

Reciprocal localization of Nod and kinesin fusion proteins indicates microtubule polarity in the *Drosophila* oocyte, epithelium, neuron and muscle

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SUMMARY

Polarization of the microtubule cytoskeleton is an early event in establishment of anterior-posterior polarity for the *Drosophila* oocyte. During stages 8-9 of oogenesis, when *oskar* mRNA is transported to the posterior pole of the oocyte, a fusion protein consisting of the plus-end-directed microtubule motor kinesin and β -galactosidase (Kin: β gal) similarly localizes to the posterior pole, thereby suggesting that plus ends of microtubules are pointed to the posterior. In this paper, we have substituted the motor domain of Kin: β gal with the putative motor domain (head) from the kinesin-related protein Nod. In cells with defined microtubule polarity, the Nod: β gal fusion protein is an *in vivo* minus-end reporter for microtubules. Nod: β gal localizes to apical cytoplasm in epithelial cells and to the poles of

mitotic spindles in dividing cells. In stage 8-10 oocytes, the Nod fusion localizes to the anterior margin, thus supporting the hypothesis that minus ends of microtubules at these stages are primarily at the anterior margin of the oocyte. The fusion protein also suggests a polarity to the microtubule cytoskeleton of dendrites and muscle fibers, as it accumulates at the ends of dendrites in the embryonic PNS and is excluded from terminal cytoplasm in embryonic muscle. Finally, the reciprocal *in vivo* localization of Nod: β gal and Kin: β gal suggests that the head of Nod may be a minus-end-directed motor.

Key words: Nod, microtubule, polarity, localization, *Drosophila*, neuron, oocyte, muscle, epithelium, kinesin

INTRODUCTION

The asymmetric segregation of cytoplasmic determinants is a general mechanism for distinguishing fates of sister cells, and requires the polar distribution of these molecules prior to cytokinesis (Horvitz and Herskowitz, 1992; Rhyu and Knoblich, 1995). The anterior-posterior (A-P) axis of the early embryo of *Drosophila melanogaster* offers an extreme example of asymmetrically distributed determinants (St Johnston and Nüsslein-Volhard, 1992). The mRNA for the anterior determinant *bicoid* (*bcd*) is localized to the anterior pole of the egg, while the mRNA for the posterior determinant *nanos* (*nos*) is localized to the 'polar plasm' at the posterior pole (Berleth et al., 1988; Driever and Nüsslein-Volhard, 1988a,b; Lehmann and Nüsslein-Volhard, 1991; Wang and Lehmann, 1991). In addition, molecules necessary for development of the future germline, such as *germ cell-less* (*gcl*) mRNA, Vasa (Vas) protein and Tudor (Tud) protein, are highly enriched in the polar plasm (Hay et al., 1988; Lasko and Ashburner, 1990; Jongens et al., 1992; Bardsley et al., 1993). The localization of these determinants to their respective poles requires the establishment of an A-P axis during oogenesis.

Genetic analysis of the 'posterior group' of maternal-effect genes has begun to unravel some of the mechanisms involved in assembling the polar plasm at the posterior pole (for reviews see St Johnston, 1993; Rongo and Lehmann, 1996). The *oskar* (*osk*) gene plays a critical role in polar plasm assembly. *osk* mRNA and Staufén (Stau) protein are the first components of

polar plasm to localize to the posterior pole; their localization occurs during stages 8 and 9 of oogenesis (Ephrussi et al., 1991; Kim-Ha et al., 1991; St Johnston et al., 1991). *osk* is essential for polar plasm assembly, and mislocalization of a chimeric *osk* mRNA to the anterior of the oocyte leads to a *stau*-independent assembly of polar plasm at the anterior pole (Lehmann and Nüsslein-Volhard, 1986; Ephrussi and Lehmann, 1992). Normal embryonic polarity therefore relies upon the efficient transport of *osk* mRNA to the posterior pole of the oocyte.

The A-P axis of the oocyte depends on at least two intercellular signalling events: one from the oocyte to the follicle cells and a second one, dependent on the first, from the follicle cells back to the oocyte. As a stage 1 egg chamber assembles in the germarium, the oocyte positions itself posterior to the nurse cells and induces a subset of the adjacent follicle cells to become posterior polar cells (González-Reyes and St Johnston, 1994; for reviews of oogenesis, see Mahowald and Kambysellis, 1980; Spradling, 1993). The signal from the oocyte requires the germline function of the genes *gurken* (*grk*), which encodes a TGF- α homolog, and *cornichon* (*cni*), while its reception requires the *Drosophila* EGF receptor (*DER* or *torpedo* [*top*]) in the follicle cells (Neuman-Silberberg and Schüpbach, 1993; González-Reyes et al., 1995; Roth et al., 1995). If the signal is disrupted by mutation of the above genes or by abnormal positioning of the oocyte in *spindle-A*, *spindle-B*, *spindle-C* or *spindle-D* (*spn-A*, *spn-B*, *spn-C* or *spn-D*) mutants, the posterior follicle cells adopt characteristics typical of anterior follicle cells (González-Reyes and St Johnston, 1994;

González-Reyes et al., 1995; Roth et al., 1995). Posterior polar cells in turn signal the oocyte to establish normal A-P polarity; this second signal requires the functions of *Notch* (*N*) and *Delta* (*Dl*) in the soma and the catalytic subunit of protein kinase A (PKA, encoded by *DCO*) in the germline (Ruohola et al., 1991; Lane and Kalderon, 1994). Perturbing the somatic signal either by transformation of the posterior follicle cells or by mutations in *N*, *Dl* or *DCO* causes mislocalization of *osk* mRNA and Stau protein to the center of the oocyte, and mislocalization of *bcd* mRNA to the posterior as well as the anterior pole (Ruohola et al., 1991; González-Reyes and St Johnston, 1994; Lane and Kalderon, 1994; González-Reyes et al., 1995; Roth et al., 1995).

How do the follicle cells direct the localization of mRNAs within the oocyte? One popular model has been that the signal from the soma triggers a reorganization of the microtubule cytoskeleton and allows formation of a microtubule array, which is polarized along the A-P axis. The evidence for a reorganization of the microtubule cytoskeleton is clear from immunofluorescence studies (Theurkauf et al., 1992, 1993). Shortly after the 16-cell cluster of the oocyte and its nurse cells forms, a microtubule organizing center (MTOC) can be detected in the oocyte, posterior to the nucleus. Microtubules emanate from this MTOC into the nurse cells and evidence from electron microscopy indicates that the centrioles from the nurse cells accumulate in the oocyte (Mahowald and Strassheim, 1970). The MTOC and the centrioles are detectable through stage 6 of oogenesis, after which they disappear and, intriguingly, the localization of *bcd* and *osk* mRNAs begins (Mahowald and Kambyzellis, 1980; St Johnston et al., 1989; Ephrussi et al., 1991; Kim-Ha et al., 1991; Theurkauf et al., 1992).

