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Cup is an eIF4E binding protein required for both the translational repression of *oskar* and the recruitment of Barentsz

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In *Drosophila* oocytes, precise localization of the posterior determinant, Oskar, is required for posterior patterning. This precision is accomplished by a localization-dependent translational control mechanism that ensures translation of only correctly localized *oskar* transcripts. Although progress has been made in identifying localization factors and translational repressors of *oskar*, none of the known components of the *oskar* complex is required for both processes. Here, we report the identification of Cup as a novel component of the *oskar* RNP complex. *cup* is required for *oskar* mRNA localization and is necessary to recruit the plus end–directed microtubule transport factor Barentsz to the complex. Surprisingly, Cup is also required to repress the translation of *oskar*. Furthermore, eukaryotic initiation factor 4E (eIF4E) is localized within the oocyte in a *cup*-dependent manner and binds directly to Cup in vitro. Thus, Cup is a translational repressor of *oskar* that is required to assemble the *oskar* mRNA localization machinery. We propose that Cup coordinates localization with translation.

Introduction

Localization of mRNAs is used by many polarized cells as a means of restricting the distribution of a protein to a particular cytoplasmic domain. One of the most extensively characterized systems for studying mRNA localization is the *Drosophila* oocyte (Bashirullah et al., 1998; Johnstone and Lasko, 2001). The basic unit of *Drosophila* oogenesis is the egg chamber, which is comprised of an oocyte and 15 nurse cells surrounded by a layer of somatic follicle cells. The oocyte is connected to the nurse cells by a network of cytoplasmic bridges called ring canals. This network allows the nurse cells to synthesize various mRNAs that are required for early embryogenesis and transport them in a microtubule-dependent manner to discrete locations within the oocyte (Pokrywka and Stephenson, 1995). The correct localization of *oskar* mRNA to the posterior pole is particularly crucial for development since this localization is essential for both posterior patterning and establishment of the germ line (Ephrussi et al., 1991). During early oogenesis (stages 1–6), *oskar* mRNA accumulates at the posterior pole of the oocyte where the minus ends of the microtubule array are concentrated (Fig. 1 A) (Ephrussi et al., 1991; Kim-Ha et al., 1991; Theurkauf et al., 1993). At stages 7 and 8, the microtubules reorganize so that microtubule nucleation occurs over most of the oocyte cortex with the majority of the minus ends being concentrated at the anterior of the oocyte (Fig. 1 A) (Cha et al., 2002). Tracking the minus ends of the microtubules, *oskar* mRNA transiently localizes to the anterior of the oocyte during these stages (Ephrussi et al., 1991; Kim-Ha et al., 1991). During stages 9 and 10, however, *oskar* mRNA transits back to the posterior pole in a plus end–directed transport step that requires *kinesin heavy chain* (*khc*) (Fig. 1 A) (Brendza et al., 2000). Once *oskar* mRNA reaches the posterior pole it is translated (Fig. 1 A). The mechanism for coupling translational activation to completion of the last step in *oskar* mRNA localization has remained elusive.

One general model for how coupling of localization and translation might occur is that there are factors common to both the localization and translational control complexes that are required to coordinate the completion of localization with translational activation. Mutations in a gene product that is common to both complexes might be predicted to

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Abbreviations used in this paper: Btz, Barentsz; eIF4E, eukaryotic initiation factor 4E; Exu, Exuperantia; Yps, Ypsilon Schachtel.

