ANALYSIS OF ZNF9 FUNCTION IN CAP-INDEPENDENT TRANSLATION AND MYOTONIC DYSTROPHY TYPE 2

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Dissertation

Submitted to the Faculty of the

Graduate School of Vanderbilt University

In partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

in

Biological Sciences

December, 2010

Nashville, Tennessee

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ACKNOWLEDGEMENTS

There is an old saying that states “it takes a village to raise a child.” This quote is especially applicable to my development and training over the past 5 years. I want to thank all of the people, my village, who have been instrumental in my transition from graduate student to scientist. Thank you all for raising me to be a genuine scientist and not the village idiot.

First and foremost, I want to thank Dr. Andrew Link, my mentor. Andy put his faith in me and let me discover my own path and explore my own project. Hopefully, I’ve repaid that faith you had in me when I walked into your office looking for a place to perform my graduate work. Thanks for everything you’ve done and I hope you find all of the success in the world.

Next, I’d like to give thanks to my committee members, Drs. Todd Graham, Lila Solnica-Krezel, Jim Patton, and Ron Emeson. I’ll admit, I was never scared or nervous to walk into a committee meeting, and I attribute that not to my lacking an innate fear response, but rather to your ability to be critical of my project, but not criticize. I truly appreciate your guidance and your helpful suggestions along the way.

I want to thank the Department of Biological Sciences and the IGP at Vanderbilt University for providing a great home and an amazing staff of people that were there to support me throughout my career. Thanks as well to Dr. Carrie Elzie, a very talented and caring scientist, who taught me to stay focused and not let every exciting new idea impede the progress of my old ideas. Finally,

many thanks to Drs. Ralf Krahe, Cristian Ruse, and Darryl Pappin, who were instrumental in providing reagents, tools, and advice that helped push this work forward.

To my friends, there is no way I can thank you enough, so I won't even try. You provided much needed relief from the daily grind. I hope you come visit me in Philadelphia. It's much nicer than Durham.

To my parents and the rest of my wonderful family, I love you all, even though it seems I only come home to eat Thanksgiving dinner and open presents at Christmas. I cannot properly thank you for all of the love and support you have given me while I chased this dream of never having a "real job". Anyone would be lucky to have a family like you.

And finally, to Leda Ramoz, I want to tell you how much your love and support means to me. You've been absolutely and unequivocally supportive of me and my career, and from the bottom of my heart, I want to thank you for all that you've done for me. Now that this dissertation is finished, I guess it's my turn to repay the favor. I can't think of anything I want to do more.

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CHAPTER I

INTRODUCTION

Molecular mechanisms of translation initiation

Protein synthesis is an essential cellular process where genomic information encoded on messenger RNA (mRNA) molecules is decoded by the translation machinery and transformed into polypeptides, or proteins. Ribosomes translate mRNA and synthesize proteins using amino acids delivered by transfer RNA (tRNA). The translation machinery consists of the ribosome and other associated regulatory proteins, known collectively as translation factors. The eukaryotic ribosome is comprised of two subunits that are named due to their different sedimentation coefficients. The 40S ribosomal subunit is a stable complex of 33 separate polypeptides and an 18S ribosomal RNA (Dinman 2008). The 60S subunit contains 46 proteins and 3 different rRNA molecules, 28S, 5.8S, and 5S (Dinman 2008). Aminoacyl tRNAs are short RNA molecules that transfer a specific amino acid to a growing polypeptide chain during translation. tRNAs contain a three base region called the anticodon that can base pair to the corresponding three base codon region on mRNA (Jackson, Hellen et al. 2010). Each tRNA can be uniquely attached to only one of the 20 amino acids. The translationally active form of the ribosome is the 80S ribosome, made up of the 40S and 60S subunits in complex with other essential translation factors, an mRNA molecule, the translation factors, and aminoacyl tRNAs.

Before a particular messenger RNA can be translated, a number of concerted molecular events need to occur. The process of protein synthesis is broken down into three distinct steps initiation, elongation, and termination. Initiation, the first step in protein synthesis, comprises all of the necessary events up to the formation of a translationally active 80S ribosome localized at the start codon base-paired with the initiation methionyl tRNA. Elongation is the active process of the ribosome decoding the information contained in the codons of the mRNA producing polypeptides through the ribosome's peptidyl transferase activity. Using the mRNA as a template, the ribosome traverses each codon of the mRNA, pairing it with the appropriate amino acid provided by a tRNA. Peptide bond formation is actually catalzyed by the 23S rRNA within the 60S subunit (Jackson, Hellen et al. 2010). Termination is the last step in the process and involves recognition of the stop codon and subsequent mRNA release and disassembly of the active 80S ribosome into its 40S and 60S subunits. While each step in protein synthesis has multiple, distinct levels of regulation, translation initiation is the most highly regulated and complex step in the process (Jackson, Hellen et al. 2010).

The mechanism of eukaryotic translation initiation is an area of intense study due to the complexity of selecting temporally and spatially which mRNA molecules should be translated. Ignoring the large number of signaling pathways that converge upon the translation initiation machinery to regulate its activity, the general mechanism of initiation is still extremely complex and an area of active research (Van Der Kelen, Beyaert et al. 2009; Jackson, Hellen et al. 2010).

The consensus mechanism for translation initiation in eukaryotes is known as the scanning model (Kozak 1989; Kozak 1999). Briefly, in this model, the 40S ribosome binds to the 5' cap structure of an mRNA molecule and translocates, or "scans" along the mRNA looking for an AUG start codon. When the scanning 40S ribosome locates the start codon and the methionyl initator tRNA base-pairs with the start codon, the 60S ribosomal subunit is recruited, thereby forming the translationally active 80S subunit (Jackson, Hellen et al. 2010).

The scanning model for translation initiation relies on the ability of the ribosome or associated translation factors to bind to the 5' region of an mRNA molecule and begin the scanning process. Prokaryotic messenger RNA contains specific sequences, known as the Shine-Dalgarno sequence, which directly recruits the small ribosomal subunit and initiation factors to internal sites on the mRNA within 5-10 nucleotides from the start codon (Shine and Dalgarno 1975). In eukaryotes, a consensus sequence that recruits the translation machinery is mediated by a 7-methyl-guanosine cap structure at the 5' end of the mRNA (Furuichi, Morgan et al. 1975; Furuichi, Muthukrishnan et al. 1975; Furuichi, Muthukrishnan et al. 1976). The eukaryotic initiation factor eIF4E, a member of the eIF4F complex, specifically recognizes and binds to the 7-methylguanosine cap (Sonenberg, Rupprecht et al. 1979). The other members of eIF4F serve essential roles in translation initiation and the recruitment of the 40S small ribosomal subunit. eIF4G acts as a scaffolding protein, mediating the recruitment of the 40S subunit -bound eIF3 to the cap-bound eIF4E. The eIF4F complex and the 40S subunit scan along the 5' untranslated region of the mRNA until the

complex reaches the initiation codon, AUG (Kozak 1980; Kozak 1980). Cellular mRNAs lacking the 7-methylguanosine cap structure are either inefficiently translated or not translated at all, suggesting that the cap structure is required for the initiation of translation (Kapp and Lorsch 2004).

Not all translation initiation in eukaryotes requires the 5' cap structure

Even though the scanning model explains the mechanistic details associated with initiating translation of 7-methyl-guanosine-capped mRNAs, it fails to explain translation of certain uncapped RNA sequences and translation of specific mRNA when cap-dependent initiation is inhibited. Certain mRNAs, such as those derived from the picornavirus family, are not capped, yet they are translated efficiently in eukaryotic cells (Pelletier and Sonenberg 1988; Jang, Davies et al. 1989). The discovery that the 5'UTR of poliovirus could initiate translation in the absence of the 5' cap complex suggested an alternative mechanism for eukaryotic translation initiation (Pelletier and Sonenberg 1988). 5'UTR elements that could support translation initiation even in the uncapped form were called internal ribosome entry sites (IRES) (Pelletier, Kaplan et al. 1988; Pelletier and Sonenberg 1988). A number of different viruses were found to contain IRES structures, that could initiate translation at internal sites of the mRNAs independent of the 5' end (Wilson, Powell et al. 2000; Liljas, Tate et al. 2002), suggesting that internal ribosome entry might represent a mechanism used by viruses to bypass eukaryotic control mechanisms. Indeed, many picornaviruses encode a protease that specifically cleaves the eukaryotic

cap-binding protein, eIF4G, rendering host cap-dependent translation inactive and stimulating translation of viral IRES-containing transcripts (Haghighat, Svitkin et al. 1996; Bushell and Sarnow 2002).

IRES-mediated translation, despite occurring in the absence of a 5' cap structure, still requires the recruitment of the ribosome and translation machinery to the mRNA. Initially, the Sonenberg group suggested that the IRES RNA sequences or structures themselves could recruit the small ribosomal subunit, much like the Shine-Dalgarno sequence functions in prokaryotic organisms. Early work with the cricket paralysis virus (CrPV) IRESs provided a completely novel mechanism for translation initiation and ribosome recruitment. The Sarnow group discovered that the CrPV IRES adopts a structure similar to the initiator tRNA molecule and directly recruits the ribosome in the absence of other protein cofactors. The IRES RNA localizes directly in the P-site of the ribosome and mimics tRNA binding, thus facilitating translation elongation (Wilson, Pestova et al. 2000; Wilson, Powell et al. 2000; Spahn, Jan et al. 2004). This method of recruitment and initiation seems to be unique to just the CrPV IRES, suggesting that other recruitment mechanisms existed for other IRESs (Pestova and Hellen 2003).

Discovery of the La protein suggested an alternative mechanism for ribosomal recruitment to IRESs (Meerovitch, Pelletier et al. 1989). The La protein is capable of binding to the IRES-containing region of the poliovirus mRNA, promoting IRES-mediated translation (Meerovitch, Pelletier et al. 1989; Meerovitch, Svitkin et al. 1993). Proteins that can bind to IRESs and facilitate

cap-independent translation are named IRES-trans-acting factors, or ITAFs (Stoneley and Willis 2004). It is unlikely that eukaryotic cells have evolved to produce proteins whose function involves helping viruses replicate and prosper. ITAFs, therefore, are proposed to perform functions that are important to eukaryotic cells unrelated to the translation of viral mRNAs that will be discussed later.

Eukaryotic cellular mRNAs also contain internal ribosome entry sites

In 1991, Sarnow and colleagues discovered that the human immunoglobulin binding protein (BiP) mRNA could be translated in a cap-independent fashion (Macejak and Sarnow 1991), providing the first evidence that IRESs were present in non-viral mRNAs. Soon after, many other human mRNAs were discovered to contain IRESs (Oh, Scott et al. 1992; Vagner, Gensac et al. 1995; Gan and Rhoads 1996). Interestingly, previously discovered ITAFs of viral IRESs also regulated the function of cellular IRESs, suggesting that viral and cellular IRESs may activate translation through similar mechanisms. Cellular and viral IRESs do not share any significant sequence homology, leading researchers to posit that structural or cofactor similarity mediates IRES function (Hellen 2010). ITAFs are believed to bind to IRES RNA and act as RNA chaperones, folding the RNA into a structure that is directly recognized by the ribosome (Stoneley and Willis 2004; Mitchell, Spriggs et al. 2005). This also suggests that cellular ITAFs may not recognize structures, but rather recognize specific RNA sequences within the IRES. Structural

characterization of cellular IRESs with associated ITAFs and translation machinery would be extremely beneficial in dissecting the mechanism of action for cellular IRESs.

Immediately after the discovery of eukaryotic cap-independent translation, researchers questioned why mRNAs contained IRESs, since even IRES-containing cellular mRNAs are capped at the 5' end. Sarnow and colleagues initially hypothesized that cellular mRNAs might contain IRESs in order to support translation of certain mRNAs during mitosis, a time when cap-dependent translation is severely reduced (Bonneau and Sonenberg 1987; Huang and Schneider 1991; Macejak and Sarnow 1991). This hypothesis was confirmed by the discovery that the IRES of the ornithine decarboxylase (ODC) mRNA was active predominantly during mitosis, and that ODC protein activity is required for progression from mitosis (Fredlund, Johansson et al. 1994; Fredlund, Johansson et al. 1995; Fredlund and Oredsson 1996; Pyronnet, Pradayrol et al. 2000). Additional physiological events, such as environmental stress, apoptosis, UV irradiation, and viral infection see increased levels of cap-independent translation ((Stoneley, Chappell et al. 2000; Stoneley and Willis 2004; Spriggs, Stoneley et al. 2008). Overall, IRES-containing mRNAs are thought to be translated in response to a situation where increased levels of the protein are needed, but cannot be synthesized through normal cap-dependent mechanisms.

Internal ribosome entry site-mediated translation of the ornithine decarboxylase mRNA

Ornithine decarboxylase (ODC) is an essential cellular enzyme that catalyzes the conversion of ornithine to putrescine, which is eventually converted into other polyamines by downstream enzymes (Kay, Singer et al. 1980). Polyamines are required for cell cycle progression and DNA synthesis, and depletion of cellular polyamines results in cell cycle arrest (Kay, Singer et al. 1980; Laitinen, Stenius et al. 1998; Shantz and Levin 2007). The mechanism of polyamine action is poorly understood, but is thought to involve a combination of DNA, RNA, and protein interactions (Berlaimont, Pauwels et al. 1997; Laitinen, Stenius et al. 1998). Supplementing growth media with polyamines confers a selective growth advantage to tissue culture cells. Mutations that increase ODC activity are associated with a number of human cancers (Leinonen, Alhonen-Hongisto et al. 1987; Polvinen, Sinervirta et al. 1988; Elitsur, Koh et al. 1995; Tamori, Nishiguchi et al. 1995; Brabender, Lord et al. 2001; Sanchez Mas, Martijnez-Esparza et al. 2002). A number of chemotherapeutic agents target the polyamine biosynthesis pathway, in particular the ODC enzyme, because inhibition of polyamine biosynthesis results in cell cycle arrest and apoptosis (Pegg, Shantz et al. 1995; Shantz and Levin 2007).

The requirement for polyamine biosynthesis during mitosis suggested that the activity or expression of these enzymes is upregulated during the cell cycle. Indeed, ODC and other polyamine biosynthetic enzymes have increased expression during G1/S phase, a period of cellular growth, and during the G2/M transition, a period of cellular stress during mitosis

(Dircks, Grens et al. 1986; Park, Wolff et al. 1993; Fredlund, Johansson et al. 1995)}. As previously discussed, the ODC mRNA was determined to contain an IRES that is active during the G2/M transition, resulting in increased levels of the ODC protein (Pyronnet, Pradayrol et al. 2000). Attaching the ODC 5'UTR to the ORF of an unrelated gene confers translation during mitosis. Addition of a stable hairpin structure directly upstream of the IRES blocks cap-dependent translation, due to an inability of the ribosome to scan, but does not affect internal ribosome entry and translation initiation. Finally, Sonenberg and colleagues mutated polypyrimidine tracts in the ODC IRES and found that they were required for internal ribosome entry (Pyronnet, Pradayrol et al. 2000). This groundbreaking work provided a *bona fide* physiological role for internal ribosome entry site mediated translation of cellular mRNAs. Despite the importance of these results, the mechanisms and proteins regulating cap-independent translation of the ODC IRES were still unclear.

Mutating the polypyrimidine tract of the ODC IRES to purines abolished cap-independent translation of ODC during the G2/M transition (Pyronnet, Pradayrol et al. 2000). This result suggests that these mutations either altered the RNA secondary structure or altered the sequence required for recognition by ITAFs to recruit the ribosome. Work by Vincent Gerbasi in Dr. Andrew Link's laboratory provided insight into the mechanism of ODC IRES-mediated translation. Using RNA affinity chromatography of the wild-type and mutated ODC IRES sequences followed by highly sensitive LC-MS/MS analysis, Gerbasi was able to isolate two proteins that bound to the wild-type, but not mutant forms

of the IRES. These proteins were identified as poly(C) binding protein 2 (PCBP2) and zinc finger 9 (ZNF9). Functional analysis of PCBP2 and ZNF9 in ODC IRES-dependent translation suggested that the two proteins acted as ITAFs. While PCBP2 is a known ITAF for viral internal ribosome entry sites, the conclusion that ZNF9 regulates cap-independent translation was a novel finding. Interestingly, little is known about the functional activity of the ZNF9 protein.

The discovery and *in vitro* biochemical characterization of the ZNF9 gene

The zinc finger 9 gene (ZNF9), also known as cellular nucleic acid binding protein, (CNBP), was initially identified as a protein capable of binding the sterol regulatory element (SRE) of the HMG-coA-reductase promoter by a yeast 1 hybrid screen (Rajavashisth, Taylor et al. 1989). Even though the protein had single stranded DNA binding activity, the protein was purposely named cellular nucleic acid binding protein because of its amino acid sequence. Primary sequence analysis of ZNF9 revealed the presence of seven CCHC zinc finger sequences (Rajavashisth, Taylor et al. 1989) and an RGG box domain. The CCHC zinc fingers have the consensus sequence (C-Ar-X-C-G-X-X-X-H-X-X-XX-C), where Ar represents an aromatic amino acid and X is a variable amino acid. While other zinc finger motifs are commonly found in DNA binding proteins, the CCHC-type zinc finger is most commonly associated with the nucleocapsid domain of the human immunodeficiency virus 1 (HIV1) (Bombarda, Grell et al. 2007; Grigorov, Decimo et al. 2007). The nucleocapsid protein binds to the HIV-1 RNA genome and regulates its stability and replication

(De Guzman, Wu et al. 1998). RGG boxes bind single stranded RNA and are found in a number of translation factors and RNA chaperones (Darnell, Warren et al. 2004; Darnell, Mostovetsky et al. 2005). The presence of these different motifs normally used to bind RNA suggested that ZNF9 may have additional functions beyond the regulation of the sterol regulator element. Indeed, follow-up studies demonstrated that rat ZNF9, identical to the human protein, could bind both single stranded RNA and DNA (Yasuda, Mashiyama et al. 1995).

The use of yeast 1 hybrid screening libraries and DNA affinity chromatography to isolate sequence specific DNA binding proteins revealed ZNF9 as a putatively promiscuous regulator of transcription. ZNF9 was reported to control transcription of the *c-myc* gene through interactions with the CT element in the c-myc promoter (Michelotti, Tomonaga et al. 1995). Additionally, ZNF9 was found to bind to an enhancer of the JC polyoma virus, the promoters of the B-myosin heavy chain and the M-CSF1 genes, further suggesting that ZNF9 functions as a transcriptional regulator (Flink and Morkin 1995; Konicek, Xia et al. 1998; Liu, Kumar et al. 1998). All of the promoters used as bait to isolate ZNF9 contain guanine-rich stretches that were postulated to be the preferred target of ZNF9.

The ability of ZNF9 to bind to G-rich stretches was supported by the discovery that ZNF9 regulates the translation of *Xenopus laevis* ribosomal proteins with G-rich 5' untranslated regions (Pellizzoni, Lotti et al. 1997). Using RNA affinity chromatography, Paola Pierandrei-Amaldi and colleagues discovered that ZNF9 along with the La protein binds to the 5'UTR of ribosomal

proteins promoting translation of these mRNAs (Cardinali, Di Cristina et al. 1993; Pellizzoni, Lotti et al. 1997; Pellizzoni, Lotti et al. 1998). Another study showed CNBP also binds to G-rich regions within nascent c-myc transcripts (Yasuda, Mashiyama et al. 1995).

Work performed mainly in the lab of Nora Calcaterra sheds light onto the *in vitro* RNA and DNA binding activity of ZNF9. Using primarily electrophoretic mobility shift assays, the Calcaterra group has systematically analyzed ZNF9 binding to DNA and RNA oligonucleotides of varying sequence and length (Lombardo, Armas et al. 2007; Armas, Nasif et al. 2008; Borgognone, Armas et al. 2010). Ultimately, ZNF9 binds to single stranded RNA or DNA, but not double stranded DNA. ZNF9 binds with approximately equal affinity to RNA or DNA, with a very strong preference for G-rich nucleic acids, similar to the probe sequences used to pulldown and identify ZNF9 in previous studies (Armas, Nasif et al. 2008). While there does not seem to be a difference in affinity for RNA or DNA, ZNF9 may form dimers during interactions with RNA, but not with DNA (Armas, Nasif et al. 2008). EMSA analysis reveals that ZNF9 may act as a nucleic acid chaperone, stimulating DNA and RNA annealing, and that this activity may explain ZNF9s ability to stimulate transcription and translation (Lombardo, Armas et al. 2007; Armas, Nasif et al. 2008; Borgognone, Armas et al. 2010). Characterization of the *in vitro* biochemistry of ZNF9 provides insight into the mechanism of action and into possible *in vivo* nucleic acid targets, but does not give any information into the functional and physiological role for the ZNF9 protein.

Studying the in vivo role of the ZNF9 protein

Soon after the initial cloning and discovery of ZNF9, a homolog was discovered in *Schizosaccharomyces pombe,* providing the first experimental data of a ZNF9 phenotype. *A* null allele of *byr3*, the putative homolog, caused drastic reductions in the sporulation efficiency of diploid yeast, a phenotype that was rescued by expression of the human *znf9* gene (Xu, Rajavashisth et al. 1992). Sporulation in *S. pombe* is controlled by the yeast *ras* homolog, suggesting that ZNF9 might be part of this important cellular pathway regulating cell growth and proliferation. Characterization of embryonic *X. laevis* development revealed that ZNF9 mRNA is consistently expressed through all developmental phases and tissues, providing evidence as to the protein's physiological importance (Flink, Blitz et al. 1998; De Dominicis, Lotti et al. 2000). ZNF9 mRNA expression data from chicken and frog embryos further supported the proposed importance of ZNF9 during embryogenesis and later development (Ruble and Foster 1998; Calcaterra, Palatnik et al. 1999).

The embryonic *ZNF9* mRNA expression patterns suggest that the protein has functional importance in the development of the embryo. Using a viral integration strategy, the Li lab created a *znf9* knockout mouse and provided unequivocal evidence of ZNF9s importance in embryonic development (Chen, Liang et al. 2003). Absence of the *znf9* gene in homozygous animals results in severe truncation of the developing forebrain, correlating well to the *ZNF9* mRNA expression pattern in the forebrain of mouse embryos (Chen, Liang et al. 2003; Shimizu, Chen et al. 2003). Knockout of *znf9* is embryonic lethal by day 13.5.

Approximately 40% of mice with one copy of the *znf9* gene show severe defects in craniofacial development, such as smaller snouts and jaws, and small or absent eyes (Chen, Liang et al. 2003). The requirement for *znf9* in craniofacial development was confirmed by siRNA knockdown of *ZNF9* mRNA in chicken embryos and morpholino knockdown in zebrafish embryos (Abe, Chen et al. 2006; Weiner, Allende et al. 2007).

The previous reports of ZNF9s function as a regulator of *c-myc* expression suggested that perhaps the craniofacial defects are caused by losses in *c-myc* in the developing embryo. *C-myc* is a well known regulator of cell growth, proliferation, and differentiation. Analysis of *c-myc* expression in the embryonic forebrain revealed completely absent or low c-myc mRNA expression in mutant *znf9* mice, suggesting that ZNF9 is required for forebrain specification through induction of *c-myc (Chen, Liang et al. 2003).* Craniofacial tissues in the developing embryo are derived from the neural crest tissues (Szabo-Rogers, Smithers et al. 2010). Zebrafish ZNF9 morphants show reduced expression of *col2A1, crestin,* and *foxD3*, important markers for the developing neural crest tissue, indicating that ZNF9 regulates the expression of these genes and ultimately might control neural crest development (Weiner, Allende et al. 2007). Regions with low or absent ZNF9 expression in ZNF9 morphants showed abnormally low cell proliferation and increased apoptosis (Weiner, Allende et al. 2007), consistent with the previous observation that overexpression of ZNF9 in mouse P19 cells increases rates of cell proliferation (Shimizu, Chen et al. 2003). Overall, this data suggests that ZNF9 controls craniofacial development through

control of cellular proliferation, potentially through induction of *c-myc* and other target genes.

Deciphering the function of the ZNF9 protein became more urgent with the discovery that mutations in the *ZNF9* gene were linked to myotonic dystrophy type 2 (DM2). 64 sets of parents and offspring that had clinical features of myotonic dystrophy but did not have the mutation responsible for myotonic dystrophy type 1 were screened for genomic rearrangements or expansions using transmission disequilibrium testing (Liquori, Ricker et al. 2001). Subsequent Southern blot analysis and DNA sequencing revealed a variable nucleotide expansion within the first intron of *znf9* in patients affected with the clinical features of myotonic dystrophy (Liquori, Ricker et al. 2001). While the first intron normally contains CCTG microsatellites of varying length, pathological expansions in this region were seen up to 48kB in length. The expansion of CCTG in *znf9* that causes DM2 is remarkably similar to the CTG expansion in the 3'UTR of *dmpk* that results in myotonic dystrophy type 1 (Brook, Mccurrach et al. 1992). Because the two diseases have extremely similar clinical manifestations and similar nucleotide expansions in seemingly unrelated genes, Ranum and colleagues hypothesized that the mechanism for the disease was likely independent of the function of either gene (Liquori, Ricker et al. 2001).

General mechanisms of nucleotide expansion repeat disorders

The myotonic dystrophies (Types 1 and 2) are classified as nucleotide (or microsatellite) repeat expansion disorders. These diseases are also commonly

referred to as diseases of unstable repeat expansions (Gatchel and Zoghbi 2005). The diseases ultimately result from errors in DNA replication within normal tandem repeat regions in the genome, leading to large expansions of the repeat region (Nakayabu, Miwa et al. 1998; Xu, Peng et al. 2000; Lahue and Slater 2003). Most of these disorders show increased pathogenicity, either earlier onset or increased severity, in subsequent generations, due to the increased likelihood of further expansions in mutated gene regions (Carpenter 1994; La Spada, Paulson et al. 1994; O'Donnell and Zoghbi 1995). Interestingly, although the mutated gene for each disease is different, all known nucleotide repeat expansion disorders target the nervous system, suggesting the inherent sensitivity of this system to perturbations of normal physiological processes (Gatchel and Zoghbi 2005).

Within the nucleotide repeat expansion disorder classification, there are three general pathogenic mechanisms. The first are diseases caused by nucleotide expansions within or near a gene that results in a loss of function for that gene. The most commonly studied disease of this type is Fragile X Syndrome, which is caused by a repeat expansion in 5'UTR of the *fmr1* gene (Calkhoven, Muller et al. 2002; Gatchel and Zoghbi 2005). In the case of Fragile X Syndrome, expansion of the 5'UTR results in transcriptional and translational silencing of the FMR1 gene (Chen, Tassone et al. 2003; Beilina, Tassone et al. 2004). The loss of function mechanism is supported by evidence that animals lacking the *fmr1* share many of the developmental and behavioral abnormalities associated with the repeat expansion (Huber, Gallagher et al. 2002; Bear, Huber

et al. 2004). Additionally, a single point mutation in *fmr1* that disrupts FMR1 translational repression gives rise to a severe form of Fragile X, suggesting that a direct loss of FMR1 activity leads to development of the disease (Feng, Absher et al. 1997).

The second general mechanism for NRE disorders is caused by a gain of function for a mutated gene. In Huntington's disease, trinucleotide repeats within the coding region of the *Huntingtin* gene give rise to a mutant protein containing a long stretch of glutamine residues (Gatchel and Zoghbi 2005). These long stretches of glutamine residues are common within nucleotide repeat expansion disorders and are commonly referred to as polyQ diseases. The mutant Huntingtin protein is inherently disordered, and misfolding of the mutant protein leads to formation of intracellular aggregates that lead to cellular toxicity (La Spada, Paulson et al. 1994; Gatchel and Zoghbi 2005). Genetic models expressing mutant forms of Huntingtin or other proteins engineered to have large glutamine expansions show similar phenotypes, giving support to the gain of function model (Watase, Weeber et al. 2002; Yoo, Pennesi et al. 2003). Polyglutamine expansion within the *Huntingtin* gene does severely decrease huntingtin protein function, but it is not clear whether this reduction in activity contributes to the disease (Dragatsis, Levine et al. 2000; Zuccato, Ciammola et al. 2001; Dragatsis, Zeitlin et al. 2004).

