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# The Cytoplasmic Dynein and Kinesin Motors Have Interdependent Roles in Patterning the *Drosophila* Oocyte

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## Summary

**Background:** Motor proteins of the minus end-directed cytoplasmic dynein and plus end-directed kinesin families provide the principal means for microtubule-based transport in eukaryotic cells. Despite their opposing polarity, these two classes of motors may cooperate in vivo. In ***Drosophila*** **circumstantial evidence suggests that dynein acts in the localization of determinants and signaling factors during oogenesis. However, the pleiotropic requirement for dynein throughout development has made it difficult to establish its specific role.**
**Results:** We analyzed dynein function in the oocyte by disrupting motor activity through temporally restricted expression of the dynactin subunit, dynamitin. Our results indicate that dynein is required for several processes that impact patterning; such processes include localization of *bicoid* (*bcd*) and *gurken* (*grk*) mRNAs and anchoring of the oocyte nucleus to the cell cortex. Surprisingly, dynein function is sensitive to reduction in kinesin levels, and germ line clones lacking kinesin show defects in dorsal follicle cell fate, *grk* mRNA localization, and nuclear attachment that are similar to those resulting from the loss of dynein. Significantly, dynein and dynactin localization is perturbed in these animals. Conversely, kinesin localization also depends on dynein activity.
**Conclusions:** We demonstrate that dynein is required for nuclear anchoring and localization of cellular determinants during oogenesis. Strikingly, mutations in the kinesin motor also disrupt these processes and perturb dynein and dynactin localization. These results indicate that the activity of the two motors is interdependent and suggest a model in which kinesin affects patterning indirectly through its role in the localization and recycling of dynein.

## Introduction

The intracellular transport of macromolecules requires the coordinated interaction of motor molecules, cargos, and signals that regulate targeted movement along networks of cytoskeletal tracks. In *Drosophila*, oogenesis and early embryogenesis are two stages of development that critically depend on correct subcellular transport. Despite an array of genetic, cell-biological, and direct experimental approaches, two major stumbling blocks have hampered analysis of the roles played by individual motors. First, the motor components are required multiple times during development, so disrupting function at an early stage prevents scrutiny of later processes. Second, the loss of many motor-associated proteins leads to cell lethality. These issues have especially impacted understanding of the role of cytoplasmic dynein, the major minus end-directed microtubule motor in late stages of oogenesis. The complete absence of dynein prevents oocyte specification, whereas in hypomorphic alleles oogenesis proceeds apparently normally [1, 2]. Thus, although circumstantial data suggest that dynein could mediate essential steps such as migration of the oocyte nucleus and localization of mRNAs, it remains an open possibility that the tasks are performed by other minus end-directed motors. We have used a targeted disruption approach to circumvent these difficulties and have obtained direct evidence that dynein is required for the subcellular localization of *grk* and *bcd* transcripts and for anchoring the oocyte nucleus.

*Drosophila* oogenesis is initiated when a germ-line stem cell divides asymmetrically to produce a daughter stem cell and a cystoblast that undergoes four further divisions to form a cyst of 16 interconnected cells (see [3] for a recent review and primary references). One cyst cell is specified to become the oocyte through unequal segregation of the fusome, a membranous organelle, while the remaining 15 differentiate into nurse cells that provide RNA and proteins to the egg. During the first six of the 14 morphologically defined stages in oogenesis, mRNA and other cargos are transported from the nurse cells to the egg along microtubules that pass through the ring canals and have their minus ends in the oocyte. At stage 6 a critical patterning event occurs when the anterior/posterior (A/P) axis of the egg is specified by the TGF-$\alpha$ ligand Grk. Grk is initially localized around the oocyte nucleus at the egg chamber's posterior, where it instructs follicle cells in close proximity to adopt a posterior fate. After differentiation, these cells signal back to the oocyte and trigger reorganization of the microtubule network and migration of the nucleus to the anterior. Grk remains associated with the oocyte nucleus at the anterior, where it signals to overlying follicle cells a second time and thus confers dorsal rather than default ventral fates. Nuclear migration as well as localization of *grk* and other mRNAs requires an intact microtubule network. Kinesin is thought to be the plus-end motor responsible for transport to the oocyte posterior because mutations in the kinesin heavy chain (Khc) affect *osk* mRNA localization to this region [4]. However, the minus-end motor responsible for movement of the nucleus and localization of *bcd* mRNA to the anterior of the oocyte has not been unambiguously identified. Mutations in the dynein-interacting proteins Lissencephaly1 (Lis1) and BicD affect nuclear positioning, making cytoplasmic dynein a logical candidate for this function [5, 6]. The fact that Swallow (Swa), a protein that anchors *bcd* mRNA at the anterior of the oocyte,

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binds the dynein light-chain subunit (Dlc) in yeast two-hybrid and immunoprecipitation assays further implicates dynein in this process [7]. However, these observations are not definitive because both Lis1 and BicD may have additional dynein-independent roles, and Dlc also interacts with the motor myosin V and other proteins with high affinity (reviewed in [8]). Furthermore, hypomorphic alleles in the *Dynein heavy chain* (*Dhc*) gene do not display overt defects in either nuclear migration or localization of *bcd*, *grk*, and *osk* mRNAs [2].

To examine the role of dynein in oogenesis, we have disrupted its function in a temporally restricted fashion. The multiprotein dynactin complex is essential for dynein activity in vivo and is thought to mediate the attachment of cargos to the motor. The largest dynactin component, Glued (Gl), binds to the dynein intermediate chain (Cdic) and thus links the two complexes. Overexpression of the p50/dynamitin (Dmn) subunit of dynactin causes dissociation of Gl from dynactin and thus interferes with cargo-anchoring functions and compromises dynein activity [9]. Disruption of dynein activity through Dmn misexpression is a well-established approach in mammalian cell culture, and injection of recombinant human Dmn protein into *Drosophila* embryos has been used successfully to demonstrate the involvement of dynein in transcript localization and chromosome movement during early embryogenesis [10–12]. Using Dmn overexpression, we have identified novel functions for dynein in anchoring the oocyte nucleus and localizing *grk* mRNA. In addition, we show that dynein is required for the localization of *bcd* transcript. Unexpectedly, we have found that kinesin is also required for nuclear positioning and D/V patterning. This appears to reflect a function that may allow kinesin to reuse the same dynein molecule for multiple rounds of minus end-directed transport within the oocyte.

