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The Roles of Ecdysoneless in Endoplasmic Reticulum Stress and Oncogenesis

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UNIVERSITY OF NEBRASKA, MEDICAL CENTER

Role of Mammalian Ecdysoneless protein in Endoplasmic Reticulum Stress and Oncogenesis

A DISSERTATION

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By

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ABSTRACT

Tumor cells are well known to exhibit ER stress as a result of their altered environment characterized by redox and calcium imbalance, deregulation of protein synthesis to meet their oncogenic demands, and decreased vascularization associated with nutrient limitation and hypoxia, all of which are conducive of ER stress. Accordingly, markers of ER stress signaling response are up-regulated in various cancers. The outcome of ER stress signaling response varies from survival and adaption to apoptosis. Although pro-apoptotic ER stress signaling molecules are up-regulated in cancer, the mechanisms by which cancer cells seem refractory to, or evade, their apoptotic signaling are not fully understood. In this study, we investigate roles of the mammalian Ecdysoneless (ECD) in ER stress and oncogenesis. Certain tumors overexpress ECD protein suggesting possible roles in oncogenesis. In fact, both normal and cancer cells require ECD for their growth and undergo cell cycle arrest when the ECD level is deficient. ECD associates with various stress response proteins such as p53, well known for genotoxic stress response such as DNA damage, and TXNIP, an oxidative stress protein recently implicated in ER stress response. Furthermore, ECD is a critical component of chaperone-like complexes such as the R2TP complex whose members have ER stress-related roles. In this study, we provide evidence that ECD is involved in ER stress.

Stress induction by multiple stimuli, including Thapsigargin, Tunicamycin, glucose starvation, H$_2$O$_2$, NOX4 overexpression, led to reduced ECD protein levels, but ECD mRNA increased, in a PERK-eIF2α dependent manner. Although a phospho-protein, ECD is not a target for a phosphorylation-mediated degradation by PERK but is rather targeted for a translation block via the PERK-eIF2α axis. To assess the functional connection
between ECD and ER stress signaling pathways, we used cells in which ECD could be inducibly depleted or overexpressed. Depletion of ECD enhanced PERK signaling and apoptosis upon ER stress induction while overexpression of ECD produced the opposite effect by inhibiting PERK signaling and increasing cell survival. IRE1α signaling was slightly affected by these changes in the cellular level of ECD, as indicated by the slight increase or decrease of its downstream target, spliced XBP-1, upon ER stress induction in the presence or absence of ECD; on the other hand, the ATF6 pathway was minimally affected.

Based on these findings, we examined the possible mechanism by which ECD regulates ER stress signaling, particularly the PERK pathway. We found that ECD co-localized and associated with all the ER stress sensors PERK, IRE1α, ATF6 and GRP78. However, ECD does not modulate the enzymatic activity of PERK toward its substrate eIF2α. Rather, ECD enhanced chaperones’ levels, predominantly, GRP78 upon ER stress induction. Finally, disruption of this chaperone-enhancing effect of ECD abrogated the attenuating effect of ECD on PERK and impaired the pro-survival effect of overexpressed ECD upon ER stress induction. Taken together, ECD regulates the levels of chaperones, predominantly, GRP78 to enhance the folding capacity of the stressed ER and provide survival advantage in a stress condition.
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Dr. Vimla Band is the principal investigator (PI) in the ECD project, which this study was a part of. She is one of the very few researchers working on ECD. Obviously, this project could not have gone anywhere without her and I would like to thank her for her active roles in the study. She did all she could to ensure completion of this project, including countless meetings and discussions. I would also like to thank my committee members for their suggestions along the way.

I thank the Band lab members for being part of the journey too. Particularly, I thank Dr. Mir Riyaz who embraced me from day one as his own student and answered every question I asked him, including technical ones.
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**ABBREVIATIONS**

ASK1: Apoptosis-Signaling Kinase 1

ATF2: Activation of Transcription Factor 2

ATF4: Activation of Transcription Factor 4

ATF6; Activation of Transcription Factor 6

ATP: Adenosine Triphosphate

Bcl-2:B-cell Lymphoma 2

BFA: Brefeldin A

CDK2: Cyclin Dependent Kinase 2

CHOP: CCAAT enhancer binding protein Homologous Protein

CK2: Casein Kinase 2

DNA: Deoxyribonucleic Acid
Dox: Doxycycline

DTT: Dithiothreitol

ECD: Ecdysoneless

EDEM: ER Degradation Enhancing α-mannosidase-like protein

eIF2α: Eukaryotic Initiation of translation Factor 2

ER: Endoplasmic Reticulum

ERAD: ER Associated Degradation

ERo1α: ER Oxidoreductin-1 alpha

G1/S: G1 phase to S phase transition

G2/M: G2 phase to M phase

GADD34: Growth Arrest and DNA Damage inducible 34

GADD53: Growth Arrest and DNA Damage inducible 153

GCN2: General Control Non-derepressible 2

GCR2: Glycolysis Regulation 2

GFP: Green Fluorescent Protein

GRP78: Glucose-Regulated Protein 78

GRP94: Glucose-Regulated Protein 94
GTP: Guanine Triphosphate

H$_2$O$_2$: Hydrogen Peroxide

hECD: Human Ecdysoneless

HRI: Heme-Regulated Inhibitor

hSGT1: Human Suppressor of GCR two 1

IRE1\(\alpha\): Inositol-Requiring Enzyme 1 alpha

IRES: Internal Ribosomal Entry Site

JNK: c-Jun N-terminal Kinase

KO: Knockout

MEF: Mouse Embryonic Fibroblast

Met: Methionine

mRNA: Messenger RNA

NOX4: NADPH Oxidase 4

O$_2$: Oxygen

P53: Protein 53

p58 IPK: Protein 58 Inhibitor of Protein Kinase

PCR: Polymerase Chain Reaction
PDI: Protein Disulfide Isomerase

p-eIF2α: phospho-eIF2α

PERK: PKR-like ER Kinase

PKR: Protein Kinase RNA-dependent

PIH1D1: Protein Interacting with Hsp90 1contining Domain 1

PP1: Protein Phosphatase 1

p-PERK: phospho-PERK

PTM: Post Translation Modification

qRT-PCR: quantitative Real-Time PCR

R2TP: RUVB1/RUVB2/Tah1/PiH containing complex

RedOx: Oxidation and Reduction

RIDD: Regulated IRE1 Dependent Decay

RNA: Ribonucleic Acid

ROS: Reactive Oxygen Species

rtTA: Reverse Tetracycline controlled Trans-activator

RUVBL1: RuvB-like AAA ATPase 1

S. Cerevisiae: Saccharomyces cerevisiae
SAPK: Stress Activated Protein Kinases

SDS-PAGE: Sodium Dodecylsulfate-polyacrylamide gel electrophoresis

siRNA: Small-Interfering RNA

SP1/2: Site ½ Proteinase

TRAF2: TNF Receptor Associated Factor 2

tRNA: transfer RNA

TXNIP: Thioredoxin-Interacting Protein

UPR: Unfolded Protein Response

WT: Wild-Type

XBP-1: X-box Binding Protein 1
CHAPTER ONE: INTRODUCTION
1.1 Overview of the Endoplasmic Reticulum (ER) and its functions in the cells

The Unfolded Protein Response (UPR), a term used interchangeably with Endoplasmic Reticulum (ER) stress response, is a reference to stress in the ER which is a membrane-bound sub-cellular organelle interconnecting other organelles (1), including the Golgi apparatus, the lysosome, the mitochondria and the nucleus. It is the largest organelle and the site for many central cellular functions such as synthesis, folding and maturation of secreted and membrane proteins, biogenesis of cholesterol, and calcium storage. Different classes of proteins mediate these functions of the ER; key among them are proteins involved in translation. In eukaryotic cells, translation occurs directly in the ER, following assembly and translocation of the ribosomal complex to the translocation channel. This complex is composed of the eukaryotic initiation factor 2 alpha (eIF2α) in a ternary complex with bound GTP and the methionine initiator tRNA (Met-tRNAi-eIF2α-GTP) (2). As the newly synthesized proteins enter the ER, they undergo post-translational modifications (PTM) in an oxidative environment. Examples of common PTM are glycosylation, disulfide bond formation and phosphorylation. Proper PTM precedes proper folding of proteins into their three-dimensional structure with the assistance of ER resident chaperones such as the Glucose-Regulated Protein 78/ Immunoglobin Binding Protein (GRP78/ Bip), GRP94, calreticulin, calnexin and protein disulfide isomerases or PDI (3). The PDIs, in particular, are oxygen-requiring ER oxidoreductase 1 (Ero1) enzymes which generate disulfide bonds de novo and transfer them to their thioredoxin motif containing the thiol/disulfide oxidoreductase site (4). Lastly, the ER chaperones are calcium binding proteins and function calcium-dependently, justifying why the ER is also
the main calcium storage in the cell, with ATP-dependent calcium pumps which influx calcium from the cytosol to the ER. The ER also participates in intra and extracellular calcium signaling (5).

1.2 Physiological and pathological conditions that perturb ER homeostasis and induce stress

Physiological conditions that induce stress in the ER, such as glucose deficiency, are cell type specific. Glycosylation, a critical PTM, is disrupted when cells are glucose-deprived, as a result of a decreased level of glycosyl group in the cells (6). Moreover, glucose deficiency also decreases ATP level in the cells, impairing crucial functions of ATP-dependent calcium pumps in the cells (6). High glucose also perturbs proper ER function in secretory cells, such as pancreatic beta cells, due to the ensuing increased production of insulin that must be processed through the ER. Other physiological perturbants that induce ER stress are calcium deprivation, which impairs the function of key ER chaperones, free fatty acids and cytokines. In pancreatic beta cells, the best model for studying ER stress, ER stress can be physiologically induced by mutant insulin (6). Thus, pathological conditions that cause increased blood glucose levels, such as diabetes, induce ER stress (7). Viral infection is another type of pathological ER stress inducer because of the increased synthesis of viral proteins that must be processed in the host ER. Ischemia, the loss of blood supply to body organs, is also known to induce ER stress (8). Finally, in order to support proper protein folding, the ER environment is highly
oxidative and is sensitive to change in the reduction-oxidation (RedOx) state of the cell (9).

### 1.3 Upstream signaling pathways of the ER stress response

ER stress is a condition in cells characterized by protein load in the ER exceeding the folding capacity of the ER. It can be caused by any of the physiological or pathological conditions mentioned above. Additionally, pharmaceutical agents such as Thapsigargin, Tunicamycin, Brefeldin A, Dithiothreitol and hydrogen peroxide also induce ER stress. Perturbation of the ER function elicits a response termed the unfolded protein response (UPR). The UPR is an adaptive pro-survival response in its initial phase but shifts to a pro-apoptotic response when the stress becomes overwhelming (10-12). Three canonical pathways mediate these two phases of the UPR of which PERK (PKR-like ER Kinase), IRE1α (Inositol Requiring Enzyme 1 alpha) and ATF6 (Activation of Transcription Factor 6) are the upstream players.

PERK is an ER transmembrane kinase with luminal, transmembrane and cytoplasmic domains. The cytoplasmic c-terminal kinase domain of PERK is activated by homo-dimerization of the protein followed by auto-phosphorylation (10,13,14). The active PERK kinase phosphorylates the eukaryotic initiation factor 2 alpha (eIF2α) (15), altering its conformation and leading to a gradual disassembly of the ternary Met-tRNAi-eIF2α-GTP complex, a critical part of the active translation complex. The resulting inhibition of the translation machinery causes a decrease in the load of protein entering the ER lumen (15) as well as cell cycle block, allowing cells time to solve the stress in the ER. Although global cap-dependent translation is blocked, a cap-independent translation of specific ER stress response genes proceeds. One such cap-independently
translated protein is Activation of Transcription Factor 4 (ATF4) (15), a transcription factor of the same family as ATF6. ATF4 translocates into the nucleus to activate a transcription enhancer gene, the CCAAT-enhancer-binding protein homologous protein (CHOP), which sensitizes cells to ER stress-induced cell death by multiple mechanisms including a down-regulation of the pro-survival Bcl-2 (B-Cell Lymphoma 2) protein family while increasing levels of members of the death receptor protein family. Moreover, CHOP is known to down-regulate glutathione levels, the cell’s anti-oxidant defense protein, leading to a surge in the levels of reactive oxygen species (ROS), a subsequent release of cytochrome c into the cytoplasm and activation of the caspase cascade, culminating in cell death (15-19). Concurrently, CHOP activates another protein, GADD34 which, together with the type 1 protein phosphatase (PP1), targets eIF2α for de-phosphorylation and resumption of protein synthesis (20-24). Alternatively, this recovery from the stress-induced translation block is also achieved in cells by cellular PERK inhibitor p58 IPK (25,26).

IRE1 exists in two isoforms, IRE1α and IRE1β. IRE1α, the widely studied isoform, has both kinase and RNAse function. Like PERK, it is activated by dimerization and auto-phosphorylation. With the RNAse activity, it is known to splice a 26 nucleotide intron from the X-box binding protein 1 (XBP-1) (16,27-30) to generate an active transcription factor, which translocates to the nucleus to activate ER response genes, one of which is the ER degradation-enhancing protein (EDEM) (16,31). In severe ER stress, EDEM activates the massive degradation of misfolded and unfolded proteins, termed ER-associated degradation (ERAD) (32,33), a process involving ER chaperone-mediated retro-translocation of proteins to the cytoplasm for proteasomal degradation. The RNAse
function of IRE1α is also associated with a degradation of ER-associated mRNA in a process termed RIDD or regulated IRE1α-dependent decay (34,35) to further inhibit protein translation into the stressed ER. With the kinase function, IRE1α is known to recruit TRAF2 (TNF-receptor activating factor 2) to activate the apoptosis-signaling kinase 1 (ASK1) and the Jun N-terminal Kinase (JNK), leading to caspase cascade and cell death (11,36).

ATF6, like IRE1, exists in two isoforms, ATF6α, the most studied isoform, and ATF6β. Upon ER stress signaling, ATF6α translocates to the Golgi apparatus where it is proteolytically cleaved by the Golgi Site 1 & 2 proteases (SP 1&2) (37,38) to generate an active transcription factor which translocates into the nucleus to activate ER stress target genes. ATF6α activates expression of chaperones upon ER stress, including GRP78, GRp94 (39).

1.4 The ER stress in Cancer: a paradox

The ER stress is the basis of diseases collectively referred to as ER stress diseases, including heart diseases, neurodegenerative diseases, diabetes and cancer (40). With regard to cancer, the ER stress response is known to promote survival mechanisms in cancer, hence driving oncogenesis. The tumor microenvironment is indeed a very hostile environment, characterized by Redox imbalance (41,42)], deregulated protein synthesis (43), and decreased vascularization associated with hypoxia and hypoglycemia (44), all of which are conducive to ER stress. Cancer cells also generate a higher level of hydrogen peroxide, a precursor of reactive oxygen species (ROS) which also perturbs ER
homeostasis. As such, markers of the ER stress response are up-regulated in various cancers [22], suggestive of fully activated and operational ER stress signaling to drive tumor progression. For instance, X-Box binding Protein 1 (XBP1) promotes survival in triple negative breast cancer by enhancing the hypoxic response pathway (45) while CHOP drives hepatocellular oncogenesis by promoting oncogenic cell growth (46). In the same vein, the chaperone GRP78 promotes tumor survival, metastasis, angiogenesis and resistance to a wide variety of therapies (47,48).

Controversially, components of the ER stress response are also implicated in negative regulatory control of tumor growth. In this context, activated PERK has been known to induce proliferation block via multiple mechanisms including blocks in DNA synthesis and protein translation (49-53). Moreover, expression of PERK leads to tissue atrophy in *Drosophila melanogaster*, a phenotype that was corrected by co-expressing GADD34, the phosphatase that represses PERK signaling (54). Although there are conflicting reports as to the role of PERK in tumor progression, the PERK pathway has a strong potential to reduce the growth of tumor cells (55). Besides PERK, a second possibility exists for the ER stress signaling to regulate tissue growth through ATF4 which has also been shown to negatively control neurogenesis in the developing mouse brain (56). ATF4 levels change during cell cycle progression and the protein undergoes a phosphorylation-dependent degradation required for cell cycle progression (56). This negative regulatory role of ATF4 on cell growth was observed in another study in which overexpression of ATF4 inhibited cell proliferation and mammary gland development (57).
The last and third level of negative regulation of cell growth by components of the ER stress response involves CHOP, also known as Growth Arrest and DNA-Damage inducible (GADD153). Induction of CHOP has been reported in in vitro differentiation experiments of 3T3-L1 cells to adipocytes, a process requiring growth arrest (58), suggesting a connection of CHOP to growth arrest as indicated by the very nomenclature of the protein (Growth Arrest and DNA-Damage inducible). Moreover, in tumors of adipose tissue, a chromosomal translocation has been found to cause a fusion protein between CHOP and a novel RNA binding protein, TLS (translocated in liposarcoma) (59-61). The TLS-CHOP fusion protein was oncogenic (62). This oncogenic gain of function of the mutant form of CHOP, ie TLS-CHOP, further implies an anti-oncogenic or growth suppressive role of the wild type CHOP. In fact, microinjection of CHOP into cells inhibited BrdU incorporation and caused proliferative block, a defect reversed by the TLS-CHOP variant mentioned above (62). Altogether, these studies provide strong evidence that the ER stress signaling has the capacity to both negatively and positively affect tumor growth. However, paradoxically, although the ER stress signaling is activated in various cancers, cells in tumors seem refractory to its growth inhibitory signaling and only proliferate, even indefinitely. This seeming tolerance of tumor cells to the stress-induced growth inhibitory signaling hints to the existence of mechanisms to desensitize and protect tumor cells against the growth inhibitory effect of the ER stress signaling.

1.5 The Ecdysoneless (ECD) protein, its oncogenic and stress-related potentials
ECD was first identified in *Drosophila* as the mutation that caused a deficiency in the steroid hormone, Ecdysone, a hormone that plays roles in normal embryogenesis, metamorphosis and reproduction (63), all of which require cell growth and proliferation. As a result, these ECD mutants understandably exhibited developmental defects. The mammalian ECD was discovered from a screen of genes that could rescue the growth defects in *S. Cerevisiae* mutant for the Growth Control Regulatory gene 2, GCR2 (64). This was the first indication that the mammalian ECD plays a role in growth. Studies from my mentor’s lab provided the most direct evidence that ECD does indeed play a key role in positively regulating cell growth and development. The lack of ECD in cells caused cell cycle to be arrested in G1 phase, a defect that was rescued by exogenously expressed ECD (65). The requirement of ECD for cell growth may justify its overexpression in various cancers and its association with poor prognosis and short patient survival (66,67). In pancreatic cancer, knock down of ECD reduced proliferation and tumorigenicity with cells arrested at G1-S phase (66). While ECD overexpression alone did not accelerate cell proliferation, its overexpression together with Ras enhanced cell proliferation in epithelial cells did (68). However, ECD overexpression in fibroblasts cells induced p53 dependent cellular senescence, by p53 stabilization and positively regulating p53 (69), implying a link to p53. Moreover, ECD interacts with p53 to stabilize it and positively regulates its transcriptional activity (69), raising the possibility that ECD may have a connection to p53 pathways. In addition to its tumor suppressive roles, p53 is also related to stress such as genotoxic stress and oxidative stress. ECD may be part of protein complexes that modulate p53 stress functions such as the oxidative stress regulator TXNIP. ECD interacts with TXNIP and together, ECD-TXNIP increases p53
activity (70). Similar to ECD, TXNIP interacts with p53 to increase its stability and function under oxidative stress (71). Significantly, a recent study found an ER-stress-related role for TXNIP in its interaction with PDI (Protein Disulfide Isomerase) to increase their enzymatic activity during ER stress thereby reducing stress (72). Furthermore, TXNIP is implicated in ER stress response as a novel component of the stress response signaling to mediate the inflammation-related axis of the stress-induced apoptosis (73). Also, TXNIP may also regulate the physiological function of the ER because it is part of the cellular redoxisome, comprising Thioredoxin, which regulates the redox state of the cell including the redox environment in the ER. The PDIs mentioned above are redox-sensitive ER oxidoreductases with Thioredoxin motifs and changes in the ER oxidative environment impair their functions (4).

