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Cup regulates *oskar* mRNA stability during oogenesis

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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.

1. 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., 2011) . 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.

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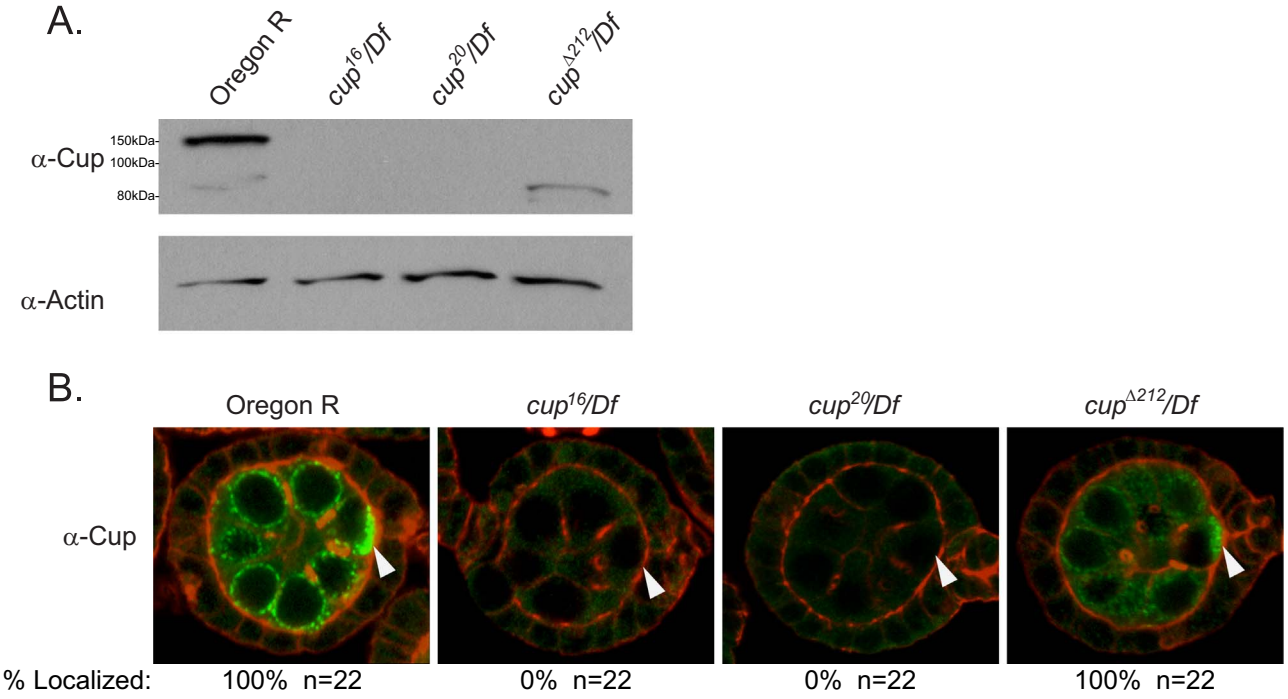


Fig. 1. Strong hypomorphic alleles, *cup*^{16} and *cup*^{20}, are protein null alleles of *cup*. (A). Ovary extract from *cup*^{16}*/Df(2L)bsc7* and *cup*^{20}*/Df(2L)bsc7* lack Cup protein, whereas *cup*$^{\Delta 212}$*/Df(2L)bsc7* mutants produce a decreased level of N-terminally truncated Cup protein lacking the eIF4E-binding region. Ovaries from Oregon R, *cup*^{16}*/Df(2L)bsc7*, *cup*^{20}*/Df(2L)bsc7,* and *cup*$^{\Delta 212}$*/Df(2 L)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 *oskar* RNP complex is comprised of 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 consists 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, 2001; Minshall and Standart, 2004; Nakamura et al., 2001, 2004; Squirrell et al., 2006; Tafuri and Wolffe, 1993; Tanaka et al., 2006; Wilhelm et al., 2005, 2003, 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 assess 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.

