Dimerization of oskar 3’ UTRs promotes hitchhiking for RNA localization in the Drosophila oocyte

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ABSTRACT
mRNA localization coupled with translational control is a highly conserved and widespread mechanism for restricting protein expression to specific sites within eukaryotic cells. In Drosophila, patterning of the embryo requires oskar mRNA transport to the posterior pole of the oocyte and translational repression prior to localization. oskar RNA splicing and the 3’ untranslated region (UTR) are required for posterior enrichment of the mRNA. However, reporter RNAs harboring the oskar 3’ UTR can localize by hitchhiking with endogenous oskar transcripts. Here we show that the oskar 3’ UTR contains a stem–loop structure that promotes RNA dimerization in vitro and hitchhiking in vivo. Mutations in the loop that abolish in vitro dimerization interfere with reporter RNA localization, and restoring loop complementarity restores hitchhiking. Our analysis provides insight into the molecular basis of RNA hitchhiking, whereby localization-incompetent RNA molecules can become locally enriched in the cytoplasm, by virtue of their association with transport-competent RNAs.

Keywords: RNA dimerization; kissing-loop interaction; oskar mRNA localization; RNA hitchhiking; oskar RNA; Drosophila oocyte

INTRODUCTION
In Drosophila, oskar (osk) mRNA is transcribed in the nurse cells of the syncytial egg chamber and transported into the developing oocyte, where it is enriched at the posterior pole (Ephrussi et al. 1991; Kim-Ha et al. 1991). osk translation is repressed during mRNA transport and activated once the mRNA reaches the posterior pole (Kim-Ha et al. 1995). Spatial restriction of osk expression is critical for proper embryonic patterning: Embryos lacking Osk protein develop no abdomen or germline, whereas embryos expressing ectopic Osk develop posterior structures in the place of the head (Lehmann and Nusslein-Volhard 1986; Ephrussi et al. 1991; Ephrussi and Lehmann 1992; Smith et al. 1992).

The signals mediating mRNA localization often reside in the 3’ untranslated region (3’ UTR) (Martin and Ephrussi 2009), and it was initially thought that the osk 3’ UTR contains all the cis-regulatory signals for posterior localization (Kim-Ha et al. 1993). However, further analysis revealed that, in addition to the 3’ UTR, splicing of the first intron is required for osk mRNA posterior enrichment (Hachet and Ephrussi 2004). Furthermore, localization of intronless lacZ-osk 3’-UTR reporter RNA was shown to depend on the presence of localized osk mRNA. It was therefore proposed that intronless osk 3’-UTR-containing messages might coassemble via their 3’ UTRs for posterior transport by hitchhiking with endogenous, transport-competent osk transcripts (Hachet and Ephrussi 2004).

osk mRNA was shown biochemically to be part of heavy particles containing RNA and protein (Wilhelm et al. 2003; Chekulaeva et al. 2006), and injected osk is transported in granules containing more than 100 molecules (Glotzer et al. 1997). Thus, hitchhiking might involve copackaging of osk RNA molecules into higher-order structures similar to the granules containing localized RNAs in other cell types (Martin and Ephrussi 2009).

osk hitchhiking could involve direct base-pairing between 3’ UTRs or their indirect association via an intermediate (Kim-Ha et al. 1993; Hachet and Ephrussi 2004; Jenny et al. 2006). Indeed, Bruno and PTB proteins have been shown to induce osk RNA oligomerization (Chekulaeva et al. 2006;...
Besse et al. 2009). In contrast, direct base-pairing of osk mRNAs has not been reported. However, dimerization of RNA molecules is associated with the packaging of genomic retroviral RNA in the virion (Kung et al. 1975; Fu et al. 1994), and, in Drosophila, bicoid mRNA dimerizes in vitro and assembles into particles upon injection into embryos (Ferrandon et al. 1997; Wagner et al. 2001).

Here we demonstrate that a stem–loop in the osk 3′ UTR is a dimerization domain and that nucleotides promoting RNA dimerization in vitro also promote osk 3′-UTR-mediated hitchhiking in vivo. Our work reveals a role of RNA–RNA interaction for coordinated transport of RNA molecules within the oocyte.

