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The *Drosophila* hnRNPA/B homologue, Hrp48, is specifically required for a novel step in *osk* mRNA localisation

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Abstract

The Staufen-dependent localisation of *oskar* mRNA to the posterior of the *Drosophila* oocyte induces the formation of the pole plasm, which contains the abdominal and germline determinants. To identify essential genes required for this process, we performed a germline clone screen for mutations that disrupt the posterior localisation of GFP-Staufen, and isolated three missense alleles in the hnRNPA/B homologue, Hrp48. These mutants specifically abolish *osk* mRNA localisation, without affecting its translational control or splicing, or the localisation of *bicoid* and *gurken* mRNAs and the organisation of the microtubule cytoskeleton. Furthermore, Hrp48 co-localises with *osk* mRNA throughout oogenesis, and binds directly to its 5′ and 3′ regulatory regions. Thus, Hrp48 is the first example of a trans-acting factor that binds to *oskar* mRNA to mediate its posterior transport. The *hrp48* alleles cause a different defect in *oskar* mRNA localisation from all other mutants, and disrupt the formation of GFP-Staufen particles. This defines a novel step in the localisation pathway, which may correspond to the assembly of Staufen/*oskar* mRNA transport particles.

Introduction

The intracellular localisation of mRNA is a common mechanism for targeting proteins to specific regions within cells, and plays an important role in establishing cellular asymmetries (Palacios, 2002; Kloc et al, 2002). For example, the localisation of *ASH1* mRNA to the bud tip in *Saccharomyces cerevisiae* leads to the asymmetric inheritance of mating type switching, while β-actin mRNA localises to the leading edge of chicken embryonic fibroblasts, where it is required for polarized cell migration (Jansen et al., 1996; Long et al., 1997; Takizawa et al., 1997). Localised mRNAs in oocytes and early embryos also play an essential role in axis formation, by functioning as localised cytoplasmic determinants that control the patterning of the embryo. This has been best characterised in *Drosophila*, where the two main body axes of the embryo are determined by the localisation of three maternal mRNAs. *gurken* mRNA localises to the dorsal-anterior corner of the oocyte, where Gurken protein signals to induce a subset of follicle cells to adopt a dorsal fate, thereby defining the dorsal-ventral axis of the embryo (Neuman-Silberberg and Schüpbach, 1993; Nilson and Schüpbach, 1998). *bicoid* mRNA localises to the anterior of the oocyte, and encodes the morphogen that patterns the head and thorax of the embryo (St Johnston et al., 1989; Driever, 1993). Finally, the localisation of *oskar* (*osk*) mRNA to the posterior of the oocyte specifies where the pole plasm forms, which contains the abdominal and germline determinants that pattern the posterior of the embryo (Ephrussi et al., 1991; Kim-Ha et al., 1991; Ephrussi and Lehmann, 1992).

mRNAs can be localised by a variety of mechanisms, but this often thought to occur via active transport along the cytoskeleton, although this has only been convincingly proven in a handful of cases. *ASH1* mRNA is transported along actin cables into the bud tip by the type V myosin, Myo4p (Bertrand et al., 1998; Beach et al., 1999; 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 localisation of *gurken* mRNA in the oocyte (Wilkie and Davis, 2001; MacDougall et al., 2003). Another transcript that is likely to be localised by

active transport is Myelin Basic protein mRNA, which has been observed to move from the cell body towards 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 line of evidence suggest that *osk* mRNA is also transported by kinesin towards MT plus ends. 1) Posterior localisation is microtubule-dependent, since it is disrupted by MT-depolymerising drugs, and by mutations that alter the polarised arrangement of MT in the oocyte (Clark et al., 1994; Theurkauf, 1994; González-Reyes et al., 1995; Roth et al., 1995; Shulman et al., 2000; Martin and St Johnston, 2003). 2) Localisation is presumably directed towards the plus ends of MT, because the plus end marker, kinesin-bgal co-localises with *osk* mRNA at the posterior pole (Clark et al., 1994). 3) *osk* mRNA is not localised in mutants for the kinesin heavy chain, and this motor protein localises with the mRNA to the posterior pole of wildtype oocytes (Brendza et al., 2000; Duncan and Warrior, 2002; Palacios and St Johnston, 2002).

For an mRNA to be localised, the cis-acting localisation elements within the RNA must be recognised by trans-acting factors that couple it to the motors that transport it. A number of such factors required for *osk* mRNA localisation have been identified in maternal screens, as mutants that disrupt the posterior localisation of *osk* mRNA, without affecting the polarity of the oocyte or the organisation of the MT cytoskeleton. The best characterised of these is the dsRNA-binding protein, Staufen (Stau), which co-localises with *osk* mRNA throughout oogenesis, and forms a crescent with the RNA at the posterior pole of the oocyte (St Johnston et al., 1991; St Johnston et al., 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 instead at the anterior of the oocyte (Ephrussi et al., 1991; Kim-Ha et al., 1991). Furthermore, the posterior localisation of Stau requires *osk* mRNA, and depends on its RNA-binding activity, strongly suggesting that Stau binds directly to the mRNA to mediate its posterior transport (Ferrandon et al., 1994; Ramos et al., 2000). A very similar phenotype is seen in hypomorphic mutants of *mago nashi* and *y14/tsunagi* and null alleles of *barentsz*, and all three proteins transiently co-localise with *osk* mRNA at the posterior of the oocyte, indicating that they are also components of the *osk* mRNA localisation

complex (Newmark and Boswell, 1994; Hachet and Ephrussi, 2001; Mohr et al., 2001; van Eeden et al., 2001). Mago and Y14 associate with each other and are part of the exon-exon junction complex that marks where introns have been excised (Kataoka et al., 2001; Le Hir et al., 2001). This suggests that the Mago/Y14 complex is loaded onto *osk* mRNA during splicing in the nucleus to control its localisation in the oocyte cytoplasm. Thus, Stau, Mago, Y14 and Btz are thought to play a direct role in coupling *osk* RNA to the factors that localise it, such as kinesin. None of these proteins have been shown to bind specifically to any sequence elements within *osk* mRNA, however, and it is unclear how the RNA is recognised.

An alternative strategy to identify proteins required for mRNA localisation is to screen for proteins that bind directly to the RNA in uv cross-linking assays, and this has led to the identification of four proteins that bind to specific regions of *osk* RNA. Surprisingly, however, all of these proteins have been implicated in the translational control of *osk* mRNA, rather than its localisation. Bruno binds to three regions of the *osk* 3'UTR, called Bruno Response Elements (BREs), and mutations in the BREs that disrupt Bruno binding allow the premature translation of *osk* mRNA before it has been localised (Kim-Ha et al., 1995; Webster et al., 1997). The ectopic Osk protein produced causes a bicaudal phenotype in the resulting embryos, in which abdominal structures form in place of the head and thorax, highlighting the importance of spatially regulating Osk translation. 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 cross-linked to the BREs, and mutations that disrupt the binding of p50, but not Bruno, also lead to premature translation (Gunkel et al., 1998). Interestingly, 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 been localised to the posterior pole. This has led to the proposal that interactions between the 5' and 3' ends of *osk* mRNA, which could be mediated by p50, are important for translational control.

Although genetic screens for maternal-effect mutations have proved an effective way to identify proteins required for *osk* mRNA localisation, these can only recover homozygous viable mutants, and would therefore miss any lethal mutations in essential genes. To circumvent this problem, we have 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 localisation of GFP-Stau (Chou and Perrimon, 1996; Martin et al., 2003). Since Stau localises to the posterior of the oocyte with *osk* mRNA, and in an *osk* mRNA-dependent manner, these screens can identify new mutants that disrupt *osk* mRNA localisation, and have two advantages over previous approaches. First, because only the germ line is homozygous, one can recover lethal alleles of genes that are also required at other stages of development. Second, by screening living oocytes for defects in the distribution of GFP-Stau, one can find mutations that could not be identified on the basis of their cuticular phenotypes, because they produce no fertilised eggs or die as early embryos. Here we report the identification of three missense alleles of Hrp48 in such a screen, which reveal a very specific function of this general HnRNP protein in *osk* mRNA localisation. Furthermore, these mutants cause a novel defect in *osk* mRNA localisation, suggesting that Hrp48 acts at a new step in the localisation pathway.

