysis of CypA mediated PRL-induced rotational/conformational change in the PRLr-ICD provides novel insight into CypA-PPIase activity in regulating structure/function relationships of the prolactin receptor in mammary/breast cancer. Importantly, understanding the mechanisms of how CypA acts as a signaling switch to regulate oncogenic progression, would enable discoveries to target this molecule and associated kinases in breast cancer.

4.2 Regulation of the structure/function relationships of the PRLr

4.2.1 PRLr ECD/ICD and structural modifications

PRLr-ECD does not go through any significant conformational change upon PRL binding based on FRET studies (Tallet, Fernandez et al. 2011). The crystal structure of the

unliganded (absence of GH) GHR-ECD, which is structurally similar to the PRLr-ECD, also demonstrates only small changes compared to the ligand (GH) bound state (Brown, Adams et al. 2005). Existing data indicates that the signal transduction mechanisms primarily rely on ICDs (Haxholm, Nikolajsen et al. 2015), mediated by ligand-induced conformational change in the ICD (Brooks, Dai et al. 2014). Our data demonstrate that the PRLr-ICD goes through a conformational change in a PRL dependent manner (Figs. 3.2-3.5). CypA mediated PRL-induced conformational change in the PRLr-ICD results in phosphorylation/activation of receptor proximal molecules including Jak2, Src, Stat5 and their association with the PRLr (Fig. 3.10). The ICDs of the PRLr and GHR share properties such as conserved Box1 and Box2 regions and intrinsically disordered region(s) that consist of a pattern of transient structure and number/position of lipid interaction domains (LIDs) (Haxholm, Nikolajsen et al. 2015). The PRLr-ICD exists mostly as monomeric and dynamic rapidly inter-converting conformers (Bugge, Papaleo et al. 2016). Our data support this observation based on CypA's function as a switch for rapidly inter-converting the PRLr-ICD from an inactive to an active conformer when stimulated with PRL (Figs. 3.3 and 3.5).

Unlike PRLr-ECD, the ICD can extend in the direction of the membrane due to the structural flexibility and large capture radius that permits it to reach and interact with diverse interaction partners (Pezet, Buteau et al. 1997). Therefore, the ICD is concurrently interacting with many kinases including Jak2, Src, Fyn, PPIAse(s) like CypA and transcription factors like Stats. Box 1 is an example of a small linear motif in the PRLr-ICD, shown to interact with the Jak2-FERM domain; as well as the domain near Box1 where CypA binds at residue P334 (Zheng, Koblinski et al. 2008). Our data confirms

that the constitutively bound prolyl isomerase can modulate ICD conformation to affect interactions of other proteins in the membrane proximal region. We have demonstrated that CypA knockdown decreases phosphorylation of Jak2, Src and Stat5 as well as their association/interaction with the PRLr (Fig. 3.10) indicating a correlation between conformational change of the ICD and phosphorylation/interaction of receptor proximal kinases. Furthermore, post-translational modification of the ICD, specifically phosphorylation may modulate the structural ensemble of the ICD (Bah, Vernon et al. 2015). We have demonstrated that PRL-induced phosphorylation of the PRLr itself, at residues 381 and 587 which are thought to play roles in the PRLr engagement of Src and Stat5, respectively (Pezet, Ferrag et al. 1997, Brooks 2012), however, there is no clear evidence whether these phosphorylation(s) play any role in modulating the structural ensemble of the PRLr-ICD.

4.2.2 CypA prolyl isomerase activity in Box 1 and its proximal region to induce conformational change of the PRLr-ICD

CypA interacts with both the long and intermediate hPRLr isoforms (Syed, Rycyzyn et al. 2003). It has been demonstrated that PPI could markedly alter PRLr-ICD structure and function by inducing a conformational change of the proline rich Box 1 or near Box 1 motifs of the PRLr (O'Neal, Chari et al. 1996). We have fluorescently tagged (CFP or YFP) intermediate isoforms of the PRLr and demonstrated that CypA induces conformational change in the PRLr-ICD in a PRL dependent manner (Figs. 3.2-3.3 and 4.1). Investigation of a peptide sequence (amino acid sequence (IFPPVPGP)) within the Box 1 motif by NMR (nuclear magnetic resonance) spectroscopy analysis has shown that a proline imide bond exists in preferred trans conformation in this motif. Interestingly, the

third proline residue tends to assume cis position in the Box 1 motif and slowly isomerizes from cis to trans conformer in the absence of PPI enzymatic activity (Chang and Clevenger 1996, Chang, Ye et al. 1998). However, the rest of the proline residues in the Box 1 motif preferred natural trans conformation and went through quick cis-trans isomerization mediated by PPI activity. Particularly, the third proline of the PRLr Box 1 motif modulated its conformation from a linear strand into a “pseudo cyclic” conformation through cis-trans isomerization (O'Neal, Chari et al. 1996). These findings indicate that the Box 1 motif or proline residue near Box 1 could alter PRLr function by changing its inherent structure (Chang and Clevenger 1996, Chang, Ye et al. 1998). Our data demonstrate that CypA binds to either Box1 or near this region of the PRLr-ICD altering its inherent structure in a PRL-induced manner. It is very likely that CypA induces a conformational change from a linear strand into “pseudo cyclic” conformation. It is probable that such a conformational change promotes increased phosphorylation/activation of receptor proximal molecules of the PRLr/Jak2 complex due to their closer proximity to each other. CypA inhibition or loss of its expression impedes such PRL-induced conformational change of the PRL-ICD (Figs. 3.2-3.5) and subsequent phosphorylation/activation of Jak2-Stat5 signaling intermediates (Figs. 3.6 and 3.8). These findings provide fundamental mechanistic understating of CypA regulation of PRL-induced structure/function relationships of the PRLr (Fig. 4.1).

