Rho-binding kinase (LET-502) and myosin phosphatase (MEL-11) regulate cytokinesis in the early *Caenorhabditis elegans* embryo

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Accepted 26 March 2002
Journal of Cell Science 115, 2271-2282 (2002) © The Company of Biologists Ltd

Summary

Rho-binding kinase and myosin phosphatase regulate the contraction of actomyosin filaments in non-muscle and smooth muscle cells. Previously, we described the role of *C. elegans* genes encoding Rho-binding kinase (*let-502*) and myosin phosphatase targeting subunit (*mel-11*) in epidermal cell-shape changes that drive morphogenesis and in spermathecal contraction. Here we analyze their roles in a third contractile event, cytokinesis within early embryos. We demonstrate that these genes function together to regulate the rate of cleavage furrow contraction, with Rho-binding kinase/LET-502 mediating contraction, whereas myosin phosphatase/MEL-11 acts as a brake to contraction: early embryonic cleavage often fails or is slowed when *let-502* is mutated, whereas *mel-11* mutations result in ectopic furrowing and faster furrow ingression. These phenotypes correspond to changes in the levels of phosphorylated regulatory non-muscle myosin light chain (rMLC). The gene products of *let-502* and *mel-11* colocalize at cleavage furrows, and their mutations alleviate one another's defects. rMLC is phosphorylated in *let-502; mel-11* double mutants, indicating that a kinase is able to phosphorylate rMLC in the absence of both LET-502 and MEL-11. Genetic and molecular epistasis experiments place LET-502 and MEL-11 in a cytokinetic pathway. LET-502 and MEL-11 regulate the activity of non-muscle myosin after actin, non-muscle myosin heavy chain/NMY-2, regulatory non-muscle myosin light chain/MLC-4 and early formin/CYK-1 have formed a contractile ring. Proteins including Rho GTPase activating protein/CYK-4 and late CYK-1, which are required for late stages of cytokinesis, function downstream of LET-502 and MEL-11.

Key words: Rho-binding kinase, Myosin phosphatase, Cytokinesis, Contraction, *C. elegans*

Introduction

Cytokinesis, the final step of mitosis, is the separation of the cytoplasm and replicated nuclear contents into daughter cells through actomyosin ring contraction. The ring forms during anaphase and contracts during telophase to form two daughter cells. Furrowing terminates after the ring fully ingresses and contacts an interzone bundle of microtubules, the central spindle (Raich et al., 1998; Field et al., 1999; Jantsch-Plunger et al., 2000; Robinson and Spudich, 2000; Glotzer, 2001). This paper focuses on genes, namely *C. elegans* Rho-binding kinase (*let-502*) and myosin phosphatase (*mel-11*), which have antagonistic activities to regulate contraction of the actomyosin ring.

Rho GTPases have been implicated in regulating the various stages of cytokinesis in eukaryotes (Drechsel et al., 1996; Mackay and Hall, 1998; Prokopenko et al., 1999; Jantsch-Plunger et al., 2000; Prokopenko et al., 2000; Glotzer, 2001). For example, inhibiting Rho activity blocks cytokinesis in both *Xenopus* and *C. elegans* embryos, suggesting that Rho is required for furrow formation (Drechsel et al., 1996; Jantsch-Plunger et al., 2000). Similarly in *Drosophila*, furrow formation fails in mutations of *pebble*, a Rho guanine exchange factor (GEF) (Prokopenko et al., 1999).

Rho-binding kinase (ROK) is a downstream Rho effector that is a candidate for modulating myosin activity during furrow ingression. Non-muscle myosin forms fibres at the onset of cytokinesis, which is concurrent with the localization of active myosin light chain kinase (MLCK) to the cleavage furrow (DeBiasio et al., 1996; Totsukawa et al., 1999; Poperechnaya et al., 2000). The regulatory non-muscle myosin light chain (rMLC) homologues (*Drosophila spaghetti-squash* and *C. elegans mlc-4*), the non-muscle myosin heavy chain homologue (*C. elegans nmy-2*) and the MLCK homologue (*Dictyostelium* MLCK-A) are all required for cytokinesis (Karess et al., 1991; Guo and Kemphues, 1996; Smith et al., 1996; Shelton et al., 1999). In smooth muscle and during stress fibre formation, contraction is induced by MLCK phosphorylation of rMLC (Gallagher et al., 1997; Totsukawa et al., 2000; Somlyo and Somlyo, 2000; Katoh et al., 2001). Myosin phosphatase blocks contraction by dephosphorylating rMLC to counteract MLCK. Rho activates ROK, which then releases the brake to contraction by phosphorylating and inhibiting the myosin phosphatase targeting subunit (MYPT) (Kimura et al., 1996; Kawano et al., 1999; Somlyo and Somlyo, 2000). ROK also directly phosphorylates rMLC in vitro (Amano et al., 1996), but it is not clear if this occurs in all in vivo systems (Sward et al., 2000). In *Drosophila*, planar cell polarity signaling activates ROK (Drok) to regulate the

actin cytoskeleton through the regulation of MLC (Spaghetti-squash) activity (Winter et al., 2001).

The precise roles that ROK and myosin phosphatase play during cytokinesis are not clear. Injection of dominant-negative ROK constructs resulted in multinuclear cells in *Xenopus* embryos and cultured mammalian cells owing to failed glial fibrillary acid protein (GFAP) disassembly, which is required for proper cell separation following cleavage (Yasui et al., 1998). However, the use of ROK inhibitors in cultured mammalian cells instead indicated its requirement for furrow contraction. Another ROK-related Rho effector, citron-k, localized to the cleavage furrow and midbody in HeLa cells, and transfection with dominant-negative constructs caused abnormal furrow contractions (Madaule et al., 1998; Yasui et al., 1998; Kosako et al., 1999; Kosako et al., 2000). However, in vivo studies indicate that citron-k is not the global regulator of cytokinesis and probably functions redundantly (Di Cunto et al., 2000). Myosin phosphatase activity is downregulated following mitosis, but the physiological relevance of this has not been shown (Totsukawa et al., 1999).

There is a need for determining ROK's role in cytokinesis using endogenous loss-of-function mutations rather than transfected dominant-negative mutations or chemical inhibitors (Madaule et al., 1998; Yasui et al., 1998; Kosako et al., 1999; Kosako et al., 2000), both of which could have effects on other (unknown) proteins. Using endogenous *let-502* and *mel-11* mutations, we previously described in vivo roles for the *C. elegans* ROK (LET-502) and MYPT (MEL-11) in morphogenesis and spermathecal function, two different contractile events in the worm (Wissmann et al., 1997; Wissmann et al., 1999; Piekny et al., 2000). In morphogenesis, LET-502 and MEL-11 together regulate the actin-mediated epidermal cell shape changes that drive elongation of the embryo (Wissmann et al., 1997; Piekny et al., 2000). During oocyte fertilization, LET-502 and MEL-11 each independently regulate the contraction of different tissues within the spermatheca (Wissmann et al., 1999). Here we use *let-502* and *mel-11* mutations to demonstrate in vivo roles for both genes in another contractile event, cytokinesis. We show that LET-502 and MEL-11 together control cytokinesis in a manner similar, but not identical to, their regulation of epidermal cell shape changes during morphogenesis. This suggests that LET-502 and MEL-11 are utilized at different stages of the life cycle for various contractile events, but each event is genetically and biochemically distinct.

Materials and Methods

Strains and alleles

C. elegans strains *var.* Bristol were maintained according to standard procedures (Brenner, 1974). The following alleles and chromosome rearrangements were used in this study (Hodgkin et al., 1988; Hodgkin, 1997): *let-502(sb106, sb108), dpy-5(e61), mel-11(it26), unc-4(e120), mnCl* [*dpy-10(e128) unc-52(e444)*] chromosome II balancer (Edgley et al., 1995), *cyk-1(t1568, t1611, s2833, or36), unc-32(e189), unc-36(e251), ncl-1(e1865), qCl* [*glp-1(q339) dpy-19(e1259)*] chromosome III balancer (Edgley et al., 1995), *him-3(e1147), him-8(e1489)* and *cyk-4(t1689)*.

Microscopy and immunofluorescence

Early embryos were dissected from gravid adult hermaphrodites. For temperature-sensitive (*ts*) alleles, hermaphrodites were upshifted to the non-permissive temperature for 1-2 hours prior to dissection. Embryos were mounted on 3% agarose pads in M9 solution (Sulston and Hodgkin, 1988) and examined by Nomarski optics on a Zeiss Axioplan microscope. Images were photographed using either Techpan film (Kodak) developed at ASA 100 or using videotape recording.

