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NMY-2 maintains cellular asymmetry and cell boundaries, and promotes a SRC-dependent asymmetric cell division

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ABSTRACT

The nonmuscle myosin II NMY-2 is required for cytokinesis as well as for the establishment of zygote asymmetry during embryogenesis in *Caenorhabditis elegans*. Here we describe two conditional *nmy-2* alleles that rapidly and reversibly inactivate the protein. We show that NMY-2 has late-cell-cycle roles in maintaining embryonic asymmetries and is also required for a surprisingly late step in the maintenance of the cytokinesis furrow. Finally, during a signaling-induced asymmetric cell division, NMY-2 is required for SRC-dependent phosphotyrosine signaling and acts in parallel with WNT-signaling to specify endoderm.

Introduction

Cells utilize numerous mechanisms to establish and maintain asymmetries. For example, during *Caenorhabditis elegans* embryogenesis, the initially symmetrical oocyte becomes polarized by sperm entry and reorganizes its cytoplasm so that cell division generates daughter cells that differ in size, cytoplasmic content and cell fate. Later, at the 4-cell stage, WNT-and SRC-mediated signaling induces an endoderm precursor to align its division axis along the polarized axis of the cell and then divide asymmetrically (for a review, see Bowerman and Shelton, 1999).

The initial establishment of polarity in a worm zygote involves actomyosin-mediated events such as cortical ruffling and cytoplasmic streaming (Hill and Strome, 1988; Hird and White, 1993). These actomyosin movements cause the PAR proteins to locate asymmetrically, which further directs the asymmetrical localization of other cell fate determinants and the mitotic apparatus (Munro et al., 2004). The non-muscle myosin, NMY-2, plays an important role in this dynamic process (Munro et al., 2004; Guo and Kemphues, 1996). Zygotes depleted of NMY-2 by RNAi fail to exhibit cytoplasmic and cortical contractions, and ultimately fail to establish nearly all of the asymmetries normally observed in pre-divisional one-cell embryos (Guo and Kemphues, 1996; Cuenca et al., 2003).

While these previous studies had implicated NMY-2 in the establishment of asymmetries in the one-cell embryo, NMY-2 is also essential for driving the cleavage furrow during cytokinesis (Guo and Kemphues, 1996; Cuenca et al., 2003). Thus it was not possible to use irreversible gene-inactivation methods such as RNAi to ask how NMY-2 regulates cellular processes beyond the zygote stage. To address this question we sought conditional alleles of *nmy-2* within a collection of temperature-sensitive embryonic lethal strains (Pang et al., 2004; Nakamura et al., 2005). Here we describe the analysis of two such alleles that result in rapid and reversible inactivation of the NMY-2 protein upon temperature shift. Using these alleles we show that NMY-2 is required not only for the establishment of polarity, but also for its maintenance, and that NMY-2 also appears to play an important role in sustaining the cell boundary. These alleles have also allowed us to examine the consequences of reducing NMY-2 function specifically during the 4-cell stage, when WNT- and SRC-signaling are required to polarize the endoderm precursor cell, EMS. Both conditional *nmy-2* mutants enhance the endoderm defects of WNT-pathway mutants, but not of SRC-pathway mutants. In addition, the SRC-dependent accumulation of phosphotyrosine at the P2/EMS cell junction is defective in *nmy-2* mutants. These findings provide new insights into NMY-2 functions in the maintenance of cellular asymmetries and cell boundaries, and implicate this conserved motor protein in a signaling-induced asymmetric cell division.

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Materials and methods

Genetics

C. elegans strains were cultured as described (Brenner, 1974). Bristol N2 was used as the wildtype strain. *ne1490* and *ne3409* were both recessive alleles and were isolated in the Hawaiian (CB4856) background in a temperature-sensitive embryonic lethal screen (Pang et al., 2004; Nakamura et al., 2005) and were outcrossed more than 6 times to be RNAi-sensitive. The TH120 (GFP::PAR-2; mCherry::PAR-6) worm strain was a kind gift from Drs. Carsten Hoege and Tony Hyman (Schonegg et al., 2007). The MG170 (*zen-4(or153)*; xsEx6[ZEN-4::GFP]) strain was obtained from the *Caenorhabditis* Genetics Center.

Temperature shift

The temperature shift/microscopy experiments were conducted on two compound microscopes located in two adjacent rooms. One room was maintained at a temperature of 15 °C, and the other at 25 °C. It took approximately 10 s to travel between rooms and less than 1 min for the sample to change temperatures between 15 °C and 25 °C on pre-equilibrated aluminum surfaces.

RNA interference (RNAi)

RNAi was performed either by injection of dsRNA into hermaphrodites (Fire et al., 1998) or by feeding with bacteria expressing dsRNA (Timmons et al., 2001). Full-length *src-1* cDNA was cut out of the yeast 2-hybrid vector *src-1* pACT2 and inserted into the vector L4440 within the multiple cloning site using the enzyme sites *Nco*I and *Xho*I. Approximately 1.5 KB (containing the ATG and 5′UTR) of *mom-5* was inserted into the vector L4440 at the *Xho*I and *Bgl*II enzyme sites. The *Escherichia coli* bacterial strain HT115 was transformed with each plasmid and either fed to worms as previously described (Timmons et al., 2001) or dsRNA was generated using the T7 MEGAscript high yield transcription kit from Ambion (Austin, TX, USA) and injected into worms.

