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A conserved role for the zinc finger polyadenosine RNA binding protein, ZC3H14, in control of poly(A) tail length

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ABSTRACT

The ZC3H14 gene, which encodes a ubiquitously expressed, evolutionarily conserved, nuclear, zinc finger polyadenosine RNA-binding protein, was recently linked to autosomal recessive, nonsyndromic intellectual disability. Although studies have been carried out to examine the function of putative orthologs of ZC3H14 in Saccharomyces cerevisiae, where the protein is termed Nab2, and Drosophila, where the protein has been designated dNab2, little is known about the function of mammalian ZC3H14. Work from both budding yeast and flies implicates Nab2/dNab2 in poly(A) tail length control, while a role in poly(A) RNA export from the nucleus has been reported only for budding yeast. Here we provide the first functional characterization of ZC3H14. Analysis of ZC3H14 function in a neuronal cell line as well as in vivo complementation studies in a Drosophila model identify a role for ZC3H14 in proper control of poly(A) tail length in neuronal cells. Furthermore, we show here that human ZC3H14 can functionally substitute for dNab2 in fly neurons and can rescue defects in development and locomotion that are present in dNab2 null flies. These rescue experiments provide evidence that this zinc finger-containing class of nuclear polyadenosine RNA-binding proteins plays an evolutionarily conserved role in controlling the length of the poly(A) tail in neurons.

Keywords: ZC3H14/dNab2/Nab2; polyadenosine RNA-binding protein; poly(A) tail; intellectual disability

INTRODUCTION

Post-transcriptional regulation of gene expression is critical for determining the spatial and temporal gene expression pattern of a cell. Such regulation is particularly critical in neurons where RNA trafficking and local translation play key roles in neuronal function (Sinnamon and Czaplinski 2011; Goldie and Cairns 2012). This post-transcriptional regulation of gene expression is mediated by numerous RNA-binding proteins. The functional importance of these RNA-binding proteins is highlighted by the growing number of proteins in this family that have been linked to human diseases (Cooper et al. 2009).

One key class of RNA-binding proteins, called polyadenosine RNA-binding proteins (Pabs), bind specifically to the poly(A) tails of mRNA transcripts and play important roles in the post-transcriptional control of gene expression (Eckmann et al. 2011). Previous studies demonstrate that Pabs can modulate transcript stability, export from the nucleus, and translation, highlighting their functions as key regulators of gene expression (Mangus et al. 2003). Recently, mutations in the human ZC3H14 gene, which encodes a zinc finger polyadenosine RNA-binding protein (Leung et al. 2009), were linked to inherited, nonsyndromic, intellectual disability revealing the critical role of this Pab protein in the brain (Pak et al. 2011; Kelly et al. 2012). The ZC3H14 protein (also termed mSUT-2) (Guthrie et al. 2009, 2011), belongs to an evolutionarily conserved, ubiquitously expressed family of Cys3His tandem zinc-finger (ZnF) polyadenosine (poly(A)) RNA-binding proteins (Anderson et al. 1993; Hector et al. 2002; Kelly et al. 2007; Leung et al. 2009; Pak et al. 2011). The mammalian ZC3H14 locus encodes three long protein isoforms (isoforms 1–3) that at steady state localize to the nucleus and one short isoform (isoform 4) that localizes to the cytoplasm (Leung et al. 2009; Kelly et al. 2012). Isoforms 1–3 are ubiquitously expressed and share a common domain structure (Fig. 1A) with both Drosophila melanogaster Nab2 (dNab2) and Saccharomyces cerevisiae Nab2, while the shorter ZC3H14 isoform 4, which is highly expressed only in testes, has a unique first exon but retains the C-terminal tandem of gene expression (Mangus et al. 2003).
ZnF RNA-binding domain (Leung et al. 2009). Interestingly, one of the ZC3H14 mutations in intellectually disabled patients (R154->termination codon) leads to loss of ZC3H14 isoforms 1–3 but leaves isoform 4 intact (Pak et al. 2011), suggesting that these longer isoforms are necessary for cognitive function in humans, while isoform 4 may perform a distinct function.

