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## Role of ECDYSONELESS in ERBB2/HER2 Mediated Breast Oncogenesis

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**ROLE OF ECDYSONELESS IN ERBB2/HER2 MEDIATED BREAST  
ONCOGENESIS**

By

Shalis Ammons

A DISSERTATION

Presented to the Faculty of the University of Nebraska Graduate College in Partial  
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Genetics, Cell Biology & Anatomy

Graduate Program

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# **ROLE OF ECDYSONELESS IN ERBB2/HER2 MEDIATED BREAST ONCOGENESIS**

**By**

**Shalis Ammons**

**University of Nebraska Medical Center, 2016**

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Breast cancer is the second leading cause of cancer related deaths in women in the United States. The human Epidermal Growth Factor 2 (ErbB2) gene amplification and/or receptor overexpression subtype of breast cancer accounts for 25% of all breast cancers. A crucial regulator of the ErbB2 signaling pathway is the heat shock protein 90 (Hsp90) and its interacting protein complex. One such complex is the R2TP/Prefoldin-like complex that is composed of four proteins, RUVBL1, RUVBL2, PIH1D1, and RPAP3 and seven prefoldin-like proteins. This complex has been shown to be involved in telomere elongation, ribosome biogenesis, protein stability; etc. We and others have recently shown that Ecdysoneless (ECD) protein functions as a mediator for interaction of HSP90 and the R2TP complex and determines which intracellular molecules the chaperone complex will regulate. Ecdysoneless, was first discovered as a *Drosophila* fly mutation and we later identified the mammalian ortholog of ECD in human epithelial cells as a binding partner of human papilloma virus 16 E6 oncoprotein. Using knockout gene strategy we demonstrated that ECD deletion is embryonic lethal and its knockdown or knockout (using fl/fl mouse embryonic fibroblasts and adenovirus cre mediated deletion) *in vitro* led to block in cell cycle progression. Subsequently, we demonstrated ECD is overexpressed in breast cancers, specifically in ErbB2+ breast cancers and its overexpression correlates with poor prognosis and poor survival in these patients.

As part of my thesis work, I investigated how ECD regulates cell cycle progression and the role of ECD in ErbB2-driven oncogenesis. We showed that ECD is phosphorylated on several serine residues by CK2 that are important for ECD's cell cycle function. In second goal, we have shown a novel interaction between ErbB2 and ECD, and knockdown of ECD deregulates the stability of the ERBB2 and HSP90 complex and leads to downregulation of ErbB2 and consequently decreased expression of downstream effectors. We speculate ECD functions as a co-oncogene in ErbB2-driven breast cancer and future studies using transgenic models will explore this possibility.

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## Abbreviations

17-AAG: 17-allylamino-17-demethoxygeldanamycin

AAA+: ATPases associated with diverse cellular activities

ACK1: Activated Cdc42-associated Kinase 1

ACTB: Beta-Actin

ANOVA: One way analysis of variance

AKT: Protein kinase B (PKB ALSO CALLED)

ATF6: activating transcription factor 6

BCL2: B-cell lymphoma 2

C-MYB: MYB- proto-oncogene

CDK: Cyclin Dependent Kinase

CDK4: Cyclin Dependent Kinase 4

CHOP: CCAAT/enhancer-binding protein-homologous protein

CLOCK: Clock Circadian Regulator

CK2: Casein Kinase 2

CRY1: Cryptochrome Circadian Clock 1 (mCry1)

C-terminal: Carboxy terminal

DAPI: 4'-6-Diamidino-2-phenylindole

DCIS: Ductal Carcinoma In Situ

DNA: Deoxyribonucleic Acid

factor 2 alpha

E6: HPV oncoprotein E6

ECD: Ecdysoneless

EGFR: Epidermal Growth Factor 1

EGFs: Epidermal Growth Factors

EMT: Epithelial to Mesenchymal Transition

ER: Estrogen Receptor

ERBB: Epidermal Growth Factor Receptor Family

FACS: Fluorescence Assisted Cell Sorting

FGF: Fibroblast Growth Factor

FGFR: Fibroblast Growth Factor Receptors

FKBP12: immunophilin FK506-binding protein

G1/S: G1 to S transition

G1: Gap 1 phase

G2/M: G2 to M transition

G2: Gap 2 phase

GATA3: Trans-acting T-cell-specific transcription factor

GCR1: Glycolysis Regulation 1

GCR2: Glycolysis Regulation 2

GLUT4: Glucose transporter type 4

HAT: Histone Acetyl Transferase

hECD; Human Ecdysoneless

HER2/ErbB2: Human Epidermal Growth Factor Receptor 2



HER3/ErbB3: Human Epidermal Growth Factor Receptor 3

HER4/ErbB4: Human Epidermal Growth Factor Receptor 4

hMECs: Human Mammary Epithelial Cells

HPV16: Human Papilloma Virus 16

hSGT1: Human Suppressor of GCR two

HRP: HorseRadish Peroxidase

HSC70: Heat Shock Chaperone 70

HSP90: Heat Shock Protein 90

hTERT: Human Telomerase Reverse Transcriptase

IDC: Infiltrating Ductal Carcinoma

IHC: Immunohistochemistry

IRE1 $\alpha$ : inositol-requiring kinase 1 alpha

KD: Knock Down

KO: Knock Out

M: Mitotic phase

mAb: Monoclonal Antibody

MAPK: Mitogen Activated Protein Kinase

MDM2: Murine Double Minute 2

MEFs: Mouse Embryonic Fibroblasts

mTOR: Mammalian Target of Rapamycin

mTORC1: Mammalian Target of Rapamycin Complex 1

mRNA: Messenger RNA

MUC4: Mucin 4

MybBP1A: Myb-Binding Protein 1A

NRGs: Neuregulins

NUFIP: Nuclear Fragile X Mental Retardation Protein Interacting Protein

N-terminal: Amino terminal

PBS: Phosphate Buffered Saline

PCR: Polymerase Chain Reaction

PERK: PKR-like ER kinase

PEST: Proline (P); Glutamic acid (E); Serine (S); Threonine (T)

PFA: Paraformaldehyde

PGC1- $\alpha$ : Peroxisome proliferator-activated receptor gamma coactivator 1-alpha

PI3K: Phosphatidylinositol-4,5-bisphosphate 3-kinase

PIH1: Protein Interacting with Hsp90 1

PIH1D1: PIH1 domain containing 1

PIKK: Phosphatidylinositol-3 Kinase related protein Kinase

POU2F1: POU class 2 homeobox 1

PR: Progesterone Receptor

PKB: Protein kinase B (AKT)

PREP1: Pbx-regulating protein-1

PRP8: Pre-mRNA processing Factor 8

OCT-1: octamer-binding transcription factor -1

R2TP: RUVB1/RUVB2/Tah1/PiH containing complex

RB: Retinoblastoma

RELA: v-Rel Avian Reticuloendotheliosis Viral Oncogene Homolg A

RNA: Ribonucleic Acid

RNAPII: RNA polymerase II complex

rRNA: Ribosomal RNA

RT: Room Temperature

RUVB1: RuvB-like AAA ATPase 1

RUVB2: RuvB-like AAA ATPase 2

S: Synthesis phase

S6K1: S6 kinase

SD: Standard Deviation

siRNA: Small- Interfering RNA

snRNP: Small Nucleolar Ribonucleoprotein

SPCA2: Secretory Pathway Calcium ATPase

Spok: Spookier

TAH1: Telomere Associated Homeobox-containing protein 1

TBST: Tris-buffered Saline with Tween-20

TCGA: The Cancer Genome Atlas Network

TEL2/TELO2: Telomere Elongation Regulation Protein

TGF-  $\alpha$ : Transforming Growth Factor- $\alpha$

TNBC: Triple Negative Breast Cancer

TPR: Tetratricopeptide Repeat

tRNA: Translational RNA

TRRAP: Transformation/Transcription Domain-Associated Protein

TRX: Thioredoxin

TXNIP: Thioredoxin Interacting Protein

VDUP1: Vitamin D3-Upregulated Protein 1

WT: Wildtype

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# **Chapter 1**

## **Introduction**

## 1.1 The Ecdysoneless Protein: Discovery and History

Numerous genetic studies of the *Ecdysoneless* (*ecd<sup>1</sup>*) mutation in *Drosophila melanogaster* have been studied for years in a variety of biological process such as embryogenesis, metamorphosis, and larval molting (Garen, Kauvar, & Lepesant, 1977) In *ecd<sup>1</sup>* mutants; that contain a temperature sensitive allele; cause larval developmental arrest when embryos are contained in a restrictive temperature. This phenomenon was thought to be caused by a deficiency in the steroid hormone, ecdysone (Garen, Kauvar, & Lepesant, 1977). In *Drosophila*, ecdysone has been implicated to be involved in reproduction, embryogenesis, and developmental processes. The *Ecdysoneless* gene encodes for the protein that has been shown to have a separate function other than steroid biosynthesis. The *ecd* protein in *Drosophila* has been molecularly identified to be involved in cell survival through mutation studies, although the mechanism of this has not been clearly defined (Gaziova, Bonnette, Henrich, & Jindra, 2004). There have been several studies in *Saccharomyces cerevisiae* that imply ECD (hSTG1 is the name used in these studies) may have a role in the transcription of glycolytic genes. One group was able to identify hSTG1 as a complement for the growth defect caused by the *GCR2* mutant in *Saccharomyces cerevisiae*. hSTG1 not only rescued the growth defect caused by the *GCR2*, it was also able to restore the glycolytic enzyme activity (Sato, Jigami, Suzuki, & Uemura, 1999). Interestingly, hSTG1 has no sequence similarity to *GCR2* and even though it may have a role in transcription, does not have structural homologue in *Saccharomyces cerevisiae* (Uemura, Koshio, Inoue, Lopez, & Baked, 1997). Human ECD was identified, in Dr. Vimla Band's laboratory, as a Human Papilloma Virus 16 E6 (HPV16 E6) binding protein using a yeast two-hybrid system. This interaction was able to open many doors about the function of ECD in cells and other ECD interacting partners.



My mentor's laboratory has published hECD as a p53, a tumor suppressor, interacting protein and this interaction helps to stabilize p53 (Zhang, 2006). Association studies using the proteasomal inhibitor MG-132 showed hECD stabilizing p53 by associating and inhibiting murine double minute-2 (MDM2)(Zhang, 2006). Overexpression of hECD lead to increased levels of p53 and an increase in the transcription of p53 target genes. Whereas, knocking down of hECD leads to a decrease in p53 expression levels(Zhang, 2006). The mode of action by which hECD does this is through its interaction with MDM2 and inhibits its' mediated degradation of p53 (Zhang, 2006). In order to study the physiological function of ECD, my mentor's laboratory generated conditional deletion ECD flox/flox mice because complete knock out of ECD is embryonically lethal to mice at the blastocyst stage (J. H. Kim et al., 2009). Mouse embryonic fibroblasts (MEFs) were derived from these mice and these cells have the ability to delete ECD upon introduction of Cre-recombinase in an *in vitro* setting. In order to study the cellular function of ECD, our laboratory deleted ECD in MEFs and observed these cells undergoing a proliferative block. This block was due to a delay in G<sub>1</sub>-S phase of the cell cycle and this phenotype was able to be rescued by introducing human ECD into these MEFs (J. H. Kim et al., 2009). ECD was shown to bind to Retinoblastoma (RB) protein and competes with E2F transcription factors for binding. ECD facilitates the dissociation of RB from E2F transcription factors and loss of ECD leads to a delay in this dissociation thus leading to a proliferative block in G<sub>1</sub>-S phase of the cell cycle and a delay in cell cycle progression(J. H. Kim et al., 2009). Loss of ECD also showed a reduction in the levels of E2F transcription factors target genes such as CDK2, Cyclin A, Cyclin B<sub>1</sub>, and Cyclin E (J. H. Kim et al., 2009). ECD plays an important role in cell cycle progression through its interaction with RB, however how ECD protein is stabilized and modified in cells and how this effects ECD function still needed to be determined.

## 1.2 Domains of the Ecdysoneless Protein

The Human *ecd* gene is located on chromosome 10 at the locus of 10q22.3. The *ecd* gene has 15 exons and its amino acid sequence has been shown to be evolutionary conserved from humans to yeast in the C-terminal region (aa 439-644)(J. H. Kim, Gurumurthy, Band, & Band, 2010). The structure of ECD is currently unknown and it does not have an identifiable DNA binding-domain. Our laboratory was able to show that ECD has an intrinsic transactivation activity and it resides in the C-terminal region of ECD (J. H. Kim et al., 2010). ECD also shuttles between the nucleus and the cytoplasm of a cell which indicates that ECD has a strong nuclear export signal, but it has not been identified yet (J. H. Kim et al., 2010). In collaboration with Dr. Mir I have shown that ECD is a phosphoprotein and it has over 100 potential phosphorylation sites (Aditya Bele Thesis, 2015; Mir et al., 2015). ECD is constitutively phosphorylated and there is an extensive list of potential kinases that possibly interact with ECD, list is located in Aditya Bele Thesis 2015 in Appendix B. We have determined that ECD is phosphorylated on several serine residues and these residues are located in functional domain that has been shown to be involved in protein-protein interactions (Hořejší et al., 2014; Mir et al., 2015). This domain contains a large number negatively charged amino acids and can be characterized as a PEST (Proline (P); Glutamic acid (E); Serine (S); Threonine (T)) domain (Aditya Bele Thesis, 2015; Mir et al., 2015). PEST domains have been shown to be involved in stability and degradation of multiple proteins (Rogers, Wells, & Rechsteiner, 1986). ECD has also been shown to contain two “DSDD” motifs which assist in determination of substrate specificity for PIH1D1, a member of the R2TP co-chaperone complex (von Morgen, Hořejší, & Macurek, 2015). ECD has also been shown to interact with PIH1D1 in a phospho-dependent manner on serine residues 505 and 518 (Horejší

et al., 2010). Our laboratory has discovered different phosphorylation sites and domains on ECD, and this has helped us understand the role ECD may be playing in cells as well as in cancer.

### **1.3 Expression of ECD in Different Cancers**

As discussed below ECD is overexpressed in several cancers, including breast cancer, the focus of my studies. Breast cancer is the second leading cause of cancer related deaths in women in the United States and it is a very heterogeneous disease (American Cancer Society, 2015). Unlike other cancers, defining the progression of breast cancer has proved difficult, but it can be classified broadly into two categories, in situ carcinoma and invasive infiltrating carcinoma. Breast carcinomas can then be further classified into two subclasses, ductal carcinoma in situ (DCIS) or lobular carcinoma in situ (LCIS) (Connolly J, Kempson R, LiVolsi V, Page D, Patchefsky A, 2004; Malhotra, Zhao, Band, & Band, 2010). Ductal carcinoma in situ is more common in patients than lobular carcinoma in situ. Currently, based on expression profiling, breast cancer is categorized into six molecular subtypes; luminal subtype A which is Estrogen Receptor (ER)/ Progesterone Receptor (PR) positive and Epidermal Growth Factor Receptor 2 (ErbB2/HER2) negative, luminal subtype B which is ER+/PR+ and ErbB2+, ErbB2+ positive, basal-like or triple negative which is ER-, PR-, and ErbB2-, claudin-low, and normal-like (Malhotra et al., 2010; Prat et al., 2010; Sorlie et al., 2003; Sørliie et al., 2001) (Table 1). Each subtype is classified according to particular set of gene expression as well as based upon the receptor expression profile. The subtype of a breast cancer patient helps to determine the prognosis, as well as the treatment options (Malhotra et al., 2010; Prat et al., 2010; Sorlie et al., 2003; Sørliie et al., 2001). Due to the heterogeneity of these tumors, there are different survival trends in these subtypes. The worst outcome, according to survival data analysis, is basal-like subtype followed by ErbB2 overexpressing subtype, claudin-low, normal-like, and the luminal

subtypes (Prat et al., 2010; Sørli et al., 2001). These subtypes have been classified based on different molecular markers, and one such marker that is highly expressed in more than one subtype of cancer is ErbB2.

Human Epidermal Growth Factor Receptor 2 (HER2/ErbB2) is a member of the Epidermal Growth Factor Receptor (ErbB) family. There are four members of the ErbB family of receptors, Epidermal Growth Factor 1 (EGFR), Human Epidermal Growth Factor Receptor 2 (HER2/ErbB2), Human Epidermal Growth Factor Receptor 3 (ErbB3/HER3), Human Epidermal Growth Factor Receptor 4 (HER4/ErbB4) (Burden & Yarden, 1997; Yarden & Sliwkowski, 2001). Each of these receptors comprise of three domains: an extracellular domain, where ligand binding and dimerization occurs; an alpha-helical transmembrane segment; and an intracellular tyrosine kinase domain (Baselga & Swain, 2009; Olayioye, Neve, Lane, & Hynes, 2000). In order to activate these receptors, an extracellular signal is received in the form of ligand binding (Baselga & Swain, 2009). Some of the common ligands that bind to these receptors include epidermal growth factors (EGFs), transforming growth factor- $\alpha$  (TGF- $\alpha$ ), and Neuregulins (NRGs) (Baselga & Swain, 2009; Olayioye et al., 2000; Yarden & Sliwkowski, 2001). Once a ligand is bound, the receptors need to undergoes a conformational change to expose the dimerization domain leading to dimerization, either homodimerization, between two of the same type of receptor, or heterodimerization, between two different ErbB family receptors (Baselga & Swain, 2009). After dimerization, the intracellular tyrosine kinases are activated through trans-phosphorylation; thus in turn causing the activation of the tightly regulated downstream signaling pathways of the ErbB family of receptors (Baselga & Swain, 2009; Yarden & Sliwkowski, 2001). These signaling pathways control a number of cellular functions such as cellular proliferation, organ development, cell to cell interactions, and organ repair (Baselga & Swain, 2009; Yarden &

Sliwkowski, 2001). The receptor tyrosine kinase family signaling pathways are tightly regulated and necessary for cells to function normally.

Table 1.1: Breast Cancer Subtypes

Subtype of Breast Cancer	Characteristic Features of Subtype	Properties	Known Markers	Prevalence	Prognosis
Luminal A	ER high, HER2 low	ER <sup>+</sup> and/or PR <sup>+</sup> , HER2 <sup>-</sup> , low Ki67	ER <sup>+</sup> and/or PR <sup>+</sup>	~40%	Best
Luminal B	ER low, PR <sup>+</sup> or PR <sup>-</sup> HER 2 <sup>+</sup> low or Her 2- with Ki-67 high	Proliferation High	ER <sup>+</sup> and/or PR <sup>+</sup> , High Ki67 when HER2- low	~20%	Good
Normal Breast Like	High expression of genes that characterize basal epithelial cells and adipose cells. ER-	Adipose tissue gene signature	CD24 low/ CD44 high Vimentin High TWIST1 high	~6-10%	Moderate
HER 2 Enriched	HER2 <sup>+</sup> , ER <sup>-</sup> , PR <sup>-</sup>	Overexpression of the HER2 receptor	HER2	~10-25%	Poor
Claudin Low	ER-, Claudin <sup>-</sup> , vimentin <sup>+</sup> , E-cadherin low High enrichment of EMT markers	Claudin low Vimentin High	Claudin 3, 4,7 low. Vimentin High	~12-14%	Poorer
Basal Like	ER <sup>-</sup> , PR <sup>-</sup> , HER2 <sup>-</sup>	EGFR <sup>+</sup>	Keratin 5 and Keratin 14 positive	~15-20%	Poorest

Table 1.1: **The classification of the six breast cancer subtypes.** This classification is based off of microarray dataset analysis of human patient tumor samples (Prat et al., 2010, 2014; Sorlie et al., 2003; Sørliie et al., 2001). The table also includes the known markers, prognostic prevalence, and predicted survival pattern of each subtype (Malhotra et al., 2010; Prat et al., 2010, 2014; Sorlie et al., 2003; Sørliie et al., 2001)

Human Epidermal Growth Factor Receptor 2 (HER2/ErbB2) is a member of the Epidermal Growth Factor Receptor family and it is the only family member that does not bind to a ligand (Baselga & Swain, 2009). ErbB2 is able to homodimerize with itself and heterodimerize with other ErbB family members, increasing the signaling potential of this receptor (Rouzier et al., 2005). There are two major signaling pathways that are activated by the ErbB family of receptors. The first is the Mitogen Activated Protein Kinase (MAPK) pathway that is involved in proliferation. Once the MAPK pathway is activated, it leads to the activation of downstream transcription factors that regulate genes that are involved in cell proliferation, migration, differentiation, and angiogenesis (Baselga & Swain, 2009). The second pathway is the Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)- Protein kinase B (AKT/PKB) pathway, which is involved in cell survival. The PI3K-AKT pathway activates several factors that are involved in cell survival and anti-apoptosis signaling (Baselga & Swain, 2009; Yarden & Sliwkowski, 2001). Both of these pathways have been shown to be deregulated in variety of cancers such as breast, lung, ovarian, and prostate (Baselga & Swain, 2009). ErbB2/HER2 enriched tumors express abnormal levels of the ErbB2 receptor (Sørli et al., 2001). ErbB2 overexpression accounts for 25% of all breast cancers and has a poor prognosis (Bertucci et al., 2004). ErbB2 overexpression has been linked to amplification of the ErbB2 gene on chromosome 17 in the regions q 12-21 (Hynes & MacDonald, 2009), increased transcriptional activity (Ehrlich et al., 2009), and an increase in the stability of the receptor (Onitilo, Engel, Greenlee, & Mukesh, 2009). *ErbB2* is a highly regulated gene, and its overexpression has been linked to the overexpression of several transcription factors that regulate its expression. The regulation of the *erbB2* gene is discussed more in Appendix B of this thesis. It is a transmembrane receptor tyrosine kinase that is involved in a variety of signal transduction pathways within the cell, some of which help promote proliferation and cell survival (Harari & Yarden, 2000).

Sustaining proliferative signaling and resisting cell death mechanisms are two hallmarks of cancer and have been shown to be a major driving forces of oncogenesis in multiple cellular systems (Hanahan & Weinberg, 2011). ErbB2, as well as the rest of the ErbB family of receptors, are trans-membrane tyrosine receptor kinases that are involved in various signaling transduction pathways that are important in normal cell development as well as oncogenesis (Yarden & Sliwkowski, 2001). Studies have shown that patients with ErbB2+ tumors are shown to have a worsen prognosis as compared to patients with ER<sup>+</sup>/PR<sup>+</sup> tumors (Prat et al., 2010; Slamon et al., 1987). A study conducted by The Cancer Genome Atlas (TCGA) Network of approximately 500 breast tumors has shown the biological heterogeneity of clinical ErbB2-overexpressing cancers (HER2<sup>+</sup>), as defined by gene amplification (Prat et al., 2014). This group further characterized these cancers by gene expression into two subclasses, HER2-enriched (HER2E) and luminal HER2<sup>+</sup> (Prat et al., 2014). HER2E and HER2<sup>+</sup> tumors exhibited higher frequencies of aneuploidy, somatic mutations, and *TP53* mutations and also show genetic amplification of Fibroblast Growth Factor Receptors (FGFRs), Epidermal Growth Factor Receptor (EGFR), cyclin dependent kinase 4 (CDK4), and Cyclin D1 in these patients (Prat et al., 2014). Luminal HER2<sup>+</sup> displayed a higher expression of a luminal gene cluster which included trans-acting T-cell-specific transcription factor (GATA3), B-cell lymphoma 2 (BCL2), and Estrogen Receptor 1 (ESR1) (Prat et al., 2014). Although, not all tumors of the HER2-enriched gene expression subtype are *erbb2* amplified, it is thought that some breast cancers with a single copy of *erbb2* gene harbor an expression signature of ErbB2 dependence and may benefit from anti-ErbB2 therapy (Prat et al., 2014). Current therapy methods have attempted to target the ErbB2 receptor through its extracellular domain using Trastuzumab. Trastuzumab is a humanized monoclonal antibody that contains two antigen-specific sites that binds to the juxtamembrane of the extracellular domain of the ErbB2 receptor and is currently the approved

treatment for patients that overexpress ErbB2 (Hudis, 2007; Nahta & Esteva, 2006; Vu & Claret, 2012).

In some ErbB2 positive breast cancer patients, there is a chance of relapse and metastasis. These patients usually develop acquired or de novo resistance to Trastuzumab that lead to metastasis, but the mechanism behind this is not yet fully understood (Nahta & Esteva, 2006; Vu & Claret, 2012). One promising therapy option that has recently come to light to combat acquired resistance are Hsp90 inhibitors (Maloney & Workman, 2002; Workman, Burrows, Neckers, & Rosen, 2007). The Hsp90 inhibitor, 17-AAG has been shown to cause the rapid degradation of ErbB2 (Goetz, Toft, Ames, & Erlichman, 2003; Modi et al., 2011; Sausville, Tomaszewski, & Ivy, 2003). Although 17-AAG seems like a potent drug, it still causes toxicity to patients. Therefore is an increasing the need for other molecular therapy targets that cause less toxicity to patients. This thesis will attempt to validate the potential of Ecd as a novel molecular therapy target in ErbB2 positive breast cancer cells.

We speculate that ECD may be one such molecule as our laboratory has shown that ECD expression was low in normal breast tissue and also in hyperplasia of breast tissue (X. Zhao et al., 2012). However, ECD levels were shown to be high in ductal carcinoma in situ (DCIS), benign breast hyperplasia, and in infiltrating ductal carcinoma (IDC) samples (X. Zhao et al., 2012). After examining a larger cohort of over 900 samples, strong positive correlations between ECD expression and higher histological grade, mitotic index, and Nottingham Prognostic Index score could be observed (X. Zhao et al., 2012). Interestingly, a positive association between ECD high expression and ErbB2/HER2 overexpression was also seen. Patients with high ECD expression and ErbB2/HER2 overexpression showed a poorer overall survival outcome compared to patients with low ECD expression and ErbB2/HER2 overexpression (X. Zhao et al., 2012).



ECD has also been shown to be overexpressed in pancreatic cancer (Dey et al., 2012).

Pancreatic cancer is one of the most aggressive and lethal malignancies seen today in patients.

It is characterized by an extremely poor prognosis, late clinical presentation, and poor response to therapy methods (Chakraborty, Baine, Sasson, & Batra, 2011). Since cell cycle regulation is highly deregulated in pancreatic cancer, ECD expression was observed for its cell cycle regulation function (Dey et al., 2012). ECD expression is low in normal pancreatic tissue but is overexpressed in ductal adenocarcinoma form of pancreatic cancer (Dey et al., 2012).

Interestingly, ECD expression correlated to primary pancreatic tumors with distant metastatic sites. ECD knockdown showed a reduction of cellular proliferation in pancreatic cells *in vitro* and a reduction of tumor size and less metastasis in mice *in vivo* (Dey et al., 2012). Another study has proposed that ECD is involved in gastric cancer epithelial to mesenchymal transition (EMT) and metastasis through Cdc-42-associated kinase 1 (ACK1)-AKT- POU class 2 homeobox 1 (POU2F1) pathway (S.-H. Xu et al., 2015). Gastric cancer is the second leading cause of cancer mortality worldwide and is usually detected at an advanced disease state for most patients (Deng et al., 2012). The authors suggest that ECD is a downstream effector of ACK1 and ECD expression is regulated by the transcription factor POU2F1 (POU2F1 is also known as octamer-binding transcription factor-1 (OCT-1))(S.-H. Xu et al., 2015). The proposed mechanism is that ACK1, which is overexpressed in gastric cancer, activates AKT and AKT in turn activated POU2F1. POU2F1 then binds to the *ecd* gene and upregulates *ecd* transcription. Overexpression of ECD promotes invasion, migration, and induces EMT in gastric cancer (S.-H. Xu et al., 2015). The authors believe the dysregulation of the signaling pathway outlined previously induces EMT in gastric cancer patients which lead to metastasis and ECD is playing a critical role in this(S.-H. Xu et al., 2015).

Figure 1.1: **Ecd is overexpressed in various breast cancer cell lines.**

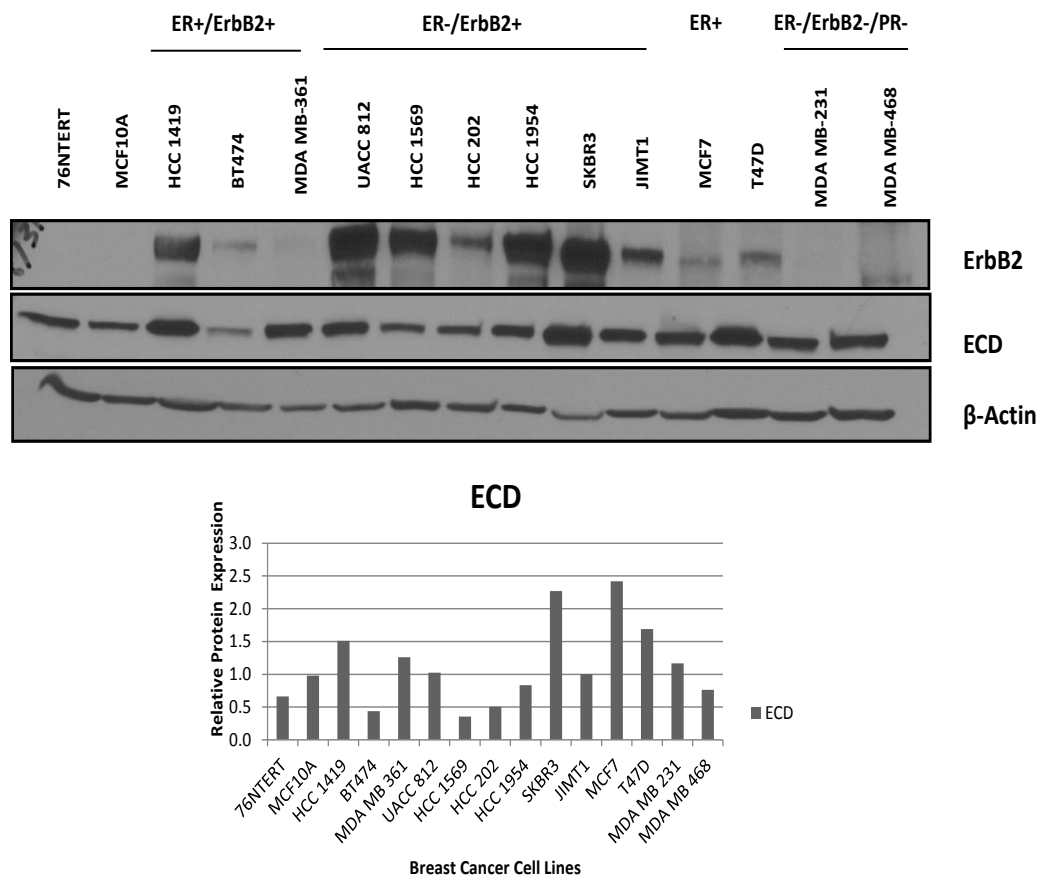


Figure 1.1: **Ecd is overexpressed in various breast cancer cell lines.** Various breast cancer cell lines were harvested using RIPA Buffer and Ecd expression was determined by western blotting using anti-Ecd, anti-ErbB2, and anti-β-actin (as loading control). 76N.TERT and MCF10A serve as normal immortalized breast epithelial cell lines. Cell lines are separated via subtype. The graph below is the densitometry analysis of ECD levels as compared to β-actin. Densitometry performed using ImageJ.

In addition, our laboratory has seen overexpression of ECD in prostate (Figure 1.2) and in cervical cancers. In prostate cancer ECD overexpression is correlated to a poorer overall survival outcome in these patients (Bele, 2015). ECD overexpression is observed in adeno carcinomas

and in squamous cell carcinomas of the cervix (Bele, 2015). In a variety of cancers, ECD overexpression is observed and this overexpression is correlated with poor prognosis for many patients. Taken together, these studies suggest a clear role for ECD overexpression in tumor cell survival and potential therapy resistance. For my thesis I focused on two questions i) what is the mechanism of ECD mediated cell cycle progression and ii) how ECD connects with ErbB2.

#### **1.4 ECD Known Protein Interactions and Pathways**

Human ECD was first identified as a Human Papilloma Virus 16 E6 (HPV16 E6) binding protein in Dr. Vimla Band's laboratory. ECD has been shown to be overexpressed in variety of cancers (Dey et al., 2012; S.-H. Xu et al., 2015; X. Zhao et al., 2012). These observations lead to our lab's desire to study the different protein interactions and functions of ECD (Figure 1.3). Previous lab members originally showed hECD as a p53 interacting protein and it was concluded that ECD stabilizes p53 by associating and inhibiting MDM2 (Zhang, 2006). Another study in the lab highlighted the role of ECD in the cell cycle as an RB binding protein (J. H. Kim et al., 2009). ECD binds to RB and competes with E2F transcription factors for binding. ECD facilitates the dissociation of RB from E2F transcription factors, which leads to cell cycle progression (J. H. Kim et al., 2009). Recently, a previous graduate student in the lab was able to show ECD has a synergistic role with the potent oncogene H-RAS and together, both proteins cause oncogenic transformation in human mammary epithelial cells (Bele et al., 2015). ECD overexpression alone was able to show some oncogenic properties but ECD+RAS overexpressing HMECs completely underwent transformation (Bele et al., 2015). ECD+RAS cells exhibited better survival, cell migration, invasion, and acinar formation in 3D Matrigel cultures as compared to vector expressing, RAS overexpressing, and ECD overexpressing HMECs alone (Bele et al., 2015).

Figure 1.2: **Ecd is overexpressed in various prostate cancer cell lines.** Various prostate cancer cell lines were harvested using RIPA Buffer and Ecd expression was determined by western blotting using anti-Ecd and anti- $\beta$ -actin (as loading control). PSV40 and PHPV18 serve as immortalized prostate epithelial cell lines.

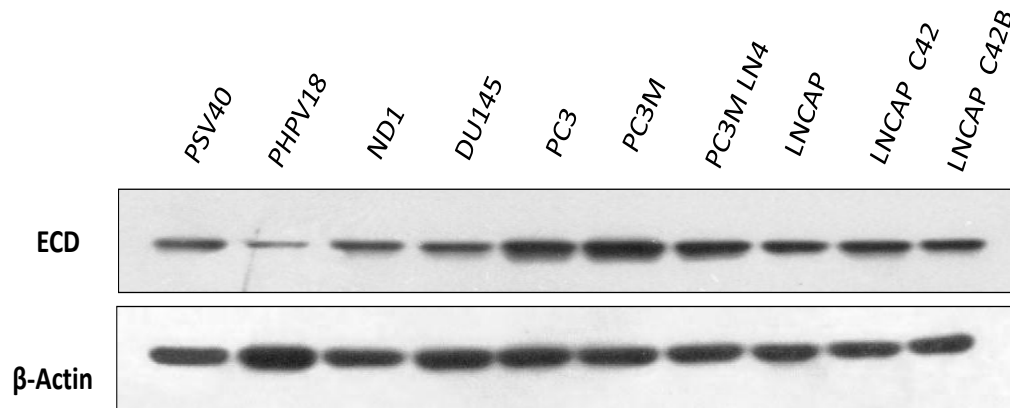


Figure 1.3: **ECD and its Different Interacting partners.**

**Figure 1.3: ECD and its Different Interacting partners.** A schematic depicting the various proteins that interact with ECD and the different phosphorylation sites and domains of the ECD protein. Ecd contains a PEST domain. It also consists of two 'DSDD' motifs. There are also several major serine residues that are phosphorylated by CK2, but only the two serine residues that interact with PIH1D1 are indicated with red boxes. (Bele, 2015; Bele et al., 2015; Claudius, Romani, Lamkemeyer, Jindra, & Uhlirova, 2014; J. H. Kim, 2009; J. H. Kim et al., 2009; Mir et al., 2015; Zhang, 2006)

ECD overexpression may be contributing to oncogenesis through the interactions. ECD may also play a role in the regulation of the Secretory Pathway Calcium ATPase (SPCA2). SPCA2 has been shown to be overexpressed in breast cancer, particularly in the ErbB2+ subtype. It plays a role in oncogenesis by promoting calcium dependent signaling through the store operated calcium channel Orai1 (Feng et al., 2010). Our lab has shown using a global microarray based expression profile, that SPCA2 expression levels are high in ECD+RAS HMECs as compared to ECD overexpressing and RAS overexpressing HMECs alone (Bele, 2015). Recently, another laboratory has published that ECD interacts with a protein called TXNIP, also known as vitamin D3 - upregulated protein 1 (VDUP1) or thioredoxin (TRX) – binding protein-2 (Suh et al., 2013). TXNIP, which is a member of the tumor suppressor family, is involved in a number of cellular processes such as apoptosis. The authors concluded that ECD interacts with TXNIP to decrease MDM2-mediated ubiquitination of p53. This interaction leads to p53 stabilization and an increase in p53 activity (Suh et al., 2013). Another laboratory has reported that in *Drosophila* cells, ECD interacts with Pre-mRNA processing Factor 8 (PRP8), a member of the U5 snRNP spliceosomal complex (Claudius et al., 2014). ECD also interacts with core components of the U5 snRNP spliceosomal complex, and along with PRP8, are necessary for cell survival in larval imaginal discs. Deletion of *ecd* or *prp8* prevents the splicing of an intron from CYP307A2/*spookier (spok)* pre-mRNA causing a block in entry to metamorphosis by the elimination of the ecdysone-biosynthetic enzyme (Claudius et al., 2014). Interestingly, human ECD was able to rescue this phenotype, further confirming the conservative nature of the ECD. These studies show ECD interactions are important in a number of cellular processes and that ECD may function similarly in a variety of species.

A previous graduate student in the lab performed a mass spectrometry analysis of human ECD and was able to show that ECD interacts with a number of proteins, and one such protein

was, Myb-Binding Protein 1A (MYBBP1A). MYBBP1A is mainly localized in the nucleolar and is a transcriptional regulator that regulates the activity of several transcription factors such as MYB- proto-oncogene (*c-myb*), Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1- $\alpha$ ), v-Rel Avian Reticuloendotheliosis Viral Oncogene Homolg A (RelA), Cryptochrome Circadian Clock 1 (*mCry1*), PREP1: Pbx-regulating protein-1 (Prep1), and Clock Circadian Regulator (CLOCK) (Díaz et al., 2007; Fan et al., 2004; Favier & Gonda, 1994; Hara et al., 2009; Tavner et al., 1998). MYBBP1A is involved in a number of cellular processes such as, mitosis, transcriptional regulation, rRNA synthesis, and ribosome biogenesis (Akaogi, Ono, Hayashi, Kishimoto, & Yanagisawa, 2013; Bele, 2015; Mori et al., 2012; Ono et al., 2013, 2014). A previous graduate student was able to observe that loss of ECD leads to a down regulation of MYBBP1A and ECD co-localizes with MYBBP1A when ECD is overexpressed (Bele, 2015). Taken together, it is plausible to conclude that ECD plays a role in MYBB1A stability and may also play a role in the functions of MYBBP1A. Our laboratory has also shown that ECD interacts with p300 and may be playing an important role in chromosome integrity. In a study using primary mouse embryonic fibroblasts (MEFs), upon ECD deletion, higher frequencies for chromosome aberrations including chromosome breaks, fragments, deletions, and translocations were observed (Bele, 2015). We hypothesize that ECD is functionally important in the maintenance of chromosomes through histone acetylation. Our laboratory has shown that ECD interacts with the histone acetyltransferase, p300 and enhances its HAT activity (J. H. Kim, 2009). ECD however, does not have a DNA binding domain and therefore is not a transcription factor, but ECD may play a role in transcription through its interaction with p300.

Recently, another graduate student in my mentor's laboratory has shown that ECD may be a key regulator in the ER stress pathway through its interaction with PKR-like ER kinase (PERK). ER stress is a stimulus for to initiate a defensive process called the unfolded protein response



(UPR). UPR is comprised of several cellular mechanisms that are aimed to either assist in cellular survival, or in cases of extreme stress, initiate mechanisms of cell death such as autophagy or apoptosis (Ma & Hendershot, 2001; Welihinda, Tirasophon, & Kaufman, 1999). ECD levels are downregulated in HMECs upon treatment of different endoplasmic reticulum stress inducing compounds. We also observe ECD interaction with several UPR pathway components, including PERK, indicating that ECD is an important regulator of UPR (This work will be discussed in more detail in Chapter 3). We have also observed in PERK knockout mouse embryonic fibroblast, ECD levels remain unchanged, indicating that ECD may play a role as a negative regulator of stress.

ECD has been shown to interact with a protein, PIH1 domain-containing protein 1 (PIH1D1) and this interaction is Casein Kinase 2 (CK2) mediated phosphorylation dependent (Hořejší et al., 2014). ECD binds to PIH1D1 on Serine residues 505 and 518 and PIH1D1 recognizes ECD as a substrate by the DpSDD motif on ECD (Hořejší et al., 2014). PIH1D1 is a member of the co-chaperone complex named R2TP/Prefoldin complex. The R2TP complex consists of four members: Rvb1, Rvb2, Phi1, and Tah1 in yeast and RUVBL1, RUVBL2, PIH1D1, and RPAP3 in humans (Kakihara & Houry, 2012). These complex components are known by different names as indicated in Table 1.2, however; for the purpose of this thesis, the gene names of each component will consistently be used. The R2TP complex was first discovered in yeast cells in 2005 by the Parsons lab (R. Zhao et al., 2005), as an heat shock protein 90, (HSP90) associated chaperone complex. This complex is conserved from yeast to humans. Proteomic analysis of HSP90 interacting partners in human samples identified RUVBL1, RUVBL2, PIH1D1, and RPAP3 and GST-pulldown and yeast two hybrid experiments were conducted to reaffirm these results (Boulon et al., 2008; Te, Jia, Rogers, Miller, & Hartson, 2007). Each component of the R2TP is well characterized and has novel functions that are separate from the complex's

functions. The R2TP complex also interacts with the seven protein members of the Prefoldin-like complex, which are Rpb5, PFDN2, PFDN6, URI, UXT, PDRG1, and WDR92 (Table 1.2) (Boulon, Bertrand, & Pradet-Balade, 2012). The Prefoldin-like complex is present only in mammalian cells and its main function is to increase the stability of the R2TP complex (Kakihara & Houry, 2012).

The R2TP/Prefoldin co-chaperone complex is composed of four proteins along with HSP90. Two of these proteins are RUVBL1 and RUVBL2 and they are closely related, highly conserved, AAA+ (ATPases associated with diverse cellular activities) super family ATPases. ATPases are characterized by the AAA+ domain containing: Walker A and B motifs, sensor domains 1 and 2, and an arginine finger (Jha & Dutta, 2009; Nano & Houry, 2013). These proteins usually function as a hexamer to hydrolyze ATP to generate energy that can be utilized in the conformation change of nucleic acids or proteins (Hanson & Whiteheart, 2005). Both of these proteins are ATP-dependent helicases that function as scaffold protein that assist in the formation of several protein complexes and are essential for their functions (Jha & Dutta, 2009). The RUVBL1 and RUVBL2 proteins have been shown to be involved in several cellular processes such as transcription, chromatin remodeling, DNA damage response, RNA polymerase II (RNAP II) assembly, apoptosis, and mitotic spindle assembly (Boulon et al., 2012; Jha & Dutta, 2009). Some of the specific complexes that RUVBL1 and RUVBL2 are known to assist in the formation of include the INO80, TIP60, and SWR/SRCAP complexes (Nano & Houry, 2013). Both of the proteins have been shown to be overexpressed in a variety of cancers including colon, lung, and breast (Huber et al., 2008; S. G. Kim et al., 2013; Nano & Houry, 2013) Studies have shown that decreasing the levels of RUVBL1 and RUVBL2 *in vitro* causes reduced tumor cell growth and an increase of apoptosis (Huber et al., 2008). In cancers, the transcription of both of these proteins is also deregulated, which is a contributing factor to the overexpression of these proteins (Nano & Houry, 2013). RUVBL1 and RUVBL2 have multiple roles in cancer including signaling,

apoptosis, modulating cellular transformation, and DNA damage response and these roles are mediated through their interaction with a number of proteins such as the tumor suppressor Hint1, and transcription factors such as  $\beta$ -catenin, c-myc, and E2F (Huber et al., 2008; Nano & Houry, 2013). Recently, our laboratory has published that ECD has a novel interaction with the R2TP complex through the protein RUVBL1 (Mir et al., 2015). Our lab was able to show that ECD interacts with RUVBL1 and this interaction is phosphorylation independent and also necessary for cell cycle progression. We showed that phosphorylation deficient mutants of ECD failed to bind to the PIH1D1 component of the R2TP complex, but were still able to interact with the complex through RUVBL1. Also, phosphorylation deficient mutants were able to partially rescue the cell cycle block observed in *ecd*<sup>flox/flox</sup> MEFs upon *ecd* deletion. We were able to conclude that ECD interaction with the R2TP is through the phosphorylation dependent interaction with PIH1D1 and the phosphorylation independent interaction with RUVBL1. We also showed that ECD interaction with RUVBL1 is essential for cell cycle progression; however, ECD interaction with PIH1D1 is not (Mir et al., 2015). I am an author in this work and the full paper can be seen in Appendix A of this thesis.

PIH1D1 is the connector protein between RuvBL1/RuVBL2 and RPAP3, which binds to the C-terminal region (Hořejší et al., 2014). It is also the complex protein that usually interacts directly with most of the known adaptor proteins. PIH1D1 has a phospho-peptide binding domain in the N-terminal region that binds preferentially to highly acidic phosphorylated proteins, which enables it to recognize specific substrates within the cell. PIH1D1 specific binding proteins, such as TEL2 and ECD, contain a conserved phospho-peptide motif, DpSDD, that is recognized by the phospho-peptide binding domain in PIH1D1 (Hořejší et al., 2014). PIH1D1, along with the R2TP complex, interacts with TEL2 to help in the stability of phosphatidylinositol-3 kinase-related protein kinases (PIKKs) and the formation of PIKKs functional complexes such as mTORC1,

mTORC2, and ATRIP (Horejsí et al., 2010; Takai, Wang, Takai, Yang, & de Lange, 2007). PIH1D1 interacts with NUFIP and is necessary for the accumulation, assembly, and stability of small nucleolar ribonucleoprotein (snoRNPs) and the core proteins of the snoRNP complex (Boulon et al., 2012). PIH1D1 also interacts with box C/D snoRNP factors Nop1, Nop58, Nop56, which are involved in snoRNP biogenesis (R. Zhao et al., 2008). PIH1D1 directly interacts with Raptor, a component of the mTORC1 and has been shown to play an important role in its complex assembly and S6 kinase activation in breast cancer cells (Kamano et al., 2013). Also, PIH1D1 binds to RPAP3 at its C-terminal domain and have been shown to be regulators of apoptosis in ovarian cancer cells (Inoue, Saeki, Egusa, Niwa, & Kamisaki, 2010). PIH1D1 is an important member of the R2TP complex and plays a role in a number of cell processes and also seems to be playing a role in oncogenesis.

RPAP3 has been shown to be component that interacts directly with Hsp90 and is the bridge protein between the R2TP complex and Hsp90 (Back et al., 2013). This protein seems to helping to stabilize the R2TP complex in cells. RPAP3 contains two tetratricopeptide repeat (TPR) motifs, which have been shown to a binding domain region where Hsp90 binds to and its co-chaperones (Kakihara & Houry, 2012). This protein has been characterized as a cell death enhancer for its role in apoptosis. RPAP3 interacts with Monad (WDR92), which is an apoptosis regulating protein (Yoshida et al., 2013). This protein is expressed in three isoforms, isoform 1, 2, and 3. RPAP3 isoform 1 has been shown to interact with PIH1D1 and downregulation of this isoform by small interfering RNA, causes downregulation of PIH1D1 protein levels (Yoshida et al., 2013). RPAP3 isoform 2 does not interact with PIH1D1 and have a dominant negative effect on the survival of the R2TP complex (Yoshida et al., 2013). RPAP3 also binds to WDR92, which is member of the prefoldin-like chaperone complex and this interaction may mediate the R2TP complex binding to the prefoldin-like chaperone complex (Back et al., 2013; Itsuki et al., 2008).

Table 1.2: R2TP Complex

Yeast Name	Human Name/ R2TP and Prefoldin	Protein Names	Gene Name
Rvb1	hRvb1	RuvB-like 1, Pontin 52, Pontin, TIP49A, 49 kDa, TBP-interacting protein (TIP49), ECP-54, NMP238, TAP54- $\alpha$ , INO80H, TIH1	<b>RUVBL1</b> , NMP238, TIP49, TIP49A
Rvb2	hRvb2	RuvB-like 2, Reptin, TIP49B, 48 kDa, TBP-interacting protein (TIP48), ECP-51, TAP54- $\beta$ , INO80J, TIH2	<b>RUVBL2</b> , TIP48, TIP49B
Tah1	Spagh	RNA polymerase II-associated protein, hSpagh, FJL21908	<b>RPAP3</b>
Pih1, Nop17	Pih1d1	PIH1 domain-containing protein 1, Nop17 homolog	<b>PIH1D1</b> , Nop17
	Rpb5	RNA polymerase I, II, and III subunit ABC1, DNA-directed RNA polymerase subunit E, XAP4	POLR2E
	PFDN2	Prefoldin subunit 2	PFDN2, PFD2
	PFDN6	Prefoldin subunit 6, protein Ke2	PFDN6, PFD6, HKE2
	URI	RPB5-mediating protein, unconventional prefoldin RPB5 interactor	RMP, URI
	UXT	Ubiquitously expressed transcript protein, ART-27	UXT, PDRG
	PDRG1	P53 and DNA damage-regulated protein 1	PDRG1, PDRG
	WDR92	WD repeat-containing protein 92, Monad	WDR92

**Table 1.2: The molecular components of the R2TP/Prefoldin Like complex with their common designations in *S. cerevisiae* and in humans.** The table includes the various protein and gene names of the R2TP/ Prefoldin Like Complex in yeast and in humans. The black texts are members of the R2TP complex and the red texts are components of the Prefoldin- like Complex. The bolded names are the ones that are used ubiquitously throughout the dissertation. (Boulon et al., 2010, 2012)

Heat shock protein 90 (HSP90) is a molecular chaperone protein that interacts with the R2TP complex by binding to RPAP3. It is highly conserved throughout eukaryotic cells and it is essential for viability. HSP90 is a dimeric chaperone protein that contains three domains, the N-Terminal Domain, the Middle Domain, and the C-terminal domain (Taipale, Jarosz, & Lindquist, 2010). The N-Terminal domain contains the ATP binding site that is critical for Hsp90 conformational change. The Middle domain is thought to be involved in client recognition and the C-Terminal Domain is used for dimerization (Taipale et al., 2010). Hsp90 is involved in the folding, maintenance, and regulation of a variety of proteins, including ErbB2. Hsp90 is involved in multiple steps in ErbB2 protein folding and maintenance. It binds to the nascent protein and is involved in the final stage of protein folding (R. Zhao et al., 2005). At the plasma membrane, HSP90 binds to the kinase domain of ErbB2, forming a stable complex and protecting it from ubiquitin-dependent proteasomal degradation (W. Xu et al., 2005). Hsp90 also plays a role in the maintenance and regulation of downstream targets of ErbB2 (Taipale et al., 2010; W. Xu et al., 2005). HSP90 assist in the proper folding and has a role in degradation of misfolded protein by the proteasome (Pratt, Morishima, Peng, & Osawa, 2010). HSP90 recognizes specific client proteins through its different co-chaperone interactions indicating that co-chaperones assist in the various roles of HSP90 (Taipale et al., 2010).