### RESULTS AND DISCUSSION

**oskar RNA dimerizes in vitro**

To investigate if osk 3′-UTR-mediated hitchhiking might involve direct RNA–RNA interaction, we first assessed if osk 3′-UTR RNA molecules (Fig. 1A) can associate in vitro, by evaluating their mobility on nondenaturing agarose gels. In vitro transcribed full-length osk 3′-UTR RNA runs as a single fast-migrating band under low-salt (buffer M) conditions that hinder RNA dimerization (Marquet et al. 1991; Ferrandon et al. 1997), whereas a band of slower mobility was detected under high-salt (buffer D) conditions (Fig. 1A,B, lanes 1,2). A salt-sensitive shift was also observed with the 3′ half of the osk 3′ UTR (Fig. 1B, lanes 5,6). Furthermore, a band of
intermediate mobility appeared upon coincubation of the full-length with the 3′ half of the osk 3′ UTR, independent of which of the two RNAs was labeled (Fig. 1B, lanes 3,4). To address whether this salt-sensitive shift truly reflects a property of the 3′ half of the osk 3′ UTR to oligomerize, we next incubated the 5′ half and 3′ half RNAs in water, buffer M, and buffer D (Fig. 1A,C). The 5′ half did not form oligomers in either water or in buffers of increasing salt concentration (Fig. 1C, lanes 4–6). In contrast, the 3′ half RNA, which in water ran as a single band, appeared as a band of slower mobility in buffer M and fully shifted to the slower migrating band in buffer D (Fig. 1C, lanes 1–3). Taken together, these results show that osk RNA dimerizes in vitro, via sequences present in the 3′ portion of the 3′ UTR.

Several osk orthologs have been identified and their RNAs shown to be localized (Webster et al. 1994; data not shown). Alignment of these sequences revealed a conserved region (nucleotides 714–827) predicted to form a stem–loop within the dimerizing fragment (Fig. 1D). Interestingly, the terminal loop consists of a conserved GC-rich palindromic sequence (rectangle in Fig. 1D), a motif previously shown to mediate direct RNA–RNA interaction between HIV-1 RNA molecules (Brunel et al. 2002). Differences between the primary sequence of the orthologs in the stem–loop either lie in predicted bulges or correspond to covarying nucleotides in the helices that should maintain the secondary structure (Fig. 1D).

To validate the predicted structure, we probed RNA molecules consisting of the 3′ half of the osk 3′ UTR with DMS and RNases T1 and T2. The data are summarized in Figure 2A. Under limiting conditions, RNase T1 cleaves unpaired (or single-stranded) guanines (Fig. 2A,B), while RNase T2 cleaves unpaired nucleotides with a preference for adenines (Fig. 2A). Dimethyl-sulfate (DMS) methylates Watson-Crick positions of residues located in single-stranded segments; adenines (N1-A) and cytosines (N3-C) (Fig. 2A). As illustrated in Figure 2A, the region of the osk 3′ UTR extending from nucleotides 714 to 827, oskDD, forms a stem–loop structure that is highly conserved among Dro sophila idae species (Fig. 1D).

Comparison of RNase T1 digestion performed in either buffer M or buffer D revealed no difference in the structure of the 3′ half of the osk3′UTR RNA under the two salt conditions, indicating that salt does not promote major secondary structure reshaping. We also tested the folding of the presumptive dimerization domain on its own (oskDD) (see Fig. 1A), which revealed an enzymatic cleavage profile indistinguishable from that of the full 3′ UTR, indicating that the stem–loop in isolation is robust (data not shown). Thus the predicted structure of the stem–loop is confirmed by the experimental data (Fig. 2A).

To identify the nucleotides that promote RNA dimerization, we compared the RNase T1 cleavage pattern of the oskDD RNA under low- and high-salt conditions; this revealed that only guanine 766, located within the palindromic loop, is protected (Fig. 2B, arrow). We also performed dimerization interference experiments, using ethylnitrosourea (ENU), which modifies the phosphates of nucleotides. Sites of modifications on oskDD monomers or dimers were identified by alkaline treatment, which induces RNA scission at the modified phosphate. Cleavages present in monomers but absent in dimers indicate the positions at which modification prevents RNA dimerization (negative interference). As shown in Figure 2C, only the modification of nucleotides within the palindromic loop impaired RNA dimerization.

