MECHANISTIC INSIGHT INTO THE PROLACTIN-/ANDROGEN-INDUCIBLE CARBOXYPEPTIDASE-D AND EDD E3 UBIQUITIN LIGASE GENES IN TRIPLE-NEGATIVE BREAST CANCER

by

Tyler Matthew MacDonald

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DEDICATION PAGE

I dedicate the entirety of this thesis to my late grandfather, Douglas Reginald Cosman.

I hope I am making you proud.

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ABSTRACT

Membrane-bound Carboxypeptidase-D (CPD) hydrolyzes C-terminal arginine residues from extracellular substrates. Released arginine, taken into cells, is converted into nitric oxide (NO). Our laboratory has shown that CPD mRNA/protein levels are upregulated by prolactin (PRL), 17β-estradiol, and androgen testosterone in breast cancer (BCa) cells, in turn, increasing NO production to promote BCa cell survival. EDD E3 ubiquitin ligase identified by differential display (EDD) is an E3 ubiquitin ligase protein normally involved in protein turnover, however it is frequently overexpressed in cancer cells. EDD has been implicated by our laboratory in the mammalian-target-of-rapamycin (mTOR) signalling pathway that regulates the initiation of protein translation. Like CPD, EDD protein levels are upregulated by hormones, specifically PRL, progesterone, and synthetic androgen R1881. This study investigated the role of PRL/androgen-inducible EDD and CPD in BCa, particularly in the aggressive triple-negative breast cancer (TNBC) subtype. This was accomplished *in vivo* by immunohistochemical analysis of EDD in archival human breast specimens as well as examining the effect of high EDD or CPD mRNA expression on the survival probability of BCa patients. A multitude of *in vitro* experiments were conducted to demonstrate hormonal upregulation of EDD or CPD and the biological effects of altered EDD or CPD gene expression in TNBC cell lines. The present study showed that high CPD mRNA expression in BCa patients correlated with a poorer probability of relapse-free survival. CPD protein expression was upregulated by PRL and R1881 in a time-/dose-dependent manner and was accompanied by increased intracellular NO production, which enhanced TNBC cell survival *in vitro*. Depletion of CPD from TNBC cell lines using small-interfering ribonucleic acid (siRNA) resulted in decreased TNBC cell viability. EDD immunostaining was shown to be low in benign human breast tissue but increased with breast cancer progression *in vivo*. High EDD mRNA expression correlated with a poorer probability of overall and relapse-free survival in BCa and TNBC patients, respectively. EDD protein expression was upregulated by PRL and R1881 in a time-/dose-dependent manner *in vitro* and was speculated to occur at the transcriptional level due to the presence of putative hormone response elements in the EDD gene promoter. SiRNA-mediated knockdown of EDD gene expression induced a variety of effects including decreased TNBC cell viability, increased expression of pro-apoptotic-associated proteins MOAP-1 and Bax, cleavage of Caspase-7 and PARP-1, decreased PRL-/R1881-induced phosphorylation of initiation factor 4E binding protein-1 (4E-BP1), and subsequent 4E release, as well as decreased resistance to anti-cancer drugs. In summary, PRL/R1881-inducible CPD increased TNBC cell survival through the production of NO. EDD levels increased with BCa progression and loss of PRL/R1881-inducible EDD decreased TORC1 signalling, promoted pro-apoptotic protein expression, and decreased anti-cancer drug resistance in BCa cells. Collectively, this work supports EDD and CPD as therapeutic targets for BCa, including TNBC, and suggest that EDD and CPD expression may predict BCa responsiveness to various anti-cancer drug treatments.

LIST OF ABBREVIATIONS AND SYMBOLS USED

4E-BP1	Eukaryotic initiation factor 4E-binding protein 1
5′-TOP	5′-terminal oligopyrimidine tract
AF-1	Activation function-1
AF-5	Activation function-5
Akt	Protein kinase B
ANOVA	Analysis of variance
AR	Androgen receptor
ARE	Androgen response element
ATP	Adenosine triphosphate
BCa	Breast cancer
BH3	BCL-2 homology 3
CPA	A-type carboxypeptidase
CPB	B-type carboxypeptidase
CPD	Carboxypeptidase-D
CS-FBS	Charcoal-stripped fetal bovine serum
Ct	Cycle threshold
DAF-2	4,5-Diaminofluorescein
DAF-2DA	4,5-Diaminofluorescein diacetate
DAF-2T	4,5-Diaminofluorescein triazole
DBD	DNA-binding domain
DETA-NONOate	(Z)-1-[N-(2-aminoethyl)-N-(2 -ammonioethyl)amino]diazen-1-ium-1,2-diolate
DHT	Dihydrotestosterone
DMEM	Dulbecco's modified eagle medium
DNase I	Deoxyribonuclease I
EDD	E3 ligase identified by differential display
eIF4E	Eukaryotic initiation factor 1
EGFR	Epidermal growth factor receptor
eNOS	Endothelial nitric oxide synthase
ER	Estrogen receptor
FAR	Furyacryloyl-alanyl-arginine
FBS	Fetal bovine serum
HECT	Homologous to E6-associated protein C-terminus
HEPES	4-(2-hydroxyethyl)-1-piperazineethane sulfuric acid
HER2	Human epidermal growth factor receptor-2
hPRL	Human prolactin
hPRLR	Human prolactin receptor

HRP	Horse radish peroxidase
IC_{50}	Half-maximal inhibitory concentration
iNOS	Inducible nitric oxide synthase
GAS	Gamma-interferon regulatory sequence
Jak2	Janus kinase 2
m^7GTP	7-methyl guanosine triphosphate
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MEM	Minimum essential medium
MOAP-1	Modulator of apoptosis protein-1
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target-of-rapamycin
mTORC1	Mammalian target-of-rapamycin complex 1
mTORC2	Mammalian target-of-rapamycin complex 2
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
P70S6K	Ribosomal protein S6 kinase beta-1
PARP-1	Poly-ADP ribose polymerase-1
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol 3-kinase
PP2Ac	Catalytic subunit of protein phosphatase 2A
PR	Progesterone receptor
PRL	Prolactin
PRLR	Prolactin receptor
Q3Q1	Top 25% vs bottom 25%
RING	Really interesting new gene
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
RT- qPCR	Reverse transcription quantitative polymerase chain reaction
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SH2	Src Homology 2
siRNA	Small-interfering ribonucleic acid
STAT	Signal transducer and activator of transcription
TBST	Tris-buffered saline tween-20

TEMED	Tetramethylethylenediamine
TNBC	Triple-negative breast cancer
UBR5	Ubiquitin Protein Ligase E3 Component N -Recognin 5

Weights and measures

ºC	Degree Celsius
λ	Wavelength
h	Hour
kDa	Kilodalton
n	Number
ml	Milliliter
µl	Microliter
M	Molar
mM	Millimolar
µM	Micromolar
nM	Nanomolar
µg	Microgram
ng	Nanogram
bp	Basepair
kbp	Kilo basepair
g	Acceleration of gravity
cm	Centimeter
nm	Nanometer
P	Probability
ρmol	Picomole
v/v	Volume/volume
w/v	Weight/volume
V	Volt

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CHAPTER 1. INTRODUCTION

1.1 Preface

Breast cancer can be cured when diagnosed early, and the options for treatment depend on the cancer subtype, of which there are four subtypes. Each subtype has different expression levels of hormone receptors. Outcomes are poor for women whose breast cancer cells lack the estrogen, HER2 and progesterone receptors, referred to as triple-negative breast cancer (TNBC). These women rely on chemotherapy for treatment since they will not respond to drugs that target these receptors. Our laboratory has shown that even in these women, their TNBC tumours have the prolactin and androgen receptors which can turn on pathways to stimulate cell division. One pathway involves a prolactin- and androgen-inducible protein called Carboxypeptidase-D (CPD). Plasma membrane-bound CPD helps to produce Nitric Oxide (NO) in the cell and NO helps cancer cells to survive. A second prolactin- and androgen-inducible protein, E3 ubiquitin ligase identified by differential display (EDD) also acts to promote cancer cell survival. Our laboratory has detected active CPD/NO and EDD pathways in TNBC. My research objective was to support the idea of blocking the prolactin and/or androgen receptor to suppress these pathways that promote cancer cell survival. This research has put forth evidence that may contribute to the design of new therapies, e.g., prolactin/androgen receptor antagonists, anti-CPD-NO and/or anti-EDD, as additional or alternative treatments to chemotherapy for aggressive breast cancer.

1.2 Breast Cancer

1.2.1 Statistics

As of 2017, cancer is the leading cause of death in Canada, with 1 in 2 Canadians diagnosed with cancer (1). There were 206,200 new cancer cases in Canada in 2017 (1), with Nova Scotia ranked as one of the top three provinces with the highest cancer incidence rates, behind New Brunswick and Newfoundland and Labrador. Breast cancer (BCa) is the most commonly diagnosed cancer among Canadian women, with 1 in 8 women diagnosed with BCa in 2017. Of these women, approximately 5,000 died from the disease (1). Provincially, it is estimated that 790 women were diagnosed with BCa out of 6,200 new Nova Scotian cancer cases in 2017 (1).

1.2.2 Subtypes of Breast Cancer

BCa is classified into four main molecular subtypes. The luminal-A subtype, represents 50-60% of all BCa, is characterized by high expression of the estrogen receptor (ER) and/or progesterone receptor (PR) but are negative for human epidermal growth factor receptor-2 (HER2), and contain low levels of proliferating cell nuclear antigen Ki-67 (2, 3). Luminal-A BCa patients have a good prognosis with a low relapse rate compared to other BCa subtypes (3). The luminal-B subtype, represents 15-20% of all BCa, is defined as ER-positive, PR-positive, HER2-positive or negative, and high levels of Ki-67 (3). Of note, the cut-off for Ki-67 low, as in luminal-A, and Ki-67 high, as in luminal-B, has not yet been standardized and, thus, is not validated for clinical application (4). The luminal-B subtype has a more aggressive phenotype, worse patient prognosis, higher recurrence rate and lower overall patient survival rate compared to luminal-A (5, 6). The HER2-positive subtype represents 15-20% of all BCa, is

characterized by high expression of the HER2 gene and genes associated with the HER2 pathway. Approximately 50% of HER2-positive tumors are ER-positive, except at lower levels (3). HER2-positive tumours present aggressively, are highly proliferative, and have a poor patient prognosis if not treated (3). Finally, the triple-negative breast cancer (TNBC) subtype, accounts for 8-37% of all BCa (7), is characterized by a lack of ER, PR and HER2 protein expression.

1.2.3 Triple-Negative Breast Cancer

TNBC is associated with higher mortality rates, as these cancers exhibit high cell proliferation, copy number imbalances and, in most cases, mutations in the *TP53* gene (8–10). TNBC is more common in women with African ancestry than any other ethnic origin (11). Further subclassification of TNBC yields two additional subtypes: basal-like and claudin-low. Basal-like tumors represent 50-75% of the TNBC subtype and express high levels of basal epithelial markers such as laminin, cytokeratin 5 and cytokeratin 14 (3). Basal-like tumors are also highly proliferative as indicated by positive staining for the proliferation marker Ki-67 using immunohistochemistry (8). Claudin-low tumors express low levels of several genes involved in epithelial cell-to-cell tight junctions, including claudin-3, -4, and -7, E-cadherin, and occludin (12). Claudin-low tumors also present with features of stem cellness and epithelial-mesenchymal transition (12). TNBC tumors, whether basal-like or claudin-low, are highly aggressive, highly metastatic to the brain and lung, and patients have a very poor prognosis (13).

1.2.4 Therapies for Breast Cancer

The main treatment modalities for BCa consist of surgery, radiation therapy, endocrine therapy, chemotherapy, and targeted therapy. For endocrine therapy

specifically, the selective estrogen receptor modulator tamoxifen is now a standard treatment for luminal-A/-B tumours (14). The standard of care for HER2-enriched BCa consists of the monoclonal antibody trastuzumab coupled with chemotherapy (15). Chemotherapy regimens are primarily recommended for TNBC and HER2-positive tumours, and is often used in combination with endocrine therapy in high-grade luminal-A/-B tumours. Typical chemotherapy regimens are administered for 12-24 weeks and are comprised of combinations of anthracyclines (e.g., doxorubicin), alkylating agents (e.g., cisplatin), taxanes (e.g., paclitaxel) , and antimetabolites (e.g., methotrexate) (14). Doxorubicin exerts its primary action by binding to DNA-associated enzymes such as topoisomerase I/II and intercalating into DNA to interfere with its uncoiling, leading to apoptosis (16). Cisplatin acts by crosslinking purine bases in DNA and interfering with DNA repair and replication mechanisms (17). Paclitaxel acts by stabilizing microtubule polymerization during mitosis whilst preventing depolymerization, resulting in arrest of cells in G2/M and subsequent apoptosis (18). Methotrexate inhibits dihydrofolate reductase, thereby preventing folic acid production and, as a result, inhibiting DNA and RNA synthesis (19). Although effective, resistance to cisplatin and doxorubicin, amongst other chemotherapeutic drugs, often occurs in many recurrent tumours.

To combat chemotherapeutic drug resistance, attention had shifted towards personalized medicine which aims to identify new targets and develop novel therapies against those targets specific to the disease of each patient. One such target, prolactin, has emerged as a potential therapeutic target given its role in tumorigenesis (see 20 for a review).

1.3 Prolactin

Prolactin (PRL) is a polypeptide hormone secreted by lactotrophs in the anterior pituitary. PRL belongs to group I of the helix bundle protein hormones, a family of cytokines that also includes growth hormone and placental lactogen (21, 22). PRL has a variety of physiological functions, including reproduction, homeostasis, metabolism, osmoregulation, and immunoregulation (see 21 for a review). PRL is encoded on chromosome six by a 10 kb gene with five exons and four introns (23, 24). Once translated, PRL is arranged as a single chain of 199 amino acids with three intramolecular disulfide bonds and a molecular mass of 23 kDa (23, 25). Alternative splicing, proteolytic cleavage, and post-translational modifications, such as phosphorylation and glycosylation, can alter the molecular weight and activity of PRL (26–28).

1.3.1 Sites of Prolactin Gene Expression

Lactotrophs are specialized cells that secrete PRL and they comprise 20-50% of the total cell population in the anterior pituitary (22). PRL is also synthesized and secreted by the brain (29), placenta (30), decidua (31), mammary gland (32), and lymphocytes in the immune system (33). Transcriptional regulation of the PRL gene occurs at two independent promoters. The proximal region controls the anterior pituitary-specific transcription, whereas a distal promoter, 5-7 kbp upstream of exon 1, controls extrapituitary transcription (34, 35).

1.3.2 Prolactin Receptor

Belonging to the class I cytokine receptor superfamily (36, 37), the PRLR is a single membrane-bound protein containing an extracellular domain, a transmembrane domain, and an intracellular domain (38). PRLR is encoded on chromosome six by a

gene over 100 kbp in length with ten exons (36, 37). Three different tissue-specific promoters exist for transcriptional regulation of the PRLR. Promoter I is specific for the gonads, promoter II is specific for the liver, and promoter III is present in gonadal and non-gonadal tissues (39). Transcription from these different promoters, as well as alternative splicing, gives rise to the many reported PRLR isoforms in a diverse range of tissues (22, 40). There are seven known PRLR isoforms in humans each of which exhibit a different signalling capacity (see 38, 39 for reviews). The major 598 amino acid PRLR isoform contains a 210 amino acid N-terminal extracellular ligand-binding domain, a 24 amino acid transmembrane domain, and a 364 amino acid intracellular domain (42). PRL and placental lactogens are known to activate the PRLR in humans. Growth hormone in primates can also activate the PRLR. Given that circulating PRL is ten-fold higher than growth hormone, and placental lactogens are restricted to gestation, PRL is considered the main physiological ligand of the hPRLR (42, 43).

1.3.3 Prolactin Receptor Signalling

Activation of the PRLR occurs through ligand-induced receptor dimerization resulting in a trimeric complex of one ligand and two PRLR moieties (39, 41) (See Figure 1). Intermolecular interactions between the high-affinity site 1 on PRL and the first PRLR moiety drive the interaction of site 2 on PRL with a second PRLR moiety. The two extracellular domains of the PRLR moieties then interact with each other (42, 45, 46). Downstream signalling then occurs through a variety of different protein kinases, the central one being Janus tyrosine kinase 2 (Jak2). Jak2 is constitutively bound to each of intracellular domains of the PRLR moieties (47). Jak2 proteins transphosphorylate each other upon receptor dimerization (48) and then phosphorylate tyrosine residues in the

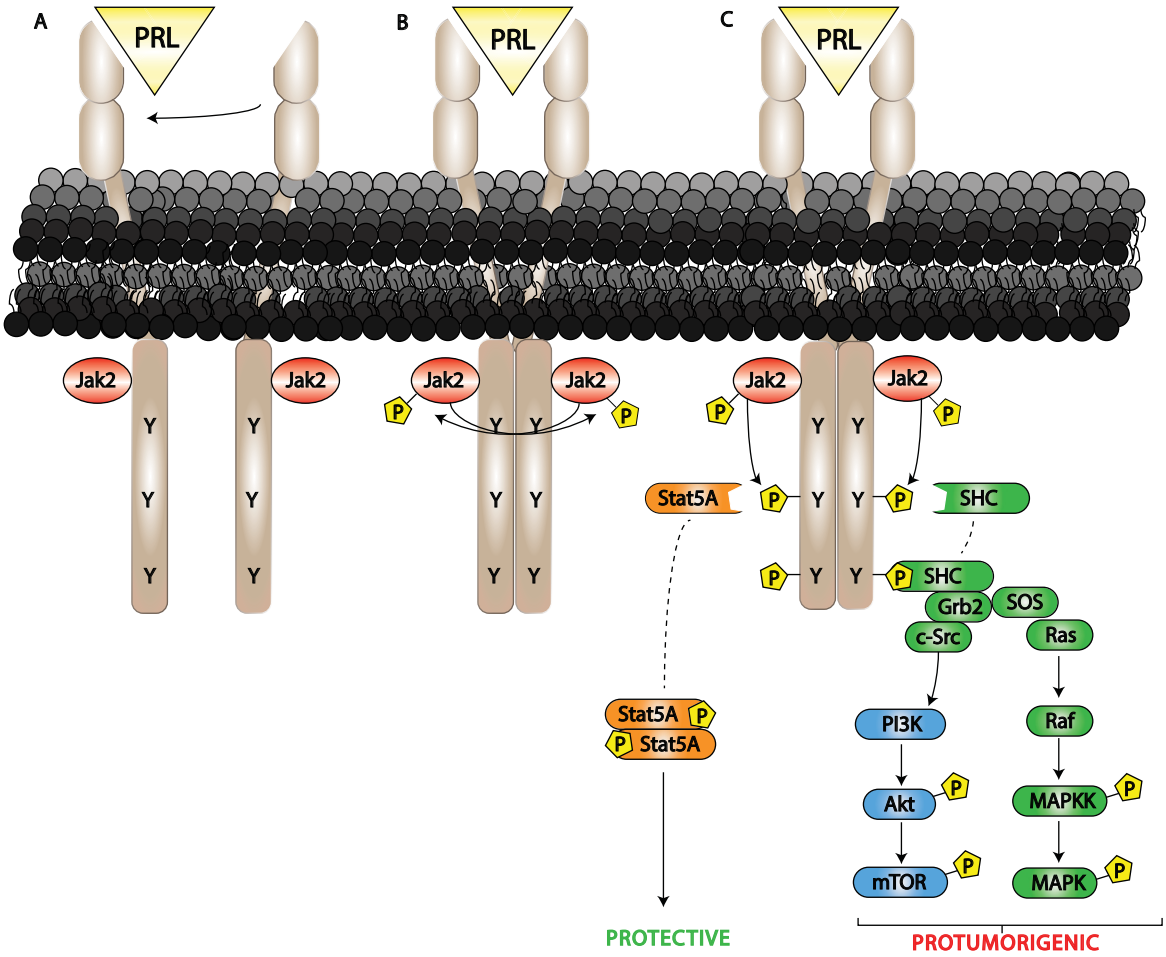

Figure 1. Protective and Pro-tumorigenic Signal Transduction Pathways Initiated by Activation of the PRLR. A) PRL's binding site one interacts with one PRLR moiety composed of an extracellular, transmembrane, and intracellular domain. Formation of the hormone-receptor complex induces the interaction of a second PRLR moiety with PRL's second binding site. B) After PRL-induced dimerization of the PRLR, Jak2 kinases, constituitively bound to conserved regions of the intracellular domain of the PRLR, are activated and transphosphorylate (P) each other. C) Activated Jak2 kinases then phosphorylate tyrosine residues (Y) in the intracellular domain of the PRLR. Stat5A is recruited to the phosphorylated tyrosine residues and is phosphorylated, homodimerizes and translocates to the nucleus where it is known to drive cell differentiation. Recruitment of other proteins to the PRLR such as SHC can induce signaling cascades leading to the activation of Akt, mTOR and MAPK which are thought to be pro-tumorigenic in breast cancer. Adapted from: (Freeman *et al*, 2000; Goffin, 2017).

