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Kinesin light chain-independent function of the *Kinesin heavy chain* in cytoplasmic streaming and posterior localisation in the *Drosophila* oocyte

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SUMMARY

Microtubules and the Kinesin heavy chain, the force-generating component of the plus end-directed microtubule motor Kinesin I are required for the localisation of *oskar* mRNA to the posterior pole of the *Drosophila* oocyte, an essential step in the determination of the anteroposterior axis. We show that the Kinesin heavy chain is also required for the posterior localisation of Dynein, and for all cytoplasmic movements within the oocyte. Furthermore, the KHC localises transiently to the posterior pole in an *oskar* mRNA-independent manner. Surprisingly, cytoplasmic streaming still occurs in *kinesin light chain* null mutants, and both *oskar* mRNA and Dynein localise to the posterior pole. Thus, the Kinesin heavy chain can function independently of the light chain in the oocyte, indicating that it associates with its cargoes by a novel mechanism.

Movies available on-line

Key words: Motor proteins, Axis formation, Cytoplasmic streaming, RNA transport, Kinesin, *Drosophila*

INTRODUCTION

In *Drosophila*, the asymmetries that specify the embryonic axes are established early during oogenesis in a process that requires the precise localisation of several transcripts (Riechmann and Ephrussi, 2001; van Eeden and St Johnston, 1999). The localisation of *oskar* mRNA to the posterior pole of the oocyte specifies where the pole plasm forms, and thus where the abdomen and the germ line will develop (Ephrussi and Lehmann, 1992; Kim-Ha et al., 1991). At early stage 9 of oogenesis, *oskar* mRNA starts accumulating in a crescent at the posterior pole of the oocyte, where it stays until the early stages of embryogenesis (Ephrussi et al., 1991; Kim-Ha et al., 1991). Treatments with microtubule-depolymerising drugs disrupt this posterior localisation, indicating that it requires an intact microtubule cytoskeleton (Clark et al., 1994). Furthermore, the localisation of *oskar* mRNA seems to correlate with the organisation of the microtubules in the oocyte. During stages 7-8, a signal from the posterior follicle cells induces reorganisation of the oocyte cytoskeleton, to form an anterior to posterior gradient of microtubules that persists until stage 10 (Lane and Kalderon, 1994; Ruohola et al., 1991; Theurkauf et al., 1992). A marker for the plus-ends of microtubules, the motor domain of Kinesin heavy chain fused to β-Galactosidase (Kin-βGal) localises to the posterior during these stages, whereas the putative minus-end marker, nod-βGal, and the MTOC component, Centrosomin, localise to the anterior (Clark et al., 1994; Clark et al., 1997; Brendza et al., 2000). In addition, the microtubules at the anterior of the oocyte are more resistant to short treatments with microtubule-depolymerising drugs, indicating that the more stable minus ends lie at this pole (Theurkauf et al., 1992). Thus, the microtubule cytoskeleton appears to be organised with the majority of the minus ends at the anterior, with the plus ends extending towards the posterior pole (Cha et al., 2001; Theurkauf et al., 1992). Mutants in the heavy chain of the plus end-directed microtubule motor protein, Kinesin I abolish the posterior localisation of *oskar* mRNA (Brendza et al., 2000). These results suggest that Kinesin I actively transports *oskar* mRNA along microtubules towards the plus ends at the posterior pole. However, Cha et al. have recently shown that microtubule minus ends are associated with most of the oocyte cortex (Cha et al., 2002). As they detect *oskar* mRNA around the entire cortex of *Kinesin heavy chain* (*Khc*) mutant germline clones, they propose that Kinesin transports the RNA away from all regions of the cortex except the posterior pole. According to this model, Kinesin functions to exclude *oskar* mRNA from the anterior and lateral cortex, and another mechanism must therefore somehow deliver it to the posterior pole.

Conventional Kinesin, Kinesin I, was the first member of the Kinesin superfamily to be identified, and is responsible for the ATP-dependent transport of several distinct cargoes along microtubules, such as vesicles, membranous organelles and pigment granules (Brady, 1985; Goldstein and Yang, 2000; Vale et al., 1985; Yang et al., 1989). Kinesin I is composed of two Kinesin heavy chains (KHC) and two Kinesin light chains (KLC), each of which is encoded by several genes in vertebrates, but single-copy genes in *Drosophila*. The KHC has an N-terminal motor domain that contains both the microtubule and ATP binding sites, a central coiled-coil domain that

dimerises to form a bipartite stalk, and a globular C-terminal tail. The KLC binds to the C-terminal region of the heavy chain stalk through its N-terminal coiled-coil domain, while its C-terminal region contains six tetra-trico peptide (TPR) domains, which interact with cargo adaptors (Bowman et al., 2000; Gauger and Goldstein, 1993; Gindhart and Goldstein, 1996; Kamal et al., 2000; Verhey et al., 2001).

In addition to the heavy chain of Kinesin I, mutants in *staufen*, *barentsz*, *mago nashi*, *tsunagi* (*Drosophila* Y14) and *non-muscular cytoplasmic tropomyosin II* (*TmII*; *Tm1* – FlyBase) block the localisation of *oskar* mRNA to the posterior pole (Erdélyi et al., 1995; Hachet and Ephrussi, 2001; Micklem et al., 1997; Mohr et al., 2001; Newmark and Boswell, 1994; Tetzlaff et al., 1996; van Eeden et al., 2001). Staufen protein colocalises with *oskar* mRNA throughout oogenesis in both wild-type and mutant egg chambers, and its posterior localisation is *oskar* mRNA-dependent and vice versa (Ferrandon et al., 1994; St Johnston et al., 1991). The protein contains five dsRNA binding domains, and this RNA-binding activity is essential for *oskar* mRNA localisation (Ramos et al., 2000; St Johnston et al., 1992). Thus, Staufen presumably binds directly to *oskar* mRNA and is a reliable marker of the localisation of the transcript. Barentsz, Mago nashi and Tsunagi/Y14 are also likely to be components of the *oskar* mRNA localisation complex, as they colocalise with the mRNA to the posterior pole (Hachet and Ephrussi, 2001; Mohr et al., 2001; Newmark et al., 1997; van Eeden et al., 2001). Kinesin I has not been shown to interact with any of these proteins, however, and it is unclear whether the motor interacts with the *oskar* mRNA complex directly.

Glotzer et al. have proposed an alternative model for *oskar* mRNA localisation, in which cytoplasmic flows circulate the mRNA around the oocyte, and it is then trapped at the posterior by a pre-localised anchor (Glotzer et al., 1997). In this model, the microtubules and kinesin would be required to generate the cytoplasmic flows that facilitate the diffusion of the mRNA towards this anchor. The bulk movement of the cytoplasm in the egg chamber has been previously studied by video-enhanced contrast microscopy, and by time-lapse films using confocal imaging of fluorescent yolk granules (Gutzeit, 1986a; Gutzeit and Koppa, 1982; Theurkauf et al., 1992; Bohrmann and Biber, 1994). From stage 7 onwards, nurse cell cytoplasm flows through the ring canals into the oocyte, where it is efficiently mixed with the ooplasm by cytoplasmic movements in the oocyte, called streaming. The ooplasmic streaming at stage 9 consists of random motions in several directions, but the nature of these movements changes dramatically at stage 10b when streaming becomes unidirectional, and around five times faster (Bohrmann and Biber, 1994). Actin-depolymerising drugs block the nurse cell-to-oocyte movements, but not streaming within the oocyte. By contrast, microtubule-disrupting drugs have no effect on the nurse cell-to-oocyte flow, but block both the slow streaming in the oocyte at stage 9 and the more rapid streaming at 10b (Gutzeit, 1986b; Gutzeit, 1986a; Bohrmann and Biber, 1994). The microtubules rearrange to form parallel arrays 5-10 μm below the oocyte cortex when the stage 10b streaming begins, and vesicles are observed in close proximity to these microtubules, suggesting that the movement is driven by vesicle transport along microtubules (Theurkauf et al., 1992).