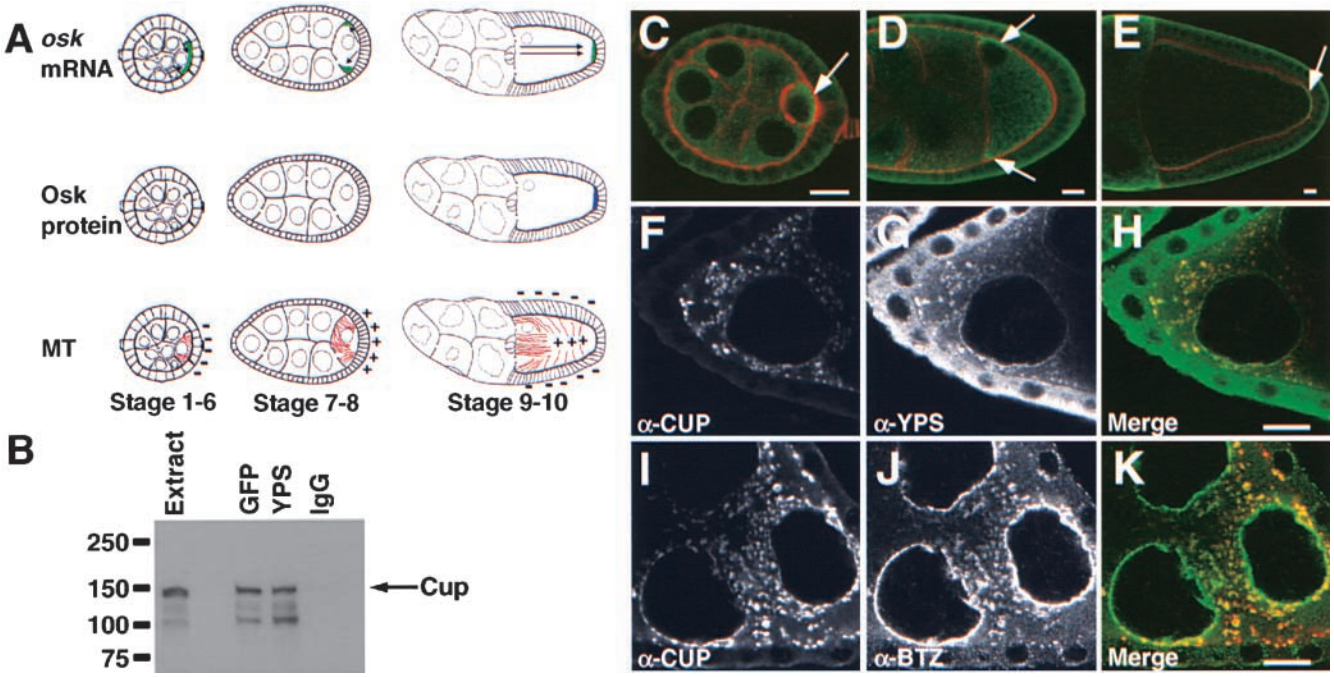

Figure 1. **Cup is a component of the *oskar* RNP complex.** (A) Diagram showing the stage specific movements of *oskar* mRNA and the corresponding changes in Oskar translation and microtubule polarity. *oskar* mRNA, green; Oskar protein, blue; microtubules (MT), red. (B) Immunoblot for Cup (arrow) of immunoprecipitates from GFP-Exu extract using α-GFP (GFP), α-Yps (YPS), or rabbit IgG (IgG) antibodies. (C) Cup protein (green; arrows) is concentrated in at the posterior of the developing oocyte in stages 1–6 (stage 5 is shown; actin is in red). (D) Cup transiently accumulates at the anterior of the oocyte during stages 7 and 8 (stage 7 is shown). (E) Cup then accumulates at the posterior pole during stages 9 and 10 (stage 9 is shown). (F–H) Cup and Yps colocalize in cytoplasmic particles in nurse cells from stage 8 egg chambers. (F) α-Cup staining. (G) α-Yps staining. (H) Merged image. Cup is in red and Yps is in green. (I–K) Cup and Btz colocalize in cytoplasmic particles in nurse cells from stage 8 egg chambers. (I) α-Cup staining. (J) α-Btz staining. (H) Merged image. Cup is in red and Btz is in green. Bars, 10 μm.

cause mislocalization of *oskar* mRNA and premature translation of the *oskar* message. However, mutants that disrupt *oskar* mRNA localization typically have phenotypes similar to those observed in *barentsz* (*btz*) mutants: failure of plus end–directed transport of the *oskar* message during stages 9 and 10, resulting in a complete lack of *oskar* translation (van Eeden et al., 2001). Conversely, a number of translational repressors of *oskar* mRNA (e.g., *BicC*, *bruno*, *ME31B*) have been identified, but their effects on *oskar* mRNA localization appear to be limited (Kim-Ha et al., 1995; Saffman et al., 1998; Nakamura et al., 2001). For instance, mutating all of the Bruno response elements in the *oskar* 3′UTR causes premature translation of *oskar* at stages 7 and 8, but does not interfere with *oskar* mRNA localization (Kim-Ha et al., 1995). Thus, although a number of components are known to be required for either localization or translational repression, no component isolated to date appears to be a part of both complexes.

To identify new components of the *oskar* RNP complex, we previously purified an eight-protein complex that contains *oskar* mRNA (Wilhelm et al., 2000). In this study, we identify the 147-kD protein of this complex as the product of the female sterile gene *cup*. Surprisingly, *cup* is required both for translational repression and localization of *oskar* mRNA. We also demonstrate that Cup binds to eukaryotic initiation factor 4E (eIF4E) and is necessary to recruit the localization factor Barentsz to the complex. Thus, Cup is a translational repressor of *oskar* that is required to assemble the *oskar* mRNA localization machinery. Because of its interactions with both the localization and translational control complexes, we propose that Cup is a likely regulatory target for the coupling machinery.

