IDENTIFYING ADDITIONAL CENTROSONE ATTACHMENT GENES AND
CHARACTERIZING CENTROSONAL LOCALIZATION OF ZYG-12

A Thesis in
Genetics
by
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Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Master of Science

August 2008
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ABSTRACT

The association between the centrosome and the nucleus is crucial for mitosis in the early Caenorhabditis elegans embryo. Four components are essential for this association: zyg-12, sun-1, microtubules and dynein complex (Reinsch and Karsenti, 1997; Malone et al., 2003). However, the molecular mechanism governing the centrosome-nucleus association is incompletely understood, and the question whether additional components are involved in this process has not been answered yet.

To address this question, I performed two genetic screens to identify additional genes that may play roles in the centrosome-nucleus association. I found additional zyg-12-like genes in the suppressor screen on a centrosome-duplication gene zyg-1. I also identified suppressors that may genetically interact with zyg-12 through another suppressor screen on gene zyg-12. The preliminary characterizations of zyg-1 suppressors suggest that gene zyg-1 may have an unrevealed function in promoting the centrosome-nucleus association. My zyg-12 suppressor screen also provided strong evidence for the existence of the additional components involving in this process.

In addition, to better understand the function of zyg-12 in the association, I investigated the mechanism of the centrosomal localization of ZYG-12. Using the microtubule binding assay, I tested the hypothesis that ZYG-12 interacts with microtubules to localize to the centrosome. However, ZYG-12 did not bind to microtubules in the assay, which suggested that it may localize to the centrosome through some other mechanism.
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INTRODUCTION

The centrosome, the major microtubule organizing center (MTOC) in animal cells, is responsible for assembling the bi-polar spindle structure during mitosis (Bornens, 2002; Doxsey, 2001). It consists of a pair of centrioles surrounded by a protein matrix of pericentriole materials (Urbani and Sterns, 1999). In *C. elegans* embryos, the centrosome usually closely apposes to the nucleus in the interphase. This association between two organelles is critical for the normal cell division in *C. elegans* early embryogenesis. The disruption of this association will lead to a failure of the mitotic spindle assembly and abnormal chromosome segregation, thus producing aneuploidy in *C. elegans* embryos (Reinsch and Gonczy, 1998; O’Connell et al., 2000; Severson et al., 2000). Research in mammalian cell cultures revealed a direct link between aneuploidy and cancer progression (Nigg, 2002). The disassociation of the centrosome from the nucleus was proposed to contribute to the mis-regulation of the centrosome cycle and the genetic instability that promotes cancer formation and progression (Doxsey, 2002; Nigg, 2002).

Previous studies of the centrosome-nucleus association revealed that this apposition is mediated by microtubules and the microtubule-dependent motor protein complex including dynein heavy, intermediate and light chains (Aronson, 1971; Reinsch and Karsenti, 1997; Reinsch and Gonczy, 1998). Also, several possible roles of the association were proposed, such as maintaining the proximity of the centrosomes to the chromosome upon the onset of mitosis. However, the mechanism and the function of this association are not thoroughly understood.
In 2003, Malone et al. first identified two essential genes for centrosome attachment to the nucleus: zyg-12 and sun-1, and confirmed that microtubules and dynein protein complex are also required for this association. This study reveals that ZYG-12, as a member of Hook protein family, localizes to both the nuclear envelope and the centrosome. Importantly, the findings of the self-association in concert with its dual localizations suggest that ZYG-12 may link the centrosome to the nucleus. As shown in this study, SUN-1 is required for ZYG-12’s localization at the nuclear envelope. Thus, it may anchor ZYG-12 at the outer nuclear membrane through direct or indirect interaction. In addition, the depletion of embryonic microtubules causes the loss of the centrosomal localization of ZYG-12 and gives rise to the centrosome detachment phenotype, which indicates microtubules are indispensable for this process. Importantly, dynein complex, a microtubule motor protein complex, requires ZYG-12 to localize to the nuclear envelope. Therefore, dynein is also involved in the association (Malone et al., 2003; Aronson, 1971; Gonczy et al, 1998; Yoder and Han, 2001).

Based on these findings, Malone et al. proposed a two-step model for the centrosome-nucleus association in *C. elegans*. The attachment process is completed through two steps: firstly, dynein complex, interacting with ZYG-12 on the outer nuclear envelope, moves along microtubules towards the centrosome to pull two organelles close. Upon the establishment of the proximity, the attachment will be initiated and maintained through the dimerization of ZYG-12 on the outer nuclear envelope and ZYG-12 at the centrosome. This model incorporates four essential components and provides important
insights into the mechanism of the centrosome-nucleus association. However, the detailed function of each component within this model remains marginally characterized. Also, an important question that has not been answered yet is whether any other essential player is required for this association.

Here I performed *zyg-1* and *zyg-12* suppressor screens to identify additional components that may have important roles in this process. I also investigated the mechanism of *ZYG-12*'s centrosomal localization to better characterize the function of *zyg-12* in this association. These studies will help to validate the proposed model and provide a better understanding of the importance and the function of the centrosome-nucleus association.
MATERIALS and METHODS

C. elegans strains

All strains were maintained as described (Brenner, 1974). The following strains were used: N2 (wild type), DH1 zyg-1(b1), BW54 zyg-12(ct350), WH198 dpy-10(e128) zyg-12(ct350); unc-119(e2498).

