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Report

LET-99, GOA-1/GPA-16, and GPR-1/2 Are Required for Aster-Positioned Cytokinesis

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Summary

At anaphase, the mitotic spindle positions the cytokinesis furrow [1]. Two populations of spindle microtubules are implicated in cytokinesis: radial microtubule arrays called asters and bundled nonkinetochore microtubules called the spindle midzone [2–4]. In *C. elegans* embryos, these two populations of microtubules provide two consecutive signals that position the cytokinesis furrow: The first signal is positioned midway between the microtubule asters; the second signal is positioned over the spindle midzone [5]. Evidence for two cytokinesis signals came from the identification of molecules that block midzone-positioned cytokinesis [5–7]. However, no molecules that are only required for, and thus define, the molecular pathway of aster-positioned cytokinesis have been identified. With RNAi screening, we identify LET-99 [8, 9] and the heterotrimeric G proteins GOA-1/GPA-16 and their regulator GPR-1/2 [10–12] in aster-positioned cytokinesis. By using mechanical spindle displacement, we show that the anaphase spindle positions cortical LET-99, at the site of the presumptive cytokinesis furrow. LET-99 enrichment at the furrow depends on the G proteins. GPR-1 is locally reduced at the site of cytokinesis-furrow formation by LET-99, which prevents accumulation of GPR-1 at this site. We conclude that LET-99 and the G proteins define a molecular pathway required for aster-positioned cytokinesis.

Results and Discussion

A Synthetic RNAi Screen for Aster-Positioned Cytokinesis Genes

We looked for genes required specifically for aster-positioned cytokinesis by screening for genes that are only required for cytokinesis in the absence of a spindle midzone. To facilitate the screening process, we disrupted the spindle midzone genetically by using *spd-1*(*oj5*) mutant embryos [6]. Because *spd-1*(*oj5*) mutants have a disrupted midzone, the midzone-positioned cytokinesis signal is also disrupted [5, 6]; likewise, we assume that if the aster-positioned cytokinesis signal is defective, the midzone-positioned furrow forms and the cell divides. We looked for genes whose depletion by RNAi prevented embryos from cleaving in a *spd-1*(*oj5*) mutant background but did not prevent embryos from cleaving in a wild-type background. Embryos were microscopically examined for multinucleated cells: Failed cytokinesis causes multinucleated cells. Gene disruptions that caused multinucleated embryos in *spd-1*(*oj5*) but not wild-type worms were further observed for determining whether the multinucleate phenotype was indeed caused by a cytokinesis failure (see the Experimental Procedures in the Supplemental Data available with this article online).

Of the 1000 genes screened by RNAi, 60 produced a multinucleated phenotype in *spd-1*(*oj5*) and the wild-type, seven produced multinucleated cells only in a *spd-1*(*oj5*) background, and the three following genes failed cytokinesis: *cls-2*, *gpr-1/2*, and *let-99* (Table S1). We did not investigate *cls-2* further. After depletion of GPR-1/2 or LET-99 in *spd-1*(*oj5*), a furrow formed but did not completely ingress, suggesting a role of these proteins in aster-positioned cytokinesis (Movies S1 and S2).

LET-99 Is Required for Cytokinesis in the Absence of a Spindle Midzone

LET-99 is a DEP domain protein, which localizes to the cortex in a band. LET-99 has been implicated in controlling microtubule-based pulling forces involved in spindle positioning, but it has not been implicated in cytokinesis [8, 9].

To confirm that the cytokinesis failure in *let-99*(RNAi); *spd-1*(*oj5*) embryos was due to the spindle midzone defect seen in *spd-1*(*oj5*) mutants and not due to another role of *spd-1*, we analyzed cytokinesis-furrow formation in *let-99* mutants with a mechanically disrupted spindle midzone. We filmed embryos expressing alphatubulin::YFP by spinning-disk microscopy to visualize spindle midzone and asters. During early anaphase, we irradiated the midzone by using a UV laser. As a result of irradiation, the two half spindles moved apart because of astral pulling forces [13]. Irradiated cells divided, consistent with the idea that the spindle midzone is dispensable for furrowing in one-cell *C. elegans* embryos (n = 10, Figure 1A) [14].

