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Zfrp8 forms a complex with Fragile-X Mental Retardation Protein and regulates its localization and function

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Abstract

Fragile-X syndrome is the most commonly inherited cause of autism and mental disabilities. The *Fmr1* (*Fragile-X Mental Retardation 1*) gene is essential in humans and *Drosophila* for the maintenance of neural stem cells, and *Fmr1* loss results in neurological and reproductive developmental defects in humans and flies. FMRP (Fragile-X Mental Retardation Protein) is a nucleo-cytoplasmic shuttling protein, involved in mRNA silencing and translational repression. Both *Zfrp8* and *Fmr1* have essential functions in the *Drosophila* ovary. In this study, we identified FMRP, Nufip (Nuclear Fragile-X Mental Retardation Protein-interacting Protein) and Tral (Trailer Hitch) as components of a Zfrp8 protein complex. We show that *Zfrp8* is required in the nucleus, and controls localization of FMRP in the cytoplasm. In addition, we demonstrate that *Zfrp8* genetically interacts with *Fmr1* and *tral* in an antagonistic manner. Zfrp8 and FMRP both control heterochromatin packaging, also in opposite ways. We propose that Zfrp8 functions as a chaperone, controlling protein complexes involved in RNA processing in the nucleus.

Keywords

Zfrp8; FMRP; Trailer Hitch; translational repression

Introduction

Stem cell maintenance is essential for the generation of cells with high rates of renewal, such as blood and intestinal cells, and for the regeneration of many organs such as the brain and skin. We have previously shown that *Zfrp8* is essential for maintaining hematopoietic, follicle, and germline stem cells (GSCs) in *Drosophila melanogaster* (Minakhina et al., 2014; Minakhina and Steward, 2010). Knockdown (KD) of *Zfrp8* in GSCs results in the loss of stem cell self-renewal, followed by the eventual loss of all germline cells (Minakhina et al., 2014). Similarly in vertebrates, the *Zfrp8* homolog, *Pdcd2*, is essential for embryonic stem cell maintenance and the growth of mouse embryonic fibroblasts; *Pdcd2* mouse

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embryos die before implantation (Granier et al., 2014; Mu et al., 2010). PDCD2 is abundantly expressed and essential in highly proliferative cells including cultured cells and clinical isolates obtained from patients with hematologic malignancies (Barboza et al., 2013). The function of Zfrp8 and PDCD2 is highly conserved, as expression of transgenic PDCD2 is sufficient to rescue *Zfrp8* phenotypes (Minakhina et al., 2014). Zfrp8 directly binds to Ribosomal Protein 2 (RpS2), a component of the small ribosomal subunit (40S), controls its stability and localization, and hence RNA processing (personal communication with Svetlana Minakhina). *Zfrp8* also interacts with the piRNA pathway, which is conserved throughout all metazoans and is also essential for the maintenance of GSCs (Cox et al., 1998).

The piRNA pathway functions in maintaining heterochromatin stability and regulating the expression levels of retrotransposons. Both processes are thought to occur through piRNA targeting of chromatin modifying factors to the DNA. Guided by piRNAs, the piRNA pathway protein Piwi and associated proteins can set repressive epigenetic modifications to block transcription of nearby genes (Klenov et al., 2007; Le Thomas et al., 2013). Levels of transposon transcripts are also controlled by cytoplasmic PIWI-piRNA complexes, which can bind complementary mRNAs and mark them for translational repression and degradation (Lim et al., 2009; Rouget et al., 2010).

Fragile-X Mental Retardation Protein (FMRP) functions as a translational repressor involved in RNA silencing [reviewed in (Pimental and Tiossi, 2014)]. FMRP is a Piwi interactor and part of the piRNA pathway (Bozzetti et al., 2015; Megosh et al., 2006). FMRP-deficient animals display phenotypes similar to piRNA pathway mutants including genomic instability and de-repression of retrotransposons (Bozzetti et al., 2015; Deshpande et al., 2006). While FMRP is predominantly localized within the cytoplasm, FMRP complexes have also been demonstrated within the nucleus. In *Xenopus*, FMRP has been shown to bind target mRNAs co-transcriptionally in the nucleus (Kim et al., 2009). Like Zfrp8, FMRP has been shown to bind ribosomal proteins prior to nuclear export (Chen et al., 2014; Taha et al., 2014). In the cytoplasm, the FMRP-containing RNP complex controls mRNAs stability, localization, and miRNA-dependent repression (Chen et al., 2014; Napoli et al., 2008). FMRP mRNA targets are not well defined, as different studies show low overlap of putative targets in neuronal tissues (Brown et al., 2001; Chen et al., 2003; Darnell et al., 2001; Darnell et al., 2011; Miyashiro et al., 2003).

In *Drosophila*, FMRP is required to maintain GSCs, and loss of *Fmr1* is associated with infertility and developmental defects in oogenesis and neural development (Callan et al., 2010; Costa et al., 2005; Wang et al., 2008; Yang et al., 2007). *Fmr1*, the gene encoding FMRP, is essential in both vertebrates and *Drosophila* for the maintenance of neural stem cells (NSCs) (Callan et al., 2010; Luo et al., 2010; Tervonen et al., 2010). In humans, loss of FMRP is associated with Fragile X-associated disorders, which cover a spectrum of mental, motor, and reproductive disabilities [reviewed in (Kidd et al., 2014; Santos et al., 2014; Sherman et al., 2014)]. Fragile X-associated disorders are the most commonly inherited cause of mental disabilities and autism (Hagerman, 2008). In vertebrates, FMRP physically interacts in the nucleus with NUFIP1 (Nuclear FMRP-Interacting Protein 1), a nucleo-cytoplasmic shuttling protein involved in ribonucleoprotein (RNP) complex formation

(Bardoni et al., 2003; Boulon et al., 2008; McKeegan et al., 2009; Rothe et al., 2014). NUFIP1 is found in the nucleus in proximity to nascent RNA, and in the cytoplasm associated with ribosomes (Bardoni et al., 2003). In the cytoplasm, FMRP co-localizes and associates with Trailer Hitch (Tral) to form a translational repressor complex (Barbee et al., 2006). The Tral complex contains a number of translational repressor proteins, which together control the initiation of translation and the stability of mRNAs, such as *gurken (grk)* (Barbee et al., 2006; Jeske et al., 2011; Rouget et al., 2010; Wilhelm et al., 2005). In *Drosophila*, loss of Tral causes ovary phenotypes similar to piRNA pathway mutants, including oocyte polarity defects and transposon activation (Kugler et al., 2009; Liu et al., 2011; Snee and Macdonald, 2009).

In this study we identified Zfrp8 interactors by performing a yeast-two hybrid screen, and also by analyzing the components of the Zfrp8 complex by mass spectrometry. The nature of the proteins in the Zfrp8 complex indicates that it is involved in mRNA metabolism and translational regulation. We found that Zfrp8, Nufip, FMRP, and Tral are all part of the complex and we show that *Zfrp8* interacts antagonistically with *Fmr1* and *tral*, suppressing their oogenesis defects. Furthermore, we determined that Zfrp8 is required within the nucleus, and controls FMRP localization within the cytoplasm. We further confirm that FMRP functions in heterochromatin silencing and that Zfrp8 is required in the same process, but has an opposite function of FMRP. We propose that Zfrp8 functions as a chaperone of the FMRP' containing RNP translational repression complex and controls the temporal and spatial activity of this complex.