Although little is known about the molecular function of human ZC3H14, both the S. cerevisiae and D. melanogaster presumptive orthologs of ZC3H14 bind polyadenosine RNA and are required to restrict poly(A) tail length in vivo (Anderson et al. 1993; Hector et al. 2002; Pak et al. 2011). As with ZC3H14 isoforms 1–3, the budding yeast and fly Nab2 proteins both localize to the nucleus at steady state (Anderson et al. 1993; Pak et al. 2011). Genetic studies reveal interactions between Nab2 and genes encoding components of the nuclear polyadenylation machinery (Kelly et al. 2010; Pak et al. 2011) suggesting that one principal Nab2 function is control of polyadenylation. Consistent with a critical role for Nab2 in post-transcriptional regulation, S. cerevisiae and D. melanogaster Nab2 are essential for cell viability and development, respectively (Anderson et al. 1993; Pak et al. 2011).

Our previous work demonstrating a requirement for D. melanogaster Nab2 in normal locomotor behavior (Pak et al. 2011) led to the hypothesis that neurons are exquisitely sensitive to defects in nuclear poly(A) tail length control. Subsequent findings that neuron-specific expression of wild-type dNab2 rescues dNab2 null developmental and locomotor defects, and that neuron-specific depletion of dNab2 otherwise wild-type animals can elicit similar defects, confirmed this hypothesis (Pak et al. 2011). These studies, coupled with the finding that humans with ZC3H14 mutations display nonsyndromic intellectual disability (Pak et al. 2011), suggest that ZC3H14 and dNab2 may perform functionally analogous roles in RNA metabolism in invertebrate and vertebrate neurons. However, no studies have been performed previously to assess the molecular function of ZC3H14.

Here we demonstrate that depletion of ZC3H14 leads to extended poly(A) tails in a cultured mouse neuronal cell line without significant accumulation of poly(A) RNA in the nucleus, supporting a model in which ZC3H14 plays a primary role in control of poly(A) tail length in mammalian cells. To extend these molecular results and assess the function of ZC3H14 in neurons in vivo, we exploit Drosophila mutant for Nab2. Our results show that neuron-specific transgenic expression of a nuclear isoform of human ZC3H14, but not the shorter, cytoplasmic isoform retained in human ZC3H14-associated intellectual disability patients (Pak et al. 2011), rescues developmental and behavioral phenotypes of Nab2 null D. melanogaster. Critically, this overt rescue by a nuclear isoform of ZC3H14 is accompanied by repair of an underlying defect in poly(A) tail length in the fly brain. These data indicate that human ZC3H14 is a functional ortholog of fly Nab2 that plays a critical role in the control of poly(A) tail length of neuronal RNAs in vivo.

RESULTS
To examine the molecular function of ZC3H14, we utilized neuro 2A (N2A) cells (Olmsted et al. 1970). Consistent with previous results (Leung et al. 2009; Guthrie et al. 2011), ZC3H14 isoforms 1–3 (Fig. 1A) are primarily localized to the nucleus in this neuronal cell line (Fig. 1B). To assess the functional consequences of loss of ZC3H14, we used siRNA-mediated depletion. The siRNAs targeted to ZC3H14 were designed to deplete all transcript variants. As a control for a protein implicated in regulation of poly(A) tail length, we also examined the consequences of siRNA-mediated depletion of the nuclear poly(A)-binding protein, PABPN1. PABPN1 modulates the processivity of poly(A) polymerase to achieve optimal poly(A) tail length (Kuhn and Wahle 2004) and previous work has shown that depletion of PABPN1 leads to shortening of bulk poly(A) tails (Apponi et al. 2010). As shown in Figure 2A, both ZC3H14-isol-3 and PABPN1 could be efficiently depleted from N2A cells. We then examined bulk poly(A) tail length in N2A cells treated with either of two independent ZC3H14 siRNAs, PABPN1 siRNA, or control scrambled siRNA (Fig. 2B,C). Depletion of ZC3H14 resulted in an increase in bulk poly(A) tail length.
FIGURE 2. ZC3H14 is required for proper poly(A) tail length control. (A) An immunoblot shows that both ZC3H14 (isoforms 1–3) and a control polyadenosine RNA-binding protein, PABPN1, are robustly depleted from N2A cells by treatment with specific siRNA with no effect of a scrambled control RNA (Ctrl). ZC3H14-iso4 is not detected as the antibody was raised to the N-terminal PWI-like domain not present in isoform 4 (see Fig. 1A). The double band observed for PABPN1 is likely due to the presence of phosphorylated PABPN1. The corresponding β-actin band is shown as a loading control. (B) ZC3H14 was depleted from N2A cells using two independent siRNAs (C, inset). As controls, cells were also treated with PABPN1 siRNA to deplete PABPN1 or scrambled control siRNA (siScramble). Total RNA samples were prepared and subjected to bulk poly(A) tail length analysis as described in Materials and Methods. A gel of the resolved bulk poly(A) samples is shown. Approximate positions of nucleotide size markers (nt) are indicated. (Inset) An immunoblot shows the level of ZC3H14 (isoforms 1–3) knockdown obtained with two independent siRNAs for this experiment. The knockdown obtained for the control polyadenosine RNA-binding protein, PABPN1, is also shown. No treatment (−) or a control Scramble siRNA has no effect on levels of ZC3H14 or PABPN1 as indicated. The relative intensity of the signal is plotted vs. poly(A) tail length as determined by size markers. The positions on the scan corresponding to 100, 200, and 300 nt are indicated. (Inset) An immunoblot shows the level of ZC3H14 (isoforms 1–3) knockdown obtained with two independent siRNAs for this experiment. The knockdown obtained for the control polyadenosine RNA-binding protein, PABPN1, is also shown. No treatment (−) or a control Scramble siRNA has no effect on levels of ZC3H14 or PABPN1. β-actin serves as a loading control. (D) The siRNA-treated samples shown in the inset in C were used for fluorescence in situ hybridization to assess poly(A) RNA localization. Total poly(A) RNA is visualized with an oligo dT probe. The corresponding DAPI and phase images are shown. Representative cell fields are shown. Results are typical of more than 50 cells analyzed for each siRNA where the distribution of poly(A) RNA in cells treated with ZC3H14 siRNA was indistinguishable from cells treated with scramble control siRNA.

