In Vivo Imaging of oskar mRNA Transport Reveals the Mechanism of Posterior Localization

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SUMMARY

oskar mRNA localization to the posterior of the Drosophila oocyte defines where the abdomen and germ cells form in the embryo. Although this localization requires microtubules and the plus end-directed motor, kinesin, its mechanism is controversial and has been proposed to involve active transport to the posterior, diffusion and trapping, or exclusion from the anterior and lateral cortex. By following oskar mRNA particles in living oocytes, we show that the mRNA is actively transported along microtubules in all directions, with a slight bias toward the posterior. This bias is sufficient to localize the mRNA and is reversed in mago, barentsz, and Tropomyosin II mutants, which mislocalize the mRNA anteriorly. Since almost all transport is mediated by kinesin, oskar mRNA localizes by a biased random walk along a weakly polarized cytoskeleton. We also show that each component of the oskar mRNA complex plays a distinct role in particle formation and transport.

INTRODUCTION

mRNA localization is a common mechanism for targeting proteins to specific regions of a cell and plays an important role in axis formation in many organisms, where localized mRNAs function as cytoplasmic determinants. This has been extensively studied in Drosophila, where both main body axes are defined by the localization of bicoid, oskar (osk), and gurken mRNAs to distinct regions of the oocyte (Bashirullah et al., 1998; St Johnston, 2005). osk mRNA moves to the posterior of the oocyte during stages 8–10 of oogenesis and is translated as soon as it is localized to the posterior pole, where Oskar protein nucleates the polar granules, which contain the abdominal and germline determinants (Ephrussi et al., 1991; Kim-Ha et al., 1991, 1995; Markussen et al., 1995).

The Drosophila egg chamber consists of a syncytium of 15 nurse cells that are connected by ring canals to the oocyte. osk mRNA localizes in the nurse cells and must first be transported into the oocyte. This localization depends on the activity of BicD and Egalitarian, which are thought to couple the mRNA to dynein, which moves the mRNAs toward the minus ends of microtubules in the oocyte (Bullock and Ish-Horowicz, 2001; Clark et al., 2007). The localization of osk mRNA within the oocyte is also microtubule dependent and is disrupted by treatments with microtubule depolymerizing drugs (Clark et al., 1994).

Microtubule stainings or live imaging of microtubule-associated proteins reveal that the oocyte contains an anterior-posterior gradient of microtubules during the stages when osk mRNA is localized, in which most microtubules are nucleated from the anterior and lateral cortex. In addition, a fusion between the plus end-directed motor, kinesin, and β-galactosidase accumulates at the oocyte posterior, suggesting that plus ends are enriched at this pole (Clark et al., 1994). However, it has been argued that the Kiniβgal fusion protein localizes to the posterior by hitchhiking since microtubules cannot be detected at the oocyte posterior (Cha et al., 2002). Thus, the organization of the microtubules in the oocyte is still controversial, and it is unclear how the microtubule network directs osk mRNA to the posterior.

Several mutants disrupt osk mRNA localization within the oocyte without affecting the cytoskeleton and are good candidates for mutants in essential trans-acting factors. These include three factors (HRP48, the exon junction complex [EJC], and Staufen) that colocalize with osk mRNA both before and after its localization and whose posterior localization is osk mRNA dependent, strongly suggesting that they are essential components of the osk mRNA localization complex. Null mutants in the hnRNPA/B protein, HRP48, are cell lethal, but three point mutations lead to a uniform distribution of osk mRNA in the oocyte (Huynh et al., 2004). Mutants in the EJC components, Mago nashi, Y14, eIF4AIII, and Barentsz, lead to the mislocalization of the
mRNA to the anterior of the oocyte (Hachet and Ephrussi, 2001; Mohr et al., 2001; Newmark and Boswell, 1994; Palacios et al., 2004). The EJC is recruited to mRNAs during splicing and marks where exons have been removed. Consistent with this, the first intron of osk is necessary for its posterior localization (Hachet and Ephrussi, 2004). Mutants in the dsRNA-binding protein, Staufen (Stau), cause a similar accumulation of osk mRNA at the anterior of the oocyte to EJC mutants, but a small amount of RNA is always localized to the posterior at stage 9 (Ephrussi et al., 1991; Kim-Ha et al., 1991; St Johnston et al., 1991; van Eeden et al., 2001). This localization is only transient, however, because Stau is also required for the translational activation of osk mRNA and its anchoring at the posterior pole (Micklem et al., 2000). While the EJC and HRP48 disappear from the oocyte posterior at stage 10 of oogenesis, Stau associates with osk mRNA throughout oogenesis (St Johnston et al., 1991; Jenny et al., 2006).