The third and final general mechanism for nucleotide repeat expansion disorders is caused by a gain of function of expanded repeat RNA. The myotonic dystrophies are the prototypical members of this family of diseases. In this

proposed mechanism, an affected gene is transcribed and translated normally, but the expanded RNA repeat forms secondary structures that render it stable in either the cytoplasm or the nucleus. RNA binding proteins interact with the stable RNA and are prevented from performing their normal cellular function. Thus far, the stable RNA is thought to interfere with normal RNA splicing, as two of the known interacting proteins, MBNL and CUGBP1 have normal roles in splicing (Fardaei, Rogers et al. 2002; Kino, Mori et al. 2004; Ho, Bundman et al. 2005; Warf and Berglund 2007). The mechanisms regulating the stable RNA and the cellular gain of function are complex and are the least understood of the nucleotide repeat expansion disorders.

Molecular mechanisms underlying the myotonic dystrophies

Myotonic dystrophy types 1 and 2 (DM1 and DM2) are caused by an expansion of different nucleotide repeats within two different genes, and have similar, but not identical, clinical manifestations (Ranum and Day 2002; Vihola, Bassez et al. 2003; Udd, Meola et al. 2006). Both diseases display muscle weakness, myotonia, progressive muscle wasting, and cataract development. Additionally, both diseases show testicular atrophy, insulin sensitivity, and sudden cardiac arrythmias and arrest (Ranum and Day 2002; Ranum and Cooper 2006; Udd, Meola et al. 2006). While there are multiple differences between the manifestations of DM1 and DM2, the main difference between the two diseases is that DM2 does not have a congenital form (Cho and Tapscott

2006). This important difference argues that although the molecular mechanisms for the two diseases may be similar, they are not identical.

Initially, the proposed mechanism for myotonic dystrophy type 1 was thought to be similar to other NREs, like Fragile-X or Huntington's disease, in that the expanded repeat was thought to inhibit DMPK expression or activity. These early studies provided evidence that DMPK mRNA levels were abnormally low and the cytoplasmic concentration of the DMPK protein were reduced in adult patients with DM1 (Carango, Noble et al. 1993; Fu, Friedman et al. 1993; Krahe, Ashizawa et al. 1995). Mouse models with reduced DMPK levels display adult onset myopathy, cardiac conduction defects, and cardiac arrythmias, similar to what is seen in DM1 patients (Jansen, Groenen et al. 1996; Reddy, Smith et al. 1996; Mounsey, Mistry et al. 2000). Despite the similarities, the symptoms seen in DMPK knockout models were thought to be too mild and too focused on only the cardiac system to explain the full scope of the myotonic dystrophy disease mechanism.

The creation of a mouse model for DM1 provided the first clear evidence of a possible RNA gain of function mechanism (Mankodi, Logigian et al. 2000; Seznec, Agbulut et al. 2001). A mouse line expressing expanded CTG repeats in the 3'UTR of the skeletal actin gene recapitulated most of the symptoms associated with DM1 in humans, suggesting that the context of the expansion was not necessarily important for disease progression (Mankodi, Logigian et al. 2000; Seznec, Agbulut et al. 2001). The DM1 mouse model fit nicely with the previous observations that large CUG RNA complexes were found in the nucleus

of DM1 patients (Taneja, McCurrach et al. 1995; Taneja 1998). The presence of stable CUG RNA complexes provided an initial clue in to the potential mechanism of the disease and led to the identification of two proteins, CUGBP1 and MBNL, that bound and colocalized with the RNA (Timchenko, Timchenko et al. 1996; Miller, Urbinati et al. 2000).

CUGBP1 activity was implicated in DM1 disease progression after the observation that CUGBP1 is a regulator of alternative splicing of the troponin T (TNNT2) mRNA. Patients with DM1 showed clear misregulation of TNNT2 splicing associated with increased CUGBP1 activity, suggesting that aberrant CUGBP1 regulation caused some of the symptoms of DM1 (Philips, Timchenko et al. 1998). Other misspliced mRNAs with relevance to DM1 symptoms were attributed to an increase in CUGBP1 activity (Charlet-B., Savkur et al. 2002; Savkur, Philips et al. 2004). CUGBP1 is activated by initial binding to RNA targets containing CUG motifs, similar to the mechanism of PKR activation (Timchenko, Timchenko et al. 1996; Philips, Timchenko et al. 1998).

As further evidence for an RNA-mediated gain of function in DM1, the protein MBNL colocalizes with nuclear CUG RNA foci (Miller, Urbinati et al. 2000). MBNL regulates alternative splicing of TNNT2 and chloride channel 1 (ClC-1) and reduced MBNL activity leads to a failure of these transcripts to mature (Kanadia, Johnstone et al. 2003). Alternative isoforms of TNNT2 and ClC-1 have reduced *in vivo* activity and are thought to underlie the myopathy and cardiac transduction defects of DM1. Interestingly, increased CUGBP1 activity opposes the action of MBNL, whose activity is thought to be reduced by CUG

repeat RNA binding. Knockout of MBNL activity in mice gives rise to many of the clinical symptoms of DM1, suggesting that a loss of MBNL activity leads to some of the symptoms of DM1 (Kanadia, Johnstone et al. 2003).

The discovery that a CCTG expansion in the first intron of *znf9* causes DM2 provided additional support to the RNA-mediated gain of function hypothesis for the myotonic dystrophies. CCUG RNA foci are found in skeletal muscle tissue from DM2 patients and show colocalization with MBNL and increased CUGBP1 levels (Liquori, Ricker et al. 2001; Margolis, Schoser et al. 2006; Salisbury, Sakai et al. 2008; Salisbury, Schoser et al. 2009). Additionally, DM2 patients show similar aberrant splicing as patients with DM1, suggesting that the two diseases share a similar underlying mechanism. Despite all of the evidence for the RNA-mediated gain of function model, most laboratories have ignored or rejected critical evidence supporting a more complex mechanism for the myotonic dystrophies.

Evidence for a combined RNA-mediated gain of function and a ZNF9 loss of function model

As previously mentioned, DM1 and DM2 share many clinical symptoms, but the main difference between the two diseases is the congenital form of DM1. This fact alone argues against a unified mechanism for DM1 and DM2, but the additional observed clinical and cellular differences between the two diseases suggests that the two diseases have different mechanisms. The RNA-mediated toxicity model explains a large number of DM1 and DM2 features, and certainly contributes to myotonic dystrophy pathogenesis. Neither the CTG expansion

model nor the MBNL knockout models for myotonic dystrophy manifest a congenital form, suggesting that the presence of the repeat expansion in another gene or loss of MBNL function are not enough to cause DM1.

Knockout of DMPK function in mice leads to myopathy and cardiac conduction defects, indicating that DMPK gene activity may be involved in DM1 disease progression (Jansen, Groenen et al. 1996; Reddy, Smith et al. 1996; Mounsey, Mistry et al. 2000). Expression of 100 CUG repeats within the DMPK 3'UTR causes severe myoblast differentiation defects, while expression of the same repeats within an unrelated 3'UTR has no observable effect on differentiation (Amack and Mahadevan 2001; Amack, Reagan et al. 2002). These data underscore the importance for the genomic context of the nucleotide expansion.

Mice homozygous for a loss in *znf9* gene function die during embryonic development (Chen, Liang et al. 2003). Approximately 40% of the heterozygous animals die shortly after birth with craniofacial and skeletal muscle defects, but initial characterization of the other 60% revealed phenotypically normal animals (Chen, Liang et al. 2003). Further examination of supposedly normal *znf9*$^{+/-}$ animals resulted in a striking observation. *znf9*$^{+/-}$ animals display late-onset muscle weakness, myotonia, cardiac conduction defects, and cataract development, symptoms seen in patients with myotonic dystrophy (Chen, Wang et al. 2007). Restoration of ZNF9 protein levels rescued the myotonic dystrophy-like defects in znf9$^{+/-}$ animals. These results provided the first clear evidence that

the RNA-mediated gain of function model for myotonic dystrophy was not fully correct and may need to include a loss of function for either DMPK or ZNF9.

X. Rationale for dissertation research

The observation that a loss of ZNF9 gene activity might contribute to the pathogenesis of myotonic dystrophy type 2 provided a clear rationale for this dissertation. Previous work in the Link lab suggested that ZNF9 functions as a regulator of cap-independent translation (Gerbasi and Link 2007). I hypothesized that if ZNF9 activity contributed to myotonic dystrophy, patients with DM2 would have reduced cap-independent translation activity due to a decrease in ZNF9 function. The key component to determining if ZNF9 activity is deficient in DM2 patients is determining the exact molecular function of the ZNF9 protein.

CHAPTER II

ZNF9 ACTIVATION OF IRES-MEDIATED TRANSLATION OF THE HUMAN ODC MRNA IS DECREASED IN MYOTONIC DYSTROPHY TYPE 2

The following chapter was published in *PLoS ONE 5(2): e9301. Feb 2010.*

Abstract

Myotonic dystrophy types 1 and 2 (DM1 and DM2) are forms of muscular dystrophy that share similar clinical and molecular manifestations, such as myotonia, muscle weakness, cardiac anomalies, cataracts, and the presence of defined RNA-containing foci in muscle nuclei. DM2 is caused by an expansion of the tetranucleotide CCTG repeat within the first intron of *ZNF9*, although the mechanism by which the expanded nucleotide repeat causes the debilitating symptoms of DM2 is unclear. Conflicting studies have led to two models for the mechanisms leading to the problems associated with DM2. First, a gain-of-function disease model hypothesizes that the repeat expansions in the transcribed RNA do not directly affect ZNF9 function. Instead repeat-containing RNAs are thought to sequester proteins in the nucleus, causing misregulation of normal cellular processes. In the alternative model, the repeat expansions impair ZNF9 function and lead to a decrease in the level of translation. Here we examine the normal *in vivo* function of ZNF9. We report that ZNF9 associates with actively translating ribosomes and functions as an activator of cap-independent translation of the human ODC mRNA. This activity is mediated by

direct binding of ZNF9 to the internal ribosome entry site sequence (IRES) within the 5'UTR of *ODC* mRNA. ZNF9 can activate IRES-mediated translation of ODC within primary human myoblasts, and this activity is reduced in myoblasts derived from a DM2 patient. These data identify ZNF9 as a regulator of cap-independent translation and indicate that ZNF9 activity may contribute mechanistically to the myotonic dystrophy type 2 phenotype.

Introduction

Myotonic dystrophy types 1 and 2 are forms of muscular dystrophy resulting from large expansions of nucleotide repeats within noncoding regions of *DMPK* and *ZNF9*, respectively (Cho and Tapscott 2007). Myotonic dystrophy type 1 (DM1) is caused by expansion of a CTG triplet repeat within the 3' untranslated region (UTR) of *DMPK*, whereas myotonic dystrophy type 2 (DM2) results from expansion of a CCTG repeat within the first intron of *ZNF9* (Aslanidis, Jansen et al. 1992; Liquori, Ricker et al. 2001). Despite the different expansions within two apparently unrelated genes, both diseases share many common clinical manifestations, including myotonia, muscle weakness, cataract development, insulin resistance, and cardiac conduction defects. The shared symptoms suggest the mechanisms causing the disease may also be shared (Ranum and Day 2002; Day, Ricker et al. 2003). Although they have similar symptoms, there are also a number of very dissimilar features making them clearly separate diseases (Udd, Meola et al. 2006). A major difference between DM1 and DM2 is that only DM1 shows a congenital form of the disorder (Cho

and Tapscott 2007). Other apparent differences are the distribution of clinical muscle weakness/atrophy and the morphological involvement of different fiber types. In DM1, weakness and atrophy involves distal, facial, bulbar and respiratory muscles, whereas in DM2 the proximal muscles are preferentially involved, and the patients have marked muscle pains (Ricker, Koch et al. 1994). DM1 type 1 fibers show atrophy changes whereas in DM2 a subpopulation of type 2 fibers are highly atrophic (Vihola, Bassez et al. 2003). These phenotypic differences suggest that other cellular and molecular pathways are involved besides the shared molecular pathomechanisms.

In both DM1 and DM2, the expanded nucleotide repeats are transcribed but are not translated. For *ZNF9*, the transcribed CCTG repeats within the first intron are apparently removed from both the wild-type and DM2 pre-mRNAs by splicing, resulting in translation of the normal ZNF9 protein (Margolis, Schoser et al. 2006). Two studies of ZNF9 mRNA and protein expression levels reported no change in diseased myoblasts or lymphoblastoid cell lines compared to unaffected cells (Botta, Caldarola et al. 2006; Margolis, Schoser et al. 2006). These results indicate that the repeat RNA does not affect normal ZNF9 function and have led to a disease model in which the expanded repeats somehow dominantly interfere with other cellular processes (Amack, Paguio et al. 1999; Seznec, Agbulut et al. 2001; Amack, Reagan et al. 2002; Mankodi, Takahashi et al. 2002). This model is supported by the observation that expression of transgenically engineered expanded CTG repeats in unrelated genes can cause some of the phenotypes seen in the myotonic dystrophies (Mankodi, Logigian et

al. 2000). This toxic-RNA gain-of-function disease model hypothesizes that the transcribed CTG or CCTG repeat expansions fold into RNA structures that are retained in the nucleus forming distinct foci. Experimental data shows the ribonuclear complexes sequester similar sets of proteins in the nucleus, apparently causing misregulation of normal cellular processes, in particular RNA splicing (Fardaei, Larkin et al. 2001; Fardaei, Rogers et al. 2002; de Haro, Al-Ramahi et al. 2006). Most prominently, the transcribed CTG and CCTG repeats both bind muscleblind-like (MBNL) and CUGBP1/ETR-3-like factors (CELF) that are involved in alternative RNA splicing.

In contrast to the toxic-RNA gain-of-function model, several studies have revealed a role for ZNF9 in DM2. Knockout of *ZNF9* in mice results in embryonic lethality due to defects in forebrain and craniofacial development (Chen, Liang et al. 2003). Other studies in chick and zebrafish show that ZNF9 deletion results in severe brain and muscle phenotypes (Abe, Chen et al. 2006; Weiner, Allende et al. 2007). Mice heterozygous for the *znf9* knockout display late-onset muscle wasting, cardiac abnormalities, cataracts, and mRNA expression defects similar to those seen in DM2 (Chen, Wang et al. 2007). These defects can be rescued by reintroduction of wild-type levels of ZNF9, suggesting that a loss of ZNF9 function likely contributes to DM2. Similar to DM2, DMPK deficient mice show a subset of the phenotypes seen in DM1 patients (Reddy, Smith et al. 1996). A recent study reports that expanded CCTG repeats in DM2 give rise to defects in ZNF9 expression and cellular localization (Huichalaf, Schoser et al. 2009). The discrepancy between this study and earlier characterizations of ZNF9 expression

and localization in DM2 cells is unclear. This study reported that decreased ZNF9 activity is linked to a downregulation of translation (Huichalaf, Schoser et al. 2009). A second study has reported that the expression of the CCTG repeats in mouse and human myoblasts alters translation and protein degradation (Salisbury, Schoser et al. 2009). These data support the notion that the ZNF9 protein may play a role in DM2 and that the RNA-mediated dominant gain-of-function model for myotonic dystrophy is not the only pathomechanism for the disease.

The lack of an *in vivo* biochemical function for ZNF9 has hindered the ability to clearly define the role that ZNF9 plays in DM2. ZNF9 has been proposed to act in a variety of cellular functions, including transcription, splicing, and translation (Michelotti, Tomonaga et al. 1995; Pellizzoni, Lotti et al. 1997; Pellizzoni, Lotti et al. 1998; Chen, Liang et al. 2003; Gerbasi and Link 2007). Recent evidence suggests that ZNF9 likely acts as a regulator of translation. ZNF9 associates with 5' terminal oligopyrimidine (TOP) mRNA elements (Cardinali, Carissimi et al. 2003), and 5' TOP mRNAs are inefficiently translated in DM2 (Huichalaf, Schoser et al. 2009). Previously, our group identified ZNF9 as a positive regulator of IRES-mediated translation for the rat ornithine decarboxylase (ODC) mRNA (Gerbasi and Link 2007), providing further evidence that ZNF9 functions in translational regulation.

We hypothesized that ZNF9 acts as an IRES *trans*-activating factor (ITAF) for cap-independent translation of the human ODC mRNA. By analyzing the *in vivo* function of ZNF9 as an activator of cap-independent translation, we sought

to determine whether a loss in ZNF9 activity contributes to DM2. The data presented here suggest that ZNF9 directly interacts with the IRES of the human ODC mRNA, associates with translating ribosomes, and activates cap-independent translation. Additionally, our data show that ZNF9 activity is reduced in myoblasts from a patient affected with DM2, providing further evidence that a loss-of-function mechanism contributes to myotonic dystrophy type 2 disease.

Materials and Methods

Cell Culture

HEK293T and HeLa cell lines were grown in DMEM containing 10% fetal bovine serum and penicillin/streptomycin solution according to standard laboratory practices (ATCC). Control and DM2 human myoblasts were grown in Complete Myoblast Medium (Promocell GmbH, Heidelberg, Germany) and cultured according to the manufacturer's recommendations. DM2 myoblasts were established from muscle biopsies from a male patient in his early 40's with clinical symptoms of DM2. The (CCTG)n repeat size was approximately 4500 repeats, as determined using established methods (Sallinen, Vihola et al. 2004).

Plasmid Construction

The full-length 5'UTR of the human ODC mRNA from clone #5088190 (Magic Consortium CloneID) was inserted between the *Renilla* and firefly luciferase genes to create pcDNA3.1-hODC-IRES. pcDNA3.1-V5-ZNF9 was constructed

by insertion of the PCR-generated full-length open reading frame of human ZNF9 into pENTR and subsequent recombination into pcDNA3.1/nV5-Dest (Invitrogen) by recombinational cloning (Invitrogen). The PCR primers for cloning ZNF9 were 5'- CACCATGAGCAGCAATGAGTGCTT-3' and 5'- TTATTAGGCTGTAGCCTCAATTGTGCA-3'. pcDNA3.1-V5-LacZ was created by recombinational cloning of pcDNA3.1/nV5-GW/*lac*Z into pcDNA3.1/nV5-Dest. An HIV –based reporter vector (hODC-IRES) was cloned by introduction of the full-length hODC-IRES coding sequence into H163 (Sundrud, Grill et al. 2003). pcDNA3.1-hODC-IRES was digested with *Kpn*I and *Not*I, blunt-ended with Klenow fragment, and inserted into the *Sma*I site of H163. HIV-ZNF9-myc and HIV-LacZ-Myc were created by digestion of pcDNA3.1-ZNF9-myc or pcDNA3.1-LacZ-myc with *Kpn*I and *Pme*I. The fragments were blunt-ended with Klenow fragment and inserted into the *Sma*I site of H163. All clones were verified by DNA sequencing.

Western Blotting

Rabbit polyclonal antibodies were generated against full-length, recombinant ZNF9 (Covance). Briefly, recombinant human ZNF9 was expressed in *E. coli* (BL21DE3) as an N-terminal GST fusion protein and purified by glutathione affinity chromatography. ZNF9 without the GST tag was released from the column by cleavage with 3C protease (Prescission Protease, GE). The purity and identity of ZNF9 were determined by Coomassie staining and mass spectrometry analysis. ZNF9 antibodies were affinity purified against full-length

recombinant ZNF9. Antibodies for immunoblotting were used at the following concentrations: ZNF9 (1:1,000), GAPDH (Millipore) (1:5,000), c-myc (Cell Signaling) (1:1,000), anti-myc epitope (Vanderbilt Monoclonal Antibody Core) (1:2,000), V5 (Invitrogen) (1:2,500).

Subcellular Fractionation

HeLa cells were transfected with either pcDNA3.1-ZNF9-myc or pcDNA3.1-LacZ-myc using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations. Transfected cells were fractionated into whole cell, cytoplasmic, and nuclear fractions using the NE-PER Nuclear and Cytoplasmic Extraction reagents (Thermo Scientific) according to the manufacturer's recommendations.

Quantitative RT-PCR

Total RNA was isolated from cultured cells using TRIzol according to manufacturer's recommendations (Invitrogen). RNA was quantified using a Nanodrop spectrophotometer (Thermo Scientific). 1μg of RNA was DNase-treated (Promega) and used in reverse transcription reactions primed with random hexamers (Superscript III, Invitrogen). cDNA samples were treated with RNase H and 5μL of each sample were used for quantitative real-time PCR reactions. Real-time PCR was performed using the Applied Biosystems 7000 and the manufacturer's protocols (Applied Biosystems). Taqman probes used were: ZNF9 (Hs00231535_m1) and GAPDH (4333764T).

Sucrose Gradient Analysis and Centrifugation

Polysome analysis was performed as previously described (Gerbasi and Link 2007). Intact cells were lysed by repeated vortexing in five pellet volumes of lysis buffer (100 mM Tris, 10 mM $MgCl_2$, 100 mM KCl, and 1% TritonX-100), and cellular debris was removed by centrifugation (4°C, 13,500x*g*). 10 O.D. units of lysate were loaded onto a 7-47% sucrose gradient. Unless indicated, sucrose solutions contained 10 mM Tris-HCl (pH 7.5), 5% cycloheximide, 5 mM $MgCl_2$, and 100 mM EDTA. Gradients were centrifuged for 2 h at 178,000x*g* in a Sorvall SW-41 swinging bucket rotor. Polysome analysis was performed by bottom displacement and monitored by UV absorbance at 254 nm. Fractions were collected for western blotting. Ribosome pelleting experiments were performed by centrifugation of the cell lysates described above through a 1 M sucrose cushion (-/+ 50 mM EDTA) at 100,000x*g* for 2 h in a TLA120.2 rotor. The resulting ribosome pellets were resuspended in ribosome solubilization buffer (20 mM TRIS pH 7.5, 5 mM $MgCl_2$, 6 M urea). Supernatant fractions were precipitated with trichloroacetic acid, washed with acetone, and resuspended in ribosome solubilization buffer. For ribosome salt wash analysis, ribosome pellets were isolated as described above, and the supernatants were discarded. Pellets were washed in ice cold PBS and resuspended in ribosome salt wash buffer containing 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM BME, and either 100 mM, 250 mM, 500 mM, or 1 M potassium acetate. Resuspended ribosomes were then centrifuged at 100,000x*g* for 2 h in a TLA120.2 rotor. Supernatant fractions were removed and TCA precipitated as described above, and pellet

fractions were resuspended in solubilization buffer. Equal volumes of lysate were separated by polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and immunoblotted for ZNF9 protein.

In vitro RNA Synthesis and Binding Assays

RNA probes were synthesized using double-stranded DNA templates with upstream T7 promoters using Ambion's MegaShortScript kit according to the manufacturer's recommendations. The DNA primers used were: 5'-TAA TAC GAC TCA CTA TAG G-3' and 5'-GAT TTC TTG ATG TTC CTA TGG AAA ACT AAG AGA TGG AAT TGA AAG AAA CCT ATA GTG AGT CGT ATT A-3'. The sequence of the synthesized RNA probe is 5'-UUUCUUUCAAUUCCAUCUCUUAGUUUUCCAUAGGAACAUCAAGAAAUC-3'. The RNA probe was purified by TRIzol extraction (Invitrogen), and the size and purity were determined by gel electrophoresis. Probes were end labeled with ^{32}P using T4 polynucleotide kinase according to the manufacturer's recommendations (NEB). All RNA binding reactions were performed with 20 pmol of labeled RNA in a reaction volume of 30 μL of RNA binding buffer (150 mM NaCl, 100 mM $NaHPO_4$, and 5% glycerol) in a clear-bottom 96 well plate at room temperature, unless otherwise indicated. UV cross-linking reactions were performed on a 302 nm UV lightbox for 5 min at room temperature. Reactions were stopped by addition of Laemmli buffer. The reactions were analyzed by polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes.

Membranes were stained with Ponceau S to measure ZNF9 protein levels, and exposed to film for imaging of the radioactive signals.

Luciferase Reporter Assays

HeLa cells were grown in 24-well plates to 70% confluence and transfected with the human ODC-IRES reporter and either pcDNA3.1-V5-LacZ or pcDNA3.1-V5-ZNF9 using Lipofectamine 2000 according to manufacturer's recommendations (Invitrogen). Cells were harvested after 24 h, and dual luciferase reporter assays were performed according to the manufacturer's recommendations (Promega) using a Turner TD-20/20 lumninometer.

Mass Spectrometry-based Proteomics

Sample preparation and mass spectrometry-based proteomics analysis were performed as described earlier (Fleischer, Weaver et al. 2006; Gerbasi and Link 2007).

Generation of shRNA Virus

Double-stranded DNA sequences were generated and cloned into pENTR/U6 using the Block-iT U6 RNAi Entry vector kit (Invitrogen). Primer sequences were ZNF9-198 Top strand 5'- CAC CGC AGC AAT GAG TGC TTC AAG TAG AGC TTG ACT TGA AGC ACT CAT TGC TGC -3', ZNF9-198 Bottom strand 5'-AAA AGC AGC AAT GAG TGC TTC AAG TCA AGC TCT ACT TGA AGC ACT CAT TGC TGC -3', ZNF9-645 Top Strand 5'-CAC CGC AAG ACA AGT GAA GTC

AAC TAG AGC TTG AGT TGA CTT CAC TTG TCT TGC -3', ZNF9-645 Bottom Strand 5'-AAA AGC AAG ACA AGT GAA GTC AAC TCA AGC TCT AGT TGA CTT CAC TTG TCT TGC -3'. Ligation products were transformed into One Shot TOP10 competent *E. coli* (Invitrogen). Minipreps were performed on selected colonies and shRNA fidelity was verified by DNA sequencing. Gateway recombination reactions were performed by transfering the shRNA constructs into the lentiviral vector system as described previously (Antons, Wang et al. 2008). Lentiviruses encoding ZNF9 or control shRNAs were generated by calcium-phosphate mediated co-transfection of HEK293T cells with the resulting shRNA-containing lentiviral vector, VSV-G, and pol/gag. VSV-G and pol/gag vectors were kind gifts of Dr. D. Unutmaz, NYU School of Medicine (Motsinger, Haas et al. 2002). The resulting viral supernatants were concentrated using Amicon 100K cut-off concentrators (Millipore).

Viral Infection and Knockdown of ZNF9

Viral supernatants were applied to $1x10^6$ HeLa cells cultured under standard conditions. Infections were performed at an MOI of 2 and were allowed to proceed for 48 h. GFP+ cells were sorted and collected using a FACSAria flow sorter (BD Biosciences) and placed back in culture. Knockdown of target genes was assessed by quantitative RT-PCR (Taqman) and western blotting directed at the target protein.

Infection of Primary Myoblasts

HIV-hODC-IRES reporter, HIV-ZNF9-myc, and HIV-LacZ-myc viral supernatants were generated by calcium phosphate mediated co-transfection of the HIV vector and pL-VSV-G into HEK293T cells cultured in DMEM with 10% FBS (Sundrud, Grill et al. 2003). The medium was changed after 6 h and replaced with myoblast complete growth medium. Supernatants were collected 48 h after transfection and were sterile filtered and stored at -80°C. Primary myoblasts were seeded at 15,000 cells per well in myoblast complete growth medium. Viral supernatants were added to the cells for 8 h, the medium was changed to standard growth medium, and the cells were subsequently assayed after 48 h for luciferase activity as described above.

Statistical Methods

Two-tailed Student's T-tests (95% confidence interval) were performed on pairwise combinations of data to determine statistical significance.

Results

ZNF9 specifically binds to a cellular mRNA IRES element

Previously, we identified ZNF9 in a screen for proteins binding to an IRES element from the rat ornithine decarboxylase (ODC) mRNA (Pyronnet, Pradayrol et al. 2000; Gerbasi and Link 2007). It was unknown whether ZNF9 directly binds the ODC's IRES RNA sequence or if it binds in the presence of other protein cofactors. We tested whether ZNF9 can bind directly to a 45 bp region in the

human ODC mRNA corresponding to the rat IRES-containing sequence. In Figure 1A, we show that ZNF9 directly binds to the 45 bp human ODC IRES RNA sequence. The interaction is sequence specific since the interaction was abolished with the addition of a 50-fold molar excess of an unlabeled competitor identical to the probe (Fig. 1). In contrast, the addition of a 50-fold molar excess of a non-specific competitor RNA did not reduce or eliminate ZNF9 binding to the ODC IRES RNA probe (Fig. 1). The ability of ZNF9 to bind to a putative IRES element in the absence of other proteins suggests that it may act as an ITAF through direct recognition of the IRES sequence. One possible mechanism for ITAF function involves ZNF9 acting as a physical scaffold between an IRES-containing mRNA and the ribosomal machinery. Tethering of the mRNA to the ribosome by an ITAF is thought to facilitate translation of the mRNA (Stoneley and Willis 2004; Spriggs, Bushell et al. 2005; Semler and Waterman 2008). In order to test this model for ZNF9, we investigated the interactions of ZNF9 with the translation machinery.