Evidence for a polarized microtubule array after stage 7 comes from two sources. First, immunofluorescence indicates that after the early MTOC disappears, oocyte microtubules form an anterior-to-posterior gradient; the high concentration of microtubules at the anterior cortex of the oocyte suggests an anterior nucleation of microtubules (Theurkauf et al., 1992). The second piece of evidence comes from experiments using a kinesin:β-galactosidase fusion protein (Kin:βgal, encoded by the fusion transgene *khc:lacZ*) (Giniger et al., 1993; Clark et al., 1994). The fusion protein contains the motor domain of the plus-end-directed microtubule motor protein kinesin (Yang et al., 1989, 1990) and localizes to regions of microtubule plus ends when expressed in cells with known cytoskeletal polarity. When expressed in the germline, Kin:βgal accumulates in the posterior of the oocyte during stages 8-9 of oogenesis, which strongly suggests microtubule plus ends point to the posterior when *osk* mRNA is being localized (Clark et al., 1994). Moreover, in *spn-A*, *spn-B*, *spn-C*, *spn-D*, *grk*, *cni*, *DER*, *N*, *Dl* or *DCO* mutants, Kin:βgal, like *osk* mRNA and Stau protein, localizes to the center of the oocyte (Clark et al., 1994; González-Reyes and St Johnston, 1994; Lane et al., 1994; González-Reyes et al., 1995; Roth et al., 1995). Since Kin:βgal localization is independent of *osk* and *stau* (Clark et al., 1994), a simple hypothesis is that the signal from the follicle cells is necessary to polarize the microtubule cytoskeleton of the oocyte, which in turn directs the localization of *osk* and *bcd* mRNAs.

While polarization of the oocyte microtubules is thought to be an output of the signal from the follicle cells, the nature of

this polarity has been questioned. According to the above model, plus ends of microtubules should point toward the posterior of stage 9 oocytes. However, both the heavy chain of cytoplasmic dynein, a minus-end-directed motor, and the Glued protein, a component of the dynein regulatory complex dynactin, are also posteriorly localized at stage 9 (Li et al., 1994; McGrail et al., 1995). This paradox creates some uncertainty about the polarity of the oocyte microtubule cytoskeleton and suggests that either Kin:βgal or dynein is not an accurate reporter of microtubule polarity of the oocyte. The localization of an independent molecule, in particular to the anterior, might help to resolve this dilemma.

In this paper, we construct a fusion protein, which localizes to minus ends of microtubules in several cell types and to the anterior cortex of stage 8-10 oocytes. The chimera is based on the kinesin-related protein (KRP) encoded by the *no distributive disjunction* gene (*nod*) (Zhang et al., 1990). *nod* is required for the proper segregation of nonexchange chromosomes via the distributive system during female meiosis in *Drosophila* (Carpenter, 1973; Grell, 1976; Hawley et al., 1993). In *nod* mutants, nonexchange chromosomes, such as chromosome IV, undergo high rates of nondisjunction. The Nod protein is distributed along the arms of meiotic chromosomes during metaphase I (stages 13-14 of oogenesis), due to an 82 amino acid region near the C terminus which contains three HMG-type DNA-binding domains (Afshar et al., 1995a,b). The N-terminal 320 amino acids of Nod shares strong sequence similarity with the motor domains of kinesin and other members of the superfamily of KRPs, which suggests that Nod may function as a microtubule motor in meiosis (Zhang et al., 1990; Goldstein, 1993). Knowing whether Nod is indeed a motor and identifying its direction is important for understanding the mechanism by which it ensures disjunction of achiasmata chromosomes. To date, however, the lack of motility of purified Nod protein along microtubules has left these questions unanswered.

We have created a Nod:β-galactosidase fusion protein (Nod:βgal) by replacing the motor domain of Kin:βgal with the putative motor domain from the N terminus of Nod. We find that, in cells with known microtubule polarities, Nod:βgal is a minus-end reporter for microtubules. Nod:βgal localizes to apical cytoplasm in polarized epithelial cells, to the poles of mitotic spindles in dividing cells and to the posterior of stage 1-6 oocytes. In stage 8-10 oocytes, Nod:βgal accumulates along the anterior cortex of the oocyte. The anterior localization of Nod:βgal in these oocytes supports the conclusions of microtubule polarity derived from the posterior localization of Kin:βgal. Furthermore, the *in vivo* localization of Nod:βgal in cells with known microtubule polarities may have implications for direction of the Nod putative motor domain.

MATERIALS AND METHODS

Fly stocks

UAS-KZ³⁰/TM3 and *UAS-KZ^{31Y}* stocks were generous gifts of P. Kolodziej. Line *KZ1071* was obtained in a screen performed by P. Kolodziej, D. Doherty, T. Jongens, I. E. C. and E. Giniger (unpublished). *GAL4* lines *24B* and *elav-GAL4* were generously provided by L. Luo. Mutant stocks *y cv swa³ v f/FM3*, *v swa¹/FM3*, *swa⁶/FM7*, *cn exu¹ bw/CyO* and *vas¹ cn exu bw/CyO* were obtained from the Bloomington stock center; alleles are described in Lindsley and Zimm (1992).

Construction of *nod:lacZ* transgene

To construct *nod:lacZ*, we made use of a *nod* cDNA plasmid, generously provided by H. Epstein and R. S. Hawley. The cDNA insert began at nucleotide -8 relative to the translation start site and was contained (from 5' to 3') between the *KpnI* and *EcoRI* sites of BluescriptIIISK (Stratagene). The *KpnI* site was destroyed and converted to an *XbaI* site by addition of a linker to create *pXba-nod*. With plasmid *khc:lacZ* (Giniger et al., 1993) as a template, we used the polymerase chain reaction (PCR) to amplify a region of the coiled-coil domain, from nucleotides 1320 to 1598 of *khc* (Yang et al., 1989), and added *HindIII* and *BamHI* sites on the 5' and 3' ends, respectively. Primers for the reaction were GGGAAGCTTAAGAACGTGGTCT-GCGTTAACG and CGGATCCGGACATATTGGCGAGAGCCG. The amplified fragment was cut with *HindIII* and *BamHI* and used to replace the corresponding fragment of *pXba-nod*, to create *pnod:khc*. Integrity of the amplified sequence was confirmed by dideoxy sequencing (USB, Sequenase 2.0). Finally, an *XbaI-SfiI* fragment of *pnod:khc* was used to replace the corresponding fragment of *pkhc:lacZ*, thereby creating *pnod:lacZ*. For enhancer trap lines, *nod:lacZ* was cloned as a *NotI-KpnI* fragment into the vector pEG117 (Giniger et al., 1993) to create the pNZ transgene; for UAS lines, the *NotI-KpnI* fragment was cloned into the vector pUAS_T (Brand and Perrimon, 1993) to create pUAS-NZ. The transgenes were introduced into a *y w* stock by P-element-mediated transformation (Rubin and Spradling, 1982).

