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Saccharomyces cerevisiae Gis2 interacts with the translation machinery and is orthogonal to myotonic dystrophy type 2 protein ZNF9

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ABSTRACT

The myotonic dystrophy type 2 protein ZNF9/CNBP 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 studies suggested that ZNF9 regulates translation and facilitates the process of cap-independent translation through interactions with mRNA and the translating ribosome. To help determine the role played by ZNF9 in the activation of translation initiation, we combined genetic and biochemical analysis of the putative *ZNF9* ortholog *GIS2*, in the budding yeast *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 acts as a cap-independent translation factor.

1. Introduction

The human disease myotonic dystrophy type 2 (DM2) is caused by a CCTG tetranucleotide repeat expansion in the first intron of the *znf9* gene, which codes for the protein ZNF9/CNBP [1]. ZNF9/CNPB is a small nucleic acid binding protein. Previous studies have implicated ZNF9 in a wide variety of molecular functions, ranging from the regulation of transcription to control of cell growth and proliferation [2–6]. Much of the ambiguity in the reported function of ZNF9 comes from the complexity of studying essential genes in mammalian model systems. A homozygous deletion of ZNF9/CNBP results in embryonic lethality in mice due to defects in brain development [3]. Reduced expression of ZNF9 results in truncated forebrains in chickens and severe craniofacial defects in zebrafish [4,5]. While these phenotypes provide evidence for the importance of the ZNF9 protein in growth and development, they do not illuminate the *in vivo* cellular functions and interactions of the protein.

Recently, we discovered that ZNF9 functions as a regulator of cap-independent or internal ribosome entry site (IRES)-mediated translation [7,8]. IRES-mediated translation in eukaryotes involves a number of factors, including IRES-specific *trans*-activating factors (ITAFs) that are thought to stabilize certain mRNA structures and facilitate their interactions with the ribosome [9–11]. 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 5′-cap complex [8]. Other groups have also observed that ZNF9 acts as a regulator of translation [2,12–15], but the full scope of ZNF9's cellular interactions and any other processes regulated by ZNF9 are not well understood.

The translation machinery of the baker's yeast *Saccharomyces cerevisiae* and other single-celled eukaryotes is well conserved. The *S. cerevisiae* genome encodes one putative homolog of mammalian *ZNF9* called *GIS2*. *GIS2* was initially cloned as a multi-copy suppressor of the Gal-phenotype in a *snf1/mig1/srb8* yeast mutant [16]. The physiological role for *GIS2* in *S. cerevisiae* is unknown.

Because ZNF9 is absolutely required for the viability of vertebrate organisms [3,17], facile genetic analysis of ZNF9 function is limited in these complex animals. To address these issues, we have begun characterization of the putative ZNF9 homolog in *S. cerevisiae*. While Gis2p contains significant sequence similarity with the *Homo sapiens* ZNF9 protein, it is unknown whether the two proteins can be considered functional orthologs.

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Using a series of biochemical and genetic assays, we demonstrate that Gis2p and ZNF9 are likely functional orthologs. Like ZNF9, Gis2p associates with translating ribosomes and copurifies with many ribosomal protein subunits as determined by mass spectrometry-based proteomics. Gis2p interaction with the ribosome is RNAse-sensitive, suggesting a mechanism for the observed ribosomal interactions. Expression of Gis2p in human cells is able to activate cap-independent translation of the ODC IRES. These data together suggest that the *S. cerevisiae* protein Gis2p and the mammalian protein ZNF9 are functional orthologs and provide a novel system in which to study the molecular functions of ZNF9 in translation and other essential cellular processes.

2. Materials and methods

2.1. Yeast strains and plasmids

Yeast genetic manipulations and media preparation were performed essentially as described [18]. The Δ*gis2* deletion strain used in this study had the entire Gis2 ORF replaced by a kanamycin cassette in the BY4743 background [19]. Knockout strains of Δ*gis2* were confirmed by PCR-based amplification across the Δ*gis2*::Kan^R locus and phenotypically by growth on G418-containing medium. The Gis2-TAP yeast strain for purification of Gis2p has been previously described [20].

