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A novel C-terminal kinesin subfamily may be involved in chromosomal movement in *Caenorhabditis elegans*[1]

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Abstract C-terminal kinesin motor proteins, such as the *Drosophila* NCD and yeast KAR3, are involved in chromosomal segregation. Previously we have described two orthologs of NCD in *Caenorhabditis elegans*, KLP-3 and KLP-17, which also participate in chromosome movement. Here we report cDNA cloning of *klp-15* and *klp-16*, and the expression pattern of the genes encoding C-terminal motor kinesins including *klp-15* and *klp-16*. Interestingly KLP-15 and KLP-16 form a unique class of C-terminal kinesins, distinct from the previously known C-terminal motors in other organisms. Using in situ hybridization and RNA interference assay, we show that although all of these motors mediate chromosome segregation, they do so in a combination of unique and overlapping manners, suggesting a complex hierarchy of kinesin motor function in metazoans.

Key words: Kinesin-like protein; Embryonic development; C-terminal motor; Cell division; Chromosomal movement; *Caenorhabditis elegans*

1. Introduction

In eukaryotes, kinesin motor proteins transport a variety of cellular cargoes on microtubule tracks. All kinesins share a motor domain that contains the highly conserved ATP and microtubule binding sites, and is generally located in the amino-terminus of the protein. However, several kinesins have been discovered in which the motor domain is located in the carboxy-terminus of the protein, such as the *Drosophila* NCD (non-claret disjunctional) [1,2] and yeast KAR3 [3,4] kinesins, that are retrograde motors and play a critical role in chromosome segregation.

To study the in vivo function of kinesins, *Caenorhabditis elegans* is an attractive model system as the classical genetics and developmental analysis can easily be applied, and an exhaustive account of the nematode's genome sequence is available [5]. The genomic sequence has allowed exhaustive search for each and every member of the kinesin superfamily in *C. elegans*. Thus, we may ask how many different kinesins participate in a given cellular process, and whether they perform a unique or redundant function. We previously identified 20 genes encoding **k**inesin-**l**ike **p**roteins (*klp*s) in *C. elegans* [6]; four of these kinesins, KLP-3, KLP-15, KLP-16 and KLP-17, contain C-terminal motors, similar to the *Drosophila* NCD and the yeast KAR3 kinesin. Members of the NCD subfamily of kinesin are known to attach with spindles and facilitate chromosome distribution to different poles during mitotic division [1–4]. Similarly, *C. elegans* KLP-3 and KLP-17 are also involved in chromosomal movement and segregation [6,7].

This report is a completion of the cloning and expression studies of the C-terminal motors in *C. elegans*, as we describe two novel cDNA clones encoding *klp-15* and *klp-16* genes. Although KLP-3, KLP-15, KLP-16 and KLP-17 are C-terminal motors, their amino acid sequence homology and secondary structure analysis suggest that KLP-15 and KLP-16 constitute a unique subgroup, distinct from KAR3 and the NCD subfamily. We have determined the pattern of expression of these genes by RNA in situ hybridization and RNA interference assay, which shows that multiple C-terminal motors participate in chromosome dynamics. These results indicate a combinatorial requirement of kinesins in the cell division process and provide novel reagents to study the dynamics of chromosome segregation in the early embryo.

2. Materials and methods

2.1. Isolation of the cDNA clones encoding kinesin-like proteins

A *C. elegans* cDNA library in a λ-ZAPII vector (kindly given by Y. Kohara) was screened using labeled probes made from two truncated cDNA clones, YK164d2 and YK1d3 (Y. Kohara) that correspond to the *klp-15* and *klp-16* genes respectively. From a screen of about 1.5×10^6 plaques, two positive cDNA clones of the *klp-15* gene were identified. Similarly, we screened 1×10^6 plaques and obtained three positive clones of the *klp-16* gene. The insert was excised from the phage as a pBluescript vector using standard protocols (see [6]). The nucleotide sequence of the cDNA clones was determined by the dideoxy method [8]. The two clones of *klp-15* and three clones of *klp-16* were purified and sequenced, and these two full length cDNA clones were named SQ#Y115 (corresponding to *klp-15*) and SQ#Y116 (corresponding to *klp-16*). The remaining three clones were missing upstream sequences at the 5′ end.

