The RhoGAP RGA-2 and LET-502/ROCK achieve a balance of actomyosin-dependent forces in C. elegans epidermis to control morphogenesis

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Embryonic morphogenesis involves the coordinate behaviour of multiple cells and requires the accurate balance of forces acting within different cells through the application of appropriate brakes and throttles. In C. elegans, embryonic elongation is driven by Rho-binding kinase (ROCK) and actomyosin contraction in the epidermis. We identify an evolutionary conserved, actin microfilament-associated RhoGAP (RGA-2) that behaves as a negative regulator of LET-502/ROCK. The small GTPase RHO-1 is the preferred target of RGA-2 in vitro, and acts between RGA-2 and LET-502 in vivo. Two observations show that RGA-2 acts in dorsal and ventral epidermal cells to moderate actomyosin tension during the first half of elongation. First, time-lapse microscopy shows that loss of RGA-2 induces localised circumferentially oriented pulling on junctional complexes in dorsal and ventral epidermal cells. Second, specific expression of RGA-2 in dorsal/ventral, but not lateral, cells rescues the embryonic lethality of rga-2 mutants. We propose that actomyosin-generated tension must be moderated in two out of the three sets of epidermal cells surrounding the C. elegans embryo to achieve morphogenesis.

KEY WORDS: ARHGAP20, C. elegans, Rho-kinase, RhoGAP, Epithelial, Morphogenesis

INTRODUCTION

Embryonic morphogenesis and organogenesis involve orderly events of cell shape changes and cell movements, such as the fusion of epithelial sheets, the directional elongation of epithelial tubes and the polarised rearrangement of cells within the plane of an epithelium (Pilot and Lecuit, 2005). The absence of overt variability in the general shape of individuals from a given species indicates that these events proceed with great precision and robustness. Such reproducibility is remarkable because morphogenesis involves many cells and, within cells, many molecular complexes whose activity must be accurately coordinated. In particular, morphogenesis involves multiple forces representing cell adhesion, internal contraction and external traction (Keller, 2006), which must be tightly balanced to avoid producing misshapen embryos.

Most morphogenetic processes are orchestrated by small GTPases, which ultimately induce cytoskeleton remodelling through the activity of various effectors (Burridge and Wennerberg, 2004; Van Aelst and Symons, 2002). Among Rho GTPase effectors, the Rho-binding kinase (ROCK) and two of its targets, the myosin regulatory light chain MLC-4, or the ROCK homologue LET-502, play a crucial role in smooth cell contraction, cytokinesis and various morphogenetic processes (Franke et al., 2005; Jacinto and Martin, 2001; Matsumura, 2005; Piekny et al., 2003; Wissmann et al., 1999; Wissmann et al., 1997; Young et al., 1993). ROCK activates myosin II by phosphorylating either the myosin regulatory light chain, or the MBS, a negative regulator of myosin II (Riento and Ridley, 2003).

How this pathway is regulated in vivo remains unclear. Some observations underscore the necessity to achieve precision in controlling myosin II activity. During Drosophila dorsal closure, myosin II generates the forces that allow cells of the leading edge to extend and amnioserosa cells to constrict (Franke et al., 2005; Jacinto et al., 2002; Young et al., 1993). When myosin II activity is not uniform along the leading edge, the lateral-epidermal sheets become misaligned at the dorsal midline, which impairs dorsal closure (Franke et al., 2005). Likewise, excessive myosin activity during Drosophila eye morphogenesis causes photoreceptor to move out of the eye disc epithelium (Lee and Treisman, 2004).

We study elongation of the C. elegans embryo as a paradigm for tube elongation. During this process, epidermal cells surrounding the embryo extend along the anterior-posterior axis of the embryo and constrict along its circumference (Chisholm and Hardin, 2005). Remodelling of circumferentially oriented actin filaments is thought to drive elongation, because treating embryos with cytochalasin D blocks elongation (Priess and Hirsh, 1986). Genetic analysis has outlined the central role played by the ROCK-myosin II pathway in elongation. Mutations affecting the myosin II heavy chain NMY-1, its regulatory light chain MLC-4, or the ROCK homologue LET-502, result in hypoelongation with embryos arresting at the 2-fold stage (Piekny et al., 2003; Shelton et al., 1999; Wissmann et al., 1997). Conversely, mutants of the MBS homologue MEL-11 are characterised by the presence of a bulge resulting from rupture of the embryo (Wissmann et al., 1999). This phenotype presumably results from myosin II hyperactivity, as let-502 and nmy-1 mutations prevent the formation of a bulge, and because mel-11 and let-502 mutations suppress each other (Piekny et al., 2003; Wissmann et al., 1999).

LET-502/ROCK and the myosin II heavy chain have a similar filamentous distribution, whereas MEL-11/MBS is localised at membranes during elongation, where it may no longer inhibit the contractile apparatus, and becomes cytoplasmic at the end of elongation (Piekny et al., 2003). This differential localisation, the observation that MEL-11/MBS is cytoplasmic in let-502 mutants

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Accepted 11 April 2007
and the genetic interactions between both genes have suggested that MEL-11/MBS acts at the end of elongation to block any further myosin II contractility (Piekny et al., 2003).