ZYG-12A::GFP, β-tubulin::GFP were expressed via the pie-1 promoter and enhancer sequences (Strome et al., 2001). WH221 (ZYG-12A::GFP), WH204 (β-tubulin::GFP) were originally created via microparticle bombardment (Praitis et al., 2001; Strome et al., 2001).

BW1809 him-5(e1467), MT3989 clr-1(e1745) bli-2(e768)(ChrII), alys4(EGL-17::GFP)(ChrI), CB78 unc-6(e78)(ChrX), mls11::GFP(ChrIV), mls10::GFP(ChrIII), kuls29::GFP(ChrV) were used in two-point mapping.

RNAi

RNAi of zyg-12 and zyg-1 were performed using the feeding RNAi vector L4440 to express double stranded RNA of the target genes in the feeding bacteria HT115(Fraser et al., 2000; Timmons and Fire, 1998). Double-stranded RNAs were synthesized in vitro from zyg-12 and zyg-1 cDNA template for micro-injection as described in Sonnichsen et al., 2005.
**Immunofluorescence and Microscopy**

Indirect immunofluorescence was performed as described (Malone et al., 2003). Primary antibodies were applied at a dilution of 1:200 to 1:1000, and secondary antibodies were applied at 1:500. For DNA staining, 0.1 μM DAPI was added in the next-to-last wash. Live imaging of embryos was performed with Nomarski DIC optics microscopy as described (Malone et al., 2003). GFP epifluorescence was observed using an Eclipse 90i microscope (Nikon, Melville, NY). GFP was excited and collected through an Endow GFP HYQ BP filter cube (Ex 470/40, DM 525, BA 525/50). Images were analyzed using SIMPLEPCI software (version 6.1.1; Compix, Sewickley, PA). For live imaging of GFP-expressing animals, embryos were mounted on 2.5% agar pads. Images were further processed with Adobe Photoshop.

**Expression Constructs**

For the constructs of MBP-ZYG-12 fusion protein, PCR-generated cDNAs encoding full-length ZYG-12 or the NH2-terminal truncations (ΔN-ZYG-121-236), which were all tagged with a MBP epitope at N-terminus, were inserted between PstI and XbaI sites of pMAL-c2 (New England Biolabs). Likewise, His-ZYG-12 fusion proteins (full length or amino acids 1-236) were generated in pET-28a (EMD biosciences) with N-terminal and C-terminal polyhistidine tags. The expression and purification of ZYG-12 fusion protein were performed according to the manufacturer’s instruction (New England Biolabs, EMD biosciences).
Suppressor Screen

Temperature-sensitive strains were maintained at permissive temperature 16° C. Six small NGM plates of L4 stage worms were washed off with M9 solution and collected via low-speed centrifugation. This parental generation was then soaked in 0.5 mM EMS for 4 hours. After 4 hours, they were recovered and washed with M9 for 3 times. Free-moving worms were then transferred to the large NGM plate and kept at 16° C until F1 generation was fertilized. The gravid F1 worms were collected and lysed with bleaching solution to release the internal eggs. These eggs were then distributed to fresh large NGM plate for the F2 worms to hatch synchronously at 16° C. At L4 stage, they were shifted to non-permissive temperature 24° C in search of suppressors in F3 generation.

Microtubule binding assays

Microtubule binding assays were performed using the microtubule-associated protein spin-down kit (Cytoskeleton) according to the manufacturer’s instructions. In brief, microtubules were assembled from purified bovine brain tubulin for 20 min at 35° C in the presence of GTP and stabilized with taxol. Assembled microtubules (10µg) were incubated with 30 µg of cytosolic proteins in total volume of 50 µl for 20 min at RT. Associated proteins and microtubules were pelleted at 100,000 g through a glycerol cushion. Pellets were dissolved in 10 µl SDS-loading buffer and compared with 10 µl from the supernatant by SDS-PAGE.
RESULTS