FIGURE 1

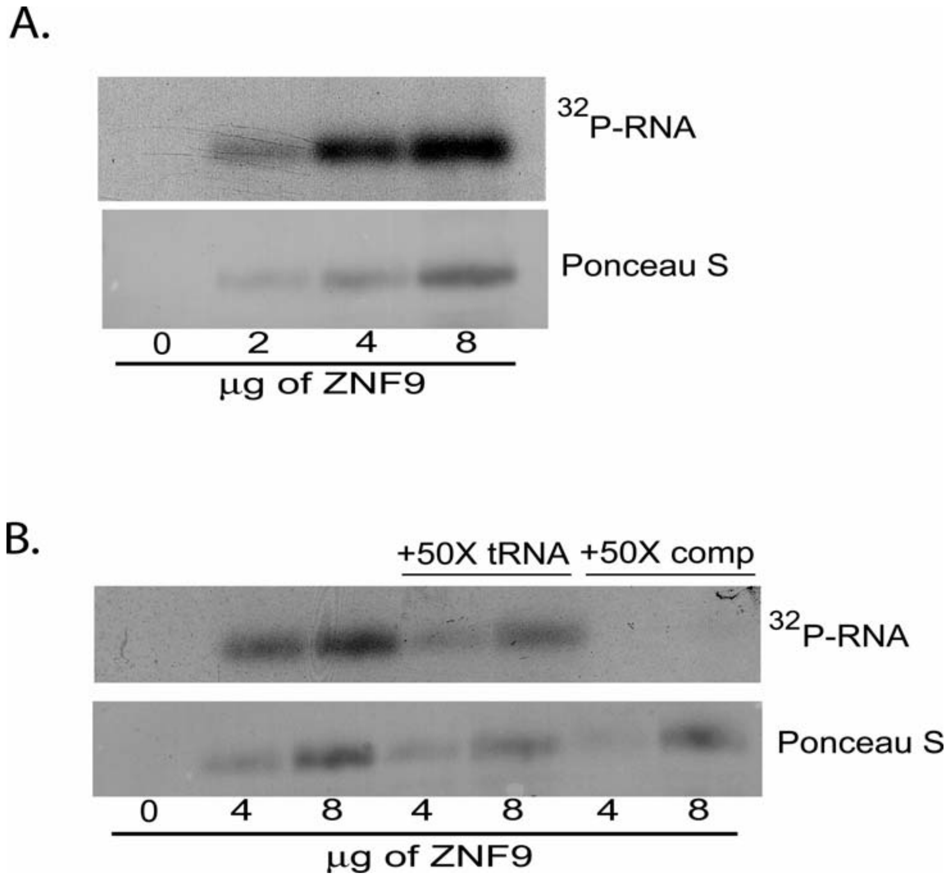


Figure 1. ZNF9 specifically binds to a human cellular mRNA IRES element. A. Increasing amounts of recombinant, full-length ZNF9 were incubated with ^{32}P-end-labeled ODC RNA probe and cross-linked. Reactions were separated by polyacrylamide gel electrophoresis, transferred to nitrocellulose and exposed to X-ray film. Membranes were stained for ZNF9 protein using Ponceau S. B. Recombinant ZNF9 was incubated with ^{32}P-end-labeled ODC RNA probe and either a 50-fold molar excess of bovine liver tRNA or a 50-fold molar excess of unlabeled ODC RNA competitor (comp) probe. Samples were processed as in *A.*

ZNF9 interacts with the translating eukaryotic ribosome

Based on its proposed role as an ITAF for cap-independent translation, we hypothesized that ZNF9 interacts with translating ribosomes. Previous work from our lab showed that ZNF9 co-sediments with the polyribosome fractions, suggesting an interaction with the ribosome and a role in translation (Gerbasi and Link 2007). Therefore, we sought to analyze the intracellular localization of ZNF9 and whether ZNF9 associates with the ribosome during active translation. Subcellular fractionation experiments revealed that the majority of ZNF9 protein is found in the cytoplasm (Fig. 2A). Both myc-tagged and endogenous ZNF9 localize to the cytoplasm (Fig. 2A). Western blotting for a known nuclear protein (c-myc) and a cytoplasmic protein (β-galactosidase) were used as controls for the selectivity of the fractionation technique. Both control proteins were found exclusively in the expected fraction. A small amount (~ 5%) of the total ZNF9 found in the cell was localized to the nucleus. While this may be carryover from the cytoplasmic fraction, the experimental and control results suggest that a small fraction of ZNF9 is nuclear. The significance and function of nuclear ZNF9 are unknown, but ZNF9 has been shown previously to have a nuclear pool (Huichalaf, Schoser et al. 2009; Salisbury, Schoser et al. 2009). The cytoplasmic localization of ZNF9 is consistent with its proposed role in translation.

Differential centrifugation of high molecular weight complexes is a well-established technique for the isolation of polyribosomes (Merrick 1979). To determine if ZNF9 copurifies with human polysomes, lysates from HeLa cells were loaded onto a sucrose cushion and the polyribosome fraction was isolated.

The majority of ZNF9 is found in the pellet (polyribosome) fraction, which suggests that ZNF9 associates with translating ribosomes (Fig. 2B). A fraction of ZNF9 protein remains in the supernatant, suggesting that a portion of ZNF9 protein is not actively engaged with the ribosome (Fig. 2B). As a control, GAPDH, a protein that does not interact with translating ribosomes, is absent from the pellet fraction and is exclusively localized to the supernatant. The addition of EDTA disrupts polyribosome formation and is often used as a control to rule out association with other high molecular weight complexes that co-sediment with ribosomes (Fleischer, Weaver et al. 2006). In the presence of EDTA, ZNF9 is found exclusively in the supernatant fraction (Fig. 2B), suggesting that the ZNF9 sedimentation in the absence of EDTA is due to an association with the ribosome and not a separate protein complex. We also analyzed ZNF9 sedimentation with ribosomes by linear sucrose gradient ultracentrifugation and confirmed that ZNF9 associates with very high molecular weight particles and that this interaction is abolished by the addition of EDTA (Fig. 2C). As seen previously, a fraction of ZNF9 does not sediment in the ribosome-containing fractions, suggesting that a pool of ZNF9 is not actively engaged in translation.

In order to further test our hypothesis that ZNF9 acts as a translation initiation factor for cap-independent translation, we sought to test how tightly ZNF9 is associated with ribosomal proteins. Ribosome salt wash experiments are often used to separate core ribosomal protein subunits from associated translation factors (Link, Fleischer et al. 2005). The assay is also used to estimate the relative strength of protein-ribosome interactions by increasing the

salt concentration (Link, Eng et al. 1999). We hypothesized that, like other translation factors, ZNF9 would be released from the polysomes with intermediate concentrations of salt. At low concentrations of potassium acetate, ZNF9 localizes exclusively to the ribosome pellet (Fig. 2D). At 500mM potassium acetate, a concentration that is sufficient to strip most translation factors from ribosomes (Merrick 1979), ZNF9 is partially dissociated from the ribosome. At 1 M potassium acetate, ZNF9 protein is completely removed from the ribosome, suggesting that ZNF9-ribosome interactions are not as strong as the core ribosomal subunits. In summary, our data indicate that ZNF9 associates with translating polysomes and that the strength and mode of the interaction is consistent with those of other translation factors (Merrick 1979; Fleischer, Weaver et al. 2006).

FIGURE 2

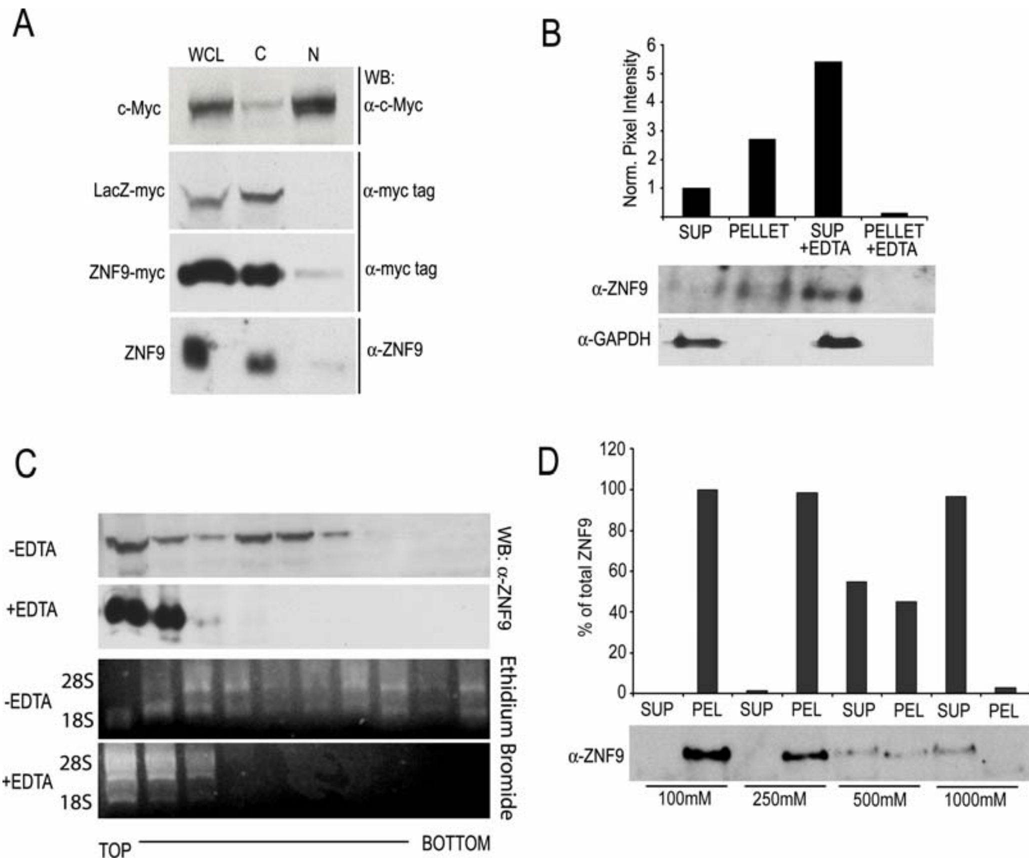


Figure 2. ZNF9 interacts with the translating human eukaryotic ribosome. A. Western blots of subcellular fractions of HeLa cells extracts transfected with either ZNF9-myc or *LacZ*-myc and probed with the indicated antibodies. 10% of each fraction was analyzed by western blotting. WCL: whole cell extract, C: cytoplasmic, N: nuclear B. ZNF9 and GAPDH western blotting of supernatant and polysome fractions of HeLa cell lysates isolated in the absence or presence of EDTA. Quantification was normalized to the levels of ZNF9 in the supernatant fraction in the absence of EDTA C. ZNF9 western blotting of sucrose gradient fractions of HeLa cell lysates in the absence or presence of EDTA. Equal cell lysate amounts (10 O.D. units) were applied to each gradient (-/+ 50 mM EDTA), and each fraction was TCA precipitated. 50% of the total precipitated protein was analyzed by western blotting. D. ZNF9 western blotting analysis of supernatant and pellet fractions from ribosome salt wash experiments. Western blots were quantified and the data are reported as ratios of ZNF9 in the pellet fraction to the amount of ZNF9 in the supernatant, normalized to the values for 100 mM potassium acetate.

ZNF9 activates cap-independent translation of the human ODC mRNA

We have determined that ZNF9 binds specifically to the 5'UTR of the human ODC mRNA and interacts with translating ribosomes. Previous data from our lab revealed that ZNF9 can activate cap-independent translation of the rat ODC mRNA, which is similar, but not identical, to the human ODC mRNA. These data suggest a direct role for ZNF9 in regulating translation of the ODC mRNA. To test if ZNF9 promotes human ODC IRES activity, a bicistronic reporter plasmid with the full-length human ODC 5'UTR was used to test the ability of ZNF9 to activate cap-independent translation (Fig. 3A). Overexpression of ZNF9 in HeLa cells resulted in an ~3-fold increase (p-value=0.0031) in translation of the second cistron of the reporter compared to control experiments with β-galactosidase overexpression (Fig. 3B). These data, along with a demonstration of direct ZNF9 binding to the 5'UTR of ODC, strongly suggests that ZNF9 acts as a translation factor for cap-independent translation of the human ODC mRNA.

In order to validate the role for ZNF9 in cap-independent translation of the human ODC mRNA, we designed specific short hairpin RNA constructs (shRNAs) to knockdown expression of ZNF9 mRNA. The short hairpin constructs were expressed in a lentiviral system for infection into HeLa cells, and infected cells were selected for stable GFP expression by flow cytometry. Cells expressing the ZNF9-specific shRNA showed ~90% reductions (p-value=2.2×10^{-5}) in ZNF9 mRNA levels and a reduction in ZNF9 protein levels compared to control shRNA-expressing cells, as assessed by quantitative RT-PCR and

western blotting (Fig. 3C and insert). These cells with dramatically reduced levels of ZNF9 have ~30% reduction (p-value=0.0014) in cap-independent translation of ODC (Fig. 3D), suggesting that ZNF9 is required for full activation of cap-independent translation of the ODC mRNA.

FIGURE 3

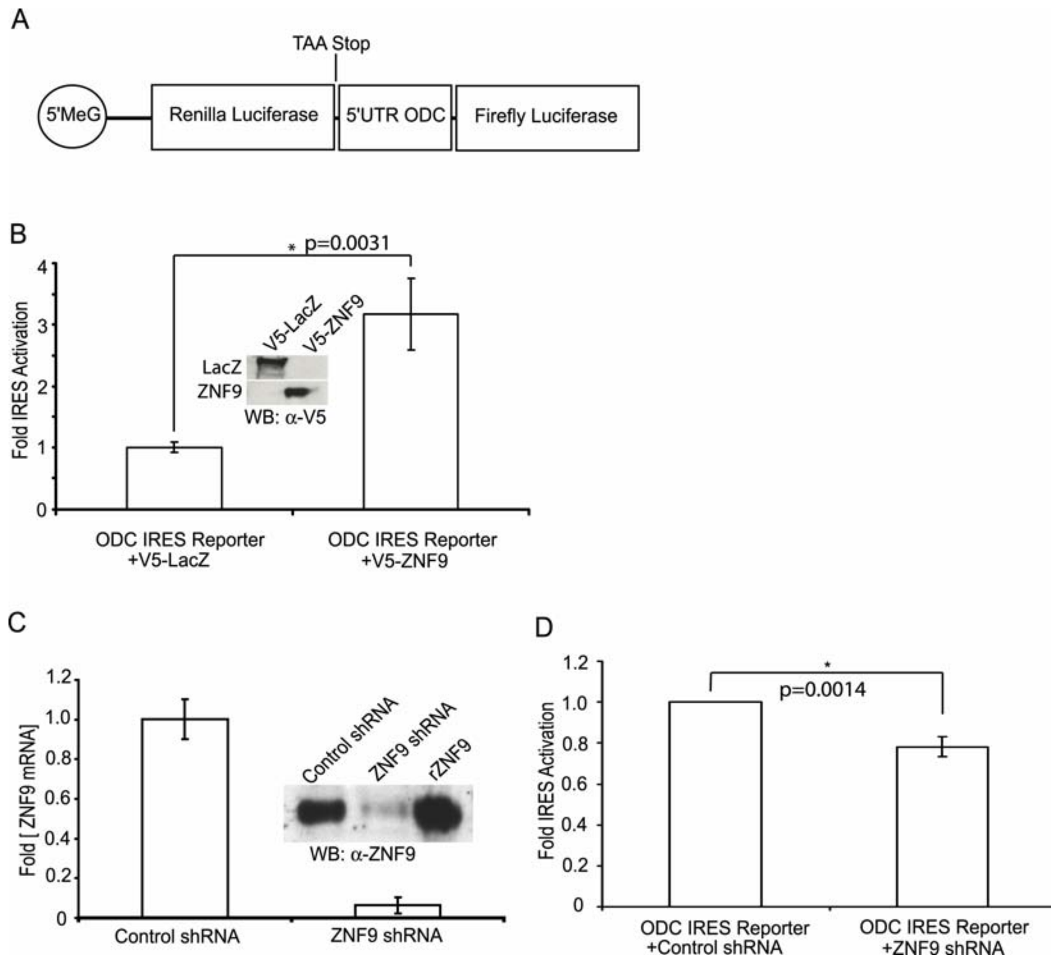


Figure 3. ZNF9 activates cap independent-translation of the human ODC mRNA. A. Diagram of the ODC bicistronic vector for measuring cap-independent translation of human ODC. 5'meG: 5'-methyl-G cap structure. B. HeLa cells expressing the ODC bicistronic vector and either V5-ZNF9 or V5-LacZ were analyzed for luciferase activity. The level of cap-independent translation is reported as a ratio of cap-independent translation (firefly) to cap-dependent translation (*Renilla*). Values are averages of 6 independent experiments. The error bars are the standard error of the mean (S.E.M). The insert figure is a representative western blot analysis showing the V5-LacZ and V5-ZNF9 levels in the HeLa cells lysates. C. ZNF9 mRNA and protein levels after RNAi of ZNF9 in HeLa cells. HeLa cells expressing either a control shRNA or a ZNF9-specific shRNA were analyzed using quantitative RT-PCR and western blotting to quantify expression of ZNF9 RNA and protein, respectively. The RT-PCR values were computed using the $\Delta\Delta$Ct method and are normalized to the levels of GAPDH mRNA. Error bars represent the standard error. The insert figure is a western blot showing the ZNF9 proteins levels. Recombinant ZNF9 (rZNF9) was used as a positive control, and equal amounts of protein were loaded in each lane as assessed by BCA protein assays. D. HeLa cells expressing the ODC bicistronic vector and either a control or ZNF9-specific shRNA were assessed for luciferase activity. The level of cap-independent translation is reported as a ratio of cap-independent translation (firefly) to cap-dependent translation (*Renilla*). Values are the averages of 3 independent experiments and are normalized to the control shRNA. The error bars are the S.E.M.

Cap-independent translation of ODC is defective in human DM2 myoblasts

Our data show that ZNF9 stimulates the cap-independent translation of the ODC mRNA. Using a biochemical assay for ZNF9 function, we sought to address the critical question of whether the repeat expansions that cause human DM2 also compromise this function of ZNF9. If ZNF9 activity is reduced in DM2, we predicted that translation of the ODC mRNA would be reduced compared to wild-type myoblasts. A bicistronic reporter (HIV-hODC) for cap-independent translation of ODC was introduced into normal and primary human DM2 myoblasts by viral infection. We found cap-independent activation of ODC function is reduced in DM2 (Fig. 4A). DM2 myoblasts show a 31% reduction (p-value=0.003) in cap-independent translation of the ODC reporter (Fig. 4A), providing evidence that cap-independent translation is reduced in DM2. Using quantitative RT-PCR for ZNF9, we determined that the DM2 myoblasts used in our analysis have ~ 50% of the ZNF9 mRNA (p-value=0.0003) found in wild-type myoblasts (Fig. 4B), similar to what was observed in an earlier study (Huichalaf, Schoser et al. 2009).

To test if the reduction in cap-independent translation results from loss of ZNF9 activity, we tested whether overexpression of ZNF9 would rescue the defect. We overexpressed ZNF9 or β-galactosidase in wild-type and DM2 myoblasts by infection with HIV-based virions. Overexpression of ZNF9 in wild-type myoblasts increased cap-independent translation 59% (p-value=0.0016) as seen previously in the HeLa cell lines (Fig. 3B). Expression of β-galactosidase (*LacZ*) in WT and DM2 myoblasts had no discernible effect on cap-independent

translation, as expected from the results shown in Figure 4A. Overexpression of ZNF9 in DM2 myoblasts results in a 12% increase (p-value=0.235) in cap-independent translation of the ODC reporter compared to DM2 myoblast controls (Fig. 4C). The level of cap-independent translation in ZNF9-overexpressing DM2 myoblasts is not restored to the level of wild-type myoblasts (Fig. 4C). The results show that ZNF9 overexpression increases cap-independent translation in both wild-type and DM2 myoblasts, but ZNF9 overxpression in DM2 myoblasts is not sufficient to restore cap-independent translation of the ODC reporter to the level seen in wild-type myoblasts.

In summary, our experiments indicate that ZNF9 associates with actively translating polysomes and activates cap-independent translation of ODC in cultured cell lines. ZNF9 activates cap-independent translation in primary human myoblasts, and this activity is reduced in myoblasts from a DM2 patient. Overexpression of ZNF9 partially restores cap-independent translation in human DM2 myoblasts (Fig. 4C), suggesting that the repeat expansions causing DM2 probably contribute to the reduction of at least one aspect of ZNF9 function. However, other modes of regulating gene expression may be involved and cannot be ruled out.

FIGURE 4

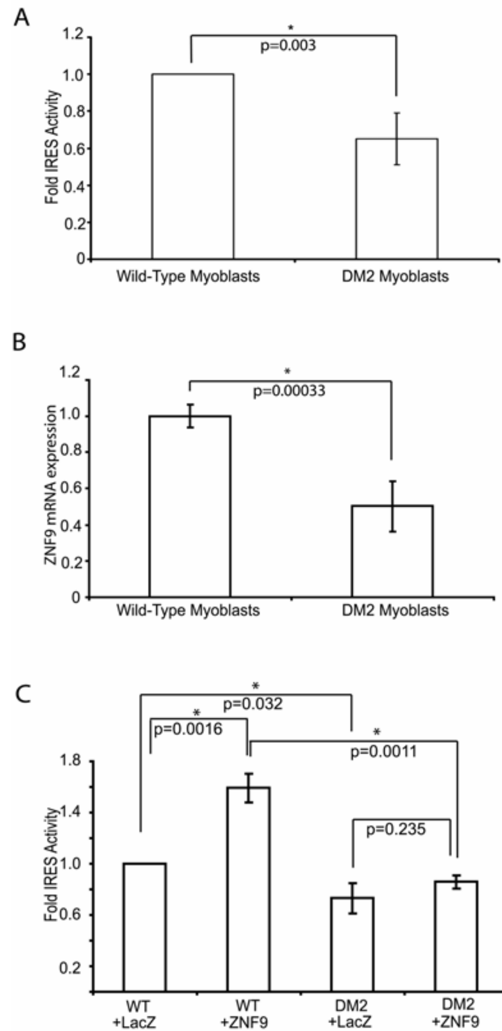


Figure 4. Cap-independent translation of ODC is defective in DM2 myoblasts.
A. IRES activity in wild-type and DM2 myoblast. Equal numbers of human wild-type or DM2 myoblasts were infected with virions expressing the bicistronic ODC reporter vector and were analyzed for luciferase activity. The level of cap-independent translation is reported as a ratio of cap-independent translation (firefly) to cap-dependent translation (*Renilla*). Values are averages of 6 independent experiments and are normalized to IRES activity in wild-type myoblasts. B. Differences in ZNF9 mRNA expression in wild-type and DM2 myoblasts. Quantitative real-time PCR was performed on equal amounts of total mRNA from wild-type and DM2 myoblasts. Values were computed using the $\Delta\Delta$Ct method and are normalized to levels of GAPDH mRNA. Error bars represent the S.E.M. C. IRES activity in wild-type and DM2 myoblast overexpressing ZNF9. Equal numbers of wild-type or DM2 myoblasts were infected with the bicistronic ODC reporter vector and vectors for either the overexpression of LacZ or ZNF9. Cell lysates were analyzed for luciferase activity as described above. Values are reported as a ratio of cap-independent translation to cap-dependent translation and are representative of 4 independent experiments. The values are normalized to the value of IRES activation in wild-type myoblasts overexpressing LacZ. Error bars represent the S.E.M.

Discussion

Misregulation of translational control is a common cause of multiple diseases, including many cancers, fragile X syndrome, and vanishing white matter disease (Holland, Sonenberg et al. 2004; Bagni and Greenough 2005; Scheper, Proud et al. 2006; Sonenberg and Hinnebusch 2009). Defects in cap-independent translation have been shown previously to be linked to diseases such as X-linked dyskeratosis congenita (Yoon, Peng et al. 2006), suggesting that defects specifically in cap-independent translation can result in human diseases. Previous investigations into ZNF9 activity showed that reduced ZNF9 protein expression results in diminished translational activity and leads to symptoms that mimic those seen in myotonic dystrophy type 2 (Chen, Wang et al. 2007; Huichalaf, Schoser et al. 2009; Salisbury, Schoser et al. 2009).

Our results support a model in which ZNF9 acts as an IRES trans-activating factor (ITAF) for cap-independent translation of ODC. ZNF9 binds directly to the 5'UTR of the ODC mRNA. ZNF9 is localized mainly to the cytoplasm and physically associates with actively translating ribosomes. Overexpression of ZNF9 activates cap-independent translation of a reporter construct in tissue culture cells. This ITAF activity is reduced in primary myoblasts from a myotonic dystrophy type 2 patient. These results are consistent with a model in which ZNF9 activity is reduced in DM2 patients.

Because ZNF9 interacts with both an mRNA and ribosomes, one likely mechanism for its ITAF activity is recognizing specific IRES sequences or structures within the 5'UTR of mRNAs and subsequently recruiting other initiation

factors and the ribosome for translation activation (Stoneley and Willis 2004; Spriggs, Bushell et al. 2005; Semler and Waterman 2008). It is unknown whether ZNF9 directly recruits the translation machinery or whether other factors are required. Both PCBP2 and La, known ITAFs, have been previously shown to interact with ZNF9 and similar RNA substrates (Pellizzoni, Lotti et al. 1997; Pellizzoni, Lotti et al. 1998; Crosio, Boyl et al. 2000; Gerbasi and Link 2007). Interestingly, ZNF9 was found to interact with an assortment of single-strand RNA binding proteins and ribosomal protein subunits in a large-scale proteomic screen, consistent with the possibility that ZNF9 acts in concert with other proteins to activate translation (Ewing, Chu et al. 2007). Further examination of the interactions of ZNF9 with mRNAs and specific translation proteins will be important in elucidating the details of ribosome recruitment during cap-independent translation, currently a poorly understood process.

The two conflicting models for the cause of DM2 symptoms stem in part from conflicting data regarding the expression of ZNF9 mRNA and protein. Two separate studies noted no change in ZNF9 mRNA and protein levels in DM2 myoblasts or lymphoblastoid cell lines (Botta, Caldarola et al. 2006; Margolis, Schoser et al. 2006). These results support the prevailing toxic-RNA gain-of-function disease model in which the repeat expansions do not reduce ZNF9 activity but rather interfere with other critical processes. In contrast, Huichalaf et *al.* used both western blotting and *in situ* antibody staining in DM2 myoblasts to detect a decrease in ZNF9 protein levels that correlated with decreased translational activity (Huichalaf, Schoser et al. 2009). These results are

consistent with the loss-of-function disease model, in which the repeat expansions compromise ZNF9 function. The discrepancies may be due to low sample numbers, the use of unaffected tissue types (lymphoblastoid), or differences in the cell line's genetic backgrounds. A larger and more comprehensive analysis of ZNF9 protein levels in DM2 is warranted to resolve the discrepancies.

Our study of ZNF9 overexpression in DM2 myoblasts suggests that neither of the two models completely describes the situation in DM2. First, we find cap-independent translation is reduced but not eliminated in myoblasts from DM2 patients compared to myoblasts from healthy controls, a result expected from loss of ZNF9 function. The residual activity in DM2 myoblasts may stem from either low levels of ZNF9, partially active ZNF9, or ZNF9-independent activity. Overexpression of ZNF9 in normal myoblasts results in an increase in cap-independent translation. Thus, it seems reasonable to expect that overexpression of ZNF9 in DM2 myoblasts would restore cap-independent translation to at least normal levels. Cap-independent translation activity was slightly increased compared to control DM2 myoblasts and did not restore wild-type levels. This result suggests that activation of cap-independent translation by ZNF9 is somehow blocked in DM2 cells, possibly through a dominant-negative action of the expanded repeat RNA. Cell-free, reconstituted system in which ZNF9 levels can be carefully controlled will be important for a clearer understanding of this phenomenon.

