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Proteomic analysis reveals CCT is a target of Fragile X mental retardation protein regulation in *Drosophila*

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Abstract

Fragile X mental retardation protein (FMRP) is an RNA-binding protein that is required for the translational regulation of specific target mRNAs. Loss of FMRP causes Fragile X Syndrome (FXS), the most common form of inherited mental retardation in humans. Understanding the basis for FXS has been limited because few *in vivo* targets of FMRP have been identified and mechanisms for how FMRP regulates physiological targets is unclear. We have previously demonstrated that *Drosophila* FMRP (dFMRP) is required in early embryos for cleavage furrow formation. In an effort to identify new targets of dFMRP-dependent regulation and new effectors of cleavage furrow formation, we used two-dimensional difference gel electrophoresis and mass spectrometry to identify proteins that are misexpressed in *dfmr1* mutant embryos. Of the 28 proteins identified, we have identified three subunits of the Chaperonin containing TCP-1 (CCT) complex as new direct targets of dFMRP-dependent regulation. Furthermore, we found that the septin Peanut, a known effector of cleavage, is a likely conserved substrate of fly CCT and is mislocalized in both *cct* and in *dfmr1* mutant embryos. Based on these results we propose that dFMRP-dependent regulation of CCT subunits is required for cleavage furrow formation and that at least one of its substrates is affected in *dfmr1*- embryos suggesting dFMRP-dependent regulation of CCT contributes to the cleavage furrow formation phenotype.

Keywords

Fragile X Syndrome; FMRP; *Drosophila*; midblastula transition; cleavage; cellularization

Introduction

Fragile X Syndrome (FXS) is the most common form of heritable mental retardation in humans as well as a leading known cause of autism. FXS results from the reduced activity of

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Fragile X Mental Retardation Protein (FMRP) as a consequence of either the transcriptional silencing of or mutations within the *FMR1* gene. FMRP is a selective RNA-binding protein that is implicated in the development and maintenance of neuron morphology and function. FXS is thought to result from the aberrant translational regulation of potentially hundreds of mRNAs causing defects in neuron morphology and synapse function (Brown et al., 2001). Efforts have been made to identify target mRNAs using a variety of approaches including bioinformatics, immunoprecipitation and microarray analysis, antibody-positioned RNA amplification, and proteome analysis (Brown et al., 2001; Darnell et al., 2001; Liao et al., 2008; Miyashiro et al., 2003; Schaeffer et al., 2001; Todd et al., 2003). While these approaches have identified many potential candidate RNAs, few direct targets have been shown to be physiologically relevant *in vivo* targets underscoring the importance of validating candidates *in vivo*.

It is still unclear how FMRP associates with its target mRNAs and at what stage of translation FMRP regulates its targets. FMRP has been reported to affect translational elongation of its target mRNAs (Khandjian et al., 2004; Stefani et al., 2004) as well as the initiation of translation of target mRNAs (Ishizuka et al., 2002; Monzo et al., 2006; Siomi et al., 1996; Zalfa et al., 2003). These differences likely reflect the complexity by which FMRP mediates its regulation, and identification of new targets will likely give new insights into mechanism of its regulation.

Drosophila has a single *fmr1* ortholog that has been characterized in the larval and adult nervous system (Zhang et al., 2001). Roles for dFMRP outside of the nervous system have been identified during oogenesis (Costa et al., 2005; Epstein et al., 2009), pole cell formation (Deshpande et al., 2006), and spermatogenesis (Zhang et al., 2004), but its function during these processes is unclear. A handful of direct targets of dFMRP regulation that have been identified and verified in neurons are know regulators of the microtubule and actin cytoskeletons: *futsch*, *Rac1*, and *chickadee* (*Profilin*) (Lee et al., 2003; Reeve et al., 2005; Xu et al., 2004; Zhang et al., 2001). Among these, *futsch* (*MAP1B*) and *Rac1* have also been identified as targets of FMRP in mice (Castets et al., 2005; Lee et al., 2003). Thus, dFMRP likely has roles in regulating the cytoskeleton in both mammalian and fly neurons through the regulation of common mRNA targets.

We have previously found a role for translational regulation by dFMRP during cleavage furrow formation at the midblastula transition (Monzo et al., 2006). This is a developmental process that is well suited for the study of dFMRP function. First, cleavage furrow formation relies on some of the same cytoskeletal proteins that are regulated by dFMRP in the neuron. In addition, the midblastula transition is characterized by a shift from maternal to zygotic genetic control, and this shift involves a tremendous amount of post-transcriptional regulation of both maternal and zygotic mRNAs (Tadros et al., 2007).

Cleavage furrow formation is a specialized form of cytokinesis, often referred to as cellularization, whereby cortically positioned nuclei are encapsulated by invaginating plasma membrane furrows. This process is known to require a dramatic reorganization of the actin and microtubule cytoskeletons, and these cytoskeletons are thought to work in concert to mediate furrow formation. It is thought that the cellular phenotype observed in *dfmr1* mutant embryos results from the cumulative misregulation of a large number of dFMRP targets.

In this study, a comparative proteomics-based approach was used to identify targets of dFMRP regulation during furrow formation at the midblastula transition. Here, we describe the results of this screen and focus on a new group of dFMRP targets, subunits of the Chaperonin containing TCP-1 (CCT) complex, and the effect of their misregulation on

Drosophila cleavage furrow formation. We have also found that the septin Peanut is a conserved substrate of CCT in fly and is misexpressed in *cct* and *dfmr1* mutant embryos. The discovery of these new targets of dFMRP regulation will likely provide insights into the mechanisms for FMRP translational regulation and the general pathways that FMRP may impinge upon to affect cellular morphologies.

Materials and methods

Stocks and Genetics

Stocks used in this study were maintained on standard corn meal molasses media at 25°C. *w; dfmr1^{3}/TM6C, Tb, Sb* was a gift from T. Jongens (University of Pennsylvania). The following stocks were provided by the Bloomington Stock Center and the gene disrupted in the stock that is relevant to this study is noted in parenthesis next the stock name.

w^{1118} and *Oregon-R* (wild type)

w^{1118}; TM3, Sb/CxD

y^{1}w; RpS13^{1}/CyO

w^{1118}; Df(3R)Exel6265, P{w[+mC]=XP-U}Exel6265/TM6B Tb, Sb^{1} (*dfmr1*)

w^{1118}; Df(3R)Exel6191, P{w[+mC]=XP-U}Exel6191/TM6B, Tb^{1} (*cct1*)

w^{1118}; Df(3R)Exel6270, P{w[+mC]=XP-U}Exel6270/TM6B, Tb^{1} (*cct3*)

w^{1118}; Df(2L)Exel6034, P{w[+mC]=XP-U}Exel6034/CyO (*cct4*)

w^{67c23}P{w[+mC]=lacW}Tcp-1zeta[G0057]/FM7c (*cct6*)

w^{1118}; Df(3R)Exel6150, P{w[+mC]=XP-U}Exel6150/TM6B, Tb^{1} (*cct7*)

cn^{1}P{ry[+t7.2]=PZ}l(2)03996/CyO; ry^{506} (*cct8*)

w^{1118}; Df(2R)Exel6052, P{w[+mC]=XP-U}Exel6052/CyO (*scra*)

To limit variation in genetic background between control and mutant embryos for the proteomic screen, single flies from *w; dfmr1^{3}/TM6C, Tb, Sb* and *w^{1118}; Df(3R)Exel6265/ TM6B Tb, Sb^{1}* and *w^{1118}* were outcrossed to *w^{1118}; CxD/TM3* over three generations, and stocks were established from single flies.