1. zyg-1 suppressor screen

1.1 Identification of the suppressors

Two essential genes for the centrosome-nucleus association: zyg-12 and sun-1 were identified through genetic screens (Malone et al., 2003). zyg-12 was originally isolated in a genomic screen in search of the temperature-sensitive embryonic lethal mutation. sun-1 was identified in a genome-wide time-lapse microscopy screen of previously identified embryonic lethal mutations (Kamath et al., 2003). In spite of a multitude of mutant hunts (Hirsh and Vanderslice, 1976; Miwa et al., 1980; Cassada et al., 1981; Kemphues et al., 1988; O’Connell et al., 1998; Gonczy et al., 1999) and exhaustive genome-wide RNAi-based screenings (Simmer et al., 2003; Sonnichsen et al., 2005), no additional genes have been implicated in the centrosome-nucleus association. Thus, existing forward and reverse genetic screening strategies may not be very effective in isolating more genes with important roles in this process. Therefore, a more focused screening strategy is needed. One such strategy is a suppressor screen whereby one screens for mutations that suppress the phenotype of an existing mutant. Genes whose products interact in either a positive or a negative way with the gene of interest can thus be identified. For lethal mutations, the suppressor screen is particularly powerful as it allows one to rapidly and efficiently select for mutations that restore some degree of viability. In addition, this method is high in throughput and efficient; hundreds of thousands of genomes could be screened at one time and suppressors could be easily isolated with certain strong selection, such as suppressing embryonic lethality.
Interestingly, one zyg-1(it25) suppressor screen performed in Kevin O’Connell’s lab yielded some unexpected findings: some of the identified zyg-1 suppressors, for example, a sun-1 allele, displayed the centrosome detachment phenotype-zyg-12-like phenotype. Known as a centrosome duplication gene, zyg-1 encodes a homolog of Ca2+/calmodulin-dependent protein kinase required for the centriole duplication (O’Connell et al., 2001). No evidence has been shown for its involvement in the centrosome-nucleus association. However, the discovery of a sun-1 allele as a suppressor of zyg-1 suggested that zyg-1 may genetically interact with sun-1 and have undiscovered function in centrosome attachment. Also, the characterizations of other zyg-1 suppressors having the centrosome detachment phenotype may yield more information about additional zyg-12-like genes. Therefore, this strategy of zyg-1 suppressor screen (Figure 1) can be employed by us to explore additional components involving in tethering the centrosome to the nucleus.

Using the same approach, I performed a suppressor screen with DH1 strain that carries a temperature sensitive zyg-1(b1) mutation. This strain is viable at 16° C but shows 100% embryonic lethality at 24° C. I have screened approximately 800,000 haploid genomes. 28 suppressors, named as SUP1 to SUP28, have been found in total.

1.2 Centrosome detachment of zyg-1 suppressors
To characterize these suppressors, I first examined their centrosome detachment phenotype using the immunostaining assay. Given the importance of this association in the early embryogenesis, it is reasonable to infer that only a weak detachment phenotype could be observed, if there is such phenotype. Thereby, I chose to perform immunostaining on a group of early suppressor embryos and searched for a low percentage of embryos displaying the centrosome detachment phenotype using fluorescence microscopy. Among sixteen suppressors I have first characterized, six have this weak detachment phenotype. Four examples are shown in figure 2.

Figure 1. Suppressor screen strategy. To identify mutations that may be involved in centrosome attachment, animals carrying the temperature-sensitive mutation zyg-1(b1) were treated with EMS. At permissive temperature, EMS-induced germ-line sup mutations are passed to the F₁ generation in the heterozygous state (sup+/+) and to the F₂ generation in the homozygous state (sup/sup). To identify sup-bearing individuals, F₂ embryos were isolated from gravid mothers, allowed to develop until L4 stage at permissive temperature, and then shifted to restrictive temperature for 3 weeks. sup-bearing lines produce multiple generations over this time period while all other individuals die off. The columns of green arrows indicate that individual pools of mutagenized lines were processed in parallel to ensure independent lines of suppressors (adapted from Figure 1 O’Connell et al., 2007).
Among these six candidates, SUP14 displayed a relatively stronger centrosome detachment phenotype. Seven of thirty-five examined one-cell embryos of SUP14 have one or two detached centrosomes at 24°C as shown in figure 3.

Figure 2. zyg-1 suppressors display a weak centrosome detachment phenotype. (A-B) suppressor embryos were stained for centrosomes with antibodies to α-tubulin (red) and DNA with DAPI (blue). (C-D) suppressor embryos were stained for DNA with DAPI (blue) and centrosomes with antibodies to α-tubulin (red) and ZYG-12 (green). (A) one SUP4 embryo (1/35) shows one detached centrosome from sperm pronuclear at 24°C. (B) two detached centrosomes were found in one SUP7(3/50) embryo at 16°C. (C) one SUP11(1/32) embryo shows a detached centrosome from the nucleus at 24°C. (D) one SUP13 embryo shows detached centrosomes at two-cell stage at 24°C. (E-F) Images of wild-type and zyg-12(ct350) carrying β-tubulin::GFP prior to nuclear envelope breakdown (Adapted from figure 1 of Malone et al, 2003). (E) Wild type has normal centrosome attachment. (F) zyg-12(ct350) shows two detached centrosomes from the nuclei.
Figure 3. SUP14 shows a relatively stronger centrosome detachment phenotype. (A and C) Embryos are stained for DNA with DAPI (blue) and centrosomes with antibodies to α-tubulin (red) and ZYG-12 (green). (B) Embryos are stained for centrosomes with antibodies to α-tubulin (red) and DNA with DAPI (blue).