Chaperone proteins, including HSP90, have been seen to be overexpressed in a number of different cancers. Hsp90 is overexpressed in breast cancer cell lines and also in breast cancers samples. In microarray data obtained from over 600 breast cancer tumor samples, higher expression of HSP90 has been correlated to ErbB2 overexpression, Estrogen Receptor overexpression, larger tumors, lymph node involvement, and a poor overall survival (Pick et al., 2007). Overexpression of chaperones has been a major contribution to oncogenesis and has been linked to providing protection to tumor cells in a nutrient deprived, hypoxic,

microenvironment (Whitesell & Lindquist, 2005). Also increased chaperone levels may allow tumor cells to adapt to the imbalanced signaling that is associated with transformation and thereby assist tumor cells in their evasion of apoptosis (Sangster, Queitsch, & Lindquist; Takayama, Reed, & Homma, 2003; Whitesell & Lindquist, 2005). Since this discovery, Hsp90 has become a new molecular therapy target in breast cancers. Currently, Hsp90 inhibitors, such as 17-allylamino-17-demethoxygeldanamycin (17-AAG), are in clinical trials for anticancer therapeutic agents (Goetz et al., 2003). 17-AAG, which is a Geldanamycin derivative, is a small molecule inhibitor that binds to the ATP pocket domain of HSP90 and inhibits its interactions with its client proteins (Sausville et al., 2003). This inhibitor causes downregulation of ErbB2 levels *in vitro* and *in vivo* and is currently used in clinical trials in combination with Trastuzumab ((Basso, Solit, Munster, & Rosen, 2002; Modi et al., 2011; Sausville et al., 2003). Interestingly, I have observed that treatment of ErbB2+ breast cancer cell lines with 17-AAG leads to downregulation of ECD, as well as some components of the R2TP complex (This work is explained more in Chapter 3). This may suggest that downregulation of ErbB2 leads to destabilization of ECD and of the R2TP complex. HSP90 inhibitors have been seen to be toxic to some patients (Goetz et al., 2003). This concern drives scientists to discover other co-chaperone complexes that interact with HSP90 and are involved in oncogenesis that could become future therapy targets.

The R2TP complex assists with the assembly of multi-molecular protein complexes involved in the following pathways: mRNA and tRNA transcription, telomerase complex assembly, small nucleolar ribonucleoprotein (snoRNP) biogenesis, phosphatidylinositol-3 kinase-related protein kinase (PIKK) stability and signaling, and translation (Boulon et al., 2012)(Kakihara & Houry, 2012)(Boulon et al., 2010)(Kakihara, Makhnevych, Zhao, Tang, & Houry, 2014)(Kamano et al., 2013)(Horejsí et al., 2010). This co-chaperone complex assists with



transcription through the assembly of the RNA polymerase II complex (RNAPII). RNAPII is an enzyme that is composed of twelve subunits, and one of these subunits, Rpb1 associates with HSP90 and the R2TP complex (Boulon et al., 2010). HSP90 and the R2TP complex are necessary to help stabilize unassembled RNAPII subunits before the assembly of the full enzyme (Boulon et al., 2010). The R2TP complex has also been shown to be involved in the assembly of RNA Polymerase I and RNA Polymerase III complexes (Boulon et al., 2012). Small nucleolar ribonucleoproteins (snoRNPs) are characterized into two families C/D and H/ACA and this characterization is determined by an RNA motif associated with a specific set of proteins (Boulon et al., 2004; Kiss, Fayet, Jády, Richard, & Weber, 2006). SnoRNPs are involved in pre-rRNA processing in the nucleolus (Kiss et al., 2006). HSP90 and the R2TP complex are required for the accumulation of snoRNPs and this complex interacts with immature snoRNPs through an adaptor protein called Nuclear Fragile X Mental Retardation Protein Interacting Protein (NUFIP) (Bizarro et al., 2015; Boulon et al., 2008). The R2TP complex interacts with NUFIP through PIH1D1, which binds to it and help in the proper folding and stabilization of snoRNP core proteins before and during assembly (Boulon et al., 2012). NUFIP acts as an adaptor protein for a larger family of RNPs named L7Ac RNPs, which include three core proteins that require HSP90 for their stability (Boulon et al., 2008). This could possibly broaden the scope of the R2TP complex's involved in snoRNPs biogenesis. The R2TP co-chaperone complex is also involved in the stabilization and assembly of the phosphatidylinositol-3 kinase-related protein kinase (PIKK) family of kinases. The PIKK family consists of six members; ATR, ATM, DNA-PKcs, mTOR, SMG1, and TRRAP; that are structurally similar to one another (Boulon et al., 2012). Each component requires the adaptor protein telomere elongation regulation protein (TEL2/TELO2) for their stability (Takai et al., 2007). TEL2 recruits the R2TP complex, by binding to PIH1D1 in a phosphorylation dependent manner, to the PIKKs to help stabilize them (Horejsí et al., 2010).

TEL2, with HSP90, also assists in the complex formation of mTORC1, mTORC2, and ATRIP (Kaizuka et al., 2010; Takai, Xie, de Lange, & Pavletich, 2010). The R2TP complex and HSP90 are required in the stability and the functional complex formations of the PIKKs.

Recently, the R2TP complex members have been shown to be overexpressed in breast cancer patients on the mRNA level and genetic level and this complex is involved in the regulation of hyper-activity of the mTOR signaling pathway (S. G. Kim et al., 2013). The HSP90 and R2TP/Prefoldin-like complex are involved protein synthesis, cell growth, and telomere elongation and thus could play an important role in oncogenesis. The complex also is a master regulator of cell proliferation and helps to form and maintain different protein complexes that are involved in cycle progression (Boulon et al., 2012). Since the members of the R2TP complex and HSP90 are involved in the stabilization and assembly of multiple complexes that are involved in protein synthesis and cell cycle, it is very likely that this complex is playing an important role in oncogenesis. Sustained signaling, uncontrolled cell proliferation, and enhanced cell survival are all considered hallmarks of cancer (Hanahan & Weinberg, 2011).

Hsp90 and the R2TP/Prefoldin-like complex interact with a number of adaptor proteins in order to perform the stabilization and assembly functions and these interactions may be important in tumor development. ECD interacts directly or is in close proximity to all four of these components in the R2TP complex, and with HSP90, as indicated by (Figure 1.4). Currently, there are some inhibitors that target specific HSP90 co-chaperone proteins and HSP90 itself, however there is a need for new potential therapy targets for combinatorial therapy treatments. ECD seems to be a novel adaptor protein of this complex and together, ECD and the R2TP complex are involved in ErbB2 receptor stability in breast cancer. ECD, HSP90, and the R2TP complex seem to be playing an instrumental role in the stabilization of ErbB2 and loss of ECD abrogates

the ErbB2 and the R2TP complex interaction and eventually leads to destabilization of ErbB2.

(This work will be discussed in detail in Chapter 2).

## **1.5 ECD potential roles through different protein-protein interactions**

ECD interacts with a number of proteins and with the HSP90 and R2TP complex and this suggest that ECD may be playing a role in a number of cellular processes. The schematic in Figure 1.5 shows our laboratory's current knowledge of ECD and depicts the biological processes ECD may potentially be involved in with the R2TP complex. The R2TP is involved in the assembly and stability of several protein complexes and adaptor proteins are necessary to facilitate the interaction between the R2TP complex and these protein complexes. ECD may be interacting with the R2TP complex an adaptor protein. ECD interacts with this complex through PIH1D1 in a phosphorylation dependent manner and domain specific manner (Mir et al., 2015; von Morgen et al., 2015). ECD also interacts with the R2TP through its interaction with RUVBL1 and this interaction is essential for ECD function as a cell cycle regulator (Mir et al., 2015). Through ECD's interaction with Retinoblastoma (RB), we know that ECD is important for cell cycle progression and proliferation (J. H. Kim et al., 2009). Upon deletion of ECD, cells undergo G1 arrest and therefore cannot continue with mitosis, indicative of ECD playing an essential role (Bele et al., 2015; J. H. Kim et al., 2009). Human ECD was first identified as a Human Papilloma Virus 16 E6 (HPV16 E6) interacting protein. Our lab has found that HPV16 E6 binds to ECD at the PEST domain and this interaction is helping to stabilize HPV16 E6, which indicates the potential role of ECD in cervical cancer oncogenesis. The lab has also observed that ECD levels are high in cervical cancer tumor samples; further strengthening the hypothesis that ECD is playing a role in this cancer. ECD has also been found to interact with p53. It helps to stabilize p53 by binding to MDM2 and inhibiting its function (Zhang, 2006). P53 is an important tumor suppressor that is mutated in some cancers. Another previous graduate student has showed that ECD is playing a

role in transformation in hTERT immortalized HMECs and in cell survival. When ECD is overexpressed with the oncogene H-RAS, it leads to hTERT immortalized HMECs undergoing transformation (Bele et al., 2015). ECD and RAS cells exhibited more migration, invasion and acinar formation in 3D matrigel as compared to ECD only, RAS only, and vector expressing cells. These cells also exhibited better survival, indicated by reduced autophagic response, under extreme cellular stress conditions (Bele et al., 2015). These results indicate that ECD may be playing a role in the cellular stress and cellular death pathways.

Further evidence confirming ECD role in a cell death pathway is the observation that ECD interacts with TXNIP. TXNIP is a tumor suppressor that is involved in apoptosis. ECD interacts with TXNIP to decrease ubiquitination of p53 leading to its stability (Suh et al., 2013). A graduate student in Dr. Vimla Band's lab has shown that ECD is involved in cellular stress. ECD interacts with PERK. PERK is a mediator of the unfolded protein response (UPR) pathway, and it is composed of several molecular mechanisms that are aimed to respond to cellular stress (Ma & Hendershot, 2001). In cases of mild stress, mechanisms for cell survival are activated and in cases of extreme stress, mechanisms for cellular death are activated (Ma & Hendershot, 2001; Welihinda et al., 1999). Different stress inducing compounds, such as Thapsigargin and Tunicamycin, cause downregulation of ECD levels in HMECs, indicating that ECD may be a regulator of the UPR pathway.

Figure 1.4: Endogenous association of ECD with HSP90, and the four R2TP components using Proximity Ligation Assay.

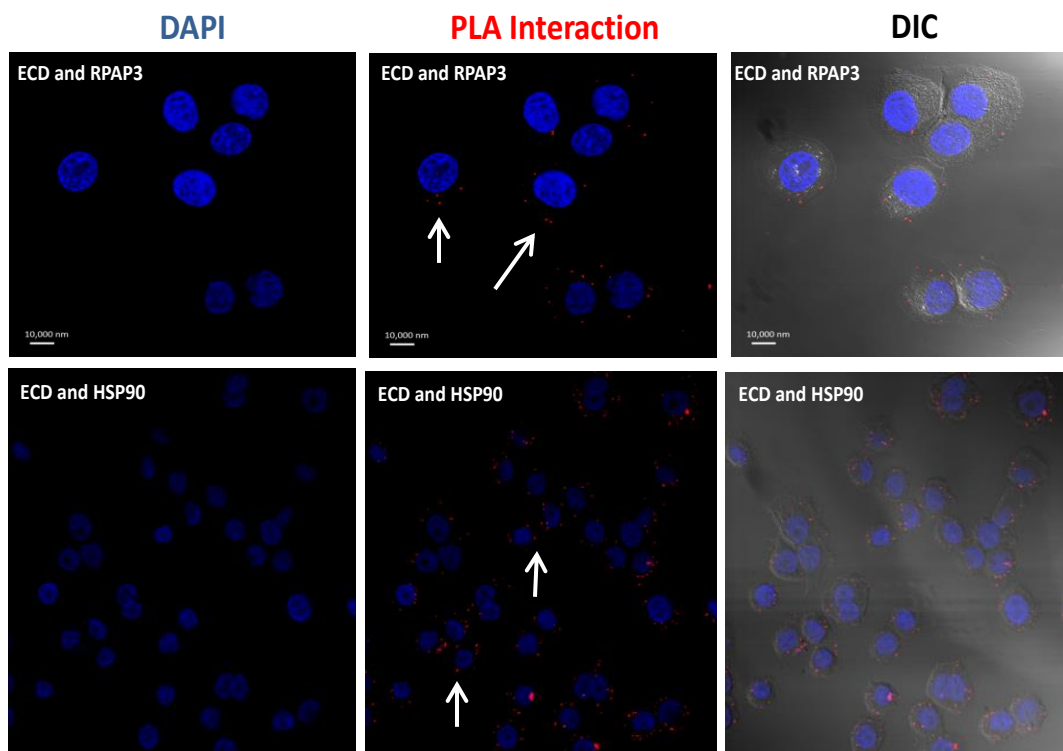
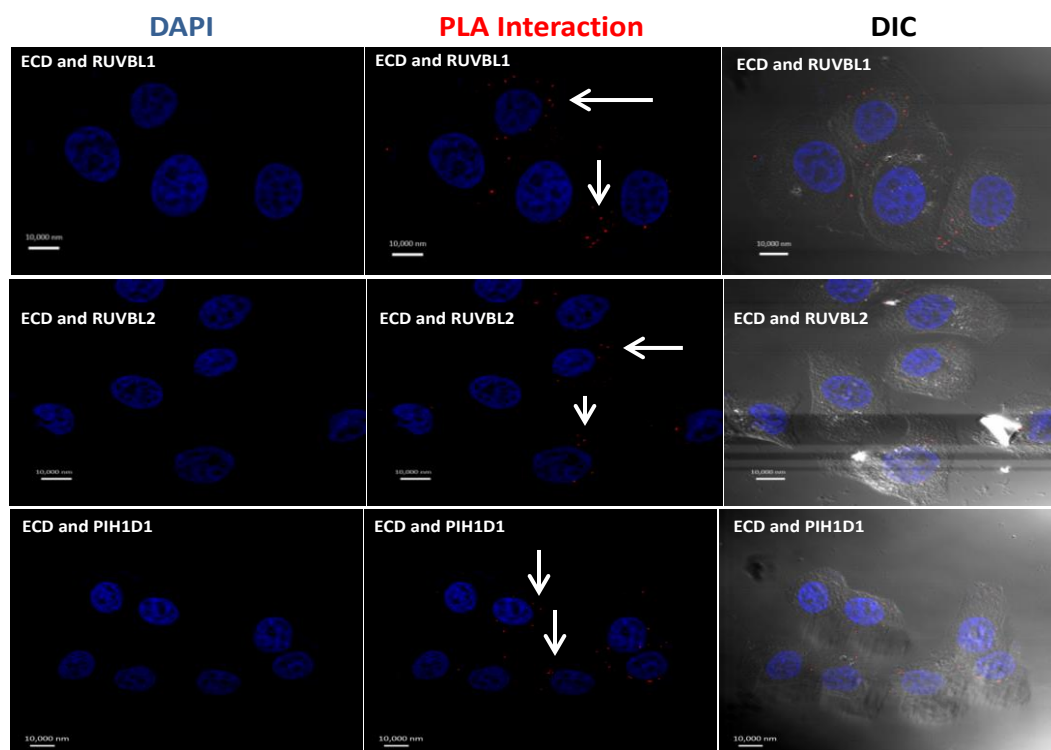
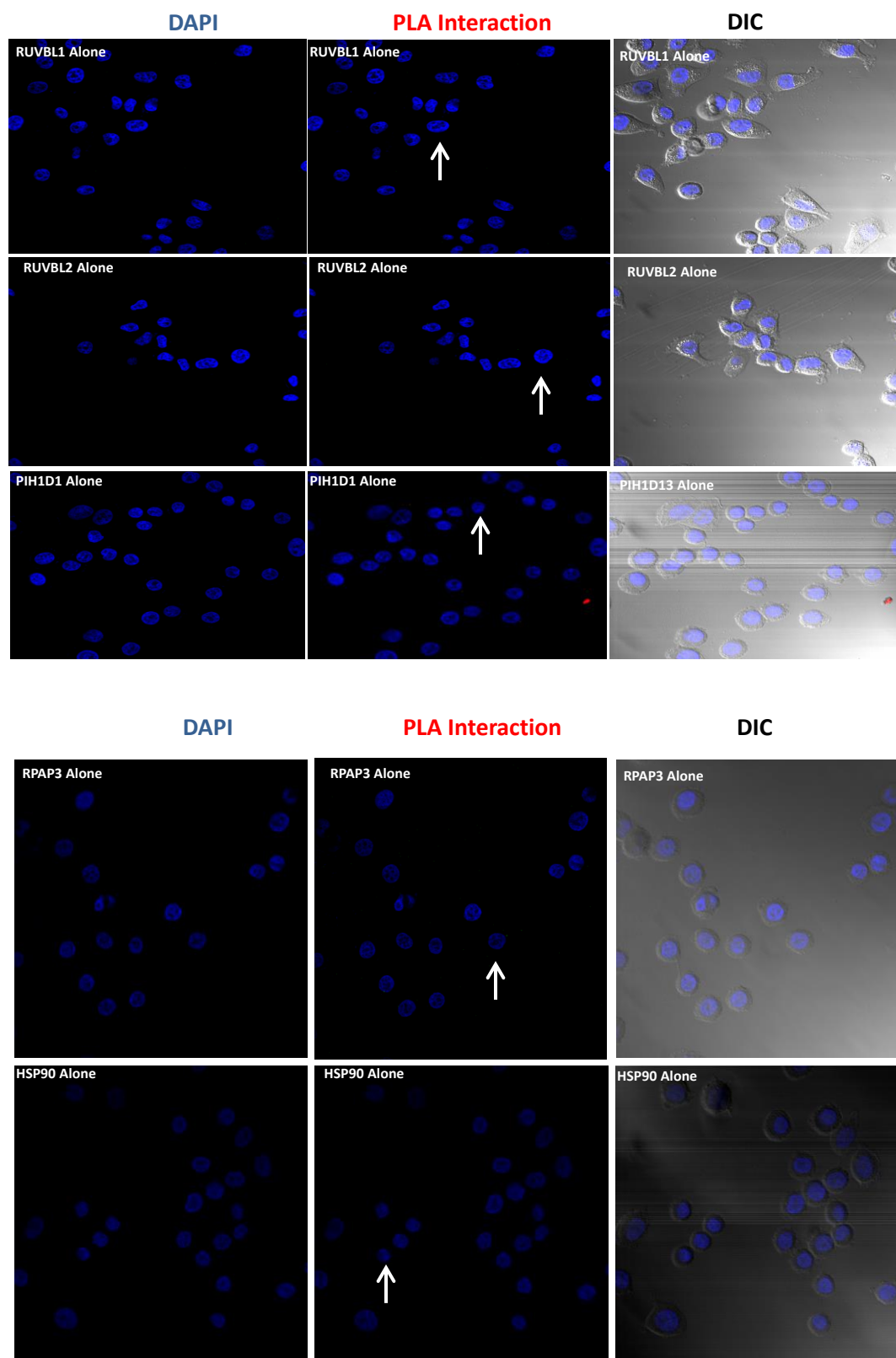
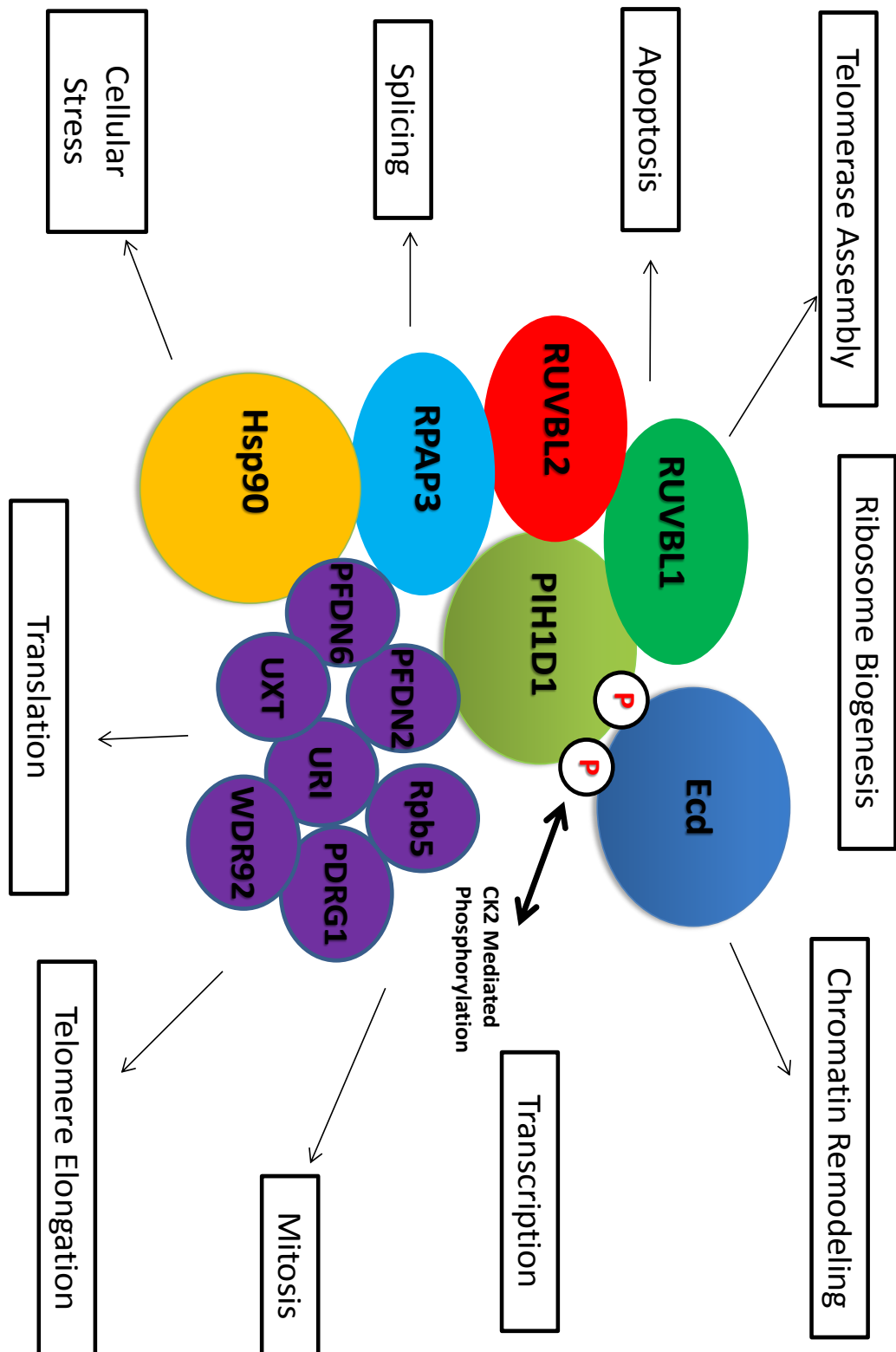


Figure 1.4: Endogenous association of ECD with HSP90, and the four R2TP components using Proximity Ligation Assay:



**Figure 1.4: Endogenous association of ECD with HSP90, and the four R2TP components using Proximity Ligation Assay:** SKBR3 cells were plated on 18mm coverslips and were fixed in 3% paraformaldehyde. Cells were then blocked in blocking buffer for 30 minutes and incubated with primary antibody for 2 hours. Coverslips were then incubated in mouse (minus probe) and rabbit (plus probe) probes for one hour, and then ligated for 30 minutes, and then probes underwent amplification using a red fluorescent dye to indicate interactions for 2 hours. Controls were ECD and HSP90 as positive control and single antibody controls as negative controls. The scale bar is 10nm in length. All the pictures with ECD and the R2TP complex members were taken on 63X objective lens. ECD and HSP90 positive control and single antibody control pictures were taken on a 40X objective lens.

Figure 1.5: Potential Functions of ECD with the HSP90 and the R2TP/Prefoldin-like Complex.





**Figure 1.5: Potential Functions of ECD with the R2TP Complex.**

A schematic representation of Ecd as a part of R2TP/Prefoldin-like complex. Together ECD and the R2TP complex interact with other proteins to perform various cellular functions. (Bele, 2015; von Morgen et al., 2015)

ECD also interacts with several molecular components of the UPR pathway, including PERK and GRP78/BIP. (This work will be discussed in more detail in Appendix B). Interestingly in PERK knockout mouse embryonic fibroblast (MEFs) that are treated with ER stress inducers, ECD levels remain unchanged. This observation indicates that ECD may play a role as a negative regulator of stress. Our lab has shown that Secretory Pathway Calcium ATPase (SPCA2) expression levels are high in ECD+RAS HMECs as compared to ECD overexpressing and RAS overexpressing HMECs alone (Bele, 2015). SPCA2 has been shown to be overexpressed in breast cancer and it plays a role in oncogenesis by promoting calcium dependent signaling (Feng et al., 2010). ECD may be regulating the expression of SPCA2 and when ECD is overexpressed, it causes upregulation of SPCA2 in certain cell lines.

ECD may also be playing a role in ribosome biogenesis through its interactions with PIH1D1, PRP8, and MYBBP1A. In *Drosophila* cells, ECD interacts with Pre-mRNA processing Factor 8 (PRP8), a member of the U5 snRNP spliceosomal complex (Claudius et al., 2014) and is necessary for cell survival in larval imaginal discs. Deletion of *ecd* or *prp8* prevents the splicing of an intron from CYP307A2/*spookier* (*spok*) pre-mRNA. This splicing defect causes a block in entry to metamorphosis (Claudius et al., 2014). Human ECD is able to rescue this phenotype which indicates that ECD may have a similar function in this process in different species. ECD also interacts with the nucleolar protein, Myb-Binding Protein 1A (MYBBP1A), which is a transcription regulator that regulates several transcription factors (Díaz et al., 2007; Fan et al., 2004; Favier & Gonda, 1994; Hara et al., 2009; Tavner et al., 1998). MYBBP1A also plays an essential role in a number of cellular processes such as, mitosis, transcriptional regulation, rRNA synthesis, and ribosome biogenesis (Akaogi et al., 2013; Bele, 2015; Mori et al., 2012; Ono et al., 2013, 2014). A previous graduate student was able to observe that loss of ECD leads to a down regulation of MYBBP1A (Bele, 2015). Upon overexpression, ECD also co-localizes with MYBBP1A

in the nucleolus (Bele, 2015). ECD may be playing a role in MYBB1A stability and may also play a role in the functions of MYBBP1A. Our laboratory has also shown that ECD interacts with p300 and may be playing an important role in chromosome integrity. Upon deletion of *ecd* in primary mouse embryonic fibroblasts (MEFs), there were frequencies for chromosome aberrations (Bele, 2015). These chromosome aberrations include: chromosome breaks, fragments, deletions, and translocations were observed. ECD is functionally important in the maintenance of chromosomes through histone acetylation. Our laboratory has shown that ECD interacts with the histone acetyltransferase, p300 and enhances its HAT activity (J. H. Kim, 2009). ECD may play a role in transcription through its interaction with p300 since it does not have a DNA binding domain. ECD seems to be playing multiple roles in mammalian cells and differential expression of ECD causes several novel phenotypes. ECD is an important factor for cellular processes such as development, cell cycle, cellular stress, stability, complex assembly, calcium signaling, ribosome biogenesis, and possibly transcription and chromosome integrity. ECD also seems to be playing an important role in oncogenesis as indicated with its overexpression in a number of cancers as well as its transformation potential in HMECs with overexpression of H-RAS (Bele, 2015; Bele et al., 2015; Dey et al., 2012; X. Zhao et al., 2012). We have also observed that ECD is associated with ErbB2 and ECD this interaction is facilitated by HSP90 (Ammons and Mirza et.al 2016 Chapter 2). Downregulation of ECD leads to downregulation of ErbB2 levels and downstream molecular targets of ErbB2 signaling. ECD knockdown also depletes the number of ErbB2 receptors on the plasma membrane. This work will be fully discussed in Chapter 2 of this dissertation. I have also observed that different chemotherapeutic agents affect ECD levels and levels of the R2TP complex. Currently, Trastuzumab and Lapatinib are two chemotherapy drugs that are used to treat patients with ErbB2 overexpressing breast cancer. 17-AAG is an HSP90 inhibitor that is currently in clinical trials for patients with ErbB2+ subtype of breast cancer.

These three drugs downregulate ECD protein levels and levels of some of the R2TP complex members indicating that ErbB2 signaling may be regulating the expression of these proteins. This work will be fully discussed in Chapter 3 of this dissertation. I also observed using data collected from the Cancer Genome Atlas, that ECD and several components of the R2TP complex are overexpressed in different subtypes of breast cancer. This work will be fully discussed in Chapter 3 of this dissertation. Lastly, using different inhibitors we have shown that ECD levels are regulated by several different proteins, such as m-TOR and AKT. When I used Rapamycin, an mTOR inhibitor, I observed downregulation of ECD and several members of the R2TP complex in ErbB2+ cell lines. Since the R2TP complex has been observed to be involved in mTOR stabilization and activity (Horejsí et al., 2010; S. G. Kim et al., 2013), it is plausible to hypothesize that ECD may be part of a stabilization feedback loop with mTOR, however more experiments need to be conducted before this can be proven. Again this work will be fully discussed in Chapter 3 of this dissertation. When I treated ErbB2+ cell lines with MK-2206, a pan-AKT inhibitor, I observed down regulation of ECD. Since knockdown of ECD leads to down regulation of AKT levels and an AKT inhibitor leads to down regulation of ECD, it is plausible to hypothesize that ECD and AKT are in a feedback loop that regulate one another's levels perhaps through different complex interactions. Interesting, ECD is not phosphorylated by AKT, according to the potential kinases of ECD list created by a previous graduate student in the laboratory (Bele, 2015). This work will also be fully discussed in Chapter 3 of this dissertation.

Although there are several mechanisms of how ECD functions in cells that still need to fully elucidated, our resources within the laboratory such as anti-ECD antibodies, *in vitro* and *in vivo* models, and different ECD constructs and ECD mutant constructs, will help us uncover the physiological and pathological role(s) of ECD in humans. Given the scope of the different protein-protein interactions of ECD, its involvement in a number of cellular processes, and its

differential expression of ECD in a number of different cancers, ECD should also be under consideration as a new molecular biomarker as well as a new potential drug target.

## 1.6 Rationale, Hypothesis, and Specific Aims

The Human Epidermal Growth Factor Receptor 2 (ErbB2) overexpression accounts for 25% of all breast cancers and is associated with a poor patient survival. A crucial component involved in the regulation of ErbB2 signaling pathway is the chaperone component heat shock protein 90 (Hsp90) and interacting protein complex. Hsp90 participates in the folding, assembly, and proteolytic turnover of many intracellular proteins, including ErbB2. Hsp90 also interacts with other chaperone complexes in order to perform its protein regulation function. One such complex that interacts with Hsp90 is the R2TP/Prefoldin-like Complex. In Eukaryotes, the R2TP complex is comprised of four proteins, RuvBL1, RuvBL2, PIH1D1, and RPAP3 which interact in concert with seven prefoldin-like proteins. Together, Hsp90 and the R2TP complex are involved in several pathways, such as telomere elongation, splicing, ribosome biogenesis, and PIKK kinase stability. The Hsp90 and the co-chaperone R2TP complex interact with an adaptor protein that facilitates the assembly of this complex and determines which intracellular molecules of the chaperone complex are regulated.

Ecdysoless (Ecd) was first discovered in *Drosophila melanogaster*, which encodes a protein whose orthologs are present in several other species including humans. My mentor's laboratory identified the mammalian ortholog of *Drosophila* Ecdysoless from a yeast two-hybrid screen of human mammary epithelial cells (hMECs). My mentor's laboratory then showed Ecd is a novel cell cycle regulator that is essential for embryogenesis and cell cycle progression (34). Our preliminary studies demonstrate Ecd is a phosphoprotein Casein Kinase 2 (CK2) is a major kinase that phosphorylates Ecd. Phosphorylation of Ecd regulates its stability.

My studies have demonstrated Ecd has a strong interaction with Hsp90 chaperone protein and with RuvBL1, a member of the R2TP/Prefoldin-like Complex. We and others have shown CK2-mediated phosphorylation of Ecd, particularly at Serine residues 505 and 518, are required for Ecd's interaction with PIH1D1, an R2TP complex member. This phosphorylation dependent interaction is known to determine the substrate specificity of this complex. Significantly, my mentor's laboratory has shown Ecd is overexpressed in breast cancers, specifically in ErbB2+ breast cancers, and its overexpression correlates with poor prognostic markers and poor survival of patients. Importantly, my recent studies demonstrate treatment of ErbB2+/Ecd+ tumor cells with HSP90 inhibitors or tyrosine kinase inhibitors (Lapatinib and Trastuzumab) dramatically down-regulated ErbB2 as well as Ecd, suggesting a link between these two proteins.

**Based on our preliminary and published results, I hypothesize that ECD, as a cell cycle regulator, could be functioning as a co-oncogene in ErbB2 driven oncogenesis.**

**SPECIFIC AIMS:**

**AIM I: Determine if Ecd is a co-oncogene in ErbB2 mediated oncogenesis.** ECD associates with ErbB2 and HSP90. Hsp90 is a chaperone protein that interacts with the R2TP/Prefoldin-like complex and together is involved in the assembly and stabilization of a variety of proteins. I hypothesize that in ErbB2+ breast cancer cells, ECD levels and association are playing a role in ErbB2 stability. In order to test this hypothesis, I will a) Determine if ECD is a mediator protein for Hsp90 and R2TP/Prefoldin-like chaperone complex in ErbB2+ breast cancer cells, b) Elucidate the biological pathway that ECD and the R2TP/Prefoldin-like complex utilizes for ErbB2 mediated breast oncogenesis.

**AIM II: Elucidate the role of ECD as part of the Hsp90 and R2TP/Prefoldin-like chaperone complex in ErbB2-mediated oncogenesis.** Studies have shown that in breast cancers, Hsp90, R2TP proteins, and Ecd are overexpressed. These components could potentially function in the

stability of other overexpressed or mutated signaling proteins that promote uncontrolled proliferation and/or cell survival of cancer cells. I hypothesize that altering phosphorylated Ecd levels within breast cancer cells will cause the complex to become hyper-active or destabilized leading to a gain or loss of uncontrolled proliferation and/or cell survival.

## **Chapter 2**

# **ECD Positively Regulates ErbB2 Expression and Function**



**ECD positively regulates ErbB2 expression and function**

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Running Title: **ECD binds to ErbB2 and regulates its mRNA and protein stability**

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559-7328;

**ABSTRACT:**

ErbB2/HER2 overexpression accounts for approximately 25-30% of all breast cancer patients and is associated with poor prognosis. Ecdysoneless (ECD), a novel cell cycle regulator, is overexpressed in breast cancer and its overexpression is correlated with poor survival outcome in ErbB2+ breast cancer patients. In this study, to assess if ECD regulates ErbB2 protein, we first analyzed if ECD and ErbB2 proteins interact with each other. Using co-immunoprecipitation, as well as proximity ligation assays, we demonstrate ECD, ErbB2 and HSP90 are present in the same complex. Furthermore, silencing of ECD by siRNA-mediated knockdown led to the downregulation of total ErbB2, phospho-ErbB2 and surface levels of ErbB2. Notably, downregulation of ErbB2 led to significant decrease in its downstream targets, ERK signaling. As expected, knockdown of ECD resulted in significant decrease in ErbB2 association with HSP90. We further demonstrate that ECD-mediated decrease in ErbB2 protein was due to a significant decrease in ErbB2 mRNA stability and in part by protein stability. Significantly, knockdown of ECD in ErbB2+ breast cancer cells resulted in decrease in invasion and migration. These results demonstrate a novel interaction of Ecdysoneless with ErbB2 and its functional consequence on ErbB2 levels and function. Future studies should address if destabilization of ECD together with anti-ErbB2 therapy could provide a better treatment strategy for ErbB2+ breast cancer patients.

**Keywords:** Breast cancer; HER2; ErbB2; Ecdysoneless; HSP90; R2TP

## INTRODUCTION:

Breast cancer is the second leading cause of cancer related deaths in women in the United States with the highest number of new estimated cases in women than any other cancer.<sup>1</sup> Among different molecular subtypes of breast cancer, Epidermal Growth Factor Receptor 2 (ErbB2/HER2) is one of the more aggressive subtypes, which accounts for approximately 25-30% of all breast cancer cases.<sup>2</sup> Although breast cancer patients with ErbB2 overexpression are ideal candidates for anti-ErbB2 Trastuzumab (Herceptin) antibody therapy<sup>3</sup>, not all patients benefit from this treatment. Approximately 15% of patients relapse after therapy due to de novo or acquired resistance<sup>4</sup> and progress into a metastatic disease.<sup>5</sup> Therefore, defining pathways that are connected to ErbB2 protein stability may provide better treatment strategies. We have identified one such candidate, called Ecdysoneless (ECD).

We identified the mammalian ortholog of *Drosophila Ecdysoneless* protein in a yeast two-hybrid screen of human mammary epithelial cells (hMECs) cDNA library using human papilloma virus 16 E6 (a highly efficient hMECs immortalizing gene in an *in vitro* setting) as a bait and hMEC cDNA library. We previously identified Ecdysoneless (ECD) as a p53 interacting protein.<sup>6</sup> Subsequent studies from our laboratory, defined an important role for ECD in cell cycle regulation. ECD interacts with retinoblastoma (RB) protein and competes with E2F transcription factor to bind to RB.<sup>7</sup> Further analyses from our laboratory demonstrated ECD protein is overexpressed in breast cancers and its overexpression is correlated with ErbB2+ breast cancers.<sup>8</sup> ECD overexpression in ErbB2+ patients correlated with poor survival of the patients, suggesting a co-oncogenic role of ECD in ErbB2-mediated oncogenesis.<sup>8</sup> Recent studies from our laboratory

confirmed the co-oncogenic role of ECD in hMECs when ECD is co-overexpressed with well-known oncogene mutant H-Ras.<sup>9</sup> H-Ras, which is widely used for *in vitro* hMECs transformation, is a known down-stream mediator of ErbB2 signaling.<sup>10,11</sup> Overexpression of ECD and mutant RAS in hMECs led to an oncogenic phenotype not only in *in vitro* cell assays but also formed tumors upon implantation in to the mammary gland of NOD/SCID mice.<sup>9</sup> Additionally, we and others have recently identified ECD is a component of a co-chaperone protein complex named R2TP-prefoldin complex.<sup>12,13</sup> The R2TP complex is comprised of four molecular components: two AAA+ ATPases RUVBL1 and RUVBL2, an adaptor protein PIH1D1, and an HSP90 interacting protein RPAP3.<sup>14</sup> We have shown that ECD interacts with two components of R2TP complex, PIH1D1 and RUVBL1, and ECD's interaction with RUVBL1 is critical for ECD's function in cell cycle progression.<sup>13</sup>

In the present study, we examined the association and potential mechanistic link between ECD and ErbB2 proteins. Using co-immunoprecipitation, as well as proximity ligation assay, we demonstrate ECD associates with ErbB2, as well as ErbB2 stabilizing protein HSP90. Furthermore, siRNA mediated knockdown of ECD downregulated total ErbB2 protein levels, surface levels of ErbB2, as well as phospho-ErbB2 protein. The association of ErbB2 with HSP90 was significantly reduced upon ECD knockdown. Significantly, ECD knockdown significantly decreased ErbB2 mRNA stability but also partly decreased turnover of the protein. Lastly, knockdown of ECD in ErbB2+ breast cancer cells resulted in a decrease in invasion and migration of cells. Thus, we present evidence that ECD associates with ErbB2 and regulates its mRNA stability, and we suggest knockdown of ECD together with Trastuzumab may provide a better treatment

strategy for ErbB2+ /ECD+ breast cancer patients.

## **RESULTS:**

### **ECD associates with ErbB2 protein**

Given our previous findings that elevated levels of ECD protein strongly correlates with overexpression of ErbB2, we speculated that ECD may directly interact with ErbB2. For this purpose, we performed two sets of experiments, in one set of experiments, endogenous ECD was immunoprecipitated using anti-ECD antibodies in ErbB2+ breast cancer cell lines, followed by immunoblotting with anti-ECD or anti-ErbB2 antibodies. These results clearly demonstrated an association between ECD and ErbB2 proteins. We also performed reciprocal experiments with immunoprecipitation with anti-ErbB2 antibodies followed by immunoblotting with anti-ECD antibodies and obtained similar association (Figure 1A and 1B). It has been shown that Heat shock protein 90 (HSP90) regulates ErbB2 stability, as it is involved in the folding, maintenance, and regulation of ErbB2.<sup>15</sup> Therefore, we assessed if ECD interacts with HSP90. Immunoprecipitation with anti-HSP90 antibody followed by western blotting with anti-HSP90 showed ErbB2 was associated with HSP90, as expected. Significantly, ECD was also associated with HSP90 (Figure 1C), suggesting that all three proteins are in a complex in ErbB2+ cells. In the second approach we used proximity ligation assay<sup>16</sup> to examine direct interaction of ECD with ErbB2 or ECD with HSP90. In these experiments ErbB2 interaction with HSP90 served as a positive control. In this assay, each dot represents one molecule of each protein interactions. As indicated by the red fluorescent dot signals (Supplementary Figure 1) and in Figure 1D, endogenous ECD is in close proximity to ErbB2, as well as

to HSP90. The Y-axis indicates the total number of signals counted per five random fields with ten cells per field. The X-axis indicates two protein interactions. Taken together, our results clearly demonstrate association of ECD with ErbB2, as well as HSP90, suggesting a tri-complex of ECD-ErbB2-HSP90 in ErbB2+ cells.

### **ECD regulates levels of ErbB2 protein**

To investigate the function of ECD and ErbB2 interaction, we knocked down ECD in ErbB2+ cell lines using two independent siRNAs against ECD, a scrambled siRNA was used as control. Significantly, knockdown of ECD showed a dramatic reduction in the phosphorylated (at residue 1248) as well as total ErbB2 protein (Figure 2A). As expected, decreased levels of ErbB2 led to decreased phosphorylated ERK levels. Notably the levels of total ERK, AKT other downstream target of ErbB2 remained unchanged indicating that the effects of ECD downregulation on ErbB2 and ERK are specific.

Next, we assessed if down regulation of ECD changes surface levels of ErbB2. For this purpose, we treated two ErbB2+ cell lines with scrambled or two different siRNA against ECD, as described before. After 48 hours, live cells were collected and incubated with Alexa Fluor 647-conjugated mouse anti-human ErbB2 or IgG and analyzed using FACS analysis. As shown in Figure 2B and 2E, there was a clear reduction in surface levels of ErbB2, indicated by the shift towards left of the two siRNA ECD peaks, as compared to the control scrambled siRNA peak. In order to quantify these shifts, the mean fluorescence intensity of ErbB2 peak was calculated (Figure 2C and 2F). Western blotting confirmed knocked down of ECD in these cells (Figure 2D and 2G). Taken together, these results suggested ECD regulates ErbB2 protein levels.

### **Knockdown of ECD decreases interaction of ErbB2 with HSP90 protein**

ErbB2 protein stability is strongly modulated by its interaction with HSP90 chaperone protein complex.<sup>10,15</sup> This interaction is essential in preventing ErbB2 ubiquitination and degradation.<sup>17,18</sup> Dissociation of this interaction is an important therapeutic approach.<sup>19</sup> To examine if ECD down regulation influences interaction of HSP90 and ErbB2 protein, we performed again proximity ligation assays as well as co-immunoprecipitation assays in ECD knockdown cells. Notably, PLA experiment (Figure 3A), as well as co-immunoprecipitation experiment (Figure 3B) showed a significant decrease in interaction of HSP90 with ErbB2 protein upon ECD knockdown, underscoring that ECD regulates ErbB2 protein.

### **ECD regulates ErbB2 mRNA and protein stability**

To ascertain whether ECD mediated decreased in ErbB2 levels is a mechanism at the transcriptional level or at the protein level, ECD siRNA expressing cells were treated with cycloheximide (CHX), a known nascent translation blocking agent at 0 time point and then harvested cells at various time points followed by western blotting of cell lysates. To start with (0 time point) the ErbB2 protein levels were low as compared to control cells. As time progress, in ECD knock down cells the ErbB2 protein turnover was faster. A clear decrease at later time points 12-24 hours of CHX treatment (Figure 3C). These experiments showed ErbB2 protein is highly stable in control cells, but has a faster turnover in ECD knockdown cells (Figure 3C). However, considering the dramatic decrease in ErbB2 protein level upon ECD knockdown and this slight increase in protein turn over may not be the entire mechanism how ECD regulates ErbB2 protein levels.

Therefore, we explored if ECD-mediated decrease in ErbB2 is due to transcriptional regulation.

### **ECD knockdown downregulates ErbB2 mRNA by altering its stability**

To assess if ECD downregulates ErbB2 at a transcriptional level, we knockdown ECD from two ErbB2+ cell lines, SKBR3 and BT474, isolated mRNA and performed real-time PCR (RT-PCR) to assess the effect on ErbB2 mRNA levels. Significantly, we observed a dramatic decrease in ErbB2 mRNA levels upon ECD knock down in both cell lines (Figure 4 A and 4 B). DHFR, a known E2F target gene and known to be decreased upon ECD KD, served as a positive control and EGFR, another family member of ErbB2 served as a negative control in these experiments.

Next, we asked if decrease in ErbB2 mRNA levels upon ECD knockdown is due to mRNA stability. For this purpose, two breast cancer cell lines, SKBR3 and BT474 were treated with actinomycin D, a transcriptional blocker agent after ECD knock down. Total RNA was isolated at indicated time points after actinomycin D treatment. qRT-PCR was performed to analyze ErbB2 mRNA levels. In ECD downregulated cells, at 0 time point the ErbB2 mRNA levels were low as compared to control cells. As time progress, the mRNA from control cells was more stable. Notably, ErbB2 mRNA half life is approximately 7 to 8 hrs in scrambled siRNA expressing SKBR3 and BT474 cells, in keeping with previously published data.<sup>20,21</sup> Significantly, ECD knockdown reduced the ErbB2 mRNA stability to approximately 4 to 5 hours (Figure 4C and 4D). Thus, the major mechanism of ECD mediated decrease in ErbB2 is transcriptional effect, however protein stability may also contribute.



## **ECD Knockdown dramatically inhibits invasion and migration of ErbB2+ breast cancer cell lines**

To demonstrate the functional role of ECD in ErbB2-driven tumorigenic phenotype, we used scrambled or ECD siRNA expressing BT474 cells and then performed commonly used cell migration and invasion assays using Boyden Chambers, as described in Material and Methods. As shown in (Figure 5A) knockdown of ECD significantly inhibited invasion of BT474 cells in both siRNAs expressing cells when compared to the control siRNA ( $p=0.002$  and  $p=0.009$ ). Silencing of ECD also resulted in reduced cell migration capacity ( $p=0.004$  and  $p=0.042$ ) (Figure 5C). Western blotting confirmed ECD knockdown in BT474 cells. These results suggest a role of ECD in ErbB2-driven oncogenesis.

Taken together, ECD associates with ErbB2 and HSP90 and regulates ErbB2 mRNA as well as protein stability. Furthermore, ECD contributes to ErbB2-mediated tumorigenic phenotype.

### **Discussion:**

Recent molecular profiling has helped classify the ErbB2+ breast cancers as a distinct subtype of breast cancer accounting for 20 to 30% of all cases.<sup>22,23</sup> Targeting of ErbB2 with humanized anti-ErbB2 monoclonal antibodies is now an important therapeutic strategy for ErbB2-overexpressing breast cancers<sup>3,24</sup>, but therapeutic resistance has emerged as a key issue<sup>4,5</sup>, providing impetus for identification of molecular pathways that regulate ErbB2 at the mRNA and protein levels, as such mechanisms may offer alternate avenues for targeting. Both physiological (*e.g.* via EGF-

induced heterodimerization with EGFR) and pharmacological (using anti-ErbB2 antibodies and HSP90 inhibitors) down-regulation of ErbB2 have been linked to induction of receptor ubiquitinylation, which apparently targets the modified receptor for lysosomal and proteasomal degradation.<sup>10,19,25-28</sup> While most of the studies focus on protein degradation, downregulation of ErbB2 mRNA which can eventually translate into lower protein levels is an area that has received less attention. Knowledge of the mRNA level control of ErbB2 expression could facilitate the development of newer targeted strategies to achieve receptor down-regulation as well as combinatorial therapeutic strategies with agents that target ErbB2 signaling.

In this study, we describe a novel interaction of ErbB2 with ECD, a positive cell cycle regulatory protein whose expression is increased in ErbB2+ breast cancers, predicting poor survival in such patients. We previously identified ECD in a yeast two-hybrid screen as a binding partner of the oncoprotein HPV16 E6, which is a highly efficient human mammary epithelial cell immortalizing protein even though HPVs are not in general linked to breast cancer. Our subsequent analyses demonstrated that ECD is required for mammalian cell cycle progression.<sup>7</sup> Furthermore, ECD interacts with RUVBL1, a component of R2TP complex<sup>13</sup>, and this interaction and serine-phosphorylation of ECD are required for its cell cycle regulatory function. The R2TP complex, composed of RUVBL1, RUVBL2, PIH1D1, and RPAP3, is an HSP90-associated co-chaperone complex.<sup>14</sup> By interacting with distinct adaptor proteins through its PIH1D1 component, the HSP90/R2TP chaperone complex facilitates the assembly of protein complexes involved in many basic biological pathways including the mRNA and tRNA synthesis, telomerase complex assembly, small nucleolar ribonucleoprotein

(snoRNP) biogenesis, phosphatidylinositol-3 kinase-related protein kinase (PIKK) signaling, and translation.<sup>14,29-35</sup> Recently, phosphorylated ECD was shown to interact with PIH1D1<sup>12</sup> suggesting that ECD may also help target the R2TP/HSP90 complex to other targets. Notably, the levels of ECD, HSP90 and components of the R2TP complex have been shown to be overexpressed in several cancers.<sup>8,36-42</sup> These published studies from others and our laboratory prompted us to investigate if mechanistic links exist between ECD and ErbB2.

