UNIVERSITY OF CALIFORNIA

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The Zinc Transporter ZIP7 in Cell Survival & Resilience

A dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy in Molecular, Cellular & Developmental Biology

By

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March 2024

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CURRICULUM VITAE

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Education

PhD in Molecular, Cellular & Developmental Biology, University of California Santa Barbara. 2018-2024. GPA: 3.93

BS in Biology, Minor in Sociology, Loyola Marymount University, Los Angeles, CA, 2014-2018. GPA: 3.82

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2016-2018 **Dr. Sarah Mitchell's Research Group**
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2018 Introduction to Biology Lab
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ABSTRACT

The Zinc Transporter ZIP7 in Cell Survival & Resilience

By

Morgan Claire Mutch

Proteotoxic stress drives the progression of numerous degenerative diseases including Type I diabetes, Huntingtin's Disease, Parkinson's Disease, and retinitis pigmentosa, a degenerative blinding disease. Cells respond to misfolded proteins by activating the unfolded protein response (UPR), including endoplasmic-reticulum-associated-protein-degradation (ERAD) and proteasomal degradation, but persistent stress triggers apoptosis. Enhancing ERAD is a promising therapeutic approach for protein misfolding diseases.

From plants to humans, loss of the zinc transporter ZIP7 causes ER stress; however, the mechanism is unknown. We used *Drosophila melanogaster* to genetically manipulate ZIP7 and investigate its function *in vivo*. Here, we show that ZIP7-mediated Zn^{2+} transport enhances ERAD and that cytosolic Zn2+ is limiting for deubiquitination of client proteins by the Rpn11 Zn^{2+} metalloproteinase as they enter the proteasome.

We then decided to test ZIP7's effects in a fly model of retinitis pigmentosa. Interestingly, we found ZIP7 overexpression rescues defective vision and photoreceptor degeneration caused by misfolded rhodopsin in Drosophila. We have begun a collaboration looking into whether ZIP7 can prevent or suppress mutant

Rhodopsin-mediated neurodegeneration in human retinal organoids. Additionally, these results prompted a screen for potential ZIP7 rescue of other aggregate-prone proteins associated with neurodegenerative diseases. So far, we found that ZIP7 rescues photoreceptor degeneration caused by Aβ42 and Vap33. Further study of ZIP7, a novel component of ERAD, holds significant promise for therapeutic advancements in protein-folding disorders.

Table of contents

I. INTRODUCTION

A. The Surprising and Multifaceted Importance of Zinc in Cell Biology

Zinc is a trace element required in all organisms. The divalent cation is involved in many aspects of cell biology, including transcription, cell division, oxidative stress, and tissue development. Zinc deficiency is implicated in pathologies ranging from skin disorders, to gastrointestinal diseases, and immunodeficiencies. Almost all intracellular zinc is bound to protein, and it is predicted that around 10% of human proteins require zinc binding [1], [2]. However, free, unbound zinc is vanishingly rare, and the intracellular zinc concentration is tightly regulated.

1. Zinc is important for protein structure and function

Zinc stabilizes the structures of a variety of proteins. Zinc finger proteins are abundant zinc-binding proteins; around 3% of genes encode for proteins with zinc finger domains [3, 4]. Many transcription factors contain zinc finger domains that facilitate DNA binding with high specificity [2]. This specificity is useful for creating artificial zinc finger proteins used for genome editing in model organisms including flies, zebrafish, and mice [3,4]. E3-ubiquitin ligases contain RING domains, a subfamily of zinc fingers, that transfer ubiquitin molecules to proteins targeted for degradation[2,4]. Zinc finger proteins are also involved in cytoskeleton remodeling and focal adhesions. Mutations in zinc finger proteins are implicated in cancer and neurodegenerative diseases[4].

Many enzymes rely on zinc for activation. Most metalloproteases require zinc, and these are enzymes that catalyze the breakdown of proteins into polypeptides or

amino acids. For example, zinc is a cofactor for matrix metalloproteinases that degrade extracellular matrix proteins to facilitate cell migration, inflammatory signaling, and angiogenesis. Additionally, zinc binding to metallothioneins in the cytosol regulates metal ions and protects against metal toxicity [1, 2, 5]. Zinc is co-factor for SOD (superoxide dismutase), which catalyzes the conversion of superoxide (free radical oxygen) to molecular oxygen and hydrogen peroxide to reduce oxidative stress [6, 7, 8].

2. Zinc ions can serve as signals

Zinc ions act as a signal for a variety of cellular processes. Interestingly, almost all zinc in cells is bound to protein, and the concentration of free cytosolic zinc is incredibly low, in the high-picomolar to low-nanomolar range [9, 10]. Despite their low concentration, free zinc ions are thought to participate in signaling pathways including immunity and neurotransmission.

Zinc has anti-inflammatory properties and participates in cellular immune responses. Zinc opposes oxidative stress through binding MTF-1 and SOD [2, 6, 11]. It has been shown that zinc negatively regulates NF-kB signaling in mice, protecting against sepsis [12]. Zinc deficiency in infected mice prevents proper bacterial clearance and upregulated inflammatory cytokines TNFα and IL-1β [12]. Zinc deficiency also leads to apoptosis of B and T cells which are responsible for adaptive immune response [13].

Neurotransmission is also dependent on zinc. Despite low levels of free zinc in cells, presynaptic terminals and synaptic vesicles in glutaminergic neurons are

concentrated with zinc ions [14]. These vesicles release zinc into postsynaptic terminals with relatively low zinc concentration, where zinc can readily bind neurotransmitter receptors. For example, zinc can bind and regulate effects of the NMDA receptor, which is involved in synaptic plasticity. Zinc is also important for proper development of the hippocampus and cerebellum [2, 15].

3. Zinc transporters regulate zinc homeostasis

Intracellular zinc homeostasis is tightly regulated through a network of zinc transporters. Humans have 24 zinc transporters that are divided into 2 families: 14 ZIPs (SLC39a), which transport zinc into the cytosol, and 10 ZnTs (SLC30a), which transport zinc out of the cytosol. Most of these transporters are specific for zinc [16]. Zinc transporters localize to distinct organelles and regions of the cell, and some are only present in specific cell types [17]. These transporters are likely releasing zinc in "waves" to participate in signaling pathways. Individual and collective contributions of each transporter to maintaining zinc homeostasis is an interesting area of study. Mutations in zinc transporters are implicated in several types of cancer, neurological disorders, diabetes, and cardiovascular disease [8, 17, 18].

B. ZIP7

1. Localization & structure

ZIP7, or SLC39A7, is located on the membrane of the endoplasmic reticulum (ER) and transports zinc ions from the endoplasmic reticulum (ER) lumen into the cytosol. It is in the LIV-1 family of ZIPs that share a unique zinc-binding motif in the 5th of 8 transmembrane domains [19]. ZIP7, like others ZIPs, has a histidine-rich N-terminal

region that binds zinc ions in the ER [20]. Zinc transport is coordinated by histidine residues in the 4th and 5th transmembrane domains.

Taylor et al. found that ZIP7 is a gated channel that opens in response to phosphorylation [21]. Ser275 and Ser276 as are the key phosphorylation sites on ZIP7. These residues reside in the cytoplasmic loop between the 3rd and 4th transmembrane domains. CK2 phosphorylates Ser275 and Ser276, as shown through co-immunoprecipitation and proximity ligation assays between CK2 and ZIP7. Additionally, pharmacological inhibition of CK2 and CK2 RNAi decreased serine phosphorylation on ZIP7 and ZIP7-mediated zinc release. However, the upstream signaling that regulates CK2's phosphorylation of ZIP7 has yet to be determined.

There is debate over ZIP7's mechanism of zinc transport. It was suggested that the ER stores zinc, and phosphorylated ZIP7 allows zinc ions to flow down their concentration gradient into the cytosol [19]. This idea is supported by a study that used a FRET-based zinc probe eCALWY and showed zinc to be significantly higher concentration in the ER compared to the cytosol [22]. However, other studies using the FRET zinc probe ZapCY1 and inductively coupled plasma mass spectrometry found zinc concentration to be much lower in the ER compared to the cytosol [10, 23, 24]. Another study observed similar zinc concentrations in the ER and cytosol, but noted there was cell-to-cell variability in ER zinc concentration [25]. The cytosol does contain more zinc-buffering agents like metallothioneins than the ER, so it is possible the ER is more sensitive to free zinc toxicity. Since there is no evidence that ZIP7 acts

through secondary active transport, local and hard-to-detect zinc concentration gradients around ZIP7 may explain ion transport.

2. ZIP7 has many reported functions

ZIP7 is implicated in multiple diverse biological processes, including cancer cell proliferation, cell death, skin development, stem cell renewal, and glucose metabolism. Yet, a unifying mechanism to explain ZIP7's role in all these processes has not been determined.

ZIP7 promotes cancer cell proliferation

The Akt signaling pathway regulates cell growth, proliferation, and survival. Extracellular ligand binding to Receptor Tyrosine Kinases initiates the signaling cascade, leading to Akt phosphorylation and activation. Akt is a master kinase that inhibits pro-apoptotic proteins like FoxO and Caspase-9, while stimulating pro-proliferation proteins like CREB and pathways like mTOR. Akt signaling is often hyperactivated in cancer cells, driving tumor progression.

ZIP7 can activate Akt signaling. Extracellular zinc treatment in 2D cell culture leads to Akt activation [26, 27, 28, 29]. Many labs have shown that ZIP7 is necessary for zinc's effect on Akt. TamR breast cancer cells treated with extracellular zinc increased phosphorylated Akt (pAkt) expression, but this effect disappeared with ZIP7 knockdown [26]. In mouse embryonic stem cells, zinc transport by ZIP7 increased pAkt expression, and upregulated pluripotency markers POU5F1 and alkaline phosphatase which are downstream of Akt activation [30]. Additionally, ZIP7 knockdown in HGC-27 cells reduced pAkt and pmTOR expression [31]. ZIP7-mediated

zinc transport most likely promotes Akt activation through inhibition of protein tyrosine phosphatases, allowing activation of Receptor Tyrosine Kinases that promote Akt activity [26, 28, 32].

Cell Death

ZIP7 protects cancer cells from apoptosis. ZIP7 overexpression in gastric cancer cell lines MGC-803 and HGC-27 promoted cell survival, proliferation, and migration through activation of Akt [31]. Inhibiting ZIP7 using the compound NVS-ZP7-4 reduced pAkt in hepatocellular carcinoma cell lines HCCLM3 and Huh7 cells and drove apoptosis [33]. ZIP7 is upregulated in cervical cancer and colon cancer cells, where it drives proliferation [34], [35]. Knockdown of ZIP7 induced apoptosis in cervical cancer cells HeLa and ME-180 [34] and colon cancer cell line HCT116 [35].

Conversely, ZIP7 may drive susceptibility to ferroptosis, another mechanism of programmed cell death. Ferroptosis occurs when excessive iron-mediated phospholipid oxidation results in the accumulation of reactive oxygen species (ROS), causing cell death [36]. Extracellular zinc treatment in MDA-MB-231 breast cancer cells increased sensitivity to ferroptosis, similarly to iron addition [37]. ZIP7 siRNA suppressed ferroptosis in cells treated with known-ferroptosis-causers erastin and cysteine depravation. Inhibiting ZIP7 with compound NVS-ZP7-4 caused ER stress, which appeared to be protective against ferroptosis. The authors concluded that upregulation of HERPUD1, a protein that responds to ER stress, confers protection against ferroptosis [37].

ZIP7 also plays a role in necroptosis. Necroptosis is an inflammatory cell death mechanism that occurs during pathogen defense, immune signaling, and tissue injury [38]. TNFα triggers necroptosis by binding the death receptor TNFR1. ZIP7 knockout in KBM7 leukemia cells caused TNFR1 to accumulate in the ER lumen [39]. The failure of TNFR1 to reach the plasma membrane prevented initiation of necroptosis. Therefore, ZIP7's role in maintaining ER homeostasis is necessary for necroptotic signaling.

Skin Development

ZIP7 is critical for proper skin tissue development. Bin et al. studied ZIP7 in mammalian dermis and connective tissue and found that ZIP7 mutant mice were smaller by weight and had thinner dermal layers than wild-type mice[40]. In human mesenchymal stem cells (hMSCs), ZIP7 knockdown in slowed proliferation [40,41]. ZIP7 knockdown also led to aggregation of PDI, a protein-folding chaperone, in the ER[41]. Gene microarrays and quantitative PCR revealed hMSCs with ZIP7 knockdown upregulated ER stress, unfolded protein response, and apoptotic genes, and downregulated cell division, proliferation, and survival genes [40,41]. ZIP7 supports skin development and maintenance through suppressing ER stress and promoting cell proliferation.

Stem Cell Renewal

ZIP7 promotes intestinal and embryonic stem cell renewal. Ohashi et al. found that ZIP7 was upregulated in mouse intestinal epithelial cells, and loss of ZIP7 in mice caused widespread apoptosis of intestinal stem cells [42]. Apoptosis was driven by ER

stress [42]. In human embryonic stem cells, ZIP7-mediated zinc release promoted pluripotency [30]. Inhibition of ZIP7 reduced pluripotency markers POU5F1 and alkaline phosphatase. The authors attributed ZIP7's role in pluripotency to its promotion of Akt signaling.

Insulin regulation and glucose metabolism

Insulin regulates glucose homeostasis through the Insulin Receptor-Akt signaling pathway. Insulin binding to Insulin Receptor leads to phosphorylation of PI3K and Akt. Akt mediates glucose uptake by initiating the movement of glucose transporters to the cell membrane and synthesis of glycogen to store glucose. Insulin resistance occurs when Insulin Receptor no longer responds appropriately to insulin, requiring more insulin for effective glucose metabolism. Pancreatic β cells produce large amounts of insulin, relying on the ER for its synthesis and folding. Insulin resistance increases the amount insulin needed to metabolize glucose, overwhelming the ER's folding capacity and causing ER stress [18]. In turn, ER stress can exacerbate insulin resistance and cause cell death, leading to Type 2 Diabetes.

ZIP7 is implicated in insulin signaling, but its role is not fully understood. Hyperglycemia led to upregulation and activation of ZIP7 in diabetic rat cardiomyocytes and H9c2 cells [43]. This may be because CK2, which phosphorylates ZIP7, is also activated in response to glucose. Tuncay et al. demonstrated that hyperglycemic cells upregulate ER chaperones GRP78 and CALR and experience ER stress in response to an increase in cytosolic zinc levels, but this seems to be irrespective of ZIP7 expression as knockdown of ZIP7 did not affect GRP78 or CALR

levels [43]. Loss of ZIP7 in skeletal muscle cells caused downregulation of glucose metabolism genes like Glut4 and insulin signaling component Akt [44, 45]. ZIP7 was found to be downregulated in insulin resistant skeletal muscle cells [45]. It is unclear whether ZIP7 contributes to insulin signaling through regulating cytosolic zinc or through its role in ER stress.

3. ZIP7 in ER Stress

Many of ZIP7's reported functions hinge on its responsibility to maintain ER function. Secretory and transmembrane proteins are translated by ribosomes on the ER, translocated into the ER lumen for proper folding, then transported to the Golgi apparatus [46, 47]. However, a significant fraction of these proteins misfold [47]. Misfolded proteins in ER are degraded through ER-associated protein degradation (ERAD), where proteins are retrotranslocated into the cytosol, tagged with ubiquitin chains, and eventually broken down the 26S proteasome [46,47]. ER stress occurs when the ER is overwhelmed with misfolded protein. Accumulation of misfolded protein triggers the Unfolded Protein Response (UPR) that attempts to relieve stress by downregulating protein synthesis and upregulating degradation machinery and protein folding chaperones. If ER stress is too great, the UPR will initiate apoptosis. ER stress is implicated in many diseases, including neurodegenerative diseases, diabetes, and heart disease [46,47]. Therefore, identifying mechanisms of reducing ER stress are of interest for developing novel therapeutics.

