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The Role of Bicoid Stability Factor in *oskar* mRNA function and regulation, and the mechanisms for *oskar* mRNA transport to the oocyte

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Dedication

To my parents

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The role of Bicoid Stability Factor in *oskar* mRNA function and regulation, and the mechanisms for *oskar* mRNA transport to the oocyte

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The University of Texas at Austin, 2015

Supervisor: Paul M. Macdonald

My dissertation is separated into two subjects. First, I examined the role of Bicoid Stability Factor (BSF) in *oskar* (*osk*) regulation. Second, I studied *cis*-acting elements involved in *osk* mRNA transport to the oocyte during early oogenesis.

Oskar (Osk) is a body patterning determinant in *Drosophila* and is highly concentrated at the posterior pole of the oocyte. This spatially-restricted deployment relies on a coordinated program of *osk* mRNA localization and translational regulation, all dependent on *cis*-acting regulatory elements located primarily in the 3' UTR of the mRNA. Notably, some of these elements, as well as sequences required for a noncoding role of *osk* mRNA, are clustered in a short region (the C region) near the 3' end of the *osk* mRNA. To better understand the role of the C region, I searched for proteins that bind specifically to this region and I found BSF. Binding assays to mutant RNAs suggested that BSF does not act in the noncoding function of *osk* mRNA. To test for a role for BSF in regulation of Osk protein expression, I used two complementary approaches, reducing the BSF level or disrupting BSF binding to the *osk* mRNA. Both

generated similar results: a reduction or loss of posterior Osk protein and *osk* mRNA in late oogenesis and early embryogenesis, while the level of *osk* mRNA was not affected. My work suggests that BSF could act in a late phase of *osk* mRNA localization or translational activation.

Localization of *osk* mRNA to the posterior pole of the oocyte is achieved by multiple transport steps. One is mRNA transport from the nurse cells to the oocyte. Although *cis*-acting elements including the oocyte entry signal (OES) in the *osk* mRNA 3' UTR have been implicated in mRNA oocyte transport, the precise mechanisms remain unknown. Here, I show that the clusters of Bru binding sites in the *osk* mRNA 3' UTR required for translational regulation confer oocyte transport on a reporter mRNA. However, neither Bru sites nor the OES are essential for oocyte transport of *osk* mRNA. This suggests that there are multiple mechanisms redundantly acting in oocyte transport.

Table of Contents

List of Figures

Chapter 1: General Introduction

Asymmetric protein accumulation is crucial for establishing body axes in animal development, and often relies on the mechanisms of mRNA localization and translational regulation during mRNA transport. We are interested in *osk* regulation. Oskar (Osk) protein is a posterior patterning determinant in *Drosophila*. Accumulation of Osk only at the posterior pole of the oocyte is required for embryonic abdomen and germline formation. This restricted pattern of Osk accumulation is achieved by multiple regulatory processes, including mRNA localization, translational repression during mRNA localization, and translational activation once the mRNA is localized at the posterior pole of the oocyte. Various *cis*-acting elements and *trans*-acting factors mediating these regulatory processes have been identified, and their functions have been studied. However, despite this progress many questions remain and there must be additional components that have not yet been identified. Obtaining a more complete understanding of this paradigm will provide further insights into the fundamental mechanisms underlying local gene expression, which is essential in polarized cells in various biological processes.

DROSOPHILA OOGENESIS

Drosophila oogenesis is a valuable model system for the detailed study of mRNA localization and translation. A single ovary contains all of the developmental stages, simply organized from stem cells to mature eggs. The cells in which the mRNAs and proteins appear are relatively large, which allows us to easily track changes in the movements and accumulation of the mRNAs

and proteins from stage to stage. In addition, availability of the powerful genetic techniques and a wide variety of mutants makes this system useful for studying the mechanisms underlying gene regulation.

Drosophila oogenesis is divided into 14 morphologically distinct stages (Fig. 1.1A). A female *Drosophila* has a pair of ovaries, each made up of ~18 ovarioles. A single ovariole is a series of egg chambers, the structural and functional units of oogenesis. At one end of the ovariole is the germarium. At the anterior tip of the germarium, germline stem cell divisions occur to generate a new stem cell and a stem cell daughter called a cystoblast. Cystoblasts undergo four mitotic divisions, with incomplete cytokinesis, to produce a cyst of 16 cells that are interconnected by cytoplasmic bridges called ring canals. This cluster of germline cells is surrounded by a single layer of somatic follicle cells, to make the egg chamber. Only one of the 16 germline cells develops into an oocyte, while the remaining 15 cells become nurse cells with polyploid nuclei (Fig. 1.1B). The nurse cells are highly active in transcription and translation, producing mRNAs and proteins that are transported into the developing oocyte through the ring canals to aid oocyte maturation. On the other hand, the oocyte nucleus forms a compact structure called the karyosome, and remains transcriptionally quiescent until the mature egg is laid and activated. Toward the end of oogenesis, the nurse cells degenerate and expel their cytoplasm into the oocyte, a process called nurse cell dumping. Finally, the nurse cells undergo apoptosis (reviewed in (Bastock and St Johnston, 2008; Riechmann and Ephrussi, 2001)).

BODY PATTERN FORMATION IN *DROSOPHILA*

Establishment of body axes requires a restricted protein accumulation, which can be initiated by localizing specific mRNAs to particular subcellular regions. mRNA localization is often coupled to translational repression to prevent ectopic or precocious expression of the protein. In *Drosophila,* embryonic body axes are specified during oogenesis, and rely on localization of four key mRNAs, *oskar* (*osk*), *nanos* (*nos*), *bicoid* (*bcd*), and *gurken* (*grk*), to particular cytoplasmic regions of the oocyte (reviewed in (Johnstone and Lasko, 2001))(Fig. 1.1B).

The anterior-posterior (AP) patterning of the *Drosophila* embryo requires localization of *bcd* mRNA to the anterior tip of the oocyte (Berleth et al., 1988) and localization of *osk* and *nos* mRNAs to the posterior pole of the oocyte (Ephrussi et al., 1991; Gavis and Lehmann, 1992; Kim-Ha et al., 1991). The *bcd* and *nos* mRNAs are present throughout oogenesis, but their translation is repressed. After fertilization, translational repression is relieved and the proteins are produced in the embryo in opposing gradients. Bcd protein, responsible for head and thorax formation, accumulates at the anterior (Berleth et al., 1988). Nos protein, responsible for germline and abdomen formation, accumulates at the posterior (Lehmann and Nusslein-Volhard, 1991). The anterior-to-posterior Bcd gradient is mainly achieved through *bcd* mRNA localization at the anterior (Dilao and Muraro, 2010; Driever and Nusslein-Volhard, 1988; Little et al., 2011). In contrast, the posterior-to-anterior Nos gradient is achieved through both mRNA localization at the posterior and translational repression of *nos* mRNA outside the posterior region (Bergsten and Gavis, 1999)(reviewed in (Lasko, 2012)).

osk mRNA localizes to the posterior pole of the oocyte (Kim-Ha et al., 1991). Translation of unlocalized *osk* mRNA is highly repressed. Only at the

posterior, it begins to be translated to produce Osk protein responsible for assembly of the germ/pole plasm, a specialized cytoplasm containing RNP complexes essential for embryonic posterior patterning and germ/pole cell formation (Ephrussi and Lehmann, 1992; Kim-Ha et al., 1995; Markussen et al., 1995). Embryos lacking Osk at the posterior fail to form both abdomen and germ cells (Lehmann and Nusslein-Volhard, 1986), while embryos having mislocalized Osk at the anterior have ectopic abdomen and germ cells (Ephrussi and Lehmann, 1992; Smith et al., 1992). Therefore, tightly regulated *osk* mRNA localization and Osk expression is required for *Drosophila* development.

grk mRNA localizes to the posterior of the oocyte during early oogenesis, and to the anterodorsal corner of the ooctye during mid-oogenesis (Johnstone and Lasko, 2001). This distinct *grk* mRNA localization contributes to both anteroposterior and dorsoventral pattern formation. Grk protein, a transforming growth factor (TGF)-α homolog, locally activates the epidermal growth factor receptor (EGFR) on the surrounding follicle cells. Early in oogenesis, posteriorly positioned Grk activates EGFR signaling in surrounding follicle cells to adopt the posterior fate, which in turn induces AP axis specification (Gonzalez-Reyes et al., 1995; Roth et al., 1995). During mid-oogenesis, anterodorsally positioned Grk activates EGFR signaling in surrounding follicle cells to adopt the dorsal fate, which in turn initiates DV axis specification (Neuman-Silberberg and Schupbach, 1993).

OSKAR MRNA LOCALIZATION

osk mRNA is transcribed in nurse cell nuclei and transported to the oocyte through the ring canals. During stages from 1 to 7, *osk* mRNA is highly

concentrated in the oocyte. At stage 8, the oocyte begins to expand, and *osk* mRNA is transiently concentrated at the anterior of the oocyte. From stage 9 onward it specifically accumulates at the posterior pole of the oocyte (Fig. 1.2A) and remains localized until early embryogenesis (Ephrussi et al., 1991; Kim-Ha et al., 1991). Therefore, *osk* mRNA localization to the posterior pole of the oocyte can be separated into different steps: transport from the nurse cell nucleus to the cytoplasm, transport from the nurse cell cytoplasm to the oocyte, transport to the posterior pole of the oocyte, and finally anchoring at the posterior cortex of the oocyte. Various *cis*-acting elements and *trans*-acting factors acting in the different steps have been identified and studied. However, the exact molecular mechanisms mediated by them still need to be elucidated. One part of my work revealed a novel form of *osk* mRNA transport from nurse cells to the oocyte during early oogenesis. Another part of my work, on the role of BSF, raises the possibility that BSF acts in *osk* mRNA localization or anchoring during late oogenesis. To provide the context for those studies, I explain what is known about the mechanisms of *osk* mRNA localization.

In the nurse cell nucleus, *osk* mRNA is transcribed and associates with several hnRNP proteins (Hrp48, Squid (Sqd), and Glorund (Glo)), to make an initial RNP complex (St Johnston, 2005). Exon junction complex (EJC) components (Mago nashi (Mago), Y14, and eIF4Alll) are associated with the *osk* mRNA/hnRNP complex (Hachet and Ephrussi, 2001; Mohr et al., 2001; Palacios et al., 2004), which is exported into the nurse cell cytoplasm in an RNA helicase UAP56-dependent manner (Meignin and Davis, 2008). For the most part, the functional significance of assembling these factors with *osk* mRNA in the nucleus remains unclear. An exception concerns the factors involved in pre-mRNA

splicing: splicing the *osk* pre-mRNA first intron and deposition of the EJC components are crucial for *osk* mRNA localization to the posterior pole of the oocyte (to be discussed in more detail below).

From the nurse cell cytoplasm*, osk* mRNA is transported to the oocyte (simply referred as oocyte transport). This step depends on sequences within the *osk* mRNA 3' UTR (Kim-Ha et al., 1993). Recently, these elements were more precisely mapped and the oocyte entry signal (OES) was identified. The secondary structure and 'A/U richness' within the OES sequences were shown to be required for the oocyte transport of the reporter mRNA (Jambor et al., 2014). However, it is still unknown how OES works. It could act as a scaffold to recruit proteins essential for oocyte transport. Although the OES was required for oocyte transport of a reporter mRNA containing a part of the *osk* 3' UTR, it remains unknown whether the OES is necessary in the context of an otherwise intact *osk* mRNA.

In addition to the *cis*-acting element, *osk* mRNA oocyte transport depends on the minus-end-directed motor Dynein complex, including Dynein heavy chain (Dhc), Bicaudal D (Bic-D), and Egalitarian (Egl). Disruption of these factors abrogated oocyte transport (Clark et al., 2007; Ephrussi et al., 1991; Mach and Lehmann, 1997; McGrail and Hays, 1997; Suter and Steward, 1991; Swan et al., 1999). In contrast, disruption of plus-end-directed transport by mutating kinesin-1 did not disrupt oocyte transport. Instead, *osk* mRNA localization to the posterior of the oocyte was affected (Brendza et al., 2000; Cha et al., 2002). These results suggest that two distinct microtubule-dependent pathways are involved in *osk* mRNA transport: first, minus-end-directed, dynein-dependent oocyte transport, and then plus-end-directed, kinesin-1-dependent posterior localization.

Once *osk* mRNA is transported from nurse cells into the oocyte, it localizes to the posterior pole of the oocyte (simply referred as posterior localization). As mentioned above, deposition of the Exon junction complex (EJC) on the *osk* mRNA is involved in mRNA posterior localization. The EJC is a multi-protein complex that binds to mRNA upstream of exon-exon junctions following splicing, and is thought to remain bound to the mRNA until the first round of translation (Tange et al., 2005). The core EJC components Mago nashi (Mago), Y14, Tsunagi, and eIF4AIII and additional cytoplasmic protein Barentz (Btz) are crucial for *osk* mRNA posterior localization. Mutation of any one of them disrupts posterior localization (Hachet and Ephrussi, 2001; Mohr et al., 2001; Palacios et al., 2004). It is uncertain how the EJC components act in *osk* mRNA posterior localization. The EJC was suggested as a candidate protein complex that links mRNA to the microtubule motor proteins (Trucco et al., 2009), although subsequent work argues against this (Trucco et al., 2010). EJC components may serve as a landmark for recruiting other essential localization factors to *osk* mRNA. This can be supported by the evidence that eIF4AIII physically interacts with cytoplasmic localization factor Btz *in vitro,* and an *eIF4AIII* mutant enhanced the defect of *osk* mRNA posterior localization caused by *btz* mutation (Palacios et al., 2004).

Splicing contributes to *osk* mRNA posterior localization not only by EJC deposition. In addition, splicing of the first intron is necessary. Splicing of the first intron creates a 28 nt stem-loop structure named spliced *osk* localization element (SOLE), by joining of the last 18 nt of exon 1 and first 10 nt of exon 2. Posterior localization is mediated by the stem-loop secondary structure of the SOLE, not

by its sequence (Ghosh et al., 2012). Taken together, splicing has a dual function in *osk* mRNA posterior localization, assembling the SOLE and loading the EJC.

Besides EJC components, *osk* mRNA posterior localization requires various *trans*-acting factors, and some of them bind directly to the *osk* mRNA 3' UTR. One example is Staufen (Stau). Stau is an RNA binding protein that colocalizes with *osk* mRNA throughout oogenesis and is required for *osk* mRNA posterior localization (Kim-Ha et al., 1991; Martin et al., 2003). Interestingly, Stau is also required for *osk* mRNA translation and this function is independent of its role in mRNA localization (Micklem et al., 2000). Although how Stau acts is still under investigation, the different domains of Stau are implicated in different roles. Stau contains five double-strand RNA-binding domains (dsRBDs). Among them, dsRBD2 is involved in *osk* mRNA posterior localization, and dsRBD5 is involved in translational activation (Micklem et al., 2000). Recently, three types of stem-loop secondary structures named Staufen recognized structures (SRS) in the 3' UTRs were predicted by genome-wide analysis of the Stau-associated mRNAs. Among three types of SRSs (type I, II, and III), *osk* mRNA carries four Type II SRSs and one Type II SRS in the 3' UTR, suggesting the direct binding of Stau to *osk* mRNA (Laver et al., 2013).

Another *trans*-acting factor is Exuperantia (Exu). Exu was shown to be present in a large *osk*-containing RNP particle. Mutation of *exu* disrupted *osk* mRNA localization both in nurse cells and the oocyte during stages 9 and 10 of oogenesis, suggesting that Exu acts in *osk* localization both in nurse cells and the oocyte. Because the *exu* mutants reduced but did not eliminate *osk* mRNA localization, the contribution of Exu is expected to be redundant with other unknown localization mechanisms (Wilhelm et al., 2000).

Once *osk* mRNA reaches at the posterior pole, it is captured and anchored at the posterior cortex to prevent its diffusion into cytoplasm. Actin-based motor Myosin-V (Myo V) is needed for anchoring of *osk* mRNA and Osk protein at the posterior cortex, and mainly associates with the *osk* mRNA transport complex at that position. Myo V appears to act in the final short-range movements in the posterior, following the long-range microtubule-based transport of *osk* mRNA. Myo V interacts with Kinesin heavy chain (Khc) and negatively regulates Kinesin activity in *osk* mRNA localization, suggesting an antagonizing role of Myo V in microtubule organization. Taken together, coordination of microtubule- and actin-based motor activities may be required for correct *osk* mRNA posterior localization and anchoring (Krauss et al., 2009).

Interestingly, Osk protein itself acts in anchoring. *osk* mRNA produces two protein isoforms, Long and Short Osk, which have distinct functions in pole plasm assembly at the posterior of the oocyte. Short Osk acts in recruiting pole plasm components (Markussen et al., 1995), while Long Osk acts in anchoring of the pole plasm components including *osk* mRNA and Short Osk at the posterior cortex (Vanzo and Ephrussi, 2002). Recently, the two Osk isoforms were shown to stimulate endocytosis at the posterior of the oocyte. This leads to initiation of actin remodeling, and the formation of long F-actin projections emanating from cortical F-actin bundles at the posterior pole of the oocyte. This event is important for anchoring of *osk* mRNA and Osk protein at the posterior cortex (Vanzo et al., 2007).

Consistent with the requirement of actin remodeling in anchoring, several actin-associated proteins such as Bifocal and Homer (Babu et al., 2004), and several proteins involved in endocytosis such as Rabenosyn-5 (Tanaka and

Nakamura, 2008) and Rab11 (Dollar et al., 2002; Jankovics et al., 2001) were found to be involved in *osk* mRNA anchoring. However, the molecular functions of them are still unknown, such as how they associate or interact with *osk* mRNA, and whether specific sequences in the *osk* mRNA (*cis*-acting elements) are involved in its anchoring.

Distinct from the motor-dependent directional transport along the cytoskeleton elements, the bulk cytoplasmic flows that occur in the oocyte during the later stages of oogenesis have been suggested to underlie another form of *osk* mRNA localization (Glotzer et al., 1997). Fluorescently labeled *osk* mRNA that is injected into live oocytes at late stage 9-11 first disperses throughout the cytoplasm, then accumulates at the posterior pole. Injected *osk* mRNA can be transiently localized at the posterior, even in the absence of Osk protein. However, this localization disappears by stage 10 if Osk protein is not present. This suggests that initial posterior localization does not require anchoring (Glotzer et al., 1997).

In summary, transport of the *osk* mRNA throughout oogenesis involves different steps and different mechanisms. Transport to the oocyte relies on the *cis*-acting elements in the 3' UTR and a minus-end-directed, dynein-dependent transport. By contrast, *osk* mRNA posterior localization depends on plus-end-directed, kinesin-1-dependent transport. Splicing of the first intron is required for posterior localization by assembling SOLE and by depositing the EJC components. Other proteins, including Btz, Stau, and Exu, mediate *osk* mRNA posterior localization. Following the microtubule-dependent long-range movement, *osk* mRNA is captured at the posterior cortex, presumably by actin-dependent short-range movement. Finally, the *osk* mRNA is tightly anchored at

the posterior cortex mediated by F-actin cytoskeleton remodeling and endocytosis. Osk protein itself acts in *osk* mRNA and Osk protein anchoring by stimulating actin dynamics and endocytosis at the posterior. In addition to directed motor-dependent movement, bulk cytoplasmic flow also contributes to the late phase of *osk* mRNA localization.

OSKAR MRNA TRANSLATION

Restricted Osk accumulation can be achieved by a complex and coordinated program of *osk* mRNA localization and translational control. Translation is divided into two phases: translational repression during mRNA transport to the posterior pole of the oocyte, and translational activation after reaching the posterior (Kim-Ha et al., 1995; Markussen et al., 1995; Rongo et al., 1995). My work on the role of BSF raises the possibility that BSF acts in *osk* mRNA translational activation during later stage of oogenesis. Here, I explain what is known about mechanisms regulating *osk* mRNA translation.

Translational repression of *osk* mRNA during its transport:

Bruno (Bru) was the first protein shown to act in *osk* mRNA translational repression (Kim-Ha et al., 1995). Bru is a nucleo-cytoplasmic shuttling protein and is thought to bind to the *osk* mRNA in the nurse cell nuclei (Snee et al., 2008). There are multiple Bru binding sequences in the *osk* 3' UTR: the Bruno response elements (BREs)(Kim-Ha et al., 1995), and the type ll and type lll binding sites (Reveal et al., 2010). All of these sites are clustered in two separate regions of the 3' UTR, named the AB and C regions. Mutation of the BREs in the

AB and C regions reduced Bru binding *in vitro* and induced precocious expression of Osk in the oocyte *in vivo* (Kim-Ha et al., 1995). This implicated Bru in repression of translation during mRNA localization. Two models have been proposed to explain how Bru acts in repression. The first model is that Bru recruits Cup, a protein that binds to and inactivates eIF4E (Nakamura et al., 2004). The second model is that Bru oligomerizes multiple *osk* mRNAs into large silencing particles that are inaccessible to the translational machinery (Chekulaeva et al., 2006).

The first model was suggested by data showing Bru interacts with Cup, which in turn interacts with eIF4E (Nakamura et al., 2004; Wilhelm et al., 2003; Zappavigna et al., 2004). Formation of the translation initiation complex is enhanced by the interaction between the cap-binding protein eIF4E and the scaffold protein eIF4G (reviewed in (Jackson et al., 2010)). Therefore, disrupting their interaction has been considered as a common way to inhibit translation (Haghighat et al., 1995; Mader et al., 1995). Cup is an eIF4E-binding protein (eIF-BP) and competes with eIF4G for binding to the same surface on eIF4E (Zappavigna et al., 2004). Mutation of *cup* caused precocious Osk expression (Nakamura et al., 2004; Wilhelm et al., 2003). This suggested that Cup is involved in repression of *osk* mRNA translation during mRNA transport. In addition, Cup physically interacts with Bru *in vivo* (Nakamura et al., 2004). Therefore, the model is that Bru binds to the *osk* mRNA 3' UTR, recruits Cup, which then inhibits the molecule of eIF4E bound to the other end of the same molecule of *osk* mRNA (Nakamura et al., 2004). This model is appealing, but has not been rigorously tested. For example, a stronger case could be made by selectively preventing the Bru/Cup interaction.

The second model was suggested by data showing that Bru promotes *in vitro* oligomerization of an RNA consisting of a short coding region (an epitope tag) fused to two copies of the AB region. When tested in an *in vitro* translation system this RNA forms a large particle with Bru, and is not associated with ribosomal subunits (Chekulaeva et al., 2006). The model that *osk* mRNA is co-packaged in a large RNP *in vivo* is supported by hitchhiking, the phenomenon in which *osk* mRNA can confer localization on another mRNA containing the *osk* 3' UTR but is not itself able to localize (Hachet and Ephrussi, 2004). This suggested a link between two RNAs, either by direct interaction or an interaction mediated by a protein. The silencing particle model implicates Bru as the protein that mediates the link between RNAs. However, a subsequent study showed that the BREs are neither required nor sufficient for hitchhiking *in vivo* (Besse et al., 2009). It isn't clear why Bru mediates RNA oligomerization *in vitro* but appears not to do so *in vivo*. One possible explanation is that the abnormally high density of Bru binding sites in the RNA used for the *in vitro* assays leads to an unnatural assembly of artificial particles.

Although Bru does not appear to oligomerize *osk* mRNA *in vivo*, another protein does appear to have this function. Polypyrimidine tract binding protein (PTB) colocalizes with *osk* mRNA during oogenesis, and directly binds to multiple regions of the *osk* 3' UTR. *heph* is the gene that encodes PTB. In a *heph* mutant the size of endogenous *osk* RNP particles was smaller, and hitchhiking of the *osk* 3'UTR containing RNAs to endogenous *osk* mRNA was reduced. Furthermore, the *heph* mutant showed premature Osk expression during stages 5-6 of oogenesis. Taken together, PTB seems to be involved in *osk* mRNA translational repression presumably through the formation of a large silencing particle (Besse

et al., 2009). Although the repression mechanism by PTB remains unclear, PTB does not seem to act in recruitment of other translational repressors because Bru binding to *osk* mRNA is not abrogated in *heph* mutant (Besse et al., 2009). Mammalian PTB has been shown to act as a chaperone promoting intra- and intermolecular RNA interactions (Auweter and Allain, 2008; Mitchell et al., 2005; Song et al., 2005). Therefore, it is hypothesized that the chaperone activity of PTB may be essential for the multimerization of *osk* mRNAs, thereby forming the densely packed *osk* RNP particles inaccessible to the translation machinery (Besse et al., 2009).

Bicaudal C (Bic-C) is another factor implicated in *osk* mRNA translational repression. Bic-C contains five copies of the KH domain, an RNA binding motif. A mutation within a single KH domain not only weakens RNA binding *in vitr*o but also showed premature Osk expression (Saffman et al., 1998). Moreover, Bic-C overexpression disrupted posterior Osk accumulation (Chicoine et al., 2007). Bic-C has been shown to directly bind to the 5' UTR of *Bic-C* mRNA and recruits the CCR4 deadenylase complex to negatively regulate its own expression (Chicoine et al., 2007). Taken together, it is possible that Bic-C binds to *osk* mRNA to repress translation by deadenylating the poly(A) tail. However, *osk* mRNA was not highly enriched among mRNAs that copurify with Bic-C, and so the cause of depression of *osk* mRNA translation in *Bic-C* mutant ovaries remains uncertain.

Translational activation of *osk* mRNA at the posterior pole of the oocyte.

Once *osk* mRNA is localized, translation begins, which allow us to detect posterior Osk from stage 9 of oogenesis (Kim-Ha et al., 1995)(Fig. 1.2B). However, this initial Osk production is not enough for posterior body patterning

and germ cell formation. Most of Osk is produced after stage 10A of oogenesis through continued expression, and this later phase of Osk production is crucial for body patterning and germ cell formation (Snee et al., 2007). With multiple forms of repression and different phases of translation, multiple forms of translational activation may exist.