We next determined the dimerization dissociation constant ($K_D$) of the full-length (data not shown) or the 3′ half of the osk 3′-UTR RNAs and of oskDD RNA alone (Fig. 2D,E; Supplemental Fig. 1A). In the context of the 3′ half RNA, we also investigated the effect of mutations in the stem–loop on the $K_D$ (Fig. 2D,E). Deletion of the complete stem–loop (osk3′half-DD) or of its distal stem (osk3′half ΔDD) strongly reduced the $K_D$ (Fig. 2D,E). Substitution of four nucleotides in the loop with a GNRA sequence, which disrupts the palindromic sequence while maintaining a loop (Brunel et al. 2002), also resulted in a reduced $K_D$, indicating that dimerization requires the palindromic sequence of the terminal loop (Fig. 2D,E). We next tested if dimerization indeed occurred in trans, by coincubating either complementary or noncomplementary RNA species. While heterodimerization did occur when the complementary RNAs oskDD and osk3′half-WT (Supplemental Fig. 1B) were coincubated, the $K_D$ was strongly reduced when oskDD was coincubated with an RNA bearing a mutation in the palindromic loop sequence (oskDD + osk3′half-GNRA) (Supplemental Fig. 1C). Finally, substitution of two nucleotides within the palindrome with UU or AA strongly reduced the $K_D$, whereas dimerization was recovered when the complementary mutant RNAs were coincubated (Fig. 2D). Taken together, we conclude that the osk 3′ UTR dimerizes in vitro via a kissing-loop interaction involving six conserved, palindromic nucleotides. We therefore refer to this region as the osk mRNA dimerization domain (oskDD).

It is interesting to note that bicoid RNA oligomerization is initiated by a reversible double loop–loop interaction that is subsequently converted into a stable dimer (Wagner et al. 2004). In contrast, our data support a kissing-loop model for osk RNA dimerization that is highly similar to that proposed for dimerization of retroviral RNAs. Indeed, dimerization of both osk and HIV-1 RNAs is initiated by a 6-nt, GC-rich palindrome (Clever et al. 1996).

oskar dimerization loop base-pairing promotes coassembly of RNA molecules in vivo

To test if the dimerization domain might contribute to the hitchhiking of osk 3′-UTR-containing reporters with endogenous osk mRNA in vivo (Hachet and Ephrussi 2004), we fused egfp to the osk 3′ UTRs tested for dimerization in vitro (Fig. 1A) and assayed the distribution of egfp RNA in the oocyte, comparing it with that of endogenous osk (see
The chimeric \( \text{egfp} \) transgenes, which contained no introns, were expressed under the control of the maternal tubulin promoter using the UAS-Gal4 system (Fig. 3A). The wild-type (\( \text{egfp-WT} \)) and mutant (\( \text{egfp-\text{UU}} \) and \( \text{egfp-\text{GNRA}} \)) RNAs were expressed at similar levels as judged by qRT-PCR analysis on whole ovaries (Fig. 3A).