Loss of ZIP7 causes ER stress in a variety of cell types including mouse intestinal stem cells [42], human mesenchymal stem cells [40, 41], rat cardiomyocytes [43],

human cancer cell lines including MG-63 and KBM7 [10, 39], retinal pigmented epithelium ARPE-19 cells [48], *Drosophila* wing imaginal disc cells [49], and *Drosophila* epithelial cells (Guo & Mutch et al, 2024). ZIP7's role in ER stress was already implicated in skin development and stem cell renewal. Other reported functions of ZIP7 can also be explained through its role in mitigating ER stress.

ZIP7 most likely regulates Akt signaling, and subsequently proliferation and growth, through maintaining ER function and suppressing ER stress. Akt/mTOR signaling is activated in response to nutrient availability to drive cell survival, growth, and proliferation [50]. ER stress and UPR overactivation inhibit Akt/mTOR signaling to reduce protein synthesis [23, 50, 51]. ZIP7 was reported to drive cancer cell proliferation through activation of Akt based on data showing knockdown of ZIP7 reduced Akt activation [26 30 28]. However, since loss of ZIP7 is known to cause ER stress, it is more likely that ZIP7 promotes Akt through suppressing ER stress rather than a direct activation of the pathway.

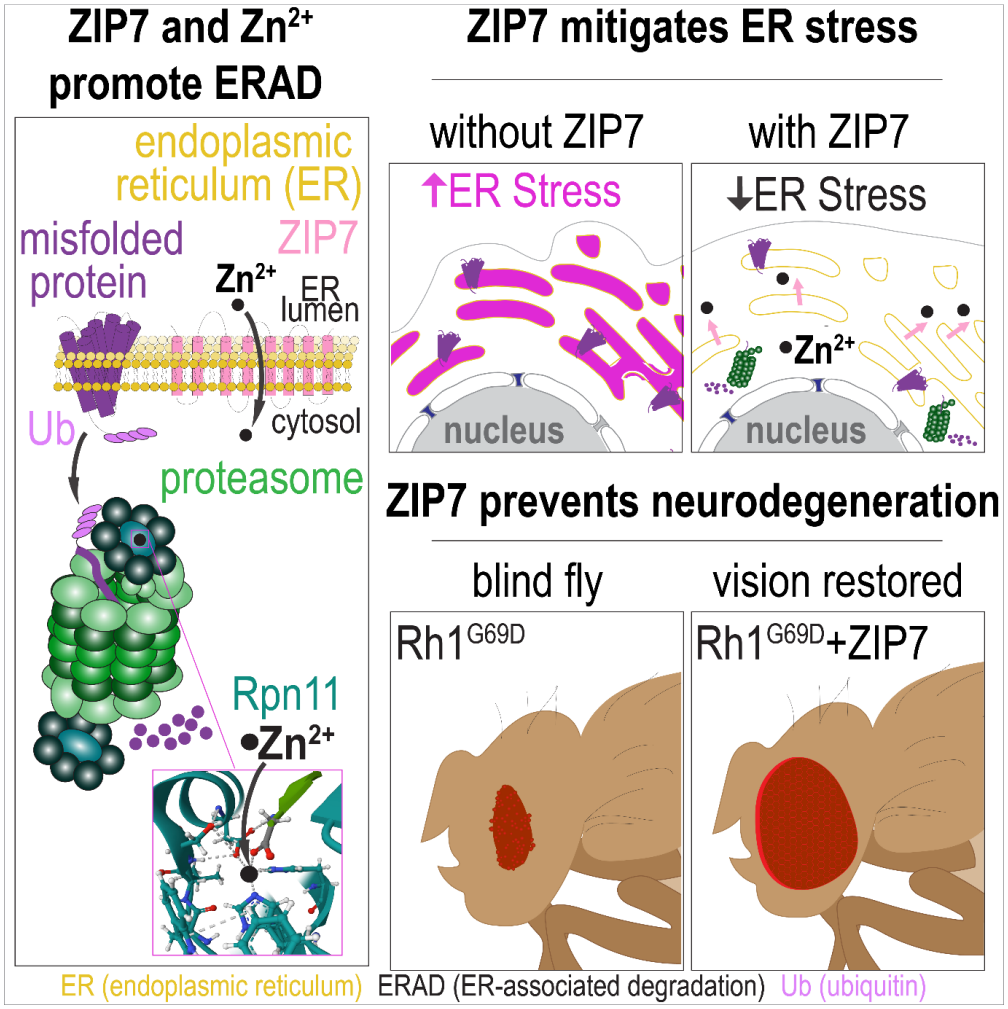
It was reported that ZIP7 is required for Notch signaling through supporting Notch trafficking to the plasma membrane [49]. Loss-of-function mutations in ZIP7 caused Notch accumulation and impaired Notch signaling in fly wing imaginal discs. ZIP7's effect on Notch signaling was determined to be due to a trafficking defect. However, we found that ZIP7 promotes degradation of misfolded Notch and it is ER stress that compromises Notch signaling (Guo & Mutch et al., 2024). These results indicate ZIP7 is promoting protein degradation rather than trafficking.

The data presented here uncover ZIP7's function in ER-associated protein degradation and reveal a unifying mechanism that contextualizes previous findings. We identified ZIP7 as a novel component of ERAD and the role of local zinc at the ER-cytoplasm interface. ZIP7 overexpression sufficiently degrades toxic, misfolded proteins and rescues ER stress. Specifically, ZIP7 enhances proteasomal degradation of misfolded proteins by providing rate-limiting zinc to Rpn11 to facilitate deubiquitination of those proteins. ZIP7 can also prevent neurodegeneration caused by misfolded protein in the fly model. These findings shed light on ZIP7's seemingly ubiquitous importance in cell biology.

II. THE ZN^{2+} TRANSPORTER ZIP7 ENHANCES ENDOPLASMIC-RETICULUM-ASSOCIATED PROTEIN DEGRADATION AND PREVENTS NEURODEGENERATION IN DROSOPHILA

This chapter is adapted from published work by: Xiaoran Guo*, Morgan Mutch*, Alba Yurani Torres, Maddalena Nano, Nishi Rauth, Jacob Harwood, Drew McDonald, Zijing Chen, Craig Montell, Wei Dai and Denise J. Montell. *Developmental Cell* (2024) *These authors contributed equally

A. ABSTRACT



B. Summary

Proteotoxic stress drives numerous degenerative diseases. Cells initially adapt to misfolded proteins by activating the unfolded protein response, including endoplasmic-reticulum-associated-protein-degradation (ERAD). However, persistent stress triggers apoptosis. Enhancing ERAD is a promising therapeutic approach for protein misfolding diseases. The ER-localized Zn^{2+} transporter ZIP7 is conserved from plants to humans and required for intestinal self-renewal, Notch signaling, cell motility and survival. However, a unifying mechanism underlying these diverse phenotypes was unknown. In studying Drosophila border cell migration, we discovered that ZIP7-mediated Zn^{2+} transport enhances the obligatory deubiquitination of proteins by the Rpn11 Zn^{2+} metalloproteinase in the proteasome lid. In human cells, ZIP7 and Zn^{2+} are limiting for deubiquitination. In a Drosophila model of neurodegeneration caused by misfolded rhodopsin (Rh1), ZIP7 overexpression degrades misfolded Rh1 and rescues photoreceptor viability and fly vision. Thus, ZIP7-mediated Zn^{2+} transport is a previously unknown, rate-limiting step for ERAD *in vivo* with therapeutic potential in protein misfolding diseases.

C. Introduction

Normal development and healthy aging require robust protein quality control. During normal cellular life, up to 30% of protein molecules misfold and are targeted for degradation by the proteasome. [52,53] Some proteins are naturally more prone to misfolding than others and some cells experience greater protein folding challenges than others. Mutations can increase the susceptibility of proteins to misfolding, as can small protein aggregates that seed larger ones. Long-lived cells like stem cells

and neurons, as well as secretory cells are especially dependent on proteasome activity for survival. Unfortunately, proteasome activity declines with age. [54,55]

Healthy cells degrade misfolded proteins. If the load of misfolded proteins increases, inducing endoplasmic reticulum (ER) stress, cells adapt by activating the unfolded protein response (UPR) [56], expanding the ER, enhancing ER-associated degradation (ERAD), reducing the rate of new protein synthesis, and altering transcriptional programs. If the stress resolves, the cell recovers. If the stress persists and overwhelms the adaptive response, cells die. There is great interest in identifying mechanisms that enhance ERAD in anticipation that such approaches will prevent or reverse degenerative diseases and promote healthy aging. [57,58] Conversely, inhibitors of proteasomal degradation of misfolded proteins are in clinical use to treat cancers, especially malignancies of highly secretory cells, such as antibody-secreting B cells.

ZIP7 is an evolutionarily-conserved, ER Zn^{2+} transporter that promotes cell survival and migration in diverse cell types and organisms. [34,35,42,49] ZIP7 promotes intestinal self-renewal, is required for B cell differentiation, and is overexpressed in multiple cancers. [42,59] Loss of ZIP7 causes ER stress in a variety of cell types within organisms as diverse as plants and yeast to flies and humans. However, the mechanism by which ZIP7 mitigates ER stress and contributes to these diverse biological functions is unknown, [20] and the effects of ZIP7 overexpression are largely unexplored.

We previously identified the *Drosophila* ortholog of ZIP7 (dZIP7, aka Catsup) in a screen for mutations that disrupt border cell migration [60] and in a border cell gene expression profile. [61] Border cells in the *Drosophila* ovary provide an *in vivo* model of collective cell migration that is amenable to unbiased genetic screening. [62] Here we show that it is by mitigating ER stress that dZIP7 promotes border cell migration. Induction of ER stress by expressing a misfolded protein ($Rh1^{G69D}$) [63,64] blocks border cell migration. Remarkably, overexpression of dZIP7 is sufficient to degrade misfolded $Rh1^{G69D}$, prevent ER stress, and thereby rescue border cell migration. We further show that ER to cytosol Zn^{2+} transport is rate-limiting for ERAD in Drosophila and human cells. dZIP7 overexpression in photoreceptor cells is sufficient to degrade $Rh1^{G69D}$ in photoreceptor cells and prevent $Rh1^{G69D}$-induced retinal degeneration. These results illuminate a previously unappreciated rate-limiting requirement for Zn^{2+} in ERAD and suggest ZIP7 overexpression as a potential gene therapy for autosomal dominant retinitis pigmentosa and other degenerative diseases.

D. Results

1. dZIP7 promotes border cell migration and prevents ER stress

Drosophila ovaries are composed of ovarioles, which are strings of egg chambers (Figure 1A) progressing through 14 stages of development, culminating with mature eggs. Each egg chamber is composed of 15 nurse cells and one oocyte (germ cells), surrounded by ~850 epithelial follicle cells. At stage 9 (Figure 1B), 4-8 border cells round up at the anterior end of the egg chamber, delaminate from the follicular epithelium, and migrate posteriorly, reaching the anterior border of the oocyte by

stage 10. Border cell clusters are composed of 4-6 migratory cells that surround and carry two non-migratory polar cells. Expression of dZIP7 RNAi in the outer, migratory border cells using *fruitlessGal4* [65] inhibited migration (Figures 1C and 1G). The defect was rescued by co-expression of UAS-dZIP7::V5 (Figures 1D and 1G). Reduction of dZIP::GFP confirmed the effectiveness of the RNAi (Figures 1E, E' and 1F, F'). Border cell migration was also impaired when dZIP7 RNAi was driven by the *c306Gal*4 (Figure 1G), which is expressed in both polar and migratory cells. FruitlessGal4-driven RNAi impaired border cell migration at least as much as c306Gal4, indicating that dZIP7 was primarily required in the outer, migratory cells (Figure 1G). This was further supported by mosaic clone analysis, in which the severity of border cell migration defects were proportional to the number of outer, migratory cells that were homozygous mutant (Figure S1A-C) and mutant cells were mostly excluded from leading positions (Figures S1D-E).

dZIP7 is the ortholog of ZIP7, a Zn^{2+} transporter that moves Zn^{2+} from the ER to the cytoplasm and suppresses ER stress in many cell types including mammalian intestinal stem cells [10,42], cancer cells [10], Drosophila imaginal discs [49] and even in plants [20]. We confirmed that dZIP7 localizes to the ER in border cells where it co-localized with the ER chaperone PDI much more significantly than with F-actin or the nucleus (Figure S2). Additionally, homozygous mutant dZIP7 border cells expressed the ER stress reporter Xbp1s::EGFP [66] whereas $dZIP7^{+/-}$ and $dZIP7^{+/+}$ cells did not (Figures 1H-H"). We also observed increased expression of the ER chaperone PDI in homozygous dZIP7 RNAi-expressing follicle cell clones (Figures 1I-I"), a phenotype that we rescued with a wild type, V5-tagged dZIP7 transgene (Figures

1J-J"). Accumulation of XBP1 and PDI are indicative of induction of an adaptive unfolded protein response (UPR). [67] We conclude that cells lacking dZIP7 experience ER stress and impaired migration, raising the question as to whether ER stress, per se, inhibits motility.