Lastly, we and others have recently shown that ECD associates with R2TP complex (74,75). The R2TP complex is involved in the assembly and remodeling of large protein-protein or protein-RNA complexes, such as RNA polymerase, small nucleolar ribonucleoproteins (snoRNPs), and phosphatidylinositol 3-kinase-related kinases (PIKKs) (76). Our recent study showed that the N-terminal region of ECD associates with the RUVBL1 component of the R2TP complex and both the phosphorylation and interaction of ECD with RUVBL1 are required for ECD’s role to promote cell cycle progression (75). The R2TP has an ER stress-related role through its RUVBL2 component (77). Notably, RUVBL2, or TIP49b has been shown to inhibit the activity of ATF2 (77), a downstream target of PERK signaling, suggesting a role of the R2TP complex component in ER stress. Overall, the multiple lines of evidence presented above

REFERENCES


CHAPTER TWO: Mammalian ECD protein functions as a novel negative regulator of the Unfolded Protein Response
2.1 Overview

The mammalian Ecdysoneless (ECD) is a highly-conserved ortholog of the Drosophila ECD gene product whose mutations impair the synthesis of Ecdysone and produce cell-autonomous survival defects but mechanisms by which ECD functions are largely unknown. This study presents evidence that ECD regulates endoplasmic reticulum (ER) stress response. Stress induction by multiple stimuli, Thapsigargin, Tunicamycin, glucose starvation, H$_2$O$_2$, NOX4 overexpression, DTT, and sorbitol, led to reduced ECD protein in levels in multiple cell lines (MCF7, MDA-468, MCF10A, Panc-1, 76NTERT, MDA231-NOX4). However, contrary to the decrease in ECD protein levels, ECD mRNA increased upon Thapsigargin treatment suggesting that the decrease in ECD protein is not at transcriptional level. To elucidate the ER stress signaling pathway regulating ECD protein levels, we induced ER stress in MEF cells in which the three ER stress pathways, PERK, IRE1α and ATF6, had been individually abrogated to produce single knock out (KO) MEFs. In IRE1α KO and ATF6 KO MEFs, the decrease in ECD protein levels was comparable with the control WT MEFs upon ER stress induction. However, in PERK KO MEFs, the levels of ECD protein did not change upon ER stress induction as compared to WT PERK. Furthermore, in PERK KO MEFs, ECD mRNA levels failed to increase, suggesting that the decrease in ECD protein and the increase in ECD mRNA may be tied to PERK activity. Since ECD is a phospho-protein and certain proteins undergo a phosphorylation-dependent degradation, we first tested the hypothesis that the decrease in ECD protein levels is PERK phosphorylation mediated. To assess that possibility, we performed an in vitro kinase assay to examine the potential that ECD could be a substrate for phosphorylation by PERK, a kinase. However, we did not find that ECD was phosphorylated by PERK. The second hypothesis for the decrease in ECD protein levels
was that ECD protein translation was blocked by PERK phosphorylation of eIF2α. To test that hypothesis, we induced ER stress in phospho-deficient eIF2α MEFs and found that the decrease in ECD levels upon ER stress induction was abrogated just as in PERK KO MEFs. The results suggested that ECD protein levels are reduced via the PERK-eIF2α axis of the ER stress signaling and that ECD may be functionally linked to that PERK-eIF2α axis. To examine that possibility, we resorted to cells in which ECD could be inducibly depleted or overexpressed. Depletion of ECD increased the levels of p-PERK, p-eIF2α, and these effects were enhanced upon ER stress induction by Thapsigargin treatment or glucose deprivation along with an increase in CHOP mRNA, and cleaved caspase 3. A slight increase in the levels of spliced XBP-1, the downstream target of IRE1α, was also observed but cleaved ATF6 did not significantly change. These effects correlated with a decrease in the colony formation of the ECD-null cells. Reciprocally, overexpression of ECD led to a marked decrease in p-PERK and its downstream targets, p-eIF2α, ATF4 and CHOP mRNA levels. Spliced XBP1 levels were slightly decreased while ATF6 did not change significantly. These effects correlated with a decrease in caspase 3 cleavage and an increase in the colony forming ability of the ECD overexpressing cells. Taken together, these results suggested that ECD functions as a repressor of stress signaling, particularly as a negative regulator of PERK signaling and that ECD and PERK are linked through a negative feedback mechanism whereby ECD inhibits PERK while, when activated, PERK inhibits ECD protein by blocking its translation.

Based on the findings presented above, we sought to investigate the possible mechanisms by which ECD regulates ER stress signaling, particularly the PERK pathway. First, we examined the localization of ECD relative to the upstream mediators of the ER stress
signaling and found that ECD localized and associated with PERK, ATF6, IRE1α and GRP78. To assess whether ECD directly regulates PERK signaling, we performed in vitro kinase assay of PERK with eIF2α in the presence of an increasing concentration of the ECD protein. However, ECD did not modulate enzymatic activity of PERK toward eIF2α. Since induction of chaperones during ER stress signaling is well known as a homeostatic mechanism aimed at restoring the ER function by clearing the stress stimulus, we next examined the effect of ECD on levels of chaperones, particularly GRP78 which is the major regulator of PERK signaling. We found that the levels of GRP78 (GRP94 and PDI to a lesser extent) were dependent on ECD and that depletion or overexpression of ECD decreased or increased GRP78 protein levels, respectively, upon ER stress induction. However, GRP78 mRNA was not affected, suggesting a post-translational effect. To determine whether a change in the levels of ECD affects GRP78 protein stability, we deleted ECD followed by cycloheximide treatment and found that the decrease in GRP78 protein level was faster in ECD-null cells. Reciprocally, in ECD overexpressing cells, the decrease in the levels of GRP78 protein was slower upon cycloheximide treatment. Finally, to determine whether GRP78 was required for ECD effects on PERK signaling, we knocked down GRP78 and found that even in the presence of overexpressed ECD, a knock down of GRP78 abrogated the attenuating effect of ECD on PERK, correlating with a decrease in colony formation of the ECD overexpressing cells. Taken together, these results argue that ECD enhances the levels of chaperones, predominantly, GRP78 to enhance the folding capacity of the stressed ER and reduce stress.
2.2 INTRODUCTION

The endoplasmic reticulum (ER) is a central subcellular organelle with essential roles in the synthesis, folding and maturation of secreted and membrane proteins, biogenesis of cholesterol, calcium homeostasis and regulation of survival and apoptosis pathways (1-10). Aberrations in these ER functions are sensed by well-conserved ER transmembrane sensors, Inositol-Requiring Enzyme 1 alpha (IRE1α), PKR-like ER kinase (PERK) and Activating Transcription Factor 6 (ATF6), that activate homeostatic signaling pathways collectively referred to as the unfolded protein response (UPR) (11,12). These ER stress sensors exhibit a dynamic and reversible interaction with the ER chaperone GRP78 (13). In un-stressed cells, GRP78 is bound to luminal domains of UPR sensors, which maintains them in an inactive state (14). During ER stress, the increased load of unfolded proteins competes for GRP78 binding, leading to activation of UPR sensors (15).

As an important chaperone, the level of GRP78 is also increased during ER stress signaling, mainly via the ATF6 pathway (16). The induction of GRP78 and other chaperones such as GRP94 (17-19) and PDI (20,21) is a homeostatic mechanism to restore normal functions in the ER and protect cells from stress-induced cell death. Part of GRP78’s cyto-protective role involves its regulation of the ER stress sensors during stress signaling to prevent their hyper-activation and ensuing cell death. To achieve that effect, GRP78 is known to reversibly bind the ER stress sensors, when induced, and inhibit their signaling (13). Consequently, overexpression of GRP78 has been shown to inhibit PERK signaling, in particular (13). Furthermore, GRP78 has been shown to physically interact with caspases (22) and prevent their cleavage (23).
The GRP78 protein is known to contain the canonical KDEL ER retention signal (24,25) which allows it to be retro-transported from the Golgi to the ER during its journey through the secretory pathway. Accordingly, GRP78 was traditionally thought of as an ER-resident protein. However, recent evidence points to a ubiquitous nature of the protein in cells. GRP78 has been found in the cytoplasm where it interacts with cytoplasmic proteins such as caspace-7 and clusterin protein (22,26); there is also evidence of GRP78 presence in the nucleus and mitochondria (27). This ubiquitous nature of GRP78 in cells suggests that the ER functions of GRP78 may not necessarily require its ER- localization, especially as studies have identified a cytoplasmic, non-KDEL containing variant of the GRP78 protein which still has ER stress-related functions via cytoplasmic regulators of the ER stress signaling (28).

The PERK pathway has emerged as a key pathway in cellular UPR response as well as homeostasis under unstressed conditions and in disease states (29-34). Release of GRP78 from PERK leads to its dimerization, auto-phosphorylation and activation (15,35,36). A major PERK substrate is the eukaryotic translation initiation factor 2 alpha (eIF2α) whose phosphorylation by PERK inactivates it leading to a block in general cap-dependent protein translation and consequent decrease in the protein load entering the ER (37); concurrently, PERK signaling selectively enhances the cap-independent translation of specific mRNAs, such as Activating Transcription Factor 4 (ATF4) (37). ATF4 induces the expression of CCAAT/enhancer-binding protein-homologous protein (CHOP) which promotes apoptosis in response to stress (37-45). PERK-induced phosphorylation of eIF2α also inhibits cell cycle progression by reducing the levels of cyclins and hence cyclin-dependent kinase 2 (CDK2) activity (46-48). Termination of PERK signaling, together
with de-phosphorylation of eIF2α, is required to re-initiate protein synthesis and resume the cell cycle. Thus, PERK-mediated UPR leads to a coordinated program of cellular protection and mitigation of stress. However, PERK also contributes to an alternate outcome through CHOP-mediated activation of a cellular death pathway to eliminate severely damaged cells (37–45). Mechanisms to fine-tune the outcomes of UPR pathways are important to mitigate the negative consequences of ER stress. This is of particular importance under conditions where cells experience ER stress as part of their physiological responses, such as antibody-secreting plasma cells or insulin-secreting pancreatic islet cells (49). We have identified ECD as a negative regulator of the PERK-mediated UPR.

The ECD gene was first identified based on genetic mutations in Drosophila that lead to reduced production of the developmentally-regulated steroid hormone Ecdysone, which is synthesized in the ER, hence the designation of Ecdysoneless for such fly mutants (50). The mammalian ECD gene was cloned based on the rescue of growth defects in S. cerevisiae mutant in the growth control regulatory gene 2 (GCR2), a glycolysis regulatory gene (51). Thus, ECD was thought to be involved in mammalian glycolysis gene expression and initially named hSGT1 (human suppressor of gcr2)(51). My lab identified the same gene in a screen for interacting partners of human papilloma virus E6 oncoprotein and found it to interact with p53 and transactivate p53-regulated genes (52,53).

To elucidate the functional role(s) of mammalian ECD, my lab generated ECD-null mice and demonstrated that homozygous deletion of ECD was early embryonic lethal while ex vivo Cre-mediated deletion of ECD in ECD<sup>fl/fl</sup> mouse embryonic fibroblasts (MEFs) led to proliferative block and a significant decrease in cell survival (54,55). ECD was found to be essential for E2F target gene expression by facilitating the dissociation of the
retinoblastoma RB protein from E2F and promoting the G1-to-S phase cell cycle progression (54). As a consequence, ECD-null MEFs showed a decrease in the levels of cyclins A, B1, E and D1, reduction in CDK2 kinase activity and were arrested in G1 phase of the cell cycle (54). Interestingly, E2F family proteins such as the E2F1 have been implicated in UPR-mediated cell death (56). In addition to its promotion of the G1/S phase, ECD also promotes G2/M phase of the cell cycle and its knockdown induced not just a G2/M arrest but also apoptosis (57). Induction of UPR not only induces apoptosis but also halts cell cycle progression in G1 and G2 phases (46-48,58) and these effects are mediated by PERK (46,47), suggesting a potential connection between ECD and the PERK arm of the UPR.

More recently, we uncovered another mechanism by which ECD regulates cellular proliferation, through its interaction with RUVBL1 and PIH1D1 components of the prefoldin co-chaperone R2TP (55) which is involved in the assembly or remodeling of a number of protein and protein-RNA complexes to regulate many physiological processes (59-65). Recently, it was reported that knock down of the RUVBL1 component of the R2TP complex induced cell cycle block and ER stress (66). Furthermore, ECD has been shown to associate with the stress response protein Thioredoxin-Interacting Protein (TXNIP) (67) which is known to bind to the ER chaperone PDI (protein disulfide bond isomerase) to increase its enzymatic activity to relieve ER stress (20). Lastly, TXNIP was recently shown to be a novel component of the UPR and is regulated by the PERK pathway (68). Based on these multiple lines of suggestive evidence pointing to a potential role of ECD in the regulation of ER stress, we investigated ECD and demonstrated that it modulates ER stress signaling, particularly the PERK arm. Induction of ER stress, using
both chemical ER stress inducers (Thapsigargin and Tunicamycin) and physiological ER stress inducers (glucose starvation), led to a reduced level of the ECD protein, and this effect was not seen in PERK KO or phospho-deficient eIF2α MEFs. Notably, ECD mRNA levels were increased, suggesting impaired ECD translation as a mechanism for reduced protein levels. ECD depletion increased the levels of p-PERK and its downstream targets, p-eIF2α and CHOP mRNA while ECD overexpression markedly decreased their levels upon ER stress induction. Spliced XBP-1, the downstream target of IRE1α and cleaved AFT6 were slightly affected. Significantly, ECD overexpression and depletion distinctly affected the survival outcome of cells upon ER stress. Additionally, we found that the levels of GRP78 (GRP94 and PDI to a lesser extent) were dependent on ECD and that depletion or overexpression of ECD decreased or increased GRP78 protein levels, respectively, upon ER stress induction. However, GRP78 mRNA was not affected, suggesting a post-translational effect. Deletion of ECD followed by cycloheximide treatment showed that the decrease in GRP78 protein level was faster in ECD-null cells. Reciprocally, in ECD overexpressing cells, the decrease in the levels of GRP78 protein was slower upon cycloheximide treatment. Finally, to determine whether GRP78 was required for ECD effects on PERK signaling, we knocked down GRP78 and found that even in the presence of overexpressed ECD, a knock down of GRP78 abrogated the attenuating effect of ECD on PERK, correlating with a decrease in the colony formation of the ECD overexpressing cells. Taken together, these results argue that ECD enhances the levels of chaperones, predominantly, GRP78 to enhance the folding capacity of the stressed ER and reduce stress.
2.3 MATERIALS AND METHODS

Reagents and antibodies

Thapsigargin, (Cat# 12758) and Tunicamycin (Cat# 12819) and Brefeldin A (cat# 9972) were from Cell Signaling and dissolved in DMSO. Dithiothreitol (cat# 9776), Sorbitol (cat# 1876) and Hydrogen Peroxide (cat# H1009) were from Sigma. A monoclonal antibody against ECD, generated in our laboratory, has been described previously (69). Antibodies against p-PERK (cat# 3179), ATF4 (cat# 11815), p-eIF2α (cat# 9721), eIF2α (cat# 9722), Caspase 3 (cat# 9662), s-XBP1 (cat# 12782), IRE1α (cat# 3294), GRP78 (cat# 3177), p-SAPK/JNK (cat# 9251) and total PERK (cat# 3192) were from Cell signaling. ATF6 antibody was purchased from Enzo Life (cat# ALX-804-381-C100).

Cell lines and culture conditions

ECD inducible MEFs were generated from the lab. 13.5 days old embryos of mice of the genotype Tet(O)-Flag-hECD-IRES-eGFP were used. rtTA was introduced retrovirally to generate Tet(O)-Flag-hECD-IRES-eGFP; rtTA. ECD fl/fl MEFs were maintained in DMEM medium supplemented with 10% fetal bovine serum and treated with adenoviruses coding for GFP (adeno-GFP, control) or Cre (adeno-Cre) as described previously (54,55). The culture condition for the ECD inducible MEFs was same as the ECD fl/fl MEFs. Panc-
1 cells have been previously described (70). For ECD overexpression studies, control MEFs or ECD over-expressing MEFs were cultured with 1μg/ml of Doxycycline (Cat # 231286, BD Biosciences) to induce expression of the ECD transgene. MEFs with a non-phosphorylatable eIF2α were obtained from Dr. Thomas Rutkowski’s lab, Carver College of Medicine, Iowa and has been described elsewhere (71). PERK knockout (KO) MEFs were from ATCC (CRL-2976) while IRE1α KO and ATF6 KO were gifts from Dr. Urano’s lab, Washington University St. Louis and Dr. Hendershot’s lab, St. Jude Children Research Hospital, Tennessee, respectively. Immortal mammary epithelial cell lines MCF-10A, 76NTERT were from the lab and was cultured in DFCI-1 medium (55). Cancer cells line MCF7 and MDA-468 were cultured in alpha media. NOX4 expressing MDA-231 were obtained from Dr. Teoh-Fitzgerald’s lab (UNMC, Biochemistry).

**Western blotting**

For western blotting, cell lysates were prepared in RIPA buffer (Cat# 89901, Thermo Scientific) supplemented with protease inhibitors (Roche, Cat# 11836145001). Lysates were resolved on SDS-PAGE gel, transferred on to nylon membrane (IPVH00010, Millipore), and subjected to ECL-based Western blotting as described (54,55).

**Colony Formation Assay**- For clonogenic assay, 1,000 cells were plated in triplicate and allowed to attach to the plate (about 8 h) followed by treatment with Thapsigargin for 24h. 10 days later, colony formation was assessed as previously described (54,55).
**In vitro kinase assay**- A total of 500 ng of purified recombinant ECD proteins or its mutants was incubated with 0.2 mM ATP, 1 µCi of [³²P]ATP (Perkin-Elmer), and 0.2 µl (10 U) of human recombinant PERK or CK2 (NEB, Beverly, MA) at 30°C for 30 min. The reaction was stopped by adding SDS-PAGE sample buffer. The ³²P-labeled proteins were detected by autoradiography after SDS-PAGE and then transfer to polyvinylidene difluoride (PVDF) membranes (Millipore).

**Induction of ER stress by glucose starvation**- Panc-1 cells were maintained in DMEM supplemented with 10% fetal bovine serum and then switched to glucose-free DMEM medium (cat# 11966-025, life technologies) for indicated times.