Results

Identification of a novel locus required for the posterior localisation of Stau.

In a germline clone screen of 4,331 EMS mutagenised lines, we identified 146 mutants on chromosome arm 2L with defects in GFP-Stau localisation within the oocyte. These include 3 new alleles of *cappuccino*, 4 alleles of *spire* and 4 alleles of *aubergine*, which are genes known to disrupt *osk* RNA localisation or anchoring (Manseau and Schüpbach, 1989; Wilson et al., 1996; Harris and Macdonald, 2001). Many of the other mutants cause other defects in the oocyte at stage 9, indicating that they disrupt more general functions, such as organisation of the cytoskeleton or cell polarity. However, a small number

specifically disrupt GFP-Stau localisation, including one lethal complementation group of three alleles , 5A2-6, 7E7-18 and 10B2-9, 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 localised throughout the oocyte cytoplasm at stage 9-10, and never localises to the posterior pole, as it does in wildtype (Fig 1A, B). These two alleles also share a second phenotype that is not observed in any other mutants that disrupt *osk* mRNA localisation. In wildtype ovaries, a significant proportion of GFP-Stau localises to large particles in the cytoplasm of the nurse cells, and smaller particles in the oocyte cytoplasm. These 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 somewhat weaker phenotype, since a small amount of GFP-Stau can sometimes be detected at the posterior of the oocyte at stage 9, and the cytoplasmic GFP-Stau particles are only mildly affected (data not shown).

All three *linha* mutations have the same effect on *osk* mRNA localisation as they do on Stau. The mRNA still accumulates in the oocyte during the early stages of oogenesis, but never shows any localisation at the posterior pole (Fig 1C, D). This indicates that these mutants have no effect on the transport of *osk*mRNA and Stau protein from the nurse cells into the oocyte, and specifically disrupt their movement within the oocyte to the posterior pole. In other *osk* mRNA localisation mutants, such as *mago nashi*, *y14*, *barentsz*, *stau* and *TmII*, *osk* mRNA shows a transient accumulation at the anterior of the oocyte (Ephrussi et al., 1991; Kim-Ha et al., 1991; Newmark and Boswell, 1994; Erdélyi et al., 1995; van Eeden et al., 2001). 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 labelling of the actin cytoskeleton reveals no other obvious defects until stage 11, when all three alleles share a second phenotype. In wild type stage 11 egg chambers, the nurse cells contract and

expel their cytoplasm into the oocyte, in a process called nurse cell dumping (Fig. 1E)(Spradling, 1993). This fails to occur in mutant germline clones, resulting in the formation of very small eggs, which are usually not laid (Fig. 1F). As a consequence of this dumpless phenotype, the eggs are never fertilised, and we have therefore been unable to analyse the effects of the mutations on embryonic patterning.

The ovoD technique cannot be used to look at phenotypes early in oogenesis, because ovoD egg chambers develop as far as stage 6, and cannot be distinguished from the homozygous mutant germline clones. We therefore generated *linha* mutant clones that marked by the loss of GFP, using an FRT nls- GFP chromosome (Luschnig et al., 2000). These clones show a wildtype accumulation of *osk* mRNA into the early oocyte, and display no other defects at these stages (Fig 1G). Furthermore, Stau immunostaining and labelling 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 localise *osk* mRNA and Stau at the posterior of the oocyte is not a consequence of an earlier defect.

linha mutations disrupt *hrp48*, which encodes a *Drosophila* A/B type hnRNP.

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 ~20kb interval in 27C4, and we therefore tested lethal P-elements inserted in the region for non-complementation of our alleles. The 7E7-18 and 10B2-9 alleles are lethal over 4 P-elements, which 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 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 (Fig. 2A) (Hammond et al., 1997). Thus, the *linha* mutations are

alleles of *hrp48*, which encodes one of the three most abundant hnRNP (heterogeneous nuclear RiboNucleoProteins) proteins 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). Sequencing of the *linha* alleles revealed that each contains a missense mutation in the *hrp48* coding region. $linha^{5A2\text{-}6}$ is a G to A transition that changes amino acid 101 from Glycine to Aspartic acid (Fig. 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 (Fig.2A).

To examine the affect of these mutations on Hrp48 protein levels, we raised a polyclonal antibody against two peptides from the C-terminus of the protein. This antibody recognises a protein doublet at 48-50kD on western blots that is identical to the doublet seen with an anti-Hrp48 monoclonal antibody (Fig. 2B) (Matunis et al., 1992b; Siebel et al., 1994). In addition, the signal could be specifically blocked by the addition of the excess peptide antigen, indicating that the antibody is specific for Hrp48. Western blots on extracts from wildtype 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 wildtype (Fig. 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 (Piñol-Roma and Dreyfuss, 1992; Dreyfuss et al., 1993; Matunis et al., 1993). Since this suggested that Hrp48 might play a general role in mRNA localisation, we examined whether the *hrp48*missense mutants alter the distribution of other mRNAs that are localised within the oocyte. In contrast to *osk* mRNA, *bcd* and *gurken* mRNAs are localised normally to the

anterior cortex and to the dorsal/ anterior margin of the oocyte in *hrp48*linha mutant germline clones (Fig 3A-D). *gurken* mRNA translation also appears to be normal in these mutants, as Gurken protein localisation is indistinguishable from wild type (Fig. 3E, F).

Although Hrp48 is an RNA-binding protein, its role in *osk* mRNA localisation could be indirect, and be mediated through an effect on the organisation of the microtubules. Indeed, the primary cause of the failure to localise *osk* mRNA in mutants in the RNA helicase, Spn-E, and the RNA-binding protein, Orb, is a disorganisation of the oocyte microtubule cytoskeleton that leads to premature cytoplasmic streaming (Martin et al., 2003). To investigate if the microtubule cytoskeleton is affected in *hrp48*linha germline clones, we examined the localisation of the Kinesin-ß-Galactosidase fusion protein, which marks the plus ends of microtubules (Clark et al., 1994). In wildtype ovaries, Kin-Bgal localises to the posterior cortex of the oocyte at stage 9, and an identical localisation is observed in mutant germline clones (Fig 4A-F). The overall organisation of the microtubule cytoskeleton is also indistinguishable from wildtype, as revealed by the distribution of the microtubule-binding protein, tau-GFP, in living oocytes (Fig 4G, H) (Micklem et al., 1997). Furthermore, time lapse films of mutant egg chambers show the normal slow and chaotic pattern of cytoplasmic streaming in the oocyte, in contrast to the rapid circular streaming observed in *spn-E*, *orb*, *cappuccino* and *spire* mutants (data not shown) (Theurkauf, 1994; Martin et al., 2003). Thus, these missense alleles of *hrp48* specifically disrupt the localisation of *osk* mRNA, and have no effect on the polarisation of the oocyte or the organisation of the microtubule cytoskeleton.

osk and *Ubx* mRNA splicing arenot affected in *hrp48*^{linha} mutant ovaries.

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 (Siebel et al., 1994; Hammond et al., 1997; Burnette et al., 1999). The localisation of *osk* mRNA is also likely to require splicing of the pre-mRNA, since it depends on the Exon-exon junction complex components, Mago nashi

and Y14, which mark where the introns have been removed (Hachet and Ephrussi, 2001; Mohr et al., 2001). To test if the *osk* mRNA localisation 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 (Fig. 5B). Hrp48 is therefore either not required for *osk*mRNA splicing, or retains its splicing activity in the $hrp48^{linha}$ mutations. To test this possibility, we analysed the ratio of Ubx isoforms, a known target of Hrp48 splicing activity (Burnette et al., 1999). We found that L3 larvae heterozygous for any of the $hrp48^{linha}$ alleles present the same pattern of Ubx isoforms as wild type L3 larvae (Figure 5C). We further checked if the ratio of isofoms was changed in larvae homozygous for any of the $hrp48^{linha}$ alleles and found no difference (Figure 5C). These results were confirmed by quantification of the RT-PCR (Figure 5D). Thus, these data indicate that the $hrp48^{linha}$ alleles do not affect Hrp48 splicing activity and suggest a direct role for Hrp48 in *oskar* mRNA localisation.

Hrp48 accumulates early into the oocyte and localises to the posterior of the oocyte with *osk* mRNA.