Microscopy and blastomere isolation

Light microscopy, immunofluorescence microscopy, and blastomere isolation were described previously (Rocheleau et al., 1997; Bei et al., 2002). Immunostaining of phosphotyrosine (PY99) and phospho-SRC (PY416, #2101 Cell Signaling, Santa Cruz, CA, USA, used at 1/50) was performed as described (Bei et al., 2002). The ZEN-4 antibody is a kind gift from Dr. Jeff Hardin and the immunostaining was performed as described (Raich et al., 1998).

Results

Point mutations in the S2 region of NMY-2 cause temperature-sensitive (ts) loss-of-function phenotypes

We identified two recessive *nmy-2* alleles in our collection of temperature-sensitive embryonic-lethal mutants, *nmy-2(ne3409)* and *nmy-2(ne1490)* (Pang et al., 2004; Nakamura et al., 2005). At the permissive temperature of 15 °C, both of these mutant strains are fully fertile and viable with brood sizes and hatching rates comparable to wildtype (Supplemental table). However, when shifted to the restrictive temperature of 25 °C at the larval L4 stage, they produce arrested embryos containing only one or a few multinucleated cells. Among the zygotes assayed at 25 °C ($n>100$), 100% failed cytokinesis at the first mitotic division. These embryos then continued to cycle through cell divisions, attempting and failing additional cleavages. Both mutants respond to temperature change rapidly and reversibly. For example, when *nmy-2(ne3409)* early embryos were shifted from 15 °C to 25 °C during cytokinesis, the cleavage furrow either instantly stopped and regressed ($n=5/10$), or stopped and retracted partially, before resuming furrow progression at a slower rate ($n=5/10$, Supplemental movies 1A, B). In embryos of this latter type, the cleavage furrow retracted within 2 min after the furrow had seemingly bisected the cell, well before mitosis of the next cell cycle (Supplemental movie 1B). These findings suggest that temperature-dependent inactivation of the *nmy-2(ts)* mutant proteins occurs rapidly.

When shifted prior to the first division, we found that mutant zygotes failed to exhibit cytoplasmic ruffling and the cortical contractions known as pseudocleavage. In addition, we found that the cortical flow of cytoplasm was much slower and that, rather than exhibiting their normal posterior displacement, both the congression of the pronuclei and the establishment of the first mitotic spindle occurred at the center of the cell. Simultaneously, all of the other asymmetries normally observed in pre-divisional one-cell embryos failed to become established, and subsequently cytokinesis always failed, resulting in multinucleated one-cell embryos (Supplemental movie 2B). Nearly identical phenotypes were previously reported for *nmy-2(RNAi)* embryos, suggesting that both of the new conditional *nmy-2* mutants are strongly impaired for actomyosin contraction. Genetic cloning and sequencing of the mutants showed that both mutations alter conserved residues in the S2 region of the NMY-2 protein (Fig. 1). The S2 domain is the dimerization region of the myosin heavy chain and is essential for the activity of the motor domain (Tama et al., 2005). Taken together these findings suggest that, at non-permissive temperature, these alleles result in strong loss-of-function phenotypes.

While previous RNAi-based studies had implicated NMY-2 in the establishment of initial asymmetries in the one-cell embryo, it was not possible, due to the irreversible nature of RNAi-mediated knockdowns, to ask if NMY-2 was required continuously for the maintenance of those asymmetries (Guo and Kemphues, 1996; Cuenca et al., 2003; Munro et al., 2004). To address this question we allowed initial asymmetries to be established and then shifted one-cell embryos up to non-permissive temperature either at the pronuclei-meeting stage or at anaphase. We found that NMY-2 activity was required continuously for the maintenance of the cortical PAR protein domains, as assayed by the localization of the posterior cortical determinant PAR-2 and the anterior cortical determinant PAR-6. In the wildtype, once polarity has been established at the pronuclei-meeting stage, PAR-6 occupies the anterior half of the cortex, and PAR-2 occupies the posterior half of the cortex throughout the first cell cycle (Hung and Kemphues, 1999; Boyd et al., 1996; Cuenca et al., 2003; Liu et al., 2004; Schonegg et al., 2007). In the *nmy-2(ts)* mutants, upshift to non-permissive temperature resulted in the extension of the PAR-6 domain toward the posterior pole of the egg, with concomitant retraction of the PAR-2 domain ($n=13/13$; Fig. 2B and Supplemental movie 3). As a control, the *nmy-2-ts* strains were maintained and analyzed at the permissive temperature (15 °C). 100% of these embryos maintained a wildtype localization pattern for PAR-6 and PAR-2 throughout mitosis at 15 °C ($n=7$; Fig. 2A and Supplemental movie 7). We then asked if a temperature downshift during mitosis would rescue the polarity defects in the mutant zygotes. Embryos maintained continuously at 25 °C from ovulation were mounted for observation. During pronuclei-meeting or early anaphase, embryos were rapidly downshifted by moving the glass slides to an aluminum surface pre-equilibrated at 15 °C. Among eight embryos analyzed after the downshift, five exhibited restoration of anterior-posterior polarity, as evidenced by retraction of the PAR-6 domain and expansion of the PAR-2 domain on the cortex (Fig. 3A and Supplemental movie 4). The PAR-6::mCherry signal did not completely disappear from the posterior cortex upon down shift to 25 °C. We think this may be an artifact of the mCherry label since 5 out of 5 PAR-6::GFP; *nmy-2(ts)* embryos examined resulted in complete

Fig. 1. Partial amino acid sequence alignment for the NMY-2 orthologs from *C. elegans* (Ce), *Drosophila melanogaster* (Dm) and *Homo sapiens* (Hs).

removal of PAR-6 from the posterior cortex upon temperature downshift (Supplemental movie 8). The remaining three *nmy-2 (ne3409)*; PAR-6::mCherry; PAR-2::GFP embryos failed to restore the A-P polarity. They never developed a posterior PAR-2 domain after downshift and instead exhibited a uniform PAR-6 localization on the entire cortex. In these embryos, strong PAR-2 signals eventually developed at the furrows during cytokinesis where they overlapped with strong PAR-6 signals (Fig. 3B and Supplemental movie 5).

