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The ER Stress-dependent Regulation of MicroRNAs in Mammals

by

Shannon Leigh Behrman

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biochemistry

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

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by

Shannon Leigh Behrman

Without question this thesis is dedicated to

my parents, David and Terri Behrman,

my brother, Derek Behrman,

and my partner in crime, Michael Poreda,

for their undying love and support

ACKNOWLEDGMENTS

First and foremost, I thank Peter Walter for taking me under his wing and believing in me throughout this entire journey, despite the fact that I was painfully naïve in my formative graduate school years. He gave me the opportunity to find the scientist within me and to rise well above my perceived limits. Also, his infectious enthusiasm and imagination proved to me that science can truly be a creative passion. Conveniently, his creative energy oftentimes transformed into some sort of tomfoolery, and Christmas will never be the same for me or any other Walter lab alum.

I also thank Peter for granting me the great fortune of being a part of the Walter lab family. It is from this incredibly talented community that I learned how to navigate the huge abyss of science and still manage to have fun. The list of past and present Walter lab folks I'd like to thank is, if you can imagine, longer than my tenure in the Walter lab, so I'll try to keep it brief (apologies to those names not included – there's just not enough space!). Exceptional post-docs that provided above-and-beyond intellectual and technical support include (in chronological order): the royal prankster, Jason Brickner, the unapologetically-true-to-herself, Kirsten Jaglo, the zen master, Pablo Aguilar, the spirited thinker, Tomas Aragon, the pragmatic guidance counselor, Sebastian Schuck, the ascetic (and hilarious) mentor, Ciara Gallagher, and the vivacious go-getter, Carmela Sidrauski. Diego Acosta-Alvear deserves special mention because his intellectual and technical expertise and tenacious guidance helped fuel this

project and give me a considerable amount of assuredness.

This lab excelled at recruiting exceptional graduate students whom many I consider lifetime companions. I still miss the daily presence of the jovial Siamese twins, Alex Engel and Sebastian Bernales. Both Han Li and Karen Moreira also painted a warm and joyous lab atmosphere: Han was my cheerleader and confidant that provided tireless enthusiastic emotional and technical support. I honestly wouldn't have made it without her. Karen was my sweet and playful accomplice in all matters of practical jokes. And, last (but certainly not least), there was Ms. Claudia Rubio, the one person who shared this entire journey with me through thick and thin. I can't thank her enough for reminding me that I'm not crazy and that life is pretty fun on the outside.

I am also grateful for the lab's support staff for all of their diligence that contributed in significant ways to my scientific progress: our lab managers, Silke Nock and Paige Nittler, the cell culture guru and lab technician extraordinaire, Fran Sanchez, the other gifted lab technician, Tenzin Kundon, and the glassware cleaner, Oya Unal. Teresa Donovan also deserves special praise for her deft administrative skills and for keeping a keener eye on my reimbursements.

My gratitude extends beyond the Walter lab borders to my thesis committee, Dave Morgan and Jeff Cox, for their scientific and academic guidance and support – Dave especially for being so accessible and optimistic. Danny Dam and the retired Sue Adams also receive praise for their exhaustive administrative support – making sure my life in grad school went smoothly. For

specific contributions to my project, I thank: David Ron, for thoughtful and insightful discussions and for essential reagents; members of the Sandler Asthma Basic Research (SABRE) Center Functional Genomics Core Facility, Rebecca Barbeau, Andrea Barczak, and David Erle, for technical support of miRNA expression arrays and analysis; Carolina Arias, for assistance in the analysis and visualization of microarray data; and, Jiashun Zheng, for assistance in bioinformatics analysis.

And finally, considering how exorbitantly expensive mammalian cell culture is, I am eternally grateful for the financial support provided by the National Science Foundation (predoctoral fellowship), the Howard Hughes Medical Institute, and the National Institute of Health.

Thank you all!

ABSTRACT

The ER Stress-dependent Regulation of MicroRNAs in Mammals

by

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MicroRNAs (miRNAs) are small, non-coding RNAs that post-transcriptionally regulate messenger RNAs through sequence-specific interactions. miRNAs have recently been shown to exert their regulatory influence during cellular stresses. Endoplasmic reticulum (ER) stress, one example of a cellular stress, stems from an imbalance in the ER's protein folding capacity, oftentimes resulting from such insults as an increase in protein load or expression of misfolding mutant proteins. Consequently, mis- or unfolded proteins accumulate within the ER, which triggers the unfolded protein response (UPR). In mammals, three UPR sensors, IRE1, ATF6, and PERK, detect the folding status of the ER, thus activating transcriptional as well as post-transcriptional programs that lead to adaptation. If ER stress is unmitigated and homeostasis is not restored, the UPR switches from a cytoprotective role to an apoptotic one.

Intriguingly, genome-wide miRNA expression analyses revealed a more complex downstream adaptive network of the UPR. Prolonged ER stress prompted the differential regulation of 11 miRNAs, 8 of which were up-regulated in the presence of ER stress-inducing drugs. The differential expression of only

one of those miRNAs, miR-708, demonstrated a dependence on the UPR transcription factor, CHOP. Curiously, mir-708 resides in the intron of *Odz4*, a gene ambiguously involved in neural development that was also previously characterized as transcriptionally activated by CHOP. The striking co-expression of both miR-708 and *Odz4* in the brain and eyes suggested a common physiological function in these tissues. Furthermore, loss- and gain-of-function experiments showed that miR-708 inhibits the expression of rhodopsin, a heavily synthesized multi-spanning transmembrane protein in photoreceptor cells of the eye. In light of this, one can speculate a cytoprotective role for miR-708 whereby it acts to prevent excessive rhodopsin from entering the ER in photoreceptor cell. Thus, miR-708 and its transcriptional activator, CHOP, are implicated in the homeostatic regulation of ER function in the mammalian visual system.

TABLE OF CONTENTS

Chapter 1

Introduction: The Unfolded Protein Response and MicroRNAs in Mammals 1

Chapter 2

A CHOP-regulated MicroRNA Controls Rhodopsin Expression 20

Chapter 3

Summary and Perspectives 61

Appendix A

On the post-transcriptional regulation of X-box binding protein-1 (*Xbp1*) 73

LIST OF FIGURES AND TABLES

Chapter 1

Figure 1-1: The Unfolded Protein Response in Mammals 16

Figure 1-2: MicroRNA Biogenesis in Metazoans 17

Figure 1-3: 24-hour time course of UPR induction in 3T3 fibroblasts................. 18

Figure 1-4: Inconspicuous changes in the expression of two miRNAs,
miR-148a and miR-27a, exposed to ER stress 19

Chapter 2

Figure 2-1ab: CHOP regulates miR-708 expression during ER stress 51

Figure 2-1cd: CHOP regulates miR-708 expression during ER stress............... 52

Figure 2-2ab: miR-708 is a conserved intronic miRNA highly expressed in
neuroectodermal tissue.. 53

Figure 2-2c-e: miR-708 is a conserved intronic miRNA highly expressed in
neuroectodermal tissue.. 54

Figure 2-3: Mature miR-708 is loaded on the RISC 55

Figure 2-4: miR-708 targets rhodopsin for post-transcriptional inhibition........... 56

Supplemental Figure 2-1: miRNA expression profiles of CHOP-deficient
cells undergoing ER stress 57

Supplemental Figure 2-2: Inconspicuous changes in the expression of
miRNAs in 3T3 fibroblasts exposed to ER stress for 10 hours......... 58

Supplemental Figure 2-3: Canonical UPR markers are observed relatively early upon induction of ER stress 59

Supplemental Table 2-1: Top 30 candidate target genes of miR-708 defined by TargetScan 60

Chapter 3

no figures

Appendix A

Figure A-1: XBP1-s protein expression increases during 10 h of ER stress 82

Figure A-2: XBP1-s protein stability does not change with ER stress 83

Figure A-3: Polysome profiles of *Xbp1-s*-expressing cells 84

Figure A-4: Deletional analysis did not reveal a mechanism for post-transcriptional regulation of *Xbp1-s* mRNA 85

Chapter 1

Introduction:

The Unfolded Protein Response and MicroRNAs in Mammals

The endoplasmic reticulum (ER) evolved as a highly fine-tuned chemical environment that allows newly synthesized proteins to fold into their proper conformations before export or transport to the cell surface. Common physiological stresses, such as an increase in a cell's protein secretory load, the disruption of ER calcium homeostasis, or protein mutations, promote the misfolding of proteins in the lumen of the ER. Accumulation of such mis- or unfolded proteins triggers the unfolded protein response (UPR), which adjusts the ER's protein folding capacity to match the increased need for protein folding machinery. Demonstrating its importance, the UPR is evolutionarily conserved from yeast to humans with higher eukaryotic organisms boasting more complex variations. Originally observed and well-characterized in *S. cerevisiae* in this lab, the UPR depends on the ER transmembrane protein, Ire1p, a Ser/Thr kinase endoribonuclease (Cox et al., 1993). Ire1p's luminal domain senses unfolded proteins, oligomerizes, and activates the cytoplasmic endoribonuclease domain, which initiates the unconventional splicing of an mRNA substrate, encoding the transcription factor Hac1p. When translated, Hac1p targets UPR genes encoding proteins involved in protein folding, secretion/transport, and degradation (Cox and Walter, 1996; Travers et al., 2000)

In contrast to yeast, the UPR in mammals depends on three distinct ER transmembrane proteins, IRE1 (inositol requiring enzyme 1), ATF6 (activating transcription factor 6), and PERK (PKR-like ER kinase) (Ron and Walter, 2007). To maintain homeostasis, the mammalian UPR unleashes both adaptive and

preventive programs: the adaptive program, largely mediated by ATF6 and IRE1, functions to 'clean up the mess,' allowing adaptation of ER function. Both pathways enlist players that try to minimize the amount of improperly folded proteins in the ER compartment, some of which include chaperones that aid in the folding of proteins, ER-associated degradation components that target rogue proteins for degradation, and lipid biosynthesis enzymes that facilitate ER expansion. The preventive program, mediated by PERK and IRE1, decreases the load of proteins in the ER by either repressing protein synthesis or degrading transcripts, respectively. In such instances where persistent ER stress becomes insurmountable, the UPR actively initiates programmed cell death or apoptosis, which is postulated to protect multicellular organisms from cells that express misfolded signaling proteins, may aberrantly respond to extracellular signals, and hence would be intrinsically dangerous to the organism.

The mechanisms for how these three ER stress sensors get activated has been subject to in depth examination by numerous labs over the last two decades. In particular, a breadth of exciting, unforeseen insights was generated from our lab and others about how both compartmental portions of IRE1 function. I will not attempt to review these findings here but rather refer you to a collection of impressive papers (Credle et al., 2005; Aragon et al., 2009; Korennykh et al., 2009; Li et al., 2010). I will, however, review how the ER stress sensors activate their respective downstream signaling pathways (Figure 1-1): mammalian IRE1 retains the same mechanism of activation as Ire1p in yeast. Its activated RNase

domain cleaves a 26-nt intron from the HAC1 mRNA equivalent, X-box binding protein-1 (*Xbp1*) mRNA, in a site-specific manner (Calfon et al., 2002; Shen et al., 2001; Yoshida et al., 2001). The 5' and 3' fragments are re-ligated to form a newly spliced *Xbp1* transcript that gets translated into a potent transcriptional activator of UPR target genes (XBP1-s). XBP1-s is a basic-region leucine zipper protein that binds to both UPR elements (UPRE) and ER stress-response elements (ERSE) controlling such genes as the ER-associated degradation component, EDEM, or the ER chaperone, BiP, respectively (Yoshida et al., 2003). Aside from instituting adaptive factors, IRE1 was recently discovered to prevent ER-client protein load by regulating the degradation of ER membrane-localized transcripts, known as the regulated IRE1-dependent decay (RIDD) (Han et al., 2009; Hollien et al., 2009; Hollien and Weissman, 2006).

ATF6 undergoes intramembrane proteolysis upon activation and is converted into a soluble protein (ATF6(N)) (Yoshida et al., 1998), reminiscent of the mechanism that was first described for the activation of sterol response element binding protein (SREBP). Proteolysis liberates ATF6(N) from its ER membrane anchor which becomes a functional transcription factor that also binds to ERSE in the upstream promoters of UPR genes including *Xbp1*.

Upon the sensing of unfolded proteins, PERK dimerizes and autophosphorylates to activate its kinase domain. The PERK kinase domain then phosphorylates the eIF2α translation initiation factor, which attenuates translation throughout the cell (Harding et al., 2000). Although translational

repression lessens the load on the ER by reducing the concentration of translated proteins, it paradoxically promotes the translation of unique mRNAs containing short upstream open reading frames, such as the mRNA encoding the transcription factor ATF4. ATF4 activates genes involved in amino acid metabolism and redox homeostasis, as well as the gene encoding the transcription factor, C/EBP homologous protein (CHOP) (Harding et al., 2003). Although CHOP expression has been linked to ER stress-induced apoptosis (Zinszner et al., 1998), its role in the UPR is broad and extends beyond this single function. It was originally noted in Zinszner et al 1998 that activation of CHOP precedes that of the apoptotic cascade by several hours in cultured mouse embryonic fibroblasts (MEFs) or days in murine kidneys *in vivo*, suggesting that CHOP does not directly turn on apoptotic components. Thus, before the onset of cell death, it is becoming increasingly apparent that CHOP also plays a cytoprotective role. This proposed role is supported by the fact that CHOP has been shown to up-regulate genes involved in survival and development. For example, CHOP regulates transcription of GADD34, a phosphatase that restores translation following PERK activation by removing the inhibitory phosphate from eIF2α (Marciniak et al., 2004). Restoring translation was shown to be important for survival of GADD34-deficient mice. Another example of a protective gene activated by CHOP is *Odz4*, a gene encoding a surface membrane protein essential in development (Wang et al., 1998) (see Chapters 2 and 3 for more information regarding *Odz4*).

Much akin to the post-transcriptional inhibitory actions of the PERK and RIDD pathways, miRNAs impede the synthesis of proteins through transcript cleavage and/or translational repression (Guo et al., 2010; Lim et al., 2005; Olsen and Ambros, 1999; Wightman et al., 1993). MicroRNAs (miRNAs), are small endogenous RNAs of about 22 nucleotides in length that play substantial gene-regulatory roles in a myriad of biological processes including cell differentiation, survival, and proliferation (Bartel, 2004). miRNAs exert their regulatory role by directing the RNA-induced silencing complex (RISC) to their target transcript. They accomplish this through sequence-specific interactions mainly centered on the 5' terminal 2-7 nucleotides of the miRNA, otherwise known as the 'seed' sequence (Lewis et al., 2003). The phylogenetically conserved genes encoding miRNAs are distributed throughout intergenic regions or within the sequences of protein-coding genes (Griffiths-Jones et al., 2006). As a result of their wide genomic dispersal, they can be under the control of autonomous promoters or intricately coupled to the transcriptional regulation of a neighboring or host gene (Baskerville and Bartel, 2005). RNA polymerase II drives transcription of a larger primary miRNA precursor, called the pri-miRNA, which is subsequently processed while being shuttled from the nucleus to the cytoplasm (Figure 1-2) (Bartel, 2004). For miRNAs that hitchhike on other transcripts, simlar precursor RNAs are produced, e.g., as byproducts of mRNA splicing. Pri-miRNAs are cleaved by Drosha RNAse III endonuclease into ~60-70 nucleotide stem loops, otherwise known as the hairpin precursor or pre-miRNAs. Ran-GTP and

Exportin-5 work in concert to actively export pre-miRNAs from the nucleus. Once in the cytoplasm, the RNase Dicer then slices the pre-miRNAs, leaving imperfect dsRNA duplexes. The miRNA-miRNA* duplexes are loaded into the RISC whereby the two strands are peeled apart freeing the 'guide strands' (miRNA) for transcript targeting. Once removed, the other 'passenger strands' (miRNA*) are degraded (Khvorova et al., 2003; Schwarz et al., 2003).