Early embryos were collected for staining by dissecting hermaphrodites on polylysine-coated slides in M9 solution (Sulston and Hodgkin, 1988). A coverslip was placed on each slide, and the embryos were frozen on dry ice for a minimum of 30 minutes. The coverslips were removed and slides were placed in –20°C MeOH for 15 minutes, followed by 5 minutes in –20°C acetone. For actin staining only, freeze-cracked slides were placed in a 3.7% formaldehyde solution (w/v) in 75% MeOH at room temperature for 10 minutes and then placed in 100% MeOH at room temperature for 5 minutes (Waddle et al., 1994). Actin staining could be performed only with the formaldehyde-based fixation procedure; however, LET-502 and MEL-11 staining could be performed only with the methanol fixation procedure. For all methods, the slides were then placed directly into 1× phosphate-buffered saline (PBS) with 0.1% Triton X-100 (PBT) buffer for a minimum of 1 hour, then incubated with appropriate dilutions of antisera in PBT with 20% normal goat or donkey serum (Jackson Immunoresearch Laboratories). Mouse anti-actin monoclonal antibodies (Sigma) were used at a 1:50 dilution, rabbit anti-NMY-2 polyclonal antibodies (K. Kemphues, Cornell University, Ithaca, NY) were used at a 1:50 dilution, rabbit anti-CYK-1 polyclonal antibodies (B. Bowerman, University of Oregon, Eugene, OR) were used at a 1:20 dilution, rabbit anti-rMLC phosphoserine-19 polyclonal antibodies (M. Walsh, University of Calgary, Calgary, AB) were used at a 1:50 dilution, P-granules were stained with mouse OIC1D4 monoclonal antibodies (S. Strome, Bloomington, IN) at a 1:1 dilution, rat anti-LET-502 polyclonal antibodies (see below) were used at a 1:100 dilution and rabbit anti-MEL-11 polyclonal antibodies (see below) were used at a 1:50 dilution. All slides were incubated with primary antibodies overnight at room temperature and washed three times with PBT prior to adding the appropriate secondary antibody. Anti-rat IgG conjugated to indocarbocyanine (Cy-3; Jackson Immunoresearch Laboratories), anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC; Jackson Immunoresearch Laboratories) or Alexa 488 (Molecular Probes) and anti-rabbit IgG conjugated to cyanine (Cy-2; Jackson Immunoresearch Laboratories) or Alexa 488 (Molecular Probes) were diluted 1:100 in PBT, and slides were incubated with the appropriate secondary antibody at room temperature for one hour. Slides were washed three times with PBT prior to being incubated with 1 μg/mL DAPI (Roche) for 10 minutes at room temperature. After one wash with PBT, any remaining liquid was removed with a kimwipe. A drop of Slowfade Light Antifade solution (Molecular Probes) and a coverslip were added to each slide and sealed with nail polish. Some images were photographed using a 35mm camera on a Zeiss AxioPlan fluorescence microscope with a 63× oil objective (embryos) or a 40× objective (gonads). Other images were collected for each fluorochrome as stacks of ~12-15× 1 μm from a Leica DM-R fluorescence microscope using either a 40× oil objective (embryos) or a 20× oil objective (gonads) with an Optivar tube set at a distance of 2 and images were collected with a Princeton Instruments 14-bit cooled charge-coupled device (CCD). The collected stacks were digitally deconvoluted using the nearest neighbor algorithm of Autoquant Autodeblur version 5.1 for Windows, and all images were analyzed using similar parameters. These images were then individually imported into Adobe Photoshop version 4.0 for Windows to add colour and merge images.

RNA-mediated interference (RNAi)

Double-stranded RNA was generated for both *let-502* and *mlc-4* as previously described (Piekny et al., 2000; Shelton et al., 1999). RNA

for the *citron* genes was made by oligo(dT)-primed synthesis (GibcoBRL) followed by PCR of the pooled cDNA. The primers used were specific to W02B8.2: the forward primer included the T3 promoter binding sequence 5′ AATTAACCCTCACTAAAGGGATGAACGAATCAATATATATAC 3′ and the reverse primer including the T7 promoter binding sequence 5′ TAATACGACTCACTATAGGGTTAGTTTTTGGATCTTTTCA 3′. Primers specific to F59A6.5 were: the forward primer, which included the T3 promoter binding sequence 5′ AATTAACCCTCACTAAAGGGATGTGTGACTCTGTTTAC 3′ and reverse primer including the T7 promoter binding sequence 5′ TAATACGACTCACTATAGGGCCCACGAAGCAATCCAAG 3′. The PCR product was then used for in vitro transcription (Megascript T3 and T7, Ambion). Concentrations of ~500-1000 μg/ml dsRNA were used, diluted in diethylpyrocarbonate-treated water. Wild-type, *let-502(sb106)* or *mel-11(it26) unc-4* L3 and L4 stage larvae were collected (~100-200) and placed into microfuge tubes with 20-40 μl of dsRNA solution and left overnight at 20°C. The soaked worms were then pipetted onto fresh *Escherichia coli* seeded plates and allowed to recover for 12-24 hours. Worms of similar stages were then placed into groups of between three and five on seeded plates and allowed to lay eggs overnight. Worms were collected from plates with low hatching rates (0-15%) and dissected as described above to observe their embryos. (Worms were soaked rather than injected as *let-502(sb106)* hermaphrodites could not recover well from injection and to allow for the collection of large numbers of embryos.)

LET-502, MEL-11 and rMLC phosphoserine-19 antisera

Rat polyclonal antibodies were raised against a HIS-LET-502 fusion using the pQE30 vector and the QiaExpression kit (Qiagen) with 103 amino acids corresponding to a region between the kinase domain and the coiled-coil region (encoded by 309 bp from the start of exon 5, using the following primers: forward 5′ GGTGGATCCAAATCGGACGATGAC 3′ and reverse 5′ GGGGTCGACTTCTCGGTTTTTCGA 3′). Antisera were affinity purified with a GST-LET-502 fusion using the same LET-502 fragment as described above cloned into the pGEX-3X vector and glutathione S-transferase (GST) expression system (Pharmacia) coupled to a cyanogen-bromide-activated Sepharose column (Pharmacia). Western blot analysis showed that the affinity-purified antiserum recognized one band at ~130 kDa (expected M_r 129 kDa) with gravid adult hermaphrodite extracts solubilized with 1 M NaCl, implying that a LET-502 isoform is preferentially associated with the cytoskeleton. This band also was detected in 1× PBS extracts, suggesting that some LET-502 isoforms are in the cytoplasm. Two other bands that differ in size by only a few kDa (~130-140 kDa) were also seen in the 1× PBS extracts, implying that several LET-502 isoforms exist (owing to alternative splicing) and/or some of the isoforms are phosphorylated or partially degraded. All bands were blocked by adding excess GST-LET-502 to the antisera. Decreased immunostaining in the *let-502* mutants further supported the conclusion that the antisera are specific (see Results).

Rabbit polyclonal antibodies were raised against a GST-MEL-11 fusion containing 64 amino acids from a portion 3′ to the ankyrin repeats (encoded by 192 bp from the start of exon 13, using forward primer 5′ CAGGATCCGCAGTCGTCCAACAGAAC 3′ and reverse primer 5′ GCGAATTCCGGCAACCGATAAAT 3′). The antisera were affinity purified using a cyanogen-bromide-activated Sepharose column coupled to the MEL-11 fragment cleaved from the GST-MEL-11 protein. Western blot analysis showed that the antisera recognize five bands in the ~110-120 kDa size region (which is consistent with the expected size range for the five splice variants described by Wissmann et al., with extracts from gravid hermaphrodites in 1× PBS (Wissman et al., 1999). Some of these bands were also visualized when solubilized in 1 M NaCl, suggesting that some of the MEL-11 isoforms are preferentially associated with the cytoskeleton, whereas others are in the cytoplasm. Multiple MEL-11 isoforms exist because of alternative splicing, as observed by Wissmann et al., but in addition, it is likely that some MEL-11 isoforms are phosphorylated or degraded (Wissman et al., 1999). All bands were blocked by incubating the antisera with excess GST-MEL-11 protein. Decreased immunostaining in *mel-11* mutants provide further evidence that the antisera are specific (see Results).

Polyclonal antibodies that specifically recognize rMLC (20 kDa light chain of myosin) phosphorylated at serine 19 were raised in rabbits by injection of a peptide (KKRPQRATS(P)NVFC) corresponding to residues 11-22 of the chicken light chain with a phosphoserine at position 19 and a C-terminal Cys coupled to KLH. Antibodies were purified from the IgG fraction by peptide affinity column chromatography and shown to be specific for rMLC phosphorylated at serine 19 by western blotting. The antibodies were generously donated by Michael P. Walsh (University of Calgary, AB) and his laboratory.

Results

let-502 and *mel-11* control cleavage furrow contraction

We previously described a role for *let-502* and *mel-11* in morphogenesis and oocyte fertilization, two different contractile events in the worm (Wissmann et al., 1997; Wissmann et al., 1999; Piekny et al., 2000). More recently, we identified two *let-502* alleles (*sb103* and *sb106*) that show strict maternal-effect embryonic lethality owing to defects in another contractile event, cytokinesis (Piekny et al., 2000). One of these alleles, *sb106*, behaves as a weak dominant-negative for the abnormal morphogenesis phenotype but acts as a hypomorph for the cytokinetic defect (Piekny et al., 2000). Here we describe *let-502*'s role in cleavage in detail by further analyzing *let-502(sb106)*'s cytokinetic phenotype. We previously described only morphogenetic and sterility phenotypes for *mel-11* (Wissmann et al., 1997; Wissmann et al., 1999; Piekny et al., 2000), and here we show that *mel-11* embryos also have non-lethal cleavage defects.

