Cell shape and Wnt signaling redundantly control the division axis of *C. elegans* epithelial stem cells

Marjolein Wildwater1,*, Nicholas Sander2, Geert de Vreede1 and Sander van den Heuvel1,*

**SUMMARY**
Tissue-specific stem cells combine proliferative and asymmetric divisions to balance self-renewal with differentiation. Tight regulation of the orientation and plane of cell division is crucial in this process. Here, we study the reproducible pattern of anterior-posterior-oriented stem cell-like divisions in the *Caenorhabditis elegans* seam epithelium. In a genetic screen, we identified an *alg-1* Argonaute mutant with additional and abnormally oriented seam cell divisions. ALG-1 is the main subunit of the microRNA-induced silencing complex (miRISC) and was previously shown to regulate the timing of postembryonic development. Time-lapse fluorescence microscopy of developing larvae revealed that reduced *alg-1* function successively interferes with Wnt signaling, cell adhesion, cell shape and the orientation and timing of seam cell division. We found that Wnt inactivation, through *mig-14* Wntless mutation, disrupts tissue polarity but not anterior-posterior division. However, combined Wnt inhibition and cell shape alteration resulted in disordered orientation of seam cell division, similar to the *alg-1* mutant. Our findings reveal additional *alg-1*-regulated processes, uncover a previously unknown function of Wnt ligands in seam tissue polarity, and show that Wnt signaling and geometric cues redundantly control the seam cell division axis.

**KEY WORDS:** *C. elegans*, microRNA, Argonaute, Wnt signaling, Cleavage plane, Asymmetric division

**INTRODUCTION**
The development of complex organisms with specialized cell types, tissues and organs requires tight coordination between cell division and differentiation. This is achieved in part through asymmetric cell divisions that segregate the potential to proliferate and the commitment to differentiate to different daughter cells (Galli and van den Heuvel, 2008; Gonczy, 2008; Knoblich, 2008). For example, asymmetric divisions allow adult stem and precursor cells to self-renew and simultaneously produce daughter cells that will go on to differentiate. By contrast, proliferative (or symmetric) cell divisions generate two essentially identical daughter cells and promote exponential increases in cell number. The proper balance between proliferative and asymmetric cell divisions is crucial in stem cell maintenance and tissue homeostasis.

Differences between daughter cells may arise during division or after completion of division (Horvitz and Herskowitz, 1992). The first mode of division is considered intrinsically asymmetric and applies, for instance, to the *C. elegans* zygote and to *Drosophila* neuroblasts (Galli and van den Heuvel, 2008; Gonczy, 2008; Knoblich, 2008). During intrinsically asymmetric division, cell-fate determinants become unequally localized according to the polarity of the cell or tissue, and the mitotic spindle aligns along the polarity axis. Consequently, cell cleavage, which takes place perpendicular to the spindle, generates two daughter cells with different constituents. As an alternative mode of asymmetric division, external signals (e.g. from a stem cell niche) may instruct the fate of daughter cells that are initially identical after division. Importantly, the axis and plane of cell cleavage are critical in both forms of asymmetric division, as they determine the distribution of determinants as well as the size and position of daughter cells.

The mechanisms that determine the axis of division in the context of a tissue are poorly understood. Seam cells in the *C. elegans* epidermis provide a powerful model for studies of stem cell-like divisions within a polarized epithelium. These cells undergo symmetric and asymmetric divisions with a reproducible anterior-posterior orientation at stereotypical times during postembryonic development (Fig. 1A) (Sulston and Horvitz, 1977). The left and right lateral sides of the first stage larva (L1) each contain a row of ten seam cells, which continue to divide during each larval stage (Fig. 1A,B). Usually, the posterior daughter cell maintains the seam cell fate, while the anterior daughter terminally differentiates and fuses with the epidermal syncytium hyp7 (in the V1-V4 and V6 lineages) or becomes a neuron or neuronal support cell (in the H2, V5 and T lineages). In the second larval stage (L2), six seam cells (V1p-4p, V6p and H1aa) also go through a proliferative division, which expands the seam cell number to sixteen. Seam cells are polarized in the apical-basal as well as anterior-posterior direction. Anterior-posterior polarity involves asymmetric localization of Wnt/β-catenin asymmetry pathway components, such as APR-1 APC, WRM-1 β-catenin and POP-1 TCF/LEF (Mizumoto and Sawa, 2007b). Apical junctions that contain cadherin-catenin complexes separate the apical and basolateral domains and connect neighboring seam cells (Labouesse, 2006; Lynch and Hardin, 2009).

Previous studies revealed that a ‘heterochronic’ pathway controls the stage-dependent timing of seam cell divisions (Ambros and Horvitz, 1984; Chalfie et al., 1981). Several heterochronic genes encode microRNAs (miRNAs), which promote the transition from the L1 to L2 (lin-4), the L2 to L3 (let-7 sister miRNAs) and the L4 to adult (let-7) stages (Abbott et al., 2005; Lee et al., 1993; Reinhart et al., 2000; Wightman et al., 1993). *lin-4* mutant larvae...
skip the L2 stage-specific proliferative divisions and show a reduced number of seam cells, whereas mutants that lack the let-7 sister (let-7s) miRNAs reiterate these divisions and contain extra seam cells (Resnick et al., 2010). Although this heterochronic pathway provides stage-specific timing, what distinguishes proliferative from asymmetric divisions and what controls the seam cell division axis remain unknown.