We then used midzone ablation to study cytokinesis-furrow formation in *let-99(RNAi)* embryos. Nonirradiated *let-99*(RNAi) embryos formed a clearly visible spindle midzone and divided. After midzone ablation in *let-99*(RNAi) embryos, the cortex showed shallow ingressions over the entire surface. However, these ruffles did not ingress and cytokinesis failed. (n = 10, Figure 1A). In the second mitotic cycle, embryos typically formed a tetrapolar spindle with midzone-like structures and cytokinesis furrows (data not shown).

Laser-mediated midzone ablation produces a stronger cytokinesis defect in *let-99*(RNAi) than the *spd-1*(*oj5*) mutant—either because *spd-1*(*oj5*) does not cause a midzone null phenotype or because mechanical midzone ablation also affects aster-positioned cytokinesis—for example, by sequestering centralspindlin, which

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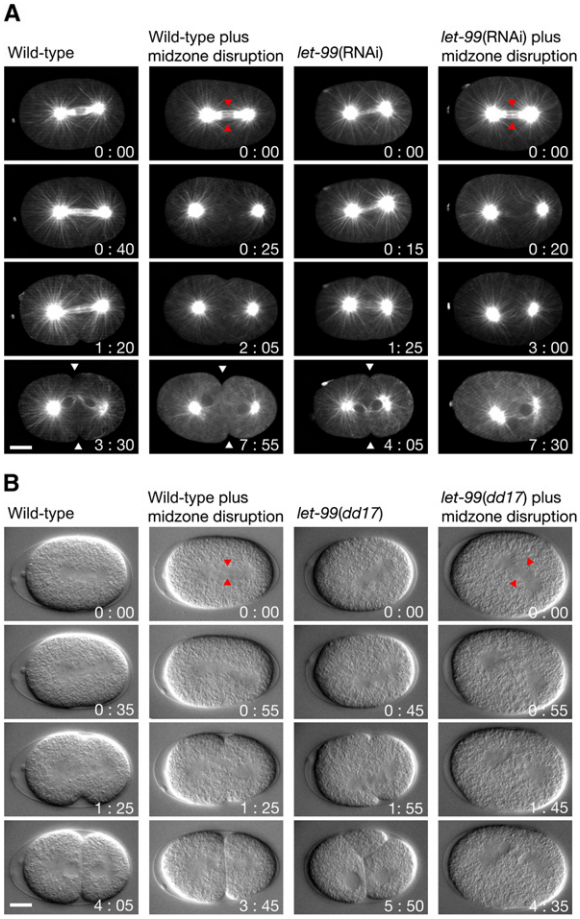

Figure 1. LET-99 Is Required for Aster-Positioned Cytokinesis but Not Midzone-Positioned Cytokinesis

(A) Mechanical spindle-midzone disruption in *let-99*(RNAi) with a UV laser observed in tubulin fluorescence. Red arrows indicate the irradiated region. The scale bar represents 10 μm. The time is shown as minutes:seconds.

(B) Mechanical spindle-midzone disruption in *let-99*(*dd17*) mutant embryos with a UV laser observed in DIC optics. Red arrows indicate the irradiated region. The scale bar represents 10 μm. The time is shown as minutes:seconds.

is required for both cytokinesis furrows [5, 6]. Aster separation is slightly reduced in *let-99* mutants. However, we do not think that the cytokinesis defect is a direct consequence of reduced aster separation [4]. The best evidence for this view is the existence of conditional *let-99* mutants that display the reduced aster separation defect but do not display a cytokinesis failure (see the Supplemental Data).