2.2. Northern blot analysis

Total RNA was extracted from healthy wild-type worms according to the kit manufacturer's instructions (Qiagen) with minor adaptation for *C. elegans*. Poly(A)$^+$ RNA was prepared from the total RNA using Oligotex-dT30 according to the kit manufacturer's instructions (Takara, CA). 5 μg of poly(A)$^+$ RNA for each gene (*klp-15* and *klp-16*) was electrophoresed on a denaturing agarose gel and was transferred to a nylon membrane. For cross-linking, the membrane was irradiated by UV light for 5 min, and baked at 80°C for 2 h. The filter was then hybridized with digoxigenin labeled probe at 65°C for 16 h

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[1] The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence data bases with accession numbers AB032080 (clone SQ#Y115), AB033380 (clone SQ#Y116), and AB031233 (clone SQ#Y117).

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as described [6]. Here *klp-15* and *klp-16* cDNAs were used as templates to make labeled probes. The signal was detected using the kit manufacturer's instruction (Boehringer Mannheim). The RNA size marker (Takara, CA) was used to measure the size of the detected signal.

2.3. Protein homology search and computer-based secondary structure prediction

The overall homology was searched using the BLAST program [9]. Protein homologies among the C-terminal motor containing kinesin-like proteins were searched by the CLUSTALW program [10]. The secondary structure of KLP-3, KLP-15, KLP-16 and KLP-17 was deduced using the published algorithm [11].

2.4. Phylogenetic tree of the NCD/Kar3 subfamily

Based on the alignment of the motor domain [10], a phylogenetic tree using the maximum likelihood (ML) method [12] was obtained. The ProML program implemented in the MOLPHY package [13] was used and adopted the JTT model [14] to compute the likelihood of a tree. First the ML distances of all pairs of sequences were computed, and a neighbor joining (NJ) tree [15] was constructed using the distances. The reliability of each internal branch of trees was evaluated using the bootstrap method [16]. The bootstrap probabilities of occurrence of internal branches were also computed using the ProtML program, which quickly computes the bootstrap probability by resembling of estimated log-likelihood method [17].

2.5. RNA in situ hybridization of nematode embryos

For the RNA in situ hybridization in *C. elegans* embryos, we followed the published method [18]. For probes, near full length cDNA clones of *klp-3, klp-15* (SQ#Y115), *klp-16* (SQ#Y116) and *klp-17* (SQ#Y117) were labeled with digoxigenin, following the manufacturer's instructions (Boehringer). Four independent samples were analyzed to confirm the staining pattern.

2.6. RNA interference assay

Double stranded (ds) RNA interference (RNAi) experiments were done according to the published method [19]. The dsRNA was injected into the gonad of young and L4 larval wild-type hermaphrodites at a concentration of ~200–500 ng/μl. Injected animals produced few unaffected progeny which were presumably fertilized before injection. The effect of RNAi was most significant after 16–24 h of injection. After the injection, the injected animals and the resulting embryos were fixed and permeabilized with Carnoy's solution (ethanol:acetic acid:chloroform = 60:30:10), incubated in a DAPI (5 μg/ml) solution for 30 min. Stained embryos were observed using Nomarski (DIC) optics in a Nikon microscope. In controls, only the injection buffer and a negative control (*osm-3*) [20,21] were injected into developing gonads, which produced normal embryos with no morphological or developmental defects.

3. Results

3.1. Isolation of klp-15 and klp-16 cDNA clones and Northern blot analysis

In our search for the members of the kinesins in *C. elegans*, we have previously identified cDNA clones, corresponding to the entire kinesin family *klp-1* to *klp-20* [6]. Among the 20 genes, four cDNA clones are derived from the *klp-3*, *klp-15, klp-16* and *klp-17* genes encoding C-terminus kinesins. Interestingly, two kinesin-like proteins, KLP-15 and KLP-16, show very high homology to each other. The *klp-15* gene maps between the *spe-11* and *let-393* loci on chromosome I. To obtain a full length *klp-15* cDNA clone, we screened a *C. elegans* cDNA library in the λ-ZAPII vector, using a probe made from the yk164d2 cDNA clone (Y. Kohara) and identified a single 2020 bp clone (SQ#Y115) with an open reading frame. Sequencing of the SQ#Y115 clone revealed that the 2020 bp clone (Fig. 1A) is indeed derived from the *klp-15* gene and encodes the complete transcript. Northern blot analysis was performed from the wild-type poly(A)$^+$ containing RNA, using the SQ#Y115 cDNA clones as probe, giving a 2.1 kb band corresponding to the *klp-15* gene (Fig. 1C), suggesting that our SQ#Y115 clone is a full length clone, containing the entire *klp-15* gene.