**FIGURE 2.** A secondary structure in the 3\(^{\prime}\) half of the \( \text{osk} \) 3\(^{\prime}\) UTR promotes dimerization. (A) Proposed secondary structure of nucleotides 714–827, the conserved region within the dimerization-competent portion of the \( \text{osk} \) 3\(^{\prime}\) UTR. Data supporting this prediction were collected by chemical (dimethyl-sulfate, DMS) and enzymatic (RNase T1 and T2) probing and are summarized in the figure. DMS (blue squares, weak; yellow circles, medium; red circles, strong modification) modifies unpaired adenines and cytosines, RNase T1 (solid circle, strong; open circle, medium; gray open circle, weak cleavage) cleaves unpaired guanines, and RNase T2 (solid square, strong; open square, medium; gray open square, weak cleavage) efficiently targets single-stranded nucleotides with a preference for adenines. (B) Enzymatic probing of the 3\(^{\prime}\) half of the \( \text{osk} \) 3\(^{\prime}\) UTR using RNase T1. Hydrolysis was conducted in buffer M or D, and the reaction was incubated for 2, 4, and 8 min. An incubation control (C) was included that did not contain the enzyme. Sequencing lanes (\( U, A, C, G \)) were run in parallel. Nucleotide 766 was specifically protected in buffer D but not in buffer M (arrow). (C) Phosphate ethylation interference by ENU. Autoradiography of the fractionation gel after modification conducted on the dimerization domain of \( \text{osk} \) RNA. (M and D) RNA extracted from the monomer and the dimer bands, respectively; (T) total population of modified RNA; (C) incubation control. Lane R corresponds to the RNase T1 ladder. Negative interference is indicated by circles: A complete disappearance of a band revealed a “strong” interference (solid circle), a slight disappearance of a band indicated a “weak” interference (open circle). (D) Summary of experiments probing the dimerization capacity (\( K_D \)) of \( \text{osk} \) 3\(^{\prime}\) half-WT RNAs with mutations in the conserved stem–loop region and \( \text{oskDD} \). Association of RNAs was strongly reduced when RNAs with partial or complete deletion of the stem–loop (\( \text{osk3} \) half-\( \text{DD} \), -\( \text{DDd} \), -\( \text{GNRA} \)) or with substitutions in the terminal loop were analyzed. Dimerization was restored when RNAs with compensatory mutations (\( \text{osk3} \) half-\( \text{AA} \) and -\( \text{UU} \)) were coincubated. \( K_D \)’s are indicated on the right. (E) Kinetics of dimerization of \( \text{osk3} \) half-WT, \( \text{osk3} \) half-\( \text{DD} \), \( \text{osk3} \) half-\( \text{DDd} \), and \( \text{osk3} \) half-\( \text{GNRA} \) RNAs as a function of RNA concentration. Indicated is the amount of nonradioactive RNA (nanomolar, nM); in addition, trace amounts of radioactive RNAs were added for visualization. Representative autoradiographs of the agarose gels show monomeric (gray arrow) and dimeric (black arrow) RNA species. Fifty percent of \( \text{osk3} \) half-WT RNA formed dimers at 72–100 nM. RNAs bearing a mutation in the dimerization domain dimerized at >400 nM.
Transport of osk mRNA from the nurse cells into the oocyte during early oogenesis is mediated by the 3' UTR (Jenny et al. 2006). Confirming that the osk 3' UTR is sufficient, and that hitchhiking is not required for this first step in transport, the egfp reporter RNAs were enriched in the oocyte at stage 6 (Fig. 3B,G,L). During mid-oogenesis, osk mRNA is transported within the oocyte to the posterior pole, and it is for this second step in localization that hitchhiking becomes critical (Hachet and Ephrussi 2004). Our analysis of the egfp reporter RNAs at stage 9 showed that mutation of the dimerization domain selectively affected the ability of the mutant reporters to localize at the posterior pole (Fig. 3C,H,M, quantification in Fig. 3Q). Localization of endogenous osk mRNA was unaffected by the presence of the reporter RNAs (Fig. 3E,J,O), and in all cases, Osk protein was detected at the posterior pole, confirming that endogenous osk RNA was correctly localized and translated (Fig. 3F,K,P). The osk mRNA and Osk protein levels in the transgenic lines are, in fact, highly similar to those in the w1118 control (Fig. 3R,S).

Both wild-type and mutant reporter RNAs could be detected at the posterior at stage 10 (Fig. 3D,I,N), when cytoplasmic streaming and Osk protein-dependent trapping result in posterior accumulation of osk 3'-UTR-containing RNAs (Glotzer et al. 1997). The reduced posterior enrichment of the mutated egfp reporter RNAs at stage 9 clearly indicates that the dimerization domain promotes the osk 3'-UTR-dependent hitchhiking of RNA molecules in vivo.

To test if osk hitchhiking involves direct base-pairing, we first generated the oskAA transgene, which bears a 2-nt AA substitution (Fig. 1A) in the dimerization loop of an otherwise wild-type, intron-containing, osk gene (oskWT) (Hachet and Ephrussi 2004). The oskWT and oskAA transgenes were crossed into the osk RNA null background, such that in each case the transgene was the only source of osk RNA in the oocyte (thereafter referred to as oskWT or oskAA oocytes). oskAA rescued the developmental arrest at stage 7 of oogenesis that is the primary defect in egg chambers completely null for osk mRNA (Jenny et al. 2006). In situ hybridization confirmed that, just like oskWT RNA, oskAA RNA was enriched in and localized at the posterior pole in the majority of osk RNA null mutant oocytes (Fig. 4A,A',B,B'). Therefore, the presence of a palindromic sequence in the dimerization loop is not essential for posterior localization of full-length, spliced osk RNA molecules, which can localize on their own (Hachet and Ephrussi 2004).