Transposition of the NZ transgene

y w; NZ37.3/CyO; +/+ virgin females were crossed to *y w; +/CyO; Sb Δ2-3/TM6,Ubx* males to generate *y w; NZ37.3/CyO; Sb Δ2-3/+* F₁ males. These males were then crossed in pair matings to *y w* virgin females. Red-eyed, Curly, non-Stubble F₂ animals represented transpositions of the NZ transgene; individual F₂ males were taken and crossed to *y w* virgin females to generate NZ stocks. In a few cases, F₂ females of this class were isolated to obtain transpositions onto the X chromosome; line *NZ137.3* is one such insertion. Ovaries from NZ lines were dissected and stained for X-gal as previously described (Clark et al., 1994).

Whole-mount in situ hybridization

Preparation of an antisense RNA probe for *lacZ* and modifications to the technique of Tautz and Pfeifle (1989) were as described previously (Clark et al., 1994).

Antibody staining

Ovaries were dissected by hand or prepared in mass and fixed by the technique of Theurkauf et al. (1992). For visualization of microtubules, ovaries were incubated for 10 minutes in cold (-20°C) methanol. Embryos were fixed for 20 minutes in a 1:1 mixture of heptane and 4% formaldehyde (Ted Pella, Inc.) in PEMS (100 mM Pipes pH 6.8, 2 mM EGTA, 1 mM MgCl₂). In cases where preservation of microtubules was necessary (e.g. spindle staining), embryos were fixed either for 2 minutes in a 1:1 mixture of heptane:37% formaldehyde (Fisher) or twice for 5 minutes each in 1:1 heptane:methanol. Antibodies used were a polyclonal anti-β-galactosidase antibody from Cappel (preabsorbed against either egg chambers or embryos as appropriate, and used at 1:5000), monoclonal antibody DM1A against α-tubulin (Sigma, used at 1:500) and monoclonal antibody mAb22C10 (generous gift of S. Benzer; used at 1:250). Anti-β-galactosidase stainings were amplified with biotinylated goat anti-rabbit IgG and either streptavidin:Cy3 (Jackson Laboratories, each at 1:200) or Vectastain ABC (Vector). Other secondary antibodies used were DTAf-conjugated donkey anti-mouse IgG (Jackson labs, 1:200). Peroxidase stained samples were dehydrated and mounted in Permount (Fisher). Fluorescent samples were mounted in SlowFade (Molecular Probes) and confocal images were obtained on a BioRad MRC600 confocal imaging system. Figures were assembled using Adobe Photoshop 3.0.5 and Adobe Illustrator 6.0.

RESULTS

The *nod* gene encodes a protein of 666 amino acids (Fig. 1A), the first 320 of which bear 36% identity to the motor domain (or 'head') of the *Drosophila* kinesin heavy chain (Zhang et al., 1990). The *in vivo* localization of Kin:βgal (Giniger et al., 1993; Clark et al., 1994) suggested an assay system to examine the function of the Nod head. Specifically, we asked whether the Nod head could functionally substitute for the kinesin head in Kin:βgal and promote localization of a fusion protein. We constructed a *nod:lacZ* fusion gene, encoding the fusion protein Nod:βgal (see Fig. 1 and Materials and Methods). The Nod:βgal fusion protein was identical to Kin:βgal except that the 333 amino acid kinesin head had been replaced by the 320 amino acid Nod head. Expression of *nod:lacZ* was accomplished by two different methods. (1) NZ lines utilized the 'enhancer trap' technique, in which tissue-specific expression of the transgene is mediated by enhancers near the site of insertion in the genome (O'Kane and Gehring, 1987; Bier et al., 1989; Grossniklaus et al., 1989). (2) UAS-NZ lines made use of the two-component expression system of Brand and Perrimon (1993), which is based on tissue-specific expression of the yeast transcriptional activator *GAL4*.

Nod:βgal localizes to apical cytoplasm in epithelial cells

To examine the behavior of the Nod:βgal fusion protein, we first analysed its subcellular distribution in epithelial cells. Epithelial cells have a microtubule cytoskeleton which is polarized along the longitudinal axis of the cell, with minus ends at the apical side and plus ends pointing toward the basal side (Bacallao et al., 1989). We used two different types of epithelial cells for this experiment: cells of the embryonic hindgut and the follicle cells of the ovary. In the hindgut, the apical membrane is toward the lumen of the gut, while in the egg chamber, the apical membrane points toward the oocyte (Mahowald and Kambyzellis, 1980; Skaer, 1993). As shown in Fig. 2, Nod:βgal localized to apical cytoplasm when expressed in either the gut or the follicle cells, whereas Kin:βgal localized to basal cytoplasm in both tissues. Thus, in epithelial cells of both the gut and the ovary, Nod:βgal and Kin:βgal exhibited reciprocal subcellular distributions.

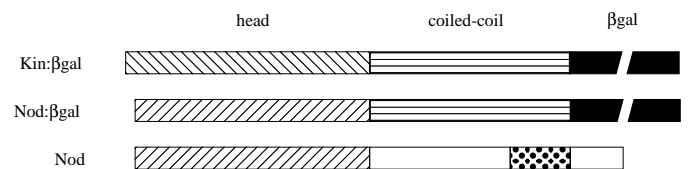


Fig. 1. Chimeras used in this study: Kin:βgal (Giniger et al., 1993) and Nod:βgal. Nod is included for reference. Hatched regions are head domains of *Drosophila* kinesin heavy chain (amino acids 1-333 of kinesin) and of Nod (amino acids 1-320 of Nod). Horizontally striped regions correspond to the N-terminal half of the coiled-coil domain of kinesin heavy chain (amino acids 334-606). Broken black bar is β-galactosidase, present at the C terminus of each fusion. Note that Kin:βgal and Nod:βgal differ only in the head domain. Stippled region corresponds to amino acids 513-594 of Nod. This region has three HMG 14/17 DNA-binding domains and mediates binding of Nod to chromosomes *in vivo* (Afshar et al., 1995a,b).