Here we characterise a new component of the C. elegans elongation pathway, RGA-2, as a member of the RhoGAP family (GTPase-activating protein). Two classes of regulatory proteins influence the state of small GTPases: guanine exchange factors (GEFs) promote conversion to the GTP-bound active form that is able to interact with various effectors, whereas GAPs accelerate the return to the GDP-bound inactive state (Moon and Zheng, 2003). Using fast time-lapse analysis, we show that excessive tension builds up in rga-2 mutant embryos. Using a genetic approach we show that RGA-2 acts as a negative regulator of LET-502/ROCK in a subset of epidermal cells. Altogether, our data suggest that different epidermal cells have different roles in elongation and underline the need to moderate actomyosin tension in dorsal/ventral cells. Moreover, our data provide genetic evidence supporting the current models of C. elegans embryonic morphogenesis as a cell seam-driven process.

**MATERIALS AND METHODS**

**Strains and genetic methods**

Control N2 and other animals were propagated at 20°C (unless noted otherwise) (Brenner, 1974). The following strains were used: NL2099, rrf-3(pk1426); CB698, van-10(e698); HR483, mel-11(s656) unc-4(e120)/mmCl[dpy-10(e128) unc-52(e444)]; KK332, mel-11(t26b) unc-4(e120) spo-1(unc-13/mmCl[dpy-10(e128) unc-52(e444)]; HR1184, +[rc32]1(on-2[6e76]); nunc-1(b115) dp-8(e130) fzt-1. Other alleles were: let-502(ca201sl54) (Wissmann et al., 1997); let-502(s118) (gift from P. Mains, University of Calgary, Calgary, Canada); nzo1[hsp-3; rho-1(G14V) tsx-3::gfp] (LG) (McMullan et al., 2006). let-502(s118) is a missense mutation in a conserved residue of the catalytic domain (P. Mains, personal communication). Recombinant let-502(s118) nzo1 animals were recovered based on the presence of the tsx-3::gfp marker. The allele rga-2(hd102) was obtained by screening a frozen mutagenesis library (which was generated by H.H. and his colleagues). It was outcrossed six times to N2 and balanced with hhn1[unc-54(h14040)]; let-502(s118) nzo-5(e61) rga-2(hd102) animals were recovered as Dpy recombinants from the progeny of let-502(s118) nzo-5(e61) rga-2(hd102) animals as a selection marker (100 ng/µl) and PBS2II at (80 ng/µl). Rescue was assessed after injecting plasmids at 1 ng/µl into rga-2(hd102); hhn1[unc-54(h14040)] animals (rescued animals did not segregate Unc progeny and had the mutant rga-2 genotype in PCR assays using a 5′ primer absent from the injected plasmid).

**Temperature-shift experiments and interactions with hs:rho-1(G14V)**

L4 larvae were picked and put at 20°C or 25.5°C overnight. The next morning, to roughly synchronise embryos, mothers were allowed to lay eggs for 1-hour time intervals on plates preheated to 20°C or 25.5°C. Wild-type embryos were collected in parallel. At regular intervals after egg-laying, embryos were picked at the desired stage under a binocular microscope and transferred to preheated plates. Unhatched embryos, arrested, dumpyshaped and normal larvae were counted the next day and 3 days after transfer. Interactions with the hs:rho-1(G14V) construct were performed in a similar way, and embryos then subjected to a 30-minute heat shock at 30°C. Plates were returned to 25.5°C for assays involving let-502(s118ts), or to 20°C for assays involving RNAi against rga-2. Heat shock was performed either 3 or 4 hours after removing mothers. Harsher heat shock (45 minutes at 31°C) induced some L1 larval lethality in the nzo1 background, but still almost no embryonic lethality with the characteristic ventral bulge of rga-2 embryos (data not shown).

**Biochemical analysis of RGA-2**

Recombinant C. elegans RHO-1, CED-10 and CDC-42 GTPases with a C-terminal 6×His tag (Jantsch-Plunger et al., 2000) were expressed overnight at 25°C and purified at 4°C using BD-Talon Resin (Clontech). A fragment encoding the GAP domain of RGA-2 (residues 219-475; the GAP domain itself corresponds to residues 261-429) was cloned into pGEX4T-1, expressed at 25°C and purified using Glutathione Sepharose 4B (Pharmacia Biotech). Proteins were dialysed overnight at 4°C into 10 mM Tris-HCl (pH 7.6), 150 mM NaCl, 2 mM MgCl2, 0.1 mM DTT, concentrated with a Centricon column (Millipore) and quickly frozen at −80°C. GAP activity assays were performed as described elsewhere (Jantsch-Plunger et al., 2000).

**Embryo staining**

C. elegans embryos carrying the rga-2::gfp F1 transgene (see above) were stained with bodipy-TRX-phallacidin (Invitrogen, Molecular Probes) diluted at 1:10 in PBS (Costa et al., 1997), without prior chitinase digestion. Stacks of 40 images every 0.25 µm (GFP fluorescence) or 6-10 images every 0.15 µm (phallacidin staining) were captured using a Leica SP2 AOB5 confocal microscope, then projected using the Tcs software (McMahon et al., 2001) and processed with Adobe Photoshop.

**Time-lapse videomicroscopy**

DIC time-lapse was performed using a Leica DMRXA2 microscope equipped with a PE120 Peeler heating stage set at 25.5°C by a PE94 controller (Linkam). Images were captured with a Coolsnap HQ camera (Roper Scientifics) and analysed using MetaMorph (Universal Imaging). Fluorescent videomicroscopy was performed in animals carrying the integrated transgene mec-46 using a Leica TCS SP5 confocal microscope (using a 100× 1.4 Plan Apochromat HCX CS objective), controlled by...
Leica LAS AF imaging software; illumination was via a 561nm DPSS yellow laser (10 mW). Acquisition was performed in resonant mode (fast scan), with a line average of four. Embryos were mounted on an agarose pad in M9 buffer and the coverslip was sealed with paraffin oil. Image mosaics were performed using MetaMorph.