Materials and Methods

Fly lines and genetic interactions

Germline expressing VALIUM22 constructs were used for RNAi experiments. *UAS-Zfrp8 RNAi* (TRiP# GL00541, BDSC# 36581) and *UAS-tral RNAi* lines (TRiP# GL00680, BDSC# 38908) were obtained from the TRiP at Harvard Medical School, Boston, MA, USA. The *hsp70-Gal4* driver (*P[GAL4-Hsp70.PB]89-2-1*), *Df(3R)Exel6265*, and PEV reporters (*P[hsp26-pt-T, hsp-70w+]118E10-C4, P[hsp26-pt-T, hsp-70w+]118E15-T4)* were obtained from the Bloomington Stock Center. The *nos-Gal4* driver (*P[GAL4::VP16-nos.UTR]*) was obtained from T. Schupbach (Princeton, NJ, USA). The $Fmr1^{3}$ line was a gift from T.A. Jongens (Philadelphia, PA, USA) and the $Fmr1^{\Delta 50M}$ line was from D.C. Zarnescu (Tucson, AZ, USA). The *Df(3R)Exel6265* line was obtained from the Bloomington Stock Center (BDSC# 7732). The Tral-GFP reporter protein trap line ($P[w^{+mC}=PTT\text{-}un1]G00140$) was received from L. Cooley and the FlyTrap Project (New Haven, CT, USA) (Morin et al., 2001). In all experiments w^{118} flies were used as wild type controls.

For egg phenotype and fertility assays, 1 day-old females and males were set up on egglay plates and were changed daily over 5 days. The number of eggs laid was counted when the plate was changed and egg phenotypes and fertility rates were assessed 2 days later. Ventralization phenotypes were scored as previously described (Li et al., 2014).

Zfrp8 constructs

The *Zfrp8* coding region was amplified by RT-PCR and cloned into a Gateway *pENTR4* (Life Technologies) vector. *Zfrp8* deletion constructs were created via PCR site-directed mutagenesis. The *Zfrp8* NLS deletion construct removes putative NLS sequences at residues 100–106 and 246–263. The *Zfrp8* NES deletion construct removes a putative NES sequence at residues 304–317. Deletion constructs were then cloned into *pUAS-TAP-mCherryW-attB* vector for injections (Hudson and Cooley, 2010).

For targeted *Zfrp8* constructs, A GFP coding sequence was then subcloned at the 5' end of *Zfrp8* to create *pENTR4-GFP-Zfrp8* (Gateway). To create membrane-localized *CD8-GFPZfrp8*, transgenic mouse *CD8a* was amplified from y^1w^**; P[UAS-mCD8::GFP.L]LL5* (BDSC #5137) and subcloned at the 5' end of the *GFP* coding sequence (Lee and Luo, 1999). Nuclear-localized *GFP-NLS-Zfrp8* and cytoplasmic-localized *GFP-NES-Zfrp8* constructs were created by amplifying *pENTR-GFP-Zfrp8* via circular PCR, using primers with extended 5' *NLS* and *NES* coding sequences, respectively. The NLS sequence encodes the SV40 Large T-antigen monopartite NLS, PKKKRKV (Kalderon et al., 1984). The NES sequence encodes the HIV-1 Rev NES, LPPLERLTLD (Fischer et al., 1995). The inserts were transferred into *pUASg-attB* plasmids using the Invitrogen Gateway Cloning System (Bischof et al., 2013). Transgenic fly lines were created via PhiC31 integrase-mediated transgenesis inserted into the attP2 landing site (Groth et al., 2004) by Genetics Services, Inc. at Cambridge, MA, USA.

For targeted rescue experiments, transgenic *Zfrp8* lines were crossed to *hsp70-Gal4* in a *Zfrp8* mutant background and raised at 25°C. Viability was calculated by comparing the number of actual eclosed adults to total expected adults. For mutational analysis and genetic interaction experiments, crosses were raised until eclosion at 29°C, and subsequently maintained as adults at 25°C until examination.

Position effect variegation

Ethanol-based pigment extraction and quantification was performed as described in Sun et al. (2004) with minor modifications. Flies were homogenized in 250ul pigment assay buffer, followed by incubation at room temperature for 1 hour for pigment extraction. A final volume of 200 ul of pigment extract was used to read OD at 480 nm. For each assay, data from 3 samples (each sample made up of twenty 3 day old flies, randomly picked from the population) were collected.

Protein purification and mass spectrometry

To isolate the Zfrp8 protein complex, tandem affinity purification (TAP) was done as described in (Burckstummer et al., 2006; Kyriakakis et al., 2008; Veraksa et al., 2005). *Zfrp8* was cloned into *pUAST-NTAP* (Veraksa et al., 2005). Transgenic flies carrying *pUAST-NTAP-Zfrp8* were generated using standard methods (Brand and Perrimon, 1993). Expression of NTAP-Zfrp8 under the *da-Gal4* driver was sufficient to rescue *Zfrp8* lethality and sterility. Extracts of *da-Gal4/UAS-NTAP-Zfrp8* and w^{118} (control) 0–12 hr embryos were used for two step affinity purification. Proteins were separated by SDS-PAGE and bands visualized by Coomassie staining. To eliminate the contribution from IgG and Zfrp8

itself, fragments from 60–200 kD and from 15–35 kD were cut from the gel and analyzed by the Biological Mass Spectrometry Facility of the University of Medicine and Dentistry of New Jersey–Rutgers for LC-MS/MS analysis. Positive proteins were represented by ≥5 peptides in Zfrp8 fractions and also ≤1 peptides in the vector only control.

Nufip was cloned into the *pMK33-NTAP* vector (Veraksa et al., 2005). *Drosophila* S2 cells were transfected with either *pMK33-NTAP-Nufip* or the vector alone (as a control). After transfection and selection of stable cell lines, cells were grown for 8 days at 18°C before lysis and tandem affinity purification. Input and immunoprecipitation fractions were probed with anti-FMRP and anti-Zfrp8 antibodies.

For co-immunoprecipitation experiments, human *NUFIP1* was cloned into a *pCDNA 3xFLAG* vector. After transfection of HEK293 cells with this construct or the empty vector, cells were grown for 72 hours at 37°C before lysis and immunoprecipitation using a Sigma-Aldrich FLAG Immunoprecipitation Kit as described in the manufacturer's instructions. Extracts from cells transfected with the empty FLAG tag vector were used as negative controls. Input and immunoprecipitation fractions were probed on western blot with anti-FLAG and anti-PDCD2 antibodies.

Yeast two-hybrid screen

The PDCD2 yeast two-hybrid screen was performed using the Matchmaker Gold Protocol Yeast-Two Hybrid System (Clontech #630489). Matchmaker uses 4 different reporters each under the control of a distinct and separate cell cycle-responsive promoter, M1-expressing *AUR1-C* (Aureobasidin A/AbA resistance), M1-expressing *MEL1* (α-galactosidase), G1-expressing *HIS3* (histidine biosynthesis) and G2-expressing *ADE2* (adenine biosynthesis). For bait, full-length human *PDCD2* was cloned into the *pGBKT7* vector. Expression of PDCD2 in yeast cells was confirmed by Western blotting using anti-PDCD2, and tested negative for auto-activation and toxicity. The *pGBKT7-PDCD2* construct was then mated to a Mate and Plate normalized mouse embryonic stem cell library (Clontech #630484). Mated yeast culture was plated onto low-stringency plates containing minimal, synthetically defined (SD) -Leu/-Trp/X-α-Gal/AbA. Positive colonies were confirmed on high-stringency plates containing (SD) -Ade/-His/-Leu/-Trp/X-α-Gal/AbA. Positive plasmids were then isolated and retested with *pGBKT7-PDCD2* on high stringency plates for final confirmation. Retested positives were then sequenced to identify PDCD2 interactors.

Protein prediction software

Ortholog prediction was completed using DIOPT- DRSC Integrative Ortholog Prediction Tool (Hu et al., 2011). Prediction of Zfrp8 nuclear localization signals was completed using cNLS Mapper (Kosugi et al., 2009). Prediction of Zfrp8 nuclear export signal was completed using ExPASy NetNES (la Cour et al., 2004).

Immunostaining and microscopy

For immunostaining, ovaries were dissected from either virgins (<12 hours) or at 7 days after eclosion, as indicated. Lymph glands were dissected from third instar larvae. For each immunostaining experiment, a minimum of 15 samples were analyzed.