To investigate the sub-cellular localisation of Hrp48 during oogenesis, we used our affinity purified polyclonal antibody, and an independent rabbit polyclonal antibody (Siebel et al., 1994). Hrp48 is expressed throughout oogenesis in the nucleus and cytoplasm of the germline and somatic cells, but its cytoplasmic localisation shows striking asymmetries in the germline (Fig. 6A-C). Firstly, 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 polarised on entering region 3 of the germarium (Fig. 6A). (Huynh et al., 2001). This localisation at the posterior of the oocyte persists during stage 1-6 of oogenesis, but disappears when the oocyte repolarises at stage 7. Secondly, Hrp48 accumulates in a crescent at the posterior pole of the oocyte at stage 9, where it remains until at least stage 10b (Fig. 6C). This posterior crescent of Hrp48 does not form in germline clones of the *hrp48* missense alleles (data not shown). The nuclear localisation of Hrp48 is

unaffected by these mutations, however, consistent with the fact that these alleles are not protein nulls.

The localisation of Hrp48 is very similar to that of *osk* mRNA, suggesting that it is associates with the mRNA and is transported with it to the posterior pole. Two lines of evidence support this view. Firstly, Hrp 48 co-localises at the posterior with other components of the *osk* mRNA localisation complex, such as Y14 (Fig 6E-G). Secondly, Hrp48 does not form a posterior crescent in mutants that disrupt *osk* mRNA localisation, such as $stau^{D3}$, although it still localises to the oocyte at early stages (Fig. 6B,D).

Hrp48 is p50 and binds to the 5′ region and 3′UTR of *osk* mRNA.

The results above indicate that Hrp48 is required *osk* mRNA localisation and co-localises with it to the posterior pole, raising the possibility that it binds directly to the mRNA to mediate its transport to the posterior. A protein of similar molecular weight, called p50, has previously been shown in uv-crosslinking assays 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 (Fig. 7A) (Gunkel et al., 1998). The major band that cross-links 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 (Fig. 7B). The accompanying manuscript from Tomaka et al demonstrates that this is indeed the case, since peptide sequences from purified p50 correspond to regions of Hrp48, and anti-Hrp48 antibodies immunoprecipitate the p50/*osk* RNA complex.

We next examined whether any of the *hrp48* missense alleles disrupt the binding Hrp48 to *osk* mRNA, by performing uv cross-linking assays with extracts from homozygous mutant larvae. All three mutant Hrp48 proteins efficiently cross-linked to radio-labelled RNAs containing the p50 binding sites in *osk* mRNA, and they showed a similar specificity to wildtype Hrp48 for the sense strand of the RNAs over the antisense RNA controls (Fig. 7B, and data not shown). We also performed RNA-affinity assays by incubating wildtype 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 co-purified with each RNA as efficiently as the wildtype protein, indicating that the RNA-binding specificity of HRP48 is not affected by these missense mutations (Fig. 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 localised to the posterior(Gunkel et al., 1998). Since this indicates that Hrp48 is required to repress the translation of *osk* mRNA until it is localised, we examined whether *hrp48* missense mutations disrupt repression and allow the translation of the unlocalised mRNA. However, we could not detect any Osk protein in Western blots or immunostainings of mutant germline clones (Fig. 7D, 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 localisation, and co-localise with it to the posterior pole, strongly suggesting that these proteins are components of the mRNA localisation complex (St Johnston et al., 1991; Newmark et al., 1997; Hachet and Ephrussi, 2001; Mohr et al., 2001; 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 a different set of proteins that bind to specific sequences in *osk* RNA, including Bruno, Apontic, p68, and p50 (Kim-Ha et al., 1995; Webster et al., 1997; Gunkel et al., 1998; Lie and Macdonald, 1999). The functions of these proteins have been difficult to analyse, either because the identity of the protein is not known, or because the existing alleles disrupt oogenesis at an early stage. Nevertheless, indirect experiments have

implicated all four proteins in the regulation of *osk* mRNA translation, but not in the localisation of the mRNA. Our results and those of Tomaka et al provide the first 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 pole of the oocyte, as well as for translational control. Germline clones of the three missense alleles of *hrp48* have no effect on the polarity of the oocyte, the organisation of the microtubules, or the localisation of *bicoid* or *gurken* mRNAs, but completely abolish the posterior localisation of *osk* mRNA.

Since Hrp48 has been shown to regulate alternative splicing, it is possible that the *oskar* mRNA localisation 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 *oskar* mRNA transport. However, several lines of evidence indicate that this is very unlikely to be the case. Firstly, these missense alleles have no effect on the alternative splicing of either the P element or Ubx transcripts. Since the insertion alleles of Hrp48 do disrupt the splicing pattern of these RNAs, the missense alleles do not appear to impair the function of Hrp48 in splicing regulation. Secondly, the $hrp48^{linha}$ mutations cause a different defect in *oskar* mRNA localisation from all other known mutations, and there are therefore no candidates RNA targets of Hrp48 splicing regulation that could account for the phenotype. Finally, Hrp48 binds to sequences in the 5' region and the 3'UTR of *osk* mRNA, and co-localises with the mRNA at the posterior. This strongly suggests that the requirement for Hrp48 in *oskar* mRNA localisation is direct, and that it functions as an essential trans-acting factor that recognises the RNA and plays a role in coupling it to the localisation machinery.

The observation that *hrp48* missense alleles produce a different defect in *oskar* mRNA localisation form all other known mutants suggest that it acts at distinct step in the localisation pathway. In *stau*, *barentsz*, *mago nashi*, *tsunagi*/*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; Kim-Ha et al., 1991; Newmark and Boswell, 1994; Erdélyi et al., 1995; van Eeden et al., 2001). Since *osk* mRNA shows a transient accumulation at the

anterior in wildtype before it localises 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 do not detect any 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 localisation, such as Stau. One argument against this interpretation is that the localisation to the anterior of the oocyte is thought to be a by-product of the transport from the nurse cells into the oocyte, which occurs normally in the *hrp48* missense alleles (Serano and Cohen, 1995). In support of this view, all mRNAs that are transported into the oocyte also localise, at least transiently, to the anterior cortex at stage 9. We therefore favour an alternative model in which Hrp48 acts downstream of Stau, Mago, Barentsz 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, such as kinesin. One should be able to distinguish between these models by examining the distribution of *osk* mRNA in egg chambers that are doubly mutant for *hrp48* and one of the *stau* class of mutants, but this has so far proved impossible, because the appropriate stocks are lethal.

Many localised mRNAs appear to move as large cytoplasmic particles, leading to the suggestion that they need to be packaged into transport granules in order to be localised. 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 behaviour 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 visualised by labelling the mRNA-binding proteins that are required for localisation of the mRNA. For example, injected *bicoid* mRNA recruits Stau into motile particles in *Drosophila* syncytial blastoderm embryos, while GFP-

tagged mouse Stau1 and ZBP-1 have been observed to form particles that move along microtubules in neuronal processes (Köhrmann et al., 1999; Wagner et al., 2001; Tiruchinapalli et al., 2003). 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, and this may account for the failure to localise the mRNA to the posterior pole in these mutants. If these particles sequester the mRNA and prevent its diffusion, this could 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 (Matunis et al., 1992a; Matunis et al., 1992b; Kelley, 1993; Matunis et al., 1993). It is therefore surprising to recover *hrp48* mutations that have such a specific effect on the localisation of *osk* mRNA. The accompanying manuscript by Tomaka et al reports that P-element insertions in *hrp48* produce a distinct phenotype. Although *osk*mRNA is often not localised to the posterior, it is sometimes found in the centre of the oocyte, which is indicative of a defect in oocyte polarity. Consistent with this, a marker for the plus ends of the microtubules, kinesin-Bgal, also localises to the centre of the oocyte in these mutants, whereas it is always shows a wildtype posterior localisation in the missense alleles. Thus, the P-element insertions presumably disrupt the regulation of another mRNA that is required for the polarisation of the oocyte microtubule network, and this may reflect the fact that these alleles, but not the missense alleles, disrupt alternative splicing. A second important difference is that the P-element alleles also 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 unlocalised, but no Osk protein is produced. Finally, P alleles of Hrp48 also affect *gurken* mRNA localisation and translation (Goodrich, Clouse, and Schüpbach, pers. comm.), whereas we observed no defects in the distribution of either *gurken* mRNA or protein in germline clones of the missense alleles. 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 have been shown to produce reduced levels of wildtype 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 localisation. 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 other HnRNPA/B family members suggests that this region functions as an oligomerisation 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 oligomerises, 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 localisation because Hrp48 oligomerisation 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 localisation. 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 wildtype 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 localisation have not been mapped, and these sites could be distinct from those involved in translational repression. Thus, one possibility is that this mutation only disrupts Hrp48 binding to a specific subset of its target sites, including unidentified sites in *osk* mRNA that are

required for localisation. 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 localisation machinery.