We isolated a full length *klp-16* cDNA clone (SQ#Y116) of size 2007 bp using a probe made from a cDNA clone yk1d3 (Y. Kohara) of size 1.36 kb (Fig. 1B). Northern blot analysis was performed using the SQ#Y116 cDNA clone as a probe. It reveals a 2.05 kb band (Fig. 1D), suggesting that our SQ#Y116 clone encodes the full length *klp-16* gene. The *klp-16* maps in the region between *mec-8* and *ceh-5* loci on the same chromosome I.

3.2. C-terminus kinesins KLP-15 and KLP-16 form a novel kinesin subgroup

Deduced amino acid sequences reveal that the newly identified kinesins *klp-15* and *klp-16* both encode proteins of 587 amino acid, whereas *klp-3* and *klp-17* encode proteins of 598 and 605 amino acid residues respectively. Their motor domains are located at the C-terminus of the protein sequences. Overall structure and the motor domain sequences of all three kinesins show homology with human kinesin heavy chain or *Drosophila* NCD. Recently, the crystal structure of the NCD has been determined [22]. Based on the structural data, we have found that conserved MT binding sites and nucleotide binding pocket sites N1, N2, N3, and N4 [22] are present in the motor domains of KLP-15 and KLP-16 (Fig. 1A,B).

The three kinesins show quite high motor domain homology among themselves; e.g. KLP-15 versus KLP-16 has 91% identity; KLP-15 versus KLP-17 has 45% identity; and KLP-16 versus KLP-17 shows 45% identity (Fig. 2A). However, they show relatively low homology with the previously known members of the C-terminus kinesin subfamily (such as NCD, KAR3 and KLP-3); e.g. amino acid identities between KLP-15 and *C. elegans* KLP-3, *Drosophila* NCD, yeast KAR3 are 31%, 36%, and 33% respectively (Fig. 2A). Our phylogenetic tree analysis suggests that KLP-15 and KLP-16 constitute a distinct subfamily from the KLP-3, KAR3 and NCD kinesins (Fig. 2B).

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Fig. 1. cDNA cloning and Northern blot analyses. A: Nucleotide sequence of a full length cDNA clone of *klp-15*. The start codon atg and the stop codon tag, located at 119 bp and 1880 bp respectively, are boxed. The deduced amino acid sequence is shown under the nucleotide sequence and shaded amino acid residues represent the microtubule (HVSYRDSKLTQ) and nucleotide binding pocket sites N1, N2, N3, N4 (GQTGSGKT, NESSSR, VDLAGSE, RVRP respectively). B: Nucleotide sequence of a full length cDNA clone of *klp-16*. The start codon atg and the stop codon tga, located at 3 bp and 1764 bp respectively, are boxed. The deduced amino acid sequence is shown under the nucleotide sequence. Shaded amino acid residues represent the microtubule (HVSYRDSKLTQ) and nucleotide binding pocket sites N1, N2, N3, N4 (GQTGSGKT, NGSSSR, VDLAGSE, RVRP respectively). C: A 2.1 kb band is seen in the Northern blot using 5 μg of the N2 (wild-type) mixed mRNA, probed with the *klp-15* cDNA (SQ#Y115). D: A 2.05 kb band is seen in the Northern blot using 5 μg of the N2 (wild-type) mixed mRNA, probed with the *klp-16* cDNA (SQ#Y116).