2.2. Tandem affinity purification and LC/MS/MS analysis

Gis2p was purified from 1 L cultures of the Gis2-TAP yeast strain grown to early stationary phase ($O.D._{600}$ 2–4). Gis2p and its associated proteins were isolated using a dual affinity protocol as previously described [21]. A 10% fraction of the eluted proteins was analyzed by SDS–PAGE on 4–12% Novex Bis-TRIS gels and silver stained. The remaining eluted proteins were reduced, alkylated, and digested with sequencing grade trypsin as previously described [21]. Trypsin-digested peptides were identified using two-dimensional microcapillary liquid chromatography coupled with an LTQ linear ion trap mass spectrometer as described previously [21]. The acquired mass spectra were correlated to a translated *S. cerevisiae* ORF database using Sequest [22]. The resulting peptide hits were processed, assembled, and analyzed using bioinformatics graphical comparative analysis tools (BIGCAT) as previously described [7,23,24].

2.3. Polysome profiling and ribosome salt wash experiments

Polysome profiling experiments were performed as previously described [21]. Ribosome pelleting was accomplished by centrifugation of whole cell lysates through a 1 M sucrose cushion (±10 mg/mL RNAse H or 50 mM EDTA) at 100,000*g* for 2 h in a TLA120.2 rotor. The resulting ribosome pellets were resuspended in ribosome solubilization buffer (20 mM TRIS–HCL (pH 7.5), 5 mM $MgCl_2$, and 6 M urea). Supernatant fractions were precipitated with TCA, washed with acetone, and resuspended in ribosome solubilization buffer. For ribosome salt wash analysis, ribosome pellets were isolated as described above. 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 β-mercaptoethanol, and either 100 mM, 250 mM, 500 mM, or 1 M potassium acetate. Resuspended ribosomes were then centrifuged at 100,000*g* for 2 h in a TLA120.2 rotor. Supernatant fractions were removed, precipitated with TCA, and analyzed by SDS–PAGE and western blotting.

2.4. Cell 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 have been previously described [8]. pcDNA3.1-V5-Gis2 was created by Gateway-mediated recombination of pcDNA3.1-V5-*ccdB* and pENTR-Gis2, which was created by cloning the *GIS2* coding sequence amplified from yeast genomic DNA using the primers 5′-CACCAT GTCTCAAAAAGCTTGTTACG-3′ and 5′-CTAAGCCTTTGGACAATCCT-3′. Cap-independent translation activity was assayed as previously described [8].

2.5. Cloning of *znf9* deletion mutants

To create deletion mutants of ZNF9, PCR products amplified using the following primers on full-length pcDNA3.1-V5-ZNF9 were cloned into pENTR-D/TOPO (Invitrogen). Antisense primer for the Δ1 and ΔRGG mutants: 5′-AGGCTGTAGCCTCAATTGTGCATTC-3′.

Sense primer for Δ1: 5′-CACCATGACTGGTGGAGGCCGTGGTCG-3′. Sense primer for ΔRGG: 5′-CACCATGATTTGTTATCGCTGTGGTGA-3′. Sense primer for the Δ7, Δ6, and Δ5 mutants: 5′-CACCATGAGCAGCAATGAGTGCTT-3′. Antisense primer for Δ7: 5′-TTATTCACTTGTCTTGCTGCAGT-3′. Antisense primer for Δ6: 5′-TTATTTGGTGCAGTCTTTTTGAA-3′. Antisense primer for Δ5: 5′-TTACTCATCTGCATGGTCGCAGT-3′. Destination clones were created by Gateway-mediated recombination of individual entry clones with pcDNA3.1-V5-*ccdB* (Invitrogen).