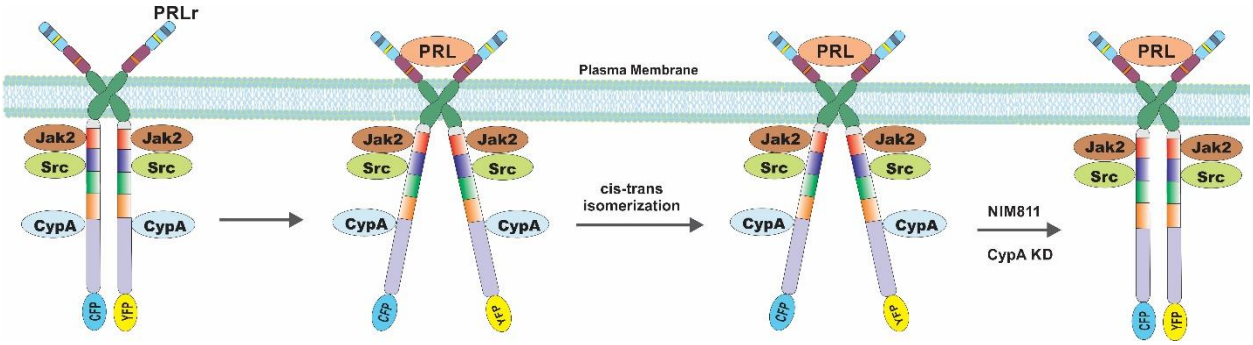


Figure 4.1 CypA mediates PRL-induced conformational change of the PRLr-ICD. Schematic demonstrates that constitutively bound CypA induces a conformational change of the PRLr-ICD through PPIase activity in a PRL dependent manner. NIM811 inhibits PRL-induced conformational change of the PRLr-ICD. C-terminal tagged PRLr-CFP, -YFP allows to assess such conformational change using FRET/FLIM strategies.

4.2.3 The consequence of the structural change of the PRLr in proximal signaling

Previous study of the growth hormone receptor structure/function using FRET and mutagenesis approaches suggested a ligand induced conformational change that affects phosphorylation and proximal signaling (Brown, Adams et al. 2005). Our lab has shown that CypA binds to a specific proline residue of the PRLr and may induce such conformational change within the receptor. Either kinase inactive (CypA-PPI) or point mutant (PRLr-P334A) resulted in decreased PRLr-CypA interactions, Jak2 phosphorylation and decreased downstream gene expression (Zheng, Koblinski et al. 2008).

Like other cytokine receptor family members, PRLr is composed of the conserved Box 1/Variable Box/Box 2/X-Box regions. Although the proline-rich Box 1 motif is known for Jak2 binding and PRL-induced phosphorylation, the carboxyl terminal proline residues

including P248 and P250 are important, since their deletion or point mutation(s) abrogate Jak2 binding and PRL-induced gene expression (Lebrun, Ali et al. 1995, Pezet, Buteau et al. 1997, He, Wang et al. 2003). On the other hand, the Box 1 region by itself is not sufficient for lactogenic signaling (Lebrun, Ali et al. 1995, Chang and Clevenger 1996), demonstrating that other proximal protein(s) like Src, Fyn, Stat5 associated with the PRLr affect Jak2 activation. Our data demonstrate that loss of CypA also blocks phosphorylation/activation of Jak2, Src and Stat5 and their association with the PRLr, as exemplified by the actions of kinase-defective Src that precludes the activation of Stat5 by Jak2 induced by PRL (Garcia-Martinez, Calcabrini et al. 2010). In this case, CypA acts as a signaling switch inducing PRL-induced conformational change that modulates activation/phosphorylation of these kinases, their association with the PRLr and perhaps their regulation of each other.

4.3 CypA regulation of Jak2/Src/Stat5 signaling and breast cancer cell functions

4.3.1 NIM811 inhibits CypA PPIase activity

The activation of the Jak2-Stat5 pathway is thought to play an important role in mammary tumorigenesis (Wagner and Rui 2008). However, inhibitors of Jak2/Stat5 or the receptors associated with this complex's activation have demonstrated limited success (Bousoik and Montazeri Aliabadi 2018), in part due to their inability to block this pathway. In an effort to find a better inhibitor of this pathway, our lab began to work with CypA, which through its prolyl isomerase activity is necessary for Jak2 activation. Use of a CypA inhibitor (CsA), both *in vitro* and *in vivo* (Zheng, Koblinski et al. 2008) has significant effects on mammary tumorigenesis and progression. However, the immunosuppressive properties of CsA, through its engagement of calcineurin, precludes the use of this agent

in breast cancer patients. Thus, the development of non-immunosuppressive cyclosporin(s) that remain fully functional as PPI inhibitors, such as NIM811, could have significant impact as therapeutic agents. Herein, we demonstrate that NIM811 is fully capable of inhibiting the PRLr-triggered activation of the Jak2-Stat5 pathway and significantly alters breast cancer cell functions.

NIM811 is structurally similar to CsA, except having a sec-butyl instead of an isobutyl group (Tang 2010). The central clinically relevant difference between CsA and NIM811 is that NIM811 is non-immunosuppressive, thus an ideal candidate to test in a clinical trial. In contrast to CsA, NIM811 does not inhibit the phosphatase activity of calcineurin, the translocation of NFAT (nuclear factor of activated T) to the nucleus or IL-2 expression, hence maintaining normal T-cell function and associated immune responses (Jauliac, Lopez-Rodriguez et al. 2002, Yiu, Kaunisto et al. 2011). NFAT has been linked to breast cancer invasion and is also involved in antagonistic crosstalk with Stat5 in breast cancer cells (Zheng, Fang et al. 2011). In contrast to CsA, the inability of NIM811 to interact with NFAT may provide another great benefit of the drug. As such, NIM811 treatment does not interfere with NFAT inhibitory regulation of Stat5 and its downstream targets in breast cancer cells, therefore maintaining its efficacy and avoiding drug resistance.

Our data suggest that non-immunosuppressive NIM811 binds and blocks the prolyl isomerase activity of CypA without altering its expression in breast cancer cells (Fig. 3.6). By blocking prolyl isomerase activity of CypA, NIM811 inhibits PRL-induced conformational change of the PRLr and activation/phosphorylation of Jak2-Stat5 signaling intermediates. Furthermore, NIM811 effectively inhibits pro-tumorigenic gene expression including CISH and Cyclin D1 and blocks breast cancer cell proliferation,

survival, anchorage independent growth and migration. The anti-cancer properties of NIM811 reside in the ability of the drug to bind and block the prolyl isomerase activity of CypA. These findings confirm the observed inhibition of signaling and anti-cancer properties of the prolyl isomerase-detective form of CypA (Zheng, Koblinski et al. 2008).

4.3.2 CypA modules breast cancer cell functions through the Jak2-Stat5 axis

The data presented here demonstrate that the PPI activity of CypA contributes to proximate PRLr receptor activation, enabling signal transduction through the PRLr/Jak2 complex. Inhibition of CypA PPI function by NIM811, blocks tyrosine phosphorylation of Jak2, Stat5 and Src at several sites that modulate the overall activity of these kinases. Similarly, NIM811 significantly reduces the phosphorylation of the PRLr itself at residues 381 and 587, which are thought to play roles in the PRLr engagement of Src and Stat5, respectively (Pezet, Ferrag et al. 1997, Brooks 2012). Like pharmacological inhibition of CypA, its knockdown also significantly suppresses phosphorylation of the PRLr, Jak2 and Src at those specific tyrosine residues (Fig. 3.8), in addition to their association with the PRLr (Fig 3.10), suggesting that CypA plays a pivotal role in binding/interaction and phosphorylation/activation of the PRLr proximal molecules, which alter breast cancer cell functions.