## RESULTS

### The RhoGAP RGA-2 is involved in the elongation process

To identify new genes required for *C. elegans* morphogenesis, we conducted a feeding RNAi screen in a strain hypersensitive to RNAi, looking for elongation defects. The predicted gene *Y53C10A.4*, which was RNAi-sensitive in wild-type animals upon injection but not feeding (Table 1A), stood out for two reasons. First, *Y53C10A.4* RNAi-resistant animals are viable but sterile; *nmy-1(1b115)* is semi-dominant. Lethality was calculated taking into account all the progeny of the corresponding heterozygous strains.

### Table 1. Genetic interactions between rga-2, let-502 and mel-11

Table 1 presents the genetic interactions between rga-2, let-502, and mel-11. Each experiment was performed at least two (A) or three (B) times.

### Control

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<th>Genotype</th>
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<th>Let</th>
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<td>417</td>
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<td>1031</td>
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<td>67</td>
<td>302</td>
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</table>

Each experiment was performed at least two (A) or three (B) times.

+ wild type.

+HS, Heat shock.

Ctrl, Control experiment.

Let, Percentage embryonic lethality, except for experiments involving let-502(sb118ts) at 25°C, in which case it reflects embryonic and larval lethality scored 72 hours after hatching.

n, The total number of larvae laid by heterozygous animals.

*RNAi feeding was performed using mel-11 unc-4[mnc1] heterozygotes; lethality was calculated taking into account viable Unc progeny (presumptive mel-11 unc-4 homozygotes). For instance, mel-11(sb56) unc-4(e120)/mnC1 animals lay 7% dead eggs, 18% sterile coiler Uncs (presumptive mel-11), 25% sterile Dpy paralysed Uncs (presumptive mnc1) and 50% wild-type animals, suggesting that dead eggs correspond to homozygous mel-11(sb56) embryos. Dead eggs had a ventral bulge. The small percentage of embryonic lethality observed in the progeny of heterozygous mel-11(sb56)/+ and mel-11(it26)/+ mothers is consistent with the original description of these alleles (Wissmann et al., 1999). We reproducibly saw the phenotype they reported for mel-11(it26) embryos laid by homozygous mel-11(it26) mothers.

††RNAi feeding was performed using mel-11 unc-4/mnC1 heterozygotes.

‡‡At 20°C, 32% of the larval progeny of heterozygous mel-11(sb56)/+ and mel-11(it26)+ mothers had the characteristic bulge of larval lethality when injected into the female germline, which was RNAi-sensitive in wild-type animals. According to this estimate, we predicted that 67% of the progeny of these heterozygous animals would be heterozygous or homozygous mel-11 mutants. The total number of larvae laid by heterozygous animals was on the same day and with the same hatching.

§§Using a different dsRNA preparation, we found 13% lethality for N2 without (nzIs1[hs::rho-1(G14V)]) and 67% after heat shock (nzIs1[hs::rho-1(G14V)]) males and hDf17/hIn1[unc-54(h1040)] hermaphrodites. Dead embryos had a ventral bulge.

*Inferred from the F1 cross progeny of rga-2(hd102)/hn1[unc-54(h1040)] heterozygotes; 25% of embryos were paralysed (presumptive hhn1[unc-54] animals), 25% of embryos did not hatch and the rest were normal; thus, >99% rga-2(hd102) embryos die.

†Inferred from the progeny of mel-11(sb56) unc-4(e120)/mnC1 animals; 25% of animals were paralysed (presumptive mnc1 animals), 25% of embryos did not hatch and the rest were normal; thus, >99% mel-11(sb56) embryos die.

‡Inferred from the progeny of mel-11(sb56) unc-4(e120)/mnC1 males and hDf17/hIn1[unc-54(h1040)] hermaphrodites. Dead embryos had a ventral bulge.

§Inferred from the F1 cross progeny of rga-2[hn102]/hn1[unc-54(h1040)] males and hDf17/hIn1[unc-54(h1040)] hermaphrodites. Dead embryos had a ventral bulge.

¶Percentage of larval lethality, dying larvae were as illustrated in Fig. 1E.

††Arrested progeny had the hypoelongation phenotype of nmy-1(1b115) larvae rather than the bulge phenotype of rga-2-deficient embryos.

‡‡Inferred from the progeny of mel-11(sb56) unc-4(e120)/mnC1 mothers; 25% of embryos die.

**At 20°C, 32% of the larval progeny of heterozygous mel-11(sb56)/+ and mel-11(it26)+ mothers had the characteristic bulge of larval lethality when injected into the female germline, which was RNAi-sensitive in wild-type animals. According to this estimate, we predicted that 67% of the progeny of these heterozygous animals would be heterozygous or homozygous mel-11 mutants. The total number of larvae laid by heterozygous animals was on the same day and with the same hatching.

***The difference between heat-shocked let-502(sb118ts) and let-502(sb118ts) nzIs1[hs::rho-1(G14V)] is statistically significant (P<0.01).

Wissmann et al. (1999). We reproducibly saw the phenotype they reported for mel-11(it26) embryos laid by homozygous mel-11(it26) mothers.

