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The *C. elegans* Hook Protein, ZYG-12, Mediates the Essential Attachment between the Centrosome and Nucleus

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Summary

The centrosome and nucleus are intimately associated in most animal cells, yet the significance of this interaction is unknown. Mutations in the *zyg-12* gene of *Caenorhabditis elegans* perturb the attachment of the centrosome to the nucleus, giving rise to aberrant spindles and ultimately, DNA segregation defects and lethality. These phenotypes indicate that the attachment is essential. ZYG-12 is a member of the Hook family of cytoskeletal linker proteins and localizes to both the nuclear envelope (via SUN-1) and centrosomes. ZYG-12 is able to bind the dynein subunit DLI-1 in a two-hybrid assay and is required for dynein localization to the nuclear envelope. Loss of dynein function causes a low percentage of defective centrosome/nuclei interactions in both *Drosophila* and *Caenorhabditis elegans.* We propose that dynein and ZYG-12 move the centrosomes toward the nucleus, followed by a ZYG-12/SUN-1-dependent anchorage.

Introduction

The centrosome and nucleus are closely apposed in most interphase cells. The association was first described at the EM level for nuclei isolated from rat liver and subsequently for a variety of cultured cells (Bornens, 1977; Kuriyama and Borisy, 1981; Nadezhdina et al., 1979). Immunolocalization studies have since extended this observation to many cell types. One possible role for the association is to maintain the proximity of centrosomes to the chromosomes upon the onset of mitosis. Abnormal positioning of the centrosomes could lead to a failure of astral microtubules to capture chromosomes upon nuclear breakdown, producing defects in the congression and segregation of chromosomes. Additionally, the association could be required for positioning the nucleus at the cell center (Aronson, 1971). Also, the force to move nuclei during nuclear and pronuclear migration may be transmitted through the asters of the centrosome (Raff and Glover, 1989; Reinsch and Gönczy, 1998). In spite of these hypothesized roles for the association, the mechanism and the importance of the association in these or any other processes are unknown.

The attachment between the centrosome and nucleus withstands both mechanical and biochemical disruption in cultured mammalian cells, including homogenization of the cell, solubilization of the nuclear envelope, enzymatic digestion of the chromatin, and disruption of the microtubule cytoskeleton using both cold shock and colcemid (Bornens, 1977; Kuriyama and Borisy, 1981; Nadezhdina et al., 1979). In contrast, sea urchin embryos first treated with colcemid (which disrupts microtubules) and subsequently centrifuged produce embryos with nuclei detached from centrosomes and mislocalized at the cortex. Regional inactivation of the colcemid allowed microtubule growth and movement of nuclei toward the centrosome (Aronson, 1971). Similar nuclear movement has been reconstituted in vitro and appears to be mediated by dynein (Reinsch and Karsenti, 1997). These observations suggest a model that dynein-mediated minus end-directed motility of nuclei along microtubules is responsible for the maintenance of the attachment of centrosomes and nuclei (Reinsch and Gönczy, 1998). The attachment is not static: centrosomes are released from the nuclei in a variety of cells during interphase, including polarized epithelium and *Drosophila* spermatocytes (Gonzalez et al., 1998; Reinsch and Gönczy, 1998).

Although no genes have been shown to be essential for the attachment of centrosomes to the nucleus, there is evidence that dynein may be involved. Depletion of cytoplasmic dynein components partially disrupts the attachment in both *Drosophila* and *C. elegans*. *Drosophila* embryos with reduced dynein function display a variety of phenotypes, including partially expressive centrosome attachment defects at the syncytial blastoderm stage (Robinson et al., 1999). RNA-mediated interference (RNAi) of the *C. elegans dhc-1* dynein heavy chain causes defects in meiosis, centrosome separation, and pronuclear migration. Additionally, in 15% of *dhc-1* RNAi embryos, the centrosomes fail to attach to the sperm pronucleus (Gönczy et al., 1999). RNAi of the *C. elegans dli-1* gene, which encodes the dynein light intermediate chain, produce similar embryonic phenotypes, including the low percentage of attachment defects (Yoder and Han, 2001). *unc-84*, a gene required for nuclear migration during *C. elegans* development, had also been hypothesized to play a role in the attachment of the centrosome and nucleus (Malone et al., 1999; Raff, 1999). However, subsequent studies of localization of nuclei and centrosomes in one cell type affected by *unc-84* mutations indicate that *unc-84* may not be required for the attachment (Lee et al., 2002).

Here we report the characterization of *zyg-12*, a gene that is absolutely required for the attachment between the centrosome and nucleus. Analysis of *zyg-12* mutations revealed that this attachment is essential. *zyg-12* is alternatively spliced and encodes members of the Hook protein family, which has been hypothesized to tether membrane bound organelles to the cytoskeleton (Walenta et al., 2001). ZYG-12 localizes to both the centrosome and nucleus during *C. elegans* development

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and, like all other Hooks, is able to bind to itself. Localization of ZYG-12 to the centrosome requires microtubules. Localization of ZYG-12 to the nuclear envelope requires the function of an additional centrosomal attachment gene, *sun-1*. In addition to its role in maintaining the attachment between the centrosome and nucleus, ZYG-12 also localizes dynein to the nuclear envelope, where dynein may act to bring the centrosome and nucleus into proximity. ZYG-12 then maintains the stable attachment of the nucleus and centrosome via a dynein-independent mechanism.

Results

zyg-12 Is Required for the Attachment of Centrosomes and Nuclei

C. elegans centrosomes closely associate with the nuclear envelope within one minute of nuclear envelope reformation and remain there during interphase (Keating and White, 1998). Using differential interference microscopy (DIC), we found that a previously isolated temperature-sensitive mutant, *zyg-12(ct350)*, was defective in this attachment (Figures 1A–1L). In wild-type embryos, nuclei are visible in the one cell embryo from their lack of refractile cytoplasmic granules. Centrosomes are also visible due to their ability to organize microtubule arrays, which exclude granules (Figures 1B and 1C). DIC observation of *zyg-12(ct350)* embryos at the nonpermissive temperature (25°C) revealed that in 100% of analyzed embryos ($n > 50$), centrosomes failed to associate with nuclei throughout interphase (Figures 1E–1H).

An additional *zyg-12* mutation, *zyg-12(or577)*, causes identical phenotypes as those seen for *zyg-12(ct350)* (Figures 1F and 1I). Both mutations are recessive, temperature sensitive, and display a strict maternal effect (data not shown) (Wood et al., 1980). Phenotypic analyses of *zyg-12* mutations over a deficiency, *zyg-12* RNAi, and *zyg-12* RNAi in a *zyg-12* mutant background gave similar phenotypes, consistent with these defects being strong loss-of-function phenotypes (Figures 1J–1L). DIC imaging of both mutations revealed defects not only in the attachment of centrosomes and nuclei, but also in pronuclear migration in 100% of embryos observed ($n > 50$) (Figures 1E–1G and 1I). Additionally, nuclei are mispositioned within the cell (Figures 1F and 1H). These embryos also had attachment and positioning defects in later cell cycles ($n > 50$). Embryos raised at a permissive temperature and transferred to the nonpermissive temperature were aberrant in both centrosome attachment and nuclear positioning within one minute of the shift, suggesting a continued requirement for *zyg-12*, not just an initial requirement during the first cell cycle ($n = 10$). *zyg-12* mutant embryos are otherwise quite normal. For example, cytokinesis and segregation of early polarity markers occurred normally (Figure 1H and data not shown). Centrosome separation and nuclear envelope breakdown occurred normally in *zyg-12* mutant embryos even though these organelles were often in quite different regions of the cell, implying that close association of the centrosome and nucleus is not required for these processes in *C. elegans* (see Supplemental Movie S1 at http://www.cell.com/cgi/content/full/115/7/825/DC1; data not shown).