Rabbit anti-Zfrp8 antibody was used at 1:2500 (Minakhina et al., 2014). Monoclonal mouse anti-FMRP 5B6 (DSHB, University of Iowa) was used at 1:1000. Monoclonal mouse anti-FLAG M2 (Sigma) was used at 1:1000. Polyclonal rabbit anti-PDCD2 (1:1000) was a gift from P.A. Sharp (Cambridge, MA, USA) (Scarr and Sharp, 2002). Alexa Fluor 488 Phalloidin (Invitrogen) and secondary antibodies (Jackson Laboratories) were used at 1:300. Hoechst 33258 (1:5000) was used to stain DNA. Ovary immunostaining images were captured using a Leica TCS SP5 laser scanning confocal microscope (at 63× oil), analyzed with Leica Microsystems software and processed using Adobe Photoshop. Egg phenotype images were captured using a Zeiss SteREO Discovery. V8 stereomicroscope (at 5×), analyzed with ProgRes Mac CapturePro 2.6 software and processed using Adobe Photoshop.

Quantitative RT-PCR

Quantitative RT-PCR was performed as described in the manufacturer instructions using the QuantiTect SYBR Green kit (Qiagen), Smart Cycler II (Cepheid) and the relative standard curve method. RNA was isolated from 10–20 virgin ovaries (<12 hours old) using Qiagen RNeasy Plus Mini kits. Confirming knockdown in the *tral RNAi* line, we quantified *tral* expression in *nos-Gal4/UAS-tral RNAi* at 0.320 ±0.045 s.d., compared to *nos-Gal4/+* expression at 1.043 ±0.053 s.d. from whole ovaries. w^{118} control ovaries used as the baseline (equal to 1). Transcript levels were normalized to those of *Gapdh1*. A minimum of two biological and two technical replicates were performed for each genotype. Primers used for *tral* qPCR were: AAATGCCACAACCGCGAC, AAAGTGGCTTTCCACTGGC

Results and Discussion

Nufip and FMRP are components of the Zfrp8 protein complex

Zfrp8 is essential for stem cell maintenance, but its molecular functions have not yet been clearly defined (Minakhina et al., 2014; Minakhina and Steward, 2010). In order to address this question we used two distinct approaches. We performed a yeast-two hybrid screen to identify direct interactors of Zfrp8 and we also characterized the components of the Zfrp8 complex by mass spectrometry.

Because of the high sequence and functional conservation of Zfrp8 (flies) and PDCD2 (mammals) (Minakhina et al., 2014), and because no stem cell-derived cDNA library exists in *Drosophila*, we decided to screen a mouse embryonic stem cell cDNA library using mammalian PDCD2 as bait (see Materials and Methods). We isolated 46 initial positives, and identified 19 potential interactors after re-testing of the positives (Supplemental Table 1).

In order to purify the Zfrp8 protein complex we established a transgenic line expressing NTAP-tagged Zfrp8 under the control of the general *da-Gal4* (*daughterless*) driver. Two-step tandem affinity purification was performed on embryonic extracts and the purified proteins were separated by SDS-PAGE electrophoresis. The proteins were eluted and analyzed by mass spectrometry (see Materials and Methods). Thirty proteins were identified as part of the Zfrp8 complex. The threshold for interactors was set to at least 5× peptide enrichment in Zfrp8 over vector control fractions (Table 1). Eighteen of the proteins are

predicted to function in ribosomal assembly or translational regulation, strongly suggestive of a function of Zfrp8 in mRNA processing (i.e. translation, localization, and stability). In the complex we found six ribosomal subunits (five 40S subunits and one 60S subunit); EF2 and eIF-4a, which are required for translation initiation and elongation; and FMRP, Tral and Glorund which function in mRNA transport and translational repression. While Zfrp8 interacts with several ribosomal proteins it does not appear to be part of the ribosome itself (Marygold et al., 2007).

No overlapping interactors were found in our yeast-two hybrid screen and mass spectrometry assay. But interestingly, FMRP was identified as part of the Zfrp8 complex by mass spectrometry and NUFIP1 in our yeast-two hybrid assay. Most likely Nufip (estimated 57 kD) was not identified as part of the Zfrp8 complex in the TAP-purification approach, because we excluded proteins with similar size to tagged Zfrp8 (~55 kD) from the mass spectrometry analysis. To investigate whether these proteins could work together in the same molecular process we confirmed the interaction of both Zfrp8 and PDCD2 with Nufip (flies) and NUFIP1 (mammals) in tissue culture cells. Immunoprecipitation of human HEK293 cell extracts expressing FLAG-tagged NUFIP1 pulled down endogenous PDCD2 (Fig. 1A). We next examined whether this protein interaction also exists in *Drosophila*. We were able to co-purify endogenous Zfrp8 with NTAP-tagged Nufip from transfected S2 cells (Fig. 1B). We then performed an additional Western blot on the purified NTAP-Nufip isolate and could show that FMRP is present in the protein complex (Fig. 1B), indicating that Nufip physically interacts with both Zfrp8 and FMRP. Our results suggest that all three proteins function together in a molecular complex which regulates RNP processing/assembly and translation. Based on these results, and the requirement of both *Zfrp8* and *Fmr1* in stem cell maintenance, we decided to characterize the genetic interaction between these genes.

Loss of *Zfrp8* suppresses *Fmr1* infertility and ovary defects

To further characterize the connection between the two genes, we examined whether the loss of *Zfrp8* can modify oogenesis defects reported for *Fmr1* females (Costa et al., 2005). Similar to what was previously reported, 100% of $Fmr1^{\Delta 50M}/Df(3R)Exel6265$ (N = 26) and 80% of $Fmr1^{\Delta 50M}/Fmr1^{3}$ (N = 22) ovaries displayed developmental defects (Costa et al., 2005). The ovarioles contained fused egg chambers (100%, 64% for each genotype, respectively; Fig.2C and 2E, bracket), aberrant nurse cell numbers (46%, 32% ; Fig.2C and 2E, arrow). We occasionally also observed egg chambers with oocyte misspecification/multiple oocytes (< 20%, not shown). Interestingly, the loss of one copy of *Zfrp8* suppressed the majority of *Fmr1* ovary defects, restoring cell division in the germline, as well as egg chamber morphology and separation (Fig. 2D, 2F). In $Zfrp8/+; Fmr1^{\Delta 50M}/Df(3R)6265$ (N=20), fusion of the first egg chamber is still observed in most germaria, but despite this, oogenesis appears to proceed normally resulting in normal looking ovarioles (Fig. 2D, bracket). $Zfrp8/+; Fmr1^{\Delta 50M}/Fmr1^{3}$ (N=33) ovaries appear almost completely normal even though these ovarioles contain no FMRP (Fig. 2F).

The loss of *Fmr1* has also been associated with a strong reduction in egg production (Bauer et al., 2008; Zhang et al., 2004). We found that similar to previous reports, $Fmr1^{\Delta 50M}/$

Df(3R)Exel6265 and $Fmr1^{\Delta 50M}/Fmr1^{3}$ mutants display a strong reduction in fertility; females laid on average of 1 and 6 eggs/day, respectively, as compared to 18 eggs/day for wildtype flies (Table 2). The removal of one copy of *Zfrp8* partially suppressed *Fmr1* infertility and resulted in 8 eggs/day from $Fmr1^{\Delta 50M}/Df(3R)Exel6265$ and 15 eggs/day from $Fmr1^{\Delta 50M}/Fmr1^{3}$ females. These results demonstrate that *Zfrp8* and *Fmr1* affect the same process and that even though they are found in the same complex, have opposing functions.

Zfrp8 is required for proper FMRP localization

To investigate the nature of the Zfrp8 interaction with FMRP, we examined the localization of the proteins within the ovary. As we have shown previously, Zfrp8 displays ubiquitous distribution in all cells and cell compartments of the wild type ovary (Fig. 3A, A'). No significant changes in Zfrp8 localization or levels are visible in *Fmr1* ovaries (Fig. 3B, B'). FMRP has a more varied distribution pattern, present in strong, cytoplasmic puncta in the cytoplasm of nurse cells and follicle cells (Fig. 3A, A", E, E'), and also in high levels in the cytoplasm of the maturing oocyte (3E, E'). FMRP is also detectable in low levels in nurse cell nuclei at stage 8 egg chambers at an average of 9.76 puncta per nucleus (N=80). As expected, *Fmr1* ovaries display no FMRP staining in either the cytoplasm or nucleus (Fig. 3B, B", F, F',).