One form of activation, called regional activation, involves the inhibition of the repressive activity of Bru. Regional activation occurs in a broad posterior region in the oocyte, does not depend on localization of the mRNA, and does not require any *cis*-acting element in the mRNA. This form of activation is disrupted by Bru mutations that prevent Bru dimerization, suggesting that dimerization of Bru inhibits its ability to repress translation. Loss of Bru dimerization does not affect translation of endogenous *osk* mRNA, and so this form of activation is not essential and is likely redundant with another form of activation (Kim et al., 2015).

Other forms of activation are mediated by *cis*-acting elements, including the IBEs and certain Bru binding sites. In addition, several proteins including Orb, Vasa, and Staufen are also implicated in activation.

Imp-binding elements (IBEs) are sequences located in the 3' UTR that are required for translational activation. The IBEs are binding sites for Imp protein, the *Drosophila* homolog of insulin growth factor ll mRNA binding protein (IMP). There are 13 copies of the consensus IBE motif (UUUAY) in the *osk* 3' UTR. Mutation of all IBEs, or of certain subsets, prevented Osk production at any stage of oogenesis, leading to a strong posterior patterning defect in embryos. The IBEs act in translation, not mRNA localization, as the initial posterior localization of the IBE mutant mRNAs was normal (Munro et al., 2006). Due to the absence of Osk, the mRNAs were not anchored properly at the posterior cortex of the

oocyte. These results suggest that IBEs are critical for activation of translation at the posterior. Although Imp directly binds to IBEs and colocalizes with *osk* mRNA, the role of IBEs in *osk* mRNA translational activation is independent of Imp (Munro et al., 2006). It is still unknown whether the IBEs mediate binding of other factors, or whether IMP serves other roles.

Interestingly, the C region BREs function not only in repression, but also have a role in translational activation (Reveal et al., 2010). While mutations of both the AB and C BREs induced precocious Osk expression in the oocyte, mutations of just the C BREs reduced posterior Osk accumulation at stage 9 onward. Because *osk* mRNA localization and translation are tightly coupled and depend on one another, it is often difficult to distinguish the two processes. However, *osk* mRNA localization was normal in the *C BREs* mutant. Therefore, the C BREs acts specifically in translation rather than localization (Reveal et al., 2010). How the C BREs contribute to activation is still unclear. As the C BREs are near the 3' end of the mRNA, polyadenylation was suggested as a possible role of the C BREs. However, the length of *osk* mRNA poly(A) tail is not changed when the C BREs are mutated. Similarly, the type ll Bru binding sites in the C region (C ll) are also required for translational activation (Reveal et al., 2010). Interestingly, the degree of the activation defect caused by the C ll mutation is stronger than caused by the C BREs mutation, even though Bru binding to the C ll is much weaker than binding to the C BREs. Therefore, another activation factor, in addition to Bru, may also bind to the C ll sites.

The role of Bru could involve recruiting other activation factors. Bru physically interacts with Vasa (Webster et al., 1997) and Orb (Castagnetti and Ephrussi, 2003), two factors implicated in *osk* mRNA translational activation.

Vasa (Vas) is involved in *osk* mRNA translational activation during the late stages of oogenesis. A *vas* mutant showed normal accumulation of Osk at stage 10 of oogenesis (Harris and Macdonald, 2001), but the total level of Osk was largely reduced in the ovary as judged by western blot analysis (Markussen et al., 1995; Rongo et al., 1995). This suggests that Vas acts in a later phase of Osk expression rather than activation at stage 9-10. It is still unknown how Vas works. Vas is an ATP-dependent, DEAD-box RNA helicase. Another DEAD Box helicase, eIF4A, melts secondary structure in the mRNA 5' UTR, which helps ribosome scanning (Gingras et al., 1999). Similarly, Vas may activate translation by restructuring the *osk* mRNA. Vas colocalizes with *osk* mRNA at the oocyte posterior from stage 9 of oogenesis. Association of Vas with *osk* mRNA could be achieved by either Bru or direct binding to *osk* mRNA. Bru was shown to directly interact with Vas (Webster et al., 1997), which suggests that Bru recruits Vas to *osk* mRNA. On the other hand, Vas was shown to bind directly and specifically to the U-rich motif within the *mei-P26* 3' UTR to activate its translation (Liu et al., 2009). Similar to this, Vas could be directly recruited to *osk* mRNA by a U-rich motif present in the *osk* 3' UTR.

Another way in which Bru could mediate activation is through recruitment of Orb to the *osk* mRNA. Orb is the *Drosophila* homolog of *Xenopus* CPEB, a protein that binds to a U-rich cytoplasmic polyadenylation element (CPE) (Hake and Richter, 1994; Stebbins-Boaz et al., 1996) to recruit and stabilize the cytoplasmic polyadenylation machinery (Mendez et al., 2000). In many species, there is a correlation between the translational status of an mRNA and the length of its poly(A) tail (reviewed in (Richter, 1999)). The mRNA active in translation has a long poly(A) tail, while the mRNA repressed in translation has a short

poly(A) tail (Lieberfarb et al., 1996; Salles et al., 1994). One study showed that *osk* mRNA requires a long poly(A) tail for the most efficient translation with *in vitro* translation systems, although how this relates to the situation *in vivo* is not entirely clear. The length of the *osk* mRNA poly(A) tail is still controversial. Orb does appear to have a role in activation of *osk* mRNA translation by regulating the poly(A) tail (Castagnetti and Ephrussi, 2003; Chang et al., 1999). In *orb* mutant ovaries *osk* mRNA has a somewhat shortened poly(A) tail and Osk protein levels are reduced. One important question is whether this function of Orb serves as a prerequisite for *osk* mRNA translation, or if it is an event that occurs specifically at the posterior pole of the oocyte for local activation of translation. A comparison of *osk* mRNAs extracted from two groups of egg chambers, early (up to stage 5, when *osk* mRNA is not translated) and late (5-14, when *osk* mRNA is translated), showed that both groups had poly(A) tails of similar lengths. This argues that Orb-dependent polyadenylation does not occur solely at the time when *osk* mRNA translation occurs, and therefore serves as a prerequisite but does not immediately trigger translation (Castagnetti and Ephrussi, 2003). Furthermore, the *osk* mRNA translation defect in *orb* mutants might be, to some extent, due to an mRNA localization defect. Over 30% of oocytes of a hypomorphic *orb* mutant showed abnormal localization of *osk* mRNA (Castagnetti and Ephrussi, 2003), and a strong *orb* mutant failed to localize *osk* mRNA to the posterior pole of the oocyte (Christerson and McKearin, 1994). In this strong *orb* mutant microtubule organization was also disrupted (Martin et al., 2003). Therefore, Orb could stimulate *osk* mRNA translation by adding or maintaining the poly(A) tail, could act in *osk* mRNA localization by regulating microtubule organization, or both.

Interestingly, Orb interacts physically with Bic-C (a negative regulator of *osk* mRNA translation, presumably acting by deadenylating *osk* mRNA)(Saffman et al., 1998) and Bru (a negative/positive regulator of *osk* mRNA translation) (Castagnetti and Ephrussi, 2003). This supports the idea that regulation of poly(A) tail is used as one of the mechanisms regulating *osk* mRNA translation, although the experiments with Bru suggest otherwise.

Like Orb, Staufen (Stau) is a protein involved in both *osk* mRNA localization and translation. Mutants of *stau* fail to localize *osk* mRNA, revealing a role in that process. Localization of *osk* mRNA is typically required for its translation, and so Stau has at least an indirect role in activation. Evidence for a more direct role came from experiments with the *osk ABC BRE*$^{-}$ transgene, which is defective in repression and produces Osk protein precociously and independent of mRNA localization. In the *stau* mutant the precocious translation of Osk from *osk ABC BRE*$^{-}$ was lost. Further evidence of a direct role for Stau in activation of *osk* mRNA translation came from analysis of engineered *stau* mutants. Stau contains five double-stranded-RNA-binding domains (dsRBDs). Mutation of dsRBD2 disrupted *osk* mRNA posterior localization. By contrast, mutation of dsRBD5 had no effect on *osk* mRNA localization, but disrupted translational activation (Micklem et al., 2000). Since dsRBD5 is not required for RNA binding, the contribution of Stau in *osk* mRNA translational activation is probably recruitment of other proteins that have not been identified yet (Micklem et al., 2000).

In summary, *osk* mRNA translation is tightly regulated by many *cis*- and *trans*-acting factors. During mRNA localization, translation is repressed. Bru, Cup, and Bic-C are involved in repression. Once RNA reaches the posterior pole

of the oocyte, repression is relieved and translation is activated. One form of activation is inhibition of the repressive activity of Bru, which occurs in the posterior region of the oocyte and does not require mRNA localization or a regulatory element in the *osk* mRNA. Other forms of activation rely on *cis*-acting elements including the IBEs and the C region Bru binding sites. *trans*-acting factors including Vas, Orb, and Stau are involved in activation. Some of the proteins are involved in multiple processes. Orb and Stau acts in both *osk* mRNA localization and translational activation. Bru acts in both *osk* mRNA translational activation and repression. How these forms of activation occur remains unclear.

OSKAR mRNA FUNCTION

RNAs are typically categorized by their coding potential. One group consists of mRNAs. These RNAs have a coding function and encode proteins. Another group has no coding function. These RNAs can be involved in variety of biological processes such as gene expression at the levels of transcription, RNA processing, and translation, protection of genomes from foreign nucleic acids, and guidance for DNA synthesis or rearrangement. Mostly, noncoding RNAs perform their function as an RNA-protein complex. Examples include the ribosome, snRNPs, telomerase, and microRNAs. A ribozyme acts in an exceptional manner, as the RNA structure itself performs the enzymatic function (reviewed in (Cech and Steitz, 2014)). *Xenopus VegT* mRNA is an unusual example of an mRNA that performs both functions. As an mRNA, it contains long open reading frame encoding a transcription factor required for endoderm formation in *Xenopus* oocyte. *VegT* mRNA itself is also required for organization of the cytokeratin cytoskeleton in the vegetal cortex of the oocyte, however VegT

protein is dispensable for this structural function (Kloc et al., 2007; Kloc et al., 2005).

Another mRNA having both coding and noncoding function is *Drosophila osk*. As an mRNA, *osk* encodes a protein required for embryonic body patterning and germ cell formation (Ephrussi et al., 1991; Kim-Ha et al., 1991). In addition to this coding function, *osk* mRNA is also required for progression of oogenesis and formation of the karyosome in the oocyte. *osk* mutants that lack *osk* mRNA (*osk* RNA null mutants) arrest oogenesis and have fragmented karyosomes (Jenny et al., 2006). Osk protein is not required for this part of *osk* gene function because *osk* nonsense mutant alleles do not arrest oogenesis (Kim-Ha et al., 1991). Furthermore, expression of the *osk* 3' UTR alone was sufficient to rescue the oogenesis arrest of *osk* RNA null mutants, indicating that the *osk* 3'UTR provides the noncoding RNA function (Jenny et al., 2006).

Recently, essential noncoding functional elements in the *osk* 3' UTR were identified (Kanke et al., 2015). The essential elements are clustered close together in the C region and are of three types. One type of essential element consists of the C region Bru binding sites. A second type of essential element is defined by two adjacent clusters of mutations, each altering 5 nucleotides. The factor or factors that bind this element is unknown. The third type of element consists of A-rich sequences, which appear to serve as binding sites for poly(A) binding protein (PABP). Mutation of these elements largely eliminated noncoding function, with phenotypes almost identical to those caused by complete absence of *osk* mRNA. Neither RNA levels nor the RNA accumulation pattern in early-stage egg chambers was affected by mutation of these elements. Therefore, loss of the *osk* RNA noncoding function is not due to affecting RNA stability or RNA

distribution (Kanke et al., 2015). How this cluster of essential elements functions is not yet known.

BICOID STABILITY FACTOR

I found that Bicoid Stability Factor (BSF) binds to the *osk* RNA 3' region involved in both regulation of *osk* expression and the noncoding function of *osk* mRNA. Here, I briefly explain BSF.

BSF was previously identified as a cytoplasmic ovarian protein which bound to *bcd* mRNA 3' UTR, and showed a redundant role in stabilization of *bcd* RNA during oogenesis (Mancebo et al., 2001). BSF is a member of the family of Leucine-rich pentatricopeptide repeat-containing (LRPPRC) proteins (Mancebo et al., 2001; Sterky et al., 2010). The pentatriocopeptide repeat (PPR) is a canonical 35 amino acid motif that functions as an RNA binding domain. It can appear in many copies in a single protein, repeated up to 30 times. The PPR protein family was initially found in plants (Small and Peeters, 2000). A large number of PPR proteins from plants have been discovered and shown to act in a wide range of RNA processing events, such as RNA editing, splicing, cleavage, and translation within chloroplasts and mitochondria (reviewed in (Schmitz-Linneweber and Small, 2008)). Mammalian genomes have only a few members of this gene family, and their functions and molecular mechanisms have been partially elucidated. BSF as a LRPPRC protein is mainly found in mitochondria and this may be the predominant location at least in some tissues (Bratic et al., 2011). Within mitochondria, reducing the level of BSF by RNAi knockdown (KD) affects mitochondrial transcription, mRNA polyadenylation, and translation (Bratic

et al., 2011). BSF was also reported to have a role in regulation of early zygotic genes expression in early embryogenesis by binding to a short consensus sequence in the 5' UTR region of the DNA (De Renzis et al., 2007).

Characterization of *bsf* gene expression patterns in *Drosophila* females shows the highest expression level in ovaries, with lower expression in other tissues (flybase website). A *bsf* mutant is lethal, and when tested in germ line clones arrests oogenesis at a very early stage of development (De Renzis et al., 2007). Therefore BSF is required for *Drosophila* oogenesis. However, it still largely remains unknown what *bsf* functions are, both in ovaries and in other tissues.

OVERVIEW OF DISSERTATION RESEARCH

The main goal of my dissertation research is to elucidate the mechanism regulating *osk* expression by BSF, a protein that I found to bind to 3' sequences in the *osk* mRNA.

We identified essential elements for *osk* RNA noncoding function positioned in a short region (the C region) near the 3' end of the *osk* mRNA. They are in close proximity to the elements required for mRNA translational activation. I initially searched for proteins that bind specifically to this region of the mRNA to better understand the roles of this region. BSF was found as a binding factor. Because two mutations in *osk* mRNA that reduce BSF binding have no effect on the noncoding function of *osk* RNA, BSF does not appear to be required for this function. However, such specific binding is strongly suggestive of a functional role. Furthermore, the close proximity of the BSF binding site to Bru binding sites

required for activation of *osk* mRNA translation raises the possibility that BSF has a similar role. I tested this possibility using two complementary approaches: reducing BSF levels and disrupting BSF binding.

Knock down (KD) of *bsf* during oogenesis caused a decrease of Osk accumulation at the posterior pole of the oocyte during later oogenesis (after stage 10), and a decrease of both Osk protein and *osk* RNA accumulation at the posterior end during early embryogenesis with a consistent correlation between reduced protein and RNA. However, neither protein nor RNA accumulation was affected by *bsf* KD during oogenesis up to stage 10. Disruption of the interaction between BSF and *osk* mRNA by mutating the BSF binding sites generated similar results. Mutating the BSF binding sites did not affect RNA levels. This suggests that BSF does not act in *osk* mRNA stability. As longer poly(A) tails typically correlate with enhanced translation, I examined poly(A) tail length to test whether BSF directly affects translation. I found no decrease in poly(A) tail length of the mutant *osk* mRNA relative to control. According to the results so far, two possible roles of BSF could be suggested. BSF could act in *osk* mRNA localization. Because only localized *osk* mRNA is competent for translation, a defect in mRNA localization would lead to loss of Osk expression. Alternatively, BSF could act in a late phase of *osk* mRNA translational activation. Because Osk is required for *osk* mRNA anchoring to the posterior cortex, a defect in Osk expression would lead to dispersal of the mRNA.

Interestingly, our results showed that the same *cis*-acting elements are required for different purposes at different times in *Drosophila* oogenesis. The essential elements for the *osk* RNA noncoding function acting in early in oogenesis are also crucial for *osk* mRNA translation later in oogenesis. It is

unknown how they act. The *trans*-acting factors bound to this region (such as Bru, BSF, and PABP) could act together for regulating *osk* translation or noncoding function. It will be worth finding binding partners of them, and it will be interesting to determine whether and how they all interact to regulate *osk*.

FIGURES

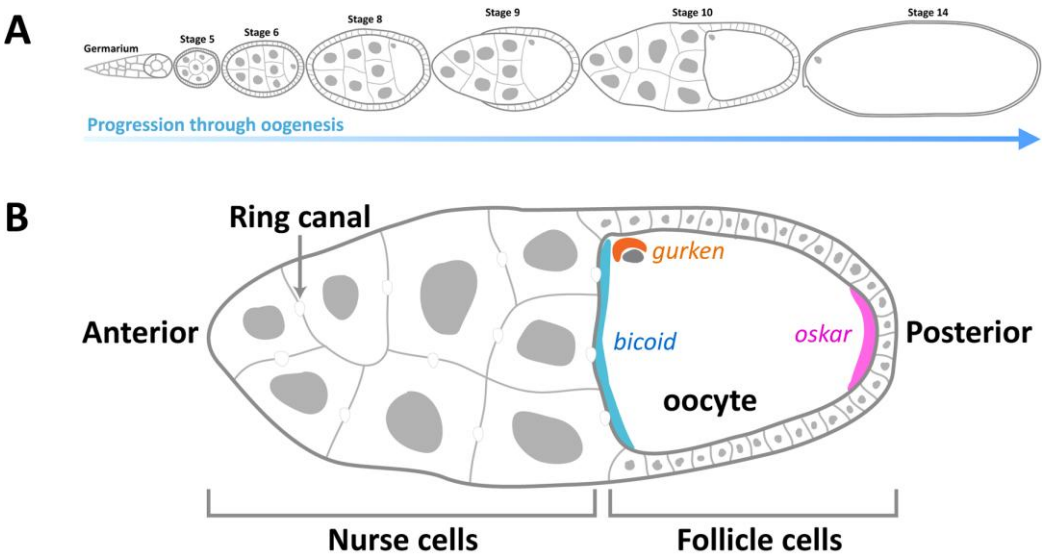

Figure 1.1. *Drosophila* oogenesis

A. A single ovariole. Egg chambers of increasing age are displayed from left to right. The germarium contains germline and somatic stem cells that produce cells in an egg chamber. Nurse cells degenerated near the end of oogenesis.
B. An egg chamber contains germline-derived nurse cells and the oocyte interconnected by ring canals, and somatic follicle cells that surround the oocyte (and nuse cells at earlier stages). Three localized determinants are shown in a stage 10 egg chamber.

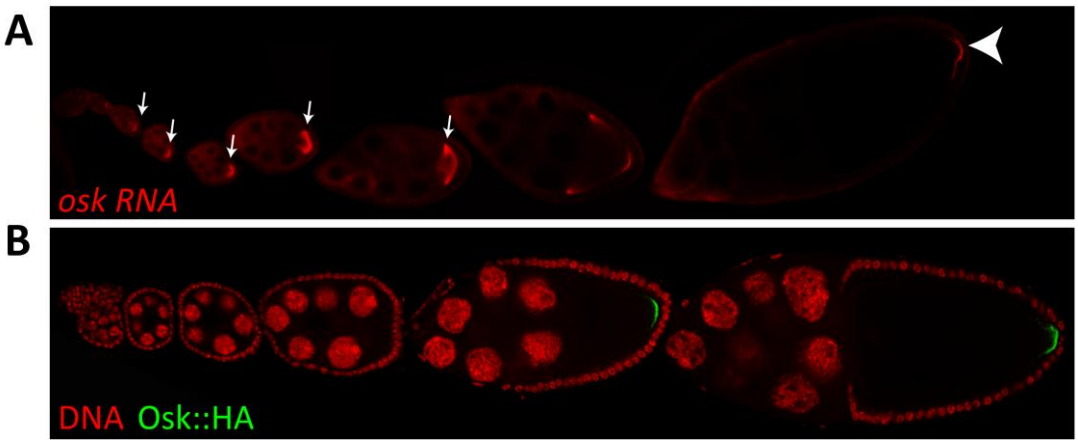

Figure 1.2. *osk* mRNA and Osk protein distributions during oogenesis

A. *osk* mRNA is initially concentrated in the oocyte (arrows) during early oogenesis, and it eventually becomes localized to the posterior pole of the oocyte (arrowhead) from stage 9 onward. *osk* mRNA is red.
B. Osk protein is not detected during early stages of oogenesis. Osk protein appears only at the posterior pole of the oocyte from stage 9 onward. DNA is red, Osk::HA is green.

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Chapter 2: RNA Sequences Required for the Noncoding Function of *oskar* RNA also Mediate Regulation of Oskar Protein Expression by Bicoid Stability Factor

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ABSTRACT

The *Drosophila oskar* (*osk*) mRNA is unusual in having both coding and noncoding functions. As an mRNA, *osk* encodes a protein which is deployed specifically at the posterior of the oocyte. This spatially-restricted deployment relies on a program of mRNA localization and both repression and activation of translation, all dependent on regulatory elements located primarily in the 3' untranslated region (UTR) of the mRNA. The 3' UTR also mediates the noncoding function of *osk*, which is essential for progression through oogenesis. Mutations which most strongly disrupt the noncoding function are positioned in a short region (the C region) near the 3' end of the mRNA, in close proximity to elements required for activation of translation. We show that Bicoid Stability Factor (BSF) binds specifically to the C region of the mRNA. Both knockdown of *bsf* and mutation of BSF binding sites in *osk* mRNA have the same consequences: Osk expression is largely eliminated late in oogenesis, with both mRNA localization and translation disrupted. Although the C region of the *osk* 3' UTR is required for the noncoding function, BSF binding does not appear to be essential for that function.

INTRODUCTION

One way to categorize RNAs is by their coding potential, or lack thereof. Members of one group, the mRNAs, have long open reading frames and are translated, thereby performing a coding function. The other group, consisting of RNAs without long open reading frames, has many members with no consistent size or organization. Such noncoding RNAs perform a wide variety of structural, regulatory and enzymatic functions (Cech and Steitz, 2014). Often, these coding

and noncoding roles are mutually exclusive. Most of the exceptions involve small ORFs, which can encode short peptides, in long noncoding RNAs (lncRNAs)(Andrews and Rothnagel, 2014; Bazzini et al., 2014; Anderson et al., 2015). Rarely, more dramatic overlap in function has been observed for conventional mRNAs with long open reading frames. The *Xenopus VegT* mRNA encodes a transcription factor required for endoderm formation in the embryo. The same mRNA also has a structural role in organization of the cytokeratin cytoskeleton (Heasman et al., 2001; Kloc et al., 2005; Kloc et al., 2007). Depletion of *VegT* mRNA leads to fragmentation of the cytokeratin network in the vegetal cortex of the oocyte. Sequences within much of the mRNA appear to act redundantly in controlling the organization of the cytokeratin network, with a functional element contained within a 300 nt portion of the 3' UTR sufficient to induce depolymerization of cytokeratin filaments (Kloc et al., 2011).

A second mRNA with essential coding and noncoding functions is *oskar* (*osk*), from *Drosophila*. Osk protein is expressed specifically at the posterior pole of the oocyte and early embryo, where it is responsible for embryonic body patterning and germ cell formation (Lehmann and Nüsslein-Volhard, 1986; Kim-Ha et al., 1991; Ephrussi et al., 1991). In the absence of Osk protein, oogenesis progresses normally except for the failure to assemble posterior pole plasm in the oocyte. Although eggs are produced, the embryos fail to form abdominal segments and die. This coding role for *osk* places substantial constraints on the mRNA sequence. The open reading frame is constrained by the need to encode Osk protein. In addition, noncoding regions are constrained by the elaborate regulation required to restrict Osk protein expression to a discrete subcellular domain: misexpression of Osk is just as lethal as loss of Osk (Ephrussi and

Lehmann, 1992; Smith et al., 1992). The *osk* mRNA is also needed, independent of its coding role, for progression through oogenesis. In the absence of *osk* mRNA a variety of defects emerge in the organization of the egg chamber, with oogenesis arrested and no eggs produced (Jenny et al., 2006; Kanke et al., 2015). These defects are present well before the developmental stage when Osk protein first appears, and the *osk* RNA function does not require the *osk* coding region. Instead, the *osk* mRNA 3' UTR mediates the noncoding function, placing constraints on the sequence of that region of the mRNA.

Deployment of Osk protein specifically at the posterior pole of the oocyte involves a complex and coordinated program of mRNA localization and translational control. *osk* mRNA is transcribed in the nurse cells and transported into the oocyte through cytoplasmic bridges. Within the oocyte, *osk* mRNA is transiently enriched at different positions, culminating in persistent posterior localization starting at stage 9; this is when Osk protein first accumulates (Kim-Ha et al., 1991; Ephrussi et al., 1991; Kim-Ha et al., 1995; Rongo et al., 1995; Markussen et al., 1995). Translational repression serves to prevent expression from *osk* mRNA that has not yet been localized, or has failed to become localized (Kim-Ha et al., 1995; Nakamura et al., 2001; Wilhelm et al., 2003; Nakamura et al., 2004; Besse et al., 2009). Once *osk* mRNA is localized, translational activation must then override repression and allow Osk protein to be made. Many factors and regulatory elements are required for this regulation (Lipshitz and Smibert, 2000; Besse and Ephrussi, 2008; Lasko, 2012), with most of the elements positioned in the 3' UTR (Kim-Ha et al., 1993; Kim-Ha et al., 1995; Munro et al., 2006; Reveal et al., 2010; Vazquez-Pianzola et al., 2011; Jambor et al., 2014). Among the elements are a number of binding sites for Bru (BREs and

others), clustered in two regions of the 3' UTR: the AB region (close to the coding region), and the C region (close to the 3' end). Mutation of all the BREs disrupts translational repression, revealing the role of Bru as a repressor (Kim-Ha et al., 1995). By contrast, mutation of only the C region cluster of BREs disrupts translational activation, implicating Bru in activation, as well as repression (Reveal et al., 2010). Similarly, mutation of the Bru type II binding sites (Reveal et al., 2011) in the C region also disrupts translational activation (Reveal et al., 2010).