Our in vitro analysis showed that osk RNA dimerizes through kissing-loop interactions (Fig. 2E). We therefore assessed the ability of oskAA RNA to associate in vivo with the complementary egfp-UU RNA and promote its localization at the posterior pole. Simultaneous expression of the egfp-UU and oskAA transgenes in the osk RNA null background resulted in posterior localization of egfp-UU RNA in the majority of oocytes (Fig. 4D'). In contrast, coexpression of the egfp-WT reporter with oskAA led to a posterior enrichment of the noncomplementary egfp-WT RNA in only a minority of oocytes (Fig. 4C'). In both cases, the presence of Staufen protein at the posterior pole confirmed that oskAA RNA was correctly localized (Fig. 4C,D), and in situ hybridization confirmed that egfp RNAs were correctly enriched in stage 6 oocytes (Fig. 4C,D).

We therefore conclude that base-pairing interactions between the dimerization loops of unspliced reporter RNA and spliced, localization-competent osk RNA molecules occur in vivo.

**CONCLUDING REMARKS**

In this study, we have shown that the dimerization domain mediates hitchhiking of osk 3'-UTR-containing reporter RNAs. In the future, it will be interesting to determine the in vivo role of this domain and of hitchhiking in the regulation endogenous osk mRNA. It is likely that dimerization is involved in other aspects of osk regulation. Interestingly,
Reveal et al. (2010) recently proposed that translational activation of osk molecules can occur in trans, which could also involve RNA dimerization.

The palindromic sequence that initiates oligomerization of osk mRNAs is not present in other transcripts enriched at the posterior pole such as nanos and cyclinB (data not shown). However, other RNAs have been shown to undergo dimerization. In the case of bicoid mRNA, in vitro dimerization requires a single stem-loop (but is not initiated by a palindromic loop), whereas particle formation upon bicoid RNA injection into living embryos also requires other parts of the 3’ UTR (Wagner et al. 2001). The HIV-1 dimerization initiation site (DIS) promotes RNA dimerization in vitro, while formation of a stable duplex requires proteins to consolidate the initial kissing-loop interaction (Russell et al. 2004; Lever 2007).

In the case of osk mRNA, both Bruno and PTB proteins have been shown to promote osk 3’ UTR RNA oligomerization (Chekulaeva et al. 2006; Besse et al. 2009). In view of the multiple means through which osk RNA molecules can interact and oligomerize, it is remarkable that the RNA–RNA interaction detected in vitro has a discernable effect on hitchhiking in vivo. The redundancy of mechanisms mediating the association of osk RNA molecules can explain the apparently normal localization of oskAA RNA at the posterior pole. Whereas a kissing-loop interaction via the di- }

FIGURE 3. The dimerization domain promotes osk RNA interaction during hitchhiking in vivo. (A) Schematic representation of the reporter constructs used in this panel. (A’–Q). Egg chambers from w118 flies expressing egfp-WT, -UU, or -GNRA under the control of a mato4-tub-Gal4 driver. (A’) qRT-PCR experiment probing the relative levels of gene expression of egfp-WT, egfp-UU, and egfp-GNRA RNAs. RNA expression is shown as the percentage of the egfp-WT RNA level. All RNA levels were normalized to rp49 RNA. (B–P, R, S) Reporter RNAs are detected using an egfp-antisense probe, endogenous osk is visualized using a probe antisense to the osk coding sequence, and Osk protein is stained with anti-Osk antibodies. All egg chambers shown were counterstained with DAPI (blue). Bar, 50 μm. The panels highlighting the posterior pole of different oocytes (D–F, J–K, N–P) show images at the same magnification as A, H, and M. (B, G, L) egfp-WT, -UU, and GNRA reporter RNAs accumulate in young oocytes (stage 6). (C, H, M) Posterior accumulation of mutant egfp-UU and -GNRA reporter RNAs is strongly reduced in comparison with egfp-WT RNA at stage 9. The arrow in C indicates the position of the posterior pole. (D, J, N) egfp-WT, and to a lesser degree also egfp-UU and -GNRA, RNAs are present at the posterior pole of stage 10 oocytes. (E, L, O) Endogenous osk mRNA at stage 9 is localized in the presence of reporter RNAs. (F, K, P) Osk protein is expressed in stage 9 oocytes in the presence of reporter RNAs. (Q) Percentage of oocytes showing a posterior accumulation of egfp-WT, -UU, or -GNRA reporter RNAs in stage 9 egg chambers. The accumulation of egfp RNAs was scored in at least two independent experiments (egfp-WT: n = 127; egfp-UU: n = 61; egfp-GNRA: n = 71). (R, S) w118 control flies, which do not express transgenic reporter3’ UTR, were also stained for osk mRNA and Osk protein for comparison.