**Statistical analysis**- for assessment of statistical significance, a Student t test was used. P values of < 0.05 were considered statistically significant

**RNA isolation and real-time quantitative PCR (qRT-PCR)**

Total RNA was isolated using the TRizol reagent (Invitrogen cat# 15596018). 1 µg of RNA was reverse- transcribed using SuperScript II reverse transcriptase (Invitrogen cat# 18064014). The qPCR was performed with primer sets indicated in the table below:
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<th>Reverse primer</th>
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2.4 Results

2.4.1 Effect of ER stress induction by Thapsigargin, Tunicamycin, Brefeldin A on ECD protein levels in MCF-7, MDA-468 and MCF-10A

To begin to explore the potential link of ECD to stress pathways, we assessed the effects of various chemical ER stress inducers on ECD protein levels. For this purpose, cancer cell lines MCF7 and MDA-468 and normal mammary epithelial cell line MCF-10A were treated with Thapsigargin, Tunicamycin and BFA, and ECD protein levels were analyzed by western blot. Treatment with either chemical increased the levels of eIF2α phosphorylation, a marker of ER stress (Figure 2.1A-C). ECD protein levels did not change much in MCF7 and MDA-468 cells with either chemical but the levels decrease more dramatically in MCF-10A (Figure 2.1 A-C)
**Figure 2.1 Effect of ER stress induction in MCF7, MDA-468 and MCF-10A:** (A-C): indicated cells were treated with Thapsigargin (Tg), Tunicamycin (tun) and Brefeldin A (BFA) and equal protein was resolved on SDS-PAGE gel followed by western blot with indicated antibodies.
2.4.2 Physiological stress by glucose deprivation led to a decrease in ECD protein levels

Given the effect of chemical ER stress inducers on ECD protein levels presented above, we next assessed whether physiological stress such as glucose starvation would have similar effects. For this purpose, we used a human pancreatic carcinoma cell line (Panc-1) and mouse beta cells (MIN-6) cells both of which are known to exhibit ER stress upon glucose starvation (21). These cells were switched from their normal complete DMEM medium to glucose-free medium for various time points and ECD protein levels were assessed by western blot. This glucose-free culture condition increased the levels of the glucose-regulated protein 78 (GRP78), PERK phosphorylation (p-PERK) and p-eIF2α as expected (21). Significantly, similar to chemical ER stress, glucose starvation-induced ER stress also exhibited reduced levels of the ECD protein (Figure 2.2 A-B)
<table>
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**Panc-1**

Glucose starvation
Figure 2.2: Physiological stress by glucose starvation reduces ECD protein levels:

(A-B) Human pancreatic carcinoma and mouse beta cell lines were cultured in glucose-free medium for indicated time points. Equal protein was resolved on SDS-PAGE gel followed by western blot with indicated antibodies.
2.4.3 Effects of other forms of stress on ECD protein levels

The effect of both chemically-induced and physiologically-induced stress on the levels of ECD protein prompted us to further examine the effects of other stresses (oxidative, osmotic, and reducing) on ECD protein levels. For that purpose, we first treated MCF10A, 76NTERT and MCF7 cells with H$_2$O$_2$, a commonly used oxidative stress inducer (72). Significantly, treatment with H$_2$O$_2$, also led to a decrease in ECD protein levels (Figure 2.3 A). To further assess these effects, we used another type of cells (MDA-231) which are under a physiologically-induced oxidative stress. These cells express the NOX4 system, which generates ROS and oxidative stress (73,74) as diagrammed in Figure 2.3 B. Again, here too, even in the absence of any exogenously-derived stress, ECD protein levels are lower in the NOX4-expressing cells as compared to the control cells. Reductive and osmotic stresses, by DTT and sorbitol treatment, respectively, also led to a decrease in the ECD protein level (Figure 2.3 C-D).
<table>
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**A**

**MCF10A**

**76NTert**

**MCF7**

$H_2O_2$ (μM) | -   | +   | -   | +   |
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<td>β-actin</td>
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The NOX4 system and Oxidative stress:

NADPH $\rightarrow$ NADP$^+$ $+$ e$^-$ $+$ H$^+$

O$_2$ $\rightarrow$ O$_2^-$ $\rightarrow$ H$_2$O$_2$ $\rightarrow$ ER Stress

Oxidative stress
Figure 2.3: Effects of other forms of stress (oxidative, reductive and osmotic) on ERCD protein level: (A) MCF-10A, 76NTERT and MCF7 cell lines were treated with indicated doses of H₂O₂, (B) MDA-231 expressing NOX4 and their control cells were lysed, (C) MCF10A and MCF7 cell lines were treated with DTT, (D) T98G cells were treated with sorbitol. Equal protein was resolved on SDS-PAGE gel followed by western blot with indicated antibodies.
2.4.4 ER stress induction by Thapsigargin and glucose deprivation led to increase in ECD mRNA levels

Given that all forms of stress led to a decrease in ECD protein levels, we assessed whether this decrease in ECD protein levels was due to reduced ECD mRNA levels. We treated MCF10A cells with Thapsigargin and measured ECD mRNA levels using qRT-PCR. Induction of CHOP mRNA was used as a control (Figure 2.4 A). Notably, ECD mRNA levels were not only not reduced but in fact showed an increase (Figure 2.4 B), suggesting that the reduction in the ECD protein level was not at the transcriptional level. Likewise, physiological stress by glucose starvation inPanc-1 cells also increased ECD mRNA levels (Figure 2.4 D) with GRP78 mRNA induction used as control (Figure 2.4 C).
A

CHOP mRNA

Relative mRNA

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B

ECD mRNA

Relative mRNA

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C

**GRP78 mRNA**

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D

**ECD mRNA**

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Figure 2.4: ER stress induction by Thapsigargin and glucose starvation led to an increase in ECD mRNA. MCF-10A cells were treated with Thapsigargin (A-B) and Panc-1 cells were cultured in glucose-free medium (C-D), then total RNA was isolated followed by qRT-PCR using primers against CHOP, GRP78, and ECD (Mean +/- SD and p-values are shown from 3 independent experiments * p<0.05). CHOP and GRP78 mRNA induction served as control for Thapsigargin-induced and glucose starvation-induced ER stress, respectively.
2.4.5 The decrease in the levels of ECD protein upon ER stress induction is PERK-eIF2α dependent

Given the results above that various stresses led to a decrease in ECD protein level but that ECD mRNA increased, we sought to determine the mechanism by which ECD protein and mRNA levels inversely correlate. All stresses mentioned above are interconnected. For instance, Oxido-reductive stress induces ER stress and vice versa, as summarized in the diagram in Figure 2.3B. ER stress activates three canonical signaling pathways summarized in Figure 1.1. To determine which pathway controls ECD protein and mRNA levels, we induced ER stress in cells in which the upstream mediators of the pathways (PERK, IREα and ATF6) have been genetically abrogated. For that purpose, we used ATF6 KO, IRE1α KO and PERK KO MEFs in which the pathways have been individually knocked out (KO). The absence of the protein was confirmed by western blot with antibody against the protein that was knocked out (Figure 2.5A-C). In ATF6 KO and IRE1 KO MEFs, the decrease in ECD protein levels was comparable to that in the control wild-type (Figure 2.5A-B). However, in PERK KO MEFs, the decrease in ECD protein levels was abrogated (Figure 2.5C), suggesting that the decrease in ECD protein levels is PERK dependent.
A  

**ATF6**

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<tr>
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- ECD
- β-actin

B  

**IRE1α**

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- ECD
- IRE1
- β-actin
Figure 2.5: The decrease in the levels of ECD protein upon ER stress induction is PERK dependent. (A-C): Wild-type (WT) ATF6, IRE1α, PERK and their knockout (KO) MEFs were treated with Thapsigargin and then cell lysates were resolved on SDS-PAGE gels followed by western blot with indicated antibodies.
As we previously observed that ECD mRNA increased upon ER stress induction but the protein levels decreased, here we sought to assess whether ECD mRNA changes in PERK WT vs. KO MEFs. Again, we treated PERK WT and KO MEFs with Thapsigargin and measured the change in ECD mRNA by qPCR. The identity of the PERK KO MEFs in this experiment was determined by analyzing the product of the PCR product obtained with primers against the PERK KO domain. As shown in Figure 2.6A, there was no PCR product in the PERK KO samples, confirming the identity of the cells. To further confirm that, we used primers against CHOP mRNA in the qPCR. As expected, while CHOP mRNA was induced in the PERK WT cells, the induction was very minimal in the PERK KO cells (Figure 2.6B), since CHOP is downstream of PERK (44). Significantly, while ECD mRNA increased in PERK WT MEFs, it did not increase much in PERK KO MEFs (Figure 2.6C). This result that ECD mRNA failed to increase when the protein levels didn’t change, suggests that ECD mRNA only increases when the protein levels are down, suggesting a feedback mechanism.
A

PERK WT

PERK KO

KO domain

β-actin

B

CHOP mRNA

Relative mRNA

PERK WT

PERK KO

UT 7h 14h
**Figure 2.6: ECD mRNA levels failed to increase in PERK KO MEFs upon ER stress.** (A): PCR was performed on RNA isolated from PERK WT and KO MEFs with primers against the KO domain. The PCR product was resolved on agarose gel. (B-C): PERK KO and control WT MEFs were treated with Thapsigargin and total RNA was isolated at indicated time points followed by qRT-PCR with primers against CHOP.
To explain the PERK-dependent decrease in ECD protein levels, we proposed two hypotheses:

1) PERK phosphorylates ECD leading to ECD degradation since (i) PERK is a kinase, (ii) ECD is a phospho-protein as a substrate for CK2 phosphorylation and (iii) certain proteins are known to undergo a phosphorylation-dependent degradation (75).

2) PERK phosphorylates eIF2α to inhibit ECD protein translation since PERK is known to inhibit translation during ER stress signaling by phosphorylating eIF2α (37).

To investigate the first hypothesis that ECD undergoes a PERK phosphorylation-dependent degradation, we first assessed whether ECD could be a substrate for PERK phosphorylation by performing an in vitro kinase assay of ECD by PERK. EIF2α was used as the positive control for PERK phosphorylation since EIF2α is a substrate for PERK (37); and CK2 was used as a positive control for phosphorylation of ECD since CK2 is known to phosphorylate ECD (55). As expected, EIF2α and ECD were phosphorylated in vitro by PERK and CK2, respectively (Figure 2.7, lanes 1-2 and 3, respectively). However, a phosphorylation of ECD by PERK was not observed (Figure 2.7, lane 4-5).
Figure 2.7: PERK does not phosphorylate ECD: In vitro kinase assay of recombinant human ECD prepared from two different sources (bacterial and insect cell derived) was performed using human recombinant GST fused PERK. Proteins were separated by SDS-PAGE, transferred to PVDF membrane, and phosphate labeling was detected by autoradiography. Phosphorylation of eIF2α by PERK was used as the positive control for PERK mediated phosphorylation while phosphorylation of ECD by CK2 was used as the positive control for ECD phosphorylation.
To investigate the second possibility that ECD translation is blocked by PERK phosphorylation of eIF2α, we resorted to eIF2α MEFs. Since PERK activation and subsequent phosphorylation of eIF2α mediates translational block in response to ER stress (37), we utilized MEFs from mice that carry a serine 51 to alanine mutation in eIF2α which prevents its phosphorylation by activated PERK and makes the cells resistant to PERK-mediated translation block upon ER stress induction (71). As expected, the levels of p-eIF2α increased in WT MEFs but not in the phospho-deficient eIF2α mutant MEFs when ER stress was induced with Thapsigargin or Tunicamycin (Figure 2.8, compare lanes 1-3 with lanes 4-6). Importantly, while ECD protein levels decreased in WT MEFs treated with ER stress inducers (Fig. 1J, compare lane 1 to lanes 2-3), the levels of ECD protein did not change in identically-treated mutant eIF2α MEFs (Figure 2.8, compare lane 4 to lanes 5-6). Together, these results support a conclusion that ECD protein levels are downregulated upon ER stress in a PERK-eIF2α dependent manner, and that ER stress upregulates ECD mRNA levels, suggesting a regulatory link between ECD and the PERK pathway of ER stress.
Figure 2.8: The decrease in ECD protein levels is abrogated in eIF2α phospho-deficient MEFs. WT eIF2α MEFs or mutant eIF2α phospho-deficient MEFs were treated with Thapsigargin (Tg, 50 nM) or Tunicamycin (Tun, 50 ng/ml) for 14 h and then cell lysates were analyzed by western blotting with indicated antibodies.
2.4.6 ECD depletion or over-expression distinctly regulate ER stress signaling upon ER stress induction

The abrogation of the decrease and increase in ECD protein and mRNA levels, respectively, upon disruption of PERK pathway (Figure 2.5-2.8) suggested that ECD may be functionally linked to UPR through PERK pathway. Therefore, we assessed the activation of the PERK pathway in the absence or presence of UPR inducers in cells in which ECD could be inducibly deleted. For this purpose, we utilized \(ECD^{fl/fl}\) MEFs which have been previously established and shown to exhibit \(ECD\) gene deletion and loss of ECD protein expression upon infection with adenovirus expressing Cre recombinase (54). Previously, it was observed that induction of \(ECD\) deletion in these \(ECD^{fl/fl}\) MEFs, aside from inducing cell cycle arrest, led to a decrease in cell survival even in the absence of any stress (54,55).

Notably, Cre-mediated deletion of \(ECD\) in the \(ECD^{fl/fl}\) MEFs led to an increase in the levels of phospho-PERK (p-PERK) and p-eIF2α, when compared to control adeno-GFP infected MEFs (Figure 2.9 compare lane 1 with 3), and these effects were further enhanced by treatment with Thapsigargin (Tg), the inhibitor of the Sarco/Endoplasmic Reticulum Calcium ATPase (Figure 2.9, compare lane 2 with 4). Spliced XBP1, the downstream target of IRE1α signaling also showed some increase in ECD null cells upon Thapsigargin treatment while ATF6 did not show a significant change (Figure 2.9).
Figure 2.9: Increased UPR in ECD-null cells upon Thapsigargin. Ecd^{+/+} MEFs were infected with adenoviruses coding for GFP (adeno-GFP for control) or Cre (adeno-Cre) for 72h. The cells were then left untreated or treated with Thapsigargin. Equal amount of proteins was resolved on SDS-PAGE gel followed by western blotting with indicated antibodies.
Consistent with the increased p-PERK levels upon ECD deletion, Thapsigargin treated ECD-depleted MEFs also exhibited increased expression of CHOP mRNA (Figure 2.10A), a downstream effector of the PERK pathway whose target genes promote cell death (37-41,43,44). The increased CHOP mRNA correlated with an increased cleaved caspases 3 in ECD null cells (Figure 2.10B, compare lane 2 with 4) and a significant reduction in cell survival, as measured by colony-forming assays (Figure 2.10C).
A

[Graph showing CHOP mRNA expression levels over time (0h, 7h, 12h) with bars representing relative mRNA levels for WT and ECD-/- genotypes.]

B

[Western blot images showing protein levels of ECD, Caspase 3, Cleaved-Caspase 3, and β-actin in WT and ECD-/- genotypes with lanes 1 to 4.]
Figure 2.10: ECD-null cells exhibited increased ER stress and cell death upon Thapsigargin treatment. (A): After adenoviruses infection as described in Figure 2.9, cells were treated with Thapsigargin. Total RNA was isolated followed by qRT-PCR with
CHOP primers; Mean +/- SD and p-values from 3 independent experiments are indicated * p<0.05. (B): After Adenoviruses infection, the cells were then left untreated or treated with Thapsigargin. Equal amount of proteins was resolved on SDS-PAGE gel followed by western blotting with indicated antibodies. (C): After adenoviruses infection as described in (A), equal number (1,000) of wild-type (WT) or ECD/- cells were plated in triplicate and treated with Thapsigargin for 24h. 10 days later, surviving colonies were assessed after crystal blue (0.5% in 25% Methanol) staining. The color retained after the wash was dissolved in 10% acetic acid and the absorbance was read at 590 nm. The graph below represents the relative absorbance; Mean +/- SD and p-values from 4 independent experiments are indicated * p<0.05; ** p≤0.002
To further assess these effects, we used physiological stress in Panc-1 cells in which ECD was knocked down followed by glucose starvation. Again, here too, a siRNA knock down of ECD plus glucose starvation led to an enhanced eIF2α phosphorylation, the downstream target of PERK, and increased cleaved caspase 3 as compared to control cells (Figure 2.11, compare lanes 1-5 with lanes 6-10), suggesting more cell death in ECD-knocked down cells upon glucose starvation. Moreover, spliced XBP-1 levels, the downstream target of IRE1α, were increased in ECD siRNA-treated cells as compared to the control cells whereas ATF6 levels were comparable (Figure 2.11, compare lanes 1-5 with lanes 6-10).
Figure 2.11: ECD knock down in Panc-1 cells upon glucose starvation led to increased UPR and cell death. Panc-1 cells were treated with control or ECD siRNA for 48h. Then, the cells were switched to glucose-free media and cell lysates were prepared at indicated time points followed by western blot with indicated antibodies.
Given the effects of ECD depletion on the UPR presented above, we used a reciprocal approach to overexpress ECD and then examined its impact on the UPR, in particular the PERK pathway, upon ER stress induction. To this end, and because of ongoing difficulties overexpressing ECD in cancer cell lines, we resorted to MEFs generated in the lab from mice that carry a Doxycycline (Dox)-inducible ECD transgene ((Tet(O)-Flag-hECD-IRES-eGFP; rtTA)) or a control transgenic mouse without rtTA ((Tet(O)-Flag- hECD-IRES-eGFP)). These MEFs were treated with Dox to induce ECD overexpression, followed by treatment with Thapsigargin. As expected, control MEFs exhibited an increase in p-PERK and CHOP mRNA; however, the MEFs with Dox-induced ECD overexpression exhibited a substantially reduced level of p-PERK and CHOP mRNA upon Thapsigargin treatment as compared to control MEFs (Figure 2.12A-B).
Figure 2.12: Decreased p-PERK and CHOP mRNA induction in ECD inducible MEFs upon Thapsigargin

**MEFs upon Thapsigargin.** (A): ECD inducible MEFs [(Tet(O)-Flag- hECD-IRES eGFP; rtTA)] or control MEFs [(Tet(O)-Flag- hECD-IRES eGFP)] were treated with...
Dox for 48 h followed by treatment with Thapsigargin. Cell lysates were prepared at indicated time points and equal amount of proteins was resolved on SDS-PAGE gel followed by western blot with indicated antibodies. (B): Following ECD induction and Thapsigargin treatment as described above, CHOP mRNA levels were assessed using qRT-PCR.
Likewise, when the ECD inducible MEF cell lines were treated with Thapsigargin for various time points (3, 6, 12 or 24h), ECD overexpressing MEFs exhibited lower levels of p-PERK compared to those in control MEFs (Figure 2.13A, compare lanes 1-5 with lanes 6-10). A corresponding reduction in the levels of ATF4, a downstream target of PERK signaling, was seen in ECD overexpressing MEFs compared to their control MEFs (Figure 2.13A, compare lanes 3-5 with lanes 8-10); s-XBP1 and ATF6 also were slightly reduced in ECD over-expressing MEFs (Figure 2.13A, compare lanes 1-5 with lanes 6-10). Similar to Thapsigargin, induction of ER stress with Tunicamycin, which induces ER stress by inhibiting glycosylation of proteins in the ER, was also associated with lower levels of p-PERK and p-eIF2α in ECD overexpressing MEFs as compared to control MEFs (Figure 2.13B, compare lanes 2-5 with lanes 7-10). Taken together, these results strongly support a conclusion that ECD negatively regulates PERK signaling.
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Figure 2.13: Decreased UPR signaling in ECD inducible MEFs correlated with in chaperones induction upon Thapsigargin treatment. (A-B): ECD inducible MEFs [(Tet(O)-Flag- hECD-IRES eGFP;rtTA)] or control MEFs [(Tet(O)-Flag- hECD-IRES eGFP)] were treated with Dox for 48 h followed by treatment with Thapsigargin. Cell lysates were prepared at indicated time points and equal amount of proteins was resolved on SDS-PAGE gel followed by western blot with indicated antibodies.
2.4.7 ECD overexpression protects cells from ER stress-induced cell death

ER stress response is initially aimed at the survival of cells (76); however severe ER stress shifts the response from a pro-survival to a pro-apoptotic response (36,44) through PERK-mediated induction of CHOP expression, a transcription factor that enhances the expression of pro-apoptotic pathway genes (37-41,43,44,77). Several studies found that inhibition of the PERK pathway protected cells against stress-induced cell death (78-81). Given our observations that ECD functions as a modulator of PERK signaling, we assessed if the level of ECD determines a differential survival vs. apoptotic cell fate upon ER stress induction. For that purpose, we treated control MEFs or Dox-inducible ECD overexpressing MEFs with Thapsigargin and then assessed the level of apoptosis induction by examining caspase 3 cleavage (82). As anticipated, a Thapsigargin dose-dependent increase in cleaved caspase 3 levels was observed in control MEFs, whereas the levels of cleaved caspase 3 were markedly lower in ECD-overexpressing MEFs (Figure 2.14). Moreover, given the role of stress-activated protein kinases SAPK/JNK in mediating ER stress-induced cell death, as a downstream target of IRE1α (44,83,84), we further assessed the levels of phospho-SAPK/JNK. Notably, while the p-SAPK/JNK level increased in response to Thapsigargin in control MEFs, no changes were observed in ECD-overexpressing MEFs (Figure 2.14, compare lane 1-3 to 4-6).
Figure 2.14: Decreased cleaved caspase 3 and p-SAPK/JNK levels in ECD overexpressing MEF upon Thapsigargin. ECD was induced as described in (Fig. 3 and 4) followed by Thapsigargin treatment for 24 h. Equal amount of proteins was resolved on SDS-PAGE gel followed by western blot with indicated antibodies.
To further assess the pro-survival effects of ECD against apoptotic cell fate upon ER stress induction, we assessed the abilities of control vs. ECD-overexpressing MEFs to form colonies after their exposure to ER stress. For this purpose, equal numbers of control or ECD overexpressing MEFs were treated with Thapsigargin for 24h, and the cells were maintained in Thapsigargin-free medium for 10 days followed by crystal violet staining and counting of surviving colonies. Notably, more colonies were observed in ECD overexpressing MEFs as compared to control MEFs (Figure 2.15), further supporting the conclusion that ECD provides survival advantage upon ER stress.
**Figure 2.15: Increased colony formation in ECD over-expressing MEFs upon Thapsigargin.**

After ECD induction, control and ECD inducible MEFs were trypsinized and equal number of cells (1,000) were plated in triplicates. 8 h later, the cells were treated with Thapsigargin for 24h. 10 days later, surviving colonies were assessed by
Crystal blue staining (0.5% in 25% Methanol). The color retained after the wash was dissolved in 10% Acetic acid and the absorbance was read at 590 nm. The graph below represents the relative absorbance; Mean +/- SD and p-values from 4 independent experiments are indicated ** p≤0.002.