Results and discussion

Cup is a component of the *oskar* RNP complex

To identify novel components that play a role in either localization or translational regulation of *oskar* mRNA, we previously purified an *oskar* RNP complex that contains Exuperantia (Exu), Ypsilon Schachtel (Yps), and six unidentified proteins (Wilhelm et al., 2000). Using mass spectrometry, we identified the 147-kD protein of this complex as Cup. To confirm that Cup is a bona fide component of the *oskar* RNP complex, we immunoprecipitated both GFP-Exu and Yps and immunoblotted with α-Cup antibody. Cup specifically coimmunoprecipitates with both GFP-Exu and Yps, demonstrating that Cup is a component of the complex (Fig. 1 B).

cup was originally identified as a female sterile mutation that forms eggs that are open at the anterior due to a failure in chorion deposition at the anterior of the oocyte (Schupbach and Wieschaus, 1991; Keyes and Spradling, 1997). This previous work established that Cup is a cytoplasmic protein that is localized early to the oocyte (Keyes and Spradling, 1997). Since Cup copurifies with components of an *oskar* RNP complex, we decided to examine the distribution

of Cup during oogenesis in more detail. Immunostaining of different stage egg chambers (see Spradling, 1993, for staging) revealed that Cup accumulates at the posterior of the oocyte during stages 1–6, consistent with previously published results (Fig. 1 C) (Keyes and Spradling, 1997). At stages 7 and 8, Cup was localized to the anterior of the oocyte (Fig. 1 D), followed by redistribution to the posterior of the oocyte during stages 9 and 10 (Fig. 1 E). Thus, Cup copurifies with components of the *oskar* RNP complex and is localized within the oocyte in a temporal–spatial pattern identical to that of *oskar* mRNA.

One of the rationales for using GFP-Exu as a biochemical handle for the purification of localization complexes is that GFP-Exu forms particles in nurse cells that move in a microtubule-dependent manner (Theurkauf and Hazelrigg, 1998). Previously, we demonstrated that Yps, which