To generate *dfmr1*- embryos, *w; dfmr1^{3}/TM6C, Tb, Sb* virgin females were crossed to *w^{1118}; Df(3R)Exel6265/TM6B Tb, Sb^{1}* males, and *dfmr1*- embryos were collected from *w; dfmr1^{3}/ Df(3R)Exel6265* virgin females mated to *Oregon-R* males.

To generate *cct*- embryos, the deficiency stocks removing *cct1* (*w^{1118}; Df(3R)Exel6191/ TM6B, Tb^{1}*) and *cct4* (*w^{1118}; Df(2L)Exel6034/CyO*) were crossed to produce *w^{1118}; Df(3R)Exel6191/+; Df(2L)Exel6034/+* progeny. Virgin females of this genotype were crossed to *w^{67c23}P(Tcp-1zeta)/FM7c* males. *cct*- embryos were collected from virgin *w^{67c23}P(Tcp-1zeta)/+; Df(2L)Exel6034/+; Df(3R)Exel6191/+* (*cct1*, *cct4*, and *cct6* triple heterozygote) virgin females mated to *Oregon-R* males.

Two dimensional difference gel electrophoresis and mass spectrometry (2D DIGE/MS)

Embryos were collected from *w^{1118}; dfmr1^{3}/Df(3R)Exel6265* (*dfmr1*-) and *w^{1118}; +/ Df(3R)Exel6265* (control), and mid-cellularizing embryos were hand sorted and frozen as described in (Gong et al., 2004). Mutant and control proteins were labeled and separated in 2D gels as described in (Gong et al., 2004; Viswanathan et al., 2006). In brief, equal masses (100-150 μg) of total protein from control and mutant embryos were labeled with Cy3-NHS

or Cy5-NHS, subjected to isoelectric focusing on 13 cm, pH 3-10 non-linear Immobiline strips according to the manufacturer's protocol (Amersham Biosciences), and separated through 10-15% SDS-PAGE gradient gels. For each experiment control and mutant proteins were reciprocally labeled in parallel: Cy3-NHS-labeled control/Cy5-NHS-labeled mutant and Cy5-NHS-labeled control/Cy3-NHS-labeled mutant. To control for loading error, 1 μg of BSA was added to each sample prior to labeling. Gels were imaged with an imager that has an integrated robotic gel cutter. Gel images were analyzed using IPLab (Scanalytics) and QuickTime and protein intensities were quantified using SExtractor (https://sourceforge.net/projects/sextractor) as described in (Viswanathan et al., 2006). Proteins of interest were excised from gels and mass spectrometry fingerprint analysis and protein identification was performed as described in (Gong et al., 2004).

Immunoprecipitations

WT and *dfmr1*- mid-cellularizing embryos were collected, dechorionated, and frozen. Because *dfmr1*- embryos are slightly developmentally delayed, *dfmr1*- embryos were allowed to age an additional 15 minutes (~2.25-3.25hr for WT and ~2.5-3.5hr for *dfmr1*-). A sample of each embryo collection was checked to ensure equivalent staging between the two genotypes. Anti-dFMRP IPs were performed using extracts made from the embryos [10,000 × *g* supernatants (S10) in TKT_{100} buffer (10 mM Tris–HCl pH 8.0, 100 mM KCl, 0.05% Triton X-100, 1 mM DTT, 50 units/ml RNasin, protease inhibitors)] as described in (Monzo et al., 2006). Anti-PNUT IPs were performed as follows. 250 μg WT and *dfmr1*- S10 was incubated with 0.75 μg Rabbit anti-PNUT antibody for 10 hrs at 4°C. A mock-IP was performed with WT extract and no antibody. 25 μl BSA-coated protein G Sepharose 4B (Sigma) was added to each mixture for 4 hrs at 4°C. After supernatants were removed, beads were washed 5 times in TKT_{100}, eluted with SDS/PAGE sample buffer at 90°C for 2 minutes, and analyzed by Western blot.

Reverse Transcription Quantitative PCR (RT-qPCR)

Total RNA was extracted from embryos, immunoprecipitated material and corresponding input S10 extracts using TRIzol-LS reagent (Invitrogen, Carlsbad, CA). 1 μg of total embryonic RNA or 100 ng of total immunoprecipitated or S10 RNA were digested with amplification grade DNase I (Invitrogen) according to manufacturer's protocol followed by reverse transcription using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems (ABI), Foster City, CA) including RNase Inhibitors (Promega, Madison, WI). Quantitative PCR was performed in 384-well plates with Power SYBR PCR Master Mix in a 7900HT Sequence Detector (ABI). Levels of specific RNAs were quantified as in (Monzo et al., 2006). Primer sequences are listed in supplemental material.

Gel Filtration Chromatography

WT and *dfmr1*- embryos were collected and extracts were prepared as described for IPs above. Between 1.0 and 1.3 mg of total protein from each genotype was separated by gel filtration chromatography on a Superose 6 column (GE Healthcare) at a rate of 0.2 ml/min with 1 ml fractions collected over the entire run. Fractions were precipitated using Trichloroacetic acid, resuspended in SDS/PAGE sample buffer, and analyzed by Western blot. Molecular weight standards were separated through column prior to embryonic extracts.

Antibodies

The following antibodies and stains were used in this study: anti-dFMRP (6A15; AbCam), anti-alpha Tubulin (DM1A, Sigma), anti-PNUT [1:500; KEKK (Field et al., 1996)], anti-PNUT (no dilution; 4C9H4, Developmental Studies Hybridoma Bank), anti-Anillin (1:500;

C. Field, Harvard Medical School), and anti-CCT1 (1:500; 91A, AbCam). Dilutions in parenthesis indicate those used for IF analysis on fixed embryos. Typically, a 100-fold dilution of the dilution for IF worked well for immunoblot analysis.

Live Analysis

Embryos were collected from wild type or mutant females mated to wild type males at 25°C. Analysis of cellularization was done as described in (Monzo et al., 2006). Embryos were imaged using a Zeiss AxioVert 200 microscope with a 40× EC Plan Neofluar objective equipped with differential interference contrast optics.

Fixed Immunofluorescence Analysis

Fixed analysis was performed as described in (Monzo et al., 2006). Rhodamine-Phalloidin (Cytoskeleton, Inc.) was used to detect actin. Primary antibodies were detected with affinity-purified Alexa Fluor® secondary antibodies (Molecular Probes). Embryos were mounted in Vectashield (Vector Laboratories) and imaged using a Leica SP2-LCS confocal microscope.

Results

Comparative proteomic analysis of WT and *dfmr1*- cleavage stage embryos

The goal of this study was to identify direct and indirect targets of dFMRP-dependent translational regulation that contribute to the morphogenesis defects observed during cleavage furrow formation in *dfmr1* mutant embryos (Monzo et al., 2006). To identify proteins that are differentially expressed in embryos derived from $fmr1^{3}/Df(3R)Exel6265$ females (after this referred to as *dfmr1*- embryos) compared to wild type cleavage stage embryos, we performed a proteomic screen using 2D difference gel electrophoresis followed by mass spectrometry (2D DIGE/MS) (Viswanathan et al., 2006). This approach has been used previously to identify dFMRP targets in fly brain and testis (Zhang et al., 2005; Zhang et al., 2004). Forty-five protein spots changed reproducibly in *dfmr1*- cleavage stage embryo lysates representing a small fraction of total protein spots observed in our gel conditions (Figure 1B). Of these, twenty-eight proteins were identified by Maldi-TOF mass spectrometry with fold changes in abundance ranging from 1.3 to 56.7 (Table 1). The majority of proteins (13/28) increased in abundance in the mutant lysates; five decreased in abundance; and ten shifted in pI and/or molecular weight. The 28 proteins can be classified into five general gene ontology categories based on reported function, including metabolism, protein stability, translation, cytoskeleton and membrane trafficking, and unknown (Figure 1D).

