Developmental Cell, Vol. 6, 625–635, May, 2004, Copyright ©2004 by Cell Press

The *Drosophila* hnRNPA/B Homolog, Hrp48, Is Specifically Required for a Distinct Step in *osk* mRNA Localization

Jean-René Huynh,[1,2] Trent P. Munro,[1] Katia Smith-Litière,[1] Jean-Antoine Lepesant,[2] and Daniel St Johnston[1,*]
[1]The Wellcome Trust/Cancer Research UK Gurdon Institute and Department of Genetics Cambridge University Tennis Court Road Cambridge CB2 1QR United Kingdom
[2]Institut Jacques-Monod CNRS Universités Paris 6 et 7 2, place Jussieu F-75251 Paris, Cedex 05 France

Summary

The Staufen-dependent localization of *oskar* mRNA to the posterior of the *Drosophila* oocyte induces the formation of the pole plasm, which contains the abdominal and germline determinants. In a germline clone screen for mutations that disrupt the posterior localization of GFP-Staufen, we isolated three missense alleles in the hnRNPA/B homolog, Hrp48. These mutants specifically abolish *osk* mRNA localization, without affecting its translational control or splicing, or the localization of *bicoid* and *gurken* mRNAs and the organization of the microtubule cytoskeleton. Hrp48 colocalizes with *osk* mRNA throughout oogenesis, and interacts with its 5′ and 3′ regulatory regions, suggesting that it binds directly to *oskar* mRNA to mediate its posterior transport. The *hrp48* alleles cause a different *oskar* mRNA localization defect from other mutants, and disrupt the formation of GFP-Staufen particles. This suggests a new step in the localization pathway, which may correspond to the assembly of Staufen/*oskar* mRNA transport particles.

Introduction

mRNA localization is a common mechanism for targeting proteins to specific regions within cells, and plays an important role in establishing cellular asymmetries (Kloc and Bilinski, 2003; Palacios and St Johnston, 2001). In *Drosophila*, the two main body axes of the embryo are determined by the localization of three maternal mRNAs within the oocyte. *gurken* mRNA localizes to the dorsal-anterior corner of the oocyte, where Gurken protein signals to polarize the dorsal-ventral axis of the embryo (Neuman-Silberberg and Schüpbach, 1993; Nilson and Schüpbach, 1998). *bicoid* mRNA localizes to the anterior of the oocyte, and encodes the morphogen that patterns the head and thorax of the embryo (Driever, 1993; St Johnston et al., 1989). Finally, the localization of *oskar* (*osk*) mRNA to the posterior of the oocyte specifies where the pole plasm forms, which contains the abdominal and germline determinants (Ephrussi et al., 1991; Ephrussi and Lehmann, 1992; Kim-Ha et al., 1991).

mRNAs can be localized by a variety of mechanisms, but this is often thought to occur via active transport along the cytoskeleton. *ASH1* mRNA is transported along actin cables into the bud tip by the type V myosin, Myo4p (Beach et al., 1999; Bertrand et al., 1998; Bohl et al., 2000; Takizawa and Vale, 2000). In *Drosophila*, *wingless* and pair rule transcripts are transported to the apical side of the embryo by the minus end-directed microtubule motor, cytoplasmic dynein, and a similar mechanism probably accounts for the anterior and dorsal localization of *gurken* mRNA in the oocyte (MacDougall et al., 2003; Wilkie and Davis, 2001). Another transcript that is likely to be localized by active transport is Myelin Basic protein mRNA, which has been observed to move from the cell body toward the distal processes of oligodendrocytes in a process that requires microtubules (MT) and the plus end-directed motor, kinesin (Ainger et al., 1993; Carson et al., 1997). Several lines of evidence suggest that *osk* mRNA is also transported by kinesin toward MT plus ends. (1) Posterior localization is microtubule dependent, since it is disrupted by MT-depolymerizing drugs and by mutations that alter the polarized arrangement of MT in the oocyte (Clark et al., 1994; González-Reyes et al., 1995; Martin and St Johnston, 2003; Roth et al., 1995; Shulman et al., 2000; Theurkauf, 1994). (2) Localization is presumably directed toward the plus ends of MT, because the putative plus end marker, kinesin-β-gal, colocalizes with *osk* mRNA at the posterior pole (Clark et al., 1994). (3) *osk* mRNA is not localized in *kinesin heavy chain* mutants, and this motor protein localizes with the mRNA to the posterior pole of wild-type oocytes (Brendza et al., 2000; Duncan and Warrior, 2002; Palacios and St Johnston, 2002).

For an mRNA to be localized, the *cis*-acting localization elements within the RNA must be recognized by *trans*-acting factors that couple it to the motors that transport it. A number of such factors required for *osk* mRNA localization have been identified in maternal screens as mutants that disrupt the posterior localization of *osk* mRNA, without affecting the polarity of the oocyte or the organization of the MT cytoskeleton. The best characterized of these is the dsRNA binding protein, Staufen (Stau), which colocalizes with *osk* mRNA throughout oogenesis, and forms a crescent with the RNA at the posterior pole of the oocyte (St Johnston et al., 1991, 1992). In *stau* mutants, *osk* mRNA accumulates normally in the oocyte during early oogenesis, but it fails to move to the posterior cortex at stage 9, and remains at the anterior of the oocyte (Ephrussi et al., 1991; Kim-Ha et al., 1991). A very similar phenotype is seen in *mago nashi,y14/tsunagi* and *barentsz* mutants, and all three proteins transiently colocalize with *osk* mRNA at the posterior of the oocyte, indicating that they are also components of the *osk* mRNA localization complex (Hachet and Ephrussi, 2001; Mohr et al., 2001; Newmark and Boswell, 1994; van Eeden et al., 2001). Mago, Y14,

*Correspondence: ds139@mole.bio.cam.ac.uk

Barentsz, and eIF4AIII are part of the exon-exon junction complex (EJC) that marks where introns have been excised (Kataoka et al., 2001; Le Hir et al., 2001; Palacios et al., 2004). This suggests that the EJC is loaded onto *osk* mRNA during splicing in the nucleus to control its localization in the oocyte cytoplasm. Although Stau and the EJC are thought to play a direct role in coupling *osk* RNA to the factors that localize it, none of these proteins has been shown to bind specifically to any sequence elements within *osk* mRNA, and it is unclear how the RNA is recognized.

Another way to identify proteins required for mRNA localization is to screen for proteins that bind directly to the RNA in UV crosslinking assays, and this has led to the identification of four proteins that bind to specific regions of *osk*. Surprisingly, all of these proteins have been implicated in the translational control of *osk* mRNA, rather than its localization. Bruno binds to three regions of the *osk* 3′UTR, called Bruno Response Elements (BREs), and mutations in the BREs that disrupt Bruno binding cause the premature translation of *osk* mRNA before it has been localized (Kim-Ha et al., 1995; Webster et al., 1997). Apontic may collaborate with Bruno to mediate repression, as it also binds to specific regions of the *osk* 3′UTR, and interacts with Bruno both physically and genetically (Lie and Macdonald, 1999). In addition, an unidentified protein, called p50, can be crosslinked to the BREs, and mutations that disrupt the binding of p50, but not Bruno, also lead to premature translation (Gunkel et al., 1998). p50 also binds to a region between the two alternative initiation codons near the 5′ end of *osk* mRNA, along with another protein called p68, and deletion of this region leads to a failure to relieve translational repression once *osk* mRNA has reached the posterior pole.

Although genetic screens for maternal-effect mutations have proved an effective way to identify proteins required for *osk* mRNA localization, these can only recover homozygous viable mutants, and would therefore miss any lethal mutations in essential genes. To circumvent this problem, we used the Dominant Female sterile technique and the FLP/FRT system to perform large-scale genetic screens in germline clones for mutations that disrupt the localization of GFP-Stau, which localizes to the posterior of the oocyte with *osk* mRNA (Chou and Perrimon, 1996; Martin et al., 2003). Here we report the identification in such a screen of three missense alleles of Hrp48, which reveal a specific function of this general HnRNP protein in *osk* mRNA localization.

Results

Identification of a Locus Required for the Posterior Localization of Stau

In a germline clone screen of 4331 EMS mutagenized lines, we identified 146 mutants on chromosome arm 2L with defects in GFP-Stau localization within the oocyte. These include three new alleles of *cappuccino*, four alleles of *spire*, and four alleles of *aubergine*, which are genes known to disrupt *osk* RNA localization or anchoring (Harris and Macdonald, 2001; Manseau and Schüpbach, 1989; Wilson et al., 1996). Among the remaining mutants, we identified a lethal complementation group of three alleles, 5A2-6, 7E7-18, and 10B2-9, that specifically disrupt GFP-Stau localization, which we named *linha*.