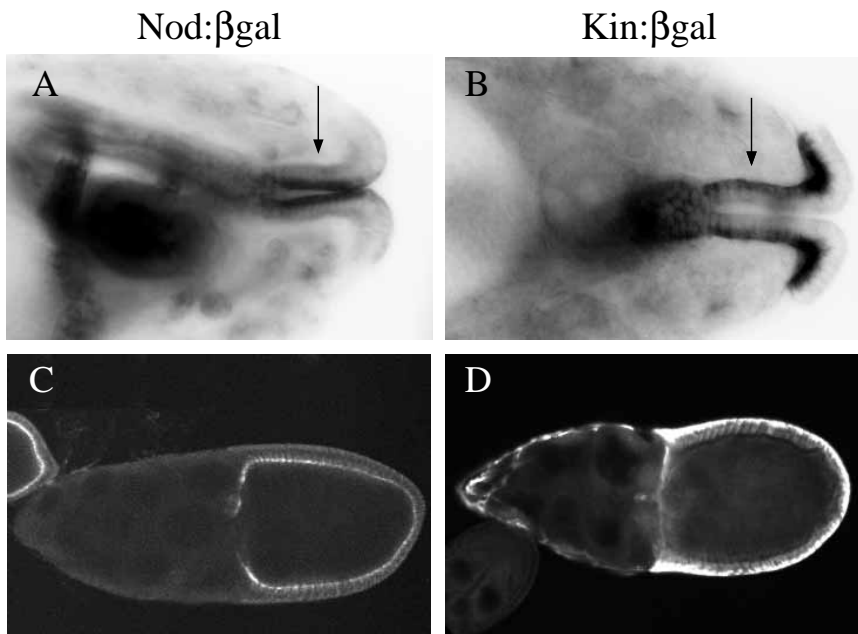


Fig. 2. Nod:βgal and Kin:βgal localize reciprocally in epithelial cells. (A,B) Dorsal views of stage 13 embryos from *NZ133.4* (A) and *KZ1071* (B), stained with anti-β-galactosidase. Hindgut epithelium indicated by arrow. Note apical and basal localization, respectively, in A and B. (C) Stage 10b egg chamber from *NZ133.4* female, stained with anti-β-galactosidase. Nod:βgal is in the follicle cell layer and is localized to the side facing the oocyte (i.e. apical). (D) Early stage 10b egg chamber from *hs-GAL4/+; UAS-KZ^{30/+}* subjected to a 30 minute heat shock at 39°C and a 3 hour recovery at 18°C. Kin:βgal is localized to the basal side, away from the follicle cells. Posterior is to the right in this and all subsequent figures.

Nod:βgal concentrates at the poles of mitotic spindles

Because Nod:βgal localized to apical cytoplasm in epithelial cells, we suspected that it might be an *in vivo* reporter for the minus ends of microtubules in polarized cytoskeletons. To test this further, we examined the distribution of Nod:βgal relative to the mitotic spindles of dividing cells. Mitotic spindle microtubules are oriented with minus ends at the spindle poles and plus ends toward the chromosomes (Euteneuer and McIntosh, 1981; Euteneuer et al., 1982). We stained 3- to 7-hour-old embryos from two enhancer trap lines, *NZ118.3* and *NZ153.1*, with antibodies to α-tubulin and β-galactosidase. Nod:βgal was strongly concentrated at the poles of metaphase and anaphase spindles (Fig. 3 and data not shown). Some Nod:βgal was also found along the spindle microtubules; this was not due simply to 'bleedthrough' from the tubulin channel, as similar staining was also detected when mitotic cells were identified with the DNA dye OliGreen (data not shown). Thus in dividing cells, as well as in the more differentiated epithelial cells, Nod:βgal accumulated in regions of cytoplasm that contained foci for microtubule minus ends.

Nod:βgal and Kin:βgal localize to opposite ends of stage 8-9 oocytes

In cells with defined microtubule polarity, Nod:βgal distribution consistently followed the positions of minus ends of microtubules. We therefore used it as an indicator for microtubule minus ends in the oocyte. Eight out of fifty transgenic *NZ* lines screened by X-gal staining of ovaries produced Nod:βgal in the germline. Subcellular distribution of Nod:βgal was consistent among these lines, despite differences in the timing and level of expression.

Both X-gal staining (not shown) and immunofluorescence demonstrated that, in

stage 1-6 egg chambers from transgenic line *NZ143.2*, Nod:βgal accumulated specifically in a single germline cell at the posterior of the chamber (Fig. 4A,B). The consistent posterior position of this cell indicated that it was the oocyte. By stage 6, Nod:βgal was concentrated against the posterior cortex. The distribution of Nod:βgal during these early stages thus mirrored the documented position of the MTOC for the nurse cell/oocyte cluster (Theurkauf et al., 1992, 1993).

By stage 8, however, Nod:βgal underwent a dramatic redistribution within the oocyte. The protein was no longer detectable at the posterior of the oocyte, but was instead concentrated in a peripheral ring along the anterior cortex of the oocyte (Fig. 4C). Within this ring, the region flanking the oocyte nucleus had the highest levels of Nod:βgal. The anterior localization sharpened during stage 9 and persisted through stage 10 (Fig. 4D and data not shown). Localization of Nod:βgal was in striking contrast to that of Kin:βgal, which,

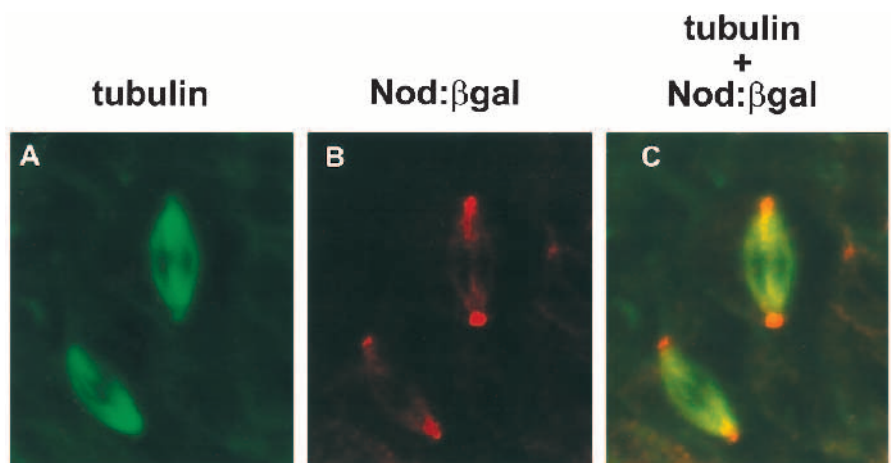


Fig. 3. Nod:βgal is concentrated at mitotic spindle poles. (A-C) Spindles from embryo of *NZ118.3*, visualized with anti-tubulin (A, green) and anti-β-galactosidase (B, red). (C) An overlay of the two images. Nod:βgal is present along the spindles, but is enriched at the poles.

as had been previously shown, localized to the posterior of stage 8 and 9 oocytes (Clark et al., 1994; Fig. 4E).