Similar temperature-shift experiments enabled us to identify a late cytokinesis role for NMY-2 in maintaining the cleavage furrow. When *nmy-2(ne3409)* one-cell embryos were shifted to non-permissive temperature during cell division, the cleavage furrow would often regress prior to the completion of cytokinesis, confirming a well-recognized role of NMY-2 for powering the actomyosin-based furrow contraction (Supplemental movie 1A). However, if the mutant was kept at the permissive temperature of 15 °C until the furrow had completed as monitored by DIC imaging, and then shifted up, an immediate furrow regression was never observed. Instead, if the mutant was shifted up during a 3-min interval after furrow completion, the daughter cells remained separated throughout interphase of the next cell cycle, but invariably underwent cleavage furrow regression and cellular refusion during the next mitosis. The onset of this delayed regression of the cleavage furrow appeared to coincide with the time during daughter-cell mitosis when the spindles reached the cortex (Figs. 4A, G and Supplemental movie 6). The refusion did not occur when embryos were shifted to non-permissive temperature greater than 3 min after cytokinesis of the first cell division (Figs. 4B, G). This phenotype is reminiscent of temperature-sensitive mutants of *zen-4*, a gene that is required for the abscission of cytokinesis. ZEN-4 encodes a kinesin-like protein and its human ortholog has been found to be part of the structure that anchors the membrane vesicles to the midbody-ring during cytokinesis abscission (Raich et al., 1998; Gromley et al., 2005). NMY-2 and ZEN-4 colocalize at the midbody, consistent with possible functional interactions. To ask if NMY-2 disruption would affect ZEN-4, we examined ZEN-4 immunolocalization in *nmy-2(ne3409)* embryos

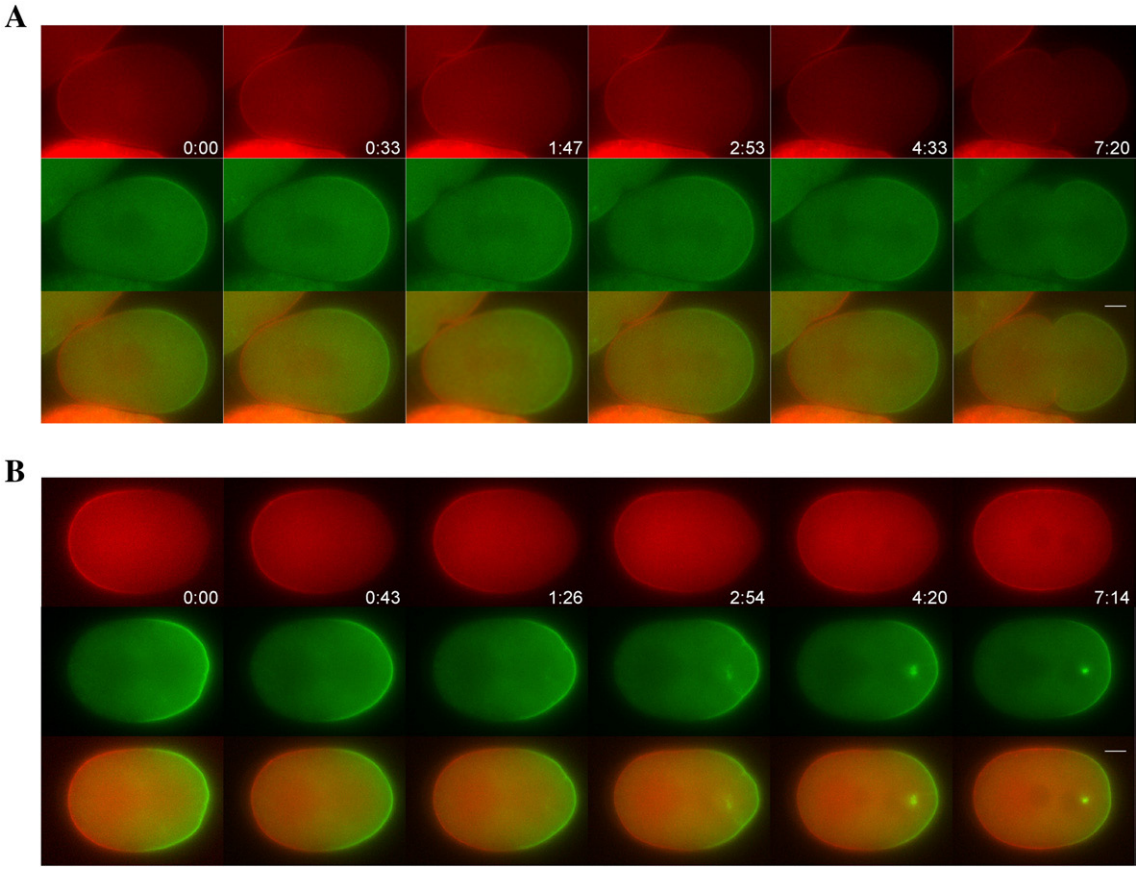


Fig. 2. Continuous 15 °C imaging (Panel A) and temperature upshift from 15 °C to 25 °C (Panel B) of anaphase *nmy-2(ne3409)*; PAR-6::mCherry; PAR-2::GFP zygotes. The anterior is to the left. Minutes:seconds were labeled. 0:00: upshift point. Each column consists of frames taken at the same time point: PAR-6::mCherry (first row), PAR-2::GFP (second row), and combined (third row). The bar indicates 10 um.