In germline clones of the 7E7-18 and 10B2-9 alleles, GFP-Stau accumulates in the oocyte normally during the early stages of oogenesis, but remains diffusely localized throughout the oocyte cytoplasm at stage 9–10, and never localizes to the posterior pole, as it does in wild-type (Figures 1A and 1B). These alleles also share a second phenotype that is not observed in any other mutants that disrupt *osk* mRNA localization. In wild-type ovaries, a significant proportion of GFP-Stau localizes to large particles in the cytoplasm of the nurse cells, and smaller particles in the oocyte cytoplasm. Both alleles cause a dramatic reduction in both the size and frequency of these particles, and the GFP-Stau signal is much more diffuse in the cytoplasm. Germline clones of the third allele, 5A2-6, produce a slightly weaker phenotype, as some Staufen is occasionally detected at the posterior pole (data not shown).

All three *linha* mutations have the same effect on *osk* mRNA localization as they do on Stau. The mRNA still accumulates in the oocyte during the early stages of oogenesis, but never shows any localization at the posterior pole (Figures 1C and 1D). In other *osk* mRNA localization mutants, *osk* mRNA shows a transient accumulation at the anterior of the oocyte. This is not the case in *linha* mutants, however, as both *osk* mRNA and Stau show a uniform distribution throughout the oocyte cytoplasm at stage 9.

Rhodamine-Phalloidin labeling of the actin cytoskeleton reveals no other obvious defects until stage 11, when all three alleles show a defect in nurse cell dumping, resulting in the formation of very small eggs, which are usually not laid (Figures 1E and 1F). As a consequence of this dumpless phenotype, the eggs are never fertilized, and we have therefore been unable to analyze the effects of the mutations on embryonic patterning.

To detect phenotypes early in oogenesis, we generated *linha* mutant clones that were marked by the loss of GFP (Luschnig et al., 2000). These clones show a wild-type accumulation of *osk* mRNA into the early oocyte, and display no other defects at these stages (Figure 1G). Furthermore, Stau immunostaining and labeling of the actin cytoskeleton revealed the same phenotypes as with the ovoD technique. This confirms that the *linha* phenotype is strictly germline dependent, and indicates that the failure to localize *osk* mRNA and Stau at the posterior of the oocyte is not a consequence of an earlier defect.

linha Mutations Disrupt Hrp48

The *linha* mutations were mapped by meiotic recombination, using a hybrid strategy that employed both visible markers and Single Nucleotide Polymorphisms (SNPs) (Martin et al., 2001). This placed the gene in an ~20 kb interval in 27C4, and we found that the 7E7-18 and 10B2-9 alleles are lethal over 4 P elements in this region that form a lethal complementation group, l(2)02814, l(2)02647, l(2)k16203, and l(2)k10413 . In addition, the 5A2-6 allele is lethal over l(2)k02647, l(2)k02814 and l(2)k10413, and female-sterile over l(2)k16203. These P elements are inserted upstream and in the first

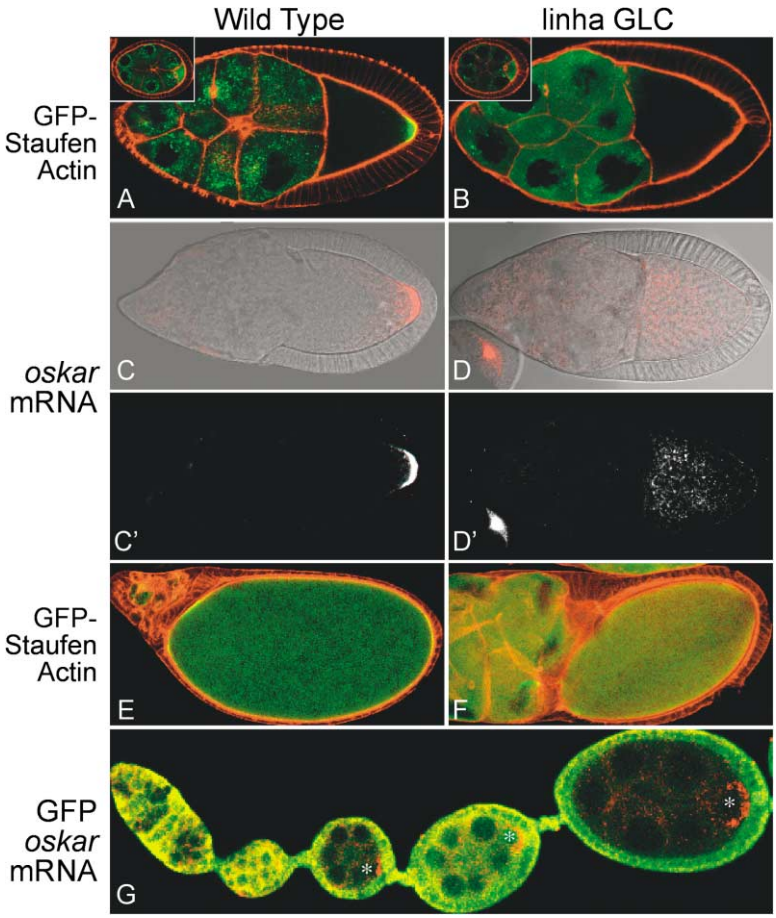

Figure 1. *linha* Mutations Abolish the Posterior Localization of Stau and *osk mRNA*

(A) Wild-type egg chambers, showing the accumulation of GFP-Stau (green) in the oocyte at stage 6 (inset) and its localization to the posterior pole at stage 9 (actin in red).

(B) In *linha* germline clones (GLC), GFP-Stau accumulates in the oocyte, but does not localize to the posterior. The particles of GFP-Staufen in the nurse cell cytoplasm are also strongly reduced in size and frequency and are absent from the oocyte.

(C and C′) *osk* mRNA localization to the posterior of a wild-type stage 10 oocyte.

(D and D′) *osk* mRNA localization in a *linha* GLC.

(E) A wild-type stage 11 egg chamber, showing the normal contraction of the nurse cells and the dumping of their cytoplasm into the oocyte (GFP-Stau, green; actin, red).

(F) A *linha* GLC at stage 11 showing the dumpless phenotype.

(G) Chimeric ovariole containing *linha* mutant germline clones marked by the loss of nuclear GFP (green). *oskar* mRNA (red) accumulates in the oocyte normally in mutant oocytes (asterisk).

intron of *hrp48* and have previously been shown to significantly reduce the level of Hrp48 protein expression, while their lethality is fully rescued by an *hrp48* transgene (Figure 2A) (Hammond et al., 1997). Thus, the *linha* mutations are alleles of *hrp48*, which encodes one of the three most abundant hnRNPs (heterogeneous nuclear RiboNucleoProteins) in *Drosophila*.

Hrp48 is a member of the hnRNPA/B family of RNA binding proteins, which consist of two N-terminal RNA-recognition motifs (RRM) and a C-terminal Glycine-rich domain (Matunis et al., 1992a). *linha*$^{5A2\text{-}6}$ is a G to A transition that changes amino acid 101 from Glycine to Aspartic acid (Figure 2A). This Glycine falls in the RNP2 motif of the second RRM, and is conserved in all hnRNPA/B family members (Birney et al., 1993). In contrast, both *linha*$^{7E7\text{-}18}$ and *linha*$^{10B2\text{-}9}$ are G to A transitions that change tryptophans (W) to asparagines (N) at amino acids 312 and 342 in the Gly-rich domain (Figure 2A).

To examine the effect of these mutations on Hrp48 protein levels, we raised a polyclonal antibody against two peptides from the C terminus of the protein. This antibody recognizes a protein doublet at 48–50 kDa on Western blots that is identical to the doublet seen with an anti-Hrp48 monoclonal antibody (Figure 2B) (Matunis et al., 1992b; Siebel et al., 1994). Western blots on extracts from wild-type and homozygous mutant third instar larvae show that the three missense *hrp48* alleles express approximately normal levels of Hrp48 protein that has the same mobility as in wild-type (Figure 2C). The missense alleles therefore disrupt protein function, and not protein stability or expression.

hrp48^{linha} Mutations Do Not Disrupt the Oocyte Polarity

HnRNP A/B family members associate with most, if not all, nascent transcripts in the nucleus, and are transported into the cytoplasm with processed mRNAs (Dreyfuss et al., 1993; Matunis et al., 1993; Piñol-Roma and Dreyfuss, 1992). Since this suggested that Hrp48 might play a general role in mRNA localization, we examined whether the *hrp48* missense mutants alter the distribution of other mRNAs that are localized within the oocyte. In contrast to *osk* mRNA, *bcd* and *gurken* mRNAs are localized normally to the anterior cortex and to the dorsal/anterior margin of the oocyte in *hrp48*^{linha} germline clones (Figures 3A–3D). *gurken* mRNA translation also appears to be normal in these mutants, as Gurken protein localization is indistinguishable from wild-type (Figures 3E and 3F).

