UNIVERSITY OF CALIFORNIA, SAN DIEGO

Spatial organization of mRNA regulation and metabolic activity

A dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy

in

Biology

by

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The Dissertation of Risa Maruyama Broyer is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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DEDICATION

This work is dedicated to

my family,

my mentors,

my colleagues,

and friends

for

your unconditional support.

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Chapter 3 is a manuscript currently under review at the journal Developmental Cell. Broyer, R.M., Sato, B.K., and Wilhelm, J.E. "The Pan gu kinase complex triggers the proteolytic disassembly of maternal RNPs to promote mRNA degradation at the maternal-to-zygotic transition." B. Sato is co-first author for this manuscript and performed the experiments in Figures 3-1, 3-2, 3-5, 3-8, 3-9, 3-10, and 3-11. J. Wilhelm wrote the manuscript. The dissertation author is the primary experimenter and author on this paper.

Chapter 4 is a manuscript in preparation and will be submitted for publication. Broyer, R.M., Monfort, E., Begovich, K., Wilhelm, J.E. "Human disease mutations in PRPP synthetase alter its ability to polymerize and disrupt the organization of the actin cytoskeleton." E. Monfort and A. Lee performed PRPS1/PRPSAP1 immunofluorescence in yeast. All other experiments, imaging, data analysis, and writing this version of the manuscript were my responsibility. J. Wilhelm is revising for publication. The dissertation author is the primary experimenter and author on this paper.

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ABSTRACT OF THE DISSERTATION

Spatial organization of mRNA regulation and metabolic activity

by

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Doctor of Philosophy in Biology

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Professor James Wilhelm, Chair

Organization of biological processes is a central principle of cell biology. However, until recently, the context of this organization has largely centered on membrane-bound organelles and their internal biochemistry. Recent discoveries of intracellular structures that organize biochemical processes such as P bodies, purinosomes, and self-assembling metabolic enzymes suggests there is much to be

uncovered in the studies of cytoplasmic organization. This thesis focuses on two mechanisms for organizing the cytoplasm: one involving the spatial regulation of key mRNA processing events in early development and the other involving polymerization of metabolic enzymes and the role it plays in connecting metabolic regulation to broader areas of cell biology. The spatial regulation of mRNA processing events is largely dependent on the role of the mRNA-associated ribonucleoprotein (RNP) complex that functions to regulate transcript translation and stability. We focus on one such RNP, Cup, that has been previously described as a translational repressor for *oskar* mRNA, the transcript that is critical for anterior-posterior patterning in *Drosophila* development. Here, we identify that Cup is also required for *oskar* mRNA stability. Conversely, we will also show a novel pathway for mRNA degradation. Previous studies have identified the role of the Pan gu kinase complex in activating the translation of the mRNA degradation machinery at the maternal-to-zygotic transition, where maternally loaded transcripts are degraded as a precursor to zygotic transcriptional control. We identify a parallel pathway that acts in concert to destabilize these maternal transcripts through ubiquitin-mediated degradation of the associated RNPs. Switching to a different mechanism of cytoplasmic organization, we will reveal the self-assembling property of the metabolic enzyme PRPP synthetase (PRPS), the enzyme responsible for synthesizing the substrate for nucleotide biosynthesis. We will also show the defects in cellular actin organization associated with the mutations in PRPS leading to a disease-state. Furthermore, we will identify that the inhibitor of PRPS associates with the polymerized form of PRPS and also with

the actin cytoskeleton; this suggests a novel method of regulation and possible mechanism behind PRPS diseases.

Chapter 1

Introduction

Introduction

Organizational principles of the cell

One of the central principles of cell biology is the compartmentalization and organization of biochemical processes. While this principle is now considered universal to all cell types, until relatively recently, complex forms of cellular organization were thought to be largely restricted to eukaryotes. Bacteria were seen as "bags of enzymes" with little need for the complex regulatory control mechanisms that intracellular organization makes possible. However, the discovery in the 1990s of bacterial cytoskeletons comprised of distant relatives of eukaryotic tubulin, actin, and intermediate filaments argued that even the simplest cell required sophisticated intracellular organization (Bi and Lutkenhaus, 1991; Bork et al., 1992; de Boer et al., 1992; Desai and Mitchison, 1998; Doi et al., 1988; Lara et al., 2005; RayChaudhuri and Park, 1992; van den Ent and Lowe, 2000).

The fact that the bacterial cytoskeleton was undiscovered for so long raised the possibility that other forms of intracellular organization remained to be identified in both prokaryotes and eukaryotes. In eukaryotes, most of the focus has been on the role of membrane-bound organelles in compartmentalizing biochemical reactions (LeDuc and Bellin, 2006; Luisi, 2002). This form or organization has been widely studied both for its regulatory possibilities and its ability to prevent reactions from causing deleterious effects. For instance, the biochemical reactions that are restricted to mitochondria (Lenaz and Genova, 2009) and peroxisomes (Veenhuis et al., 2000) prevent the release of oxidative and damaging free radicals to the rest of the cell. In contrast, the cytoplasm was largely viewed as a "protein soup" where these membrane

compartments existed. In spite of this commonly held view, many "special cases" of cytoplasmic organization were known in higher eukaryotes. For example, mRNA localization during oogenesis and embryogenesis was known to be important for establishing gradients that determined the anterior-posterior and dorsal-ventral body axes (Rongo and Lehmann, 1996; St Johnston and Nusslein-Volhard, 1992). While this form of mRNA localization is entirely cytoplasmic, it was largely considered an oddity resulting from the fact that the early *Drosophila* embryo develops as a multinucleate syncytium. However, later studies have found this mRNA localization is necessary for migration of fibroblasts (Latham et al., 1994; Latham et al., 2001; Sundell and Singer, 1991), cell fate determination in *S. cerevisiae* (Shepard et al., 2003), and learning and memory in neurons (Dubowy and Macdonald, 1998; Eberwine et al., 2001). This argues that forms of cytoplasmic organization often get used repeatedly throughout evolution, even if they are initially discovered in organisms with unusual biological features.

The last decade has seen an explosion in the discovery of novel intracellular structures, including processing bodies (Sheth and Parker, 2006), U bodies (Liu and Gall, 2007), and purinosomes (An et al., 2008). Furthermore, the identification of the self-assembling nature of many metabolic enzymes (Ingerson-Mahar et al., 2010; Liu, 2010; Narayanaswamy et al., 2009; Noree et al., 2010) reveals that there are many more structures to be found. These intracellular structures all share a common set of features: they lack a membrane, they behave as either phase-separated liquids or as polymeric filaments, and they are often evolutionarily conserved. Unfortunately, they

also share another common feature—their functions within the cell remain largely unclear.

This thesis focuses on two mechanisms for organizing the cytoplasm: one involving spatial regulation of key mRNA processing events in early development, and the other involving polymerization of metabolic enzymes and the role it plays in connecting metabolic regulation to broader areas of cell biology. Studies on spatial regulation of mRNA processing will be presented in the first two chapters: the role of the key ribonucleoprotein (RNP) Cup in mRNA stability will be presented (Chapter 2) as well as the identification of a novel pathway of proteolytic RNP degradation as a prerequisite for mRNA destabilization (Chapter 3). Switching to a very different mechanism of cytoplasmic organization, Chapter 4 will describe the identification of conserved polymerization of metabolic enzymes with a focus on PRPP synthetase, the enzyme responsible for catalyzing the reaction to create the initial substrate for nucleotide biosynthesis.

mRNA processing

mRNA can assume many fates following the completion of transcription. To date, most of the focus has been on post-transcriptional mRNA modifications, such as capping, splicing, and the addition of a poly-A tail that affects mRNA stability and translation. The set of proteins that bind to an mRNA transcript are equally important in determining whether a given transcript is translated, degraded, stored, or localized to a particular site in the cell. Interestingly, a set of the novel organelles discovered in the past two decades, such as stress granules (Kedersha et al., 1999; Nover et al.,

1989) and processing bodies (P-bodies) (Sheth and Parker, 2003; Teixeira et al., 2005) are in fact believed to be sites for mRNA modification, degradation or sequestration/protection. Moreover, many of the proteins that associate with mRNA in the nucleus and regulate mRNA fate also play roles in targeting the transcripts to these novel structures; this is consistent with these structures having critical functions in post-transcriptional gene regulation. In Chapter 2 of this thesis, I will discuss the role of a major protein of the *oskar* RNP, Cup, that is required for translational control and transcript stability.

The localization of *oskar* mRNA to the posterior pole of the developing *Drosophila* oocyte is critical for establishing the posterior body plan. However, it is equally important that unlocalized *oskar* mRNA be protected from degradation until localization is completed and that this unlocalized *oskar* mRNA remains translationally silenced. Thus, *oskar* mRNA requires the careful integration of translation, localization, and stability in order for it to properly pattern the posterior of the embryo. While protection and translational silencing are important for regulating *oskar* mRNA during oogenesis, a number of other developmental processes are dependent on mRNA degradation. The maternal-to-zygotic transition, which occurs within the first two hours of *Drosophila* embryogenesis, is one such event where maternally loaded mRNA in the embryo is rapidly degraded at the transition to pave the way for zygotic transcription and control. Chapter 3 will discuss my finding of a novel mechanism of mRNA degradation that is involved in the targeted degradation of protective elements of mRNA transcripts by the ubiquitin-proteasome system. It will

be shown that ubiquitin-mediated degradation of RNPs is a prerequisite for proper maternal mRNA degradation.

Metabolic enzymes and polymerization

Examination of RNA biology often provides a window into some of the most ancient functions of the cell. The number of novel intracellular structures with a role in RNA regulation led us to speculate that this form of compartmentalization could have functioned as an early form of enzyme regulation. Strikingly, the last decade has seen the discovery of a number of cytoplasmic macromolecular structures comprised of metabolic enzymes. These include enzymes that act in consecutive steps in biosynthetic pathways ranging from purine biosynthesis to fatty acid and central carbon metabolism (An et al., 2008; Campanella et al., 2005; Ishikawa et al., 2004). Such multi-enzyme complexes are thought to be beneficial in a variety of ways: first, the activity of the enzymes and flux through the pathway would seemingly increase when the enzymes are assembled in a common structure. Second, the association could allow for substrate channeling or tunneling so that the intermediate metabolites are able to be shielded from diffusion or recruitment by competitive sequestration (Conrado et al., 2008; Dunn, 2012; Lee et al., 2012; Zhang, 2011). In addition, research has proposed that the oligomerization is thought to be used to regulate enzyme activity, by providing a structure for the binding of substrates, cofactors, and allosteric effectors or inhibitors (Fairman et al., 2011; Kim and Raushel, 2001), and also can be a mechanism for cells to initiate a rapid response to changes in

extracellular environments (An et al., 2008; Buchan et al., 2008; Narayanaswamy et al., 2009; Noree et al., 2010).

There are two broad classes of these novel structures composed of metabolic enzymes: 1) filaments, where it is believed that the physical chemistry of polymerization can be leveraged to regulate enzyme activity and 2) "puncta," where many enzymes have phase separated into distinict liquid "droplets" similar to those recently involved in RNA regulatory structures, such as the nucleolus or processing body. Our lab has identified 59 metabolic enzymes that assemble into structures *in vivo* (Noree et al., in preparation). Ten form filaments, while the remaining 49 are puncta. Interestingly, 17 of these puncta-forming enzymes are actually recruited to RNA stress granules, suggesting that these stress granules might serve to broadly integrate a number of cellular functions. This is a particularly intriguing idea since it would help explain why many inborn errors of metabolism cause many highly specific symptoms that are difficult to explain in terms of the known biochemical function of the enzyme. In Chapter 4, I explore this possibility by focusing on PRPP synthetase. I have found that PRPP synthetase forms filaments that are conserved from yeast to humans. This result suggested that studies of the cell biology of the PRPP synthetase could lend insights into the unusual human genetics and pathophysiology of PRPP synthetase syndromes caused by mutation. My work has defined a potential pathway that leads from perturbation of PRPP synthetase organization to disruption of the actin cytoskeleton, suggesting a possible explanation for the sensorineural deafness seen in patients with mutations in PRPP synthetase. I will discuss this work in the context of

the importance of understanding how different forms of cellular organization interact dynamically to regulate cell shape and function.

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Chapter 2

Cup regulates *oskar* mRNA stability during oogenesis

Abstract

The proper regulation of the localization, translation, and stability of maternally deposited transcripts is essential for embryonic development in many organisms. These different forms of regulation are mediated by the various protein subunits of the ribonucleoprotein (RNP) complexes that assemble on maternal mRNAs. However, while many of the subunits that regulate the localization and translation of maternal transcripts have been identified, relatively little is known about how maternal mRNAs are stockpiled and stored in a stable form to support early development. One of the best characterized regulators of maternal transcripts is Cup - a broadly conserved component of the maternal RNP complex that in *Drosophila* acts as a translational repressor of the localized message *oskar*. In this study, we have found that loss of *cup* disrupts the localization of both the *oskar* mRNA and its associated proteins to the posterior pole of the developing oocyte. This defect is not due to a failure to specify the oocyte or to disruption of RNP transport. Rather, the localization defects are due to a drop in *oskar* mRNA levels in *cup* mutant egg chambers. Thus, in addition to its role in regulating *oskar* mRNA translation, Cup also plays a critical role in controlling the stability of the *oskar* transcript. This suggests that Cup is ideally positioned to coordinate the translational control function of the maternal RNP complex with its role in storing maternal transcripts in a stable form.

Introduction

Post-transcriptional regulation of maternally deposited mRNAs plays a central role in embryonic patterning in many metazoans. This regulation takes a number of forms, including the spatial and temporal regulation of transcript localization, translation, and stability. The ultimate fate of a particular maternal message is controlled by the set of proteins that are recruited to the transcript forming a ribonucleoprotein complex (RNP). At the core of the maternal RNP complex are four subunits that are associated with maternal transcripts in *Drosophila melanogaster*, *Caenorhabditis elegans*, and *Xenopus laevis*: a Y box family RNA binding protein (Boag et al., 2005; Mansfield et al., 2002; Matsumoto et al., 1996; Wilhelm et al., 2000; Yurkova and Murray, 1997), an RNA helicase (Audhya et al., 2005; Boag et al., 2005; Ladomery et al., 1997; Minshall and Standart, 2004; Minshall et al., 2001; Nakamura et al., 2001), an Lsm domain protein (Audhya et al., 2005; Boag et al., 2005; Squirrell et al., 2006; Tanaka et al., 2006; Wilhelm et al., 2005), and an eIF4E binding protein (Li et al., 2009; Minshall et al., 2007; Nakamura et al., 2004; Semotok et al., 2005; Wilhelm et al., 2003). Thus, one of the central questions in understanding post-transcriptional control of development is defining the role of each RNP subunit in regulating RNA fate.

The regulation of *oskar* mRNA during *Drosophila* oogenesis is one of the most extensively characterized systems for examining how different subunits of the maternal RNP might regulate maternal transcripts (Kugler and Lasko, 2009). Indeed, the *oskar* transcript is ideal for these studies as it is subject to multiple levels of regulation that must often be coordinated with one another in order for proper

development to take place. The correct localization of *oskar* mRNA to the posterior pole of the *Drosophila* oocyte is particularly crucial for embryonic development, since this localization is essential for both posterior patterning and establishment of the future germ line (Ephrussi et al., 1991; Kim-Ha et al., 1991). The *oskar* transcript is also subjected to an additional level of translational control: localization-dependent translation, where only the correctly localized message is actively translated (Rongo et al., 1995). In contrast to mRNA localization and translational control, the regulation of *oskar* mRNA stability is poorly understood. *oskar* mRNA, like many maternal transcripts, has a short poly(A) tail that should destabilize the message (Lie and Macdonald, 1999). However, the only known factor that contributes to *oskar* mRNA stability is the poly(A) binding protein (PABP) which is known to protect the poly(A) tail from degradation (Vazquez-Pianzola et al.) . Thus, *oskar* is an excellent model transcript for analyzing how the different aspects of the localization, translation, and stability of maternal mRNAs are controlled at the molecular level.

The *oskar* RNP complex is comprised a core complex whose subunits are common to maternal RNP complexes in many species as well as several sequence-specific RNA-binding proteins. In *Drosophila,* this core complex is comprised of the RNA helicase, Me31B, the eIF4E binding protein, Cup, the Y-box family RNA binding protein, YPS, and the LSm domain protein, Trailer Hitch (Tral) (Audhya et al., 2005; Boag et al., 2005; Ladomery et al., 1997; Li et al., 2009; Mansfield et al., 2002; Minshall et al., 2007; Minshall and Standart, 2004; Minshall et al., 2001; Nakamura et al., 2001; Nakamura et al., 2004; Squirrell et al., 2006; Tafuri and Wolffe, 1993; Tanaka et al., 2006; Wilhelm et al., 2005; Wilhelm et al., 2003;

Wilhelm et al., 2000). Of the proteins in the core complex, the biochemical function of Cup is the best understood. Cup is a translational repressor of *oskar* mRNA that is recruited to the message by the sequence-specific RNA-binding protein, Bruno (Bru) (Kim-Ha et al., 1995; Nakamura et al., 2004; Webster et al., 1997; Wilhelm et al., 2003). Once Cup is recruited to the message, it acts to translationally repress *oskar* mRNA by binding the translation initiation factor, eIF4E (Nakamura et al., 2004; Wilhelm et al., 2003). Since eIF4E binding to the 5'cap of the transcript is normally the first step in assembling a functional translation initiation complex, the formation of a 5'cap-eIF4E-Cup complex blocks translation by sequestering the 5'cap of the message (Nakamura et al., 2004; Nelson et al., 2004; Wilhelm et al., 2003). *In vitro* studies of Cup in *Drosophila* S2 cells suggest that Cup might also regulate transcript stability in a manner that is separate from its eIF4E binding activity. These studies found that when Cup is tethered to a reporter transcript it promotes poly(A) tail shortening without destabilizing the message (Igreja and Izaurralde, 2011). Furthermore, these studies found that Cup binds directly to the CAF1–CCR4–NOT deadenylase complex. Thus, Cup has roles at both the 5' and 3' end of its target mRNAs (Igreja and Izaurralde, 2011). Recent studies have also described a bipartite binding mechanism of eIF4E through a canonical and non-canonical binding domain of Cup, suggesting that both domains are required for proper localization and repression (Igreja et al., 2014; Kinkelin et al., 2012). However, the role of these additional functions in regulating *oskar* mRNA stability and/or translation *in vivo* remains unexplored.

The fact that Cup participates in regulation of the 5'cap, the poly(A) tail, and makes direct contact with two other subunits of the core complex, Me31B and Tral, suggested that Cup might regulate multiple roles within the RNP complex (Nakamura et al., 2004; Tritschler et al., 2008; Wilhelm et al., 2003). In order to identify these additional functions, in this study we surveyed the known alleles of *cup* and identified two alleles, cup^{16} and cup^{20}, that are apparent protein null alleles of *cup*. Utilizing these alleles, we have found that in the absence of *cup* many of the known subunits of the *oskar* RNA-protein complex fail to be localized to the developing oocyte. This localization defect in ovaries that lack detectable levels of Cup protein is not due to a failure to determine the oocyte. To determine whether the failure to localize subunits of the *oskar* RNP to the posterior pole is due to a transport defect or to a defect in *oskar* mRNA stability, we combined a quantitative *in situ* approach with measurements of *oskar* mRNA levels. This analysis revealed that the loss of Cup protein causes a decrease in *oskar* mRNA levels and a corresponding decrease in the localization of *oskar* mRNA to the oocyte - a decrease in localization that is masked when traditional enzyme-linked *in situs* are used. We conclude that, in addition to its role in regulating *oskar* mRNA translation, Cup also plays a critical role in controlling the stability of the *oskar* transcript.

Results

cup^{16} and *cup*^{20} are apparent protein null alleles of *cup*

cup was originally identified by Schüpbach and Wieschaus in a screen for mutations that cause sterility in females (Schupbach and Wieschaus, 1989). This screen identified an unusually high number of *cup* alleles that caused oogenesis to arrest anywhere between stage 5 and 14 depending on the strength of the allele (Keyes and Spradling, 1997). However, subsequent studies of a *cup* allele, *cup*$^{\Delta 212}$, where the canonical eIF4E-binding site of Cup is deleted, oogenesis progressed to stage 14 (Nakamura et al., 2004). The fact that strong hypomorphic alleles of *cup* cause oogenesis to arrest much earlier than *cup* alleles where eIF4E binding is compromised suggested that Cup has an additional role in oogenesis that that is separate from translational control of *oskar* transcripts.

In an attempt to uncover these additional roles of *cup*, we first sought to identify protein null alleles of *cup*. Flies carrying the deficiency, *Df(2L)bsc7*, which deletes the *cup* locus, were crossed to flies bearing each of the available *cup* alleles to generate *cup/Df(2L)bsc7* females. Ovaries from the hemizygous females were dissected and analyzed for the presence of Cup protein by both immunoblot (Figure 2-1A) and immunofluorescence (Figure 2-1B). This screen identified two alleles, *cup*^{20} and *cup*^{16}, where there was no detectable Cup protein in the hemizygous ovaries by either technique. In contrast, *cup*$^{\Delta 212}$*/Df(2L)bsc7* females express a shortened form of Cup that is localized correctly to the oocyte (Figure 2-1A,B). Thus, based on our analysis, both *cup*^{20} and *cup*^{16}, appear to be protein null alleles of *cup*.

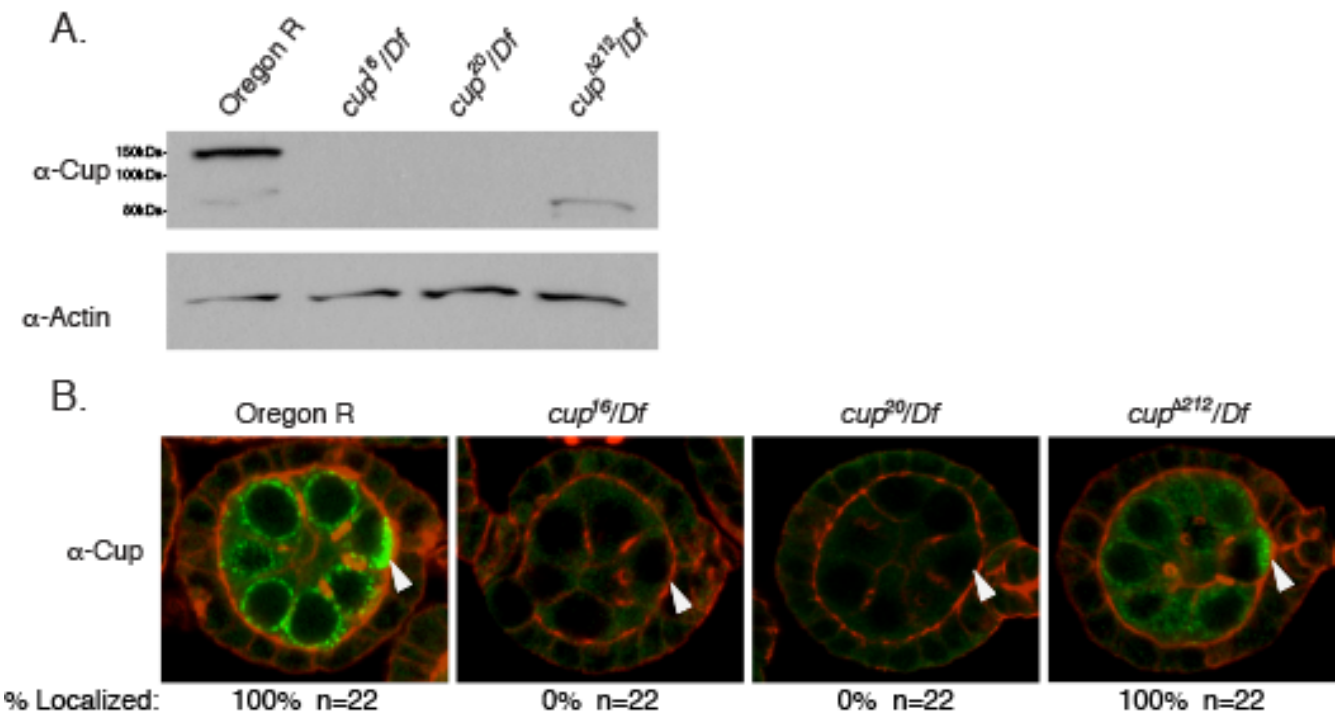
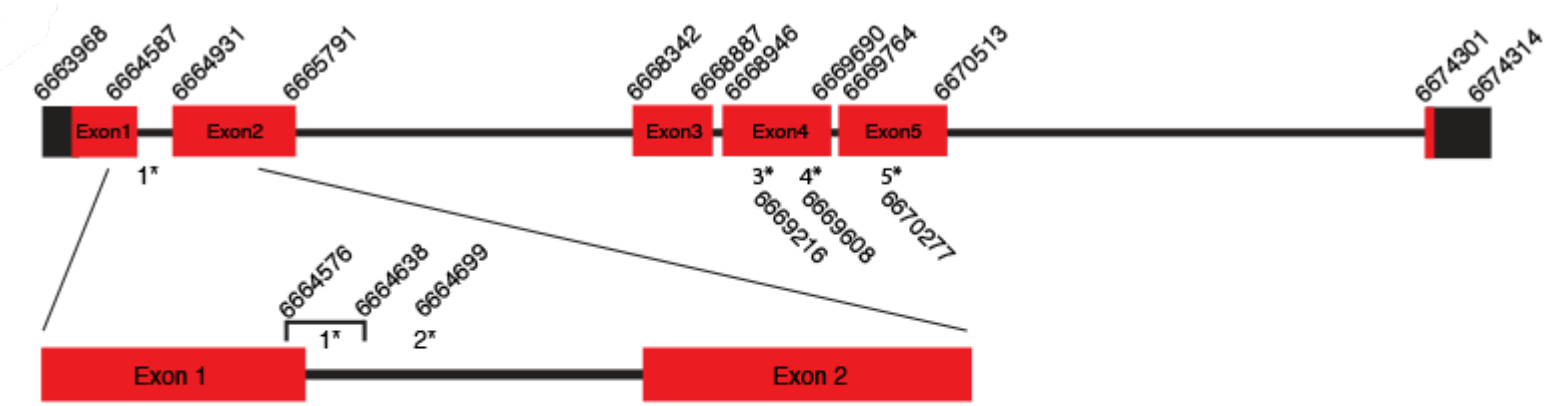


Figure 2-1 Strong hypomorphic alleles, *cup*^{16} and *cup*^{20}, are protein null alleles of *cup*

(A) Ovary extract from *cup*^{16}*/Df(2L)bsc7* and *cup*^{20}*/Df(2L)bsc7* lack Cup protein, whereas *cup* $^{\Delta 212}$*/Df(2L)bsc7* mutants produce a decreased level of N-terminally truncated Cup protein lacking the eIF4E-binding region. Ovaries from Oregon R, *cup*^{16}*/Df(2L)bsc7*, *cup*^{20}*/Df(2L)bsc7,* and *cup*$^{\Delta 212}$*/Df(2L)bsc7* were dissected from fattened flies, lysed in sample buffer, and subsequently analyzed by immunoblotting with the indicated antibody.
(B) Cup protein in Oregon R is normally localized to the posterior of the stage 4 egg chamber. Cup protein is undetectable in the protein null alleles, *cup*^{16} and *cup*^{20}*,* but properly localized in *cup*$^{\Delta 212}$ mutants. Ovaries were dissected from fattened flies and analyzed by immunofluorescence using the indicated antibody.

The large number of *cup* alleles of varying allelic strengths suggested that identifying the molecular lesion in these alleles could be useful in structure-function studies of Cup. In order to identify the basis for loss of the Cup protein in cup^{20} and cup^{16} as well as the nature of the mutation in several weaker, commonly studied *cup* alleles (cup^{8}, cup^{21}, cup^{32}), we sequenced the entire *cup* locus from flies that were hemizygous for each of the 5 cup alleles. This sequencing also included the coding regions of genes that overlapped partially or entirely with the cup transcription unit. We identified five sequence changes from the reference sequence (Figures 2-2, 2-3). However, each of these variants is present in all of the alleles of *cup* that we examined (cup^{8}, cup^{21}, cup^{32}, cup^{16}, cup^{20}). The fact that these 5 alleles exhibit widely varying strengths argues that these alterations are likely polymorphisms in the *cup* locus (Keyes and Spradling, 1997).

