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# The translational regulator Cup controls NMJ presynaptic terminal morphology

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ABSTRACT

During oogenesis and early embryonic development in *Drosophila*, translation of proteins from maternally deposited mRNAs is tightly controlled. We and others have previously shown that translational regulatory proteins that function during oogenesis also have essential roles in the nervous system. Here we examine the role of Cup in neuromuscular system development. Maternal Cup controls translation of localized mRNAs encoding the Oskar and Nanos proteins and binds to the general translation initiation factor eIF4E. In this paper, we show that zygotic Cup protein is localized to presynaptic terminals at larval neuromuscular junctions (NMJs). *cup* mutant NMJs have strong phenotypes characterized by the presence of small clustered boutons called satellite boutons. They also exhibit an increase in the frequency of spontaneous glutamate release events (mEPSPs). Reduction of eIF4E expression synergizes with partial loss of Cup expression to produce satellite bouton phenotypes. The presence of satellite boutons is often associated with increases in retrograde bone morphogenetic protein (BMP) signaling, and we show that synaptic BMP signaling is elevated in *cup* mutants. *cup* genetically interacts with two genes, *EndoA* and *Dap160*, that encode proteins involved in endocytosis that are also neuronal modulators of the BMP pathway. Endophilin protein, encoded by the *EndoA* gene, is downregulated in a *cup* mutant. Our results are consistent with a model in which Cup and eIF4E work together to ensure efficient localization and translation of endocytosis proteins in motor neurons and control the strength of the retrograde BMP signal.


## 1. Introduction

Most key developmental regulatory proteins are regulated by multiple mechanisms. These include transcriptional regulation, mRNA localization, translational control, protein localization, and protein lifetime. In *Drosophila*, many proteins involved in oogenesis and early development are translated from maternal mRNAs. Because these mRNAs are deposited in the egg, their translation must be precisely regulated in order to allow production of their protein products at the appropriate times and places. Most genes encoding maternally expressed regulators are also zygotically transcribed later in development, and some of these genes function during nervous system development. The translational control mechanisms used to regulate expression and localization of these maternal gene products are also operative in the nervous system. However, translational regulatory circuits in neurons can have different organizations from those that operate during early development.

Pumilio (Pum) and Nanos (Nos) are key maternal translational regulators. They form a complex that binds to the 3′ UTR of *hunchback (hb)* mRNA and represses its translation in the posterior part of the embryo (Sonoda and Wharton, 1999). We and others have shown that zygotic Pum and Nos are also required for neural development and function (Baines, 2005; Menon et al., 2009; Menon et al., 2004; Muraro et al., 2008; Ye et al., 2004). Pum, Nos, and the general translational initiation factor eIF4E are components of a regulatory circuit in the neuromuscular system that controls postsynaptic translation of glutamate receptor (GluR) mRNAs. In postsynaptic muscles, Pum binds to the 3′ UTRs of the *eIF4E* (Menon et al., 2004), *GluRIIA*, and *nos* mRNAs (Menon et al., 2009) and represses their translation. Postsynaptic Nos represses expression of the alternate GluR subunit, GluRIIB, by an unknown mechanism that is not dependent on Pum (Menon et al., 2009). In neurons, the Pum/Nos complex binds to and represses translation of *paralytic (para)* mRNA, which encodes a voltage-gated sodium channel (Muraro et al., 2008). Pum and Nos are also required for normal development of neuromuscular junction (NMJ) presynaptic terminals (Menon et al., 2009; Menon et al., 2004), and they regulate branching of the dendrites of peripheral sensory neurons (Ye et al., 2004).

Since Pum and Nos function in the nervous system, we wished to investigate molecules that interact with these translational regulators during oogenesis or early embryonic development and define their roles at the larval NMJ. In this paper, we examine the zygotic functions of Cup, which is a maternal regulator of *nos* mRNA translation in oocytes. Cup also binds to eIF4E (Nakamura et al., 2004; Nelson et al.,

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2004; Wilhelm et al., 2003; Zappavigna et al., 2004), and eIF4E expression is controlled by Pum in the neuromuscular system (Menon et al., 2004). Thus, we were interested in determining whether Cup is also important for neuromuscular system development.