Aside from micromanaging general developmental processes, these small, non-coding RNAs have recently been demonstrated to regulate cellular stress responses or processes impinging on the function of the ER, most notably hypoxia, insulin secretion, and B-cell differentiation (Huang et al., 2009; Poy et al., 2004; Vigorito et al., 2007). Because of the overlap in function with these processes and the broad spectrum of genes that the UPR ultimately regulates, I hypothesized that the UPR unleashes a miRNA regulatory network that contributes to its preventive program by reducing the load of ER-destined proteins. To begin to address my hypothesis, I started by generating a miRNA expression profile using expression arrays from MEFs treated with the ER stress inducers tunicamycin (Tm) and thapsigargin (Tg) for 10 hours. This was a pilot experiment therefore, no biological replicates were included disallowing any statistical analysis. I subjected the cells to 10 hours of ER stress because this provided a point of maximal induction for most UPR markers observed, notably *Xbp1-s* and *Chop* (Fig 1-3). Also in this pilot analysis, I included *Xbp1*-deficient MEFs along with its WT counterparts to see if XBP1 specifically activates miRNA

transcription. XBP1 was chosen because it up-regulates a significant portion of the UPR transcriptome (Lee et al., 2003).

In contrast to my expectations, no miRNAs showed significant (i.e. more than 2-fold) up-regulation in both Tm and Tg-treated cells in either the *Xbp1* +/+ or *Xbp1* -/- MEFs (see Supplemental Fig 2-2; data not shown). There were a handful of candidates with more than 1.5-fold differential expression in cells treated with either of the ER stress-inducers. I tested several of these 'lackluster' candidates biochemically in longer, more detailed time courses but found none of them showed any significant change of expression after 24 hours of ER stress (Fig 1-4). Additionally, there were two miRNAs that demonstrated considerable down-regulation, miR-503 and miR-1959. Although these two miRNAs could have been interesting to investigate, I decided to remain focused on unlocking the transcriptional control of miRNAs as governed by a UPR transcription factor, i.e. the ER stress-dependent transcriptional activation of miRNAs. Further contributing to my disregard of these miRNAs was the fact that not much is currently known about the down-regulation of miRNAs. I am certain that with time our knowledge of miRNA negative regulation will advance, and miR-503 and miR-1959 will be exciting to further investigate in this regard.

In Chapter 2, I describe the story that unfolded from my second attempt at miRNA expression profiling. Using genome-wide miRNA expression analyses, bioinformatics and biochemical assays, I provide evidence that miR-708, a mammalian intronic miRNA, is CHOP-dependent and controls rhodospin

expression. The physiological implications of such regulation link the UPR to the mammalian visual system, more specifically to the development of the retina. I discuss these implications along with the many future directions conceived during this project more in depth in the concluding chapter, Chapter 3.

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Figure Legends

Figure 1-1. The Unfolded Protein Response in Mammals

The unfolded protein response is comprised of three ER-transmembrane sensors, IRE1, PERK, and ATF6, which initiate both transcriptional and post-transcriptional programs aimed at cytoprotection (see text for details). *Xbp1-u* denotes unspliced *Xbp1*; *Xbp1-s* denotes spliced *Xbp1*.

Figure 1-2. MicroRNA Biogenesis in Metazoans

The biogenesis of a metazoan miRNA begins with the transcription of an endogenous miRNA coding sequence and ends with the mature guide strand (miRNA) loaded on the RISC (see text for details).

Figure 1-3. 24-hour time course of UPR induction in 3T3 fibroblasts

3T3 fibroblasts treated with 5 μg/ml Tm or 500 nM Tg for 24 h. Top panels: Western blots showing canonical UPR markers XBP1-s, BiP, and protein disulfide isomerase (PDI). GAPDH was used as a loading control. Bottom panels: semi-quantitative RT-PCR assays showing total *Xbp1*, spliced *Xbp1* (*Xbp1-s*), *ERdj4*, and *Chop* transcripts. In the total *Xbp1* RT-PCR panel, the middle band represents the unspliced (u) mRNA, the bottom band the spliced (s), and the top band an unspliced-spliced hybrid (*). *Actin beta* was used as a loading control.

Figure 1-4. Inconspicuous changes in the expression of two miRNAs, miR-148a and miR-27a, exposed to ER stress

RNase protection assays looking at the expression levels of two representative miRNAs, miR-148a and miR-27a, from the array dataset of 3T3 cells treated with Tm or Tg for 10 h that were more than 1.5-fold up-regulated. 3T3 fibroblasts were treated with 5 μg/ml Tm over a 24 h time course. Loading control, miR-16, not shown.

The Mammalian Unfolded Protein Response

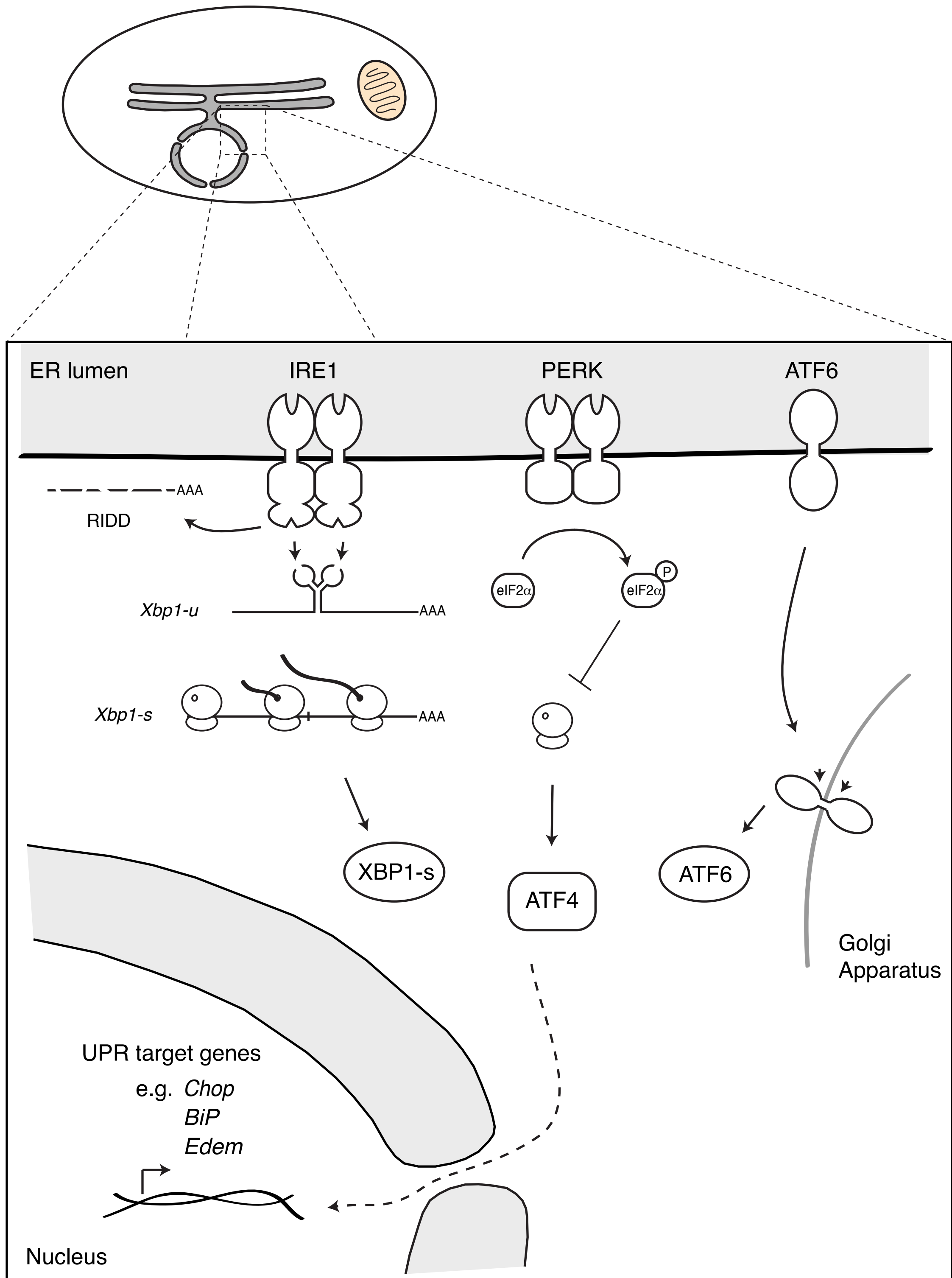


Figure 1-1

MicroRNA Biogenesis in Metazoans

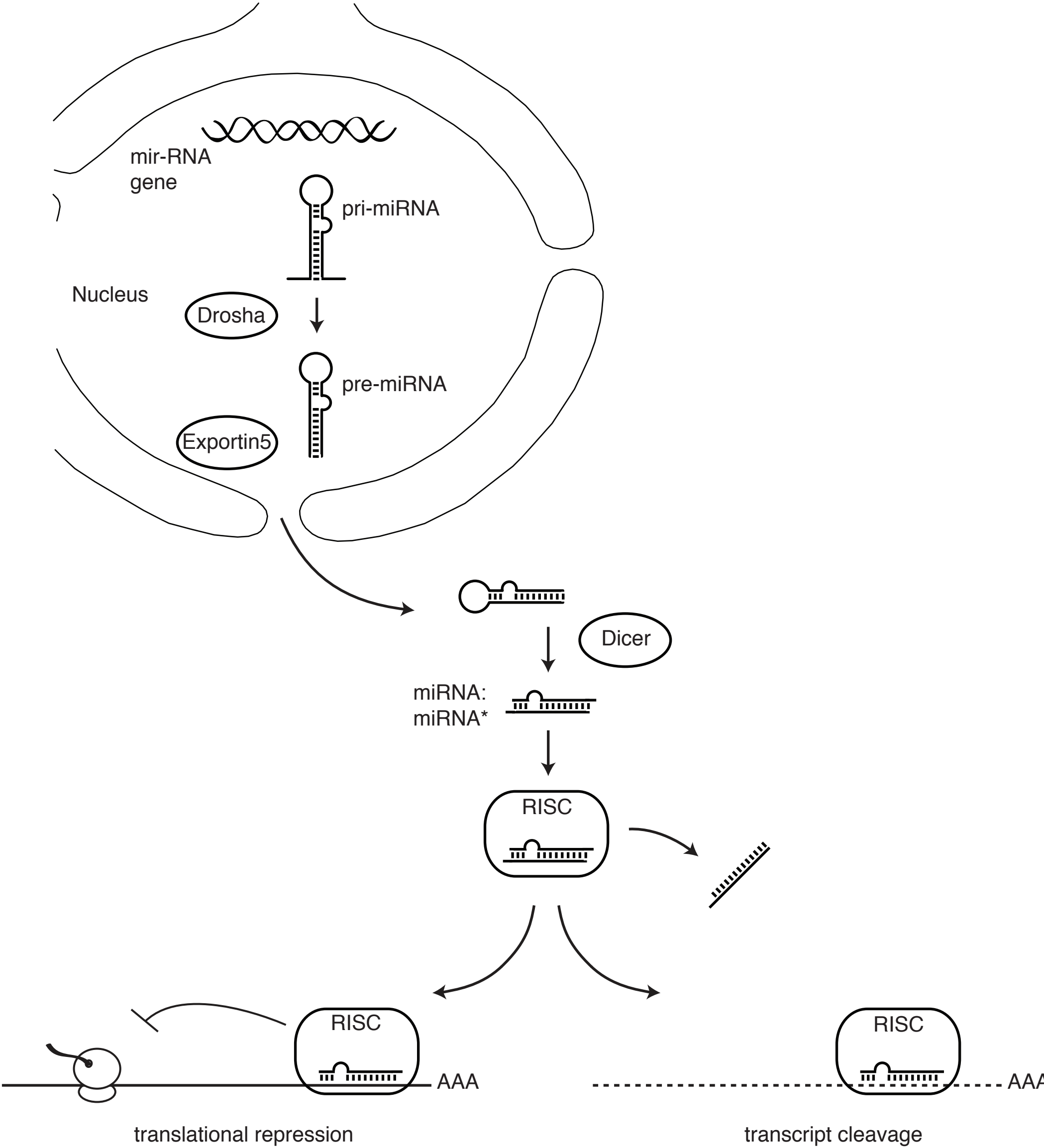
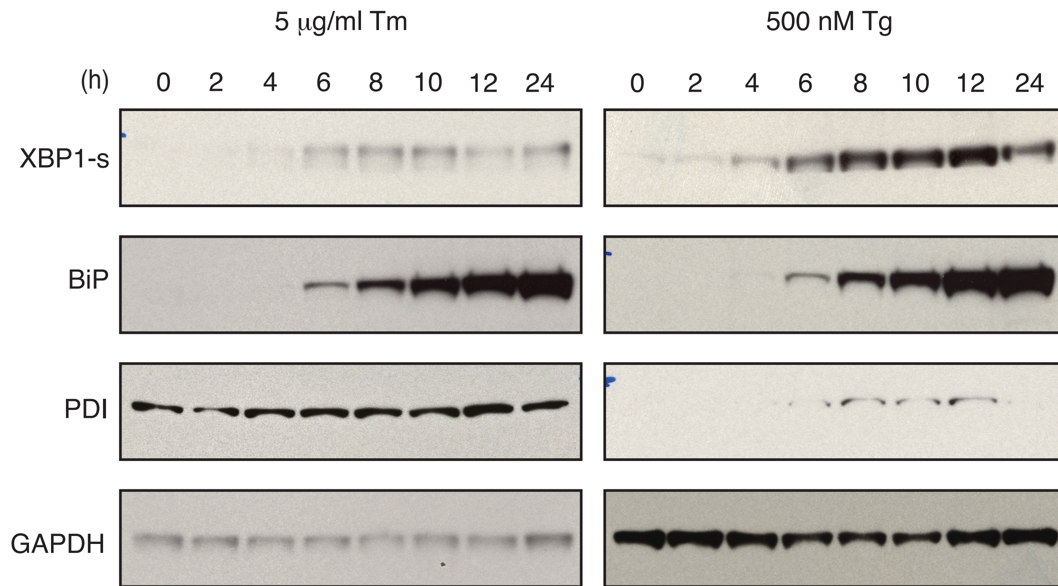


Figure 1-2

Westerns:



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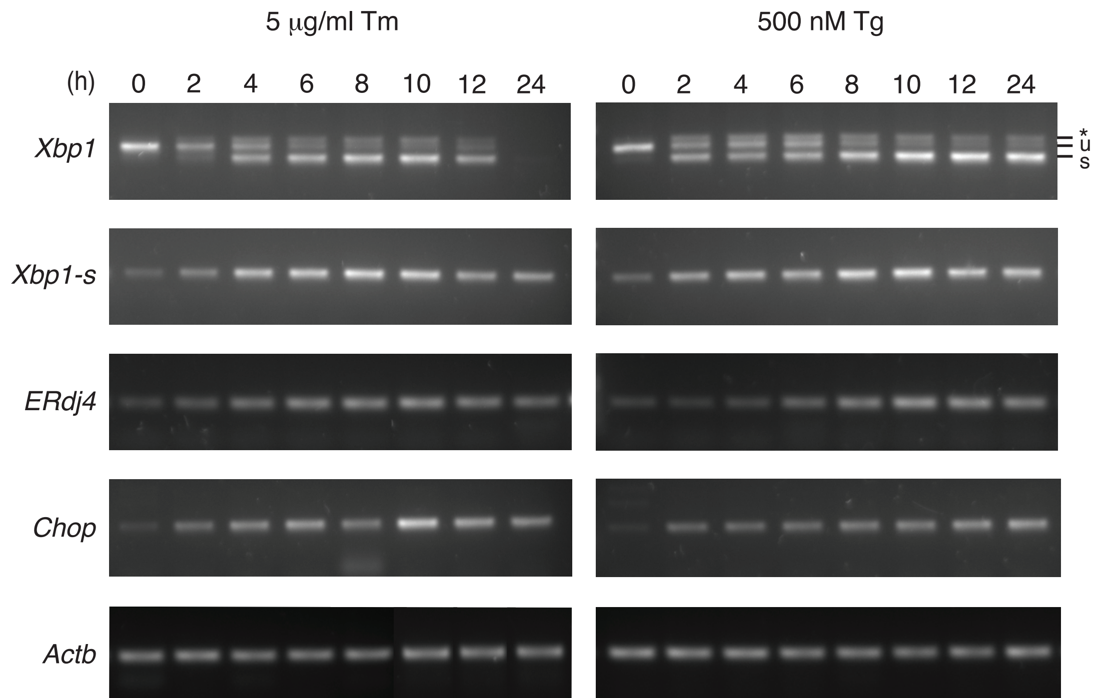

Figure 1-3

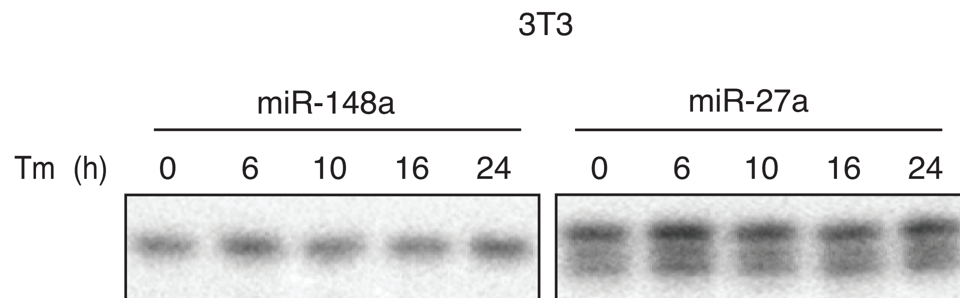

Figure 1-4

Chapter 2

A CHOP-regulated MicroRNA Controls Rhodopsin Expression

A CHOP-regulated MicroRNA Controls Rhodopsin Expression

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Running title: miR-708 Controls Rhodopsin Expression

Key words: miR-708, CHOP, Rhodopsin, Odz4, ER-stress

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Abstract

Using genome-wide miRNA expression profiling, bioinformatics and biochemical analyses, we identified miR-708, an ER stress-inducible miRNA whose expression is regulated by the transcription factor CHOP. miR-708 is encoded in an intron of the CHOP-regulated gene, *Odz4*. ODZ4 is a member of the highly conserved teneurin family of developmental regulators. CHOP-deficient fibroblasts fail to regulate expression of both *Odz4* and mir-708, explaining the dependency of mir-708 on CHOP. *Odz4* and mir-708 are co-expressed in the brain and eyes of the mouse, suggesting common physiological functions in these tissues. Among thirty predicted candidate targets of miR-708, we validated rhodopsin through loss- and gain-of-function experiments. Together, our data implicate miR-708 in the homeostatic regulation of ER function in the mammalian visual system, whereby miR-708 may help avert excessive rhodopsin load entering the ER. miR-708 may hence function analogous to other UPR controls that throttle protein influx into the ER, such as general translational down-regulation by PERK or membrane-bound mRNA decay by IRE1.