A

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gtagttgactacggtaatccattcgatttggcgggaattttcagaggcggagttcaactttgagcgggaaaatgtcaatgaaggggctgatttcctcatttttgtcccaa 110
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          M  N  V  A  R  R  R  S  G  L  F  R  S  T  I  G  A  T  P  K  I  T  R  G  R  A  A  A  P  S  T  K  E  A  34
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B

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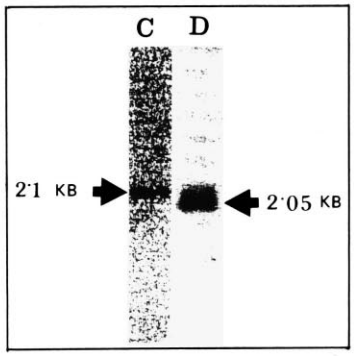

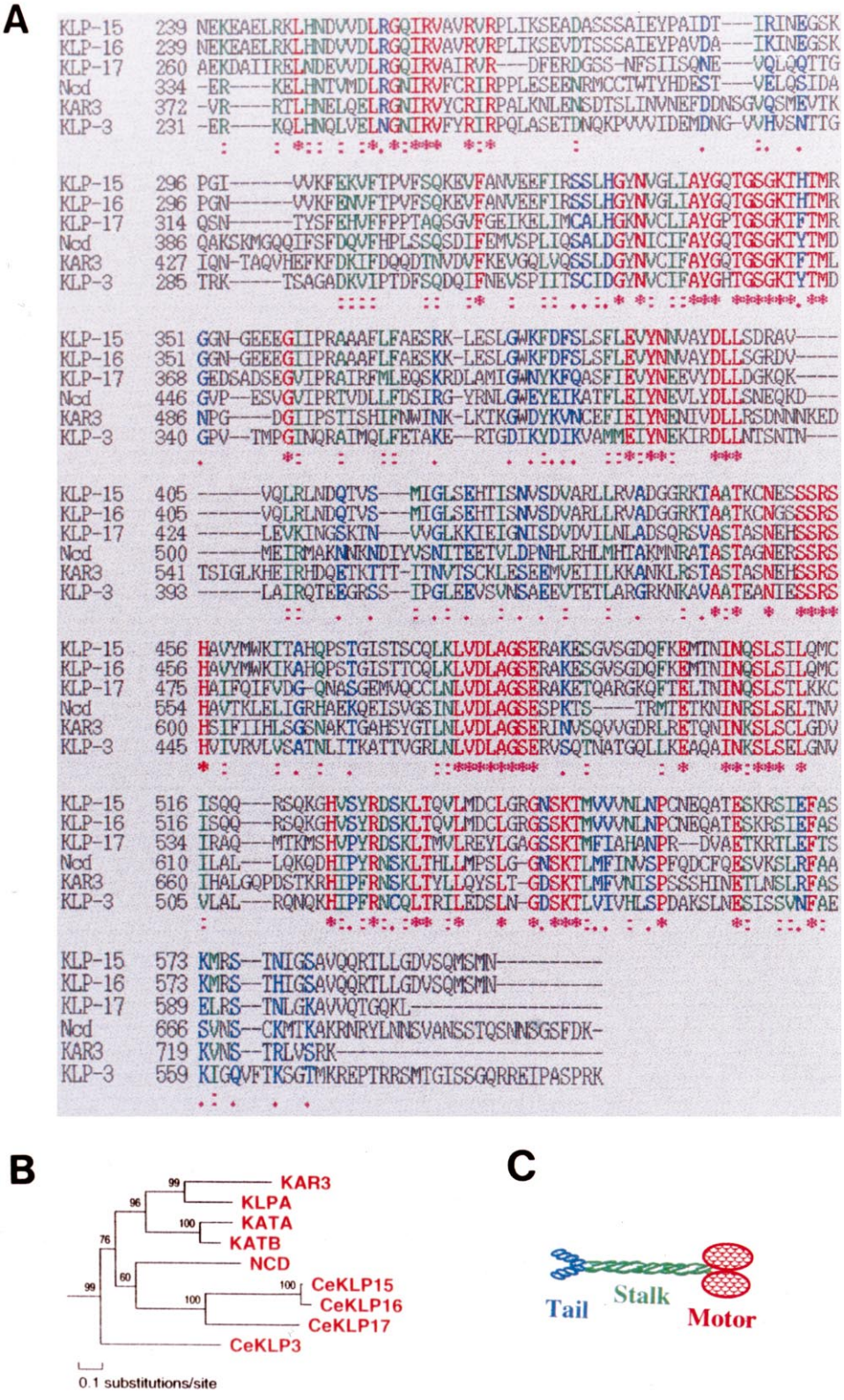

Fig. 2. Amino acid sequence homology among the C-terminal motor kinesins. A: Motor domain sequence identities between KLP-15 and KLP-16, KLP-17, KLP-3, *Drosophila* NCD, yeast KAR3 are respectively 91%, 45%, 31%, 36%, and 33%. Similarly, homologies between KLP-16 and KLP-17, KLP-3, *Drosophila* NCD, yeast KAR3 are 45%, 31%, 34%, 33% respectively. B: Phylogenetic tree of the C-terminal motor kinesins in *C. elegans*. KLP-15 and KLP-16 form a subgroup in the C-terminal motor subfamily. C: A representative model for C-terminal motor KLP that shows a globular motor (a dimer) domain located in the C-terminal (red), a helical structure stalk domain in the middle of the protein (green) and a small globular tail domain (blue) (for review, see [24]).

