The Dissertation Committee for Goheun Kim Certifies that this is the approved version of the following dissertation:

Investigating the Role of Bruno Interactions with *oskar* Regulatory Proteins

Committee:

Paul Macdonald, Supervisor

Karen Browning

Janice Fischer

David Stein

Scott Stevens

Investigating the Role of Bruno Interactions with *oskar* Regulatory Proteins

by

Goheun Kim, B.S.

Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas at Austin

August 2014

Dedication

To my grandparents

Acknowledgements

First, I would like to thank my supervisor Dr. Paul M. Macdonald for giving me the opportunity to work in his lab, for sharing his expertise in research, for inspiring me to be a good scientist, and for putting up with me throughout my graduate career. I would also like to thank the members of my committee: Dr. Karen Browning, Dr. Janice Fischer, Dr. David Stein, and Dr. Scott Stevens, for their valuable comments, advice and their time over the years. I also thank the past and present members of the Macdonald lab, not only for their help and discussions on my research, but also for their friendship and moral support. Thanks to my good friends in the graduate school, especially those that I have made in recent years and who accompanied me through this long tunnel, sharing our stories. Thanks also to my dear friends at the University Catholic Center – I could not have done this without your spiritual support and guidance. Finally, I would like to thank my family who have loved and cared for me all the while and for the wonderful opportunities my parents offered me throughout my life. I thank and give praise to God for surrounding me with good people and gifts in this world and for the beauty of science.

Investigating the Role of Bruno Interactions with *oskar* Regulatory Proteins

Goheun Kim, Ph.D

The University of Texas at Austin, 2014

Supervisor: Paul M. Macdonald

Oskar (Osk) is a posterior body-patterning determinant in Drosophila melanogaster and is highly concentrated at the posterior pole of the oocyte. *osk mRNA* is translationally repressed until it reaches the posterior of the oocyte where Osk protein is made. Bruno (Bru) represses translation during *osk* mRNA localization by direct binding, but how Bru-mediated repression is relieved at the posterior of the oocyte is unknown. Two types of Bru protein interactions are implicated in repression of *osk*: Bru-Cup interaction and Bru dimerization. By mapping the Bru domains that are important for these interactions, I found that the amino-terminal domain of Bru contributes to both interactions, and deletion of this domain caused a defect in translational repression. However point mutations, within the amino-terminal domain, that disrupt both types of interaction *in vitro* did not interfere with translational repression *in vivo*. The difference may be due to other factors stabilizing the Bru-Cup interaction *in vivo*, as the mutant Bru still associates with Cup *in vivo*. My work supports the model of repression that relies on Bru interaction with Cup. I also build a new model in which Bru dimerization promotes translational activation of *osk*, based on my unexpected results:

dimerization-defective Bru only weakly accumulated Osk::GFP fusion protein encoded by an *osk::GFP* reporter RNA bearing a Bru-binding region, while dimerization-competent Bru showed the opposite effect. This suggests that dimerization may contribute to switching Bru from a repressor to an activator, with dimerization controlled *via* a post-translational modification. Consistent with this, I found that a small fraction of Bru in ovaries is phosphorylated. PKA is a positive regulator of *osk* expression and phosphorylates Bru *in vitro*. To test if PKA regulation of *osk* is mediated through Bru, I examined the effect of altering PKA activity on Bru phosphorylation and Bru-mediated repression. Modulating PKA activity caused small, yet detectable changes in Bru phosphorylation and Bru-dependent translational repression using an *osk::GFP* reporter. However, while the studies with Bru mutants suggest that phosphorylation promotes repression by Bru, these studies argue for a role in promoting activation. Further work will be required to explain these phenomena.

Table of Contents

List of Tables

List of Figures

Chapter 1: General Introduction

AXIAL PATTERNING IN EARLY DROSOPHILA DEVELOPMENT

Establishment and maintenance of cell polarity can be achieved by asymmetric accumulation of proteins to distinct subcellular compartments. Both mRNA localization and regulated translation during transport are mechanisms used to restrict gene expression in space and time. Embryonic axis formation in many vertebrate and invertebrate organisms is often achieved by actions of maternal RNA determinants that are localized to distinct compartments within eggs or oocytes (Kumano, 2011; Medioni et al., 2012).

In Drosophila, localized RNAs specify patterning along both anteroposterior and dorsoventral axes (Fig 1.1). The first axis to be specified is the anteroposterior axis, which is first established by localization of the *gurken* (*grk*) mRNA to the posterior pole of the oocyte during early oogenesis (González-Reyes et al., 1995; Roth et al., 1995). The *grk* mRNA encodes a transforming growth factor (TGF)-α homologue. Upon Grk signaling-dependent microtubule rearrangement, localization of the *bicoid* (*bcd*) mRNA to the anterior (Berleth et al., 1988) and the *oskar* (*osk*) and *nanos* (*nos*) mRNAs to the posterior (Ephrussi et al., 1991; Kim-Ha et al., 1991; Gavis and Lehmann, 1992) of the oocyte establish the anteroposterior axis. The *bcd* and *nos* mRNAs are translationally repressed in late-stage oocytes, and this repression is relieved upon fertilization. Bcd and Nos proteins are produced in opposing gradients in embryos to direct formation of head and thorax (Bcd; Driever and Nüsslein-Volhard, 1988), and abdomen (Nos; Lehmann and Nüsslein-Volhard, 1991). Following specification of the anteroposterior axis, the *grk* mRNA moves to the anterodorsal corner of the

growing oocyte to specify the dorsoventral axis (Neuman-Silberberg and Schüpbach, 1993).

The *osk* mRNA is translated at the posterior pole of the oocyte during mid oogenesis to initiate assembly of the germ/pole plasm, a specialized cytoplasm containing RNAs and proteins required for abdominal patterning and germ/pole cell formation (Ephrussi and Lehmann, 1992; Kim-Ha et al., 1995; Markussen et al., 1995). Embryos lacking maternal Osk activity fail to form both abdomen and germ cells (Lehmann and Nüsslein-Volhard, 1986). Moreover, overexpression or misexpression of Osk at the anterior leads to ectopic abdomen and germ cells (Ephrussi and Lehmann, 1992; Smith et al., 1992). Thus *osk* must be tightly regulated for the viability of the embryo.

The dorsoventral axis is established by a signaling pathway mediated by uniformly distributed Toll receptors, which are activated only on the ventral side of the embryo by a ventrally restricted serine protease cascade (Roth, 1994; Stein, 1995). Grk signaling in the anterodorsal corner of the oocyte restricts the expression of the *pipe* mRNA to the ventral cells of the follicular epithelium that surrounds the oocyte (Sen et al. 1998). Pipe generates a ventral cue that locally activates the serine protease cascade to generate the active ligand for Toll (Zhang et al., 2009; Cho et al., 2010).

DROSOPHILA OOGENESIS

Oogenesis is development of the egg in female ovaries and is arbitrarily divided into 14 morphologically distinct stages in Drosophila (Fig 1.2; Spradling et al., 1993). The egg chamber is the functional unit of oogenesis and is produced

in the germarium containing germline and somatic stem cells. At the anterior tip of the germarium, the germline stem cell divides to give a new stem cell and a cystoblast. Cystoblasts in turn divide four times, with incomplete cytokinesis, to give rise to a cyst of 16 cells that are interconnected by cytoplasmic bridges called ring canals. The nurse cells produce macromolecules and organelles, which are transported to the developing oocyte through the ring canals to aid oocyte maturation, while the oocyte nucleus remains largely quiescent (Spradling et al., 1993; de Cuevas et al., 1997). Toward the end of oogenesis, the nurse cells degenerate and expel their bulk cytoplasm into the oocyte ("nurse cell dumping"). The 16-cell cyst is surrounded by a single, epithelial layer of somatic follicle cells, which secrete the eggshell and play crucial roles in signaling pathways that underlie oocyte polarity. The egg chambers mature in an ovariole, in which progressively later stages of egg chambers are found toward the posterior. A female Drosophila has two ovaries, each of which holds approximately 18 ovarioles (Riechmann and Ephrussi, 2001; Bastock and St. Johnston, 2008).

Maternal RNAs and proteins are stored in the oocyte to govern early embryonic development (Davidson, 1986). They are transported from the nurse cells to the oocyte in a microtubule-dependent manner (Pokrywka and Stephenson, 1995), but almost all components of the cytoplasm are also transported by a microfilament-dependent process during nurse cell dumping. Asymmetric distribution of several maternal RNAs in the oocyte depends on microtubules. (Riechmann and Ephrussi, 2001; Kugler and Lasko, 2009, Gaspar, 2011).

mRNA LOCALIZATION COUPLED WITH TRANSLATIONAL REGULATION

mRNA localization and translational regulation are two mechanisms to achieve asymmetric protein accumulation in polarized cells. These processes occur in situations that require spatial and temporal regulation of gene expression, such as asymmetric cell division, cell fate specification, cell motility, embryonic axis formation, and synaptic plasticity (Johnstone and Lasko, 2001; Condeelis and Singer, 2005; King et al., 2005; Martin and Zukin, 2006; Paquin and Chartrand, 2008). The two processes are generally thought to be coupled by assembling ribonucleoprotein (RNP) complexes containing RNAs and associated *trans*-acting factors that function in localization and/or translational regulation. Transport RNP complexes are usually considered to form co-transcriptionally and undergo dynamic remodeling during transport by numerous protein rearrangements, to dictate the fate of the RNP at each step of its maturation (Lewis and Mowry, 2007; Besse and Ephrussi, 2008). RNPs form by binding of *trans*-acting factors (proteins, as well as miRNAs) to *cis*-regulatory elements in RNAs. RNP complexes engage with motors for directed transport along microtubules and microfilaments. Localizing RNAs are often translationally silenced, and their translation is derepressed upon localization or activated in response to a specific signal (Besse and Ephrussi, 2008; Medioni et al., 2012).

The *osk* mRNA is transcribed from the nurse cell nuclei, and many *trans*-acting factors involved in localization and/or translational repression of *osk* are nucleo-cytoplasmic shuttling proteins that may first bind *osk* RNA in the nurse cell nuclei (Kugler and Lasko, 2009; Kato and Nakamura, 2012). Following pre-mRNA processing events, the *osk* mRNA is exported into the nurse cell cytoplasm. In a process dependent on microtubules (Pokrywka and Stephenson,

1995), the *osk* mRNA is transported into the oocyte through the ring canals at stage 1 of oogenesis and remains highly concentrated in the oocyte. After transient concentration at the oocyte anterior at stage 8, *osk* message gets tightly localized to the posterior of the oocyte by stage 9 and remains localized until early embryogenesis (Ephrussi et al., 1991; Kim-Ha et al., 1991). The *osk* mRNA is translated only after it is localized to the posterior pole (Kim-Ha et al., 1995; Markussen et al., 1995; Rongo et al., 1995). Usage of alternative start codons that are in the same reading frame within the coding sequence makes two Osk isoforms, which have distinct functions (Fig 1.3; Markussen et al., 1995). The Long Osk is required for anchoring of the *osk* mRNA and short Osk protein. The Short Osk induces formation of germ plasm containing determinants for both posterior patterning and germ cell formation (Markussen et al., 1995; Vanzo and Ephrussi, 2002).

A large number of *trans*-acting factors that regulate different steps in localization and translational regulation of *osk* have been identified. Some of these factors are involved in both localization and translational control, while others involved in only one process nevertheless interact with each other, indicative of coupling of the two processes (Kugler and Lasko, 2009; Kato and Nakamura, 2012). Mechanisms of *osk* localization and translation have been extensively studied, but many aspects of post-transcriptional regulation of *osk* still remain to be elucidated.

OSKAR MRNA TRANSPORT

The *osk* mRNA transport to the posterior of the oocyte requires microtubules (Pokrywka and Stephenson, 1995). Prior to stage 7, a microtubule-organizing center (MTOC) at the oocyte's presumptive posterior nucleates microtubules, which extend to the nurse cells through the ring canals (Theurkauf et al., 1993). After Grk signaling to the posterior follicle cells at stage 7, the microtubule network undergoes a drastic rearrangement. The MTOC at the posterior disassembles, and microtubules are nucleated from anterior and lateral cortices. This creates a decreasing gradient of microtubules from anterior to posterior, with plus ends enriched at the posterior (Theurkauf et al., 1992; González-Reyes et al., 1995; Roth et al., 1995; Cha et al., 2002).

Motor proteins move along microtubules toward either a minus end or plus end. The minus-end-directed microtubule motor, Dynein, is required for *osk* transport into the oocyte but dispensable for posterior localization (Januschke et al., 2002). Kinesin is the plus-end-directed microtubule motor. Kinesin heavy chain (KHC) is the force-producing subunit of the tetrameric Kinesin I, the conventional kinesin (Martin et al., 1999). In the khc^{27} mutant, the *osk* mRNA is transported to the oocyte, but not localized to the posterior (Brendza et al., 2000). From the combined data, *osk* seems to switch from Dynein motor in nurse cells, to Kinesin I in oocyte.

Live imaging of the *osk* mRNA in the oocyte further supports this model. An *osk* mRNA bearing bacteriophage MS2 sites was constructed. The *osk-MS2* RNA was expressed in flies that also express MS2-nls-GFP fusion protein, which tethers to MS2 sites and displays the characteristic *osk* mRNA localization pattern (Zimyanin et al., 2008). GFP/*osk-MS2* particles move fast in all directions

at stage 9, with a small posterior bias due to plus-end-directed movement along weakly polarized microtubules. Consistent with Kinesin-dependent posterior transport, there was a great reduction in fast, directed movement seen in *khc* mutants, but no effect seen in *dynein heavy chain* (*dhc*) mutants (Zimyanin et al., 2008).

Microtubule motor proteins interact with adaptor proteins that recruit cargoes for directed transport. A candidate protein complex that could link mRNAs to motors is the Exon Junction Complex (EJC). The EJC is a multi-protein complex that binds the mRNA upstream of exon-exon junctions concomitant with splicing, and is thought to remain bound to the mRNA until the first round of translation (Tange et al., 2005). The core protein components of the Drosophila EJC are eIF4AIII, Barentz, Mago Nashi, and Y14/Tsunagi, which are all required for *osk* localization to the posterior (van Eeden et al., 2001; Hachet and Ephrussi, 2001; Mohr et al., 2001; Palacios et al., 2004). Consistent with the requirement for splicing in EJC deposition, posterior localization of the *osk* transcript was shown to depend on the presence of the first intron (Hachet and Ephrussi, 2004), in addition to the 3' UTR (Kim-Ha et al., 1993). Splicing of the first intron, in turn, results in formation of a 28nt stem-loop structure termed SOLE (spliced *oskar* localization element), made by joining of the last 18nt of exon1 and first 10nt of exon2, and EJC loading. The structural, rather than sequence, integrity of the SOLE is required for efficient *osk* RNP motility for posterior localization (Ghosh et al., 2012). In summary, splicing in the nurse cell nucleus is required for correct localization of the *osk* mRNA in the oocyte cytoplasm, and the EJC components loaded upon splicing play a pivotal role in *osk* mRNA localization.

The *osk* 3' UTR is also required for correct localization of *osk*, and different elements within the 1043nt 3' UTR mediate distinct steps in localization (Kim-Ha et al., 1993). Many *trans*-acting factors are required for correct localization of *osk*, and some bind directly to the 3' UTR. Hrp48 is a Drosophila hnRNP A/B homolog required for posterior localization of *osk*, which colocalizes with the *osk* transcript throughout oogenesis (Huynh et al., 2004; Yano et al., 2004). Heterogeneous nuclear RNP (hnRNP) proteins are a family of RNA-binding proteins involved in many aspects of RNA regulation, and many are nucleo-cytoplasmic shuttling proteins that first bind RNA in the nucleus (Dreyfuss et al., 2002). Therefore Hrp48 may bind *osk* in the nurse cell nuclei and remain associated until localization, similar to the EJC complex. Hrp48 directly binds *osk* 3' UTR, and three missense alleles disrupt posterior localization without affecting splicing or microtubule polarity (Huynh et al., 2004; Yano et al., 2004). Furthermore, formation of the *osk*-containing particles is disrupted in two of these missense mutants, implicating Hrp48 in *osk* RNP assembly (Huynh et al., 2004; Mhlanga et al., 2009).

Staufen (Stau) is another protein that colocalizes with *osk* mRNA throughout oogenesis and is required for proper localization of *osk* and *bcd* mRNAs (St. Johnston et al., 1991). In *stau* mutants, *osk* mRNA is not properly localized at the posterior (Ephrussi et al., 1991; Kim-Ha et al., 1991), and no Osk protein is made (Kim-Ha et al., 1995; Markussen et al., 1995; Rongo et al., 1995). Stau has 5 double-stranded RNA (dsRNA)-binding domains, four of which bind dsRNA. Stau is thought to bind directly to *osk* mRNA, but biochemical proof has been difficult to obtain because of insolubility of the intact, full-length protein (St. Johnston et al., 1992; Micklem et al., 2000). Stau and *osk* are interdependent for

their localization to the posterior pole (Ferrandon et al., 1994), most likely through interaction of Stau with the *osk* 3' UTR, as the 3' UTR is required for Stau transport into the oocyte (Jenny et al., 2006). A recent genome-wide analysis of the dsRNA-binding activity of Stau revealed that the 3' UTRs of Stau-bound transcripts are highly enriched for three types of secondary structures, two of which are present within the *osk* 3' UTR: Type II and III SRSs (Staufen-recognized structures), which are motifs containing 19 (Type II) or 12 (Type III) contiguous bases that have 4 or 2 unpaired/mismatched bases at maximum, respectively. The *osk* 3' UTR carries four Type II SRSs and one Type III SRS (Laver et al., 2013). Stau is also implicated in translational activation of *osk*, independent of its role in *osk* RNA localization (Kim-Ha et al., 1995; Micklem et al., 2000).

After posterior localization, the *osk* mRNA is anchored at the posterior cortex (Ephrussi et al., 1991; Kim-Ha et al., 1991). Anchoring of the *osk* mRNA depends on the Long Osk protein, which also anchors the Short Osk protein; the Long-Osk-mediated anchoring is essential for efficient germ cell formation (Vanzo and Ephrussi, 2002). Anchoring of the *osk* mRNA also depends on the F-actin cytoskeleton and a number of F-actin associated proteins (Polesello et al., 2002; Babu et al., 2004; McNeil et al., 2009; Suyama et al., 2009). Consistent with this, Osk stimulates actin dynamics at the posterior pole. In *osk* protein-null mutants, cortical actin projections show decrease in number and length (Vanzo et al., 2007). Therefore, *osk* promotes its own localization in a positive feedback loop involving actin dynamics.

Consistent with a role of actin cytoskeleton in *osk* anchoring, Myosin-V (Myo V) is required for posterior localization of *osk*. *didum* encodes the unique

Drosophila class-V unconventional myosin, which is an actin-dependent motor (Bonafé and Sellers, 1998; MacIver et al., 1998). In *didum* mutants, the *osk* mRNA and Osk protein are not tightly anchored at the cortex (Krauss et al., 2009). MyoV interacts with *osk in vivo* and may direct the final short-range actomyosin V-dependent translocation or entrapment, followed by a long-range microtubule-based transport. Interestingly, Myo V interacts with Khc in a yeast-two-hybrid assay, and they genetically interact with each other in an antagonistic manner (Krauss et al., 2009). Therefore, coordination of microtubule- and actin-based motors is essential for correct localization and anchoring of *osk* mRNA at the posterior pole.

To summarize, transport of the *osk* mRNA from the nurse cells to the oocyte and localization to the posterior pole depend on the microtubule cytoskeleton and motors. The first intron and the 3' UTR of *osk* are essential for correct localization, and many proteins bind directly or indirectly near/to these elements to mediate localization. Stau, Hrp48 and the EJC complex disrupt posterior localization without affecting oocyte transport. Other proteins that interact with the dynein-dynactin complex have been implicated in oocyte transport of the *osk* mRNA (Bullock and Ish-Horowicz, 2001; Dienstbier et al., 2009). Therefore the *osk* RNP contains many *trans*-acting factors, of which each mediates a distinct step of localization; and it is plausible to think that the *osk* RNP undergoes remodeling during its lifetime, such as recruitment/displacement or post-translational modifications of factors to orchestrate a complicated localization process.

TRANSLATIONAL REPRESSION OF *OSKAR* DURING TRANSPORT

Only localized *osk* mRNA makes Osk protein, implicating that it is under translational regulation (Kim-Ha et al., 1995; Markussen et al., 1995; Rongo et al., 1995). Bruno (Bru; encoded by the *arrest* gene) was the first protein shown to directly bind and translationally repress *osk* RNA (Kim-Ha et al., 1995). Bru is a nucleo-cytoplasmic shuttling protein and may first bind *osk* in the nurse cell nuclei (Snee et al., 2008). Bru binds the *osk* 3' UTR in two regions: AB close to the coding sequence and C close to the poly(A) tail (Fig 1.3). Each region contains multiple Bru-binding sites, including the Bruno Response Elements (BREs) (Kim-Ha et al., 1995; Reveal et al., 2011). Mutating the BREs reduces Bru binding *in vitro* and leads to precocious expression of Osk *in vivo*, suggesting that Bru binding is essential to prevent premature translation (Kim-Ha et al., 1995). A direct evidence for translational repression comes from *in vitro* assays using cell-free translation systems from ovary extract, which recapitulates Bru- and BRE-dependent repression (Lie and Macdonald, 1999; Castagnetti et al., 2000). Currently, there are two models for how Bru represses Osk translation. In the first model, Bru recruits Cup, an eIF4E-binding protein, blocking initiation of translation (Nakamura et al., 2004). In the second model, Bru oligomerizes multiple *osk* mRNAs into large particles that are inaccessible to the translational machinery (Chekulaeva et al., 2006).

The first model comes from data showing that Bru interacts with Cup (Nakamura et al., 2004), which in turn interacts with eukaryotic translation initiation factor 4E (eIF4E; Wilhelm et al., 2003; Nakamura et al., 2004; Zappavigna et al., 2004). Translational initiation requires interaction of several initiation factors prior to recruitment of the small ribosomal subunit. eIF4E binds

the 5’ cap of the mRNA and the scaffold protein eIF4G, which recruits the 43S preinitiation complex to the mRNA *via* interacting with eIF3 (Gebauer and Hentze, 2004). Cup binds eIF4E on the same surface as eIF4G does, through a conserved 4E-binding motif, and competes with eIF4G (Nakamura et al., 2004; Zappavigna et al., 2004). Thus the eIF4E-eIF4G interaction is crucial for translational initiation and is blocked by Bru-Cup-eIF4E interaction. In *cup* mutants, Osk is prematurely and ectopically translated although the *osk* mRNA is localized to the posterior (Wilhelm et al., 2003; Nakamura et al., 2004).

The second model is built upon data that Bru’s RNA binding promotes formation of the heavy RNP particles, termed silencing particles, that are inaccessible to ribosomes (Chekulaeva et al., 2006). Reporters bearing two copies of the AB region were used in a cell-free translation system, and particles were fractionated by centrifugation in a sucrose density gradient. The heavy (50-80S) silencing particles formed in a BRE-dependent manner and were devoid of ribosomes. Furthermore, *osk* oligomerization required the presence of Bru, and the silencing particles contained Cup and Me31B proteins, both involved together with Bru in translational repression of *osk* (Chekulaeva et al., 2006). Altogether, the data suggest that Bru mediates repression through a mechanism that involves packaging of multiple *osk* transcripts and sequestering them from the translational machinery, thus blocking initiation of translation. A concern about the significance of these results is that the mRNAs used for *in vitro* translation and fractionation experiments do not resemble the native *osk* transcript, consisting of a short coding region (an epitope tag) and two copies of the *osk* 3' UTR AB region (Chekulaeva et al., 2006). The AB region is comprised almost entirely of sequences shown to bind Bru (Kim-Ha et al., 1995; Reveal et al.,

2011). As a consequence, the mRNA in these experiments consists mostly of Bru-binding sites, which may lead to assembly of artificial and non-physiological particles. A direct testing of the role of Bru in *osk* RNP formation has been unsuccessful because strong *arrest* (*aret*) mutants arrest oogenesis early (Schüpbach and Wieschaus, 1991), and the large size of the *aret* locus (>100 kb) creates a significant barrier to making a rescuing transgene.

Other proteins that associate with the Bru-Cup-eIF4E complex are also involved in translational regulation of *osk* (Wilhelm et al., 2000; Nakamura et al., 2001). Maternal Expression at 31B (Me31B) is another component of the *osk* RNP and is required for translational repression of *osk* (Nakamura et al., 2001). In *me31B*-mutant egg chambers, Osk expression is seen ectopically in nurse cells during early stages. Me31B is a DEAD-box RNA helicase (De Valoir et al., 1991), and the yeast homolog of Me31B, Dhh1p, is an mRNA-decapping activator in P bodies (Coller and Parker, 2005). P bodies are large RNPs devoid of ribosomes and are found in eukaryotes from budding yeasts to humans (Eulalio et al., 2007). Although P bodies were originally identified as sites of mRNA decay (Sheth and Parker, 2003), additional studies revealed a broader role in many aspects of RNA regulation, such as mRNA storage and translational repression (Brengues et al., 2005; Bhattacharyya et al., 2006). The components of the P bodies are highly conserved across species and are shared with the sponge bodies of Drosophila (Kato and Nakamura, 2012). GFP-Me31B forms cytoplasmic particles in the nurse cells and oocyte and colocalizes with the *osk* mRNA in sponge bodies (Nakamura et al., 2001). Sponge bodies are amorphous, electron-dense structures that contain tubular ER-like membrane-cisternae (Wilsch-Brauninger et al., 1997) and a large number of post-

transcriptional regulatory factors and localizing mRNAs including *osk* (Snee and Macdonald, 2009). Sponge bodies change their composition rapidly upon entry into the oocyte from the nurse cells (Snee and Macdonald, 2009), suggesting that the *osk* RNP could be a dynamic particle undergoing remodeling of *trans*-acting factors in different regions within the egg chamber.

TRANSLATIONAL ACTIVATION OF *OSKAR* UPON POSTERIOR LOCALIZATION

It still remains unknown how translation of Osk protein is activated once the *osk* transcript reaches the posterior pole of the oocyte. There must be derepression by removal or inactivation of Bru and other repressors. In addition, there may also be activation by recruitment of an activator. Whatever the mechanism, spatial cues likely mediate localized translation since activation is coordinated with localization.

Interestingly, Bru-binding sites are not only required for repression, but also required for activation of translation (Reveal et al., 2010). When only the C-region BREs are mutated, activation of translation is defective. As noted earlier, mutation of both AB- and C-region BREs disrupts repression, and so the C-region BREs have roles in both repression and activation. The fact that mutation of all BREs leads to a loss of repression and excess Osk indicates that the C-region-mediated activation is not required when repression is defective. Since the BREs are *bona fide* Bru binding sites, and mutating Bru binding sites other than the BREs in the C region also leads to similar defects, Bru is the most likely candidate for an activator (Reveal et al., 2010). Since the contribution of the C-region BREs in activation is most pronounced in late-stage oocytes and early

embryos, Bru may switch its function from a repressor to an activator during late oogenesis. How this occurs is not known, but could involve a post-translational modification.

There are additional *cis* elements important for translational activation of *osk*: Imp-binding elements (IBEs) and a 5' activating element (Fig 1.3). IBEs are motifs found in multiple copies within the *osk* 3' UTR and binding sites for the Drosophila homolog of insulin growth factor II mRNA-binding protein (IMP). Mutating subsets of IBEs results in defects in Osk accumulation and posterior patterning (Munro et al., 2006), although *Imp* mutants have no defects in *osk* regulation (Geng and Macdonald, 2006; Munro et al., 2006). The 5' activating element is a poorly characterized region within the coding sequence between the start codons for Long and Short Osk. Inversion of the 3' half within this 130nt region abrogates binding of two ovarian proteins: p50, later identified as Hrp48; and p68, whose identity remains unknown (Gunkel et al., 1998; Yano et al., 2004). The effect of the inversion on expression was monitored using a *lacZ* reporter mRNA, which contains the *osk* 5' region up to the downstream AUG for the Short Osk, fused to the *lacZ* coding sequence, followed by the entire *osk* 3' UTR. In this reporter, the inversion mutation prevents posterior accumulation of the β-galactosidase activity (Gunkel et al., 1998). Although the 5' element and IBEs function in activation of translation, the *trans*-acting proteins that bind them to mediate activation are unknown.

One way in which Bru could mediate activation is through recruitment of Vasa (Vas) to the *osk* mRNA. Vas is an ATP-dependent, DEAD-box RNA helicase, and is recruited to the posterior pole to function in posterior patterning and germ cell formation, similar to Osk (Schüpbach and Wieschaus, 1986; Hay et

al., 1988; Lasko and Ashburner, 1990). Bru interacts with Vas directly (Webster et al., 1997) and may recruit Vas to *osk*. Consistent with this idea, Vas colocalizes with the *osk* transcript to the posterior pole of the oocyte from stage 9 of oogenesis (Liang et al., 1994), and is required for efficient Osk translation (Markussen et al., 1995; Rongo et al., 1995). The defect in Osk accumulation only appears late in oogenesis (Harris and Macdonald, 2001). It has been shown that the RNA-binding and helicase activities of Vas are dispensable for its posterior localization (Liang et al., 1994), and thus protein-protein interaction may account for localization of Vas. Another possibility is that Vas binds *osk* directly to activate translation. Vas binds directly and specifically to a U-rich motif within the *mei-P26* 3' UTR to activate its translation (Liu et al., 2009). Similarly, Vas could be recruited to *osk* by a U-rich motif present in the *osk* 3' UTR. vas^{o11} and vas^{o14} mutants, which are defective in RNA binding *in vitro*, are unable to support pole cell formation, although these Vas mutants localize to the posterior pole like the wild type (Liang et al., 1994). Therefore interaction of Vas with *osk* mRNA and subsequent RNA-unwinding activity may be the key to efficient Osk translation and pole cell formation.

Another way in which Bru could mediate activation is through recruitment of Orb to the *osk* mRNA. Orb is a Drosophila homolog of Xenopus CPEB, a protein that binds the U-rich cytoplasmic polyadenylation element (CPE; Hake and Richter, 1994; Stebbins-Boaz et al., 1996) and presumably recruits and stabilizes the cytoplasmic polyadenylation machinery (Mendez et al., 2000). In many species, mRNAs with a long poly(A) tail are translationally active while those with a short poly(A) tail are translationally inactive. In Drosophila, no canonical CPE has been identified, but a protein that comigrates with Orb can be

UV-crosslinked to the *osk* 3' UTR (Chang et al., 1999). Alternatively, Orb could be recruited to *osk* via interaction with Bru, with which Orb interacts physically and genetically (Castagnetti and Ephrussi, 2003). Orb colocalizes with the *osk* mRNA, and the *osk* mRNA is mislocalized in strong *orb* mutants (Christerson and McKearin, 1994; Lantz et al., 1994). In weak *orb* mutants, the poly(A) tail of *osk* is shorter in length, and posterior Osk is greatly reduced at stages 9 and 10 (Chang et al., 1999; Castagnetti and Ephrussi, 2003), even in egg chambers that display correct Stau localization (Castagnetti and Ephrussi, 2003). Therefore, Orb could stimulate Osk translation by elongating or maintaining (*via* protection from degradation) the poly(A) tail.

Like Orb, Staufen (Stau) is involved in both *osk* mRNA localization and translational activation, two processes that are coupled and hard to tease apart when assessing the mutant phenotype. However, two processes are uncoupled in mothers expressing the *oskBRE-* transgene, which has mutations in both AB and C regions and thus causes defects in repression and produces bicaudal embryos (Kim-Ha et al., 1995). Embryos from *orb-* or *stau*-mutant mothers have abdominal deletions (Schüpbach and Wieschaus, 1986; Christerson and McKearin, 1994), due to defects in *osk* mRNA localization (Ephrussi et al., 1991; Kim-Ha et al., 1991; Christerson and McKearin, 1994; Lantz et al., 1994). Although the *orb*-mutant phenotype is rescued by the *oskBRE-* transgene, the *stau*-mutant phenotype is not (Kim-Ha et al., 1995); this result suggests that *stau* has a role in activation of translation, which is required in the absence of repression, independent of its role in *osk* localization. Consistent with this, the dsRNA-binding domain 5 (dsRBD5) of Stau does not bind dsRNA *in vitro*, unlike

four other dsRBDs that do, yet dsRBD5 is required for translation of localized *osk* mRNA at the posterior (Micklem et al., 2000).