A conserved role for hnRNPA/B proteins in mRNA localisation and translation

Two other members of the hnRNP A/B family have also been implicated in mRNA localisation. *Drosophila* Squid, which is most closely related to hnRNPA1, binds directly to *gurken* mRNA, and is required for its localisation to the anterior-dorsal corner of the oocyte (Kelley, 1993; Norvell et al., 1999). As is the case for *osk* mRNA, this localisation is microtubule-dependent, and is disrupted by mutations in kinesin (Brendza et al., 2002; Januschke et al., 2002; MacDougall et al., 2003). Squid is also required to repress the translation of unlocalised *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 homologues to *Drosophila*Hrp48, plays a very comparable role in the localisation of MBP mRNA in mammalian 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, since localisation is inhibited by antisense oligonucleotides directed against the kinesin heavy chain(Ainger et al., 1993; Carson et al., 1997). This localisation requires the specific binding of hnRNP A2 to a 21nt ARE2 element in the MBP 3'UTR that is necessary and sufficient for localisation and efficient translation, and antisense depletion of hnRNPA2 inhibits MBP mRNA localisation (Hoek et al., 1998; Munro et al., 1999). In addition, recent work has shown that the microtubule-dependent localisation 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, which disrupts *osk* mRNA localisation without affecting RNA-binding, falls in the equivalent domain of the *Drosophila* protein. Thus, there are striking

parallels between the functions of Hrp48, Squid and hnRNP A2 in mRNA localisation and translational control, suggesting that these hnRNP A/B proteins play a conserved role in these processes in flies and mammals.

The nuclear history of *osk* mRNA determines its localisation 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 localisation 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). For example, the transport of ASH1 mRNA to the bud tip in S. cerevisiae requires the binding of She2p and Loc1p in the nucleus (Long et al., 2001; Kruse et al., 2002); one of the factors necessary for the localisation of Vg1 mRNA to vegetal pole of the *Xenopus* oocyte, Vg1RBP60, is the homologue of the mammalian nuclear RNA-binding protein hnRNPI/PTB (Cote et al., 1999); while the nuclear shuttling protein, ZBP-2/KSRP binds to the localisation element of β-actin mRNA, and has been implicated in the cytoplasmic localisation of the mRNA in chicken fibroblasts and neurons(Gu et al., 2002). In the case of *osk* mRNA, cytoplasmic localisation seems to require the binding of at least three distinct proteins in the nucleus; Hrp48, which probably associates with the RNA co-transcriptionally, and Mago and Y14, which are recruited to the RNA during splicing as part of the exon-exon junction complex. The mRNA must then recruit additional essential localisation factors in the cytoplasm, such as Stau and Barentsz proteins, before it is competent to localise to the posterior pole of the oocyte. Thus, the assembly of a functional *osk* RNA localisation complex is a complicated process that requires the stepwise recruitment of multiple nuclear and cytoplasmic proteins, all of which are essential for posterior localisation. 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 kinesin motor that presumably mediates its transport to the posterior pole.

Methods and Materials.

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 localisation of GFP-Stau, which was performed using the FRT 40A and FRT40A ovoD chromosomes (Chou and Perrimon, 1996), following the procedure described in Martin et al (2003b). 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. SNPs were identified by amplifying 200-300 bp DNA fragments from intergenic regions by PCR , and running the fragments on SSCP gels (Amersham) (Martin et al., 2001). Bands that appeared different between the two genotypes were confirmed by sequencing. We found 41 SNPs, of which 18 were also RLFPs, with an average interval between 2 SNPs of 500 kb.

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 analysing 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, which were identified by the absence of the CyO GFP Balancer.

Fly stocks

We used the following stocks for the genetic screen:
w-; P[ry+; hsp70:neo; FRT40A] (Xu and Rubin, 1993),
w-; FRT[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) P[w+; *kinesin-LacZ* (KZ2030)] (Clark et al., 1994) transgenes was transposed onto the *y*, *w,* P[ry+; hsFLP] chromosome to examine the microtubule organisation in *hrp48* mutant germline clones.

We also used the following mutant alleles:
$stau^{D3}$(Schüpbach and Wieschaus, 1986); $cappuccino^{RK}$; $spire^{R9}$(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 all P insertions in *hrp48*.

Hrp48 Antibody production

Polyclonal antibodies were raised against Hrp48 by immunising Rabbit with two peptides; 1: N-CRTGPGNSASKSGSEY-C and 2: N-EGASNYGAGPRSAYGNC-C, which correspond to non-conserved regions in the C-terminal glycine rich domain. The serum was then affinity purified on peptide columns following standard procedures.
To determine Hrp48 protein levels in the *linha* mutants, homozygous larvae were homogenised in squishing buffer (25 mM HEPES (pH7.5), 400 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 10% Glycerol and diluted *Complete*™ protease inhibitor cocktail (Roche)). Equal amounts of protein were loaded onto 10% SDS PAGE and Western blotted. The membranes were probed with a 1:3000 dilution of the Rabbit anti-Hrp48, and a monoclonal anti-tubulin antibody (clone DM1A) as a loading control (Sigma).

Western blot and RT-PCR analysis of *osk*.

For Western analysis, ovaries were dissected in PBT and directly transferred into a boiling C buffer (0.125 M Tris[pH 6,8], 2% SDS, 4 M urea, 5% β-mercaptoethanol) and frozen at –20°C. 1X Laemli buffer was added before loading onto a 8% SDS-polyacrylamide gels, and western blotting. The blots were probed with rabbit anti-Osk antibody at 1/20 000 (Markussen et al., 1995) or rabbit anti-MBF1 at 1/20 000 as a loading control.
For RT-PCR analysis, ovaries were dissected into PBT and frozen at –80°C. 80 μg of wildtype or mutant ovaries were homogenised into 160 μl of Trizol® reagent solution and RNA extracted as per manufacturers instructions (Invitrogen). Reverse Transcription was performed using the AMV RT-PCR kit (Roche).

Immunostainings and in situ *hybridisation*

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 visualise the actin cytoskeleton (Molecular Probes).
In situ hybridisations were performed as described in (González-Reyes et al., 1995) using RNA probes labelled with dig-UTP (Roche). Fluorescent in situ for *oskar* mRNA were done using a directly labelled probe (see Trent) or a TSA-plus Cy3 system (NEN).

UV crosslinking assay.

The UV crosslinking assays were performed using Method 2 of Gunkel et al (1998), as this optimises 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 wildtype Hrp48 protein in the follicle cells. Homozygous mutant larvae were placed on ice and homogenised with a plastic pipette pestle with three volumes of 25 mM HEPES (pH7.5), 400 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 10% Glycerol and diluted *Complete*™ protease inhibitor cocktail (Roche)). ^{32}P (Cytidine 5'-

[a-32P] triphosphate, Amersham) labelled 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, 1995 #20} using the Promega Riboprobe® system.

RNA affinity pulldown assays.

RNA was transcribed in the presence of biotin-16-UTP using the Biotin RNA labelling mix (Roche) and 20 U of RNAsin (Promega). Unincorporated nucleotides were removed with Sephadex G50 mini spin columns (Roche) and the RNA analysed as above. Streptavidin magnetic particles (Roche) were prepared by washing once with Buffer 1(100 mM NaOH, 100 mM NaCl), twice with Buffer 2 (100 mM NaCl) and twice with RNA binding buffer (10 mM Tris-HCl,100 mM NaCl, 1 mM EDTA, pH7.5). An equal amount of biotinylated RNA was then bound to 40 µl of washed beads in a final volume of 250 µl per binding reaction for 20 min at room temperature. Unbound RNA was subsequently removed by three washes with Buffer 3 (10 mM 10 mM Tris-HCl, 1 M NaCl, 1 mM EDTA, pH7.5) 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 prepared as above were diluted to a final volume of 100 µl with Interaction buffer and incubated with the beads prepared as above for 1 hour rotating at 4 C. The magnetic particles were then washed five times for five minutes each with interaction buffer. Bound proteins were eluted with 60 µl of SDS PAGE loading buffer (0.5 M Tris-HCl, 10% (w/v) SDS, 2-mercaptoethanol, 0.05% (w/v) bromophenol blue, 10% Glycerol, pH 6.8) by boiling for 5 min. The eluted proteins 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; and Antoine Guichet, the Guichet lab and the Lepesant Lab for helpful discussions. This work was supported by an European Union Marie Curie Fellowship and EMBO fellowship (J.R.H.), a Human Frontiers

Programme Collaborative Grant (T. P. M.) and by the Wellcome Trust (K.S-L.and D. St J.).