Based on yeast-two hybrid and immunoprecipitation assays, CypA binds to the prolactin receptor constitutively in both T47D, and MCF7 cells (Syed, Rycyzyn et al. 2003, Zheng, Koblinski et al. 2008) and activates in response to prolactin to induce phosphorylation of Jak2 and subsequent Stat5 (Figs 3.6 and Fig 3.8). Through its prolyl isomerization, CypA is inducing a structural change in the prolactin receptor to modulate signal transduction (Figs 3.3 and 3.5). CypA not only modulates Jak2, Src and Stat5 (Fig. 3.6 and 3.8), but

also other substrates or components of the PRLr signaling downstream network including, Akt and MAPK (Fig. 3.7). In this case, PRLr-associated Fyn and Ras may contribute to the PRL-induced activation of the Akt and MAPK (Rodriguez-Viciana, Warne et al. 1994, al-Sakkaf, Dobson et al. 1997). Moreover, PRL induces association of Vav1 with the PRLr, and Vav1 interacts with the tyrosine kinases Fyn (Chang, Ye et al. 1998) and Tec (Kline, Moore et al. 2001). The interaction of the PRLr/Jak2/Stat5 complex with associated kinases are context dependent, and may rely on mutual interactions among each other, perhaps resulting in an overall increase in the affinity of the individual members of this complex for each other. CypA likely enhances such interactions and activations to induce oncogenesis.

Our data demonstrate that, at least in human breast cancer cells, CypA inhibition targets the proximal Jak2/Src kinases, through its inhibition of the PPI activity of CypA. However, Jak2 is not fully committed to the residues surrounding the tyrosine it phosphorylates, hence many other PRLr-associated signaling proteins such as SHP2, Fyn, Tec and Vav may serve as substrates. These associated phosphatase/kinases may compensate or co-operatively work with the PRLr/Jak2/Src complex to alter both proximal and downstream signaling/functions of breast cancer cells. We demonstrate that the loss of Jak2 and Src activity mediated by CypA inhibition leads to loss of PRL-induced activation of downstream signaling molecules such as Stat5, Akt and MAPK, and the concomitant downregulation of PRL-induced oncogene expression including EGR3, DUSP6, LIN28A, Cyclin D1 and GBP1 that readily explain the observed decrease in proliferation, growth, and migration of breast cancer cells. Notably, our CypA genetic deletion and rescue overexpression studies presented here validates that the effects on signaling and

proliferation are comparable to those observed following NIM811 treatment. These complimentary approaches bolster the relevance of the Jak2-Stat5 axis and associated kinases for signaling in breast cancer pathobiology and indicate that the inhibition of this pathway may be critical for impeding breast cancer progression.

4.3.3 Role of Src in PRLr/Jak2 complex signaling and breast cancer cell functions

PRL induced activation of the PRLr stimulates Jak2 and SFK catalytic activity, resulting in promotion of differentiation, proliferation and other functions (Clevenger and Medaglia 1994, Berlanga, Fresno Vara et al. 1995). PRL induces the association of c-Src to the PRLr and its catalytic activity to enhance proliferative signals in rodents (Berlanga, Fresno Vara et al. 1995). Although there may be interrelationships between Jak2 and Src, Jak2 is not required for Src association and activation of the PRLr. Substitution of proline residues to alanine within the Box 1 domain that is required for Jak2 interaction/activation, does not interfere with PRLr activation of c-Src. It has been demonstrated that c-Src lacking kinase activity can prevent PRL-induced stimulation of Stat5 by Jak2 in MCF7 breast cancer cells (Garcia-Martinez, Calcabrini et al. 2010). Our data clearly demonstrate that CypA increases PRL-induced phosphorylation of Src and its association with the PRLr in breast cancer cells. In contrast, NIM811 treatment or loss of CypA significantly downregulates Src phosphorylation/activation in breast cancer cells (Figs. 3.6 and 3.8). Moreover, inhibition of c-Src tyrosine kinase activity does not block PRL activation of Jak2/Stat5 (Fresno Vara, Caceres et al. 2001). However, mutation of the adapter domain(s) of c-Src abrogates PRL stimulation of the Jak2/Stat5 signaling pathway (Garcia-Martinez, Calcabrini et al. 2010). These data indicate that s-Src can modulate activation of the Jak2/Stat5 pathway by its functional adapter domains but not

through its catalytic activity. CypA mediated PRL-induced conformational change alters Src interaction with the PRLr and may modulate its interaction with Jak2/Stat5 as well. The dynamic structural changes of the PRLr mediated by CypA likely has functional consequences to the receptor's proximal signaling molecules and likely affects their interaction with each other. It is possible that CypA modulation of the PRLr-ICD indirectly affects Src adaptor function or its ability to bind the receptor and/or Jak2/Stat5.

4.3.4 CypA inhibition modulates global gene expression signatures

NIM811 had significant effects on PRL-induced gene expression at both global and individual gene levels. The CISH and Cyclin D1 genes, which are involved in the pathogenesis of breast cancer (Arnold and Papanikolaou 2005, Borges, Moudilou et al. 2008), both demonstrated significantly reduced expression at the RNA and protein levels (Fig. 3.11), after NIM811 treatment. Transcriptomic analysis revealed the global contribution of CypA to PRLr-induced gene expression identifying several novel PRL-induced and NIM811-inhibited genes, for example, EGR3, KLF4, and LIN28A (Fig. 3.12, Table 3). Each of these genes has been found to contribute to the pathobiology of breast cancer, as follows: i) ERG3 is a regulator of estrogen mediated invasion and a potent prognostic factor in breast cancer (Suzuki, Inoue et al. 2007), ii) KLF4 is required for maintenance of breast cancer stem cells, migration and invasion (Yu, Li et al. 2011), and iii) Lin28A facilitates breast cancer metastasis and promotes the cell cycle by regulating Cyclin D1 (Xiong, Zhao et al. 2017). Functional analyses our microarray data revealed cancer, cellular proliferation/growth, migration/invasion and gene expression as the top molecular phenotypes affected by NIM811 treatment. The fact that NIM811 down-regulated 57% of the top 100 PRL-induced genes involved in a proliferative and