While this result suggests that the various *cup* alleles have mutations in transcriptional control elements that lie outside the *cup* locus, our analysis also identified a region (6664576-6664638) where sequence could not be obtained despite repeated attempts and in spite of the fact that this region gave high quality sequence using genomic DNA from Oregon R flies. The fact that all *cup* alleles examined have an unsequenceable region near the end of exon 1 suggests that the insertion of a transposable element in the *cup* locus might be responsible for the high frequency of *cup* alleles in the original screen for female sterile mutations (Schupbach and Wieschaus, 1989). While the mechanism for how such an insertion could lead to a collection of *cup* alleles with a wide variety of strengths is unclear, our results argue



	Coordinates	Nucleotide	Protein
1	6664576...6664638	Unsequenceable region in *cup* mutants	
2	6664699	GATC deletion in *cup* mutants	
3	6669216	G>A	N/A
4	6669608	AT>GG	H>R
5	6670277	A>C	K>Q

Figure 2-2 The *cup* phenotype is a result of a mutation outside of the cup locus

Genomic sequencing of the *cup* locus in Oregon R, $cup^{8}/Df(2L)BSC7$, $cup^{21}/Df(2L)BSC7$, $cup^{32}/Df(2L)BSC7$, $cup^{16}/Df(2L)BSC7$, and $cup^{20}/Df(2L)BSC7$ revealed several nucleotide polymorphisms and an unsequenceable region spanning the end of exon 1 into the first intron in the *cup* alleles. The unsequencable region was only present in the mutants, whereas Oregon R provided high quality sequence.

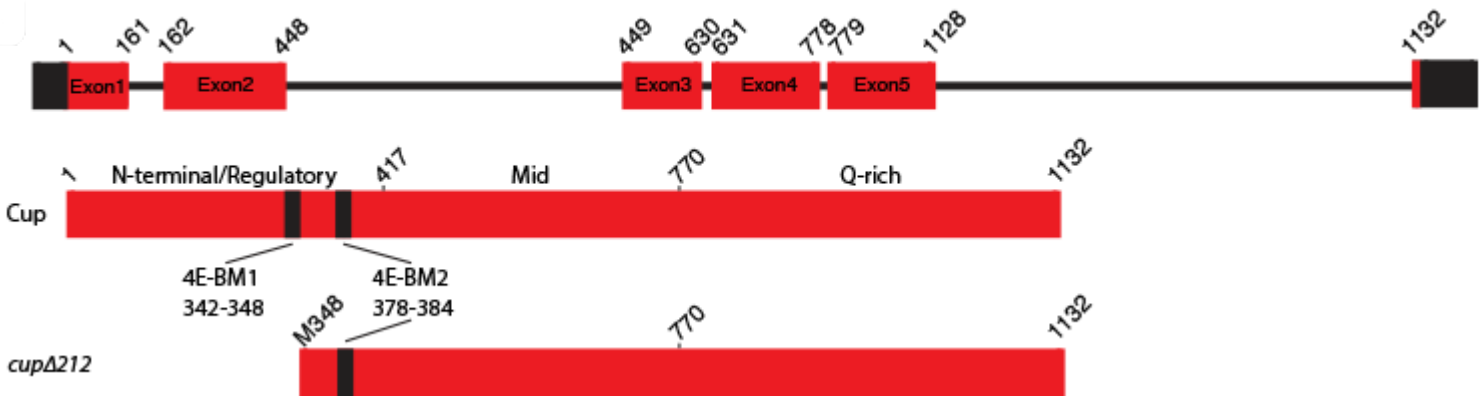


Figure 2-3 Cup consists of three domains

The Cup protein consists of three different domains; the N-terminal, or regulatory domain, followed by the mid and Q-rich regions which comprise the effector domain. The canonical and non-canonical eIF4E binding sites, 4E-BM1 and 4E-BM2, respectively, lie in the N-terminal regulatory region. The $cup^{\Delta 212}$ mutation is a 347 amino acid deletion of the N-terminal region that effectively separates the function of the eIF4E canonical binding site from the non-canonical binding site.

that attempts to leverage the existing collection of EMS-generated *cup* alleles to identify novel functional domains/motifs of Cup are unlikely to be effective.

Loss of Cup protein strongly disrupts the oocyte localization of the protein components of the *oskar* RNP complex.

Because our sequence analysis of several *cup* alleles did not yield additional insights into the functional domains of Cup, we next focused on utilizing our two putative null alleles of *cup* to determine the role of Cup protein in RNP localization and assembly. The fact that Cup binds directly to two of the highly conserved core components of the *oskar* RNA protein (RNP) complex, the RNA helicase Me31B and the Lsm domain protein Trailer hitch (Tral) and appears to have separate domains that control eIF4E binding and poly(A) tail length suggested that Cup might play a role in the recruitment of other components of the *oskar* RNP (Igreja and Izaurralde, 2011; Nakamura et al., 2004; Tritschler et al., 2008; Wilhelm et al., 2003). In order to test this possibility, we first examined whether *cup* was required for either the correct localization of three classes of proteins in the *oskar* RNP complex: the core proteins (Tral, Me31B, YPS), the RNA binding proteins that specifically recognize the *oskar* transcript (Bruno, Orb), and the RNA degradation factor, DCP1 (Chang et al., 1999; Kim-Ha et al., 1995; Mansfield et al., 2002; Nakamura et al., 2001; Nakamura et al., 2004; Wilhelm et al., 2005; Wilhelm et al., 2003). Ovaries from $cup^{16}/Df(2L)bsc7$ and $cup^{20}/Df(2L)bsc7$ females were dissected and immunostained with antibodies against each of these *oskar* RNP subunits (Figures 2-4, 2-5). In wild type egg chambers, each of these proteins is localized to the posterior pole of the developing oocyte and their

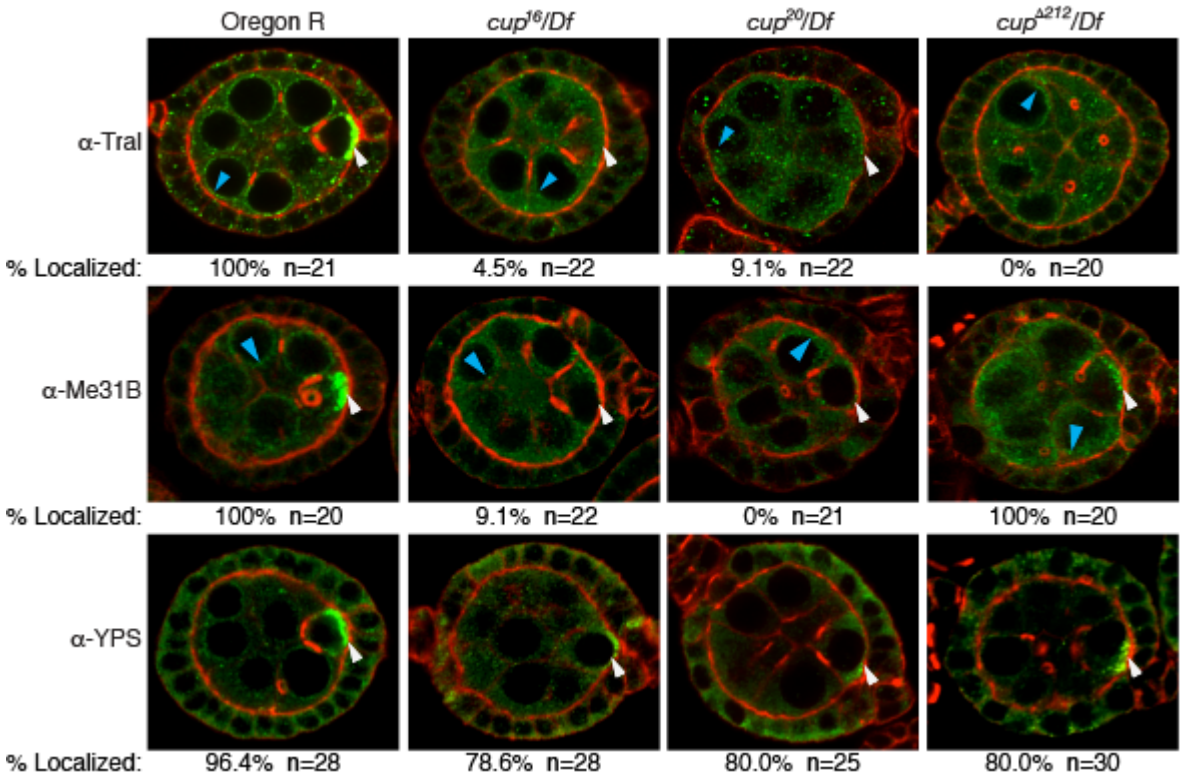


Figure 2-4 Localization of *osk* RNP complex components Tral, Me31B and YPS are disrupted in *cup* mutants

Immunofluoresence in stage 4 Oregon R and *cup*^{16}*/Df(2L)bsc7*, *cup*^{20}*/Df(2L)bsc7*, and *cup*$^{\Delta 212}$*/Df(2L)bsc7* egg chambers against *osk* RNP subunits Trailer hitch (Tral), Me31B, YPS. Proper oskar RNP localization appears at the posterior of the oocyte (white arrow), with RNP presence also on the nurse cell nuclear membrane (blue arrows). Ovaries were dissected from fattened flies and analyzed by immunofluorescence using the indicated antibody. Percentages indicate egg chambers with localized protein. Staining indicates a lack or severely reduced level of localization of all osk RNP subunits in *cup*^{16}*/Df(2L)bsc7* and *cup*^{20}*/Df(2L)bsc7,* and slightly reduced localization of all *osk* RNP subunits in *cup*$^{\Delta 212}$*/Df(2L)bsc7* except for Tral, which is completely absent.

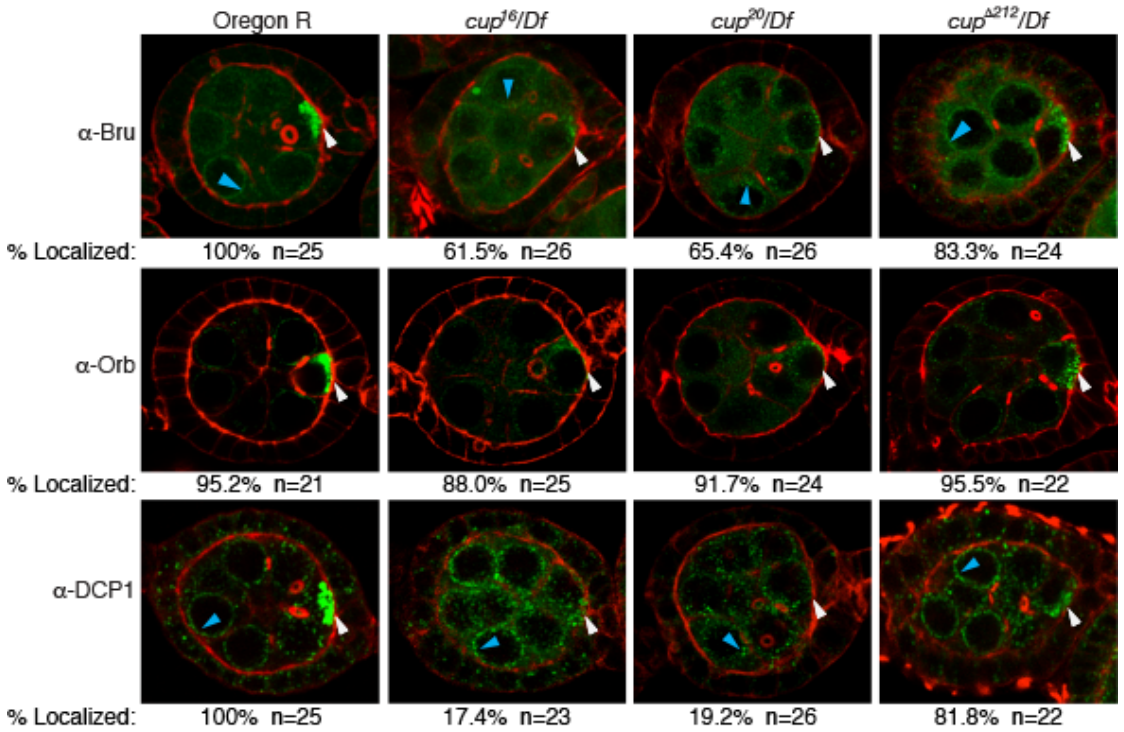


Figure 2-5 Localization of *osk* RNP complex components Bru, Orb, and DCP1 are disrupted in *cup* mutants

Immunofluoresence in stage 4 Oregon R and *cup*^{16}*/Df(2L)bsc7*, *cup*^{20}*/Df(2L)bsc7*, and *cup*$^{\Delta 212}$*/Df(2L)bsc7* egg chambers against *osk* RNP subunits Bruno (Bru), Orb, and DCP1. Proper *oskar* RNP localization appears at the posterior of the oocyte (white arrow), with RNP presence also on the nurse cell nuclear membrane (blue arrows). Ovaries were dissected from fattened flies and analyzed by immunofluorescence using the indicated antibody. Percentages indicate egg chambers with localized protein. Staining indicates a lack or severely reduced level of localization of all osk RNP subunits in *cup*^{16}*/Df(2L)bsc7* and *cup*^{20}*/Df(2L)bsc7,* and slightly reduced localization of all *osk* RNP subunits in *cup*$^{\Delta 212}$*/Df(2L)bsc7* except for Tral, which is completely absent.

localization tracks the localization of *oskar* mRNA throughout oogenesis (Figures 2-4, 2-5; Oregon R column). However, in *cup*^{16}*/Df(2L)bsc7* and *cup*^{20}*/Df(2L)bsc7* egg chambers, we found that a large percentage of egg chambers either failed to localize/accumulate the *oskar* RNP subunits. The most severe defects were for the core subunits Tral and Me31B, which are known to make direct contact with Cup. Only 4.5% of *cup*^{16}*/Df(2L)bsc7* egg chambers localized Tral protein correctly to the oocyte, while only 9.1% localized Me31B correctly. In contrast, the weakest effects on localization were on the RNA binding proteins, YPS (78.6% correct localization) and Orb (88% correct localization) (Figures 2-4, 2-5; *cup*^{16}*/Df* column). Comparable effects on *oskar* RNP subunit localization were also seen in *cup*^{20}*/Df(2L)bsc7* egg chambers (Figures 2-4, 2-5; *cup*^{20}*/Df* column). However, for all of the *oskar* RNP subunits tested, even when the protein was localized correctly to the oocyte the amount of protein detected within the oocyte was severely reduced as compared to wild type egg chambers (Figures 2-4, 2-5).

One possible explanation for the failure to localize all of these proteins is that Cup is required for either the expression or stability of these subunits. To test this possibility, we dissected ovaries from *cup*^{16}*/Df(2L)bsc7* and *cup*^{20}*/Df(2L)bsc7* females and immunoblotted for all of the proteins that exhibited defective localization to the posterior pole of the oocyte (Figure 2-6). We observed two distinct effects of the loss of Cup on the other subunits of the *oskar* RNP. Loss of Cup had no effect on the protein levels of either YPS or Bruno, arguing that the localization defects for these two proteins are not due to decrease in expression. In contrast, we observed a slight

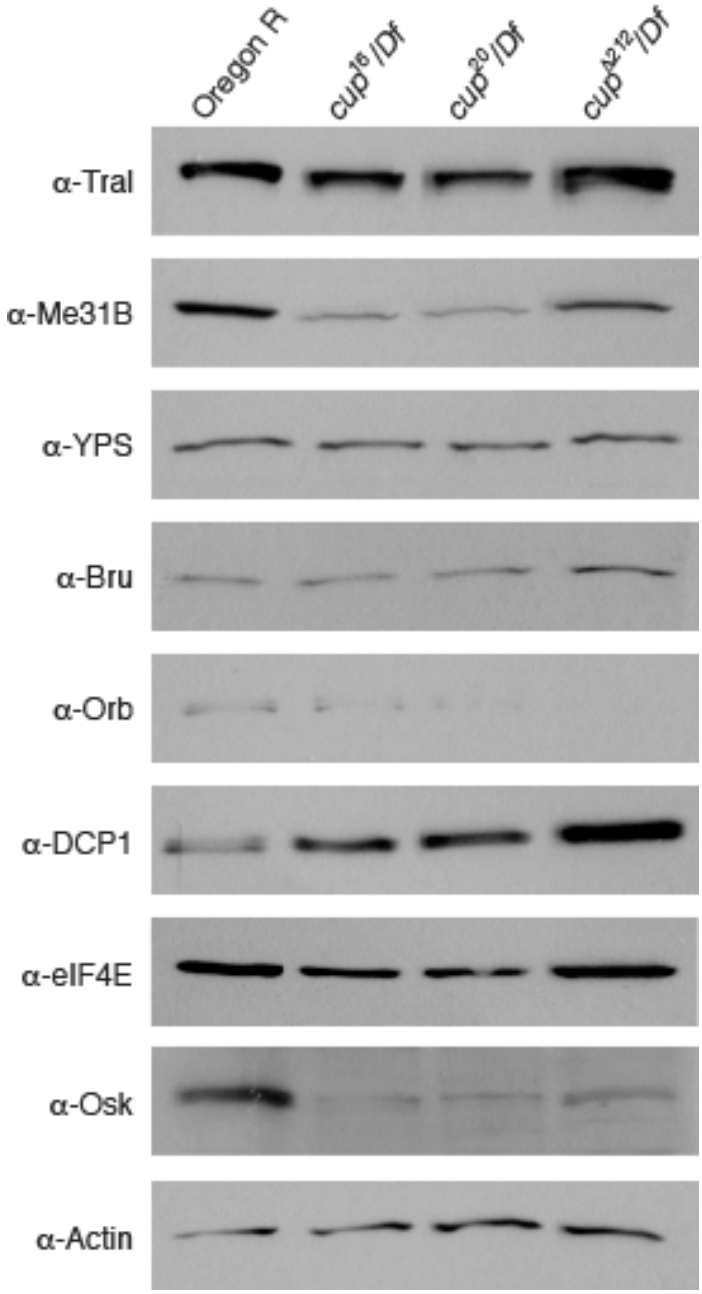


Figure 2-6 Cup is not required for the expression or stability of *osk* RNP subunits.

Protein expression levels of Tral, Me31B, YPS, Bru, Orb, DCP1, eIF4E, Osk, and actin in Oregon R and *cup*^{16}*/Df(2L)bsc7*, *cup*^{20}*/Df(2L)bsc7*, and *cup*$^{\Delta 212}$*/Df(2L)bsc7* mutant ovary extracts. Extracts were made from ovaries of fattened flies and analyzed by immunoblotting with the corresponding antibodies.

decrease in the protein levels of Tral, Me31B, and Orb and an increase in the levels of DCP1 in ovaries of all the mutant *cup* alleles. This suggested that while Cup was required for the full expression and/or stability of a subset of these proteins, its effects on protein levels were likely insufficient to explain the protein localization defect we observe in *cup* mutant egg chambers. Consistent with this interpretation, we also noted that the nurse cell perinuclear localization of Tral, Me31B, Bru, and DCP1 was comparable in wild type and *cup* mutant egg chambers (Figures 2-4, 2-5; blue arrows), while the signal within the oocyte was absent (Figures 2-4, 2-5; white arrows). Thus, while the overall level of Tral and Me31B protein was reduced in *cup* mutant egg chambers, loss of Cup selectively eliminated the accumulation of these proteins in the oocyte. Therefore, we conclude that Cup is required for the oocyte localization of Tral, Me31B, YPS, Bruno, Orb, and DCP1.

This novel requirement for Cup in the localization of multiple RNP components to the posterior pole of the developing oocyte raised the question of which domains of Cup are required for this phenotype. Since the existing EMS alleles of *cup* are not useful for such studies, we focused on the $cup^{\Delta 212}$ allele, which deletes the first 347 amino acids of Cup including the canonical eIF4E-binding site (Figure 2-3). Previous studies of the $cup^{\Delta 212}$ egg chambers found that this truncated form of Cup causes premature translation of *oskar* at stage 5 of oogenesis without affecting *oskar* mRNA localization (Nakamura et al., 2004). While this mutation appears to cleanly separate localization from translational control, $cup^{\Delta 212}$ egg chambers were never examined for defects in the localization of other components of the *oskar* RNP, including eIF4E, whose localization should be completely blocked in these egg

chambers. As predicted, we found that eIF4E accumulation in the developing oocyte was completely eliminated in *cup*^{16}*/Df(2L)bsc7* and *cup*^{20}*/Df(2L)bsc7* egg chambers and was greatly reduced in *cup*$^{\Delta 212}$*/Df(2L)bsc7* egg chambers where only 15.8% of egg chambers showed any eIF4E localization to the oocyte (Figure 2-7). Interestingly, when we immunostained ovaries from *cup*$^{\Delta 212}$*/Df(2L)bsc7* females for each of the components of the *oskar* RNP we found that the deletion of the amino terminal 347 amino acids of Cup, causes a complete loss of Tral localization to the developing oocyte (Figures 2-4, 2-5; *cup*$^{\Delta 212}$ column). Furthermore, while the remaining components of the *oskar* RNP (Me31B, YPS, Bru, Orb, and DCP1) were properly localized, the amount of protein that accumulates in the oocyte is greatly reduced (Figures 2-4, 2-5; *cup*$^{\Delta 212}$ column). None of these defects are due to alterations in protein level since expression levels of these proteins in *cup*$^{\Delta 212}$*/Df(2L)bsc7* ovaries are comparable to wild type ovaries with the exception of Orb, whose levels are greatly reduced in *cup*$^{\Delta 212}$*/Df(2L)bsc7* ovaries (Figure 2-6). Thus, while loss of the amino terminal 347 amino acids of Cup containing the canonical eIF4E binding site strongly disrupts Tral and eIF4E localization to the oocyte, this truncation does not phenocopy the spectrum of RNP subunit localization defects that we observe in putative null alleles of *cup*.

cup mutants do not have defects in oocyte determination

One possible explanation for the failure to localize Tral, Me31B, YPS, Bruno, Orb, and DCP1 to the oocyte is that Cup is required for proper oocyte formation and

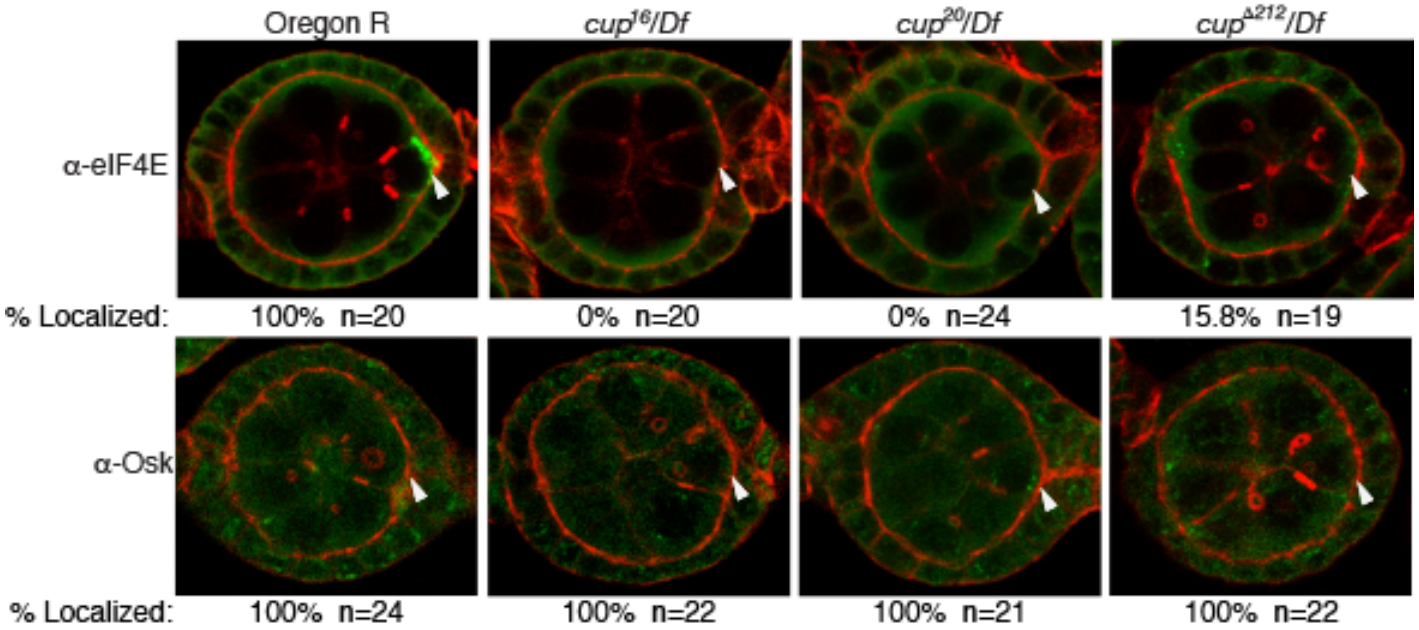


Figure 2-7 eIF4E is not properly localized in *cup* alleles but remains translationally silenced

Immunofluoresence in stage 4 Oregon R and *cup*^{16}*/Df(2L)bsc7*, *cup*^{20}*/Df(2L)bsc7*, and *cup*$^{\Delta 212}$*/Df(2L)bsc7* egg chambers against eIF4E show that its localization is either lost or drastically reduced. Oskar protein levels remain translationally silenced in mutant alleles.

that in these strong *cup* alleles the oocyte was not determined correctly. If this were the case, instead of 15 nurse cells and one oocyte, one would expect *cup* egg chambers that lack detectable Cup protein to have 16 nurse cells and no oocyte. To test this possibility, we immunostained ovaries from $cup^{16}/Df(2L)bsc7$, $cup^{20}/Df(2L)bsc7$, and $cup^{\Delta 212}/Df(2L)bsc7$ for the oocyte determination marker, C3G, which is present in the synaptonemal complex of the meiotically active oocyte (Page and Hawley, 2001). We found that C3G staining was restricted to the oocyte in $cup^{16}/Df(2L)bsc7$, $cup^{20}/Df(2L)bsc7$, and $cup^{\Delta 212}/Df(2L)bsc7$ egg chambers (Figure 2-8), indicating that the defect in *oskar* RNP localization is not due to a failure to specify an oocyte.

cup mutants show reduced levels of _oskar_ mRNA

The fact that the oocyte was properly specified suggested that *cup* mutants might have a defect in RNP assembly and/or transport. As a first step towards distinguishing between these possibilities, we used enzyme-linked RNA *in situ* hybridization to determine if *cup* was also required for the localization of *oskar* mRNA. While *osk* mRNA was correctly localized in $cup^{\Delta 212}/Df(2L)bsc7$ egg chambers as previously reported (Nakamura et al., 2004), surprisingly, we found that *oskar* mRNA localization to the posterior pole of the oocyte was also normal in both $cup^{16}/Df(2L)bsc7$ and $cup^{20}/Df(2L)bsc7$ egg chambers (Figure 2-9). This result seemed paradoxical since Bruno, which is known to bind directly to the 3' UTR of *oskar* mRNA (Kim-Ha et al., 1995), is not localized properly in the oocyte in either $cup^{16}/Df(2L)bsc7$ or $cup^{20}/Df(2L)bsc7$ egg chambers (Figure 2-5). Furthermore, it

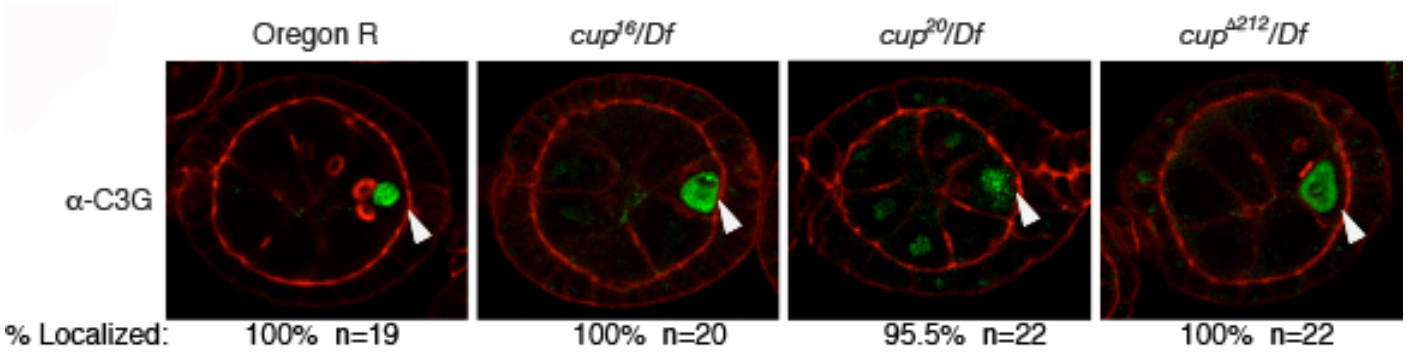


Figure 2-8 Failure to localize *oskar* RNP complex is not due to oocyte determination defects

C(3)G staining of Oregon R, *cup*^{16}*/Df(2L)bsc7*, *cup*^{20}*/Df(2L)bsc7*, and *cup* $^{\Delta 212}$*/Df(2L)bsc7* was used to detect the meiotically active oocyte. C(3)G presence was detected in stage 4 egg chambers in all *cup* alleles.