The noncoding role of *osk* mRNA is mediated by the 3' UTR (Jenny et al., 2006). One part of this role is to sequester Bru, and this relies on the Bru binding sites that also mediate regulation (Kanke et al., 2015). Of greater importance to the noncoding requirement for *osk* mRNA are sequences positioned close to the mRNA 3' end in the C region, including the Bru binding sites that activate translation. These C region Bru binding sites contribute to sequestration of Bru, but also play a separate and essential role in *osk* noncoding function (Kanke et al., 2015). Additional sequences essential for the noncoding function, which do not bind Bru, are positioned nearby. Some of the sequences in this region appear to act by binding poly(A) binding protein (PABP) (Vazquez-Pianzola et al., 2011; Kanke et al., 2015). However, the mutations which most strongly disrupt *osk* RNA function are not PABP binding sites, and the factor expected to bind them has not been identified (Kanke et al., 2015).

To better understand the roles of the C region of the *osk* mRNA we searched for proteins which bind specifically to the essential sequences. Here we show that Bicoid Stability Factor (BSF), a protein previously found to act in stabilizing the *bicoid* mRNA (Mancebo et al., 2001), binds to the *osk* C region,

with binding dependent on sequences most critical for *osk* RNA function early in oogenesis. Surprisingly, we find that the same sequences are also required again, late in oogenesis, for regulation of *osk* expression. BSF mediates this later function, as shown in two complementary approaches. However, binding of BSF to the C region does not appear to be responsible for the early function, as certain mutations which substantially reduce BSF binding have no effect on the noncoding role of *osk* mRNA. Why regulatory and functional elements should be superimposed in the RNA sequence is an intriguing question, as the *osk* 3' UTR is quite large and thus does not seem to be constrained in size.

RESULTS

Proteins that bind close to the 3' end of the *osk* mRNA

To identify proteins that bind to the C region of the *osk* mRNA 3' UTR, an affinity purification approach with a streptavidin-binding aptamer (*S1* aptamer)(Walker et al., 2008) was used. Transcripts consisting of the final 150 nt of the *osk* mRNA (the C region) fused to the *S1* aptamer (*oskC::S1*) were bound to streptavidin beads and mixed with ovary extracts to allow assembly of RNP complexes. After washing and recovery of the beads (the pellet fraction), many proteins co-purified (Fig. 2.1A). These are expected to include a large number of non-specific binding proteins, in addition to proteins bound specifically. Indeed, purification using just the *S1* RNA yielded a very similar collection of proteins, as judged by staining of total protein (Fig. 2.1A) and by detection of individual proteins (Fig. 2.1B). As an initial test to identify proteins bound specifically to the *osk* C region RNA, a parallel purification was performed with a mutant version of

the RNA, carrying the *osk3'977-981* mutation that (in the context of the intact *osk* mRNA) largely abolishes *osk* RNA function (Kanke et al., 2015). One protein not bound to *oskC3'977-981::S1*, or to *S1* alone, migrated with an apparent size of 150 kDa. Mass spectrometry of the 150 kDa band identified Bicoid Stability Factor (BSF) as the most abundant protein. Western blot analysis of samples from the affinity purifications confirmed this identification (Fig. 2.1B). The western blots were also probed for Bru, which binds to sites within the C region. As expected, Bru did not bind the *S1* RNA, but did bind both *oskC::S1* and *oskC3'977-981::S1* (Fig. 2.1B; Kanke et al., 2015)(the 977-981 mutation does not affect the Bru binding sites; Fig. 2.1D).

Additional transcripts bearing other mutations were tested for BSF binding to examine whether the correlation between loss of BSF binding (Fig. 2.1C) and loss of *osk* RNA function (Fig. 2.1D) would be extended. Mutant *oskC3'984-988::S1* was as strongly defective as *oskC3'977-981::S1*, with BSF binding reduced to a background level. Mutants *oskC3'970-974::S1* and *oskC3'997-1001::S1* also had substantially reduced BSF binding, although still above background. The remaining mutants, *oskC3'990-994::S1* and *oskC3'1004-1008::S1,* had strong BSF binding, similar to the wild type level (Fig. 2.1C). Comparison of the effects of mutations on BSF binding and noncoding *osk* RNA function reveals that two mutants - *oskC3'970-974* and *oskC3'997-1001* - were clearly impaired for BSF binding (with the stronger effect for *oskC3'970-974*), yet both retained a wild type level of *osk* RNA function (Fig. 2.1D; Kanke et al., 2015). Thus, BSF binding appears not to be required for the noncoding *osk* RNA function, although we cannot exclude the possibility that the residual weak binding of these mutants is sufficient for this function.

bsf is required for accumulation of Osk protein

Although BSF binding to the *osk* C region does not appear to be essential for the noncoding *osk* RNA function, such specific binding is strongly suggestive of a functional role. Furthermore, the close proximity of the BSF binding site to Bru binding sites required for activation of *osk* mRNA translation raises the possibility that BSF has a similar role. Characterization of Osk protein expression in a *bsf* mutant is problematic, as oogenesis is blocked at an early stage (De Renzis et al., 2007), while translation of *osk* mRNA occurs much later in oogenesis. A knock down (KD) approach could avoid this problem, as depletion of BSF might not occur quickly enough to interfere with its role early in oogenesis. The TRiP-*bsf* transgene (a strain from the Transgenic RNAi Project (TRiP), see Methods) was expressed in the female germ line under control of GAL4 drivers. In initial experiments the *nosGAL4::VP16-nos.UTR* driver, which is active at the earliest stages of oogenesis (Van Doren et al., 1998), was used. Most egg chambers failed to develop to the later stages (Fig. 2.6), consistent with the prior analysis of the *bsf* mutant (De Renzis et al., 2007). We therefore used the *matalpha4-GAL-VP16* Gal4 driver, which is not strongly active very early in oogenesis. To assess the efficiency of *bsf* KD, BSF protein was monitored in ovaries and early stage embryos (prior to the onset of zygotic transcription) by western blot analysis. The level of BSF was reduced in ovaries, with a much stronger reduction in early-stage embryos (Fig. 2.2A).

Osk protein expression was monitored in the *bsf* KD ovaries by immunodetection of endogenous Osk, or of Osk::HA expressed from an epitope-tagged *osk* transgene (Kim et al., 2015; Kanke and Macdonald, 2015); both

methods gave similar results. In the *bsf* KD ovaries, Osk protein expression appeared normal up to stage 10 (Fig. 2.2B, Fig. 2.7), when deposition of the vitelline membrane interferes with antibody accessibility. Further reducing BSF levels in flies also heterozygous for the *bsf* ^{SH1181} mutation also had no effect (Fig. 2.2B, Fig. 2.7). Consistent with this, the accumulation of *osk* RNA was also similar to that in wild type egg chamber up to stage 10 (Fig. 2.2C).

To examine the later stages of oogenesis, when most of Osk protein is made, an *osk::GFP* transgene with the entire *osk* gene and regulatory elements was used (Fig. 2.2D)(Snee et al., 2007). Detection of GFP fluorescence circumvents the problem of the vitelline membrane restricting access for antibodies. In a wild type background Osk::GFP appeared at the posterior pole of stage 9 oocytes, just as for endogenous Osk. This distribution persisted for the remainder of oogenesis, with the signal strength increasing at the later stages. For the *bsf* KD egg chambers, the early pattern of Osk::GFP was similar to that in wild type. However, at later stages of oogenesis defects in Osk::GFP expression appeared: although most late stage oocytes retained the high level typically found in wild type, some had either weak or undetectable levels (Fig. 2.2E). To further enhance depletion of BSF, the same experiments were also performed with flies heterozygous for *bsf* ^{SH1181}; the defects were stronger, with most oocytes having no detectable Osk::GFP (Fig. 2.2E).

We also monitored localization of *osk::GFP* mRNA in the *bsf* KD. Because the effect on Osk::GFP protein was variable, double-labeling experiments were performed to detect both the mRNA and protein in the same samples and thus reveal any correlation in defects. For this analysis early embryos were used, as the vitelline membrane of late stage egg chambers interferes with the *in situ*

hybridization method. In wild type embryos both Osk::GFP protein and *osk::GFP* RNA were present at high levels at the posterior pole. By contrast, most embryos from *bsf* KD mothers heterozygous for *bsf* ^{SH1181} had weak or undetectable levels of both Osk::GFP protein and *osk::GFP* mRNA, with a consistent correlation between reduced mRNA and reduced protein (Fig. 2.2F).

The knockdown results implicate BSF in posterior accumulation of Osk protein and *osk* RNA. BSF could have an indirect effect, although the binding of BSF to the *osk* mRNA suggests a direct role. Furthermore, BSF is present at the posterior pole of the oocyte where *osk* mRNA is localized (Fig. 2.2G), consistent with persistent binding and a direct role. This posterior crescent of BSF was substantially reduced in the *bsf* KD (Fig. 2.2G).

Loss of BSF binding affects Osk protein expression but not *osk* mRNA stability

To complement the studies in which BSF protein is depleted, we also examined the consequences of mutating the *osk* mRNA to disrupt BSF binding. Similar results from both approaches would strengthen the argument that BSF regulates Osk expression. Furthermore, defects in *osk* expression associated with loss of BSF binding would strongly support the model that BSF acts directly.

Four mutations in the *osk* 3' UTR C region affected BSF binding: the *3'970-974* and *3'997-1001* mutations reduced binding, and the *3'977-981* and *3'984-988* mutations further reduced binding to background levels (Fig. 2.1C). For analysis of Osk expression, the standard approach is to place a mutant transgene in an *osk* RNA null background so that it is the only source of *osk*

mRNA and protein. Two features of *osk* mRNA create complications for analysis of these transgenes.

One complication involves the bifunctionality of *osk* mRNA, with its coding and noncoding roles. The noncoding function of *osk* mRNA is required for progression through oogenesis: in the absence of *osk* mRNA, oogenesis is arrested at stage 6/7, before the normal onset of Osk protein expression (Jenny et al., 2006). When tested in the *osk* RNA null background, most *osk* transgenes provide full *osk* RNA function, enabling progression through oogenesis and evaluation of Osk expression. However, the two mutations that most strongly disrupt BSF binding, *3'977-981* and *3'984-988*, essentially eliminate *osk* RNA function (Kanke et al., 2015). Consequently, oogenesis remains arrested when *osk* transgenes with these mutations are tested in *osk* RNA null flies, and an effect on Osk protein expression cannot be evaluated. The solution is to co-express these mutants with a helper RNA, an *osk* mRNA that provides the noncoding function, but cannot itself make Osk protein. Thus, oogenesis progresses normally, and the ability of a mutant transgene to make Osk protein (or embryonic patterning activity) can be monitored. A helper *osk* mRNA that has full RNA function but is protein null and cannot make Osk protein is osk^{54}, which has a small insertion in the first exon coding region that alters the reading frame and introduces a stop codon (Kim-Ha et al., 1991).

The second complication is that some forms of *osk* translational control are subject to the phenomenon of regulation in *trans* (Reveal et al., 2010). The concept of regulation in *trans* has been invoked to explain how translational control elements on one RNA molecule can influence the translational regulation of another RNA molecule. We hypothesized that regulation in *trans* is made

possible by assembly of the RNAs in particles, placing them in close proximity. Under these circumstances, long range interactions that underlie certain forms of regulation (e.g. between molecules bound to distant parts of an mRNA, such as the 5' cap and the 3' UTR) would now have the potential to occur between different RNA molecules. As an example, the phenomenon of regulation in *trans* is displayed by *osk* transcripts with mutations in the C region Bru binding sites. When expressed as the only *osk* mRNA in an *osk* RNA null background, *osk C BRE*$^{-}$ is strongly defective in activation of translation (Fig. 2.3A). However, this defect is largely eliminated by co-expression with other *osk* transcripts which have wild type C region BREs, but are themselves unable to make functional Osk protein (e.g. *osk*^{54})(Fig. 2.3A)(Reveal et al., 2010). The only source of functional Osk protein in this experiment was from *osk C BRE*$^{-}$, and so its activation defect must have been suppressed.

Just as the *osk C BRE*$^{-}$ regulatory defect can be rescued in *trans*, a regulatory defect of *osk3'977-981* and *osk3'984-988* mutants might also be rescued in *trans*. The problem is that we can't test the *osk3'977-981* and *osk3'984-988* mutants in the absence of other *osk* mRNAs (to eliminate the possibility of rescue in *trans*), because a helper *osk* mRNA is required to provide the noncoding *osk* RNA function these mutants lack, and thus allow developmental progression to the later stages of oogenesis when Osk protein is expressed.

A solution to this experimental challenge is to use *osk IBE*$^{-}$ transgenes to provide the helper RNA. These transgenes have mutations in binding sites for Imp, which are spread throughout the *osk* 3'UTR. Mutation of certain subsets of IBEs or all IBEs have the same effects: a complete absence of Osk protein, with

the noncoding *osk* RNA function remaining intact (Munro et al., 2006). Importantly, the *osk IBE*$^{-}$ mutant does not participate in regulation in *trans*: its activation defect is not rescued at all by the presence of *osk* mRNAs with intact IBEs (Reveal et al., 2010), and it does not strongly rescue the activation defects of other *osk* mutants (e.g. *osk C BRE*$^{-}$, Fig. 2.3A; unpublished). Therefore, the *osk IBE*$^{-}$ mRNA can be used to provide the early noncoding *osk* RNA function that mutants *osk3'977-981* and *osk3'984-988* lack, but the *osk IBE*$^{-}$ mRNA will not influence the ability of these mutants to make Osk protein later in oogenesis.

Transgenes with mutations that disrupt BSF binding, or with the interdigitated mutation that does not affect BSF binding, were tested for their ability to provide *osk* patterning activity in the presence of either *osk*^{54} or *osk IBE*$^{-}$ helper RNAs (Fig. 2.3A). The mutant with normal BSF binding, *osk3'990-994*, is largely defective in the noncoding *osk* RNA function, and produces very few eggs (Kanke et al., 2015)(Fig. 2.3A). The *osk*^{54} helper RNA fully rescued the noncoding function of *osk3'990-994*, allowing production of embryos, and these embryos had no patterning defects. Similar results were obtained with *osk3'990-994* in combination with the *osk IBE*$^{-}$ helper RNA. From these results we conclude that the *osk3'990-994* mutation affected only the noncoding *osk* RNA function, and did not substantially alter regulation of *osk* expression.

By contrast, the mutants with BSF binding defects all displayed evidence of reduced *osk* patterning activity (Fig. 2.3A). The *osk3'977-981* and *osk3'984-988* mutants have the strongest defects in BSF binding. Because these mutants are completely defective in the noncoding *osk* RNA function (Kanke et al., 2015), they produce no embryos in the absence of a helper *osk* mRNA. When these mutants were co-expressed with the *osk*^{54} helper RNA, patterning was effectively

wild type. However, when co-expressed with the *osk IBE*$^{-}$ helper RNA, only very low levels of *osk* patterning activity were produced. From these results we conclude that the *osk3'977-981* and *osk3'984-988* mutants each had defects in *osk* expression, as observed when co-expressed with the *osk IBE*$^{-}$ helper RNA. Furthermore, these defects could be rescued in *trans*, as shown by the absence of significant patterning defects when the *osk3'977-981* and *osk3'984-988* mutants were co-expressed with the *osk*^{54} helper RNA. Thus, the mutants with the strongest defects in BSF binding had reduced or almost undetectable *osk* activity.

The *osk3'970-974* and *osk3'997-1001* mutants also interfere with BSF binding but to a lesser degree. These mutants can produce embryos without a helper RNA, because neither affects the noncoding *osk* RNA function (Kanke et al., 2015). The embryos from both mutants were missing abdominal segments, the phenotype caused by reduced levels of *osk* activity. The severity of the patterning defects of the *osk3'970-974* and *osk3'997-1001* mutants correlated with the effect on BSF binding, although the weaker *osk3'997-1001* mutant also has a lower level of mRNA (Kanke et al., 2015). Using the helper RNAs to test for regulation in *trans*, the patterning defects of *osk3'970-974* and *osk3'997-1001* were fully rescued by co-expression with *osk*^{54}, and only very weakly rescued by co-expression with *osk IBE*$^{-}$. Thus, all of the mutants with defects in BSF binding had patterning defects consistent with reduced *osk* activity, and all displayed regulation in *trans*.

To confirm that the loss of *osk* patterning activity of the mutants unable to bind BSF was due to a defect in Osk protein expression, we monitored Osk::GFP produced by *oskT140::GFP* transgenes, either a wild type version or versions

bearing the *3'977-981* or *3'984-988* mutations (which most strongly affect BSF binding)(Fig. 2.4A). The transgenes were co-expressed with the *osk IBE*$^{-}$ helper to provide noncoding *osk* RNA function. The results were similar to those obtained with the *bsf* KD. No change in Osk::GFP expression between the wild type and mutants was detected up to stage 10 of oogenesis (Fig. 2.4B). However, in late stage oocytes and early stage embryos the mutants had dramatically reduced levels of Osk::GFP (Fig. 2.4C,D).

One explanation for the defects caused by the *3'977-981* and *3'984-988* mutations is destabilization of the mRNA, an option suggested by the role of *bsf* in stabilizing *bcd* mRNA (Mancebo et al., 2001). To ensure that transgene mRNA levels were not influenced by differences in transcription, all of the *oskT140::GFP* transgenes used in Fig. 2.4 were introduced by phiC31 transgenesis to the same target site in the genome, thus avoiding variation in transcription due to insertion site. Neither of the mutants had decreased mRNA levels relative to the control (Fig. 2.4E), demonstrating that mutation of the BSF binding sites does not cause destabilization of the mRNA.

Close proximity in Bru and BSF binding

The *3'970-974* mutation disrupts a type II Bru binding site (Reveal et al., 2010; Reveal et al., 2011), interferes with BSF binding (Fig. 2.1D), and causes reduced *osk* patterning activity (Fig. 2.3A). A mutant with both of the *osk* C region type II Bru binding sites disrupted (*osk C II*$^{-}$) has a strong defect in activation of *osk* mRNA translation (Reveal et al., 2010)(Fig. 2.3B), and the patterning defect of the *osk C II*$^{-}$ mutant had been suggested to result from the loss of Bru binding

to the type II sites (Reveal et al., 2010). Now, knowing that mutation of the 3' type II site also affects BSF binding, we reevaluated this conclusion.

Patterning activities were compared for transgenes with the different combinations of mutant type II Bru binding sites: both sites mutated (*osk3'920-923,970-974*, which is *osk C II*$^{-}$), only the 5' site mutated (*osk3'920-923*, or *osk C 5'II*$^{-}$), or only the 3' site mutated (*osk3'970-974*, or *osk C 3'II*$^{-}$)(Fig. 2.3B). The original *C II*$^{-}$ mutant with both sites mutated has the weakest patterning activity, even though this mRNA is present at a higher level than the others (Fig. 2.3C). Comparing the transgenes with single type II sites mutated revealed that they make unequal contributions to *osk* activity: mutation of just the 5' site had no effect on patterning, while mutation of just the 3' site led to partial *osk* activity (as already described above). This suggests some degree of redundancy between the two sites, but with the 3' site having a more important contribution. Redundancy could reflect the fact that both sites bind Bru (Reveal et al., 2010; Reveal et al., 2011), with Bru bound to either site providing at least partial function. A similar argument cannot be made for BSF, as mutation of the 5' site alone had no effect on BSF binding, nor did mutation of the 5' site enhance the reduction of BSF binding caused by mutation of the 3' site (Fig. 2.3D; note that the effects on Bru binding in this assay are subtle, as the high affinity BREs are also present in the C region RNA substrate). The more substantial contribution of the 3' site to *osk* activity could be due to its role in binding both Bru and BSF. An understanding of how this sequence both constitutes a Bru binding site and is required for strong BSF binding will require an in-depth analysis of BSF binding properties. Nevertheless, there is no need to invoke competition for Bru and BSF binding to the same sequence. Instead, Bru bound to the type II site could

enhance or facilitate interaction of BSF with adjacent sequences, which are essential for BSF binding.

BSF and *osk* mRNA polyadenylation

The position of the BSF binding site in the *osk* mRNA, close to the poly(A) tail, raises the possibility that BSF activates translation by promoting cytoplasmic polyadenylation. This possibility is also suggested by the role of BSF in processing and adding poly(A) tails to polycistronic mitochondrial RNAs, although this reaction is substantially different from cytoplasmic polyadenylation of conventional eukaryotic mRNAs (Bratic et al., 2011). To measure poly(A) tail lengths for the mRNAs with mutations in the BSF binding site, the assay must be able to distinguish between those mRNAs and the *osk IBE*$^{-}$ helper mRNA (which must be present to provide *osk* noncoding RNA function). One useful approach relies on circularization of the mRNA, followed by mRNA-specific reverse transcription and PCR (using a primer complementary to GFP sequences present only in the mRNAs with the mutated BSF binding site), and sequencing (Fig. 2.5A, Methods). The results showed a range of poly(A) tail length from 20 to almost 100 in all genotypes, consistent with a previous direct measurement of wild type *osk* mRNA (Lie and Macdonald, 1999). Neither *oskT140::GFP 3'977-981* nor *oskT140::GFP 3'984-988* had consistently shorter poly(A) tails than the *oskT140::GFP* mRNA control (Fig. 2.5B,C). This suggests that BSF function in *osk* expression is not by controlling polyadenylation.

DISCUSSION

Here we focus on two features of the work. One is the identification of BSF as a factor required for the complex post-transcriptional regulation of *osk* mRNA. The second is the discovery that RNA elements with seemingly unrelated responsibilities, and acting at different times during oogenesis, are superimposed in the *osk* mRNA.

BSF is a one of a family of proteins harboring the pentatricopeptide (PPR) motif, which acts as an RNA binding domain (Barkan et al., 2012; Filipovska and Rackham, 2013; Yin et al., 2013). The vast majority of PPR proteins are from plants and have multiple copies of the motif, typically with no other recognizable domain (Lurin et al., 2004). When BSF was first characterized, methods for detection of protein motifs reported seven copies of the PPR in BSF: four tandem copies near the amino terminus, and three separated copies in the carboxyl-terminal half (Mancebo et al., 2001). By the current definition of the PPR domain, presumably refined with the large number of additional examples identified since 2001, BSF has only a single PPR domain. However, the entire amino-terminal region containing the four tandem repeats is highly conserved with the closest human homolog of BSF, Leucine-rich PPR motif-containing protein (LRPPRC), in which all four PPRs continue to be identified (Mili et al., 2001; Sterky et al., 2010; Ruzzenente et al., 2012). Given the divergence of the PPR-like domains of BSF, it is possible that not all have RNA binding activity.

PPR-containing proteins have been implicated in a wide range of processes, ranging from transcription, to mRNA export from the nucleus (Mili et al., 2001; Topisirovic et al., 2009; Tsuchiya et al., 2004), to various forms of post-transcriptional regulation in both cytoplasm and organelles (Sasarman et al.,

2010; Gohil et al., 2010). Thus, the presence of this motif suggests a function in nucleic acid transactions, but is not indicative of a specific role. Except for the PPR proteins in which other domains dictate function (Zehrmann et al., 2011), proteins of this type may serve primarily in binding to nucleic acids, with the outcome of that binding probably specified by associated factors.

BSF was initially identified as a cytoplasmic ovarian protein which bound to *bicoid* mRNA, playing a redundant role in stabilization of the mRNA (Mancebo et al., 2001). BSF also binds DNA, and was purified on the basis of specific binding to a short DNA sequence found in the promoter region of genes transcribed very early in embryogenesis (De Renzis et al., 2007). Whether BSF binds to and activates transcription of associated genes *in vivo* remains uncertain: another protein, Zelda, binds the same sequence and is required for early transcription (Liang et al., 2008). In flight muscle BSF appears in sarcomeric bands. BSF, like LRPPRC, is also found in mitochondria and this may be the predominant location at least in some tissues (Bratic et al., 2011). Within mitochondria, reducing the level of BSF affects mitochondrial transcription, mRNA polyadenylation, and translation (Bratic et al., 2011).

We have now identified a further role for BSF, in regulation of *osk* mRNA. Complementary approaches, either disrupting BSF binding to the mRNA or reducing the level of BSF, had similar effects: reduction or loss of both posteriorly-localized *osk* mRNA and Osk protein late in oogenesis and in embryos. The defects were strongest with *osk* transgenes bearing mutations that reduced BSF binding to background levels, and less severe with KD of *bsf*. This is not surprising, as the KD does not eliminate BSF. Regulation of *osk* mRNA occurs at several levels, and several options can explain the observed defects.

We have ruled out one option, an effect on *osk* mRNA stability. Two other options are most likely for the loss of Osk protein accumulation: disruption of mRNA localization or translational activation. Neither mutation of the BSF binding site nor KD of *bsf* affects the initial phase of *osk* mRNA localization to the posterior pole of the oocyte. However, the mechanism of localization can change as oogenesis progresses and BSF could act in a later phase, having a role in capture of mRNA circulated in the oocyte by cytoplasmic flow (Glotzer et al., 1997). Because only localized *osk* mRNA is efficiently translated, a defect in mRNA localization would result in loss of Osk protein expression. Alternatively, BSF could act in a late phase of translational activation, when the bulk of Osk protein accumulates (Snee et al., 2007). Because Osk protein is required for anchoring of *osk* mRNA, a defect in production of Osk protein would result in dispersal of the mRNA. Distinguishing between possible roles in mRNA localization or translational activation is simple if one defect clearly precedes the other, but no difference in the timing of the defects has been detected.

If BSF more directly affects translation, a possible mechanism involves the poly(A) tail. Typically, longer tails are correlated with enhanced translation, and the length of the tail can be extended in the cytoplasm (Jacobson, 1996; Wickens et al., 1996; Preiss and Hentze, 1998). As first shown in *Xenopus*, CPEB protein bound to a regulatory element (CPE) positioned close to the poly(A) tail directs cytoplasmic polyadenylation and translational activation (Hake and Richter, 1994). We found no decrease in poly(A) tail length for *osk* mRNAs with mutations in the BSF binding sites, but instead a small increase. Thus it appears that BSF does not activate *osk* mRNA translation by extension of the poly(A) tail. A caveat

to that conclusion is that an effect on a very small subpopulation of *osk* transcripts would not have been detected.