2. Results

2.1. 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 pro-

gressed 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 is separate from translational control of *oskar* transcripts.

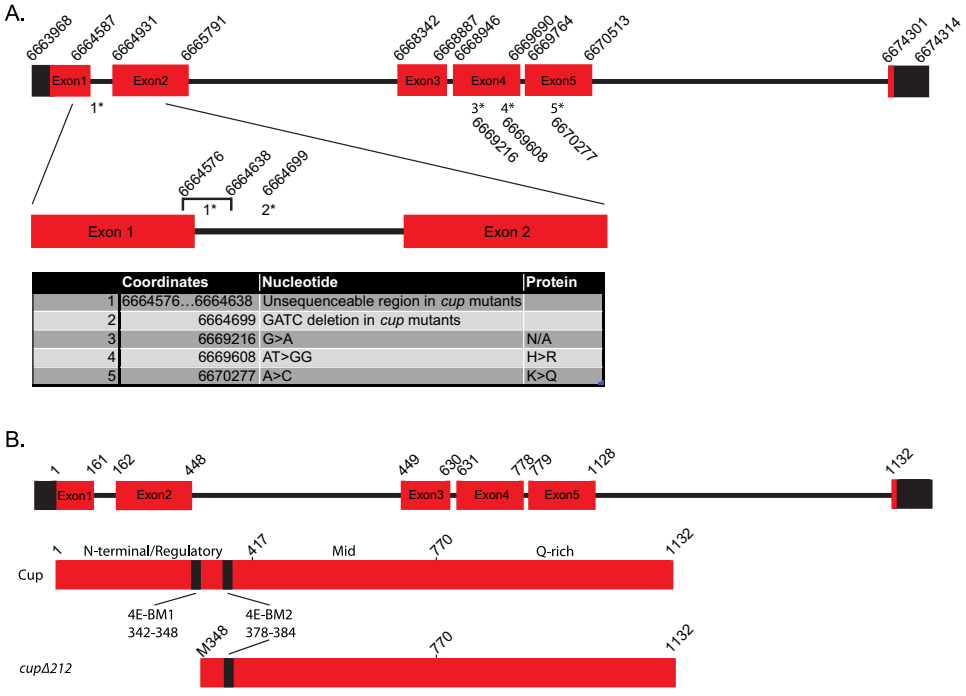
In an attempt to uncover these additional roles of *cup*, we first sought to identify protein null alleles of *cup*. Flies carrying the deficiency, *Df(2L)bsc7*, which deletes the *cup* locus, were crossed to flies bearing each of the available *cup* alleles to generate *cup/Df(2L) bsc7* females. Ovaries from the hemizygous females were dissected and analyzed for the presence of Cup protein by both immunoblot (Fig. 1A) and immunofluorescence (Fig. 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 (Fig. 1A, B). Thus, based on our analysis, both cup^{20} and cup^{16}, appear to be protein null alleles of *cup*.

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 (Fig. 2A). 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 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.

2.2. 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, 2004; Wilhelm et al., 2005, 2003). Ovaries from $cup^{16}/Df(2L)bsc7$ and



	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

Fig. 2. The *cup* phenotype is a result of a mutation outside of the cup locus. (A). 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. (B). 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.