Cup is encoded by the female-sterile gene *fs(2)cup*, which is required for production of functional oocytes (Keyes and Spradling, 1997; Schupbach and Wieschaus, 1991). Cup's major role during oogenesis is to regulate translation of mRNAs that are localized to specific domains within the oocyte. *oskar (osk)* mRNA is localized to the posterior pole of the oocyte and is required for the establishment of the germ line and for posterior patterning (Ephrussi et al., 1991). Cup is required for *osk* mRNA localization, and it represses translation of *osk* mRNA until it reaches its final location. Translation of *osk* mRNA is prematurely derepressed in *cup* mutants, resulting in the expression of Osk protein at the wrong pole of the oocyte (Wilhelm et al., 2003). Cup itself does not bind to mRNAs, but engages with *osk* mRNA by forming a complex with Bruno, a sequence-specific RNA-binding protein (Nakamura et al., 2004). Cup also represses translation of *nos* mRNA that is not localized at the posterior pole of the embryo. It engages with *nos* mRNA through its interactions with Smaug, another sequence-specific RNA-binding protein (Nelson et al., 2004). Cup was also identified as a binding partner of Nos in a yeast two-hybrid screen (Verrotti and Wharton, 2000).

Cup represses translation through a variety of mechanisms. One proposed mechanism is to obstruct the formation of the elongation initiation factor 4F (eIF4F) complex. The eIF4F complex includes an RNA helicase, eIF4A, a scaffolding protein, eIF4G, and the cap-binding protein, eIF4E. eIF4E is the target for translational repressors known as eIF4E-binding proteins (4E-BPs). By competing with eIF4G for binding to eIF4E, 4E-BPs inhibit the recruitment of the 43S preinitiation complex and block translation (Wilhelm and Smibert, 2005). Cup is a 4E-BP, and contains two eIF4E-binding motifs located within its N-terminal domain (Nakamura et al., 2004; Nelson et al., 2004; Wilhelm et al., 2003; Zappavigna et al., 2004). In addition to binding each other directly in vitro and in cell culture assays, *eIF4E* and *cup* genetically interact to regulate ovary development. Cup is also required for localizing eIF4E to the posterior pole in developing oocytes (Zappavigna et al., 2004).

Since Nos and Cup interact and function together in the germline, and Cup regulates *nos* mRNA translation, we anticipated that if zygotically expressed Cup has a function at the NMJ, zygotic *cup* mutants might have phenotypes that resembled either loss-of-function (LOF) or gain-of-function (GOF) *nos* phenotypes (Menon et al., 2009). In the present study, we show that Cup is indeed expressed by motor neurons and localized to NMJ presynaptic terminals. However, we found that the *cup* zygotic phenotype is quite distinct from those of *nos* LOF or GOF mutants. There was also no obvious change in Nos protein levels in *cup* mutants.

Given these results, we decided to perform a broader analysis of the functions of Cup and eIF4E at the NMJ. We find that *cup* mutant NMJs contain many small, clustered boutons. These are known as satellites (reviewed by Menon et al., 2013; O'Connor-Giles and Ganetzky, 2008; Oh and Robinson, 2012). The frequency of spontaneous glutamate release events (minis) is altered in *cup* mutants. *cup* and *eIF4E* genetically interact, as in the ovary (Zappavigna et al., 2004), and the nature of these interactions suggest that Cup is a positive regulator of eIF4E function in motor neurons. We also show that, like other genes for which mutations produce satellite bouton phenotypes, Cup is a negative regulator of the retrograde Bone Morphogenetic Protein (BMP) signaling pathway, in which Glass Bottom Boat (Gbb), a BMP ligand secreted by the muscle, activates BMP receptors on the presynaptic terminal and thereby increases expression of genes required for synaptic growth (Marques and Zhang, 2006). Cup appears to indirectly affect BMP signaling via positive regulation of endocytic proteins that are neuronal modulators of the pathway.