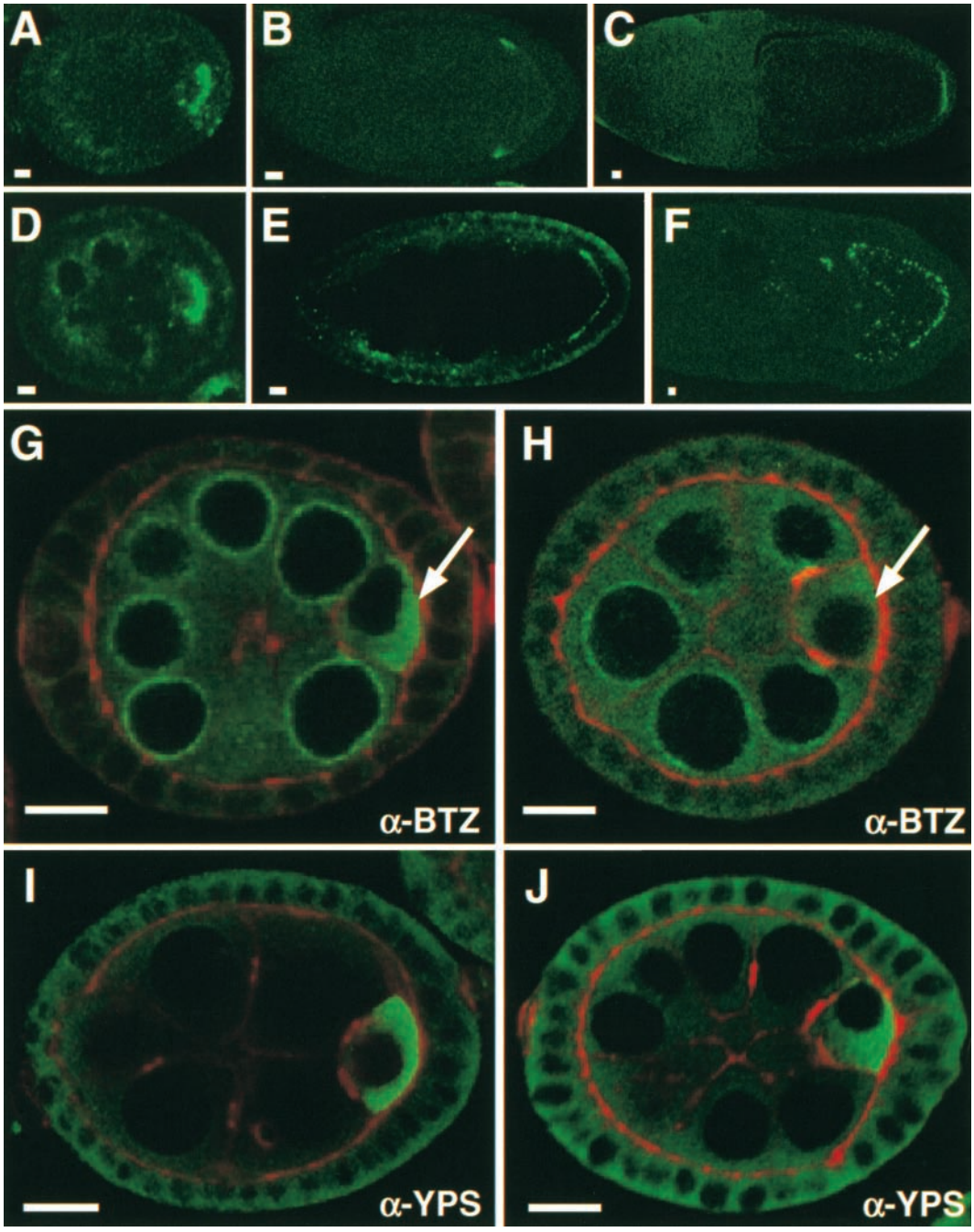

Figure 2. ***Cup* mutations specifically disrupt *oskar* mRNA localization and Btz recruitment.** Localization of *oskar* mRNA in ovaries from *yw* females during stage 4 (A), stage 7 (B), and stage 10 (C). Localization of *oskar* mRNA in cup^{1}/cup^{4506} females during stage 5 (D), stage 8 (E), and stage 9 (F). *oskar* mRNA is distributed along the cortex of the oocyte in stages 8–10 in cup^{1}/cup^{4506} egg chambers. (G) Btz (green) is localized to the nuclear envelope as well as the posterior pole of the oocyte (arrow) in *yw* egg chambers. (H) In cup^{1}/cup^{4506} egg chambers, Btz accumulates at the nuclear envelope, but is only weakly present at the posterior pole of the oocyte (arrow). Yps (green) is localized normally to the posterior pole of the oocyte in both *yw* (I) and cup^{1}/cup^{4506} (J) egg chambers. Actin is in red. Bars, 10 μm.

binds directly to Exu, localizes to these motile particles (Wilhelm et al., 2000). To determine if Cup is also a component of these particles, we immunostained egg chambers for both Cup and Yps. The particulate staining observed for both Cup and Yps in the nurse cells showed a high degree of overlap, indicating that Yps and Cup are part of the same particles in vivo (Fig. 1, F–H). Recently, a novel component of the *oskar* mRNA localization machinery, Btz, was identified that has a staining pattern that is strikingly similar to that of Cup (van Eeden et al., 2001). We immunostained egg chambers for both Cup and Btz to determine if they were also present in the same nurse cell particles. Most cytoplasmic particles contained both Cup and Btz (Fig. 1, I–K). Interestingly, Btz protein that localized tightly to the nuclear rim did not display a large amount of overlap with Cup (Fig. 1 K), indicating that this pool of Btz might be part of a separate complex. Thus, Cup is present in motile RNP particles that contain Btz, a known component of the *oskar* mRNA localization machinery.

Cup is required for *oskar* mRNA localization.

Since Cup colocalizes and copurifies with components of the *oskar* RNP complex, we next asked if Cup plays a role in *oskar* mRNA localization. For this and subsequent experiments, we focused our attention on the heteroallelic combination of cup^{1}/cup^{4506} since the combination of the strong cup^{4506} allele with the intermediate strength cup^{1} allele allowed oogenesis to proceed far enough to assay *oskar* mRNA localization. This allelic combination yielded results that were representative of other heteroallelic combinations (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200309088/DC1) and also allowed us to minimize the effects of secondary mutations since cup^{1} and cup^{4506} were isolated in separate screens. In situ hybridization of *oskar* mRNA in cup^{1}/cup^{4506} egg chambers revealed that although *oskar* mRNA localization is normal in stages 1–7 of oogenesis (Fig. 2, A and B, D and E), during stages 8- 10, *oskar* mRNA is predominantly cortical with some enrichment at the posterior pole (Fig. 2, C and F). This dispersed localization pattern is similar to that observed in weak alleles of *btz* where low levels of *oskar* mRNA are localized to the posterior pole (van Eeden et al., 2001).

Cup is required to recruit the localization factor Btz

Because *btz* mutants display a late stage *oskar* mRNA localization defect similar to that of *cup* mutants (van Eeden et al., 2001), we next examined the effect of *cup* mutants on the distribution of Btz. Normally, Btz protein is present on the nuclear envelope in nurse cells and colocalizes with *oskar* mRNA in the oocyte (Fig. 2 G). However, in cup^{1}/cup^{4506} egg chambers, the accumulation of Btz protein within in the oocyte is greatly reduced from stage 1 onward, whereas the Btz present on the nuclear envelope in the nurse cells is unaffected (Fig. 2 H). The failure in the transport of Btz to the oocyte is not due to a general defect in assembly of the *oskar* RNP since cup^{1}/cup^{4506} egg chambers localize Yps and *oskar* mRNA normally during early oogenesis (Fig. 2, I and J, D and E; Figs. S1 and S2, available at http://www.jcb.org/cgi/content/full/jcb.200309088/DC1). Thus, Cup is specifically required to localize Btz to the oocyte. This result, together with the findings that Cup and Btz colocalize as well as sharing similar *oska*r mRNA localization defects, argues that *cup* mutants fail to localize *oskar* mRNA because Cup is required to recruit Btz to the complex.