We have analysed the role of Kinesin I in the oocyte and showed that it is required for all cytoplasmic movements and posterior localisations in the oocyte. Surprisingly, the Kinesin light chain is not required for any of the functions of kinesin in the oocyte, indicating that it associates with its cargo by a novel mechanism.

MATERIALS AND METHODS

Fly stocks and germline clones

All germline clones were generated by the FLP recombinase system (Chou et al., 1993; Chou and Perrimon, 1996) using either the lack of GFP as a marker for homozygous clones, or the ovoD system, in which only homozygous mutant oocytes develop further than stage 4/5 of oogenesis. Two *Kinesin light chain* alleles, the null allele klc^{8ex94} and the hypomorphic allele klc^{1} (P-element insertion) (Gindhart et al., 1998), and a null *sunday driver* allele, syd^{z4} (Bowman et al., 2000), were recombined into the FRT2A chromosome. The presence of the null allele klc^{8ex94} in the chromosome used to induce the germline clones was confirmed by PCR analysis and lethality of the homozygous larvae. The null allele btz^{2} was recombined into the FRT82B chromosome (van Eeden et al., 2001). Germline clones were generated by heat shocking second instar larvae for 2 hours at 37°C for 3 consecutive days. Other fly stocks used were $stau^{D3}$ (St Johnston et al., 1991), $TmII^{gs}$ (Erdélyi et al., 1995), $mago^{1}/Df(2R)F36$ (Boswell et al., 1991), $Dhc64c^{6-6}$, $Dhc64c^{6-12}$ (McGrail and Hays, 1997), $Dhc64C^{greco}$ (Bolivar et al., 2001) and FRT42B c Khc^{27}/CyO (Brendza et al., 2000). A hs-FLP, GFP-Staufen line created by mobilising a GFP-Staufen transgene (Schuldt et al., 1998) onto a hs-FLP X chromosome was also used (K. Litière and D. St J., unpublished).

Whole-mount in situ hybridisation and antibody staining

Females were fattened for 24 hours, and the ovaries dissected in PBT (PBS+0.1%Tween), fixed for 20 minutes in 4% paraformaldehyde/PBT, washed with PBT and kept in methanol at –20°C.

For antibody staining, the ovaries were then washed with PBT, blocked with PBT-10 (PBT +10% BSA) for 1 hour and incubated with the antibody in PBT-1 (+1% BSA) for 12 hours. After washing the ovaries with PBT-1 several times for 30 minutes, they were incubated with the secondary antibody for at least 3 hours. They were finally washed three times with PBT for 15 minutes and mounted in Vectashield (Vector). All steps were performed at room temperature. The following antibodies were used: rabbit anti-Staufen (1:1000) (St Johnston et al., 1991), monoclonal P1H4 anti-Dhc64C (1:200) (McGrail and Hays, 1997) and rabbit anti-KHC (1:250, Cytoskeleton company).

For RNA in situ hybridisation, the ovaries were rehydrated after the methanol treatment with PBT, post-fixed for 5 minutes with 4% paraformaldehyde/PBT, and washed three times for 10 minutes with PBT. They were then incubated in the pre-hybridisation buffer (50% formamide, 5×SSC, 0.1% Tween-20 pH 4.5) for 1 hour at 70°C, and then overnight at 70°C with the *oskar* mRNA antisense digoxigenin (DIG)-UTP probe in hybridisation buffer (pre-hybridisation + Boerhinger Calf tRNA 0.1 mg/ml + heparin 0.05 mg/ml). After several washes at 70°C with a 1:1 mix of pre-hybridisation:PBT, and at room temperature with PBT, the ovaries were incubated for 1 hour with an anti-DIG antibody coupled to either Alkaline Phosphatase (Roche) or to Cy3 (Jackson ImmunoResearch). In the first case, the ovaries were then washed with PBT three times for 20 minutes and once with staining solution (100 mM NaCl, 50 mM $MgCl_2$, 100 mM Tris pH 9.5, 0.01% Tween-20). The reaction was developed by the addition of NBT:BCIP kit from Promega following the manufacturer's instructions. Finally, they were washed several times with PBS and mounted in 50% glycerol. In the second case, the ovaries were washed and mounted as previously described for the antibody staining.

Analysis of cytoplasmic movements

Females were fattened for 24 hours and dissected in 10S Voltalef oil (Altachem). All time-lapse movies of living egg chambers were taken using either a Nipkow spinning disk confocal or a BioRad confocal MRC1024 and a Nikon inverted microscope. On the Nipkow spinning disk confocal we made 15 minutes time lapse films by collecting images every 2 seconds. On the BioRad confocal, the time-lapse movies were obtained by collecting *z* series of five sections at 2 µm intervals every minute for 45 minutes. Three distinct type of particles were analysed: GFP-Staufen particles, uncharacterised vesicles that were visualised by exciting the sample with the 568 nm wavelength light, and collecting the emission through a OG515 or OPEN filter; and the yolk granules. The tricolor images shown in Fig. 2A-J were obtained by creating a Photoshop RGB file in which three consecutive images of a time lapse movie were inserted in the red, green and blue channel, respectively. The Kalman images shown in Fig. 2K-P, Fig. 3 and Fig. 6 were obtained by using the Kalman averaging function of the confocal microscope to merge successive scans in one composite image. The oocytes were laser scanned either once (1) or in a continuous manner for 15 (15) or 30 (30) times, and the composite image is shown.

RESULTS

To determine whether kinesin functions in the same step of *oskar* mRNA localisation as the other proteins required for this process, we compared the distribution of the RNA in germline clones of a null allele of the Kinesin heavy chain, *Khc^{27}*, to *barentsz*, *staufen* and *mago nashi* mutants. Although no *oskar* mRNA reaches the posterior of the stage 9 oocyte in *Khc^{27}*, there is a clear difference in the distribution of the mRNA from that observed in the other mutants, such as *barentsz*. In the latter, *oskar* mRNA remains tightly localised at the anterior cortex, whereas it is found throughout the anterior half of the oocyte in *Khc^{27}* mutant clones (Fig. 1A-D). We also performed fluorescent in situ hybridisation to examine the distribution of *oskar* mRNA in the *Khc* mutant in more detail, using confocal microscopy. This reveals an anterior-to-posterior gradient of mRNA with an enrichment along the lateral cortex (Fig. 1E,F). Consistent with this, antibody staining for Staufen protein show an identical distribution to *oskar* mRNA (Fig. 1G,H). These results suggest that the *Khc* mutant blocks *oskar* mRNA localisation after it has been released from the anterior cortex, whereas all of the other factors are required for this release.

The Kinesin heavy chain is required for cytoplasmic streaming in the oocyte

One way to distinguish between the models for *oskar* mRNA localisation is to observe how the mRNA moves to the posterior in living oocytes. A GFP-Staufen fusion protein rescues the *oskar* mRNA localisation defect of a *staufen* null mutant and localises to the posterior (Fig. 2A) (Schuldt et al., 1998), and we attempted to use this fusion to visualise the movement of Staufen/*oskar* mRNA complexes in vivo. To detect particles that move at similar speeds to kinesin-dependent fast axonal transport, we made 15 minute time-lapse films by collecting images every 2 seconds on a Nipkow spinning disc confocal microscope (see Movie 1 at http://www.welc.cam.ac.uk/~dstjlab/isa_movies/isa_mov_index.htm). Although many GFP-Staufen particles undergo rapid movements in the nurse cells, we could not detect any fast-moving particles in the oocyte, and observed that most particles move at much slower rates that are hard to see at this time scale. We therefore generated slower time lapse films by imaging once a minute for 45 minutes on a standard confocal microscope, and observed bright GFP-Staufen particles that move from the anterior towards the posterior of the oocyte (see Movie 2 at http://www.welc.cam.ac.uk/~dstjlab/isa_movies/isa_mov_index.htm). These movements require functional kinesin, as most particles remain stationary in *Khc^{27}* mutant germline clones (see Movie 3 at http://www.welc.cam.ac.uk/~dstjlab/isa_movies/isa_mov_index.htm). This can be easily visualised by importing three successive images from the time lapse films into the red, green and blue channels of a Photoshop file (Fig. 2A-D) (Theurkauf and Hazelrigg, 1998). If a GFP-Staufen particle moves between scans, it appears as separate red, green and blue dots, and the distance between them can then be measured to calculate its speed. However, stationary