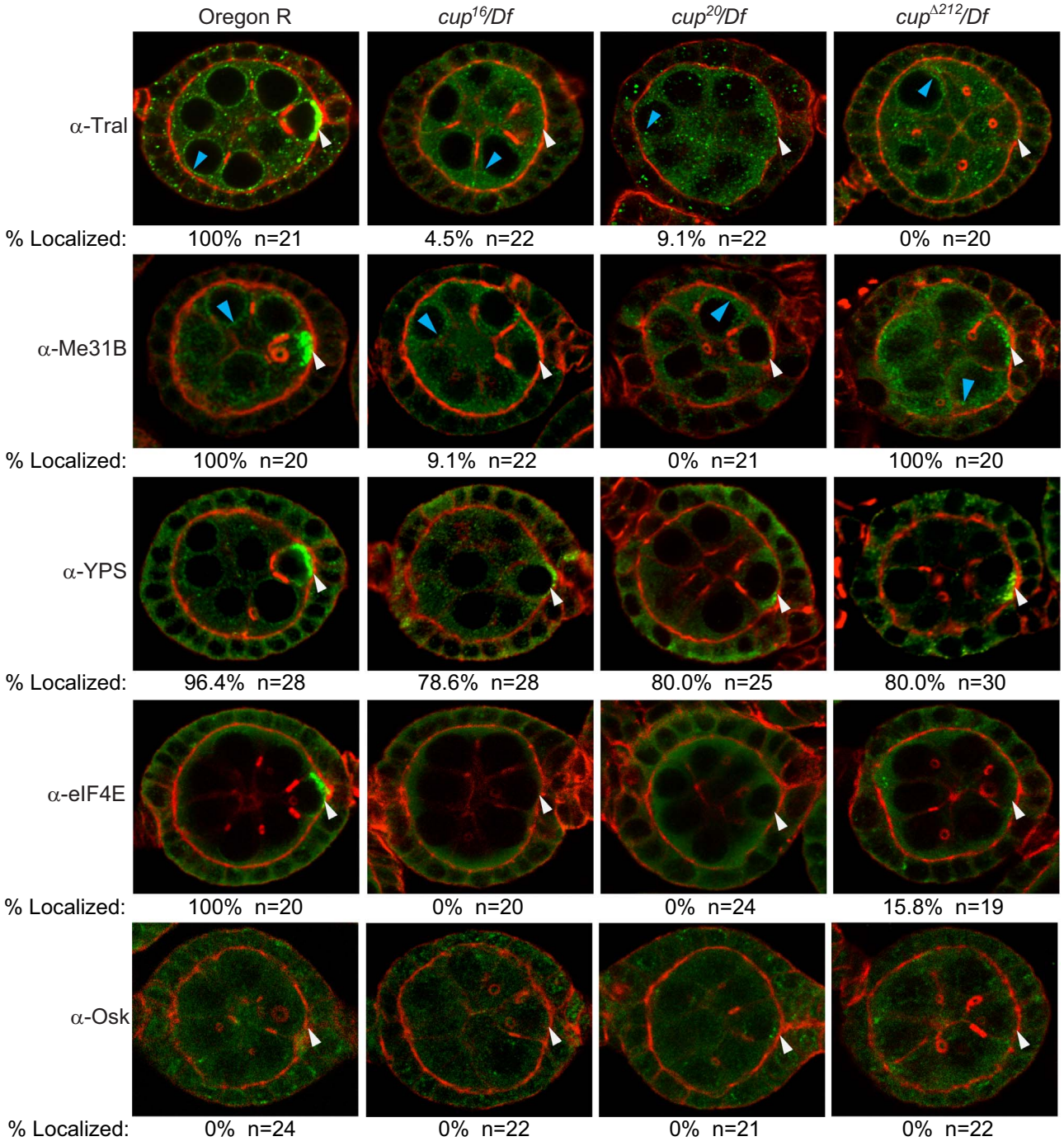


Fig. 3. *oskar* RNP complex localization is disrupted in *cup* mutants. Immunofluoresence in stage 4 Oregon R and $cup^{16}/Df(2L)bsc7$, $cup^{20}/Df(2L)bsc7$, and $cup^{\Delta 212}/Df(2L)bsc7$ egg chambers against core *osk* RNP subunits Trailer hitch (Tral), Me31B, and YPS, as well as eIF4E and Osk. Proper *oskar* RNP localization appears at the posterior of the oocyte (white arrows), 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 *oskar* RNP subunits in $cup^{16}/Df(2L)bsc7$ and $cup^{20}/Df(2L)bsc7$, and slightly reduced localization of all *oskar* RNP subunits in $cup^{\Delta 212}/Df(2L)bsc7$ except for Tral, which is completely absent. 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 does not accumulate to a significant degree at stage 4 egg chambers in any of these genotypes consistent with prior studies of $cup^{\Delta 212}/Df(2L)bsc7$.