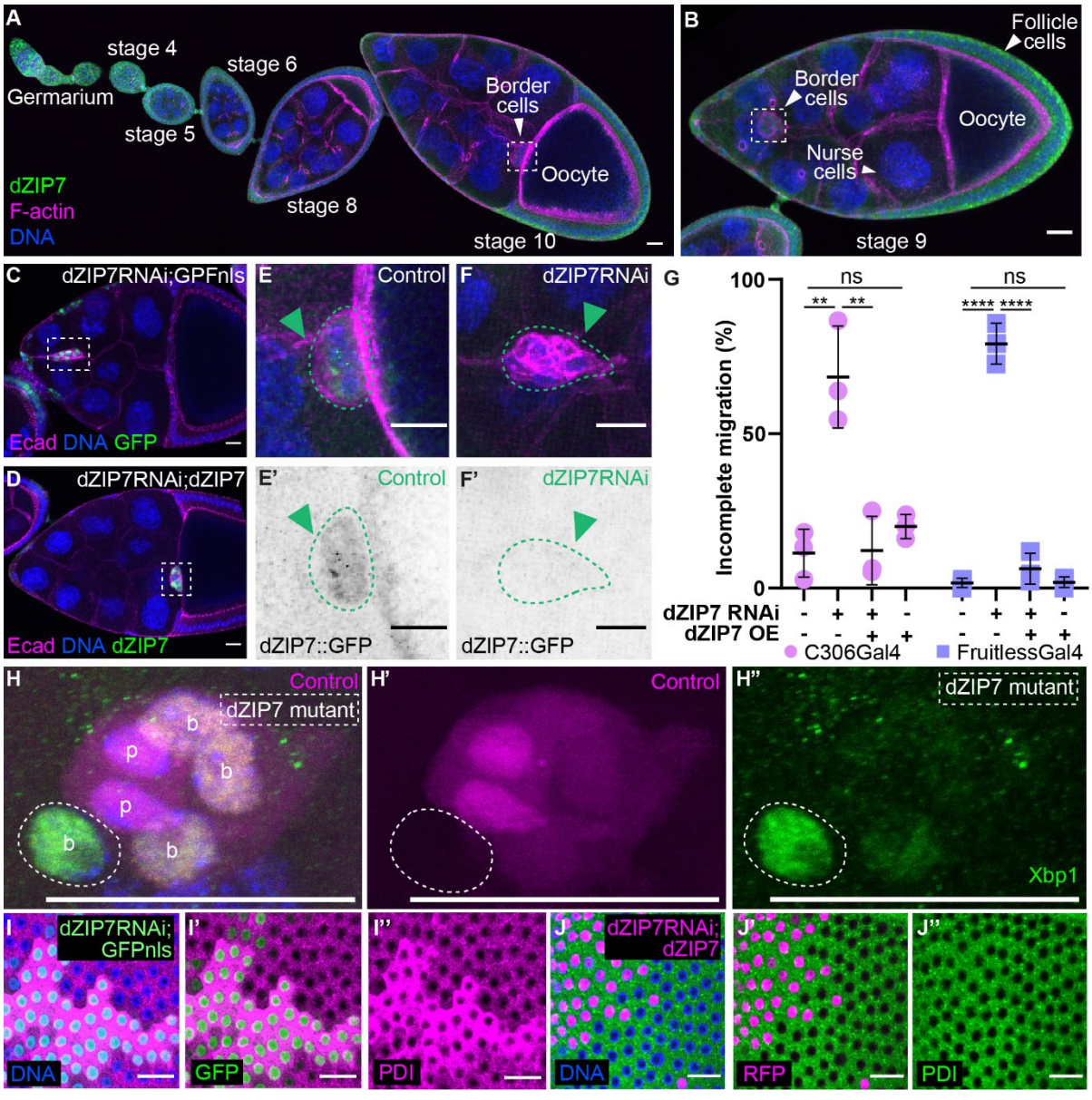


Figure 1: dZIP7 knockdown causes border cell migration defects and ER stress. (A, B) Developing Drosophila egg chambers expressing dZIP7::GFP. DNA is in blue. F-actin is in magenta. Border cells migrate during stage 9. (B) and complete

migration by stage 10 (A). (C, D) Stage 10 egg chambers with fruitlessGal4 driving expression of UAS-dZiP7RNAi and (C) UAS-GFPnls or (D) UAS-dZIP7::V5 in outer, migratory border cells. (E-F') dZIP7::GFP expression (black/gray) in control border cells (E, E') or c306Gal4>dZIP7RNAi (F, F'). E' and F' are the same clusters as E and F but shown in inverted grayscale. (G) Quantification of stage 10 migration defects in c306Gal4 (magenta) and fruitlessGal4 (purple) driving the indicated transgenes. Each dot represents the average of >24 egg chambers (n=3 independent experiments). Error bars=SEM. (H-H'') A mosaic border cell cluster composed of some control cells (RFP+, which can be dZIP7+/+ or dZIP7-/+, magenta, H') and one homozygous dZIP7 mutant cell (RFP-, outlined). Polar cells (p) express higher levels of RFP compared to outer border cells. Xbp1::EGFP (green, H") is a marker for ER stress. DNA is in blue. (I-I'') Anti-PDI antibody staining (magenta) reveals that clones of cells expressing dZIP7RNAi and GFPnls exhibit ER expansion. (J-J'') Mosaic clones expressing dZIP7RNAi and dZIP7::V5 and RFP (magenta) show similar PDI staining as neighboring wild type cells. Scale bars=20μm.

2. dZIP7 is limiting for ERAD

To address whether ER stress inhibits migration, we expressed a misfolded rhodopsin protein, $Rh1^{G69D}$, known to induce ER stress. [68,69] Misfolded rhodopsin accumulates in the ER and causes retinal degeneration in flies and humans. [69–72] We found that $Rh1^{G69D}$ accumulated to high levels intracellularly in border cells, induced ER stress, and blocked their migration (Figures 2A-2E). Similarly, RNAi against the protein chaperone HSC70-3, which is known to cause ER stress, inhibited border cell migration in 92% of egg chambers (n=3 independent experiments). Thus, ER stress caused by unrelated mechanisms - either loss of dZIP7, expression of a misfolded protein, or inhibition of a chaperone – correlates with impaired motility.

Since loss of dZIP7 caused ER stress, we wondered if dZIP7 overexpression might suppress ER stress, so we co-expressed $Rh1^{G69D}$ and dZIP7::V5 in border cells. Interestingly, dZIP7::V5 expression restored normal border cell migration (Figures 2B and 2C), reduced ER stress (Figures 2B and 2D) and virtually

eliminated Rh1^{G69D} protein (Figures 2B and 2E). Together, these results suggest that dZIP7 is a limiting factor for degrading misfolded Rh1, which is known to be degraded by ERAD [23-25], though a role for ZIP7 in ERAD has not previously been described.

If ZIP7 enhances ERAD, the proteasome inhibitor MG132 should block the ability of dZIP7 overexpression to promote Rh1^{G69D} degradation. In the absence of MG132, ZIP7 overexpression virtually eliminated Rh1^{G69D} protein (Figures 2B and 2E). In contrast, MG132 largely prevented the ability of ZIP7 to promote Rh1^{G69D} degradation (Figures 2F-2H). MG132 also prevented dZIP7-mediated rescue of the Rh1^{G69D} border cell migration defect (Figure 2G compared to Figure 2B). If ZIP7 normally promotes migration by promoting ERAD, then inhibiting ERAD should block migration. So we inhibited expression of the ERAD component known as Ubiquitin fusion-degradation 1-like (Ufd1), which is known to be required for degradation of Rh1^{G69D}. Ufd RNAi also inhibited border cell migration (Figure 2I). We conclude that ZIP7 likely promotes ERAD.

Misfolded rhodopsin is ubiquitinated by ER membrane-localized ubiquitin ligases (HRD1 and SORDD1/2), extracted from the ER membrane via the p97 chaperone TER94, and degraded by the proteasome (Figure 3A). [35,37] Our data described above implicate ZIP7 and cytosolic Zn^{2+} as limiting for this process. To investigate which step of the ERAD process requires dZIP7, we used an antibody that recognizes ubiquitinated proteins to compare wild type, dZIP7-overexpressing, and dZIP7 RNAi cells. One hypothesis was that dZIP7 might provide Zn^{2+} to ubiquitin ligases such as Hrd1 and SORDD1/2, which are Zn^{2+}-binding proteins that

reside in the ER membrane (Figure 3A). If this were true, we might expect dZIP7 overexpression to increase - and dZIP7 RNAi to decrease - the abundance of polyubiquitinated proteins (PUBs). However, we observed the opposite effect. dZIP7 RNAi increased polyubiquitinated protein abundance (Figures 3B, 3B') compared to the lacZ control (Figures 3C, #C' and S3A-S3B'). Conversely, dZIP7 overexpression essentially eliminated detectable polyubiquitinated proteins (Figures 3D and 3E). We also found that $Rh1^{G69D}$ expression increased the accumulation of PUBs compared to the control (Figures S3A-B' and D), and ZIP7 overexpression suppressed the effect (Figures S3C, S3C', and S3D). Since PUBs accumulated in the absence of dZIP7, we conclude that ZIP7 functions downstream of ubiquitination to enhance ERAD.

dZIP7 reduced the abundance of polyubiquitinated proteins suggesting that it might be required instead for deubiquitination. There are many deubiquitinating enzymes (DUBs), but Rpn11 stood out as a top candidate. Whereas deubiquitination by some DUBs can rescue proteins from degradation, deubiquitination by Rpn11 is an essential prerequisite for entry of client proteins into the proteasome core and thus, like dZIP7, is essential for misfolded protein degradation (Figure 3A). [73] Furthermore, unlike most DUBs, Rpn11 requires Zn^{2+} for catalysis [74–76]. In fact, Rpn11 is the only Zn^{2+}-requiring DUB implicated in ERAD. [77] *In vitro*, when ubiquitinated substrates and the 26S proteasome assemble into complexes, chelating Zn^{2+} inactivates Rpn11. Addition of Zn^{2+} is sufficient to reactivate Rpn11 and stimulate deubiquitination [74]. Since dZIP7 transports Zn^{2+} to the cytosol [10] and

promotes loss of ubiquitinated proteins and degradation of $Rh1^{G69D}$, we hypothesized that dZIP7 might be limiting for Rpn11 activity.

In vitro, when Rpn11 is activated by addition of Zn^{2+} but the 20S core proteases are blocked, deubiquitinated substrates stall and accumulate. [74] So we tested the effect of ZIP7 overexpression in the presence of MG132. We observed that ZIP7 still stimulated deubiquitination (Figures 3F-3H). Together with the observation that ZIP7 overexpression failed to degrade $Rh1^{G69D}$ in the presence of MG132 (Figures 2F-2H), this result supports the idea that ZIP7 enhances deubiquitination of misfolded proteins upstream of 20S proteasomal degradation, possibly by providing Zn^{2+} to Rpn11.

If ZIP7 promotes Rpn11-mediated deubiquitination, an Rpn11 inhibitor should block the ability of dZIP7 to enhance deubiquitination of misfolded proteins. To test this prediction, we used the potent and selective Rpn11 inhibitor capzimin, which blocks the Zn^{2+} within the Rpn11 catalytic site. [78] Capzimin largely prevented the effects of ZIP7 on deubiquitination (Figures 3I-3K'). Rpn11 RNAi also caused a severe border cell migration defect (Figure S3E). We conclude that dZIP7 enhances the obligatory Zn^{2+}- and Rpn11-dependent deubiquitination of misfolded proteins prior to proteasomal degradation.

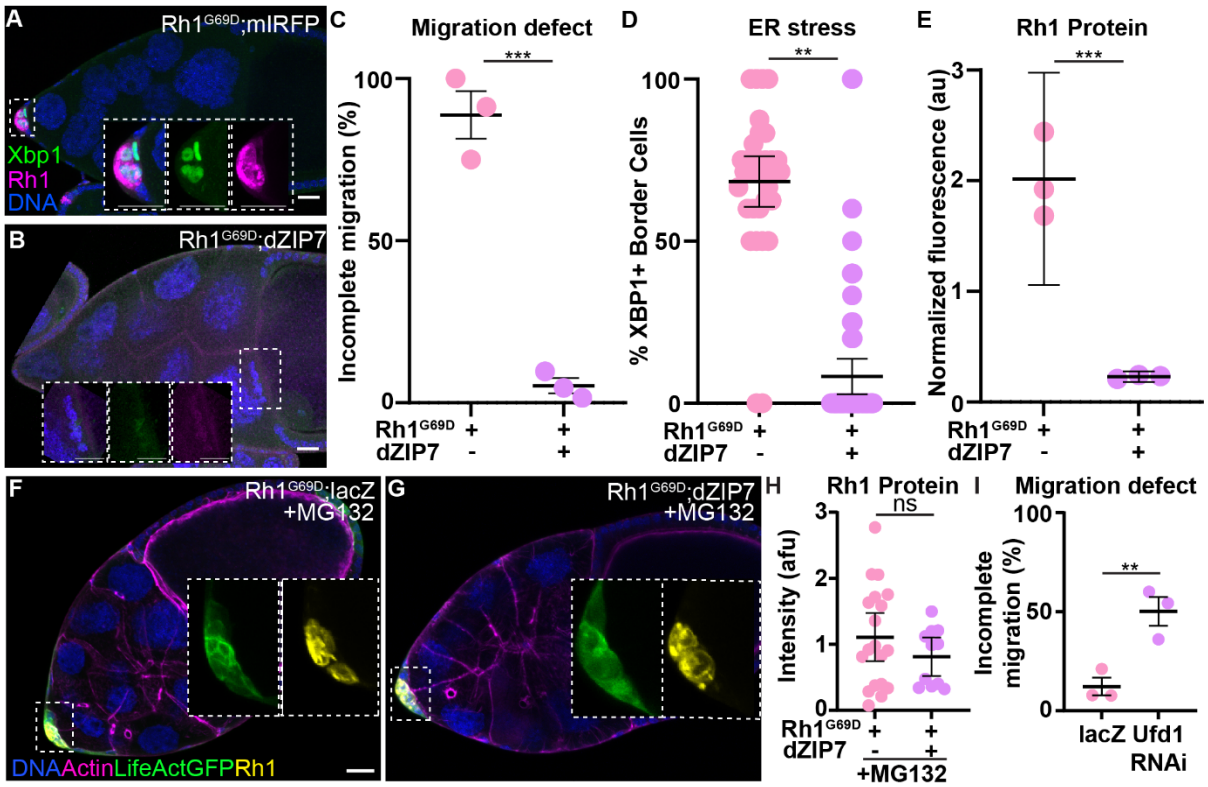


Figure 2: dZIP7 enhances proteasomal degradation of Rh1^{G69D}. (A) Stage 10 egg chamber expressing Rh1^{G69D} (magenta) and the ER stress sensor Xbp1::EGFP (green) in border cells using c306Gal4. Rh1^{G69D} accumulation induced ER stress and blocked migration. (B-D) Co-expressing dZip7 rescued migration (B-C) and reduced Xbp1::EGFP (B-D) and Rh1^{G69D} (B-E). (C) Each dot represents one experiment (n>17 clusters). Error bars=SEM. (D) Each dot represents an individual border cell. Error bars=95% confidence intervals. (E) Each dot represents one experiment (n>17 clusters). Error bars=95% confidence intervals. (F-H) Stage 10 egg chambers expressing misfolded Rh1^{G69D} in border cells (insets) treated with 10μM of the MG132 proteasome inhibitor for 5 hours and stained with an antibody against Rh1. dZip7 co-expression with Rh1^{G69D} did not reduce Rh1 protein levels in the presence of MG132 showing that the dZIP7-mediated degradation of Rh1^{G69D} is mediated by the proteasome and that dZIP7 functions upstream of the 20S core enzyme blocked by MG132. (I) Knocking down the ERAD component Ufd1 in border cells causes migration defects. Each dot represents one experiment (n>30 clusters). Error bars=95% confidence intervals. *P≤0.05, ** P≤0.01, *** P≤0.001. Scale bars=20μm.

3. ZIP7-mediated Zn2+ transport is limiting for ERAD in Drosophila

ZIP7 resides in the ER membrane and transports Zn^{2+} from the ER to the cytosol [10,21]. To test whether the Zn^{2+} transporter activity of dZIP7 is important for border cell

migration, we introduced point mutations, H315A and H344A, which replace histidine residues that are required for Zn^{2+} transport [79] (Figure 4A, purple) and are conserved between ZIP7 and a more distant family member from *Arabidopsis* IRT1 (Figure S4A). As controls, we engineered dZIP7^{H187A} and dZIP7^{H183A} mutants (Figure 4A, green), which are not located in the ZIP7 core transmembrane domains involved in Zn^{2+} transport and are predicted not to affect transport. [79] We generated transgenic flies expressing the mutants under Gal4/UAS control and included a V5 tag so that we could monitor protein abundance and localization. We then co-expressed each of these RNAi-resistant transgenes with dZIP7 RNAi and evaluated protein expression and border cell migration. All the proteins were stably expressed and correctly localized to the ER (Figures 4B and 4C and S4H and S4I). The point mutations predicted not to disrupt Zn^{2+} transport, dZIP7^{H187A} and dZIP7^{H183A}, rescued border cell migration to nearly wild type levels whereas neither dZIP7^{H344A} nor dZIP7^{H315A} provided significant rescue (Figures 4B and 4C and S4H and S4I), as quantified in Figure 4D.