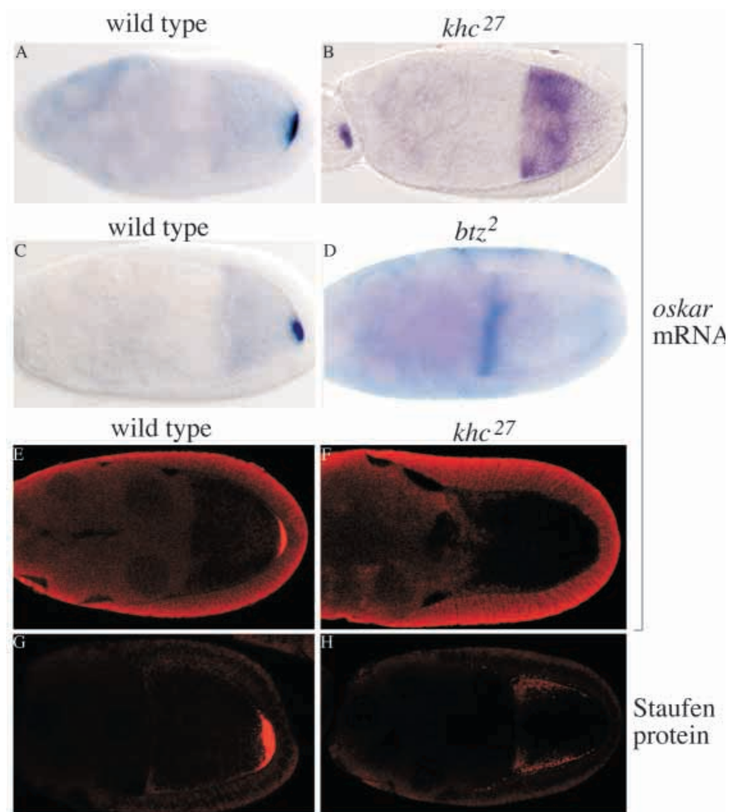

Fig. 1. *Kinesin heavy chain* (*Khc*) mutants block *oskar* mRNA localisation after the transcript has been released from the anterior cortex. Localisation of *oskar* mRNA (A-F) and Staufen protein (G,H) at stage 9 of oogenesis in wild-type and *Khc* mutant ovaries. In wild-type egg chambers, *oskar* mRNA (A,C,E) and Staufen protein (G) localise to the posterior pole of the oocyte and remain anchored there throughout oogenesis. This localisation is completely abolished in ovaries that are mutant for *Khc*, and both *oskar* mRNA and Staufen protein are detected at the anterior and throughout the anterior half of the oocyte (B,F,H). By contrast, *oskar* mRNA is found at the anterior cortex in *barentsz* (D). *staufen* and *mago nashi* mutants show an identical phenotype (I. M. P. and D. St J., unpublished). *oskar* mRNA in situ hybridisation was performed with an anti-DIG antibody coupled either to alkaline phosphatase (A-D) or to Cy3 (E,F). Anterior is towards the left, posterior towards the right.