Embryos from hermaphrodites mutant for *mel-11(it26)* showed ectopic furrowing during both pseudocleavage (the incomplete cleavage that occurs during pronuclear migration after fertilization) and during early cell divisions (Fig. 1, compare wild-type A-F with mutant G-L). *mel-11(it26)* is a temperature-sensitive allele that behaves as a null at the restrictive temperature. Cells in *mel-11(it26)* embryos initiated their nuclear divisions and cleavage furrow ingressions at the correct time, but furrow ingression was completed approximately twice as fast as in wild-type embryos (Fig. 2). For example in the *mel-11(it26)* embryo in Fig. 1T, the AB cell (the anterior blastomere) completed division prior to the initiation of the division of the P1 cell (the posterior blastomere). In wild-type embryos (Fig. 1S), AB completed its cleavage only after cleavage was initiated in P1. *mel-11*'s maternal-effect lethality is rescued by mating to wild-type males (Kemphues, 1988; Wissmann et al., 1999). Because the observed cytokinetic defects occur too early to be rescued by a paternally contributed wild-type allele, *mel-11*'s maternal-effect lethality must primarily stem from defects in elongation rather than cytokinesis.

let-502(sb106) embryos displayed phenotypes opposite to those seen in *mel-11(it26)*, with embryos showing little or no pseudocleavage and slowed or failed cytokinesis (Fig. 1M-R). In *let-502(sb106)* embryos with successful first divisions, the AB cell furrow initiated at the correct time but ingressed at only half

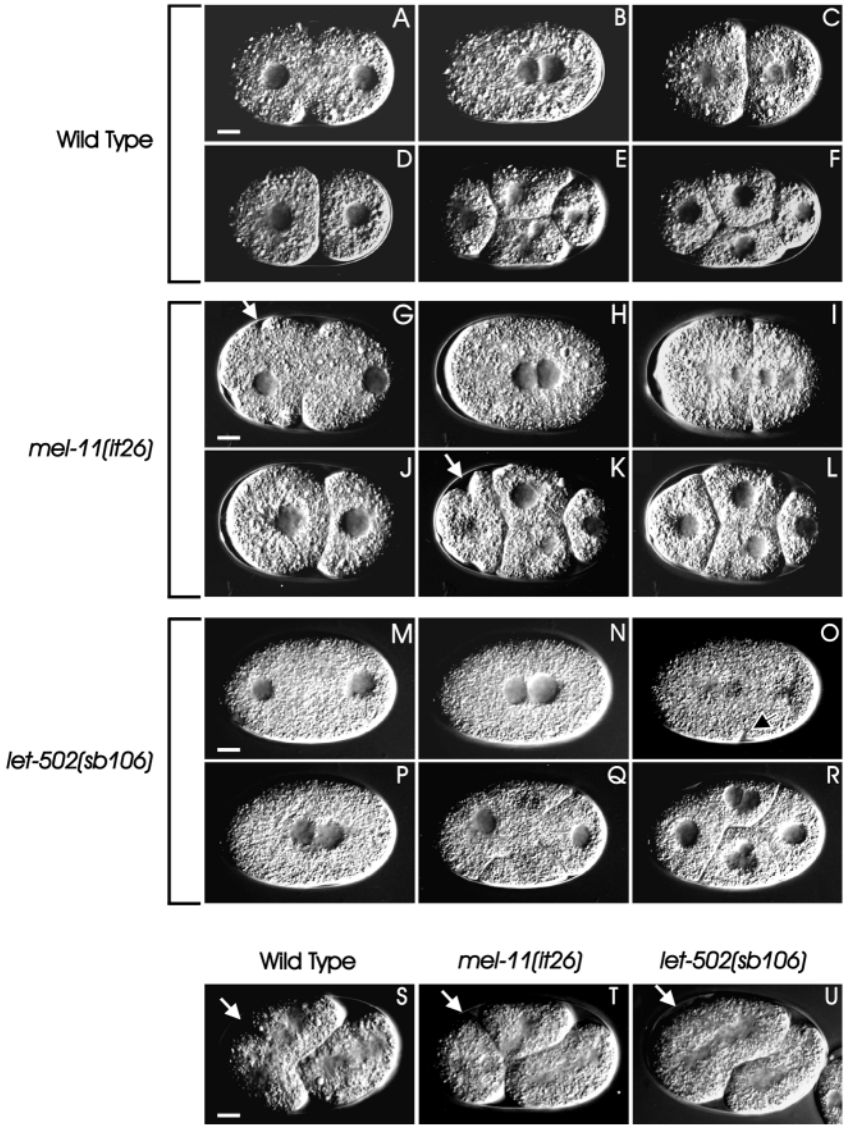

Fig. 1. *let-502(sb106)* and *mel-11(it26)* embryos display defects during pseudocleavage and early cleavages. (A-F) A wild-type embryo during pseudocleavage, pronuclear fusion, late first cell division, two-cell stage, late second division and four-cell stage, respectively. (G-L) A *mel-11(it26)* embryo during similar stages. White arrows indicate ectopic furrows. (M-R) *let-502(sb106)* embryo at stages similar to the wild-type embryo in (A-F). Pseudocleavage furrows either do not form or are small in comparison with wildtype (compare A wtih M). Embryos that have unsuccessful cleavages still form short furrows as indicated by the black arrowhead (O), but these regress. This particular embryo underwent successful cleavages during the next round of cell division, forming an abnormal four-cell embryo (R). Wild-type (S), *mel-11(it26)* (U) and *let-502(sb106)* (V) embryo dividing from the two- to the four-cell stage. All are at the same cell cycle stage as judged by nuclear and spindle morphology and the time since the previous division. Arrows indicate the AB cell furrow, which completes cleavage early in *mel-11(it26)* and late in *let-502(sb106)* relative to wildtype. Bars, 7 μm.

the wild-type speed (Fig. 2). For example, in the *let-502(sb106)* embryo in Fig. 1U, P1 completed its division prior to AB. In *let-502(sb106)* cells that failed to complete cytokinesis, furrows initiated at the correct time and ingressed slightly but then regressed (Fig. 1O,P). The ability of furrows to ingress even slightly could be because of residual LET-502 activity or may reflect the activity of a partially redundant pathway.

Failed cell divisions occur (apparently) at random in *let-502(sb106)* embryos, with many divisions being normal. Again, this could be the result of either residual LET-502 activity or a partially redundant pathway. We examined *let-502(sb106)* embryos treated with dsRNA (used for RNAi) as they presumably would have LET-502 levels depleted to a greater extent. Indeed, *let-502(sb106RNAi)* embryos had decreased embryonic viability (i.e. at 25°C, *let-502(sb106)* had 41% hatching and *let-502(sb106RNAi)* had 0%). However, divisions still failed at random with the number of successful cell divisions at 25°C as follows: six had no divisions, two had one division, four had two divisions, five had four divisions and the remaining 35 embryos all arrested prior to morphogenesis (probably indicating that at least one fatal division error occurred prior to that time). Therefore *let-502*(+) is required for the embryo to complete all of its cell divisions correctly but is not required for the successful completion of every cell division.

The above results suggest that *let-502* and *mel-11* have antagonistic activities during cleavage furrow ingression. We predict that mutations in *let-502* and *mel-11* should alleviate one another's cleavage defects, as previously we had reported that both *let-502*'s early cleavage defects and elongation phenotype (which are genetically separable) are suppressed by *mel-11*. At 25°C, *let-502(sb106)* had 41% hatching, *mel-11(sb55)* had 25.2% hatching and *let-502(sb106); mel-11(sb55)* had 90% hatching (Wissmann et al., 1997; Piekny et al., 2000). Indeed, combining a hypomorphic *let-502* allele, *sb106* or *sb108* (the latter displays no cleavage defects) with *mel-11(it26)* resulted in furrow ingression times near to wild-type rates, with few failed divisions (Fig. 2).

In summary, *let-502* mutants have slow furrow ingression, implying that LET-502(+) is required for contraction to proceed. In contrast, *mel-11* mutants show faster furrow ingression and ectopic furrows, suggesting that MEL-11(+) acts as a brake to contraction and prevents furrow formation at inappropriate locations. The genetic interactions between *let-502* and *mel-11* imply that they influence each other's activities in the furrow. Together, LET-502 and MEL-11 regulate the rate or force of cleavage furrow contraction, which is essential for a high fidelity of successful cell divisions.

LET-502 and MEL-11 proteins are enriched in cleavage furrows

Our evidence indicates that *let-502* and *mel-11* regulate

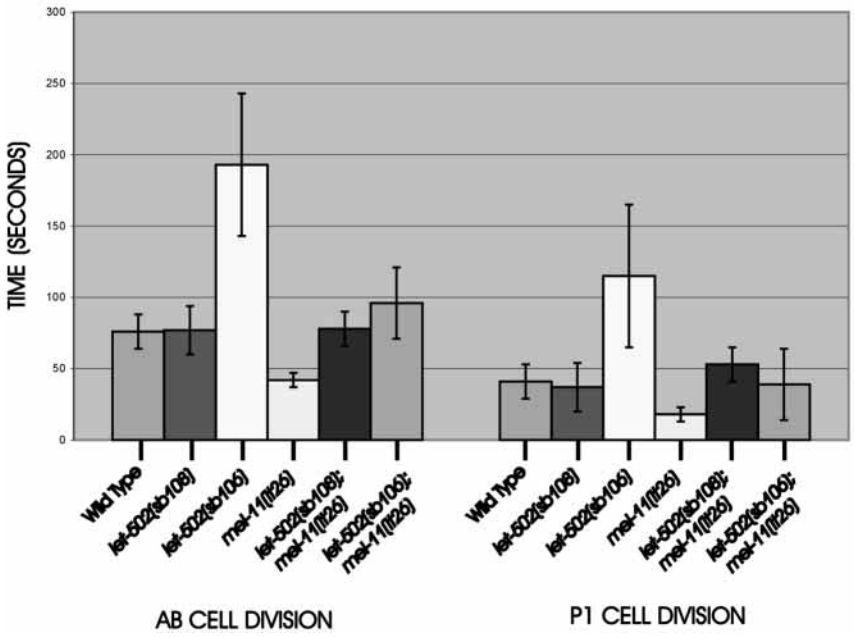

Fig. 2. Furrow ingression times for two- to four-cell stage embryos undergoing AB (left) and P1 (right) cell divisions. AB and P1 cell cleavage furrows that ingress slower in *let-502(sb106)* were normal in *let-502(sb108)* but were faster in *mel-11(it26)* embryos. *let-502(sb108* or *sb106)* and *mel-11(it26)* suppresses one another's cleavage defects in double mutants, resulting in furrow ingression times closer to wildtype. Error bars show 1 standard deviation. Failed cell divisions are not included in these data. Wildtype: *n*=7; *let-502(sb106)*: *n*=9; *let-502(sb108)*: *n*=9; *mel-11(it26)*: *n*=9; *let-502(sb108) mel-11(it26)*: *n*=10; *502(sb106) mel-11(it26)*: *n*=6.