2.4.8 ECD co-localizes and associates with PERK, ATF6, IRE1α and GRP78

Given the roles of ECD in modulating ER stress as presented above, we sought to define the possible mechanism(s). Previously, ECD has been reported to localize, predominantly, in the cytoplasm with a fast nuclear export, allowing it to shuttle through the nucleus (85). Given the roles of ECD in attenuating ER stress signaling, particularly PERK, we first asked whether ECD could also be localizing to the ER or co-localizes with the upstream mediators of the ER stress signaling, especially as previous works from the lab showed a peri-nuclear localization for ECD, which is the classic definition of an ER localization. Therefore, we performed a proximity ligation assay (PLA) to assess the localization of ECD relative to PERK and GRP78. Known ECD interaction with PIH1D1 component of the R2TP served as the positive control (55,59). Indeed, ECD and PIH1D1 formed distinct foci detectable by PLA (Figure 2.15B, red dots). Significantly, PLA signals were also observed for ECD and GRP78 or ECD and PERK (Figure 2.15 C-D). Negative controls (IgG) did not show any PLA signal (Figure 2.15 E).
IgG Mouse + IgG Rabbit
Figure 2.15: ECD co-localizes with PERK and GRP78. MCF-10A cells were fixed with 3% PFA and stained with indicated antibodies (all antibodies were generated in rabbit except anti-ECD mouse antibody) followed by species-specific secondary antibodies linked to complementary DNA probes to allow fluorescent probe-based detection of the PCR amplification product as distinct foci. Incubation with Proximity Ligation Assay plus and minus probes, followed by ligation and amplification, was carried out according to the manufacturer protocol. Red dots indicate interaction. ECD and PIH1D1 served as the positive control.
The localization of ECD close to the ER stress signaling mediators prompted us to assess their association. For that purpose, we carried out immunoprecipitation (IP) of ECD from lysates from MCF-10A cell followed by western blotting for PERK, GRP78, IRE1α, and ATF6 antibodies to assess their association. IP of ECD co-immunoprecipitated PIH1D1 (Figure 2.16) as expected based on known interaction of ECD with PIH1D1 (55,59). Importantly, PERK, GRP78, IRE1α and ATF6 also co-immunoprecipitated with ECD (Figure 2.16).
Figure 2.16: ECD associates with PERK, GRP78, IRE1α and ATF6. Lysates from MCF-10A cells were subjected to IP with anti-ECD antibody overnight followed by Western blotting with indicated antibodies. ECD and PIH1D1 served as the positive controls.
To further assess these results, we carried out a GST pull down experiment by incubating bacterially purified GST-fused full length ECD, C-terminal and N-terminal deletion mutants ECD with cell lysate containing overexpressed GRP78, IRE1α, PERK and ATF6 followed by western blot with the respective antibodies; this GST pull down experiment will help define the domain(s) on ECD required for their association. Known interaction of full length GST-ECD with RUVBL1/Pontin was used as a positive control (55). As expected, full length GST-ECD pulled down RUVBL1/Pontin (Figure 2.17). Significantly, full length ECD interacts with GRP78, IRE1α and ATF6. N-terminal and C-terminal deletions of ECD abrogated interaction with GRP78 and ATF6 (Figure 2.17) while only the C-terminal deletion of ECD abrogated interaction with IRE1α (Figure 2.17), suggesting that both the N and C termini are required for interaction with GRP78 and ATF6 but only the C-terminus is required for interaction with IRE1α. GST-ECD did not pull down PERK.
Figure 2.17: Defining the domain(s) on ECD required for interaction. 3µg of bacterially purified GST fused ECD full length (FL) and its mutants were incubated with lysates from 293T cells overexpressing GRP78, IRE1α and ATF6 for 3h at 4°. The GST beads were washed and resolved on SDS-PAGE gel followed by western blot with indicated antibodies. Pontin and ECD interaction served as a positive control. Below the blot is the ponceau.
2.4.9 ECD associates with GRP78 and PERK upon ER stress induction

We further examined the ER stress-related roles of ECD by assessing its association with PERK and GRP78 upon ER stress induction. For that purpose, we carried out immunoprecipitation (IP) of ECD from lysates from the MCF-10A cell, treated or left untreated with Thapsigargin, followed by western blotting for PERK and GRP78 antibodies to assess their association. IP of ECD co-immunoprecipitated PIH1D1 (Figure 2.18A), as expected based on known interaction of ECD with PIH1D1 (55,59). Importantly, PERK and GRP78 also co-immunoprecipitated with ECD (Figure 2.18A). A reverse IP of GRP78 also co-immunoprecipitated ECD, with PERK used as a positive control (Figure 2.18B). Taken together, these results suggest that ECD associates with PERK and GRP78.
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Figure 2.18: ECD association with PERK and GRP78 upon ER stress induction.

Lysates from MCF-10A cells, treated or left untreated with Thapsigargin, were subjected to IP with anti-ECD antibody (A) or anti-GRP78 (B) followed by Western blotting with indicated antibodies. ECD and PH1D1(A) or GRP78 and PERK (B) served as positive controls.
2.4.10 ECD does not modulate enzymatic activity of PERK in vitro

Given that ECD associates with PERK and modulates its signaling, we hypothesized that ECD could be affecting PERK signaling either directly or indirectly. To test the direct hypothesis, we assessed the possibility that ECD affects PERK enzymatic activity. We performed an in vitro kinase assay of PERK with its substrate eIF2α in the presence of an increasing concentration of pure ECD protein. This experiment showed that eIF2α phosphorylation did not change in the presence of an increasing concentration of ECD (Figure 2.19), suggesting that ECD does not directly modulate PERK enzymatic activity.
Figure 2.19: ECD does not modulate enzymatic activity of PERK toward eIF2α. *In vitro* kinase assay of recombinant human GST fused PERK with its substrate eIF2α in the presence of increasing concentration of pure ECD protein. Proteins were separated by SDS-PAGE, transferred to PVDF membrane, and phosphate labeling was detected by autoradiography.
2.4.11 ECD positively regulates levels of chaperones, predominantly, GRP78 protein

The results above that ECD does not modulate the enzymatic activity of PERK ruled out the possibility of a direct effect on PERK signaling. Therefore, we examined the next possibility of an indirect mechanism whereby ECD acts through another protein to modulate PERK. GRP78 is a repressor of the three arms of the ER stress signaling, including PERK, of which it is known to bind to the luminal domain to repress its activation in unstressed cells (13,14). The dissociation of GRP78 from PERK upon ER stress is key to activation of PERK signaling, but it is also a reversible process, as GRP78 is also known to bind back to PERK to inhibit its signaling when the stress is relieved. Accordingly, we asked whether ECD could be cooperating with GRP78 to modulate PERK signaling especially since ECD and GRP78 associate (Figure 2.16-2.18). To explore that possibility, we first examined the changes in the levels of GRP78 in the presence and absence of ECD upon ER stress induction. We first exposed the wild-type MEFs or MEFs with ECD deletion induced by adeno-Cre to Thapsigargin, and then assessed the levels of GRP78 protein, at various time points, by western blotting. The levels of GRP78, over time, were reduced in ECD-null MEFs compared to GRP78 levels in control MEFs (Figure 2.20A, compare lanes 3-5 with 8-10). Likewise, using physiological stress, a knock down of ECD in Panc-1 cells led to a decrease in GRP78 levels upon glucose starvation (Figure 2.20B, compare lanes 1-5 with lanes 6-10).
**Figure 2.20: ECD deletion or knock down led to a reduced induced levels of GRP78.**

(A): Ecd floxed MEFs were treated with adenovirus as previously described and then the cells were treated with Thapsigargin (50 nM); (B): ECD was knocked down by siRNA (20 nM) in Panc-1 cells followed by exposure to glucose-free media. Cell lysates were collected at indicated time points followed by western blot with indicated antibodies.
To further examine these effects, we used a reciprocal approach by overexpressing ECD and assessing GRP78 protein levels upon ER stress induction. In the presence of overexpressed ECD, upon induction of ER stress by Thapsigargin treatment, the levels of GRP78 were robustly elevated (Figure 2.21A). GRP94 and PDI, two other chaperones with ER stress-related functions, were also increased although not as robustly as GRP78 (Figure 2.21A). Similar results were obtained with Tunicamycin treatment, another ER stress inducing chemical (Figure 2.21B).
Figure 2.21: ECD overexpression led to a robust increase in chaperone levels, predominantly, GRP78. ECD inducible MEFs [((Tet(O)-Flag- hECD-IRES eGFP; rtTA))] or control MEFs [((Tet(O)-Flag- hECD-IRES eGFP))] were treated with Dox for 48 h followed by treatment with Thapsigargin (50 nM, A) or Tunicamycin (50 ng/ml, B). Cell lysates were prepared at indicated time points and equal amount of proteins was resolved on SDS-PAGE gel followed by western blot with indicated antibodies.
2.4.12 ECD does not affect GRP78 transcription or mRNA stability

Based on the distinct effect of ECD depletion or overexpression on GRP78 protein levels, we next examined GRP78 mRNA induction in the presence or absence of ECD. We carried out qPCR analyses of mRNA isolated from WT or ECD-null MEFs treated with Thapsigargin. As expected, GRP78 mRNA levels increased in WT MEFs upon Thapsigargin treatment (Figure 2.22A); however, the levels of GRP78 mRNA induction in ECD-null MEFs was comparable to that in control MEFs (Figure 2.22A). Similarly, GRP78 mRNA levels in control vs. ECD overexpressing MEFs treated with Thapsigargin were comparable (Figure 2.22B). Likewise, we examined the post-transcriptional effect of ECD on GRP78 mRNA stability by treating cells with Actinomycin D, to inhibit mRNA synthesis, and collected mRNA at various time points followed by qPCR with primers against GRP78 mRNA. The rate of decrease of GRP78 mRNA over time is comparable between ECD depleted or overexpressed cells and their respective controls (Figure 2.23A-B), suggesting that ECD does not affect GRP78 mRNA stability. Taken together, these results suggest that ECD does not affect GRP78 mRNA transcription or post-transcriptionally.
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**GRP78 mRNA**

- **Relative mRNA**
- **WT**
- **ECD-/-**

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Figure 2.22: **Effect of ECD on GRP78 mRNA induction.** Following ECD deletion by Adeno-CRE infection (A) or ECD overexpression (B) and Thapsigargin treatment, the levels of GRP78 mRNA were assessed in WT (control) vs. ECD-/-(Cre-adenovirus treated) or control vs. ECD overexpressing MEFs using qRT-PCR.
A

GRP78 mRNA stability

Relative mRNA

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B

GRP78 mRNA stability

Relative mRNA

Act D:

- **WT**
- **ECD-/−**

- **Linear (WT)**
- **Linear (ECD-/−)**

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Figure 2.23: Effect of ECD on GRP78 mRNA stability. Following ECD deletion by Adeno-CRE infection (A) or ECD overexpression (B) and Thapsigargin treatment, the cells were treated with Actinomycin D and mRNA was collected at indicated time points. The levels of GRP78 mRNA were assessed in WT (control) vs. ECD-/-(Cre-adenovirus treated) or control vs. ECD overexpressing MEFs using qRT-PCR.
2.4.13 ECD enhances GRP78 protein stability and this effect is required for ECD to attenuate ER stress signaling

The results above showing that ECD does not regulate GRP78 protein at mRNA level raised a strong possibility that ECD might instead regulate GRP78 at protein level especially since the two protein associated. To determine whether GRP78 may be less stable in ECD null cells or more stable in ECD overexpressing cells, we first knocked down ECD in Panc-1 cells, which have high levels of GRP78, followed by inhibition of protein synthesis by cycloheximide treatment over various time points and GRP78 protein levels were analyzed by western blot. Significantly, the time dependent decrease in GRP78 protein levels following cycloheximide treatment was faster in ECD-knocked down cells compared to their control cells (Figure 2.24A). Next, to examine the possibility that ECD overexpression is associated with increased GRP78 protein stability, control or ECD overexpressing cells were treated with Thapsigargin to induce GRP78 protein, followed by cycloheximide (CHX) treatment over various time points. Notably, the time-dependent decrease in GRP78 levels was slower in ECD overexpressing MEFs as compared to control MEFs (Figure 2.24B). Finally, to determine whether the increase in GRP78 protein stability upon ECD overexpression was required for the attenuation of PERK activation seen upon ER stress induction, we depleted GRP78 using siRNA in both control and ECD overexpressing MEFs followed by Thapsigargin treatment. Notably, even in the presence of overexpressed ECD, the attenuating effect of ECD overexpression on PERK phosphorylation upon Thapsigargin treatment was abrogated by GRP78 knockdown (Figure 2.24C; compare lane 5-6 with 7-8). Furthermore, this knock down of GRP78 reduced the survival effect of ECD overexpression by reducing the
colonies’ ability to form the ECD overexpressing cells (Figure 2.24D, compare lane 2 and 4). Taken together, these results support the conclusion that ECD positively regulates GRP78 protein level upon ER stress induction to attenuate PERK activation and provide survival advantage.
### Panc-1

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<td>β-actin</td>
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**Graph:**

- **Ctrl siRNA**
  - $T_{1/2} > 36h$
- **ECD siRNA**
  - $T_{1/2} ~ 24h$

**Legend:**
- **ECD**
- **GRP78**
- **β-actin**
B

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**ECD**

**GRP78**

**β-actin**

Lane: 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10

Relative GRP78

**Tet(O)-Flag- hECD-IRES eGFP**

**Tet(O)-Flag- hECD-IRES eGFP; rtTA**

CHX, h: - 2 6 12 15

0 0.2 0.4 0.6 0.8 1 1.2

Relative GRP78

0 0.2 0.4 0.6 0.8 1 1.2

**Tet(O)-Flag-hECD-IRES eGFP**

**Tet(O)-Flag-hECD-IRES eGFP; rtTA**
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**Figure D**

- **1μg/ml Dox:** + + + + +
- **GRP78 SiRNA:** - - + + +
- **Scrambled:** + + - - -
- **Tg:** - + - + +

**Tet(O)-Flag- hECD -IRES eGFP**

**Tet(O)-Flag- hECD -IRES eGFP; rtTA**

**Lane:** 1 2 3 4

**Relative survival**

- **Tg:** - + - +
  - **Scrambled**
  - **GRP78**

- **Tet(O)-Flag- hECD -IRES eGFP**
- **Tet(O)-Flag- hECD -IRES eGFP; rtTA**
**Figure 2.24: ECD mediated GRP78 protein stability is required for ECD ER stress function.** (A): ECD was knocked down in Panc-1 cells followed by cycloheximide treatment (25 uM). Cell lysates were prepared at indicated time points followed by western blot with indicated antibodies. (B): ECD inducible MEFs and their control MEFs were treated with Dox, as described previously, followed by treatment with Thapsigargin and then Cycloheximide treatment (25 uM) for indicated time points. Cell lysates were prepared followed by western blotting with indicated antibodies. (C): ECD overexpressing and their control MEFs were treated with GRP78 SiRNA (30 nM) or control siRNA (scrambled). 24 h later, the cells were treated with Dox for 48 h to induce ECD overexpression followed by Thapsigargin treatment (50 nM). Equal amount of proteins was resolved on SDS-PAGE gel followed by western blotting with indicated antibodies. (D): After ECD induction, control and ECD inducible MEFs were trypsinized and equal number of cells (1,000) were plated in triplicates. 8 h later, the cells were treated with Thapsigargin for 24h. 10 days later, surviving colonies were assessed by crystal blue staining (0.5% in 25% Methanol). The color retained after the wash was dissolved in 10% Acetic acid and the absorbance was read at 590 nm. The graph below represents the relative absorbance; Mean +/- SD and p-values from 3 independent experiments are indicated **p≤0.002
2.5 DISCUSSION

The ER-localized stress response pathway referred to as the UPR is a well-conserved response to a number of cellular stresses such as unfolding of proteins in the ER. The UPR elicits a spectrum of downstream responses whose outcomes range from restoration of homeostasis to cellular apoptosis if the stress is extreme and prolonged (36,44). The UPR thus represents a double-edged sword and must be intricately regulated to prevent inappropriate cellular outcomes. Mechanisms that help modulate the magnitude and type of the UPR in response to physiological or pathological stress stimuli are not fully understood. In this study, we provide evidence that ECD protein is a repressor of stress response.

Several lines of circumstantial evidence discussed in the Introduction section suggested a potential involvement of ECD in UPR but direct support for such a role has been lacking. To explore a connection of ECD to stress response pathways, we first assessed the effects of multiple stress stimuli on ECD protein levels. Induction of stress both chemically (Thapsigargin, Tunicamycin, BFA, DTT, sorbitol, and H₂O₂) and physiologically (glucose starvation and NOX4 expression) in multiple cell lines led to downregulation of ECD protein levels (Figure 2.1-2.3). The concomitant increase in ECD mRNA levels (Figure 2.4) suggests that the protein was transcriptionally induced. By using AFT6 KO, IRE1α and PERK kinase knockout (KO), we found that PERK is the upstream mediator of the decrease in ECD protein levels and that in PERK KO MEFs, ECD protein and mRNA levels did not change upon ER stress induction (Figure 2.5-2.6). Since ECD is not a substrate for PERK phosphorylation (Figure 2.7) but ECD protein levels did not
change when eIF2α phosphorylation, the downstream effect of PERK activation, was disrupted (Figure 2.8), the results strongly suggest that ECD protein levels are regulated via the PERK-eIF2α axis upon ER stress induction. Since that axis controls translation upon stress induction, then the results further suggest that ECD protein undergoes a PERK-eIF2α-dependent translation block upon ER stress. The decrease in ECD protein levels in other forms of stress, including oxido-reductive and osmotic stresses, further argues that induction of stress in general leads to an eIF2α-dependent decrease in ECD protein levels. In fact, all forms of cellular stress, including viral infection and nutrient limitation in addition to those mentioned above, converge to a common node, eIF2α. Moreover, they are interconnected. For instance, viral infection induces ER stress (86) due to the increase in viral protein synthesis. Oxidative stress and nutrient limitation also induce ER stress (87-89).

