The Conserved, Disease-Associated RNA Binding Protein dNab2 Interacts with the Fragile X Protein Ortholog in Drosophila Neurons

Rick S. Bienkowski, Emory University
Ayan Banerjee, Emory University
J. Christopher Rounds, Emory University
Jennifer Rha, Emory University
Omotola F. Omotade, Emory University
Christina Gross, University of Cincinnati
Kevin J. Morris, Emory University
Sara Leung, Emory University
ChangHui Pak, Emory University
Stephanie K. Jones, Emory University

Only first 10 authors above; see publication for full author list.

Journal Title: Cell Reports
Volume: Volume 20, Number 6
Publisher: Elsevier (Cell Press): OAJ | 2017-08-08, Pages 1372-1384
Type of Work: Article | Final Publisher PDF
Publisher DOI: 10.1016/j.celrep.2017.07.038
Permanent URL: https://pid.emory.edu/ark:/25593/s4rjj

Final published version: http://dx.doi.org/10.1016/j.celrep.2017.07.038

Copyright information:
© 2017 The Author(s)
This is an Open Access work distributed under the terms of the Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Accessed November 21, 2018 2:59 PM EST
The Conserved, Disease-Associated RNA Binding Protein dNab2 Interacts with the Fragile X Protein Ortholog in *Drosophila* Neurons

**Highlights**

- dNab2 is the fly ortholog of a human RBP lost in inherited intellectual disability

- A cytoplasmic pool of dNab2 interacts with the fragile X homolog dFMRP

- dNab2 regulates the CamKII mRNA and supports memory with dFMRP

- dFMRP and dNab2 both restrict poly(A) length of neuronal mRNAs

**Authors**

Rick S. Bienkowski, Ayan Banerjee, J. Christopher Rounds, ..., Gary J. Bassell, Anita H. Corbett, Kenneth H. Moberg

**Correspondence**

acorbe2@emory.edu (A.H.C.), kmoberg@emory.edu (K.H.M.)

**In Brief**

*Drosophila* dNab2 is an ortholog of an RNA binding protein lost in an inherited intellectual disability, but its neuronal role is undefined. Bienkowski et al. present evidence that dNab2 interacts with the fly fragile X ortholog (dFMRP), a key regulator of neuronal mRNA translation, and co-regulates neurodevelopment and function with dFMRP.
The Conserved, Disease-Associated RNA Binding Protein dNab2 Interacts with the Fragile X Protein Ortholog in Drosophila Neurons

Rick S. Bienkowski,1,2 Ayan Banerjee,2,3 J. Christopher Rounds,1,2 Jennifer Rha,2 Omotola F. Omotade,1 Christina Gross,6 Kevin J. Morris,2,4 Sara W. Leung,2,3 ChangHui Pak,1,2 Stephanie K. Jones,2,3 Michael R. Santoro,4 Stephen T. Warren,2,4,5 James Q. Zheng,1 Gary J. Bassell,1 Anita H. Corbett,2,3,* and Kenneth H. Moberg1,7,*

1Department of Cell Biology
2Department of Biochemistry
3Department of Biology
4Department of Human Genetics
5Department of Pediatrics
Emory University and Emory University School of Medicine, Atlanta, GA 30322, USA
6Department of Pediatrics, University of Cincinnati, Cincinnati, OH 45229, USA
7Lead Contact
*Correspondence: acorbe2@emory.edu (A.H.C.), kmoberg@emory.edu (K.H.M.)
http://dx.doi.org/10.1016/j.celrep.2017.07.038

SUMMARY

The Drosophila dNab2 protein is an ortholog of human ZC3H14, a poly(A) RNA binding protein required for intellectual function. dNab2 supports memory and axon projection, but its molecular role in neurons is undefined. Here, we present a network of interactions that links dNab2 to cytoplasmic control of neuronal mRNAs in conjunction with the fragile X protein ortholog dFMRP. dNab2 and dfmr1 interact genetically in control of neurodevelopment and olfactory memory, and their encoded proteins co-localize in puncta within neuronal processes. dNab2 regulates CaMKII, but not futsch, implying a selective role in control of dFMRP-bound transcripts. Reciprocally, dFMRP and vertebrate FMRP restrict mRNA poly(A) tail length, similar to dNab2/ZC3H14. Parallel studies of murine hippocampal neurons indicate that ZC3H14 is also a cytoplasmic regulator of neuronal mRNAs. Altogether, these findings suggest that dNab2 represses expression of a subset of dFMRP-target mRNAs, which could underlie brain-specific defects in patients lacking ZC3H14.

INTRODUCTION

RNA binding proteins (RBPs) play important roles in the biogenesis and expression of virtually all types of eukaryotic RNAs, including protein-coding mRNAs (Moore, 2005). Despite these broad roles, mutations in genes that encode RBPs often lead to tissue-specific disease pathology, particularly within the brain and nervous system (Castello et al., 2013). Examples of this link include the fragile X mental retardation protein (FMRP) and the spinal muscular atrophy protein SMN (Edens et al., 2015; Gross et al., 2012). The prevalence of neurological disorders caused by defects in RBPs likely reflects the enhanced role post-transcriptional mechanisms play in translational control within distal neuronal processes.

The ZC3H14 (zinc-finger CysCysCysHis [CCCH]-type 14) gene encodes a ubiquitously expressed RBP that is lost in an inherited form of autosomal, recessive, non-syndromic intellectual disability (Pak et al., 2011). Patients homozygous for nonsense mutations in ZC3H14 have reduced IQ but lack associated dysmorphic features. Loss of the ubiquitously expressed Drosophila ZC3H14 homolog, dNab2, produces defects in adult viability, motor function, and brain morphology that are fully rescued by neuronal dNab2 re-expression and partially rescued by human ZC3H4 expression (Kelly et al., 2014, 2016; Pak et al., 2011). These data reveal an important, and evidently conserved, role for human ZC3H14 and fly dNab2 in neurons.