Localization of Nod: β gal was dependent upon the integrity of the microtubule cytoskeleton. To induce microtubule depolymerization, we fed *NZ143.2* flies colchicine at a concentration of 100 μ g/ml. After 4 hours of feeding, germline

microtubules were undetectable and anterior localization of Nod: β gal was observed in only 8.1% ($n=86$) of stage 8-9 oocytes, as compared to 90% ($n=120$) from untreated flies (Fig. 5).

Whole-mount in situ hybridization with an antisense RNA probe for *lacZ* demonstrated that neither the early localization of Nod: β gal to the oocyte nor the later localization to the anterior of the oocyte was due to localization of the *nod: lacZ* mRNA (data not shown). The anterior localization of Nod: β gal was also independent of the genes *exuperantia* (*exu*) and *swallow* (*swa*), both of which are required for anterior localization of *bcd* mRNA during these stages (data not shown; St Johnston et al., 1989).

Nod: β gal accumulates at dendritic terminals

Microtubules in axons have a uniform orientation, with plus ends pointing distally from the cell body (Heidemann et al., 1981). Dendrites, in contrast, have been shown in vertebrates to contain a mixture of microtubules, with either plus or minus ends oriented distal to the cell body (Baas et al., 1988; Burton, 1988; Sharp et al., 1995). Since Nod: β gal and Kin: β gal acted as in vivo reporters for microtubule minus and plus ends, respectively, we used them to assess the overall polarity of the dendritic microtubule cytoskeleton. We crossed flies bearing either a *UAS-NZ* transgene or a *UAS-KZ* transgene to flies containing the pan-neural *GAL4* transgene *elav-GAL4* (Luo et al., 1994). Embryos produced by these crosses were collected and stained with both anti- β -galactosidase and the monoclonal antibody 22C10, which labels the cell membranes of all PNS neurons (Zipursky et al., 1984).

Fig. 6 shows the result of this experiment for the lateral cluster of chordotonal neurons. As expected, Nod: β gal was effectively excluded from the axons, a result consistent with the idea that Nod: β gal is a minus-end reporter. Surprisingly, though, Nod: β gal strongly accumulated at the distal tips of the dendrites, with some of the fusion protein also detectable along the shafts of the dendrites and in the cell bodies (Fig. 6A-C). In contrast, however, Kin: β gal efficiently filled the axon bundle for the chordotonal neurons, but only poorly labeled the dendrites, with no accumulation at the dendritic termini (Fig. 6D-F; see also Giniger et al., 1993). Similar observations were obtained for external sensory neurons (data not shown). These results suggest that the overall polarity of dendritic microtubules in the chordotonal and external sensory neurons of *Drosophila* is opposite to that of axons.

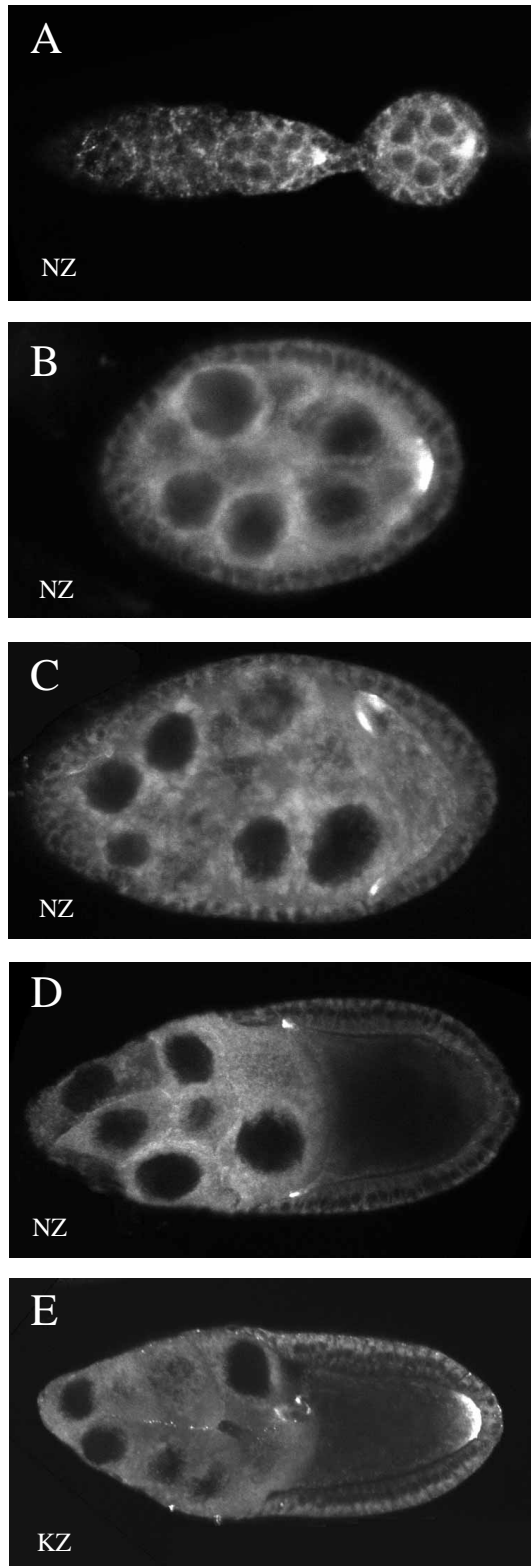


Fig. 4. Nod: β gal localization in germline during oogenesis. Ovaries of line *NZ143.2* (A-D) or *KZ503* (E) were stained with anti- β -galactosidase and analyzed with a BioRad MRC600 confocal microscope. (A) Germarium and stage 2 egg chamber. Nod: β gal localizes to the oocyte. (B) Stage 6 egg chamber. Nod: β gal is concentrated against the posterior cortex of the oocyte. (C) Stage 8 egg chamber. Nod: β gal is seen in cross section at the anterior corners of the oocyte and flanks both sides of the oocyte nucleus. (D) Late stage 9 egg chamber. Nod: β gal is tightly localized to the anterior corners of the oocyte. Some fusion protein is also present in the nurse cells. For comparison, a late stage 9 egg chamber expressing Kin: β gal is shown in E. Kin: β gal localizes efficiently to the posterior of the oocyte (see also Clark et al., 1994). Note that line *KZ503* also expresses Kin: β gal in border cells, a set of follicle cells that migrate to the anterior margin of the oocyte by stage 9. Images in A,B represent three stacked sections roughly 1 μ m apart; those in C-E represent stackings of five sections.