In ZIP7 knockdown cells, membrane proteins, such as Notch and EGFR, accumulate abnormally in the ER [9]. We also observed this in clones of border cells expressing dZIP7 RNAi (Figures S5A and S5B'). In Drosophila follicle cells, as in imaginal disc cells, the effect is specific to some but not all membrane proteins. For example, E-cadherin does not accumulate abnormally in dZIP7 RNAi-expressing cells (Figures S5C and S5C' and [9]).

To test whether Zn^{2+} transport is essential to prevent this accumulation, we assessed Notch and EGFR staining in clones of ZIP7 RNAi cells co-expressing the

mutant forms of ZIP7, compared to neighboring wild type cells within the same cluster as an internal control (Figures 4E-4J and S4J-S4M'). the Zn^{2+}-transport-proficient proteins ($dZIP7^{H183A}$ and $dZIP7^{H187A}$) exhibited similar levels of Notch and EGFR as wildtype cells in the same cluster (Figures 4E-F', 4I and 4J, and S4J-S4K'). By contrast, the Zn^{2+}-transport-deficient proteins ($dZIP7^{H344A}$ and $dZIP7^{H315A}$) failed to rescue and accumulated more Notch and EGFR than wild type cells of the same cluster (Figures 4G-J and S4L-M'). From these experiments, we conclude that Zn^{2+} transport is an essential function of dZIP7 in promoting ERAD.

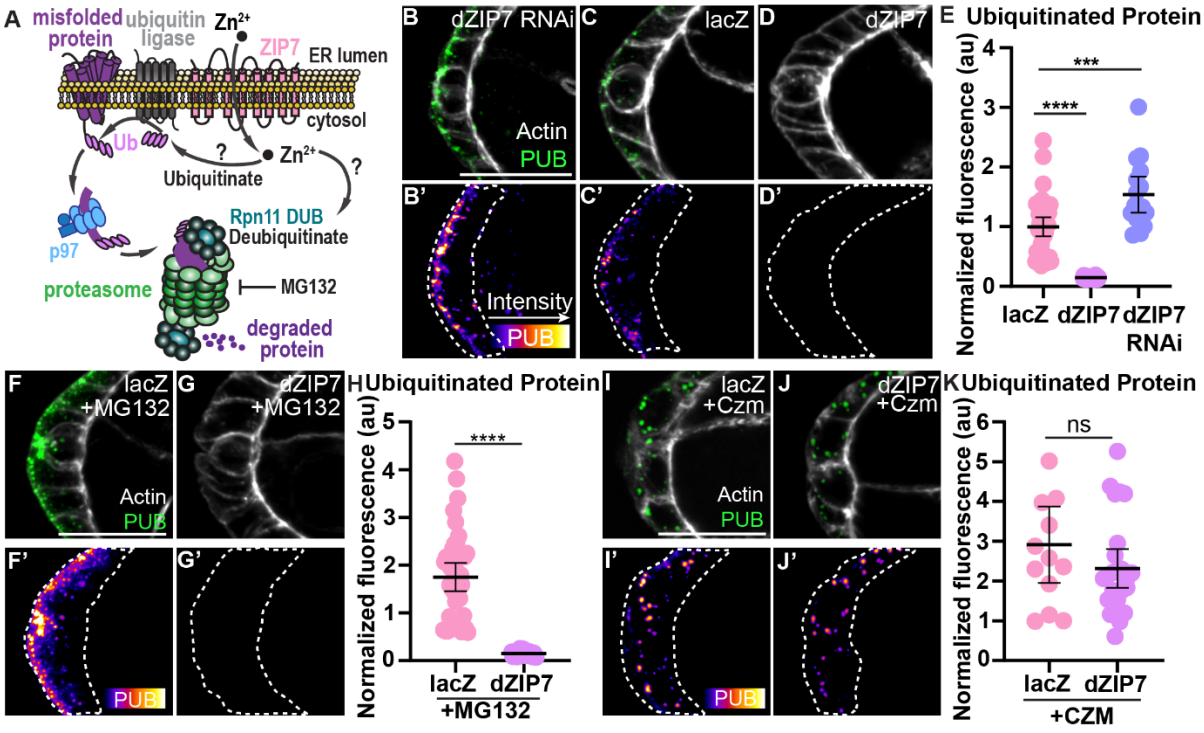


Figure 3: dZIP7 enhances ERAD and promotes Rpn11-mediated deubiquitination of proteins required for proteasome entry. (A) Schematic of proteasomal processing of misfolded proteins. (B-D') Representative images of stage 8 egg chambers stained with an antibody against ubiquitinated proteins (PUB, green in B-D, FIRE LUT in B'-D'). (E) Quantification of fluorescence intensity of PUB staining in control (lacZ), dZIP7 overexpression and dZIP7RNAi expressing border cells. (F-G') dZip7 overexpression reduced ubiquitinated protein levels in egg chambers treated with MG132 (10μM, 5 hours). Relative fluorescence intensity is

quantified in (H). (I-J') dZip7 overexpression does not prevent ubiquitinated protein buildup in egg chambers treated with Rpn11 inhibitor Capzimin (Czm, 20μM, 5 hours); this effect is quantified in (K). Dots represent individual border cell clusters. Error bars=95% confidence intervals. *** P≤0.001, **** P≤0.0001. Scale bars=20μm.

4. Zn2+ and ZIP7 are limiting for deubiquitination of proteasome client proteins in human cells

ZIP7 is nearly ubiquitously expressed in cells from organisms as diverse as plants and animals [20]. Our results, combined with the observations that the proteasome is highly abundant [73] whereas cytosolic $[Zn^{2+}]_{free}$ is extremely low (~100pM - 1 nM) [10], suggested that Zn^{2+} might be rate limiting for proteasome activity, particularly when cells are stressed. So, we tested the ability of a Zn^{2+} ionophore, pyrithione (an organic salt of zinc capable of permeating cell membranes) [10] to enhance deubiquitination of proteins in human cells.

This ionophore has previously been shown to rescue ER stress in ZIP7-deficient HeLa cells [10], indicating that an important function of ZIP7 is to increase cytosolic Zn^{2+} (rather than decreasing it in the ER). So, we incubated HeLa cells with MG132 to induce ER stress and increase the abundance of polyubiquitinated proteins (Figures 4K and 4L). We then tested the effect of the Zn^{2+} ionophore. The added Zn^{2+} reduced the accumulation of polyubiquitinated proteins in the MG132-treated cells (Figures 4K and 4L). We conclude that cytosolic Zn^{2+} is limiting for proteasomal degradation of misfolded proteins in cells with ER stress.

To test whether overexpression of human ZIP7 could enhance protein deubiquitination in HeLa cells as it does in border cells, we generated HeLa cells expressing doxycycline-inducible ZIP7 (Figure S4N). Inhibition of the proteasome

with MG132 increased the abundance of ubiquitinated proteins and dox-induced ZIP7 overexpression significantly reduced that effect (Figures 4M and 4N). We conclude that in Drosophila and human cells, ZIP7 provides limiting Zn^{2+} for deubiquitination of proteasome client proteins.

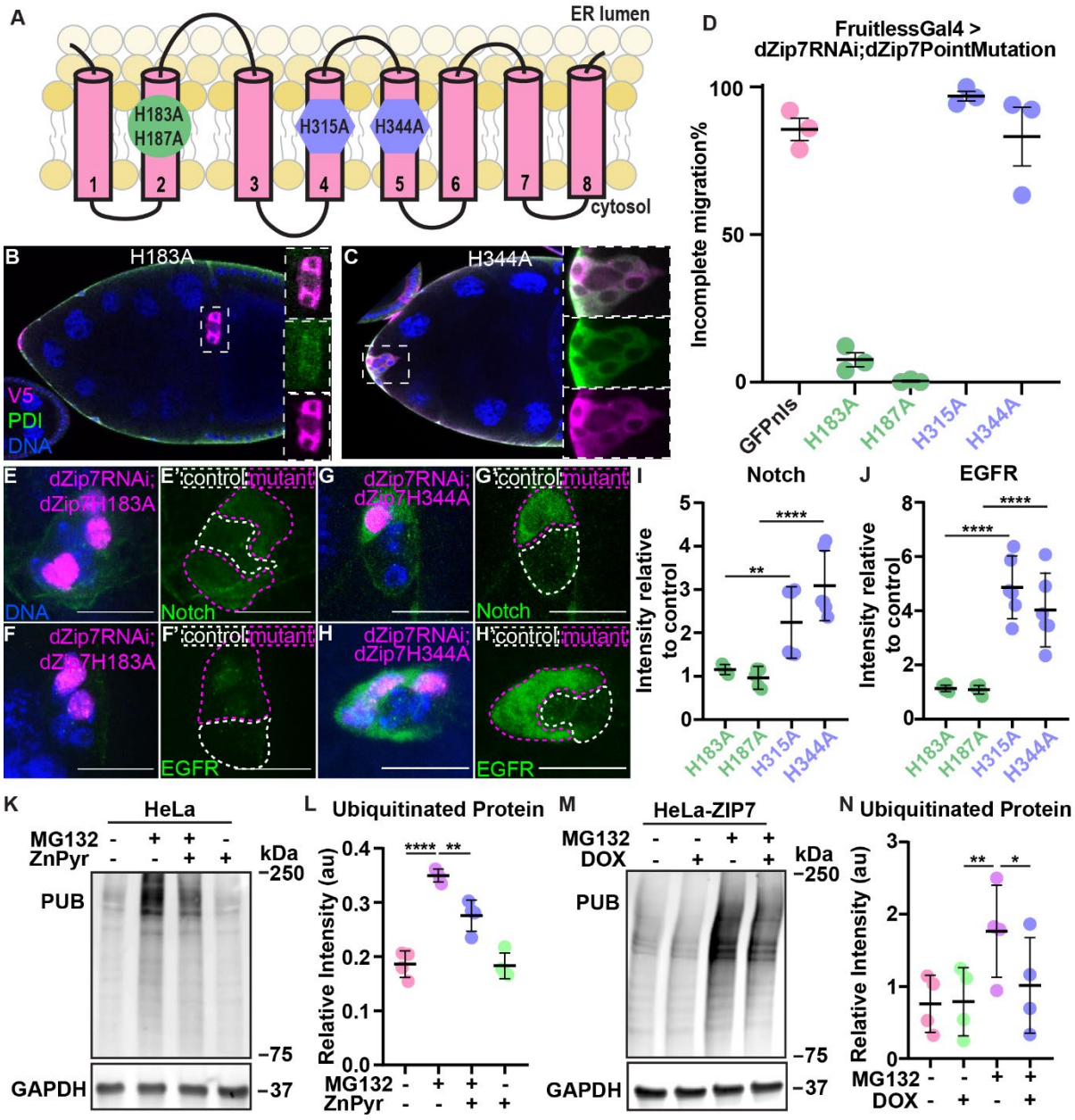


Figure 4: Cytosolic Zn^{2+} is limiting for ERAD. (A) Schematic of transmembrane domains and topology of dZIP7. Point mutations H183A and H187A res within the

second transmembrane domain while H315A and H344A are within the highly conserved HELP domain and CHEXPHEXGD motif on the fourth and fifth transmembrane domains required for Zn2+ transport. (B-C) Co-localization of V5-tagged, RNAi-resistant dZIP7 mutants with the ER marker PDI (green) in border cells. (D) Quantification of incomplete migration at stage 10 in egg chambers expressing dZIP7RNAi with the indicated mutant forms. N=3 independent experiments. Error bars=SEM. (E-H') Mosaic expression of dZIP7RNAi together with the indicated mutant forms of dZIP7 marked by RFPnls (magenta) and stained for Notch or EGFR (green). Scale bars=20 μm. Quantification of the fold change of Notch (I) and EGFR (J) expression in dZIP7 mutants compared to control cells.(K) Representative western blot on HeLa cell protein extract probed for polyubiquitinated protein and GAPDH. Cells were treated with/without proteasome inhibitor MG132 and zinc pyrithione (ZnPyr), a zinc ionophore. Treatments from left to right: DMSO, 500nM MG132, 500nM MG132 + 1μM ZnPyr, 500nM ZnPyr. (L) Adding ZnPyr to MG132-treated cells reduces polyubiquitinated protein levels. N= 4 experiments. (M) Western blot on HeLa cells with DOX-inducible ZIP7 that were treated with/without doxycycline and with/without MG132 and probed for polyubiquitinated protein and GAPDH. (N). ZIP7 overexpression reduces polyubiquitinated protein in HeLa cells treated with MG132. N= 4 experiments. Error bars= standard deviation. *P≤0.05, **P≤0.01, ****P≤0.0001.

5. ZIP7 is required to degrade misfolded Notch and EGFR whereas ER stress independently inhibits Notch transcriptional responses

Notch and EGFR accumulate abnormally in the ER in ZIP7-deficient fly wing disc cells [49] and human cancer cells. [80] We also observed abnormal accumulation of Notch (Figures 5A, 5A' and S5A, A') and EGFR (Figures 5B, B' and S5B, B') but not E-cadherin (Figures S5C and S5C') in dZIP7 knockdown follicle cell clones, supporting the generality of the phenomenon. Accumulation of Notch and EGFR following ZIP7 knockdown or inhibition in flies and mammalian cells has previously been attributed to a defect in protein trafficking [9,28]. However, our results implicate defective ERAD as the likely cause of abnormal intracellular Notch and EGFR in ZIP7 k.d. cells.