ZC3H14 and dNab2 are predominantly localized to the nucleus but are members of a conserved protein family whose founding member, S. cerevisiae Nab2, shuttles between the nucleus and the cytoplasm (Green et al., 2002; Leung et al., 2009; Pak et al., 2011). ZC3H14 and dNab2 share a domain structure of an N-terminal PWI (proline/tryptophan/isoleucine)-like domain, a nuclear localization sequence, and five well-conserved C-terminal CCCH-type zinc fingers (ZnFs) (Leung et al., 2009). These ZnF domains bind synthetic polyadenosine RNA probes in vitro (Kelly et al., 2010; Pak et al., 2011), implying that dNab2 and ZC3H14 interact with adenosine-rich tracts in vivo. In support of this hypothesis, ZC3H14 co-localizes with poly(A) mRNA speckles in rodent hippocampal neurons (Pak et al., 2011), and its loss increases bulk poly(A) tail (PAT) length among RNAs in cultured N2a cells (Kelly et al., 2010; Pak et al., 2011), implying that dNab2 and ZC3H14 interact with adenosine-rich tracts in vivo. In support of this hypothesis, ZC3H14 co-localizes with poly(A) mRNA speckles in rodent hippocampal neurons (Pak et al., 2011), and its loss increases bulk poly(A) tail (PAT) length among RNAs in cultured N2a cells (Kelly et al., 2010; Pak et al., 2011), implying that dNab2 and ZC3H14 interact with adenosine-rich tracts in vivo. In support of this hypothesis, ZC3H14 co-localizes with poly(A) mRNA speckles in rodent hippocampal neurons (Pak et al., 2011), and its loss increases bulk poly(A) tail (PAT) length among RNAs in cultured N2a cells (Kelly et al., 2010; Pak et al., 2011), implying that dNab2 and ZC3H14 interact with adenosine-rich tracts in vivo.
nervous system (PNS) and CNS replicates almost all phenotypes resulting from zygotic loss of dNab2, while dNab2 depletion from motor neurons does not (Pak et al., 2011). Moreover, pan-neuron dNab2 depletion impairs short-term memory and disrupts axon projection into the α/β lobes of the mushroom bodies (MBs) (Kelly et al., 2016), twin neuropil structures in the brain required for associative olfactory learning and memory (Heisenberg, 2003). In dNab2 mutants, β axons misproject across the brain midline and α axons show a high frequency of branching defects (Kelly et al., 2016). Selective depletion of dNab2 in Kenyon cells, which give rise to MB α/β axons (Armstrong et al., 1998), is sufficient to phenocopy these dNab2 zygotic defects, and dNab2 re-expression in these cells is sufficient to rescue them (Kelly et al., 2016). However, there is little evidence of how dNab2 regulates bound RNAs and whether this regulation occurs exclusively in the nucleus, as suggested by the nuclear steady-state localization of dNab2, Nab2, and ZC3H14 (Anderson et al., 1993; Leung et al., 2009), or involves a role for dNab2 in cytoplasm.

Here, we describe a genetic screen for dNab2-interacting factors in the Drosophila eye that uncovers physical and functional interactions between dNab2 and the Drosophila ortholog of the FMRP. The FMRP RBP is lost in fragile X syndrome (FXS), the most common genetic cause of intellectual disability (Bassell and Warren, 2008). FMRP undergoes nucleo-cytoplasmic shuttling but is enriched in the cytoplasm at steady state. Cytoplasmic FMRP regulates ~800 polyadenylated neuronal mRNAs, allowing for finely tuned pre- and post-synaptic translation of their encoded proteins (Darnell et al., 2011; Richter et al., 2015). Genetic interactions between dNab2 and the Drosophila FMRP gene (dfmr1) correspond at a molecular level to an RNase-resistant physical association of dNab2 and Drosophila FMRP (dFMRP) proteins in neurons. Within brain neurons, dNab2 and dFMRP co-localize in the soma but are also detected within discrete messenger ribonucleoprotein (mRNP)-like foci distributed along neuronal processes. A corresponding memory defect in dNab2/+; dfmr1/+ trans-heterozygotes indicates that dNab2 may co-regulate a subset of mRNAs bound by dFMRP. dNab2 associates with the dFMRP-regulated mRNA encoding CaMKII (calmodulin-dependent protein kinase-II) and is required for repression of a CaMKII translational reporter in neurons. By contrast, dNab2 does not appear to regulate a second dFMRP-target mRNA encoding Futsch/Map1β, implying that the spectrum of dNab2-regulated mRNAs only partially overlaps with dFMRP. Moreover, we find evidence that dFMRP and FMRP restrict PAR1 length of neuronal mRNAs in a manner similar to dNab2 and ZC3H14. Finally, we show that ZC3H14 is present in hippocampal axons and dendrites, where it is enriched in ribonucleoprotein (RNP) and 80S ribosomal fractions. Altogether, these data represent a significant advance in understanding dNab2/ZC3H14 by defining a role for these disease-associated RBPs in translational control of neuronal mRNAs that, in Drosophila, occurs in conjunction with the dFMRP protein.

RESULTS

dfmr1 Alleles Interact with a dNab2 Transgene in the Eye
To identify factors that interact with dNab2 in neurons, we exploited the finding that dNab2 expression in Drosophila retinal cells (GMR-Gal4, UAS-dNab2, hereafter referred to as GMR-dNab2) produces a rough-eye phenotype that is readily modified (Figure 1A) (Pak et al., 2011). GMR>dNab2 eyes (dNab2 overexpression [o/e] in Figure 1B) are reduced in size, lack full pigmentation, and have disorganized ommatidia, presumably due to effects of excess dNab2 on endogenous retinal RNAs. A selected group of 200 alleles (loss of function, RNAi depletion, or EP-type overexpression), corresponding to 135 genes that encode factors with (1) established roles in neurodevelopment or neuronal function, (2) RNA binding activity, or (3) roles in mushroom body (MB) development, were evaluated for modification of the GMR-dNab2 phenotype. This approach identified 15 enhancers corresponding to 10 genes and 28 suppressors corresponding to 16 genes (Figure 1A; Table S1). Among the modifiers are alleles of the previously defined dNab2-interacting genes poly(A) binding protein 2 (PABP2) and hiiragi (poly(A) polymerase) (Pak et al., 2011), in addition to previously undefined interactors like the FXS mental retardation ortholog (dfmr1), cytoplasmic poly(A) binding protein (PABPC1), and the elongation factor-1α (EF-1α), and elf-4e translation factors.