Although Hrp48 is an RNA binding protein, its role in *osk* mRNA localization could be indirect, and mediated through an effect on the organization of the microtubules. To investigate if the microtubule cytoskeleton is affected in *hrp48*^{linha} germline clones, we examined the localization of the Kinesin-β-galactosidase fusion protein (Clark et al., 1994). In wild-type ovaries, Kin-β-gal localizes to the posterior cortex of the oocyte at stage 9, and an identical localization is observed in mutant

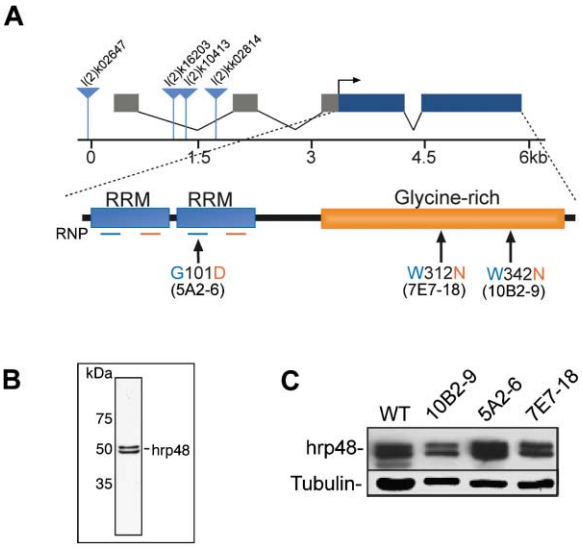

Figure 2. *linha* Mutations Are Missense Alleles of *hrp48*

(A) The structure of the *hrp48* gene and protein, showing the positions of the P element alleles and the three linha missense alleles. The noncoding exons are shown in gray and the coding region in blue. The Hrp48 protein is shown below and consists of two RNA recognition motifs (RRMs), each made of two consensus RNPs, and a C-terminal Glycine-rich region. The three amino acids changes corresponding to the three alleles found in the screen are shown (numbers indicate the amino acid position).

(B) A Western blot with Hrp48 peptide antibody showing a doublet of Hrp48 protein bands at 48–50 kDa.

(C) A Western blot of extracts from wild-type and mutant 3rd instar larvae probed with anti-Hrp48 and anti-tubulin as a loading control.

germline clones (Figures 4A–4F). The overall organization of the microtubule cytoskeleton is also indistinguishable from wild-type, as revealed by the distribution of the microtubule binding protein, tau-GFP, in living oocytes (Figures 4G and 4H) (Micklem et al., 1997). Furthermore, time lapse films of mutant egg chambers show the normal slow and chaotic pattern of microtubule-dependent cytoplasmic streaming. Thus, these missense alleles of *hrp48* specifically disrupt the localization of *osk* mRNA, and have no effect on the polarization of the oocyte or the organization of the microtubule cytoskeleton.

The *hrp48*^{linha} Mutations Do Not Affect the Splicing of *osk* and *Ubx* mRNAs

Hrp48 has been shown to regulate the differential splicing of the P element in the germline and the soma, and the alternative splicing of Ubx pre-mRNA (Burnette et al., 1999; Hammond et al., 1997; Siebel et al., 1994). The localization of *osk* mRNA is also likely to require splicing of the pre-mRNA, since it depends on the Exon-exon junction complex (Hachet and Ephrussi, 2001; Mohr et al., 2001). To test if the *osk* mRNA localization phenotype of *hrp48* is due to a defect in splicing, we examined whether each of three small introns within the *osk* coding region is correctly removed from the pre-mRNA in germline clones of the *hrp48*^{linha} alleles. RT-PCR with primers on both sides of each intron revealed an identical pattern of bands in wild-type and all three mutants (Figure 5B). Hrp48 is therefore either not required for *osk* mRNA splicing, or retains its splicing activity in the *hrp48*^{linha} mutations.

Since Hrp48 regulates alternative splicing, it is possible that the failure to localize *oskar* mRNA in the *hrp48*^{linha} mutants is an indirect consequence of a defect in the splicing pattern of some other transcript. We therefore analyzed whether the *hrp48*^{linha} alleles disrupt the ratio of splice variants of Ubx, a known target of Hrp48. In contrast to heterozygotes for the P insertions in *hrp 48*, which show a marked increase in the abundance of the IIa splice variant (Burnette et al., 1999), *hrp48*^{linha} heterozygotes have a similar pattern of Ubx isoforms to wild-type L3 larvae (Figure 5C). We also examined the ratios of the different Ubx splice variants in L2 larvae homozygous for the *hrp48*^{linha} alleles (Figure 5C). Although the pattern of alternative splicing of Ubx is quite

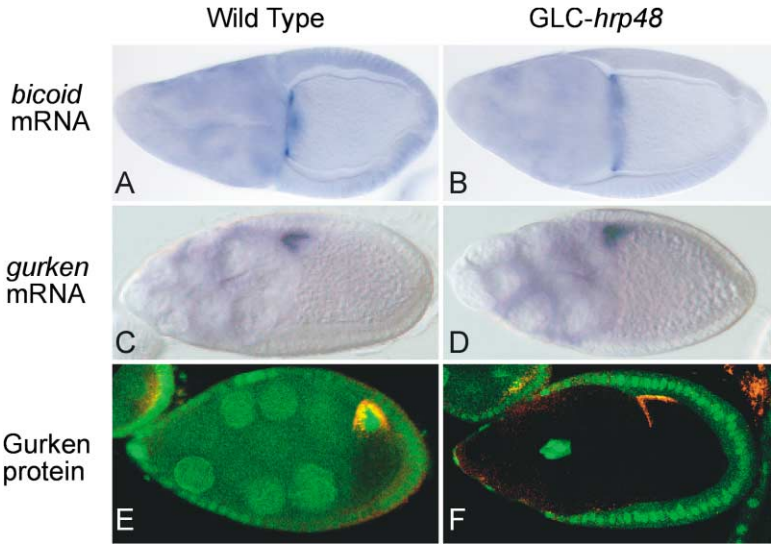

Figure 3. *hrp48* Missense Mutations Do Not Affect the Localization of *bicoid* or *gurken* mRNAs

(A and B) In situ hybridizations for *bcd* mRNA in wild-type (A) and *hrp48* GLC mutant egg chambers (B); *bcd* mRNA localizes normally at the anterior of the oocyte.

(C and D) In situ hybridization for *grk* mRNA in wild-type (C) and *hrp48* mutant egg chambers(D). *grk* mRNA is localized normally to the dorsal-ventral corner of the oocyte, above the nucleus.

(D and E) Gurken protein (red) is correctly localized in wild-type (D) and *hrp48* mutant germline clones (E), which are marked by the absence of a GFP (green).

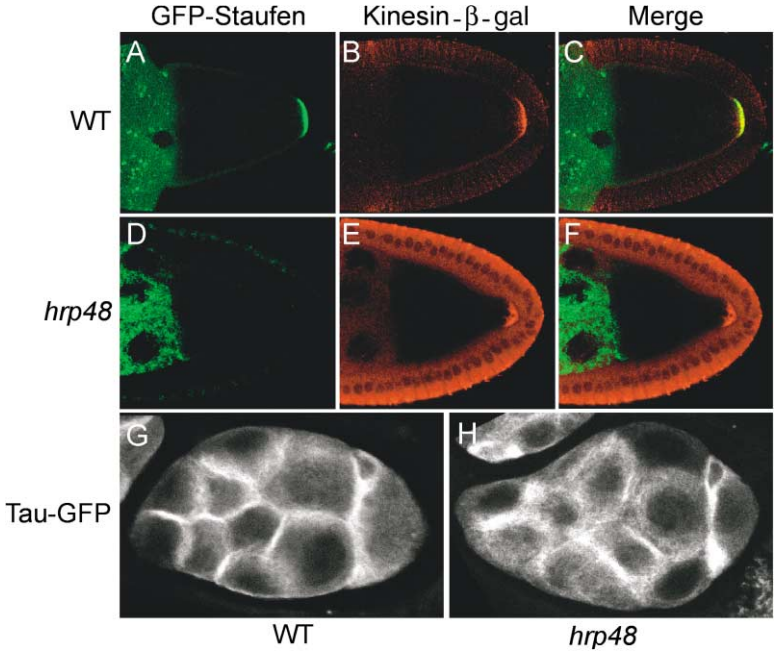

Figure 4. *hrp48* Missense Mutations Do Not Affect Microtubule Organization

(A–C) Wild-type stage 9 egg chamber, showing the posterior localization of GFP-Stau (A; green) and Kinesin-β-gal (B; red). The merge of the two images is shown in (C).

(D–F) An *hrp48* GLC. GFP-Stau (D) is not localized to the posterior, but Kinesin-β-gal (E) localizes normally.

(G and H) Tau-GFP labeling of the microtubules in wild-type (G) and *hrp48* mutant stage 9 egg chambers. The microtubule organization in the mutant is identical to the wild-type, with the highest concentration of MT along the anterior cortex of the oocyte.

different in L2 and L3 larvae, the mutant larvae also show an identical pattern to wild-type at this stage (Figures 5C and 5D). Thus, the *hrp48*^{linha} alleles do not appear to affect the function of Hrp48 in alternative splicing.