Cup is required to maintain translational repression of *oskar* mRNA

Since all mutations isolated to date that disrupt *oskar* mRNA localization also block *oskar* translation, we next examined the role of *cup* in *oskar* translation. To our surprise, Oskar protein accumulated prematurely in the oocyte during stages 6 and 7 in cup^{1}/cup^{4506} egg chambers, indicating that *cup* is required to translationally repress *oskar* mRNA during these stages (Fig. 3, A and B; Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200309088/DC1). It is also worth noting that in *cup* mutants we only observe accumulation of Oskar protein at those sites where *oskar* mRNA is most enriched (Fig. 3 B; Fig. S3). This may be due to the fact that the *cup* alleles used in this study are hypomorphic alleles. The effects of *cup* are specific for *oskar* mRNA since the localized translation of *gurken* mRNA at the dorsal anterior region of the oocyte during stage 9 is unaffected in a cup^{1}/cup^{4506} mutant background (Fig. 3, C and D). Thus, *cup* is not a general translational regulator of localized messages.

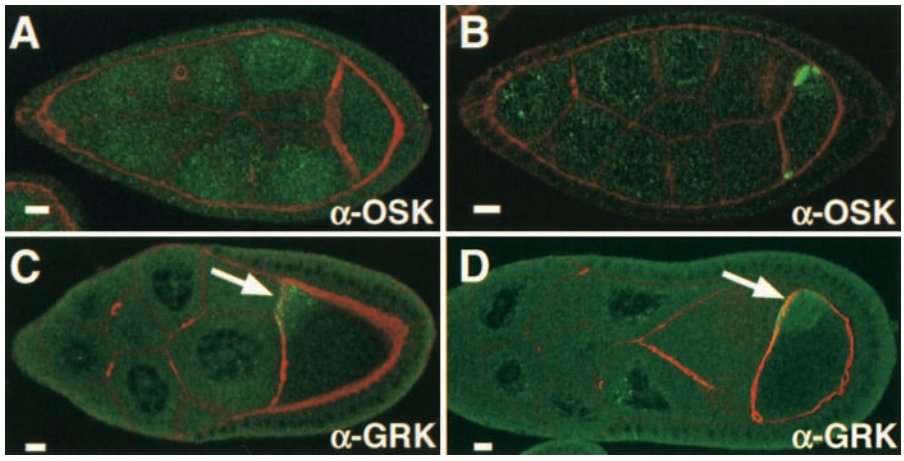

Figure 3. ***Cup* is required for translational repression of *oskar* mRNA.** (A) Oskar protein (green) is not present in stage 7 *yw* egg chambers. Actin is in red. (B) Oskar protein is prematurely translated at the anterior of the oocyte in stage 7 cup^{1}/cup^{4506} egg chambers. The distribution of Gurken protein (arrows) is normal in both *yw* (C) and cup^{1}/cup^{4506} (D) egg chambers. Gurken is green. Actin is red. Bars, 10 μm.