Multiple GMR-dNab2 modifier alleles correspond to factors that act within a translational pathway centered on dfmr1 (in bold in Table S1). The dfmr1<sup>450</sup> and dfmr1<sup>4173</sup> loss-of-function alleles each dominantly suppress GMR-dNab2, as does co-expression of a dfmr1 RNAi transgene, indicating that dFMRP is required for excess dNab2 to disrupt eye morphology (Figure 1B). Moreover, UAS-dNab2 and UAS-dfmr1 transgenes are individually viable but synthetically lethal when co-expressed with GMR-Gal4 in retinal neurons. The basis for this synthetic effect could be the enhancement of dfmr1-induced apoptosis reported in earlier studies (Wan et al., 2003). dfmr1-interacting genes also modify the GMR-dNab2 phenotype (Table S1), including the microRNA (miRNA) components Ago1 and Gw182, the Rm62/dmp68 RNA helicase, the RBPs staufen and Ataxin-2, and Timp, a protease inhibitor implicated in synaptic FXS overgrowth in mice and flies (Barbee et al., 2006; Cziko et al., 2009; Jin et al., 2004; Siller and Broadie, 2011; Sudhakaran et al., 2014). This pattern of genetic links suggests that dNab2 may interact with the dFMRP pathway in retinal neurons.

dfmr1 Alleles Modify Locomotor and Mushroom Body Phenotypes Caused by dNab2 Loss
Interactions between dNab2 and dfmr1 alleles were examined in two additional neuronal contexts: locomotor behavior and MB development. Pan-neuronal RNAi of dNab2 (elavC155> dNab2<sub>RNAi</sub>) causes a locomotor defect in a negative geotaxis assay (Pak et al., 2011) that is dominantly enhanced by the dfmr1<sup>4173</sup> null allele (Figure 1C), which is consistent with its suppressive effect on gain-of-function GMR-dNab2. This dNab2<sub>RNAi</sub> locomotor defect is enhanced by overexpression of dfmr1, indicating that dFMRP and dNab2 are not redundant in this context (Figure S1). Endogenous dNab2 and dFMRP are both expressed within Kenyon neurons whose axons branch to form the MB lobes (Bosseie et al., 1992; Kelly et al., 2016; Michel et al., 2004). Null alleles of dNab2 and dfmr1 (dNab2<sup>exc</sup> and dfmr1<sup>450</sup>) elicit similar MB defects, including missing or thinned α lobes, that occur with similar severity and penetrance (Figures 1D and 1E) (Kelly et al., 2016).
and are reciprocally sensitive to the genetic dose of the other factor: \( d\text{Nab2}^\text{ex3} \) and the weaker \( d\text{Nab2}^\text{D113} \) allele (Michel et al., 2004) dominantly increase the frequency of \( \alpha \) lobe defects in \( d\text{Nab2}^\text{ex3} \) mutants, while \( d\text{Nab2}^\text{ex3} \) dominantly rescues \( \alpha \) lobe defects in \( d\text{FMR1}^\text{D50} \) and \( d\text{FMR1}^\text{D113} \) mutants (Figures 1D, 1E, and S2). These opposing effects imply that \( d\text{Nab2} \) is required for normal \( \alpha \) lobe development but supports aberrant \( \alpha \) lobe development in the absence of \( d\text{FMRP} \). This dependence on \( d\text{FMRP} \) status could reflect linked or sequential roles for \( d\text{Nab2} \) and \( d\text{FMRP} \) on a shared cohort of RNAs. The lack of genetic interactions between \( d\text{Nab2} \) and \( d\text{FMR1} \) in \( \beta \) lobe axons (Figures 1F and S2) could indicate that \( d\text{Nab2} \)-\( d\text{FMRP} \) co-regulate \( \alpha \) lobe-specific RNAs. Expression of a \( U\text{AS-dFMR1} \) transgene in \( d\text{Nab2}^\text{ex3} \) neurons severely disrupts MBs (Figure S2), again arguing that \( d\text{FMRP} \) and \( d\text{Nab2} \) may interact functionally but are not redundant.

**dNab2 Co-localizes with dFMRP in Neurites**

The genetic links between \( d\text{Nab2} \) and \( d\text{FMR1} \) suggest that their encoded proteins might associate within neurons. At steady state, \( d\text{Nab2} \) localizes to nuclei (Kelly et al., 2012; Pak et al., 2011), while \( d\text{FMRP} \) is cytoplasmic (Santos et al., 2014). However, homologs of both proteins undergo nucleocytoplasmic shuttling in association with bound RNAs (Feng et al., 1997; Michel et al., 2004) and are reciprocally sensitive to the genetic dose of the other factor: \( d\text{FMR1}^\text{D50} \) and the weaker \( d\text{FMR1}^\text{D113} \) allele (Michel et al., 2004) dominantly increase the frequency of \( \alpha \) lobe defects in \( d\text{Nab2}^\text{ex3} \) mutants, while \( d\text{Nab2}^\text{ex3} \) dominantly rescues \( \alpha \) lobe defects in \( d\text{FMR1}^\text{D50} \) and \( d\text{FMR1}^\text{D113} \) mutants (Figures 1D, 1E, and S2). These opposing effects imply that \( d\text{Nab2} \) is required for normal \( \alpha \) lobe development but supports aberrant \( \alpha \) lobe development in the absence of \( d\text{FMRP} \). This dependence on \( d\text{FMRP} \) status could reflect linked or sequential roles for \( d\text{Nab2} \) and \( d\text{FMRP} \) on a shared cohort of RNAs. The lack of genetic interactions between \( d\text{Nab2} \) and \( d\text{FMR1} \) in \( \beta \) lobe axons (Figures 1F and S2) could indicate that \( d\text{Nab2} \)-\( d\text{FMRP} \) co-regulate \( \alpha \) lobe-specific RNAs. Expression of a \( U\text{AS-dFMR1} \) transgene in \( d\text{Nab2}^\text{ex3} \) neurons severely disrupts MBs (Figure S2), again arguing that \( d\text{FMRP} \) and \( d\text{Nab2} \) may interact functionally but are not redundant.