To examine the null phenotype of *let-99*, we used PCR to screen an EMS mutagenized *C. elegans* library and obtained an N-terminal deletion, *let-99*(*dd17*). *let-99*(*dd17*) is likely to be a null because the N terminus of the gene including the start codon and the DEP domain are deleted (Figure 1B and the Supplemental Experimental Procedures). All *let-99*(*dd17*) embryos examined completed cytokinesis (25/25). When we ablated the spindle midzone and observed embryos by time-lapse DIC microscopy, all *let-99*(*dd17*) embryos (15/15) showed small ingressions over the entire cortex but failed to form a cytokinesis furrow (Figure 1B and Movies S3–S6). Taken together, our results suggest that embryos can cleave without a spindle midzone but that in the absence of a midzone, the aster-positioned cytokinesis furrow is essential. *Let-99* is not required for the midzone-positioned cytokinesis furrow: *let-99* null embryos undergo cytokinesis in the presence of a midzone but fail to cleave in the absence of a midzone. Assuming that cytokinesis consists of two pathways—aster-positioned cytokinesis and midzone-positioned cytokinesis—we conclude that LET-99 is required specifically for aster-positioned cytokinesis.

LET-99 Contributes to Contractile-Ring Formation and the Timing of Cytokinesis

The two cytokinesis signals from the mitotic spindle reorganize the acto-myosin cortex and thus lead to the formation of a contractile ring [1]. We looked at the role of LET-99 in the kinetics of contractile-ring contraction by imaging fluorescently tagged nonmuscle myosin 2 (NMY-2::GFP) embryos end-on by using spinning-disk microscopy [15, 16] (Supplemental Experimental Procedures). NMY-2::GFP was concentrated at the leading edge of the ring in *let-99* mutants as well as in the wild-type. The rate of ring contraction was similar in *let-99* mutant and wild-type embryos (0.52% ± 0.03% embryo diameter per s [n = 5] for the wild-type and 0.59% ± 0.03% embryo diameter per s [n = 5] in *let-99*(*or204ts*) embryos). However, compared with the circular contractile ring in the wild-type, the contractile ring in *let-99* mutant embryos was irregularly shaped in all embryos observed (n = 5), and we observed ectopic furrows, which often caused cytoplast formation (Figures 2A and 2B). We also found that the furrow formed later in *let-99* mutant embryos compared to wild-type embryos (wild-type: 84 ± 2 s after anaphase onset; *let-99*(*dd17*): 155 ± 8 s after anaphase onset [n = 5 for both *let-99*(*dd17*) and the wild-type]; the Supplemental Experimental Procedures and Figure 2C).

One possible explanation for the late formation and the inhomogeneity of the contractile ring in midzone-only cytokinesis embryos is that microtubules of the asters are touching and evenly distributed over the cortex, whereas the spindle midzone is located in the center of the cell. The direct contact between microtubules and the cortex might be more suitable for providing a precise and homogenous signal required for a regularly shaped ring. Furthermore, a midzone signal might take longer to reach the cortex compared with a direct signal from the asters. This idea could explain why the aster-positioned signal seems more prominent in the large blastomeres of early embryos [17].

LET-99 Localizes to the Site of Cytokinesis-Furrow Formation

We next examined whether the localization of LET-99 corresponds to its role in cytokinesis. Previous work with immunostaining for endogenous LET-99 has shown that LET-99 is enriched in a cortical posterior band [9]. However, the relationship between band formation and cytokinesis had not been studied. For this purpose, we generated transgenic embryos expressing a LET-99 fusion construct with either YFP (YFP::LET-99) or mCherry (mCherry::LET-99) [18]. Both constructs rescued known *let-99* mutants (Supplemental Data). We recorded

