Copyright

by

Matthew Robert Kanke

2014

The Dissertation Committee for Matthew Robert Kanke Certifies that this is the approved version of the following dissertation:

Characterization of *oskar* translational activation and the *oskar* RNA function

Committee:

Paul Macdonald, Supervisor

Janice Fischer

Arlen Johnson

Kimberly Raab-Graham

David Stein

Characterization of *oskar* translational activation and the *oskar* RNA function

by

Matthew Robert Kanke, 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

December 2014

Dedication

To my parents,

for their enduring support and affection.

Acknowledgements

I would like to thank my advisor, Dr. Paul Macdonald, for allowing my participation in his research, providing guidance, and showing patience as I developed into a scientist. He is an exempliary scientist to model oneself after. I would like to thank the members of my dissertation committee: Dr. Janice Fischer, Dr. Arlen Johnson, Dr. Kimberly Raab-Graham, and Dr. David Stein, for advice offered and time spent at my committee meetings. I would like to thank the past and present members of the Macdonald lab and the greater UT fly community, for the support and advice on my projects over the years. Finally, I would like to thank my family and friends. Over the years, the disappointments would have been more bitter and the successes less sweet without you in my life.

Characterization of *oskar* translational activation and the *oskar* RNA function

Matthew Robert Kanke, Ph.D

The University of Texas at Austin, 2014

Supervisor: Paul M. Macdonald

Oskar (Osk) protein is required for posterior body patterning and establishment of the germline in *Drosophila*. Coordination of *osk* mRNA localization and translational regulation ensures Osk protein expression is confined to the oocyte posterior. Proper expression requires repression of *osk* RNA during transport and activation upon localization. Once activated, *osk* mRNA is translated into two protein isoforms, Long and Short Osk. Here I describe an element in the 5' end of *osk* mRNA that is highly conserved across multiple *Drosophila* species and required for *osk* translational activation. This 5' element is located in a region that is also protein coding for the longer Osk isoform and assays were designed to disentangle the effects that mutations had on protein and RNA function. The 5' element is needed for efficient Osk translation, but only in the absence of Long Osk translation from the same transcript, suggesting a redundant role. Although the 5' element was previously implicated in a posterior-specific relief of repression, here I provide evidence that

the 5' element acts as a general enhancer of translation, independent of localization and repression.

In addition to its protein coding role, *osk* mRNA has a non-coding function. Egg chambers lacking *osk* mRNA fail to form a karyosome and arrest mid-oogenesis. RNA function depends on the presence of the *osk* 3' UTR in the oocyte. Here I demonstrate that *osk* mRNA influences distribution of regulators. In the absence of *osk* mRNA these regulators dissociate from ribonucleoproteins in the germ cells and accumulate in the follicle cells. I find that the *osk* 3' UTR performs multiple roles contributing to RNA function. Multiple binding sites act to sequester the translational repressor Bruno in one role. Another involves sequences not bound by Bruno near the 3' end of *osk*. In contrary to disruption of Bruno sequestration, which requires mutation of multiple binding sites, mutation of a single site was sufficient to disrupt RNA function. However, disruption of either role recapitulates the failure of karyosome formation and the accumulation of regulators in the follicle cells.

Table of Contents

List of Figures xi

Chapter 1: General Introduction 1

DROSOPHILA OOGENESIS 1

ESTABLISHING THE BODY PATTERN IN DROSOPHILA 2

OVERVIEW OF *oskar* mRNA LOCALIZATION AND TRANSLATIONAL REGULATION 4

LOCALIZATION OF *oskar* mRNA 4

TRANSLATIONAL REGULATION OF *oskar* mRNA 7

TRANSLATION FROM ALTERNATE START CODONS 13

A NON-CODING FUNCTION OF *oskar* mRNA 17

OVERVIEW OF THE DISSERTATION RESEARCH 19

REFERENCES 24

Chapter 2: A 5' region of the *oskar* mRNA plays both regulatory and protein-coding roles in enhancing posterior accumulation of Oskar protein 33

ABSTRACT 34

INTRODUCTION 35

RESULTS 39

Mapping a regulatory element in the *osk* 5' region 39

The *oskar* 5' regualtory element activates translation 41

The *oskar* 5' activation element is normally dispensable 45

The 5' element enhances protein accumulation independent of repression or mRNA localization 46

Does the 5' element mediate oocyte-specific translational activation? 48

DISCUSSION 50

MATERIALS AND METHODS 55

Flies and Transgenes 55

Cuticle analysis 56

Determination of sequence conservation 56
Western blotting 57
Whole mount immunodetection 57
RNA analysis 58
Acknowledgments 58
FIGURES 60
REFERENCES 73

Chapter 3: *oskar* RNA plays multiple non-protein coding roles to support oogenesis and maintain integrity of the germline/soma distinction .. 76
ABSTRACT 77
INTRODUCTION 78
RESULTS 80
Bruno binding sites are required for *oskar* RNA function 80
oskar mRNA acts to sequester Bruno 81
A second component to *oskar* RNA function 83
oskar RNA function and karyosome formation 87
Absence of *oskar* mRNA alters the distribution of germline-specific or -enriched proteins 89
DISCUSSION 91
MATERIALS AND METHODS 97
Flies and Transgenes 97
Egg laying assays 97
RNA detection 98
Measurement of egg chamber length 98
RNA binding 99
Whole mount immunodetection 99
Acknowledgments 100
FIGURES 101
REFERENCES 118

Chapter 4: Future directions 121
Future directions related to chapter 2 122
Future directions related to chapter 3 124
REFERENCES 127

Appendix: Investigating Osk isoform accumulation preference 128
INTRODUCTION 129
RESULTS 129
Slightly increasing 5' UTR length or altering *osk* start codon context do not affect ratio of isoforms, but do affect the overall Osk protein level 129
Substantial lengthening of the 5' UTR increases Long Osk production 131
MATERIALS AND METHODS 132
Flies and Transgenes 132
Western blotting 132
RNA detection 133
FIGURES 134
REFERENCES 137

References 138

Vita 150

List of Figures

Figure 1.1. Egg chamber progression during *Drosophila* oogenesis....... 21

Figure 1.2. Maternal mRNA localization in a *Drosophila* egg chamber ... 22

Figure 1.3. Translational regulatory elements in *osk* mRNA and the Osk protein isoforms 23

Figure 2.1. Mapping an RNA element required for *osk* activity 60

Figure 2.2. Sequence conservation in the 5' region of the *osk* gene....... 62

Figure 2.3. The 5' element is required for translational activation 63

Figure 2.4. The 5' element is required for the early phase of Osk protein accumulation 65

Figure 2.5. Effects of mutating the 5' element on translation and Long Osk function 67

Figure 2.6. The 5' element activates translation independent of mRNA localization and translational repression................................ 69

Figure 2.7. *osk* 5' sequences confer oocyte enrichment on reporter expression 72

Figure 3.1. BREs are required for the RNA function of *osk*.................... 101

Figure 3.2. Sequences in the *osk* 3' UTR C region are essential for *osk* RNA function, independent of Bru binding 103

Figure 3.3. Mapping regions of the *osk* 3' UTR that contribute to *osk* RNA function 105

Figure 3.4. Fine scale mapping of *osk* RNA function elements............. 107

Figure 3.5. Karyosome defects of *osk* RNA null mutants 109

Figure 3.6. Redistribution of Bru and other germline proteins in *osk* RNA null ovaries 111
Figure 3.7. Confirmation of Bru redistribution in *osk* RNA null ovaries.. 113
Figure 3.8. Mutants defective in *osk* RNA function fail to rescue the Bru redistribution phenotype 114
Figure 3.9. Reducing levels of *osk* mRNA and PABP causes karyosome defects 116
Figure 4.1. 5' UTR and AUG context contribute to Osk protein accumulation 134
Figure 4.2. Extended 5' UTR causes modest increase in Long Osk accumulation 136

Chapter 1: General Introduction

DROSOPHILA OOGENESIS

Oogenesis is the process by which a germ cell matures into an egg, and, in *Drosophila*, has been divided into 14 developmental stages to assist in discussion (Figure 1.1). A female has two ovaries, each composed of 16-18 ovarioles. The ovariole is a progression of egg chambers in the various stages of oogenesis, with the anterior in the germarium and the posterior near fully developed. Beginning in the germarium, a single germline stem cell divides to produce a replacement stem cell and a cystoblast. This cystoblast undergoes four rounds of mitosis coupled with incomplete cytokinesis to form a single cyst, which comprises 16 cells connected by cytoplasmic bridges (known as ring canals) surrounded by a monolayer of somatic follicle cells. One of the two cells from the first mitotic division accumulates the factors necessary to differentiate into the oocyte. The remaining 15 cells, called nurse cells, become polyploid and will supply the oocyte with the nutrients and organelles needed to develop properly. The 15 nurse cells and oocyte surrounded by a layer of follicular cells form the unit known as the egg chamber (Figure 1.2). In contrast to the nurse cell nuclei, the oocyte nucleus condenses the chromatin into a compact structure known as the karyosome. As development progresses, the oocyte increases in size due to the deposition of nutrients, proteins and cytoplasm by the nurse cells. As oogenesis concludes, the nurse cells, in a process known as “dumping”, degrade and deposit their bulk cytoplasm into the oocyte. The mature egg will arrest initially at prophase I of meiosis before progressing to a prolonged arrest in metaphase I until fertilization occurs. Following fertilzation, meiosis completes,

yielding the fertilized ovum and three polar bodies, of which the three polar bodies will degenerate (reviewed in (Bastock and St Johnston, 2008; Riechmann & Ephrussi, 2001)).

ESTABLISHING THE BODY PATTERN IN DROSOPHILA

Organization of the basic body plan is one of the initial steps of morphogenesis. Determination of body patterning in many cases requires the asymmetric distribution of mRNAs and proteins. Proper deployment of these determinants often relies on coordinated programs of RNA localization and translational regulation. These programs can be executed autonomously or in response to an external cue. In mammalian systems, the establishment of the body pattern occurs post-fertilization, with establishment of the anterior-posterior axis occurring in response to sperm entry. (Lu et al., 2001). In other organisms, formation of some body axes can occur prior to zygote formation, by the asymmetric accumulation of maternally-provided mRNAs. In *Xenopus*, the localization of *VegT* and *Vg1* to the vegetal pole of the egg dictates the animal-vegetal hemispheres, however the sperm entry site defines the dorsal-ventral axis (Heasman, 2006). *Drosophila* represents one extreme where the egg is fully patterned prior to fertilization.

In *Drosophila*, embryonic axis formation relies on localization of three key mRNAs within the oocyte (Riechmann & Ephrussi, 2001). Dorsal-ventral patterning is ordered by the localization of *gurken* (*grk*) mRNA to the anterior-lateral corner. The anterior-posterior patterning requires localization of *bicoid*

(*bcd*) mRNA to the anterior and *oskar* (*osk*) mRNA to the posterior (van Eden & St Johnston, 1999).

Grk signaling at the posterior establishes the polarized microtubule (MT) network necessary for localization of these mRNAs within the oocyte. Here, Grk signals the follicle cells to assume a posterior fate (González-Reyes et al., 1995; Roth et al., 1995). The follicle cells in turn signal the oocyte and cause the disassembly of the posterior MT organizing center (MTOC), resulting in a repolarization of the MT network, with the MTs emanating from the anterior portion of the oocyte (Steinhauer, J. & Kalderon, D., 2006). Transport along the repolarized MTs localizes the oocyte nucleus and *grk* mRNA at the anterior-dorsal corner of the oocyte (Macdougall et al., 2003), where Grk signal induces the overlaying follicle cells to adopt a dorsal fate (Neuman-Silberberg & Schupbach, 1993). *bcd* mRNA accumulates as an anterior to posterior gradient in the oocyte (Spirov et al., 2009). *bcd* mRNA is translated post-fertilization and Bcd protein is necessary for anterior patterning (Driever & Nusslein-Volhard, 1988).

Contrasting *bcd* and *grk*, *osk* mRNA is localized to the posterior pole of the oocyte (Kim-Ha et al., 1991). At the posterior, *osk* is translated from alternate, in-frame translational start sites into two protein isoforms, Long and Short Osk (Kim-Ha et al., 1995; Markussen et al., 1995; Rongo et al., 1995). Long Osk anchors Short Osk and *osk* mRNA, while Short Osk nucleates the formation of the pole plasm, a differentiated cytoplasm containing factors necessary for posterior patterning and establishment of the germline (Markussen et al., 1995; Vanzo & Ephrussi, 2002). *nos* localization and translation at the posterior of the embryo depends on pole plasm formation (Ephrussi et al., 1991). Nos protein

diffuses to form a posterior to anterior gradient necessary for posterior patterning (Gavis & Lehmann, 1992).

OVERVIEW OF *OSKAR* MRNA LOCALIZATION AND TRANSLATIONAL REGULATION

osk mRNA is synthesized in the nurse cells, transported to the oocyte, and localized to the posterior of the oocyte prior to translation (Kim-Ha et al., 1995; Markussen et al., 1995; Rongo et al., 1995). Posterior expression of Osk is necessary for formation of the posterior abdominal segments and formation of pole cells, the precursors to the germ line (Ephrussi & Lehmann, 1992; Lehmann & Nusslein-Volhard, 1986). The lack of posterior Osk results in loss of posterior patterning while either ectopic or excessive Osk results in too much posterior patterning and loss of anterior body patterning (Ephrussi and Lehman, 1992; Lehmann & Nusslein-Volhard, 1986; Smith et al., 1992; Wharton & Sthrul, 1991). The dire consequences of *osk* misregulation require that stringent controls exist for *osk* mRNA localization, translational repression, and translational activation. Here I discuss the underlying mechanisms.

LOCALIZATION OF *OSKAR* MRNA

Localized mRNAs assemble into ribonucleoproteins (RNP) complexes composed of the RNA and numerous *trans*-acting factors engaged with the RNA. These RNPs contain factors necessary for both localization as well as translational regulation of the mRNA. The RNPs are directed to subcellular locations by the binding of *trans*-factors to *cis*-RNA elements, known as

localization signals, which are often stemloop structures found in the 3' UTR. Localized RNAs can contain multiple localization signals to direct transport through different cellular regions. Movement of RNPs along microfilaments and microtubules requires coupling to molecular motors (reviewed in (Besse & Ephrussi, 2008; Martin & Ephrussi, 2009)).

osk mRNA is synthesized in the nurse cells. From here, *osk* RNA must be transported to the oocyte, localized to the posterior pole, and maintained at the posterior for proper deployment of the Osk protein. *cis*-acting elements and *trans*-acting factors assemble a localization-competent RNP complex. Many *osk* mRNAs and proteins can combine to form large particles (Snee & Macdonald, 2009). *cis*-elements in the 3' UTR and the *trans*-acting factors polypyrimidine track binding (PTB) and Bru are proposed to contribute to assembly of these large particles (Besse et al., 2009; Chekulaeva et al., 2006). Directed transport relies on *cis*-acting *osk* RNA localization signals and coupling of the RNPs to MT-based motors (Besse and Ephrusi, 2008; Kugler and Lasko, 2009). Maintenance at the posterior requires the action of Osk protein, microfilaments, and an actin-based molecular motor (Babu et al., 2004; Krauss et al., 2009; Vanzo et al., 2007).

Assembly of the localization-competent *osk* RNP begins prior to export from the nurse cell nuclei. Here, splicing of *osk* is likely coupled with the deposition of the exon junction complex (EJC) upstream of the splice site (Hachet & Ephrussi, 2004). Splicing is essential to localization, as the removal of the first intron forms a localization signal, the spliced *osk* localization element (SOLE) (Hachet & Ephrussi, 2004; Ghosh et al., 2012). The SOLE and EJC components are required for the posterior localization in the oocyte, but not the initial transport

to the oocyte (Hachet & Ephrussi, 2001; Ghosh et al., 2012; Mohr et al., 2001; Palacios et al., 2004; Van Eden et al., 2001). The *osk* RNP is exported to the cytoplasm in a process that could involve the association of more factors; the *osk* RNP contains several heterogeneous nuclear RNP (hnRNP) proteins, a group of proteins that first bind the RNA in the nucleus, such as Bruno (Bru) and Hrp48 (Huynh et al., 2004; Snee et al., 2008). The *osk* RNP is coupled to the MT minus-end-directed motor Dynein and is transported to the oocyte along MTs emanating from the posterior of the oocyte. This oocyte transport is provided by a localization signal, the oocyte enrichment signal (OES), found in the *osk* 3' UTR as well as the Bicaudal D and Egalitarian components of the *osk* RNP granule (Bullock & Ish-Horowicz, 2001; Clark et al., 2007; Jambor et al., 2014; Mach & Lehmann, 1997).

Posterior accumulation of the *osk* RNP relies on the SOLE, located in the *osk* coding region, and the *osk* 3' UTR (Ghosh et al., 2012; Hatchet & Ephrussi, 2004; Kim-Ha et al., 1993). Localization requires the association with the plus-end-directed motor Kinesin (Brendza et al., 2000). The *osk* RNP is transported in all directions, but a slight bias towards an enrichment of microtubule plus ends at the posterior of the oocyte is enough for localization (Zimyanin et al., 2008).

At the posterior, *osk* mRNA is translated into two protein isoforms, Long and Short Osk, beginning at stage 9 of oogenesis (Markussen et al., 1995). Long Osk is necessary for anchoring of Short Osk and *osk* mRNA at the posterior (Vanzo & Ephrussi, 2002). In conjunction, the Osk isoforms stimulate actin filament growth into the oocyte, and these actin projections have been proposed to maintain pole plasm at the posterior (Vanzo et al., 2007). Consistent with this hypothesis, mutation of factors necessary for actin cytoskeleton maintenance

disrupts Osk anchoring (Jankovics et al., 2002; Dahlgaard et al., 2007), and a class-V myosin (MyoV) motor, which moves along actin filaments, is needed for the tight posterior crescent of *osk* mRNA and Osk protein (Krauss et al., 2009).

A late phase accumulation of *osk* mRNA at the posterior occurs by a mechanism distinct from directive MT transport. At the end of stage 10A, the microtubule network restructures, thus disrupting the Kinesin-dependent path of *osk* localization (Theurkauf et al., 1992). At the same time, MT-dependent movement of the oocyte cytoplasm begins to occur (known as cytoplasmic streaming) (Theurkauf et al., 1992). Fluorescently labeled *osk* mRNA was transported and anchored to the posterior during cytoplasmic streaming in a process dependent on endogenous *osk* expression (Glotzer et al., 1997). This suggests a mechanism where unlocalized *osk* mRNA is moved around the oocyte by cytoplasmic streaming, but entrapped at the posterior by Osk anchoring. While Osk protein begins to accumulate at stage 9, the majority of Osk is produced during a later phase, around stage 13/14 (Snee et al., 2007), and the entrapment of previously unlocalized *osk* mRNA could contribute to this enrichment.

TRANSLATIONAL REGULATION OF *OSKAR* MRNA

Temporal and spatial control of protein expression requires coupling of mRNA localization with translational regulation. Translational regulation occurs in two phases: translational repression during transport of mRNA and translational activation once localization is achieved (Besse and Ephrussi, 2008). Both phases are crucial, as ectopic expression of Osk due to faulty translational

repression results in anterior patterning defects (Kim-Ha et al., 1995). Conversely, lack of Osk accumulation due to faulty translational activation results in posterior patterning defects (Kim-Ha et al., 1995). Translational activation occurs either by turning off repression by the subtraction or inactivation of a repressor, bypassing repression by recruiting a protein to activate translation, or a combination of the two.

In the nurse cells, various repressors are assembled in the *osk* RNP. Translational repression is collaborative, and missing repressive components can lead to precocious translation of *osk* mRNA (Nakamura et al., 2004; Saffman et al., 1998; Wilhelm et al., 2003). Mechanisms exist to block access to *osk* mRNA by translational machinery, such as the interaction between Bru, Cup, and the eukaryotic translational initiation factor 4E (eIF4E) (Jackson et al., 2010; Nakamura et al., 2004). Canonical translational initiation requires the binding of eIF4E to eIF4G at the 5' cap of the mRNA, and the suggested competition for the eIF4E binding site by Cup would disrupt translational initiation complex recruitment (Nakamura et al., 2004). In a more indirect approach, *osk* mRNA is assembled into large RNP particles and these particles are hypothesized to silence translation by blocking access to *osk* by the translational initiation complex (Besse et al., 2009; Chekulaeva et al, 2006). Conversely, mechanisms exist that directly alter *osk* mRNAs' ability to be used as a translational substrate. In many cases, a long poly(A) tail is required for efficient translation, and *osk* lacking a poly(A) tail is poorly translated independent of repression (Castagenetti and Ephrussi, 2003).

POTENTIAL MECHANISMS FOR OSK TRANSLATIONAL ACTIVATION

Antagonistic counterparts exist to these repressive mechanisms to activate translation. Phosphorylation is a key component to expression of many localized mRNAs, as phosphorylation of repressors can decrease RNA or protein binding affinities (Besse and Ephrussi, 2008). For example, phosphorylation of the repressor ZBP1 by Src kinase permits translation of β-actin at cell boundaries (Huttelmaier et al., 2005). Similarly in yeast, phosphorylation of repressors Khd1 and Puf6 reduces RNA binding and permits translation of Ash1p in the budding daughter cell (Deng et al., 2008; Paquin et al, 2007). cAMP-dependent Protein Kinase (PKA) activity is necessary for proper spatial expression of *osk* (Yoshida et al., 2004). PKA is composed of two phosphorylating, catalytic subunits and two regulatory subunits that inhibit the catalytic subunits (Taylor et al., 1990). A cAMP signal causes dissociation of the catalytic and regulatory subunits, allowing for the phosphorylation of PKA targets.

Mutations in the regulatory subunit, PKA-R1, cause precocious, ectopic Osk expression, indicating that excessive phosphorylation disrupts *osk* translational repression (Yoshida et al., 2004). Bru is a target of PKA and mutation of predicted PKA phosphorylation sites in Bru that mimic phosphorylation disrupt Bru dimerization and weaken the interaction with the eIF4 binding protein (eIF4-BP) Cup *in vitro* (Kim et al., 2014). The same mimetic mutations do not affect translational repression *in vivo*, although binding to Cup is not disrupted *in vivo* either (Kim et al., 2014). Phosphorylation of additional sites in Bru could be required *in vivo* for relief of repression.

A long poly-A tail is a prerequisite for efficient translation of many RNAs during development, including the axis determinants *nos* and *bcd* (Lieberfarb et

al., 1996; Zaessinger et al., 2006). Translational repression can be accomplished by the recruitment of deadenylases to shorten the tail, such as CCR4/NOT1 complex (Piccioni, 2005). Translational fate depends on the dynamics of the poly(A) tail length. Bicaudal-C (BicC) recruits the CCR4/NOT1 complex to RNAs (Chicoine et al., 2007). BicC is a negative regulator of *osk* and could conceivably mediate repression by the recruitment of CCR4/NOT1 to *osk* mRNA (Chicoine et al., 2007; Saffman et al., 1998). Indeed, a long poly(A) tail is needed for efficient *osk* translation (Castagenetti and Ephrussi, 2003). The *Drosophila* CPEB homolog oo18 RNA binding (Orb) is necessary for elongation of poly(A) tails through the recruitment of poly(A) polymerases (Benoit et al., 2008; Juge et al., 2002). Orb is a component of the *osk* RNP, and in *Orb* mutants, *osk* poly(A) tail length and Osk protein levels are reduced, indicating Orb is needed for *osk* polyadenylation and translation (Chang et al., 1999; Castagenetti and Ephrussi, 2003). Orb is in a complex with BicC and these proteins could be acting antagonistically on *osk* poly(A) tail length, as the defects associated with BicC mutant are suppressed when combined with an *orb* mutant (Castagenetti and Ephrussi, 2003).

CIS-ACTING OSK TRANSLATIONAL ACTIVATION ELEMENTS

osk translational activation is mediated by *cis*-acting elements identified in multiple regions of the *osk* mRNA. These include a Bruno binding sites in the C region of the *osk* 3' UTR, Imp Binding Elements (IBE) dispersed throughout the *osk* 3' UTR, and 5' element in the *osk* coding region (Figure 1.3) (Gunkel et al., 1998, Reveal et al., 2010; Munro et al., 2006).

Bru binds to multiple sites in two regions of the *osk* 3' UTR, the AB and C regions (Figure 1.3), and binding to both regions contributes to translational repression (Kim-Ha et al., 1995; Reveal et al., 2011). Mutation of one type of Bruno binding site, the Bruno response elements (BREs), alleviates repression and results in ectopic expression of Osk (Kim-Ha et al., 1995). Interestingly, Bru binding sites have been shown to have an additional contribution to relief from repression (Reveal et al., 2010). Mutation of Bru binding sites only in the C region severely reduces Osk expression at the posterior, indicating the C region Bru binding sites are necessary for translational activation as well (Reveal et al., 2010). Because *osk* is translated in the absence of BREs, the loss of Osk expression when C region binding sites are mutated suggest a role for the Bru binding sites in relief from Bru-mediated repression (Reveal et al., 2010).

The *Drosophila* homolog of insulin growth factor II mRNA-binding protein (IMP) binds to the consensus sequence UUUAY, termed the Imp Binding Element (IBE). These IBEs are found in 13 locations dispersed throughout the *osk* 3' UTR (Figure 1.3) (Munro et al., 2006). Mutation of 3' UTR IBEs results in a loss of posterior localization of Imp and mutation of certain subsets of these IBEs cause a loss of *osk* translation (Munro et al., 2006). Although the Imp-binding sites are required for *osk* translation, the Imp protein itself is dispensable, as *osk* is translated in *Imp* loss-of-function mutants (Geng and Macdonald, 2006; Munro et al., 2006). Therefore IBEs are necessary for *osk* translation by a yet unknown, Imp-independent mechanism.

The *osk* 5' regulatory element was identified by the removal of nucleotides 39-288 from a genomic *osk* transgene and the observation that the deletion mutant abolished Osk activity (Gunkel et al., 1998). To further characterize the

element, an *osk::lacZ* reporter transgene was constructed containing the 5' portion of *osk* fused to the *lacZ* coding region and regulated by the *osk* 3' UTR. In the context of the reporter transgene, a 62 base pair inversion eliminated β-galactosidase activity (Figure 1.3) (Gunkel et al., 1998). An *osk::lacZ* transgene containing a truncated *osk* 3' UTR fused to anterior localization signal localized to the anterior of the oocyte, but failed to be translated, leading to the conclusion that the 5' element acts specifically at the posterior of the oocyte (Gunkel et al., 1998). However, the deleted portion of the *osk* 3' UTR is required to antagonize Bru-mediated repression (Reveal et al., 2010), and whether the 5' element enhances translation elsewhere remains unknown.

FACTORS MEDIATING OSK TRANSLATIONAL ACTIVATION

Several other proteins are known to be involved in *osk* translation, but how they function remains unresolved. Vasa (Vas) colocalizes with the *osk* transcript to the posterior pole of stage 9 oocytes (Liang et al., 1994), and *vas* mutants cause a decrease in Osk expression (Markussen et al., 1995; Rongo et al., 1995). Vas is an ATP-dependent, DEAD-box RNA helicase (Hay et al., 1988). Vas^{O14} and Vas^{O11} contain mutations that disrupt the RNA binding capabilities, and these mutants fail to produce a germline (Liang et al., 1994). Vas could bind to *osk* and enhance expression by removing RNA structural complexity (Hay et al., 1988). Alternatively, Vas binds eIF5B, an essential eIF necessary for ribosomal subunit joining, and Vas/eIF5B binding is essential for germline formation (Carrera et al., 2000; Johnstone and Lasko, 2004). Disruption of the binding does not affect Osk accumulation in stage 10 oocytes (Johnstone and Lasko, 2004). However, Vas is not required for initial Osk accumulation, and the

late phase of Osk accumulation could require the Vas/eIF5B interaction (Harris & Macdonald, 2001; Snee et al., 2007).

Stau and *osk* localize to the posterior pole in a process that is interdependent (St Johnston et al., 1991). Staufen contains five double-stranded RNA binding domains (dsRBD), of which dsRBD2 and dsRBD3 have been implicated in localization (Micklen et al., 2000; Ramos et al., 2000; St Johnston et al., 1992). However, the dsRBD5 is not required for localization, as the *osk* mRNA accumulates at the posterior of the oocyte when the domain is deleted, yet no Osk protein is produced (Micklem et al., 2000). The dsRBD5 domain must either alleviate repression or activate translation. Insight comes from *osk BRE-* mRNA, which accumulates Osk ectopically due to defective translational repression, but doesn't accumulate any Osk, either ectopically or at the posterior, in a *stau* mutant (Kim Ha et al., 1995). The failure to accumulate Osk in a repression-defective situation indicates that Stau acts in translational activation of *osk*.

TRANSLATION FROM ALTERNATE START CODONS

Translational initiation involves the assembly of an elongation-competent 80s ribosome on an mRNA, which generally occurs at the site of an initiation codon, or AUG. Recognition of the AUG is by a 48S initiation complex, composed of the mRNA, the 40S ribosomal subunit, the tRNA anticodon, and various eukaryotic initiation factors (eIFs). The binding of the anticodon to AUG stimulates the attachment of the 48S initiation complex to the 60S ribosomal subunit, thus forming of the 80S ribosome (Jackson et al., 2010).

Scanning-dependent translational initiation requires the assembly of the initiation complex on the 5' cap of the mRNA. Initiation complex assembly requires the eIF4F complex, composed of the cap-binding eIF4E, the RNA helicase eIF4A, and the protein scaffold eIF4G, associating with the 40S ribosomal subunit and associated eIFs. Scanning by the complex requires the unwinding of mRNA secondary structure by eIF4A and movement of the ribosome in the 3' direction. At the first AUG encountered, eIF2 hydrolysis of GTP commits the complex to initiation and eIF5B mediates the release of various eIFs and the joining of the 60S subunit (Jackson et al., 2010).

Despite the general mechanism for translational initiation, it isn't always the first AUG that is used. There are multiple mechanisms to allow translation to initiate from a downstream AUG (Kochetov, 2008). In cap-dependent translation, AUG recognition by the scanning initiation complex depends on the nucleotide sequences surrounding the AUG (known as the context), and a poor context can inhibit AUG recognition by some initiation complexes (Kozak, 1997). This leaky initiation results in complexes continuing to scan the mRNA, where they are free to then initiate translation at alternative, downstream AUGs.