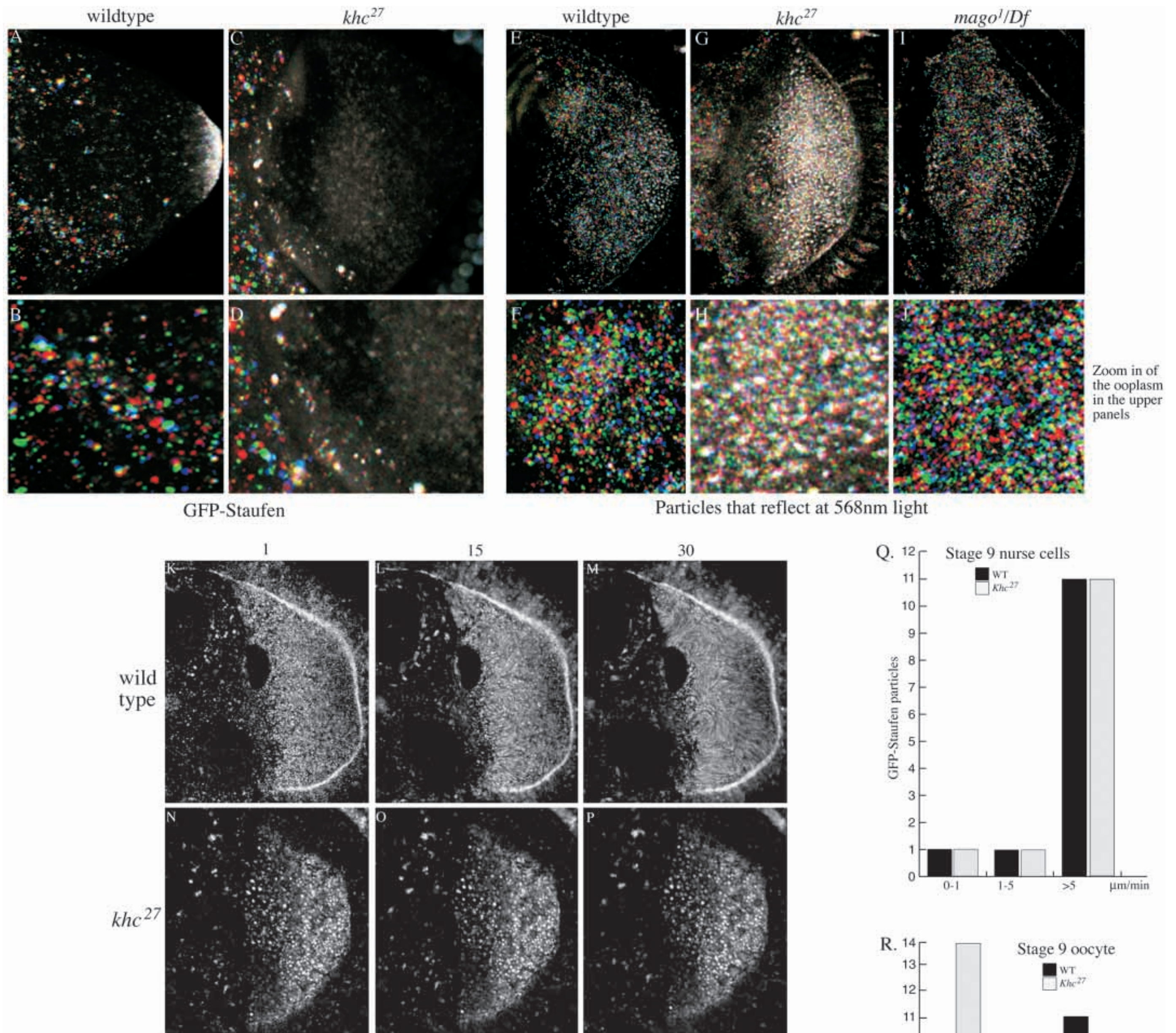

Fig. 2. The Kinesin heavy chain is required for ooplasmic movement at stage 9. (A-D) Movement of GFP-Staufen particles in wild-type (A,B) and *Khc* mutant (C,D) egg chambers. Each image shows three consecutive time points of a time lapse movie that have been imported into the red, green and blue channels of a Photoshop file. In the nurse cell cytoplasm, the movement of GFP-Staufen particles in the *Khc* mutant is indistinguishable from that in wild type. In the ooplasm, however, most of the GFP-Staufen particles are static in the *Khc* mutants (white particles), and the ones that do move lie close to the ring canals. (E-J) Movement of particles that reflect 568 nm light in wild-type (E,F), *Khc* (G,H) and *mago nashi* (I,J) mutant egg chambers. In the *Khc* mutant, most of the particles in the oocyte are stationery (white particles), whereas all the particles in wild type and in *mago nashi* mutant oocytes move between time points. In *staufen*, *barentsz* and *Tropomyosin II* mutant oocytes, these particles also show the same movement as in wild type (I. M. P. and D. St J., unpublished). (K-P) Movement of particles that reflect 568 nm light in wild type (K-M) and *Khc* mutant (N-P) egg chambers. The oocytes were scanned either once (1; K,N) or in a continuous manner using the Kalman function of the confocal microscope for 15 (15; L, O) or 30 (30; M, P) scans, and the composite image is shown. Each scan lasts 7 seconds, so the images represent either 7 seconds (K,N), 105 seconds (L,O) or 210 seconds (M,P) of real time. The ooplasmic streaming observed in a stage 9 wild-type oocyte (K-M) is completely abolished in the *Khc* mutant oocyte (N-P). (Q,R) Quantitation of the movement of GFP-Staufen particles in wild-type and *Khc* mutant egg chambers. Movement of GFP-Staufen particles in wild-type (black boxes) and *Khc* mutant (grey boxes) egg chambers. The graphic shows the number of particles (*x*-axis) that move with a certain speed (*y*-axis) in both the nurse cells (Q) and the oocyte (R). In *Khc* mutant egg chambers, no movement of GFP-Staufen particles within the oocyte was observed, although the motion of these particles within the nurse cells was unaffected. This analysis does not consider the movements of the particles in the *z*-axis, but a more detailed analysis of the time lapse movies reveals that the particles move much less in this dimension.

particles appear white, due to the superposition of the red, green and blue signals. Although almost all particles in wild-type oocytes are motile, the majority of particles in *Khc*[27] oocytes are stationary, except for those in the vicinity of the ring canals (Fig. 2A-D,Q, R). This defect on the movement of GFP-Staufen particles was completely rescued by a wild-type *Khc* transgene. Kinesin is not required, however, for the motion of these particles in the nurse cells, or for their movement from the nurse cells into the oocyte. Quantification of these data reveals that the particles show similar movements in the nurse cells of wild-type and mutant egg chambers (average velocities of 5.0 μm per minute), but are much less motile in mutant oocytes (5 μm compared with 0.5 μm per minute). Indeed, the majority of particles that do move in mutant oocytes lie in the vicinity of the ring canals, and may be carried by the flow of cytoplasm from the nurse cells.

Unfortunately, the GFP-Staufen particles are unlikely to represent Staufen/*oskar* mRNA complexes in transit to the posterior, because untagged Staufen is not found in large particles unless it is overexpressed, and the GFP-Staufen is expressed at much higher levels than the endogenous protein, which is already present in excess over *oskar* mRNA (Ferrandon et al., 1994). Furthermore, the GFP-Staufen particles do not accumulate at the anterior of the oocyte in *mago nashi* and *barentsz* mutants, in contrast to *oskar* mRNA and endogenous Staufen (I. M. P. and D. St J., unpublished). Thus, they most probably correspond to aggregates of overexpressed protein. Although a proportion of the GFP-Staufen must associate with *oskar* mRNA to mediate its posterior localisation, and accumulates with it at the posterior pole, we have been unable to determine how it gets there, presumably because it localises in particles that are either too small, too dim or too rare to image in this way.

Although the particles probably do not reflect the localisation of *oskar* mRNA, they do undergo dramatic kinesin-dependent movements in the oocyte, which could be caused either by active transport or by cytoplasmic flows. To distinguish between these possibilities, we compared their movements with those of the surrounding cytoplasm, which can be follow by visualising a particle in the oocyte that reflects 568 nm light, and which may correspond to a peroxisome. In wild-type oocytes, the red particles always move in the same direction and at the same speed as the GFP-Staufen particles in their vicinity, even though their relative positions rule out the possibility that they are attached to the same motor or microtubule (see Movie 4 at http://www.welc.cam.ac.uk/~dstjlab/isa_movies/isa_mov_index.htm). These observations, and the fact that the particles move much slower than in other kinesin-dependent transport processes indicate that these movements correspond to cytoplasmic flows. As is the case for GFP-Staufen, the removal of kinesin blocks virtually all movement of the red particles in the oocyte, but has no effect on their motion within the nurse cells or on nurse cell to oocyte transport (Fig. 2E-J; see Movie 5 at http://www.welc.cam.ac.uk/~dstjlab/isa_movies/isa_mov_index.htm). The yolk vesicles also fail to move in mutant oocytes, although the uptake of the yolk from the follicle cells is unaffected, and the oocyte grows at the normal rate (I. M. P. and D. St J., unpublished).

As an alternative way to visualise the cytoplasmic movements in the oocyte, we used the Kalman averaging function of the confocal microscope to merge successive scans into a composite image. Although Kalman averaging is normally used to reduce noise in images of stationary objects, moving particles become increasingly blurred as the number of scans is increased, and the direction of blurring indicates the orientation of their motion. Using this technique to image the red fluorescent particles in wild-type egg chambers reveals multiple flows in different directions, which vary from oocyte to oocyte (Fig. 2K-P). The fastest flows are always seen at the anterior of the oocyte, while the region around the posterior pole remains fairly quiescent (Fig. 2K-M). By contrast, no flows can be detected in *Khc* mutant oocytes, and the particles gradually become fuzzy because of Brownian motion as the number of scans increases (Fig. 2N-P).

These results show that the Kinesin heavy chain is required for all detectable cytoplasmic movements within the oocyte. This raises the question of the relationship between these flows and *oskar* mRNA localisation, and we therefore examined the movements of the red fluorescent particles in other mutants that block the posterior localisation of *oskar* mRNA. The ooplasmic movements are indistinguishable from those in wild type in *staufen*, *mago nashi*, *barentsz* and *TmII* mutant egg chambers (Fig. 2G,H; see Movie 6 at http://www.welc.cam.ac.uk/~dstjlab/isa_movies/isa_mov_index.htm). These results show that the kinesin-dependent ooplasmic flows are caused by the movement of some other structure than the *oskar* mRNA localisation complex.

When the nurse cells transfer most of their contents into the oocyte at stage 10b, the oocyte cytoplasm starts to flow unidirectionally around the oocyte at a speed that is about five times faster than at stage 9 (Fig. 3A-C). Although rapid streaming at stage 10b is distinct from the stage 9 flows, it also depends on the Kinesin heavy chain, as it does not occur in stage10b *Khc* mutant oocytes (Fig. 3D-F). This streaming defect is completely rescued by a wild-type *Khc* transgene. This effect is apparent in an even single time section. Normally, the material that enters the anterior of the oocyte from the nurse cells is efficiently dispersed by streaming throughout the ooplasm. In *Khc* mutants, however, this material does not mix with the rest of the cytoplasm, and the oocyte constituents become stratified. For example, fluorescent particles that are transported from the nurse cells into the oocyte accumulate at the anterior, while yolk spheres, which are endocytosed from the follicle cells, are excluded from the anterior and remain cortical. Thus, Kinesin is required for both the slow chaotic cytoplasmic flows at stage 9, and the faster directional flows at stage 10b, suggesting that a common mechanism generates both movements.