## Results

In order to investigate the effects of targeted disruption of dynein activity, we generated heat shock-inducible (*hsDmn*) and Gal4-responsive (*UAS-Dmn*) transgenic lines (see Experimental Procedures). The *hsDmn* transgene permits tight temporal control of misexpression, whereas *UAS-Dmn* allows spatially restricted transcription when coupled with the appropriate Gal4 drivers [13]. After mapping the insert position, we assayed *hsDmn* flies for the ability to induce Dmn expression by probing immunoblots with polyclonal antisera against the *Drosophila* protein (see Experimental Procedures). A single band migrating at 45 kDa, close to the predicted size of the endogenous protein, was detected in untreated control flies. This band was present at approximately 5- to 10-fold higher levels in animals that had been heat shocked for 60 min (Figure 1A). A time course of induction showed that elevated Dmn levels are present 15 min after heat shock and persist for at least 6 hr (our unpublished data). To test whether *Dmn* overexpression perturbs the stability or localization (or both) of the dynein/dynactin complex in vivo, we stained egg chambers with antisera against Dmn, Gl (the largest subunit of dynactin), and the dynein intermediate chain

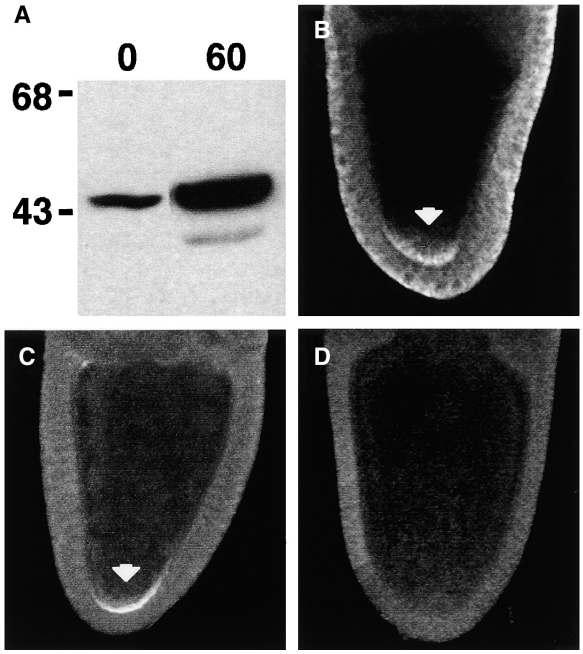


Figure 1. Western Blot Analysis and Immunolocalization to Assess *hsDmn* Induction

(A) Antisera against Dmn recognize an approximately 45 kDa band in extracts from *hsDmn* larvae prior to induction. A marked increase in Dmn expression is apparent after 60 min of heat shock. Both Dmn (B) and the dynactin component Gl (C) are enriched at the posterior cortex (arrowhead) in wild-type stage 10 oocytes. (D) The posterior localization of Gl is abolished by Dmn overexpression.

(Cdic). In the wild-type, Dmn preferentially accumulates in the oocyte during early oogenesis and shows both perinuclear and cortical staining through stage 8. By stage 9 Dmn is enriched in a crescent at the posterior cortex as well as in lateral regions (Figure 1B). Overall, this distribution mimics that of Gl and the dynein intermediate and heavy chains (Figure 1C and our unpublished data; [14–16]). Within 60 min of *hsDmn* induction, high levels of Dmn were detected throughout the oocyte, nurse cells and follicle cells (data not shown). In contrast, Gl and Cdic staining was undetectable in stage 9/10 oocytes and was strongly reduced at earlier stages (Figure 1D and our unpublished data), demonstrating that Dmn overexpression disrupts the localization of the dynein/dynactin complex.

### *Dmn* Overexpression Affects *bcd* mRNA Localization without Compromising Microtubule Organization

To determine whether abrogation of dynein/dynactin activity affects mRNA localization, we examined the asymmetric distribution of *bcd* and *osk* transcripts that are transported to opposite ends of the oocyte. mRNA for *bcd* that is confined to the anterior of stage 9/10 oocytes in the wild-type was unaffected in 97.5% ($n = 200$) of egg chambers from control females 12 hr after heat shock (Figure 2A). In contrast *bcd* mRNA was dispersed over the anterior third of the oocyte in 26.8% ($n = 149$) of stage 9/10 egg chambers from *hsDmn* females, dem-

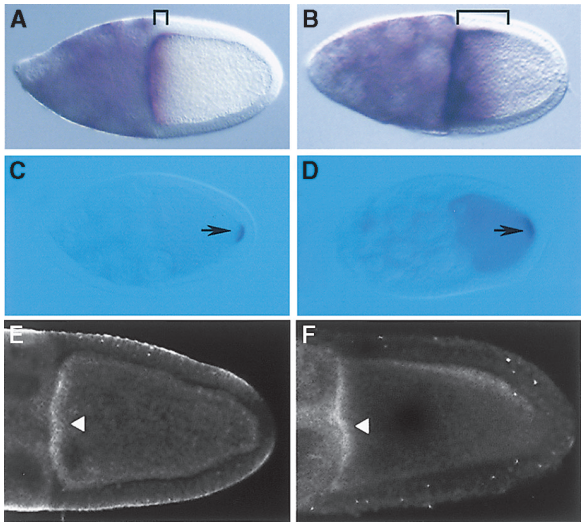


Figure 2. Effect of Dmn Upregulation on *bcd* and *osk* Transcript Localization

Egg chambers are at stage 9/10 and are oriented with the anterior to the left. In situ hybridization shows that *bcd* mRNA is tightly localized to the anterior in the wild-type (A), in strong contrast to the dispersed distribution over one-third the length of the oocyte after *hsDmn* overexpression (B). Brackets indicate the extent of transcript distribution along the A/P axis. *osk* mRNA is typically confined to the posterior in the wild-type ([C], see arrow), but its distribution is perturbed in response to *hsDmn* induction (D). Although a focus of *osk* transcript is clearly visible at the posterior, low-levels of mRNA are also present throughout the stage 9 egg chamber. Antibodies against Cnn indicate microtubule organization and show staining around the cortex with enrichment at the anterior both in wild-type ([E], see arrowhead) and after Dmn induction (F).

onstrating that dynein activity is required for *bcd* mRNA transcript localization (Figure 2B). *osk* mRNA is normally confined to the posterior of the oocyte by a process that requires both kinesin and an intact microtubule cytoskeleton. In the wild-type, *osk* message is detected in the oocyte from stage 1 onward and is associated with the posterior cortex adjacent to the nucleus until stage 7. After breakdown of the posterior microtubule-organizing center (MTOC), *osk* mRNA is transiently located at the anterior and center of the oocyte before forming a tight crescent at the posterior cortex from stage 9 onward (Figure 2C, [4, 17]). Egg chambers dissected one hour after *hsDmn* induction show a clear focus of *osk* mRNA at the posterior pole. However, lower levels of transcript can be detected throughout the oocyte, especially during stages 8 and 9 (Figure 2D). Thus, *hsDmn* expression perturbs but does not abolish *osk* transcript localization.