The range of phenotypes observed for *zyg-12* mutants is a subset of those observed for loss of function of the dynein heavy chain gene, *dhc-1*, and the dynein light intermediate chain gene, *dli-1* (Gönczy et al., 1999; Yoder and Han, 2001). All three have detached centrosomes and a complete failure of pronuclear migration. In addition, *dhc-1* RNAi produces many phenotypes that are never seen in *zyg-12* mutants, including meiotic defects, yolk granule movement defects, centrosome separation failure, and aberrant attachment of centrosomes to the cell cortex (data not shown). Since *zyg-12* is involved in the attachment of centrosomes to the nucleus in the wild-type, we determined if *zyg-12* is required for the aberrant cortical attachment of centrosomes in *dhc-1* RNAi embryos. *dhc-1* RNAi, *zyg-12(ct350)* embryos raised at the nonpermissive temperature have centrosomes that are still attached to the cortex indicating that *zyg-12* is not required for this aberrant cortical attachment ($n = 5$).

Centrosomes Are Normal Except for Attachment Defect

Expression of β-tubulin::GFP revealed normal astral and spindle microtubule organization in both wild-type (Figures 1M–1O) and *zyg-12* mutant embryos in both interphase and mitosis ($n = 14$) (Figures 1Q–1S). In addition, the accumulation of γ-tubulin::GFP at the centrosome in *zyg-12* mutant embryos was identical to that of wild-type embryos (Figures 1X and 1Z) (Strome et al., 2001). Also, immunolocalization of SPD-5 and ZYG-9 indicate that both of these centrosomal markers localize normally in *zyg-12* mutant embryos (Figures 1W and 1Y, data not shown) (Hamill et al., 2002; Matthews et al., 1998). Wild-type and *zyg-12* mutant centrosomes looked identical by electron microscopy: both were clear of cytoplasmic granules, had microtubules associated with the periphery, and had normal centrioles (Figures 1U and 1V). Given that *zyg-12* mutant embryos are otherwise normal, we conclude that *zyg-12* is specifically required for the attachment of the centrosome and nucleus.

To investigate which aspects of the mutant phenotypes cause the embryonic lethality, we observed spindle dynamics in *zyg-12* mutants using timelapse imaging of β-tubulin::GFP, HIS-11::GFP (a GFP-tagged histone protein) expressing embryos (Figure 1P). At the onset of anaphase in wild-type embryos, two chromatin masses moved toward the opposite poles of the spindle (Figure 1P). In *zyg-12* mutant embryos, the spindle often incorporated the chromatin from only one pronucleus (see Supplemental Movie S2 on *Cell* website). During anaphase, this resulted in disorganized chromosome alignment, ultimately producing segregation defects (Figure 1T), which probably explains the lethality of *zyg-12* mutants. Segregation defects caused by mutations in other genes, such as *hcp-1*, *sep-1*, and *abc-1* have been shown to produce similar embryonic lethality (O'Connell et al., 2000; Severson et al., 2000; Siomos et al., 2001). To determine if the lethality is due specifically to the pronuclear migration defects, *zyg-12* embryos were shifted at the two or four cell stage, when pronuclear migration is already complete. Nine of ten *zyg-12(ct350)* embryos shifted at the two cell or four cell stage failed to hatch, while only 3 of 20 wild-type embryos failed to

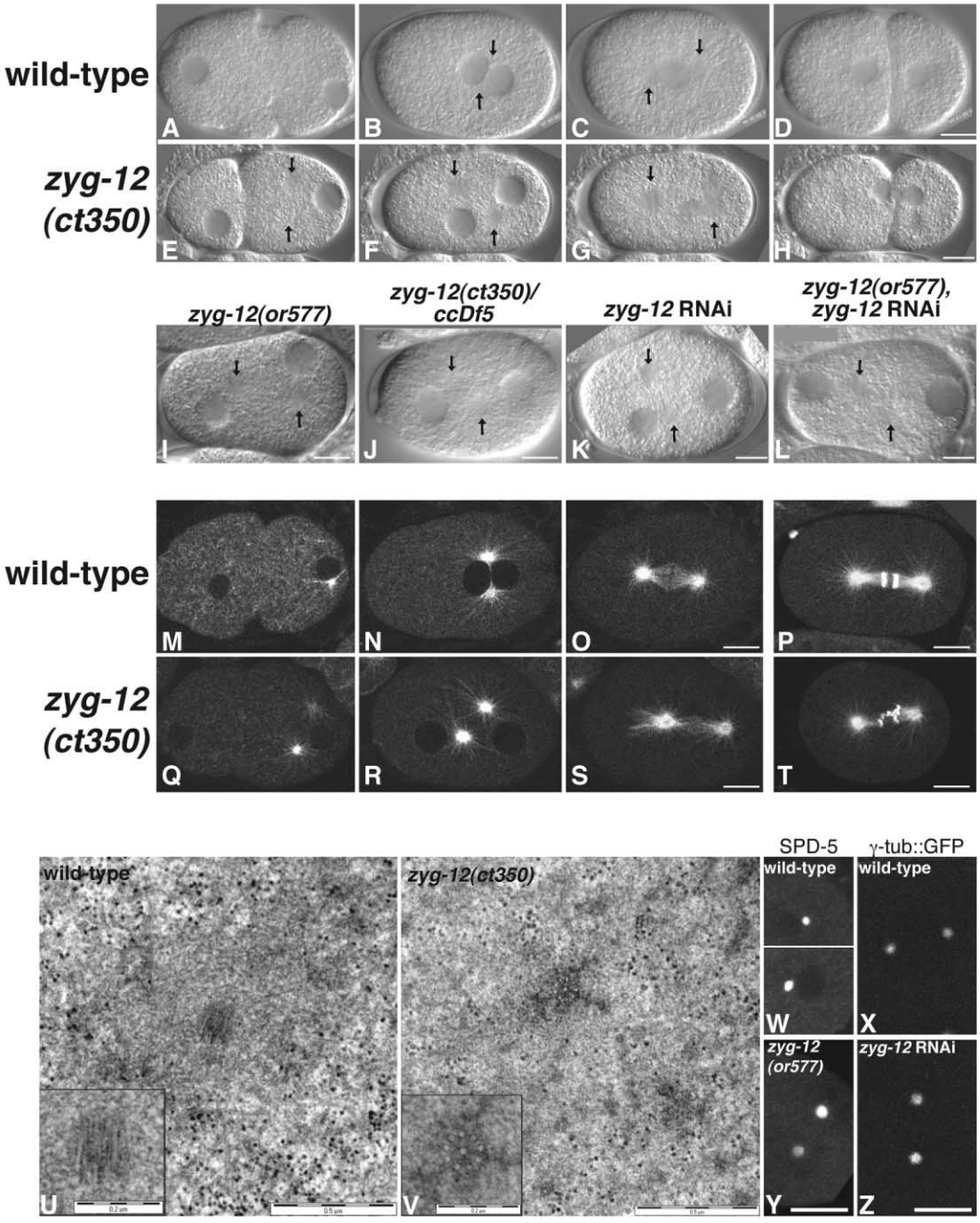

Figure 1. *zyg-12* Is Required for the Attachment of the Centrosome and Nucleus

Timelapse Nomarski differential interference microscopy (DIC) images of wild-type (A–D) and *zyg-12(ct350)* (E–H) embryos. (A and E) Initiation of pronuclear movement, (B and F) just prior to nuclear envelope breakdown, (C and G) prophase, and (D and H) two cell embryo. (I–L) Nomarski DIC images of embryos just prior to nuclear envelope breakdown. (I) *zyg-12(or577)*, (J) *zyg-12(ct350)/ccDf5*, (K) *zyg-12* RNAi, (L) *zyg-12(or577)*, *zyg-12* RNAi. (M–O and Q–S) Timelapse images of β-tubulin::GFP expressing strains. (P and T) Images of β-tubulin::GFP, HIS-11::GFP expressing strains. (M–P) Wild-type. (Q–T) *zyg-12(ct350)*. (M and Q) initiation of pronuclear movement, (N and R) prior to nuclear envelope breakdown, (O and S) metaphase, (P and T) early anaphase. For movies corresponding to Figures 1M–1O and 1Q–1S, see Supplemental Movies S1A and S1B on the *Cell* website; for movies corresponding to Figures 1P and 1T, see Supplemental Movies S2A and S2B. All embryos in this and subsequent figures are oriented with anterior to the left. (U, W, and X) Wild-type. (V, Y, and Z) *zyg-12* mutant or RNAi embryo. (U and V) Electron microscopy of centrosomes with the centrioles presented at higher magnification in the inset. (W and Y) SPD-5 localization. (W) Two focal planes from same embryo. (X and Z) γ-tubulin::GFP localization. Bar, 10 μm (except in [U] and [V]).

hatch, indicating that the embryonic lethality exhibited by *zyg-12* mutant embryos is not due specifically to pronuclear migration defects and is likely due to aberrant chromosomal congression and segregation.