Secondary analysis of candidate targets to identify direct targets

The 28 proteins identified by 2D DIGE/MS represent potential direct and indirect targets of dFMRP-dependent translational regulation. We performed a secondary screen to identify, among the candidate targets, the proteins whose mRNAs specifically associate with dFMRP and could represent direct targets of dFMRP regulation. The amount of mRNA corresponding to the difference proteins in dFMRP immunoprecipitations (IPs) from WT and *dfmr1*- cellularizing embryo extracts was quantified and normalized to the amount of a standard control mRNA (*RpL32*) (Figure 2). Based on previous studies, we considered an enrichment of at least two-fold of the mRNA in WT compared to *dfmr1*- IPs significant and suggests the mRNA associates with dFMRP and is subject to direct translational regulation by dFMRP. The fold enrichment of a previously described target, *trailer hitch* (*tral*), was measured as a positive control (Monzo et al., 2006). In these experiments *tral* mRNA was enriched 3.5 fold in WT IPs (Figure 2). The mRNAs for five candidates were at least two-fold enriched: *CG5525*, *Cctγ*, *Tcp-1η*, *β'cop*, and *Aats-gly*. Although this analysis does not

exclude the possibly that the mRNAs indirectly associate with dFMRP through the direct association with another RNA-binding protein, it does suggest that the mRNAs are present in a dFMRP-containing ribonucleoprotein complex. In addition, the mRNA sequence of all the potential candidates was analyzed using the algorithm RNABOB (www.genetics.wustl.edu/eddy/software) for G-quartet motifs, a known dFMRP binding motif (Darnell et al., 2001). This analysis revealed putative G-quartets in the coding sequence of *CG5525*, *Cctγ*, *Tcp-1η*, and *β'cop* mRNAs (Table 2), however we have not tested if these sequences contribute to their association with dFMRP. Together, these observations suggest that *CG5525*, *Cctγ*, *Tcp-1η*, *β'cop*, and *Aats-gly* are potential direct targets of dFMRP regulation.

Three subunits of the CCT complex are misregulated in *dfmr1-* embryos

Protein sequence analysis showed that *CG5525*, *Cctγ*, and *Tcp-1η* encode three of the eight subunits of the Chaperonin Containing TCP-1 (CCT) complex [also referred to as TCP-1 or TCP-1 Ring Complex (TRiC)]. The genes encoding the other five subunits were also identified within the *Drosophila* genome based on protein sequence homology (Figure 3). In this study, we will refer to the complex as CCT and the independent eight subunits as CCT1-8 for simplicity. CCT is a group II chaperonin that assists in the folding or in the assembly of protein substrates in an ATP-dependent manner (Liou and Willison, 1997). The CCT holocomplex is composed of two rings containing eight distinct subunits, and each subunit is expressed stoichiometrically from an individual gene. The eight different cct genes encode conserved regions and a variable region, termed the apical domain, which confers substrate specificity (Liou and Willison, 1997). Although CCT was initially thought to exclusively fold actin and tubulin, between 2-7% of cytosolic proteins have been recently identified to interact with CCT in yeast and mammalian cells (Dekker et al., 2008;Yam et al., 2008). Most of these newly identified CCT substrates are functionally and structurally diverse and cannot be easily predicted based on function or sequence alone. All eight of the *cct* genes are essential for eukaryotic cell viability, and it has been shown that temperature sensitive (ts) alleles of *cct4* in budding yeast cause cytokinesis defects, a cellular process analogous to *Drosophila* cellularization (Dekker et al., 2008). A comprehensive comparison of the *D. melanogaster* CCT protein sequences with those of *S.cerevisiae* and *H. sapiens* reveals a high degree of conservation for each (Figure 3), suggesting conserved functionality. Interestingly, the *D. melanogaster cct* sequences are more conserved with the *cct* sequences of *H. sapiens* than *S.cerevisiae* (Figure 3). We tested for the association of dFMRP with the mRNAs for five subunits not identified in the screen (*cct1*, *2*, *5*, *6* and *8*) and found the mRNAs do not associate with dFMRP during the cleavage stage, suggesting dFMRP does not regulate all subunits of the CCT complex (Figure 2). Because three subunits (CCT3, CCT4, and CCT7) of this complex are misexpressed in *dfmr1-* embryos, we chose to examine if their misregulation is relevant to the *dfmr1-*furrow formation phenotype.

CCT is required for proper cleavage furrow formation

In order to assess the requirement for CCT during fly development, we examined cleavage furrow formation in embryos laid by females lacking a single copy of one or three different cct genes. While all WT embryos completed furrow formation in about 55 minutes, half of the embryos examined from females simultaneously lacking a copy of *cct1*, *cct4*, and *cct6* (*cct-*) displayed a delay in furrowing rate (Figure 4A) similar to what is observed in the majority of *dfmr1-* embryos (Monzo et al., 2006). One embryo displayed moderate disruptions in nuclear morphology although furrowing rates appeared normal (data not shown). Furrowing proceeded normally in embryos derived from single heterozygous females of *cct1*, *cct3*, *cct4*, *cct6*, *cct7*, and *cct8* (data not shown). If the decrease in CCT4 abundance is relevant to the *dfmr1-* phenotype, we expected to observe an enhancement of

the phenotype. Significantly, when a single copy of *cct4* is removed in a *dfmr1*- background, the percentage of embryos displaying severe disruptions in furrow formation was enhanced from 25% (*dfmr1*-) to 75% (*cct4-/+*, *dfmr1*-) (Figure 4B). A similar effect was observed in *cct6-/+*, *dfmr1*- (Figure 4B). Genetic interactions between *dfmr1* and *cct3* or *cct7*, the other CCT subunits identified in the screen, were not tested because all three genes reside on the same chromosome resulting in difficulties generating necessary stocks. The interactions with *cct4* and *cct6* were specific as removing a copy of a general translational regulator, ribosomal protein gene (*RpS13*), or a known effector of cleavage furrow formation, the scraps gene, which encodes Anillin, does not enhance the *dfmr1*- severe furrow formation phenotype (Figure 4B). Together, these observations suggest that the misregulation of CCT in *dfmr1*- embryos is an important contributing factor to the furrow formation phenotype.

CCT holocomplex assembly is disrupted in *dfmr1*- embryos

Based on the initial observation that dFMRP is required for the proper expression of CCT subunits and that CCT is required for proper cleavage furrow formation, we predicted that CCT function would be compromised in *dfmr1*- embryos. To test if the CCT holocomplex is disrupted in *dfmr1*- cleavage stage embryos, embryonic extracts were separated by gel filtration chromatography and subjected to immunoblot analysis. The CCT1 antibody used in this study was previously generated against a rat CCT1 peptide and detects a single band with a molecular weight of the predicted size of approximately 60 kDa in cleavage stage embryo extracts (Figure 5A). The abundance of CCT1 was not affected in *dfmr1*- embryos, as quantified in 1D SDS-PAGE gels (Figure 5A). The majority of CCT1 in WT and *dfmr1*- extracts fractionated near 660 kDa (fraction 11/20, Figure 5B). Interestingly, about 40% of CCT1 in *dfmr1*- extracts fractionated near 60 kDa (Figure 5B). This fractionation profile was observed in multiple runs. Consistent with these results, it was previously shown that reduction of a single CCT subunit by siRNA in mouse fibroblasts disrupts assembly of the other CCT subunits into the holocomplex (Grantham et al., 2006). These observations suggest that in WT cleavage stage embryos the vast majority of CCT1 is incorporated into a large holocomplex, and in *dfmr1*- embryos the assembly of CCT holocomplex is at least partially disrupted with some CCT1 left in monomeric or unassembled form.