Superimposition of RNA elements for function and regulation

osk mRNA has both coding and noncoding functions. In the absence of *osk* mRNA there are multiple defects beyond those caused later in oogenesis by the absence of Osk protein: the karyosome fails to condense, multiple proteins are displaced from large RNPs (nuage and sponge bodies), the same proteins become enriched in the somatic follicle cells, and oogenesis is arrested (Jenny et al., 2006; Kanke et al., 2015). How the presence of *osk* RNA prevents these problems is only partially understood. It is noteworthy that sequences critical for *osk* RNA function overlap with sequences which mediate regulation by Bru and BSF. This raises the question of whether the same RNA elements serve both roles, which might imply some mechanistic commonalities in RNA function and regulation, or if different types of RNA elements are superimposed on one another.

One biochemical role of *osk* mRNA in its noncoding function is to sequester Bru, presumably preventing Bru from binding inappropriately to low affinity sites in normally unbound mRNAs, and thereby altering their regulation (Kanke et al., 2015). Mutations which reduce Bru binding also cause the other defects associated with absence of *osk* mRNA (karyosome formation, protein distributions, arrest of oogenesis), but to a lesser degree. Thus, sequestration of Bru is one component of *osk* RNA function, but not the main component.

The C region Bru binding sites have an additional role in the noncoding *osk* RNA function, with mutation of all C region sites being effectively equivalent

to complete absence of *osk* mRNA. By contrast, mutation of all AB region Bru binding sites only reduces the noncoding *osk* RNA function, and so there must be a unique property of the C region sites. It is not higher affinity for Bru, as AB region sites bind Bru more strongly (Kim-Ha et al., 1995; Kanke et al., 2015). Rather, the special feature of the C region sites is most likely their close proximity to other sequences essential for noncoding *osk* RNA function. Curiously, the C region Bru binding sites can also be distinguished from those in the AB region by their role in translational activation: mutation of C region sites disrupts activation, while mutation of AB regions sites does not.

Other sequences in the *osk* 3' region also have roles in both regulation and noncoding *osk* RNA function. A-rich sequences (ARSs) close to the poly(A) tail bind PABP (Vazquez-Pianzola et al., 2011). Mutations affecting some or all of the ARSs cause partial defects in both the noncoding function and *osk* mRNA regulation (Vazquez-Pianzola et al., 2011; Kanke et al., 2015)(unpublished). How the ARSs contribute to either RNA function or regulation is not entirely understood. In *pabp* mutants *osk* mRNA is destabilized, but it is uncertain if this reflects loss of PABP binding to the poly(A) tail or to the ARSs (Vazquez-Pianzola et al., 2011). However, mutation of an ARS does affect *osk* regulation without altering mRNA stability (Kanke et al., 2015). Thus, it is unknown if the mechanisms used by PABP and the ARSs for *osk* regulation and noncoding *osk* function are shared or distinct.

Mutations which disrupt BSF binding have defects in *osk* regulation, and the evidence presented here strongly supports the conclusion that BSF mediates this regulation. Two of these mutations, *osk3'977-981* and *osk3'984-988*, also have severe effects on noncoding *osk* RNA function, almost identical to absence

of *osk* mRNA. However, other mutations which disrupt BSF binding (*osk3'970-974* and *osk3'997-1001)*, albeit to a lesser degree, have no detectable effect on noncoding *osk* RNA function (Kanke et al., 2015). Thus, the ability to bind BSF does not correlate with the ability to provide noncoding *osk* RNA function. Instead, the evidence suggests that two different functions are superimposed in the critical *osk* 3' UTR C region. One function, mediated by BSF binding, acts in regulation of *osk* mRNA expression late in oogenesis. A second function, which presumably involves interaction with another factor, is essential for the action of *osk* as a noncoding RNA early in oogenesis.

The high density of RNA elements for function or regulation in the C region of the *osk* 3' UTR is paralleled by an extremely high degree of phylogenetic conservation. The segment containing the cluster of Bru, BSF and PABP binding sites has the highest level of sequence conservation across the entire 3' UTR, and is more conserved than much of the *osk* coding region (UCSC genome browser phastCons analysis). An intriguing question is why the functional and regulatory elements are so densely packed and superimposed. Reuse of the same sequences for multiple purposes is a well known feature of certain viruses, imposed by size constraints (Sanger et al., 1977). A similar explanation seems unlikely for *osk*, as the 3' UTR is quite large (over 1000 nucleotides) and there is no obvious reason why a small further enlargement would pose a problem. It will be interesting to determine if these elements are subject to a positional constraint, relying on mechanisms which require close proximity to the 3' end of the mRNA and thus forcing them to be superimposed.

SUMMARY & FUTURE STUDY

Here, I showed that BSF associates with a 3' region of the *osk* mRNA 3' UTR and is required for either a late phase of *osk* mRNA localization or a late phase of *osk* mRNA translational activation, or both.

In this chapter, I suggested that BSF is not required for *osk* noncoding RNA function, because two mutations in *osk* mRNA that strongly reduce BSF binding (although still above the background level) did not affect the noncoding function. However, we still cannot exclude the possibility that the remaining BSF binding is enough for noncoding function, and thus BSF may be involved in noncoding function. There may be other factor(s) important for the noncoding function that are recruited by BSF to the *osk* mRNA. An immunoprecipitation-based approach can be used to test this possibility, ideally using protein extracts prepared from early-stage egg chambers, as noncoding function is required early in oogenesis.

Bru and PABP binding sites are involved in both *osk* mRNA translational activation and the *osk* noncoding RNA function (Reveal et al., 2010; Kanke et al., 2015; Vazquez-Pianzola et al., 2015; unpublished data). Interestingly, BSF binding sites are very close to or overlap with the Bru or PABP binding sites. Therefore, it is possible that BSF acts in its role as a complex with either Bru or PABP, or both. We can test their interaction by using a co-immunoprecipitation approach.

MATERIALS AND METHODS

Flies and transgenes

For *bsf* KD, TRiP line *y sc v; P{TRiP.HMS01022}attP2* (Bloomington stock 34550) was used. Mutants were *bsf* ^{SH1181} (De Renzis et al., 2007), *osk*^{54} (Lehmann and Nüsslein-Volhard, 1986), *osk*^{A87} (Jenny et al., 2006), *osk*^{0} (Kanke et al., 2015), and *Df(3R)osk* (Reveal et al., 2010). The *osk IBE*$^{-}$ transgene used in Figs. 2.4 and 5 has the A set of IBEs mutated, and the version used in Fig. 2.3 has all IBEs mutated. Both behave identically, with a complete absence of Osk protein produced (Munro et al., 2006), and the same very low level of ability to rescue in *trans* the activation defect of *osk C BRE*$^{-}$ (unpublished). The different versions of *osk IBE*$^{-}$ were used to facilitate fly stock construction. The *osk::HA* transgene reproduces endogenous *osk* expression and function (Kim et al., 2015; Kanke and Macdonald, 2015). The *osk::GFP* transgene used in Fig. 2.2 (Snee et al., 2007), had been remobilized in the lab of Liz Gavis to restore expression. The *osk* transgenes with 3' UTR C region mutations were those described (Kanke et al., 2015), or had the same mutations but with *mGFP6* (Haseloff, 1999) inserted after T140 (M139 is the aminoterminal end of Short Osk). The latter set were constructed in vector pGE-attB (Huang et al., 2009) and inserted by phiC31 transgenesis into a second chromosome site at 51C (Bloomington stock 24482).

Antibodies

Antibodies for western blot analysis: mouse anti-Bru (1:8,000); rat anti-BSF (1:1,000); rat anti-Cup (1:2,000); rabbit anti-Growl (1:2,000); rabbit anti-Hrp48 (1:10,000); rabbit anti-La (1:1,000); chicken anti-NS1 (1:1,000); rabbit anti-Tral (1:10,000); mouse anti-α-Tubulin (Sigma)(1:1,000); alkaline phosphatase-

conjugated goat anti-rat IgG (Sigma)(1:5,000); alkaline phosphatase-conjugated goat anti-mouse IgG (Applied Biosystem)(1:5,000). Antibodies for imaging: mouse anti-HA (Covance)(1:1,000); rabbit anti-Osk (1:2,000); mouse anti-Orb (4H8)(1:1); rabbit anti-BSF (1:1,000); Alexa Fluor 488 goat anti-mouse IgG (Invitrogen)(1:800); Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen)(1:800).

oskC::S1 aptamer fusion RNAs for affinity purification

A plasmid with the S1 aptamer and a T7 promoter (from Craig Smibert) was modified by addition of the final 150 bp of the *osk* 3' UTR (wt or mutant; amplified by PCR). Plasmids were linearized with EcoRl (NEB), purified by phenol extraction and ethanol precipitation, and used as templates for transcription reactions. Fusion RNA transcripts were synthesized and purified with the MEGAscript T7 Kit according to the manufacturers instructions (Ambion).

Preparation of ovary extract

Ovaries from young females (w^{1118}) fed on yeast for 3~4 days were dissected in 1X PBS on ice, washed once with ice cold COEB100 (50 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 100 mM NaCl, 0.1% Triton X-100, 10% Glycerol, 0.1 mM DTT, Complete, Mini, EDTA-free protease inhibitor cocktail tablet (Roche)) and frozen at -80 °C. Ovaries were thawed (all subsequent steps on ice or at 4 °C) and homogenized with a plastic pestle in a 1.5 ml microfuge tube followed by adding 10 µl of COEB100 per pair of ovaries. The lysate was cleared by centrifugation at 12,000 rpm for 25 min and the supernatent saved. Protein concentration in the extract was determined by Bradford (Bio-rad) method, and

the extract diluted to 1 mg/ml with COEB100. After centrifugation at 12,000 rpm for 25 min, extract was pre-incubated with 5 µg of soluble avidin (Sigma) per 1 mg of protein for 15 min.

RNA affinity purification

Streptavidin agarose beads (Sigma) were equilibrated by washing several times with OEB100 (50 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 100 mM NaCl, 0.1% Triton X-100, 10% Glycerol, 0.1 mM DTT). RNA for purification (40 µg) was incubated with 35 µl of beads in 100 µl ice-cold OEB100 supplemented with 40 units/ml RNasin (Promega) for 1 hr at 4 °C with gentle rotation. The beads were spun down, the supernatant removed, and 1 mg of pre-cleared ovary extract (1 ml) added followed by incubation for 2 hr at 4 °C with rotation. After incubation, the beads were spun down, saving both pellet and supernatant (S) fractions. The pellet fraction was washed several times with ice-cold OEB100, and boiled in 35 µl of 6X protein sample buffer for 10 min to elute bound proteins (P). S and P fractions were separated by SDS-PAGE and analyzed by coomassie staining and western blot.

Whole-mount ovary staining

Sample preparation and antibody staining were performed as described (Kim-Ha et al., 1995; Reveal et al., 2010). TO-PRO-3 Iodide (Invitrogen)(1:1,000) was used to stain nuclei. Microscopy of all samples made use of a Leica TCS-SP laser scanning confocal microscope.

In situ hybridization

In situ hybridization in ovary whole mount preparation was performed as described (Jambor et al., 2014). Fluorescent RNA probes were synthesized with DIG RNA labeling mix (Roche).

Cuticle analysis

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

RNA-protein double staining in embryos

RNA-protein double staining was modified from (Lécuyer et al., 2008). Briefly, early-stage embryos were dechorionated with bleach and fixed with paraformaldehyde/heptane mix for 20 min. The embryos were post-fixed two times, and then incubated with RNA probe overnight. After washing steps, the embryos were incubated with the mouse anti-GFP (Santa Cruz) diluted at 1:200 for 2 hr, followed by incubation with Alexa Fluor 488 goat anti-mouse (Invitrogen) diluted at 1:800 for 1 hr. After washing the embryos were incubated with 1/50 diluted Cy5 tyramide (PerkinElmer) for 30 min and mounted on slides with Vectashield Mounting Medium (Vector Labs), and imaged with Leica TCS-SP laser scanning confocal microscope.

RNA preparation and qPCR analysis

Ovaries from young females fed on yeast for 3-4 days were dissected into

ice-cold PBS and late-stage egg chambers isolated. Approximately 100 early-stage embryos were collected from females in the process of oviposition and dechorionated. Total RNAs were isolated using Tri Reagent-LS (Molecular Research Center) according to the manufacturer's protocol. RNA samples were treated with DNase I (Qiagen) and purified with RNeasy MiniElute Cleanup Kit (Qiagen). 1 µg of RNAs were used to synthesize cDNA using iScript cDNA synthesis kit (Bio-rad) according to the manufacturer's protocol. qPCR was carried out on a ViiA™ 7 Real-Time PCR system (Applied Biosystem) using iTaq™ Universal SYBR® Green Supermix (Bio-rad). Primers: *α-tubulin*, 5'-atgcgcgaagtagtctccatc-3' and 5'-aggtgtcgtgacccacact-3'; *mGFP6*, 5'-tttcactggagttgtcccaa-3' and 5'-ggccatggaacaggtagttt-3' (set1) and 5'-aggacgacgggaactacaag-3' and 5'-taagctcgatcctgttgacg-3' (set 2).

RNA circularization and RT-PCR

RNAs were prepared as for qPCR. Gene specific oligo/RNase H treatment was performed to remove mRNA 5' caps. 2 µg RNA and 2.5 µg oligo 1 (5'-ggaattcacttgtgactgcg-3') were mixed in 20 µl of 10 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA (pH 8.0), incubated for 10 min at 70 °C and gradually cooled to 25 °C. The samples were incubated for 1 hr at 37 °C with 2.5 U of RNase H (NEB) and 20 U of RNase Inhibitor, Human Placenta (HPRI, NEB) in 50 µl of RNase H buffer (NEB). Decapped RNAs were purified with RNeasy MiniElute Cleanup Kit (Qiagen).

For circularization, decapped RNA (1.5 µg) was incubated with 10 U of T4 RNA ligase (NEB) and 20 U of HPRI in 200 µl T4 RNA ligase buffer, 0.1 mM ATP, and 10% PEG8000 for over 16 hr at 16 °C. Circularized RNAs (cRNAs)

were precipitated with isopropanol after extraction with phenol/chloroform, the cRNA pellets were washed with 70% ethanol, and dissolved 8 µl nuclease free-dH_2O. cDNA was synthesized with the GeneAmp RNA PCR Kit (Applied Biosystems), using 3 µl of cRNA and oligo 2 (5'-ggccatggaacaggtagttt-3'). PCR with oligo 2 and oligo 3 (5'-tctggatccttctggcgtaatttacag-3') was performed using Plantium PCR SuperMix (Invitrogen). PCR products were purified using ExoSAP-IP (Affymetrix) for direct sequencing and for cloning into pCRll-TOPO vector (Invitrogen).

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FIGURES

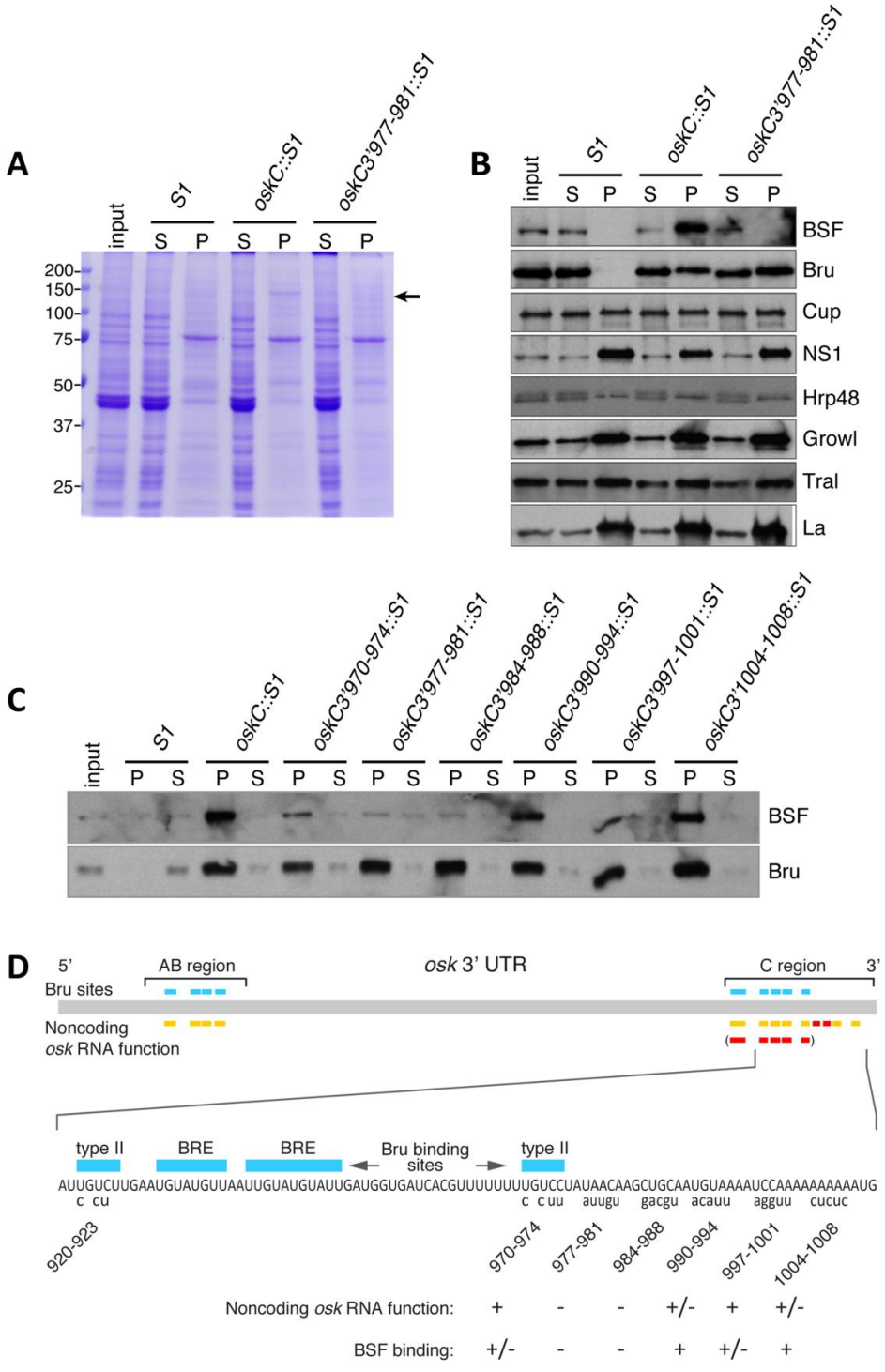
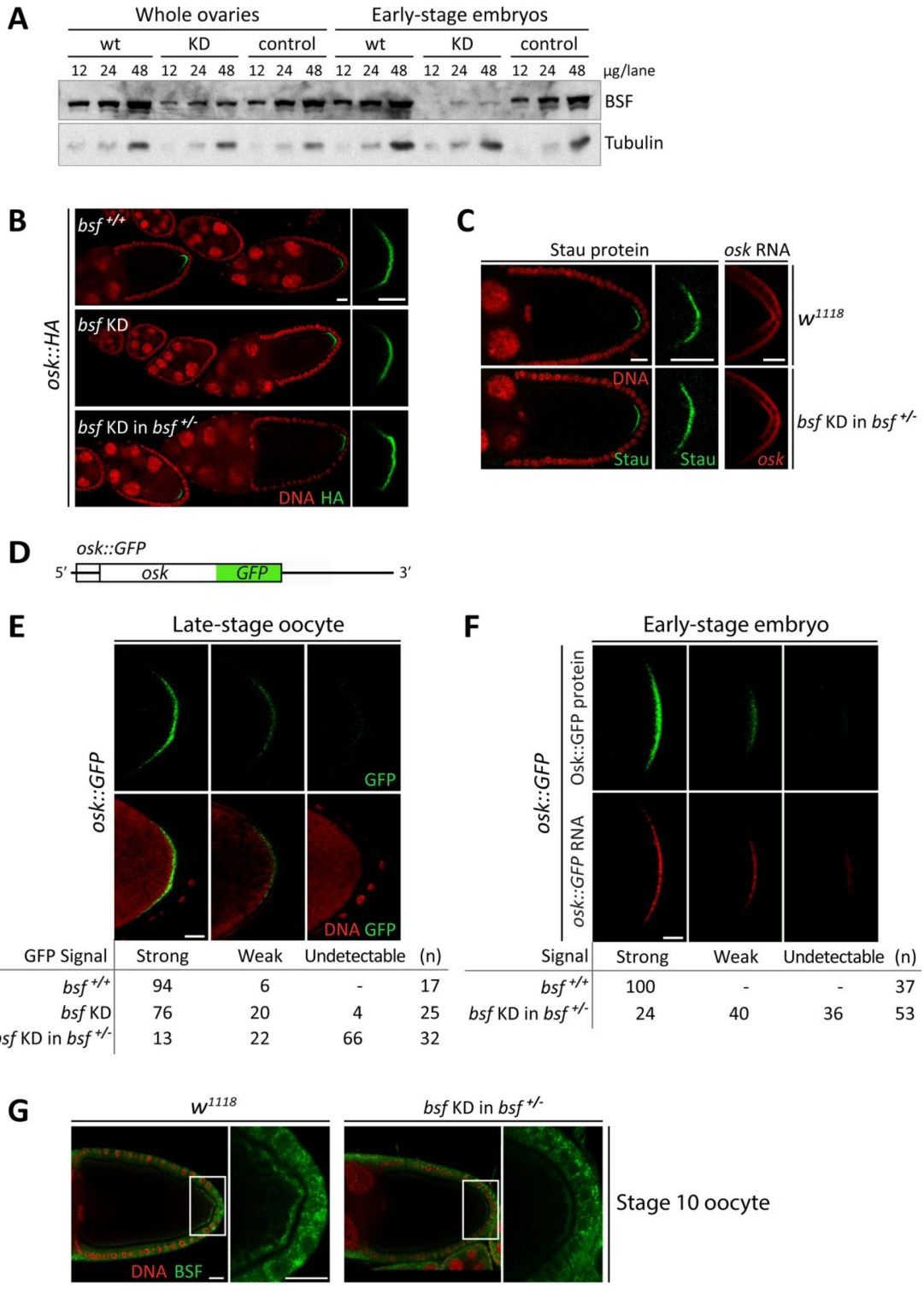


Figure 2.1. BSF binds close to the 3' end of the *osk* mRNA.

A. Coomassie-stained gel of ovarian proteins from affinity purification. Input is the starting material. S and P are supernatent and pellet fractions following purification with the RNAs indicated at top (the *osk* RNA segment is the C region, the final 150 nt of the 3' UTR, either wild type or with the indicated mutations). Amounts loaded, relative to the extract used for one purification are: input and supernatent, 0.8%; pellet, 33.3%. The arrow indicates a ~150 kDa protein which binds only to wild type *oskC::S1* RNA. Because of the small amount of supernatent loaded, loss of binding does not lead to a high level of protein detected in the supernatent fraction.
B. Western blot analysis of proteins in fractions from the affinity purifications.
C. BSF and Bru binding, detected by western blot, to *osk* 3' region mutant RNAs. The amounts loaded were as in panel A. Mutations 977-981 and 984-988 have the strongest effect on BSF binding, 970-974 and 997-1001 are intermediate (with 977-1001 retaining the most binding activity), and 990-994 and 1004-1008 have no detectable effect. Names for these mutations reflect location of the changes in the 3' UTR, with position 1 being the first nucleotide after the stop codon.
D. Diagram of the *osk* mRNA 3' UTR with the sequence of *osk* C region showing mutations and Bru binding sites. The 3' UTR is shown as a grey rectangle with the AB and C regions indicated above (Kim-Ha et al., 1995). Individual Bru binding sites (BREs and type II sites) are shown above the 3' UTR as blue bars. Mutations that disrupt noncoding *osk* RNA function are shown below the 3' UTR as yellow or red bars. The yellow bars indicate a lesser effect of individual mutations (multiple Bru sites must be mutated to strongly disrupt the noncoding function). The red bars indicate mutations that effectively eliminate that function. Simultaneous mutation of all C region Bru binding sites (as indicated by the red bars in parentheses) also eliminates the noncoding *osk* RNA function. The C region is enlarged below to show the RNA sequence, the positions of Bru binding sites, mutations (below the sequence in lower case) and their effects on *osk* RNA function and BSF binding.



GFP Signal	Strong	Weak	Undetectable	(n)
bsf +/+	94	6	-	17
bsf KD	76	20	4	25
bsf KD in *bsf* +/-	13	22	66	32

Signal	Strong	Weak	Undetectable	(n)
bsf +/+	100	-	-	37
bsf KD in *bsf* +/-	24	40	36	53

Figure 2.2. *bsf* is required for a late phase of Osk protein expression.