3. Results

3.1. *S. cerevisiae Gis2p copurifies with the eukaryotic ribosome*

Phylogenetic and sequence alignment analysis using the ClustalW algorithm suggests ZNF9/CNBP sequences are conserved in all eukaryotic organisms including *S. cerevisiae* (Fig. 1A and B) [25]. Human ZNF9/CNBP shares 36% sequence identify and 59% similarity with yeast Gis2p. Primary sequence analysis of *ZNF9/CNBP* and yeast Gis2p revealed the presence of seven CCHC zinc finger sequences (Fig. 1C) [26]. While an RGG box motif is identified in higher eukaryotic organisms, the RGG motif is noticeably absent from the *S. cerevisiaie* Gis2p protein. We sought to determine whether the high level of sequence similarity translated into functionally similar protein activities.

Previously, ZNF9 was found to associate with the translating ribosome and act as a regulator of cap-independent translation [7,8]. To examine whether Gis2p and ZNF9 have conserved interactions with the ribosome, we used tandem affinity purification 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 [24,27]. Following TAP-isolation, the eluted proteins were separated by SDS–PAGE and visualized by silver staining (Fig. 2A). Affinity purification of Gis2p yielded a large set of co-purifiying proteins, the majority of which were below 40 kDa. We identified the co-purifying peptides and proteins by LC–MS/MS analysis (Supplemental Table 1) [21]. Label-free, semi-quantitative protein abundance factors (PAF) were computed for each identified protein to give a relative measure of the abundance of each protein (Supplemental Table 1 and Supplemental Fig. 1) [28]. Based on this method for comparing LC–MS/MS experiments, the most abundant co-purifying proteins were ribosomal subunits and other translation related proteins (Fig. 2B and Supplemental