Because the failed recognition of an AUG in a suboptimal context allows for initiation of translation at a downstream initiation codon, if the downstream AUG is in the same reading frame, a single mRNA can produce two protein isoforms differing only in the amino-terminal end. Yeast Glutaredoxin 2 (Grx2) reduces oxidative species that can prove toxic at high concentrations. In yeast, two Grx2 isoforms with distinct subcellular localizations are produced from a single mRNA (Pedrajas et al., 2002). The long isoform localizes to the mitochondria. The short isoform is translated from an in-frame AUG downstream

from the long *GRX2* AUG and is cytoplasmic. Expression of short *GRX2* depends on a poor initiation codon context at the upstream AUG; mutation of the upstream AUG to the optimal context eliminates expression of short *GRX2* (Porras et al., 2006). By maintaining a suboptimal context at the long AUG, *GRX2* is able to produce two isoforms with discrete localization patterns, providing oxidative reductase activity to multiple cellular localities.

Translation can also reinitiate downstream of a short upstream open reading frame (uORF). In some cases, translation will initiate at a short uORF, but the entire ribosome does not detach following translational termination of that uORF. Translation termination is usually followed by the dissociation from the mRNA of the ribosome and associated eIFs from the mRNA. At a low rate, the 40S ribosomal subunit and an incomplete set of eIFs can stay bound to the mRNA and continue scanning, re-associating with the necessary initiation components along the way. Once reconstitution of the initiation complex is achieved, AUG recognition can occur (Kochetov, 2008). Therefore, the distance from the uORF and the availability of replenishing eIFs dictate recognition of a downstream AUG. Perhaps the most extensively studied example of this type of alternative start codon usage is with the yeast *GCN4* mRNA. GCN4 is a transcriptional activator of genes involved in amino acid biosynthesis (Mueller & Hinnebusch, 1986). *GCN4* mRNA contains four upstream open reading frames (uORFs). Under normal conditions, re-initiation after translation of the first uORF will occur at the other uORFs, greatly reducing the number of 48S complexes reaching the GCN4 AUG (Mueller & Hinnebusch, 1986). However, under amino acid starvation conditions, the low levels of GTP-primed eIF2 needed for a competent initiation complex increases the time before AUG recognition can

occur (Abastado et al., 1991; Dever et al., 1995). By the time the initiation complex is reconstituted, it has already bypassed the most 3' uORF and will instead initiate at the GCN4-producing AUG, leading to induction of amino acid synthesis (Abastado et al., 1991).

Canonical cap-dependent translational initiation can by bypassed by the use of internal ribosome entry sites (IRESs). First identified in viruses, but since identified in many cellular mRNAs, IRESs allow for the loading of ribosomes on the mRNA at an internal site. IRESs bypass the need for the cap-binding eIF4E by forming specific tertiary structures recognized either by certain initiation complex components or by the 40S ribosome. The formation of an internal initiation complex is similar to the use of alternate AUGs (Jackson et al., 2010).

The use of IRESs can be combined with traditional translational initiation to produce multiple protein species. PITSLRE protein kinases are proposed to act in tumor suppression and cell cycle progression (Cornelis et al., 2000). The two PITSLRE isoforms, $p110^{PITSLRE}$ and $p58^{PITSLRE}$, are produced from a single transcript. $p110^{PITSLRE}$ is translated by conventional cap-dependent translational initiation throughout the cell cycle (Cornelis et al., 2000). $p58^{PITSLRE}$ is translated specifically in the G2/M phase of the cell cycle and influences cellular growth, and expression of $p58^{PITSLRE}$ is reliant on an IRES (Cornelis et al., 2000). The use of an IRES at a specific time point allows for combination of canonical and non-canonical translational initiation mechanisms to temporally regulate isoform expression.

osk mRNA is translated from two alternative start codons to produce Long and Short Osk. The isoforms have distinct localization patterns and functions

with in the egg. How the alternate *osk* start codons are used is unknown, but could occur by the mechanisms described above.

A NON-CODING FUNCTION OF *OSKAR* MRNA

Coding RNAs are RNAs that associate with ribosomes and produce a protein product. Non-coding RNAs are transcribed, but contain no predicted protein coding region. Non-coding RNAs can be involved in protein synthesis, such as the well-known ribosomal RNAs (rRNAs) and transfer RNAs (tRNAs). Non-coding RNAs can also regulate gene expression, splicing, editing, transport, and degradation (Cech & Steitz, 2014). In the field of RNA research, the function of RNAs is often parsed into coding or non-coding. The methods used to define an RNA as coding or non-coding are imperfect, leading to the mis-categorization of coding RNAs as non-coding and *vice versa* (Dinger et al., 2008). More importantly, this (false) dichotomy fails to address those RNAs having both an RNA function and a protein coding function, an example of which is *osk* mRNA. In addition to the role of Osk protein in body axis formation, *osk* mRNA has a non-coding function necessary for progression through oogenesis. The *osk* RNA functional element resides in the 3' UTR, and the absence of the *osk* 3' UTR in the oocyte arrests development at mid-oogenesis (Jenny et al., 2006).

Some non-coding RNAs are non-functional and can be attributed to transcriptional noise. Considering only the functional species, various non-coding RNAs have been grouped based on length, biogenesis, structure, and function, such as small nucleolar RNAs, PIWI RNAs, and micro RNAs (Cech and Steitz, 2014). However, the long non-coding RNAs (lncRNAs) represent one

group that defies distinct classification due to disparities in length, sequence conservation, and functionality (Mercer et al., 2009).

lncRNAs are broadly defined as non-coding transcripts over 200 nucleotides (Rinn & Chang, 2009). lncRNA-mediated regulation is most often exhibited in the nucleus and on transcription, and transcriptional influences can range from regulation of a single gene to a large genomic region . In the nucleus, lncRNAs can act in *cis* or *trans*, where a *cis*-regulatory lncRNA functions on nearby genes on the same allele, while a *trans*-regulatory lncRNA exerts its function elsewhere (Guttman & Rinn, 2012). The simple act of lncRNA transcription can influence the transcriptional rate of nearby genes, either positively or negatively. However, lncRNAs often function through their interaction with proteins. lncRNAs can induce conformational change of proteins, recruit regulatory complexes to specific genomic loci, directly interact with transcriptional machinery, sequester protein regulators, and act as molecular scaffolds for assembly of RNPs (Guttman & Rinn, 2012; Mercer et al., 2009). The later two functions have been proposed as mechanisms for *osk* RNA function.

lncRNAs can act as 'decoy' RNAs to bind and sequester regulatory factors, thus inhibiting regulation elsewhere. For example, NF-Y is a pro-apoptotic transcription factor. The lncRNA PANDA contains NF-Y binding sites and will sequester NF-Y away from the promoters of apoptosis-inducing genes, thus promoting cell survival (Hung et al., 2011). *osk* mRNA contains *cis*-acting regulatory elements throughout the 3' UTR. Some of these elements are repeated multiple times, allowing multiple regulators to bind to a single transcript (Kim-Ha et al., 1995; Munro et al., 2006; Reveal et al., 2011). Thus, the *osk* 3'

UTR could act as a regulatory sponge to prevent excessive regulation of other target RNAs.

lncRNAs can act as molecular scaffolds to assemble a functional RNP. The lncRNA HOTAIR is necessary for repression of *Hox* genes during development. HOTAIR contains binding sites for two complexes involved in chromatin modification, which are required together for function (Tsai et al., 2010). *osk* 3' UTR could be acting similarly to assemble factors necessary for progression through oogenesis.

OVERVIEW OF DISSERTATION RESEARCH

My dissertation research investigates two separate aspects of *osk*. The first is the translational activation required to produce Osk protein at the posterior of the oocyte, while the second is the non-coding RNA function provided by *osk* RNA.

Translational activation of *osk* is multifaceted and requires both relief of repression and activation *per se*. Key *cis*-acting elements regulate translational activation. Of these, a 5' element in the *osk* coding region is necessary for *osk* activity, and proposed to be active only at the posterior of the oocyte. Here, I found that the 5' element overlaps with coding sequences necessary for Long Osk function. To disentangle protein and RNA effects, transgenes were used that didn't produce Long Osk. Using deletion mutants, I mapped the element to a region that has high sequence conservation in many *Drosophila* species. The 5' element is necessary for translation of *osk*, and has the greatest influence on late stage accumulation of Osk. However, the 5' element is required only when Long

Osk is not translated, suggesting a redundant role. Mutation of the 5' element decreases translation of an unlocalized *osk::GFP* reporter that contains no *osk* sequences involved in translational repression, indicating that the element acts to activate translation independent of localization and repression. This is contradictory to the previous assertion of a posterior specific role in relief from repression.

The non-coding *osk* RNA function is required for formation of the karyosome and for progression through oogenesis, and is provided by the *osk* 3' UTR in the oocyte. Here I further characterize the *osk* RNA-null phenotype. In the absence of *osk* RNA, regulatory proteins dissociate from germline RNPs and acquire access to the somatic follicle cells. I found that the RNA function is provided by multiple *osk* 3' UTR sequences, with distinct contributions to RNA function. One role is to sequester the translational repressor Bru by providing multiple binding sites in two separate regions. Mutation of the high affinity Bru binding sites produces the RNA-null phenotype, which can be partially rescued by reducing Bru protein levels or adding additional binding sites. Another role was uncovered by precise mapping near the *osk* 3' end that found additional, non-Bruno binding sequences required for RNA function. The disruption of either role disturbs karyosome formation and the proper distribution of germline regulators.

FIGURES

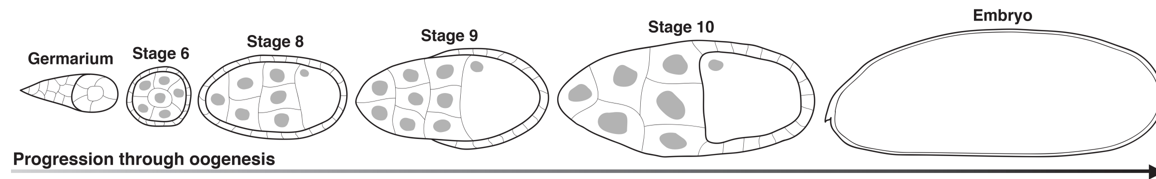


Figure 1.1. Egg chamber progression during *Drosophila* oogenesis

Egg chambers at various stages of oogenesis as development progresses in an ovariole. The egg chambers begin as stem cells at the anterior (left) of the germarium, which divide to form a cyst of germline-derived cells surrounded by somatic, follicle cells. The cyst buds off from the posterior of the germarium.

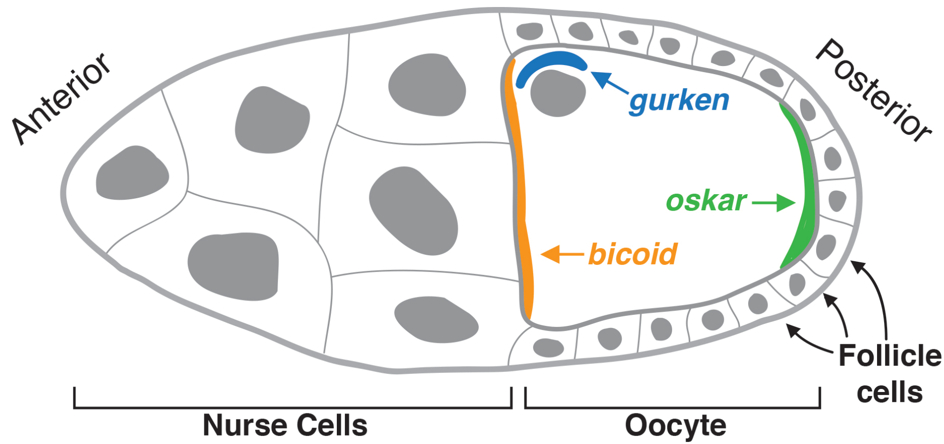


Figure 1.2. Maternal mRNA localization in a *Drosophila* egg chamber

Diagram of a *Drosophila* egg chamber. The nurse cells are at the anterior. The oocyte is the large cell at the posterior, with an overlaying layer of follicle cells. Within the oocyte, *gurken* mRNA is localized to the anterior-dorsal corner, *bicoid* mRNA is localized to the anterior margin, and *osk* mRNA is localized to the posterior, and localization of these maternally-provided mRNAs is required for normal axial patterning.

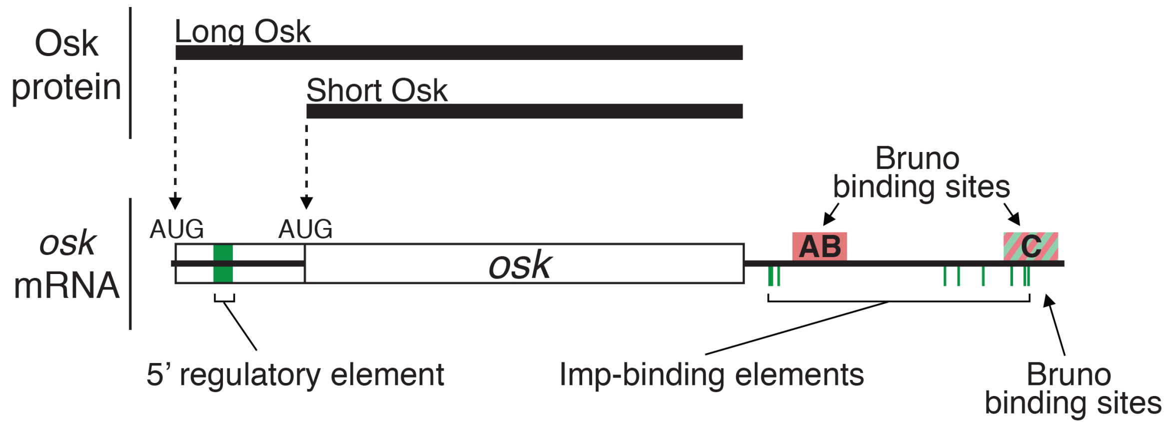


Figure 1.3. Translational regulatory elements in *osk* mRNA and the Osk protein isoforms

Bottom: the *osk* mRNA, with the coding region indicated by the white box and the UTRs indicated by the solid black lines. *osk* mRNA contains *cis*-acting elements that regulate translational repression (red; labeled above *osk* mRNA) or translational activation (green; labeled below *osk* mRNA). Top: the Osk protein isoforms, Long and Short Osk, with the dotted line indicating the AUG from which translational initiation occurs.

REFERENCES

Abastado, J., Miller, P., Jackson, B., & Hinnebusch, A. (1991). Suppression of ribosomal reinitiation at upstream open reading frames in amino acid-starved cells forms the basis for GCN4 translational control. Molecular and Cellular Biology, 11(1), 486-496.

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), 1082-1087.

Benoit, P., Papin, C., Kwak, J., Wickens, M., & Simonelig, M. (2008). PAP- and GLD-2-type poly(A) polymerases are required sequentially in cytoplasmic polyadenylation and oogenesis in Drosophila. Development, 135(11), 1969-1979.

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

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.

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.

Cech, T. and Steitz, J. (2014). The noncoding RNA revolution- trashing old rules to forge new ones. Cell, 157(1), 77-94.

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.

Chicoine, J., Benoit, P., Gamberi, C., Paliouras, M., Simonelig, M., and Lasko, P. (2007). Bicaudal-C recruits CCR4-NOT deadenylase to target mRNAs and regulates oogenesis, cytoskeletal organization, and its own expression. Developmental Cell, 13(5), 691-704.

Clark, A., Meignin, C., & Davis, I. (2007). A Dynein-dependent shortcut rapidly delivers axis determination transcripts into the Drosophila oocyte. Development 134(10) 1955-1965.

Carrera, P., Johnstone, O., Nakamura, A., Casanova, J., Jackle, H., & Lasko, P. (2000). VASA mediates translation through interaction with a Drosophila yIF2 homolog. Molecular Cell, 5(1), 181-187.

Cornelis, S., Bruynooghe, Y., Denecker, G., Van Huffel, S., Tinton, S., & Beyaert, R. (2000). Identification and characterization of a novel cell cycle-regulated internal ribosome entry site. Molecular Cell, 5(4), 597-605.

Dahlgaard, K., Raposo, A., Niccoli, T., & St Johnston, D. (2007). Capu and Spire assemble a cytoplasmic actin mesh that maintains microtubule organization in the Drosophila oocyte. Developmental Cell, 13(4), 539-553.

Deng, Y., Singer, R., & Gu, W. (2008). Translation of ASH1 mRNA is repressed by Puf6p-Fun12p/eIF5B interaction and released by CK2 phosphorylation. Genes & Development, 28(20), 1037-1050.

Dever, T., Yang, W., Astrom, S., Bystrom, A., & Hinnebusch, A. (1995). Modulation of tRNA(iMet), eIF-2, and eIF-2B expression shows that GCN4 translation is inversely coupled to the level of eIF-2.GTP.Met-tRNA(iMET) ternary complexes. Molecular and Cellular Biology, 15(11), 6351-6363.

Dinger, M., Pang, K., Mercer, T., & Mattick, J. (2008). Differentiating protein-coding and noncoding RNA: challenges and ambiguities. PLoS Computational Biology, 4(11), 1-5.

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.

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

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.

Glotzer, J., Saffrich, R., Glotzer, M., & Ephrussi, A. (1997). Cytoplasmic flows localize injected oskar RNA in Drosophila oocytes. Current Biology, 7(5), 326-337.

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.

Guttman, M. & Rinn, J. (2012). Modular regulatory principles of large non-coding RNAs. Nature, 482(7385), 339-346.

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.

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.

Heasman, J. (2006). Patterning the early Xenopus embryo. Development, 133(7), 1205-1217.

Hung, T., Wang, Y., Lin, M., Koegel, A., Kotake, Y., Grant, G. et al. (2011). Extensive and coordinated transcription of noncoding RNAs within cell-cycle promoters. Nature Genetics, 43(7), 621-629.

Huttelmaier, S., Zenklusen, D., Lederer, M., Dictenberg, J., Lorenz, M., Meng, X., et al. (2005). Spatial regulation of β-actin translation by Src-dependent phosphorylation of ZBP1. Nature, 438, 512-515.

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.

Jackson, R., Hellen, C, & Pestova, T. (2010). The mechanism of eukaryotic translation initiation and principles of its regulation. Nature Reviews, 11(2), 113-127.

Jambor, H., Mueller, S., Bullock, S., & Ephrussi, A. (2014). A stem-loop structure directs oskar mRNA to microtubule minus ends. RNA, 20(4), 429-439.

Jankovics, F., Sinka, R., Lukacsovich, T., & Erdelyi, M. (2002). MOESIN crosslinks actin and cell membrane in Drosophila oocytes and is required for OSKAR anchoring. Current Biology, 12(23), 2060-2065.

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. (2004). Interaction with eIF5B is essential for Vasa function during development. Development, 131(17), 4167-4178.

Juge, F., Zaessinger, S., Temme, C., Wahle, E., & Simonelig, M. (2002). Control of poly(A) polymerase level is essential to cytoplasmic polyadenylation and early development in Drosophila. The EMBO Journal, 21(23), 6603-6613.

Kim, G. (2014). Investigating the role of Bruno interactions with oskar regulatory proteins (Doctoral dissertation). University of Texas at Austin, Austin, Texas.

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.

Kochetov, A. (2008). Alternative translation start sites and the hidden coding potential of eukaryotic mRNAs. Bioessays, 30(7), 683-691.

Kozak, M. (1997). Recognition of AUG and alternative initiator codons is augmented by G in position +4 but is not generally affected by the nucleotides in positions +5 and +6. The EMBO Journal, 16(9), 2482-2492.

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

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.

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.

Lieberfarb, M., Chu, T., Wreden, C., Theurkauf, W., Gergen, J., & Strickland, S. (1996). Mutations that perturb poly(A)-dependent maternal mRNA activation block the initiation of development. Development, 122, 579-588.

Lu, C, Brennan, J., & Robertson, E. (2001). From fertilization to gastrulation: axis formation in the mouse embryo. Current Opinion in Genetics and Development, 11(4), 384-392.

Macdougall, N., Clark, A., Macdougall, E., & Davis, I. (2003). Drosophila gurken (TGFα) mRNA localizes as particles that move within the oocyte in two dynein-dependent steps. Developmental Cell, 4(3), 307-319.

Mach, J., & Lehmann, R. (1997). An Egalitarian-BicaudalD complex is essential for oocyte specification and axis determination in Drosophila. Genes and Development, 11, 423-435.

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. & Ephrussi, A. (2009). mRNA localization: gene expression in the spatial dimension. Cell, 136(4), 719-730.

Mercer, T., Dinger, M., & Mattick, J. (2009). Long non-coding RNAs: insights into functions. Nature Reviews Genetics, 10(3), 155-159.

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.

Mueller, P., & Hinnebusch, A. (1986). Multiple upstream AUG codons mediate translational control of GCN4. Cell, 45, 201-207.

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., Menade, 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.

Pedrajas, J., Porras, P., Martinez-Galisteo, E., Padila, C., Miranda-Vizuete, A., & Barcena, J. (2002). Two isoforms of Saccharomyces cerevisiae glutaredoxin 2 are expressed in vivo and localize to different subcellular compartments. Biochemical Journal, 264(3), 617-623.

Pestova, T., Kolupaeva, V., Lomakin, I., Pilipendo, E., Shatsky, N., Agol, V., & Hellen, C. (2001). Molecular mechanisms of translation initiation in eukaryotes. Proceedings of the National Academy of the Sciences, 98(13), 7029-7036.

Piccioni, F., Zappavigna, V., & Verrotti, A. (2005). Translational regulation during oogneesis and early development: the cap-poly(A) tail relationship. Comptes Rendus Biologies 328, 863-881.

Porras, P., Padilla, A., Krayl, M., Voos, W., & Barcena, J. (2006). One single in-frame AUG codon is responsible for a diversity of subcellular loacalizations of Glutaredoxin 2 in Saccharomyces cerevisiae. The Journal of Biological Chemistry, 281(24), 16551-16562.

Ramos, A., Grunert, S., Adams, J., Micklem, D., Proctor, M., Freund, S., et al. (2000). RNA recognition by a Staufen double-stranded RNA-binding domain. The EMBO Journal, 19(5), 997-1009.

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

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.

Rinn, J. & Chang, H. (2012). Genome regulation by long noncoding RNAs. Annual Review of Biochemistry, 81, 145-166.

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.

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

Saffman, E., Styhler, S., Rother, K., Li, W., Richard, S., & Lasko, P. (1998). Premature translation of oskar in oocytes lacking the RNA-binding protein Bicaudal-C. Molecular and Cellular Biology, 18(8), 4855-4862.

Smith, J., Wilson, J., & Macdonald, P. (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., Harrison, D., Yan, N., & Macdonald, P. (2007). A late phase of Oskar accumulation is crucial for posterior patterning of the Drosophila embryo, and is blocked by ectopic expression of Bruno. Differentiation, 75(3), 246-255.

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

Steinhauer, J. & Kalderon, D. (2006). Microtubule polarity and axis formation in the Drosophila oocyte. Developmental Dynamics, 235(6), 1455-68.

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., Brown, N., Gall, J., & Jantsch, M. (1992). A conserved double-stranded RNA-binding domain. Proceedings of the National Academy of Sciences, 89(22), 10979-10983.

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., 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.

Tsai, M., Manor, O., Wan, Y., Mosammaparast, N., Wang, J., Lan, F. et al. (2010). Long noncoding RNA as modular scaffold of histone modification complexes. Science, 329(5992), 689-693.

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., & 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.

Wharton, R., & Struhl, G. (1991). RNA regulatory elements mediate control of Drosophila body pattern by the posterior morphogen nanos. Cell, 67(5), 955-967.

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.

Zaessinger, S., Busseau, I., & Simonelig, M. (2006). Oskar allows nanos mRNA translation in Drosophila embryos by preventing its deadenylation by Smaug/CCR4. Development, 133(22), 4573-4583.

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: A 5' region of the *oskar* mRNA plays both regulatory and protein-coding roles in enhancing posterior accumulation of Oskar protein

Matt Kanke[1] and Paul M. Macdonald[1]

[1]Department of Molecular Biosciences

Institute for Cellular and Molecular Biology

The University of Texas at Austin

ABSTRACT

Local translation of *oskar* (*osk*) mRNA at the posterior pole of the Drosophila oocyte is essential for axial patterning of the embryo, and is achieved by a program of translational repression, mRNA localization, and translational activation. Multiple forms of repression are used to prevent Oskar protein from accumulating at sites other than the oocyte posterior. Activation is mediated by several types of *cis*-acting elements, which presumably control different forms of activation. We characterize a 5' element, positioned within the coding region for the Long Osk protein isoform and previously implicated in posterior-specific release from repression. We show that changes in Long Osk from mutations in the element disrupt the Long Osk anchoring function, a confounding influence on interpretation of experiments. Using assays which separate the effects of mutations on RNA regulatory elements and protein coding capacity, we find that the element enhances translation independent of either repression or the process of mRNA localization. The 5' element has a redundant role, and is required only when Long Osk is not translated from the same mRNA. This suggests that Long Osk can act in *cis* to facilitate initiation of translation at the downstream Short Osk start codon, or that ribosomes initiated at the Long Osk start codon can facilitate ribosome assembly at the downstream Short Osk start codon.

INTRODUCTION

Local translation has emerged as a fundamental mechanism for establishing the subcellular distribution of proteins (Jung et al., 2014). A large fraction of all mRNAs appear to exhibit some degree of localization (Lécuyer et al., 2007), and regional differences in mRNA abundance alone can create corresponding differences in protein levels. If the localized mRNAs are subject to translational repression of such mRNAs before they are localized, and release from repression after localization, regional differences in protein levels can be greater. The activity of localized mRNAs can be further regulated, such that localization alone is not sufficient for translation, which only occurs in response to a stimulus.

A variety of mechanisms have been implicated in translational activation of localized mRNAs. One general theme involves changes in the activity of eIF4E, the protein that binds to the mRNA 5' cap in a complex with other initiation factors (Gingras et al., 1999). If bound by a member of a family of eIF4E binding proteins, the 4E-BPs, eIF4E is prevented from forming the eIF4F complex, and translation is blocked. Activation occurs when the 4E-BPs are phosphorylated, which prevents them from binding to eIF4E.

Another mechanism that allows local translation is inhibition of repressors, which are bound to mRNAs and block their translation during localization. In several examples, it is the ability of the repressors to bind RNA that is inhibited, promoting release from the mRNA and thus activation of translation. Reduction of RNA binding affinity can involve phosphorylation of the repressors (Huttelmaier et al., 2005; Paquin et al, 2007; Deng et al., 2008), or binding to another protein

(Zaessinger et al., 2006; Jeske et al., 2011). This general type of mechanism affords a higher degree of specificity than controlling the activity of translation initiation factors, as only the repressed mRNAs are affected.

Specificity in activation is also possible though use of *cis*-acting elements, as only the mRNA bearing the element should be affected. Often, such elements control the polyadenylation state of the mRNA. This allows for both positive and negative regulation: lengthening the poly(A) tail is generally associated with enhanced translation, while deadenylation can lead to mRNA degradation.

Local translation plays a critical role in early *Drosophila* development. Specification of the embryonic body plan relies on the actions of a few key mRNAs localized to discrete regions of the oocyte. For anterior/posterior patterning, *bicoid* (*bcd*) mRNA is localized to the anterior margin of the oocyte and the anterior of the embryo, and for posterior patterning *oskar* (*osk*) and *nanos* (*nos*) mRNAs are localized to the posterior pole of the oocyte and later the embryo. Each of these mRNAs is translationally regulated, and each has different properties that require different types of regulation. The *bcd* and *nos* mRNAs are localized well before they are translated, and, especially for *bcd*, timing is a primary role of translational regulation: translation is off during oogenesis, and activated following egg laying. This control appears to be exerted largely, if not exclusively, at the level of the poly(A) tail. The situation is more complex for *nos* mRNA. Although the timing of translational activation is similar to that for *bcd*, the bulk of *nos* mRNA is not localized in the embryo and must be repressed. Accordingly, mechanisms exist to repress translation, and to locally activate translation.

The *osk* mRNA differs from *bcd* and *nos* in that protein accumulation does not lag substantially behind mRNA localization. Instead, Osk protein first appears effectively coincident with localization of the mRNA to the posterior pole of the oocyte at stage 9 of oogenesis. This suggests that a simple temporal control of translational activation is not used. Instead, a regional form of activation, or mechanistic coupling of localization and translational activation, or both, could be in operation. Repression of *osk* mRNA relies on Bruno (Bru), a protein that binds to multiple sites in the *osk* mRNA 3' UTR. Multiple other factors are involved in repression, some also binding the mRNA (e.g. Polypyrimidine Tract Binding protein, PTB), some acting in concert with Bru (e.g. Cup), one with a role in control of poly(A) tail length (Bicaudal-C) and others whose roles are less well defined (e.g. Me31B). Repression by these various proteins, which act using different mechanisms, must be overcome at the posterior pole of the oocyte. As more than one form of repression appears to be used, it seems likely that more than one form of activation may be required. Several proteins have been implicated in translational activation of osk mRNA. One is Orb, which is required to provide *osk* mRNA with a long poly(A) tail. Although Orb has been suggested to act specifically at the posterior of the oocyte, it has not been possible to rule out a role a more general requirement, with the poly(A) tail serving as a prerequisite for efficient translation, but not necessarily extended only after mRNA localization. For other proteins, including Staufen and Vasa, the specifics of how they promote *osk* mRNA translation remain uncertain.

Translational activation of *osk* depends on *cis*-acting elements in the *osk* mRNA. The first of these to be identified lies in the 5' part of the mRNA, in the region between the alternate translation initiation codons used to make Long Osk

and Short Osk proteins. These proteins differ only in the amino-terminal extension unique to Long Osk, which is required for cortical anchoring at the posterior of the oocyte. The 5' activation element was reported to mediate derepression, and to function only at the posterior pole of the oocyte. Two types of elements in the *osk* 3' UTR required for activation of translation have been identified. The Imp Binding Elements (IBEs) are short sequences present in multiple copies; mutation of a subset of these eliminates Osk protein production. Finally, a subset of the binding sites for the Bru translational repressor, those in the *osk* 3' UTR C region, play a second role in translational activation.

Here we provide a more detailed characterization of the *osk* 5' activation element. The original work on this element made use of mutants which affected both the Long Osk protein and the mRNA, and potential effects of disrupting Long Osk function were not considered. Furthermore, subsequent work showing the role of the other 3' UTR activation elements require a re-evaluation of experiments interpreted to indicate that the 5' element acts specifically at the posterior of the oocyte. We confirm the presence of a translational activation element, but find that it acts as a general enhancer of translation. Mutations of the element also disrupt Long Osk function, explaining, in part, the differences in our conclusions from the earlier work. In addition, we find that the 5' element has a redundant role, and is required only when Long Osk is not translated from the same mRNA.