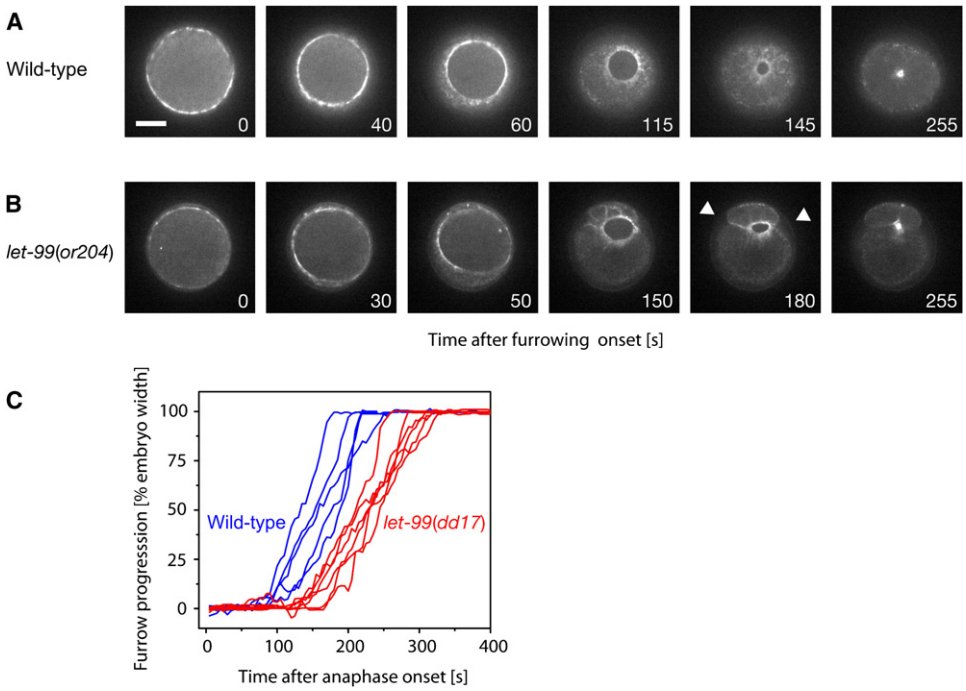

Figure 2. LET-99 Is Required for Organizing the Contractile Ring

(A and B) Contractile ring in the wild-type (A) and *let-99*(*or204ts*) mutant (B) embryos visualized by NMY-2::GFP with end-on spinning-disk microscopy. The time is shown in s; note that time 0 corresponds to furrowing onset. The scale bar represents 5μm.

(C) Kinetics of furrow ingression taken from side-view movies, which allows for identification of anaphase onset. Plotted are furrow progression (% embryo diameter) versus time (s); note that time 0 corresponds to anaphase onset. Shown are the wild-type (blue) and *let-99*(*dd17*) (red).

time-lapse images of YFP::LET-99 embryos from metaphase to the completion of cytokinesis. Confirming previous observations, LET-99 appeared as a cortical band during the first cell division [9] (Figure 3A).

We quantified cortical LET-99 intensity as a function of embryo length (Figure 3A; n = 20 cortices). LET-99 distribution at metaphase peaked in the middle of the cell (50% ± 1% embryo length) and covered about 55% of the embryo surface (27% ± 2% to 83% ± 1% embryo length). At anaphase, the LET-99 band shifted to the posterior (56% ± 1% embryo length) so that the peak LET-99 intensity now coincided with the position of the presumptive cytokinesis furrow (55% ± 1% embryo length). Our time-lapse analysis of LET-99 suggests that LET-99 distribution is dynamic, with a significant change of overall distribution occurring during anaphase. The peak intensity of cortical LET-99 correlates with the site at which the furrow eventually ingresses, consistent with a role for LET-99 in cytokinesis.

The Mitotic Spindle Positions Cortical LET-99 at Anaphase

At anaphase, both the spindle and the peak maximum are displaced toward the posterior, suggesting that the spindle may position the LET-99 band. To test this idea, we used a glass needle to alter the position of the spindle and followed the localization of cortical YFP::LET-99 (Supplemental Experimental Procedures and Movie S7). From the beginning of the manipulation until metaphase, the LET-99 peak was similar to unmanipulated cells and did not correlate with the position of the displaced spindle. At anaphase, cortical LET-99 moved toward the displaced spindle before furrow ingression was visible, and the LET-99 peak was positioned midway between the asters. The cytokinesis furrow formed at the site of the LET-99 peak, 20–30 s after repositioning of the LET-99 band. (see Figures 3B–3D, n = 6 [posterior displacement], n = 8 [anterior displacement]).

We thus conclude that before anaphase, a spindle-independent mechanism determines the cortical position of LET-99. During anaphase, the position of the mitotic spindle determines the cortical position of LET-99. This is consistent with the classic view that cytokinesis signals are active at anaphase and not before [19, 20]. What positions LET-99 before anaphase? LET-99 was initially described for its role in cell polarity. Cell polarity is required for LET-99-band formation, and the position of the band correlates with the boundary between anterior and posterior PAR domains. ([9] and Figure S1). Consistent with a role in cytokinesis, an anaphase LET-99 band was also observed in polarity defective embryos [9]. Furthermore, the cytokinesis role of LET-99 appears to be independent of cortical polarity: Midzone ablation in polarity defective embryos does not cause cytokinesis failure (Figure S2). Thus, the anaphase but not metaphase LET-99 band appears to correspond to the function of LET-99 in cytokinesis.