To further characterize the nature of the disruption of the CCT complex in *dfmr1*-embryos, we analyzed fixed cleavage stage *dfmr1*- embryos to assess subcellular localization of the CCT complex. In WT embryos CCT, as detected with the antibody against CCT1, was observed in a diffuse punctate pattern throughout the cytoplasm and to a somewhat lesser extent in the nuclei (Figure 5C). This general pattern was very similar in *dfmr1*- embryos except that larger ectopic puncta of CCT were observed in the apical cytoplasm (Figure 5C). These ectopic puncta were never observed in WT and may represent CCT1 that is not assembled into CCT holocomplex, consistent with the fractionation profile of CCT1 in *dfmr1*- embryos. Together the results of the CCT fractionation and fixed analysis of *dfmr1*- embryos suggest that CCT holocomplex does not assemble properly in *dfmr1*- embryos due to a misregulation of individual subunit stoichiometry which may lead to reduced or abnormal CCT function.

The septin Peanut is mislocalized in *cct*- and *dfmr1*- cleavage stage embryos

CCT substrates are functionally diverse. Although CCT was initially thought to exclusively fold actin and tubulin, between 2-7% of cytosolic proteins have been recently identified to interact with CCT in yeast and mammalian cells (Dekker et al., 2008; Yam et al., 2008). Recently, the septins and septin effectors were identified as a new class of physiologically relevant substrates in yeast by virtue of their physical association and genetic interaction with CCT and *cct* genes (Dekker et al., 2008). The septins belong to an important protein family that interacts with the actin and microtubule cytoskeletons and also functions to

organize cellular membranes (Weirich et al., 2008). Septins are essential for cell cycle progression and cytokinesis (Hartwell, 1971), and cleavage furrow formation defects have been reported in mutants of the *Drosophila* septin, *peanut* (Adam et al., 2000). Conditional alleles of *cct* genes cause ectopic localization of the septin Cdc3p and have low penetrance cytokinesis defects in budding yeast (Dekker et al., 2008). In addition, CCT loss of function in yeast does not affect *de novo* septin polypeptide folding, and it appears to be involved in regulating the assembly of septin filaments (Dekker et al., 2008).

To test if the septins are conserved substrates of CCT in the fly, we assessed Peanut (PNUT) localization in *cct*- cleavage stage embryos. In fixed WT embryos, the septin PNUT tightly localized to the leading edge of the furrow front along with F-actin. In fixed *cct*- embryos, the majority of PNUT localized to the furrow front as it did in WT, in addition to ectopic accumulation along the lateral membranes (Figure 6A). This ectopic accumulation is reminiscent of what is observed in yeast *cct4* ts mutants. In WT budding yeast cells undergoing division septins tightly localize to the neck between the mother and bud. In *cct4* ts mutants expressing a Cdc3p-GFP at the restrictive temperature, the GFP is seen as the neck as well at other parts of the plasma membrane (Dekker et al., 2008). We also assessed PNUT localization in *dfmr1*- embryos. Again, PNUT was seen localized to the furrow front as in WT embryos but also ectopically localized along the lateral membranes to an even greater extent than what was observed in *cct*- embryos (Figure 6B). Importantly, F-actin and other F-actin binding proteins, such as Anillin and Myosin II, that normally localize to the furrow front were not affected in these mutants (6A and B and data not shown), suggesting that the effect on PNUT localization is specific and is not a secondary effect from a general disorganization of the cortical membrane. In addition to F-actin, microtubules appear normal by immunofluorescence in fixed *dfmr1*- embryos (data not shown) suggesting that any effect on CCT function in the mutants does not perturb its known requirement for folding these substrates.

Finally, to further investigate the possibility that PNUT is a *bona fide* substrate of CCT in flies, we performed IPs to test if CCT and PNUT physically associate. A small amount of CCT1 specifically co-IPd with PNUT in WT cleavage stage embryos, consistent with PNUT being a substrate of CCT (Figure 6C). This interaction appears to be slightly disrupted in *dfmr1*- embryos as the amount of CCT that co-IPd with PNUT was slightly decreased (Figure 6C). Together, these observations suggest that septins are a likely conserved substrate of CCT in flies and that the ectopic localization of PNUT in *dfmr1*- embryos is due to the misregulation of CCT and may at least partially contribute to the *dfmr1*- cleavage furrow formation phenotype.

Discussion

The identification of new targets of FMRP translation regulation will allow for a better understanding of how loss of function of FMRP affects various cellular processes. To date, a limited number of physiologically relevant targets have been identified. Here, a comparative proteomic approach in *Drosophila* cleavage stage embryos was used to identify new targets of dFMRP-dependent regulation. Twenty-eight proteins showed differences in abundance between WT and *dfmr1*- embryos, representing potential direct and indirect targets of dFMRP. We focused on the misregulation of the chaperonin CCT in *dfmr1*- cleavage stage embryos because three of the eight CCT subunits are misregulated in the absence of dFMRP, suggesting the regulation of this complex is potentially relevant during the cleavage stage. Interestingly, the identification of three subunits of the CCT complex as targets of dFMRP regulation suggests that dFMRP is required for the coordinated expression of various subunits of a multi-subunited complex. In addition, because CCT has a complex set of substrates itself, these results are consistent with the possibility of a tiered or hierarchical

model for regulation by FMRP, whereby modulation of expression of one target affects the expression of an additional class of secondary targets. Consistent with this, we have previously shown that dFMRP regulates the expression of a known translational regulator, *tral*, whose targets are at this point unknown (Monzo et al., 2006).

dFMRP and classes of targets

The majority of targets identified in this screen are generally involved in metabolism. Many proteins that can be easily and reproducibly identified using 2D DIGE/MS are present at relatively high abundance and include many metabolic proteins. Such targets are still potentially relevant based on observations made in other studies. Three targets identified in this screen were also identified in 2D DIGE screens performed using *Drosophila* testis or heads (Table 1). One of these proteins, Jafrac1 or Thioredoxin, is involved in oxidative stress response, and it has been observed that *FMR1* mutant mice are sensitive to oxidative stress suggesting that the stress response is perturbed in the mutants (el Bekay et al., 2007). Two other proteins with known or predicated roles in oxidative stress response, Superoxide dismutase (SOD) and the protein encoded by CG6045, were found to be misregulated in *dfmr1*- embryos in this screen, suggesting that modulation of the oxidative stress response pathways may be regulated at least in part by dFMRP. Furthermore, *SOD1* was previously identified as a target of FMRP in mouse neurons (Miyashiro et al., 2003). The identification of these targets is consistent with reported observations of known FMRP targets.

About 18% of the identified targets are involved in regulation of the actin and microtubule cytoskeletons and membrane trafficking, pathways that are known to be important for cleavage furrow formation and neuron morphology. *β′cop* represents a potentially interesting target of dFMRP. β′COP is a subunit of the COPI coat which is required for forming vesicles that are destined for retrograde trafficking from the Golgi to the endoplasmic reticulum (Girod et al., 1999). In addition to the reorganization of the actin and microtubule cytoskeletons, microtubule-based targeted membrane secretion is required for *Drosophila* embryonic cleavage furrow formation (Lecuit and Wieschaus, 2000; Sisson et al., 2000). Proper trafficking between the various endomembrane compartments is crucial for proper furrow formation, and this trafficking also seems to be important in the maintenance of neuronal morphology and function (Kennedy and Ehlers, 2006). An effector of COPI vesicle formation, the GTPase activating protein Arf1 GAP, is essential for dendritic growth and outbranching (Moore et al., 2007). FMRP is also involved in dendritic growth and branching, suggesting a possible connection between the two pathways. Validation and further characterization of *β′cop* as a target of dFMRP could be important for understanding how FMRP affects membrane trafficking.