There are examples of kinases that locally phosphorylate RNA-binding proteins to release them from targets and lead to translational activation (Besse and Ephrussi, 2008). One candidate kinase that could provide the spatial and temporal cue for Osk translation is cAMP-dependent Protein Kinase (PKA). PKA is the key mediator of the ubiquitous second messenger, cAMP, in various signaling events. The PKA holoenzyme consists of two regulatory subunits (R) and two catalytic subunits (C). Upon cAMP binding, the regulatory subunits release the catalytic subunits, which are freed to phosphorylate their substrates (Taylor et al., 1990). Regulation of PKA activity has been shown to be crucial for spatial restriction of Osk. *Pka-R1* mutants, which display increased PKA catalytic activity, produce bicaudal embryos and cause premature and ectopic expression of Osk; this patterning defect is suppressed by reducing the catalytic gene dosage (Yoshida et al., 2004). PKA-R1 is expressed in the cytoplasm of the nurse cells, follicle cells, and the oocyte and enriched at membranes (Yoshida et al., 2004). PKA also regulates microtubule polarity in the oocyte. *DC0* encodes the PKA catalytic subunit in Drosophila (Lane and Kalderon, 1993). *DC0* mutants alter oocyte microtubule distribution, such that a high density of microtubules are ectopically seen at the posterior of stage 7 and 8 oocytes, concomitant with aberrant localization of Kin:β-gal, the plus-end marker, to the center of the oocyte (Lane and Kalderon, 1994). Signaling between the oocyte and somatic follicle cells is essential for correct anteroposterior axis specification *via* reorganization of microtubules during mid oogenesis (González-Reyes et al., 1995; Roth et al., 1995). Correct localization of Kin:β-gal at the posterior of the oocyte depends on

PKA activity in the germline, rather than soma, and this finding led to the postulation that PKA activity is required in the germline for transducing a temporal signal from the posterior pole at stage 6/7 for reorganization of the microtubules (Lane and Kalderon, 1994). This germline requirement of PKA at the time of oocyte polarity formation coincides with subsequent *osk* localization and translation. Therefore, PKA is an ideal candidate to locally inactivate Bru, directly or indirectly, and lead to derepression of *osk* translation.

SUMMARY AND CONCLUSION

Embryonic axis formation is mediated through subcellular localization and translational control of maternal determinants. Correct spatial and temporal control of Osk expression is essential for axial patterning of the embryo, and is achieved by coupling of mRNA localization and translational regulation. The *osk* RNP particles are thought to form in the nurse cells and move to their final destination within the oocyte. Many *trans*-acting factors associated with the *osk* RNP are essential for localization and/or translational regulation, allowing coupling of the two processes. Once the *osk* mRNA is localized to the posterior of the oocyte, translational repression must be relieved to allow for Osk accumulation. This could occur by inactivation or displacement of repressors, most likely *via* post-translational modifications. In addition to derepression, translation of Osk may also require activation, which could involve enhancement of translation by elongating the poly(A) tail, for example. Local signaling is likely involved to activate translation of *osk*. Although many translational regulatory factors have been identified, it still remains mysterious how these factors

coordinate their actions to execute a spatially and temporally precise outcome. Therefore elucidating the mechanism of translational control of *osk* would contribute to unraveling a layer of post-transcriptional control exerted on developmentally critical RNAs.

OVERVIEW OF THE DISSERTATION RESEARCH

The main goal of my dissertation research is to elucidate the regulatory mechanisms of *osk* translation. I used two approaches: mutating Bru to disrupt interactions with negative regulators of *osk* and altering the activity of a positive regulator of *osk*.

Bru interacts with Cup and with itself, and the current models of repression likely depend on Bru dimerization and Cup binding. I found that the amino-terminal domain of Bru contributes to both interactions, and deletion of this domain causes a defect in translational repression. However point mutations, within the amino-terminal domain, that disrupt both types of interaction *in vitro* did not interfere with translational repression *in vivo*. Moreover, the same mutations made in the context of the endogenous *arrest* gene, which encodes Bru, did not cause any patterning defects associated with misregulation of *osk*. Therefore, according to the results of my *in vitro* binding assays, Bru-Cup and Bru-Bru interactions could act redundantly with another form of repression that also acts through the amino-terminal domain. Alternatively, the defects in binding *in vitro* may exaggerate the defects *in vivo*, and residual interactions could suffice for efficient repression. Interestingly, dimerization-defective Bru only weakly accumulated Osk::GFP fusion protein encoded by an *osk::GFP* reporter RNA

bearing a Bru-binding region, while dimerization-competent Bru showed the opposite effect. These unexpected results suggest a new role of dimerization in translational activation.

A small fraction of Bru in ovaries is phosphorylated. PKA is a positive regulator of *osk* expression and phosphorylates Bru *in vitro*. To test if PKA regulation of *osk* is mediated through Bru, I examined the effect of altering PKA activity on Bru phosphorylation and Bru-mediated repression. Modulating PKA activity had small, yet detectable changes in Bru phosphorylation and Bru-dependent translational repression using an *osk::GFP* reporter. While the studies with Bru mutants suggest that phosphorylation promotes repression by Bru, however, the positive effect of PKA on *osk* expression may occur by phosphorylating additional targets. It also remains possible that there are additional sites of phosphorylation that inactivates Bru's repressive activity.

FIGURES

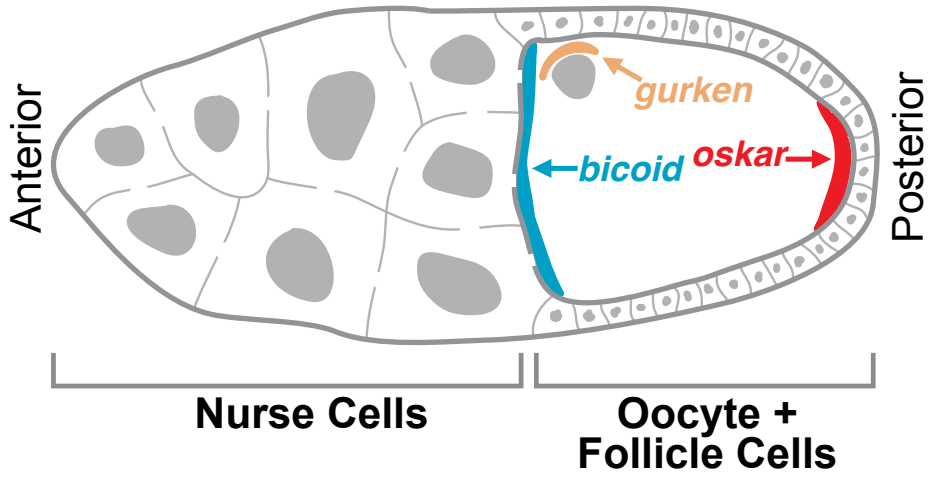


Figure 1.1. Localization of maternal RNAs that specify axial patterning in a Drosophila oocyte

An egg chamber contains germline-derived nurse cells and oocyte, and somatic follicle cells that surround the oocyte (and nurse cells at earlier stages). Three localized determinants are shown in a stage 10 egg chamber.

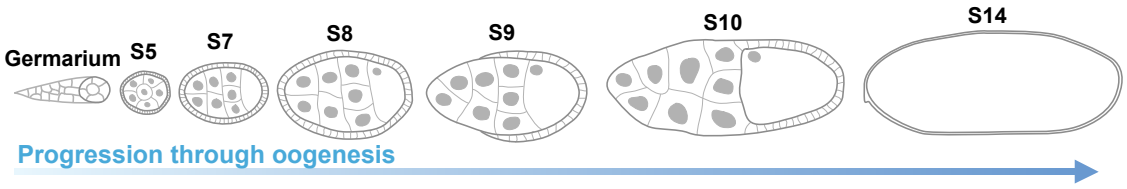


Figure 1.2. Egg chamber progression through oogenesis from germarium to stage 14

Egg chambers develop as they move toward the posterior of an ovariole within Drosophila ovaries. The germarium holds germline and somatic stem cells that give rise to cells in an egg chamber, which buds off from the tip of the germarium. Nurse cells degenerate near the end of oogenesis.

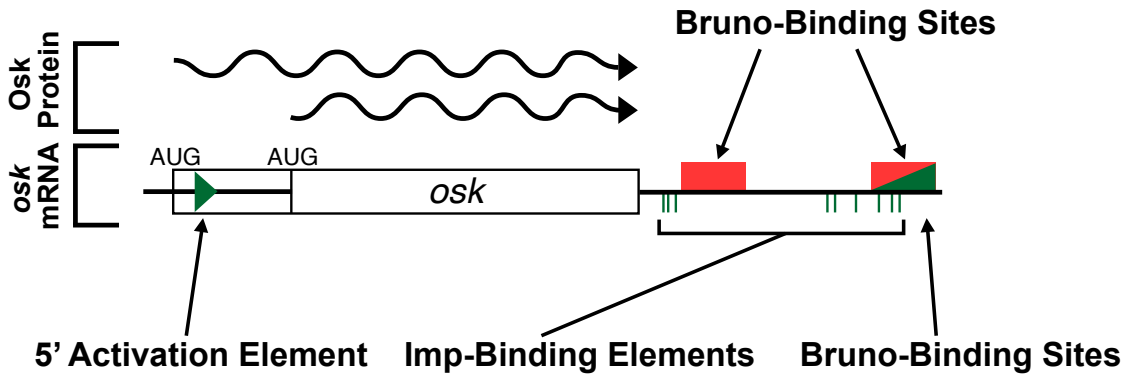


Figure 1.3. A schematic diagram of *oskar* mRNA and Oskar protein

Two large boxes represent the coding sequence, and two horizontal lines are the 5' or 3' UTRs. The wavy lines represent two Osk isoforms made from alternative start codons in the same reading frame. *Cis* elements involved in repression are depicted in red, and *cis* elements involved in activation are in green. Bru-binding sites are embedded in two regions within the 3' UTR: AB (red box) and C (red/green box).

REFERENCES

Babu, K., Cai, Y., Bahri, S., Yang, X., & Chia, W. (2004). Roles of Bifocal, Homer, and F-actin in anchoring Oskar to the posterior cortex of Drosophila oocytes. Genes & Development, 18(2), 138–143.

Bastock, R., & St Johnston, D. (2008). Drosophila oogenesis. Current Biology, 18(23), R1082-R1087.

Berleth, T., Burri, M., Thoma, G., Bopp, D., Richstein, S., Frigerio, G., et al. (1988). The role of localization of bicoid RNA in organizing the anterior pattern of the Drosophila embryo. The EMBO Journal, 7(6), 1749–1756.

Besse, F., & Ephrussi, A. (2008). Translational control of localized mRNAs: restricting protein synthesis in space and time. Nature Reviews Molecular Cell Biology, 9(12), 971–980.

Bhattacharyya, S. N., Habermacher, R., Martine, U., Closs, E. I., & Filipowicz, W. (2006). Relief of microRNA-Mediated Translational Repression in Human Cells Subjected to Stress. Cell, 125(6), 1111–1124.

Bonafe, N., & Sellers, J. R. (1998). Molecular characterization of myosin V from Drosophila melanogaster. Journal of Muscle Research and Cell Motility, 19(2), 129–141.

Brendza, R. P., Serbus, L. R., Duffy, J. B., & Saxton, W. M. (2000). A function for kinesin I in the posterior transport of oskar mRNA and Staufen protein. Science, 289(5487), 2120–2122.

Brengues, M., Teixeira, D., & Parker, R. (2005). Movement of eukaryotic mRNAs between polysomes and cytoplasmic processing bodies. Science, 310(5747), 486–489.

Bullock, S. L., & Ish-Horowicz, D. (2001). Conserved signals and machinery for RNA transport in Drosophila oogenesis and embryogenesis. Nature, 414(6864), 611–616.

Castagnetti, S., & Ephrussi, A. (2003). Orb and a long poly (A) tail are required for efficient oskar translation at the posterior pole of the Drosophila oocyte. Development, 130(5), 835–843.

Castagnetti, S., Hentze, M. W., Ephrussi, A., & Gebauer, F. (2000). Control of oskar mRNA translation by Bruno in a novel cell-free system from Drosophila ovaries. Development, 127(5), 1063–1068.

Cha, B.-J., Serbus, L. R., Koppetsch, B. S., & Theurkauf, W. E. (2002). Kinesin I-dependent cortical exclusion restricts pole plasm to the oocyte posterior. Nature Cell Biology, 4(8), 592–598.

Chang, J. S., Tan, L., & Schedl, P. (1999). The Drosophila CPEB homolog, orb, is required for oskar protein expression in oocytes. Developmental Biology, 215(1), 91–106.

Chekulaeva, M., Hentze, M. W., & Ephrussi, A. (2006). Bruno Acts as a Dual Repressor of oskar Translation, Promoting mRNA Oligomerization and Formation of Silencing Particles. Cell, 124(3), 521–533.

Cho, Y. S., Stevens, L. M., & Stein, D. (2010). Pipe-Dependent Ventral Processing of Easter by Snake Is the Defining Step in Drosophila Embryo DV Axis Formation. Current Biology, 20(12), 1133–1137.

Christerson, L. B., & McKearin, D. M. (1994). orb is required for anteroposterior and dorsoventral patterning during Drosophila oogenesis. Genes & Development, 8(5), 614–628.

Coller, J., & Parker, R. (2005). General Translational Repression by Activators of mRNA Decapping. Cell, 122(6), 875–886.

Condeelis, J., & Singer, R. H. (2005). How and why does beta-actin mRNA target? Biology of the Cell / Under the Auspices of the European Cell Biology Organization, 97(1), 97–110.

Davidson, E. H. (1986). Gene activity during oogenesis. Gene activity in early development, 305-407.

de Cuevas, M., Lilly, M. A., & Spradling, A. C. (1997). Germline cyst formation in Drosophila. Annual Review of Genetics, 31(1), 405–428.

de Valoir, T., Tucker, M. A., Belikoff, E. J., Camp, L. A., Bolduc, C., & Beckingham, K. (1991). A second maternally expressed Drosophila gene encodes a putative RNA helicase of the “DEAD box” family. Proceedings of the National Academy of Sciences, 88(6), 2113–2117.

Dienstbier, M., Boehl, F., Li, X., & Bullock, S. L. (2009). Egalitarian is a selective RNA-binding protein linking mRNA localization signals to the dynein motor. Genes & Development, 23(13), 1546–1558.

Dreyfuss, G., Kim, V. N., & Kataoka, N. (2002). Messenger-RNA-binding proteins and the messages they carry. Nature Reviews Molecular Cell Biology, 3(3), 195–205.

Driever, W., & Nusslein-Volhard, C. (1988). The bicoid protein determines position in the Drosophila embryo in a concentration-dependent manner. Cell, 54(1), 95–104.

Ephrussi, A., Dickinson, L. K., & Lehmann, R. (1991). Oskar organizes the germ plasm and directs localization of the posterior determinant nanos. Cell, 66(1), 37–50.

Ephrussi, A., & Lehmann, R. (1992). Induction of germ cell formation by oskar. Nature, 358(6385), 387–392.

Eulalio, A., Behm-Ansmant, I., & Izaurralde, E. (2007). P bodies: at the crossroads of post-transcriptional pathways. Nature Reviews Molecular Cell Biology, 8(1), 9–22.

Ferrandon, D., Elphick, L., Nüsslein-Volhard, C., & St Johnston, D. (1994). Staufen protein associates with the 3′UTR of bicoid mRNA to form particles that move in a microtubule-dependent manner. Cell, 79(7), 1221–1232.

Gaspar, I. (2011). Microtubule-based motor-mediated mRNA localization in Drosophilaoocytes and embryos. Biochemical Society Transactions, 39(5), 1197–1201.

Gavis, E. R., & Lehmann, R. (1992). Localization of nanos RNA controls embryonic polarity. Cell, 71(2), 301–313.

Gebauer, F., & Hentze, M. W. (2004). Molecular mechanisms of translational control. Nature Reviews Molecular Cell Biology, 5(10), 827–835.

Geng, C., & Macdonald, P. M. (2006). Imp Associates with Squid and Hrp48 and Contributes to Localized Expression of gurken in the Oocyte. Molecular and Cellular Biology, 26(24), 9508–9516.

Ghosh, S., Marchand, V., Gaspar, I., & Ephrussi, A. (2012). Control of RNP motility and localization by a splicing-dependent structure in oskar mRNA. Nature Structural & Molecular Biology, 19(4), 441–449.

González-Reyes, A., Elliott, H., & St Johnston, D. (1995). Polarization of both major body axes in Drosophila by gurken-torpedo signalling. Nature, 375(6533), 654–658.

Gunkel, N., Yano, T., Markussen, F. H., Olsen, L. C., & Ephrussi, A. (1998). Localization-dependent translation requires a functional interaction between the 5“ and 3” ends of oskar mRNA. Genes & Development, 12(11), 1652–1664.

Hachet, O., & Ephrussi, A. (2001). Drosophila Y14 shuttles to the posterior of the oocyte and is required for oskar mRNA transport. Current Biology, 11(21), 1666–1674.

Hachet, O., & Ephrussi, A. (2004). Splicing of oskar RNA in the nucleus is coupled to its cytoplasmic localization. Nature, 428(6986), 959–963.

Hake, L. E., & Richter, J. D. (1994). CPEB is a specificity factor that mediates cytoplasmic polyadenylation during Xenopus oocyte maturation. Cell, 79(4), 617–627.

Harris, A. N., & Macdonald, P. M. (2001). Aubergine encodes a Drosophila polar granule component required for pole cell formation and related to eIF2C. Development, 128(14), 2823–2832.

Hay, B., Jan, L. Y., & Jan, Y. N. (1988). A protein component of Drosophila polar granules is encoded by vasa and has extensive sequence similarity to ATP-dependent helicases. Cell, 55(4), 577–587.

Huynh, J.-R., Munro, T. P., Smith-Litière, K., Lepesant, J.-A., & Johnston, D. S. (2004). The Drosophila hnRNPA/B Homolog, Hrp48, Is Specifically Required for a Distinct Step in osk mRNA Localization. Developmental Cell, 6(5), 625–635.

Januschke, J., Gervais, L., Dass, S., Kaltschmidt, J. A., Lopez-Schier, H., St Johnston, D., et al. (2002). Polar transport in the Drosophila oocyte requires Dynein and Kinesin I cooperation. Current Biology, 12(23), 1971–1981.

Jenny, A., Hachet, O., Závorszky, P., Cyrklaff, A., Weston, M. D. J., Johnston, D. S., et al. (2006). A translation-independent role of oskar RNA in early Drosophila oogenesis. Development, 133(15), 2827–2833.

Johnstone, O., & Lasko, P. (2001). Translational regulation and RNA localization in Drosophila oocytes and embryos. Annual Review of Genetics, 35, 365–406.

Kato, Y., & Nakamura, A. (2012). Roles of cytoplasmic RNP granules in intracellular RNA localization and translational control in the Drosophila oocyte. Development, Growth & Differentiation, 54(1), 19–31.

Kim-Ha, J., Kerr, K., & Macdonald, P. M. (1995). Translational regulation of oskar mRNA by Bruno, an ovarian RNA-binding protein, is essential. Cell, 81(3), 403–412.

Kim-Ha, J., Smith, J. L., & Macdonald, P. M. (1991). oskar mRNA is localized to the posterior pole of the Drosophila oocyte. Cell, 66(1), 23–35.

Kim-Ha, J., Webster, P. J., Smith, J. L., & Macdonald, P. M. (1993). Multiple RNA regulatory elements mediate distinct steps in localization of oskar mRNA. Development, 119(1), 169–178.

King, M. L., Messitt, T. J., & Mowry, K. L. (2005). Putting RNAs in the right place at the right time: RNA localization in the frog oocyte. Biology of the Cell / Under the Auspices of the European Cell Biology Organization, 97(1), 19–33.

Krauss, J., López de Quinto, S., Nüsslein-Volhard, C., & Ephrussi, A. (2009). Myosin-V Regulates oskar mRNA Localization in the Drosophila Oocyte. Current Biology, 19(12), 1058–1063.

Kugler, J.-M., & Lasko, P. (2009). Localization, anchoring and translational control of oskar, gurken, bicoid and nanos mRNA during Drosophila oogenesis. Fly, 3(1), 15–28.

Kumano, G. (2012). Polarizing animal cells via mRNA localization in oogenesis and early development. Development, Growth & Differentiation, 54(1), 1–18.

Lane, M. E., & Kalderon, D. (1993). Genetic investigation of cAMP-dependent protein kinase function in Drosophila development. Genes & Development, 7(7A), 1229–1243.

Lane, M. E., & Kalderon, D. (1994). RNA localization along the anteroposterior axis of the Drosophila oocyte requires PKA-mediated signal transduction to direct normal microtubule organization. Genes & Development, 8(24), 2986–2995.

Lantz, V., Chang, J. S., Horabin, J. I., Bopp, D., & Schedl, P. (1994). The Drosophila orb RNA-binding protein is required for the formation of the egg chamber and establishment of polarity. Genes & Development, 8(5), 598–613.

Lasko, P. F., & Ashburner, M. (1990). Posterior localization of vasa protein correlates with, but is not sufficient for, pole cell development. Genes & Development, 4(6), 905–921.

Laver, J. D., Li, X., Ancevicius, K., Westwood, J. T., Smibert, C. A., Morris, Q. D., & Lipshitz, H. D. (2013). Genome-wide analysis of Staufen-associated mRNAs identifies secondary structures that confer target specificity. Nucleic Acids Research, 41(20), 9438–9460.

Lehmann, R., & Nüsslein-Volhard, C. (1986). Abdominal segmentation, pole cell formation, and embryonic polarity require the localized activity of oskar, a maternal gene in drosophila. Cell, 47(1), 141–152.

Lehmann, R., & Nusslein-Volhard, C. (1991). The maternal gene nanos has a central role in posterior pattern formation of the Drosophila embryo. Development, 112(3), 679–691.

Lewis, R. A., & Mowry, K. L. (2007). Ribonucleoprotein remodeling during RNA localization. Differentiation, 75(6), 507–518.

Liang, L., Diehl-Jones, W., & Lasko, P. (1994). Localization of vasa protein to the Drosophila pole plasm is independent of its RNA-binding and helicase activities. Development, 120(5), 1201–1211.

Lie, Y. S., & Macdonald, P. M. (1999). Translational regulation of oskar mRNA occurs independent of the cap and poly(A) tail in Drosophila ovarian extracts. Development, 126(22), 4989–4996.

Liu, N., Han, H., & Lasko, P. (2009). Vasa promotes Drosophila germline stem cell differentiation by activating mei-P26 translation by directly interacting with a (U)-rich motif in its 3' UTR. Genes & Development, 23(23), 2742–2752.

MacIver, B., McCormack, A., Slee, R., & Bownes, M. (1998). Identification of an essential gene encoding a class-V unconventional myosin in Drosophila melanogaster. European Journal of Biochemistry, 257(3), 529–537.

Markussen, F.-H., Michon, A.-M., Breitwieser, W., & Ephrussi, A. (1995). Translational control of oskar generates short OSK, the isoform that induces pole plasma assembly. Development, 121(11), 3723–3732.

Martin, K. C., & Zukin, R. S. (2006). RNA trafficking and local protein synthesis in dendrites: an overview. The Journal of Neuroscience : the Official Journal of the Society for Neuroscience, 26(27), 7131–7134.

Martin, M. A. E., Hurd, D. D., & Saxton, W. M. (1999). Kinesins in the nervous system. Cellular and Molecular Life Sciences : CMLS, 56(3-4), 200–216.

McNeil, G. P., Kaur, M., Purrier, S., & Kang, R. (2009). The Drosophila RNA-binding protein Lark is required for localization of Dmoesin to the oocyte cortex during oogenesis. Development Genes and Evolution, 219(1), 11–19.

Medioni, C., Mowry, K., & Besse, F. (2012). Principles and roles of mRNA localization in animal development. Development, 139(18), 3263–3276.

Mendez, R., Murthy, K. G., Ryan, K., Manley, J. L., & Richter, J. D. (2000). Phosphorylation of CPEB by Eg2 mediates the recruitment of CPSF into an active cytoplasmic polyadenylation complex. Molecular Cell, 6(5), 1253–1259.

Mhlanga, M. M., Bratu, D. P., Genovesio, A., Rybarska, A., Chenouard, N., Nehrbass, U., & Olivo-Marin, J.-C. (2009). In Vivo Colocalisation of oskar mRNA and Trans-Acting Proteins Revealed by Quantitative Imaging of the Drosophila Oocyte. PLoS ONE, 4(7), e6241.

Micklem, D. R., Adams, J., Grünert, S., & St Johnston, D. (2000). Distinct roles of two conserved Staufen domains in oskar mRNA localization and translation. The EMBO Journal, 19(6), 1366–1377.

Mohr, S. E., Dillon, S. T., & Boswell, R. E. (2001). The RNA-binding protein Tsunagi interacts with Mago Nashi to establish polarity and localize oskar mRNA during Drosophila oogenesis. Genes & Development, 15(21), 2886–2899.

Munro, T. P., Kwon, S., Schnapp, B. J., & St Johnston, D. (2006). A repeated IMP-binding motif controls oskar mRNA translation and anchoring independently of Drosophila melanogaster IMP. The Journal of Cell Biology, 172(4), 577–588.

Nakamura, A., Amikura, R., Hanyu, K., & Kobayashi, S. (2001). Me31B silences translation of oocyte-localizing RNAs through the formation of cytoplasmic RNP complex during Drosophila oogenesis. Development, 128(17), 3233–3242.

Nakamura, A., Sato, K., & Hanyu-Nakamura, K. (2004). Drosophila cup is an eIF4E binding protein that associates with Bruno and regulates oskar mRNA translation in oogenesis. Developmental Cell, 6(1), 69–78.

Neuman-Silberberg, F. S., & Schüpbach, T. (1993). The Drosophila dorsoventral patterning gene gurken produces a dorsally localized RNA and encodes a TGFα-like protein. Cell, 75(1), 165–174.

Palacios, I. M., Gatfield, D., St Johnston, D., & Izaurralde, E. (2004). An eIF4AIII-containing complex required for mRNA localization and nonsense-mediated mRNA decay. Nature, 427(6976), 753–757.

Paquin, N., & Chartrand, P. (2008). Local regulation of mRNA translation: new insights from the bud. Trends in Cell Biology, 18(3), 105–111.

Pokrywka, N. J., & Stephenson, E. C. (1995). Microtubules are a general component of mRNA localization systems in Drosophila oocytes. Developmental Biology, 167(1), 363–370.

Polesello, C., Delon, I., Valenti, P., Ferrer, P., & Payre, F. (2002). Dmoesin controls actin-based cell shape and polarity during Drosophila melanogaster oogenesis. Nature Cell Biology, 4(10), 782–789.

Reveal, B., Garcia, C., Ellington, A., & Macdonald, P. (2011). Multiple RNA binding domains of Bruno confer recognition of diverse binding sites for translational repression. RNA Biology, 8(6), 1047–1060.

Reveal, B., Yan, N., Snee, M. J., Pai, C.-I., Gim, Y., & Macdonald, P. M. (2010). BREs Mediate Both Repression and Activation of oskar mRNA Translation and Act In trans. Developmental Cell, 18(3), 496–502.

Riechmann, V., & Ephrussi, A. (2001). Axis formation during Drosophila oogenesis. Current Opinion in Genetics & Development, 11(4), 374–383.

Rongo, C., Gavis, E. R., & Lehmann, R. (1995). Localization of oskar RNA regulates oskar translation and requires Oskar protein. Development, 121(9), 2737–2746.

Roth, S. (1994). Axis Determination: Proteolytic generation of a morphogen. Current Biology, 4(8), 755–757.

Roth, S., Shira Neuman-Silberberg, F., Barcelo, G., & Schüpbach, T. (1995). cornichon and the EGF receptor signaling process are necessary for both anterior-posterior and dorsal-ventral pattern formation in Drosophila. Cell, 81(6), 967–978.

Schüpbach, T., & Wieschaus, E. (1986). Maternal-effect mutations altering the anterior-posterior pattern of the Drosophila embryo. Roux's Archives of Developmental Biology, 195(5), 302–317.

Schüpbach, T., & Wieschaus, E. (1991). Female Sterile Mutations on the Second Chromosomofe Drosophila melanogaster. 11. Mutations Blocking Oogenesis or Altering Egg Morpholog. Genetics, 129(4), 1119–1136.

Sen, J., Goltz, J. S., Stevens, L., & Stein, D. (1998). Spatially restricted expression of pipe in the Drosophila egg chamber defines embryonic dorsal-ventral polarity. Cell, 95(4), 471–481.

Sheth, U., & Parker, R. (2003). Decapping and decay of messenger RNA occur in cytoplasmic processing bodies. Science, 300(5620), 805–808.

Smith, J. L., Wilson, J. E., & Macdonald, P. M. (1992). Overexpression of oskar directs ectopic activation of nanos and presumptive pole cell formation in Drosophila embryos. Cell, 70(5), 849–859.

Snee, M., Benz, D., Jen, J., & Macdonald, P. M. (2008). Two distinct domains of Bruno bind specifically to the oskar mRNA. RNA Biology, 5(1), 1–9.

Snee, M. J., & Macdonald, P. M. (2009). Dynamic organization and plasticity of sponge bodies. Developmental Dynamics, 238(4), 918–930.

Spradling, A. C. (1993). Developmental genetics of oogenesis. In M. Bate, & A. Martinez-Arias (Eds.), *The Development of Drosophila melanogaster* (pp. 1-70). New York , NY: Cold Spring Harbor Laboratory Press.

St Johnston, D., Beuchle, D., & Nusslein-Volhard, C. (1991). Staufen, a gene required to localize maternal RNAs in the Drosophila egg. Cell, 66(1), 51–63.

Stebbins-Boaz, B., Hake, L. E., & Richter, J. D. (1996). CPEB controls the cytoplasmic polyadenylation of cyclin, Cdk2 and c-mos mRNAs and is necessary for oocyte maturation in Xenopus. The EMBO Journal, 15(10), 2582–2592.

Stein, D. (1995). Pattern Formation: The link between ovary and embryo. Current Biology, 5(12), 1360–1363.

Suyama, R., Jenny, A., Curado, S., Pellis-van Berkel, W., & Ephrussi, A. (2009). The actin-binding protein Lasp promotes Oskar accumulation at the posterior pole of the Drosophila embryo. Development, 136(1), 95–105.

Tange, T. Ø., Shibuya, T., Jurica, M. S., & Moore, M. J. (2005). Biochemical analysis of the EJC reveals two new factors and a stable tetrameric protein core. RNA, 11(12), 1869–1883.

Taylor, S. S., Buechler, J. A., & Yonemoto, W. (1990). cAMP-dependent protein kinase: framework for a diverse family of regulatory enzymes. Annual Review of Biochemistry, 59, 971–1005.

Theurkauf, W. E., Alberts, B. M., Jan, Y. N., & Jongens, T. A. (1993). A central role for microtubules in the differentiation of Drosophila oocytes. Development, 118(4), 1169–1180.

Theurkauf, W. E., Smiley, S., Wong, M. L., & Alberts, B. M. (1992). Reorganization of the cytoskeleton during Drosophila oogenesis: implications for axis specification and intercellular transport. Development, 115(4), 923–936.

van Eeden, F. J., Palacios, I. M., Petronczki, M., Weston, M. J., & St Johnston, D. (2001). Barentsz is essential for the posterior localization of oskar mRNA and colocalizes with it to the posterior pole. The Journal of Cell Biology, 154(3), 511–523.

Vanzo, N. F., & Ephrussi, A. (2002). Oskar anchoring restricts pole plasm formation to the posterior of the Drosophila oocyte. Development, 129(15), 3705–3714.

Vanzo, N., Oprins, A., Xanthakis, D., Ephrussi, A., & Rabouille, C. (2007). Stimulation of Endocytosis and Actin Dynamics by Oskar Polarizes the Drosophila Oocyte. Developmental Cell, 12(4), 543–555.

Webster, P. J., Liang, L., Berg, C. A., Lasko, P., & Macdonald, P. M. (1997). Translational repressor bruno plays multiple roles in development and is widely conserved. Genes & Development, 11(19), 2510–2521.

Wilhelm, J. E., Hilton, M., Amos, Q., & Henzel, W. J. (2003). Cup is an eIF4E binding protein required for both the translational repression of oskar and the recruitment of Barentsz. The Journal of Cell Biology, 163(6), 1197–1204.

Wilhelm, J. E., Mansfield, J., Hom-Booher, N., Wang, S., Turck, C. W., Hazelrigg, T., & Vale, R. D. (2000). Isolation of a ribonucleoprotein complex involved in mRNA localization in Drosophila oocytes. The Journal of Cell Biology, 148(3), 427–440.

Wilsch-Brauninger, M., Schwarz, H., & Nusslein-Volhard, C. (1997). A sponge-like structure involved in the association and transport of maternal products during Drosophila oogenesis. The Journal of Cell Biology, 139(3), 817–829.

Yano, T., de Quinto, S. L., Matsui, Y., Shevchenko, A., Shevchenko, A., & Ephrussi, A. (2004). Hrp48, a Drosophila hnRNPA/B Homolog, Binds and Regulates Translation of oskar mRNA. Developmental Cell, 6(5), 637–648.

Yoshida, S., Müller, H.-A. J., Wodarz, A., & Ephrussi, A. (2004). PKA-R1 spatially restricts Oskar expression for Drosophila embryonic patterning. Development, 131(6), 1401–1410.

Zappavigna, V., Piccioni, F., Villaescusa, J. C., & Verrotti, A. C. (2004). Cup is a nucleocytoplasmic shuttling protein that interacts with the eukaryotic translation initiation factor 4E to modulate Drosophila ovary development. Proceedings of the National Academy of Sciences of the United States of America, 101(41), 14800–14805.