Introduction

MicroRNAs (miRNAs), are small endogenous RNAs of about 22 nucleotides in length that post-transcriptionally regulate gene expression in a myriad of biological processes, including cell differentiation, survival, and proliferation (Bartel, 2004). miRNAs direct the ribonucleoproteic RNA-induced silencing complex (RISC) to their target transcripts to repress translation, degrade the transcript or both (Guo et al., 2010; Lim et al., 2005; Olsen and Ambros, 1999; Wightman et al., 1993). They accomplish this feat through sequence-specific interactions, utilizing a mRNA complementary 2-7 nucleotide seed sequence that is present at their 5' termini (Lewis et al., 2003). Genes encoding miRNAs are distributed both in intergenic regions and within protein-coding genes (Griffiths-Jones et al., 2006). As a result of this wide genomic dispersal, miRNAs can be under the control of autonomous promoters or depend on the transcriptional regulation of a neighboring or host gene (Baskerville and Bartel, 2005). Transcription initially produces a larger miRNA precursor, which is subsequently processed into a mature RNA duplex by the specific RNAses Drosha and Dicer (Bartel, 2004). This miRNA-miRNA* duplex is loaded into the RISC whereby the two strands are peeled apart freeing the 'guide strand' (miRNA) for transcript targeting. Once removed, the other 'passenger strand' (miRNA*) is degraded (Khvorova et al., 2003; Schwarz et al., 2003).

miRNAs have been linked to a variety of cellular stresses, among them processes that impinge directly on the function of the endoplasmic reticulum (ER) (e.g. hypoxia, insulin secretion, B-cell differentiation) (Huang et al., 2009; Poy et al., 2004; Vigorito et al., 2007). ER stress stems from an imbalance of the ER's protein folding capacity, which can be caused, for example, by an increased protein load or by expression of mutant proteins that enter the ER but cannot fold properly. Accumulation of mis- or unfolded proteins within the ER results in the activation of the unfolded protein response (UPR). Three distinct classes of ER-transmembrane sensors—comprised of i) ATF6 (activating transcription factor 6), ii) IRE1 (inositol-requiring enzyme 1), and iii) PERK (PKR-like ER kinase)—sense the condition in the ER and regulate the UPR in metazoans (Ron and Walter, 2007). Together, these pathways activate an adaptive transcriptional program that adjusts ER abundance and its folding capacity according to need. If ER stress remains unmitigated and homeostasis is not re-established, the UPR switches from a cytoprotective function to apoptosis (Lin et al., 2007), perhaps as a means to prevent cells from secreting or displaying on their surface malfunctioning signaling molecules that might endanger multicellular organisms.

The transcription factors ATF6 and XBP1, a downstream effector of IRE1, coordinate the expression of chaperones and foldases, as well as genes required for ER expansion and ER-associated degradation (ERAD) (Lee et al., 2003; Okada et al., 2002), leading to adaptation. Likewise, the transcription factor ATF4, a downstream effector of PERK, activates genes involved in amino acid

metabolism and redox homeostasis, as well as the transcription factor C/EBP homologous protein (CHOP) (Harding et al., 2003). At the same time, signals emanating from the UPR sensors minimize protein load in the ER. IRE1, for example, also mediates degradation of ER-bound mRNAs through a process known as regulated IRE1-dependent decay (RIDD) (Han et al., 2009; Hollien et al., 2009), and PERK globally reduces protein synthesis by phosphorylation of the translation initiation factor eIF2α (Harding et al., 2000).

CHOP expression is linked to ER stress-induced apoptosis (Zinszner et al., 1998), yet its role in the UPR extends beyond this function. For example, CHOP regulates transcription of GADD34, a component of a phosphatase acting on eIF2α to restore translation following PERK activation (Marciniak et al., 2004) as well as ODZ4, a plasma membrane protein essential in development (Wang et al., 1998).

Thus the three branches of the UPR form a complex network through which signals are processed to produce a stress and cell type appropriate response. Here we further increase this complexity in mammals through the discovery of the UPR controlled miRNA, miR-708. Our evidence suggests that miR-708 helps balance the ER protein folding capacity with the load of newly synthesized rhodopsin molecules entering the ER during retinal development.

Materials and Methods

Cells, cell culture and drug treatments

SV40 large T antigen transformed human embryonic kidney cells 293T (kind gift of M. Bassik, J. Weissman lab, UCSF), spontaneously immortalized wild-type mouse embryo fibroblasts (MEFs) (kind gift of L. Glimcher, Harvard Univeristy School of Public Health), and MEFs derived from CHOP deficient animals and their wild-type genetic counterparts (kind gift of D. Ron, New York University School of Medicine) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS), 2 mM L-glutamine and penicillin/streptomycin. For induction of ER stress, cells were treated with thapsigargin (Sigma-Aldrich) or tunicamycin (EMD Chemicals).

miRNA expression profiling

Total RNA was prepared using TRIzol reagent (Invitrogen) following manufacturer's recommendations. Sample preparation, labeling, and array hybridizations were performed according to standard protocols from the UCSF Shared Microarray Core Facilities and Agilent Technologies (http://www.arrays.ucsf.edu and http://www.agilent.com). Total RNA quality was assessed using a Pico Chip on an Agilent 2100 Bioanalyzer (Agilent Technologies). RNA was labeled with Cy3-CTP using the miRCURY LNA microRNA power labeling kit (Exiqon), according to manufacturer's protocol. Labeled RNA was hybridized to Agilent custom UCSF miRNA v3.4 multi-species

8 × 15K Ink-jet arrays. Hybridizations were performed for 16 h. Arrays were scanned using the Agilent microarray scanner and raw signal intensities were extracted with Feature Extraction v10.1 software (Agilent). The dataset was normalized using the *quantile* normalization method (Bolstad et al., 2003). The median feature pixel intensity was used as the raw signal before normalization. All procedures were carried out using functions in the R package *limma in Bioconductor* (Gentleman et al., 2004).

RNA protection assays, semi-quantitative PCR (RT-PCR), real-time RT-PCR (qRT-PCR) and TaqMan miRNA assay

Total RNA was prepared using TRIzol reagent (Invitrogen) following the manufacturer's recommendations. For the RNase protection assay, both the mirVana miRNA probe construction kit and the mirVana miRNA detection kit were used (Ambion). The miR-708-specific probe was generated using the oligonucleotide 5'- AAGGAGCTTACAATCTAGCTGGGCCTGTCTC-3' as a template for *in vitro* transcription. Gel-purified probes were then used for hybridization, digestion, and precipitation. The protected fragments were resolved on 15% polyacrylamide/8M urea gels and visualized using a phosphorimager. Densitometric analyses of digital images were performed with Image J (NIH).

RT-PCR and qRT-PCR were performed on total RNA prepared using TRIzol reagent. 250 ng of RNA were reverse transcribed with the SuperScript III First-

Strand Synthesis System for RT-PCR kit (Invitrogen) and 1% of the resulting cDNA was utilized for PCR reactions with gene-specific oligonucleotide primers. Forward and reverse primers are as follows: 5'-GAGCCAGACCACTCGGCCCT-3', 5'-GCCGGGTCAGCGAGCGATAG-3' (mouse *Odz4*); 5'-CTGCCTTTCACCTTGGAGAC-3', 5'-GATGTGCGTGTGACCTCTGT-3' (mouse *Chop*); 5'-GAACCAGGAGTTAAGAACACG-3', 5'-AGGCAACAGTGTCAGAGTCC-3' (mouse *Xbp1*, total); 5'-ATAAACCCCGATGAGGCTGT-3', 5'-AGCAGGAGGAATTCCAGTCA-3' (mouse *Grp78*); 5'-GCCATCCATAGCAAGGTTGT-3', 5'-GCCTCTTTACATGGGCTTTG-3' (mouse *Rps26*); 5'-CAGCTTCTTTGCAGCTCCTT-3', 5'-CACGATGGAGGGGAATACAG-3' (mouse *Actin beta*); 5'-CACTTGGAGGTGAAATCGCCC-3', 5'-TCCAGGTGAAGACCACACCC-3' (mouse Rho); 5'-AGCCACACCGCTCAGACAC-3', 5'-TGGAAGATGGTGATGGGATT-3' (human *GAPDH*). RT-PCR reactions were resolved on 2% agarose gels stained with SYBR safe (Invitrogen). Densitometric analyses of digital images were performed with the Image Analysis software Image J (NIH). qRT-PCR reactions were performed using iQ SYBR Green Supermix (Bio-Rad) and run in a DNA Engine Opticon 2 cycler (Bio-Rad) using the Opticon Monitor v3 software (Bio-Rad). For TaqMan miRNA assays, 500 ng of total RNA was reverse transcribed with miRNA-specific primers (hsa-miR-708 and snoRNA 202) using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) following manufacturer's recommendations. Reactions

were run in a DNA Engine Opticon 2 cycler using the Opticon Monitor v3 software.

Generation of stable cell lines, immunoprecipitations, transient transfections and immunoblotting.

The fragment encoding FLAG-tagged Ago2 of human origin was excised from pIRES-neo FLAG/HA-Ago2 (kind gift of T. Tushl) with EcoRI and HindIII and subcloned into the corresponding sites of the retroviral vector pLPCX (Clontech). Retroviruses were produced in Phoenix ecotropic cells (Orbigen) and high-titer retroviral supernatants were used to transduce 3T3 cells. Stable expressors were selected by culturing the transduced cells in the presence of puromycin. To immunoprecpitate Ago2, cells expressing FLAG-Ago2 were lysed in 0.5% NP-40, 150 mM KCl, 25 mM Tris-HCl pH 7.4, 5 mM DTT supplemented with Complete Protease Inhibitor tablets (Roche) and 100 u/ml SUPERasIN (Ambion) for 30 min on ice. Following centrifugation at 14,000 rpm at 4°C for 30 min, 1 mg of lysate was incubated with anti-FLAG M2-Agarose (Sigma) in a rotating platform at 4°C for 4 h, and the immune complexes were recovered by washing 4 times with 0.5% NP-40, 150 mM KCl, 25 mM Tris-HCl pH 7.4. To assess the abundance of miRNAs in the immunoprecipitates, total RNA was isolated using TRIzol reagent and used for TaqMan miRNA assays. The efficacy of the immunoprecipitation was determined by immunoblotting after separation of immunoprecipitates on 10% SDS-PAGE gels. The resolved peptides were transferred onto nitrocellulose

membranes and probed with a mouse monoclonal anti-Ago2 antibody (ab57113, AbCam).

Transient transfections in 293T cells with plasmids encoding rhodospin (pSPORT6-mRHO, Open Biosystems) and GFP (pcDNA3.1/NT-GFP, Invitrogen) along with a miR-708 antagomir or mimic were performed with Lipofectamine 2000 (Invitrogen) following manufacturer's recommendations. miR-708 antagomir or mimic, including their respective negative controls, were purchased from Ambion: 'Anti-miR inhibitor' miR-708 (AM11161), 'Cy3-labeled Anti-miR negative control' scramble (AM17011), 'Pre-miR miRNA precursor' miR-708 (PM11161), and 'Cy3-labeled Pre-miR negative control' (AM17120). Immunodetection of rhodospin expressed in 293T cells was performed 36 h following transfection. Cells were lysed in RIPA buffer (50 mM Tris HCl pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) for 30 minutes at 4°C and clarified for 5 minutes. Lysates were separated on 4-12% SDS-PAGE gels and transferred onto nitrocellulose membranes which were then probed with a mouse monoclonal 1D4 anti-RHO (Abcam), a mouse monoclonal anti-GFP (Roche), a rabbit polyclonal anti-GAPDH (Abcam), or a rabbit polyclonal anti-BiP (Cell Signaling Technology) antibodies. For pulse-labeling experiments, 293T cells were transfected with the abovementioned plasmid encoding rhodopsin along with either the antagomir or scrambled control, pulsed-labeled with 300 μCi ^{35}S-methionine for 1 h before lysis in RIPA buffer. 2 μg of 1D4 anti-RHO antibody

were added to lysates, incubated for 2 h in a rotating platform at 4°C, followed by the addition of Affi-Prep Protein A Support (Bio-Rad) for an additional 2 h to immunoprecipitate radiolabeled RHO. Immune complexes were washed 3 times with RIPA buffer, boiled for 3 minutes and separated on a 4-12% SDS-PAGE gel. Autoradiograms were visualized using phosphorimager and quantified using the image analysis software Image J (NIH).

Results

CHOP controls miR-708 transcription during prolonged ER stress

To determine if CHOP regulates expression of a subset of miRNAs, we induced ER stress in *Chop* +/+ and *Chop* -/- mouse embryo fibroblasts (MEFs). miRNA expression profiles performed with RNA extracted from these cells revealed 12 miRNAs that were differentially expressed by greater than 2-fold during ER stress (Fig. 2-1A; Supplemental Fig. 2-1). Eight miRNAs of this cadre exhibited an increase in expression 24 hours after induction of ER stress (miR-689, miR-708, miR-711, miR-1867-3p, miR2137, miR-762, miR-712*, miR-2132), while three showed a decrease in expression (miR-503, miR-351, miR-322). Interestingly, expression of only one of these, miR-708, was strictly dependent on CHOP (Fig. 2-1B). miR-708 levels increased greater than 3-fold with the addition of either Tm or Tg in *Chop* +/+ MEFs, and this induction was not observed for either drug in *Chop* -/- MEFs (Figs. 2-1AB). Expression of miR-708 was restricted to a late window upon induction of ER stress. Indeed, after only 10 hours, 3T3 mouse

fibroblasts in which we induced ER stress under identical conditions showed no significant expression changes of miR-708, or any other miRNA (Supplemental Fig. 2-2).

To validate the conclusions drawn from the microarray data, we examined the expression levels of miR-708 using an RNase protection assay. The analyses confirmed the ER-stress mediated activation of miR-708 expression and its late onset (Fig. 2-1C). miR-708 expression increased 11-fold in MEFs treated with tunicamycin (Tm) and 8-fold in MEFs treated with thapsigargin (Tg), two different UPR inducers. Interestingly, the increased expression of miR-708 was delayed when compared to canonical markers of UPR activation, such as the induction of the ER chaperone *Grp78* or the splicing of *Xbp1* mRNA (Supplemental Fig. 2-3), indicating that expression of miR-708, like that of CHOP, is associated with a late-phase UPR.