Hrp48 Localizes to the Posterior of the Oocyte with *osk* mRNA

Hrp48 accumulates into the oocyte as soon as it can be identified in region 2b of the germarium, and moves to the posterior when the oocyte becomes polarized on entering region 3 of the germarium (Figure 6A) (Huynh et al., 2001). This localization at the posterior of the oocyte persists during stage 1–6 of oogenesis, but disappears when the oocyte repolarizes at stage 7. Hrp48 then accumulates in a crescent at the posterior pole of the oocyte at stage 9, where it remains until at least stage 10b (Figure 6C). This posterior crescent does not form in germline clones of the *hrp48* missense alleles (data not shown). The nuclear localization of Hrp48 is unaffected by these mutations, however, consistent with the fact that these alleles are not protein nulls.

The localization of Hrp48 is very similar to that of *osk* mRNA, suggesting that it is associated with the mRNA and is transported with it to the posterior pole. Two lines of evidence support this view. First, Hrp 48 colocalizes at the posterior with other components of the *osk* mRNA localization complex, such as Y14 (Figures 6E–6G). Second, Hrp48 does not form a posterior crescent in mutants that disrupt *osk* mRNA localization, such as *stau*^{D3}, although it still localizes to the oocyte at early stages (Figures 6B and 6D).

Hrp48 Is p50 and Binds to the 5′ Region and 3′UTR of *osk* mRNA

The results above show that Hrp48 is required for *osk* mRNA localization and colocalizes with it to the posterior pole, raising the possibility that it binds directly to the mRNA to mediate its posterior transport. A protein of similar molecular weight, called p50, has previously been shown to bind to the three BREs in the *osk* 3′UTR, as well as to a 5′ translational activation element (5′act) near the 5′ end of the mRNA (Figure 7A) (Gunkel et al., 1998). The major band that crosslinks to these regions of *osk* RNA migrates at an identical position to the Hrp48 doublet detected by the antibody, suggesting that they are the same protein (Figure 7B), and the accompanying manuscript from Yano et al. (2004) confirms that this is indeed the case.

We examined whether any of the *hrp48* missense alleles disrupt the binding Hrp48 to *osk* mRNA, by performing UV crosslinking assays with extracts from homozygous mutant larvae. All three mutant Hrp48 proteins efficiently crosslink to RNAs containing the p50 binding sites in *osk* mRNA, and show a similar specificity to wild-type Hrp48 for the sense strands over the antisense RNA controls (Figure 7B and data not shown). We also performed RNA-affinity assays by incubating wild-type and mutant protein extracts with biotinylated RNAs that correspond to the 5′-act (derepressor) element, the entire *osk* 3′UTR, BREs A and B, or BRE C. In all cases, the mutant Hrp48 proteins copurified with each RNA as efficiently as the wild-type protein, indicating that the RNA binding specificity of Hrp48 is not affected (Figure 7C).

A small deletion adjacent to the proximal BREs of *osk* mRNA specifically reduces the binding of p50/Hrp48, but not of Bruno, and leads to the premature translation of the mRNA before it is localized to the posterior (Gunkel et al., 1998). Since this indicates that Hrp48 is required to repress the translation of *osk* mRNA until it is localized, we examined whether *hrp48* missense mutations disrupt repression and allow the translation of the unlocalized mRNA. However, we could not detect any Osk protein in Western blots or immunostainings of mutant germline clones (Figure 7D and data not shown).

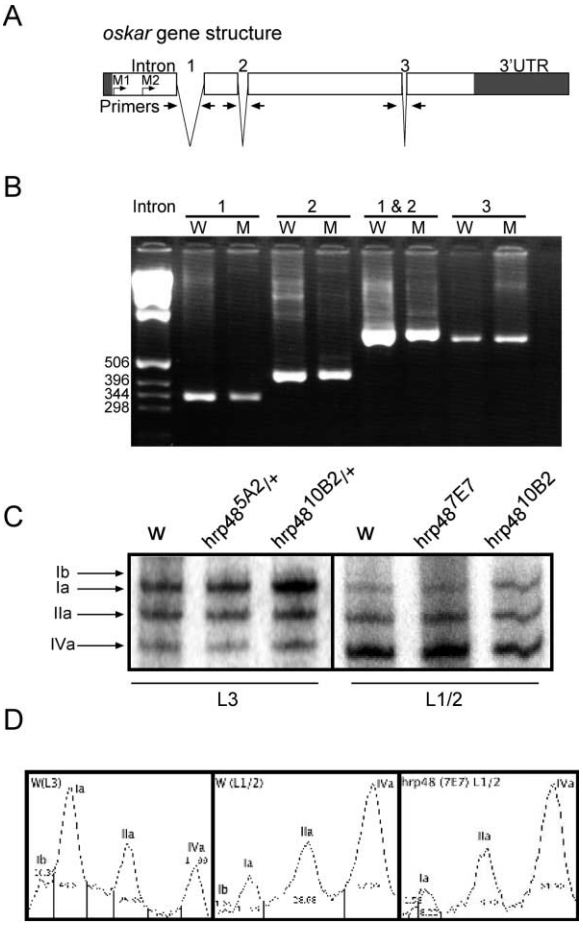

Figure 5. *osk* and *Ubx* mRNA Splicing in Wild-Type and *hrp48* Mutant Ovaries

(A) The *osk* gene contains three small introns. The arrows indicate the primers to RT-PCR each intron.

(B) RT-PCR analysis of *osk* mRNA splicing in wild-type (W) and *hrp48* mutant ovaries (M). PCR across each intron produces a single band of the same size in wild-type and mutant ovaries, indicating that *osk* is correctly spliced in *hrp48* missense mutants.

(C) Quantitative RT-PCR analysis of *Ubx* mRNA splicing in wild-type (w) and *hrp48* mutant larvae. The four main *Ubx* isoforms are represented (Ib, Ia, IIa, and IVa). $hrp48^{linha}$ mutant larvae die at the L1/L2 stage; it is therefore not possible to analyze homozygous mutant L3 larvae. Heterozygous mutant L3 larvae show the same pattern of bands as wild-type L3, with the main isoform being Ia. IVa is the main isoform in L1/L2 wild-type and homozygous *hrp48* mutant larvae.

(D) Quantification of the RT-PCR shows a clear change in the ratio of isoforms between wild-type L1/L2 larvae and L3 larvae. However, this ratio is not affected in *hrp48* mutant larvae at each stage (right panel and data not shown).

Thus, the mutant Hrp48 proteins can still mediate the translational repression of *osk* mRNA, consistent with observation that they all bind normally to the BREs.

Discussion

Previous genetic approaches have shown that Stau, Barentsz, Mago Nashi, and Y14 are required for *osk* mRNA localization, and colocalize with it to the posterior pole, strongly suggesting that these proteins are components of the mRNA localization complex (Hachet and Ephrussi, 2001; Mohr et al., 2001; Newmark et al., 1997; St Johnston et al., 1991; van Eeden et al., 2001). However, none of these proteins have been shown to bind directly to *osk* mRNA, although this seems likely to be the case for the RNA binding proteins, Stau and Y14. Biochemical strategies, on the other hand, have led to the identification of Bruno, Apontic, p68, and p50 as proteins that bind to specific sequences in *osk* RNA, all of which have been implicated in the regulation of *osk* mRNA translation but not in the localization of the mRNA (Gunkel et al., 1998; Kim-Ha et al., 1995; Lie and Macdonald, 1999; Webster et al., 1997). Our results and those of Yano et al. (2004) provide a link between these two approaches, by demonstrating that p50 corresponds to Hrp48 and that it is specifically required for the transport of the mRNA to the posterior of the oocyte. Germline clones of the three missense alleles of *hrp48* have no effect on the polarity of the oocyte, the organization of the microtubules, or the localization of *bicoid* or *gurken* mRNAs, but abolish the posterior localization of *osk* mRNA.

Since Hrp48 has been shown to regulate alternative splicing, it is possible that the *oskar* mRNA localization phenotype of the $hrp48^{linha}$ mutations is an indirect consequence of a defect in the splicing of another mRNA that encodes a factor that is directly involved in *osk* mRNA transport. However, this is unlikely to be the case for two reasons. First, these missense alleles have no effect on the alternative splicing of Ubx transcripts. Since the insertion alleles of Hrp48 do disrupt the Ubx splicing pattern, the missense alleles do not appear to impair the function of Hrp48 in splicing regulation. Second, Hrp48 binds to sequences in the 5′ region and the 3′UTR of *osk* mRNA, and colocalizes with the mRNA at the posterior. This strongly suggests that the requirement for Hrp48 in *osk* mRNA localization is direct, and that it functions as an essential *trans*-acting factor that recognizes the RNA and plays a role in coupling it to the localization machinery.