As well as these RNA-binding proteins, Tropomyosin II (TmlII) and the Kinesin heavy chain (Khc) are also required for osk mRNA localization. A P-element allele of TmlII leads to the mislocalization of osk mRNA to the anterior (Erdelyi et al., 1995). Tropomyosin stabilizes F-actin and modulates its interaction with other actin-binding proteins, but there is no other evidence for a direct role of actin in osk mRNA localization, and Tropomyosin does not colocalize with osk mRNA. Null alleles of the Khc cause a different phenotype, in which osk RNA localizes around the anterior and lateral cortex (Brendza et al., 2000; Cha et al., 2002). Kinesin is enriched at the oocyte posterior at stage 9, although this localization is not osk mRNA dependent, unlike the RNA-binding proteins mentioned above (Palacios and St Johnston, 2002).

The role of the Khc suggests a simple model for osk mRNA localization, in which this plus end-directed motor is linked to the mRNA posteriorly by a prelocalized anchor (Glotzer et al., 1997). This model is based on the observation that injected osk mRNA accumulates at the posterior of late-stage oocytes without showing any directed movement toward the posterior pole. Instead, the RNA is washed around the oocyte by the cytoplasmic flows at these stages. Both the strong cytoplasmic flows in late oocytes and the weaker “seething” movements at stage 9 depend on kinesin (Serbus et al., 2005). The motor might therefore play an indirect role in osk mRNA localization by generating flows that facilitate its diffusion, thereby ensuring its efficient delivery to a posterior anchor.

Cha et al. have proposed a third model in which kinesin transports osk mRNA away from all regions of the cortex except the posterior (Cha et al., 2002). This cortical exclusion model is based on the observation that the mRNA accumulates in the center of the oocyte at stage 8 but is found all round the cortex in Khc germline clones. According to the model, osk mRNA localization is driven by the lack of microtubules emanating from the posterior cortex, thereby preventing kinesin from removing the RNA from this region. Both the central accumulation of osk mRNA at stage 8 and its posterior localization at stage 9 are delayed in Khc hypomorphs that reduce the speed of the motor without affecting its other properties (Serbus et al., 2005). This has led to the proposal that kinesin is required for both steps in osk mRNA localization: it first transports the mRNA to the center of the oocyte and then facilitates its targeting to the posterior, perhaps by directed transport along a specific subset of microtubules or by catalyzing random movements that are somehow biased toward the posterior.

One way to distinguish between the models for osk mRNA localization is to observe its movement in living oocytes. The most common approach for visualizing mRNA localization has been injection of fluorescently labeled transcripts (Cha et al., 2001; MacDougall et al., 2003; Weil et al., 2006; Wilkie and Davis, 2001). This technique cannot be used for osk mRNA, however, because splicing of the first intron is required for its localization. An alternative approach is to fuse GFP to an RNA-binding protein that associates with the mRNA of interest, and ZBP-1-GFP and Exu-GFP have been used in this way to track β-actin and bcd mRNA movements (Oleynikov and Singer, 2003; Theurkauf and Hazelrigg, 1998). One caveat with this method is that most RNA-binding proteins bind to more than one mRNA, making it important to demonstrate that the tagged RNA-binding protein only associates with the appropriate transcript.

An elegant way to solve this problem is to target GFP to the mRNA by inserting binding sites for the MS2 phage coat protein into the mRNA and coexpressing an MS2-GFP fusion protein that also includes a nuclear localization signal (Bertrand et al., 1998). Since MS2 does not bind any endogenous RNAs in Drosophila, the fusion protein is targeted to the nucleus unless it binds to the MS2-binding sites in the transcript of interest and is exported with the RNA into the cytoplasm. This method has been used successfully to study Ash1 mRNA localization in yeast (Beach et al., 1999; Bertrand et al., 1998), CaMKIIα3 and β-actin mRNA localization in cultured mammalian cells (Fusco et al., 2003; Rook et al., 2000), and nanos and bicoid mRNA in Drosophila oocytes (Forrest and Gavis, 2003; Weil et al., 2006).

Here we report the use of two labeling strategies to image the dynamics of osk mRNP particles in living oocytes. Our analysis of their movements rules out all of the proposed mechanisms for osk mRNA localization and leads us to propose a revised model for how the mRNA is transported to the posterior of the oocyte.