To determine whether *Zfrp8* functions in FMRP regulation, we depleted *Zfrp8* in the germline by expressing *Zfrp8* RNAi under the control of the *nos-Gal4* driver (Minakhina et al., 2014), and assessed changes in FMRP expression. In control *nos-Gal4* ovaries, FMRP levels and distribution were similar to that in wild-type ovaries (Fig. 3C, C", G, G'). However, in *Zfrp8* KD ovaries, aberrant FMRP localization is observed in the germline; FMRP is more uniformly distributed throughout the cytoplasm and puncta are strongly diminished (Fig. 3D, D", H, H'). Remaining FMRP puncta appear fragmented, reduced in intensity, size and number (~10% of wild-type; N=22 egg chambers counted). These results indicate a *Zfrp8* requirement for proper FMRP localization to the cytoplasm. FMRP normally functions by shuttling mRNA cargo from the nucleus to the cytoplasm, where it represses the translation of bound mRNA. The observed change of FMRP localization in Zfrp8 KD ovaries therefore may indicate a regulatory function for Zfrp8 in the nuclear export and localization of FMRP.

Zfrp8 is required in the nucleus

Zfrp8 protein is present in both the cytoplasm and nucleus (Fig. 3A, A') (Minakhina et al., 2014) and, as demonstrated above, controls the distribution of FMRP in the cytoplasm. We decided to investigate the cell compartment in which Zfrp8 is required, in order to elucidate how Zfrp8 regulates FMRP. To do so, we examined the capability of Zfrp8 deletion constructs to rescue mutant lethality. Expression of human *PDCD2* cDNAs driven by the general driver *da-Gal4* is fully capable of rescuing *Zfrp8* lethality (Barboza et al., 2013; Minakhina et al., 2014). We created mutated Zfrp8 constructs, removing either the two putative NLSs or the putative NES domains. These proteins were expressed under the *da-Gal4* driver, and while clearly overexpressed on Western blots, failed to rescue mutant lethality, suggesting that the three domains are essential for the function of the protein (not shown).

In an alternative approach, we assayed the function of Zfrp8 proteins targeted to a distinct cell compartment. We expressed four N-terminal GFP-tagged transgenic proteins encoding a wild-type Zfrp8, nuclear-localized NLS-Zfrp8, cytoplasmic-localized NES-Zfrp8, and cell membrane-localized CD8-GFP-Zfrp8. Transgenic Zfrp8 subcellular localization is visible when the proteins are strongly overexpressed (Supplemental Fig. 1A–D). When we expressed the transgenes at lower levels, similar to the endogenous levels, with the *hsp70-Gal4* driver at 25°C, both wild-type and nuclear-localized Zfrp8 were able to rescue mutant lethality at similar rates, whereas the cytoplasmic- and membrane-localized proteins did not show rescue (Supplemental Table 2). These results show that Zfrp8 is required in the nucleus and suggest that like FMRP, Zfrp8 may function by shuttling between nuclear and cytoplasmic compartments.

Zfrp8 suppresses the *tral* oogenesis phenotypes

We have shown that FMRP and Zfrp8 are present in the same protein complex. In addition to FMRP, our mass spectrometry results have also identified other translational regulators, such as Tral. Tral has previously been shown to function in conjunction with FMRP to control the translation of mRNAs (Barbee et al., 2006).

To determine whether Zfrp8 functions in Tral/FMRP-associated translational regulation, we investigated the genetic interaction between *Zfrp8* and *tral*. Tral regulates dorsal-ventral (D/V) patterning through the localization and translational control of *gurken (grk) m*RNA (Wilhelm et al., 2005). Eggs laid by *tral* females display ventralized chorion phenotypes, due to the aberrant Gurken morphogen gradient. If *Zfrp8* functions to regulate the translational activity of FMRP/Tral, a suppression of the *tral* ventralized phenotypes should be apparent when Zfrp8 is reduced. We depleted Tral in the germline by expressing a TRiP RNAi line (see Material and Methods) under the control of the *nos-Gal4* driver. *Tral* KD resulted in similar ventralized egg phenotypes as previously observed in eggs laid by $tral^{1}$ females (Wilhelm et al., 2005): 1% of eggs displayed two normal dorsal appendages (Wt), 36% had fused appendages (category V2/V3), and 63% had no dorsal appendages (category V4, see Materials and Methods) (Fig. 4A–B). Removing one copy of *Zfrp8* in the *tral* KD background suppressed the *tral* phenotypes (17% Wt, 53% V2/V3, and 30% V4, Fig. 4A–B). This genetic interaction suggests that in addition to controlling the localization of FMRP in the cytoplasm, Zfrp8 also influences the translational control by Tral, essential for formation of dorsal-ventral polarity in the egg (Wilhelm et al., 2005).

We investigated whether Zfrp8 regulates Tral localization as it does FMRP by examining the distribution of GFP-fusion Tral protein trap line (Morin et al., 2001). Tral protein was uniformly present in cytoplasmic compartments of germline and somatic cells, with stronger granules surrounding nuclei, and was highly enriched within the oocyte (Supplemental Fig. 3A and 3C). *Zfrp8* KD results in loss of oocyte identity (Minakhina et al., 2014), and the distribution of Tral was significantly altered in those cells. But in all other germline cells Tral distribution remained unaffected (Supplemental Fig.3B and 3D). Tral and its orthologs are cytoplasmic proteins (Wilhelm et al., 2005) and examination of the Tral protein sequence identifies no NLSs. Zfrp8 may therefore interact only indirectly with Tral and not regulate its localization.

Zfrp8 and Fmr1 control Position Effect Variegation

piRNA pathway genes have been shown to be essential for heterochromatin packaging in position effect variegation (PEV) experiments (Brower-Toland et al., 2007; Gu and Elgin, 2013). PEV measures expression of endogenous or reporter genes inserted within or adjacent to heterochromatin. *Fmr1* is specifically required for chromatin packaging as loss of a single copy of *Fmr1* is sufficient to inhibit heterochromatin silencing of a *white* reporter (w^+) inserted into the pericentric heterochromatin region 118E10 on the 4th chromosome (Deshpande et al., 2006).

We analyzed PEV of *Zfrp8 heterozygotes, Fmr1* heterozygotes and *Fmr1, Zfrp8* transheterozygotes using 118E10 (4th chromosome centromeric) and an additional w^+ reporter, inserted into heterochromatin region 118E15 (4th chromosome telomeric). While the w^+ reporters in $Zfrp8^{null}$/+ eyes were expressed at levels comparable to those in wild-type controls, expression in $Fmr1^{\Delta 50M}$ /+ of both w^+ reporters was strongly enhanced. But, the removal of one copy of both *Zfrp8* and *Fmr1* decreased expression of the reporters back to the *Zfrp8*/+, near wild-type levels, indicating restored heterochromatin silencing of both 4^{th} chromosomal insertions (Fig. 5). These findings suggest that in normal eyes, *Zfrp8* functions upstream of *Fmr1* and controls *Fmr1* effects on heterochromatin packaging.

A connection between regulation of heterochromatin silencing and Piwi has clearly been established and our results show that Zfrp8 and FMRP are part of the mechanism that controls heterochromatin silencing (Brower-Toland et al., 2007; Gu and Elgin, 2013). Heterochromatin is established at the blastoderm stage in Drosophila embryos and is subsequently maintained throughout development. Thus, FMRP and Zfrp8 function together in heterochromatin packaging in the early embryo in the same way as they do during oogenesis.