compared with cells treated with control siRNA (Fig. 2B,C). As expected, depletion of PABPN1 led to shortened poly(A) tails (Apponi et al. 2010). Similar results were obtained with both siRNAs directed against ZC3H14 ensuring that results obtained were not due to off-target effects of the siRNA used. As the S. cerevisiae Nab2 protein has been implicated in both control of poly(A) tail length and poly(A) RNA export from the nucleus (Hector et al. 2002), in parallel with the bulk poly(A) tail length assays, we used fluorescence in situ hybridization (FISH) to assess the localization of bulk poly(A) RNA in cells depleted of ZC3H14. Consistent with what has been reported for dNab2 mutant flies (Pak et al. 2011), little or no accumulation of poly(A) was detected in the nuclei of cells treated with ZC3H14 siRNA (Fig. 2D). In contrast, as expected (Apponi et al. 2010), PABPN1 depletion led to significant nuclear accumulation of poly(A) RNA. These results provide molecular evidence that ZC3H14 plays a role in poly(A) tail length control but do not directly address the function of ZC3H14 in vivo.

Given that patients with mutations that eliminate the nuclear forms of ZC3H14 display intellectual disability without other reported symptoms (Pak et al. 2011; Kelly et al. 2012), we sought to assess the function of ZC3H14 in neurons. For these studies, we exploited a Drosophila model that is null for Nab2 and shows neuronal-specific defects in both development and behavior (Pak et al. 2011). As shown in Figure 1A, the sole isoform of Drosophila Nab2 (dNab2), which is localized to the nucleus (Pak et al. 2011), shares a similar domain structure with the nuclear isoforms of ZC3H14, isoforms 1–3. To directly assess whether H. sapiens ZC3H14 and D. melanogaster Nab2 are functional orthologs, we used tissue-specific transgenic expression. Multiple transgenes encoding either human ZC3H14 isoform 1 (UAS-ZC3H14-iso1Flag), which is localized to the nucleus (Leung et al. 2009), or human ZC3H14 isoform 4 (UAS-ZC3H14-iso4Flagex3), which is localized to the cytoplasm (Leung et al. 2009), were tested for the ability to rescue developmental and behavioral defects in flies homozygous for the protein and mRNA null allele dNab2mut (hereafter referred to as dNab2mut) (Pak et al. 2011). Only a small percentage (~3%) of homozygous dNab2mut flies eclose as adults (Fig. 3A; Pak et al. 2011). Transgenic expression of ZC3H14-iso1 specifically in D. melanogaster neurons using a pan-neuronal driver (elav-Gal4, UAS-ZC3H14-iso1Flag) rescues the eclosion rate of these dNab2mut animals from ~3% to ~78% (Fig. 3A). This rescue by ZC3H14-iso1 is comparable to the rescue conferred by transgenic, neuronal re-expression of dNab2, which restores the eclosion rate to ~93% (Fig. 3A). In contrast, pan-neuronal expression of UAS-ZC3H14-iso4Flagex3 had no ameliorating effect on eclosion rates of dNab2mut animals (elav-GAL4/+; UAS-ZC3H14-iso4Flagex3/+; dNab2mut) (Fig. 3A). Immunoblot analysis confirms expression of Flag-tagged dNab2 and ZC3H14 isoforms in head lysates of transgenic animals (Fig. 3B); however, the relative level of expression of ZC3H14-iso4 attained
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**DISCUSSION**