migratory/invasive phenotype is strengthened by our observation that NIM811 also decreased the proliferation, anchorage independent growth, and migration of breast cancer cells. Furthermore, when NIM811-inhibited genes were compared with the gene signature from kinase perturbation experiments in the GEO database, significant similarities between Jak2 knockdown, LRRK2 (kinase) mutant, GSK3β knockdown, SYK inhibition and IGF1 inhibition and NIM811-treated cohorts were observed. Not surprising, the Jak2 knockdown array generated the most similar gene signature as our CypA-inhibition array, further confirming the relationship between these enzymes. It is interesting to note that the leucine-rich repeat kinase 2 (LRRK2) mutant that induces progressive degeneration of human neural stem cells (Liu, Qu et al. 2012), is also associated with breast cancer (Waro and Aasly 2018), but its mechanistic role is poorly understood. Glycogen synthase kinase 3β (GSKβ) interacts with the PRLr (Plotnikov, Li et al. 2008) and is activated by the PRLr, hence the relation between it and the NIM811 gene set. Likewise, SYK kinase appears to be activated by the PRLr (Saha, Gonzalez et al. 2009). Similarly, IGF signaling directly impacts PRLr transduction and has a recognized role in breast cancer pathobiology (Carver, Piazza et al. 2010). Taken together, these findings suggest that CypA is a key regulator of PRL-induced global genes that are implicated in inducing malignant phenotypes in breast cancer.

4.4 CypA in mammary gland development, tumorigenesis and metastases

4.4.1 CypA and its functional role in mammary gland development

The developmental defect of mammary glands observed in $CypA^{-/-}$ mice and similarities to other loss of function mouse models including PRLr, Jak2 and Stat5 (Miyoshi, Shillingford et al. 2001, Shillingford, Miyoshi et al. 2002, Cui, Riedlinger et al. 2004)

demonstrates a role for CypA in physiologic regulation of mammary gland development and function. CypA$^{-/-}$ mice developed defective alveoli that continued during pregnancy, and it was primarily due to a lack of luminal clearing of the alveoli and secretory granule formation in the epithelium (Volker, Hedrick et al. 2018), as observed in other models mentioned earlier. However, CypA$^{-/-}$ mice were able to properly lactate and nurse their pups as assessed by the weight and viability of litters compared to other genetic deletion models. This phenotype is explained by a significant loss of Jak2/Stat5 signaling, but not complete loss, in mammary epithelium and breast cancer cells both *in vivo* and *in vitro* (Fig. 3.8)(Zheng, Koblinski et al. 2008, Volker, Hedrick et al. 2018).

Like PRLr$^{-/-}$ mice (Brisken, Kaur et al. 1999, Lee and Ormandy 2012), the defect in alveologenesis and lactatogenesis of CypA$^{-/-}$ mice are epithelial in nature, not stromal (Volker, Hedrick et al. 2018). Besides genetic knockout of CypA, treatment of CypA$^{+/+}$ mice with NIM811 led to the inhibition of alveolar budding as seen with the CypA$^{-/-}$ mice (Volker, Hedrick et al. 2018). Delayed or inhibited maturation of the epithelial hierarchy of stem, progenitor and mature luminal cells in the mammary gland is associated with defective mammary gland development (Asselin-Labat, Sutherland et al. 2007, Owens, Rogers et al. 2014). In CypA$^{-/-}$ mice, there is a decrease in mature luminal cells, whereas a significant increase in both stem and progenitor cell populations were observed (Volker, Hedrick et al. 2018), suggesting that there is a inhibition of progression to the mature luminal state resulting in developmental delays in these mice. It is likely that the action of CypA on mammary gland development is not only dependent on PRLr mediated Jak2 activation, but is also associated with kinases like Src, and downstream Akt and MAPK as demonstrated (Figs. 3.6 and 3.7).

Mammary gland development during pregnancy seems to be normal in Src $^{-/-}$ mice compared to Src $^{+/+}$ mice. It is likely that other Src family members, like Fyn, compensate in this process during pregnancy. Although PRL levels in the knockout mice are not changed compared to Src $^{+/+}$ mice, PRLr expression is decreased along with associated Stat5 phosphorylation, and expression of milk protein genes (Watkin, Richert et al. 2008). Such alternation in PRLr expression may be due to Src/Fyn association with other PRLr-associated proteins such as Stat5, PI3K, Tec and Vav (Clevenger and Kline 2001). Furthermore, the alterations in signaling lead to decreased secretory activity at lactation and epithelial involution, demonstrating that Src/Fyn also plays a crucial role for PRLr expression and functionality in mammary gland development (Watkin, Richert et al. 2008). In this study, we demonstrated that loss of CypA blocks Src association with the PRLr in a PRL dependent manner in breast cancer cells (Fig. 3.10), and such mechanism is likely involved in modulating PRLr expression and associated signaling during mammary gland development (Volker, Hedrick et al. 2008).

4.4.2 CypA regulation of TNBC metastasis

Two independent models of mammary carcinoma were examined; i) human xenografts and ii) the PyMT mouse model. Each model has its advantages and disadvantages: while human xenograft models are performed in an immunocompromised model that lacks an immune system, the PyMT model is purely murine, but has an intact immune system.

Treatment of the murine xenograft model of TNBC breast cancer with NIM811 resulted in decreased lymph node metastasis and induction of necrosis, which is likely due to changes in receptor proximal signaling (Figs. 3.6 and 3.8), gene expression (Figs. 3.11 and 3.12) and migration (Fig. 3.20). Interestingly, like CsA, NIM811 therapy did not have

a statistically significant effect on tumor weight *in vivo*. However, treatment of these xenografts with NIM811 induced central tumor cell death and the outgrowth of macro-metastasis was completely inhibited (Fig 5.2). There are a few potential mechanisms through which these NIM811-mediated effects on xenograft progression may have been executed. First, its blockade of the PRLr-Jak2-Stat5 pathway (Fig. 3.6), the activity of which has been linked to malignant phenotypes of human breast cancer (Holtkamp, Nagel et al. 1984); second, NIM811 treatment significantly reduced expression of the PRL-responsive regulatory genes CISH, Cyclin D1 (Fig. 3.11); and third, NIM811 inhibited PRL-induced global genes/network involved in promoting cancer malignancies (Fig. 3.12)(Table 3).