A. Efficiency of *bsf* KD in ovaries and early embryos, as detected by western blot analysis. w^{1118} was used as a control. Samples were wt (w^{1118}), KD (TRiP-*bsf* transgene with *matalpha4-GAL-VP16*), and control (TRiP-*bsf* transgene without GAL4 driver). Proteins were detected using anti-BSF (top) or anti-α-Tubulin (bottom).
B. Osk expression is normal in the *bsf* KD up to stage 10 of oogenesis. Panels show detection of nuclei and Osk::HA (Methods). Higher magnification images of just Osk::HA from other examples of egg chambers are shown at right. *bsf* KD was as in panel A. For *bsf* KD in $bsf^{+/-}$, the flies were heterozygous for bsf^{SH1181}. DNA, red; Osk::HA, green. Scale bars in this and other panels are 20 µm. Genotypes used were as follows (only relevant genes are indicated). $bsf^{+/+}$ is +/+; *osk::HA matalpha4-GAL-VP16* osk^{A87}*/TM3Sb*. *bsf* KD is +/+*; osk::HA matalpha4-GAL-VP16* osk^{A87}*/TRiP-bsf. bsf* KD in $bsf^{+/-}$ is bsf^{SH1181}/+; *osk::HA matalpha4-GAL-VP16* osk^{A87}*/TRiP-bsf*.
C. *osk* mRNA is localized normally in *bsf* KD up to stage 10 of oogenesis. Left and middle panels show Stau protein (which colocalizes with *osk* mRNA; middle panel at higher magnification) and the right panels show *osk* mRNA. For the Stau panels DNA is red and Stau is green. For the *in situ* hybridization panels *osk* RNA is red (the signal in follicle cells is background).
D. Schematic diagram of the *osk::GFP* transgene for examination of late-stages of oogenesis, which is a genomic *osk* gene with GFP appended to the end of the *osk* coding region. All *osk* sequences, including the 3' UTR and transcriptional control elements, remain present.
E. Osk::GFP expression in late-stage egg chambers. Panels show the posterior portion of stage 13-14 egg chambers, with examples of the different levels of posterior Osk::GFP accumulation. Upper panels are just the Osk::GFP signal, while lower panels also show DNA (and background TOPRO-3 staining). The table below the images shows the percentage of oocytes in each category for the same genotypes as in panel B. DNA, red; Osk::GFP, green.
F. Osk::GFP protein (upper) and *osk::GFP* mRNA (lower) double-labeling in early-stage embryos. *osk::GFP* expressed from single copies in $bsf^{+/+}$ or *bsf* KD in $bsf^{+/-}$ background. Osk::GFP protein and *osk::GFP* RNA (upper and lower panels, respectively) in early-stage embryos (each pair of panels is from the same embryo). This correlation between levels of localized protein and mRNA was observed in all embryos. The table below the images is as in panel E, with the values applying to both protein and mRNA.
Genotypes for the samples in E and mothers of samples in F were as follows. $bsf^{+/+}$: *osk::GFP/+; matalpha4-GAL-VP16* osk^{A87}*/TM2*. *bsf* KD: *osk::GFP/+; matalpha4-GAL-VP16* osk^{A87}*/TRiP-bsf. bsf* KD in $bsf^{+/-}$ background: *osk::GFP/*bsf^{SH1181}*; matalpha4-GAL-VP16* osk^{A87}*/TRiP-bsf*.
G. Immunodetection of BSF in wild type and in *bsf* KD. For each genotype the posterior of an egg chamber is shown at left, with the boxed posterior region shown at higher magnification at the right. Although the anti-BSF antibody shows poor penetration into the egg chamber, a crescent of BSF is detected specifically at the posterior pole of the oocyte (lower levels appear along the entire cortex). Note that the follicle cell BSF is not affected by the *bsf* KD, which is limited to the germline cells by use of the *matalpha4-GAL-VP16* driver. DNA (TOPRO-3), red; BSF, green.

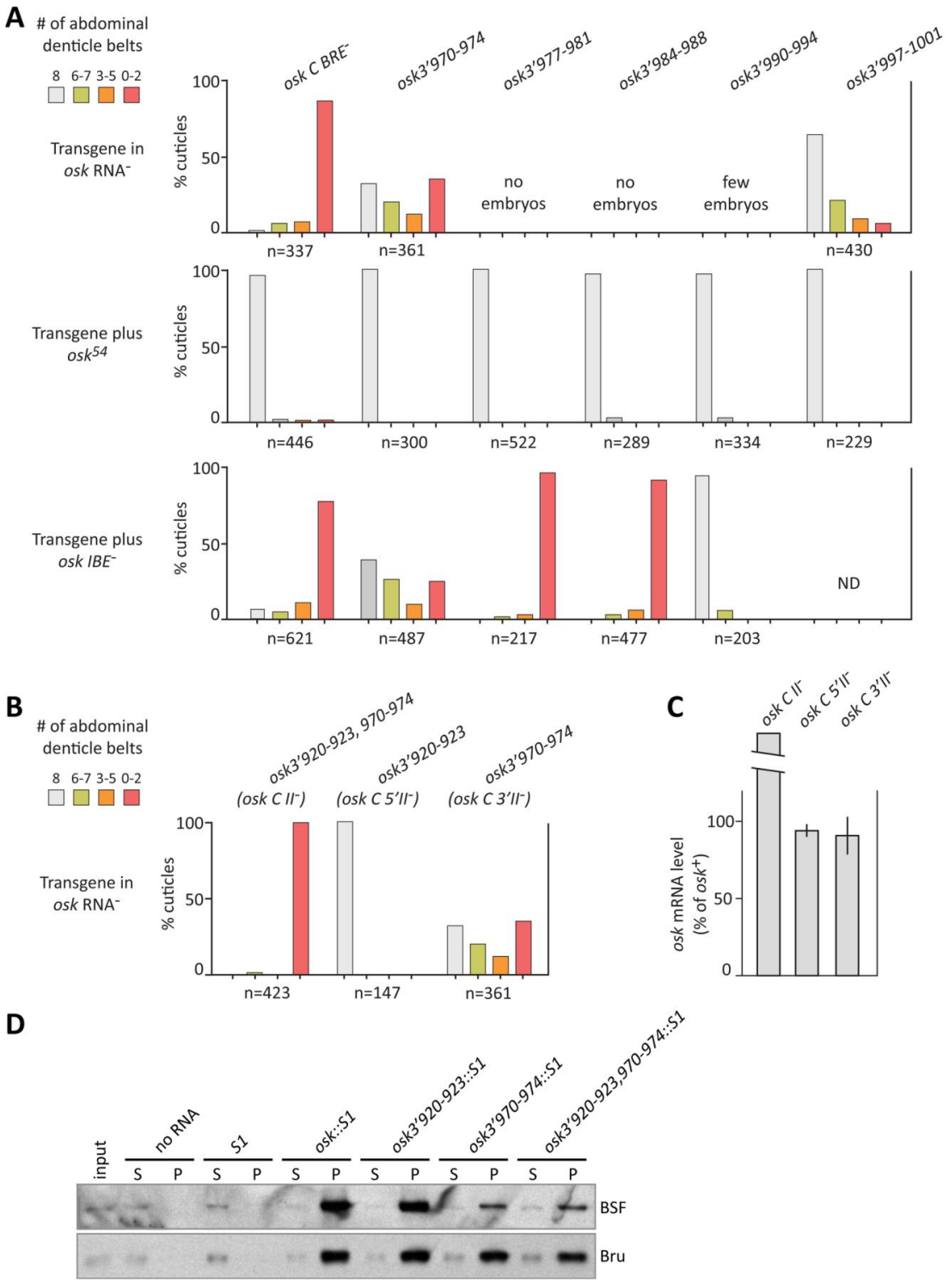


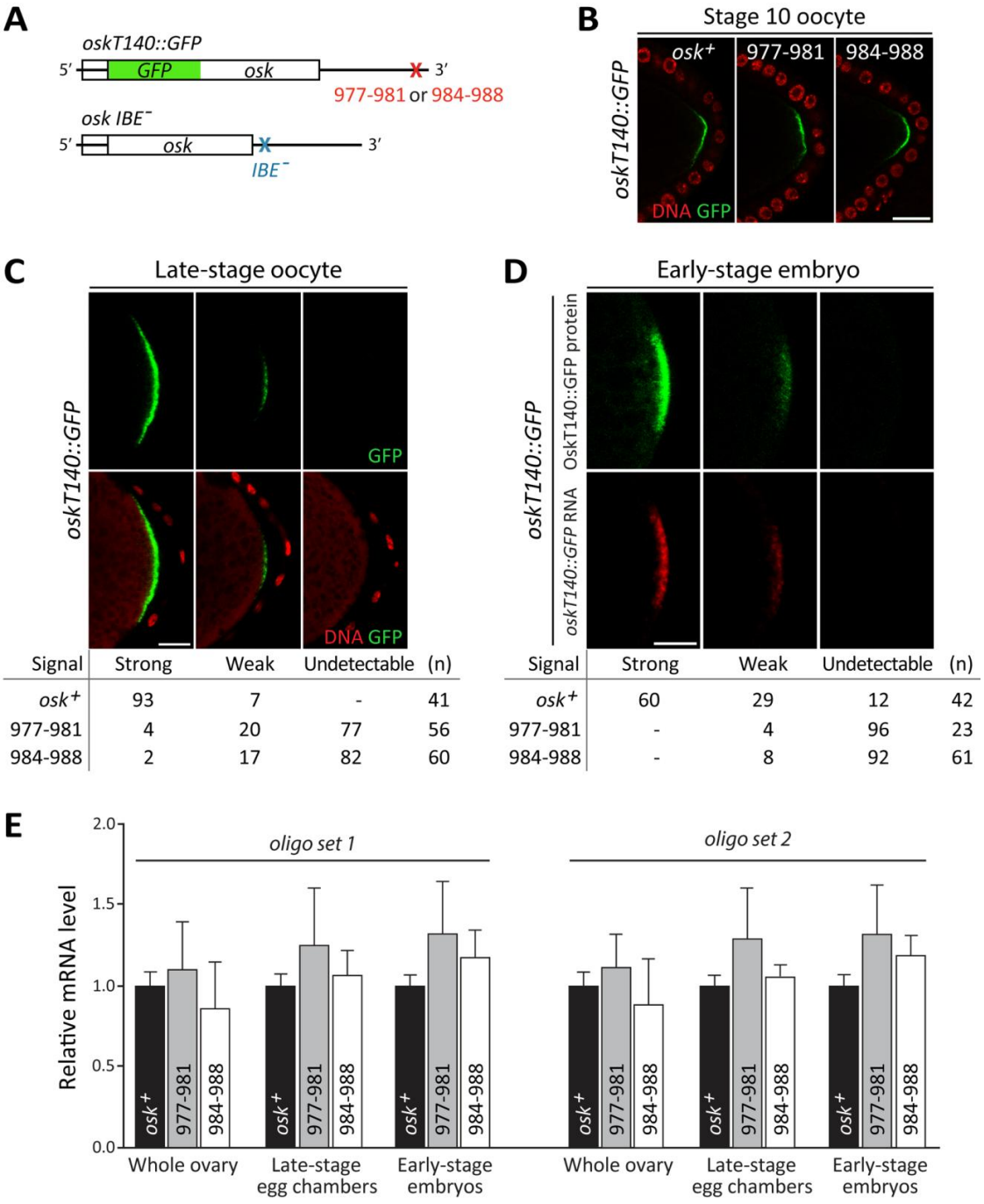
Figure 2.3. Effects of mutating Bru and BSF binding sites on *osk* activity and protein binding.

A. Patterning activity of *osk* transgenes with 3' UTR C region mutations, tested as single copies in the $osk^{A87}/Df(3R)osk$ background (*osk* RNA^-)(top), with osk^{54} (middle) or *osk* IBE^- (bottom; this transgene has all IBEs mutated) also present. For each genotype, the fraction of cuticles with the number of abdominal segments indicated by colors is given as a percentage. The number of abdominal segments corresponds to the level of *osk* activity, with wild type embryos having eight. Mutants which produce no or few embryos lack the noncoding *osk* RNA function required for progression through oogenesis. ND is not determined (because of the very modest patterning defects of the *osk3'997-1001* mutant). Transgene mRNA levels differ somewhat (Kanke et al., 2015), but the strong levels of *osk* patterning activity they produce when co-expressed with the osk^{54} mRNA (and thus subject to regulation in *trans*) provides a point of reference for interpreting their activities in the absence of regulation in *trans*.

B. Patterning activity of *osk* transgenes, tested as single copies in the $osk^{A87}/Df(3R)osk$ background (*osk* RNA^-).

C. Levels of *osk* mRNAs for the transgenes in panel B, relative to the amount from a wild type *osk* transgene. Data are reproduced from (Kanke et al., 2015).

D. BSF and Bru binding, detected by western blot, to *oskC::S1* RNAs (the final 150 nt of the *osk* 3' UTR) with mutated type II Bru binding sites. The amounts loaded were as in Fig. 1A. As in Fig. 1, the 970-974 mutation reduced BSF binding but not to the background level seen with the *S1* RNA. Mutation of the Bru type II sites did not dramatically reduce Bru binding, as the high affinity BREs are also present in all of the *oskC::S1* RNAs.



Signal	Strong	Weak	Undetectable	(n)
osk⁺	93	7	-	41
977-981	4	20	77	56
984-988	2	17	82	60

Signal	Strong	Weak	Undetectable	(n)
osk⁺	60	29	12	42
977-981	-	4	96	23
984-988	-	8	92	61

Figure 2.4. Loss of BSF binding affects Osk protein expression but not *osk* mRNA stability.

A. Schematic diagram of *osk* mRNAs used in the assays. The *oskT140::GFP* transgene has wild type or mutant versions of the 3' UTR. All *oskT140::GFP* transgenes were inserted at the same genomic site by phiC31 transgenesis to avoid position effects that might alter transcription levels. The *osk IBE*$^{-}$ transgene has the A subset of IBE mutations (Munro et al., 2006) in the 3' UTR.

B. OskT140::GFP in stage 10 oocytes. All *oskT140::GFP* transgenes were co-expressed with *osk IBE*$^{-}$ (to provide the noncoding *osk* RNA function for the mutants) in the *osk*^{0}/*Df(3R)osk* background. Panels show the posterior portion of stage 10 egg chambers; Left panel is from *osk*$^{+}$; Middle panel is from *oskT140::GFP-977-981*; Right panel is from *oskT140::GFP-984-988*. DNA in red and OskT140::GFP in green. The scale bar is 20 µm.

C. OskT140::GFP in stage 13-14 egg chambers. The panels are examples of the different level of OskT140::GFP accumulation at the posterior of the oocyte: strong, weak, or very weak/undetectable. The upper panels are OskT140::GFP only, and the lower panels show both OskT140::GFP and DNA. The table below the images shows the percentage of oocytes in each category for the *oskT140::GFP* transgenes with wild type or mutant *osk* 3' UTRs. DNA in red and OskT140::GFP in green. The scale bars for B, C and D are 20 µm.

D. OskT140::GFP protein and *osk::GFP* RNA (upper and lower panels, respectively) in early-stage embryos (each pair of panels is from the same embryo). This correlation between levels of localized protein and mRNA was observed in all embryos. The table below the images is as in panel B, with the values applying to both protein and mRNA.

E. Levels of mRNA measured by qPCR. RNA levels were measured with m*GFP6* oligos (two different oligo pairs) to detect only *oskT140::GFP* transgene mRNAs. Error bars indicate standard deviation, n is 9 for each sample. The *oskT140::GFP* transgenes make use of *mGFP6* (Methods).

Genotypes for B-E (maternal genotypes for embryos) were as follows. *osk*$^{+}$: *oskT140::GFP*/*osk IBE*$^{-}$*; osk*^{0}*/Df(3R)osk.* 977-981: *oskT140::GFP 3'977-981*/*osk IBE*$^{-}$*; osk*^{0}*/Df(3R)osk.* 984-988: *oskT140::GFP 3'984-988*/*osk IBE*$^{-}$*; osk*^{0}*/Df(3R)osk*.

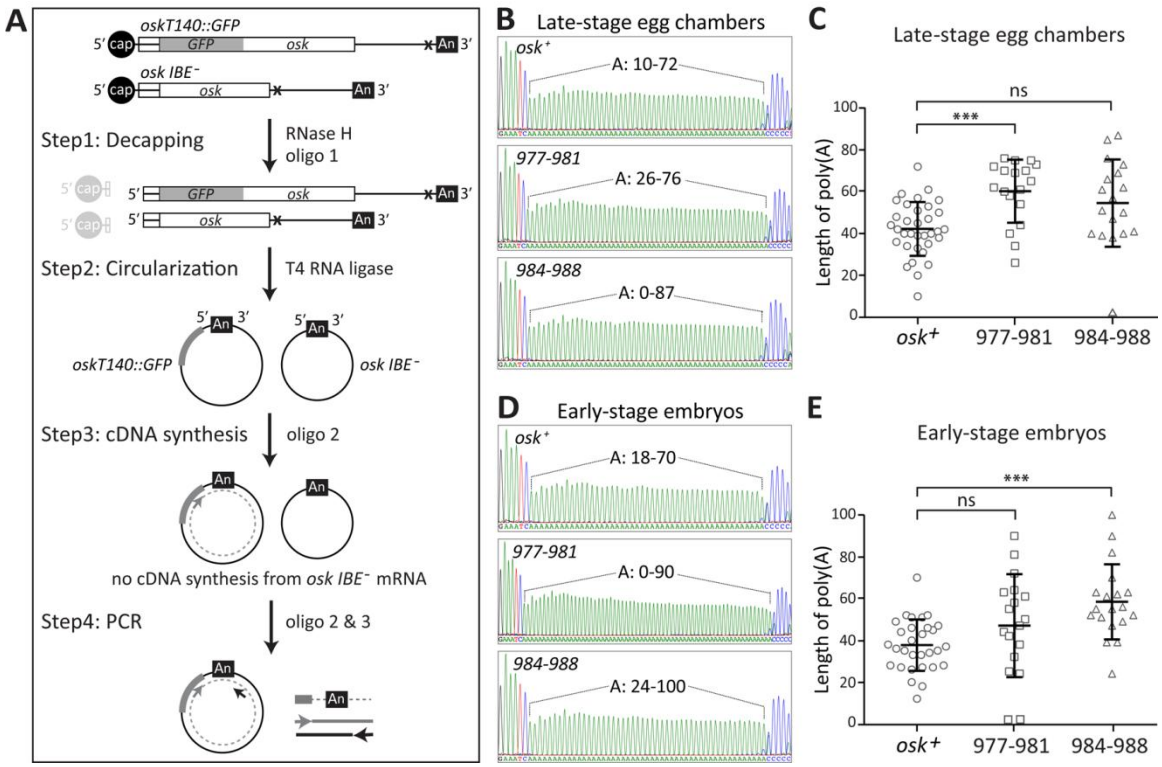


Figure 2.5. BSF binding is not required to maintain poly(A) tail length.

A. Schematic diagram of the circularized RT-PCR (cRT-PCR) assay, to selectively measure poly(A) tail length for *oskT140::GFP* mRNAs when co-expressed with *osk IBE*$^{-}$ mRNA. The *osk IBE*$^{-}$ transgene has the A subset of IBE mutations (Munro et al., 2006) in the 3' UTR. The use of a primer (oligo 2) which anneals to GFP ensures that only the *oskT140::GFP* mRNAs are substrates for both cDNA synthesis and PCR in the assay.
B-E. Lengths of poly(A) tails for individual cloned PCR products from late-stage egg chambers (B-C) and early-stage embryos (D-E). For panels B and D the sequence traces are for a single clone, with the range in poly(A) tail length given above. P values are indicated by *** P<0.001; ns, not significant with p>0.05. Genotypes are the same as in Fig. 4.

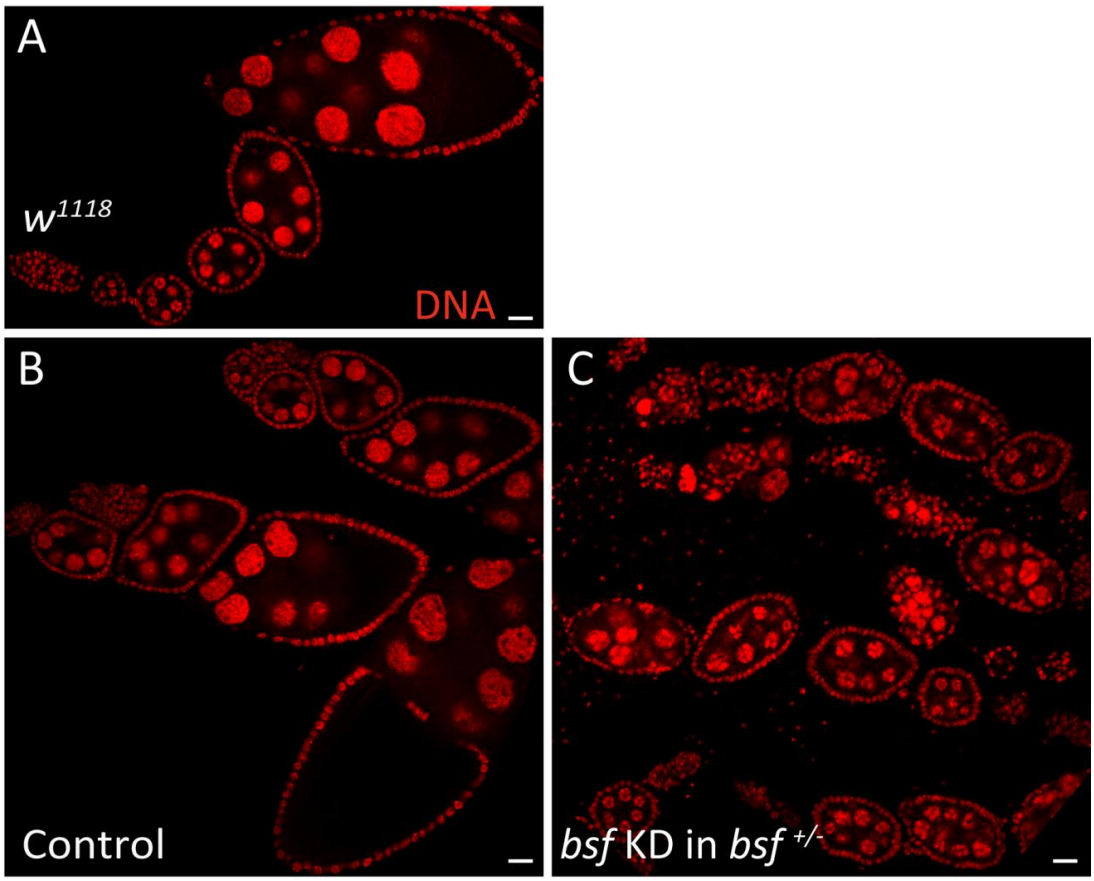


Figure 2.6. Knock down of *bsf* blocks oogenesis.

Each panel shows ovarioles stained with TOPRO3 for detection of nuclei. A, wild type (w^{1118}). B, control with the GAL4 driver (*nos-GAL4::VP16-nos.UTR)*. C, *bsf* KD in *bsf* heterozygote (bsf^{SH1181}/+; *TRiP-bsf*/*nos-GAL4::VP16-nos.UTR*). The scale bar is 20 µm.

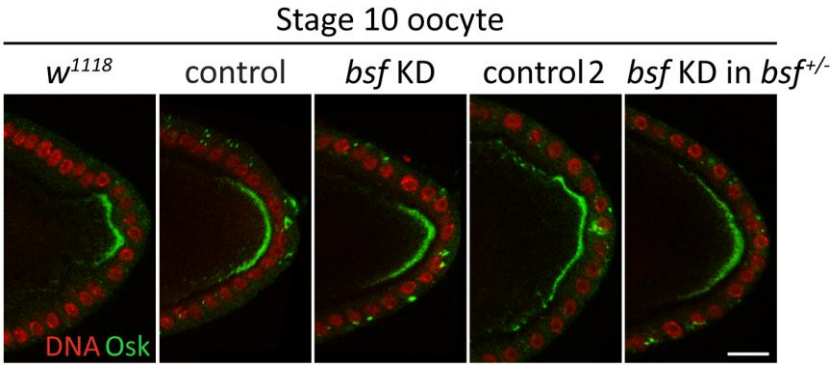


Figure 2.7. Osk expression is normal in *bsf* KD up to stage 10 of oogenesis.

Each panel shows detection of nuclei and endogenous Osk. Genotypes are as follows. w^{1118}: bsf^{+}/bsf^{+}. Control: *TRiP-bsf/TRiP-bsf* (no GAL4 driver). *bsf* KD: *matalpha4-GAL-VP16/TRiP-bsf.* Control 2: bsf^{SH1181}/+; TRiP-*bsf*/TM2 (no GAL4 driver). *bsf* KD in $bsf^{+/-}$: bsf^{SH1181}/+; *TRiP-bsf*/*matalpha4-GAL-VP16*. DNA, red; Osk, green. The scale bar is 20 µm.

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Chapter 3: Multiple mechanisms, including a novel role for translational regulator Bruno, direct transport of *oskar* mRNA to the oocyte

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INTRODUCTION

Analysis of early stages of animal development highlighted the phenomenon of mRNA localization. Proteins responsible for patterning certain insect and amphibian embryos are asymmetrically distributed within oocytes, and the patterns of protein distribution can be traced to the prior localization of the encoding mRNAs (St Johnston 1995). Localization of these mRNAs relies on *cis*-acting signals, which serve to associate the mRNAs with factors involved in directed transport and anchoring (Lipshitz and Smibert 2000). Thus far, there appears to be a correlation between the complexity of the localization program and the complexity of the localization signal. At one extreme, exemplified by the *Drosophila bicoid* (*bcd*) and *oskar* (*osk*) mRNAs, localization involves multiple steps and the mRNAs are delivered to mRNA-specific destinations (St Johnston et al. 1989; Kim-Ha et al. 1991; Ephrussi et al. 1991). Signals that localize these mRNAs are complex, with multiple different elements needed (Macdonald 1990; Macdonald 1993; Macdonald 1997; Ferrandon 1997; KimHa 1993; Ghosh 2012). Some other programs of mRNA localization, such as apical localization in early *Drosophila* embryos (Davis and Ish-Horowicz 1991) or localization to the distal regions of leading lamellae and filopodia of cultured chicken embryonic fibroblasts (Lawrence and Singer 1986), are comparatively simple. For these mRNAs the localization signals are also comparatively simple, typically limited to one discrete element that may bind a single localization factor (Ross et al. 1997; Dienstbier et al. 2009). Notably, the signals for simple localization programs are modular: they can be used in different cell types and for multiple mRNAs (Bullock and Ish-Horowicz 2001)(Snee et al. 2005), and they can be incorporated into more complex localization signals (Macdonald et al. 1993). The TLS localization

signal from the *Drosophila fs(1)K10* mRNA is a paradigm for such modularity (Serano and Cohen 1995).