*Y53C10A.4 rga-2 as its predicted translation product belongs to the RhoGAP family, with predicted Pleckstrin homology (PH) and GAP domains (Fig. 1G). RGA-2 displays strong homology (49% similarity) over its PH and RhoGAP domains to the vertebrate ARHGAP20 (also called RA-RhoGAP), but lacks the Ras-association domain (RA) and the RhoGAP domains to the vertebrate ARHGAP20 (also called RA-RhoGAP), but lacks the Ras-association domain (RA) and the two annexin-like repeats of ARHGAP20 (see Fig. S1 in the supplementary material). ARHGAP20 has recently been implicated in neurite outgrowth, integrating RAP1 (RAP1a – Mouse Genome Informatics) and RHOA GTPase signalling (Yamada et al., 2005).
strong recessive loss-of-function or null allele because its phenotype did not become more severe in trans to the deficiency hDf17 (Table 1A). DIC microscopy (Fig. 1C,D) and time-lapse analyses (Fig. 1J) showed that rga-2(hd102) embryos elongated normally until the 1.8-fold stage (Fig. 1H); then, their head generally failed to constrict, and most of them ruptured ventrally before the 2.3-fold stage, causing a highly penetrant embryonic lethality. For comparison, mel-11(it26) mutants usually ruptured around the 1.6- to 1.7-fold stage (Fig. 1H,K) and let-502(sb118ts) hatching at the non-permissive temperature. Arrows, ventral bulge. (G) Physical map of Y53C10A.4/rga-2 with position of the hd102 deletion indicated. Predicted domains of the putative 908-residue RGA-2 protein along with its closest mammalian homologue, ARHGAP20. PH, Pleckstrin Homology; RhoGAP, GTPase-activator protein for Rho-family GTPases; RA, Ras-association (RalGDS/AF-6); ANXL, annexin-like. The percentages of identity and similarity are indicated (for alignments, see Fig. S1 in the supplementary material). (H) Elongation rates for the mutants presented in I-L based on at least four embryos per genotype. (I-L) Still images from DIC videos for wild-type (I), rga-2(hd102) (J), mel-11(it26) (K) and let-502(sb118ts) (L) embryos maintained at 25.5°C. Timing (in minutes) is shown above each column. Arrows point to areas where rga-2 and mel-11 embryos appear to rupture; double-headed arrows, failure to constrict the head. Most rga-2(hd102) embryos (18/21) ruptured before the 2.3-fold stage (5/21 embryos ruptured before the 2-fold stage) in the anterior ventral midline (17/21) or less frequently posteriorly (3/21). Consistent with previous findings (Wissmann et al., 1999), 12/13 mel-11(it26) embryos ruptured at about the 1.6-fold stage. For originals, see Movies 1-4 in the supplementary material. Scale bar: 10 μm.
RGA-2 colocalises with circumferential actin bundles

To determine the distribution and subcellular localisation of RGA-2, we generated a C-terminal fusion between RGA-2 and the green fluorescent protein (GFP). This reporter construct is functional, as it rescued the lethality of *rga-2(hd102)*. We observed GFP expression in the epidermis from the 1.2-fold stage of elongation to the end of embryogenesis. The GFP formed a circumferential filamentous pattern (Fig. 2A-C) that was spatially and temporally strikingly reminiscent of the circumferential actin microfilament pattern (Priess and Hirsh, 1986). Indeed, actin staining in a strain expressing the RGA-2::GFP construct showed that the two networks coincide (Fig. 2D). In larvae and adults, the circumferential filamentous pattern did not persist, but expression was seen in a subset of head (Fig. 2F) and tail (not shown) socket cells, the vulva (Fig. 2H), more faintly the uterus (not shown), and the rectum (Fig. 2J).

To identify the RGA-2 domain responsible for colocalisation with actin, we generated smaller GFP constructs. The N-terminal PH domain conferred a punctate distribution (Fig. 3A,B, construct F2), which could represent localisation to membranes and is consistent with the known lipid-binding properties of PH domains. Constructs lacking the final 46 residues (constructs F3, P6, P8, P9, P12, P13), including one with the GAP domain alone (F4), had a mostly cytoplasmic distribution with a faint superimposed filamentous distribution (Fig. 3A,C,C′,D). By contrast, constructs containing the final 46 residues had a filamentous distribution (Fig. 3A,E,E′) like that of the full-length protein (see Fig. 2C). We conclude that RGA-2 has a C-terminal domain conferring strong association with actin microfilaments, and probably a second domain of lower affinity for microfilaments that could correspond to the GAP domain itself. The C-terminal part of RGA-2 bears no sequence similarity to any known protein; hence, we do not know whether association with actin is direct or indirect.

Fig. 2. RGA-2 colocalises with actin microfilaments in the epidermis. (A-C) Confocal lateral images of *C. elegans* embryos carrying the rescuing *rga-2::gfp* fusion F1 (see Fig. 3). (A) 1.2-fold, (B) 1.5-fold and (C) 3-fold stage embryos. GFP expression was restricted to the epidermis; it initially highlighted cell-cell junctions (white arrows), plus thin and short filaments that progressively became organised into a circumferential filamentous network. (D) Confocal image of a small area of the body from a *rga-2::gfp* transgenic 3-fold embryo showing GFP fluorescence (green, left), bodipy-phallacidin staining to visualise actin (red, middle) and merged picture (right) showing colocalisation. Arrows, actin in underlying muscles; arrowheads, epidermal cell-cell junctions. (E-J) DIC (top) and GFP fluorescence (bottom) pictures of adults, illustrating expression in sensory support cells of the head (E,F), vulval cells (G,H) and rectal cells (I,J), which are epithelial. Scale bars: 10 μm.
for viability, or, as indicated above, that the second weaker microfilament-association domain is sufficient for activity. Since the latter might correspond to the GAP domain, we could not directly remove it without affecting RGA-2 function. To test whether association with microfilaments is crucial for function, we inserted the prenylation signal (CAAX box) of MIG-2 (Portereiko and Mango, 2001) at the C-terminus of the P13 construct to force membrane binding. Presence of the CAAX box, but not of a control microfilament-association domain is sufficient for activity. Since the latter might correspond to the GAP domain, we could not directly interpret these indentations as a manifestation of an extreme actomyosin tension pulling on the plasma membrane at a position where actin bundles are tethered by a cadherin-catenin complex. Interestingly, we could partially rescue the rupturing phenotype of 