As HSP90 is a molecular chaperone that assists in the proper folding and maintenance of the mature, folded states a variety of client proteins, including ErbB2, and HSP90 overexpression in breast cancers is correlated with a poor overall survival outcome<sup>36</sup>, we speculated that ECD may be part of a complex with HSP90 and that this complex may help stabilize the ErbB2 protein. Co-immunoprecipitation and proximity ligation assays clearly demonstrated that ECD is in a complex with ErbB2, as well as with HSP90 proteins in ErbB2+ breast cancer cell lines (Figure 1). Using well established ECD specific siRNA, we demonstrate that ECD knockdown decreases the levels of total as well as phospho-ErbB2 protein, as well as surface ErbB2 levels (Figure 2). As the reduction in ErbB2 levels upon ErbB2 knockdown was seen under conditions of cycloheximide block of new proteins synthesis, these results suggest that ECD functions in part by promoting ErbB2 protein stability (Figure 3C and D). ECD knockdown was associated with a decrease in the association between ErbB2 and HSP90 (Figure 3A and B), suggesting that ErbB2 stabilizing function of ECD may be mediated through HSP90.

Notably, the reduction in total ErbB2 levels upon ECD knockdown was considerably more robust than the modest decrease in ErbB2 protein half-life, suggesting

that additional mechanisms were involved in how ECD regulates ErbB2. Analysis of ErbB2 mRNA levels demonstrated a significant reduction in ECD knockdown cells (Figure 4 A and B), suggesting a second mechanism by which ECD regulates ErbB2 protein expression. Furthermore, treatment of control or ECD knockdown cells with Actinomycin D to block new mRNA synthesis demonstrated a reduction in the half-life of ErbB2 mRNA (Figure 4 C and D); supporting the idea that ECD promotes ErbB2 mRNA stability.

How ECD promotes the ErbB2 mRNA stability will be of great interest to dissect through future analyses. In this regard, several recent studies have shown the regulation of ErbB2 mRNA levels to be a key component of ErbB2 protein expression in tumor cells. Heregulin was reported to negatively regulate the transcription of ErbB2/3 without influencing EGF receptor transcription.<sup>21</sup> An ErbB3-interacting protein EBP1 has been shown to reduce the expression of ErbB2 by affecting mRNA stability.<sup>43</sup> ErbB2 mRNA stability has also been shown to be regulated negatively by the binding of microRNAs, such as miR-125a and miR-125b in the 3'-UTR<sup>44-46</sup>, and positively by binding to adjacent sequences of HuR protein.<sup>47,48</sup> Some studies have reported a counter-regulatory relationship between microRNA-dependent destabilization and HuR-mediated stabilization.<sup>47</sup> ErbB2 expression is also under active transcriptional control and has been linked to positive regulation by breast cancer-overexpressed coactivator SRC-3<sup>49,50</sup> and transcription factor ERR $\alpha$ <sup>51,52</sup> as well as negative regulation by PAX2 or FOXP3.<sup>53-56</sup> Future studies should help delineate the roles of these known pathways or other mechanism of mRNA stability and/or transcription of ErbB2 in how ECD functions positively in the maintenance of ErbB2 levels in breast cancer cells.

Given our prior work establishing ECD as a positive regulator of cell cycle progression <sup>7</sup>, its overexpression in ErbB2+ breast cancers <sup>8</sup>, and promotion of an oncogenic phenotype in human mammary epithelial cells by overexpression of ECD together with mutant H-Ras <sup>9</sup>, we assessed if ErbB2-driven oncogenic phenotype requires the presence of ECD. Indeed, we observed a dramatic downregulation of invasion and migration of ErbB2+ breast cancer cells when ECD was downregulated, underscoring a functional role for ECD in ErbB2-driven oncogenesis.

In conclusion, the present study demonstrates a novel interaction of ECD with ErbB2 and HSP90 which appears to positively regulate ErbB2 stability and in addition reveal a novel role of ECD in the stability of ErbB2 mRNA. Future studies of *in vivo* cooperation of ErbB2 and ECD in oncogenesis, using ECD plus ErbB2 transgenic mouse models currently being engineered in our laboratory, will be of great interest to extend our current findings that clearly support an important role of ECD in ErbB2-driven oncogenesis. Furthermore, targeting ECD in ErbB2+ cells along with anti-ErbB2 therapy, trastuzumab may provide a better treatment strategy.

## **Materials and methods:**

### **Cell Culture, Regents, and Transfections:**

SKBR3 and BT474 cells were cultured and maintained in RPMI medium supplemented with 10% fetal bovine serum. For proximity ligation assays, SKBR3 cells were grown to 30% confluence on glass cover slips in 12-well plates. Cells were transfected with either 50 nM of control, ECD siRNA1, ECD siRNA2 described previously <sup>6</sup>, using the DharmaFECT 1 Transfection Reagent (T-2001-03, Dharmacon, Pittsburgh, PA). CHX

(Sigma, St. Louis, MO) Actinomycin D (Sigma, St. Louis, MO) is commercially available.

### **Duolink in situ proximity ligation assay**

Anti-mouse PLA probe plus, anti-rabbit PLA probe minus, and detection kit Res 563 were purchased from OLink Bioscience (Uppsala, Sweden). 3% Paraformaldehyde fixed cells were blocked with Blocking buffer received from the kit for 30 minutes, and incubated with primary antibodies for ECD and ErbB2 (c-8) (sc-284), ErbB2 (c-8) (sc-284), and HSP90 (H-114) (sc-7947), ErbB2 and Ada3, and IgG mouse (sc-2025) and IgG rabbit(sc-2763) for 2 hours at 37°C, as previously from Santa Cruz Biotechnology (Dallas, TX) described in detail <sup>16</sup>. PLA probes were diluted 1:5 in antibody diluent solution. Ligation and Amplification steps were followed from the Duolink Proximity Ligation Assay Protocol. Detection of the PLA signals was carried out with LSM 510 META and LSM 710 META Confocal fluorescence microscope (Zeiss).

### **FACS Analysis**

For live-cell surface ErbB2 staining, cells trypsinized using Trypsin from Sigma (St. Louis, MO). After the trypsin was inactivated, the cells were then spun down at 1000 RPM at 4 °C. Cells were washed three times with FACS buffer and incubated for 1 h on ice in the dark with Alexa Fluor 647-conjugated mouse anti-human ErbB2 monoclonal antibodies (mAb) (cat no,3244412) from Biolegend (San Diego, CA) Inc or mouse IgG (control) diluted in FACS buffer. Following the incubation, the cells were washed three times using the same buffer. Cells were fixed at room temperature in 4% PFA for 10 min,

run on a BD FACScalibur flow cytometer and analyzed with CellQuest software as done previously.<sup>57</sup>

### **Immunoprecipitation and Western Blotting**

For immunoprecipitation cells were washed in 1X PBS and lysed in CHAPS buffer {[0.3% CHAPS, 20 mM Tris-HCl (pH 7.4), 120 mM NaCl, 10% glycerol, 5mM EDTA] supplemented with protease and phosphatase inhibitor (Roche, Indianapolis, IN). Lysates equivalent to 2mg of total protein were subjected to immunoprecipitation, as described in figure legends. For western blotting, cell extracts were prepared in RIPA buffer (Cat# 89901, ThermoFisher Scientific, Waltham, MA) supplemented with protease inhibitor (Roche, Indianapolis, IN). Lysate equivalent to 15-25 $\mu$ g of protein was resolved on SDS-PAGE gel followed by western blotting with indicated antibodies. Antibody against ECD was described previously<sup>7,8</sup>. Rabbit anti-ErbB2 pAb (c-8) (sc-284) from Santa Cruz Biotechnology (Dallas, TX) and rabbit anti-HSP90  $\alpha/\beta$  pAb (H-114) (sc-7947), from Santa Cruz Biotechnology (Dallas, TX). Conjugated secondary antibodies for Western blotting are from Invitrogen (Grand Island, NY).

### **Transwell Migration and Invasion Assays**

The migration assay was done using BD BioCoatTranswell chambers (#354578) (San Jose, CA). In BT474 cells ECD was knockdown using specific siRNA as described above. Before plating cells were starved in 0.1% serum. 10,000 cells were plated in the top chambers. After 2 h, 10% fetal calf serum containing medium was added to the lower chamber and then incubation was carried out for 24 h. Cells were fixed with ice-cold methanol and stained with propidium iodide. Similar protocol was followed as described.<sup>9</sup> The migrated cells on the bottom surface of filters were then mounted on

coverslips and observed at 20X magnification under rhodamine filter using 710 LSM at confocal microscopy. Five random fields were counted from each well of triplicates. Invasion assay was performed using BD Matrigel invasion chambers (#354480) (San Jose, CA). Cells were plated, processed and counted similar to migration assay.

### **RNA Isolation and Quantitative Real Time PCR**

Total RNA was isolated from SKBR3 and BT474, control and siRNA treated samples using TRIzol reagent, according to the manufacturer's protocol. 1 µg RNA was reverse transcribed using SuperScript™ II reverse transcriptase (Invitrogen, ThermoFisher Scientific, Waltham, MA) and oligo-dT primers (18MER) and quantitative real-time PCR (qRT-PCR) analyses with specific primer sets for *ECD*<sup>6</sup> Forward: 5' - ACTTTGAAACACACGAACCTGGCG-3' and Reverse: 5' - TGATGCAGGTGTGTGCTAGTTCCT-3', *DHFR* Forward: 5'- TAAACTGCATCGTCGCTGTGT-3' and Reverse: 5' - AGGTTGTGGTCATTCTCTGGAAA-3', *ErbB2* Forward: 5'- AGCCTTGCCCCATCAACTG-3' and Reverse: 5'-AATGCCAACCACCGCAGA-3', *EGFR* Forward: 5'-TGCCATCCAAACTGCACCTA-3' and Reverse: 5' - CTGTGTTGAGGGCAATGAG-3'. *GAPDH*, Human Forward: 5'- GTCATCCATGACAAGTTTGG-3' and Reverse: 5' - TGCCAGTGAGCTTCCCGTTC-3', were ordered from (Sigma, St. Louis, MO). qRT PCR were carried out in an Applied Biosystems 7500 Real Time PCR system using Power SYBR Green master mix from Applied Biosystems (ThermoFisher Scientific, Waltham, MA).

### **mRNA Stability Assays**



Cells were seeded in 6 well plates (200,000 cells per well). After 48 hrs of ECD knockdown, followed by addition of Actinomycin D (5 $\mu$ g/ml) (Sigma, St. Louis, MO) at time zero time point. Total RNA was isolated at indicated time point, 0, 2, 4, 6, 8 and 12 hours of Actinomycin D with Trizol as per the manufacture's protocol. First-strand cDNA was synthesized using SuperScript III reverse transcriptase, followed by qPCR with ErbB2, ECD, and GAPDH primers, as described above.

### **Figure Legends:**

**Figure1. ECD associates with ErbB2:** Co-immunoprecipitation shows association of endogenous ErbB2, ECD and HSP90 (A, B & C). Two ErbB2+ cell lines, SKBR3 or BT474, cell lines were lysed in CHAPS buffer and 2 mg of lysates were immunoprecipitated (IP) with anti-ErbB2, anti-ECD or anti-HSP90 specific antibodies. Mouse (Ms) and Rabbit (Rb) IgG served as negative controls. HSP90 interaction was used as a positive control. The bound proteins were subjected to Western blotting analysis with indicated antibodies in A, B & C. (D) Proximity ligation assays were performed between ECD and HSP90 or ECD and ErbB2(as described in materials and methods). Quantification was performed by Duolink Image tool. This tool is able to detect the number of signals per cell. We took 5 distinctive jpeg images of the cells under 63X magnification. These images are uploaded and checked for quality using the Duolink ImageTool. The nuclei channel is automatically labeled blue and the user selects the signal channel, and in this case it is red). The tool then counts approximately 10 individual cells per image, therefore we have about 50 cells that we counted. The histograms represent the total number of signals of each image.

**Figure 2. ECD regulates levels of ErbB2 protein.** (A) ErbB2<sup>+</sup> cell lines, SKBR3 and BT474 were transfected with either 40 nM of control or two different ECD siRNA, using the DharmaFECT 1 Transfection Reagent. Lysates were harvested after 48 hours of transfection and immunoblotted with indicated antibodies. B-actin was used as a loading control. Data shown here are mean of  $\pm$  S.E.(error bars ) from three independent experiments and p values were calculated using student's t test  $p \leq 0.05$  is considered significant \*. **(B) ECD knockdown reduces the surface levels of ErbB2.** SKBR3 and BT474 cells were treated with scrambled or two different siRNA specific for ECD, as discussed previously. After 48 hours of transfection these cells were labeled with Alexa flour 488- conjugated anti-ErbB2 antibody for 1 hour at 4C. Anti- mouse IgG alone served as a negative control (B & E). Cell surface levels of ErbB2 were quantified using flow cytometry and then analyzed with BD cellquest<sup>TM</sup> software. (C & F) The Y axis represents MFI (mean fluorescence intensity) (C & F) Lysates were harvested after 48 hours of transfection and then immunoblotted with indicated antibodies.  $\beta$ -actin was used as a loading control (D and G).

**Figure 3. Knockdown of ECD decreases interaction of ErbB2 with HSP90** (A) Proximity Ligation Assay depicting interactions between ErbB2 and HSP90 upon ECD knockdown. Proximity ligation assay was carried out, as described above. Quantification of proximity signals was carried out using Duolink Image Tool Software. Analysis of >50 cells per interaction is shown here. These figures and graphs are representative images. **(B)** SKBR3 cell line was transfected with scrambled and siRNA against ECD, as discussed previously. After 48 hours of transfection these cells were lysed in CHAPS buffer and 2 mg of lysates were immunoprecipitated (IP) with anti-HSP90 antibody. The

bound proteins were subjected to Western blotting analysis first with anti-ErbB2 antibody and after stripping blotted again with anti-HSP90 antibody. **(C)** SKBR3 cell line was transfected with scrambled or siRNA against ECD, 48 hours later cells were treated with cycloheximide (CHX) at 100 ( $\mu\text{g/ml}$ ) (0 time point), and then cell lysates were collected at indicated time periods for Western blotting. Down regulation of ECD was confirmed by western blotting and  $\alpha$ -tubulin was used as a loading control. **(D)** ErbB2 protein degradation in SKBR3 was measured by using Image J software after normalizing with corresponding  $\alpha$ -tubulin band intensities. The values were converted as percentages; log value was calculated and plotted on Y axis. X-axis represents times after CHX treatment.

**Figure 4: ECD knockdown decreases ErbB2 mRNA levels by enhancing its turnover**

ECD knockdown was performed using specific siRNA against ECD in ErbB2 positive, BT474 and SKBR3 cell lines. Total RNA was isolated after 48 hour of transfection and cDNA was made. Levels of ECD and ErbB2 mRNA were detected by quantitative real-time RT-qPCR; DHFR was used as a positive control and GAPDH as an internal control. Fold change over GAPDH was calculated and plotted **(A)** BT474 and **(B)** SKBR3 cells. **ECD down regulation decreases ErbB2 mRNA stability.** The stability of mRNA was analyzed in SKBR3 **(C)** and BT474 cells **(D)**. After ECD down regulation by siRNA, cells were treated with actinomycin D (5  $\mu\text{g/ml}$ ) at 0 time point, and then total RNA was isolated at various time points (0, 2, 4, 6, 8 and 12 hours) after actinomycin D treatment. cDNAs were made and levels of ErbB2 were measured by RT-qPCR. GAPDH was used as normalization control.

**Figure 5: Knockdown of ECD decreases invasion and migration ability of ErbB2 positive breast cancer cell lines.** Western blotting shows downregulation of ECD protein upon siRNA knockdown (**B & D**), these cells when plated on Boyden chambers show decrease in their ability to invade (**A**) and migrate (**C**). The bar diagrams represent number of cells migrated or invaded. Data shown here are mean  $\pm$  S.E. (standard error bars) from three independent experiments, and p values were calculated using student's t test,  $p \leq 0.05$  is considered significant \*.

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### **Authors' Contributions**

Conception and design: HB, VB

Development of methodology: SM, SAA, AS, AB, RAM, AO

Acquisition of data: SM, SAA, AS, AB, RAM, AO

Analysis and interpretation of data: HB, VB, SM, SAA

Writing, review, and/or revision of the manuscript: HB, VB, SM, SAA, AS

Administrative, technical, or material support:

Study supervision: HB, VB

CONFLICT OF INTEREST: The authors declare no conflict of interest.

## Figures:

### Figure 1:

Figure 1.

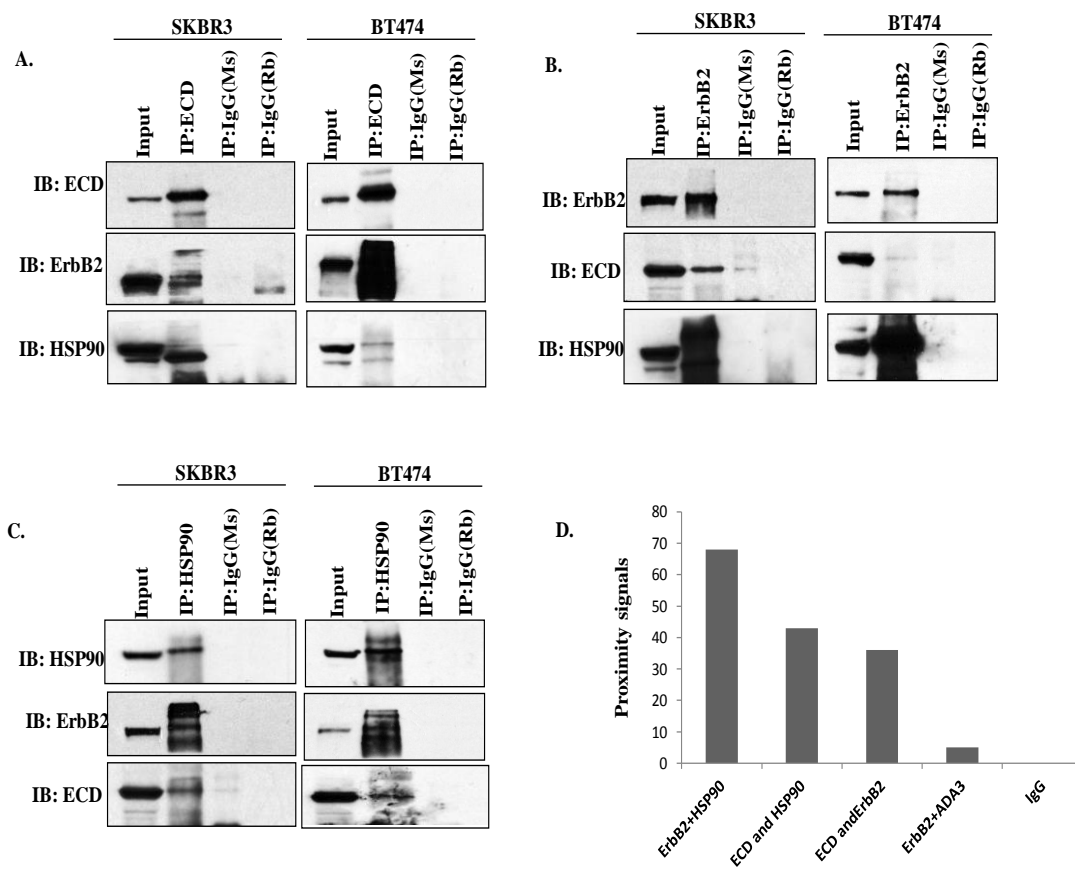


Figure 2:

Figure 2.

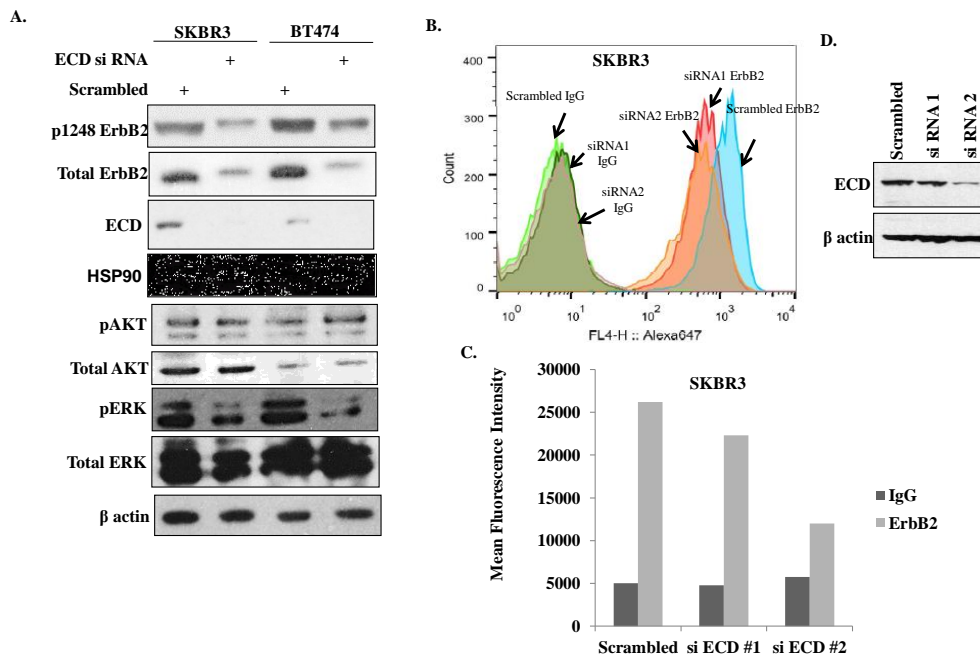


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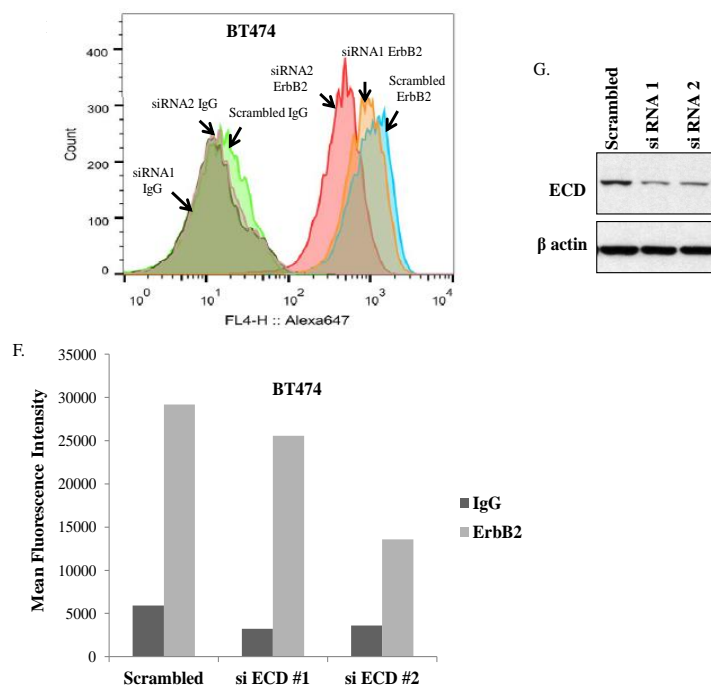


Figure 3:

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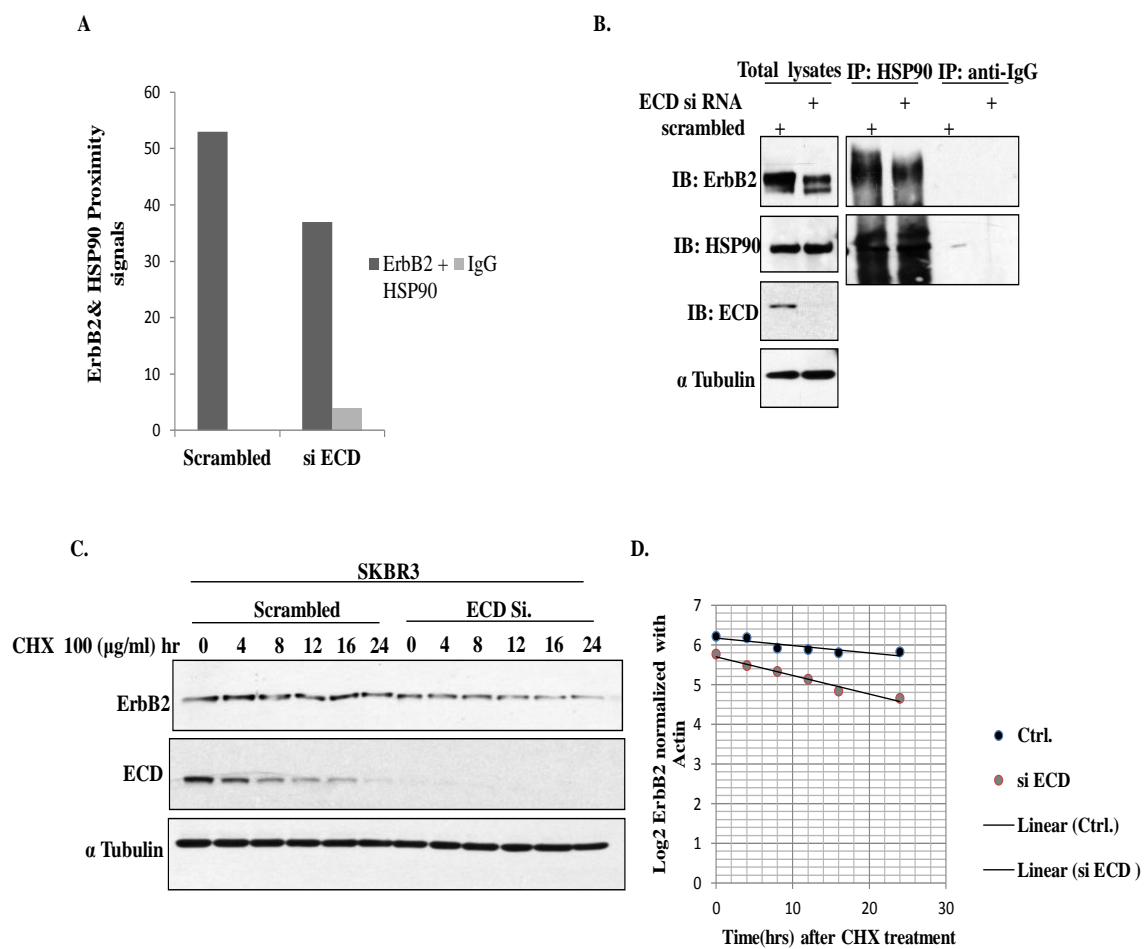




Figure 4:

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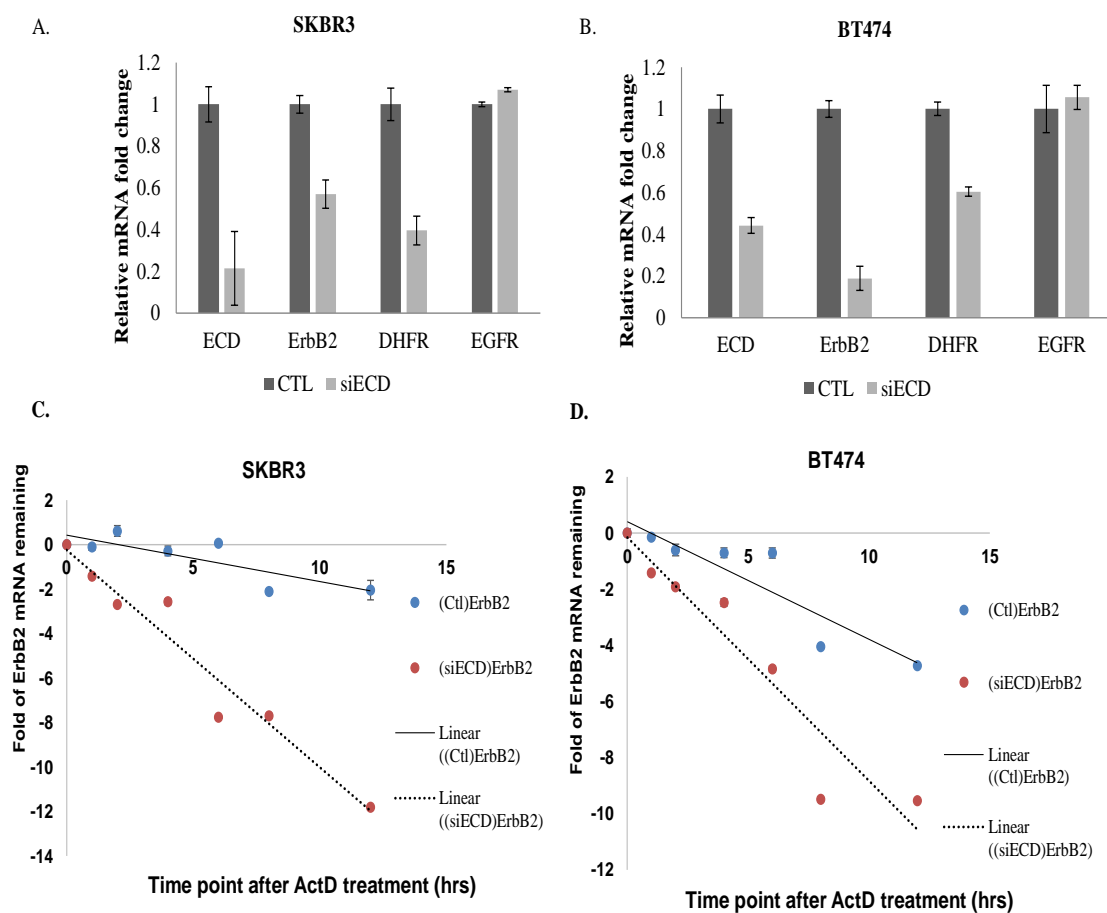
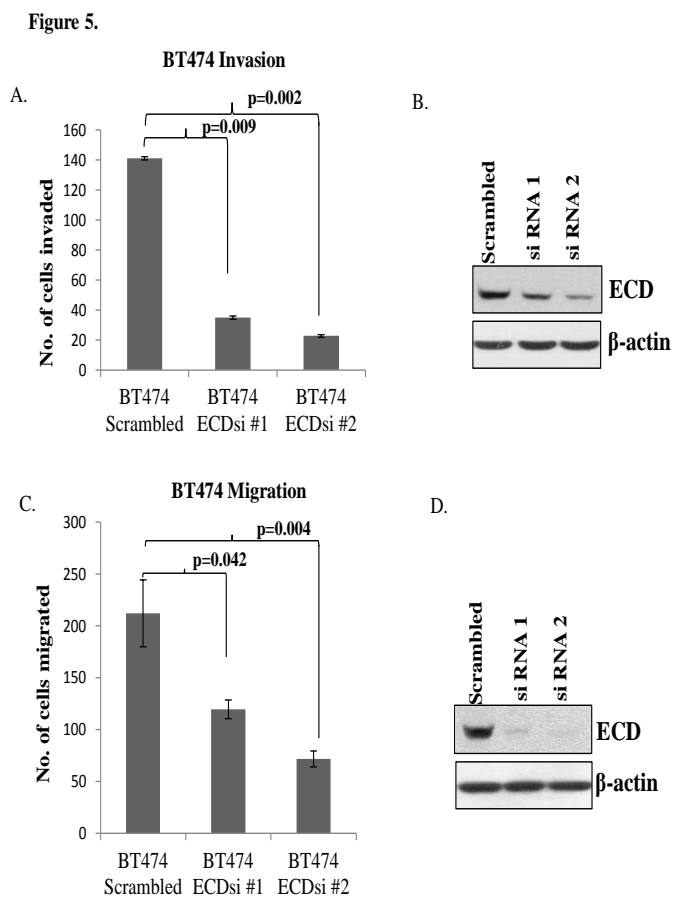


Figure 5



## Supplementary Figure Legends

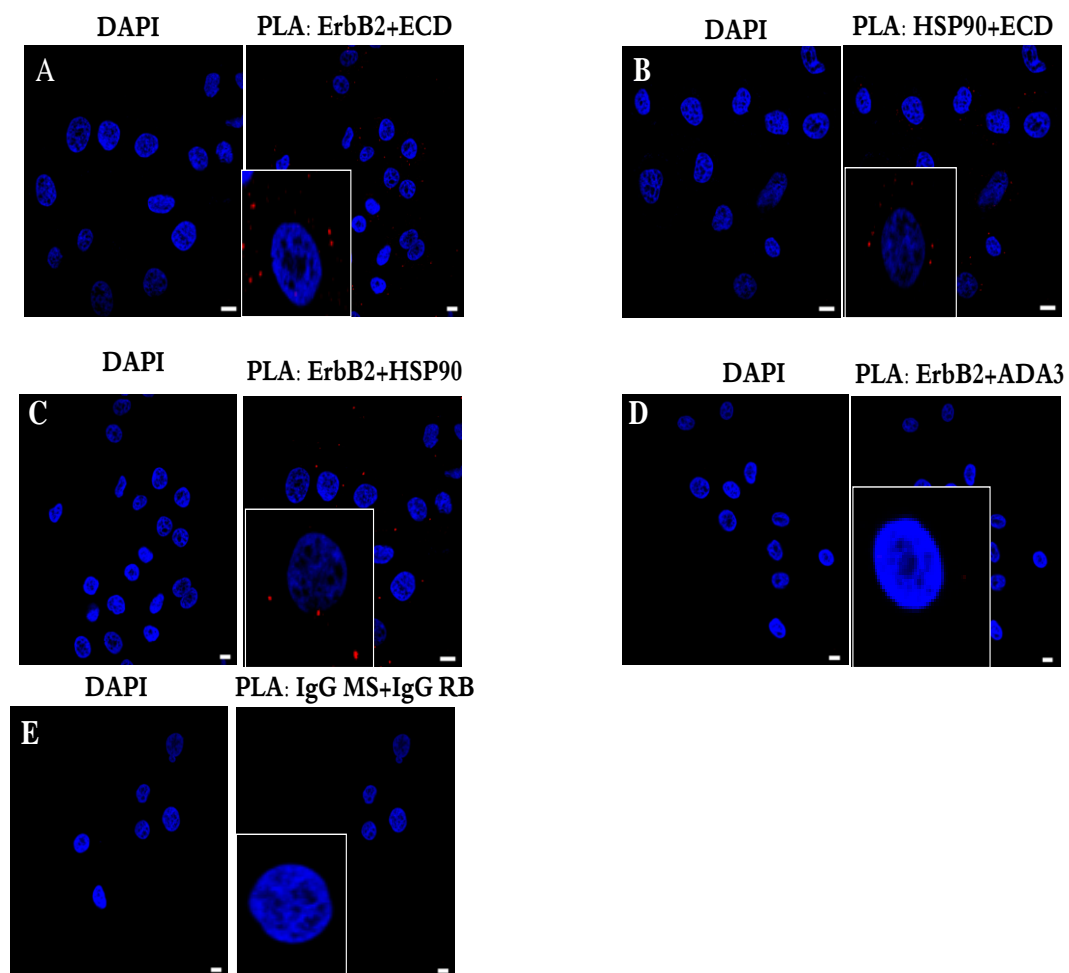
Supplementary Figure 1: A-B) Proximity Ligation Assay depicting Interaction between ECD and ErbB2, and ECD and HSP90. SKBR3 cells were plated on 18mm coverslips and were fixed in 3% paraformaldehyde. Cells were then blocked in blocking buffer for 30 minutes and incubated with primary antibody for 2 hours. Coverslips were then incubated in mouse (minus probe) and rabbit (plus probe) probes for one hour, then ligated for 30 minutes, then probes underwent amplification using a red fluorescent dye to indicate interactions for 2 hours. Each red fluorescent signal indicates one protein-protein interaction. Controls were C) HSP90 and ErbB2 as positive control, D) ErbB2 and ADA3 and E) IgG antibodies used as as negative controls. Pictures were taken at 63x magnification and 40X magnification for controls on the Zeiss 510 and 710 Confocal Microscopes. Scale bars represent 10 $\mu$ m. Insets are of representative cell taken from the image.

Supplementary Figure 2: A-B) Proximity Ligation Assay depicting Interaction between ErbB2 and HSP90 when ECD knockdown and control samples. BT474 cells were plated on 18mm coverslips and were transfected with either 40 nM of control or ECD siRNA 2, using the DharmaFECT 1 Transfection Reagent. After 48 hours of transfection cells were fixed in 3% paraformaldehyde. Cells were then blocked in blocking buffer for 30 minutes and incubated with primary antibody for 2 hours. Coverslips were then incubated in mouse (minus probe) and rabbit (plus probe) probes for one hour, then ligated for 30 minutes, then probes underwent amplification using a red fluorescent dye to indicate interactions for 2 hours. Each red fluorescent signal indicates one protein-protein interaction. C-D) IgG antibodies controls as negative controls in C) control siRNA cells

and D) siRNA ECD cells. Scale bars represent 10 $\mu$ m. Pictures were taken at 63x magnification on the Zeiss 510 and 710 Confocal Microscopes. Insets are of representative cell taken from the image.

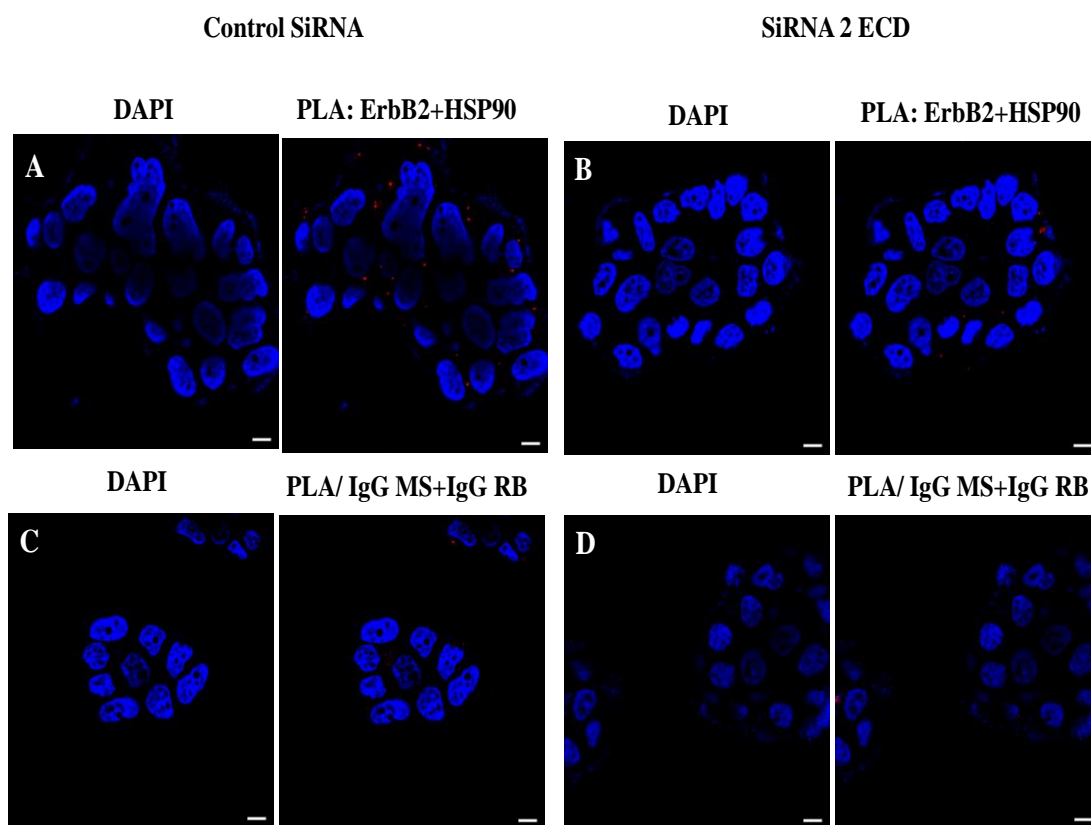
**Supplementary Figures:**

Supplementary Figure 1



## Supplementary Figures

Supplementary Figure 2



## **Chapter 3**

# **Ongoing Studies and Future Directions**

### **3.1 ECD localization and expression in ERBB2 exogenous overexpressed cell lines.**

In order to explore the role of ECD assisting in the stabilization of ERBB2 *in vitro*, we wanted to observe if ECD localization differed in ERBB2+ breast cancer cell lines. Our laboratory has shown that ECD is mainly localized in the cytoplasm; however ECD has the ability to shuttle to the nucleus. ECD does not have a nuclear localization signal, but it does have a fast nuclear export (J. H. Kim et al., 2010). ERBB2 is mainly localized at the plasma membrane and is stabilized by HSP90 and other co-chaperones (Yarden & Sliwkowski, 2001). HSP90, in complex with other co-chaperones, bind to the tyrosine kinase domain of ERBB2 in the cytoplasm and protects the receptor from ubiquitination and degradation (Ehrlich et al., 2009; W. Xu et al., 2005). ERBB2 also undergoes a recycling process called endocytosis; however the pathway has not been fully elucidated. Endocytosis is an active transport pathway that transports molecules into the cell by engulfing them and there are several pathways the cell can utilize to complete this function (Marsh, 1999). Interestingly, in some cell lines the ERBB receptors have been shown to activate several biochemical signaling pathways, such as ERK and AKT, during endocytosis (Liu, Tao, Woo, Xiong, & Mei, 2007; Yang, Huang, Xiong, & Mei, 2005). Since ERBB2 and ECD both may have functions in the cytoplasm, I wanted to see if ECD and ERBB2 colocalized in any compartment of the cell when ERBB2 is overexpressed. In order to test this question, I performed several immunofluorescence experiments in several different ERBB2+ cell lines. I used human anti-ECD antibody that



the lab has generated and human anti-ERBB2 antibody from Santa Cruz. As shown in Figure 3.1, ECD does not colocalize with ERBB2 at the plasma membrane. However, it is difficult to determine if ECD and ERBB2 colocalize in the cytoplasm upon ERBB2 overexpression and further test will need to be performed to confirm this phenomenon.

Since we have previously published that ECD overexpression correlates with ERBB2 overexpression in breast cancer patients (X. Zhao et al., 2012), we wanted to see if we can recapitulate this correlation *in vitro*. We first observed localization of these two proteins in ERBB2+ cell lines, and we were not able to see distinctive colocalization in any cell compartment. I then wanted to observe how exogenous ERBB2 effected protein and mRNA levels in immortalized human mammary epithelial cell lines (HMECs) and in MCF7 breast cancer cells which are ER+. All these cell lines have basal levels of ERBB2, so I transfected either pMSCV-vector puromycin or pMSCV-ERBB2 puromycin into these cells using retroviral infection and treated cells with puromycin selection. After selection, I harvested protein lysates and mRNA from these cells and observed levels of ECD and ERBB2. Interestingly, ECD protein levels, compared to vector control, increased after exogenous ERBB2 transfection (Figure 3.2) in all three cell lines. This result in conjunction with ECD overexpression observed in breast cancer cell lines shown in Figure 1.1, indicate that ECD levels are regulated by ERBB2 and elevated ECD is necessary in ERBB2 overexpressed cell lines. ECD mRNA however, does not seem to be significantly changed in vector or ERBB2 overexpressing cells (Figure 3.3). From these results, we are able to conclude that ECD is overexpressed in ERBB2 overexpressed cell lines on protein level and not mRNA level, indicating that ECD is becoming more

Figure 3.1: ECD and ERBB2 Localization in ERBB2+ Breast Cancer Cell Lines.

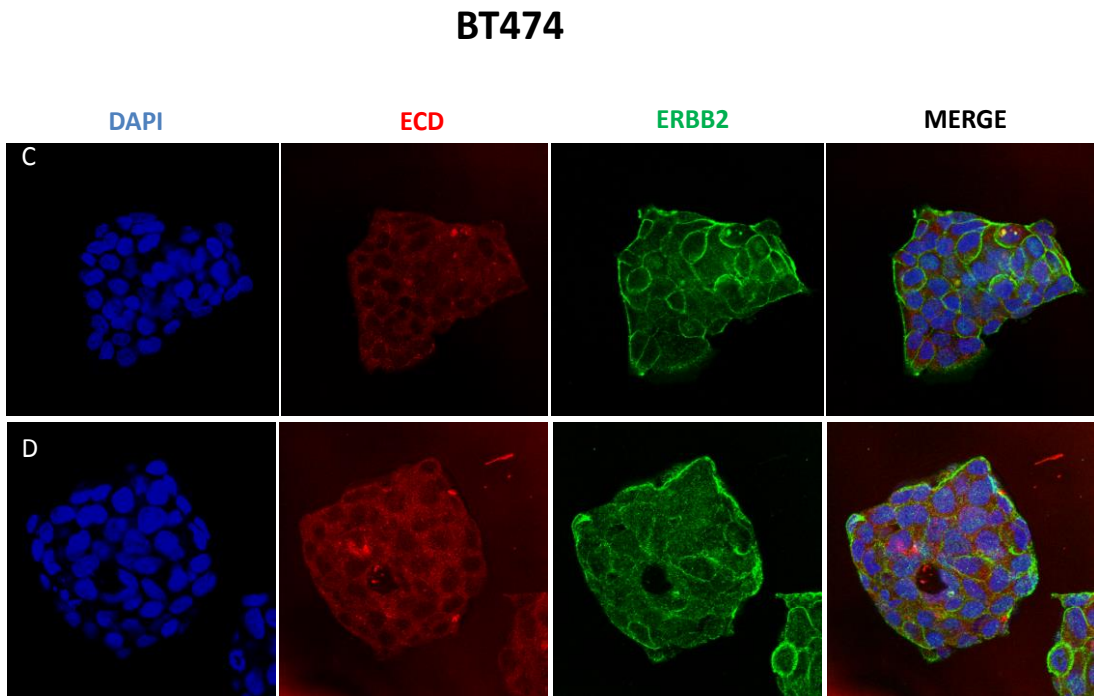
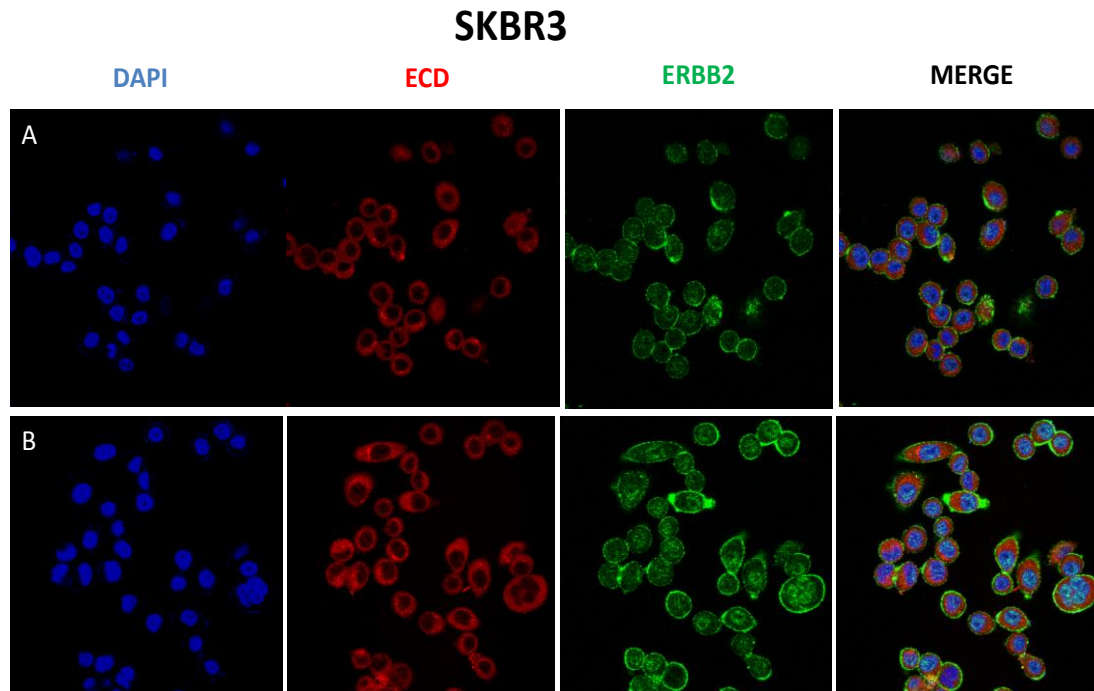
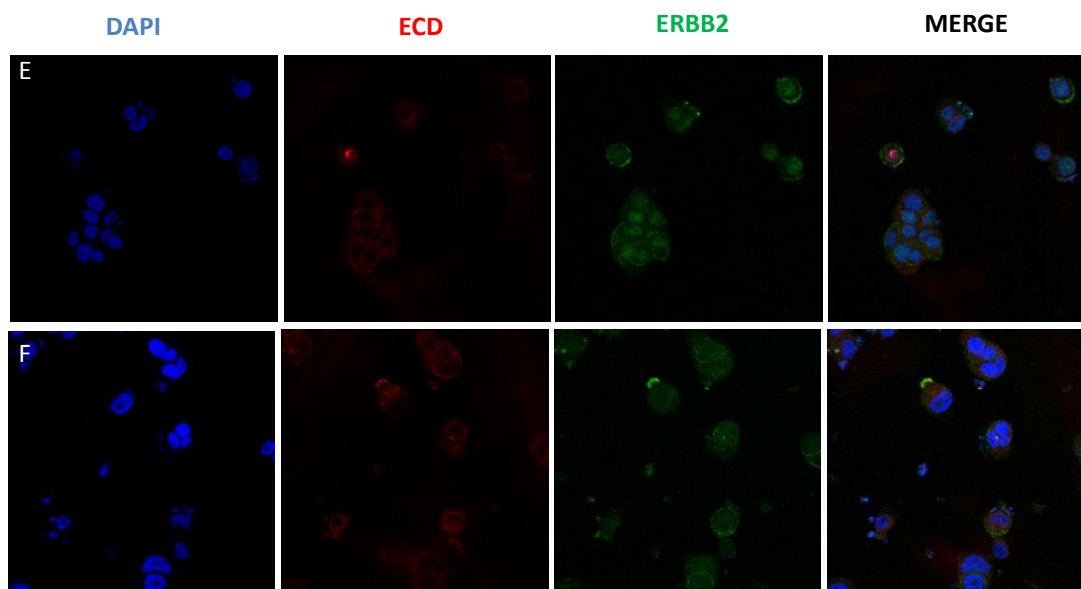


Figure 3.1: ECD and ERBB2 Localization in ERBB2+ Breast Cancer Cell Lines (continued).