RESULTS

Mapping a regulatory element in the *oskar* 5' region

To characterize the role in Osk expression of sequences in the 5' portion of the *osk* mRNA, we used transgenes in which the start codon for translation of Long Osk, M1, was mutated (*oskM1R*). Because Long Osk is not produced in *oskM1R* (Vanzo & Ephrussi, 2002), mutations that lie between the start codons for Long Osk and Short Osk will only alter the mRNA sequence, and not the protein coding sequence (Figure 2.1A). Thus, any change in *osk* activity between *oskM1R* and mutants with lesions in the extended 5' UTR (all sequences upstream from the Short Osk AUG) must be due to a change in *osk* expression, not a structural defect in Osk protein. As reported previously (Vanzo & Ephrussi, 2002), *oskM1R* provides essentially wild type levels of *osk* patterning activity, and almost all embryos from mothers expressing only *oskM1R* have normal segmentation.

A 62 nt inversion, similar to that used previously with an *osk::lacZ* reporter transgene (Gunkel et al, 1998), was introduced into *oskM1R* to make *oskM1R INV121-182*. The mutant had dramatically reduced *osk* patterning activity (Figure 2.1B). These results confirmed the presence of an element required for *osk* expression, and provisionally mapped it to the region affected by the inversion. The inversion could directly alter a regulatory element, by a change in the sequence. Alternatively, the inversion could have an indirect effect, with a change in mRNA structure affecting a regulatory element in nearby sequences. To distinguish between these options, and to test other parts of the *osk* 5' region for a role in *osk* expression, a series of deletion mutants covering most of the extended 5' UTR was tested (Figure 2.1A). Most of the mutants retained normal

levels of *osk* patterning activity. Only *oskM1R Δ91-120* and *oskM1R Δ121-150* were defective, with extremely low levels of *osk* patterning activity (Figure 2.1B). For these mutants, as well as *oskM1R INV121-182*, mRNA levels remained close to that of the endogenous gene (Figure 2.1C). Although there was a slight reduction in the amount of mRNA, this could not be the cause of the defects, as mutant *oskM1R Δ261-310* had a similar mRNA level but retained normal *osk* patterning activity. These results indicated that the *osk* 5' regulatory element resides in the region from nucleotides 91-150 of the *osk* mRNA. Because deletions of much of the remainder of the extended *osk* 5' UTR did not affect *osk* activity, there are no other required regulatory elements, or if there are elements they must act redundantly.

To address the possibility that multiple 5' regulatory elements exist and act redundantly, a further transgene was tested. The *oskM1R Δ40-84 Δ196-423* mutant retains the 91-150 region and flanking sequences, but lacks most of the rest of the extended *osk* 5' UTR (Figure 2.1A). Despite the extensive deletions, the mutant had a level of *osk* patterning activity similar to that of *oskM1R* (Figure 2.1B). Thus, within the region tested there is only a single RNA segment with important regulatory information.

Although the 5' regulatory element could occupy much of the defined 120 nt region, it could also be contained in a more compact region overlapping the junction of the Δ91-120 and Δ121-150 deletions. Because the regulatory element is expected to be conserved in evolution, we compared the relevant sequences from a number of sequenced *Drosophila* species. In one approach, conservation was assessed by *phastCons*, which computes conservation scores for aligned sequences based on phylogeny, a model of the nucleotide substitution process,

and a propensity for conservation to be similar at adjacent sites along the genome (Siepel et al., 2005). With the default settings of the UCSC genome browser, almost the entire *osk* 91-150 region is shown to be highly conserved, as is a more 5' region and various shorter segments of the extended *osk* 5' UTR (Figure 2.2). We also surveyed the same region for clusters of highly conserved sequences (Materials and methods). This screening was more stringent, with many fewer regions of high conservation identified (Figure 2.2). Notably, the longest stretch of highly conserved nucleotides in the entire extended 5' UTR lies exactly at the junction of the Δ91-120 and Δ121-150 deletions. Within the conserved region, it appears that the exact sequence is conserved, not simply the protein coding sequence. If maintaining the Long Osk protein sequence provided the only selective pressure, some DNA sequence variation would be expected from use of alternate codons; this is not the case.

To test the importance of the highly conserved sequences, mutant *oskM1R Δ118-135* was constructed and tested. Just as for the mutants with deletions of nt 91-120 and 121-150, *osk* patterning activity was dramatically reduced (Figure 2.1B). This defect was not due to a reduction in mRNA level (Figure 2.1C). Thus, the highly conserved sequences are crucial for function of the 5' regulatory element.

The *oskar* 5’ regulatory element activates translation

The strong embryonic patterning defects from deletion of osk mRNA sequences 91-120, 121-150 and 118-135 suggested that Osk protein levels would be correspondingly low. This was indeed the case, as shown by western

blot analysis of ovary proteins (Figure 2.3A). Moreover, the mutant retaining the crucial regions but lacking much of the rest of the extended *osk* 5' UTR retained a high level of Short Osk (Figure 2.3B). Before considering the options for why protein levels are reduced, it is noteworthy that one mutant - *oskM1R Δ311-360* - produces two proteins, one the size of Short Osk, and one larger. Contained between the initiator codons for Long and Short Osk are two additional AUG codons, not in the Long/Short Osk reading frame. Each is followed by a short open reading frame (internal ORF, or iORF; 27 or 13 codons, respectively) and a stop codon. The Δ311-360 deletion removes the stop codon for the second of these iORFs, and shifts its reading frame to that used for Long and Short Osk (Figure 2.3C). The size of the unusual protein is consistent with use of this AUG for initiation of translation, strongly suggesting that at least one, and possibly both, of the short iORFs is translated. How initiation of Short Osk translation normally occurs is unknown, and could involve scanning of preinitiation complexes formed at the mRNA cap, or a form of internal ribosome entry. Knowing that an intermediate AUG, positioned upstream of the AUG for Short Osk, is used to initiate translation in the Δ311-360 deletion mutant may be useful in elucidating the mechanism of Short Osk translational initiation.

The protein accumulation defects of the *osk* 5' mutants must result from inefficient translation, as mRNA levels were close to normal, and the mutations do not affect the encoded proteins. However, because posterior localization of *osk* mRNA is normally required for Osk protein accumulation, a defect in *osk* mRNA localization can disrupt translation indirectly. We examined the mRNA distribution of the *oskM1R Δ118-135* mutant, using *oskM1R* for comparison. As for the assays of *osk* activity (above), these experiments were performed in *osk*

RNA null flies lacking any other source of *osk* mRNA. Consequently, there is no possibility that an mRNA localization defect would be masked by piggybacking, the phenomenon in which localization-defective *osk* transcripts co-localize with wild type *osk* transcripts (Hachet & Ephrussi, 2004). In the absence of Long Osk, which provides an anchoring function, *oskM1R* transcripts were not restricted as tightly to the posterior pole of the oocyte as normal (Figure 2.3E). For *oskM1R*, as well as *oskM1R Δ61-90* (a deletion mutant with normal osk patterning activity and Osk protein levels), the mRNA was present in puncta positioned at or near the posterior of the oocyte (Figure 2.3F). A comparable distribution was observed for *oskM1R Δ118-135* (Figure 2.3G). Thus, a defect in mRNA localization was not responsible for the reduced protein level of the *oskM1R Δ118-135* mutant, and translation must have been disrupted.

Although the western blot analysis revealed defects in Osk protein accumulation for the 5' region mutants, this represents an average of Osk throughout the ovary and including all stages of oogenesis. The accumulation of Osk during oogenesis begins at stage 9, but the bulk of Osk protein is made late (Snee et al, 2007). Consequently, there may be different forms of translational activation that operate at different stages. Indeed, mutations in the *osk* 3' UTR C region BREs most severely disrupt the later phase of Osk accumulation (Reveal et al, 2010). To determine if the 5' region mutants disrupted the early phase of translation, proteins were monitored by immunodetection and confocal microscopy. Initial experiments were performed, as above, with transgenes expressed in an *osk* RNA null background. Because Long Osk was not provided by the *oskM1R*-based transgenes, Short Osk protein was not properly anchored at the posterior cortical region and appeared in puncta which were often

displaced from the cortex (data not shown). This presented a challenge for quantitation, as the puncta were distributed unevenly through multiple focal planes. As an alternate approach, we tested the transgenes, all of which include a 3xHA epitope tag for detection, in an *osk*$^+$ background. In this situation the epitope-tagged Osk protein made by the mutant transgene could be detected and distinguished from endogenous Osk, and was anchored to the cortex by endogenous Long Osk to facilitate quantitation (Figure 2.4B).

Using this assay, we found that the *osk* 5' region mutants with normal patterning activity produced Osk protein at levels similar to *oskM1R* (Figure 2.4C,G,H,I). The mutants with greatly reduced patterning activity showed a clear reduction in protein levels at stage 9, although the reduction was less severe than detected in the western analysis (Figure 2.4D,E,F,I). By image analysis the *oskM1R Δ91-120*, *oskM1R Δ118-135* and *oskM1R Δ121-150* mutants had roughly half the normal level of Osk at stage 9 of oogenesis (Figure 2.4I), while the western analysis revealed a larger reduction for each.

There are two likely explanations for different amounts of Osk detected in the imaging and western assays. First, the *osk* 5' region mutations could disrupt the late phase of *osk* translation more strongly than the early phase, just as observed for the *osk* C region BRE- mutants (Reveal et al, 2010). If so, the western analysis, which includes late stage egg chambers, would show a stronger defect, as is observed. Alternatively, the presence of endogenous Osk in the imaging experiments could have enhanced translation of the *oskM1R*-based mutants (although there is no enhancement of *oskM1R*). If endogenous Osk does enhance translation of the mutants, this should also be detected in western blot analysis. We repeated the western blot analysis, but with the

transgenes in an *osk+* background (Figure 2.4J). Just as observed in the absence of endogenous Osk, the defective mutants showed a greater reduction than seen in the image analysis. We infer that the *osk* 5' region translational activation element influences translation most strongly during the late phase of Osk accumulation, in addition to the defects seen earlier.

The *oskar* 5' activation element is normally dispensable

We have shown that, in the context of *oskM1R*, the 5' activation element is necessary for efficient translation of Short Osk. To determine if the element is also required for translation of Long Osk, we constructed and tested the *osk Δ121-150* mutant, which retains wild type M1 (Figure 2.5A). This mutant was compared to an *osk*$^+$ transgene, and to the *oskM1R Δ121-150* mutant, all tested in the osk RNA null background. Surprisingly, the consequences of the Δ121-150 mutation varied dramatically, depending on whether *oskM1* was wild type or mutant. In the context of wild type *oskM1*, deletion of the *osk* 121-150 sequences had almost no effect: *osk* patterning activity was effectively wild type (Figure 2.5C), and the levels of Short and Long Osk proteins were reduced only slightly (Figure 2.5B). This stands in sharp contrast to the effects of deleting nt 121-150 in the context of *oskM1R*: *osk* patterning activity was almost eliminated (Figure 2.5C), and the level of Short Osk was substantially reduced (Figure 2.5B). The only clear defect caused by deleting nt 121-150 in the context of *oskM1* was in the distribution of the protein, which was not tightly restricted to the posterior cortex (Fig. 5D). Coexpression of *oskΔ121-150* with *osk*$^+$ corrected this defect, as expected (Fig. 5D). This behavior indicated that the Long Osk

anchoring function was disrupted by the 121-150 deletion, validating the concern that mutation of this region could have affected properties of reporter proteins. Why mutation of the 5' activation element should have such different consequences for *osk* and *oskM1R* is not clear. The most obvious difference - one makes Long Osk and one does not - could suggest that the presence of Long Osk protein suppressed the Δ121-150 regulatory defect. However, we have shown that the reduced level of Short Osk from *oskM1R Δ121-150* persists even in the presence of a source of Long Osk (above). We consider possible explanations in the Discussion.

The 5' element enhances protein accumulation independent of repression or mRNA localization

Our results show that the 5' activation element enhances translation, at least in the context of *oskM1R*. Two central questions about translational activation of osk mRNA apply to the 5' element. First, does it serve to override translational repression, which normally limits expression of unlocalized osk mRNA? Second, is this form of activation coupled to mRNA localization, either mechanistically or by acting only at the site of localization? Osk protein normally accumulates only from mRNA localized to the posterior pole of the oocyte, and there may be an activation mechanism coordinated with localization. Indeed, the 5' element has been argued to mediate localization-dependent translation (Gunkel et al, 1998)(see Discussion). To further characterize the 5' activation element we tested its effect on translation in the absence of either translational repression or mRNA localization. The 5' part of the *osk* mRNA (nt 1-534), including the activation element, was fused to *GFP* to make *UAS-osk1-534::GFP*.

The *osk1-534::GFP* mRNA is transcribed under UAS/GAL4 control (Kim et al, submitted). This mRNA lacks the *osk* 3' UTR and thus does not include various elements mediating translational repression (Kim-Ha et al, 1995; Reveal et al, 2010). The *osk1-534::GFP* mRNA also lacks the SOLE, a signal for posterior localization of *osk* mRNA that is positioned in the *osk* coding region just 3' to position 534 (Ghosh et al, 2012). The *osk1-534::GFP* mRNA is not localized to any region within the egg chamber (Figure 2.6B), and is translated to produce Osk::GFP protein (Figure 2.6A).

The *osk1-534::GFP* mRNA produces three proteins, as detected by western blotting (Figure 2.6C). Two are the sizes expected from use of the Long and Short Osk start codons. The third is smaller, approximately the size of GFP alone and variable in both amount and exact position of migration in SDS-PAGE. To confirm the origin of the Long and Short protein isoforms, and to explain the origin of the smallest proteins, we tested transgenes with mutated start codons. Mutation of M1 eliminated the Long Osk::GFP form, as well as the short, GFP-sized proteins. Mutation of M139 eliminated the Short Osk::GFP form. When both M1 and M139 were mutated, no proteins were detected (Figure 2.6C). These results confirmed the assignments of the isoforms initiated from the alternate Osk start codons. In addition, the short, GFP-sized protein must have been produced by partial degradation of the Long Osk::GFP protein; degradation is consistent with variation in its exact size and amount.

The INV121-182 mutation was introduced into *osk1-534::GFP* to test for an effect on translation. Levels of both Long and Short isoforms were reduced (Figure 2.6D; also compare Figure 2.6J,K with H,I), with no corresponding change in mRNA levels (Figure 2.6E). The reduction in the Long isoform could be

due to a change in expression or a change in protein stability, as the INV mutation disrupts the coding region. The mutation does not, however, affect the sequence of Short Osk::GFP, and so the reduced levels can be attributed to a change in expression from disruption of the 5' element. Although this effect was not as dramatic as for Short Osk from the *oskM1R INV121-182* mutant, this result does show that the 5' activation element enhances translation in a fashion that does not involve relief from repression. Furthermore, activation is independent of mRNA localization.

Does the 5' element mediate oocyte-specific translational activation?

There are two notable features of the Osk::GFP protein distribution. First, it is concentrated at cortical regions and cell boundaries, both in the nurse cells and in the oocyte (Figure 2.6A,H,I). Second, the protein is enriched in the oocyte (Figure 2.6A, Figure 2.7), despite the absence of any localization of the mRNA to that cell (Figure 2.6B). GFP expressed alone showed no similar enrichment in the oocyte, nor was it concentrated at cell boundaries (Figure 2.7, Figure 2.6F,G). The concentration at cortical regions and cell boundaries appears to be equivalent to the anchoring of Osk protein. Just as Long Osk mediates anchoring, only the Long Osk::GFP isoform supporting cortical and cell boundary enrichment. Protein produced from *UAS-M1R osk1-534::GFP*, which makes only Short Osk::GFP, was not anchored (data not shown). The INV121-182 mutation disrupts anchoring of Osk::GFP (Figure 2.6J,K), consistent with the loss of Long Osk anchoring from the Δ121-150 mutation (Figure 2.5D).

For understanding translational activation, the oocyte enrichment conferred by the 5' region of osk mRNA is of interest. One interpretation of the oocyte enrichment of Osk::GFP is that translational activation mediated by the 5' element is specific to, or most effective in, the oocyte. Consistent with this view, the oocyte enrichment was lost when the element was inactivated by INV121-182 (Figure 2.7). However, it is also possible that translation, independent of any *osk* sequences in the mRNA, is simply stronger in the oocyte, but that this spatial difference in translation efficiency cannot be detected unless the protein is anchored. In this scenario, the absence of oocyte enrichment for protein made from the *UAS-GFP* transgene would be due to diffusion of GFP between the interconnected nurse cells and oocyte. To ask if GFP expressed in the oocyte would freely diffuse unless anchored, reporter mRNAs were modified by addition of the *K10* Transport and Localization Signal (TLS). The TLS confers efficient transport to the oocyte, and later localization to the anterior margin of the oocyte (Serano & Cohen, 1995). The anchored Osk::GFP protein made from *osk1-534::GFP-TLS* mRNA was largely restricted to the oocyte (Figure 2.6M), as expected from the combination of mRNA localization and protein anchoring. However, unanchored GFP made from *GFP-TLS* mRNA displayed no substantial enrichment in the oocyte (Figure 2.6L). Thus, GFP can freely move between the nurse cells and oocyte. Because sequences which act at the RNA level to activate translation also encode a domain required for protein anchoring, we cannot use mutations that disrupt activation to determine where activation occurs. Thus, oocyte enrichment of the Osk::GFP protein may be due to oocyte-specific translational activation, but other explanations are possible.

Discussion

We have shown that mutations within the 5' portion of the *osk* mRNA, which both encodes Long Osk and is included in an extended 5' UTR for Short Osk, affect both protein function and translational regulation. The amino-terminal domain unique to the Long Osk isoform is required for cortical anchoring of Osk (Vanzo & Ephrussi, 2002), and is sufficient for anchoring an Osk::ß-galactosidase fusion protein (Gunkel et al, 1998). The question of which parts of this domain are required for anchoring had been unknown. Our results show that deletion of amino acids 36-45 (in the *oskΔ121-150* mutant) disrupts this function. We have also tested deletion versions of UAS-osk1-534::GFP transgenes, and find that of deletions spanning the interval of amino acids 7-65 (nt 33-210 in the numbering scheme used for the mutations), which encompasses the two largest blocks of high sequence conservation in the amino-terminal domain (Fig. 2), it is only deletion of amino acids 26-35 and 36-45 that disrupts anchoring (data not shown). These are the two deletion mutants which define the extent of the translational activation element (Δ91-120 and Δ121-150), a curious coincidence. Long Osk associates with endocytic membranes along the oocyte cortex (Vanzo et al, 2007). The molecular basis for this association is unknown, but it is notable that prediction of potential palmitoylation sites (data not shown) identifies a candidate (amino acid 37) positioned almost at the junction of the two deletions. Palmitoylation could contribute to membrane association (Smotrys & Linder, 2004), and might be important for Long Osk function. The predicted palmitoylation site is contained within the most highly conserved portion of the extended *osk* 5' UTR, which is required for the regulatory function contained in

the mRNA. Independent of whether palmitoylation occurs, this sequence appears to be under selection both for protein and RNA functions.

Characterization of the translational regulatory element in the extended *osk* 5' UTR presents a challenge, in that mutations will affect both the mRNA and the encoded protein, confounding interpretation of results. Our results show that this is not simply a hypothetical concern, as the mutations that disrupt the RNA element also disrupt Long Osk function and thus Osk protein anchoring. Consequently, conclusions from previous work on this element, in which effects on the encoded protein were not addressed (Gunkel et al, 1998), require reevaluation.

The combination of extensive mutational analysis and sequence comparisons reported here indicate that there is a single regulatory element in the extended *osk* 5' UTR, and strongly suggest that it is contained, in part or whole, in a short, very highly conserved sequence. The interpretation that this work identifies a regulatory element rests on the assumption that the mutations are loss-of-function, and disrupt the element. Alternatively, the defective mutants could be gain-of-function, interfering with *osk* expression because of unusual RNA sequences or structures formed by the mutations. The possibility for creation of novel binding sites for disruptive factors seems extremely unlikely, as three different deletion mutations juxtapose three different sets of sequences: the likelihood of one fusion sequence having this disruptive property is not high, and for all three to do so seems highly improbable. Predicted folding of wild type and mutant RNAs does not reveal any consistent, substantial change in stability, and so that possibility also appears very unlikely. . However, we note that the Δ121-150 deletion is predicted to increase stability of a stem-loop, which might explain

why this mutation can have slightly stronger defects than the other defective mutants. The high conservation of sequences in the region implicated in regulation also argues that the mutations lack an element, although this argument is tempered by the fact that the same region must be conserved for the function of Long Osk protein.

To characterize the role of the 5' regulatory element we used transgenes in which only the mRNA was altered, not the encoded protein. In this manner we showed that the element functions in translation, not mRNA localization or stability. Previous work also concluded that the role was in activation of translation. However, for the experiments that most directly addressed mechanism, the assay scored posterior localization of a Long Osk::ß-galactosidase fusion protein (Gunkel et al, 1998), and the failure to detect localized protein could have been due to an anchoring defect. Indeed, we have shown that GFP expressed from an mRNA localized to the oocyte retains no evidence of its place of origin in the absence of anchoring (Figure 2.6J).

Osk protein first appears during stage 9 of oogenesis, with the bulk of the protein accumulating later. Mutation of the 5' regulatory element lowered the amount of Osk made during the initial phase, but had the strongest effect on later accumulation. This pattern is similar to the effects of mutating the *osk* 3' UTR C region BREs (Reveal et al, 2010). This similarity raises the question of whether an early decrease in the amount of Osk causes a further reduction later, or if translation is more sensitive to defects in activation (or certain types of activation) at different stages. A positive feedback loop stimulates Osk production (Zimyanin et al, 2007), so any translation activation defect might become more pronounced later in development. However, we find that even when 5' element

mutants are tested in presence of endogenous Osk, which would stimulate the later production, a substantial defect in translation remains.

Using *osk::GFP* transgenes we have shown that the 5' regulatory element exerts an effect in the absence of translational repression or mRNA localization. Thus, the element can act as a general enhancer of translation, and is not limited to the more specialized function of overriding repression or being coordinated with mRNA localization. It is possible that this function may be provided primarily in the oocyte, as protein from the transgene accumulates preferentially there despite no corresponding localization of the mRNA. However, we are unable to perform a definitive test because of technical limitations. The effect of loss of the element in the context of *osk::GFP* mRNA is not as strong as the effect on Short Osk from *osk* transgenes, and so the element may serve an additional role not operational in the *osk::GFP* transgenes. Other possible roles include those proposed by Gunkel et al (1998). They argued that the element mediates posterior localization-dependent activation of translation. The evidence for this conclusioni comes from an experiment in which an *osk* mRNA derivative was misdirected to a different region of the oocyte, and failed to be translated there. More recent studies established that a portion of the *osk* 3' UTR removed from that mislocalized mRNA included sequences essential for translation (IBEs and the *osk* C region Bru binding sites) (Munro et al, 2006; Reveal et al, 2010), which can explain the failure of the mislocalized mRNA to be translated. Therefore, while it remains possible that the element has posterior localization-dependent or other specialized roles, the more general enhancement of translation function shown here is the only confirmed role.

A puzzling aspect of our results is the finding that the 5' activation element is dispensable unless the translation start codon for Long Osk is mutated. Mutating the Long Osk start codon has two consequences: loss of the Long Osk protein, and loss of assembled ribosomes moving along the mRNA towards and through the start codon for Short Osk. Presumably, one or both of these features can substitute for the function of the 5' element. The simple presence of Long Osk is not sufficient, as coexpression of Long Osk fails to rescue the translation defect of the 5' element mutants. A more arcane version of a requirement for Long Osk is that the nascent protein must be provided in *cis*, perhaps immediately associating with the template mRNA to perform a function which compensates for loss of the 5' activation element. This function could be similar to that proposed below.

Another option is that the element acts redundantly with transiting ribosomes for translation from the Short Osk start codon. Assuming that translation of both Long and Short Osk relies on scanning initiation, in which preinitiation complexes assemble at the 5' cap and move along mRNA, then both ribosomes and preinitiation complexes will transit towards the Short Osk initiation codon, where preinitiation complexes can be converted to assembled ribosomes. For the *oskM1R* mutant mRNA, only preinitiation complexes would be engaged. RNA secondary structures impede movement of the preinitiation complex (Kozak, 1986), and helicase activity is required to unwind these obstacles (Pisareva et al, 2008). By contrast, the ribosome has an intrinsic mRNA helicase activity, which can disrupt very stable downstream helices and provides a high degree of processivity (Takyar et al, 2005). An explanation of our results is that there are barriers to 48S progress in the *osk* extended 5' UTR, either bound

factors or RNA secondary structures (although no strongly stable structures are predicted), and these must be removed for translation of Short Osk. The ribosome would be able to perform this function, allowing a trailing preinitiation complex to proceed to the start codon for Short Osk. By contrast, a preinitiation complex would be unable to efficiently do so on its own. In this model the 5' element could act by recruiting a helicase to assist the preinitiation complex. It is not certain that translation of Short Osk relies on a cap-dependent scanning mechanism, but even with a form of internal initiation the same principle could apply: initiation requires the removal of an interfering factor, and is achieved either by a ribosome making Long Osk, or by a factor recruited by the 5' activation element.

Progress in understanding the function of the 5' element will require identification of the factor it binds. We have not yet detected any protein that binds specifically to the element. Gunkel et al (1998) described proteins that bind to a larger part of the *osk* 5' region. However, no good correlation exists between regions showing strong binding and regions we have shown to be important for activation, and so these proteins are not strong candidates for activators.

MATERIALS AND METHODS

Flies and Transgenes

w^{1118} flies were used as wild-type. *Df(3R)osk* (Reveal et al, 2010) and *osk*^{A87} were used for all *osk* rescue experiments. Genomic *osk* transgenes included a 3xHA epitope tag, inserted after amino acid 140, to facilitate western blot analysis and, in some cases, to distinguish the protein from endogenous Osk

(J Jones and PMM, submitted). This tag does not detectably alter *osk* expression or activity. The deletion and inversion mutations were constructed using PCR, and introduced into the *osk* rescuing transgene (Kim-Ha et al, 1991) by standard methods. The *UAS-osk1-534::GFP* transgene (Kim et al submitted) was modified in the same manner, including insertion of the *fs(1)K10 TLS* sequence. The version of GFP in this transgene is mGFP6 (Haseloff, 1999).

Cuticle analysis

Cuticle preparations (Wieschaus & Nüsslein-Volhard, 1986) were mounted in Hoyer's Mounting Medium and viewed with a Nikon Eclipse E600 microscope.

Determination of sequence conservation

Sequence conservation across the 5' portion of *osk* was assessed on the UCSC Genome Browser based on the conservation scores calculated by *phastCons* (Siepel et al., 2014). *phastCons* scores short, highly-conserved regions similarly to long, moderately conserved regions. Additionally, gaps in the alignment are treated as missing data, which may overestimate conservation. As another measure of conservation, the 5' portions of *osk* from 11 *Drosophila* species (*D. ananassae*, *D. erecta*, *D. grimshawi*, *D. melanogaster*, *D. mojavensis*, *D. persimilis*, *D. pseudoobscura, D. sechellia*, *D. simulans*, *D. virilis*, *D. yakuba*) were aligned using the standard settings in *clustalW2*, and analyzed for consecutive instances of perfect nucleotide conservation across all species. For the diagram in Figure 2.2, a vertical line was drawn at every position in the

sequence in which a nucleotide and the adjoining nucleotide were identical for all species.

Western blotting

Ovaries from females raised on yeast for 3-4 days were dissected in ice-cold PBS and prepared as described (Kim-Ha et al, 1991) using ice cold lysis buffer (25mM Tris-Cl pH6.8, 1mM $MgCl_2$, 100mM KCl, 1mM DTT, and 0.1% Triton X-100). Lysates were run on a SDS-PAGE gel and transferred to PVDF membrane. Antibodies were used at the following dilutions: mouse anti-HA (1:1000, Covance), mouse anti- α-tubulin (1:2000, Sigma), and mouse anti-GFP (1:1000, Santa Cruz).

Whole mount immunodetection

Sample preparation, antibody staining, and microscopy were performed as described (Kim-Ha et al, 1995; Reveal et al, 2010). Quantitations were performed on samples fixed, processed and imaged with identical settings in parallel. To estimate levels of protein localized to the posterior pole of oocytes, the fluorescent signal in a single focal plane, judged to be the strongest, was traced and the sum of intensity measured using Fiji. A crescent of similar size and shape was traced at the anterior of the oocyte where Osk is not expressed. The anterior mean intensity was subtracted from that of the posterior. For comparison of signal intensities between the oocyte and nurse cells, the outline of each region was traced and mean signal intensities determined with Fiji. The UAS/GAL4 system produces a small proportion of egg chambers in which some

nurse cells have no expression. These nurse cells were not included for the analysis.

RNA analysis

In situ hybridization with ovary samples was performed as described (Snee & Macdonald, 2009). Fluorescent RNA probes for *osk* and *GFP* were synthesized using the DIG RNA labeling mix (Roche). Samples were mounted on slides with Vectasheild Mounting Medium (Vector Labs), and imaged with the Leica TCS-SP laser scanning confocal microscope.

For RNase protection assays, RNA was isolated from 3-4 day old females using Tri Reagent-LS (Molecular Research Center) as per the manufacturers instructions followed by phenol/chloroform extraction. Assays were performed using the RPA III Kit (Ambion). Following electrophoresis of products in denaturing gels, signals were detected by phosphorimaging with the Typhoon laser scanner (GE Healthcare) and quantitated using Image J. At least three assays were performed for each transgene.

Acknowledgments

We thank Anne Ephrussi for a plasmid and the osk^{A87} flies, the Bloomington stock center for flies, and members of the Macdonald lab for comments on the manuscript. This work was supported by NIH grant GM54409.

Author contributions

M.K. and P.M.M. designed experiments, performed experiments, analyzed data generated from the experiments, and wrote the paper.