Microtubules Are Required for the Attachment of the Centrosome and Nucleus

We next investigated whether microtubules are required for the attachment between the centrosome and nu-

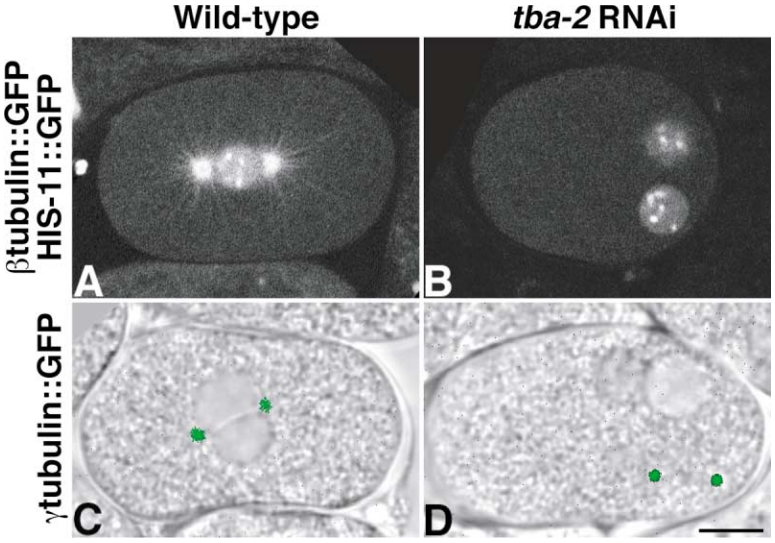

Figure 2. Microtubules Are Required for the Attachment of the Centrosome and Nucleus

Two-photon images of embryos expressing β-tubulin::GFP and HIS-11::GFP (A and B) or γ-tubulin::GFP merged with the transmitted light image (C and D). (C and D) Nuclei are visible as clearings in the cytoplasm, γ-tubulin::GFP is green. (A and C) Wild-type embryos and (B and D) *tba-2* RNAi embryos just prior to nuclear envelope breakdown. Bar, 10 μm.

cleus. Complete disruption of *C. elegans* embryonic microtubules using drugs such as nocodazole has proven difficult to achieve (Hyman and White, 1987). To overcome these experimental difficulties, we used RNAi to disrupt microtubules, using sequence from the α-tubulin *tba-2*, which would be predicted to deplete both *tba-1* and *tba-2*, the two α-tubulins expressed in the embryo (Hill et al., 2000). In a strain expressing both β-tubulin::GFP and HIS-11::GFP for labeling of microtubules and chromatin, *tba-2* RNAi depleted microtubules to below a detectable level (Figures 2A and 2B). We observed a range of phenotypes after RNAi, from low levels of organized tubulin at the centrosome to undetectable levels (Figure 2B). Consistent with this observed microtubule depletion, RNAi of *tba-2* has been shown to cause pronuclear migration defects and apparent spindle defects in otherwise wild-type embryos (Zipperlen et al., 2001).

To track the location of the centrosomes in *tba-2* RNAi embryos, we expressed the centrosomal marker γ-tubulin::GFP (Strome et al., 2001). As seen in the wild-type, the RNAi embryos have two normal foci of γ-tubulin (Figures 2C and 2D). We identified 19 centrosomes in timelapse recordings of 9 *tba-2* RNAi embryos during the first or second cell cycle. Of these, seven centrosomes were not attached to the nucleus, implicating microtubules in the attachment of the centrosome to the nucleus (Figure 2D). This low frequency may be due to variability in the depletion of microtubules, given the importance of microtubules for the localization of ZYG-12 suggested by observations discussed below.

zyg-12 Encodes a Hook Protein

zyg-12 maps to the left arm of chromosome II between *unc-85* and *dpy-10* (Wood et al., 1980). We further mapped *zyg-12* to a region of approximately 660 kb. To identify *zyg-12*, we searched for predicted open reading frames in this region that result in a *zyg-12*-like phenotype by RNAi. Only one, ZK546.1, produced phenotypes similar to those caused by mutations in *zyg-12* (Figure 3A). Expression of the genomic fragment encoding the ZK546.1 open reading frame under the control of a heterologous promoter rescued the centrosome attachment defects of both *zyg-12(ct350)* and *zyg-12(or577)*. Additionally, single missense mutations were found in ZK546.1 in both *zyg-12(ct350)* (A to C at position +131 of the transcripts) and *zyg-12(or577)* (A to C at position +1100 of the transcripts). Taken together, these data are consistent with *zyg-12* encoding ZK546.1. Sequencing cDNAs encoded by *zyg-12* identified five different transcripts produced by alternative splicing (Figure 3A). Three *zyg-12* transcripts encode the same open reading frame (ORF), while the remaining two transcripts produce different ORFs, for a total of three ORFs encoded by *zyg-12* (Figure 3A).

The three predicted ZYG-12 isoforms are identical over the first 730 amino acids (Figure 3B). In addition to the 730 amino acids in common, ZYG-12 A, B, and C have an additional 3, 44, and 28 amino acids at the C terminus, respectively. All ZYG-12 isoforms encode a central coiled-coil domain. The C-terminal extension of both B and C encode predicted transmembrane domains (Figure 3). The two mutations, *zyg-12(ct350* and *or577)*, both of which cause identical phenotypes, are missense mutations in distinct domains of ZYG-12. *zyg-12(ct350)* changes a glutamine to proline at amino acid 44, disrupting a short coiled-coil prediction at the N terminus. *zyg-12(or577)* changes a glutamine to proline at amino acid 367, disrupting the central coiled-coil prediction.

The common 730 N-terminal amino acids of the ZYG-12 isoforms share structural and sequence identity with the Hook family of proteins. Hook family members have been hypothesized to act as linker proteins between membrane compartments and the microtubule cytoskeleton, based primarily upon studies of human Hook3 (Kramer and Phistry, 1999; Walenta et al., 2001). The ZYG-12-mediated attachment between the nucleus and centrosome in *C. elegans* can be viewed as the linking of the membrane bound nucleus with the centrosome and its associated microtubules, suggesting an evolutionarily conserved role for the Hooks. These pro-