1.3 Characterizations of sup-14

To continue the characterization, I chose to focus on SUP14, as it shows a relatively stronger zyg-12-like phenotype in the immunostaining study. Given that alleles of zyg-12 and sun-1 produce a centrosome detachment phenotype, I first examined the possibility that sup-14 is an allele of zyg-12 or sun-1. I sequenced zyg-12 (upstream 250 bp and the entire genomic coding sequence) and sun-1 (the entire genomic coding sequence) in the SUP14 strain. The results suggested that sup-14 is unlikely to be another allele of zyg-12 or sun-1. I then proceeded to determine the molecular identity of the suppression mutation using the traditional two-point mapping. In consideration of the fact that the suppression may only exist in the presence of the original mutation, I constructed a background strain CY10 that carries the original b1 mutation linked with a genetic marker: unc-4 (Figure 4A). SUP14 was then crossed into CY10 to ensure the presence of b1 (Figure 4B and 4C). On this step, none of Unc F2 could produce suppressed progeny, which indicated that the suppression mutation(s) occurs on ChrII.
Figure 4. Genetic scheme for constructing CY10 and marking zyg-1(b1) in SUP14. (A) Constructing CY10 by crossing DH1 carrying original zyg-1 mutation into SP49 carrying dpy-10 and unc-4 on ChrII and selecting Unc but non-Dpy F2 recombinant. (B and C) two scenarios of crossing CY10 into SUP14 to mark zyg-1 with unc-4 (B) scenario 1: when the suppression mutation(s) occurs in other chromosomes rather than ChrII, where zyg-1 is, 1/4 of the F2 generation will produce suppressed progeny at 24° C. (C) scenario 2: when the suppression mutation(s) occurs in ChrII, none of the F3 generation is viable at 24° C.
In light of the fact that zyg-1 is also on ChrII, I further examined the possibility that sup-14 is another allele of zyg-1, though zyg-1 has not been implicated in the centrosome-nucleus association. I sequenced the upstream 350 bp and the entire coding region of gene zyg-1 in the SUP14 strain. Interestingly, a unique single-base-pair substitution was found at the locus of the original zyg-1(b1) mutation, which converts the mutant amino acid from Leucine to Phenylalanine (Proline in wild-type) as shown in figure 5.

<table>
<thead>
<tr>
<th></th>
<th>DNA Sequence</th>
<th>Amino Acid Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type</td>
<td>gaagaattgattacgtaacgaacat</td>
<td>DRTVRGRNGEELITLTNNFVYTSQMPKEVQNDYRML...451</td>
</tr>
<tr>
<td>zyg-1(b1)</td>
<td>gaagaattgattacgtaacgaacat</td>
<td>DRTVRGRNGEELITLTNNL</td>
</tr>
<tr>
<td>sup-14</td>
<td>gaagaattgattacgtaacgaacat</td>
<td>DRTVRGRNGEELITLTNNFVYTSQMPKEVQNDYRML...451</td>
</tr>
</tbody>
</table>

Figure 5. DNA and amino acid sequences of b1 and sup-14. b1 allele contains a C-to-T transition at position 1321 that converts proline 432 to leucine. sup-14 allele contains two mutations, a C-to-T transition at position 1320 and a C-to-T transition at position 1321, converting proline 432 to phenylalanine.

As known, suppressors can work through different mechanisms. Bypass suppressors reconstruct the process to eliminate the requirement for the gene being suppressed. Other suppressors restore the activity of the suppressed gene or lower the requirement for that gene. In this study, bypass suppressors work in a manner that would produce suppression completely independent of zyg-1. Alternatively, nonbypass suppressors would restore the normal process of duplication utilizing the residual zyg-1
activity present in the *zyg-1(b1)* mutant. To determine by which mechanism *sup-14* works, I removed the residual *zyg-1* activity present in SUP14 using *zyg-1* RNAi and then examined the suppression. After *zyg-1* RNAi treatment, SUP14 shows almost 100% embryonic lethality, while it is complete healthy without the treatment. Thus, *sup-14* does not bypass *zyg-1* (Table 1).

<table>
<thead>
<tr>
<th>EMB%</th>
<th>zyg-1 RNAi</th>
<th>NGM</th>
</tr>
</thead>
<tbody>
<tr>
<td>N2 (n=15)</td>
<td>81.7%</td>
<td>0</td>
</tr>
<tr>
<td>SUP14(n=15)</td>
<td>97.6%</td>
<td>0</td>
</tr>
<tr>
<td>SUP4(n=15)</td>
<td>52.0%</td>
<td>9.6%</td>
</tr>
<tr>
<td>SUP7(n=15)</td>
<td>97.5%</td>
<td>0</td>
</tr>
<tr>
<td>SUP2(n=15)</td>
<td>49.6%</td>
<td>34.2%</td>
</tr>
</tbody>
</table>

Table 1. *sup-14* does not bypass *zyg-1(b1)*. Five L4-stage worms were fed with or without *zyg-1* RNAi on each plate. Adults are transferred to the new plate every day. Eggs on the plate are counted at the time when the worms are transferred. The unhatched eggs on the plate are counted again after 24 hrs. Worms are followed for four consecutive days. SUP14 shows 100% embryonic lethality after *zyg-1* RNAi. Thus, *sup-14* depends on the residual activity of *zyg-1* to produce the suppression.