FIGURES

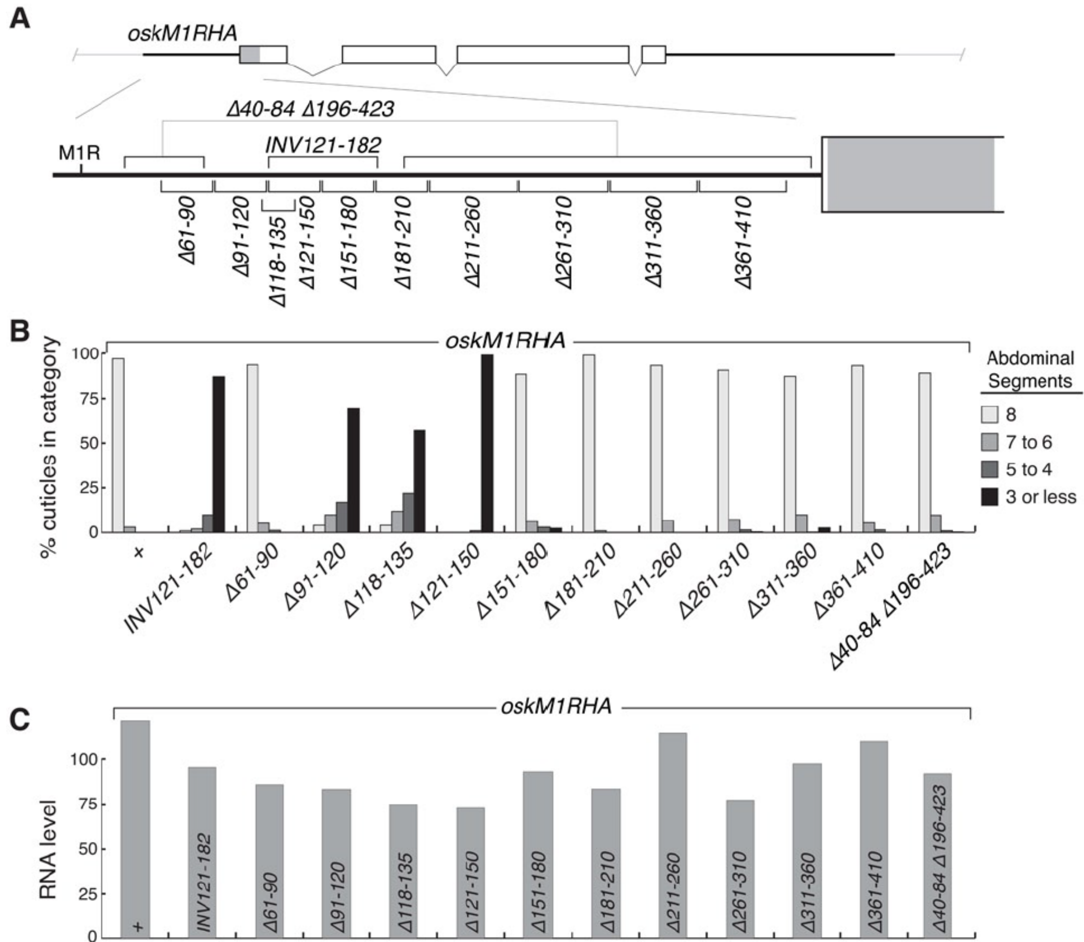


Figure 2.1 - Full caption next page

Figure 2.1. Mapping an RNA element required for *osk* activity

A. Diagram of the 5' region of the *osk* mRNA bearing the M1R mutation, which eliminates translation of Long Osk. The extended 5' UTR is shown as a black line, and the Short Osk coding region as a rectangle. The *oskM1RHA* transgene has the M1R mutation and contains 3 copies of the HA epitope tag, inserted after residue T140 (the Short Osk start codon is M139). Deletions are indicated.

B. Patterning activity of *osk* transgenes, tested as single copies in the *oskA87/Df(3R)osk* background (RNA null). The number of abdominal segments corresponds to the level of *osk* activity, with wild type embryos having eight.

C. Levels of *osk* mRNA produced from a single copy of the indicated transgenes. All values are normalized against the level of mRNA from a single copy of the *oskHA* transgene, which is identical to *oskM1RHA* except that it has the wild type M1 codon. Levels of *rp49* were monitored to normalize for amount of RNA used in each assay.

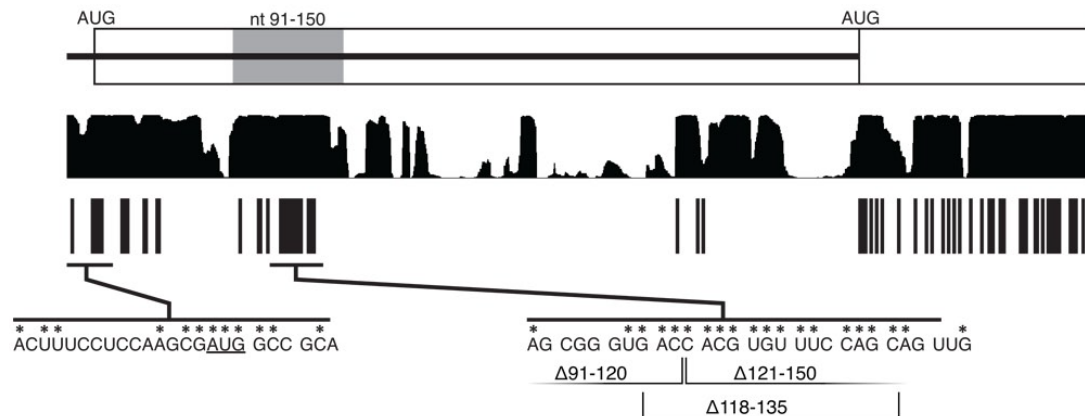


Figure 2.2. Sequence conservation in the 5' region of the *osk* gene

The diagram at top shows the 5' region of osk, with the extended 5' UTR as a black line and the osk coding region as a rectangle. The AUG start codons for Long and Short Osk are shown, and the region containing the 5' activation element is shaded. The two analyses of conservation are shown below, with the phastCons output above and the clusters of perfectly conserved positions shown below. For the latter, each vertical line indicates the presence of 2 consecutive positions that are perfectly conserved among the species analyzed (Methods and Materials). At bottom are segments of the osk sequence showing the short regions most highly conserved in the extended 5' UTR. Within the coding region, codons are indicated by spacing, and perfectly conserved positions are identified with asterisks. The endpoints of the indicated deletion mutations are marked.

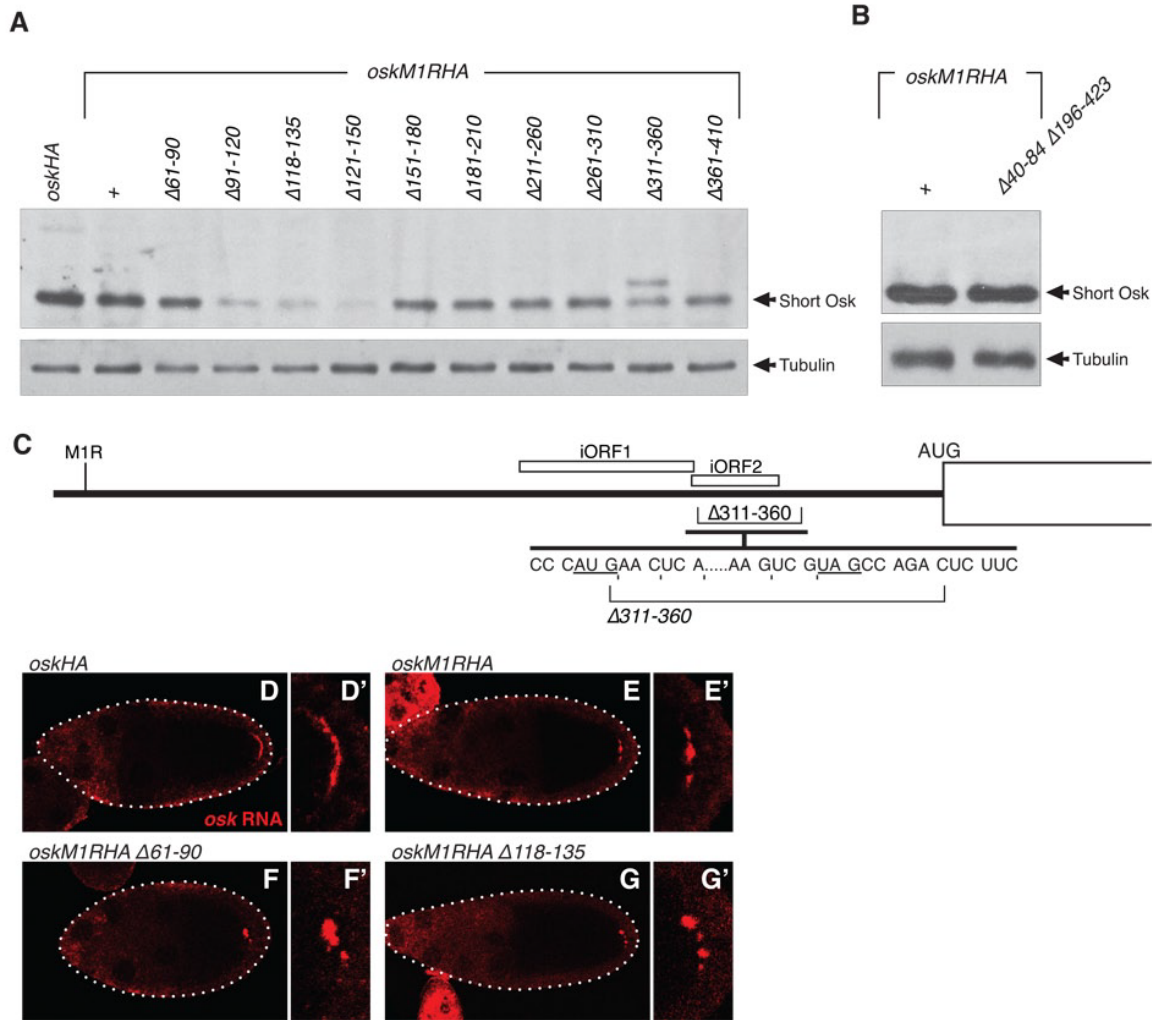


Figure 2.3 - Full caption next page

Figure 2.3. The 5' element is required for translational activation

A and B. Western blot analysis of transgenes expressed as single copies in the *osk*^{A87}*/Df(3R)osk* background. Tubulin is detected as a loading control.

C. Diagram of the *osk* 5' region, showing the positions of the two iORFs and how the Δ311-360 deletion fuses iORF2 to the Osk reading frame, and thus can produce the novel protein band detected in A.

D-G. In situ hybridization to detect transgene mRNAs in the *osk*^{A87}*/Df(3R)osk* background (panels D'-G' are magnified views of the posterior region to better show the mRNA distributions). Egg chambers are outlined with dotted lines. For the *oskHA* transgene, which makes both Long and Short Osk, the mRNA is tightly restricted to a posterior crescent (D,D'). The *oskM1R* transgene lacks Long Osk and its anchoring function, and the mRNA has a more punctate distribution (E,E'). Similarly, both of the mutants tested, one with normal *osk* activity (F,F'; the Δ61-90 deletion) and one largely lacking *osk* activity (G,G'; the Δ118-135 deletion), have the same punctate distribution of mRNA.

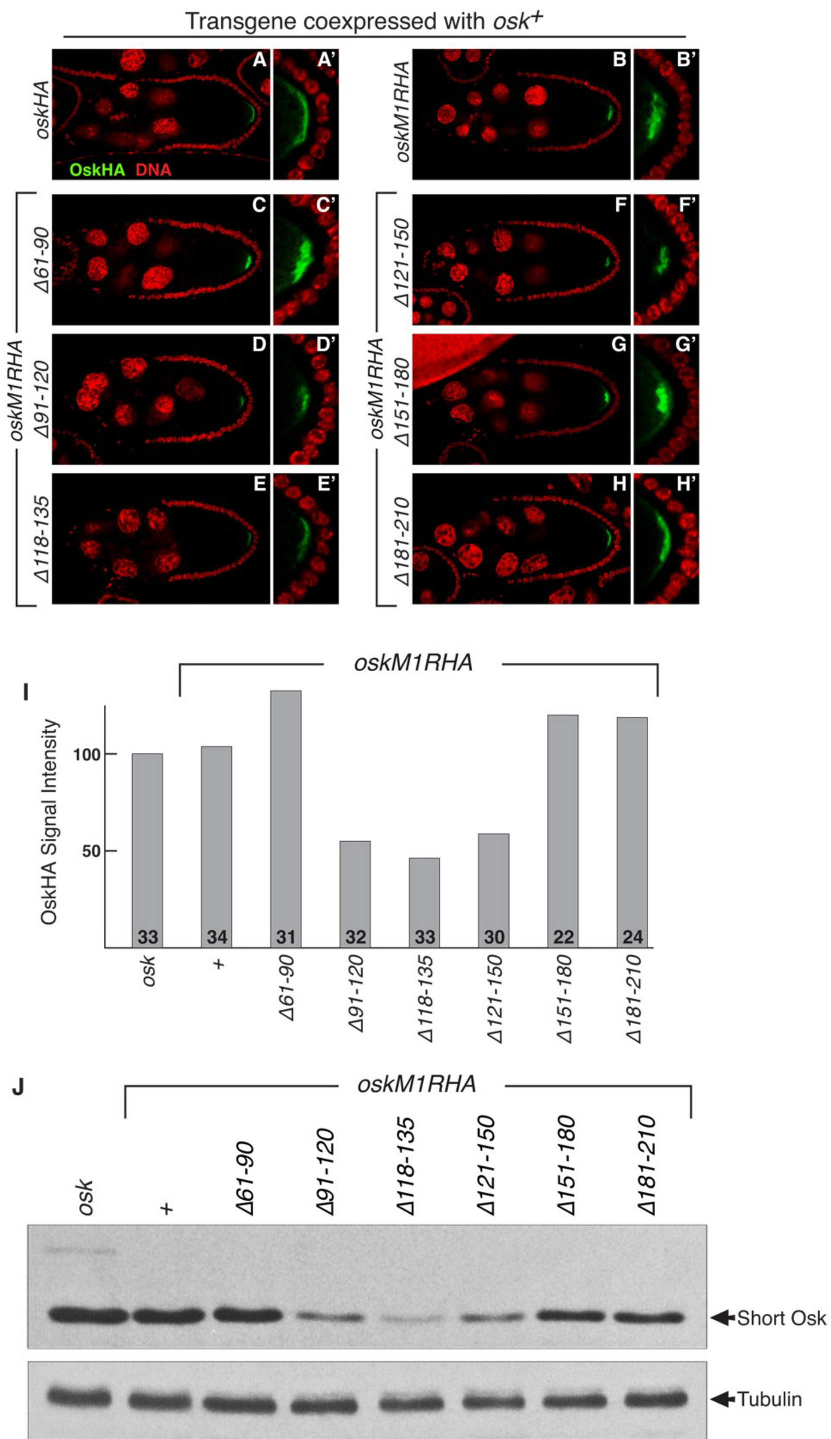


Figure 2.4 - Full caption next page

Figure 2.4. The 5’ element is required for the early phase of Osk protein accumulation

A-H,A'-H'. Detection of transgenic Short OskHA protein expressed from single copies of the indicated transgenes in the presence of endogenous Long Osk for anchoring. Panels A'-H' are magnified views of the posterior of the oocyte to better show the proteins. Green is OskHA and red is DNA detected with ToPro-3.

I. Quantification of protein levels from the imaging experiments of A-H. OskHA signal intensities (Methods and Materials) are shown normalized to that from the *oskHA* transgene. The number of oocytes scored is indicated at the bottom of each bar.

J. Western blot analysis of transgenes expressed as single copies in the presence of a wild type copy of *osk*. Only the transgenic Osk protein is detected using anti-HA antibodies. Tubulin is detected as a loading control.

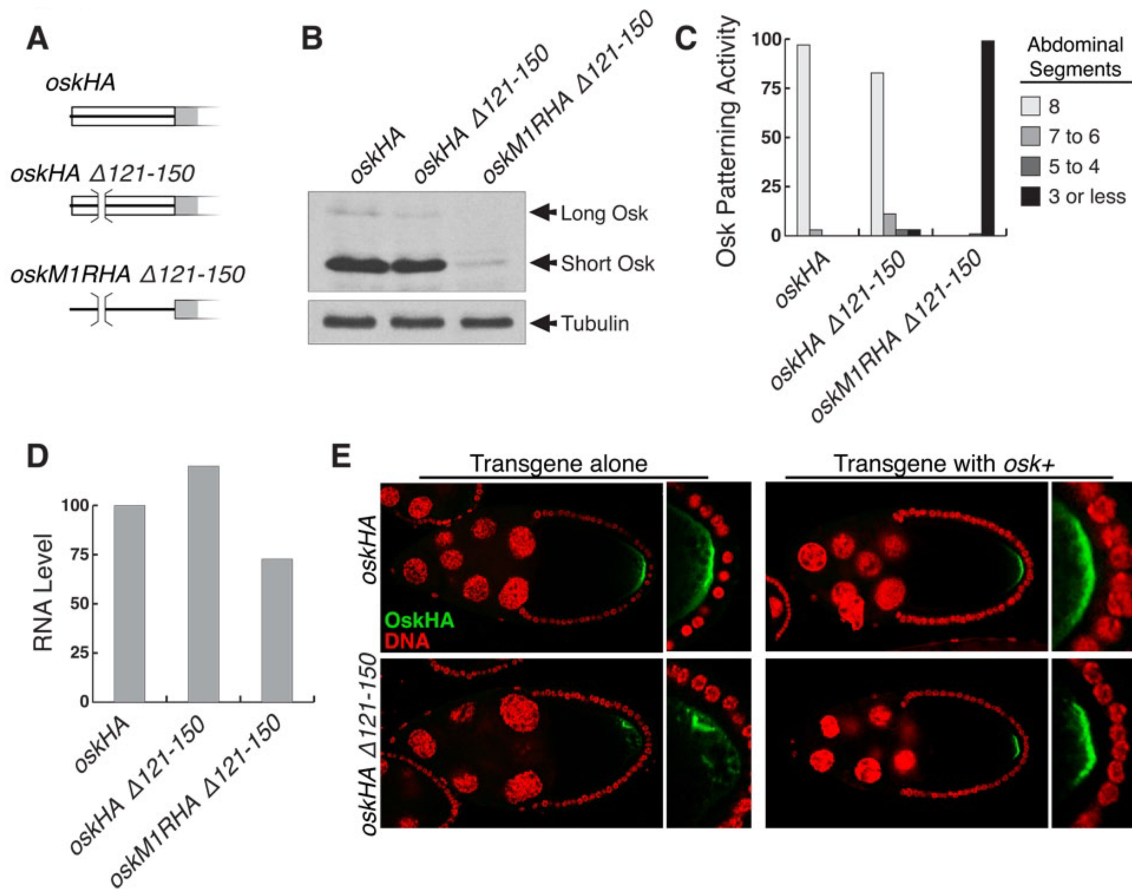


Figure 2.5 - Full caption next page

Figure 2.5. Effects of mutating the 5' element on translation and Long Osk function

A. Diagram of the 5' region of the *osk* transcripts, using the conventions from Figs. 1 and 2.

B. Western blot analysis of transgenes expressed as single copies in the *osk^{A87}/Df(3R)osk* background.

C. Patterning activity of osk transgenes, tested as single copies in the *osk^{A87}/Df(3R)osk* background (RNA null). The number of abdominal segments corresponds to the level of *osk* activity, with wild type embryos having eight.

D. Levels of *osk* mRNA produced from a single copy of the indicated transgenes. All values are normalized against the level of mRNA from a single copy of the *oskHA* transgene. Levels of *rp49* were monitored to normalize for amount of RNA used in each assay.

E. Detection of transgenic OskHA expressed from single copies of the indicated transgenes. For the panels at left, the transgenes were tested in the *osk^{A87}/Df(3R)osk* background, revealing the anchoring defect of the OskHAΔ121-150 mutant, which lacks aa 36-45. This defect is rescued when coexpressed with wild type Long Osk, as shown in the panels at right.

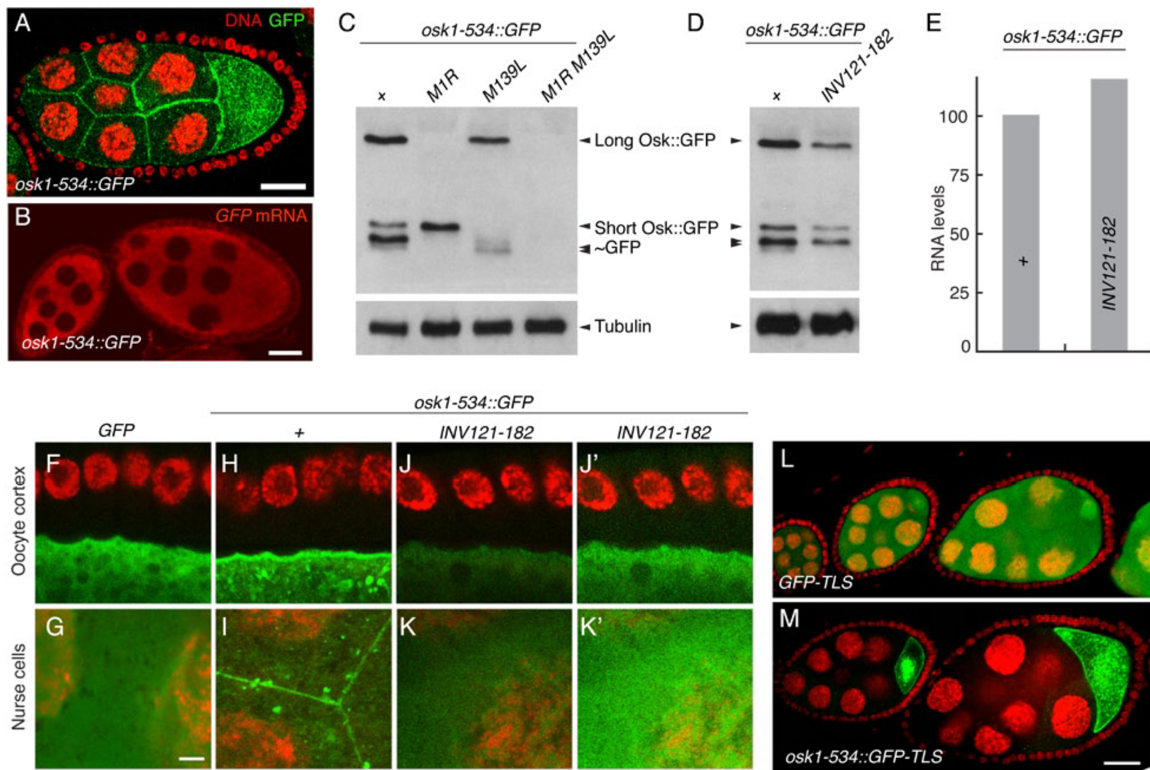


Figure 2.6 - Full caption next page

Figure 2.6. The 5' element activates translation independent of mRNA localization and translational repression

A. Distribution of the Osk1-534::GFP fusion protein. Scale bars in A and B are 25 μm.

B. In situ hybridization to detect the *osk1-534::GFP* mRNA.

C-D. Western blot analysis of transgenes expressed as single copies in the *osk^{A87}/Df(3R)osk* background. All are versions of *UAS-osk1-534::GFP*, with the start codons for Long (M1) and Short Osk (M139) as indicated in C, and the INV121-183 mutation included in D. The cluster of fastest migrating bands vary in appearance in separate experiments; the examples shown here are typical.

E. Levels of mRNA produced from a single copy of the indicated transgenes. All values are normalized against the level of mRNA from a single copy of the *UAS-osk1-534::GFP* transgene. Levels of *rp49* were monitored to normalize for amount of RNA used in each assay.

Caption continued on next page.

F-K. Detection of Osk::GFP fusion proteins in stage 10 egg chambers. All panels are the same magnification and the scale bar is 5 μm. For panels F,H,J and J' the images show a portion of the lateral cortical region, with the follicle cell nuclei separate from the oocyte. Osk::GFP protein is restricted to the oocyte, as the GAL4 driver (maternal a-tub 4) is germ line-specific. For panels G,I,K,and K' the images show a portions of several nurse cells and the boundaries between them. Signal intensities can only be compared between panels H-K, which were imaged under identical conditions. Panels J' and K' are identical to J and K except that the green signal was enhanced to better show the absence of any anchoring. The level of protein from the *UAS-GFP* transgene is much higher than from the *UAS-osk1-534::GFP* transgenes, and lower intensity laser settings were used to obtain images in F and G with signal intensity comparable to H and I. Anchoring of the Osk1-534::GFP protein is manifested in the enrichment at the cortex, along nurse cell boundaries, and the punctate appearance in the cytoplasm. Neither GFP alone nor the Osk1-354 INV121-182::GFP protein shows any similar anchoring.

L and M. Detection of GFP (L) and Osk1-534::GFP (M) fusion proteins expressed from mRNAs bearing the K10 TLS and thus localized to the oocyte. The oocytes in M are readily identified by the intense green signal. The egg chambers in L are oriented similarly, and the oocytes appear largely indistinguishable from the nurse cells.

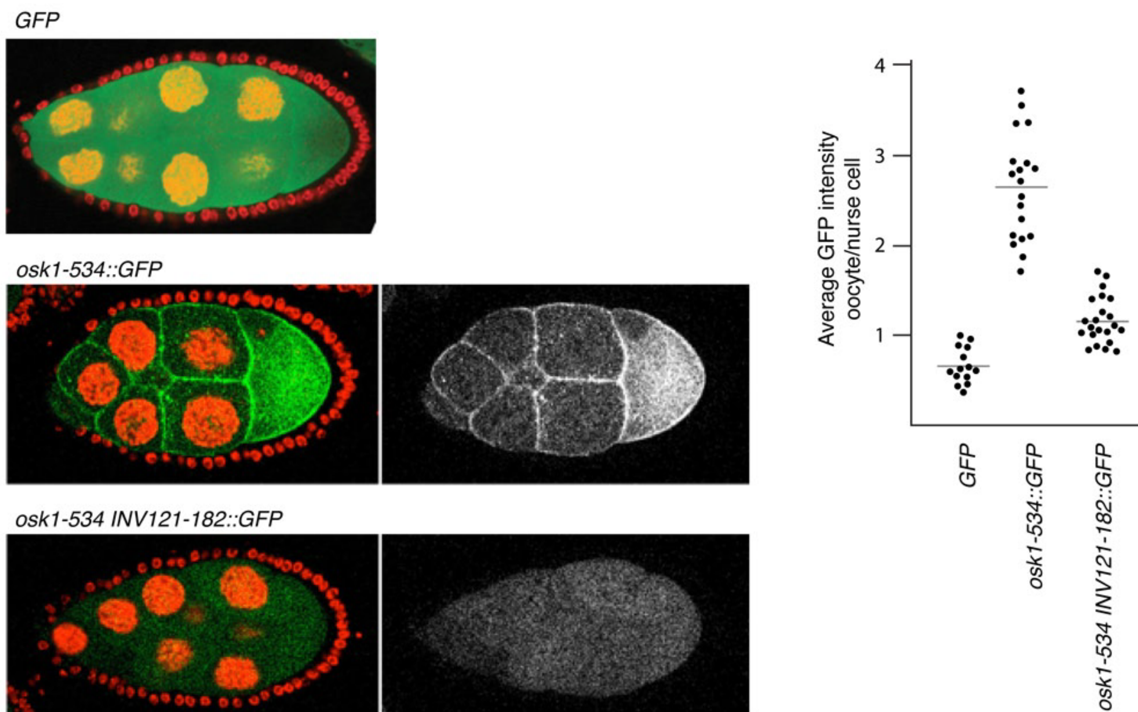


Figure 2.7. *osk* 5' sequences confer oocyte enrichment on reporter expression

Panels at left show expression patterns for the transgenes indicated. To better show the anchoring and oocyte enrichment, the GFP channels for the lower two transgenes are shown alone in the center. GFP levels for the *UAS-osk1-534::GFP*-based transgenes are directly comparable, but the more highly expressed *UAS-GFP* transgene was imaged with lower laser intensity to allow better comparison of distributions.

The graph at right shows quantitation of the degree of oocyte enrichment for each transgene. Quantitation was performed with ImageJ and the data graphed in Kallediagraph.

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 and Development, 18(2), 138-143.

Benoit, P., Papin, C., Kwak, J., Wickens, M., & Simonelig, M. (2008). PAP and GLD-2-type poly(A) polymerases are required sequentially in cytoplasmic polyadenylation and oogenesis in Drosophila. Development, 135(11), 1969-1979.

Besse, F., Lopez de Quinto, S., Marchand, V., Trucco, A., & Ephrussi, A. (2009). Drosophila PTB promotes formation of high-order RNP particles and represses oskar translation. Genes and 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.

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

Chicoine, J., Benoit, P., Gamberi, C., Paliouras, M., Simonelig, M., & Lasko, P. (2007). Bicaudal-C recruits CCR4-NOT deadenylase to target mRNAs and regulates oogenesis, cytoskeletal organization, and its own expression. Developmental Cell, 13(5), 691-704.

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.

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

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

Jung, H., Gkogkas, C., Sonenberg, N., & Holt, C. (2014). Remote control of gene function by local translation. Cell, 157(1), 26-40.

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.

Kozak, M. (1986). Influences of mRNA secondary structure on initiation by eukaryotic ribosomes. Proceedings of the National Acadamy of Sciences, 83(9), 2850-2854.

Lasko, P. (2011). Posttranscriptional regulation in Drosophila oocytes and early embryos. Wiley Interdisciplinary Reviews RNA, 2(3), 408-416.

Lasko, P. (2012). mRNA localization and translational control in Drosophila oogenesis. Cold Spring Harbor Perspectives in Biology, 4(10).

Lecuyer, E., Yoshida, H., Parthasarathy, N., Alm, C., Babak, T., Cerovina, T., Hughes, T. et al. (2007). Global analysis of mRNA localization reveals a prominent role in organizing cellular architecture and function. Cell, 131(1), 174-187.

Munro, T., Kwon, S., Schnapp, B., & St Johnston, D. (2006). A repeated IMP-binding motif controls oskar mRNA translation and anchoring independently of Drosophila melanogaster IMP. Journal of Cellular Biology, 172, 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.

Pisareva, V., Pisareva, V., Komar, A., Hellen, C., & Pestova, T. (2008). Translation initiation on mammalian mRNAs with structured 5'-UTRs requires DExH-box protein DHX29. Cell, 135(7), 1237-1250.

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.

Saffman, E., Styhler, S., Rother, K., Li, W., Richard, S., & Lasko, P. (1998). Premature translation of oskar in oocytes lacking the RNA-binding protein Bicaudal-C. Molecular and Cellular Biology, 18(8), 4855-4862.

Serano, T., & Cohen, R. (1995). A small predicted stem-loop structure mediates oocyte localization of Drosophila K10 mRNA. Development, 121(11), 3809-3818.