To examine whether disruption of the dynein/dynactin complex affects transcript localization indirectly by altering microtubule organization, we stained egg chambers with antisera against Centrosomin (Cnn), a MTOC component that marks microtubule minus ends [18]. In both wild-type and *hsDmn* females, Cnn localization to the oocyte cortex and the anterior was unaffected by heat shock treatment (Figures 2E and 2F). In a second set of experiments, the distribution of a Tau-GFP fusion protein that decorates microtubules in the oocyte [1, 19] was also unaltered after Dmn overexpression, confirming that the integrity and polarity of oocyte microtubules is maintained (our unpublished data). In summary, Dmn overexpression strongly affects *bcd* mRNA localization and perturbs the restriction of *osk* transcript without grossly disrupting microtubule organization.

## Upregulation of *Dmn* in the Oocyte Affects Nuclear Positioning

Grk signaling from the oocyte to overlying follicle cells depends on the tight association of its mRNA and protein with the oocyte nucleus. Thus, conditions that affect nuclear location or *grk* activity result in alterations in the morphology and position of chorionic structures such as the posterior aeropyle and the paired dorsal appendages (DA) that derive from anterior dorsal follicle cells on either side of the midline (Figure 3A$_{i}$; reviewed in [20]). To determine if dynein activity is required for nuclear localization, we subjected *hsDmn* females to heat shock. We then collected eggs and examined them by darkfield microscopy. Dmn overexpression resulted in eggs with dorsal patterning defects of varying severity that were apparent in the position and shape of the DA. In the majority of affected eggs, the DA were fused at the base or along their entire length to form a single composite structure (Figure 3A$_{ii}$). In all of these cases, the DA were correctly positioned along the A/P axis. In some instances, however, the DA were highly reduced or absent, and their rudiments were displaced toward the posterior of the egg (Figure 3A$_{iii}$, arrowhead), reminiscent of mutants in which the oocyte nucleus is mispositioned because of a failure in nuclear migration or anchoring [5, 6, 21–23]. The highest frequency of patterning defects was in eggs collected 12–36 hr post-heat shock, indicating that oocyte development is sensitive to disruption of dynein activity during a temporally restricted window (see below). The fact that eggs from wild-type females subjected to the same regimen developed normally argues that the defects were not caused by stress from the heatshock.

The *hsDmn* transgene drives expression both in the oocyte and in the follicle cells, so the patterning defects could result from disruption of dynein activity in either germ line or somatic follicle cells. To distinguish between these possibilities, we drove *UAS-Dmn* expression in a restricted manner by using a follicle cell-specific driver [24]. Females of the genotype *C355-Gal4*;*UAS-Dmn* produced eggs with normal DA, indicating that the defects in morphology cannot be attributed to altered dynein function in follicle cells (our unpublished data). To confirm that the UAS constructs were functional, we drove expression in the larval nervous system by using an *elav-Gal4* driver, resulting in severe defects in axonal transport similar to those described for mutations in *Dhc* (our unpublished data; [25]). Thus, the lack of a phenotype from expression of Dmn in follicle cells cannot be attributed to ineffectiveness of the UAS construct.

We next examined egg chambers from flies carrying *hsDmn* and a β-Gal enhancer trap expressed in the germinal vesicle to directly observe the position of the oocyte nucleus after Dmn misexpression. Anterior migra-

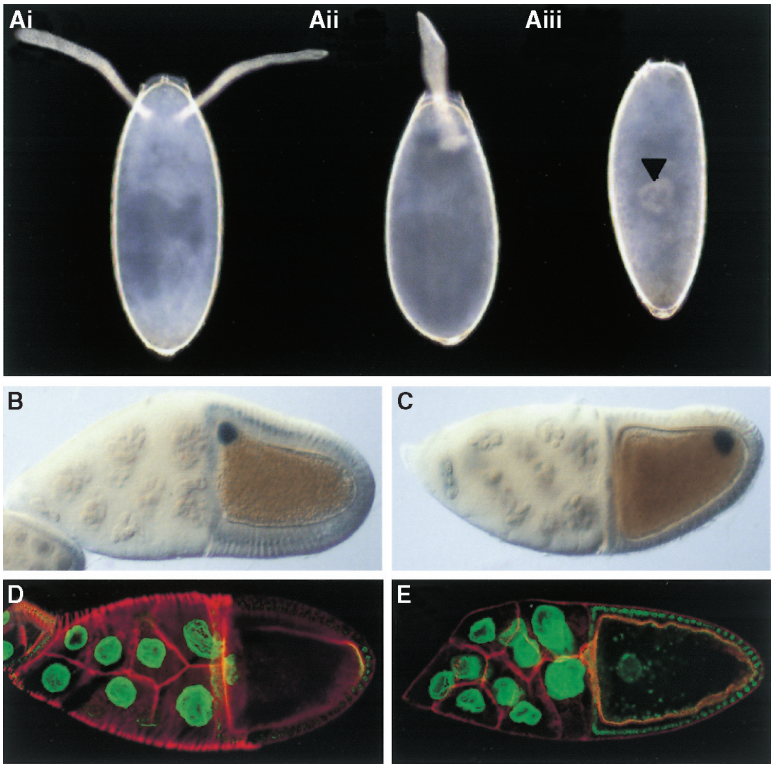


Figure 3. Dmn Overexpression Results in Dorsal Patterning Defects in the Oocyte

Eggs in (A) are oriented with the anterior to the top and are viewed dorsally. Dark-field micrograph of a wild-type egg with paired dorsal appendages (DA) located anteriorly (Ai). A majority of the eggs laid by *hsDmn* mothers display moderate ventralization in which the dorsal appendages are fused along their length ($A_{ii}$). In a small but significant number of cases, the egg cases are severely ventralized ($A_{iii}$), and the DA are reduced to a single patch of appendage material and displaced toward the posterior (arrowhead). Egg chambers in (B)–(E) are oriented with the dorsal end up and the anterior to the left. (B) Control stage 10 egg chambers dissected 12 hr after heat shock show the oocyte nucleus at its correct dorsal anterior location. (C) In contrast, in similarly treated *hsDmn* flies the nucleus is severely mispositioned in 4%–8% of the egg chambers. The oocyte nucleus was visualized with a β-Gal enhancer trap line. (D,E) Confocal images of egg chambers stained to visualize the nuclear lamina (Alexa 488 conjugated wheat germ agglutinin, in green) and cortical F-actin (rhodamine phalloidin, in red). In wild-type egg chambers (D) the nuclei are apposed to the cortex (yellow color denotes overlap). (E) In egg chambers after Dmn overexpression, the displaced nuclei are often found separated from the cortical actin.