Interestingly, neither Notch (Figures 5C and 5C') nor EGFR (Figures 5D and 5D') accumulated abnormally in $Rh1^{G69D}$-expressing follicle cells, including border

cells. Yet, both ZIP7 knockdown cells (Figures 5E and 5 E') and Rh1^{G69D}-expressing cells (Figures 5G and 5G') exhibited reduced Notch transcriptional responses (Figures 5F and 5H). We conclude that dZIP7 knockdown causes two independent effects on Notch: accumulation of misfolded protein in the ER due to reduced ERAD and inhibition of Notch transcriptional activity, presumably as a consequence of the ER stress response. [81–83]

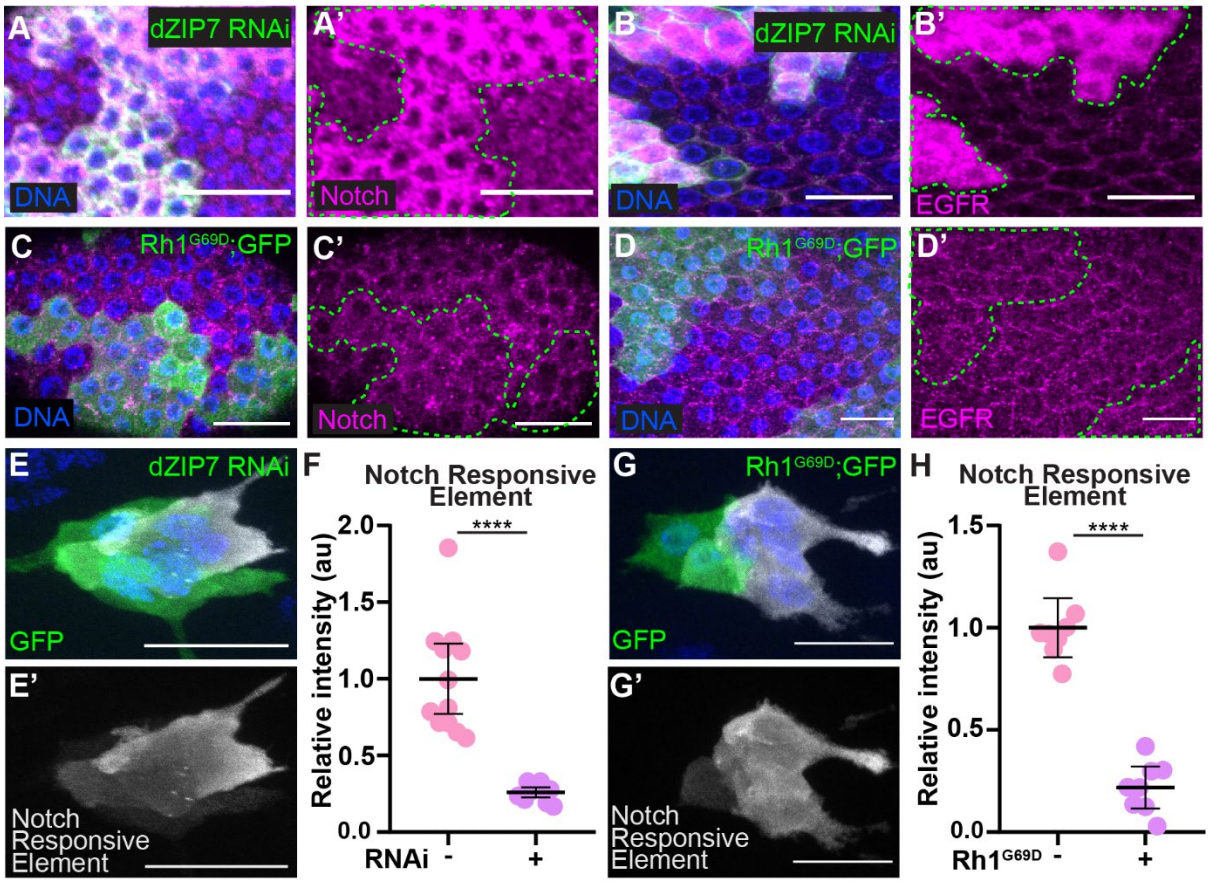


Figure 5: dZIP7 knockdown results in Notch & EGFR accumulation and reduced Notch transcriptional activity. (A-B') Intracellular Notch (A, A') and EGFR (B, B') accumulated in epithelial follicle cell clones expressing dZIP7RNAi (GFP+, green) relative to neighboring wild type cells. (C-D') Mosaic clones of follicle cells expressing Rh1G69D and GFP. Rh1G69D expression does not cause accumulation of Notch (C, C') or EGFR (D, D') relative to wild type cells. (E-H) Notch transcriptional activity visualized with a Notch responsive element reporter (white). (E-F) Notch in dZIP7RNAi expressing cells (GFP+) compared to neighboring wild

type (GFP-) cells. (G-H) Notch in Rh1G69D- expressing cells (GFP+) compared to wild type cells (GFP-). **** P≤0.0001. Scale bars=20μm.

6. dZIP7 overexpression prevents retinal degeneration caused by Rh1G69D

The observations that dZIP7 overexpression is sufficient to degrade $Rh1^{G69D}$, reduce ER stress, and rescue border cell migration and the ubiquity of ZIP7 and proteasomes suggested that dZIP7 overexpression might also be effective at suppressing retinal degeneration due to folding-defective rhodopsin. To test this hypothesis, we co-expressed UAS-dZIP7::V5 with UAS-$Rh1^{G69D}$ in fly photoreceptor cells using GMR-Gal4. Eye morphology was normal in flies expressing dZIP7 alone (Figures 6A, 6B and 6G) whereas $Rh1^{G69D}$ causes severe disruption of eye morphology compared to controls [63,68–70,72] (Figures 6C, 6D and 6G). Co-expression of $Rh1^{G69D}$ and dZIP7 fully rescued eye morphology in the majority of flies examined (Figures 6E, 6F and 6G).

To determine whether the mechanism of dZIP7 overexpression was the same in photoreceptor cells as border cells, we stained eye discs from third instar larvae with an antibody that labels all neuronal nuclei (Elav) and an antibody against Rh1. Rhodopsin is normally not expressed in larval photoreceptor cells so any Rh1 detected $Rh1^{G69D}$ expressed from GMR-Gal4. Rh1 was abundantly expressed in the cytoplasm of photoreceptor cells in GMR-Gal4;UAS-$Rh1G^{69D}$;UAS-lacZ discs (Figures 6H and 6H'), whereas it was nearly undetectable in the majority of GMR-Gal4;UAS-$Rh1^{G69D}$;UAS-dZIP7 discs (Figures 6I-6J). We conclude that dZIP7 overexpression promotes the degradation of $Rh1^{G69D}$ in photoreceptors as it does in border cells, demonstrating the generality of the effect in disparate cell types.

To test whether dZIP7 overexpression could restore visual function, we carried out electroretinogram (ERG) recordings, which measures the summed responses of all retinal cells to light. Control flies display a corneal negative receptor potential upon turning on a light stimulus, which quickly decays to baseline upon termination of the light (Figure 6K). The large maintained component of the ERG results principally from activation of the phototransduction cascade. The on- and off-transient responses, which are nearly coincident with the initiation and cessation of the light stimulus (Figure 6K), depends on synaptic transmission from the photoreceptor cells to postsynaptic cells in the optic lobes. Expression of $Rh1^{G69D}$ in photoreceptor cells greatly diminished the amplitude of the ERG, and eliminated the on- and off-transients (Figures 6L and S6). Of note, overexpression of dZIP7 in $Rh1^{G69D}$ photoreceptor cells restored a normal ERG, including a full receptor potential and synaptic transmission, as evidenced by the on- and off-transients (Figures 6M and S6). As a control, we found that expression of dZIP7 alone in photoreceptor cells had no measurable adverse effects on the ERG (Figure 6N), as quantified in Figures 6O and S6. These data demonstrate that over-expression of dZIP7 prevents the deleterious impact of $Rh1^{G69D}$ on the response of retinal cells to light without side effects.

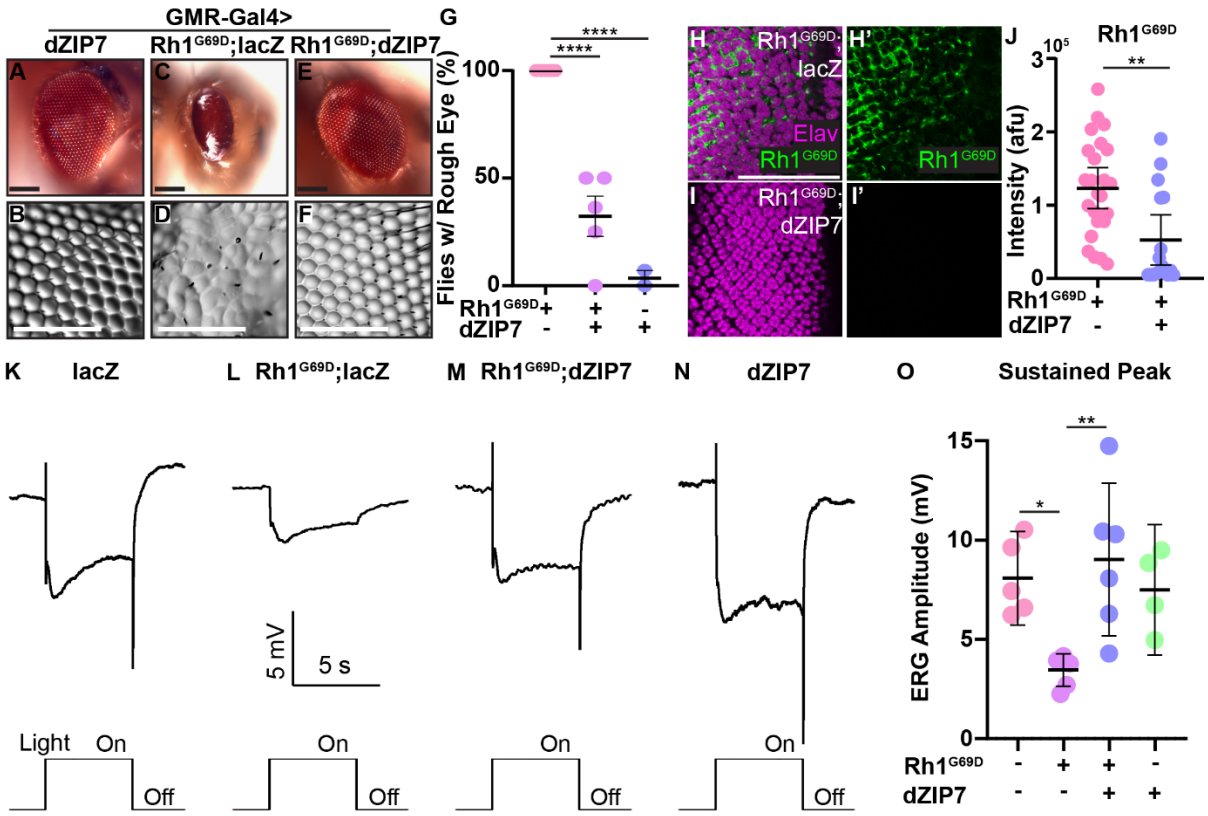


Figure 6: dZIP7 overexpression prevents Rh1^{G69D} retinal degeneration. (A, C, E) Representative light photomicrographs of retinal morphology. (B, D, F) DIC images of retinal imprint morphology. (G) Quantification of number of flies with rough eye. dZIP7 overexpression allows ~70% of flies to develop with normal eyes. Each dot represents an average of >10 flies observed in each experiment. (H-I') Larval eye discs expressing Rh1^{G69D} stained with antibodies against photoreceptors (Elav, magenta) and Rh1 (green). (J) dZIP7 expression significantly reduces Rh1^{G69D} protein accumulation. (K-N) Representative ERG recordings of one-week-old flies. (O) Quantification of ERG recordings. dZIP7 co-expression with Rh1^{G69D} returns ERG amplitude to control levels. Error bars represent 95% confidence intervals. *P≤0.05, ** P≤0.01, **** P≤0.0001.

E. Discussion

1. A model for dZIP7 function: Zn^{2+} transport from the ER to the cytosol is limiting for ERAD and mitigation of ER stress

dZIP7 is a conserved protein that goes by names including ZRT1 in yeast, IRT1 in plants, dZIP7 or *Catsup* in Drosophila, and SLC39a7/Zip7/Ke4 in mammals. While

many studies come to a common conclusion that loss or inhibition of ZIP7 disrupts ER homeostasis in cells from plants to flies and humans [10,20,42,43,80,84], the mechanism has been unclear. [20] Moreover it has been unclear whether the disparate phenotypes caused by ZIP7 knockdown, which include defects in intestinal self-renewal, B cell differentiation, cell motility and survival amongst others, have a common underlying cause or represent pleiotropic activities of the ZIP7 protein. The data presented here provide evidence for an unanticipated and possibly unifying mechanism. Our data support a model in which dZIP7 promotes ERAD and prevents ER stress by providing free Zn^{2+} to enhance the catalytic activity of the Rpn11 DUB in the proteasome lid (see graphical abstract). This role for dZIP7 is critical since free Zn^{2+} is present at exceedingly low intracellular levels [10], and therefore could be limiting. In the absence of ZIP7, misfolded/unfolded proteins accumulate and cause ER stress. Although Rpn11 is a top candidate for ZIP7-provided Zn^{2+}, our experiments cannot rule out that other Zn^{2+} binding proteins may contribute to the beneficial effects of ZIP7. For example, the chaperone p97 extracts misfolded and ubiquitinated proteins from the ER and transfers them to the 26S proteasome, and one subunit of p97, Npl4, is a Zn^{2+} finger protein. Our experiments do not rule out the possibility that ZIP7 promotes deubiquitination by enhancing p97, in addition to, or instead of Rpn11 activity.

It is striking that dZIP7 overexpression is sufficient to enhance proteasomal degradation of misfolded proteins, including $Rh1^{G69D}$, preventing the harmful effects of ER stress including blindness. In contrast to earlier work that suggested that ZIP7 primarily promotes trafficking of membrane proteins such as Notch and EGFR [49,80],

our results show that release of Zn^{2+} from the ER to the cytosol via ZIP7 is limiting for ERAD.

We favor the model that the step in ERAD that is most sensitive to Zn^{2+} is deubiquitination of client proteins by Rpn11 and/or p97-mediated transfer of misfolded proteins to the proteasome. [74,75] Our *in vivo* genetic and pharmacological studies are concordant with *in vitro* biochemistry. Rpn11 requires Zn^{2+} to deubiquitinate client proteins. [74–76,78] This is an essential step so that the client protein can enter into the 20S proteasome for degradation by trypsin, chymotrypsin, and caspase-like endoproteases. Rpn11 also enhances proteasomal degradation by allowing ubiquitin to be recycled. Worden et al [74] were able to assemble a complex *in vitro* composed of a ubiquitinated substrate and the 26S proteasome, including Rpn11. In the presence of a Zn^{2+} chelator, the complex assembles but Rpn11 is catalytically inactive, so ubiquitinated substrates accumulate. Upon addition of Zn^{2+}, Rpn11 deubiquitinates the client protein. In the presence of the 20S protease inhibitor epoxomicin, Rpn11 deubiquitinates the client but it is not degraded, so deubiquitinated protein accumulates.

We observe remarkably similar effects by manipulating dZIP7 *in vivo* as Worden et al observed by manipulating Zn^{2+} *in vitro*. [74] In the absence of dZIP7, ubiquitinated proteins accumulate, whereas upon overexpression of dZIP7 in the presence of MG132, deubiquitinated substrate proteins (e.g. $Rh1^{G69D}$) accumulate. It is reasonable to propose that cytosolic free Zn^{2+} could be rate-limiting because proteasomes are abundant, whereas cytosolic free Zn^{2+} is vanishingly rare at ~1 nM [10], which is ~100-fold less than the typical free cytosolic [Ca^{2+}]. We propose that

ZIP7 provides rate-limiting Zn^{2+} to p97 and/or Rpn11, and thus that the level of ZIP7 determines a cell's capacity to degrade misfolded proteins. In support of this idea and the generality of the mechanism proposed here, we found that increasing intracellular Zn^{2+} enhanced deubiquitination of proteins in a human cell line in the presence of MG132.

Why is ZIP7-mediated Zn^{2+} transport dispensable for ubiquitinating misfolded proteins even though the E3 ubiquitin ligases that catalyze that reaction are Zn^{2+}-binding proteins? In contrast to Rpn11, which can fold and assemble into proteasome/substrate complexes even in the presence of a Zn^{2+} chelator, Zn^{2+} is a structural element of the RING finger domains of the ubiquitin ligases. We suspect that the ligases would fail to fold in its absence and therefore likely have a high affinity for Zn^{2+}.

Despite the fact that Zn^{2+} is the second most abundant divalent cation in cells, free Zn^{2+} is exceptionally rare in the cytosol because nearly all Zn^{2+} is bound to proteins. While an essential trace element, excess cytosolic Zn^{2+} can be toxic [85], yet ZIP7 overexpression did not cause detectable harm either to follicle cells in the ovary or to photoreceptor cells in the eye. This suggests that the Zn^{2+} transported to the cytosol via ZIP7 might predominantly exert its effects locally near the ER. ZIP7 may not directly bind to the ERAD machinery though because it was not detected in an extensive proteomic analysis. [86] The human genome encodes 24 Zn^{2+} transporters, 14 of which belong to the ZIP family which move Zn^{2+} into the cytoplasm from outside the cell or from inside an organelle while 10 are members of the ZnT family which transport Zn^{2+} out of the cell or into organelles. [87,88] The large

sizes of these families are consistent with the idea that local Zn^{2+} sources may be important for promoting necessary Zn^{2+}-dependent processes without increasing global levels, which would be toxic.