cleavage furrow ingression. Therefore, we examined LET-502 and MEL-11 localization at furrows using immunofluorescence (IF) with antibodies raised against each protein (see the Materials and Methods). In wild-type embryos, LET-502 and MEL-11 both localized to cleavage furrows at early stages of furrow ingression and remained there during later stages of ingression (Fig. 3iA-F). Anti-MEL-11 also faintly stained the central spindle during late anaphase, but this structure was not noticeably altered in *mel-11(it26)* mutant embryos stained with anti α-tubulin (data not shown).

We examined LET-502 and MEL-11 localization in mutant embryos to verify that their molecular and genetic phenotypes are consistent and to determine whether they are co-dependent on one another for their localization. Cytoplasmic LET-502 levels were decreased but not eliminated in *let-502(sb106)* embryos, consistent with the interpretation that *let-502(sb106)* is a hypomorph (Fig. 3iG,I). In *let-502(sb106RNAi)* embryos, LET-502 levels were depleted to a greater extent than in *let-502(sb106)* embryos, and indeed LET-502 was often undetectable (see Fig. 5F, Fig. 7D below). In addition, MEL-11's intensity at furrows appeared to weaken in *let-502(sb106)* embryos (Fig. 3iH). Although MEL-11 staining was severely depleted in *mel-11(it26)* embryos raised at the non-permissive temperature, LET-502 localization was unaffected (Fig. 3iJ-L). Therefore, LET-502 is not dependent on MEL-11 for its localization to cleavage furrows, but MEL-11 may be partially dependent on LET-502.

We used deconvolution microscopy to determine the extent of overlap between LET-502 and MEL-11 at cleavage furrows. Initially we used wild-type embryos (Fig. 3iiA-C); however, high cytoplasmic LET-502 often obscured our visualization of LET-502 at the cleavage furrow. Using the hypomorph *let-502(sb106)*, which has decreased LET-502 in the cytoplasm (see above), LET-502 clearly overlapped with MEL-11 at the furrow (Fig. 3iiD-F). Embryos from hermaphrodites heterozygous for the dominant-negative mutation *let-502(ca201)* gave similar patterns of LET-502 and MEL-11 colocalization (Fig. 3iiG-I). Kosako et al. found similar effects in cell culture experiments where the presence of ROK inhibitors decreased levels of ROK in the cytoplasm, more clearly revealing its presence at the furrow (Kosako et al., 2000).

We conclude that LET-502 and MEL-11 colocalize to ingressing cleavage furrows, implying that LET-502 and MEL-11 together regulate cleavage furrow ingression.

LET-502 and MEL-11 are not required to form cleavage furrows

The *let-502* and *mel-11* phenotypes suggest that they regulate the rate of cleavage furrow ingression and are not involved in furrow formation. LET-502 colocalized with NMY-2 myosin thick filaments at the cleavage furrow (Fig. 4A-C), implying that LET-502 (and by implication MEL-11) is associated with the contractile ring. [We were not able to examine the localization of LET-502 and MEL-11 with actin because of incompatible fixation requirements (see Materials and Methods)]. LET-502's association with the thick filaments of the contractile ring is consistent with LET-502 targeting either rMLC or MEL-11 (and MEL-11 targeting rMLC) to regulate its activity during furrow contraction.

rho-1(RNAi) leads to failure of furrow formation in *C. elegans* embryos (Jantsch-Plunger et al., 2000). Since LET-502/ROK is a Rho effector, we determined whether LET-502 and MEL-11 also are involved in the initial formation of the actomyosin ring. NMY-2 and actin retained their localization at the site of furrow formation in all *let-502(sb106)* (*n*=16) and *let-502(sb106RNAi)* (*n*=16) embryos examined at the one, two and four cell stages (Fig. 4D-I), despite the fact that 33% of *let-502(sb106)* embryos have visible cleavage defects at these times. NMY-2 and actin similarly retained their location in *mel-11(it26)* embryos (Fig. 4J-O; *n*=29). Therefore, *let-502* and *mel-11* probably are not required to properly localize components of the cleavage furrow to its site of formation. Interestingly, NMY-2 localized to the mitotic spindle during metaphase but disappeared as the cell cycle progressed (Fig. 4G,I).

In summary, LET-502 colocalizes with NMY-2 in the contractile ring, but LET-502 and MEL-11 are not required for initial furrow formation and probably are recruited to the furrow after the actomyosin ring is formed.

LET-502 and MEL-11 regulate cleavage furrow contraction by altering the levels of active rMLC

The *let-502* and *mel-11* phenotypes imply that they regulate the

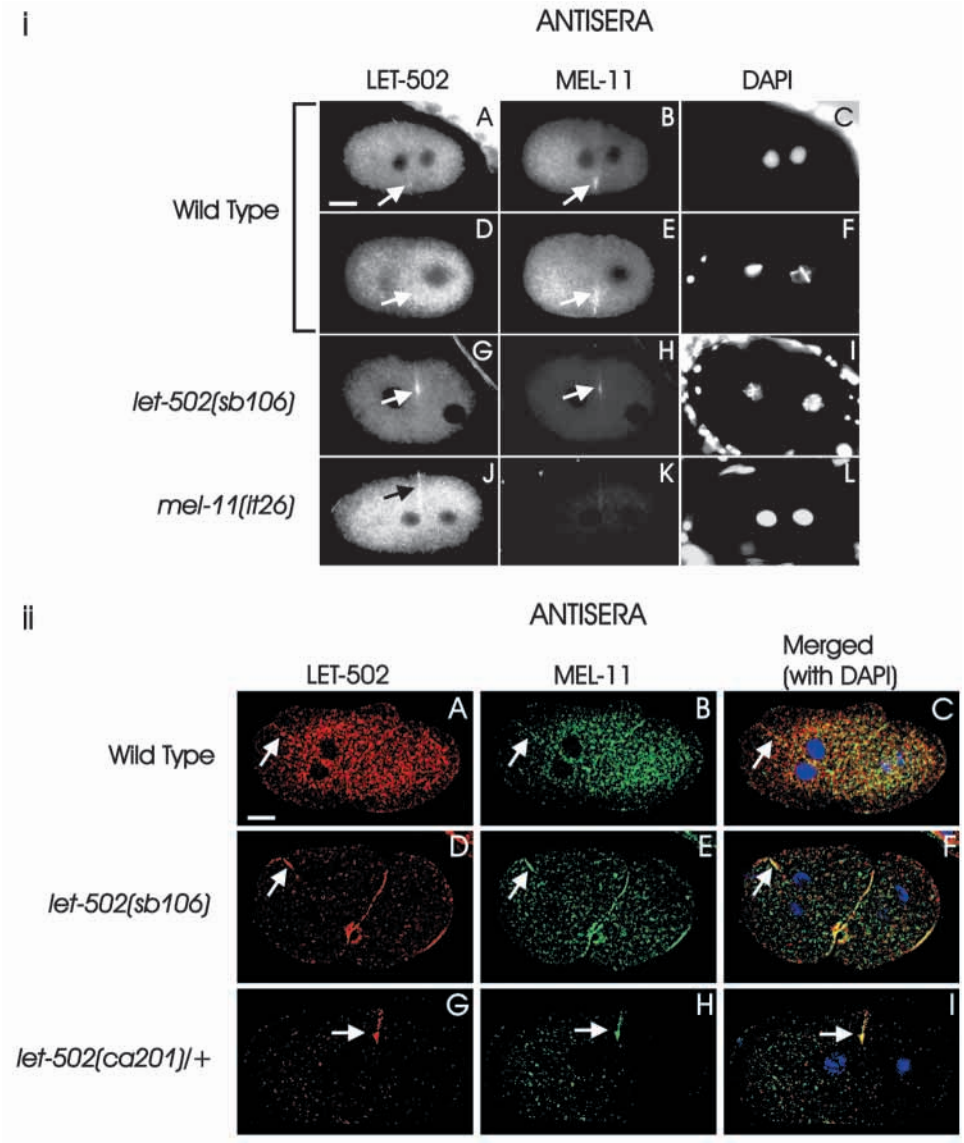

Fig. 3. LET-502 and MEL-11 localize at cleavage furrows. (i) IF of LET-502, MEL-11 and DAPI in wild-type, *let-502(sb106)* or *mel-11(it26)* embryos. (A-C) Arrows indicate that both LET-502 and MEL-11 are enriched at the furrow during early stages of wild-type cleavage and are found throughout the furrow as it ingresses (D-F). They remain at cell boundaries after cell divisions are completed. (G,I) Cytoplasmic LET-502 is decreased in *let-502(sb106)* mutant embryos. (H,I) MEL-11 staining is weakened in *let-502(sb106)* in comparison with wild-type embryos and is almost entirely depleted in *mel-11(it26)* embryos (K,L); however, LET-502 retained its location (J,L) in *mel-11* mutants. Bar, 9 μm. (A-F) n=21, (G,I) n=52, (H,I) n=40, (K,L) n=15, (J,L) n=38. (ii) LET-502 and MEL-11 localization in wild-type and *let-502(sb106)* embryos using digital deconvolution microscopy. Arrows indicate the ingressing cleavage furrows. (A-C) LET-502 and MEL-11 overlap at the ingressing furrow in wild-type, *let-502(sb106)* (D-F) and embryos from *let-502(ca201)/+* hermaphrodites (G-I). Bar, 6.5 μm. (A-C) n=7, (D-F) n=4 and (G-I) n=4.