PRLR intracellular domains (49). Once activated, the PRLR signals through a variety of other kinases and transcription factors. Notably, the canonical signalling pathway involves the Signal Transducer and Activator of Transcription (STAT) family of proteins which interact with the phosphorylated tyrosine residues on the PRLR through a highly conserved Src Homology 2 (SH2) domain (22, 50). Specifically, it is STAT5A and STAT5B that play an important role as transducers of PRLR signal transduction (48, 49).

1.3.4 The Role of Prolactin in Breast Cancer

PRL has historically been viewed as a promoter of mammary tumorigenesis (42). Indeed the majority of studies state that the PRLR is overexpressed in BCa, however, it is not clear whether BCa or genetic alterations in cancer cells give rise to this overexpression (42). Evidence has been put forth to support both the promoting and protective effects of PRLR signalling in BCa (see 39 for a review). These differing effects are resultant of the many PRLR signalling cascades that have been identified in BCa, which may help to explain the versatility of PRL action in BCa. For instance, STAT5A/B signalling has been shown to be both protective and pro-tumorigenic (see 50 for a review) whereas mitogen-activated protein kinase (MAPK)/phosphatidylinositol 3-kinase (PI3K)/protein kinase B(Akt)/mammalian-target-of-rapamycin (mTOR) signalling has been implicated in pro-tumorigenic processes such as cell proliferation, invasion and chemoresistance (39) (See Figure 1). Recent reports (54, 55) have shown that increased stiffness of the extracellular matrix, as seen in BCa progression, shifts the balance of protective versus pro-tumorigenic signalling by enhancing pro-tumorigenic PRLR-elicited signalling whilst suppressing protective STAT5A signalling. Interestingly, Jak2/STAT5A is the only signalling cascade initiated by the PRLR in prostate cancer

and, furthermore, STAT5A nuclear localization in prostate cancer has been positively correlated with a higher Gleason score (56), cancer recurrence (57), and a lower progression-free 15-year survival rate (56). Collectively, it has been suggested that PRLR inhibition remains a promising strategy in prostate cancer, whereas BCa may benefit more from inhibition of selective pathways regulated by the PRLR (42).

1.4 Androgens

Androgens are essential male sex steroid hormones that have many physiological functions including the development of the male sex organs and secondary sex characteristics as well as the initiation of spermatogenesis (58). The primary circulating androgen is testosterone which is derived from cholesterol and produced by Leydig cells in the testes. Testosterone can be irreversibly metabolized into another androgen, dihydrotestosterone (DHT), by the microsomal enzymes 5α-reductase type 1 and type 2. DHT has a two-fold greater affinity for the androgen receptor (AR) compared to testosterone (59) and is essential for development of the prostate and differentiation of male external genitalia (58, 60). Both testosterone and DHT elicit their effects through binding to the AR, a ligand-activated nuclear transcription factor.

1.4.1 Androgen Receptor

The AR belongs to a superfamily of ligand-activated nuclear receptors that includes the ER, PR, thyroid hormone receptors and peroxisome proliferator-activated receptors, among others (61, 62). The AR gene is located on chromosome X at the locus Xq11-Xq12 and consists of eight exons, resulting in a 110 kDa protein composed of 919 amino acids (63, 64). The AR contains a 555-amino acid N-terminal domain, a 69-amino acid DNA-binding domain (DBD), a 43-amino acid flexible hinge region, and a 255-

amino acid C-terminal ligand-binding domain. The N-terminal domain is encoded by exon 1, the DBD is encoded by exons 2 and 3, and the ligand-binding domain is encoded by exons 4-8 (62) (See Figure 2).

The N-terminal domain of the AR harbours the major transcriptional activation sites: the essential activation function-1 (AF-1) and the non-essential activation function-5 (AF-5) domains (66). In addition to transcriptional regulation, the N-terminal domain plays a role in stabilization of the ligand-AR complex. It does so by binding to the C-terminal ligand-binding domain, which results in slowing ligand dissociation and AR turnover (67, 68). The highly-conserved DBD is composed of two zinc-finger domains, where the most N-terminal zinc-finger determines androgen response element (ARE) recognition and the remaining zinc-finger is involved in protein-protein interactions (60). Finally, the conserved C-terminal ligand-binding domain has been shown to be essential in the repression of AR activation since deletion of the ligand-binding domain leads to constitutive activation of the AR (69).

1.4.2 Androgen Receptor Signalling

The AR is localized to the cytoplasm in the absence of its ligand and is associated with heat-shock proteins-90, -70, and -56, as well as other cytoskeletal proteins (70). Upon ligand binding, the ligand-binding domain of the AR undergoes a conformational change, causing the dissociation of heat-shock proteins and enabling receptor dimerization through intramolecular and intermolecular interactions between the N-terminal domain and ligand-binding domain (71). The ligand-bound dimerized AR translocates to the nucleus and binds to specific ARE (consensus: 5′-

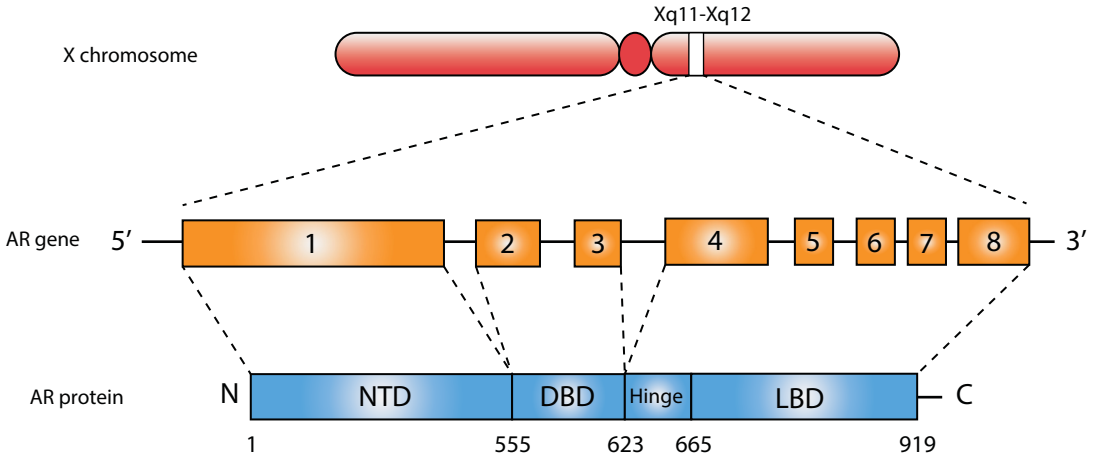

Figure 2. Organization of the Human Androgen Receptor Gene and Functional Domain Structure of the Androgen Receptor Protein. The eight exons of the human androgen receptor encoded on chromosome Xq11-Xq12 and their corresponding functional protein domains [N-terminal domain (NTD), DNA-binding domain (DBD), and C-terminal ligand-binding domain (LBD)]. Adapted from: (Tan *et al*, 2015).

GGA/TACAnnnTGTTCT-3′) in the promoter and/or enhancer regions of target genes and recruits specific cofactors to modulate target gene transcription (71).

1.4.3 Androgen in Breast Cancer

The AR is estimated to be expressed in 77% of BCa patients with expression levels varying widely by BCa subtype (72). Of particular interest is the correlation of improved clinicopathological symptoms and AR expression in patients with the aggressive TNBC subtype (73). Currently, there is not a clear agreement between the correlation of BCa risk and the level of circulating androgens (74). The primary circulating androgen in females is testosterone produced by both the ovaries and adrenal glands. Importantly, testosterone in the breast can be converted to the more potent DHT by 5α-reductase or to 17β-estradiol by aromatase, thus enabling the dichotomous activation of either the AR or the ER (75). In a similar manner to PRLR signalling, there is conflicting evidence surrounding the association between AR signalling and BCa development and progression. For instance, reductions in mammary ductal extension and branching were seen in AR knock-out mice (76), whereas accelerated mammary growth was seen in an *in vivo* model of AR transcriptional inactivation (77). Additionally, *in vitro* studies add to this lack of clarity. For instance, multiple groups have shown that AR signalling is inhibitory toward BCa cell survival (78, 79) whereas others have shown that AR signalling promotes proliferation of BCa cells (80).

1.5 Carboxypeptidases

Carboxypeptidases are proteases that hydrolyze the C-terminal peptide bond in peptides and proteins to release one amino acid per hydrolysis reaction. Carboxypeptidases are classified into two main groups: serine- and metallo-

carboxypeptidases (81). The serine-carboxypeptidases are classic serine proteases in that they utilize a catalytic triad of serine, aspartic acid, and histidine. In contrast, the metallo-carboxypeptidases use glutamic acid and a zinc atom for hydrolysis of the peptide bond (81). Further classification of the carboxypeptidases yields the carboxypeptidase-A (CPA)-type enzymes which preferentially hydrolyze C-terminal hydrophobic residues and the carboxypeptidase-B (CPB)-type enzymes which preferentially hydrolyze C-terminal arginine or lysine residues (81). CPA and CPB enzymes are primarily involved in digestion and are relatively small in molecular weight (~50 kDa) (81).

1.5.1. Carboxypeptidase-D

Carboxypeptidase-D (CPD) is a 180 kDa membrane-bound B-type metallo-carboxypeptidase that is localized to the *trans*-Golgi network as well as the plasma membrane (82, 83). Mammalian CPD is distributed in a wide range of tissues, including the ovaries, testes, spinal cord, kidney, gut, lung, pituitary, and hippocampus (83, 84). Of note, a PRL-inducible nuclear-targeted CPD isoform with a truncated N-terminal domain (160 kDa; (85) was discovered by differential display in rat PRL-dependent Nb2 and Nb2-Sp T-lymphoma cell lines (86). Like other B-type carboxypeptidases, CPD hydrolyzes C-terminal arginine or lysine residues from peptides, with a preference of alanine being in the penultimate position (87). The human CPD gene is 88.3 kbp in size, contains 21 exons and 21 introns, and is encoded on chromosome 17q11.2 (88). CPD contains three 320-amino acid carboxypeptidase repeat extracellular domains (domain I, II and III), a 20-amino acid transmembrane domain, and a 60-amino acid cytoplasmic domain. Domain I and II are enzymatically active. Domain III is inactive but it binds and presents standard CPD substrates to domain I and II (89, 90). These three domains

account for the exceptional size of CPD (180 kDa) as compared to CPA and CPB enzymes (~50 kDa).

Plasma membrane localization of CPD is suggestive of CPD acting as a cell surface enzyme. Indeed, it was shown in a mouse macrophage cell line that extracellular addition of CPD substrates increased intracellular production of nitric oxide (NO) by six-fold. The CPD-released arginine is transported into the cell where it is converted to NO by nitric oxide synthase (91). Cleavage of C-terminal arginine from synthetic CPD substrates has also been reported in rat micro-vascular endothelial cells (92).

A novel isoform of CPD, denoted as CPD-N (160 kDa), was cloned from a human leukemic cDNA library and found to be expressed in a rat PRL-dependent Nb2 lymphoma cell line (86). CPD-N expression was enhanced following PRL or interleukin-2 treatment of Nb2 cells. CPD (180 kDa) expression was also shown to be PRL-inducible in MCF-7 luminal-A BCa cells (86). This upregulation of CPD occurred at the transcriptional level, since the CPD promoter region was found to contain putative gamma-interferon regulatory sequences (GAS) and androgen response elements. CHIP-seq and luciferase reporter assays demonstrated that the sequences, which bind STAT5A and AR, respectively, are active in MCF-7 BCa cells (93). These findings indicate that hormonal upregulation of CPD occurs at the transcriptional level.

Furthermore, PRL was shown to upregulate nitric oxide synthase (NOS) expression in rat Nb2 (94) and human MCF-7 cells (95), which in turn, promoted cell survival and inhibited cell apoptosis. By abrogating PRL-stimulated NO production using small-interfering ribonucleic acid (siRNA) targeting CPD, it was found that CPD, not NOS, was the major contributor of intracellular NO production (95). CPD and NO

upregulation by PRL and androgens has also been reported in breast (93) and prostate cancer cell lines (96).

1.6 Nitric Oxide

Nitric oxide synthase enzymes catalyze the NADPH- and oxygen-dependent conversion of L-arginine to L-citrulline and NO. The three NOS isoforms are: endothelial NOS (eNOS), inducible NOS (iNOS) and neuronal NOS (nNOS). NO, a highly reactive free-radical, was the first recorded gaseous signalling messenger (97). Up until the 1980s, NO was only known as a constituent of smog and also being a respiratory irritant (97). This changed upon the discovery that the pathway by which macrophages kill bacteria and cancer cells is arginine-dependent (98). NO was then recognized as one of the most important cytotoxic molecules and a variety of different physiological roles of NO began to emerge (99). The effects of NO are typically classified into direct effects resulting from reactions with NO itself, or indirect effects resulting from NO-derived reactive oxygen species such as peroxynitrite and dinitrogen trioxide (100). Generally, the direct effects of NO prevail under conditions of low and/or brief NO production to promote protective functions. Whereas indirect effects of NO occur under high and/or sustained NO production leading to toxic consequences (100).

1.6.1 Nitric Oxide and Cancer

NO may play a protective role in cancer, by promoting apoptosis and inhibiting cell proliferation (101, 102). Several studies have reported that enhanced NO production results in a dramatic decrease in metastasis in melanoma and lymphoma cells (103–105).

NO and NO metabolites, such as the previously mentioned peroxynitrites, play important roles in mediating cellular genotoxicity. Genotoxic effects of NO include

protein and DNA damage, gene mutation, loss of protein function, apoptosis, and necrosis (106, 107). Several studies have linked NO production to initiating or promoting tumourigenesis (e.g., 100, 101), thus, attention has shifted towards elucidating how the NO signalling axis promotes cell proliferation and cell survival. Evidence has implicated mTOR in NO signalling pathway since iNOS was shown to activate mTOR (110) and mTOR activation was necessary for the phosphorylation and activation of eNOS (111).

1.7 Mitochondrial-Mediated Apoptosis

Apoptosis, originally described during insect development (112), is a process where cells shrink with intact, but ruffled, plasma membranes, and the cell nuclei become condensed and fragmented (113). Apoptosis plays a key role in animal development. For instance, activated lymphocytes and non-functional neurons are removed by apoptosis. Two different apoptotic pathways have been identified in mammalian systems: the intrinsic (mitochondrial) and extrinsic pathways (114).

The mitochondrial pathway to apoptosis (see Figure 3) is regulated through members of the Bcl-2 family, a family comprised of three different subfamilies: (1) pro-apoptotic BCL-2 homology 3 (BH3)-only initiator proteins (Bim, Bid, Noxa, Hrk, Bmf, and Bad), (2) pro-survival guardian proteins (Bcl-2, Bcl-xL, Mcl-1, A1, and Bcl-B), and (3) pro-apoptotic effector proteins (Bax and Bak) (115). Normally, in healthy cells, pro-apoptotic effectors Bax and Bak are inhibited by pro-survival guardian proteins (e.g., Mcl-1). In response to certain stimuli, such as stress or intracellular damage, the pro-apoptotic BH3-only initiator proteins (e.g., Bim) are upregulated, which then activate Bax and Bak directly, or indirectly through inhibition of the pro-survival guardian proteins (113, 115). Bax, a cytosolic pro-apoptotic protein, associates with modulator of

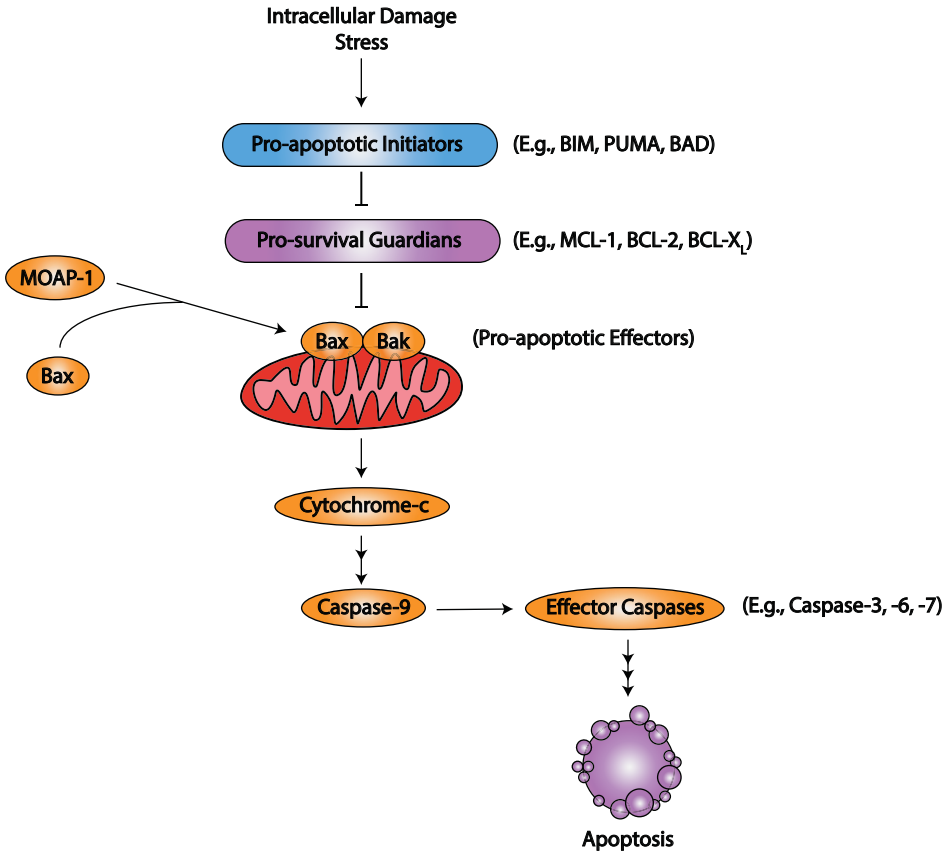

Figure 3. The Mitochondrial-mediated Pathway to Apoptosis. Intracellular damage and stress are some examples that can stimulate activation of the pro-apoptotic initiator proteins which, once activated, will inhibit pro-survival guardian proteins. Inactivation of the guardian proteins will allow pro-apoptotic effector Bax to be directed from the cytosol to the mitochondrion by MOAP-1, where Bax will then insert itself into the outer mitochondrial membrane and oligomerize with a second pro-apoptotic effector protein Bak. Bax and Bak will both permeabilize the outer mitochondrial membrane, releasing cytochrome-c into the cytosol where it will lead to the activation of caspase-9 and subsequent activation of effector caspases. The activated effector caspases then promote cellular apoptosis.
Adapted from: (Czabotar *et al*, 2014)

apoptosis protein-1 (MOAP-1) upon apoptotic induction. MOAP-1 facilitates the translocation of Bax from the cytosol to the mitochondria, where Bax will homo-oligomerize and insert into the outer mitochondrial membrane (115). Mitohondrial membrane-bound Bak, will also homo-oligomerize, and together, the multimeric Bax and Bak permeabilize the outer mitochondrial membrane through an unconfirmed mechanism (115–117). Mitochondrial outer membrane permeabilization leads to the release of cytochrome-c and other apoptogenic factors (118) into the cytosol to promote activation of caspase-9. Caspases (cysteine aspartyl proteases) are zymogens that undergo specific cleavage of their N-terminal prodomain during either apoptosis or pyroptosis (113, 119). During apoptosis, caspase-9 activation will, in turn, lead to the cleavage and activation of effector caspase-3, -6, and -7 which cleave more than 1,300 substrates to promote cellular apoptosis (113, 115).

Evasion of apoptosis is one way in which cancer cells can develop resistance to anti-cancer drugs. Resistance may also result from inactivation or increased metabolism of the drug, enhanced DNA repair, decreased drug absorption or increased drug efflux (120).

1.8 Mammalian Target-of-Rapamycin (mTOR)

mTOR is a protein serine/threonine kinase that significantly regulates cell growth, proliferation and survival through integrating inputs from several extracellular and intracellular cues such as growth factors, amino acids, cellular energy, stress, and oxygen levels (121–123). mTOR occurs as two structurally and functionally different multiprotein complexes, mammalian target-of-rapamycin complex 1 (mTORC1) and mammalian target-of-rapamycin complex 2 (mTORC2), which are found downstream of

the PI3K/Akt pathway. The mTORC1 protein complex has been described as the master regulator of cell growth and metabolism (124). Little is known about the biology of mTORC2, however, it has a major role in cytoskeletal organization and plays a role in cell survival, proliferation, and metabolism (121).

The mTORC1 pathway regulates initiation of translation by directly phosphorylating/activating ribosomal protein S6 kinase beta-1 (p70S6K) and phosphorylating/inactivating eukaryotic initiation factor 4E (eIF4E) binding protein 1 (4E-BP1; 113) which, in turn, promotes the initiation of protein synthesis (126). The activation of p70S6K, through multiple effectors, leads to translational initiation of 5′-terminal oligopyrimidine tract (5′-TOP) mRNAs leading to an increase in ribosomal biogenesis and (121). The 4E-BP1 protein occurs as phosphorylated isoforms α (hypophosphorylated), β, and γ (hyperphosphorylated; 115, 116). The phosphorylation of 4E-BP1 induces its dissociation from eIF4E, enabling eIF4E to participate in the formation of the eIF4F (eIF4A, B, G, E) complex that is required for initiating translation from the 5′-7-methyl guanosine triphosphate (m^7GTP) cap of mRNAs (121).