tion of the nucleus occurs during stages 7–8, when the size of the oocyte is comparable to the nuclear diameter and it is thus difficult to unambiguously score defects in positioning. However, by stage 9 a dramatic increase in oocyte size allows nuclear location to be assayed reliably. In control heat-shocked females, the oocyte nucleus was positioned at the anterior cortex without exception (Figure 3B). After *Dmn* overexpression, a small but significant percentage (4%–9%) of stage 9 and 10 egg chambers contained severely mispositioned nuclei that either remained associated with the cortex (Figure 3C) or were dislodged from the cortical cytoskeleton (see below). To precisely determine nuclear location relative to the oocyte cortex, the nuclear lamina and F-actin were simultaneously labeled with conjugated wheat germ agglutinin and rhodamine-phalloidin, respectively, and examined by confocal microscopy. In the wild-type at stage 10, the nucleus is closely apposed to the dense layer of microfilaments that lines the cortex (Figure 3D, yellow color denotes overlap). Confocal sectioning revealed that about half of the displaced nuclei in *hsDmn* egg chambers were clearly separated from the cortical actin (Figure 3E). Thus, disruption of dynein function can lead to dissociation of the nucleus from the cortical layer of the oocyte.

To determine the temporal requirement for dynein activity during oogenesis, we heat shocked females and collected eggs at 5 hr intervals. Oogenesis proceeds according to a defined sequence, and the average duration of each stage allows an estimate of the developmental time point when the egg chamber experienced an increase in Dmn levels (Figure 4, data for hsDmn/+ in red; [26]). We found that the frequency of dorsal appendage defects was highest in the 20–25 and 25–30 hr collections (45%, n = 211 and 33.3%, n = 222, respectively), although patterning defects were also apparent in eggs collected 35 and 40 hr after heat shock (18.7%, n = 257 and 3.7%, n = 301, respectively). Interestingly, the time period that showed the highest frequency of defects corresponds to stage 8/9 oocytes that should have completed nuclear migration at the time of heat shock. This finding argues that the requirement for dynein extends beyond its involvement in nuclear movement. Consistent with this, we found that although patterning defects could be seen in up to 45% of eggs (Figure 4), the frequency of severely ventralized phenotypes (indicative of nuclear displacement) did not exceed 5% of the affected sample.

### Loss of Dynein Activity Transiently Interrupts Grk Signaling by Preventing Transcript Localization

An alternative mechanism by which *Dmn* overexpression could affect dorsal patterning is through disruption of *grk* expression/localization. Unlike most oocyte mRNAs that are transported from the nurse cells, *grk* is primarily expressed in the oocyte nucleus and is associated with the germinal vesicle throughout oogenesis [27]. Prior to stage 7, *grk* mRNA is located between the nucleus and the posterior follicle cells. After reorientation of the microtubule network and nuclear migration, *grk* transcript accumulates in a ring along the anterior cortex before refining to form a tight cap around the nucleus at the prospective anterior dorsal region during stages 9 and 10 [27, 28]. We simultaneously visualized

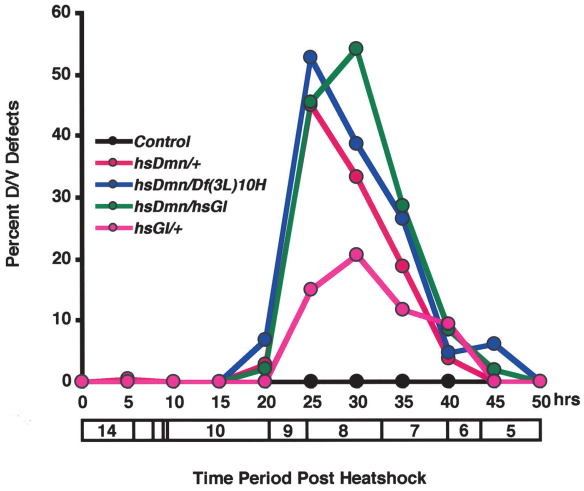


Figure 4. Disruption of Dynein Activity at Different Stages of Oogenesis and in Genetic backgrounds that Compromise Dynein Function

The lower panel shows a timeline of oogenesis (according to [26]) correlated with the relative hours after heat shock. Females carrying one copy of the *hsDmn* transgene in the backgrounds denoted were heat shocked, and eggs were collected over successive 5 hr periods and scored for DV patterning defects. The highest frequency of defects was centered on the 20–25 hr interval, i.e., egg chambers that were at stage 9 at the time of heat shock. The effective period for Dmn induction extended to the 35–45 hr interval that includes stage 6/7, when nuclear migration is initiated. Heterozygosity for dynein (*Df(3L)10H*) and coexpression of *hsGl* enhanced the frequency of defects.

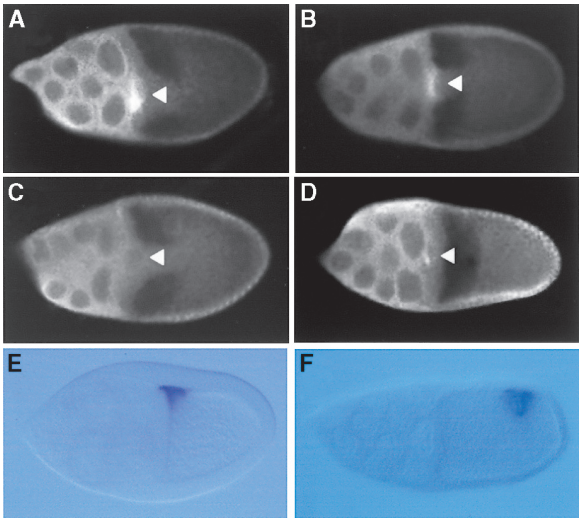


Figure 5. Effect of Dynamitin Upregulation on *grk* and *BR-C* Expression

Egg chambers in (A)–(D) are oriented with their anterior to the left and their dorsal side facing the viewer. *grk* message was visualized with a fluorescent substrate, whereas *BR-C* was detected with conventional histochemistry. The nucleus is marked with an arrowhead in (A)–(D). In wild-type controls (A) as well as *hsDmn* animals (B) dissected immediately after heat shock (0 hr time point), *grk* mRNA can be seen associated with the nucleus in a dorsal anterior location flanked by two dorsolateral domains of *BR-C* that appear as dark patches. *grk* transcript was undetectable in *hsDmn* egg chambers 6 hr after heat shock, although *BR-C* expression is present in two separate domains (C). At 12 hr after heat shock, reduced levels of *grk* mRNA are associated with the nucleus. However, *BR-C* transcription is in a single domain indicative of an earlier loss in *grk* signaling (D). Egg chambers in (E) and (F) are oriented with their dorsal side up. Visualization of digoxygenin-labeled *grk* mRNA reveals anterior dorsal localization in the wild-type (E). After *hsDmn* upregulation, approximately 5% of egg chambers show a mispositioned nucleus that continues to expresses *grk*. Some of these nuclei remain associated with the cortex (F).