The phenotype of the *hrp48* missense alleles differs from that of the other mutants that disrupt *osk* mRNA localization, suggesting that it acts at a distinct step in the localization pathway. In *stau, barentsz, mago, Y14*, and *tropomyosinII* mutants, *osk* mRNA also fails to reach the posterior, but most of the RNA remains at the anterior cortex (Ephrussi et al., 1991; Erdélyi et al., 1995; Kim-Ha et al., 1991; Newmark and Boswell, 1994; van Eeden et al., 2001). Since *osk* mRNA shows a transient accumulation at the anterior in wild-type before it localizes to the posterior, these proteins may be required to release *osk* mRNA from the anterior, and to couple it to the posterior transport pathway. In contrast, we detect no accumulation of *osk* mRNA at the anterior of the oocyte in the *hrp48* missense mutants, and the mRNA shows a uniform distribution throughout the oocyte cytoplasm. This raises the possibility that Hrp48 is required for the transient anterior accumulation of *osk* mRNA, and acts upstream of the other proteins required for posterior localization, such as Stau. One argument against this interpretation is that the localization to the anterior of the oocyte is thought to be a by-product of the transport from the nurse cells into the oocyte (Serano and Cohen,

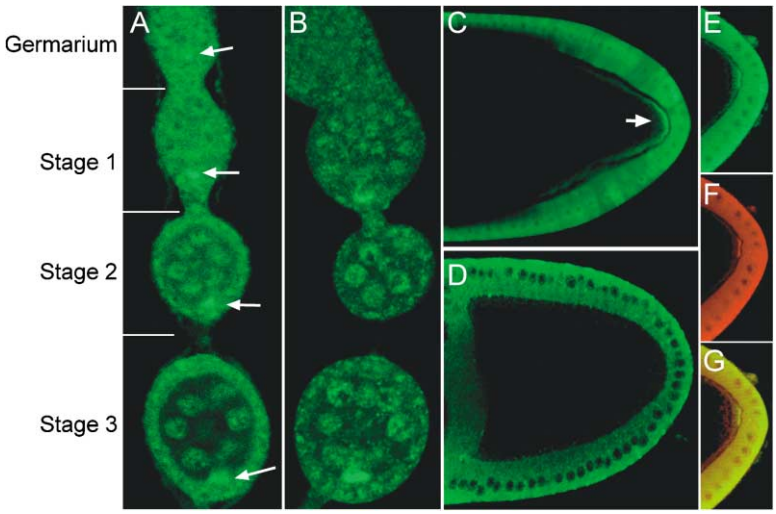

Figure 6. Hrp48 Colocalizes with *osk* mRNA throughout Oogenesis

(A and B) Hrp48 staining of wild-type (A) and *stau*^{D3} (B) ovarioles. Hrp48 accumulates in the oocyte (arrows) from region 2 of the germarium onward. Note that Hrp48 is both nuclear and cytoplasmic.

(C and D) Hrp48 staining of wild-type (C) and *stau*^{D3} (D) stage 10 egg chambers. Hrp48 is localized in a crescent at the posterior of the oocyte in wild-type, but not in *stau* mutants.

(E–G) The posterior of a wild-type stage 10 oocyte stained for Hrp48 (E, green) and Y14 (F, red). The two proteins colocalize at the posterior pole in the merged image (G).

1995), which occurs normally in the *hrp48* missense alleles. In support of this view, all mRNAs that are transported into the oocyte also localize, at least transiently, to the anterior cortex at stage 9. We therefore favor an alternative model in which Hrp48 acts downstream of Stau, Mago, etc. In this case, the *stau* class of mutants might block the release of *osk* mRNA from the anterior cortex, whereas the *hrp48* alleles might stimulate this release, but prevent the subsequent association of the mRNA with the factors that transport it to the posterior pole.

Many localized mRNAs appear to move as large cytoplasmic particles, leading to the suggestion that they must be packaged into transport granules in order to be localized. For example, when pair rule, *wingless*, or *bicoid* mRNAs are injected into *Drosophila* embryos, they assemble into particles that move in a microtubule-dependent manner, and fluorescent MBP mRNA shows a similar behavior when introduced into cultured oligodendrocytes (Ainger et al., 1993; Ferrandon et al., 1994; Lall et al., 1999; Wilkie and Davis, 2001). The formation of these transport particles has also be visualized by labeling proteins that bind to localized mRNAs. For example, injected *bicoid* mRNA recruits Stau into motile particles in *Drosophila* syncytial blastoderm embryos, while GFP-tagged mouse Stau1 has been observed to form particles that move along microtubules in neuronal processes (Köhrmann et al., 1999; Tiruchinapalli et al., 2003; Wagner et al., 2001). In this context, it is very striking that two of the *hrp48* missense mutants strongly reduce the formation of GFP-Stau particles in the nurse cell and oocyte cytoplasm. This suggests that Hrp48 plays a role in the formation of Stau-containing *osk* mRNA transport particles, which could account for the failure to localize the mRNA to the posterior pole in these mutants. If these particles sequester the mRNA and prevent its diffusion, this may also explain why *osk* mRNA remains at the anterior in the *stau* class of mutants, but not in the *hrp48* missense alleles.

Hrp48 is one of the three most abundant HnRNPs in *Drosophila*, along with Hrp40(Squid) and Hrp38, and is thought to bind most, if not all, nascent transcripts in the nucleus (Kelley, 1993; Matunis et al., 1992a, 1993, 1992b). It is therefore surprising to recover *hrp48* mutations that have such a specific effect on the localization of *osk* mRNA. Yano et al. (2004) report that P element insertions in *hrp48* produce a distinct phenotype. Although *osk* mRNA is often not localized to the posterior, it is sometimes found in the center of the oocyte, which is indicative of a defect in oocyte polarity. Consistent with this, Kin-β-gal also localizes to the center of the oocyte in these mutants, whereas it always shows a wild-type posterior localization in the missense alleles. Thus, the P element insertions presumably disrupt the regulation of another mRNA that is required for the polarization of the oocyte microtubule network. A second important difference is that the P element alleles cause the premature translation of *osk* mRNA, which is consistent with the identification of Hrp48 as p50, which binds to sites in the *osk* BREs (Gunkel et al., 1998). In contrast, the missense alleles have no discernable effect on translational repression, as *osk* mRNA is completely unlocalized, but no Osk protein is produced. P alleles of Hrp48 also affect *gurken* mRNA localization and translation (Goodrich et al., 2004), whereas we observed no defects in the distribution of either *gurken* mRNA or protein in germline clones of the missense alleles. Finally, the P alleles, but not the missense alleles, disrupt the alternative splicing of Ubx RNAs. These differences presumably reflect the distinct nature of the molecular lesions in the two types of allele. All of the P element insertions fall in the promoter or an intron in the 5′UTR of the *hrp48* gene, and produce reduced levels of wild-type Hrp48 protein (Hammond et al., 1997). In contrast, each of the missense alleles expresses approximately normal levels of mutant Hrp48, in which a single amino acid has been changed.

The comparison between the phenotypes of the two classes of *hrp48* mutations indicates that the missense alleles affect regions of the protein that are specifically required for *osk* mRNA localization. This is particularly interesting in the case of the two Trp to Asn mutations in the Glycine-rich domain (GRD), since this domain is not involved in RNA binding, and neither mutant affects the interaction of Hrp48 with *osk* mRNA. Evidence from

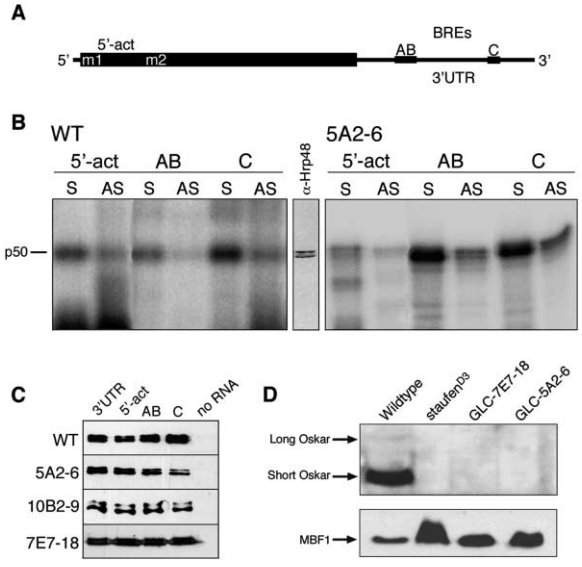

Figure 7. Hrp48 Binds 5′ and 3′ Elements in *osk* mRNA

(A) A diagram of *osk* mRNA, showing the alternate start codons, m1 and m2, and the BREs in the 3′UTR (AB and C). The 5′-act region between m1 and m2 and the AB and C regions containing the BREs were used as probes in the binding experiments below.

(B) UV crosslinking assays using wild-type and *hrp48* mutant protein extracts, with probes corresponding to the regions shown in (A). The major band that crosslinks to these probes at approximately 50 kDa, comigrates with Hrp48 (Western blot in the center). The pattern of bands is identical in the *hrp48* mutant extracts, indicating that the Hrp48 missense alleles do not disrupt RNA binding.