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Figure Legends

Figure 1: *linha* mutations abolish the posterior localisation of Stau and *osk mRNA*

(A) Wildtype egg chambers, showing the accumulation of GFP-Stau (Green) in the oocyte at stage 6 (inset) and its localisation 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 localise to the posterior. The particles of GFP-Stafeun in the nurse cell cytoplasm are also strongly reduced in size and frequency and are absent from the oocyte.

(C) and **(C')** *osk* mRNA localisation to the posterior of a wildtype stage 10 oocyte.

(D) and **(D')** *osk* mRNA localisation in a *linha* GLC.

(E) A wildtype stage 11egg 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) Chimaeric 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).

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 non-coding exons are shown in grey 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 48-50kD.

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

Figure 3: *hrp48* missense mutations do not affect the localisation of *bicoid* or *gurken* mRNAs.

(A, B) *In situ* hybridisations for *bcd* mRNA in wild type (A) and *hrp48* GLC mutant egg chambers (B), *bcd* mRNA localises normally at the anterior of the oocyte.
(C, D *In situ* hybridisation for *grk* mRNA in wild type (C) and *hrp48* mutant egg chambers(D). *grk* mRNA is localised normally to the dorsal-ventral corner of the oocyte, above the nucleus.
(D, E), Gurken protein (red) is correctly localised 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 organisation.

(A-C) Wild type stage 9 egg chamber, showing the posterior localisation of GFP-Stau (A; green) and Kinesin-βgal (B; red). The merge of the two images is shown in (C)
(D-F) An *hrp48* GLC. GFP-Stau (D) is not localised to the posterior, but Kinesin-βgal **(E)** localises normally.
(G, H) Tau-GFP labelling of the microtubules in wildtype (G) and *hrp48* mutant stage 9 egg chambers. The microtubule organisation in the mutant is identical to the wild type, with the highest concentration of MT along the anterior cortex of the oocyte.

Figure 5: *osk* and *Ubx* mRNA splicing in wild type and *hrp48* mutant ovaries

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

(B) RT-PCR analysis of *osk* mRNA splicing in wildtype (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 wildtype (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 analyse homozygous mutant L3 larvae. Heterozygous mutant L3 larvae show the same pattern of bands as wildtype 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 dramatic change in the ratio of isoforms between wildtype L1/L2 larvae and L3 larvae. However, at each stage this ratio is not affected in *hrp48* mutant larvae (right panel and data not shown).

Figure 6: Hrp48 co-localises with *osk* mRNA throughout oogenesis

(A , B) Hrp48 staining of wildtype (A) and $stau^{D3}$ (B) ovarioles. Hrp48 accumulates in the oocyte (arrows) from region 2 of the germarium onwards . Note that Hrp48 is both nuclear and cytoplasmic.
(C, D) Hrp48 staining of wildtype (C) and $stau^{D3}$ (D) stage 10 egg chambers Hrp48 is localised in a crescent at the posterior of the oocyte in wildtype, 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 co-localise at the posterior pole in the merged image (G).

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 wildtype and *hrp48* mutant protein extracts, with probes corresponding to the regions shown in (A). The major band that

cross-links to these probes at approximately 50 kDa, co-migrates with Hrp48 (western blot in the centre). 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 co-purify with *osk* 5′ and 3′ regions in affinity pull-down assays. Biotinylated *osk* RNA probes were used to affinity purify interacting proteins from wildtype and mutant larval extracts, and the bound proteins were then run on western blots and probed for Hrp48. The missense mutant proteins co-purify with the *osk* 3′UTR, 5′-act and BREs to the same extent as the wildtype protein.

(D) A western blot of ovarian extracts from wildtype, $stau^{D3}$, 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 unlocalised *osk* mRNA.

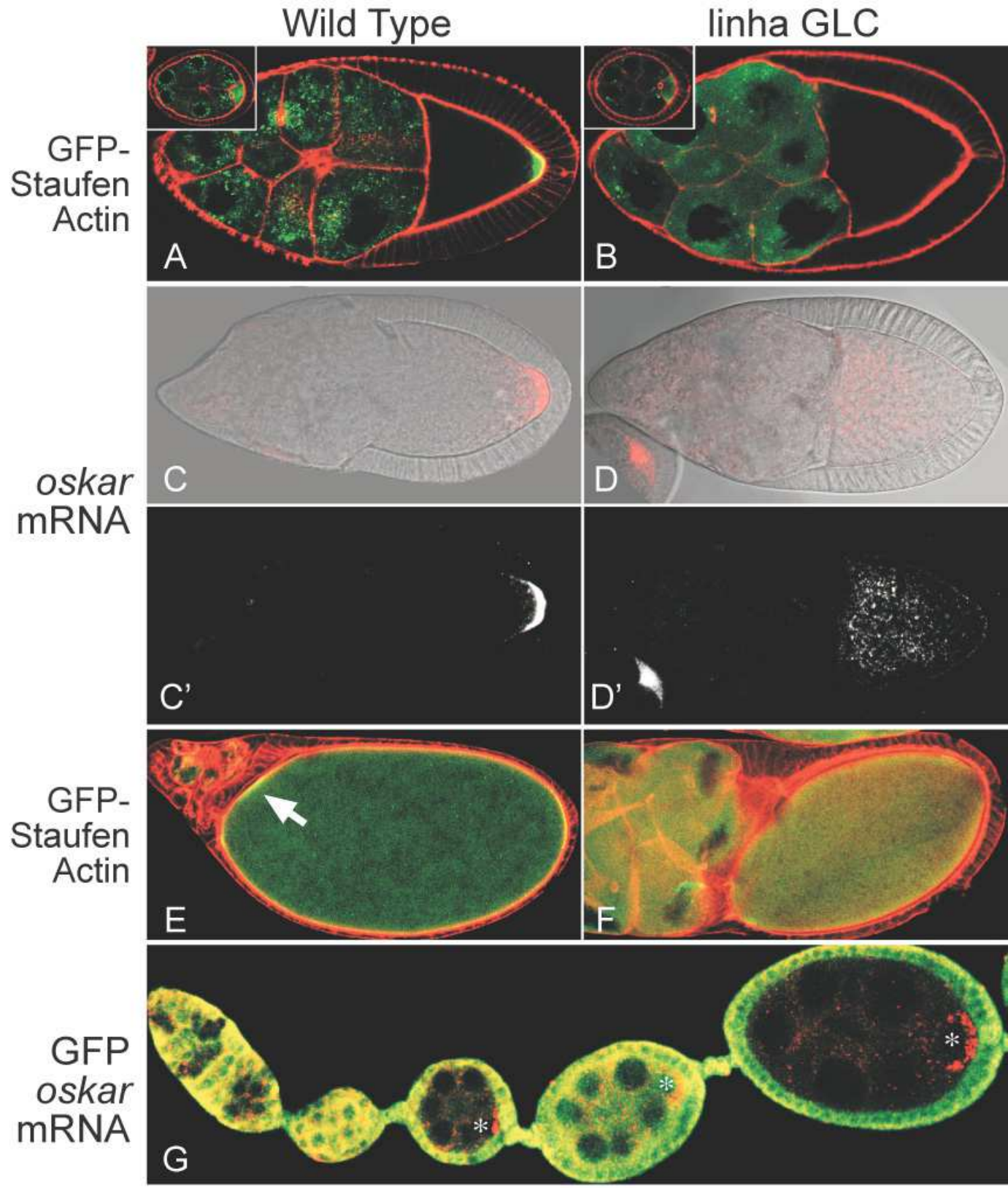
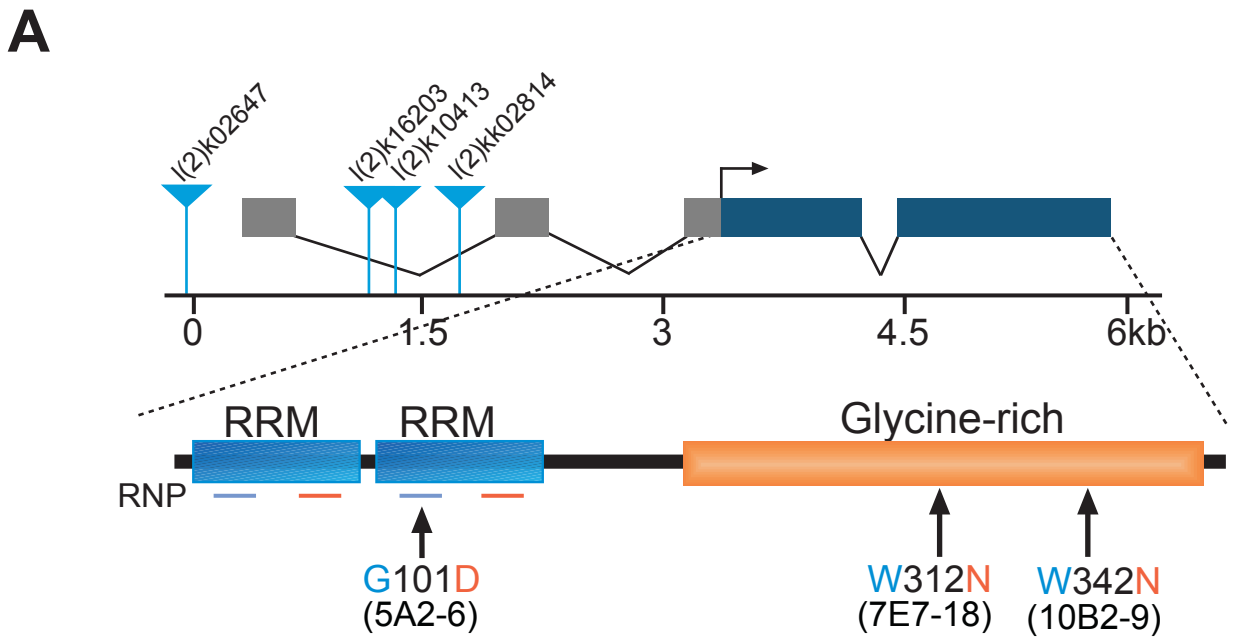