Our data presented here and previous observations suggest that a combination of an RNA-mediated dominant effect and ZNF9 loss-of-function contribute to DM2. Salisbury *et al.* have shown that transcribed CCTG repeats are found in both the nucleus and the cytoplasm of DM2 myoblasts and that the RNA repeats misregulate translation (Huichalaf, Schoser et al. 2009; Salisbury, Schoser et al. 2009). ZNF9 may be inhibited by the presence of transcribed CCTG repeats in the cytoplasm, much as the RNA splicing factor MBNL activity is thought to be inhibited by the presence of transcribed CTG and CCTG RNA in the nucleus (Fardaei, Rogers et al. 2002; Warf and Berglund 2007; Yuan, Compton et al. 2007). Both proteins contain multiple zinc finger motifs to mediate binding to specific RNAs and may be inhibited by similar substrates. In future work, it will be important to determine whether ZNF9 and other RNA binding proteins are inhibited by the presence of transcribed CCTG repeats in either the nucleus or the cytoplasm.

CHAPTER III

SACCHAROMYCES CEREVISIAE GIS2 INTERACTS WITH THE EUKARYOTIC TRANSLATION MACHINERY AND IS ORTHOLOGOUS TO MAMMALIAN ZNF9

Abstract

The myotonic dystrophy type 2 (DM2) protein ZNF9 is a small nucleic acid binding protein proposed to act as a regulator of transcription and translation. The precise functions and activity of this protein are poorly understood. Previous work described in Chapter II suggested that ZNF9 facilitates the process of cap-independent translation through interactions with mRNA and the translating ribosome. To determine the role played by ZNF9 in the activation of this specialized form of translation initiation, I aimed to combine genetic and biochemical analysis of a putative ZNF9 ortholog, Gis2, in *Saccharomyces cerevisiae*. Purification of the Gis2p protein followed by mass spectrometry based-proteomic analysis identified a large number of co-purifying ribosomal subunits and translation factors, strongly suggesting that Gis2p interacts with the protein translation machinery. Polysome profiling and ribosome isolation experiments confirm that Gis2p physically interacts with the translating ribosome. Interestingly, expression of yeast Gis2p in HEK293T cells activates cap-independent translation driven by the 5'UTR of the ODC gene. These data suggest that Gis2 is functionally orthologous to ZNF9 and may act as a cap-independent translation factor. Genetic analysis of Gis2p function in

Saccharomyces cerevisiae can provide insight into the molecular mechanism of the mammalian ZNF9 protein.

Introduction

The study of human pathologies and disease states are complicated by the complexity of human biology. This is especially true when dealing with human genetic disorders that can result from the loss of a single gene's function, like in cystic fibrosis, or through a combination of multiple genetic changes, like in cancer and neurological disorders. Therefore, researchers often seek to study related cellular processes in more genetically tractable systems, such as *Saccharomyces cerevisiae.* In fact, the use of systems such as *S. cerevisiae* has led to a much greater understanding of quite a few uniquely human disease states, such as Diamond Blackfan anemia, Parkinson's disease, cancer, and amyotrophic lateral sclerosis (Xu, Pin et al. 2004; Leger-Silvestre, Caffrey et al. 2005; Cooper, Gitler et al. 2006; Gitler, Bevis et al. 2008; Hemphill, Bruun et al. 2008; Johnson, McCaffery et al. 2008).

The human disease myotonic dystrophy type 2 (DM2) is caused by a tetranucleotide repeat expansion in the first intron of the *znf9* gene, which codes for the protein ZNF9. ZNF9 is a small nucleic acid binding protein believed to function as a regulator of either transcription or translation, and has been proposed to act as a regulator of cellular growth and proliferation in vertebrates. Much of the ambiguity in the reported functions of ZNF9 comes from the complexity in studying essential genes in mammalian model systems. Deletion

or mutation of *ZNF9* results in embryonic lethality in mice and chickens. While this provides evidence for the importance of the ZNF9 protein in growth and development, it does not illuminate the *in vivo* cellular functions and interactions of the protein.

Recently, we discovered that ZNF9 functions as a regulator of a cap-independent, or internal ribosome entry site (IRES)-dependent, translation (Sammons, Antons et al. 2010). IRES-mediated translation in eukaryotes is mediated by a number of factors, including IRES-specific *trans*-activating factors (ITAFs) that are thought to act through stabilize certain mRNA structures and facilitate interactions with the ribosome. ZNF9 directly binds to the IRES sequence in the 5'UTR of the ornithine decarboxylase (ODC) mRNA and facilitates the translation of this mRNA independent of the cap complex (Sammons, Antons et al. 2010). Other groups have also observed that ZNF9 acts as a regulator of translation (Pellizzoni, Lotti et al. 1997; Pellizzoni, Lotti et al. 1998; Crosio, Boyl et al. 2000; Schlatter and Fussenegger 2003; Huichalaf, Schoser et al. 2009), but the full scope of ZNF9's cellular interactions and any other processes regulated ZNF9 are not well understood. The use of other model systems and lines of inquiry are required to gain a full understanding of ZNF9 function.

Previous studies in the zebrafish, mouse, and chick model systems have provided insight into the molecular function of the ZNF9 protein. ZNF9 mRNA is present in the developing embryos of mice, chickens, and zebrafish, with the highest level expression being found in regions that will develop into craniofacial

structures. In mice, homozygous *znf9* null mutants exhibit embryonic lethality due to abnormalities in the developing craniofacial system and developing brain. Mice heterozygous for *znf9* develop various symptoms, including muscle weakness and sudden arrhythmia, which mimic the symptoms of DM2. Knockdown experiments in chicks, zebrafish, and *Xenopus* confirmed that loss of ZNF9 activity leads to craniofacial and brain development phenotypes. Further studies in these model systems suggest that ZNF9 regulates cell proliferation or apoptosis, although the mechanism of action in these processes is unknown and unexplored.

The translation machinery of the baker's yeast *Saccharoymces cerevisiae* and other single-celled eukaryotes is well conserved. Yeast offers many advantages to vertebrate model systems for the genetic study of individual proteins or genetic pathways. Genes required for cell viability, such as many eukaryotic translation initiation factors and ribosomal proteins, can still be genetically perturbed, allowing for sophisticated gene replacement studies. The *S. cerevisiae* genome encodes one putative homolog of mammalian ZNF9 called Gis2p. *GIS2* was initially cloned as a multi-copy suppressor of the Gal^- phenotype exhibited by *snf1/mig1/srb8* mutants, but no other evidence for Gis2p function has been reported. SNF1, a homolog of human AMPK, is part of a signaling cascade that regulates the transcriptional activation of the genes required for galactose utilization. SNF1 is a kinase and phosphorylates MIG1, a DNA-binding protein that represses transcription of galactose genes (Ostling and Ronne 1998; Treitel, Kuchin et al. 1998). Phosphyorylated MIG1 is inactive and

depresses transcription or the GAL genes, controlled in part by SRB8, a component of the Mediator complex (Ostling and Ronne 1998; Treitel, Kuchin et al. 1998). Deletion of MIG1 allows for growth on galactose, but deletion of SRB8 in *mig1* cells creates Gal- cells (Balciunas and Ronne 1995; Balciunas and Ronne 1999). While *GIS2* may in fact function to regulate the expression of the genes required for galactose utilization, the result has never been replicated or followed-up.

In this study, we show that the *S. cerevisiae* protein Gis2p shares considerable sequence homology with the mammalian ZNF9 protein and is likely a functional ortholog. Gis2p associates with translating ribosomes and copurifies with many ribosomal protein subunits as determined by mass spectrometry based proteomics. Finally, Gis2p is able to activate cap-independent translation of the ODC IRES in human tissue culture cells. These data together suggest that the *S. cerevisiae* protein Gis2p and the mammalian protein ZNF9 are functional orthologs and provide a novel system to study the molecular function of ZNF9 in translation and other essential cellular processes.

Materials and methods

Yeast strains and plasmids

Yeast genetic manipulations and media preparation were performed essentially as described. The g*is2 d*eletion strain used in this study had the entire *gis2* ORF replaced by a kanamycin cassette in the BY4743 background

(*MAT**a**/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/LYS2 MET15/met15Δ0 ura3Δ0/ura3Δ0)* (Winzeler, Shoemaker et al. 1999). Knockout strains of *gis2* were confirmed by PCR-based amplification across the kan^R locus and growth of the strains on G418-containing medium. Yeast strains with the Gis2p tandem-affinity-purification tags have been described (Ghaemmaghami, Huh et al. 2003).

Tandem Affinity Purification

Gis2p was purified from 1 L cultures of a Gis2p-TAP-tagged yeast strain (Ghaemmaghami, Huh et al. 2003) grown to early stationary phase ($O.D._{600}$ 2-4). Gis2p and associated proteins were isolated using a dual affinity protocol as previously described (Link, Fleischer et al. 2005). 10% of the eluted proteins were analyzed by SDS-PAGE on 4-12% Novex Bis-TRIS gels (Invitrogen) and silver stained. The remaining eluted proteins were reduced with DTT, alkylated with iodoacetamide, and digested with sequencing grade trypsin (Promega) for LC-MS/MS analysis.

Western Blotting

Anti-TAP serum (OPEN Biosystems) was used at a 1:1000 dilution. Anti-HA-HRP (Pierce) was used at 1:10,000. All primary antibodies were diluted in 5% non-fat dry milk and TBST (TRIS-buffered saline and 0.1% TWEEN-20) supplemented with 0.04% NaN_3.

Mass Spectrometry-based Proteomics and Data Analysis

Trypsin-digested peptides were identified using two dimensional microcapillary liquid chromatography coupled with an LTQ linear ion trap mass spectrometer (Thermo Electron, Inc) as described previously (Link, Eng et al. 1999; Link, Fleischer et al. 2005). Acquired mass spectra were correlated to a translated *Saccharomyces cerevisiae* ORF database using Sequest (Eng, McCormack et al. 1994). The resulting peptide hits were analyzed, clustered, and visualized using BIGCAT ((McAfee, Duncan et al. 2006).

Polysome Profiling

For the isolation of ribosomes by sucrose gradient centrifugation, Gis2-TAP-tagged strains were grown in 10 mL of SC-HIS to an $O.D._{600}$ of 0.8. Cycloheximide was added to a concentration of 50 μg/mL, and the cultures were placed on ice for 10 min. Cells were pelleted, washed with cold phosphate buffered saline, and lysed with 0.5 mm glass beads in 500 μL of lysis buffer (10 mM Tris-HCl, pH 8.0, 140 mM NaCl, 1.5 mM MgCl2, 0.5% NP40, 10 mM DTT, 300 μg/mL heparin, 100 μg/mL cycloheximide and 250 U/mL RNASIN (Promega). Cellular debris was cleared by centrifugation at 20,000 x g for 10 min. Supernatants (100μl) were layered on top of 10-40% sucrose gradients in 10 mM Tris-HCl, pH 7.5, 140 mM NaCl, 1.5 mM MgCl2, 10 mM DTT, 100 μg/mL cyclohexamide, and 0.5 mg/ml heparin. Gradients were centrifuged in a Beckman SW-41 swinging bucket rotor for 60 min at 38,000 rpm. 1 mL fractions were collected by bottom displacement with on-line UV_{280} measurements

(Agilent). Fractions were TCA-precipitated and 50% of the total protein was analyzed by SDS-PAGE and western blotting using anti-TAP serum (OPEN Biosystems) at 1:1000.

Ribosome pelleting and ribosome salt wash experiments

Ribosome pelleting experiments were performed by centrifugation of whole cell lysates described above through a 1 M sucrose cushion (-/+ 10mg/mL RNAse H or 50mM EDTA) at 100,000x*g* for 2 h in a TLA120.2 rotor. The resulting ribosome pellet was resuspended in ribosome solubilization buffer (20 mM TRIS pH 7.5, 5 mM $MgCl_2$, 6 M urea). Supernatant fractions were precipitated with trichloroacetic acid, washed with acetone, and resuspended in ribosome solubilization buffer. For ribosome salt wash analysis, ribosome pellets were isolated as described above, and the intial supernatants were discarded. Pellets were washed in ice cold PBS and resuspended in ribosome salt wash buffer containing 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM BME, and either 100 mM, 250 mM, 500 mM, or 1 M potassium acetate. Resuspended ribosomes were then centrifuged at 100,000x*g* for 2 h in a TLA120.2 rotor. Supernatant fractions were removed and TCA precipitated as described above, and pellet fractions were resuspended in solubilization buffer. Equal volumes of lysate were separated by polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and immunoblotted using α-TAP antibodies.

Growth defect and translation inhibitor selection assays

Yeast strains were grown in either SC or SC+G418 (0.2mg/ml) to an $O.D._{600}$ of 0.6. Cells were counted with a hemocytometer, adjusted to equal concentration, and serially diluted (10 fold). Five µL of each 10-fold serial dilution was spotted onto the appropriate test plates. Growth assays to test salt sensitivity were performed with either 0.5M or 1M NaCl. Translation inhibitors were added to the media at the following concentrations: hygromycin (100 µg/mL), paromomycin (0.4 mg/mL), anisomycin (100 µg/mL), rapamycin (25 ng/mL), and cycloheximide (0.2 µg/mL and 2 µg/mL). For growth on galactose, standard YPD media was prepared replacing 20g/L dextrose with 20g/L of galactose. For heat shock experiments, log-phase yeast cells were incubated in prewarmed (45°C) YPD for 15 minutes, and then plated on YPD plates as described above. The plates were incubated for 48 hours and then photographed.

Tissue culture assay for cap-independent translation

HEK293T cells were cultured in 10 cm tissue culture-treated plates using DMEM and 10% fetal bovine serum at 37°C and 5% CO_2. pcDNA3.1-hODC-IRES, pcDNA3.1-V5-LacZ, and pcDNA3.1-V5-ZNF9 were previously described (Sammons, Antons et al. 2010). pcDNA3.1-V5-Gis2 was created by Gateway-mediated recombination of pcDNA3.1-V5-*ccdB* (Invitrogen) and pENTR-Gis2, which was created by cloning the *gis2* coding sequence amplified from yeast genomic DNA using the sense primer 5'- CACCAT

GTCTCAAAAAGCTTGTTACG-3’ and the antisense primer 5’-CTAAGCCTTTGGACAATCCT-3’. Equal amounts of pcDNA3.1-hODC-IRES and a plasmid expressing either ZNF9, Gis2p, or β-galactosidaese were transfected into HEK293T cells using Lipofectamine (Invitrogen) following themanufacturer’s recommendations. After 24 hours, cells were collected and processed for luciferase assays according to the manufacturer’s recommendations (Promega, Dual Luciferase Assay Kit). Cap-independent translation activity is reported as previously described (Sammons, Antons et al. 2010).

Cloning of Znf9 deletion mutants

To create deletion mutants of ZNF9, PCR amplified products using the following primers on full-length pcDNA3.1-V5-ZNF9 were cloned into pENTR-D/TOPO (Invitrogen). For the ∆7, ∆6, and ∆5 mutants, the sense primer was 5’ CACCATGAGCAGCAATGAGTGCTT-3’.

Antisense ∆7: 5’- TTATTCACTTGTCTTGCTGCAGT-3’.

Antisense ∆6: 5’-TTATTTGGTGCAGTCTTTTTGAA-3’.

Antisense ∆5: 5’-TTACTCATCTGCATGGTCGCAGT-3’. The antisense primer for the ∆1 and ∆RGG mutants was 5’-AGGCTGTAGCCTCAATTGTGCATTC-3’. Sense primer for ∆1: 5’-CACCATGACTGGTGGAGGCCGTGGTCG-3’. Sense primer for ∆RGG: 5’-CACCATGATTTGTTATCGCTGTGGTGA-3’. Destination clones were created by Gateway-mediated recombination of individual entry clones with pcDNA3.1-V5-*ccdB* (Invitrogen). All plasmids were sequence verified by the Vanderbilt DNA Sequencing Facility. Fusion with the V5-tag was verified

by western blotting analysis using α-V5 monoclonal antibody (Invitrogen). The ability of the ZNF9 mutants to activate cap-independent translation was assessed as described above.

Results

ZNF9 is evolutionarily conserved in eukaryotes

Previous studies have implicated ZNF9 in a wide variety of molecular functions, ranging from the regulation of transcription to the control of cell growth and proliferation (Pellizzoni, Lotti et al. 1997; Chen, Liang et al. 2003; Abe, Chen et al. 2006; Weiner, Allende et al. 2007; Armas, Aguero et al. 2008). Because ZNF9 is absolutely required for the viability of vertebrate organisms (Chen, Liang et al. 2003; Shimizu, Chen et al. 2003), genetic analysis of ZNF9 function is impossible in these complex animals. The exact roles or molecular functions of ZNF9 are unknown, partially due to a lack of a simple model organism in which to study ZNF9 biochemical and genetic activity. Using the NCBI tool Homologene, which searches for homologous proteins throughout all fully sequenced and annotated eukaryotic genomes, we found putative ZNF9 orthologs in multiple organisms (Figure 5) Interestingly, the *Saccharomyces cerevisiae* protein Gis2p has high levels of homology to ZNF9 in both sequence and in domain architecture (Figure 6). ZNF9 and its vertebrate homologs contain seven CCHC-type zinc fingers, with an RGG box between the first and second zinc finger (Figures 5 and 6). ZNF9 homologs seem to cluster into specific groups,

vertebrate, cartilaginous fish, insects, and fungi. While the putative fungi homologs, including Gis2p have seven CCHC-type zinc finger domains, neither Gis2p nor the *S. pombe* homolog contains an RGG box.

While Gis2p contains significant sequence similarity with the *Homo sapiens* ZNF9 protein, it is unknown whether the two proteins can be considered functional orthologs. In order to demonstrate orthology, we determined whether Gis2p has biochemical properties and interactions similar to ZNF9. Previously, ZNF9 was found to associate with the translating ribosome and act as a regulator of cap-independent translation (Sammons, Antons et al. 2010).

Figure 5

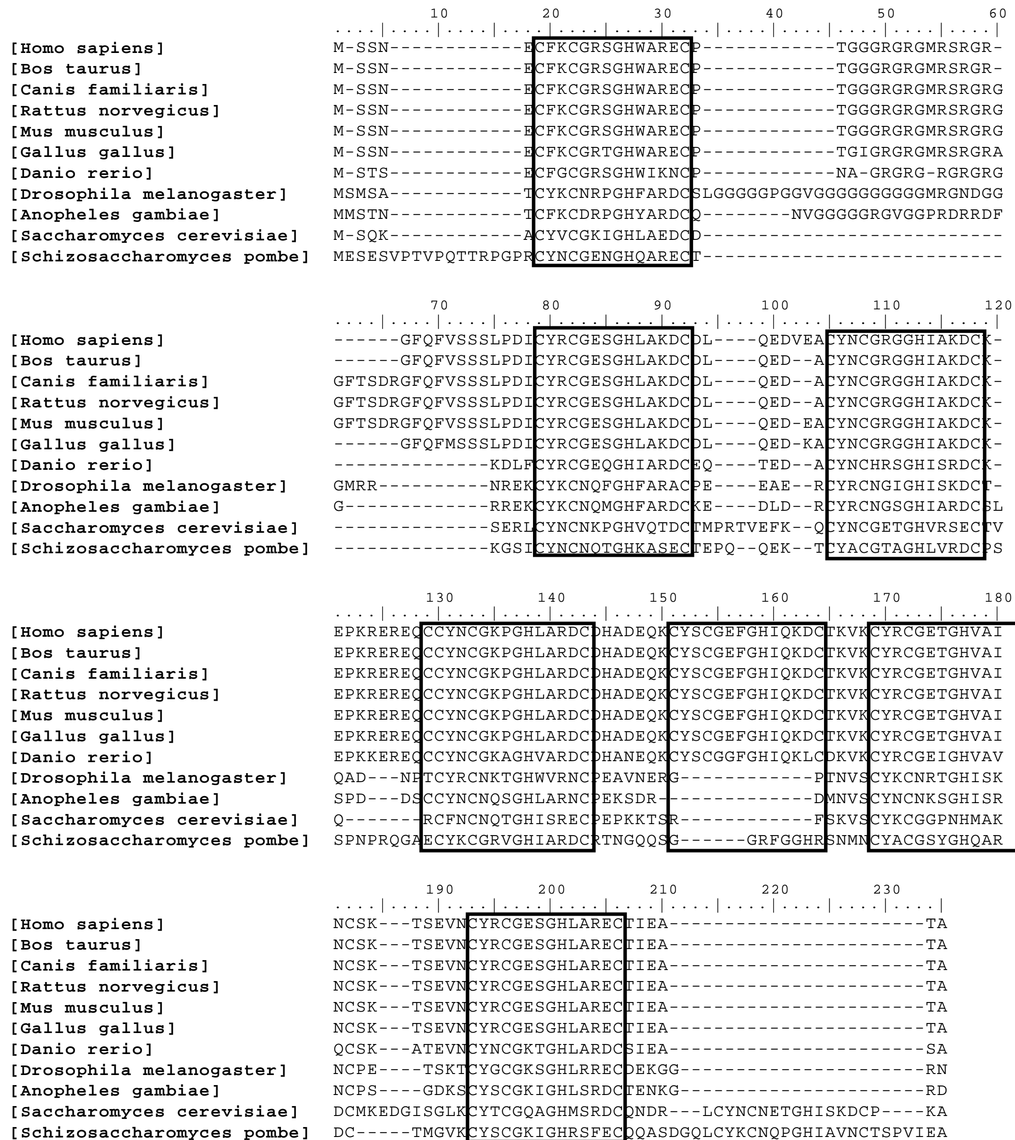
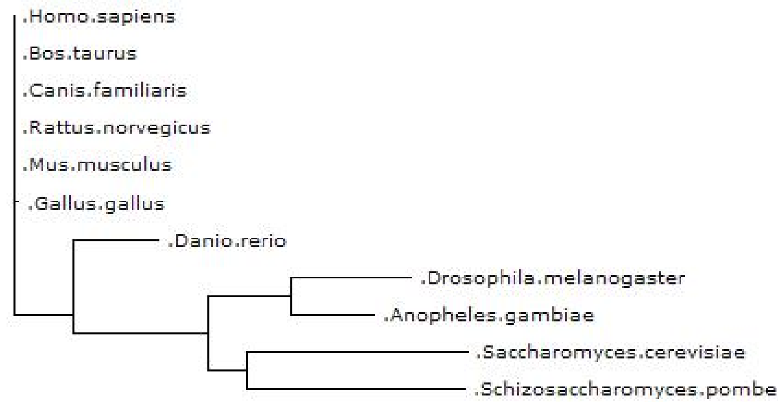


Figure 5. Multiple sequence alignment of putative ZNF9 homologs. Using the National Center for Biotechnology Information's Homologene software, putative homologs to the human ZNF9 protein were identified. Amino acid sequences of all putative homologs were aligned using MAFFT (Multiple Alignment by Fast Fourier Transform (Katoh, Misawa et al. 2002)). Boxed regions represent aligned CCHC-type zinc finger motifs.

Figure 6

A



B

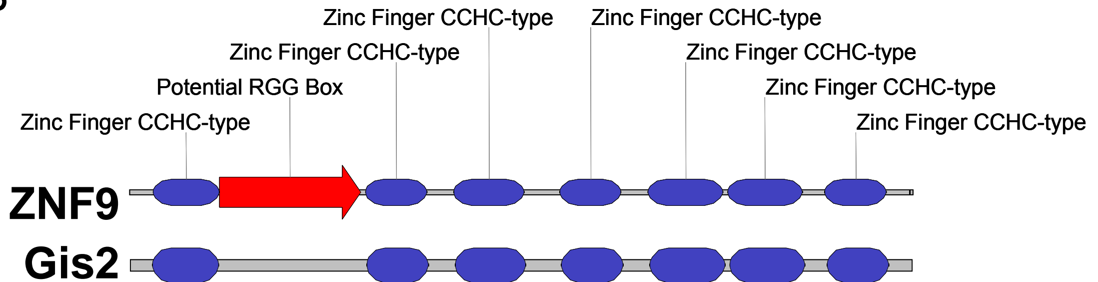


Figure 6. Phylogenetic analysis and functional domain analysis reveal a *Saccharomyces cerevisiae* homolog to the human ZNF9 protein. A. Phylogenetic analysis (Katoh, Misawa et al. 2002) groups the putative homologs of ZNF9 (Figure 3-1) based upon their divergence from the human ZNF9 amino acid sequence. Vertebrate ZNF9 homologs are all identical to the human protein, while cartilaginous fish, insects, and fungi are in their own distinct groups. B. ZNF9 and the Gis2 share very similar domain architectures and sequences, suggesting that they may be functional orthologs.

Saccharomyces cerevisiae Gis2p copurifies with the eukaryotic ribosome

To examine whether Gis2p and ZNF9 have conserved interactions with other proteins, we used tandem affinity purification (TAP) followed by tandem mass spectrometry to identify proteins that associate with Gis2p. This approach has been extensively used to identify and characterize protein:protein interactions in yeast (Gavin, Bosche et al. 2002; Gavin and Superti-Furga 2003; Gavin, Aloy et al. 2006). Following TAP-isolation, the eluted proteins were separated by SDS-PAGE and visualized by silver staining, as seen in Figure 7. Affinity purification of Gis2p yielded a large set of co-purifiy proteins, the majority of which were below 40kDa. We identified the co-purifying proteins by shotgun LC-MS/MS analysis as previously described (Link, Eng et al. 1999; Link, Fleischer et al. 2005; Fleischer, Weaver et al. 2006). The mass spectrometry data were processed and analyzed using the BIGCAT software suite (McAfee, Duncan et al. 2006). Semi-quantitative protein abundance factors (PAF) were computed for each identified protein to give a relative measure of the abundance of each protein (McAfee, Duncan et al. 2006). Based on this quantification method, the most abundant co-purifying proteins were ribosomal subunits and other translation related proteins (Figure 7 and Appendix A, Table A1). Hierarchal clustering of data from three replicate experiments revealed that Gis2p reproducibly copurifies with ribosomes and other translation factors, but also copurifies with other proteins not found in ribosome isolations (Figure 7 and Appendix A, Table A1). Similarly, immunoprecipitation of ZNF9 from HeLa cells followed by shotgun proteomic analysis revealed interactions with the human

ribosome machinery (Appendix A, Table A2). These data suggest that Gis2p copurifies with components of the eukaryotic ribosome and provide evidence of biochemical interactions are conserved between Gis2p and ZNF9.

FIGURE 7

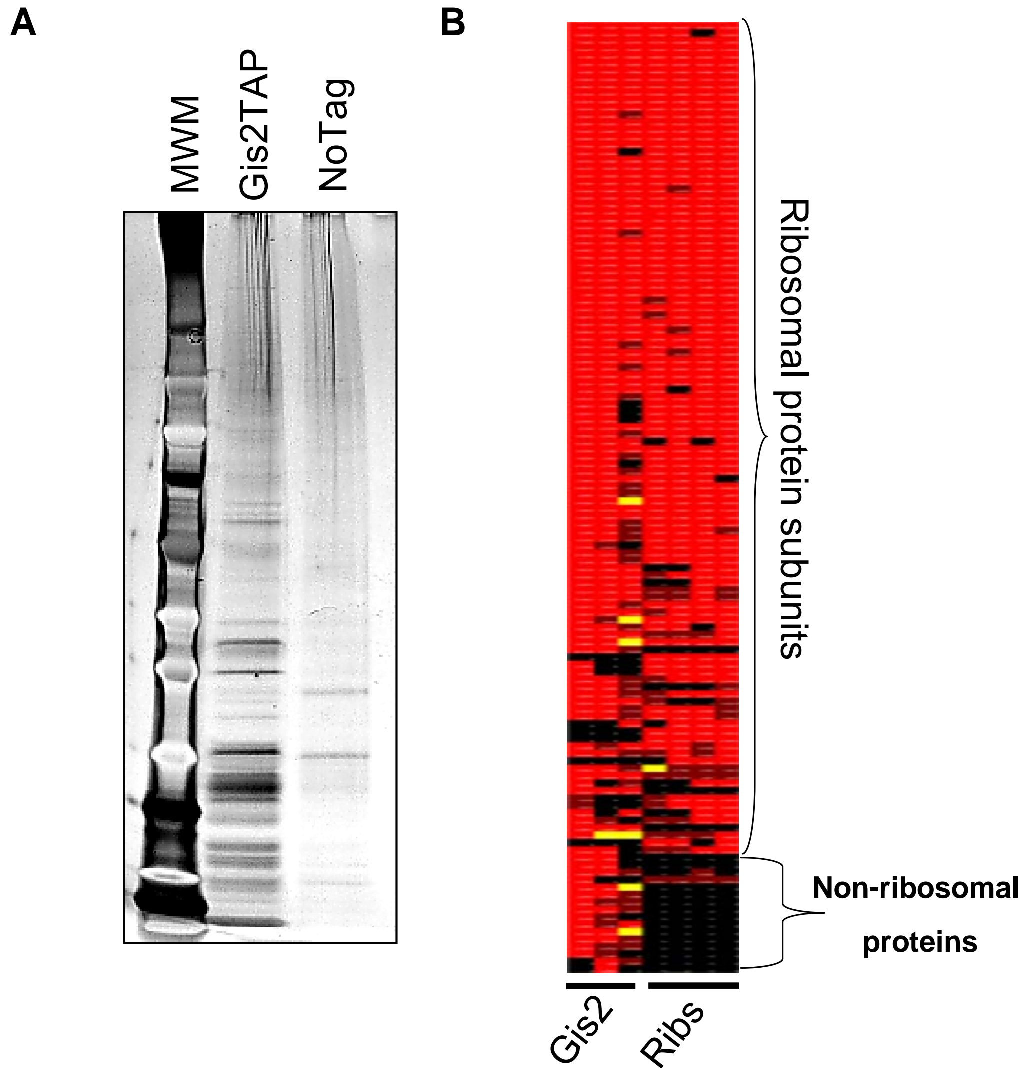


Figure 7. Purification of Gis2-TAP reveals the interactome of Gis2p.
A.Eluted proteins from TAP protocol from a Gis2-TAP expressing strain and an untagged parental strain. 10% of eluted proteins were analyzed by SDS-PAGE and silver stained. MWM-molecular weight markers. B. Heirarchal clustering of 3 replicate Gis2-TAP purifications compared to 4 replicate isolations of ribosomes from mass spectrometry data. On the heat map, protein abundance is measured from black representing no protein identified to bright red meaning high abundance. Notice the significant overlap in the proteins copurified with Gis2-TAP and purified ribosomes.