## UACC 812



## HCC 1419

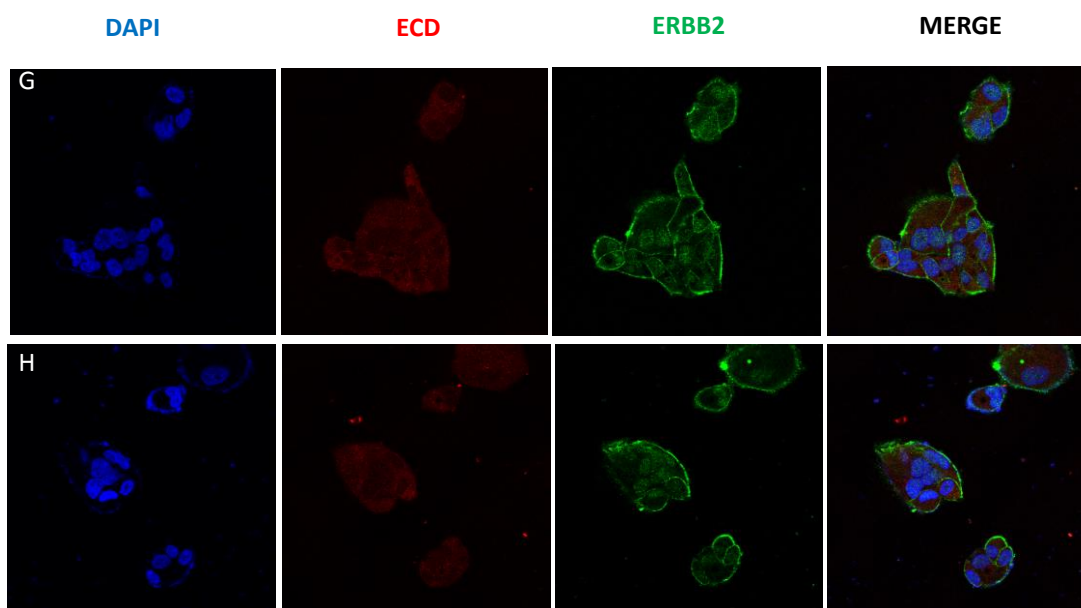
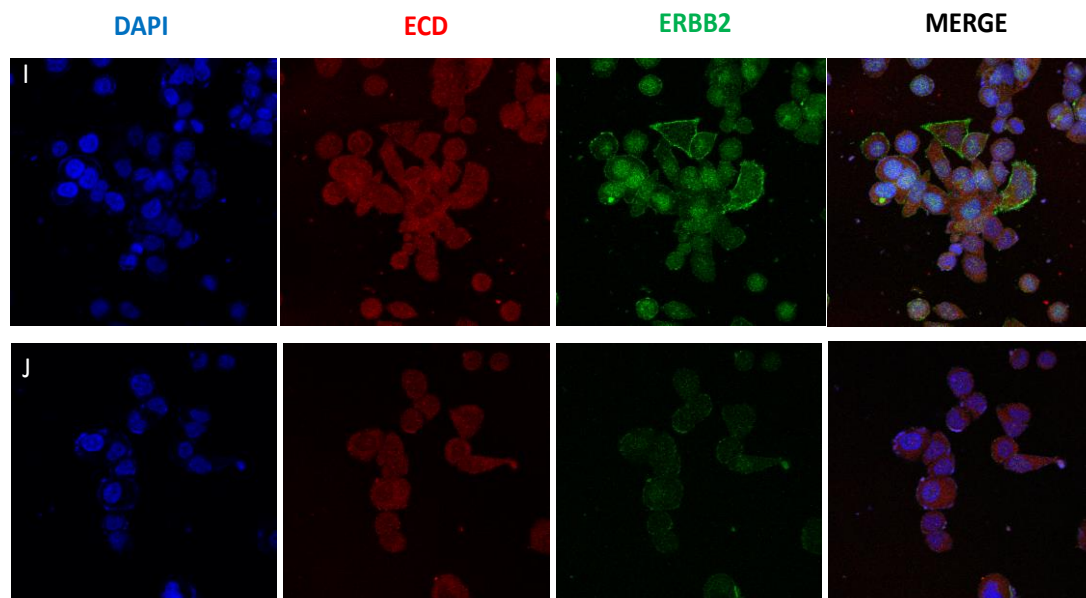
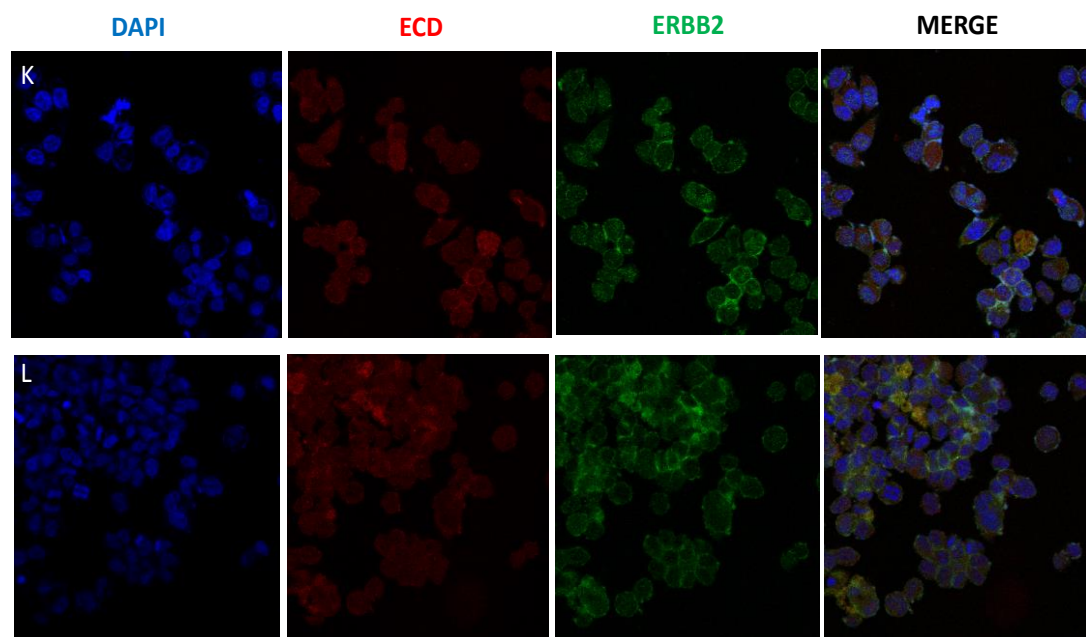


Figure 3.1: ECD and ERBB2 Localization in ERBB2+ Breast Cancer Cell Lines (continued)

**HCC 202****UACC 893**

**Figure 3.1: ECD and ERBB2 Localization in ERBB2+ Breast Cancer Cell Lines.** Different ERBB2+ breast cancer cell lines, SKBR3 (A-B), BT474 (C-D), UACC 812 (E-F), HCC 1419 (G-H), HCC 202 (I-J), and UACC 893 (K-L) were plated on cover slips were fixed in 4% paraformaldehyde for 20 minutes. The primary antibodies used were human anti-ERBB2 antibody, and anti-ECD antibody. Secondary antibodies used were Alexa fluor 488 and Alexa fluor 594 from Life Technologies. Nuclei were counterstained with DAPI. The cover slips were then placed on slides using the mounting medium. Fluorescent images were captured using LSM 510 META Confocal fluorescence microscope at 40X magnification (Zeiss).

Figure 3.2: ECD is overexpressed in overexpressed ERBB2 transfected cell lines.

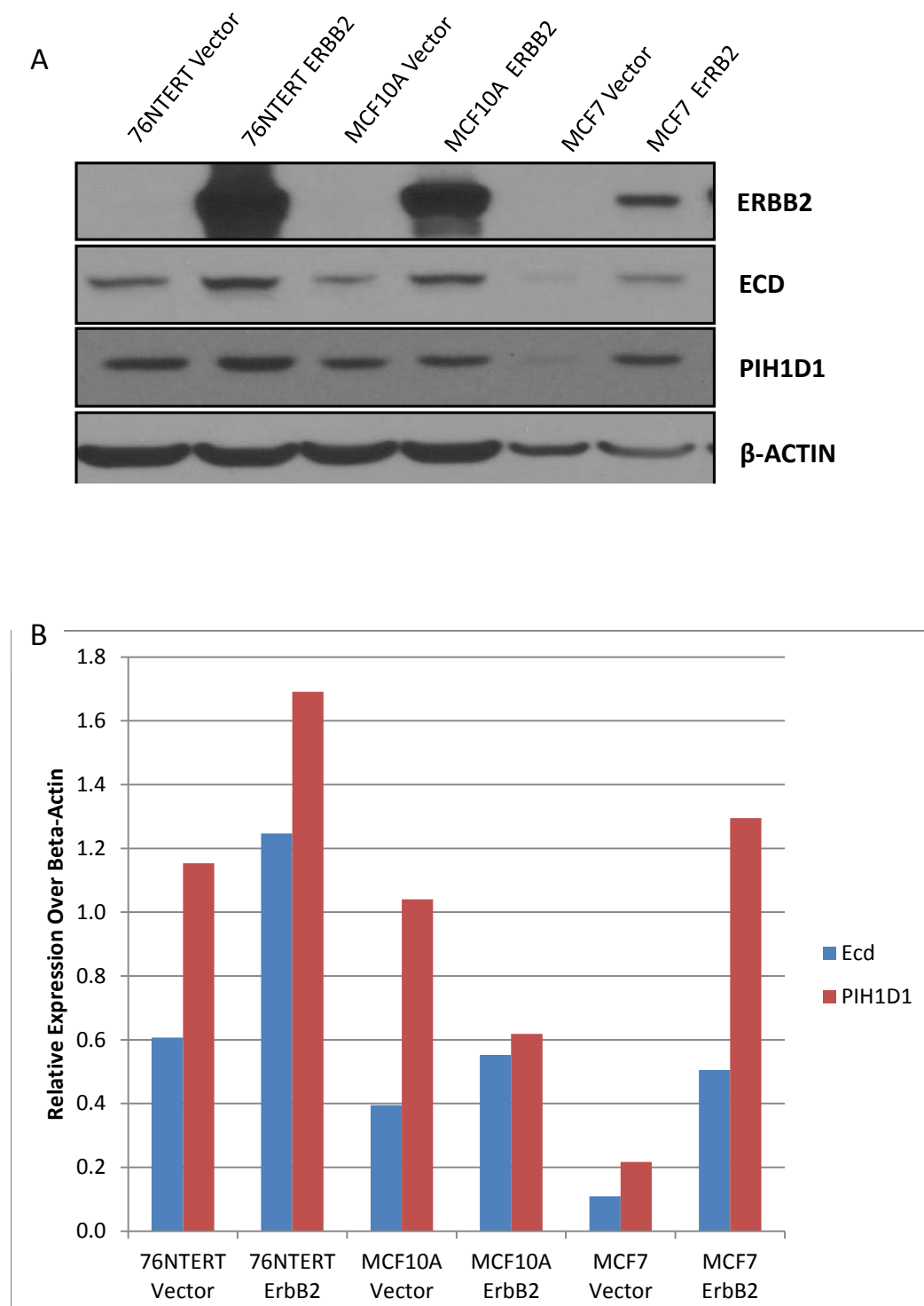


Figure 3.2: **ECD is overexpressed in overexpressed ERBB2 transfected cell lines.** A)

76NTERT, MCF10A, and MCF 7 cell lines were transfected with either vector plasmid or ErbB2 plasmid. Cell lines were treated with puromycin and were harvested using RIPA Buffer and ECD expression was determined by western blotting using anti-ECD, anti-ERBB2, anti-PIH1D1, and anti- $\beta$ -actin (as loading control). B) The graph below is the densitometry analysis of ECD levels as compared to  $\beta$ -ACTIN. Densitometry performed using ImageJ.

Figure 3.3: **ECD mRNA is unaffected in overexpressed ERBB2 transfected cell lines.**

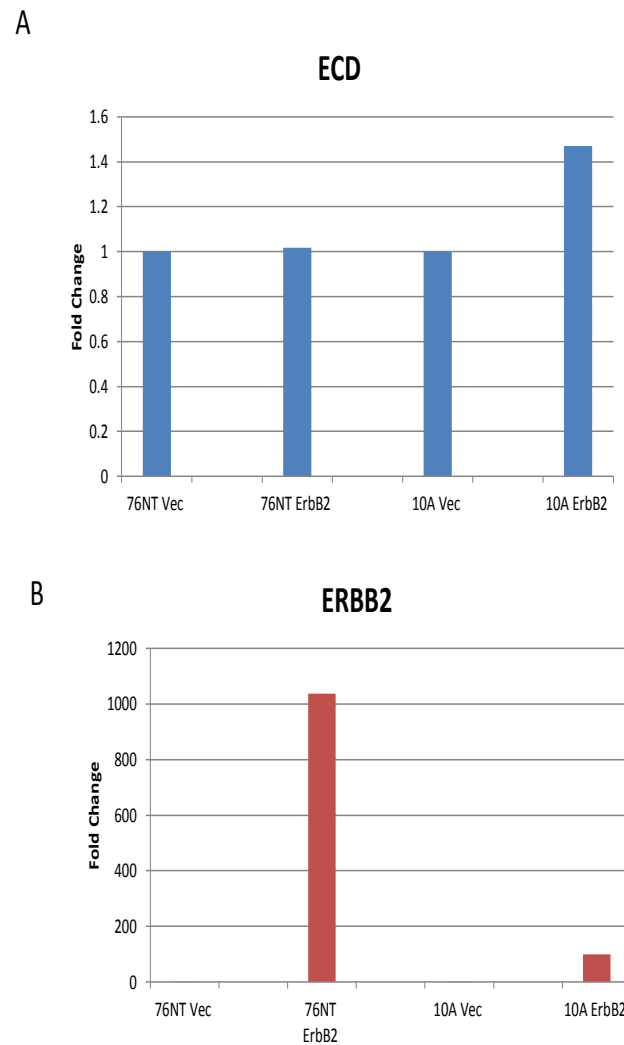


Figure 3.3: **ECD mRNA is unaffected in overexpressed ERBB2 transfected cell lines. A-B)**

76NTERT and MCF10A cell lines were transfected with either vector plasmid or ErbB2 plasmid and mRNA was harvested using Trizol. Real Time PCR analysis was performed and the data is represented in the bar graphs above for A) ECD or B) ERBB2.



stabilized in these cell lines and this stabilization in turn is helping in the stabilization of the ERBB2 receptor.

### **3.2 Potential role of ECD and the R2TP complex in ErbB2 signaling pathway.**

As a continuation of the work discussed in Chapter 2, we wanted to see how different chemotherapeutic agents targeting ErbB2 affected ECD and members of the HSP90/R2TP co-chaperone complex. The R2TP complex is comprised of four molecules: PIH1D1, RUVBL1, RUVBL2, and RPAP3 and the complex interact with the chaperone protein, HSP90. HSP90 is a chaperone protein that assist in the folding, maintenance, and assembly of many proteins and many protein complexes in cells (R. Zhao et al., 2005). Interestingly, we have shown that ECD and HSP90 interact with one another through immunoprecipitation and proximity ligation assay. We have also seen that ECD and HSP90 co-localize with one another in SKBR3 cells, an ERBB2 overexpressing breast cancer cell line (Figure 3.4). HSP90 has been shown to be overexpressed in breast cancer and it is an important chaperone that helps to stabilize ERBB2 at the plasma membrane, we wanted to see how ECD and HSP90 are interacting with the R2TP complex in ERBB2+ breast cancer cells.

The HSP90/ R2TP complex has been shown to be involved in the assembly and stabilization of a number of protein complexes such as RNA polymerase II assembly, PIKKs, box C/D small nucleolar ribonucleoprotein (snoRNP) biogenesis, telomere activity, apoptosis, and chromatin remodeling (Kakihara & Houry, 2012). Recent reports have shown overexpression of the R2TP components in different cancers, such as breast and colon(S. G. Kim et al., 2013). We then wanted to observe the levels of the R2TP complex and ECD in different breast cancer cell lines.

Figure 3.4: **ECD and HSP90 Co-localization in SKBR3 cells.**

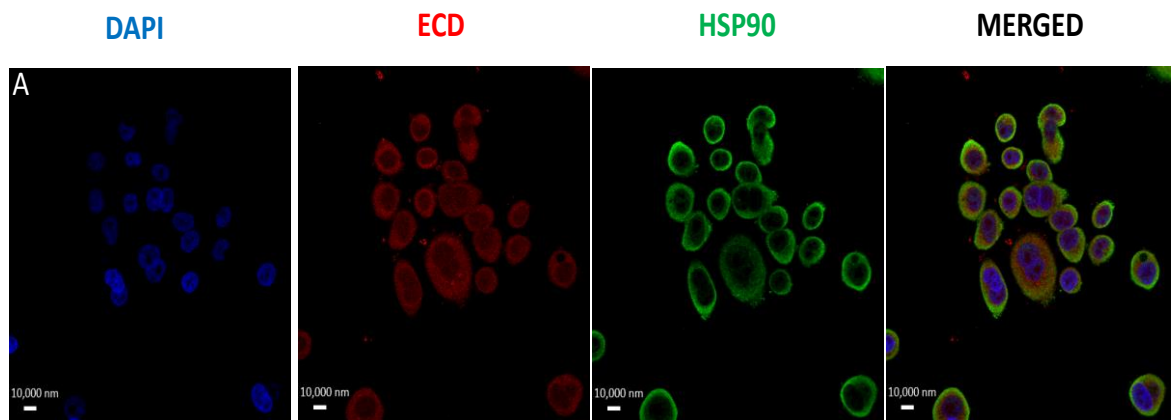
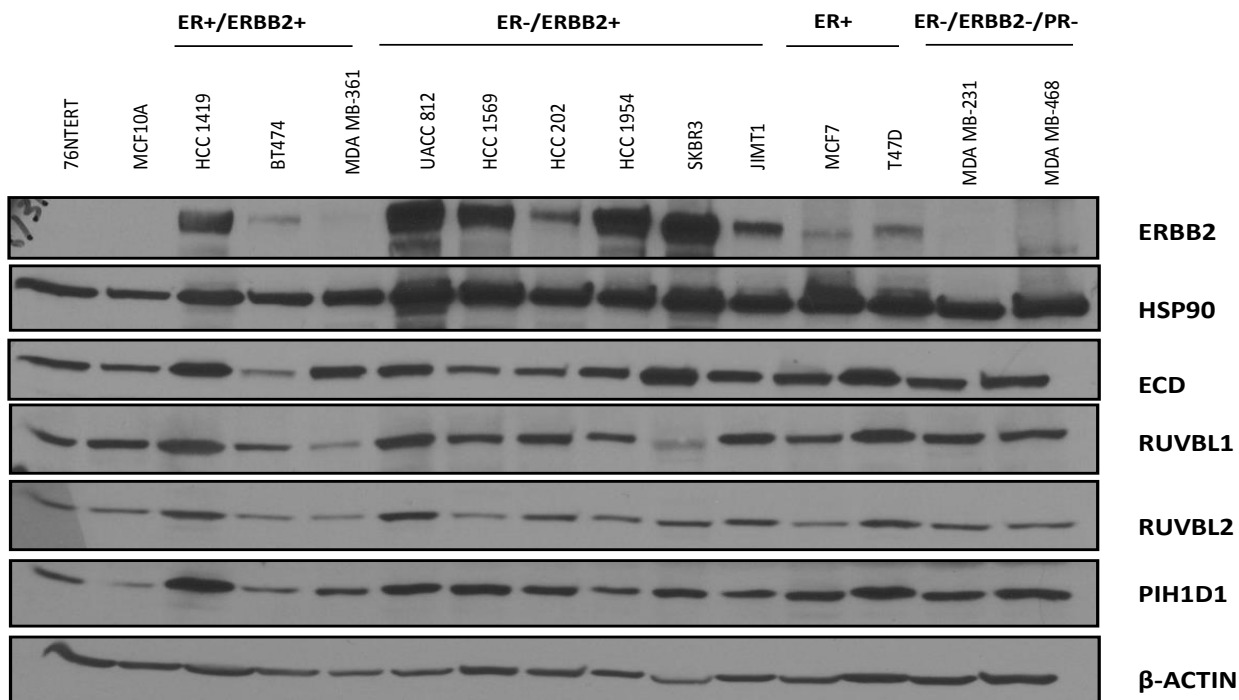


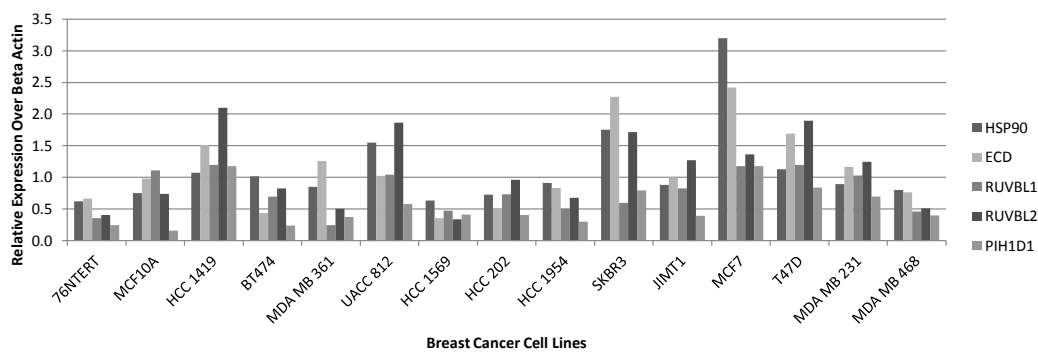
Figure 3.4: **ECD and HSP90 Co-localization in SKBR3 cells.** A) SKBR3 cells were plated on cover slips were fixed in 4% paraformaldehyde for 20 minutes. The primary antibodies used were human anti-HSP90 antibody, and anti-ECD antibody. Secondary antibodies used were Alexa fluor 488 and Alexa fluor 594 from Life Technologies. Nuclei were counterstained with DAPI. The cover slips were then placed on slides using the mounting medium. Fluorescent images were captured using LSM 510 META Confocal fluorescence microscope at 40X magnification (Zeiss).

Figure 3.5: ECD, HSP90, and members of the R2TP complex is overexpressed in various breast cancer cell lines.

A



B



**Figure 3.5: ECD, HSP90, and members of the R2TP complex is overexpressed in various breast**

**cancer cell lines.** A) Various breast cancer cell lines were harvested using RIPA Buffer and ECD

expression was determined by western blotting using anti-ECD, anti-ERBB2, anti-HSP90, anti-

PIH1D1, anti-RUBVL1, anti-RUVBL2, and anti- $\beta$ -ACTIN (as loading control). 76NTERT and MCF10A

serve as normal immortalized breast epithelial cell lines. Cell lines are separated via subtype. B)

The graph below is the densitometry analysis of ECD levels as compared to  $\beta$ -ACTIN.

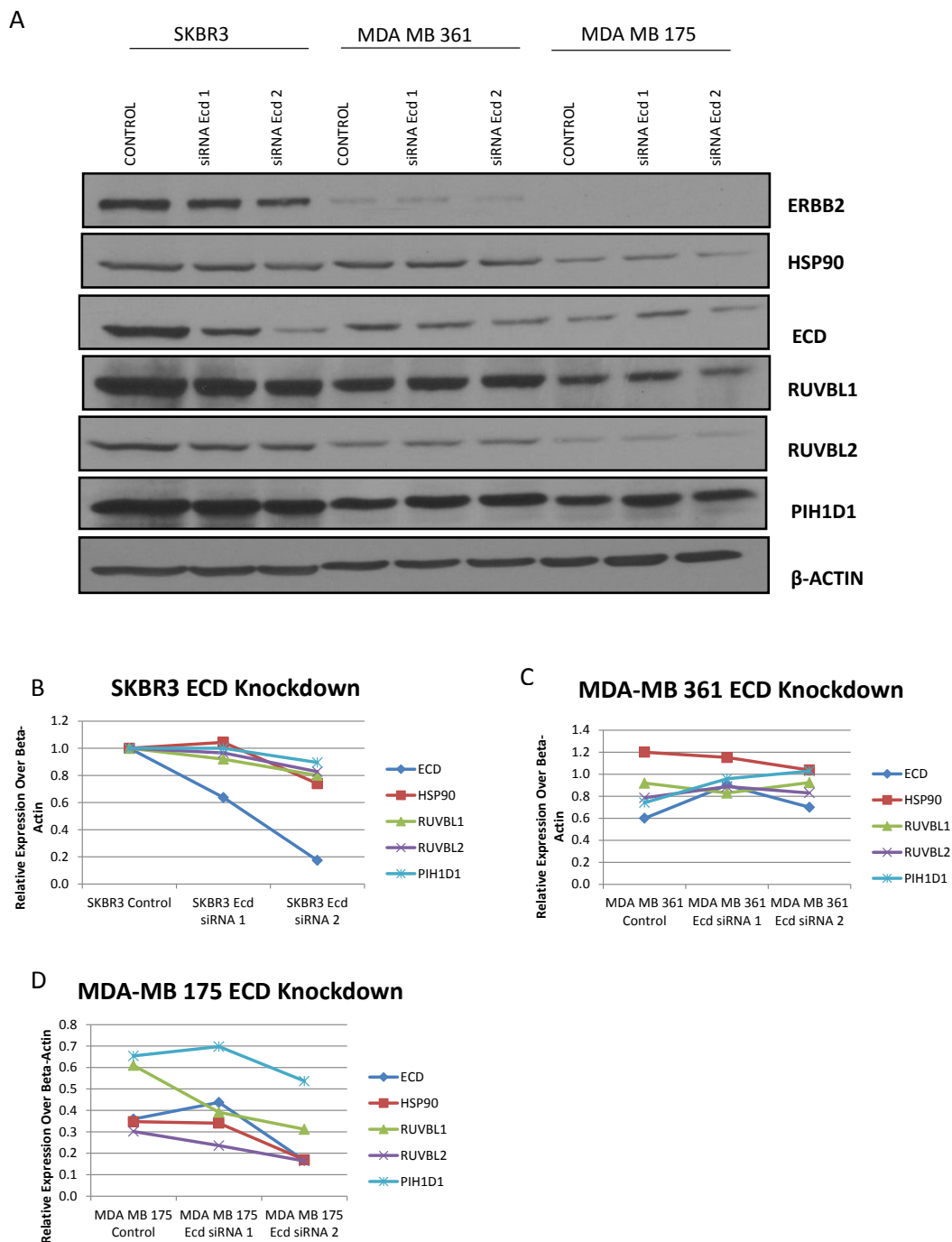
Densitometry performed using ImageJ.

As shown in Figure 3.5, ECD and different members of the R2TP are overexpressed in different breast cancer cell lines. There are some cell lines that have more expression of the R2TP components, and the overexpression seems to correlate with ECD overexpression. This observation needs to be explored further though to see if ECD overexpression correlates with R2TP complex members' overexpression in cancers.

There have been several speculations about the role of the HSP90/R2TP complex in oncogenesis. The HSP90/R2TP complex plays important role in several cellular functions such as proliferation, division, and survival. The complex is involved with the assembly of the human telomerase RNP complex, which is necessary for telomere elongation and cellular division (Boulon et al., 2008; Venteicher, Meng, Mason, Veenstra, & Artandi, 2008). The HSP90/R2TP complex is also involved in the stability of the mTOR kinase, which is involved in cellular survival. Over-activation of the mTOR kinase and upregulation of telomerase have been observed in a number of cancers and it is hypothesized that the HSP90/R2TP complex is helping to stabilize or assembly these complexes in cancer cells (Zoncu, Efeyan, & Sabatini, 2011). In Chapter 2, we observed that knockdown of ECD leads to downregulation of ERBB2 and downstream targets of ERBB2; we wanted to see if knockdown of ECD had any effect on the levels of HSP90 or the R2TP complex. We treated three breast cancer cell lines with siRNA scrambled control, siRNA ECD 1, or siRNA ECD 2 for 48 hours. We collected lysates and ran them on an SDS-PAGE gel. As shown in Figure 3.6, knock down of ECD does not seem to have any effect on levels of HSP90 or members of the R2TP complex.

Since knock down of ECD did not have any effect on levels of HSP90/R2TP complex, I wanted to observe if downregulation of ERBB2, either by downregulation of ERBB2 protein levels or inhibition of ERBB2 signaling pathway, had any effect on the complex. In order to

Figure 3.6: ECD siRNA mediated knockdown does not downregulate members HSP90/R2TP complex in various breast cancer cell lines.



**Figure 3.6: ECD siRNA mediated knockdown does not downregulate members HSP90/R2TP complex in various breast cancer cell lines.** A) Various breast cancer cell lines were transfected with siRNA scrambled control or siRNA ECD 1 or 2 and incubated for 48 hours. Cells were harvested using RIPA Buffer and ECD expression was determined by western blotting using anti-ECD, anti-ERBB2, anti-HSP90, anti-PIH1D1, anti-RUBVL1, anti-RUVBL2, and anti- $\beta$ -ACTIN (as loading control). 76NTERT and MCF10A serve as normal immortalized breast epithelial cell lines. Cell lines are separated via subtype. B-D) Quantitative density analysis of ECD, HSP90, RUVBL1, RUVBL2, and PIH1D1 normalized to  $\beta$ -ACTIN in SKBR3, MDA-MB 361, and MDA-MB 175 breast cancer cell lines where ECD was knockdown with two different siRNAs. Densitometry performed using ImageJ.

achieve this, we treated ERBB2+ cell lines with different chemotherapy agents that target ERBB2. The most common chemotherapy drug used in cancer patients with ERBB2 overexpression is Trastuzumab (Herceptin). Trastuzumab is a human monoclonal antibody that has two specific antigen sites that bind to the extracellular juxtamembrane of the ERBB2 receptor and prevents activation of the intracellular tyrosine kinase domain (Hudis, 2007). There are several mechanisms of action of Trastuzumab may utilize to decrease signaling. These mechanisms include decreasing ERBB2 receptor dimerization, activation of the immune system, increasing the degradation of the ERBB2 receptor by increasing the endocytic recycling pathway, and inhibiting the shedding of the extracellular domain (Hudis, 2007; Vu & Claret, 2012).

Trastuzumab has a high efficacy rate in breast cancer patients and it is the most common form of therapy in ERBB2+ breast cancers. However, increasing instances of acquired and primary resistance proves to be quite a challenge for clinicians. The mechanism of Trastuzumab resistance has not been fully elucidated. One potential mechanism of resistance is the disruption between Trastuzumab with ERBB2 leading to steric hindrance. MUC4 is a glycoprotein that has been shown to interact with ERBB2 and it is also overexpressed in breast cancers. MUC4 binding to ERBB2 does not allow Trastuzumab to interact with the receptor and shut down ERBB2 signaling (Nahta & Esteva, 2006). It is also hypothesized that overlapping signaling pathways such as SRC or PI3K/AKT pathway become overactive in patients with Trastuzumab resistance (Vu & Claret, 2012). Since Trastuzumab resistance is a growing concern for clinicians and scientists alike, there is a need for new biomolecules that can be used as potential therapy targets. Therefore, in order to explore the possibility of ECD becoming a new potential biomarker, we wanted to see how levels of ECD and the HSP90/R2TP complex members are affected when cells are treated with Trastuzumab. ERBB2+ cell lines, SKBR3 and BT474, were treated with different dosages of Trastuzumab for 72 hours. After 72 hours, lysates



were collected and run on an SDS-page gel. As shown in Figure 3.7, Trastuzumab causes down regulation of phosphorylated ERBB2 and total ERBB2, which has previously been shown. Interestingly, Trastuzumab also causes the down regulation of ECD and the four components of the R2TP complex. This indicates that downregulation of ERBB2 leads to the destabilization of ECD and the R2TP complex. In conjunction, we treated two Trastuzumab resistant cell lines, SKBR3 TR and BT474 TR, with different dosages of Trastuzumab for 72 hours. In Figure 3.8 we observed that Trastuzumab had no effect on ECD levels or levels of the four R2TP components. These results indicate that abrogation of ERBB2 signaling and levels leads to the destabilization of the R2TP complex and ECD. Since the R2TP complex is emerging as an important co-chaperone scaffolding complex in cells and in cancers, it is important to understand how the complex is regulated in oncogenesis. In the future, ECD and the R2TP complex may potentially be used as new chemotherapy targets. By combining Trastuzumab and inhibitors to ECD and the R2TP complex may help increase the efficacy of Trastuzumab and become new treatments for patients with Trastuzumab resistance.

In order to determine if downregulation of ECD was a cause of ERBB2 signaling abrogation or downregulation of ERBB2 protein levels, we decided to use another chemotherapy agent that targets the ERBB2 signaling pathway. Lapatinib (GW572016) is a chemotherapy agent that targets the intracellular tyrosine kinase domain of Epidermal Growth Factor Receptor 1 (EGFR) and ERBB2. It is a dual kinase inhibitor that binds to the tyrosine kinase domain of these receptors and inhibits phosphorylation and down regulates their signaling pathways (Wood et al., 2004). Research has shown that combination of Trastuzumab and Lapatinib treatment to patients with ERBB2 overexpressing subtype of breast cancer, improved rates of pathological response as compared to single agent treatments (de Azambuja et al., 2014). Lapatinib however, has some cytotoxic effects on patients and some patients acquire resistance, thus

Figure 3.7: ERBB2+ cell lines treated with Trastuzumab causes downregulation of ECD and members of the R2TP complex.

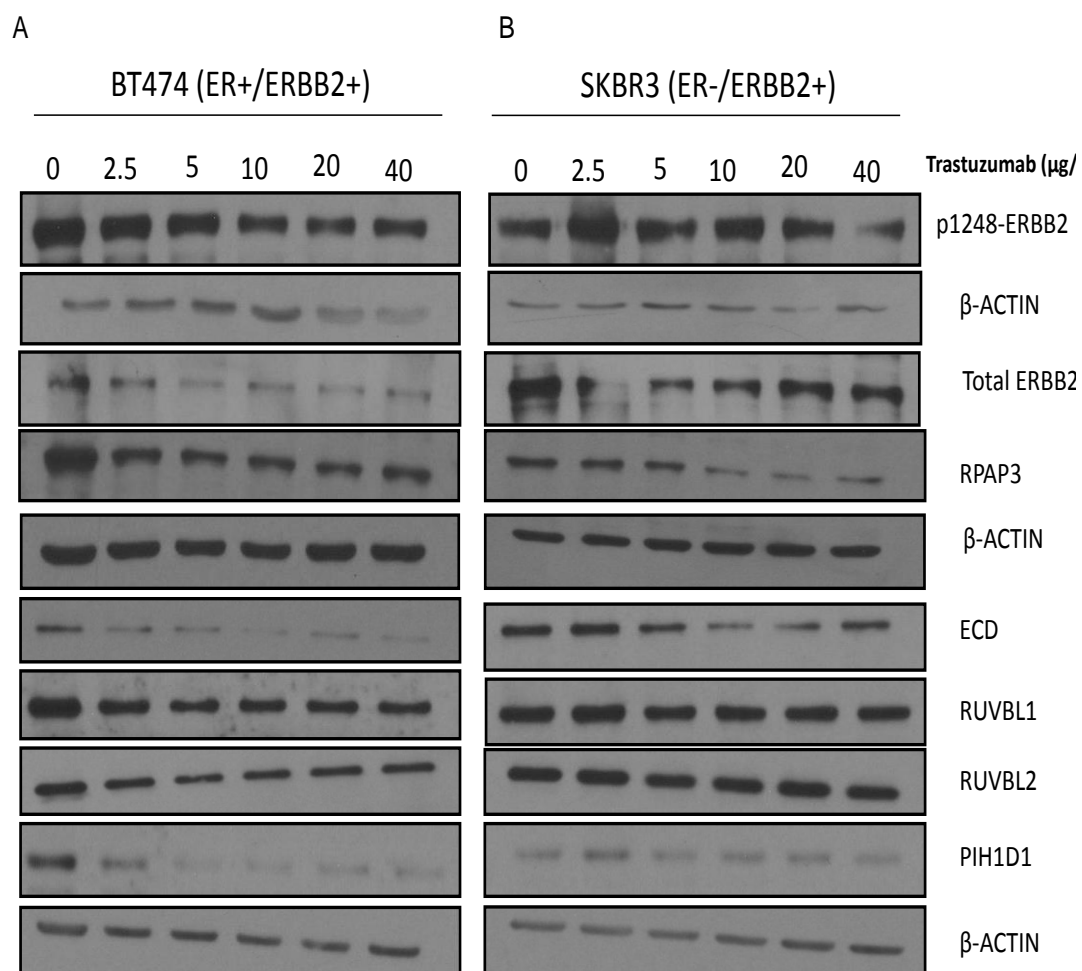
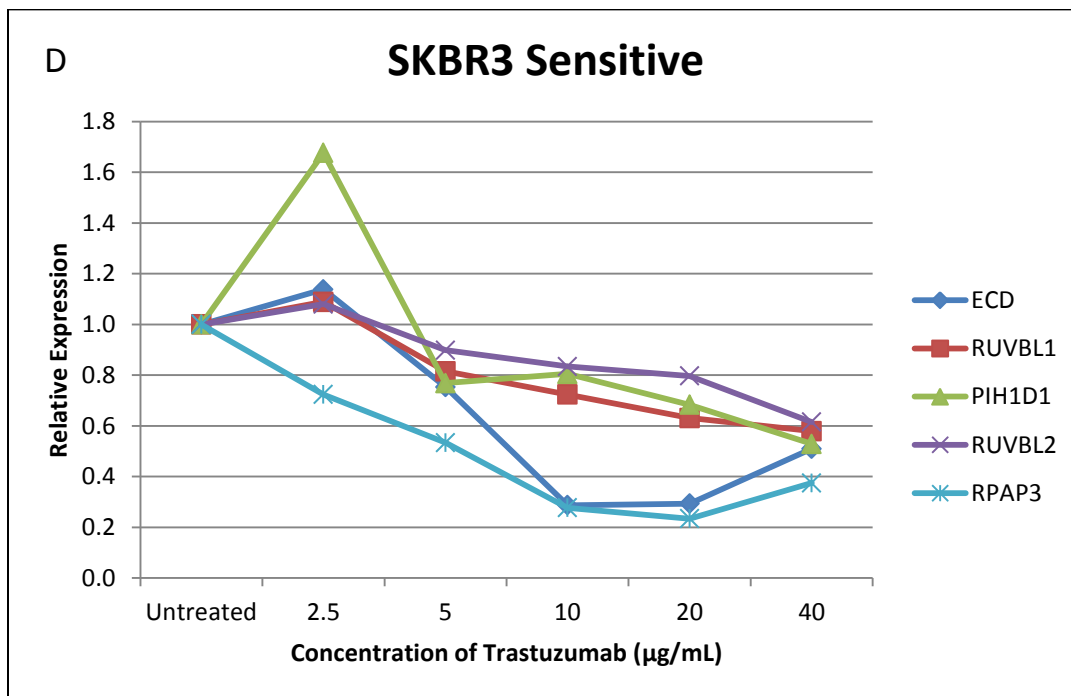
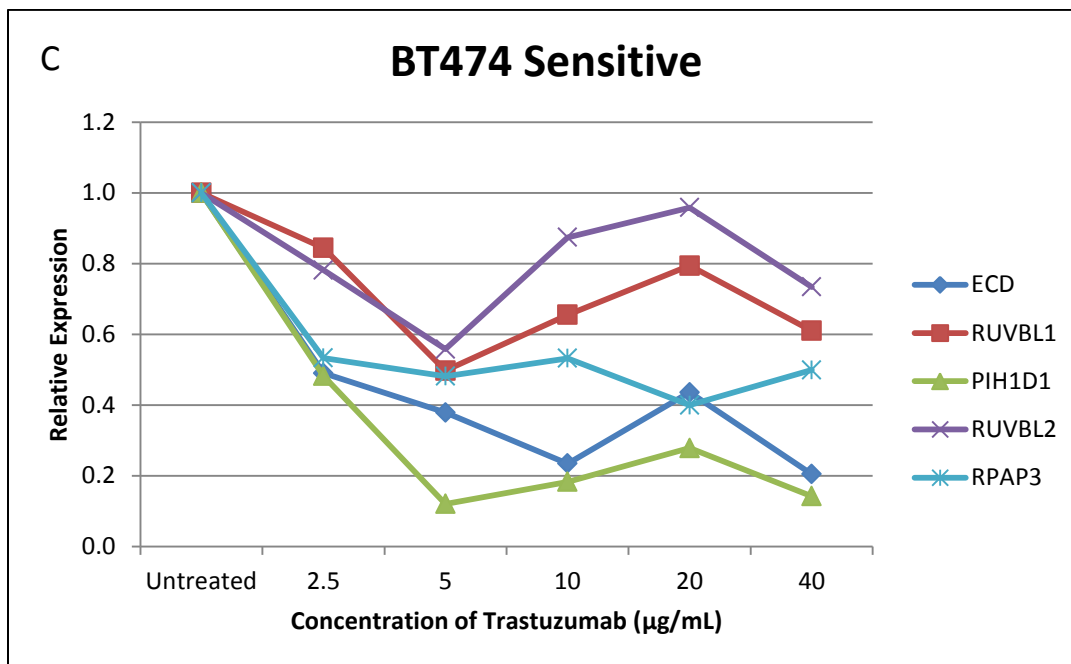


Figure 3.7: ERBB2+ cell lines treated with Trastuzumab causes downregulation of ECD and members of the R2TP complex (continued).



**Figure 3.7: ERBB2+ cell lines treated with Trastuzumab causes downregulation of ECD and members of the R2TP complex.** A-B) BT474 and SKBR3 cells were treated with different dosages of Trastuzumab (Herceptin) in  $\mu\text{g}/\text{mL}$  and lysates were collected after 72 hours of treatment. Cells were harvested using RIPA Buffer and ECD expression was determined by western blotting using anti-ECD, anti-ERBB2, anti-PIH1D1, anti-RUBVL1, anti-RUBVL2, anti-RPAP3, and anti- $\beta$ -ACTIN (as loading control). C-D) Quantitative density analysis of ECD, RUBVL1, RUBVL2, and PIH1D1 normalized to  $\beta$ -ACTIN in BT474 and SKBR3 breast cancer cell lines. Densitometry performed using ImageJ.

Figure 3.8: ERBB2+ Trastuzumab resistant cell lines treated with Trastuzumab does not change ECD and members of the R2TP complex levels.

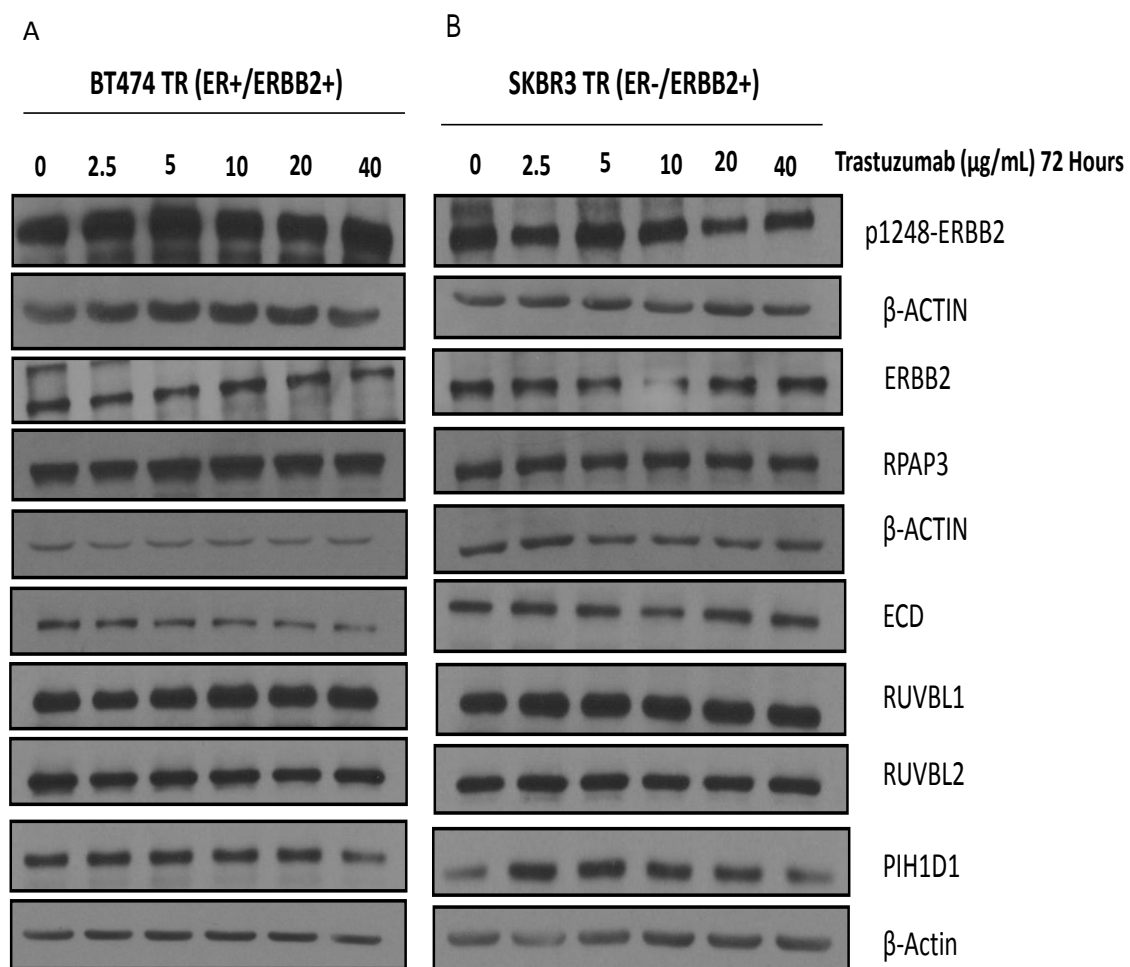
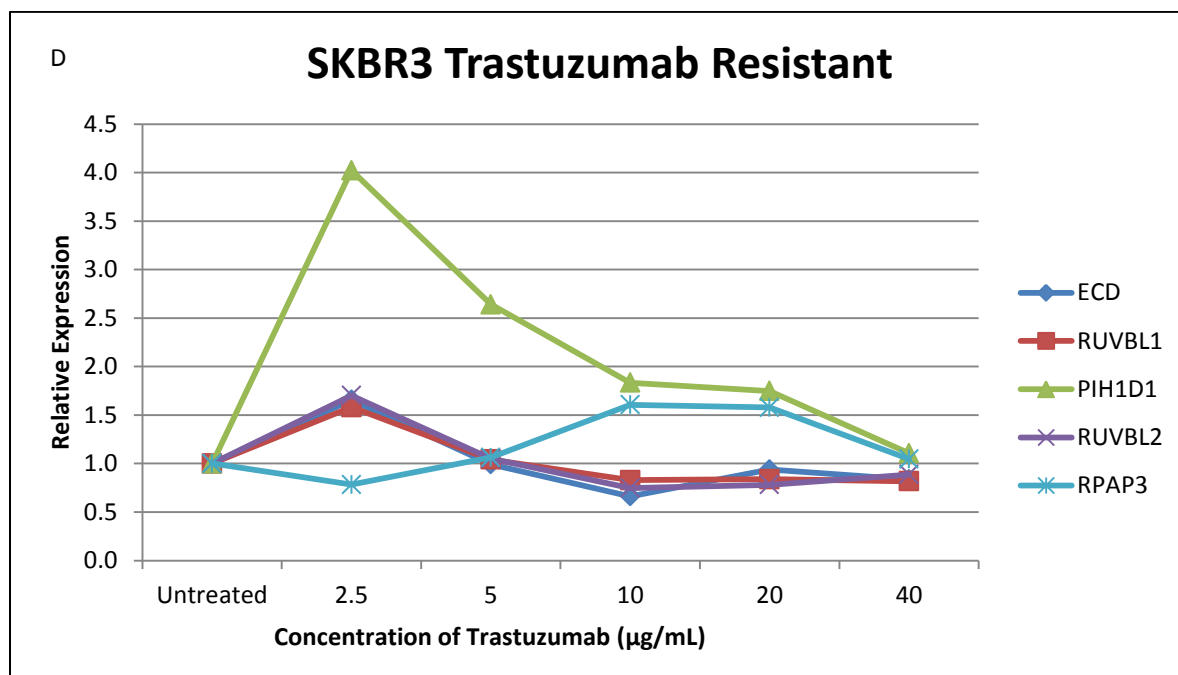
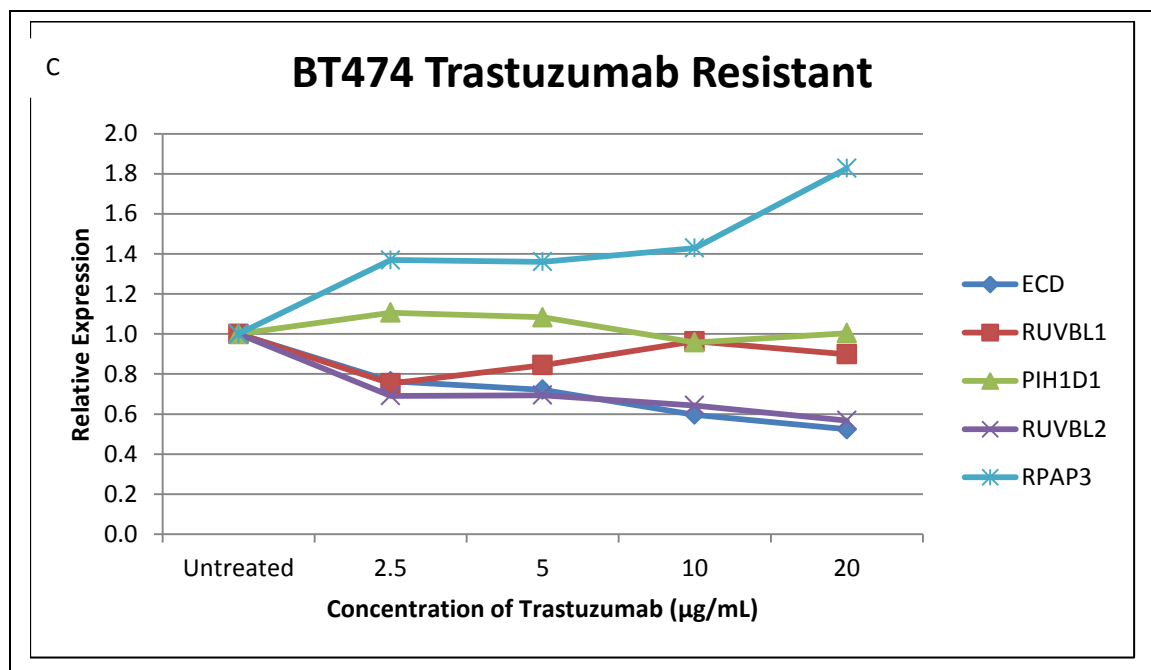


Figure 3.8: ERBB2+ Trastuzumab resistant cell lines treated with Trastuzumab does not change ECD and members of the R2TP complex levels (continued).