RESULTS

Visualization of osk mRNPs in Living Oocytes

We have previously tried to visualize osk mRNA by following the movement of a GFP-tagged version of Stau that colocalizes with the mRNA throughout oogenesis and fully rescues the osk mRNA localization defects of stau null mutants (Figures 1A–1C) (Palacios and St Johnston, 2002). Although we observed large GFP-Stau granules in the oocyte cytoplasm by confocal microscopy,
Furthermore, these large granules only form when the protein is expressed in the same direction as other organelles in the surrounding cytoplasm. The velocity of these granules never exceeded the speed of cytoplasmic flows \((0.1 \mu\text{m/s})\), suggesting that they were moving along the same microtubule or actin filament (Movie S2). Surprisingly, these movements occurred in all directions. In contrast to these fast movements, many particles moved only slowly along irregular paths \((>0.1 \mu\text{m/s})\), suggesting that they were being carried by the slow cytoplasmic flows and undergoing Brownian motion (Figure 1H and data not shown).

The lack of a clear bias in the direction of GFP-Stau particle movements was unexpected given the models for osk mRNA localization and raised the question of whether all of the particles contain osk mRNA. Stau associates with other transcripts at other stages of development, and it is therefore possible that some of the particles in the stage 9 oocyte represent Stau complexes with another mRNA, or free Stau aggregates (St Johnston, 2005). We therefore used the MS2 system to label osk mRNA directly by generating an osk genomic rescuing construct in which 10 MS2-binding sites were inserted immediately after the stop codon. When this transgene is crossed to flies expressing MS2-nls-GFP, the MS2-GFP-labeled osk mRNA accumulates at the posterior of the oocyte in an identical pattern to endogenous osk mRNA (Figure 1D). oskMS2 rescues the early oogenesis defect of an osk mRNA null mutant and still localizes normally to the posterior of the oocyte at stage 9 in the absence of endogenous osk, confirming that the introduction of MS2-binding sites does not disrupt its localization signal (Figure S1A). Unlike GFP-Stau, oskMS2 RNA does not form large cytoplasmic aggregates, confirming that the latter are probably an overexpression artifact.

When visualized at high magnification in living oocytes, oskMS2 mRNA labeled small particles throughout the oocyte cytoplasm, which showed fast movements in all directions similar to those of the small GFP-Stau particles (Movies S3 and S4). Indeed, we could detect even more moving particles of oskMS2 mRNA, presumably because this labeling system introduces up to 20 GFPs per osk mRNA and is therefore more sensitive than the single GFP attached to Stau. Nevertheless, it appears that most if not all small Stau particles contain osk mRNA since GFP-Stau particles were also labeled by oskMS2/MS2-GFP, and alternate imaging of the red and green channels revealed that the two labels move in concert on the same particle (Figures 1G and 1G’; Movie S5). Thus, GFP-Stau is a reliable marker for osk mRNA particles, suggesting that the protein does not associate with other RNAs to form particles at stage 9 of oogenesis.
Movement of osk mRNP Particles Requires Microtubules

Injection of the microtubule-destabilizing drug, colcemid, into the oocyte abolished almost all fast, directed movements of the particles, which instead underwent random Brownian oscillations (Movie S6). Feeding flies with colcemid caused a similar cessation of the movement of the GFP-Stau particles and abolished their accumulation at the posterior pole (Figure S2A). The fast movements of the GFP-Stau particles resumed after the colcemid was inactivated with a short UV-pulse, and the fusion protein showed a visible accumulation at the posterior pole within 50 min (Figures S2B and S2C and Movie S7). Neither feeding, immersion of the ovaries, nor direct injection of Latrunculin A into the oocyte stopped the motility of GFP-Stau and oskMS2 particles (Movie S8). Thus, most, if not all, of the fast particle movements are microtubule rather than actin dependent.

The results above indicate that osk mRNA particles are transported along microtubules, strongly suggesting that these movements represent the microtubule-dependent step in its posterior localization. However, the behavior of the particles does not correspond to the predictions of the main models for the localization of the mRNA. The movement of the particles in all directions rules out the model in which kinesin transports osk mRNA along a highly polarized microtubule cytoskeleton to the posterior. Two aspects of our results also argue against the cortical exclusion model. First, osk mRNP particles frequently undergo fast, directed movements to the posterior pole (for example, Figure 1L), indicating that they are not simply transported away from the anterior and lateral cortex. Second, the osk mRNP particles show a similar frequency of fast microtubule-dependent movements close to the posterior pole as they do in other areas of the oocyte, indicating that a significant number of microtubules extend into this region. Finally, the active transport of the particles suggests that the mRNA does not localize by passive diffusion and anchoring. However, random fast movements could facilitate the diffusion of the mRNA throughout the oocyte so that it can be efficiently captured by a posterior anchor.

osk mRNA Particle Movement Shows a Weak Posterior Bias

To determine whether there are any anterior-posterior biases in the behavior of osk mRNP particles that might contribute to their delivery to the posterior, we analyzed the particle movements in more detail. We first determined the proportion of particles undergoing active transport at any moment by tracking all moving and “nonmoving” particles in several oocytes. Most particles move along irregular paths at the same speed as the yolk granules in the surrounding cytoplasm (≤0.1 μm/s), indicating that they are being carried by the Khc-dependent slow seething of the cytoplasm. However, in any given 5 s period, 13.4% of the oskMS2 particles (91/689 particles, 7 movies) and 13.1% of the GFP-Stau particles (178/1111, 6 movies) move in a directed and processive manner at speeds that are indicative of active transport (See Figures 1E−1L).