2. *zyg-12* suppressor screen

2.1 Identification of the suppressors
To explore more genes that may have roles in the centrosome-nucleus association, I performed another suppressor screen on gene \textit{zyg-12}, whereby genes that genetically interact with \textit{zyg-12} can be identified. Given that \textit{zyg-12} is absolutely required for the centrosome-nucleus association, this screen elicits interesting questions about the underlying mechanism of the possible extragenic suppression. We envisioned several possibilities: the additional suppression mutation may modify the interacting components of ZYG-12, such as linker proteins between ZYG-12 and SUN-1 or the scaffolding proteins of ZYG-12 at the centrosome, to partially restore its activity. Extragenic mutations could also function in regulatory pathways, for example, to diminish upstream negative signals controlling the attachment, which in turn can suppress the phenotypes caused by \textit{zyg-12} mutation.

BW54 strain that carries a temperature sensitive \textit{zyg-12} mutation (\textit{ct350}) at the N-terminus was used in this screen. It is fertile at 16° C but becomes sterile at 24° C. I followed the same procedure of \textit{zyg-1} suppressor screen as described above (Figure 1), except one difference in selecting suppressors: I did not only search for viable F\textsubscript{3} progeny but also fertilized F\textsubscript{2} as suppressors, because the suppressor stains may have restored fertility, but cannot produce healthy progeny due to egg-laying defects or embryonic lethality. To isolate such suppressor, I shifted these gravid F\textsubscript{2} back to permissive temperature in hope that they could produce viable progeny at 16° C for the further analysis. However, in all of such cases, no viable progeny could be collected.
In sum, I screened approximately 500,000 haploid genomes and identified 9 suppressors, named as SUPA to SUPI. All of these suppressors produce viable progenies at 24° C.

2.2 Characterizations of zyg-12 suppressors

To characterize these suppressors, I first examined the centrosome phenotype in the suppressor embryos to determine if the centrosome-nucleus association was restored in addition to the fertility. All of suppressor strains showed the normal centrosome attachment in the live embryo imaging. Five examples are shown in figure 6.

I then proceeded to investigate the nature of the suppressive interaction. For the same reason as I applied zyg-1 RNAi on zyg-1 suppressors, I first used zyg-12 RNAi to determine if the suppressors can bypass zyg-12. I found all of nine suppressors were susceptible to the RNAi treatment. The phenotype of SUPA and SUPE is comparable to the wild type after zyg-12 RNAi (Table 2). The other seven suppressors, however, are hyper-susceptible to the treatment (Table 2). They have the enhanced phenotype compared with wild type after RNAi. The most likely explanation for this result is that the ZYG-12-dependent centrosome attachment pathway is indispensable for proper embryonic cell division. These results also indicate that all zyg-12 suppressors work by increasing the residual activity of the zyg-12(ct350) allele or by lowering the requirement of zyg-12 for the successful centrosome attachment. I further sequenced upstream 200bp and the entire coding region of zyg-12 in suppressor strains and found that SUPA and
SUPE are revertants of \textit{zyg-12(\textit{ct350})}. One additional silent mutation was found in SUPD. No additional mutation of \textit{zyg-12} was found in the rest suppressor strains.

Figure 6. \textit{zyg-12} suppressors have normal centrosome attachment. (A-E) DIC images of five \textit{zyg-12} suppressor embryos having normal centrosome-nucleus association (For each suppressor stain, n>20). Asterisks denote position of nuclei. (F-G) Images of wild-type and \textit{zyg-12(\textit{ct350})} carrying \(\beta\)-tubulin::GFP prior to nuclear envelope breakdown (Adapted from figure1 of Malone et al, 2003). (F) Wild type has normal centrosome attachment. (G) \textit{zyg-12(\textit{ct350})} shows two detached centrosomes from the nuclei.

In the examination of plate phenotypes of these suppressors, I found that three suppressor strains: SUPB, SUPC, and SUPD have a Him (high incidence of male) phenotype as well as a smaller brood size. It suggested that \textit{zyg-12} may have pleiotropic functions in \textit{C. elegans} development, playing different roles in different processes. Possibly, one suppressor can specifically suppress one phenotype caused by \textit{zyg-12} mutation in one process, but not the defects in other processes. In this case, SUPB, C and D may effectively suppress the centrosome detachment phenotype in embryogenesis but
not the chromosome segregation defect caused by \textit{zyg-12} in oogenesis. It is also possible that these suppressor mutations may produce Him phenotype and a small brood size on their own, in addition to suppressing the phenotypes caused by \textit{zyg-12}.

<table>
<thead>
<tr>
<th>EMB% (n=15)</th>
<th>N2</th>
<th>SUPA</th>
<th>SUPB</th>
<th>SUPC</th>
<th>SUPD</th>
<th>SUPE</th>
<th>SUPF</th>
<th>SUPG</th>
<th>SUPH</th>
<th>SUPI</th>
</tr>
</thead>
<tbody>
<tr>
<td>NGM</td>
<td>0</td>
<td>0</td>
<td>3.1%</td>
<td>9.4%</td>
<td>4.5%</td>
<td>1.0%</td>
<td>1.3%</td>
<td>0</td>
<td>2.3%</td>
<td>0.4%</td>
</tr>
<tr>
<td>\textit{zyg-12} RNAi</td>
<td>67.8%</td>
<td>64.0%</td>
<td>96.5%</td>
<td>87.2%</td>
<td>92.2%</td>
<td>65.6%</td>
<td>90.2%</td>
<td>84.8%</td>
<td>88.9%</td>
<td>97.8%</td>
</tr>
</tbody>
</table>

Table 2. None of \textit{zyg-12} suppressors bypasses \textit{zyg-12}(ct350). EMB percentage was counted as described before (Table 1). After \textit{zyg-12} RNAi, SUPA and SUPE have the comparable EMB percentages with the wild type. The rest suppressors, however, have elevated EMB percentages, compared to the wild type. The results indicate all of suppressors depend on the residual activity of \textit{zyg-12} to produce the suppression.