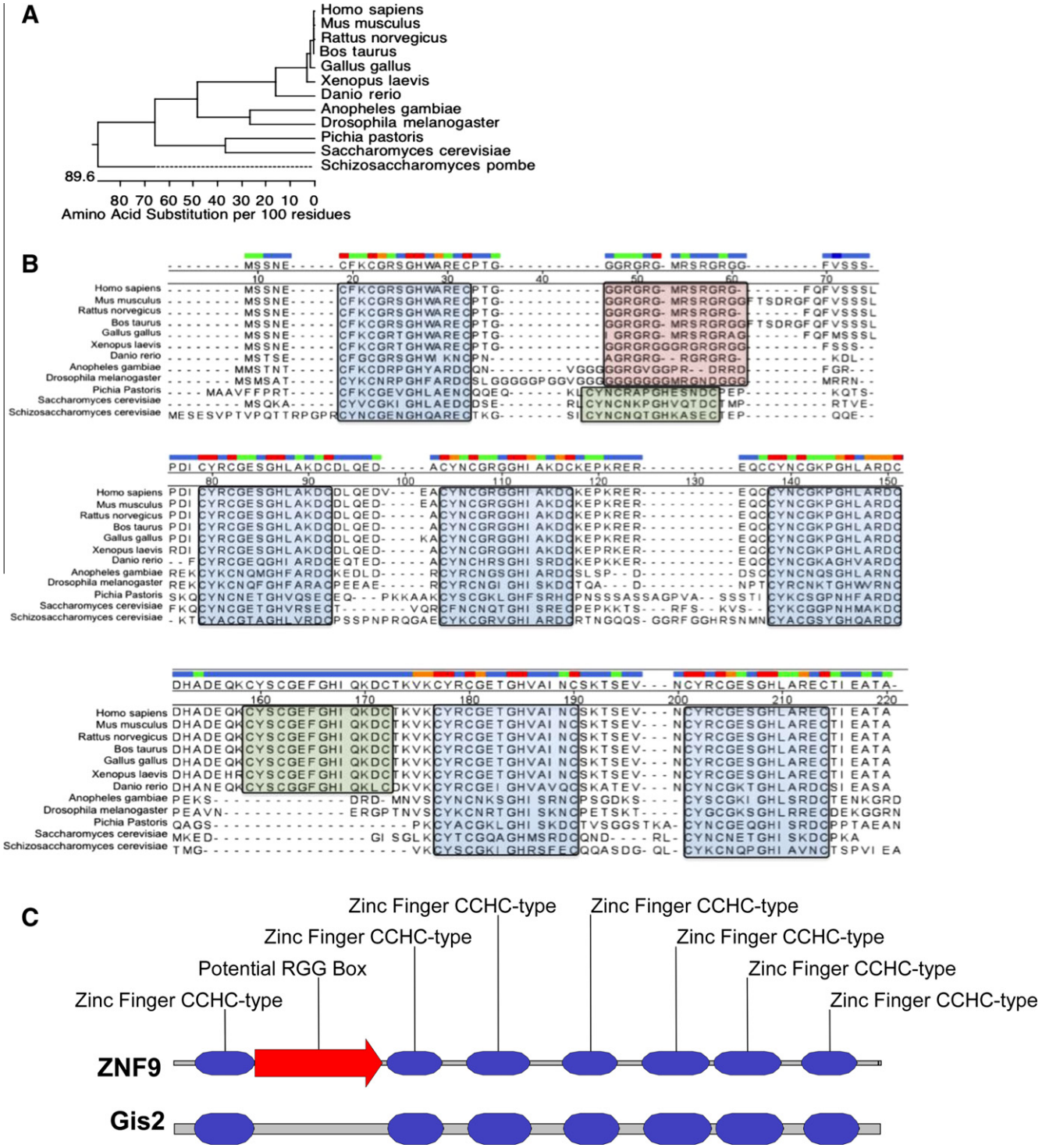

Fig. 1. Phylogenetic and sequence analysis of ZNF9/CNBP homologs. (A) RefSeq protein models of ZNF9/CNBP homologs for each organism were analyzed for sequence similarity using the ClustalW algorithm. Divergence and distance are graphed as the number of amino acid differences per 100 residues. (B) Sequence alignment of putative ZNF9 orthologs. Protein sequence alignment of ZNF9/CNBP homologs was performed using the ClustalW algorithm and visualized using MegAlign (DNAStar Lasergene). Blue boxes represent shared CCHC zinc finger motifs found in all protein models. The red box represents the RGG domain, which is only found in multicellular organisms, while the green boxes represent the unaligned CCHC domains differentially located in the unicellular and multicellular groups. (C) Alignment of human ZNF9 and yeast Gis2p functional domains.

Fig. 1). Clustering of data from three replicate Gis2-TAP isolates and four replicate purified ribosomes revealed that Gis2p reproducibly copurifies with ribosomes and other translation factors. In earlier studies, ZNF9 copurifies with a number of ribosomal proteins in HeLa cell lines, and proteomic analysis of immunoprecipitated ZNF9 revealed a large number of copurifiying ribosomal and RNA-binding proteins [8,29]. These data suggest that Gis2p copurifies with components of the eukaryotic ribosome and provide evidence that biochemical interactions are conserved between Gis2p and ZNF9.