In the ovary, *fs(1)K10* mRNA is synthesized in the nurse cells and then efficiently transported to the oocyte and enriched along the anterior margin. The TLS is located within the *fs(1)K10* mRNA 3'UTR and consists of an A/U-rich stem-loop (Serano and Cohen 1995), which adopts an unusual helical structure (Bullock et al. 2010). Egalitarian (Egl) protein binds the TLS to mediate association with Dynein for transport along microtubules (Dienstbier et al. 2009). Deletion of the TLS eliminates localization of *fs(1)K10* mRNA (Serano and Cohen 1995), while addition of a minimal TLS (44 nt) to other mRNAs robustly confers the same program of oocyte transport and anterior localization (Serano and Cohen 1995; Bullock and Ish-Horowicz 2001). The TLS also supports apical localization when microinjected into early *Drosophila* embryos, with localization dependent on the same proteins as in the ovary (Bullock and Ish-Horowicz 2001). Strikingly, a number of embryonic mRNAs that display this pattern of apical localization in early embryos undergo transport to the oocyte when ectopically expressed in ovaries. These mRNAs have stem-loop structures required for localization, often similar in composition to the TLS (Bullock and Ish-Horowicz 2001).

Transport mediated by the TLS/Egl transport system can be useful in the context of more complex localization programs. Both *bcd* and *osk* mRNAs must be transported to the oocyte, and both appear to use this system. A stem loop structure within the *bcd* localization signal mediates transport to the oocyte and is sufficient to direct apical localization in the early embryo. Both localization programs are sensitive to the same point mutation in the signal (Macdonald et al.

1993; Bullock and Ish-Horowicz 2001). The situation for *osk* mRNA is more nuanced. Just as for mRNAs with the TLS, the *osk* mRNA is very strongly enriched in the oocyte. This property relies on a localization element within the 3' UTR (Kim-Ha et al. 1993; Kim et al. 2014). Sequences which appear to be important for the function of this element, called the OES, have been more precisely mapped to part of a stem loop structure which resembles the TLS in having an A/U-rich stem. Mutation of this part of the stem disrupts OES activity, and compensatory changes restore activity (Jambor et al. 2014). Despite this similarity between the TLS and the OES, the OES is depauperate by comparison: it is not active in isolation, and only directs a reporter mRNA in the context of larger regions of the *osk* 3'UTR. Furthermore, Egl-dependent apical localization of *osk* mRNA in early embryos is substantially weaker than for other RNAs with TLS-like signals (Bullock and Ish-Horowicz 2001; Jambor et al. 2014). These observations raise the possibility that additional localization elements contribute to transport of *osk* mRNA to the oocyte.

The phenomenon of mRNA localization initially appeared to be limited in scope, but it is now clear that many mRNAs are subject to this form of regulation (Lécuyer et al. 2007; Holt and Bullock 2009). In addition, there are many different distribution patterns. Does each different pattern require a unique localization signal, to be bound by a protein dedicated to that program of localization? Combinatorial localization mechanisms might reduce the number of required signals and factors. Nevertheless, the great diversity in localization patterns suggests the existence of many localization factors. Here we show that a regulatory circuit used to control translation - both the binding sites in the mRNA and the binding factor - also mediates mRNA localization. Translational

regulation of *osk* mRNA relies on Bruno (Bru), which binds to multiple sites in the 3' UTR (Kim-Ha et al. 1995; Reveal et al. 2010; Reveal et al. 2011). One cluster of Bru binding sites in the AB region, near the 5' end of the 3' UTR, acts in translational repression. A second cluster of Bru binding sites, near the mRNA 3' end in the C region, acts in both repression and translational activation. We find that either cluster of Bru binding sites confers oocyte transport on a reporter mRNA, as do isolated Bru binding sites. However, neither Bru sites nor the OES are essential for oocyte transport of *osk* mRNA. We suggest that the use of a suboptimal TLS/Egl oocyte transport system is an important feature of the complex localization of *osk* mRNA: weak association with that system would facilitate the subsequent transfer to other systems for delivery of the mRNA to the posterior pole of the oocyte, while strong and persistent association would prevent that later step in localization.

RESULTS

The AB and C regions of the *osk* 3' UTR confer oocyte localization on reporter mRNAs

UAS-GFP reporter transgenes bearing either of the two *osk* 3' UTR regions with Bru binding sites - the AB and C regions - are translationally repressed during oogenesis. The *GFP*, *GFP-AB* and *GFP-C* mRNAs are present at similar levels, but the amounts of GFP protein produced by *GFP-AB* and *GFP-C* mRNAs are dramatically lower than for *GFP* mRNA (Reveal et al. 2010). Comparison of the distribution of these mRNAs by in situ hybridization revealed a further difference that was most prominent during the previtellogenic stages of oogenesis. In combination with a germ line-specific GAL4 driver, the *GFP* mRNA

was present evenly throughout the germ line cells. By contrast, the *GFP-AB* and *GFP-C* mRNAs were substantially enriched in the oocyte (Fig. 3.1), revealing that the AB and C regions provided localization activity similar to that of the TLS. However, folding predictions suggested that neither region adopts a stem-loop structure, a characteristic of TLS and TLS-like signals. Thus, each of these regions appears to contain a novel form of transport signal.

Bru binding sites confer oocyte localization on reporter mRNAs

Because the AB and C regions both mediated enrichment in the oocyte, any feature shared by these regions would be a likely candidate for the transport signal. The AB and C regions were initially defined by their ability to bind Bru, a repressor of *osk* mRNA translation (Kim-Ha et al. 1995). To ask if Bru binding sites are required for oocyte enrichment, we made use of reporter transgenes originally constructed to study translational regulation by Bru. The *UAS-osk1-534::GFP* reporter includes the first 534 bp of the *osk* transcription unit (most of the first exon, and encoding the N-terminal 173 amino acids) (Kim et al. 2015). Versions of this reporter transgene (referred to as *UAS-osk::GFP* for simplicity) include the *osk* 3’ UTR AB or C regions, in wild type form or with Bru binding sites mutated. The *UAS-osk::GFP* reporter alone, without *osk* 3’ UTR sequences, produced an mRNA that was not enriched in the oocyte (Fig. 3.2A). Just as for the GFP reporter, addition of the *osk* 3' UTR AB or C regions conferred substantial oocyte enrichment on the *osk::GFP* mRNA (Fig. 3.2B,C). Notably, mutation of the Bru binding sites in either *UAS-osk::GFP-AB* or *UAS-osk::GFP-C* eliminated oocyte enrichment (Fig. 3.2E,F).

Although these results showed that Bru binding sites are required for

enrichment of the mRNAs in the oocyte, the Bru sites might not be sufficient, and other proteins which also bind to the AB and C regions might also be required. To ask if Bru binding sites alone confer oocyte enrichment, two additional transgenes were tested. Each has multiple copies of Bru binding sites inserted in the reporter mRNA 3' UTR, and each is synthetic and not derived from the *osk* mRNA.

The *UAS-GFP-anti-Bru aptamer* transgene includes the template for multiple copies of the bru.4 and bru.18 aptamers. These anti-Bru aptamers were selected *in vitro* for their ability to bind recombinant Bru protein, and they confer translational repression on the GFP reporter mRNA (Reveal et al. 2011). The *GFP-anti-Bru aptamer* mRNA was enriched in the oocyte (Fig. 3.2G).

The *UAS-GFP-4xBRE* transgene includes 4 copies of the TGTTTTATATGT motif, which corresponds to a BRE-type Bru binding site. Inclusion of these Bru binding sites confers Bru-dependent translational repression on the *GFP-4xBRE* mRNA (Reveal et al. 2011). These binding sites also resulted in enrichment of the mRNA in the oocyte (Fig. 3.2H).

From these results we conclude that the presence of Bru binding sites in an mRNA is sufficient for oocyte enrichment. For both the *GFP-anti-Bru aptamer* and *GFP-4xBRE* mRNAs the degree of oocyte enrichment was lower than conferred by the *osk* 3' UTR AB or C regions. However, a lower degree of enrichment is expected, because the synthetic Bru binding sites are not as effective as the *osk* AB or C regions in conferring translational repression on the reporter mRNA, and the synthetic binding sites have lower affinity for Bru than the AB and C region RNAs (Reveal et al. 2010).

Bru mediates oocyte RNA enrichment independent of endogenous *osk* mRNA

A possible mechanism for Bru-dependent oocyte enrichment is hitchhiking, in which the reporter mRNAs would be transported to the oocyte via association with endogenous *osk* mRNA. Such a mechanism has been established for mRNA localization to the posterior of the oocyte later in oogenesis, with Polypyrimidine Tract Binding protein (PTB) mediating association of *osk* mRNA and the hitchhiking mRNAs (Hachet and Ephrussi 2004; Besse et al. 2009). By analogy, Bru could bridge between the reporter and *osk* mRNAs. To test this possibility, we monitored the distribution of the *osk::GFP-AB* reporter mRNA in ovaries lacking *osk* mRNA. Even in the absence of *osk* mRNA, substantial oocyte enrichment of the *osk::GFP-AB* mRNA was detected (Fig. 3.2I). Thus, Bru-dependent RNA enrichment in the oocyte was not achieved by hitchhiking on *osk* mRNA.

Degeneracy in oocyte transport of *osk* mRNA

We have shown that either of two regions of the *osk* mRNA 3' UTR was sufficient to direct Bru-dependent transport into the oocyte. A different transport signal was initially mapped by deletion analysis (Kim-Ha et al. 1993), with a recent study showing that a highly conserved portion of that region was important for transport (Kim et al. 2014). Another recent report also addressed the function of this signal, using reporter mRNAs to identify sequences sufficient for transport and to define the Oocyte Entry Signal or OES (Jambor et al. 2014). The OES is active in the context of overlapping segments of the *osk* 3' UTR (positions 499-759 and 630-1028). Within the overlapping region is a stem-loop structure, SL2b,

whose loop sequences mediate RNA dimerization. When inserted in the 3' UTR of a *GFP* mRNA, each of the overlapping segments confers strong oocyte enrichment. Deletion of the loop of SL2b does not affect transport, and so this process is independent of RNA dimerization. Significantly, mutation of either stem strand in the distal portion of SL2b disrupts oocyte transport of the GFP reporter mRNA, while mutation of both strands to restore base pairing restores transport. These mutations define the OES, which has some similarity to the TLS, being an A/U rich region of double-stranded RNA.

The experiments with the OES and the Bru binding sites demonstrate that multiple elements in the *osk* 3' UTR can direct transport of reporter mRNAs to the oocyte. What these experiments do not test is whether either individual type of transport element is essential for oocyte transport of the *osk* mRNA (as opposed to a reporter mRNA not containing all *osk* mRNA sequences). To address this issue we mutated the different elements in *osk* transgenes, which were then tested in flies lacking endogenous *osk* mRNA to eliminate the possibility of hitchhiking. The *osk ABC all*$^{-}$ mRNA has the Bru binding sites in both AB and C regions mutated (Reveal et al. 2010). The same mutations abolished the ability of these regions to confer oocyte transport of the *GFP-AB* and *GFP-C* mRNAs, but had no discernible effect on transport of *osk* mRNA to the oocyte: the *osk ABC all*$^{-}$ mRNAs showed strong oocyte enrichment (Fig. 3.3). To inactivate the OES in the *osk* mRNA, we used a deletion mutant, ***osk3'*Δ*665-685,*** that lacks one strand of the stem shown to be required for OES function, as well as the loop which mediates RNA dimerization. The ***osk3'*Δ*665-685*** mutant was strongly enriched in the oocyte (Fig. 3.3). Thus, neither the Bru binding sites nor the OES are required for transport of *osk* mRNA to the oocyte, suggesting degeneracy in this

process. To assess the consequences of eliminating both Bru- and OES-dependent transport, an *osk* transgene bearing both the Bru site mutations and the OES deletion, *osk ABC all⁻ 3'Δ665-685*, was tested. Despite the loss of both types of oocyte transport signals, the mRNA was still strongly enriched in the oocyte (Fig. 3.3). Thus, there must be another contribution to oocyte transport.

The likely location for an additional transport element is close to the OES in SL2b. In the initial mapping of *osk* sequences important for transport, two deletion mutants, lacking 3' UTR nt 534-684 or 683-796, had strong defects in transport to the oocyte (Kim-Ha et al. 1993). Each mutant removes one stem of SL2b. Both of the more recent studies also found that deletions within this region strongly affected transport. Mutant *GFP-osk$^{\Delta 61}$* deletes all of one strand of the SL2b (Kim et al. 2014), and *UAS-gfp-osk3'UTR Δ2b* deletes all of SL2b (Jambor et al. 2014).

To better understand the contributions of the different parts of SL2b to oocyte transport, we tested several additional mutants. Three of these have small deletions that, in combination with *osk3'Δ665-685*, collectively remove or disrupt all of SL2b (Fig. 3.4A,B). All of the small deletion mutants show clear enrichment of *osk* mRNA in the oocyte, although *osk3'Δ706-723* is not as strongly enriched as the wild type control (Fig. 3.4C). Given the absence of a strong transport defect for any of the mutants removing only parts of SL2b, we also tested a mutant in which the entire SL2b was precisely deleted. This mutant, *osk3'Δ634-742*, was strongly impaired for transport, but the vast majority of egg chambers retained unambiguous enrichment of the mRNA in the oocyte (Fig. 3.4D). These results lead to two conclusions. First, there must be degeneracy or redundancy within SL2b, as mutations equivalent to those used to disrupt OES function in

reporter transgenes had no substantial effect on *osk* mRNA transport. Second, although SL2b is an important contributor to oocyte transport of *osk* mRNA, it is not the only contributor.

Mutation of stem-loop 2b impairs the noncoding *osk* RNA function

The *osk* mRNA has a noncoding function, independent of its role in producing Osk protein. Sequences which provide the noncoding function are contained within the *osk* mRNA 3' UTR, and they require localization to the oocyte for their activity (Jenny et al. 2006; Kanke et al. 2015). Consequently, mutations which impair transport to the oocyte may also impair *osk* noncoding function. Each of the mutants with deletions in SL2b was tested for rescue of the *osk* RNA null phenotype. In the absence of *osk* mRNA, oogenesis is arrested at stage 6/7 and no eggs are produced. Partial rescue of this phenotype allows oogenesis to progress further and restores some egg laying, with the rate of egg laying providing a measure of noncoding *osk* RNA function (Jenny et al. 2006; Kanke et al. 2015).

A wild type *osk* transgene, when present in a single copy, provides strong noncoding *osk* RNA function with an egg laying rate of 2.5 or more eggs per female per hour. Each of the small deletions within SL2b resulted in a lower rate of egg laying. For mutants *osk3'*Δ*636-652*, *osk3'*Δ*665-685* and *osk3'*Δ*688-705* the reduction was modest, with rates between half and two thirds of wild type. For mutant *osk3'*Δ*706-723*, which had the strongest defect in RNA transport to the oocyte, the reduction was more severe with only 0.1 eggs laid per hour per female. Even with two copies of the transgene the rate was still affected at 1.4 eggs/hr/female. Not surprisingly, deletion of all of SL2b had the strongest effect:

no eggs were laid with one copy of the transgene, and with two copies the rate remained low at 0.7/hr/female. Among the eggs laid, many had a spindle morphology, a phenotype also found in other situations in which rescue of the osk RNA null phenotype is incomplete.

DISCUSSION

There are two main conclusions from these studies. The first is that Bru, a translational regulator, plays an additional role in mRNA transport. The ability of a factor with another primary function to also confer RNA localization suggests an explanation for the considerable diversity of mRNA localization patterns, and a general mechanism by which these patterns can be achieved. The second main conclusion is that multiple mechanisms contribute to transport of *osk* mRNA to the oocyte. We suggest that this is not simply to ensure transport, but provides the means to transition from this phase of mRNA localization to the subsequent movement within the oocyte to the posterior pole.

Bru was originally identified for its role in translational repression (Kim-Ha et al. 1995). Subsequent studies also implicated Bru in translational activation (Reveal et al. 2010). Now we have found that the presence of Bru binding sites in mRNAs confers a specific form of mRNA localization, transport to the oocyte during early stages of oogenesis. The distribution of Bru in the ovary fits well with the new role. At early stages Bru protein is highly concentrated in the oocyte, despite a more uniform distribution of the mRNA (Webster et al. 1997). Bru must therefore be moved to this destination, a process that would allow for colocalization of associated RNAs.

It is noteworthy that a large fraction of all mRNAs display some form of

localization. Within the ovary about 10% are concentrated in early stage oocytes, a pattern similar to the localization conferred by Bru (Dubowy and Macdonald 1998). This high incidence of RNA transport to the oocyte could represent, at least in part, colocalization with proteins that function primarily in some other role, but facilitate a form of mRNA localization as they are themselves moved to the oocyte. Recent studies have highlighted secondary roles in RNA binding for various proteins more primarily implicated in other functions (Castello et al. 2012). We propose a related form of secondary function: RNA binding proteins acting in some form of regulation or metabolism can have the secondary function of RNA transport. This paradigm could apply to the remarkable prevalence of mRNA localization found for embryonic mRNAs in *Drosophila*, of which about 70% exhibit some form of localization (Lécuyer et al. 2007). For some or even many of these mRNAs, localization could rely on association with proteins whose primary role is not mRNA transport, but can nevertheless provide this function as a consequence of their own subcellular localization. Notably, very often the degree of mRNA localization is not high, implying a low efficiency which would be consistent with the model. If this model is correct, an extremely large and diverse set of RNA binding sites will function as mRNA localization signals.

The work presented here highlights the value of combining different types of assays to explore mechanisms of mRNA localization. Testing the ability of an RNA element to confer localization on reporter mRNAs reveals what the element can do. Testing the effects of removing the element from its natural setting, the host RNA, reveals whether that function is essential. In this manner we have shown that multiple elements share the ability to direct the *osk* mRNA to the oocyte, but that no single element is essential.

The system for transport of *osk* mRNA to the oocyte displays both degeneracy and redundancy. Degeneracy refers to the ability of structurally distinct elements to perform the same function (Tononi et al. 1999). The Bru sites and the OES have degenerate roles in RNA transport. Redundancy involves the use of multiple iterations of the same element, each performing the same function (Tononi et al. 1999). Here the different Bru binding sites act redundantly, with either of two clusters of sites conferring oocyte localization on a reporter mRNA. Similarly, the different parts of SL2b could act redundantly. The previous mapping of the OES tested the distal portion of the SL2b (Jambor et al. 2014). We have now shown that multiple parts of SL2b contribute to OES function, with a central part being most sensitive to deletion. However, the entire SL2b must be deleted to substantially reduce mRNA transport to the oocyte. A likely explanation is that SL2b provides multiple, redundant binding sites for Egl, and that all must be deleted to strongly affect mRNA transport to the oocyte.

Both redundancy and degeneracy can provide robustness to a biological process. While having robust transport of *osk* mRNA is important - this localization step is required both for the noncoding *osk* RNA function and as a prerequisite for further localization within the oocyte - there may be another reason why *osk* relies on its particular combination of transport mechanisms. A suggestive and intriguing feature of the OES is its relative frailty, as compared to the archetype for this class of localization signal, the TLS of f*s(1)K10* mRNA. The TLS is largely insensitive to sequence context: a short segment containing just the TLS stem-loop has been shown to confer strong oocyte transport on multiple different mRNAs (Serano and Cohen 1995). This robustness suggests that formation of the TLS structure, which is critical for its function and binding Egl

(Dienstbier et al. 2009; Bullock et al. 2010), is highly favored. By contrast, the OES cannot function on its own: even the complete stem-loop 2b does not support RNA localization of a reporter mRNA, requiring flanking *osk* 3' UTR sequences (either 131 nt to the 5' side or 269 nt to the 3' side) for this activity (Jambor et al. 2014). Sensitivity to sequence context raises the possibility that alternate folding options constrain the ability of the OES to adopt the structure suitable for Egl binding. The contrast between the TLS and the OES is also evident in their different abilities to direct mRNA localization in an ectopic setting. When tested for apical localization in early embryos, the TLS is highly active, while the OES is weakly active with only a fraction of the *osk* RNA being localized (Bullock and Ish-Horowicz 2001; Jambor et al. 2014). Such incomplete localization could be due to only a fraction of the RNA having the required structure, or to inherently weaker affinity of the structure for Egl, or both. Why would evolution of *osk* have produced an etiolated TLS-like transport signal? Notably, the TLS not only directs the *fs(1)K10* mRNA to the oocyte, it also restrains the mRNA at the site of entry into the oocyte at the anterior margin (Serano and Cohen 1995). Although *osk* mRNA is also concentrated at the anterior margin of the oocyte, this distribution is transient and the mRNA undergoes a further step in localization to the posterior pole, a process that relies on a different localization signal, the SOLE (Kim-Ha et al. 1991; Ephrussi et al. 1991; Ghosh et al. 2012). We suggest that the need to switch between different localization systems - one for transport to the oocyte, and one for movement within the oocyte - demands that the machinery mediating the initial step not be tenaciously associated with the mRNA.

SUMMARY & FUTURE STUDY

Here, we showed a new role of Bru binding sites in the *osk* mRNA 3' UTR in mRNA oocyte transport. Unlike the OES, previously reported as the oocyte transport element located in the *osk* mRNA 3' UTR (Jambor et al., 2014), Bru binding sites do not seem to adopt a stem-loop structure and are active in isolation. Bru binding sites or the OES are sufficient to direct oocyte transport of a reporter mRNA. However, neither of them is required for oocyte transport of *osk* mRNA. Therefore, there must be another contribution to oocyte transport. We suggest that there are multiple degenerate mechanisms acting in oocyte transport of *osk* mRNA.

Interestingly, deletion of a stem-loop structure (SL2b) in the OES not only greatly reduced oocyte transport of *osk* mRNA, but also impaired *osk* noncoding function. Preliminary results indicate that loss of *osk* noncoding RNA function is not due to reduced *osk* mRNA levels. These results are consistent with an earlier observation (Kanke et al ref) that transport of *osk* mRNA to the oocyte is required for its activity in noncoding function.

To confirm that the role for SL2b in *osk* noncoding RNA function is to provide transport of the mRNA to the oocyte, we can ask if the TLS (a well-characterized signal for oocyte transport) can substitute for SL2b. The TLS confers strong oocyte transport on multiple different mRNAs (Serano and Cohen, 1995). Therefore, adding the TLS to an *osk* mRNA lacking SL2b should restore oocyte transport of *osk* mRNA, and is expected to also restore *osk* noncoding RNA function. If the noncoding function is not restored, this would argue that SL2b has a role in that function distinct from transport of the mRNA to the oocyte.

The same transgene leads to a further experiment, in which we can ask if a strongly active oocyte transport signal will interfere with the subsequent steps in *osk* mRNA localization. If posterior localization is disrupted by replacement of SL2b with the TLS, this would support our model that *osk* mRNA uses weak mechanisms for oocyte transport to facilitate the subsequent transfer to the machinery involved movement of mRNA to the posterior pole of the oocyte.

FIGURES

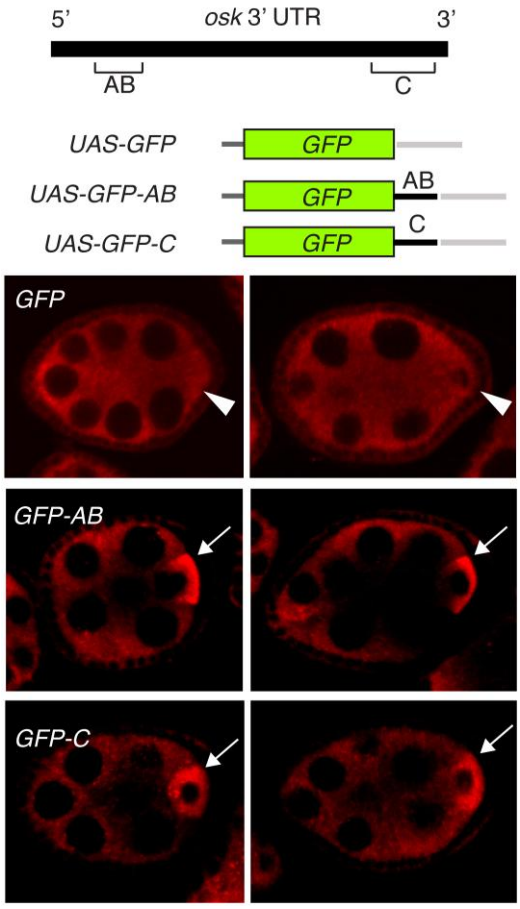

Figure 3.1. Oocyte transport directed by the AB and C regions of the *osk* mRNA 3' UTR.

The diagram at top shows the *osk* mRNA 3' UTR, with the two regions containing Bru binding sites (AB and C) indicated, as well as the transgenes tested. Below are panels showing the distribution of transgene mRNAs. For each egg chamber the position of the oocyte is indicated with an arrowhead (if no enrichment of the mRNA) or an arrow (if the mRNA is enriched).

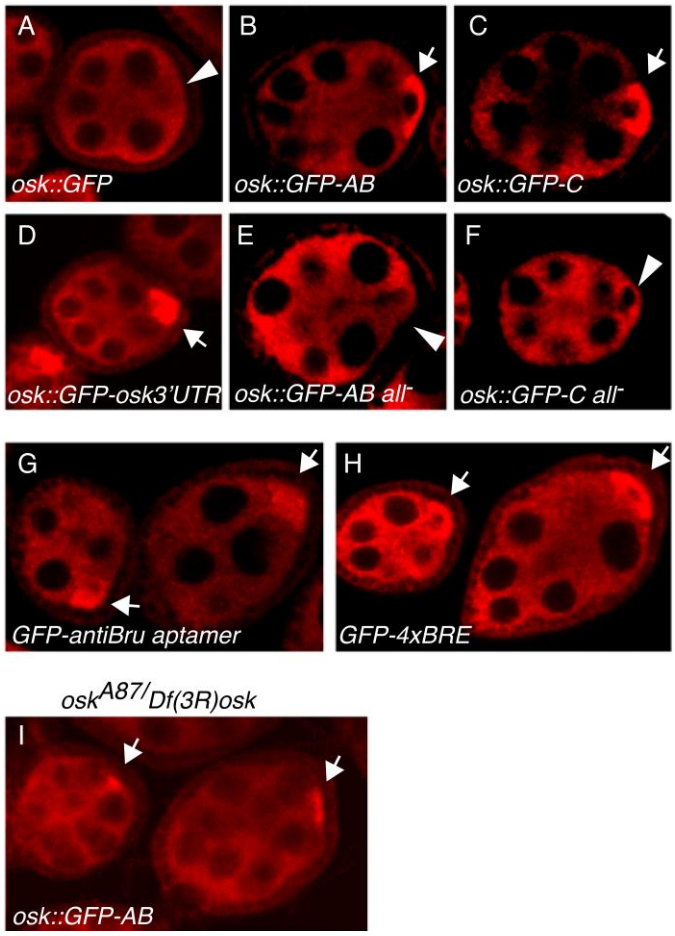

Figure 3.2. Bru binding sites mediate RNA transport to the oocyte.