$cup^{20}/Df(2L)bsc7$ females were dissected and immunostained with antibodies against each of these *oskar* RNP subunits (Figs. 3 and 4). In wild type egg chambers, each of these proteins is localized to the posterior pole of the developing oocyte and their localization tracks the localization of *oskar* mRNA throughout oogenesis (Figs. 3 and 4; 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) (Figs. 3 and 4; cup^{16}/Df column). Comparable effects on *oskar* RNP subunit localization were also seen in $cup^{20}/Df(2L)bsc7$ egg chambers (Figs. 3 and 4;

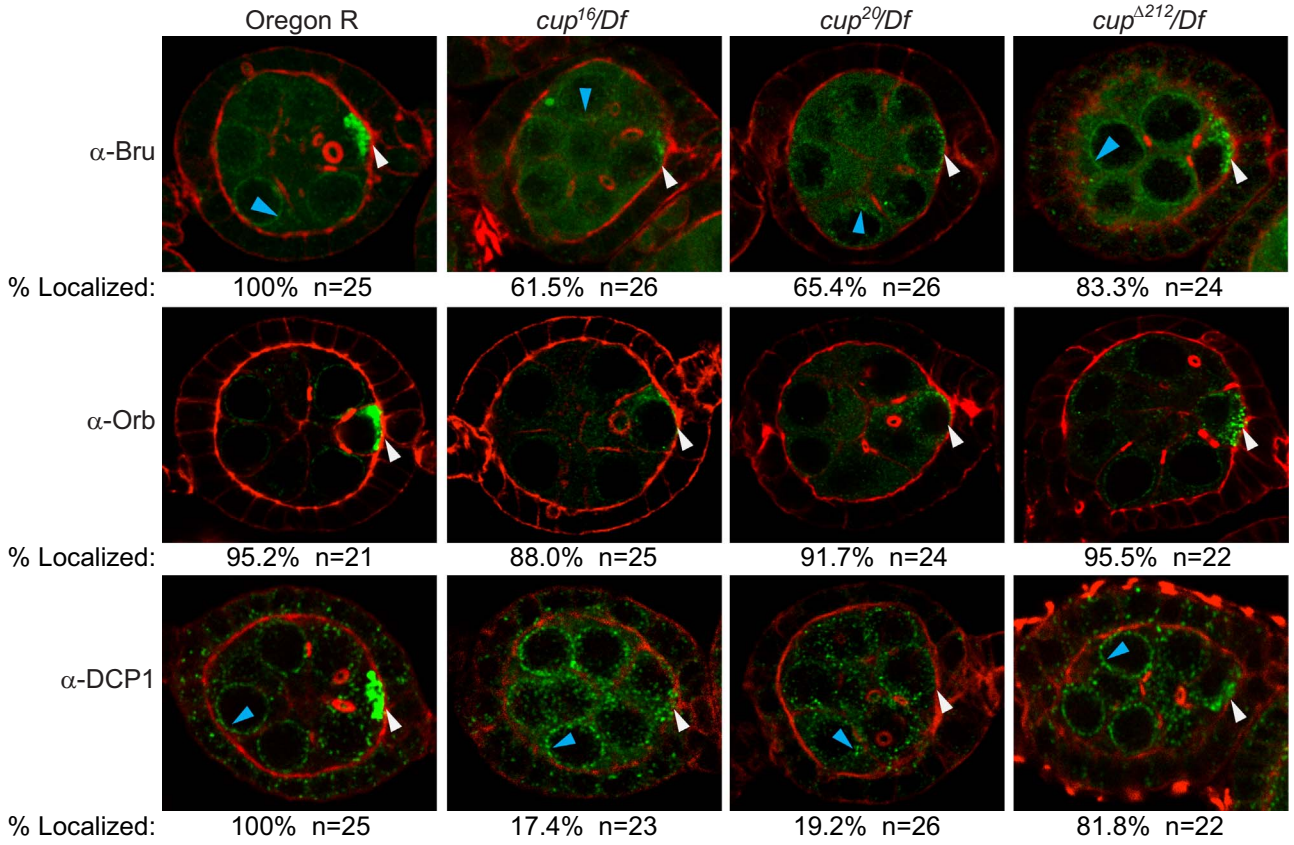


Fig. 4. Localization of known components of the osk RNP is disrupted in *cup* mutants. Immunofluoresence in stage 4 Oregon R and $cup^{16}/Df(2L)bsc7$, $cup^{20}/Df(2L)bsc7$, and $cup^{\Delta 212}/Df(2L)bsc7$ egg chambers against *osk* RNP subunit DCP1 and the *osk* mRNA binding proteins, Bruno (Bru) and Orb. The localization of all three subunits is either lost or drastically reduced.

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 (Figs. 3 and 4).