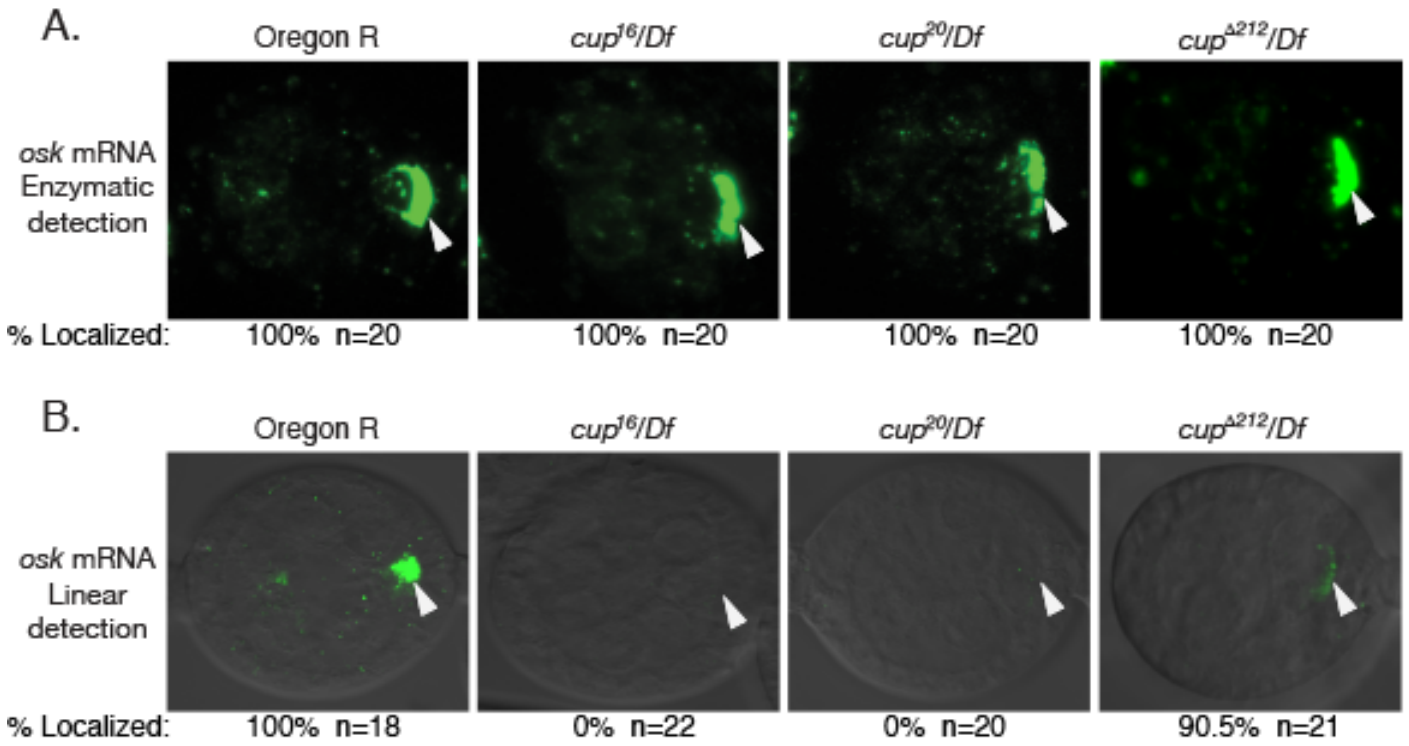


Figure 2-9 Enzyme-linked RNA *in situ* hybridization methods may mask changes in levels of transcript

(A). Enzyme-linked RNA *in situ* hybridization of *osk* transcripts shows that *osk* mRNA is localized similar to wild type levels at the posterior of the oocyte in all *cup* alleles. **(B).** Using a linear RNA *in situ* hybridization method to detect *osk* transcripts, it becomes evident that there is a drastic decrease in *osk* mRNA at the posterior in the *cup* alleles. *cup*^{16}*/Df(2L)bsc7* and *cup*^{20}*/Df(2L)bsc7* have no apparent localization of *osk*, while there is a severely decreased level in *cup*$^{\Delta 212}$*/Df(2L)bsc7*.

seemed unlikely that *oskar* mRNA could be transported to the oocyte in the absence of most of the known proteins that make up the *oskar* RNP. Thus, we explored the possibility that the non-linearity that is inherent in an enzyme-linked RNA *in situ* was masking a profound defect in *oskar* mRNA localization. To test this, we reanalyzed *oskar* mRNA localization in $cup^{16}/Df(2L)bsc7$, $cup^{20}/Df(2L)bsc7$, and $cup^{\Delta 212}/Df(2L)bsc7$ egg chambers using a quantitative, linear detection RNA *in situ* that uses a fluorophore-conjugated secondary antibody to detect the presence of the *oskar* RNA probe. Consistent with our hypothesis, while *oskar* mRNA was strongly localized to the oocyte in wild type egg chambers using this more quantitative approach, we found that the *oskar* mRNA signal was barely detectable in the oocytes from both $cup^{16}/Df(2L)bsc7$ and $cup^{20}/Df(2L)bsc7$ egg chambers (Figure 2-9). Furthermore, we were able to detect a strong decrease in the amount of *osk* mRNA localized to the oocyte in $cup^{\Delta 212}/Df(2L)bsc7$ egg chambers. This result likely explains the decrease in RNP subunit localization that we observe in $cup^{\Delta 212}/Df(2L)bsc7$ egg chambers, and also argues that the eIF4E binding domain of Cup is required for the accumulation of *oskar* mRNA to wild type levels in the developing oocyte. Together, these observations suggest that Cup is required for the proper localization of both the mRNA and protein components of the *oskar* RNP.

The defect that we observe in *oskar* RNP localization to the developing oocyte in *cup* mutant egg chambers could be due to either a failure to transport the RNP complex to the oocyte or to destabilization of the *oska*r message throughout the egg chamber. If Cup were required for transport into the oocyte, one would expect loss of Cup to cause a loss of *oskar* mRNA signal in the oocyte as well as a corresponding

increase in *oskar* in the nurse cells. However, neither the linear nor the more sensitive enzyme-linked *in situs* detected any increase in signal in the nurse cells in *cup*^{16}*/Df(2L)bsc7*, *cup*^{20}*/Df(2L)bsc7*, or *cup*$^{\Delta 212}$*/Df(2L)bsc7* egg chambers (Figure 2-9). This suggested that the defect was not a transport defect and instead was likely due to destabilization of *oskar* mRNA throughout the egg chamber. To further test this possibility, we used quantitative real-time PCR to assess the levels of *oskar* transcript in *cup*^{16}*/Df(2L)bsc7* , *cup*^{20}*/Df(2L)bsc7*, and *cup*$^{\Delta 212}$*/Df(2L)bsc7* ovaries relative to wild type ovaries. Using *rpLP2* as a control transcript to normalize the level of *oskar* mRNA in each of the samples, we found that *oskar* mRNA levels were decreased 238-fold in *cup*^{16}*/Df(2L)bsc7* ovaries and 52-fold in *cup*^{20}*/Df(2L)bsc7* ovaries as compared to wild type ovaries (Figure 2-10). We also observed a 1.74-fold decrease in *oskar* mRNA levels in *cup*$^{\Delta 212}$*/Df(2L)bsc7* ovaries suggesting that crippling eIF4E binding can significantly destabilize the *oskar* message, but not as drastically as a complete loss of Cup. This result, together with our *in situ* analysis argues that Cup is required to stabilize and accumulate *oskar* mRNA during oogenesis.

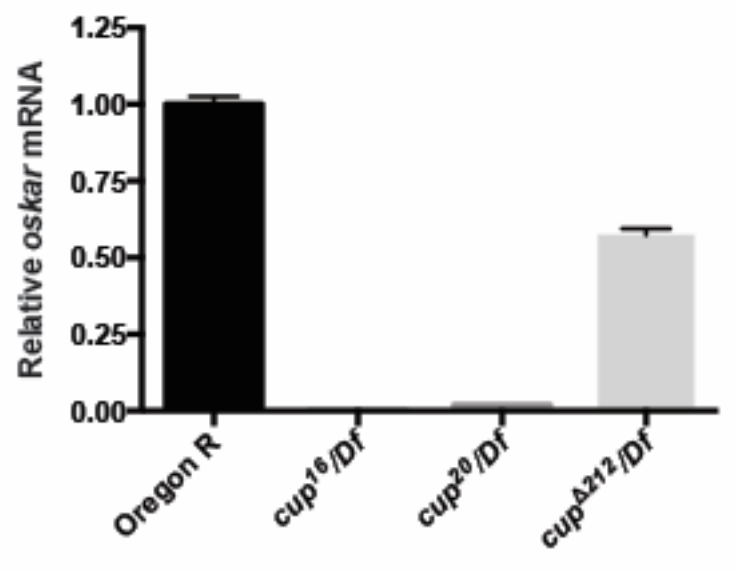


Figure 2-10 Cup is required for *osk* mRNA stability

Levels of *oskar* mRNA were measured using quantitative PCR in Oregon R, cup^{16}/Df(2L)bsc7, cup^{20}/Df(2L)bsc7, and $cup^{\Delta 212}$/Df(2L)bsc7. Levels of *osk* transcript were reduced 238-fold in cup^{16}/Df(2L)bsc7 ovaries and 52-fold in cup^{20}/Df(2L)bsc7 ovaries relative to *osk* in Oregon R. However, there was only a 1.74-fold decrease in *oskar* mRNA levels in $cup^{\Delta 212}$/Df(2L)bsc7.

Discussion

Early embryonic development is critically dependent on the post-transcriptional regulation of maternal mRNAs. While the importance of regulating the localization, translation, and stability of these messages is well established, the question of how particular RNP subunits contribute to each of these forms of post-transcriptional regulation has long been difficult to address. Previous biochemical and genetic analysis suggested that Cup might have additional functions distinct from its role as a translational repressor of *oskar* mRNA. In this study, we have found that loss of *cup* disrupts the accumulation of both the *oskar* mRNA and its associated proteins in the developing oocyte. This defect is not due to a failure to specify the oocyte or in RNP transport. Rather, the localization defects are due to a drop in *oskar* mRNA levels in *cup* mutant egg chambers. Thus, in addition to Cup's well-established role in translationally regulating *oskar* mRNA, Cup is also required to stably accumulate *oskar* mRNA during oogenesis.

These observations provide insights into the long-standing question of how maternal mRNAs are stored for extended periods of time. Previous studies have found that maternally deposited mRNAs are highly stable with a half-life estimated to be greater than two weeks (Gurdon et al., 1973). This extreme stability is thought to be due to the action of the conserved core maternal RNP complex which in *Drosophila* is comprised of the RNA helicase, Me31B, the eIF4E binding protein, Cup, the Y-box family RNA binding protein, Yps, and the LSm domain protein, Tral (Audhya et al., 2005; Boag et al., 2005; Ladomery et al., 1997; Li et al., 2009; Mansfield et al., 2002; Minshall et al., 2007; Minshall and Standart, 2004; Minshall et al., 2001; Nakamura et

al., 2001; Nakamura et al., 2004; Squirrell et al., 2006; Tafuri and Wolffe, 1993; Tanaka et al., 2006; Wilhelm et al., 2005; Wilhelm et al., 2003; Wilhelm et al., 2000). However, while previous genetic studies in *Drosophila* have found roles for these four subunits in mRNA localization and translational control, they have not identified a role for these proteins in promoting mRNA stability during oogenesis (Mansfield et al., 2002; Nakamura et al., 2001; Nakamura et al., 2004; Wilhelm et al., 2005; Wilhelm et al., 2003). Our results argue that Cup is a critical subunit for allowing maternal mRNAs to stably accumulate during oogenesis providing functional evidence for the long hypothesized role of the complex in promoting maternal RNA stability.

The fact that previous studies of *me31B*, *trailer hitch*, and *yps* failed to identify any *oskar* mRNA localization defect in early oogenesis would suggest that Cup is the only subunit of the core RNP complex that regulates *oskar* mRNA stability. However, our results argue that these previous studies may have missed effects on *oskar* mRNA levels for technical reasons. All of these previous studies utilized standard enzyme-linked RNA *in situ* protocols which we have found completely mask even severe defects in *oskar* mRNA accumulation (Figure 5 B,C). A reexamination of mutations in the core subunits of the *oskar* RNP using RNA *in situs* based on a linear detection methodology should be quite informative in uncovering whether other subunits of the RNP also contribute to *oskar* mRNA stability.

This type of analysis has already proven useful for understanding the effects of the classic the $cup^{\Delta 212}$ allele on *oskar* mRNA regulation. Much of our understanding of the *in vivo* relevance of Cup-eIF4E binding has come from studies of the $cup^{\Delta 212}$ allele where the amino terminal 347 amino acids containing the canonical eIF4E

binding site is deleted. Previous studies of *cup*$^{\Delta 212}$ mutant egg chambers found that *oskar* was translated prematurely at stage 5 of oogenesis while *oskar* mRNA localization appeared to be unimpaired. Additionally, while the *cup*$^{\Delta 212}$ mutation only deletes one of two characterized eIF4E binding sites, the truncated Cup protein fails to co-immunoprecipiate with eIF4E arguing that the deletion disrupts most or all eIF4E binding activity *in vivo*. The fact that eliminating eIF4E binding only affected translation and not mRNA stability seemed to rule out the simplest mechanism for protecting maternal mRNAs: Cup sequestering the 5'cap of *oskar* mRNA via its interactions with eIF4E (Nakamura et al., 2004; Wilhelm et al., 2003). However, our analysis of the *cup*$^{\Delta 212}$ allele using a linear *in situ* protocol and quantitative RT-PCR revealed that *oskar* mRNA only accumulates to 50% of its normal levels in *cup*$^{\Delta 212}$ */Df(2L)bsc7* egg chambers and that its localization to the developing oocyte is strongly impaired. This result suggests that the canonical eIF4E binding motif and/or other elements in the amino terminal 347aa region of Cup are required for full protection of the *oskar* transcript.

While our results implicate 5'Cap sequestration via eIF4E binding as a major mechanism for stabilizing the *oskar* mRNA, the fact that loss of eIF4E binding *in vivo* does not cause complete destabilization of the *oskar* message argues that other domains of Cup likely contribute to protecting the *oskar* transcript in vivo. This interpretation is supported by the recently described role of Cup in regulating translation via changes in poly(A) tail length (Igreja and Izaurralde, 2011). When full length Cup is tethered to a reporter mRNA, it recruits the deadenylase complex to the message causing the poly(A) tail to shorten. While a decrease in poly(A) tail length

normally leads to transcript degradation, Cup also stabilized its target by interfering with decapping of the message (Igreja and Izaurralde, 2011). Furthermore, the ability to prevent decapping was not dependent on the canonical eIF4E-binding site (Igreja and Izaurralde, 2011). This stabilizing role was interpreted as a necessary, secondary function of a protein that regulates translation via deadenylation. However, our finding that loss of Cup causes loss of *oskar* mRNA and early arrest of oogenesis argues that the reverse is true and that Cup is required for message stability even when it is not present to trigger deadenylation. This suggests that there may be additional factors that regulate *oskar* mRNA stability in egg chambers that may be lacking in the *Drosophila* S2 cells which have been used for structure-function studies of Cup (Igreja and Izaurralde, 2011).

One of the most perplexing aspects of Cup's role in *oskar* regulation is that several different biochemical functions have been assigned to the protein utilizing a variety of *in vivo* and *in vitro* systems. Most of these studies have focused on whether Cup mediates translational repression via binding to eIF4E, deadenylation, or the formation of a multimeric silencing complex (Chekulaeva et al., 2006; Igreja and Izaurralde, 2011; Nakamura et al., 2004; Wilhelm et al., 2003). One goal of our studies was to identify the earliest essential function of *cup* in oogenesis in order to provide a foundation for future structure-function studies to dissect the relative contributions of each of these mechanisms to *oskar* mRNA regulation. However, our discovery that the earliest function of Cup is to stabilize the *oskar* message suggests that such studies of *cup* will provide a novel entry point for dissecting how one protein can regulate both the translation and stability of its target transcript.

Materials and Methods

Fly Stocks

Fly stocks were cultured at 22°C-25°C on standard food. The wild-type fly strain used was Oregon R. *cup*^{16} and *cup*^{20} alleles were from the EMS mutagenesis screen described in Schupbach and Wieschaus (1991). *w*^{1118}*; Df(2L)BSC7/CyO* stock was obtained from Bloomington Stock Center at Indiana University. *cup*$^{\Delta 212}$*/CyO* stock was a gift from the lab of Dr. Nakamura (Nakamura et al., 2004).

Antibody Generation

Me31B antibody was prepared by cloning the full-length coding region into the pGEX-6P-2 vector to express amino-GST tagged Me31B recombinant protein. The protein was expressed in *E. coli* and purified using an Affi-Gel column. The protein was injected into rabbits for antiserum production (Covance). Antiserum was affinity purified using an Affi-gel column coupled with GST-Me31B protein.

Immunoblot analysis

The following antibodies were used for immunoblot analysis: anti-Me31B (this manuscript), anti-Cup (Keyes and Spradling, 1997), anti-Tral (Wilhelm et al., 2005), anti-DCP1 (Barbee et al., 2006), anti-YPS (Wilhelm et al., 2000), and anti-Bru (Webster et al., 1997). Mouse anti-Orb (6H4-s) and mouse anti-Actin (JLA20) are from the Developmental Studies Hybridoma Bank.

Immunoblot analysis was performed as previously described (Wilhelm et al., 2000) with the following modifications: Primary antibodies used were anti-Cup (1:1000),

anti-Tral (1:2000), anti-DCP1 (1:1000), anti-Me31B (1:2000), anti-YPS (1:2000), anti-Orb (1:2000), anti-Bru (1:1000), and anti-Actin (1:100). Protein was detected by chemiluminescence using HRP-conjugated donkey anti-rabbit IgG (GE Healthcare) at 1:10,000, HRP-conjugated sheep anti-mouse IgG (GE Healthcare) at 1:2,500, or HRP-conjugated goat anti-rat IgG (GE Healthcare) at 1:10,000.

Immunostaining and fluorescence microscopy

Immunostaining and microscopy was performed as previously described (Wilhelm et al., 2003) using the following primary antibody concentrations: anti-CupRat (1:1000), anti-Tral (1:1000), anti-DCP1 (1:1000), anti-Me31B (1:1000), anti-YPS (1:1000), anti-Orb (1:20), and anti-Bru (1:1000). The following secondary antibodies were used: goat anti-rabbit AlexaFluor488 (1:200) and goat anti-mouse AlexaFluor488 (1:200). Samples were mounted in Vectashield (Vector Laboratories). Microscopy was performed using Leica TCS SP2 confocal microscope.

***In situ* hybridization**

In situ hybridization protocol was performed as previously described (Wilkie and Davis, 2001) with the following modifications: Prehybridization solution contained 50ug/mL tRNA and 100ug/mL salmon sperm DNA. Prehybridization and hybridization steps were carried out at 55°C. DIG labeled probes were prepared from *osk* cDNA and hybridized probes were detected using 1:300 mouse anti-DIG antibody (Roche). Enzymatic detection was performed using TSA Fluorescein System (Perkin

Elmer). Linear detection was performed using 1:200 AlexaFluor488 goat anti-mouse secondary antibody.

RT-PCR Analysis

Flies were fattened on wet yeast for 1 day and ovaries were dissected in Grace's media (Gibco). RNA was extracted from ovaries using homogenization and TRIzol Reagent (Invitrogen) as described in the product manual. cDNA was prepared from isolated RNA using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). The cDNA was then used for qRT-PCR analysis by detection using QuantiTect SYBR Green (QIAGEN) on the iCycler (BioRad). Primer sequences are available upon request.

Sequencing

Five flies from the following strains were collected for each sample: *cup*^{8}*/Df(2L)BSC7*, *cup*^{21}*/Df(2L)BSC7*, *cup*^{32}*/Df(2L)BSC7*, *cup*^{16}*/Df(2L)BSC7*, *cup*^{20}*/Df(2L)BSC7*, and Oregon R. The flies were homogenized using a microfuge tube and pestle in 50uL Squishing Buffer (10mM Tris-Cl pH8.2, 1mM EDTA, 25mM NaCl, and 0.2mg/mL Proteinase K) and incubated at room temperature for 30 minutes. The samples were incubated at 95°C for 2minutes and then transferred to ice. 1uL of this crude fly prep was used as PCR template with primers flanking each of the individual exons and the product was sent for sequencing. Primer sequences are available upon request.

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Chapter 3

The Pan gu kinase complex triggers the proteolytic disassembly of maternal RNPs to promote mRNA degradation at the maternal-to-zygotic transition

Abstract

Maternal mRNA is stored in highly stable RNP complexes during oogenesis. This extreme mRNA stability, however, poses a problem for the maternal-to-zygotic transition when many maternal messages must be degraded to pave the way for the control of development by zygotic transcription. As a result, it has been suspected that maternal RNP complexes are remodeled to facilitate mRNA degradation. We have identified a set of maternal RNP subunits are degraded in the early *Drosophila* embryo via ubiquitin-mediated proteolysis. Furthermore, we find that disrupting the ubiquitin-proteasome system prevents mRNA degradation in the early embryo and that RNP subunit degradation is controlled by the protein kinase, *pan gu*, which also activates the mRNA degradation machinery in the early embryo. Interestingly, *pan gu* controls RNP subunit degradation via a pathway that is dependent on the meiotic anaphase promoting complex (APC) but separate from the one used to activate the mRNA degradation machinery. Thus, *pan gu* controls maternal mRNA degradation via two parallel pathways: activating mRNA degradation machinery and the removal of protective RNP subunits via ubiquitin-mediated proteolysis. Together, these findings provide new insights into how protein degradation can trigger transcript destruction suggesting a novel interplay between two major degradation pathways that were previously thought to be separate.

Introduction

The spatial and temporal regulation of maternally deposited RNA and protein drives many of the early events of embryogenesis in *Drosophila* and other organisms. This is due to the fact that transcription is not activated until later in development. Thus, the ability to stably accumulate large amounts of RNA and protein during oogenesis is critical for many developmental processes. Consistent with this, while mRNA in most systems has a relatively short half-life, maternally deposited mRNAs are highly stable with a half-life estimated to be greater than two weeks (Gurdon et al., 1973). This extreme stability is believed to be due to the action of a conserved core maternal RNP complex which in *Drosophila* is comprised of the RNA helicase, Me31B, the eIF4E binding protein, Cup, the Y-box family RNA binding protein, Yps, and the LSm domain protein, Trailer Hitch (Audhya et al., 2005; Boag et al., 2005; Ladomery et al., 1997; Li et al., 2009; Mansfield et al., 2002; Minshall et al., 2007; Minshall and Standart, 2004; Minshall et al., 2001; Nakamura et al., 2001; Nakamura et al., 2004; Squirrell et al., 2006; Tafuri and Wolffe, 1993; Tanaka et al., 2006; Wilhelm et al., 2005; Wilhelm et al., 2003; Wilhelm et al., 2000). One of the ways in which this complex is thought to stabilize transcripts is via the eIF4E binding protein subunit of the complex sequestering the 5' cap of the transcript and preventing its removal by deadenylation dependent decapping (Igreja and Izaurralde, 2011). The ability of the core maternal RNP complex to block mRNA degradation presents the early embryo with a critical problem - how to eliminate highly stable maternal messages as a prelude to the onset of zygotic control of development at the maternal-to-zygotic transition (MZT).

Previous genetic studies have identified a maternal mRNA degradation pathway in *Drosophila* that controls transcript elimination at the MZT (Tadros et al., 2007; Tadros et al., 2003). In this pathway, egg activation stimulates a complex consisting of the protein kinase Pan gu (Png), and two accessory subunits, Giant nuclei (Gnu) and Plutonium (Plu) (Tadros et al., 2007). Activation of the Png kinase in turn triggers translation of the RNA binding protein, Smaug (Smg). Smg binds to specific maternal mRNAs and recruits the CCR4/Twin deadenylase to initiate message degradation (Semotok et al., 2005; Tadros et al., 2007).

However, the presence of Smg alone is not sufficient for mRNA degradation. Previous studies found that the expression of s*mg* in embryos lacking Png does not trigger mRNA degradation - a result that suggested the existence of an additional *png*-dependent pathway that is required for maternal mRNA degradation (Tadros et al., 2007). These observations raised the possibility that *png* regulates both the activation of mRNA degradation factors and the inactivation of protective subunits of the maternal RNP.

Our studies have found that a subset of maternal RNP subunits are eliminated via ubiquitin-mediated degradation during early embryogenesis and that disrupting the ubiquitin-proteasome pathway stabilizes maternal messages. Furthermore, these protein degradation events are controlled by the Png kinase complex. We have also identified a subset of mRNA degradation mutants that are defective in RNP subunit degradation, but do not affect Png activation or *smg* translation. Together, these results argue that *png* coordinates activation of the mRNA degradation machinery with

degradation of the stabilizing proteins on maternal RNPs to trigger mRNA turnover at the maternal-to-zygotic transition.

Results

Cup, Tral, and Me31B are specifically degraded via ubiquitin-mediated proteolysis during the early phase of the maternal-to-zygotic transition.

While a great deal is known about the mechanism of mRNA degradation during the early phase of the maternal-to-zygotic transition, relatively little is known about the fate of the proteins that are associated with maternal messages. In order to address this question, we measured the levels of the major components of maternal RNPs during the first four hours of embryogenesis by immunoblot. These experiments revealed that the levels of many proteins that are associated with maternal mRNAs, such as Dcp1, Yps, and Edc3, are not down-regulated in concert with the degradation of maternal transcripts (Figure 3-1). However, three proteins were found to be down-regulated during the initial phases of the maternal-to-zygotic transition – Cup, Tral, and Me31B (Figure 3-1). These results argue that there is not a global homeostatic mechanism for down-regulating all of the proteins subunits of the maternal RNP as messages are destroyed, but rather only a specific subset of those proteins are targeted for elimination from the early embryo.