Polysome analysis reveals Gis2p colocalizes with the ribosome

To test whether copurification of Gis2p with ribosomal subunits and the translation machinery is a result of *in vivo* association with the ribosome during translation, we isolated ribosomes by differential centrifugation and assayed the presence of Gis2p. Initially, lysates from a Gis2p-TAP-tagged strain were loaded onto a sucrose cushion and subjected to ultracentrifugation to isolate polyribosomes. As seen in Figure 8B, the large majority of Gis2p is found in the polysome pellet, which suggests that Gis2p associates with the translating ribosome. We confirmed this result in linear sucrose gradient ultracentrifugation experiments. As seen in Figure 8A, Gis2p is found in the heavy polysome-containing fractions. In both centrifugation strategies, a small population of Gis2p was found in the non-ribosomal fractions, suggesting that not all of the cellular Gis2p is actively associated with the ribosome. Human ZNF9 shows the same pattern of localization: predominantly ribosomal, with a small non-ribosome pool (Sammons, Antons et al. 2010).

Ribosome salt wash experiments have been used to separate core ribosomal subunits from associated translation factors, and were used to determine the relative strength of human ZNF9's ribosome interaction (Sammons, Antons et al. 2010). We reasoned that Gis2p and ZNF9 should elute from the ribosome at similar salt concentrations if they are biochemical and functional orthologs. As seen previously for human ZNF9, Gis2p is absent from the wash fractions at low salt concentrations (100mM and 250mM potassium acetate) (Figure 8C). At the 500mM salt concentration, Gis2p begins to elute

from the ribosome pellet, and at 1M salt, all of the Gis2p is found in the wash fraction. These results are similar to what was previously observed with human ZNF9 and suggest that the Gis2p interaction with the ribosome is similar to that of other translation factors but not quite at the strength of core ribosomal subunits (Sammons, Antons et al. 2010). The similar ribosomal interactions of Gis2p and ZNF9 provide evidence that they are putative biochemical orthologs and suggest that they function in the essential cellular process of translation.

FIGURE 8

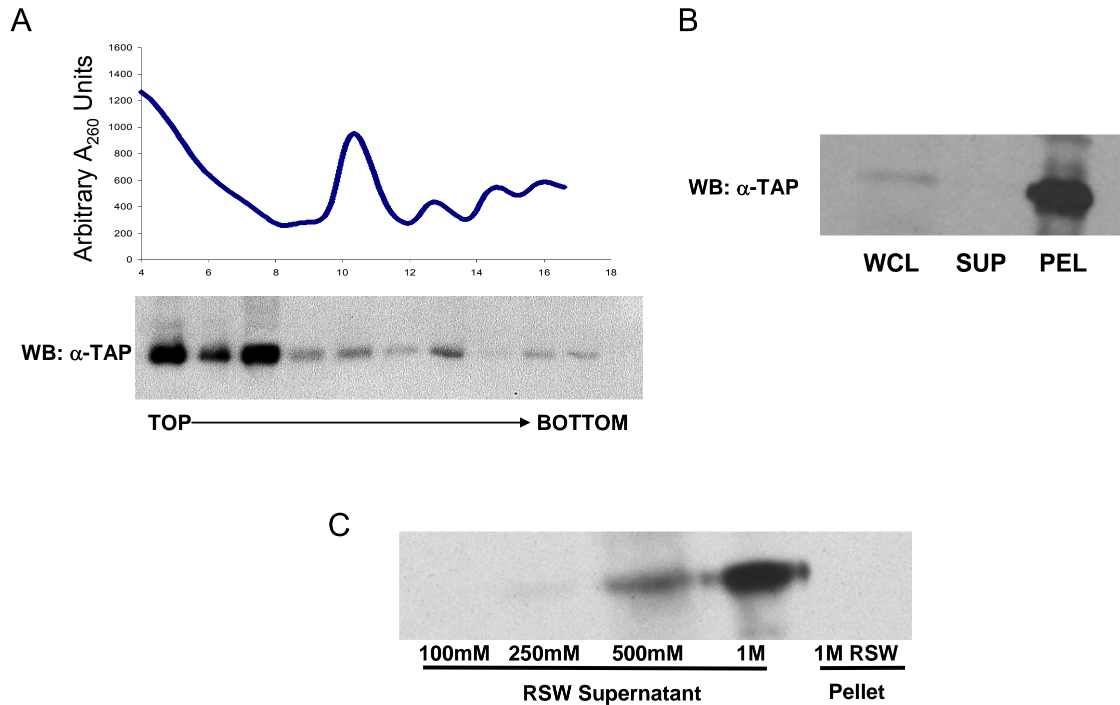


Figure 8. *S. cerevisiae* Gis2p interacts with the ribosome.
A. Polysome analysis of sucrose gradient ultracentrifugation fractions for Gis2p were measured by bottom displacement and on-line UV_{260} measurements. 10% of each fraction was analyzed by western blotting for the presence of Gis2. B. Western blot analysis for the presence of Gis2p in whole cell lysates (WCL), supernatant (SUP), or pellet (PEL) fractions after isolation of polysomes from Gis2-TAP-tagged cells. C. Ribosome salt wash and western analysis for Gis2p. Potassium acetate was used as the salt in salt wash buffers.

Gis2p-Ribosome interactions are sensitive to RNAse

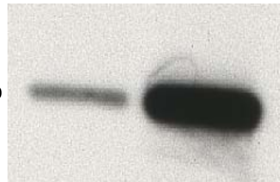
The data shown in Figure 8 strongly suggests that Gis2p interacts with the translating ribosome, but the data do no suggest a mechanism behind the interaction. Because of the polysomal localization of Gis2p, we tested whether Gis2p's ribosome association required tethering through mRNA by treatment with either RNAse A. RNAse A specifically cleaves single-stranded RNA. Lysates of Gis2-TAP expressing cells were mock-treated or treated with 50μg/mL of RNAse A for 30 minutes at room temperature and then Gis2-TAP was purified and subjected to western blot analysis and mass spectrometry based proteomics. Interestingly, even though more Gis2p was purified in RNAse-treated samples, less ribosomal proteins were identified by mass spectrometry. This result suggests that RNAse A-treatment releases Gis2-TAP from the ribosome and makes the purification scheme more efficient. To confirm that RNAse A-treatment releases Gis2p from ribosomes, ribosomes were pelleted and then resuspended in ribosome wash buffer supplemented with either RNAse A or EDTA. EDTA disrupts polyribosomes and has been shown to disrupt the interaction between certain RNA binding proteins and the ribosome, including human ZNF9 (Sammons, Antons et al. 2010). Ribosomes were then reisolated through a sucrose cushion and analyzed for the presence of Gis2p. As seen in Figure 9B, treatment of ribosomes with RNAse or EDTA results in a large decrease in Gis2p association, suggesting that Gis2p interacts with actively translating ribosomes and that this interaction requires an interaction with either mRNA or RNAse-sensitive rRNA.

FIGURE 9

A

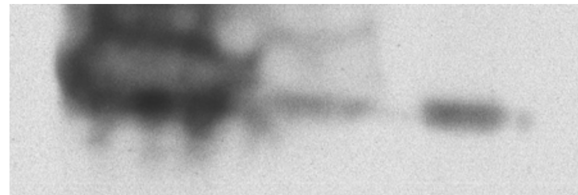
Name	-RNAse	+RNAse
GI2	1.1694	1.7541
RPS13	1.7617	1.1745
RPS0A	2.8547	0
RPL8B	1.7786	1.0672
RPS30A	2.8098	0
RPL9A	1.8545	0.9273
RPL36A	2.6969	0
RPL36B	2.6942	0

WB: α-TAP



B

WB: α-TAP



+EDTA +RNase

Figure 9. Gis2p interacts with the translating ribosome through associations with single-stranded RNA. A. Protein abundance factors of a subset of proteins isolated by purification of Gis2-TAP in the presence or absence of RNAse. Also, western blot analysis of eluted proteins shows the abundance of isolated Gis2-TAP in the presence or absence of RNAse. B. Western blot analysis for Gis2-TAP of solubilized polysome pellets in the presence of EDTA or RNase.

Knockout of Gis2p function results in no obvious growth phenotype

In an effort to identify the function of Gis2p, we analyzed the phenotypes of a *gis2* mutant. An *S. cerevisiae gis2* knockout mutant was created previously and no obvious growth defect or lethality was observed (Winzeler, Shoemaker et al. 1999). Because of Gis2p's proposed role in translation, we reasoned that this mutant would be sensitive to conditions that perturb normal cellular translation. Using traditional spot dilution growth assays, we tested growth of a homozygous diploid *gis2* null mutant and its parental strain under many conditions known to disturb translation, such as high temperature, high osmolarity, a poor carbon source, heat shock. We also tested several chemicals that specifically inhibit translation. Deletion of *gis2* does not cause any discernible growth defect when compared to the parental strain under any of the tested conditions (Figure 10).

Figure 10

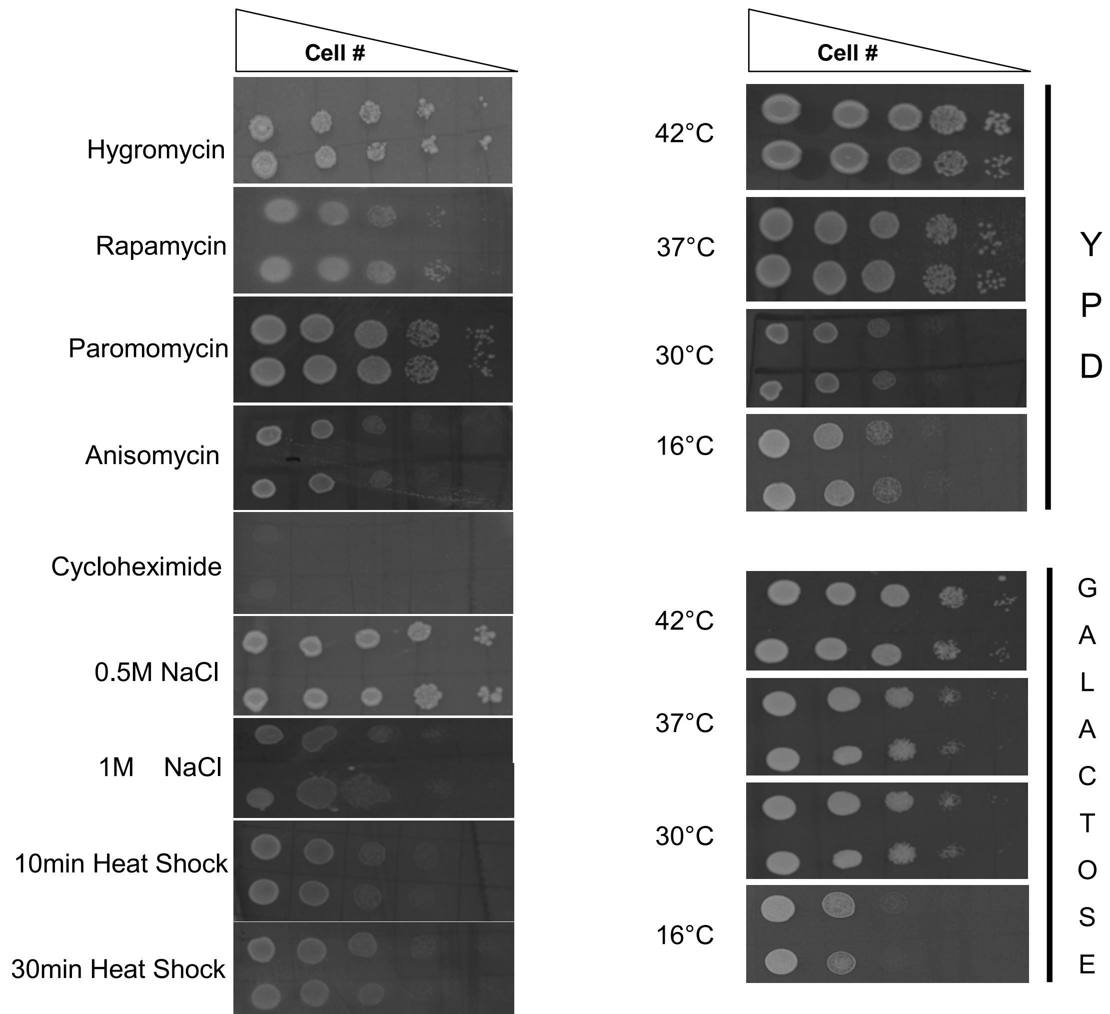


Figure 10. Spot dilution growth assays of *gis2* mutants.
Growth assays of either wild-type parental strains (top of panel) or *gis2* knock-out strains (bottom of panel) under a variety of conditions that perturb translation. Conditions are described in the *Materials and Methods* section.

Expression of Gis2p activates cap-independent translation of the ODC IRES in HEK293T cells

In analyzing whether Gis2p is an ortholog of ZNF9, one of our critical considerations was function. We wanted to test whether Gis2p and ZNF9 can functionally substitute for each other. Because we did not identify any phenotypes resulting from loss of Gis2p, we were unable to do this experiment in yeast. Instead, we used human tissue culture cells. Previously, we showed that ZNF9 functions as an activator of cap-independent translation in mammalian cells. We reasoned that if Gis2p were a true ortholog of ZNF9, it might also be able to facilitate activation of IRES targets, namely the ODC IRES. To measure cap independent translation, we used a previously characterized bicistronic reporter plasmid system (Sammons, Antons et al. 2010). The ODC-IRES reporter encodes the *Renilla* luciferase gene upstream of the firefly luciferase gene. The full-length 5'UTR of the ODC gene is fused between the two open reading frames. The full-length coding sequence of Gis2p, ZNF9, and an unrelated protein (β-galactosidase) were placed downstream of a V5 epitope tag in a mammalian expression plasmid. Co-expression of the ODC-IRES reporter with V5-ZNF9 in HEK293T cells activated translation of the ODC IRES, whereas co-expression with V5-β-Gal did not. Interestingly, expression of V5-Gis2p activated cap-independent translation of the reporter at levels similar to V5-ZNF9 (Figure 11). These results show that Gis2p is an activator of IRES-dependent ODC translation and provide further evidence that Gis2p and ZNF9 are functional and biochemical orthologs.

Figure 11

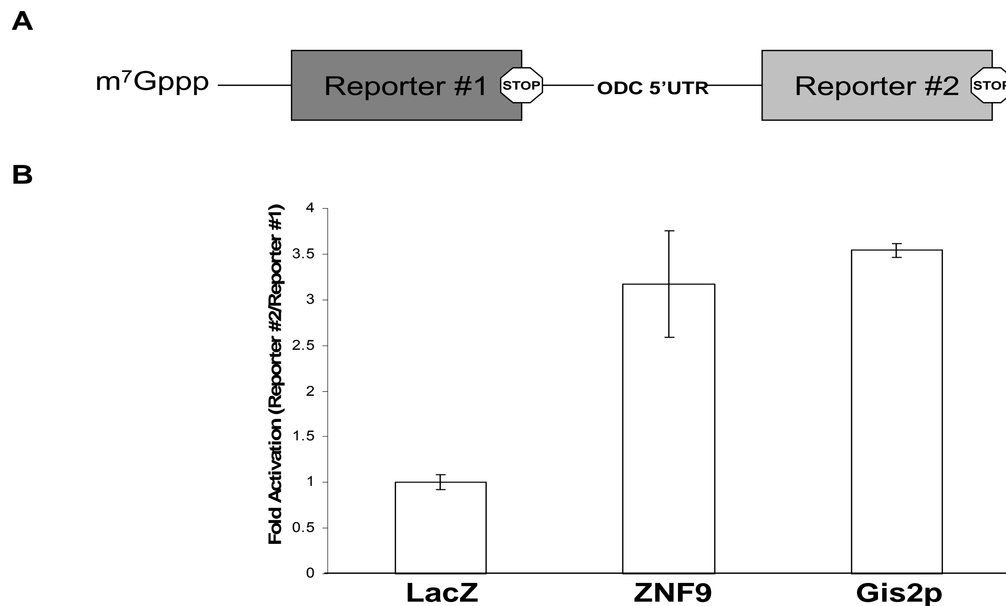


Figure 11. Expression of Gis2p in HEK293T cells activates IRES-dependent translation. A. Schematic representation of the bicistronic ODC-IRES reporter. B. HEK293T cells expressing the bicistronic reporter and either LacZ, ZNF9, or Gis2p were analyzed for luciferase activity. Cap-independent translation is reported as a ratio of Cap-independent translation (reporter #2, firefly luciferase) to cap-dependent translation (reporter #1, *Renilla* luciferase). Data represents 3 independent experiments, and error bars represent the standard deviation.

A conserved C-terminal region of ZNF9 is required for full cap-independent translation activation

We reasoned that the region of Gis2p responsible for cap-independent translation activity is found in the evolutionarily conserved regions or domains shared with ZNF9. Gis2p contains 7 sequential CCHC-type zinc finger motifs and no other conserved functional domains. ZNF9 additionally contains an RGG box motif between the first and second zinc fingers. To test the regions of ZNF9 that are required for cap-independent translation activity, we created a series of N and C-terminal deletion mutants, as shown in Figure 12. Each of these mutants was co-expressed with the ODC IRES reporter and cap-independent translation activity was measured. Deletion of either the first CCHC-zinc finger or the first zinc finger in combination with the RGG box had no effect on the ability of ZNF9 to activate cap-independent translation. The Δ5 mutant, lacking the fifth, sixth, and seventh zinc fingers, fails to fully activate cap-independent translation, whereas the Δ6 mutant, lacking only the sixth and seventh zinc fingers, shows no difference in activity compared to the wild-type ZNF9. These data demonstrate that the RGG box of ZNF9, which is not found in Gis2p, is not important for cap-independent translation activity, but that an intact fifth CCHC-zinc finger is necessary for full activation of translation. Collectively, the data presented here show that Gis2p is a functional ortholog of the mammalian ZNF9 protein and suggest that information gleaned from the study of Gis2p in yeast is relevant to the understanding of ZNF9.

FIGURE 12

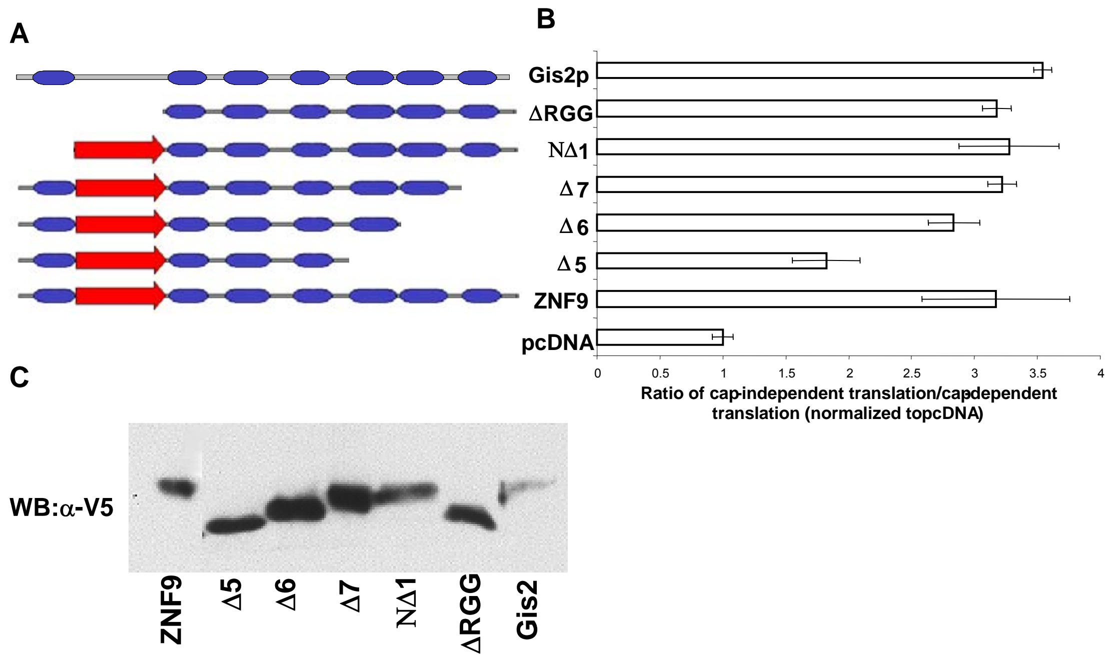


Figure 12. A conserved C-terminal region of ZNF9 is required for full cap-independent translation activity. A. Schematic representation of the various ZNF9 mutants. B. HEK293T cells were transfected with the ODC-IRES reporter and the indicated plasmids and cap-independent translation activity was measured. Cap-independent translation activity is depicted as a ratio of cap-independent translation to cap-dependent translation. Data represents 3 independent experiments. Error bars represent the standard deviation. C. Western blot analysis of V5-tagged ZNF9 mutant and Gis2p protein expression in HEK293T cells.

Discussion

Gis2p has evolutionarily conserved functions in translation

Because proteins are the critical catalysts of all cellular functions, eukaryotic cells have sophisticated and diverse mechanisms to temporally and spatially control protein synthesis. This work provides evidence that the *Saccharomyces cerevisiae* protein Gis2p is part of an evolutionarily conserved mechanism to control translation of specific mRNA molecules that contain internal ribosome entry sites. IRES-driven translation is theorized to have developed in viruses as a mechanism to allow for translation of viral mRNAs after the shutdown of the canonical cap-dependent translation pathway. Multiple studies have demonstrated cap-independent translation in *S. cerevisiae,* of both viral and endogenous cellular mRNA molecules (Thompson, Gulyas et al. 2001; Komar, Lesnik et al. 2003; Komar and Hatzoglou 2005; Gilbert, Zhou et al. 2007). IRES-mediated translation of viral mRNAs is thought to occur through conserved secondary structure inherent to the IRES itself, but IRES-mediated translation of cellular RNAs is believed to require sequence specific cofactors, or ITAFs. The Doudna laboratory demonstrated that cap-independent translation of certain mRNAs is required for haploid invasive growth, the first demonstrated physiological role for cap-independent translation in yeast (Gilbert, Zhou et al. 2007). Since Gis2p functions in cap-independent translation in human cells, it would be interesting to test whether *gis2* mutants are defective in invasive growth. It remains to be seen whether Gis2p functions as an ITAF for any of the

known yeast IRES-containing mRNAs, but the conserved function of Gis2p in mammalian IRES-mediated translation suggests that Gis2p likely acts as an ITAF in yeast. No mRNA-specific ITAFs have been identified yet in yeast (Thompson, Gulyas et al. 2001; Komar, Lesnik et al. 2003; Komar 2005; Landry, Hertz et al. 2009). Further investigation into what, if any, yeast cellular IRESs are regulated by ITAFs, and Gis2p in particular, will be needed to fully understand the physiological role of cap-independent translation in yeast and other eukaryotic organisms.

Gis2p and ZNF9 share common biochemical interactions

As demonstrated here and previously (Sammons, Antons et al. 2010), Gis2p and ZNF9 associate with the eukaryotic ribosome and act as regulators of cap-independent translation. ZNF9 interacts with other known ITAFs, such as PCBP2 and the La protein (Pellizzoni, Lotti et al. 1998; Cardinali, Carissimi et al. 2003; Schlatter and Fussenegger 2003; Gerbasi and Link 2007) that may have vital roles in regulating the cap-independent translation activity of ZNF9. Interestingly, the *S. cerevisiae* protein Sro9p is a putative ortholog of the mammalian La protein and was identified as a potential Gis2p interactor in our studies (Table 3-1). The Michnick group recently showed a putative interaction between Sro9p and Gis2p by genome-wide protein-fragment complementation assay (Tarassov, Messier et al. 2008). Sro9p was initially identified as a multicopy suppressor of an RNA export defect and was shown to associate with polysomes (Kagami, Toh-e et al. 1997; Sobel and Wolin 1999). Recently, Sro9p

was shown to associate with nascent transcripts and form part of an mRNP complex that is exported to the cytoplasm, where Sro9p then acts as a regulator of translation (Rother, Burkert et al. 2010). Our current hypothesis is that Gis2p and Sro9p directly interact, similar to ZNF9 and La, and that a protein complex containing these proteins work together to stimulate translation in yeast. The yeast model system offers intriguing opportunities to study Gis2p and Sro9p and their potentially conserved cellular roles in translation.

Using S. cerevisiae as a model system to study ZNF9 function and the regulation of cap-independent translation

The eukaryotic translation machinery is extremely well-conserved from yeast to man. The rapid growth rate and the ease of identifying mutants make yeast a very tractable model system in which to study the essential cellular process of protein synthesis. Many important discoveries, including the identities and activities of various translation factors and the identities of all of the components of the eukaryotic ribosome, were first made in *S. cerevisiae*. While the role that ZNF9 plays in the progression of myotonic dystrophy type 2 is best studied in the context of mammalian cells, the role of ZNF9 in cap-independent translation can be addressed using a variety of approaches yeast to identify novel regulators and functions of the ZNF9 homolog, Gis2p. As previously discussed, the conservation of ITAF activity between ZNF9 and Gis2p suggests that the molecular and biochemical interactions and activities of Gis2p in yeast are directly relevant to our understanding of ZNF9 function. Recently developed genetic tools, such as E-MAP (Collins, Schuldiner et al. 2006; Fiedler, Braberg et

al. 2009), should allow for the further identification of potential Gis2p biochemical interactors and genetic pathways in which Gis2p is involved.

Although previous work suggested that ZNF9 plays a role in cell proliferation, *gis2* mutants grew at a similar rate under all conditions tested, suggesting that any role of ZNF9 in cell proliferation is not shared by Gis2p. One interesting possibility is that the RGG box of ZNF9, which is not found in Gis2p, functions in the control of cell proliferation and growth. Further studies are needed to understand what role the different conserved and unique functional domains play in the multiple roles of ZNF9 and Gis2p.

CHAPTER IV

PROTEOMIC ANALYSIS OF SKELETAL MUSCLE FROM MYOTONIC DYSTROPHY TYPE 2 MURINE MODELS

Abstract

Myotonic dystrophy type 2 is caused by a tetranucleotide expansion in the first intron of the *znf9* gene. The prevailing disease mechanism suggests that transcribed repeat RNA causes cellular toxicity, leading to pathological symptoms. Despite the prevalence of the RNA toxicity model, alternative models for the mechanism of DM2 have not been ruled out or tested. In this chapter, I test the model that ZNF9 activity is reduced in DM2 and contributes to DM2 disease progression by examining changes in protein expression in murine models of myotonic dystrophy using quantitative mass spectrometry-based proteomics.