Because we needed to image as quickly as possible to detect the moving particles, we could only collect data in a single focal plane and could therefore follow only a segment of the trajectory of most fast-moving particles. Nevertheless, both GFP-Stau and oskMS2 particles showed similar average track lengths (2.4 and 2.8 μm, respectively), and the longest in focus movements were over 10 μm, indicating that the transport is processive. There were no significant differences between the track lengths of particles moving toward the anterior and those moving toward the posterior (Table S1).

Both GFP-Stau and oskMS2/MS2-GFP particles also moved with very similar average velocities toward the anterior or posterior of the oocyte (Table S1). In both cases, however, particles in the anterior half of the oocyte moved with a higher average speed than those in the posterior half (Table S1). Plotting the velocity distributions of particles in both halves of the oocyte revealed that particles in the anterior have a bimodal distribution of velocities, indicating that a small population of particles move considerably faster than the rest (Figure 2B). Since osk mRNA is transported from the nurse cells into the oocyte by dynin, this fast population may represent particles that have just entered the oocyte and are still coupled to this pathway (Bullock and Ish-Horowicz, 2001; Clark et al., 2007).

We used the angle of the vector between the start and finish of each particle movement as a measure of their net direction and compared the number of particles traveling toward the posterior or the anterior (Figure 2C). The number of tracks in each 180° sector showed a significant excess of movements toward the posterior, with 56.5% (277/491, p < 0.005) of the GFP-Stau tracks and 57% (155/272, p < 0.025) of oskMS2/MS2-GFP tracks having a posterior orientation (Figure 2C, ii and iv; Table S1). We used the direction and velocity of each particle from the tracking data to calculate the net displacement for the average particle. This gives a net posterior displacement of 0.03 ± 0.01 μm/s for GFP-Stau particles and 0.04 ± 0.02 μm/s for oskMS2/MS2-GFP particles (Figure 2D; Table S1). The bias therefore gives the transport of osk RNP an overall posterior vector.

mago, btz, and Tmll Mutants Reverse the Directional Bias in osk mRNA Movement

The results above raise the question of whether the weak posterior bias in osk mRNP particle movement is necessary for the posterior localization of the mRNA, or whether this active transport merely facilitates the diffusion of the mRNA so that it can be efficiently trapped at the posterior. To distinguish between these models, we examined the effects of mutants that disrupt osk mRNA localization. If the bias is important, one would expect it to be altered in some of these mutants, whereas the facilitated diffusion and anchoring model predicts that mutants should disrupt either the motility or the anchoring, but not the bias itself.

No GFP-Stau particles are visible in the oocyte cytoplasm of germline clones of hrp48<sup>10B2-9</sup> or hrp48<sup>17E7-18</sup> (Figures 3A–3B). Thus, HRP48 appears to be essential for the formation of osk mRNA transport particles, which may account for the uniform distribution of the mRNA in these mutants.

In contrast, particle formation appears normal in btz, mago, Tmll<sup>959S</sup> mutants. In these mutants, osk mRNA forms a diffuse gradient extending away from the anterior cortex of the oocyte, when visualized either by in situ hybridization or by GFP-Stau or oskMS2 (Figures 3C–3E). There is about a 5-fold reduction in the proportion of particles that undergo fast, directed
movements in these mutants, although there is still a significant amount of active transport in each case (Figure 3G, Table 1, and Movies S9, S10, and S11). Thus, the EJC and Tropomyosin affect the behavior of the osk RNP particles but are not required for their assembly.

To determine whether any of these mutants also affect the directional bias, we tracked a large number of particles and measured the vector of their movement. This revealed that mago, btz, and Tmlf\textsuperscript{pp} mutants cause a highly significant reversal in the directional bias, with 57%–59% of particles moving anteriorly, compared to only 43% in wild-type (Figures 3H and 3I). This results in a net anterior displacement of the particles that may explain the steady-state localization of the mRNA at the anterior of mutant oocytes.

Stau Does Not Affect the Speed and Directionality of Particle Movements

\textit{stau} mutants also contain normal numbers of oskMS2/MS2-GFP particles, but the majority of these are tightly localized at the oocyte anterior in a narrower region than the shallow anterior gradient seen in mago, btz, and Tmlf\textsuperscript{pp} mutants (Figure 3F; Movie S12). In addition, a trace amount of osk mRNA always localizes to the posterior of stau mutant oocytes at stage 9 (van Eeden et al., 2001).