With regard to ER stress, these findings linked ECD to the PERK-eIF2α pathway. A functional connection of ECD to the PERK arm of the UPR is supported by the distinct modulation of the PERK-mediated responses elicited by perturbations of the cellular levels of ECD. Depletion of ECD sensitized cells to PERK signaling in response to Thapsigargin treatment, with increase in p-PERK and p-eIF2α levels, as well as an increase in the downstream effector of PERK, the transcription factor CHOP, altogether led to increased caspase 3 cleavage and reduced survival of these ECD depleted MEFs (Figure 2.10). Similarly, physiological stress by glucose deprivation in a human pancreatic carcinoma cell line (Panc-1) upon ECD knock down resulted in increased ER stress with enhanced p-eIF2α, spliced XBP-1 and cell death (Figure 2.11). Reciprocally, upregulation of ECD reduced PERK signaling upon induction of ER stress (Figure 2.12). As activation of the
PERK pathway promotes cell death in response to ER stress, primarily through CHOP-dependent expression of pro-apoptotic genes (37-45) and abrogation of PERK signaling protects cells against stress-induced cell death (78-80), our results supported the likelihood that ECD functions to modulate PERK pathway activity and promote cell survival during ER stress. Indeed, assessment of cellular survival in response to ER stress showed that reduction in ECD levels impaired cell survival to ER stress (Figure 2.10) while overexpression of ECD promoted cell survival (Figure 2.14).

Collectively, these results support the conclusion that ECD and PERK are linked through a negative feedback mechanism whereby ECD exerts an inhibitory effect on PERK pathway signaling and activated PERK in turn reduces ECD protein levels via eIF2α-dependent translational block. Consistent with a reciprocal negative feedback relation between ECD and PERK, activated PERK negatively regulates cell growth (46-48); conversely, ECD positively regulates cell growth (54) and is overexpressed in human breast and pancreatic cancer specimens, correlating with poor prognostic markers and shorter survival (69,90). While the physiological benefit of an increased ECD mRNA upon ER stress is not yet understood, a speculation is that this effect may reflect a feedback response to the decrease in ECD protein levels whereby cells may try to compensate for the loss of ECD but ECD translation is blocked. Although ECD has been reported to play roles in pre-mRNA splicing in drosophila (91), it is unlikely that ECD alters its own mRNA splicing upon ER stress because ECD protein levels decrease upon ER stress.

Modulation of ECD levels using knockdown or overexpression demonstrated that ECD is a positive regulator of GRP78 levels (Figure 2.20 and 2.21). While slight increases in GRP94 and PDI were observed, the effect on GRP78 was more dramatic. However, this
effect may be post-translational since ECD did not have a transcriptional or post-transcriptional effect on GRP87 mRNA. The slight increase in GRP94 and PDI, both of which have ER stress-related functions, further suggests that ECD may affect chaperones’ levels, in general, when overexpressed upon ER stress induction.

As increased expression of GRP78 and other chaperones is known to promote the clearing of ER stress-causing unfolded protein load and reduce the activation of UPR sensors (13-15, 20), this increase in chaperones levels in the presence of ECD upon ER stress induction argue that ECD may be repressing stress by elevating chaperone levels. Indeed, a knock down of GRP78 abrogated the attenuating effect of ECD on PERK signaling even in the presence of overexpressed ECD (Figure 2.24C). Notably, GRP78 is required for cell survival not only in response to ER stress (23, 92-94) but also in other stressful and hostile conditions such as glucose deficiency encountered in tumor microenvironments (95-100). Given that the PERK arm of the UPR is also activated in cancer (77, 101, 102) and both ECD and GRP78 are overexpressed in cancer (69, 90, 95-100), we suggest that ECD overexpression may play a similar role to mitigate the negative consequences of elevated PERK signaling found in cancer. Given the mechanistic link between ECD and GRP78 in inhibiting the PERK pathway to promote cell survival, it will be of great interest to explore if GRP78 and ECD are co-overexpressed in tumors that use the UPR to promote tumor cell survival and hence may be suitable targets for UPR-directed therapeutic agents.
REFERENCES


CHAPTER 3: Summary and Conclusion
ECD protein has been known for many years as an important cell growth regulatory protein. The overexpression of ECD in cancer, including breast, prostate and pancreatic cancers, strongly suggests oncogenic roles for ECD. In this study, we presented evidence for an involvement of ECD in ER stress in cancer. Normal or cancer cell lines with low levels of ECD exhibited increased ER stress and cell death upon ER stress induction. Conversely, ECD overexpressing cells exhibited a decreased ER stress and cell death upon ER stress. Together, these distinct effects of ECD knock down or overexpression on cells argue that ECD may be functioning as a buffering protein against stress in cells and as a regulator of sensitivity of cells to ER stress. The increase in ECD mRNA upon ER stress indicates a transcriptional induction of ECD and further suggests an ER stress-related role.

The functional connection of ECD to the PERK-eIF2α arm of the ER stress signaling further argues in favor of the pro-survival roles for ECD in stress conditions, since that axis mediates cell death during ER stress signaling via CHOP induction (1-8). ECD inhibits PERK-eIF2α signaling while PERK-eIF2α inhibits ECD protein translation, thus establishing a negative feedback loop between ECD and the PERK arm of the ER stress signaling.

Mechanistically, ECD may be exerting its effects by multiple ways. Particularly, the ECD-GRP78 axis is a novel pathway for regulating ER stress. ECD enhances the level of GRP78, a key chaperone and inhibitor of PERK signaling. As increased expression of GRP78 and other chaperones is known to promote the clearing of ER stress-causing unfolded protein load and reduce the activation of UPR sensors (9-12), this increase in chaperones’ levels in the presence of ECD upon ER stress induction argues that ECD may be attenuating stress by elevating chaperone levels and enhancing the folding capacity in
the stressed ER. Indeed, a disruption of the chaperone-enhancing effect of ECD abrogated the attenuating effect of ECD on PERK signaling even in the presence of overexpressed ECD. Notably, GRP78 is required for cell survival not only in response to ER stress (13-16) but also in other stressful and hostile conditions such as glucose deficiency encountered in tumor microenvironments (17-22). Given that the PERK arm of the UPR is also activated in cancer (8,23,24) and both ECD and GRP78 are overexpressed in cancer (17-22,25,26), we suggest that ECD overexpression may play a similar role to mitigate the negative consequences of elevated PERK signaling found in cancer. Given the mechanistic link between ECD and GRP78 in inhibiting the PERK pathway to promote cell survival, it would be of great interest to explore if GRP78 and ECD are co-overexpressed in tumors that use the UPR to promote tumor cell survival and hence may be suitable targets for therapeutic agents directed against ER stress in cancer.

Conclusion:

- Stress induction by multiple stimuli, Thapsigargin, Tunicamycin, glucose starvation, H$_2$O$_2$, NOX4 overexpression, DTT, and sorbitol, led to a reduced ECD protein in multiple cell lines (MCF7, MDA-468, MCF10A, Panc-1, 76NTERT, MDA231-NOX4).
- ECD mRNA increased upon Thapsigargin treatment.
- In PERK KO MREFs, the levels of ECD protein did not change upon ER stress induction as compared to WT PERK.
• In PERK KO MEFs, ECD mRNA levels failed to increase as compared to WT PERK.

• PERK does not phosphorylate ECD.

• The decrease in ECD protein levels is via the PERK-eIF2α axis of the ER stress signaling.

• Loss of ECD in cells increased the levels of p-PERK, p-eIF2α, and these effects were enhanced upon ER stress induction by Thapsigargin treatment or glucose deprivation along with an increased in CHOP mRNA, and cleaved caspase 3.

• Loss of ECD slightly increased the levels of spliced XBP-1, the downstream target of IRE1α but cleaved ATF6 did not significantly change.

• Loss of ECD correlated with a decrease in the colony formation of ECD-null cells upon ER stress induction.

• Overexpression of ECD led to a marked decrease in p-PERK and its downstream targets, p-eIF2α, ATF4 and CHOP mRNA levels upon ER stress induction

• Overexpression of ECD upon ER stress induction slightly increased the levels of spliced XBP1 but ATF6 did not change significantly.

• Overexpression of ECD upon ER stress induction correlated with a decrease in caspase 3 cleavage and an increase in the colony formation of ECD overexpressing cells

• ECD localized and associated with PERK, ATF6, IRE1α and GRP78.

• ECD did not modulate enzymatic activity of PERK toward eIF2α.

• Depletion of ECD upon ER stress induction was associated with decreased GRP78 protein levels.
• Overexpression of ECD upon ER stress induction increased the levels of GRP78 protein.

• GRP78 mRNA was not affected by deletion or overexpression of ECD upon ER stress induction.

• Knock down of ECD followed by cycloheximide treatment was associated with a faster decrease in GRP78 protein level as compared to control cells with intact cellular ECD levels.

• ECD overexpression plus cycloheximide treatment correlated with a slower decrease in GRP78 protein levels.

• A knock down of GRP78 abrogated the attenuating effect of ECD on PERK, even in the presence of overexpressed ECD. This effect further correlated with a decrease in the colony formation of ECD overexpressing cells.
REFERENCES


CHAPTER 4: Future Directions
• **Characterize ER stress elements (ERSE) in ECD gene promoter**

The increase in ECD mRNA upon ER stress induction suggests existence of ERSE in ECD gene promoter. A follow-up study to characterize these ERSE would be interesting.

• **Assess potential roles of ECD in autophagy through GRP78**

In addition to further examining the ECD-GRP78 connection in tumor context, it would also be interesting to assess whether ECD pro-survival effect upon ER stress is via GRP78-mediated autophagy. The rationale is that ECD enhanced GRP78 while GRP78 has been implicated in autophagy. Although a clear mechanism was not presented, it was reported in numerous studies that GRP78 was required for autophagy induction and that inhibition of GRP78 inhibited autophagy induction (1-3).

• **Assess potential ECD-R2TP connection in regulating ER stress**

The R2TPcomplex, composed of RUVBL1, RUVBL2, PIH1D1 and RPAP3 is a multi-protein complex which regulates the assembly and stability of many complexes (4). A study identified a role for RUVBL2 in the regulation of ATF2 in response to stress through direct interaction with ATF2 (5). ECD is a component of the R2TP complex (6,7). ECD interacts with the RUVBL1 component of the
R2TP complex, an interaction required for ECD roles in cell growth (33). It is reported that knock of RUVBL1 affected cellular level of GRP78 an induced ER stress (8). Therefore, it would be curious to examine the potential ECD-R2TP connection in ER stress regulation, especially as ECD overexpression correlated with enhanced of both GRP78 and RUVBL1 upon ER stress induction (Figure 4.1 below).

**Figure 4.1: ECD overexpression correlates with enhancement with RUVBL1 and GRP78.** ECD overexpressing and their control MEFs were treated with Tunicamycin and protein lysate was prepared at indicated time points followed by western blot with indicated antibodies.
• **Assess the requirement of ECD in chaperone (GRP78, GRP94)-mediated ER calcium regulation.** The ER is well known as a site for calcium storage in cells. ECD regulates the levels of the critical chaperone proteins (GRP78 and GRP94) that mediate that function of the ER (9,10).

• **Assess the roles of ECD in protein translation through GRP78.** GRP78 is known to be involved in protein translation not only by binding nascent protein in the ER but importantly by forming the translocation channel in complex with Sec61 (11,12).

• **Explore in vivo studies of the ECD-GRP78 axis in regulating oncogenesis-induced ER stress in mouse models.** Works in the lab indicated potential oncogenic roles of ECD, as its overexpression induced tumors. Tumor initiation and progression are complex processes characterized by ER stress as a result of the increased bioenergetics demands in the tumor. Overcoming this early ER stress is paramount for tumor progression. It would be interesting to assess whether the ECD-mediated upregulation in the levels of GRP78 is required for tumor initiation and progression.
REFERENCES


Chapter 5: Appendix


Fig. 1:

A) MCF10A
Tg: 0h, 12h, 24h
ECD
p-eIF2α
β-actin

B) MCF10A
Tun: 0h, 12h, 24h
ECD
p-eIF2α
β-actin

C) Panc-1
Glucose starvation
Tg: 0h, 8h, 12h, 24h
ECD
p-eIF2α
β-actin

D) CHOP mRNA
Relative mRNA
Tg: 0h, 8h, 12h, 24h

E) ECD mRNA
Relative mRNA
Tg: 0h, 8h, 12h, 24h

F) ECD mRNA
Relative mRNA
Glucose starvation: 0h, 12h, 24h, 48h, 72h

G) WT PERK
KO
ECD
p-PERK
β-actin
Lane: 1, 2, 3, 4

H) CHOP mRNA
Relative mRNA
UT, 7h, 14h

I) ECD mRNA
Relative mRNA
UT, 7h, 14h

J) eIF2α
Wild-type
Phospho-deficient
ECD
p-eIF2α
β-actin
Lane: 1, 2, 3, 4, 5, 6

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Fig. 2:

A

PERK
GRP78
DAPI
MERGE

B

ECD
ECD
PERK
GRP78
DAPI
DAPI
MERGE
MERGE
Merged

C

ECD
GRP78
DAPI
MERGE

D

Ms IgG
Ms IgG
DAPI
Merged

E

Rb IgG
Rb IgG
DAPI
Merged

F

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Lane: 1 2 3 4

B

CHOP mRNA

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E

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Fig. 3: continued

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**Fig. 4:**

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**B**

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**F**

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**G**

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Fig. 5:

A. Western blot analysis of Tg (nM) treatment.

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B. CHOP mRNA expression levels.

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C. Relative survival analysis.

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Mammalian ECD protein is a novel negative regulator of the PERK arm of unfolded protein response.

Appolinaire A. Olou1, Aniruddha Sarkar1, Aditya Bele1, C.B. Gurumurthy1, Riyaz A. Mir1, Shalis A. Ammons1, Sameer Mirza1, Irfana Saleem2, Fumihiko Urano6,7, Hamid Band1,3,5 and Vimla Band1,5#

Departments of 1Genetics, Cell Biology and Anatomy, 2Biochemistry and Molecular Biology 3Pathology & Microbiology, College of Medicine; 4Eppley Institute for Research in Cancer and Allied Diseases; and 5Fred & Pamela Buffett Cancer Center, University of Nebraska Medical Center, 985805 Nebraska Medical Center, Omaha, NE 68198, 6Division of Endocrinology, Metabolism and Lipid Research, and 7Department of Pathology, Washington University School of Medicine, St. Louis, Missouri.

Running Title: ECD modulates PERK pathway

# Corresponding author: Email: vband@unmc.edu; Phone: (402)-559-8565; Fax: (402)-559-7328

Keywords: ECD, ER, PERK, UPR, Cell survival, GRP78

Word count: Material and Method: 765
Introduction, Results and Discussion: 4368
ABSTRACT

The mammalian Ecdysoneless (ECD) is a highly-conserved ortholog of the Drosophila Ecd gene product whose mutations impair the synthesis of Ecdysone and produce cell-autonomous survival defects but mechanisms by which ECD functions are largely unknown. Here, we present evidence that ECD regulates endoplasmic reticulum (ER) stress response. ER stress induction led to a reduced ECD protein but this effect was not seen in PERK KO or phospho-deficient eIF2α MEFs; moreover, ECD mRNA levels were increased, suggesting impaired ECD translation as the mechanism for reduced protein levels. ECD co-localizes and co-immunoprecipitates with PERK and GRP78. ECD depletion increased the levels of p-PERK, p-eIF2α, and these effects were enhanced upon ER stress induction. Reciprocally, overexpression of ECD led to a marked decrease in p-PERK, p-eIF2α and ATF4 levels, but a robust increase in GRP78 protein levels. However, GRP78 mRNA levels were unchanged, suggesting a post-transcriptional event. Knockdown of GRP78 reversed the attenuating effect of ECD over-expression on PERK signaling. Significantly, overexpression of ECD provided a survival advantage to cells upon ER stress induction. Taken together, we demonstrate that ECD promotes survival upon ER stress by increasing GRP78 protein levels to enhance the adaptive folding protein in the ER to attenuate PERK signaling.
INTRODUCTION

The endoplasmic reticulum (ER) is a central subcellular organelle with essential roles in the synthesis, folding and maturation of secreted and membrane proteins, biogenesis of cholesterol, calcium homeostasis and regulation of survival and apoptosis pathways (1-10). Aberrations in these ER functions are sensed by well-conserved ER transmembrane sensors, Inositol-Requiring Enzyme 1 alpha (IRE1α), PKR-like ER kinase (PERK) and Activating Transcription Factor 6 (ATF6), that activate homeostatic signaling pathways collectively referred to as the unfolded protein response (UPR) (11,12). These ER stress sensors exhibit a dynamic and reversible interaction with the ER chaperone GRP78 (13). In un-stressed cells, GRP78 is bound to luminal domains of UPR sensors, which maintains them in an inactive state (14). During ER stress, the increased load of unfolded proteins competes for GRP78 binding, leading to activation of UPR sensors (15) which, through intermediate signaling, evoke overlapping as well as pathway-specific responses to restore ER homeostasis and promote cell survival or alternatively eliminate stressed cells through apoptosis if homeostasis cannot be restored.

The PERK pathway has emerged as a key pathway in cellular UPR response as well as homeostasis under unstressed conditions and in disease states (16-21). Release of GRP78 from PERK leads to its dimerization, auto-phosphorylation and activation (15,22,23). A major PERK substrate is the eukaryotic translation initiation factor 2 alpha (eIF2α) whose phosphorylation by PERK inactivates it leading to a block in general cap-dependent protein translation and consequent decrease in the protein load entering the ER (24); concurrently, PERK signaling selectively enhances the cap-independent translation of specific mRNAs, such as Activating Transcription Factor 4 (ATF4) (24). ATF4 induces the expression of CCAAT/enhancer-binding protein-homologous protein (CHOP) which promotes apoptosis in response to stress (24-32).
PERK-induced phosphorylation of eIF2α also inhibits cell cycle progression by reducing the levels of cyclins and hence cyclin-dependent kinase 2 (CDK2) activity (33-35). Termination of PERK signaling, together with de-phosphorylation of eIF2α, is required to re-initiate protein synthesis and resume cell cycle. Thus, PERK-mediated UPR leads to a coordinated program of cellular protection and mitigation of stress. However, PERK also contributes to an alternate outcome through CHOP-mediated activation of a cellular death pathway to eliminate severely damaged cells (24-32). Mechanisms to fine-tune the outcomes of UPR pathways are important to mitigate the negative consequences of ER stress. This is of particular importance under conditions where cells experience ER stress as part of their physiological responses, such as antibody-secreting plasma cells or insulin-secreting pancreatic islet cells (36). We have identified ECD as a negative regulator of the PERK-mediated UPR.

The Ecd gene was first identified based on genetic mutations in Drosophila that lead to reduced production of the developmentally-regulated steroid hormone Ecdysone, which is synthesized in the ER, hence the designation of ecdysoneless for such fly mutants (37). The mammalian Ecd gene was cloned based on the rescue of growth defects in S. cerevisiae mutant in the growth control regulatory gene 2 (GCR2), a glycolysis regulatory gene (38). Thus, ECD was thought to be involved in mammalian glycolysis gene expression and initially named hSGT1 (human suppressor of gcr2)(38). We identified the same gene in a screen for interacting partners of human papilloma virus E6 oncoprotein and found it to interact with p53 and transactivate p53-regulated genes (39,40).

To elucidate the functional role(s) of mammalian ECD, we generated Ecd-null mice and demonstrated that homozygous deletion of Ecd was early embryonic lethal while ex vivo Cre-mediated deletion of Ecd in Ecdfl/fl mouse embryonic fibroblasts (MEFs) led to proliferative
block and a significant decrease in cell survival (41,42). ECD was found to be essential for E2F target gene expression by facilitating the dissociation of the retinoblastoma RB protein from E2F and promoting the G1-to-S phase cell cycle progression (41). As a consequence, Ecd-null MEFs showed a decrease in the levels of cyclins A, B1, E and D1, reduction in CDK2 kinase activity and were arrested in G1 phase of the cell cycle (41). Interestingly, E2F family proteins such as the E2F1 have been implicated in UPR-mediated cell death (43). In addition to its promotion of the G1/S phase, ECD also promotes G2/M phase of the cell cycle and its knockdown induced not just a G2/M arrest but also apoptosis (44). Induction of UPR not only induces apoptosis but also halts cell cycle progression in G1 and G2 phases (33-35,45) and these effects are mediated by PERK (33,34), suggesting a potential connection between ECD and the PERK arm of the UPR.