Our data are consistent with a model in which Cup and eIF4E facilitate localization and translation of endocytic proteins at the proper time(s) and place(s). Cup is known as a translational repressor and competes with eIF4G for binding to eIF4E (Nelson et al., 2004). However, Cup's binding to eIF4E is not necessarily a repressive mechanism. Based on studies in cultured cells, it was recently proposed that eIF4E binding to Cup tethers eIF4E to mRNAs destined for specific locations, so that translation can begin promptly when Cup dissociates from the mRNA complex (Igreja and Izaurralde, 2011). In this way, Cup can both repress translation of mRNAs via a non-eIF4E-dependent mechanism, and positively control translation by facilitating rapid assembly of the eIF4F complex when repression is relieved. We suggest that the second mechanism operates in motor neurons. It is consistent with our findings that Cup is a positive regulator of direct and indirect modulators of the BMP signaling pathway and works in concert with eIF4E to facilitate normal development and function of NMJ presynaptic terminals.

## 2. Results

### 2.1. Cup protein localizes to NMJ presynaptic terminals

To evaluate Cup's functions during NMJ development, we first determined whether Cup is expressed in the nervous system. Third-instar larvae were dissected and stained with antibodies against Cup and visualized by confocal microscopy. Three polyclonal antibodies made against different regions of the protein by different research groups were tested for immunoreactivity. Rabbit and mouse anti-Cup antibodies were made against amino acids 595–975 of Cup, while the rat anti-Cup antibody was generated against amino acids 201–499 (Keyes and Spradling, 1997; Nakamura et al., 2004). We found that all three antibodies stained the larval NMJ (Figs. 1 and S1). The postsynaptic marker Discs-Large (Dlg) was used to outline the boutons (Fig. 1A2). Rabbit (Fig. 1A1) and rat and mouse (Fig. S1) Cup antibodies all showed punctate Cup staining within the presynaptic bouton and to a lesser extent on the postsynaptic muscle membrane. In addition to staining at the NMJ, Cup is also found in the cytoplasm of neuronal cell bodies in the ventral nerve cord (VNC) (Figs. 1A4 and S1). These cells were identified as neurons using antibodies against horseradish peroxidase (HRP), which selectively stain the surfaces of insect neurons and are commonly used as neuronal markers.

Presynaptic localization of Cup at the NMJ was confirmed by double staining with antibodies for the synaptic markers Fasciclin 2 (Fas2), a cell adhesion molecule in the periactive zone, and Bruchpilot (Brp), an active zone protein (Fig. 1B and C, respectively). Anti-Fas2 stains in a network-like pattern within the presynaptic bouton, and also stains postsynaptically around the bouton (Fig. 1B2). Brp shows punctate staining marking active zones (Fig. 1C2). Cup puncta inside presynaptic boutons show partial overlap with both Fas2 and Brp (insets in B3 and C3). Cup is enriched in the type 1b boutons and localizes faintly at type 1 s boutons (asterisk in Fig. S1). Anti-Cup does not stain the axon trunks.

To confirm that the presynaptic staining we observed is due to Cup protein, we next examined Cup immunoreactivity at NMJs in *cup* mutants and in larvae where Cup was knocked down using transgenic RNAi. The *cup* alleles used in this study are $cup^{\Delta 212}$ (Nakamura et al., 2004), a P-element imprecise excision line that produces an N-terminally truncated Cup protein, two ethyl methane sulfonate (EMS)-induced alleles ($cup^{20}$ and $cup^{8}$), and two deficiencies that remove *cup*, *Df(2L)BSC7* (26D10-27C1) and *Df(2L)BSC187* (26F3-27A1). The two EMS-induced mutations are not protein nulls, and their sequences are not known (Keyes and Spradling, 1997). During oogenesis, $cup^{20}$ and $cup^{8}$ have stronger defects in egg chamber development than does $cup^{\Delta 212}$ (Nakamura et al., 2004).

We observed a decrease in Cup protein levels at the NMJ relative to wild-type, as visualized with the rabbit antibody, in $cup^{20}$/*Df(2L)BSC7* transheterozygotes (Fig. S2). Wild-type and *cup* NMJs exhibited similar staining intensities with anti-HRP (Fig. S2). The residual Cup antibody immunoreactivity seen in the mutant is likely due to expression of