**dNab2 Co-localizes with dFMRP in Neurites**

The genetic links between \( d\text{Nab2} \) and \( d\text{FMRP} \) suggest that their encoded proteins might associate within neurons. At steady state, \( d\text{Nab2} \) localizes to nuclei (Kelly et al., 2012; Pak et al., 2011), while \( d\text{FMRP} \) is cytoplasmic (Santos et al., 2014). However, homologs of both proteins undergo nucleocytoplasmic shuttling in association with bound RNAs (Feng et al., 1997; Michel et al., 2004) and are reciprocally sensitive to the genetic dose of the other factor: \( d\text{FMR1}^\text{D50} \) and the weaker \( d\text{FMR1}^\text{D113} \) allele (Michel et al., 2004) dominantly increase the frequency of \( \alpha \) lobe defects in \( d\text{Nab2}^\text{ex3} \) mutants, while \( d\text{Nab2}^\text{ex3} \) dominantly rescues \( \alpha \) lobe defects in \( d\text{FMR1}^\text{D50} \) and \( d\text{FMR1}^\text{D113} \) mutants (Figures 1D, 1E, and S2). These opposing effects imply that \( d\text{Nab2} \) is required for normal \( \alpha \) lobe development but supports aberrant \( \alpha \) lobe development in the absence of \( d\text{FMRP} \). This dependence on \( d\text{FMRP} \) status could reflect linked or sequential roles for \( d\text{Nab2} \) and \( d\text{FMRP} \) on a shared cohort of RNAs. The lack of genetic interactions between \( d\text{Nab2} \) and \( d\text{FMR1} \) in \( \beta \) lobe axons (Figures 1F and S2) could indicate that \( d\text{Nab2} \)-\( d\text{FMRP} \) co-regulate \( \alpha \) lobe-specific RNAs. Expression of a \( U\text{AS-dFMR1} \) transgene in \( d\text{Nab2}^\text{ex3} \) neurons severely disrupts MBs (Figure S2), again arguing that \( d\text{FMRP} \) and \( d\text{Nab2} \) may interact functionally but are not redundant.

**dNab2 Co-localizes with dFMRP in Neurites**

The genetic links between \( d\text{Nab2} \) and \( d\text{FMRP} \) suggest that their encoded proteins might associate within neurons. At steady state, \( d\text{Nab2} \) localizes to nuclei (Kelly et al., 2012; Pak et al., 2011), while \( d\text{FMRP} \) is cytoplasmic (Santos et al., 2014). However, homologs of both proteins undergo nucleocytoplasmic shuttling in association with bound RNAs (Feng et al., 1997; Michel et al., 2004).

---

**Figure 1. Genetic Interactions between dNab2 and dfmr1**

(A) Schematic of the GMR-dNab2 screen. GMR-Gal4 overexpression (o/e) of dNab2 from the dNab2<sup>EP3716</sup> allele leads to a rough-eye phenotype that was enhanced by 14 and suppressed by 30 of the 200 candidate alleles.

(B) Adult eyes from control (GMR-Gal4/+), dNab2 o/e (GMR-Gal4/+;dNab2<sup>EP3716</sup>/+), dNab2 o/e+dfmr1 heterozygote (GMR-Gal4/+;dfmr1<sup>D50</sup>/dNab2<sup>EP3716</sup> and GMR-Gal4/+;dfmr1<sup>D113</sup>/dNab2<sup>EP3716</sup>), dNab2 o/e+dfmr1 RNAi (GMR-Gal4/+;UAS-dfmr1<sup>RNAi</sup>/dNab2<sup>EP3716</sup>), and dfmr1 heterozygote (GMR-Gal4/+;dfmr1<sup>D50</sup>+) adult females.

(C) Negative geotaxis behavior of 5-day-old control (elav<sup>C155</sup>-Gal4), pan-neuron dNab2 RNAi (elav<sup>C155</sup>-Gal4,UAS-dNab2<sup>RNAi</sup>), pan-neuron dNab2 RNAi+dfmr1 heterozygote (elav<sup>C155</sup>-Gal4,UAS-dNab2<sup>RNAi</sup>,dfmr1<sup>D113M</sup>/+), or dfmr1 heterozygote (elav<sup>C155</sup>-Gal4,dfmr1<sup>D113</sup>+/+) flies. Data represent the percentage of flies reaching the cylinder top at each time point. Each genotype represents ≥ 10 independent trials (10 flies/trial). Error bars, SD.

(D) Anti-Fas2 stained wild-type (WT; isogenic precise excision pex41 of the element used to create dNab2<sup>ex3</sup>), dNab2 null (dNab2<sup>ex3/ex3</sup>), dfmr1 null (dfmr1<sup>D50/D50</sup>), dNab2 null with one copy of dfmr1 (dNab2<sup>ex3</sup>,dfmr1<sup>D50</sup>/dNab2<sup>ex3</sup>), dfmr1 null lacking one copy of dNab2 (dNab2<sup>ex3</sup>,dfmr1<sup>D50</sup>+/dNab2<sup>ex3</sup>), or trans-heterozygote (dNab2<sup>ex3</sup>,dfmr1<sup>D50</sup>+/+) brains.

(E and F) Penetrance of (E) \( \alpha \) lobe or (F) \( \beta \) lobe defects in the same genotypes as (C), with individual lobes counted as discrete events (≥ 24 brains per genotype).