As in the other localization mutants, the frequency of fast, directed oskMS2 particle movements in the oocyte cytoplasm is reduced about 4-fold in \textit{stau} null mutants (Figure 3G). Despite the visible anterior accumulation of oskMS2/MS2-GFP, there is no change in the directional bias of particle movements, with 59% moving toward the posterior, resulting in a positive net posterior displacement of the particles of 0.08 \textmu m/s (Table 1). Most osk mRNP particles appear to be trapped at the anterior of the oocyte, but those that escape the anterior move with a similar average velocity (0.47 \textmu m/s) and posterior bias to wild-type. Since Stau is required for the posterior anchoring and translation of osk mRNA, the weak accumulation of the mRNA at the posterior of \textit{stau} oocytes at stage 9 cannot be explained by random transport and localized anchoring. This therefore suggests that this posterior enrichment is caused solely by the posterior bias in particle movements.

Most osk RNP Particles Move toward Microtubule Plus Ends

The discovery that osk RNP particles move in all directions with a slight posterior bias raises the question of how this bias arises. This is difficult to address because the organization of the microtubules in the oocyte is unclear, but one can imagine two extreme scenarios. In the first, the microtubule cytoskeleton is
highly polarized along the anterior-posterior axis with the plus ends at the posterior, but the particles undergo transport toward both the plus and minus ends of the microtubules, with a slight excess of plus end-directed transport. This type of bidirectional transport has been observed for several organelles, such as mitochondria in axons, and lipid droplets and pair rule transcripts in the Drosophila embryo (Bullock et al., 2006; Welte, 2004). At the other extreme, osk RNP particles could undergo exclusively plus end-directed transport along a weakly polarized microtubule cytoskeleton, in which microtubules extend in all directions, with slightly more having their plus ends pointing posteriorly.

The models for the bias in osk mRNP movement can be distinguished by quantifying the proportion of particles that move toward the plus or minus ends of the microtubules. Although this is not possible in wild-type oocytes because of the complexity of the microtubule organization, treatments with actin-depolymerizing drugs induce premature streaming of the oocyte cytoplasm, which washes the microtubules into alignment around the oocyte cortex (Manseau et al., 1996). Cytoplasmic streaming requires kinesin and is thought to result from the plus end-directed transport of large organelles or vesicles (Dahlgaard et al., 2007; Serbus et al., 2005). The parallel arrays of microtubules generated by the flows are therefore aligned with their plus ends pointing in the direction of the flow. We took advantage of this to examine the movement of osk mRNPs along microtubules of known polarity by imaging oskMS2 in wild-type oocytes that had been treated with Latrunculin A (Movie S8). As shown in Figure 4E, 82% of the fast-moving particles move in the same direction as the flow. Assuming that Latrunculin does not affect the behavior of osk mRNP particles, this suggests that the vast majority of movements are plus end-directed.

The obvious candidate for a plus end-directed motor that moves osk mRNPs is kinesin 1. We therefore imaged particle movement in Khc27 germline clones (Movie S13). The proportion of particles that showed fast, directed movements during a 5 s period was only 2.4%, which is more than a 5-fold reduction compared to wild-type (Figures 5A–5F). Some movements can still be detected, however, indicating that other motors can move the particles processively (Table 1).

Although these data indicate that more than 80% of osk mRNA particle movements are kinesin dependent, this does not necessarily mean that kinesin directly transports the particles because the loss of kinesin could affect the activity of other motors. For example, kinesin is required for the localization of cytoplasmic dynein to the posterior of the oocyte, suggesting that the two motors are associated (Januschke et al., 2002; Palacios and St Johnston, 2002). Thus, the removal of the Khc could alter dynein function, either by disrupting a kinesin/dynein complex or by disrupting dynein localization.

We therefore examined the behavior of oskMS2 particles in Dhc6–12, a viable mutant combination that significantly delays the localization of gurken mRNA and halves the speed at which dynein transports mRNAs in the nurse cells (Clark...
et al., 2007; MacDougall et al., 2003; Mische et al., 2007). The posterior localization of oskMS2 was not affected in this mutant combination, and the particles underwent fast movements toward the anterior and posterior of the oocyte with similar frequencies to wild-type, giving a normal net posterior displacement (Figures 5E–5H; Table 1). The speed of particle movement in both directions was slightly increased compared to wild-type (0.53 μm/s). Since one would expect the dynein hypomorphs to reduce the speed of any dynein-dependent particle movements, these data argue against a direct role for dynein in the transport of osk mRNPs within the oocyte.