(C) Mutant Hrp48 proteins copurify with *osk* 5′ and 3′ regions in affinity pull-down assays. Biotinylated *osk* RNA probes were used to affinity purify interacting proteins from wild-type and mutant larval extracts, and the bound proteins were then run on Western blots and probed for Hrp48. The missense mutant proteins copurify with the *osk* 3′UTR, 5′-act, and BREs to the same extent as the wild-type protein.

(D) A Western blot of ovarian extracts from wild-type, *stau*^{D3}, and *hrp48*$^{7E7\text{-}18}$ and *hrp48*$^{5A2\text{-}6}$ germline clones, probed with an antibody for Osk protein. The *hrp48* mutant extracts contain no detectable Long or Short Osk, indicating that these alleles do not disrupt the translational repression of unlocalized *osk* mRNA.

other HnRNPA/B family members suggests that this region functions as an oligomerization domain. For example, the GRD of Human HnRNP A1 has been shown to self-associate in vitro, and this interaction depends on large aromatic residues embedded with the Glycine-rich region (Cartegni et al., 1996). Thus, it is possible that Hrp48 also oligomerizes, and that this is disrupted by mutating the aromatic Tryptophans in the GRD, which could explain why both mutations impair the formation of GFP-Stau particles. These mutations may therefore abolish RNA localization because Hrp48 oligomerization is required to form high-order *osk* RNP complexes, which are the substrate for posterior transport.

It is more difficult to explain why the 5B2-6 allele disrupts *osk* mRNA localization. Although one would expect a mutation in a conserved residue in the RNA recognition motif to disrupt RNA binding, the mutant protein binds to *osk* mRNA in vitro as well as the wild-type protein, in both UV-crosslinking and pull-down assays. Furthermore, it presumably also associates with *osk* mRNA in vivo, because it mediates normal translational repression, which requires binding to the sites in the BREs. The Hrp48 binding sites in *osk* mRNA that are necessary for localization have not been mapped, and these sites could be distinct from those involved in translational repression. Thus, this mutation may only disrupt Hrp48 binding to a specific subset of its target sites, including unidentified sites in *osk* mRNA that are required for localization. Since the glycine that is mutated is not directly involved in RNA binding, another possibility is that the mutation disrupts the interaction of Hrp48 with another protein that is required to couple *osk* mRNA to the localization machinery.

A Conserved Role for hnRNPA/B Proteins in mRNA Localization and Translation

Two other members of the hnRNP A/B family have also been implicated in mRNA localization. *Drosophila* Squid, which is most closely related to hnRNPA1, binds directly to *gurken* mRNA, and is required for its localization to the anterior-dorsal corner of the oocyte (Kelley, 1993; Norvell et al., 1999). Squid is also required to repress the translation of unlocalized *grk* mRNA, and interacts with Bruno, a translational repressor of both *grk* and *osk* mRNAs (Norvell et al., 1999). Since Hrp48 binds to the Bruno Response elements in *osk* mRNA, and is required to repress its premature translation, these two *Drosophila* hnRNP A/B proteins perform remarkably similar functions in the regulation of *osk* and *gurken* mRNAs. Furthermore, hnRNP A2, which is one of the closest mammalian homologs of *Drosophila* Hrp48, plays a comparable role in the localization of MBP mRNA in oligodendrocytes. When MBP mRNA is injected into oligodendrocytes, it forms large particles that move along microtubules into the distal processes, and, like *osk*, the mRNA is probably transported by kinesin (Ainger et al., 1993; Carson et al., 1997). This localization requires the specific binding of hnRNP A2 to a 21 nt A2RE element in the MBP 3′UTR that is necessary and sufficient for localization and efficient translation (Hoek et al., 1998; Munro et al., 1999). In addition, recent work has shown that the microtubule-dependent localization into oligodendrocyte processes is mediated by the second RRM of hnRNP A2 (Brumwell et al., 2002), and it is intriguing that the *hrp48* 5B2-6 mutation falls in the equivalent domain of the *Drosophila* protein. These clear parallels between the functions of Hrp48, Squid, and hnRNP A2 suggest that these hnRNP A/B proteins play a conserved role in mRNA localization and translational control in flies and mammals.

The Nuclear History of *osk* mRNA Determines Its Localization in the Cytoplasm

Hrp48 is a predominantly nuclear protein that associates with nascent transcripts, and regulates alternative pre-mRNA splicing. It therefore seems very likely that Hrp48 binds to *osk* mRNA in the nucleus, and is exported into the cytoplasm with the RNA, where it regulates localization and translation. This adds to the growing body of evidence that the cytoplasmic fate of mRNAs is determined in part by their nuclear history (Farina and Singer, 2002). In the case of *osk* mRNA, cytoplasmic localization seems to require the binding of at least four distinct

proteins in the nucleus; Hrp48, which probably associates with the RNA cotranscriptionally, and Mago, Y14, and eIF4AIII, which are presumably recruited to the RNA during splicing as part of the exon-exon junction complex. The mRNA must then recruit additional essential localization factors in the cytoplasm, such as Stau and Barentsz, before it is competent to localize to the posterior pole of the oocyte. Thus, the assembly of a functional *osk* RNA localization complex requires the stepwise recruitment of multiple nuclear and cytoplasmic proteins, all of which are essential for posterior localization. One of the main challenges for the future will be to determine how this complex is assembled, and how these proteins act together to link the mRNA to the motor that mediates its transport to the posterior pole.

Experimental Procedures

Isolation and Mapping of *hrp48^{linha}* Mutations

The three *hrp48^{linha}* alleles were identified in a germline clone screen for mutations on chromosome arm 2L that affect the localization of GFP-Stau, following the procedure described in Martin et al. (2003). To map the mutants, we generated a low-density map of Single Nucleotide Polymorphisms (SNPs) between the FRT40A chromosome and the *al,dp,b,pr,c,px,sp* marker chromosome. *linha* mutations were initially mapped between *al* and *dp*, and 570 recombinants were generated between those two visible markers (one recombinant per 25 kb). The genotype of each recombinant line was determined by extracting DNA from a single male, and analyzing the SNP pattern by PCR amplification. This placed the mutations between two SNPs in 27B4 and 27D1, with 9 recombinants falling into this region of 177 kb. None of these recombinants separated the mutations from an additional RFLP in 27C4, indicating that *linha* lies in a ~20 kb interval around this marker. The three alleles were identified by sequencing the Hrp48 coding region that had been amplified from DNA extracted from homozygous mutant larvae.

Fly Stocks

We used the following stocks for the genetic screen: *w-*; P[ry+; hsp70:neo; FRT40A] (Xu and Rubin, 1993), *w-*; P[ry+; hsp70:neo; FRT40A] P[w+; ovoD1]/CyO (Chou and Perrimon, 1996), *y*, *w*, P[w+; mat tub a4:GFP-Stau] P[ry+; hsFLP]; Bl, L/CyO.

Germline clones marked by the loss of GFP were generated using *w-*; P[ry+; hsp70:neo; FRT40A] P[w+; ubi:GFPnls] (Luschnig et al., 2000).

P[w+; *Tau*-GFP] (Micklem et al., 1997) and P[w+; *kinesin-LacZ* (KZ2030)] (Clark et al., 1994) transgenes were transposed onto the *y*, *w,* P[ry+; hsFLP] chromosome to examine the microtubule organization in *hrp48* mutant germline clones.

We also used the following mutant alleles: *stau^{D3}*(Schüpbach and Wieschaus, 1986); *cappuccino^{RK}*; *spire^{RP}*(Manseau and Schüpbach, 1989) *aubergine^{QC}*(Schüpbach and Wieschaus, 1991) and l(2)k02647, l(2)k16203, l(2)k10413, and l(2)k02814 (Hammond et al., 1997; Spradling et al., 1999), which are P insertions in *hrp48*.

Hrp48 Antibody Production

Polyclonal antibodies were raised against Hrp48 by immunizing Rabbits with two peptides; 1: N-CRTGPGNSASKSGSEY-C and 2: N-EGASNYGAGPRSAYGNC-C, which correspond to nonconserved regions in the C-terminal Glycine-rich domain. The serum was affinity purified on peptide columns following standard procedures.

Western Blot and RT-PCR Analysis of *osk*

Western analysis of Oskar protein was performed as described by Markussen et al. (1995). Blots were probed with rabbit anti-Osk antibody at 1/20,000. rabbit anti-MBF1 was used at 1/20,000 as a loading control.

For RT-PCR analysis, 80 μg of wild-type or mutant ovaries was homogenized into 160 μl of Trizol reagent solution and RNA extracted as per manufacturer instructions (Invitrogen). Reverse transcription was performed using the AMV RT-PCR kit (Roche).