Conclusion

Here we show that Zfrp8 is part of a complex that is involved in RNA processing, i.e. translation, localization, and stability. We propose that Zfrp8 likely forms a ribonucleoprotein complex with Nufip, FMRP and select mRNAs in the nucleus, and is required for localization of this complex in the cytoplasm. After nuclear export, mRNAs within the complex are targeted for translational control and repression by FMRP and Tral. The suppression of the *Fmr1* and *tral* phenotypes in a *Zfrp8* heterozygous background, occurs in the absence of *Fmr1* and the strong reduction of *tral*. This suggests that Zfrp8 function is not protein specific, but rather that it controls the FMRP and Tral-associated complex, even in the absence of each of the two proteins. Our hypothesis is consistent with Zfrp8 actively controlling the localization of FMRP to cytoplasmic foci, as this localization is affected in *Zfrp8* germ cells.

We have previously identified a piRNA pathway protein, Maelstrom (Mael), that is controlled by Zfrp8 in a similar manner as FMRP. Zfrp8 forms a protein complex with Mael, genetically suppresses the loss of *mael*, and controls Mael localization to the nuage, a perinuclear structure (Minakhina et al., 2014). But the *Zfrp8* phenotype is stronger and appears earlier than that of *mael, tral, Fmr1*, or other piRNA pathway regulatory genes we

have studied so far. Zfrp8 may therefore control a central step in the regulation of specific RNPs. Consistent with this hypothesis, our TAP purification and mass spectrometry analysis identified a number of Zfrp8-associated proteins, the majority of which function in ribosomal assembly or translational regulation, such as the ribosomal protein RpS2. And *Zfrp8* KD in the germ line and partial loss of *rps2* result in a similar "string of pearls phenotype", caused by developmental arrest in early stages of oogenesis (Cramton and Laski, 1994; Minakhina et al., 2014). In addition, a recent study has shown that Zfrp8 and PDCD2 contain a TYPP (TSR4 in yeast, YwqG in E. coli, PDCD2 and PDCD2L in vertebrates and flies) domain, which has been suggested to perform a chaperone-like function in facilitating protein-protein interactions during RNA processing (Burroughs and Aravind, 2014). These observations lead us to hypothesize that *Zfrp8* functions as a chaperone essential for the assembly of ribosomes and the early recruitment and localization of ribosomal-associated regulatory proteins, such as FMRP, Tral and Mael (Fig. 6).

Zfrp8 negatively controls the functions of *Fmr1* and *tral*. In the absence of FMRP and Tral the temporal and spatial control of translation of their associated RNPs is lost. We propose that reducing the level of Zfrp8 diminishes the availability of these RNP-complexes in the cytoplasm resulting in suppression of the *Fmr1* and *tral* phenotypes.

Zfrp8, *Fmr1* and *tral* have all been shown to genetically and physically interact with components of the piRNA pathway, and to regulate the expression levels of select transposable elements (Liu et al., 2011; Megosh et al., 2006; Minakhina et al., 2014). Transposon de-repression is often associated with the loss of heterochromatin silencing. The molecular mechanisms underlying heterochromatin formation appear to involve maternally contributed piRNAs and piRNA pathway proteins that control the setting of epigenetic marks in the form of histone modifications, maintained throughout development (Gu and Elgin, 2013). But transposon expression can also be controlled post-transcriptionally by cytoplasmic PIWI-piRNA complexes, suggesting that transposon deregulation and heterochromatin silencing phenotypes seen in FMRP and Zfrp8 may be linked to translational de-repression (Lim et al., 2009; Rouget et al., 2010). We propose that by facilitating the early assembly of ribosomes with specific translational repressors, *Zfrp8* regulates several developmental processes during oogenesis and early embryogenesis including dorsal-ventral signaling, transposon de-repression, and position effect variegation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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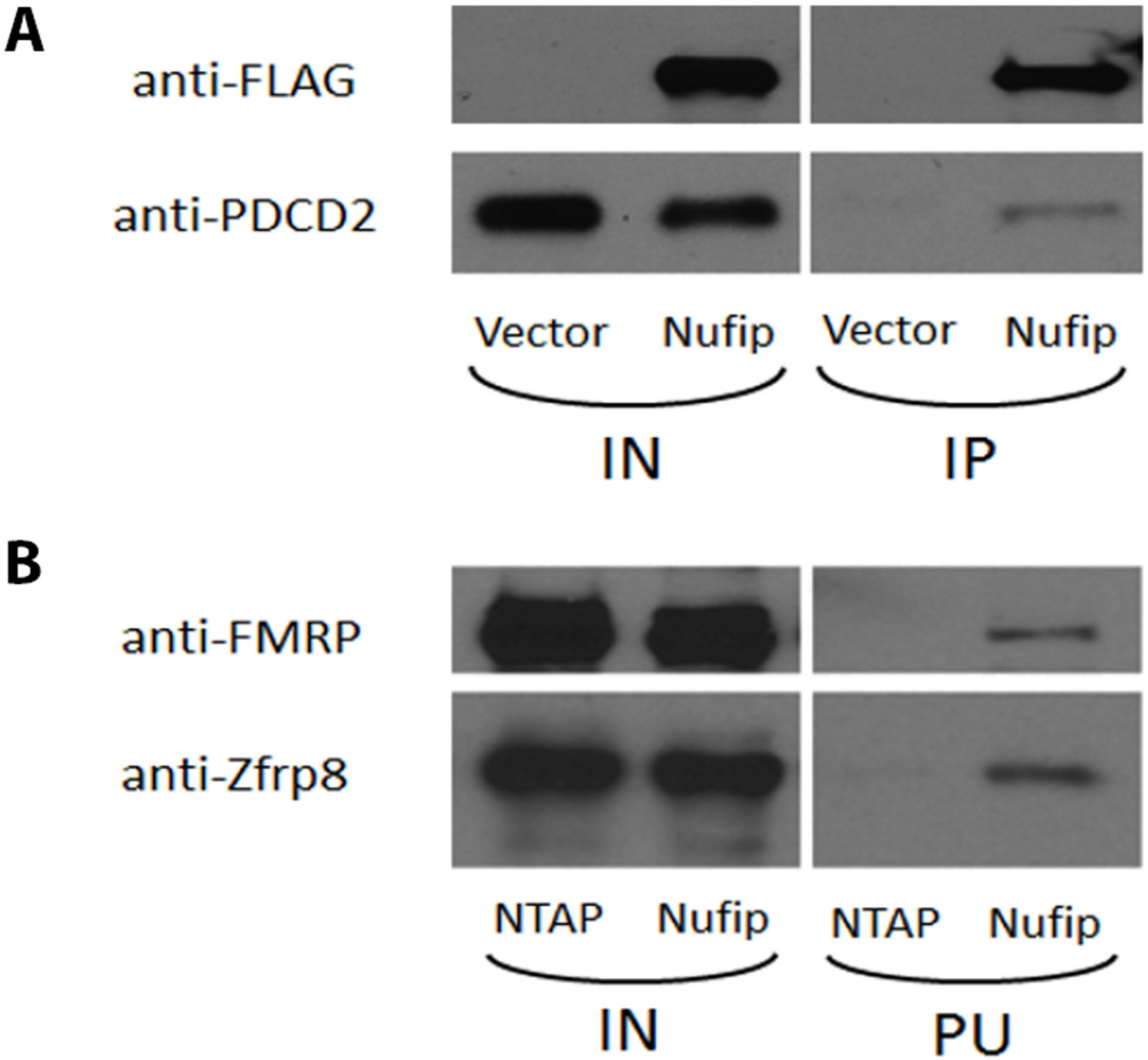


Figure 1. Nufip and FMRP are components of the Zfrp8 complex

A. Western blot displaying the immunoprecipitation of human PDCD2 with FLAG-tagged NUFIP1 from HEK293 cell extracts. Negative control: extract from cells transfected with the empty FLAG-tag vector. **B.** NTAP-tagged *Drosophila* Nufip was expressed in S2 cells and purified. Protein complex is visualized on western blot with anti-FMRP and anti-Zfrp8 antibodies. Negative control: extract from cells transfected with the empty NTAP-tag vector. IN – total extract, IP immunoprecipitate, PU purified complex.