Distributions of transgene mRNAs were detected by in situ hybridization. For each egg chamber the position of the oocyte is indicated with an arrowhead (if no enrichment of the mRNA) or an arrow (if the mRNA is enriched). A-F are the *osk::GFP* reporter, with additions as indicated. G-H are the *GFP* reporter (Fig. 3.1) with additions as indicated. I shows the distribution of the *osk::GFP-AB* mRNA in egg chambers lacking *osk* mRNA.

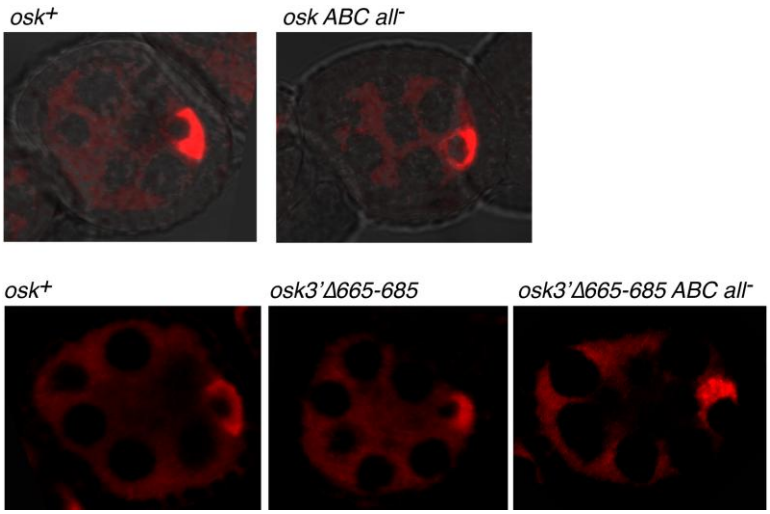

Figure 3.3. Persistent oocyte transport when both Bru binding sites and the OES are mutated.

All panels are in situ hybridization to detect *osk* mRNAs, as indicated. All transgenes were tested in an *osk* RNA null background.

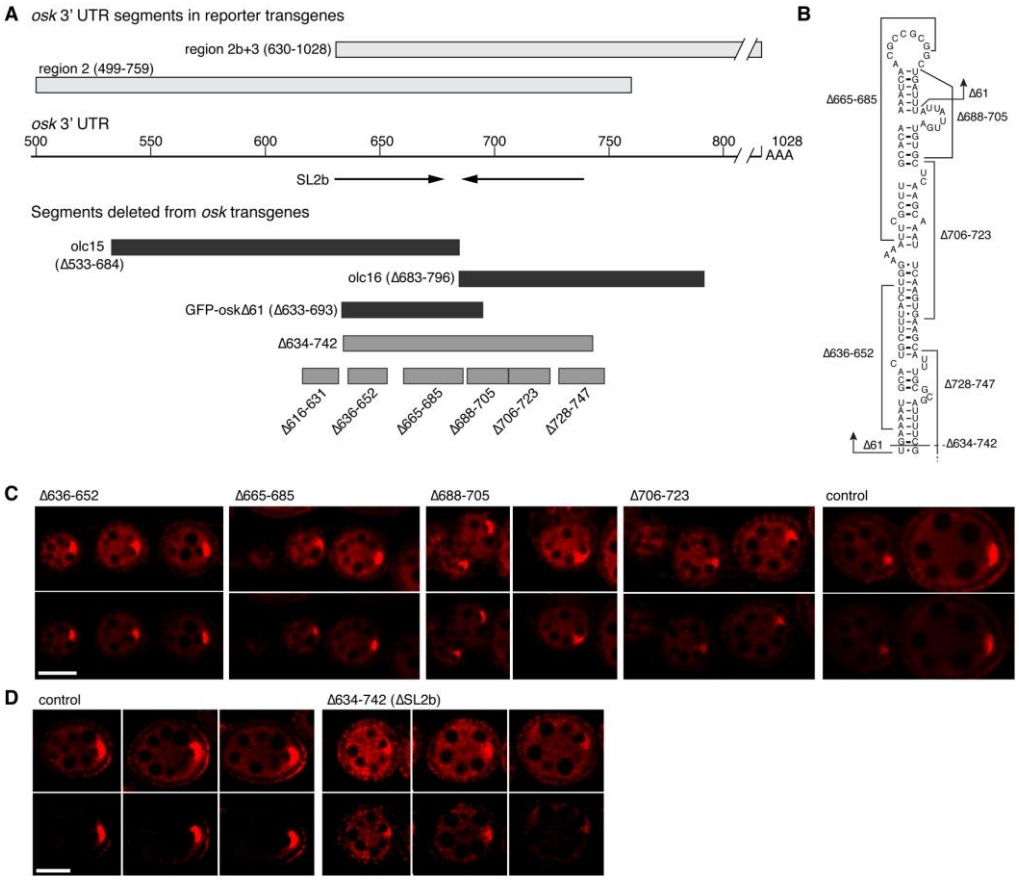

Figure 3.4. The role of stem-loop 2b in transport of *osk* mRNA to the oocyte.

A. Diagram of the 3' half of the *osk* 3' UTR, with the position of SL2b marked for comparison to reporter transgenes with 3' UTR segments (indicated above with light gray bars; (Jambor et al. 2014)) or to deletions introduced into *osk* transgenes (indicated below with dark and medium gray bars). Those with dark bars are from Kim-Ha et al. (olc15 and olc16) or Kim et al. (GFP-oskΔ61). Those with medium gray bars are from this study.
B. Proposed structure of SL2b (Jambor et al. 2011) with the positions of deletions indicated.
C. Effects of small deletions on *osk* mRNA transport to the oocyte. Each transgene was tested as a single copy in the *osk* RNA null background, with the transgene RNA detected by in situ hybridization. The upper and lower panels are identical, except that the signal intensity was increased for the upper panels to display the entire egg chambers.
D. Effect of deleting the entire SL2b on *osk* mRNA transport to the oocyte. The panels are as in C. Although the oocyte enrichment is low for the mutant, all of the egg chambers (n=30) had detectable concentration in the oocyte as shown by the representative examples.

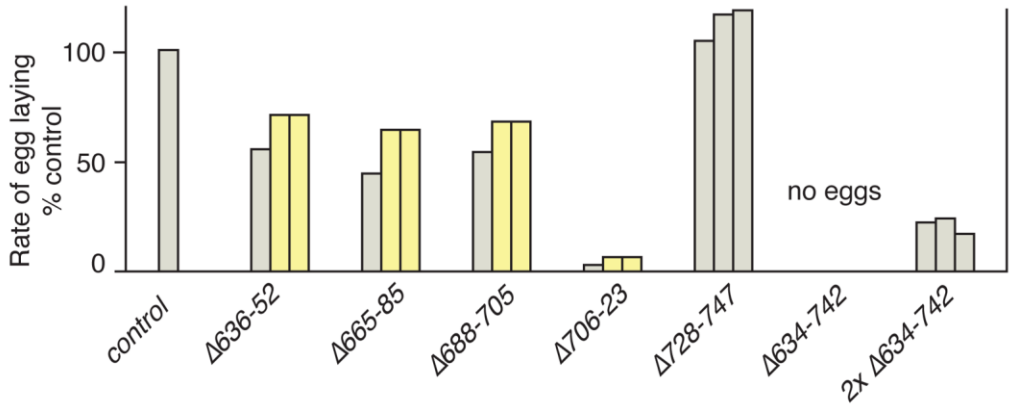

Figure 3.5. Stem-loop 2b is required for the noncoding function of *osk* mRNA.

Egg laying rates for *osk* transgenes tested in *osk* RNA null background. Each vertical bar indicates the results of a separate experiment.

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Appendix: Additional Information of Bicoid Stability Factor in *oskar* Regulation

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RESULTS

Inducing *bsf* KD in different developmental stages of oogenesis does not affect Osk accumulation up to stage 10 of oogenesis.

The *bsf* KD shown in the Chapter Two was induced by the *matalpha4-GAL-VP16* Gal4 driver which activates gene expression during mid oogenesis. In parallel with using the *matalpha4-GAL-VP16* driver, I also tested other GAL4 drivers: the *nosGAL4::VP16-nos.UTR* driver which activates gene expression in the female germarium and then again later in oogenesis, and the *MTD-GLA4 (MTD)* driver which activates gene expression in the female germarium and throughout oogenesis. To assess the efficiency of the *bsf* KD, BSF protein level was monitored in ovaries by western blot analysis. The level of BSF reduction by the *nosGAL4::VP16-nos.UTR* driver in ovaries was similar to that by the *matalpha4-GAL-VP16* driver (Fig. A.1A).

Osk protein expression was monitored in the *bsf* KD ovaries by immunodetection of endogenous Osk (Fig. A.1B, C, and D), or of Osk::HA (Fig. A.1E,F) expressed from an epitope-tagged *osk* transgene; both methods gave similar results. The amount of Osk was similar to that in the wild type egg chambers up to stage 10 (Fig. A.1B). Different from using the *matalpha4-GAL-VP16* driver in the bsf^{SH1181} heterozygous mutant background to further reduce BSF level, using the *nosGAL4::VP16-nos.UTR* driver in the same genetic background disrupted oogenesis (Fig. A.1C,E). Most of the ovarioles showed arrested or abnormal progression of oogenesis (Fig. A.1C,E, top panels), and few ovarioles showed normal oogenesis (Fig. A.1C,E, bottom panels). However, the amount of Osk was similar to that in the wild type egg chambers up to stage 10 (Fig. A.1D, left panels and Fig. A.1F). Consistent with this, the accumulation of

osk RNA was also similar to that in the wild type egg chambers up to stage 10 (Fig. A.1D, middle and right panels).

BSF localizes mainly at the posterior pole of the oocyte

BSF was initially identified as a cytoplasmic ovarian protein which bound to *bicoid* mRNA. BSF appeared in particles within the cytoplasm of both the nurse cells and the oocyte during oogenesis up to stage 7-8 (Mancebo et al., 2001). BSF was found in mitochondria and this may be the predominant location at least in some tissues (Bratic et al., 2011). Since I found that BSF was associated with *osk* mRNA and involved in posterior Osk accumulation during late oogenesis, I wanted to re-examine its subcellular distribution within the oocyte during late oogenesis. First, I tried immunodetection of BSF using a new anti-BSF antibody to examine stage 9-10 egg chambers. During early-stages of oogenesis (< stage 5), BSF was present throughout the egg chamber appearing in regions surrounding the nuclei, consistent with the previous result (Mancebo et al., 2001)(Fig. A.2A). During mid-oogenesis (stage 6-8), BSF was not detected either in the nurse cells or the oocyte. This might be because of an antibody issue since the anti-BSF antibody showed poor penetration into the egg (Fig. A.2A). In stage 10 egg chambers, BSF mainly appeared at the posterior pole of the oocyte with lower levels detected along the entire cortex of the oocyte (Fig. A.2B-D); however BSF was not detected in the nurse cells probably due to an antibody penetration problem. Although individual differences were seen within the same genotype, generally a lower level of BSF crescent was detected in the $bsf^{+/-}$ (Fig. A.2C-C'') and *bsf* KD in $bsf^{+/-}$ oocytes (Fig. A.2D-D'') compared to the wild-type oocytes (Fig. A.2B-B'').

Next, I tried expressing the *UAS-bsf::GFP* transgenes using the *matalpha4-GAL-VP16* driver to examine the later-stages of oogenesis (after stage 10), when immunodetection becomes impossible due to deposition of the vitelline membrane. Interestingly, overexpression of BSF also disrupted progression of oogenesis, although it is also possible that BSF::GFP as a fusion protein could cause this problem. In the $bsf^{+/+}$ background, BSF::GFP expression caused abnormal progression of oogenesis. Even though the overall ovary phenotype seemed to be arrested in development, several late-stage egg chambers could be seen in the ovary. Four *UAS-bsf::GFP* transgenic lines showed similar expression pattern. During early-stages of oogenesis (< stage 5), a strong GFP signal was detected throughout the egg chamber and found in regions surrounding the nuclei, consistent with the immunodetection results. During mid-oogenesis (stage 6-8), the GFP signal appeared as mosaic pattern or disappeared. During late-stages of oogenesis (stage 9-10), no GFP signal was detected throughout the egg chamber. Overall the line-1 and line-2 showed a weaker GFP signal than the line-3 and line-4. Corresponding to this, the line-1 and line-2 showed a milder defect in progression of oogenesis than the line-3 and line-4 (Fig. A.3A-D). The correlation between the BSF::GFP expression level and degree of the defect in progression of oogenesis suggests an early function of BSF in oogenesis.

If the excess of BSF was what caused the abnormal progression of oogenesis, this defect could be relieved/rescued by reducing the BSF level. Therefore, I tried expressing the *UAS-bsf::GFP* transgene in the $bsf^{+/-}$ background using the *matalpha4-GAL-VP16* driver or the *nosGAL4::VP16-nos.UTR* driver. In the $bsf^{+/-}$ background, using the *matalpha4-GAL-VP16* did not

reduce the defect in progression of oogenesis, and showed a similar expression pattern/level to that in the *bsf*$^{+/+}$ background (Fig. A.3E-E' versus Fig. A.3B). In the *bsf*$^{+/-}$ background, using the *nosGAL4::VP16-nos.UTR* somewhat rescued the defect in progression of oogenesis, and the GFP signal was detected in late-stage egg chambers; a mosaic pattern was shown in the nurse cells, but no GFP signal was detected in the oocyte at all (Fig. A.3F-F').

BSF and Bru are not co-immunoprecipitated

Both BSF and Bru bind to the *osk* RNA 3' region. Among three BSF binding sites, two (977-981 and 984-988) are very close to and one (970-974) overlaps with the Bru binding site. All of these three sites appeared to be involved in Osk expression. Therefore, it is conceivable that BSF and Bru interact with each other to activate translation of *osk* mRNA. To test this possibility I performed an immunoprecipitation (IP) assay. Unexpectedly, neither BSF nor Bru was co-immunoprecipitated with either the anti-Bru or anti-BSF antibody, respectively (Fig. A.4A). It could simply suggest that BSF and Bru do not interact. Since BSF and Bru bind to the *osk* RNA 3' region directly (Fig. A.4B), it is also possible that their interaction is RNA-dependent and our experimental condition was not proper to detect this (since I did not add RNase inhibitors to the samples during the IP assay, there could have been degradation of RNA during antibody incubation with ovary extract overnight). It is worth repeating the IP assay in different experimental conditions such as adding RNase inhibitors, adjusting the salt/detergent concentration.

Loss of BSF binding affects Osk protein expression

In the Chapter Two, I showed that loss of BSF binding to the *osk* RNA affects Osk expression in the later-stage oocytes and early-stage embryos by imaging approach. I also monitored the level of Osk protein by western blot analysis. Same transgenes used for the imaging approach were tested (Fig. A.5A). The whole ovary extract and extract from the later-stage egg chambers were each divided into a soluble fraction and an insoluble fraction (see Methods). In the soluble fraction, both endogenous Long Osk and Short Osk were detected by the anti-Osk antibody in extract from the $osk^{+/+}$ (w^{1118}) and $osk^{+/-}$ (IBE^{-}; $osk^{+/-}$) ovaries. Although I could not detect the OskT140::GFP protein in the soluble fraction using the anti-Osk or anti-GFP antibodies (Fig. A.5B), I could in the insoluble fraction. As expected, the protein expression level of the mutant transgenes (977-981 or 984-988) was dramatically reduced compared to that of the wild type transgene (osk^{+}) (Fig. A.5C). The degree of reduction was stronger in extract from the later-stage egg chambers, consistent with the imaging results showing no change in the expression of the mutant transgenes up to stage 10 of oogenesis. Similar results were obtained from using the *osk::HA* transgenes (Fig. A.5D,E).

BSF also binds to the *osk* RNA 5' region, however the correlation between osk translational activation and BSF binding to the *osk* RNA 5' region is not established

Translational activation of *osk* is dependent on the *cis*-acting elements in *osk* mRNA. One of these to be initially identified lies in the 5' portion of the mRNA, in the regions between the alternate translation initiation codons used to make the Long Osk and Short Osk proteins (Gunkel et al., 1998). Recently, we

reevaluated the 5’ portion in order to characterize the role of particular sequences in Osk expression. Deletion of the *osk* mRNA sequences 91-120, 118-135 and 121-150 resulted in low protein levels and embryonic patterning defects ((Kanke and Macdonald, 2015), Fig. A.6A). Interestingly, we found that BSF also binds to the *osk* RNA 5’ portion containing sequences initially considered to be important for translational activation. BSF binding to the 5’ RNA was as strong as binding to the 3’ RNA (Fig. A.6B,C). Therefore, I wanted to test the transcripts bearing deletion mutations (Fig. A.6D) for loss of BSF binding and consequently any loss of translational activation. The mutants Δ61-90 and Δ91-120 were strongly defective in BSF binding. The remaining mutants, Δ118-135, Δ121-150, Δ151-180, and *osk5’*(*M1R*) (this is a control for Δ118-135 which also has the M1R mutation) had no defect in BSF binding (Fig. A.6E). The mutant Δ61-90 had no defect in Osk expression and embryonic body patterning in the absence of BSF binding. This suggests that BSF binding to the *osk* RNA 5’ region is not involved in the *osk* translational activation.

MATERIALS AND METHODS

Flies and Transgenes

The genomic *osk* transgenes included 3XHA epitope tag inserted after T140 (M139 is the aminoterminal end of Short Osk). This tag does not detectably alter *osk* expression or patterning activity. Expression of UAS transgenes was tested by the *nosGAL4::VP16-nos.UTR* or *MTD-GAL4* (Bloomington stock 31777) driver.

Antibodies

Antibodies for western blot analysis: mouse anti-HA (Covance)(1:1,000); mouse anti-Bru (1:8,000); rat anti-BSF (1:1,000); rabbit anti-Osk (1:2,000); mouse anti-GFP (Sigma)(1:2,500); mouse anti-α-Tubulin (Sigma)(1:1,000); alkaline phosphatase-conjugated goat anti-rat IgG (Sigma)(1:5,000); alkaline phosphatase-conjugated goat anti-mouse IgG (Applied Biosystem)(1:5,000). Antibodies for imaging: mouse anti-HA (Covance)(1:1,000); rabbit anti-Osk (1:2,000); rabbit anti-BSF (1:1,000); Alexa Fluor 488 goat anti-mouse IgG (Invitrogen)(1:800); Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen)(1:800).

Immunoprecipitation

Protein extract was prepared as for RNA affinity purification. Protein A/G beads (Santa Cruz) were equilibrated by washing several times with OEB100 (50 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 100 mM NaCl, 0.1% Triton X-100, 10% Glycerol, 0.1 mM DTT). 1 mg of ovary extract (1ml) was incubated with 10 ul of anti-BSF (1:100) or 1 ul of anti-Bru (1:1,000) overnight at 4 °C with rotation. And then, 20 ul of equilibrated protein A/G beads were added followed by incubation for 1 hr at 4 °C with rotation. After incubation, the beads were spun down, saving both pellets and supernatant (S) fractions. The pellet fraction was washed several times with ice-cold OEB150 (50 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 150 mM NaCl, 0.1% Triton X-100, 10% Glycerol, 0.1 mM DTT), and boiled in 20 µl of 6X protein sample buffer for 10 min to elute bound proteins (P). S and P fractions were separated by SDS-PAGE and analyzed by western blot.

Preparation of ovary extract for western blot analysis

Ovaries from young females fed on yeast for 3~4 days were dissected in 1X PBS on ice, washed once with ice cold COEB100 (50 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 100 mM NaCl, 0.1% Triton X-100, 10% Glycerol, 0.1 mM DTT, Complete, Mini, EDTA-free protease inhibitor cocktail tablet (Roche)). 20 pairs of ovaries and 200 late-stage egg chambers from each genotype were collected. After washing, they were homogenized with a plastic pestle in a 1.5 ml microfuge tube in the presence of 60 ul (whole ovaries) or 20 ul (late-stage egg chambers) COEB100. The lysates were separated the soluble fraction (in the supernatant) from the insoluble fraction (in the pellet) by centrifugation at 12,000 rpm for 25 min. Protein concentration of the soluble fraction was determined by Bradford (Bio-rad) method. Insoluble fractions were boiled in 100 µl (whole ovaries) or 50 µl (late-stage egg chambers) of 6X protein sample buffer for 15 min and 10 µl was separated by SDS-PAGE for western blot analysis.

UV cross-linking assay

The *osk* RNA probes were transcribed from the plasmids used in RNA affinity purification using MAXIscript kit (Ambion) and uniformly labeled with [α-^{32}P]UTP (800Ci/mmol, Perkin Elmer) according to the manufacturers instructions. After phenol/chroloform extraction, unincorporated nucleotides were removed using NucAway spin column (Ambion) and the RNA probes were precipitated with ammonium acetate/ethanol. UV cross-linking assay was performed as described (Kim-Ha et al., 1995), except the different composition of 10X binding buffer (60 mM HEPES, pH7.9, 300mM KCl, 20 mM $MgCl_2$, and Complete, Mini EDTA-free protease inhibitor cocktail tablet (Roche)). After electrophoresis of

cross-linked adducts, gels were dried using GelAir Drying system (Bio-rad) and exposed to a Phosphor Screen (Molecular Dynamics) for 12 hr. The screen was then analyzed with a Typhoon lase scanner (GE Healthcare).

FIGURES

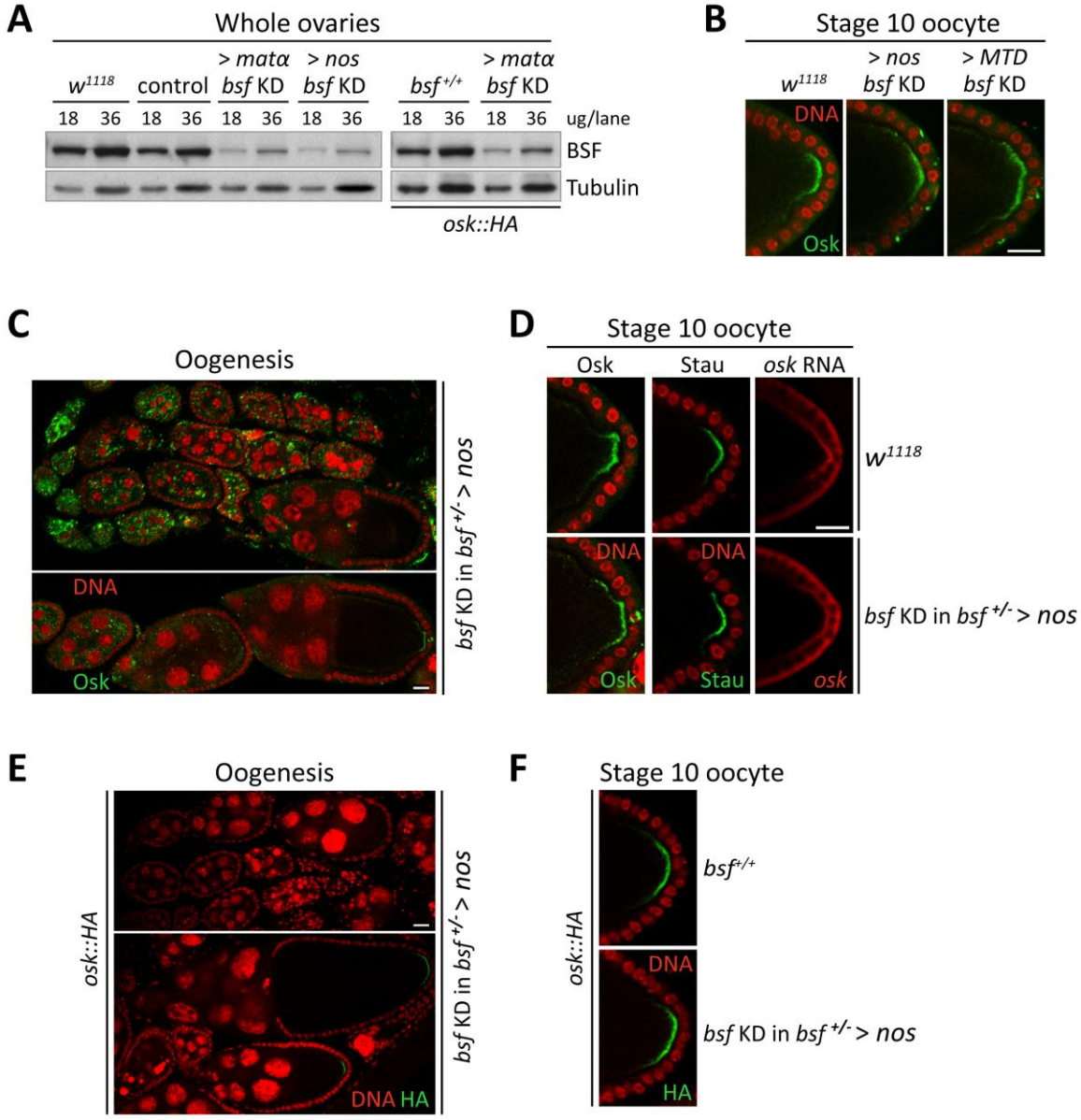

Figure A.1. Inducing *bsf* KD in different developmental stages does not affect Osk accumulation during oogenesis up to stage 10.