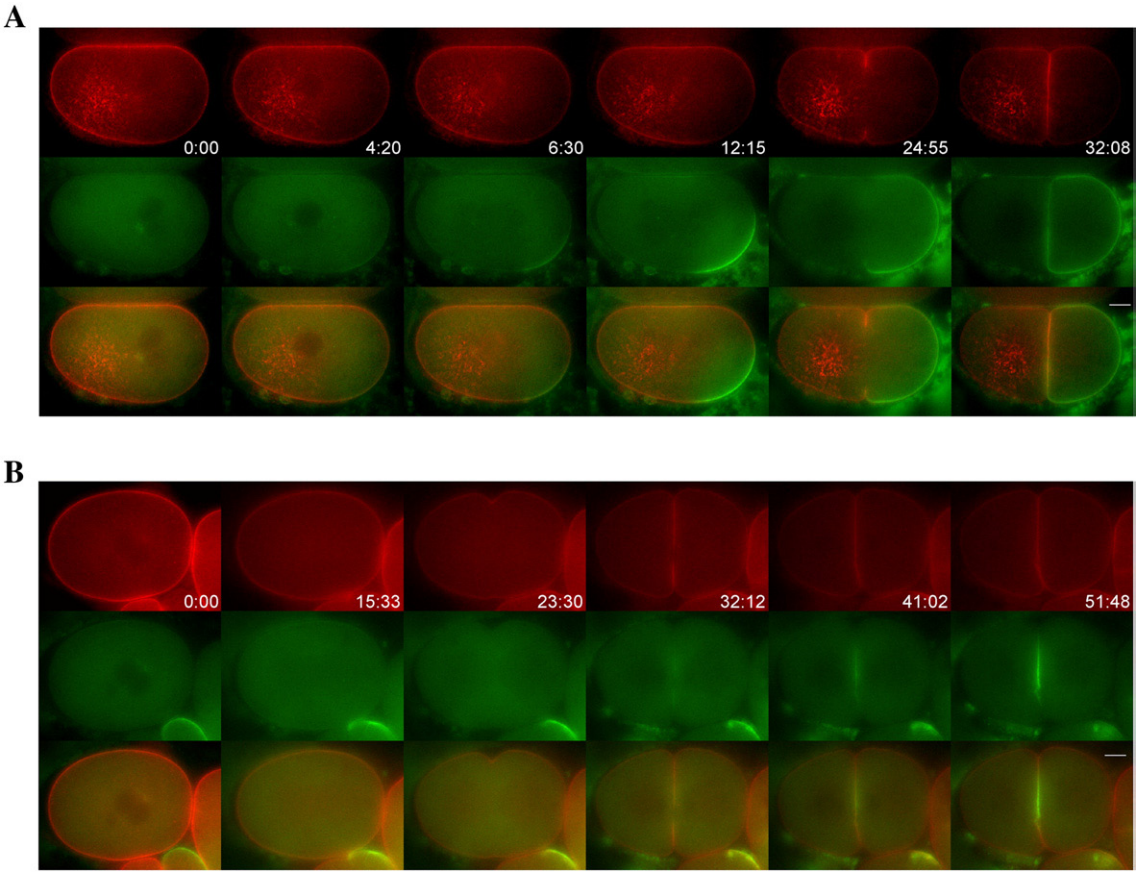


Fig. 3. Temperature downshift from 25 °C to 15 °C of prometaphase *nmy-2(ne3409)*; PAR-6::mCherry; PAR-2::GFP zygotes. The anterior is to the left. (A) An embryo that developed complementary PAR-6 and PAR-2 cortical signals during downshift. (B) An embryo that failed to develop an apical PAR-2 but later developed a PAR-2 signal at the cleavage furrow. Minutes:seconds were labeled. 0:00: downshift point. Each column consists of frames taken at the same time point: PAR-6::mCherry (first row), PAR-2::GFP (second row), and combined (third row). The bar indicates 10 μm.

upshifted to 25 °C at different points in the cell cycle. After a 3-min temperature pulse at 25 °C, embryos were immediately frozen and fixed for immunostaining. We found that when upshifted and fixed during metaphase and anaphase, the ZEN-4 localizations in the mutant were similar to those of the wildtype (data not shown). However, when upshifted immediately after the cytokinesis furrow was complete and well before the membrane refusion began, as monitored by DIC imaging, the ZEN-4 pattern was dramatically different. While ZEN-4 was concentrated on the midbody in the wildtype ($n=5/5$), it dispersed to a large circle around the cell boundary ($n=5/5$, Fig. 5, see Discussion).

NMY-2 activity was also required for maintaining the nascent cell boundary for other sister-cell pairs. For example, in upshift experiments on the daughters of the 2-cell stage blastomeres AB and P1, and of the 28-cell stage blastomeres Ea and Ep, we found that NMY-2 activity was likewise required for the maintenance of the separation of the daughter-cell pairs (Fig. 4, and data not shown). Surprisingly, however, the daughters of AB and P1 differed markedly in the duration of the NMY-2 requirement post division. As observed after division of the one-cell embryo, a period of 3 min at permissive temperature was sufficient to maintain the division of the anterior sister cells, ABa and ABp ($n=15$, Figs. 4C, D, G). In contrast, a period of 9 min at permissive temperature was required to maintain the division of the posterior sister cells, P2 and EMS ($n=17$, Fig. 4E–G).

nmy-2 interacts genetically with WNT signaling components

The extended requirement of NMY-2 activity for the maintenance of the P2/EMS cell boundary overlaps with a critical period during which these sister cells are competent to signal to one another to induce cell polarity and to specify endoderm (Goldstein, 1993). This P2/EMS signaling event was previously shown to involve parallel inputs from both the WNT and the SRC signaling pathways (Lin et al., 1995; Rocheleau et al., 1997; Thorpe et al., 1997; Bei et al., 2002). In order to examine the possible role of NMY-2 in the signaling interaction between P2 and EMS, we first needed to design experimental conditions that permitted the two cells to remain separate and intact, so that the outcome of signaling could be interpreted unambiguously.