Wild Type
linha GLC
GFP-
Staufen
Actin
A
B
oskar
mRNA
C
D
C'
D'
GFP-
Staufen
Actin
E
F
GFP
oskar
mRNA
G

Figure 1

Figure 2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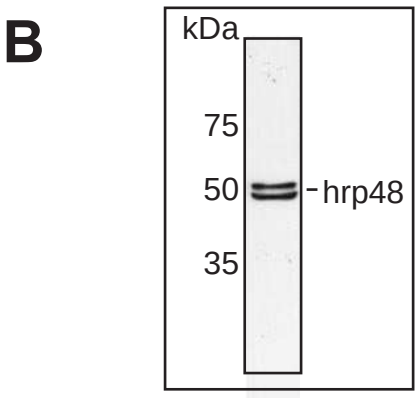
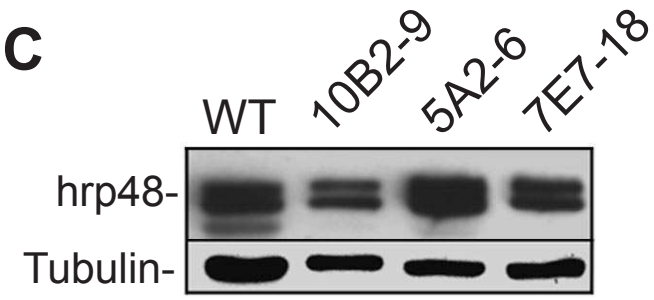
l(2)k16203
l(2)k10413
l(2)kk02814
0
1.5
3
4.5
6kb
RRM
RRM
Glycine-rich
RNP
G101D
(5A2-6)
W312N
(7E7-18)
W342N
(10B2-9)