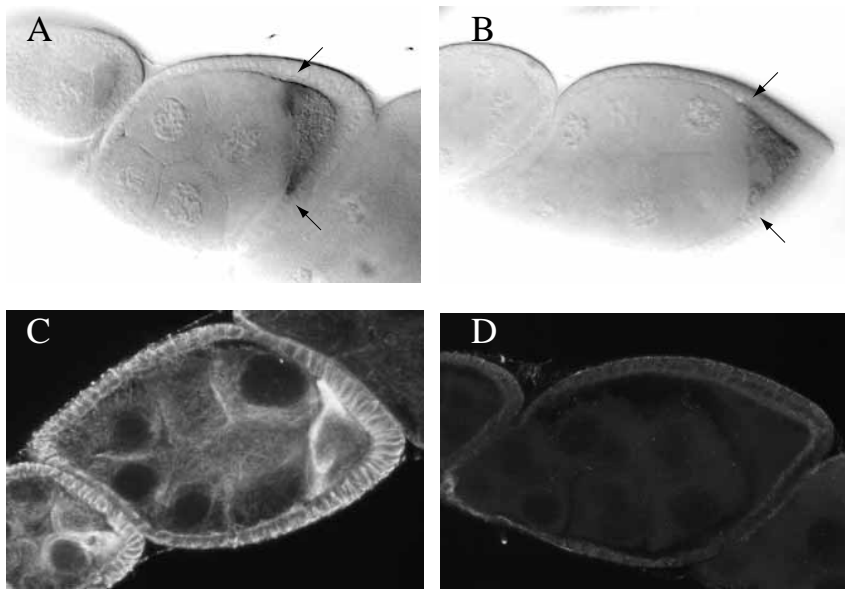


Fig. 5. Anterior localization of Nod:βgal requires microtubule integrity. *NZ143.2* flies were fed 100 μg/ml colchicine for 4 hours, at which point ovaries were dissected and stained for Nod:βgal with X-gal (A,B) or for microtubules with α-tubulin antibody DM1A (Sigma) (C,D). Stage 8 egg chambers are shown in all panels. After 4 hours of treatment, Nod:βgal was lost from the anterior margin (arrowheads) and microtubules were completely destroyed in the germline. (Compare A and C with B and D.) Images in C,D were obtained with identical settings on the confocal microscope.

Reciprocal localization of Nod:βgal and Kin:βgal in muscle

In addition to their reciprocal distributions in the oocyte, epithelial cells and neurons, Nod:βgal and Kin:βgal localized in complementary manners in embryonic muscles. We crossed *UAS-NZ* and *UAS-KZ* lines to the pan-mesoderm *GAL4* transgene *24B*, in order to express Nod:βgal and Kin:βgal in the embryonic musculature. As shown in Fig. 7A, Nod:βgal was excluded from the muscle attachment regions, but was detectable at high levels in the interior of the muscle fibers. In contrast, Kin:βgal concentrated at muscle attachment sites, and was present at only low levels throughout the remainder of the fibers (Fig. 7B and unpublished observations, E. Giniger and Y. N. J.).

DISCUSSION

A 2-reporter system for detecting microtubule polarity in vivo

The results presented here, together with previous results (Giniger et al., 1993; Clark et al., 1994), demonstrate that the Nod:βgal and Kin:βgal fusion proteins are in vivo reporters for polarized microtubule cytoskeletons. The two proteins, which differ only in the identity of the motor domain, localize in a reciprocal manner in a variety of different cell types. Furthermore, in cells in which the polarity of the microtubule cytoskeleton is known, such as epithelial cells, mitotic cells and neurons, the localization of Nod:βgal and/or Kin:βgal accurately reflects the polarity of the microtubules.

The localization of Nod:βgal during oogenesis mirrors the dynamic nature of the microtubule cytoskeleton. Early in oogenesis, as a discrete egg chamber forms in region 3 of the germarium, both