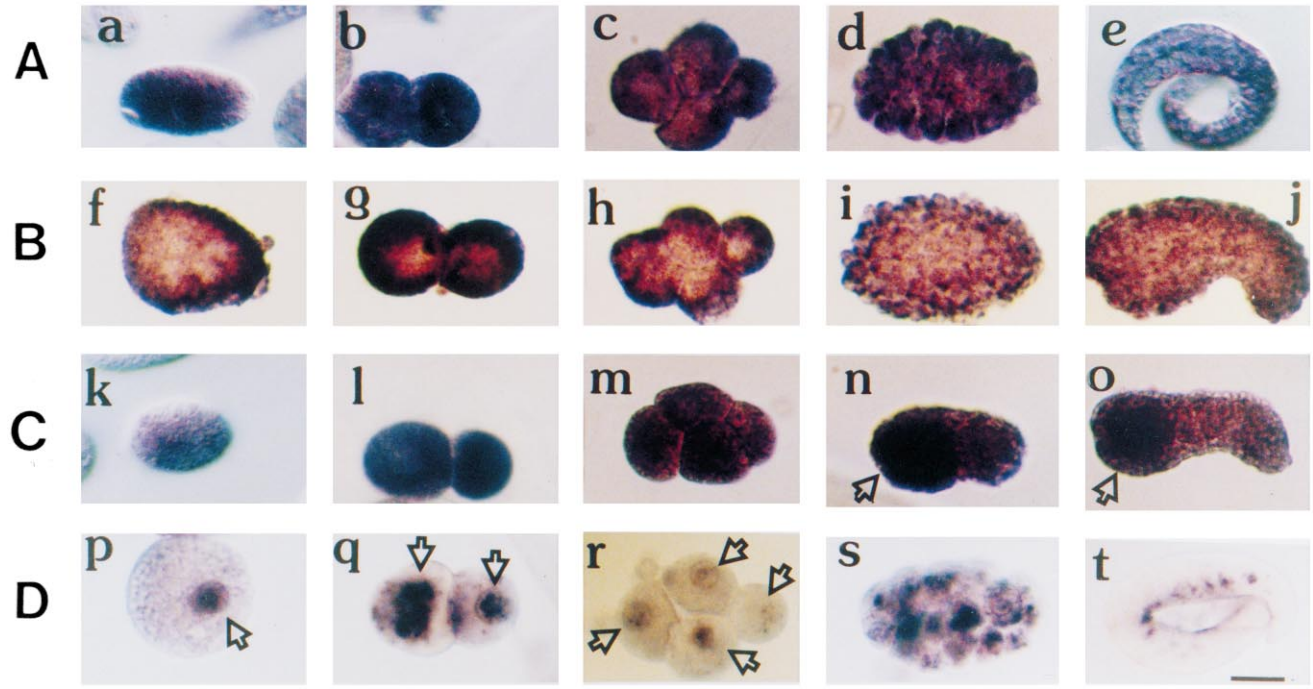

Fig. 3. The mRNA in situ hybridization expression pattern. A: The expression pattern of *klp-3*. Staining in a one cell (a), a two cell (b), and a four cell (c) stage embryo, multiple cells (d), young larval stage (e). B: Expression pattern of *klp-15*. Staining is mostly in the periphery of the cell (f–j). C: Expression patterns of *klp-16*, of one cell, two cell, and four cell stage embryos show uniform expression in all cells (k–m), expression in the late embryos and young larvae is predominant in the anterior region (n, o). D: Expression of *klp-17* in the cell nuclei (p–t). Scale bar represents 8 μm.