Since early *Drosophila* development is regulated by post-transcriptional mechanisms, we first tested whether the down-regulation of the proteins was due to one such mechanism: ubiquitin-mediated proteolysis. If the degradation of Cup, Tral, and Me31B occurs in a ubiquitin-dependent manner, one would predict that blocking

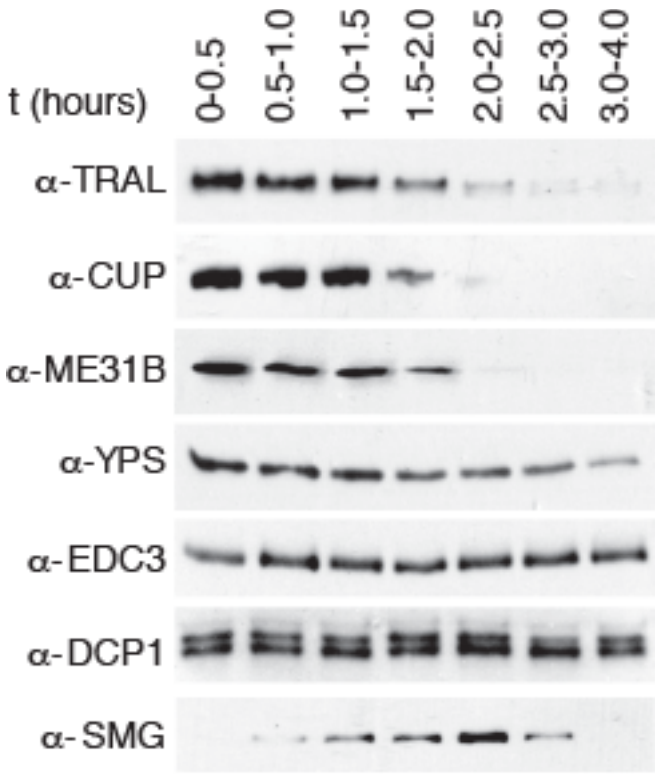

Figure 3-1 A subset of maternal RNP components are degraded via ubiquitin-mediated proteolysis at the MZT

Tral, Cup and Me31B are eliminated during early embryonic development. Embryos from wild type flies grown at room temperature were collected for 30-minute intervals and aged for the indicated time period. Equal numbers of embryos were then collected, dechorionated and lysed in sample buffer, after which the lysates were analyzed by immunoblotting with the indicated antibody.

proteasome function would stabilize Cup, Tral, and Me31B. To test this possibility, we treated permeabilized *Drosophila* embryos with the proteasome inhibitor lactacystin and measured the levels of Cup, Tral, and Me31B by immunoblot. Lactacystin treatment stabilized all three subunits, arguing that Cup, Tral, and Me31B are degraded by the proteasome during the MZT (Figure 3-2A).

In order to confirm that our results were not due to indirect effects of lactacystin treatment, we also disrupted ubiquitin-mediated proteolysis using a genetic approach. *uba1* is essential for ubiquitin-mediated proteolysis since it is the only E1 conjugating enzyme in the *Drosophila* genome (Lee et al., 2008). $Pros26^{1}$ is a temperature-sensitive allele of the β6 -subunit of the proteasome that dominantly interferes with protein degradation (Neuburger et al., 2006). However, embryos that are homozygous for either $uba1^{H42}$, a null allele of *uba1*, or $Pros26^{1}$ only have larval phenotypes due to the maternally loaded Uba1 and proteasome subunits rescuing the mutant phenotype in the early embryo. In order to disrupt ubiquitin-mediated proteolysis in the early embryo, we generated strains that were heterozygous for $uba1^{H42}$ and also expressed the dominant-negative $Pros26^{1}$ allele. Cup, Tral, and Me31B demonstrated increased stability in embryos from mothers that were heterozygous for both $uba1^{H42}$ and $Pros26^{1}$ (Figure 3-2B). These results, together with the fact that lactacystin treatment stabilizes all three RNP subunits, argue that ubiquitin-mediated proteolysis is responsible for the elimination of Cup, Tral, and Me31B from the early embryo at the MZT.

The fact that proteasome-mediated degradation is required for the down-regulation of Cup, Tral, and Me31B, raised the question of whether these three

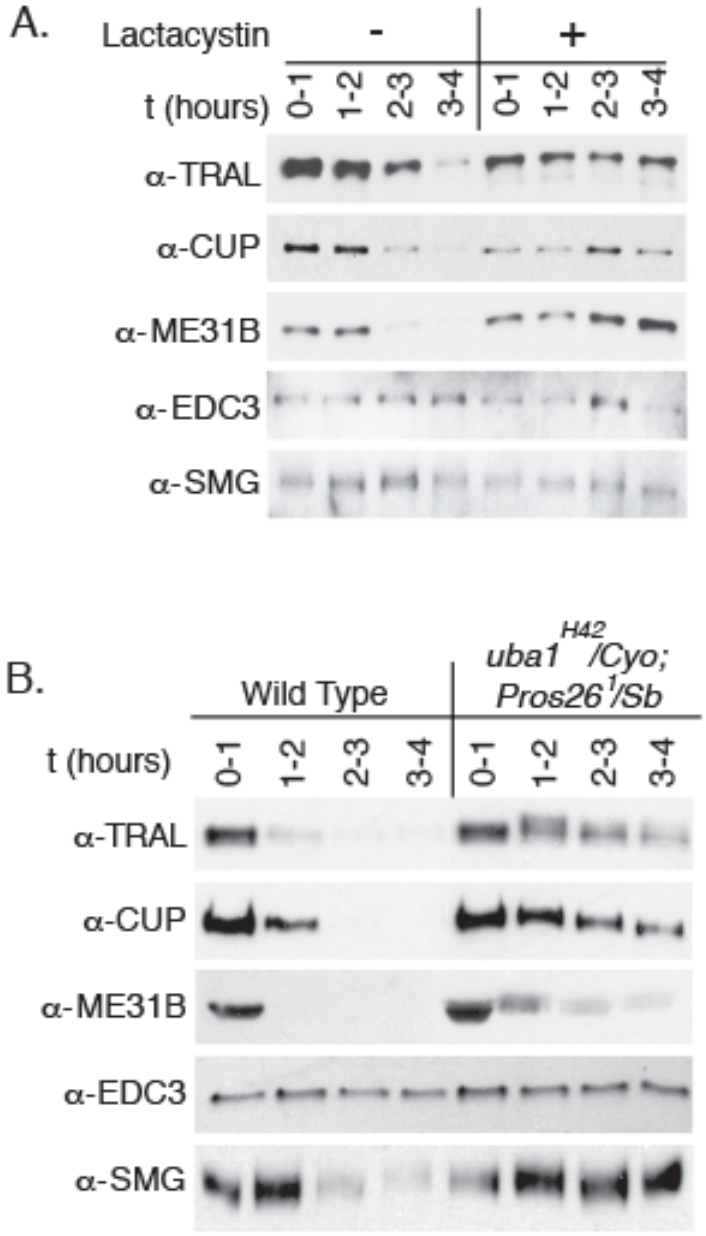

Figure 3-2 Maternal RNP degradation can be chemically or genetically inhibited

(A) Tral, Cup and Me31B degradation is blocked by proteasome inhibitor treatment. Embryos were collected for 1 hour and dechorionated. They were then incubated in Schneider's media with or without 10 μM lactacystin for the indicated time period, followed by lysis and analysis by immunoblotting.
(B) Genetic disruption of the ubiquitin-proteasome pathway interferes with Tral, Cup and Me31B degradation. Embryos were collected as previously described from wild type or *uba*^{H42}*/Cyo; Pros26*^{1}*/Sb* flies.

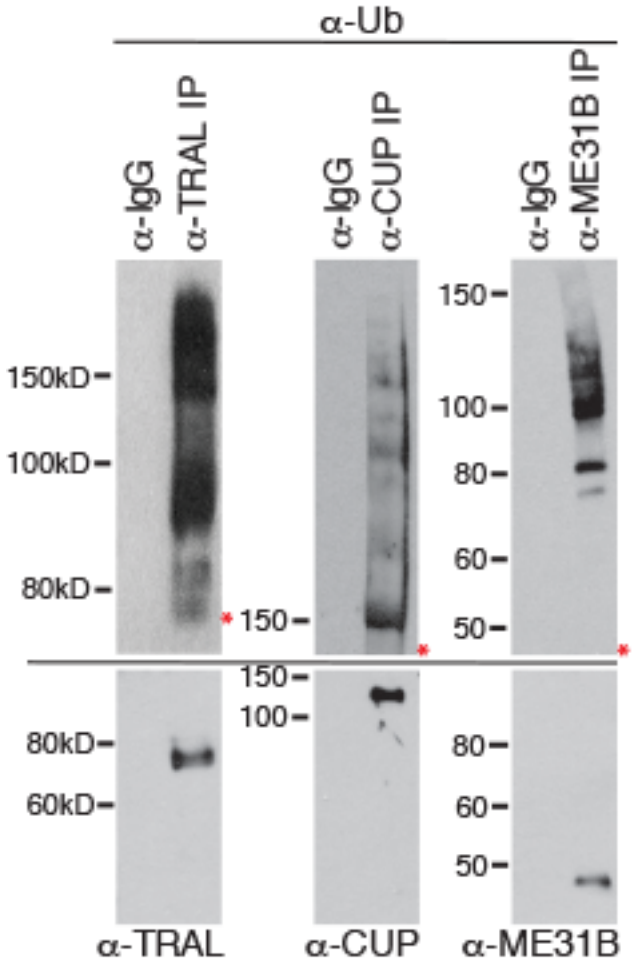

Figure 3-3 Maternal RNPs are ubiquitinated in the early embryo

Tral, Cup and Me31B are ubiquitinated in early embryos. Embryos were collected for 2 hours from wild type flies, dechorionated and lysed in DXB50 buffer. Beads covalently attached to the indicated antibody were incubated with the embryo extract and resulting immunoprecipitate was analyzed by immunoblotting with an anti-Ub antibody (Top Panels) and the antibody used in the pull-down (Bottom Panels). Molecular weight of non-ubiquitinated RNP is indicated with red asterisk.

subunits are direct targets of the ubiquitination machinery. If the ubiquitin-proteasome pathway acts directly on Cup, Tral, and Me31B at the maternal-to-zygotic transition, one would expect these proteins to be ubiquitinated in the early embryo. To examine this, we immunoprecipitated Cup, Tral, and Me31B from embryo extracts and immunoblotted for ubiquitin. Consistent with our model, all three proteins were poly-ubiquitinated in the early embryo (Figure 3-3).

The degradation of Cup, Tral, and Me31B occurs independently of maternal mRNA degradation.

One possible explanation for these results is that Cup, Tral, and Me31B are unstable when they are not bound to mRNA and that the degradation of these three proteins is a secondary consequence of the large-scale elimination of maternal messages during the first two hours of the MZT. If this model were true, one would expect that mutations that stabilize maternal messages, such as *smg* or *twin* (the catalytic subunit of the 3' exonuclease), would also stabilize Cup, Tral, and Me31B (Semotok et al., 2005). To test this possibility, we first asked whether any of their messages were targets of the maternal mRNA degradation pathway since any effect on protein levels might be caused by changes in *cup*, *tral*, and *me31B* transcript stability in these mutants. Quantitative RT-PCR was used to measure *cup*, *tral*, and *me31B* transcripts levels in embryos from $smg^{1}/Df\text{-}Scf^{R6}$ females. While mutations in *smg* had no effect on *cup* transcript levels during the first two hours of the MZT (Figure 3-4), the levels of both *tral* and *me31B* transcripts are significantly increased (Figure

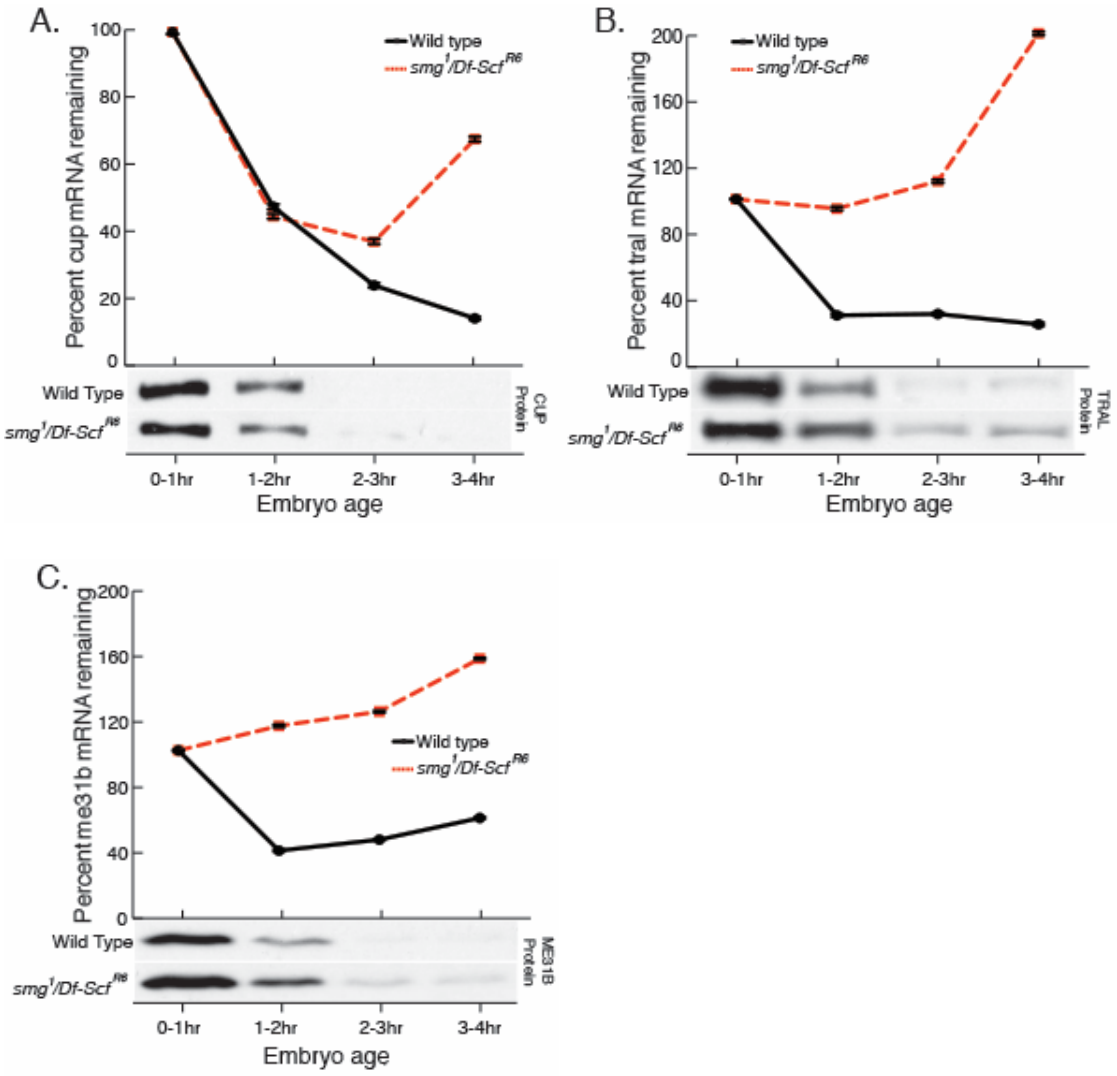

Figure 3-4 Protein degradation is not secondary to mRNA degradation

(A) Mutations in *smg* have no effect on *cup* mRNA degradation or Cup protein degradation during the first two hours of the MZT. Mutations in *smg* stabilize both **(B)** *tral* and **(C)** *me31b* transcripts during the first two hours of the MZT, while both Tral and Me31B proteins continue to be degraded.

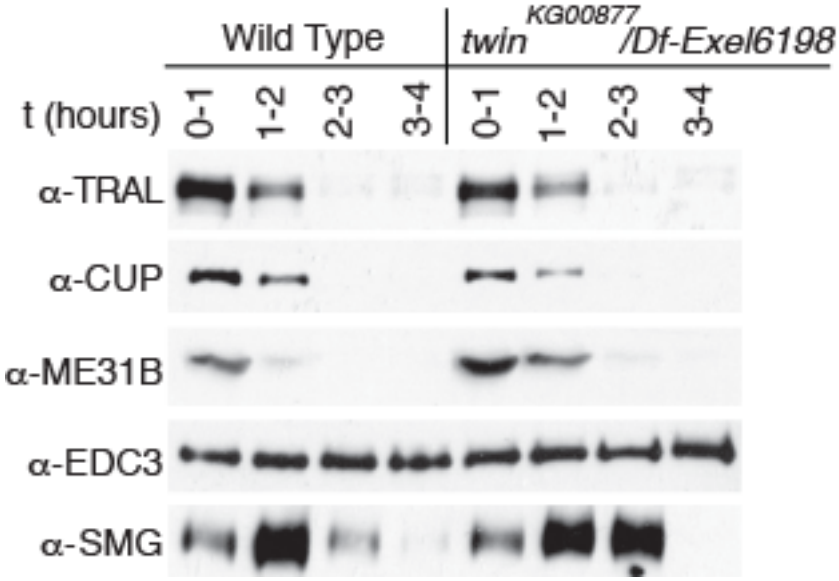

Figure 3-5 Protein degradation occurs in mutants that have defects in mRNA degradation

Tral, Cup, and Me31B degradation is not affected *twin* mutant flies that are defective in maternal mRNA degradation. Embryos were collected and analyzed as previously described from wild type, Smg^{1}/Df, or $twin^{KG00877}/Df$ flies.

3-4) arguing that *tral* and *me31B* mRNAs are targets of the maternal mRNA degradation machinery.

Interestingly, in spite of the increased levels of *tral* and *me31B* transcripts, the levels of Cup, Tral, and Me31B protein were still all strongly down-regulated in embryos from $smg^{1}/Df\text{-}Scf^{R6}$ females (Figure 3-4), similarly to wild type. The levels of all three proteins were also down-regulated in embryos from $twin^{KG00877}/Df\text{-}Exel6198$ females which are also defective in maternal mRNA degradation (Figure 3-5). Together these results argue that the elimination of Cup, Tral, and Me31B in the early embryo is not a secondary consequence of maternal mRNA degradation.

Degradation of maternal transcripts is dependent on ubiquitin-mediated proteolysis

The fact that degradation of specific subunits of maternal RNP complexes occurs even when maternal mRNA degradation is blocked argues that the destruction of maternal transcripts does not cause the destabilization of the proteins that make up the maternal RNP. As a result, we next examined whether protein degradation is necessary for maternal mRNA degradation. If RNP subunit removal is necessary for maternal mRNA degradation, one would predict that mRNA degradation would be impaired in mutant backgrounds, such as $uba1^{H42}/+;\ Pros26^{1}/+$, that interfere with RNP subunit degradation. To test this, we examined the stability of two well characterized mRNA targets of *smg*-mediated mRNA degradation, *nanos* and *hsp83*, in both wild type and $uba1^{H42}/+;\ Pros26^{1}/+$, embryos. Both *nanos* and *hsp83* transcripts were stabilized in $uba1^{H42}/+;\ Pros26^{1}/+$ embryos arguing that ubiquitin-

mediated RNP subunit degradation contributed to maternal mRNA degradation (Figure 3-6A,B).

The Png kinase complex acts in early embryogenesis to coordinate the elimination of both protein and mRNA components of maternal RNPs

The fact that disrupting the ubiquitin-proteasome pathway stabilizes maternal messages during the early phase of the MZT suggested that two possibilities. First, the known steps of maternal mRNA degradation might be regulated via ubiquitin-mediated proteolysis. Alternatively, Cup, Tral, and Me31B protein degradation might be regulated by the same pathways that trigger mRNA degradation machinery in the early embryo. If the first possibility were correct, the disruption of ubiquitin-medieated proteolyis in *uba1/+; Pros26*^{1}*/+* embryos could stabilize maternal messages by either blocking or delaying egg activation, Png kinase activity or and *smg* translation (Tadros et al., 2007). However, Smg is translated in *uba1/+; Pros26*^{1}*/+* embryos (Figure 3-2). This argues that disrupting ubiquitin-mediated proteolysis does not stabilize maternal transcripts by altering any of the known steps of the maternal mRNA decay pathway. As a result, we turned our attention to the question of whether maternal RNP protein degradation was controlled by any of the steps that regulate maternal mRNA degradation.

One of the hallmarks of the maternal mRNA degradation pathway in *Drosophila* is that it can be initiated by egg activation in the absence of fertilization (Tadros et al., 2003). Egg activation in turn triggers the Png kinase complex that regulates translation of the mRNA degradation factor, Smg (Tadros et al., 2007).

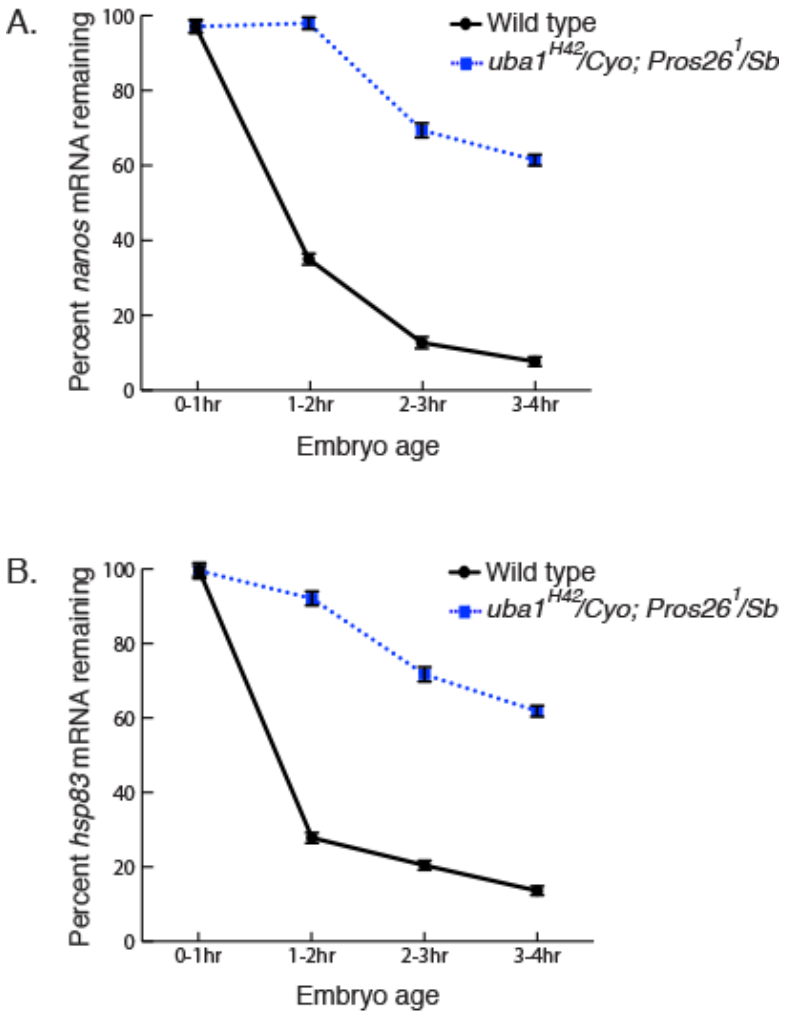

Figure 3-6 Degradation of maternal transcripts *nanos* and *hsp83* are dependent on the ubiquitin-proteasome system.

(A) *nanos* and **(B)** *hsp83* mRNA degradation is inhibited in flies partially impaired in the ubiquitin-proteasome pathway. Embryos were collected for 1 hour from wild type or *uba*^{H42}*/Cyo; Pros26*^{1}*/Sb* flies and aged for the indicated time. Embryos were then dechorionated and lysed to isolate RNA. The RNA was used to synthesize cDNA, which acted as the template for quantitative RT-PCR reactions, from which we determined the relative amounts of *nanos* or *hsp83* message. *nanos* and *hsp83* levels were normalized to a control RNA (*rpLP2*).

In order to determine the relationship between the RNP subunit degradation and the events that lead to maternal mRNA degradation, we first tested whether egg activation was sufficient to trigger the degradation of Cup, Me31B and Tral. All three proteins are degraded in both fertilized and unfertilized eggs that have been activated *in vivo* (Figure 3-7A). Thus, RNP subunit degradation is downstream of egg activation in the maternal mRNA degradation pathway. Furthermore, since no transcription occurs in activated, unfertilized eggs (Anderson and Lengyel, 1979), RNP subunit degradation is regulated purely by post-transcriptional mechanisms.

The Pan gu kinase is a key player in the post-transcriptional regulatory program triggered by egg activation and is the most upstream component of the maternal mRNA degradation pathway (Kronja et al., 2014; Tadros et al., 2007; Tadros et al., 2003). Thus, we next tested whether Png was required for RNP subunit degradation. When the levels of Cup, Tral, and Me31B are measured in embryos from png^{50} females, all of the proteins are maintained at a constant level demonstrating that *png* function is required to degrade these three proteins (Figure 3-7B). Similar results were also obtained when another subunit of the Pan gu kinase complex, Giant nuclei (Gnu), is disrupted arguing that the Png kinase complex coordinately regulates the degradation of both mRNA and protein components of maternal RNPs (Figure 3-8).

Given that degradation of the proteins of the maternal RNP are regulated by Png kinase, we next investigated the relationship between RNP subunit degradation and known pathways controlled by Png. While proteomics studies have implicated Png kinase broadly in translational regulation, there are two distinct Png-dependent pathways that have been well characterized in the activated egg. In the first pathway,

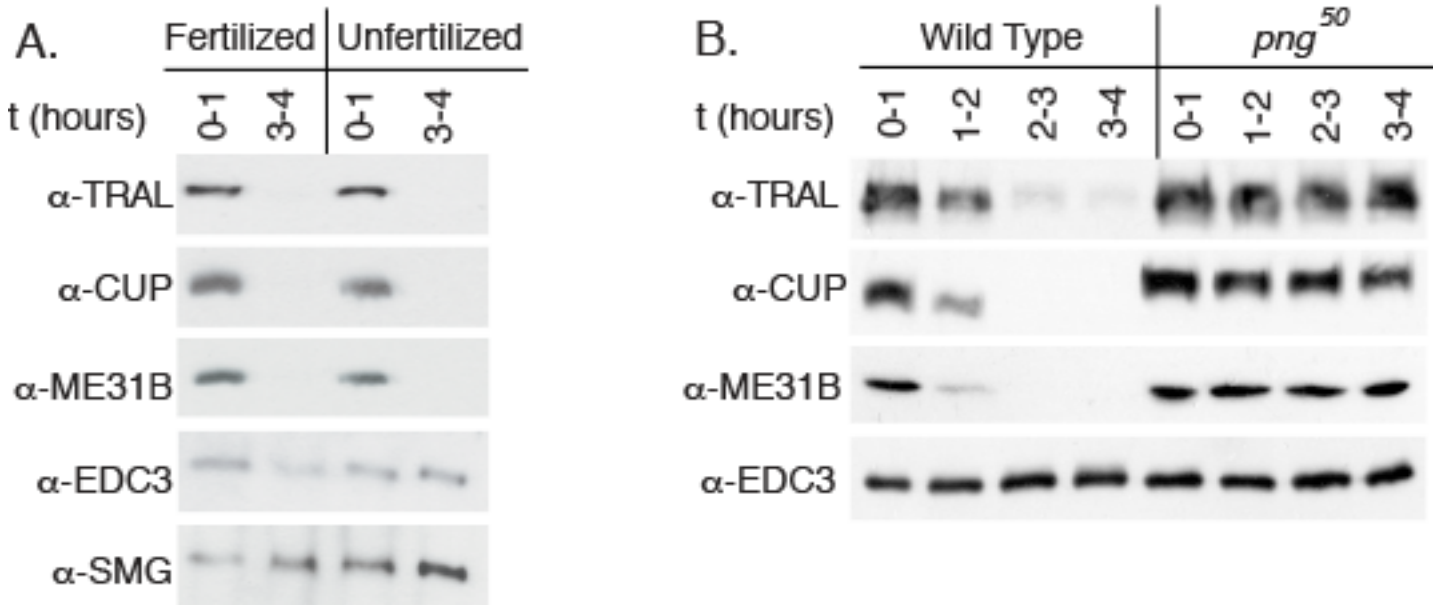

Figure 3-7 The Png kinase complex is required for the degradation of maternal RNP components

(A) Tral, Cup and Me31B are degraded in unfertilized embryos. **(B)** Degradation of Tral, Cup and Me31B is inhibited in *png*. Embryos were collected and analyzed as previously described from wild type or *png*^{50}

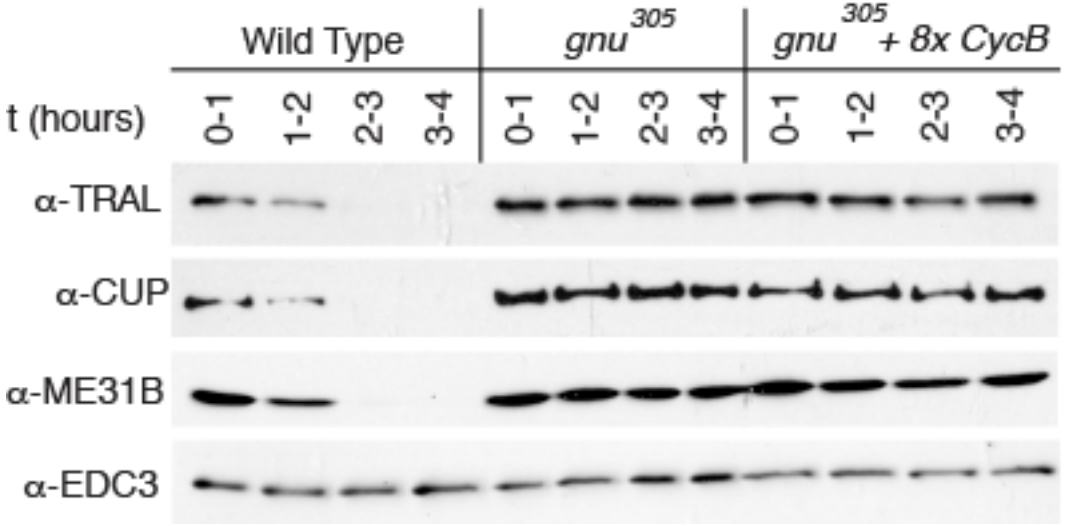

Figure 3-8 Png kinase complex mutants have defects in the degradation of maternal RNP components even when mitotic cell cycle is restored

Degradation of Tral, Cup, and Me31B is inhibited in *gnu* mutant flies. Embryos were collected and analyzed as previously described from wild type, gnu^{305} or gnu^{305} with 8 additional copies of *CycB* flies.