Aats-gly, also known as *glycyl-tRNA synthetase* (*gars*), was also identified as a target of dFMRP regulation. In the screen, Aats-gly was identified as a protein that shifts in pI in *dfmr1*- embryos, and overall protein abundance decreases in the mutant. We also showed that the *Aats-gly* mRNA likely associates with a dFMRP complex, suggesting it is a direct target of dFMRP. Glycyl-tRNA synthetase is important for catalyzing tRNA aminoacylation, a critical step in protein translation. *Aats-gly* is the ortholog of the human *GARS* gene that is associated with Charcot-Marie-Tooth neuropathy type 2D (CMT2D), a heritable disease that causes defects in motor and sensory neurons (Chihara et al., 2007). Similar to *dfmr1* mutants, *Aats-gly* mutants have defects in dendritic arborization as a result of perturbing protein translation (Chihara et al., 2007). The relationship between FMRP and Aats-gly expression may be relevant for both cleavage furrow formation during the midblastula transition as well as aspects of development in the nervous system.

Together, these findings suggest that genes normally thought of as functioning in a general ‘housekeeping’ capacity, such as those functioning in metabolic processes and protein

synthesis, may have more specific functions during development and cell maintenance and their precise regulation is important for cellular processes.

CCT and dFMRP regulation

Three subunits of CCT were identified in this screen suggesting that regulation of this complex is particularly important. Moreover, their mode of regulation by dFMRP is complex. CCT3 was identified as shifting in pI to a more basic state in the mutant extracts; CCT4 was identified as decreasing 2.6 fold in mutant extracts; and CCT7 was identified as increasing 1.4 fold in mutant extracts (Table 1). It should be noted that overall abundance of CCT3 was difficult to assess because the labeled spots were not well resolved. Despite the differences in how protein abundance is affected, dFMRP appears to associate with all three mRNAs, which all contain a known FMRP-binding motif, suggesting that dFMRP is conferring different types of regulation on the different transcripts. Further biochemical analysis of the interaction between the mRNAs and dFMRP will address if the observed association is direct or indirect.

The increase in abundance of CCT7 is most consistent with the prevailing idea that FMRP primarily functions as a negative regulator of translation and is required for the normal repression of *cct7*. If dFMRP directly regulates the expression of CCT4 or any of the proteins identified as decreasing in abundance in the mutant, it would suggest that dFMRP normally activates the translation of these targets. Although there have been reports consistent with the possibility that FMRP is a translational activator, there has been no demonstration of direct activation of target mRNAs (Brown et al., 2001). To test this, the translational competency of specific transcripts will need to be assessed. Many (~35%) of the proteins, including CCT3, identified shift in molecular weight and/or pI suggesting that normal post-translational modification is altered in the mutant lysates. This would likely result from indirect regulation by dFMRP, possibly by directly affecting the translation of a protein required for the normal post-translational modification of the identified proteins. Given our observation that dFMRP associates with the *cct3* mRNA, an alternative hypothesis is that *cct3* represents a normal direct target of dFMRP that is translated inappropriately in the wrong subcellular compartment in the absence of dFMRP. This translation could lead to an accumulation of an inappropriately post-translationally modified form of CCT3 in the absence of dFMRP. Alternatively, the overall abundance of the protein may be affected although this is difficult to discern due to the resolution of the different protein spots. It is known that proper stoichiometry of the subunits of the complex is important for CCT function, so it is formally possible that the misregulation of just one of the subunits is sufficient to disrupt the function of the complex in the *dfmr1*- embryos. Although the proposed models of regulation by dFMRP above suggest that expression of targets is primarily affected at the level of translation, it is possible that mRNA stability is affected in the *dfmr1* mutants.

Regardless of the mechanism of regulation of the *cct* subunits by dFMRP, the genetic data presented suggests that disruption of the CCT complex is relevant to the cleavage furrow formation phenotype observed in *dfmr1*- embryos. We have shown that CCT function is required for proper furrow formation. We have also shown that affecting CCT function by reducing the copy number of *cct4* or *cct6* enhances the severe *dfmr1*- cleavage furrow formation phenotype previously described (Monzo et al., 2006). It is possible that the perturbation of the levels of any of the subunits will affect CCT function, although we were unable to test for genetic interactions with all of the *cct* subunits due to technical limitations in generating necessary stocks. Taken together, we suggest that dFMRP likely uses different mechanisms to coordinately regulate the expression of different subunits of a common complex to affect cleavage furrow formation. FMRP may also employ this type of regulation to affect other forms of cell morphogenesis.

Septins and CCT

The septins are a known class of CCT substrates in yeast and are themselves required for cytokinesis and cleavage furrow formation (Adam et al., 2000; Field et al., 1996). We have found that the septins are a conserved family of CCT substrates in fly, and specifically, that the septin PNUT depends on CCT and dFMRP for its proper localization in the fly embryo. This observation is consistent with previous reports that in early *dfmr1*- embryos PNUT is mislocalized (Deshpande et al., 2006), although it is still unclear if the misregulation of the septin contributes to the furrow formation defects observed in both *cct*- and *dfmr1*- embryos. In addition, an interesting low penetrant phenotype that we have observed in *dfmr1*- embryos that was also reported to occur in *pnut*- embryos is a defect in the migration of the posterior midgut (Adam et al., 2000). Normally when furrow formation is almost complete the posterior midgut migrates over the dorsal side of the embryo, however in the mutants the migration occurs ventrally or to either lateral side. These observations are consistent with the idea that the misregulation of PNUT in *dfmr1*- has morphological consequences and that the indirect regulation of PNUT by CCT is critical for the formation of proper cleavage furrows during cellularization.

The genetic and biochemical relationships between FMRP, CCT, and septins may have significance in the nervous system as septins are known to localize to dendritic protrusions and branch points. Misregulation of septins also causes defects in dendritic morphology, similar to what is observed in FMRP mutants and FXS patients (Tada et al., 2007; Xie et al., 2007). It is tempting to speculate that misregulation of CCT could cause septin defects in neurons of *dfmr1* mutants and possibly in FXS patients.

Conclusions

Our work suggests that three of the eight *cct* mRNAs are misregulated in *dfmr1*-cleavage stage embryos. This appears to alter the normal stoichiometry of CCT subunits and in turn affects CCT complex assembly. The aberrant expression of CCT holocomplex in turn affects the assembly of some number of its substrates that are required for proper furrow formation, suggesting a multi-tiered regulatory system. We have identified at least one of these substrates as the septin PNUT. The misregulation of PNUT results in ectopic accumulation of PNUT to lateral membranes, where it may be affecting/impeding how the furrow forms and ingresses. It seems likely that the cleavage furrow formation phenotypes observed in *dfmr1*- embryos are not due to the misregulation of a single transcript or protein but rather the cumulative misregulation of many factors that are regulated in a hierarchical manner that contributes to the phenotype. Although this screen revealed an important set of targets, it was certainly not to saturation. Other strategies will need to be employed to get closer to identifying as many relevant targets as possible. The identification and characterization of new targets of FMRP will certainly be important for a more complete understanding the function of FMRP and the etiology of FXS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We dedicate this paper to the vibrant life and scientific career of Dr. John C. Sisson. John embraced all aspects of science with unparalleled tenacity and humor. We are all better people for having him as a colleague. He will be dearly missed.