Zhang, Z., Stevens, L. M., & Stein, D. (2009). Sulfation of Eggshell Components by Pipe Defines Dorsal-Ventral Polarity in the Drosophila Embryo. Current Biology, 19(14), 1200–1205.

Zimyanin, V. L., Belaya, K., Pecreaux, J., Gilchrist, M. J., Clark, A., Davis, I., & St Johnston, D. (2008). In Vivo Imaging of oskar mRNA Transport Reveals the Mechanism of Posterior Localization. Cell, 134(5), 843–853.

Chapter 2: Role of Bruno Protein Interactions in Translational Regulation of *oskar*[1]

ABSTRACT

Oskar (Osk) is a posterior body-patterning determinant in Drosophila and is highly concentrated at the posterior pole of the oocyte. Tight spatial and temporal restriction of the Osk patterning activity is essential for proper development of the embryo. Bruno (Bru) directly binds to the *osk* mRNA and represses translation during mRNA localization to the posterior pole. After *osk* mRNA localization, repression must be alleviated to allow accumulation of Osk protein. In one model for repression, Bru bound to *osk* mRNA recruits Cup, which in turn binds eIF4E and prevents its interaction with eIF4G. In another model, Bru promotes oligomerization of multiple *osk* mRNAs into large particles that are inaccessible to the translational machinery. The interactions of Bru with RNA and proteins must underlie its repressive activity, and may be disrupted for release from repression. We show that Bru dimerizes, with the amino-terminal domain contributing to both dimerization and interaction with Cup. Deletion of this domain disrupts translational repression by Bru in an *in vivo* assay. We also show that a

---

[1] Goheun Kim and Paul M. Macdonald, Department of Molecular Biosciences, Institute for Cellular and Molecular Biology, The University of Texas at Austin; Keiji Sato and Akira Nakamura, Department of Germline Development, Division of Organogenesis, Institute of Molecular Embryology and Genetics, Kumamoto University; Maria D. Person, Division of Pharmacology and Toxicology, Institute for Cellular and Molecular Biology, The University of Texas at Austin

small fraction of ovarian Bru is phosphorylated. Several predicted sites of phosphorylation by PKA in Bru lie within the amino-terminal domain, with at least one of these being phosphorylated by PKA *in vitro*. Phosphomimetic mutations in these sites interfere with Bru binding to both Cup and itself *in vitro*, while the corresponding phosphosilent mutations have no effect. However the phosphomimetic mutations do not interfere with translational repression *in vivo*. Surprisingly, Bru dimerization appears to be important for translational activation, not repression, an implication at odds with one model of repression.

INTRODUCTION

Post-transcriptional gene regulation fine-tunes the amount and location of many gene products that are essential for development. In Drosophila, localization of several determinants within a single large cell, the oocyte, is required for correct axis formation and body patterning (reviewed in Lipshitz and Smibert, 2000; Palacios and St. Johnston, 2001).

Oskar (Osk) is a posterior determinant that specifies formation of the embryonic abdomen and germline (reviewed in St Johnston and Nüsslein Volhard, 1992). In the absence of Osk, no abdomen and germ cells form (Lehmann and Nüsslein Volhard, 1986). Conversely, overexpression of Osk can cause a range of defects, from anterior patterning defects at a low level to a mirror-image duplication of abdominal segments (the bicaudal phenotype) at a high level (Smith et al., 1992). Similarly, specific misexpression of Osk at the anterior efficiently produces bicaudal embryos (Ephrussi and Lehmann, 1992). Thus, proper deployment of Osk is critical for embryonic pattern formation, viability and fertility.

The *osk* mRNA is transcribed in nurse cells and transported into the oocyte early in oogenesis. Then the mRNA is localized to the posterior pole at stage 9 of oogenesis and remains anchored there until early embryogenesis (Kim-Ha et al., 1991; Ephrussi et al., 1991; Rongo et al., 1995, Vanzo and Ephrussi, 2002). Translation of *osk* is repressed until the mRNA is localized, and this coupling of RNA localization and translation is essential for restricting the Osk activity to the posterior pole (Kim-Ha et al., 1995; Rongo et al., 1995; Markussen et al., 1995).

Bruno (Bru; encoded by the gene *arrest* or *aret*) is an RNA-binding protein containing three RNA Recognition Motifs (RRMs) commonly found in many RNA-binding proteins. The two tandem RRMs are positioned near the amino terminus, separated from a third RRM at the carboxyl terminus by a linker region (Webster et al., 1997; Snee et al., 2008). Bru directly binds the *osk* 3' UTR in two regions: AB close to the coding sequence and C close to the poly(A) tail. Each region contains multiple Bru-binding sites, including the Bruno Response Elements (BREs) (Kim-Ha et al., 1995; Reveal et al., 2011). Mutation of the BREs disrupts Bru binding to *osk* mRNA, resulting in excess Osk activity and bicaudal phenotype, and implicating Bru as a translational repressor (Kim-Ha et al., 1995; Reveal et al., 2010). Direct evidence for translational repression comes from *in vitro* assays using cell-free translation systems from ovary extracts, which recapitulate Bru- and BRE-dependent repression (Lie and Macdonald, 1999; Castagnetti et al., 2000).

Currently, there are two models for repression of *osk* translation. In the first, Bru recruits Cup, and Cup binds eIF4E through the conserved eIF4E-binding motif shared by many eIF4E-binding proteins, including eIF4G (Nakamura et al., 2004; Zappavigna et al., 2004). Consequently, the Cup-eIF4E interaction competitively blocks the eIF4E-eIF4G interaction, which is necessary for translational initiation. Cup interacts with both Bru and eIF4E in the absence of RNA, and several *cup* mutants express Osk precociously and ectopically (Wilhelm et al., 2003; Nakamura et al., 2004). In the second model, Bru promotes *osk* mRNA oligomerization and formation of large silencing particles, which are inaccessible to the ribosomes. Using a reporter mRNA consisting of a FLAG epitope tag and two copies of the *osk* 3' UTR AB region, Chekulaeva et al

showed that translational repression was accompanied by the formation of unusually heavy RNPs containing Bru. In separate assays, they also showed that Bru and BREs are required to form the *osk* oligomers (Chekulaeva et al., 2006). In summary, the ability of Bru to bind Cup is essential for one model of repression, and the ability of Bru to oligomerize *osk* RNA is essential for the other model, but these functions have not been directly tested for an effect on translational repression.

Regulation of cAMP-dependent Protein Kinase (PKA) activity is critical for regulation of *osk* mRNA. Upregulating the activity of PKA causes a bicaudal phenotype, due to precocious and ectopic expression of Osk. Downregulating PKA activity results in reduced Osk expression despite correct mRNA localization (Yoshida et al., 2004). Posterior localization of *osk* mRNA requires microtubules. Early in oogenesis, the microtubules are nucleated from a microtubule-organizing center (MTOC) at the oocyte's presumptive posterior (Theurkauf et al., 1993). During stage 6/7, the microtubule network undergoes a drastic rearrangement - the MTOC at the posterior disassembles, and microtubules are nucleated from anterior and lateral cortices, with plus ends enriched at the posterior of the oocyte (Theurkauf et al., 1992; Lane and Kalderon, 1994). Reorganization of microtubules requires two sequential signaling events between the oocyte and somatic follicle cells: from the oocyte to the follicle cells, and then from the follicle cells back to the oocyte (González-Reyes et al., 1995; Roth et al., 1995). PKA activity is required in the germline to establish the correct polarity of microtubules, which is essential for proper RNA localization including *osk* (Lane and Kalderon, 1994). Thus, it has been suggested that PKA activity in the oocyte transduces a temporal signal from the posterior follicle cells to reorganize the microtubule

network during mid oogenesis (Lane and Kalderon, 1994; González-Reyes et al., 1995; Roth et al., 1995). This germline requirement of PKA at the time of oocyte polarity formation coincides with subsequent *osk* localization and translation. Although PKA activity positively regulates Osk expression, it is not known if PKA acts directly on regulators of *osk* translation, by inactivating a repressor or activating an activator *via* phosphorylation.

Here we demonstrate that Bru dimerizes, and characterize the binding of Bru to itself and to Cup. We show that Bru is phosphorylated *in vivo*, and that PKA phosphorylates Bru *in vitro*. Phosphomimetic mutations eliminate Bru dimerization and reduce the Bru-Cup interaction. Notably, these mutations do not disrupt translational repression *in vivo*. Surprisingly, Bru dimerization appears to be important for translational activation, not repression, an implication at odds with the silencing particles model of repression.

RESULTS

Bruno dimerizes *via* a domain that also mediates Cup binding

A GST pull-down assay was used to test for the ability of Bru to dimerize. Full-length Bru was expressed as a fusion to GST, and incubated with Bru bearing a His_6 tag. Following affinity purification of GST::Bru with glutathione agarose beads, copurification of His_6::Bru was tested by Western blot analysis using the anti-His_6 antibody. By this assay, Bru did dimerize, while His_6::Bru did not bind to GST alone (Fig 2.1A).

To map the domain of Bru responsible for dimerization, deletion derivatives of Bru (Fig 2.2B) were tested in the GST::Bru pull-down assay. The

three RRMs all function in RNA binding (Snee et al., 2008), so we focused on the other domains. Deletion of the Bru amino-terminal domain (aa1-146) eliminated binding to GST::Bru, while deletion of most of the linker domain between RRMs 2 and 3 (aa334-416) had no effect. The amino terminal domain is not only required for dimerization with Bru, but is also sufficient: the isolated domain bound GST::Bru (Fig 2.2A and 2.2B). The ability of Bru to dimerize provides a simple explanation for how Bru oligomerizes *osk* mRNA: a molecule of Bru bound to one *osk* mRNA could dimerize with a second molecule of Bru bound to a different *osk* mRNA. With the many Bru-binding sites in the *osk* mRNA 3' UTR, formation of large, highly interconnected protein-RNA assemblies is possible. This suggests that the proposed use of *osk* mRNA oligomerization as a mechanism of translational repression likely relies on Bru dimerization.

A second Bru interaction, with Cup, provides the basis for the other proposed mechanism of translational repression, in which Bru recruits Cup to the *osk* mRNA (Nakamura et al., 2004). We used a GST::Cup pull-down assay to monitor interaction with Bru. As expected, full-length Bru (Bru+) bound GST::Cup. Deletion of either aa1-146 or aa334-416 had no dramatic effect on binding, but deletion of both domains eliminated binding. Just as for Bru dimerization, the isolated amino-terminal domain was sufficient for binding to GST::Cup (Fig 2.2A and 2.2B).

The results showing that deletion of the Bru amino-terminal domain did not disrupt the interaction between Bru and Cup proteins were obtained using purified recombinant proteins. We also performed similar assays using unpurified Bru proteins, expressed in the same manner but still in the bacterial extracts where other proteins can compete for binding interactions. In this modified assay,

deletion of the amino-terminal domain did reduce binding to Cup, while deletion of aa334-416 had no dramatic effect. This suggests that the Bru amino-terminal domain is more important for Cup binding than is the linker domain (Fig 2.1B).

Deletion of the amino-terminal domain interferes with Bruno-dependent translational repression

Our evidence that the amino-terminal domain of Bru is essential for dimerization and contributes to Cup binding suggests that this domain is likely to play an important role in repression. To test this prediction we established an *in vivo* tethering assay, in which we monitor translation of a *GFP-MS2* reporter mRNA. The 3' UTR of the *GFP-MS2* mRNA includes 18 copies of the bacteriophage *MS2* stem loop, which is a binding site for the MS2 coat protein (MCP; Bardwell and Wickens, 1990). Forms of Bru are expressed as fusions to MCP, so that they become bound to the reporter mRNA. Both the reporter mRNA and tethered Bru proteins are expressed in Drosophila ovaries using the UAS/GAL4 system. Levels of the MCP::Bru fusion proteins were determined by Western blot; all transgenic lines used here were expressed at similar levels (Fig 2.3A).

The *GFP-MS2* reporter by itself was expressed throughout the germline cells of the egg chamber (Fig 2.4A). Coexpression of tethered Bru dramatically reduced GFP level (10 fold; Fig 2.4C), with a more modest reduction in the *GFP-MS2* mRNA level (1.7 fold; Fig 2.3C). Therefore, in this assay Bru is both repressing translation and reducing mRNA stability, although repression is the stronger effect (compare Fig 2.4J and Fig 2.4L). By contrast, a control MCP fusion protein did not cause a reduction in GFP (Fig 2.4B).

Testing mutants of Bru in this assay revealed that deletion of the amino-terminal domain of Bru led to a substantial increase in GFP (Fig 2.4F). Deletion of the linker domain by itself had no strong effect on GFP level (Fig 2.4G), but did enhance the effect of deleting the amino-terminal domain (Fig 2.4H). To determine to what extent the changes in GFP level were due to effects on translational repression and mRNA stability, reporter mRNA levels were measured. Both mutants with enhanced GFP also had elevated *GFP-MS2* mRNA (Fig 2.4L), although the changes were strongest at the protein level (Fig 2.4J and 2.4K). Thus, the deletions interfere with both activities of Bru in this assay: translational repression and destabilization of mRNA.

We also tested the amino-terminal domain by itself in the tethering assay. This domain supported an intermediate level of repression (Fig 2.4I).

The results of the tethering assay are consistent with either of the current models of translational repression by Bru, since disrupting dimerization and interaction with Cup are predicted to disrupt repression. Our observation of an effect of Bru on mRNA stability also fits with evidence showing that Cup-dependent repression involves a change in mRNA stability in Drosophila S2 cells (Igreja and Izaurralde, 2011). An effector domain of Cup was shown to destabilize reporter mRNAs to which it was tethered *via* deadenylation-dependent decapping and degradation, in addition to translational repression independent of deadenylation (Igreja and Izaurralde, 2011).

Bruno is a phosphoprotein

To allow translation of the *osk* mRNA once it has been localized to the posterior pole of the oocyte, there must be a release from repression. How this is accomplished is not known, but one possibility is that Bru is post-translationally modified to inhibit its repressive activity. To ask if Bru is phosphorylated, the conventional approach of testing for phosphatase-dependent changes in electrophoretic mobility of the protein was used. In untreated ovary extract, Bru appeared by Western blot analysis as a major band, with a faint lower-mobility band. Treatment with phosphatase eliminated the weak band. By contrast, addition of phosphatase inhibitors enhanced the minor band, consistent with the interpretation that this small fraction of Bru is phosphorylated (Fig 2.5A left).

To make a more compelling case for phosphorylation, we also tested the MCP::HA_3::Bru 1-146 protein from above, which at 32 kDa is substantially smaller than Bru (64 kDa) and thus might display a larger change in mobility from phosphorylation. This was indeed the case, and the difference between the major Bru band and the slower migrating fraction was more dramatic (Fig 2.5A right). Bru phosphorylation was also analyzed using the phosphate-affinity SDS-PAGE with the acrylamide-pendant Phos-tag, which separates different phosphoprotein isoforms. Using this approach, multiple, different phosphorylated species could be detected (Fig 2.6A).

Bruno is phosphorylated by PKA

To identify candidate phosphorylation sites in Bru, we used the NetPhosK 1.0 and KinasePhos prediction programs. Both report multiple sites for many

different kinases, although none of the candidate sites had scores suggesting a high probability of phosphorylation (data not shown). Nevertheless, Bru is phosphorylated and so even sites with modest scores remain as candidates. Several amino acids are predicted to be targets for the Protein Kinase A (PKA), an interesting option since alteration of PKA activity affects *osk* expression pattern and embryonic body patterning (Yoshida et al., 2004).

To evaluate PKA, *in vitro* phosphorylation assays were performed using the PKA catalytic subunit and full-length Bru. Bru was strongly phosphorylated, while BSA (a negative control) was not (Fig 2.7A). By contrast, neither Casein Kinase I (CK1) nor Calmodulin-dependent Protein Kinase II (CaMKII), which have similar recognition motifs to that of PKA, supported detectable phosphorylation of full-length Bru (Fig 2.7B).

To map the sites of phosphorylation, the Bru deletion proteins used to map interaction domains were used as substrates (Fig 2.2B). The results demonstrate that PKA phosphorylates one or more sites in the amino-terminal region of Bru: deletion of this domain greatly reduced phosphorylation, and the isolated domain was itself phosphorylated. The linker region is not required for phosphorylation, as deletion of this region did not reduce the level of phosphorylation. Deletion of both the amino-terminal and linker domains reduced phosphorylation to the same low level as seen with deletion of just the amino-terminal domain (Fig 2.5B).

To identify where PKA phosphorylates Bru in its amino-terminal domain, we performed a tandem mass spectrometry (MS/MS) analysis of Bru phosphorylated *in vitro*. Although four candidate sites are predicted, only phosphoserine at position 7 (S7) was identified with high confidence and no ambiguity (Fig 2.5D). A majority of peptides containing either S4 or T135 was

detected as unphosphorylated; S88 could not be tested since aa36-119 was undetectable due to a low coverage of MS/MS (see Materials and Methods). Nevertheless, there is still a possibility of weak phosphorylation below the limit of detection at either S4 or T135, and we cannot rule out phosphorylation within the aa36-119 region. Consistent with S7 phosphorylation, mutation of S7 to alanine (S7A) substantially reduced phosphorylation by PKA *in vitro*. Because the S7A mutant retained a low level of phosphorylation, we also tested additional mutations in other predicted sites, either alone or in combinations. Of the mutants tested, the protein with all three mutations, S4A/S7A/T135A, was most resistant to phosphorylation (Fig 2.5C). All mutations except for S4A were also made in the context of the full-length Bru to test their effect on phosphorylation, and similar results were obtained (Fig 2.6B).

Phosphomimetic mutations disrupt Bruno dimerization and Cup binding

Since the amino-terminal domain of Bru is important for both dimerization and interaction with Cup - interactions that underlie the models for how Bru represses translation -, the potential phosphorylation of one or more residues within this region might inhibit repression. As a first step in testing this possibility we asked if phosphomimetic mutations would interfere with Bru protein interactions.

Pull-down assays were performed with GST::Cup and GST::Bru, using Bru mutants with phosphosilent alanine (A) or phosphomimetic glutamate (E) substitutions at one or more of the three residues that affect phosphorylation by

PKA: S4, S7, and T135. The full-length protein with no mutations (Bru+) served as a positive control, and Δ1-146 Δ334-416 as a negative control.

None of the phosphosilent mutants showed reduced binding to GST::Bru (Fig 2.8A) or GST::Cup (Fig 2.8B), demonstrating that mutation of the affected residues does not inherently disrupt the protein interactions. By contrast, the phosphomimetic mutants did display reduced or undetectable binding, with dimerization being more sensitive to the changes. The S7E mutation significantly reduced dimerization, and the S4E/S7E double mutant retained only a very low level of dimerization. Including the T135E mutation did not obviously further reduce dimerization by S7E (in S7E/T135E), but did reduce dimerization of the triple mutant (S4E/S7E/T135E) to below the level of detection (Fig 2.8A and 2.8C).

Bru binding to Cup was less sensitive to the phosphomimetic mutations. The S7E single mutation did not affect binding, and the double mutation combinations caused only modest defects. Binding to Cup was, however, strongly reduced for the S4E/S7E/T135E triple mutant (Fig 2.8B and 2.8C).

Since S4E/S7E/T135E triple mutant has the most severe defect in both types of Bru interaction, we might expect to see a corresponding defect in translational repression given the existing models.

Phosphomimetic mutations in the candidate phosphorylation sites impair Bruno's RNA-binding activity

Cooperative binding is a common strategy to enhance affinity for a substrate. Dimerization of Bru might facilitate cooperative binding to RNA, and if

so, the mutations inhibiting dimerization are expected to impair Bru's RNA-binding activity.

A UV-crosslinking assay was used to test Bru proteins for their ability to bind the *osk* 3' UTR *AB* region RNA, which has multiple Bru-binding sites. The two mutants most strongly defective in dimerization, S4E/S7E and S4E/S7E/T135E, showed compromised RNA binding (Fig 2.9A). After quantitation, normalization for protein levels and statistical analysis of three independent experiments, both mutants were considered to have a significant change in their RNA-binding ability when compared to their ala-mutant (dimerization-competent) counterparts (Fig 2.9B).

In addition to disrupting known protein interactions underlying the current models of repression, phosphomimetic glu mutations may interfere with translational repression by simply reducing the affinity for the substrate mRNAs.

Mutations that inhibit Bruno dimerization and Cup binding do not affect repression

Our analysis of PKA phosphorylation of Bru *in vitro* suggests that PKA may also modify the protein *in vivo*. Testing this prediction has proven to be challenging, and we have not been able to obtain a conclusive answer because we were not able to make an antibody against the phospho-S7 peptide. Nevertheless, the Bru mutants with defects in dimerization and Cup binding provide useful tools to test the importance of these interactions for Bru function. As one such test, we made use of the tethering assay to monitor translational repression. Surprisingly, none of the mutants showed any notable decrease in repression: repression by wild-type Bru reduced the GFP signal from the reporter

mRNA close to the background level, and the results for each of the mutants were similar (Fig 2.10). All Bru proteins were expressed at similar levels (Fig 2.3B), and the *GFP-MS2* RNA level was similar in all cases (Fig 2.3C).

MCP is known to dimerize (LeCuyer et al., 1995). This property could substitute for Bru dimerization, and thus neutralize the effect of the dimerization-defective mutations. To rule out a dimerization artifact associated with the MCP-MS2 system, we used another tethering system which relies on binding of a bacteriophage lambda N peptide to the *boxB* stem-loop RNA (De Gregorio et al., 1999). In this case the reporter mRNA is *GFP* with 6 copies of the *boxB* sequence in the 3' UTR (*GFP-boxB*), and the Bru proteins are expressed as fusions to λN peptide. Just as with the other system, tethered Bru repressed translation of the reporter mRNA (compare Fig 2.11A and B). Notably, the triple glu mutant did not affect repression (Fig 2.11C), confirming that dimerization is not required for translational repression.

Dimerization-defective Bruno mutants show normal Oskar expression pattern and body patterning activity

Although two different tethering assays each show that mutations disrupting Bru dimerization have no effect on translational repression, the ideal *in vivo* test would be to assay these mutations in the context of intact Bru. To do so we used homologous recombination (HR) to exchange exons that encode the amino-terminal region of Bru. The replacement regions were *wild type* (*aret*$^{+}$), *S4A/S7A/T135A* (*aret*^{ala}), or *S4E/S7E/T135E* (*aret*^{glu}). Loss of *aret* function leads to an early arrest of oogenesis, with no oocyte specified. Females, in which the HR replacement alleles provided the only copy of *aret,* all displayed normal

progression through oogenesis. Embryos obtained from these females were tested for patterning defects. Although misregulation of *osk* expression - either too little or too much Osk activity - causes striking patterning defects, no such defects were found for any of the *aret* HR alleles. The only phenotype detected was for the *aret^{glu}* mutant, and was unrelated to any known *osk* defect: an increase in the proportion of embryos that fail to develop (Table 2.1).

To directly test if *ala* and *glu* mutations have an effect on Osk expression, Osk was monitored during oogenesis. We used two *osk* transgenes for this analysis, both based on a rescuing genomic DNA *osk* transgene. The *osk::HA* transgene is tagged with an epitope for sensitive immunodetection. The *osk::GFP* transgene allows detection of Osk during late stages of oogenesis, when deposition of the vitelline membrane prevents access by antibodies and thus precludes the use of whole-mount immunofluorescent detection. These transgenes were introduced into the *aret^{+}*, *aret^{ala}* or *aret^{glu}* backgrounds.

Like endogenous Osk, Osk::HA (Fig 2.12D) and Osk::GFP (Fig 2.12G) appear in a tight posterior crescent from stage 9 onward. There was no defect in repression in stage 8 (Fig 2.12A-C) egg chambers, and we did not detect any difference in Osk expression pattern among the three replacement lines in stage 9 (Fig 2.12D-F) or later (Fig 2.12G-I) egg chambers, consistent with the absence of any patterning defects (Table 2.1).

Bruno mutants competent or defective in dimerization have opposing effects on Osk1-173::GFP expression

The *osk* mRNA contains a large 3' UTR bound by many proteins implicated in activation or repression. To better reveal any regulatory defects

caused by loss of Bru dimerization, it would be ideal to monitor an mRNA regulated only by Bru, and thus free of potential sources of redundancy in regulation. For this purpose we used the *UAS-osk1-173::GFP-AB* reporter, which displays two regulatory features: Bru-dependent repression throughout the nurse cells and oocyte; and stage-dependent release from repression (or activation) in the oocyte. The latter effect is most dramatic for posterior-specific release from repression at stage 9, but there is also an overall increase in fluorescence throughout the oocyte beginning at stage 7/8.

The *osk1-173::GFP-AB* reporter mRNA (Fig 2.13D) includes an *osk* 5' region, which encodes an anchoring domain (Vanzo and Ephrussi, 2002) and serves to restrict movement of the protein (and thus reveals where it is translated), and the *osk* 3' UTR *AB* region, which contains Bru binding sites and mediates Bru-dependent translational repression (Kim-Ha et al., 1995; Reveal et al., 2011). For the experiments described below, expression of the reporter mRNA was driven by the *matα4-GAL-VP16* driver (Martin and St. Johnston, 2003).

In the *aret*$^{+}$ background at stages 9 and 10, most egg chambers have Osk1-173::GFP in a gradient emanating from posterior of the oocyte (Fig 2.13A, Table 2.2). In the *aret*^{ala} background, the intensity of Osk1-173::GFP in the gradient was as strong as, or sometimes stronger, than for *aret*$^{+}$ (Fig 2.13B), and the fraction of egg chambers showing the gradient was higher (Table 2.2). In the *aret*^{glu} background, the Osk1-173::GFP gradient was frequently weaker than for *aret*$^{+}$ (Fig 2.13C), and most egg chambers had no detectable gradient (Table 2.2). These results were unexpected, since loss of Bru dimerization is predicted to interfere with repression (see Discussion). Instead, the results show that the

mutant unable to dimerize is defective in posterior expression, which could be due to enhancement or unnatural persistence of Bru-dependent repression, or to loss of Bru-dependent activation of translation.

The S4E/S7E/T135E mutations impair Cup binding and eliminate dimerization *in vitro* but do not cause any detectable loss of translational repression *in vivo*. These interactions underlie the current models for translational repression by Bru, and two general explanations can account for the apparent discrepancy. First, one or both models may be wrong. Second, the mutations in Bru may have a stronger defect on *in vitro* protein interactions than on *in vivo* interactions. In particular, the Bru-Cup interaction is only partially defective *in vitro*, and might be stabilized or enhanced by other factors *in vivo*. To address the latter option, we wanted to test the two interactions - Bru-Bru and Bru-Cup - *in vivo*. Bru dimerization has not previously been detected *in vivo*. In an attempt to detect this interaction, we made use of flies expressing a Bru::GFP or GFP::Bru fusion protein. Immunoprecipitates obtained with anti-GFP antibodies were tested for the presence of Bru. Although Bru::GFP (Fig 2.14A) or GFP::Bru (Fig 2.14B) were readily detected with anti-Bru antibodies, Bru was not. We do not know why dimerization *in vivo* is not detected, but difficulty in detecting such an interaction limits our ability to test the Bru mutants.

By contrast, Bru and Cup can be coimmunoprecipitated. Assays with extracts from the *aret*$^{+}$ and *aret*-mutant ovaries revealed that each of the mutants retained a robust interaction with Cup (Fig 2.14C).

DISCUSSION

Translational regulation of *osk* mRNA is critical for development. Repression of translation ensures that the protein does not accumulate where it could disrupt embryonic patterning. Activation of translation, coordinated with localization of the mRNA, provides a local source of Osk protein for posterior patterning and germ cell formation. Multiple factors are implicated in this regulation. Some provide specificity, binding selectively to the mRNA (Kim-Ha et al., 1995; Gunkel et al., 1998; Munro et al., 2006; Besse et al., 2009; Reveal et al., 2010; Vazquez-Pianzola et al., 2011). Others, the effector factors, are recruited by the specificity factors and function to interact or interfere with the translation machinery (Nakamura et al., 2001; Castagnetti and Ephrussi, 2003; Nakamura et al., 2004). The best characterized of the specificity factors is Bru, with well-defined binding sites (Kim-Ha et al., 1995; Reveal et al., 2011), compelling evidence of a role in repression (Lie and Macdonald, 1999; Castagnetti et al., 2000), and proposed models for how it mediates repression (Nakamura et al., 2004; Chekulaeva et al., 2006). In addition, Bru is also implicated in activation of translation (Reveal et al., 2010).

Here we have characterized Bru protein interactions. Binding to the effector factor Cup is key to one model of repression, and Bru dimerization offers an explanation for how Bru could oligomerize *osk* mRNA in the other model of repression. We have also shown that Bru is phosphorylated *in vivo*, and have characterized phosphorylation of Bru *in vitro* by PKA. Although we have not been able to confirm that PKA performs the same phosphorylation *in vivo*, the Bru mutants used to map sites of PKA phosphorylation are valuable tools for interfering with Bru interactions *via* subtle changes in the protein.

Prior work with *in vivo* assays of Bru function has been limited to use of mutants obtained by classical forward genetic screens, as well as overexpression studies. The ability to introduce engineered mutations was constrained by the large size of *aret* (the gene encoding Bru), and the size restrictions of standard methods of transgenesis. We developed two types of assay systems to study, *in vivo*, the effects of mutations that disrupt Bru protein interactions. The first of these is the tethering assay, in which Bru is targeted to a reporter mRNA. Because the RNA-binding activity of Bru itself is dispensable in this assay, we could include mutations in the Bru RNA-binding domains. This reduces or eliminates Bru binding to mRNAs other than the reporter, and thus abrogates the developmental defects, which are caused by ectopic Bru expression, that complicate interpretation of results of such experiments (Filardo and Ephrussi, 2003; Snee et al., 2008). A notable feature of this assay is that it showed two effects of Bru binding: translational repression and reduced mRNA stability. Although Bru can repress translation in ovary extracts with no effect on mRNA stability (Lie and Macdonald, 1999), regulation by Cup does alter mRNA stability (Igreja and Izaurralde, 2011). Therefore, when Bru recruits Cup to an mRNA, some reduction of mRNA stability is expected.

The second general type of assay systems involved homologous recombination to introduce engineered mutations into the *aret* locus. These novel alleles can be used to study *osk* in its native context, as well as the *osk* transgenes modified to facilitate detection but under all of the normal transcriptional and post-transcriptional regulation. Because of the complexity of *osk* regulation, with extensive opportunities for redundancy, we also developed an mRNA substrate, *osk1-173::GFP-AB*, whose regulation predominantly or

exclusively relies on Bru as a specificity factor. By using a version of GFP fused to the Osk protein-anchoring domain, the movement of the protein was restricted, thereby revealing where it was translated. Strikingly, we found that translational repression of this mRNA was specifically alleviated at the posterior of the oocyte, independent of mRNA localization. Because the Osk1-173::GFP fusion protein does not itself migrate to the posterior of the oocyte, its appearance there must be due to local translation. This strongly argues that it is not the process of mRNA localization that is coordinated with this form of activation of translation, it is simply the outcome of the process that matters: mRNA at the posterior is more efficiently translated than mRNA at other positions.

Using each of these assays we asked what happens when Bru has the three amino-acid changes, in the amino-terminal domain, that disrupt dimerization and reduce binding to Cup *in vitro*. Consistently, there was no substantial reduction in translational repression, a result seemingly at odds with both models proposed for repression. However, despite the reduction in Cup binding *in vitro*, the mutant Bru retained a sufficiently strong interaction with Cup *in vivo* to allow coimmunoprecipitation. It is possible that other ovarian proteins, not present in the *in vitro* binding assays, stabilize or enhance the Bru-Cup interaction *in vivo*.

A more intriguing result was obtained with the *osk1-173::GFP-AB* reporter mRNA, which allows us to monitor regulation with Bru as the only, or predominant, specificity factor and thus independent of other forms of control. In the *aret*$^{+}$ background, translation of Osk1-173::GFP was strongly repressed in the nurse cells and the oocyte, but with release from repression (or initiation of activation) specifically at the posterior of the oocyte beginning around stage 9

(coincident with the onset of productive *osk* mRNA translation). By contrast, the Bru mutant defective in protein interactions, *aret*^{glu}, showed greatly reduced posterior expression. A provocative aspect of our results is the different consequences of mutations in Bru that either mimic or would prevent phosphorylation: while the phosphomimetic mutant (*aret*^{glu}) had greatly reduced posterior Osk1-173::GFP, the mutant resistant to phosphorylation (*aret*^{ala}) showed enhanced posterior Osk1-173::GFP. As noted earlier, we have been unable to confirm that the affected Bru amino acids are *bona fide* sites for phosphorylation *in vivo*. Nevertheless, obtaining opposite results from mimicking or preventing phosphorylation does suggest that this modification occurs *in vivo*, and that the modification serves to inhibit activation or maintain Bru in a repressive form. Although PKA, the kinase that phosphorylates the amino-terminal domain of Bru *in vitro*, has been implicated in enhancing Osk expression, while the Bru phosphomimetic mutant interferes with translation, PKA could have multiple effects on the pathways involved in regulation of *osk*. Alternatively, it may be that a different kinase phosphorylates Bru *in vivo*, or PKA phosphorylation of additional sites is specifically involved in activation.