Previous studies have shown that signaling can be reconstituted in partial embryos assembled from isolated P2 and EMS blastomeres (Goldstein, 1992; Goldstein, 1993; Goldstein, 1995a,b). Therefore, we cultured *nmy-2* mutant embryos at 15 °C until the 4-cell stage and then immediately separated the newly formed P2 and EMS cells. We then moved the separated blastomeres to 25 °C and put P2 and EMS cells back in contact with each other. Physically separating the cells prevented the cleavage furrow from regressing at the restrictive temperature, and allowed us to ask whether the *nmy-2-ts* mutants are impaired in the signaling required to induce the rotation of the EMS nuclear centrosome complex. Using this protocol, we found that 80% of the partial embryos examined ($n=15$) exhibited proper EMS spindle orientation. This percentage is slightly lower but is not dramatically different from the 90% frequency ($n=10$) of A–P divisions observed for wildtype blastomeres under identical conditions (Schlesinger et al., 1999; Goldstein, 1995a,b; this study). These results suggest that polarity signaling that directs A/P division in EMS remains largely intact in the *nmy-2* mutant embryos examined.

As a second approach to look for synergy between *nmy-2* alleles and previously identified P2/EMS signaling factors we used genetically sensitized backgrounds. To do this we first determined semi-permissive temperatures at which early cell-division and polarity are not compromised in the *nmy-2(ne3409)* and *nmy-2(ne1490)* single-mutant strains. We then constructed double mutants, either through genetics or RNAi, with previously identified P2/EMS signaling factors and examined the double-mutant embryos for endoderm specification when cultured at the threshold temperature for *nmy-2* activity. We found that, under these conditions, the *nmy-2* mutants significantly enhanced the endoderm to mesoderm transformations observed in WNT-pathway mutants (Table 1). For example, the number of embryos lacking endoderm increased from 21% in the *mom-2(ne141)* single mutant embryos to over 90% in *nmy-2(ts); mom-2(ne141)* double mutant embryos cultured at the semi-permissive temperature of 20 °C (Table 1). In contrast, when *src-1* or *mes-1* were inactivated in the *nmy-2(ts)* mutant backgrounds, we observed no significant increase in the frequency of gutless embryos.

Consistent with our findings from the blastomere isolation studies, we failed to observe any significant *nmy-2*-threshold-dependent enhancement of the EMS division axis defect when combined with mutants or RNAi targeting WNT or SRC pathway components (data not shown). Taken together, these findings support a role for *nmy-2* in promoting endoderm specification (but not division orientation), perhaps by functioning in a pathway that parallels the WNT-signaling pathway.