*grk* and *Broad Complex* (*BR-C*), a zinc-finger transcription factor that serves as a molecular readout of dorsal follicle cell fates because it is expressed in two dorsal anterior patches corresponding to the future DA in response to Grk signaling [29]. The effect of *Dmn* overexpression was assayed in egg chambers dissected before (0 hr) as well as 6 and 12 hr after heat shock induction. Prior to heat shock, *grk* transcript is associated with the nucleus and is flanked by *BR-C* expression in the overlying follicle cells (Figure 5A). Six hours after induction, *grk* localization was unaffected in wild-type, but could not be detected in 63.2% (n = 19) of stage 9/10 *hsDmn* egg chambers (Figure 5B, C). Twelve hours after induction, only 22% (n = 18) of stage 10 egg chambers did not have *grk* mRNA associated with the nucleus, whereas the remaining egg chambers showed reduced levels suggesting partial restoration of localization (Figure 5D). This reveals that dynein is required for patterning the dorsal region of the oocyte in part because of its role in transporting or anchoring *grk* mRNA. We also observed that *Dmn* induction altered the spatial expression of *BR-C*, albeit with a time delay relative to its effect on *grk*. *BR-C* expression was unaffected 6 hr after Dmn induction (Figure 5C). However, in stage 10 egg chambers 12 hr after the heat pulse, the normally distinct *BR-C* domains were fused into a single patch of expression centered above the nucleus (Figure 5D). This lag is likely to reflect the time required for EGFR signaling to direct the change in the transcriptional pattern of its downstream targets. It is worth noting that the reappearance of nuclear-associated *grk* mRNA was independent of the position of the nucleus 12 hr after Dmn induction (Figures 5E and 5F). However, when nuclei were apposed to the side of the oocyte, *grk* transcript was present at the cortex as well as in the perinuclear region (Figure 5F, compare with wild-type in 5E). In summary, our data reveal that *grk* transcript localization and follicle cell fate (illustrated by *BR-C* expression) display high sensitivity to Dmn overexpression. This correlates well with the high incidence of dorsal patterning defects in eggs laid by *hsDmn* females (see Figure 4) and suggests that mislocalization of *grk* mRNA makes a significant contribution to the ventralized phenotype. In addition, the fact that *grk* transcript localization is altered at stage 9/10 is consistent with the temporal profile of D/V patterning defects (see Figure 4). It is significant that the nucleus was usually centered beneath the domains of BR-C expression (see Figures 5B–5D), indicating that Dmn misexpression does not cause a transient displacement of the germinal vesicle and subsequent reattachment to the cortex at a random location. Consistent with the effects on the message,

*grk* protein levels were also highly reduced 6 hr after Dmn induction (our unpublished data).

### Mutations that Reduce Dynein and Dynactin Activity Enhance the Effects of Dmn Overexpression

Although both *grk* and *bcd* mRNA localization are sensitive to Dmn misexpression, the threshold for the disruption of nuclear position appears to be higher. To test if enhancing Dmn levels could increase the penetrance of nuclear positioning defects, we subjected flies containing four copies of *hsDmn* to a rigorous regimen of five successive 30 min heat shock pulses interspersed over a 5 hr period. Although this increased the incidence of DA fusion to 78%, DA displacement along the A/P axis (indicative of nuclear mispositioning) was essentially unchanged (data not shown). The low frequency of displaced nuclei suggested that heatshock Dmn expression might not completely inhibit dynein activity. We therefore introduced the *hsDmn* transgene into different sensitized backgrounds that compromised dynein function before inducing expression and collecting eggs at 5 hr intervals (see Figure 4). Wild-type controls (indicated in black) were unaffected by the heat shock, whereas a single copy of *hsDmn* (red line) resulted in dorsal patterning defects in 45% (n = 211) of the eggs laid at 25 hr post heat shock. Removal of one copy of *Dhc* (*Df(3L)10H*, blue line) increased the frequency of D/V defects to 53% at 25 hr (n = 214), consistent with the idea that Dmn overexpression does not completely abolish dynein function. Interference with dynactin function by expression of a truncated form of the *Gl* protein that acts in a dominant-negative manner (*hsp70Gl*, pink line [30]) was significantly less effective than *hsDmn* in disrupting dorsal patterning (see Figure 4). However, coexpression of *hsDmn* and *hsp70Gl* (green line) increased the percentage of eggs with DA defects. Remarkably, no corresponding elevation in A/P defects with respect to the position of the DA were observed, and dissection of stage 10 egg chambers from all genotypes 1, 6, and 12 hr after *Dmn* induction failed to detect a significant increase in mispositioning of the oocyte nucleus (our unpublished data).

### Reduction in Kinesin Levels Potentiates the Effect of Dmn Overexpression

We next tested whether dynein function was sensitive to reduction in the level of kinesin, the major plus-end motor. *hsDmn* expression was induced in females heterozygous for null alleles of *Kinesin heavy chain* (*Khc*), which encodes the motor domain, or *Kinesin light chain* (*Klc*), which in turn mediates interaction of the motor with its cargo. These females showed only a modest increase in the percentage of eggs with D/V patterning defects, but we observed a dramatic enhancement in the severity of these patterning defects. As mentioned previously, the majority of eggs from *hsDmn*/+ females displayed moderate ventralization with partial or complete fusion of the DA (see Figures 3A and 4). In contrast, 25–30 hr after heat shock, 55% of the eggs (n = 150) from $Khc^{8}$/+;*hsDmn*/+ transheterozygotes displayed D/V patterning defects; of these, 72.5% had severely ventralized eggshells with displaced or highly reduced DA. Similarly, in eggs from *hsDmn*/*Klc* females, 52% (n = 150) were affected and 76% of these were severely ventralized. The strong dominant genetic interaction observed between the opposite polarity dynein and kinesin motors indicates that they may be acting in concert to impact a common process.