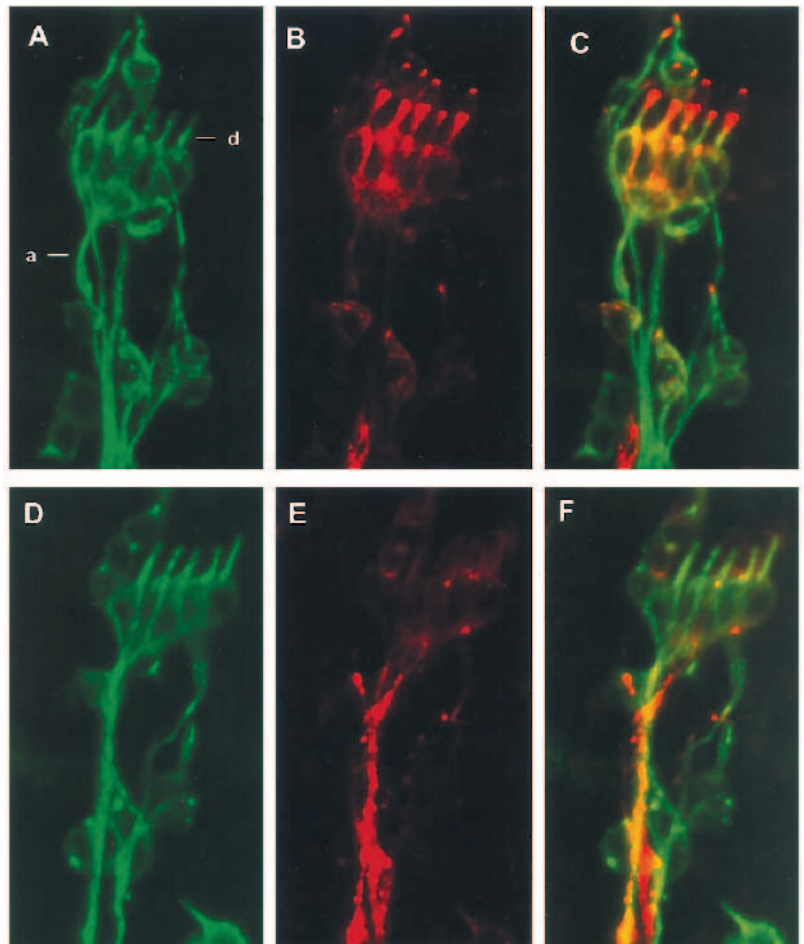


Fig. 6. Nod:βgal (A-C) and Kin:βgal (D-F) in lateral chordotonal neurons. Sample in A-C is from an *elav-GAL4/+; UAS-NZ^{B3.3}/+* embryo; sample in D-F is from an *elav-GAL4/+; UAS-KZ^{31Y}/+* embryo. The surface of the chordotonal neurons is visualized with mAb22C10 (green, A and D), while the Nod:βgal and Kin:βgal fusion proteins are detected with anti-β-galactosidase (red, B and E). Note the differences in dendritic (d) and axonal (a) distributions of the two fusion proteins.

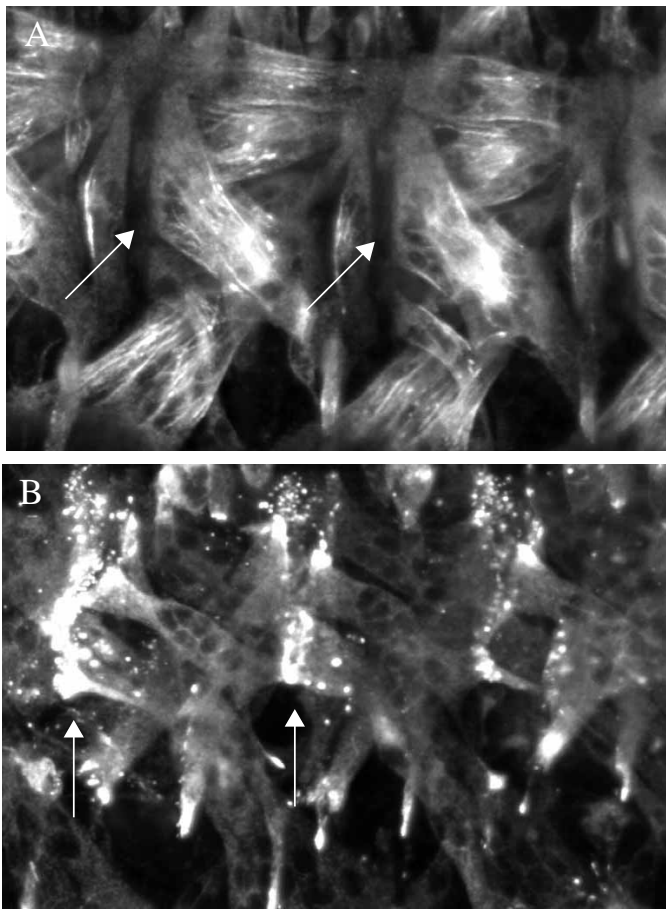


Fig. 7. Localization of Nod: β gal and Kin: β gal in embryonic muscle. Embryos of genotype *GAL4-24B/+; UAS-NZ^{B3.3}/+* (A) or *GAL4-24B/+; UAS-KZ^{31Y}/+* (B) stained with anti- β -galactosidase. 5 stacked 1 μ m optical sections are shown for each. (A) Nod: β gal is present at high concentrations in the interior of the myotubes, but is notably absent from the terminal regions at the attachment sites (arrows). (B) Kin: β gal, in contrast, is almost exclusively at the attachment sites (arrows).

the MTOC and Nod: β gal are found in the oocyte (Theurkauf et al., 1992, 1993; and Fig. 2A). At stage 7, the MTOC breaks down and the microtubule cytoskeleton reorganizes, and by stage 8, Nod: β gal localizes to the anterior cortex of the oocyte. The localization of Nod: β gal to the anterior of stage 8 oocytes indicates that by this time, microtubule minus ends point predominantly to the anterior. This finding supports the models of Theurkauf et al. (1992) and Clark et al. (1994), by which microtubules of stage 8-9 oocytes are polarized along the A-P axis, with minus ends at the anterior and plus ends at the posterior.

Like Nod: β gal, cytoplasmic dynein heavy chain and Glued protein both localize to the oocyte in stage 1-6 egg chambers and to the apical side of follicle cells. In stage 9 oocytes, however, dynein and Glued resemble Kin: β gal in that they localize to the posterior pole (Li et al., 1994; McGrail et al., 1995). It is still unclear why dynein localizes to the posterior of stage 9 oocytes. Given that Nod: β gal does not concentrate at the posterior pole at stage 9, we find it unlikely that dynein

localizes to the posterior by minus-end-directed transport along the majority of microtubules, although such a mechanism is sufficient to explain its early accumulation in the oocyte. It is also unlikely that posterior localization of dynein is achieved by attachment to a specific site on the cortex at the posterior, since dynein, like *osk* mRNA, Stau protein and Kin: β gal, mislocalizes to the center of the oocyte in *N* mutants (Li et al., 1994). If dynein localizes to the posterior by minus-end-directed motility along a special subset of oppositely oriented microtubules, the organization of these microtubules would also have to be altered in the cytoskeletons of *N* mutant oocytes.

It is worth noting that subcellular localization of dynein may be mediated not only by its motor activity, but also by activities involved in recycling or binding to specific cargo. Indeed, in vertebrates, cytoplasmic dynein has been found at kinetochores in mitotic cells (Pfarr et al., 1990; Steuer et al., 1990). It may be that dynein and Glued are brought to the posterior by association with one or more endogenous plus-end-directed motors. Alternatively, the direction of dynein motility may be regulated *in vivo*, so that its localization to the posterior occurs as a result of its own plus-end-directed motor activity. There is to date no evidence to support either of these models, and understanding the posterior localization of the dynein/Glued complex will await further analysis of its function in oogenesis.