I next worked on determining the molecular identities of these suppressors using the conventional two-point mapping method. I first crossed the suppressors into a background strain WH198, which carries \textit{zyg-12}(ct350) linked with \textit{dpy-10}, to mark the original mutation. I then proceeded to assign each suppressor to one chromosome using five marker strains: \textit{ayls4}(EGL-17::GFP)(ChrI), \textit{mIs11}:GFP(ChrIV), CB78 \textit{unc-6(e78)}(ChrX), \textit{mIs10}:GFP(ChrIII), \textit{kuls29}:GFP(ChrV). The preliminary results of two-point mapping are summarized in Table 3.
Table 3. Results of two-point mapping of zyg-12 suppressors. Suppressors were first crossed into WH198 to mark zyg-12(ct350) with dpy-10. Then these constructed strains were crossed into marker strains: ayls4(EGL-17::GFP)(ChrI), CB78 unc-6(e78)(ChrX), mIs11::GFP(ChrIV), mIs10::GFP(ChrIII), kuls29::GFP(ChrV) to assign the suppression mutation to one chromosome. The analysis indicated sup-b localizes on ChrII; sup-c on ChrX; sup-d on either ChrIV or ChrI or ChrX(undecided); sup-f on ChrX or ChrV(undecided); sup-g on ChrIII, ChrI or ChrX(undecided); sup-h on ChrX, and sup-i on ChrV or ChrX(undecided).

3. Mechanism of ZYG-12’s centrosomal localization

3.1 N-terminus of ZYG-12 does not directly bind to microtubules in vitro

zyg-12 is an essential gene involved in the centrosome-nucleus association. It encodes three alternatively spliced isoforms: ZYG-12A, B and C (Figure 7). Three isoforms share the same N-terminal domain and the central coiled-coil domain. ZYG-12B and C have the additional transmembrane domain and conserved KASH domain at the C-terminus. Previous work showed that all three ZYG-12 isoforms depend on microtubules to localize to the centrosome (Malone et al., 2003). After the depletion of α-tubulin, the centrosomal localization of ZYG-12 is lost. Also, the N-terminus of ZYG-12 is homologous to the microtubule binding domain of human hook protein. Hook proteins function in organelle positioning and vesicle trafficking, linking membrane-bounded compartments to microtubules. For example, human hook3 protein binds to microtubules
with its N-terminus and mediates the interaction between the Golgi apparatus and microtubules (Kramer and Phistry, 1999; Walenta et al., 2001). Given the similarity between the sequence of ZYG-12 and Hhk3, N-terminal domain of ZYG-12 may also interact with microtubules. In addition, other studies in our lab found that GFP tagged C-terminal of ZYG-12 could localize to nuclear envelope, but not to the centrosome. This suggested that N-terminal domain and coiled-coil domain are important for the centrosomal localization of ZYG-12.

Based on the information, we first hypothesized that ZYG-12 may directly interact with microtubules with its N-terminus to localize to the centrosome. I used \textit{in vitro} microtubule binding assay to test this hypothesis. Firstly, the fusion protein pMG1 containing the first 236 amino acid of ZYG-12A tagged with maltose binding protein (MBP) was expressed and purified. Identically, mpMG1 was generated, which contains the first 236 amino acid as well as \textit{zyg-12(ct350)} mutation. The microtubule binding assay was then performed according to supplier’s instructions (Cytoskeleton). In brief, microtubules were assembled and stabilized \textit{in vitro}. The purified fusion protein was incubated with microtubules for 30 minutes at room temperature. High-speed (100,000 g) air centrifugation was applied to each reaction to pellet microtubules and the associated proteins after the incubation. Fractions of the supernatant and the precipitate were then collected and examined in the SDS-PAGE. The results indicated that N-terminal 236 aa of ZYG-12 does not directly associate with microtubules (Figure 8 7A/9A). Thus, the hypothesis of directing binding to microtubules with its N-terminal domain was not supported by the analysis.
Figure 7  Schematic of three isoforms of ZYG-12. The N-Terminus of ZYG-12 shares the similarity with the microtubule binding domain of Hhk3 protein.