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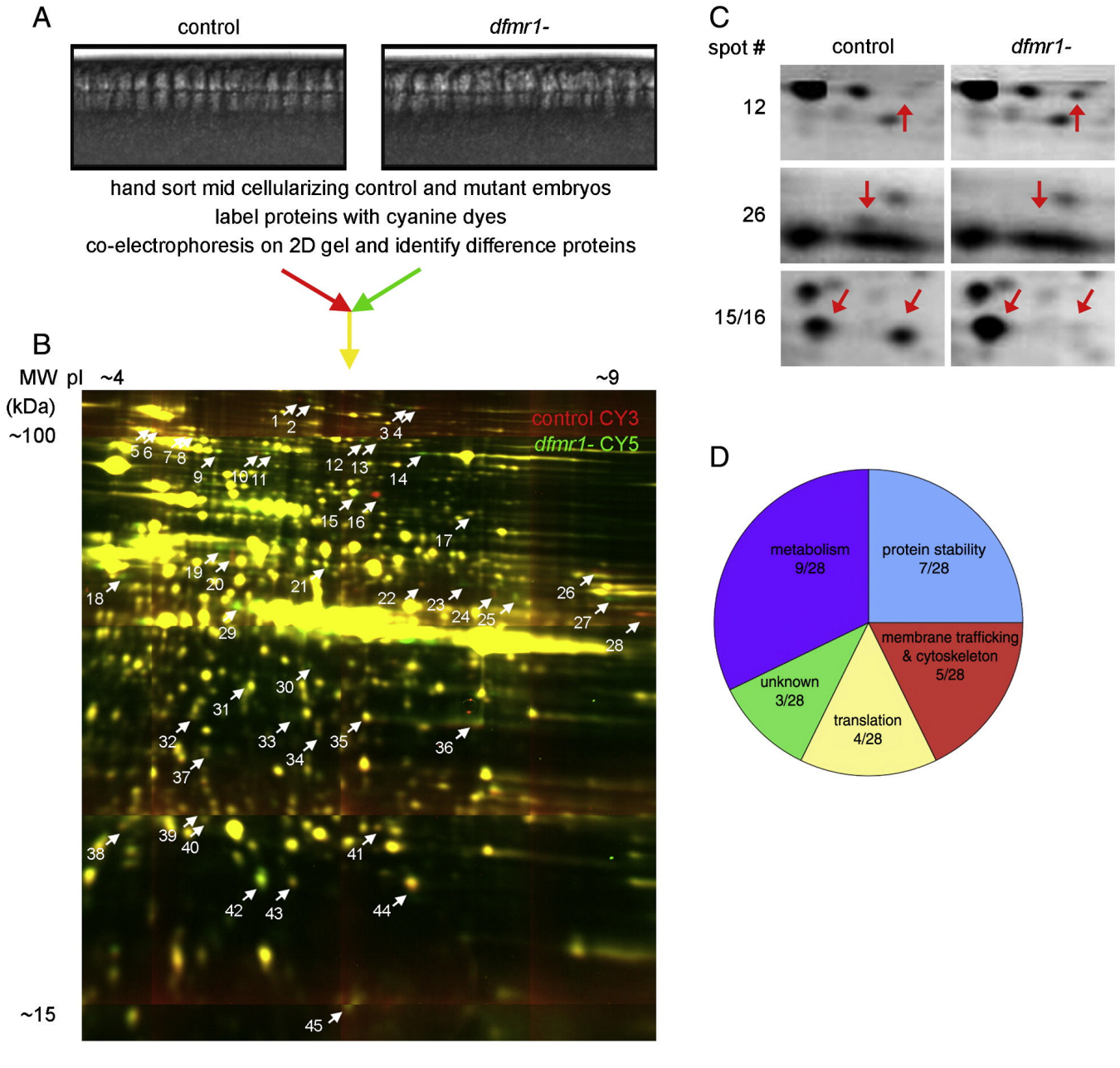

Figure 1. Comparative proteomic analysis of control and *dfmr1*- cleavage stage embryos

(A) Schematic showing general procedure for 2D DIGE analysis. Differential interference contrast (DIC) images of control and *dfmr1*- embryos show the morphological stage of sorted embryos. (B) A master gel is pseudocolored with control extracts labeled with CY3 (red) and *dfmr1*- extracts labeled with CY5 (green). The approximate isoelectric point (pI) is indicated at the top of the gel and the approximate molecular weight (MW) is indicated to the left. Difference spots are indicated with white arrow and numbered. The labeled difference spots were observed in at least four gel replicates. (C) High magnification examples of difference spots are shown with red arrows, and corresponding spot number in (C) is indicated to left. (D) The 28 difference proteins identified by mass spectrometry are categorized by proposed gene ontology.

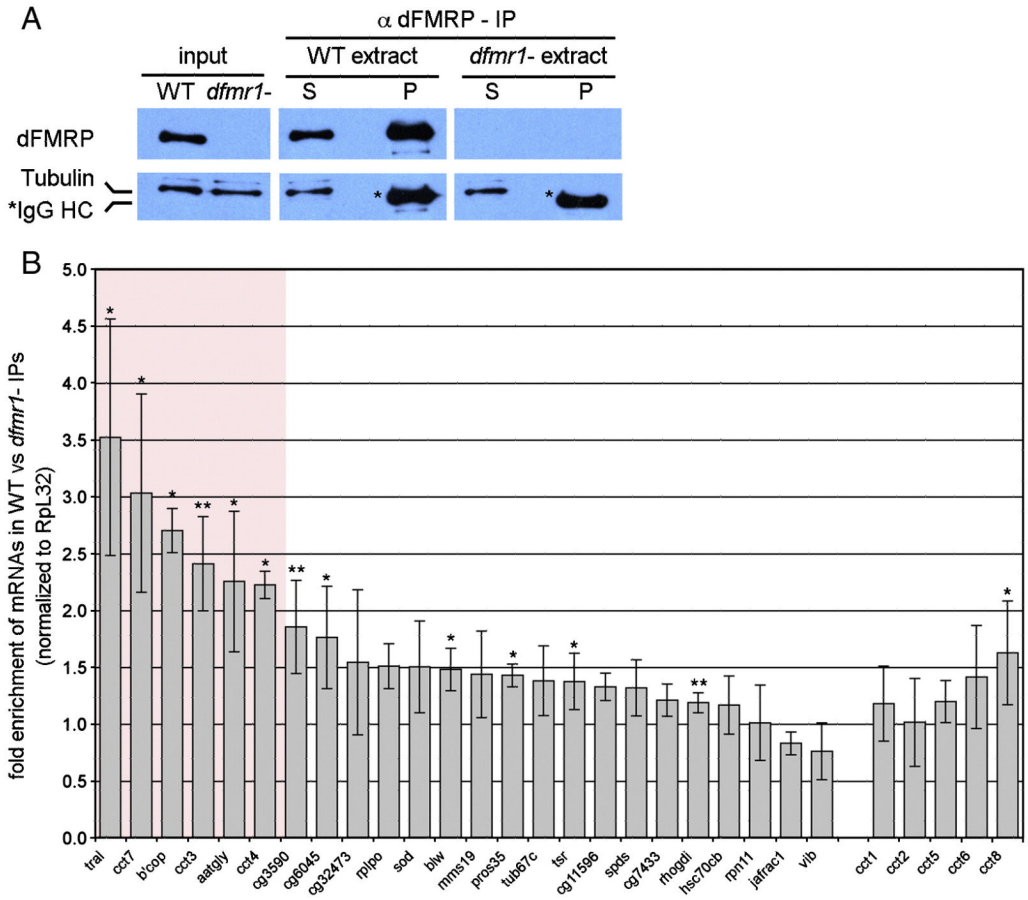

Figure 2. Immunoprecipitation of candidate target mRNAs with dFMRP

(A) Immnuoblots showing input and supernatant (S) and pellet (P) fractions of IPs performed using an anti-dFMRP antibody with WT and *dfmr1*- extracts probed with dFMRP and Tubulin antibodies. Although no Tubulin co-IPd with dFMRP, IgG heavy chain (HC) was present in equal volumes in the P fraction of each IP as indicated with asterisks. (B) RNA was extracted from P fractions shown in (A) and subjected to qRT-PCR. Histogram shows the fold enrichment of each mRNA in WT vs. *dfmr1*- IPs normalized to *RpL32*. *tral* mRNA is a know target of dFMRP and its enrichment was tested as a positive control in these experiments. Error bars indicate standard deviations (SD). Significance was assessed using the Student's *t* test (*, $P \leq 0.05$ and **, $P \leq 0.005$). Red box highlights those mRNAs that are at least 2-fold in enriched.