osk 3’ UTR

A Sty1–MaelII fragment of the plasmid pBS-oks 3’ UTR was subcloned into the Stul site of the pUT7 vector, a pUC18 derivative containing a T7 RNA polymerase. osk3’half (nucleotides 1–616) and osk3’-half (nucleotides 616–1151) of osk 3’ UTR were cloned into the Stul site of the pUT7 vector. oskDD (the dimerization domain, nucleotides 714–827) was PCR-amplified from the osk 3’ UTR DNA. osk 3’ UTR-ΔDD, ΔDDD, -GNRA, -AA, and -UU were generated by PCR mutagenesis (Quick Change PCR kit; QIAGEN). The complete dimerization domain is deleted in osk 3’ UTR-ΔDD (nucleotides 715–827), while only the distal stem is deleted in osk 3’ UTR-ΔDDD (nucleotides 736–802). In osk 3’ UTR-AA, -UU, and -GNRA, nucleotides of the terminal loop are substituted (AA, nucleotides 768–769; UU, nucleotides 764–765; GNRA, nucleotides 765–768).

osk 3’-UTR orthologs from D. yakuba, D. pseudobscura, D. immigrans, D. mercatorum, and Z. sepsoides

Total ovarian RNA was isolated (TRizol; Invitrogen), poly(A) RNAs were enriched (mRNA Purification Kit; DynaBeads), and cDNAs were generated (Superscript First Strand Synthesis Kit; Invitrogen). Sequences were amplified with a specific primer against the osk coding region and an oligo(TT) primer. PCR products were TA-cloned and sequenced. Sequences were aligned (ClustalW), and the secondary structures were predicted with mfold v2.3 at 25°C (Zuker 2003).

MATERIALS AND METHODS

Plasmids

osk 3’ UTR

A Sty1–MaellII fragment of the plasmid pBS-oks 3’ UTR was subcloned into the Stul site of the pUT7 vector, a pUC18 derivative containing a T7 RNA polymerase. osk3’half (nucleotides 1–616) and osk3’-half (nucleotides 616–1151) of osk 3’ UTR were cloned into the Stul site of the pUT7 vector. oskDD (the dimerization domain, nucleotides 714–827) was PCR-amplified from the osk 3’ UTR DNA. osk 3’ UTR-ΔDD, ΔDDD, -GNRA, -AA, and -UU were generated by PCR mutagenesis (Quick Change PCR kit; QIAGEN). The complete dimerization domain is deleted in osk 3’ UTR-ΔDD (nucleotides 715–827), while only the distal stem is deleted in osk 3’ UTR-ΔDDD (nucleotides 736–802). In osk 3’ UTR-AA, -UU, and -GNRA, nucleotides of the terminal loop are substituted (AA, nucleotides 768–769; UU, nucleotides 764–765; GNRA, nucleotides 765–768).
FIGURE 4. osk RNAs engage in direct interactions in the oocyte. Egg chambers from osk RNA null flies (osk<sup>es1</sup>/Df(3R)p<sup>X110</sup>) expressing full-length transgenic osk mRNA under the control of pCog and nos-Gal4 drivers. (A–A''', B–B''') RNA expressed from osk<sup>WT</sup> (A–A''') and osk<sup>AA</sup> (B–B''') transgenes is transported into the oocyte (stage 6) and localizes at the posterior pole of stage 9 egg chambers (osk<sup>WT</sup>, 93%; osk<sup>AA</sup>, 91%). In a subset of the egg chambers (osk<sup>WT</sup>, 30%; osk<sup>AA</sup>, 39%), ectopic osk mRNA is additionally detected. Staufen protein is equally enriched at the posterior pole of egg chambers. As established by qRT-PCR, osk<sup>WT</sup> and osk<sup>AA</sup> were overexpressed to similar levels in comparison to osk levels of <i>w<sup>1118</sup></i> flies. osk RNAs were detected using an osk (coding region)–antisense probe (red) immuno-stained with anti-Staufen antibodies (white). (C–C'', D–D'') Egg chambers coexpressing osk<sup>AA</sup> with either egfp-WT or egfp-<i>UU</i>. Both reporter RNAs enrich in young oocytes (C,D), but posterior pole accumulation of the egfp-WT is strongly reduced (C' (16% ± 13%) compared with the egfp–<i>UU</i> (D') (91% ± 5%) at stage 9 of oogenesis. Staufen enriches equally in the presence of either RNA (C'',D'). A summary of reporter<sup>3</sup>UTR RNA hitchhiking at stage 9 of oogenesis is shown below. All egg chambers were simultaneously stained with egfp-antisense probe (red) to specifically detect the reporter RNAs and anti-Staufen antibodies (white) to also detect full-length osk mRNA. The accumulation of egfp-reporter RNA at the posterior pole was scored in at least two independent experiments, analyzing at least 15 egg chambers per experiment. All egg chambers were stained for RNA (red) by whole-mount in situ hybridization and counterstained with DAPI (blue). Bar, 50 μm.