Taqman miRNA assays detected the mature form of miR-708 in *Chop* +/+ and *Chop* -/- MEFs undergoing prolonged ER stress (Fig. 2-1D). Corroborating the RNase protection data, we observed an 11-fold increase in miR-708 expression in *Chop* +/+ MEFs but not in *Chop* -/- MEFs. Together, our data show that miR-708 expression is regulated by CHOP during the late-phase of the ER stress response.

mir-708* is an intronic miRNA residing within the CHOP-inducible gene *Odz4

The gene encoding miR-708 resides in intron 1 of *Odz4*, an evolutionarily conserved gene (Fig. 2-2A). The encoded protein, ODZ4, is the vertebrate homolog of the teneurins, which are cell surface signaling molecules important in a plethora of developmental processes (Ben-Zur et al., 2000). *Odz4* was originally characterized as one of several genes regulated by CHOP (Wang et al., 1998). mir-708 may therefore be an indirect transcriptional target of CHOP carved out of the *Odz4* transcript produced late upon UPR induction (Fig. 2-1).

Bioinformatics analyses indicated strict evolutionary conservation of mir-708 in mammals only, suggesting that any functional importance of mir-708 is restricted to this clade (Fig. 2-2B). Further sequence alignments failed to identify homologs of mir-708 in other vertebrates. As *Odz4* and its ancestral homologs are conserved in bilateral animals (Fig. 2-2C), mir-708 appears to be more recently evolved. Its localization within *Odz4* suggests that mir-708 has co-opted the ER stress-dependent regulation of *Odz4*. Therefore, we asked if miR-708 is indeed co-expressed with *Odz4*. RT-PCR analyses in *Chop* +/+ and *Chop* -/- MEFs subjected to ER stress indicated a CHOP-dependent expression of *Odz4* (Fig. 2-2D) that mirrored the kinetics of accumulation of miR-708 (compare Figs. 2-1C, 2-1D, and 2-2D). Like miR-708, *Odz4* expression occurred late after induction of ER stress, indicative of transcriptional regulation fitting with the kinetics of

accumulation of CHOP. Moreover, expression of miR-708 correlated well with the expression of *Odz4* in adult mouse tissues (Fig. 2-2E). Strikingly, we observed a significant accumulation of both transcripts in tissues of neuroectodermal origin (e.g. brain and eyes), strongly suggesting a physiological role for miR-708 in tissues where *Odz4* is expressed.

miR-708 is loaded on the RISC

Since miR-708 is induced by ER stress, we reasoned that a functional, mature form of the miRNA should be loaded onto the RISC. Argonaute 2 (Ago2) is an essential component of the RISC, which is required for miRNA-directed post-transcriptional regulation. To test whether miR-708 is loaded onto the RISC, we performed immunoprecipitations in 3T3 cells stably expressing Ago2 tagged with the FLAG epitope (FLAG-Ago2) followed by Taqman-based detection of miR-708 (Fig. 2-3A). Analyses in untreated cells revealed a 75-fold enrichment of miR-708 loaded onto the RISC, indicating that even the low steady state level of miR-708 is efficiently loaded onto the complex (Fig. 2-3B). In ER-stress induced cells, the enrichment by immunoprecipitation resulted in a 500-fold increase in RISC-associated miR-708 indicating that the strong transcriptional induction of mir-708 upon ER stress results in a significant increase of miR-708 loaded onto the RISC (Fig. 2-3B). Together, these results show that miR-708 is engaged with the cellular components expected for a functional miRNA.

Rhodopsin is a functional target of miR-708

To address the biological role of miR-708, we used the miRNA target prediction program TargetScan (Friedman et al., 2009; Lewis et al., 2005) to generate a list of candidates (Supplemental Table 2-1). Gene ontology analyses on the predicted targets revealed a notable enrichment of genes involved in phototransduction (*RHO, RCVRN*), or in retinal maintenance/development (*RHO, RCVRN, AAK1, GPM6A*). Since Taqman-based analyses revealed enhanced expression of *Odz4* and miR-708 in the eyes (Fig. 2-2E), we reasoned that rhodopsin may be targeted by miR-708. Since rhodopsin is a multi-spanning transmembrane protein, UPR-regulated miR-708 expression may serve to keep its synthesis aligned with the protein folding capacity of the ER (see Discussion). Moreover, bioinformatics analyses revealed a highly conserved, putative miR-708 binding site in the 3'UTR of rhodopsin, which, like miR-708 itself, is highly conserved among mammals (Fig. 2-4A).

To test this notion, we asked whether miR-708 regulates the expression of rhodopsin. To this end, we performed loss-of-function experiments by transiently transfecting 293T cells with a plasmid encoding full-length rhodopsin and a control plasmid encoding GFP along with a chemically modified single-stranded antisense inhibitor (antagomir) that directly hybridizes to miR-708 or a scrambled control. In complimentary gain-of-function experiments, we co-transfected the abovementioned plasmids along with dsRNA designed to mimic the miR-708-

miR-708* (‘Pre-miR’) duplex, or a dsRNA duplex scrambled control. Since 293T cells exhibit much higher basal levels of miR-708 than MEFs (Fig. 2-4B), we expected that it would allow us to examine the effects of miR-708 on rhodopsin expression even in the absence of ER stress to boost its expression. Indeed, altering the levels of endogenous miR-708 with the antagomir resulted in a net accumulation of rhodopsin of almost 7-fold (Fig. 2-4C, left) and a net increase in the mRNA encoding it as compared to the scrambled control (Fig. 2-4D, left). Conversely, addition of a miR-708 mimic resulted in a rhodopsin decrease of more than 2.5-fold (Fig. 2-4C, right) and an accompanying decrease of the mRNA encoding it (Fig. 2-4D, right). Notably, GFP levels were unaffected by either the miR-708 antagomir or mimic, indicating that the effects observed were specific to rhodopsin mRNA. To observe miR-708’s effect on newly synthesized rhodopsin, we performed a pulse-labeling experiment in 293T cells transiently transfected with a plasmid encoding the full-length rhodopsin along with either an antagomir or scrambled control. Consistent with our immunoblotting data, we observed a net increase of rhodopsin (~2.1-fold) in cells transfected with the antagomir (Fig. 2-4E). Taken together, our results show that miR-708 targets rhodopsin mRNA, resulting in its decreased expression in mammalian cells.

Discussion

Here we show that the intronic miRNA miR-708 is regulated by ER stress and provide evidence that one of its roles is to control expression of rhodopsin. mir-

708 resides within the first intron of *Odz4*, a target of the ER-stress regulated transcription factor CHOP (Wang et al., 1998). *Odz4* and its paralogs (*Odz1-3*) encode large surface transmembrane proteins required in developmental processes likely involved in the regulation of neurite growth, cell adhesion, and retinal development (Kinel-Tahan et al., 2007; Zhou et al., 2003). They are part of the ancestral teneurin gene family, which are ubiquitously expressed in the developing nervous system (Young and Leamey, 2009). Indeed, Odz4 is expressed in the developing eye (Ben-Zur et al., 2000) as well as in the adult brain and eyes (Fig. 2-2E). While little is known about the role of *Odz4* during ER stress, our data show that miR-708 expression is co-regulated with *Odz4* downstream of CHOP, thereby linking its regulation to the UPR.

miR-708 evolved late in evolution, lacking identifiable homologs outside of mammals. Thus, it is attractive to speculate that hitchhiking along the CHOP-regulated ancestral *Odz4*, mir-708 acquired ER stress-regulated expression of its own. Our results support coupled expression of *Odz4* and miR-708 in cells undergoing ER stress, and the marked co-expression of miR-708 and *Odz4* in brain and eyes suggests a physiological function of miR-708 in the same tissues (Lutter et al., 2010). As such, miR-708 joins other miRNAs that hitchhike in introns of pre-mRNAs, such as miR-33 encoded within an intron of *SREBP*, a gene encoding a transcription factor important in the cholesterol biogenesis pathway (Marquart et al., 2010; Najafi-Shoushtari et al., 2010; Rayner et al.,

2010). Similarly, the identification of rhodopsin as a target of miR-708 links ER-stress, and the PERK pathway through CHOP, to the regulation of rhodopsin biosynthesis in the eye.

Bioinformatics analyses suggest miR-708 targets several genes involved in the visual system. We focused on rhodopsin since the newly synthesized transmembrane protein must traverse the secretory pathway, heavily relying on ER function for its delivery to the disc membrane of outer segments in rod photoreceptor cells (Mendes et al., 2005). Indeed, a modest increase in *Chop*, and to a lesser extent *Grp78*, are observed in the developing retinas of rats (Lin et al., 2007), indicating heightened ER function and activation of the UPR. This suggests that an upsurge in *Odz4* and mir-708 transcripts may have also followed, although future experiments are required to address such expression changes. Thus, it is plausible to assume that miR-708 may have evolved as an additional safeguard mechanism controlling the synthesis of rhodopsin, thereby balancing demand with protein folding capacity of the ER ensuring homeostasis. In this way, miR-708 function would be conceptually similar to that of the RIDD pathway (Han et al., 2009; Hollien et al., 2009), which reduces protein influx by degrading membrane associated transcripts, or eIF2α phosphorylation in the PERK pathway, which achieves the same goal by down-tuning translation (Harding et al., 2000).

Moreover, miR-708 may also play an important role in retinal degenerative diseases that lead to blindess (e.g. retinitis pigmentosa). In some cases of retinitis pigmentosa (RP), single missense mutations compromise the folding and trafficking of rhodopsin leading to retention in the ER and eventual photoreceptor cell death (Anukanth and Khorana, 1994; Tam and Moritz, 2006). In such instances, UPR hyperactivation has been implicated in the apoptotic fate of the photoreceptor (Kosmaoglou et al., 2009). Indeed, genetic models of retinitis pigmentosa show a late-phase burst of *Chop* activation (Lin et al., 2007), which, coupled to increased miR-708 production, may be a last attempt to reinstate homeostasis on an already burdened ER. It will be interesting to explore whether less severe folding mutations are silent only because miR-708 keeps mutant rhodopsin expression levels low enough to prevent or delay cell death. Together, our data assign CHOP a cytoprotective function beyond its pro-apoptotic role. CHOP therefore resides at the vertex of a complex bi-phasic equilibrium contributing to the delicate balance that determines the outcome of the life/decision made be the UPR.

Acknowledgments

We thank members of the Walter laboratory for their critical review of the manuscript, both Walter lab members and David Ron for thoughtful insights and essential reagents, and Han Li and Fran Sanchez for technical support. We also thank the members of the Sandler Asthma Basic Research (SABRE) Center Functional Genomics Core Facility, Rebecca Barbeau, Andrea Barczak, and David Erle, for technical support of miRNA expression arrays and analysis. Array experiments were funded in part by NIH/NCRR UCSF-CTSI Grant number ULI RR024131. We thank Carolina Arias for assistance in the analysis and visualization of microarray data and Jiashun Zheng for assistance in bioinformatics analysis. This worked is supported by a National Science Foundation predoctoral fellowship (S.L.B.) and the Irvington Institute Postdoctoral Fellowship of the Cancer Research Institute (D.A.A.). P.W. is an investigator of the Howard Hughes Medical Institute.

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Figure Legends

Figure 2-1. CHOP regulates miR-708 expression during ER stress

A) Heatmaps and Venn diagrams illustrating miRNAs differentially regulated during ER stress in CHOP +/+ and CHOP -/- MEFs. The applied criterium for differential expression (DE) was a more than 2-fold change (FC) in treated versus untreated conditions, represented as logarithmic values in the heatmap. Red, increase in DE during ER stress; Green, decrease in DE during ER stress. miR-708 was the only miRNA that did not show any expression changes in *Chop* -/- MEFs. Cells were treated with 5 μg/ml tunicamycin (Tm) or 500 nM thapsigargin (Tg) for the indicated time. B) Scatter plots illustrating the changes in expression of the miRNAs in panel A. miR-708 is indicated by the arrowhead. C) RNase protection assay in *Chop* +/+ MEFs treated with 5 μg/ml Tm or 500 nM Tg for 24 h. Loading control: miR-16. Bottom panel indicates a densitometric quantification of the data (miR-708/miR-16). Error bars: standard deviations from two independent experiments. D) Taqman miRNA assay of miR-708 in *Chop* +/+ and *Chop* -/- MEFs treated with 5 μg/ml Tm. Data was normalized to snoRNA 202. Error bars: standard deviations from three independent experiments.

Figure 2-2. miR-708 is a conserved intronic miRNA highly expressed in neuroectodermal tissues.

A) Schematic representation of the locus encoding *Odz4* indicating that mir-708 resides within the first intron of the host gene. UCSC Genome Browser

conservation in mammals is shown below the diagram. B) Sequence alignment of the mir-708 hairpin in mammals (Mmu, mouse; Rno, rat; Hsa, human; Ppy, orangutan; Cfa, dog; Eca, horse). The guiding strand (miR-708) and passenger strand (miR-708*) sequences are outlined in black boxes. (B) Phylogenetic tree of bilateral animals indicating the conservation of the ancestral Odz/Teneurin homologs in different clades. Chop and miR-708 appear later in evolution with Chop homologs annotated in amphipians and mammals. miR-708 homologs are found only in mammals (blue box). D) Semi-quantitative RT-PCR analyses in *Chop* +/+ and *Chop* -/- MEFs treated with 5 μg/ml Tm for 24 h. Both *Chop* and *Odz4* transcripts are not detected in *Chop* -/- MEFs. Relative abundance of *Grp78* mRNA indicates activation of the UPR. Loading control: *Actin beta*. E) Gene expression analyses of miR-708 (Taqman miRNA assay) and *Odz4* (qRT-PCR) in adult mouse tissues normalized to snoRNA 202 and *Rps26*, respectively.

Figure 2-3. Mature miR-708 is loaded on the RISC

A) Immunoprecipitation of Ago2 tagged with the FLAG epitope (FLAG-Ago2) from 3T3 fibroblasts stably expressing it. Right panel, identical experiment in 3T3 cells transduced with an empty vector. FT, flow-through. B) Taqman miRNA assay of miR-708 from FLAG-immunoprecipitated fractions obtained from lysates of the cells in panel A. Error bars: standard deviations from two independent experiments. * p value <0.0005, ** p value <0.008

Figure 2-4. miR-708 targets rhodopsin for post-transcriptional inhibition

A) Top panel: Schematic representation of full-length mouse rhodopsin mRNA. Red box, putative miR-708 binding site in the 3'UTR. Bottom panel: sequence alignment of the region containing the predicted conserved miR-708 site in different mammals. Red box, seed sequence; black dotted line encircles entire putative site. Mmu, mouse; Hsa, human; Ptr, chimpanzee; Mml, rhesus; Rno, rat; Ocu, rabbit; Cfa, dog; Fca, cat; Bta, cow; Dno, armadillo. B) Relative basal levels of miR-708 in each cell line (Taqman miRNA assay). Error bars: standard deviations from three independent experiments. * p value < 0.02 C) Western blots of lysates from 293T cells transfected with plasmids encoding full-length mouse *Rho* and GFP along with a mir-708 mimic or an antisense inhibitor (antagomir) targeting mir-708. Overexpressed RHO expectedly formed aggregates when resolved in SDS-PAGE. BiP was used to show activation of the UPR. GFP was used as control for transfection efficiency and off-target effects of the antagomir/mimic. Loading control: GAPDH. Numbers indicate the relative fold-changes in expression normalized to GAPDH. Scrambled, negative control for antagomir; Anti-miR-708, antagomir for miR-708; Pre-miR NC, negative control for miR-708 mimic; Pre-miR-708, miR-708 mimic. D) Semi-quantitative RT-PCR analysis from the experiment in panel C. Numbers indicate the relative expression levels of *Rho* mRNA normalized to *GAPDH* mRNA**.** E) Autoradiograms from 239T cells transfected with a plasmid encoding full-length

mouse *Rho* along with and antagomir or scrambled control and pulse-labeled with ^{35}S-methionine (^{35}S-Met) for 1 hour. Left panel: lysates immuoprecipitated with an anti-rhodopsin antibody. Right panel: total lysates. Numbers indicate relative amounts of radiolabeled rhodopsin normalized to total lysate.

Supplemental Data

Supplemental Figure 2-1. miRNA expression profiles of CHOP-deficient cells undergoing ER stress

Heat map of miRNA expression from CHOP +/+ and CHOP -/- MEFs treated with either 5 μg/ml Tm or 500 nM Tg for 24 h. The applied criterium for differential expression (DE) was a more than 2-fold change (FC) in treated versus untreated conditions, represented as logarithmic values. Red, increase in DE during ER stress; Green, decrease in DE during ER stress. Inserts represent the window of miRNAs that met the above criterium.