The Heterotrimeric G Proteins GOA-1/GPA-16 and Their Regulator GPR-1/2 Are Required for Aster-Positioned Cytokinesis

How does LET-99 localize to the cytokinesis furrow? Our screen for genes that prevented formation of a cleavage furrow in a *spd-1* mutant background identified *gpr-1/2*

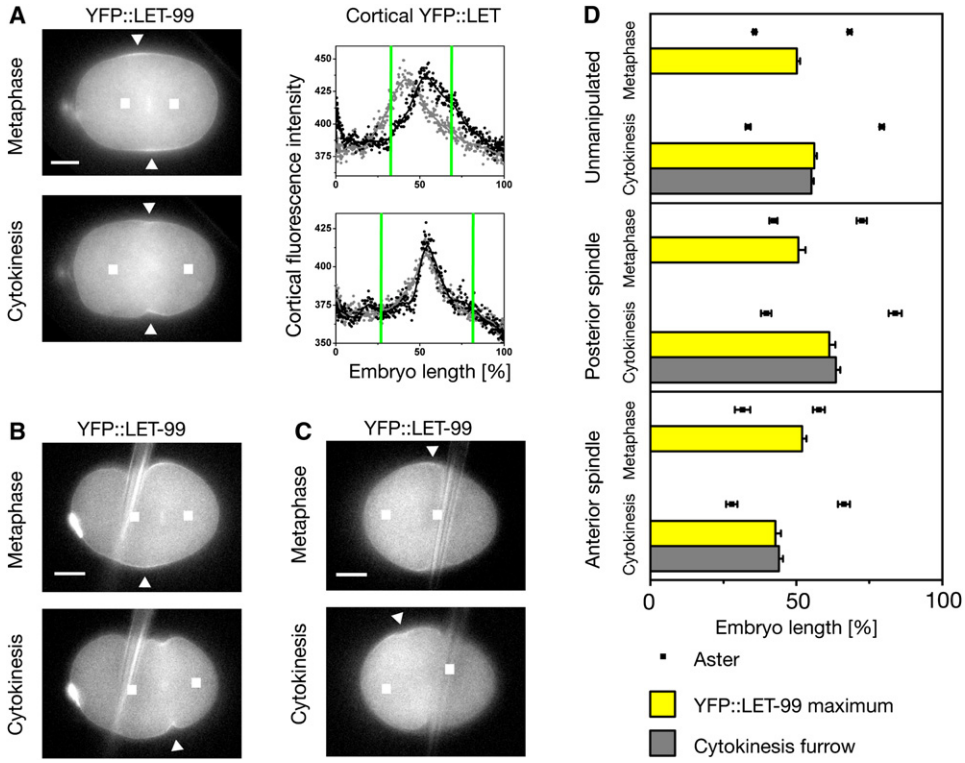

Figure 3. The Mitotic Spindle Positions Cortical LET-99 at Anaphase but Not at Metaphase

(A) LET-99 localization. Shown is YFP::LET-99 epifluorescence during metaphase (top) and cytokinesis (bottom). White squares indicate position of aster centers, and arrowheads indicate cortical LET-99. The scale bar represents 10 μm. LET-99 localizes to the cortex, the polar body, metaphase chromosomes, and the spindle midzone. Plots display cortical fluorescence intensity versus embryo length. The anterior is to the left, and the posterior is to the right.

(B and C) Spindle displacement assay. (B) A glass needle is pressed on the anterior of the cell. The spindle is hyperdisplaced to the posterior compared with wild-type embryos. (C) A glass needle is pressed on the posterior of the cell. The spindle is displaced to the anterior of the cell. Shown are YFP::LET-99 fluorescence during metaphase and cytokinesis. White squares indicate position of aster centers, and arrowheads indicate cortical LET-99. Errors are SEM. Scale bars represent 10 μm.