Secondary structure analysis of KLP-15 and KLP-16 suggests that they have highly similar structures (data not shown). Both proteins have a globular motor domain (residues 230–587) at the C-terminus of the protein sequence connected with a small globular domain (residues 77–133) via an α-helical stalk domain. Their N-terminus domain (residues 1–76) is rich in coiled-coil structure. In comparison, the N-terminal region of KLP-17 is small and globular, mostly in α-helical and coiled-coil conformation intercepted by β-sheet turns. The globular N-terminal region is connected to the globular motor domain (residues 260–605), via a coiled-coil stalk region, which is largely in α-helical conformation [7]. Likewise, KLP-3 contains an α-helical coiled-coil region (residues 10–91) at its N-terminus which is connected to a C-terminal globular motor domain (residues 231–598) via a stalk domain [6].

3.3. RNA expression using in situ hybridization

To reveal the in vivo function of the novel kinesins, we performed RNA in situ hybridization on embryos (Fig. 3) and the dissected gonads (data not shown) from young adults. Near full length cDNA probes of *klp-3, klp-15, klp-16* and *klp-17* were used to see the pattern of mRNA expression of these genes. Although the amino acid sequence homology between KLP-15 and KLP-16 is very high, their mRNA expressions are quite different. The *klp-15* expression signal appears in the periphery of embryos, apparently in the hypodermal cells during development (Fig. 3B). However, the *klp-16* signal is localized in the anterior nervous system in the head region in late embryos and is not seen in early embryos (Fig. 3C). These observations suggest that *klp-16* is involved in early development of the nervous system in embryos, whereas the *klp-3* RNA hybridization signal is detected in the entire cytoplasm throughout embryogenesis (Fig. 3A).

Remarkably, the expression of *klp-17* is highly localized in the cell nuclei of embryos (Fig. 3D) [7]. The hybridization signal remains fairly strong during the entire development, including the cell proliferation and morphogenetic phase. Since this pattern of gene expression is highly unusual, we repeated all these observations several times. Several control experiments were conducted to ascertain the specificity of the hybridization signal for *klp-3, klp-15, klp-16* and *klp-17* genes. Removing the coding region, and using only the Bluescript vector as a probe, does not show staining except a little background. As a negative control, we used the *dpy-20* probe [23] for RNA in situ hybridization and found no staining in embryos.

3.4. RNA interference causes arrested embryos

To study the in vivo gene function of a given gene in *C. elegans*, the RNAi technique, which disrupts gene function, has provided a powerful tool [19]. The phenotype caused by RNAi is generally very specific and reproducible. We have used RNAi to eliminate the expression of KLP-3, KLP-15, KLP-16 and KLP-17 by injecting the corresponding dsRNA into the maternal germ lines of hermaphrodites [19]. To study the effect of RNAi, injected animals were observed using Nomarski optics, following 16–24 h of the time of injection. About 500 animals (L4 larval and young adult stages) were injected for each gene to observe the effect of RNAi of specific genes. Among the injected animals, about 80% (average) show the effect of RNAi in 16–24 h following injection. We found that *klp-15* RNAi resulted in arrested embryos at the one cell stage (Fig. 4C,G) but *klp-16* RNAi causes arrested embryonic