Supplemental Figure 2-2. Inconspicuous changes in the expression of miRNAs in 3T3 fibroblasts exposed to ER stress for 10 hours

Heat map of miRNA expression from 3T3 cells treated with either 5 μg/ml Tm or 500 nM Tg for 10 h. The applied criterium for differential expression (DE) was a more than 2-fold change (FC) in treated versus untreated conditions, represented as logarithmic values. Red, increase in DE during ER stress; Green, decrease in DE during ER stress. Only miR-1959 and miR-503 showed a decrease in DE. No miRNAs showed an increase in DE.

Supplemental Figure 2-3. Canonical UPR markers are observed relatively early upon induction of ER stress

RT-PCR assay of Chop +/+ MEFs showing splicing of *Xbp1* mRNA and accumulation of the *Grp78* transcript. (S), spliced form of XBP1; (U) un-spliced variant. Loading control, *Actin beta.*

Supplemental Table 2-1. Top 30 candidate target genes of miR-708 defined by TargetScan

Top 30 predicted target genes of miR-708 defined by TargetScan. Gene symbols, names, total context scores, and manually curated gene function/process for each candidate are indicated. Rhodopsin is highlighted in yellow.

* Gene symbols are indicated for human homologs.

** Score is the context score as assigned by TargetScan.

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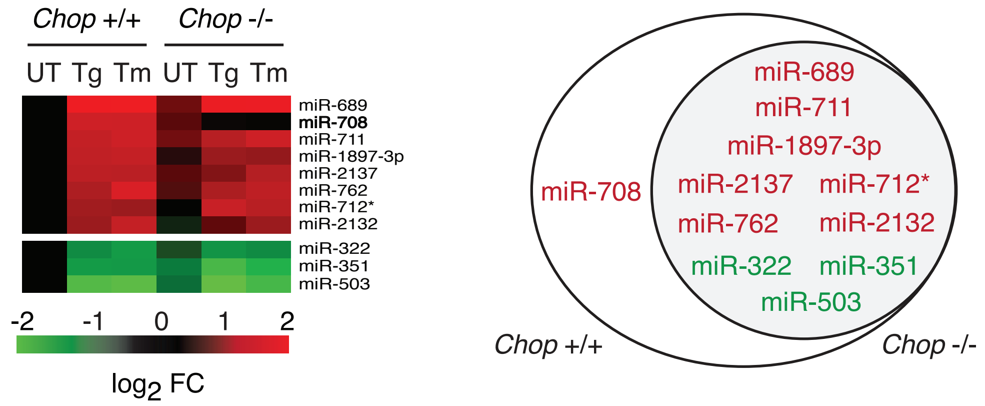

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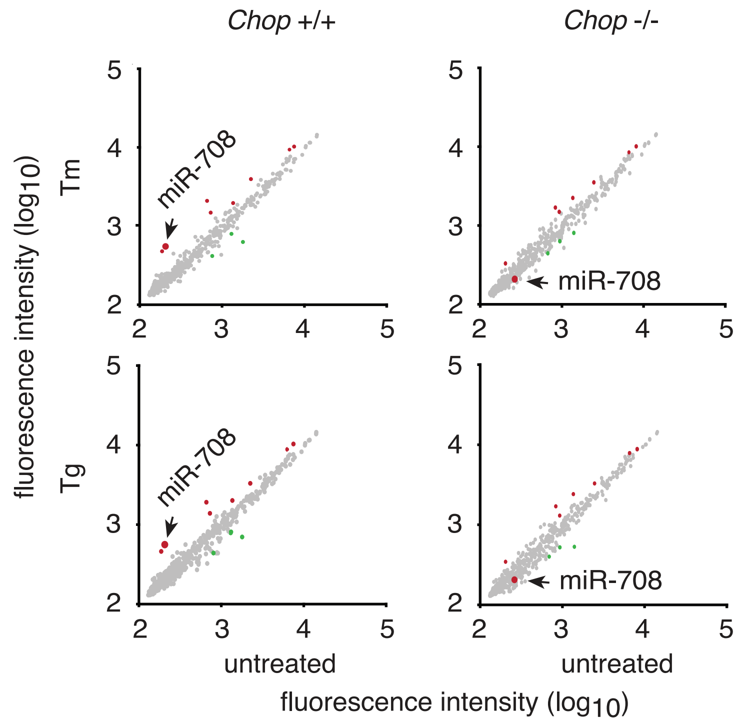
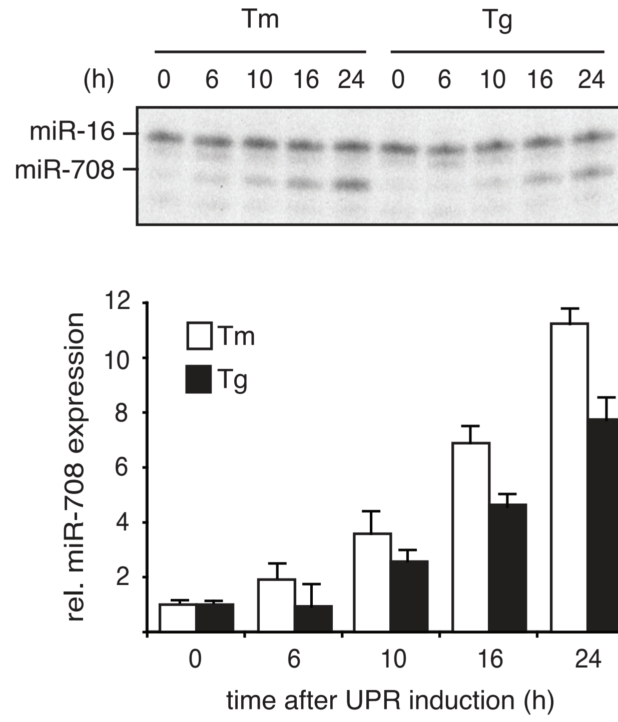


Figure 2-1 AB

C



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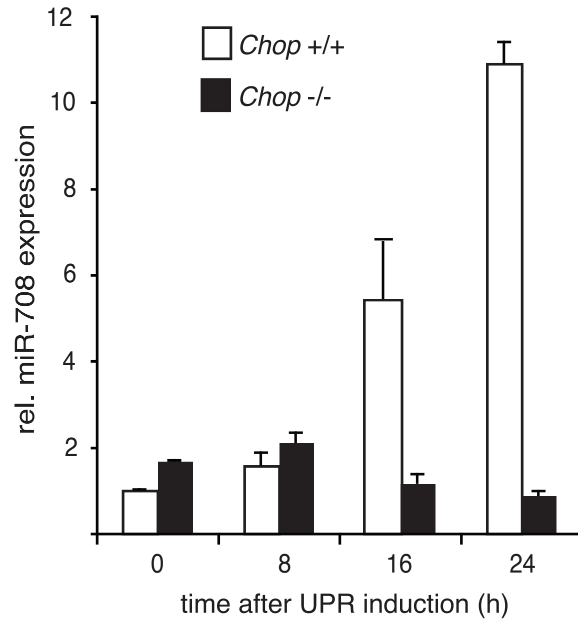

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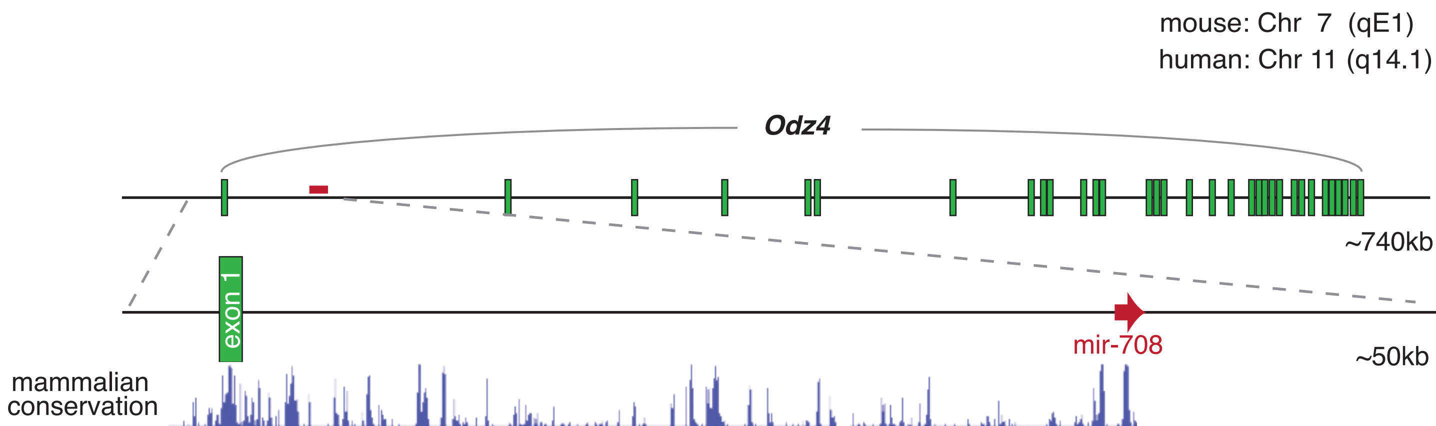

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Figure 2-2 AB

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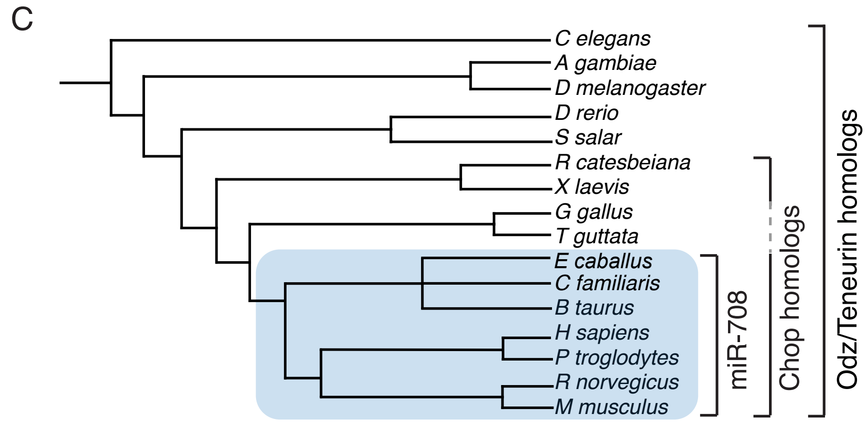

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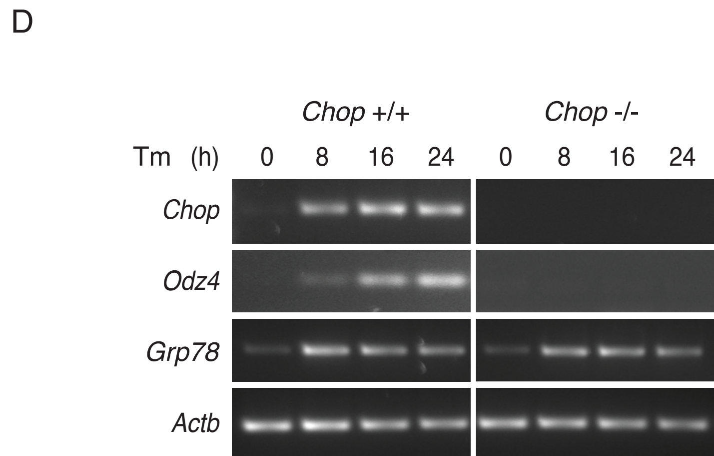

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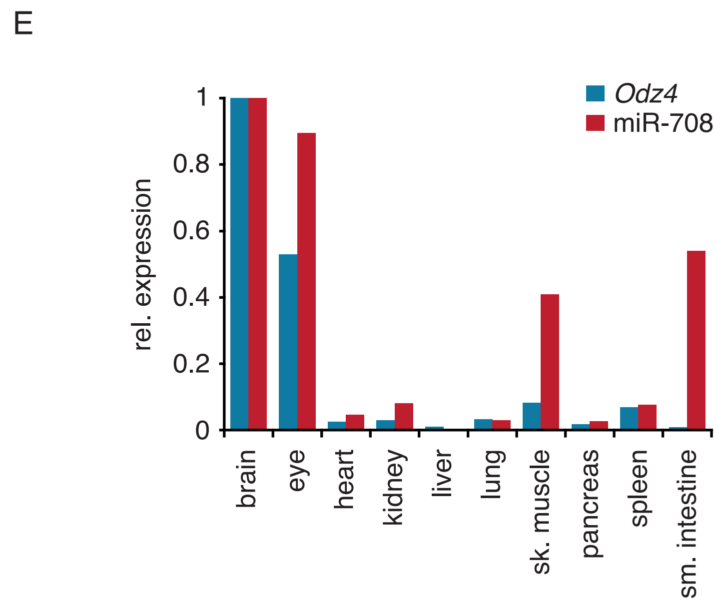

Figure 2-2 CDE

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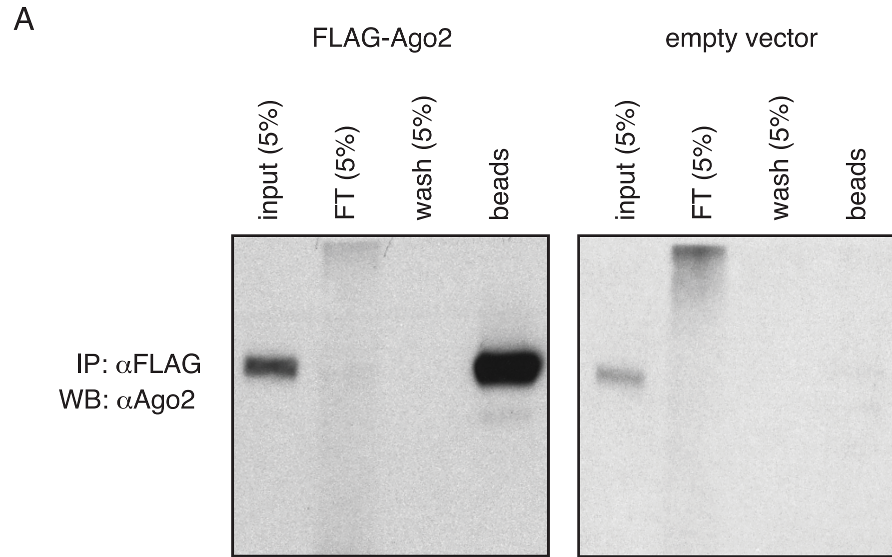