Smotrys, J., & Linder, M. (2004). Palmitoylation of intracellular signaling proteins: regulation and function. Annual Review of Biochemistry, 73, 559-587.

Snee, M., Harrison, D., Yan, N., & Macdonald, P. (2007). A late phase of Oskar accumulation is crucial for posterior patterning of the Drosophila embryo, and is blocked by ectopic expression of Bruno. Differentiation, 75(3), 246-255.

Takyar, S., Hickerson, R., & Noller, H. (2005). mRNA helicase activity of the ribosome. Cell, 120(1), 49-58.

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.

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

Wieschaus, E., & Nüsslein-Volhard, C. (1986). Looking at embryos. In Drosophila: A practical approach, Roberts DB (eds) pp199-227. Washington, D.C.: IRL Press.

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

Zimyanin, V., Lowe, N., & St Johnston, D. (2007). An oskar-dependent positive feedback loop maintains the polarity of the Drosophila oocyte. Current Biology, 17(4), 353-359.

Chapter 3: *oskar* RNA plays multiple non-protein coding roles to support oogenesis and maintain integrity of the germline/soma distinction

Matt Kanke[1], Helena Jambor[2,3], John Reich[1], Brittany Marches[1], Ronald Gstir[2,4], Young Hee Ryu[1], Anne Ephrussi[2], and Paul M. Macdonald[1]

[1]Department of Molecular Biosciences

Institute for Cellular and Molecular Biology

The University of Texas at Austin

[2]European Molecular Biology Laboratory

Heidelber, Germany

[3]Max-Planck Institute of Molecular Cell Biology and Genetics

Dresden, Germany

[4]Division of Genomics & RNomics

Innsbruck Medical University

Innsbruck, Austria

ABSTRACT

The *Drosophila oskar* (*osk*) mRNA is unusual in that it has both coding and noncoding functions. As an mRNA, *osk* encodes a protein required for embryonic patterning and germ cell formation. Independent of that function, the absence of *osk* mRNA disrupts formation of the karyosome and blocks progression through oogenesis. Here we show that loss of *osk* mRNA also affects the distribution of regulatory proteins, relaxing their association with large RNPs within the germline, and allowing them to accumulate in the somatic follicle cells. This and other noncoding functions of the *osk* mRNA are mediated by multiple sequence elements with distinct roles. One role, provided by numerous binding sites in two distinct regions of the *osk* 3' UTR, is to sequester the translational regulator Bruno, which itself controls translation of *osk* mRNA. This defines a novel regulatory circuit, with Bruno restricting the activity of *osk*, and *osk* in turn restricting the activity of Bruno. Other functional elements, which do not bind Bru and are positioned close to the 3' end of the RNA, act in the oocyte and are essential. Despite the different roles played by the different types of elements contributing to RNA function, mutation of any leads to accumulation of the germline regulatory factors in the follicle cells.

INTRODUCTION

The fundamental role of an mRNA is to serve as a template for translation, and thus encode a protein. To ensure that this protein is produced at the desired level and in the appropriate location, the mRNA may also contain a variety of regulatory signals. These are often located within the noncoding parts of the mRNA, the 5' and 3' untranslated regions (UTRs). In particular, the 3' UTR is a common position for signals that control mRNA translation, stability, localization and length of the poly(A) tail.

Additional RNAs perform noncoding functions. Some of these RNAs were recognized early, notably the ribosomal and transfer RNAs that work in conjunction with mRNAs during translation. Other noncoding RNAs perform myriad functions, with the breadth and diversity of this group only becoming recognized in recent years. Some of the noncoding RNAs share substantial similarities in structure and function. Examples include certain types of small regulatory RNAs, such as microRNAs (miRNAs) which influence stability and translation of bound mRNAs (Bartel, 2009). But other noncoding RNAs defy classification, with many grouped solely by size into the category of long noncoding RNAs (lncRNAs). Some lncRNAs act in cis, influencing transcription close to their site of synthesis. Others act in trans, by a variety of mechanisms including, but not limited to, control of transcription. A lncRNA can act as a scaffold for assembly of multiprotein complexes, and it can act as a decoy to bind and inhibit the action of a protein. Interactions with other RNAs are also possible via base pairing. Identification of lncRNAs has far outpaced characterization of their roles and how they function, and the range of mechanisms can only expand.

Given their large size, there is no reason to assume that all lncRNAs will be constrained to perform a single function (reviewed by (Cech and Steitz. 2014)).

The Drosophila *oskar* (osk) mRNA is unusual, even by these standards, in that it performs both coding and noncoding functions. *osk* mRNA is expressed at high levels during oogenesis, where it appears only in the germline cells of the ovary, and this maternal mRNA is contributed to the embryo, where it is rapidly degraded at the midblastula transition (Kim-Ha et al., 1991; Ephrussi et al., 1991). A role for *osk* in axial patterning of the embryo was established with classical mutants affecting the Osk protein. Protein null alleles cause a strong maternal-effect phenotype: no obvious defects exist during oogenesis but all of the resulting embryos fail to develop abdominal segments (Lehmann and Nüsslein-Volhard. 1986; Kim-Ha et al. 1991). More recently, new *osk* alleles were identified in which insertion of a transposon largely abolishes production of *osk* mRNA (Jenny et al. 2006). Surprisingly, these *osk* RNA null mutants revealed a further phenotype: progression through oogenesis was blocked. In wild type ovaries *osk* mRNA is present continually from the very earliest stages, but its translation is repressed until the later stages. Osk protein is not required earlier in oogenesis, suggesting that the *osk* mRNA had a separate noncoding role. Indeed, dissection of the *osk* mRNA revealed that the RNA activity resides in the 3' UTR (Jenny et al. 2006).

Here we show that there are multiple components to *osk* RNA function. One role is to sequester the translational regulator Bruno (Bru), an interaction that defines a newly described regulatory circuit. In addition, sequences clustered near the 3' end of the *osk* 3' UTR are crucial for its function and appear to comprise multiple different elements. This organization would be consistent with

a scaffolding function, with the different elements serving to bind factors. One surprising consequence of the absence of *osk* mRNA is enhanced accumulation in the somatic follicle cells of proteins thought to be restricted to the germline, a phenomenon that may contribute to the arrest of oogenesis.

RESULTS

Bruno binding sites are required for *oskar* RNA function

BREs, one class of binding site for Bruno (Bru), are clustered in two separate regions of the *osk* mRNA 3' UTR, the AB and C regions (Figure 3.1A). BREs mediate translational regulation, and mutation of all of the BREs results in precocious expression of Osk protein and disruption of embryonic patterning. This phenotype was first observed when testing an *osk ABC BRE-* transgene in an *osk* protein null background (Kim-Ha et al. 1995). More recently, identification of *osk* RNA null mutants (Jenny et al. 2006) allowed the same transgene to be tested as the only source of *osk* mRNA (Reveal et al. 2010). In addition to the embryonic patterning defects, a second phenotype was observed: mutation of all BREs substantially reduces the rate of egg laying. Reduced egg laying is consistent with partial disruption of the *osk* noncoding RNA function, such that progression through oogenesis is impaired but not abolished. Examination of ovaries supported this interpretation: many ovarioles had egg chambers that arrested development (data not shown). To explore this phenomenon we compared rates of egg laying for females lacking endogenous *osk* mRNA (osk^{A87}/*Df(3R)osk*) and expressing transgenic *osk* mRNAs with all BREs intact (*osk+*), with AB or C region BREs mutated (*osk AB BRE-* and *osk C BRE-*,

respectively), or with all BREs mutated (*osk ABC BRE-*). Quantitative assays showed that mutation of any set of BREs reduced egg laying, with loss of all BREs having the strongest effect (Figure 3.1B). The different activities were not due to different levels of *osk* mRNA, as the various mRNAs were present at similar levels (Figure 3.1C).

To further confirm that provision of Bru binding sites constitutes at least part of *osk* RNA function, we asked if impairment of *osk* RNA function caused by loss of Bru binding sites in the *osk* mRNA could be offset by providing Bru binding sites in another mRNA (Figure 3.1D). The *UAS-GFP-4xBRE* transgene has four copies of an isolated 12 bp BRE-type binding site, but no other *osk* mRNA sequences, and the *GFP-4xBRE* mRNA is translationally repressed in ovaries (Reveal et al., 2011). Germline expression of this mRNA with a GAL4 driver resulted in a substantial increase in the rate of egg laying relative to *osk ABC BRE-* alone. By contrast, expression of a control *UAS-GFP* transgene lacking Bru binding sites did not increase egg laying.

oskar **mRNA acts to sequester Bruno**

Two models have been proposed for the noncoding function of *osk* mRNA (Jenny et al., 2006). In one model, the *osk* mRNA (and more specifically the *osk* mRNA 3' UTR, which is both necessary and sufficient for this function (Jenny et al., 2006)) serves as a scaffold for assembly of an RNP particle that in some manner facilitates progression through oogenesis. In the second model the *osk* mRNA 3' UTR sequesters a regulatory factor that would otherwise inhibit oogenesis. Our data clearly demonstrate a role for Bru binding sites in the

function of *osk* mRNA. In the context of the two models, Bru would either be a component of the assembled RNP particle, or be sequestered and thus limited in activity. For the first model a reduction in the level of Bru would be expected to interfere with RNP assembly, potentially enhancing the *osk* RNA null phenotype. By contrast, for the second model a reduction in the level of Bru would serve the same purpose as sequestration of Bru, and should suppress the *osk* RNA null phenotype.

To test these predictions we adopted two approaches. The first was to reduce the level of Bru activity in ovaries lacking *osk* mRNA; this approach could reveal a suppression of the RNA null phenotype. In females lacking *osk* mRNA and heterozygous for either of two *aret* alleles (*aret* encodes Bru (Webster et al. 1997)) oogenesis was still arrested, but often progressed further as judged by elongation of the egg chambers (Figure 3.1F). The degree of rescue corresponded to the severity of the *aret* allele: *aretPA*, a missense mutant which retains some activity, had a weaker suppressive effect, while *aretQB*, a stronger nonsense mutant more strongly suppressed the *osk* RNA null phenotype. These results argue that *osk* mRNA acts to sequester Bru.

In a second approach we used females expressing the *osk ABC BRE-* mRNA, such that partial *osk* RNA function is provided and either enhancement or suppression of the phenotype is possible. Mutating one copy of *aret* in this background substantially suppressed the defect in egg laying (Figure 3.1E). The results of both approaches support the model (see Discussion) that the non-coding function of *osk* mRNA is, at least in part, to sequester Bru.

A second component to *oskar* RNA function

Mutation of the BREs reduces but does not abolish *osk* RNA function. The residual *osk* RNA activity could be due to residual Bru binding to the *osk* mRNA, or *osk* mRNA could perform a function in addition to sequestration of Bru, or both. The BREs are not the only type of Bru binding site, and other classes of binding sites have been identified. The type II and type III sites are, like the BREs, clustered in the AB and C regions of the *osk* mRNA 3' UTR (Reveal et al., 2010). Mutation of all AB region sites (the BREs and the single type II site) in *osk AB all-* caused a moderate disruption of *osk* RNA function, similar to that for *osk AB BRE-* (Figure 3.1B). In striking contrast, mutation of all C region sites (the BREs, the two type II sites, and the single type III site) in *osk C all-* eliminated *osk* RNA function: no eggs were laid (Figure 3.1B) and oogenesis was arrested just as in the absence of *osk* mRNA (Figure 3.2C). Similarly, the *osk ABC all-* mRNA (with both AB and C sites mutated), also lacked *osk* RNA function (Figure 3.1B, data not shown). The dramatic loss of *osk* RNA function from mutating the C region Bru binding sites was not due to loss of the type III site (found only in the C region), as mutation of this site alone did not affect egg laying ((Reveal et al., 2010) and below). Likewise, mutation of the two type II sites in the C region did not interfere with egg laying ((Reveal et al., 2010) and below).

That the *osk ABC all-* mutant was more defective in *osk* RNA function than the *osk ABC BRE-* mutant was not surprising, since the all- mutant should be less able to sequester Bru. However, the differences in *osk* RNA activity of the *osk AB all-* and *osk C all-* mutants was unexpected: the AB region binds Bru more strongly than does the C region ((Kim-Ha et al., 1995); Figure 3.2D), and loss of the AB binding sites should therefore lead to a stronger phenotype if the

sole noncoding function of *osk* mRNA is to sequester Bru. This inconsistency between the strength of Bru binding and strength of *osk* RNA function suggests that the C all- mutations have consequences beyond reduction of Bru binding. Specifically, they appear to disrupt an additional noncoding function of the *osk* mRNA. Presumably, this other function is mediated by sequences overlapping with, or close to, the C region Bru binding sites.

A complementary mapping approach also revealed the importance of the *osk* 3' region. Portions of the *osk* 3' UTR were appended to a *GFP* mRNA and expressed under UAS/GAL4 control in the germline of *osk* RNA null ovaries, and assayed for rescue of *osk* RNA function, including progression through oogenesis and the ability to lay eggs (Figure 3.3A). When the *osk* 3' UTR was divided into three segments, none provided the missing *osk* RNA function. A possible explanation is that the *osk* mRNA must be concentrated in the oocyte for at least one aspect of its function. Only the central fragment, within which an oocyte entry signal (OES) has been mapped (Jambor et al., 2014), conferred oocyte enrichment. Testing larger fragments, containing the central region and either 5' or 3' flanking sequences, showed that the combination of the central and 5' parts still failed to rescue egg laying, even though the mRNA was enriched in the oocyte (Figure 3.3B). By contrast, the fragment with the central and 3' parts did rescue to the extent that the females could lay eggs. The simplest interpretation of these results is that sequences critical for the *osk* RNA function are provided by a 3' segment of the UTR, and that the OES serves to deliver this RNA to the oocyte where their function is performed. Consistent with this model, the OES and the 3’ region must be coupled in *cis*, as co-expression of the two RNA segments from two independent transgenes showed no rescuing effect

(data not shown). To prove that the OES is required for oocyte enrichment, and does not make another essential contribution to *osk* RNA function, we replaced the OES with the TLS, an element which promotes enrichment of *fs(1)K10* mRNA in the oocyte (Serano and Cohen, 1995). An mRNA containing the essential *osk* 3' region fused to the TLS was efficiently enriched in the oocyte and rescued the *osk* RNA null phenotype to the point where at least some eggs were laid. Using the TLS to provide oocyte enrichment, the 3' segment was further truncated to identify a 119 nt minimal 3' region (Figure 3.3A). This minimal 3' region includes all of the Bru binding sites in the C region, except for the single type III site.

Both of the approaches described above highlight the importance for *osk* RNA function of a 3' part of the *osk* 3' UTR, which acts together with sequences elsewhere in the 3' UTR (e.g. the AB region BREs) to provide full *osk* RNA function. To more precisely map functional elements near the *osk* mRNA 3' end, a scanning mutagenesis of this region was performed (Figure 3.4). Fifteen sets of mutations, most having 5 contiguous nucleotides altered, were each introduced into a genomic *osk* transgene which, in wild type form, provides full *osk* function and completely rescues all defects associated with absence of *osk* mRNA (Figure 3.1B and below). The mutants were named *osk3'x-y*, where x and y refer to the first and last positions of mutations within the *osk* 3' UTR.

As an initial test of the scanning mutants for *osk* RNA function, the egg laying assay was used (Figure 3.4A), with a single copy of a mutant transgene in the *osk*^{A87}*/Df(3R)osk* background. Most of the mutants support *osk* RNA function: eggs were laid at a rate similar to that found with a wild type *osk* transgene, indicating that progression through oogenesis is efficient. However, mutation of sequences close to the 3' end of *osk* mRNA interferes with *osk* RNA function:

mutants *osk3'977-981* and *osk3'984-988* failed to lay any eggs, and mutants *osk3'990-994* and *osk3'1004-1008* had greatly reduced egg laying.

The mutations from the scanning mutagenesis that interfered with *osk* RNA function do not affect known Bru binding sites, which are all positioned more 5' in the mRNA (Reveal et al., 2010). Furthermore, Bru does not bind appreciably to the region of the 3' UTR bearing the defective mutations (Kim-Ha et al., 1995). Nevertheless, the mutations might indirectly affect Bru binding, and thereby disrupt *osk* RNA function. To address this possibility, RNA binding assays were performed (Figure 3.2E). Neither of the mutants lacking all *osk* RNA function (*osk3'977-981* and *osk3'984-988*) affected Bru binding. Similarly, a mutant with impaired *osk* RNA function (*osk3'990-994*) retained full Bru RNA binding. By contrast, the *osk3'970-974* mutant, in which one of the type II Bru binding sites is disrupted, showed reduced Bru binding. Thus, although one component of the *osk* RNA function is to bind Bru, mutations near the 3' end of the *osk* mRNA define other key elements that contribute to *osk* RNA function by a different mechanism.

Failure of certain scanning mutants to provide *osk* RNA function could owe to a defect in the RNA element that provides the function. Alternatively, the RNA could be unstable or not enriched in the oocyte. Although most mutant mRNAs were present at levels similar to the wild type mRNA, several with mutations near the 3' end of the *osk* mRNA were less abundant (Figure 3.4A). This group includes mutants with normal *osk* RNA function, so a lower mRNA level is not by itself sufficient to abrogate this function. Nevertheless, lower levels of mutant *osk* mRNAs could be partially responsible for *osk* RNA function defects. To address this possibility, we increased mRNA levels using additional copies of the

transgenes. Notably, even when the levels of the *osk3'977-981* and *osk3'984-988* mutant mRNAs were equal to the wild type *osk* mRNA (Figure 3.4B), no eggs were laid, and oogenesis was arrested at a stage similar to that when no *osk* mRNA is present (Figure 3.4C,D). Thus, these mutants have extreme effects on *osk* RNA function independent of any effect they may have on *osk* mRNA stability. We also examined the distribution of representative mutant mRNAs. Of the mutants tested, two provided normal *osk* RNA function, two provided partial function, and two were most seriously affected. Each of these six mutants displayed the normal pattern of *osk* mRNA accumulation in early stage egg chambers, with the mRNA highly concentrated in the oocyte (Figure 3.4E). Thus, loss of *osk* RNA function by the affected mutants cannot be attributed to a defect in nuclear export or transport to the oocyte.

***oskar* RNA function and karyosome formation**

During oogenesis the meiotic chromosomes of the oocyte form a single compact cluster - the karyosome - within the nucleus. In the absence of *osk* mRNA the karyosome does not form properly, and the chromosomes usually appear in multiple zones within the oocyte nucleus ((Jenny et al., 2006), Figure 3.5C). This phenotype raised the possibility that the oogenesis arrest of *osk* RNA null mutants might be caused by defects known to affect the karyosome.

The karyosome phenotype of *osk* RNA null egg-chambers is strikingly similar to that of mutants defective in repair of DNA damage (Ghabrial et al., 1998). When damaged DNA is not repaired, a checkpoint is activated and karyosome morphology is altered. These karyosome defects can be suppressed

by mutation of genes responsible for execution of the checkpoint, such as *mei41* (Ghabrial and Schupbach, 1999). To ask if the karyosome defects in the absence of *osk* mRNA are due to activation of this checkpoint, we tested *osk* RNA null females that were also homozygous for *mei41*^{D3}. There was no rescuing effect on either oogenesis arrest or karyosome morphology, suggesting that an ectopically activated DNA damage checkpoint is not the cause for the *osk* RNA null phenotype (data not shown).

We have identified two contributions to osk RNA function: Bru binding sites which act, at least in part, to sequester Bru; and 3' sequences that do not bind Bru and must have a separate role. To ask if the karyosome defects can be assigned to Bru sequestration or to the separate action of the 3' sequences, we did two types of experiments. In one, the effect of reducing *aret* activity was monitored. Just as for progression through oogenesis (Figure 3.1E), the karyosome phenotype was suppressed, although to a much larger degree (Figure 3.5A). For the second type of experiment we tested the *osk* mutants that have defects in progression through oogenesis for karyosome morphology (Figure 3.5B). Mutation of either Bru binding sites or the essential 3' sequences interfered with karyosome formation, just as for progression through oogenesis. However, the severity of the two phenotypes was not perfectly correlated. In particular, mutant *osk3'990-994* retained a low level of egg laying, yet had a karyosome defect similar to that of mutants *osk3'977-981* and *osk3'984-988*, both of which laid no eggs and were strongly arrested in oogenesis. In addition, even the most seriously affected scanning mutants produced a slightly higher fraction of normal karyosomes than in the absence of *osk* mRNA. This suggests that none of the mutants tested is completely defective in *osk* RNA function, which is

not surprising given that multiple elements contribute to this function and each mutant retains a subset of the elements.

Absence of *oskar* mRNA alters the distribution of germline-specific or -enriched proteins

Bru protein normally appears in the germline cells of the ovary, where it is enriched in the oocyte at early stages (Webster et al., 1997). Immunodetection of Bru in *osk* RNA null females by confocal microscopy revealed two changes in distribution. First, the normal strong enrichment of Bru in cytoplasmic particles, both perinuclear nuage and sponge bodies (Snee and Macdonald, 2009), was diminished. The particles were readily visible in Figure 3.6A, and prominent at higher magnification (Figure 3.6B), but substantially less pronounced in *osk* RNA null ovaries (Figure 3.6 C,D). Second, the very low level of signal normally detected in the somatic follicle cells, previously assumed to be background, was enhanced. As a quantitative measure of this change, fluorescence intensity levels were determined along lines drawn across the follicle cell/nurse cell boundary (Figure 3.6E), revealing a consistent increase in follicle cell signal when *osk* mRNA is missing (Figure 3.6F). We also measured average fluorescence intensity in multiple areas of follicle cells (Figure 3.6F), an approach that minimizes variation (see Materials and Methods). The difference in follicle cell signal intensity for wild type and *osk* RNA null egg chambers was substantial.

Detection of Bru in follicle cells was unexpected, as expression of the protein in the ovary was thought to be restricted to the germline (Webster et al., 1997). This surprising discovery raised a number of questions. One key issue was whether this phenomenon was caused by absence of *osk* mRNA. Notably,

this defect was fully rescued by addition of an *osk* transgene retaining full *osk* RNA function (Figure 3.6G). Furthermore, two different *osk* RNA null genotypes, osk^{A87}*/Df(3R)osk* and osk^{0}/osk^{0}, showed the same changes in Bru distribution (below). Therefore, this defect is indeed due to loss of *osk* mRNA, and cannot be attributed to other mutations present in the *osk* RNA null flies. A second question was whether the protein detected in the follicle cells is really Bru. Ideally, a mutant lacking Bru protein would be used to confirm the specificity of the immunodetection. However, *aret* null mutants arrest oogenesis too early to perform the experiment (Schüpbach and Wieschaus, 1989). As an alternative, the ovaries were stained with different anti-Bru antibodies. The same expanded distribution of Bru was detected (Figure 3.7).

One interpretation of these results is that in the absence of *osk* mRNA Bru escapes from the germline cells, and in some manner enters the adjacent follicle cells. To determine if the change in distribution was exclusive to Bru, or more general, an additional protein was tested. Within the ovary Orb is thought to be expressed exclusively in the germline (Lantz et al. 1994). Immunodetection of Orb in wild type and *osk* RNA null ovaries revealed no striking difference in pattern, with the signal seen only in the germline cells (Figure 3.6H and I). However, quantifying signal intensity revealed a small but statistically significant enhancement in the follicle cells of *osk* RNA null egg chambers (Figure 3.6J). This difference between wild type and *osk* RNA null ovaries could be visualized by enhancing the signal intensity, revealing a halo of Orb staining in the follicle cells. The halo was clearly visible and restricted to the follicle cell region in the *osk* RNA null ovaries (Figure 3.6I'), but weak or not visible in wild type (Figure 3.6H'). The Orb protein is present in sponge bodies and enriched in nuage (Snee

and Macdonald, 2009), although to a lesser extent than Bru. As for Bru, the particulate distribution of Orb was reduced in the *osk* RNA null ovaries (Figure 3.6H and I).

To determine if the sequences in *osk* mRNA that mediate other aspects of *osk* RNA function are also required to ensure the normal distribution of Bru protein, mutant *osk* transgenes were tested for this phenotype (Figure 3.8B). Mutants of the *osk* 3' UTR C region which are deficient in *osk* RNA function all displayed elevated Bru signal in follicle cells (Figure 3.8D-G). The *osk ABC BRE-* mutant, which retains an intermediate level of *osk* RNA function as measured by progression through oogenesis, showed an intermediate phenotype in Bru redistribution: the level of Bru in follicle cells was elevated as compared to wild type, but less so than in the absence of any *osk* mRNA.

Discussion

A striking property of the *osk* mRNA is its dual function. In the guise of an mRNA it encodes the Osk protein, which is essential for axial patterning of the oocyte and embryo. Acting as a lncRNA, the *osk* mRNA is required for progression through oogenesis. Here we have characterized the noncoding role of *osk* mRNA. This has revealed a surprising feature of what goes wrong when *osk* mRNA is not present, and shows that *osk* mRNA performs multiple noncoding functions using different sequence elements.

The initial characterization of mutants lacking *osk* mRNA revealed three defects: at stage 2 of oogenesis the karyosome fails to form, appearing in fragments; Stau protein fails to become enriched in the oocyte, consistent with its

transport there in a complex with *osk* mRNA; and oogenesis is arrested at stage 7, after which the egg chambers degenerate (Jenny et al, 2006). We have found two additional defects. One is the infrequent appearance of egg chambers with too many nurse cells, suggesting an extra round of cell division (data not shown). More notably, we discovered that two germ line-specific proteins, Bru and Orb, appear in the somatic follicle cells when the flies lack *osk* mRNA. These proteins also change their distribution within germline cells, as both show a reduced enrichment in nuage, a germline RNP.

The mechanism by which these proteins accumulate in the follicle cells is not clear, and neither is a secreted protein that could be delivered by endocytosis. Ring canals allow for movement of macromolecules between nurse cells and from nurse cells to the oocyte (Robinson and Cooley, 1996), as well as between subsets of follicle cells (McLean and Cooley, 2013). By contrast, portals for exchange of components between germline and somatic cells have been thought to be limited to gap junctions, which allow only for transit of small molecules (Bohrmann and Haas-Assenbaum, 1993; Zhu et al, 2007). A recent report of germline-derived proteins appearing in the follicle cell epithelium independent of endocytosis raises the possibility of alternate pathways of protein exchange, although in this example the germline proteins do not appear to actually enter into individual follicle cells (Furriols and Casanova, 2014). One explanation of the appearance of the germline proteins in the follicle cells of *osk* RNA null egg chambers invokes enhanced transcription in the follicle cells, not protein movement. Attempts to monitor levels of the mRNAs in follicle cells by *in situ* hybridization have been inconclusive. This approach produces significant background staining, making it difficult to detect what would very likely be an

extremely low level of transcription (based on the observed levels of proteins in the follicle cells).

Independent of how germline proteins appear in the somatic follicle cells in the absence of *osk* mRNA, and why these proteins are less enriched in germline RNPs, these changes in protein distribution could underlie the other *osk* RNA null phenotypes. The protein distribution defect is present from the earliest stages of oogenesis, and is completely penetrant. The inappropriate presence of post-transcriptional regulators could certainly alter the follicle cell proteome, perhaps causing the arrest of oogenesis. Likewise, changes in the germline RNPs could affect the proper regulation of germline transcripts, and thereby cause some or all of the *osk* RNA null phenotypes.

Further insights into the noncoding function of the *osk* mRNA come from identification of required sequence elements. Mutation of the BREs, the primary Bru binding sites in *osk* mRNA, causes a partial disruption of *osk* RNA function. Although the Bru binding sites mediate translational repression of *osk* mRNA (Kim-Ha et al, 1995), loss of repression from mutation of these sites cannot account for the arrest of oogenesis due to absence of *osk* mRNA, because unregulated translation of an *osk* mRNA lacking the 3' UTR does not interfere with progression through oogenesis (Vanzo and Ephrussi, 2002). Instead, this role of Bru binding sites in *osk* RNA function can be explained by either of two likely models: sequestration of a factor - Bru - that would otherwise disrupt oogenesis; and formation of a critical RNP required for progression through oogenesis. The models make very different predictions for the consequences of reducing the level of Bru, and our data argue very strongly for the sequestration model. Although there are examples of RNAs functioning as decoys to bind and

inhibit proteins (reviewed by (Cech and Steitz, 2014)), the *osk*/Bru interplay is unusual in that the known activity of Bru - to control *osk* translation - is not inhibited. Instead, association of Bru with *osk* mRNA is expected to constrain the binding of Bru to lower affinity targets. In the absence of *osk* mRNA, promiscuous binding by Bru may occur, presumably leading to misregulation of those mRNAs.

Sequestration of Bru is not the only noncoding function of *osk* mRNA. Experiments to address what portion of the *osk* 3' UTR can provide at least partial RNA function identified the extreme 3' end, along with a signal for transport to the oocyte. The 3' region does contain Bru binding sites, but other included elements play an even more important role: while mutation of the main Bru binding sites substantially reduces egg laying, certain mutations near the 3' end can have more drastic effects, with the most severe abolishing egg laying and closely resembling a complete loss of *osk* mRNA. The tight proximity of these inhibitory mutations - all are contained within a 30 nt region - might suggest that a single element is disrupted, but all indications argue for two or more different functional elements.

One type of functional element in the 3' region has already been reported. (Vazquez-Pianzola et al, 2011) showed that a cluster of A-rich sequences (ARS) at the extreme 3' end of *osk* mRNA are bound by Poly(A) binding protein (PABP). In a rescue assay similar to that used here for one set of experiments (Figure 3.3), UAS/GAL4 expression of the wild type *osk* 3' UTR restored some egg laying to *osk* RNA null flies, but *ΔAR* mutants lacking parts of the ARS region did not. Although this was taken as evidence for a role for the ARS elements and PABP in osk RNA function, there are two issues which complicate that interpretation. First, the *osk 3' UTR ΔAR* RNAs are present at reduced levels relative to the wild

type. Because even the wild type *osk* 3' UTR does not fully rescue egg laying to the wild type level in the UAS/GAL4 assay, a reduced level of the RNA might disrupt activity. Second, the deletions in the *ΔAR* mutants impinge on the other region we have found to be critical for *osk* RNA function (below), with a 1 nt overlap between the *ΔAR* deletions and the positions mutated in the very strongly defective *osk3'984-988* mutant. Thus, an effect on the element defined by *osk3'984-988* could disrupt egg laying in this assay. Despite these issues, two lines of evidence do support a role for the ARS elements and PABP in *osk* RNA function. First, the partial loss of *osk* RNA activity by our mutant *osk3'1004-1008* would be consistent with a role for the ARS elements in *osk* RNA function. This mutant disrupts part of one ARS: 5 of a run of 10 A residues are altered. Because this mutant has both egg laying and karyosome defects in the robust *osk* genomic transgene assay (in which a wild type transgene provides complete rescue and fully restores the normal rate of egg laying), there is clearly a disruption of *osk* RNA function. Whether mutating more of the ARS elements would completely abolish *osk* RNA function remains uncertain. Second, reducing levels of both *osk* mRNA and PABP activity substantially enhances karyosome defects similar to those due to absence of *osk* mRNA (Figure 3.9).