\*p = 4.8 \times 10^{-1} and **p = 1.5 \times 10^{-5} (chi-square test).
Green et al., 2002; Kim et al., 2009). As previously reported (Pak et al., 2011), dNab2 is enriched in neuronal nuclei of 3-day-old cultured adult brain neurons co-stained with anti-dNab2 antibody and anti-horseradish peroxidase (anti-HRP) to visualize neuronal membranes (three examples in Figures 2A–2C). However, two-thirds of neurons also contain a punctate pool of dNab2 distributed into the cytoplasm of neuronal processes (Figures 2A–2C, right panels are magnified views of single processes) that is absent in dNab2 null neurons (Figure 2D). Approximately 80% of cultured, CD8:GFP-labeled Kenyon cells (OK107-Gal4, UAS-CD8:GFP) also contain dNab2 puncta in processes (Figure 2E). The absence of cytoplasmic dNab2 in some Kenyon cells could reflect lobe-specific differences (e.g., α, β, γ lobe) or developmental age (e.g., early- versus late-born neurons) (Kunz et al., 2012). In aggregate, these data reveal that dNab2 localizes to the nuclei and distal processes of neurons.

Given the genetic interactions between dNab2 and dfmr1, antibodies to these two RBPs were used to assess their co-localization in the cytoplasm of cultured brain neurons. As described previously, dFMRP is detected at low levels in the nucleus, at higher levels in the cell body cytoplasm, and in puncta that distribute along the length of processes (Figures 2F–2H) (Barbee et al., 2006; Cziko et al., 2009; Feng et al., 1997; Wan et al., 2000). These puncta resemble reported dFMRP-containing mRNPs that contain other RNA processing factors such as PABC (Cziko et al., 2009), which is a genetic modifier of GMR>dNab2 (Table S1). Significantly, dNab2 co-localizes with dFMRP puncta in the cell body (yellow arrows in Figure 2G and corresponding magnified cell body views in Figure 2H) and in neuronal processes (boxed regions in Figure 2G and corresponding magnified neuronal process views in Figure 2H). Quantification of this overlap within processes indicates that ~20% of dNab2 overlaps with dFMRP-positive puncta, while ~25% of dFMRP overlaps with dNab2-positive puncta (by Manders overlap co-efficient, n = 12 processes). These data suggest that dNab2 is a component of some dFMRP granules in brain neurons and provide a potential molecular context for the observed genetic interactions between dNab2 and dfmr1.

### The dNab2 and dFMRP Proteins Associate and Support Olfactory Memory

An adapted version of the RNA-tagging technique (Yang et al., 2005) was used to assess physical interaction of dNab2 and dFMRP in brain neurons. Head lysates of flies expressing either FLAG-dNab2 or FLAG-hPABP (human poly(A) RNA binding protein) in neurons (elavC155>UAS-FLAG-Nab2 or FLAG-hPABP) were precipitated with anti-FLAG (Figure 3A), and then probed to detect recovery of FLAG-dNab2 or FLAG-hPABP (anti-FLAG panel), or with anti-dFMRP (6A15) (Morales et al., 2002) to detect co-purifying endogenous dFMRP (anti-dFMRP panel). dFMRP is detected in FLAG-dNab2 precipitates but not in FLAG-hPABP or control (elavC155>UAS-FLAG-Nab2 or FLAG-hPABP) precipitates. Addition of RNase does not block recovery of dFMRP with FLAG-dNab2, indicating that this association is RNase resistant (Figure S3).

To confirm the dNab2-dFMRP association and biochemically test its localization, flies expressing neuronal FLAG-dNab2 were
separated into nuclear (Nuc) and cytoplasmic (Cyto) fractions and then subject to immunoprecipitation (IP) for endogenous dFMRP (Figure 3C). Fractionation was confirmed with lamin-D (nucleus) and β-tubulin (cytoplasm) antibodies. Although dNab2 and dFMRP show inverse patterns of enrichment in the nucleus and cytoplasm, dNab2 is recovered in association with dFMRP from both compartments (Figure 3C). These biochemical data support the microscopy data in Figures 2 A–2C and provide additional evidence that dNab2 physically associates with dFMRP in multiple neuronal compartments.

\[ \text{dNab2 is required for courtship conditioning (Kelly et al., 2016), suggesting that it may regulate memory in conjunction with dFMRP.} \]

\[ \text{We therefore used an aversive olfactory conditioning assay (Figure 3D) to test whether heterozygosity for dNab2 could sensitize memory circuits to loss of a single copy of dfmr1 (i.e., trans-heterozygotes). Control adult flies (Figures 3E and 3F, white bars) display a strong positive response to light (phototaxis) when these stimuli are tested individually but impaired MCH-induced suppression of phototaxis relative to control wild-type animals (white bars) or those carrying only the dNab2\textsuperscript{ex3} allele (light gray bar) or dfmr1\textsuperscript{150} (gray bars) allele. The memory defect in dNab2\textsuperscript{ex3}, dfmr1\textsuperscript{150} trans-heterozygotes is enhanced relative to the mild defect in dfmr1\textsuperscript{D50} heterozygotes (Figure 3F) (Cziko et al., 2009; Sudhakaran et al., 2014), indicating that reduced dFMRP renders olfactory memory pathways sensitive to dNab2 dosage. A hypomorphic allele of dnc (dnc\textsuperscript{1}), which encodes a cyclic AMP phosphodiesterase required for memory (Tully and Quinn, 1985), also shows a memory defect in phototaxis suppression (Figures 3E and 3F, black bars), confirming the utility of the assay.} \]
dNab2 thus appears to show in vivo specificity in its association with polyadenylated transcripts. The evidence of physical association of dNab2 with CamKII was complemented by analysis of an in vivo reporter that detects regulatory inputs into 3’ sequences of the CamKII mRNA. This reporter contains the CamKII-3’ UTR fused to a Gal4-inducible eYFP coding sequence and is sensitive to dFMRP-mediated repression in antennal lobe projection neurons (ALPNs) (Ashraf et al., 2006; Sudhakaran et al., 2014). Expression of the CamKII reporter (GH146-Gal4>UAS-eYFP:CamKII-3’ UTR) in ALPNs leads to enhanced yellow fluorescent protein (eYFP) fluorescence in the cell bodies and dendrites (Figure 4B). As described previously (Sudhakaran et al., 2014), dFMRP RNAi increases eYFP fluorescence in ALPNs approximately 2-fold, while RNAi of the NMDA receptor (NR1) has no effect (Figures 4C and 4D). RNAi of dNab2 in ALPNs elevates eYFP expression to a similar extent as dfmr1 RNAi but has no effect on an unrelated reporter composed of EGFP fused to SV40-3’ UTR. qPCR confirms that the effects of dNab2 and dFMRP RNAi on eYFP fluorescence occur without a substantial effect on steady-state levels of the hybrid eYFP:CamKII-3’ UTR mRNA (Figure 4E). dNab2 overexpression does not suppress the effect of dfmr1 RNAi on eYFP:CamKII-3’ UTR expression (Figure S4), indicating that dNab2 is not redundant to dFMRP in translational effects mediated though the CamKII-3’ UTR.