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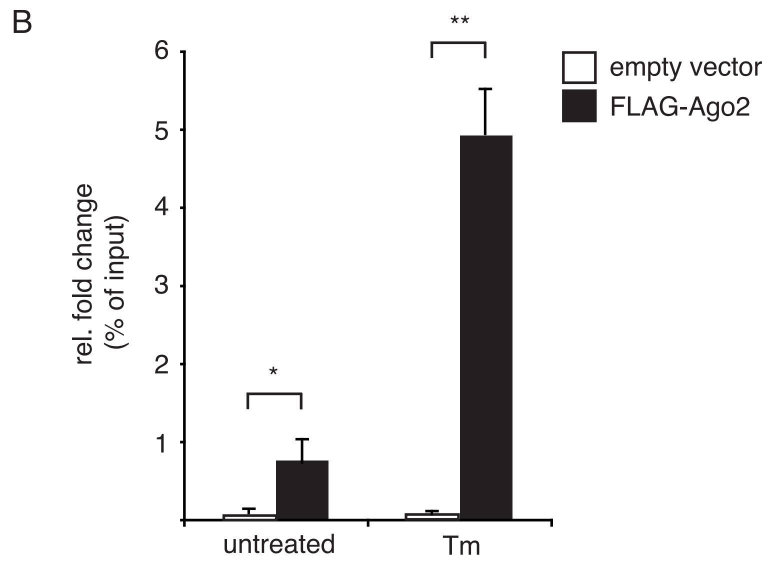
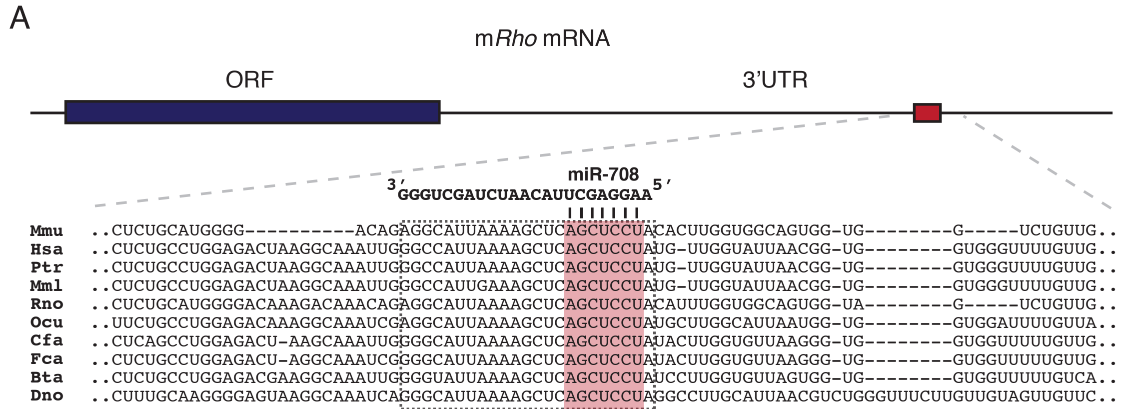
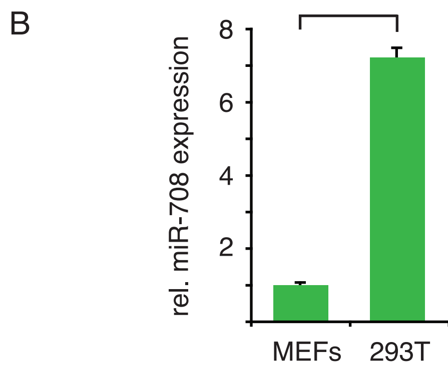
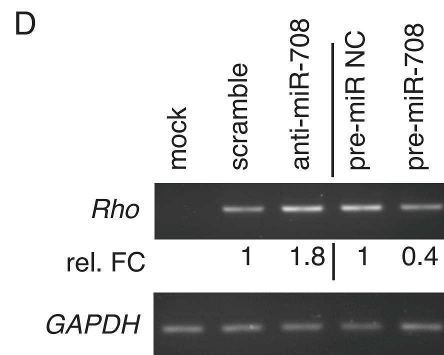
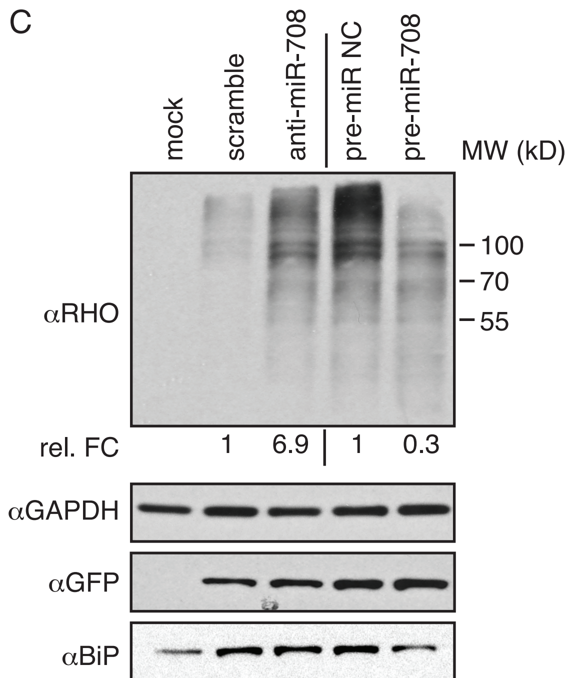


Figure 2-3









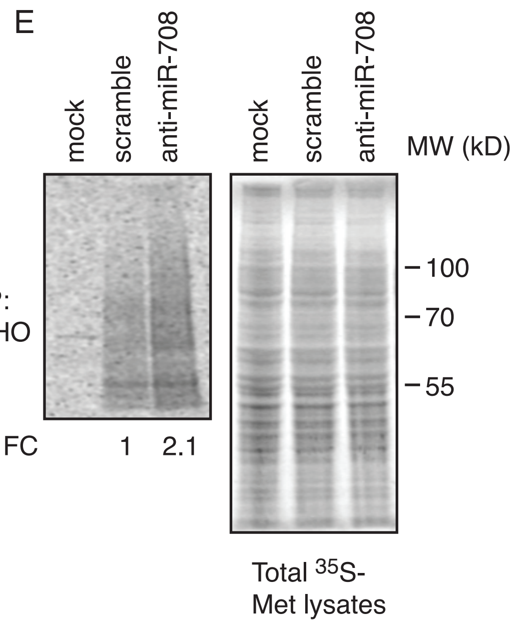
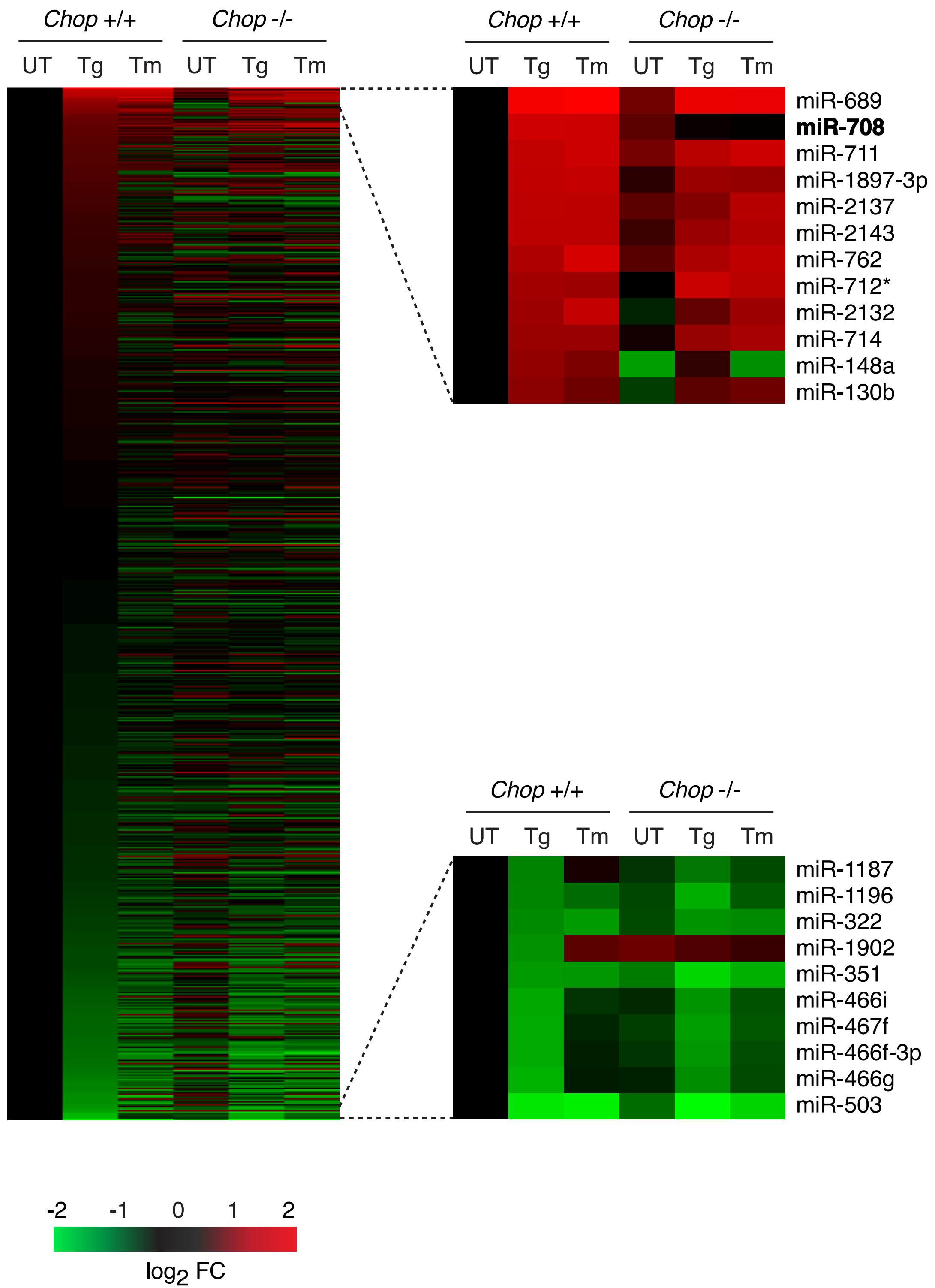
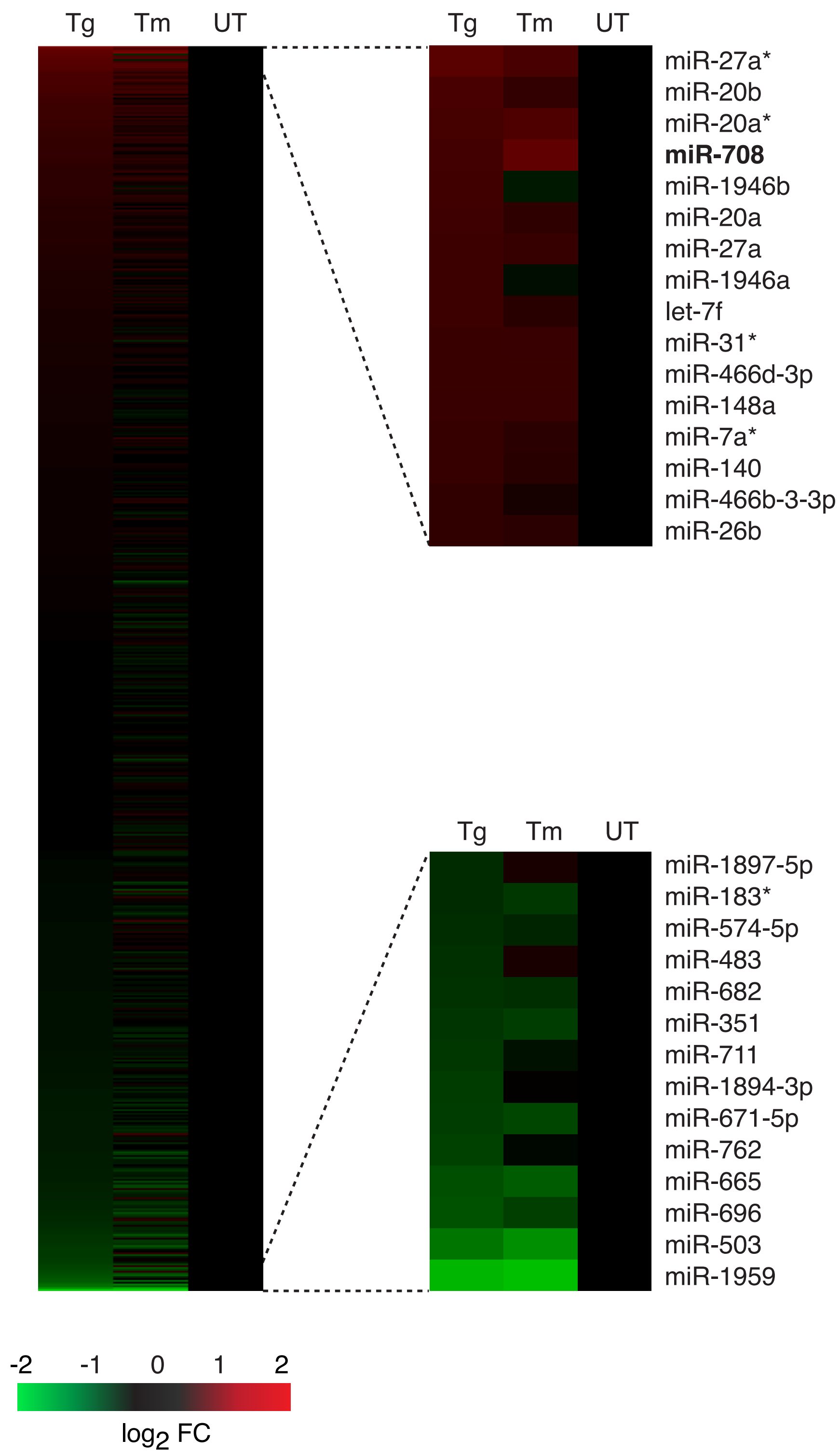


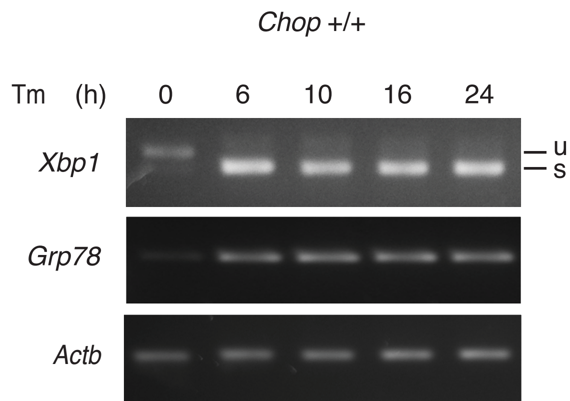
Figure 2-4



Supplemental Figure 2-1



Supplemental Figure 2-2



Supplemental Figure 2-3

TargetScanHuman 5.1 predictions for mmu-miR-708

Gene symbol*	Gene name	Score**	Function/Process
SHPRH	SNF2 histone linker PHD RING helicase	-0.82	DNA repair
KIAA0355	KIAA0355	-0.68	unknown
GPM6A	glycoprotein M6A	-0.44	neuronal processes
BAMBI	BMP and activin membrane-bound inhibitor	-0.43	TGF-β receptor signaling
NNAT	neuronatin	-0.42	brain development
AAK1	AP2 associated kinase 1	-0.41	receptor-mediated endocytosis
IQSEC2	IQ motif and Sec7 domain 2	-0.40	ARF guanyl-nucleotide exchange factor
EN2	engrailed homeobox 2	-0.40	nervous system development
HOXB3	homeobox B3	-0.38	development
RAP1B	RAP1B, member of RAS oncogene family	-0.37	GTPase activity
hCG1757335	hCG1757335	-0.37	unknown
FOXJ3	forkhead boxJ3	-0.37	transcription
CHL	cell adhesion molecule -homology to L1CAM	-0.34	neural cell adhesion
ETF1	eukaryotic translation termination factor 1	-0.33	translation
TMEM200B	transmembrane protein 200B	-0.33	unknown
TNS3	tensin 3	-0.32	cell migration/proliferation
RPGRIP1L	RPGRIP1-like	-0.32	development
DKK3	dickkopf homolog 3 (*X laevis*)	-0.32	inhibitor of Wnt signaling pathway
DGCR14	DiGeorge syndrome critical region gene 14	-0.30	RNA splicing/nervous system dev.
SETDB1	SET domain, bifurcated 1	-0.29	chromatin modification
SLAMF6	SLAM family member 6	-0.29	natural killer cell activation
RHO	rhodopsin	-0.28	phototransduction
RPP14	ribonuclease P/MRP 14kDa subunit	-0.28	tRNA processing
GRIA4	glutamate receptor, ionotrophic, AMPA 4	-0.28	synaptic transmission
SRPRB	signal recognition particle receptor, B subunit	-0.27	putative co-translation
C14ORF101	chromosome 14 ORF 101	-0.27	unknown
CNTFR	ciliary neurotrophic factor receptor	-0.26	nervous system development
RNF165	ring finger protein 165	-0.24	metal ion binding
RCVRN	recoverin	-0.24	phototransduction
C12orf101	chromosome 12 orf	-0.24	unknown

Supplemental Table 2-1

Chapter 3

Summary and Perspectives

It was only in 1993 that the first miRNA, lin-14, was first discovered in *C. elegans* (Lee et al., 1993). Seven years passed before another miRNA, let-7, also in *C. elegans*, came to light (Reinhart et al., 2000). Serendipitously, let-7 is highly conserved among bilateral animals, and this finding sparked the discovery of miRNAs in other organisms including flies and humans. Now, a mere 17 years later, our understanding of the importance of miRNAs in biology and the scope of their regulatory influence continues to expand exponentially, revealing a whole new universe of gene expression control.