We propose a model for the posterior-specific switch in Bru-mediated repression based on the properties of the *aret* mutants. In the model, dimerization of Bru makes the protein competent for translational activation, either by inhibiting the repressive activity or converting it to an activator. The model is based on control of dimerization, since the phosphomimetic mutations most strongly affect that interaction, but control of other interactions (e.g. with an as yet uncharacterized binding partner) is possible. Phosphorylation of Bru inhibits dimerization, and helps maintain Bru in the repressive state. Reduced

phosphorylation of Bru, which we hypothesize occurs preferentially at the posterior of the oocyte starting at stage 9, alters the activity of Bru. With the phosphomimetic mutant, Bru is fixed in the repressive state, and the reporter regulated only by Bru has weakened posterior activation. The same is not true for *osk* mRNA, as forms of translational activation mediated by other specificity factors (Gunkel et al., 1998; Munro et al., 2006; Vazquez-Pianzola et al., 2011) contribute to expression. With the phosphosilent mutant, phosphorylation is prevented, enhancing activation of the reporter for which Bru is the only specificity factor. Because this mRNA is not translated throughout the egg chamber, there must be other regulatory factors, acting in concert with Bru for activation, which are limiting and perhaps restricted in their distribution.

MATERIALS AND METHODS

Flies and Transgenes

w^{1118} flies were used as the wild type. Transgenic fly stocks were established by standard methods. Expression of the UAS transgenes was driven by the *nosGAL4VP16* (Van Doren et al., 1998) or *matα4-GAL-VP16* driver (Martin and St. Johnston, 2003), as indicated. The *P[UAS-GFP-MS2$_{18}$]* and *P[UAS-GFP-boxB$_6$]* transgenes were generated by first inserting mGFP6 (Haseloff, 1999) into the Asp718 site of pUASp (Rorth, 1998). Then 18 copies of the MS2 binding sites from p8486 or 6 copies of the boxB binding sites from p8918 were inserted as BamHI-BglII fragment into the BamHI site from the pUASp vector.

P[UAS-MCP::HA$_3$::bru2$^-$3$^-$] was generated in multiple steps. First, a short 5' UTR and the coding sequence of the *MCP* gene were amplified by PCR to introduce terminal restriction sites, Asp718 (5') and BamHI (3') in frame. This Asp718-BamHI fragment was inserted into the pUASp vector using the same restriction sites. Then three copies of the HA epitope tag bearing BglII (5') and BamHI (3') at termini were inserted into the BamHI site from the pUASp vector, creating *P[UAS-MCP::HA$_3$]*. Finally, the full-length or partially deleted *bru* cDNA that are bearing point mutations in RRM2 (K239A, F241A) and RRM3 (N521A, F523A) (Snee et al., 2008) was cloned into the BamHI (5') and XbaI (3') sites as either BglII-XbaI (full length and Δ334-416) or BamHI-XbaI (Δ1-146 and Δ1-146 Δ334-416) fragment. Deleting the BamHI-XbaI fragment from *P[UAS-MCP::HA$_3$::bru2$^-$3$^-$]* made *P[UAS-MCP::HA$_3$::bru2$^-$3$^-$, 1-146]*. *P[UAS-λN::HA$_3$::bru2$^-$3$^-$]* was generated by Paul Macdonald.

The *P[UAS-osk1-173::GFP-AB]* transgene was generated by Ginny Pai. *P[osk::HA$_3$]* and *P[osk::GFP]* were constructed by Paul Macdonald and both transgenes rescue the *osk* RNA-null phenotype but only *osk::HA* rescues the protein-null phenotype. *osk^0* was made by Brittany Marches *via* a modified ends-out targeting. *osk^0* was recombined with *P[osk::HA$_3$]* or *P[osk::GFP]* to keep the correct dosage of *osk.*

Point mutations in the *in silico* PKA phosphorylation sites were independently made by site-directed mutagenesis within the 5' region of the *bru* cDNA, which encodes Bru 1-146, and bearing BglII (5') introduced by PCR and internal BamHI (3') at termini. The BglII-BamHI fragment of *bru* was then inserted into the BamHI site in *P[UAS-MCP::HA$_3$::bru2$^-$3$^-$, Δ1-146]* to make various full-length MCP::HA$_3$::Bru2$^-$3$^-$ proteins bearing different point mutations.

Cloning, Expression and Purification of Recombinant Bru and Cup Proteins

GST::Bru was constructed by subcloning full-length *bru* cDNA into pGEX-2TK (GE Healthcare). GST::Cup577-947 was a gift from Robin Wharton (Verrotti and Wharton, 2000). The Bru proteins used for binding were tagged at the amino terminus with six histidine residues provided by the pET-15b (Novagen) vector and used for purification. Internal BamHI site and the EcoRI site in the 3' UTR were used to make *Δ1-146*. Deleting the PflM1-NotI or BamHI-EcoRI fragment from the full-length *bru* cDNA made *Δ334-416* or *1-146*, respectively.

Point mutations in the *in silico* PKA phosphorylation sites were independently made by site-directed mutagenesis within the 5' region of the *bru* cDNA, which encodes 1-146, bearing NdeI (5') introduced by PCR and internal BamHI (3') at termini. NdeI-BamHI fragment was then replaced with equivalent fragment from either *wild-type* or *Δ334-416 bru* cDNA to make various full-length or Δ334-416 proteins bearing different point mutations.

Proteins were expressed in CodonPlus (Stratagene) *E. coli*, after induction with IPTG. Pelleted cells were resuspended in ice-cold lysis buffer (50mM Tris-Cl pH8.0, 150mM NaCl, 1mM EDTA, 1mM DTT, 2mg/ml lysozyme, and 0.1% IGEPAL-CA-630) supplemented with Complete, Mini, EDTA-free protease inhibitor cocktail tablet (Roche). Following incubation on ice for 20 min, cells were lysed by sonication and lysates were centrifuged at 17,000g for 30 min at 4°C to remove debris. Glycerol was added to the supernatant to 20% final volume and the extracts were stored at -70°C.

A different buffer was used to make extracts for protein purification using the His_6 tag. After induction, pelleted cells were resuspended in ice-cold lysis buffer (50mM $NaH_2PO_4 \cdot H_2O$ pH8.0, 300mM NaCl, 20mM imidazole, 0.01% β-Mercaptoethanol, and 2mg/ml lysozyme) supplemented with Complete, Mini, EDTA-free protease inhibitor cocktail tablet (Roche). Following incubation on ice for 20 min, cells were lysed by sonication and lysates were centrifuged at 17,000g for 30 min at 4°C to remove debris. 250µl Ni-NTA Agarose (Quiagen) in 50% slurry was added per 1ml supernatant, and the reaction was incubated for 1-2 hr at 4°C on a rotator. The lysate-Ni-NTA mixture was then loaded into a disposable column equilibrated with the lysis buffer to remove flow-through and washed three times with increasing concentrations of imidazole in lysis buffer (up to 40mM). The proteins were eluted five times with 250mM imidazole in lysis buffer. Glycerol was added to the supernatant to 20% final volume and the extracts were stored at -70°C.

GST Pull-down Assay

Equivalent amount of GST::Bru, GST::Cup or GST was first immobilized on Glutathione Sepharose 4B (GE Healthcare) prepared in 50% slurry in binding buffer (50mM Tris-Cl pH7.5, 150mM NaCl, 10% glycerol, 1mM DTT, and 0.1% IGEPAL-CA-630) supplemented with Complete, Mini, EDTA-free protease inhibitor cocktail tablet (Roche), by incubating with the extract overnight at 4°C on a rotator. The beads were spun down, washed three times and resuspended in binding buffer to make 50% slurry. Then 20µl of this slurry was incubated with ~100ng of each of the N-terminally His_6-tagged Bru proteins in 80µl reaction

containing binding buffer for 2-3 hr at room temperature with rotation. The beads were spun down, washed three times with binding buffer, and boiled in 5µl 2X SDS loading buffer to elute the bound proteins. Eluates were separated by SDS-PAGE and analyzed by Western blot. The mouse anti-His antibody (ABGENT) diluted at 1:2000 and alkaline phosphatase-conjugated goat anti-mouse antibody (Applied Biosystems) diluted at 1:5000 were used to detect Bru proteins.

In Vitro Phosphorylation Assay

Phosphorylation reactions (20µl) contained ~250ng of purified substrate, 1-2 unit of recombinant PKA catalytic subunit, CK1 or CaMKII (all from NEB), and 0.2mM [γ-^{32}P]ATP (adjusted to 250µCi/µmol, Perkin Elmer) in kinase buffer (buffer for PKA: 50mM Tris-Cl pH7.5, 100mM KCl, 5mM $MgCl_2$, and 2.4mM DTT; buffers for CK1 or CaMKII provided by NEB) supplemented with Complete, Mini, EDTA-free protease inhibitor cocktail tablet (Roche). Reactions were incubated at 30°C for 30 min and terminated by addition of 3X SDS loading buffer. Proteins were separated by SDS-PAGE, and gels were dried (Bio-Rad) and exposed to a Phosphor Screen (Molecular Dynamics) for 12 hr. The screen was then analyzed with a Typhoon laser scanner (GE Healthcare).

RNA Binding Assay

The *osk* 3' UTR *AB* probe was transcribed from p8391 using MAXIscript Kit (Ambion) and uniformly labeled with [α-^{32}P]UTP (800Ci/mmol, Perkin Elmer). After phenol/chloroform extraction, unincorporated nucleotides were removed using NucAway spin column (Ambion) and the probe was precipitated with

ammonium acetate/ethanol. UV cross-linking assay was performed as described (Kim-Ha et al., 1995), except that 10X binding buffer consisted of 60mM Hepes pH7.9, 300mM KCl and 20mM $MgCl_2$, and was supplemented with Complete, Mini, EDTA-free protease inhibitor cocktail tablet (Roche). ~500ng of purified recombinant Bru proteins were used. After electrophoresis of cross-linked adducts, gels were dried (Bio-Rad) and exposed to a Phosphor Screen (Molecular Dynamics) for 12 hr. The screen was then analyzed with a Typhoon laser scanner (GE Healthcare).

RNase Protection Assay

Ovaries from young females fed on yeast for 3-4 days were dissected into ice-cold PBS and RNA samples prepared using Tri Reagent-LS (Molecular Research Center) according to the manufacturer's instruction. Assays were performed using the RPA III kit (Ambion) according to the manufacturer's instruction and the results quantitated using ImageJ. Probes were transcribed *in vitro* using the MAXIscript Kit (Ambion) and uniformly labeled with [α-^{32}P]UTP (800Ci/mmol, Perkin Elmer). Unincorporated nucleotides were removed using NucAway spin column (Ambion). After electrophoresis of probes, the gel was transferred to filter paper, covered with plastic wrap and exposed to a Phosphor Screen (Molecular Dynamics) for at least 2 hr. The screen was then analyzed with a Typhoon laser scanner (GE Healthcare).

Detection of Phosphorylated Bruno in the Ovary

Ovaries from young females, fed on yeast for 3-4 days, were dissected and extract was prepared as previously described (Kim-Ha et al., 1995) in ice-cold lysis buffer (50mM Hepes pH7.9, 150mM KCl, and 1% IGEPAL-CA-630) supplemented with Complete, Mini, EDTA-free protease inhibitor cocktail tablet (Roche). Reactions (20µl) contained 10-15µg of ovary extract in phosphatase buffer (50mM Hepes pH7.9, 100mM NaCl, 10mM $MgCl_2$, and 1mM DTT), and where indicated, one or more of the following components were added: 1-2 units of alkaline phosphatase (calf intestinal, NEB), 1.6M beta-glycero phosphate, and 40mM sodium vanadate. Reactions were incubated at 30°C for 1 hr and terminated by addition of 3X SDS loading buffer. Proteins were separated by SDS-PAGE and analyzed by Western blot.

For phosphate-affinity SDS-PAGE using acrylamide-pendant Phos-tag (WAKO), 50µM Phos-tag and 200µM $MnCl_2$ were added to both stacking and separating gels in solution.

The mouse anti-Bru antibody (from Nakamura lab) diluted at 1:8000 and mouse anti-HA antibody (Covance) diluted at 1:1000 were used to detect endogenous and transgenic Bru proteins, respectively. The alkaline phosphatase-conjugated goat anti-mouse antibody (Applied Biosystems) diluted at 1:5000 was used together.

Whole-Mount Ovary Staining

Ovaries from young females fed on yeast for 3-4 days were dissected into PBS at room temperature and fixed in 4% formaldehyde in PBS for 20 min with

gentle mixing. The ovaries were then washed for 30 min in four changes of PBT (PBS plus 0.1% Triton X-100) and blocked for 2 hr in four changes of PBT containing 5% goat serum. For detection of GFP *in situ*, nuclei were counterstained with TO-PRO-3 Iodide (642/661, Invitrogen) diluted 1:1000 in PBT containing 1% goat serum for 1 hr at room temperature. For HA immunostaining, ovaries were incubated with anti-HA antibody (Covance) diluted 1:1000 in PBT containing 1% goat serum for 1-2 hr at room temperature, followed by an overnight rotation at 4°C. Ovaries were washed several times over 3 hr in PBT, then incubated with Alexa Fluor 488 goat anti-mouse antibody (Invitrogen) diluted 1:800 and TO-PRO-3 Iodide (642/661, Invitrogen) diluted 1:1000 in PBT containing 1% goat serum for 2 hr at room temperature in dark. Finally, stained ovaries were washed several times over 3 hr in PBT and then mounted in Vectashield (Vector Labs). Microscopy of all samples made use of a Leica TCS-SP laser scanning confocal microscope.

Quantitation of GFP levels was done using the Macnification software from images obtained using a single plane of focus. The average green fluorescence was sampled from four different regions in the nurse cell cytoplasm of each of 10 to 11, stage 5 or 6 egg chambers.

Co-immunoprecipitation

Ovaries from young females, fed on yeast for 3-4 days, were dissected and extract was prepared as previously described (Kim-Ha et al., 1995) except in ice-cold DXB-100 (25mM Hepes-K pH6.8, 250mM sucrose, 1mM $MgCl_2$, 100mM KCl, 1mM DTT, and 0.1% Triton X-100) supplemented with Complete, Mini,

EDTA-free protease inhibitor cocktail tablet (Roche). To 1ml of 1mg lysate, 2µg of one of the following antibodies was added: rabbit anti-HA tag (Rockland), mouse anti-GFP (Santa Cruz), mouse anti-Bru (from Nakamura lab) and mouse anti-IgG (Santa Cruz). Reactions were incubated at 4°C overnight with rotation to form the immune complex. 13µl of Protein A/G Magnetic Beads (Pierce) was added to each reaction, followed by incubation at 4°C for 1-2 hr. Then the resin was washed three times with DXB-150 (same as DXB-100 except 150mM KCl). After adding 30µl of 2X SDS loading buffer to the resin, samples were vortexed, centrifuged and boiled. Proteins were separated by SDS-PAGE and analyzed by Western blot.

The mouse anti-Bru antibody (from Nakamura lab) diluted at 1:8000 and mouse anti-HA antibody (Covance) diluted at 1:1000 were used to detect endogenous and transgenic Bru proteins, respectively, together with the alkaline phosphatase-conjugated goat anti-mouse antibody (Applied Biosystems) diluted at 1:5000. The rat anti-Cup219 antibody (from Smibert lab) diluted 1:2000 and the alkaline phosphatase-conjugated goat anti-rat antibody (Sigma) diluted at 1:10,000 were used to detect Cup.

Phosphopeptide Analysis of His_6-Tagged Bruno 1-148

Experimental: Trypsin in-gel digest was performed and followed by desalting with a µC18 ziptip (Millipore). The samples were run by LC-MS/MS on a Thermo Orbitrap Elite mass spectrometer equipped with an ultra-high-pressure Dionex Ultimate 3000 RSLC nano-LC system with buffer A (0.1% (v/v) formic acid in water) and buffer B (0.1% (v/v) formic acid in acetonitrile). Peptides were

concentrated onto an in-house packed 100-nm-inner diameter x 2-cm C18 column (Magic C18, 3 μm, 100 Å, Michrom Bioresources Inc.), and then separated on a 75-nm-inner diameter x 15-cm C18 fused silica column (Dionex). Liquid chromatography was performed using a gradient of 3 to 27 to 45% buffer B over 0 to 69 to 80 min, with a total run time of 98 min. Data-dependent acquisition in the orbitrap detector used scan cycles of one MS1 scan (300-1700) at a resolution of 60,000 followed by MS2 at resolution 15,000 on the 10 most intense ions using CID fragmentation with 3 *m/z* isolation width and normalized collision energy of 35. Singly charged ions were excluded. Dynamic exclusion was used with 1 repeat count, 24 sec repeat duration, 12 sec exclusion duration and exclusion list of 500 ions. Technical replicates were performed for each sample.

Peptide/protein identification was performed with ProteomeDiscoverer 1.4 using search nodes SEQUEST_HT (Thermo) and MS-Amanda (Protein Chemistry Facility, Vienna, Austria, http://ms.imp.ac.at/?goto=pd-nodes). The initial search was conducted with SwissProt database (Jan 2014 version with 456701 sequences) and the search parameters used were as follows: two missed cleavages were permitted, static modifications on cysteine carbamidomethylation, dynamic modifications on oxidized methionine, 10 ppm precursor tolerance and 0.02 Da MS/MS tolerance. Peptide identifications were filtered using the Target Decoy validation node where decoy database of reversed sequences is generated for calculating false discovery rates. A high confidence FDR 1% filtering was applied. The contaminant proteins were determined from the initial search, and then a custom database of 10-contaminant trypsin and human keratin protein sequences, and the Bruno

sequence were concatenated with the E. coli database (from Jan 14, 2014) to create a custom database with 4963 sequences. The data was searched with the custom and a matched decoy database using the same parameters as before with the addition of dynamic modification of phosphorylation of serine and threonine and maximum 2 modifications/peptide. The search results were validated with the target decoy and, in parallel, routed to phosphoRS v. 3.0, where scores above 50 are considered good scores. PhosphoRS also assigns a probability for likelihood of a particular phosphorylation site on a given peptide on those peptide identifications made using SEQUEST HT. The results of all searches were filtered for high confidence peptides (FDR 1%), with 2 peptides/protein.

Results: Bruno was observed with 3657 spectral counts from the LC-MS/MS runs searched with SEQUEST_HT and MS-Amanda, along with 27 contaminant proteins having much lower spectral counts. Overexpressed Bruno with His_6 tag had spectral counts covering 50% of the sequence. There is one sequence stretch not amenable to proteolytic digest, which was not observed, but all other peptides were observed with tens of spectral counts. Several phosphopeptides were observed with high confidence only in the kinase-treated sample. (6)ASFLANRR(13) was observed as a phosphopeptide in 3 spectral counts, and the site assignment of phosphorylation of S7 is unambiguous due to the single serine residue. The (6)ASFLANR(12) S7 phosphopeptide is also detected. The peptides (-3)GSHMFTSRASFLANR(12) and (-3)GSHMFTSRASFLANR(13) are also detected as phosphopeptides at high confidence. In this case the site assignment is ambiguous, with phosphoRS assigning equal probability for phosphorylation on T3, S4, and S7. Further

examination of the SEQUEST_HT results reveals that 8 of the 17 MS/MS have a higher XCorr value for the S7 site assignment than any other assignment, and 7 have equivalent XCorr (scores differ by < 0.1) for the T3, S4 and S7 or the S4 and S7 assignments, with the XCorr for all assignments shown in BrunoProtein sheet Bruno phosphopeptides. It is unclear how MS-Amanda site assignments are determined, so those spectral count site assignments are not included. Thus, the parsimonious explanation of the data restricts the assignment of phosphorylation to S7, though it does not rule out the possibility of phosphorylation of S4 and T3. In addition, at least 2 phosphorylation sites are detected on the 18-amino-acid, His-tag sequence in one or both samples.

Genomic Engineering of the *arrest* Locus

Since the entire *aret* locus is too big to target (over 100kb), we targeted a 2.1kb region (12,270,445 to12,272,531; the gene annotation based on Release 5.48) including the first two protein-coding exons that encode the amino terminus of female Bru (up to aa193). First the founder knock-out line was made by a modified ends-out targeting. The targeting donor DNA fragment contained 5' and 3' homologous arms flanking the targeted region, and a loxP-flanked *white+* (*w+*) transgenic marker juxtaposed by an attP site of *Φ*C31, as in pGX-attP (Huang et al., 2009). The 5' arm (12,265,973 to 12,270,438) was made by PCR using the *w1118* genomic DNA. The 4.5kb 5' arm was cloned in three fragments into pCRII-TOPO vector (Invitrogen). The 5'-most fragment was amplified to introduce a 5' NotI site and included the KpnI site near the 3' end. The middle fragment was amplified to include the KpnI near the 5' end and the AflII near the 3' end.

The 3'-most fragment was amplified to introduce a 3' KpnI site and included the AflII site near the 5' end. Finally, these three fragments were ligated together into pGX-attP using NotI and KpnI within the 5' MCS.

The 3' arm (12,272,538 to 12,276,637) was made by PCR using p4045 as a template. The plasmid was modified by converting XhoI at the 5' end of the 3' arm sequence into XbaI by cutting with XhoI, filling in the ends, and ligating with XbaI linker. Similarly, the plasmid was further modified by converting AflIII at the 3' end of the 3' arm sequence into BamHI. Then the XbaI-BamHI fragment was cloned into pGX-attP using SpeI and BglII within the 3' MCS.

Transgenic stocks were established by standard methods. Virgins carrying the targeting donor DNA were crossed to males carrying *hs-flp* and *hs-ISceI* (Bloomington 6935), and the resulting progeny were heat-shocked three times over three days as embryos. Many mosaic-eyed progeny were obtained, indicating ends-out targeting worked. Then the mosaic-eyed virgins were again crossed to males carrying *P{70flp}10* (Bloomington 6938) in order to reduce the number of false positives. The resulting progeny were heat-shocked once three days later as embryos. Red-eyed males were selected and balanced over CyO chromosome after segregation testing. Homozygous females were sterile, as expected for *aret* phenotype. Deletion was further verified by PCR.

The *w+* marker in between the 5' and 3' arms was removed by crossing the knock-out males to virgins carrying *hs-Cre* (Bloomington 1092). There was no heat shock involved because *Cre* was supposed to be constitutively active. The desired progeny, which have the knock-out chromosome and *hs-Cre*-bearing chromosome, were orange-eyed due to the marker present in *hs-Cre*. The orange-eyed males were then crossed to virgins carrying *vasa-ΦC31*

(Bloomington 24749). Balanced stocks were established from a single male and sent for injection with various replacement plasmids in pGE-attB vector. The *w+* marker and extra vector sequences were removed by Cre recombinase, as described above, and balanced stocks were established from a single male. Correct integration and presence of appropriate mutations were verified by PCR.

To produce *aret* mutants containing triple *ala* or *glu* mutations, we performed site-directed mutagenesis with two rounds of PCR for each of *S4/S7* and *T135* mutations. Each of the two PCR fragments were synthesized in the first PCR reaction using a mutagenic primer, a primer that anneals to either the 5' or 3' end of the replacement DNA, and *w1118* DNA as the template. Terminal restriction sites, EcoRI (5') and KpnI (3'), were introduced. The two PCR fragments were purified, and a second PCR was performed using a mix of these two fragments and the two terminal primers used previously. The final mutagenized PCR fragment (~2.1kb) was first cloned into pCRII-TOPO, and EcoRI-KpnI fragment was subcloned into pSP73. Internal EcoRV site (12,271,334) was used to cut out and ligate the 5' EcoRI-EcoRV fragment carrying *S4/S7* mutation, and the 3' EcoRV-KpnI fragment carrying *T135* mutation, into pGE-attB vector using EcoRI and KpnI.

All PCR reactions were carried out using Phusion High-Fidelity PCR Master Mix (Finnzymes) to minimize the error rate.

Acknowledgments

We thank Akira Nakamura and Craig Smibert for antibodies, Anne Ephrussi and Janice Fischer for fly stocks, Kristin Patterson for generously

guiding with the genomic engineering, and members of the Macdonald lab for advice and comments on the manuscript. This work was supported by NIH grant GM54409.

FIGURES

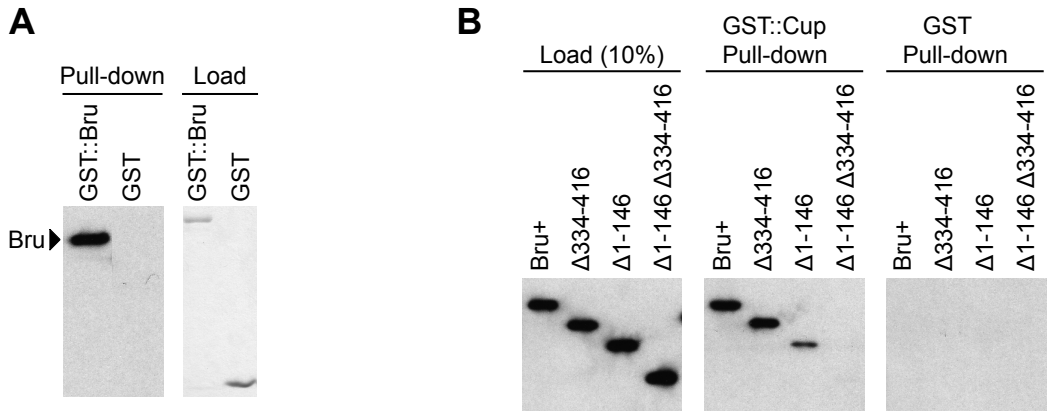


Figure 2.1. Bru dimerizes and Bru domain 1-146 is important for Cup binding

(A) GST pull-down assays using GST::Bru or GST, and the full-length Bru protein. Left panel is a Western blot probed with anti-His_6 antibody, which detects Bru (but not GST::Bru). Right panel is a Coomassie staining of GST::Bru and GST to show the amounts used for the experiment.

(B) GST pull-down assays using GST::Cup or GST, and the Bru proteins as labeled. Each panel is a Western blot probed with anti-His_6 antibody, which detects the Bru proteins (but not GST::Bru).

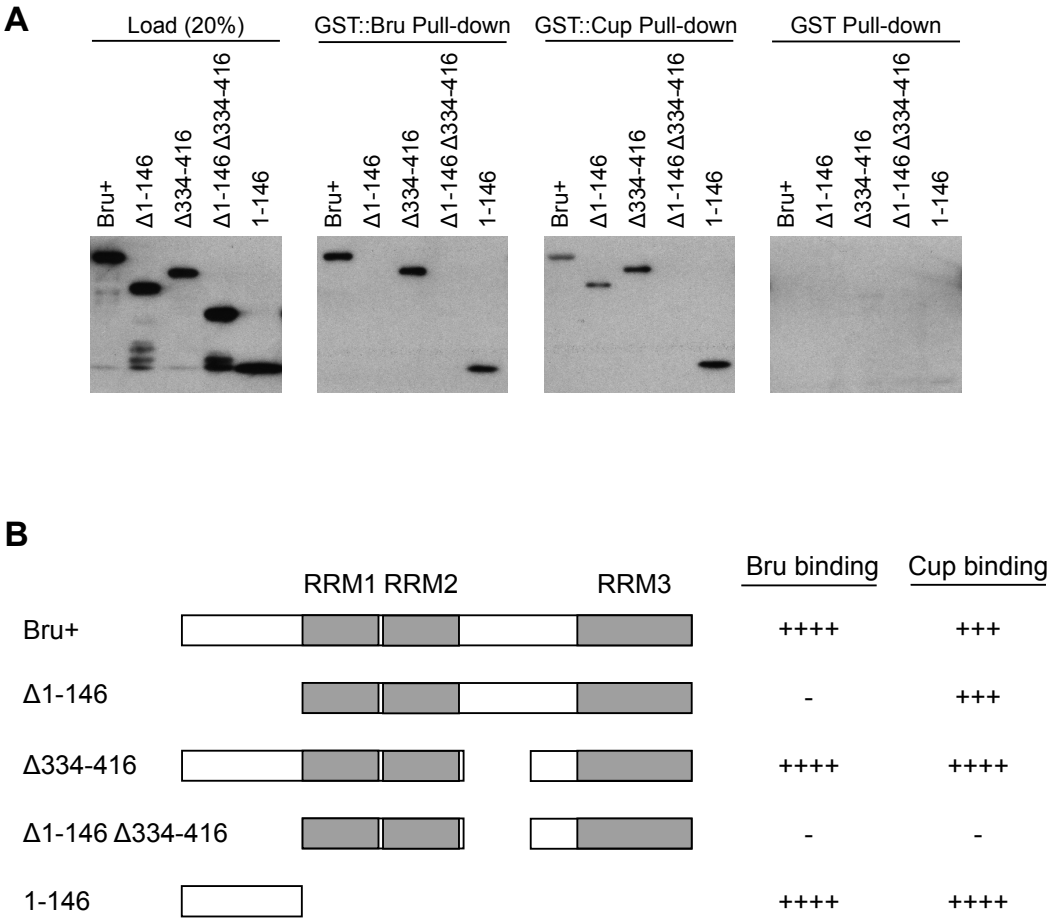


Figure 2.2. Bru domain 1-146 is important for both Bru and Cup binding

(A) GST pull-down assays using the GST fusion proteins indicated at top, and the purified Bru proteins as labeled. Each panel is a Western blot probed with anti-His_6 antibody, which detects the Bru proteins (but not GST::Bru).

(B) A schematic diagram of Bru proteins used in part A. The three RNA Recognition Motifs (RRMs) of Bru are shown as gray boxes; RRM3 is an extended RRM and is thus larger (Lyon et al., 2009). A summary of results from the pull-down experiments is shown on the right. ++++ indicates a wild-type level

of binding, - is no detectable binding, and the intermediate values indicate the relative strengths of impaired binding.

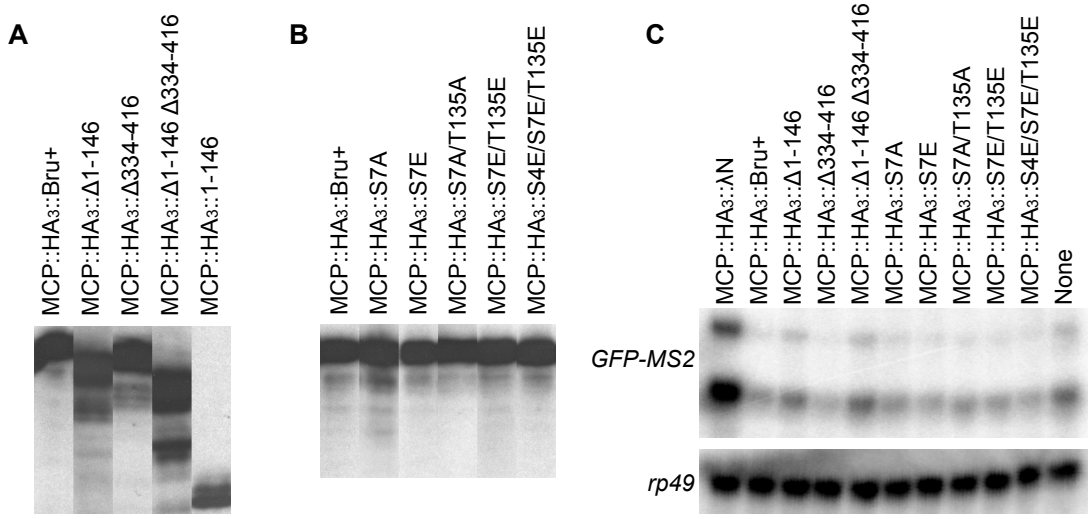


Figure 2.3. Levels of tethered Bru proteins and *GFP-MS2* reporter RNA in ovaries

(A and B) Western blot of ovary extract from flies expressing MCP::HA_3::Bru proteins as labeled. Expression of the UAS transgenes was driven by the *matα4-GAL-VP16* driver (Martin and St. Johnston, 2003). Proteins were detected using anti-HA antibody.

(C) RNase protection assays of *GFP-MS2* and *rp49* mRNAs from ovaries expressing MCP::HA_3::Bru proteins as labeled. Expression of the UAS transgenes was driven by the *nosGAL4VP16* driver (Van Doren et al., 1998). Quantitation of the RNA levels is shown on Fig 2.4L.