2. Biomedical implications of the role of ZIP7 in ERAD

Our finding that dZIP7 overexpression alleviates ER stress and cellular death due to $Rh1^{G69D}$ expression has some general biomedical implications. Dominant mutations in rhodopsin that impair folding and cause accumulation in the ER cause retinal degeneration in human patients [71], for which there is no effective prevention or therapy. Over-expression of proteins that enhance ERAD is a promising therapeutic strategy. Additionally, toxic protein aggregates have been proposed to kill neurons by inhibiting ERAD in numerous neurodegenerative diseases including Huntington's, Altzheimer's, Parkinson's, frontotemporal dementia, and others, even when the toxic protein is not localized in the ER. [89,90] Thus, strategies to enhance ERAD may be useful in treating multiple degenerative diseases.

The suppression of ER stress and border cell migration by dZIP7 overexpression is consistent with the observation that ZIP7 is over-expressed in numerous cancers where it promotes survival, proliferation and migration and correlates with disease progression, invasion, and metastasis. [26,34,35,59] A ZIP7 inhibitor was identified in a screen for drugs to treat Notch-dependent cancers, based on the model that ZIP7 is important for Notch trafficking [80]. We show that ER stress impairs Notch transcriptional activity independent of any trafficking defect because $Rh1^{G69D}$ inhibits Notch signaling without abnormal Notch or EGFR protein

accumulation. Precisely how ER stress or the UPR inhibits Notch signaling is not yet clear, but the observation that a pharmacological inhibitor of ZIP7 was identified as a suppressor of Notch signaling by the Notch intracellular domain (NICD) in cultured U2OS osteosarcoma cells [80] suggests that there is a deeply conserved requirement for ZIP7 for Notch transcriptional activity. Nolin et al [80] showed that ZIP7 inhibition causes accumulation of full length Notch and a decrease in the NICD, and concluded that Notch activation by proteolysis was likely perturbed upon inhibition of ZIP7. An alternative interpretation is that full-length Notch accumulates in the ER lumen due to inhibition of ERAD, and that the NICD activity is inhibited by the global ER stress response.

Our results suggest that ZIP7 inhibitors might be effective against cancers that rely especially heavily on proteasomes. Proteasome inhibitors such as bortezomib are approved for the treatment of B cell malignancies including multiple myeloma and mantle cell lymphoma [91]. Our results suggest that ZIP7 inhibitors might be repurposed to treat those cancers as well, especially considering that resistance typically develops against a single therapeutic agent. Moreover, by inhibiting ERAD specifically rather than all proteasomes generally, ZIP7 inhibition may be less toxic than bortezomib. Interestingly, hypomorphic mutations in ZIP7 cause a B cell deficiency due to defects in B cell differentiation in human patients [92]. Although the mechanism underlying this phenotype is unknown, our results implicate ZIP7 in the UPR, and mutations that compromise the UPR also cause B cell deficiency due to defective B cell differentiation. So, the results presented here suggest a possible link between these otherwise disparate observations. B cell

development appears to depend upon a functional UPR and ER stress response, perhaps to ensure resilience to the natural ER stress B cells experience when they secrete large quantities of antibody.

Finally, the similarities in dZIP7 functions and phenotypes across disparate cells, tissues, and organisms suggests that the border cell system offers an excellent model for deciphering the fundamental and conserved effects of this protein *in vivo.*

3. Limitations of the Study

The data do not currently distinguish whether Zn^{2+} is limiting only for Rpn11 activity or also for Npl4, which is a zinc finger domain protein and subunit of the p97 chaperone complex. The data also do not currently exclude the possibility that ZIP7 promotes border cell migration through a mechanism other than promoting ERAD, such as transit through the ER or protein folding within the ER, though the data are consistent with the model that a primary and general function of ZIP7 is to stimulate ERAD by providing Zn^{2+} to ER-associated proteasomes. Further work will be required to determine if the ZIP7-mediated Zn^{2+} ions remain in the local vicinity of the ER, or diffuse to activate proteasomes throughout the cell. Whether ZIP7 overexpression can prevent retinitis pigmentosa in mammals including humans remains to be tested. Whether ZIP7 overexpression can prevent neurodegenerative diseases other than retinitis pigmentosa also remains to be investigated.

F. SUPPLEMENTARY FIGURES

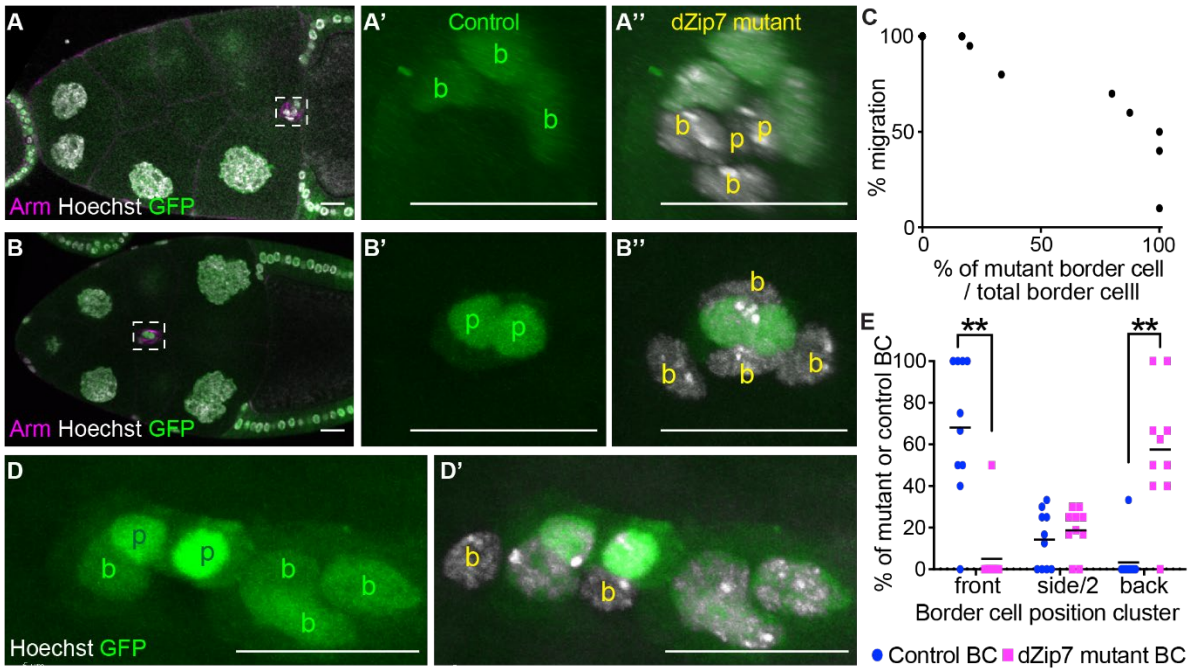


Figure S1, related to Figure 1: dZIP7 functions in migratory border cells. (A-A") An egg chamber with homozygous dZip7 mutant cells (GFP-). Both polar cells (p) and two border cells (b) are mutant. (B-B") An egg chamber in which all outer border cells are GFP-/- (homozygous dZip7 mutant). (C) Migration distance expressed as a percentage of the migration path for mosaic border cell clusters as a function of the proportion of homozygous mutant cells in each cluster. (D) High magnification view showing the spatial distribution of dZip7+ (GFP+) and dZip7-/- (GFP-/-) cells in a migrating cluster. (E) Quantification of the percentage of dZip7+ vs dZip7-/- border cells in the front, side, or back of the border cell cluster showing that dZip7-/- cells are more likely to occupy a rear position. "p" indicated polar cells, "b" indicated border cells, green labels control cells, yellow labels mutant cells. ** P≤0.01, *** P≤0.001, **** P≤0.0001. Scale bars=20 μm.

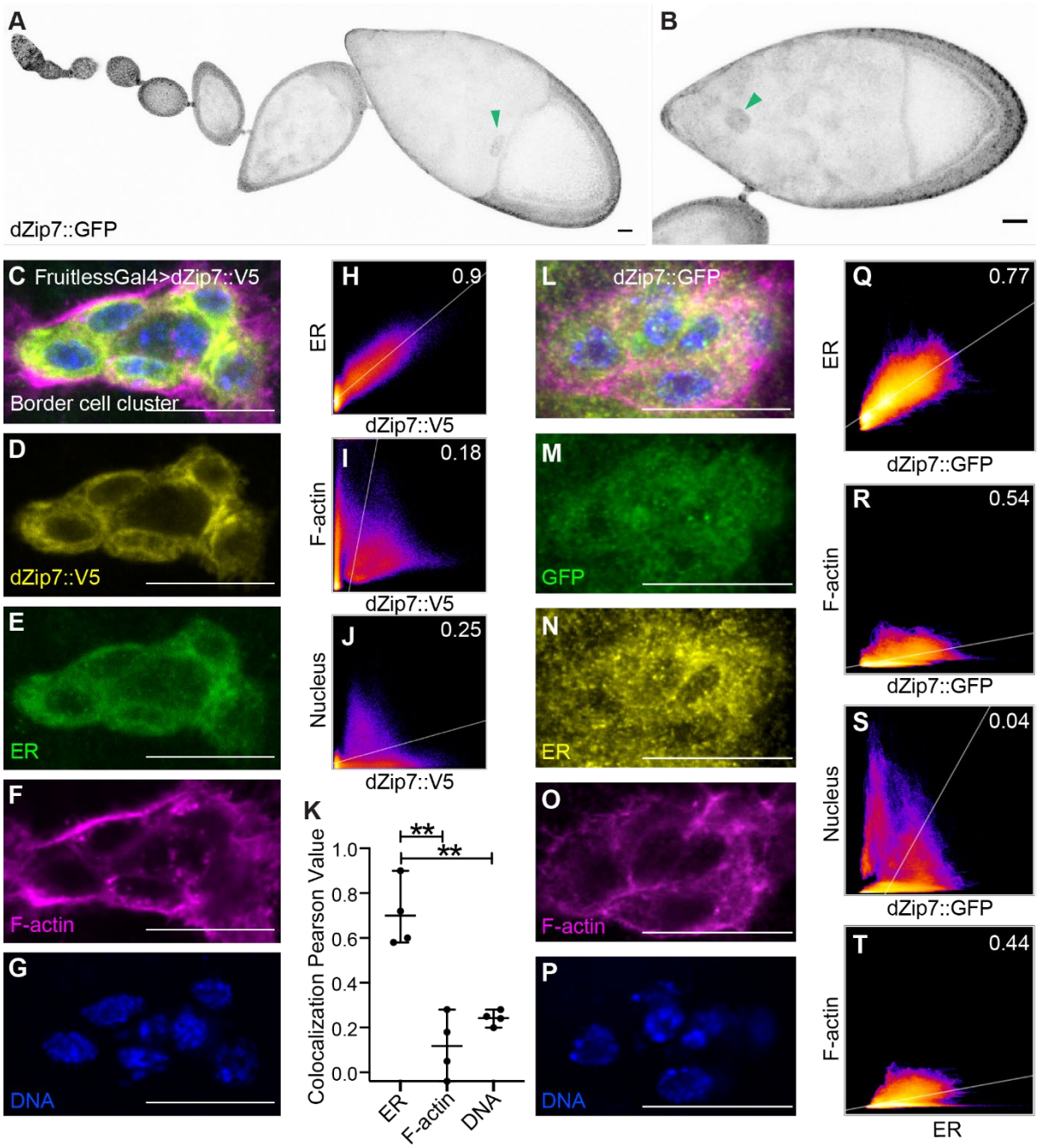


Figure S2, related to Figure 1: dZIP7 localizes to the ER. (A, B) dZip7::GFP grayscale single channel corresponding to Figure 1A. (C-G) High magnification of a border cell cluster showing the localization of overexpressed dZip7::V5 (yellow), anti-PDI staining for ER (green), phalloidin (magenta), and Hoechst (blue). (H-J) 2-dimensional intensity histograms showing localization of dZip7::V5 relative to ER, F-actin, and DNA. The colocalization regression Pearson's coefficient is displayed in the upper right corner. (K) Comparison of Pearson's coefficients (average of 4 border cell clusters). ** P value< 0.01. (L-P) High magnification of a border cell cluster

expressing dZip7::GFP under the control of endogenous regulatory sequences (green), ER (PDI, yellow), F-actin (phalloidin, magenta) and DNA (Hoechst, blue). (Q-T) 2-dimensional intensity histograms showing colocalization and Pearson's coefficient for dZip7::GFP relative to ER, F-actin, nuclei, as well as ER relative to F-actin. Scale bars=20 μm.

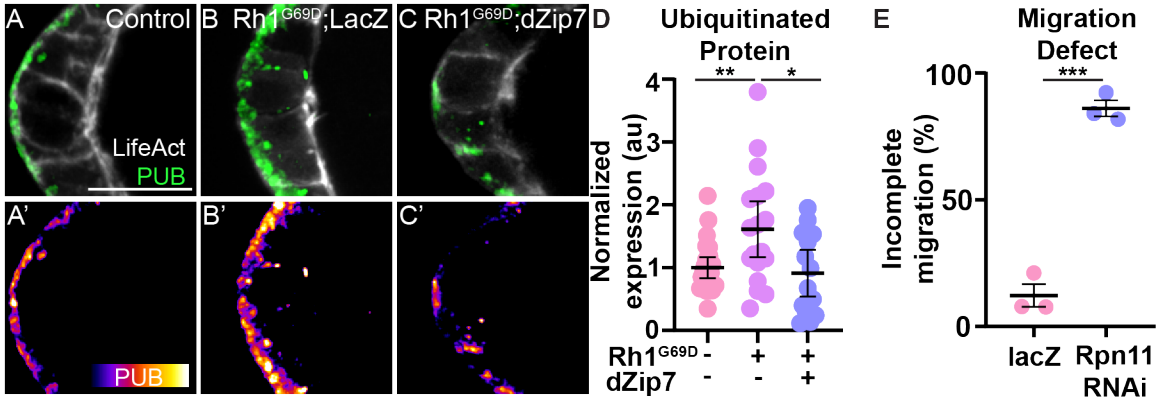


Figure S3, related to Figure 3: dZIP7 promotes deubiquitination of misfolded proteins including Rh1G69D. (A-C') Representative images of stage 8 egg chambers stained with an antibody against ubiquitinated proteins (PUB). Scale bars= 20μm. (D) Quantification of fluorescence intensity of ubiquitinated proteins. Expressing Rh1G69D causes buildup of ubiquitinated proteins compared to the control, but this effect is suppressed by co-expressing dZip7. Dots represent individual border cell clusters. (E) Rpn11 knockdown significantly impairs border cell migration. Each dot represents an experiment of n>15 border cell clusters). Error bars represent the 95% confidence intervals. *P≤0.05, ** P≤0.01.