The Kinesin heavy chain localises to the posterior pole of the oocyte

To further understand the function of Kinesin I in *oskar* mRNA localisation and cytoplasmic streaming within the oocyte, we examined the distribution of the KHC itself, and found that it is strongly localised at the posterior of the oocyte from stage 9-10a. This staining is specific, as it is not observed in *Khc* mutant germline clones (Fig. 4A,C). The microtubule cytoskeleton in the stage 9 oocyte is polarised with the plus-ends towards the posterior, and this is therefore where one would expect an active plus-end directed motor, like Kinesin I, to accumulate. The activity of Kinesin I is believed to require cargo binding (Coy et al., 1999; Friedman and Vale, 1999). This localisation therefore strongly suggests that the motor

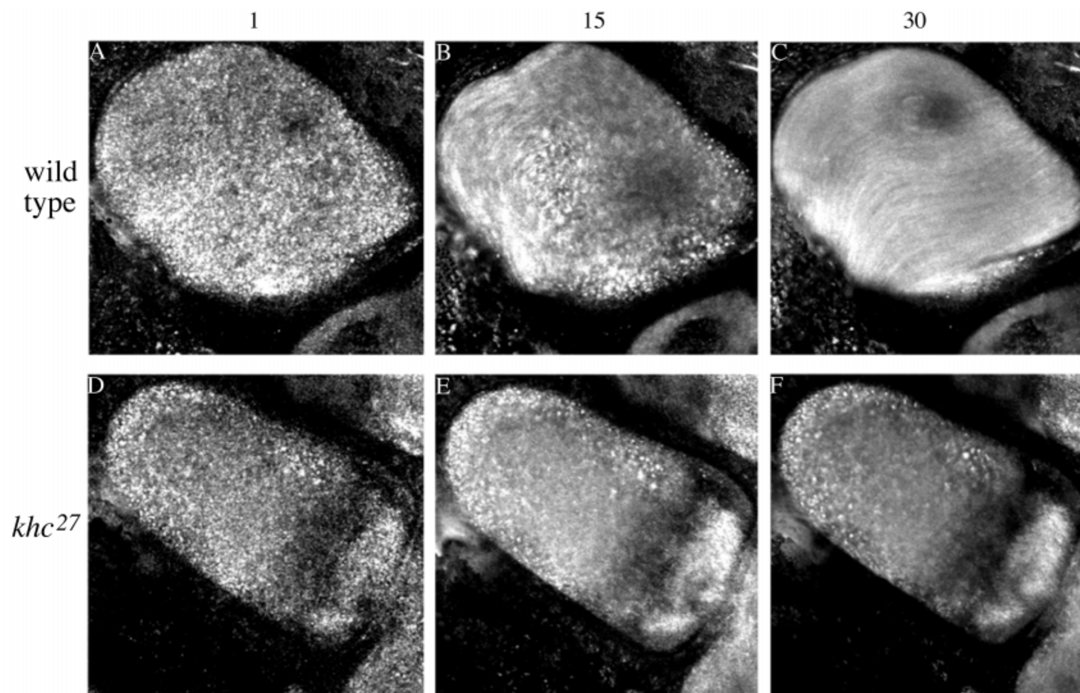

Fig. 3. The Kinesin heavy chain is required for ooplasmic movement at stage 10b. Movement of particles that reflect 568 nm light in wild-type (A-C) and *Khc* mutant (D-F) egg chambers. The oocytes were scanned either once (1; A,D) or continuously using the Kalman function of the confocal microscope for 15 (15; B,E) or 30 (30; C,F) scans. The ooplasmic streaming observed in wild-type oocyte is completely blocked in *Khc* mutant oocytes.

protein transports something along microtubules to the posterior pole. However, the KHC still accumulates at the posterior both in *staufen* mutant oocytes, in which *oskar* mRNA is not localised (Fig. 4B). Thus, Kinesin I presumably transports something else to the posterior pole, in addition to *oskar* mRNA.

The Kinesin heavy chain is required for Dynein heavy chain localisation to the posterior pole of the oocyte

Another candidate cargo for Kinesin I is the Dynein heavy chain (Dhc64C, DHC), which also localises to the posterior of the oocyte during stage 9 of oogenesis (Li et al., 1994) (Fig. 4D). DHC localisation is not required for the posterior localisation of Staufen and *oskar* mRNA, as they both localise normally in a combination of hypomorphic *Dhc64C* alleles that abolishes the posterior localisation of DHC (McGrail and Hays, 1997). Furthermore, DHC localisation is independent of the posterior localisation of *oskar* mRNA, because it shows a wild-type accumulation at the posterior in *staufen*, *barentsz* and *mago nashi* mutant egg chambers (Fig. 4E; I. M. P. and

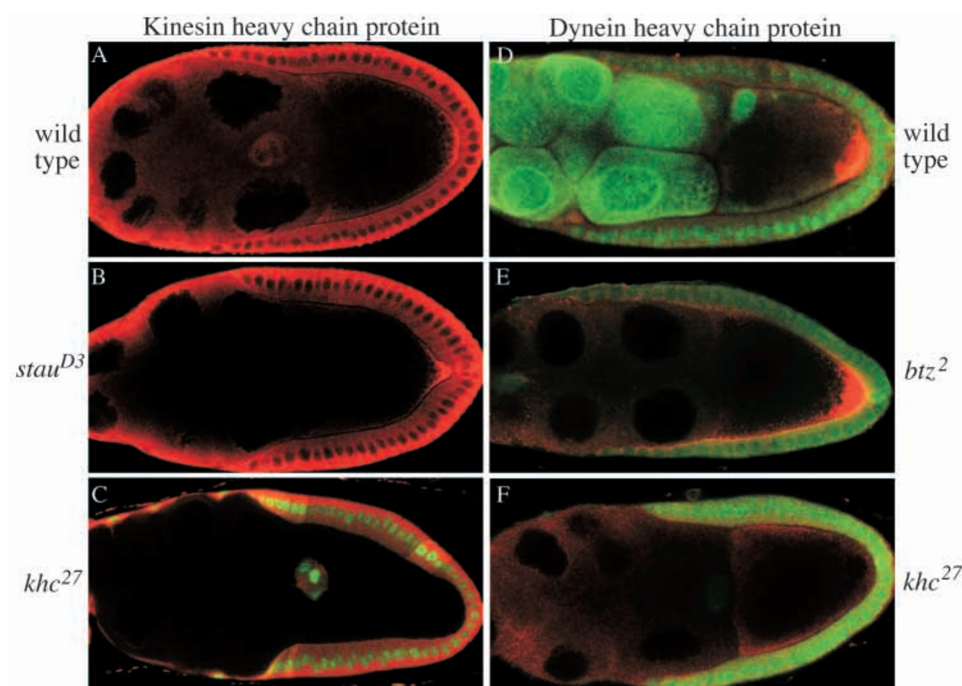

Fig. 4. The Kinesin heavy chain and the Dynein heavy chain localise to the posterior pole of the oocyte. (A-C) Localisation of the KHC in wild-type (A), *staufen* (B) and *Khc* (C) mutant egg chambers. KHC localises to the posterior of the oocyte at the stage when *oskar* mRNA is localised to this pole (A). The posterior localisation of KHC does not depend on *oskar* mRNA, however, as KHC is also detected at the posterior in *staufen* mutant egg chambers (B). KHC staining is absent in the *Khc* null germline clones, demonstrating the specificity of the antibody (C). (D-F) Localisation of DHC to the posterior pole of wild-type (D), *barentsz* (E) and *Khc* (F) mutant oocytes. DHC localises to the posterior pole of the stage 9 oocyte (D). This localisation is completely abolished in the *Khc* mutant oocytes (F), but it is the same as in wild type in *barentsz* (E). DHC localisation is also the same as in wild type in *staufen* and *mago nashi* mutant egg chambers (I. M. P. and D. St J., unpublished). The mutant clones are marked by the absence of nuclear GFP.

D. St J., unpublished). By contrast, DHC shows no posterior enrichment in Khc^{27} germline clones (Fig. 4F). This localisation defect is completely rescued by a wild type *Khc* transgene. Thus, the KHC is required for the posterior localisation of both DHC and *oskar* mRNA, even though neither is required for the localisation of the other. The KHC still accumulates at the posterior in *Dhc64C* mutant oocytes (I. M. P. and D St J., unpublished). Thus, Kinesin I presumably transports something else to the posterior pole, in addition to *oskar* mRNA and DHC.

The functions of kinesin and dynein appear to be interdependent in neurones, because mutants or inhibitors of either motor block both anterograde and retrograde fast axonal transport (Brady et al., 1990; Martin et al., 1999; Stenoien and Brady, 1997; Waterman-Storer et al., 1997). To test whether this is also the case in the oocyte, we examined whether the hypomorphic *Dhc64C* mutant combination has any effect on streaming. The cytoplasmic flows still occur in this mutant, but they are significantly slower than normal. Although this is consistent with the idea that dynein and kinesin are interdependent, it is not possible to test whether the cytoplasmic flows would be completely abolished in the absence of dynein, because the null mutants block oocyte determination (McGrail and Hays, 1997).