Introduction

A nucleotide expansion in the znf9 gene causes the human disease myotonic dystrophy type 2

Nucleotide repeat expansion (NRE) disorders define a large class of human pathologies that share the common characteristic of large, mutated tracts of repeat DNA that confer either a loss or gain of function phenotype. The human disease myotonic dystrophy type 2 (DM2) is caused by an expansion of

the CCTG tetranucleotide repeat in the first intron of the *ZNF9* gene (Liquori, Ricker et al. 2001). A similar disorder, myotonic dystrophy type 1 (DM1), results from the expansion of a CTG trinucleotide repeat in the 3'UTR of the *DMPK* gene (Brook, Mccurrach et al. 1992). The nucleotide expansion occurs in untranslated regions of the ZNF9 transcripts, leading to the production of seemingly normal protein products (Margolis, Schoser et al. 2006). Expression of the expanded repeats in unrelated genes causes myotonic dystrophy like symptoms, suggesting that the mere presence of the expansion, rather than the context of the mutation, gives rise to the myotonic dystrophies (Mankodi, Logigian et al. 2000; Amack and Mahadevan 2001; Seznec, Agbulut et al. 2001). These observations collectively have led to the development of the RNA toxicity model for myotonic dystrophy. The hypothesis suggests that the transcribed, but untranslated nucleotide repeat expansions form stable RNA structures that are toxic to the cell. The toxicity is believed to stem from the ability of specific cellular proteins, such as MBNL and CUGBP1, to bind to the aberrant RNA structures (Fardaei, Larkin et al. 2001; Kanadia, Johnstone et al. 2003; de Haro, Al-Ramahi et al. 2006; Hino, Kondo et al. 2007). Interaction with the stable RNA substrates is thought to either inactivate certain proteins, such as the RNA splicing factor MBNL, or confer greater activity upon them, such as in the case of the translation factor CUGBP1 (Timchenko, Timchenko et al. 1996; Fardaei, Larkin et al. 2001; Ho, Charlet et al. 2004).

A role for the ZNF9 protein in myotonic dystrophy type 2

Unlike the prevailing model for myotonic dystrophy, most other nucleotide expansion repeat disorders result from the inactivation of one particular gene due to the mutated nucleotide repeats. In Fragile X mental retardation, nucleotide expansions are translated in-frame with the FMRP gene, giving rise to a mutated form of the protein that lacks endogenous activity (Brown, Jin et al. 2001). In the case of DM2, the tetranucleotide repeat expansion is not translated and while it is transcribed, several studies suggest the expansion does not affect RNA stability, processing, or export. These reports, along with a series of review papers, have led to the hypothesis that there is no involvement of ZNF9 gene function in the progression of the DM2 disease state. Recent evidence from the Link, Krahe, and Timchenko groups has brought this conclusion into question and has reignited the study of ZNF9 activity and function in DM2 (Huichalaf, Schoser et al. 2009; Sammons, Antons et al. 2010)(R. Krahe, personal communication).

What is the function and activity of the ZNF9 protein?

The initial cloning and characterization of the *ZNF9* gene suggested that ZNF9 may act as a transcription factor for steroid regulated promoters (Rajavashisth, Taylor et al. 1989). Further characterization provided evidence that ZNF9 might regulate both transcription and translation through its *in vitro* nucleic acid binding activity (Michelotti, Tomonaga et al. 1995; Pellizzoni, Lotti et al. 1997). *In vivo* functional analyses were undertaken in multiple systems, but a consensus function was never attributed to the protein. Because of this lack of

consensus, the function of the ZNF9 protein in DM2 was never tested. Gerbasi et al showed an *in vivo* role for the ZNF9 protein, in the process of cap-independent translation (Gerbasi and Link 2007). Following up on this observation, I determined that ZNF9's cap-independent translation activity is reduced in myoblasts from DM2 patients (Sammons, Antons et al. 2010). The Timchenko group observed that ZNF9 translational targets are downregulated in DM2, further suggesting that the *in vivo* activity of ZNF9 is aberrant, despite earlier reports that ZNF9 protein and mRNA levels were normal in DM2 (Margolis, Schoser et al. 2006). Additionally, the Krahe group has noted a reduction in ZNF9 mRNA and protein levels in a number of DM2 patients (R. Krahe, personal communication, manuscript in preparation). Misregulated translational control due to ZNF9 loss-of-function would have a profound effect on the expression of a large number of proteins in DM2.

Tools for the analysis of ZNF9's role in DM2

Our hypothesis is that ZNF9 is an RNA binding protein that functions as a translation factor, and ZNF9 protein levels or activity are misregulated in myotonic dystrophy type 2. As a result, proteins regulated by ZNF9 are expected to have aberrant expression. Gaining more precise knowledge of the proteins whose translation is regulated by ZNF9 is critical in understanding the contribution of ZNF9 to DM2 and the distinct molecular mechanisms and cellular pathways that cause the DM2 disease state. The overarching question of this work asks what are the changes in protein expression in myotonic dystrophy type

2 compared to the non-disease state. Does a decrease in ZNF9 expression underlie the disease mechanism of DM2?

In the following chapter, we sought to analyze the expression of ZNF9 protein in newly developed mouse models for myotonic dystrophy. In collaboration with Ralf Krahe's group at the University of Texas Health Sciences Center and the Darryl Pappin's group at Cold Spring Harbor Laboratory, we assessed protein expression changes in the skeletal muscle from these new mouse models using quantitative mass spectrometry. These newly developed tools and experimental strategies discussed in this chapter, along with the results presented here, should be directly applicable and relevant to the study of ZNF9's function in myotonic dystrophy type 2.

Materials and methods

Western blotting

Whole cell lysates from mouse skeletal muscle were lysed on ice for 30 minutes using standard RIPA buffer (50mM TRIS, 150mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 1 Roche EDTA-free mini Protease inhibitor tablet/mL). Whole cell lysates were centrifuged at 16,5000 rpm for 15 minutes at 4C to pellet insoluble fractions. Soluble protein was assayed for protein concentration using the BCA assay method. 50μg of cellular protein were fractionated by SDS-PAGE analysis using 10% BisTRIS polyacrylamide gels (Novex, Invitrogen) and transferred to nitrocellulose for western blotting analysis.

Polyclonal ZNF9 antibodies were previously described (Sammons, Antons et al. 2010). GAPDH antibodies (Millipore) were used at 1:5,000 concentration. Antibodies were diluted in 1X TBST and 5% non-fat dry milk.

Myotonic dystrophy type 2 mouse models

The Krahe laboratory has generated a knockin mouse model (DM2-CCTG-KI) containing a $(CCTG)_{189}$ expansion in the first intron of the mouse *znf9* gene, which mimics the causative mutation resulting in DM2 in humans (R. Krahe, unpublished data). The original transgenic knockin mouse was created by injection of a *neo* cassette with the $(CCTG)_{189}$ expansion targeting the 1st intron of *znf9.* The neo cassette was flanked with loxP sites, and the cassette was removed by cre-mediated recombination. Both the DM2-*ZNF9ki-neo* and DM2-CCTG-KI mice are viable and recapitulate the major features of DM2 (R. Krahe, personal communication). A DM2tgCCTG transgenic line was created that expresses 121 CCTG repeats within the first intron of the human skeletal actin gene (R. Krahe, unpublished data). The DM1tgCTG mouse line was a gift of Dr. Charles Thornton. It expresses 250 CTG repeats in the 3'UTR of the human skeletal actin gene (Osborne, Lin et al. 2009).

Isolation and processing of mouse skeletal muscle

Mouse skeletal muscle was stored at -80C until used. Muscle was cut into 1-2mm pieces using a clean razor blade and placed into a 2.0mL eppendorf tube. 250uL of 100mM triethylammonium biocarbonate and 250uL of

2,2,2-trifluoroethanol were added to the tube. Muscle tissue was solubilzed by 3X-1 min sonication steps (power 2, micro tip) with 1 min of rest on ice between each round. Tubes were then shaken at 1600rpm for 1 hr at 60C, followed by another round of sonication. The tubes were then spun at 165000 rpm for 15 min at room temperature in a microcentrifuge to pellet all insoluble protein. Soluble protein was removed and analyzed for protein concentration using the BCA assay method. 100ug of protein was reduced with 10mM DTT for 10 min at 60C and alkylated with iodoacetemide for 30 min at 30C. Samples were then digested overnight at 37C with sequencing grade trypsin (Promega). Peptides were then snap-frozen in liquid nitrogen and stored at -80C until mass spectrometry analysis.

Quantitative mass spectrometry analysis

Desalted and trypsinized peptides were labeled using the iTRAQ 4-plex labeling kit (Applied Biosystems) according to previously published protocols (Ross, Huang et al. 2004). Labeled peptides were then pooled and analyzed by LC-MS/MS using a quadropole-time of flight mass spectrometer (Q-TOF) by the Cold Spring Harbor Laboratories Proteomic Facility (Cristian Ruse and Darryl Pappin). Peptides and proteins were identified by searching the acquired mass spectrometry data against the mouse protein database using the MASCOT algorithm (Perkins, Pappin et al. 1999). Peptide abundance measurements were derived from the iTRAQ reporter ions using the SCAFFOLD program (Lundgren, Martinez et al. 2009; Searle 2010).

Results

ZNF9 protein expression in murine models of myotonic dystrophy

Previous models of myotonic dystrophy express expanded nucleotide repeats in the 3'UTR of the *HSA* gene or rely on knockout of downstream pathways to mimic the DM1 state (Mankodi, Logigian et al. 2000; Seznec, Agbulut et al. 2001). These models do not take into account the molecular and cellular pathways affected by expression of CCUG expanded repeats within the context of the *znf9* gene. There have been no investigations into the expression of ZNF9 protein in response to a DM2-like expansion in mice. In collaboration with Ralf Krahe's lab (University of Texas Health Sciences Center at Houston), we undertook an analysis of ZNF9 protein expression levels in the skeletal muscle of mice with 189 CCTG repeats in the first intron of the mouse *znf9* gene. A second model mouse line, expressing the expansion along with a loxP-flanked *neomycin* cassette, was analyzed in parallel. Control mice were litter, age, and sex matched. Whole cell lysates from the thigh skeletal muscle of matched mice were analyzed by SDS-PAGE and western blotting for ZNF9 protein expression. Western blot analysis for GAPDH was used as a control for protein loading. As seen in Figure 13, ZNF9 protein levels are slightly reduced in DM2-CCTG-KI muscle compared to controls (22% reduction, as measured by densitometry). ZNF9 protein expression in unfloxed DM2-CCTG-KI muscle is severely reduced compared to wild-type control muscle, indicating that the presence of the *neo* cassette affects ZNF9 expression. This result is likely due to transcription of the

neo gene on the opposite orientation across the *znf9* transcription start site (R. Krahe, personal communication). Interestingly, the decrease in ZNF9 protein expression in DM2-CCTG-KI muscle is similar to the decreases in ZNF9 expression in myoblasts from DM2 patients (R. Krahe, personal communication; manuscript in review), suggesting that expansion of CCUG nucleotide repeats within the first intron of *znf9* reduces the level of ZNF9 protein (Huichalaf, Schoser et al. 2009; Sammons, Antons et al. 2010). While the exact molecular mechanism(s) for the reduction in ZNF9 protein expression is unknown, it is likely due to the expanded CCTG repeat in the context of the 1st intron of the *znf9* gene misregulating the processing of *znf9* transcripts.

In order to test the hypothesis that the reduction in ZNF9 levels is attributed to the CCTG expansion within the first intron of *znf9*, another set of mouse models were developed. The new transgenic line expresses 121 CCTG repeats in the first intron of the *HSA* gene, analogous to the DM1 model mouse with 250 CTG repeats in the 3'UTR of the *HSA* gene. These mice can be used to directly test whether the genomic context of the nucleotide repeat expansion (*znf9, dmpk,* or another gene) is important for disease progression. We specifically wanted to understand whether expression of CTG or CCTG expansion RNA alone would result in decreased ZNF9 protein expression in skeletal muscle. Western blot analysis of skeletal muscle tissue lysates from control, DM1tgCTG or DM2tgCCTG mice is shown in Figure 13B. ZNF9 protein levels are consistent across all samples relative to GAPDH controls. These data strongly suggest that a reduction in ZNF9 protein levels in DM2 patients is likely

due to the genomic location of the CCTG expansion and not the presence of CCUG or CUG nuclear RNA foci proposed in the RNA gain of function model.

FIGURE 13

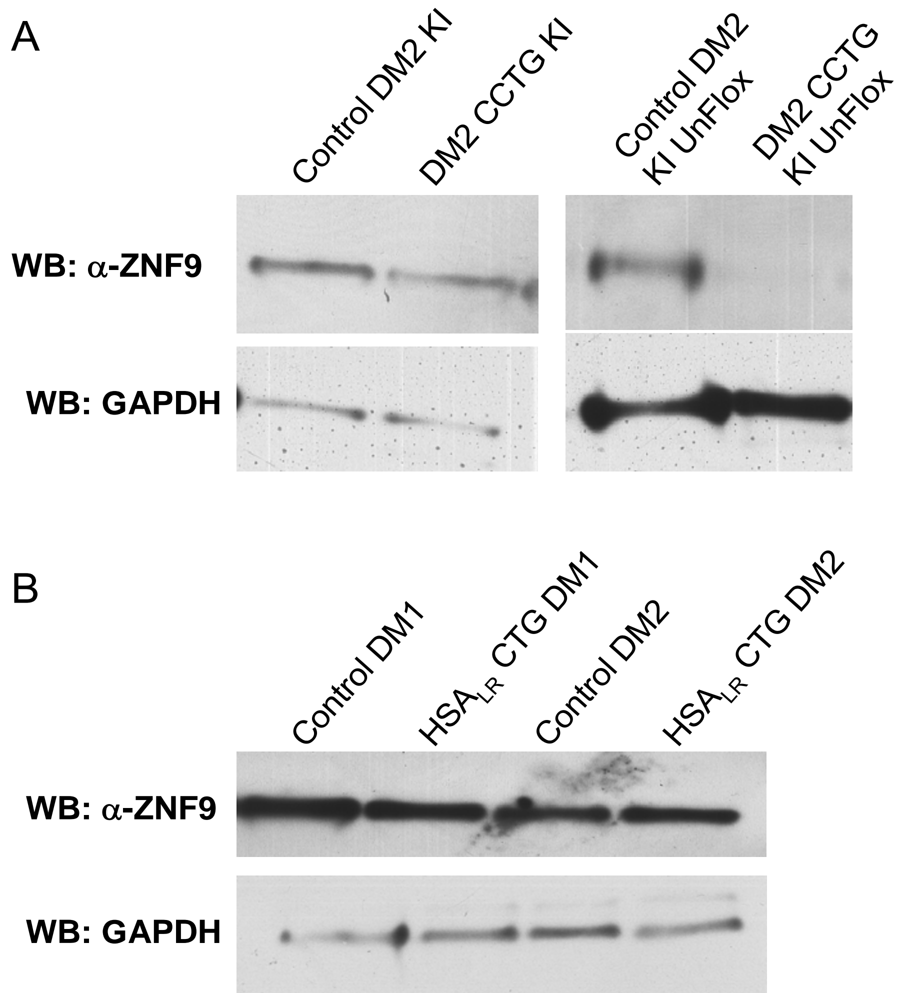


Figure 13. Western blot analysis of skeletal muscle from mouse models of myotonic dystrophy. A. Western blot analysis of ZNF9 and GAPDH levels in control and DM2 CCTG KI mice (see *Materials and Methods)*. B. Western blot analysis of ZNF9 and GAPDH levels in control and DM1tgCTG or DM2tgCCTG (see *Materials and Methods).*

Quantitative proteomic analysis of protein abundance in myotonic dystrophy mouse models

In order to develop effective therapies and treatments for the myotonic dystrophies, it is critical to fully understand the cellular and molecular pathways perturbed in the disease state. Very little is known about the differences in gene expression in DM2 and non-affected individuals. Gene expression analysis of myotonic dystrophy has focused on the missplicing events due to decreased MBNL expression or changes in mRNA expression due to expression of CTG repeats (Osborne, Lin et al. 2009; Du, Cline et al. 2010). Most previous analyses looked specifically for changes in splicing patterns or mRNA abundance in DM1 mouse models. Here, we perform an unbiased quantitative mass spectrometry-based proteomic analysis to measure protein abundance changes in various mouse models of myotonic dystrophy. We chose to use the iTRAQ method for quantitative proteomics because it labels proteins after lysis of cells or tissues, unlike other metabolic labeling techniques like SILAC (Farley and Link 2009). Skeletal muscle tissue (quadriceps) from age, sex, and litter-matched mice was processed for iTRAQ labeling and mass spectrometry analysis as discussed in *Materials and Methods.*

As is common in large gene expression data sets, a method for statistical analysis is needed to identify proteins that are likely to be relevant. For each group comparison, we expressed changes in protein abundance as a ratio of the calculated protein abundance score in the experimental sample versus the score of the control sample. By converting all measurements to a ratio of experimental to control samples, we focused on relative abundance between samples. The

resulting values were then converted to log2 and a group mean score was computed for all identified proteins in that comparison. The standard deviation of each group was calculated and any protein with a ratio outside 2 standard deviations of the mean, either positively or negatively, was considered significant.

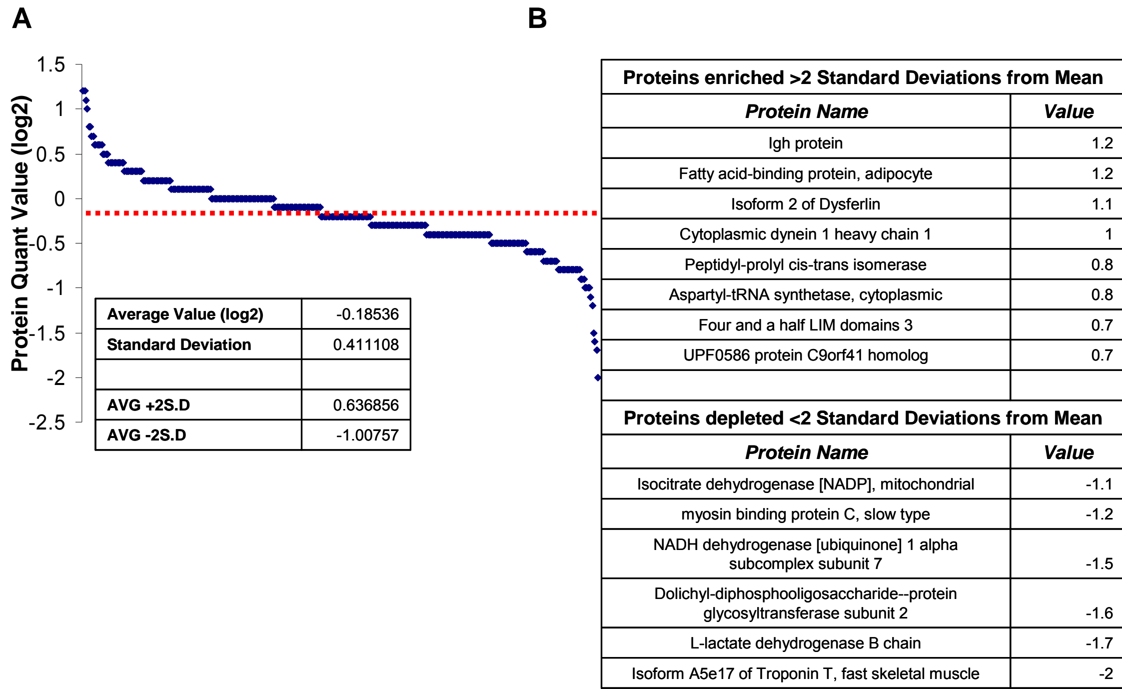
Comparison of DM2-CCTG-KI and wild-type control muscle

We were interested in measuring the protein abundance changes in DM2-CCTG-KI mouse skeletal muscle because we believed this model to be the most relevant to the actual human disease. Abundance measurements for 369 different proteins were obtained in this analysis. A total of 8 proteins, listed in Figure 14B, were upregulated over 2 standard deviations from the mean abundance in DM2-CCTG-KI skeletal muscle compared to wild-type muscle. The protein showing the largest increase in abundance between samples was the heavy chain of immunoglobulin class G (IgG-HC) (Figure 14B), which is significantly upregulated in DM2-CCTG-KI mouse muscle. Patients with myotonic dystrophy have reduced levels of serum IgG (hypoglobulinemia), but levels of IgG in the muscle are unknown (Nakamura, Kojo et al. 1996). The protein FHL3 is also upregulated in mouse models of cardiomyopathy and is specifically expressed in cardiac and skeletal muscle subtypes (Chu, Ruiz-Lozano et al. 2000). FHL3 overexpression in skeletal muscle progenitors suppresses myotube formation and may lead to decreased muscle regeneration (Cottle, McGrath et al. 2007; Meeson, Shi et al. 2007). Finally, the adipocyte fatty acid binding protein (FABP4) was found in significantly higher abundance in

DM2-CCTG-KI mouse muscle than in controls. Despite its name, FABP4 is normally expressed in skeletal muscle and increased expression of FABP4 suppresses normal muscle contraction in rats (Lamounier-Zepter, Look et al. 2009). It is tempting to speculate that these upregulated proteins directly contribute to the skeletal and cardiac muscle phenotypes seen in patients with myotonic dystrophy type 2.

Identifying proteins that are less abundant in DM2-CCTG-KI mice than wild-type controls can provide significant insight into the molecular mechanisms that are absent in DM2. In our analysis, expression of 6 proteins was significantly reduced in DM2-CCTG-KI mice, as shown in Figure 14B. Of particular interest is Troponin T, which is the most significantly reduced protein in our analysis. Troponin T is an essential protein responsible for contraction of skeletal and cardiac muscle (Gerhardt and Ljungdahl 1998). Most importantly, Troponin T mRNA is a direct target of MBNL and CUGBP1, and Troponin T missplicing gives rise to a large, inactive isoform of Troponin T in the skeletal and cardiac muscle of adult myotonic dystrophy patients (Philips, Timchenko et al. 1998). Unfortunately, the peptides identified by mass spectrometry do not distinguish between the two isoforms. This result suggests that perhaps additional misregulation of troponin T contributes to myotonic dystrophy. Further examination into troponin T protein expression in the myotonic dystrophies is warranted.

FIGURE 14



Average Value (log2)	-0.18536
Standard Deviation	0.411108
AVG +2S.D	0.636856
AVG -2S.D	-1.00757

Proteins enriched >2 Standard Deviations from Mean	
Protein Name	***Value***
Igh protein	1.2
Fatty acid-binding protein, adipocyte	1.2
Isoform 2 of Dysferlin	1.1
Cytoplasmic dynein 1 heavy chain 1	1
Peptidyl-prolyl cis-trans isomerase	0.8
Aspartyl-tRNA synthetase, cytoplasmic	0.8
Four and a half LIM domains 3	0.7
UPF0586 protein C9orf41 homolog	0.7
Proteins depleted <2 Standard Deviations from Mean	
Protein Name	***Value***
Isocitrate dehydrogenase [NADP], mitochondrial	-1.1
myosin binding protein C, slow type	-1.2
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 7	-1.5
Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 2	-1.6
L-lactate dehydrogenase B chain	-1.7
Isoform A5e17 of Troponin T, fast skeletal muscle	-2

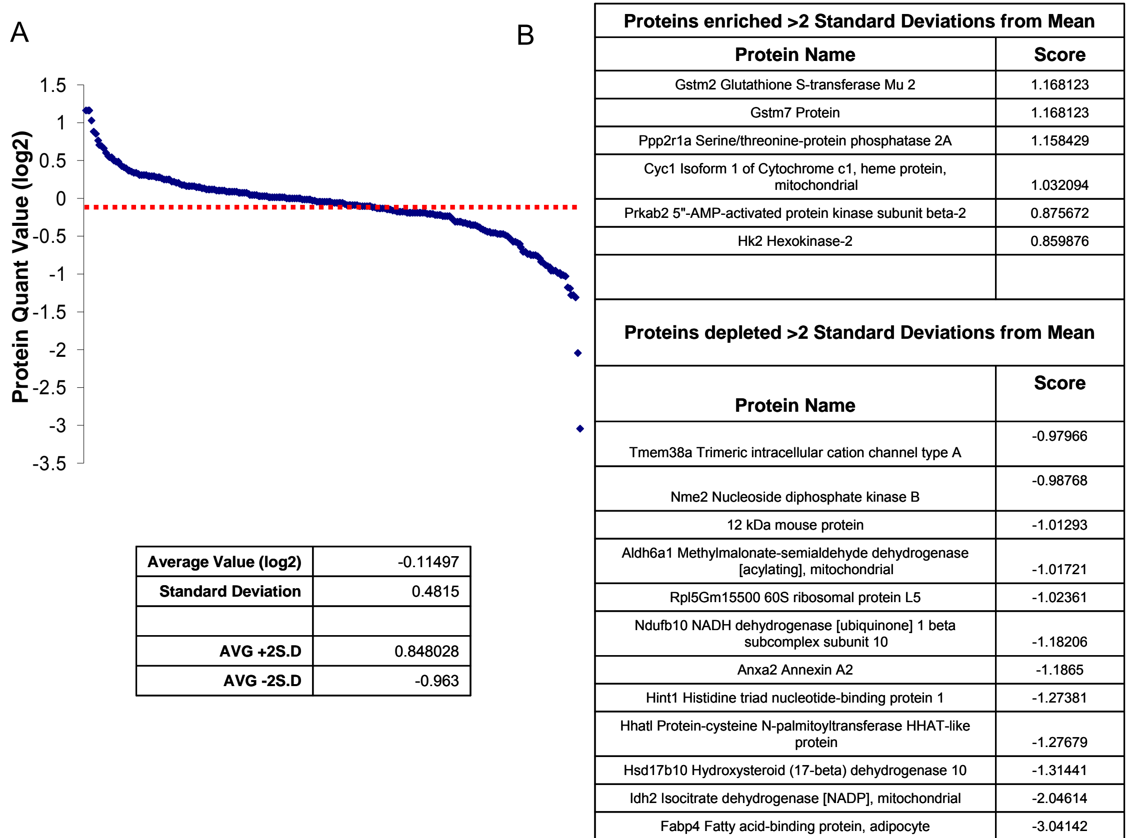
Figure 14. Quantitative proteomic analysis of protein abundance in DM2-CCTG-Knockin mice versus controls. A. Abundance values for each identified and quantified protein displayed as log2(DM2-CCTG-KI ^{score}/Control $_{score}$). The inset table displays the average abundance value, the standard deviation, and the values used as a cutoff to determine significance for enriched and depleted proteins. The average abundance value of all proteins discovered is represented by the dotted line. **B.** Table of proteins that were enriched or depleted over 2 standard deviations from the mean abundance.

Proteomic analysis of skeletal muscle from DM2tgCCTG and DM1tgCTG mice

A direct comparison between DM1 and DM2 using gene expression analysis has not yet been reported in the literature. Most analyses focus on the different models or tissue types from one particular disease (Osborne, Lin et al. 2009; Du, Cline et al. 2010). We sought to use mouse models of DM1 and DM2 to analyze protein abundance changes in the skeletal muscle in response to nucleotide repeat expansions in unrelated genes. First, we compared the protein abundance changes in the DM2tgCCTG mouse, which expresses 121 CCTG repeats in the first intron of the HSA gene, to wild-type control muscle. Unfortunately, while these data cannot be directly compared to the DM2-CCTG-KI model with any statistical significance, we can still observe general trends in protein abundance changes between the two DM2 models. The most striking difference between the data is the downregulation of FABP4 in DM2tgCCTG mice, which was upregulated in DM2-CCTG-KI mice compared to controls. These particular experiments were not set up to measure absolute protein abundance. Nonetheless, there is a marked reduction in FABP4 relative to control samples in the DM2tgCCTG muscle. I hypothesize that the difference between the expression of FABP4 in the two mouse models lies in the genomic context of the CCTG nucleotide expansion. Additional experiments, such as an alternative means to measure absolute protein concentrations, are needed to better understand this result. Ideally, the two mouse models would be analyzed in the same experiment, allowing us to elucidate relative changes in protein abundance between the two models.