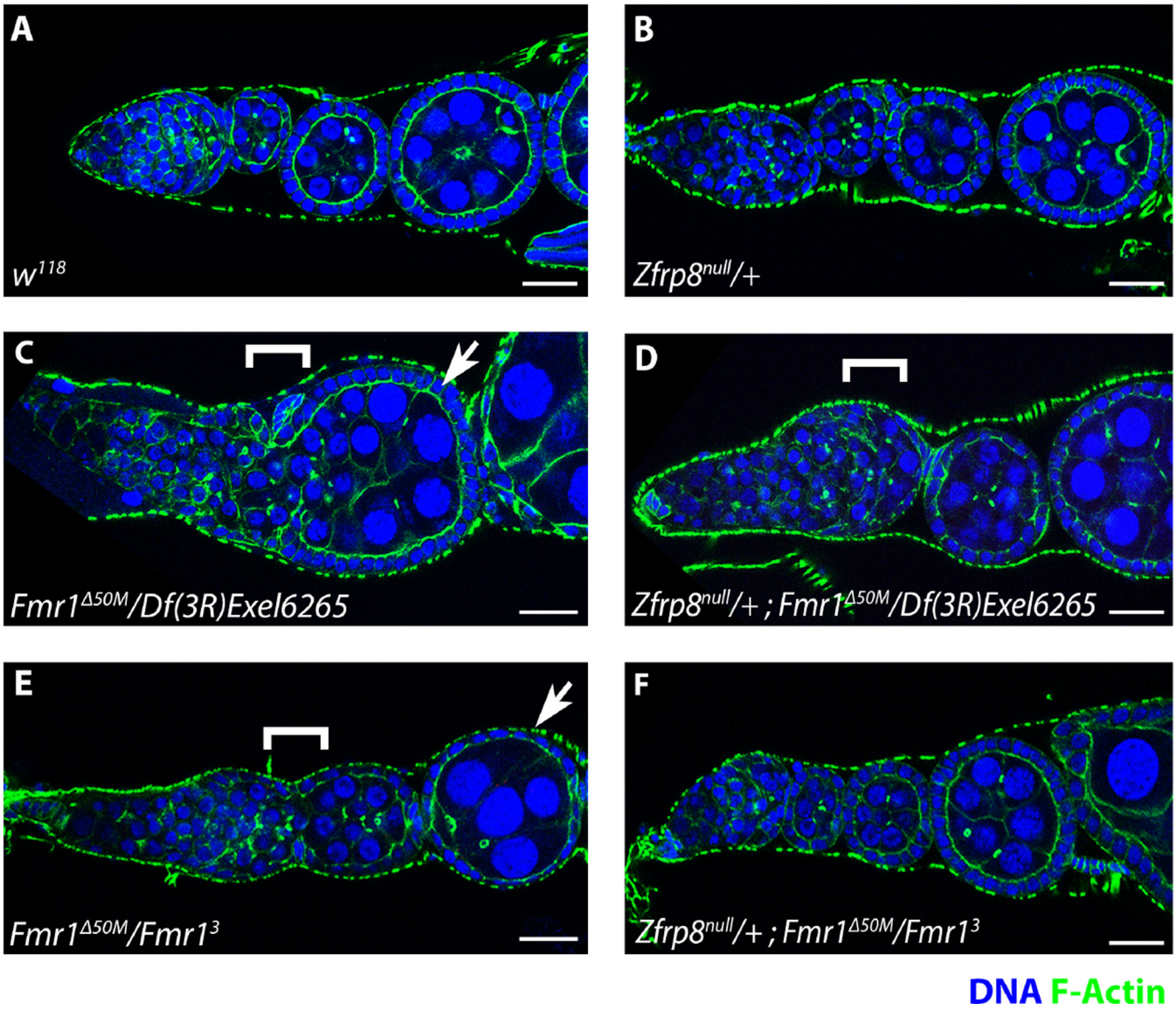


Figure 2. Loss of *Zfrp8* suppresses *Fmr1* infertility and ovary defects

A. Wild-type ovariole comprised of a normal germarium and early egg chambers. **B.** $Zfrp8^{null}/+$ heterozygote ovarioles do not display any morphological defects. **C.** $Fmr1^{\Delta 50M}/Df(3R)Exel6265$ ovarioles displays a disorganized germarium, often fused to an egg chamber containing more than the normal 15 nurse cells (bracket and arrow). **D.** $Zfrp8^{null}/+; Fmr1^{\Delta 50M}/Df(3R)Exel6265$ ovarioles show suppression of the $Fmr1^{\Delta 50M}/Df(3R)Exel6265$ morphological defects. While the first egg chamber often remains fused to the germarium (bracket), the later stages of oogenesis are normal. **E.** $Fmr1^{\Delta 50M}/Fmr1^{3}$ ovariole containing a fused germarium-egg chamber (bracket) and an egg chamber with abnormal numbers of nurse cells (arrow). **F.** $Zfrp8^{null}/+ ; Fmr1^{\Delta 50M}/Fmr1^{3}$ exhibit morphologically normal ovarioles. DNA (blue); filamentous actin-phalloidin (green). Confocal sections are shown; scale bar: 10 uM.

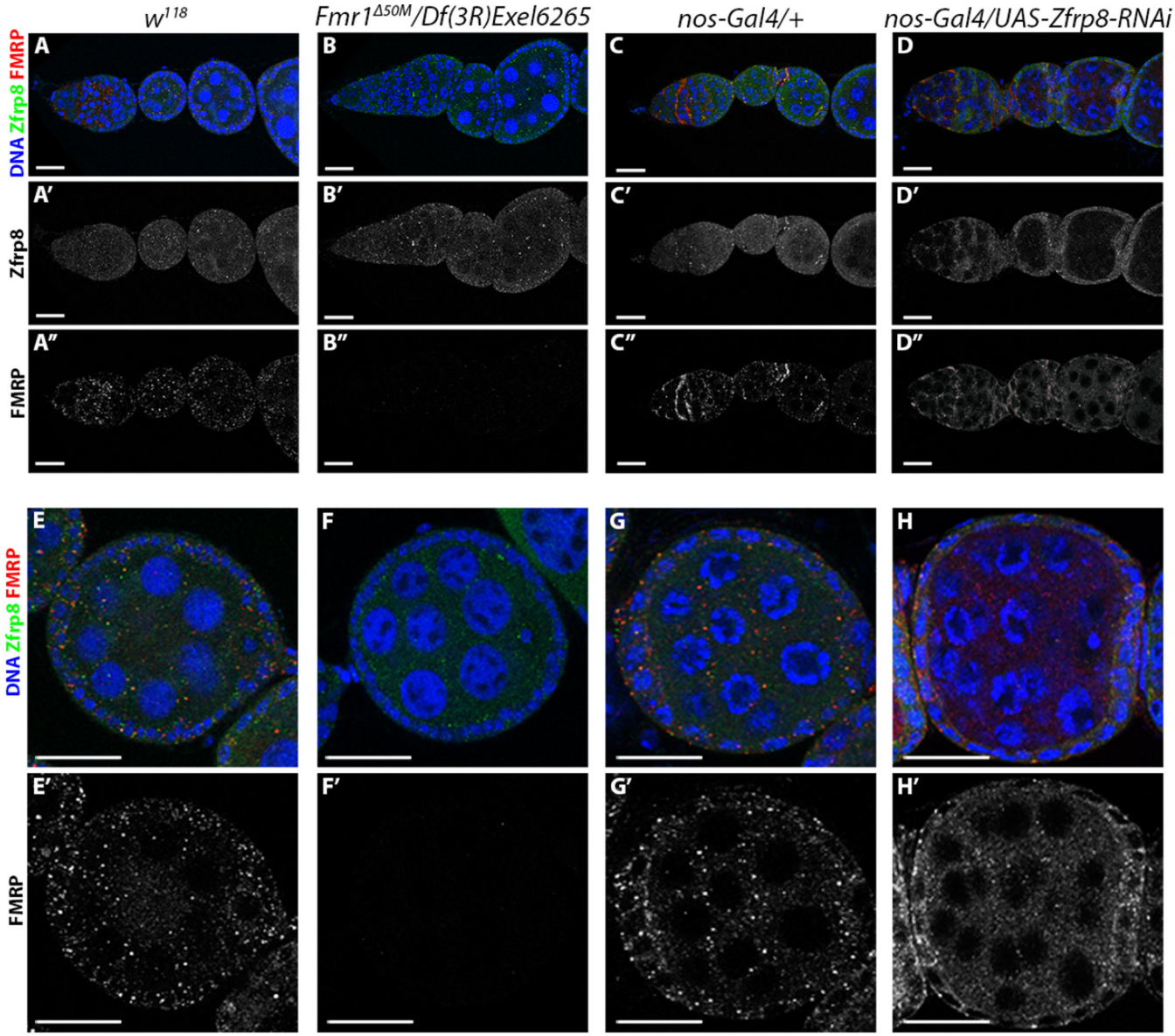
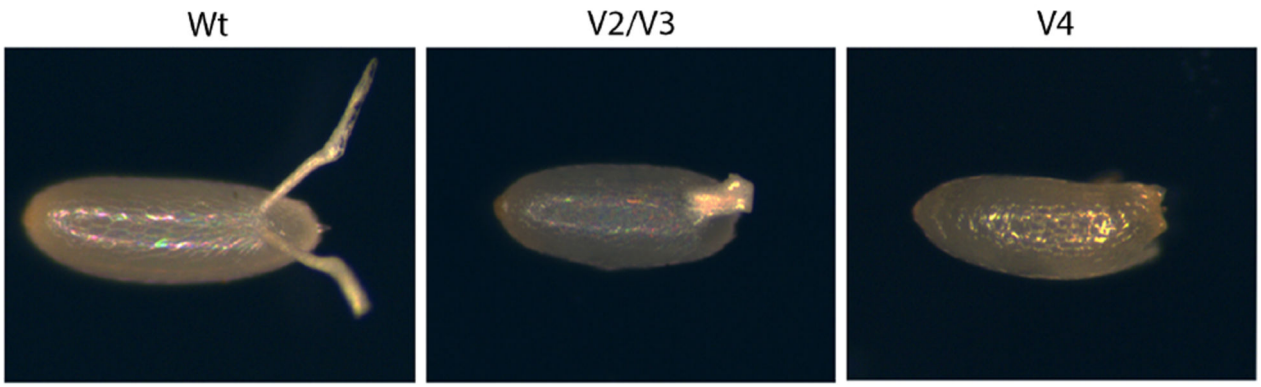