To address the role of kinesin in osk mRNA transport without the complications of indirect effects caused by the null allele, we examined two missense mutations in the kinesin motor domain, Khc23 and Khc27, which reduce the speed of the motor without having any detectable effects on its other functions (Brendza et al., 1999; Serbus et al., 2005). As previously reported, osk mRNA still localizes in germline clones of both alleles, but there is a delay in its posterior accumulation (Figures 5B and 5C). Unlike the Khc null mutation, Khc27 and Khc23 have little effect on the frequency of particle movements, consistent with the idea that the mutant motor proteins associate normally with their cargoes (Figure 5F). More importantly, there is a significant reduction in the speed of oskMS2 particle movement in these mutants, with values of 0.35 μm/s (p < 0.001) for Khc27 and 0.25 μm/s (p < 0.001) for Khc23 (Table 1; Figure 5G). This slower movement proves that kinesin transports osk mRNPs along microtubules.

Since the reduced speed in Khc27 and Khc23 provides a direct indication of kinesin-dependent transport, it allowed us to ask whether kinesin moves osk mRNPs specifically toward the posterior of the oocyte, or both anteriorly and posteriorly. The velocity profile of particles moving toward the anterior showed a pronounced shift to lower speeds in both mutants that was indistinguishable from that seen in particles moving toward the posterior (Figures 5I and 5J). Thus, kinesin mediates the majority of fast particle movements in both directions, indicating that the posterior bias is chiefly due to a weak bias in the orientation of the microtubules, rather than bidirectional transport along a more strongly polarized cytoskeleton.

**DISCUSSION**

The mechanism of osk mRNA localization has been controversial, and a number of competing models have been proposed to explain its targeting to the posterior. Here, we have observed directly how the RNA travels to the posterior by tracking the movements of osk mRNA particles at high temporal resolution in living oocytes. Surprisingly, our results are inconsistent with the existing models, leading us to propose a new mechanism for the localization of the mRNA.

First, it is clear that the mRNA is not transported in a highly directed fashion toward the posterior since the particles move in all directions with only a slight posterior bias. Second, the rapid transport of the osk mRNPs argues against a role for passive diffusion (Glotzer et al., 1997). Third, our results are inconsistent with the two-step model for osk mRNA localization, in which kinesin first transports the RNA away from the anterior and lateral cortex to the oocyte center before it is translocated to the posterior in a second step (Cha et al., 2002). osk mRNA particles show a similar behavior in all regions of the oocyte at stage 9, with a consistent small excess of particles moving posteriorly, and this is incompatible with the idea that particles are first transported to the center. Moreover, slow kinesin mutants have an identical effect on the speeds of particle movements in all regions of the oocyte, strongly arguing that kinesin transports the mRNA in a one-step pathway all of the way to the posterior pole.

Instead, our data suggest that osk mRNA is localized by a biased random walk, in which each particle undergoes a large number of active movements in many different directions, with
a small excess of movements toward the posterior. After hundreds of movements, the 14% excess of posterior movements results in a large net posterior displacement that delivers the mRNA to its destination. Given that 13% of particles are moving at any one time, the average osk mRNA will undergo a net posterior displacement during the 6–10 hr of stage 9 of 112–187 μm (6–10 × 3600 s × 0.13 × 0.04 μm/s). Since this is more than 1.5 times the length of the oocyte (80 μm), this is more than sufficient to produce a robust posterior localization of osk mRNA by the end of stage 9.

This model is supported by the observation that the direction of the bias correlates perfectly with the site of osk mRNA accumulation; wild-type oocytes show a posterior bias and posterior localization of the mRNA, whereas the bias is reversed in mago, TmII, and btz mutants, and osk mRNA accumulates at the anterior. The effectiveness of the biased random walk in localizing the RNA is even more clearly demonstrated by staun mutants: most of the mRNA is trapped at the anterior of staun mutant oocytes, and the mRNA that is released into the oocyte cytoplasm moves four times less frequently than in wild-type. Nevertheless, the small number of movements that occur show a normal posterior bias, which leads to a transient posterior enrichment of the mRNA that is lost at later stages because the RNA is not anchored.

Similar biased bidirectional transport has been described for lipid droplets in the Drosophila embryo and for many other particles and organelles in other systems (Welte, 2004). In most cases, the bias depends on the competing activities of motors that move in opposite directions. By contrast, our results indicate that the vast majority of osk mRNA movements are directed toward microtubule plus ends and are mediated by kinesin. First, when the microtubules are aligned around the cortex by premature cytoplasmic streaming, over 80% of fast-moving particles move in the same direction as the cytoplasmic flows, i.e., toward the plus ends. Second, more than 80% of movements are abolished by null mutations in the Khc. Third, point mutations in kinesin reduce the speed of particle movements in all directions. Fourth, we have never observed any particles that show a clear 180° reversal in the direction of their movement out of more than 3000 particle tracks analyzed, indicating that particles rarely switch between plus and minus end-directed motion.