There is a large body of evidence implicating the mTOR pathway in cancer pathogenesis. For instance, components of the PI3K signalling pathway, upstream of both mTORC1 and mTORC2, are often mutated in human cancers (121). Loss of function of tumour suppressor p53, a commonality in many cancers, promotes the activation of mTORC1 (129). 4E-BP1/eIF4E also mediates the oncogenic effects of Akt signalling on cell growth and tumour progression (130). Interestingly, the contribution to oncogenic Akt effects by p70S6K are limited, indicating that the two downstream protein synthesis signalling branches of mTORC1 are not equally required for cancer progression (130,

131). It is suggested that the mTORC1/4E-BP1/eIF4E axis contributes to cancer progression by promoting the translation of specific mRNAs of pro-oncogenic proteins which regulate energy metabolism, cell survival, and cell-cycle progression (121). It has been shown that PRL stimulates mTOR activity in lymphoma (132) and BCa (133) cells and that the α4-catalytic subunit of protein phosphatase 2A (PP2Ac) complex regulates TORC1 signalling through 4E-BP1/eIF4E and p70S6K to initiate protein translation, cell cycle progression, and cell proliferation (124, 134) (See Figure 4).

1.9 E3 Ubiquitin Ligases

Ubiquitin ligases control every aspect of eukaryotic biology by attaching ubiquitin chains to proteins, targeting them for proteasomal degradation (135). Ubiquitin, a 76-amino acid protein that is highly conserved amongst eukaryotes, is conjugated to protein substrates to form polyubiquitylated chains linked at lysine-48 or lysine-11 residues (136, 137). The polyubiquitylated proteins serve as a signal to be degraded by the proteasome, a highly regulated multi-catalytic protease complex essential for a diverse range of cellular functions (138, 139). First discovered in the 1980s (140), the tagging of proteins with ubiquitin moieties and subsequent degradation by the adenosine triphosphate (ATP)-dependent proteasome is known as the ubiquitin proteasome system (124). Three enzymes are required for the conjugation of ubiquitin to proteins. The first is the ubiquitin-activating enzyme (E1) (126), which consumes ATP and forms a thioester linkage between the last glycine residue in ubiquitin and a cysteine residue in E1 (142, 143). A second enzyme, E2 (141), catalyzes the transfer of ubiquitin to a third enzyme, E3. E3 catalyzes the ligation of ubiquitin to the substrate protein at a specific site, known as a degron (141–143). E3 ubiquitin ligases have received the most attention, as they are

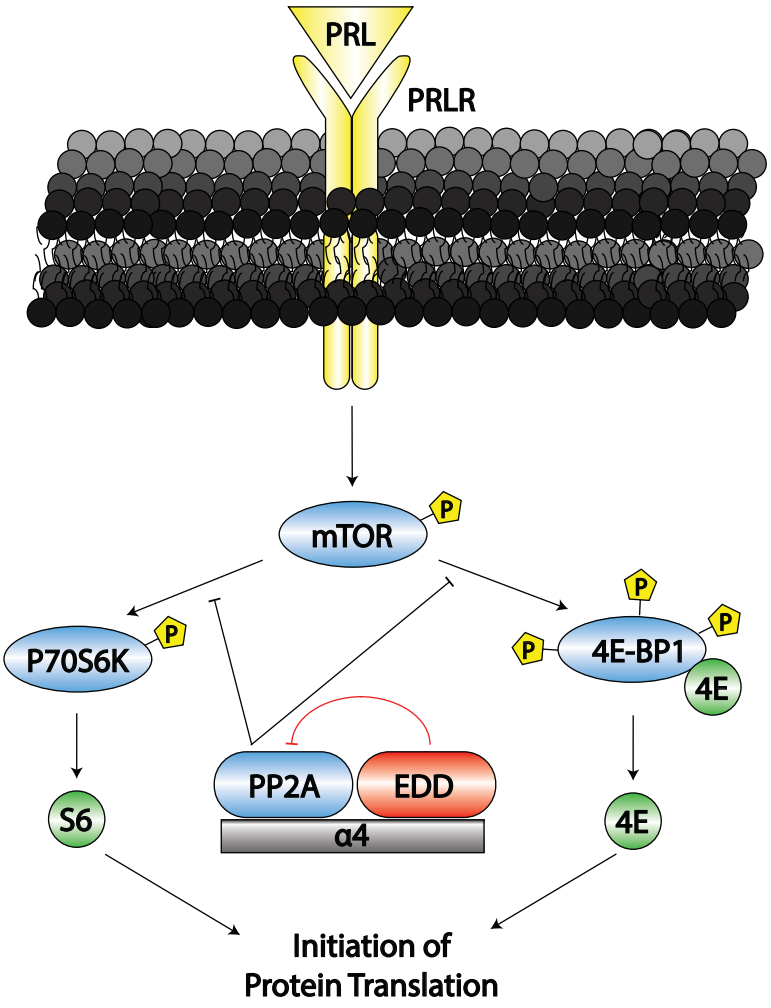

Figure 4. The Role of EDD in PRL-mediated mTOR Signalling. The activated PRLR leads to the eventual phosphorylation and activation of mTOR, in turn, leading to the phosphorylation and activation or inactivation of downstream signalling components P70S6K and 4E-BP1, respectively. Activation of mTOR culminates in initiating protein translation. EDD polyubiquitinates PP2A, a negative regulator of mTOR signaling, for degradation by the proteasome. Therefore, EDD indirectly stimulates mTOR signalling activity.

responsible for determining substrate specificity for ubiquitylation (142), thus, making them promising drug targets for a variety of diseases (144–146). There are two primary classes of E3 ligases: Homologous to E6-associated protein C-Terminus (HECT) and Really Interesting New Gene (RING) ligases, each with a different mode of action (147). The ubiquitin-proteasome system controls many basic cellular processes – such as the cell cycle (148), signal transduction (149), the immune response (150), metabolism (151), and protein quality control (152).

In addition to polyubiquitylation, proteins can also be monoubiquitylated on one or multiple lysine residues (153). Monoubiquitylation has been shown to play a key role in the budding of retroviruses from the plasma membrane, endocytosis, and histone regulation (153). Ubiquitin chains can be removed from proteins by deubiquitylating enzymes (154), suggesting that ubiquitylation is dynamic and not a terminal process. Moreover, ubiquitin has also been found to play a key role in transcriptional regulation of a variety of genes (155).

1.9.1 E3 Ubiquitin Ligase Identified by Differential Display

The 280-kDa *HYD* protein in *Drosophila melanogaster* imaginal discs was initially found to cause imaginal disc overgrowth (156). Further analyses revealed that this protein is responsible for the regulation of cell proliferation during development in *Drosophila melanogaster* (156). A 300-kDa mammalian ortholog of the *HYD* was isolated from the human T47D luminal-A BCa cell line and shown to be progestin-inducible (157). This human ortholog, named E3 ubiquitin ligase identified by differential display (EDD), was found in a variety of tissues and organs, and the EDD (or UBR5) gene is localized to chromosome 8q22.3 (157).

1.9.2 EDD and Breast Cancer

EDD is overexpressed in many solid tumours which implies a role in tumorigenesis (158, 159). EDD has emerged as a key regulator of various cellular processes in cancer, including gene expression, genome integrity, and chemoresistance (160). High nuclear EDD expression in a cohort of 151 women with serous ovarian carcinoma was associated with increased risk of disease recurrence following first-line chemotherapy, and siRNA-knockdown of EDD gene expression partially restored cisplatin sensitivity in cisplatin-resistant ovarian cancer cells *in vitro* (161). Loss of EDD induced cell-cycle arrest at G1 through upregulation of tumour suppressor p53 and p21 proteins in osteosarcoma cells *in vitro* (162). Recent analysis of primary TNBC by whole-exon sequencing showed strong EDD gene amplification. EDD overexpression was confirmed in TNBC tissues and, using a murine TNBC model, CRISPR/cas9-mediated EDD deletion dramatically abrogated tumour growth and metastasis (163). EDD has recently been linked to cisplatin drug resistance in ovarian cancer cells by polyubiquitylating MOAP-1 for degradation by the proteasome (164). Importantly, in cisplatin-resistant cell lines, EDD knockdown increased MOAP-1 expression and re-sensitized these cells to cisplatin (164).

Using yeast two-hybrid analysis, the Too laboratory identified EDD as a novel protein partner of a mTOR/TORC1-associated protein complex comprising α4-phosphoprotein and PP2Ac (149). The α4 protein physically interacted with PP2Ac and EDD at its N- and C-termini, respectively (166). Furthermore, the Too laboratory showed that EDD polyubiquitinated PP2Ac for proteasomal degradation (165) (See Figure 4). Treatment of human MCF-7 and T47D BCa cell lines with progesterone and PRL

upregulated EDD mRNA and protein levels, with a concomitant decrease in PP2Ac levels (165), further supporting a role for EDD in PP2Ac turnover.

1.10 Objectives

The present study investigated the role of EDD and CPD in BCa. Specifically, the roles of EDD and CPD *in vivo* was determined by examining expression in benign and malignant human breast specimens using IHC and KM-plot analyses as well as *in vitro* effects of EDD and CPD using gene knockdown (siRNA, shRNA) and gene overexpression.

CHAPTER 2. MATERIALS AND METHODS

2.1 Antibodies

The dilutions and sources of primary antibodies used were: Custom-made affinity-purified rabbit anti-CPD (1:500) from ThermoFisher Scientific (Waltham, MA); mouse mAb anti-EDD (1:200; sc515494), anti-Poly-ADP ribose polymerase (PARP; 1:1000; sc74470), and anti-Bax (1:200; sc20067) from Santa Cruz Biotechnologies (Dallas, TX); rabbit anti-caspase 7 (1:1000; cs9492), anti-4E-BP1 (1:1000; cs9452), and anti-eIF4E (1:1000; cs9742S) from Cell Signalling (Danvers, MA); rabbit anti-modulator of apoptosis protein-1 (MOAP-1; sb100970-T32) from Sino Biologicals (Beijing, China); rabbit anti-β-tubulin (1:10,000; sT8328), anti-β-actin (1:20,000; sA2066), and the secondary antibody conjugates goat anti-mouse (1:5000) and goat anti-rabbit (1:5000) horse radish peroxidase (HRP) from Sigma-Aldrich (Oakville, ON).

2.2 Cell Culture

Human breast cell lines were maintained as follows: Luminal-A MCF-7 cells were maintained in high glucose Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal bovine serum (FBS), 1 mM sodium pyruvate, 2 mM L-glutamine, 1X minimum essential medium (MEM) non-essential amino acids, 100 μg/ml streptomycin, and 1% penicillin-streptomycin. HER2-enriched SKBR3 and MDA-MB-231 TNBC cells were maintained in high glucose DMEM containing 10% heat-inactivated FBS, 2 mM L-glutamine, 5 mM 4-(2-hydroxyethyl)-1-piperazineethane sulfuric acid (HEPES), and 1% penicillin-streptomycin. MDA-MB-468 TNBC cells were maintained in high glucose DMEM containing 10% heat-inactivated FBS. MDA-MB-436 TNBC cells were maintained in Leibovitz L-15 medium containing 10% heat-inactivated

FBS, 1X transferrin-selenium-ethanolamine, 16 μg/ml glutathione, and 1% penicillin-streptomycin. MDA-MB-436 cells were incubated in atmospheric air at 37ºC whereas all other cell lines were incubated in 5% CO_2 at 37ºC. Before R1881, a synthetic androgen that does not undergo aromatization to estrogen, and/or PRL treatment, cells were made quiescent (to induce cell-cycle synchronization and reduce hormone production) in DMEM containing 1% heat-inactivated, charcoal-stripped FBS (CS-FBS) and all additives of the maintenance medium for 24 hours (h). In experiments examining NO production, cells were made quiescent in arginine-free DMEM containing 1% CS-FBS for 24 h prior to treatment with PRLR and AR antagonists, PRL, and/or R1881. In experiments using a synthetic NO donor, cells were cultured in DMEM containing 5% FBS (positive control) or 5% CS-FBS for 24 h prior to NO donor treatment. All cell lines were confirmed mycoplasma-free.

2.3 Preparation of Total Cell Lysates

Pelleted cells were harvested in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% IGEPAL/octylphenoxypolyethoxyethanol, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate [SDS]) containing 100:1 (v/v) protease inhibitor cocktail P8340 (Sigma-Aldrich), 1 mM sodium orthovanadate and 1 mM phenylmethylsulphonyl fluoride. Passage through a 21-gauge needle was used to disrupt cells. Cell lysates were incubated at 4 ºC for 30 minutes and then centrifuged at 13,000 x *g* for 20 minutes at 4 ºC. The supernatant was analyzed by a Bradford Protein Assay.

2.4 Bradford Protein Assay

Protein concentrations of cell lysates were determined by using a Quick Start™ Bradford Protein Assay according to the manufacturer's protocol (Bio-Rad Laboratories, Mississauga, Ontario, Canada). Absorbances were measured at 595 nm using bovine serum albumin for standard curves.

2.5 Transfection of Small Interfering Ribonucleic Acid

The siRNAs used were Silencer® Select Negative Control #1 and #2 siRNA, Pre-designed Ubiquitin Protein Ligase E3 Component N-Recognin 5 (UBR5) Silencer® Select siRNA s28025 (siEDD1) and s28024 (siEDD2) and pre-designed Silencer® siRNA 103996 (siCPD1) and 103997 (siCPD2) which were all purchased from ThermoFisher Scientific (Waltham, MA). Prior to transfection, MCF-7, MDA-MB-231 or MDA-MB-468 cells in complete medium were seeded at a density of 3-5 x 10^5 cells per 3.48 cm well for 24 h. Cells were transfected with 40 ρmol of siRNA per well using RNAiMAX™ (ThermoFisher Scientific) according to the manufacturer's protocol. After 18 h, cells were either lysed or made quiescent for 24 h prior to hormone treatment.

2.6 Transfection of EDD Expression Plasmids

MDA-MB-436 cells in complete medium were seeded at 5 x 10^4 cells per 1.58 cm well. Twenty-four hours later cells were washed with phosphate-buffered saline (PBS; pH 7.4) and re-incubated in serum-free Leibovitz L-15 medium. Cells were then transfected with pCMV-Tag2B.EDD (a gift of Drs. Darren Saunders and Charles Watts of the Garven Institute of Medical Research in Austrailia; Addgene plasmid #37188) or pCMV-Tab2B control vector using GenJet Transfection Reagent, according to the manufacturer's protocol (Version II; FroggaBio, Toronto, Canada. After 18 h, cells were

washed with PBS and treated with half-maximal inhibitory concentration (IC_{50}) of cisplatin (0.3125 μM), doxorubicin, (0.023 μM), rapamycin (16.59 μM) or INK128 (14.38 nM) for five days or left untreated (controls). On day five, viable cells were counted.

2.7 Generation of shEDD-MCF-7 Clones

MCF-7 cells were stably transduced with shRNA targeting EDD through infection with lentiviral particles (sc-43733-V, Santa Cruz Biotechnology), following the manufacturer's protocol. Puromycin (4 μg/ml)-resistant clones were isolated with cloning rings. EDD mRNA/protein expressions were variable, ranging from 0 to 63% knockdown by reverse-transcription quantitative-polymerase chain reaction (RT-qPCR) analysis, and from 0 to 100% knockdown by immunoblotting analysis. Specifically, EDD protein level was reduced by over 90% in clone 1.1C as compared to uninfected cells, but only by 22% in clone 1.1A. Clone 1.1A was used as a non-knockdown control cell line for comparison with clone 1.1C.

2.8 Trypan Blue Cell Viability Assay

Cells were detached using TrypLE™ Express (ThermoFisher Scientific). Trypsin was then inactivated using an equal volume of complete growth medium before addition of 0.4% trypan blue solution (1:1). Viable cells were counted using a TC20 cell counter (Bio-Rad Laboratories)

2.9 RNA Extraction and Reverse Transcription-Polymerase Chain Reaction

Total RNA was extracted from cell lysates using an EZ-10 DNAaway RNA Mini-Preps Kit (BioBasic, Markham, ON, Canada) according to the manufacturer's protocol. RNA was treated with DNase I and used (1 μg/reaction) for semi-quantitative polymerase

chain reaction (PCR) or RT-qPCR using the Comparative Cycle Threshold (Ct) method (93). All PCR reactions used β-actin as a control. The following forward and reverse primer sets were used: Human EDD: 5′-GAC-GCG-AGA-ACT-CTT-GGA-AC-3′ and 5′-TTC-AAA-TGG-ATT-TGG-GGG-TA-3′ (196 bp amplicon; for RT-qPCR) or 5′-AGA-TGC-TGA-CCC-TTC-TTC-TCT-CCT-GC-3′ and 5′-GCA-CCC-AAT-TTC-CAG-TCT-TC-3′ (209 bp; for semi-quantitative PCR); human β-actin: 5′-AAA-CTG-GAA-CGG-TGA-AGG-TG-3′ and 5′-AGA-GAA-GTG-GGG-TGG-CTT-TT-3′ (172 bp amplicon).

2.10 Immunoblot Analysis

Previously prepared protein samples (10-30 μg/lane) diluted in 3X sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) buffer were loaded onto 6-15% SDS-polyacrylamide gels (0.375 M Tris-HCl, pH 8.8, 0.1% SDS, 0.05% ammonium persulfate and 0.05% tetramethylethylenediamine [TEMED]). Protein samples were resolved at 150 V in SDS-PAGE running buffer (0.02 M Tris-HCl, pH 8.3, 0.2 M glycine and 0.1% SDS). Proteins resolved in SDS-PAGE were transferred onto a BioTrace™ Nitrocellulose Blotting Membrane (PALL Life Sciences, New York, NY) by applying 30 V overnight at 4 ºC in western transfer buffer (25 mM Tris, pH 8.3, 192 mM glycine, and 20% methanol). Nitrocellulose membranes were blocked for 1 h at room temperature in skim milk powder (10% w/v) in Tris-buffered saline-Tween 20 (TBST; 0.02 M Tris-HCl, pH 7.6, 0.2 M NaCl, 0.05% Tween 20). Nitrocellulose membranes were incubated with primary antibody diluted in skim milk powder (10% w/v) in TBST overnight at 4 ºC. Afterward, the membranes were washed thrice with TBST for 15 minutes each time. The membranes were incubated with the appropriate secondary antibody HRP-conjugate prepared in skim milk powder (10% w/v) in TBST for 1 h at

room temperature. The membrane was washed using the same washing cycle and immunoreactive signals were detected by chemiluminescence using a Clarity Western ECL Substrate Kit (Bio-Rad Laboratories). Densitometry was performed using ImageJ64 with β-actin or β-tubulin as a control.

2.11 Cap-binding Assay

Immobilized γ-aminophenyl-m^{7}GTP (C_{10}-spacer) agarose beads (Jena Biosciences, Germany) were pre-washed thrice with freshly-prepared RIPA lysis buffer containing 100:1 (v/v) of protease inhibitor cocktail P8340 (Sigma-Aldrich), 1 mM sodium orthovanadate and 1 mM phenylmethylsulphonyl fluoride. Cell lysates were prepared from cells treated ± PRL (10 ng/ml) or ± R1881 (10 nM) and transfected ± siNT or siEDD1. Each sample of cell lysate (100 µg protein) and 25 µl of pre-washed m^{7}GTP-agarose beads were incubated together in a microfuge tube, gently agitated on a nutator at 4 ºC for 3 h, then centrifuged at 500 x *g* for 1 minute, and the supernatants were removed. The protein-bound m^{7}GTP-agarose beads in each tube were washed thrice with 1 ml RIPA buffer by inversion, re-centrifuged at 500 x *g*, and the supernatants were removed. The protein-bound m^{7}GTP-agarose beads, in 50 µl of 1X SDS-PAGE buffer, were boiled at 100 ºC for 15 minutes to elute m^{7}GTP cap-binding proteins. Eluted fractions and the respective whole cell lysates were resolved by SDS-PAGE and analyzed by western blotting for eIF4E, 4E-BP1, and β-actin.

2.12 DAF2-DA Assay for Nitric Oxide

MDA-MB-231 or MDA-MB-468 cells were seeded at 25,000 - 50,000 cells/well onto CC^2 glass 4-chambered slides (Lab-Tek, Rochester, NY). Twenty-four hours later, cells were made quiescent in arginine-free DMEM containing 2% CS-FBS for 48 h.

Quiescent cells were treated with PRL (20 ng/ml) and/or R1881 (10 nM), in the presence or absence of the PRLR antagonist Δ1-9-G129R-hPRL (40 ng/ml) and/or the AR antagonist flutamide (20 μM). Cells were washed thrice with PBS and incubated with 4,5-diaminofluorescein diacetate (DAF2-DA; 10 μM; Abcam) for 30 minutes. When taken into the cell, nonfluorescent DAF-2DA is hydrolyzed by intracellular esterases to membrane-impermeable 4,5-diaminofluorescein (DAF-2), which reacts with NO to produce fluorescent 4,5-diaminofluorescein triazole (DAF-2T). Following incubation, cells were washed with PBS and treated with synthetic CPD substrate furyacryloyl-alanyl-arginine for 30 minutes (FAR; 4 mM; Bachem, [Bubendorf, Switzerland]). Fluorescence was examined using a Zeiss Aviovert 200M inverted microscope (Zeiss, Jena, Germany). Images were captured using a Hamamatsu Orca-R2 camera (Hamamatsu Photonics, Hamamatsu Cite, Japan) and analyzed using Cell Profiler™ image analysis software.