rate of cleavage furrow contraction, most probably by regulating rMLC, which is activated by phosphorylation of serine 19 in higher eukaryotes (which corresponds to serine 18 in *C. elegans*). We found that an antibody raised against rMLC phosphoserine 19 in chickens (M. Walsh, University of Calgary, personal communication) specifically crossreacted with MLC-4 in *C. elegans*. Using this reagent, we observed that high amounts of rMLC phosphoserine 19/18 were detectable in the cytoplasm and the cortex and in the cleavage furrow of wild-type embryos, and rMLC phosphoserine 19/18 colocalized with LET-502 at all locations (Fig. 5A). Furrow and cytoplasmic rMLC phosphoserine 19/18 levels were elevated in *mel-11(it26)* mutant embryos (Fig. 5B), whereas these levels were greatly decreased in *let-502(sb106)* mutant embryos (Fig. 5C). The antisera were specific for the rMLC encoded by *mlc-4* (Shelton et al., 1999), which is required for early *C. elegans* cleavages, since rMLC phosphoserine 19/18 was either non-detectable (Fig. 5D) or present a very low levels (Fig. 5E) in *mlc-4(RNAi)* embryos. In addition, the antisera recognized a single band at the appropriate molecular weight on western blots (data not shown). rMLC phosphoserine 19/18 levels were restored close to wild-type levels in *let-502(sb106RNAi) mel-11(it26)* embryos (Fig. 5F).

In summary, LET-502 appears to positively regulate contraction either by direct phosphorylation of MLC-4 or indirectly by inhibiting MLC-4 dephosphorylation by MEL-11. Probably, there is a threshold level of active rMLC required for successful cytokinesis, and cells that lack this level fail to undergo cleavage. The double mutants restored sufficient amounts of active rMLC in the furrow for successful cleavages to occur. This suggests that another kinase, other than LET-502/ROK, phosphorylates rMLC in these circumstances.

Where do LET-502 and MEL-11 fit into the cytokinetic pathway?

Our evidence suggests that LET-502 and MEL-11 regulate contraction of the actomyosin ring after furrow formation but prior to the end of cleavage furrow ingression. We performed molecular and genetic epistasis experiments and placed LET-502 and MEL-11 in a pathway with respect to other proteins known to be involved in furrow progression. We tested genes

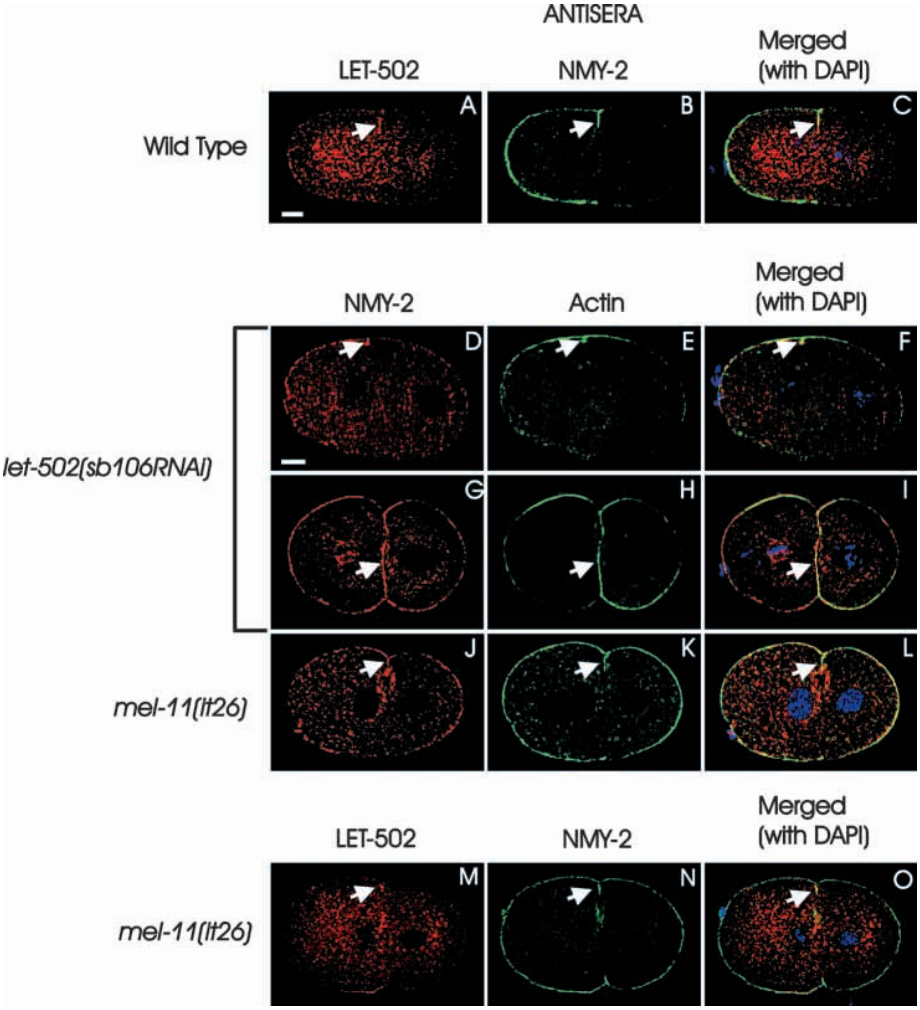

Fig. 4. Localization of LET-502 with myosin (NMY-2) in wild-type embryos, and localization of NMY-2 and actin in *let-502* and *mel-11* mutant embryos using digital deconvolution microscopy. Arrows indicate the ingressing or newly completed cleavage furrows. (A-C) LET-502 and NMY-2 colocalize at the ingressing furrow during cleavage. (D-I) NMY-2 and actin still accumulate and colocalize to the putative site of furrow formation in all of the *let-502(sb106)* and *let-502(sb106RNAi)* embryos examined. Note that NMY-2 also localizes to the spindle during metaphase (G). NMY-2 and actin (J-L) and LET-502 and NMY-2 (M-O) retain their localization at the furrow in a *mel-11(it26)* mutant embryo during cleavage. (A-C) Bar, 5 μm. n=3 by deconvolution and n=13 by IF. (D-I) Bar, 7 μm. n=11 *let-502(sb106RNAi)* stained for both NMY-2 and actin by deconvolution and n=5 *let-502(sb106RNAi)* embryos stained for NMY-2 only by IF. (J-L) n=4 stained for both NMY-2 and actin by deconvolution, n=23 stained for NMY-2 only by IF and n=2 stained for actin only by IF. (M-O) n=2 by deconvolution and n=23 by IF.

encoding MLC-4 (rMLC), early and late CYK-1 (formin) activity and CYK-4 [Rho GTPase activating protein (GAP)]. Early CYK-1 is required for actin polymerization and the earliest stages of furrow formation (A. Severson and B. Bowerman, University of Oregon, Eugene, personal communication). Actin polymerization then is followed by or occurs in conjunction with the incorporation of NMY-2 and MLC-4 to form an actomyosin ring prior to furrow ingression (Shelton et al., 1999). Late CYK-1 and CYK-4 are required for the final stages of furrow ingression and/or termination (Swan et al., 1998; Jantsch-Plunger et al., 2000).

We examined LET-502 and MEL-11 localization in embryos defective for early *cyk-1* function using embryos from *cyk-1* heteroallelic hermaphrodites carrying a weak allele (showing late cytokinetic defects on its own) and a strong (sterile) allele (A. Severson and B. Bowerman, University of Oregon, Eugene, personal communication). LET-502 and MEL-11 failed to localize in this genetic background (Fig. 6A-C). Therefore, since early CYK-1 activity is involved in actin polymerization and contractile ring formation, we conclude that LET-502 and MEL-11 require a properly formed contractile ring for their localization.