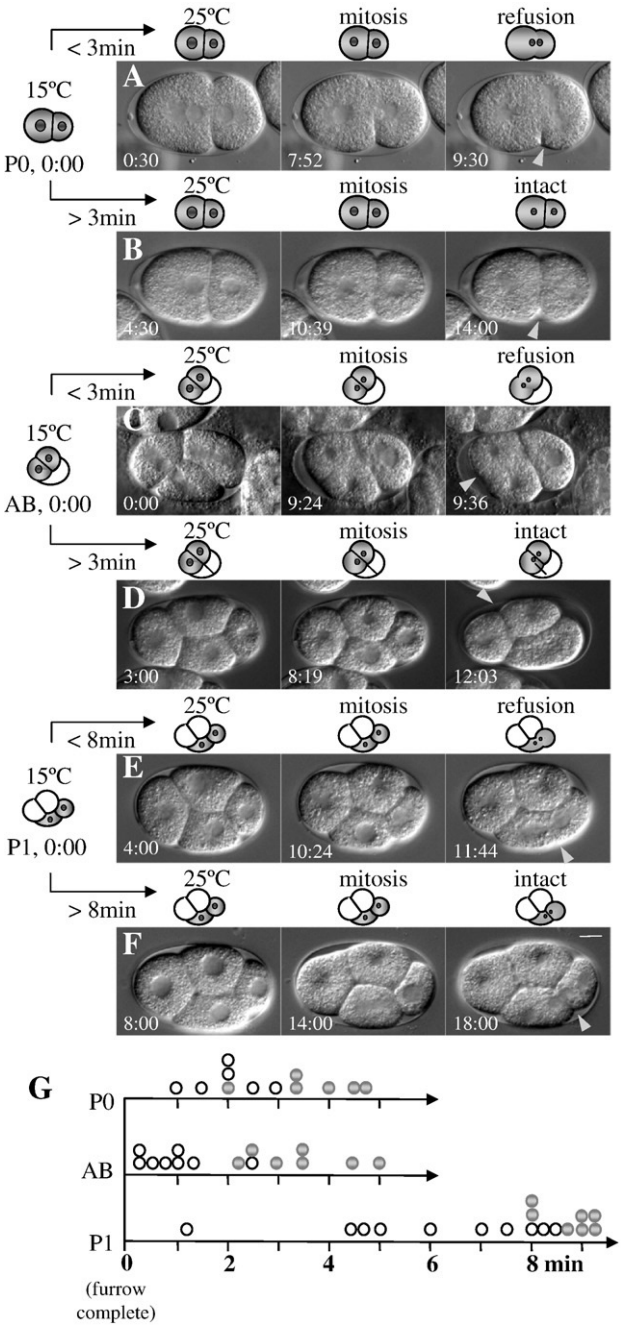


Fig. 4. NMY-2 is required for the maintenance of nascent cell boundaries. (A–F) Diagrams and micrographs depicting the results of representative temperature-shift experiments performed on *nmy-2(ne3409)* embryos. Each row of three micrographs depicts a single embryo maintained at 15 °C until cell cleavage was complete (time 0), and then shifted to 25 °C at the time indicated in the first micrograph of each row (minutes:seconds). (G) Summary of all 44 embryos assayed as described in A-F. Blank circles represent embryos whose furrows later fused back during the next mitosis, and grey circles represent embryos whose furrows never fused back. The position of each circle on the *X*-axis indicates the time at which the corresponding embryo was shifted up. The bar indicates 10 μm.

SRC-dependent phosphotyrosine is impaired in nmy-2(ts) mutants

P2/EMS signaling leads to the accumulation of an intense phosphotyrosine signal at the junction between P2 and EMS. This phosphotyrosine signaling depends on *mes-1* and *src-1*, and can be visualized using the antibody PY99 (Bei et al., 2002). Activated SRC kinase itself is also tyrosine phosphorylated (for a review, see Bjorge et al., 2000). A SRC_PY416 antibody is available that specifically recognizes this activated and phosphorylated form of SRC. We found that this antibody stains the cell cortex in wildtype embryos and that this signal completely disappears in *src-1* RNAi embryos ($n>10$; Figs. 6A, C, F). Importantly, the signal is elevated at the P2/EMS junction in the wildtype, in a pattern that is identical to that observed for PY99 staining. When staining the *mes-1(bn74)* mutants with the SRC_PY416 antibody, the elevated signal at the P2/EMS junction was lost in all the embryos ($n=17$; Figs. 6B, F). Consistent with a role for *nmy-2* in the SRC pathway, we found that 35% ($n=40$) of *nmy-2* mutant embryos failed to exhibit an elevated SRC_PY416 signal, while 73% ($n=26$) failed to exhibit an elevated PY99 signal at the P2/EMS junction when maintained at the semi-permissive temperature of 21 °C (Figs. 6D–G). These defects are unlikely to be secondary consequences of a defective initial polarity, because both PAR-2::gfp and PAR-6::gfp localizations are normal in the 4-cell stage *nmy-2(ts)* embryos at 21 °C (data not shown). These findings suggest that NMY-2 activity is required for efficient SRC-1 activation.

Discussion

The *C. elegans* PAR proteins represent conserved components of a cortical mechanism for the establishment of cellular asymmetry (Goldstein and Macara, 2007). Previous studies have suggested that the cortical PAR localizations are established by cortical actomyosin flow powered by NMY-2 (Munro et al., 2004). After establishment of initial cortical asymmetries the cortical flow ceases, and it is thought that anterior-posterior polarity is maintained by the protein PAR-2, which excludes the PAR-6 protein from the posterior cortex (Cuenca et al., 2003). Here, using conditional alleles of *nmy-2,* we have shown that NMY-2 is required throughout the cell cycle to maintain the PAR-2 and PAR-6 localizations. Thus, both the establishment and the maintenance of PAR-2 in the posterior domains and restriction of PAR-6 to the anterior domain of the embryo are dependent on NMY-2 activity. In wild-type embryos, an NMY-2-dependent cortical flow of cytoplasm coincides with pronuclear migration and the establishment of the PAR domains. After this period of cortical flow, the cytoplasmic movement is largely driven by the anaphase spindle elongation, and NMY-2 signal is reduced in the posterior cortex (Guo and Kemphues, 1996; Cuenca et al., 2003; Munro et al., 2004). In downshifted mutant

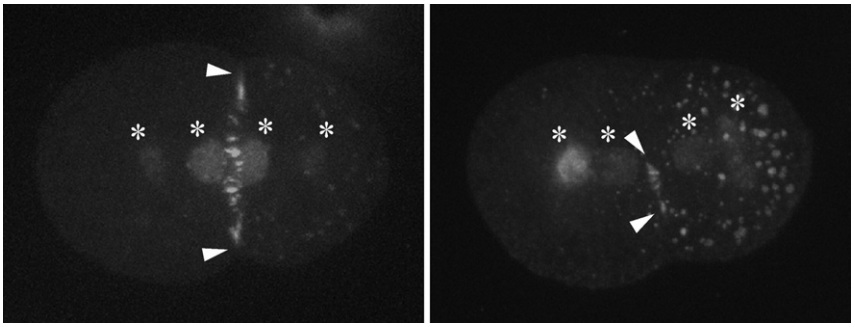


Fig. 5. ZEN-4 staining in an *nmy-2(ne3409)* (left) and a wildtype (right) embryos. Arrowheads demarcate the extent of the ZEN-4 signal. "*" denotes variable and probably nonspecific spindle pole and chromosome stainings.

embryos, cortical PAR domains were re-established, however the cytoplasmic movement still correlated with the anaphase spindle elongation instead of the change in cortical PAR domains. These indicate that some other NMY-2 dependent process is driving PAR-protein asymmetries. Perhaps PAR-2 localization modifies the posterior cortex in a way that promotes the NMY-2-dependent shuffling of PAR-6 from the posterior cortex to the cytoplasm with a concomitant expansion of the PAR2 domain, thus establishing a positive feedback of PAR-2.