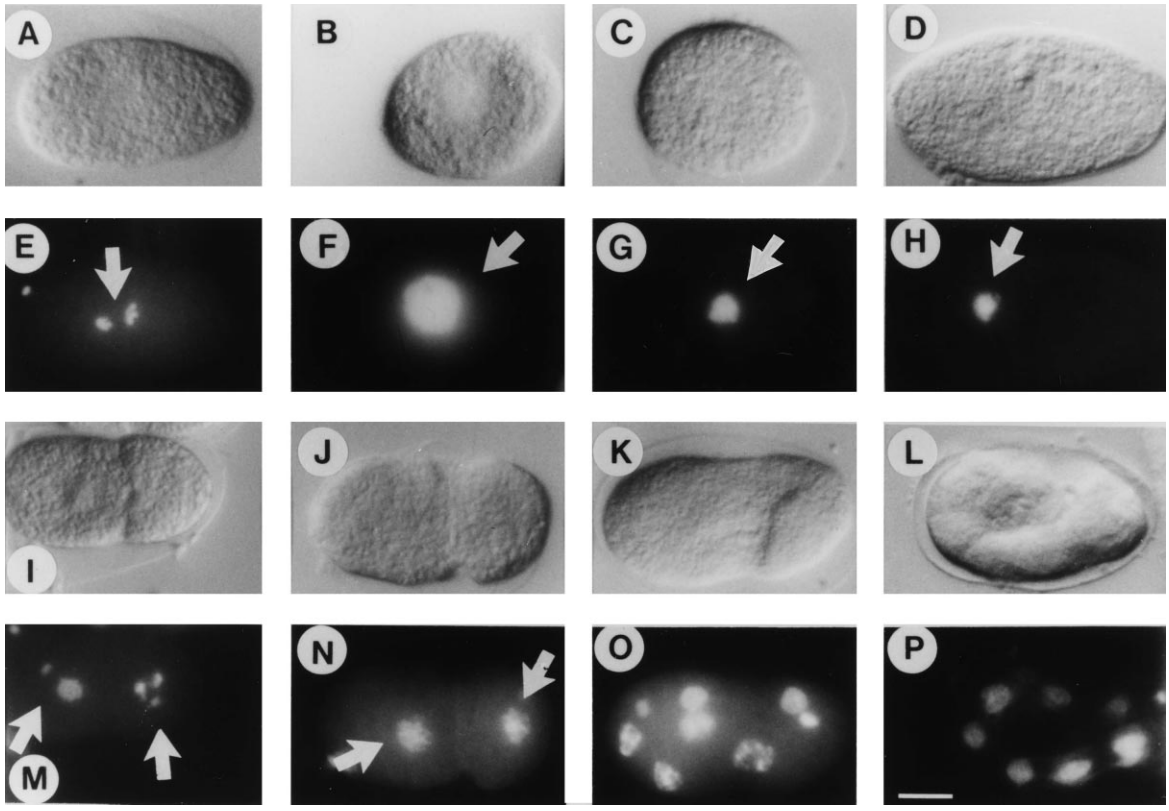

Fig. 4. RNA interference pattern in arrested embryos for the *klp-3*, *klp-15*, and *klp-16* genes, as viewed by Nomarski optics. A: A wild-type, one cell normal embryo. B–D: The effect of *klp-3, klp-15* and *klp-16* RNAi on one cell stage embryos. E–H: Same embryos as shown in A–D respectively, stained with the DNA-staining dye DAPI. I: A two cell stage wild-type embryo. J: The *klp-16* RNAi, two cell stage arrested embryo. K: An eight cell stage (approximately) wild-type embryo. L: *klp-16* RNAi, an eight cell (approximately) stage arrested embryo. M–P: The same embryos as shown in I–L, stained with DAPI, showing normal chromosome. Scale bar represents 12 μm.

phenotype at the one cell to about eight cell stages (Fig. 4D,H,J,L,N,P). On the other hand *klp-17* RNAi treated animals produced arrested embryos at the one and two cell stages [7]. We have previously shown that the antisense RNA of *klp-3* under the control of a heat shock promoter caused the one cell arrest embryo phenotype [6], which is consistent with the RNAi result (Fig. 4B,F). Arrested embryos in each case were stained with DAPI to visualize chromosomes. DAPI staining showed that the chromosomes in the arrested embryos are polyploid and the spindles are disorganized. These results could suggest a role of the *klp-3*, *klp-15, klp-16* and *klp-17* genes in chromosomal distribution, during early cell divisions in embryos. However, further analysis of spindle orientation and organization using Nomarski optics or the use of specific antisera to visualize spindle shape, organization and orientation will be needed to establish the in vivo function of these C-terminal kinesins in chromosomal segregation in *C. elegans*.

4. Discussion

The genome sequencing project has provided the basic framework to elucidate the role of the kinesin superfamily in *C. elegans* and allowed the identification of each and every member of the kinesin superfamily in *C. elegans* [5]. The nematode genome encodes 20 kinesin-like proteins. We have identified cDNA clones corresponding to most of these genes [6,7,20,21]. Among the 20 kinesin-like proteins in *C. elegans*, four proteins, KLP-3, KLP-15, KLP-16, and KLP-17, are C-terminal motor kinesins. An analysis of kinesin function in *C. elegans* will provide useful insight into intracellular transport in metazoans. The NCD group of motor proteins is involved in spindle attachment and chromosomal movement. In *C. elegans*, we have earlier reported the C-terminal motor kinesins KLP-3 and KLP-17, orthologs of NCD, and their role in chromosome movement [6,7]. Here, we have described two novel members of the NCD family, KLP-15 and KLP-16, which form a distinct subfamily of the C-terminal motor proteins, not found in any other organism in the databases. What role do KLP-15 and KLP-16 play in vivo?