### Kinesin and Dynein Motors Cooperate in Mediating Nuclear Anchoring and D/V Patterning

It has previously been shown that *Khc* is essential for the localization of *osk* RNA and protein to the posterior of the oocyte [4]. However, the potent enhancement of the Dmn misexpression phenotype by a 50% reduction in either *Khc* or *Klc* activity suggested that kinesin could have additional roles in patterning the D/V axis of the egg. Because null mutations in both *Khc* and *Klc* are lethal [31, 32], we used the FLP-FRT system to generate females completely lacking *Khc* activity in the germ line. We found that eggs laid by $Khc^{27}$ null females showed highly penetrant defects in follicle cell patterning (98%, n = 200), suggestive of attenuated *grk* signaling. The eggs could be grouped into three categories based on the severity of the D/V patterning defects (Figure 6A). The first group (37%) consisted of eggs with fused DA similar to the predominant class of defects caused by Dmn overexpression (Compare Figure $6A_{ii}$ with Figure 3A). The second group (43%) had reduced posteriorly displaced DA (Figure $6A_{iii}$), and a third group (28%) showed an even stronger phenotype in which no DA material was detected (Figure $6A_{iv}$). Eggs in all three categories contained an aeropyle at the posterior, indicating that reception of the *grk* signal by posterior follicle cells during stages 6 and 7 was not affected by the lack of kinesin.

We next examined if the patterning defects resulting from altered nuclear positioning in *Khc* mutant egg chambers. In agreement with a previous report [4], we found that the majority of mutant egg chambers proceed normally through early stages of oogenesis. However, simultaneous visualization of the nuclear lamina and cortical actin cytoskeleton revealed that the oocyte nucleus was clearly mispositioned along the A/P axis or displaced from the cortex in 62% of stage 9/10 oocytes (n = 74) (compare Figure 6B with the wild-type in Figure 3D). Interestingly, an ectopic focus of phalloidin staining was often adjacent to the displaced nucleus (Figure 6B, arrow), suggesting that a segment of F-actin is associated with the nucleus even when it is detached from the cortex. Nuclear mislocalization could also be detected during stage 8, although with a lower frequency (our unpublished data). This may simply be a consequence of the difficulty in scoring nuclear displacement at this early stage; alternatively, it may indicate a differential requirement for *Khc* in maintaining nuclear position. The large percentage of oocytes with displaced nuclei is in agreement with the high frequency of severely ventralized eggs laid by *Khc* germ line null females.

To determine how *grk* mRNA and protein localization were perturbed by the loss of *Khc* activity, we probed mutant egg chambers. Staining with Grk antisera re-

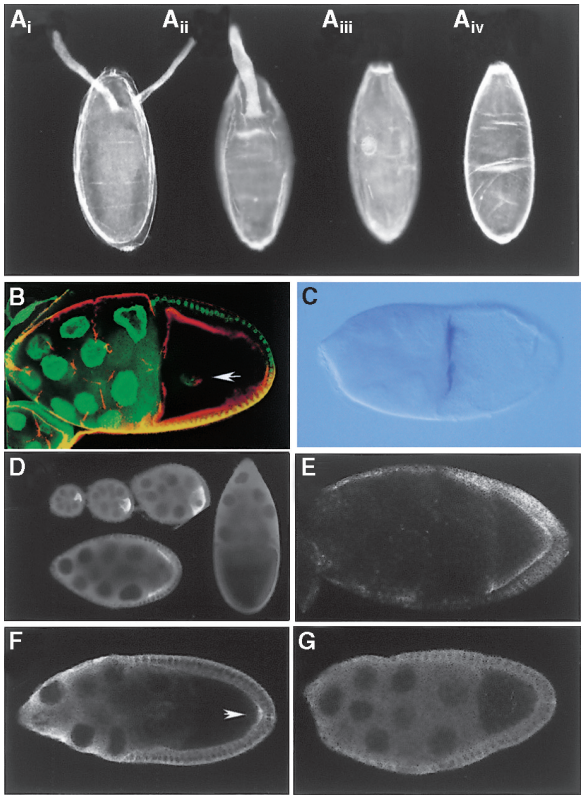


Figure 6. Loss of Kinesin Affects D/V Patterning in the Oocyte

Eggs in (A) are oriented with their anterior to the top and are viewed dorsally. Dark-field micrograph of a wild-type egg ($A_i$) shows paired DA, whereas eggs produced by females with *Khc*$^{27}$ germ line clones show moderate ($A_{ii}$) to severely ventralized ($A_{iii}$,$A_{iv}$) phenotypes. In ($A_{ii}$), DA are fused along their entire length, whereas in ($A_{iii}$) the DA is reduced in size and displaced posteriorly. DA are completely absent in the severely ventralized eggs in ($A_{iv}$). (B) A stage 10 egg chamber from *Khc*$^{27}$ germ line clones was stained for visualization of the nuclear lamina (green) and F-actin (red). The nucleus is displaced from the cortical F-actin, but a small patch of phalloidin-positive material (arrow) often remains associated with the nucleus (compare with wild-type in Figure 3D). (C) In *Khc* mutants, *grk* mRNA does not refine and remains dispersed along the anterior cortex even in stage 10 oocytes (see wild-type in Figure 5E for comparison). A string of *Khc* null egg chambers from the same ovariole (D) stained with antisera against Dmn shows normal distribution of the protein up to stage 8 but a failure to localize to the posterior cortex in stage 9/10 (stages are as marked, see Figure 1C for wild-type comparison). Wild-type (E,F) and *hsDmn* egg chamber (G) stained with antisera against Khc. At early stage 8 (E) kinesin can be detected at the oocyte cortex as well as in the overlying follicle cells, whereas from late stage 8 onward (F) it shows enriched accumulation (arrowhead) at the posterior cortex. (G) In egg chambers after *hsDmn* induction, localization of kinesin was strongly reduced.

vealed normal localization through stage 7 of oogenesis but highly reduced levels of protein in the majority of egg chambers at stage 9/10, even when the nuclei were appropriately located at the anterior (our unpublished data). In situ hybridization to visualize *grk* transcript provided a potential explanation for the reduction in protein levels. In wild-type egg chambers after migration of the nucleus, *grk* mRNA is transiently distributed in an anterior ring adjacent to the nurse cells before refining into a tight crescent around the nucleus at the future dorsal anterior region of the egg (Figure 5E). We found *grk* transcript distribution to be essentially unaltered prior to stage 8. However, in stage 9/10 egg chambers, *grk* mRNA remained in a diffuse band at the anterior of the oocyte, although it showed slight enrichment around the nucleus (Figure 6C). This result implicates Khc activity in the refinement and restriction of *grk* transcript during late stages of oocyte development.