Here we present the first functional characterization of the mammalian polyadenosine RNA-binding protein, ZC3H14. The data couple evidence from a cultured neuronal cell line with functional studies exploiting a Drosophila model and support a role for ZC3H14 in modulating poly(A) tail length.
Significantly, we also find evidence that members of this class of zinc finger polyadenosine RNA-binding proteins are functional orthologs in metazoan neurons. Results presented here reveal that the ZC3H14 protein, which is lost in cases of human intellectual disability (Pak et al. 2011), is required for proper control of poly(A) tail length. Consistent with the observation that human patients lacking ZC3H14 display brain-specific defects, expression of human ZC3H14 solely in the neurons of dNab2 mutant flies is sufficient to rescue both behavioral defects and an underlying molecular defect that ZC3H14-iso4 is highly expressed only in the testes (Leung et al. 2009). Second, expression studies suggest that ZC3H14-iso4 is likely unaffected by ZC3H14 loss. A key question that remains is to understand the functional consequences of extended poly(A) tails on neuronal development and function, as well as whether the observed neuronal phenotypes are due to changes in specific RNAs or classes of RNAs.

To assess the function of human ZC3H14 in vivo, we have taken advantage of a Drosophila model in which functional rescue conferred by tissue-specific expression can be readily assessed. D. melanogaster has been utilized to identify conserved genes and signaling pathways that function in a variety of human diseases ranging from cancer (Pan 2010) to muscular dystrophy (Chartier et al. 2006; Lloyd and Taylor 2010) and has proven particularly useful in defining the genetic requirements of higher cognitive function (Narro et al. 2007). Genetic and molecular data presented here support a model in which the human ZC3H14-iso1 and Drosophila Nab2 proteins function at a common step in RNA processing, and that human ZC3H14 protein can maintain molecular interactions with critical RNAs and proteins in Drosophila neurons, and consequently can elicit similar downstream effects on cellular physiology and development.

Several lines of evidence suggest that the ZC3H14-iso1-3 proteins may have a function distinct from ZC3H14-iso4. First, the protein isoforms localize to different cellular compartments with ZC3H14-iso1 located in the cytoplasm, while ZC3H14-iso4 is localized to the nucleus at steady state, while ZC3H14-iso4 is located in the cytoplasm (Leung et al. 2009). Second, expression studies suggest
of ZC3H14-iso1 (see Fig. 3B). Furthermore, the transgenic proteins are both N-terminally epitope-tagged which could interfere with protein function. Thus, our results suggest that ZC3H14-iso4 is not a functional ortholog of dNab2 but further studies would be required to directly address this point.

Our analysis provides evidence that rescue of both organismal and behavioral defects in the dNab2 mutant flies correlates with a rescue of a molecular defect in polyadenylation. However, this work does not directly demonstrate that altered polyadenylation underlies the *Drosophila* phenotypes. Given the interdependent nature of transcription, RNA processing, export, and translation, dNab2/ZC3H14 could play direct roles in functions other than control of poly(A) tail length. Indeed, recent work has defined roles for the *S. pombe* Nab2 protein in regulated RNA turnover (Grenier St-Sauveur et al. 2008; Ibrahim et al. 2011; Smibert et al. 2012). Future studies investigating the precise molecular role of ZC3H14 and the neuronal target RNAs that it binds and regulates will be critical in defining how defects in Nab2/ZC3H14-dependent posttranscriptional regulation of gene expression contribute to neuronal dysfunction.

**MATERIALS AND METHODS**

**Cell culture**

Neuro 2A (N2A) cells (Olmsted et al. 1970) were grown in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, 0.1 mg/mL streptomycin, and 100 units/mL penicillin. DNA plasmids were transfected into cultured cells using Lipofectamine2000 (Invitrogen) according to manufacturer’s protocol.