RGA-2 negatively controls LET-502/ROCK

Since RGA-2 associates with actin microfilaments, we considered two possibilities to explain why rga-2 embryos rupture. The bulge could result from a loss of epidermal integrity, as observed in mutants affected in genes affecting microfilament anchoring (Bosher et al., 2003; Costa et al., 1998; Köppen et al., 2001; McMahon et al., 2001), or from a disruption of the epidermal cytoskeleton as observed in mutants (Wissmann et al., 1999). Alternatively, it could be due to hypercontraction of the actomyosin network during embryogenesis, as occurs in mel-11 mutants (Wissmann et al., 1999). We did not detect any major integrity defect of epidermal junctions nor of the actin cytoskeleton (see Fig. S2 in the supplementary material), arguing against the first possibility.

Two sets of experiments showed that rga-2 controls actomyosin activity. First, to examine the architecture of rga-2(hd102) embryos when they rupture, we performed fast time-lapse imaging of the adherens junction marker dlg-1::rfp (Köppen et al., 2001; McMahon et al., 2001). Starting at the 1.9-fold stage of elongation (time point 1500 seconds in Fig. 4A), deep indentations of the

Table 2. rga-2 acts in dorsal/ventral cells and can be suppressed by stronger junctions

<table>
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<tr>
<th>Genotype</th>
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<td>e4</td>
<td>62</td>
<td>197</td>
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RGA-2 negatively controls LET-502/ROCK

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DLG-1::RFP signal could be seen exclusively in dorsal and ventral cells of rga-2(hd102) but not wild-type embryos (Fig. 4C,D). We interpret these indentations as a manifestation of an extreme actomyosin tension pulling on the plasma membrane at a position where actin bundles are tethered by a cadherin-catenin complex. Interestingly, we could partially rescue the rupturing phenotype of rga-2(hd102) mutants by increasing the DNA concentration of the dlg-1::rfp construct from 1 to 10 ng/µL in injection mixes, indicating that more DLG-1 can strengthen junctions against tension (Table 2). For comparison, we examined mel-11(it26) embryos (Fig. 4B), and noticed that seam cells were often deformed at the 1.5-fold stage: instead of being rectangular they were W-shaped, indicating that there was an imbalance of tension between dorsal/ventral and seam cells. In addition, although we observed fewer indentations than in rga-2(hd102) embryos, they protruded both within dorsal/ventral and seam cells and were often larger (Fig. 4C,D). Therefore, we suggest that mel-11 acts earlier than rga-2 and in all epidermal cells.

Second, we examined whether rga-2 could act in the ROCK and myosin II pathway defined by let-502, mmy-1 and mel-11. Since rga-2 and mel-11 mutations lead to similar defects, lowering RGA-2 and MEL-11 activities together might result in synergistic effects. Conversely, as rga-2 and let-502 mutations result in seemingly opposite phenotypes, if they act in the same pathway then lowering gene activity for both genes might restore normal elongation and viability. Using RNAi by feeding, which does not induce a phenotype in wild-type animals, we found that RNAi against rga-2 increased the embryonic lethality of mel-11(sb56) and mel-11(it26) mutants to almost 100% (Table 1A). At the cellular level, we observed the sum of defects resulting from loss of each gene separately (Fig. 4B), causing embryos to rupture earlier with more severe head defects. These observations suggest that mel-11 and rga-2 affect a similar process.
To test for genetic interactions between \textit{rga-2} and \textit{let-502}, we used \textit{let-502(sb118ts)}, a thermosensitive allele (P. Mains, personal communication) that shows no defect at 20°C, but at 25°C displays the strong elongation defects and L1-stage lethality characteristic of other \textit{let-502} alleles (Piekny et al., 2000) (Fig. 1F, Table 1A). We found that \textit{rga-2} and \textit{let-502} partially suppressed each other. For instance, RNAi against \textit{rga-2} by feeding reduced the lethality of \textit{let-502(sb118ts)} at 25°C from 95% to 33%, resulting in slightly shorter animals than normal, probably because elongation was slightly impaired. These observations were confirmed with another \textit{let-502} allele (Table 1A), \textit{let-502(ca201sb54)} (Piekny et al., 2000), showing that suppression is not allele-specific. Moreover, using the nearly 100% lethal allele \textit{rga-2(hd102)}, we could build a \textit{let-502(sb118ts) rga-2(hd102)} double-mutant, which was 68% viable at 20°C but non-viable at 25°C. At 20°C, non-viable \textit{let-502(sb118ts) rga-2(hd102)} embryos ruptured like \textit{rga-2(hd102)} embryos, whereas at 25°C they did not rupture and displayed the hypoelongation phenotype of \textit{let-502(sb118ts)} embryos alone. Thus, partial reduction of \textit{LET-502/ROCK} activity can suppress \textit{rga-2} lethality and, conversely, \textit{let-502} lethality can only be suppressed by partial reduction of \textit{rga-2} function.