A. Efficiency of the *bsf* KD in ovaries using the *matalpha4-GAL-VP16* (*matα*) or the *nosGAL4::VP16-nos.UTR* (*nos*) driver detected by western blot analysis. w^{1118} was used as a wild type control. Genotypes used as follows. Control is +/+;

TRiP-bsf/TRiP-bsf. *bsf* KD>*matα* is *+/+; TRiP-bsf/matalpha4-GAL-VP16. bsf* KD>*nos* is *+/+; TRiP-bsf/nosGAL4::VP16-nos.UTR.* For *osk::HA* transgenic, *bsf* $^{+/+}$ is *+/+; osk::HA matalpha4-GAL-VP16 osk*^{A87}*/+. bsf* KD>*matα* is *+/+; osk::HA matalpha4-GAL-VP16 osk*^{A87}*/TRiP-bsf*.

B. Osk accumulation is normal in the *bsf* KD driven by different GAL4 drivers up to stage 10 of oogenesis. Panels show the posterior portion of stage 10 egg chambers. DNA, red; Osk, green. The scale bars in this and other panels are 20 μm. Genotypes are as follows. *bsf* KD>*nos* is *+/+; TRiP-bsf/nosGAL4::VP16-nos.UTR. bsf* KD>*MTD* is *otu-GAL4-VP16; nos-GAL4-NGT/+; TRiP-bsf/nos-GAL4-VP16.*

C. Ovary phenotypes of *bsf* KD in *bsf* $^{+/-}$ by the *nosGAL4::VP16-nos.UTR* driver. The examples of the ovarioles showing arrested oogenesis (top) and normal oogenesis (bottom). DNA, red; Osk, green.

D. The *bsf* KD in the *bsf* $^{+/-}$ by the *nosGAL4::VP16-nos.UTR* driver does not affect Osk expression and *osk* RNA accumulation up to stage 10 of oogenesis. Left panels show detection of endogenous Osk protein. Middle panels show Stau protein (which colocalizes with *osk* mRNA). Right panels show *osk* mRNA. Top panels from *w*^{1118} and bottom panels from *bsf* KD in *bsf* $^{+/-}$ by the *nosGAL4::VP16-nos.UTR* driver. For the Osk and Stau protein panels, DNA is red and protein is green. For the in situ hybridization panels *osk* RNA is red (the signal in follicle cells is background).

Genotypes in C-D is as follows. *bsf* KD in *bsf* $^{+/-}$ *>nos* is *bsf*^{SH1181}*/+; TRiP-bsf/ nosGAL4::VP16-nos.UTR.*

E. Ovary phenotypes of the *bsf* KD in the *bsf* $^{+/-}$ by the *nosGAL4::VP16-nos.UTR* driver. Top panel shows arrested oogenesis ovarioles. Bottom panel shows late-stage egg chambers having normal Osk::HA accumulation.

F. Osk expression is normal in the *bsf* KD in the *bsf* $^{+/-}$ by the *nosGAL4::VP16-nos.UTR* driver up to stage 10 of oogenesis. Panels show posterior portion of stage 10 egg chambers. Top panel shows *bsf*$^{+/+}$ and bottom panel shows *bsf* KD in *bsf* $^{+/-}$ by the *nosGAL4::VP16-nos.UTR* driver. DNA, red; Osk::HA, green.

Genotypes in E-F are as follows. *bsf* $^{+/+}$ is *osk::HA/+; nosGAL4::VP16-nos.UTR osk*^{A87}*/+. bsf* KD in *bsf* $^{+/-}$ *>nos* is *bsf*^{SH1181}*/ osk::HA; nosGAL4::VP16-nos.UTR osk*^{A87}*/TRiP-bsf*. DNA, red: Osk::HA, green.

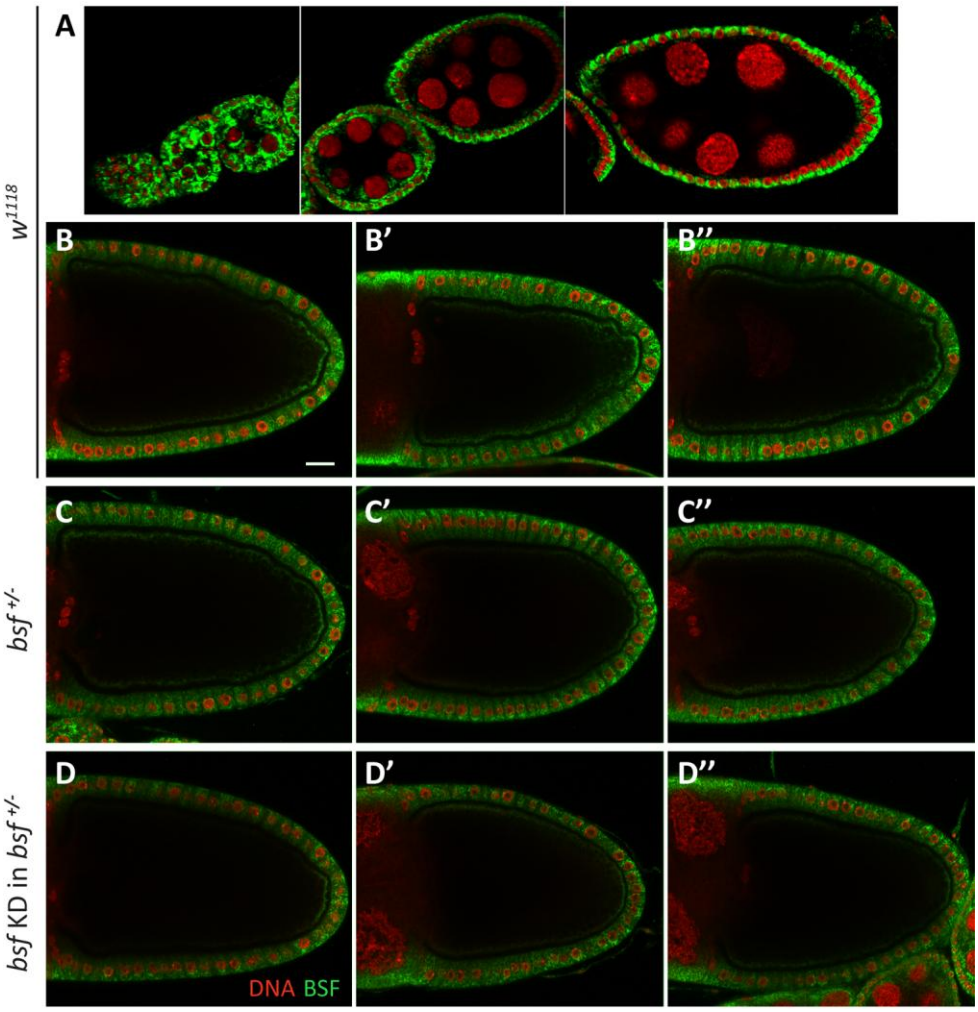

Figure A.2. Within the oocyte, BSF is mainly localized at the posterior pole

Immunodetection of BSF expressed from w^{1118} (A-B''), $bsf^{+/-}$ (C-C''), and *bsf* KD in $bsf^{+/-}$ (D-D'') egg chambers. All panels show only the portion of the oocyte; each panel from the same genotype shows individual variation in BSF expression. Although the anti-BSF antibody shows poor penetration into the egg chamber, a crescent of BSF is detected specifically at the posterior pole of the oocyte (lower levels appear along the entire cortex). DNA, red; BSF, green. Scale bar is 20 μm. Genotypes used as follows. $bsf^{+/-}$ is bsf^{SH1181}*/CyO; TRiP-bsf/TM2*. *bsf* KD in $bsf^{+/-}$ is bsf^{SH1181}*/+; TRiP-bsf/matalpha4-GAL-VP16*.

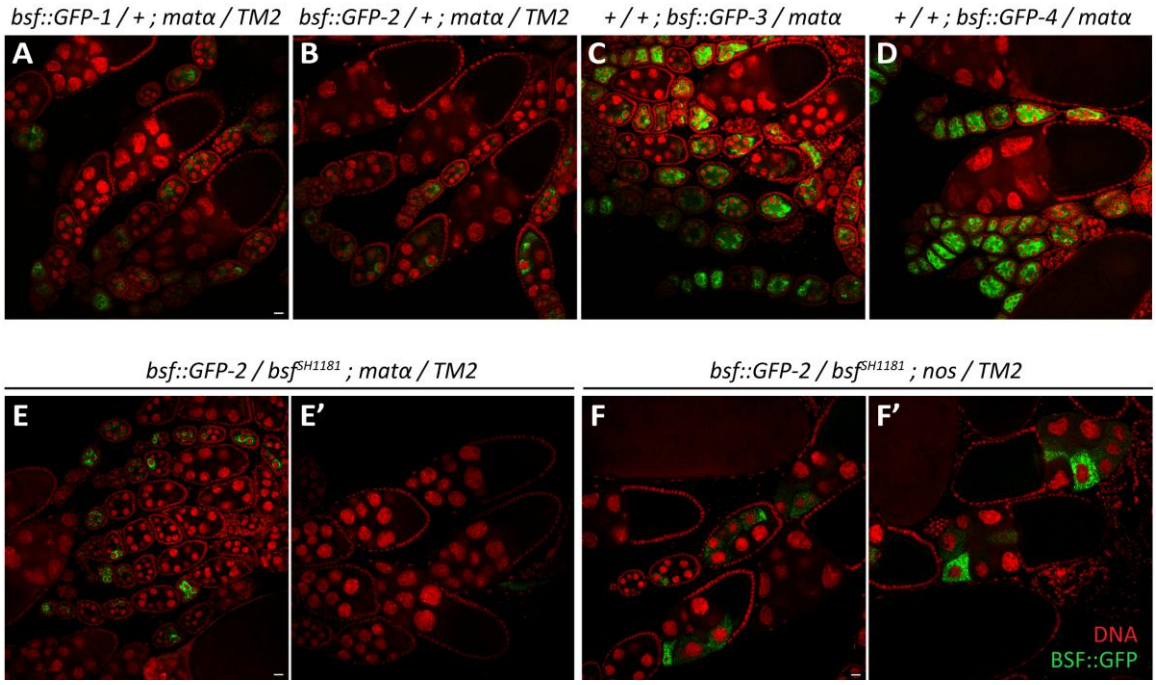

Figure A.3. Overexpression of BSF::GFP disrupts progression of oogenesis

A-D. Ovary phenotypes of the *UAS-bsf::GFP* expression by the *matalpha4-GAL-VP16 (matα)* driver in the $bsf^{+/+}$ background. Four lines of the *UAS-bsf::GFP* were tested. Each panel is from each transgenic line and the genotypes are indicated at top. DNA, red; BSF::GFP, green. The scale bar in this and other panels are 20 μm.
E-E'. The *UAS-bsf::GFP* expression by the *matalpha4-GAL-VP16 (matα)* driver in the $bsf^{+/-}$ background. Early-stage egg chambers are shown in E. Mid- and late-stage egg chambers are shown in E'. The genotype is indicated at top. DNA, red; BSF::GFP, green.
F-F'. The *UAS-bsf::GFP* expression by the *nosGAL4::VP16-nos.UTR (nos)* driver in the $bsf^{+/-}$ background. Early- and mid-stage egg chambers are shown in F. Late-stage egg chambers are shown in F'. The genotype is indicated at top. DNA, red; BSF::GFP, green.

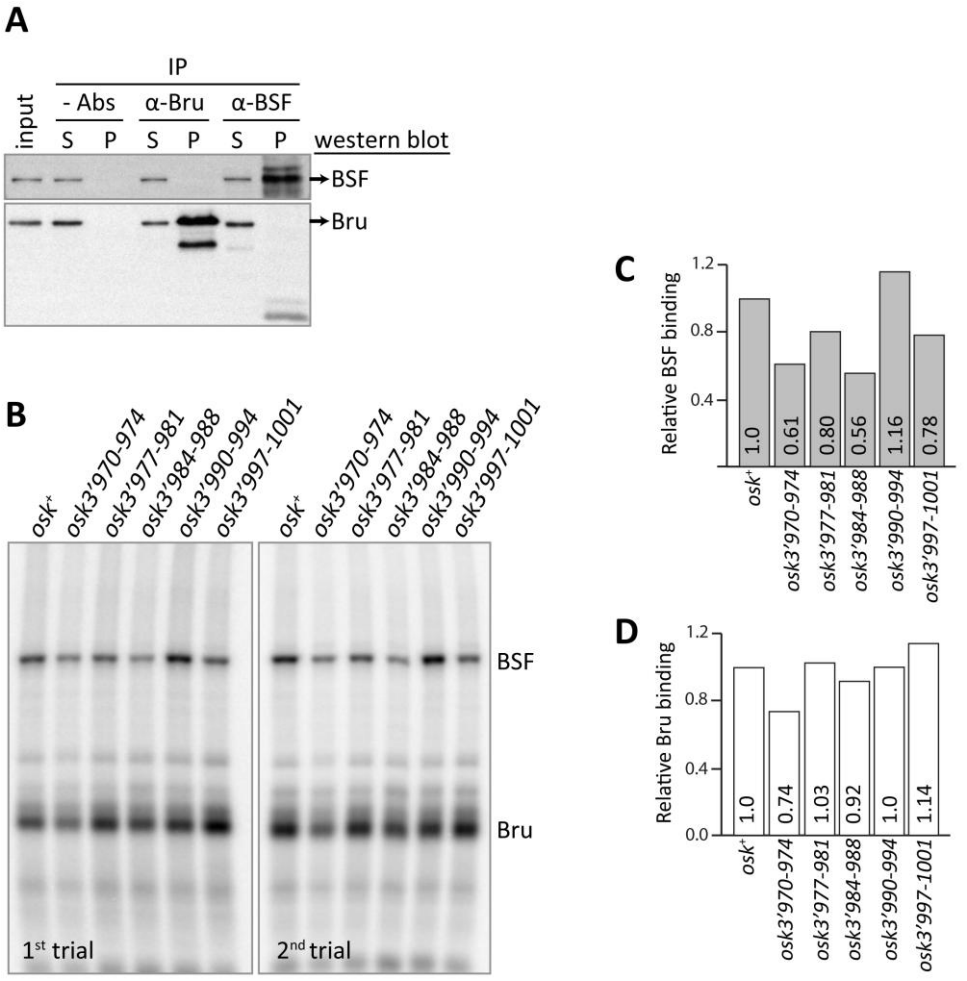

Figure A.4. BSF and Bru are not co-immunoprecipitated

A. Upper panel is a western blot probed with the anti-BSF antibody, and the lower panel is a western blot probed with the anti-Bru antibody. Input is the starting material. S and P are supernatant and pellet fractions following immunoprecipitating with the anti-BSF or anti-Bru antibodies indicated at top. Amounts loaded are: input and supernatant, 0.8%; pellet, 40%. The extra bands detected by the anti-Bru might be a degraded Bru or nonspecific bindings of the antibody.

B. BSF directly binds to *osk* RNA. BSF and Bru binding, detected by the UV cross-linking assay, to the radiolabeled *osk* 3' region mutant RNAs. The two blot images show the results from independent experiments. Mutants 970-974 and 984-988 have the strongest effect on BSF binding, 977-981 and 997-1001 are intermediate, and 990-994 has no detectable effect.

C-D. Quantification of BSF (C) and Bru (D) binding levels from the UV cross-linking assay.

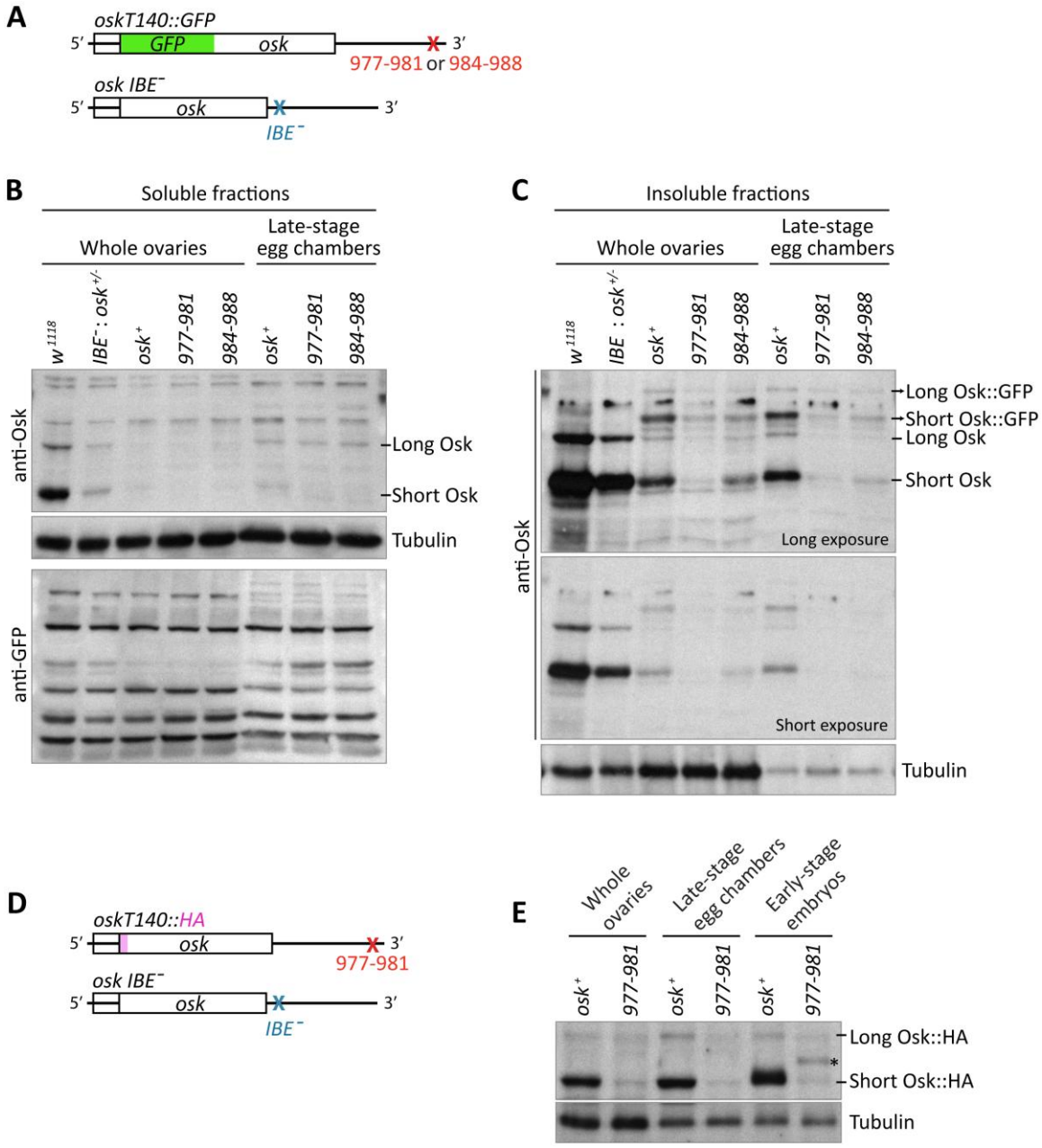

Figure A.5. Loss of BSF binding affects Osk protein expression

A. Schematic diagram of *osk* transgenes used in the assay. The *oskT140::GFP* transgene has wild type or mutant versions of the 3' UTR. All *oskT140::GFP* transgenes were inserted at the same genomic site by phiC31 transgenesis to avoid position effects that might alter expression levels. The *osk IBE*$^{-}$ transgene has the A subset of IBE mutations (Munro et al., 2006) in the 3' UTR.

B. Western blot results of the soluble fractions probed with the anti-Osk (top), anti-α-Tubulin (middle), and anti-GFP (bottom). 24 µg of soluble proteins were used for probing the anti-Osk and α-Tubulin antibodies, and 48 µg of soluble proteins were used for probing the anti-GFP antibody which detects OskT140::GFP protein. OskT140::GFP proteins were not detected by the anti-GFP antibody. All bands in bottom panel result from non-specific antibody binding.

C. Western blot results of the insoluble fractions probed with the anti-Osk (top) and anti-α-Tubulin (bottom). Long Osk and Short Osk bands from the *oskT140::GFP* transgenes are degradation products.

Genotypes used as follows. *IBE-; osk*$^{+/-}$ is *osk*$^{IBE-}$*/osk*$^{IBE-}$*;Df(3R)osk/TM2*. *osk*$^{+}$ is *oskT140::GFP/osk IBE*$^{-}$*; osk*^{0}*/Df(3R)osk.* 977-981 is *oskT140::GFP3'977-981/osk IBE*$^{-}$*; osk*^{0}*/Df(3R)osk.* 984-988 is *oskT140::GFP3'984-988/osk IBE*$^{-}$*; osk*^{0}*/Df(3R)osk*.

D. Schematic diagram of the *osk* transgenes used in the assay. The *osk::HA* transgene has wild type or mutant version, 977-981 of the 3' UTR. The *osk IBE*$^{-}$ transgene has the A subset of IBE mutations (Munro et al., 2006) in the 3' UTR.

E. Western blot results probed with the anti-HA (top) which detects Osk::HA protein, and the anti-α-Tubulin (bottom). 24 µg of soluble proteins were used. The band indicated by an asterisk was not appeared after anti-HA probing, it was appeared only after the anti-α-Tubulin probing. Therefore, this band might be a background or nonspecific binding of the anti-α-Tubulin.

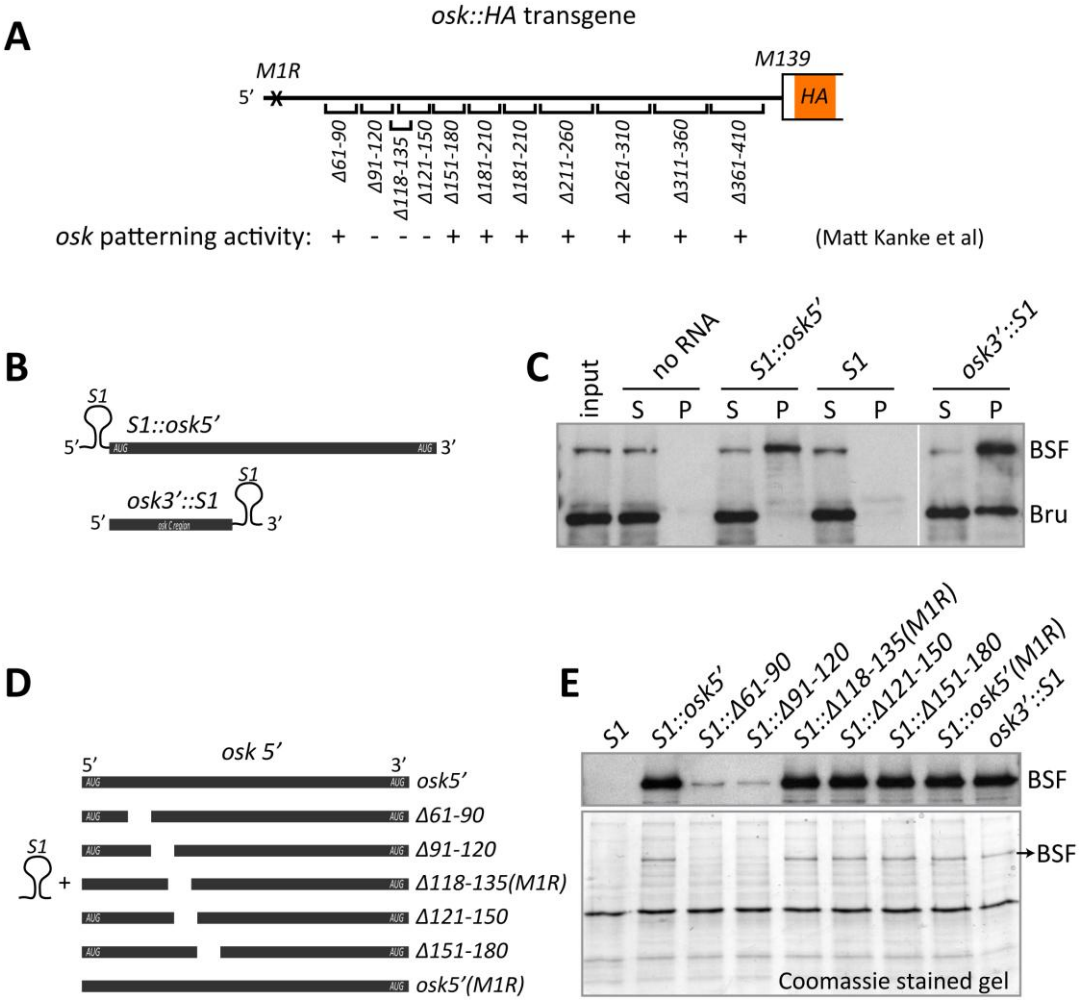

Figure A.6. BSF also binds to the *osk* RNA 5' region, however the correlation between *osk* translational activation and BSF binding to the *osk* RNA 5' region is not established

A. Diagram of the *osk*::HA transgenes carrying deletion mutations in the 5' region. The first start codon was mutated, and HA was inserted next to the second start codon. Regions implicated in embryonic body patterning activity corresponding to the translational activation are indicated. The Δ91-120, Δ118-135, and Δ121-150 showed the strongest defect in embryonic body patterning. Other mutations had no detectable defect (Kanke and Macdonald, 2015).
B. Schematic diagram of the *osk* 5' region of RNA fused to S1 aptamer (*S1::osk5'*) and *osk* 3' region of RNA fused to S1 aptamer (*osk3'::S1*) used in S1 aptamer RNA purification assay.
C. BSF and Bru binding, detected by western blot analysis, to the *osk* 5' or 3' region RNAs. Input is the starting material. S and P are supernatant and pellet

fractions following purification with the RNAs indicated at top. Amounts loaded are: input and supernatent, 0.8%; pellet, 33.3%.
D. Schematic diagram of the *osk* 5' region deletion mutant RNAs used in RNA purification assay to test BSF binding.
E. BSF binding detected by western blot (top) and coomassie staining (bottom) to the *osk* 5' region mutant RNAs. The amounts loaded were as in panel C. The Δ61-90 and Δ91-120 have the strongest effect on BSF binding. Other mutations have no detectable effect. The arrow in coomassie stained gel indicates the bands of BSF judged by size.

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Vita

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