**dNab2 Plays a Minor Role in futsch Regulation**

The interaction between dNab2 and CamKII mRNA prompted analysis of futsch, a second dFMRP-target mRNA. Futsch is an ortholog of the microtubule-associated protein-1β (Map1β) and its mRNA is a conserved target of dFMRP and FMRP (Hummel et al., 2000; Lu et al., 2004; Zhang et al., 2001). Excess Futsch promotes synaptic growth at the larval neuromuscular junction (NMJ) of dfmr1 mutant larvae (Roos et al., 2000; Zhang et al., 2001). dNab2 alleles have no effect on NMJ growth (Pak et al., 2011), suggesting that dNab2 may not regulate Futsch in vivo. Consistent with this hypothesis, the levels of Futsch protein are unaltered in dNab2 null brain neurons as assessed by anti-Futsch staining intensity (Figures 5A–5C), a technique used previously to assess the dFMRP regulation of Futsch at NMJs (Coyne et al., 2015). Futsch mRNA is also not significantly enriched in immunoprecipitates of neuronal FLAG-dNab2 relative to control brains (Figure S5), suggesting that futsch mRNA does not associate with dNab2 in brains. Consistent with its role as a repressor of Futsch translation (Zhang et al., 2001), dFMRP expression reduces Futsch levels in cultured brain neurons relative to controls (Figures 5A and 5B), especially in shafts of major neuronal processes (yellow arrows). dNab2 loss suppresses this effect without affecting dfmr1 transgene expression (Figures 5A–5D), arguing that dNab2 may be ectopically recruited to regulate futsch when dFMRP is overexpressed. However, the lack of effect of dNab2 alleles on Futsch levels argues that dNab2 is not normally required to repress futsch mRNA in neurons.

dFMRP and FMRP Restrict PAT Length

The differential requirement for dNab2 and dFMRP in Futsch regulation prompted analysis of dNab2 or dFMRP loss on futsch PAT length using the extended poly(A) tail (ePAT) assay (Figure 6A). Consistent with the observation that dNab2 loss does not elevate Futsch protein levels in individual neurons, loss of dNab2 had no effect on futsch PAT length relative to controls. Thus, futsch is an mRNA whose PAT length is regulated independently of dNab2. By contrast, futsch PAT length is extended in dfmr1 mutant heads (Figure 6A, gel lane 3 and graph). Given the large number of FMRP/dFMRP mRNA targets, we next...
tested the effect of dFMRP/FMRP loss on bulk PAT length in adult Drosophila heads and cultured mouse N2a neuroblastoma cells. dfmr1 mutant adult heads and FMRP-depleted N2a cells both show elongated PAT lengths to a degree that mirrors or exceeds the effect of dNab2/ZC3H14 loss (Figures 6B and 6C). These data indicate that the role of dFMRP in control of futsch expression is paralleled by a role in limiting futsch PAT length in vivo that is not shared by dNab2 and that dFMRP/FMRP appears to be required to restrict bulk PAT length in neurons.

ZC3H14 Localizes to Axons and Dendrites and Associates with RNPs

The finding that dNab2 localizes to neurons prompted analysis of the subcellular distribution of ZC3H14 in cultured hippocampal neurons. An anti-ZC3H14 antibody (Leung et al., 2009) detects ZC3H14 not only in hippocampal nuclei (as described in Pak et al., 2011) (Figures 7A and 7B) but also in cytoplasmic processes of differentiated hippocampal neurons after either 5 or 21 days in vitro (DIV) culture. In 5 DIV neurons, cytoplasmic ZC3H14 is enriched in Tau-positive axons relative to Map2-positive dendrites (Figure 7A). At 21 DIV, ZC3H14 is distributed into dendrites and postsynaptic density-95 (PSD95)-positive dendritic spines with well-elaborated dendritic arbors (Figure 7B, arrowheads). ZC3H14 is also detected in the cytoplasmic fraction of murine brains (Figure 7C, doublet). Recovery of THOC1, a nuclear RBP (Li et al., 2005), in the nuclear fraction confirms that biochemical evidence of cytoplasmic ZC3H14 is not a non-specific pattern common to all RBPs. Anti-ZC3H14 specificity was confirmed with lysates generated from ZC3H14(ex13/ex13) knockout mouse brains (Rha et al., 2017).

The pool of ZC3H14 protein that distributes into distal hippocampal processes is likely to be part of larger mRNP complexes that modulate mRNA processing and translation (Donlin-Asp et al., 2017). This hypothesis was tested by linear sucrose density gradient fractionation of cytoplasmic post-natal day (P) 13 mouse brain lysates generated in the presence or absence of the Ca^{2+} chelator EDTA, which disrupts mRNP complexes, including mono- and polyribosomes (Figure 7D) (Stefani et al., 2004). In untreated Cyto brain lysates, ZC3H14 co-sediments into multiple fractions across the sucrose density gradient, showing enrichment in fractions that contain 80S monoribosomes (Figure 7D, left panel). Addition of EDTA results in a dramatic shift of ZC3H14 into lighter fractions and disruption of RNP complexes, as indicated by the loss of polyribosome peaks in the RNA absorption profile and a shift of the ribosomal S6 protein, a component of the 40S subunit (Figure 7D, right panel) (Roux et al., 2007). A parallel analysis of cytoplasmic lysates generated from cultured cells confirms the effect of EDTA on ZC3H14-containing complexes and the P0 protein, a subunit of the 60S ribosomal subunit (Figure S6). Addition of puromycin, which disrupts translating ribosomes (Franklin and Godfrey, 1966), also depletes a fraction of ZC3H14 that co-sediments with polyribosomes (Figure S6, asterisks, lanes 6–9). In aggregate, these data indicate that endogenous ZC3H14 localizes to nuclei, cell bodies, and distal neuronal compartments, including pre-synaptic axons and post-synaptic dendrites and spines, where it is principally found in RNPs and 80S ribosomal complexes with likely roles in regulating RNA translation.