Figure 3. *Zfrp8* is required for proper FMRP localization

A–A", E–E'. In wild-type (w^{118}) ovarioles FMRP is seen as puncta throughout the cytoplasm and at low levels in nurse cell nuclei of stage 8 egg chambers at an average of 9.76 puncta per nucleus (N=80), while Zfrp8 is uniformly distributed in the cytoplasm and nuclei. **B–B", F–F'.** In $Fmr1^{\Delta 50M}/Df(3R)Exel6265$ ovarioles FMRP protein is absent as expected both in the cytoplasm and in nuclei (N=81), while Zfrp8 expression and distribution is not significantly changed. **C–C", G–G'.** Control *nos-Gal4/+* ovarioles appear similar to wild-type, FMRP localizes to cytoplasmic foci;. **D–D", H–H'.** Within *Zfrp8* KD egg chambers FMRP localization to cytoplasmic puncta is disrupted and the protein is more uniformly distributed throughout the cytoplasm. Anti-Zfrp8 (green), anti-FMRP (red), DNA (blue). Confocal sections are shown; scale bar: 10 uM.

A



B

Genotype	Wt	V2/V3	V4	Eggs Counted
nos-Gal4/+	100%	0%	0%	n= 422
nos-Gal4/UAS-tral RNAi	1%	36%	63%	n= 499
Zfrp8^{null}/+ ; nos-Gal4/UAS-tral RNAi	17%	53%	30%	n= 271

Figure 4. *Zfrp8* suppresses *tral* oogenesis phenotypes

A. *w^{118}* (Wt) egg displaying two normally spaced dorsal appendages. A ventralized egg displaying only one wider, fused dorsal appendage (V2/V3) (Li et al., 2014). A fully ventralized egg displaying no dorsal appendages (V4). **B.** Loss of one copy of *Zfrp8* suppresses the *tral* dorsal-ventral egg phenotype.

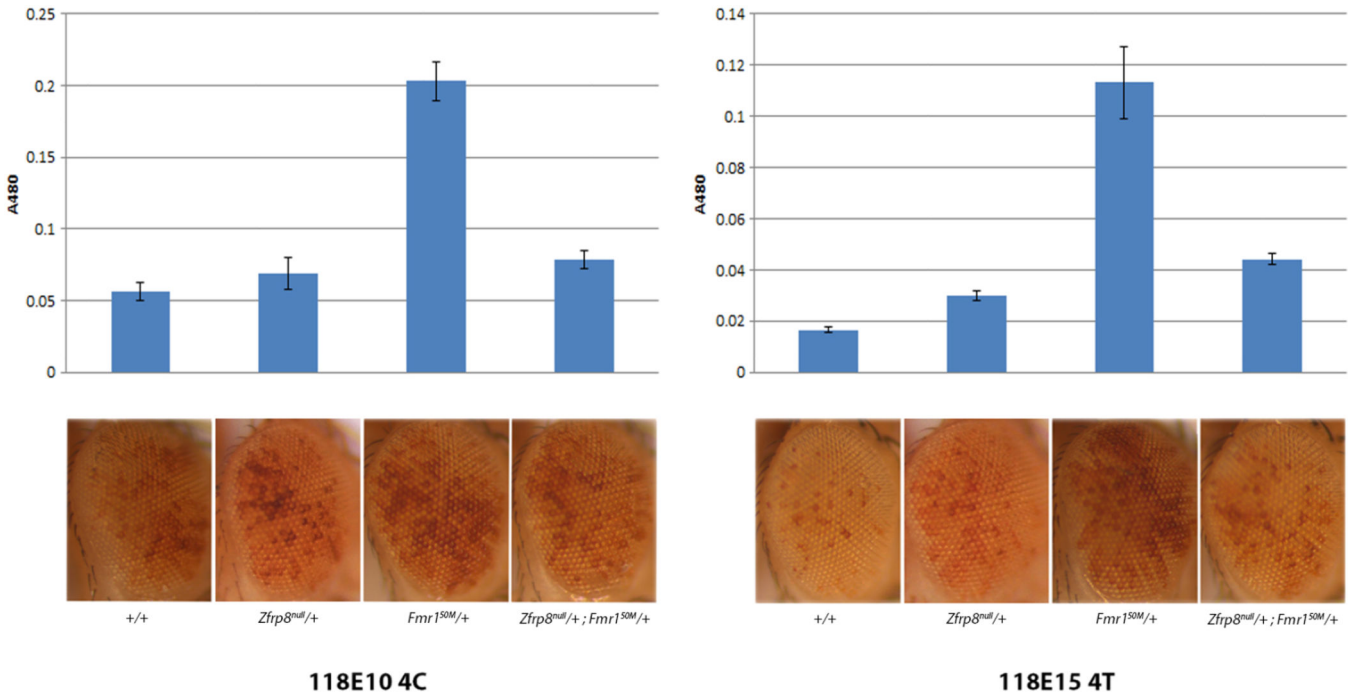


Figure 5. *Zfrp8 and Fmr1* control Position Effect Variegation (PEV)

A. Eyes of fly expressing the PEV reporter [w^+]118E15-4T in *w; w, Zfrp8*^{null}*/+; w, Fmr1*^{50M}*/+; w, Zfrp8*^{null}*/+, Fmr1*^{50M}*/+* backgrounds. **B.** Eyes of fly expressing the PEV reporter [w^+]118E10-4C in *w; w, Zfrp8*^{null}*/+; w, Fmr1*^{50M}*/+; w, Zfrp8*^{null}*/+, Fmr1*^{50M}*/+* backgrounds. The expression of both reporters is unchanged in the *Zfrp8*^{null}*/+* background, while the expression is enhanced in the *Fmr1*^{50M}*/+* background. In a *Zfrp8*^{null}*/+, Fmr1*^{50M}*/+* background, expression is suppressed compared to *Fmr1*^{50M}*/+* alone. Measurement of eye color (OD 480nm) of extracts from corresponding fly heads support the result.