4.4.3 Complete loss of CypA blocks tumorigenesis and metastases in a transgenic mouse model

In precedent data, erbB2 x $CypA^{-/-}$ mice showed markedly delayed tumor latency/survival and reduced tumor multiplicity in comparison with the erbB2 x $CypA^{+/+}$ group. As observed in the TNBC xenografts and erbB2 x $CypA^{-/-}$ mice, CypA knockout mice crossed into a spontaneous tumor model also significantly altered mammary cancer progression (Volker, Hedrick et al. 2018). Similarly, when crossed into a PyMT model of transgene-driven mammary carcinogenesis (Muller, Sinn et al. 1988), PyMT x $CypA^{-/-}$ mice demonstrated a highly significant decrease in tumor burden in comparison with the PyMT x $CypA^{+/+}$ cohorts (Fig. 3.22). While this is likely due to the loss of phosphorylation of Jak2 and Stat5, and Stat5 driven gene expression, it is probable that the loss of interaction with other signaling proteins such as Tec, Vav and Fyn may also contribute to this phenotype (Kline, Roehrs et al. 1999). Loss of CypA in the PyMT mice demonstrated a

significant difference in lymph node metastasis compared to the wild-type control, even after correcting for tumor burden. These mice significantly reduced both number and area of metastasis. Although the CypA$^{-/-}$ and Jak2$^{-/-}$ mice show some parallels in their effects on tumor initiation and primary tumor growth (Zheng, Koblinski et al. 2008, Sakamoto, Lin et al. 2009, Sakamoto, Triplett et al. 2010), the inhibition of outgrowth of metastasis by the CypA knockout was highly significant unlike Jak2$^{-/-}$ mice. It is likely that CypA has other targets that are necessary for the metastatic phenotype. For example, the association of Vav family members with the PRLr, could alter PRL-induced cytoskeletal reorganization and motility/migration of human breast cancer cells (Maus, Reilly et al. 1999). The PRLr mediates activation of both Src and Fyn (Clevenger and Kline 2001) like Jak2. However, PRLr-associated Src or Fyn activation is not dependent on the association/activation of Jak2 with the PRLr (Fresno Vara, Carretero et al. 2000). As such, Jak2/Stat5 signaling is significantly decreased in mammary glands isolated from Src$^{-/-}$ mice compared to mammary glands from Src$^{+/+}$ mice (Watkin, Richert et al. 2008, Garcia-Martinez, Calcabrini et al. 2010). Like Jak2, Src kinase activity and its association with the PRLr inhibited by CypA knockout may co-operatively reduce Jak2/Stat5 signaling and mammary malignancies. In terms of mammary tumorigenesis, it can be inferred that the loss of CypA function may have a broader action on parallel signaling pathways in addition to those that are associated to PRLr/Jak2/Src complex. Although, our model of CypA regulation of the PRLr signaling is focused on the activation of Jak2/Src by CypA, a few Jak2 targets such as Akt, MAPK are also modulated by CypA PPI activity (Fig. 3.7)(Zheng, Koblinski et al. 2008).

4.4.4 The Jak2-Stat5 axis and Stat5 duality in mammary cancer progression

Constitutive activation of the Jak/Stat pathway is a hallmark of many cancers including breast, prostate, head and neck, and lymphoma (Yu and Jove 2004). Stat3 and Stat5 are activated in a significant proportion of breast tumors (Cotarla, Ren et al. 2004, Diaz, Minton et al. 2006) and both have been demonstrated to be mammary oncogenes (Barbieri, Pensa et al. 2010, Vafaizadeh, Klemmt et al. 2010). We have demonstrated that PRL induces tyrosine phosphorylation of Stat5, whereas CypA inhibition significantly blocks pYStat5 (Fig. 3.6 and 3.8). Our data indicated that erbB2 x $CypA^{+/+}$ has significantly higher levels of pYStat5 and CISH expression compared to erbB2 x $CypA^{-/-}$ mice (Volker, Hedrick et al. 2018), confirming the importance of pYStat5 in tumorigenesis. However, the role of Stat5 during mammary oncogenesis and metastasis is debatable. Nuclear localized pYStat5 has been investigated in breast cancer tissues with less than 25% of breast cancer expressing pYStat5 with the lowest levels during the metastatic stage. The link that nuclear-localized pYStat5 is connected to a favorable prognosis (Nevalainen, Xie et al. 2004, Peck, Witkiewicz et al. 2011) led to the hypothesis that Stat5 is not involved in breast cancer progression. However, this assumption failed to acknowledge that Stat5 promotes the survival and anchorage independent growth of human breast cancer *in vitro* (Tang, Zuo et al. 2010), while mouse models have demonstrated that gain- and loss-of function of Stat5 led to enhanced vs. decreased mammary tumorigenesis (Humphreys and Hennighausen 2000, Ren, Cai et al. 2002, Iavnilovitch, Cardiff et al. 2004). The duality of Stat5 (oncogenesis vs differentiation) can be explained by the observation that pYSTAT5 is not a perfect surrogate for Stat5 induced gene expression. In contrast to pYStat5, un-phosphorylated Stat5 (upYStat5) is found in the nucleus of more than 75% of primary and metastatic breast cancers (Watson and

Miller 1995, Cotarla, Ren et al. 2004). Furthermore, pYStat and upYStat have merely 30% of the global gene activation sets (Yang, Chatterjee-Kishore et al. 2005) and upStats can dimerize in the absence of ligand-induced tyrosine phosphorylation, nuclear translocation and activation of target genes unlike pYStat5 (Braunstein, Brutsaert et al. 2003, Yang and Stark 2008). We have demonstrated that CypA inhibition significantly suppressed more than 50% of the PRL-induced Stat5 target genes in breast cancer cells (Fig. 3.13). Although Stat5 tyrosine phosphorylation is important for tumorigenesis, the upStat5 may be driving the observed lymph node and lung metastasis in the PyMT x $CypA^{+/+}$ mice (Fig. 3.23) by regulating Stat5 target genes. Therefore, Stat5 can function both as a differentiating factor and oncogene in mammary oncogenesis, depending upon its phosphorylation status.

4.4.5 Jak/Stat and/or Src targeted therapy in combination with NIM811 and their potential in breast cancer

Jak-Stat signaling is critical for cancer development in both tumor cells and the tumor microenvironment, and both Jak and Stat have emerged as important targets for cancer treatment (Yu and Jove 2004). Ruxolitinib, tofacitinib, and fludarabine are the molecularly targeted drugs against Jak/Stat pathways that have shown limited efficacy in breast cancer therapy (Bousoik and Montazeri Aliabadi 2018). However, dual inhibition of Jaks and SFKs by the novel compound E738 blocks phosphorylation of Jak2 and Src leading to an induction of tumor cell death and inhibition of Stats (Nam, Wen et al. 2013). Strategies that disrupt protein-protein interactions needed for Stat phosphorylation or inhibit Stat-DNA binding have shown promise in preclinical studies by inducing apoptosis and decreasing proliferation/growth both *in vitro* and *in vivo*. For example, withacnistin

prevents the recruitment of Stats for phosphorylation by inhibiting binding of Stat3 and Stat5 to the cytoplasmic region of receptors (Zhang, Blaskovich et al. 2014). NIM811 effectively also blocks phosphorylation of Jak2/Src/Stat5, downstream gene expression, lymph node/lung metastasis, while inducing central tumor necrosis, making this drug an attractive targeted therapy in combination with Jak2/Src/Stat5 inhibitors for breast cancer patients. However, further pre-clinical/clinical studies need to be conducted to demonstrate the efficacy of these drugs in combination.