DISCUSSION

Here, we report the results of a candidate-based screen for factors that interact genetically with the Drosophila dNab2 gene, which encodes an RBP whose human ortholog is lost in an inherited intellectual disability. Identified interacting genes include components of the translation machinery (PAB1, EF-1α, and
eIF-4e and elements of a pathway centered on the Drosophila ortholog of the FMRP translational repressor (dfmr1 itself, Argonaute-1, Gw182, Rm62, staufen, and Ataxin-2), suggesting that dNab2 functions within the dFMRP pathway. Additional genetic tests support this hypothesis. dfmr1 alleles suppress a rough-eye phenotype caused by transgenic expression of dNab2 in retinal neurons, while dfmr1 alleles enhance a locomotor defect caused by neuronal RNAi of dNab2. Genetic interactions also occur in the CNS, where dfmr1 heterozygosity enhances the frequency of MB α lobe defects in dNab2 mutants. dNab2 heterozygosity suppresses MB α lobe defects in dfmr1 mutants, implying a functional hierarchy in which dNab2 effects are dependent on dFMRP status. The inability of either RBP to rescue phenotypes caused by loss of the other argues for a model in which dNab2 and dFMRP participate in a common mechanism or mechanisms but are not functionally redundant.

Genetic interactions between the dNab2 and the dfmr1 genes are paralleled by a dNab2:dFMRP protein complex detected in neurons. This dNab2:dFMRP interaction, which could involve other factors, includes a cytoplasmic pool of dNab2 that partially co-localizes with dFMRP in mRNP-like granules in neuronal processes, suggesting that the two RBPs may associate with some of the same RNAs. dNab2 can interact with and regulate the CaMKII mRNA, a dFMRP target, but is not required to regulate futsch, a second dFMRP target. The finding that trans-heterozygosity for dNab2 and dfmr1 impairs olfactory memory provides additional evidence that dNab2:dFMRP co-regulate some neuronal mRNAs. Finally, we find that murine ZC3H14 is present in axons and dendrites of murine hippocampal neurons and associates with mRNPs and elements of the translational machinery. FMRP also localizes to dendrites and axons and regulates filopodial dynamics and motility of axonal growth cones (e.g., Antar et al., 2006). In aggregate, these data significantly advance our understanding of the role of dNab2/ZC3H14 proteins in neurons by defining a cytoplasmic pool of these proteins associated with translational control of mRNAs that, in Drosophila, occurs in conjunction with dFMRP.

This study highlights the dNab2:dFMRP association but also suggests that dNab2 can function independently of dFMRP. For example, dNab2 and dFMRP are each required for MB αβ lobe structure (Kelly et al., 2016; Michel et al., 2004), yet dosage-sensitive interactions between dNab2 and dfmr1 alleles are only evident in α lobes, suggesting that dNab2 and dFMRP may co-regulate RNAs within specific axon branches. In addition, dNab2 selectively regulates CaMKII, but not futsch, and that asymmetry is reflected at the level of the futsch PAT, which

---

**Figure 6. Effect of dFMRP/FMRP Loss on PAT Length**

(A) Schematic of extended poly(A) tail (ePAT) length assay using linker PCR amplification of futsch PAT and the TvN control fragment (12 adenosines, with

---

the size given as adenosine [A] 12) from control, dNab2 null (dNab2ex3), or dfmr1 null (dfmr1D50/D50) heads. Size standards are indicated (A200/A300). The right panel shows the densitometry trace of the PCR products.

(B) Bulk PAT length among total RNAs harvested from adult heads of the same genotypes in (A). Short and long exposures (with size “ladder”) are shown, along with densitometry traces of each lane normalized for band intensity.

(C) Bulk PAT length in N2a cells treated with ZC3H14, Fmr1, or scramble small interfering RNAs (siRNAs) and accompanying densitometry trace. Sizes are indicated. The boxed region highlights elongated PATs in the ~A400 size range in ZC3H14 and Fmr1 siRNA cells. Western confirmation of siRNA knockdown is shown.
is unchanged in dNab2 mutant brains but extended in dfmr1 mutant brains. The failure of dNab2 alleles to alter Futsch protein levels is consistent with their lack of effect on the Futsch-dependent process of NMJ development (Pak et al., 2011). Altogether, these data suggest that the futsch mRNA is not a physiological target of dNab2 and that dNab2 only regulates a subset of dFMRP-bound transcripts.

dFMRP protein is a well-established translational repressor, but the data reveal a previously unappreciated requirement for dFMRP/FMRP to inhibit mRNA PAT length, which in the case of futsch, is likely to stem from direct binding by dFMRP. This effect on PAT length could simply be a secondary consequence of enhanced futsch translation in dfmr1/Fmr1 mutant cells. However, loss of the cytoplasmic polyadenylation element binding protein (CPEB), which promotes cytoplasmic PAT extension in mammals and flies (Cziko et al., 2009; Keleman et al., 2007; Mastushita-Sakai et al., 2010; Udagawa et al., 2012), rescues FXS phenotypes in Fmr1 knockout mice (Udagawa et al., 2013). One interpretation of this result is that inappropriate PAT elongation contributes to excess translation in Fxs, similar to the positive correlation between PAT length and translation observed among germline and embryonic mRNAs (Eichhorn et al., 2016; Subtelny et al., 2014). These data thus raise the possibility that altered mRNA polyadenylation may be an unappreciated feature of translational dysregulation in neurons lacking dfmr1/Fmr1.