**D. melanogaster stocks and genetics**

All crosses and stocks were maintained in standard conditions unless otherwise noted. The *Nab2ex3* null allele (referred to here as *Nab2null*) has been described previously (Pak et al. 2011). Complete open reading frames of *ZC3H14* isoform 1 (NCBI #NM_024824.4) and *ZC3H14* isoform 4 (NCBI #NM_207662) were cloned into a Gateway (Invitrogen) entry vector. The Gateway system was then used to transfer either *ZC3H14-iso1* or *ZC3H14-iso4* into a plasmid encoding an N-terminal Flag epitope (plasmid pTFW). Plasmids were then sequenced to ensure that the correct reading frame was maintained and that no mutations had been introduced during the cloning process. Sequenced plasmids were then injected by...
siRNA-mediated depletion

Stealth RNAi (Invitrogen) was used to knockdown ZC3H14 and PABPN1 expression in cultured N2A cells. Cells were plated in GM at a density of 1 x 10^5 cells per well on collagen-coated six-well plates and after 6 h, duplexed siRNAs at the final concentration of 80 nM were used to transfect cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions in GM. After overnight incubation, medium containing transfection complexes were replaced by fresh GM. PABPN1 knockdown was assessed by immunoblotting using an antibody anti-ZC3H14 (Leung et al. 2009) or anti-PABPN1 (Apponi et al. 2010) antibody after 40 h of transfection. Cells were transfected with scrambled control or one of two ZC3H14 siRNAs (Invitrogen). ZC3H14-1, TGAC TGACCTGAGTGGCGACAGAA; ZC3H14-2, GAAAGAGCCTCG ATATCTGACTCCAAA. PABPN1 siRNAs were previously described (Apponi et al. 2010).

Immunoblotting

Analyses of protein levels were performed by immunoblotting using standard procedures (Green and Sambrook 2012). For N2A cells, cells were lysed with RIPA-2 (50 mM Tris-HCl at pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS) containing protease inhibitors (Mini Complete, Roche) and equal amounts of total protein (5–20 μg) were resolved by SDS-PAGE, transferred to nitrocellulose, and the desired protein was detected by immunoblotting with appropriate antibody (polyclonal ZC3H14) (Leung et al. 2009) or PABPN1 (Apponi et al. 2010) diluted 1:5000. For flies, 20–40 h of transfection. Cells were transfected with scrambled control or one of two ZC3H14 siRNAs (Invitrogen). ZC3H14-1, TGAC TGACCTGAGTGGCGACAGAA; ZC3H14-2, GAAAGAGCCTCG ATATCTGACTCCAAA. PABPN1 siRNAs were previously described (Apponi et al. 2010).

Determination of bulk poly(A) tail length

Bulk poly(A) tails were analyzed using a standard PAT assay (Chekanova et al. 2001) as described previously (Apponi et al. 2010). Total RNA was isolated from either cultured N2A cells or 250–300 fly heads of 5–6 d old flies of the selected genotype. Total RNA was 3′-end labeled with [32P]pCp using T4 RNA ligase, and digested with a cocktail of RNase A/T1. The resulting tracts of poly(A) RNA were resolved by gel electrophoresis and imaged by film or PhosphorImager. At least three biological replicates were performed for each fly genotype analyzed. Using ImageJ software, densitometry analysis of each experiment was performed in order to obtain profile curves for each sample that was analyzed. Profiles of the relative signal intensity at a given location on the gel were plotted in Microsoft Excel and a moving average (period = 25) calculated.

Fluorescence in situ hybridization (FISH)

The protocol used for poly(A) RNA localization was previously described (Farny et al. 2008). Briefly, cells were allowed to adhere to ECL-coated coverslips in DM for 6 h. Cells were fixed with 4% paraformaldehyde in PBS for 10 min, permeabilized with 100% cold methanol for 10 min and 70% ethanol at 4°C overnight. Cells were hybridized for 1–2 h at 37°C with DIG-Oligo-dT(30) probe (Integrated DNA Technologies) at 0.2 mM in hybridization buffer (25% formamide, 10% dextran sulfate, 0.005% BSA, 1 mg/mL yeast tRNA in 2X SSC). Following washes with 2X SSC, cells were incubated with FITC-conjugated anti-DIG antibody (Molecular Probes) at RT for 1 h and with Hoechst 33325 dye (Sigma) for 5 min. Images were obtained using an Olympus IX81 microscope with a 0.3 NA 100X Zeiss Plan-Neofluor objective. Images were captured using a Hamamatsu digital camera with Slidebook software (version 5.0).

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