Immunostainings and In Situ Hybridization

Anti-Orb (6H4 and 4H8) at 1/250 (DSHB, Iowa) (Lantz et al., 1994); mouse anti-Gurken at 1/10 (Neuman-Silberberg and Schüpbach, 1996); rat anti-Y14 at 1/50 (Hachet and Ephrussi, 2001); rabbit anti-Stau at 1/5000 (St Johnston et al., 1991); rabbit anti-Hrp48 at 1/1000 (Siebel et al., 1994); rabbit anti-β-gal 1/1000 (ICN Pharmaceuticals); mouse anti-GFP at 1/200 (Roche). Texas-Red- and FITC-conjugated secondary antibodies were used at 1/100 (Molecular Probes). Rhodamine-conjugated Phalloidin was used to visualize the actin cytoskeleton (Molecular Probes).

In situ hybridizations were performed as described in González-Reyes et al. (1995) using RNA probes labeled with dig-UTP (Roche), or allyl-UTP followed by treatment with Cy3 monoreactive dye (Amersham).

UV Crosslinking Assay

The UV crosslinking assays were performed using Method 2 of Gunkel et al. (1998), as this optimizes detection of p50/Hrp48. The protein extracts were prepared from larvae, as egg chambers containing germline clones of the *hrp48* missense alleles express significant amounts of wild-type Hrp48 protein in the follicle cells. ^{32}P (Cytidine 5′-[α-32P] triphosphate, Amersham) labeled RNA probes were transcribed from templates prepared by PCR from the *osk* cDNA, corresponding to fragment G (nucleotides 40–170 of the 417 nt region between M1 and M2) (Gunkel et al., 1998), and the BREs (AB and C) (Kim-Ha et al., 1995) using the Promega Riboprobe system.

RNA Affinity Pulldown Assays

RNA was transcribed in the presence of biotin-16-UTP using the Biotin RNA labeling mix (Roche) and 20 U of RNAsin (Promega). An equal amount of biotinylated RNA was then bound to 40 μl of washed Streptavidin magnetic particles (Roche) in a final volume of 250 μl for 20 min at room temperature. Unbound RNA was removed by three washes with 10 mM Tris-HCl, 1 M NaCl, and 1 mM EDTA (pH 7.5), and the beads further washed twice with Interaction buffer (10 mM HEPES, 100 mM NaCl, 1 mM EDTA, 5% (v/v) Glycerol, 10 g/l Heparin, and 5 g/l tRNA). Larval protein extracts were diluted to a final volume of 100 μl with Interaction buffer, and incubated with the beads for 1 hr rotating at 4°C. The magnetic particles were then washed five times for 5 min with interaction buffer. Bound proteins were eluted with 60 μl of boiling SDS-PAGE buffer, and were separated by SDS-PAGE, and Western blotted using affinity purified anti-Hrp48.

Acknowledgments

We wish to thank Anne Ephrussi, Don Rio, and Trudi Schüpbach for fly stocks and antibodies. We are very grateful to Vitaly Zimyanin for his initial observation that GFP-Stau particles are markedly reduced in *hrp48* mutants, and to the Guichet and Lepesant labs for helpful discussions. This work was supported by a European Union Marie Curie Fellowship and EMBO fellowship (J.-R.H.), by a Human Frontiers Programme Collaborative Grant (T.P.M.), and by the Wellcome Trust (K.S.-L. and D.S.).

Received: August 18, 2003
Revised: February 13, 2004
Accepted: March 29, 2004
Published: May 10, 2004

References

Ainger, K., Avossa, D., Morgan, F., Hill, S.J., Barry, C., Barbarese, E., and Carson, J.H. (1993). Transport and localization of exogenous myelin basic protein mRNA microinjected into oligodendrocytes. J. Cell Biol. *123*, 431–441.

Beach, D.L., Salmon, E.D., and Bloom, K. (1999). Localization and anchoring of mRNA in budding yeast. Curr. Biol. *9*, 569–578.

Bertrand, E., Chartrand, P., Schaefer, M., Shenoy, S.M., Singer, R.H., and Long, R.M. (1998). Localization of *ASH1* mRNA particles in living yeast. Mol. Cell *2*, 437–445.

Birney, E., Kumar, S., and Krainer, A.R. (1993). Analysis of the RNA-recognition motif and RS and RGG domains: conservation in metazoan pre-mRNA splicing factors. Nucleic Acids Res. *21*, 5803–5816.

Bohl, F., Kruse, C., Frank, A., Ferring, D., and Jansen, R.P. (2000). She2p, a novel RNA-binding protein tethers *ASH1* mRNA to the Myo4p myosin motor via She3p. EMBO J. *19*, 5514–5524.

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

Brumwell, C., Antolik, C., Carson, J.H., and Barbarese, E. (2002). Intracellular trafficking of hnRNP A2 in oligodendrocytes. Exp. Cell Res. *279*, 310–320.

Burnette, J.M., Hatton, A.R., and Lopez, A.J. (1999). Trans-acting factors required for inclusion of regulated exons in the Ultrabithorax mRNAs of *Drosophila melanogaster*. Genetics *151*, 1517–1529.

Carson, J.H., Worboys, K., Ainger, K., and Barbarese, E. (1997). Translocation of myelin basic protein mRNA in oligodendrocytes requires microtubules and kinesin. Cell Motil. Cytoskeleton *38*, 318–328.

Cartegni, L., Maconi, M., Morandi, E., Cobianchi, F., Riva, S., and Biamonti, G. (1996). hnRNP A1 selectively interacts through its Gly-rich domain with different RNA-binding proteins. J. Mol. Biol. *259*, 337–348.

Chou, T.-B., and Perrimon, N. (1996). The autosomal FLP-DFS technique for generating germline mosaics in *Drosophila melanogaster*. Genetics *144*, 1673–1679.

Clark, I., Giniger, E., Ruohola-Baker, H., Jan, L., and Jan, Y. (1994). Transient posterior localisation of a kinesin fusion protein reflects anteroposterior polarity of the *Drosophila* oocyte. Curr. Biol. *4*, 289–300.

Dreyfuss, G., Matunis, M.J., Pinol-Roma, S., and Burd, C.G. (1993). hnRNP proteins and the biogenesis of mRNA. Annu. Rev. Biochem. *62*, 289–321.

Driever, W. (1993). Maternal control of anterior development in the *Drosophila* embryo. In The Development of *Drosophila melanogaster*, M. Bate, and A. Martínez-Arias, eds. (New York: Cold Spring Harbor Laboratory Press), pp. 301–324.

Duncan, J.E., and Warrior, R. (2002). The cytoplasmic Dynein and Kinesin motors have interdependent roles in patterning the *Drosophila* oocyte. Curr. Biol. *12*, 1982–1991.

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

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

Erdélyi, M., Michon, A., Guichet, A., Bogucka Glotzer, J., and Ephrussi, A. (1995). A requirement for *Drosophila* cytoplasmic tropomyosin in *oskar* mRNA localization. Nature *377*, 524–527.

Farina, K.L., and Singer, R.H. (2002). The nuclear connection in RNA transport and localization. Trends Cell Biol. *12*, 466–472.

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

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

Goodrich, J.S., Clouse, N., and Schüpbach, T. (2004). Hrb27C, Sqd and Otu cooperatively regulate *gurken* RNA localization and mediate nurse cell chromosome dispersion in *Drosophila* oogenesis. Development *131*, 1949–1958.

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

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

Hammond, L.E., Rudner, D.Z., Kanaar, R., and Rio, D.C. (1997). Mutations in the *hrp48* gene, which encodes a *Drosophila* heterogeneous nuclear ribonucleoprotein particle protein, cause lethality and developmental defects and affect P-element third-intron splicing in vivo. Mol. Cell. Biol. *17*, 7260–7267.

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

Hoek, K.S., Kidd, G.J., Carson, J.H., and Smith, R. (1998). hnRNP A2 selectively binds the cytoplasmic transport sequence of myelin basic protein mRNA. Biochemistry *37*, 7021–7029.

Huynh, J.R., Shulman, J.M., Benton, R., and St Johnston, D. (2001). PAR-1 is required for the maintenance of oocyte fate in *Drosophila*. Development *128*, 1201–1209.

Kataoka, N., Diem, M.D., Kim, V.N., Yong, J., and Dreyfuss, G. (2001). Magoh, a human homolog of *Drosophila* Mago nashi protein, is a component of the splicing-dependent exon-exon junction complex. EMBO J. *20*, 6424–6433.

Kelley, R.L. (1993). Initial organization of the *Drosophila* dorsoventral axis depends on an RNA-binding protein encoded by the *squid* gene. Genes Dev. *7*, 948–960.

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

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

Kloc, M., and Bilinski, S.M. (2003). RNA localization and its role in the spatially restricted protein synthesis. Folia Histochem. Cytobiol. *41*, 3–11.

Köhrmann, M., Luo, M., Kaether, C., DesGroseillers, L., Dotti, C.G., and Kiebler, M.A. (1999). Microtubule-dependent recruitment of Staufen-Green Fluorescent Protein into large RNA-containing granules and subsequent dendritic transport in living hippocampal neurons. Mol. Cell. Biol. *10*, 2945–2953.