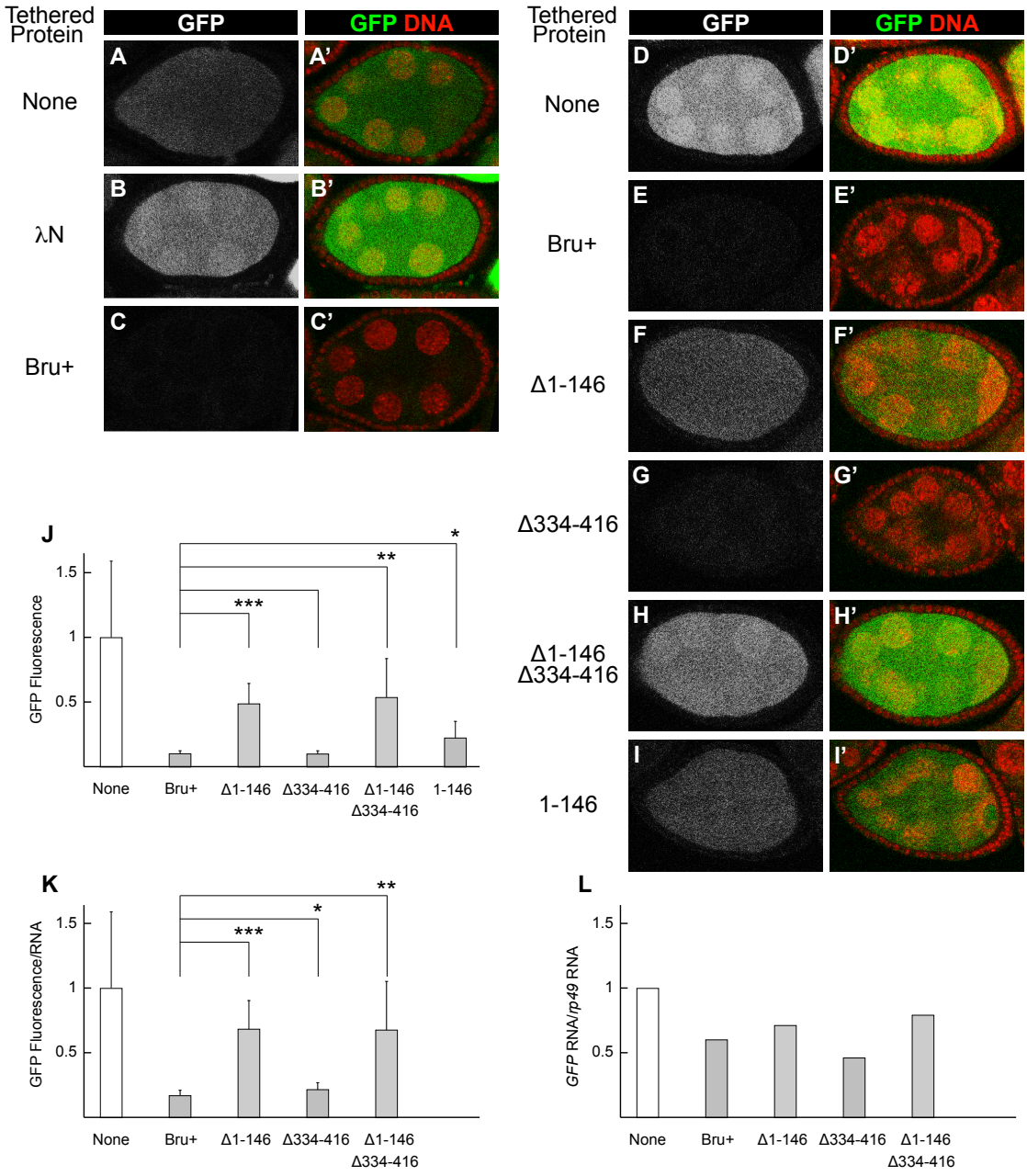


Figure 2.4. The Bru amino-terminal domain is essential for translational repression in a tethering assay

(A-C, A'-C') show egg chambers expressing the *GFP-MS2* reporter mRNA. (B-C, B'-C') also express either MCP::HA_3::λN (B, B') or MCP::HA_3::Bru+ (C, C') protein. All Bru proteins used include point mutations in RRM2 and RRM3 to inhibit the Bru's RNA-binding activity (this has no effect on tethering, which relies on the MCP's RNA-binding activity). Ectopic expression of Bru causes developmental defects early in oogenesis (Filardo and Ephrussi, 2003; Snee et al., 2008), which would complicate the assay. By inhibiting Bru's RNA binding, these defects are eliminated (Snee et al., 2008). (A-C, A'-C') were fixed in parallel and imaged together under the same settings. A phage lambda N protein fused to MCP did not render repression of the reporter but rather caused elevated GFP level (panel B), partly due to an increase in the *GFP-MS2* RNA level (Fig 2.3C).

(D-I, D'-I') show egg chambers expressing the *GFP-MS2* reporter mRNA. (E-I, E'-I') also express MCP::HA_3::Bru proteins, of the type shown at left. (D-I, D'-I') were fixed in parallel and imaged together under the same settings. Expression of the UAS transgenes was driven by the *nosGAL4VP16* driver (Van Doren et al., 1998).

(J) GFP fluorescence was quantitated using Macnification and the value for none, which lacks any MCP::HA_3::Bru proteins, was set to one. The mean and standard deviation were calculated from roughly 40 samples per genotype. The asterisks indicate the Bru proteins with the GFP protein level differing significantly from the Bru+, using the student's T test (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$).

(K) GFP fluorescence was normalized for the *GFP-MS2* RNA levels, which were normalized using the *rp49* RNA levels as in panel (L). The value for none, which lacks any MCP::HA_3::Bru proteins, was set to one. The mean and standard

deviation were calculated from roughly 40 samples per genotype. The asterisks indicate the Bru proteins with the GFP protein/RNA level differing significantly from the Bru+, using the student's T test (*p≤0.05, **p≤0.01, ***p≤0.001).

(L) *GFP-MS2* RNA levels in Fig 2.3C were quantified by ImageJ and normalized using the *rp49* signal. The value for none, which lacks any MCP::HA_3::Bru proteins, was set to one.

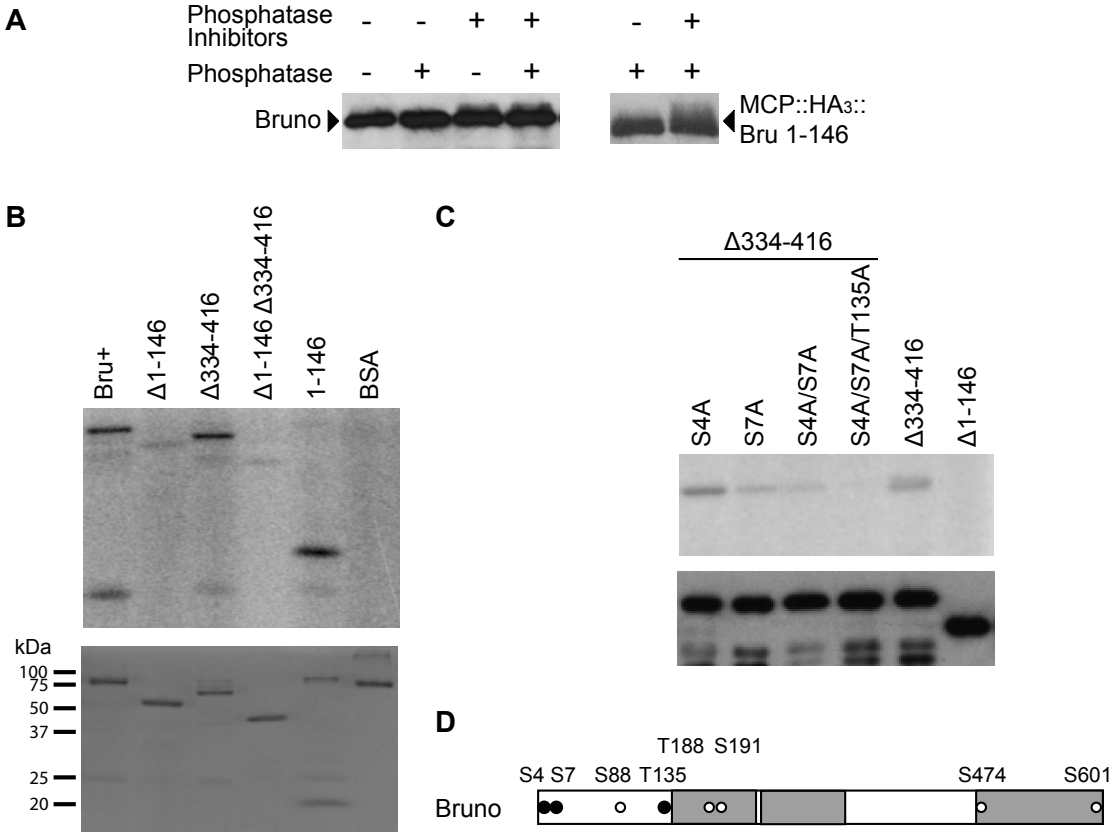


Figure 2.5. PKA phosphorylates Bru in the amino-terminal domain and Bru phosphosilent mutations disrupt phosphorylation by PKA

(A) (Left) Western blot of *wild-type* ovary extract after incubation with phosphatase and/or phosphatase inhibitors as indicated above. Proteins were detected using anti-Bru antibody. (Right) Western blot of ovary extract from flies expressing MCP::HA_3::Bru1-146 protein, with treatments noted as above. Proteins were detected using anti-HA antibody. Inhibitors used were sodium vanadate and beta-glycero phosphate, which are competitive inhibitors of the alkaline phosphatase.

(B) *In vitro* phosphorylation assay using gamma ^{32}P-ATP, purified mouse PKA catalytic subunit and purified Bru proteins as labeled (as in Fig 2.2B). BSA was

used as a negative control. Top: autoradiogram to detect phosphorylation. Bottom: Coomassie staining of proteins used for the phosphorylation assay to show the relative amounts of input proteins. The upper band in 1-146 lane is a contaminating bacterial protein.

(C) *In vitro* phosphorylation assay using gamma ^{32}P-ATP, purified mouse PKA catalytic subunit and purified phosphosilent (Ala) mutant Bru proteins as labeled. The positions of amino acids predicted to be candidates for phosphorylation by PKA are shown in the schematic (D). The point-mutated Bru proteins have the Δ334-416 deletion, which does not affect phosphorylation (panel B). Top: autoradiogram to detect phosphorylation. A similar assay using the same mutations in the context of the full-length Bru is shown in Fig 2.6B. Bottom: Western blot of proteins used in the phosphorylation assay to show the relative amounts of input proteins.

(D) A schematic diagram of Bru showing PKA phosphorylation sites predicted by NetPhosK and KinasePhos. Three amino acids, S4, S7 and T135, depicted as black circles, were tested in different experiments by mutating them to either alanine (phosphosilent) or glutamate (phosphomimetic).

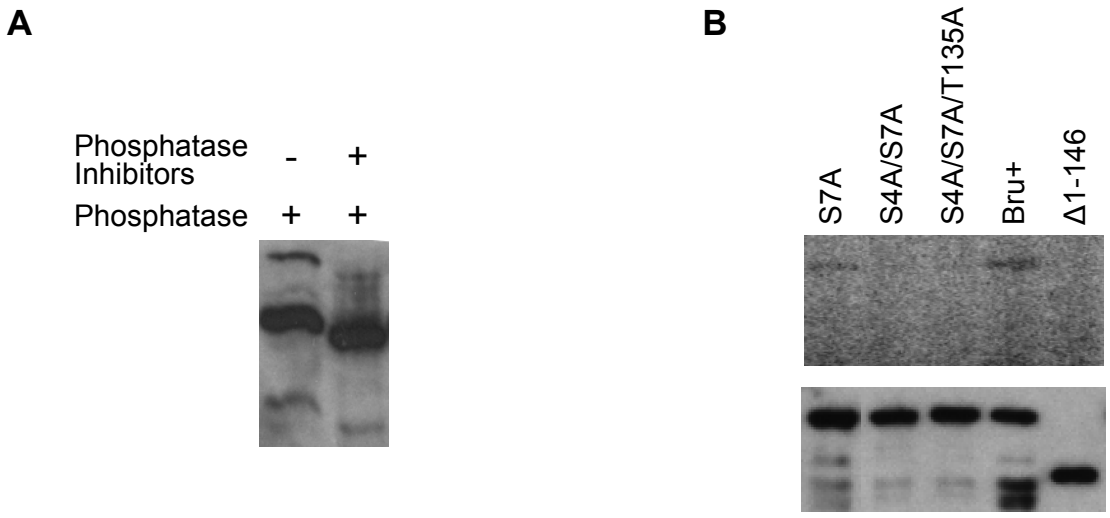


Figure 2.6. Bru is phosphorylated at multiple sites and Bru phosphosilent mutations disrupt phosphorylation by PKA

(A) Phosphate-affinity SDS-PAGE using acrylamide-pendant Phos-tag that separates different phosphoprotein isoforms, followed by Western blot to detect proteins using anti-Bru antibody. When the *wild-type* ovary extract is treated with phosphatase alone, a major Bru band and two upper bands, of which one more distinct and running higher than the other, are seen. When inhibitors are also present, there is a visible smudge consisting of multiple bands above the major Bru band. Inhibitors used were sodium vanadate and beta-glycero phosphate, which are competitive inhibitors of the alkaline phosphatase.

(B) *In vitro* phosphorylation assay using gamma ^{32}P-ATP, purified mouse PKA catalytic subunit and purified phosphosilent (Ala) mutant Bru proteins as labeled. The positions of amino acids predicted to be candidates for phosphorylation by PKA are shown in the schematic Fig 2.5D. The Bru proteins are full length and used at concentrations less than the Δ334-416 Bru proteins in Fig 2.5C. Top: autoradiogram to detect phosphorylation and show that compared to Bru+, S7A

mutant has reduced phosphorylation. Phosphorylation of both S4A/S7A and S4A/S7A/T135A mutants is undetectable as with Δ1-146. Bottom: Western blot of proteins used in the phosphorylation assay to show the relative amounts of input proteins.

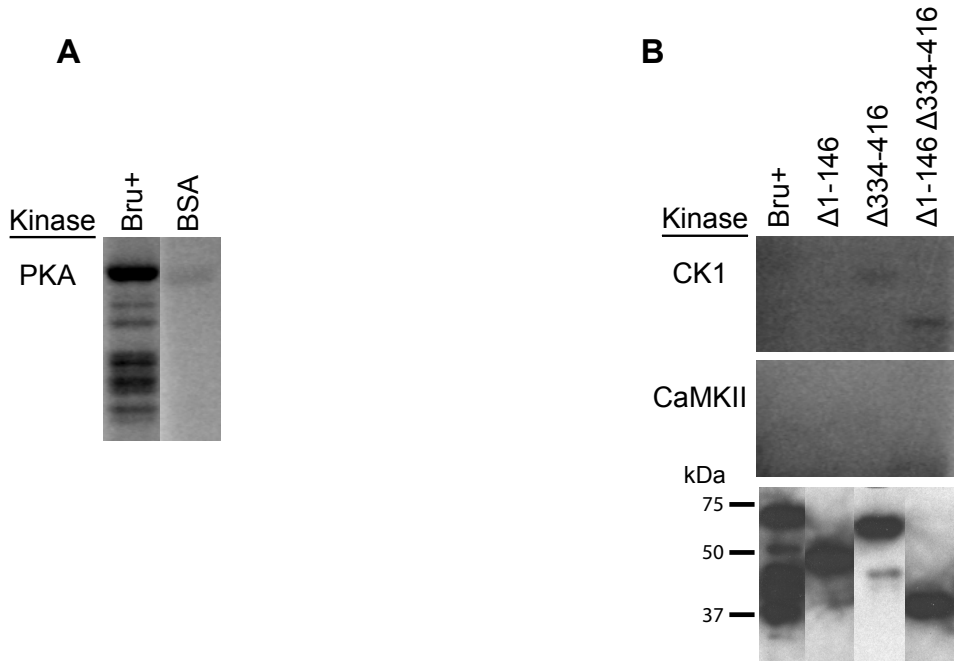
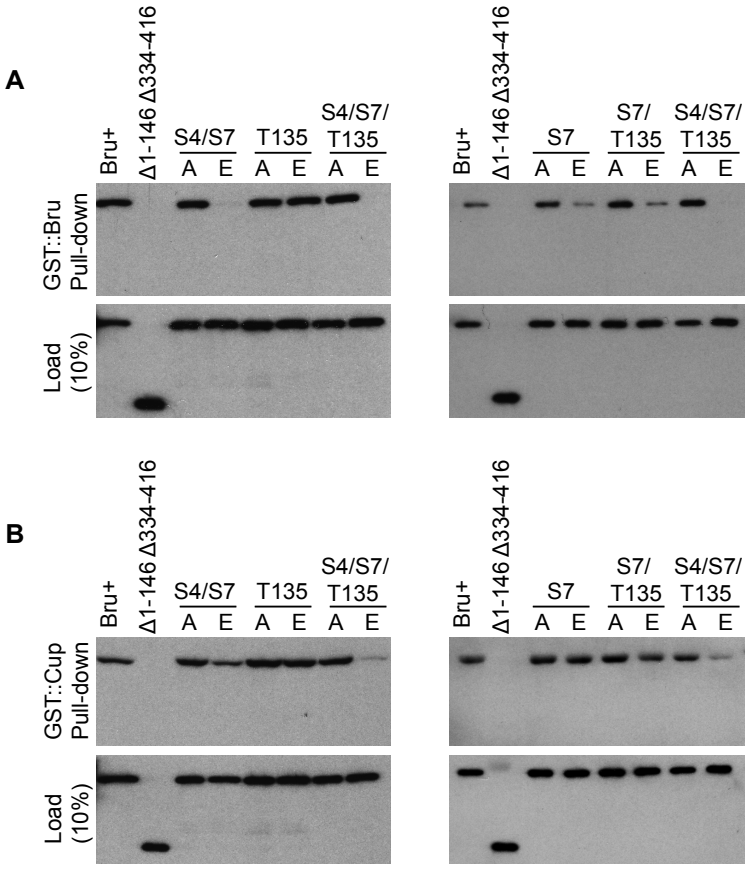


Figure 2.7. Bru is phosphorylated by PKA but not by CK1 or CaMKII

(A) *In vitro* phosphorylation assay using gamma ^{32}P-ATP, purified mouse PKA catalytic subunit, and purified Bru or BSA. The amount of substrates used was equivalent, but a lot higher than that shown in Fig 2.5B. The autoradiogram shows that compared to BSA, Bru+ is strongly phosphorylated.

(B) *In vitro* phosphorylation assay using gamma ^{32}P-ATP, purified rat CK1 (top) or purified rat CaMKII (middle), and purified Bru proteins as labeled. Top and middle: autoradiograms to detect phosphorylation. Both Δ334-416 and Δ1-146 Δ334-416 proteins show a low amount of phosphorylation by CK1. Bottom: Western blot of proteins used in the phosphorylation assay to show the relative amounts of input proteins. The amount of Bru+ used was equivalent in both panels.



C

Mutated Site(s)	Mutation	Bru binding	Cup binding
S7	A	++++	++++
	E	++	++++
T135	A	++++	++++
	E	++++	++++
S7/T135	A	++++	++++
	E	++	+++
S4/S7	A	++++	++++
	E	-	++
S4/S7/T135	A	++++	++++
	E	-	+

Figure 2.8. Bru phosphomimetic mutations additively impair both Bru-Cup and Bru-Bru interactions

GST::Bru (A) and GST::Cup (B) pull-down assays to detect interactions with Bru proteins. The input proteins are indicated at top, with Δ1-146 Δ334-416 as a negative control. Each panel is a Western blot probed with anti-His_6 antibody, which detects the Bru proteins (but not GST::Bru).

(C) A summary of results from the pull-down assay. ++++ indicates a wild-type level of binding, - is no detectable binding, and the intermediate values indicate the relative strengths of impaired binding.

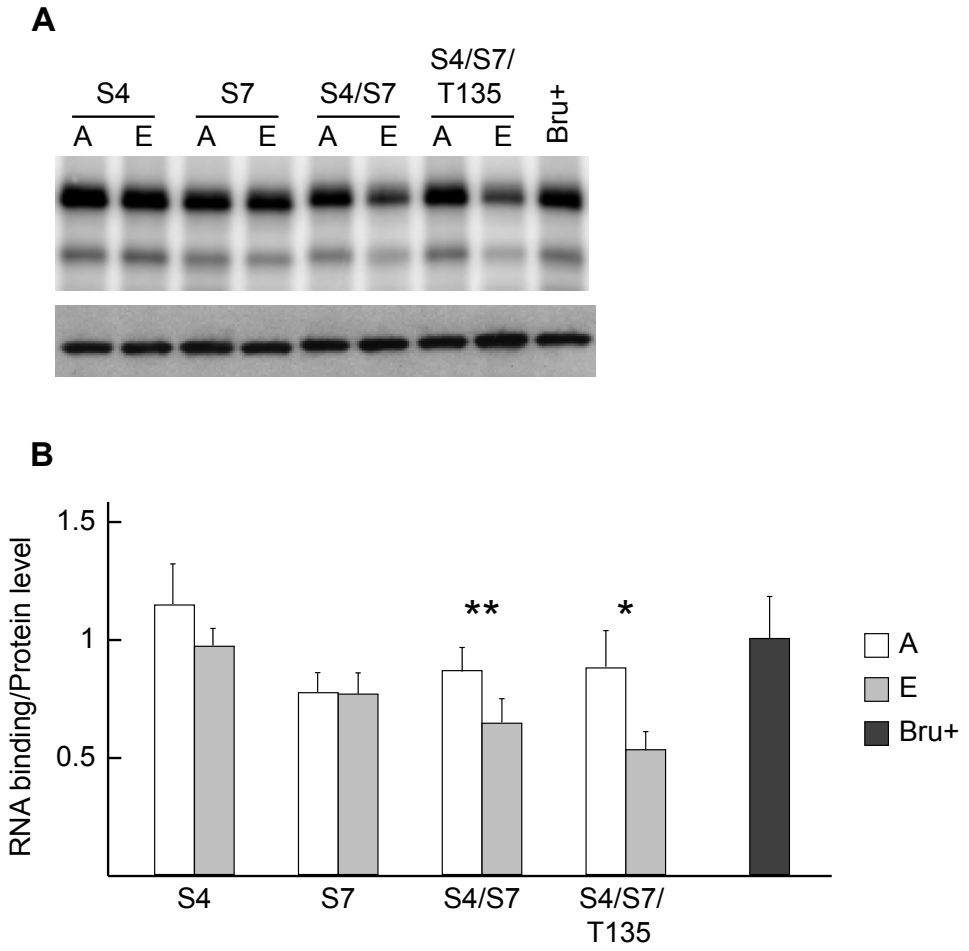


Figure 2.9. Bru phosphomimetic mutations impair Bru's *osk* RNA-binding activity

(A) UV-crosslinking assay of Bru binding to the radiolabeled *osk* 3' UTR AB region RNA. The Bru proteins used are indicated above the autoradiogram showing crosslinked Bru. At bottom is a Western blot of input proteins showing the relative amounts used in the assay.

(B) The *osk* RNA-binding activity and Bru protein levels were quantitated using ImageJ. The RNA binding was normalized for the protein level, and the value for Bru+ was set to one. The mean and SEM were calculated from three

independent experiments. The change in RNA-binding activity in a pair-wise comparison was considered significant in S4/S7 and S4/S7/T135 using the student's T test (*$p \leq 0.05$; **$p \leq 0.01$). The change in RNA binding of S4E/S7E (26% from the ala counterpart with $p=0.008$) was considered more statistically significant than that of S4E/S7E/T135E (40% decrease from the ala counterpart with $p=0.04$), due to a greater sample variation of S4/S7/T135.

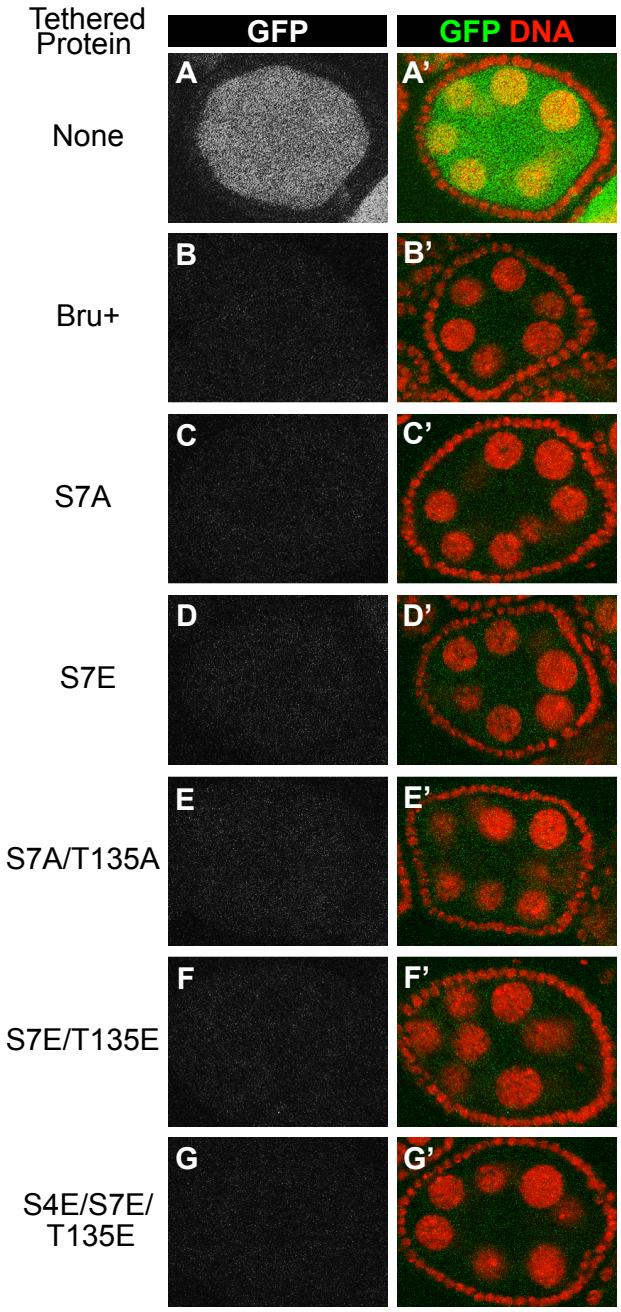


Figure 2.10. Interaction-defective Bru mutants retain strong repressive activity in the tethering assay

(A-G, A'-G') show egg chambers expressing the *GFP-MS2* reporter mRNA. (B-G, B'-G') also express MCP::HA_3::Bru proteins, of the type shown at left. All Bru proteins include point mutations in RRM2 and RRM3 to inhibit the Bru's RNA- binding activity (this has no effect on tethering, which relies on the MCP's RNA-binding activity). All samples were fixed in parallel and imaged together under the same settings. Expression of the UAS transgenes was driven by the *nosGAL4VP16* driver (Van Doren et al., 1998).

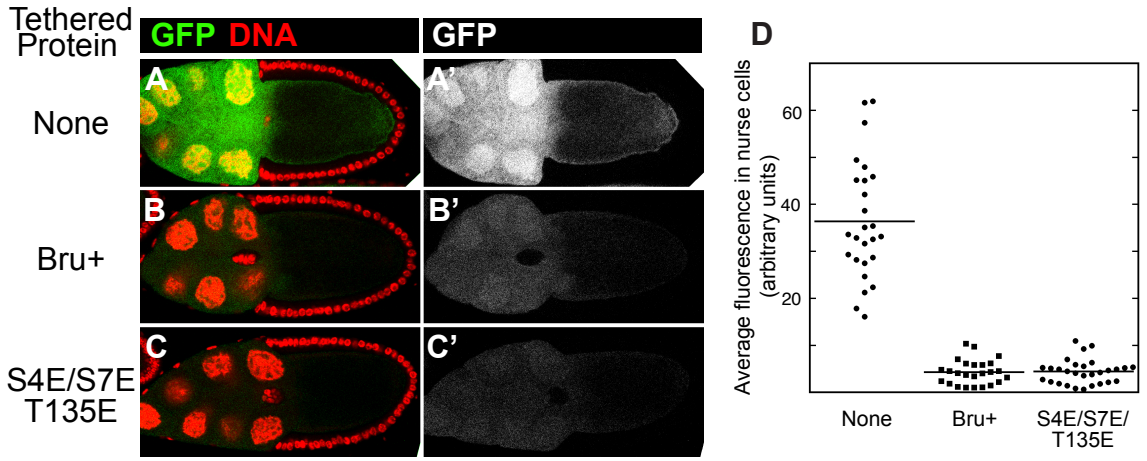


Figure 2.11. Interaction-defective Bru mutants can repress translation of the *GFP-boxB* reporter in a tethering assay

(A-C, A'-C') show egg chambers expressing the *GFP-boxB* reporter mRNA. (B-C, B'-C') also express λN::HA_3::Bru proteins, of the type shown at left. All Bru proteins include point mutations in RRM2 and RRM3 to inhibit the Bru's RNA-binding activity (this has no effect on tethering, which relies on the λN's RNA-binding activity). All samples were fixed in parallel and imaged together under the same settings. Expression of the UAS transgenes was driven by the *matα4-GAL-VP16* driver (Martin and St. Johnston, 2003). The levels of the MCP::Bru fusion proteins are expected to be similar since they were inserted at the same genomic location *via* ΦC31-mediated DNA integration, and the mutations in Bru did not alter Bru protein stability in the other tethering assay.

(D) GFP fluorescence was quantitated using Macnification. The mean was calculated from over 20 samples per genotype.

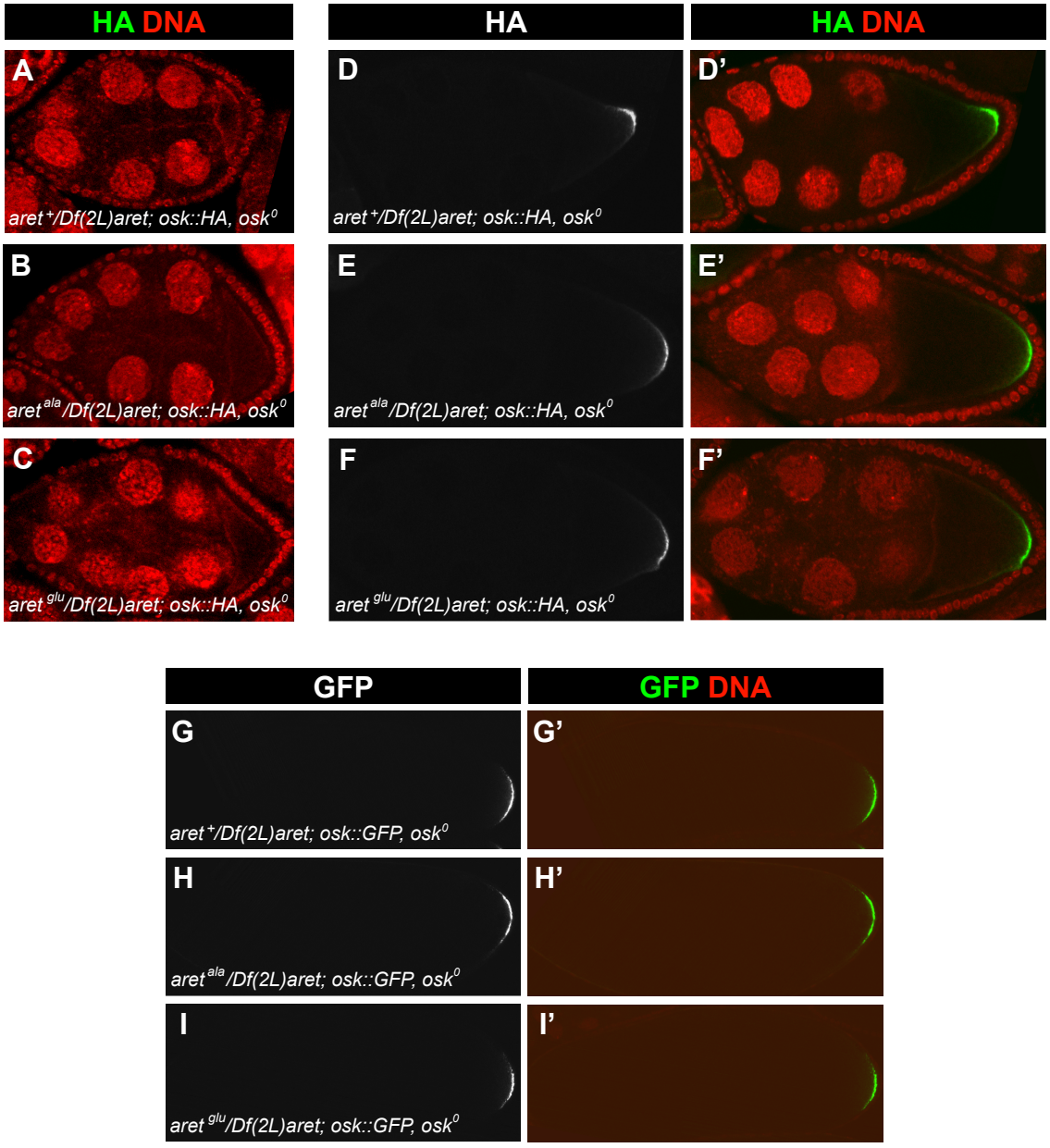


Figure 2.12. The Osk expression pattern in *aret* replacement lines

(A-F, D'-F') show egg chambers expressing the *osk::HA* genomic transgene in different genetic backgrounds with a distinct, genetically engineered *aret* gene, as labeled. All have a single copy of *aret* in *trans* to *Df(2L)aret* and two copies of

the *osk* transgene. (A-C) are stage 8, and (D-F) are stage 9 egg chambers. (D'-F') show HA in green and nuclei in red. All samples were fixed in parallel and imaged together under the same settings.