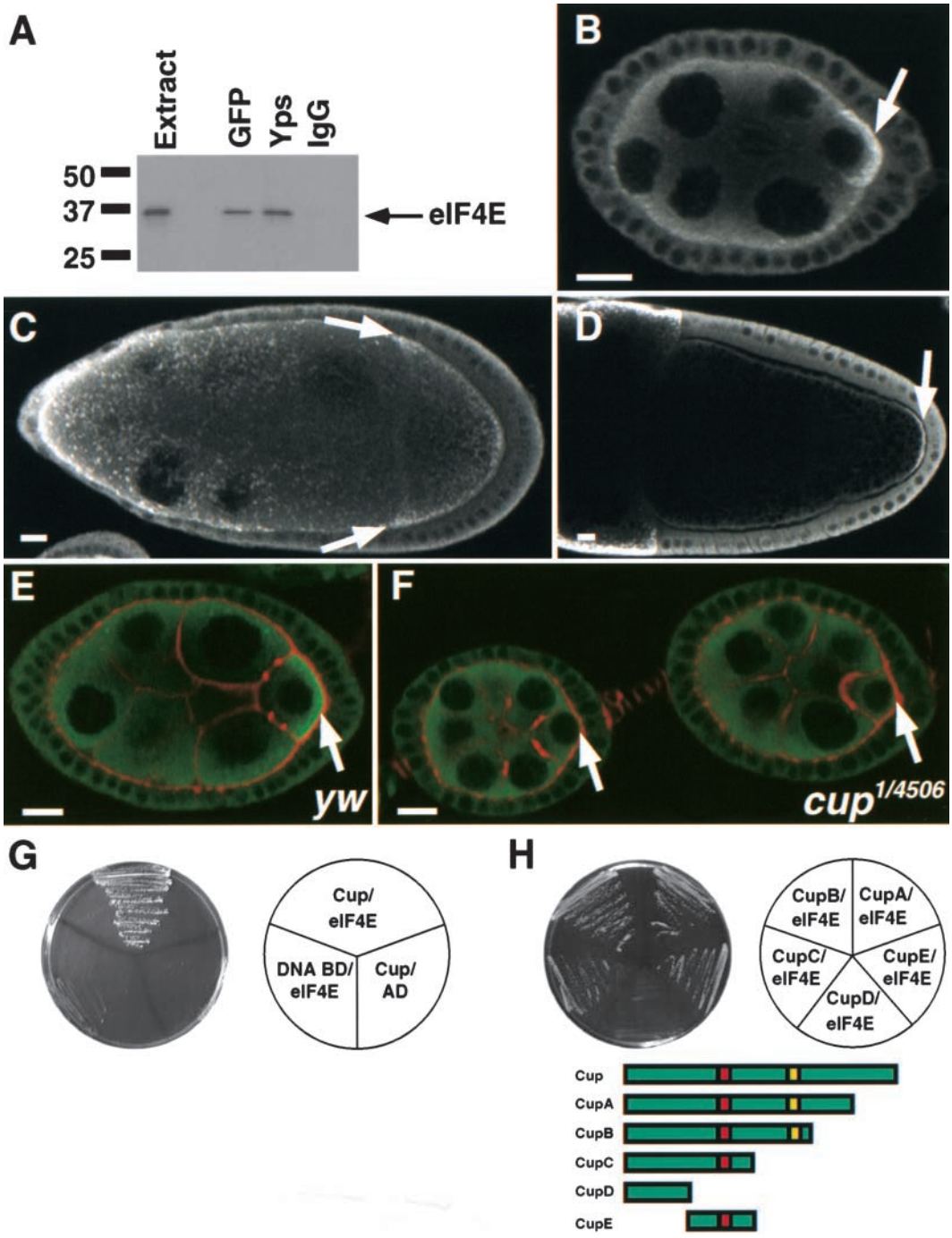

Figure 4. **eIF4E is localized to the posterior pole in a *cup*-dependent manner.** (A) Immunoblot for eIF4E of immunoprecipitates from GFP-Exu extract using α-GFP (GFP), α-Yps (YPS), or rabbit IgG (IgG) antibodies. (B) eIF4E protein (arrows) is concentrated at the posterior of the developing oocyte in stages 1–6 (stage 6 is shown). (C) eIF4E transiently accumulates at the anterior of the oocyte during stages 7 and 8 (late stage 8 is shown). (D) eIF4E then accumulates at the posterior pole during stages 9 and 10 (stage 10 is shown). (E) In *yw* egg chambers, eIF4E protein (arrow) is concentrated at the posterior of the oocyte (stage 6 is shown). (F) In cup^{1}/cup^{4506} egg chambers, eIF4E is distrubuted homogenously thoughout the oocyte and nurse cells and is not localized to the posterior pole (arrows; stages 4 and 6 are shown). (G) Doubly transformed yeast expressing a GAL4 DNA binding domain Cup fusion in combination with either a transcriptional activation domain (AD) fusion to eIF4E or the activation domain alone. All interactions were scored based on growth on his^{-} ade^{-} media. (H) Deletion analysis of Cup to identify regions required for eIF4E binding. The yellow box is the region of homology with 4E-T, a mouse eIF4E binding protein. The red box is the site of the canonical eIF4E binding motif YXXXXLϕ, where X is any amino acid and ϕ is any hydrophobic amino acid. Bars, 10 μm.

eIF4E is localized to the posterior pole in a *cup*-dependent manner

To better understand the role of Cup in maintaining the translational repression of *oskar* mRNA, we first sought to identify components of the translation machinery that were present in the complex by testing likely candidates. Immunoprecipitation of GFP-Exu and Yps showed that eIF4E, the 5′ cap binding component of the translation initiation

complex, is specifically associated with these components of the *oskar* RNP complex (Fig. 4 A). eIF4E and other components of the translation initiation machinery are generally thought of as being homogenously distributed due to their critical role in translation throughout the cell. Surprisingly, we found that eIF4E is localized in a dynamic pattern within the oocyte. eIF4E is localized to the posterior of the oocyte early in oogenesis during stages 1–6 (Fig. 4 B). At stages 7 and 8, eIF4E redistributed to the anterior of the oocyte (Fig. 4 C), and during stages 9 and 10, eIF4E accumulated at the posterior of the oocyte (Fig. 4 D). This pattern of localization was also observed with a GFP-eIF4E protein trap line (unpublished data). Thus, eIF4E localizes in a temporal–spatial pattern identical to that of Cup, suggesting that it is a component of the complex in vivo.