Closely paralleling the recent history and burgeoning significance of miRNAs is that of the unfolded protein response. Although much seminal work regarding the discovery and characterization of the ER chaperones BiP and GRP94 occurred in the 1980s, it wasn't until 1992 when the unfolded protein response (UPR) received its designation following the discovery that overexpression of the unfolding variant of the influenza hemagglutinin protein up-regulated both BiP and GRP94 (Gething and Sambrook, 1992). A year later, both Cox et al. from this lab and Mori et al. from the Gething/Sambrook lab identified the first UPR component, Ire1p, in a screen using the cloned UPR promoter element, thus, igniting a rapid discovery of more UPR players (Cox et al., 1993; Mori et al., 1993). Now, much like miRNAs, its importance in biology and scope of influence is also wildly expanding, continuously being linked to an increasing number of developmental processes and diseases. As we are

learning, just about any process that relies on healthy ER function relies on the UPR.

In this study, I merged both the miRNA and UPR fields by identifying a novel ER stress-inducible miRNA, miR-708, whose expression is regulated by the transcription factor CHOP. Genomics analysis revealed that mir-708 resides in the intron of *Odz4*, a gene that is also regulated by CHOP. I validated their CHOP-mediated co-regulation and also showed they have strikingly similar expression patterns with considerable enrichment in neuroectodermal tissue, i.e. the brain and eyes. These findings thus begged the question: Are miR-708 and *Odz4* involved in functionally related ER stress-dependent processes in these tissues? Because the function of *Odz4* was as yet undetermined, I needed to approach this question by defining the role of miR-708. More specifically, I needed to uncover the gene(s) targeted for inhibition by miR-708.

Using the online miRNA target prediction program TargetScan (Lewis et al., 2005), 4 out of 30 top candidate genes were expressed in the retina, an essential component of the eye. Interestingly, one of these retina-related genes was the light-sensing protein, rhodopsin. Rhodopsin was an attractive candidate for miR-708 targeting since its synthesis must heavily rely on ER function, and its expression in both cell culture and in the developing mouse retina was previously linked to the UPR (Lin et al., 2007). Indeed, loss or gain-of-function studies demonstrated that miR-708 controls rhodopsin expression, which prompted the parsimonious speculation that miR-708 may help prevent excessive rhodopsin

load from entering the ER. In that sense, miR-708 may function analogous to other UPR controls that throttle protein influx into the ER, such as general translational down-regulation by PERK or membrane-bound mRNA decay by IRE1. Admittedly, these results were a bit unexpected considering miR-708's late-phase induction is regulated by the 'pro-apoptotic' factor, CHOP. However, as mentioned previously, CHOP's designated role as executioner has incited some previous reconsideration. As we continue to research CHOP and its downstream effectors, such as miR-708, we get a clearer picture for its overall function in the UPR. Together, my data implicated miR-708 and the UPR transcription factor, CHOP, in the homeostatic regulation of ER function in the mammalian visual system.

Undoubtedly, this project has opened several doors along the way with great potential for new discoveries. What proceeds is an outline delineating those questions and suggestions for future directions.

What role does miR-708/Odz4/CHOP play in the physiological context of the retina?

Dissecting the relationship between the UPR, miR-708, and rhodopsin in the physiological context of the mammalian visual system is the next logical step of this project. Without question, miR-708 must be demonstrated to regulate expression of endogenous rhodopsin in the eye. Initially, I propose to characterize rhodopsin, miR-708, *Odz4*, and *Chop* expression in the developing

retinas of rodents to see if they are indeed temporally and spatially coordinated. It's especially important to show correlative expression of miR-708/*Odz4*/*Chop* in the same cells as rhodopsin-expressing cells, i.e. rod photoreceptor cells. If miR-708 is confirmed as coordinately expressed with rhodopsin, then loss- and gain-of-function experiments using antagomirs and mimics, respectively, can address their regulatory relationship, much as I did here in this study with overexpressed rhodopsin.

Retinitis pigmentosa (RP) is a retinal degenerative disorder oftentimes caused by single missense mutations in rhodopsin that muddle its folding, trafficking, or activation (Mendes et al., 2005). Because rhodopsin comprises more than 30% of synthesized protein in rod photoreceptor cells, these mutants create huge disruptions in cellular function, eventually leading to cell death and blindness. Considering the cytoprotective function of the UPR, it will also be interesting to explore if miR-708 affects photoreceptor cell viability in both healthy and RP models. Ideally, an RP model expressing a rhodopsin-misfolding variant that is phenotypically less severe than others should be used. That way, the effect miR-708 may have on cell viability and, therefore, RP pathology would likely be more apparent. Delaying or preventing cell death as a result of repressing misfolded rhodopsin expression could establish miR-708 has a potential drug therapy for RP. As a disease of the eye, potential pharmaceuticals could be administered locally, thus side-stepping many complications that may arise from systemic treatments with UPR modulators.

Aside from rhodopsin, there were 3 other genes expressed in retinas that were also predicted to be targets of miR-708 (Supplemental Table 2-1). If miR-708 is expressed in the retina, then almost certainly it has targets other than rhodopsin. Observing the regulatory effect miR-708 has on these genes could provide even more insight into the general role miR-708 plays in the visual system. Coincidentally, two of those genes, recoverin and AP2 associated kinase 1, are intimately involved in rhodopsin regulation. Recoverin (*RCVRN*) regulates the photoresponse by preventing the endocytosis of light-activated rhodopsin (RHO*) (Gorodovikova et al., 1994). AP2 associated kinase 1 (*AAK1*) regulates the adaptor protein AP2, which has also been linked to RHO*-mediated endocytosis through its interactions with arrestin (Orem et al., 2006).

What other genes does miR-708 regulate?

In Chapter 2, I presented a table of the top 30 predicted targets of miR-708 (Supplemental Table 2-1). Interestingly, there were a handful of genes on that list involved in nervous system development, including rhodopsin (*GPM6A*, *NNAT*, *EN2*, *CHL*, *DGCR14*, *RHO*, *GRIA4*, *CNTFR*, and *RCVRN*). Considering both miR-708 and *Odz4* are predominately enriched in adult brain tissues, these neural-related genes are begging for investigation into possible post-transcriptional regulation as mediated by miR-708. Although the effects were quite modest, very recent *Chop* loss-of-function studies in neuronal cells suggests a cytoprotective role for CHOP in hypoxia-induced neurons (Halterman

et al., 2010), further implicating its downstream effectors, *miR-708* and *Odz4,* as agents of cytoprotection in neurons as well.

Does Odz4 functionally overlap with miR-708?

The findings I made here strongly suggest miR-708 contributes to rhodopsin regulation in the mammalian retina. Assuming a regulatory relationship is established for rhodopsin, miR-708, and *Chop* in the developing retina (as proposed above), then it is highly conceivable *Odz4* functionally overlaps with miR-708 in regulating rhodopsin, or at least in regulating photoreceptor cell homeostasis. *Odz* family members encode cell surface transmembrane proteins whose predicted structure closely resembles that of receptors, hinting at possible involvement in cell-cell signaling (Kinel-Tahan et al., 2007). Loss of function mutations in the *Odz4* homolog in *Drosophila* (*odz*) resulted in depletion of precursor ommatidial cells, photoreceptor cell loss, ommatidial disorder and fusions, as well as several other defects in the developing compound retina. These debilitating defects in the eye, as Kinel-Tahan et al. surmise, suggest the need for *odz* in cell proliferation and/or survival. As we know from my experiments, *Odz4* is also quite abundantly expressed in the eyes of the adult mouse. Ideally, loss- or gain-of-function studies could address the question of *Odz4* function, however, several technical problems arise: (1) *Odz4* -/- mice are embryonic lethal, (2) full-length *Odz4* cDNA is large (~13kb) making it technically challenging to clone into a plasmid and transfect,

(3) most *Odz4* mutants have extremely severe or even lethal developmental phenotypes not necessarily relating to the eye, as this gene is also required for earlier developmental processes (Lossie et al., 2005), and, finally, (4) knocking down the *Odz4* transcript with siRNA or shRNAs could inadvertently down-regulate its intronic partner gene, miR-708. Assuming technical feasibility, I recommend using eye tissue-specific, developmentally controlled conditional knockouts of *Odz4* for loss-of-function experiments.

What are the evolutionary implications of an exclusively mammalian miRNA regulating an ancestral gene?

Bioinformatics analyses suggest the regulation of rhodopsin expression by miR-708 is a recent evolutionary feature since the mature miRNA is only conserved in mammals. This is seemingly irreconcilable insofar as rhodopsin belongs to the ancient gene family comprised of opsins. Recent structural and functional comparisons of opsin subfamilies have come to light which calls into question how ancestrally similar opsin subfamilies truly are (Nilsson, 2005; Terakita, 2005). For instance, beyond a common topology of seven transmembrane segments, vertebrate rhodopsin shares very little sequence homology with invertebrate rhodopsin, nor do they share homology in the visual systems that encase them. These differences in visual systems include the morphology of the photoreceptor cell and membranes, ontogeny, and signal transduction components (Nilsson, 2005). Thus, the current thinking is that both

vertebrate and invertebrate rhodopsin fall into quite different ancestral subclasses. Taking such homology comparisons into consideration, it is easy to see how the evolutionary gap between the appearance of rhodopsin and its regulation by miR-708 may not be so much a of conundrum but rather pose an appealing biological question aimed at establishing the differences between mammals and other vertebrate clades where CHOP has evolved (e.g. amphibians and mammals). Although I was only able to bioinformatically detect the mature miR-708 in mammals, it remains possible that ancestral forms of miR-708 pre-dating the evolution of mammals may control the expression of opsins in taxonomic groups. Along the same line of thought, divergent evolution may hold the key as to the regulation of rhodopsin expression in other vertebrates.

What about the other miRNAs regulated by ER stress, independent of CHOP?

In my miRNA expression profiling analysis, I generated a list of 12 candidates whose expression varied more than 2-fold upon ER stress induction (Figure 2-1). Only the ER stress-mediated expression of miR-708 proved dependent on CHOP, but what about the other 10 miRNAs that are regulated by ER stress? Mining the upstream regions of their respective genomic miRNA coding sequence for known consensus promoter elements of UPR transcription factors (e.g. ATF6, XBP1, ATF4) may reveal candidates. If biochemically validated, I recommend pursuing miR-689 because it exhibited the highest

induction in both conditions after 24 h of ER stress (5.5-fold with Tm; 6.4-fold with Tg). miR-762 is also interesting because it resides, although in antisense, within the gene, *Bcl7c*. The Gene Ontology database suggests the *Bcl7c* gene may encode a protein involved in apoptosis, however, this remains to be validated. Additionally, I highly encourage investigating the 3 miRNAs that are significantly down-regulated during ER stress: miR-503, miR-322, and miR-351. All three of these miRNAs are part of an intergenic miRNA cluster on chromosome X of the mouse. It is plausible to propose that miRNAs of the same cluster would collectively be down-regulated during ER stress because they have overlapping functions antithetical to the UPR.

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Appendix A:

On the post-transcriptional regulation of X-box binding protein-1 (*Xbp1*)

Appendix A: On the post-transcriptional regulation of X-box binding protein-1 (*Xbp1*)

As discussed in Chapter 1, the mammalian UPR is comprised of three ER-transmembrane sensors, one of which includes the broadly conserved IRE1. Upon sensing unfolded proteins, IRE1 oligomerizes and autophoshporylates, thus activating its cytosolic RNase domain. The activated RNase, in turn, cleaves a 26-nt intron from X-box binding protein-1 (*Xbp1*) mRNA, in a site-specific manner (Calfon et al., 2002; Shen et al., 2001; Yoshida et al., 2001). The 5' and 3' fragments are re-ligated to form a newly spliced *Xbp1* transcript that gets translated into a potent transcriptional activator of UPR target genes (XBP1-s). XBP1-s is a basic-region leucine zipper protein that binds to both UPR elements (UPRE) and ER stress-response elements (ERSE) controlling such genes as the ER-associated degradation component, EDEM, or the ER chaperone, BiP, respectively (Yoshida et al., 2003). Demonstrating its overall importance in animal development, homozygous mutants exhibit markedly impaired liver development resulting in severe anemia, necrosis of cardiac myocytes, morphological abnormalities of the neural tube, and fetal death around embryonic day 14 (Reimold et al., 2000).

When I joined the lab, *Xbp1* mRNA had just been discovered the previous year as the metazoan substrate of IRE1. Although *Xbp1* mRNA contained in both sequence and structure the IRE1 splice sites and encoded a homologous bZIP domain, it shared no overall homology with its yeast counterpart, HAC1

mRNA. *In vitro* and *in vivo* data demonstrated *Xbp1* not only was a bona fide IRE1 substrate, but that it also gets swiftly transcriptionally activated by ATF6, another ER stress sensor. Following UPR induction, XBP1-s expression increases exponentially, likely as a result of both rapid transcriptional up-regulation as well as splicing. Interestingly, though, this rapid expression occurred coincidently during prime PERK activity, i.e. when global protein synthesis is largely repressed. How was it, then, that spliced *Xbp1* mRNA finagled translation during the UPR, overcoming this apparent translation hurdle? Scanning its mRNA sequence and predicted structure revealed no overt clues as to a mechanism of post-transcriptional regulation. There was no evidence of any short upstream open reading frames (uORF), as is the case for *Atf4*, and nor any internal ribosome entry sites (IRES), as is the case for *Grp78*.

I set off by testing my hypothesis of ER stress-mediated *Xbp1* post-transcriptional regulation through overexpression studies. To test the regulation of *Xbp1* independent of both its transcriptional and splicing controls, I cloned a full-length CMV promoter-driven mouse N-terminally Flag-tagged *Xbp1* without its 26-nt intron (Flag-*Xbp1-s*) from David Ron's pCMV2-Flag-*Xbp1-u* plasmid (Calfon et al., 2002). I transiently transfected this full-length Flag-*Xbp1-s* plasmid into HEK293 cells and induced the UPR using ER stress-inducing drugs, Tg or Tm, more than 24 h post-transfection. Over a 10 h time course of ER stress, I observed an increase in XBP1-s expression despite the fact that its equivalent mRNA levels slightly decreased (Fig. A-1). XBP1-s expression appeared to max

out between 6-8 h (Fig. A-1B). To ensure this was not a result of an overall translational artifact during transient transfection, I performed the same experiment with GFP and saw no change in protein expression following a UPR time course (data not shown). These results suggested that *Xbp1-s* mRNA was being post-transcriptionally regulated, independent of IRE1-mediated splicing.

To determine if this potential post-transcriptional regulation involved the enhancement of XBP1-s protein stability following ER stress, I performed a synthesis shut-off assay before and after addition of Tg in these Flag-*Xbp1-s*-transfected cells (Fig A-2). Using GAPDH as an internal loading control, XBP1-s displayed similar protein half-lives in either condition, with $T_{1/2}$ (untreated) = 29 minutes (+/- 2 min) and $T_{1/2}$ (6 h Tg) = 32 min (+/- 2 min). These similarities in half-lives removed protein stabilization as a plausible explanation for the increase in XBP1-s expression observed in the above experiments (Fig. A-1).

To investigate whether *Xbp1* mRNA was indeed undergoing translational regulation, I performed sucrose gradient sedimentation with lysates from these Flag-*Xbp1-s*-transiently transfected cells (Fig. A-3). Sucrose gradients allow one to observe the ribosomal occupancy of a specific mRNA under varying conditions. The more ribosomes (or polysomes) associating with an mRNA, the more it is believed to be actively translating. In these experiments, I observed the ribosomal association of overexpressed *Xbp1-s* mRNA and endogenous *GAPDH* mRNA, an actively translating housekeeping gene, in either the presence or absence of ER stress (3 h Tg) (Fig. A-3, left panels). In the

untreated condition, the cells exhibited a typical polysome profile of normal healthy cells. Interestingly, *Xbp1-s* mRNA predominately co-migrated with the lighter fractions (i.e. soluble, subunits, and mono- and disomes), suggesting a slower rate of translation. In contrast, GAPDH mRNA predominately co-migrated with the heavier fractions (i.e. high order polysomes).