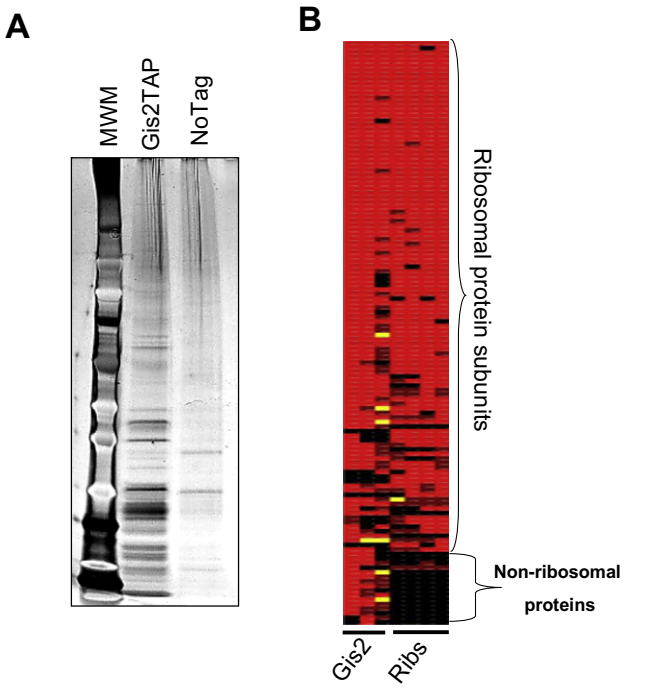

Fig. 2. Analysis of proteins isolate by Gis2p-targetted TAP reveals the interactome of Gis2p. (A) Eluted proteins purified by a TAP protocol from a Gis2-TAP expressing strain and an untagged parental strain. 10% of the eluted proteins were analyzed by SDS–PAGE and silver stained (MWM: molecular weight markers). (B) Clustering of proteins identified by tandem mass spectrometry from 3 replicate Gis2p-TAP purifications compared to 4 replicate isolations of ribosomes. On the heat map, protein abundance is represented by color, from black representing no protein identified to bright red meaning high PAF value. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2. Polysome analysis reveals Gis2p colocalizes with the ribosome

To independently 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 for the presence of Gis2p. Initially, lysates from a Gis2-TAP-tagged strain were loaded onto a sucrose cushion and subjected to ultracentrifugation to isolate polyribosomes. As seen in Fig. 3A, the large majority of Gis2p is found in the polysome pellet, which suggests that Gis2p associates with the translating ribosome. We confirmed this result using linear sucrose gradient ultracentrifugation experiments (Fig. 3B). With 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 [8].

Ribosome salt wash (RSW) experiments are often used to separate core ribosomal subunits from associated translation factors. Previously, we used RSW to determine the strength of human ZNF9's ribosome interaction [8]. 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 remains associated with ribosomes at low salt concentrations (100 mM and 250 mM potassium acetate) (Fig. 3C). With 500 mM salt, Gis2p begins to elute from the ribosome pellet, and at 1 M salt, all of the Gis2p is found in the wash fraction. These results are similar to what we previously observed with human ZNF9 [8]. Gis2p's interaction with the ribosome is similar to that of other translation factors and not quite as strong as that of core ribosomal subunits. 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.

3.3. Gis2p–ribosome interactions are sensitive to RNAse

Next, we investigated the mechanism of Gis2p's interaction with ribosomes. Because of the polysomal localization of Gis2p, we tested whether Gis2p's ribosome association required tethering through RNA by treatment with RNAse A. We treated lysates of Gis2-TAP expressing cells with 50 μg/mL of RNAse. Then, Gis2p was purified and subjected to western blot analysis and mass spectrometry-based proteomics (Fig. 3D). Interestingly, even though more Gis2p was purified from RNAse-treated samples compared to mock-treated controls, less ribosomal protein was identified by mass spectrometry. These results suggest that RNAse A treatment releases Gis2p from the ribosome and makes the purification more efficient. To confirm that RNAse A treatment releases Gis2p from ribosomes, we purified ribosomes were by ultracentrifugation and treated them with RNAse A. As a control, in parallel, we treated ribosomes with EDTA, which disrupts polyribosomes and their interactions with certain RNA binding proteins, including human ZNF9 [8]. Ribosomes were then reisolated through a sucrose cushion and analyzed by western blotting for the presence of Gis2p. As seen in Fig. 3E, 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 RNA.