A

Chaperonin Containing T-Complex (CCT) or TCP-1 Ring Complex (TRiC) subunite									
Flybase gene name	CG Identifier	Subunit		MW (kDa)	PI	cytological position	%identity/similarity to *S. cerevisiae* homologs	%identity/similarity to human homologs	change in *dfmr1-*
Tcp1-like	CG5374	CCT1	TCP-1α	59.6	6.3	3R: 94B1	63 / 79	73 / 85	
	CG7033	CCT2	TCP-1β	58.1	5.6	X: 8C8	63 / 79	71 / 84	
Cctγ	CG8977	CCT3	TCP-1γ	59.4	6.9	3R: 89D6	59 / 76	69 / 83	shift right
	CG5525	CCT4	TCP-1δ	57.1	7.6	2L:33F3	58 / 79	70 / 86	decrease
Cct5	CG8439	CCT5	TCP-1ε	59.3	6.1	2R: 48E4	61 / 79	71 / 88	
T-cp1ζ	CG8231	CCT6	TCP-1ζ	58.2	6.6	X: 13E13-14	52 / 71	69 / 85	
Tcp-1η	CG8351	CCT7	TCP-1η	59.4	6.3	3R: 85B1-2	62 / 79	76 / 87	increase
	CG8258	CCT8	TCP-1Θ	59.4	4.9	2R: 44F5	42 / 64	62 / 79	

B

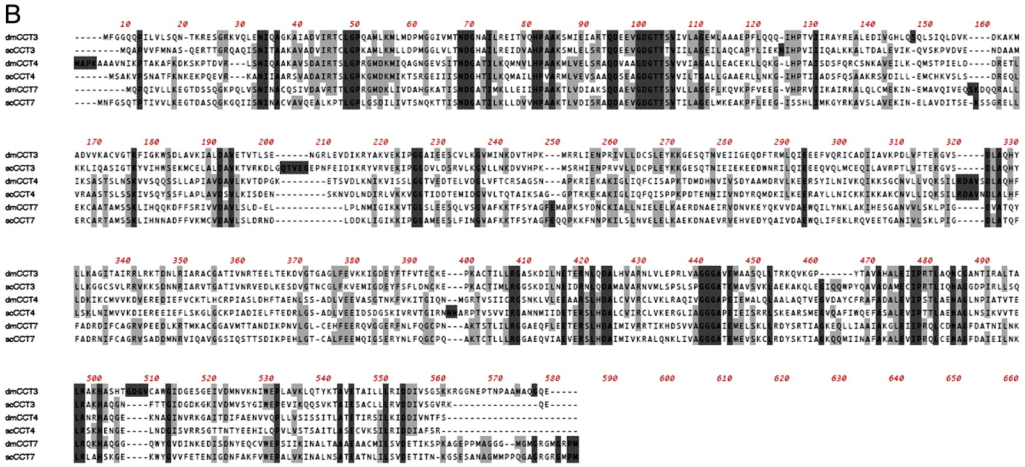

Figure 3. CCT subunits in *Drosophila melanogaster*

(A) Table shows known genetic and molecular information for each of the eight CCT subunits in fly. Percentages of identity and similarity in protein sequence for each of the subunits was compared between fly and budding yeast and fly and human. The protein difference detected in *dfmr1-* embryos the 2D DIGE gels is indicated for those proteins identified in the screen. (B) A Clustal-W alignment of the protein sequences of the fly (dm) and budding yeast (sc) CCT subunits affected in *dfmr1-* embryos shows regions of high conservation.

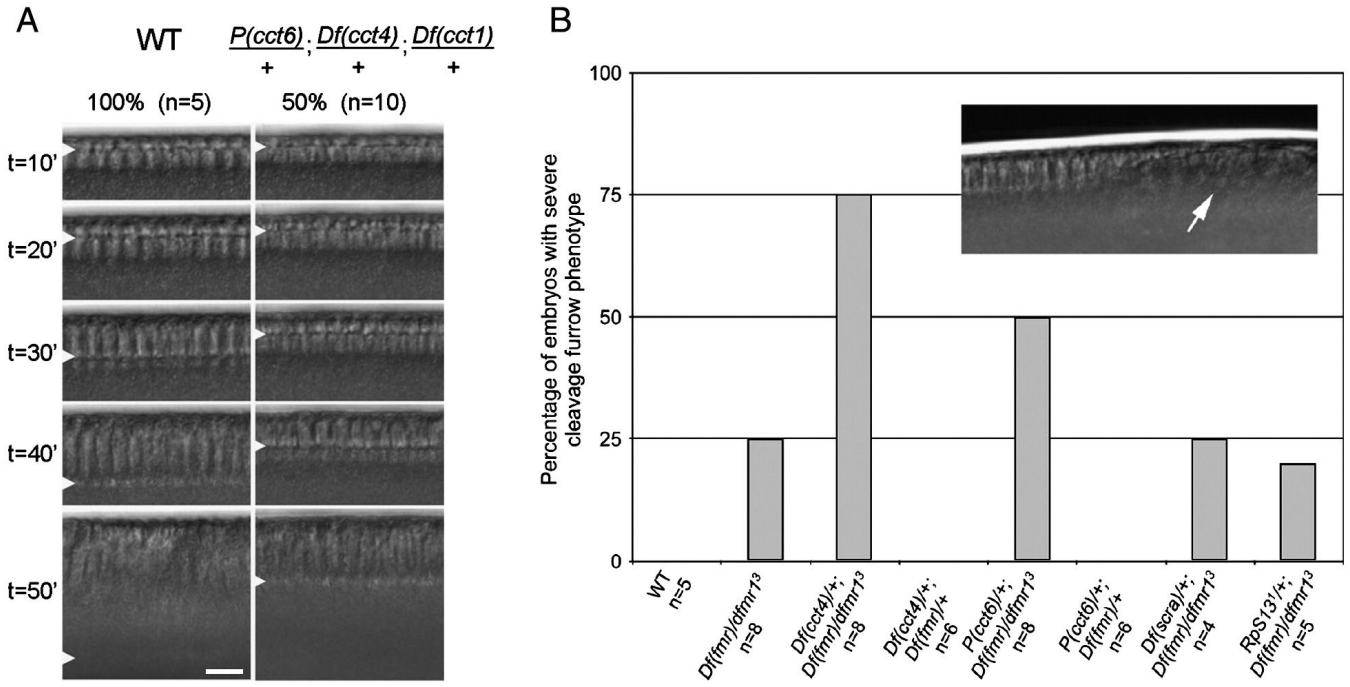

Figure 4. CCT is required for proper cleavage furrow formation and loss of *cct* enhances *dfmr1*-phenotype

(A) Frames from representative DIC movies of WT and *cct*- cleavage stage embryos. Time (t) is in minutes from the start of interphase of nuclear cycle 14. Percentage and number of embryos examined (n) with shown phenotype is indicated at top. Arrowheads and brackets indicate the furrow front position and nuclear elongation, respectively. Scale bar indicates 10 μm. (B) Bar graph shows percentage of embryos with severe cleavage furrow phenotype. Genotypes are indicated at the bottom with the total number of embryos observed (n). The inset is a representative DIC image of an embryo with a severe furrowing phenotype. Arrow indicates where furrow is completely disrupted.