The traditional view of the oocyte microtubule cytoskeleton is that it is polarized along the anterior-posterior axis with minus ends at the anterior and plus ends at the posterior. This view is based on the localization of fusion proteins containing the motor domains of Nod and kinesin to the anterior and posterior of the oocyte, respectively, and the assumption that these act as minus and plus end markers (Clark et al., 1994, 1997). The microtubule organization appears much more complex, however, when visualized directly: the microtubules appear to be nucleated from both the anterior and lateral cortex and extend in all directions to form an anterior-posterior gradient (Cha et al., 2001; MacDougal et al., 2003). Our data are consistent with this latter view because osk mRNA particles move in all directions in every region of the oocyte. More importantly, because almost all osk mRNA movements are plus end directed, each RNA track provides a snapshot of the polarity of a microtubule segment. The observation that 57% of tracks have a net posterior vector therefore indicates that the microtubules have only a weak orientation bias toward the posterior. Even if all 10%–20% of kinesin-independent osk mRNA movements are minus end directed, this would still give a posterior bias in microtubule polarity of only 62%. Thus, our data suggest a revised view of the organization of the cytoskeleton, in which the microtubules extend in all directions from the anterior and lateral cortex, with about 20% excess of microtubules with their plus ends pointing posteriorly. One appealing aspect of this model is that it can reconcile the two opposing views of the microtubule organization. Kinjøgal is an unregulated motor that constitutively moves toward the plus ends of microtubules, and we propose that it accumulates at
the posterior by following a biased random walk similar to osk mRNA. According to this view, it is not a marker for microtubule plus ends but for regions where plus ends are most enriched.

Mutants in different components of the osk mRNA localization complex produce very similar phenotypes when analyzed by in situ hybridizations to fixed samples. However, they have different effects on the dynamics of osk mRNA particles. First, hp48 mutants abolish the formation of visible osk mRNA particles, indicating a requirement for this HnRNP/A-like protein in the assembly of functional transport particles.

Mutants in the EJC components, Mago nashi and Btz, do not affect osk mRNP particle formation but reduce the frequency of particle movement and reverse the bias, so that the particles accumulate at the oocyte anterior. This behavior is what one would expect if the movement is primarily mediated by a minus end-directed motor. Furthermore, the anterior accumulation of osk mRNA in these mutants resembles that of many other mRNAs that are transported into the oocyte by the dynein/Bic-D/Egl pathway, and which are thought to localize to the anterior by default, because this pathway remains active in the oocyte (Serano and Cohen, 1995). Thus, the EJC may be required to turn off the dynein/Bic-D/Egl pathway when osk mRNA enters the oocyte so that it can then associate with the kinesin pathway.

*Tml* mutants have the same effects on osk mRNP dynamics as EJC mutants, suggesting that Tropomyosin is required for the same step in localization. This raises the possibility that Tropomyosin plays a role in either the recruitment of the EJC to osk mRNA or the subsequent activity of the EJC in switching from the anterior to the posterior localization pathway.

Stau seems to function downstream of the EJC since osk mRNA is either trapped at the anterior or moves with a normal posterior bias toward the posterior pole. Our results suggest that Staufen regulates several aspects of osk mRNA behavior once it enters the oocyte. First, it seems to be required for the efficient release of the mRNA from the anterior. This may reflect a role of Staufen in the coupling of the mRNA to the kinesin-dependent posterior transport pathway, osk mRNA particles that escape the anterior move with a normal bias but a reduced frequency, suggesting that Stau is also required for full kinesin activity. Finally, Stau is essential for the activation of osk mRNA translation once the mRNA has reached the posterior pole (Micklem et al., 2000). Thus, the in vivo analysis of osk mRNA dynamics reveals that different components of the osk mRNP complex are required for at least three distinct steps in the localization pathway, namely particle formation, uncoupling from the dynein/BicD pathway, and release from the anterior and coupling to the kinesin pathway, and this may begin to explain why so many trans-acting factors are required for the localization of this mRNA.