2.13 Nitric Oxide Donor for Cell Viability

MDA-MB-231 or MDA-MB-468 cells in complete medium were seeded at a density of 1 x 10^5 cells per 3.48 cm well for 24 h. Culture medium was then changed to arginine-free medium containing 5% CS-FBS for 24 h. Cells were subsequently treated with increasing concentrations of synthetic NO donor (Z)-1-[N-(2[aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (DETA-NONOate). On day 3, viable cells were counted.

2.14 Annexin-V Staining for Apoptosis

Annexin-V-FLUOS staining (Roche Diagnostics, Mennheim, Germany) was performed following the manufacturer's protocol. Briefly, cells were seeded at 1 x 10^5

cells per 3.48 cm well. After 24 h, the culture medium was changed to arginine-free medium containing 5% CS-FBS for 24 h. Subsequently, cells were treated ± PRL (10 ng/ml) and/or ± FAR (4 mM). On day 2, both floating and adherent cells were collected, washed once with PBS, pelleted and resuspended in 100 µl of incubation buffer containing Annexin-V-FLUOS labeling reagent and propidium iodide at the recommended concentrations. The samples were incubated in the dark for 15 minutes and diluted with 300 µl of incubation buffer before flow cytometry at λexcitation 488 nm and detection at λemission 515 nm (Annexin-V) and 600 nm (propidium iodide). Data was analyzed using FCS Express-6 DeNovo Software (Glendale, CA). Percentage cell death was calculated from cells that stained positive for either Annexin-V-FLUOS (early apoptosis) or Annexin-V-FLUOS and propidium iodide (late apoptosis).

2.15 Kaplan-Meier Survival Analysis

An online database (http://ec2-52-201-246-161.compute 1.amazonaws.com/kmexpress/index.php) was used to determine the relevance of individual EDD mRNA expression to the overall patient survival. Survival curves for EDD mRNA expression in BCa patients from the TCGA database (n = 943) were bifurcated by top 25% vs bottom 25% (Q3Q1). A second online database (http://kmplot.com/analysis/) was used to determine the relevance of individual EDD and CPD mRNA expression to the relapse-free survival. Survival curves for EDD were bifurcated by median and plotted for high grade (3) basal BCa patients (n = 293). Survival curves for CPD were bifurcated by median and plotted for basal BCa patients (n = 618). Survival hazard ratio and 95% confidence intervals, as well as log rank P, were calculated and displayed on the webpage.

2.16 Statistics

All statistical analyses were performed using Graphpad prism, and the results were expressed as mean ± SEM ($n \geq 3$) unless otherwise stated in the figure legends. Student's unpaired, one-tailed t-tests and one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison tests were performed to compare mean values. A *P*-value of ≤ 0.05 was considered statistically significant.

CHAPTER 3. RESULTS

For a complete summary of all results, see Figure 24.

3.1 High CPD mRNA Expression Correlates with Poor Patient Survival

Recently published work by Dr. Too's group (167) demonstrated that CPD immunostaining increases with BCa progression. I sought to determine if BCa patient survival is different between patients expressing high or low CPD mRNA expression. To do this, I used Kaplan-Meier-Plotter analysis to show that TNBC patients with high CPD expression had a significantly lower probability of relapse-free survival (Figure 5).

3.2 CPD is Upregulated by Hormones in TNBC and HER2-Enriched Cell Lines

Previous work in our laboratory has shown that hormonal treatment of Luminal-type BCa cells results in CPD upregulation of mRNA and protein expression (93, 95). To see if CPD expression is altered in non-luminal-type cell lines, I arrested TNBC MDA-MB-231, MDA-MB-468, and MDA-MB-436 cell lines, as well as the HER2-enriched SKBR3 cell line and treated cells with PRL or R1881 for 3 and 6 hours. Cell lysates were immunoblotted for CPD. On average in MDA-MB-231 cells, PRL upregulated protein levels of CPD by 2.35-fold after 3 hours. R1881 also upregulated protein levels of CPD in MDA-MB-231 cells by 2.23-fold after 3 hours (Figure 6A). Fold-increases in upregulation of CPD levels were similar for MDA-MB-468 cells (from 1.57 to 4.39-fold) (Figure 6B) and SKBR3 cells (from 1.19 to 3.21-fold) (Figure 6C). In contrast, MDA-MB-436 cells did not respond to hormonal treatment and CPD protein levels were unchanged by either PRL or R1881 (Figure 6D).

The cell lines and timepoints showing the highest fold-increase in CPD expression were used in experiments examining intracellular NO production.

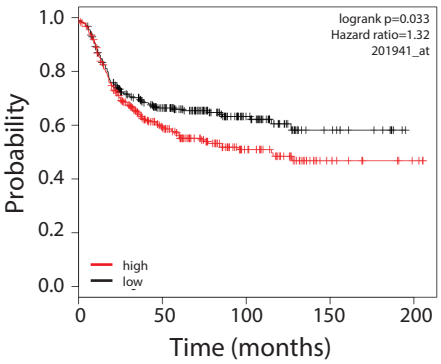

Figure 5. Determination of the Prognostic Value of CPD mRNA Expression.
An online database (http://kmplot.com/analysis/) was used to determine the relevance of individual CPD mRNA expression to the relapse-free survival. Survival curves for CPD Affymetrix ID: 201941_at (CPD) are plotted for basal TNBC patients (n = 618). Patient samples were bifurcated by the median into low (black) and high (red) expression groups for CPD. Survival Hazard ratio (HR) and 95% confidence intervals, as well as log rank P were calculated and displayed on the webpage. A p value of <0.05 was considered to be statistically significant.

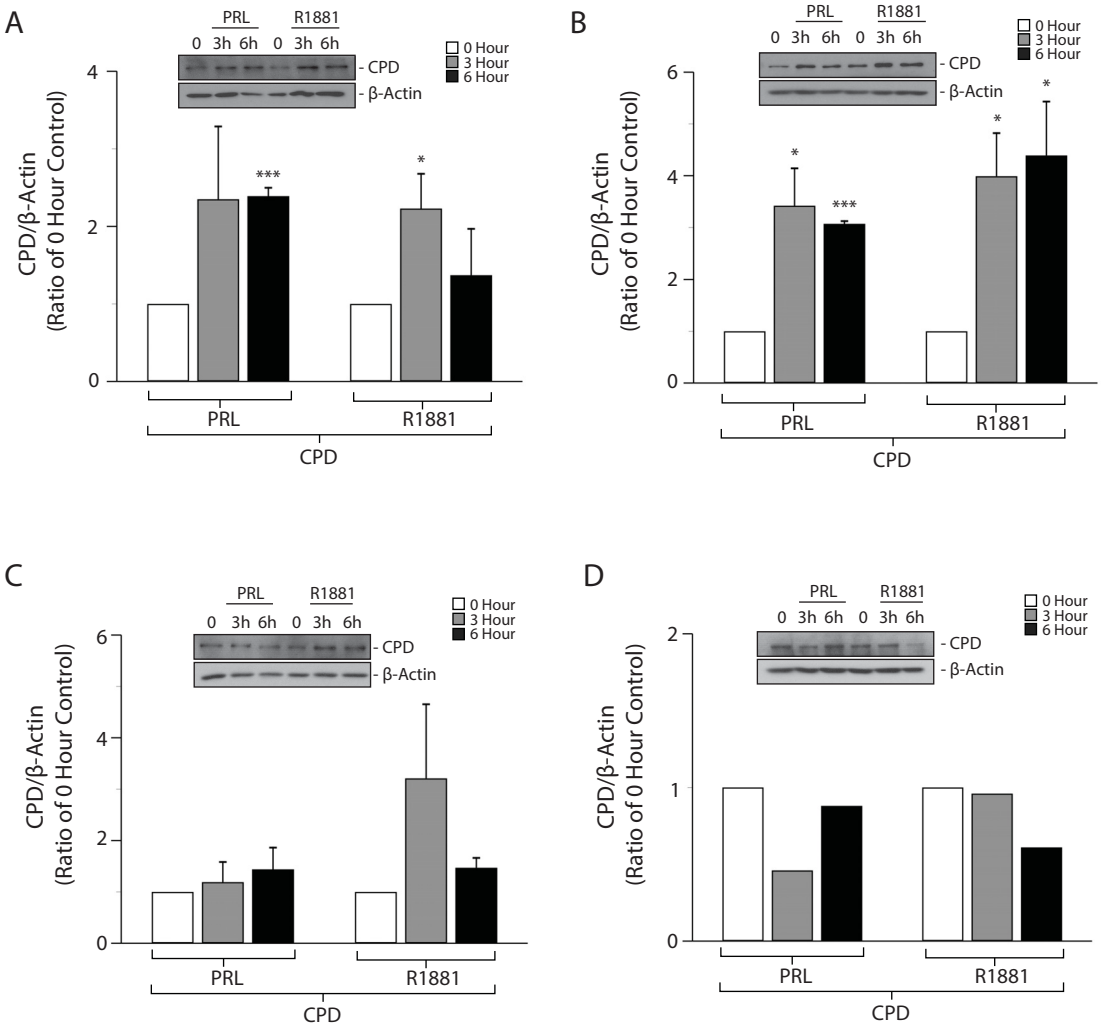

Figure 6. PRL and R1881 Upregulate CPD in TNBC and HER2-enriched Cells.
A) Triple-negative MDA-MB-231, B) MDA-MB-468, and C) HER2-enriched SKBR3, and triple-negative D) MDA-MB-436 cells were made quiescent in DMEM supplemented with 1% charcoal-stripped FBS for 24 hours. Cells were treated with PRL (10 ng/ml) or R1881 (10 nM) for the indicated times. After treatment, cell lysates were collected and analyzed by SDS-PAGE and western blotting as described in Materials and Methods. All values are expressed as mean ± SEM of at least three independent experiments, with the exception of (D; n=1).
* $p<0.05$; ** $p<0.01$; *** $p<0.001$ compared to 0 h control.

3.3 Loss of CPD Decreases TNBC Cell Viability

The Too laboratory, using well-established methods to knockdown CPD mRNA and protein, has previously shown that siRNA-mediated knockdown of CPD increases cellular apoptosis in luminal-A MCF-7 BCa cells (95). In my thesis, I used siRNA to knockdown CPD (siCPD1 and siCPD2) in two TNBC cell lines. The loss of CPD following siRNA transfection was confirmed in MDA-MB-231 (Figure 7A) and MDA-MB-468 (Figure 7C) cells using RT-qPCR. The decrease in CPD gene expression was detected on Day 2 to Day 4. Viable cell counts demonstrated a significant decrease in the viability of MDA-MB-231 (Figure 7B) and MDA-MB-468 (Figure 7D) cells transfected with siCPD1 or siCPD2, as compared to the non-targetting siNT1- or siNT2-transfected controls. SiCPD2 was chosen for the remainder of experiments as siCPD2 was consistently more effective than siCPD1.

3.4 PRL and R1881 Increase CPD-mediated NO Production and Survival in TNBC Cells

Previously published work by the Too laboratory found that PRL-upregulated CPD increases intracellular NO production and inhibits apoptosis in luminal-A MCF-7 BCa cells (95). To determine if similar results are seen in TNBC cell lines, I arrested MDA-MB-231 and MDA-MB-468 cells. These cells, cultured in arginine-free medium, displayed low or undetectable levels of intracellular NO. The addition of Furyacryloyl-alanyl-arginine (FAR), a synthetic CPD substrate, increased NO levels in both cell lines (Figure 8A, Figure 9A). In the presence of FAR, NO production was enhanced by the addition of PRL and/or R1881. Up to a 20-fold increase in NO production was seen in the MDA-MB-231 cells following FAR, PRL, and R1881 treatment, as compared to cells

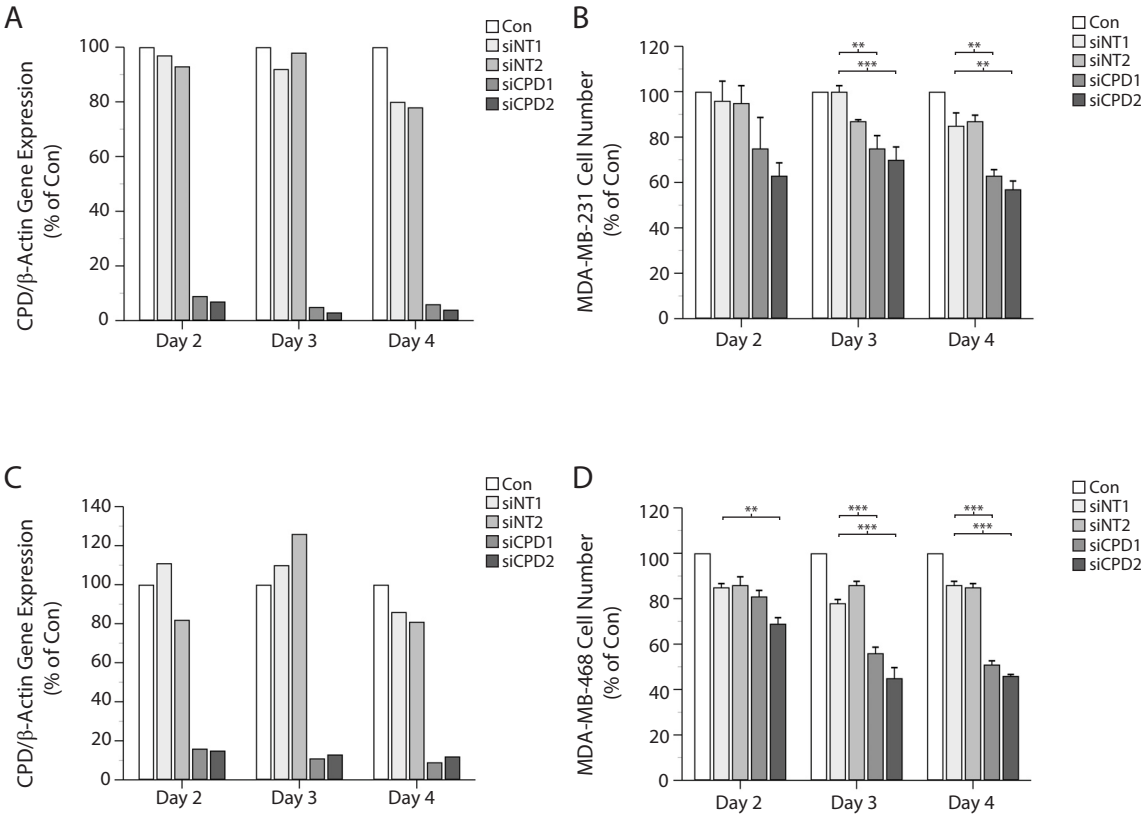

Figure 7. Knockdown of CPD mRNA Expression Decreases TNBC Cell Viability.
A) MDA-MB-231 and C) MDA-MB-468 TNBC cells were either untransfected (Con) or transfected with siNT1, siNT2, siCPD1 or siCPD2 as described in Materials and Methods. RNA was collected on day 2, 3, and 4. CPD and β-actin gene expression was measured using RT-qPCR. Concurrently, viability for B) MDA-MB-231 and D) MDA-MB-468 cells was assessed post-transfection by trypan blue exclusion cell counting on day 2, 3, and 4 as described in Materials and Methods. All values are expressed as mean ± SEM of at least three independent experiments, with the exception of A) and C) (n=1)
** $p<0.01$; *** $p<0.001$ compared to non-targeting siNT-transfected cells.

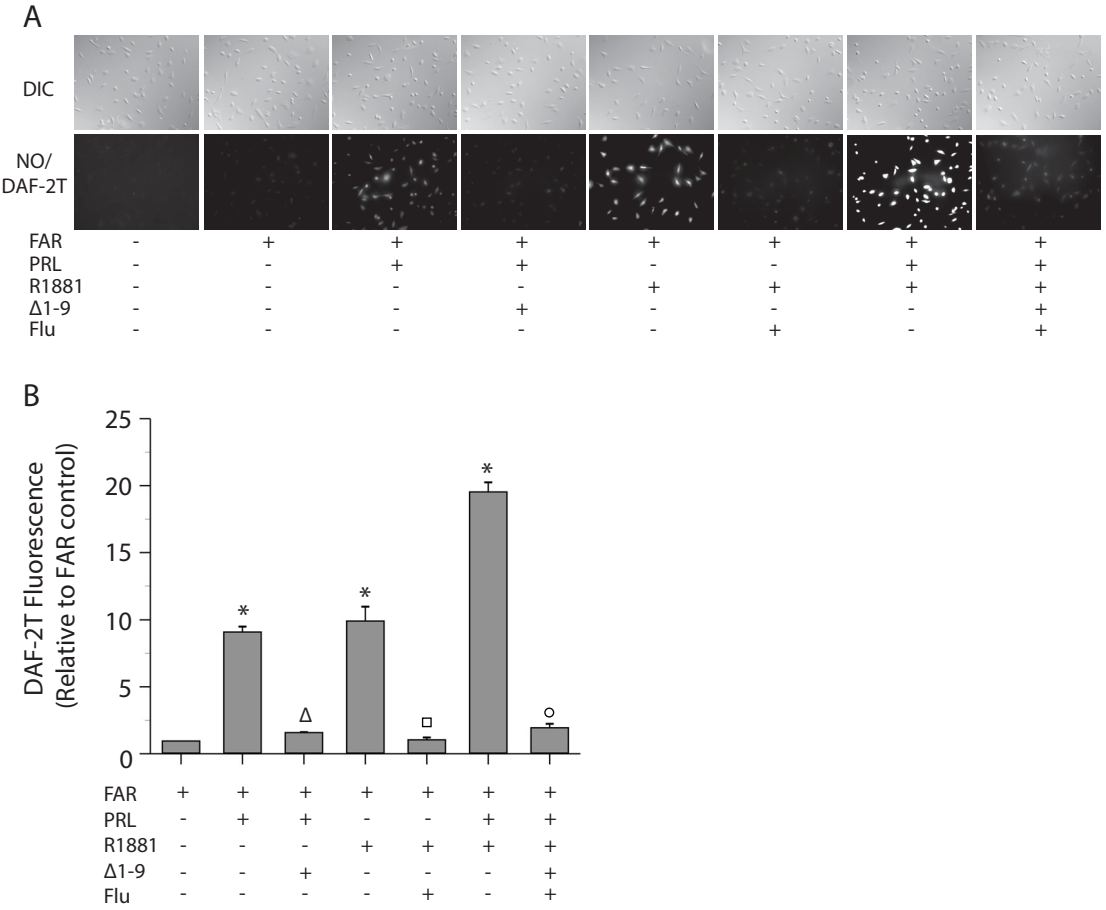

Figure 8. PRL and R1881 Increase NO Production in MDA-MB-231 TNBC Cells. Quiescent MDA-MB-231 cells were treated for 3 h with PRL (20 ng/ml) and/or R1881 (10 nM). Cells were assayed for NO levels as described in Materials and Methods. Shown in A) are representative differential interference contrast (DIC) and fluorescence microscopy images depicting increased NO production following treament with PRL and/or R1881 as well as decreased NO production after hormone blocking action with PRLR and AR antagonists Δ1-9-G129R-hPRL (Δ1-9; 40 ng/ml) and flutamide (Flu; 20 μM), respectively. In B), DAF-2T intracellular fluorescence intensity was quantified using CellProfiler and expressed as a ratio of the FAR control. All values are expressed as mean ± SEM of at least three independent experiments. * $p < 0.001$ as compared to FAR Control; Δ $p < 0.001$ as compared to + PRL; □ $p < 0.001$ as compared to + R1881; ○ $p < 0.001$ as compared to + PRL + R1881

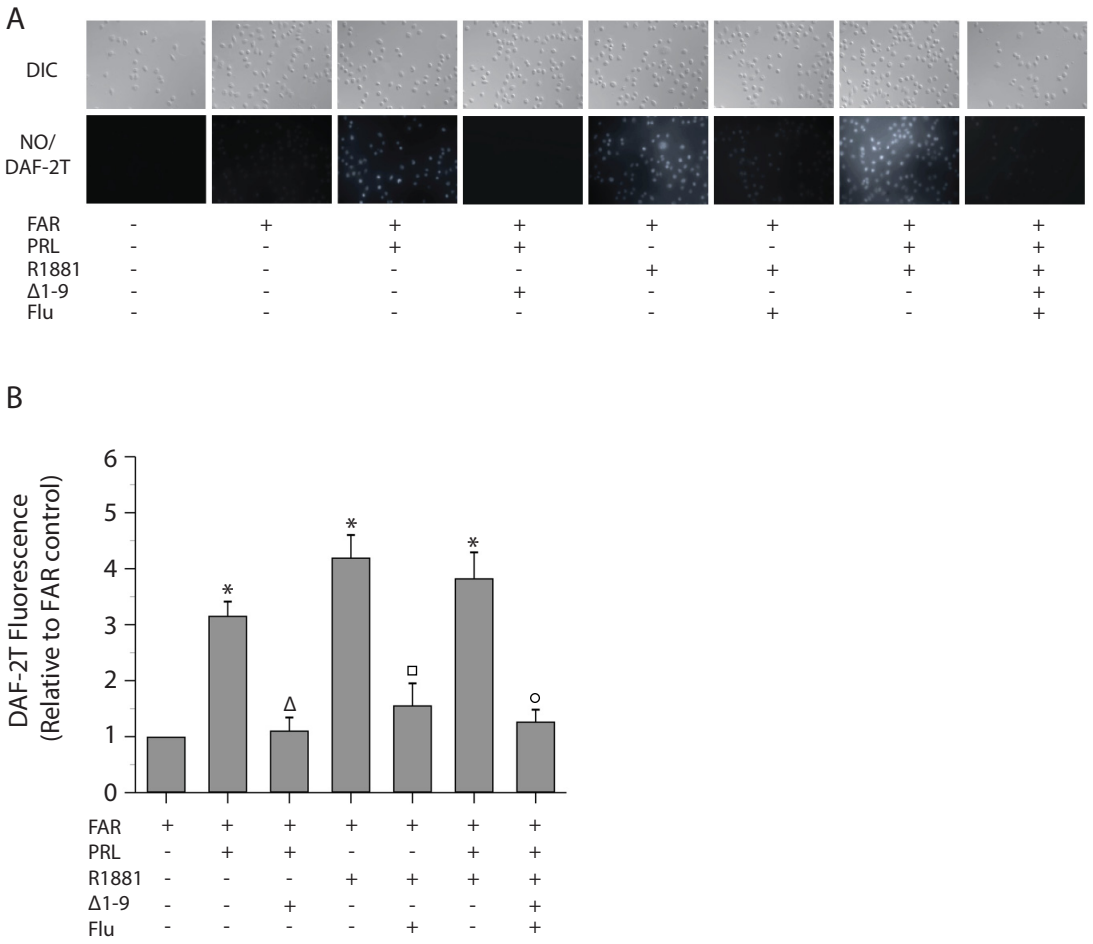

Figure 9. PRL and R1881 Increase NO Production in MDA-MB-468 TNBC Cells. Quiescent MDA-MB-468 cells were treated for 3 h with PRL (20 ng/ml) and/or R1881 (10 nM). Cells were assayed for NO levels as described in Materials and Methods. Shown in A) are representative differential interference contrast (DIC) and fluorescence microscopy images depicting increased NO production following treament with PRL and/or R1881 as well as decreased NO production after hormone blocking action with PRLR and AR antagonists Δ1-9-G129R-hPRL (Δ1-9; 40 ng/ml) and flutamide (Flu; 20 μM), respectively. In B), DAF-2T intracellular fluorescence intensity was quantified using CellProfiler and expressed as a ratio of the FAR control. All values are expressed as mean ± SEM of at least three independent experiments. * $p < 0.001$ as compared to FAR Control; Δ $p < 0.001$ as compared to + PRL; □ $p < 0.001$ as compared to + R1881; ○ $p < 0.001$ as compared to + PRL + R1881

treated with FAR only (Figure 8A, 8B). The MDA-MB-468 cells showed a 4-fold increase in NO production under identical conditions (Figure 9A, 9B). In addition, this enhanced NO production was markedly decreased in the presence of the PRLR antagonist Δ1-9-G129R-hPRL and/or the AR antagonist flutamide, indicating that PRLR and AR blockade decreased NO production.