To provide further evidence that \textit{rga-2} acts in the same pathway as \textit{let-502}, we examined whether mutations in the non-muscle myosin NMY-1 could suppress the rupturing phenotype of \textit{rga-2} embryos. \textit{rga-2(RNAi)} by injection in heterozygous \textit{nmy-1(sb115)} animals resulted in hypoelongated embryos (presumptive \textit{nmy-1} mutants) that did not rupture (Table 1A), suggesting that rupturing depends on actomyosin contraction. We conclude that \textit{let-502} and
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**Fig. 5. The GAP domain of RGA-2 enhances GTP hydrolysis by RHO-1 and CDC-42.** Results of GAP assays obtained by incubating purified GTPases and GST-RGA-2GAP fusion protein in the presence of \( \alpha^{32P}\)GTP (see Materials and methods), resolving the reaction products by thin layer chromatography (see Fig. S3 in the supplementary material), and quantifying the GTP/GDP fraction with a phosphorimager. Each time point represents the fraction of GTP hydrolysed after 2 minutes as a function of RGA-2GAP concentration. The graph is representative of four independent experiments.

RGA-2 acts in vitro and in vivo on the GTPase RHO-1
The sequence of the RGA-2 GAP domain suggests that it acts on Rho family GTPases. If, as outlined above, RGA-2 acts in the LET-502/ROCK pathway, we expect that RGA-2 should specifically act on RHO-1, as in vertebrates ROCK is a RHOA effector (Ishizaki et al., 1996; Leung et al., 1996; Matsui et al., 1996). To test this prediction, we performed in vitro GAP activity assays using a recombinant protein spanning the RGA-2 GAP domain (residues 219-475) and C. elegans RHO-1, CED-10 (RAC1) and CDC-42 recombinant fusion proteins. The GAP domain of RGA-2 activated GTP hydrolysis only in the presence of RHO-1 and CDC-42 (Fig. 5, and see Fig. S3 in the supplementary material), and required a 40 times lower RGA-2 GAP concentration to activate RHO-1 than CDC-42 (10 versus 400 nM). Interestingly, the mammalian homolog ARHGAP20 also displays stronger specificity towards RHOA (Yamada et al., 2005).

The data presented above are compatible with a model whereby RGA-2 activity maintains RHO-1 in the GDP-bound state, thereby preventing LET-502 activation. To test this model, we could not use RNAi against rho-1 because it blocks the first embryonic division (Jantsch-Plunger et al., 2000), nor could we use a rho-1 mutation as there is none available. Instead, we used a strain with an integrated transgene expressing a constitutively-active form of RHO-1 (G14V) under a heat-shock promoter (McMullan et al., 2006), expecting that it should enhance the lethality of a partial reduction of rga-2 function or reduce the lethality of let-502(sb118ts) embryos. In a wild-type background, expression of the RHO-1(G14V) protein after heat shock had only a moderate effect, presumably because the wild-type RHO-1 was still present (Table 1B). RNAi against rga-2 in this strain more than doubled the rate of lethality, even in the absence of heat shock (Table 1B). We do not think that this strain is hypersensitive to RNAi because RNAi against rga-2 by feeding was as poorly efficient as in wild-type animals (Table 1B). Instead we reason that the heat-shock promoter is slightly active even at 20°C. Heat-shock expression of RHO-1(G14V) significantly reduced the lethality of let-502(sb118ts) animals (Table 1B). Taken together, our data are consistent with the RhoGAP RGA-2 acting through RHO-1 in vivo, although we cannot exclude the possibility that it can also act through another GTPase, and support the notion that RHO-1 activates LET-502/ROCK during elongation.

RGA-2 acts mainly in dorsal/ventral epidermal cells
Our data suggest that the RhoGAP RGA-2 acts to negatively regulate RHO-1 and LET-502/ROCK, and ultimately myosin II. Since another negative regulator of myosin II, MEL-11, was already known, the identification of RGA-2 raises the issue of whether it acts in the same cells and at the same time as LET-502.

The lateral epidermal seam cells are thought to play a central role in embryonic elongation (Pickney et al., 2003; Priess and Hirsh, 1986; Shelton et al., 1999; Wissmann et al., 1999). In this framework, we asked whether rga-2 acts in seam and/or dorsal/ventral cells. We expressed a rga-2 cDNA under the control of promoters active only in seam cells (ceh-16) (Cassata et al., 2005), or in dorsal/ventral epidermal cells (elt-3) (Gilleard et al., 1999). The ceh-16p:rga-2 could not rescue the lethality of rga-2(hd102) embryos, whereas the elt-3p:rga-2 construct could rescue about 75% of transgenic embryos after correcting for transgene transmission frequency (Table 2). Joint presence of both constructs did not significantly improve rescue. We conclude that rga-2 acts mainly in dorsal/ventral epidermal cells, presumably to moderate contraction as it is occurring.