The dynamics of osk mRNA particles have several features in common with the behavior of MS2-labeled mRNAs in mammalian cells. Fusco et al. (2003) found that RNA particles undergo stochastic movements in COS cells, in which they switch between fast microtubule-dependent movements, diffusion, and stationary phases. Furthermore, an RNA containing the β-actin localization signal showed a 5-fold higher frequency of fast movements than a random RNA. This is very similar to the behavior of osk mRNA, which undergoes fast, direct movements 4–5 times more frequently in wild-type oocytes than in EJC, *Tml*, and stalk mutants. MS2-GFP has also been used to image Cam-KHz mRNA in the dendrites of cultured neurons and labels particles that show similar bidirectional movements to osk mRNA (Rook et al., 2000; Kanai et al., 2004). Since dendrites contain microtubules of mixed orientations and Stau, Barentsz, and kinesin...
have been implicated in dendritic mRNA localization, it will be interesting to determine whether CamKIIα mRNA localizes by a biased random walk similar to osk mRNA (Kanai et al., 2004; Macchi et al., 2003).

**EXPERIMENTAL PROCEDURES**

**Drosophila Stocks**

GFP-Stau was expressed from the maternal atu-tubulin promoter from a transgene on an X chromosome that also carries hsFLP (Martin et al., 2003). To generate the oskMS2 construct, we inserted 10 MS2-binding sites into an Spel site that was introduced immediately after the osk stop codon in a full-length osk genomic rescue construct (Munro et al., 2008). oskMS2 transgene inserts were recombined with MS2-GFP transgenes on the same chromosome (Forrest and Gavis, 2003).

Germline clones were generated using the evoD/Flp system by heat-shock second to third instar larvae for 2 hr at 37°C for 3 consecutive days (Chou and Perrimon, 1996).

Other fly strains used were FRT82B btau² (Palacios and St Johnston, 2002), TmPpE (Erdelyi et al., 1995), mago¹/FRT3F36 (Boswell et al., 1991), FRT 42B c Khc27/Cyo, FRT 42B c Khc27/Cyo, FRT 42B c Khc27/Cyo (Brendza et al., 2000), FRT40A hpr41020-9/Cyo, FRT40A hpr41020-9/Cyo (Huynh et al., 2004), oskFRT³/FRT3RipXT103 (Jenny et al., 2006), and Dhc6-8/Dhc6-17 (McGrail and Hays, 1997).

**Fast Imaging and Deconvolution**

Egg chambers were dissected directly onto coverslips in 10S Voltalef oil (Altac), imaging was performed on a wide field DeltaVision microscope (Applied Precision). Out-of-focus light was reassigned to its point of origin by iterative deconvolution.

**Particle Tracking and Image Analysis**

Moving particles were tracked manually using the Metamorph (Universal Imaging Corporation) image analysis software. To avoid bias in the selection of particles, we tracked all visible particles in each movie. We analyzed an average of 240 particle tracks per genotype from 3 to 50 oocytes. A custom program was used to calculate the average speed, distance, and directionality of each track from the original tracking data. A vector was taken between the first and last points of each track to calculate the overall direction of the movement. The proportion of moving particles was calculated by counting the number of particles in a selected 300 x 300 pixel region (19.89 μm x 19.89 μm) and determining how many of these particles underwent active movements during the next 10 frames.

The comparisons of speeds and net posterior displacements were performed using t tests and Wilcoxon rank-sum tests. The normality of the distribution was tested using the Jacque-Bera normality test. When normal, the significance of the difference in the means was assessed using a standard Student’s t test for distributions with equal variance, or the Welch-Satterthwaite-corrected Student’s t test for distributions with unequal variance.

The difference in the posterior versus anterior bias was assessed using the difference in the posterior versus anterior bias was assessed using the difference in the means was assessed using a standard Student’s t test for distributions with unequal variance, or the Welch-Satterthwaite-corrected Student’s t test for distributions with unequal variance.

The distribution was non-normal, we compared medians using the non-parametric counterpart of the Student’s t test, the Wilcoxon rank-sum test.

The difference in the posterior versus anterior bias was assessed using the standard χ² test. To test whether the net posterior displacement in wild-type was significantly larger than zero we performed a Wilcoxon 1-sample test.

**Drug Treatments**

The microtubule cytoskeleton was depolymerized by treating egg chambers with colchicine or colchicine (Sigma). Wild-type flies were starved for 1 day after hatching and then fed fresh yeast paste containing 100 μg/ml colchicine overnight (Pokrywka and Stephenson, 1995). Colchicine was inactivated by a 10 s pulse of UV light (Theurkauf and Haezling, 1998). For immersion experiments, we dissected ovaries in 20 μg/ml colchicine in Schneider’s media. Colchicine was injected into the oocyte at 100 μg/ml in water.

F-actin was depolymerized either by feeding females 200 μg/ml Latrunculin A in yeast paste or by dissecting ovaries in 50 μg/ml Latrunculin A.

**Supplemental Data**

Supplemental Data include one table, two figures, and thirteen movies and can be found with this article online at http://www.cell.com/cgi/content/full/134/5/843/DC1/.

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