**Figure 3.8: ERBB2+ Trastuzumab resistant cell lines treated with Trastuzumab does not change ECD and members of the R2TP complex levels.** A-B) BT474 and SKBR3 Trastuzumab resistant cells were treated with different dosages of Trastuzumab (Herceptin) in  $\mu\text{g}/\text{mL}$  and lysates were collected after 72 hours of treatment. Cells were harvested using RIPA Buffer and ECD expression was determined by western blotting using anti-ECD, anti-ERBB2, anti-PIH1D1, anti-RUBVL1, anti-RUVBL2, anti-RPAP3, and anti- $\beta$ -ACTIN (as loading control). C-D) Quantitative density analysis of ECD, RUVBL1, RUVBL2, RPAP3, and PIH1D1 normalized to  $\beta$ -ACTIN in BT474 and SKBR3 breast cancer cell lines. Densitometry performed using ImageJ

indicating the need other molecular targets (D'Amato et al., 2015). We treated SKBR3 and BT474 cells with Lapatinib and collected lysates at different time points, and observed the effect of the chemotherapeutic agent on ECD levels and levels of different R2TP components. As shown in Figure 3.9, Lapatinib strongly downregulates the phosphorylation of ERBB2, which we used as a positive control. Levels of ECD and levels of different R2TP components were down regulated upon Lapatinib treatment in both ERBB2+ cell lines. Since Lapatinib is a dual kinase inhibitor that affects EGFR as well as ERBB2, we wanted to determine if the downregulation was specific to ERBB2 signaling. Therefore, we treated MCF7 cells which are Estrogen Receptor positive breast cancer cells that have a basal level of ERBB2 and EGFR and MDA MB 231 cells, which is a triple negative (ER-/PR-/HER2-) EGFR+ breast cancer cell line. Interestingly, upon treatment of Lapatinib in these cell lines did not alter the levels of ECD or members of the R2TP complex (Figure 3.10). These results indicate that deregulation of ECD and the R2TP complex is specifically tied to the downregulation of the ERBB2 receptor.

After observing these results, we wanted to see how downregulation of protein levels of ERBB2 would affect ECD and members of the R2TP components. We therefore treated cells with a chemotherapeutic agent that targets HSP90 and leads to ERBB2 degradation. The Hsp90 inhibitor, 17-AAG has been shown to cause the rapid degradation of ErbB2 (Maloney & Workman, 2002; Pick et al., 2007; Taipale et al., 2010; Whitesell & Lindquist, 2005; Workman et al., 2007). 17-AAG, which is a derivative of Geldanamycin, is 17-AAG is a small molecule inhibitor that binds to the ATPase activity domain of the Hsp90 dimeric protein, inhibiting its ATP activity and prevents its interaction with client proteins (Workman et al., 2007). This inhibitor has a certain sensitivity to mature ErbB2 receptors that is not seen in other Epidermal Growth Factor Receptor family members. Even though 17-AAG has been shown to be more promising than its predecessor, some clinicians fear the potential of



FIGURE 3.9: ERBB2+ cell lines treated with Lapatinib causes downregulation of ECD and members of the R2TP complex

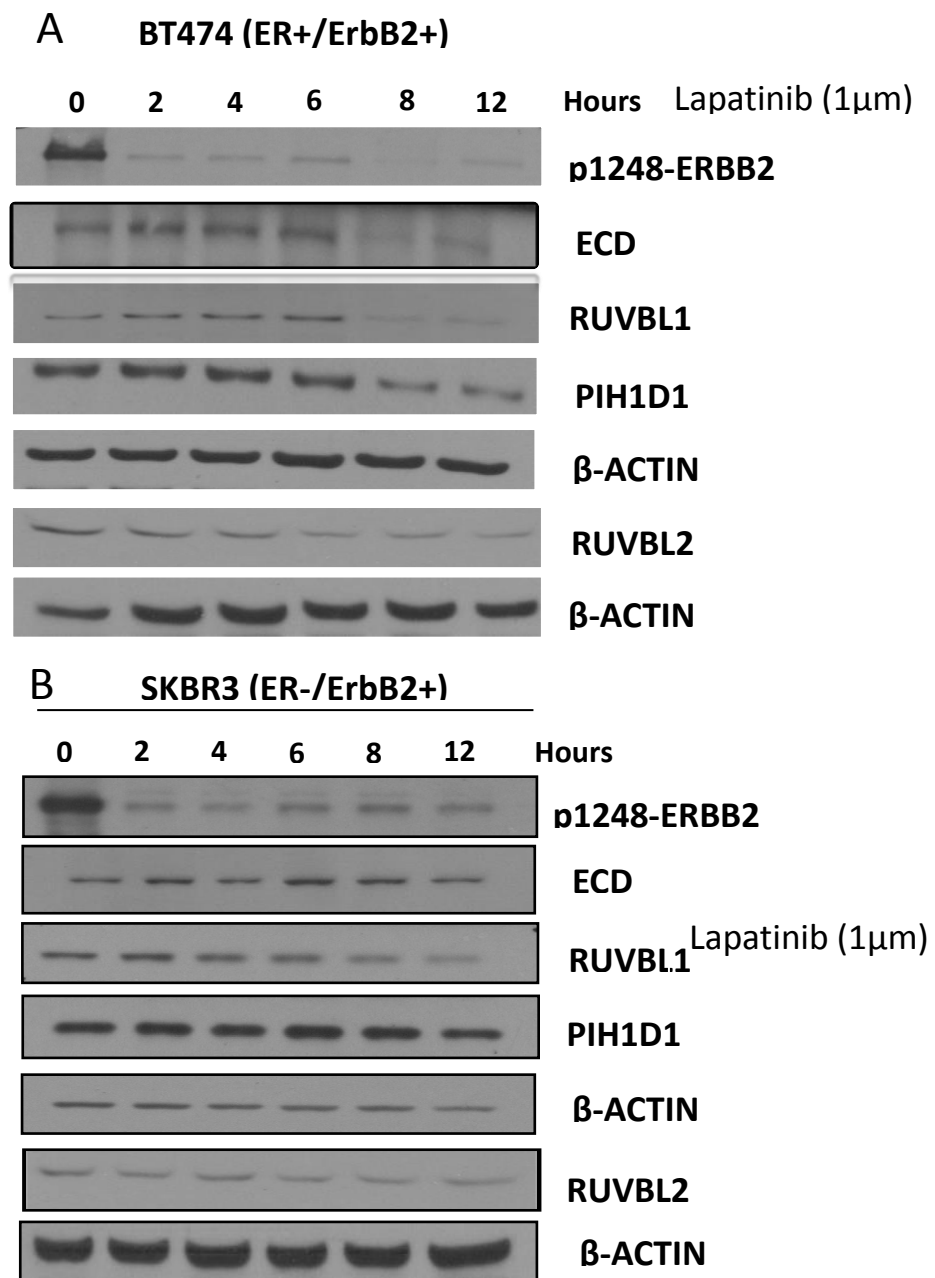
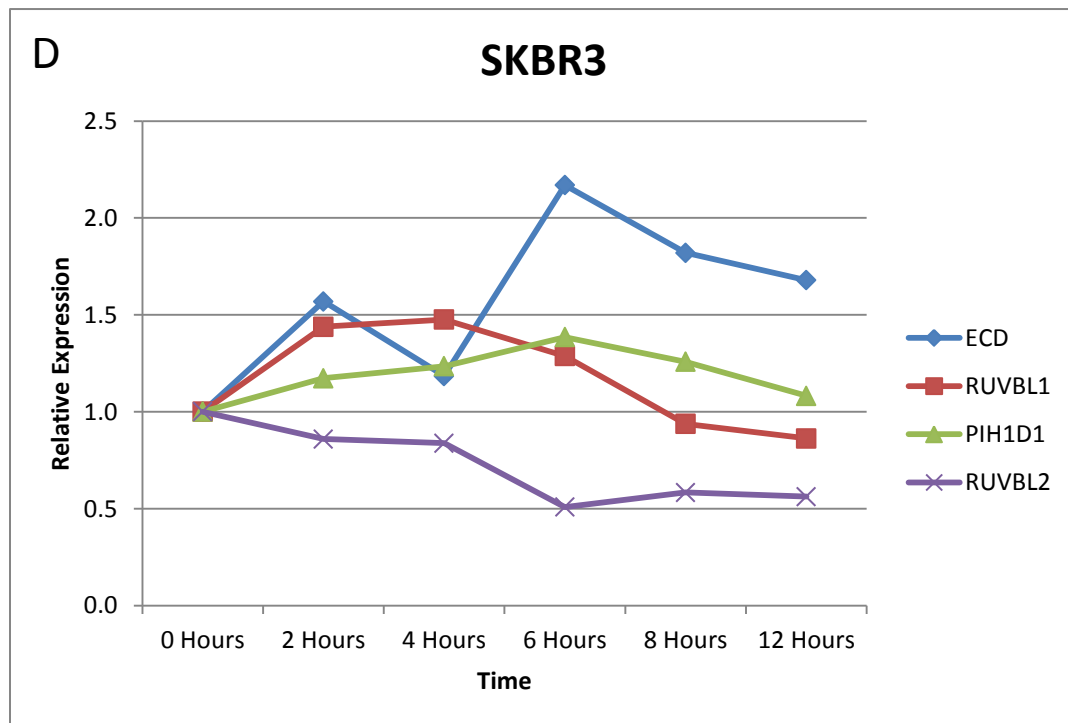
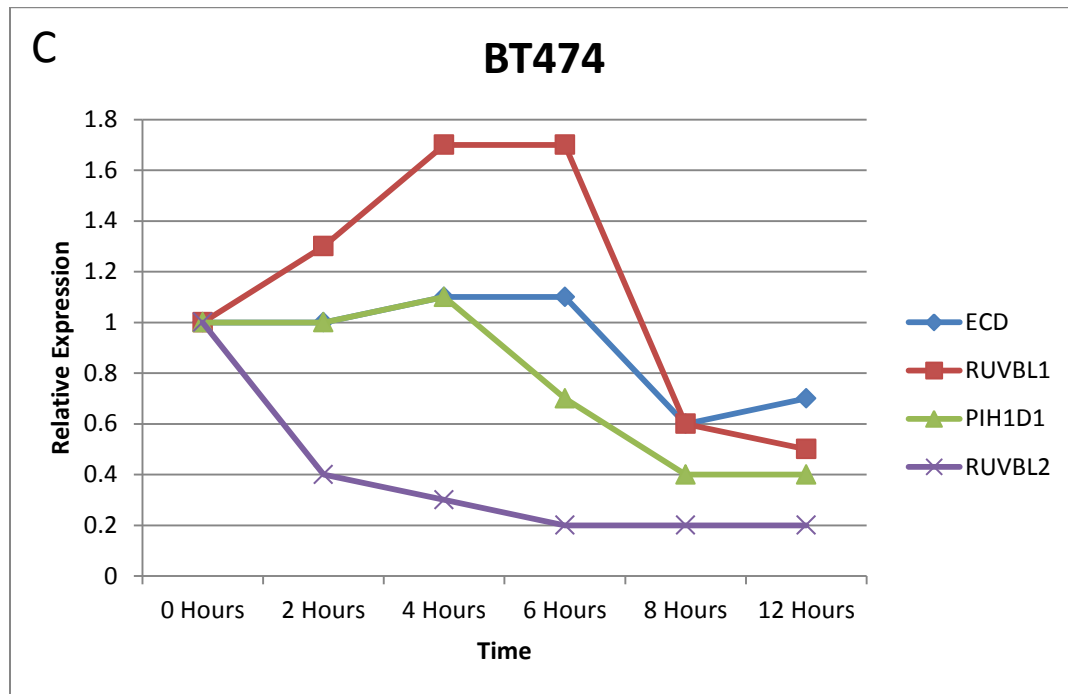
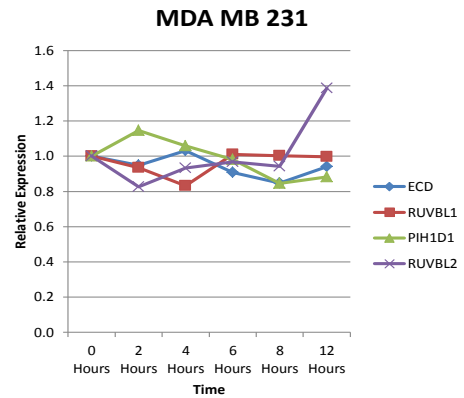
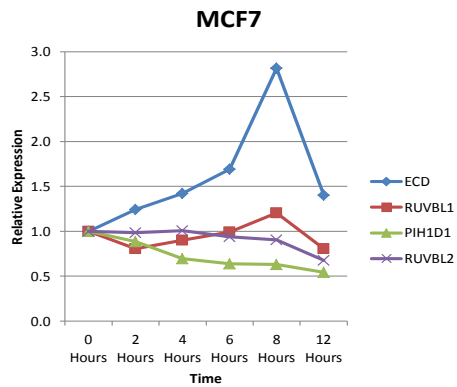
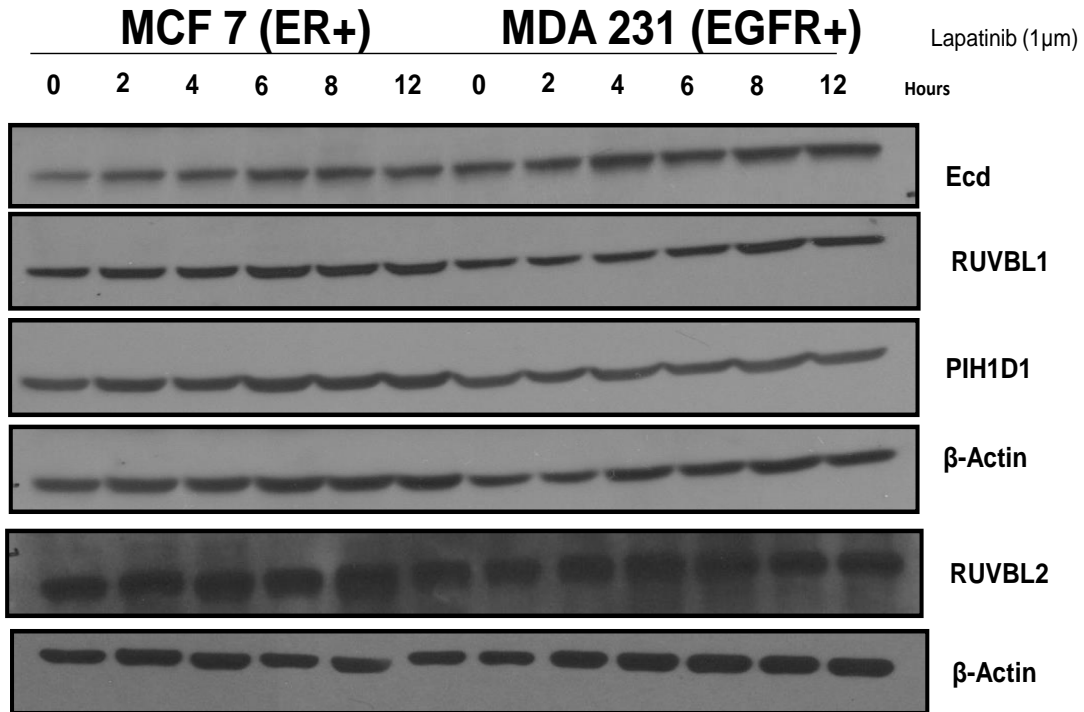


FIGURE 3.9: ERBB2+ cell lines treated with Lapatinib causes downregulation of ECD and members of the R2TP complex (continued)



**FIGURE 3.9: ERBB2+ cell lines treated with Lapatinib causes downregulation of ECD and members of the R2TP complex** A-B) BT474 and SKBR3 cells were treated with 1 $\mu$ m of Lapatinib and lysates were collected at the specified time points. Cells were harvested using RIPA Buffer and ECD expression was determined by western blotting using anti-ECD, anti-phospho-ERBB2, anti-PIH1D1, anti-RUBVL1, anti-RUVBL2, and anti- $\beta$ -ACTIN (as loading control). C-D) Quantitative density analysis of ECD, RUVBL1, RUVBL2, and PIH1D1 normalized to  $\beta$ -ACTIN in BT474 and SKBR3 breast cancer cell lines. Densitometry performed using ImageJ.

Figure 3.10: MCF7 and MDA MB 231 cell lines treated with Lapatinib causes no downregulation of ECD and members of the R2TP complex



**FIGURE 3.10 MCF7 and MDA MB 231 cell lines treated with Lapatinib causes no downregulation of ECD and members of the R2TP complex** A) MCF7 (ER+) and MDA-MB-231 (ER-/ERBB2-/PR-) breast cancer cells were treated with 1 $\mu$ m of Lapatinib and lysates were collected at the specified time points. Cells were harvested using RIPA Buffer and ECD expression was determined by western blotting using anti-ECD, anti-PIH1D1, anti-RUBVL1, anti-RUVBL2, and anti- $\beta$ -ACTIN (as loading control). B) Quantitative density analysis of ECD, RUVBL1, RUVBL2, and PIH1D1 normalized to  $\beta$ -ACTIN in BT474 and SKBR3 breast cancer cell lines. Densitometry performed using ImageJ.

hepatotoxicity (Workman et al., 2007). Geldanamycin proved to be highly toxic and caused hepatotoxicity, and although 17-AAG has been shown to be less toxic (Whitesell & Lindquist, 2005; Workman et al., 2007), there is still a possibility that this drug will not be suitable for some patients. We therefore wanted to see how 17-AAG affects ECD levels in ERBB2+ cell lines. I took BT474 and MDA 361 cells and treated them with different dosages of 17-AAG. As shown in Figure 3.11, ECD levels are decreased upon introduction of 17-AAG in both breast cancer cell lines. To reconfirm this result, I performed a time dependent experiment on BT474 and blotted the western with PIH1D1, which is a member of the R2TP complex. I observed a gradual down regulation of ECD and PIH1D1 in this experiment (Figure 3.12). I also performed another time dependent experiment on BT474 and a dose dependent experiment on 21MT2 cells, which is another ERBB2+ breast cancer cell line. I observed down regulation of ECD, ERBB2, as well as down regulation of RUVBL1, another member of the R2TP complex (Figure 3.13). However, HSP90 levels were unchanged in 21MT2 cells. In conclusion, 17-AAG treatment leads to downregulation of ECD and two components of the R2TP complex. This downregulation seems to be caused by the downregulation of ERBB2 levels in ERBB2+ breast cancer cell lines. This indicates that ECD and the R2TP complex become destabilized when ERBB2 is downregulated indicating that this complex may be acting downstream of this receptor. By understanding how ECD and the R2TP complex is stabilized *In vitro* could eventually help design small molecule inhibitors that target these protein and destabilize them in ERBB2+ breast cancers and in chemotherapy resistant tumors.

Figure 3.11: ERBB2+ cell lines treated with 17-AAG leads to downregulation of ECD.

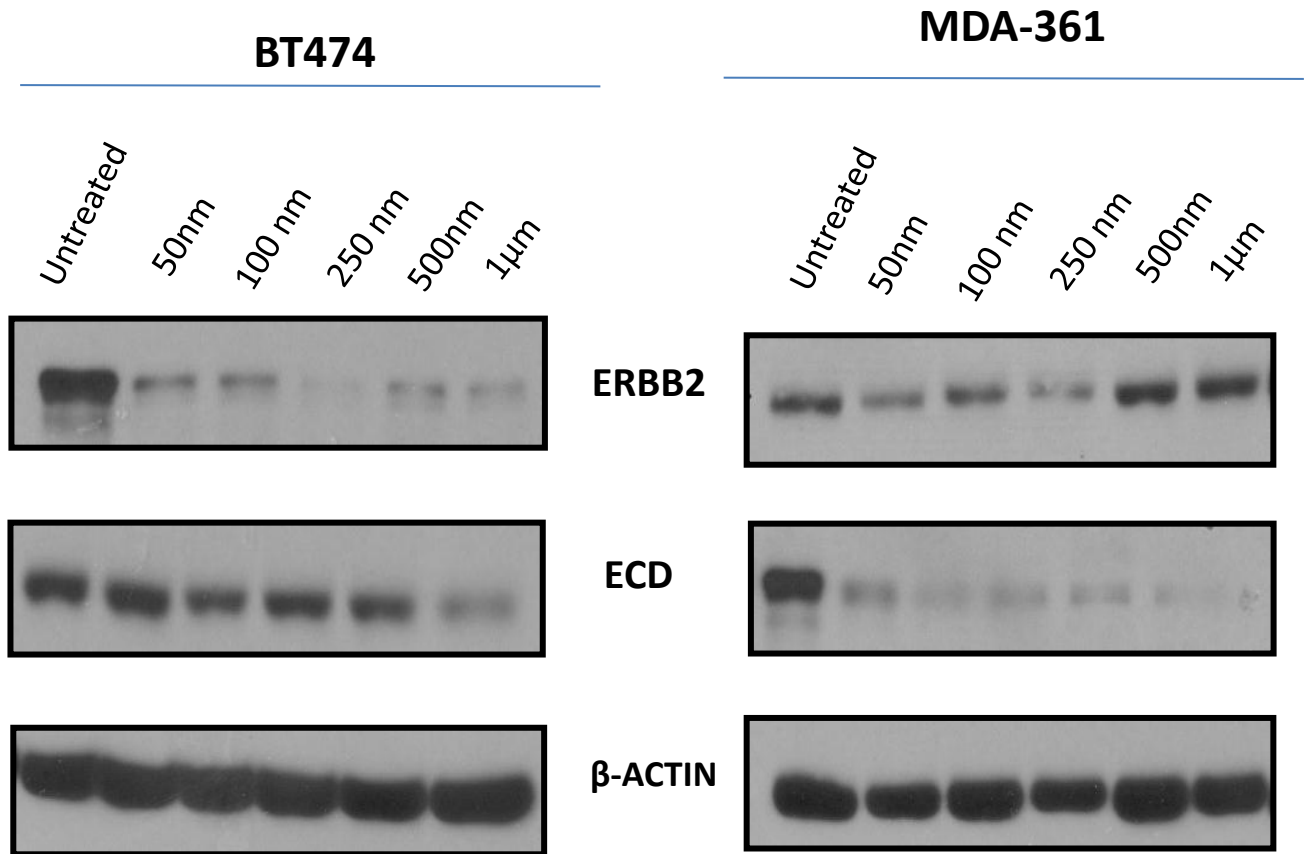


Figure 3.11: ERBB2+ cell lines treated with 17-AAG leads to downregulation of ECD. A-B) BT474 and MDA 361 cells were treated with different dosages of 17-AAG and lysates were collected after 9 hours of treatment. Cells were harvested using RIPA Buffer and ECD expression was determined by western blotting using anti-ECD, anti-ERBB2, and anti- $\beta$ -ACTIN (as loading control).

Figure 3.12: BT474 ERBB2+ breast cancer cell line treated with 17-AAG lead to downregulation of ECD and members of the R2TP complex

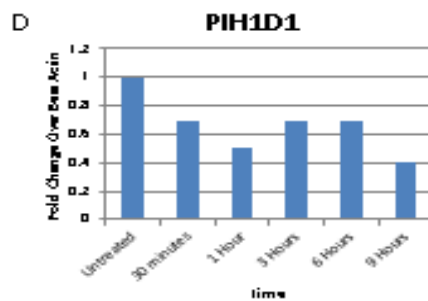
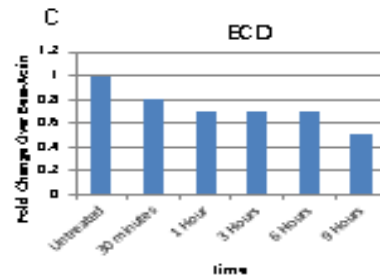
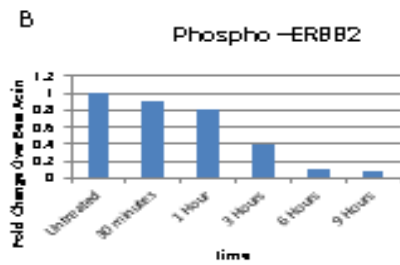
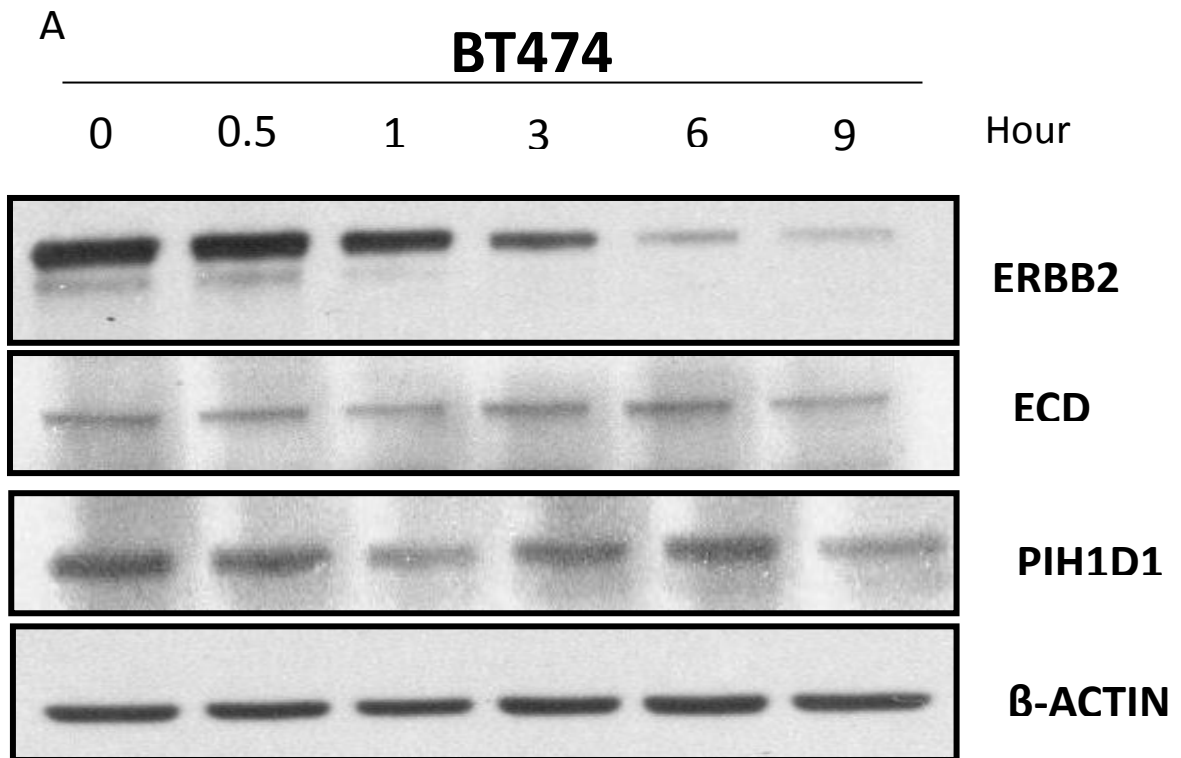




Figure 3.12: **BT474 ERBB2+ breast cancer cell line treated with 17-AAG leads to downregulation of ECD and members of the R2TP complex.** A) BT474 were treated with 100nm of 17-AAG and lysates were collected after 9 hours of treatment. Cells were harvested using RIPA Buffer and ECD expression was determined by western blotting using anti-ECD, anti-ERBB2, anti-PIH1D1, and anti- $\beta$ -ACTIN (as loading control). B-D) Quantitative density analysis of B) phospho-ERBB2, C) ECD, and D) PIH1D1 was normalized to  $\beta$ -ACTIN. Densitometry performed using ImageJ.

Figure 3.13: ERBB2+ cell lines treated with 17-AAG leads to downregulation of ECD and members of the R2TP complex

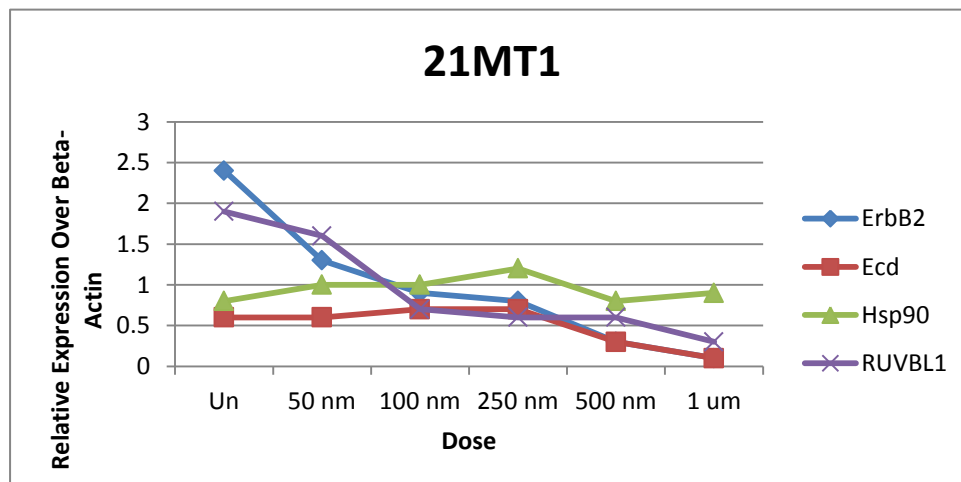
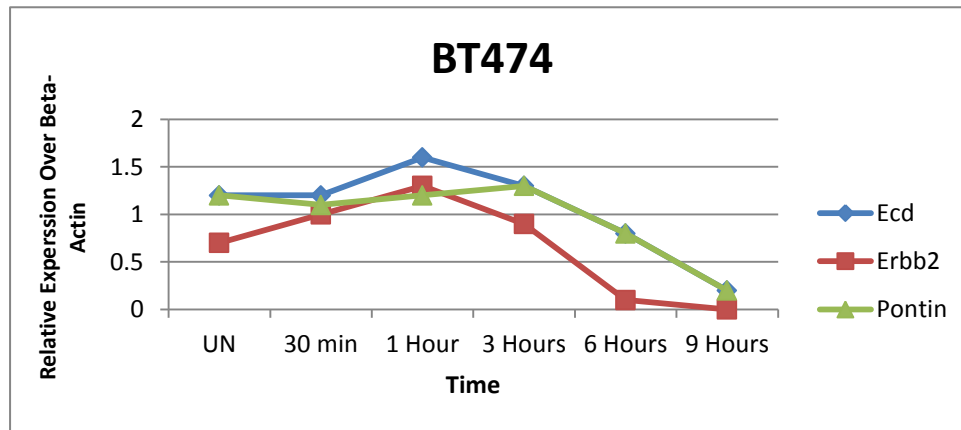
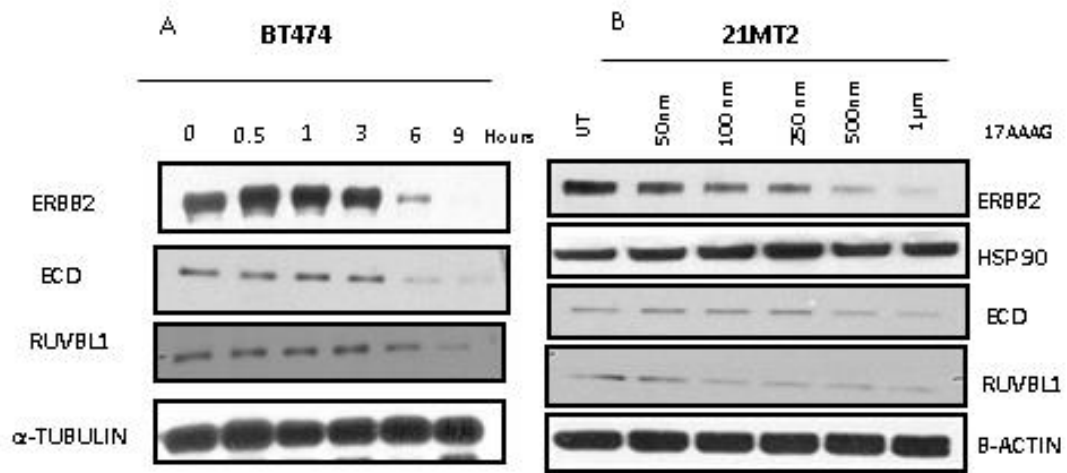


Figure 3.13: **ERBB2+ cell lines treated with 17-AAG leads to downregulation of ECD and members of the R2TP complex.** A) BT474 were treated with 100nm of 17-AAG and lysates were collected after 9 hours of treatment. B) 21MT2 cells were treated with different dosages of 17-AAG and lysates were collected after 9 hours of treatment. Cells were harvested using RIPA Buffer and ECD expression was determined by western blotting using anti-ECD, anti-ERBB2, anti-HSP90, anti-RUBVL1, and anti- $\beta$ -ACTIN (as loading control). C-D) Quantitative density analysis of ECD, RUVBL1, and HSP90 was normalized to  $\beta$ -ACTIN. Densitometry performed using ImageJ.

### 3.3 Role of ECD in downstream ErbB2 Signaling Pathway

In Chapter 2, we observed that siRNA mediated knock down of ECD caused down regulation of downstream components of ERBB2 signaling pathway. In order to assess the role ECD may be playing in different signaling pathways, we used a panel of inhibitors against specific kinases and observed if ECD levels were altered. We first looked at the effect Rapamycin, an mTOR inhibitor in ERBB2+ breast cancer cell lines. Mammalian target of rapamycin (mTOR) is a member of the phosphoinositide-kinase-related kinase (PIKK) family, and is a master regulator of cellular growth and proliferation (Bjornsti & Houghton, 2004; Hay & Sonenberg, 2004). Two downstream effectors of mTOR that regulates protein synthesis by phosphorylation and inactivation of the repressor of mRNA translation, eukaryotic initiation factor 4E-binding protein (4E-BP1), and through the phosphorylation and activation of S6 kinase (S6K1) (Hay & Sonenberg, 2004). The mTOR kinase also responds to environmental stimuli and nutrient conditions which help the kinase to regulate several cellular processes such as protein turnover, transcription, actin cytoskeleton organization, repress autophagy, and initiate translation (Bjornsti & Houghton, 2004). mTOR signaling is regulated by growth factors, amino acids, ATP and oxygen levels, and possibly by mitochondrial stress. Two of the upstream effectors of mTOR are the P13K/AKT kinases, which are downstream of the ERBB family of receptors (Hay & Sonenberg, 2004). Since mTOR is involved in a number of cellular processes and since it is deregulated in cancer, it began to gain interest as a cancer therapy target. Rapamycin, which is an mTORC1 and mTORC2 inhibitor, is a macrolide antibiotic that was first discovered to be a potent immunosuppressive (Bjornsti & Houghton, 2004; Pohanka, 2001; Saunders, Metcalfe, & Nicholson, 2001). Developing Rapamycin for a cancer therapy has been slow due to fears about formulation and stability of these inhibitors in patients. The mechanism of action for Rapamycin

is that the inhibitor first binds to immunophilin FK506-binding protein (FKBP12) and together, the complex inhibits the autophosphorylation of mTOR (Bjornsti & Houghton, 2004). Since mTOR is downstream of the ERBB receptor tyrosine kinases, I wanted to determine the effect Rapamycin had on ECD levels as well as levels for the R2TP complex. As shown in Figure 3.14, treatment of ERBB2+ cell lines with different dosages of Rapamycin for 72 hours caused downregulation of ECD and the R2TP components. The R2TP complex is involved in the stability of mTOR and other family members of the PIKK family (Horejsí et al., 2010). This data indicates that the R2TP complex perhaps in conjunction with ECD become destabilized when mTOR is inhibited.

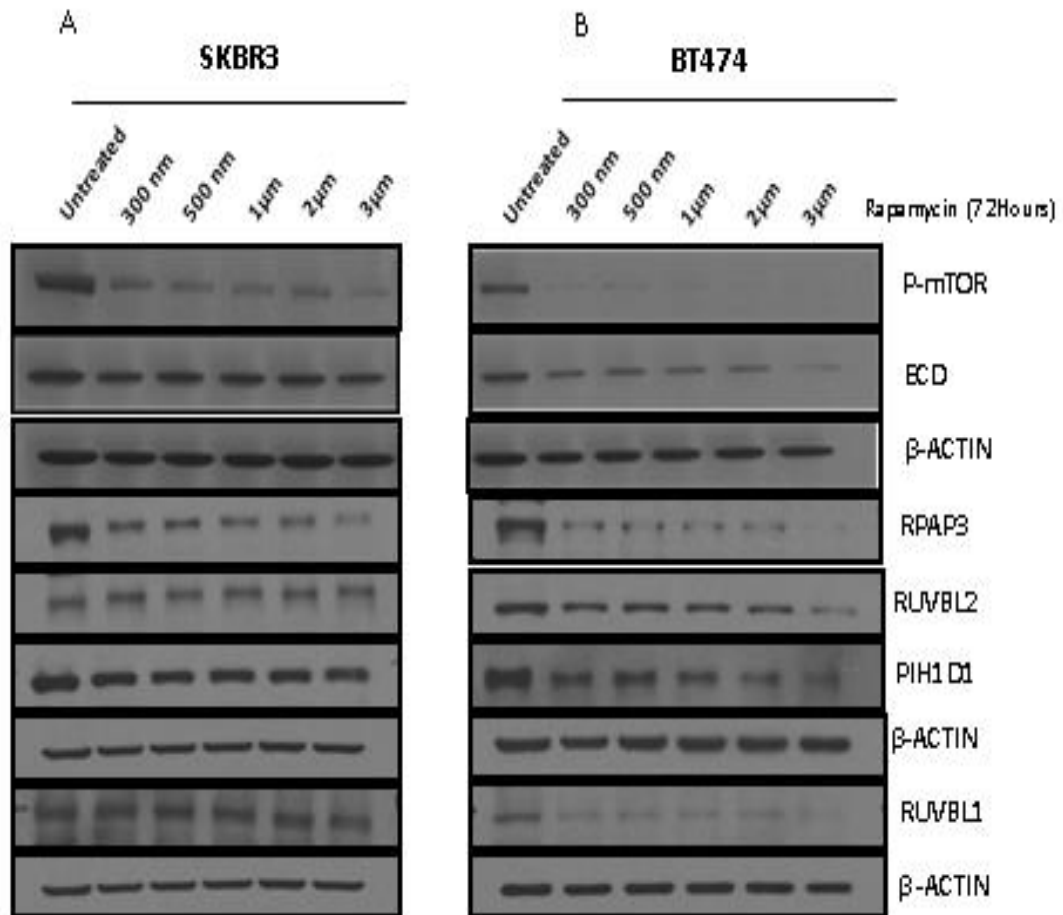
An immediate downstream signaling pathway that is activated by ERBB2 is the P13K/AKT pathway. The phosphatidylinositol 3 –kinase (PI3K)-Akt signaling pathway is activated by receptors with protein tyrosine kinase activity and by G protein-coupled receptors. Once PI3K is activated it converts the plasma membrane lipid PI (4,5)P<sub>2</sub> to PI(3,4,5)P<sub>3</sub> (Osaki, Oshimura, & Ito, 2004). AKT is a serine/ threonine kinase that preferentially binds PI (3, 4, and 5) P<sub>3</sub> over other PIs. Once PI (3, 4, and 5) P<sub>3</sub> is produced by PI3K, it begins to accumulate at the plasma membrane. AKT then translocates to the plasma membrane and binds to the phospholipids. Once bound to PI(3,4,5)P<sub>3</sub>, AKT undergoes a conformational change to expose its two phosphorylation sites and allows it to be phosphorylated (Osaki et al., 2004). The P13K/AKT pathway regulates fundamental cellular functions such as transcription, translation, proliferation, growth, and survival (Osaki et al., 2004). This pathway is also highly deregulated in cancer through over-activation. PI3K/AKT leads to the activation of a number of signaling cascades that lead to cell growth, proliferation, survival, and motility that is necessary to drive tumor progression (Vivanco & Sawyers, 2002). By understanding molecules that affect the levels of P13K or AKT in cells will help in the development of novel chemotherapy agents that will be

effective in combating these cancers. In order to understand the role of ECD in this pathway, I treated two ERBB2+ breast cancer cell lines with MK-2206 HCl. MK-2206 HCl is an AKT 1, AKT 2, and AKT 3 inhibitor. As shown in Figure 3.15, upon treatment of MK-2206 HCl, ECD levels were downregulated. The effect seemed more dramatic in BT474 cells which are ER+/ERBB2+ as compared to SKBR3 cells which are ER-/ERBB2+. This may be caused by the expression of ER in these cells, but this hypothesis needs to be explored at a later time.

### **3.4 Potential Role of ECD in Different Subtypes of Breast of Cancer**

Our laboratory has shown that ECD is overexpressed in ERBB2+ tumors and this overexpression is correlated with ERBB2 overexpression (X. Zhao et al., 2012). HSP90 has also been shown to be overexpressed in breast cancers and this overexpression is correlated with poor survival outcome (Pick et al., 2007). Recent studies have shown that the R2TP components, and TEL2 which is a known adapter protein in the complex, are overexpressed in breast and colon cancer patient samples (S. G. Kim et al., 2013). However, no one has shown if overexpression of ECD and the four R2TP components correlate with one another in different breast cancer subtypes. Therefore, I collaborated with another graduate student, Kristin Wipfler, in the bioinformatics laboratory of Dr. Babu Guda to observe the levels of ECD and the R2TP complex are overexpressed in similar breast cancer subtypes. In order to achieve this she first downloaded RNASeqV2 mRNA expression data from the Cancer Genome Atlas (TCGA) for 712 breast invasive carcinoma patients. The sequencing to generate this data was performed with the Illumina HiSeq 2000 platform and was analyzed with the version 2 pipeline, which uses MapSplice for the alignment and RSEM for quantitation. She then divided the samples into ER+, HER2+, ER+/HER2+, and triple negative subtypes. The expression data was then filtered to examine six genes, ECD, RUVBL1, RUVBL2, PIH1D1, RPAP3, and TELO2. Table 3.1 is a compilation

Figure 3.14: ERBB2+ cell lines treated with Rapamycin causes downregulation of ECD and members of the R2TP complex.



**Figure 3.14: ERBB2+ cell lines treated with Rapamycin causes downregulation of ECD and members of the R2TP complex.** A-B) SKBR3 and BT474 cells were treated with different dosages of Rapamycin and lysates were collected after 72 hours of treatment. Cells were harvested using RIPA Buffer and ECD expression was determined by western blotting using anti-ECD, anti-phospho-mTOR, anti-PIH1D1, anti-RUBVL1, anti-RUVBL2, anti-RPAP3, and anti- $\beta$ -ACTIN (as loading control).



Figure 3.15: ERBB2+ cell lines treated with MK-2206 HCl causes downregulation of ECD.

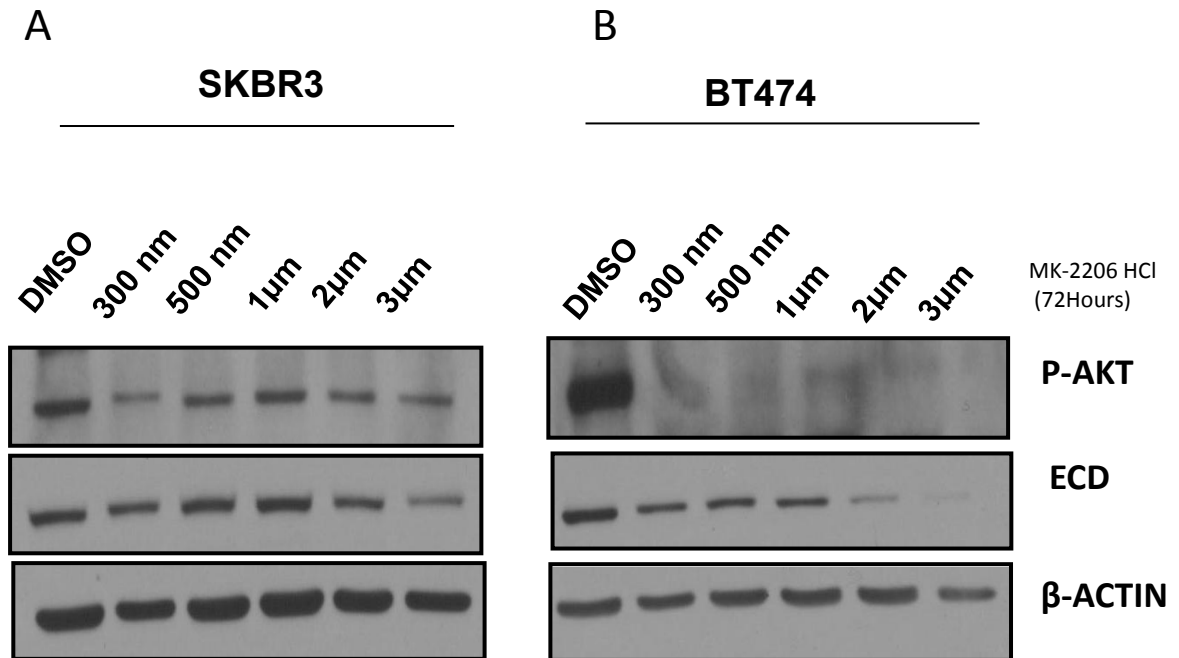


Figure 3.15: ERBB2+ cell lines treated with MK-2206 HCl causes downregulation of ECD. A-B) SKBR3 and BT474 cells were treated with different dosages of MK-2206 HCl and lysates were collected after 72 hours of treatment. Cells were harvested using RIPA Buffer and ECD expression was determined by western blotting using anti-ECD, anti-phosphorylated AKT, and anti- $\beta$ -ACTIN (as loading control).

of the summary results that are common for each group among the six genes. Kristin then performed ANOVA tests comparing the expression between the four subtypes for each of the six genes: ECD (Table 3.2), RUVBL1 (Table 3.4), RUVBL2 (Table 3.6), PIH1D1 (Table 3.7), RPAP3 (Table 3.9), and TELO2 (Table 3.11). Five of the ANOVA tests were significant, indicating that at least one of the four groups was significantly different from the others for five of the six genes. For the five genes with significant ANOVA results, she then performed student's t-tests for each possible combination of subtypes to identify which subtypes had significantly different expression levels from the others (ECD (Table 3.3), RUVBL1 (Table 3.5), PIH1D1 (Table 3.8), RPAP3 (Table 3.10), and TELO2 (Table 3.12)). Remarkably, ECD, RUVBL1, PIH1D1, RPAP3, and TELO2 all have the same two groups of subtypes where there is a significant difference between the groups. One group is a comparison between ER+ and Triple Negative subtypes and the second group is ER+/HER2+ and Triple Negative. By observing the Transcripts per million (TPM) number on the charts, we are able to determine if there are more transcripts or less transcripts in one subtype compared to the other. We can also add or subtract the variance to see the range of transcript differences between the subtypes. There are also other subtype groups that are significant between each of the six genes that we looked at, but the ones highlighted above are trending patterns for all genes. This data indicates that there may be a correlation of ECD mRNA expression and the four R2TP components mRNA expression in different subtypes of breast cancer. This data is very interesting and needs to be explored further.

### **3.5 Role of ECD in Endoplasmic Reticulum Stress Pathway**

Recently, another graduate student in my mentor's laboratory has shown that ECD may be a key regulator in the ER stress pathway through its interaction with PERK. ER stress is a stimulus; such as hypoxia, nutrient deprivation, or misfolded proteins; for the cell to initiate a

Table 3.1: Summary of experimental results for TCGA samples for ECD, TEL2, and R2TP components.

Groups	Count	Average	Variance	Transcripts per Million (TPM)
ER+	435	3.32147E-05	2.50368E-10	33.21466841
ER+/HER2+	121	3.11382E-05	2.84927E-10	31.13821112
HER2+	40	2.90911 E-05	1.83211E-10	29.09105854
ER-/PR-/HER2-	116	2.54898E-05	1.27466E-10	25.48980237

Table 3.1: **Summary of experimental results for TCGA samples for ECD, TEL2, and R2TP components.** mRNA expression data from different breast cancer patients was collected from The Cancer Genome Atlas. The patient samples were separated based on molecularly classified subtypes, such as ER+, HER2+, ER+/HER2+, and ER-/PR-/HER2- (triple negative). Each patient sample in each subtype was then filtered for six genes: A) ECD, B) RUVBL1, C) RUVBL2, D) PIH1D1, E) RPAP3, and F) TELO2. The summary data includes the counts (the number of patient samples taken from each group), the averages, the variances, and transcripts per million.

- ❖ This work and statistical analysis was performed by Kristin Wipfler, B.S. from Dr. Babu Guda laboratory, in collaboration with myself.
- ❖ The dataset was provided by ©The Cancer Genome Atlas

Table 3.2: An ANOVA test was then performed on each gene, starting with ECD, between each subtype group to determine if there was any significant change in mRNA expression for ECD.

Source of Variation	SS	df	MS	F	P-Value	Significance
Between groups	5.70536E-09	3	1.90179E-09	8.177497092	2.34214E-05	YES
Within Groups	1.64655E-07	708	2.32563E-10			

Table 3.3: Student T-Test was then performed for each gene between each subtype to determine if there was a significant change in mRNA expression for ECD.

GROUP1 compared to GROUP 2	P-Value	Significance
ER+ To ER+/HER2+	0.208863782441418	NO
ER+ To HER2+	0.111370684530581	NO
ER+ To ER-/PR-/HER2-	0.0000010779541448405	YES
ER+/HER2+ to HER2+	0.487362470329568	NO
ER+/HER2+ to ER-/PR-/HER2-	0.00285254895147712	YES
HER2+ to ER-/PR-/HER2-	0.100855856548926	NO

Table 3.4: An ANOVA test was then performed on each gene between each subtype group to determine if there was any significant change in mRNA expression for RUVBL1.

