Developmental Biology 410 (2016) 202–212

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

DB Letters

Zfrp8 forms a complex with fragile-X mental retardation protein and regulates its localization and function

William Tan, Curtis Schauder, Tatyana Naryshkina, Svetlana Minakhina, Ruth Steward *

Rutgers University, Department of Molecular Biology, Cancer Institute of New Jersey, Waksman Institute, 190 Frelinghuysen Road, Piscataway, NJ 08854, USA

ARTICLE INFO

Article history:
Received 9 November 2014
Received in revised form
13 November 2015
Accepted 9 December 2015
Available online 7 January 2016

Keywords:
Zfrp8
FMRP
Trailer Hitch
Translational repression

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.

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 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.,

* Corresponding author.
E-mail address: steward@waksman.rutgers.edu (R. Steward).

http://dx.doi.org/10.1016/j.ydbio.2015.12.008
0012-1606/

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, 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.

2. Materials and methods

2.1. 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 $Fmr1^{\Delta 50\ M}$ 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-un1]G00140)* was received from L. Cooley and the FlyTrap Project (New Haven, CT, USA) (Morin et al., 2001). In all experiments w^{1118} 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).

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-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-GFP-Zfrp8*, transgenic mouse *CD8a* was amplified from *$y^{1}w^{*}$; 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.

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-NTAP* (Veraksa et al., 2005). Transgenic flies carrying *pUAST-NTAP-Zfrp8* were generated using standard methods (Brand and