Two experiments were performed to determine whether the decreased cell viability was a result of decreased NO production due to CPD loss. Firstly, TNBC cells were treated with DETA-NONOate, a synthetic NO donor, to mimic CPD-mediated NO production. Both MDA-MB-231 (Figure 10A) and MDA-MB-468 (Figure 10B) cells cultured in arginine-free and stress-induced conditions (5% CS-FBS) showed increased cell viability following treatment with 25 μM DETA-NONOate. Secondly, MDA-MB-231 cells cultured in arginine-free medium and treated with FAR and PRL displayed a significant ($p < 0.001$) decrease in the percentage of apoptotic cells (58.82±2.19%) compared to untreated control cells (77.94±2.51%; Figure 10C).

3.5 EDD Immunostaining Increases with BCa Progression *in vivo*. High EDD mRNA Expression Correlates with Poor Patient Survival

EDD immunostaining during BCa progression was determined using matched benign human breast tissues, ductal carcinoma in-situ (DCIS), low-grade (LG), high-grade (HG) and TNBC breast tumours (Figure 11A). EDD staining, as a percent of total area, increased from 17.63±6.25% in benign tissues to 25.65±10.49, 45.13±10.45, 71.29±5.50, and 65.85±7.71% in DCIS, LG, HG and TNBC breast tumours, respectively (Figure 11B, *left panel*). LG, HG, and TNBC tumours had significantly higher levels of EDD staining, as compared to benign breast tissues. EDD was detected in the nucleus,

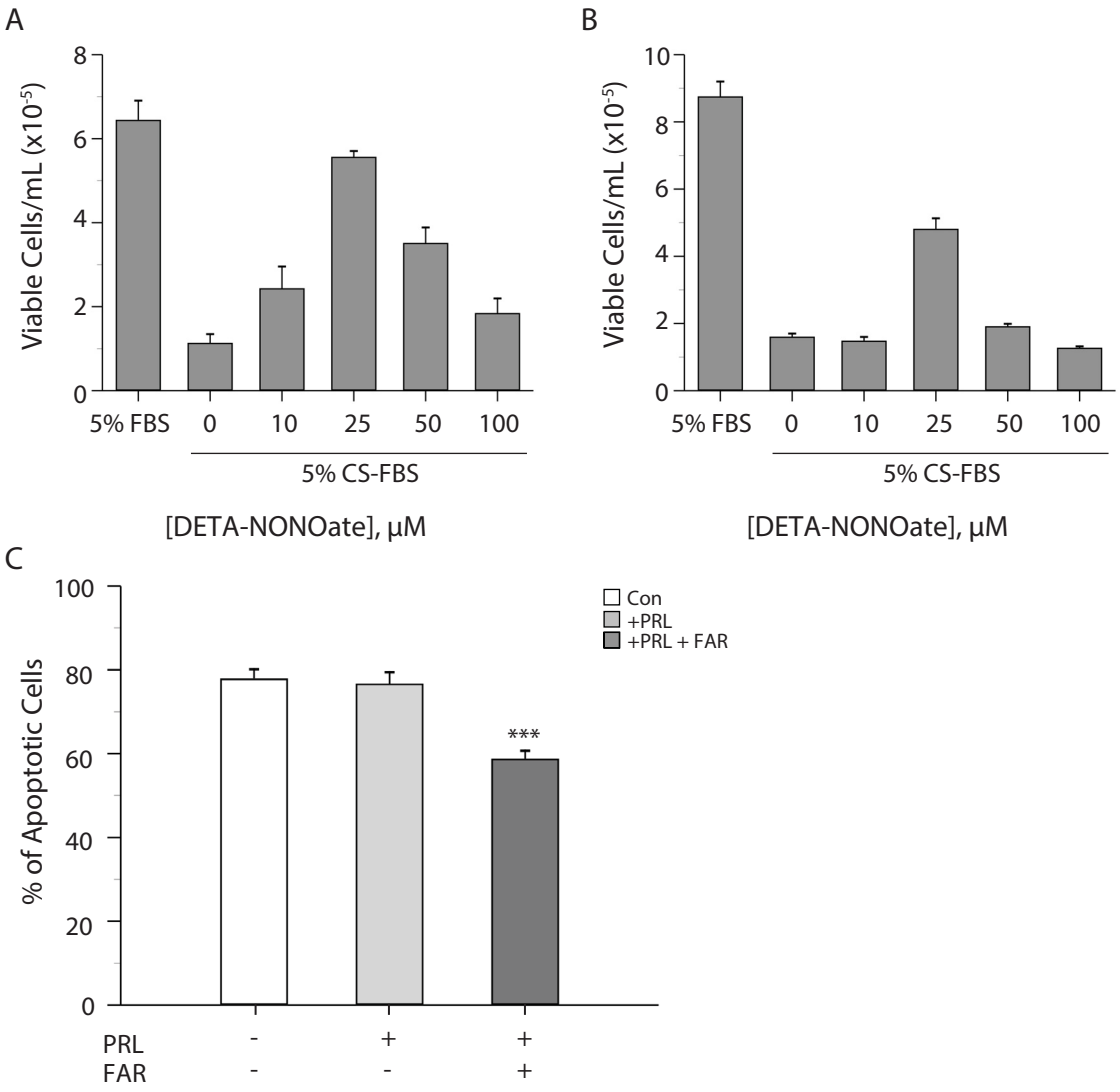

Figure 10. NO Donor DETA-NONOate Increases Cell Viability and Arginine-derived NO Reduces Apoptosis. A) MDA-MB-231 and B) MDA-MB-468 TNBC cells grown in arginine-free medium containing 5% heat-inactivated charcoal-stripped FBS for 24 h were treated with increasing concentrations of DETA-NONOate as described in Materials and Methods. Cell viability was assessed by trypan blue exclusion cell counting after 3 days. All values are expressed as mean ± SEM (n = 4). C) MDA-MB-231 cells ± PRL and/or ± synthetic CPD substrate FAR were stained with Annexin-V and propidium iodide and analyzed by flow cytometry. All values are expressed as mean ± SEM of at least three independent experiments. *** $p<0.001$ compared to Con (-PRL, -FAR).

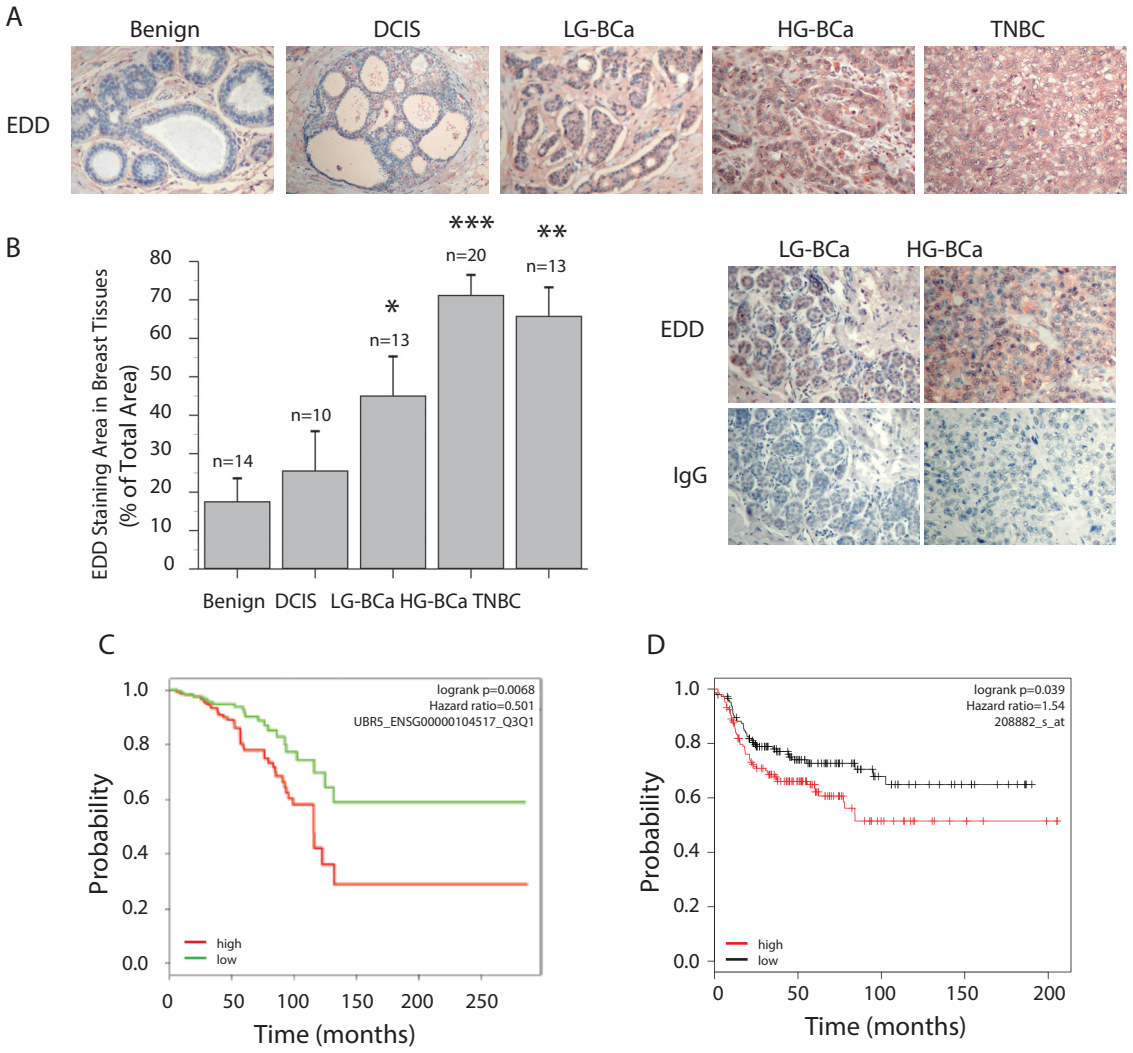

Figure 11. EDD Levels Increase with BCa Progression *in vivo* and Correlates with Poor Patient Survival. A) Immunostaining for EDD was performed using mouse anti-EDD mAb and the percent of EDD area stained (red) was determined in benign tissues, DCIS, low-grade (LG), high-grade (HG) BCa, and in TNBC, as described in Materials and Methods. B) EDD-stained area, as a percent of total area, of individual specimens was plotted. Mean ± SEM, * significantly differentfrom benign ($p < 0.05$, n=13), ** significantly different from benign and DCIS ($p < 0.05$, n=13), *** significantly different from benign, DCIS and LG-BCa ($p < 0.05$, n = 20). C) An online database (http://ec2-52-201-246-161.compute-1.amazonaws.com/kmexpress/index.php) was used to determine the relevance of individual EDD mRNA expression to the overall patient survival. Survival curves for EDD mRNA expression in BCa patients from the TCGA database (n = 943) were bifurcated by Q3Q1 (top 25% in red versus bottom 25% in green). D) An online database (http://kmplot.com/analysis/) was used to determine the relevance of individual EDD mRNA expression to the relapse-free survival. Survival curves for EDD Affymetrix ID: 208882_s_at (UBR5) are plotted for high-grade (3) basal BCa patients (n = 293). Patient samples were bifurcated by the median into low (black) and high (red) expression groups for EDD. Survival Hazard ratio (HR) and 95% confidence intervals, as well as log rank P were calculated and displayed on the webpage. A p value of <0.05 was considered to be statistically significant.

but was mainly in the cytoplasm, especially in HG and TNBC tumours. The absence of specific staining with IgG in matched areas of LG and HG BCa that were also immunostained with mouse anti-EDD indicated specificity of the EDD antibody (Figure 11B, *right panel*).

Moreover, analysis of the TCGA database using Kaplan-Meier-Express software showed that BCa patients (n=943) with high EDD (UBR5) mRNA expression had a significantly lower probability of overall survival (Figure 11C). In addition, Kaplan-Meier-Plotter analysis showed that HG-TNBC patients with high EDD (UBR5) mRNA expression had a significantly lower probability of relapse-free survival (Figure 11D).

3.6 EDD is Upregulated by Hormones in TNBC and HER2-Enriched Cell Lines

EDD expression has been reported to be upregulated by PRL and progesterone in luminal-A T47D cells (165). I therefore sought to determine if EDD expression is altered following hormonal treatment of TNBC cell lines. Quiescent TNBC MDA-MB-231, MDA-MB-468, and MDA-MB-436 cells, as well as the HER2-enriched SKBR3 cells were treated with PRL or R1881 for 3 and 6 hours. Cell lysates were immunoblotted for EDD. On average in MDA-MB-231 cells, PRL upregulated protein levels of EDD by 4.01-fold after 3 hours. R1881 also upregulated protein levels of EDD by 4.36-fold after 3 hours (Figure 12A). Fold-increases in EDD levels were similar for MDA-MB-468 cells (from 1.57 to 4.39-fold) (Figure 12B) and SKBR3 cells (from 1.19 to 3.21-fold) (Figure 12C). In contrast, MDA-MB-436 cells did not respond to hormonal treatment and of EDD protein levels were unchanged by either PRL or R1881 (Figure 12D). The cell lines with low or high EDD expression were used for overexpression or knockout/knockdown experiments, respectively.

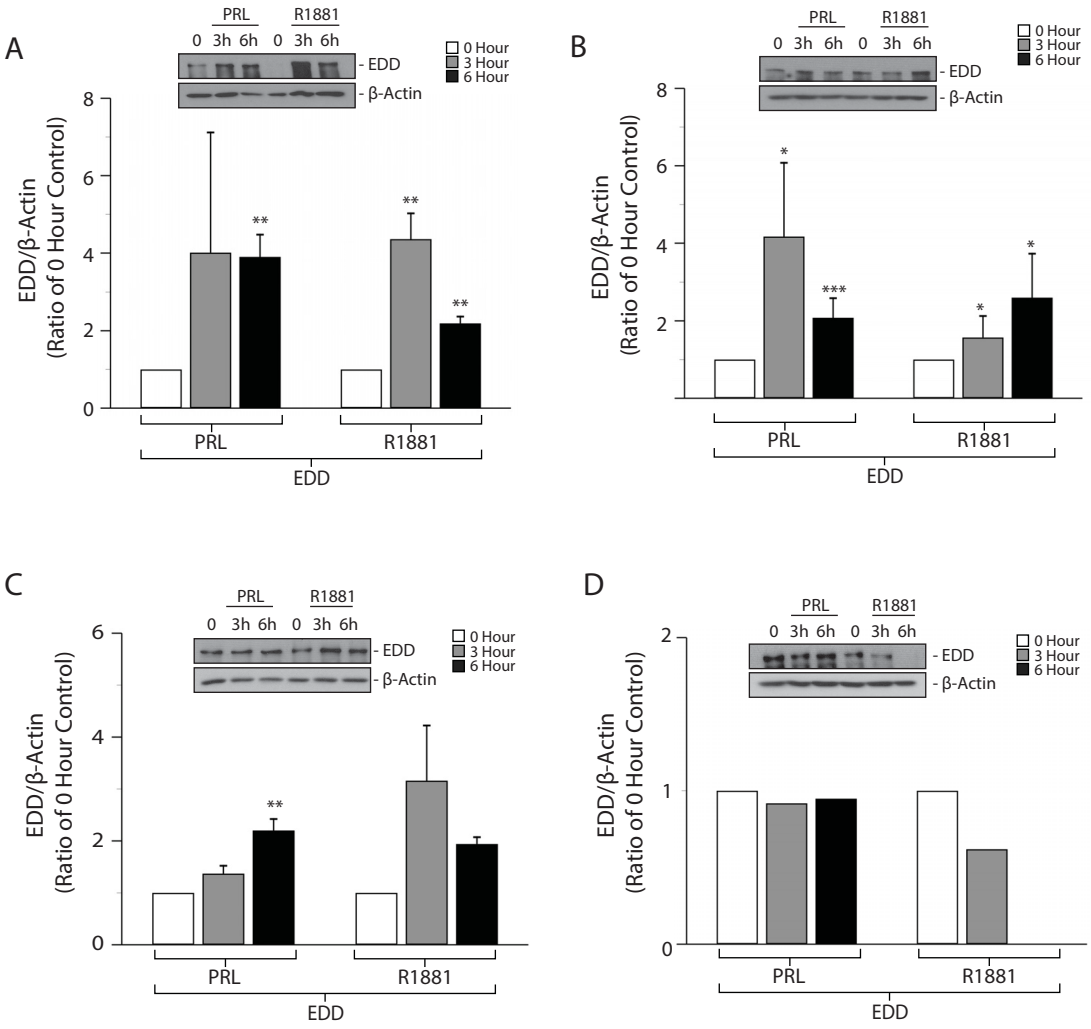

Figure 12. PRL and R1881 Upregulate EDD in TNBC and HER2-enriched Cells.
A) Triple-negative MDA-MB-231, B) MDA-MB-468, and C) HER2-enriched SKBR3, and triple-negative D) MDA-MB-436 cells were made quiescent in DMEM supplemented with 1% charcoal-stripped FBS for 24 hours. Cells were treated with PRL (10 ng/ml) or R1881 (10 nM) for the indicated times. After treatment, cell lysates were collected and analyzed by SDS-PAGE and western blotting as described in Materials and Methods. All values are expressed as mean ± SEM of at least three independent experiments, with the exception of (D; n=1).
* $p<0.05$; ** $p<0.01$; *** $p<0.001$ compared to 0 h control.

Preliminary analysis of the EDD promoter revealed several putative ARE and GAS sequences as well as Sp-1, a transcription factor reported to be upregulated by PRL in Nb2 cells (168), binding sites (Figure 13). The presence of these putative response elements or binding sites suggest that EDD may be upregulated by PRL and R1881 at the transcriptional level.

3.7 Loss of EDD Decreases TNBC Cell Viability

Knockdown of EDD gene expression using siRNA has previously been shown by the Too laboratory to decrease cell viability in luminal-A MCF-7 and T47D BCa cells (MacDonald et al, *In Press*). Using siRNA targetting EDD (siEDD1 and siEDD2), loss of EDD following siRNA transfection was confirmed in MDA-MB-231 (Figure 14A) and MDA-MB-468 (Figure 14C) cells using RT-qPCR. The decrease in EDD gene expression was detected on Day 2 to Day 4. Viable cell counts demonstrated a significant decrease in the viability of MDA-MB-231 (Figure 14B) and MDA-MB-468 (Figure 14D) cells transfected with siEDD1 or siEDD2, as compared to the non-targetting siNT1- or siNT2-transfected controls. SiEDD1 was chosen for the remainder of experiments as siEDD1 was consistently more effective than siEDD2.