(D) Quantification of spindle displacement. The peak of LET-99 intensity is in the middle of the embryo at metaphase, regardless of spindle position, but shifts to the position midway between the asters at anaphase. Black squares represent aster centers. The yellow histogram shows YFP::LET-99 peak maximum position, and the gray histogram shows the cytokinesis-furrow position. Errors are SEM.

as being potentially required for the astral signal. GPR-1/2 is a G protein regulator that acts through the redundant G proteins GOA-1 and GPA-16. The G proteins are required for the generation of microtubule-based cortical pulling forces necessary for spindle positioning [10–12]. *Gpr-1/2*(RNAi) and *Goa-1/gpa-16*(RNAi) embryos all divided successfully in the presence of a spindle midzone (5/5). UV-laser-based midzone ablation in both *gpr-1/2*(RNAi) and *goa-1/gpa-16*(RNAi) prevented a cytokinesis furrow from forming (5/5, Figures 4A and 4B). Thus, the G protein pathway is required for aster-positioned cytokinesis.

GOA-1/GPA-16 Are Required for LET-99 Furrow Localization

Previous results have shown that LET-99 and G proteins act in the same pathway [21]. We therefore tested whether GOA-1/GPA-16 are involved in furrow localization of LET-99. We depleted GOA-1/GPA-16 by using RNAi in embryos expressing mCherry::LET-99. *goa-1/gpa-16*(RNAi) embryos formed a LET-99 band visible from metaphase until early anaphase. At the time of cytokinesis-furrow formation, we did not observe the enrichment of cortical LET-99 at the site of the cytokinesis furrow (n = 6, Figures 4C and 4D). Thus, we conclude that the G proteins position the anaphase LET-99 band. The metaphase and the anaphase LET-99 bands are positioned by different mechanisms, and they appear to be molecularly distinct.

Cortical GPR-1 Is Reduced at the Site of Cytokinesis-Furrow Formation by LET-99

We next examined whether the localization of GPR-1/2 corresponds to its role in cytokinesis. Previous work with immunostaining for endogenous GPR-1/2 has shown that GPR-1/2 localizes to microtubule asters and the cortex, consistent with the idea that GPR-1/2 is required for aster-positioned cytokinesis [10–12]. GPR-1/2 localization has been shown to depend on LET-99 [21]. However, the relationship between GPR-1/2 localization and cytokinesis-furrow formation had not been studied. We recorded time-lapse images of YFP::GPR-1 embryos from anaphase onset to the completion of cytokinesis (Supplemental Experimental Procedures). Confirming previous observations, GPR-1 localized to the cortex and microtubule asters. We quantified and averaged cortical GPR-1. The cortical distribution of GPR-1 at the time of cytokinesis-furrow formation was