The genes of interest (osk<sup>3</sup>UTR-WT, -GNRA, -<i>UU</i>, and -region2, <i>D. pseudoobscura</i> and <i>D. virilis</i> osk<sup>3</sup> UTRs) were PCR-amplified from pU7 templates with primers attaching BamHI restriction sites, and TA-cloned. BamHI fragments were then subcloned into pUASpGWΔK10 to generate the respective egfp RNAs. To generate pUASpGWΔK10, the K10 terminator was removed from pUASp (Rorth 1998) by XbaI and PstI digestion, bluntling, and religation of pUASp. The egfp open reading frame (ORF) was amplified from pCRII-TOPO-EGFP with primers attaching KpnI and NotI sites, digested, and cloned to KpnI NotI-digested pUASpΔK10.

osk<sup>WT</sup> was previously described (Hachet and Ephrussi 2004). To generate osk<sup>AA</sup>, an AflI–BstZ171 fragment of pSP72-osk<sup>WT</sup> was replaced with the respective fragment from pUC18T7-osk<sup>3</sup>UTR.

osk<sup>AA</sup> was then PCR-amplified attaching BamHI restriction sites and ligated to BamHI-digested pUASpΔK10. Primer sequences are available upon request.

RNA synthesis and in vitro dimerization

In vitro transcription was conducted as previously described (Wagner et al. 2001, 2004) using BamHI-linearized plasmid DNA as template. RNA dimerization was performed as previously described (Wagner et al. 2001). Dimerization buffer D contained 50 mM sodium cacodylate (pH 7.5), 300 mM KCl, and 5 mM MgCl<sub>2</sub>, while monomers were obtained in buffer M (50 mM sodium cacodylate at pH 7.5, 50 mM KCl, 0.1 mM MgCl<sub>2</sub>). Large RNA fragments were analyzed as described (Wagner et al. 2001), while short RNA fragments were resolved at 4°C by electrophoresis using 12% acrylamide/(bis-acrylamide 1/30) gels.

<sup>K<sub>4</sub></sup> determination

The apparent dissociation constant (<i>K<sub>4</sub></i>) of the dimer was determined by mixing increasing concentration of unlabeled RNA (between 4.6 nM and 300 nM) with a negligible constant concentration of labeled RNA (<i><i>c</i></i>1 nM) (Wagner et al. 2004). Dimerization was conducted under standard dimerization conditions (see above). The concentration of unlabeled RNA in the experiments ranged from 0.15 to 720 nM. All experiments were performed at least twice.

Probing and footprinting experiments

RNA probing was carried out as previously described (Wagner et al. 2004). osk RNA (750 ng), in the presence of trNA (1.5 μg), was incubated in buffers allowing or prohibiting dimerization, respectively, before subjecting it to RNase hydrolysis or chemical modification. Typically, incubation in buffer D yielded 50% of dimeric and 50% of monomeric RNA, while 100% monomers were formed in buffer M. Enzymatic hydrolysis was performed with 0.2 units of RNase T1 (Pharmacia), 0.1 unit of RNase T2 (Sigma), and 0.1 unit of RNase V1 (Pharmacia). Dimethylsulfate (DMS; Aldrich) reaction was allowed to proceed for 2, 4, or 8 min. Modifications were detected using 10% polyacrylamide/(bis-acrylamide 1/30) gels.