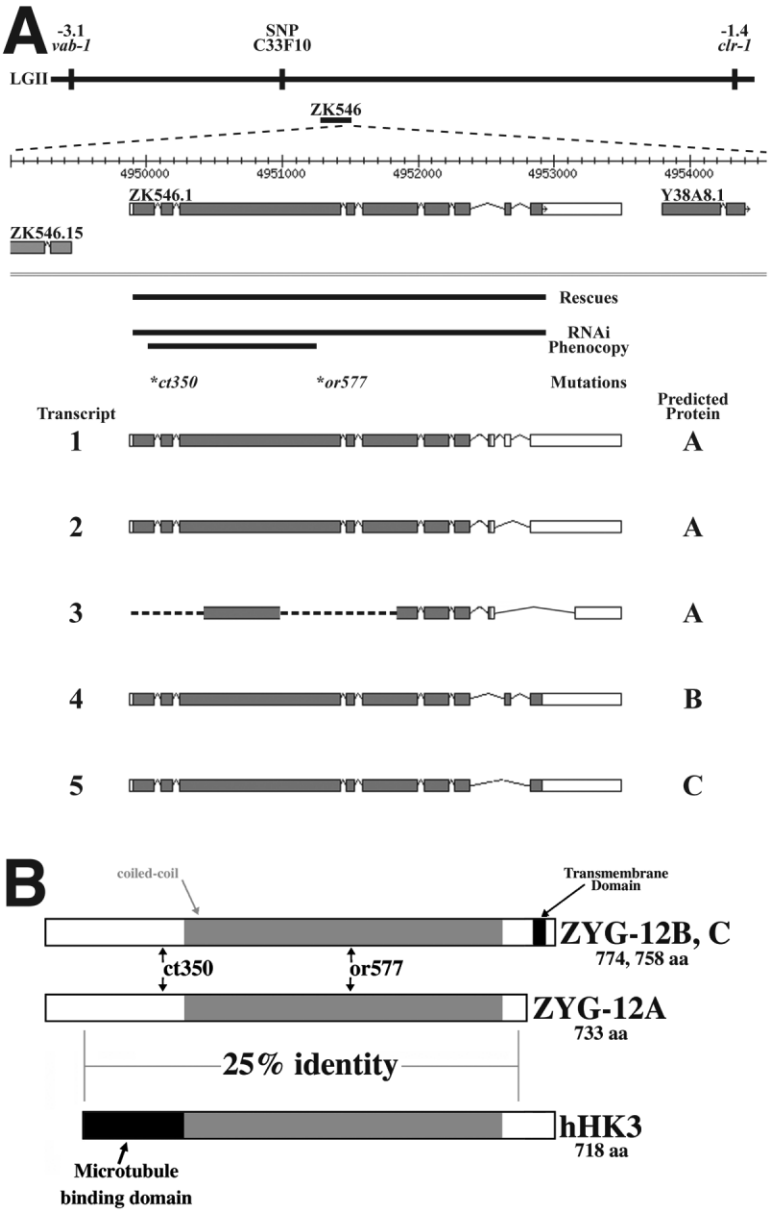

Figure 3. Positional Cloning and Gene Structure of *zyg-12*, which Encodes a Member of the Hook Protein Family

(A) *zyg-12* was mapped to the left arm of chromosome II between *vab-1* and *clr-1*. Further mapping placed *zyg-12* to the right of a SNP on cosmid C33F10. Three-point mapping with *vab-1* and the C33F10 SNP predicted *zyg-12* to be in the region of ZK546. A single open reading frame, ZK546.1, when subjected to RNAi, produced phenotypes identical to that of *zyg-12* mutations. Additionally, expression of the genomic fragment encoding ZK546.1 under a heterologous promoter rescued the *zyg-12* phenotypes. Missense mutations for both *ct350* and *or577* were identified within ZK546.1. The sequencing of seven cDNAs and analysis of two ESTs from this locus were performed to determine the gene structure. Five classes of transcripts were identified that encode three predicted proteins that differ at the C terminus. Boxes represent exons, closed boxes indicate predicted translated regions, dashed lines in transcript three are unsequenced.

(B) ZYG-12 isoforms share overall structural similarity and 25% identity with the Hook family of mammalian proteins (represented here by hHK3). Sequence of ZYG-12 isoforms A, B, and C have been deposited in Genbank under accession numbers AY487140, AY487141, and AY487142, respectively.

teins, including ZYG-12, have a central coiled-coil domain, which in *Drosophila* and humans has been shown to homodimerize. A microtubule binding domain is present in the N terminus of all previously identified Hooks. In the case of human Hook3, the C terminus mediates binding to the Golgi apparatus, the membranous organelle that hHook3 has been implicated in organizing (Walenta et al., 2001). This C-terminal function is intriguing because two of the three ZYG-12 isoforms contain sequences that are predicted to act as a membrane-spanning domain. *Drosophila* and human Hooks do not encode transmembrane domains (Walenta et al., 2001).

ZYG-12 Localizes to the Nuclear Envelope and Centrosomes

To determine the localization of ZYG-12, we expressed ZYG-12 isoforms tagged with GFP and raised antibodies to ZYG-12. The GFP reporters were expressed using a *pie-1*-based expression system, which drives expression in the germline and early embryo (Strome et al., 2001). The transgene ZYG-12A,B,C::GFP was constructed with *zyg-12* genomic DNA from the start codon to the stop codon in exon 10. This genomic construct contains all identified exons and introns of *zyg-12* and should express all three isoforms. Expression of this transgene completely rescued all mutant phenotypes, including defects in attachment, pronuclear migration, and nuclear positioning in *zyg-12(ct350)* and *zyg-12(or577)* early embryos shifted to the nonpermissive temperature (n = 20). We also expressed ZYG-12A::GFP individually and ZYG-12B,C::GFP together. Neither of these constructs was able to rescue the embryonic mutant phenotypes, indicating that all isoforms, or at least a combination of A and B or A and C, are required in the early embryo.

ZYG-12A,B,C::GFP localized to the nuclear envelope

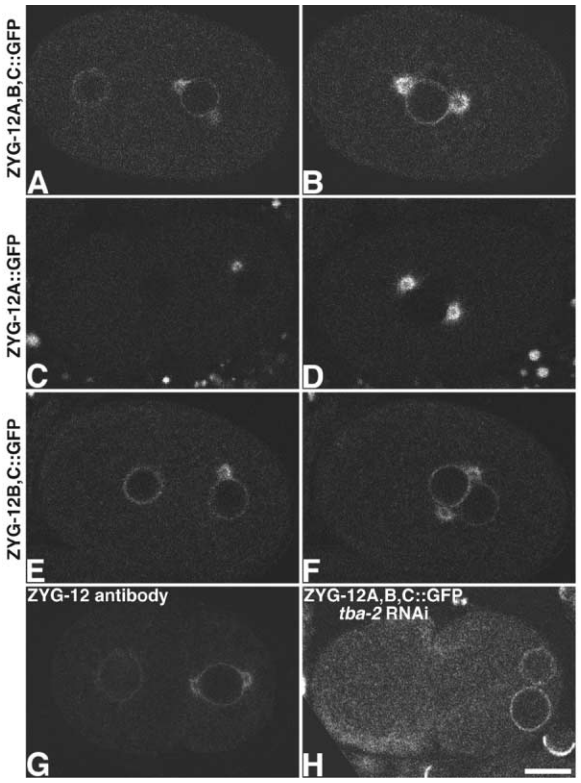

Figure 4. ZYG-12 Isoforms Localize to Both the Centrosome and Nucleus

Timelapse two-photon images of embryos expressing GFP-tagged ZYG-12 isoforms. Embryos are expressing (A and B) ZYG-12A,B,C; (C and D) ZYG-12A; (E and F) ZYG-12B,C tagged with GFP. (A), (C), and (E) are at the pronuclear migration stage and (B), (D), and (F) are at the centration stage. (C and E) Only one of two centrosomes visible in focal plane. (G) Wild-type embryo at the two cell stage stained with ZYG-12 antibodies. Centrosomes in the anterior cell AB are out of the plane of focus. (H) *tba-2* RNAi embryo expressing ZYG-12A,B,C::GFP. All embryos shown are wild-type for the genomic copy of *zyg-12*. For movie corresponding to Figures 4A and 4B, see Supplemental Movie S3 on *Cell* website. Bar, 10 μm.

in the syncytial gonad, oocytes, and all nuclei during the development of the early embryo (Figures 4A and 4B). The tagged ZYG-12 isoforms also localized to the region around the centrosome (Figures 4A and 4B). This hollow sphere of localization appeared similar to that of the proximal ends of microtubules organized by the centrosome (Figures 1M and 1N). Labeled microtubules extended in a linear fashion to the cortex, whereas the ZYG-12 labeling was concentrated at the centrosome and did not extend more than a few μm away from the centrosome. This may suggest that isoforms of ZYG-12 are able to colocalize with microtubules but only at the minus end or only near a centrosome. ZYG-12A::GFP alone localized to only the centrosome and was not detected at the nuclear envelope (Figures 4C and 4D). Expression of ZYG-12B,C::GFP resulted in localization to both the nuclear envelope and centrosomes (Figures 4E and 4F). Expression of either ZYG-12B::GFP or ZYG-12C::GFP alone resulted in identical localization as ZYG-12B,C::GFP (Figures 4E, 4F, and 5C; data not shown). We raised polyclonal antibodies against ZYG-12 to investigate the localization of the endogenous ZYG-12. ZYG-12 localized to both the centrosome and nuclear envelope in a pattern identical to that seen with ZYG-12A,B,C::GFP (Figure 4G). The predicted transmembrane domains of ZYG-12B and C suggest that the localization to the nucleus may be as an integral nuclear envelope protein. Given that ZYG-12A::GFP, which does not contain a predicted transmembrane domain, does not rescue the mutant phenotypes, the predicted transmembrane domain of ZYG-12B and C may be functionally important.