Png kinase regulates the cell cycle by activating *cyclin B* translation in order to facilitate the S/M transition after meiosis (Lee et al., 2001; Lee et al., 2003; Vardy and Orr-Weaver, 2007). In the second pathway, Png kinase controls two parallel processes that must be activated for maternal mRNA degradation to occur: the translation of the RNA binding protein Smg and a second poorly characterized pathway - the *png*-dependent, *smg*-indepenent branch of the mRNA degradation pathway (Tadros et al., 2007).

To determine which of these steps/pathways might be used to regulate maternal RNP protein degradation, we exploited the extensive genetic reagents available for the cell cycle and mRNA decay pathways. We first tested whether RNP degradation was downstream of Png's role in cell cycle regulation. Early embryos from *png* mutant females exhibit mitotic defects that can be partially suppressed by increasing the gene dosage of either *cyclin B* or *cyclin B3* (Lee et al., 2001). However, the mRNA degradation defects in these embryos cannot be suppressed by increasing *cyclin B* copy number consistent with mRNA degradation and cell cycle regulation being separate Png-regulated pathways (Tadros et al., 2003). We applied the same approach to examine whether mutations in the Png kinase complex fail to degrade Cup, Tral, and Me31B due to a reduction CDK/cyclin activity. When 8 copies of *cyclin B* are introduced into a gnu^{305} mutant background, we observe no rescue of the Cup/Tral/Me31B degradation defect (Figure 3-8). This result argues that the Png kinase complex regulates Cup, Tral and Me31B levels independent of its role in regulating *cyclin B* translation. Furthermore, because our initial studies of RNP subunit degradation found that RNP subunits are degraded normally in *smg* mutant

embryos (Figure 3-4), RNP subunit degradation is independent of Smg function. Thus, the degradation of the protein subunits of the maternal RNP is controlled via a *png*-dependent, *smg*-independent pathway making it an excellent candidate for the missing branch of the maternal mRNA decay pathway.

In order to test this possibility further, we sought to define when the Png kinase complex is required to regulate RNP subunit degradation. While the Png kinase complex is present in both oogenesis and embryogenesis, previous studies have shown that Png activity is only required in the embryo in order to trigger maternal mRNA degradation (Tadros et al., 2007). If the Png kinase complex coordinates the translation of Smg with the degradation of RNP subunits, one would expect that activation of Png kinase within the embryo would be sufficient to trigger RNP subunit degradation as well. To test this, we utilized a transgenic strain where the *png* ORF was expressed under the control of the cis-acting elements of *bicoid* (*bcd5' UTR-png-bcd 3' UTR*) in a png^{158} mutant background (Tadros et al., 2007). The cis-acting elements of *bicoid* have two key features that make it ideal for this experiment. First, while *bicoid* is transcribed during oogenesis, it is only translated after egg activation and this translational regulation is controlled by the *bcd* 3'UTR. Thus, Png protein in this strain is absent throughout oogenesis, but is then restored after egg activation, allowing its role in oogenesis to be separated from its role in the early embryo. Second, the *bicoid* RNA localization elements target messages to the anterior of the embryo. As a result, the *bcd5' UTR-png-bcd 3' UTR* mRNA from the transgene will be restricted to the anterior pole, generating a gradient of rescue activity. Thus, if Png is only required in the embryo for RNP subunit degradation, one would expect that

RNP subunits would be selectively degraded at the anterior of the embryo where Png is present.

To test this prediction, we immunostained embryos from *png*^{158}*; bcd5' UTR-png-bcd 3' UTR* females for both Smg and Cup. Consistent with previous studies using this strain, we found that Smg is expressed in an anterior to posterior gradient as predicted by the requirement for Png to translationally activate Smg (Figure 3-9) (Tadros et al., 2007). Cup, however, was found in a posterior to anterior gradient. The loss of Cup signal at the anterior of the embryo, where the Png kinase is found, argues that Png kinase is only required in the embryo for RNP subunit degradation. Thus, both RNP subunit degradation and maternal mRNA degradation are coordinately regulated by the Png kinase complex in the early embryo.

Identification of mRNA degradation mutants that activate Smaug translation, but are defective in RNP subunit degradation

The timing of RNP subunit degradation combined with the fact that it is *png*-dependent, but *smg*-independent suggested that this might be the missing branch of the maternal mRNA degradation pathway. If this were the case, the mutations in genes that act in this branch of the mRNA degradation pathway should be defective for both RNP subunit degradation and mRNA degradation, but would express Smg normally. To identify such mutants, we rescreened a collection of maternal mRNA degradation pathway mutants and uncharacterized maternal effect lethals (Tadros et al., 2003) to determine if they showed defects in Cup, Tral, and Me31B protein degradation. We found that none of the uncharacterized maternal effect lethals had defects in RNP

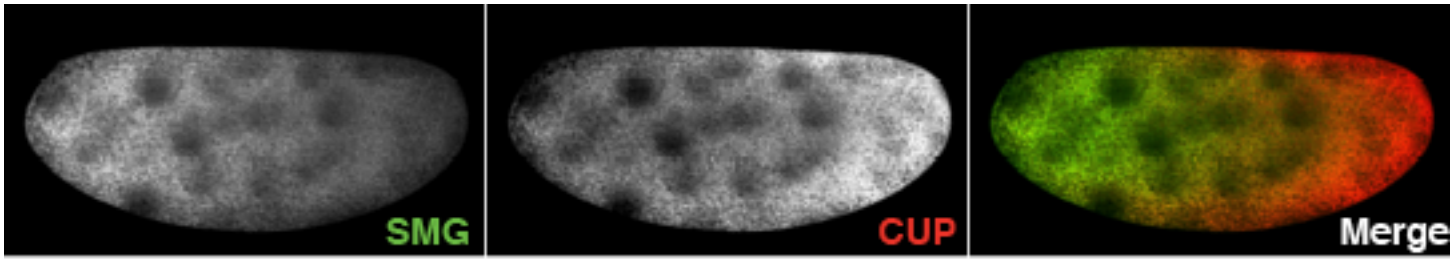

Figure 3-9 Png kinase dependent degradation of maternal RNP components can be visualized in the embryo

Cup degradation at the posterior is inhibited when Png is localized to the anterior of the embryo. Embryos were collected for 30 minutes and aged for 2 additional hours from flies expressing a transgene which contains the *png* open reading frame flanked by the 5' and 3' *bcd* UTRs. The embryos were dechorionated, permeabilized and stained with α-Smg and α-Cup antibodies.

subunit degradation. However, we identified 3 additional mRNA degradation pathway components that are also required for RNP subunit degradation: *grauzone* (*grau*), *wispy* (*wisp*), and *cortex* (*cort*) (Figure 3-10A,B,C). Since all three of these mutants were previously shown to have defects in mRNA degradation, we next tested whether these mutants disrupted Smg translation (Tadros et al., 2003). Consistent with previous studies of *wisp,* we found that Smg translation was activated normally in *wisp* mutants arguing that *png* signaling and *smg* activation are not affected in this mutant (Figure 3-10B) (Cui et al., 2008). Similarly, we found that *grau* and *cort* mutants also activate *smg* translation normally (Figure 3-10A,C). These results argue that *grau*, *wisp*, and *cort* all regulate both RNP subunit and maternal mRNA degradation via a *smg*-independent pathway.

Both the *cort* and *fzy* meiotic anaphase promoting complexes are required for degradation of RNP subunits and maternal transcripts.

One feature common to mutations in *grau*, *wisp*, and *cort* is that they all affect meiotic APC function. *cort* is a CDC20 family member that acts as a targeting subunit of the meiotic anaphase promoting complex (APC) (Chu et al., 2001; Pesin and Orr-Weaver, 2007; Swan and Schupbach, 2007). *grau* is a transcription factor whose only essential target is *cort*, while *wisp* is a GLD-2 family poly(A) polymerase that regulates the translation of *cort* during meiosis (Cui et al., 2008; Harms et al., 2000). The fact that all three mutants have a defect in *cort* regulation or activity raised the question of whether the defects in RNP subunit degradation in these mutants are due to a meiotic arrest or are specific to defects in meiotic APC activity. While previous

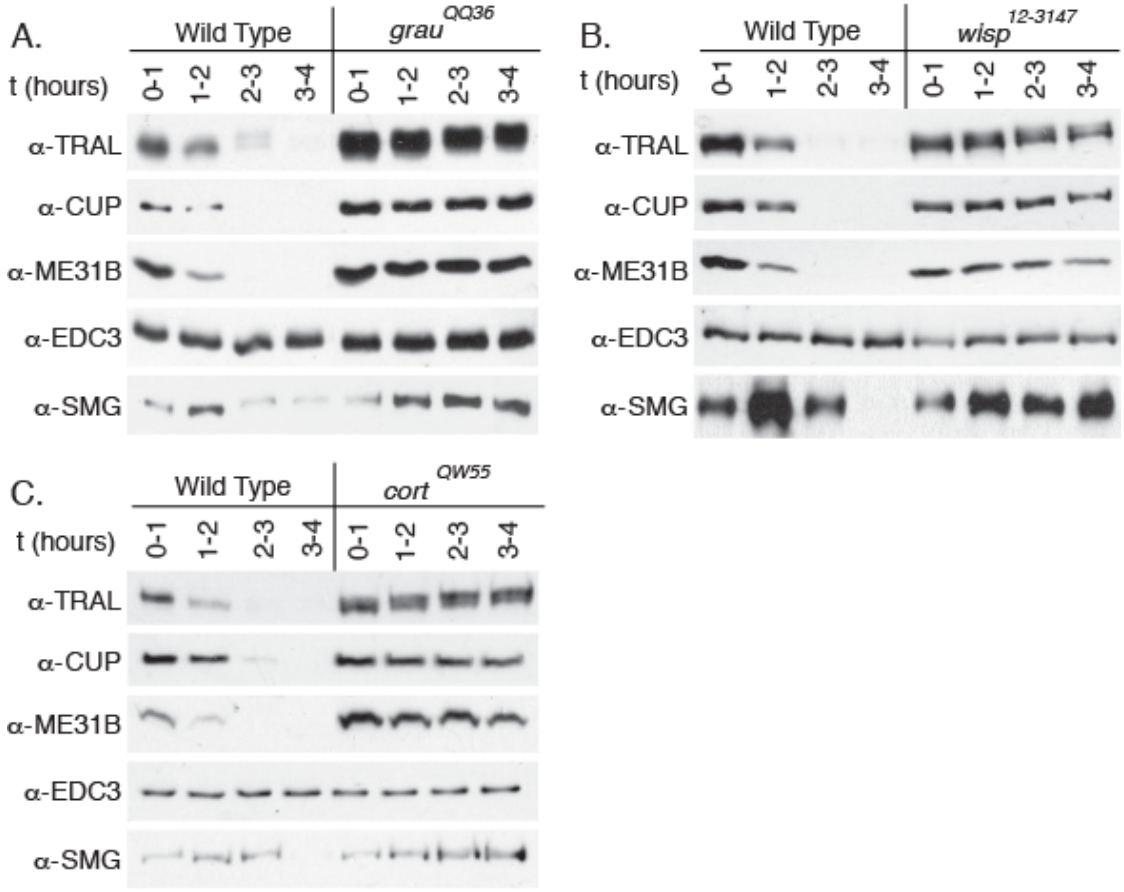

Figure 3-10 Identification of mRNA degradation mutants that express Smg but are defective in RNP protein degradation

Tral, Cup and Me31B degradation is blocked in **(A)** *grauzone*, **(B)** *wispy* and **(C)** *cortex* mutants. Embryos were collected and analyzed as previously described from wild type, $grau^{QQ36}$, $wisp^{12\text{-}3147}$ and $cort^{QW55}$ flies.

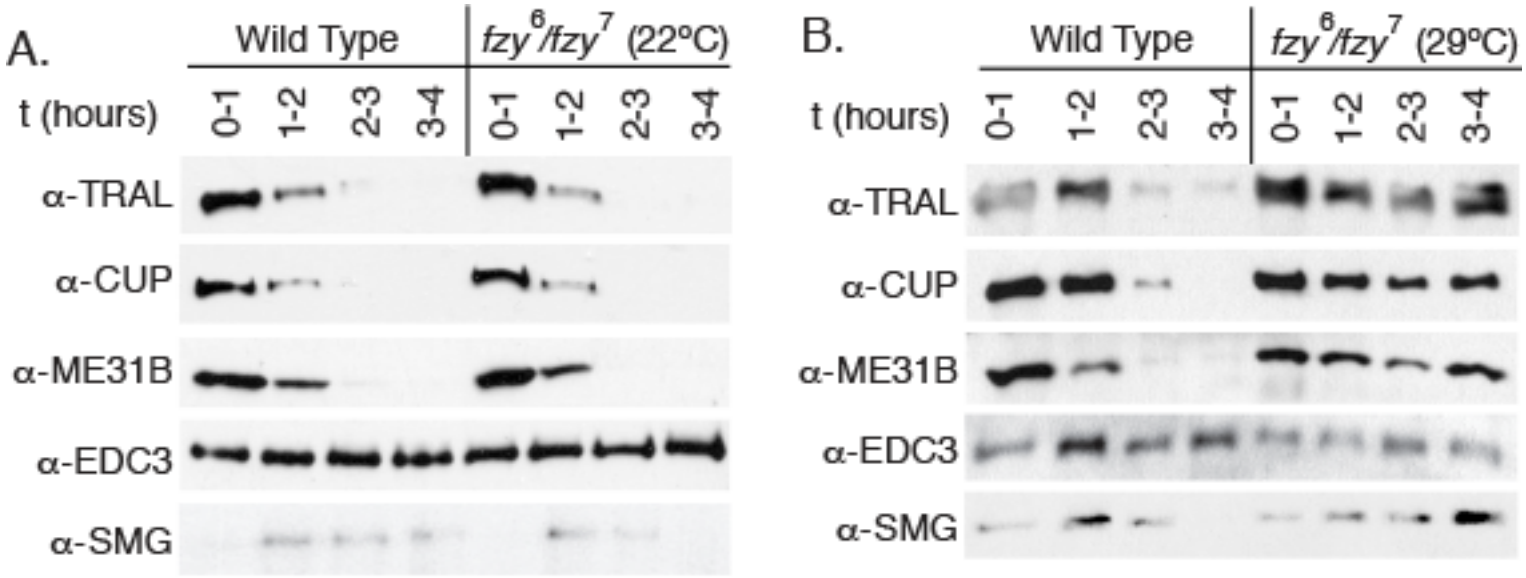

Figure 3-11 The meiotic APC is required for maternal RNP degradation

Tral, Cup and Me31B degradation is blocked in *fzy*^{6}/*fzy*^{7} mutant flies when meiotic APC function is restricted, but not when mitotic APC function is restricted. Embryos were collected from wild type and *fzy*^{6}/*fzy*^{7} mutant flies incubated at either **(A)** room temperature or **(B)** the non-permissive temperature of 29°C.

studies of *grau* and *cort* found that null mutations in either of these genes cause eggs to arrest in metaphase II of meiosis, *wisp* mutants complete female meiosis and exhibit defects primarily in pronuclear fusion (Cui et al., 2008; Harms et al., 2000; Swan and Schupbach, 2007). Since all three mutations cause the same block in RNP degradation, these phenotypes suggest that the defect in RNP degradation is not secondary to meiotic cell cycle arrest.

Because the defect in RNP degradation in these three mutants did not appear to be due to an arrest in the meiotic cell cycle, we next examined whether RNP degradation was solely dependent on *cort* or on APC function generally. *Drosophila* is unusual in that there are two CDC20 family members that function during meiosis: Cort and Fizzy (Fzy) (Swan and Schupbach, 2007). Interestingly, Fzy is also the targeting subunit for the mitotic APC during early embryogenesis. The mitotic and meiotic functions of *fzy* can be separated by raising *fzy* mutant flies at different temperatures; this provided us with a unique tool to address the role of different meiotic and mitotic APC complexes in Cup, Tral, and Me31B degradation (Swan and Schupbach, 2007). To test the role of mitotic Fzy-APC complexes in RNP subunit degradation, we used immunoblotting to measure the levels of Cup, Tral, and Me31B in embryos laid by fzy^{6}/fzy^{7} mutant mothers raised at 22°C – the restrictive temperature for the mitotic functions of *fzy* (Figure 3-11A). However, all three proteins were degraded normally under these conditions arguing that the mitotic APC is not required for RNP subunit degradation.

In order to address whether the meiotic Fzy-APC contributes to RNP subunit degradation, we repeated our measurements of the stability of Cup, Tral, and Me31B

using embryos laid by *fzy*^{6}/*fzy*^{7} mutant mothers raised at 29°C – the restrictive temperature for meiotic functions of *fzy* (Figure 3-11B). In contrast to mitosis, when meiotic *fzy* function is disrupted, all three RNP subunits failed to be degraded. These results argue that both the Fzy and Cort forms of the meiotic APC are necessary for the degradation of RNP subunits.

cort, fzy, and png are all required for RNP subunit ubiquitination

Since mutations *png*, *cort*, *fzy*, *grau*, and *wisp* all prevent the degradation of the protein components of maternal RNPs, we sought to define the requirements for each gene product in the RNP degradation pathway. Because *grau*, *wisp*, *cort, and fzy* all contribute to meiotic APC activity, we focused our efforts on characterizing more precisely the role of *cort* and *png* in maternal RNP subunit degradation. Ubiquitin-mediated proteolysis requires two distinct steps: ubiquitination of the target protein and recognition of the ubiquitinated protein by the proteasome. In order to determine which step in the ubiquitin-mediated proteolysis of RNP subunits is defective in embryos laid by *png* females, we made extracts from embryos laid by either wild type or *png*^{50} mutant females and then examined Tral and Me31B ubiquitination. While both Tral and Me31B were ubiquitinated in wild type embryos, virtually all ubiquitination of both proteins was blocked in embryos from *png*^{50} mutant mothers (Figure 3-12A,B). We found similar results when the ubiquitination of Tral and Me31B was measured in embryos from *cort* females (Figure 3-13A,B). Thus, we conclude that *cort* and *png* are each required for the ubiquitination of maternal RNP subunits.

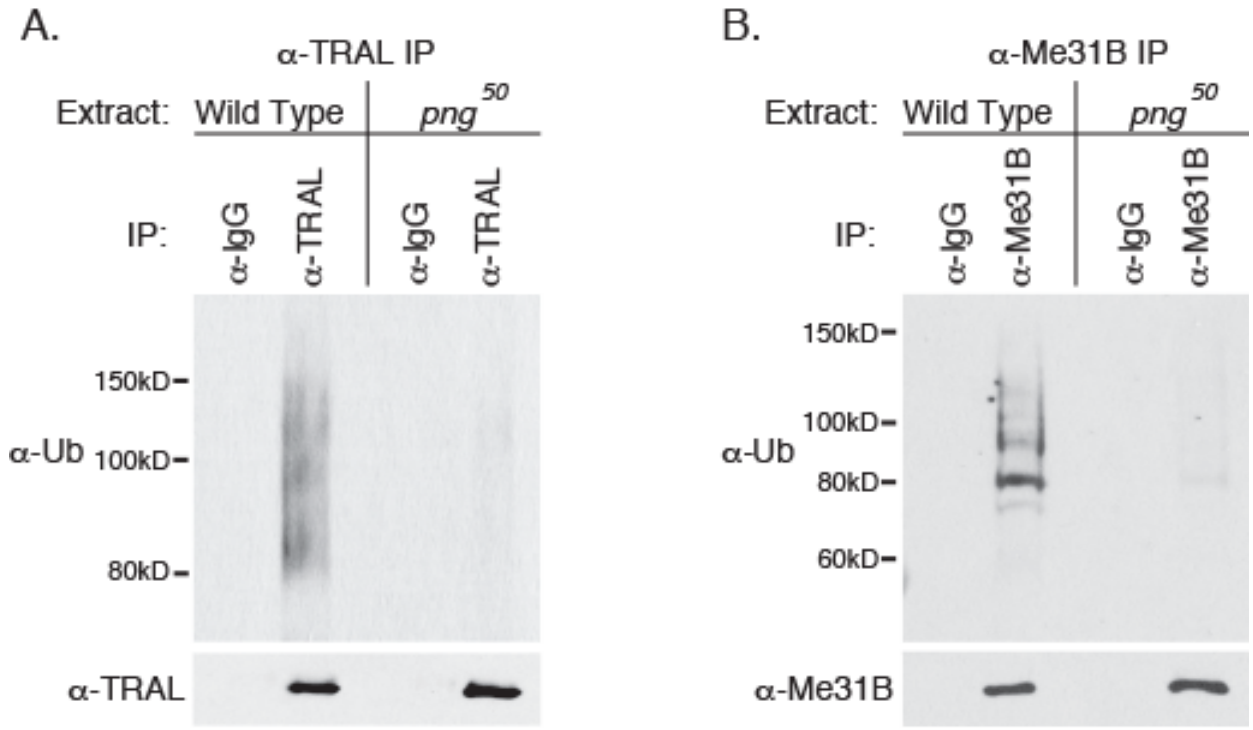

Figure 3-12 Ubiquitination of maternal RNP components requires functional Pan gu

Tral and Me31B ubiquitination is impaired in *png* mutants. Embryos were collected as previously described from wild type or png^{50} flies. Extracts were immunprecipitated with (A) α-Tral and (B) α-Me31B antibodies and probed for ubiquitin.

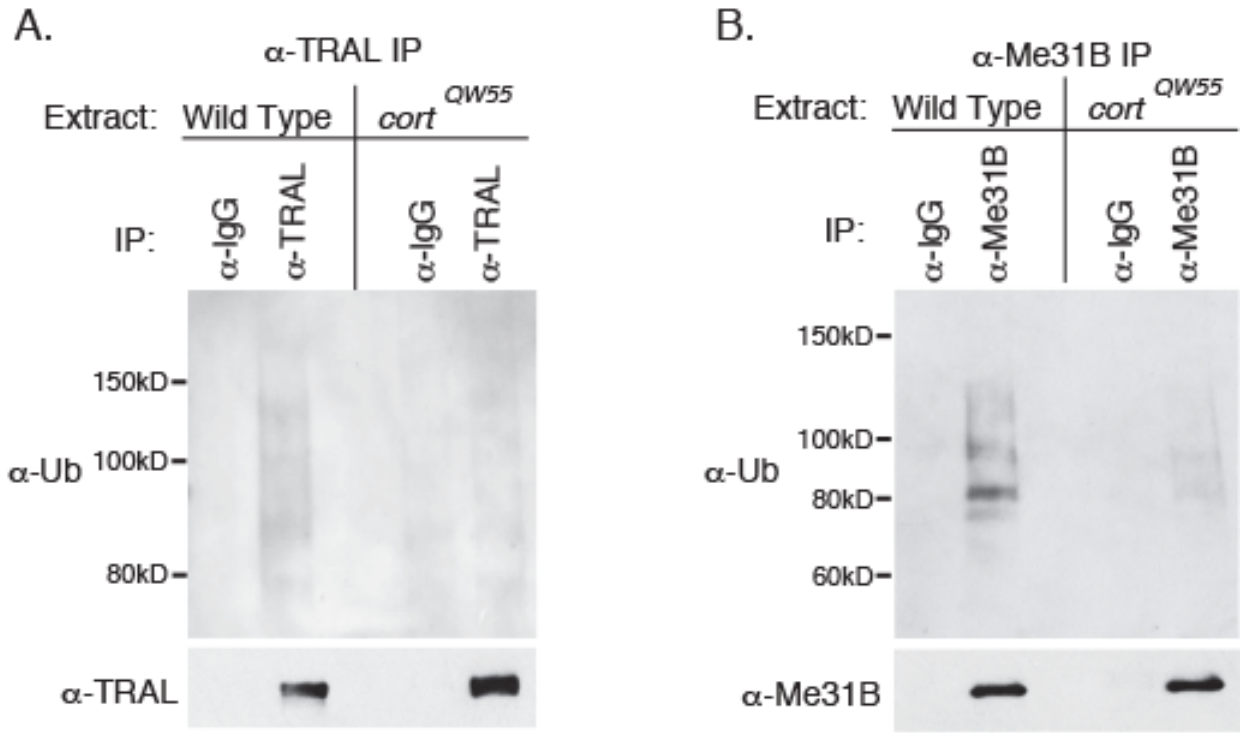

Figure 3-13 Ubiquitination of maternal RNP components requires functional Cortex

Tral and Me31B ubiquitination is impaired in *png* mutants. Embryos were collected as previously described from wild type or $cort^{QW55}$ flies. Extracts were immunprecipitated with (A) α-Tral and (B) α-Me31B antibodies and probed for ubiquitin.

Discussion

One of the central problems of the *Drosophila* maternal-to-zygotic transition is how the maternal transcriptome is converted from a highly stable state that allows deposition and storage in the developing oocyte to one where 20% of the messages are eliminated within hours of egg activation (Tadros et al., 2007). Here, we have identified three maternal RNP subunits - the translational repressor, Cup, the RNA helicase, Me31B, and the LSm domain protein, Tral, - that are degraded via ubiquitin-mediated proteolysis in the early embryo coincident with the onset of maternal mRNA degradation. Furthermore, disruption of RNP subunit degradation using either mutations in the core ubiquitin-proteasome machinery or mutations that block RNP subunit ubiquitination (i.e *cort*, etc.) causes maternal mRNA to be stabilized. Together, these results argue that maternal RNP complexes must be disassembled in order to allow the maternal transcript to be degraded.