Phosphate modification interference

The appropriate 5’-end-labeled fragment of the osk dimerization domain (10 ng) was alkylated on its phosphate groups by ethynitrosourea (ENU) essentially as described before (Wagner et al. 2004). Modifications were detected using 10% polyacrylamide/1/20 M urea gels.

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2004). Modifications were allowed for 1 min at 90°C with 0.32 volume of ENU-saturated ethanol in 25 μL of 50 mM sodium cacodylate (pH 6.5), 50 mM KC1, and 1 mM EDTA, in the presence of 1 μg of total tRNA. End-labeled RNA fragments were separated on a 12% polyacrylamide/8 M urea gel. To control RNA modification, the end-labeled RNA was submitted to the phosphate modification procedure using the same modification conditions, then directly submitted to the ethylated phosphate cleavage procedure without purification of the monomeric and dimeric species. Experiments were conducted twice.

**qRT-PCR**

Quantitative RT-PCR was performed on cDNAs generated from ovaries of flies expressing the respective transgenes. Amplified product was detected using the SYBRGreen (Ambion) system on an ABI Prism 7500 real-time PCR apparatus. The egfp portion of the transgenic RNAs was amplified using primers specific for the EGFP ORF and normalized to rp49. The amplification efficiency was determined using serial dilutions of a cDNA mix from all samples.

**Assaying osk RNA hitchhiking in egg chambers**

Transgenes were expressed in the germline using the maternal-α-tubulin-Gal4 driver. For expression of oskAA and coexpression of oskAA with egfp-reporter variants in osk RNA null flies, the pCog and nano-Gal4 drivers were combined. Lines with equivalent mRNA expression levels, as judged by in situ hybridization and qRT-PCR, were used. To score hitchhiking, posterior enrichment of the egfp reporter RNAs in the oocyte at stage 9 was measured. In each experiment, posterior enrichment was monitored in at least 20 egg chambers. Only egg chambers with posterior Staufen protein, indicative of posterior osk mRNA localization, were scored. At least two individual transgenic insertion lines were analyzed in parallel, and experiments were repeated at least three times.

**Whole-mount in situ hybridization**

In situ hybridization was performed as described (Hachet and Ephrussi 2004). Antisense probes for the egfp and osk coding sequences (cds) were generated by in vitro transcription (Ambion; Megascript) and detected using HRP-conjugated sheep anti-DIG antibody (1:200; Roche) followed by Cy3-tyramide signal amplification (Perkin Elmer).

**Whole-mount immuno/in situ hybridization**

Immunostaining coupled with in situ hybridization was carried out as previously described (Vanzo and Ephrussi 2002), and antigens were detected using rabbit anti-Staufen (1:1500) and rat anti-Osk (1:3000) antibodies. Cy5 (Jackson Immuno Research Laboratories) and AlexaFluor488 (Invitrogen) coupled secondary antibodies were used at 1:500 dilution.

**Fly stocks**

The following wild-type flies were used: w1118 (wild-type control); D. yakuba and D. pseudoobscura (a gift from M. Ashburner); and D. immigrans, D. mercatorum, and Z. sepsoides (a gift from R. Lehmann). The following mutant fly stocks were used: oskαAT (Vanzo and Ephrussi 2002) and Df(3R)3X703 (Lehmann and Nusslein-Volhard 1986), which in combination are null for osk RNA (Jenny et al. 2006). UAS-coupled genes were expressed in the germline using pCog-Gal4:VP16 (Rorth 1998), nano-Gal4:VP16 (Rorth 1998), and mat-α4-tub-Gal4:VP16 (Bloomington stock #7062) promoters.

**Microscope**

For all analyses, a confocal microscope (DMR-E; Leica) equipped with a scan head (TCS SP2 AOBS; Leica) and an oil-immersion 20×, 1.4 NA objective was used. Images were acquired at a 2× zoom and edited with Adobe Photoshop CS.

**SUPPLEMENTAL MATERIAL**

Supplemental material is available for this article.

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