(G-I, G'-I') show late-stage egg chambers expressing the *osk::GFP* genomic transgene in different genetic backgrounds with a distinct, genetically engineered *aret* gene, as labeled. All have a single copy of *aret* in *trans* to *Df(2L)aret* and two copies of the *osk* transgene. (G'-I') show GFP in green and nuclei in red. All samples were fixed in parallel and imaged together under the same settings.

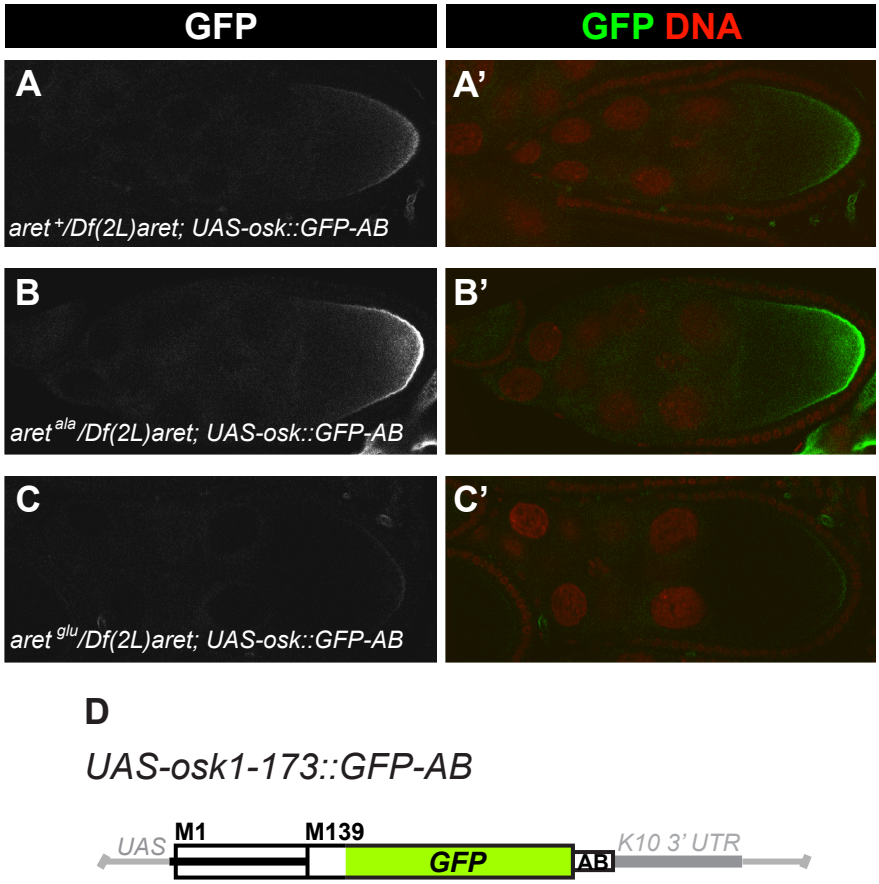


Figure 2.13. The *osk1-173::GFP-AB* reporter expression in *aret* replacement lines

(A-C, A'-C') show egg chambers expressing the *osk1-173::GFP-AB* reporter in different genetic backgrounds with a distinct, genetically engineered *aret* gene, as labeled. All have a single copy of *aret* in *trans* to *Df(2L)aret* and a single copy of the reporter. (A'-C') show GFP in green and nuclei in red. All samples were fixed in parallel and imaged together under the same settings. Expression of the UAS transgene was driven by the *matα4-GAL-VP16* driver (Martin and St. Johnston, 2003).

(D) A schematic diagram of the *UAS-osk1-173::GFP-AB* reporter. The large rectangle comprises a part of the *osk* coding sequence including two start codons and up to T173 and a full *GFP* coding sequence as labeled. The *osk 3' UTR AB* region, which contains the *BRE*s, is depicted as the small rectangle. The thick black line includes the 15nt *5' UTR* common to both Osk isoforms and *5' UTR* specific for the short isoform. The thick gray line is the 0.4kb *K10 3' UTR* fragment, part of the pUASp vector sequence in gray (Rorth, 1998).

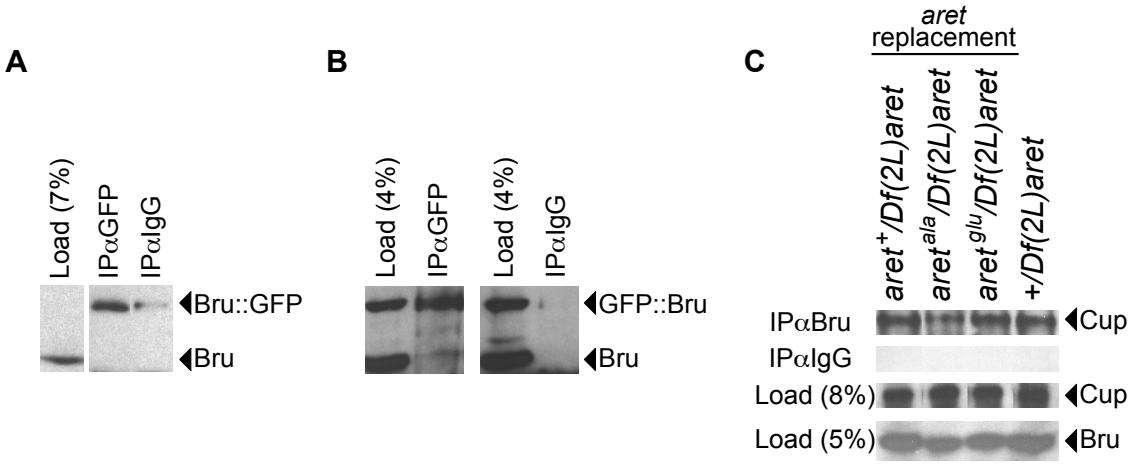


Figure 2.14. Bru amino-terminal mutations do not disrupt Cup binding *in vivo*

(A) Immunoprecipitation from ovary extract expressing Bru::GFP using the antibodies as labeled. Each panel is a Western blot probed with anti-Bru antibody, which detects the Bru proteins. Expression of the UAS transgene was driven by the *matα4-GAL-VP16* driver (Martin and St. Johnston, 2003).

(B) Immunoprecipitation from ovary extract expressing GFP::Bru using the antibodies as labeled. Each panel is a Western blot probed with anti-Bru antibody, which detects the Bru proteins. Expression of the UAS transgene was driven by the *matα4-GAL-VP16* driver (Martin and St. Johnston, 2003).

(C) Immunoprecipitation from the *wild-type* (+) or genetically engineered *aret* ovary extracts as labeled, using the antibodies as labeled. All have a single copy of *aret* in *trans* to *Df(2L)aret.* Each panel is a Western blot probed with anti-Cup or anti-Bru antibody.

TABLES

Maternal Genotype [a]	Cuticular Phenotype (%) [b] Wild Type	No Cuticle Development	N
aret^{+}/*Df(2L)aret*	99	1	686
aret^{ala}/*Df(2L)aret*	95	5	455
aret^{glu}/*Df(2L)aret*	82	18	421

Table 2.1. Embryonic development of various *aret* replacement lines

[a] All have a single copy of *aret* in *trans* to *Df(2L)aret*, which removes much of *aret* and some of *bru-2*. *aret*$^{+}$ is the *wild-type* replacement. Most of the replacement lines, when homozygous, showed maternal-effect lethality. This was not due to the *aret* mutations, since this phenotype was not present for hemizygotes. Consequently, all replacement lines used here and in other experiments were tested in a hemizygous background.

[b] Embryos had either wild-type (with 8 abdominal denticle belts) or no cuticle.

Maternal Genotype [a]	Oocyte GFP level (%) [b]			
	Strong	Weak	Undetectable	n
aret^{+}*/Df(2L)aret; UAS-osk1-173::GFP-AB*	55	21	24	29
aret^{ala}*/Df(2L)aret; UAS-osk1-173::GFP-AB*	60	23	17	30
aret^{glu}*/Df(2L)aret; UAS-osk1-173::GFP-AB*	15	23	62	26

Table 2.2. Expression of *UAS-osk1-173::GFP-AB* in various *aret* replacement lines

a All have a single copy of *aret* in *trans* to *Df(2L)aret*, which removes much of *aret* and some of *bru-2*. *aret*$^{+}$ is the *wild-type* replacement.

b The number of stage 9/10 egg chambers displaying strong, weak or undetectable GFP signal was scored.

REFERENCES

Bardwell, V. J., & Wickens, M. (1990). Purification of RNA and RNA-protein complexes by an R17 coat protein affinity method. Nucleic Acids Research, 18(22), 6587–6594.

Besse, F., López de Quinto, S., Marchand, V., Trucco, A., & Ephrussi, A. (2009). Drosophila PTB promotes formation of high-order RNP particles and represses oskar translation. Genes & Development, 23(2), 195–207.

Castagnetti, S., & Ephrussi, A. (2003). Orb and a long poly (A) tail are required for efficient oskar translation at the posterior pole of the Drosophila oocyte. Development, 130(5), 835–843.

Castagnetti, S., Hentze, M. W., Ephrussi, A., & Gebauer, F. (2000). Control of oskar mRNA translation by Bruno in a novel cell-free system from Drosophila ovaries. Development, 127(5), 1063–1068.

Chekulaeva, M., Hentze, M. W., & Ephrussi, A. (2006). Bruno Acts as a Dual Repressor of oskar Translation, Promoting mRNA Oligomerization and Formation of Silencing Particles. Cell, 124(3), 521–533.

De Gregorio, E. (1999). Translation driven by an eIF4G core domain in vivo. The EMBO Journal, 18(17), 4865–4874.

Doren, M. V., Williamson, A. L., & Lehmann, R. (1998). Regulation of zygotic gene expression in Drosophila primordial germ cells. Current Biology, 8(4), 243–246.

Ephrussi, A., Dickinson, L. K., & Lehmann, R. (1991). Oskar organizes the germ plasm and directs localization of the posterior determinant nanos. Cell, 66(1), 37–50.

Ephrussi, A., & Lehmann, R. (1992). Induction of germ cell formation by oskar. Nature, 358(6385), 387–392.

Filardo, P., & Ephrussi, A. (2003). Bruno regulates gurken during Drosophila oogenesis. Mechanisms of Development, 120(3), 289–297.

González-Reyes, A., Elliott, H., & St Johnston, D. (1995). Polarization of both major body axes in Drosophila by gurken-torpedo signalling. Nature, 375(6533), 654–658.

Gunkel, N., Yano, T., Markussen, F. H., Olsen, L. C., & Ephrussi, A. (1998). Localization-dependent translation requires a functional interaction between the 5“ and 3” ends of oskar mRNA. Genes & Development, 12(11), 1652–1664.

Haseloff, J. (1999). GFP variants for multispectral imaging of living cells. Methods in Cell Biology, 58, 139–151.

Huang, J., Zhou, W., Dong, W., Watson, A. M., & Hong, Y. (2009). Directed, efficient, and versatile modifications of the Drosophila genome by genomic engineering. Proceedings of the National Academy of Sciences of the United States of America, 106(20), 8284–8289.

Igreja, C., & Izaurralde, E. (2011). CUP promotes deadenylation and inhibits decapping of mRNA targets. Genes & Development, 25(18), 1955–1967.

Kim-Ha, J., Kerr, K., & Macdonald, P. M. (1995). Translational regulation of oskar mRNA by Bruno, an ovarian RNA-binding protein, is essential. Cell, 81(3), 403–412.

Kim-Ha, J., Smith, J. L., & Macdonald, P. M. (1991). oskar mRNA is localized to the posterior pole of the Drosophila oocyte. Cell, 66(1), 23–35.

Lane, M. E., & Kalderon, D. (1994). RNA localization along the anteroposterior axis of the Drosophila oocyte requires PKA-mediated signal transduction to direct normal microtubule organization. Genes & Development, 8(24), 2986–2995.

LeCuyer, K. A., Behlen, L. S., & Uhlenbeck, O. C. (1995). Mutants of the bacteriophage MS2 coat protein that alter its cooperative binding to RNA. Biochemistry, 34(33), 10600–10606.

Lehmann, R., & Nüsslein-Volhard, C. (1986). Abdominal segmentation, pole cell formation, and embryonic polarity require the localized activity of oskar, a maternal gene in drosophila. Cell, 47(1), 141–152.

Lie, Y. S., & Macdonald, P. M. (1999). Translational regulation of oskar mRNA occurs independent of the cap and poly(A) tail in Drosophila ovarian extracts. Development, 126(22), 4989–4996.

Lipshitz, H. D., & Smibert, C. A. (2000). Mechanisms of RNA localization and translational regulation. Current Opinion in Genetics & Development, 10(5), 476–488.

Lyon, A. M., Reveal, B. S., Macdonald, P. M., & Hoffman, D. W. (2009). Bruno protein contains an expanded RNA recognition motif. Biochemistry, 48(51), 12202–12212.

Markussen, F.-H., Michon, A.-M., Breitwieser, W., & Ephrussi, A. (1995). Translational control of oskar generates short OSK, the isoform that induces pole plasma assembly. Development, 121(11), 3723–3732.

Martin, S. G., & St Johnston, D. (2003). A role for Drosophila LKB1 in anterior-posterior axis formation and epithelial polarity. Nature, 421(6921), 379–384.

Munro, T. P., Kwon, S., Schnapp, B. J., & St Johnston, D. (2006). A repeated IMP-binding motif controls oskar mRNA translation and anchoring independently of Drosophila melanogaster IMP. The Journal of Cell Biology, 172(4), 577–588.

Nakamura, A., Amikura, R., Hanyu, K., & Kobayashi, S. (2001). Me31B silences translation of oocyte-localizing RNAs through the formation of cytoplasmic RNP complex during Drosophila oogenesis. Development, 128(17), 3233–3242.

Nakamura, A., Sato, K., & Hanyu-Nakamura, K. (2004). Drosophila cup is an eIF4E binding protein that associates with Bruno and regulates oskar mRNA translation in oogenesis. Developmental Cell, 6(1), 69–78.

Palacios, I. M., & St Johnston, D. (2001). Getting the message across: the intracellular localization of mRNAs in higher eukaryotes. Annual Review of Cell and Developmental Biology, 17, 569–614.

Reveal, B., Garcia, C., Ellington, A., & Macdonald, P. (2011). Multiple RNA binding domains of Bruno confer recognition of diverse binding sites for translational repression. RNA Biology, 8(6), 1047–1060.

Reveal, B., Yan, N., Snee, M. J., Pai, C.-I., Gim, Y., & Macdonald, P. M. (2010). BREs Mediate Both Repression and Activation of oskar mRNA Translation and Act In trans. Developmental Cell, 18(3), 496–502.

Rongo, C., Gavis, E. R., & Lehmann, R. (1995). Localization of oskar RNA regulates oskar translation and requires Oskar protein. Development, 121(9), 2737–2746.

Roth, S., Shira Neuman-Silberberg, F., Barcelo, G., & Schüpbach, T. (1995). cornichon and the EGF receptor signaling process are necessary for both anterior-posterior and dorsal-ventral pattern formation in Drosophila. Cell, 81(6), 967–978.

Rørth, P. (1998). Gal4 in the Drosophila female germline. Mechanisms of Development, 78(1-2), 113–118.

Smith, J. L., Wilson, J. E., & Macdonald, P. M. (1992). Overexpression of oskar directs ectopic activation of nanos and presumptive pole cell formation in Drosophila embryos. Cell, 70(5), 849–859.

Snee, M., Benz, D., Jen, J., & Macdonald, P. M. (2008). Two distinct domains of Bruno bind specifically to the oskar mRNA. RNA Biology, 5(1), 1–9.

St Johnston, D., & Nusslein-Volhard, C. (1992). The origin of pattern and polarity in the Drosophila embryo. Cell, 68(2), 201–219.

Theurkauf, W. E., Alberts, B. M., Jan, Y. N., & Jongens, T. A. (1993). A central role for microtubules in the differentiation of Drosophila oocytes. Development, 118(4), 1169–1180.

Theurkauf, W. E., Smiley, S., Wong, M. L., & Alberts, B. M. (1992). Reorganization of the cytoskeleton during Drosophila oogenesis: implications for axis specification and intercellular transport. Development, 115(4), 923–936.

Vanzo, N. F., & Ephrussi, A. (2002). Oskar anchoring restricts pole plasm formation to the posterior of the Drosophila oocyte. Development, 129(15), 3705–3714.

Vazquez-Pianzola, P., Urlaub, H., & Suter, B. (2011). Pabp binds to the osk 3'UTR and specifically contributes to osk mRNA stability and oocyte accumulation. Developmental Biology, 357(2), 404–418.

Verrotti, A. C., & Wharton, R. P. (2000). Nanos interacts with cup in the female germline of Drosophila. Development, 127(23), 5225–5232.

Webster, P. J., Liang, L., Berg, C. A., Lasko, P., & Macdonald, P. M. (1997). Translational repressor bruno plays multiple roles in development and is widely conserved. Genes & Development, 11(19), 2510–2521.

Wilhelm, J. E., Hilton, M., Amos, Q., & Henzel, W. J. (2003). Cup is an eIF4E binding protein required for both the translational repression of oskar and the recruitment of Barentsz. The Journal of Cell Biology, 163(6), 1197–1204.

Yoshida, S., Müller, H.-A. J., Wodarz, A., & Ephrussi, A. (2004). PKA-R1 spatially restricts Oskar expression for Drosophila embryonic patterning. Development, 131(6), 1401–1410.

Zappavigna, V., Piccioni, F., Villaescusa, J. C., & Verrotti, A. C. (2004). Cup is a nucleocytoplasmic shuttling protein that interacts with the eukaryotic translation initiation factor 4E to modulate Drosophila ovary development. Proceedings of the National Academy of Sciences of the United States of America, 101(41), 14800–14805.

Chapter 3: Investigating the Role of PKA in Bruno-dependent Translational Regulation[2]

ABSTRACT

Translational control of localizing mRNAs is a common and an efficient way to restrict protein synthesis in cells. In Drosophila, several RNAs are localized to a distinct subcellular location within an oocyte and embryo to direct body patterning. Oskar (Osk) is a posterior-patterning determinant in Drosophila and is highly concentrated at the posterior pole of the oocyte. Tight spatial and temporal restriction of the Osk patterning activity is essential for proper development of the embryo. Bruno (Bru) directly binds to the *osk* mRNA and represses translation during mRNA localization to the posterior pole. After *osk* mRNA localization, repression must be alleviated to allow accumulation of Osk protein. How this is achieved is unclear, but local inactivation of repressors at the site of protein synthesis is an option that has been previously reported for localized mRNAs in other systems. cAMP-dependent protein kinase (PKA) is expressed in the ovary, and the loss-of-function mutants in the type 1 regulatory subunit (*Pka-R1*) are defective in repression of *osk* translation. Here we test the role of PKA on Bru phosphorylation and Bru-mediated repression. Altering PKA activity causes small, yet detectable changes in Bru phosphorylation and Bru-dependent translational repression using an *osk::GFP* reporter bearing a Bru-binding region. Notably, however, the changes detected by altering PKA activity

---

[2] Goheun Kim and Paul M. Macdonald, Department of Molecular Biosciences, Institute for Cellular and Molecular Biology, The University of Texas at Austin

are very different from those using Bru phosphomimetic and phosphosilent mutations. The reason for this difference is not known. PKA must phosphorylate many different proteins, with the potential for both direct and indirect effects on Bru and on *osk* regulation.

INTRODUCTION

A vast number of mRNAs are localized to a specific subcellular location to establish functionally distinct compartments. Localizing mRNAs are also translationally regulated, such that they are repressed during transport and derepressed or activated upon localization (reviewed in Besse and Ephrussi, 2008; Kugler and Lasko, 2009; Martin and Ephrussi, 2009). Localizing mRNAs function in a diverse set of cellular events such as epithelial cell polarity (Horne-Badovinac and Bilder, 2008; Li et al., 2008), cell migration (Condeelis and Singer, 2005), cell fate specification (Long et el., 1997; Lambert and Nagy, 2002; Kugler and Lasko, 2009), synaptic plasticity (Sutton and Schuman, 2006) and guidance of axonal growth cones (Lin and Holt, 2007). In addition to providing spatial restriction of gene expression, mRNA localization provides a high temporal resolution given that a local stimulus can induce protein synthesis on-site rather than a relatively delayed nuclear response (Martin and Ephrussi, 2009).

Localizing mRNAs are packaged into Ribonucleoproteins (RNPs) containing *trans*-acting, RNA-binding proteins that bind *cis*-acting, regulatory elements in RNA and mediate localization and/or translational repression. RNA-binding proteins that repress translation are often phosphorylated at the final destination by a pre-localized kinase or in response to a specific signal (Besse and Ephrussi, 2008). For example, Puf6 and Khd1 are proteins that bind the *ASH1* mRNA and repress translation in budding yeast. Phosphorylation of Puf6 by Ck2 (casein kinase-II) and Khd1 by Yck1 (type I casein kinase) reduce their RNA binding affinity for *ASH1*, leading to translational derepression (Paquin et al., 2007; Deng et al., 2008). Both Ck2 and Yck1 localize to the bud cortex where

ASH1 is translated. In neurons, translation of localized mRNAs occurs in response to specific external signals, such as synaptic activation and axonal guidance cues (Bramham and Wells, 2007; Lin and Holt, 2007). For instance, Glu stimulation induces Aurora kinase-dependent phosphorylation of CPEB, after which it switches from being a translation repressor to an activator of cytoplasmic polyadenylation element (CPE)-containing *CAMKIIα* target mRNA, by promoting elongation of the poly(A) tail (Huang et al., 2002).

The *oskar (osk)* mRNA is transcribed in nurse cells and transported into the oocyte early in oogenesis (Ephrussi et al., 1991; Kim-Ha et al., 1991). Then the mRNA is localized to the posterior pole at stage 9 of oogenesis, when Osk protein is first made (Kim-Ha et al., 1995; Markussen et al., 1995; Rongo et al., 1995). Usage of alternative start codons that are in the same reading frame within the coding sequence makes two Osk isoforms, which have distinct functions (Markussen et al., 1995). Long Osk is required for anchoring of *osk* RNA and Short Osk protein. Short Osk induces formation of the germ plasm containing determinants for both posterior patterning and germ cell formation (Markussen et al., 1995; Vanzo and Ephrussi, 2002).

Translational repression of *osk* during transport is mediated by Bruno (Bru), which directly binds the *osk* 3' UTR in two regions: AB close to the coding sequence and C close to the poly(A) tail. Each region contains multiple Bru-binding sites, including the Bruno Response Elements (BREs) (Kim-Ha et al., 1995; Reveal et al., 2011). Direct evidence for translational repression comes from *in vitro* assays using cell-free translation systems from ovary extract, which recapitulates Bru- and BRE-dependent repression (Lie and Macdonald, 1999; Castagnetti et al., 2000).

There are several *cis* elements important for translational activation of *osk*: BREs in the C region, Imp-binding elements (IBEs), and a 5' activating element. Mutating the C region BREs causes defects in Osk accumulation and posterior patterning, implicating C region BREs in activation of translation, in addition to a redundant role in repression (Reveal et al., 2010). IBEs are motifs found in multiple copies within the *osk* 3' UTR, and are binding sites for the Drosophila homolog of insulin growth factor II mRNA-binding protein (IMP). Mutating subsets of IBEs results in defects in Osk accumulation and posterior patterning, although *Imp* mutants have no defects in *osk* regulation (Geng and Macdonald, 2006; Munro et al., 2006). The 5' activating element is a poorly characterized region within the coding sequence between the start codons for Long and Short Osk. Inversion of the 3' half within this 130nt region abrogates binding of two ovarian proteins: p50, later identified as Hrp48; and p68, whose identity remains unknown (Gunkel et al., 1998; Yano et al., 2004). The effects of the inversion on expression was monitored using a *lacZ* reporter mRNA, which contains the *osk* 5' region up to the downstream AUG for the Short Osk, fused to the *lacZ* coding sequence, followed by the entire *osk* 3' UTR. In this reporter, the inversion mutation prevents posterior accumulation of the β-galactosidase activity (Gunkel et al., 1998). Although the 5' element and IBEs function in activation of translation, the *trans*-acting proteins that bind them to mediate activation are unknown. For the BREs in the C region, Bru is a candidate activator, which could switch its role from a repressor at the right time by recruiting other factors specifically to the C region to mediate activation.

The cAMP-dependent protein kinase (PKA) is involved in many signaling events and is only transiently activated in response to cAMP. The PKA

holoenzyme complex is a tetramer composed of two catalytic subunits and a regulatory subunit dimer. The regulatory subunits bind the catalytic subunits to sequester them in an inactive state in the absence of cAMP. Upon binding cAMP, the regulatory subunits undergo a conformational change and release the catalytic subunits from inhibition (Taylor et al., 2004 and 2012). In Drosophila, there are two regulatory subunits (*Pka-R1*, *Pka-R2*; FlyBase) and three catalytic subunits (*Pka-C1*, *Pka-C2*, *Pka-C3*; FlyBase). *DC0* (*Pka-C1*) is a Drosophila PKA gene that has the highest homology to the mammalian PKA, and measurement of the kinase activity in adult extracts showed that *DC0* is the source of all or most cAMP-dependent kinase activity. *DC0* is an essential gene and mutants are lethal. During oogenesis, loss of *DC0* disrupts microtubule polarity, and the actin structures around ring canals that connect nurse cells (Lane and Kalderon, 1993). *Pka-R1*-mutant ovaries that have increased catalytic activity show precocious and ectopic translation of Osk, and the resulting embryos have anterior patterning defects such as head defects and the bicaudal phenotype (a mirror-image duplication of abdominal segments), due to excess Osk activity (Yoshida et al., 2004). Translation of both short and long isoforms of Osk are increased although the mRNA level remains the same. Both PKA-C1 and PKA-R1 proteins are enriched on the germ cell membranes but expression in follicle cells is also detectable (Lane and Kalderon, 1995; Yoshida et al., 2004).

DC0 (*Pka-C1*) is required in the germline to establish correct microtubule polarity during mid oogenesis, during which the microtubule network undergoes a rearrangement prior to *osk* mRNA localization (Lane and Kalderon, 1994). Reorganization of microtubules requires two sequential signaling events between the oocyte and somatic follicle cells: from the oocyte to the follicle cells, and then

from the follicle cells back to the oocyte (González-Reyes et al., 1995; Roth et al., 1995). Thus it has been postulated that PKA transduces a temporal signal from the posterior follicle cells for reorganization of the microtubule network (Lane and Kalderon, 1994; González-Reyes et al., 1995; Roth et al., 1995). It is also plausible that this temporal cue is responsible for local translation *via* phosphorylation of Bru or other regulators of *osk*. Interestingly, PKA phosphorylates Bru *in vitro*, and mutating the candidate sites of phosphorylation reduces the ability to be phosphorylated by PKA (see Chapter Two). Therefore, Bru could be one of the targets of PKA in the *osk* regulatory pathway, but it is unlikely to be the sole target since we did not see any patterning defects associated with *osk* regulation when the candidate sites were mutated (see Chapter Two).

Here we investigate the role of PKA in Bru-dependent translational control using *osk::GFP* reporter mRNAs. We alter the catalytic activity by several approaches: using the *Pka-R1* loss-of-function mutant, and using flies expressing constitutively active, regulatory (R*) or catalytic (C*) subunits. We find that altering PKA activity has no consistent detectable effect on Bru phosphorylation. Expression of an *osk::GFP* reporter mRNA, which is under Bru-mediated repression but shows oocyte-specific translation, is sensitive to an increase in PKA activity. Together, these results suggest that PKA is under a tight regulation, and a small change in the catalytic activity can lead to a substantial defect in *osk* regulation.

RESULTS

Modulating PKA activity does not dramatically alter the amount of Bruno phosphorylation

Increasing PKA catalytic activity causes a defect in translational repression of *osk* mRNA prior to its localization, and the resulting embryos are bicaudal (Yoshida et al., 2004). PKA activity can be modulated by mutating the regulatory or catalytic subunits. Yoshida et al. identified two strong loss-of-function alleles of *Pka-R1, Pka-R1*^{E1} and *Pka-R1*^{18304}*,* through genetic screens and showed that extracts of mutant, adult flies displayed increased kinase activity as compared to wild-type (the increase was only 1.2 fold without addition of exogenous cAMP, but 1.5-2 fold with addition of 5μM cAMP). Similarly, transgenic expression of the constitutively active, catalytic subunit (C*), which bears mutations that render it insensitive to regulation by the regulatory subunit (Orellana and McKnight, 1992), is expected to cause increased catalytic activity. On the other hand, we expect to see decreased catalytic activity by expressing the dominant-negative, regulatory subunit (R*), because of its inability to bind cAMP and subsequently, constitutive inhibition of the catalytic subunit (personal communication with Daniel Kalderon).

We reproduced the anterior patterning defects reported for *Pka-R1* mutants. However, UAS/GAL4-driven expression of *Pka-C** in the ovary caused only minor patterning defects in progeny embryos, and the defects were of a different type from that seen with the *Pka-R1* mutant. Testing three different *UAS-Pka-C** lines and using the *matα4-GAL-VP16* driver, only one produced any bicaudal embryos, and even then only at a very low frequency (<1%). On the other hand, when PKA activity was decreased in the ovary by expressing *UAS-*

*Pka-R**, the majority of progeny embryos (98-100%) did not develop cuticles (see Table 3.1 for phenotypic analysis and Fig 3.1 for representative images).

It is not surprising that altering PKA activity would disrupt embryonic development, as PKA has roles in oogenesis in addition to its role in *osk* regulation, such as maintenance of nurse cell boundaries and microtubule reorganization in the oocyte (Lane and Kalderon, 1994; Lane and Kalderon, 1995). Why the *Pka-R1* mutant and enhanced PKA activity from *Pka-C** expression have such different effects is not clear, although it seems likely that *UAS-Pka-C** expression does not dramatically enhance PKA activity.

We next wanted to see if altering PKA activity resulted in a change in Bru phosphorylation. To detect Bru phosphorylation, the conventional approach of testing for phosphatase-dependent changes in electrophoretic mobility of the protein was used. In phosphatase-treated, *wild-type* ovary extract, Bru appeared by Western-blot analysis as a major band. When ovary extract was treated with phosphatase inhibitors, a faint lower-mobility band above the major Bru band was apparent. In *Pka-R1*-mutant ovary extract, however, there is no dramatic change in the amount of the phosphoisoform although for this set of samples, a small increase is evident (Fig 3.2A; compare lanes 2 and 4).

Bru phosphorylation was further analyzed by the phosphate-affinity SDS-PAGE with the acrylamide-pendant Phos-tag, which separates different phosphoprotein isoforms. Using this approach, multiple different phosphorylated species could be detected in both *wild type* and *Pka-R1* mutant. When *wild-type* ovary extract is treated with phosphatase alone, a major Bru band and two upper bands, of which one more distinct and running higher than the other, are seen. When inhibitors are also present, there is a visible smudge consisting of multiple

bands above the major Bru band. Again, there seems to be a slight increase of the phosphoisoforms in *Pka-R1*-mutant extract, but no significant increase overall (Fig 3.2B; compare lanes 1 and 3).

To make a more extensive comparison, ovary extracts from *Pka-R1* mothers were made on five different days, but we did not see a consistent change in phosphorylation from the *wild-type* control (Fig 3.2C).

UAS/GAL4 expression of the dominant-negative, regulatory (*Pka-R**) subunit also did not cause a significant change in the amount of the phosphoisoforms. However, a small increase in abundance of the lower-mobility band was visible in *Pka-C** compared to *Pka-R** ovaries across different lines tested (Fig 3.2D). Although the levels were more consistently higher with the *UAS-Pka-C** lines, this could be due to random variation in samples, just as observed for the *Pka-R1* mutant flies. Alternatively, loss of PKA activity may weakly destabilize Bru.

The failure to detect any consistent change in Bru phosphorylation may be because only a small fraction of Bru is phosphorylatable by PKA or because an intermediate kinase activated by PKA is rate-limiting (see Discussion).

Increasing PKA activity leads to a minor change in Bruno-dependent translational repression

Translational regulation of *osk* mRNA is complex. Multiple proteins are implicated in repression, and multiple proteins are implicated in activation. To focus specifically on Bru-dependent control of *osk* mRNA translation, we used *osk1-173::GFP-AB* reporter mRNAs (Fig 3.3E) that display two regulatory features: Bru-dependent repression throughout the nurse cells and oocyte; and

stage-dependent release from repression in the oocyte. The latter effect is most dramatic for posterior-specific release from repression at stage 9, as described in Chapter 2, but there is also an overall increase in fluorescence throughout the oocyte beginning at stage 7/8. The *osk1-173::GFP-AB* reporter mRNAs include an *osk* 5' region, which encodes an anchoring domain (Vanzo and Ephrussi, 2002) and serves to restrict movement of the protein (and thus reveals where it is translated), and the *osk* 3' UTR *AB* region, which contains Bru binding sites and mediates Bru-dependent translational repression (Kim-Ha et al., 1995; Reveal et al., 2011).