As described earlier, MLC-4 is probably a substrate for MEL-11 and/or LET-502 (Shelton et al., 1999; Piekny et al., 2000), and indeed LET-502 and MEL-11 failed to localize in *mlc-4(RNAi)* embryos (Fig. 6D-F). It is likely that LET-502 and MEL-11 are recruited to the furrow to act on MLC-4 as a target. Consistent with both *let-502* and *mlc-4* acting during cytokinesis, decreased

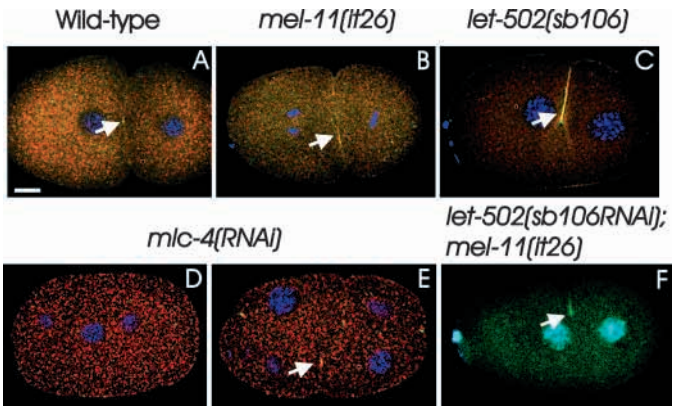

Fig. 5. Localization of active rMLC and LET-502 in embryos using digital deconvolution microscopy. All images shown are merged with rMLC posphoserine 19/18 in green, LET-502 in red and DAPI in blue, and arrows point to ingressing cleavage furrows. (A) rMLC phosphoserine 19/18 and LET-502 colocalize in wild-type embryos. (B-C) The intensity of rMLC posphoserine 19/18 decreases in *let-502(sb106)* embryos and increases in *mel-11(it26)* embryos in comparison with wild-type embryos. (D,E) rMLC phosphoserine 19/18 is not detectable or is present at low levels in *mlc-4(RNAi)* embryos. (F) rMLC phosphoserine 19/18 levels are restored in *let-502(sb106RNAi); mel-11(it26)* embryos. Bar, 6 μm. (A) n=4, (B) n=4, (C) n=2, (D) n=1, (E) n=1 and (F) n=6.

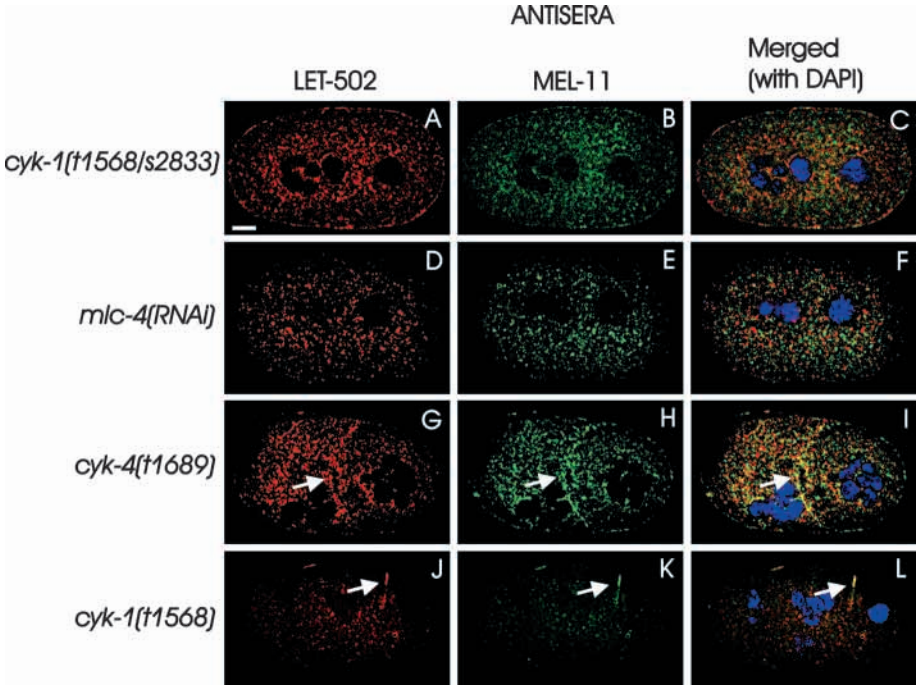

Fig. 6. LET-502 and MEL-11 require MLC-4 and early CYK-1 function for their localization but do not require late CYK-1 function or CYK-4. (A-C) LET-502 and MEL-11 no longer localize to the site of cleavage furrow formation in *cyk-1(s2833/t1568)* embryos, which show the early *cyk-1* phenotype, or in *mlc-4(RNAi)* embryos (D-F). (G-I) LET-502 and MEL-11 retain their localization in *cyk-4(t1689)* embryos. (J-L) LET-502 and MEL-11 retain their localization in *cyk-1(t1568)* embryos, which display the late *cyk-1* phenotype. *cyk-1(t1611)* embryos gave similar results (data not shown). Bar, 6 μm. (A-C) n=5 by IF, (D-F) n=10 by IF, (G-I) n=7 by deconvolution and n=6 by IF and (J-L) n=8 by deconvolution.

activity of these genes enhance (exacerbate) one another's defects. Weak *mlc-4(RNAi)* (using lower concentrations of dsRNA) results in 43% hatching, while in *let-502(sb106)* embryos show ~85% hatching at 20°C. If there were no genetic interactions between *let-502* and *mlc-4*, the predicted hatching rate would be ~37%, but the observed value was 13%, implying that the genes are genetically influencing the same process.

CYK-4 is a Rho GAP required for a late step in cytokinesis (Jantsch-Plunger et al., 2000). We examined LET-502 and MEL-11 localization in *cyk-4* mutant embryos, which form furrows that ingress substantially but then regress (Jantsch-Plunger et al., 2000). LET-502 and MEL-11 retained their location in *cyk-4* mutant embryos, implying that LET-502 and MEL-11 are not dependent on CYK-4 (Fig. 6G-I). However, *let-502* and *cyk-4* do interact genetically: at 15°C, *let-502(sb106* or *sb108) cyk-4(t1689)* animals were sterile owing to germline proliferation defects (see next section). *cyk-4* also was epistatic to *mel-11*, with embryos displaying the *cyk-4* phenotype (arresting at the one cell stage).

CYK-1's late role in cytokinesis may be to stabilize ingressed cleavage furrows until they can be disassembled, since weak *cyk-1* mutations result in embryos that form cleavage furrows that ingress substantially but then regress (Swan et al., 1998). LET-502 and MEL-11 distributions were not altered in weak

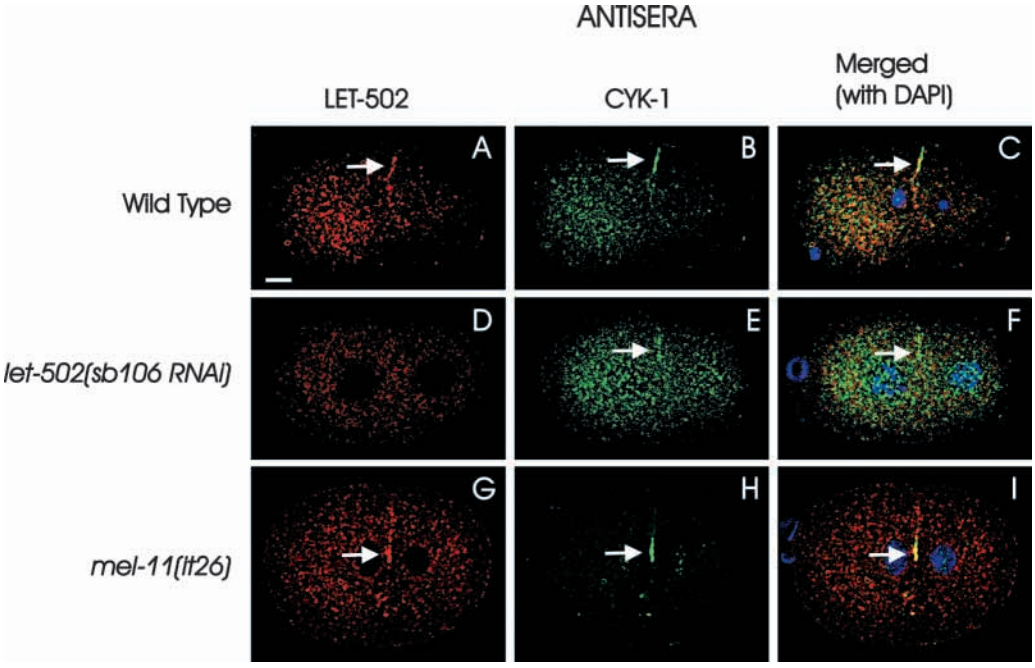

Fig. 7. CYK-1 is dependent on LET-502 but not on MEL-11 for furrow localization. (A-C) CYK-1 colocalized with LET-502 in wild-type embryos, but its localization was partially disrupted in *let-502(sb106RNAi)* embryos (D-F). (G-I) CYK-1 retained its localization in *mel-11(it26)* embryos. Bar, 6 μm. (A-C) n=2 by deconvolution and n=4 by IF, (D-F) n=3 by deconvolution and n=2 by IF and (G-I) n=1 by deconvolution.