More recently, we uncovered another mechanism by which ECD regulates cellular proliferation, involving its interaction with RUVBL1 and PIH1D1 components of the prefoldin co-chaperone R2TP (42) which is involved in the assembly or remodeling of a number of protein and protein-RNA complexes to regulate many physiological processes (46-52). Recently, it was reported that knock down of the RUVBL1 component of the R2TP complex induced cell cycle block and ER stress (53). Furthermore, ECD has been shown to associate with the stress response protein Thioredoxin-Interacting Protein (TXNIP) (54) which is known to bind to the ER chaperone PDI (protein disulfide bond isomerase) to increase its enzymatic activity to relieve ER stress (55).

Lastly, TXNIP was recently shown to be a novel component of the UPR and is regulated by the PERK pathway (56). Thus, multiple lines of suggestive evidence pointed to a potential role of ECD in the regulation of ER stress. In this study, we demonstrate that induction of ER stress, using both chemical ER stress inducers (Thapsigargin and Tunicamycin) and physiological ER
stress inducers (glucose starvation), led to a reduced ECD protein, and this effect was not seen in PERK KO or phospho-deficient eIF2α MEFs. Notably, ECD mRNA levels were increased, suggesting impaired ECD translation as a mechanism for reduced protein levels. Moreover, ECD localizes and co-immunoprecipitates with the unfolded protein response mediators PERK and GRP78. While ECD depletion increased the levels of p-PERK and its downstream targets, p-eIF2α and ATF4, ECD overexpression markedly decreased their levels upon ER stress induction, whereas induction of GRP78 protein robustly increased, suggesting an enhanced adaptive folding capacity of the ER. Knockdown of GRP78 reversed the attenuation of PERK signaling seen upon ECD overexpression. Significantly, ECD over-expression and depletion distinctly affected the survival outcome of cells upon ER stress.

MATERIALS AND METHODS

Reagents and antibodies - Thapsigargin, (Cat# 12758) and Tunicamycin (Cat# 12819) were from Cell Signaling, dissolved in DMSO and used at indicated concentrations. SiRNA against GRP78 was from Santa Cruz (Sc-35522) and Cycloheximide was purchased from Sigma (cat# 7698). A monoclonal antibody against ECD, generated in our laboratory, has been described previously (57). Antibodies against p-PERK (cat# 3179), ATF4 (cat# 11815), p-eIF2α (cat# 9721), eIF2α (cat# 9722), GRP78 (cat# 3177), Caspase 3 (cat# 9662), GRP94 (cat# 2104), s-XBP1 (cat# 12782), RCAS1 (cat# 6960), PDI (cat# 3501) and total PERK (cat# 3192) were from Cell signaling. ATF6 and SERCA2 antibodies were purchased from Enzo Life (cat# ALX-804-381-C100) and abcam (ab2861), respectively. PERK and GRP78 used for immunofluorescence and proximity ligation assay (PLA) were from Santa Cruz (cat# sc-9477) and abcam (ab21685),
respectively. PIH1D1 was from Santa Cruz (18Y9 sc-101000). GRP78 for IP/WB was purchased from abcam (cat#21685).

Establishment of MEFs with doxycycline-inducible ECD over-expression from Ecd transgenic mice - MEFs were generated from 13.5 days old embryos of mice of the genotype Tet(O)-Flag-hEcd-IRES-eGFP used as control. rtTA was introduced retrovirally to generate Tet(O)-Flag-hEcd-IRES-eGFP; rtTA (the derivation of the transgenic mice will be described separately).

Cell lines and culture conditions - Ecd fl/fl MEFs were maintained in DMEM medium supplemented with 10% fetal bovine serum and treated with adenoviruses coding for GFP (adeno-GFP, control) or Cre (adeno-Cre) as described previously (41,42). Panc-1 cells have been previously described (58). For ECD overexpression studies, control MEFs or ECD over-expressing MEFs were cultured with 1μg/ml of Doxycycline (Cat # 231286, BD Biosciences) to induce expression of the Ecd transgene. MEFs with a non-phosphorylatable eIF2α were obtained from Dr. Thomas Rutkowski’s lab, Carver College of Medicine, Iowa and has been described elsewhere (59). PERK knockout (KO) MEFs were from ATCC (CRL-2976). Immortal human mammary epithelial cell line MCF-10A was cultured in DFCI-1 medium (42).

Cellular Fractionation and Immunoprecipitation - Subcellular fractionation of MCF-10A cells into soluble (GAPDH was used as a marker(42,60)) and microsomal fractions (PERK and SERCA2 were used as a marker) was carried out according to the manufacturer protocol (Sigma
Aldrich, Cat# ER0100)(61). For immunoprecipitation (I.P), cells were washed in Phosphate Buffered Saline (PBS) and lysed in CHAPS buffer [(0.3% CHAPS, 20 mM Tris-HCl (pH 7.4), 120 mM NaCl, 10% glycerol, 5 mM EDTA supplemented with protease and phosphatase inhibitor (Roche, Cat# 04906837001 and 11836145001)].

**Western blotting**- For western blotting, cell lysates were prepared in RIPA buffer (Cat# 89901, Thermo Scientific) supplemented with protease inhibitors (Roche, Cat# 11836145001). Lysates were resolved on SDS-PAGE gel, transferred on to nylon membrane (IPVH00010, Millipore), and subjected to ECL-based Western blotting as described (41,42).

**Immunofluorescence analysis**- Immortal MEFs were cultured on coverslips, fixed for 30 min in 3% Paraformaldehyde (PFA) and permeabilized in PBS (containing 0.5 % Triton X-100 and 10% goat serum) for 20 min at room temperature, followed by blocking in PBS (containing 10% goat serum) for 1 hour at room temperature. The cells were then incubated with primary antibodies in blocking buffer overnight at 4°C followed by 1-hour incubation with their corresponding secondary antibodies in blocking buffer at room temperature. Cells were then washed in PBS (containing 0.1% Tween-20) and then mounted for confocal with the super-resolution structure-illuminated microscopy (SIM). Confocal images were obtained using the Carl Zeiss LSM 510 microscope and 3D-SIM images were collected with a Zeiss ELYRA PS.1 illumination system (Carl Zeiss).
Proximity Ligation Assay (PLA)- PLA was previously described (62). Briefly, MCF-10A cells were fixed with 3% PFA, stained with the indicated antibodies and incubated with Proximity Ligation Assay plus and minus probes followed by ligation and amplification reaction according to the manufacturer’s protocol (DuoLink Sigma).

Colony Formation Assay- For clonogenic assay, 1,000 cells were plated in triplicate and allowed to attach to the plate (about 8 h) followed by treatment with Thapsigargin for 24h. 10 days later, colony formation was assessed as previously described (41,42).

RNA isolation and real-time quantitative PCR (qRT-PCR)- Total RNA was isolated using the TRizol reagent (Invitrogen cat# 15596018). 1 µg of RNA was reverse-transcribed using SuperScript™II reverse transcriptase (Invitrogen cat# 18064014). The qPCR was performed with primer sets indicated in the table:

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tbody>
<tr>
<td>mouse CHOP</td>
<td>CTGCTTTCCACCTTGGGAGAC</td>
<td>CGTTTCCTGGGGATGAGATA</td>
</tr>
<tr>
<td>human CHOP</td>
<td>CATGGCCTTTTCCTTCCGGG</td>
<td>CCAGAGAAGCAAGCTCAAGA</td>
</tr>
<tr>
<td>mouse GRP78</td>
<td>AGTGGTGGCCACTAATGGAG</td>
<td>CAATCCTTGCTTGATGCTGA</td>
</tr>
<tr>
<td>mouse Ecd</td>
<td>CCGTCTGGCACACAACCTTCTGCTG</td>
<td>AGGGTCGAAACATCCCTCCATCGA</td>
</tr>
<tr>
<td>human Ecd</td>
<td>ACTTTGAAACACGAACCTGGCG</td>
<td>TGATGCAAAGTGCTAGTTCTC</td>
</tr>
</tbody>
</table>
Induction of ER stress by glucose starvation- Panc-1 cells were maintained in DMEM supplemented with 10% fetal bovine serum and then switched to glucose-free DMEM medium (cat# 11966-025, life technologies) for indicated times.

Statistical analysis- for assessment of statistical significance, a Student t test was used. P values of < 0.05 were considered statistically significant.

RESULTS

Induction of ER stress leads to reduced ECD protein expression in a PERK-eIF2α-dependent manner- To begin to explore the potential link of ECD to ER stress, we asked if the levels of ECD protein are affected by induction of ER stress. For this purpose, immortal human mammary epithelial cell line MCF-10A was treated with Thapsigargin or Tunicamycin, two commonly used chemical ER stress inducers (63), and ECD protein levels were analyzed by western blotting. Treatment with both ER stress inducers increased the level of p-eIF2α as expected (63) (Fig. 1A&B). Notably, a concomitant time-dependent decrease in ECD protein levels was observed upon treatment with either Thapsigargin (Fig. 1A) or Tunicamycin (Fig. 1B).

Given the effect of chemical ER stress inducers on ECD protein levels, we next assessed whether physiological stress such as glucose starvation would have similar effects. For this purpose, we used human pancreatic carcinoma cell line (Panc-1) which is known to exhibit ER stress upon glucose starvation (63). Significantly, similar to chemical ER stress, glucose starvation-induced
ER stress also exhibited a reduced levels of ECD protein (Fig. 1C). To assess whether the decrease in ECD protein levels was due to reduced ECD mRNA levels, we measured ECD mRNA levels using qRT-PCR. Induction of CHOP mRNA was used as a control (Fig. 1D). Notably, ECD mRNA levels were not only not reduced but in fact showed an increase (Fig. 1E), suggesting that the reduction in ECD protein level was not at the transcriptional level; likewise, physiological stress by glucose starvation also increased ECD mRNA levels (Fig. 1F). Since PERK activation and subsequent phosphorylation of eIF2α mediates translational block in response to ER stress (24), we used MEFs in which this pathway is genetically abrogated. First, we treated Wild-Type (WT) MEFs or MEFs from PERK kinase domain knockout (PERK-KO) mice (64) with Thapsigargin and analyzed ECD protein levels by western blotting. The expected lack of PERK pathway activation in PERK-KO MEFs was confirmed by lack of induction of PERK phosphorylation in PERK-KO MEFs compared to WT MEFs (Fig. 1G). Significantly, while a decrease in ECD protein levels was observed in WT MEFs treated with Thapsigargin, the levels of ECD protein were unchanged in PERK-KO MEFs (Fig. 1G). Then, ECD mRNA were assessed in PERK KO and control MEFs to determine whether the levels are altered upon Thapsigargin treatment. Again, CHOP mRNA induction was used as a positive control (Fig. 1H). As expected, in PERK KO MEFs, CHOP mRNA is very minimally induced upon Thapsigargin treatment as compared to control cells (Fig. 1H), since CHOP is downstream of PERK (24-32). Notably, while ECD mRNA increased in control WT MEFs, in PERK KO MEFs, induction of ECD mRNA is low as compared to control WT PERK MEFs (Fig. 1I).

To further examine the role of the PERK pathway in the downregulation of ECD protein expression, we utilized MEFs from mice that carry a serine 51 to alanine mutation in eIF2α which prevents its phosphorylation by activated PERK and makes the cells resistant to PERK-
mediated translation block upon ER stress induction (59). As expected, the levels of p-eIF2α increased in WT MEFs but not in the phospho-deficient eIF2α mutant MEFs when ER stress was induced with Thapsigargin or Tunicamycin (Fig. 1J, compare lanes 1-3 with lanes 4-6).

Importantly, while ECD protein levels decreased in WT MEFs treated with ER stress inducers (Fig. 1J, compare lane 1 to lanes 2-3), the levels of ECD protein did not change in identically-treated mutant eIF2α MEFs (Fig. 1J compare lane 4 to lanes 5-6). Together, these results support a conclusion that ECD protein levels are downregulated upon ER stress in a PERK-eIF2α dependent manner, and that ER stress upregulates ECD mRNA levels, suggesting a regulatory link between ECD and the PERK pathway of ER stress.

ECD co-localizes and associates with PERK and GRP78- To further explore a potential link between ECD and the ER stress pathway, we assessed the localization of ECD. We first performed immunofluorescence using Super-resolution Structured Illumination Microscopy (SIM), a technique that offers a much higher resolution than conventional confocal microscopy (65,66). Co-localization of PERK and GRP78 served as a positive control since PERK and GRP78 are known interacting partners (13). Indeed, we observed that endogenous PERK and GRP78 were co-localized in a punctate distribution (Fig. 2A). Significantly, ECD also co-localized with PERK and GRP78 (Fig. 2B & C), although not as extensive as GRP78-PERK co-localization. Negative controls rabbit and mouse IgGs showed no staining (Fig. 2 D & E). Next, we performed sub-cellular fractionation of MCF-10A cells to biochemically assess the ER localization of ECD. This analysis showed expected presence of ECD in the soluble fraction (40,42). Importantly, ECD was prominently present in the ER-containing microsomal fraction (Fig. 2F). Given the co-localization of ECD with PERK and GRP78 (Fig. 2A-C), we assessed if
ECD associates with PERK and/or GRP78. First, we used PLA to assess the proximity of ECD relative to GRP78 and PERK in MCF-10A cells. Known ECD interaction with PIH1D1 component of the R2TP served as positive control (42,46). Indeed, ECD and PIH1D1 formed distinct foci detectable by PLA (Fig. 2G, red dots). Significantly, PLA signals were also observed for ECD and GRP78 or ECD and PERK (Fig. 2H-I). Finally, we carried out immunoprecipitation (IP) of ECD from lysates from MCF-10A cell, treated or left untreated with Thapsigargin, followed by western blotting for PERK and GRP78 antibodies to assess their association. IP of ECD co-immunoprecipitated PIH1D1 (Fig. 2K), as expected based on known interaction of ECD with PIH1D1 (42,46). Importantly, PERK and GRP78 also co-immunoprecipitated with ECD (Fig. 2K, left panel). A reverse IP of GRP78 also co-immunoprecipitated ECD, with PERK used as a positive control (Fig. 2K right panel). Taken together, these results suggest that ECD associates with PERK and GRP78.

ECD regulates the PERK arm of the UPR: The association of ECD with PERK and GRP78 (Fig. 2) and abrogation of the decrease in ECD levels in PERK KO cells upon ER stress (Fig.1) suggested that ECD may be functionally linked to UPR through PERK pathway. Therefore, we assessed the activation of the PERK pathway in the absence or presence of UPR inducers in cells in which ECD could be inducibly deleted. For this purpose, we utilized Ecd\({}^{fl/fl}\) MEFs which have been previously established and shown to exhibit Ecd gene deletion and loss of ECD protein expression upon infection with adenovirus expressing Cre recombinase (41). Previously, we have observed that induction of Ecd deletion in these Ecd\({}^{fl/fl}\) MEFs, aside from inducing cell cycle arrest, led to a decrease in cell survival even in the absence of any stress (41,42).
Notably, Cre-mediated deletion of Ecd in the Ecd<sup>fl/fl</sup> MEFs led to an increase in the levels of phospho-PERK (p-PERK) and p-eIF2α, when compared to control adeno-GFP infected MEFs (Fig. 3A, compare lane 1 with 3), and these effects were further enhanced by treatment with Thapsigargin (Tg), the inhibitor of the Sarco/Endoplasmic Reticulum Calcium ATPase (Fig. 3A, compare lane 2 with 4). Spliced XBP1 also showed some increase in ECD null cells upon Thapsigargin treatment while ATF6 did not show a significant change (Fig. 3A). Consistent with the increased p-PERK levels upon ECD deletion, Thapsigargin treated ECD-depleted MEFs also exhibited increased expression of CHOP mRNA (Fig. 3B), a downstream effector of the PERK pathway whose target genes promote cell death (24-28,30,31). The increased CHOP mRNA correlated with an increased cleaved caspases 3 in ECD null cells (Fig. 3C, compare lane 2 with 4) and a significant reduction in cell survival, as measured by colony-forming assays (Fig. 3D).

To further assess these effects, we used physiological stress in Panc-1 cells (Fig. 3E) in which ECD was knocked down followed by glucose starvation. Again, here too, a siRNA knock down of ECD plus glucose starvation led to an enhanced eIF2α phosphorylation, the downstream target of PERK, and increased cleaved caspase 3 as compared to control cells (Fig. 3E, compare lanes 1-5 with lanes 6-10), suggesting more cell death in ECD-knocked down cells upon glucose starvation.

In a reciprocal approach, we overexpressed ECD and then examined its impact on the UPR, in particular the PERK pathway, upon ER stress induction. To this end, we generated MEFs from mice that carry a Doxycycline (Dox)-inducible Ecd transgene ((Tet(O)-Flag-hECD-IRES-eGFP; rtTA)) or a control transgenic mouse without rtTA ((Tet(O)-Flag-hECD-IRES-eGFP)). These MEFs were treated with Dox to induce ECD overexpression, followed by treatment with Thapsigargin. As expected, control MEFs exhibited an increase in p-PERK and CHOP mRNA;
however, the MEFs with Dox-induced ECD overexpression exhibited a substantially reduced level of p-PERK and CHOP mRNA upon Thapsigargin treatment as compared to control MEFs (Fig. 3F & G). Likewise, when the MEF cell lines were treated with Thapsigargin for various time points (3, 6, 12 or 24h), ECD over-expressing MEFs exhibited lower levels of p-PERK compared to those in control MEFs (Fig. 3H, compare lanes 1-5 with lanes 6-10). A corresponding reduction in the levels of ATF4, a downstream target of PERK signaling, was seen in ECD over-expressing MEFs compared to their control MEFs (Fig. 3H, compare lanes 3-5 with lanes 8-10); s-XBP1 and ATF6 also were slightly reduced in ECD over-expressing MEFs (Fig. 3H, compare lanes 1-5 with lanes 6-10). Importantly, ECD overexpression was associated with a substantially higher level of GRP78 following Thapsigargin treatment as compared to control MEFs (Fig. 3H compare lanes 2-5 to lanes 7-10); GRP94 protein and PDI, two other chaperones, were also slightly increased in ECD over-expressing MEFs but not as dramatically increased as GRP78 upon Thapsigargin treatment (Fig. 3H compare lanes 1-5 to lanes 6-10).

Similar to Thapsigargin, induction of ER stress with Tunicamycin, which induces ER stress by inhibiting glycosylation of proteins in the ER, was also associated with lower levels of p-PERK and p-eIF2α in ECD overexpressing MEFs as compared to control MEFs (Fig. 3I compare lanes 2-5 with lanes 7-10). Furthermore, higher levels of GRP78 protein was also observed in ECD overexpressing MEFs as compared to control MEFs upon Tunicamycin treatment (Fig. 3I compare lanes 2-5 with lanes 7-10). Taken together, these results strongly support a conclusion that ECD negatively regulates PERK signaling while enhancing the adaptive capacity of the cells by increasing levels of chaperones, predominantly, GRP78 protein in response to ER stress.