Expectedly, the polysome profile shifted to the lighter portion of the gradient upon the addition of Tg for 3 hours, suggesting PERK is still operating to repress global translation. Under this condition, *Xbp1-s* mRNA also demonstrated a slight shift, although to heavier fractions. This surprising result suggested an increase in ribosomal occupancy for *Xbp1-s* mRNA and, hence, an increase in its translation. Also under Tg treatment, *GAPDH* mRNA moved slightly to the lighter fractions as would be predicted for a transcript without ER stress-dependent translational regulation. To ensure the co-migration patterns for both *Xbp1-s* and *GAPDH* transcripts were the result of association with actual ribosomes and not some large protein complex with coincident migration, I added EDTA, a Mg^{+2} chelator, which disrupts ribosome complexes (Fig. A-3, right panels). As a result of EDTA addition, both mRNAs shifted to left in both conditions. However, *Xbp1-s* mRNA did appear to linger more in the lighter (insoluble) fractions much as it did before in the untreated condition. Assuming this observation is true, these results suggest that *Xbp1-s* mRNA associates with a non-ribosomal complex in the untreated condition. <u>Let me emphasize, though, that these results must be reproduced before solid conclusions are drawn, as I</u>

never managed to overcome countless technical issues in my subsequent experiments. All things considered, these results indicated that a mechanism of ER stress-dependent translational regulation of *Xbp1-s* mRNA possibly existed.

With this in mind, I performed deletional analyses to locate the region of *Xbp1-s* mRNA that was responsible for this apparent form of translational regulation (Fig. A-4). In doing so, I made a countless constructs, including GFP reporters, all derived from the original Flag-*Xbp1-s* plasmid. For simplistic purposes, I elected to show results from only four of those Flag-*Xbp1-s* constructs: 5'UTR deleted, 5'UTR replaced with β-globin 5'UTR, and 3'UTR replaced with β-globin 3'UTR, and the full-length *Xbp1-s*, as a control. Much as before, I transiently transfected each of these constructs into HEK293 cells, observing protein and RNA expression following a certain period of ER stress. With the small, ~30-nt long 5'UTR deleted, the translational privilege of XBP1-s appeared to be lost following the addition of Tg (Fig. A-4, top left panel). This was exciting as it suggested the 5'UTR of *Xbp1* played a role in translational regulation. Obfuscating this result, however, was the result from the *Xbp1* construct with its 5'UTR replaced with the β-globin 5'UTR - an increase in protein expression occurred following 6 h of Tg despite the fact that RNA levels remained relatively constant (Fig. A-4, bottom left panel; RNA data not shown). More importantly, when the 3'UTR was replaced with the β-globin 3'UTR, the change in expression still remained, although the kinetics appeared to change maxing at 3 h (Fig. A-4, top right panel). This suggested that the 3'UTR did not

play a role in *Xbp1* translational regulation. Oddly (and quite sadly), the full-length *Flag-Xbp1-s* control no longer showed the increase in XBP1-s expression after 6 h of Tg (Fig. A-4, bottom right panel). This result was further supported by duplicate experiments and by *Xbp1-s* stably expressing MEFs (data not shown). Together, these experiments gave me entirely confounding results putting into question my original observation.

So why did the results differ so greatly from the first to the last experiment when I used the same construct? I think two problems existed: (1) Lipid-based transient transfections, in this case of Lipofectamine 2000 (Invitrogen), can produce highly variable levels of gene expression from plasmids regardless of transcript level. Perhaps, this variability seemingly extended into my UPR time course more than 24 h post-transfection. I still can't explain why I didn't see the artifact with my GFP control, however. (2) Western blots can also produce highly variable results depending on the quality of transfer and application of the ECL substrate. It is highly plausible this was also the issue with my Western blots. To get around these issues, it would have been better to perform stable transfections of full-length *Xbp1-s* and to visualize immunoprecipitated ^{35}S-labeled XBP1-s in the presence and absence of ER stress.

Taken together, these results do not support my initial hypothesis that *Xbp1* mRNA experiences post-transcriptional regulation during ER stress. Despite the fact that I was unable to demonstrate a mechanism for *Xbp1* mRNA post-transcriptional regulation, it is quite possible one does exist. The shift in the

co-migration pattern of *Xbp1-s* mRNA from the lighter monosome- and disome-containing fractions to the heavier polysome-containing fractions during ER stress suggests there may be an ER stress-mediated enhancement of its translation rate. However, as I mentioned previously, these results must be repeated. I also think it would be better to simply just observe the behavior of the endogenous *Xbp1* mRNA, rather than the overexpressed, as transient transfections clearly proved deceptive in determining expression changes.

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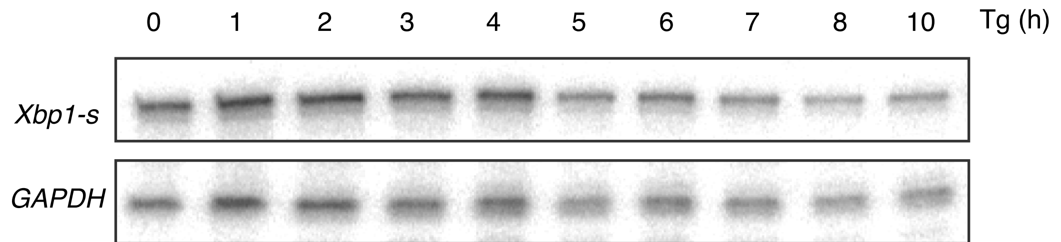
XBP1-s protein expression increases during 10 h of ER stress

A. RNase protection assay of overexpressed *Xbp1-s* mRNA



B. Western blot of overexpressed FLAG-XBP1-s protein

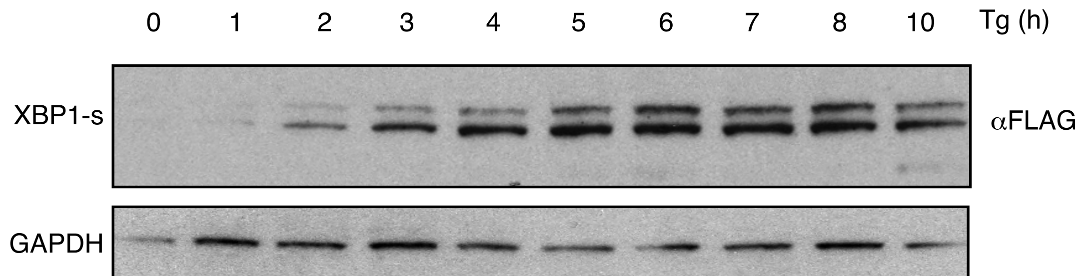


Figure A-1

XBP1-s protein stability does not change with ER stress

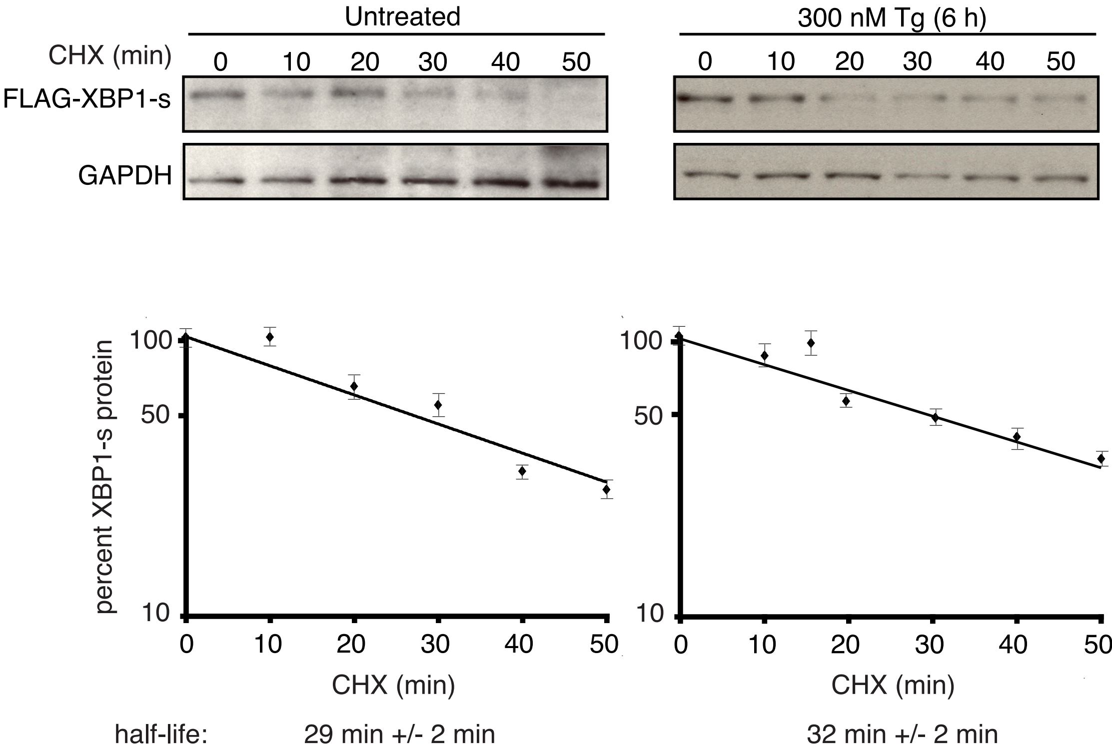


Figure A-2

Polysome profiles of *Xbp1-s*-expressing cells

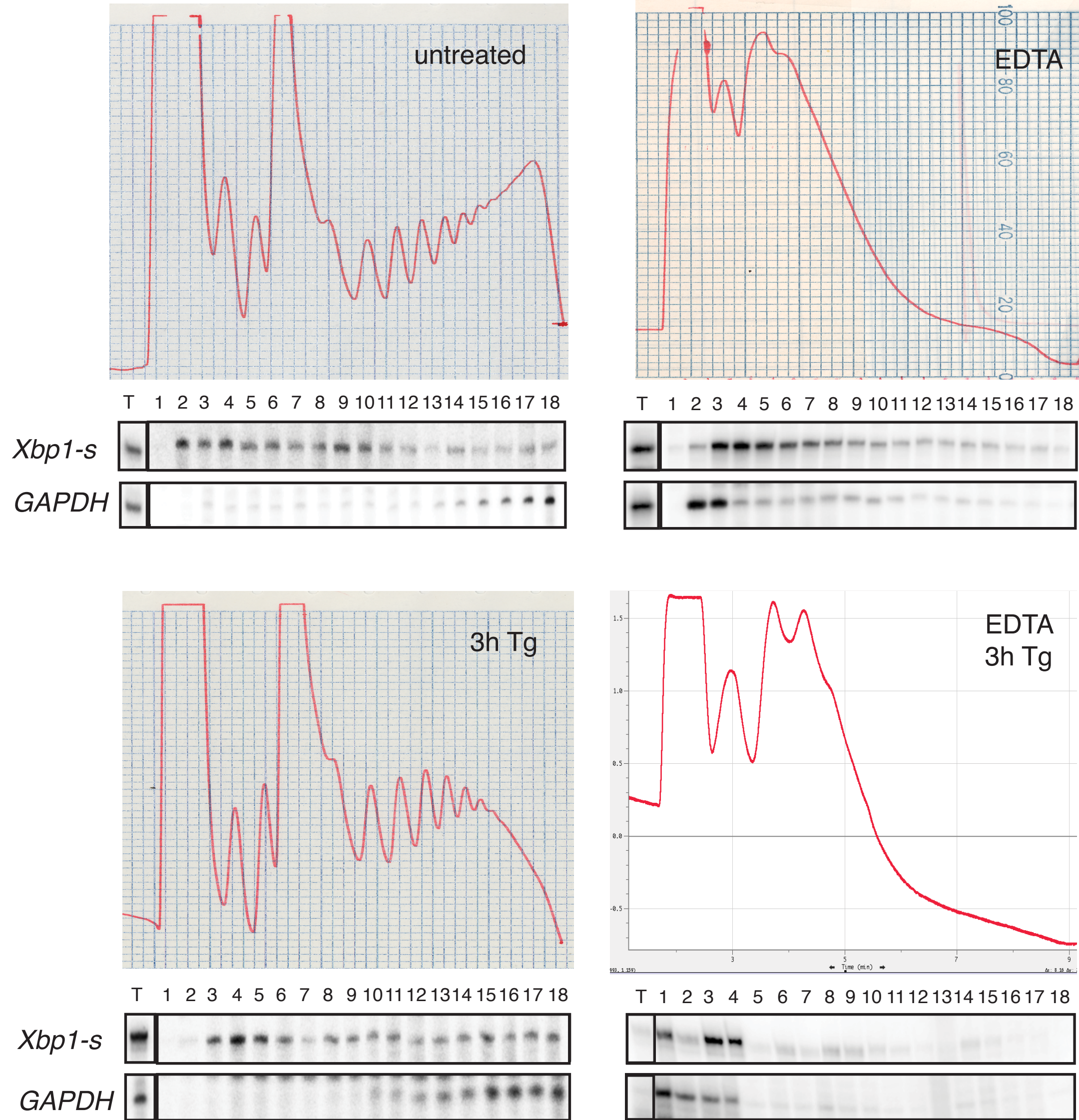


Figure A-3

Deletional analysis did not reveal a mechanism for post-transcriptional regulation of *Xbp1-s* mRNA

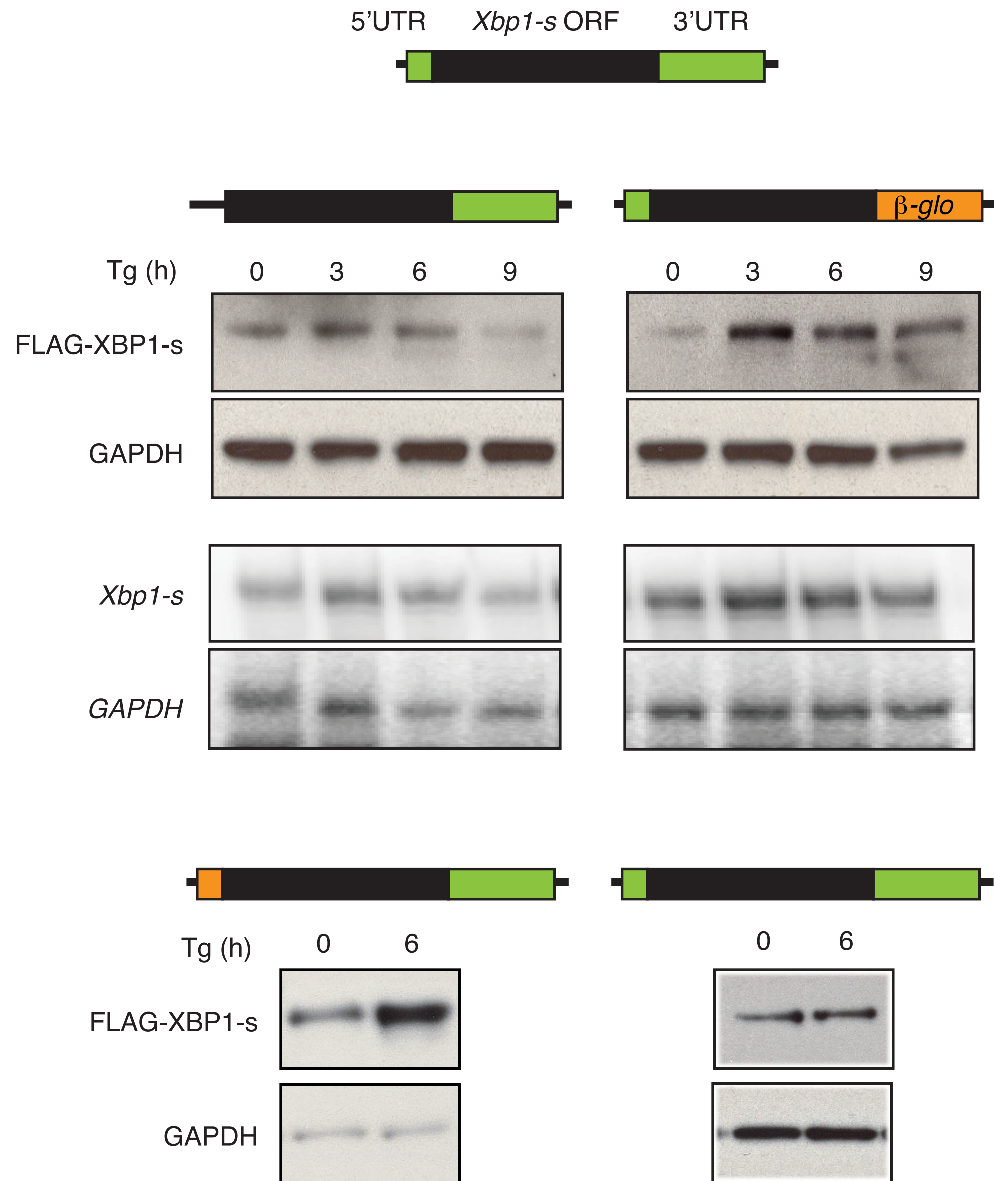


Figure A-4

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