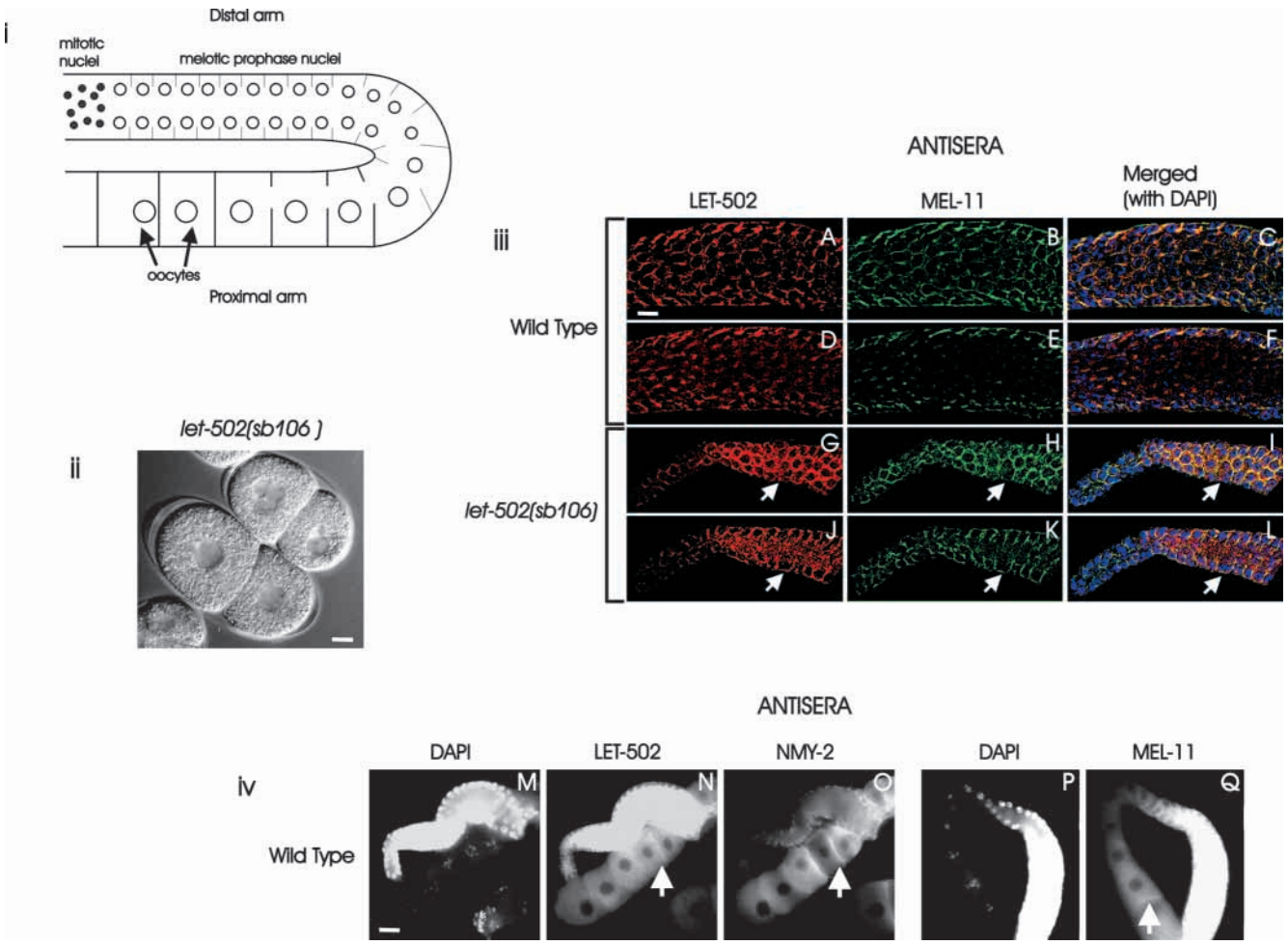

Fig. 8. *let-502* affects oocyte cellularization, and LET-502 and MEL-11 localize to gonad membranes. (i) A schematic outlines the structure of a gonad arm from an adult hermaphrodite. (ii) Nomarski photograph of two embryos of very different sizes collected from a *let-502(sb106)* hermaphrodite. Compared with wildtype, which has a very narrow size distribution, *let-502* embryos vary substantially above and below the mean (data not shown). Bar, 7.5 μm. (iii) The distal portion of the arm, consisting of a syncitium of nuclei in meiotic prophase, was immunostained for deconvolution. Partitions outline each nucleus (as observed from *z* sections taken superficially near the surface of the gonad arm in A-C and G-I), but these partitions do not extend into the central core (as observed by more central *z* sections D-F and J-L) where the nuclei are connected by a common cytoplasm. (A-F) LET-502 and MEL-11 colocalize at the membrane that surrounds the syncitial nuclei in wild-type gonads. (A-C) is a superficial *z* section and (D-F) is a *z* section from near the centre of the arm. (G-L) Gonads from *let-502(sb106)* hermaphrodites are thinner compared with a corresponding region of the wild-type gonad and are sometimes multinucleate, as indicated by the arrow (G-I superficial view versus J-L central view). Bar, 10 μm. (iv) (M-Q) LET-502, NMY-2 and MEL-11 localize to oocyte boundaries after cellularization. Bar, 25 μm. (A-F) n=4, (M-O) n=6 stained for both LET-502 and NMY-2 and n=23 stained for LET-502 only, (P-Q) n=8.

cyk-1 mutant embryos, suggesting that LET-502 and MEL-11 are not dependent on late CYK-1 function (Fig. 6J-L). A CYK-1 antibody was used for the reciprocal experiment to determine whether LET-502 and MEL-11 are required to localize CYK-1 for its late role. CYK-1 colocalized with LET-502 in wild-type embryos (Fig. 7A-C), and it was still present at furrows in *let-502(sb106RNAi)* embryos, although at less intense levels than in wild-type embryos (Fig. 7D-F). Therefore, CYK-1's late role in cytokinesis may be partly dependent on LET-502. Genetically, *cyk-1* and *let-502* mutants strongly enhanced one another: at 20°C *let-502(sb106* or *sb108) cyk-1(t1568* or *t1611* or *or36)* animals were sterile owing to germline proliferation defects (see next section). CYK-1's location at furrows was not altered in *mel-11(it26)* embryos (Fig. 7G-I), but *cyk-1* also was epistatic to *mel-11*, with embryos displaying the *cyk-1* phenotype (arresting at the one cell stage). Therefore, CYK-1 is not dependent on MEL-11, consistent with 'normal', albeit rapid, furrow ingression in *mel-11*.

Function of *let-502* in oocyte cellularization

The *C. elegans* hermaphrodite gonad contains mitotically proliferating nuclei within a syncytium (Fig. 8i). These nuclei progress through meiosis and become fully cellularized into oocytes as they move through the gonad in a distal to proximal direction (relative to the vulva) (Kimble and Ward, 1988). Membranes invaginate into the cytoplasm around each nucleus in the distal mitotic region but do not fully enclose the nuclei. The membranes eventually pinch off to cellularize nuclei into separate oocytes, which may occur by a mechanism similar to cytokinesis.

Genes that are required for cytokinesis, such as *mlc-4* and *cyk-1*, also affect oocyte formation within the hermaphrodite gonad,

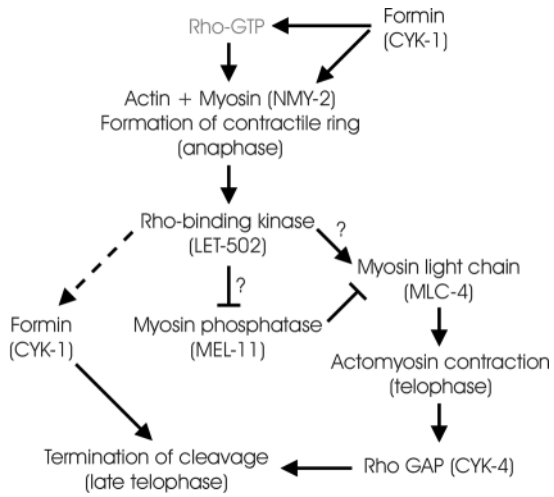

Fig. 9. Pathway showing how *let-502* and *mel-11* act with respect to other genes to regulate cytokinesis. During anaphase, structural components of the furrow begin to associate into a contractile bundle, including NMY-2, MLC-4 and actin. Cleavage furrow contraction is regulated by MLC-4 phosphorylation. LET-502 and MEL-11 localize to the furrow and together regulate the rate of its contraction. MEL-11 would prevent cleavage furrow contraction from occurring until the proper signal (Rho-GTP) is produced to increase LET-502's activity. Active LET-502 causes the downregulation of MEL-11 and/or the direct activation of MLC-4, allowing the actomyosin ring to contract. After the ring contracts, proteins such as CYK-4 and late CYK-1 regulate furrow termination.

and mutations result in varying levels of sterility (Swan et al., 1998; Shelton et al., 1999) (A. Severson and B. Bowerman, University of Oregon, Eugene, personal communication). This effect raises the question as to whether *let-502* regulates oocyte cellularization, which would be similar to its role in cytokinesis. *let-502(sb106)* hermaphrodites laid eggs of noticeably variable sizes in comparison to the uniform range seen in wildtypes. Some embryos (although not obviously polyploid) were more than twice the normal length, whereas others were smaller than the wildtype (Fig. 8ii). This variation was also reflected in the size of oocytes within the gonad where the most mature oocyte was not always the largest (as is seen in wildtype), indicating that the membranes are probably not forming at proper distances from one another (data not shown). Some oocytes were multinuclear, indicating a failure to cellularize properly. Embryo sizes were normal in *mel-11* mutants, and *mel-11* mutants suppressed the size defect of *let-502* mutants (data not shown).

LET-502's role in cellularization is consistent with its localization in the gonad, where LET-502 and MEL-11 colocalized at the membrane invaginations surrounding each nucleus within the mitotically dividing syncitium (Fig. 8iiiA-F). The membrane invaginations still formed in *let-502(sb106)* gonad arms; however, the gonad arms were much thinner, and some of the membrane invaginations appeared to be disrupted, with some surrounding multiple nuclei (Fig. 8iiiG-L). In addition, LET-502 localized to oocyte cell boundaries as they became cellularized in a pattern similar to the MEL-11 and NMY-2 distribution (Fig. 8ivM-Q). rMLC phosphoserine 19/18 also localized to oocyte cell boundaries (data not shown). Therefore, LET-502's expression pattern within the gonad combined with its phenotype is consistent with *let-502* being involved in oocyte cellularization. Despite the lack of an obvious gonad phenotype in *mel-11* mutants, MEL-11 was present at cell boundaries, suggesting that *mel-11* may also play a non-essential in oocyte formation.