Our observation that the proteolysis of maternal RNP subunits is regulated by the Pan gu kinase complex also suggests that maternal RNP subunit degradation is integrated into the maternal mRNA degradation pathway. Previous work on the maternal-to-zygotic transition primarily focused on the pathway required to activate mRNA degradation machinery. In this pathway, the Png kinase complex activates translation of the RNA binding protein, Smg, which recruits mRNA degradation enzymes to specific transcripts causing their destruction (Semotok et al., 2005; Tadros et al., 2007; Tadros et al., 2003). However, these studies also revealed the existence of a second Png kinase-dependent pathway that acted in parallel with Smg activation to trigger transcript destruction at the MZT (Tadros et al., 2007). Our finding that

maternal RNP subunits are degraded in *png*-dependent, but *smg*-independent manner suggests that a major role of this second, uncharacterized pathway is the regulated disassembly of maternal RNP complexes (Figure 3-14). Thus, we conclude that maternal mRNA degradation requires at least two Png regulated events: the activation of mRNA degradation factors such as Smg and the destabilization of the RNP used to store maternal messages during oogenesis.

Role of the meiotic APC in maternal mRNA degradation

Previous genetic screens for mutants defective in mRNA degradation identified *cort*, *grau*, and *wisp* as genes that are required for mRNA degradation (Tadros et al., 2003). While all three of these genes regulate meiotic APC activity, connecting the meiotic APC to the existing mRNA degradation pathway has been difficult. One of the reasons for this difficulty is that the block in mRNA degradation does not appear to be due to a defect in meiotic progression. *wisp* mutant eggs complete female meiosis , but fail to degrade maternal mRNA, while other meiotic mutants disrupt meiosis, but do not have defects in maternal mRNA degradation (Cui et al., 2008; Tadros et al., 2003). Thus, there does not appear to be a simple relationship between meiotic cell cycle arrest and the failure to degrade maternal mRNA. Furthermore, since Smg translation is normal in *wisp*, *cort,* and *grau* mutant embryos, the mRNA degradation defects observed in meiotic APC mutants are not due to a failure to activate the mRNA degradation machinery (Cui et al., 2008). Our finding that *wisp*, *cort,* and *grau* mutant embryos all have defects in RNP degradation provides an

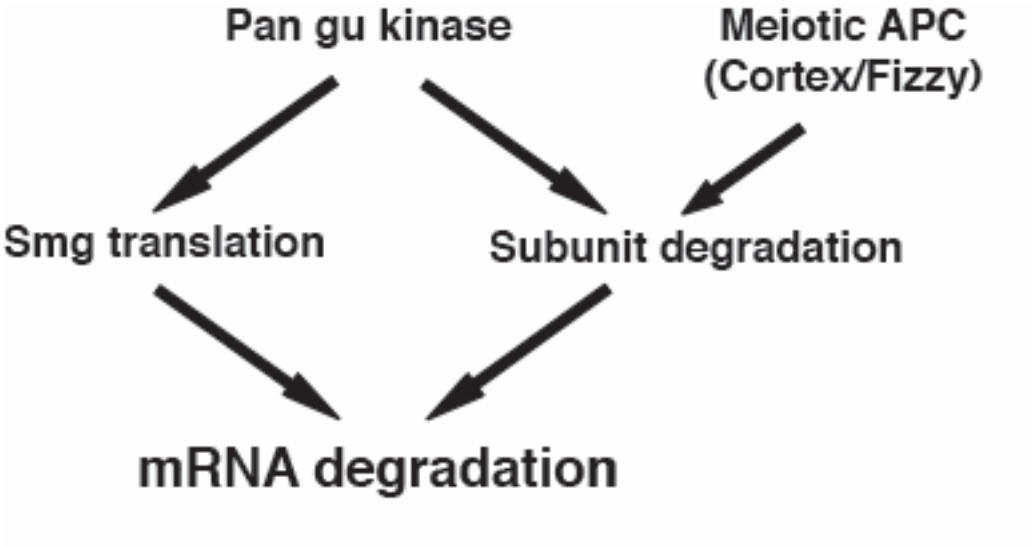

Figure 3-14 Model of the maternal mRNA degradation pathway

explanation for how mutations that disrupt meiotic APC activity can activate the maternal mRNA degradation machinery, but still fail to degrade maternal messages.

Our observations of the role of the meiotic APC in the maternal mRNA degradation pathway also help to explain how the progress through two critical early events, egg activation and meiosis, are monitored before triggering the irreversible destruction of a large fraction of maternal transcripts. While Pan gu kinase is the most upstream component of the maternal mRNA degradation pathway, it only monitors egg activation not meiotic progression as evidenced by the fact that Smg translation occurs in *cort*, *grau*, and *wisp* mutant embryos (Cui et al., 2008). Thus, meiotic APC activity provides a second input into the mRNA degradation pathway to prevent premature mRNA destruction if meiosis is altered. Furthermore, the requirement for Pan gu kinase to initiate RNP degradation coupled with the fact that this activity is only supplied after egg activation ensures that the meiotic APC cannot cause the premature degradation of maternal RNPs during oogenesis. Thus, RNP subunit degradation is under the control of two independent regulatory triggers, Pan gu kinase and the meiotic APC (Figure 3-14).

Because the maternal-to-zygotic transition is a conserved feature of higher metazoans, one might expect that some of the components of the pathway we have identified might also play a comparable role in other developmental systems. While *pan gu* does not have clear orthologs in other systems, the meiotic APC has, in fact, been implicated in the regulation of maternal protein degradation during the oocyte-to-egg transition in *C. elegans*. During the oocyte-to-egg transition in *C. elegans*, a set of meiotic proteins, egg maturation factors, and RNA binding proteins are targeted for

ubiquitin-mediated degradation (DeRenzo et al., 2003; Pellettieri et al., 2003). The activation of this degradation pathway is dependent on two components of the meiotic cell cycle: CDK-1 and the meiotic APC. CDK-1 activates a downstream kinase, MBK-2, that works in concert with a Cullin/SCF family E3 ubiquitin ligase to degrade substrates, while the meiotic APC controls MBK-2 activity by regulating the stability of a cortical anchoring protein, EGG-3 (Cheng et al., 2009; DeRenzo et al., 2003; Pellettieri et al., 2003; Stitzel et al., 2007; Stitzel et al., 2006). Thus, while the maternal protein degradation pathways in *Drosophila* and *C. elegans* are both dependent on a kinase and the APC, in *C. elegans* both regulators are integral components of the cell cycle. One possible reason for the differences in these two systems is the fact that egg activation in *Drosophila* is separable from fertilization making it necessary to have regulatory input from both the egg activation pathway and the cell cycle before committing to RNP degradation. Future work directed at identifying degradation pathway components that lie downstream of either *pan gu* or the meiotic APC in *Drosophila* is likely to provide insights into how different organisms solve the common problem of large scale transcriptome and proteome remodeling posed by the maternal-to-zygotic transition.

Disassembly of RNP complexes via ubiquitin-mediated proteolysis

While our work provides new insights into the early events of the maternal-to-zygotic transition, it also suggests a novel role for proteolysis in the regulation of mRNA stability. The regulation of mRNA stability is largely controlled by the composition of the ribonucleoprotein complex that assembles on the transcript.

However, the mechanisms involved in complex formation and rearrangement are poorly understood. Previous work has focused on the role of RNA helicases in displacing RNP components to allow mRNA degradation enzymes to access their target transcripts (Franks et al., 2010). Our work argues that a second mechanism for RNP disassembly, ubiquitin-mediated proteolysis, can also be used to facilitate mRNA degradation. Furthermore, RNP subunit degradation and the recruitment/activation of mRNA decay factors are coordinately regulated during the MZT in *Drosophila*. Given that many of the components of the maternal RNP in *Drosophila* have functional equivalents in somatic cells, it is possible that many regulated mRNA decay events require both the activation of mRNA decay machinery and the removal of stabilizing proteins from the message via proteolysis. Interestingly, while the mechanism of stabilization is unknown, the observation that inhibiting ubiquitin-mediated proteolysis stabilizes AU-rich cytokine mRNAs that undergo rapid turnover (Deleault et al., 2008; Franks et al., 2010). While future studies of this and other systems will be necessary to determine which regulated mRNA decay events couple proteolysis of stabilizing factors with recruitment of mRNA degradation factors to regulate transcript stability, the interplay between ubiquitin-mediated proteolysis and mRNA decay opens a number of novel possibilities for post-transcriptional gene regulation.

Materials and Methods

Fly stocks

Fly stocks were cultured at 22°C unless otherwise noted. Wild type stocks were Oregon-R. Mutants used in the paper were *png*^{50} (Fenger et al., 2000), *gnu*^{305} (Freeman et al., 1986), *gnu*^{305} + *8x* cycB (Lee et al., 2001), *uba1*^{H42} (Lee et al., 2008), *Pros26*^{1} (Holden and Suzuki, 1973), *smg*^{1} (Dahanukar et al., 1999), *twin*^{KG00877} (Temme et al., 2004), *cort*^{QW55} (Schupbach and Wieschaus, 1989), *grau*^{QQ36} (Schupbach and Wieschaus, 1989), *wisp*$^{12\text{-}3147}$ (Brent et al., 2000), *fzy*^{6} and *fzy*^{7} (Dawson et al., 1993), and *bcd5' UTR-png-bcd 3' UTR* (Tadros et al., 2007).

Embryo extract collection

Embryos were collected and aged for the times indicated and rinsed with water, dechorionated in 50% bleach for 2 minutes, and then rinsed again in water. For time courses, a fixed number of embryos were collected (30-50 depending on the experiment) into a microfuge tube at each time point, and 100ul of 2X sample buffer (4% SDS, 20% glycerol, 120mM Tris pH6.8, 0.01% bromophenol blue, 5% β-mercaptoethanol) was added. Samples were homogenized with a dounce and heated at 95°C for 5 minutes.

For immunoprecipitations, embryos were collected similarly but dounced in Drosophila extract buffer (DXB, 25mM Hepes, pH6.8, 50mM KCl, 1mM $MgCl_2$, 1mM DTT, 250mM sucrose) with protease inhibitors and N-ethyl maleimide (NEM)

followed by two rounds of centrifugation at 14,000 x g for 15 minutes at 4°C. Extracts were then frozen in liquid nitrogen.

Immunoblotting

Samples were loaded onto a polyacrylamide gel and transferred to nitrocellulose by semi-dry transfer. Membranes were blocked in 10% milk in TBST (20mM Tris-HCl, pH7.5, 200mM NaCl, and 0.1% Tween-20). The indicated primary antibodies were diluted 1:2000 in a solution of TBST with 5% BSA. Secondary antibodies were diluted in TBST with 5% milk.

For ubiquitin blots, nitrocellulose membranes were autoclaved for 30 minutes on liquid cycle followed by 10 minutes on gravity cycle after transfer. Membranes were blocked in 10% BSA in TBST with 0.3% Tween-20. Both primary (anti-ubiquitin, Invitrogen) and secondary antibodies were incubated in TBST with 0.3% Tween-20 and 5% BSA.

Immunoprecipitation

Immunoprecipitations utilized Protein-A agarose beads (Invitrogen) covalently crosslinked with dimethyl pimelidate to the indicated antibody. Embryo extracts were diluted in DXB with 1% SDS, 0.5% NP-40, 0.5% deoxycholate, protease inhibitors, and NEM, and added to the Protein-A beads. Extracts and beads were incubated on a rocker for 2 hours at 4°C, followed by 5 washes with DXB immunoprecipitation buffer. The bound material was removed from the beads by the addition of 50μl

sample buffer and 5 minutes of heating at 95°C. Samples were then loaded on a polyacrylamide gel and immunoblotted.

Lactacystin treatment

Embryos were collected for 1 hour, then dechorionated as described above, and blotted dry. Embryos for the 0-1 time point were dounced in sample buffer immediately, while embryos for later time points were added to Schneider's drosophila media either with or without 10μg/ml lactacystin. Following a one (1-2 hr time point) or two (2-3 hr time point) hour room temperature incubation, embryos were removed and processed as above.

Immunofluorescence

Embryos were collected for 30 minutes and aged for the indicated times. They were washed, dechorionated in 50% bleach for 2 minutes, and rinsed with embryo wash buffer (7% NaCl and 0.5% tritonX-100) and water. Dechorionated embryos were added to a vial containing 2m heptane and 2ml 3.7% formaldehyde in PEM buffer (0.1M PIPES, 1mM $MgCl_2$ pH6.9). The embryos were shaken followed by a 20-minute incubation. The formaldehyde layer was removed, embryos were rinsed with methanol, and the heptane layer was then removed. After another methanol wash, the embryos were incubated in a 50% methanol, 50% PBTA (1X PBS, 1% BSA, 0.05% tritonX-100, 0.02% sodium azide) solution, followed by blocking in PBTA. The primary antibody was incubated in PBTA overnight at 4°C, followed by PBTA

washes and a 2-hour secondary antibody incubation at room temperature. After PBS washes, the embryos were mounted on a slide in mounting media.

RNA isolation and cDNA synthesis

RNA was isolated from embryos that were collected for 1 hour, aged to the appropriate time, and dechorionated similarly to the protein assays above. Embryos were then dounced in 1ml Trizol (Invitrogen). 200µl chloroform was added followed by 15 seconds of mixing and a 2-minute incubation at room temperature. The tube was centrifuged at 4°C for 15 minutes at 14,000 x g. The aqueous layer was combined with 200µl isopropanol and incubated at room temperature for 10 minutes, followed by a second round of centrifugation. The RNA containing pellet was washed with 80% ice-cold ethanol and centrifuged at 4°C for 6 minutes. The pellet was resuspended in water and DNase treated after which the RNA was precipitated again using same procedure. The isolated RNA was used to synthesize cDNA with the SuperScriptIII First-Strand Synthesis System for RT-PCR (Invitrogen). For each cDNA reaction, 0.25ug RNA was used.

Quantitative RT-PCR

Each qPCR sample had a total volume of 20µl and consisted of 2µM forward and reverse primers, 10µl QuantiTect SYBR Green (Qiagen), and 6µl cDNA diluted 1:10 in water. A Bio-Rad iCycler was used for amplification with the following settings:

Cycle 1 Step 1 95°C 3 minutes, Cycle 2 (40X) Step 1 95°C 10 seconds, Step 2 55°C 30 seconds real time detection, Cycle 3 (81X) Step 1 55°C 10 seconds melt curve.

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Chapter 4

Metabolic enzyme polymerization and its role in orphan genetic disease

Introduction

The spatial organization of metabolic pathways is one of the hallmark achievements of cell biology. In fact, much of our knowledge about organelles such as mitochondria and peroxisomes is rooted in the initial discovery that particular metabolic enzymes are partitioned into specific membrane-bound compartments (LeDuc and Bellin, 2006; Lenaz and Genova, 2009; Luisi, 2002; Veenhuis et al., 2000). While most of the focus historically has been on how metabolic enzymes are targeted to particular membrane-bound compartments and the role of that compartmentalization in regulating metabolism, the cell biology of metabolic enzymes that reside in the cytoplasm has received little examination. Recent work on metabolic enzymes in a variety of organisms has found that many metabolic enzymes are capable of forming polymers or other higher order structures suggesting that pathway organization is as important in the cytoplasm as it is in organelles (An et al., 2008; Ingerson-Mahar et al., 2010; Liu, 2010; Narayanaswamy et al., 2009; Noree et al., 2010). Much of this recent work has concentrated on the role of metabolic enzyme organization in regulating metabolic pathway activity. For instance, in *S. cerevisiae*, CTP synthase polymerizes into filaments and puncta (Noree et al., 2010) and does so in response to end product inhibition (Noree et al., 2014). In human tissue culture cells enzymes in the *de novo* purine biosynthetic pathway co-assemble together into a large structure called purinosomes, which are believed to facilitate pathway flux (An et al., 2008). Thus, the role of such large enzyme assemblages in regulating metabolism is becoming increasingly clear. However, the existence of these large structures has raised the possibility that they may have acquired additional cell biological functions,

much in the same way that mitochondria play roles in both oxidative phosphorylation and apoptosis (Macip et al., 2003).

One of the long-standing mysteries of "inborn errors of metabolism" has been how mutations that disrupt important metabolic pathways can often result in highly specific phenotypes. For instance, mutations in PRPP synthetase, the enzyme that produces the substrate for the first step in *de novo* purine biosynthesis, cause deafness and ataxia (Becker et al., 1988; Nyhan et al., 1969; Simmonds et al., 1985). In contrast, mutations in the enzyme that catalyzes a later step of *de novo* purine biosynthesis, IMP dehydrogenase, causes retinal degeneration (Bowne et al., 2002; Kennan et al., 2002). Two hypotheses have been advanced to explain the genetics of these diseases: 1) the existence of a subset of cells that are particularly sensitive to the build up of specific metabolic intermediates, or 2) that certain metabolic enzymes have acquired important secondary "moonlighting" functions in the cell that are modulated by metabolic activity. In order to explore the possibility that metabolic enzyme structures might have additional cell biological functions, we have focused on a set of syndromes caused by mutations in PRPP synthetase, whose genetics are difficult to explain solely in terms of their effect on enzyme activity. There are three classes of mutations in PRPP synthetase. First, there are overexpression mutations in PRPP synthetase that cause gout (Becker et al., 1988; Zoref et al., 1975) — a phenotype that is easily explained as a result of the increased level of purine production. Secondly, there are loss of mutations in PRPP synthetase that cause sensorineural deafness, progressive ataxia and, in more severe alleles, intellectual disability (Arts et al., 1993; Kim et al., 2007; Rosenberg and Chutorian, 1967; Zoref et al., 1975) — phenotypes

that are difficult to connect directly to purine biosynthesis. Third, and even more perplexingly, there are "superactivity" mutations, which disrupt the feedback inhibition of PRPP synthetase, causing phenotypes associated with both the overexpression and loss of function mutations: gout, sensorineural deafness, and ataxia (Ahmed et al., 1999; Akaoka et al., 1981; Becker et al., 1982; Becker et al., 1986; Becker et al., 1988; Becker et al., 1980; Becker and Seegmiller, 1975; Becker et al., 1995; Becker et al., 1996; Zoref et al., 1975) The fact that "superactivity" mutations and loss of function mutations in PRPP synthetase cause the same set of neurological phenotypes suggested that this enzyme might have a deeper connection to cellular function than previously thought.

Here, we demonstrate that PRPP synthetase forms novel filaments in the nucleus, in a manner that is conserved from yeast to humans. Furthermore, we have found that "superactivity" mutations cause PRPP synthetase to promiscuously polymerize in the cytoplasm. Interestingly, the formation of these cytoplasmic filaments is responsive to small molecule intermediates and products of the purine biosynthetic pathway. We also discovered that both superactivity and loss of function mutations in PRPP synthetase cause comparable disruption of actin stress fibers, suggesting a common mechanism for affecting the actin cytoskeleton. Coincidentally, PRPSAP1, which is a known inhibitor of PRPP synthetase and is also structurally related to the enzyme, forms filaments that co-localize with actin stress fibers. The levels and localization of PRPSAP1 protein are disrupted in both loss of function and superactivity mutations in PRPP synthetase. Together these results suggest that metabolic enzyme structures are more deeply integrated with cellular functions than

previously thought and suggest a novel framework for thinking about the pathophysiology of inborn errors of metabolism.

Results

PRPP Synthetase forms filaments that are conserved from yeast to humans

Given the unusual genetics of human PRPP synthetase syndromes, we first examined whether PRPP synthetase assembled into large cytoplasmic structures by utilizing the yeast GFP strain collection. One of the advantages of using the yeast GFP strain collection is that every tagged protein is expressed at its endogenous locus, eliminating artifacts due to over-expression (Huh et al., 2003). In *S. cerevisiae*, PRPP synthetase is in fact encoded by 5 genes: PRS1, PRS2, PRS3, PRS4, and PRS5. Past biochemical studies found that only certain combinations of the five subunits of PRPP synthetase can assemble into an active enzyme complex: PRS1/2/5, PRS1/4/5, PRS2/4/5, and PRPS1/3 (Carter et al., 1997; Hernando et al., 1999; Hernando et al., 1998). Interestingly, it was recently found that only Prs3p-GFP and Prs5p-GFP form cytoplasmic filaments (Noree et al., in preparation), suggesting that polymerization might be a novel mechanism for regulating access to key PRPP synthetase subunits in yeast.

In most higher eukaryotes, PRPP synthetase is comprised of a homohexamer of a single subunit and is encoded by a single gene, PRPS1. To test whether PRPP synthetase could form filaments in higher eukaryotes, we generated an affinity-purified antibody against human PRPP synthetase (PRPS1) and assayed a variety of cell types and organisms for the presence of PRPS1 filaments by immunofluorescence.

We found that PRPP synthetase encoded by PRPS1 is able to form filaments in *S. cerevisiae*, *Drosophila* egg chambers, rat hippocampal neurons, and human primary fibroblasts (Figure 4-1). This argues that the ability to form filaments is a conserved feature of PRPP synthetase. Interestingly, the PRPS1 filaments that form in higher eukaryotes are restricted to the nucleus, suggesting that PRPS1 activity and/or the PRPS1 filaments have a previously unappreciated nuclear role.

The superactivity mutation D182H causes promiscuous polymerization of PRPS1 in the cytoplasm

Given the unusual genetics of PRPS1 syndromes, we next investigated whether human disease mutations in PRPS1 alter the localization or the assembly behavior of the PRPS1 filaments. Utilizing primary human fibroblasts from two patients with loss of function (LOF1 and LOF2) mutations in PRPS1 and one patient with a feedback resistance/superactivity mutation (SM) with a single nucleotide substitution resulting in a aspartic acid to histidine change at position 182 (Roessler et al., 1991; Roessler et al., 1993), we examined the effect of the mutations on the frequency of filament formation and the location of the filaments within the cell (Figure 4-2). Both LOF1 and LOF2 mutations show a decrease in PRPS1 protein levels (Figure 4-2B). As a result, we predicted that both of these mutations would severely decrease PRPS1 filament formation in the nucleus. Indeed, when examined, only 2.4% of LOF1 and 1.7% for LOF2 cells had nuclear filaments, while 22.2% of wild type fibroblasts had nuclear filaments (Figure 4-2A).

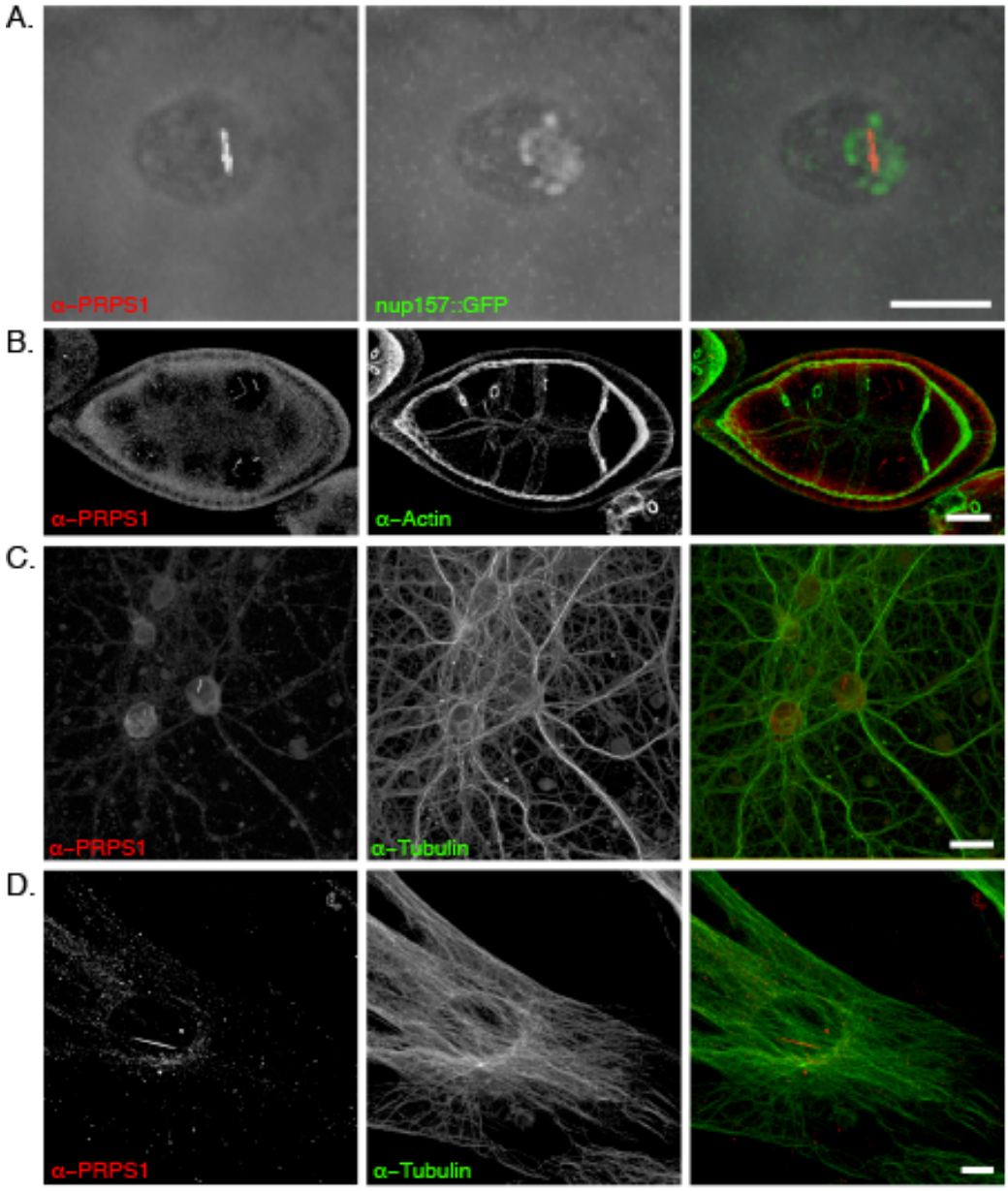

Figure 4-1 PRPP synthetase forms nuclear filaments that are conserved from yeast to humans

Immunofluoresence staining reveals that PRPP synthetase 1 (PRPS1) forms nuclear filaments in **(A)** *S. cerevisiae*, **(B)** *D. melanogaster* egg chambers, **(C)** rat hippocampal neurons, and **(D)** wild type human primary fibroblasts. PRPS1 is indicated in red across all cell types and counter stained with indicated antibody (green). Scale bar is (A) 5μm (B) 25μm (C) 15μm (D) 5μm.