Lall, S., Francis-Lang, H., Flament, A., Norvell, A., Schüpbach, T., and Ish-Horowicz, D. (1999). Squid hnRNP protein promotes apical cytoplasmic transport and localization of *Drosophila* pair-rule transcripts. Cell *98*, 171–180.

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

Le Hir, H., Gatfield, D., Braun, I.C., Forler, D., and Izaurralde, E. (2001). The protein Mago provides a link between splicing and mRNA localization. EMBO Rep. *2*, 1119–1124.

Lie, Y.S., and Macdonald, P. (1999). Apontic binds the translational repressor Bruno and is implicated in regulation of *oskar* mRNA translation. Development *126*, 1129–1138.

Luschnig, S., Krauss, J., Bohmann, K., Desjeux, I., and Nüssleín-Volhard, C. (2000). The *Drosophila* SHC adaptor protein is required for signaling by a subset of receptor tyrosine kinases. Mol. Cell *5*, 231–241.

MacDougall, N., Clark, A., MacDougall, E., and Davis, I. (2003). *Drosophila gurken* (TGFα) mRNA localizes as particles that move within the oocyte in two dynein-dependent steps. Dev. Cell *4*, 307–319.

Manseau, L.J., and Schüpbach, T. (1989). *cappuccino* and *spire*: two unique maternal-effect loci required for both the anteroposterior and dorsoventral patterns of the *Drosophila* embryo. Genes Dev. *3*, 1437–1452.

Markussen, F.H., Michon, A.M., Breitwieser, W., and Ephrussi, A. (1995). Translational control of *oskar* generates short Osk, the isoform that induces pole plasm assembly. Development *121*, 3723–3732.

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

Martin, S.G., Dobi, K.C., and St Johnston, D. (2001). A rapid method to map mutations in *Drosophila*. Genome Biology *2*, research0036. 0031–0012.

Martin, S.G., Leclerc, V., Smith-Litière, K., and St Johnston, D. (2003). The identification of novel genes required for *Drosophila* anterior-posterior axis formation in a germline clone screen using GFP-Staufen. Development *130*, 4201–4215.

Matunis, E.L., Matunis, M.J., and Dreyfuss, G. (1992a). Characterization of the major hnRNP proteins from *Drosophila melanogaster*. J. Cell Biol. *116*, 257–269.

Matunis, M.J., Matunis, E.L., and Dreyfuss, G. (1992b). Isolation of hnRNP complexes from *Drosophila melanogaster*. J. Cell Biol. *116*, 245–255.

Matunis, E.L., Matunis, M.J., and Dreyfuss, G. (1993). Association of individual hnRNP proteins and snRNPs with nascent transcripts. J. Cell Biol. *121*, 219–228.

Micklem, D.R., Dasgupta, R., Elliott, H., Gergely, F., Davidson, C., Brand, A., González-Reyes, A., and St Johnston, D. (1997). The *mago nashi* gene is required for the polarisation of the oocyte and the formation of perpendicular axes in *Drosophila*. Curr. Biol. *7*, 468–478.

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

Munro, T.P., Magee, R.J., Kidd, G.J., Carson, J.H., Barbarese, E., Smith, L.M., and Smith, R. (1999). Mutational analysis of a heterogeneous nuclear ribonucleoprotein A2 response element for RNA trafficking. J. Biol. Chem. *274*, 34389–34395.

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

Neuman-Silberberg, F.S., and Schüpbach, T. (1996). The *Drosophila* TGF-α like protein Gurken: expression and cellular localization during *Drosophila* oogenesis. Mech. Dev. *59*, 105–113.

Newmark, P.A., and Boswell, R.E. (1994). The *mago nashi* locus encodes an essential product required for germ plasm assembly in *Drosophila*. Development *120*, 1303–1313.

Newmark, P.A., Mohr, S.A., Gong, L., and Boswell, R.E. (1997). *mago nashi* mediates the posterior follicle cell-to-oocyte signal to organize axis formation in *Drosophila*. Development *124*, 3197–3207.

Nilson, L.A., and Schüpbach, T. (1998). Localized requirements for *windbeutel* and *pipe* reveal a dorsoventral prepattern within the follicular epithelium of the *Drosophila* ovary. Cell *93*, 253–262.

Norvell, A., Kelley, R.L., Wehr, K., and Schüpbach, T. (1999). Specific isoforms of squid, a *Drosophila* hnRNP, perform distinct roles in Gurken localization during oogenesis. Genes Dev. *13*, 864–876.

Palacios, I., and St Johnston, D. (2001). Getting the message across: the intracellular localisation of mRNAs in higher eukaryotes. Annu. Rev. Cell Dev. Biol. *17*, 569–614.

Palacios, I.M., and St Johnston, D. (2002). Kinesin light chain-independent function of the Kinesin heavy chain in cytoplasmic streaming, and posterior localization in the *Drosophila* oocyte. Development *129*, 5473–5485.

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

Piñol-Roma, S., and Dreyfuss, G. (1992). Shuttling of pre-mRNAbinding proteins between nucleus and cytoplasm. Nature *355*, 730–732.

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

Schüpbach, T., and Wieschaus, E. (1986). Maternal-effect mutations altering the anterior-posterior pattern of the *Drosophila* embryo. Rouxs Arch. Dev. Biol. *195*, 302–317.

Schüpbach, T., and Wieschaus, E. (1991). Female sterile mutations on the second chromosome of *Drosophila melanogaster*. II. Mutations blocking oogenesis or altering egg morphology. Genetics *129*, 1119–1136.

Serano, T.L., and Cohen, R.S. (1995). Gratuitous messenger RNA localization in the *Drosophila* oocyte. Development *121*, 3013–3021.

Shulman, J.M., Benton, R., and St. Johnston, D. (2000). The *Drosophila* homolog of *C. elegans* PAR-1 organizes the oocyte cytoskeleton and directs *oskar* mRNA localisation to the posterior pole. Cell *101*, 1–20.

Siebel, C.W., Kanaar, R., and Rio, D.C. (1994). Regulation of tissue-specific P-element pre-mRNA splicing requires the RNA-binding protein PSI. Genes Dev. *8*, 1713–1725.

Spradling, A.C., Stern, D., Beaton, A., Rhem, E.J., Laverty, T., Mozden, N., Misra, S., and Rubin, G.M. (1999). The Berkeley *Drosophila* Genome Project gene disruption project: Single *P*-element insertions mutating 25% of vital *Drosophila* genes. Genetics *153*, 135–177.

St Johnston, D., Driever, W., Berleth, T., Richstein, S., and Nüsslein-Volhard, C. (1989). Multiple steps in the localization of *bicoid* RNA to the anterior pole of the *Drosophila* oocyte. Dev. Suppl. *107*, 13–19.

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

St Johnston, D., Brown, N.H., Gall, J.G., and Jantsch, M. (1992). A conserved double-stranded RNA-binding domain. Proc. Natl. Acad. Sci. USA *89*, 10979–10983.

Takizawa, P.A., and Vale, R.D. (2000). The myosin motor, Myo4p, binds Ash1 mRNA via the adapter protein, She3p. Proc. Natl. Acad. Sci. USA *97*, 5273–5278.

Theurkauf, W.E. (1994). Premature microtubule-dependent cytoplasmic streaming in *cappuccino* and *spire* mutant oocytes. Science *265*, 2093–2096.

Tiruchinapalli, D.M., Oleynikov, Y., Kelic, S., Shenoy, S.M., Hartley, A., Stanton, P.K., Singer, R.H., and Bassell, G.J. (2003). Activity-dependent trafficking and dynamic localization of zipcode binding protein 1 and β-actin mRNA in dendrites and spines of hippocampal neurons. J. Neurosci. *23*, 3251–3261.

van Eeden, F.J.M., Palacios, I.M., Petronczki, M., Weston, M.J.D., and St Johnston, D. (2001). Barentsz is essential for the posterior localization of *oskar* mRNA and colocalizes with it to the posterior. J. Cell Biol. *154*, 511–524.

Wagner, C., Palacios, I., Jaeger, L., St Johnston, D., Ehresmann, B., Ehresmann, C., and Brunel, C. (2001). Dimerization of the 3′UTR of bicoid mRNA involves a two-step mechanism. J. Mol. Biol. *313*, 511–524.

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

Wilkie, G.S., and Davis, I. (2001). *Drosophila wingless* and pair-rule transcripts localise apically by dynein-mediated transport of RNA particles. Cell *105*, 209–219.

Wilson, E., Connell, J.E., and Macdonald, P.M. (1996). Aubergine enhances *oskar* translation in the *Drosophila* ovary. Development *122*, 1631–1639.

Xu, T., and Rubin, G. (1993). Analysis of genetic mosaics in developing an adult *Drosphila* tissues. Development *117*, 1223–1237.

Yano, T., López de Quinto, S., Matsui, Y., Shevchenko, A., Shevchenko, A., and Ephrussi, A. (2004). Hrp48, a *Drosophila* HnRNP A/B homolog, binds and regulates translation of *oskar* mRNA. Dev. Cell *6*, this issue, 637–648.