It is interesting that there were two different outcomes in the mutant downshift experiments. In five of the eight embryos analyzed, re-activating NMY-2 at pronuclei-meeting or anaphase restored PAR-6 /PAR-2 polarity on the cortex, while in the remaining three embryos, polarity was not re-established. There was no correlation between the stages of the downshift (whether it was pronuclei-meeting or anaphase) and the resulting pattern. However, we noticed that 4/5 of embryos that restored polarity had a small and faint PAR-2 cortical patch at the beginning of the downshift, while the three embryos that failed to restore polarity did not have any preexisting cortical PAR-2 signal. These data suggest that after a zygote has entered mitosis, the PAR-6/PAR-2 boundary is largely regulated by existing cortical PARs instead of mitotic centrosome or microtubules.

Our findings suggest that NMY-2 is required to maintain cell boundaries well into the next cell cycle. When newly-divided cells were upshifted to non-permissive temperature, the nascent cell boundary always regressed during the next cell cycle. When fixed well before the membrane regression, the midbody protein ZEN-4 was found to be mislocalized. Double staining of ZEN-4 and the plasma membrane were attempted using several approaches but were unsuccessful (data not shown), therefore we were not able to determine if the fixation procedure itself artificially induced membrane refusion and if the nascent cell boundary regressed before ZEN-4 mislocalization in the fixed samples. Since the ZEN-4::GFP; *nmy-2 (ts)* embryos failed to provide sufficiently bright GFP signal at the midbody to allow study of the ZEN-4 dynamics in live fluorescent imaging (data not shown), we used another approach to ask if the mid-body localization of ZEN-4 depends directly on NMY-2 activity. We stained isolated blastomeres that were physically separated from one another to prevent membrane regression. Curiously, in these experiments the ZEN-4 signals were largely lost in the *nmy-2* mutants but not in wildtype isolated balstomeres, suggesting that inactivating NMY-2 rendered ZEN-4 localization unstable (data not shown). However due to a high nonspecific staining of ZEN-4 in blastomeres treated in this way, this conclusion is only tentative.

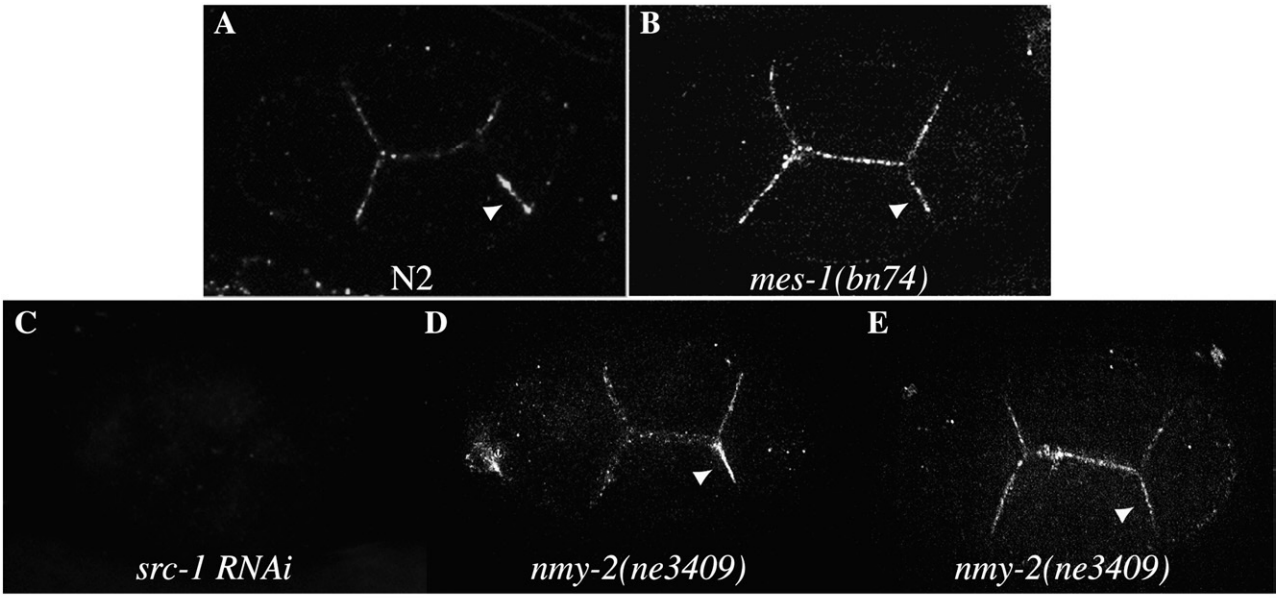
Cellular abscission is the final step in cytokinesis and is thought to involve the rapid fusion of membrane vesicles near the midbody to create a membrane seal (Gromley et al., 2005). Perhaps after the initial rapid NMY-2-dependent ingression of the furrow, a ZEN-4-dependent midbody complex maintains the furrow for several minutes, while a second or slower NMY-2-dependent mechanism facilitates the final abscission of the cells and stabilizes the boundary. For example, apart from stabilizing the ZEN-4/midbody ring structure, NMY-2 might promote the transport and fusion of vesicles to the mid-body region.

It is interesting that stabilization of the P2/EMS boundary requires NMY-2 activity for a significantly longer time than observed for other cell boundaries. It is also intriguing that this extended time period coincides with the interval during which P2 and EMS are competent to signal for endoderm induction. These observations raise the question of whether this sensitivity to refusion may reflect a structural feature of the P2/EMS boundary, perhaps the presence of an "open" configuration of the midbody ring complex that facilitates the signaling interaction. Alternatively, NMY-2 could be involved in transporting signaling molecules such as MES-1 and SRC-1 to the P2/EMS boundary with the result that sufficient NMY-2 activity is available to participate in stabilizing the boundary only after signaling is complete.