3.2 Full-length ZYG-12A does not directly bind to microtubules in vitro

In consideration of the possible importance of the coiled-coil domain in the centrosomal localization of ZYG-12, I further analyzed if full-length ZYG-12A could directly associate with microtubules using the same approach. Likewise, full length ZYG-12A was constructed, expressed and purified. However, the SDS-PAGE analysis indicated that it does not directly bind to microtubule either (Figure 9 3A). In this experiment, we found that a small amount of pMG1 and mpMG1 pellets with microtubules (Figure 9A and 9A). However, they also present in the pellet fraction when microtubules were not present in the reactions (Figure 9C 10p and 11p). These results suggested that the presence of the small amount of ZYG-12 segment in the pellet was probably due to the protein degradation or sample overloading.
Figure 8. N-terminus of ZYG-12 does not bind to microtubules, as shown in the SDS-PAGE analysis of the pellet (A) and the supernatant (B) of each reaction (table). Proteins are detected by coomassie staining. Positive control MAP2 presents in the pellet (1A), but not in the supernatant (1B). Negative control BSA stays in the supernatant (2B), but not in the pellet (2A). Both pMG1 (N-terminal 236 aa of ZYG-12) and mpMG1 (N-terminal 236 aa of ZYG-12 carrying ct350 mutation) only present in the supernatant (7B and 9B), but not in the pellet (7A and 9A).
Figure 9. Full-length ZYG-12A does not bind to microtubules, as shown in the SDS-PAGE analysis of the pellet (A and 10p, 11p in C) and of the supernatant (B and 10s, 11s in C) each reaction (table). Proteins are detected by coomassie staining. Positive control MAP2 presents in the pellet (1A), but not in the supernatant (1B). Negative control BSA presents in the supernatant (2B), but not in the pellet (2A). Full length ZYG-12A stays in the supernatant (3A), but not in the pellet with MT (3B). A small amount of pMG1 and mpMG1 presents in the pellet with MT (8A and 9A). However, this may be due to the large loading amount, as they also present in the pellet without MT (10p and 11p).
3.3 ZYG-12A does not bind to microtubules in the presence of the cytosol

The results of the microtubule binding assay indicated that neither N-terminal domain nor the full-length ZYG-12A binds to microtubules. Given that microtubules are required for the centrosomal localization of ZYG-12, I next examined the possibility of the indirect association between ZYG-12 and microtubules. As shown in previous research (Malone et al., 2003), ZYG-12 only localizes to the centrosome and its close vicinity within a few μm, which suggests that ZYG-12 may only stay specifically at the minus end of the microtubule. Therefore, we hypothesized that additional protein(s) may link ZYG-12 to the minus end of the microtubule. To test this hypothesis, I prepared worm lysates and added them into the reactions in the microtubule binding assay to provide the possible linker protein(s). I then examined if full-length ZYG-12A and N-terminal segment of ZYG-12 could associate with microtubules in the presence of cytosol.

Worm lysates of WH221 (ZYG-12A::GFP) and N2 (wild-type) were made by the homogenization of 1ml of packed worms in 4ml lyse buffer using the mortar and the pestle in liquid nitrogen. The microtubule binding assay was performed as described above. The SDS-PAGE analysis indicated that neither N-terminus segment nor full-length ZYG-12A binds to microtubules in the presence of N2 or WH221 cytosol (Figure 10 and data not shown).
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Figure 10  Neither Full-length nor N-terminus of ZYG-12A binds to microtubules in the presence of the cytosol, as shown in the SDS-PAGE analysis of the pellet (A and B) and the supernatant (C and D) of each reaction (table). The results showed that full length ZYG-12A does not bind to microtubules in the presence of the cytosol (16B). In this experiment, pMG1 pellets with microtubules in 3A, 7A and 11B. However, this may be due to the large loading amount of the protein, since without microtubule, the similar amount of the protein still presents in the pellet (3C, 7C and 11D). Thus, N-terminal segment of ZYG-12A does not associate with MT in the presence of the cytosol either.
DISCUSSION

1. zyg-1 suppressor screen

To characterize sup-14, I backcrossed SUP14 to the original zyg-1(b1) line to remove extraneous mutations produced during EMS treatment, and found the suppression mutation(s) is on ChrII. I then sequenced zyg-1 in SUP14 and confirmed that a missense mutation occurs at the locus of original zyg-1(b1) mutation and converts the mutant amino acid from Leucine to Phenylalanine (Proline in wild-type). Thus, sup-14 is likely to be a novel allele of zyg-1. If this specific zyg-1 allele truly causes the weak centrosome detachment phenotype of SUP14, the identification of a gene that participates in the centrosome duplication was an unexpected outcome of our screen. It is interesting to rethink about the role of zyg-1 in linking the centrosome–nuclear attachment to the centrosome duplication. Previous studies of the centrosome duplication revealed that ZYG-1 kinase functions at the very upstream place of epistasis pathways. It is involved in the signal cascades regulating the centrosome duplication cycle as well as the cell cycle (Delatte et al, 2006; Pelletier et al, 2006). Thus, it is possible that zyg-1 also functions in the signaling pathway regulating the centrosome attachment to the nucleus (Figure 11).