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

In analyzing whether Gis2p is an ortholog of ZNF9/CNBP, one of our critical considerations was function. We tested whether a diploid Δ*gis2* yeast strain display slow growth or lethal phenotypes after a number of insults, including a series of translation inhibitors (Supplemental Fig. 2). Growth rate of Δ*gis2* cells was comparable to wild type under all conditions tested, suggesting that Gis2 is non-essential for survival in these conditions. Previously, we showed that ZNF9 functions as an activator of cap-independent translation in mammalian cells, specifically the ODC IRES [7,8]. If Gis2p is a true ortholog of ZNF9, it should facilitate activation of IRES targets. To measure cap-independent translation, we used a previously characterized bicistronic reporter plasmid system (Fig. 4A) [8]. 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 (Fig. 4B). Consistent with our hypothesis, expression of V5-Gis2p activated cap-independent translation of the reporter at levels similar to V5-ZNF9 (Fig. 4B). 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.

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

We hypothesized that regions of Gis2p critical for its cap-independent translation activity are in 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

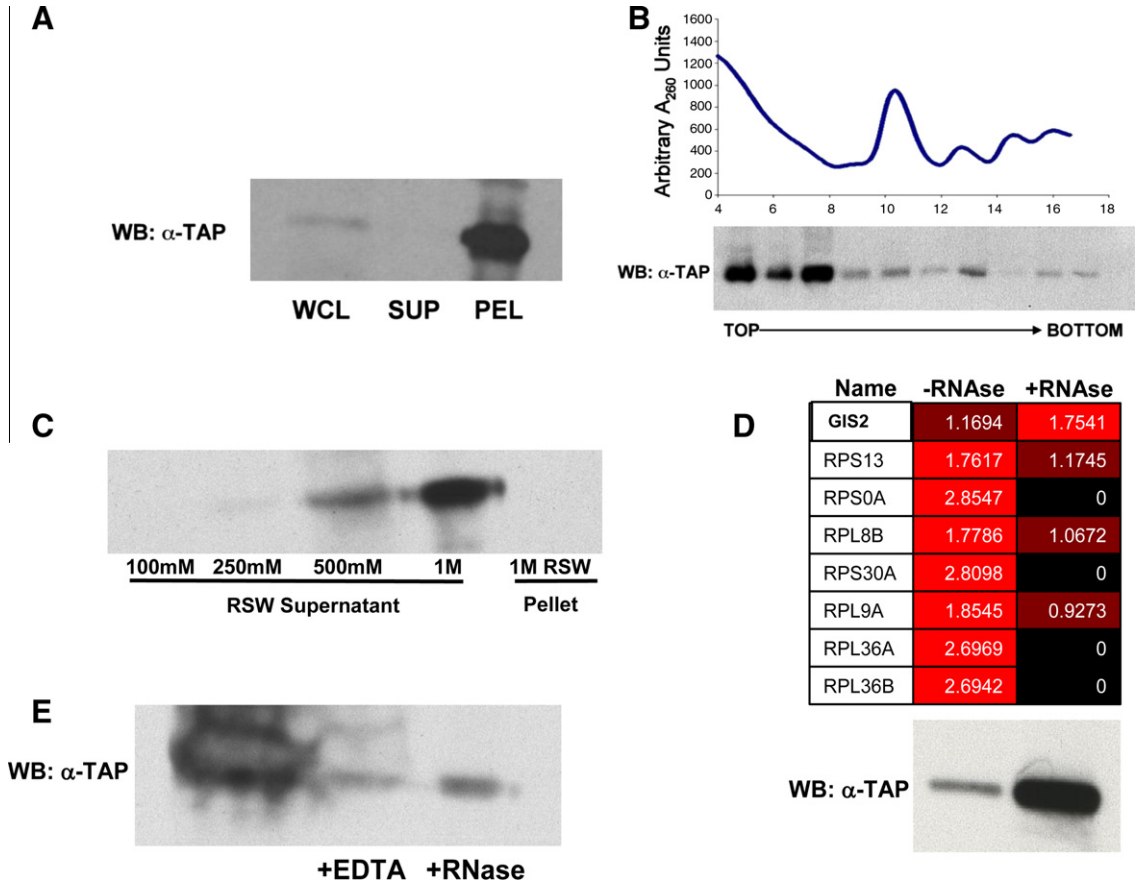

Fig. 3. *S. cerevisiae* Gis2p interacts with the ribosome. (A) Western blot analysis for the presence of Gis2p in whole cell lysates (WCL), supernatant (SUP), or pellet (PEL) fractions after isolation of polysomes from Gis2-TAP-tagged yeast cells. (B) Polysome analysis of the Gis2-TAP strain was measured using on-line UV_{260} absorbance. 10% of each fraction was analyzed by western blotting for the presence of Gis2p. (C) Western analysis for Gis2p in ribosome salt washes of increasing concentrations of potassium acetate. (D) Changing abundance of a subset of proteins isolated by purification of Gis2-TAP in the presence or absence of RNAse. (E) Western analysis for Gis2-TAP in solubilized polysome pellets treated with EDTA or RNAse.

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 (Fig. 4C). Western analysis of the various ZNF9 constructs in HEK293T cells showed equivalent expression of the Wt and deletion constructs (Fig. 4D). Each mutant was co-expressed with the ODC IRES reporter in HEK293T cells, and cap-independent translation activity was measured (Fig. 4E). 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.

4. Discussion

4.1. 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 *S. cerevisiae* protein Gis2p is part of an evolutionarily conserved mechanism to control translation of specific mRNA molecules that contain internal ribosome entry sites. Multiple studies have demonstrated cap-independent translation in *S. cerevisiae* of both viral and endogenous cellular mRNA molecules [30–33]. 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. It remains to be seen whether Gis2p functions as an ITAF for any of the known yeast IRES-containing mRNAs. 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 to date in yeast [30–32,34]. 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.

4.2. Gis2p and ZNF9 share common biochemical interactions

As demonstrated here and previously [7,8], 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 [7,12,14,35], which 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