Given that ZYG-12 isoforms contain a region with similarity to the microtubule binding domain of other Hook proteins and that ZYG-12 isoforms can localize to the centrosome, a microtubule organizing structure, we wanted to determine if ZYG-12 localization is dependent on the presence of microtubules (Walenta et al., 2001). When we depleted microtubules via *tba-2* RNAi, we observed that centrosomal localization of ZYG-12A,B,C::GFP was lost although the nuclear envelope localization was completely normal (Figures 2A, 2B, and 4H).These data are consistent with localization of ZYG-12 to the centrosome arising by direct or indirect binding of ZYG-12 to microtubules.

sun-1 Is Required for Centrosomal Attachment and the Localization of ZYG-12 to the Nuclear Envelope

We found an additional gene, *sun-1*, with *zyg-12*-like phenotypes in a genome-wide videorecording screen of previously identified embryonic lethals (Kamath et al., 2003). In 100% of embryos observed, both centrosomes were unattached to the nuclei after *sun-1* RNAi ($n = 20$) (Figure 5B). Like *zyg-12*, *sun-1* RNAi does not affect the ability of centrosomes to organize microtubules (Figure 5B).

SUN-1 is a 473 amino acid predicted protein with the two hallmarks of a SUN protein: a putative transmembrane domain and a SUN domain at the C terminus (Figure 5A). The SUN domain was originally identified by sequence comparison between the *S. pombe* gene *sad1* and the *C. elegans* gene *unc-84* (*sad1, unc-84* SUN) (Malone et al., 1999). The domain is also found in several mammalian proteins (Figure 5A). UNC-84 is a nuclear envelope protein required for nuclear migration and positioning during late embryonic and larval *C. elegans* development (Malone et al., 1999). UNC-84 is required for the localization of two other nuclear envelope proteins, UNC-83 and ANC-1, which are individually involved in nuclear migration and positioning, respectively (Starr and Han, 2002; Starr et al., 2001). The mammalian SUN family currently includes four proteins, SUN1 (also called UNC84A), SUN2 (also called UNC84B), SPAG4, and MGC33329 (data not shown; Malone et al., 1999). Human SUN1 and SUN2 are also components of the nuclear envelope (Dreger et al., 2001; Schirmer et al., 2003).

To investigate the role of SUN-1 in the mechanism of centrosomal attachment, we tested the effect of *sun-1* RNAi on ZYG-12 localization. We assayed strains that expressed all three ZYG-12 isoforms tagged with GFP or just ZYG-12C::GFP, which contains a putative transmembrane domain. Using either strain, ZYG-12 localiza-

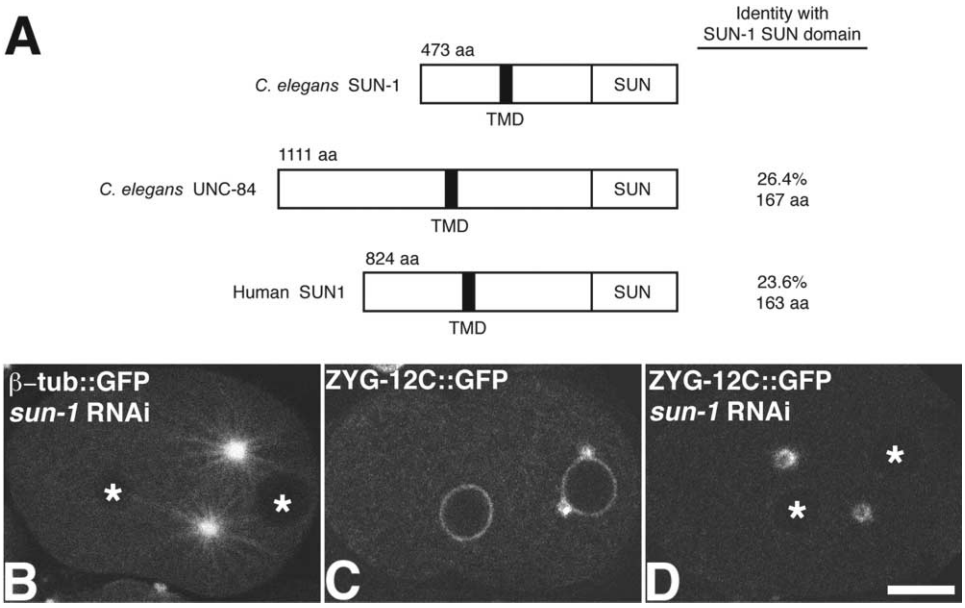

Figure 5. *sun-1*, a Second Gene Required for Centrosomal Attachment, Localizes ZYG-12 to the Nuclear Envelope but Not the Centrosome

(A) SUN-1 contains a putative transmembrane domain and a conserved SUN domain. All SUN domain containing proteins such as UNC-84 and SUN-1 also include a predicted transmembrane domain. (B) *sun-1* RNAi disrupts the attachment of centrosomes to nuclei in 100% of embryos, indicating that, like *zyg-12*, *sun-1* is required for the attachment. (C and D) *sun-1* RNAi disrupts the localization of ZYG-12C::GFP to the nuclear envelope but not centrosomes. Asterisks denote position of nuclei. Bar, 10 μm.

tion to the centrosome was normal, but the localization to the nuclear envelope was severely reduced or eliminated (n = 13) (Figures 5C and 5D; data not shown).

ZYG-12 Can Bind to Itself and the Dynein Light Intermediate Chain in the Two-Hybrid Assay

Given that ZYG-12 is required for the attachment of the centrosome to the nucleus and that ZYG-12 isoforms localize to both organelles, a simple mechanism for the attachment could be homodimerization of ZYG-12. This seems possible, given that all human Hook proteins are able to homodimerize (Walenta et al., 2001). We used a two-hybrid assay to test this hypothesis. The ZYG-12A isoform was used in these experiments because it lacks the C-terminal predicted transmembrane domain of the ZYG-12B and ZYG-12C, making it more amenable to two-hybrid analysis. Full-length ZYG-12A was able to bind to itself in the assay. By analyzing deletions, this interaction domain was mapped to the central coiled-coil domain (Figure 6A). We tested the effect of introducing both the *ct350* and *or577* mutations into both the bait and prey constructs and assayed binding. Mutant proteins were temperature sensitive for the binding assay, consistent with the interaction having physiological relevance. To investigate if ZYG-12 can interact with itself in vivo, we immunoprecipated GFP-tagged ZYG-12 using a GFP-specific antibody. Probing the immunoprecipated material with ZYG-12 antiserum demonstrated that nontagged endogenous ZYG-12 was pulled down with the tagged protein, consistent with dimerization in vivo (Figure 6B).

Using a two-hybrid screen, we identified additional proteins that can bind ZYG-12A. Of particular interest were two independent clones encoding either full-length or the C-terminal two-thirds of the *C. elegans* dynein light intermediate chain, DLI-1 (Figure 6A). RNAi suppression of DLI-1 or the dynein heavy chain, DHC-1, give identical postmeiotic defects and are the only other genes previously identified with any role in the attachment of the centrosome and nucleus (Gönczy et al., 1999; Yoder and Han, 2001). Mapping of the interaction indicated that the N-terminal 236 amino acids of ZYG-12 are sufficient for binding to DLI-1 (Figure 6A). This is the same domain that binds microtubules in both *Drosophila* and mammalian Hook proteins. *zyg-12(ct350)*, which maps to the DLI-1 binding domain, confers temperature sensitivity to the two-hybrid interaction (Figure 6A). Although *zyg-12(or577)* does not affect the ability of ZYG-12 to bind DLI-1 in vitro, it disrupts the ability of ZYG-12 to interact with itself in the two-hybrid assay, which may explain the strong loss-of-function phenotype of this allele.