3.8 Loss of EDD Increases Expression of Pro-apoptotic Initiator and Effector Proteins

To assess whether the decreased cell viability found in this thesis was a result of increased apoptosis, I immunoblotted for apoptosis effector protein Bax, its cytoplasmic-to-mitochondrion translocator MOAP-1, as well as downstream caspase-7 and caspase substrate PARP-1 of the mitochondrial-mediated apoptotic pathway. Luminal-A MCF-7 cells were selected for these experiments as we have shown that siEDD1-transfected

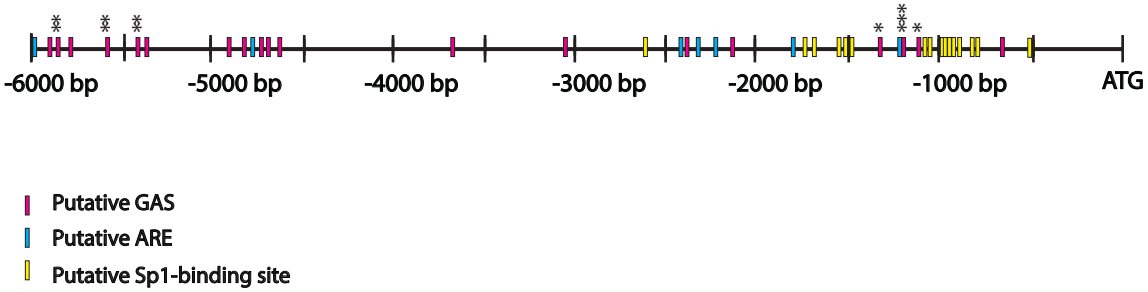

Figure 13. The EDD Gene Promoter Contains Putative Hormone Response Elements and Sp-1 Binding Sites. A depiction of the EDD promoter containing putative gamma-interferon regulatory sequences (GAS), androgen response elements (ARE) and Sp-1 binding sites. ⁂ STAT5-binding in T-cells; ⁑ Active motif; * Active motif with nnn

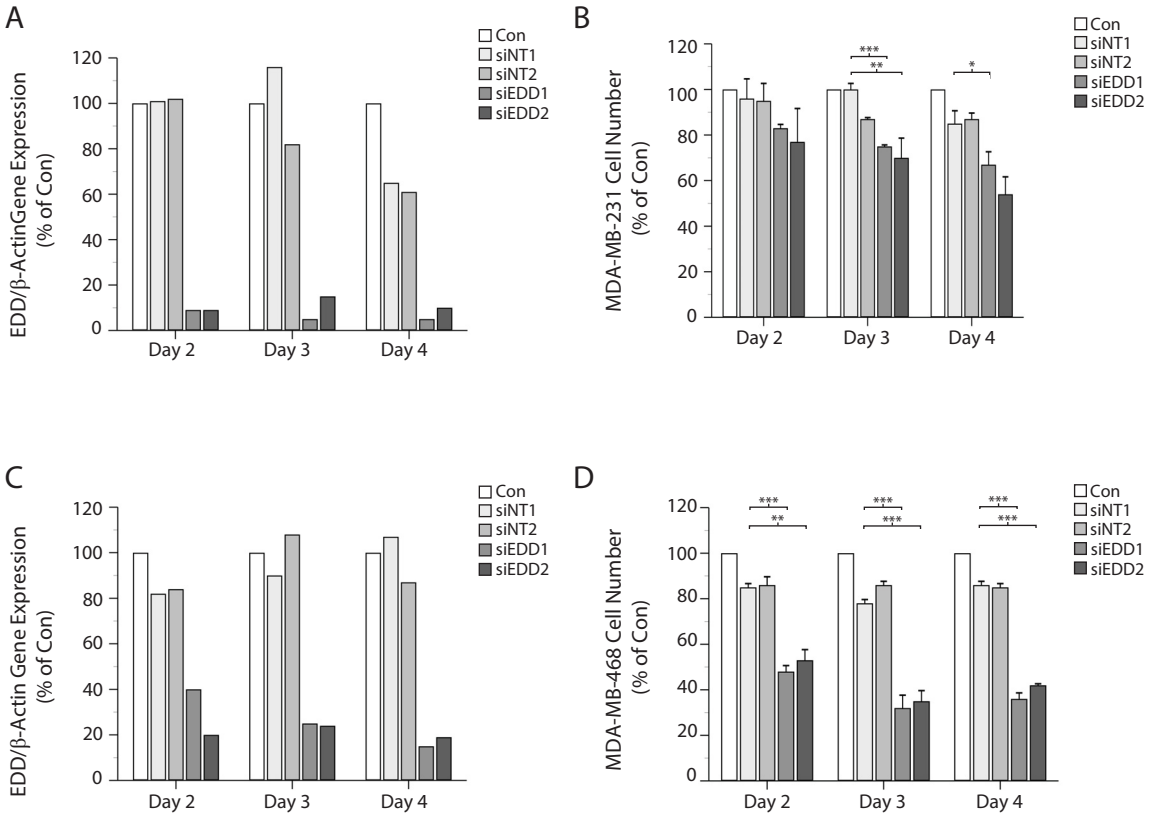

Figure 14. Knockdown of EDD mRNA Expression Decreases TNBC Cell Viability.
A) MDA-MB-231 and C) MDA-MB-468 TNBC cells were either untransfected (Con) or transfected with siNT1, siNT2, siEDD1 or siEDD2 as described in Materials and Methods. RNA was collected on day 2, 3, and 4. EDD and β-actin gene expression was measured using RT-qPCR. Concurrently, viability for B) MDA-MB-231 and D) MDA-MB-468 cells was assessed post-transfection by trypan blue exclusion cell counting on day 2, 3, and 4 as described in Materials and Methods. All values are expressed as mean ± SEM of at least three independent experiments, with the exception of A) and C) (n=1)
* $p<0.05$; ** $p<0.01$; *** $p<0.001$ compared to non-targeting siNT1-transfected cells.

MCF-7 cells arrest in G2/M of the cell cycle and undergo early- and late-stage apoptosis which was accompanied by increased expression of pro-apoptotic Bak and Bim proteins (Performed by Lynn Thomas in MacDonald et al, *In Press*). Loss of EDD following siEDD1 transfection resulted in a 2-fold elevation in both MOAP-1 and Bax protein levels (Figure 15A, 15B). This was accompanied by a time-dependent increase in cleaved caspase-7 with up to a 6-fold cleavage after 24 h and up to a 14-fold increase in PARP-1 cleavage after 48 h (Figure 16).

3.9 Loss of EDD Decreases PRL-/R1881-Induced Phosphorylation of 4E-BP1 and eIF4E Release

Our laboratory has previously reported that PRL stimulates the phosphorylation of 4E-BP1 in PRL-dependent rat Nb2 lymphoma cells (132, 169). The effect of EDD loss on PRL-/R1881-induced phosphorylation of 4E-BP1 was assessed by transfecting quiescent MCF-7 cells with siEDD1 48 h prior to acute treatment of these cells with PRL or R1881 for 1 h. SiEDD1-transfected cells displayed a 90% loss of both EDD mRNA (Figure 17A) and protein (Figure 17B), as compared to siNT-transfected and untransfected controls. In both siNT and untransfected control cells, immunoblots for 4E-BP1 showed doublet α-4E-BP1 and β-4E-BP1 bands (Figure 17C, 18A, and 19A, lane 1). PRL- or R1881-induced hyperphosphorylation of 4E-BP1 giving rise to a higher molecular weight, hyperphosphorylated γ-band after 1 h (Figure 17C, 18A, and 19A, lanes 2 and 3). This PRL- or R1881-induced γ-4E-BP1 band was attenuated in siEDD1-transfected cells (Figure 17C, 18A, and 19A, lane 4). Densitometric analyses of several independent experiments demonstrated that PRL significantly increased γ-4E-BP1 in siNT and untransfected control cells but this increase was significantly attenuated with

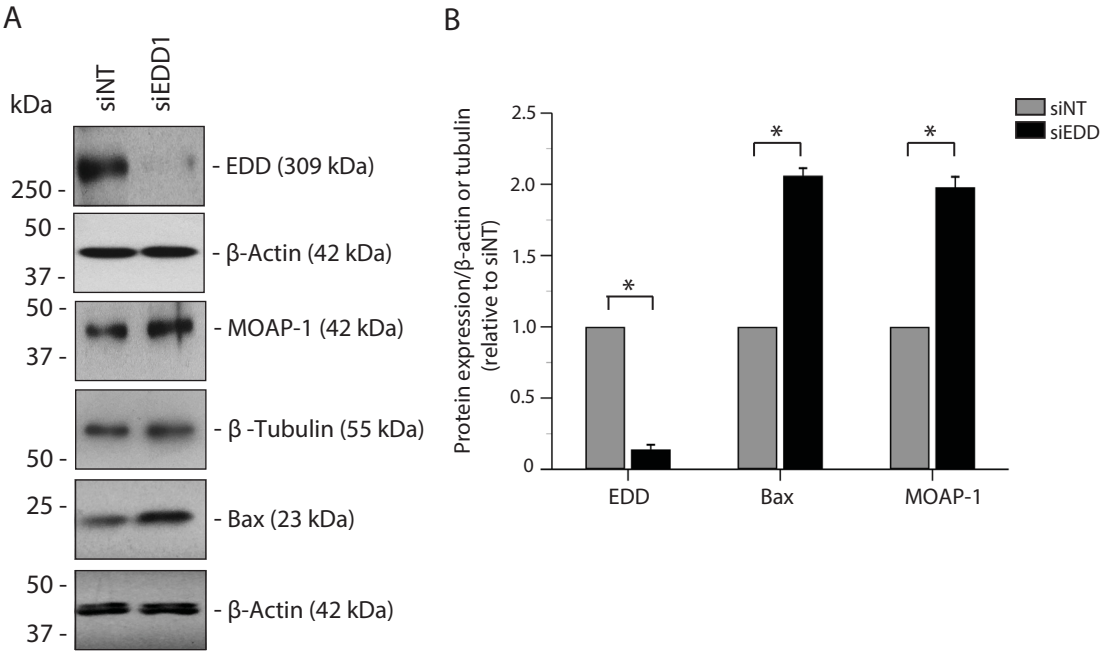

Figure 15. EDD Loss Increases Pro-apoptotic Bax and Modulator of Apoptosis Protein-1 (MOAP-1) Protein Expression. Quiescent MCF-7 cells were transfected with siEDD1 or siNT for 24 h as described in Materials and Methods. Cell lysates were subjected to SDS-PAGE and Western analysis. A) Representative Western blots. B) Densitometric analyses of EDD, Bax and MOAP-1 protein levels were expressed as a ratio of either β-actin or β-tubulin and normalized to siNT control cells. All values are expressed as mean ± SEM (n = 3-6) * $p<0.05$ compared to siNT control.

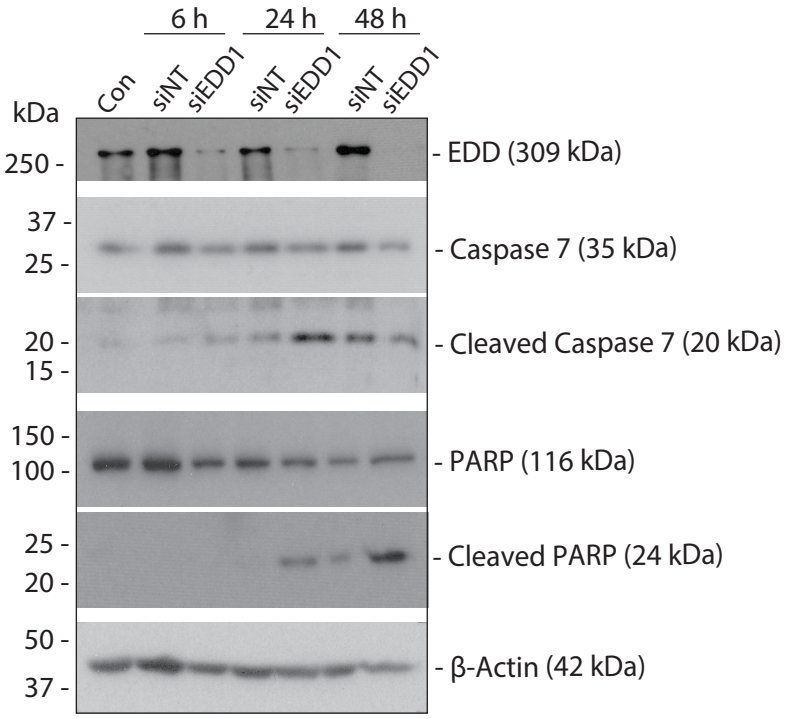

Figure 16. Loss of EDD Increases Caspase-7 and PARP-1 Cleavage in Luminal A MCF-7 Cells. MCF-7 cells were transfected with siEDD1, siNT or left untransfected (Con) for up to 48 h as described in Materials and Methods. Cell lysates were subjected to SDS-PAGE and Western analysis. Immunoblots shown are representative of at least two independent experiments.

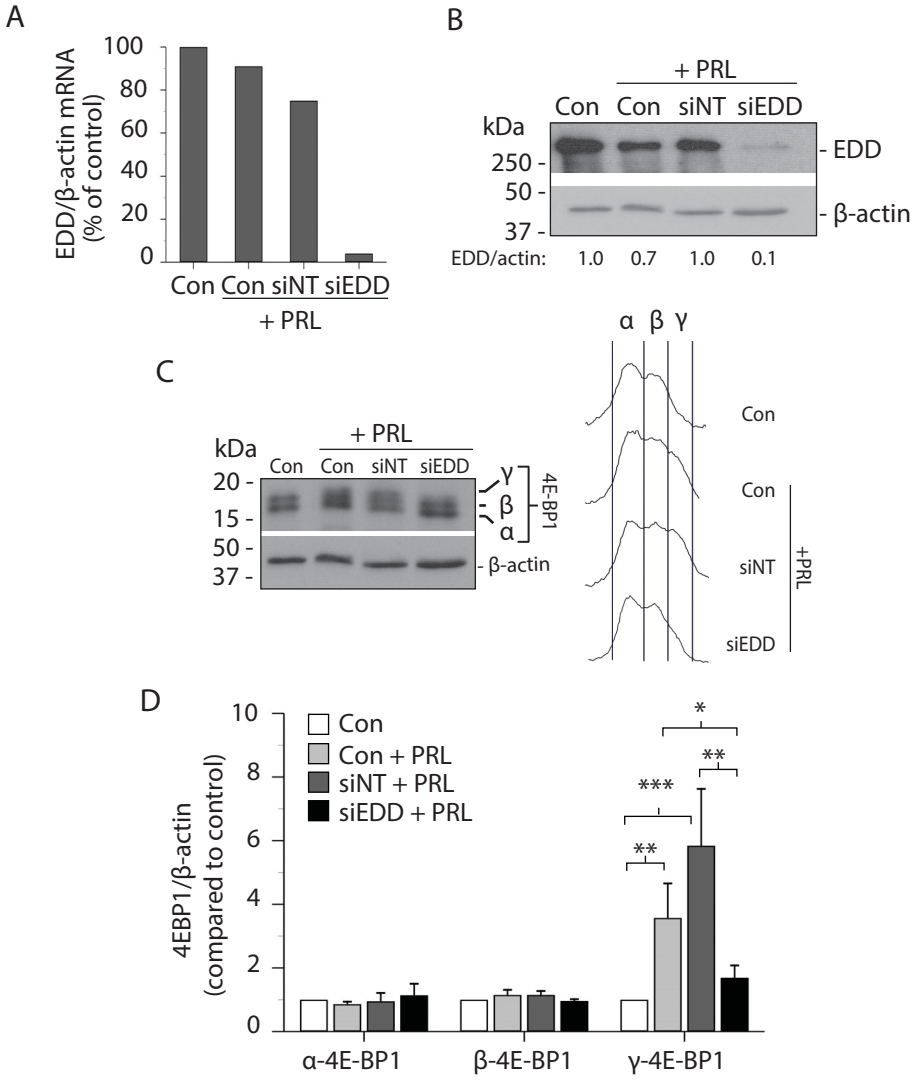

Figure 17. Loss of EDD Decreases PRL-stimulated Phosphorylation of 4E-BP1.
Quiescent MCF-7 cells were transfected with siEDD1, siNT or left untransfected (Con) for 48 h as described in Materials and Methods. Cells were treated with PRL (10 ng/ml) for 1 h. A) Total RNA was extracted for RT-qPCR analysis and B) cell lysates were subjected to SDS-PAGE and Western analysis. C) Densitometric analysis was carried out on the α-, β-, and γ-4EBP1 bands. In D), densitometric analyses of the α-, β-, and γ-4EBP1 bands were expressed as a ratio of β-actin and normalized to control cells. All values are expressed as mean ± SEM of at least four independent experiments. * $p<0.05$; ** $p<0.01$; *** $p<0.001$ as indicated.

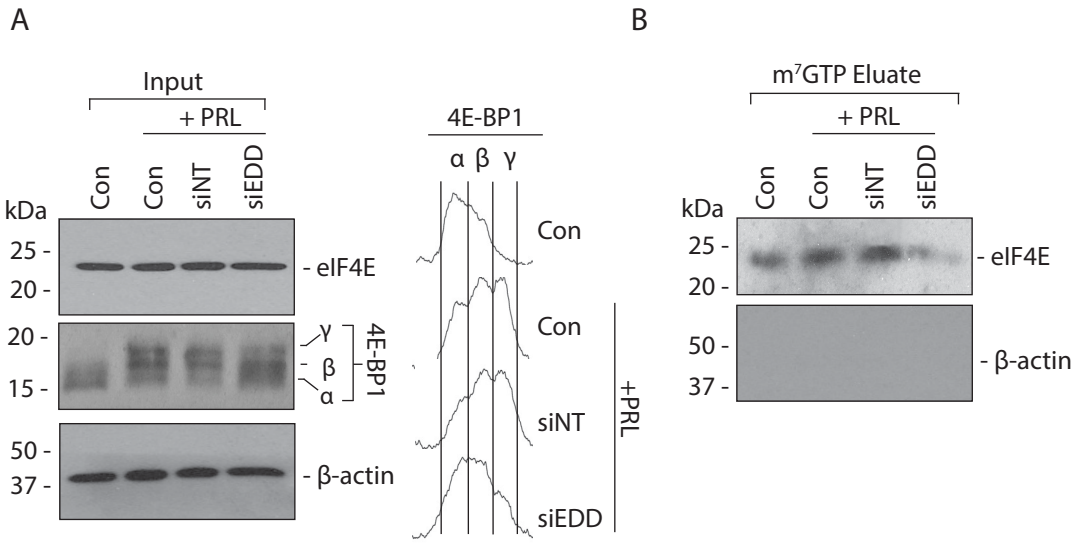

Figure 18. Loss of EDD Decreases PRL-stimulated Dissociation of eIF4E from 4E-BP1
Quiescent MCF-7 cells were transfected with siEDD1, siNT or left untransfected (Con) for 48 h as described in Materials and Methods. Cells were treated with PRL(10 ng/ml) for 1 h. A) Cell lysates (input) were subjected to SDS-PAGE and Western analysis followed by densitometric analysis of the α-, β-, and γ-4EBP1 bands. In B), cell lysates were incubated with m^7GTP agarose beads as decribed in the Materials and Methods and lysates were subsequently washed, eluted and subjected to SDS-PAGE and Western analysis of translation initation factor eIF4E (m^7GTP Eluate). β-Actin was used as a control to confirm sufficient washing of the agarose affinity resin. Immunoblots shown are representative of at least three independent experiments.

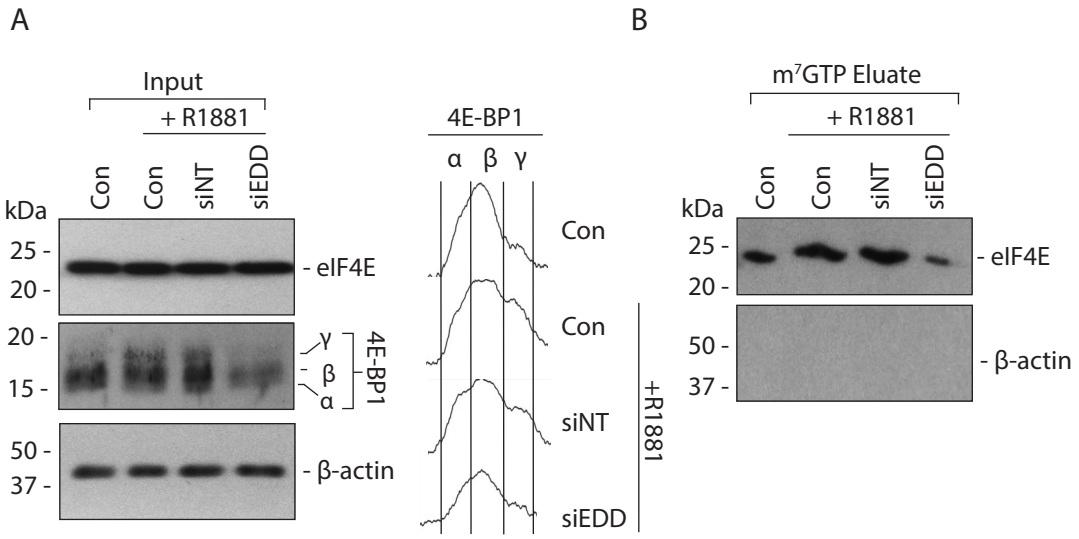

Figure 19. Loss of EDD Decreases R1881-stimulated Dissociation of eIF4E from 4E-BP1
Quiescent MCF-7 cells were transfected with siEDD1, siNT or left untransfected (Con) for 48 h as described in Materials and Methods. Cells were treated with R1881 (10 nM) for 1 h. A) Cell lysates (input) were subject to SDS-PAGE and Western analysis followed by densitometric analysis of the α-, β-, and γ-4EBP1 bands. In B), cell lysates were incubated with m^7GTP agarose beads as decribed in the Materials and Methods and lysates were subsequently washed, eluted and subject to SDS-PAGE and Western analysis of translation initation factor eIF4E (m^7GTP Eluate). β-Actin was used as a control to confirm sufficient washing of the agarose affinity resin. Immunoblots shown are representative of at least three independent experiments.