4.5 Implication of NIM811 therapy in breast cancer

NIM811 treatment significantly inhibits proliferation, anchorage-independent growth and migration of breast cancer cells. Induction of apoptosis including activation of cleaved-PARP and -Caspase are observed only at very high doses of NIM811. In clinic, having minimal drug related side effects could possibly translate into an advantage in either dose titration or usage in combination with other chemotherapeutic drugs.

One of the first studies that provided data linking CsA treatment with reduction in breast cancer incidence had over 23,000 female patients, of whom around 19,000 received CsA after kidney transplant surgery. In this longitudinal study, the CsA treated cohort was followed for 1-11 years and 86 de novo breast cancer cases were observed compared to the 114 expected cases. The decrease in breast cancer incidence in the female kidney transplant patients was rather significant compared to the general population in the first post-transplantation year (Stewart, Tsai et al. 1995). Although there was a clear benefit in decreasing breast cancer incidence among transplant patients receiving CsA, there may be other cancer risk associated with CsA treatment. There have been studies in animals and humans indicating an increased risk of gastrointestinal, epidermal and

lymphoid cancer following CsA treatment (Penn and First 1986, Hojo, Morimoto et al. 1999). This increased incidence in cancer may be due CsA mediated immunosuppression and associated side effects. In this context, the non-immunosuppressive NIM811 is a superior alternative to CsA. NIM811 was well tolerated, with no significant toxicity during our *in vivo* study of breast cancer xenografted mice. NIM811 therapy markedly inhibited tumor growth, metastasis and associated signaling in breast cancer cells and mammary cancer models. These significant findings have a few important implications. First, these data support an *in vivo* biologic role for CypA and its PPI activity. Second, they address the crucial role for CypA as a metastasis promoter and potential regulator of the central tumor microenvironment. Our studies have elucidated the fundamental mechanisms of CypA action on PRLr structure and functional relationships as well as proximal signaling in breast cancer cells. This sheds light onto the prospects for translational opportunities for development of NIM811 analogs and/or novel PPI-inhibitory therapies directed against CypA in human breast cancer. NIM811 or an analogue may become a best in class targeted therapy for ER/PR$^{-/+}$ breast cancer patients, based on its specificity, minimal side-effects, non-immunosuppressive quality and efficacy in blocking mammary cancer metastasis.

A few analogues of cyclosporine, besides NIM811, have shown efficacy in recent pre-clinical /clinical studies. One of these such non-immunosuppressive selective inhibitors of CypA is Alisporivir (Debio025) that demonstrated an anti-viral effect with pegylated interferon-alpha (pIFN-α) in patients with genotype 1 and 4 HCV (Flisiak, Feinman et al. 2009). Another potent CypA inhibitor is MM218, which can selectively inhibit the extracellular CypA with no detectable adverse effects in an animal model of acute lung

injury and has stronger anti-inflammatory effects than CsA (Balsley, Malesevic et al. 2010). Overall, these data suggest that targeting CypA and its associated pathway are highly relevant for human breast cancer therapeutics.

4.6. Proposed Model

Our data presented here elucidate the fundamental mechanisms of how CypA regulates PRL-induced conformational change of the PRLr that modulates receptor proximal signaling in breast/mammary cancer progression. In response to PRL stimulation, CypA induces a conformational change in the PRLr-ICD and phosphorylates/activates Jak2-Stat5 signaling intermediates. Phosphorylated/activated PRLr, Jak2, and Src promote nuclear translocation of Stat5, which propagates signals to induce global gene expression, including CISH and Cyclin D1, and modulates breast cancer cell functions. Thus, CypA regulates PRL-induced conformational change of the PRLr-ICD, leading to activation of the PRLr/Jak2/Src complex that promotes signaling, gene expression, breast cancer cell malignancies, mammary tumorigenesis and metastasis (Fig. 4.2).

4.7 Future Direction

NIM811 therapy had profound effects on tumorigenesis, metastasis and associated signaling in breast cancer cells and mammary cancer models. As a therapeutic agent, NIM811 has minimal toxicity and has successfully undergone patient trials for safety in patients with genotype 1 HCV patients (Lawitz, Godofsky et al. 2011). Additional pre-clinical studies with existing and novel breast cancer therapeutics is warranted to identify additional synergies, but trials in breast cancer patients could occur soon. Our future work will include a pre-clinical drug study using the MMTV-PyMT model, patient-derived

xenograft studies (PDX) as well as a phase II clinical trial for breast cancer patients. Since the original developer (Novartis) of NIM811 is not actively pursuing the drug for pre-clinical/clinical studies, we will utilize the NIM811 analogue, Debio025 (Debio) for both animal studies and the human clinical trial. We will also employ an FDA approved PI3K inhibitor, Alpelisib (Novartis) use with Debio025, as for combination therapy. The rational for using Alpelisib is because of clinically meaningful benefit of this drug for patient with advanced or metastatic breast cancer in a phase III trial. Furthermore, Alpelisib targets PRLr downstream PI3K/Akt signaling pathway and has potential for synergistic benefits for patient when use with Debio025, as combination therapy.

Debio025 therapy for MMTV-PyMT mice

Although we have generated and used the $PyMT^{+}$ x $CypA^{-/-}$ model to investigate the role of CypA in mammary tumorigenesis and metastasis, the efficacy of the NIM811 analogue/Debio025 in an immune-competent mouse model is yet to be determined. To assess the effects of Debio025 therapy in mammary cancer progression, MMTV-PyMT mice will be treated with vehicle control and ED100 dose of Debio025. Cohorts (n=15) will undergo therapy at different times such as 4 weeks, 8 weeks and 11 weeks to assess if the drug treatment can inhibit the progression of the mammary cancers from one stage to the next, such as i) pre-invasive to invasive and/or ii) invasive to metastasis. After 4-6 weeks of therapy, tumor sections will be harvested for H & E staining/analyses and tumor lysates prepared to measure phospho/total levels of PRLr, Jak2, Stat5, and Src, in order to determine expression of downstream genes (Cyclin D1, CISH etc.) and assess Ki67 and cleaved caspase 3 *in vitro*. As seen in the PyMT x $CypA^{-/-}$ model, we anticipate that

Debio025 therapy will markedly reduce tumor burden and inhibit lymph node and lung metastases.