RGA-2 and LET-502 act during the same embryonic period
Several observations indicate that rga-2 acts during the first part of elongation: our time-lapse recordings show that membrane indentations appear around the 1.9-fold stage and that embryos rupture by the 2.2-fold stage (Figs 1, 4). To determine until what stage rga-2 is required, we took advantage of the let-502(sb118ts) rga-2(hd102) double mutant, which is mostly viable at 20°C but non-viable above 25°C (Table 1A). We first determined when LET-502/ROCK is essential during embryonic development. We found that let-502(sb118ts) embryos up-shifted to the non-permissive temperature once they had reached the 2-fold stage elongated normally (Fig. 6A). Conversely, embryos down-shifted to the permissive temperature at the lima-bean stage elongated normally, whereas they did not recover when transferred beyond that stage. These observations strongly suggest that LET-502/ROCK is required for elongation between the lima-bean and 2-fold stages. We thus reasoned that the double mutant let-502(sb118ts) rga-2(hd102) should allow us to test whether RGA-2 is essential beyond the 2-fold stage. Temperature-shift experiments showed that let-502(sb118ts) rga-2(hd102) embryos are viable at the non-permissive temperature once they have reached the 2-fold stage (Fig. 6A), which we interpret to mean that RGA-2 is dispensable beyond the 2-fold stage. This, RGA-2 and LET-502 are likely to act during the same developmental period, at the beginning of elongation.
Actomyosin in *C. elegans* morphogenesis

**RGA-2 acts in the LET-502/ROCK elongation pathway**

Our data strongly suggest that RGA-2 acts in the same pathway as LET-502 and myosin II (Fig. 6B). First, let-502 mutations partially suppress the lethality of rga-2 mutants. Since let-502(sb118ts) rga-2(hd102) double mutants display the elongation defects of single let-502(sb118ts) animals at the non-permissive temperature, we conclude that *let-502* is epistatic to *rga-2*. Second, consistent with the expectation that the small GTPase RHO-1 should activate the Rho-binding kinase LET-502, RGA-2 acts preferentially on RHO-1 in vitro, whereas in vivo expression of a constitutively active RHO-1 (mutation G14V) enhances the phenotype of *rga-2* and partially suppresses that of *let-502*. These experiments not only identify RHO-1 as a crucial GTPase during *C. elegans* elongation, but also suggest that RHO-1 acts between RGA-2 and LET-502. Third, the ventral rupture, resulting from excessive actomyosin tension that pulls on the junctions (Fig. 4) in *rga-2* mutants, is suppressed by loss of NMY-1 myosin heavy chain. We favour the notion that *rga-2* and *mel-11* act in parallel to regulate myosin activity, because of the epistasis and biochemical results summarised above. Moreover, RGA-2 colocalised with actin microfilaments, whereas MEL-11 is at junctions and remained so in *rga-2* mutants (data not shown). Interestingly, the myosin heavy chain NMY-1 forms filamentous-like structures similar to actin (Piekny et al., 2003) and LET-502 is interspersed with these structures, consistent with the idea that RGA-2, LET-502 and myosin II act together to control actomyosin contractility.

Some RhoGAP proteins can act as effectors rather than downregulators of small GTPases (Kozma et al., 1996; Zheng et al., 1994). In particular, the RGA-2 homologue ARHGAP20 behaves as a RAP1 GTPase effector during neurite outgrowth (Yamada et al., 2005). We do not believe that RGA-2 acts like this, as it does not possess a clear RA domain and because the *C. elegans* RAP1 homologues mainly affect larval secretion (Pellis-van Berkel et al., 2005).

Our observations suggest that RGA-2 contains two domains for colocalisation with epidermal actin: a major C-terminal domain that appears dispensable for function, and a minor domain that coincides with the GAP domain. Forcing membrane localisation of RGA-2 to sequester it away from actin affects its activity, suggesting that RGA-2 association with microfilaments is important for function. Whether RGA-2 colocalisation with actin reflects direct or indirect binding to actin remains to be determined. The colocalisation of other RhoGAPs to actin stress fibres, focal contacts, filopodia or lamellipodia has been reported before (Fauchereau et al., 2003; Hildebrand et al., 1996; Kozma et al., 1996; Lavelin and Geiger, 2005). The targeting of RhoGAPs to diverse subcellular localisations might provide specificity and explain the requirement for more GTPases than GTPases (22 GTPases versus six Rho family GTPases in *C. elegans* genome).

Functional studies in tissue culture cells indicate that RhoGAPs can affect the actin cytoskeleton, leading to loss of stress fibres or the formation of filopodia and/or lamellipodia (Kozma et al., 1996; Lavelin and Geiger, 2005; Yamada et al., 2005). Our genetic analysis did not reveal a role for *rga-2* in maintaining cytoskeleton integrity. Rather, the finding that *rga-2* acts as a negative regulator of ROCK strongly suggests that RGA-2 activity influences actomyosin contractility. It will be interesting to determine whether its closest vertebrate homologue, ARHGAP20, also acts in the ROCK pathway, particularly in cancer, because ROCK has been recognised to promote transcellular invasion of tumour cells (Itoh et al., 1999).

**DISCUSSION**

Contraction of the actomyosin cytoskeleton within the lateral epidermal seam cells is thought to play a crucial role during elongation of *C. elegans* embryos (Piekny et al., 2003; Priess and Hirsh, 1986; Shelton et al., 1999). Two proteins are known to regulate myosin II contractility during this process, the ROCK homologue LET-502, and MEL-11, the regulatory myosin-binding subunit of PP1 phosphatase (Piekny et al., 2003; Piekny et al., 2000; Wissmann et al., 1999; Wissmann et al., 1997). Our work identifies a microfilament-associated RhoGAP, RGA-2, as a new key regulator of LET-502 and brings novel insights into the balance of forces controlling elongation.