Since Cup is required for the correct localization of Btz to the oocyte, we next investigated whether Cup is required for eIF4E localization. Immunostaining of cup^{1}/cup^{4506} mutant egg chambers revealed that Cup is required for localization of eIF4E to the posterior of the oocyte from stage 1 onward (Fig. 4, E and F). Disruption of *cup* function did not significantly affect the level of unlocalized eIF4E (Fig. 4, E and F), indicating that the defect is primarily in the recruitment of eIF4E to the complex.

Because Cup shares limited homology with 4E-T, a known eIF4E binding protein and a translational repressor in mammals (Dostie et al., 2000), we tested whether Cup binds to eIF4E using a two-hybrid interaction assay. This assay showed a direct interaction between Cup and eIF4E (Fig. 4 G). Cup interacted equally with both isoforms of eIF4E (unpublished data). Deletion analysis of Cup using the two-hybrid assay identified an eIF4E interaction domain that contains a canonical eIF4E binding motif (Fig. 4 H). This motif is found in eIF4G as well as translational repressors (e.g., 4E-T) that block translation by preventing the eIF4E–eIF4G interaction (Mader et al., 1995). Thus, Cup is an eIF4E binding protein that acts directly to repress *oskar* translation.

Although mRNA localization in *Drosophila* has been the subject of extensive genetic analysis, only a few attempts have been made to characterize biochemically the proteins associated with localized messages. In this study, we have biochemically identified Cup as a novel component of the *oskar* RNP complex. This assignment is based on a number of findings. First, Cup copurifies with both Exu and Yps, which have both been shown to be in a biochemical complex with *oskar* mRNA. Second, Cup protein exhibits the same dynamic localization pattern as that seen for *oskar* mRNA as well as other components of the complex. Third, Cup colocalizes with Yps and Btz particles, indicating that this these proteins form a complex in vivo. Finally, the relevance of the biochemical association is supported by genetic studies of *cup* function, demonstrating a role for *cup* in translational repression of *oskar* mRNA as well as recruitment of Btz and eIF4E to the RNP complex.

A model for coupling *oskar* localization to translational derepression

Because Cup is a translational repressor that is also required to assemble the *oskar* mRNA localization machinery, we propose that the coupling between localization and translation occurs by regulating these two functions of Cup. In this model, Cup is required early in the assembly of the transport complex in order to recruit components, such as Btz, that will later be used to dock to kinesin (Fig. 5 A). This is consistent with our results that *cup* is required to localize Btz to the posterior pole and that *cup* mutants exhibit *oskar* mRNA localization defects comparable to those observed in *btz* mutants. The fact that mammalian Btz and 4E-T are nucleocytoplasmic shuttling proteins suggests that the defect in particle assembly in *cup* mutants may occur in the nucleus rather than in the cytoplasm (Dostie et al., 2000; Macchi et al., 2003). However, further studies will be necessary to determine the site of assembly.

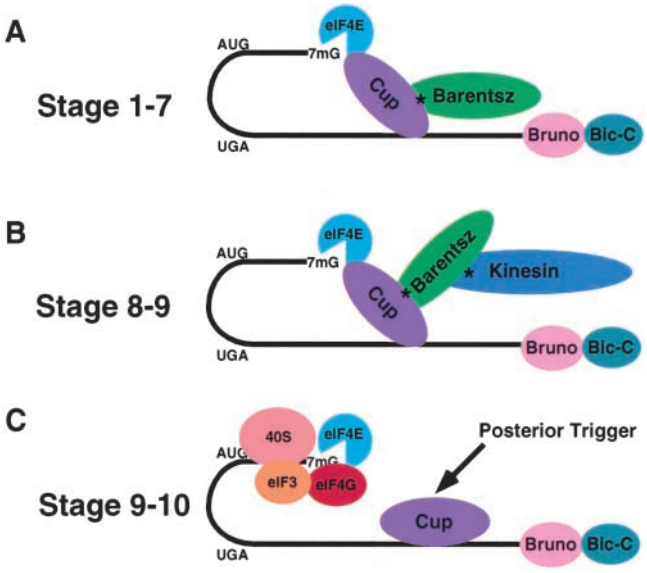

Figure 5. **A model for coupling oskar mRNA localization and translational activation via Cup.** (A) During stages 1–7, Cup is required to recruit plus end–directed transport factors, such as Btz. (B) During stages 8 and 9, the *oskar* RNP rearranges so that Btz can recruit kinesin. (C) During stages 9 and 10, *oskar* mRNA localizes to the posterior pole and is anchored there. This anchoring event or a posterior localized signal acts on Cup to cause partial disassembly of the complex and breaks the interaction between Cup and eIF4E allowing translation. Asterisks (*) mark interactions that may not be direct.

Because Btz is normally part of the transport complex throughout oogenesis even though it is only required for the kinesin-mediated transport step during stages 9 and 10 (van Eeden et al., 2001), we further propose that the complex undergoes rearrangement in order to activate Btz and switch from minus end–directed transport to kinesin-mediated transport (Fig. 5 B). Since we have yet to establish the direct binding of Cup to Btz or Btz to kinesin it is unclear how many components of the complex may be involved in this reorganization.