EDD loss. There was no difference between the level of α-4E-BP1 and β-4E-BP1 amongst the four treatment groups (Figure 17D).

Hyperphosphorylation of 4E-BP1 results in the release of eIF4E which then binds to the 5′-m^{7}GTP cap of newly synthesized mRNAs to initiate protein translation (170). I performed a series of cap-binding assays which showed that the disappearance of the PRL-/R1881-induced γ-4E-BP1 band in siEDD1-transfected cells correlated with a decrease in eIF4E binding to the m^{7}GTP agarose (Figure 18B and 19B, lane 4). As expected, no β-actin was recovered from the cap-binding assay, which demonstrated adequate washing of the agarose beads prior to immunoblotting.

3.10 Ectopic EDD Promotes Drug Resistance in TNBC Cells

EDD overexpression has been linked to platinum drug resistance in ovarian cancer cells (161, 164). On that basis, I looked to determine if EDD overexpression is implicated in anti-cancer drug resistance in BCa. The TNBC MDA-MB-436 cells express low EDD protein levels (Performed by Lynn Thomas in MacDonald et al, *In Press*). Therefore, MDA-MB-436 cells were transfected with increasing plasmid concentrations of pCMV-Tag2B.EDD or control pCMV-Tag2B to vary ectopic EDD levels. Ectopic EDD expression was confirmed using RT-qPCR (Figure 20A, 20B, *upper panels*) and Western blotting (Figure 20B, *lower panels*). Transfected cells were treated with IC_{50} doses of cisplatin (0.3125 μM; Figure 20C, *left panel*) or doxorubicin (0.023 μM; Figure 20D, *left panel*) for five days. Untreated control cells which received pCMV-Tag2B.EDD above 250 ng/ml, showed a decrease in viable cell number compared to drug-free pCMV-Tag2B controls (Figure 20C, 20D, *middle panels*). As a result, a maximum plasmid concentration of 500 ng/ml was used for subsequent transfection experiments. Viable cell

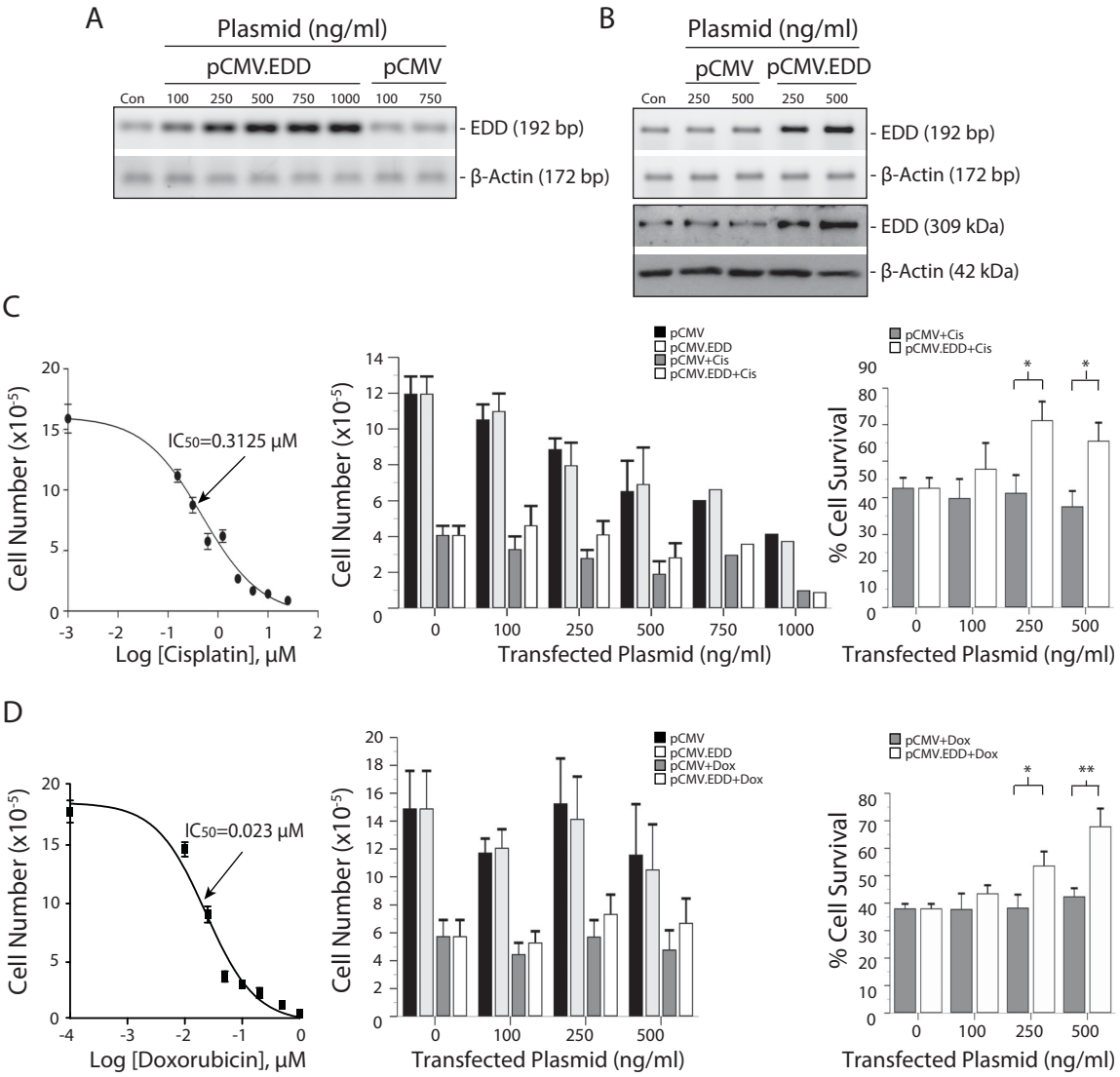

Figure 20. Ectopic EDD Increases Cisplatin/Doxorubicin Resistance in TNBC Cells. MDA-MB-436 cells were transfected with plasmid pCMV-Tag2B or pCMV-Tag2B.EDD at the indicated concentrations or left untransfected. A,B, (*upper panel*) After two days, the cells were harvested for semi-quantitative PCR or B, (*lower panel*) Western analysis. MDA-MB-436 cells were treated with increasing doses of C) cisplatin and D) doxorubicin for five days and were counted using trypan blue exclusion cell counting to determine the IC_{50} values (*left panels*). MDA-MB-436 cells were treated with the IC_{50} dose of C) cisplatin or D) doxorubicin and counted by trypan blue exclusion cell counting and plotted as cell number ± drug treatment (*middle panels*) or as percent survival of drug-treated transfectants to untreated controls (*right panels*). All values are expressed as mean ± SEM of at least three independent experiments. * $p<0.05$; ** $p<0.01$ indicates significance between the groups compared.

numbers for pCMV-Tag2B-transfected controls were further decreased with cisplatin (Figure 20C, *middle panel*) and doxorubicin (Figure 20D, *middle panel*) treatment. The pCMV-Tag2B.EDD-transfected cells were more drug resistant than control cells. Specifically, the percent cell survival was significantly increased in cisplatin- (Figure 20C, *right panel*) and doxorubicin-treated (Figure 20D, *right panel*) cells that received 250 or 500 ng/ml pCMV-Tag2B.EDD, as compared to their respective pCMV-Tag2B control.

Given that EDD modulates PRL stimulation of mTOR signalling (Figures 1, 4, and 17-17), I investigated the sensitivity of MDA-MB-436 cells to rapamycin (TORC1 inhibitor) and INK128 (TORC1/2 inhibitor). IC_{50} doses of rapamycin and INK128 were 16.59 μM (Figure 21A, *left panel*) and 0.014 μM (Figure 21B, *left panel*), respectively. As before, viable cell counts decreased after pCMV-Tag2B.EDD transfection for five days and further decreased with rapamycin (Figure 21A, *middle panel*) and INK128 (Figure 21B, *middle panel*) treatment. However, pCMV-Tag2B.EDD-transfected cells were more resistant to each inhibitor as compared to controls, with the percent surviving rapamycin (Figure 21A, *right panel*) or INK128 (Figure 21B, *right panel*) treatment significantly increased. Therefore, ectopic EDD expression increased cell resistance to chemotherapy drugs, TORC1, and TORC1/2 inhibitors.

3.11 Loss of EDD Decreases Drug Resistance in Luminal-A BCa Cells

Since anti-cancer drug resistance occurred in cells with overexpressed EDD, I sought to determine whether the reverse was true as well, that is, were EDD-depleted cells less resistant to anti-cancer drugs. To examine this, lentiviral shRNA-EDD gene knockout was performed in luminal-A MCF-7 cells which we have previously shown to

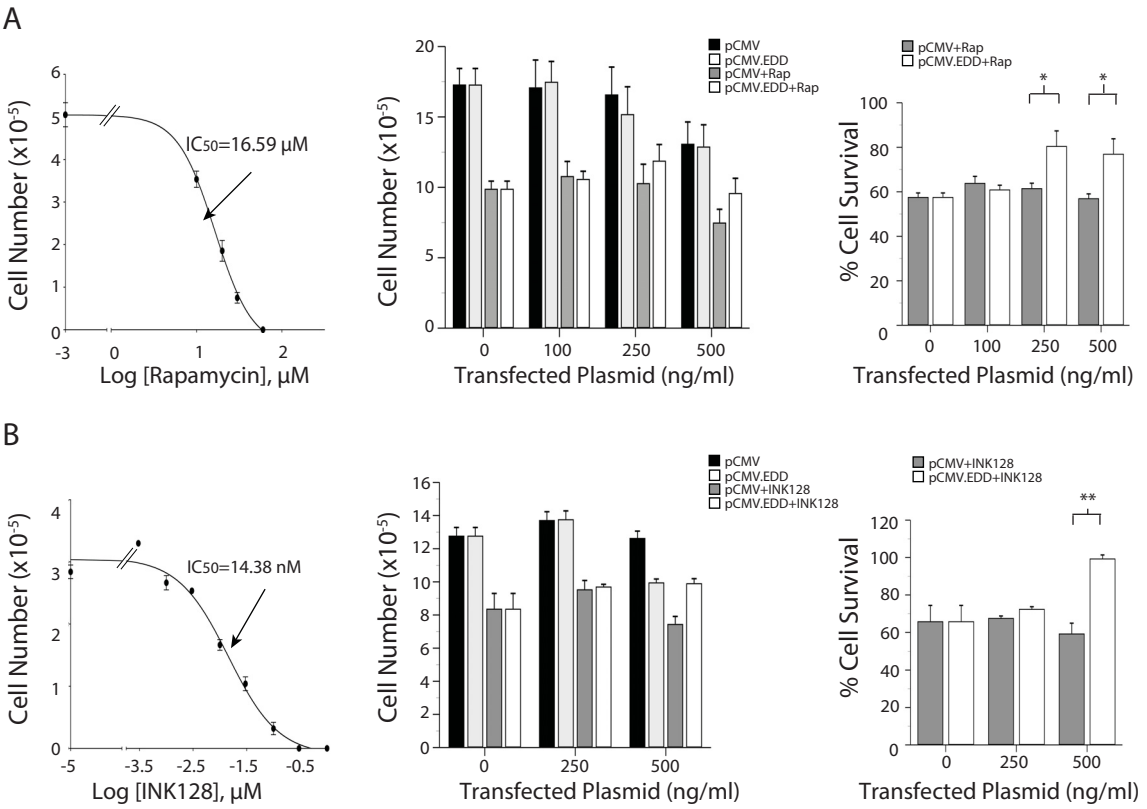

Figure 21. Ectopic EDD Increases Rapamycin/INK128 Resistance in TNBC Cells. MDA-MB-436 cells were transfected with plasmid pCMV-Tag2B or pCMV-Tag2B.EDD at the indicated concentrations or left untransfected. MDA-MB-436 cells were treated with increasing doses of A) rapamycin and B) INK128 for five days. Viable cells were counted using trypan blue exclusion cell counting to determine the IC_{50} values (*left panels*). MDA-MB-436 cells were treated with the IC_{50} dose of A) rapamycin or B) INK128, and counted via trypan blue exclusion cell counting and plotted as cell number ± drug treatment (*middle panels*) or as percent survival of drug-treated transfectants to pCMV-Tag2B + drug (*right panels*). All values are expressed as mean ± SEM of at least three independent experiments. * $p<0.05$; ** $p<0.01$ indicates significance between the groups compared.

express high levels of EDD (Performed by Lynn Thomas in MacDonald et al, *In Press*). Western analysis of several stable puromycin-resistant clones showed EDD knockout clones 1.1C and 3.1A, as well as clones 1.1A, 1.1B and 3.1C (Figure 22A). Clones 1.1C and 1.1A were selected as the EDD knockout and control clones, respectively. Clones 1.1C and 1.1A were treated with IC_{50} doses of cisplatin (0.3125 μM), doxorubicin (0.023 μM), rapamycin (16.59 μM) or tamoxifen (20 μM), and viable cells were counted over five days. In the absence of drug treatment, EDD-knockout clone 1.1C grew at a slower rate than control clone 1.1A (Figure 22B and 22C, *left panels*; Figure 23A and 23, *left panels*). All four drug treatments decreased the growth of each clone (Figure 22B and 22C, *left panels*; Figure 23A and 23B, *left panels*). Regardless of which drug was used, clone 1.1C was less resistant to the drug than clone 1.1A on Day 4 and/or Day 5 (*middle panels*). The percentage of clone 1.1C cells surviving drug treatment significantly decreased on Day 5 compared to clone 1.1A (*right panels*). Therefore, EDD loss decreased cell resistance to chemotherapy drugs, cisplatin and doxorubicin, as well as to TORC1 inhibitor rapamycin, and estrogen-receptor modulator tamoxifen.

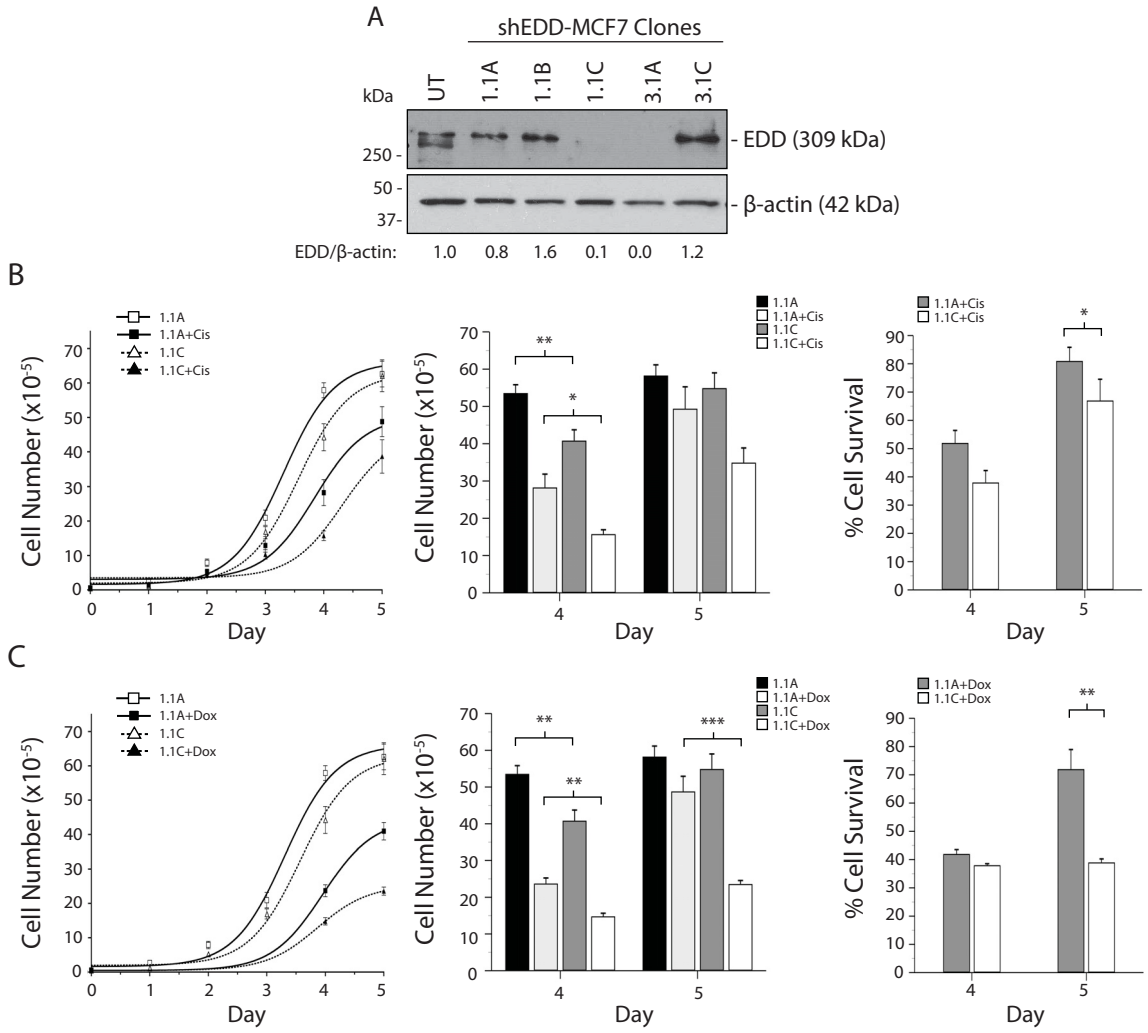

Figure 22. Loss of EDD Decreases Resistance to Cisplatin/Doxorubicin in Luminal A MCF-7 Cells. MCF-7 cells were infected with shRNA lentiviral particles for EDD knockout as described in Materials and Methods. In A), western analysis demonstrated knockout of EDD in clone 1.1C but not in clone 1.1A. Each clone was treated with B) 0.3125 μM cisplatin or C) 0.023 μM doxorubicin over five days and cell viability was assessed by trypan blue exclusion cell counting (*left panels*). Day 4 and 5 cell counts were replotted to clearly show statistical significance (*middle panels*) between drug-treated/untreated clone 1.1A and clone 1.1C or expressed as percent survival comparing drug-treated clones 1.1A and 1.1C (*right panels*). All values are expressed as mean ± SEM of at least three independent experiments.
* $p<0.05$; ** $p<0.01$; *** $p<0.001$ indicates significance between the groups compared.