Kinesin light chain and *Sunday driver* are not required for ooplasmic streaming and posterior localisation

The discovery that the KHC is required in the oocyte for the posterior localisation of *oskar* mRNA, the posterior localisation of DHC and for cytoplasmic streaming raises the question of whether these reflect three independent functions of the motor, or whether they all depend on a common underlying process. One way to address this question is to determine whether the three functions require different factors to couple kinesin to its cargoes. One of the main cargo adaptors in *Drosophila* neurones is Sunday driver (Syd), as *syd* mutants cause the same defects in axonal transport as null mutants in either the Kinesin heavy chain or light chain (Bowman et al., 2000). We therefore examined the phenotypes of *syd* null germline clones. The posterior localisations of both Staufen and DHC are indistinguishable from wild-type in *syd* mutants (Fig. 5A,B,D,E). Furthermore, the absence of Syd has no effect on the rate of cytoplasmic streaming at either stage 9 or stage 10b (Fig. 6A-C; I. M. P. and D. St J., unpublished) (Table 1).

The lack of a phenotype in *syd* germline clones prompted us to investigate the role of the KLC in these processes, by generating germline clones of a hypomorphic allele, klc^{1}, or a null allele, klc^{8ex94}, which is a deletion of the entire coding region (Gindhart et al., 1998). Staufen still forms a normal posterior crescent in 100% of both the klc^{1} and the klc^{8ex94} mutant egg chambers (Fig. 5C). Occasionally, however, it can also be found in a dot near the posterior of the oocyte, which is often connected to the posterior crescent (I. M. P. and D. St J., unpublished). DHC also localises to the posterior in the absence of the KLC, although the amount is reduced compared with wild type (Fig. 5F). These subtle phenotypes are completely different from those caused by loss of the KHC, and indicate that the light chain may be required for the efficiency of posterior localisation, but that it is not essential for this process. One possible explanation for the lack of a requirement for the KLC is that the phenotype is rescued by the perdurance of the wild-type protein that is synthesised in the heterozygous germline stem cells before the clones were induced. To test this possibility, we examined egg chambers from germline clones that had been induced two weeks earlier, and observed the same effects on *oskar* mRNA and DHC localisation. As the germline stem cells divide every 12-16 hours, they should have gone through over 20 divisions in this period, ruling out the possibility that any wild-type protein survives.

Finally, we examined the cytoplasmic streaming in *Klc* mutant oocytes. Unlike the heavy chain, the KLC is not required for streaming at either stage 9 or stage 10b, although the movements are often less vigorous than in wild type (Fig. 6D-F; I. M. P. and D. St J., unpublished). Thus, the KLC is therefore dispensable for the three KHC-dependent processes that we have examined in the oocyte, suggesting that they may be related.

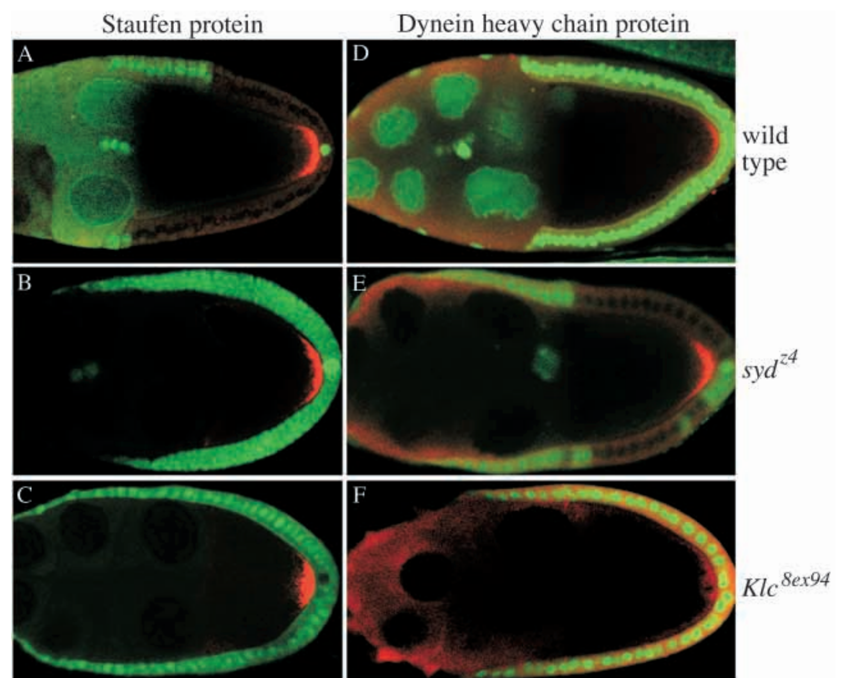

Fig. 5. The *Kinesin light chain* (*Klc*) and *sunday driver* are not essential for the posterior localisation of Staufen and Dynein. Localisation of Staufen (A-C) and the dynein heavy chain (D-F) at the posterior pole of the oocyte in wild-type (A,D), *sunday driver* (*syd*; B,E) and *Klc* (C,F) mutant egg chambers. In *syd* mutant oocytes, the localisation of Staufen (B) and DHC (E) proteins is indistinguishable from wild type (A,D). In *Klc* mutant egg chambers, Staufen (C) and DHC (F) are transported from the anterior to the posterior as in wild type, although the Staufen posterior crescent is occasionally not as tight as in wild type (I. M. P. and D. St J., unpublished), and the amount of DHC at the posterior is reduced. The mutant clones are marked by the absence of nuclear GFP.

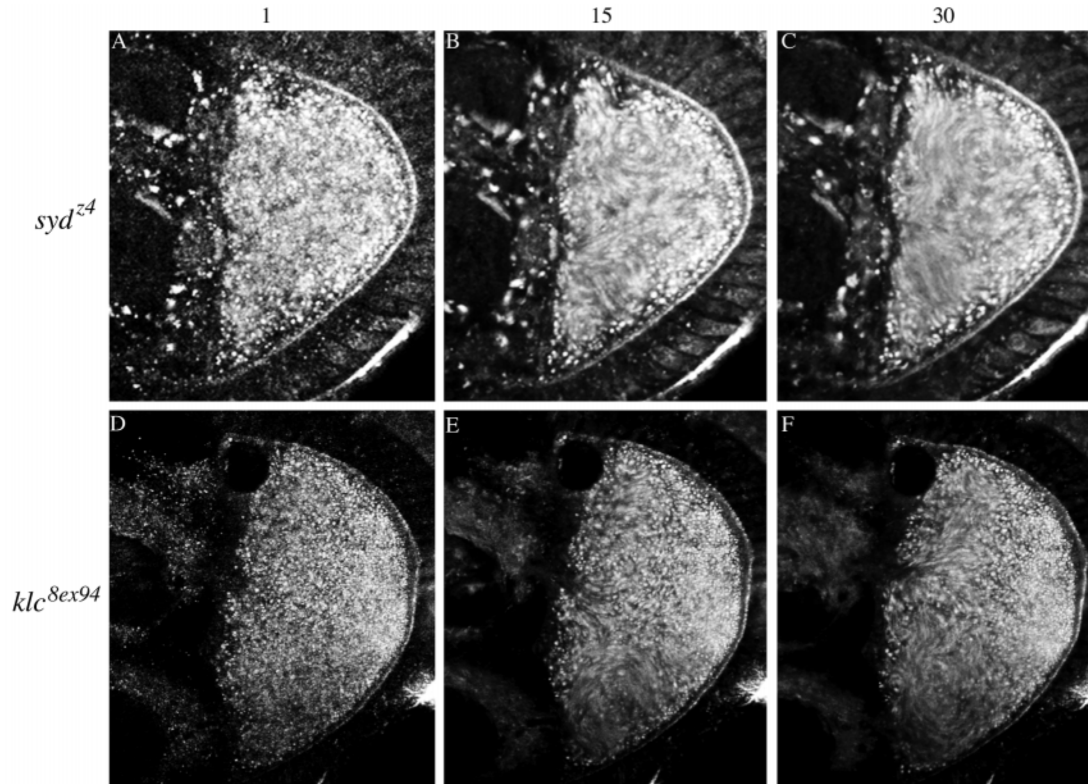

Fig. 6. The *Kinesin light chain* (*Klc*) and *sunday driver* are not required for ooplasmic streaming. Movement of red fluorescent particles in *sunday driver* (*syd*; A-C) and *Klc* (D-F) stage 9 mutant egg chambers. The oocytes were scanned either once (1; A,D) or continuously using the Kalman function of the confocal microscope for 15 (15; B,E) or 30 (30; C,F) scans. The cytoplasmic streaming observed in *Klc* and *syd* stage 9 mutant oocytes is analogous to wild type (see Fig. 2K-M). In *Klc* mutant oocytes, however, the streaming seems to be less vigorous, as the cytoplasmic movements in the most posterior region of the oocyte are reduced.