LET-502 and MEL-11 are not required for early embryonic polarity

Rho GTPases (Rho and Cdc42) and non-muscle myosin (NMY-2 and MLC-4) are also involved in cell polarity (Guo and Kemphues, 1996; Shelton et al., 1999; Prokopenko et al., 2000; Kay and Hunter, 2001). However, *let-502* and *mel-11* mutant embryos did not appear to have any polarity defects, that is, the asymmetrical events of pronuclear fusion, placement of the mitotic spindle and segregation of P granules all were normal in their cellular position, structure and timing (A.P., unpublished).

Discussion

The function of ROK during cytokinesis has been investigated in vitro; however, its precise role is not clear. One report suggested that ROK is required late, for cell separation via targeting GFAP for disassembly (Yasui et al., 1998), while another indicated that ROK regulated cleavage furrow contraction by altering myosin activity (Kosako et al., 2000). These experiments employed dominant-negative mutations or drugs that could have unknown effects on other proteins. In addition, no experiments have examined the function of MYPT in cytokinesis. We show here, through loss-of-function studies of the endogenous genes, that *C. elegans* ROK (*let-502*) and MYPT (*mel-11*) together regulate cleavage furrow contraction during cytokinesis. This assertion is supported by the *let-502* and *mel-11* mutant phenotypes coupled with altered levels of phosphorylated (i.e. active) rMLC within these mutants: *let-502* mutants have slow or failed furrow ingression and lower levels of phosphorylated rMLC, whereas *mel-11* mutants have faster furrow contraction and higher levels of phosphorylated rMLC. Simultaneous loss of *let-502* and *mel-11* restores normal cleavage and close to wild-type levels of phosphorylated rMLC. This indicates that *let-502* and *mel-11* function antagonistically and probably redundantly with another pathway to regulate furrow contraction, and a kinase other than ROK/LET-502 can phosphorylate rMLC. *let-502* and *mel-11* probably are not required for cell separation since in *let-502* and in *let-502; mel-11* double mutant embryos, cells that successfully complete cleavage appear to separate as in wild-type embryos.

To define further the roles of *let-502* and *mel-11* during furrow contraction, we examined the molecular epistasis between *let-502, mel-11* and other gene products known to regulate cytokinesis in *C. elegans.* We propose that *let-502* and *mel-11* function downstream of actin, myosin and early formin (CYK-1) activity to regulate cytokinesis (Fig. 9). Actin and myosin (NMY-2) retain their localization at furrows in the absence of LET-502 and MEL-11, and LET-502 and MEL-11 localize to furrows only after ingression is initiated. In addition, LET-502 and MEL-11 no longer localize in embryos mutant for early *cyk-1* function. CYK-1/formin may regulate actin polymerization and functions early during contractile ring assembly (A. Severson and B. Bowerman, University of Oregon, Eugene, personal communication). Actin and NMY-

2 are then incorporated into the contractile ring, followed by recruitment of LET-502 and MEL-11.

The likely target of LET-502/ROK and MEL-11/MYPT is MLC-4/rMLC, whose phosphorylation triggers contraction in smooth muscle and stress fibre formation (Amano et al., 1996; Kimura et al., 1996; Kawano et al., 1999; Somlyo and Somlyo, 2000; Sward et al., 2000). In higher eukaryotes, ROK induces contraction by either activating rMLC by direct phosphorylation (Amano et al., 1996) (see also Sward et al., 2000) or indirectly by downregulating MYPT, which itself inhibits rMLC by dephosphorylation (Kimura et al., 1996; Kawano et al., 1999). Similarly, our previous genetic studies showed that MLC-4 is probably a target of LET-502 and MEL-11 during morphogenesis, the contractile event that drives elongation (Piekny et al., 2000). In this report we have shown that levels of phosphorylated (active) rMLC are dependent on LET-502 and MEL-11 and that LET-502 and MEL-11 localizations are disrupted when MLC-4 is removed. Therefore, we propose that LET-502 and MEL-11 regulate cytokinesis in a manner similar to their homologues in smooth muscle contraction and stress fibre formation in higher eukaryotes. LET-502 and MEL-11 regulate MLC-4/rMLC activity to control contraction. The balance of LET-502 and MEL-11 is crucial; when this balance is disrupted, as in the single mutants, cleavage fails to occur properly.

Furrow ingression is terminated through the downregulation of Rho by CYK-4/Rho GAP when the contractile ring contacts the central spindle (Jantsch-Plunger et al., 2000). Consistent with this, LET-502 and MEL-11 retain their localization in *cyk-4* mutant embryos, which have furrows that ingress extensively but then regress. Therefore, CYK-4 may function after LET-502. CYK-1/formin also functions late in cleavage, possibly by regulating the stability of the ingressed furrow until it can be disassembled (Swan et al., 1998). LET-502 and MEL-11 are still localized to furrows in embryos mutant for late *cyk-1* function. LET-502 could be involved in regulating CYK-1's localization for its late function, as CYK-1 localization is partially disrupted in *let-502* mutant embryos.

A caveat in our work is that the mutations and RNAi may not completely eliminate LET-502 activity in early embryos. LET-502 is involved in oocyte cellularization, and completely depleting LET-502 might prevent proper oocyte partitioning, precluding the examination the role of LET-502 during the subsequent mitotic cleavages. Therefore, low levels of LET-502 sufficient for oocyte formation could be present in the embryos and also may be responsible for the cleavage furrows we sometimes observed in those embryos. However, we favour the possibility that residual oocyte formation and the sometimes normal mitotic cleavages may reflect the existence of a parallel pathway that can regulate cleavage furrow contraction in the absence of *let-502*.

In both cytokinesis and morphogenesis, simultaneous loss of *mel-11* and *let-502* resembles wildtype (Wissmann et al., 1997; Wissmann et al., 1999; Piekny et al., 2000). This suggests that redundant pathways regulate contraction in the absence of both *let-502* and *mel-11*. During elongation, this redundant pathway includes *fem-2* [PP2c phosphatase (Pilgrim et al., 1995; Chin-Sang and Spence, 1996; Piekny et al., 2000)]; however, genetic experiments indicate that *fem-2* is not involved in the redundant pathway during cytokinesis (P.M., unpublished). In higher eukaryotes, the ROK-related kinase, citron-k, is involved in cytokinesis (Madaule et al., 1998). The *C. elegans* citron-like genes (F59A6.5 and W02B8.2) do not encode kinase domains, and RNAi to either gene in wild-type or *let-502(sb106)* worms has no effect on cytokinesis (A.P., unpublished). Future work will be needed to uncover the elements of the cytokinetic pathway acting in parallel to *let-502* and *mel-11*.

C. elegans ROK (LET-502) and MYPT (MEL-11) are used repeatedly for different contractile events in the worm. They regulate morphogenesis, the process whereby actin-mediated contractions within epidermal cells cause the cells to change shape to drive elongation of the embryo (Priess and Hirsh, 1986; Wissmann et al., 1997; Piekny et al., 2000). In *let-502* mutants, the epidermal cells fail to contract, whereas in *mel-11* mutants, the epidermal cells hypercontract. *let-502* and *mel-11* mutants suppress one another's defects, implying that they function together (and antagonistically) to regulate the cell shape changes (Wissmann et al., 1997; Piekny et al., 2000). *let-502* and *mel-11* also regulate contraction of the spermatheca, an organ used for oocyte fertilization (Wissmann et al., 1999), and weak alleles of either gene cause sterility owing to failed oocyte fertilization. However, unlike elongation, *let-502* and *mel-11* mutants are not able to suppress one another's sterility and indeed are expressed in different regions of the spermatheca (Wissmann et al., 1999). In the present work, we describe the role of *let-502* and *mel-11* in another contractile event, cytokinesis. As in morphogenesis, *let-502* and *mel-11* function together to regulate cleavage furrow ingression. However, genes involved in cytokinesis, such as *cyk-1, nmy-2* and *cyk-4* have no apparent role in morphogenesis (Swan et al., 1998; Guo and Kemphues, 1996; Janstch-Plunger et al., 2000), whereas genes involved in morphogenesis, such as *fem-2, unc-73* and *mig-2* do not appear to function in cytokinesis (Pilgrim et al., 1995; Steven et al., 1998; Zipkin et al., 1997; Wissmann et al., 1999; Piekny et al., 2000) (P.M., unpublished). Therefore, *let-502* and *mel-11* act in concert with different genes during elongation and cytokinesis, suggesting that the 'contractile cassette' functions with different genes in different tissues.

We thank B. Bowerman and A. Severson for the *cyk-1* strains, CYK-1 antibody, *mlc-4* cDNA and for communicating their unpublished results and helpful discussions. We thank K. Kemphues for the NMY-2 antibody, M. Walsh for the rMLC phosphoserine-19 antibody and S. Strome for the OIC1D4 P-granule antibody. We thank members of the McGhee and Mains laboratories and M. Walsh for discussion. This work was supported by grants from the Canadian Institutes of Health Research and the Alberta Heritage Foundation for Medical Research to P.E.M. We received nematode strains from T. Steirnagle at the *Caenorhabditis* Genetics Center, funded by the National Institutes of Health Center for Research Resources.

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