B
kDa
75
50
35
hrp48


C
WT
10B2-9
5A2-6
7E7-18
hrp48-
Tubulin-

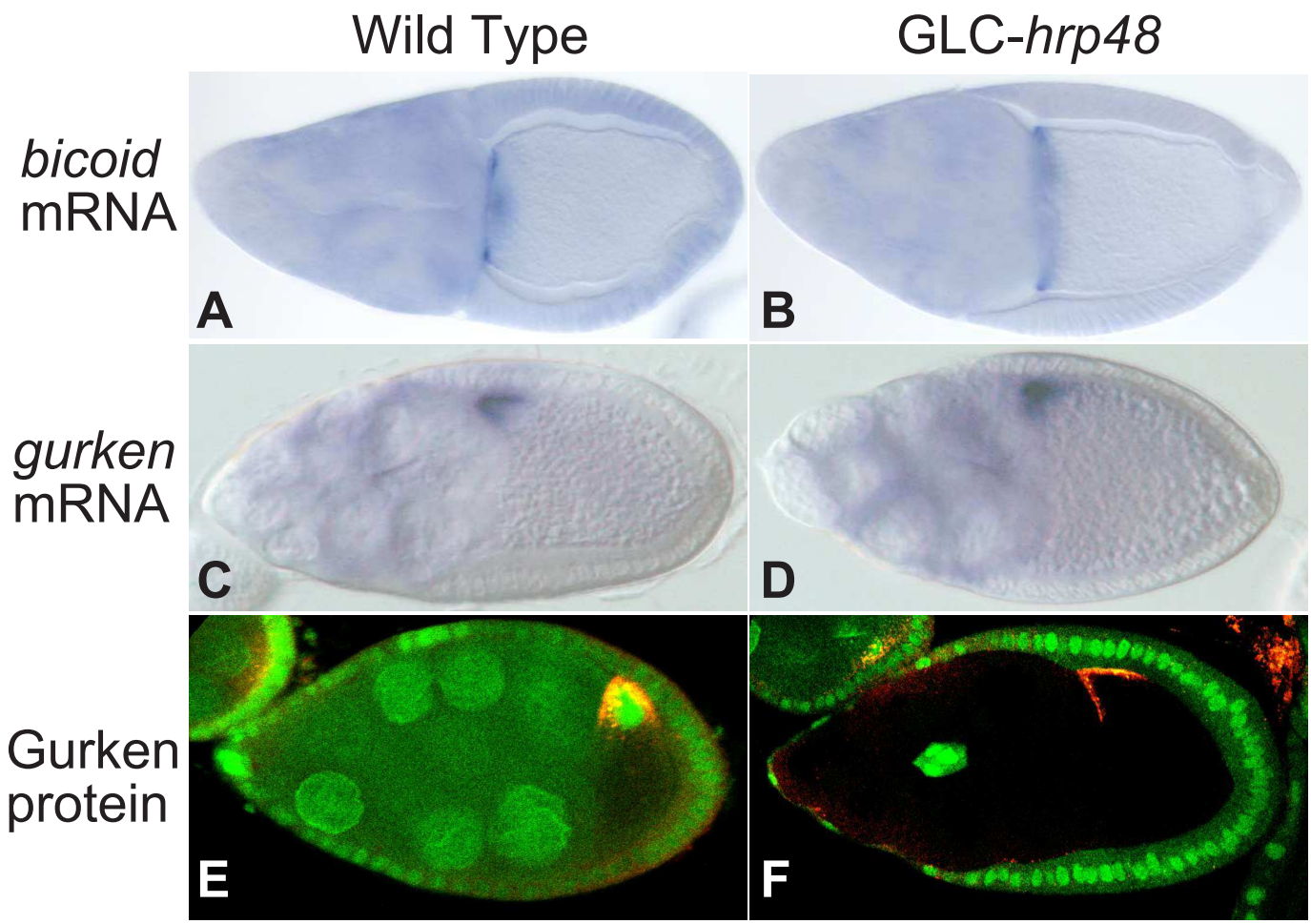

Wild Type
GLC-*hrp48*
bicoid mRNA
gurken mRNA
Gurken protein
A
B
C
D
E
F

Figure 3

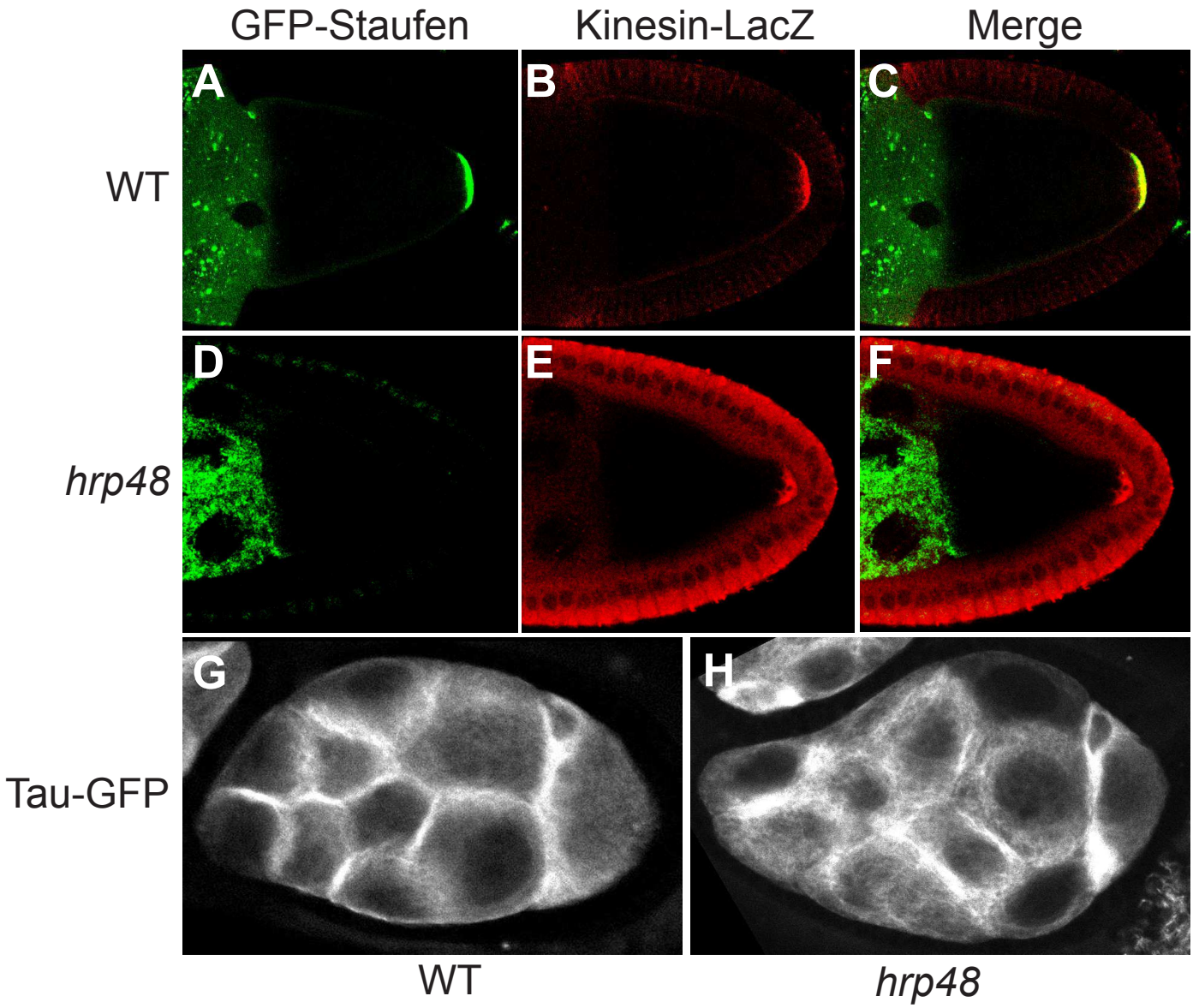

GFP-Staufen
Kinesin-LacZ
Merge
A
B
C
WT
D
E
F
hrp48
G
H
Tau-GFP
WT
hrp48

Figure 4

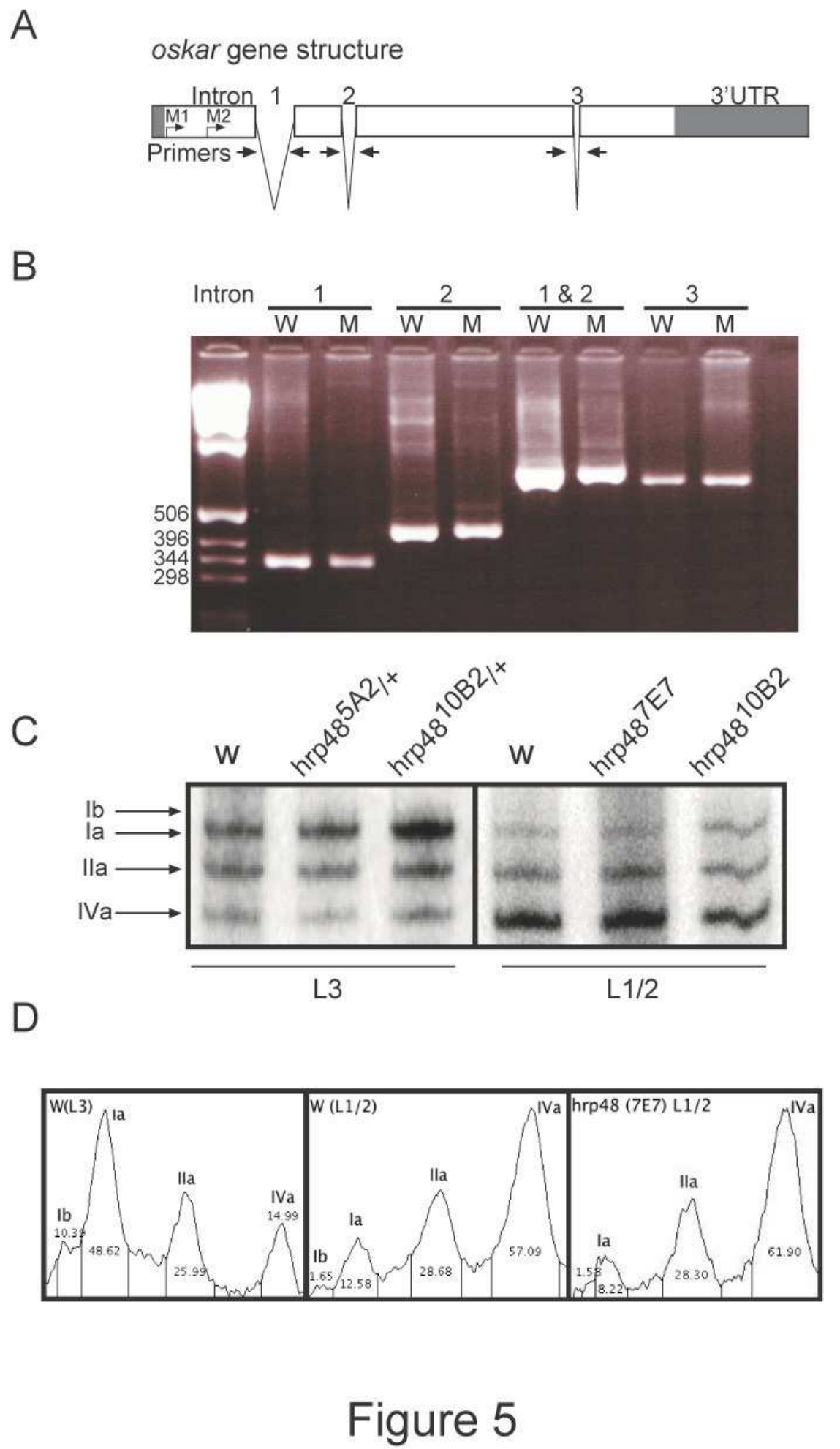

A
oskar gene structure
Intron
1
2
3
3'UTR
M1
M2
Primers
B
Intron
1
2
1 & 2
3
W
M
W
M
W
M
W
M
506
396
344
298
C
w
hrp48 5A2/+
hrp48 10B2/+
w
hrp48 7E7
hrp48 10B2
Ib
Ia
IIa
IVa
L3
L1/2
D
W(L3)
Ia
IIa
IVa
Ib
10.39
48.62
25.99
14.99
W (L1/2)
IVa
IIa
Ia
Ib
1.65
12.58
28.68
57.09
hrp48 (7E7) L1/2
IVa
IIa
Ia
1.58
8.22
28.30
61.90