*The increased induction of GRP78 is required for ECD to attenuate PERK signaling.*

Induction of GRP78 expression during ER stress is a homeostatic mechanism to reduce the
unfolded protein load in the ER, thereby reducing the activation of UPR sensors. As a major regulator of PERK, overexpression of GRP78 has been shown to reduce PERK signaling upon ER stress induction (13). Given the enhanced induction of GRP78, together with attenuation of PERK signaling in ECD overexpressing MEFs exposed to ER stress (Fig. 3H-I), we examined the possibility that the increased GRP78 levels upon ECD overexpression may be mechanistically linked to the reduction of PERK signaling. To investigate this possibility, we first examined changes in the levels of GRP78 in ECD deleted MEFs where we observed increased PERK phosphorylation (Fig. 3A). To assess if ECD was required for optimal GRP78 protein levels upon ER stress, we exposed the wild-type MEFs or MEFs with Ecd deletion induced by adeno-Cre (as in Fig. 3A-D) to Thapsigargin, and then assessed the levels of GRP78 protein, at various time points, by western blotting. The levels of GRP78, over time, were reduced in ECD-null MEFs compared to GRP78 levels in control MEFs (Fig. 4A, compare lanes 3-5 with 8-10). Likewise, using physiological stress, a knock down of ECD in Panc-1 cells led to a decrease in GRP78 levels upon glucose starvation (Fig. 4B, compare lanes 1-5 with lanes 6-10). To examine whether the reduced induction of GRP78 in ECD-null MEFs was a result of reduced GRP78 mRNA expression, we carried out qPCR analyses of mRNA isolated from WT or Ecd-null MEFs treated with Thapsigargin. As expected, GRP78 mRNA levels increased in WT MEFs upon Thapsigargin treatment (Fig. 4C); however, the levels of GRP78 mRNA induction in Ecd-null MEFs was comparable to that in control MEFs (Fig. 4C). Similarly, GRP78 mRNA levels in control vs. ECD over-expressing MEFs treated with Thapsigargin were comparable (Fig. 4D). To determine whether GRP78 may be less stable in ECD null cells, we first knocked down ECD in Panc-1 cells, which have high level of GRP78, followed by inhibition of protein synthesis by cycloheximide treatment over various time points and GRP78
protein levels were analyzed by western blot. Significantly, the time dependent decrease in GRP78 protein levels following cycloheximide treatment was faster in ECD-knocked down cells compared to their control cells (Fig. 4E). Next, to examine the possibility that ECD overexpression is associated with increased GRP78 protein stability, control or ECD over-expressing cells were treated with Thapsigargin to induce GRP78 protein, followed by cycloheximide (CHX) treatment over various time points. Notably, the time-dependent decrease in GRP78 levels was slower in ECD over-expressing MEFs as compared to control MEFs (Fig. 4F). Finally, to determine whether the increase in GRP78 protein levels upon ECD overexpression was required for the attenuation of PERK activation seen upon ER stress induction, we depleted GRP78 using siRNA in both control and ECD over-expressing MEFs followed by Thapsigargin treatment. Notably, the attenuating effect of ECD overexpression on PERK phosphorylation upon Thapsigargin treatment was abrogated by GRP78 knockdown (Fig. 4G; compare lane 5-6 with 7-8). Taken together, these results support the conclusion that ECD positively regulates GRP78 protein level upon ER stress induction to attenuate PERK activation.

**ECD overexpression protects cells from ER stress-induced cell death**- ER stress response is initially aimed at the survival of cells (67); however severe ER stress shifts the response from a pro-survival to a pro-apoptotic response (23,31) through PERK-mediated induction of CHOP expression, a transcription factor that enhances the expression of pro-apoptotic pathway genes (24-28,30,31,68). Several studies found that inhibition/attenuation of the PERK pathway protected cells against stress-induced cell death (69-72). Given our observations that ECD functions as a modulator of PERK signaling, we assessed if the levels of ECD determine a differential survival vs. apoptotic cell fate upon ER stress induction. For that purpose, we treated
control MEFs or Dox-inducible ECD overexpressing MEFs with Thapsigargin and then assessed
the level of apoptosis induction by examining caspase 3 cleavage (73). As anticipated, a
Thapsigargin dose-dependent increase in cleaved caspase 3 levels was observed in control
MEFs, whereas the levels of cleaved caspase 3 were markedly lower in ECD-overexpressing
MEFs (Fig. 5A). Real-time qPCR analyses demonstrated that Thapsigargin-induced expression
of CHOP, a PERK-regulated mediator of cell death (24-28,30-32), was lower in ECD
overexpressing MEFs as compared to control MEFs (Fig. 5B). To further assess the pro-survival
effects of ECD against apoptotic cell fate upon ER stress induction, we assessed the abilities of
control vs. ECD-overexpressing MEFs to form colonies after their exposure to ER stress. For this
purpose, equal numbers of control or ECD overexpressing MEFs were treated with Thapsigargin
for 24h, and the cells were maintained in Thapsigargin-free medium for 10 days followed by
crystal violet staining and counting of surviving colonies. Notably, more colonies were observed
in ECD overexpressing MEFs as compared to control MEFs (Fig. 5C), further supporting the
conclusion that ECD provides survival advantage upon ER stress.

**DISCUSSION**

The ER-localized stress response pathway referred to as the UPR is a well-conserved
response to a number of cellular stresses such as unfolding of proteins in the ER. The UPR elicits
a spectrum of downstream responses whose outcomes range from restoration of homeostasis to
cellular apoptosis if the stress is extreme and prolonged (23,31). The UPR thus represents a
double-edged sword and must be intricately regulated to prevent inappropriate cellular outcomes.
Mechanisms that help modulate the magnitude and type of the UPR in response to physiological
or pathological stress stimuli are not fully understood. In this study, we provide evidence that ECD protein is a negative regulator of the PERK arm of the UPR through GRP78.

Several lines of circumstantial evidence discussed in the Introduction section suggested a potential involvement of ECD in UPR but direct support for such a role has been lacking. We established that induction of ER stress, using both chemical ER stress inducers (Thapsigargin and Tunicamycin) and physiological ER stressors (glucose starvation), leads to downregulation of ECD protein levels while the mRNA levels were elevated (Fig. 1A-F). By using PERK kinase knockout (KO) or phosphorylation-deficient eIF2α MEFs (Fig. 1G-J), we established that ECD was linked to the PERK pathway of the UPR. The association of ECD with PERK and GRP78 (Fig. 2) further linked ECD to the PERK arm of the UPR. A functional connection of ECD to the PERK arm of the UPR is supported by the distinct modulation of the PERK-mediated responses elicited by perturbations of the cellular levels of ECD. Depletion of ECD sensitized cells to PERK signaling in response to ER stress, with increase in p-PERK and p-eIF2α levels, as well as an increase in the downstream effector of PERK, the transcription factor CHOP, leading to a reduced survival of these ECD depleted MEFs (Fig. 3A-E). Reciprocally, upregulation of ECD reduced PERK signaling upon induction of ER stress (Fig. 3F-I). As activation of the PERK pathway promotes cell death in response to ER stress, primarily through CHOP-dependent expression of pro-apoptotic genes (24-32) and abrogation of PERK signaling protects cells against stress-induced cell death (69-71), our results supported the likelihood that ECD functions to modulate PERK pathway activity and promote cell survival during ER stress. Indeed, assessment of cellular survival in response to ER stress showed that reduction in ECD levels impaired cell survival to ER stress (Fig. 3C-E) while overexpression of ECD promoted cell survival (Fig. 5). Collectively, these results support the conclusion that ECD and PERK are
linked through a negative feedback mechanism whereby ECD exerts an inhibitory effect on PERK pathway signaling whereas activated PERK in turn reduces ECD protein levels via eIF2α-dependent translational block. Consistent with a reciprocal negative feedback relation between ECD and PERK, activated PERK negatively regulates cell growth (33-35); conversely, ECD positively regulates cell growth (41) and is overexpressed in human breast and pancreatic cancer specimens, correlating with poor prognostic markers and shorter survival (57,74). While the physiological benefit of an increased ECD mRNA upon ER stress is not yet understood, a speculation is that this effect may reflect a feedback response to the decrease in ECD protein levels to compensate for the loss of ECD but ECD translation is blocked. Although ECD has been reported to play roles in pre-mRNA splicing in drosophila (75), it is unlikely that ECD alters its own mRNA splicing upon ER stress because ECD protein levels decrease upon ER stress.

Modulation of ECD levels using knockdown or overexpression demonstrated that ECD is a positive regulator of GRP78 levels (Fig. 3H-I; Fig 4.). While slight increases in GRP94 and PDI were observed, the effect on GRP78 was more dramatic. As increased expression of GRP78 and other chaperones is known to promote the clearing of ER stress-causing unfolded protein load and reduce the activation of UPR sensors (13-15,55), we surmised that upregulation of GRP78 and other chaperones (Fig. 3H) may represent one potential mechanism by which ECD negatively regulates the PERK pathway activation and relieves ER stress. Indeed, knockdown of GRP78 eliminated the ability of overexpressed ECD to attenuate PERK pathway signaling (Fig. 4G). Notably, GRP78 is required for cell survival not only in response to ER stress (76-79) but also in other stressful and hostile conditions such as glucose deficiency encountered in tumor microenvironment (80-85). Given that the PERK arm of the UPR is also activated in cancer
and both ECD and GRP78 are overexpressed in cancer (57, 74, 80-85), we suggest that ECD overexpression may play a similar role to mitigate the negative consequences of elevated PERK signaling found in cancer. Given the mechanistic link between ECD and GRP78 in inhibiting the PERK pathway to promote cell survival, it will be of great interest to explore if GRP78 and ECD are co-overexpressed in tumors that use the UPR to promote tumor cell survival and hence may be suitable targets for UPR-directed therapeutic agents.

Acknowledgements: We thank the members of the Band laboratory for helpful discussions. The WT and mutant eIF2α MEFs were a gift from Dr. Thomas Rutkowski’s lab, Carver College of Medicine, Iowa.

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Figure Legends:
Figure 1. Induction of ER stress leads to reduced ECD protein expression in a PERK-eIF2α-dependent manner. (A-C): MCF-10A cells were treated with Thapsigargin (50 nM, A) or Tunicamycin (50 ng/ml, B); Panc-1 (C) were cultured in glucose-free medium and then cell lysates were prepared at the indicated time points. Equal amount of proteins was resolved on SDS-PAGE followed by western blotting with indicated antibodies. Increase in the levels of p-eIF2α served as marker for induction of ER stress. (D-F): MCF-10A cells were treated with Thapsigargin (D-E) and Panc-1 cells were cultured in glucose-free medium (F), then total RNA was isolated followed by qRT-PCR using CHOP primers and ECD primer (Mean +/- SD and p-values are shown from 3 independent experiments * p<0.05). CHOP mRNA induction served as control for Thapsigargin-induced ER stress. (G): Wild-type (WT) PERK and PERK kinase domain knockout (PERK-KO) MEFs were treated with Thapsigargin (50 nM) for 14 h and then cell lysates were resolved on SDS-PAGE gels followed by western blot with indicated antibodies. (H-I): PERK KO and control WT MEFs were treated with Thapsigargin and total RNA was isolated at indicated time points followed by qRT-PCR with primers against CHOP (Fig. H) or ECD (Fig. I). (J): WT eIF2α MEFs or mutant eIF2α phospho-deficient MEFs were treated with Thapsigargin (Tg, 50 nM) or Tunicamycin (Tun, 50 ng/ml) for 14 h and then cell lysates were analyzed by western blotting with indicated antibodies.

Figure 2: ECD co-localizes and associates with PERK and GRP78. (A-E): Immortal MEFs were fixed in 3% paraformaldehyde (PFA) and stained with indicated antibodies and mounted for analyses with structure-illuminated microscopy (SIM). PERK and GRP78 co-localization served as positive control. DAPI was used to stain the nucleus. (F): MCF-10A cells were fractionated into soluble and microsomal fractions. The purity of the fractions was assessed by
GAPDH (a marker for the soluble/cytoplasmic fraction), PERK and SERCA (both markers for microsomal fraction) and RCAS1 (a Golgi-predominant protein) (88). (G-J): MCF-10A cells were fixed with 3% PFA and stained with indicated antibodies (all antibodies were generated in rabbit except anti-ECD mouse antibody) followed by species-specific secondary antibodies linked to complementary DNA probes to allow fluorescent probe-based detection of the PCR amplification product as distinct foci. Incubation with Proximity Ligation Assay plus and minus probes, followed by ligation and amplification, was carried out according to the manufacturer protocol. Red dots indicate interaction. ECD and PIH1D1 served as positive control. (K): Lysates from MCF-10A cells, treated or left un-treated with Thapsigargin, were subjected to IP with anti-ECD antibody (left panel) or anti-GRP78 (right panel) followed by Western blotting with indicated antibodies. ECD and PIH1D1(left panel) or GRP78 and PERK (right panel) served as positive controls.

Figure 3. ECD regulates the PERK pathway of the UPR. (A & C): Ecd^{fl/fl} MEFs were infected with adenoviruses coding for GFP (adeno-GFP for control) or Cre (adeno-Cre) for 72h. The cells were then left un-treated or treated with Thapsigargin (50 nM). Equal amount of proteins was resolved on SDS-PAGE gel followed by western blotting with indicated antibodies. (B): After adenoviruses infection as described in (A), cells were treated with Thapsigargin. Total RNA was isolated followed by qRT-PCR with CHOP primers; Mean +/- SD and p-values from 3 independent experiments are indicated * p<0.05. (D): After adenoviruses infection as described in (A), equal number (1,000) of wild-type (WT) or ECD^{-/-} cells were plated in triplicate and treated with Thapsigargin for 24h. 10 days later, surviving colonies were assessed after crystal blue (0.5% in 25% Methanol) staining. The color retained after the wash was dissolved in 10%
acetic acid and the absorbance was read at 590 nm. The graph below represents the relative absorbance; Mean +/- SD and p-values from 4 independent experiments are indicated * p<0.05; ** p≤0.002. (E): Panc-1 cells were treated with control or ECD siRNA for 48h. Then, the cells were switched to glucose-free media and cell lysates were prepared at indicated time points followed by western blot with indicated antibodies. (F; H-I): ECD inducible MEFs [(Tet(O)-Flag- hECD-IRESeGFP;rtTA)] or control MEFs [(Tet(O)-Flag- hECD-IRESeGFP)] were treated with Dox for 48 h followed by treatment with Thapsigargin (50 nM) or Tunicamycin (50 ng/ml). Cell lysates were prepared at indicated time points and equal amount of proteins was resolved on SDS-PAGE gel followed by western blot with indicated antibodies. (G): Following ECD induction and Thapsigargin treatment as described above, CHOP mRNA levels were assessed using qRT-PCR.

**Figure 4. The increased induction of GRP78 expression is required for ECD to downregulate PERK signaling.** (A): Ecd^{fl/fl} MEFs were treated with adenovirus as described in (Fig. 3A-C) and then the cells were treated with Thapsigargin (50 nM). Cell lysates were prepared at the indicated time points. Equal amount of proteins was resolved on SDS-PAGE gel followed by western blotting with indicated antibodies. (B): ECD was knocked down by siRNA (20 nM) in Panc-1 cells followed by exposure to glucose-free media and cell lysates were collected at indicated times points followed by western blot with indicated antibodies. (C-D): Following ECD deletion (C) or ECD overexpression (D) and Thapsigargin treatment as described above, the levels of GRP78 mRNA were assessed in WT (control) vs. ECD-/- (Cre-adenovirus treated) or control vs. ECD over-expressing MEFs using qRT-PCR. (E): ECD was knocked down in Panc-1 cells followed by cycloheximide treatment (25 uM). Cell lysates were...
prepared at indicated time points followed by western blot with indicated antibodies. (F): ECD inducible MEFs and their control MEFs were treated with Dox, as described previously, followed by treatment with Thapsigargin and then Cycloheximide treatment (25 uM) for indicated time points. Cell lysates were prepared followed by western blotting with indicated antibodies. (G): ECD overexpressing and their control MEFs were treated with GRP78 SiRNA (30 nM) or control siRNA (scrambled). 24 h later, the cells were treated with Dox for 48 h to induce ECD overexpression followed by Thapsigargin treatment (50 nM). Equal amount of proteins was resolved on SDS-PAGE gel followed by western blotting with indicated antibodies.

**Figure 5. ECD overexpression provides survival advantage.** (A): ECD was induced as described in (Fig. 3 and 4) followed by Thapsigargin treatment for 24 h. Equal amount of proteins was resolved on SDS-PAGE gel followed by western blot with indicated antibodies. (B): Following ECD induction with Dox and Thapsigargin treatment as described above, total RNA was isolated and CHOP mRNA was assessed by qPCR; Mean +/- SD and p-values from 3 independent experiments are indicated * p<0.05. (C): After ECD induction, control and ECD inducible MEFs were trypsinized and equal number of cells (1,000) were plated in triplicates. 8 h later, the cells were treated with Thapsigargin for 24h. 10 days later, surviving colonies were assessed by Crystal blue staining (0.5% in 25% Methanol). The color retained after the wash was dissolved in 10% Acetic acid and the absorbance was read at 590 nm. The graph below represents the relative absorbance; Mean +/- SD and p-values from 4 independent experiments are indicated ** p≤0.002.
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A Novel Interaction of Ecdysoneless (ECD) Protein with R2TP Complex Component RUVBL1 Is Required for the Functional Role of ECD in Cell Cycle Progression

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Ecdysoneless (ECD) is an evolutionarily conserved protein whose germ line 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 posttranslational modifications or protein-protein interactions. Since phosphorylated ECD was recently shown to interact with the PIH1D1 adaptor component of the R2TP coohaperone 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 and yet exhibited a reduced ability to rescue Ecd-deficient cells from cell cycle arrest. Biochemical analyses demonstrated 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.

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 (melting) (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 HSGT1 (human suppressor of Gcr2) and was suggested to function as a coactivator 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 papillomavirus 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+/+ mouse embryonic fibroblasts (MEFs) led to a G1/S cell cycle arrest, and this phenotype was rescued by the 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 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 pulldown screen using the phospho-peptide-binding do-


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main 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 multisubunit complexes, including the small nucleolar ribonucleoproteins, RNA polymerase II, and phosphatidylinositol 3-kinase-related kinases 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). Germ line deletion of Ruvbl1 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 Ruvbl1 in Ruvbl1<sup>abr</sup> cells also led to G<sub>1</sub>/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 (65A) disabled for CK2-mediated phosphorylation exhibits reduced ability to rescue the cell cycle arrest caused by Ecd gene deletion. Notably, whereas 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 phosphomimetic mutant (65S/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 although 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.** A protein phosphatase (catalog no. P9614) was purchased from Sigma-Aldrich USA, and the treatment was given according to the manufacturer’s instructions. 12.5% SuperSep Phos-tag (50 μmol/liter) was purchased from Wako Laboratory Chemicals (catalog no. 195-16391). Electrophoresis was performed according to the manufacturer’s protocol. PreScission protease was purchased from GE Healthcare Life Sciences.

**Cell cultures.** HEK-293T, MEFs, and T98G glioblastoma cell lines were grown in DMEM (Life Technologies, Grand Island, NY) 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 α-minimum essential medium (α-MEM). The CK2 inhibitor TBB (4,5,6,7-tetramethoxy-2-azabenimidazole) was dissolved in dimethyl sulfoxide and used at 50 μM.