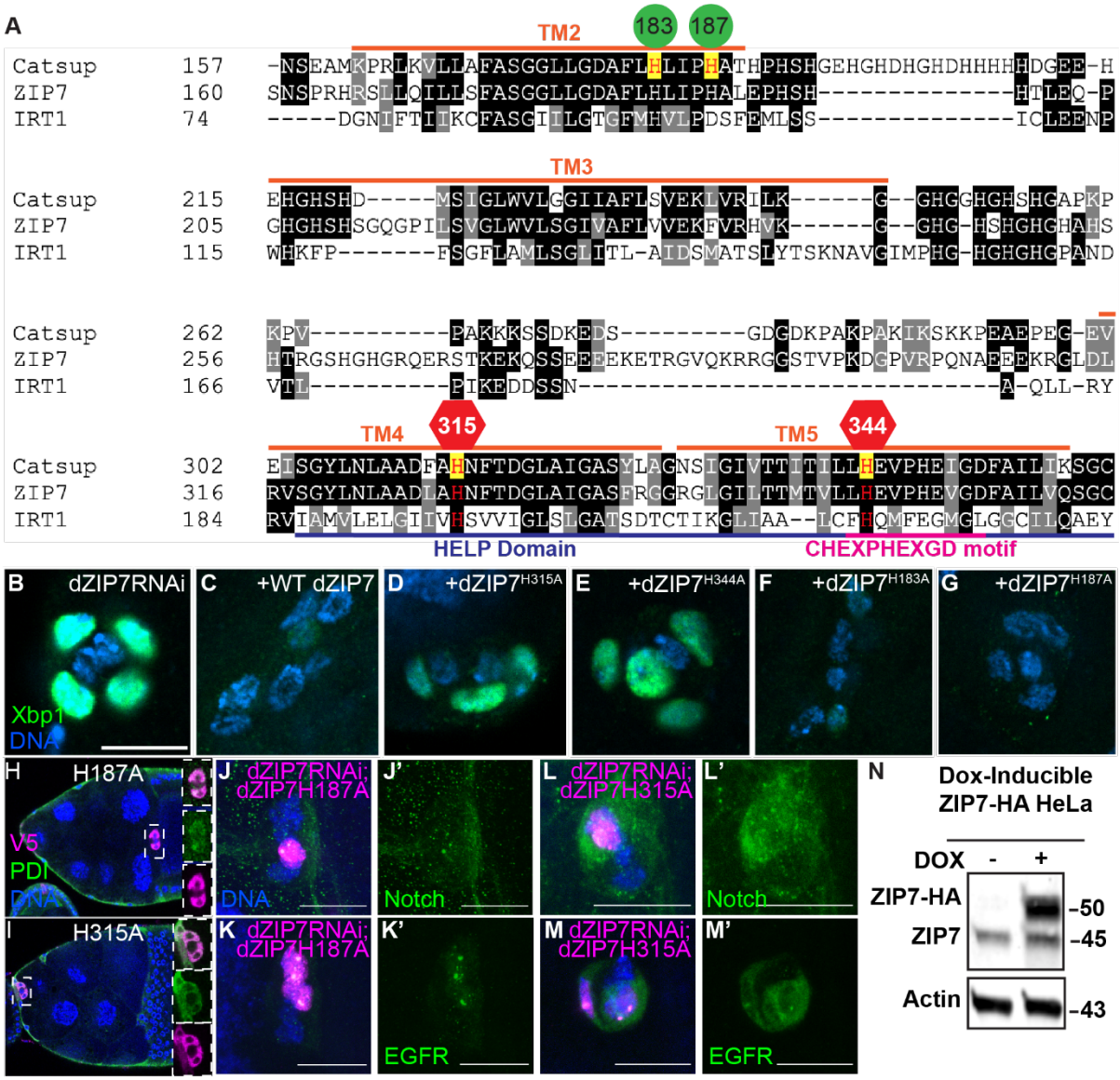


Figure S4, related to Figure 4: Zn2+ transport is required for ZIP7 function. (A) Histidine to alanine mutations predicted to disrupt Zn2+ transport (red) or not (green) indicated on the sequence alignment between plant IRT1, human ZIP7, and Drosophila dZIP7 (Catsup). (B-G) Images of border cell clusters with C306-Gal4 driving dZIP7 RNAi and Xbp1-GFP. (B) ZIP7 RNAi induces the ER stress sensor Xbp1 (green). (C) Wild type ZIP7 rescues ER stress. (D, E) Mutations predicted to disrupt Zn2+ transport block rescue. (F, G) Mutations predicted not to disrupt Zn2+ transport do not block rescue. (H-I) Co-localization of V5-tagged, RNAi-resistant dZIP7 mutants with the ER marker PDI (green) in border cells. (J-M') Mosaic expression of dZIP7RNAi together with the indicated mutant forms of dZIP7 marked by RFPnls (magenta) and stained for Notch or EGFR (green). (N) Western blot of HeLa cells expressing doxycycline-inducible HA-tagged ZIP7. Scale bar= 20μM

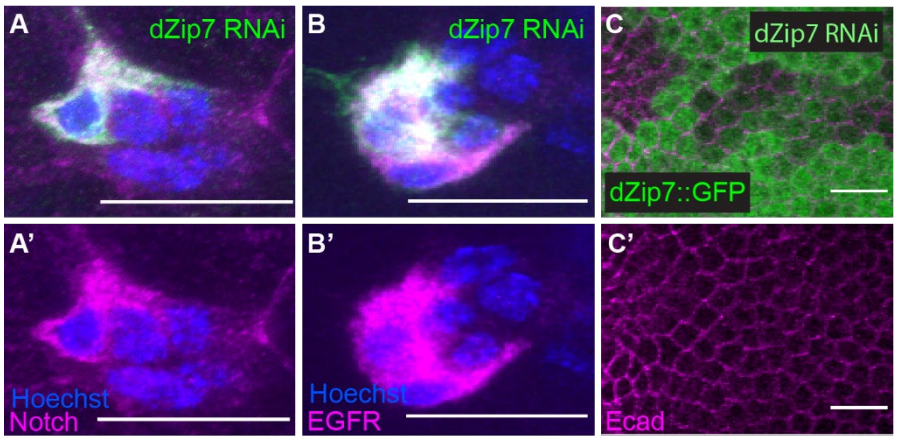


Figure S5, related to Figure 5: dZIP7 knockdown causes ER accumulation of Notch and EGFR but not Ecadherin. (A-A') dZIP7RNAi-expressing clones (GFP+, green) accumulate intracellular Notch protein in border cells relative to neighboring wild type cells. (B, B') Accumulation of EGFR (magenta) in dZIP7RNAi-expressing border cells. (C, C') c306Gal4>dZip7RNAi reduces dZip7::GFP expression but does not cause E-cadherin (magenta) intracellular accumulation. Scale bars=20μm.

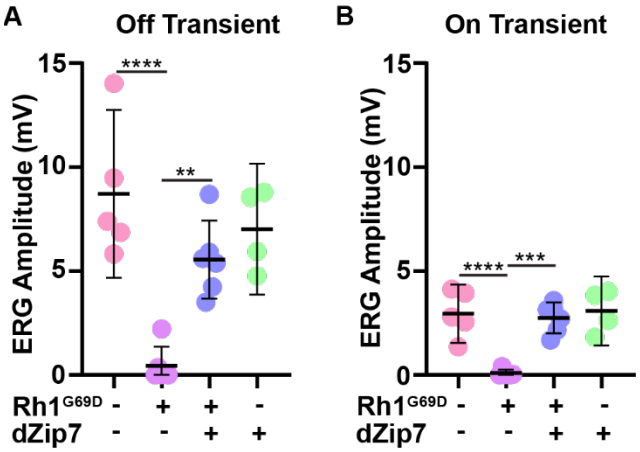


Figure S6, related to Figure 6: dZIP7 co-expression with Rh1G69D returns ERG amplitude to control levels. (A-B) Quantification of ERG recordings of response to off transient (A) and on transient (B) light. Error bars represent 95% confidence intervals. ** P≤0.01, *** P≤0.001, **** P≤0.0001.

III. INVESTIGATING ZIP7 IN CELL BIOLOGY & DISEASE

A. ZIP7 IN NEURODEGENERATIVE DISEASE & AGING

1. ZIP7 prevents neurodegeneration caused by Aβ42 & VAPB in Drosophila

ZIP7 enhances ERAD to promote degradation of $Rh1^{G69D}$, a protein that misfolds and accumulates, causing ER stress and cell death (Fig. 2,3). ZIP7 overexpression prevented $Rh1^{G69D}$-induced neurodegeneration in the fly retina (Fig. 6). Abnormal protein accumulation is a hallmark of many neurodegenerative diseases, and flies are a fantastic model for genetic analysis of protein aggregation [93] [94] [95]. Table 1 lists the human neurodegenerative disease-associated genes that have been studied in flies and are commercially available as UAS lines. Because protein aggregation and ER stress are common features of neurodegenerative diseases, we decided to test whether ZIP7 could prevent neurodegeneration caused by other disease-associated proteins.

Disease	Gene (UAS Lines)
Alzheimer's Disease	APH1A & APH1B (2), APP (39), APLP1 & APLP2 (11), BACE1 (2), HSD17B10/ERAB (3), MARK1/2/3/4 (5), NCSTN (1), PSEN1/2 (9), PEN2/PSENEN (2), Tau (MAPT) (18)
Amyotrophic lateral sclerosis	SOD1 (15), VAPB (3), TARDBP (15), ATXN2 (4), UBQLN2 (4), TAF15 (1), EWSR1 (1)
Frontotemporal Dementia	Tau (MAPT) (18), TARDBP (15)
Hereditary spastic paraplegias	L1CAM (4), SPG3A (3), KIF5A (1), REEP1 (5)
Huntington Disease	HTT (68), HIP14 (1), General polyglutamine peptides (5)
Neuronal ceroid lipofuscinosis	CTSD (1)
Parkinson's Disease	SNCA (7), PARK2 (4), PINK1 (3), DJ1/PARK7 (2), VPS35 (1), GBA (3)
Spinocerebellar ataxia	ATXN1 (5), ATXN2 (4), CACNA1A (5), General polyglutamine peptides (5)

Table 1: Human neurodegenerative disease-associated genes that have been studied in flies.

We set up a screen to test whether ZIP7 expression could suppress or prevent photoreceptor degeneration caused by toxic proteins. We chose to screen rough eye phenotypes because they give a visual readout of neurodegeneration, and these experiments can be performed fairly quickly. We initially screened 6 disease-associated genes that have been shown to induce a rough eye phenotype in flies: Aβ42, Tau, VAPB, TDP-43, Htt, and α-synuclein. We found that ZIP7 co-expression was able to prevent the rough eye phenotype caused by Aβ42 (Fig. 7A) and dVAPB (Fig. 7B). However, ZIP7 did not prevent the rough eye phenotype caused by Tau, TDP-43, Htt, or α-synuclein.

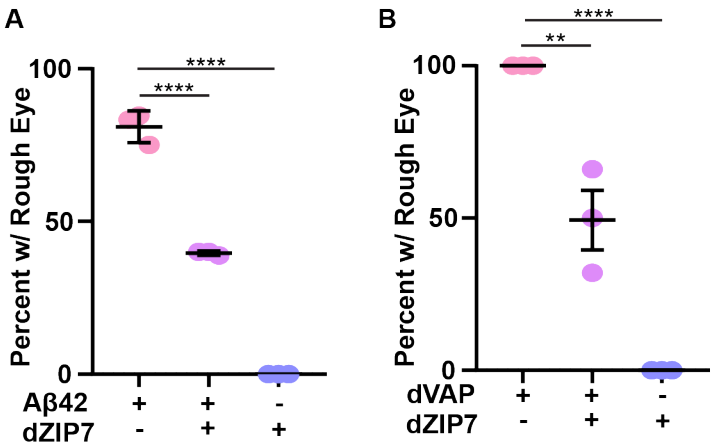


Figure 7: dZIP7 prevents neurodegeneration in the fly retina caused by Aβ42 and dVAP. (A-B) Quantification of the number of flies with rough eye phenotype when using GMR-Gal4 to express Aβ42 (A) or dVAP (B) with and without dZIP7 co-expression. Error bars represent 95% confidence intervals. ** P≤0.01, **** P≤0.0001.

Interestingly, the identified proteins that ZIP7 rescues all misfold in the ER, while the others misfold in the cytosol. This suggests that ZIP7 is particularly important for suppressing the toxicity of ER-misfolding proteins. ZIP7 is an ER

protein required for ERAD, so it follows that ZIP7 would facilitate degradation of ER misfolded proteins. We are currently screening other disease-associated proteins in the fly eye for potential ZIP7 rescue, paying extra attention to those proteins that misfold in the ER. Future studies will be performed in disease-appropriate neurons to model more aspects of the human disease in flies.

2. ZIP7 extends lifespan in Drosophila

We found that ZIP7 enhances ERAD by providing rate-limiting zinc to Rpn11, facilitating deubiquitination and promoting proteasome activity. Proteasomal degradation is critical for mitigating toxic, misfolded protein accumulation and maintaining proteostasis. Interestingly, proteasome capacity is known to decline with age [96]. This is due to lower expression of proteasomal subunits, decreased proteasome assembly, and age-related diseases often involving toxic protein aggregates that congest degradation machinery. Expressing proteasomal subunits has been shown to extend lifespan in flies and C. elegans [96]. Additionally, fibroblasts derived from long-lived humans have higher proteasomal activity levels compared to controls [97]. These studies imply that boosting proteasome activity may be the key to longevity.

Since ZIP7 potently enhanced proteasomal degradation of misfolded proteins, we asked whether ZIP7 had any effect on normal aging. Tonoki et al. found that Rpn11 overexpression in flies extended lifespan by enhancing proteasome capacity [54]. ZIP7 appears to enhance proteasome activity through activating Rpn11, so we thought ZIP7 could have a similar effect on lifespan. Using a driver expressed in all

somatic cells, we found that ZIP7 knockdown reduced lifespan and ZIP7 overexpression extended lifespan by about 30% compared to the control (Fig. 8). Amazingly, overexpressing both ZIP7 and Rpn11 essentially doubled lifespan compared to control flies.

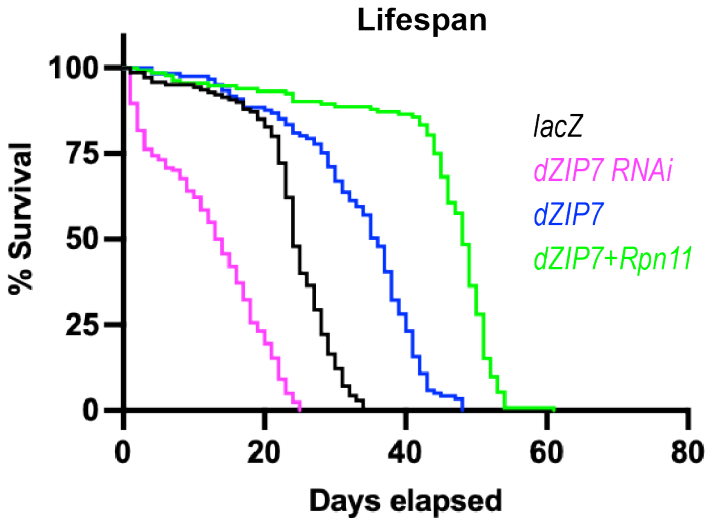


Figure 8: dZIP7 overexpression extends lifespan in flies. Da-Gal4 with a temperature-sensitive Gal4 repressor ($Gal80^{ts}$) was used to drive transgenes. Flies were grown at 18°C until pupation, then moved to 29°C for adulthood. Flies expressing a control UAS gene (lacZ, black) lived around 35 days. dZIP7 RNAi (pink) reduced lifespan to around 25 days. Overexpression of dZIP7 (blue) increased lifespan to around 50 days, and co-expressing dZIP7 and Rpn11 (green) extended lifespan to almost 60 days. *Data generated by Sreesankar Easwaran*

B. COLLABORATIONS

ZIP7 prevented $Rh1^{G69D}$-induced neurodegeneration in the fly model, so we decided to expand our research into mammalian models of Retinitis pigmentosa (RP). RP is a genetic disease where rod photoreceptors degenerate, eventually causing blindness. RP is caused by mutations in the photoreceptor protein Rhodopsin (RHO), and the most common mutation in North America is RHO^{P23H} [98].