The potent enhancement of Dmn misexpression by reduction in Khc or Klc and the nuclear positioning defects in *Khc* germ line clones could be a consequence of independent inputs from kinesin and dynein/dynactin on nuclear anchoring and migration. Alternatively, the effect of kinesin may be mediated through effects on dynein/dynactin localization or transport. To distinguish between these possibilities, we examined the distribution of the dynein and dynactin complexes in egg chambers lacking kinesin. In the wild-type, Dmn and other components of the dynactin complex are present at high levels in the oocyte through stage 7 and by stage 8 are enriched in the oocyte cortex (see Figures 1B and 1C; [14–16]). Staining with antisera against Dmn showed that its localization is independent of kinesin activity during early and mid-oogenesis. However, in stage 9/10 egg chambers the overall level of Dmn is reduced, and the protein cannot be detected in the posterior crescent, where it is normally highly enriched (Figure 6D). Staining with antisera against Cdic revealed a similar loss of dynein localization at the posterior, although staining was still apparent at the anterior oocyte-nurse cell boundary (our unpublished data). The alteration in Dmn and Cdic distribution in *Khc* mutant egg chambers demonstrates that kinesin activity is required for the posterior localization of the dynein/dynactin complex during late oogenesis.

Given the strong genetic interactions between the two motors, we wished to determine whether kinesin localization in turn requires activity of the dynactin complex. It has recently been shown that Khc is expressed at high levels in germ line cells in the ovary as well as in follicle cells [33]. In the oocyte Khc is localized at the cortex from stage 8 onward, and like dynein/dynactin shows posterior enrichment at stage 9/10 as well as low levels of perinuclear staining (Figures 6E, 6F, and our unpublished data; [33]). We found that in egg chambers dissected from females 1 hr after Dmn induction, the posterior focus of Khc accumulation could not be detected in stage 9/10 oocytes and staining at the cortex was also highly reduced (Figure 6G). The fact that disruption of dynein or kinesin activity alters the distribution of the opposite polarity motor suggests that their localization in the oocyte is interdependent.

## Discussion

### Dynein and Kinesin Have Interdependent Roles in Oogenesis

Our results demonstrate that the dynein/dynactin complex has at least two essential roles in late oogeneis: anchoring the nucleus to the cell cortex and mediating the localization of several transcripts critical for patterning the oocyte and the embryo. Compromising

kinesin activity enhances the effects of inhibiting dynein, suggesting that the plus end-directed motor is involved in the same processes. There is a precedent for a linkage between motors with opposite polarity because antibodies against either kinesin or dynein/dynactin components inhibit both plus-end and minus-end transport in extruded squid axoplasm, and mutations in *Drosophila* kinesin or dynein/dynactin perturb both anterograde and retrograde axonal transport (reviewed in [34]). Furthermore, in *Gl* and *Dhc* mutants lipid droplet transport toward microtubule plus ends in the embryo is severely affected [35], and dynamitin misexpression abolishes bidirectional movement of lipid vesicles in mammalian fibroblasts [36].

One explanation for the strong genetic interaction observed between kinesin and *Dmn* overexpression could be that kinesin is required to transport dynein toward microtubule plus ends. This would allow individual dynein complexes to be reused for multiple rounds of minus end-directed motion. A reduction in kinesin levels may compromise this recycling and decrease the pool of available dynein; it would thus affect dynein's ability to translocate cargo toward microtubule minus ends. This model provides a mechanistic basis for why processes that involve dynein, such as nuclear attachment and *grk* RNA localization, could be severely impacted in oocytes lacking *Khc*. It is also consistent with our observation that in *Khc* mutant egg chambers the dynein/dynactin complex is not localized to the lateral cortex of the oocyte after stage 8 (see Figure 6). Significantly, localization of *grk* transcript and protein are relatively unaffected prior to stage 8, when defects in dynein/dynactin localization first become apparent.

In this context, it is interesting that the distribution of Khc in the oocyte resembles that of dynein and dynactin components; i.e., it is enriched at the cortex and the perinuclear region, where microtubule minus ends are expected to be most abundant (see Figure 6; [33]). Such a pattern is consistent with a role for kinesin in recycling dynein from the cortex, similar to its proposed function in transporting *osk* mRNA [17], but raises the paradoxical question of how kinesin localization is established. We have found that after *hsDmn* induction cortical staining for Khc is reduced, suggesting that Khc localization is in turn dependent on dynein activity. Transport of kinesin to the cortex could occur as a result of a direct physical interaction between the two motors. Alternatively, kinesin and dynein could bind common cargoes or adaptor proteins; this would be analogous to the situation in the embryo, where both dynein and a so-far-unidentified plus-end motor associate with individual lipid droplets. Transport of the particles and the associated motors could occur in either direction if the activity of the opposite polarity motors is appropriately regulated [35, 37]. Interaction with a common intermediate anchored to the posterior cortex could also explain why kinesin, dynein, and dynactin colocalize in this region. The recent finding that dynein-associated structures move rapidly along microtubules in both directions in *Dictyostelium* suggests that motor recycling may be a common mechanism for enhancing optimal utilization of a limited pool of these mechanochemical enzymes [38].

### Dynein Activity Is Required for Transcript Localization

Our results indicate a role for dynein in *grk* transcript localization. The fact that *grk* mRNA cannot be detected in late-stage oocytes 1–6 hr after Dmn induction (see Figure 5C) argues that dynein could be required for both the transport and anchoring of *grk* message. When microtubules are depolymerized, *grk* mRNA forms aggregates on the oocyte nuclear lamina, suggesting that this represents a site where it is anchored [27]. It might therefore be expected that if dynein functions exclusively in transport, inhibition of its activity would cause an increase in the perinuclear concentration of *grk* mRNA. Furthermore, transcripts that were already at the cortex should not have been disrupted. In oocytes assayed 1 or 6 hr after Dmn induction, *grk* message was absent from the nuclear periphery and the cortex, irrespective of where the nucleus was positioned. However, 12 hr after Dmn induction, *grk* mRNA localization to the nucleus had partially recovered (see Figures 5D and 5F). Interestingly, when the oocyte nucleus was incorrectly positioned along the A/P axis but remained cortically attached, *grk* transcript was also detected at the cortex. This argues that nuclear position and proximity to the cortex are primary determinants of *grk* localization. It is notable that *grk* message is insensitive to Dmn overexpresssion and the absence of kinesin in earlier-stage egg chambers, when it may be transported by a diffusion-based mechanism and is known to accumulate even in the absence of microtubules or microfilaments [27]. Localization of *bcd* message at the anterior of the oocyte is also highly susceptible to Dmn misexpression (see Figures 2A and 2B). Although our results cannot distinguish between inhibition of transport or anchoring of the mRNA, other data argue that dynein is likely to be involved in both of these aspects [7, 11, 39, 40]. Resolution of this issue may require direct observation of RNA localization in live egg chambers after *hsDmn* induction.