The dNab2:dFMRP complex suggests that dNab2 may regulate gene expression through its interaction with dFMRP. FMRP inhibits translational initiation (Napoli et al., 2008; Schenck et al., 2001, 2003), blocks ribosome movement along polyribosome-associated mRNAs (Darnell and Klann, 2013), and interacts with elements of the miRNA machinery (Sozzetti et al., 2015; Caudy et al., 2002; Ishizuka et al., 2002; Muddashetty et al., 2011). The dNab2-sensitive CaMKII-3’UTR GFP sensor is also regulated by the miRNA pathway (Ashraf et al., 2006; Sudhakaran et al., 2014), and multiple factors involved in miRNA-induced silencing interact genetically with dNab2 (Table S1). The precise role dNab2 plays on bound mRNAs is not clear. PAT elongation induced by dNab2 loss could enhance recruitment of cytoplasmic PABPs that promote translation-coupled circularization of mRNAs (Preiss and Hentze, 1999). dNab2 and its ortholog ZC3H14 both repress PAT length and may thus indirectly limit the binding of cytoplasmic PABPs to key transcripts. Alternatively, they may directly compete with these PABPs for binding to polyadenosine tails and thus occlude access of other factors involved in translation.

Consistent with the role of dNab2 in translational regulation, its ortholog ZC3H14 localizes to axons, dendrites, and dendritic spines in hippocampal neurons and co-sediments with 80S ribosomes. FMRP is primarily associated with polysomes and can inhibit translation by ribosome stalling (e.g., Darnell et al., 2011). The FMRP-target CamKII mRNA is enriched in anti-ZC3H14 precipitates, and CamKII levels increase in the hippocampus of Zc3h14−/− brains compared to control mice (Rha et al., 2017), raising the possibility that Drosophila and vertebrate CaMKI mRNAs are conserved targets of dNab2/ZC3H14. The FMRP-related protein Fxr1 (Morales et al., 2002; Stackpole et al., 2014) co-precipitates with the zinc-finger domain of ZC3H14 (Hu and Gao, 2014), suggesting that ZC3H14 may interact with FMRP family members in a manner analogous to dNab2 and dFMRP.

Altogether, the data presented here provide evidence that dNab2 localizes to both the nucleus and the cytoplasm of...
**Drosophila neuronal processes and that it interacts physically and functionally with the dFMRP protein. Additional data provide evidence of an equivalent pool of cytoplasmic ZC3H14 that interacts with RNP complexes found in the axons and dendrites in the mouse brain. Given the link between FMRP and intellectual disability in humans (Santoro et al., 2012), these interactions raise the possibility that defects in translational silencing of mRNAs transported to distal sites within neuronal processes contribute to neurodevelopmental and cognitive defects in Drosophila lacking dNab2 or in humans lacking ZC3H14.

**EXPERIMENTAL PROCEDURES**

**Drosophila Genetics**

Crosses were maintained in 25 °C humidified incubators with 12 hr light-dark cycles. The ex3, pex41 (precise excision 41), and UAS-FLAG-dNab2 alleles have been described previously (Pak et al., 2011). Modifier stocks are identified by source or stock in Table S1. Drivers: GMR (BL1350), elav<sup>T155</sup> (BL458), OK107 (BL854), and D1H146 (BL30026). Alleles: dNab2<sup>2Egpt771</sup> (UAS-dNab2, BL17159), dfmr1<sup>a0</sup> (BL6930), dfmr1<sup>1213M</sup> (BL6929), dc<sup>r</sup> (BL6020), UAS-NR1IR<sup>W+</sup>, dnc1<sup>UAS-dfmr1</sup> (gift of T. Jongens), Krashes and Waddell, 2011). Males were outcrossed to Oregon-R w+, dnc1<sup>UAS-NR1IR</sup> (VDRC 27487), w++, dnc1<sup>UAS-dfmr1IR</sup> (BL458), w++, dnc1<sup>UAS-dfmr1IR</sup> (BL25941), w++, dnc1<sup>UAS-CD8-GFP</sup> (BL35200), Pabp2<sup>EP2264</sup>, Pabp2<sup>EP2264</sup>, dNab2<sup>EP3716</sup>, elav<sup>C155</sup> (gift of M. Simonelig), Pabp2<sup>EP2264</sup>, dNab2<sup>EP3716</sup>, elav<sup>C155</sup> (gift of M. Simonelig), dNab2<sup>EP3716</sup> (BL6020), and Pabp2<sup>EP2264</sup>, dNab2<sup>EP3716</sup> (BL6930).

**Behavioral Assays**

Aversive olfactory conditioning was performed essentially as described (Pak et al., 2011). Male flies were aged overnight (o/n) in fresh vials and then tested for light:dark preference in a fresh T-maze for 1 min. Performance indices (PIs = R<sup>C</sup>/R<sup>C</sup> + R<sup>C</sup>/R<sup>C</sup>) were calculated for each trial (≥4 trials per condition). The ePAT assay was performed exactly as described (Chartier et al., 2017). Bulk PAT length analysis was performed as described (Apponi et al., 2010).

**Western Blotting**

Samples were run on 5% SDS-PAGE gels, transferred to polyvinylidene fluoride (PVDF) membrane (Bio-Rad), blocked, and then probed with antibodies: anti-FLAG-M2 (Sigma) at 1:1,000, anti-dFMR1 mAb 6A15 at 1:1,500, anti-lamin (DSHB) at 1:2,000, anti-histone H3 at 1:100, and anti-THOC1 at 1:100.

**Hippocampal Culture and Imaging**

Neuronal isolation and culture were performed as described (Kaesche and Banker, 2000). P1 hippocampi were dissociated, dissociated, and plated on poly-D-lysine-treated coverslips (EMD Millipore) in neurobasal medium with B-27 and GlutaMAX (Invitrogen). Neurons were fixed with 4% paraformaldehyde, washed, permeabilized with 0.2% Triton X-100, and then blocked with 4% BSA, 1% normal goat serum (NGS), and 0.1% Triton X-100. Antibodies were anti-ZC3H14 (1:500; Leung et al., 2009), Map2 (1:500; Sigma, M1406), and Tau (Chemicon, MAB3420). Anti-rabbit or anti-mouse Alexa 488/546 antibodies were used as secondary antibodies. Cells were imaged between 4 and 6 or 20 and 22 DIV using a Nikon TIE inverted microscope.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, six figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2017.07.038.