Endoderm specification at the 4-cell stage in *C. elegans* depends on synergistic inputs from both WNT and SRC-1/MES-1 signaling (Bei et al., 2002). Our findings suggest that NMY-2 promotes SRC-1/MES-1 signaling. Like mutations in *src-1* and *mes-1*, mutations in *nmy-2* enhance the endoderm-specification defects associated with all of the WNT mutants analyzed (including *mom-2*/Wnt, *mom-5*/Frizzled, and *dsh-2;mig-5*/Disheveled). Conversely, *nmy-2(ts)* mutants failed to enhance the endoderm-specification defects associated with *src-1* or *mes-1* mutants, suggesting that the gut defects observed in the *wnt; nmy-2(ts)* double mutants was not likely a secondary consequence of

Table 1
Genetics of P2/EMS signaling.

Embryo type	% Embryos lacking intestine at 20 °C (n)
nmy-2(ne1490)	0 (n>500)
nmy-2(ne3409)	0 (n>500)
mom-2(ne141)	21 (n=182)
mom-2(RNAi)	53 (n=232)
nmy-2(ne3409); mom-2(ne141)	91 (n=486)
nmy-2(ne3409); mom-2(RNAi)	94 (n=140)
nmy-2(n1490); mom-2(RNAi)	95 (n=262)
mes-1(bn74); mom-2(RNAi)	99 (n=150)
mom-5(zu193)	9 (n=163)
mom-5(RNAi)	4 (n=299)
nmy-2(ne3409) mom-5(zu193)	38 (n=381)
nmy-2(ne3409) mom-5(RNAi)	62 (n=189)
nmy-2(n1490); mom-5(RNAi)	62 (n=166)
mes-1(bn74); mom-5(RNAi)	97 (n=216)
dsh-2; mig-5(RNAi)	3 (n=110)
nmy-2(ne3409) dsh-2; mig-5(RNAi)	40 (n=253)
nmy-2(n1490); dsh-2; mig-5(RNAi)	28 (n=184)
mes-1(bn74); dsh-2; mig-5(RNAi)	95 (n=134)
src-1(RNAi)	5 (n=243)
nmy-2(n1490); src-1(RNAi)	7 (n=107)
nmy-2(n3409); src-1(RNAi)	7 (n=182)
mes-1(RNAi)	0 (n>200)
nmy-2(n1490); mes-1(RNAi)	0 (n>200)
nmy-2(n3409); mes-1(RNAi)	0 (n>200)



F

	% of elevated SRC	% of uniform SRC
N2 (n = 27)	100	0
dpy-5 nmy-2(ne1490) (n = 20)	55	45
dpy-5 nmy-2(ne3409) (n = 20)	75	25
mes-1(bn74) (n = 17)	0	100

G

	% of elevated PY99	% of uniform PY99
N2 (n = 22)	82	18
dpy-5 nmy-2(ne1490) (n = 14)	29	71
dpy-5 nmy-2(ne3409) (n = 12)	25	75

Fig. 6. NMY-2 is required for elevated phosphotyrosine staining at the P2/EMS junction. (A through E) Representative micrographs showing SRC_PY416 staining in Wildtype N2 (A); *mes-1(bn74)* (B); *src-1* dsRNA-injected N2 (C); and *nmy-2(ne3409)* embryos shifted to 25c for 2 min (D) for an elevated SRC at P2/EMS junction similar to the wildtype (A); and (E) for an uniform SRC pattern similar to (B). Arrowheads indicate the P2/EMS junction. (F and G): Tabulation of the percentage of embryos analyzed with elevated or uniform SRC_PY416 signal (F), and PY99 signal (G), for each embryo type.

impaired cytokinesis. Taken together these findings indicate a separable role of NMY-2 in endoderm specification and place NMY-2 in the SRC-1/MES-1 branch of the P2/EMS signaling pathway.

We did not observe an increase in the mis-orientation of the EMS spindle axis in *nmy-2* single mutants or in any of the double mutants analyzed. This apparent lack of synergy with respect to the regulation of division orientation could indicate that NMY-2 functions downstream of a branch in the SRC-1-signaling pathway that is specific for endoderm induction. However, it is also possible that the threshold for SRC-1 signaling to control axis specification is simply lower than the threshold for endoderm induction and that, in the conditional mutants analyzed, NMY-2 activity is not sufficiently compromised to disrupt the proper orientation of the EMS division axis.

Consistent with a placement of NMY-2 upstream of SRC-1 activation, we found that NMY-2 activity is required for localization of activated SRC-1, and for PY99-phosphotyrosine staining at the P2/EMS cell contact. These findings suggest that NMY-2 is either required for SRC-1 activation or for transport or maintenance of the activated kinase at the junction.

Previous studies suggest that SRC-family kinases and the actomyosin complex interact during signaling. For example, one study found that the targeting of v-SRC to focal adhesions required myosin activity (Fincham et al., 2000). Other studies linked SRC-family members to specialized lipid domains, called lipid rafts, whose activity and recycling appear to be regulated by the actomyosin cytoskeleton (Lajoie and Nabi, 2007; Holowka et al., 2005). Understanding how NMY-2 regulates SRC-1 activity, and how these signaling pathways integrate with WNT signaling and the cell-cycle machinery to induce endoderm and cellular polarity, will no doubt shed light on conserved signaling pathways that regulate cell fate and cell polarity in both normal development and disease.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2009.12.041.

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