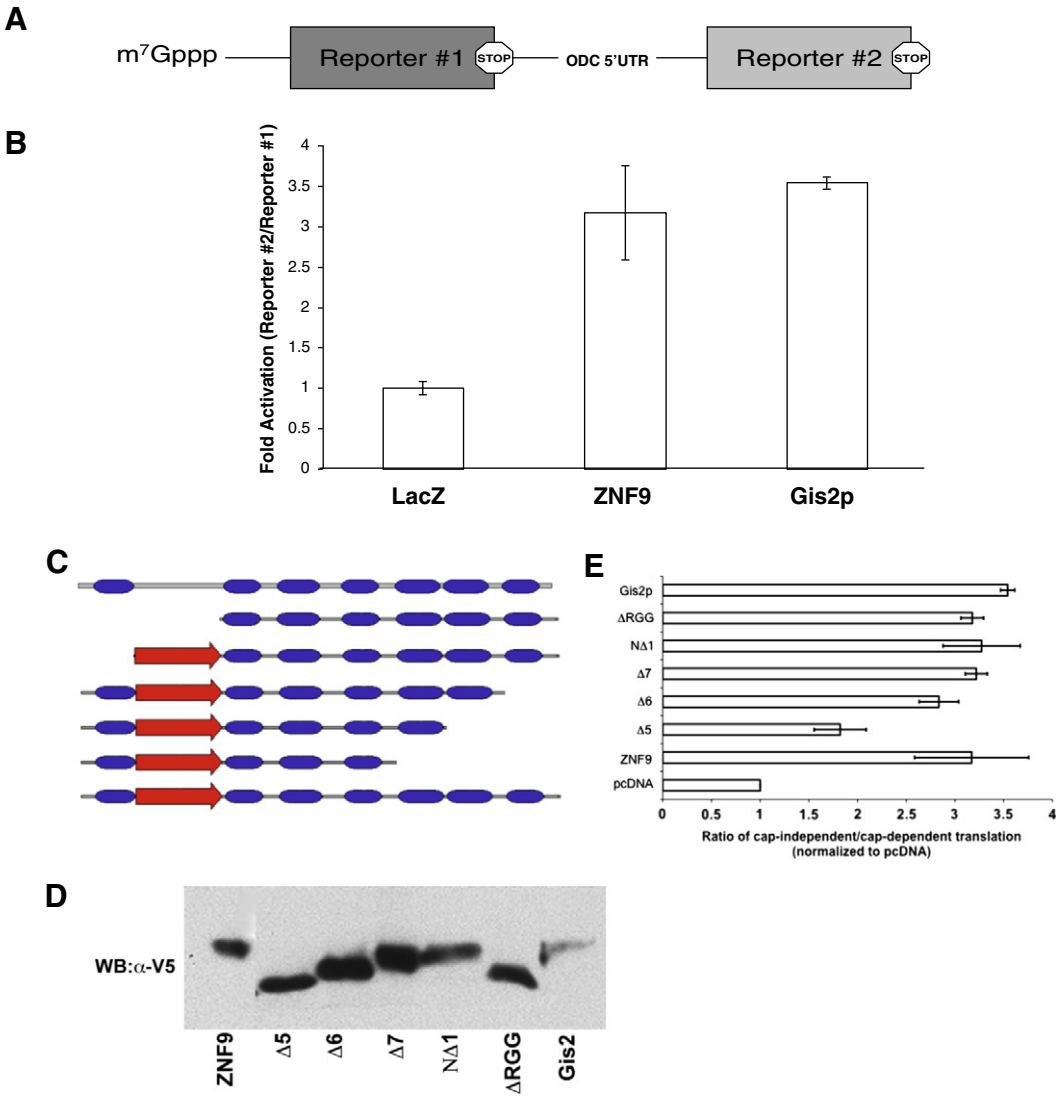

Fig. 4. Expression of Gis2p and ZNF9 deletion mutants in HEK293T cells activates IRES-dependent translation. (A) Schematic representation of the bicistronic ODC-IRES reporter. (B) Cap-independent translation in HEK293T cells expressing the bicistronic ODC-IRES reporter and either LacZ, ZNF9, or Gis2p. Cells were analyzed for luciferase activity, and cap-independent translation is reported as a ratio of cap-independent translation (reporter #2, firefly luciferase) to cap-dependent translation (reporter #1, *Renilla* luciferase). Three independent experiments were averaged. The error bars show the standard deviations. (C) Schematic representation of Gis2p and various ZNF9 deletion mutants. (D) Western blot analysis using anti-V5 monoclonal antibody against V5-tagged ZNF9 constructs and Gis2p protein expression in HEK293T cells (the lane corresponding to the wild-type ZNF9 construct was cropped from another gel for clarity). (E) Cap-independent translation in HEK293T cells expressing the ODC-IRES reporter and the Gis2p and ZNF9 plasmid constructs. The plasmid constructs shown in C. are aligned with the translation assay results, respectively. Cells were analyzed for cap-independent activity as described above. Three independent experiments were averaged. The error bars represent the standard deviations.

as a potential Gis2p interactor in our studies (Supplemental Fig. 2). The Michnick group recently showed a putative interaction between Sro9p and Gis2p by genome-wide protein-fragment complementation assay [36]. Sro9p was initially identified as a multi copy suppressor of an RNA export defect and was shown to associate with polysomes [37,38]. Sro9p associates with nascent transcripts and forms part of an mRNP complex that is exported to the cytoplasm, where Sro9p then acts as a regulator of translation [39].

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

The yeast model system offers intriguing opportunities to study Gis2p and Sro9p and their potentially conserved cellular roles in 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. While the role that ZNF9 plays in the progression of DM2 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 in 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 in understanding ZNF9 function.

Although previous work suggested that ZNF9 plays a role in cell proliferation, Δ*gis2* deletion mutants grew at a rate similar to the wild type rate under all conditions tested, suggesting that ZNF9's role 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 [6]. Further studies are needed to understand what role the different conserved

and unique functional domains play in the multiple roles of ZNF9 and Gis2p. 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.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.01.086.

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