ZYG-12 Is Required for the Localization of DHC-1 to the Nuclear Envelope

To determine whether the physical interaction between ZYG-12 and the DLI-1 seen in the two-hybrid assay may be functionally important, we asked whether ZYG-12 or the dynein complex is required for the localization of the other. DHC-1, the dynein heavy chain component of the dynein complex, localizes to the nuclear envelope as well as the cytoplasm and other structures (Gönczy et al., 1999). Given that ZYG-12 also localized to the nuclear envelope, it seemed possible for a physical interaction between ZYG-12 and DLI-1 to be involved in the localization of these proteins. Using DHC-1 antibodies, we determined that DHC-1 did not localize to the nuclear envelope in *zyg-12(ct350)* mutant embryos (Figures 7A

A

BD	AD	Growth? 30°	25°	15°
ZYG-12	ZYG-12	yes	yes	yes
ZYG-12(ct350)	ZYG-12(ct350)	no	no	yes
ZYG-12(or577)	ZYG-12(or577)	no	no	yes
ZYG-12 N-term	ZYG-12 N-term	no		
ZYG-12 C-C	ZYG-12 C-C	yes		
ZYG-12 C-C	ZYG-12 N-term	no		
ZYG-12	DLI-1	yes		
ZYG-12	DLI-1ΔNterm	yes		
ZYG-12(ct350)	DLI-1	no	no	yes
ZYG-12(or577)	DLI-1	yes	yes	yes
ZYG-12 N-term	DLI-1	yes		
ZYG-12 C-C	DLI-1	no		

B

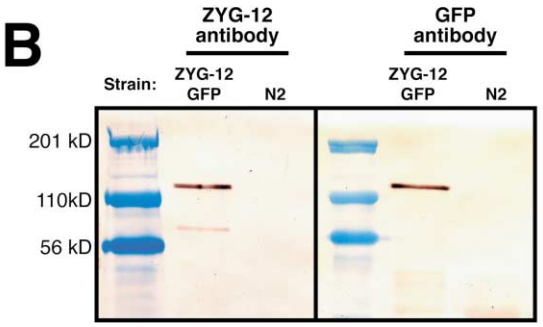

Figure 6. ZYG-12 Binds to Itself and Dynein Light Intermediate Chain

(A) ZYG-12 binds to itself and dynein light intermediate chain in the two-hybrid assay. ZYG-12 N-term: 1-236aa, ZYG-12 C-C (coiled-coil): 230–734(stop of ZYG-12A), DLI-1ΔN-term: 124–404(stop), all data reported assayed on -leu -trp -his -ade, BD: binding domain construct, AD: activation domain construct.

(B) ZYG-12 binds to itself in vivo. Lysates from wild-type (N2) and ZYG-12A,B,C::GFP expressing strains were immunoprecipated with GFP antibodies. Eluted proteins were Western blotted and probed with ZYG-12 and GFP antibodies. ZYG-12::GFP (upper band) and endogenous ZYG-12 (lower band) were detected in the ZYG-12::GFP containing immunoprecipated material.

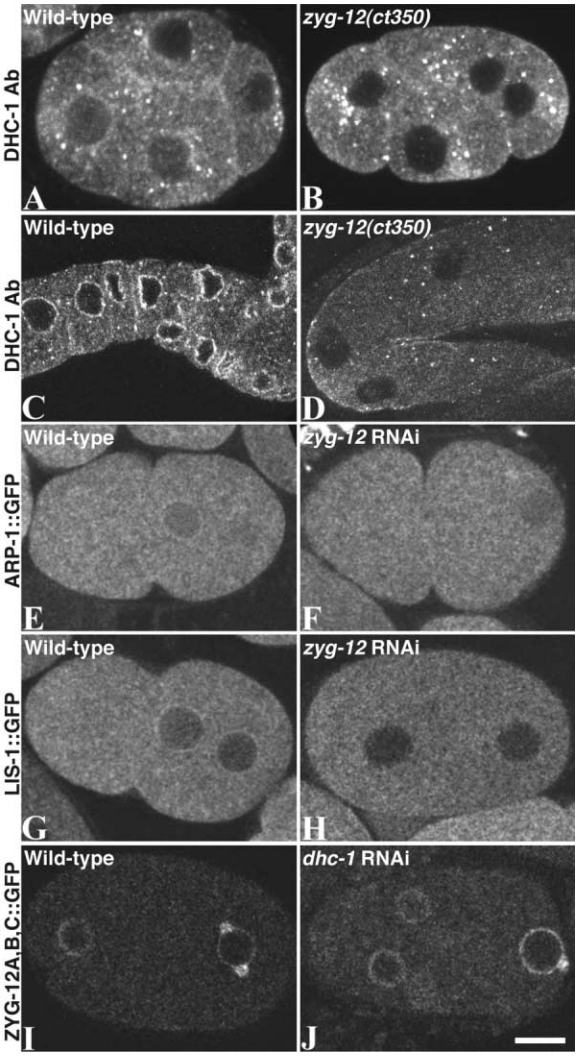

Figure 7. *zyg-12* Is Required for the Localization of Dynein Heavy Chain to the Nucleus but Dynein Heavy Chain Is Not Required for ZYG-12 Localization

(A–D) DHC-1 antibody staining in embryos (A and B) and extruded gonad (C and D), (E and F) ARP-1::GFP localization, (G and H) LIS-1::GFP localization, (I and J) ZYG-12A,B,C::GFP localization. (A, C, E, G, and I) Wild-type, (B and D) *zyg-12(ct350)*, (F and H) *zyg-12* RNAi, (J) *dhc-1* RNAi. For movies corresponding to Figures 7G and 7H, see Supplemental Movies S4A and S4B on *Cell* website. Bar, 10 μm.

and 7B). ZYG-12 localized to the nuclear envelope in the gonad (data not shown) and is required for DHC-1 localization to gonadal nuclei (Figures 7C and 7D). Consistent with the DHC-1 localization data, the dynein-associated protein LIS-1 and the dynactin component ARP-1 both localized to the nuclear envelope and cytoplasm in wild-type embryos but not to the nuclear envelope in *zyg-12* RNAi embryos (Figures 7E–7H). Both LIS-1::GFP and ARP-1::GFP localization is dynein dependent (data not shown). In contrast, localization of ZYG-12A,B,C::GFP was normal in a *dhc-1* RNAi embryo (Figures 7I and 7J). We propose that ZYG-12 independently localizes to the nuclear envelope where it recruits dynein possibly via the ability of ZYG-12 to bind to the dynein component DLI-1.

Discussion

Our observations of the phenotypic consequences of lack of centrosome attachment to the nucleus in *zyg-12* mutants demonstrate that ZYG-12 mediates an essential attachment between these organelles. The disassociated centrosomes cause a failure in pronuclear migration in the one cell embryo and mispositioning of nuclei and centrosomes during all early cell cycles, which gives rise to defective congression and segregation. The DNA segregation defects result in aneuploidy, which has been demonstrated to cause embryonic death in *C. elegans* (O'Connell et al., 2000; Severson et al., 2000; Siomos et al., 2001). Aneuploidy has also been linked to cancer progression in mammals (Nigg, 2002). Recently, there has been a renewed interest in the role of misregulation of centrosome number with regard to aneuploidy and the progression of cancer (Doxsey, 2002; Nigg, 2002). Possibly a loss of centrosomal attachment and

the resulting aneuploidy could also play a role in cancer progression.

The requirement for ZYG-12-dependent positioning of nuclei in mitotic cells is in contrast to the recently characterized, nonessential, ANC-1-dependent nuclear positioning within the differentiated syncytia of *C. elegans*. Animals lacking ANC-1 are otherwise wild-type (Hedgecock and Thomson, 1982; Starr and Han, 2002). Although ZYG-12 is required during early embryonic divisions, it is not required during later embryonic cell divisions. One possible explanation is that with decreasing cell size as development progresses, centrosomes are physically constrained to lie in fairly close proximity to the nucleus, allowing normal congression and segregation of the chromosomes. Alternatively, the spindle assembly checkpoint may become more effective during later cell cycles and allow time for congression and segregation defects to be resolved (Kitagawa and Rose, 1999).