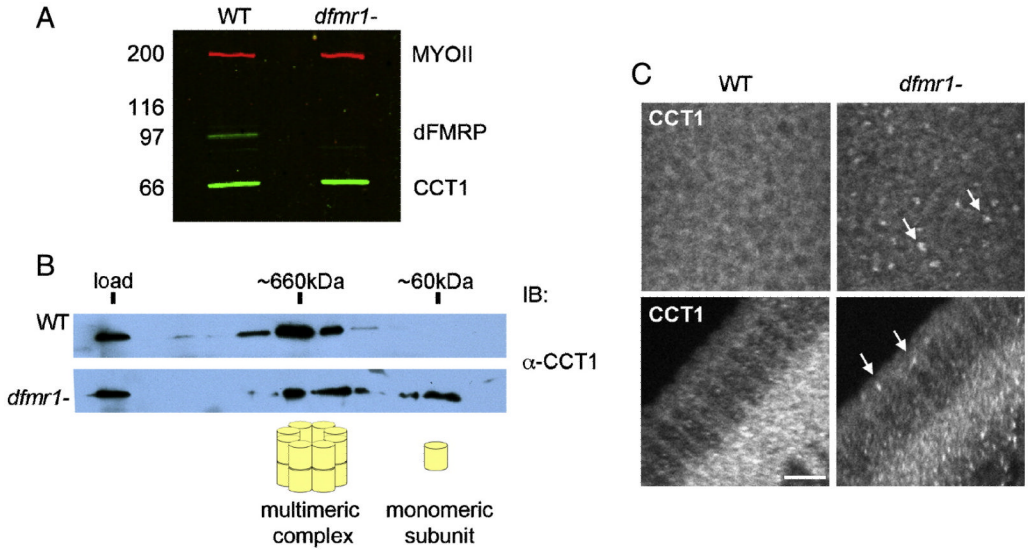

Figure 5. CCT holocomplex assembly is disrupted in *dfmr1*- embryos

(A) Quantitative immunoblot (IB) of WT and *dfmr1*- cleavage stage embryo extracts probed with antibodies against CCT1, dFMRP, and Myosin II (MYOII, loading control) indicated to right. MW in kDa is indicated to the left. (B) IB of fractions collected from 1.3 mg of WT and *dfmr1*- cleavage stage embryo extracts separated using a Superose 6 column and probed with anti-CCT1 antibody. 10 mg of protein loaded onto column is indicated at far left of blots. 660kDa complex was present in fraction 11 of 20. Cartoon representation of possible state of CCT complex formation indicated below blots. (C) Immunofluorescence (IF) analysis of fixed cleavage stage WT and *dfmr1*- embryos shows CCT1 localization in surface (top) and sagital (bottom) views. Arrows indicate abnormal accumulation of CCT1. Scale bar indicates 10 μm.

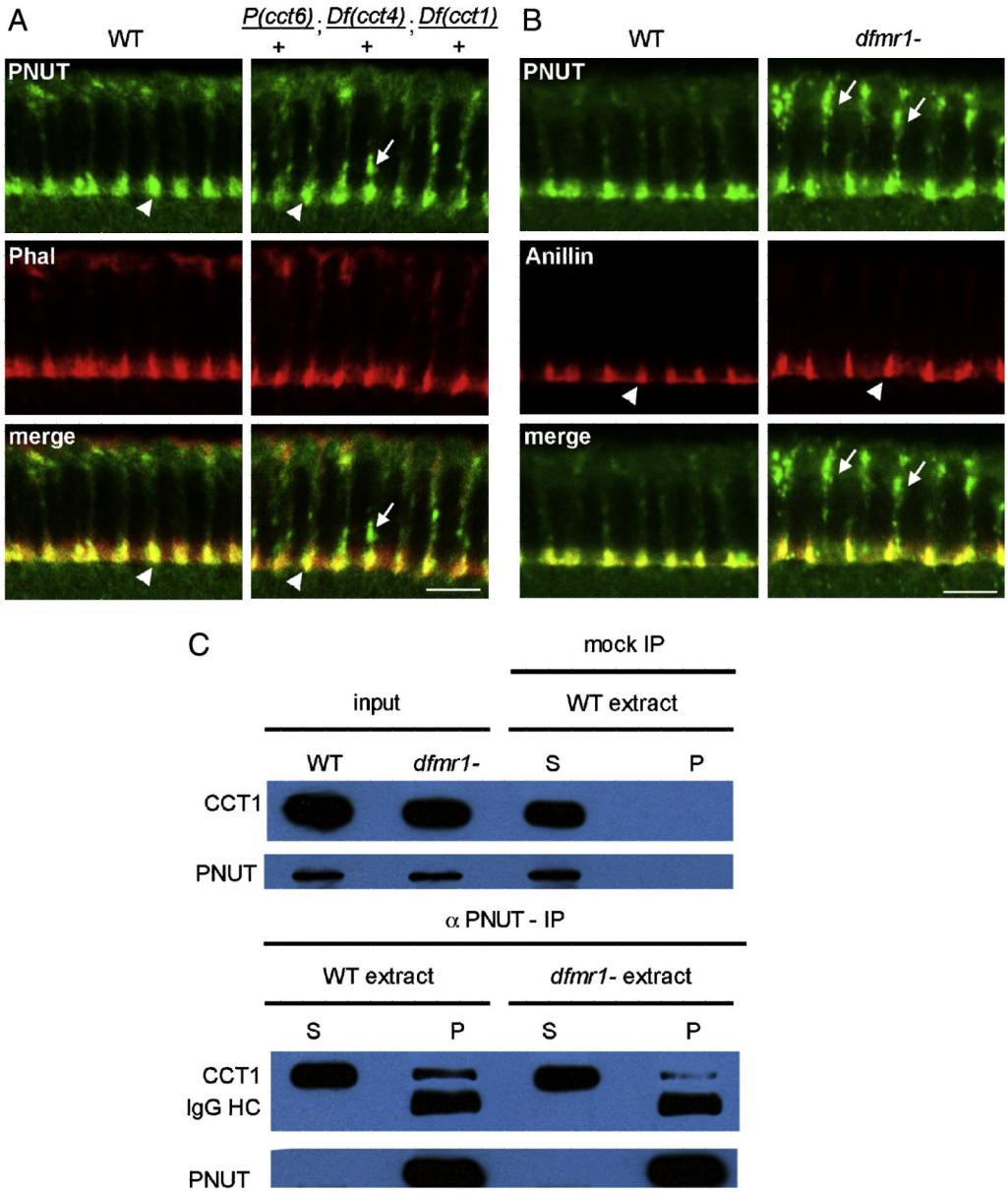

Figure 6. PNUT localization is dependent on CCT and is a likely substrate of CCT

(A) IF analysis of fixed cleavage stage WT and *cct*- embryos shows PNUT and F-actin (Phalloidin) localization. (B) IF analysis of fixed WT and *dfmr1*- embryos shows PNUT and Anillin localization. Arrow heads indicate normal localization of PNUT and Anillin to furrow fronts, and arrows indicate abnormal accumulation of PNUT along lateral membrane. Scale bar indicates 10 μm. (C) Immunoblots showing PNUT IPs from WT and *dfmr1*- extracts. 10 μg starting extract was loaded in input lanes (4% of total input). 10 μg of supernatants (S) and 50% of pellet (P) from mock and anti-PNUT IPs was loaded in indicated lanes. Proteins probed for indicated to left.