DISCUSSION

The Kinesin heavy chain functions independently of the light chain in the oocyte

Several lines of evidence have suggested that the light chain is essential for the function of conventional kinesin in vivo. Mutants in the *Drosophila light chain* are lethal, and produce the same block in fast axonal transport as mutants in the *kinesin heavy chain*, leading to axonal swelling and progressive posterior paralysis (Gindhart et al., 1998; Hurd and Saxton, 1996; Saxton et al., 1991). Mutants in one of the mouse KLC genes also interfere with the function of the heavy chain, by causing its aberrant accumulation near the *cis*-Golgi (Rahman et al., 1999). One proposed role for the light chain is to regulate the activity of the motor domain. The light chain inhibits the ATPase activity of the motor in vitro, and co-transfection experiments in tissue culture cells have demonstrated that it represses the binding of the heavy chain to microtubules (Hackney, 1994; Hackney et al., 1991; Kuznetsov et al., 1989; Stewart et al., 1993; Verhey et al., 1998). As the phenotypes of *Klc* mutants indicate that it also plays a positive role in kinesin function, it may inhibit motor activity in the absence of cargo,

Table 1. Analysis of Staufen and Dynein heavy chain posterior localisation and ooplasmic streaming in several mutant backgrounds

	Ooplasmic streaming at stage 9	Ooplasmic streaming at stage10b	Staufen at the posterior	DHC at the posterior
Khc^{27}*	−−−	−−−	−−−	−−−
$stau^{D3}$	+++	+++	−−−	+++
btz^{2}*	+++	+++	−−−	+++
$mago^{1}/Df$	+++	+++	−−−	+++
$TmII^{gs}$	+++	+++	−−−	nd
syd^{z4}*	+++	+++	+++	+++
$Khc^{27}/+;Klc^{8ex94}/+$	+++	nd	+++	+++
$Khc^{27}/+;Dhc64C^{greco}/+$	+++	nd	+++	+++
$Dhc64C^{6\text{-}6}/Dhc64C^{6\text{-}12}$	++	nd	+++	−−−
Klc^{8ex94}*	++	++	++	+/++

*Germline clones.
nd, not defined.

but activate it upon cargo binding (Kamal and Goldstein, 2000).

A second essential function of the light chain is to couple the heavy chain to its cargoes (Kamal and Goldstein, 2002). A genetic screen for mutants that disrupt axonal transport led to the identification of a highly conserved membrane protein, Syd, which binds to the TPR repeats of the KLC (Bowman et al., 2000). As *syd* mutants cause the same defects as null mutants in the *Klc* and *Khc*, and the protein is found on vesicles, it is likely to be a major cargo adaptor in axonal transport. The mammalian homologue of Syd, JIP3/JSAP1, was isolated in an independent screen for cargo adaptors, along with two other Jun N-terminal kinase interacting proteins, JIP1 and JIP2, which also bind to the TPR domains of the light chain and link the motor to several cytoplasmic and transmembrane protein cargoes (Verhey et al., 2001). The amyloid precursor protein (APP) and the related APP-like proteins constitute a second family of cargo adaptors that bind to the same region of the light chain (Kamal et al., 2000). Studies in both mice and *Drosophila* have shown that these proteins are required to couple kinesin to specific vesicular cargoes that are transported along axons (Gunawardena and Goldstein, 2001; Kamal et al., 2001). The heavy chain probably makes some contribution to cargo-binding, because it has been shown to interact with microsomal membranes (Skoufias et al., 1994). In addition, the microsomal transmembrane protein Kinectin co-purifies with kinesin, and binds directly to the C-terminal region of the heavy chain (Kumar et al., 1995; Ong et al., 2000; Toyoshima et al., 1992). There is no Kinectin homologue in *C. elegans* or *Drosophila*, however, whereas the mouse Kinectin knock out is viable and fertile, and shows no obvious defects in kinesin-dependent transport processes (Plitz and Pfeffer, 2001). Thus, all known specific cargo interactions with kinesin are mediated by the light chain.

In light of the results above, it is very surprising that the light chain is dispensable for the three functions of kinesin in the *Drosophila* female germline. One trivial explanation is that there is a second light chain gene in *Drosophila*, but this seems highly unlikely for several reasons. First, the protein is not redundant in the nervous system, as a strong axonal transport phenotype is observed in *Klc* mutants (Gindhart et al., 1998). Second, there is only one light chain gene in the 'complete' *Drosophila* genome sequence (63% sequence identity to human kinesin light chain 1), and all of the light chain cDNAs in the extensive *Drosophila* EST collections correspond with this gene (Adams et al., 2000; Goldstein and Gunawardena, 2000). Third, the 'complete' genome sequence of another Dipteran insect, the mosquito *Anopheles gambiae*, also contains only a single *Klc* gene. Although it is possible that there is a second light chain gene in the small region of each genome that has not been sequenced, it seems very improbable that this would be the case in both organisms. Thus, our results strongly suggest that the kinesin heavy chain can function without a light chain in the oocyte, and that it must therefore interact with its cargo or cargoes in some other way.

Although there is no precedent for light chain independent activities of the KHC in higher eukaryotes, the distantly related kinesin heavy chains of fungi, such as *Neurospora crassa*, function without any associated light chains (Steinberg and Schliwa, 1995). Mutagenesis studies on the *N. crassa* kinesin have identified a putative cargo-binding domain in the tail, and this region has been conserved in animal KHCs (Seiler et al., 2000). It may therefore represent an alternative cargo-binding domain that could account for the light chain independence of the KHC functions in the oocyte. Interestingly, the glutamate receptor interacting protein, GRIP1, has recently been shown to bind to this region of the mouse KHC (Setou et al., 2002). GRIP1 has been proposed to target kinesin to dendrites, and it is not yet known whether it functions as a cargo adaptor, or plays a role in light chain independent transport.

Kinesin heavy chain is required for all ooplasmic streamings during oogenesis

Twenty years ago it was suggested that the vigorous ooplasmic streaming and the cytoplasmic movements in the nurse cells in stage 10b egg chambers are independent processes (Gutzeit and Koppa, 1982). Our results demonstrate that this is indeed the case, not only at stage 10b, but also earlier in oogenesis, as ooplasmic streaming is completely abolished in *Khc* mutant egg chambers, whereas the cytoplasmic movements in the nurse cells and from the nurse cells into the oocyte are unaffected. It is unclear how kinesin creates these cytoplasmic flows in the oocyte. Given its role in vesicle transport in other systems, an attractive model is that it transports some organelle or vesicle along microtubules, and that this then generates flows in the surrounding cytoplasm, because of its viscosity. It seems unlikely that kinesin is directly transporting any of the particles or vesicles that we have visualised in our assays, as these particles move at speeds of about 0.1 μm/second at stage 9, which is significantly slower than other reported kinesin-dependent transport processes (Goldstein and Yang, 2000). This suggests that kinesin generates streaming by transporting some other organelle or vesicle more rapidly along the microtubules.

