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.

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1. 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 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 hematoïc 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; Tahara 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., 2009).
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 eggglay plates and were changed daily for 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. Ventrinalization phenotypes were scored as previously described (Li et al., 2014).

3. 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-mCherry-W-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-GFP-Zfrp8, transgenic mouse CD8a was amplified from y’w+; P[UsCD8::GFP] L5 (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.

4. 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 250 ul pigment assay buffer, followed by incubation at room temperature for 1 h 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.

5. 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-NTP (Veraksa et al., 2005). Transgenic flies carrying pUAST-NTP-Zfrp8 were generated using standard methods (Brand and
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 w118 (control) 0–12 h 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.

Zfrp8 nuclear localization signals was completed using cNLS reporter, full-length human PDCD2 was transfected into S2 cells. Positive plasmids were then isolated and retested with RA-Gal4/UAS-NTAP-Zfrp8 driver was sufficient to rescue Zfrp8 lethality and sterility. Extracts of da-Gal4/UAS-NTAP-Zfrp8 and w118 (control) 0–12 h 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 h 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.

6. 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 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 pGBK7-PDCD2 construct was then mated to a M9 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 pGBK7-PDCD2 on high stringency plates for final confirmation. Retested positives were then sequenced to identify PDCD2 interactors.

7. Protein prediction software

Ortholog prediction was completed using DIOPT- DRSC Integrative Ortholog Prediction Tool (Hu et al., 2011). Prediction of Zfrp8 nuclease 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).

8. Immunostaining and microscopy

For immunostaining, ovaries were dissected from either virgins (< 12 h) 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 SPS 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.

9. 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 h 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 ± 0.045s.d., compared to nos-Gal4/+ expression at 1.043 ± 0.053s.d. from whole ovaries. w118 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.

10. Results and discussion

10.1. 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 purge the Zfrp8 protein complex we established a transgenic line expressing NTAP-tagged Zfrp8 under the control of the general da-Gal4 (daughtersless) 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 elf-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 it does not appear to be part of the ribosome itself (Marygold et al., 2007).

Table 1

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<th>Symbol</th>
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<th>Human Ortholog</th>
<th># Peptides matched</th>
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<td>8</td>
<td>51.3</td>
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* For all proteins, ≤ 1 peptides were found in the vector-only TAP control.

11. 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 Fmr1Δ50M/Fmr1Δ50M,Df(3 R)Xel6265 (N = 26) and 80% of Fmr1Δ50M/Fmr1Δ50M,Fmr1Δ50M (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 E, bracket), aberrant nurse cell numbers (46%, 32%; Fig. 2C and E, 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 and F). In Zfrp8''/+; Fmr1''/+; 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 in normal looking ovarioles (Fig. 2D, bracket). Zfrp8''/+; Fmr1''/+; Df(3R)6265 (N = 33) ovarioles 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''/+; Df(3R) Exel6265 and Fmr1''/+; Df(3R)Exel6265 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 wild-type flies (Table 2). The removal of one copy of Zfrp8 partially suppressed Fmr1 infertility and resulted in 8 eggs/day from Fmr1''/+; Df(3R)Exel6265 and 15 eggs/day from Fmr1''/+; Df(3R)Exel6265 and 15 eggs/day from Fmr1''/+; Df(3R)Exel6265 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.

12. Zfrp8 is required for proper FMRP localization

To determine whether Zfrp8 functions in FMRP regulation, we investigated the genetic interaction between Zfrp8 and Fmr1 using genetic Fmr1''/+; Df(3R)Exel6265 and 15 eggs/day from Fmr1''/+; Df(3R)Exel6265 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.

13. 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.

14. Zfrp8 suppresses the Trail 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 Trail. Trail 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 Trail/FMRP-associated translational regulation, we investigated the genetic interaction between Zfrp8 and trail. Trail regulates dorsal-ventral (D/V)
patterning through the localization and translational control of gurken (grk) mRNA (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 tral1 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 and 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 and 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 C). 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 D). 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.

15. 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

### Table 2

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Hatch rate</th>
<th>Eggs laid per day</th>
<th>Eggs counted</th>
</tr>
</thead>
<tbody>
<tr>
<td>w118</td>
<td>95.10%</td>
<td>18.01</td>
<td>n = 1801</td>
</tr>
<tr>
<td>Fmr1Δ50M/+</td>
<td>91.68%</td>
<td>16.64</td>
<td>n = 317</td>
</tr>
<tr>
<td>Df[3R]Exel6265/+</td>
<td>90.79%</td>
<td>12.25</td>
<td>n = 956</td>
</tr>
<tr>
<td>Fmr1Δ50M/Df[3R]Exel6265</td>
<td>94.85%</td>
<td>0.97</td>
<td>n = 97</td>
</tr>
<tr>
<td>Zfrp8αβ/+ ; Fmr1Δ50M/Df[3R]Exel6265</td>
<td>85.99%</td>
<td>8.28</td>
<td>n = 236</td>
</tr>
<tr>
<td>Fmr1Δ50M/Fmr1Δ50M</td>
<td>89.55%</td>
<td>5.74</td>
<td>n = 145</td>
</tr>
<tr>
<td>Zfrp8αβ/ + ; Fmr1Δ50M/Fmr1Δ50M</td>
<td>78.48%</td>
<td>15.14</td>
<td>n = 336</td>
</tr>
</tbody>
</table>

Fig. 2. Loss of Zfrp8 suppresses Fmr1 infertility and ovary defects. A. Wild-type ovariole comprised of a normal germarium and early egg chambers. B. Zfrp8αβ/+ heterozygote ovarioles do not display any morphological defects. C. Fmr1Δ50M/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. Zfrp8αβ/+ ; Fmr1Δ50M/Df[3R]Exel6265 ovarioles show suppression of the Fmr1Δ50M/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. Fmr1Δ50M/Fmr1Δ50M ovariole containing a fused germarium-egg chamber (bracket) and an egg chamber with abnormal numbers of nurse cells (arrow). F. Zfrp8αβ/+ ; Fmr1Δ50M/Fmr1Δ50M exhibit morphologically normal ovarioles. DNA (blue); filamentous actin-phalloidin (green). Confocal sections are shown; scale bar: 10 μM.
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 Zfrp8mut/+ eyes were expressed at levels comparable to those in wild-type controls, expression in Fmr1Δ50M/+ 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 4th 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.

16. 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 miRNAs 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 de-regulation 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.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2015.12.008.

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