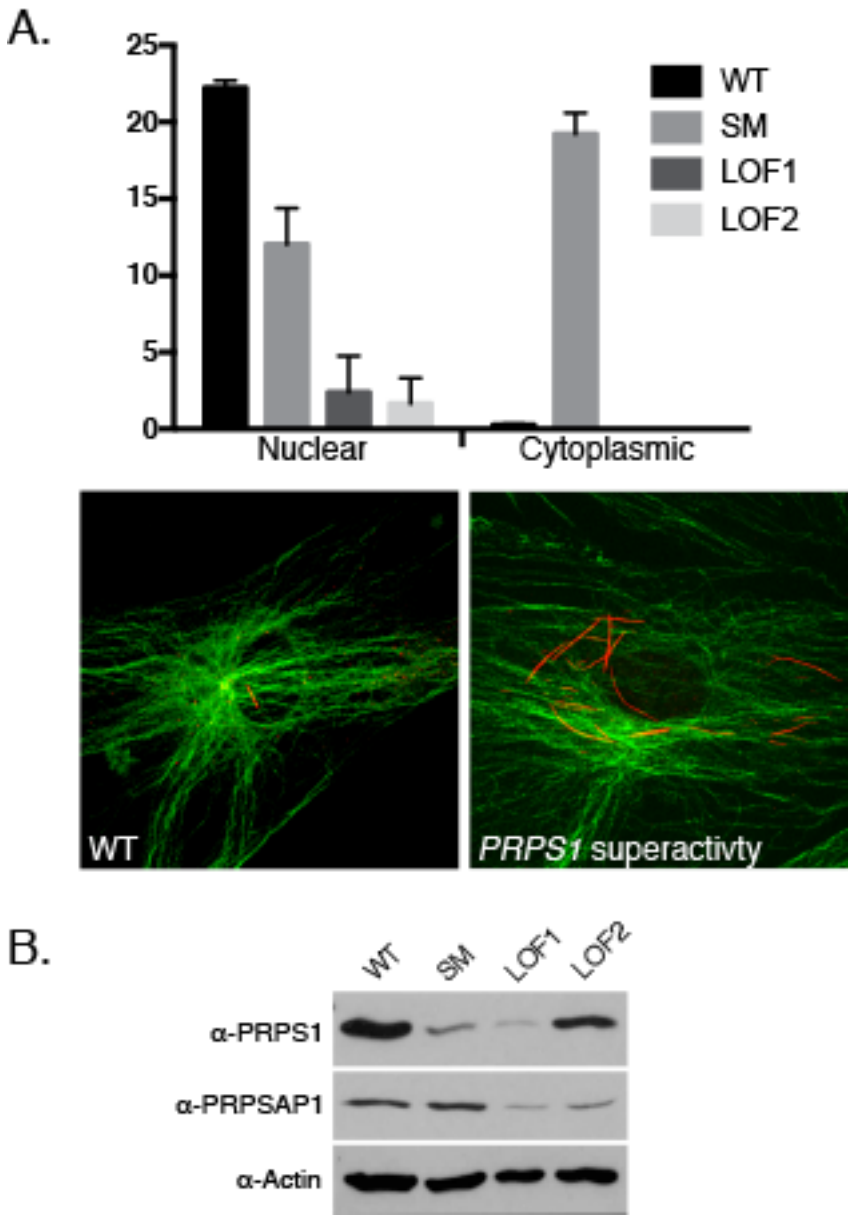

Figure 4-2 PRPP synthetase normally forms nuclear filaments in wild type fibroblasts while superactivity mutation D182H results in promiscuous polymerization in the cytoplasm

(A) PRPP synthetase 1 (PRPS1) normally forms nuclear filaments in 22.2% of wild type fibroblasts and does not form cytoplasmic filaments. However, in fibroblasts with the superactivity mutation D182H, *PRPS1* forms nuclear filaments in only 12.0% of the cells, but also forms promiscuous filaments in the cytoplasm in 19.2% of the cells. Loss of function cells form nuclear filaments in only 2.4% (LOF1) or 1.7% (LOF2) of total cells. **(B)** The aberrant polymerization of D182H *PRPS1* in the cytoplasm of SM cells is not due to upregulated protein levels as seen by western blot.

Since we discovered that the "superactivity" mutation (SM) decreases the level of cellular PRPS1 (Figure 4-2B), we expected a comparable decrease in nuclear filament formation. Accordingly, we observed that only 12.0% of SM fibroblasts had a nuclear PRPS1 filament in comparison to 22.2% in wild type fibroblasts (Figure 4-2A). However, the most striking discovery was that 19.2% of the SM fibroblasts had numerous cytoplasmic PRPS1 filaments. These are never observed in either wild type or loss of function mutant fibroblasts (Figure 4-2A). Furthermore, the cytoplasmic PRPS1 filaments did not appear to mirror any cytoskeletal structures. Thus, while the PRPS1 superactivity mutation has minimal effects on the formation of nuclear filaments, it causes unexpected promiscuous polymerization of PRPS1 in the cytoplasm (Figure 4-2A).

PRPS1 cytoplasmic filaments respond to changes in ATP and GTP

The fact that the pathogenic mutation that causes resistance to end product inhibition (D182H) triggers PRPS1 filament formation in the cytoplasm raised the question of whether the PRPS1 filaments in the nucleus and/or cytoplasm are responsive to levels of metabolites known to regulate PRPS1. Previous studies identified multiple factors that regulate PRPP synthetase activity: ATP, ADP, AMP, P_i, Mg^{2+}, PRPP concentration as well as the inhibitory proteins (Fox and Kelley, 1971; Li et al., 2007; Planet and Fox, 1976; Switzer and Sogin, 1973; Tatibana et al., 1995a). Given that the D182H mutation known to make PRPP synthetase resistant to end product inhibition by ATP or GTP also triggers promiscuous polymerization, as shown here, we first examined whether increasing ATP or GTP levels transiently would

affect PRPS1 filaments. WT and SM fibroblasts were treated with 0.5 mM adenosine, guanosine, or cytidine for 30 minutes in order to transiently increase ATP or GTP levels in the cell. Interestingly, we found that adenosine and guanosine addition completely eliminated the cytoplasmic PRPS1 filaments from SM fibroblasts. Cytidine treatment decreased the number of cells with cytoplasmic PRPS1 filaments 10-fold (Figures 4-3, 4-4B). Thus, cytoplasmic PRPS1 filaments in SM fibroblasts are responsive to changes in the regulatory metabolites adenosine, guanosine, and cytidine. This finding also clearly demonstrates that PRPS1 filaments are not the result of nonspecific protein aggregation. Interestingly, while cytoplasmic PRPS1 filaments are strongly responsive to changes in ATP or GTP levels, the nuclear filaments in both wild type and SM fibroblasts are largely unresponsive to changes in nucleotide levels (Figures 4-3, 4-4A), suggesting that nuclear PRPS1 filaments are either unusually stable or protected from changes in metabolite levels.

Both superactivity and loss of function mutations in *PRPS1* cause similar defects in actin stress fiber organization

It has long been mysterious how both loss of function and "superactivity" mutations in PRPS1 cause sensorineural deafness and progressive ataxia. As a first step towards building a bridge from the cell biology of PRPS1 to the disease phenotype, we focused on the effects of both loss of function and superactivity mutations on the actin cytoskeleton, as many forms of inherited sensorineural deafness can be traced back to disruption of cellular actin organization (Morin et al., 2009; Rendtorff et al., 2006; Zhu et al., 2003). In order to determine if actin organization is

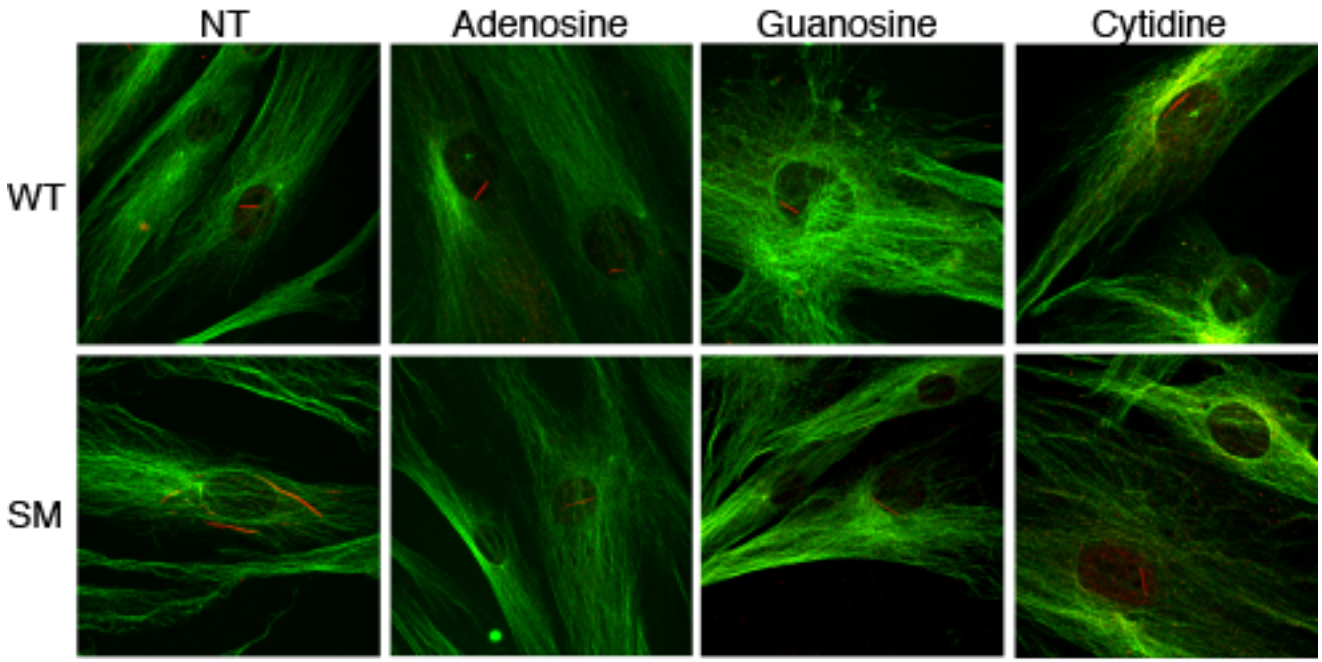

Figure 4-3 Treatment of fibroblasts with the PRPP synthetase superactivity mutation (SM) using nucleosides eliminates *PRPS1* filaments in the cytoplasm but not the nucleus as revealed by immunofluorescence

Treatment of wild type (WT) and superactivity mutation (SM) fibroblasts with adenosine, guanosine, or cytidine does not affect the nuclear PRPS1 filaments in the WT or mutant cells. Treatment of SM fibroblasts with adenosine or guanosine completely eliminates the cytoplasmic filaments. Cytidine treatment of SM fibroblasts decreased the percentage of cells with cytoplasmic filaments 10-fold. WT cells treated with nucleoside remain unchanged due to their initial lack of cytoplasmic filaments. Immunofluorescence of PRPS (red), and tubulin (green) is shown.
Note: Cells were plated on coverslips and allowed to recover for 9 days before treatment with nucleoside. Cell count greater than n=500 was observed for each condition and the experiment was performed four times.

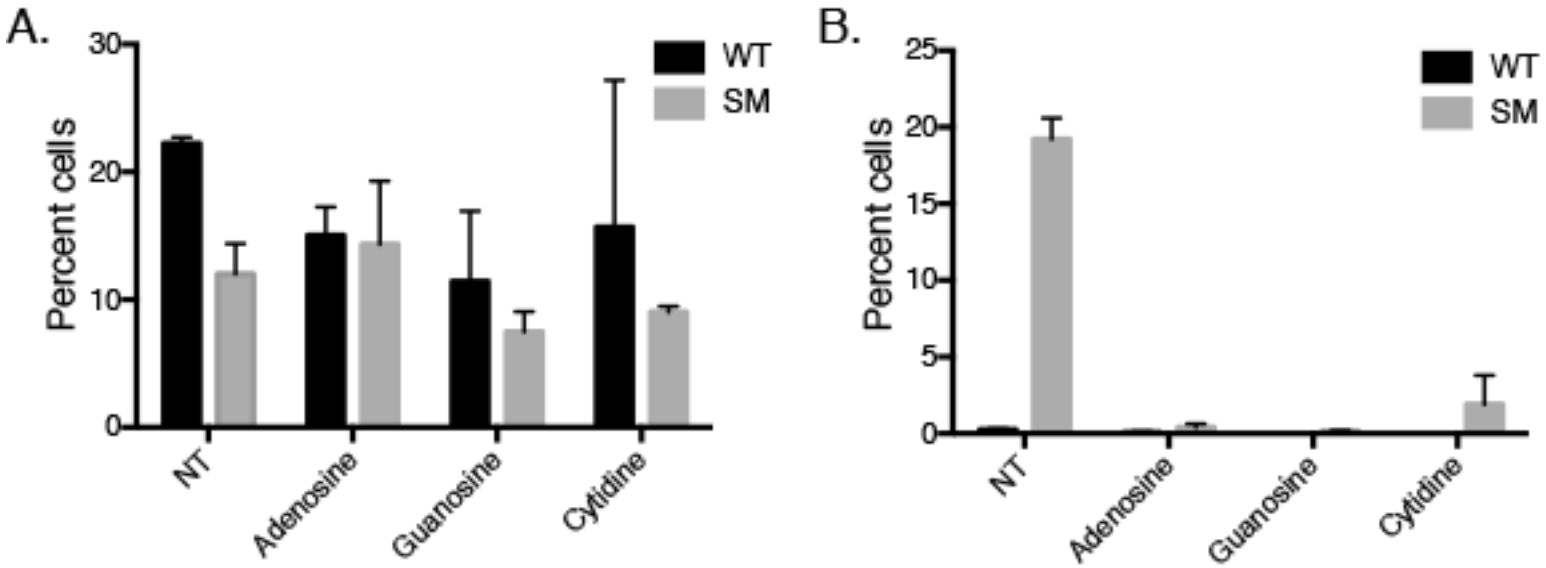

Figure 4-4 Treatment of fibroblasts with the PRPP synthetase superactivity mutation (SM) using nucleosides eliminates *PRPS1* filaments in the cytoplasm but not the nucleus as revealed by quantification

(A) Treatment of wild type (WT) and superactivity mutation D182H (SM) fibroblasts with adenosine, guanosine, or cytidine does not affect the nuclear PRPS1 filaments in WT or mutant cells. **(B)** Treatment of SM fibroblasts with adenosine and guanosine completely eliminates the cytoplasmic filaments. Cytidine treatment of SM fibroblasts decreases the percentage of cells with cytoplasmic filaments 10-fold. WT cells remain unchanged by nucleoside addition due to initial lack of cytoplasmic filaments.
Note: Cells were plated on coverslips and allowed to recover for 9 days before treatment with nucleoside. Cell count greater than n=500 was observed for each condition and the experiment was performed four times for statistical

disrupted in either superactivity or loss of function PRPS1 mutant cell lines, we examined the actin stress fibers and their polarity within the cell at different stages of confluency for wild type fibroblasts as well as SM and LOF patient fibroblasts. When wild type cells are 50% confluent, the actin stress fibers were largely oriented along a single axis (Figures 4-5, 4-6). This can be visualized in Figure 4-5, WT panel and can also be graphically represented by a polarity plot (Figure 4-6, blue line). The actin stress fibers in LOF and SM cells that are 50% confluent were also tightly aligned, arguing that PRPS1 mutants do not affect the ability of the cells to polarize along an axis (Figures 4-5, 4-6). However, as the cells became progressively more confluent, the actin stress fibers in both the LOF1 and SM cells assumed a more circumferential distribution and largely failed to align along a single axis (Figures 4-7, 4-8). The LOF2 mutation showed disorganized actin filaments at both 50% and 100% confluency (Figures 4-5, 4-6, 4-7, 4-8). Thus, both the superactivity and loss of function mutations have a similar deleterious effect on the organization of actin stress fibers.

PRPSAP1 forms novel filaments that associate with actin stress fibers

While both superactivity and loss of function mutations in PRPS1 cause disruptive effects on actin organization, the mechanism leading from alterations in PRPS1 activity or filament formation to regulation of the actin cytoskeleton was unclear. To address this, we examined known PRPS1-interacting proteins for possible links to the actin cytoskeleton. PRPS Associated Protein 1 (PRPSAP1) was originally

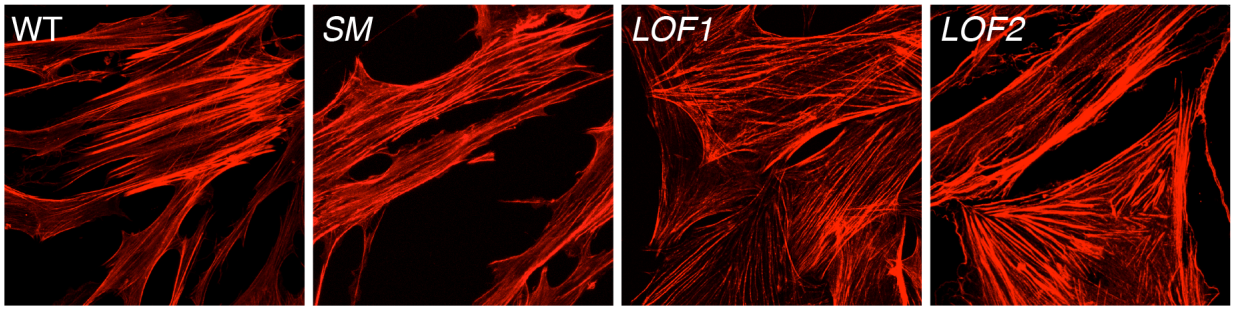

Figure 4-5 Immunofluorescence showing actin distribution in fibroblasts recovered 5 days post-plating

Wild type, SM fibroblasts, LOF1 fibroblasts, and LOF2 fibroblasts were immunostained for analysis of actin stress fiber orientation. At 5 days recovery post-plating, SM fibroblasts exhibit the greatest polarity with actin stress fibers oriented closest to the central axis, when compared to WT and LOF1 and LOF2 fibroblasts. Representative image of actin staining is shown.
Note: Cells were plated and allowed to recover for 5 days before processing for immunofluorescence and quantification of actin stress fibers. Cell counts of greater than n=45 were counted per cell line from randomized fields and each experiment was performed in triplicate.

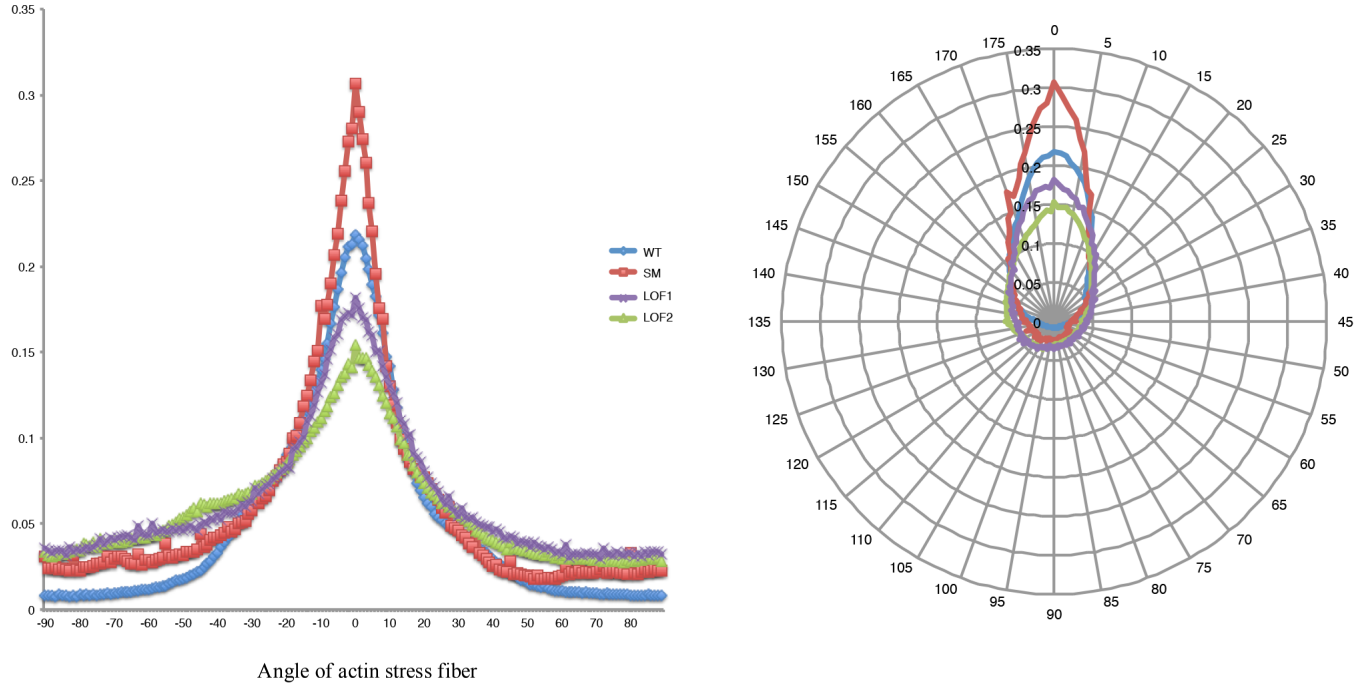

Figure 4-6 Quantification of actin distribution in fibroblasts recovered 5 days post-plating

Wild type, SM fibroblasts, LOF1 fibroblasts, and LOF2 fibroblasts were analyzed for angle of actin stress fiber. At 5 days recovery post-plating, SM fibroblasts exhibit the greatest polarity with actin stress fibers oriented closest to the central axis, when compared to WT, LOF1 and LOF2 fibroblasts. This timepoint is approximately 50% confluency.
Note: Cells were plated and allowed to recover for 5 days before processing for immunofluorescence and quantification of actin stress fibers. Cell counts of greater than n=45 were counted per cell line from randomized fields and each experiment was performed in triplicate.

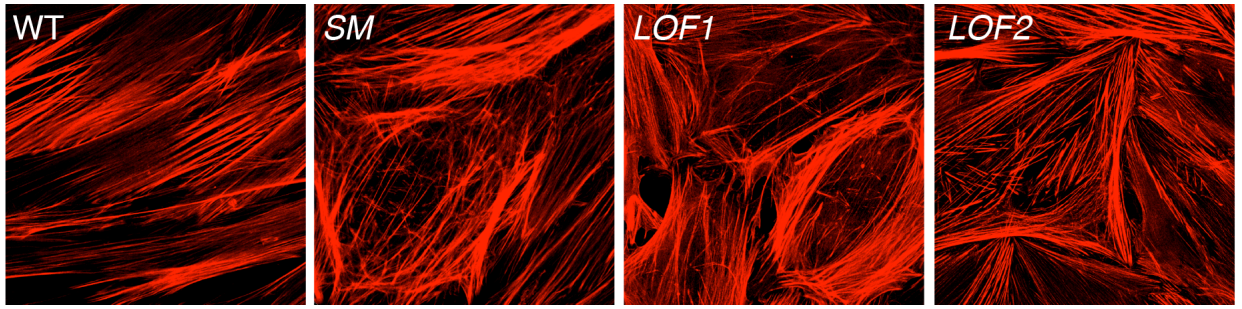

Figure 4-7 Immunofluorescence showing actin distribution in fibroblasts recovered 9 days post-plating

Wild type, SM fibroblasts, LOF1 fibroblasts, and LOF2 fibroblasts were immunostained to analyze actin stress fiber orientation. At 9 days recovery post-plating, which is at 100% confluency, WT fibroblasts exhibit the greatest polarity with actin stress fibers oriented closest to the central axis; SM and LOF1 and LOF2 fibroblasts appear to have a large degree of disorganization of actin stress fibers. Representative image of actin staining is shown. Note: Cells were plated and allowed to recover for 9 days before processing for immunofluorescence and quantification of actin stress fibers. Cell counts of greater than n=45 were counted per cell line from randomized fields and each experiment was performed in triplicate.

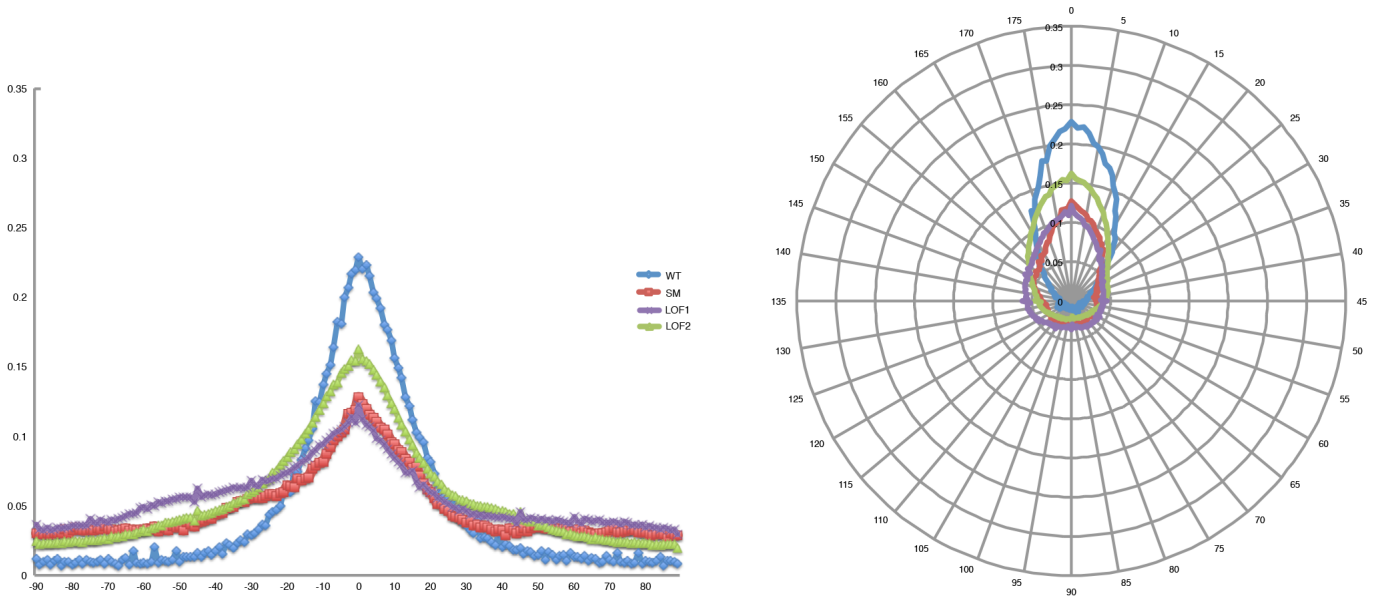

Figure 4-8 Quantification of actin distribution in fibroblasts recovered 9 days post-plating

Wild type, SM fibroblasts, LOF1 fibroblasts, and LOF2 fibroblasts were analyzed for angle of actin stress fibers with respect to the longest cell axis. At 9 days recovery post-plating which is 100% confluency, WT fibroblasts exhibit the greatest polarity with actin stress fibers oriented closest to the central axis. SM and LOF1 changed to have largely disorganized actin networks at this time point. LOF2 showed disorganization at both 50% and 100% confluency. Note: Cells were plated and allowed to recover for 9 days before processing for immunofluorescence and quantification of actin stress fibers. Cell counts of greater than n=45 were counted per cell line from randomized fields and each experiment was performed in triplicate.

identified as an inhibitor of PRPP synthetase (PRPS1) (Kita et al., 1994). Human PRPSAP1 is structurally related to PRPS1 with 42% sequence identity and has been hypothesized to inhibit enzyme activity by acting as a dominant negative subunit in the active hexameric PRPS1 enzyme (Kita et al., 1994; Tatibana et al., 1995b). This structural similarity raised the question as to whether PRPSAP1 could also form filaments. To test this possibility, we generated an antibody to human PRPSAP1 and used immunofluorescence microscopy to determine the distribution of PRPSAP1 in human fibroblasts. Interestingly, we found that PRPSAP1 co-localized with the actin stress fibers in fibroblasts (Figure 4-9). To ask if PRPSAP1 forms a novel filament network that co-aligns with actin fibers, or if it is instead an actin-binding protein and binds to the preexisting actin filaments, we expressed PRPSAP1 in *S. cerevisiae* to determine if it was capable of forming filaments independent of its interaction with actin. We found that PRPSAP1 formed robust filaments in yeast that did not recruit or co-localize with actin (Figure 4-10). This suggests that PRPSAP1 is part of a novel filament network that co-aligns with actin in fibroblasts, but does not require actin to form filaments.

Mutations in PRPS1 alter the expression and localization of PRPSAP1

These above results suggested that alterations in PRPS1 might alter actin organization via an effect of PRPS1 on PRPSAP1. If PRPSAP1 contributes to the actin phenotypes that we observe in PRPS1 mutant fibroblasts, we would expect that PRPS1 mutations should disrupt PRPSAP1 organization or expression. Immunofluorescence and immunoblot analysis revealed that in LOF1 and LOF2

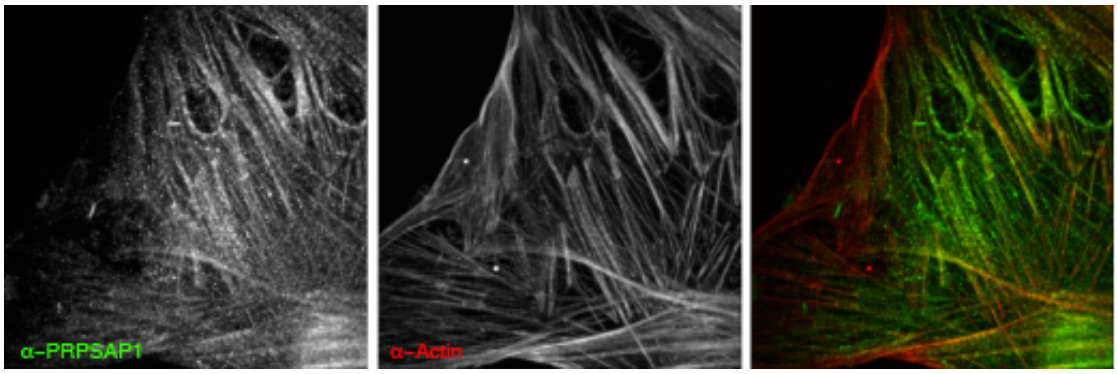

Figure 4-9 Immunofluorescence of PRPSAP1 in fibroblasts shows that PRPSAP1 colocalizes with the actin cytoskeleton

Fibroblasts immunostained for PRPSAP1 (green) and counter stained with actin (red) show that the PRPSAP1 filament network and the actin cytoskeleton colocalize (merge). Wild type fibroblasts were fixed and stained as described in Methods.