The scanning mutants with the strongest defects in *osk* RNA function are *osk3'977-981* and *osk3'984-988*, which are positioned adjacent to one another and do not alter A-rich sequences. Because each of these mutants almost completely lacks *osk* RNA function, they must define a critical element (or elements). Like the Bru binding sites, this element could act in sequestration of one or more factors. Alternatively, it could play a scaffolding function. We have assayed for proteins that bind this region, but have not detected any whose

binding to the scanning mutants correlates with their effects on *osk* RNA function (unpublished).

The other mutant with such severe defects is *osk C all-*, in which all of the C region Bru binding sites are mutated. None of these mutations have a similar effect when tested individually. The most obvious explanations for the severity of this mutant invoke redundant binding sites or a critical RNA structure. While the C all- mutations do affect Bru binding sites, consistent with redundancy, the strong phenotype cannot be attributed to loss of Bru sequestration, since mutation of the higher affinity AB region Bru binding sites does not have an equivalent effect. However, Bru bound to sites in the C region might facilitate the binding or action of another factor or factors brought into close proximity by binding to the nearby essential *osk* RNA function element. The combined effects of the multiple mutations of the *osk C all-* mutant could also alter the structure of the RNA, even if no individual mutation alone has this effect. Folding predictions do differ for the wild type and mutants, but none are predicted to form highly stable structures in this region of the 3' UTR.

In conclusion, we have discovered a striking consequence of loss of *osk* mRNA - the altered distribution of germline regulatory factors, including enrichment in somatic follicle cells - and we have identified sequence elements required for *osk* RNA function. Although different sequence elements have very different functions, one set acting to sequester Bru and others acting in different but as yet incompletely defined roles, mutation of either type is sufficient to bring about the changes in protein distribution. Although misregulation of transcripts in both somatic and germline cells resulting from theses changes in protein distribution could account for the other *osk* RNA null phenotypes, it remains a

puzzle how the different types of *osk* RNA functional elements elicit the same effect, and whether a common underlying mechanism is responsible.

MATERIALS AND METHODS

Flies and Transgenes

aret^{Z2286} was from Mary Lilly. The *osk*^{0} allele (RNA null) was generated by homologous recombination (Gong and Golic, 2003), and has sequences R3:8935117-8938212 (r6.01) deleted. Transgenes of genomic *osk* DNA with mutated Bru binding sites are from previous studies (Kim-Ha et al., 1995; Reveal et al., 2010). Scanning mutations in the *osk* C region were introduced by PCR, and incorporated into genomic *osk* transgenes essentially the same as for the Bru binding site mutations. Transgenes *UAS-GFP* and *UAS-GFP-4xBRE* (4 copies of the BRE sequence TGTTTTATATGT) have been described (Reveal et al., 2011). For these transgenes the GFP is mGFP6 (Haseloff, 1999). Some of the *UAS-egfp* transgenes with *osk* 3' UTR sequences (Figure 3.3) are from (Jambor et al., 2014). For the new transgenes, the *osk* 3'UTR segments used are provided in the Supplemental Materials. Transgene *K10-TLS 119 of 3b* differs from the others in Figure 3.3 in that it lacks *egfp*.

Egg laying assays

Newly eclosed flies were collected, aged for 2-3 days (when the first eggs appeared) and placed in cages with yeasted apple juice plates. Collections were performed over 60 hours, and the total number of eggs scored. All assays were performed at least twice. The females were typically *osk*^{A87}*/Df(3R)osk* with one

copy of an osk transgene, unless otherwise noted. A wild type osk transgene was used as a standard, and egg laying rates are all relative to that standard. Other genetic elements (*UAS-GFP* transgenes, the *nosGAL4::VP16* driver, and $aret^{Z2286}$) were included as indicated in the figures. For the results in Figure 3.3, a simplified assay was used, scoring for the presence of any laid eggs.

RNA detection

To measure RNA levels, ovaries were dissected from 3-4 day old females, RNA purified using Trizol according to the manufacturers instructions, probed for *osk* and *rp49* mRNAs by RNase protection assay (Ambion RPAIII), and quantified by phosphorimaging. Assays were performed 3 or more times. The *rp49* signal was used to normalize for the amount of RNA in each preparation.

In situ hybridization to detect *osk* or *egfp* mRNA in ovary whole mount preparations was performed as previously described (Jambor et al., 2014). For the *K10-TLS 119 of 3b* transcripts, the probe was from transcribed UASp vector sequences. Ovaries were imaged with a confocal microscope (DMR-E, Leica; TCS SP2 AOBS scan head; Leica) or a wide-field microscope (Axioplan imaging2, Zeiss) equipped with an optical sectioning device (DSD1, Andor) in Figure 3.3 and 4 respectively.

Measurement of egg chamber length

Newly eclosed flies were collected and incubated with fresh yeast at 25ºC until eggs were laid or for a maximum of 4 days. The ovaries were then dissected and fixed. To determine egg-chamber length, we measured the length of the

major axis of the oldest egg-chamber (including the follicle cell layer) per ovariole using Fiji (Schindelin et al., 2012). Egg-chambers with visibly degraded nurse cell nuclei were excluded from the analysis.

RNA binding

To monitor Bru binding to *osk* 3' UTR segments by UV crosslinking, the RNA probes, ovary extract preparation, and assays were all as described previously (Kim-Ha et al., 1995). For the affinity capture assays, a segment of the *osk* gene 3' UTR (the final 150 nt of the 3' UTR) in wild type or mutant forms was fused to DNA encoding the S1 aptamer which binds streptavidin (Walker et al., 2008). Transcripts were mixed with ovary extract and streptavidin beads. After washing, the beads were recovered, associated proteins separated by SDS-PAGE and Bru detected by western blotting (unpublished).

Whole mount immunodetection

Immunostaining of ovaries was largely as described previously (Kim-Ha et al., 1995), except that secondary antibodies were labeled with Alexafluor 488 (Invitrogen). In addition, for analysis of ovaries with arrested oogenesis the ovarioles were teased apart with a tungsten ultra micro needle (Ted Pella, Inc). Primary antibodies were used at the following dilutions: mouse anti-Lamin Dm0 (ADL84.12), 1:100; mouse anti-Orb (4H8), 1:1; mouse anti-Hts (1B1), 1:1 ; rabbit anti-CG9925, 1:2000; rabbit anti-Bru 4005 S2789-1 and -2, 1:1000. Samples were mounted on slides with Vectashield Mounting Medium (Vector Labs) and imaged with a Leica TCS-SP laser scanning confocal microscope.

Quantitation of levels made use of samples fixed and processed in parallel from flies grown in parallel and of the same age. Signal intensities along lines crossing the nurse cell/follicle cell boundary and avoiding nuclei in both nurse cells and follicle cells (Bru and the other proteins tested are predominantly cytoplasmic) were measured in Fiji. Because of the granularity in the signal, there is substantial variation along in signal intensity along the lines. Measurements of mean intensity in follicle cells were made in Fiji, on regions from the central portion of the follicle cell layer. Each region tested covered multiple follicle cells to minimize variation due to different proportions of nuclei and cytoplasm. To visualize low signals in the follicle layer (Figure 3.6 panels H'-L'), pairs of images (H and I, K and L) were adjusted identically with the levels function of Adobe Photoshop.

Acknowledgments

We thank Mary Lilly for flies and Goheun Kim and Kristin Patterson for advice on homologous recombination. The ADL84.12, 4H8 and 1B1 monoclonal antibodies were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242. This work was supported by grants GM054409 and GM096730 from the National Institutes of Health. RG and HJ were supported by fellowships from the European Molecular Biology Laboratory (EMBL) Internal PhD Programme.

FIGURES

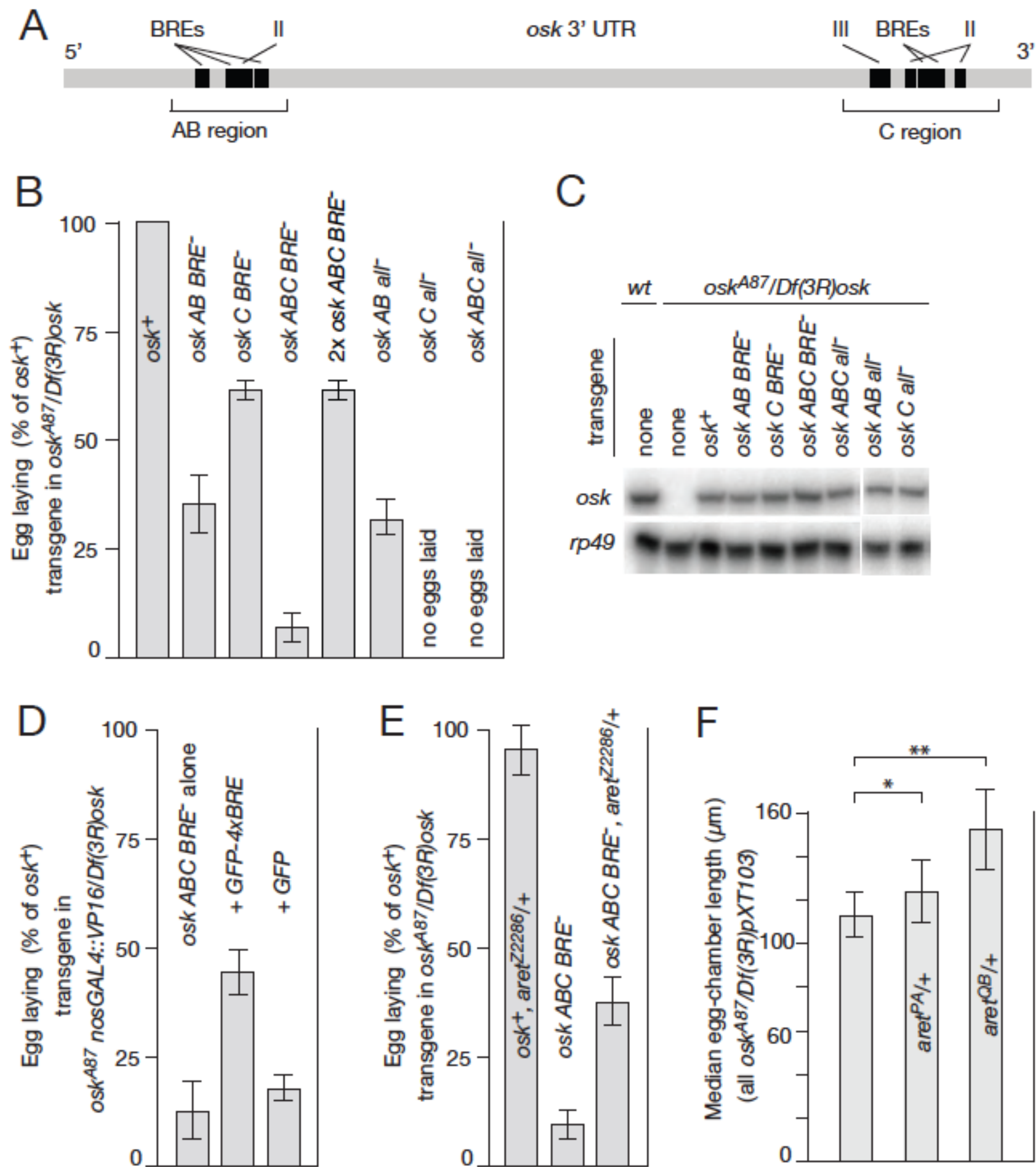


Figure 3.1 - Full caption next page

Figure 3.1. BREs are required for the RNA function of *osk*

A. Schematic of the *osk* 3' UTR indicating the locations of Bru binding sites (BREs, type II and type III). The sites are clustered in the AB and C regions.

B. Rescue of the *osk* RNA null egg laying defect by *osk* transgenes. Rates of egg laying (see Materials and Methods) for females lacking endogenous *osk* mRNA [osk^{A87}/*Df(3R)osk*] but carrying a single copy of an *osk* transgene, as indicated. The rate obtained with a single copy of the *osk+* transgene was set at 100%.

C. Transcript levels for *osk* transgenes. The *rp49* mRNA was used as a control to ensure that similar amounts of ovarian RNA were used for each genotype.

D. Addition of Bru binding sites rescues the egg laying defect of *osk ABC BRE-*. In all cases females lack endogenous *osk* mRNA and carry the *nosGAL4::VP16* driver and a single copy of the *osk ABC BRE-* transgene, which provides only partial rescue of egg laying. A *UAS-GFP* transgene or *UAS-GFP-4xBRE* transgene was also present, as indicated.

E. Reducing Bru activity rescues the egg laying defect of *osk ABC BRE-*. Rates of egg laying for females lacking endogenous *osk* mRNA [osk^{A87}/*Df(3R)osk*] but carrying a *osk+* or *osk ABC BRE-* transgene were determined, testing the consequences of reducing Bru activity by heterozygosity for $aret^{z2286}$.

F. Reducing Bru activity partially suppresses the oogenesis progression defect of *osk* RNA null females. Median egg chamber lengths were measured for the genotypes shown (n for osk^{A87}/*Df(3R)pXT103*, 16; with $aret^{PA}$/+, 37; with $aret^{QB}$/+, 36. P values derived from the Kolmogorov-Smirnov Test: *, <0.05; **, <0.01.

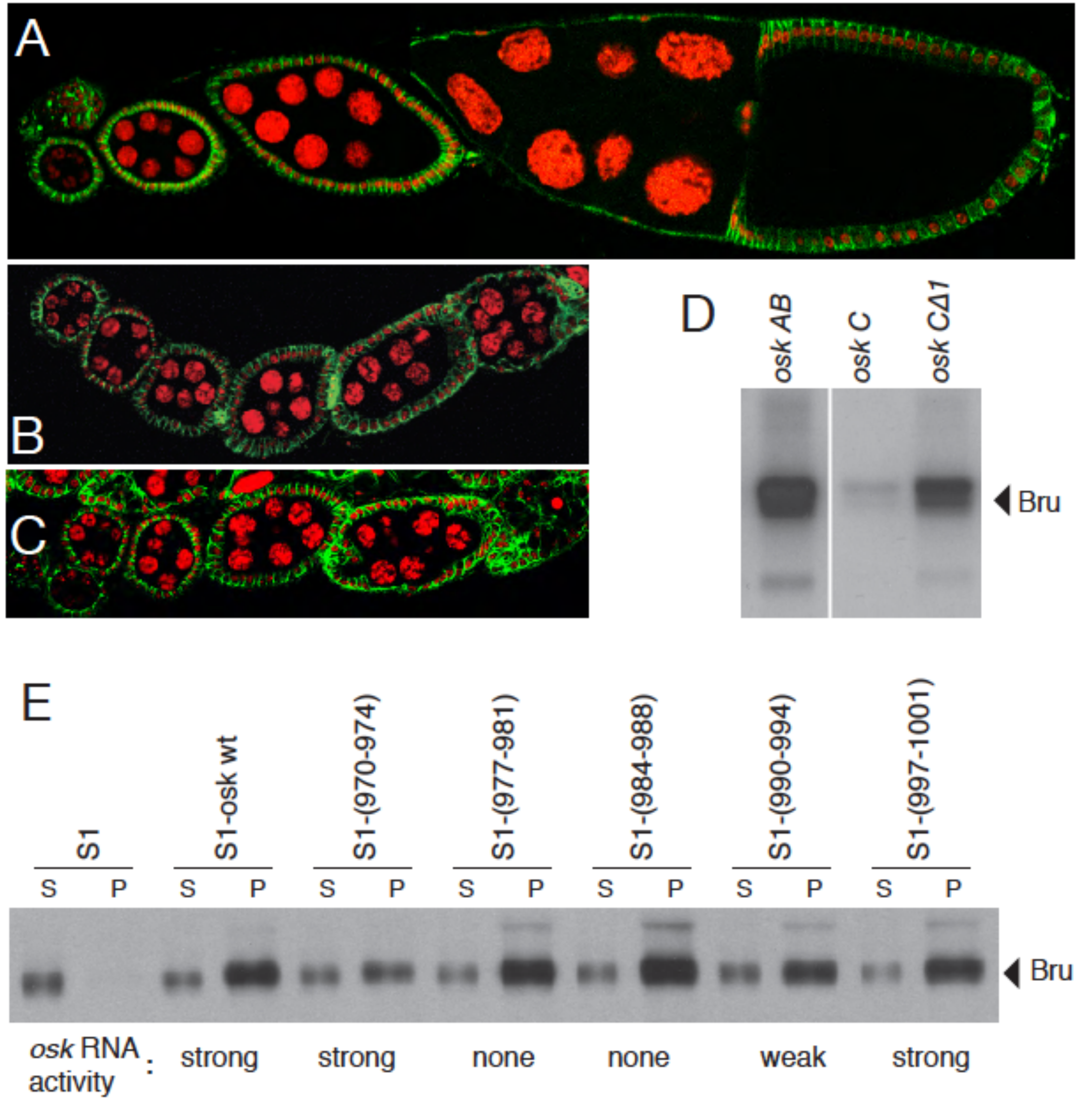


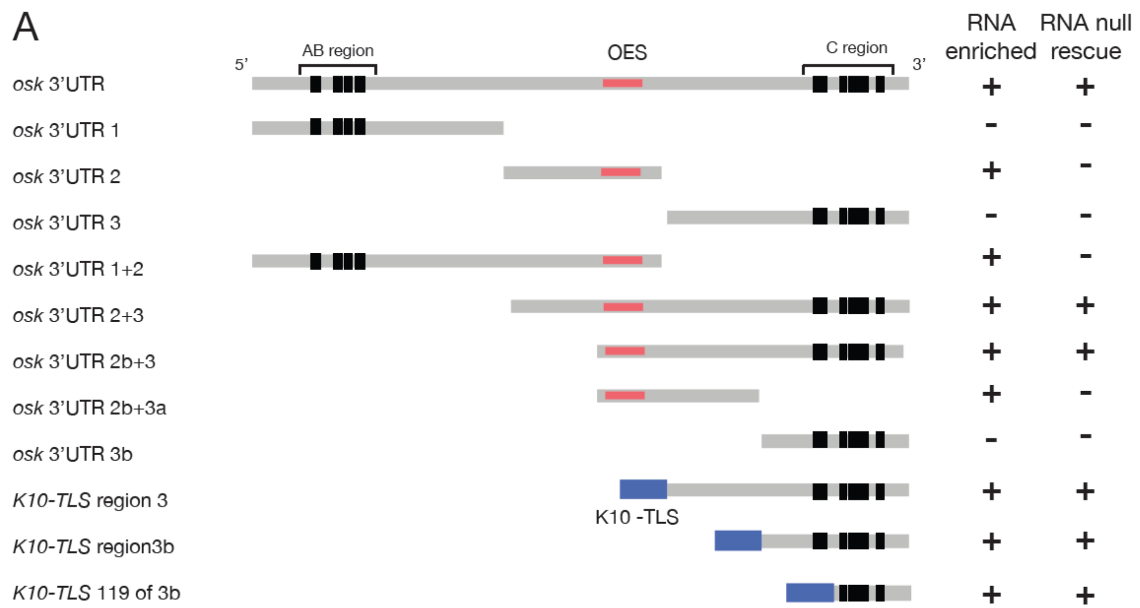
Figure 3.2 - Full caption next page

Figure 3.2. Sequences in the *osk* 3' UTR C region are essential for *osk* RNA function, independent of Bru binding

A-C. Ovarioles from wild type (A), osk^{A87}*/Df(3R)osk* (B), or osk^{A87}*/Df(3R)osk* expressing the *osk C all-* transgene (C). The ovarioles were stained with ToPro for DNA (red) and anti-Hts for Adducin-like (green).

D. UV crosslinking assay of Bru in ovarian extract binding to *osk* RNA probes. The AB and C regions contain the Bru binding sites, and are as previously defined (Kim-Ha et al., 1995). Deletion of part of the C region enhances Bru binding, perhaps by altering secondary structure that would otherwise limit accessibility. Even with the enhanced binding, the C region binds substantially less Bru than does the AB region. Similar amounts of each probe were used. All lanes are from the same autoradiogram of a single experiment and gel, with irrelevant lanes removed.

E. Affinity capture assay of Bru binding to *osk* C region RNAs, either wild type or with scanning mutations. The *osk* RNAs are fused to the S1 aptamer (the first two lanes are the aptamer alone). After incubating with ovary extract, the RNAs and bound proteins were recovered by affinity purification to generate supernatent (S; unbound) and pellet (P; bound) fractions, and the presence of Bru was determined by western blotting. How well each version of osk mRNA supports the *osk* RNA function is indicated at bottom.



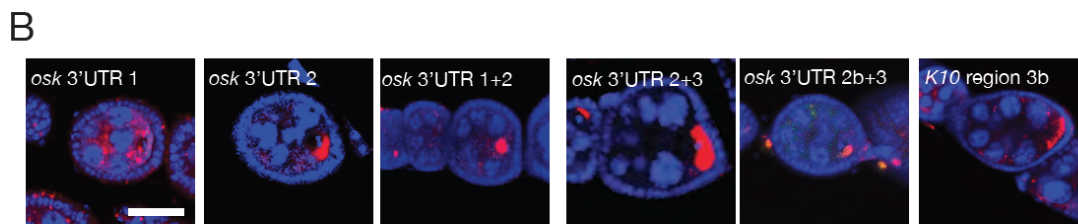
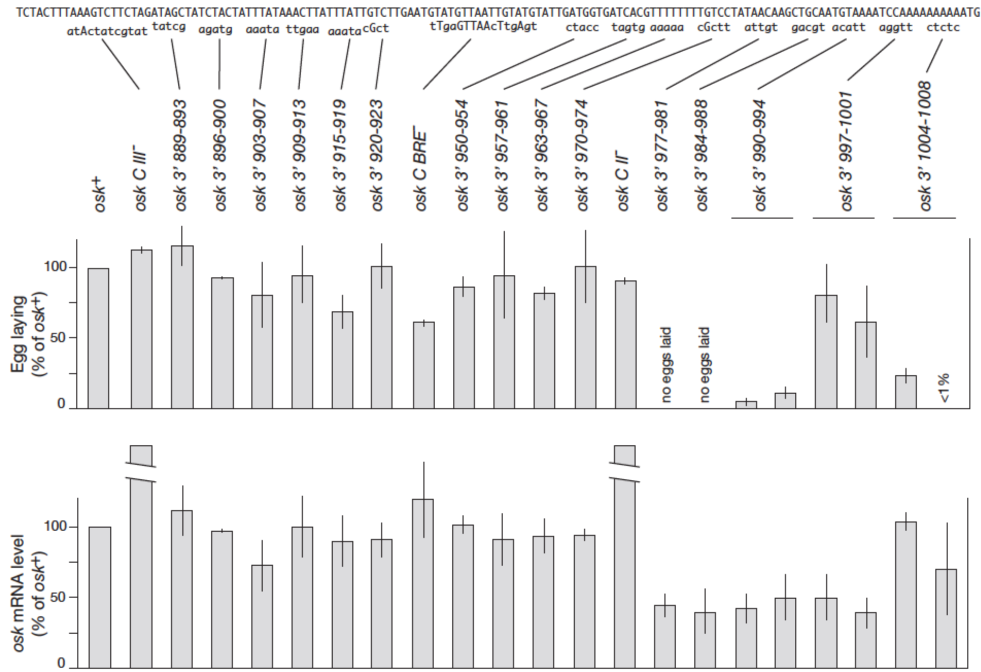


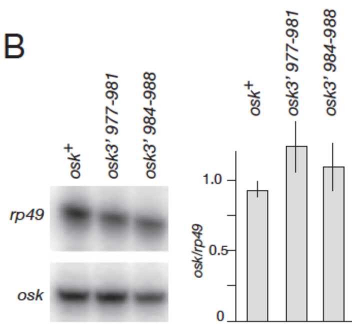
Figure 3.3 - Full caption next page

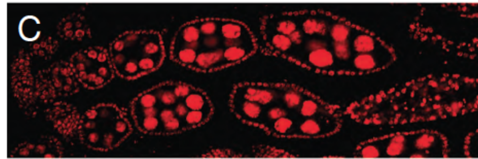
Figure 3.3. Mapping regions of the *osk* 3' UTR that contribute to *osk* RNA function

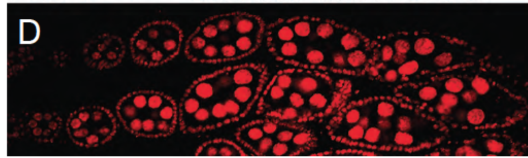
A. The *osk* 3' UTR is shown in schematic form, and the fragments tested in UAS transgenes and containing the ORF of GFP are shown as horizontal bars. The positions of the Bru binding sites and the OES (the signal that mediates transport of the mRNA to the oocyte) are indicated. The K10-TLS, the oocyte entry signal of *fs(1)K10* is indicated as a blue bar. Each transgene was tested in the osk^{A87}*/Df(3R)pXT103* background with the *pCog-Gal4:VP16* (Rørth et al., 1998) and *nanos-Gal4:VP16* (Van Doren et al., 1998) drivers. Results of the assays are indicated at right. RNA enriched: +, strong enrichment of the RNA in the oocyte; -, no enrichment in the oocyte. RNA null rescue: +, eggs laid; -, no eggs laid. The presence of eggs was scored, not the frequency of egg laying.

B. Representative in situ hybridizations against the GFP portion of the transgenic construct. Transgenic RNA signal in red and DNA in blue (scale bar, 30 μm).









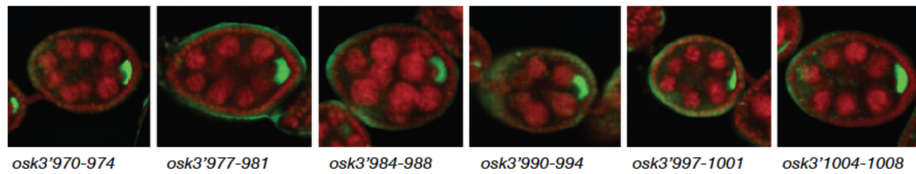


Figure 3.4 - Full caption next page

Figure 3.4. Fine scale mapping of *osk* RNA function elements

A. Mutations in the *osk* 3' region. The sequence of the region is shown, with black bars above indicating minimal fragments tested in Figure 3.3 (the final 18 nt of the *osk* 3' UTR are not shown, but are present in the minimal fragments along with a further 8 nt of genomic DNA). Beneath the sequence are shown the mutations (lower case) introduced into genomic *osk* transgenes. The *osk CII-* mutant has the mutations of both *osk 3'920-923* and *osk 3'970-974*. Single copies of each transgene were tested in the osk^{A87}*/Df(3R)osk* background for rate of egg laying and mRNA level. At least two independent transgenic lines were tested for mutants with substantial defects. The additional lines of mutants *osk3' 977-981* and *osk3' 984-988* also lacked detectable activity (data not shown). RNA levels were determined as in Figure 3.1, using *rp49* as an internal control.

B. Increasing transgene dosage to raise *osk* mRNA levels for selected mutants. The RNase protection assays are shown at left with transgenic *osk* mRNAs indicated (all were in the osk^{A87}*/Df(3R)osk* background), and the quantitation by phosphorimaging at right (samples in the same order).

C-D. Ovarioles stained with TOPRO-3 to detect nuclei. Both are osk^{A87}*/Df(3R)osk*, with D expressing two copies of the *osk3' 977-981* transgene (same genotype as in panel B).

E. Distribution of mutant *osk* mRNAs. All egg chambers are from osk^{A87}*/Df(3R)osk* females expressing a single copy of the transgene indicated. *osk* mRNA (green) was detected by in situ hybridization, with DNA (red) labeled with DAPI.

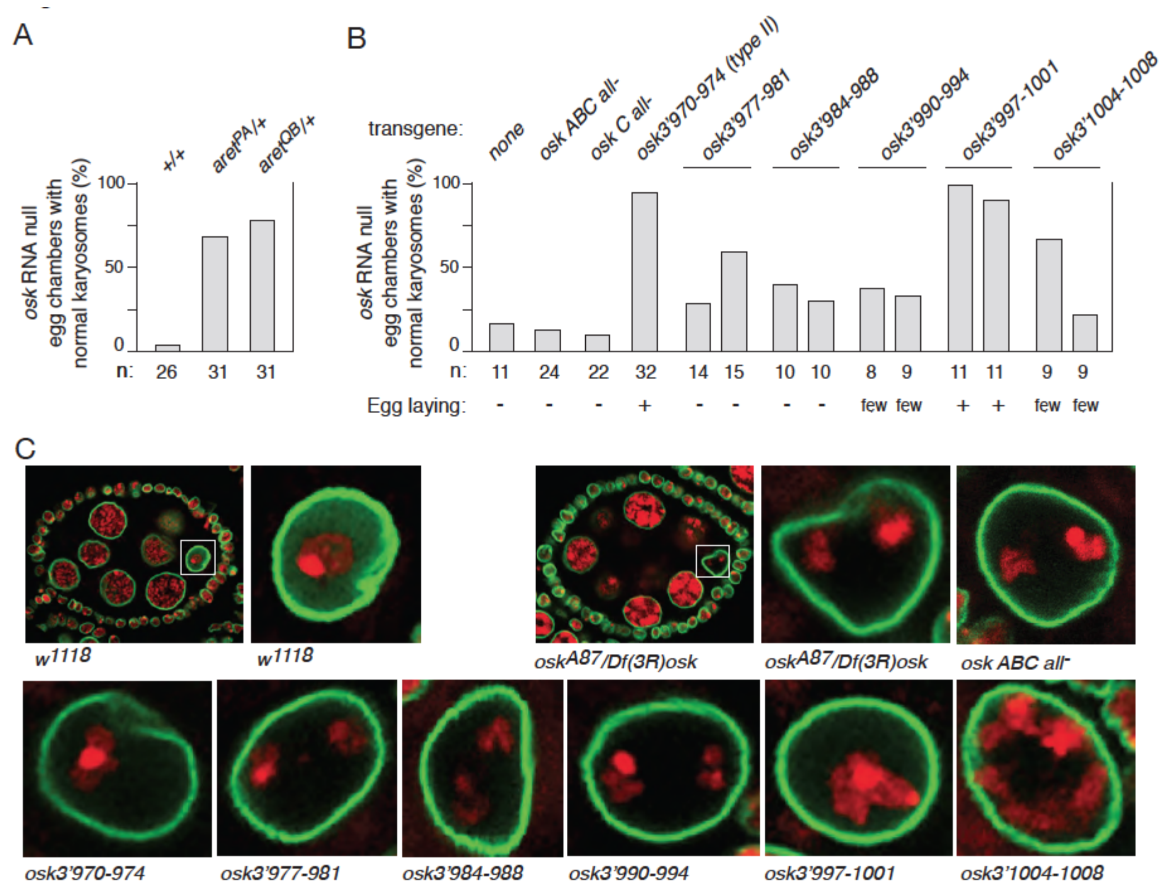


Figure 3.5 - Full caption next page

Figure 3.5. Karyosome defects of *osk* RNA null mutants

A. Suppression of karysome defects by reducing *aret* activity. Females are all *osk*^{A87}*/Df(3R)pXT103*, with the *aret* alleles indicated at top.