Figure 5

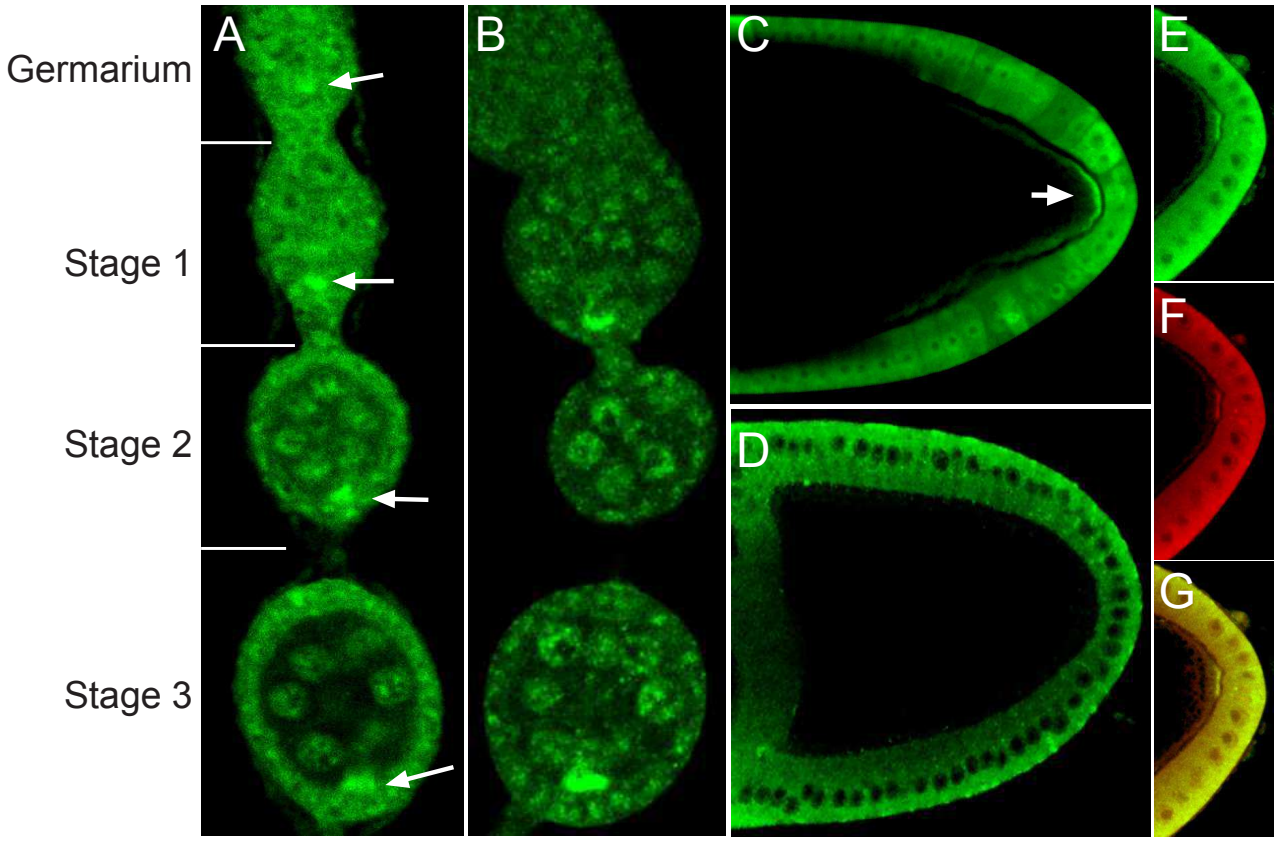


Figure 6

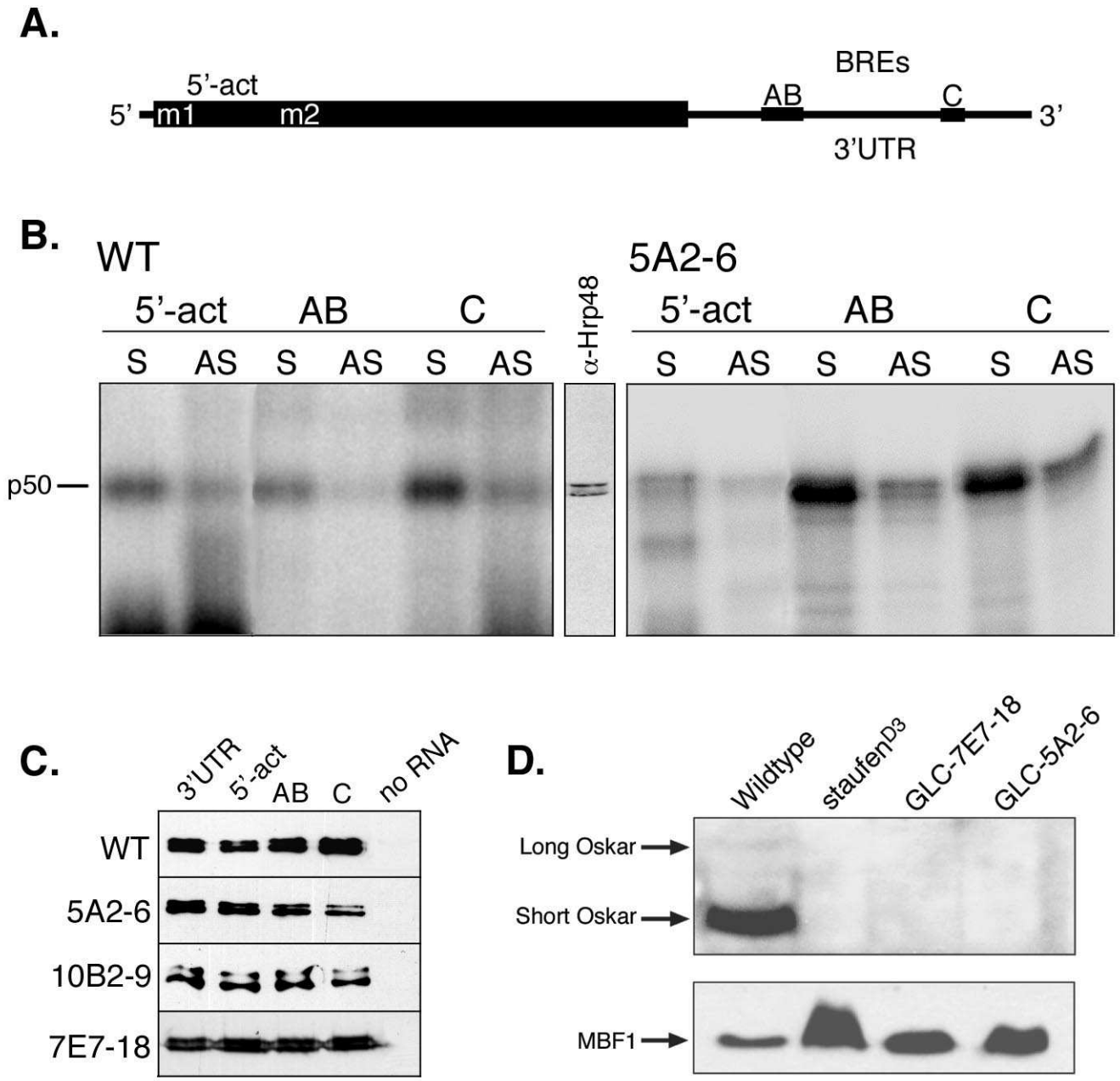

A.
5'-act
BREs
5'
m1
m2
AB
C
3'
3'UTR
B.
WT
5A2-6
5'-act
AB
C
S
AS
α-Hrp48
p50
C.
3'UTR
5'-act
AB
C
no RNA
WT
5A2-6
10B2-9
7E7-18
D.
Wildtype
staufen^D3
GLC-7E7-18
GLC-5A2-6
Long Oskar
Short Oskar
MBF1

Figure 7