Source of Variation	SS	df	MS	F	P-Value	Significance
Between groups	2.40731456 553593E-08	3	8.024381 88511978 E-09	11.041378 4479648	4.319641727811 68E-07	YES
Within Groups	5.14542853 633642E-07	708	7.267554 4298537E -10			

Table 3.5: Student T-Test was then performed for each gene between each subtype to determine if there was a significant change in mRNA expression for RUVBL1.

GROUP1 compared to GROUP 2	P-Value	Significance
ER+ To ER+/HER2+	0.151587657479842	NO
ER+ To HER2+	0.966167060065643	NO
ER+ To ER-/PR-/HER2-	3.41243885161866E-08	YES
ER+/HER2+ to HER2+	0.415493285299253	NO
ER+/HER2+ to ER-/PR-/HER2-	0.00265785411576765	YES
HER2+ to ER-/PR-/HER2-	0.0119944935269851	YES

Table 3.6: An ANOVA test was then performed on each gene between each subtype group to determine if there was any significant change in mRNA expression for RUVBL2.

Source of Variation	SS	df	MS	F	P-Value	Significance
Between groups	8.08644E-09	3	2.69548E-09	1.485313521	0.217238877	YES
Within Groups	1.28485E-06	708	1.81475E-09			

Table 3.7: An ANOVA test was then performed on each gene between each subtype group to determine if there was any significant change in mRNA expression for PIH1D1.

Source of Variation	SS	df	MS	F	P-Value	Significance
Between groups	2.41692E-08	3	8.05642E-09	8.411006568	1.69093E-05	YES
Within Groups	6.78152E-07	708	9.57842E-10			

Table 3.8: Student T-Test was then performed for each gene between each subtype to determine if there was a significant change in mRNA expression for PIH1D1.

GROUP1 compared to GROUP 2	P-Value	Significance
ER+ To ER+/HER2+	0.016332892251614	YES
ER+ To HER2+	0.165896496198448	NO
ER+ To ER-/PR-/HER2-	0.0000114492940005427	YES
ER+/HER2+ to HER2+	0.0997459616333197	NO
ER+/HER2+ to ER-/PR-/HER2-	0.000189602787837004	YES
HER2+ to ER-/PR-/HER2-	0.209932151716371	NO



Table 3.9: An ANOVA test was then performed on each gene between each subtype group to determine if there was any significant change in mRNA expression for RPAP3.

Source of Variation	SS	df	MS	F	P-Value	Significance
Between groups	1.28564E-09	3	4.28548E-10	9.161193948	5.93721E-06	YES
Within Groups	3.31193E-08	708	4.67786E-11			

Table 3.10: Student T-Test was then performed for each gene between each subtype to determine if there was a significant change in mRNA expression for RPAP3.

GROUP1 compared to GROUP 2	P-Value	Significance
ER+ To ER+/HER2+	0.336966676998174	NO
ER+ To HER2+	0.0192161582183761	YES
ER+ To ER-/PR-/HER2-	0.0000116854768042364	YES
ER+/HER2+ to HER2+	0.0080458484175475	YES
ER+/HER2+ to ER-/PR-/HER2-	0.0000147519103811155	YES
HER2+ to ER-/PR-/HER2-	0.699636362488147	NO

Table 3.11: An ANOVA test was then performed on each gene between each subtype group to determine if there was any significant change in mRNA expression for TEL02.

Source of Variation	SS	df	MS	F	P-Value	Significance
Between groups	3.54096E-09	3	1.18032E-09	9.144590003	6.07634E-06	YES
Within Groups	9.13838E-08	708	1.29073E-10			

Table 3.12: Student T-Test was then performed for each gene between each subtype to determine if there was a significant change in mRNA expression for TEL02.

GROUP1 compared to GROUP 2	P-Value	Significance
ER+ To ER+/HER2+	0.946889582010066	NO
ER+ To HER2+	0.0000315307342747369	YES
ER+ To ER-/PR-/HER2-	0.000991791813725305	YES
ER+/HER2+ to HER2+	0.000172951485415618	YES
ER+/HER2+ to ER-/PR-/HER2-	0.00518486999426572	NO
HER2+ to ER-/PR-/HER2-	0.0063119727470464	YES

Table 3.1-3.12: mRNA expression data from different breast cancer patients was collected from The Cancer Genome Atlas. The patient samples were separated based on molecularly classified subtypes, such as ER+, HER2+, ER+/HER2+, and ER-/PR-/HER2- (triple negative). Each patient sample in each subtype was then filtered for six genes: A) ECD, B) RUVBL1, C) RUVBL2, D) PIH1D1, E) RPAP3, and F) TELO2. An ANOVA test was then performed on each gene between each subtype group to determine if there was any significant change in mRNA expression. Upon a significant change in the p-value from the ANOVA test, a student T-Test was then performed for each gene between each subtype to determine if there was a significant change in mRNA expression.

- ❖ This work and statistical analysis was performed by Kristin Wipfler, B.S. from Dr. Babu Guda laboratory in collaboration with myself.
- ❖ The dataset was provided by ©The Cancer Genome Atlas

defensive process called the unfolded protein response (UPR). UPR is comprised of several cellular mechanisms that are aimed to either assist in cellular survival, or in cases of extreme stress, initiate mechanisms of cell death such as autophagy or apoptosis (Ma & Hendershot, 2001; Welihinda et al., 1999). The UPR involves three stress sensors: activating transcription factor 6 (ATF6), PKR-like ER kinase (PERK) and inositol-requiring kinase 1 alpha (IRE1 $\alpha$ ), that are kept in an inactive state through their interaction ER chaperone GRP78 (also known as Bip) (Walter & Ron, 2011). Misfolded proteins accumulate in the ER lumen during ER stress, and then these proteins bind to GRP78. This interaction promotes the ER-Golgi transport of ATF6, where its cleavage releases the transcription factor domain for translocation to the nucleus to induce the transcription of specific genes, such as GRP78, to promote ER protein folding (Walter & Ron, 2011; Welihinda et al., 1999). Then PERK is activated by phosphorylation and this in turn inactivates the eukaryotic initiation factor 2 alpha (eIF2 $\alpha$ ). The inactivation of eIF2 $\alpha$  causes a repression in general protein synthesis in order to decrease the amount of proteins entering the ER lumen while selectively inducing the translation of specific mRNAs such as ATF4. ATF4 induces the expression of CCAAT/enhancer-binding protein-homologous protein (CHOP) (Walter & Ron, 2011). The multiple arms of UPR mediate a coordinated program of cellular protection and mitigation of stress and under some conditions such as prolonged ER stress, promotes apoptosis downstream of UPR (Ma & Hendershot, 2001; Walter & Ron, 2011; Welihinda et al., 1999).

Cancer tumors exhibit increased ER stress with activation of UPR pathways and cause different cellular responses to help survive harsh environments. The responses to ER stress includes suppression of new protein synthesis, exit from cell cycle and apoptosis, processes that are not pro-oncogenic (Lee & Hendershot, 2006; Moenner, Pluquet, Bouchecareilh, & Chevet, 2007). Cancer cells must enact adaptive mechanisms to eliminate the inhibitory outcomes of the UPR

while in turn rely on the pathway's protective aspects, such as increased antioxidant defenses and increased anti-apoptotic and autophagic mechanisms (Moenner et al., 2007). Current experiments in the lab have shown that ECD levels are downregulated in HMECs upon treatment of different endoplasmic reticulum stress inducing compounds, such as Thapsigargin and Tunicamycin. I have performed a proximity ligation assay where I observed ECD interaction with several UPR pathway components, including PERK, GRP78, and ATF6 (Figure 3.16 and Figure 3.17). These results indicate that ECD is an important regulator of UPR. Interestingly, in PERK knockout mouse embryonic fibroblast (MEFs) that are treated with ER stress inducers, ECD levels remain unchanged. This observation indicates that ECD may play a role as a negative regulator of stress.

Since the UPR pathway is dysregulated in cancer, we wanted to observe how an ER stress inducer, Bortezomib, effects ECD levels in ERBB2+ breast cancer cell lines. Bortezomib (PS-341, Velcade), is a potent proteasome inhibitor (Nawrocki, Carew, Dunner, et al., 2005; Nawrocki, Carew, Pino, et al., 2005). The proteasome is an organelle that is responsible the degradation for most proteins within the cell. Bortezomib is a proteasome inhibitor that induces ER stress by preventing the passage of misfolded proteins from the ER (Nawrocki, Carew, Dunner, et al., 2005). Bortezomib is also currently in clinical trials as a chemotherapy agent that targets solid tumors (Nawrocki, Carew, Dunner, et al., 2005; Nawrocki, Carew, Pino, et al., 2005). Remarkably, when BT474 and SKBR3; two ERBB2+ breast cancer cell lines; ECD levels are upregulated in a time dependent manner (Figure 3.18). Our laboratory has shown that ECD is degraded through the proteasome, but we have not been able to show a compound that increases ECD levels *in vitro*. Combinational treatment of cells with Bortezomib and other ER stress inducers could possible elucidate the role ECD is playing in ER Stress.

Figure 3.16: Proximity Ligation Assay depicting Interaction between ECD and ER Stress

Components.

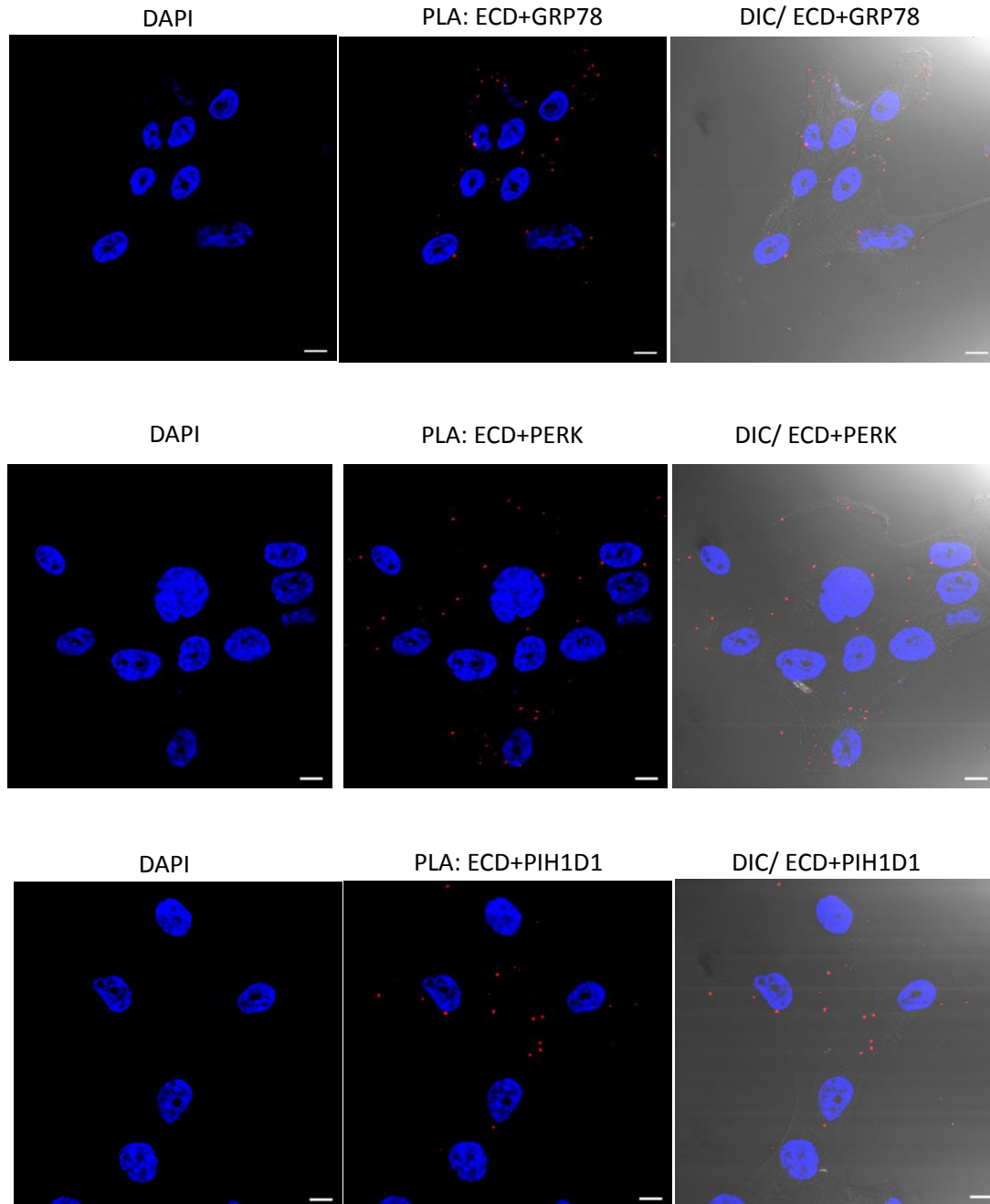
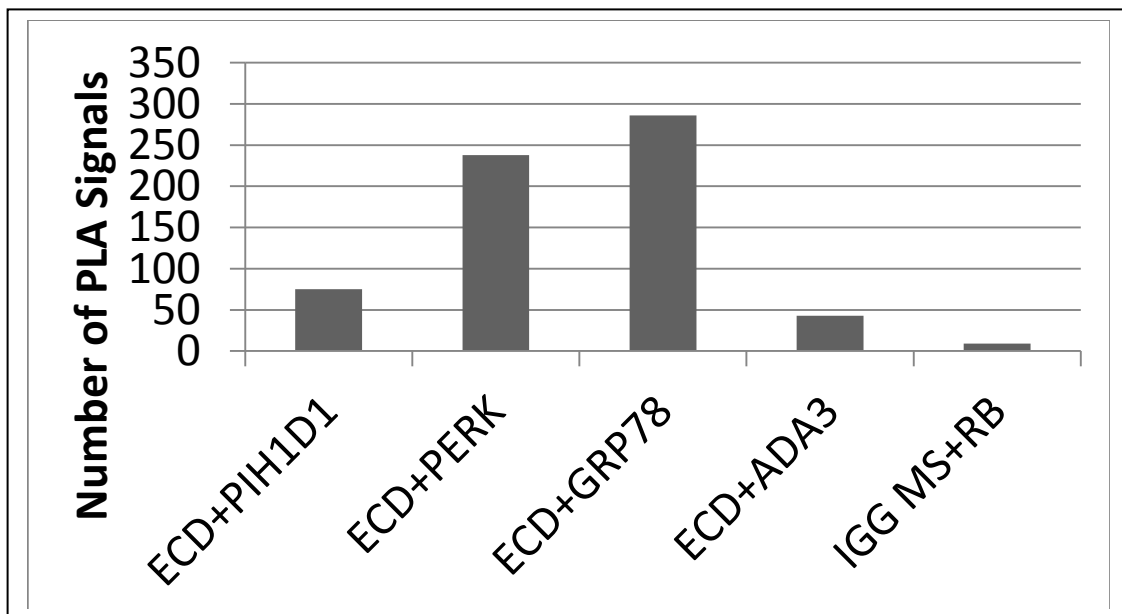
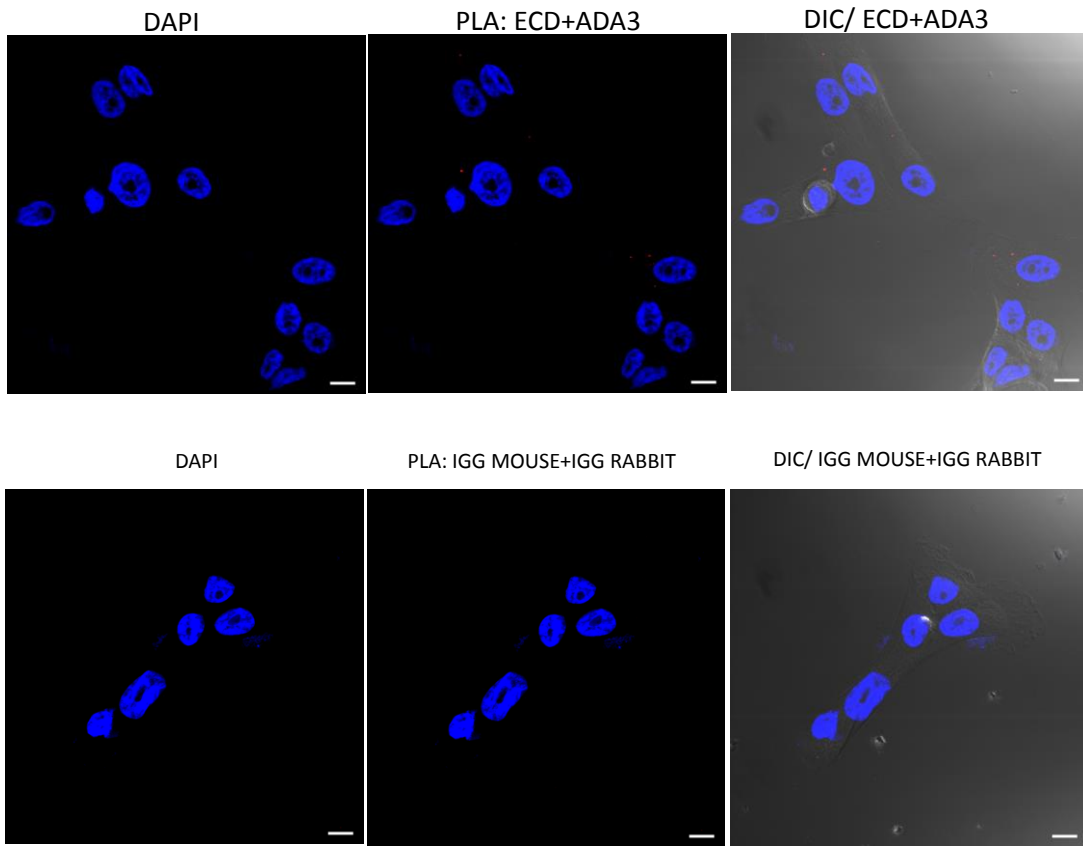


Figure 3.16: Proximity Ligation Assay depicting Interaction between ECD and ER Stress Components (continued).

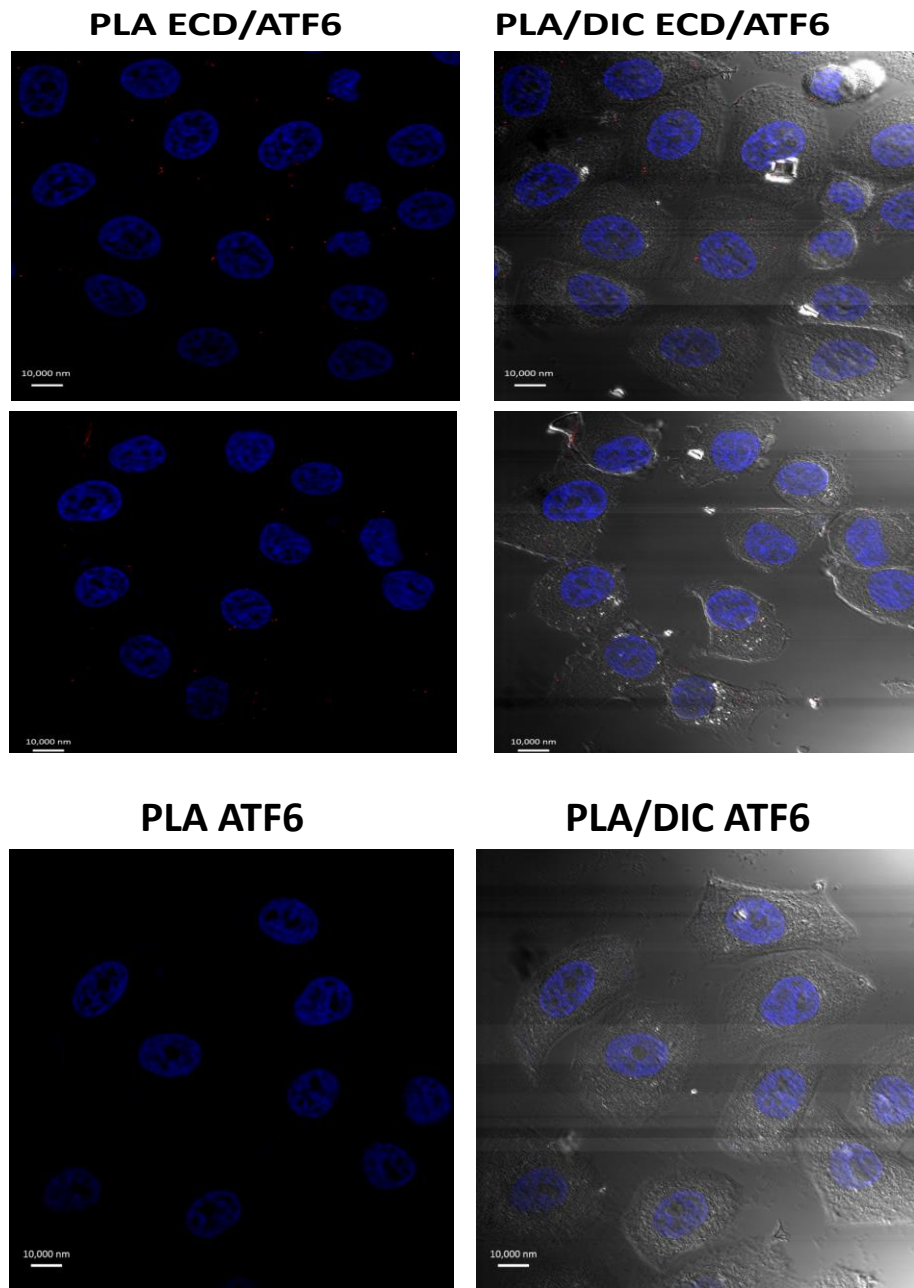


**Figure 3.16: Proximity Ligation Assay depicting Interaction between ECD and ER Stress**

**Components.** MCF10A cells were plated on 18mm coverslips and were fixed in 3% paraformaldehyde. Cells were then blocked in blocking buffer for 30 minutes and incubated with primary antibody for 2 hours. Coverslips were then incubated in mouse (minus probe) and rabbit (plus probe) probes for one hour, and then ligated for 30 minutes, then probes underwent amplification using a red fluorescent dye to indicate interactions for 2 hours. Controls were ECD and PIH1D1 as positive control; ECD and ADA3 interaction, IgG, and single antibody controls as negative controls. Quantification of proximity signals was completed using Duolink Image Tool Software. Analysis of >50 cells per interaction. These figures and graph are representative images of three independent experiments. Scale bars represent 10 $\mu$ m.



Figure 3.17: Proximity Ligation Assay depicting Interaction between ECD and ER Stress Component ATF6.



**Figure 3.17: Proximity Ligation Assay depicting Interaction between ECD and ER Stress Component ATF6.** SKBR3 cells were plated on 18mm coverslips and were fixed in 3% paraformaldehyde. Cells were then blocked in blocking buffer for 30 minutes and incubated with primary antibody for 2 hours. Coverslips were then incubated in mouse (minus probe) and rabbit (plus probe) probes for one hour, then ligated for 30 minutes, then probes underwent amplification using a red fluorescent dye to indicate interactions for 2 hours. Control was ATF6 alone as negative controls.

Figure 3.18: ERBB2+ cell lines treated with Bortezomib causes upregulation of ECD.

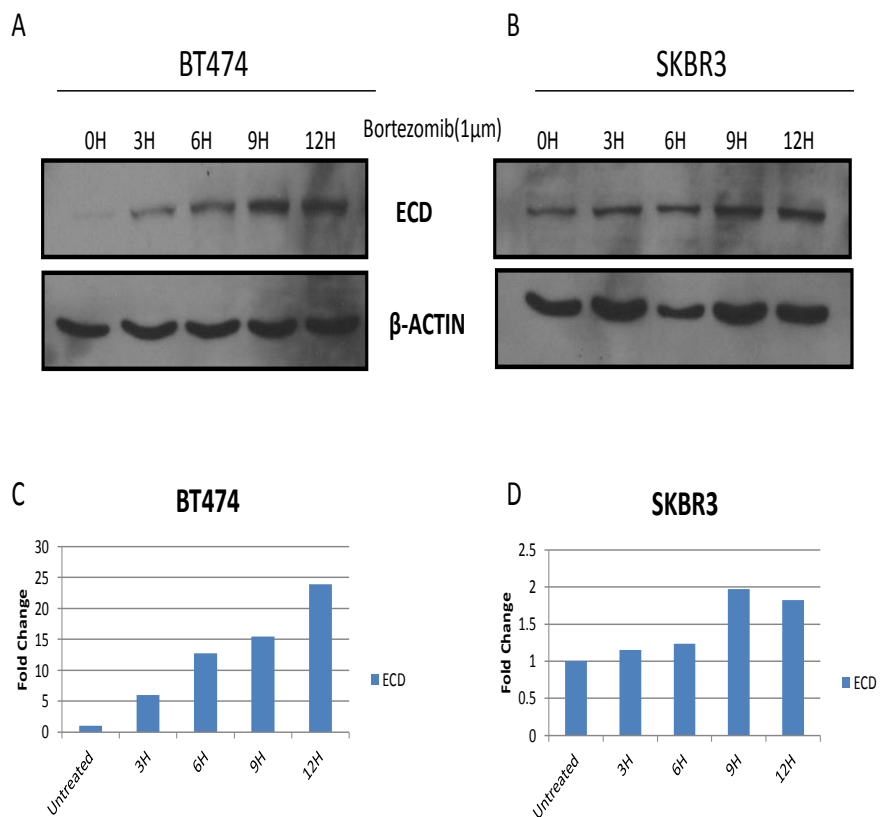
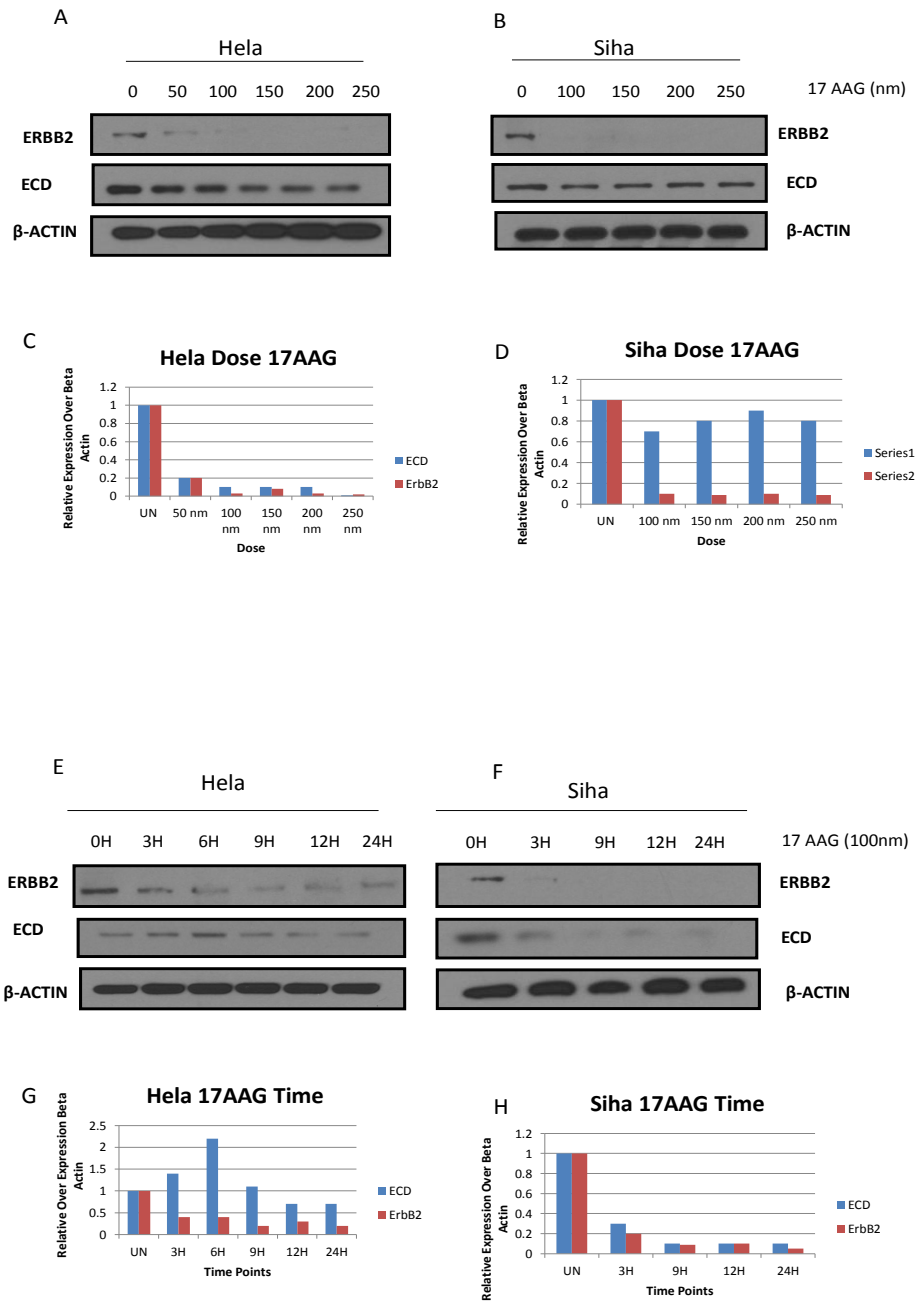


Figure 3.18: ERBB2+ cell lines treated with Bortezomib causes upregulation of ECD. A-B) BT474 and SKBR3 cells were treated with 1 μM of Bortezomib and lysates were collected at different time points. Cells were harvested using RIPA Buffer and ECD expression was determined by western blotting using anti-ECD and anti-β-ACTIN (as loading control). C-D) Quantitative density analysis of ECD was normalized to β-ACTIN. Densitometry performed using ImageJ.

### 3.6 ECD potential role as a therapy target in cervical cancer

Previously, we have shown that ECD is overexpressed in breast cancer and in pancreatic cancer (Dey et al., 2012; X. Zhao et al., 2012). Dr. Band's laboratory also identified that ECD is an HPV 16 E6 binding protein. Therefore, we wanted to examine the expression in HPV positive cancers such as cervical cancer. Cervical cancer is a malignant neoplasm arising from the uterine cervix. About 80% of all cervical cancers are of the squamous subtype; and the remainder are either adenocarcinomas, adenosquamous carcinomas, or other rare types (Waggoner, 2003). Two laboratory members in Dr. Band's lab, Dr. Aditya Bele and Dr. Sameer Mirza, have shown that ECD is overexpressed in both squamous cell carcinoma as well as adenocarcinoma of cervix and that ECD knockdown in cervical cancer cell lines leads to decrease in invasion and migration (Bele, 2015). After these results, I wanted to test how a chemotherapy agent that has already been proven to cause down regulation of ECD affected two cervical cancer cell lines. HeLa and SiHa cells that are both HPV E6 positive, were treated with different dosages of 17-AAG. As shown in Figure 3.19, treatment of 17-AAG causes down regulation of ECD in cervical cancer cell lines. These observations suggest that ECD overexpression is specifically linked to certain oncogenes and that ECD may be part of an unknown pathway that is critical for cellular functions. This makes ECD an excellent therapy target which can be applicable for a wide variety of cancers.

Figure 3.19: Cervical cancer cell lines treated with 17-AAG causes downregulation of ECD in a dose dependent and time dependent manner.



**Figure 3.19: Cervical cancer cell lines treated with 17-AAG causes downregulation of ECD in a dose dependent and time dependent manner.** A-B) HeLa and SiHa cells were treated with different dosages of 17-AAG and lysates were collected after 9 hours of treatment. Cells were harvested using RIPA Buffer and ECD expression was determined by western blotting using anti-ECD, anti-ERBB2, and anti- $\beta$ -ACTIN (as loading control). C-D) Quantitative density analyses of ECD and ERBB2 were normalized to  $\beta$ -ACTIN. Densitometry performed using ImageJ. E-F) HeLa and SiHa cells were treated with 100 nm of 17-AAG and lysates were collected after different time points indicated. Cells were harvested using RIPA Buffer and ECD expression was determined by western blotting using anti-ECD, anti-ERBB2, and anti- $\beta$ -ACTIN (as loading control). G-H) Quantitative density analyses of ECD and ERBB2 were normalized to  $\beta$ -ACTIN. Densitometry performed using ImageJ.

## **Chapter 4:**

# **Summary and Conclusions**

Breast cancer is a heterogeneous disease that has been responsible for the deaths of over two hundred thousand women in the past year in the United States (Siegel et al., 2015). There are six subtypes of breast cancer that is categorized based on the gene expression profile and the molecular properties of the tumor (Prat et al., 2010, 2014; Sorlie et al., 2003). Cancer patients with overexpressing ERBB2 have a poorer overall survival rate compared to patients that overexpress the Estrogen Receptor and/or the Progesterone Receptor (Prat et al., 2014; Sorlie et al., 2003). ERBB2 is a member of the ERBB family of tyrosine kinase receptors are involved in multiple cellular pathways and have a number of downstream targets (Hynes & MacDonald, 2009). Since ERBB2 has the ability to homodimerize with itself and heterodimerize with other receptor family members, the signaling potential increases exponentially in cancers where ErbB2 is overexpressed (Bertucci et al., 2004; Hynes & MacDonald, 2009; Onitilo et al., 2009). Current therapy utilizes the monoclonal antibody Trastuzumab, which specifically targets and inhibits the signaling of the ErbB2 receptor (Hudis, 2007; Prat et al., 2014; Yarden & Sliwkowski, 2001). In some ErbB2 positive breast cancer patients, there is a chance of relapse and metastasis. These patients usually develop acquired resistance to Trastuzumab as well, but the mechanism behind this is not yet fully understood (Nahta & Esteva, 2006; Vu & Claret, 2012). There is a strong need to find novel chemotherapy targets that cause downregulation of ERBB2 in resistant patients.

Our laboratory has previously shown that ECD is an essential regulator of cell cycle progression through its function as a regulator of the RB-E2F interaction (J. H. Kim et al., 2009). We have also previously shown that ECD interacts with p53 and regulates its expression (Zhang, 2006). We have also shown that complete knockout of ECD leads to embryonic lethality in mice and deletion of ECD in ECD<sup>flox/flox</sup> mouse embryonic fibroblast causes G1/S cell cycle arrest; but exogenous expression of ECD has the ability to rescue this phenotype (J. H. Kim et al., 2009).



Our laboratory has also shown that ECD is overexpressed in breast cancer and this overexpression is correlated with ERBB2 overexpression. We have also shown that overexpression of ECD and ERBB2 is correlated with a poor overall survival outcome (X. Zhao et al., 2012).

In my thesis, I wanted to explore the relationship between ERBB2 and ECD *in vitro* and uncover the mechanism of how ECD is working in these cells. First, we (a joint collaboration between myself and Dr. Sameer Mirza) performed immunoprecipitation and proximity ligation assays to determine the interaction between ECD and ERBB2. We were able to see some interaction, but we hypothesized that perhaps this is not a direct interaction and ECD may be interacting with ERBB2 through another protein interaction. Therefore, we decided to use this assays to assess ECD and HSP90 interaction and we were able to see a more direct interaction (Chapter 2). This result allowed us to conclude that ECD and ERBB2 have a proximity interaction that is mediated through both proteins interaction to HSP90. Interestingly, we have observed that exogenous overexpression of ERBB2 in HMECs leads to protein overexpression of ECD (Chapter 3). This indicates that cells with ERBB2 overexpression require higher levels of ECD, perhaps to help stabilize these receptors. In order to test this, we downregulated ECD expression by siRNA in ERBB2+ breast cancer cells and we saw that ERBB2 levels were decreased. We also observed that ECD knockdown downregulates some downstream effectors of ERBB2 signaling (Chapter 2). ECD is assisting in the stabilization of ERBB2 and downregulation of ECD causes a decrease in ERBB2, AKT, and ERK levels. ECD downregulation decreases the surface level of ERBB2 on ERBB2+ breast cancer cells and this downregulation leads to increase of ubiquitination of ERBB2. Interesting, we have also seen that downregulation of ECD leads to decrease in the number of interactions between ERBB2 and HSP90 (Chapter 2) possibly leading

to the destabilization of ERBB2. All of these results validate the potential of Ecd as a novel molecular therapy target in ErbB2 positive breast cancer.

Recently, it was discovered that ECD is a substrate of a protein called, PIH1D1 which is one of the members of a co-chaperone protein complex named R2TP (Hořejší et al., 2014). The R2TP complex is comprised of four molecular components: two AAA+ ATPases RUVBL1 and RUVBL2, an adaptor protein PIH1D1, and an HSP90 interacting protein RPAP3 (Boulon et al., 2012). We have shown that outside of ECD interaction with PIH1D1, ECD has a novel interaction with RUVBL1 and this interaction is critical for ECD's function in cell cycle progression (Mir et al., 2015). In my thesis, I have shown that ECD is in close proximity of all four of the components in the R2TP complex (Chapter 1). Chaperone complexes, particularly Hsp90 and the R2TP/ Prefoldin-like, have been exciting new topics of research due to promising evidence of their involvement in number of molecular pathways. Hsp90 directly stabilizes ErbB2 and protects the receptors from proteasomal degradation (Maloney & Workman, 2002; W. Xu et al., 2005). Hsp90 also works in tandem with the R2TP/Prefoldin-like complex in stabilizing PIKKs, ribosome biogenesis, mRNA transcription, tRNA transcription, and translation (Boulon et al., 2010, 2012; Horejsí et al., 2010; Kakihara & Houry, 2012; Prieto et al., 2015; Yoshida et al., 2013). In breast cancer samples, Hsp90 and the components of the R2TP/Prefoldin-like complex have been shown to be overexpressed (S. G. Kim et al., 2013; Maloney & Workman, 2002). To date, the field of determining the mechanisms chaperone complexes is involved in and discovering adaptor proteins that are facilitating these interactions and mechanisms, is still relatively new. In my research I have shown direct correlations between ERBB2 and the R2TP complex in cells (Chapter 3) and in the future, it would be beneficial to explore the mechanism this complex is playing in ERBB2+ breast cancer.

In order to explore how ERBB2 affects levels of HSP90, ECD, and R2TP complex, I used three different chemotherapy drugs that target ERBB2 to see their effect on ECD and the R2TP complex. I used Trastuzumab; a humanized monoclonal antibody against ERBB2; Lapatinib; a dual intracellular tyrosine kinase inhibitor that blocks the downstream signaling pathway of ERBB2 and EGFR; and 17-AAG. 17-AAG is a small molecule inhibitor that binds to the ATPase activity domain of the Hsp90 dimeric protein, inhibiting its ATP activity. This mechanism of action causes the rapid degradation of ErbB2 (Maloney & Workman, 2002; Modi et al., 2011; Sausville et al., 2003). Each of these drugs has their own drawbacks that have made the need to discover new molecular therapy targets critical for scientist. Trastuzumab and Lapatinib, both have had patients whose tumor cells have acquired resistance against these agents (D'Amato et al., 2015; Nahta & Esteva, 2006). Resistance is becoming a major problem for clinicians and patients. Scientists have been working on elucidating the mechanisms of resistance but it has still been a work in progress. 17-AAG has been shown to be more promising than its predecessor; Geldanamycin; some clinicians fear the potential of hepatotoxicity (Workman et al., 2007). Geldanamycin proved to be highly toxic and caused hepatotoxicity, and although 17-AAG has been shown to be less toxic, there is still a possibility that this drug will not be suitable for some patients (Whitesell & Lindquist, 2005; Workman et al., 2007). Therefore is an increasing the need for other molecular therapy targets that cause less toxicity to patients. By elucidating the mechanisms of resistance and toxicity, we would be able to develop novel chemotherapeutic targets that will be effective in cancer patients and with fewer side effects.

In Chapter 3, I have shown that ECD and R2TP complex members are down regulated in ERBB2+ cell lines upon treatment of Trastuzumab, Lapatinib, and 17-AAG (Chapter 3). Interestingly, there is no effect on HSP90 levels, in resistant cell lines, and in non-ERBB2+ cell lines. When ERBB2+ cell lines are treated with phosphorylate AKT inhibitor and mTOR kinase

inhibitor, leads to downregulation in ECD and the four members of the R2TP complex (Chapter 3). Taken together, we can infer that ECD, along with the R2TP complex, may be working downstream of these signaling pathways and that destabilization of these kinases leads to destabilization of ECD and this complex. When we downregulated ECD, we did not see any change in members of the R2TP complex. We did observe downregulation of ERBB2 and AKT, indicating that ECD may be part of a feedback loop with these kinases that help stabilize one another. In Chapter 3, in collaboration with another graduate student Kristin Wipfler, showed using data obtained from The Cancer Genome Network, that ECD and members of the R2TP complex have mRNA expression that correlates with one another in different subtypes of breast cancer. This will need to be explored further to see if these components are upregulated or downregulated in these subtypes of breast cancer. ECD, HSP90, and the R2TP complex are involved in multiple cellular processes and assist in the stabilization and assembly of a number of proteins and protein complexes. The data collected indicates that ECD is playing an important role in the stabilization of ERBB2, but ERBB2 is also playing an important role in stabilizing ECD and the R2TP complex. By understanding the role ECD and the R2TP complex is playing in ERBB2+ breast cancers, can help provide scientists with new potential therapy targets that are not just effective in breast cancer, but in other ERBB2+ cancers such as ovarian. I have shown that ECD is also in close proximity to a number of ER stress markers. ER stress is a defensive process cells undergo when they are exposed to a stimulus; such as hypoxia, nutrient deprivation, or misfolded proteins (Welihinda et al., 1999). Cancer tumors exhibit increased ER stress with activation of UPR pathways and cause different cellular responses to help survive harsh environments. ECD seems to be a novel regulator of ER stress and could potentially be a novel therapy target.

**CONCLUSIONS:**

- ECD is overexpressed in a number of cancers including breast and prostate.
- ECD interacts directly or is in close proximity with the four members of the R2TP complex: RUVBL1, RUVBL2, PIH1D1, and RPAP3. Downregulation of ECD does not affect levels of the R2TP complex.
- ECD has a novel interaction with ERBB2/HER2 in breast cancer cells and this interaction may be mediated through HSP90.
- SiRNA mediated downregulation of ECD leads to downregulation of ERBB2 protein levels, but not mRNA levels in endogenous ERBB2 overexpressing cell lines and exogenous ERBB2 overexpressing cell lines.
- ECD knockdown also leads to a decrease in levels of downstream signaling targets such as AKT and phosphorylated ERK.
- ECD knockdown reduces the surface levels of ERBB2 in ERBB2 positive cell lines.
- ECD downregulation leads to destabilization of ERBB2 and increases the ubiquitination of ERBB2.
- ECD does not co-localize with ERBB2 in ERBB2+ cell lines, but exogenous overexpression of ERBB2 in human immortalized mammary epithelial cells increases endogenous levels of ECD protein but not ECD mRNA.
- Different chemotherapy agents against ERBB2 receptor, such as Trastuzumab, Lapatinib, and 17-AAG leads to downregulation of ECD, RUVBL1, RUVBL2, PIH1D1, and RPAP3.
- Different inhibitors that target the ERBB2 signaling pathway, such as AKT and mTOR, leads to downregulation of ECD, RUVBL1, RUVBL2, PIH1D1, and RPAP3.
- ECD interacts or is in close proximity with several members of the UPR pathway, such as PERK, ATF6, and GRP78

## Chapter 5 References

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## **APPENDIX A**



## Transcriptional Regulation of ErbB2 in Breast Cancer

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Keywords: breast cancer, oncogenes, Neu/Her2,

## Summary:

Breast cancer is the second most frequent type of cancer occurring in women in the United States. Approximately 25-30% of breast cancer patients have overexpression of ErbB2, which is associated with a poor prognosis. In 20-25% of ErbB2<sup>+</sup> breast cancers, ErbB2 overexpression is caused by gene amplification of chromosome 17 regions q 12-21 and by enhanced transcription. Therefore understanding the molecular mechanisms of transcriptional regulation of *erbB2* is a significant area of research. A number of transcription factors are known to positively regulate *erbB2* gene transcription, such as SRC-3 or ERR $\alpha$  that are discussed in detail in this review article. These transcriptional factors themselves are overexpressed in certain cases of breast cancers and are potential therapy targets. In addition a group of transcription factors, such as PAX2 or FOXP3 negatively regulate *erbB2* gene. This review will present a comprehensive literature summary of transcriptional regulation of the *erbB2* gene in normal and cancer mammary cells followed by discussion on current views. Understanding the transcriptional regulation of *erbB2* may provide other therapy options for ErbB2<sup>+</sup> breast cancer patients in addition to Trastuzumab, particularly in Trastuzumab resistance cases of metastatic disease.

## Introduction:

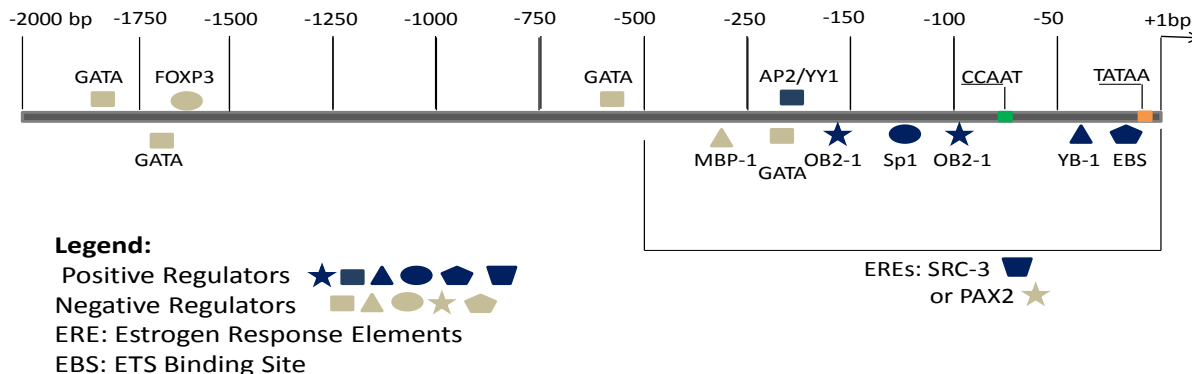
Breast cancer is the second most prevalent cause of cancer related deaths in women in the United States (1). Currently, based on expression profiling, breast cancer is categorized into six molecular subtypes; luminal subtype A which is Estrogen Receptor (ER)/ Progesterone Receptor (PR) positive and Epidermal Growth Factor Receptor 2 (ErbB2/HER2) negative, luminal subtype B which is ER+/PR+ and ErbB2+, ErbB2+ positive, basal-like or triple negative which is

ER-, PR-, and ErbB2-, claudin-low, and normal-like (2-5). Each subtype is classified according to particular set of gene expression, as well as based upon the receptor expression profile (1 - 6). The current knowledge on subtype of a breast cancer patient helps to determine the prognosis, as well as the treatment options (2-7). Currently, Trastuzumab (Herceptin) is a humanized monoclonal antibody that is most prevalently used for ErbB2+ subtype of breast cancer patients, a portion of these patients may present with more aggressive tumor and later develop resistance to the therapy (8).

ErbB2 is a member of the receptor tyrosine kinase superfamily and is involved in various signaling transduction pathways (9, 10). The RTK members all have three regions, an extracellular ligand-binding region, a single membrane-spanning region, and a cytoplasmic tyrosine kinase containing domain. ERBB2 does not have a known ligand, but, like the other receptors, can heterodimerize or homodimerize with other receptors to activate the intrinsic tyrosine kinase domain. Once activated, ERBB2 can activate the PI3K-AKT and MAPK pathways (10). Previous studies demonstrated ErbB2 is amplified in about 20-25% of breast cancers and this amplification of chromosome 17 regions q12-21 leads to overexpression of the receptor (11). Patients with ErbB2+ tumors have shown worse prognosis than patients with ER<sup>+</sup>/PR<sup>+</sup> tumors (12). A more recent study of approximately 500 breast tumors conducted by The Cancer Genome Atlas (TCGA) Network has shown the biological heterogeneity of clinical ErbB2-overexpressing cancers (HER2<sup>+</sup>), as defined by gene amplification. This group further characterized these cancers by gene expression into two subtypes, HER2-enriched (HER2E) and luminal (13). HER2E and HER2<sup>+</sup> tumors exhibited higher frequencies of aneuploidy, somatic mutations, and *TP53* mutation (13). These patients also exhibited genetic amplification of FGFRs, EGFR, CDK4, and Cyclin D1. Luminal- ErbB2-overexpressing breast cancers displayed a

higher expression of a luminal gene cluster which included GATA3, BCL2, and ESR1. Although, not all tumors of the HER2-enriched gene expression subtype are *erbb2* amplified, it is thought that some breast cancers with a single copy of *erbb2* gene harbor an expression signature of ErbB2 dependence and may benefit from anti-ErbB2 therapy (13). ErbB2 overexpression in tumors leads to enhanced proliferation and an increase in cell survival (14). ErbB2 overexpression, however, is not only caused by gene amplification, but also by an upregulation of *erbb2* transcription. Aaronson's lab has shown that on the *erbb2* promoter there is an acquired H3K4me3 mark and in ErbB2 overexpressing tumors, shown an enrichment of this mark. By using ErbB2 overexpressing and ErbB2 amplified cell lines and ChiP analyses, they were able to show that H3K9 is acetylated in ErbB2 amplified cells but not in *Erbb2* overexpressing cells. This indicates that epigenetic changes that are acquired effect the activation of ErbB2 transcription (15).

The *erbb2* promoter was initially characterized by Ishii et al. and Tal et al. (16, 17) *erbb2* gene transcription is controlled by at least two known promoters, the distal and the proximal which are separated from one another by 12 kilo bases (kb) (16). Although the distal promoter remains poorly defined, several regulatory elements have been characterized within the proximal promoter and its 5'-flanking sequence up to the 6 kb upstream of the major transcription start site (9, 11-13, 16; Fig. 1).



**Figure 1:** A schematic representation of part of the proximal promoter of the *ErbB2* gene spanning +1 base pairs to -2000 base pairs. The colored boxes represent the binding sites of the regulatory factors of transcription discussed in this review. The ERE section represents the Estrogen Response Elements that is sporadically spaced in the region of +1 base pairs to -500 base pairs. ERE is where SRC-3 and PAX2 bind with ER $\alpha$ . EBS is the Ets Binding Site where Ets transcription factors bind.