B. Frequency of karyosome defects for *osk* mutants. Egg chambers from *osk*^{A87}*/Df(3R)osk* females expressing a single copy of the transgene indicated were scored for karyosome morphology. Mutants *osk3'889-893*, *osk3'896-900*, *osk3'903-907*, *osk3'909-913*, *osk3'915-919*, *osk3'920-923*, *osk3'950-954*, *osk3'957-961* and *osk3'963-967*, which are not included in the diagram, all had 100% normal karyosomes with n values of 9 or greater. Two independent transgenic lines were tested for the scanning mutants shown. To facilitate comparison, the results of the egg laying tests (Figure 3.4A) are summarized below the graph.

C. Examples of karyosome morphology. Complete egg chambers are shown for examples of wild type (*w*^{1118}) and *osk*^{A87}*/Df(3R)osk* (left and center images in the top row). For the other panels only the oocyte is shown. Samples were labeled with TOPRO-3 for DNA (red) and anti-lamin (green).

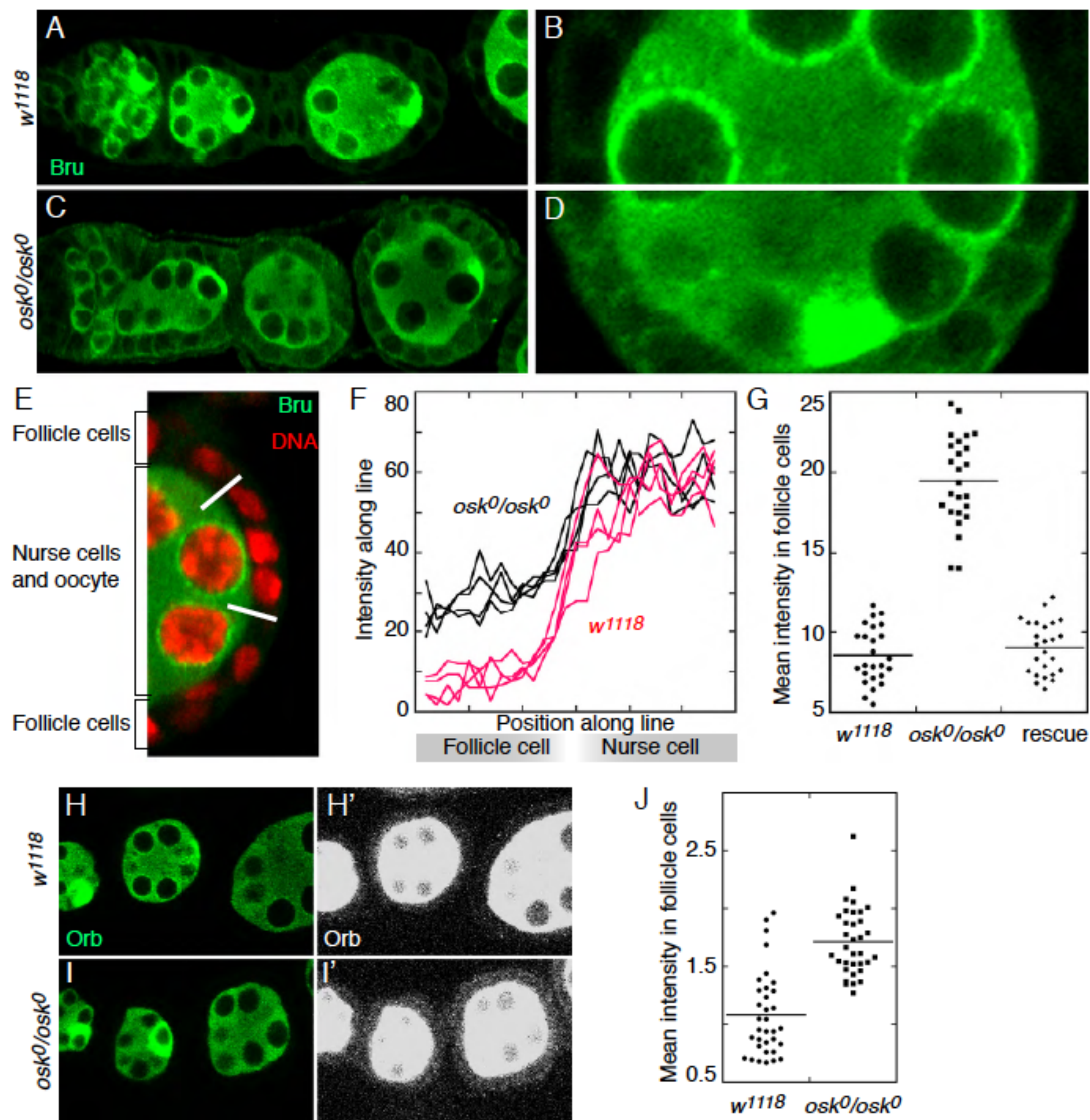


Figure 3.6 - Full caption next page

Figure 3.6. Redistribution of Bru and other germline proteins in *osk* RNA null ovaries

A-D. Immunodetection of Bru in wild type (A,B) and *osk* RNA null (osk^0/osk^0) ovaries (C,D). The higher magnification samples (B,D) show the punctate distribution of Bru, which is reduced in the *osk* RNA null mutant. The level of Bru is much greater in the oocyte than the nurse cells, as can be seen in egg chambers in which the confocal section includes the oocyte (examples in A, C, D).

E. Organization of the egg chamber. Half of an egg chamber is shown, with the different cell types indicated at left. To monitor Bru signal in nurse cells and follicle cells, single lines were drawn, as shown by the white lines, for measurement of signal intensity with Fiji (Materials and Methods).

F. Traces of signal intensity are shown along four lines for both wild type and *osk* RNA null egg chambers. The relative positions of follicle and nurse cells along the lines are indicated below.

G. Quantitation of Bru signal intensity in follicle cells. Each data point represents a region crossing multiple follicle cells (thus including a mixture of nuclei and cytoplasm) and avoiding the inner (adjacent to the germline cells) and outer boundaries of the follicle cell layer. The 'rescue' sample was osk^0/osk^0 with an *osk* transgene providing full rescue of the oogenesis arrest phenotype.

H and I, immunodetection of Orb in wild type and *osk* RNA null ovaries. For H' and I', signal intensities were adjusted identically in Photoshop to enhance the signal.

J. Quantitation of signal intensity in areas of follicle cells, as G. The difference is statistically significant: from an unpaired two-tailed Student's *t* test, $p<0.001$.

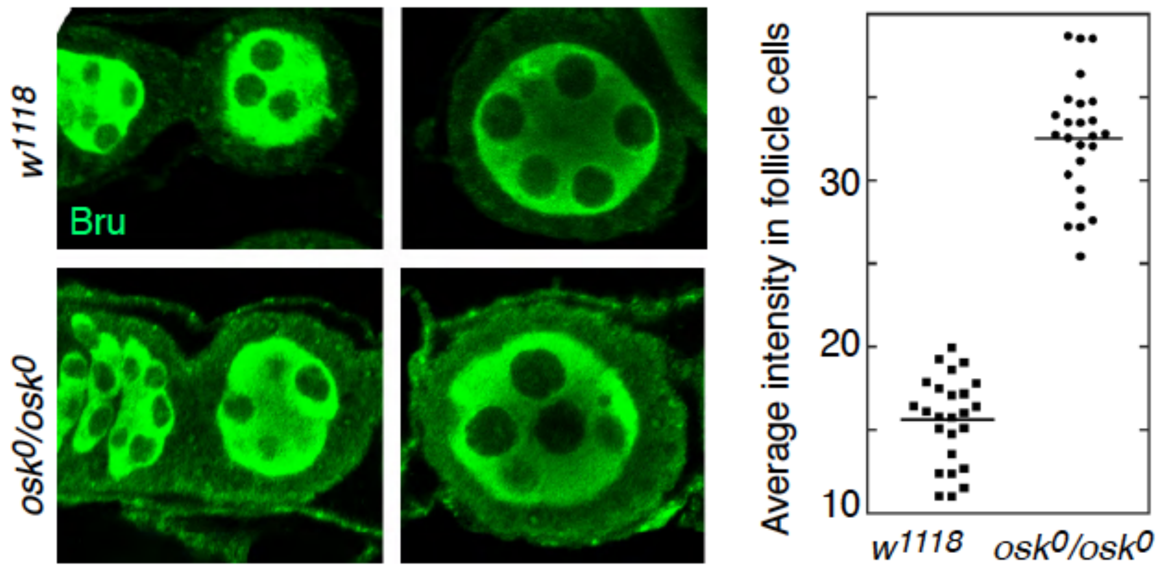
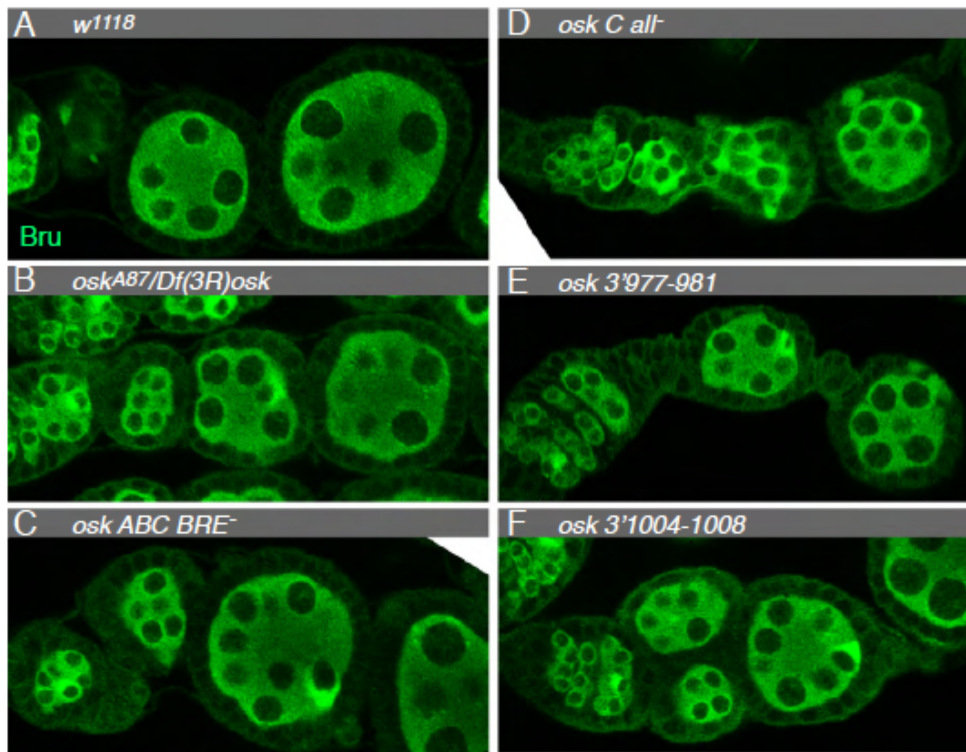


Figure 3.7. Confirmation of Bru redistribution in *osk* RNA null ovaries

Experiments of Figure 3.6 were repeated with different Bru antibodies, with the same enhancement of Bru signal in follicle cells of *osk* RNA null egg chambers.



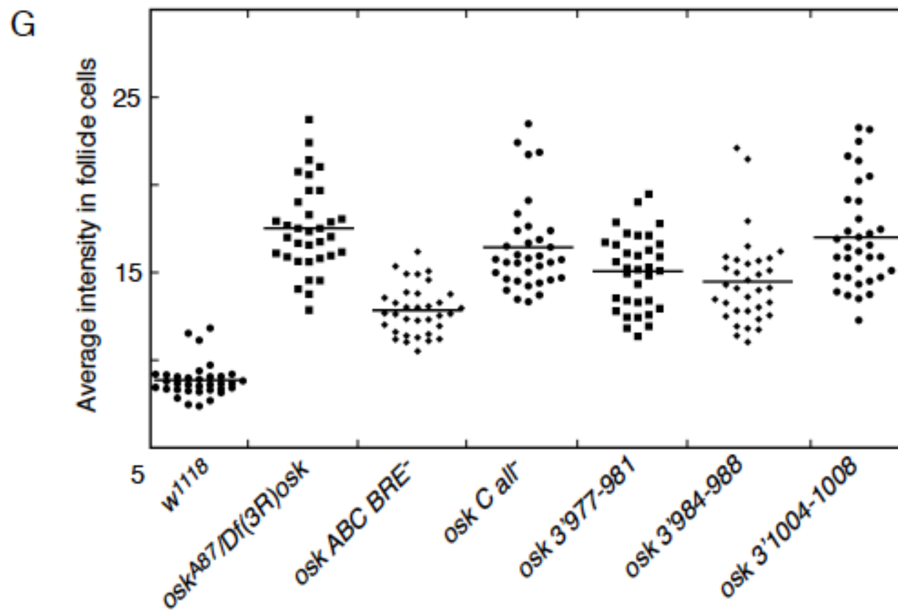


Figure 3.8 - Full caption next page

Figure 3.8. Mutants defective in *osk* RNA function fail to rescue the Bru redistribution phenotype

A-F, immunodetection of Bru. Genotypes are shown at top for A and B. For panels C-F, the *osk* transgenes (indicated at top) are in the osk^{A87}*/Df(3R)osk* background.

G. Quantitation of signal intensity in areas of follicle cells, as in Figure 3.5.

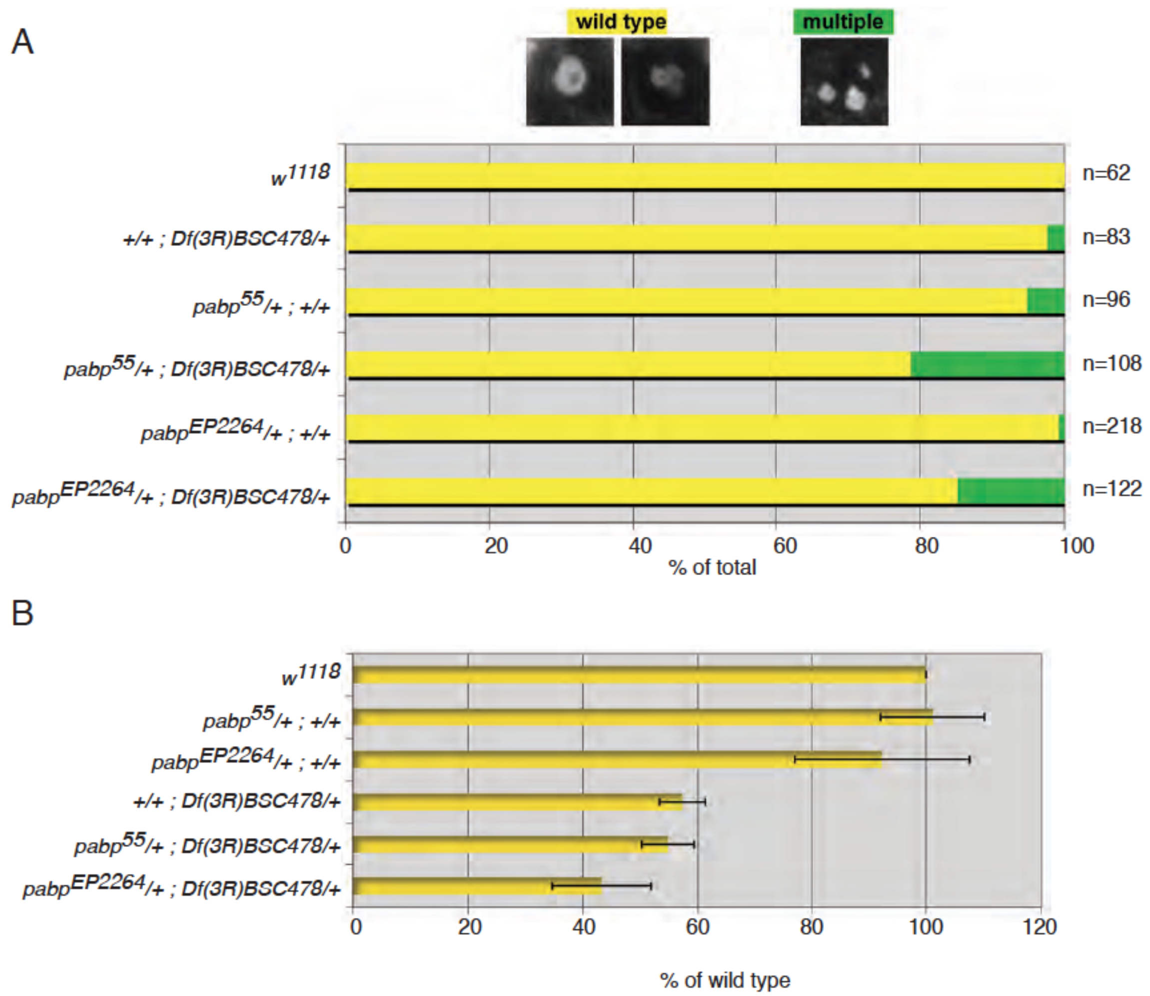


Figure 3.9 - Full caption next page

Figure 3.9. Reducing levels of *osk* mRNA and PABP causes karyosome defects

A. Females with the genotypes indicated were tested for karyosome morphology. *Df(3R)BSC478* is a deficiency chromosome with the *osk* gene deleted.

B. *osk* mRNA levels for genotypes analyzed in A. As expected, deletion of one copy of *osk* (in *Df(3R)BSC478/+*) reduces *osk* mRNA level by about half. When also heterozygous for a *pabp* mutant allele, there is no substantial further reduction in *osk* mRNA. Notably, the strongest karyosome defects are found with the *pabp* allele that has the weaker effect on *osk* mRNA level, arguing that the karyosome defects are not simply due to an inadequate amount of *osk* mRNA.

REFERENCES

Bartel, D. (2009). MicroRNAs: target recognition and regulatory functions. Cell, 136(2), 215-233.

Bohrmann, J., & Hass-Assenbaum, A. (1993). Gap junctions in ovarian follicles of Drosophila melanogaster: inhibition and promotion of dye-coupling between oocyte and follicle cells. Cell Tissue Research, 273(1), 163-173.

Cech, T. and Steitz, J. (2014). The noncoding RNA revolution- trashing old rules to forge new ones. Cell, 157(1), 77-94.

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.

Furriols, M., & Casanove, J. (2014). Germline and somatic vitelline proteins colocalize in aggregates in the follicular epithelium of Drosophila ovaries. Fly, 8.

Ghabrial, A., Ray, P., & Schupbach, T. (1998). okra and spindle-B encode components of the RAD52 DNA repair pathway and affect meiosis and patterning in Drosophila oogenesis. Genes and Development, 12(17), 2711-2723.

Ghabrial, A., & Schupbach, T. (1999). Activation of a meiotic checkpoint regulates translation of Gurken during Drosophila oogenesis. Nature Cell Biology, 1(6), 354-357.

Gong, W., & Golic, K. (2003). Ends-out, or replacement, gene targeting in Drosophila. Proceedings of the National Academy of the Sciences, 100(5), 2556-2561.

Haseloff, J. (1999). GFP variants for multispectral imaging of living cells. Methods Cell biology, 58(1), 139-151.

Jambor, H., Mueller, S., Bullock, S., & Ephrussi, A. (2014). A stem-loop structure directs oskar mRNA to microtubule minus ends. RNA, 20(4), 429-439.

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.

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.

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

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.

McLean, P., & Cooley, L. (2013). Protein equilibration through somatic ring canals in Drosophila. Science, 340(6139), 1445-1447.

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.

Robinson, D., & Cooley, L. (1996). Stable intercellular bridges in development: the cytoskeleton lining the tunnel. Trends in Cellular Biology, 6(12), 474-479.

Rorth, P., Szabo, K., Bailey, A., Laverty, T., Rehm, J., Rubin, G., et al. (1998). Systematic gain-of-function genetics in Drosophila. Development, 125(6), 1049-1057.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., et al. (2012). Fiji: an open-source platform for biological-image analysis. Nature Methods, 9(7), 676-682.

Schüpbach, T., & Wieschaus, E. (1989). Female Sterile Mutations on the Second Chromosome of Drosophila melanogaster. 1. Maternal effect mutations. Genetics, 121(1), 101-117.

Serano, T., & Cohen, R. (1995). A small predicted stem-loop structure mediates oocyte localization of Drosophila K10 mRNA. Development, 121(11), 1995.

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

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

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.

Walker, S., Scott, F., Srisawat, C., & Engelke, D. (2008). RNA affinity tags for the rapid purification and investigation of RNAs and RNA-protein complexes. Methods in Molecular Biology, 488(1), 23-40.

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.

Zhu, X., Stevens, L., & Stein, D. (2007). Synthesis of the sulfate donor PAPS in either the Drosophila germline or somatic follicle cells can support embryonic dorsal-ventral axis formation. Development, 134(8), 1465-1469.

Chapter 4: Future directions

Matt Kanke[1] and Paul M. Macdonald[1]

[1]Department of Molecular Biosciences

Institute for Cellular and Molecular Biology

The University of Texas at Austin

CHAPTER 2: A 5' REGION OF THE *OSKAR* MRNA PLAYS BOTH REGULATORY AND PROTEIN-CODING ROLES IN ENHANCING POSTERIOR ACCUMULATION OF OSKAR PROTEIN

The *osk* 5' element presumably acts to activate translation through the binding of a *trans*-acting factor, as no stable RNA structure is predicted for the element (Lorenz et al., 2011). Gunkel et al. (1998) identified two proteins, Hrp48 and an unknown protein, bound to the 5' portion of *osk*, but the binding regions of these proteins do not correspond well with the mapping of the *osk* 5' element. We attempted to identify factors bound to the 5' element by two separate methods (RNA pulldown and UV crosslinking), but were unsuccessful. Identification of the binding factor is an important part of future research on the *osk* 5' element.

Disruption of the *osk* 5' element reduces translation of an unlocalized *osk* reporter. Enrichment of the GFP signal in the oocyte when *osk::GFP* is expressed, and loss of this enrichment when the *osk* 5' element is disrupted, could suggest that the *osk* 5' element is active only in the oocyte. The loss of the oocyte enrichment could also be due to the loss of anchoring, as mutations that disrupt the 5' element disrupt anchoring, and in the absence of anchoring, Osk::GFP is free to diffuse throughout the egg chamber. The construction of an *osk::GFP* transgene that disrupts the 5' element, yet retains the anchoring function could differentiate between these two possibilities. One approach would be to introduce silent mutations that alter the nucleotide sequence of the 5' element without changing the overlaying amino acid sequence, which could disrupt the 5' element function without affecting anchoring. If silent mutations are unable to alter the 5' element sequence enough to disrupt its function, larger mutations that do disrupt its function, yet do not disrupt anchoring would need to

be tested, but this would require the Osk amino acids necessary for anchoring to be identified. These amino acids could be determined by general mutational analysis of all amino acids coded by the 5' element, but a potential palmitoylation site within the highly conserved sequence of the 5' element is an option for a more directed mutational approach. Palmitoylation can increase the association with membranes, and could be required for the Long Osk anchoring function (Basu, 2004). Osk amino acid 37 is a predicted palmitoylation site, and mutants that lack amino acid 37 are strongly defective in anchoring. An *oskT124C::GFP* transgene would change amino acid 37 to a non-palmitoylatable amino acid, which could disrupt anchoring. If anchoring is retained in the *oskT124C::GFP* mutant, the more general mutational approach would be required.

Two internal open reading frames (iORFs) exist between the long and short *osk* AUGs (called iORF1 and iORF2). The translation of the *oskM1R Δ311-360*, which removes the iORF2 stop codon and shifts the reading frame to that of long and short *osk*, reveals that translation of iORF2 occurs. Although the data from the *oskM1R Δ311-360* mutant shows iORF translation, how well the iORF2 AUG is recognized in the absence of a large deletion isn't known. Furthermore, no data exist addressing translation of iORF1. One way to determine translational rates is by examining the amount and location of ribosomes on specific transcripts, by a method known as ribosomal profiling. As ribosomal profiling data from *Drosophilia* ovaries becomes available, translational rates of the iORFs could be ascertained based on the amount of ribosomes in the ORF region as compared to the amount in the long *osk* reading frame alone. An increase in the amount of ribosomes in the iORF region would indicate that translation of the iORF is occurring. Another method to determine if translation of

the iORF occurs would involve removing the iORF stop codons while shifting the reading frame to that of short *osk*, and could be accomplished by the deletion of two nucleotides of the iORF1 stop codon or one nucleotide from the iORF2 stop codon. The appearance of new Osk species on a western blot would indicate usage of the iORFs.

Translation of upstream ORFs (uORFs) generally results in a decrease in translation from downstream AUGs. The effect of translation of the *osk* iORFs on the translation of short *osk* is not known. Mutation of the *osk* iORF AUGs, either individually or together, coupled with detection of Short Osk by western blot could determine if iORF usage is affecting short *osk* translation. Construction of *osk225A>C* and *osk319T>C* transgenes will disrupt the initiation codon for iORF1 and iORF2, respectively, while retaining the amino acid sequence of Long Osk. Differences in Short Osk protein level, as compared to *osk* alone, will reveal the individual effects of the iORF translation. Construction of the *osk225A>C&319T>C* transgene will address the combinatorial effects of iORFs usage on short *osk* translation.

CHAPTER 3: *osk* RNA PLAYS MULTIPLE NON-PROTEIN CODING ROLES TO SUPPORT OOGENESIS AND MAINTAIN INTEGRITY OF THE GERMLINE/SOMA DISTINCTION

One non-coding RNA function of *osk* mRNA is the sequestration of the translational repressor Bru by sites in the *osk* 3' UTR. In addition to *osk*, Bru binds to *cyclin A*, *sex-lethal, germ cell-less*, and *gurken* mRNAs (Filardo & Ephrussi, 2003; Moore et al., 2009; Sugimura & Lilly, 2006; Wang & Lin, 2007). Isolation of Bru and identification of the bound RNAs would reveal unknown Bru

targets, which could include RNAs involved in oogenesis progression. In the absence of *osk* RNA, Bru could have a greater binding affinity for its other targets or for mRNAs normally not bound by Bru, leading to misregulation of those RNAs. Repeating the isolation and identification of Bru-bound RNAs in the absence of *osk* RNA could reveal shifts in Bru binding affinity for certain targets, or the identity of novel targets bound only under *osk* RNA-null conditions.

The identification of 3' sites that abolish *osk* RNA function would suggest that a factor binds to that region. The sequestration of Bru, which represents one non-coding function of *osk* RNA, requires multiple Bru binding sites found in distinct regions of the 3' UTR. As the 3' sequences fall in one small, discrete region, sequestration of a factor seems unlikely for this other role. More likely, the 3' sequences would bind to a factor to perform a specific function. Identification of the *trans*-acting factor might provide insight into what other function *osk* RNA is providing for progression through oogenesis.

The germline/follicle cell membrane prevents the exchange of large molecules under normal conditions (Bohrmann & Hass-Assenbaum, 1993). In the absence of *osk* mRNA, the germ line-specific proteins, Bru and Orb, are enriched in the follicle cells. Enrichment of *osk* germline proteins could be due to the movement of the proteins to the follicle cells, which would require changes in the membrane integrity between the germline and follicle cells, or by increased transcription of the germline genes in the follicle cells. Tracking these proteins in live egg chambers would differentiate between these two possibilities. Using Bru or Orb fused to photoactivatable GFP (GFP that functions only after exposure to a certain wavelength), the GFP reporter could be activated within the germline, and the ability of the fusion protein to reach the follicle cells could be determined

by tracking the GFP signal. If *osk* RNA functions to maintain membrane integrity, the reporter would enter the follicle cells only in its absence. If the reporter fails to reach the follicle cells in the absence of *osk* RNA, then transcription of germ line-specific genes must be increased or the fusion to photoactivatable GFP changes the permeability of the germline protein.

The follicular enrichment could be specific to Bru and Orb, or a general characteristic of germline-specific proteins in the absence of *osk* mRNA. To determine if the enrichment in the follicle cells is specific to certain proteins, or represents a degeneration of the membrane integrity between the follicle cells and the germline, the distribution of more germline-specific proteins should be examined in the absence of *osk* mRNA. Germline proteins that could be tested include Vasa, Tudor, Valois, Me31B, Aubergine, and Bic-C (Schisa, 2012).

On a final note, our work provides further proof that protein-coding mRNAs can provide multiple non-coding functions. Often phenotypes associated with gene knockouts have been attributed to the loss of the protein; however, the absence of the mRNA coding for the protein and not the protein itself could conceivably be the cause of a phenotype. This requires a reassessment of the literature, with the knowledge that protein-coding mRNAs can possess non-coding functions.

REFERENCES

Basu, J. (2004). Protein palmitoylation and dynamic modulation of protein function. Current Science, 87(2), 212-217.

Bohrmann, J., & Hass-Assenbaum, A. (1993). Gap junctions in ovarian follicles of Drosophila melanogaster: inhibition and promotion of dye-coupling between oocyte and follicle cells. Cell Tissue Research, 273(1), 163-173.

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

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.

Lorenz, R., Bernhart, S., Honer, Z., Siederissen, C., Tafer, H., Flamm, C., Stadler, P., & Hofaker, I. (2011). ViennaRNA package 2.0. Algorithms in Molecular Biology, 6.

Moore, J., Han, H., & Lasko, P. (2009). Bruno negatively regulates germ cell-less expression in a BRE- independent manner. Mechanisms of Development, 126(7), 503-516.