Patient-derived xenografts (PDX)

To further asses the efficacy of Debio025 in a human breast cancer relevant model, we will perform a drug study using PDX models. We will xenograft HCIO3 (ER^+/luminal B) and the WHIM2 (TNBC) lines that express high levels of PRLr, and also contain CFP/luciferase expression constructs for live imaging in mice (Alzubi, Turner et al. 2019). Xenografted mice will be treated with i) vehicle control, ii) monotherapy (Debio025) or iii) combination therapy (Debio025 + Alpelisib) using methods as described (Alzubi, Turner et al. 2019). Upon completion of treatment regimens, tissue/tumors will be characterized to assess signaling, tumorigenesis and metastases. Furthermore, RNA sequencing of tumors, metastases, and normal non-metastatic organs will be performed to identify cancer- and organ-specific genomic properties that mediated metastasis. Lastly, gene set enrichment analyses will be conducted to determine gene signatures that are activated in metastases across TNBC PDXs.

Phase II breast cancer study

Based on the effects of i) Debio025 or ii) Debio025 + Alpelisib therapy on the progression of early or late stage lesions in the PDXs and existing data from a phase I study, we will design a phase II clinical trial for breast cancer patient following progression on or after an endocrine-based regimen. We will design a randomized three-arm phase II trial: i) standard of care chemotherapy, ii) standard of care chemotherapy + Debio025 and iii) standard of care chemotherapy + Debio025 + Alpelisib. This randomized trial is to assess

the effectiveness of Debio025 or combination therapy in treating patients with invasive breast cancer. The primary objective is to determine whether addition of Debio025 or both Debio025 + Alpelisib would improve disease or progression-free survival. We would expect minimal adverse events for patients based on the remarkable pharm/tox profile of NIM811/Debio025. We anticipate that addition of Debio025 or combination therapy with standard of care chemotherapy would significantly improve disease- or metastasis-free survival for breast cancer patients.

4.8 Summary and concluding remarks

CypA induces PRL-dependent conformational change of the PRLr-ICD, whereas inhibition of CypA or loss of its expression, impedes PRL-induced conformational change of the receptor. PRL-induced conformational change in the PRLr-ICD leads to an increase in the phosphorylation/activation of PRLr, Jak2, Src and Stat5 at specific tyrosine residues that are believed to be important for interaction/signaling in breast cancer cells. However, CypA inhibition or knockdown significantly reduces phosphorylation/activation of these signaling intermediates. Loss of CypA not only decreases phosphorylation, but also the association of Jak2, Src and Stat5 with the PRLr. A microarray analysis of the global gene expression profile indicated that NIM811 inhibited about 66% of the PRL induced genes in ER/PR$^+$ (T47D) breast cancer cells. These proximal signaling events leading to regulation of downstream genes had profound effects on biological functions of breast cancer cells including cell proliferation, survival, anchorage independent growth and migration *in vitro*. Subsequent pre-clinical testing of NIM811 in relevant mouse mammary cancer models has revealed that NIM811 treatment of a TNBC xenograft inhibited primary tumor growth and induced central tumor necrosis. Furthermore, loss of

CypA in the MMTV-PyMT mouse model demonstrated inhibition of tumorigenesis with significant reduction in lung and lymph node metastases. Overall, these results indicate that the PPI activity of CypA modulates PRLr/Jak2 complex signaling/functions in breast cancer and mammary epithelium, identifying this isomerase as a novel target for therapeutic intervention.

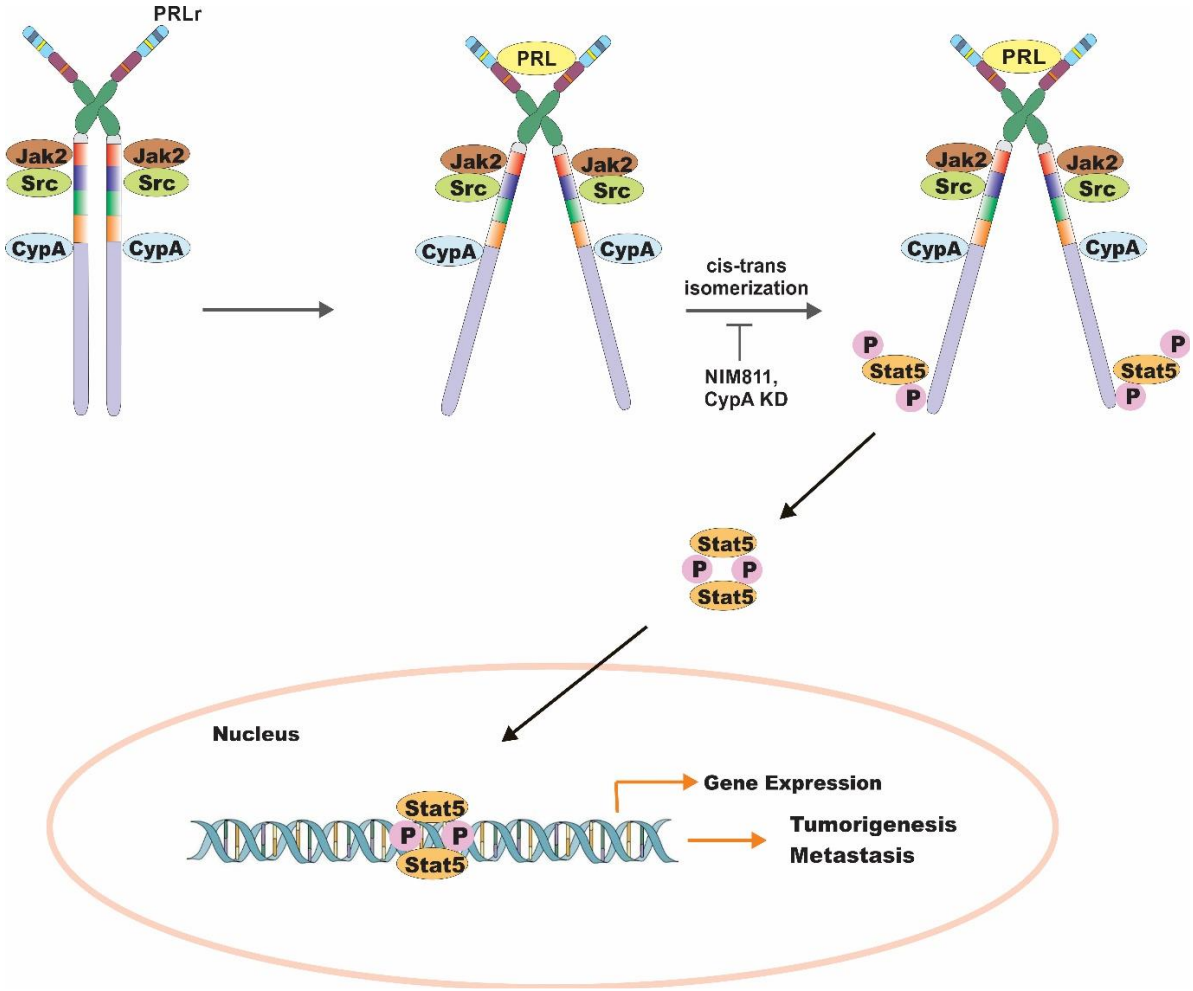


Figure 4.2 CypA as a signaling switch regulating prolactin receptor mediated signaling, mammary tumorigenesis and metastasis. Following prolactin stimulation, CypA induces a conformational change of the PRLr-ICD and Jak2 is activated through autophosphorylation. Phosphorylation of the PRLr-ICD recruits Stat5 to the PRLr and subsequently phosphorylated by Jak2. Phosphorylated Stat5 dimerizes and translocates to the nucleus. In the nucleus, it induces expression of its target genes and promotes tumorigenesis/metastasis in breast cancer.