In addition to the role of *zyg-12* in the attachment of centrosomes and nuclei, disruption of microtubules via *tba-2* RNAi has demonstrated that microtubules are also required for the attachment. Microtubules may have multiple roles during the establishment and maintenance of centrosome attachment. In addition to a role in localizing ZYG-12 to the centrosome, microtubules may be required for initially bringing the centrosomes and nuclei into proximity in some embryos (Hannak et al., 2002). SUN-1 is also required for the attachment. This SUN domain containing protein is required for the localization of ZYG-12 to the nuclear envelope. The requirements of microtubules and SUN-1 for the localization of ZYG-12 address the interesting question of how ZYG-12 isoforms, which are required at both the centrosome and nucleus for centrosomal attachment, obtain these distinct cellular positions. There are therefore at least four components involved in the attachment: ZYG-12, SUN-1, dynein (*dhc-1/dli-1*), and microtubules.

SUN-1, like its paralog UNC-84, is required for the localization of proteins to the nuclear envelope. UNC-84 localizes UNC-83, which functions in nuclear migration, and ANC-1, which functions in nuclear positioning and binds f-actin (Starr and Han, 2002; Starr et al., 2001). SUN-1 localizes ZYG-12, which functions in centrosomal attachment and the nuclear localization of dynein. One model that explains these data is that UNC-84 and SUN-1 are integral proteins of the inner nuclear envelope proteins that can interact, either directly or indirectly, with outer nuclear envelope proteins to specify the outer nuclear envelope as a unique domain. This specification could be accomplished by the same diffusion-retention model proposed for the inner nuclear envelope (Soullam and Worman, 1995). This would create a class of proteins that have not been previously identified, those that can be restricted to the outer nuclear envelope and not the endoplasmic reticulum (Voeltz et al., 2002). This would allow for the nucleus, distinct from the endoplasmic reticulum, to interact with the cytoplasm.

Our phenotypic analysis of *zyg-12* mutations has identified an additional function for the recently discovered Hook family. The only *Drosophila* Hook family member plays a nonessential role in the organization of the endocytic pathway (Kramer and Phistry, 1999). Since the *Drosophila* Hook is not essential, it is possible that *zyg-12* plays a similar role in *C. elegans* vesicle trafficking; however, no suggestive phenotypes have been observed. Of the three human Hooks, hHK3 is the most thoroughly studied. hHK3 appears to be necessary for the integrity of the Golgi apparatus, which is organized by microtubules emanating from the centrosome (Walenta et al., 2001). ZYG-12 differs from other Hooks in several important ways. Unlike *Drosophila* and human Hooks, ZYG-12 has a predicted transmembrane domain, which may be important for localization. Although the N termini of *Drosophila* and human Hooks bind microtubules, we have not yet determined if ZYG-12 is a microtubule binding protein. The only function demonstrated for the ZYG-12 N terminus is the binding of the dynein component DLI-1 in a two-hybrid assay.

Binding to ZYG-12 may be a mechanism for dynein to bind cargo. We have shown that ZYG-12 is required for the nuclear localization of dynein and that ZYG-12 binds the dynein component DLI-1 in a two-hybrid assay. These data are consistent with the mechanism for dynein binding to its cargo, the nucleus, being via the direct binding of dynein light intermediate chain to the integral nuclear envelope protein, ZYG-12. A similar mechanism has been proposed for pericentrin-dependent localization of dynein to the centrosome (Purohit et al., 1999). In another example, localization of dynein to the *Drosophila* kinetochore requires ZW10 and may be accomplished by the binding of the p50 subunit of dynactin by ZW10 (Starr et al., 1998). These data suggest that there are a variety of proteins that can localize dynein to cargo either by directly binding to dynein or indirectly binding via dynactin.

In most *C. elegans* one cell embryos, the centrosome is immediately adjacent to the newly formed sperm pronucleus due to their common point of entry. In a low percentage of embryos, the centrosome is not adjacent to the pronucleus (C.M., unpublished observations). In these embryos, it is likely that translocation of nuclear dynein along centrosomal microtubules results in the movement of the centrosome and the nucleus toward each other. Consistent with this model is our observation that RNAi depletion of microtubules cause attachment defects. Additionally, depletion of γ-tubulin causes a delay in the organization of robust microtubule asters by the centrosome. In these one cell embryos, a low percentage have unattached centrosomes early during the cell cycle. After microtubules begin to grow, the centrosome begins to move toward the nucleus (Hannak et al., 2002).

We propose a two-step model for centrosome attachment in *C. elegans*. In the first step, proximity is established between the centrosome and nucleus via dynein-mediated translocation of the nucleus toward the minus ends of astral microtubules emanating from the centrosome (Figure 8A). Once proximity has been established, we propose that attachment is initiated and maintained by ZYG-12 (Figure 8B). It is likely that the presence of ZYG-12 at both the centrosome and nucleus is required for attachment as ZYG-12A, which localizes to only the centrosome, is not sufficient. In this model, microtubules are required in both the establishment and maintenance steps, initially for dynein-mediated proximity and subsequently for the localization of ZYG-12 to centrosomes where ZYG-12 is required for attachment. One of many

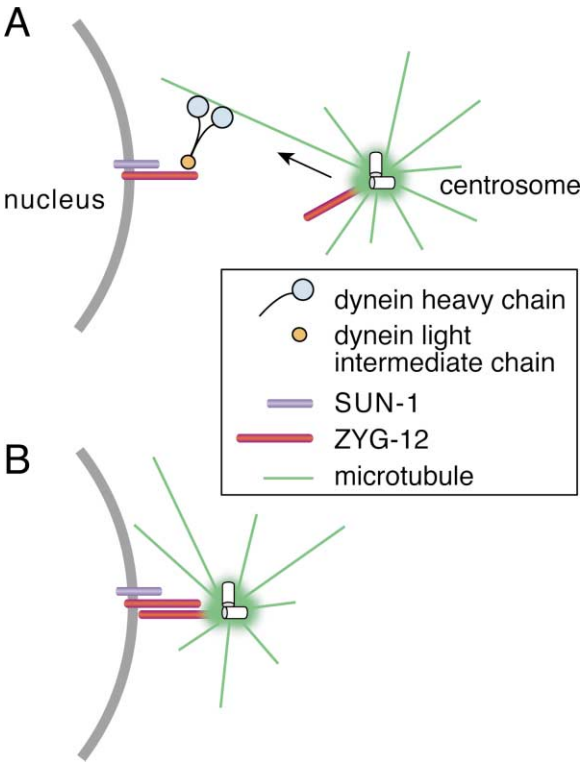

Figure 8. A Two-Step Model for Centrosome Attachment to the Nucleus

(A) The close proximity of the centrosome and nucleus can be initially established through the recruitment of dynein to the nucleus by ZYG-12 isoforms that are inserted in the nuclear envelope. Dynein translocates toward the minus ends of microtubules organized by the centrosome, bringing the centrosomes and nuclei proximal to each other. This first step is only required in a small subset of *C. elegans* embryos due to the fact that centrosomes are typically adjacent to nuclei while the nuclear envelope forms.

(B) In a dynein-independent mechanism, ZYG-12 mediates the attachment of the centrosome and nucleus. ZYG-12 isoforms are localized to the nuclear envelope as integral membrane proteins in a SUN-1-dependent manner. ZYG-12 also localizes to the centrosome via a microtubule-dependent mechanism. Localizing to both organelles, ZYG-12 may directly mediate the attachment via its ability to homodimerize. The position of ZYG-12 and SUN-1 with regards to the specific membranes of the nuclear envelope remains to be addressed, so we do not specify individual membranes in the diagram.

possible explanations for the role of ZYG-12 in the stable attachment of centrosomes to nuclei is that ZYG-12 at the nucleus and ZYG-12 at the centrosome may directly bind to each other. Consistent with this model, dynein is not required for the maintenance of the interaction.

Experimental Procedures

Preparation and Microscopy of Living and Fixed Embryos

Live imaging of embryos was performed with Nomarski DIC optics and two photon microscopy as described (Strome et al., 2001). The temperature of the stage was maintained at 25°C or room temperature (22°C). γ-tubulin::GFP images were superimposed in the green channel of simultaneously acquired transmitted light images to demonstrate the position of centrosomes with regard to the nucleus using Photoshop (Adobe Systems). The nuclei and centrosomes of the embryo imaged in Figure 2D were at different focal planes. Images of both focal planes were acquired with 20 s of each other and compiled for the figure. Using data from intermediate time points, we concluded that the image presented represents the actual cellular configuration.