Schisa, J. (2012). New insights into the regulation of RNP granule assembly in oocytes. International Review of Cellular and Molecular Biology, 295, 233-289.

Sugimura, I., & Lilly, M. (2006). Bruno inhibits the expression of mitotic cyclins during the prophase I meiotic arrest of Drosophila oocytes. Developmental Cell, 10(1), 127-135.

Wang, Z., & Lin, H. (2007). Sex-lethal is a target of Bruno-mediated translational repression in promoting the differentiation of stem cell progeny during Drosophila oogenesis. Developmental Biology, 302(1), 160-168.

Appendix: Investigating Osk isoform accumulation preference

Matt Kanke[1] and Paul M. Macdonald[1]

[1]Department of Molecular Biosciences

Institute for Cellular and Molecular Biology

The University of Texas at Austin

INTRODUCTION

Given a limited gene pool, a variety of mechanisms have evolved to increase the size of the proteome. Well-characterized mechanisms to increase protein diversity involve multiplying the mRNA species created from a single gene through the use of alternative promoters or mRNA splicing. Mature mRNA can further expand the proteome through the use of alternate start codons.

osk mRNA produces two protein isoforms from a single transcript by alternative start codon usage (Markussen et al., 1995; Rongo et al., 1995). Long Osk is translated from the first AUG encountered and Short Osk is translated from a second, in-frame AUG found several hundred nucleotides downstream from the first (Markussen et al., 1995). Short Osk is the predominant isoform, accumulating to a much higher degree than Long Osk (Markussen et al., 1995). Both isoforms have identical sequences throughout most of the protein and only differ in the amino-terminal end, yet display different subcellular localization and function (Vanzo and Ephrussi, 2002; Vanzo et al., 2007).

RESULTS

Altering 5' UTR length or long *osk* AUG context cause overall decrease in Osk protein with marginal effects on isoform ratio

Several features of *osk* mRNA near the long *osk* AUG could prevent recognition by the initiation complex. Failure to recognize the AUG would result in continued scanning of the complex with the possibility of recognition of the short *osk* AUG, and this could account for the predominance of the Short Osk isoform. Binding by the initiation complex spans ~30nt, and a 5' untranslated

region (UTR) shorter than that could inhibit translational initiation (Pestova et al., 2001). Long *osk* has a 15nt 5' UTR, well below the average length for *Drosophila melanogaster* (Misra et al., 2002), and 5' UTRs slightly shorter than that of *osk* reduce initiation complex recognition of the first AUG in vitro (Kozak, 1991). To test if the short 5' UTR is influencing translational initiation of long *osk*, a transgene increasing the 5' UTR to 35 nucleotides was constructed, *osk 5'+35* (Figure 4.1A), and expression was compared to genomic *osk*. The extended 5' UTR did not greatly affect the isoform ratio (Figure 4.1B), but did cause an overall reduction in Osk protein production (Figure 4.1D).

Recognition can also be affected by the nucleotide composition around the AUG (start codon context) and could account for the lower Long Osk levels (Kozak, 1997). The most common nucleotides proceeding an AUG in *Drosophila melanogaster* are CAAA, with the A at the -3 position being the most enriched (Nakagawa et al., 2007). The context of the short *osk* AUG (CAAC) differs by one nucleotide while the long *osk* (AGCG) differs at all positions. A transgene was constructed with the long *osk* AUG context altered to mimic that of short *osk, oskCAAC* (Figure 4.1A). The isoform ratio was not greatly altered, but the overall Osk protein level was reduced as compared to *osk* expression. The extended 5' UTR and altered AUG context were combined (*osk 5'+35&CAAC*) to test if both alterations are needed for long *osk* AUG recognition. Again, no substantial effect on isoform ratio was observed, but the Osk levels were decreased to a greater degree than with either alteration alone (Figure 4.1B,D).

The decrease in overall protein could occur if Long Osk is translated to a higher degree, but a mechanism exist to dispose of it once a threshold is exceeded, thus keeping Long Osk levels low. If short *osk* translation requires

initiation complex scan through, the increased translation at long *osk* would decrease the amount of complexes reaching the short *osk* AUG, and this could account for the overall reduction in Osk protein.

Substantial lengthening of 5' UTR increases Long Osk production

The additional nucleotides at the 5' end of the *osk 5'+35* transgene only increases the *osk* 5' UTR to 50 nucleotides. To test if the additional 35 nucleotides were not a substantial enough increase in the 5' UTR length for recognition by the initiation complex, we inserted the *osk* coding region into a UASp vector, *UASp-osk* (Figure 4.2A). The UASp vector extends the 5' UTR to 202 nucleotides (after splicing of an introduced intron), and places the transgene under the control of the UAS/GAL4 expression system. We compared *UASp-osk* to a similar transgene that only differed in 5' UTR length, *UASpmin-osk*. *UASpmin-osk* retains a 5' UTR equal in length, but not sequence, to the endogenous *osk* 5' UTR (Figure 4.2A). Long Osk from *UASp-osk* is expressed at a slightly higher level than from *UASpmin-osk* (Figure 4.2B). This suggests that the extended 5' UTR provides enough space between the 5' cap and the long *osk* AUG for recognition by the scanning translation initiation complex, resulting in increased expression of Long Osk. This conclusion comes with the caveat that this assay was conducted once, and repetition is needed to disregard experimental variability as a possibility.

The concomitant increase in Long Osk protein level with increased length of 5' UTR contrasts with the results from *osk 5'+35*, where Long Osk levels were slightly reduced. The UAS transgenes express much more Osk protein then

genomic *osk*. If a mechanism exists to dispel Long Osk once a threshold is reached, the mechanism might only have a capacity to deal with excessive levels Long Osk that could occur endogenously. The UAS/GAL4 system increases expression of Osk, both Long and Short, well above levels detected for endogenous *osk* expression. A possible explanation would be that the abnormally high Long Osk levels associated with UAS/GAL4 expression is exceeding the capacity of mechanisms responding to excessive Long Osk. The suppression in Long Osk from the endogenously expressed transgenes fits this model, with the mechanism being activated to reduce Long Osk levels that are marginally above a threshold, with the slight reduction indicative of a lag in turning off the mechanism after the proper Long Osk level had been reached.

MATERIALS AND METHODS

Flies and Transgenes

Genomic *osk* transgenes included a 3xHA epitope tag, inserted after amino acid 140, to facilitate western blot analysis (J Jones and PMM, submitted). This tag does not detectably alter *osk* expression or activity. *UASp-osk* contains the full *osk* coding region inserted into the UASp vector. The 5' UTR of *UASpmin-osk* retains 8 nucleotides from the 5' end of the UASp vector and 7 nucleotides upstream of the long *osk* AUG.

Western blotting

Ovaries from females raised on yeast for 3-4 days were dissected in ice-cold PBS and prepared as described (Kim-Ha et al, 1991) using ice cold lysis

buffer (25mM Tris-Cl pH6.8, 1mM $MgCl_2$, 100mM KCl, 1mM DTT, and 0.1% Triton X-100). Lysates were run on a SDS-PAGE gel and transferred to PVDF membrane. Antibodies were used at the following dilutions: mouse anti-HA (1:1000, Covance) and mouse anti- α-tubulin (1:2000, Sigma).

RNA detection

To measure RNA levels, ovaries were dissected from 3-4 day old females, RNA purified using Trizol according to the manufacturers instructions, probed for *osk* and *rp49* mRNAs by RNase protection assay (Ambion RPAIII), and quantified by phosphorimaging. Assays were performed 3 or more times. The *rp49* signal was used to normalize for the amount of RNA in each preparation

FIGURES

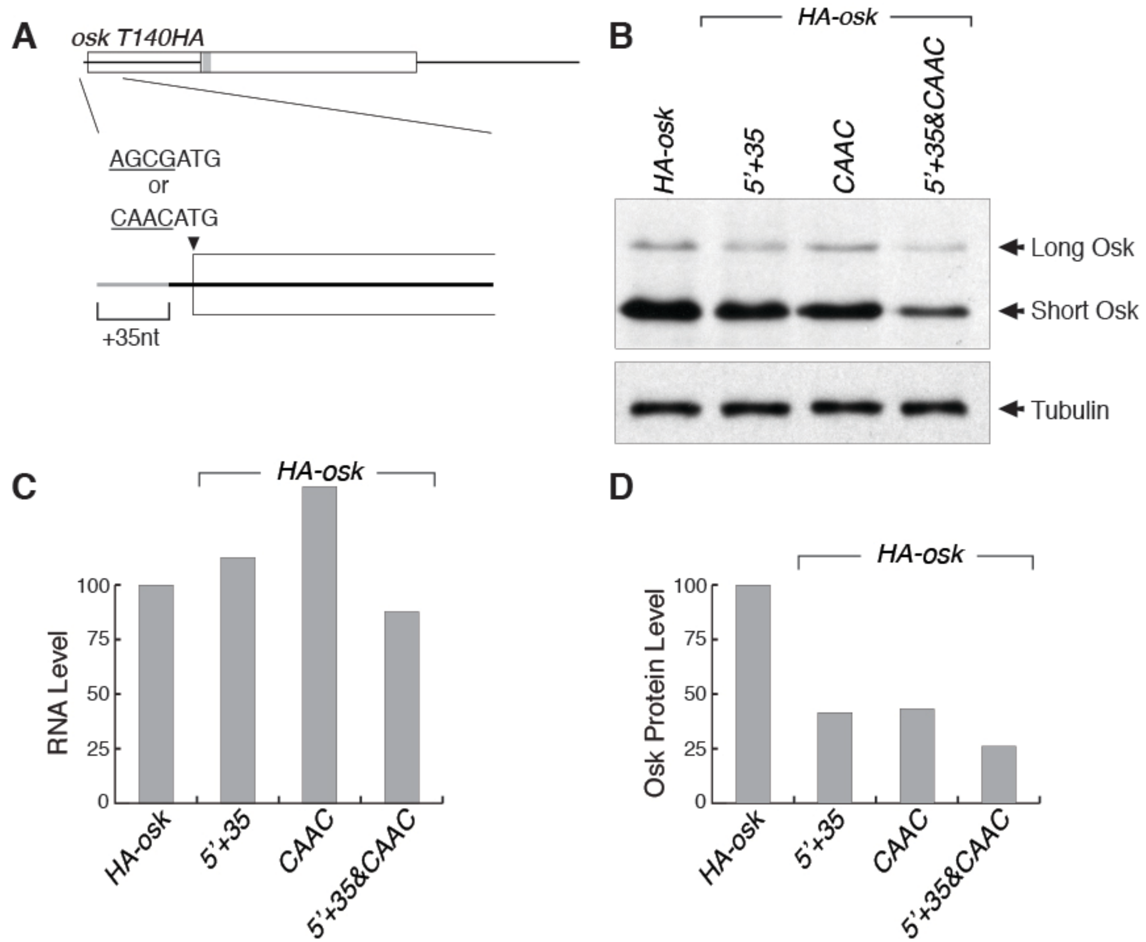


Figure 4.1 - Full caption next page

Figure 4.1. 5' UTR and AUG context contribute to Osk protein accumulation

A. Diagram of the *osk* mRNA with expanded 5' region. The endogenous UTR's (black line) and the added 5' sequences (grey line) are indicated, as is the coding region (rectangle). The grey box represents 3 copies of the HA epitope tag. The possible long *osk* start codon contexts and the additional 5' UTR represent the possible changes to *osk T140HA*.

B. Western blot analysis of transgenes expressed as single copies. Tubulin was included as a loading control.

C. Levels of *osk* mRNA produced from a single copy of the indicated transgenes. All values are normalized against the level of mRNA from a single copy of the *oskHA* transgene, which is identical to *oskM1RHA* except that it has the wild type M1 codon. Levels of *rp49* were monitored to normalize for amount of RNA used in each assay.

D. Level of total Osk protein expressed from transgenes expressed as single copies. Protein accumulation was adjusted according to the loading control and the RNA level. Values were normalized to adjusted Osk accumulation produced by *osk T140HA*.

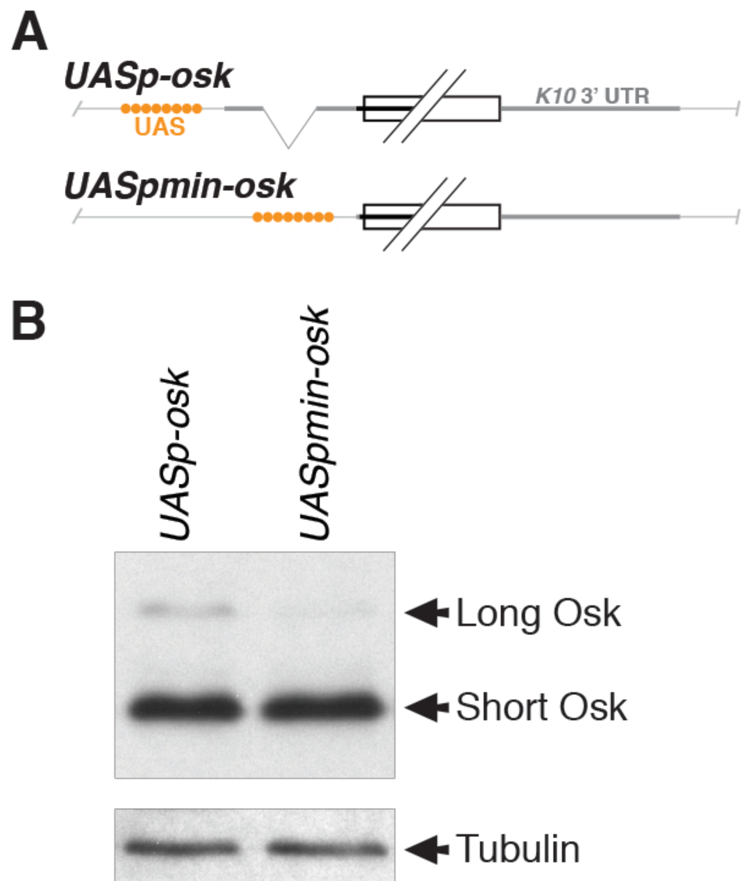


Figure 4.2. Extended 5' UTR causes modest increase in Long Osk accumulation

A. Diagram of *UASp-osk* transgenes, using conventions from Figure 4.1. Diagonal gap indicates identical portions of *osk* coding region between the transgenes that is not shown.

B. Western blot analysis of transgenes. Tubulin was included as a loading control.

REFERENCES

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.

Kozak, M. (1991). A short leader sequence impairs the fidelity of initiation by eukaryotic ribosomes. Gene Expression, 1(2), 111-115.

Kozak, M. (1997). Recognition of AUG and alternative initiator codons is augmented by G in position +4 but is not generally affected by the nucleotides in positions +5 and +6. The EMBO Journal, 16(9), 2482-2492.

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.

Misra, S., Crosby, M., Mungall, C., Matthews, B., Campbell, K., Hradecky, P. et al (2002). Annotation of the Drosophila melanogaster euchromatic genome: a systematic review. Genome Biology, 3(12), 1-22.

Nakagawa, S., Niimura, Y., Gojobori, T., Tanaka, H., & Miura, K. (2007). Diversity of preferred nucleotide sequences around the translation initiation codon in eukaryote genomes. Nucleic Acids Research, 36(3), 861-871.

Pestova, T., Kolupaeva, V., Lomakin, I., Pilipendo, E., Shatsky, N., Agol, V., & Hellen, C. (2001). Molecular mechanisms of translation initiation in eukaryotes. Proceedings of the National Academy of the Sciences, 98(13), 7029-7036.

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

Vanzo, N., & 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.

References

Abastado, J., Miller, P., Jackson, B., & Hinnebusch, A. (1991). Suppression of ribosomal reinitiation at upstream open reading frames in amino acid-starved cells forms the basis for GCN4 translational control. Molecular and Cellular Biology, 11(1), 486-496.

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 and Development, 18(2), 138-143.

Bartel, D. (2009). MicroRNAs: target recognition and regulatory functions. Cell, 136(2), 215-233.

Basu, J. (2004). Protein palmitoylation and dynamic modulation of protein function. Current Science, 87(2), 212-217.

Benoit, P., Papin, C., Kwak, J., Wickens, M., & Simonelig, M. (2008). PAP- and GLD-2-type poly(A) polymerases are required sequentially in cytoplasmic polyadenylation and oogenesis in Drosophila. Development, 135(11), 1969-1979.

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

Bohrmann, J., & Hass-Assenbaum, A. (1993). Gap junctions in ovarian follicles of Drosophila melanogaster: inhibition and promotion of dye-coupling between oocyte and follicle cells. Cell Tissue Research, 273(1), 163-173.

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.

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.

Cech, T. and Steitz, J. (2014). The noncoding RNA revolution- trashing old rules to forge new ones. Cell, 157(1), 77-94.

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.

Chicoine, J., Benoit, P., Gamberi, C., Paliouras, M., Simonelig, M., and Lasko, P. (2007). Bicaudal-C recruits CCR4-NOT deadenylase to target mRNAs and regulates oogenesis, cytoskeletal organization, and its own expression. Developmental Cell, 13(5), 691-704.

Clark, A., Meignin, C., & Davis, I. (2007). A Dynein-dependent shortcut rapidly delivers axis determination transcripts into the Drosophila oocyte. Development 134(10) 1955-1965.

Carrera, P., Johnstone, O., Nakamura, A., Casanova, J., Jackle, H., & Lasko, P. (2000). VASA mediates translation through interaction with a Drosophila yIF2 homolog. Molecular Cell, 5(1), 181-187.

Cornelis, S., Bruynooghe, Y., Denecker, G., Van Huffel, S., Tinton, S., & Beyaert, R. (2000). Identification and characterization of a novel cell cycle-regulated internal ribosome entry site. Molecular Cell, 5(4), 597-605.

Dahlgaard, K., Raposo, A., Niccoli, T., & St Johnston, D. (2007). Capu and Spire assemble a cytoplasmic actin mesh that maintains microtubule organization in the Drosophila oocyte. Developmental Cell, 13(4), 539-553.

Deng, Y., Singer, R., & Gu, W. (2008). Translation of ASH1 mRNA is repressed by Puf6p-Fun12p/eIF5B interaction and released by CK2 phosphorylation. Genes & Development, 28(20), 1037-1050.

Dever, T., Yang, W., Astrom, S., Bystrom, A., & Hinnebusch, A. (1995). Modulation of tRNA(iMet), eIF-2, and eIF-2B expression shows that GCN4 translation is inversely coupled to the level of eIF-2.GTP.Met-tRNA(iMET) ternary complexes. Molecular and Cellular Biology, 15(11), 6351-6363.

Dinger, M., Pang, K., Mercer, T., & Mattick, J. (2008). Differentiating protein-coding and noncoding RNA: challenges and ambiguities. PLoS Computational Biology, 4(11), 1-5.

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.

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

Furriols, M., & Casanove, J. (2014). Germline and somatic vitelline proteins colocalize in aggregates in the follicular epithelium of Drosophila ovaries. Fly, 8.

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

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.

Ghabrial, A., Ray, P., & Schupbach, T. (1998). okra and spindle-B encode components of the RAD52 DNA repair pathway and affect meiosis and patterning in Drosophila oogenesis. Genes and Development, 12(17), 2711-2723.

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.

Glotzer, J., Saffrich, R., Glotzer, M., & Ephrussi, A. (1997). Cytoplasmic flows localize injected oskar RNA in Drosophila oocytes. Current Biology, 7(5), 326-337.

Gong, W., & Golic, K. (2003). Ends-out, or replacement, gene targeting in Drosophila. Proceedings of the National Academy of the Sciences, 100(5), 2556-2561.

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.

Guttman, M. & Rinn, J. (2012). Modular regulatory principles of large non-coding RNAs. Nature, 482(7385), 339-346.

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.

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 Cell biology, 58(1), 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.

Heasman, J. (2006). Patterning the early Xenopus embryo. Development, 133(7), 1205-1217.

Hung, T., Wang, Y., Lin, M., Koegel, A., Kotake, Y., Grant, G. et al. (2011). Extensive and coordinated transcription of noncoding RNAs within cell-cycle promoters. Nature Genetics, 43(7), 621-629.

Huttelmaier, S., Zenklusen, D., Lederer, M., Dictenberg, J., Lorenz, M., Meng, X., et al. (2005). Spatial regulation of β-actin translation by Src-dependent phosphorylation of ZBP1. Nature, 438, 512-515.

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.

Jackson, R., Hellen, C, & Pestova, T. (2010). The mechanism of eukaryotic translation initiation and principles of its regulation. Nature Reviews, 11(2), 113-127.

Jambor, H., Mueller, S., Bullock, S., & Ephrussi, A. (2014). A stem-loop structure directs oskar mRNA to microtubule minus ends. RNA, 20(4), 429-439.

Jankovics, F., Sinka, R., Lukacsovich, T., & Erdelyi, M. (2002). MOESIN crosslinks actin and cell membrane in Drosophila oocytes and is required for OSKAR anchoring. Current Biology, 12(23), 2060-2065.

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. (2004). Interaction with eIF5B is essential for Vasa function during development. Development, 131(17), 4167-4178.

Juge, F., Zaessinger, S., Temme, C., Wahle, E., & Simonelig, M. (2002). Control of poly(A) polymerase level is essential to cytoplasmic polyadenylation and early development in Drosophila. The EMBO Journal, 21(23), 6603-6613.

Jung, H., Gkogkas, C., Sonenberg, N., & Holt, C. (2014). Remote control of gene function by local translation. Cell, 157(1), 26-40.

Kim, G. (2014). Investigating the role of Bruno interactions with oskar regulatory proteins (Doctoral dissertation). University of Texas at Austin, Austin, Texas.

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.

Kochetov, A. (2008). Alternative translation start sites and the hidden coding potential of eukaryotic mRNAs. Bioessays, 30(7), 683-691.

Kozak, M. (1986). Influences of mRNA secondary structure on initiation by eukaryotic ribosomes. Proceedings of the National Acadamy of Sciences, 83(9), 2850-2854.

Kozak, M. (1997). Recognition of AUG and alternative initiator codons is augmented by G in position +4 but is not generally affected by the nucleotides in positions +5 and +6. The EMBO Journal, 16(9), 2482-2492.

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

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

Lasko, P. (2011). Posttranscriptional regulation in Drosophila oocytes and early embryos. Wiley Interdisciplinary Reviews RNA, 2(3), 408-416.

Lasko, P. (2012). mRNA localization and translational control in Drosophila oogenesis. Cold Spring Harbor Perspectives in Biology, 4(10).

Lecuyer, E., Yoshida, H., Parthasarathy, N., Alm, C., Babak, T., Cerovina, T., Hughes, T. et al. (2007). Global analysis of mRNA localization reveals a prominent role in organizing cellular architecture and function. Cell, 131(1), 174-187.

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.

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.

Lieberfarb, M., Chu, T., Wreden, C., Theurkauf, W., Gergen, J., & Strickland, S. (1996). Mutations that perturb poly(A)-dependent maternal mRNA activation block the initiation of development. Development, 122, 579-588.

Lorenz, R., Bernhart, S., Honer, Z., Siederissen, C., Tafer, H., Flamm, C., Stadler, P., & Hofaker, I. (2011). ViennaRNA package 2.0. Algorithms in Molecular Biology, 6.

Lu, C, Brennan, J., & Robertson, E. (2001). From fertilization to gastrulation: axis formation in the mouse embryo. Current Opinion in Genetics and Development, 11(4), 384-392.

Macdougall, N., Clark, A., Macdougall, E., & Davis, I. (2003). Drosophila gurken (TGFα) mRNA localizes as particles that move within the oocyte in two dynein-dependent steps. Developmental Cell, 4(3), 307-319.

Mach, J., & Lehmann, R. (1997). An Egalitarian-BicaudalD complex is essential for oocyte specification and axis determination in Drosophila. Genes and Development, 11, 423-435.

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. & Ephrussi, A. (2009). mRNA localization: gene expression in the spatial dimension. Cell, 136(4), 719-730.

McLean, P., & Cooley, L. (2013). Protein equilibration through somatic ring canals in Drosophila. Science, 340(6139), 1445-1447.

Mercer, T., Dinger, M., & Mattick, J. (2009). Long non-coding RNAs: insights into functions. Nature Reviews Genetics, 10(3), 155-159.

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.

Moore, J., Han, H., & Lasko, P. (2009). Bruno negatively regulates germ cell-less expression in a BRE- independent manner. Mechanisms of Development, 126(7), 503-516.

Mueller, P., & Hinnebusch, A. (1986). Multiple upstream AUG codons mediate translational control of GCN4. Cell, 45, 201-207.

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., Menade, 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.

Pedrajas, J., Porras, P., Martinez-Galisteo, E., Padila, C., Miranda-Vizuete, A., & Barcena, J. (2002). Two isoforms of Saccharomyces cerevisiae glutaredoxin 2 are expressed in vivo and localize to different subcellular compartments. Biochemical Journal, 264(3), 617-623.

Pestova, T., Kolupaeva, V., Lomakin, I., Pilipendo, E., Shatsky, N., Agol, V., & Hellen, C. (2001). Molecular mechanisms of translation initiation in eukaryotes. Proceedings of the National Academy of the Sciences, 98(13), 7029-7036.

Piccioni, F., Zappavigna, V., & Verrotti, A. (2005). Translational regulation during oogneesis and early development: the cap-poly(A) tail relationship. Comptes Rendus Biologies 328, 863-881.

Pisareva, V., Pisareva, V., Komar, A., Hellen, C., & Pestova, T. (2008). Translation initiation on mammalian mRNAs with structured 5'-UTRs requires DExH-box protein DHX29. Cell, 135(7), 1237-1250.

Porras, P., Padilla, A., Krayl, M., Voos, W., & Barcena, J. (2006). One single in-frame AUG codon is responsible for a diversity of subcellular loacalizations of Glutaredoxin 2 in Saccharomyces cerevisiae. The Journal of Biological Chemistry, 281(24), 16551-16562.

Ramos, A., Grunert, S., Adams, J., Micklem, D., Proctor, M., Freund, S., et al. (2000). RNA recognition by a Staufen double-stranded RNA-binding domain. The EMBO Journal, 19(5), 997-1009.

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

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.

Rinn, J. & Chang, H. (2012). Genome regulation by long noncoding RNAs. Annual Review of Biochemistry, 81, 145-166.

Robinson, D., & Cooley, L. (1996). Stable intercellular bridges in development: the cytoskeleton lining the tunnel. Trends in Cellular Biology, 6(12), 474-479.

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

Rorth, P., Szabo, K., Bailey, A., Laverty, T., Rehm, J., Rubin, G., et al. (1998). Systematic gain-of-function genetics in Drosophila. Development, 125(6), 1049-1057.

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.

Saffman, E., Styhler, S., Rother, K., Li, W., Richard, S., & Lasko, P. (1998). Premature translation of oskar in oocytes lacking the RNA-binding protein Bicaudal-C. Molecular and Cellular Biology, 18(8), 4855-4862.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., et al. (2012). Fiji: an open-source platform for biological-image analysis. Nature Methods, 9(7), 676-682.

Schisa, J. (2012). New insights into the regulation of RNP granule assembly in oocytes. International Review of Cellular and Molecular Biology, 295, 233-289.

Schüpbach, T., & Wieschaus, E. (1989). Female Sterile Mutations on the Second Chromosome of Drosophila melanogaster. 1. Maternal effect mutations. Genetics, 121(1), 101-117.

Serano, T., & Cohen, R. (1995). A small predicted stem-loop structure mediates oocyte localization of Drosophila K10 mRNA. Development, 121(11), 1995.

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.

Smotrys, J., & Linder, M. (2004). Palmitoylation of intracellular signaling proteins: regulation and function. Annual Review of Biochemistry, 73, 559-587.

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., Harrison, D., Yan, N., & Macdonald, P. (2007). A late phase of Oskar accumulation is crucial for posterior patterning of the Drosophila embryo, and is blocked by ectopic expression of Bruno. Differentiation, 75(3), 246-255.

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

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., Brown, N., Gall, J., & Jantsch, M. (1992). A conserved double-stranded RNA-binding domain. Proceedings of the National Academy of Sciences, 89(22), 10979-10983.

Sugimura, I., & Lilly, M. (2006). Bruno inhibits the expression of mitotic cyclins during the prophase I meiotic arrest of Drosophila oocytes. Developmental Cell, 10(1), 127-135.

Takyar, S., Hickerson, R., & Noller, H. (2005). mRNA helicase activity of the ribosome. Cell, 120(1), 49-58.

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., 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.

Tsai, M., Manor, O., Wan, Y., Mosammaparast, N., Wang, J., Lan, F. et al. (2010). Long noncoding RNA as modular scaffold of histone modification complexes. Science, 329(5992), 689-693.

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

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., & 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.

Walker, S., Scott, F., Srisawat, C., & Engelke, D. (2008). RNA affinity tags for the rapid purification and investigation of RNAs and RNA-protein complexes. Methods in Molecular Biology, 488(1), 23-40.

Wang, Z., & Lin, H. (2007). Sex-lethal is a target of Bruno-mediated translational repression in promoting the differentiation of stem cell progeny during Drosophila oogenesis. Developmental Biology, 302(1), 160-168.

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.

Wharton, R., & Struhl, G. (1991). RNA regulatory elements mediate control of Drosophila body pattern by the posterior morphogen nanos. Cell, 67(5), 955-967.

Wieschaus, E., & Nüsslein-Volhard, C. (1986). Looking at embryos. In Drosophila: A practical approach, Roberts DB (eds) pp199-227. Washington, D.C.: IRL Press.

Wilhelm, J., Hilton, M., Amos, Q., & Henzel, W. (2003). Cup is an eIF4E binding protein required for both the translational repression of oskar and the recruitment of Barentsz. 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.

Zaessinger, S., Busseau, I., & Simonelig, M. (2006). Oskar allows nanos mRNA translation in Drosophila embryos by preventing its deadenylation by Smaug/CCR4. Development, 133(22), 4573-4583.

Zhu, X., Stevens, L., & Stein, D. (2007). Synthesis of the sulfate donor PAPS in either the Drosophila germline or somatic follicle cells can support embryonic dorsal-ventral axis formation. Development, 134(8), 1465-1469.

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.

Vita

Matthew Robert Kanke was born in Wisconsin. He was awarded the degree of Bachelor of Science in Biology from the University of Wisconsin - Stevens Point in May of 2006. Matthew enrolled in the Cell and Molecular Biology graduate program in August of 2007 and joined the Macdonald Lab in May of 2008.

Permanent e-mail address: Matt.Kanke@gmail.com

This dissertation was typed by Matthew Robert Kanke