Chapter 5: Appendices

5.1 Stable expression of CypA shRNAs specifically knockdown CypA and modulates PRLr-Stat5 signaling intermediates

Pharmacological inhibition of CypA PPI activity by NIM811 has significant effects in phosphorylation/activation of the PRLr, Jak2, Stat5 and Src in a PRL dependent manner. To assess whether CypA knockdown could modulate Jak2-Stat5 signaling intermediates, we knockdown CypA expression in T47D cell using CypA shRNA(s) and compared to the control shRNA. To demonstrate specificity, we have utilized two different shRNA sequences targeting CypA. Compared to shRNA control, both sequences targeting CypA significantly knockdown its expression. Following PRL stimulation for 15 minutes, pPRLr-Y381, pPRLr-Y587, pJak2-Y1007/1008, pSrc-Y416 and pStat5-Y694 are markedly induced in shRNA control condition in a PRL dependent manner compared to un-stimulated condition. In contrast, both shRNA constructs effectively downregulate phosphorylation/activation of the above signaling intermediates in a PRL dependent manner compared to PRL-induced control shRNA. These results demonstrate both CypA shRNA sequences effectively knockdown CypA expression and downregulate activation/phosphorylation of PRLr-Stat5 signaling intermediates.

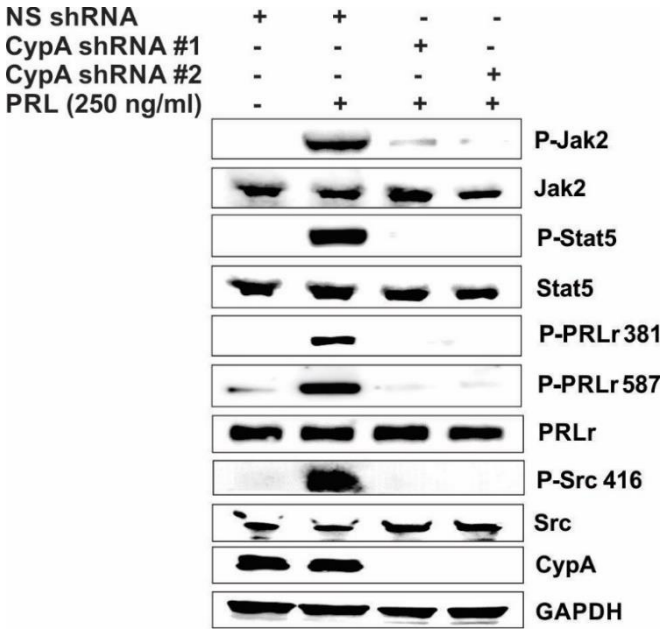


Figure 5.1 Stable expression of CypA shRNAs specifically knockdown CypA and modulates PRLr-Stat5 signaling intermediates. Stable expression of non-silencing control or CypA shRNA in T47D were serum starved for 16-24 hours, stimulated with PRL (250 ng/ml) for 15 minutes and blots were probed with the indicated antibodies. Representative blots of at least two independent experiments.

5.2 NIM811 therapy prevents macrometastasis of breast cancer xenograft.

To assess whether NIM811 can prevent metastasis of breast cancer, GFP-labeled MCF7 cells were injected in the fourth lactiferous ducts of nude mice as performed earlier. When xenograft tumors were formed for 2 weeks, mice were treated with either carrier control or NIM811 (50 mg/kg/day) for 6 weeks. Interestingly, while individual GFP-positive MDA-231 cells were observed in xenograft lymph nodes, the outgrowth of macro-metastasis was completely inhibited in these mice (Fig. 5.2) based on IF analyses of GFP-labeled xenograft and IHC confirmation with a human HLA1 antibody.

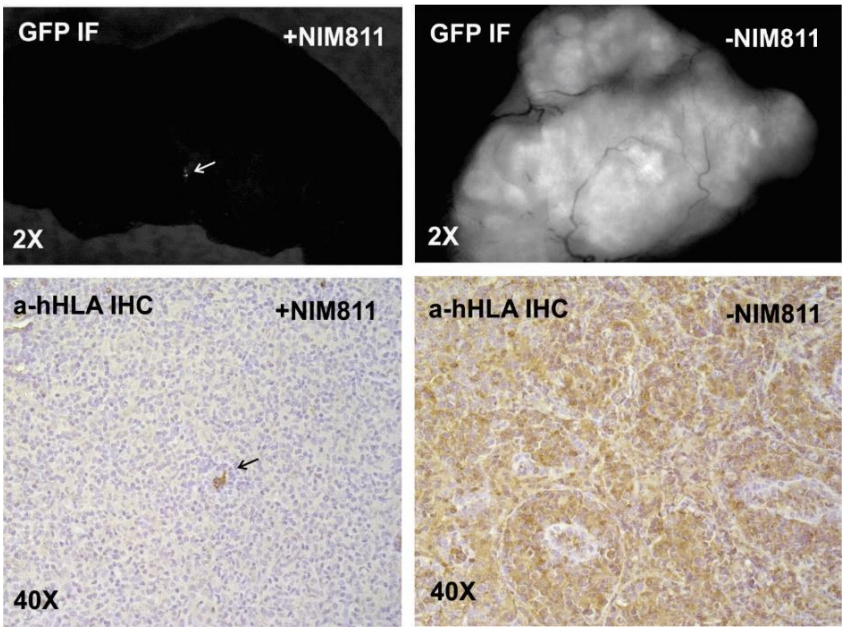


Figure 5.2 NIM811 therapy prevents macrometastatic outgrowth in GFP-labeled MCF7 xenografts. IF analyses for GFP label present in the xenografts treated 6 weeks with 50 mg/kg/day NIM811 (or carrier control; treatment initiated after 2 weeks of outgrowth) is presented in the upper panels; IHC confirmation with a-human HLA1 antibody is seen in lower panels. Magnification as indicated.

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