Once the complex reaches the posterior pole, we argue that the localization machinery is disassembled and the interaction between Cup and eIF4E is broken to allow translational activation (Fig. 5 C). Because Cup is stably maintained at the posterior pole after stage 9, whereas Btz is not (this study; van Eeden et al., 2001), we propose that the trigger that disrupts the binding of Cup to eIF4E also leads to partial disassembly of the localization machinery via Cup.

The molecular trigger for such rearrangements is unknown, however, the ability of 4E-T to bind eIF4E is regulated by phosophorylation (Pyronnet et al., 2001). Studies directed at identifying regulators of the Cup–eIF4E interaction might lead to greater mechanistic insights into the coupling mechanism.

One of the attractive features of this model is that it suggests how coupling might be accomplished in other systems. Recent work in neurons on the translational regulator CPEB suggests that it can promote the transport of mRNA into dendrites (Huang et al., 2003). Since CPEB represses translation by recruiting the eIF4E binding protein, maskin, to transcripts (Stebbins-Boaz et al., 1999), it is possible that the observed transport effect is due to a requirement for maskin to assemble the localization machinery. Thus, Cup may be representative of a general class of eIF4E binding proteins whose role is to couple mRNA localization to translational activation.

Materials and methods

Drosophila strains and culture

Fly stocks were cultured at 22–25°C on standard food. The cup^{1}, cup^{4}, cup^{1355}, and cup^{4506} alleles have been previously described (Schupbach and Wieschaus, 1991; Keyes and Spradling, 1997). The y^{1} w^{67c23} strain is described in FlyBase.

Extract preparation, immunoblots, and immunoprecipitations

All protein work was performed as previously described (Wilhelm et al., 2000). For immunoblot analysis, primary antibodies were used at a 1:1,000 dilution of α-Cup rat antibody (Keyes and Spradling, 1997) or 1:1,000 α-eIF4E rabbit antibody (a gift from P. Lasko, McGill University, Montréal, Canada).

Identification of p147

p147 was resolved by SDS-PAGE and mass spectrometry performed as described (Wang et al., 1999).

Immunostaining and fluorescence microscopy

Immunostaining and microscopy was performed as previously described (Cox and Spradling, 2003) with the following modifications: the washes immediately following fixation consisted of PBT (1 × PBS, 0.2% Triton X-100). All subsequent washes or incubations were done in PBT + 5% BSA; primary antibodies were diluted in PBT + 5% BSA as follows: rat α-Cup 1:1,000 (Keyes and Spradling, 1997), rabbit α-Osk 1:3,000 (a gift from A. Ephrussi, European Molecular Biology Laboratory, Heidelberg, Germany), rabbit α-Btz 1:1,000 (van Eeden et al., 2001), rabbit α-Yps 1:1,000 (Wilhelm et al., 2000), 1:1 mouse α-Grk (1D12, Developmental Studies Hybridoma Bank), rabbit α-eIF4E 1:1,000 (a gift from P. Lasko). The following secondary antibodies were used: goat α-rabbit and α-rat AlexaFluor488 (1:200) and goat α-rat AlexaFluor568 (1:200). Samples were mounted in Vectashield. Confocal analysis was performed using the PL APO40X 1.25NA and 100X 1.40NA objectives on the Leica TCS NT confocal microscope at 25°C.

In situ hybridization

In situ hybridization and detection were performed as described (Wilkie et al., 1999).

Two-hybrid analysis of cup and eIF4E

The Rf cassette (Invitrogen) was inserted into the two-hybrid vectors, pGADT7 and pGBKT7 (CLONTECH Laboratories, Inc.), to facilitate cloning via the Gateway cloning system (Invitrogen). The following deletion constructs were generated by PCR and were cloned into into the appropriate vector for analysis: CupA 1–912 aa, CupB 1–652 aa, CupC 1–457 aa, CupD 1–233 aa, CupE 233–457 aa. Transformants were tested for positive interactions based on their ability to grow on leu^{-} trp^{-} his^{-} ade^{-} plates as described in the protocols for the Clontech matchmaker system (CLONTECH Laboratories, Inc.). The expression of all constructs was confirmed by immunoblot of yeast lysate with either α-myc (9E10) or α-HA (12CA5) antibodies.

Online supplemental material

Online supplemental figures are available at http://www.jcb.org/cgi/content/full/jcb.200309088/DC1. Fig. S1 shows the effect of other heteroallelic combinations of *cup* on *oskar* mRNA localization and localization of Btz. Fig. S2 shows the localization of Yps and eIF4E in a variety of stages of cup^{1}/cup^{45066} egg chambers. Fig. S3 shows *oskar* derepression in cup^{1}/cup^{45066} egg chambers during stages 6–9.

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