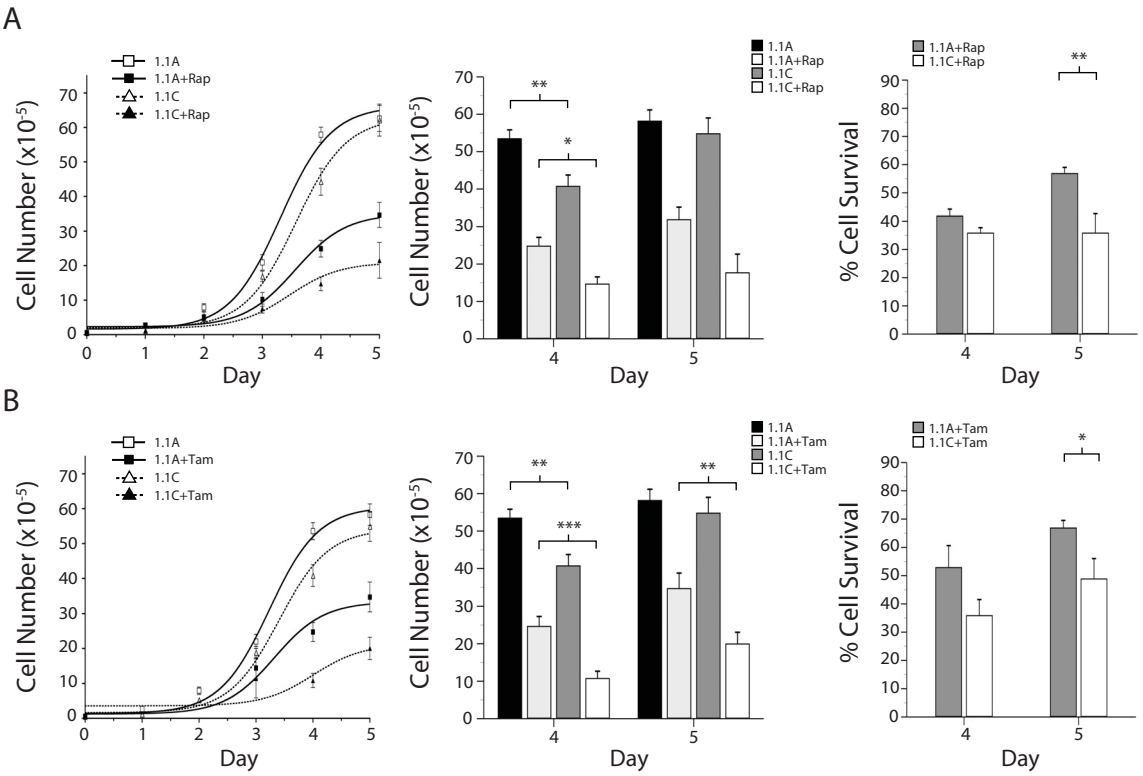

Figure 23. Loss of EDD Decreases Resistance to Rapamycin/Tamoxifen in Luminal A MCF-7 Cells. MCF-7 cells were infected with shRNA lentiviral particles for EDD knockout as described in Materials and Methods. Each clone was treated with A) 16.59 μM rapamycin or B) 20 μM tamoxifen over five days and cell viability was assessed by trypan blue exclusion cell counting (*left panels*). Day 4 and 5 cell counts were replotted to clearly show statistical significance (*middle panels*) between drug-treated/untreated clone 1.1A and clone 1.1C or expressed as percent survival comparing drug-treated clones 1.1A and 1.1C (*right panels*). All values are expressed as mean ± SEM of at least three independent experiments.
* $p<0.05$; ** $p<0.01$; *** $p<0.001$ indicates significance between the groups compared.

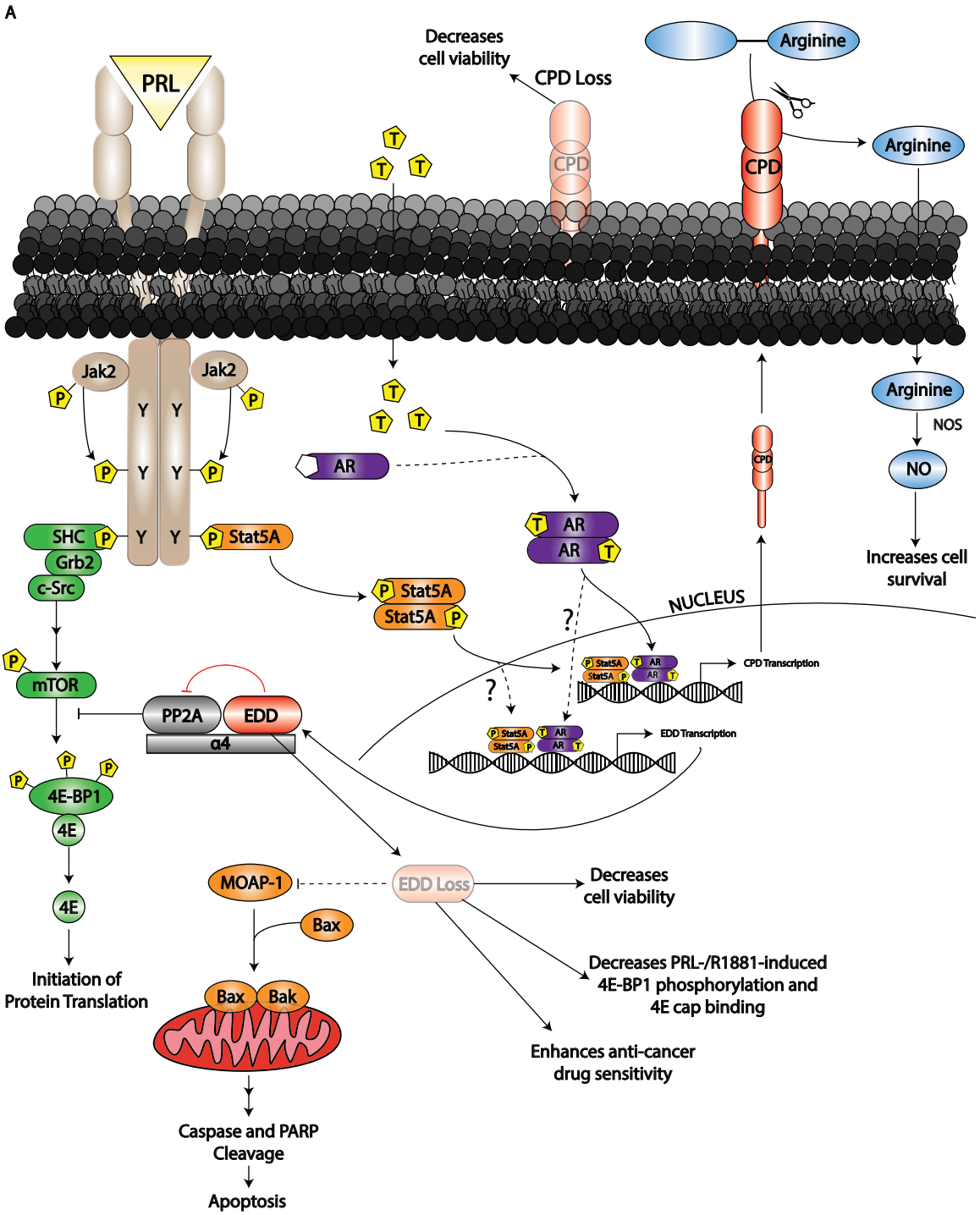

Figure 24. Complete Summary of PRL-/R1881- Mediated EDD and CPD Effects on BCa Cells. In brief, activation of PRLR/STAT5A signaling or the AR/androgen (R1881) complex results in the upregulation of CPD and EDD gene expression at the transcriptional level through binding of the dimerized STAT5A and the AR/androgen binding to gamma-interferon regulatory sequences and androgen response elements found in the CPD gene promoter and possibly in the EDD gene promoter region, respectively. Upregulation of CPD, in turn, increases intracellular nitric oxide concentrations to promote cell viability. In contrast, loss of CPD decreases cell viability. Upregulation of EDD enhances PRL/R1881-induced activation of mTOR/4E-BP1 signaling to promote the initiation of protein translation. EDD loss has a variety of cellular effects, such as decreases in cell viability, 4E cap-binding and drug resistance.

CHAPTER 4. DISCUSSION

4.1 High EDD and CPD mRNA Correlate with Poor Patient Prognosis

Several publicly accessible databases containing BCa patient survival data were accessed to examine differences in EDD and CPD mRNA expression levels. In my present study, I found that TNBC patients with high CPD mRNA expression correlated with a poorer relapse-free BCa patient survival when compared to patients with low CPD mRNA expression. In line with this finding, recent research from our laboratory found that CPD expression levels increases with BCa progression (167).

I also found that high EDD mRNA expression levels in both BCa and TNBC patients was correlated with a poorer overall and relapse-free survival, respectively. I complemented these findings by conducting immunohistochemical analysis of primary breast specimens showing that EDD expression levels also increase with BCa progression.

4.2 Hormonal Upregulation of EDD and CPD

PRL or R1881 treatment resulted in the upregulation of CPD and EDD protein expression levels in TNBC (MDA-MB-231 and MBA-MB-468) and HER2-enriched (SKBR3) cells (Figure 6 and Figure 12). Several studies have shown that CPD is upregulated by cytokines such as interferon gamma (INF-γ) and lipopolysaccharide (LPS) (92), and transforming growth factor beta-1 (TGF-β1) (171). CPD has also been shown by our laboratory to be upregulated by PRL, 17β-estradiol, and androgens (e.g., testosterone and synthetic R1881) in luminal-type BCa cells and in prostate cancer cells (86, 93, 96). Our laboratory has also used luciferase reporter and CHIP-PCR assays to demonstrate that PRL and R1881 cooperatively regulate CPD gene expression at the

transcriptional level and identified in the CPD promoter active STAT5-binding GAS and AR/ligand-binding ARE motifs (93). Although estrogen response elements were not identified in the CPD gene promoter, the ER can exert non-genomic effects though the MAPK pathway, cross-talk with STAT5 and/or the PRL-upregulated Sp-1 transcription factor (168, 172, 173).

EDD, initially described as a progestin-inducible gene (157), has been shown by our laboratory to be upregulated by PRL, progesterone, and 17β-estradiol in luminal-type BCa cells (165). In my current study, a preliminary analysis of the EDD promoter has revealed several putative GAS and ARE sites, suggesting potential transcriptional regulation of EDD by PRL/STAT5A and R1881/AR, respectively (Figure 13). The EDD gene promoter also contains several putative Sp-1-binding sites which may be activated indirectly by PRL (168).

4.3 CPD Loss Decreases Cell Viability and CPD Upregulation Promotes NO Production and Increased Cell Survival

In my present study, PRL or R1881 upregulation of CPD led to increased intracellular NO production, which correlated with increased cell survival and reduced apoptosis. In contrast, knockdown of CPD gene expression decreased NO production and decreased cell viability. Previous work in our laboratory show that in PRL-dependent rat Nb2 lymphoma cells, that is, these cells are critically dependent on PRL for cell proliferation, arginine treatment stimulated NO production and enhanced Nb2 cell proliferation (94). In addition, arginine and PRL deprivation, but followed by treatment of Nb2 cells with a synthetic NO donor, DEA/NO, inhibited apoptosis. Likewise, treatment of arginine-depleted MCF-7 cells with DEA/NO led to increased cell survival

and inhibition of apoptosis (95). It is possible that the increased cell survival due to induction of NO production is due to hormonal upregulation of nitric oxide synthase, instead of CPD. Although this was not explicitly tested in my present study, previous work in our laboratory has demonstrated that inhibition of CPD by MGTA, an inhibitor of carboxypeptidase activity, abrogated NO production and decreased MCF-7 cell survival. Furthermore, siRNA knockdown of CPD decreased arginine release, abrogated NO production, and decreased subsequent NO-mediated cell survival (95). Together, these results confirmed that it is CPD, and no other carboxypeptidase isoforms nor NOS, that enhances NO production and cell survival.

In the last two decades, interests regarding NO as a regulator of carcinogenesis and tumor progression have increased. Interestingly, the role of NO in cancer is controversial as NO either promotes or prevents cancer progression, which is determined in a concentration-dependent manner (174, 175). Specifically, low concentrations of NO (< 100 nM) promote angiogenesis and cell proliferation. Moderate concentrations of NO (100-500 nM) promote invasiveness, metastasis, cytoprotection, and repression of apoptosis. High concentrations of NO (>500 nM) promote DNA damage, oxidative stress, cytotoxicity, and apoptosis (176). Elevated NO has also been linked to chemoresistance in BCa cells, correlating with poor patient prognosis (177), and the induction of tamoxifen resistance in luminal-type BCa (178), and doxorubicin and paclitaxel resistance in TNBC cells (179). This drug resistance is suggested to occur through the concentration-dependent NO-mediated upregulation of the p-glycoprotein drug efflux pump (179). My present findings demonstrated that NO produced by hormonal upregulation of CPD increases cell viability and survival, suggesting that the

amount of intracellular NO is likely in the 100-500 nM range. Similarly, treatment of BCa cells with 25 μM of NO donor DETA-NONOate (equating to a NO concentration of 155 nM) (180), significantly ($p < 0.001$) reduced cellular apoptosis. Therefore, my current findings suggest that CPD or the CPD-Arg-NO pathway is a promising therapeutic target for BCa.

4.4 EDD Loss Decreases Cell Viability and Correlates with Increased Expression of Pro-apoptotic Mediators

In response to apoptotic stimuli, the intrinsic mitochondrial death pathway commits cells to either survive or undergo apoptosis (115). This commitment is dictated through interactions on the mitochondrial outer membrane between the BCL-2 homology 3 (BH3)-only proteins (Bim, Bid, Noxa, Hrk, Bmf, and Bad), pro-survival guardian proteins (Bcl-2, Bcl-xL, Mcl-1, A1, and Bcl-B), and pro-apoptotic effector proteins (Bax and Bak). If apoptotic thresholds are reached, inhibition of pro-survival guardians by pro-apoptotic initiators allows pro-apoptotic Bax and Bak to oligomerize and permeabilize the outer mitochondrial membrane. This, in turn, results in the release of cytochrome-c into the cytosol, leading to the serial activation of pro-apoptotic effector caspases-3, -6, and -7, and consequently, apoptosis.

Recently, siRNA-mediated knockdown of EDD has been reported to reduce cell viability, increase apoptosis, and enhance PARP-1 cleavage in ovarian cancer cell lines (181). Similarly, loss of EDD in my current study was accompanied by increased protein expression of MOAP-1 and Bax, and followed by increased cleavage of caspase-7 and the caspase substrate PARP-1. These results suggest that elevated EDD gene expression

BCa cells thereby decreases the levels of pro-apoptotic initiators and effectors in order to evade apoptosis.

Anti-cancer drug resistance frequently occurs and may develop due to cellular evasion of apoptosis. Pro-apoptotic Bax is translocated to the outer mitochondrial membrane by MOAP-1 (164). Importantly, MOAP-1 expression was low in cisplatin-resistant ovarian cancer cell lines, however, EDD gene knockdown increased MOAP-1 expression, enhanced Bax activation, and re-sensitized these cells to cisplatin-induced apoptosis. Additionally, ovarian cancers from cisplatin-resistant patients had higher EDD expression levels, compared to cisplatin-responsive patients (164), implicating EDD in cisplatin-resistance, thereby, making EDD an attractive therapeutic target for this cancer type (120). My present study also showed that BCa cells with EDD loss expressed elevated MOAP-1 and Bax protein levels, and these events could potentially contribute to a decrease in drug resistance in BCa cells.

4.5 PRL-/R1881-Induced Phosphorylation of 4E-BP1 is Decreased in EDD-depleted Cells

Previous research using rat Nb2 lymphoma cells in our laboratory demonstrated that PRL stimulated the PI3K-mediated activation of mTOR, Akt, P70S6K, and the hyperphosphorylation of 4E-BP1 (132). Interactions between PP2Ac and P70S6K or 4E-BP1 suggested that P70S6K and 4E-BP1 are substrates of PP2A (132). Moreover, our laboratory identified EDD as a binding partner of the α4 phosphoprotein-PP2Ac phosphatase complex that regulates TORC1 signalling and also determined that EDD targets PP2Ac for proteasomal degradation (165). Collectively, these results suggest that EDD may play a role in PP2Ac turnover, and indirectly, in 4E-BP1 phosphorylation.

Indeed, EDD loss, that is, PP2Ac gain, decreased PRL-/R1881-induced phosphorylation of 4E-BP1, which decreased release of eIF4E, resulting in reduced binding of the freed eIF4E to the 5′-m^{7}GTP cap of newly-synthesized mRNAs. Therefore, EDD expression activates TORC1 signalling by promoting growth factor or hormone-induced phosphorylation of 4E-BP1, culminating in the initiation of translation in BCa cells.

4.6 EDD Loss Decreases, but EDD Gain Increases, Cell Resistance to Anti-cancer Drugs

My present study showed that ectopic EDD expression in low-EDD expressing MDA-MB-436 cells promoted cell resistance to chemotherapeutic agents and TORC1/2 inhibitors. In contrast, shRNA-mediated loss of EDD in EDD-expressing MCF-7 cells decreased cell resistance to these drugs and anti-estrogen tamoxifen. Additionally, EDD loss increased pro-apoptotic Bax and MOAP-1 protein expression. Together, these results imply that EDD may confer anti-cancer drug resistance, in part, by downregulating expression of apoptotic mediators to evade apoptosis (see Figure 3). EDD overexpression and gene amplification, in serous ovarian carcinoma, have been linked to platinum resistance (161). MOAP-1, but not EDD, gene expression was directly correlated with relative cisplatin sensitivity in ovarian cancer cells. However, sensitivity to cisplatin was partially restored in platinum-resistant cells following MOAP-1 upregulation as a result of EDD knockdown (161). EDD overexpression in recurrent, platinum-resistant ovarian cancers also suggest a role in tumour survival and/or platinum-resistance (181). The enhanced cisplatin sensitivity in ovarian cancer cells as a result of EDD loss, coupled

with the activity of EDD as a regulator of cell survival and drug resistance supports EDD as a therapeutic target.

Drug resistance may also occur due to the loss of PP2A activity. EDD regulates PP2A stability (165) and PP2A has been shown to regulate the activity of proteins involved in multidrug resistance. The main proteins that confer multidrug resistance are P-glycoprotein (PGP/ABCB1/MDR-1) and breast cancer resistance protein (BCRP/ABCG2) (182). Pim-1, an oncogenic serine/threonine kinase, phosphorylates both PGP and BCRP (183, 184). Ser-683 phosphorylation of PGP protects PGP from proteasomal degradation, enables PGP glycosylation and cell surface translocation (183). Pim-1 inhibition decreases PGP expression and re-sensitizes PGP-overexpressing multidrug resistant ovarian cancer cells to doxorubicin (183). Similarly, Thr362 phosphorylation of BRCP by Pim-1 promotes the multimerization of BRCP and subsequent translocation of BRCP to the plasma membrane in prostate cancer cells (184). Knockdown of Pim-1 in drug-resistant prostate cancer cells abolishes BRCP multimer formation and re-sensitizes these cells to chemotherapeutic drugs (184). Importantly, the Pim kinases, which promote the stabilization of PGP and BRCP, are substrates of PP2A. Upregulation of PP2A, which could be induced by the downregulation of EDD, has been shown to reduce the levels of Pim proteins whereas inhibition of PP2A stabilizes the Pim proteins (185).

Interestingly, PRLR blockade appears to confer benefits to patients undergoing chemotherapy (186, 187). Moreover, inhibition of mTOR provides another avenue of anti-cancer treatment and can aid in preventing resistance to chemotherapeutic agents by intersecting crosstalk between the ER and PRLR (188). EDD targeting, such as with

siRNA, in combination with chemotherapy, for this disease may therefore prove to be beneficial (181). My current findings support EDD as a promising therapeutic target for BCa.

4.7 Summary and Conclusion

In this research, I have shown that EDD immunostaining increased from low levels in benign breast issues and DCIS, to progressively higher levels in LG, HG and TNBC tumours. Kaplan-Meier analyses showed that high EDD as well as high CPD mRNA expression independently correlates with lower probability of patient survival. Both EDD and CPD were upregulated after 3-6 h following PRL or R1881 hormonal treatment. Hormonal upregulation of CPD led to a significant increase in intracellular NO production which correlated with increased cell survival due to fewer cells undergoing apoptosis. Loss of CPD or EDD decreased cell viability. In the case of EDD, loss of EDD correlated with increased expression of pro-apoptotic initiators MOAP-1 and Bax as well as increased activation of downstream events such as cleavage of caspase-7 and PARP-1 after 24-48 h. Moreover, loss of EDD inhibited mTOR signalling by decreasing PRL- or R1881-induced phosphorylation of 4E-BP1 and decreasing eIF4E binding to the m^7GTP agarose, which represented the 5′-m^7GTP cap of mRNAs. Lastly, gain of EDD in low-EDD expressing MDA-MB-436 cells promoted cell resistance to chemotherapeutic agents and TORC1/2 inhibitors, whereas loss of EDD in EDD-expressing MCF-7 cells decreased cell resistance to these drugs and anti-estrogen tamoxifen. Collectively, this work supports EDD and CPD as therapeutic targets for BCa and suggest that EDD and CPD expression may predict BCa responsiveness to various drug treatments. Furthermore, since EDD and CPD are PRL-inducible genes (93, 165), PRLR blockade

holds promise as an additional therapy for aggressive BCa, including the TNBC-subtype that may express the PRLR.

4.8 Limitations and Future Studies

The findings of this thesis should be interpreted with several limitations in mind. Most of the experiments were performed *in vitro* and therefore may not translate directly to *in vivo* systems. Additionally, heterogeneity of cell types within the breast may complicate the utilization of the findings herein given that the *in vitro* experiments performed were done in isolation in cell lines comprised of a homogenous population of cells. Lastly, it is important to emphasize that metastasis of TNBC is primarily responsible for mortality and the *in vitro* experiments conducted did not examine metastasis (e.g., migration and cell invasion assays), however, future studies should look to examine metastasis as an endpoint in these aggressive TNBC cell lines.

Future studies on EDD should look to determine if the GAS and ARE sequences, found in the EDD promoter as described in the present findings, are transcriptionally active. Previous research in TNBC cell lines has demonstrated that NO activates the epidermal growth factor receptor (EGFR), in turn, activating proto-oncogene tyrosine-protein kinase Src. Activated Src was shown to directly stimulate mTOR activity and indirectly stimulate mTOR activity through inhibition of PP2A. Furthermore, NO was shown to upregulate the expression of PGP, resulting in enhanced resistance to chemotherapeutic drugs doxorubicin and paclitaxel (179). A future study on CPD should look to examine whether NO produced by hormonal upregulation of CPD has any effect on this EGFR-Src-mTOR pathway and investigate potential crosstalk between CPD and EDD given the overlap in signalling proteins.

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