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 (Fig. 5). 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 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 (Figs. 3 and 4; blue arrows), while the signal within the oocyte was absent (Figs. 3 and 4; 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 (Fig. 2B). 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 $cup^{16}/Df(2\ L)bsc7$ 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 (Fig. 3). 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 (Fig. 3; $cup^{\Delta 212}$ 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 (Figs. 3 and 4; *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 (Fig. 5). 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*.

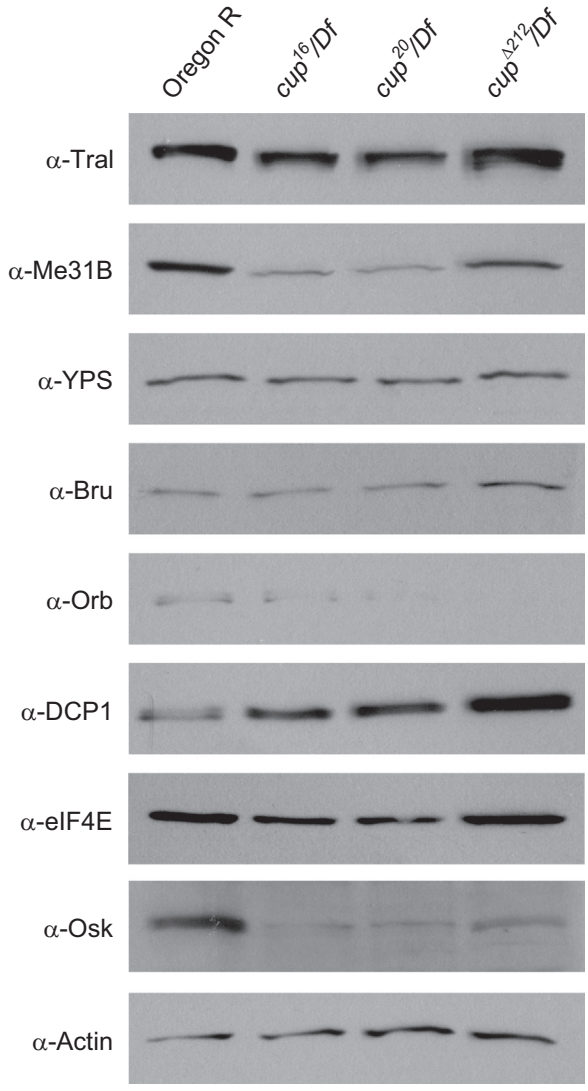


Fig. 5. Cup is not required for the expression or stability of *oskar* RNP subunits. Protein expression levels of Tral, Me31B, YPS, Bru, Orb, DCP1, eIF4E, Osk, and actin in Oregon R and $cup^{16}/Df(2L)bsc7$, $cup^{20}/Df(2L)bsc7$, and $cup^{\Delta 212}/Df(2L)bsc7$ mutant ovary extracts. Extracts were made from ovaries of fattened flies and analyzed by immunoblotting with the corresponding antibodies.