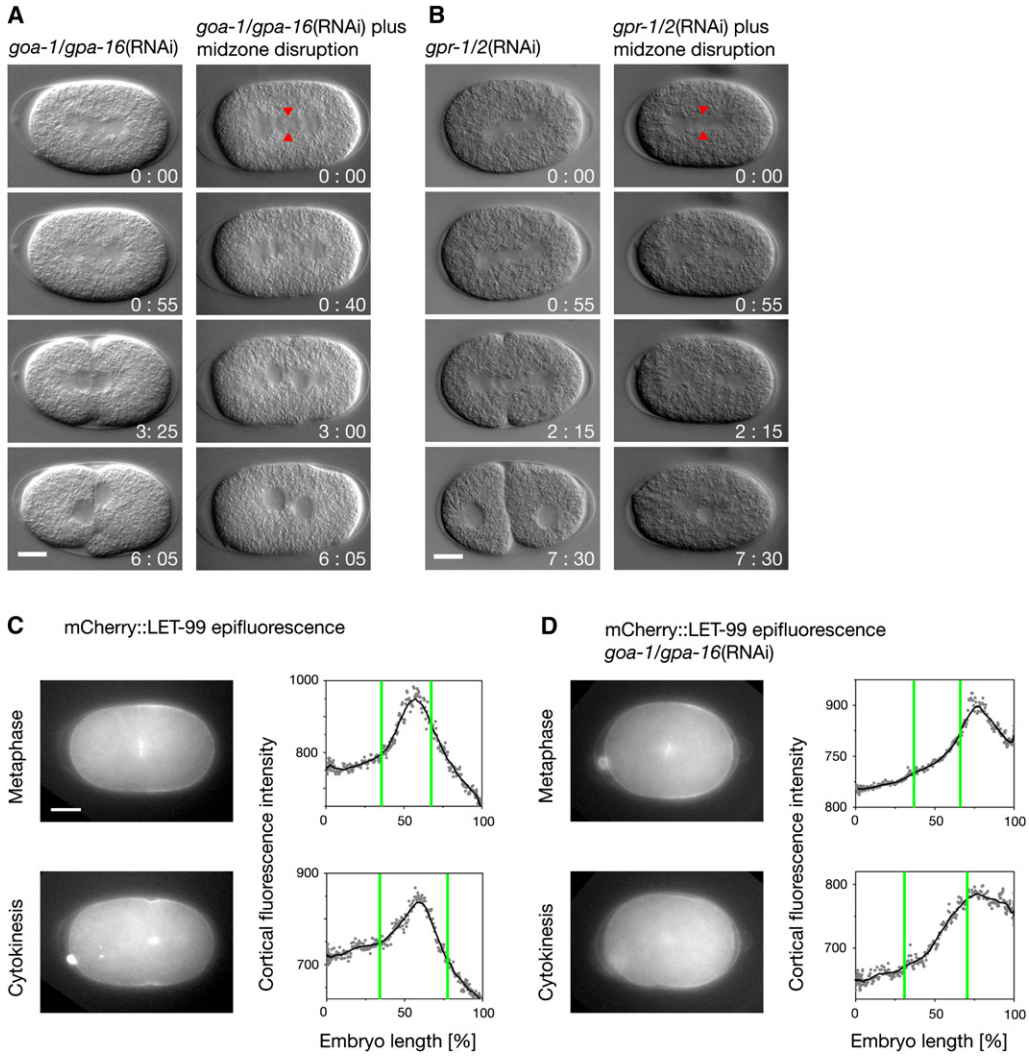

Figure 4. The G Protein Pathway Is Required for Aster-Positioned Cytokinesis and LET-99 Furrow Localization

(A and B) Mechanical spindle-midzone disruption in *goa-1/gpa-16*(RNAi) (A) and *gpr-1/2*(RNAi) (B) with a UV laser observed with DIC microscopy. Red arrows indicate the irradiated region. The scale bar represents 10 μm. The time is shown as minutes:seconds. The anterior is to the left, and the posterior is to the right.

(C and D) mCherry::LET-99 localization in the wild-type (C) and *goa-1/gpa-16*(RNAi) (B). Shown are metaphase (top) and cytokinesis (bottom). The scale bar represents 10 μm. Plots display cortical fluorescence intensity versus embryo length. Vertical green lines indicate aster position. The anterior is to the left, and the posterior is to the right.

nonuniform: GPR-1 was reduced at the poles and at the site of cytokinesis-furrow formation (Figure 5A and the Supplemental Experimental Procedures). Thus, at the time of cytokinesis-furrow formation, cortical GPR-1 localization can be described as two broad peaks flanking the site of furrow formation. The two peak maxima roughly correlated with the position of the asters and the minimum between the two asters correlated with the site of cytokinesis-furrow formation.

We next examined whether LET-99 was required for the reduction of GPR-1 at the site of furrow formation. We depleted LET-99 by using RNAi in embryos expressing YFP::GPR-1 and imaged embryos as before. GPR-1 still localized to asters and the cortex in *let-99*(RNAi) embryos. At the time of cytokinesis-furrow formation, cortical GPR-1 was not excluded from the site of cytokinesis-furrow formation. Instead, cortical GPR-1 was enriched at the site of furrow formation similarly to LET-99, and the maximum of cortical GPR-1 coincided with the site of furrow formation (Figure 5B). Thus, in the absence of LET-99, GPR-1 localization is inverted: Instead of being reduced at the site of furrow formation, it is enriched at this site and is similar to the localization of LET-99 in wild-type embryos. Taken together, these data show that LET-99 and components of the G protein pathway depend on each other for their localization: LET-99 excludes GPR-1 from the site of cytokinesis-furrow formation, and the G proteins localize LET-99 to the site of cytokinesis-furrow formation.