Potential roles for microtubule polarity in mRNA localization

The localizations of Nod: β gal and Kin: β gal during oogenesis mimics the localizations of several endogenous mRNAs and proteins, including *bcd*, *osk*, Stau, *orb* and *K10* (St Johnston et al., 1989; Ephrussi et al., 1991; Kim-Ha et al., 1991; St Johnston et al., 1991; Cheung et al., 1992; Lantz et al., 1992). A simple hypothesis for the different types of mRNA localization during oogenesis is that early accumulation of specific mRNAs in the oocyte and later localization to the anterior pole are mediated by minus-end-directed microtubule motors, whereas localization to the posterior pole is mediated by plus-end-directed motors (Pokrywka and Stephenson, 1991; Theurkauf et al., 1992, 1993; Clark et al., 1994). While such models remain speculative, they suggest several steps at which mRNA localization may be regulated: (1) association of an mRNA with the appropriate motor, (2) activation of the motor-mRNA complex and (3) polarization of the microtubule cytoskeleton to specify the direction of transport.

Although microtubule motors that function in mRNA transport have yet to be reported, there is considerable experimental evidence to support a role for microtubules in mRNA localization (for reviews see Wilhelm and Vale, 1993; St Johnston, 1995). Microtubule inhibitor treatments prevent localization of mRNAs in oocytes from both *Drosophila* and *Xenopus* (Yisraeli et al., 1990; Pokrywka and Stephenson, 1991, 1995; Theurkauf et al., 1992, 1993; Clark et al., 1994). Localized mRNAs in both systems have been found biochemically to associate with microtubules *in vitro* (Yisraeli et al., 1990; Pokrywka and Stephenson, 1994). Particles of *bcd* mRNA and Stau protein, of an Exu-GFP fusion protein, and of myelin basic protein mRNA have been found to colocalize with microtubules *in vivo* (Ainger et al., 1993; Ferrandon et al., 1994; Wang and Hazelrigg, 1994). Finally, in *spn-A*, *spn-B*, *spn-C*, *spn-D*, *grk*, *cni*, *top*, *N*, *DI* and *DCO* mutants, the

mislocalization of *bcd* mRNA to both poles, and of both Kin: β gal and *osk* mRNA to the center of the oocyte, indicate a strong correlation between microtubule polarity and localization of maternal mRNAs for the A-P axis. As efficient localization of *osk* mRNA requires cytoskeletal non-muscle tropomyosin II, however, it is likely that the actin cytoskeletal also plays a critical role in the localization of maternal RNAs (Erdelyi et al., 1995; Tetzlaff et al., 1996).

Implications of Nod: β gal localization for *nod* function

Meiosis in wild-type females arrests in the first metaphase; exchange chromosomes are tightly aligned at the metaphase plate, while nonexchange chromosomes, such as the small fourth chromosome, are slightly displaced toward the spindle poles. In *nod* mutant females, however, the nonexchange chromosomes move precociously and excessively toward the poles, and are often ejected from the spindle as it is forming (Theurkauf and Hawley, 1992). *nod* is therefore required to prevent premature poleward migration of nonexchange chromosomes, either by exerting an anti-poleward force on the chromosomes or by providing a resistance to the poleward forces.

Our results suggest that the head of Nod encodes a minus-end-directed motor. Nod: β gal and Kin: β gal differ only in their heads, and yet the two proteins exhibit complementary localizations in a variety of cell types. We have not excluded the possibility that Nod: β gal localization is mediated by an association with an endogenous minus-end-directed motor. However, such a motor would have to be present in all cell types in which we see localization of the fusion protein and it would also have to interact specifically with Nod: β gal but not with Kin: β gal. Nevertheless, the complexity of the in vivo system precludes any strong conclusions about the direction of Nod and resolving this question will await the results of in vitro studies with purified or recombinant Nod protein. It is also possible that cofactors and/or chromosomes influence the direction of the Nod head in its native complex, which would complicate results from studies of recombinant proteins.

Afshar et al. (1995b) have found that the Nod head alone binds to microtubules in vivo without any obvious concentration at plus or minus ends, which suggests that other regions of the protein are necessary for motor activity. Motility of Nod: β gal may be facilitated by the kinesin coiled-coil domain or by the tetrameric β -galactosidase moiety at the C terminus. As Nod lacks a coiled-coil domain, it must either function as a monomer, like the KRP KIF1B (Nangaku et al., 1994), or form multimers in vivo without the benefit of its own coiled-coil. Multimerization could be achieved by the binding of accessory factors to Nod or by the binding of multiple molecules of Nod to adjacent sites on DNA.

Could Nod function in meiosis as a minus-end-directed motor? The polarities of meiotic spindle microtubules, which in female *Drosophila* are organized by the chromosomes, and of interchromosomal arrays of microtubules, which run between the nonexchange chromosomes at metaphase I, are both unknown (Theurkauf and Hawley, 1992; Hawley et al., 1993). If either of these microtubules arrays were oriented with minus ends toward the metaphase plate, a minus-end-directed protein could supply an anti-poleward force. A minus-end-directed motor could also be part of a 'sliding ratchet,'

coupling nonexchange chromosomes to the plus ends of growing microtubules; in such a model, the anti-poleward force would come from polymerization of the spindle microtubules themselves (Desai and Mitchison, 1995). Models based on Nod providing a resistance to poleward forces are also consistent with a minus-end direction; such models require that Nod or other chromosomal kinesins be regulated to allow the progression of chromosomes to the pole upon release from the metaphase I arrest.

Finally, it is worth noting that the only KRPs which have thus far been shown to be minus-end-directed motors (by in vitro motility of bacterially expressed protein) are the members of the KAR3/Ncd/CHO2 subfamily, all of which bear the motor domain at the C-terminus (McDonald et al., 1990; Walker et al., 1990; Endow et al., 1994; Kuriyama et al., 1995). However, in vitro studies on chimeras of kinesin heavy chain and the minus-end-directed motor protein Ncd indicate that direction is intrinsic to the head domain, and is not dictated by the position of the head at the N terminus or C terminus (Stewart et al., 1993; Chandra et al., 1993). Our observations of reciprocal in vivo localizations of two proteins differing only in their heads are consistent with the idea that motor polarity is encoded within the head. The mechanism by which direction is specified for microtubule motors is still unknown. Some clues may come from the recently reported crystal structures of kinesin and Ncd heads (Kull et al., 1996; Sablin et al., 1996). It is possible that Nod and another potential minus-end-directed N-terminal kinesin, CENP-E (Thrower et al., 1995), may help shed additional light on this problem. Because Nod and CENP-E belong to kinesin subfamilies different from each other and from Ncd, determining their directions with certainty and comparing their structures with Ncd may eventually help reveal the structural basis for polarity of microtubule motors.

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