In the presence of normal PKA activity, Osk1-173::GFP expression was slightly enriched in the oocyte from as early as stage 8 (Fig 3.3A) and formed a gradient emanating from the posterior at stage 9 (see Chapter 2). When *Pka-C** was coexpressed with the *osk1-173::GFP-AB*, no substantial change in the posterior gradient of Osk1-173::GFP was detected. However, the earlier, more uniform expression of Osk1-173::GFP throughout the oocyte was slightly altered. Most stage 8 egg chambers (80%) normally show this pattern, and the frequency increased to 97% when *Pka-C** was expressed. To determine if this effect involves Bru, the experiment was also performed with *the osk1-173::GFP-AB BRE-*, which has mutations in the BREs that largely eliminate Bru-dependent repression. In the absence of repression by Bru, the *osk1-173::GFP-AB BRE-* mRNA produces Osk1-173::GFP throughout the nurse cells and oocyte. When *Pka-C** is coexpressed with the reporter, no consistent change in the Osk1-173::GFP level was detected. This suggests that the effect of *Pka-C** expression on the *osk1-173::GFP-AB* mRNA is mediated by action of PKA on Bru, or on some other factor involved in Bru-dependent translational repression. However,

the effect is weak, other evidence suggests that this transgene may not have much effect on PKA activity, and thus the significance of this result is uncertain.

DISCUSSION

Regulation of PKA by PKA-R1 is required for *oskar* regulation and correct body patterning

Strong *Pka-R1* mutants have anteroposterior patterning defects, and the *Pka-R1*^{18304} hemizygous egg chambers display premature and ectopic Osk expression and an overall increase in the Osk protein level, but the *osk* RNA level and localization are normal. The bicaudal phenotype of the *Pka-R1* mutants was fully suppressed by either reducing the dosage of *DC0 (Pka-C1)* or germline expression of the RA isoform of *Pka-R1*, suggesting the patterning defect is caused by excess catalytic activity (Yoshida et al., 2004).

PKA activity may regulate either translational activation or Osk protein stability. In this regard, phosphorylation of Osk or a regulator of *osk* would promote its translation or stability. Bru directly binds *osk* mRNA and represses translation (Kim-Ha et al., 1995; Lie and Macdonald, 1999; Castagnetti et al., 2000), and is phosphorylated *in vivo*. Moreover, PKA phosphorylates Bru *in vitro* (see Chapter 2). Therefore phosphorylation of Bru could be a mechanism of depression of *osk* translation at the posterior of the oocyte. However, mutating the candidate sites of PKA phosphorylation in Bru did not result in any patterning defects, leaving open additional substrate possibilities.

It is also possible that Osk protein may be stabilized, directly or indirectly by PKA-mediated phosphorylation. Osk is destabilized by Par-1/GSK-3

phosphorylation and subsequent E3-ubiquitin-ligase-mediated targeting to the proteasome (Morais-de-Sá et al., 2013). PKA could stabilize Osk *via* direct phosphorylation or by counteracting Par-1/GSK-3 pathway that phosphorylates and degrades Osk.

Both R1 and C1 subunits of PKA are enriched on the germ cell membranes (Lane and Kalderon, 1995; Yoshida et al., 2004), likely by interaction of PKA-R1 with an A kinase anchoring protein (AKAP) that regulates spatial distribution of the PKA holoenzyme (Pidoux and Tasken, 2010; Wong and Scott, 2004). Moreover, PKA activity is required in the germline for reorganization of the microtubule polarity and correct localization of Kin::β-gal, a microtubule plus-end marker, at the posterior of the oocyte (Lane and Kalderon, 1994). Hence PKA activity is required just before *osk* mRNA is localized, and may colocalize and coincide with Osk expression. Therefore, PKA may transduce a signal from the posterior follicle cells for oocyte polarity, and this local activation of PKA may be responsible for localized expression of Osk.

Does PKA phosphorylate Bruno?

We did not see a dramatic change in Bru phosphorylation in response to increased PKA catalytic activity. *Pka-R1*^{E1} and *Pka-R1*^{18304} are both strong loss-of-function alleles of *Pka-R1*, and embryos from *Pka-R1*^{E1}*/Pka-R1*^{18304} mothers have strong patterning defects (Yoshida et al., 2004). Yet there was no consistent detectable increase of Bru phosphorylation in the *Pka-R1*^{E1}*/Pka-R1*^{18304} mutant when the total amount of Bru protein is comparable between the wild type and mutant. Similarly, there was no consistent detectable change in Bru

phosphorylation for transgenic flies expressing a constitutively active catalytic subunit (C*). One issue with these studies is that Bru appears to be phosphorylated at multiple positions, as detected by altered mobility of Bru in denaturing gel electrophoresis. However, not all phosphorylations will lead to a change in mobility, and the phosphomimetic mutants characterized here are of this type. Therefore, the failure to detect changes in Bru phosphorylation by the mobility assay does not reveal whether PKA phosphorylates Bru *in vivo* at the same amino acids it phosphorylates *in vitro*.

A more revealing indication of whether PKA phosphorylates Bru *in vivo* might come from phenotypic assays. When this work was initiated, a working hypothesis was that posterior-specific phosphorylation of Bru by PKA could be responsible for changing Bru from a repressor to an activator of translation. This would be consistent with the observed effect of excess PKA activity on embryonic body patterning. However, the results of Chapter 2 suggest that phosphorylation of Bru by PKA promotes its repressive activity, exactly the opposite of what was hypothesized. Based on these results, the effect of the *Pka-R1* mutant on Osk expression would likely occur by an effect on a different target. Because kinases can have many targets, this does not rule out action by PKA on Bru. It would be interesting to see how reduction of PKA activity affects body patterning, and whether the results are consistent with the model for PKA action on Bru from Chapter 2. However, a majority of embryos from *Pka-R**-expressing mothers do not develop to a point of developing cuticles. Likewise, females heterozygous for a strong and a weak *DC0* allele fail to lay eggs (Lane and Kalderon, 1993; Lane and Kalderon, 1994).

Another approach to examine phenotypes involves use of the *osk1-173::GFP-AB* reporter, which encodes an anchored protein (to reveal where it is translated) and includes the *osk* 3' UTR *AB* region with its multiple Bru binding sites for repression (Kim-Ha et al., 1995; Reveal et al., 2011). This allows repression to be predominantly or exclusively dependent on Bru binding to the AB region and may be more sensitive to any defect in repression than an mRNA with the entire *osk* 3' UTR bound by several other proteins. In addition, this reporter displays posterior Osk::GFP expression, due to either local inhibition of Bru repression or shift of Bru from a repressor to an activator.

We saw a very small increase in the proportion of stage 8/9 egg chambers expressing the oocyte Osk1-173::GFP when PKA activity was elevated using the *UAS-Pka-C** approach. Interpretation of this result is not straightforward, given other indications that this transgene does not reproduce the effect of the *Pka-R1* mutant. It would be worthwhile to express the *osk1-173::GFP-AB* reporter in the *Pka-R1*-mutant background to more definitively test the effect of increasing the catalytic activity on Bru-dependent translational regulation. Similarly, it would be useful to test the effect of reducing PKA activity with the *UAS-Pka-R** transgene.

MATERIALS AND METHODS

Flies and Transgenes

w^{1118} flies were used as the wild type. *Pka-R1* flies used were transheterozygous for $Pka\text{-}R1^{E1}$ and $Pka\text{-}R1^{18304}$, which are strong loss-of-function alleles identified in genetic screens for maternal-effect mutations in anteroposterior patterning of the embryo (Yoshida et al., 2004). Transgenic fly

stocks were established by standard methods. Expression of the UAS transgenes was driven by the *matα4-GAL-VP16* driver (Martin and St. Johnston, 2003).

The *P[UAS-osk1-173::GFP-AB]* transgene was generated by Ginny Pai. The *P[UAS-Pka-R*]* transgene was generated by inserting, into pUASp (Rorth, 1998) using the same restriction sites, the Asp718-XbaI fragment (from p8931 or BDK2 from Daniel Kalderon), which has the full-length *Pka-R1* coding sequence, with mutations in each of the two cAMP-binding sites as well as a duplication of the first three amino acids as a cloning artifact. The *P[UAS-Pka-C*]* transgene was generated by inserting, into pUASp using the same restriction sites, the NotI-XbaI fragment (from p8928 from Daniel Kalderon), which has the full-length mouse *Pka-C* coding sequence with H87Q and W196R mutations (Orellana and McKnight, 1992), as well as the 3' UTR from the human growth hormone (hGH) gene (DeNoto et al., 1981). The point mutations render PKA-C* insensitive to regulation by PKA-R (Orellana and McKnight, 1992).

Detection of Phosphorylated Bruno in the Ovary

Ovaries from young females, fed on yeast for 3-4 days, were dissected and extract was prepared as previously described (Kim-Ha et al., 1995) in ice-cold lysis buffer (50mM Hepes pH7.9, 150mM KCl, and 1% IGEPAL-CA-630) supplemented with Complete, Mini, EDTA-free protease inhibitor cocktail tablet (Roche). Reactions (20µl) contained 10-15µg of ovary extract in phosphatase buffer (50mM Hepes pH7.9, 100mM NaCl, 10mM $MgCl_2$, and 1mM DTT), and where indicated, one or more of the following components were added: 1-2 units

of alkaline phosphatase (calf intestinal, NEB), 1.6M beta-glycero phosphate, and 40mM sodium vanadate. Reactions were incubated at 30°C for 1 hr and terminated by addition of 3X SDS loading buffer. Proteins were separated by SDS-PAGE and analyzed by Western blot.

For the phosphate-affinity SDS-PAGE using acrylamide-pendant Phos-tag (WAKO), 50μM Phos-tag and 200μM $MnCl_2$ were added to both stacking and separating gels in solution.

Mouse anti-Bru antibody (from Nakamura lab) diluted at 1:8000 and mouse anti-αTubulin antibody (Sigma) diluted at 1:1000 were used for Western blot, together with the alkaline phosphatase-conjugated goat anti-mouse antibody (Applied Biosystems) diluted at 1:5000.

Whole-Mount Ovary Staining

For detection of GFP *in situ*, ovaries from young females fed on yeast for 3-4 days were dissected into PBS at room temperature and fixed in 4% formaldehyde in PBS for 20 min with gentle mixing. The ovaries were then washed for 30 min in four changes of PBT (PBS plus 0.1% Triton X-100) and blocked for 2 hr in four changes of PBT containing 5% goat serum. Nuclei were counterstained with TO-PRO-3 Iodide (642/661, Invitrogen) diluted 1:1000 in PBT containing 1% goat serum for 1 hr at room temperature. Egg chambers were washed several times over 3 hr in PBT and then mounted in Vectashield (Vector Labs). Microscopy of all samples made use of a Leica TCS-SP laser scanning confocal microscope.

Phenotypic Analysis

Embryos were collected from young females in small population cages, aged for at least 24 hr, and cuticles were prepared and mounted for examination, as described (Wieschaus and Nüsslein-Volhard, 1986).

Acknowledgments

We thank Akira Nakamura for antibodies, Anne Ephrussi and Daniel Kalderon for DNAs or fly stocks, and members of the Macdonald lab for advice and comments on the manuscript. This work was supported by NIH grant GM54409.

FIGURES

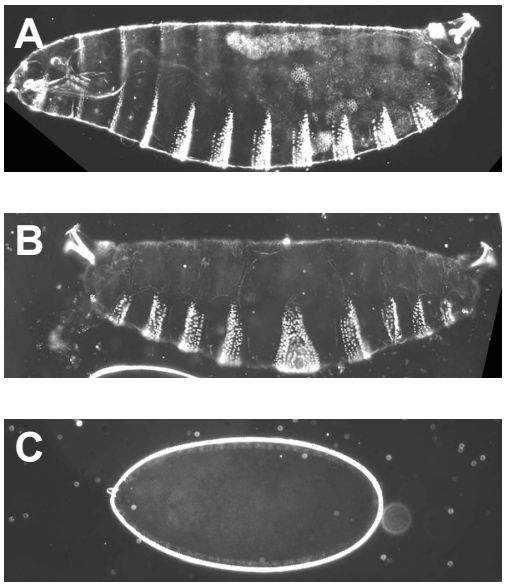


Figure 3.1. Examples of embryonic phenotypes of *Pka* mutant and transgenics

Each panel shows a cuticle of an embryo from either *Pka-R1*-mutant mothers or mothers expressing one copy of the *Pka* transgenes. (A) is phenotypically wild type having eight abdominal denticle belts and a normal head at the anterior (left) and tail at the posterior (right). (B) is a bicaudal embryo that occurs with increasing or ectopic Osk patterning activity and lacks a head at the expense of a duplicated abdomen and tail. It is the most commonly observed phenotype of embryos from *Pka-R1* mothers. Note that typically, high level of Osk overexpression produces bicaudal embryos with many fewer abdominal segments remaining than what is shown here. This cuticle pattern with multiple duplicated abdominal segments suggests that it is more than simply overexpression of Osk that is responsible. (C) is an embryo that did not develop far enough to produce a cuticle and is the most commonly observed phenotype of embryos from *Pka-R** mothers.

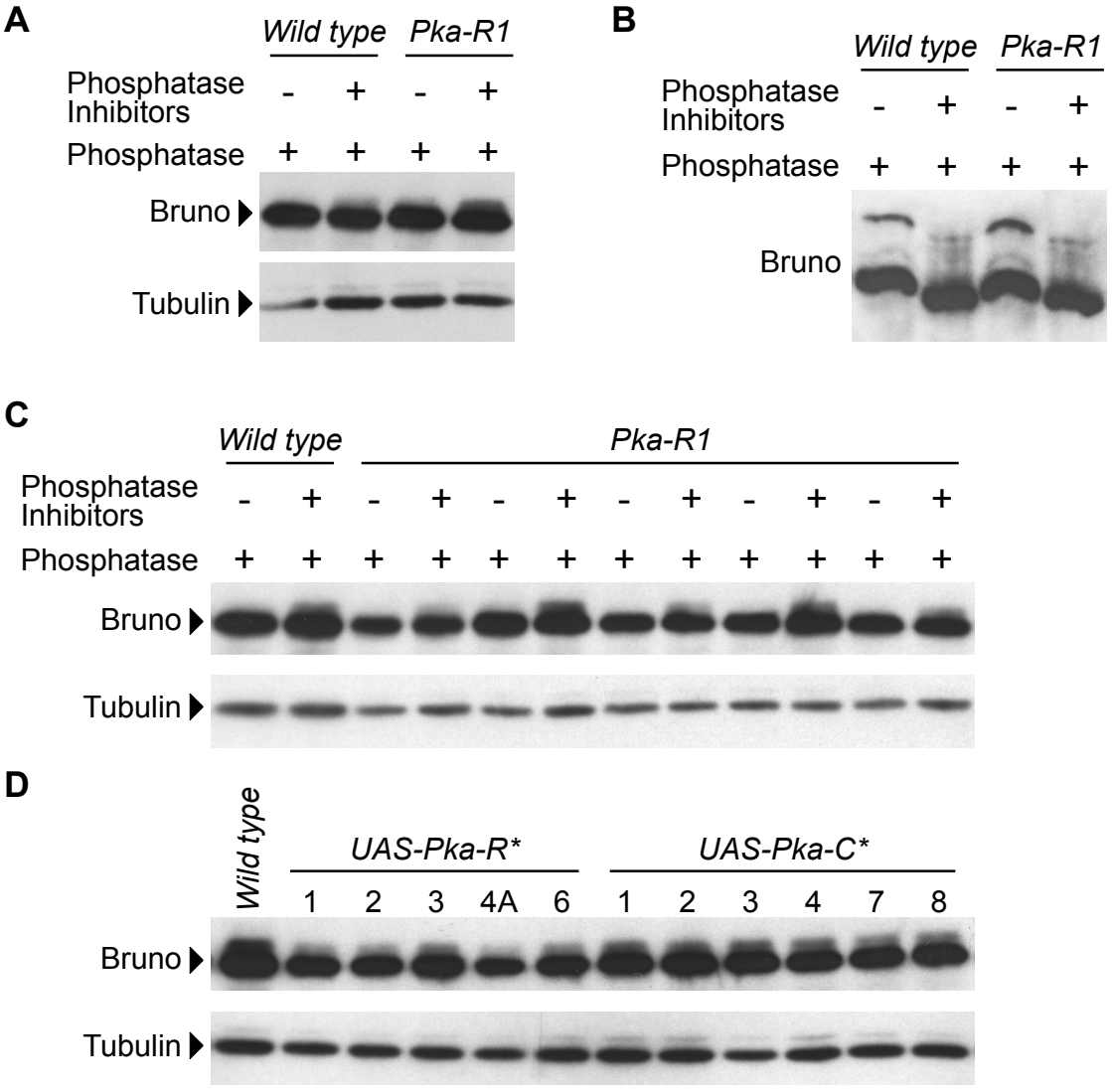


Figure 3.2. Bru phosphorylation in response to change in PKA activity

(A) Western blot of *wild-type* and *Pka-R1*-mutant ovary extract after incubation with Calf intestine alkaline phosphatase, either alone or together with phosphatase inhibitors, as indicated above. Proteins were detected using anti-Bru (top) or anti-Tubulin (bottom) antibody. Inhibitors used were sodium vanadate and beta-glycero phosphate, which are competitive inhibitors of alkaline

phosphatase. *Pka-R1* mutants used are transheterozygous for two strong, loss-of-function alleles, *Pka-R1*^{E1} and *Pka-R1*^{18304}.

(B) *Wild-type* and *Pka-R1*-mutant ovary extract incubated with Calf intestine alkaline phosphatase, either alone or together with phosphatase inhibitors, as indicated above, was run on phosphate-affinity SDS-PAGE using acrylamide-pendant Phos-tag that separates different phosphoprotein isoforms, followed by Western blot to detect proteins using anti-Bru antibody. Inhibitors used are as noted in (A).

(C) Ovary extract from *Pka-R1* mutants was prepared on five different days and incubated with, along with *wild-type* ovary extract, Calf intestine alkaline phosphatase, either alone or together with phosphatase inhibitors, as indicated above. Proteins were detected by Western blot using anti-Bru (top) or anti-Tubulin (bottom) antibody. Inhibitors used are as noted in (A).

(D) Ovary extract from multiple lines of *UAS-Pka-R**- or *UAS-Pka-C**-expressing flies, as indicated above, was prepared along with *wild-type* ovary extract. Proteins were detected by Western blot using anti-Bru (top) or anti-Tubulin (bottom) antibody.

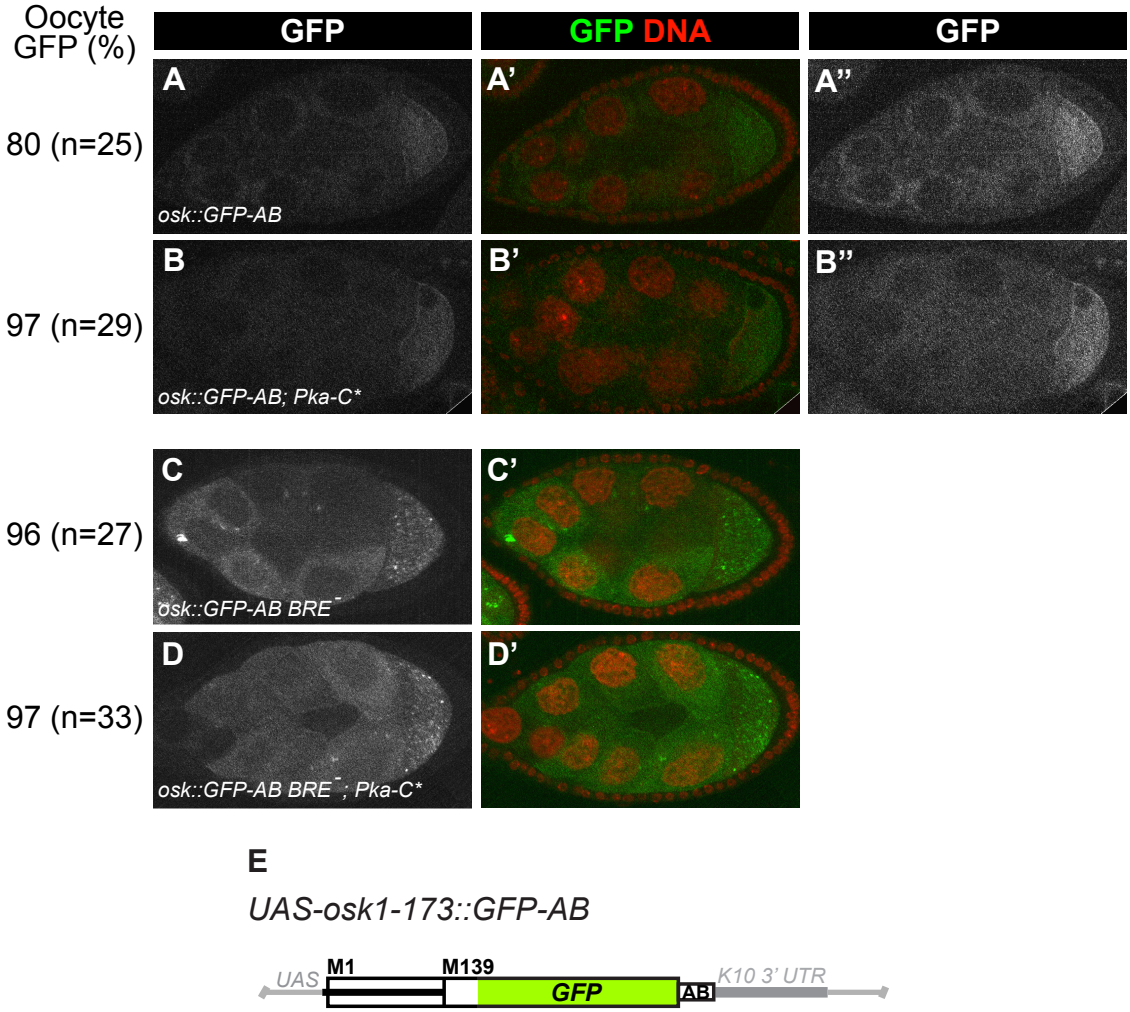


Figure 3.3. PKA-dependent change in the *osk::GFP* reporter expression

(A-D, A'-D') show egg chambers expressing the *osk::GFP* reporter containing the *osk 5'* region and either *wild-type* (A-B, A'-B') or *mutant* (C-D, C'-D') *AB* region. (B, B', D, D') also express the *Pka-C** transgene. All have a single copy of each of the transgenes. (A'-D') show GFP in green and nuclei in red. All samples were fixed in parallel and imaged together, and each pair of images in comparison was taken under the same settings. The GFP signal in (A and B)

was elevated to (A'' and B'') to highlight the oocyte enrichment. Expression of the UAS transgenes was driven by the *matα4-GAL-VP16* driver (Martin and St. Johnston, 2003).

(E) A schematic diagram of the *UAS-osk::GFP-AB* reporter. The large rectangle comprises a part of the *osk* coding sequence including two start codons and up to T173 and a full *GFP* coding sequence as labeled. The *osk 3′ UTR AB* region, which contains the *BRE*s, is depicted as the small rectangle. The thick black line includes the 15nt *5' UTR* common to both Osk isoforms and *5' UTR* specific for the short isoform. The thick gray line is the 0.4kb *K10 3' UTR* fragment, part of the pUASp vector sequence in gray (Rorth, 1998).

TABLES

Maternal Genotype[a] or Transgene	Line (chromosome)	Cuticular Phenotype (%)[b] Wild Type	Head Defect	Bicaudal	No Cuticle Development	n
Pka-R1^{E1}/Pka-R1^{18304}		22	1	41	36	318
*UAS-Pka-C**	2 (X)	53	0	0	47	214
	4 (II)	69	0	0.3	31	642
	8 (III)	59	0	0	41	198
*UAS-Pka-R**	1 (X)	2	0	0	98	886
	4A (III)	1	0	0	99	622
	6 (III)	0	0	0	100	577
w^{1118}		82	0	0	18	612

Table 3.1. Embryonic development of *Pka* mutant and transgenics

[a] *E1* and *18304* are strong loss-of-function alleles of the *Pka-R1* gene encoding the regulatory subunit of PKA. * denotes a gain-of-function mutation in the catalytic or regulatory subunit of PKA. *w^{1118}* was used as a wild-type control. Expression of the UAS transgenes was driven by the *matα4-GAL-VP16* driver (Martin and St. Johnston, 2003).

[b] Wild-type embryonic cuticles have eight abdominal denticle belts and a normal head. This category includes a very small percentage of embryos missing a single abdominal segment, which is common for embryos from females with the *matα4-GAL-VP16* driver. Embryos with head defects do not have head structures. Bicaudal embryos lack head and anterior abdominal segments at the expense of duplicated posterior segments. See Figure 3.1 for a representative image for each phenotype.

REFERENCES

Besse, F., & Ephrussi, A. (2008). Translational control of localized mRNAs: restricting protein synthesis in space and time. Nature Reviews Molecular Cell Biology, 9(12), 971–980.

Bramham, C. R., & Wells, D. G. (2007). Dendritic mRNA: transport, translation and function. Nature Reviews Neuroscience, 8(10), 776–789.

Castagnetti, S., Hentze, M. W., Ephrussi, A., & Gebauer, F. (2000). Control of oskar mRNA translation by Bruno in a novel cell-free system from Drosophila ovaries. Development, 127(5), 1063–1068.

Condeelis, J., & Singer, R. H. (2005). How and why does beta-actin mRNA target? Biology of the Cell / Under the Auspices of the European Cell Biology Organization, 97(1), 97–110.

Deng, Y., Singer, R. H., & Gu, W. (2008). Translation of ASH1 mRNA is repressed by Puf6p-Fun12p/eIF5B interaction and released by CK2 phosphorylation. Genes & Development, 22(8), 1037–1050.

DeNoto, F. M., Moore, D. D., & Goodman, H. M. (1981). Human growth hormone DNA sequence and mRNA structure: possible alternative splicing. Nucleic Acids Research, 9(15), 3719–3730.

Ephrussi, A., Dickinson, L. K., & Lehmann, R. (1991). Oskar organizes the germ plasm and directs localization of the posterior determinant nanos. Cell, 66(1), 37–50.

Geng, C., & Macdonald, P. M. (2006). Imp Associates with Squid and Hrp48 and Contributes to Localized Expression of gurken in the Oocyte. Molecular and Cellular Biology, 26(24), 9508–9516.

González-Reyes, A., Elliott, H., & St Johnston, D. (1995). Polarization of both major body axes in Drosophila by gurken-torpedo signalling. Nature, 375(6533), 654–658.

Gunkel, N., Yano, T., Markussen, F. H., Olsen, L. C., & Ephrussi, A. (1998). Localization-dependent translation requires a functional interaction between the 5“ and 3” ends of oskar mRNA. Genes & Development, 12(11), 1652–1664.

Horne-Badovinac, S., & Bilder, D. (2008). Dynein Regulates Epithelial Polarity and the Apical Localization of stardust A mRNA. PLoS Genetics, 4(1), e8.

Huang, Y.-S., Jung, M.-Y., Sarkissian, M., & Richter, J. D. (2002). N-methyl-D-aspartate receptor signaling results in Aurora kinase-catalyzed CPEB

phosphorylation and alpha CaMKII mRNA polyadenylation at synapses. The EMBO Journal, 21(9), 2139–2148.

Kim-Ha, J., Kerr, K., & Macdonald, P. M. (1995). Translational regulation of oskar mRNA by Bruno, an ovarian RNA-binding protein, is essential. Cell, 81(3), 403–412.

Kim-Ha, J., Smith, J. L., & Macdonald, P. M. (1991). oskar mRNA is localized to the posterior pole of the Drosophila oocyte. Cell, 66(1), 23–35.

Kugler, J.-M., & Lasko, P. (2009). Localization, anchoring and translational control of oskar, gurken, bicoid and nanos mRNA during Drosophila oogenesis. Fly, 3(1), 15–28.

Lambert, J. D., & Nagy, L. M. (2002). Asymmetric inheritance of centrosomally localized mRNAs during embryonic cleavages. Nature, 420(6916), 682–686.

Lane, M. E., & Kalderon, D. (1993). Genetic investigation of cAMP-dependent protein kinase function in Drosophila development. Genes & Development, 7(7A), 1229–1243.

Lane, M. E., & Kalderon, D. (1994). RNA localization along the anteroposterior axis of the Drosophila oocyte requires PKA-mediated signal transduction to direct normal microtubule organization. Genes & Development, 8(24), 2986–2995.

Lane, M. E., & Kalderon, D. (1995). Localization and functions of protein kinase A during Drosophila oogenesis. Mechanisms of Development, 49(3), 191–200.

Li, Z., Wang, L., Hays, T. S., & Cai, Y. (2008). Dynein-mediated apical localization of crumbs transcripts is required for Crumbs activity in epithelial polarity. The Journal of Cell Biology, 180(1), 31–38.

Lie, Y. S., & Macdonald, P. M. (1999). Translational regulation of oskar mRNA occurs independent of the cap and poly(A) tail in Drosophila ovarian extracts. Development, 126(22), 4989–4996.

Lin, A. C., & Holt, C. E. (2007). Local translation and directional steering in axons. The EMBO Journal, 26(16), 3729–3736.

Long, R. M., Singer, R. H., Meng, X., Gonzalez, I., Nasmyth, K., & Jansen, R. P. (1997). Mating type switching in yeast controlled by asymmetric localization of ASH1 mRNA. Science, 277(5324), 383–387.

Markussen, F.-H., Michon, A.-M., Breitwieser, W., & Ephrussi, A. (1995). Translational control of oskar generates short OSK, the isoform that induces pole plasma assembly. Development, 121(11), 3723–3732.

Martin, K. C., & Ephrussi, A. (2009). mRNA Localization: Gene Expression in the Spatial Dimension. Cell, 136(4), 719–730.

Martin, S. G., & St Johnston, D. (2003). A role for Drosophila LKB1 in anterior-posterior axis formation and epithelial polarity. Nature, 421(6921), 379–384.

Morais-de-Sá, E., Vega-Rioja, A., Trovisco, V., & St Johnston, D. (2013). Oskar is targeted for degradation by the sequential action of Par-1, GSK-3, and the SCF-Slimb ubiquitin ligase. Developmental Cell, 26(3), 303–314.

Munro, T. P., Kwon, S., Schnapp, B. J., & St Johnston, D. (2006). A repeated IMP-binding motif controls oskar mRNA translation and anchoring independently of Drosophila melanogaster IMP. The Journal of Cell Biology, 172(4), 577–588.

Orellana, S. A., & McKnight, G. S. (1992). Mutations in the catalytic subunit of cAMP-dependent protein kinase result in unregulated biological activity. Proceedings of the National Academy of Sciences of the United States of America, 89(10), 4726–4730.

Paquin, N., Ménade, M., Poirier, G., Donato, D., Drouet, E., & Chartrand, P. (2007). Local Activation of Yeast ASH1 mRNA Translation through Phosphorylation of Khd1p by the Casein Kinase Yck1p. Molecular Cell, 26(6), 795–809.

Pidoux, G., & Tasken, K. (2010). Specificity and spatial dynamics of protein kinase A signaling organized by A-kinase-anchoring proteins. Journal of Molecular Endocrinology, 44(5), 271–284.

Reveal, B., Garcia, C., Ellington, A., & Macdonald, P. (2011). Multiple RNA binding domains of Bruno confer recognition of diverse binding sites for translational repression. RNA Biology, 8(6), 1047–1060.

Reveal, B., Yan, N., Snee, M. J., Pai, C.-I., Gim, Y., & Macdonald, P. M. (2010). BREs Mediate Both Repression and Activation of oskar mRNA Translation and Act In trans. Developmental Cell, 18(3), 496–502.

Rongo, C., Gavis, E. R., & Lehmann, R. (1995). Localization of oskar RNA regulates oskar translation and requires Oskar protein. Development, 121(9), 2737–2746.

Roth, S., Shira Neuman-Silberberg, F., Barcelo, G., & Schüpbach, T. (1995). cornichon and the EGF receptor signaling process are necessary for both

anterior-posterior and dorsal-ventral pattern formation in Drosophila. Cell, 81(6), 967–978.

Rørth, P. (1998). Gal4 in the Drosophila female germline. Mechanisms of Development, 78(1-2), 113–118.

Sutton, M. A., & Schuman, E. M. (2006). Dendritic Protein Synthesis, Synaptic Plasticity, and Memory. Cell, 127(1), 49–58.

Taylor, S. S., Ilouz, R., Zhang, P., & Kornev, A. P. (2012). Assembly of allosteric macromolecular switches: lessons from PKA. Nature Reviews Molecular Cell Biology, 13(10), 646–658.