Development of gene therapies targeting causative genes in RP is a promising area of therapeutic research.

We are collaborating with Dr. Mohamed Faynus in Dr. Dennis Clegg's lab at UC Santa Barbara to study ZIP7 in a human retinal organoid model of RP. RHO^{P23H} retinal organoids recapitulate many aspects of the disease, including age-dependent photoreceptor degeneration and induction of ER stress (Fig. 9). RHO^{P23H} organoids develop normally until around day 200 when their photoreceptors start to degenerate and lose normal morphology. We plan to test whether addition of a ZIP7 overexpression construct can slow of prevent this degeneration in RHO^{P23H} organoids. We are using adeno-associated viruses (AAV) to deliver the ZIP7 gene therapy to organoids as AAVs are already clinically approved for use in the eye [98]. We will add the ZIP7-AAV at different timepoints to RHO^{P23H} organoids to determine if: (1) ZIP7 overexpression can prevent photoreceptor degeneration and (2) the time at which ZIP7 needs to be overexpressed to treat disease. If these experiments work, we plan to test a ZIP7 gene therapy in a mouse model of RP.

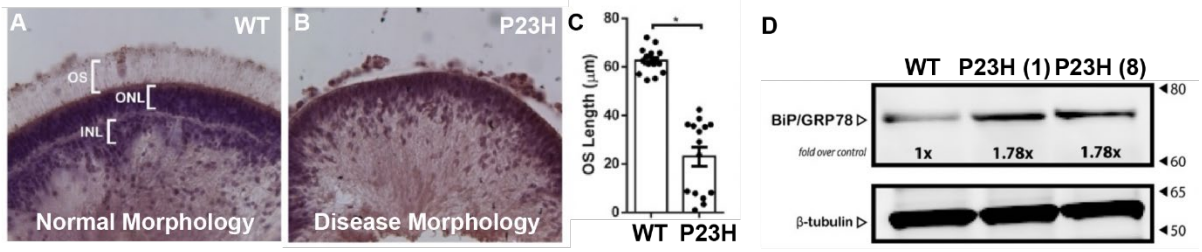


Figure 9: RHO^{P23H} retinal organoids degenerate and experience ER stress. (A, B) H&E staining of sections of a wild type (A) and RhoP23H/+ (B) human retinal organoids at 300 days of differentiation. The rod outer segments (OS), outer nuclear layer (ONL) and inner nuclear layer (INL) are labeled in A. C) Quantification of the rod outer segment lengths. Each dot represents a measurement from one organoid.

D) Western blot showing induction of ER stress in two RHOP23H organoids derived from independent gene edited hESC lines. *Data generated by Mohamed Faynus*

C. Open Questions

Does ZIP7 have a universal mechanism?

Our study identified a clear role of ZIP7 in ERAD and proteostasis. We believe this unifies existing research on ZIP7 and explains its role in cell survival, proliferation, stem cell renewal, and tissue development. However, it is possible that ZIP7 has functions outside of ERAD. Further study is needed to distinguish the effects of ZIP7 versus cytosolic zinc in certain cell processes.

For example, ZIP7 is required for B cell development, but its role appears unrelated to ER stress [92]. B cells are a type of white blood cell that produce antibodies, secrete cytokines, and bind antigens to initiate an immune response. They undergo positive and negative selection during their development, and failure at these quality control checkpoints results in cell death. Anzilotti et al. observed that hypomorphic mutations in ZIP7 in mice prevented maturation of B cells. Interestingly, these cells neither activated UPR signaling, nor experienced ER stress. The authors postulated that loss of ZIP7 lead to dysregulation of cytosolic zinc that impaired B Cell Receptor signaling. Importantly, another study found that activated B cells upregulate ZIP7 protein expression and increase intracellular zinc [99]. It may be cytosolic zinc, rather than ZIP7's promotion of ERAD, that explains involvement in B cell development.

On the other hand, ZIP7's role in ERAD could explain its effects on Akt signaling. Multiple studies have shown that ZIP7 activity promotes Akt signaling [26] [32]

[33] [31] [30]. Rather than direct activation of Akt, ZIP7 could promote Akt activity by regulating cytosolic zinc and alleviating ER stress. Extracellular zinc treatment has been shown to activate Akt signaling in multiple cell types [28] [41] [26] [29]. Cytosolic zinc inhibits protein tyrosine phosphatases, allowing activation of cellular kinases like Akt [32]. Additionally, ER stress is known to inhibit Akt signaling [23] [50] [51]. ZIP7 expression most likely promotes Akt signaling through mitigating ER stress and creating a cellular environment more conducive to growth and proliferation.

How is ZIP7 regulated?

ZIP7 phosphorylation by CK2 leads to its activation and zinc flux into the cytosol [21]. However, overexpression of ZIP7 sufficiently increases its activity (Guo & Mutch et al., 2024, [31] [28]. It is unclear how CK2 is regulated, and whether it is necessary for ZIP7 function in every context. ZIP7 and CK2 may participate in a positive feedback loop where activation of one increases activation of the other. CK2 may be constitutively active and ready to phosphorylate all available ZIP7. ER stress may enhance CK2 phosphorylation to drive ZIP7 activity. Future studies could examine the phosphorylation state of ZIP7 in different cell types and organism models.

D. Implications for Disease Biology

1. Chronic ER stress causes cellular dysfunction & disease

Types 1 & 2 Diabetes mellitus

Pancreatic β-cells produce and secrete large amounts of insulin to regulate glucose metabolism. β-cells have ER systems that are adapted to process, fold, and traffic all that protein, but sustained ER stress in the cells drives disease [100]. In Type 1

Diabetes, T cells attack β-cells, causing dysfunction and cell death. The tissue then must overcompensate to produce enough insulin, thereby increasing pressure on the ER and causing ER stress. Prolonged ER stress will induce apoptosis. Additionally, mutations in insulin cause its precursors to accumulate in the ER, inducing ER stress and eventual β-cell apoptosis [47]. Insulin resistance in Type 2 Diabetes also increases the insulin load on cells, promoting ER stress and cell death.

Hyperglycemia in retinal blood vessels leads to Diabetic Retinopathy (DR), a leading cause of blindness [101]. Hyperglycemia overwhelms the ER's protein folding capacity by upregulating insulin and collagen production. In DR, oxidative stress and ER stress positively regulate each other to aggravate cell damage. ER stress was also shown to promote inflammation in an animal model of DR [102]. ER stress, oxidative stress, and inflammation damage blood vessels in the blood-retinal barrier, prevent nutrient uptake in retinal cells, and cause cell death and eventual blindness.

Neurodegenerative diseases

Neurodegenerative diseases are characterized by the accumulation of misfolded and aggregated proteins in the brain [47,103]. Chronic deposits of protein aggregates cause sustained UPR activation and exhaust cellular degradation machinery. ER stress markers like ATF4 have been found in post-mortem brain tissue in disease patients [47,100,104]. ER stress also correlates to the amount of protein aggregation and disease severity [104].

Amyloid-β and Tau are proteins known to aggregate in Alzheimer's Disease. Both proteins are known to trigger ER stress and UPR activation in neurons [105]. Amyloid precursor protein is cleaved in the ER to produce amyloid-β oligomers that aggregate and induce ER stress [105] [106]. ER stress can also promote further tau misfolding by upregulating GSK-3β whose activity promotes hyperphosphorylation and misfolding of tau. Pharmacological inhibitors of UPR signaling are of interest in the treatment of Alzheimer's Disease.

Familial ALS, a degenerative disease affecting motor neurons, can be caused by mutations in SOD1 or VAPB. SOD1 is a protein that reduces oxidative stress. ER stress in disease neurons causes mutant SOD1 to aggregate [107]. VAPB is a regulator of protein trafficking and involved in UPR signaling via IRE1/XBP1. Mutations in VAPB cause it to aggregate in the ER and become nonfunctional, subsequently dysregulating UPR signaling and inducing apoptosis [108].

Retinal cells are also sensitive to ER stress. Age-related macular degeneration (AMD) causes blindness through degeneration of central photoreceptor neurons [109]. Retinal pigment epithelium (RPE) cell damage caused by inflammation and oxidative stress contributes to disease progression. Chronic ER stress has been shown to exacerbate both inflammation and oxidative stress in RPE and cause cell death. Retinitis pigmentosa (RP) is another blinding disease caused by rod-cone photoreceptor degeneration [109]. Most cases of RP are caused by rhodopsin mutants that misfold and accumulate in the ER and cause ER stress. It was shown in animal models of RP that retinal cells activate pro-apoptotic signaling

through the PERK arm of the UPR [110]. Enhancing ERAD and overexpressing ER chaperones has been shown to protect against RP [111].

Cancer

Cancer cells survive and proliferate in less-than-optimal conditions. One way cancer cells do this is through upregulating UPR and ERAD signaling [47]. For example, GRP78 is an ER chaperone that regulates pro-survival signaling of the UPR and is often upregulated in cancer. Loss of GRP78 in cancer cells caused apoptosis and prevented tumor growth [47 112]. Moreover, cancer cells often overexpress proteasomal subunits, leading to enhanced proteasome activity and reduced sensitivity to ER stress-induced apoptosis [113 114]. Proteasome inhibitors like bortezomib are currently used in chemotherapy drugs to treat certain cancers [47].

2. ZIP7 regulation in disease

Neuronal ceroid lipofuscinoses

Neuronal ceroid lipofuscinoses (NCLs, also known as Batten disease) are a group of lysosomal storage diseases that cause vision loss, motor defects, seizures, and death. These disorders often manifest as autosomal recessive conditions in children, but symptoms can manifest in adulthood. Lysosomal storage diseases are caused when defects in lysosomal enzymes impair lysosomes in degrading waste products in the cytosol. Lysosomal dysfunction prevents autophagy, causes ER stress, and promotes apoptosis. In NCLs, mutations in CLN genes trigger lysosomal dysfunction and lipofuscin, a lipid-protein complex degraded by lysosomes, accumulates in neurons [115–117]

Loss of ZIP7 drives cellular dysfunction in disease models with CLN6 mutations. CLN6 is a protein on the ER membrane that recruits lysosomal enzymes for lysosome synthesis [118]. CLN6 mutant mice abnormally accumulate zinc in the cerebral cortex, cerebellum, spinal cord, heart, and liver [115]. Sheep with CLN6 mutations similarly showed zinc accumulation in the central nervous system [117116]. Therefore, CLN6 drives dysregulation of zinc homeostasis. It was found in these CLN6 mutant sheep that ZIP7 expression in neurons dramatically declined with age [116]. Upregulating ZIP7 in primary cortical neurons with Cln6 mutations restored zinc homeostasis and promoted dendritic growth. Pharmacological activators of ZIP7 or gene therapies targeting ZIP7 may be useful in treating CLN6-associated NCL.

Type 2 Diabetes

The pancreas regulates blood sugar by releasing insulin, a hormone that regulates glucose storage in cells. Type 2 Diabetes occurs when pancreatic β-cells fail to produce adequate insulin in response to glucose in the blood. Insulin resistance precedes disease and causes ER stress, which in turn further exacerbates dysregulation of insulin signaling [47]. Insulin resistance and prolonged ER stress eventually cause β-cell apoptosis. Zinc and ZIP7 have been shown to help regulate insulin secretion and glucose metabolism in normal and diabetic tissue.

ZIP7 maintains zinc homeostasis in β-cells and promotes insulin secretion. Insulin forms a hexamer with 2 zinc ions before being secreted by β-cells [119,120]. Consequently, β-cell zinc homeostasis must be maintained for proper insulin folding and exocytosis. Liu et al. found that ZIP7 is abundantly expressed in in vitro mouse

pancreatic MIN6 cells and in vivo in mouse and human islets tissue compared to other zinc transporters [119]. ZIP7 knockdown also reduced insulin exocytosis. Finally, the authors found ZIP7 was necessary for regulating cytosolic zinc levels in healthy β cells. These results indicate that ZIP7 is critical for normal β-cell function.

Multiple studies have shown that cells upregulate ZIP7 in hyperglycemic conditions. Bellomo et al. observed that ZIP7 mRNA, but not protein, expression increases with glucose levels in mouse pancreatic islets [121]. On the other hand, ZIP7 protein expression is upregulated in glucose-treated C2C12 mouse skeletal muscle cells [45]. Additionally, hyperglycemia led to upregulation and activation of ZIP7 protein in rat cardiomyocytes and H9c2 cells [43]. Glucose-induced ZIP7 expression may serve 2 purposes; it relieves ER stress caused by increased insulin production and promotes Akt signaling that facilitates glucose uptake.

Loss of ZIP7 in skeletal muscle or β cells is related to glucose metabolism, dysfunctional insulin regulation, and insulin resistance. Myers et al. observed that ZIP7 knockdown in C2C12 cells downregulated glucose metabolism genes and Glut4, a glucose transporter that mediates glucose storage as glycogen [44]. Knockdown of ZIP7 in MIN6 cells led to ROS accumulation and prevented insulin secretion in glucose-treated cells [119]. Norouzi et al. found that while glucose treatment in C2C12 cells upregulated ZIP7 expression, insulin resistant C2C12 cells downregulated ZIP7 expression despite glucose treatment [45]. Downregulation of ZIP7 correlated with decreased pAkt and Glut4 expression, indicating impaired glucose metabolism. Further studies are needed to determine whether ZIP7 overexpression is a possible therapeutic strategy for insulin resistance.

Cancer

While loss of ZIP7 is associated with neurodegeneration and diabetes, multiple cancers upregulate ZIP7. Tamoxifen resistant (TamR) MCF-7 breast cancer cells have higher ZIP7 protein expression compared to basal MCF-7 cells, and ZIP7 knockdown prevents cell migration [26]. Phosphorylated ZIP7 is also highly expressed in patient breast cancer samples, indicating it could be a biomarker for anti-hormone resistant breast cancer [122,123]. ZIP7 is also upregulated in cervical carcinoma and gastric cancer tissue [31,34]. Colorectal carcinomas express ZIP7 at much higher levels than normal intestinal tissue, and high ZIP7 expression in tumors correlated with poor prognosis [124]. Therefore, inhibiting ZIP7 is a potential therapeutic approach to treating these cancers.

3. Therapeutic potential of ZIP7

ZIP7 is a strong candidate for therapeutic development. ZIP7 overexpression potently enhances ERAD and promotes cell survival. ZIP7 gene therapies could aid in the treatment of the many diseases that involve defective ERAD. A gene therapy approach could be used to upregulate ZIP7 activity in the neurons of patients with Neuronal ceroid lipofuscinoses and insulin-resistant β-cells in Diabetes. We are currently testing an AAV-ZIP7 gene therapy in a human retinal organoid model of Retinitis pigmentosa. Future research could investigate the effectiveness of a ZIP7 gene therapy in models of Alzheimer's Disease and ALS.

ZIP7, as well as proteasome activity, are upregulated in several different cancers to promote aberrant proliferation, migration, and survival. Proteasome inhibitors like

bortezomib are already used in certain chemotherapies. ZIP7 inhibitors may be an effective treatment against certain cancers. Further study of ZIP7 promises an adventure into understanding ER function, proteostasis, and disease biology that will lead to development of novel therapeutics.

IV. BIBLIOGRAPHY

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