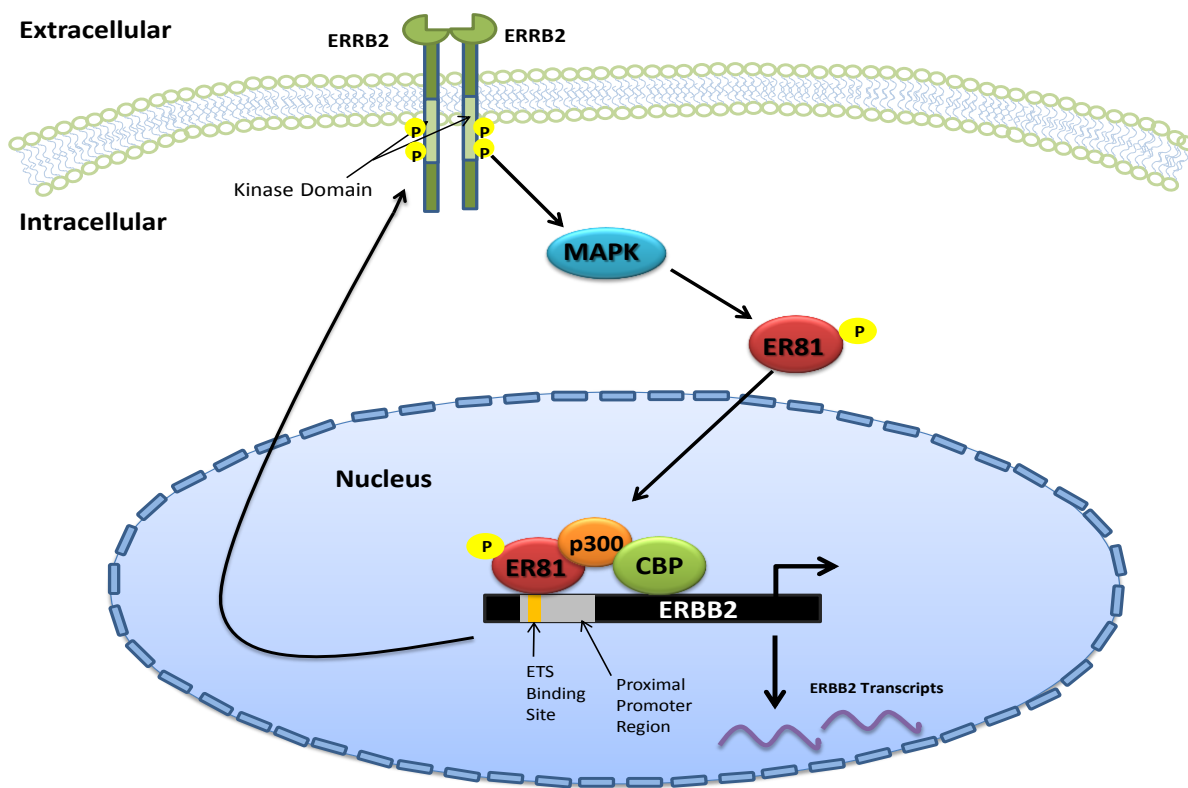
Studies suggest that several associated trans-activators or trans-repressors are involved in the increased transcription of the *erbB2* gene in breast cancer cells. These factors include: the E26 transformation-specific (ETS) transcription Factors (18-27), Activator Protein -2 (AP-2) (28-38), Estrogen Related Receptor  $\alpha$  (ERR  $\alpha$ ) (39-45), Y-Box binding protein-1 (YB-1) (46-51), Ying Yang 1 (YY1) (52-56), Specificity Protein 1 (Sp1) (19, 57-61), ER $\alpha$  with amplified in breast cancer-1 (SRC-3/AIB-1)(35, 62-65), which all positively regulate *erbB2* transcription. In addition several negative regulators, such as ER $\alpha$  with paired box 2 gene product (PAX2)(14, 68-70), Myc Promoter-Binding Protein Factor (MYB-1) with Histone Deacetylase 1 (HDAC1)(71-77), forkhead

box P3 (FOXP3) (78-79), and GATA Binding Protein 4 (GATA4) (79-80) downregulate *erbb2* gene expression. These factors along with recruitment of co-activators or co-repressors successfully turn on or off transcription of *erbb2* gene, respectively. By understanding the components that regulate *erbb2* transcription could pave the way for novel therapeutic targets and prognostic markers. Below we describe in details each factor that regulates *erbb2* gene expression.

### **Transcriptional factors/coregulators that positively regulate ErbB2 transcription**

**ETS Family of Transcription Factors (ETS)** proteins are a group of evolutionary related Transcription factors that have a conserved 85 amino acid long DNA binding domain and they regulate gene expression by binding to promoter and/or enhancer regions. ETS proteins are involved in the transcriptional activation or repression of genes that are involved in cellular processes such as proliferation, differentiation, development, transformation, and apoptosis (23). ETS transcription factors are downstream components of the RAS-MAPK pathway (24). In most ETS proteins there is a highly conserved regulator domain in the N-Terminal and a C-Terminal DNA binding domain (19). In order to activate the transcription factor, a conserved threonine residue in the regulatory domain is phosphorylated by MAPK (24). Once activated, these proteins facilitate the assembly of transcriptional complexes (19, 24). These proteins also play a critical role in the regulation of the transcription of receptor tyrosine kinases (18). There is an ETS binding domain located in the proximal promoter region of *erbb2* where an ETS transcription factor can bind and upregulate the expression of ErbB2 (18). The ETS binding site on the *erbb2* promoter is located close to the TATA major start site and when an ETS transcription factor is bound, it causes a severe bend in the DNA and creates a transcription start site at -69bp (25).

An ETS transcription factor that plays an important role in *erbB2* transcription and tumorigenesis is Polyoma Enhancer Activator 3 (PEA3). PEA3 overexpression is seen in ErbB2+ tumors and was first observed by *In situ* hybridization in paraffin embedded tumor samples (20). This overexpression correlated with ErbB2+ overexpression Immunoprecipitation studies showed that PEA3 directly interacts with ErbB2 in cells when both are exogenously expressed (20)



**Figure 2:** This schematic depicts the ETS transcription factor, ER81, and its positive regulation of the ErbB2 gene (18-27).

ETS transcription factors have been shown to be involved in a variety of cancers such as breast, prostate, thyroid, and leukemia (21-23). In ErbB2 overexpressing breast cancers, ETS transcription factors have been shown to be overexpressed and activated through phosphorylation (24). Some ETS transcription factors, particularly ETS-1, Fli-1, ER81, and other

family members, are overexpressed in breast cancer and are involved in invasion and metastasis (21, 22). Some of these positive regulators such as, ER81 (as shown in Figure 2) are downstream targets of the ErbB2 receptor tyrosine activity (18). ER81 interacts with the ETS binding site on the *erbB2/neu* promoter and activates *erbB2/neu* transcription with help of co-activators like p300 and CBP (18). This activity is enhanced by stimulation of MAPK pathways and by the ErbB2/Neu protein itself. ErbB2 activates ER81 indirectly by modulating the activity of signaling pathways that regulate ER81 phosphorylation (25, 26). Thus, ER81 forms a part of a positive regulatory feedback mechanism where oncogenic ErbB2/Neu activates ER81, and ER81 along with p300/CBP as well as activated MAPK pathway further enhances the *erbB2/neu* gene expression (26). Also, ER81 regulates transcription of matrix metallo proteases like MMP-1 which may play a role in tumor metastasis (27).

**Activator Protein-2 Family (AP-2)** is composed of three highly homologous transcription factor members, AP-2 $\alpha$ , AP-2 $\beta$ , and AP-2 $\gamma$ . All three members can bind to the proximal promoter, 218bp upstream of the major transcriptional start site, of the *erbB2* gene and activate its transcription (28). AP-2 has two binding sites on the *erbB2* proximal promoter located 213bp (29) and 495bp upstream from the transcription start site (30-32). These sites are however not conserved on the Neu promoter (33, 34). The Estrogen Receptor  $\alpha$  (ER $\alpha$ ) is a ligand dependent receptor and can only perform its function when it is bound to a ligand. In the presence of estrogen, ER $\alpha$  can bind to estrogen and then bind to the same binding site as AP-2 proteins on the *erbB2* gene and repress *erbB2* transcription (35). This binding creates a competition between ER $\alpha$  and the AP-2 transcription factors (35). It has been shown that in ErbB2 overexpressing tumors show relatively high levels of AP-2 $\alpha$  and AP-2 $\beta$  (34). The high expression of AP-2 $\alpha$  and AP-2 $\beta$  leads to increase of *erbB2* transcripts within a cell because the AP-2 proteins are able to compete out ER  $\alpha$  for binding to the *erbB2* gene (35, 36). In the presence of



Tamoxifen and other anti-estrogens, this competition is abolished and both AP-2 and ER bound to Tamoxifen or an anti-estrogen can upregulate *erbB2* transcription (37).

AP-2 $\alpha$  has a higher binding affinity to the *erbB2* promoter region than its other two family members, AP-2 $\beta$ , and AP-2 $\gamma$  (28). AP-2 proteins can also recruit cAMP binding element proteins to the promoter region (28, 37). AP-2 has been shown to be a functionally critical transcription factor involved in the transcriptional regulation of the proto-oncogene, ErbB2 (35, 38). Some studies demonstrate AP-2 is overexpressed in breast cancers and the high expression of nuclear AP-2 and ErbB2 over expression correlates with worse prognosis in patients compared to patients with low nuclear AP-2 levels despite of ErbB2 overexpression (35, 38). Consistently, suppression of AP-2 transcription factors downregulates the *erbB2* transcript levels in ErbB2+ breast cancer cell lines, thus further, underscoring an important role of AP-2 in positively regulating *erbB2* transcription.

**Estrogen Related Receptor Alpha (ERR $\alpha$ )** is a gene that is related to the ER $\alpha$  and is a nuclear orphan receptor. It binds to the estrogen response element and is involved in estrogenic actions within a cell (39). It shares features from ER $\alpha$ , including its structure and function. ERR $\alpha$  expression inversely correlates with the expression of ER $\alpha$  in breast cancer (40). Studies have shown that ERR $\alpha$  binds to the *erbB2* amplicon and regulates its transcription at different sites within the 17q12-21 chromosomal region (41). The *erbB2* amplicon is a genomic region of amplified genes that is composed of over 50 genes and some of these are thought to be driver genes of oncogenesis (42). When ERR $\alpha$  binds to the *erbB2* amplicon, it recruits its coactivator Peroxisome proliferator-activated receptor  $\gamma$  coactivator 1 $\beta$  (PGC-1 $\beta$ ) (41). PGC-1 $\beta$  is a coactivator of nuclear receptors and other transcription factors. It has been shown to function in the regulation of different components in the energy metabolism pathway (43). After ERR $\alpha$

binds to the *erbB2* promoter, it recruits its coactivator to the *erbB2* amplicon and together these components recruit RNA polymerase II to the promoter region of the gene. RNA polymerase II then transcribes the *erbB2* gene, as well as the neighboring genes (41).

ERR $\alpha$  signaling is shown to be involved in cancer progression in ErbB2<sup>+</sup> and ER<sup>+</sup> breast cancers (40, 41). ERR $\alpha$  levels are higher in ER- tumors and in ErbB2<sup>+</sup> tumors, indicating a more aggressive prognosis. When ERR $\alpha$  expression is ablated, *erbB2* amplicon transcripts are reduced (41). ERR $\alpha$  has been identified as an important contributor in the development and/or progression of breast cancer, ovarian cancer and osteopenic disorders (44-45). In ovarian cancer patients, high levels of ERR $\alpha$  correlates to a poorer survival rate (44). In breast cancer, a study has shown that ERR $\alpha$  levels are higher in ER negative tumors and in ErbB2<sup>+</sup> tumors, indicating a more aggressive prognosis. It has also been shown that ERR $\alpha$  is the most abundant nuclear receptor in a subset of tumors which lack ER $\alpha$  (40, 41). In addition, it has been observed that ErbB2 driven tumorigenesis was delayed upon silencing of ERR $\alpha$  in ErbB2<sup>+</sup> mice (40).

**Y-Box binding protein-1 (YB-1)** is an oncogenic transcription factor that binds to the *erbB2* promoter region. It was first discovered through a screening of an expression profile library for DNA binding proteins that interact with the *erbB2* gene (46). YB-1 can regulate ErbB2 expression by binding to potential YREs (YB-1 Response Elements) located about 2 Kbs from the start site. YB-1 may also regulate *erbB2* transcription by binding to other transcription factors, such as AP-2 (47). Using a transgenic mouse model, YB-1 was discovered to create genomic instability through centrosome amplification and mitotic failure in different breast carcinomas (48). YB-1 has little to no expression in normal cells but is highly expressed in a variety of cancers, including breast (49). YB-1 has also been speculated to be involved in acquired chemotherapy resistance in a number of cancers such as breast (50) and multiple myeloma (51).

In a clinical study where postoperative patients that received chemotherapy for breast cancer, but relapsed; the tumors of these patients showed a higher expression of YB-1. These patients also acquired therapy resistance. However, the tumors that showed low expression of YB-1; these patients did not relapse and therefore did not acquire resistance (50).

**Ying Yang 1 (YY1)** is a conserved zinc-finger DNA binding transcriptional factor that regulates the transcription for a number of genes that are involved in cellular processes such as cell growth, differentiation, and development. (52-53). YY1 a nuclear cofactor that interacts with the AP-2 factors, especially with AP-2 $\alpha$  (54). A clinical study using tissue arrays of different breast carcinomas were characterized by staining them using YY1, AP-2 $\alpha$ , AP-2 $\beta$ , ErbB2, and other biomarkers in order to observe possible correlations and prognostic markers. This group showed a correlation between AP-2 $\alpha$  and YY1 with ErbB2 protein expression. YY1 also correlated with ErbB2 gene expression and there were several cases that showed a difference between ErbB2 gene and protein expression (54). YY1 has the ability to interact with a number of proteins indicating that its function is dependent on its protein-protein interactions. It interacts with the AP-2 factors through a highly conserved domain and enhances the transcriptional activity at the *erb2* promoter in cells (52-56).

**Specificity Protein 1 (Sp1)** is yet another transcription factor that has been shown to regulate ERB2 expression. Sp1 is a transcription factor that is highly conserved in eukaryotic cells and binds to a variety of promoters to regulate transcription (19). Sp1 are known for their affinity to bind to GC-rich promoter regions (57). The *erb2* promoter has two GC-rich sequence elements that have been determined as distinct Sp1 binding sites approximately 50 to 130 bp upstream from the transcription start site (58). Sp1 is ubiquitously expressed in cells and it regulates different house-keeping genes that involved in cell cycle, receptor signaling, cellular growth

(59). Sp1 activation is tightly regulated through phosphorylation, glycosylation, and SUMOylation (60). A study has shown that Betulinic acid, which induces apoptosis and inhibits cell growth in ErbB2-overexpressing cells, was able to decrease levels of Sp1, Sp2, Sp3, and Sp4, causing a downregulation of ErbB2 through YY1 repression (61).

**Amplified in Breast Cancer-1 Transcription Factor SRC-3 (AIB-1)** is a member of the SRC family, which are nuclear receptor coactivators. SRC-3 binds to ER $\alpha$  at a conserved LXXLL motif at the nuclear receptor interaction domain (62). When ER $\alpha$  is bound to estrogen at its ligand binding domain, the receptor dimerizes with another Estrogen Receptor and changes its conformation to recruit coactivators such as SRC-3 to bind to the estrogen response element in the *erbB2* gene promoter region (35, 63). The overexpression of AIB1/SRC-3 was found to be correlated with increased HER2/*neu* expression and resistance to tamoxifen in ER+ breast cancer patients (62, 63). These findings suggest that the biological roles of AIB1/SRC-3 and HER2/*neu* are linked in breast cancer and that AIB1/SRC-3 may increase the sensitivity of breast cancer cells to HER2/*neu*-driven tumorigenesis (64). Cross talks between the Estrogen Receptor and ErbB2 pathways have been implicated to play important roles in Tamoxifen resistance (65, 66). However, a molecular mechanism linking estrogen receptor signaling, *erbB2* expression and tamoxifen resistance has remained elusive. Carroll and colleagues first reported that *erbB2* expression is controlled by the balance between the estrogen receptor co-activator AIB-1 and the co-repressor transcription factor PAX2 (67). Both factors competitively bind to the same *ebb2* regulatory element (67).

### **Transcription Factor Negatively Regulates ErbB2 Transcription**

**Paired Box 2 Gene Product (PAX2)** was originally discovered to regulate Wilms tumor suppressor gene, WT1, a gene that regulates the development of fetal urogenital system,

spleen, and mesothelium (68). In normal breast cell, PAX2 works in tandem with ER $\alpha$  to bind to the cis-regulatory region of the *erbB2* gene to down-regulate its transcription (14, 69).

Mutations or siRNA knockdown of PAX2 has been shown to prevent estrogen mediated transcriptional inhibition of *erbB2*, indicating a role for PAX2 in the transcriptional repression (69). PAX2 has also been shown to be a critical regulator in cell proliferation in the mammary gland (70). It has been shown that the response of breast cancer cells to Tamoxifen is regulated by competition between AIB-1 and PAX2 binding to cis-regulatory elements in *erbB2*. Indeed, a decrease in PAX2 expression in Tamoxifen-resistant (Tam-R) cells correlated with an increase in *erbB2* expression (69). The ER was still recruited to *erbB2* gene, but the PAX2 binding decreased, making Tam-R cells as responsive to Tamoxifen as tamoxifen-sensitive cells (69). Conversely, AIB-1 binding increased in Tam-R cells in response to Tamoxifen treatment. Overexpression of PAX2 reduced AIB-1 binding to the *erbB2* gene and restored Tamoxifen-mediated repression of ErbB2 and inhibited cellular proliferation (14, 69, and 70)

**Myc Promoter-Binding Protein Factor (MBP-1)** is a 48 kDA protein that binds to the c- MYC P2 promoter region and negatively regulates its transcription (71). Nuclear MBP-1 protein is correlated with breast cell transformation, since loss of its nuclear expression seems to be correlated with a worsened prognosis in patients with invasive ductal breast carcinoma (72). MBP-1 binds to the *erbB2* gene at a region that is located in its proximal promoter and recruits Histone Deacetylase 1, HDAC1, (72, 73). HDAC1 then deacetylates Histone H4 causing a repression of *erbB2* gene transcription. When ErbB2 is overexpressed, MBP-1 and HDAC1 expression decrease significantly (73).

Histone Deacetylase inhibitors are promising new therapy options for a variety of cancers. HDACs have been shown to be responsible for modifying a number of proteins such as

histones, transcription factors, and signal transduction mediators (74). HDACs are also differentially expressed in breast tumors and have been considered as therapeutic targets as well as potential prognostic markers (75). Earlier studies using a promoter-reporting cell screen, in ErbB2 overexpressing breast cancer cells, HDAC inhibitors have been shown to repress ErbB2 gene transcription by two mechanisms; direct transcriptional repression by inhibiting synthesis of ErbB2 mRNA and by accelerating the decay mature ErbB2 transcripts (76). In a recent publication using copy number and RNA transcript data, it was found that HDAC1 are able to repress highly amplified genes including ErbB2 by targeting RNA polymerase II elongation (77).

**Forkhead Box P3 (FOXP3)**, a member of the forkhead/winged helix transcription factor family, is an X-linked gene whose expression has been shown to negatively correlate with ErbB2 overexpressing mammary tumors (78). FOXP3 is a breast cancer suppressor gene and it is important for the regulation of the *erbB2* gene. FOXP3 is the first X-linked breast cancer transcription factor that binds to a consensus sequence in the 5' *erbB2* gene promoter region and represses transcription (78, 79).

**GATA Binding Protein 4 (GATA4)** is a member of the zinc finger transcription factors and is known to play a role in the regulation of several genes involved in embryogenesis and myocardium differentiation and function (80). In cardiomyocytes, GATA4 is activated via the mitogen-activated protein kinase pathway, which in turn is activated in the ErbB2 signaling pathway (80). GATA4 recognizes and binds to the GATA motif, several of these motifs are located in the *erbB2* proximal promoter region. GATA4 becomes activated through the MAPK pathway and once it is activated, it binds to the GATA motif of the *erbB2* gene repressing its transcription (80). When GATA4 is overexpressed in ErbB2 overexpressing cells, ErbB2 protein

levels are decreased and when GATA4 is silenced by siRNAs in cells, ErbB2 mRNA and protein levels increases (79, 80).

## Discussion:

In this comprehensive review we focused on various transcriptional factors or co-regulators that are associated with ErbB2 by directly or indirectly binding to its promoter and thus regulating its expression. The mechanism behind ErbB2 overexpression has been extensively studied and previously only attributed to the amplification of the *erbB2* gene (81). However, it is now documented that ErbB2 overexpression occurs without the *erbB2* gene being amplified (82). As outlined above there are multiple transcription factors that positively or negatively regulate *erbB2* transcription. ETS transcription factors have been shown to positively regulate the ERBB2 gene by binding to the ETS Response Element located in the ERBB2 promoter region (18, 25). ETS transcription factor, ER81 has been shown to positively regulate the ERBB2 gene through a positive feedback loop in the MAPK pathway. Activation of this pathway leads to phosphorylation of ER81, which in turn binds to the ERBB2 gene and activates its transcription (18). This feedback loop opens the door for a possible therapy target for patients who are ERBB2<sup>+</sup>. The ER81 transcription factor has been shown to be overexpressed or constitutively active in ERBB2<sup>+</sup> tumors (83).

AP-2 is a transcription factor that competes with estrogen to bind to an enhancer on the *erbB2* promoter region to turn on transcription (29). AP-2 is a well-studied regulator of *erbB2* transcription and has potential as a new prognosis factor in ErbB2<sup>+</sup> patients (32). High nuclear expression of AP-2 has been correlated with a worsened prognosis in ErbB2<sup>+</sup> overexpressed breast cancer patients (33). ERR $\alpha$  is a nuclear orphan receptor that binds to the estrogen response element on the *erbB2* amplicon and recruits PGC-1 $\beta$  to upregulate ErbB2's

transcription (41).  $ERR\alpha$  has recently been shown a role in tumorigenesis because knockdown of  $ERR\alpha$  shows a reduction of tumor progression in  $ErbB2^+$  mice (40). YB-1, YY1, and Sp1, all have binding sites located on the proximal promoter of the *erbB2* gene (46-61). YB-1 has been shown to have higher expression in breast cancer cells, particularly in  $ErbB2$  overexpressing cells as compared to normal (46). The *erbB2* gene is also involved in another positive feedback loop with  $ER\alpha$  and its coactivator, SRC-3 (62-67). SRC-3 overexpression has been seen in  $ErbB2$  overexpressing cells and studies have shown that overexpression of SRC-3 and  $ErbB2$  in  $ER^+$  tumor patients, leads to a poorer prognosis and a poorer survival outcome in those patients even when treated with Tamoxifen (67). In Tamoxifen treated tumor cells, SRC-3 still has the ability to bind to  $ER\alpha$  and activate the *erbB2* gene transcription leading scientists to think of alternative strategies when treating patients with  $ErbB2^+$  and  $ER^+$  tumors (14, 66, 67, and 68).

The *erbB2* gene is also regulated in a negative fashion by various factors. Some of which, are PAX-2, MBP-1 and HDAC1, GATA4 and FOXP3. PAX2 competes with SRC-3 for binding of the  $ER\alpha$  receptor when it is bound to its ligand and the complex binds to the cis-regulatory region of the *erbB2* gene and causes a repressive effect on its transcription (14). In  $ErbB2$  overexpression tumors where SRC-3 expression is high, PAX-2 expression is low (14, 66, 67, and 68). In a study to test the ability of PAX-2 to increase or decrease sensitivity to tamoxifen, MCF 7 a breast cancer  $ER^+/ErbB2^-$  cells were treated in the presence of tamoxifen or estrogen where PAX-2 was silenced by siRNA. It was observed that the PAX-2 siRNA cells showed an increase in  $ErbB2$  mRNA expression (68).

MBP-1 and HDAC1 also negatively regulate *erbB2* expression by binding to its proximal promoter region (71-77). MBP-1 recruits HDAC1 to the proximal promoter region of the *erbB2* gene causing the deacetylation of histone H4 and transcriptional repression of *erbB2* (73). Recent



studies have shown that ErbB2 expression levels have an inverse correlation to MBP-1 and HDAC expression levels in ErbB2<sup>+</sup> tumor samples (72). Interestingly, inverse correlations of repressor factors of the *erb2* gene to upregulation factors has been seen in a number of ErbB2<sup>+</sup> tumor samples (68, 72, 78, and 80). This could lead to new possible therapy ideas where exogenous expression of these repressive factors may help to decrease tumor progression of these ErbB2<sup>+</sup> tumors. FOXP3 is an X-linked gene that binds to a consensus sequence in the 5' *erb2* gene promoter region and represses transcription (78). FOXP3 expression has been shown to negatively correlate with ErbB2 overexpressing mammary tumors (78, 79). GATA4 is a member of the zinc finger transcription factors and it binds to the GATA motif (s) located in the *erb2* proximal promoter region and downregulates *erb2* transcription (80). GATA4 gene expression was also shown to be negatively correlated with ErbB2 overexpression (79). By understanding the transcriptional regulation of ErbB2, can pave the way for thinking about possible new therapy targets and prognosis markers in ErbB2<sup>+</sup> breast cancers.

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## **APPENDIX B**

**A novel interaction of ECD protein with R2TP complex component RUVBL1 is required for the functional role of ECD in cell cycle progression**

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*Key words:* Cell cycle/CK2/Ecdysoless/R2TP/ RUVBL1/RB

**Running title:** ECD-RUVBL1 interaction regulates cell cycle progression.

### **Abstract**

**Ecdysoneless (ECD) is an evolutionarily-conserved protein whose germline deletion is embryonic lethal. Deletion of *Ecd* in cells causes cell cycle arrest which is rescued by exogenous *ECD*, demonstrating a requirement of *ECD* for normal mammalian cell cycle progression. However, the exact mechanism by which ECD regulates cell cycle is unknown. Here, we demonstrate that ECD protein levels and subcellular localization are invariant during cell cycle progression, suggesting a potential role of post-translational modifications or protein-protein interactions. As phosphorylated ECD was recently shown to interact with the PIH1D1 adaptor component of the R2TP co-chaperone complex, we examined the requirement of ECD phosphorylation in cell cycle progression. Notably phosphorylation-deficient ECD mutants that failed to bind to PIH1D1 *in vitro*, fully retained the ability to interact with the R2TP complex, yet exhibited a reduced ability to rescue *Ecd*-deficient cells from cell cycle arrest. Further biochemical analyses demonstrated that an additional phosphorylation-independent interaction of ECD with the RUVBL1 component of the R2TP complex, and this interaction is essential for ECD's cell cycle progression function. These studies demonstrate that interaction of ECD with RUVBL1, and its CK2-mediated phosphorylation, independent of its interaction with PIH1D1, are important for its cell cycle regulatory function.**



## Introduction

Precisely regulated cell proliferation is essential for embryonic development as well as homeostasis in adult organs and tissues, whereas uncontrolled cell proliferation is a hallmark of cancer (1). A more in-depth understanding of the regulatory controls of cell cycle progression is therefore of great interest.

The *Ecd* gene was originally inferred from studies of *Drosophila melanogaster* *ecdysoneless* (or *ecd*) mutants that exhibit defective development due to reduced production of the steroid hormone ecdysone (2). Subsequent cloning of drosophila *ecd* helped identify a cell-autonomous role of ECD protein in cell survival aside from its non-cell autonomous role in ecdysis (molting) (3). However, the molecular basis of how ECD functions remains unknown (3). The human *ECD* homologue was initially identified in a screen of human open reading frames that complemented the *S. cerevisiae* mutants lacking *Gcr2* (Glycolysis regulation 2) gene, and it rescued the growth defect caused by reduced glycolytic enzyme activity in *Gcr2* mutants. The human gene was initially designated as *HSGT1* (human suppressor of *Gcr2*), and was suggested to function as a co-activator of glycolytic gene transcription (4). However, ECD protein bears no structural homology to *Gcr2* and a true ECD orthologue is absent in *S. cerevisiae*, suggesting that ECD likely functions by distinct mechanisms.

We identified human ECD in a yeast two-hybrid screen of human mammary epithelial cell cDNA-encoded proteins for novel binding partners of the Human Papilloma Virus 16 (HPV16) E6 oncogene (5). We showed that deletion of *Ecd* gene in mice causes embryonic lethality, identifying an essential role of ECD during early embryonic development (6). Notably, Cre-mediated conditional deletion of *Ecd* in *Ecd<sup>fl/fl</sup>*

mouse embryonic fibroblasts (MEFs) led to a G1/S cell cycle arrest, and this phenotype was rescued by ectopic expression of human *ECD* (6), indicating an essential role of *ECD* in promoting cell cycle progression. We showed that *ECD* can interact with the retinoblastoma (RB) protein and reduces the repression of RB on E2F transcription factors, providing a novel mechanism by which *ECD* functions as a positive factor of mammalian cell cycle progression (6). Recently, *ECD* was shown to play a vital role in pre mRNA splicing by interacting with the splicing factor Pre-mRNA-processing-splicing factor 8 (PRPF8) (7). We and others have shown that *ECD* shuttles between nucleus and the cytoplasm, with a predominantly cytoplasmic steady-state localization due to rapid nuclear export (7, 8). Consistent with these key cellular roles of *ECD*, we found that *ECD* is significantly overexpressed in breast and pancreatic cancers, and its overexpression correlates positively with poor prognostic factors and poor patient survival (9, 10).

A pull-down screen using the phospho-peptide-binding domain of PIH1D1, the adaptor component of the evolutionarily-conserved prefoldin-like co-chaperone complex R2TP, recently identified *ECD* as one of the binding partners (11). This interaction was shown to require dual phosphorylation of Ser-505 and Ser-518 on *ECD* (11), suggesting that *ECD* phosphorylation may mediate its interaction with the R2TP complex. To date, this interaction has not been demonstrated in the context of endogenous *ECD* nor has a functional role of this interaction been determined. The core R2TP complex is composed of four proteins, PIH1D1, RPAP3, RUVBL1, and RUVBL2 (each with a number of other names) (12). The R2TP complex is involved in the assembly of multi-subunit complexes, including the small nucleolar ribonucleoproteins (snoRNPs), RNA

polymerase II, and phosphatidylinositol 3-kinase-related kinases (PIKKs) and their complexes (13-15). As such, the R2TP complex is involved in a number of essential cellular processes. The closely-related RUVBL1 and RUVBL2 proteins are AAA+ (ATPases associated with diverse cellular activities) that are essential for R2TP function (16). Recent studies have shown that RUVBL1 (Pontin) plays an important role in cell cycle regulation (17, 18). Germline deletion of *Ruvb1* was shown to be early embryonic lethal (18, 19). Depletion of RUVBL1 in AML1-ETO fusion oncogene-expressing leukemic cells was shown to cause cell cycle arrest (17) and Cre mediated deletion of *Ruvb1* in *Ruvb1<sup>fl/fl</sup>* cells also led to G1/S cell cycle arrest (18). The apparent similarities in the embryonic lethality and cell cycle arrest phenotypes imparted by the loss of ECD or RUVBL1 expression suggested the likelihood that the recently described interaction with the R2TP complex (11) may underlie the functional requirement of ECD in cell cycle progression.

In this study, we extensively analyzed the mechanism of ECD-R2TP interaction and how disabling this interaction by mutations in ECD affects the latter's role in cell cycle progression. We demonstrate that ECD levels and localization do not vary during cell cycle progression. We show that Casein Kinase 2 (CK2) phosphorylates ECD in cells at 6 major sites and a mutant ECD (6S/A) disabled for CK2-mediated phosphorylation exhibits reduced ability to rescue the cell cycle arrest caused by *Ecd* gene deletion. Notably, while ECD can interact with PIH1D1, loss of this interaction by mutating CK2 phosphorylation sites did not impact the ECD-R2TP association in cells. We identified a novel interaction of ECD with RUVBL1, independent of ECD's interaction with PIH1D1, which we show to be essential for ECD's cell cycle progression function.

Notably, a phospho-mimetic mutant (6S/D) of ECD failed to bind PIH1D1 and was incompetent at rescuing the cell cycle arrest caused by *Ecd* gene deletion, suggesting a potential accessory role for PIH1D1-ECD interaction. Taken together, our results demonstrate that while CK2-mediated phosphorylation of ECD is important for its role in cell cycle progression, ECD's interaction with PIH1D1 is dispensable, suggesting that the novel RUVBL1-ECD interaction that we identified is particularly critical for ECD's function in cell cycle.

## **Materials and Methods**

### **Reagents**

$\lambda$  protein phosphatase (# P9614) was purchased from Sigma Aldrich USA and the treatment was given according to manufacturers' instruction. 12.5% SuperSep Phos-tag™ (50  $\mu$ mol/L) was purchased from Wako Laboratory Chemicals (catlog # 195-16391). Electrophoresis was performed according to manufacturer's protocol. PreScission protease was purchased from GE health care life sciences.

### **Cells culture**

HEK-293T, MEFs and T98G glioblastoma cell lines were grown in DMEM (Life Technologies, Grand Island, N.Y.) supplemented with 10% fetal calf serum. Immortal mammary epithelial cell line 76NTERT was cultured in DFCI-1 medium, as described previously (20). U2OS cell line was cultured in  $\alpha$ -MEM medium. CK2 inhibitor TBB was dissolved in DMSO and used at 50  $\mu$ M concentration.

### **Plasmid constructs, site-directed mutagenesis and transfection**

Generation of the pMSCV-puro (Clontech)-based expression constructs for FLAG-ECD and its truncated versions has been described previously (6, 8). The pMSCV-puro construct expressing ECD with deletion of amino acids 499-527 was generated using a three-fragment ligation into BglIII and HpaI sites. C-terminally His<sub>6</sub>-tagged ECD truncations (1-567, 1-534, 1-432) were generated through PCR amplification, cloning into XbaI and SalI sites of pET-28b+ vector (Invitrogen) and recombinant proteins were purified after expression in *E. coli* BL21DE3 strain using Nickel affinity column (GE Healthcare). C-terminally His<sub>6</sub>-tagged full-length ECD was generated by cloning *ECD* coding sequence into SalI and NotI sites of the pFastBac1 vector (Invitrogen), expressed in Sf21 insect cells and purified using Nickel affinity column. The PIH1D1-specific and control siRNAs (Santa Cruz, Sc-97385) were transfected into sub-confluent cells using DharmaFECT<sup>®</sup>1 transfection reagent (Thermo Scientific). GFP tagged full-length or truncated ECD expression constructs in the pGen2 vector were generated by replacing the ST6-GalI insert in the ST6-GalI-pGen2 construct (ST6GAL1-pXLG-NtermTCMhisStrep-DEST) (21) with PCR-amplified ECD coding sequences at the EcoRI and HindIII sites by infusion cloning kit (Clontech). The primer sequences used for cloning are indicated in Table S2. Human PIH1D1 cDNA sequences (Origene, clone SC321317) were subcloned into BamHI and XhoI sites of pGEX-6p-1 for expression as a GST fusion protein in *E. coli* BL21 strain. A recombinant GST-PERK kinase domain was expressed in BL21DE3 cells purified as GST-fusion protein.

Point mutants of ECD were generated using a PCR-based commercial kit (GENEART Site-Directed mutagenesis system, Invitrogen), according to the manufacturer's instructions, cloned into the pET28b+ vector for His-tagged recombinant

protein expression and purified using Nickel affinity column. The PCR primer sequences are listed in table S2. All constructs were verified by sequencing.

DNA constructs were transfected in HEK-293T cells using the X-tremeGENE transfection reagent (Roche). Retroviral infection was carried out as described previously (6).

### **Flow Cytometry for Cell cycle Analysis and Biochemical Fractionation**

76NERT cells were plated at  $5 \times 10^5$  cells per 100-mm dish for 12 hours, subjected to growth factor deprivation by culturing in growth factor-free DFCI-3 medium for 72 hours (20) and released from synchrony using growth factor-containing DFCI-1 medium (20). Half of the cells were fixed for fluorescence-activated cell sorter (FACS) analysis after fixation in chilled 70% ethanol and staining with propidium iodide; the remaining cells were used for western blotting. G2/M to G1 progression in MEFs was similarly assessed using FACS analysis following Nocodazole (100 ng/ml)-dependent arrest in early G2/M phase of cell cycle (22). Nuclear and cytoplasmic fractions were prepared from cells at various times points during cell cycle progression using the NEPER<sup>TM</sup> kit (Thermo Scientific, cat# 78833). To assess the mitotic index of *Ecd<sup>fllox/fllox</sup>* MEFs, infected with adeno-Cre-GFP or control Adeno-GFP. Cells were collected and fixed as described above. Cell pellet was resuspended in 100  $\mu$ l of PBS containing 1% BSA and 0.25  $\mu$ g of phospho-H3-S10 (abcam cat # ab14955) and then incubated for 1 h at room temp. Cells were washed in 150  $\mu$ l of PBS and resuspended in Alexa Fluor<sup>®</sup> 647 (A212235, Life Technologies) conjugated goat anti-mouse antibody diluted at a ratio of 1:300 in 100  $\mu$ l of PBS containing 1% BSA and incubated at room temperature in the dark for 30 min, followed by FACS analysis. The Median Fluorescence Intensity (MFI)

of GFP-positive cells at 488 and 633 nm wavelengths was recorded as an indicator of mitosis in the control and *Ecd-null* cells.

### **Immunoblotting and Immunoprecipitation**

Cells were lysed in radio-immunoprecipitation assay (RIPA) buffer (20 mM Tris, pH 7.2, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS]) and protein concentration was measured using the BCA protein assay reagent (Pierce). Immunoblotting was performed with primary antibodies against ECD (9), RB (554136, Pharmingen), anti-phospho-Ser (05-1000, Millipore), anti-phospho-Th (AB1607, Millipore), PIH1D1 (sc-101000 or sc-390810, Santa Cruz), RUVBL1 (12300S, Cell Signaling or SAB4200194, SIGMA) RUVBL2 (ab36569, Abcam), RPAP3 (HPA038311, SIGMA), PARP (sc-8007, Santa Cruz), Histone H3 (06-755, Millipore), PRPF8 (ab137694, Abcam),  $\beta$ -actin (A5441, SIGMA) or  $\alpha$ -tubulin (T6199, SIGMA), as indicated. For immunoprecipitations, cells were lysed in NP-40 lysis buffer (20 mM Tris-HCl (pH 7.5), 200 mM NaCl, 0.5% Nonidet P-40 [NP-40], 1 mM NaF, 0.1 mM Na<sub>3</sub>VO<sub>4</sub> and protease inhibitor mixture (Roche Applied Science)) and then immunoprecipitated with 3  $\mu$ g of antibodies against ECD or 35  $\mu$ l of Ezview red anti FLAG M2 affinity gel, (SIGMA) for 2 h to overnight at 4 °C. The immune complexes were captured with protein A/G-agarose (sc-2003, Santa Cruz Biotechnology). To elute FLAG-tagged proteins from anti-FLAG beads before analysis, immune complexes were incubated with 150 ng/ $\mu$ l of 3X FLAG peptide (SIGMA) for 15 min at room temp and supernatants were collected for SDS-PAGE. For PIH1D1 interaction with ECD, cell lysates were prepared in CHAPS lysis buffer (0.3% CHAPS, 0.20 mM Tris-HCl (pH 7.4), 120 mM NaCl, 10% glycerol, 5 mM EDTA) supplemented with protease and phosphatase inhibitor (Roche).

RUVBL1 immunoprecipitation to assess association with ECD was carried out using a monoclonal anti-RUVBL1 antibody (cat# SAB4200194-200UL SIGMA, 2  $\mu$ g). Immunoprecipitated RUVBL1 (close to IgG heavy chain) was detected by western blotting with an anti-RUVBL1 antibody (cat#12300s, cell signaling) that was conjugated to HP using the Lightning-Link® HP Conjugation Kit (NOVUS BIOLOGICALS).

### ***In vitro* kinase assay**

500 ng of purified recombinant ECD proteins or its mutants were incubated with 0.2 mM ATP, 1 $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P]ATP (PerkinElmer) and 0.2  $\mu$ l (10 units) human recombinant CK2 (NEB, Beverly, MA) at 30°C for 30 min, or as indicated. The reaction was stopped by adding SDS-PAGE sample buffer. The [<sup>32</sup>P] labeled proteins were detected by autoradiography following SDS-PAGE and then transfer to PVDF membranes (Millipore). Once the radioactive signals had decayed, the membranes were blotted with anti-p-Ser antibodies. 10 ng of recombinant GST-PERK kinase domain was autophosphorylated in kinase assay buffer (50 mM HEPES [pH 8.0], 10 mM MgCl<sub>2</sub>, 2.5 mM EGTA ) supplemented with 20  $\mu$ M cold ATP (NEB).5 ng were loaded on SDS page and subjected to western blotting with anti-p-Ser and anti-p-Thr antibodies.

### **[<sup>32</sup>P] metabolic labeling and immunoprecipitation**

Exponentially-growing or serum-deprived T98G cells were washed with phosphate-free DMEM supplemented with 10% dialyzed fetal bovine serum, and incubated in the same medium for 1 hour before adding 0.1 mCi [<sup>32</sup>P] orthophosphate (NEN) per 10-cm plate. Cells were labeled for 4 hours at 37°C (or for 2, 5 or 16 hours for cell cycle analyses), rinsed once in ice-cold PBS and lysed in ice-cold lysis buffer (250



mM NaCl, 1% NP-40, 20 mM Tris-HCl at pH 7.4, 1 mM EDTA, 2 µg/ml of aprotinin, 2 µg/ml of leupeptin, 1 µg/ml of pepstatin, 2.5 µg/ml of antipain, 1 µg/ml of chymostatin, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 10 mM NaF, 1 mM sodium molybdate, and 0.5 mM PMSF). Labeled ECD was immunoprecipitated with affinity-purified mouse anti-Ecd monoclonal antibody or anti-FLAG beads overnight at 4°C and Protein-G Plus/Protein-A agarose beads added for 1 h. The beads were washed three times with ice-cold wash buffer (150 mM NaCl, 1% NP-40, 20 mM Tris-HCl at pH 7.4, 1 mM EDTA, and protease inhibitors). The immunoprecipitated proteins were resolved on 7.5 % SDS–polyacrylamide gels, transferred to PVDF membrane and visualized by autoradiography.

### ***In vitro* binding assays**

GST-or His-tagged protein pull-downs were performed as described previously (6). FLAG-tagged WT ECD or its mutants (3S/A and 6S/A) were expressed by transient transfection in 293T cells and lysed in CHAPS Lysis buffer as described above. 1000 µg of lysate protein was incubated with 2 µg bead-bound purified GST-PIH1D1 for 2 hours at room temperature, washed five times, and then bound proteins were detected by western blotting with anti-FLAG antibody. Membranes were stained with Ponceau S to visualize GST fusion proteins. *In-vitro* TAP was performed as described (23) using purified recombinant ECD with a C-terminal FLAG tag.

### **Cell proliferation and colony formation assays**

The cell proliferation was analyzed as described (6). Briefly, *Ecd*<sup>flox/flox</sup> MEFs were infected with adenoviruses encoding GFP-Cre or GFP (control) (University of Iowa Gene Transfer Vector Core) and plated at 10<sup>4</sup> cells/well in 6-well plates, followed by counting

of cells at the indicated time points. For colony-formation assay, infected cells were plated at 5,000 or 1,000 per well in 6-well plates for 10 days, the colonies were stained with crystal violet (0.5% crystal violet in 25% methanol), solubilized in 10% acetic acid, and then quantitative measure of colony formation was measured by absorbance at 590 nm.

### **Statistical analysis**

A generalized estimating equation method was used to assess the differences among cell types accounting for the correlated measurement within a sample. Comparisons between WT and other cell types at a given time were made with Simulation's correction. Some results were analyzed using paired two-tailed student's-t test. p values of  $\leq 0.05$  were considered statistically significant.

### **Results**

#### **ECD levels and localization do not change during cell cycle progression**

Given the requirement of ECD for cell cycle progression and its direct association with RB (6), we assessed if ECD levels or localization are altered during cell cycle progression. For this purpose, an immortal mammary epithelial cell line, 76NTERT, was arrested in the G1 cell cycle phase by growth factor deprivation and the cells were then allowed to proceed synchronously through cell cycle phases by culture in regular growth factor-containing medium. FACS analyses showed that a majority of growth factor-deprived cells were growth arrested, with 98% cells in the G1 phase, and only 0.75% cells in the S and 1.25% cells in the G2/M phases (Fig. 1A). Western blotting of lysates showed no significant differences in the levels of ECD protein in cells at various times

during cell cycle progression (Fig. 1B). Analysis of nuclear and cytoplasmic fractions prepared at various times during cell cycle progression showed that ECD localizes primarily in the cytoplasm (Fig. 1C), consistent with its rapid nuclear export, as previously reported (8). Overall, our results indicate that ECD levels and its subcellular localization do not change significantly during cell cycle progression.

**ECD is phosphorylated on serine residues but overall phosphorylation does not change during cell cycle progression**

Given the known roles of phosphorylation in regulating the cell cycle machinery (24), we asked if ECD is a phosphoprotein and whether its phosphorylation varies with cell cycle progression. For these analyses, T98G cells (a human brain glioblastoma cell line that expresses a wild-type RB) (25) were cultured in low serum medium for 48 hours to induce growth arrest and then allowed to progress through cell cycle by adding serum-containing medium with [<sup>32</sup>P] labelled sodium orthophosphate. Autoradiography of anti-ECD immunoprecipitates showed that ECD is indeed a phosphoprotein; however, the levels of phosphorylation were comparable at various time points during cell cycle progression (Fig. 2A). As a control, RB showed an expected cell cycle related increase in phosphorylation at the 16 h and 20 h time points (Fig. 2A). Further analyses using anti-FLAG IPs from [<sup>32</sup>P]orthophosphate-labeled cells expressing an exogenous FLAG-tagged ECD protein confirmed the phosphorylation of ECD in cells (Fig. 2B). These analyses demonstrate that ECD is a phosphoprotein, however the phosphorylation levels do not change during cell cycle progression.

It was reported that a peptide sequence derived from ECD was phosphorylated by casein kinase 2 (CK2) *in vitro* (11). To assess if the phosphorylation of ECD corresponds to phospho-serine (p-Ser) or phospho-threonine (p-Th) residues, anti-FLAG immunoprecipitates of T98G cells expressing a FLAG-tagged ECD were blotted with anti-p-Ser or anti-p-Thr antibodies. A recombinant GST-PERK kinase domain, known to undergo auto phosphorylation on serine and threonine residues during an *in vitro* kinase reaction (26), was used as a positive control for serine and threonine phosphorylation. Indeed, both p-Ser and p-Th signals were detected by blotting of autophosphorylated GST-PERK kinase domain (Fig. 2C). While no signals were detected with anti-p-Thr antibody blotting of anti-ECD immunoprecipitation, even after long exposures, a specific band was observed with the anti-p-Ser antibody (Fig. 2C). These results suggested that cellular ECD is predominantly phosphorylated on serine residues.

### **CK2-mediated phosphorylation of ECD is important for its cell cycle regulation function**

In view of our results presented above, and a recent study that used an array of spotted peptides to identify CK2 phosphorylation of an ECD peptide on Ser-505 and Ser-518 (11), we performed a detailed analysis of potential phosphorylation sites on ECD using the publically available KinasePhos 2.0 tool (<http://kinasephos2.mbc.nctu.edu.tw/>). This analysis identified multiple sites on ECD that could be phosphorylated by various Ser/Th kinases. Among these, CK2 was predicted to preferentially phosphorylate multiple serine residues, and this was of obvious interest in view of our results that cellular ECD is primarily phosphorylated on Ser residues (Fig. 2C). The potential CK2 phosphorylation sites near the C-terminus, including Ser-505 and Ser-518 reported in the peptide array

screen (11), were predicted with the highest confidence (Fig. 3A); . To directly assess if ECD is a CK2 substrate, we performed an *in vitro* kinase assay with purified CK2 and recombinant full-length ECD protein or its C-terminal truncated versions. Phosphorylation was observed with full-length ECD (1-644) and its fragments encompassing residues 1-567 or 1-534, whereas substantially less phosphorylation was observed with the ECD 1-432 fragment (Fig. 3A & B). These results indicated that ECD was indeed a substrate for CK2 *in vitro*, and that CK2-dependent phosphorylation occurs predominantly within the C-terminal region of ECD.

CK2 is known to phosphorylate its substrates in clusters, with phosphorylation at one site priming the substrate for phosphorylation at additional sites (27, 28). The C-terminal region contains two potential Ser clusters, a proximal cluster of S503, S505 and S518, and a distal cluster of S572, S579 and S584 (Fig. 3C). To assess the contribution of these clusters to CK2-dependent phosphorylation of ECD, we introduced Ser to Ala mutations in these residues, individually as well as in combinations (Fig. 3C & D). While S>A mutations of the Ser residues in the distal cluster (S572A, S579A and S584A; designated as 3'S/A) had no appreciable impact on the level of phosphorylation in the *in vitro* kinase assay, similar mutations in the proximal cluster (S503A, S505A and S518A; designated as 3S/A) led to a considerable reduction in the CK2-mediated phosphorylation (Fig. 3D). Importantly, Ala mutations of all six residues (S503, S505, S518, S572, S579 and S584; designated 6S/A) nearly completely abolished the CK2-mediated *in vitro* phosphorylation of ECD (Fig. 3D). The autoradiography results were confirmed by subjecting the same filters to blotting with anti-p-Ser antibody (Fig. 3D). Since we did not observe a shift in the mobility of the 6S/A mutant on regular SDS-PAGE, perhaps

reflecting a mechanism previously reported by Lee et al. (29), we performed gel analysis of *in vitro* phosphorylated WT, 6S/A and phosphatase treated WT ECD after reacting these with Phos-tag<sup>R</sup> a dinuclear metal complex that acts as a phosphate-binding tag and can produce a mobility shift (30). As expected, the Phos-tag-bound WT ECD resolved as a slower migrating band compared to its phosphatase-treated sample; notably, the 6S/A mutant exhibited a faster mobility compared to phosphorylated WT ECD (Fig. S1A). Collectively, these results show that the C-terminal Ser clusters of ECD, especially the proximal one, can be phosphorylated by CK2. The small residual phosphorylation signal observed with the 6S/A mutant of ECD may reflect an additional minor site of CK2-mediated phosphorylation.