2.3. 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 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 (Fig. 6A), indicating that the defect in *oskar* RNP localization is not due to a failure to specify an oocyte.

2.4. 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 (Fig. 6B). 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 (Fig. 4). Furthermore, it 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 (Fig. 6C). 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 *oskar* 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 (Fig. 6B, C). 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 (Fig. 7). 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.

3. 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

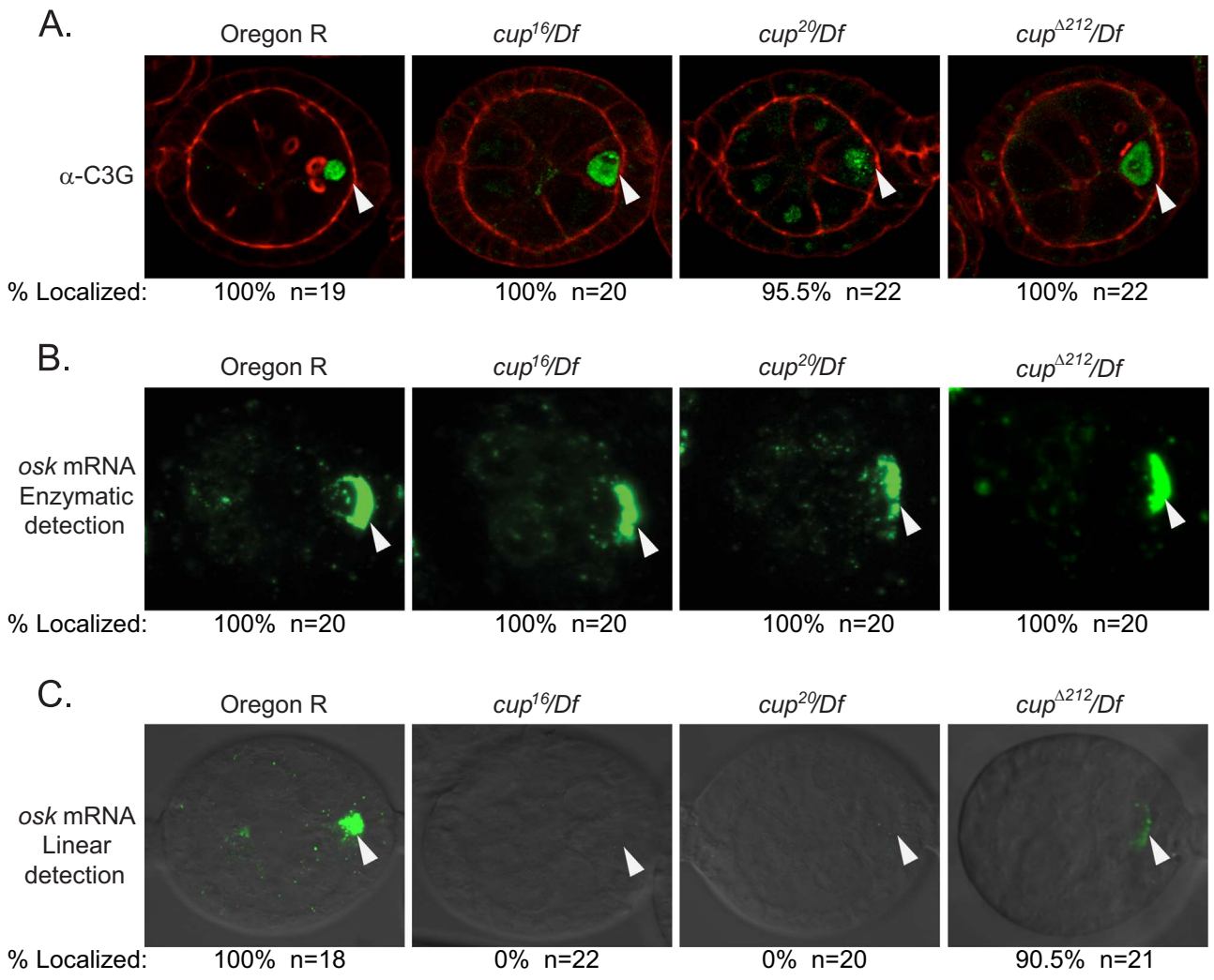


Fig. 6. Failure to localize *oskar* RNP complex is not due to oocyte determination defects. (A). C(3)G staining of Oregon R, $cup^{16}/Df(2L)bsc7$, $cup^{20}/Df(2L)bsc7$, and $cup^{\Delta 212}/Df(2L)bsc7$ was used to detect the meiotically active oocyte. C(3)G presence was detected in stage 4 egg chambers in all *cup* alleles. (B). Enzyme-linked RNA *in situ* hybridization of *oskar* transcripts shows that *oskar* mRNA is localized similar to wild type levels at the posterior of the oocyte in all *cup* alleles. (C). Using a linear RNA *in situ* hybridization method to detect *osk* transcripts, it becomes evident that there is a drastic decrease in *oskar* mRNA at the posterior in the *cup* alleles. $cup^{16}/Df(2L)bsc7$ and $cup^{20}/Df(2L)bsc7$ have no apparent localization of *oskar*, while there is a severely decreased level in $cup^{\Delta 212}/Df(2L)bsc7$.

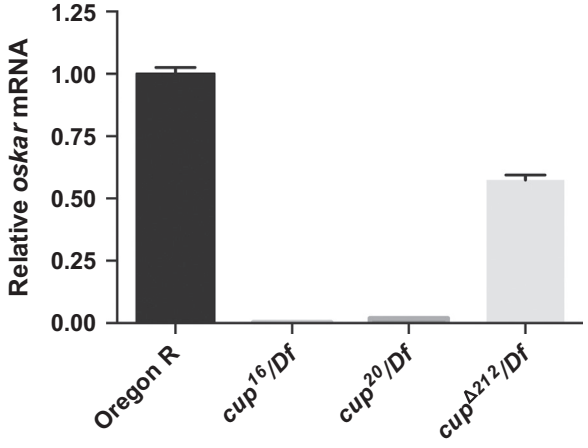


Fig. 7. Cup is required for *oskar* mRNA stability. Levels of *oskar* mRNA were measured using quantitative PCR in Oregon R, $cup^{16}/Df(2L)bsc7$, $cup^{20}/Df(2L)bsc7$, and $cup^{\Delta 212}/Df(2L)bsc7$. Levels of *oskar* transcript were reduced 238-fold in $cup^{16}/Df(2L)bsc7$ ovaries and 52-fold in $cup^{20}/Df(2L)bsc7$ ovaries relative to *oskar* in Oregon R. However, there was only a 1.74-fold decrease in *oskar* mRNA levels in $cup^{\Delta 212}/Df(2L)bsc7$.

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, 2001; Minshall and Standart, 2004; Nakamura et al., 2001, 2004; Squirrell et al., 2006; Tafuri and Wolffe, 1993; Tanaka et al., 2006; Wilhelm et al., 2005, 2003, 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, 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 (Fig. 6B, 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.

4. Materials and methods

4.1. Fly stocks

Fly stocks were cultured at 22–25 °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).

4.2. 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.

4.3. 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-eIF4E (Nelson et al., 2004), anti-Oskar (Chekulaeva 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), anti-eIF4E (1:2000), anti-Oskar (1:2000), 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.

4.4. 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), anti-eIF4E (1:200), anti-Oskar (1:200), 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.

4.5. In situ hybridization

In situ hybridization protocol was performed as previously described (Wilkie and Davis, 2001) with the following modifications: Prehybridization solution contained 50 µg/mL tRNA and 100 µg/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.

4.6. 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.

4.7. 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 50 µL Squishing Buffer (10 mM Tris-Cl pH8.2, 1 mM EDTA, 25 mM NaCl, and 0.2 mg/mL Proteinase K) and incubated at room temperature for 30 min. The samples were incubated at 95 °C for 2 min and then transferred to ice. 1 µL 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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