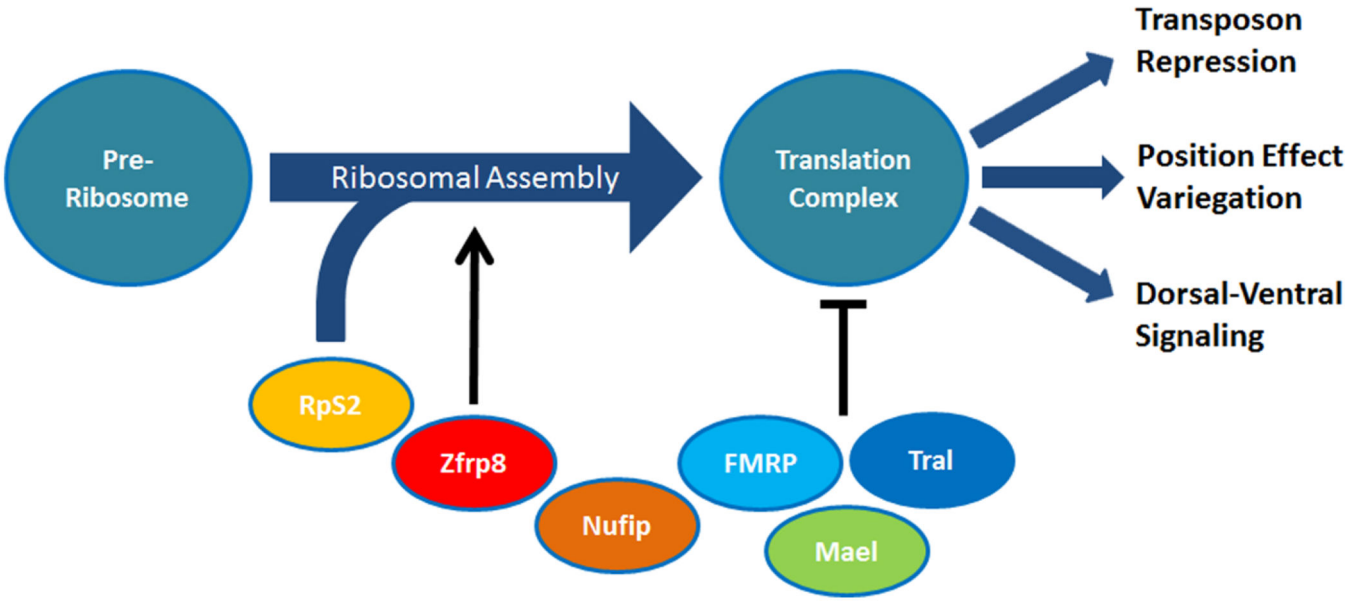


Figure 6. Model of the requirement of Zfrp8 and associated proteins

Table 1

Zfrp8 interactors identified by TAP purification followed by mass spectrometry

Symbol	Protein	Human Ortholog	# Peptides Matched*	Estimated Mass (kD)	Molecular Function
Act42A	Actin 42A	ACTB/G1	33	41.8	cytoskeletal protein
AP-2α	AP-2 complex subunit alpha	AP2A2	10	105.6	endocytosis
CkIα	Casein kinase I isoform alpha	CSNK1A1	21	39.5	protein kinase
Ef1γ	Elongation factor 1-gamma	EEF1G	23	49.0	regulation of organelle transport (Serpinskaya et al., 2013)
EF2	Elongation factor 2	EEF2	18	94.5	translation elongation factor
eIF-4a	Eukaryotic initiation factor 4A	EIF4A1/2	24	45.9	translation initiation factor
FK506-bp1	39 kDa FK506-binding nuclear protein		12	39.3	prolyl isomerase
FMRP	Fragile-X mental retardation protein	FMR1	8	72.0	Translational regulation, Rm62-interactor (Zhang et al., 2001; Ishizuka et al., 2002)
Glo	Glorund	HNRNPH1	39	61.4	Gurken mRNA localization and translational repression (Kalifa et al., 2009)
Hrb27C	Heterogeneous nuclear ribonucleoprotein 27C	DAZAP1	26	44.8	Gurken mRNA localization (Goodrich et al., 2004)
Hrb87F	Heterogeneous nuclear ribonucleoprotein 87F	HNRNPA2B1	50	39.5	ribonucleoprotein, NonA-interactor (Reim et al., 1999)
Hsc70-4	Heat shock 70 kDa protein cognate 4		23	71.1	chaperone protein
Hsp83	Heat shock protein 83	HSP90AA1/B1	8	81.9	chaperone protein, regulation of piRNA pathway (Gangaraju et al., 2011)
Map205	205 kDa microtubule-associated protein		25	126.7	cytoskeletal protein
Nep1	Neprilysin 1	MME/L1	31	96.5	peptidase
NonA	Protein no-on-transient A	SFPQ	163	77.0	RNP nucleo-cytoplasmic shuttling (Kozlova et al., 2006)
Nop60B	Nucleolar protein at 60B	DKC1	54	56.8	ribosomal RNA processing (Giordano et al., 1999)
Noppl40	Noppl40	NOLC1	34	70.5	ribosome assembly and repression of transposon expression (He et al., 2014)
Pug	Pugilist	MTHFD1	20	103.5	tetrahydrofolate conversion
Rm62	Rm62	DDX17	68	50.0	RNA helicase, FMRP-interactor (Ishizuka et al., 2002)
RpL7A	Ribosomal protein L7A	RPL7A	7	30.7	ribosomal protein
RpS2	Ribosomal protein S2	RPS2	224	28.9	ribosomal protein (Cramton and Laski, 1994)
RpS3	Ribosomal protein S3	RPS3	15	27.5	ribosomal protein
RpS4	Ribosomal protein S4	RPS4	32	29.1	ribosomal protein

Symbol	Protein	Human Ortholog	# Peptides Matched*	Estimated Mass (kD)	Molecular Function
RpS5a	40S ribosomal protein S5a	RPS5	11	25.4	ribosomal protein
RpS7	Ribosomal protein S7	RPS7	18	22.2	ribosomal protein
Top2	DNA topoisomerase 2	TOP2A/B	59	164.4	topoisomerase
Tral	Trailer hitch	LSM14A/B	80	69.3	Secretory pathway regulation; Repression of transposon expression, FMRP-interactor (Kugler et al., 2009; Liu et al., 2011)
αTub84B	Alpha-tubulin at 84B	TUBA3C/D	5	49.9	cytoskeletal protein
βTub56D	Beta-tubulin at 56D	TUBB4B	8	51.3	cytoskeletal protein

*For all proteins, ≤1 peptides were found in the vector-only TAP control

Table 2

Zfrp8 suppresses *Fmr1* egg laying defects

Genotype	Hatch Rate	Eggs Laid per Day	Eggs Counted
w^{118}	95.10%	18.01	n= 1801
$Fmr1^{\Delta 50M}/+$	91.68%	16.84	n= 317
Df(3R)Exel6265/+	90.79%	12.75	n= 956
$Fmr1^{\Delta 50M}/Df(3R)Exel6265$	94.85%	0.97	n= 97
$Zfrp8^{null}/+$; $Fmr1^{\Delta 50M}/Df(3R)Exel6265$	85.99%	8.28	n= 236
$Fmr1^{\Delta 50M}/Fmr1^{3}$	89.55%	5.74	n= 145
$Zfrp8^{null}/+$; $Fmr1^{\Delta 50M}/Fmr1^{3}$	78.48%	15.14	n= 336