To further examine this possibility, I will check the localization and expression level of ZYG-12 in SUP14 and determine if sup-14 actually disrupts the function of ZYG-12 to produce the detachment phenotype. Though my preliminary characterization of sup-14 did not indicate the altered localization of ZYG-12, the protein expression level needs to be further assayed. With the ZYG-12::GFP strain previously constructed in the
lab, the levels of GFP signal in living embryos can be tracked and quantified using the fluorescence microscopy. Also, Western blotting with ZYG-12 antibody in the worm lysate of SUP14 strain can be used to detect the protein level in comparison to the wild type. If zyg-1 is demonstrated to affect the function of zyg-12, their interaction could be investigated next. Given that ZYG-1 works at the very upstream place of pathways in the centrosome duplication, it may direct the recruitment of ZYG-12 to the centrosome. GFP signals at the centrosome in ZYG-12::GFP embryos can be tracked after the treatment of zyg-1 RNAi to determine if zyg-1 is required for the recruitment of ZYG-12 to the centrosome. Likewise, ZYG-1::GFP stain and zyg-12 RNAi can be used in answering the question whether zyg-12 is required for the proper recruitment of ZYG-1. In addition, the protein-protein interaction can be analyzed with different methods, such as the pull-down experiment of purified ZYG-1 and ZYG-12 in vitro. Also, Co-Immunoprecipitation assay can be performed to assess the interaction in vivo. These studies will help to unravel a potential novel role of zyg-1 in regulating the centrosome-nucleus association, different from its known functions in the centrosome duplication.

**Figure 11** Potential novel function of zyg-1. ZYG-1 is involved in the signal cascades regulating centriole duplication and PCM assembly. Its recruitment to the centrosome depends on spd-2, a gene involving in the centriole duplication. SPD-2 interacts with ZYG-12 in yeast two-hybrid (Malone, et al., 2003). Now we
found that *zyg-1* allele shows the centrosome detachment phenotype. Therefore, it is possible *zyg-1* functions in linking the centrosome-nucleus association with the centrosome duplication.

2. *zyg-12* suppressor screen

As mentioned before, *zyg-12* suppressor screen could elicit interesting questions about the underlying mechanism of the possible extragenic suppressions. It may yield useful information about the components implicated in the centrosome-nucleus association. For example, the interacting factors of ZYG-12, such as mediator proteins between ZYG-12 and SUN-1 or the scaffolding proteins of ZYG-12 may be discovered. Components functioning in the pathways regulating this process could be found as well.

In our preliminary study, we have already identified six extragenic suppressors: SUPC, D, F, G, H, and I. The suppressive interactions will be further studied. Three-point mapping and other genetic analysis will be performed to determine the molecular identities of the suppression mutations. Then their functions can be further investigated.

In addition to the studies of the localization and functions of the suppressor mutation, we need focus on characterizing the interaction between the identified gene and *zyg-12* to interpret the mechanism of the suppression. Different approaches can be used to achieve the goal. For example, RNAi experiment can be performed to examine the loss of function phenotype of the identified suppressor gene on its own. Any additional phenotype will be considered as an indicator of its cellular function. In addition, the localization and expression of ZYG-12 and SUN-1 need to be checked after the new gene
is knocked down, and *vice versa*. The physical interaction between ZYG-12 and the protein encoded by the identified gene can be further examined by pull-down and Co-Immunoprecipitation experiments.

The identification of *zyg-12* suppressors has provided us the opportunity to address the mechanisms that regulate centrosome–nucleus attachment. Further analysis of these suppressors should allow us to quickly dissect the regulatory pathways involved. Some of the suppressors may have novel phenotypes, such as Him seen in SUPB, SUPC, and SUPD, suggesting their novel cellular functions. Thus the analysis of these suppressors is likely to provide important insights into the centrosome-nucleus association.

**3. The mechanism of the centrosomal localization of ZYG-12**

Our data of the microtubule binding assay did not support the hypothesis that ZYG-12 binds to microtubules directly or indirectly in localizing to the centrosome. However, several factors are worthy of note. First, the sensitivity of our detecting method may be low. If only a very small amount of protein binds to microtubules, it could not be detected in SDS-PAGE. Second, the full-length or N-terminal segment of ZYG-12 synthesized in the *E. coli* expression system may not be functional. They may fail to bind to microtubules due to the lack of the post-translational modifications.
Therefore, to better test our hypothesis using the microtubule binding assay, proteins could be synthesized in the cell-free expression system to ensure the proper post-translational modification. More sensitive methods such as the silver staining or western blotting with ZYG-12 antibody could be utilized in detecting the presence of the associated protein with microtubules. Alternatively, using ZYG-12 enriched in the worm lysates of the strain carrying ZYG-12A::GFP instead of the synthesized protein is probably better in our study to avoid the potential problems of expressing proteins in bacteria.

Importantly, other approaches can be sought to investigate the mechanism of ZYG-12’s centrosomal localization. For example, microparticle bombardment can be performed to generate different transgenic worms expressing GFP tagged ZYG-12 fragments. GFP signal in the centrosome can be tracked to determine the minimum domain of ZYG-12 required for its centrosomal localization. The information yielded in this domain mapping is helpful for the further analysis. For instance, this minimum domain can be used in the yeast two-hybrid experiment in search for its interacting components, which may also be involved in localizing ZYG-12 to the centrosome.

Those studies will help us to elucidate the mechanisms of the centrosomal localization of ZYG-12 and identify more components functioning in the centrosome-nucleus association.
REFERENCES