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**Fig. 6. LET-502 and RGA-2 are dispensable beyond the 2-fold stage.** (A) The percentage of lethality reflects larval and embryonic lethality. Temperature-shifts were performed at least three times as described in the Materials and methods. More than 60 individuals were sampled for each time point. Full genotypes: squares, *let-502(sb118ts)*; circles, *let-502(sb118ts) dpy-5(e61) rga-2(hd102)*. *let-502(sb118ts) dpy-5(e61)* behaved like *let-502(sb118ts)* but was omitted for clarity (data not shown). Black symbols, 25.5 to 20°C downshift; white symbols, 20 to 25.5°C upshift. LB, lima-bean stage (no elongation); C, comma. (B) Genetic model for the position of *rga-2* in the *C. elegans* embryonic elongation pathway, based on Tables 1 and 2 and on Fig. 5. (C) Developmental model for the role of RGA-2 in elongation (see Discussion).
**RGA-2 inhibits actomyosin tension in dorsal/ventral epidermal cells**

Twenty years ago, Priess and Hirsh proposed that the lateral seam cells should play the leading role in embryonic elongation (Priess and Hirsh, 1986). Since then, the identification of let-502 and mlc-4 (encoding the myosin regulatory light chain), and the demonstration using GFP reporters that they are primarily expressed in seam cells, provided molecular data to reinforce the seam-based elongation model (Shelton et al., 1999; Wissmann et al., 1999). However, let-502 and mlc-4 have not yet been shown to act only in seam cells. In particular, a caveat of GFP reporters is that they only reveal the zygotic expression of genes with a maternal requirement (which is the case for mlc-4 and let-502). Furthermore, dorsal/ventral epidermal cells might also play a role in elongation, as they are linked to muscles, which are essential to progress beyond the 2-fold stage (Williams and Waterston, 1994). Indeed, immunofluorescence analysis clearly indicates that LET-502 and NMY-1 are also present in dorsal and ventral cells (Piekny et al., 2003).

Our work provides the strongest genetic evidence so far that the seam-based model of elongation is accurate, at least until the 2-fold stage. Three observations support this notion: (1) LET-502/ROCK and RGA-2 have opposite functions; (2) they act during the same developmental period, as they both proved dispensable beyond the 2-fold stage; (3) RGA-2 is functionally required in dorsal and ventral epidermal cells, and excessive pulling on junctional complexes of rga-2 mutants is only observed in these cells. We interpret the pulling as a result of excessive actomyosin tension, as let-502 and nmy-1 are epistatic to rga-2, and imagine that it eventually causes junctions to rupture. Interestingly, we did not observe indentations in let-502(sb118) rga-2(hd102) embryos (not shown), suggesting that let-502 activity in dorsal/ventral cells is needed for their occurrence. The simplest model is that RGA-2 negatively regulates LET-502 in dorsal/ventral cells by keeping RHO-1 in the GDP-bound form (Fig. 6C). Hence, we suggest that to reach the 2-fold stage the embryo must activate actomyosin in seam cells and repress it in dorsal/ventral cells to moderate tension. Whether RGA-2 completely inhibits or simply moderates actomyosin tension in dorsal/ventral cells is unclear yet. RGA-2 is also present in seam cells, although at lower levels, and we do not know whether it is inactive therein or whether it has a minor role to ensure appropriate contraction. Interestingly, the RhoGAP crossveinless c asymmetrically inactivates actomyosin along the apicobasal axis of tracheal and spiracle cells during *Drosophila* morphogenesis (Brodu and Casanova, 2006; Simoes et al., 2006). A general feature of morphogenesis might thus be the asymmetric activation of myosin II, either within a cell or among different cells.

Our findings have implications for the roles of LET-502 and MEL-11 during elongation. Temperature-shift experiments indicate that LET-502 is not required beyond the 2-fold stage, the stage at which muscle mutants arrest. Although this conclusion relies on a single allele, let-502(sb118ts), affecting a conserved residue in the kinase domain (P. Mains, personal communication), it should reflect when LET-502 is active. Hence, beyond the 2-fold stage, either LET-502 acts redundantly with other proteins, or the shortening of actin microfilaments does not entirely depend on antiparallel sliding through myosin II activity and might involve other molecular mechanisms possibly implying a muscle-dependent pathway.

Our identification of a second negative regulator of ROCK raises the issue of why embryos would need two negative regulators of actomyosin activity with distinct distributions. It is thought that LET-502 phosphorylates MEL-11 during elongation, inducing its sequestration to membranes, away from the actomyosin apparatus (Piekny et al., 2003). Our time-lapse recordings indicate that defects in mel-11 and rga-2 mutants become visible at slightly different stages (Fig. 1). Since we observed tension in all epidermal cells of *mel-11* embryos, MEL-11 should act, in part, in the same cells as RGA-2. One possibility could be that MEL-11 and RGA-2 act sequentially: MEL-11 could initially prevent the premature onset of elongation and then become inactive when recruited to junctions (Piekny et al., 2003), whereas RGA-2 would subsequently take the relay. Alternatively, MEL-11 could remain active when junctional, implying that MEL-11 and RGA-2 would ultimately affect different pools of myosin II located either at junctions (MEL-11) or along circumferential actin filaments (RGA-2). We note that in vertebrate epithelia, disproportionate ROCK activity can disrupt adherens junctions (Sahai and Marshall, 2002). By analogy, the presence at junctions of an inhibitor of actomyosin, such as MEL-11, could help maintain junction integrity during *C. elegans* elongation. Further analysis of MEL-11 and RGA-2 should determine which model or models (they are not mutually exclusive) is the most likely.

In conclusion, we have identified a microfilament-associated RhoGAP that acts as a negative regulator of ROCK to moderate tension in a subset of epidermal cells during morphogenesis. It will be interesting to see whether the RGA-2 homologue in vertebrates also acts in the ROCK pathway.

**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/13/2469/DC1

**References**