Taylor, S. S., Yang, J., Wu, J., Haste, N. M., Radzio-Andzelm, E., & Anand, G. (2004). PKA: a portrait of protein kinase dynamics. Biochimica Et Biophysica Acta (BBA) - Proteins and Proteomics, 1697(1-2), 259–269.

Vanzo, N. F., & Ephrussi, A. (2002). Oskar anchoring restricts pole plasm formation to the posterior of the Drosophila oocyte. Development, 129(15), 3705–3714.

Wieschaus, E. and Nüsslein-Volhard, C. (1986). Looking at Embryos. In D.B. Roberts (Ed.), *Drosophila, a practical approach* (pp. 199-226). Oxford: IRL Press.

Wong, W., & Scott, J. D. (2004). AKAP signalling complexes: focal points in space and time. Nature Reviews Molecular Cell Biology, 5(12), 959–970.

Yano, T., de Quinto, S. L., Matsui, Y., Shevchenko, A., Shevchenko, A., & Ephrussi, A. (2004). Hrp48, a Drosophila hnRNPA/B Homolog, Binds and Regulates Translation of oskar mRNA. Developmental Cell, 6(5), 637–648.

Yoshida, S., Müller, H.-A. J., Wodarz, A., & Ephrussi, A. (2004). PKA-R1 spatially restricts Oskar expression for Drosophila embryonic patterning. Development, 131(6), 1401–1410.

References

Babu, K., Cai, Y., Bahri, S., Yang, X., & Chia, W. (2004). Roles of Bifocal, Homer, and F-actin in anchoring Oskar to the posterior cortex of Drosophila oocytes. Genes & Development, 18(2), 138–143.

Bardwell, V. J., & Wickens, M. (1990). Purification of RNA and RNA-protein complexes by an R17 coat protein affinity method. Nucleic Acids Research, 18(22), 6587–6594.

Bastock, R., & St Johnston, D. (2008). Drosophila oogenesis. Current Biology, 18(23), R1082-R1087.

Berleth, T., Burri, M., Thoma, G., Bopp, D., Richstein, S., Frigerio, G., et al. (1988). The role of localization of bicoid RNA in organizing the anterior pattern of the Drosophila embryo. The EMBO Journal, 7(6), 1749–1756.

Besse, F., & Ephrussi, A. (2008). Translational control of localized mRNAs: restricting protein synthesis in space and time. Nature Reviews Molecular Cell Biology, 9(12), 971–980.

Besse, F., López de Quinto, S., Marchand, V., Trucco, A., & Ephrussi, A. (2009). Drosophila PTB promotes formation of high-order RNP particles and represses oskar translation. Genes & Development, 23(2), 195–207.

Bhattacharyya, S. N., Habermacher, R., Martine, U., Closs, E. I., & Filipowicz, W. (2006). Relief of microRNA-Mediated Translational Repression in Human Cells Subjected to Stress. Cell, 125(6), 1111–1124.

Bonafe, N., & Sellers, J. R. (1998). Molecular characterization of myosin V from Drosophila melanogaster. Journal of Muscle Research and Cell Motility, 19(2), 129–141.

Bramham, C. R., & Wells, D. G. (2007). Dendritic mRNA: transport, translation and function. Nature Reviews Neuroscience, 8(10), 776–789.

Brendza, R. P., Serbus, L. R., Duffy, J. B., & Saxton, W. M. (2000). A function for kinesin I in the posterior transport of oskar mRNA and Staufen protein. Science, 289(5487), 2120–2122.

Brengues, M., Teixeira, D., & Parker, R. (2005). Movement of eukaryotic mRNAs between polysomes and cytoplasmic processing bodies. Science, 310(5747), 486–489.

Bullock, S. L., & Ish-Horowicz, D. (2001). Conserved signals and machinery for RNA transport in Drosophila oogenesis and embryogenesis. Nature, 414(6864), 611–616.

Castagnetti, S., & Ephrussi, A. (2003). Orb and a long poly (A) tail are required for efficient oskar translation at the posterior pole of the Drosophila oocyte. Development, 130(5), 835–843.

Castagnetti, S., Hentze, M. W., Ephrussi, A., & Gebauer, F. (2000). Control of oskar mRNA translation by Bruno in a novel cell-free system from Drosophila ovaries. Development, 127(5), 1063–1068.

Cha, B.-J., Serbus, L. R., Koppetsch, B. S., & Theurkauf, W. E. (2002). Kinesin I-dependent cortical exclusion restricts pole plasm to the oocyte posterior. Nature Cell Biology, 4(8), 592–598.

Chang, J. S., Tan, L., & Schedl, P. (1999). The Drosophila CPEB homolog, orb, is required for oskar protein expression in oocytes. Developmental Biology, 215(1), 91–106.

Chekulaeva, M., Hentze, M. W., & Ephrussi, A. (2006). Bruno Acts as a Dual Repressor of oskar Translation, Promoting mRNA Oligomerization and Formation of Silencing Particles. Cell, 124(3), 521–533.

Cho, Y. S., Stevens, L. M., & Stein, D. (2010). Pipe-Dependent Ventral Processing of Easter by Snake Is the Defining Step in Drosophila Embryo DV Axis Formation. Current Biology, 20(12), 1133–1137.

Christerson, L. B., & McKearin, D. M. (1994). orb is required for anteroposterior and dorsoventral patterning during Drosophila oogenesis. Genes & Development, 8(5), 614–628.

Coller, J., & Parker, R. (2005). General Translational Repression by Activators of mRNA Decapping. Cell, 122(6), 875–886.

Condeelis, J., & Singer, R. H. (2005). How and why does beta-actin mRNA target? Biology of the Cell / Under the Auspices of the European Cell Biology Organization, 97(1), 97–110.

Davidson, E. H. (1986). Gene activity during oogenesis. Gene activity in early development, 305-407.

de Cuevas, M., Lilly, M. A., & Spradling, A. C. (1997). Germline cyst formation in Drosophila. Annual Review of Genetics, 31(1), 405–428.

De Gregorio, E. (1999). Translation driven by an eIF4G core domain in vivo. The EMBO Journal, 18(17), 4865–4874.

de Valoir, T., Tucker, M. A., Belikoff, E. J., Camp, L. A., Bolduc, C., & Beckingham, K. (1991). A second maternally expressed Drosophila gene encodes a putative RNA helicase of the “DEAD box” family. Proceedings of the National Academy of Sciences, 88(6), 2113–2117.

Deng, Y., Singer, R. H., & Gu, W. (2008). Translation of ASH1 mRNA is repressed by Puf6p-Fun12p/eIF5B interaction and released by CK2 phosphorylation. Genes & Development, 22(8), 1037–1050.

DeNoto, F. M., Moore, D. D., & Goodman, H. M. (1981). Human growth hormone DNA sequence and mRNA structure: possible alternative splicing. Nucleic Acids Research, 9(15), 3719–3730.

Dienstbier, M., Boehl, F., Li, X., & Bullock, S. L. (2009). Egalitarian is a selective RNA-binding protein linking mRNA localization signals to the dynein motor. Genes & Development, 23(13), 1546–1558.

Doren, M. V., Williamson, A. L., & Lehmann, R. (1998). Regulation of zygotic gene expression in Drosophila primordial germ cells. Current Biology, 8(4), 243–246.

Dreyfuss, G., Kim, V. N., & Kataoka, N. (2002). Messenger-RNA-binding proteins and the messages they carry. Nature Reviews Molecular Cell Biology, 3(3), 195–205.

Driever, W., & Nusslein-Volhard, C. (1988). The bicoid protein determines position in the Drosophila embryo in a concentration-dependent manner. Cell, 54(1), 95–104.

Ephrussi, A., Dickinson, L. K., & Lehmann, R. (1991). Oskar organizes the germ plasm and directs localization of the posterior determinant nanos. Cell, 66(1), 37–50.

Ephrussi, A., & Lehmann, R. (1992). Induction of germ cell formation by oskar. Nature, 358(6385), 387–392.

Eulalio, A., Behm-Ansmant, I., & Izaurralde, E. (2007). P bodies: at the crossroads of post-transcriptional pathways. Nature Reviews Molecular Cell Biology, 8(1), 9–22.

Ferrandon, D., Elphick, L., Nüsslein-Volhard, C., & St Johnston, D. (1994). Staufen protein associates with the 3′UTR of bicoid mRNA to form particles that move in a microtubule-dependent manner. Cell, 79(7), 1221–1232.

Filardo, P., & Ephrussi, A. (2003). Bruno regulates gurken during Drosophila oogenesis. Mechanisms of Development, 120(3), 289–297.

Gaspar, I. (2011). Microtubule-based motor-mediated mRNA localization in Drosophilaoocytes and embryos. Biochemical Society Transactions, 39(5), 1197–1201.

Gavis, E. R., & Lehmann, R. (1992). Localization of nanos RNA controls embryonic polarity. Cell, 71(2), 301–313.

Gebauer, F., & Hentze, M. W. (2004). Molecular mechanisms of translational control. Nature Reviews Molecular Cell Biology, 5(10), 827–835.

Geng, C., & Macdonald, P. M. (2006). Imp Associates with Squid and Hrp48 and Contributes to Localized Expression of gurken in the Oocyte. Molecular and Cellular Biology, 26(24), 9508–9516.

Ghosh, S., Marchand, V., Gaspar, I., & Ephrussi, A. (2012). Control of RNP motility and localization by a splicing-dependent structure in oskar mRNA. Nature Structural & Molecular Biology, 19(4), 441–449.

González-Reyes, A., Elliott, H., & St Johnston, D. (1995). Polarization of both major body axes in Drosophila by gurken-torpedo signalling. Nature, 375(6533), 654–658.

Gunkel, N., Yano, T., Markussen, F. H., Olsen, L. C., & Ephrussi, A. (1998). Localization-dependent translation requires a functional interaction between the 5“ and 3” ends of oskar mRNA. Genes & Development, 12(11), 1652–1664.

Hachet, O., & Ephrussi, A. (2001). Drosophila Y14 shuttles to the posterior of the oocyte and is required for oskar mRNA transport. Current Biology, 11(21), 1666–1674.

Hachet, O., & Ephrussi, A. (2004). Splicing of oskar RNA in the nucleus is coupled to its cytoplasmic localization. Nature, 428(6986), 959–963.

Hake, L. E., & Richter, J. D. (1994). CPEB is a specificity factor that mediates cytoplasmic polyadenylation during Xenopus oocyte maturation. Cell, 79(4), 617–627.

Harris, A. N., & Macdonald, P. M. (2001). Aubergine encodes a Drosophila polar granule component required for pole cell formation and related to eIF2C. Development, 128(14), 2823–2832.

Haseloff, J. (1999). GFP variants for multispectral imaging of living cells. Methods in Cell Biology, 58, 139–151.

Hay, B., Jan, L. Y., & Jan, Y. N. (1988). A protein component of Drosophila polar granules is encoded by vasa and has extensive sequence similarity to ATP-dependent helicases. Cell, 55(4), 577–587.

Horne-Badovinac, S., & Bilder, D. (2008). Dynein Regulates Epithelial Polarity and the Apical Localization of stardust A mRNA. PLoS Genetics, 4(1), e8.

Huang, J., Zhou, W., Dong, W., Watson, A. M., & Hong, Y. (2009). Directed, efficient, and versatile modifications of the Drosophila genome by genomic engineering. Proceedings of the National Academy of Sciences of the United States of America, 106(20), 8284–8289.

Huang, Y.-S., Jung, M.-Y., Sarkissian, M., & Richter, J. D. (2002). N-methyl-D-aspartate receptor signaling results in Aurora kinase-catalyzed CPEB phosphorylation and alpha CaMKII mRNA polyadenylation at synapses. The EMBO Journal, 21(9), 2139–2148.

Huynh, J.-R., Munro, T. P., Smith-Litière, K., Lepesant, J.-A., & Johnston, D. S. (2004). The Drosophila hnRNPA/B Homolog, Hrp48, Is Specifically Required for a Distinct Step in osk mRNA Localization. Developmental Cell, 6(5), 625–635.

Igreja, C., & Izaurralde, E. (2011). CUP promotes deadenylation and inhibits decapping of mRNA targets. Genes & Development, 25(18), 1955–1967.

Januschke, J., Gervais, L., Dass, S., Kaltschmidt, J. A., Lopez-Schier, H., St Johnston, D., et al. (2002). Polar transport in the Drosophila oocyte requires Dynein and Kinesin I cooperation. Current Biology, 12(23), 1971–1981.

Jenny, A., Hachet, O., Závorszky, P., Cyrklaff, A., Weston, M. D. J., Johnston, D. S., et al. (2006). A translation-independent role of oskar RNA in early Drosophila oogenesis. Development, 133(15), 2827–2833.

Johnstone, O., & Lasko, P. (2001). Translational regulation and RNA localization in Drosophila oocytes and embryos. Annual Review of Genetics, 35, 365–406.

Kato, Y., & Nakamura, A. (2012). Roles of cytoplasmic RNP granules in intracellular RNA localization and translational control in the Drosophila oocyte. Development, Growth & Differentiation, 54(1), 19–31.

Kim-Ha, J., Kerr, K., & Macdonald, P. M. (1995). Translational regulation of oskar mRNA by Bruno, an ovarian RNA-binding protein, is essential. Cell, 81(3), 403–412.

Kim-Ha, J., Smith, J. L., & Macdonald, P. M. (1991). oskar mRNA is localized to the posterior pole of the Drosophila oocyte. Cell, 66(1), 23–35.

Kim-Ha, J., Webster, P. J., Smith, J. L., & Macdonald, P. M. (1993). Multiple RNA regulatory elements mediate distinct steps in localization of oskar mRNA. Development, 119(1), 169–178.

King, M. L., Messitt, T. J., & Mowry, K. L. (2005). Putting RNAs in the right place at the right time: RNA localization in the frog oocyte. Biology of the Cell /

Under the Auspices of the European Cell Biology Organization, 97(1), 19–33.

Krauss, J., López de Quinto, S., Nüsslein-Volhard, C., & Ephrussi, A. (2009). Myosin-V Regulates oskar mRNA Localization in the Drosophila Oocyte. Current Biology, 19(12), 1058–1063.

Kugler, J.-M., & Lasko, P. (2009). Localization, anchoring and translational control of oskar, gurken, bicoid and nanos mRNA during Drosophila oogenesis. Fly, 3(1), 15–28.

Kumano, G. (2012). Polarizing animal cells via mRNA localization in oogenesis and early development. Development, Growth & Differentiation, 54(1), 1–18.

Lambert, J. D., & Nagy, L. M. (2002). Asymmetric inheritance of centrosomally localized mRNAs during embryonic cleavages. Nature, 420(6916), 682–686.

Lane, M. E., & Kalderon, D. (1993). Genetic investigation of cAMP-dependent protein kinase function in Drosophila development. Genes & Development, 7(7A), 1229–1243.

Lane, M. E., & Kalderon, D. (1994). RNA localization along the anteroposterior axis of the Drosophila oocyte requires PKA-mediated signal transduction to direct normal microtubule organization. Genes & Development, 8(24), 2986–2995.

Lane, M. E., & Kalderon, D. (1995). Localization and functions of protein kinase A during Drosophila oogenesis. Mechanisms of Development, 49(3), 191–200.

Lantz, V., Chang, J. S., Horabin, J. I., Bopp, D., & Schedl, P. (1994). The Drosophila orb RNA-binding protein is required for the formation of the egg chamber and establishment of polarity. Genes & Development, 8(5), 598–613.

Lasko, P. F., & Ashburner, M. (1990). Posterior localization of vasa protein correlates with, but is not sufficient for, pole cell development. Genes & Development, 4(6), 905–921.

Laver, J. D., Li, X., Ancevicius, K., Westwood, J. T., Smibert, C. A., Morris, Q. D., & Lipshitz, H. D. (2013). Genome-wide analysis of Staufen-associated mRNAs identifies secondary structures that confer target specificity. Nucleic Acids Research, 41(20), 9438–9460.

LeCuyer, K. A., Behlen, L. S., & Uhlenbeck, O. C. (1995). Mutants of the bacteriophage MS2 coat protein that alter its cooperative binding to RNA. Biochemistry, 34(33), 10600–10606.

Lehmann, R., & Nüsslein-Volhard, C. (1986). Abdominal segmentation, pole cell formation, and embryonic polarity require the localized activity of oskar, a maternal gene in drosophila. Cell, 47(1), 141–152.

Lehmann, R., & Nusslein-Volhard, C. (1991). The maternal gene nanos has a central role in posterior pattern formation of the Drosophila embryo. Development, 112(3), 679–691.

Lewis, R. A., & Mowry, K. L. (2007). Ribonucleoprotein remodeling during RNA localization. Differentiation, 75(6), 507–518.

Li, Z., Wang, L., Hays, T. S., & Cai, Y. (2008). Dynein-mediated apical localization of crumbs transcripts is required for Crumbs activity in epithelial polarity. The Journal of Cell Biology, 180(1), 31–38.

Liang, L., Diehl-Jones, W., & Lasko, P. (1994). Localization of vasa protein to the Drosophila pole plasm is independent of its RNA-binding and helicase activities. Development, 120(5), 1201–1211.

Lie, Y. S., & Macdonald, P. M. (1999). Translational regulation of oskar mRNA occurs independent of the cap and poly(A) tail in Drosophila ovarian extracts. Development, 126(22), 4989–4996.

Lin, A. C., & Holt, C. E. (2007). Local translation and directional steering in axons. The EMBO Journal, 26(16), 3729–3736.

Lipshitz, H. D., & Smibert, C. A. (2000). Mechanisms of RNA localization and translational regulation. Current Opinion in Genetics & Development, 10(5), 476–488.

Liu, N., Han, H., & Lasko, P. (2009). Vasa promotes Drosophila germline stem cell differentiation by activating mei-P26 translation by directly interacting with a (U)-rich motif in its 3' UTR. Genes & Development, 23(23), 2742–2752.

Long, R. M., Singer, R. H., Meng, X., Gonzalez, I., Nasmyth, K., & Jansen, R. P. (1997). Mating type switching in yeast controlled by asymmetric localization of ASH1 mRNA. Science, 277(5324), 383–387.

Lyon, A. M., Reveal, B. S., Macdonald, P. M., & Hoffman, D. W. (2009). Bruno protein contains an expanded RNA recognition motif. Biochemistry, 48(51), 12202–12212.

MacIver, B., McCormack, A., Slee, R., & Bownes, M. (1998). Identification of an essential gene encoding a class-V unconventional myosin in Drosophila melanogaster. European Journal of Biochemistry, 257(3), 529–537.

Markussen, F.-H., Michon, A.-M., Breitwieser, W., & Ephrussi, A. (1995). Translational control of oskar generates short OSK, the isoform that induces pole plasma assembly. Development, 121(11), 3723–3732.

Martin, K. C., & Ephrussi, A. (2009). mRNA Localization: Gene Expression in the Spatial Dimension. Cell, 136(4), 719–730.

Martin, K. C., & Zukin, R. S. (2006). RNA trafficking and local protein synthesis in dendrites: an overview. The Journal of Neuroscience : the Official Journal of the Society for Neuroscience, 26(27), 7131–7134.

Martin, M. A. E., Hurd, D. D., & Saxton, W. M. (1999). Kinesins in the nervous system. Cellular and Molecular Life Sciences : CMLS, 56(3-4), 200–216.

Martin, S. G., & St Johnston, D. (2003). A role for Drosophila LKB1 in anterior-posterior axis formation and epithelial polarity. Nature, 421(6921), 379–384.

McNeil, G. P., Kaur, M., Purrier, S., & Kang, R. (2009). The Drosophila RNA-binding protein Lark is required for localization of Dmoesin to the oocyte cortex during oogenesis. Development Genes and Evolution, 219(1), 11–19.

Medioni, C., Mowry, K., & Besse, F. (2012). Principles and roles of mRNA localization in animal development. Development, 139(18), 3263–3276.

Mendez, R., Murthy, K. G., Ryan, K., Manley, J. L., & Richter, J. D. (2000). Phosphorylation of CPEB by Eg2 mediates the recruitment of CPSF into an active cytoplasmic polyadenylation complex. Molecular Cell, 6(5), 1253–1259.

Mhlanga, M. M., Bratu, D. P., Genovesio, A., Rybarska, A., Chenouard, N., Nehrbass, U., & Olivo-Marin, J.-C. (2009). In Vivo Colocalisation of oskar mRNA and Trans-Acting Proteins Revealed by Quantitative Imaging of the Drosophila Oocyte. PLoS ONE, 4(7), e6241.

Micklem, D. R., Adams, J., Grünert, S., & St Johnston, D. (2000). Distinct roles of two conserved Staufen domains in oskar mRNA localization and translation. The EMBO Journal, 19(6), 1366–1377.

Mohr, S. E., Dillon, S. T., & Boswell, R. E. (2001). The RNA-binding protein Tsunagi interacts with Mago Nashi to establish polarity and localize oskar mRNA during Drosophila oogenesis. Genes & Development, 15(21), 2886–2899.

Morais-de-Sá, E., Vega-Rioja, A., Trovisco, V., & St Johnston, D. (2013). Oskar is targeted for degradation by the sequential action of Par-1, GSK-3, and the SCF-Slimb ubiquitin ligase. Developmental Cell, 26(3), 303–314.

Munro, T. P., Kwon, S., Schnapp, B. J., & St Johnston, D. (2006). A repeated IMP-binding motif controls oskar mRNA translation and anchoring independently of Drosophila melanogaster IMP. The Journal of Cell Biology, 172(4), 577–588.

Nakamura, A., Amikura, R., Hanyu, K., & Kobayashi, S. (2001). Me31B silences translation of oocyte-localizing RNAs through the formation of cytoplasmic RNP complex during Drosophila oogenesis. Development, 128(17), 3233–3242.

Nakamura, A., Sato, K., & Hanyu-Nakamura, K. (2004). Drosophila cup is an eIF4E binding protein that associates with Bruno and regulates oskar mRNA translation in oogenesis. Developmental Cell, 6(1), 69–78.

Neuman-Silberberg, F. S., & Schüpbach, T. (1993). The Drosophila dorsoventral patterning gene gurken produces a dorsally localized RNA and encodes a TGFα-like protein. Cell, 75(1), 165–174.

Orellana, S. A., & McKnight, G. S. (1992). Mutations in the catalytic subunit of cAMP-dependent protein kinase result in unregulated biological activity. Proceedings of the National Academy of Sciences of the United States of America, 89(10), 4726–4730.

Palacios, I. M., Gatfield, D., St Johnston, D., & Izaurralde, E. (2004). An eIF4AIII-containing complex required for mRNA localization and nonsense-mediated mRNA decay. Nature, 427(6976), 753–757.

Palacios, I. M., & St Johnston, D. (2001). Getting the message across: the intracellular localization of mRNAs in higher eukaryotes. Annual Review of Cell and Developmental Biology, 17, 569–614.

Paquin, N., & Chartrand, P. (2008). Local regulation of mRNA translation: new insights from the bud. Trends in Cell Biology, 18(3), 105–111.

Paquin, N., Ménade, M., Poirier, G., Donato, D., Drouet, E., & Chartrand, P. (2007). Local Activation of Yeast ASH1 mRNA Translation through Phosphorylation of Khd1p by the Casein Kinase Yck1p. Molecular Cell, 26(6), 795–809.

Pidoux, G., & Tasken, K. (2010). Specificity and spatial dynamics of protein kinase A signaling organized by A-kinase-anchoring proteins. Journal of Molecular Endocrinology, 44(5), 271–284.

Pokrywka, N. J., & Stephenson, E. C. (1995). Microtubules are a general component of mRNA localization systems in Drosophila oocytes. Developmental Biology, 167(1), 363–370.

Polesello, C., Delon, I., Valenti, P., Ferrer, P., & Payre, F. (2002). Dmoesin controls actin-based cell shape and polarity during Drosophila melanogaster oogenesis. Nature Cell Biology, 4(10), 782–789.

Reveal, B., Garcia, C., Ellington, A., & Macdonald, P. (2011). Multiple RNA binding domains of Bruno confer recognition of diverse binding sites for translational repression. RNA Biology, 8(6), 1047–1060.

Reveal, B., Yan, N., Snee, M. J., Pai, C.-I., Gim, Y., & Macdonald, P. M. (2010). BREs Mediate Both Repression and Activation of oskar mRNA Translation and Act In trans. Developmental Cell, 18(3), 496–502.

Riechmann, V., & Ephrussi, A. (2001). Axis formation during Drosophila oogenesis. Current Opinion in Genetics & Development, 11(4), 374–383.

Rongo, C., Gavis, E. R., & Lehmann, R. (1995). Localization of oskar RNA regulates oskar translation and requires Oskar protein. Development, 121(9), 2737–2746.

Roth, S. (1994). Axis Determination: Proteolytic generation of a morphogen. Current Biology, 4(8), 755–757.

Roth, S., Shira Neuman-Silberberg, F., Barcelo, G., & Schüpbach, T. (1995). cornichon and the EGF receptor signaling process are necessary for both anterior-posterior and dorsal-ventral pattern formation in Drosophila. Cell, 81(6), 967–978.

Rørth, P. (1998). Gal4 in the Drosophila female germline. Mechanisms of Development, 78(1-2), 113–118.

Schüpbach, T., & Wieschaus, E. (1986). Maternal-effect mutations altering the anterior-posterior pattern of the Drosophila embryo. Roux's Archives of Developmental Biology, 195(5), 302–317.

Schüpbach, T., & Wieschaus, E. (1991). Female Sterile Mutations on the Second Chromosomofe Drosophila melanogaster. 11. Mutations Blocking Oogenesis or Altering Egg Morpholog. Genetics, 129(4), 1119–1136.

Sen, J., Goltz, J. S., Stevens, L., & Stein, D. (1998). Spatially restricted expression of pipe in the Drosophila egg chamber defines embryonic dorsal-ventral polarity. Cell, 95(4), 471–481.

Sheth, U., & Parker, R. (2003). Decapping and decay of messenger RNA occur in cytoplasmic processing bodies. Science, 300(5620), 805–808.

Smith, J. L., Wilson, J. E., & Macdonald, P. M. (1992). Overexpression of oskar directs ectopic activation of nanos and presumptive pole cell formation in Drosophila embryos. Cell, 70(5), 849–859.

Snee, M., Benz, D., Jen, J., & Macdonald, P. M. (2008). Two distinct domains of Bruno bind specifically to the oskar mRNA. RNA Biology, 5(1), 1–9.

Snee, M. J., & Macdonald, P. M. (2009). Dynamic organization and plasticity of sponge bodies. Developmental Dynamics, 238(4), 918–930.

Spradling, A. C. (1993). Developmental genetics of oogenesis. In M. Bate, & A. Martinez-Arias (Eds.), *The Development of Drosophila melanogaster* (pp. 1-70). New York , NY: Cold Spring Harbor Laboratory Press.

St Johnston, D., Beuchle, D., & Nusslein-Volhard, C. (1991). Staufen, a gene required to localize maternal RNAs in the Drosophila egg. Cell, 66(1), 51–63.

St Johnston, D., & Nusslein-Volhard, C. (1992). The origin of pattern and polarity in the Drosophila embryo. Cell, 68(2), 201–219.

Stebbins-Boaz, B., Hake, L. E., & Richter, J. D. (1996). CPEB controls the cytoplasmic polyadenylation of cyclin, Cdk2 and c-mos mRNAs and is necessary for oocyte maturation in Xenopus. The EMBO Journal, 15(10), 2582–2592.

Stein, D. (1995). Pattern Formation: The link between ovary and embryo. Current Biology, 5(12), 1360–1363.

Sutton, M. A., & Schuman, E. M. (2006). Dendritic Protein Synthesis, Synaptic Plasticity, and Memory. Cell, 127(1), 49–58.

Suyama, R., Jenny, A., Curado, S., Pellis-van Berkel, W., & Ephrussi, A. (2009). The actin-binding protein Lasp promotes Oskar accumulation at the posterior pole of the Drosophila embryo. Development, 136(1), 95–105.

Tange, T. Ø., Shibuya, T., Jurica, M. S., & Moore, M. J. (2005). Biochemical analysis of the EJC reveals two new factors and a stable tetrameric protein core. RNA, 11(12), 1869–1883.

Taylor, S. S., Buechler, J. A., & Yonemoto, W. (1990). cAMP-dependent protein kinase: framework for a diverse family of regulatory enzymes. Annual Review of Biochemistry, 59, 971–1005.

Taylor, S. S., Ilouz, R., Zhang, P., & Kornev, A. P. (2012). Assembly of allosteric macromolecular switches: lessons from PKA. Nature Reviews Molecular Cell Biology, 13(10), 646–658.

Taylor, S. S., Yang, J., Wu, J., Haste, N. M., Radzio-Andzelm, E., & Anand, G. (2004). PKA: a portrait of protein kinase dynamics. Biochimica Et Biophysica Acta (BBA) - Proteins and Proteomics, 1697(1-2), 259–269.

Theurkauf, W. E., Alberts, B. M., Jan, Y. N., & Jongens, T. A. (1993). A central role for microtubules in the differentiation of Drosophila oocytes. Development, 118(4), 1169–1180.

Theurkauf, W. E., Smiley, S., Wong, M. L., & Alberts, B. M. (1992). Reorganization of the cytoskeleton during Drosophila oogenesis: implications for axis specification and intercellular transport. Development, 115(4), 923–936.

van Eeden, F. J., Palacios, I. M., Petronczki, M., Weston, M. J., & St Johnston, D. (2001). Barentsz is essential for the posterior localization of oskar mRNA and colocalizes with it to the posterior pole. The Journal of Cell Biology, 154(3), 511–523.

Vanzo, N. F., & Ephrussi, A. (2002). Oskar anchoring restricts pole plasm formation to the posterior of the Drosophila oocyte. Development, 129(15), 3705–3714.

Vanzo, N., Oprins, A., Xanthakis, D., Ephrussi, A., & Rabouille, C. (2007). Stimulation of Endocytosis and Actin Dynamics by Oskar Polarizes the Drosophila Oocyte. Developmental Cell, 12(4), 543–555.

Vazquez-Pianzola, P., Urlaub, H., & Suter, B. (2011). Pabp binds to the osk 3'UTR and specifically contributes to osk mRNA stability and oocyte accumulation. Developmental Biology, 357(2), 404–418.

Verrotti, A. C., & Wharton, R. P. (2000). Nanos interacts with cup in the female germline of Drosophila. Development, 127(23), 5225–5232.

Webster, P. J., Liang, L., Berg, C. A., Lasko, P., & Macdonald, P. M. (1997). Translational repressor bruno plays multiple roles in development and is widely conserved. Genes & Development, 11(19), 2510–2521.

Wieschaus, E. and Nüsslein-Volhard, C. (1986). Looking at Embryos. In D.B. Roberts (Ed.), *Drosophila, a practical approach* (pp. 199-226). Oxford: IRL Press.

Wilhelm, J. E., Hilton, M., Amos, Q., & Henzel, W. J. (2003). Cup is an eIF4E binding protein required for both the translational repression of oskar and the recruitment of Barentsz. The Journal of Cell Biology, 163(6), 1197–1204.

Wilhelm, J. E., Mansfield, J., Hom-Booher, N., Wang, S., Turck, C. W., Hazelrigg, T., & Vale, R. D. (2000). Isolation of a ribonucleoprotein complex involved

in mRNA localization in Drosophila oocytes. The Journal of Cell Biology, 148(3), 427–440.

Wilsch-Brauninger, M., Schwarz, H., & Nusslein-Volhard, C. (1997). A sponge-like structure involved in the association and transport of maternal products during Drosophila oogenesis. The Journal of Cell Biology, 139(3), 817–829.

Wong, W., & Scott, J. D. (2004). AKAP signalling complexes: focal points in space and time. Nature Reviews Molecular Cell Biology, 5(12), 959–970.

Yano, T., de Quinto, S. L., Matsui, Y., Shevchenko, A., Shevchenko, A., & Ephrussi, A. (2004). Hrp48, a Drosophila hnRNPA/B Homolog, Binds and Regulates Translation of oskar mRNA. Developmental Cell, 6(5), 637–648.

Yoshida, S., Müller, H.-A. J., Wodarz, A., & Ephrussi, A. (2004). PKA-R1 spatially restricts Oskar expression for Drosophila embryonic patterning. Development, 131(6), 1401–1410.

Zappavigna, V., Piccioni, F., Villaescusa, J. C., & Verrotti, A. C. (2004). Cup is a nucleocytoplasmic shuttling protein that interacts with the eukaryotic translation initiation factor 4E to modulate Drosophila ovary development. Proceedings of the National Academy of Sciences of the United States of America, 101(41), 14800–14805.

Zhang, Z., Stevens, L. M., & Stein, D. (2009). Sulfation of Eggshell Components by Pipe Defines Dorsal-Ventral Polarity in the Drosophila Embryo. Current Biology, 19(14), 1200–1205.

Zimyanin, V. L., Belaya, K., Pecreaux, J., Gilchrist, M. J., Clark, A., Davis, I., & St Johnston, D. (2008). In Vivo Imaging of oskar mRNA Transport Reveals the Mechanism of Posterior Localization. Cell, 134(5), 843–853.