**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 to 527 was generated using a three-fragment ligation into BglII and Hpal sites. C-terminal His<sub>x</sub>-tagged ECD truncations (1 to 567, 1 to 534, and 1 to 432) were generated through PCR amplification and cloning into XbaI and SalI sites of pET28b+ vector (Invitrogen), and recombinant proteins were purified after expression in *Escherichia coli* BL21(DE3) strain using a nickel affinity column (GE Healthcare). C-terminal His<sub>x</sub>-tagged full-length ECD was cloned by deleting ECD coding sequence into SalI and NotI sites of the pFastBac1 vector (Invitrogen), expressed in S21 insect cells, and purified by using a nickel affinity column. The PIH1D1-specific and control siRNAs catalog no. Sc-97385; Santa Cruz) were transfected into subconfluent cells using DharmaFECT1 transfection reagent (Thermo Scientific). Green fluorescent protein (GFP)-tagged full-length or truncated ECD expression constructs in the pGEn2 vector were generated by replacing the ST6-Gal1 insert in the ST6-Gal1-pGEn2 construct (ST6Gal1-pXLG-NtermTCMhis-Strep-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 listed in Table S2 in the supplemental material. Human PIH1D1 cDNA sequences clone SC321317; OriGene) were subcloned into BamHII and Xhol sites of pGEX-6p-1 for expression as a glutathione S-transferase (GST) fusion protein in *E. coli* BL21. A recombinant GST-PERK kinase domain was expressed in BL21(DE3) cells purified as a 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 by using a nickel affinity column. The PCR primer sequences are listed in Table S2 in the supplemental material. 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.** 76NTERT cells were plated at 5 × 10<sup>4</sup> cells per 100-mm dish for 12 h, subjected to growth factor deprivation by culturing in growth factor-free DFCI-1 medium for 72 h (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. G<sub>1</sub>/M-to-G<sub>2</sub>/M progression in MEFS was similarly assessed using FACS analysis after nocodazole (100 μg/ml)-dependent arrest in the early G<sub>2</sub>/M phase of cell cycle (22). Nuclear and cytoplasmic fractions were prepared from cells at various times points during cell cycle progression using the NE-PER kit (Thermo Scientific, catalog no. 78833). Ecd<sup>lox/lox</sup> MEFS were infected with adeno-Cre-GFP or control adeno-GFP to assess the mitotic index. Cells were collected and fixed as described above. The cell pellet was resuspended in 100 μl of phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 0.25 μg of phospho–H3-S10 (catalog no. ab14953; Abcam) and then incubated for 1 h at room temperature. Cells were washed in 150 μl of PBS, resuspended in Alexa Fluor 647 (catalog no. A212235; Life Technologies)-conjugated goat anti-mouse antibody diluted at a ratio of 1:300 in 100 μl of PBS containing 1% BSA, and incubated at room temperature in the dark for 30 min, followed by FACS analysis. The median fluores-
cence intensity (MIPI) of GFP-positive cells at 488- and 633-nm wave-lengths was recorded as an indicator of mitosis in the control and Ecd-null cells.

**Immunoblotting and IP.** Cells were lysed in radioimmunoprecipitation 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 the protein concentration was measured using the bicinchoninic acid (BCA) protein assay reagent (Pierce). Immunoblotting was performed with primary antibodies against ECD (9), RB (catalog no. 554136; Pharmingen), anti-phospho-Ser (05-1000; Millipore), anti-phospho-Thr (AB1607; Millipore), PIH1D1 (sc-101000 or sc-398010; Santa Cruz), RUVBL1 (12300S [Cell Signaling] or SAB4200194 [Sigma]) RUVBL2 (ab36569; Abcam), RPAP3 (HPA035811; Sigma), PARP (sc-8007; Santa Cruz), histone H3 (06-755; Millipore), PRPF8 (ab137694; Abcam), β-actin (A5414; Sigma), or α-tubulin (T6199; Sigma), as indicated. For immunoprecipitations (IPs), the 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 Na3VO4, and protease inhibitor mixture [Roche Applied Science]) and then immunoprecipitated with 3 μg of antibodies against ECD or 35 μl of E2F4 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 of 3× FLAG peptide (Sigma)/μl for 15 min at room temperature, and the supernatants were collected for SDS-PAGE. For PIH1D1 interaction with ECD, the cell lysates were prepared in CHAPS [3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate] 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 (catalog no. SAB4200194-200UL [Sigma]; 2 μg). Immunoprecipitated RUVBL1 (close to IgG heavy chain) was detected by Western blotting with an anti-RUVBL1 antibody (catalog no. 12300s; Cell Signaling) that was conjugated to horseradish peroxidase (HRP) using the Lightning-Link HRP conjugation kit (Novus Biologicals).

**In vitro kinase assay.** A total of 500 ng of purified recombinant ECD proteins or its mutants was incubated with 0.2 mM ATP, 1 μCi of [γ-32P]ATP (Perkin-Elmer), and 0.2 μl (10 U) of 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 32P-labeled proteins or its mutants were incubated with 0.2 mM ATP, 1 μM MgCl2, 2.5 mM EGTA supplemented with 20 μM cold ATP (NEB). Next, 0.5 μg was loaded onto SDS-PAGE gels and subjected to Western blotting with anti-p-Ser and anti-p-Thr antibodies. Ten nanograms of recombinant GST-PERK kinase domain was autophosphorylated in kinase assay buffer (50 mM HEPES [pH 8.0], 10 mM MgCl2, 2.5 mM EGTA) supplemented with 20 μM cold ATP (NEB). Next, 0.5 μg was loaded onto SDS-PAGE gels and subjected to Western blotting with anti-p-Ser and anti-p-Thr antibodies.

**32P metabolic labeling and immunoprecipitation.** Exponentially growing or serum-deprived T98G cells were washed with phosphate-free Dulbecco modified Eagle medium (DMEM) supplemented with 10% dialyzed fetal bovine serum and incubated in the same medium for 1 h before adding 0.1 μCi of [32P]orthophosphate (NEN) per 10-cm plate. The cells were labeled for 4 h at 37°C (or for 2, 5, or 16 h 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 [pH 7.4], 1 mM EDTA, 2 μg of aprotinin/ml, 2 μg of leupeptin/ml, 2 μg of pepstatin/ml, 2.5 μg of antipain/ml, 1 μg of chymostatin/ml, 1 mM Na3VO4, 10 mM NaF, 1 mM sodium molybdate, 0.5 mM phenylmethylsulfonyl fluoride). 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 were 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 [pH 7.4], 1 mM EDTA, and protease inhibitors). The immunoprecipitated proteins were resolved on 7.5% SDS-polyacrylamide gels, transferred to PVDF membranes, and visualized by autoradiography.

**In vitro binding assays.** GST- or His-tagged protein pulldowns were performed as described previously (6). FLAG-tagged wild-type (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. Then, 1,000 μg of lysate protein was incubated with 2 μg of bead-bound purified GST-PIH1D1 for 2 h at room temperature, followed by five washes, and then the bound proteins were detected by Western blotting with anti-FLAG antibody. Membranes were stained with Ponceau S to visualize the GST fusion proteins. In vitro tandem affinity purification (TAP) was performed as described previously (23) using purified recombinant ECD with a C-terminal FLAG tag.

**Cell proliferation and colony formation assays.** The cell proliferation was analyzed as described previously (6). Briefly, EcdR123Y MEFs were infected with adenoviruses encoding GFP-Cre or GFP (control) (University of Iowa Gene Transfer Vector Core) and plated at 104 cells/well in six-well plates, followed by counting of cells at the indicated time points. For assessing colony formation ability, infected cells were plated at 5,000 or 1,000 per well in six-well plates for 10 days, the colonies were stained with crystal violet (0.5% crystal violet in 25% methanol) and solubilized in 10% acetic acid, and then the extent of colony formation was measured by determining the 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 the WT and other cell types at a given time were made with a simulation correction (random sampling from a probability distribution). Some results were analyzed using a paired two-tailed Student t test. P values of ≤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 whether 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 though 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 phase and 1.25% cells in the G2/M phase (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), which is 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 sought to determine whether 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 h to induce growth arrest and then allowed to progress through cell cycle by adding serum-containing medium with 32P-labeled 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- and 20-h time points (Fig. 2A). Further analyses using anti-FLAG immunoprecipitations (IPs) from [32P]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 whether the phosphorylation of ECD corresponds to phosphoserine (p-Ser) or phosphothreonine (p-Thr) 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 autophosphorylation 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-Thr signals were detected by blotting of autophosphorylated GST-PERK kinase domain (Fig. 2C). Although 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 sites on Ser-505 and Ser-518 (11), we performed a detailed analysis of potential phosphorylation sites on ECD using the publicly 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/Thr 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 whether 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 (residues 1 to 644) and its fragments encompassing residues 1 to 567 or 1 to 534, whereas substantially less phosphorylation was observed with the ECD 1-432 fragment (Fig. 3A and 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 and D).
While S→A mutations of the Ser residues in the distal cluster (S572A, S579A, and S584A; designated 3S/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 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, 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 and can produce a mobility shift (30). Expected, the Phos-tag-bound WT ECD resolved as a slower-migrating band compared to its phosphatase-treated sample 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 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 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 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 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 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 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 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 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 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 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 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 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 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 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 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 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 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 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 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 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 and can produce a mobility shift (30). As expected, the Phos-tag

Although these experiments confirmed and extended the concept of CK2-mediated phosphorylation of ECD in vitro, to relate this posttranslational modification to ECD function, it was important to assess whether 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 FLAG-tagged wild type or 3S/A or 6S/A mutants of ECD. These constructs were expressed in T98G cells, and cells were metabolically labeled with [32P]orthophosphate. Equal amounts of radioactive extracts (based on the counts per minute) were subjected to anti-FLAG IP, followed by autoradiography. Although 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 whether 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 versus 3S/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 versus 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 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 the 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 on June 23, 2017 by UNIV OF NEBRASKA MED CTR
FIG 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 the 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 (aa 1 to 644) ECD and various C-terminal deletion fragments (aa 1 to 567, 1 to 534, and 1 to 432) with human recombinant CK2 were separated by SDS-PAGE, transferred to PVDF membranes, and subjected to autoradiography to detect 32P signals. The 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 six 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. 32P labeling was detected using autoradiography, and the filters were subsequently subjected to Western blotting with anti-p-Ser antibody and reprobed with anti-ECD antibody for equal loading. The 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 32P, as described above. FLAG-tagged ECD and its mutants were immunoprecipitated and subjected to autoradiography. IP of cells expressing WT ECD subjected to phosphatase treatment is shown. The blot was reprobed...
by other kinases, depending on the cell type or varying functional states.

Next, we assessed whether 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 Ecdflox/flox 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 phosphodefective mutants. In initial experiments, we expressed the WT human ECD or its 3S/A or 6S/A mutants in Ecdflox/flox MEFs (Fig. 3G) and then assessed the ability of the 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, the deletion of Ecd in Ecdflox/flox 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; also see Fig. S2A in the supplemental material). 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) (all P values are shown in Table S1 in the supplemental material). Furthermore, we examined a mutant in which serine residues 503, 505, and 518 were removed by deletion (Δ499–527) in the cell cycle rescue experiment and observed that this mutant behaved similarly to WT ECD in rescuing the proliferation block (see Fig. S2A and B in the supplemental material). Next, we generated a phosphomimetic mutant in which the six serine residues identified to be phosphorylated were mutated to aspartic acid residues (6S/D). Notably, the phosphomimetic mutant 6S/D was completely defective in cell cycle rescue experiment (Fig. 3I and J). In these experiments, we also examined the 3S/A mutant and observed a partial rescue with this mutant (Fig. 3I). Although the complete lack of rescue seen with the 6S/D mutant of ECD was surprising, it has been reported that aspartic acid phosphomimics 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.

Phosphodefective 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 SS03/50S/51S residues, we first reexamined the previously reported dependence of ECD binding to the isolated phoso-reader domain of PIH1D1 (11). For this purpose, GST-PIH1D1 pulldown 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 and 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 ECD (Fig. 4E; see also Fig. S3A in the supplemental material), 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, see also Fig. S3A in the supplemental material), as well as for the four R2TP complex components (Fig. 4D and E; see also Fig. S3A in the supplemental material). Since a phosphorylated DSpD/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 (Δ499–527) of ECD that lacks the DSpD/E motif in addition to the 6S/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 and E). Notably, compared to the levels of endogenous ECD co-IP with PIH1D1 in vector control lanes, increased amounts of ectopically expressed ECD were coimmunoprecipitated from WT ECD-transfected cell lysates. Unexpectedly, however, the WT and mutant ECD proteins were coimmunoprecipitated with PIH1D1 to comparable levels (Fig. 4D and E; see also Fig. S3A in the supplemental material). These results demonstrate that ECD interacts with the R2TP complex in cells but that 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, treated these IPs with lambda phosphatase, and then assessed the levels of coimmunoprecipitated PIH1D1. Notably, although the phosphatase treatment robustly eliminated the phosphorylation signal on ECD (anti-p-Ser blot), no reduction in PIH1D1 or ECD coimmunoprecipitation was seen (see Fig. S3B and C in the supplemental material). Thus, while CK2-phosphorylated ECD can directly interact with PIH1D1, as reported previously (11), this interaction is not required for the association of ECD with the R2TP complex. Next, we examined the ability of 6S/D or 3S/A mutants of ECD to interact with PIH1D1. For this purpose, lysates from 293T cells expressing FLAG-tagged 3S/A or 6S/D mutants were used for an in vitro pulldown assay with GST-PIH1D1. As expected, GST-PIH1D1 was able to pull down the 3S/A mutant but failed to pull...
down the 6S/D mutant of ECD (see Fig. S3D in the supplemental material), confirming that 6S/D does not mimic WT ECD for its interaction with PIH1D1.

**Novel phosphoindependent 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 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 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 and 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 and E) Lysates of HEK-293T cells transfected with untagged WT or VIPA-tagged ECD were subjected to immunoprecipitation with anti-PIH1D1 antibody and immunoblotted with the indicated antibodies.

**ECD-RUVBL1 Interaction Regulates the Cell Cycle**

**FIG 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 the 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. The 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 h earlier were subjected to anti-ECD IP followed by blotting with antibodies against the indicated proteins. V, vector-transfected cells.
tion (see Fig. S3E in the supplemental material). 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 coimmunoprecipitation experiments with an anti-RUVBL1 antibody. Both the WT and the 6S/A ECD proteins were coimmunoprecipitated 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-IP of RUVBL1 in both control and PIH1D1 knockdown 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. Germ line 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. To test this hypothesis, we first expressed GFP-tagged ECD or its several C-terminal deletion mutants in HEK-293T cells and performed co-IP experiments using an anti-RUVBL1 antibody. Notably, only full-length ECD protein (amino acids [aa] 1 to 644) coimmunoprecipitated with RUVBL1, whereas none of the C-terminal deletions of ECD was able to coimmunoprecipitate with RUVBL1 (see Fig. S4A and B in the supplemental material). 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 pulldown with glutathione-Sepharose or nickel beads. As shown in (Fig. 6A and B; see also Fig. S4C in the supplemental material), 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 (residues 150 to 438, 438 to 644, 1 to 438, and 150 to 644) (see Fig. S4D in the supplemental material) with wild-type ECD (residues 1 to 644) for their abilities to rescue the growth arrest of Ecdfl/fl 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; see also Fig. S4D in the supplemental material). As expected, deletion of Ecd in Ecdfl/fl MEFs arrested cell proliferation, and ectopic WT human ECD significantly rescued the cells from growth arrest (Fig. 6D). However, none of the deletion mutants was able to rescue the cell proliferation block imposed by endogenous ECD depletion. These

![FIG 6 Interaction with RUVBL1 is important for ECD function in cell cycle progression. (A and 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 Ecdfl/fl 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. Ecdfl/fl MEFs expressing full-length WT ECD (aa 1 to 644) or its truncations were infected with ctrl or Cre adenoviruses, colonies were stained with crystal violet after 10 days, and the solubilized dye absorbance was read at 590 nm. A histogram shows the relative rescue efficiency of each construct compared to vector control cells. Error bars represent the means ± the standard deviations of three independent experiments. A statistical comparison used a Student 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 pulldown, followed by anti-FLAG blotting. V, vector-transfected cells.](http://mcb.asm.org/)

![A.](http://mcb.asm.org/)  
![B.](http://mcb.asm.org/)  
![C.](http://mcb.asm.org/)  
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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; see also Fig. S4D in the supplemental material). Notably, two deletion fragments (residues 438 to 644 and 150 to 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). Reprobing of the same membrane with antibodies against RUVBL1 and RUVBL2 showed the expected interaction of PIH1D1 with RUVBL1 or RUVBL2 (see Fig. S4E in the supplemental material).

Our previous studies showed that ECD interacts with RB, a function important for the ECD role in cell cycle progression (6). Notably, in addition to the expected interaction of full-length ECD with RB, one mutant (aa 150 to 644) 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 (see Fig. S4F in the supplemental material).

**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 Ecdfl/fl MEFs and measured the 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 pH 3 (S10) compared to control cells (89.8) (Fig. 7A), indicating that Ecd deletion cells are cell cycle arrested prior to entering mitosis. Low levels of pH 3 (S10) were further confirmed by Western blotting (Fig. 7B). Next, we assessed the G2/M to G1 progression of MEFs arrested in the S phase by nocodazole treatment (Fig. 7C). Flow cytometry analysis revealed a significant impairment in G2/M-to-G1-phase transition upon Ecd deletion compared to control, in addition to a higher percentage of Ecd-deleted MEFs in the G1 phase (Fig. 7C to E). Taken together, these results demonstrate a critical role of ECD in both G1-to-S and G2/M-to-G1 transitions. 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 the 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 ecdysoneneless* gene, is required for embryonic development and progression of mammalian cells through the G1-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 RUVEBL1 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. In contrast 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 the phosphorylation of ECD positively regulates its function in promoting the cell cycle progression. Bioinformatics analysis, followed by mass spectrometry-based phosphoproteomics, 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. 3D and 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, however, 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. Although the overall levels and the subcellular localization of ECD remain invariant during cell cycle progression (Fig. 1 and 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 and 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 impair PIH1D1 binding (3S/A and Δ499–527) had no impact on its ability to function in cell cycle progression. However, 6S/A and 6S/D were defective in a 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). We have shown that ECD overexpression in cells leads to p53 stabilization and increased p53-dependent target gene expression and to the 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 the regulation of glucose uptake, oxidative stress, and endoplasmic reticulum stress-induced apoptosis (41, 42). Thus, ECD phosphorylation and interaction with PIH1D1 may play a role in regulating these functions. The availability of Ecd−/− MEFs in which ECD can be conditionally deleted, together with the 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: first, what are the determinants of ECD-R2TP association, 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, RUVEBL1 (Fig. 5 and 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 RUVEBL1, with only the full-length ECD competent at both functions (Fig. 6A and B; see also Fig. S4B and C in the supplemental material). Interestingly, the Δ499–527 mutant which interacts with RUVEBL1, but not PIH1D1, was able to rescue the cell cycle arrest caused by Ecd deletion (see Fig. S2A in the supplemental material). Thus, our studies identify a novel interaction of ECD with RUVEBL1 and suggest that this mode of interaction with the R2TP complex is a key to the regulation of cell cycle progression by ECD. The delineation of sequences in ECD and RUVEBL1 that mediate their interaction should help directly test whether 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 RUVEBL1 within the R2TP complex. Interestingly, mouse ECD and RUVEBL1 knockouts are phenotypically similar, since both are embryonic lethal at the blastocyst stage (6, 19). RUVEBL1 is essential for cellular proliferation as seen in knockout cells or upon knockdown of RUVEBL1 expression (18). A recent study demonstrated that RUVEBL1 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 coactivator function (8). Thus, ECD may function in close coordination with RUVEBL1.

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, RUVEBL1/2 are also parts of other functionally relevant
complexes, such as chromatin remodeling complexes TIP60, SWR1/SRCAP, and INO80 and the Fanconi anemia core complex that controls DNA interstrand cross-link repair and function, and regulate telomerase biogenesis and mitosis (19, 43–45). Given the PH1D1-independent interaction of ECD with RUVBL1, the 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. Since a key mechanism by which the R2TP complex regulates biochemical processes is by facilitating protein complex remodeling, we speculate that the interaction of ECD with the R2TP complex through RUVBL1 may facilitate the ECD-RB complex formation and helps dissociate RB from E2Fs, thereby derepressing 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, since 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 that 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) data sets (46) revealed that expression of many RUVBL complex genes was significantly higher in breast and colorectal carcinomas 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 RNA 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 glands 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 PRP8. The R2TP complex regulates mRNA and ribosome biogenesis by facilitating the assembly of small nucleolar ribonucleoproteins (snRNP s), which are known to be involved in spliceosome modification (51, 52). Upregulation of R2TP and snRNP 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 requires further investigation. Taken together, the findings presented here demonstrate that CK2-mediated phosphorylation and interaction with RUVBL1 are essential for ECD’s ability to regulate cell cycle progression.

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