These C-terminal motor kinesin family members mediate meiotic and early mitotic chromosome segregation by attaching to spindles. For example, the nuclear fusion proteins KAR3 in *Saccharomyces cerevisiae*, KatC in *Arabidopsis thaliana*, KLPA in *Aspergillus nidulans*, HSET in *Homo sapiens* are known to function in chromosomal movement (for review, see [24]). The NCD tail domain can promote MT assembly and stabilize MTs against conditions that induce MT disassembly. This suggests that NCD may influence MT dynamics within the spindle [25]. The yeast Kar3 mutants have abnormally long cytoplasmic microtubules consistent with the observation that Kar3 destabilizes microtubules at the minus end in vitro [24]. Another C-terminal motor kinesin protein from *Arabidopsis*, KatA, may act in spindle pole organization in plant cell mitotic division [26]. Thus the C-terminal motor KLPs are likely to modulate microtubule dynamics and could contribute to pole-ward chromosome movement by maintaining micro-

tubule attachment to poles while simultaneously promoting their shortening.

The data reported here on RNAi reveal that all four *klps* (*klp-3*, *klp-15, klp-16* and *klp-17*) perhaps function in chromosome distribution in early embryonic cell divisions. The dsRNA treatment results in arrested embryos and polyploid cells in the treated embryos. Although the RNAi assay is transient, it does provide a highly gene-specific and potent method to reveal the knockout phenotype of the gene in question [19]. These observations raise the question whether these genes provide a unique function, temporally and spatially, i.e. whether they act in the same set of cells at the same time in development. In situ RNA hybridization data reveal that although all of the C-terminal motor kinesins are abundantly expressed during embryogenesis, their pattern of expression is not identical. For example, *klp-3* is expressed throughout embryogenesis in the cytoplasm; the *klp-17* transcripts appear to be highly localized in the cell nucleus. In contrast, *klp-15* is expressed in the cytoplasm of cells found in the periphery of embryos. Likewise, *klp-16* expression is quite distinct and shows uniform expression from the one cell to the eight cell stage in all cells, but later expression is more localized in the anterior region of the embryo. These data suggest that the different C-terminal motors play unique and perhaps non-overlapping roles during embryogenesis. Since we have not tested the post-embryonic pattern of expression of these genes, it remains to be seen whether KLP-3, KLP-15, KLP-16 and KLP-17 participate in any overlapping function. Some evidence of a possible overlap in function is described below.

We have previously shown that *klp-3* over-expression partially rescues a defect in the temperature-sensitive mutation in the *him-14* gene that is involved in the non-disjunction of the X-chromosome [6]. This is like the reported suppression of the bimC4 mitotic spindle defect by a deletion mutation in the *klpA* kinesin, an ortholog of the yeast Kar3 in *A. nidulans* [27]. The RNAi-based kinesin gene knockout and the in situ hybridization pattern for various C-terminal kinesin genes in *C. elegans* suggest that these motors perform pleiotropic functions, some of which are unique whereas other in vivo functions may be redundant. We are seeking deletion knockout mutants for different members of the kinesin gene family for which stable mutant lines are currently not available. It is possible that such mutants may already exist as lethal, as these genes may be essential for viability. The genetic map positions of *klp-15* and *klp-16* are on linkage group I, whereas *klp-3* and *klp-17* are located on linkage group II. Several lethal and maternal genes affecting embryogenesis and larval development are located in this chromosomal region, which may be putative candidates for the KLP-15 or KLP-16 kinesins. Using germline transformation rescue, we are attempting to rescue these mutants with *klp-15* and *klp-16* genomic DNA.

In summary, we have cloned and characterized a novel C-terminal motor kinesin subfamily in *C. elegans*, which is involved in chromosomal distribution during embryogenesis. More experiments are needed to further explore the nature of the mutant phenotype in the absence of these novel kinesin proteins. Further analysis of these *C. elegans* kinesin genes may elucidate diverse in vivo functions of the C-terminal motor proteins involved during mitotic cell division in metazoan development.

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