While these experiments confirmed and extended the concept of CK2 mediated phosphorylation of ECD *in vitro*, to relate this post-translational modification to ECD function it was important to assess if ECD is phosphorylated in cells on the same sites and whether such phosphorylation is important for its function. Thus, we generated pMSCV-puro vector-based retroviral constructs encoding the FLAG-tagged wild type (WT), 3S/A or 6S/A mutants of ECD. These constructs were expressed in T98G cells, and cells were metabolically-labeled with <sup>32</sup>P-orthophosphate. Equal amounts of radioactive extracts (based on counts per minute) were subjected to anti-FLAG IP followed by autoradiography. While the phosphorylation signal observed with the 3S/A mutant was comparable to that on the WT ECD, the level of phosphorylation on the 6S/A mutant was markedly reduced (Fig. 3E). To ascertain if the defective phosphorylation of the cell-expressed 6S/A mutant reflects simply a lack of phosphorylation of the distal serine cluster, we compared the levels of phosphorylation of FLAG-tagged WT vs 3'S/A

mutant by anti-p-Ser immunoblotting of anti-FLAG IPs of lysates of T98G cells transfected with the respective constructs. We did not observe any significant differences in the anti-p-Ser signals of WT ECD vs its 3S/A mutant (Fig. S1B). These results establish that the two serine clusters in ECD identified *in vitro* as CK2 substrate sites are the major sites of phosphorylation in cells. Next, T98G cells expressing FLAG-tagged WT, 3S/A and 6S/A ECD proteins were left untreated or treated with a CK2-specific inhibitor **4,5,6,7-Tetrabromo-2-azabenzimidazole** (TBB) and their anti-FLAG IPs were blotted with anti-p-Ser antibody (Fig. 3F). Notably, CK2 inhibition reduced the phosphorylation signal in cells expressing the WT ECD or its 3S/A mutant, however cells expressing the 6S/A mutant did not exhibit any change in phosphorylation (Fig. 3F), indicating that CK2 is the primary cellular kinase responsible for phosphorylation of ECD on two major serine clusters characterized here. However, it remains possible that the additional Ser or Thr residues of ECD are phosphorylated by other kinases depending on the cell type or varying functional states.

Next, we assessed if the phosphorylation of ECD on CK2-dependent serine clusters is relevant to its cell cycle regulatory function. We have previously demonstrated that introduction of Cre recombinase in *Ecd<sup>fl/fl</sup>* MEFs, using adenovirus Cre, causes G1 cell cycle arrest that is largely rescued by introducing human ECD (6). We used this approach to compare the extent of the rescue of cell cycle arrest induced by endogenous ECD deletion upon introducing the wild-type ECD or its phospho-defective mutants. In initial experiments, we expressed the WT human ECD or its 3S/A or 6S/A mutants in *Ecd<sup>fl/fl</sup>* MEFs (Fig. 3G), and then assessed the ability of cells to progress through cell cycle without or with Cre-induced *Ecd* deletion. In each case, the expression of exogenous

human ECD proteins and the depletion of endogenous mouse *Ecd* were confirmed by western blotting (Fig. 3G). As expected, deletion of *Ecd* in *Ecd*<sup>fl/fl</sup> MEFs arrested proliferation with no recovery during the entire observation period (Fig. 3H) and ectopic WT ECD significantly rescued the cells from growth arrest (Fig. 3H & S2A). Notably, while the 3S/A mutant behaved comparably to WT ECD in rescuing cells from growth arrest, the 6S/A mutant only exhibited a partial rescue in comparison to that seen with WT ECD in repeated experiments ( $p < 0.001$ ; Fig. 3H) ( $p$  values shown in Supplemental Table S1). Furthermore, we examined a mutant in which serine residues 503, 505 and 518 were removed by deletion ( $\Delta 499-527$ ) in the cell cycle rescue experiment and observed that this mutant behaved similar to WT ECD in rescuing the proliferation block (Fig. S2A & B). Next, we generated a phospho-mimetic mutant in which the six serine residues identified to be phosphorylated were mutated to aspartic acid residues (6S/D). Notably, the phospho-mimetic mutant 6S/D was completely defective in cell cycle rescue experiment (Fig. 3I & J). In these experiments, we also examined the 3'S/A mutant and observed a partial rescue with this mutant (Fig. 3J). While the complete lack of rescue seen with the 6S/D mutant of ECD was surprising, it has been reported that aspartic acid phospho-mimics are unsuitable for biological readouts due to different chemical properties of the two residues (31, 32). Taken together, our results underscore the importance of ECD phosphorylation for cell cycle progression.

**Phospho-defective ECD mutants retain their ability to interact with PIH1D1 protein, as well as with other components of the R2TP complex**

In view of the complete lack of any functional impact of mutating ECD on S503/505/518 residues, we first re-examined the previously reported dependence of ECD binding to the



isolated phospho-reader domain of PIH1D1 (11). For this purpose, GST-PIH1D1 pull-down was carried out with lysates of HEK-293T cells transiently-transfected to express WT ECD or its 3S/A and 6S/A mutants. Confirming previous findings (11), WT ECD but not its 3S/A or 6S/A mutant was pulled down with GST-PIH1D1 (Fig. 4A). These results suggested that either the R2TP association was unnecessary for ECD function in cell cycle progression or an alternate mechanism may recruit ECD to the R2TP complex. To distinguish between these possibilities, we first carried out anti-ECD IPs of U2OS and MEF cell lysates, followed by anti-PIH1D1 blotting. These analyses confirmed the interaction of endogenous ECD and PIH1D1 (Fig. 4B & C). To examine the nature of ECD/PIH1D1 interaction in cells, we carried out anti-PIH1D1 immunoprecipitations from HEK-293T cells expressing untagged (Fig. 4D) or GFP-tagged (Fig. 4E & S3A) WT ECD or its phosphorylation site mutants (defective in binding to PIH1D1 in the pull-down assay) followed by blotting for ECD (Fig. 4D) or GFP (Fig. 4E & S3A) as well as for the four R2TP complex components (Fig. 4D, 4E & S3A). Since a phosphorylated DS<sub>p</sub>DD/E motif, conserved between human and mouse ECD proteins (Fig. 4F), was previously found to promote the interaction of ECD with PIH1D1 *in vitro* (11), we also examined a deletion construct ( $\Delta$ 499-527) of ECD that lacks the DSDD/E motif in addition to the S6/A mutant lacking all CK2-phosphorylated sites. As expected, PIH1D1 IPs were able to co-IP RPAP3, RUVBL1 or RUVBL2 to a similar extent in all lanes (Fig. 4D & E). Notably, compared to the levels of endogenous ECD co-IP with PIH1D1 in vector control lanes, increased amounts of ectopically expressed ECD were co-IPed in WT ECD transfected lanes. Unexpectedly, however, the WT and mutant ECD proteins were co-IPed with PIH1D1 to comparable levels (Fig. 4D, 4E & S3A). These results

demonstrate that ECD interacts with the R2TP complex in cells but the phosphorylation-dependent interaction of ECD with PIH1D1 is dispensable for this association. In further support of this conclusion, we carried out anti-ECD and anti-PIH1D1 IPs and treated these IP's with lambda phosphatase, and then assessed the levels of co-immunoprecipitated PIH1D1. Notably, while the phosphatase treatment robustly eliminated the phosphorylation signal on ECD (anti-p-Ser blot), no reduction in PIH1D1 or ECD co-immunoprecipitation was seen (Fig. S3B & C). Thus, while CK2-phosphorylated ECD can directly interact with PIH1D1, as reported (11), this interaction is not required for the association of ECD with the R2TP complex. Next, we examined the ability of 6S/D or 3'S/A mutants of ECD to interact with PIH1D1. For this purpose, lysates from 293T cells expressing FLAG tagged 3'S/A or 6S/D mutants were used for an *in vitro* pull-down assay with GST-PIH1D1. As expected, GST-PIH1D1 was able to pull down the 3'S/A mutant but failed to pull down the 6S/D mutant of ECD (Fig. S3D), confirming that 6S/D does not mimic WT ECD for its interaction with PIH1D1.

### **A novel phospho-independent interaction of ECD with R2TP complex through RUVBL1**

Since disabling ECD binding to PIH1D1 in the 3S/A mutant had no impact on ECD association with the R2TP complex in cells or on ECD function during cell cycle progression, we used an unbiased approach to identify potential mediators of ECD's interaction with the R2TP complex. We used an *in vitro* tandem affinity purification approach (23) to identify ECD interacting partners. For this purpose, full-length ECD was tagged with GST on the N-terminus and with FLAG epitope on the C-terminus and the twin-tagged recombinant protein was prepared in a glutathione-Sepharose bead-bound

form. Cell lysates prepared from 76NTERT cells were incubated with these beads and proteins in the complex were eluted by cleaving the ECD-FLAG part of the GST-ECD-FLAG fusion on beads with PreScission Protease. The eluted ECD-FLAG, in complex with cellular proteins, was subjected to a second round of affinity purification using anti-FLAG antibody beads, and the protein complexes were eluted with a FLAG epitope peptide. The proteins in the complex were separated by SDS-PAGE and visualized by silver staining (Fig. 5A), and bands only seen in lanes where recombinant protein was incubated with cell lysates were excised and subjected to mass spectrometry. Proteins with Mascot scores of >50 were considered potential interacting partners.

These analyses identified a known ECD binding partner PRPF8 (7) and in addition revealed several new binding partners. Interaction of PRPF8 with ECD was confirmed by immunoprecipitation (Fig. S3E). Among the new partners, RUVBL1 was one of the top candidate proteins with a Mascot score of 168. To validate the purification results, we expressed the FLAG-tagged WT ECD or its 6S/A mutant in 293T cells, and performed co-immunoprecipitation experiments using an anti-RUVBL1 antibody. Both the WT and 6S/A ECD proteins were co-immunoprecipitated with RUVBL1, suggesting that ECD interacts with RUVBL1 and that this interaction is independent of ECD phosphorylation (Fig. 5B). To further establish that ECD-RUVBL1 interaction is independent of PIH1D1, we knocked down the endogenous PIH1D1 with siRNA and then performed a co-IP experiment using an anti-ECD antibody. Notably, we observed equal co-immunoprecipitation of RUVBL1 in both control and PIH1D1 knock-down cells, confirming that ECD interaction with RUVBL1 is PIH1D1-independent (Fig. 5C). Taken together, these results demonstrate that ECD associates with the R2TP complex

though a novel interaction with RUVBL1, independent of ECD's interaction with PIH1D1.

### **Interaction with RUVBL1 is important for the role of ECD in cell cycle progression**

Germline deletion of *Ecd* or *Ruvbl1* is embryonic lethal (6, 18) and silencing of either protein in cells leads to cell cycle arrest (6, 17, 18), suggesting that interaction of ECD with RUVBL1 may play a role in the cell cycle regulation function of ECD. Towards testing this hypothesis, we first expressed GFP tagged ECD or its several C-terminal deletion mutants in HEK-293T cells and performed co-immunoprecipitation experiments using an anti-RUVBL1 antibody. Notably, only full length ECD protein (aa 1-644) co-immunoprecipitated with RUVBL1, while none of the C-terminal deletions of ECD were able to co-immunoprecipitate with RUVBL1 (Fig. S4A & B). To validate these results, we constructed several GST-tagged and His-tagged ECD deletion fragments based on the predicted secondary structure (by Garnier Robson predictions and PONDR VL-XT secondary structure prediction) and then examined the direct interaction of these ECD mutant proteins with FLAG-RUVBL1 or endogenous RUVBL1, using pull-down with glutathione-Sepharose or Nickel beads. As shown in (Figs. 6A, B & S4C), only the full-length ECD interacts with RUVBL1, whereas all C-terminal or N-terminal deletions rendered ECD defective in binding to RUVBL1. Next, we compared various FLAG-tagged deletion fragments of ECD (150-438, 438-644, 1-438 and 150-644) (Fig. S4D) with wild type ECD (1-644) for their abilities to rescue the growth arrest of *Ecd*<sup>fl/fl</sup> MEFs upon adeno-Cre-mediated endogenous *Ecd* deletion, by analyzing cell proliferation by cell counting or colony formation (Fig. 6D). In each case, the expression of exogenous human ECD proteins and loss of expression of endogenous mouse *Ecd* in Cre-expressing

cells was confirmed using Western blotting (Fig. 6C & S4D). As expected, deletion of *Ecd* in *Ecd<sup>fl/fl</sup>* MEFs arrested cell proliferation, and ectopic WT human ECD significantly rescued the cells from growth arrest (Fig. 6D). However, none of the deletion mutants were able to rescue the cell proliferation block imposed by endogenous Ecd depletion. These results demonstrate that only the full-length ECD, which interacts with RUVBL1, supports cell cycle progression. The lack of rescue with ECD deletion fragments was not due to lack of their expression (Fig. 6C & S4D). Notably, two deletion fragments (438-644 and 150-644) that failed to rescue cell cycle arrest, still retained their ability to interact with PIH1D1 (Fig. 6E), further underscoring the conclusion that interaction of ECD with PIH1D1 is dispensable while its interaction with RUVBL1 is indispensable for a role in cell cycle progression. PIH1D1 is known to directly interact with other components of the R2TP complex, such as RUVBL1 (33). Re-probing of the same membrane with antibodies against RUVBL1 and RUVBL2 showed the expected interaction of PIH1D1 with RUVBL1 or RUVBL2 (Fig. S4E).

Our previous studies showed that ECD interacts with RB, a function important for ECD function in cell cycle progression (6). Notably, in addition to the expected interaction of full-length ECD with RB, one mutant (150-644 aa) that is defective in rescuing cell cycle arrest (Fig. 6D) was earlier shown to retain its ability to interact with RB (6), suggesting that interaction with RUVBL1 is required for ECD to promote cell cycle progression while interaction with RB in the absence of interaction with RUVBL1 is insufficient for this function. In further support of this conclusion cell cycle function competent (3S/A) and deficient mutant (6S/A) of ECD show comparable interaction with RB (Fig. S4F).

### ***Ecd* deletion leads to reduced mitotic index and delayed mitotic progression**

We have previously reported that the proliferation arrest upon *Ecd* deletion is not associated with any increase in apoptosis (6). To examine the effect of *Ecd* deletion on mitosis, we used adeno-Cre to delete *Ecd* in *Ecd<sup>fl/fl</sup>* MEFs and measured the Median Fluorescence Intensity (MFI) of phospho histone H3 (S10) as an indicator of the proportion of cells in mitosis using flow cytometry (34). *Ecd* deleted cells showed a marked decrease in the MFI (45.7) of pH3 (S10) as compared to control cells (89.8) (Fig. 7A), indicating that *Ecd* deleted cells are arrested prior to entering mitosis. Low levels of pH3 (S10) were further confirmed by western blotting (Fig. 7B). Next, we assessed the G<sub>2</sub>/M to G<sub>1</sub> progression of MEFs arrested in the S phase by nocodazole treatment (Fig. 7C). Flow cytometry analysis revealed a significant impairment in G<sub>2</sub>/M to G<sub>1</sub> phase transition upon *Ecd* deletion as compared to control, in addition to a higher percentage of *Ecd* deleted MEFs in the G<sub>1</sub> phase (Fig. 7C, D & E). Taken together, these results demonstrate a critical role of ECD in both G<sub>1</sub> to S and G<sub>2</sub>/M to G<sub>1</sub> transition. These results are consistent with the known function of CK2 and RUVBL1 in cell cycle regulation (35-37).

### **Discussion**

Precise regulation of the entry into, progression through and exit from cell cycle is fundamental to developmental programs and maintenance of adult tissues in multicellular organisms. Notably, components of the cell cycle machinery and the pathways that regulate their functions are commonly altered in cancer and other diseases (1). Thus,

elucidating how the cell cycle machinery is controlled is an important area of research in cell and cancer biology.

We have previously shown that ECD, the mammalian orthologue of *Drosophila ecdysoneless* gene, is required for embryonic development and progression of mammalian cells through the G<sub>1</sub>-S phase of cell cycle progression (6). Here, we identify a novel mechanism by which ECD functions as an essential element of mammalian cell cycle progression. Using multiple complementary approaches, we demonstrate a novel interaction of ECD with the R2TP chaperone complex, mediated by the RUVBL1 component of R2TP, which we establish is required for ECD to promote cell cycle progression. We also identify a role for the CK2-dependent phosphorylation of ECD in cell cycle progression, but contrary to predictions from a previous study (11), this role is independent of the ECD interaction with PIH1D1, the phospho-reader component of the R2TP complex.

Our findings establish that phosphorylation of ECD positively regulates its function in promoting the cell cycle progression. Bioinformatics analysis followed by mass spectroscopy-based phospho-proteomics identified a number of sites that could be phosphorylated by cellular kinases but we focused on two clusters of potential CK2 phosphorylated serine residues since a recent study (11) showed that CK2-mediated phosphorylation of two such serine residues in the context of a peptide created a binding site for the phospho-reader subunit of the R2TP complex. CK2-dependent phosphorylation of site-directed mutants of ECD *in vitro*, and in cultured cells, identified six serine residues in two spatially separated clusters to be the major CK2 phosphorylation sites on ECD. Notably, however, ECD phosphorylation does not change

during cell cycle progression. This is not entirely surprising since our *in vitro* analyses as well as phosphorylation studies in cells in the presence of a CK2 inhibitor (Fig. 3 D & F) establish that CK2 is the predominant kinase that phosphorylates ECD; CK2 is considered to be constitutively-active and ubiquitous serine/threonine protein kinase (38). Despite its constitutive activity though, numerous studies point to a role for CK2 in cell proliferation and survival (39). Yet, the molecular pathways that mediate the function of CK2 in cell proliferation are largely unknown. We suggest that phosphorylation of ECD by CK2 provides one mechanism for CK2's role in cell proliferation. While the overall levels and the subcellular localization of ECD remain invariant during cell cycle progression (Fig. 1 & 2), it remains possible that ECD phosphorylation at specific sites may vary during cell cycle progression. As phospho specific antibodies against specific serine residues on ECD become available, it should be feasible to test this notion further.

Our findings that ECD is indeed a CK2 substrate *in vitro* (Fig. 3) suggested that CK2-dependent phosphorylation and subsequent interaction of ECD with the R2TP complex could provide a potential mechanism by which ECD could promote the cell cycle transit. Our co-IP studies in cell cultures demonstrate that ECD in fact is in a complex that includes the four core subunits of the R2TP complex (Fig 4D & E). Remarkably, however, multiple mutant ECD proteins, rendered incapable of directly interacting with PIH1D1, including mutations of critical serine residues in the 3S/A mutant or the 6S/A mutant or deletion of the region incorporating the major CK2 phosphorylation sites and the acidic motif DSDD that facilitates PIH1D1 interaction (11), fully retained the ability to associate with the R2TP complex. Furthermore, the ECD-R2TP association was retained in PIH1D1-depleted cells (Fig 5C). Thus, our results



support a PIH1D1-independent mechanism of ECD association with the R2TP complex. Importantly, S>A mutation of ECD residues that impart PIH1D1 binding (3S/A & Δ499-527) had no impact on its ability to function in cell cycle progression. However, 6S/A and 6S/D were defective in cell cycle rescue experiment, underscoring the importance of ECD phosphorylation for its function that encompasses amino acids beyond PIH1D1 interaction. Thus, the role of phosphorylation in regulating ECD function during cell cycle appears to be independent of mediating an interaction with PIH1D1. It remains possible, however, that phosphorylation-dependent interaction of ECD with PIH1D1, and consequently with the R2TP complex, is required for other functions of ECD aside from its role in promoting cell cycle progression (6). For example, we have shown that ECD overexpression in cells leads to p53 stabilization and increased p53-dependent target gene expression, and induction of a senescence phenotype in primary fibroblasts (5). ECD was also found to interact with thioredoxin-interacting protein (TXNIP), which was shown to promote p53 stabilization (40). TXNIP has a number of other functions including regulation of glucose uptake, oxidative stress and ER stress-induced apoptosis (41, 42), Thus ECD phosphorylation and interaction with PIH1D1 may play a role in regulating these functions. The availability of *Ecd*<sup>fl/fl</sup> MEFs in which ECD can be conditionally deleted together with phosphorylation-defective mutants that we have characterized here should allow these notions to be tested in the future.

In view of a novel, PIH1D1-independent mechanism of ECD association with the R2TP complex, we sought to answer two key mechanistic questions: one, what the determinants of ECD-R2TP association are, and second, whether this unique mode of interaction is functionally relevant in the context of cell cycle progression role of ECD?

Unbiased proteomics analysis of cellular proteins that interacted with a recombinant full-length ECD protein, followed by biochemical analyses in cells, demonstrated that ECD interacts with another component of the R2TP complex, RUVBL1 (Fig 5 & 6). Structure-function studies of ECD using deletion fragments demonstrated a strong correlation between the cell cycle progression function of ECD and its ability to interact with RUVBL1, with only the full-length ECD competent at both functions (Fig. 6A, B, S4B & C). Interestingly, the  $\Delta 499-527$  mutant which interacts with RUVBL1 but not PIH1D1, was able to rescue the cell cycle arrest caused by *Ecd* deletion (Fig. S2A). Thus, our studies identify a novel interaction of ECD with RUVBL1 and suggest that this mode of interaction with the R2TP complex is a key to the regulation of cell cycle progression by ECD. Delineation of sequences in ECD and RUVBL1 that mediate their interaction should help directly test if selective abrogation of this interaction is functionally critical in cell cycle progression as well as to assess the potential role of ECD in other roles of RUVBL1 within the R2TP complex. Interestingly, mouse ECD and RUVBL1 knockouts are phenotypically similar, both being embryonic lethal at the blastocyst stage (6, 19). RUVBL1 is essential for cellular proliferation as seen in knockout cells or upon knockdown of RUVBL1 expression (18). A recent study demonstrated that RUVBL1 functions as a critical factor for p300 recruitment to OCT4 target genes (18). It is of interest that ECD also interacts with p300 and promotes its transcriptional co-activator function (8). Thus, ECD may function in close coordination with RUVBL1.

Given the evidence we present that ECD can physically interact with two distinct components of the R2TP complex, it is conceivable that certain ECD functions require both modes of interaction. Recent studies have shown that, aside from the R2TP

complex, RUVBL1/2 are also parts of other functionally-relevant complexes, such as chromatin remodeling complexes TIP60, SWR/SRCAP, INO80, and Fanconi anemia core complex that controls DNA inter-strand crosslink repair and function, and regulate telomerase biogenesis and mitosis(19, 43-45). Given the PIH1D1-independent interaction of ECD with RUVBL1, potential roles of ECD via these alternative complexes will be of great future interest.

An essential role of ECD in cell cycle progression was established by our previous observation that ECD is essential for embryogenesis and its conditional deletion in MEFs leads to a G1-S cell cycle arrest together with an inability to initiate an E2F-dependent transcriptional program essential for cell cycle progression (6). Notably, we demonstrated that ECD competes with E2F for binding to the pocket domain of RB and that the cell cycle progression defect in *Ecd*-null MEFs could be overcome by removing the Rb-mediated suppression of E2F using a pocket-binding oncogene HPV16 E7. As a key mechanism by which the R2TP complex regulates biochemical processes is by facilitating protein complex remodeling, we speculate that interaction of ECD with the R2TP complex, through RUVBL1, facilitates the ECD-RB complex formation and helps dissociate RB from E2Fs, thereby de-repressing the E2F-mediated transcription and promoting cell cycle progression. Consistent with this speculative model, our previous studies showed that binding to RB was not sufficient for the cell cycle progression function of ECD, as we identified one ECD mutant that was able to interact with RB but was defective in cell cycle rescue.

Our previous studies demonstrated that ECD is overexpressed in breast and pancreatic cancer patient tissues, and ECD overexpression correlates with poor prognosis

and poor survival in breast cancer patients (9, 10). It is noteworthy that several components of the R2TP/prefoldin complex, including PIH1D1, RUVBL1 and RUVBL2, are also overexpressed in various cancers and are predicted to play important roles in oncogenesis (46, 47). A comprehensive meta-analysis of The Cancer Genome Atlas (TCGA) datasets (46) revealed that expression of many RUVBL complex genes was significantly higher in breast and colorectal carcinomas when compared to their normal tissue controls. These investigations suggested a correlation between RUVBL complex component overexpression and increased mTORC1 signaling and metabolic processes necessary for tumor cell growth (46). Another study demonstrated that PIH1D1 is overexpressed in various breast cancer cell lines where it plays a major role in rRNA transcription (48). Our recent studies showed a co-oncogenic role of ECD with Ras when introduced into immortal human mammary epithelial cells (49), further suggesting the potential collaborative role of ECD and the R2TP or other RUVBL-containing complexes in cell cycle regulation and oncogenesis.

A positive role of ECD in pre-mRNA splicing was reported recently based on rescue of splicing defects in the prothoracic gland of *Ecd* deficient flies by human ECD and interaction of ECD with a complex containing the spliceosome component PRP8 (7, 50). Our affinity purification/Mass Spectrometry analyses confirmed the interaction of ECD with PRPF8. The R2TP complex regulates mRNA and ribosome biogenesis by facilitating the assembly of small nucleolar ribonucleoproteins (snoRNPs), which are known to be involved in spliceosome modification (51, 52). Upregulation of R2TP and snoRNP components is thought to promote ribosome synthesis in cancer cells (47). Whether overexpressed ECD in tumors may function in concert with R2TP and other

RUVBL1-containing complexes to promote oncogenesis needs further investigation. Taken together, studies presented here demonstrate that CK2-mediated phosphorylation and interaction with RUVBL1 are essential for ECD's ability to regulate cell cycle progression.

### **Acknowledgements**

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### **Figure Legends**

**Figure 1: ECD localization and expression do not change during cell cycle progression.** 76NTERT cells were cell cycle arrested by culturing in growth factor-free DFCI-3 medium for 72 h and then switched to growth factor-containing DFCI-1 medium to initiate cell cycle progression. (A) Cells were fixed in 70% ethanol at the indicated time points, stained with propidium iodide and subjected to FACS analysis. (B) Lysates were collected at the indicated time points and subjected to western blotting for ECD or  $\beta$ -Actin. ImageJ software was used to quantify ECD signals at various time points during

cell cycle progression and expressed as relative to  $\beta$ -Actin signals. (C) Nuclear and cytoplasmic fractions were isolated from cells at various time points during cell cycle progression and subjected to western blot analysis with anti-ECD antibody. PARP and GAPDH served as positive controls for nuclear and cytoplasmic proteins respectively. All experiments were carried out in triplicates. H.E., higher exposure.

**Figure 2: ECD is phosphorylated, predominantly on serine residues.** (A) T98G cells were serum-deprived for 48 h, last 4 h in phosphate-free medium, and then cultured for indicated times in complete DMEM medium containing 100  $\mu$ Ci sodium [ $^{32}$ P]-orthophosphate per 10-cm plate. ECD or RB (used as positive control) were immunoprecipitated, resolved by SDS PAGE, transferred to PVDF membrane and subjected to autoradiography to detect phosphorylation signal. (B) T98G cells transiently transfected to express FLAG-tagged ECD were cultured for 6 h in phosphate-free DMEM and then metabolically [ $^{32}$ P]-labeled, as described above. Anti-FLAG IPs were visualized by autoradiography. (C) Anti-FLAG IPs of T98G cells transfected with FLAG-tagged ECD were western blotted with anti-p-Ser or anti-p-Thr antibodies. *In vitro* phosphorylated GST-PERK kinase domain was used as a positive control for serine and threonine phosphorylation. The extra band observed in GST-PERK lane is likely a cleavage product of PERK. Arrows point to bands of interest.

**Figure 3: Phosphorylation of ECD is important for its ability to rescue cell cycle arrest in *Ecd* null MEFs.** (A) Schematic of ECD protein, its C-terminal deleted constructs, and CK2 phosphorylation sites predicted by KinasePhos 0.2 tool (<http://kinasephos2.mbc.nctu.edu.tw/>). (B) ECD is predominantly phosphorylated near its C-terminus. *In vitro* kinase reactions of full length (1-644) ECD and various C-

terminal deletion fragments (1-567, 1-534, and 1-432) with human recombinant CK2 were separated by SDS-PAGE, transferred to PVDF membrane and subjected to autoradiography to detect [<sup>32</sup>P] signals. Purity of proteins was assessed by Coomassie brilliant blue staining (CBB). (C) Schematic representation of various point mutants. Black rectangles, WT Ser residues; white rectangles, mutant Ala residues. (D) CK2 phosphorylates ECD at 6 sites. His-tagged wild type ECD or its point mutants were purified by Nickel affinity purification and subjected to an *in vitro* kinase assay, as described above. [<sup>32</sup>P] labeling was detected using autoradiography and the filters were subsequently subjected to western blotting with anti-p-Ser antibody and re-probed with anti-ECD antibody for equal loading. Purity of recombinant proteins was assessed by CBB staining. (E) CK2-dependent phosphorylation of ECD at multiple residues in cultured cells. T98G cells expressing FLAG-tagged ECD or its phosphorylation site mutants 3S/A or 6S/A were metabolically labeled with [<sup>32</sup>P], as described above. FLAG-tagged ECD and its mutants were immunoprecipitated and subjected to autoradiography. IP of cells expressing WT ECD were subjected to phosphatase treatment is shown. The blot was re-probed with anti-ECD antibody for equal IP loading. (F) Inhibition of CK2 reduces ECD phosphorylation. T98G cells expressing FLAG-tagged ECD or its phosphorylation site mutants 3S/A and 6S/A were treated with CK2 inhibitor TBB (50 μM) for 4 h, subjected to anti-FLAG IP and western blotted with anti-p-Ser or anti-FLAG antibodies. The intensity of anti-p-Ser signals was quantified using the ImajeJ software and normalized relative to FALG-ECD signals. (G) Phosphorylation of ECD at six CK2 sites is important for its cell cycle progression function. *Ecd*<sup>fllox/fllox</sup> MEFs stably expressing vector control, wild type human ECD or its indicated mutants were infected

with control (ctrl) adeno-GFP or adeno-GFP-Cre (cre) viruses for the indicated times and lysates were analyzed by anti-ECD and  $\alpha$ -tubulin (loading control). Note that human ECD-reconstituted cells express both endogenous mouse (mEcd higher band) and ectopic hECD or its mutants (hECD, lower band). (H-J) Analysis of hECD or its mutants for rescue of *Ecd<sup>fl/fl</sup>* MEFs from cell cycle arrest induced by Cre-mediated *Ecd* deletion. *Ecd<sup>fl/fl</sup>* MEFs expressing vector (V), WT hECD or its 3S/A, 6S/A, 6S/D or 3'S/A mutants were infected with control (ctrl) or Cre adenoviruses, followed by cell counting at the indicated time points. The Cre/Ctrl cell number ratios at each time point were plotted to assess the level of rescue relative to vector-expressing MEFs. Simulation's correction was applied to control for multiple testing in the calculation of the mean ratio. The experiment is representative of three repeats.

**Figure 4: ECD Phosphorylation in living cells is dispensable for its interaction with PIH1D1 and other components of the R2TP complex.** (A) *In vitro* interaction of GST-PIH1D1 with ECD and its mutants. GST-PIH1D1 was immobilized on glutathione-sepharose beads and incubated with lysates of HEK-293 cells expressing FLAG-tagged WT ECD or its 3S/A or 6S/A mutant. (B & C) Interaction between endogenous ECD and PIH1D1 was confirmed by immunoprecipitation of ECD from U2OS or MEFs followed by western blotting with anti-PIH1D1 antibody. (D & E) Lysates of HEK-293T cells transfected with untagged (D) or GFP-tagged (E) WT, 3S/A, 6S/A or  $\Delta$ 499-527 ECD were subjected to immunoprecipitation with anti-PIH1D1 antibody and immunoblotted with the indicated antibodies. (F) Alignment of mouse and human ECD sequences in the region containing the DSDD/E motif. 'V' is vector transfected cells.



**Figure 5: ECD interacts with RUVBL1.** (A) Tandem affinity purification identified RUVBL1 as an ECD-interacting protein. 76NTERT cell lysates were incubated with glutathione-sepharose bead bound GST-ECD-FLAG and protein complexes eluted by cleaving ECD-FLAG portion with PreScission protease. Eluted ECD-FLAG in complex with bound proteins was further affinity purified using FLAG beads and then eluted using excess FLAG peptide. The presented gel corresponds to 5% of the final eluates visualized by silver staining. Arrows point to the gel slices that were analyzed by mass spectrometry. Top and middle arrows point to slices that identified PRPF8 and RUVBL1, respectively. (B) Interaction between endogenous RUVBL1 and ECD. Lysates of HEK-293T cells expressing FLAG-tagged WT ECD or its 6S/A mutant were subjected for anti-RUVBL1 IP followed by anti-FLAG blotting. (C) PIH1D1 knockdown does not affect RUVBL1-ECD association. Lysates of U2OS cells transfected with PIH1D1 or scrambled control siRNA 48 hours earlier were subjected to anti-ECD IP followed by blotting with antibodies against the indicated proteins. ‘V’ is vector transfected cells.

**Figure 6: Interaction with RUVBL1 is important for ECD function in cell cycle progression.** (A & B) Interaction between FLAG-RUVBL1 and ECD. GST-tagged or His-tagged full length ECD or its truncated mutants immobilized on glutathione-sepharose or nickel beads respectively, were incubated with lysates of HEK-293T cells expressing FLAG-tagged RUVBL1. (C) Western blotting to show the expression of WT human ECD or its deletion mutants in *Ecd<sup>fl/fl</sup>* MEFs with control or Cre adenovirus infection (arrowheads point to the human ECD or mutants). Also note that anti-ECD antibody blot does not detect the C-terminally deleted ECD 1-438 mutant. (D) Colony formation assay. *Ecd<sup>fl/fl</sup>* MEFs expressing full length WT ECD (1-644) or its truncations

were infected with ctrl or Cre adenoviruses, colonies were stained with crystal violet after 10 days and solubilized dye absorbance was read at 590 nm. Histogram shows the relative rescue efficiency of each construct as compared to vector control cells. Error bars represents mean  $\pm$  SD of the independent experiments. Statistical comparison used student's two tailed t test. (E) Interaction of FLAG-tagged ECD or its deletion fragments with PIH1D1. Lysates of HEK-293T cells expressing the indicated FLAG-tagged ECD fragments were used for GST-PIH1D1 pull-down followed by anti-FLAG blotting. 'V' is vector transfected cells.

**Figure 7: *Ecd* deletion leads to a mitotic block.** (A) *Ecd*<sup>fllox/fllox</sup> MEFs infected with control (blue) or Cre adenoviruses (red) were fixed in 70% ethanol, stained with anti-pH3(S10) and analyzed by flow cytometry. Median Fluorescence Intensity (MFI), representing the peak channel number on the X-axis, is shown. (B) Lysates of control (Ctrl) or *Ecd* deleted (Cre) *Ecd*<sup>fllox/fllox</sup> MEFs were blotted with the indicated antibodies. (C) Control (Ctrl) or *Ecd* deleted (Cre) MEFs were treated with 100 ng/ml nocodazole for 20 h and cells switched to nocodazole-free medium to initiate cell cycle progression for the indicated time points. Cells were stained with propidium iodide and analyzed by FACS for cell cycle analysis. (D) Graph shows the percentage of cells entering the G<sub>1</sub> phase after release from nocodazole treatment at various time points. Data points are mean  $\pm$  S.D. of results from three independent experiments (\*  $p \leq 0.05$  by two tailed Student's *t* test). (E) Deletion of *Ecd* in the experiment shown in C confirmed by western blotting.

Figures:

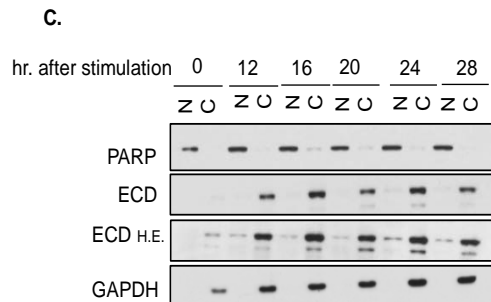
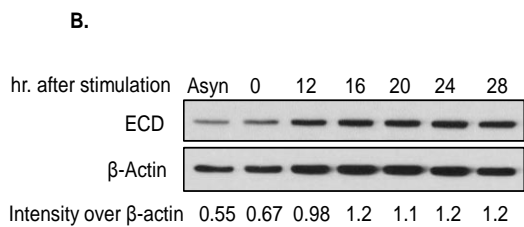
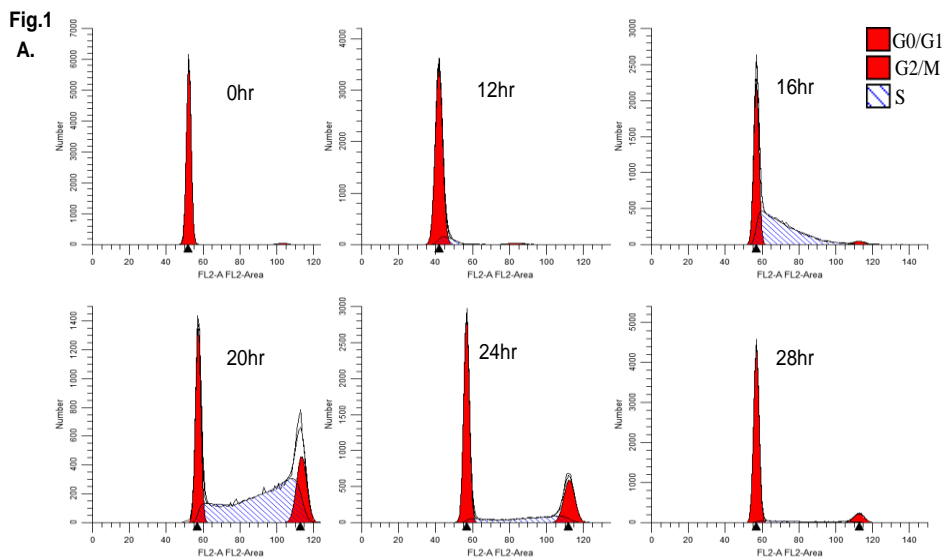


Fig. 2

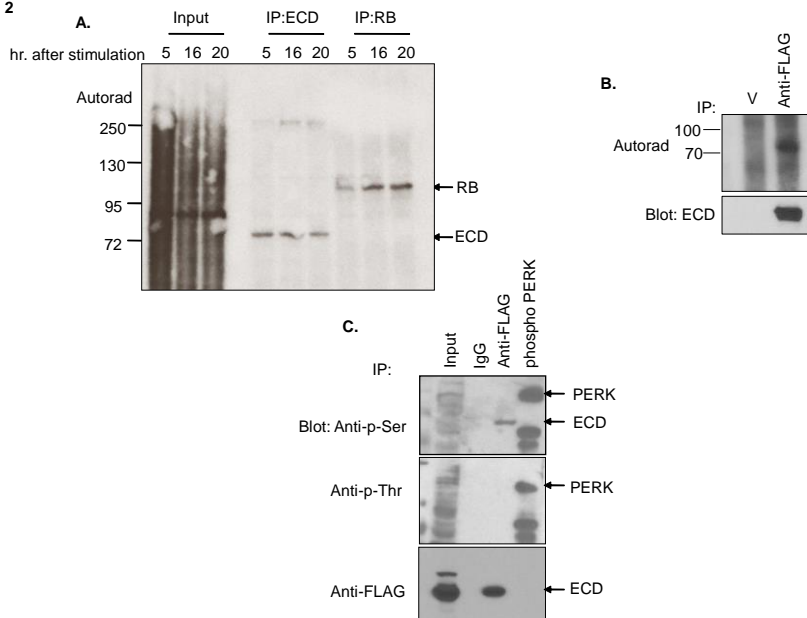


Fig. 3

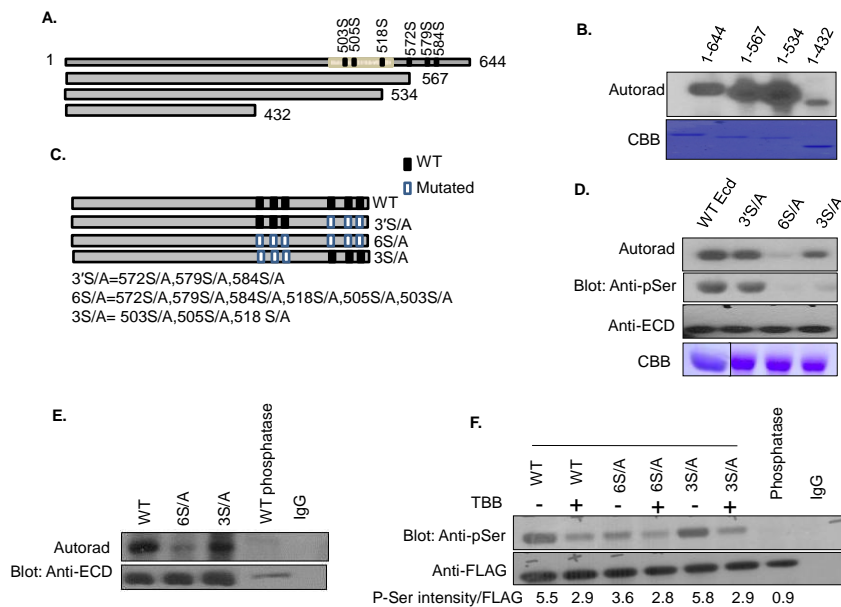


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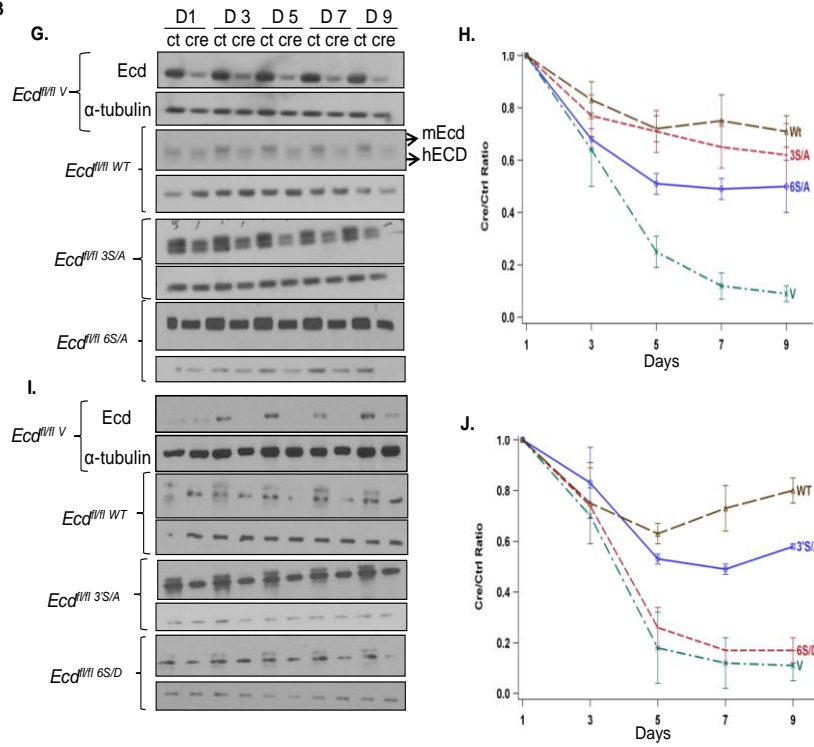


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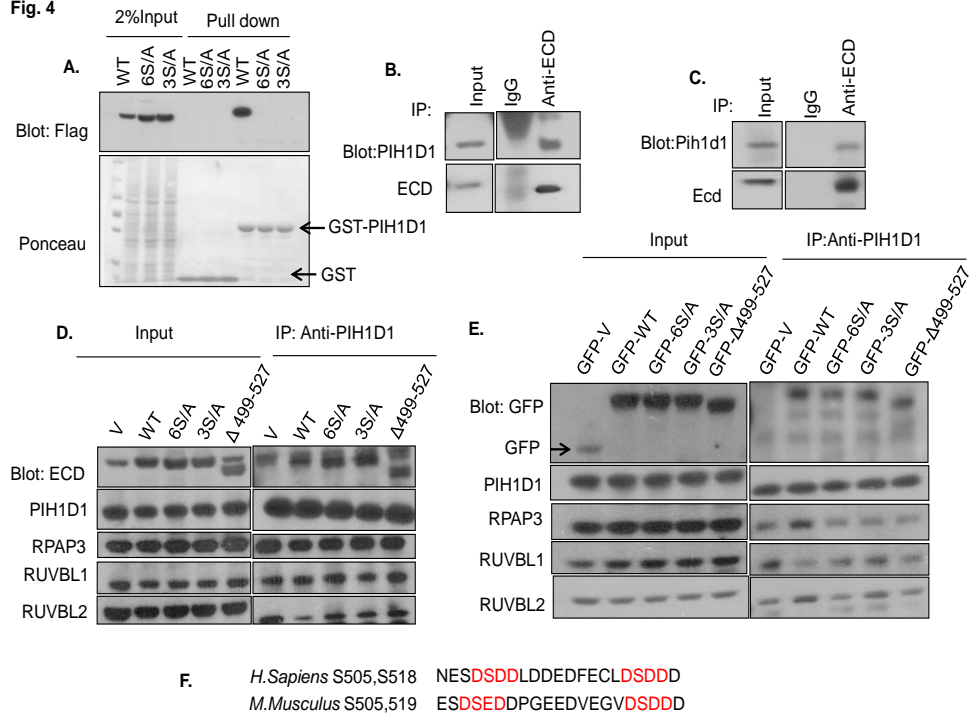


Fig. 5

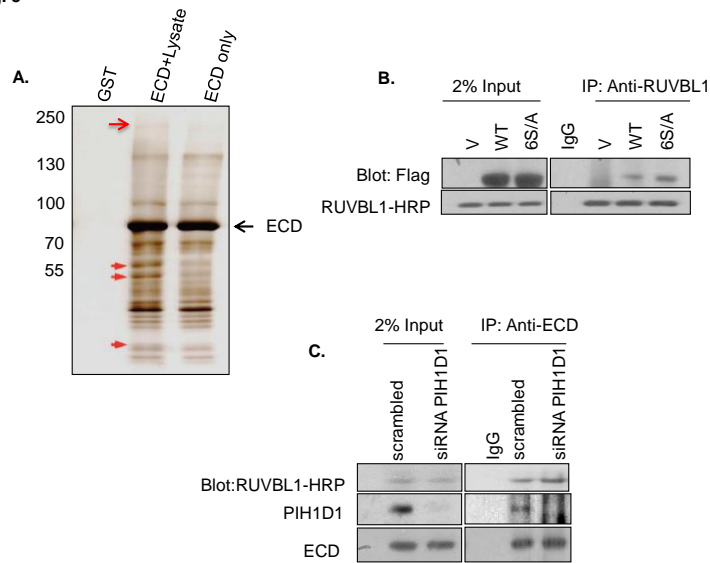


Fig. 6

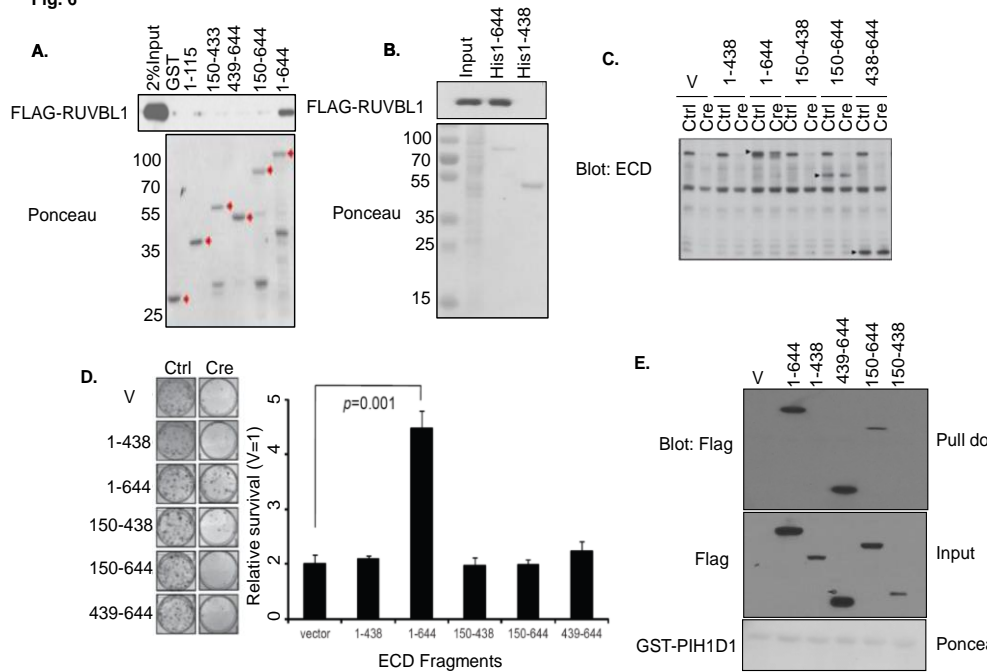
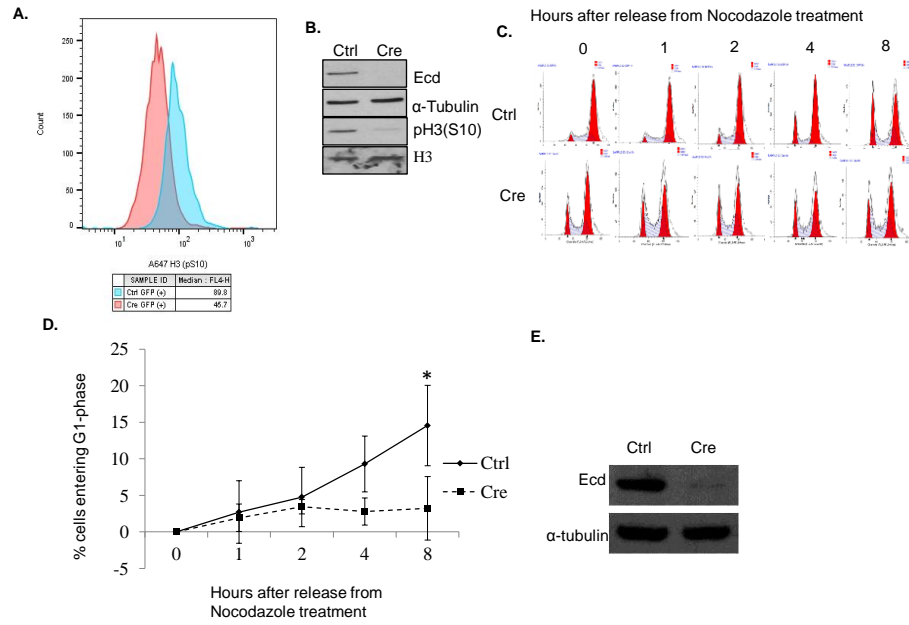


Fig. 7



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