The nature of the cytoplasmic flows in the oocyte is variable and temporally regulated (Theurkauf, 1994). The ooplasmic streaming at stage 9 is slow and uncoordinated, whereas the movements at stage 10b are faster and unidirectional, and resemble those of a 'washing machine'. As both types of ooplasmic streaming are completely abolished in *Khc* mutants, these differences cannot be due to the motor protein. The type of streaming probably depends, at least in part, on the organisation of the microtubule cytoskeleton, which changes completely at the beginning of stage 10b, but kinesin may also have distinct cargoes at the two stages, which could influence the strength of the cytoplasmic flows.

The role of the Kinesin heavy chain in *oskar* mRNA localisation?

In an attempt to understand the mechanism for *oskar* mRNA transport to the posterior, we analysed the movement of a GFP-Staufen fusion protein in living oocytes. Although this fusion protein localises to the posterior with *oskar* mRNA and rescues the *oskar* mRNA localisation defect of a *staufen* null mutant, we have been unable to resolve any movements that unambiguously correspond to posterior transport. One possible explanation for this failure is that most of the fluorescent GFP-Staufen particles do not contain *oskar* mRNA, which is expressed at much lower levels than the fusion protein. Thus, the relevant *oskar* mRNA/GFP-Staufen complexes may be too rare or too weakly fluorescent to follow in time-lapse films. Although we have been unable to determine how GFP-Staufen

reaches the posterior, our results do reveal several important features of this process that are relevant to the discussion of the models for the mechanism of *oskar* mRNA localisation.

One model proposes that cytoplasmic flows circulate *oskar* mRNA around the oocyte, so that it can then be efficiently trapped at the posterior by a pre-localised cortical anchor (Glotzer et al., 1997). Indeed, this mechanism would account for our failure to detect any directed transport of GFP-Staufen to the posterior pole. Our observation that the KHC is required for all cytoplasmic flows in the oocyte also supports this model, as it provides an explanation for why the KHC is required to localise *oskar* mRNA. However, several other considerations make this mechanism unlikely. First, the cytoplasmic flows are much weaker at the posterior of the oocyte than elsewhere, presumably because there are fewer microtubules in this region, and many oocytes show little or no cytoplasmic movement near the posterior pole. It is therefore hard to imagine how cytoplasmic flows could efficiently deliver the mRNA to a posterior anchor. Second, the hypothetical anchor would have to localise to the posterior before *oskar* mRNA and in an *oskar* mRNA independent manner, and no proteins that meet these criteria have been identified so far. Indeed, the only proteins that fulfil the second criterion are the KHC and the components of the dynein/dynactin complex. Third, *oskar* mRNA localises to the centre of the oocyte in mutants that alter the organisation of the microtubule cytoskeleton, such as *gurken*, *pka* and *par-1*, and it is hard to reconcile this with trapping by a cortical anchor, as there is no plasma membrane or cortical cytoskeleton in this region (González-Reyes et al., 1995; Lane and Kalderon, 1994; Roth et al., 1995; Shulman et al., 2000; Tomancak et al., 2000). The localisation of *oskar* mRNA still correlates with the position of microtubule plus ends in these mutants, because Kin-βGal forms a dot in the centre of the oocyte with the mRNA, and this is more consistent with the model in which *oskar* mRNA is transported along microtubules towards the posterior pole. Finally, the KHC accumulates at the posterior during the stages when *oskar* mRNA and DHC are localised, strongly suggesting that it plays a direct role in transporting them there.

Another model for *oskar* mRNA localisation proposes that the KHC functions to transport the RNA away from the minus ends of the microtubules at the anterior and lateral cortex towards the plus ends in the interior of the oocyte, and that the lack of microtubules at the posterior somehow allows the mRNA to accumulate at this pole (Cha et al., 2002). Two aspects of our data do not fit this cortical exclusion model. First, unlike Cha et al., we never saw any *oskar* mRNA or Staufen at the posterior of the oocyte in *Khc* germline clones, regardless of whether we performed fluorescent or whole-mount in situ hybridisation or antibody staining. This observation seems incompatible with a model in which kinesin removes *oskar* mRNA from the anterior and lateral cortex, but is not required for its localisation to the posterior pole. Second, the demonstration that endogenous kinesin localises to the posterior cortex, like kinesin-βGal, provides further evidence that the plus ends of the microtubules are enriched in this region, and strongly suggests that kinesin mediates transport to this pole. These localisations are not visible until stage 9, however, which is when *oskar* mRNA starts to accumulate at the posterior. Thus, our results can be reconciled with those of Cha et al., by proposing that the plus ends lie in the middle of the oocyte at stage 8, when they observe a kinesin-dependent accumulation of *oskar* mRNA in the central dot, and that they are only recruited to the posterior at stage 9, coincident with the onset of *oskar* mRNA localisation.

In light of the posterior localisation of endogenous kinesin, we think it most likely that this motor does transport *oskar* mRNA to the posterior of the oocyte, even though we have been unable to see this movement. The link between the KHC and the *oskar* mRNA localisation complex need not be direct, however. The KHC probably transports something else to the posterior of the oocyte, in addition to *oskar* mRNA and dynein, because mutants that abolish either *oskar* mRNA localisation (such as *staufen* and *barentsz*) or DHC localisation ($Dhc64C^{6-6}/Dhc64C^{6-12}$) have no effect on the posterior localisation of the KHC, even though the motor activity of the KHC is thought to require binding to a cargo. The KHC is also required for cytoplasmic streaming, and presumably induces these flows by moving a large structure, such as a vesicle or organelle, along microtubules. This structure should therefore accumulate at the posterior of the oocyte during stage 9, because this is where the microtubule plus ends and the KHC itself localise. Thus, *oskar* mRNA and dynein could reach the posterior at stage 9 by hitch-hiking on the large cargo that drives streaming. This proposal is consistent with several other observations. First, the fact that cytoplasmic streaming, *oskar* mRNA localisation and dynein localisation all share the very unusual property of being light chain independent suggests that they all depend on a single KHC-mediated transport process, which could be the transport of the cargo that induces streaming to the posterior. Second, it has been shown in a number of other systems that plus and minus end directed microtubule motors, such as kinesin and dynein, are found on the same organelles (Gross et al., 2002; Martin et al., 1999; Welte et al., 1998). Third, if dynein and *oskar* mRNA interact with the kinesin cargo independently of each other, this would explain why both their posterior localisations require the KHC, but do not require each other. Finally, there is already evidence that links *oskar* mRNA localisation with vesicle trafficking, as mutants in *rab11*, a small GTPase implicated in the regulation of endocytic vesicle recycling, disrupt the posterior localisation of *oskar* mRNA (Dollar et al., 2002; Jankovics et al., 2002). Furthermore, Rab11 itself localises to the posterior of the oocyte. The effect of Rab11 on *oskar* mRNA localisation may be indirect, however, as these mutants also disrupt the organisation of the microtubule cytoskeleton.

It is unclear why dynein localises to the posterior, but one possibility is that it is needed to recycle kinesin to the minus ends of the microtubules, so that it can mediate another round of posterior localisation. The only known phenotype of the *Dhc64C* mutants that specifically disrupt the posterior localisation of DHC is a reduction in the rate of cytoplasmic streaming, and this may due to the gradual depletion of the pool of KHC available for transport. However, this localisation may be important for recycling dynein away from the minus ends of microtubules, so that it can mediate further rounds of minus end-directed transport.

If the hitch-hiking model for *oskar* mRNA localisation is correct, Staufen, Barentsz, Mago nashi and Y14 would be required to couple the mRNA to the vesicle or organelle that is transported by kinesin. In this context, it is interesting to note that mammalian Staufen homologues have been shown to

associate with the endoplasmic reticulum (Kiebler et al., 1999; Marión et al., 1999; Wickham et al., 1999). The localisation of Vg1 mRNA to the vegetal pole of *Xenopus* oocytes requires the RNA-binding protein VERA/Vg1 RBP, which co-fractionates with markers for the endoplasmic reticulum, and this has led to the suggestion that Vg1 mRNA is transported in association with ER vesicles (Deshler et al., 1997). Thus, hitchhiking on vesicles may represent a general mechanism for mRNA transport.

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