The DHC-1 antibody was obtained from P. Gönczy. Antibody staining of embryos and extruded gonads was performed by methanol fixation followed by staining with a 1:100 dilution of anti-DHC-1 (Gönczy et al., 1999) or a 1:500 dilution of anti-ZYG-12 (see below). Confocal images were obtained using a Bio-Rad MRC1204.

Whole young adult worms containing embryos were prepared for electron microscopy by high-pressure freezing followed by freeze substitution (McDonald, 1999). The substitution was carried out in two stages, incubating in the primary medium for 68 hr at −90°C, warming to −60°C, rinsing and replacing with the secondary medium for an additional 26 hr as the samples were slowly warmed to 0°C. The primary medium was 2% glutaraldehyde dissolved in 98% Acetone/2% water. The secondary medium was 2% osmium tetroxide dissolved in 98% acetone/2% water (Walther and Ziegler, 2002). Serial longitudinal sections 65 nm thick were collected and stained with saturated aqueous uranyl acetate followed by 0.4% lead citrate. Imaging was performed on a Phillips CM 120 at 80 Kv to visualize early embryonic centrosomes.

Strains

zyg-12(ct350) was originally identified as the allele that caused the temperature-sensitive lethality observed in the wild isolate Bergerac strain of *C. elegans*. *zyg-12(ct350)* was extensively outcrossed to the wild-type *C. elegans* strain N2 (Wood et al., 1980). *zyg-12(or577)* was isolated in a mutagenesis of a strain derived from N2 (B. Nash and B. Bowermann, personal communication). The deficiency *ccDf5* was used for genetic tests of *zyg-12(ct350)* (Chen et al., 1992). β-tubulin::GFP, γ-tubulin::GFP, and HIS-II::GFP were expressed via the *pie-1* promoter and enhancer sequences (Strome et al., 2001). β-tubulin::GFP and HIS-II::GFP were originally transformed via microparticle bombardment (Praitis et al., 2001; Strome et al., 2001). γ-tubulin::GFP is expressed from a complex array to obtain germline expression (Strome et al., 2001).

RNAi of *zyg-12*, *tba-2*, *sun-1*, and *dhc-1* was performed using the feeding RNAi vector L4440 to express double-stranded RNA of the respective gene in the feeding bacteria (Fraser et al., 2000; Timmons and Fire, 1998). Injection of double-stranded RNAi synthesized in vitro from *zyg-12* cDNA template caused identical phenotypes as the feeding RNAi of *zyg-12* (data not shown). The severe microtubule depletion seen in *tba-2* RNAi, β-tubulin::GFP strains may be caused by a cumulative effect of destabilizing microtubules through the addition of a GFP tag to the β-tubulin and depleting the α-tubulins (Figure 2B). Rescue of *zyg-12(ct350)* and *zyg-12(or577)* was performed in strains in which the *zyg-12* chromosome was marked with the closely linked recessive mutation *dpy-10(e128)* to confirm that the strain was homozygous for the *zyg-12* mutant chromosome. After assaying rescue, the strain was outcrossed to confirm the presence of the *zyg-12* mutation.

Positional Cloning and Molecular Characterization of *zyg-12*, Identification and Characterization of *sun-1*

Traditional three-point mapping and single nucleotide polymorphism mapping were performed to place *zyg-12* between the cosmid C33F10 and the cloned gene *clr-1* in the region of cosmid ZK546 (Brenner, 1974; Jakubowski and Kornfeld, 1999). All predicted ORFs in this region were subject to RNAi via the RNAi feeding vector L4440 (Timmons and Fire, 1998). Expression of the genomic region encoding the candidate ORF ZK546.1 under the control of *pie-1* promoter and enhancer sequences was performed to test for rescue of both *zyg-12(ct350)* and *zyg-12(or577)* (Strome et al., 2001). Genomic DNAs encoding the ORF ZK546.1 from the mutant strains were PCR amplified and sequenced to identify molecular lesions. To confirm the gene structure of *zyg-12*, we sequenced seven cDNA clones produced by the *C. elegans* EST project (Y. Kohara, National Institute of Genetics, Mishima, Japan). This sequence analysis revealed an additional exon not predicted in WormBase (http://www.wormbase.org, release WS89).

A genome-wide RNAi screen identified an additional gene, F57B1.2, with *zyg-12*-like phenotypes that we have named *sun-1*

based upon the presence of the conserved SUN domain (Malone et al., 1999). Sequencing of cDNAs from the *C. elegans* EST project confirmed the gene prediction in WormBase (http://www.wormbase.org, release WS111).

Comparison of predicted ZYG-12 and SUN-1 protein sequences with the database was performed via BLAST (Altschul et al., 1997). Transmembrane domain predictions were performed using Biology Workbench 3.2 (http://workbench.sdsc.edu/) (Persson and Argos, 1994). Coiled-coil predictions were made using the COILS server (http://www.ch.embnet.org/software/COILS_form.html) (Lupas et al., 1991). In addition to the similarity with Hook proteins, ZYG-12 also shares weak similarity to the *C. elegans* ORF ZC8.4 in the coiled-coil region.

Generation of GFP Reporters and Two-Hybrid Constructs

GFP fusion proteins were expressed in the germline and early embryos using the *pie-1* promoter/enhancer system (Strome et al., 2001). GFP reporter constructs that expressed all three ZYG-12 isoforms (ZYG-12A,B,C) or only ZYG-12A were made using inserts derived from wild-type *zyg-12* genomic DNA cloned in the SpeI site of the vector. A reporter construct that expressed only the ZYG-12B and C isoforms (ZYG-12B,C) was made by removing exon eight (along with the splice donor and acceptor for exon eight) from the ZYG-12A,B,C reporter using the QuickChange Kit (Stratagene). Reporters expressing mutant ZYG-12A,B,C GFP fusion proteins were created using genomic DNA from mutant strains as the template. A reporter expressing LIS-1::GFP was created using the genomic fragment encoding *C. elegans* LIS-1. Bombardment transformation of the ZYG-12::GFP and LIS-1::GFP constructs was performed using a Bio-Rad PDS-1000/He according to published protocols (Praitis et al., 2001).

ZYG-12 GAL4 based two-hybrid experiments were performed according to Matchmaker 3 supplier instructions (Clontech). Inserts were cloned into the Nde1 and EcoRI sites of both pGADT7 and pGBKT7. The following inserts were used: cDNA encoding ZYG-12A from the start to stop codon, cDNA encoding an N-terminal fragment of ZYG-12A from the start codon to amino acid 236, and cDNA encoding the ZYG-12 coiled-coil containing C-terminal fragment from amino acid 230 to the stop codon. The mutations of *zyg-12(ct350)* and *zyg-12(or577)* were introduced to these constructs via the QuickChange Kit (Stratagene). Yeast transformation and assays were performed according to supplier instructions (Clontech). The two-hybrid screen was performed using both oligo-dT and random primed libraries. Thirty-six million clones were screened and 37 validated positives were identified. Two isolates were different clones of DLI-1. ZYG-12 itself was not identified in the screen.

ZYG-12 Antibodies and Coimmunoprecipation of ZYG-12

To raise ZYG-12 antibodies, we expressed amino acids 1 to 236 tagged with MBP. Purified protein was injected in two rats. The second bleed from both rats produced identical staining patterns in whole-mount embryos and specifically recognized both GFP-tagged ZYG-12 and endogenous ZYG-12 in immunoprecipitated material.

C. elegans lysates were made by homogenization of 1 ml of packed worms in 4 ml of RIPA lysis buffer using a mortar and pestle in liquid nitrogen. Both N2 and WH233, which expresses ZYG-12A,B,C::GFP, were prepared. Immunoprecipitation was performed with 3E6 GFP antibodies and protein A agarose beads from Sigma. Immunoprecipitated materials were Western blotted with GFP antibodies or ZYG-12 polyclonal serum. The lower band in the ZYG-12::GFP lysate lane probed with the ZYG-12 antibody corresponds precisely to ZYG-12 in a whole lysate (Supplemental Figure S5 on *Cell* website). The band was depleted from whole lysates when prepared from *zyg-12* RNAi animals, confirming that it is ZYG-12 (Supplemental Figure S5).

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