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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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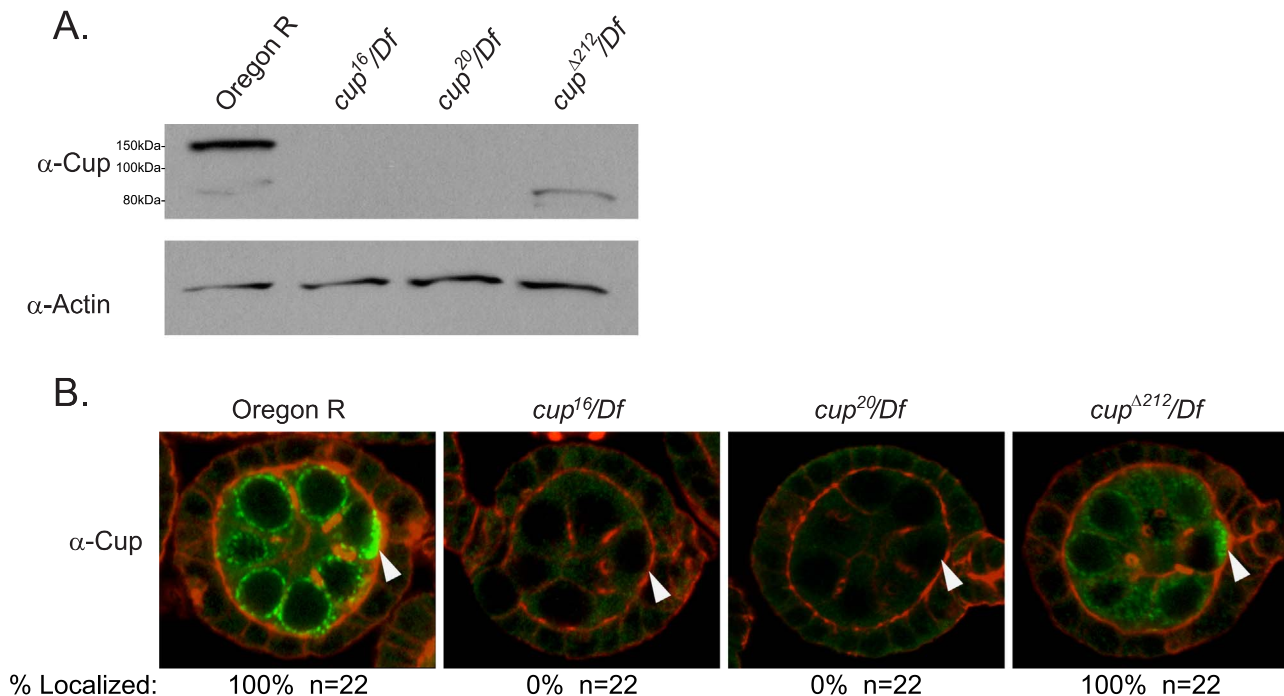

Fig. 1. Strong hypomorphic alleles, *cup*^{16} and *cup*^{20}, are protein null alleles of *cup*. (A). Ovary extract from *cup*^{16}/*Df(2L)bsc7* and *cup*^{20}/*Df(2L)bsc7* lack Cup protein, whereas *cup*$^{\Delta 212}$/*Df(2L)bsc7* mutants produce a decreased level of N-terminally truncated Cup protein 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-

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