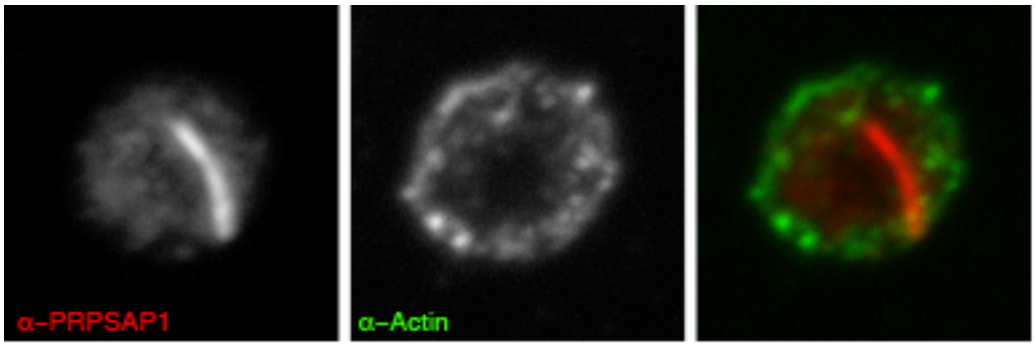

Figure 4-10 Immunofluorescence of human PRPSAP1 in *S. cerevisiae* reveals PRPSAP1 filament formation independent of yeast actin network

Human PRPSAP1 introduced into yeast forms a filament in the cell that does not co-localize with actin. This indicates that human PRPSAP1 (red) forms an independent filament structure from that of actin (green). A merge is shown at the right. Transfection and immunofluorescence were performed as described in Methods.

fibroblasts, the levels of PRPSAP1 are greatly reduced (Figure 4-2B). Interestingly, while PRPSAP1 levels are not affected in SM fibroblasts (Figure 4-2B), PRPSAP1 is recruited to the cytoplasmic PRPS1 filaments that we observe in the SM cells (Figure 4-11). Lastly, we have found that the number of cells with cytoplasmic PRPS1 filaments increases as they become progressively more confluent (Figure 4-12)—a result that provides a plausible explanation for why actin becomes increasingly disorganized in SM cells.

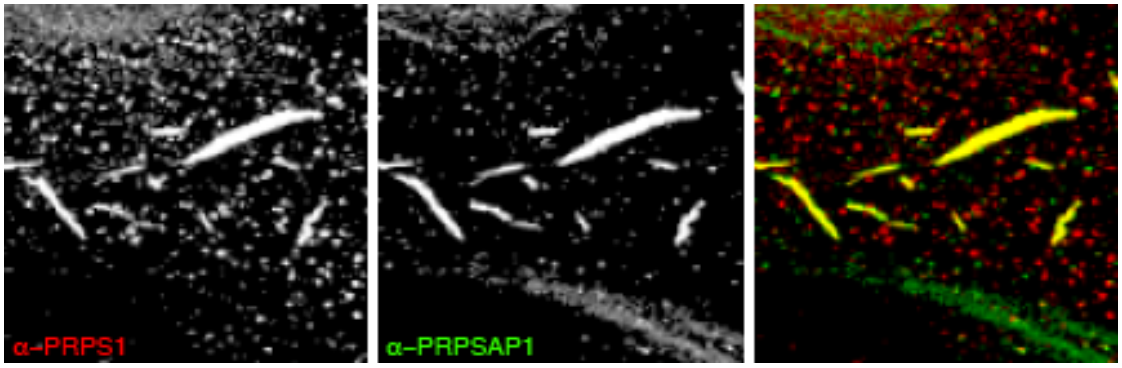

Figure 4-11 Immunofluorescence of PRPS1 and PRPSAP1 reveals that they colocalize in the cytoplasm of PRPS1 superactivity mutant (SM) fibroblasts

PRPS1 superactivity mutant fibroblasts were immunostained for PRPS1 (red) and PRPSAP1 (green). PRPS1 and PRPSAP1 filaments in the cytoplasm of SM fibroblasts colocalize with each other as shown in the merged image to the right. Immunofluorescence was performed as described in Methods.

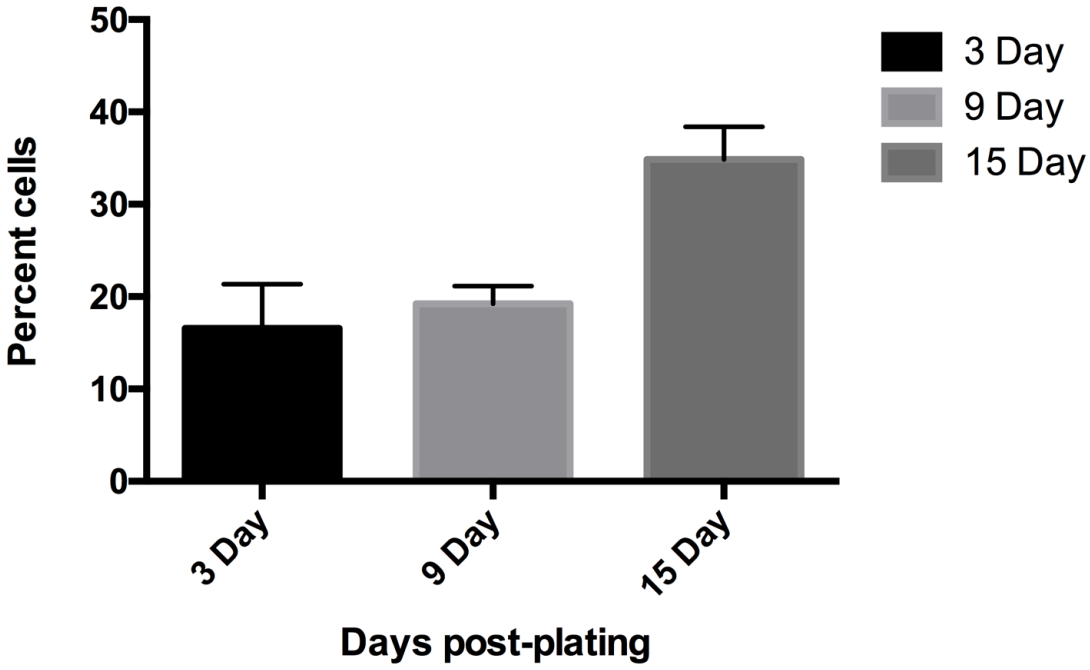

Figure 4-12 Quantification of PRPP synthetase superactivity mutation fibroblasts with aberrant cytoplasmic polymerization of *PRPS1* at 5, 9, and 15 days of recovery after plating

PRPS1 superactivity mutant fibroblasts were grown to 100% confluency and plated on coverslips. Immunostaining against PRPS1 was performed after 5, 9, or 15 days of recovery to analyze the percentage of cells with promiscuous polymerization of PRPS1 in the cytoplasm. After 3 days of recovery, 16.6% of SM cells had aberrant cytoplasmic polymerization of PRPS1, at 9 days of recovery, 19.2% of cells had aberrant cytoplasmic polymerization of PRPS1, and at 15 days of recovery, 34.8% of cells had aberrant cytoplasmic polymerization of PRPS1.
Immunofluorescence was performed as described in Methods. Cells were counted with n=500 or greater for each time point. Experiment was performed in triplicate.

Discussion

The recent discovery of numerous intracellular structures comprised of metabolic enzymes has raised two key questions: 1) what role do these structures play in regulating metabolic activity, and 2) have these structures acquired additional cell biological functions. Here, we have identified two novel intracellular filament systems that provide insight into both of these questions. One system is comprised of the enzyme PRPP synthetase, which forms filaments in the nucleus that are conserved from yeast to humans. The second is comprised of PRPSAP1, an inhibitor of PRPP synthetase, that forms filaments aligning with actin stress fibers. We have found that loss of function mutations in PRPS1 causes loss of the nuclear PRPS1 filaments (Figure 4-2), loss of PRPSAP1 filaments, and misorganization of actin stress fibers (Figures 4-7, 4-8). Given the prominent role of actin and actin regulatory proteins in hair cell function, our results provide a potential mechanism that could explain why sensorineural deafness is a common symptom in human PRPP synthetase syndromes. In order to explore this hypothesis further, we assayed the effect of the PRPS1 superactivity mutation (D182H) on PRPS1 filaments, PRPSAP1 filaments, and the actin cytoskeleton. Interestingly, this superactivity mutation caused actin organization defects comparable to those we observe in the loss of function mutations. Furthermore, the superactivity mutation causes PRPS1 to also polymerize promiscuously in the cytoplasm (Figure 4-2A) where it sequesters PRPSAP1 (Figure 4-11), disrupting its ability to interact with actin stress fibers. Together, the above results suggest that the organization of metabolic enzymes and their regulators may well be more deeply integrated with other cellular systems than previously thought - a

finding that has numerous implications for our understanding of inborn errors of metabolism.

The role of PRPS1 filaments in the nucleus

Given the role of PRPS1 in *de novo* purine biosynthesis, the fact that it forms filaments in the nucleus was unexpected. Furthermore, these filaments are particularly unusual, since unlike the majority of metabolic filaments that have been characterized, the nuclear filaments are refractory to treatments that alter known regulators of the enzyme. This behavior is in stark contrast to the behavior of the cytoplasmic PRPS1 filaments seen in the SM mutant. These cytoplasmic PRPS1 filaments readily disassemble in response to nucleotides known to trigger end product inhibition (Figures 4-3, 4-4B). Based on these results, we propose that the PRPS1 polymer is the active form of the enzyme and that nuclear filaments are preferentially stabilized in the active state. It is currently unclear whether the demand for PRPP is particularly high in the nucleus such that it would require locking PRPS1 in the active state, or if the nuclear filaments have acquired a second "moonlighting" function. It is worth noting, however, that while PRPP is most commonly considered a key intermediate in nucleotide biosynthesis, it is also required for the NAD salvage pathway (Magni et al., 1999; Preiss and Handler, 1958). Since many chromatin modifications, such as methylation and histone deacetylation, are dependent on NAD, supplying a constant source of its substrate, PRPP, might be critical for proper gene regulation. Further studies directed at understanding how PRPS1 is localized to the nucleus and how the

nuclear filaments are stabilized are likely to help resolve whether PRPS1 filaments have assumed a novel function or satisfy a specific metabolic need in the nucleus.

PRPSAP1 - a novel filament network associated with actin

In addition to identifying a novel nuclear filament, our work has also identified a filament network comprised of PRPSAP1 that aligns with actin stress fibers. PRPSAP1 was originally identified as an inhibitory subunit of PRPP synthetase in mammals (Kita et al., 1994). However, given its structural similarity to PRPS1, we investigated whether or not it was capable of polymerization. The fact that PRPSAP1 co-localized with actin stress fibers suggested that it was either a novel actin binding protein or was capable of forming its own novel filament network that aligned with actin stress fibers. Our observation that PRPSAP1 forms filaments that do not co-assemble with actin in *S. cerevisae* argues that PRPSAP1 has the ability to form a novel filament network (Figure 4-10). The discovery of the PRPSAP1 polymer network that interacts with actin opens a new area of investigation for linking the classic cytoskeleton with metabolic enzyme polymerization.

The cell biology of PRPP synthetase syndromes

Mutations in PRPS1 cause four distinct syndromes: X-linked Charcot-Marie-Tooth disease-5 (CMTX5), Arts Syndrome, X-linked nonsyndromic sensorineural deafness (DFN2), and PRS-1 superactivity disorder. The first three of these syndromes are characterized by the graded severity of the loss of function mutation in PRPS1. Sensorineural deafness is a hallmark of DFN2 patients, while CMTX5 and

Arts syndrome patients also have mental retardation, hypotonia, ataxia, and optic atrophy in addition to deafness. Surprisingly, “superactivity” mutations that disrupt feedback inhibition of the enzyme by ADP or GDP causes deafness and ataxia as well as the expected gout resulting from purine overproduction. Previously, there has not been a model for how mutations that increase or decrease activity of PRPS1 could cause both deafness and ataxia. This study provides the first evidence of how such syndromes might work. One model is that alterations in PRPS1 lead to either loss of function or a gain of function that results in sequestration of PRPSAP1 in an effort to normalize PRPS1 activity. In turn, sequestration of PRPSAP1 causes disruption in the actin cytoskeleton, leading to sensorineural deafness, since the hair cells of the inner ear are highly dependent on actin-enriched stereocilia (Tilney et al., 1983). One prediction of this model is that loss of PRPSAP1 would phenocopy many of the aspects of PRPS1 syndromes. While testing this model in primary culture fibroblasts is currently unfeasible due to the fragility of the cells, assaying the effects of disrupting PRPS1 and PRPSAP1 on hair cell function in a model organism, such as zebrafish, would provide insight into the role of these filament networks in PRPS1 syndromes.

Materials and Methods

Cell culture

Human primary fibroblasts were cultured in MEM media lacking glutamine (Gibco) and containing 10% FBS, 5% L-glutamine, 5% Penicillin-Streptomycin using standard cell culture incubation conditions. Rat hippocampal neurons were maintained in B27-supplemented neurobasal medium (Invitrogen) in standard conditions (Patrick et al., 2003). Human primary fibroblast cells of the genotype WT, SM, LOF1, or LOF2 were gifts from Dr. William Nyhan (UCSD) and Dr. Robert Naviaux (UCSD). Rat hippocampal neurons were gifts from the laboratory of Dr. Gentry Patrick (UCSD).

Antibody Generation

PRPP synthetase antibody was prepared by cloning the human full-length PRPS1 coding region into the pProEx-HTc vector to produce amino-terminally fused 6xHis-recombinant protein. The protein was expressed in *E. coli* and purified using a Ni-NTA agarose (Invitrogen) column. The protein was injected into rabbits for antiserum production (Covance). The antiserum was purified against 6xHis-PRPS1-GFP protein on a CnBr-activated sepharose 4B (GE Healthcare) column.

PRPSAP1 antibody was prepared against the combination of two synthesized peptides (Covance) CTELD and PMVKN that were injected into rabbits for antiserum production (Covance). Antiserum was purified using the peptides on a CnBr-activated sepharose 4B (GE Healthcare) column.

Immunostaining and fluorescence microscopy

For yeast immunostaining, a nup157::GFP strain was obtained from the yeast GFP collection (Howson et al., 2005) and grown at 30°C in YPD (2% peptone, 1% yeast extract, 2% dextrose). Yeast immunofluoresence fixation and antibody detection was performed as previously described (Noree et al., 2010). Immunofluorescence microscopy was performed using a DeltaVision Restoration Microscopy System (Applied Precision) and an IX70 Olympus microscope using SoftWoRx (Applied Precision) and 100X objective.

For fibroblast immunostaining, fibroblasts were cultured and plated on coverslips in MEM minus glutamine supplemented with 10% FBS, 5% L-glutamine, 5% Penicillin-Streptomycin. Fibroblasts were fixed in 1X PBS, 4% Paraformaldehyde for 10 minutes at room temperature. Coverslips were then rinsed with 1X PBS, and washed twice for 5-minutes in 1X PBS. Cells were then incubated in permeabilization solution (1X PBS, 1% goat serum, 0.5% TritonX-100) for 15 minutes at room temperature, then washed 3 times for 5 minutes in wash solution (1X PBS, 1% goat serum). Coverslips were incubated overnight at 4°C in primary antibody diluted in wash solution. Coverslips were then washed twice for 5-minutes in wash solution, then incubated at room temperature, covered for two hours, in secondary antibody diluted in wash solution. Coverslips were aspirated of any media and incubated for 7 minutes in DAPI (10mg/mL) diluted 1:5000 in wash solution. For actin staining, coverslips were quickly rinsed in wash solution and then incubated 20 minutes in Rhodamine Phalloidin (200 units/mL) (ThermoFisher) diluted 1:200 in 1X PBS. Following this, all

coverslips were washed three times for 5-minutes in wash solution, and washed once with deionized RNase-free, DNase-free water. All liquid was aspirated, coverslips were allowed to air dry, then mounted on slides in Vectashield (Vector Laboratories). Microscopy was performed using Leica TCS SP5 laser confocal microscope at 63X as previously described (Noree et al., 2010).

For neuronal staining, neurons were dissected from the hippocampus of rat embryos, plated on cover slips and cultured for 14 days using standard conditions (Patrick et al., 2003). Neurons on coverslips were then washed twice in PBS-MC (1X PBS, 1mM $MgCl_2$, 0.1mM CaCl2) and fixed in 1X PBS, 4% Paraformaldehyde, 4% Sucrose for 10 minutes at room temperature. The cover slips were next washed twice with 1mL PBS- MC, followed by additional fixation in 1mL 100% MeOH (-20°C) for 2 minutes at -20°C. The cover slips were then washed twice with 1mL PBS-MC, followed by blocking with 1mL blocking/permeablization solution (1X PBS-MC with 2% BSA, 0.2% Triton X-100) for 20 minutes. The blocking solution was removed and the cover slips were incubated overnight at 4°C in 1X PBS-MC, 2% BSA with primary antibody. The slides were then rinsed three times with PBS-MC, followed by three 5-minute PBS-MC washes at room temperature while rotating. The cover slips were then incubated with secondary antibody in PBS-MC, 2% BSA for 1 hour at room temperature. The secondary antibody was removed by one 5-minute wash in PBS-MC at room temperature while rotating. The resulting coverslips were incubated 10 minutes in PBS-MC with 2μg/mL DAPI, followed by one 5-minute wash at room

temperature with PBS-MC while rotating. Lastly, the cover slips were mounted on slides using Vectashield (Vector Laboratories) and imaged using a Leica TCS SP5 laser confocal microscope at 63X. Primary antibodies were used at the following concentrations: rabbit α–PRPS1 at 1:1000 and mouse α–tubulin-FITC at 1:250 (Sigma). Alexa Fluor 568-conjugated α-rabbit secondary antibody (Invitrogen) was used at 1:200. Rhodamine Phalloidin (200 units/mL) (Molecular Probes) was used at 1:200 to stain actin filaments.

Immunoblot analysis

Cell extracts were prepared from primary fibroblast cultures after growing them to confluency in appropriate media as described above. Adhered cells were washed twice in 1X PBS and the buffer was completely aspirated. The cell culture dishes were placed on ice and the cells were scraped into RIPA buffer (50 mM Tris pH7.5, 150 mM NaCl, 0.1% SDS, 0.5% Deoxycholate, 1% NP40) with 1:1000 protease inhibitor cocktail (Sigma) and transferred to a microfuge tube. 80uL of 2X SDS-PAGE buffer and 50uL of glass beads were added to the tube and vortexed vigorously. Samples were boiled for 5 minutes at 95°C, then incubated on ice for 5 minutes. The samples were centrifuged at 10,000 RPM for 1 minute before loading onto 10% SDS-PAGE. Proteins in the gel were transferred to a nitrocellulose membrane (GE Amersham) by electroblotting (Owl HEP-1; Thermo Fisher Scientific) and a standard protocol for Western blot was performed as previously described (Wilhelm et al., 2000). The following antibodies at the noted concentrations were used for blotting: rabbit α–

PRPS1 (1:1000), rabbit α–PRPSAP1 (1:1000), mouse α–tubulin 12G10 (1:1000) (Developmental Studies Hybridoma Bank) and mouse α–actin JLA20 (1:100) (Developmental Studies Hybridoma Bank).

Metabolite treatment

Cell cultures plated on coverslips were incubated in 50 mM nucleotide (adenosine, guanosine, or cytidine) with growth media for 30 min or 1 hour and then the immunofluorescence protocol described above was used for PRPP synthetase detection. The cells, washed and fixed as above, were visualized using a Zeiss Axiovert 200M microscope. Random fields were chosen for counting. The total number of cells counted exceeded n=500 per count per sample. Those cells with nuclear filaments and those with cytoplasmic filaments were scored. Each experiment was repeated four times for graphing and statistical analysis (Mean ± SEM).

Actin analysis

WT, SM, LOF1, and LOF2 fibroblast cultures were grown in conditions described above to 100% confluency in flasks. The cells were trypsinized using 0.25% Trypsin (Gibco), then plated on coverslips in MEM media (as described above). Cells were allowed to recover for 5, 7, or 9 days. The fixation and immunofluorescence protocol described above was used for actin visualization. Nine random fields were selected for each condition and an entire Z-axis was imaged with 0.8um sections using a Leica TCS SP5 laser confocal microscope. Each stack was analyzed for actin stress fiber directionality using FIJI (Fiji Is Just ImageJ) Local Gradient Analysis. The angles of

stress fibers were normalized to the general polarity direction of each cell and graphically represented.

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Chapter 5

Summary and future directions

While most of the membrane bound organelles and cytoskeletal structures within the cell are known, our understanding of how the cytoplasm might be organized and partitioned remains poorly understood. My thesis work has focused on two types of novel cytoplasmic organization: 1) spatial regulation of mRNA processing, and 2) regulation of metabolic enzymes via polymerization and partitioning.

The first two parts of this thesis focus on how information in the form of mRNA is spatially regulated in the cytoplasm. In Chapter 2, I identified a novel role for the ribonucleoprotein (RNP) Cup in stabilizing and protecting *oskar* mRNA, a transcript that is critical for the anterior-posterior patterning during *Drosophila* oogenesis. Maternal transcripts are well known for their unusual stability, however, the mechanism underlying this stability is unknown. It has long been thought that the maternal RNA protein complex might be responsible for stabilizing maternal messages, but most of the known subunits of these complexes have been implicated in either mRNA localization or translational control rather than mRNA stability (Audhya et al., 2005; Boag et al., 2005; Ladomery et al., 1997; Li et al., 2009; Mansfield et al., 2002; Minshall et al., 2007; Minshall and Standart, 2004; Minshall et al., 2001; Nakamura et al., 2001; Nakamura et al., 2004; Squirrell et al., 2006; Tafuri and Wolffe, 1993; Tanaka et al., 2006; Wilhelm et al., 2005; Wilhelm et al., 2003; Wilhelm et al., 2000). In *Drosophila*, the eIF4E-binding protein, Cup, had been identified as a translational repressor of *oskar* mRNA that also has roles in mRNA localization (Nakamura et al., 2004; Nelson et al., 2004; Wilhelm et al., 2003). My thesis work focused on identifying the earliest observed phenotype of *Drosophila cup* mutants. This work revealed that Cup is required to stabilize the *oskar* transcript and

that localization defects are likely secondary to loss of the message. Thus, Cup appears to function as both a translational repressor and as a stabilzation factor for *oskar* mRNA.

While maternal mRNA in the oocyte is initially incredibly stable, it is rapidly degraded after fertilization/egg activation. My work in Chapter 3 investigated how the maternal RNP might be remodeled to destabilize maternal transcripts in the embryo. My work identified a role of the multi-protein Pan gu kinase complex in triggering ubiquitin-mediated degradation of RNPs to facilitate mRNA degradation at the maternal-to-zygotic transition in early embryogenesis. I identified a set of maternal RNP subunits that are associated with stabilized maternal RNA, but I show that they become degraded in the early embryo through ubiquitin-mediated proteolysis. Importantly, I showed that blocking this natural proteolysis prevents the degradation of the maternal mRNA associated with these RNPs. We also found that Pan gu controls RNP subunit degradation through a pathway dependent on the meiotic anaphase promoting complex (APC), but separate from the Pan gu kinase pathway used to activate mRNA degradation. The identification of the maternal RNP subunits required to be degraded prior to mRNA degradation suggests that there are two parallel pathways that Pan gu controls for maternal transcript degradation—the activation of mRNA degradation machinery (Tadros et al., 2007), and the removal of the RNPs complexed with the mRNA by ubiquitin-proteasome degradation.

While my work has laid the groundwork for this previously uncharacterized proteolysis-dependent pathway required for mRNA degradation, much remains to be addressed. We have yet to discover the downstream effectors of the APC or the Pan gu

kinase on initiating the RNP subunit proteolysis, or the degrons used for the various RNP subunit proteolysis. While this study focused on the degradation of maternal mRNA in the embryo, it remains to be seen whether it translates to other cellular mRNA degradation or processing events.

My work in Chapter 4 addresses a second form of cytoplasmic organization: enzyme polymerization. With the discovery that many metabolic enzymes are capable of forming intracellular polymers, it has become apparent that polymerization may be a widely employed method of organizing and/or regulating metabolic pathways. This portion of my thesis work has focused on PRPP synthetase (PRPS) polymerization as it relates to inborn errors of metabolism. I first showed that PRPS polymerization is conserved throughout higher order metazoans, and most notably found that it naturally forms nuclear filaments from yeast to humans. Examining patient fibroblasts from those affected with a *PRPS* feedback resistance, or "superactivity" disorder, I found that this mutant PRPS enzyme not only forms the normal nuclear filament, but also promiscuously polymerizes in the cytoplasm. Furthermore, I found that the cytoplasmic filaments are disassembled by nucleotides or other known regulators of PRPS. This metabolite control strongly indicates that the filaments are not simple aggregates. In addition to these discoveries, I found that the polarity of the actin cytoskeleton is disrupted in cells with *PRPS* disorders. Analysis of the directionality of actin stress fibers from three different patients with PRPS disorders showed that while cell shape generally conforms to an axis of polarity, the actin stress fibers in the cells with the disorder have no common directionality. Moreover, I discovered that PRPSAP1, an inhibitor of PRPS1, colocalizes with PRPS1 as well as with the actin

cytoskeleton, showing that there is a correlation between PRPS dysfunction and actin regulation. Because PRPS1 superactivity disorder has been characterized clinically as presenting with both loss of function phenotypes of ataxia and deafness, as well as the gain of function phenotype of gout from the overproduction of purines (Ahmed et al., 1999; Akaoka et al., 1981; Becker et al., 1982; Becker et al., 1986; Becker et al., 1988; Becker et al., 1980; Becker and Seegmiller, 1975; Becker et al., 1995; Becker et al., 1996; Zoref et al., 1975), the enzyme compartmentalization as well as its role in regulating actin through PRPSAP1 presents a novel approach to gaining insight into the disease.

Prior to my studies of PRPS1, there was little evidence to support that the biochemistry of the enzyme was connected to any distinct cellular structure. My studies have demonstrated that alterations in PRPS1 enzyme polymerization in various PRPS1 syndromes lead to alterations in both actin organization and the association of PRPSAP1 with the actin cytoskeleton. These connections provide a new way of conceptualizing many inborn errors of metabolism and potentially developing new therapeutic approaches. The key next step is to identify the function of the PRPS1 and PRPSAP1 filaments. *In vitro* polymerization assays coupled with enzyme assays will help establish whether the nuclear filament represents the active form of the enzyme. We also do not yet know how PRPSAP1 filaments associate with the actin cytoskeleton. The development of PRPSAP1/actin bundling assays will be of help in understanding what factors mediate this association *in vivo*. Additionally, the fact that wild type PRPS1 forms filaments exclusively in the nucleus raises questions such as whether there is a specific nuclear function for the PRPS filament and how filament

assembly is restricted to the nucleus. The fact that the nuclear filaments appear to be refractory to alteration by the substrates/products tested to date suggests that these filaments might be capped. A nucleator, similar to the Arp2/3 nucleator of actin filaments, if found, might explain the targeting of the filaments to the nucleus as well as their apparent lack of dynamic behavior. Finally, connecting how both PRPS1 and PRPSAP1 function in the hair cells, responsible for vertebrate hearing and balance, and other tissues will expand our knowledge beyond my studies or the pathophysiology of the PRPS1 syndromes.

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