Not surprisingly, many of the protein expression changes between the DM2tgCCTG mouse and control are complementary. For example, PRKAB2, an AMPK regulatory subunit, has higher protein abundance in DM2tgCCTG muscle than in controls, while proteins such as HSD17B10 and ALDH6A1 show lower expression. Increased PRKAB2 expression is implicated in the shutdown of cholesterol and fatty acid synthesis, while HSD17B10 and ALDH6A1 are both critical enzymes within these synthesis pathways (Vasiliou and Pappa 2000; Iseli, Walter et al. 2005; Yang, He et al. 2005). AMPK signaling is also implicated in metabolic and oxidative stress, which is likely increased in myotonic dystrophy (Polekhina, Gupta et al. 2003). Fittingly, multiple glutathione-S-transferase enzymes are upregulated in DM2tgCCTG muscle, suggesting that increased oxidative stress may contribute to DM2 pathogenesis. Lastly, the RPL5 protein, regulated translationally by a 5'TOP mRNA sequence and the ZNF9 protein, is also downregulated in DM2tgCCTG mice (Pellizzoni, Lotti et al. 1997). Previously, the Timchenko group observed that 5'TOP containing mRNAs are poorly translated in DM1 and DM2. They hypothesize that this is caused by reductions in ZNF9 activity (Huichalaf, Schoser et al. 2009; Huichalaf, Sakai et al. 2010). Taken together, the protein abundance changes between DM2tgCCTG muscle and control muscle provide potential clues into the variety of cellular and molecular processes that are misregulated in myotonic dystrophy type 2.

FIGURE 15



Average Value (log2)	-0.11497
Standard Deviation	0.4815
AVG +2S.D	0.848028
AVG -2S.D	-0.963

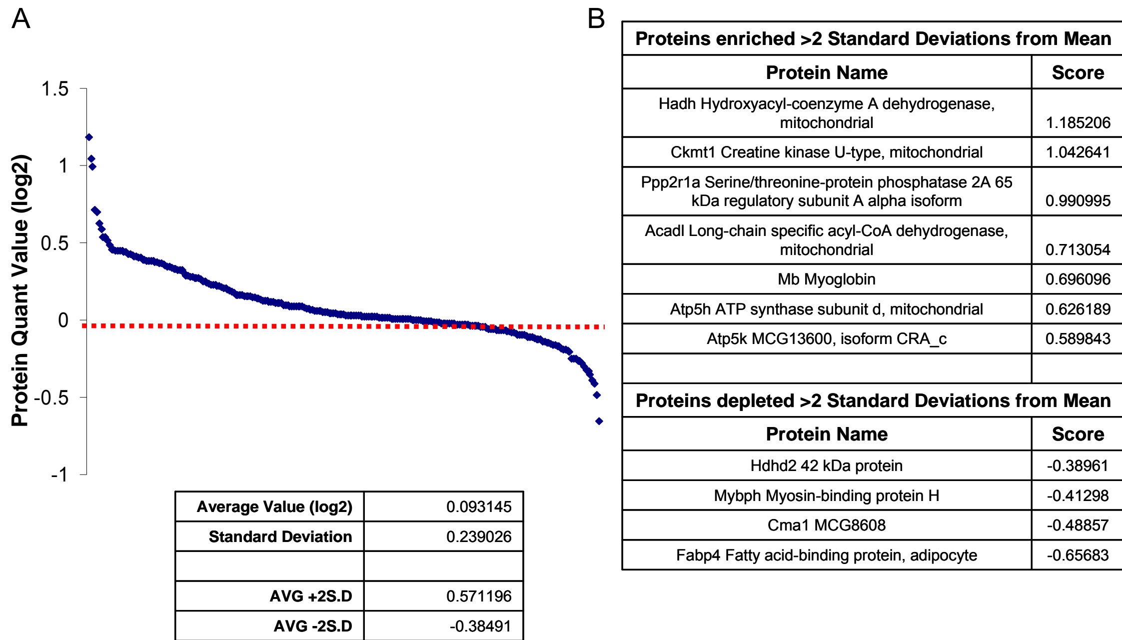
Proteins enriched >2 Standard Deviations from Mean	
Protein Name	**Score**
Gstm2 Glutathione S-transferase Mu 2	1.168123
Gstm7 Protein	1.168123
Ppp2r1a Serine/threonine-protein phosphatase 2A	1.158429
Cyc1 Isoform 1 of Cytochrome c1, heme protein, mitochondrial	1.032094
Prkab2 5"-AMP-activated protein kinase subunit beta-2	0.875672
Hk2 Hexokinase-2	0.859876

Proteins depleted >2 Standard Deviations from Mean	
Protein Name	**Score**
Tmem38a Trimeric intracellular cation channel type A	-0.97966
Nme2 Nucleoside diphosphate kinase B	-0.98768
12 kDa mouse protein	-1.01293
Aldh6a1 Methylmalonate-semialdehyde dehydrogenase [acylating], mitochondrial	-1.01721
Rpl5Gm15500 60S ribosomal protein L5	-1.02361
Ndufb10 NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 10	-1.18206
Anxa2 Annexin A2	-1.1865
Hint1 Histidine triad nucleotide-binding protein 1	-1.27381
Hhatl Protein-cysteine N-palmitoyltransferase HHAT-like protein	-1.27679
Hsd17b10 Hydroxysteroid (17-beta) dehydrogenase 10	-1.31441
Idh2 Isocitrate dehydrogenase [NADP], mitochondrial	-2.04614
Fabp4 Fatty acid-binding protein, adipocyte	-3.04142

Figure 15. Quantitative proteomic analysis of protein abundance in DM2tgCCTG mice versus controls. A. Abundance values for each identified and quantified protein displayed as log2($DM2tgCCTG_{g}$ ^{score}/Control $_{score}$). The inset table displays the average abundance value, the standard deviation, and the values used as a cutoff to determine significance for enriched and depleted proteins. The average abundance value of all proteins discovered is represented by the dotted line. **B.** Table of proteins that were enriched or depleted over 2 standard deviations from the mean abundance.

The RNA gain of function model for the myotonic dystrophies, if true, would predict that similar gene expression signatures would be present in both DM1 and DM2. If radically different patterns of gene expression exist, then the two diseases may be caused by different pathogenic mechanisms. In order to examine these possibilities, we analyzed two groups of samples for significant changes in protein abundance. The first group compared DM2tgCCTG muscle protein abundance to those of the DM1tgCTG mouse. The DM2 model (DM2tgCCTG) showed significant upregulation (>2 SD) of a number of glycolytic enzymes and proteins involved in cellular energy production compared to the DM1 model (Figure 16B). DM2tgCCTG muscle showed significant downregulation of Cma1, also known as mast chymase, which has antiproliferative activity in rat cardiomyocytes(Hara, Matsumori et al. 1999). Satellite cells from DM1 patients were previously shown to display reduced proliferative capacity compared to DM2 myoblasts (Pelletier, Hamel et al. 2009). The lack of a large number of significant differences between the DM2tgCCTG and DM1tgCTG transgenic mice is not suprising, as the two diseases are very similar. Additionally, the nucleotide expansions are not in their proper cellular context (the *znf9* and *dmpk* genes, respectively) and does not account for gene-specific effects of the expansions. Expanded repeats of CCTG and CTG are predicted to adapt very similar secondary structure and both are found in nuclear foci in DM2 and DM1, respectively. These observations strongly suggest that the RNA-mediated cellular toxicity is especially relevant to these particular mouse models.

FIGURE 16



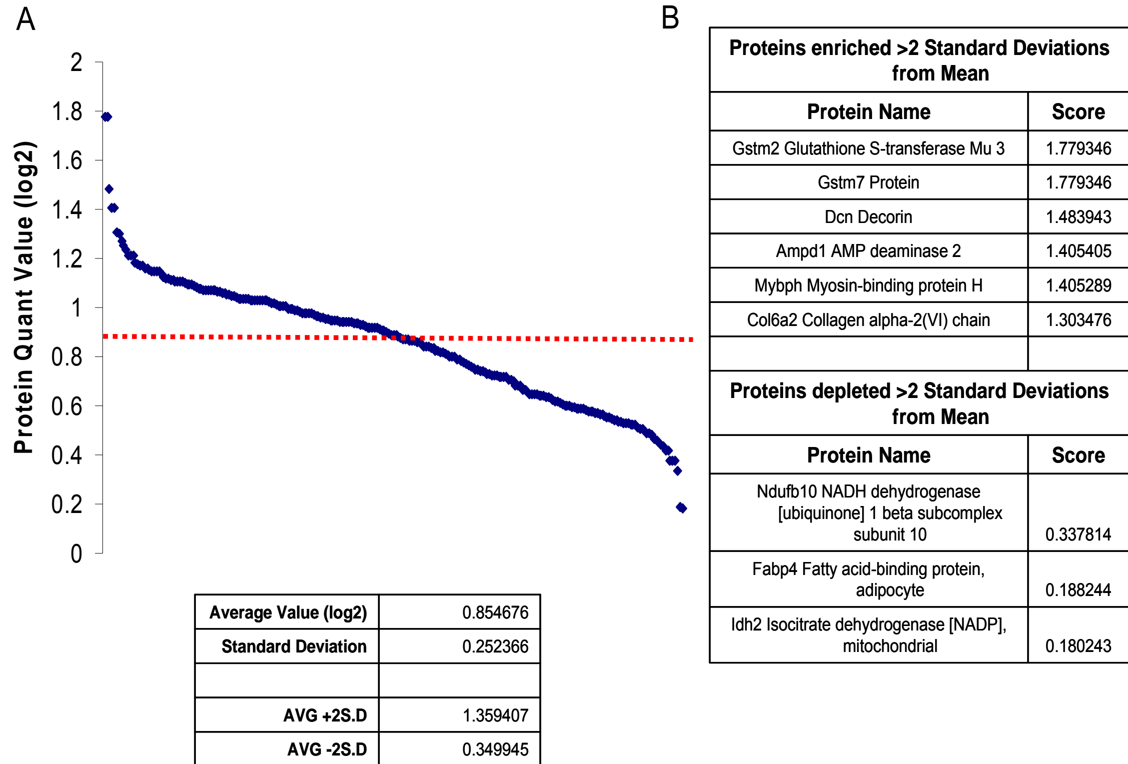
Average Value (log2)	0.093145
Standard Deviation	0.239026
AVG +2S.D	0.571196
AVG -2S.D	-0.38491

Proteins enriched >2 Standard Deviations from Mean	
Protein Name	**Score**
Hadh Hydroxyacyl-coenzyme A dehydrogenase, mitochondrial	1.185206
Ckmt1 Creatine kinase U-type, mitochondrial	1.042641
Ppp2r1a Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform	0.990995
Acadl Long-chain specific acyl-CoA dehydrogenase, mitochondrial	0.713054
Mb Myoglobin	0.696096
Atp5h ATP synthase subunit d, mitochondrial	0.626189
Atp5k MCG13600, isoform CRA_c	0.589843
Proteins depleted >2 Standard Deviations from Mean	
Protein Name	**Score**
Hdhd2 42 kDa protein	-0.38961
Mybph Myosin-binding protein H	-0.41298
Cma1 MCG8608	-0.48857
Fabp4 Fatty acid-binding protein, adipocyte	-0.65683

Figure 16. Quantitative proteomic analysis of protein abundance in DM2tgCCTG mice versus DM1tgCTG mice. A. Abundance values for each identified and quantified protein displayed as log2(DM2tgCCTG ^{score}/DM1tgCTG $_{score}$). The inset table displays the average abundance value, the standard deviation, and the values used as a cutoff to determine significance for enriched and depleted proteins. The average abundance value of all proteins discovered is represented by the dotted line. **B.** Table of proteins that were enriched or depleted over 2 standard deviations from the mean abundance.

A comparison of the similarities and differences between DM2tgCCTG and DM1tgCTG transgenic mouse skeletal muscle protein abundance could yield important cellular pathways that are disrupted in both DM1 and DM2 patients. Indeed, compared to wild-type control animals, expanded repeat expression results in a significant upregulation of a number of proteins involved in the cellular response to oxidative stress, like the glutathione-s-transferases. As muscles age and become damaged, reactive oxygen species (ROS) are produced and are neutralized by enzymes like the glutathione-s-transferases (Rossi, Marzani et al. 2008). Upregulation of enzymes to combat ROS during muscle damage and aging is common, and is likely a possible therapeutic target for muscle regeneration (Jackson 2009). Additionally, mouse muscle expressing expanded repeats shows significant and increased expression of decorin, a small proteoglycan, that is associated with actively regenerating muscle (Li, Li et al. 2007). Overall, these data support the notion that mouse models for myotonic dystrophy type 1 and type 2 share similar gene expression patterns that indicate damaged or aging muscle, but that differences between the two expression patterns may explain some of the differences between DM1 and DM2.

FIGURE 17



Average Value (log2)	0.854676
Standard Deviation	0.252366
AVG +2S.D	1.359407
AVG -2S.D	0.349945

Proteins enriched >2 Standard Deviations from Mean	
Protein Name	**Score**
Gstm2 Glutathione S-transferase Mu 3	1.779346
Gstm7 Protein	1.779346
Dcn Decorin	1.483943
Ampd1 AMP deaminase 2	1.405405
Mybph Myosin-binding protein H	1.405289
Col6a2 Collagen alpha-2(VI) chain	1.303476
Proteins depleted >2 Standard Deviations from Mean	
Protein Name	**Score**
Ndufb10 NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 10	0.337814
Fabp4 Fatty acid-binding protein, adipocyte	0.188244
Idh2 Isocitrate dehydrogenase [NADP], mitochondrial	0.180243

Figure 17. Quantitative proteomic analysis of protein abundance in DM2tgCCTG and DM1tgCTG mice compared to wild-type controls. A. Abundance values for each identified and quantified protein displayed as log2(Average of DM2tgCCTG$_{score}$ and DM1tgCTG $_{score}$ / Average of Wild-type controls$_{score}$). The inset table displays the average abundance value, the standard deviation, and the values used as a cutoff to determine significance for enriched and depleted proteins. The average abundance value of all proteins discovered is represented by the dotted line. **B.** Table of proteins that were enriched or depleted over 2 standard deviations from the mean abundance.

Discussion

In this chapter, I undertook a quantitative proteomic analysis of skeletal muscle from mouse models of myotonic dystrophy types 1 and 2. The goal of this work was to better understand the changes induced by expanded nucleotide repeats on gene expression, specifically within the *znf9* gene. Three mouse models for myotonic dystrophy were used to assess the changes in protein abundance in response to nucleotide repeat expansions, including a never-before-used knock-in model that expressed CCTG repeats within their proper cellular context.

Comparison of the protein abundance changes between the DM2-CCTG-KI mouse and its wild-type control revealed that a protein (troponin T) previously known to be misregulated in DM2 is reduced in the disease state. Follow-up experiments examining the protein expression of different troponin T isoforms, specifically the isoforms found in DM2 (those containing exon 5), will provide additional insight into the mechanism of myotonic dystrophy (Philips, Timchenko et al. 1998). All protein expression changes should be validated using other techniques, such as western blotting if appropriate antibodies are available. In cases where specific antibodies are unavailable, as is the case with many alternatively spliced protein isoforms, targeted proteomics approaches can be used. Methods like selected reaction monitoring (SRM) take advantage of isotopically-labeled peptides and can provide absolute quantification of almost any peptide or protein (Lange, Picotti et al. 2008; Picotti, Bodenmiller et al. 2009).

For example, an absolute quantification analysis of the adult and embryonic alternatively spliced troponin T isoforms would provide a clearer picture into the role of this protein (Philips, Timchenko et al. 1998; Seznec, Agbulut et al. 2001). SRM experiments are especially applicable to the analysis of proteins, like ZNF9, where there have been controversial reports on the actual abundance of the protein within DM2, because the technique is unbiased with respect to antibody:antigen binding (Margolis, Schoser et al. 2006).

While a large number of proteins showed changes in protein expression in this analysis, we focused only on those whose expression changed more than 2 standard deviations from the average protein abundance in order to target the largest differences between samples. The proteins with large changes between samples are those likely to be important in the molecular events that lead to or sustain myotonic dystrophy-like symptoms. Large changes, though, are not necessarily the most significant to the disease. In fact, extremely small perturbations in protein expression can result in large physiological changes. Increasing the number of experimental replicates would allow for the use of more sophisticated statistical analysis methods, such as those commonly used for microarray data analysis (Eisen, Spellman et al. 1998; Pan 2002). Analyses such as these would allow us to pick out small, but statistically significant, changes in protein abundance important for myotonic dystrophy disease progression.

The use of mass spectrometry-based proteomics to assess changes in protein expression is widely-used, but the design of many the experiments do not

allow for the assessment of the mechanism of these changes in protein abundance. For example, although we report a number of protein abundance changes in myotonic dystrophy mouse models, we do know whether the protein expression changes at the level of transcription, RNA processing, translation, or protein stability. Experiments combining mass spectrometry-based proteomics and genomic strategies, such as microarray or RNAseq should be used to better understand the mechanism behind such changes. This is especially applicable to the study of myotonic dystrophy because of the hypothesized roles of splicing and translation in the disease mechanism (Philips, Timchenko et al. 1998; Huichalaf, Schoser et al. 2009; Huichalaf, Sakai et al. 2010; Sammons, Antons et al. 2010).

Mice with reduced expression of either ZNF9 or DMPK develop symptoms directly mimicking the myotonic dystrophies, indicating that these genes are likely misregulated in the diseases (Jansen, Groenen et al. 1996; Reddy, Smith et al. 1996; Chen, Wang et al. 2007). The newly-developed model for DM2, the DM2-CCTG-KI mouse, will be an extremely valuable tool for determining the role of genomic context in the development of the myotonic dystrophies. Comparing the physiological, cellular, and molecular changes in this animal compared to animals expressing CCTG repeats in an unrelated gene will provide critical information into the molecular mechanism of DM2. Likewise, using a context-sensitive DM1 model alongside animals expressing CTG repeats from a transgene will help elucidate signaling and cellular pathways involved in DM1. In the future, mice with reduced ZNF9 and DMPK expression should be included in

any analysis of DM2 and DM1 molecular mechanisms. These mouse models will be critical in either further supporting the RNA toxicity model or revealing a context-dependent mechanism for the disease. Only more time and more effort will allow us to reveal the complex mechanism underlying the myotonic dystrophies.

CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

This dissertation investigated the function of the ZNF9 protein and its role in the human disease myotonic dystrophy type 2. In this chapter, I will analyze the significance of my findings and discuss their relevance to the study of myotonic dystrophy and the overall field of molecular biology. I will discuss what my work has contributed to the scientific world and propose other experimental strategies that would be beneficial to researchers following-up on this work.

ZNF9 activates cap-independent translation of the ODC IRES

Over 20 years since its initial cloning and characterization, the function of the human protein ZNF9 is unclear (Rajavashisth, Taylor et al. 1989). The initial aim of my dissertation research was to identify and validate the function of this protein, as its importance is underscored by its involvement in the disease myotonic dystrophy type 2. My work has led to a newly published report on ZNF9's *in vivo* function (Sammons, Antons et al. 2010). This report, along with other evidence presented in this dissertation, indicates that ZNF9 functions as an internal ribosome entry site *trans*-acting factor, or ITAF. Previous evidence, especially studies from the laboratory of Paola Pierandrei-Amaldi and the graduate work of Vincent Gerbasi, suggested that ZNF9 may function as a translation factor, and my work continued this line of inquiry.

Vincent Gerbasi and Andrew Link noted that overexpression of the ZNF9 protein activated cap-independent translation of a reporter gene fused to the 5'UTR of the rat ornithine decarboxylase mRNA (Gerbasi and Link 2007). I initially began my work trying to ascertain how ZNF9 might perform such a function. From previous work, ZNF9 was known to associate with the La protein and PCBP2, both known ITAFs, on different mRNA substrates (Pellizzoni, Lotti et al. 1997; Pellizzoni, Lotti et al. 1998; Gerbasi and Link 2007), but it was unknown if ZNF9 directly interacted with mRNA or required an adaptor protein. We focused specifically on the 5'UTR of the human ODC mRNA as a target site, as we were interested in the function of the ZNF9 protein in humans because of its relationship to DM2. ZNF9 specifically binds to a 45bp RNA sequence from the ODC 5'UTR, and does so independent of other proteins (Sammons, Antons et al. 2010). This data suggested that ZNF9 might associate with the translation machinery, which was confirmed by polysome profiling, cosedimentation analysis, and ribosome salt wash experiments (Sammons, Antons et al. 2010). Using a bicistronic reporter system, I discovered that ZNF9 activates cap-independent translation through the human ODC IRES (Sammons, Antons et al. 2010). Data presented in Chapter III on a potential ZNF9 homolog indicates that the ribosome interactions are evolutionarily conserved, indicating that ZNF9 is likely a *bona fide* translation factor. Taken together, these data strongly suggests that ZNF9 acts as an ITAF *in vivo.*

Work from the laboratory of Lubov Timchenko (Baylor College of Medicine) bolsters the hypothesis that ZNF9 acts to regulate translation through

interaction with 5' untranslated regions. In a series of UV crosslinking experiments, ZNF9 was found to associate with the 5'UTRs of a number of mRNAs involved in the regulation of translation (Huichalaf, Sakai et al. 2010), and that knockdown of ZNF9 expression reduces the incorporation of S35 into actively synthesizing peptides (Huichalaf, Sakai et al. 2010). Interestingly, the mRNAs studied all encode either ribosomal protein subunits or translation factors. It would be interesting if ZNF9, in addition to its ability to activate cap-independent translation of human ODC, could facilitate the synthesis of certain translation factors through cap-dependent mechanisms. Regulating translation through controlling the synthesis of the translation machinery is not uncommon (Tang, Hornstein et al. 2001; Guertin, Guntur et al. 2006), and this possibility needs to be investigated further to better understand how ZNF9 controls cellular protein synthesis. Taken together, the work presented in this dissertation and from the Timchenko group clearly indicates that ZNF9 binds to the 5'UTR of multiple mRNAs and is involved in the regulation of translation.

Potential mechanisms of ZNF9 mediated cap-independent translation activation

The full scope of the cellular interactions and mechanisms by which ZNF9 activates cap-independent translation are unclear, but work presented in Chapters II and III provide some intriguing details. ZNF9 directly binds to the ODC mRNA independent of other cofactors, but whether ZNF9 can activate cap-independent translation independent of other ITAFs is unknown. Coimmunoprecipitation experiments show that ZNF9 associates with the ITAFs

La and PCBP2, and that other ITAFs, such as UNR and polypyrimidine-tract binding protein (PTB), can bind to the ODC IRES (Gerbasi and Link 2007). Because these proteins are all known ITAFs, it was initially postulated that ZNF9 may be part of an ITAF complex that acts together to activate cap-independent translation. The initial screen that identified ZNF9 as a putative ITAF provides clues into the requirement for other proteins during IRES activation. ZNF9 and PCBP2 interacted with only the wild-type IRES sequence, but UNR and PTB interacted with both the wild-type sequence and a mutant sequence that does not support internal ribosome entry (Pyronnet, Pradayrol et al. 2000; Gerbasi and Link 2007). This suggests that the loss of either ZNF9 or PCBP2 binding to the wild-type IRES stops cap-independent translation from the ODC IRES. Because ZNF9 directly binds to the wild-type internal ribosome entry site of the ODC 5'UTR, it is likely that ZNF9 itself is a central regulator of cap-independent translation. The roles for the other members of this potential ITAF complex in ODC IRES regulation are unknown, but both UNR and PTB have been shown to repress cap-independent translation during mitosis, when the ODC IRES is most active (Pyronnet, Pradayrol et al. 2000; Schepens, Tinton et al. 2007). PTB and UNR may act to repress ODC translation by blocking ZNF9 access to their shared binding sites.

The main question remaining is how ZNF9 binding to an IRES recruits the ribosome and activates translation. Association of ZNF9 with the translation machinery is dependent upon actively translating polysomes. The putative ortholog Gis2p shows reduced ribosome association when polysomes are

treated with RNAse A, suggesting that the protein requires intact mRNA to associate with the ribosome. This is similar to what is seen for other translation factors, like FMRP (Tamanini, Meijer et al. 1996; Feng, Absher et al. 1997), suggesting that ZNF9 may first associate with an mRNA and then recruit the ribosome through an unknown mechanism. Other activators of IRES-dependent translation are believed to act as RNA chaperones and induce conformational changes in RNA secondary structure which are then recognized by the ribosome (Song, Tzima et al. 2005). This particular mechanism is mainly speculative, but would make sense based upon the mechanisms of viral IRES function. Viral IRESs activate translation almost entirely through RNA secondary structures that mimic rRNA or tRNA (Fraser, Hershey et al. 2009). It is possible that ITAFs bind to specific sequences and then induce those RNA sequences to adapt a certain structure which causes ribosome recruitment, but this has not been directly shown by experimental methods. Interestingly, ZNF9 is a known nucleic acid chaperone and could fulfill this role during IRES-mediated translation (Armas, Nasif et al. 2008). Structural studies of the interaction of ZNF9 and the ODC IRES would provide unequivocal evidence of these putative RNA chaperone activities and IRES conformational changes.

Future directions for the study of ZNF9 function and cap-independent translation

Future investigations into the mechanism of ZNF9's activity as an activator of cap-independent translation are likely given the proteins role in myotonic dystrophy type 2. The most pressing needs for ZNF9 biology are further

elucidation of the mechanism of RNA binding and ribosome recruitment and the discovery of novel mRNA targets. The mechanistic details of internal ribosome entry site mediated translation of viral mRNAs are far ahead that of cellular eukaryotic mRNAs, and the study of ZNF9 provides the opportunity to shore up our understanding of eukaryotic cellular IRES biology. To study the interaction between ZNF9 and the ribosome, a series of deletion mutants similar to those shown in Chapter III should be tested for their ability to bind to the ODC IRES and associate with the translating ribosome in an *in vitro* system. Mutations that reduce either RNA binding or ribosome association are very likely to reduce IRES activation. As previously mentioned, structural studies (X-ray crystallography, NMR) probing the ZNF9:ODC IRES interaction are especially warranted, given the lack of information on structural rearrangements in cellular IRESs. Experiments such as these are straightforward, but are likely to provide a wealth of insight into the function of ZNF9 and the function of cellular IRES-mediated translation.

It is highly probable that ZNF9 regulates the translation of additional cellular mRNAs beyond those currently known. Identification of these mRNAs will provide additional information into the cellular pathways regulated by ZNF9. The use of a RIP-CHIP or RIP-RNASeq experimental strategy can be used to identify mRNAs that associate with ZNF9 (Brown, Jin et al. 2001; Tenenbaum, Lager et al. 2002). Isolation of ZNF9 from relevant tissues, such as skeletal muscle, would coprecipitate interacting mRNAs, allowing for their identification by microarray or next-generation DNA sequencing. While these experiments can be

performed, they are still biased by the ability of the researcher to isolate ZNF9 efficiently and to isolate potentially transient RNA interactions. Alternatively, a polyribosome profiling approach can be used to identify mRNAs that are translated in the presence and absence of the ZNF9 protein. Ideally, cell lines completely lacking ZNF9 would be used for this purpose, but cell lines with reduced expression of the protein of interest have been used in the past for this purpose (Marash, Liberman et al. 2008). While the immunoprecipitation approach is preferred due to the smaller amount of data analysis required, both approaches are valid and can and should be used to identify mRNA targets of ZNF9. Identification of additional mRNA targets of ZNF9 has direct relevance to the study of myotonic dystrophy, as decreased ZNF9 activity would reduce translation of these targets, which may cause some of the observed pathologies in the disease.

The regulation of ZNF9 and its activity are aberrant in DM2

The most intriguing, and arguably most important, finding of this dissertation is that ODC IRES-dependent translation is reduced in DM2 myoblasts due to a decrease in ZNF9 expression. Previous mechanistic models for the myotonic dystrophies completely discounted the role of ZNF9 (and DMPK) gene activity in the pathogenesis of the disease. In this work, I determined that ZNF9 mRNA expression is reduced approximately 30% in myoblasts from a DM2 patient and that the activity of ZNF9 is also reduced (Sammons, Antons et al. 2010). Recent publications from the Timchenko lab further this observation, as

they observe reduced ZNF9 protein levels and aberrant protein localization in DM2 myoblasts (Huichalaf, Sakai et al. 2010). Additionally, the Krahe and Udd labs, in a survey of mature skeletal muscle from DM1 and DM2 patients, saw a marked reduction in ZNF9 protein expression in DM2 patients (R. Krahe, personal communication), further bolstering the hypothesis that ZNF9 protein expression is reduced in DM2. Of course, a reduction in a given proteins expression in a disease state does not necessarily implicate the function of that protein the underlying mechanism of that disease. Evidence for a direct role of decreased ZNF9 activity came from the lab of Yi-Ping Li, where mice with one copy of the *znf9* gene show late-onset muscle wasting, cataract formation, and cardiac arrhythmias, all symptoms of human patients with DM2 (Chen, Wang et al. 2007). These data, taken together, suggest that ZNF9 protein expression and activity are likely components of the DM2 disease mechanism.