LET-99 contains a conserved DEP domain [9, 22, 23]. DEP domains are thought to be required for membrane targeting and are present in regulators of G protein signaling [24–26]. Blast search reveals homologs of LET-99 in higher organisms that are putative orthologs (multiple

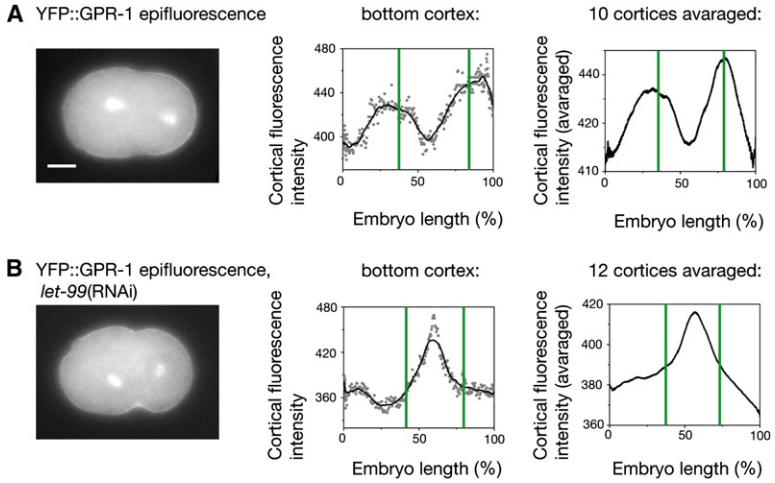

Figure 5. The G Protein Regulator GPR-1 Is Reduced at the Site of Cytokinesis-Furrow Formation by LET-99

YFP::GPR-1 localization at cytokinesis observed by epifluorescence in wild-type (A) and *let-99*(RNAi) embryos (B). The scale bar represents 10 μm. Plots display cortical fluorescence intensity versus embryo length for an individual cortex and the averaged intensity from several embryos. Vertical green bars indicate aster position. The anterior is to the left, and the posterior is to the right.

sequence alignment in Figure S4). Both vertebrates and invertebrates use G protein signaling for spindle positioning and astral pulling forces [10–12, 27, 28]. Both GPR-1/2 and its mammalian homolog LGN have been shown to act through LIN-5/Numa, a dynein regulator [10, 27]. Dynein and dynactin are thought to serve as cortical anchors of microtubules required for spindle positioning [29–31] and may also have a role in aster-positioned cytokinesis. Consistent with this idea, an accumulation of the dynein regulator $p150^{glued}$ at the site of cytokinesis was found in sea-urchin eggs [32].

How do asters position the cytokinesis furrow? We hypothesize that microtubule-based pulling forces provide a mechanical signal required for aster-positioned cytokinesis. One possible source for a mechanical signal could come from the fact that microtubules contact the cortex with different angles. Microtubules pulling on the cortex would generate lateral forces that depend on the angle on the cortex, and such forces could serve as mechanical signals. The mechanical signal caused by lateral forces could for instance be lateral cortical tension, which may be highest between the asters. This model predicts the existence of a mechanosensing pathway. LET-99 and the G proteins have been implicated in generating cortical forces, but they may also sense cortical mechanical signals. For instance, LET-99 could act by localizing to the region of highest tension, and such localization could lead to the assembly of contractile-ring components. If any furrow itself generates lateral cortical tension and if LET-99 localizes to the region of highest tension, we would expect that LET-99 would localize to any furrow, even a midzone-positioned furrow. Consistent with this hypothesis, LET-99 localizes also to a midzone-positioned furrow (Figure S3).

Supplemental Data

Supplemental Data include Experimental Procedures, four figures, one table, and seven movies and can be found with this article online at http://www.current-biology.com/cgi/content/full/17/2/185/DC1/.

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