In contrast to the dramatic effect of *Dmn* overexpression on *grk* and *bcd* transcripts, *osk* mRNA distribution is altered in a more subtle fashion. The increased level of *osk* mRNA in the cytoplasm after Dmn overexpression (see Figures 2E and 2F) is consistent with the proposal that *osk* transcript binds to cortex throughout the oocyte and that kinesin transports it toward the interior in the anterior and lateral regions [17]. Accordingly, dynein may contribute to *osk* localization by transporting transcripts toward the cortex or in their maintenance.

### Dynein and Kinesin Are Required to Correctly Position the Oocyte Nucleus

The requirement for dynein activity in positioning the oocyte nucleus at the anterior cortex could reflect a role in nuclear anchoring alone or in both nuclear migration and anchoring. We find misplaced nuclei in stage 10 egg chambers dissected 1 hr after heat shock (Figure 3) even though nuclear migration would have occurred 13–25 hr earlier (at stage 7/8). This clearly shows that reduction of dynein activity disrupts nuclear anchoring through a mechanism that is still unclear. One possibility is that sustained activity of perinuclear dynein (acting

on microtubules oriented with minus ends toward the cortex) is required to maintain nuclear position. Alternatively, cortically localized dynein may have to be continually active to keep the nucleus "reeled in" through a subset of microtubules that have the opposite orientation. In either case, the nucleus would be predicted to fall away from the cortex in the absence of dynein activity. With respect to nuclear migration, there is considerable evidence that dynein motors power such a process in fungi. Our data do not permit a firm conclusion as to whether dynein also performs this role in the oocyte. Although severely ventralized eggs were obtained 40 hr after *hsDmn* expression, suggesting a failure of nuclear migration, they could also result from defects in anchoring after migration because of perdurance of excess Dmn.

Compared to oocytes in which dynein activity has been disrupted, those lacking kinesin show a higher frequency of nuclear-positioning defects. One explanation could be that kinesin function is completely abolished in Khc null clones, whereas residual dynein activity remains after *hsDmn* induction. Alternatively, it is conceivable that kinesin is the primary motor involved in nuclear positioning and that dynein plays an accessory role. In either event, the similarity in nuclear localization defects is consistent with a model in which the function of the two motors is linked. Dynein- and kinesin-related motors also act cooperatively to bring about nuclear migration in *S. cerevisiae*. Deletion of either of the kinesin-related proteins Kip2p and Kip3p or the dynein heavy chain results in nuclear migration defects [41]. Epistatic analysis suggests that Kip2p acts cooperatively with dynein, whereas Kip3p may affect nuclear migration through an independent pathway involving Kar9p [42]. Similarly, in Aspergillus, where nuclear migration is primarily thought to be dynein mediated, it has recently been shown that kinesin mutations affect nuclear movement and distribution in the hyphae [43].

## Conclusions

We have found that both dynein and kinesin are required for nuclear anchoring and localization of cellular determinants during oogenesis. The subcellular localization of dynein and dynactin is perturbed in kinesin mutants, and kinesin distribution is affected by Dmn misexpression. The interdependence of the two motors suggests a model in which kinesin affects patterning by localizing and recycling dynein and thus maximizing its utilization.

### Experimental Procedures

#### Mutant Alleles and Transgenic Lines

Null alleles for *Khc* (*Khc*$^{8}$), *Klc* (*Df(3L)8ex94*) [32]), *hsp70Glued* [30], and UAS-Tau-GFP [19] lines were used in this analysis. Germ line clones were generated with *P{w+, FRT}42B Khc*$^{27}$ as previously described [4]. The remaining mutant stocks (*elav-Gal4*, *C355-Gal4*, *Df(3L)10H)* were obtained from the *Drosophila* Stock Center at Bloomington.

#### Generation of Transgenic Lines for Ectopic Dmn Expression

A BamH1 site was introduced upstream of the full-length *Dmn* ORF from cDNA clone LD07994 (Genome Systems) with the primer CGTTGCCAAAGGATCCCGAACACCG. We then subcloned the cDNA into BglII/StuI sites in pCaSpeR-hsp70 [44] and into BglII/KpnI sites in pUAST [13] to generate *hsDmn* and *UAS-Dmn*, respectively. Multiple independent transgenic lines were obtained, inserts were mapped, and homozygous stocks were established.

#### Production of Dmn Antisera

The *Dmn* coding sequence was cloned into pRSET-A (Invitrogen) to induce protein expression in *E. coli* strain AD494(DE3)pLysS. Recombinant Dmn was batch purified under denaturing conditions, dialyzed against water, and outsourced to Covance Research Products for immunization of rats.

#### Western Blot Analysis

Homozygous *hsDmn* larvae (L3) were heat shocked for 60 min in a 37°C water bath, harvested at different time points spanning 12 hr, homogenized in 2× SDS gel loading buffer and analyzed on a 12.5% SDS-PAGE gel. Westerns were probed with anti-Dmn (1:200) and HRP-conjugated Donkey anti-rat secondary (1:20,000) antibodies before being developped with SuperSignal West Pico Chemiluminescent Substrate (Pierce Chemical).

#### Immunostaining

Ovaries were dissected, fixed, and stained as described previously [6]. Antisera used were as follows: rat anti-Dmn (1:100), goat anti-p150$^{Glued}$ (1:50; sc-9802, Santa Cruz Biotechnology), rabbit anti-*Drosophila* Khc (1:250; Cytoskeleton Inc), monoclonal anti-dynein intermediate chain 70.1 (1:50; Chemicon), monoclonal anti-Grk 4D4 (1:200; [45], Developmental Studies Hybridoma Bank), rabbit anti-Cnn (1:50; [18]), Alexa 488 conjugated WGA (1:1000, Molecular Probes), Rhodamine-Phalloidin (1:400, Molecular Probes), Alexa 488 goat anti-rat (1:200; Molecular Probes), and Cy3 donkey anti-goat (1:200; Jackson ImmunoResearch Laboratories). Images were acquired on BioRad MRC-600 and MRC-1024 confocal microscopes and processed with Adobe Photoshop 5.0.

#### In Situ Hybridization

Newly eclosed adults were placed in yeasted vials for three days prior to dissection and fixing for hybridization [6]. Sequential hybridization was initiated with a digoxigenin-labeled probe for *BR-C*, which was visualized with NBT/BCIP. Ovaries were washed and hybridized with a second digoxigenin-labeled probe for *grk* and developed with Vector Red fluorescent alkaline phosphatase substrate.

#### Heat Shock Treatment and Egg Collection

Freshly hatched females were placed in yeasted vials for 72 hr prior to transfer to food vials without yeast. Heat shocks were carried out by submerging the vial in a water bath at 37°C for 1 hr, except when noted otherwise. Histological analysis of ovaries was carried out at 1, 6, and 12 hr intervals after heat shock treatment.

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