Discrepancies in the RNA gain-of-function model

The prevailing model, RNA gain-of-function, suggests that stable CUG or CCUG RNA repeats are retained in "nuclear foci" and sequester essential cellular proteins whose subsequent misregulation causes both myotonic dystrophy type 1 and type 2. The model was initially developed because of these RNA foci and to explain the very similar clinical manifestations of DM1 and DM2. This model is supported by a large body of literature and certainly explains a large number of the molecular observations and clinical features of the disease. It should be said that the large majority of the work in the field has focused on the more prevalent

DM1. Unfortunately, there are still quite a few discrepancies between the proposed model and the observed data (and clinical manifestations), suggesting that an important piece is missing. I propose that the missing piece of the puzzle is the effect of nucleotide expansion within the *znf9* gene in DM2, and by extension, the effect of a nucleotide repeat expansion within *dmpk* in DM1. Here, I will attempt to combine the disparate data and reconcile the current RNA gain-of-function model with the observations in this dissertation and in literature.

Toxic CUG and CCUG RNA foci are certainly present in DM1 and DM2, respectively, and their presence does result in the misregulation of splicing. Nuclear repeat RNA sequesters MBNL, reducing the activity and inhibiting the splicing of target pre-mRNAs (Fardaei, Larkin et al. 2001; Kanadia, Johnstone et al. 2003). Reduced MBNL activity has been shown to be an important component of the myotonic dystrophy disease model, as knockout of the *mbnl* gene phenocopies transgenic mice expressing expanded CTG repeats in the 3'UTR of the HSA gene (Mankodi, Logigian et al. 2000; Seznec, Agbulut et al. 2001; Kanadia, Johnstone et al. 2003). Missplicing and myotonia can be rescued by overexpression of MBNL or the use of oligonucleotides that displace MBNL from RNA foci (Kanadia, Shin et al. 2006; Arambula, Ramisetty et al. 2009; Mulders, van den Broek et al. 2009; Mulders, van Engelen et al. 2010), but these treatments do not restore other phenotypes associated with DM1 (and DM2). These results suggest that although reduced MBNL activity contributes to symptoms of myotonic dystrophy, it is not a primary, underlying cause.

CUGBP1 activity is increased in response to expression of expanded CUG or CCUG repeat RNA, and significant evidence implicates increased CUGBP1 in the development of myotonic dystrophy. Mice overexpressing CUGBP1 in skeletal muscle show severe muscle wasting and cardiac abnormalities, and chemical inhibition of PKC, the kinase that activates CUGBP1, reduces these effects (Wang, Kuyumcu-Martinez et al. 2009; Koshelev, Sarma et al. 2010; Ward, Rimer et al. 2010). While increased CUGBP1 expression and activity underlie a number of myotonic dystrophy clinical manifestations, it does not explain all of them, even in combination with reduced MBNL levels.

An important tool in myotonic dystrophy research has been the use of transgenic mouse models for the disease. The first set of mice expressed CTG repeats in the 3'UTR of the human skeletal actin gene and recapitulated many disease symptoms (Mankodi, Logigian et al. 2000; Seznec, Agbulut et al. 2001). These mice, along with the (at the time) recent discovery that DM2 was caused by an expansion of CCUG repeats within the *znf9* gene, pointed towards a gene-independent mechanism for the diseases. Interestingly, expansion of CTG repeats in the 3'UTR of the *dmpk* gene, the proper genomic context, leads to more severe myotonic dystrophy-like symptoms, suggesting that perhaps DMPK is involved in the disease mechanism. To date, no mouse models for DM2 have been published, but characterization of the DM2-CCTG-KI mouse discussed in Chapter IV is underway and appears to recapitulate DM2 symptoms (R. Krahe and B. Udd, personal communication).

Of course, the question of how ZNF9 expression and activity are reduced in DM2 remains. The most obvious, but as-of-yet untested, mechanism would be that either transcription or processing of ZNF9 mRNA is reduced due to the nucleotide expansion. Two early reports, looking at myoblasts and transformed lymphoblastoid cell lines from DM2 patients, suggested that transcription, mRNA processing, and translation are all normal in DM2 (Botta, Caldarola et al. 2006; Margolis, Schoser et al. 2006). These results are in striking opposition to work in this dissertation and published elsewhere that ZNF9 mRNA and protein levels are reduced in DM2 myoblasts. Only careful analysis of mRNA and protein levels in DM2 will resolve these discrepancies. Another possible mechanism involves an interaction of ZNF9 with the expanded CCUG RNA repeat. Although initially thought to be retained solely in the nucleus, the repeat RNA is also found in the cytoplasm with ZNF9 (Salisbury, Schoser et al. 2009; Huichalaf, Sakai et al. 2010). ZNF9 and the CCUG RNA do not colocalize by *in situ* hybridization, but this does not rule out a transient interaction, as CUGBP1 interacts with CUG and CCUG expansion RNA, but does not colocalize by immunofluorescence. Further testing of this mechanism and the reduced ZNF9 expression mechanism are warranted in light of these recent findings.

Future directions and experimental strategies for elucidating the involvement of ZNF9 in the myotonic dystrophy disease mechanism

As a result of the data presented here and from the Timchenko laboratory, a further experimental investigation into the role played by the ZNF9 protein in myotonic dystrophy would be prudent. Beyond a more careful examination of

ZNF9 mRNA expression, processing, and translation in the muscle from DM2 patients, a number of experiments should be performed. The first step was recently performed, creation of a mouse model for DM2, reported in Chapter IV. This mouse (DM2-CCTG-KI) recapitulates the exact genomic expansion seen in DM2 patients, with a large CCTG expansion in the first intron of the *znf9* gene. As seen in Chapter IV, this expansion does cause a small decrease in ZNF9 protein levels, and the Krahe lab reports decreases in transcription, certain pre-mRNA species, and ultimately, translation in these mice (R. Krahe, personal communication). The DM2-CCTG-KI mouse can be directly compared to the mouse expressing CCTG expansions in the *HSA* gene, to determine how the context of the expansion affects ZNF9 expression and ultimately, myotonic dystrophy-like symptoms. Special care should be taken to make sure that the expansions are of a similar size in all comparisons, as larger expansions cause more severe symptoms in DM2 patients. Ultimately, these mice need to be compared to the ZNF9$^{+/-}$ mouse reported by the Li group (Forsythe Institute, Boston, MA) to determine if ZNF9 functional activity is aberrant in the animals expressing the expanded repeats. A comprehensive gene expression analysis of adult mouse skeletal muscle from these three mice would provide a wealth of information into the cellular and molecular pathways governing the development of myotonic dystrophy. Unfortunately, these mice are not readily shared by the Li group, so these experiments are not likely to be carried out soon.

The RNA gain of function model, too, can be directly tested by the use of transgenic animals and quantitative proteomics. If the RNA gain of function

model is correct, one would predict that an identical set of proteins are activated or sequestered by expression of CUG and CCUG repeats. Differences in protein binding or activity induced by either CUG or CCUG repeats would be expected to account for the differences in clinical manifestations of the disease. A quantitative differential proteomics analysis of CUG and CCUG RNA binding proteins would provide information into the differences in repeat RNA binding that can be predicted to account for differences between DM1 and DM2. Skeletal muscle from adult mice should be used as a source of the RNA binding proteins, as this is the most commonly affected muscle type in myotonic dystrophy and likely contains proteins that aberrantly regulated due to CUG or CCUG repeats. Specific RNA binding proteins can be tested for colocalization or reduced function or expression in DM1 or DM2 mouse models to determine if they are truly part of the disease mechanism.

An additional set of mouse models would ultimately determine whether the RNA gain of function model is accurate. It is already known that animals expressing either CTG or CCTG expansions in the *HSA* gene show similar, but not identical, myotonic dystrophy-like symptoms, mimicking DM1 and DM2, respectively. Mice expressing identical length CCTG expansions in the 3'UTR of *dmpk* and CTG expansions in the 1st intron of *znf9* should be created. If the RNA gain of function model is accurate, the mice should switch phenotypes; mice expressing CCTG expansions in *dmpk* should exhibit more DM2-like phenotypes and vice versa.

I undertook a quantitative proteomic analysis of various mouse models of DM1 and DM2 in order to examine if any changes in protein abundance would account for the similarities and differences between DM1 and DM2. Ideally, these experiments will be repeated in DM1 and DM2 context-sensitive mouse models (ie those with expansions within *dmpk* or *znf9)* to gain the most relevant disease-specific data. Regardless, experiments examining expression (western blotting, SRM proteomics) of the significantly up- or downregulated proteins in each comparison set should be undertaken as a follow-up to my work. As discussed in Chapter IV, microarray or RNA-Seq-based mRNA abundance measurements should be performed in order to attribute changes in protein abundance to either transcription or mRNA stability. Differentially expressed proteins whose respective mRNAs show equal expression are likely regulated at the level of translation or protein stability. It would be especially interesting (and relevant) to perform a quantitative proteomics analysis on adult skeletal muscle from the DM2-CCTG-KI mouse and the ZNF9$^{+/-}$ mouse. This type of analysis would elucidate the changes in protein abundance resulting from CCTG expansions and from a loss of ZNF9 function. A combination of genetic models, gene expression analysis, and common molecular biology techniques will reveal whether or not ZNF9 protein function is truly aberrant in myotonic dystrophy type 2.

The work and data reported in this dissertation lay the groundwork for the continued study of the function of the ZNF9 protein and its role in the development of myotonic dystrophy type 2. Increased focus on the *in vivo*

function of ZNF9 will yield additional knowledge into the still poorly understood process of cap-independent translation. I also describe a potential new model system in which to study the function of the ZNF9 protein, which will allow for the identification of potential regulatory proteins by high-throughput genetic and proteomic technologies. Finally, I provide an atlas of protein expression changes in mouse models for DM2 which may be relevant to the development and maintenance of the DM2 disease state. Ultimately, this dissertation provides a clear motivation and framework for future researchers to follow in order to better understand the role of ZNF9 in myotonic dystrophy type 2. Hopefully, we, as a scientific community, will solve the complex mechanism of the myotonic dystrophies and develop effective treatments for this debilitating disease.

APPENDIX A

Table A1 –Protein abundance factors (PAF) from replicate Gis2-Tap purifications

Accession #	Gene	tot # spectra	tot non-redun spectra	tot non-redun peptides	# unique	% AA covg	PAF
YLR344W	RPL26A	138	21	13	3	61.72	14.75341
YGR034W	RPL26B	144	19	12	2	53.85	13.00568
YNL255C	GIS2	216	20	13	13	74.03	11.69385
YLL045C	RPL8B	134	28	20	3	67.32	9.960159
YOR096W	RPS7A	373	21	12	6	72.25	9.71233
YKL006W	RPL14A	47	14	10	1	61.87	9.230566
YMR194W	RPL36A	27	10	7	1	49.5	8.989572
YPL249C-A	RPL36B	27	10	7	1	49.5	8.980692
YDL061C	RPS29B	22	6	4	4	45.61	8.917955
YNL302C / YOL121C **	RPS19B / RPS19A	139	14	10	0	58.62	8.810018
YNL096C	RPS7B	122	19	12	6	60.21	8.782472
YDL136W / YDL191W	RPL35B / RPL35A	81	12	9	0	38.84	8.626887
YDL075W	RPL31A	270	11	6	1	51.75	8.492241
YGR027C / YLR333C **	RPS25A / RPS25B	59	10	5	0	42.2	8.306338
YKL180W	RPL17A	163	17	9	1	50.81	8.272909
YBR031W	RPL4A	521	32	17	2	55.37	8.185818
YHL033C	RPL8A	106	23	16	1	60.31	8.177778
YHR203C / YJR145C	RPS4B / RPS4A	121	24	18	0	48.09	8.16049
YDR382W	RPP2B	22	9	6	6	74.77	8.144796
YFR031C-A / YIL018W	RPL2A / RPL2B	194	22	15	0	47.06	8.026853
YML073C	RPL6A	77	16	12	6	63.28	8.01563
YDR012W	RPL4B	496	31	17	2	55.37	7.936102
YJR094W-A	RPL43B	70	8	6	0	49.46	7.927857
YLR075W	RPL10	139	20	17	17	61.71	7.886124
YJL177W	RPL17B	143	16	9	1	50.81	7.785509
YLR406C	RPL31B	265	10	6	1	51.75	7.711884
YML063W	RPS1B	144	22	16	4	59.77	7.635707
YDR450W / YML026C	RPS18A / RPS18B	53	13	10	0	61.9	7.630004
YDR447C /	RPS17B	105	12	8	0	54.01	7.593495

Table A1 – Continued

Accession #	Gene	tot # spectra	tot non-redun spectra	tot non-redun peptides	# unique	% AA covg	PAF
YDL082W / YMR142C **	RPL13A / RPL13B	115	17	13	0	51.5	7.537466
YLR448W	RPL6B	88	15	10	4	55.93	7.505254
YBL092W	RPL32	41	11	8	8	49.62	7.447025
YGL147C	RPL9A	67	16	10	1	59.9	7.418054
YLR441C	RPS1A	140	21	15	3	57.42	7.306127
YER074W / YIL069C	RPS24A / RPS24B	70	11	8	0	38.24	7.175941
YBR189W / YPL081W **	RPS9B / RPS9A	54	16	11	0	37.24	7.17521
YDL083C / YMR143W	RPS16B / RPS16A	90	11	9	0	51.39	6.941377
YNL067W	RPL9B	67	15	10	1	59.9	6.926167
YCR031C / YJL191W **	RPS14A / RPS14B	91	10	7	0	50.72	6.878998
YGL076C / YPL198W **	RPL7A / RPL7B	100	19	14	0	55.92	6.874593
YOR063W	RPL3	202	30	19	19	49.74	6.855889
YJL190C / YLR367W **	RPS22A / RPS22B	57	10	7	0	61.83	6.837139
YER056C-A / YIL052C **	RPL34A / RPL34B	20	9	7	0	25.41	6.598724
YMR242C / YOR312C **	RPL20A / RPL20B	79	14	9	0	40.78	6.592579
YGR085C / YPR102C **	RPL11B / RPL11A	78	13	9	0	52	6.582278
YOL127W	RPL25	47	10	7	7	61.54	6.345983
YMR194W	RPL36A	36	7	6	0	46.53	6.2927
YGL123W	RPS2	103	17	12	12	63.14	6.193078
YGL103W	RPL28	53	10	8	8	40.67	5.980146
YDR064W	RPS13	106	10	7	7	43.42	5.872335
YNL209W	SSB2	212	39	27	3	62.21	5.856296
YLR325C	RPL38	43	5	3	3	32.91	5.664439
YLR287C-A / YOR182C	RPS30A / RPS30B	34	4	3	0	28.13	5.619556

Table A1 – Continued

Accession #	Gene	tot # spectra	tot non-redun spectra	tot non-redun peptides	# unique	% AA covg	PAF
YOL039W	RPP2A	25	6	5	5	78.5	5.583473
YDL229W	SSB1	176	37	26	2	57.82	5.555389
YAL038W	CDC19	86	30	25	23	60.88	5.500046
YBR118W / YPR080W	TEF2 / TEF1	122	27	17	0	47.06	5.396438
YMR121C	RPL15B	59	13	9	2	38.54	5.323069
YGR192C	TDH3	139	19	14	5	65.77	5.315131
YNL178W	RPS3	73	14	12	12	55.6	5.282421
YHL001W / YKL006W **	RPL14B / RPL14A	46	8	6	0	38.85	5.279483
YGL030W	RPL30	41	6	4	4	38.68	5.256242
YJR123W	RPS5	99	13	9	9	51.33	5.191901
YOR369C	RPS12	20	8	6	6	46.53	5.170631
YDR471W / YHR010W **	RPL27B / RPL27A	47	8	6	0	43.07	5.159626
YGL031C / YGR148C **	RPL24A / RPL24B	42	9	6	0	22.44	5.109572
YOL040C	RPS15	54	8	5	5	51.75	4.999375
YNL069C	RPL16B	34	11	9	4	36.68	4.944042
YBR191W	RPL21A	23	9	5	1	28.57	4.93367
YBL072C / YER102W	RPS8A / RPS8B	46	11	7	0	34.83	4.891063
YBL087C / YER117W	RPL23A / RPL23B	108	7	5	0	44.2	4.836592
YBR181C / YPL090C	RPS6B / RPS6A	123	13	9	0	39.24	4.815528
YBR048W / YDR025W	RPS11B / RPS11A	48	8	6	0	33.76	4.507296
YLR029C	RPL15A	58	11	9	2	38.54	4.504136
YLR388W	RPS29A	9	3	3	3	31.58	4.503828
YGL135W / YPL220W	RPL1B / RPL1A	190	11	9	0	37.16	4.492363
YLR061W	RPL22A	225	6	3	2	46.72	4.381801
YER165W	PAB1	111	28	23	23	57.96	4.35161
YLR048W	RPS0B	35	12	8	1	51.38	4.291539
YGR214W / YLR048W **	RPS0A / RPS0B	42	12	8	0	25.3	4.282044
YBL027W / YBR084C-A	RPL19B / RPL19A	79	9	6	0	33.16	4.146701

Table A1 – Continued

Accession #	Gene	tot # spectra	tot non-redun spectra	tot non-redun peptides	# unique	% AA covg	PAF
YDR418W / YEL054C	RPL12B / RPL12A	36	7	5	0	42.17	3.927509
YGR214W	RPS0A	28	11	8	1	43.87	3.925207
YDL075W / YLR406C **	RPL31A / RPL31B	60	5	4	0	35.96	3.86011
YPL131W	RPL5	62	13	9	9	41.61	3.855851
YLR340W	RPP0	56	13	10	10	39.94	3.855622
YGR118W / YPR132W	RPS23A / RPS23B	82	6	5	0	32.19	3.741115
YFL034C-A	RPL22B	19	5	3	2	46.34	3.616113
YIL133C	RPL16A	25	8	8	3	36	3.603441
YBR009C / YNL030W	HHF1 / HHF2	11	4	3	0	28.85	3.518649
YNL301C / YOL120C	RPL18B / RPL18A	27	7	7	0	29.41	3.404173
YPL143W	RPL33A	10	4	3	1	29.63	3.291098
YOR234C	RPL33B	13	4	3	1	29.63	3.287311
YHR121W	LSM12	27	7	5	5	35.11	3.284226
YHR141C / YNL162W	RPL42B / RPL42A	30	4	2	0	14.95	3.275467
YHR073C-B	ORF	16	1	1	1	40.74	3.256268
YER131W	RPS26B	12	4	4	1	32.5	2.974641
YPL271W	ATP15	2	2	2	2	46.03	2.966039
YGL189C	RPS26A	12	4	4	1	32.5	2.961866
YOR204W	DED1	102	19	16	14	45.29	2.898418
YLL024C	SSA2	69	20	16	1	40.16	2.878941
YHL015W	RPS20	13	4	4	4	37.7	2.876249
YGR148C	RPL24B	13	5	4	1	21.15	2.84949
YML056C	IMD4	44	16	13	6	41.33	2.837181
YDL081C	RPP1A	20	3	2	2	56.07	2.750275
YCL043C	PDI1	92	16	13	13	36.71	2.747866
YOR332W	VMA4	15	7	5	5	26.5	2.644403
YBR084W	MIS1	65	28	26	25	39.86	2.636113
YNL307C	MCK1	35	11	9	9	30.59	2.550074
YJR009C	TDH2	25	9	9	2	41.74	2.51067
YGR215W	RSM27	6	3	3	3	38.74	2.420721
YMR230W / YOR293W **	RPS10B / RPS10A	4	3	2	0	22.64	2.355158
YER177W	BMH1	23	7	5	1	30.22	2.326277

Table A1 – Continued

Accession #	Gene	tot # spectra	tot non-redun spectra	tot non-redun peptides	# unique	% AA covg	PAF
YLR167W	RPS31	14	4	3	2	22.22	2.32342
YML028W	TSA1	9	5	5	5	36.04	2.315887
YLR432W	IMD3	41	13	12	2	45.04	2.297429
YCL037C	SRO9	43	11	9	9	40.69	2.288806
YER131W / YGL189C **	RPS26B / RPS26A	7	3	2	0	20	2.230981
YMR188C	MRPS17	14	6	6	6	29.41	2.17116
YAL005C	SSA1	40	15	13	3	32.81	2.150014
YBL003C / YDR225W **	HTA2 / HTA1	9	3	2	0	24.06	2.144542
YHL034C	SBP1	24	7	6	6	37.29	2.121855
YLR185W	RPL37A	2	2	2	2	21.35	2.030457
YDR500C	RPL37B	3	2	1	1	7.87	2.026753
YKL016C	ATP7	10	4	3	3	24.57	2.019182
YDR381W	YRA1	14	5	4	4	15.42	2.003526
YCR009C	RVS161	6	6	6	6	39.47	1.983471
YKR006C	MRPL13	19	6	6	6	40	1.982095
YGL068W	MNP1	23	4	3	3	20.51	1.937046
YNL071W	LAT1	47	10	10	10	27.95	1.929831
YDL130W	RPP1B	13	2	2	2	46.73	1.874766
YKL167C	MRP49	6	3	2	2	15.94	1.872542
YGL147C / YNL067W **	RPL9A / RPL9B	14	4	4	0	20.31	1.854513
YPL127C	HHO1	12	5	5	5	25.87	1.798302
YJL138C / YKR059W	TIF2 / TIF1	18	8	8	0	19.7	1.789829
YGR178C	PBP1	40	14	13	13	28.22	1.777078
YNR036C	ORF	4	3	3	3	40.26	1.773364
YBL030C	PET9	17	6	6	5	23.82	1.742869
YOR196C	LIP5	18	8	8	8	31.08	1.729842
YOL111C	TMA24	7	4	4	4	31.46	1.685488
YJL052W	TDH1	17	6	6	1	26.73	1.678322
YLR150W	STM1	25	5	4	4	28.1	1.666944
YNL112W	DBP2	16	10	10	9	27.79	1.639371
YLR029C / YMR121C **	RPL15A / RPL15B	27	4	4	0	23.9	1.637867
YFL016C	MDJ1	27	9	8	8	25.98	1.619841
YDR385W / YOR133W	EFT2 / EFT1	38	15	13	0	16.25	1.607907
YDR148C	KGD2	25	8	7	7	25.43	1.586326
YJL205C	NCE101	1	1	1	1	37.04	1.578283

Table A1 – Continued

Accession #	Gene	tot # spectra	tot non-redun spectra	tot non-redun peptides	# unique	% AA covg	PAF
YNR051C	BRE5	26	9	9	9	25.19	1.560495
YJL122W	ORF	6	3	3	3	28.41	1.555452
YPL106C	SSE1	42	12	11	9	22.05	1.551069
YDR432W	NPL3	19	7	6	6	24.34	1.541613

TABLE A1- Protein abundance factors (PAF) from replicate Gis2-Tap purifications

In 3 replicate experiments, Gis2-TAP was isolated from yeast whole cell lysate and coeluting proteins were analyzed by LC-MS/MS. Proteins were identified by searching the acquired mass spectrometry data against a non-redundant database of yeast proteins using the Sequest algorithm (Eng, McCormack et al. 1994). The resulting output files were processed and analyzed using the BIGCAT software suite (McAfee, Duncan et al. 2006). Proteins are ranked by their protein abundance factor (PAF), which is a non-isotopic method of quantifying protein abundance in mass spectrometry data.

TABLE A2- Proteins copurifying with ZNF9 in immunoprecipitation experiments

Gene	Score	tot # spe ctra	tot non-redun spectra	tot non-redun peptides	# unique	% AA covg	PAF
ZNF9	-75.08	11	3	3	3	23.16	1.5414
HSPB1	-69.46	11	6	5	5	30.24	2.6335
RPL13 / RPL13	-60.3	8	3	3	0	13.74	1.2366
KRT6A	-59.2	10	7	7	1	14.36	1.1658
RPS20	-57.54	9	3	3	3	22.69	2.2433
RPS25 / LOC401206	-53.09	7	3	3	0	16	2.1831
PRSS2	-49.42	7	4	3	1	15.38	1.5101
RPSA / RPSA	-48.88	7	3	3	0	15.25	0.9131
RPS3A / RPS3A	-48.46	7	3	3	0	12.88	1.0018
HNRPA2B1 / HNRPA2B1	-44.11	7	3	3	0	12.02	0.8332
RPS2 / LOC286444	-44	6	3	3	0	11.6	0.9577
RPS19	-38.76	6	3	3	2	17.93	1.868
RPS5	-36.84	4	1	1	1	7.35	0.4371
HNRPC / HNRPC	-34.25	5	2	2	0	7.59	0.6257
RPL27A / LOC389435	-32.74	6	3	3	0	25.68	1.8115
HSPA8 / HSPA8	-31.87	5	3	3	0	6.35	0.4231
RPL31 / LOC391581	-30.4	4	1	1	0	7.2	0.6914
HNRPA1 / HNRPA1	-30.38	5	3	3	0	12.81	0.8773
KRT2A	-30.03	5	4	4	3	7.2	0.6113
RPS28 / LOC441618	-25.55	3	1	1	0	17.39	1.2753
RPL18	-25.43	3	2	2	2	13.83	0.9245
RPL8 / RPL8	-25.24	5	3	3	0	9.34	1.0705
HNRPU / HNRPU	-24.13	3	2	2	0	2.98	0.2248
RPL12 / LOC344471	-23.37	4	1	1	0	5.45	0.5612
RPS23	-22.54	3	2	2	1	16.08	1.2652
RPL29 / LOC283412	-22.34	4	2	2	0	14.47	1.1266
TUBB2	-19.91	4	3	3	1	8.09	0.602
LOC389127	-19.51	3	1	1	1	10.77	0.6686
HIST1H4I / HIST1H4A	-19.34	3	2	2	0	21.36	1.7595

TABLE A2 – Continued

Gene	Score	tot # spectra	tot non-redun spectra	tot non-redun peptides	# unique	% AA covg	PAF
THOC4	-19.07	3	2	2	2	10.51	0.7438
ALDOA / ALDOA / ALDOA	-18.42	2	1	1	0	3.85	0.2537
RPL21 / LOC387753	-18.42	2	1	1	0	9.38	0.5386
CCT2	-18.42	2	1	1	1	2.99	0.1739
RPS11	-18.02	3	2	2	2	11.39	1.0851
HNRPH1	-17.89	3	2	2	1	5.12	0.4063
RPS8	-16.22	2	1	1	1	6.25	0.4131
NSEP1	-16.22	2	1	1	1	5.86	0.2784
FLJ33718	-15.78	3	1	1	1	4.92	0.2691
FAU	-15.46	3	2	2	2	8.27	1.3899
RUVBL1	-15.13	3	2	2	2	4.82	0.3982
ILF3 / ILF3	-14.84	2	1	1	0	1.42	0.1315
SFRS2 / SRP46	-14.53	2	1	1	0	3.62	0.3925
RPLP2	-14.41	3	2	2	2	26.96	1.7145
RPS21	-14.27	3	2	2	2	18.07	2.1951
RPL24	-12.88	2	1	1	1	5.1	0.5625
PRSS3	-12.55	2	2	2	1	11.34	0.7491
LOC388255 / LOC390714	-11.9	2	1	1	0	0.37	0.048
RPL14	-11.44	2	2	2	1	13.89	0.851
RPS4X	-11.02	2	2	2	1	8.37	0.6757
HSPD1 / HSPD1	-10.19	2	2	2	0	3.49	0.3276
GLDC	-9.13	2	1	1	1	1.47	0.0886
RPS27 / LOC389112	-9.11	2	1	1	0	13.1	1.057
RPLP1 / LOC440927	-8.36	2	1	1	0	14.04	0.8685

TABLE A2- Proteins copurifying with ZNF9 in immunoprecipitation experiments ZNF9 was immunoprecipitated from HeLa cell lysates and the coeluting proteins were analyzed by LC-MS/MS. Proteins were identified by searching the acquired mass spectrometry data against a non-redundant database of human proteins using the Sequest algorithm (Eng, McCormack et al. 1994). The resulting output files were processed and analyzed using the BIGCAT software suite (McAfee, Duncan et al. 2006). Proteins were considered significant if over 2 spectra were acquired and confidently matched.

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