In this study, we investigate the reproducible anterior-posterior orientation of the stem cell-like seam cell divisions. For advanced analysis, we created transgenic reporter strains that allow time-lapse fluorescence microscopy of seam cell divisions. We identified a mutant with large clusters of abnormally positioned seam cells and traced the mutation to the alg-1 Argonaute gene. We show that alg-1 not only contributes to the heterochronic developmental timing pathway, but also to the orientation of seam cell division from the L3 stage onward. We discovered that alg-1 inhibition also creates defects in the Wnt/β-catenin asymmetry pathway and in seam cell contacts and shape, which could potentially underlie the abnormal orientation of seam cell divisions. However, interfering with Wnt signaling, seam cell contacts or cell shape individually did not alter the division axis. Loss of Wnt secretion resulted in a random pattern of seam versus non-seam fates, indicating that Wnt ligands instruct tissue polarity in this system. However, when combined with seam cell rounding, Wnt inactivation also caused abnormally oriented seam cell divisions. These findings point to robust control of the cell division axis through a combination of Wnt signaling and geometric constraints.

**MATERIALS AND METHODS**

**Nematode strains**

We used the wild-type *Caenorhabditis elegans* strain N2 and the derivatives listed in Table 1.

**EMS screening and mapping**

Mutants with increased numbers or abnormally positioned seam nuclei were selected in a clonal EMS mutagenesis screen of ~3500 haploid genomes (Brenner, 1974) with *scm::GFP* transgenic animals (Terns et al., 1997). Our strategy aimed to evade maternally contributed larval lethality but allow identification of temperature-sensitive alleles. Adult F1 animals were washed two to three times to remove bacteria, and single animals allowed to lay eggs in 10 μl M9 containing 0.05% Tween 20 overnight at 15°C. Synchronized L2 progeny were fed, placed at 25°C and examined after 24 hours. At this time, wild-type animals finished the L3 seam divisions and nuclei could be scored prior to hyp7 fusion of anterior daughter cells. Mutants were backcrossed multiple times. SNP mapping was performed according to Davis et al. (Davis et al., 2005).

**Immunofluorescence**

Synchronized animals on poly-l-lysine-coated slides were freeze cracked and fixed with methanol (5 minutes at −20°C) and acetone (20 minutes at −20°C) (Duerk, 2006). Primary antibodies used were MH27 (1:20 supernatant or 1:200 concentrate; mouse, Developmental Studies Hybridoma Bank) and rabbit anti-GFP (1:100; Molecular Probes). Secondary antibodies used were Alexa 568 goat anti-mouse and Alexa 488 goat anti-rabbit (both 1:500; Invitrogen). Worms were mounted in Prolong Anti-Fade Gold (Invitrogen) supplemented with 2 μg/ml 4′,6-diamidino-2-phenylindole (DAPI; Sigma).

**RNAi-mediated interference**

Starved L1s were transferred onto alg-1 RNAi feeding plates and grown at 20°C. For strong knockdown by RNAi, we either injected double-stranded RNA into young adults or transferred synchronized L3 stage larvae to RNAi feeding plates and analyzed the next generation. For the dpy-11 RNAi experiments, several generations of animals were grown on RNAi food to ensure the most severe dumpy phenotype.

**Molecular cloning**

A 1.6 kb wrt-2 promoter fragment was amplified by PCR (primers 5′-CTCAAGCTTCAAGTCGACTCCAGGTAATT-3′ and 5′-TAGGATCCCCGAGAAACAATTGCGAGTTG-3′) from plasmid pPD95.69 containing *Pwrt-2::gfp* [a kind gift from Thomas Bürglin (Aspöck et al., 1999)], and cloned into the PCGSI expression vector with HindIII and BamHI. Expression constructs for GFP::H2B and GFP::PH were recloned from pPS132 (*Ppie-1::GFP::H2B*) and pAA1 into the BamHI site of the *Pwrt-2-containing* PCGSI vector. Constructs were injected into N2 animals at 5 ng/μl together with *Plin-48::mCherry* (15 ng/μl) and digested λ DNA (60 ng/μl). The alg-1 rescue construct was created by combining independent PCR reactions of an 8.3 kb genomic alg-1 fragment containing the alg-1 promoter, intron/exon sequences and 3′UTR. The PCR mix was injected into he210 animals (3-5 ng/μl) together with *Pplin-48::tdTomato* (15 ng/μl) and digested λ DNA (60 ng/μl).

**Microscopy**

Time-lapse movies of larval development were recorded at 30-second intervals for 2-7 hours at 20°C using a 63× 1.4 N/A PlanApochromat objective and a motorized microscope (Zeiss Axioplan). Animals were sedated with 10 mM muscimol (M1523 Sigma) and mounted on 5% agarose. Coverslips were sealed with Zeiss Immersol 518N oil to prevent liquid evaporation. GFP excitation light from the X-Cite source was filtered through a 470 nm long pass filter and a 490 nm cutoff filter. The emission was transmitted through a 520 nm long pass filter and a 590 nm cutoff filter. The images were visualized using a CoolSNAP HQ camera (Photometrics, Tucson, Arizona) linked to a Leica TCS SP2 laser scanning confocal microscope (Leica Microsystems, Bannockburn, Illinois) equipped with an HCX APO 63×/1.40 N/A objective (Leica). Images were acquired using the Leica Confocal Software. For confocal microscopy, the DAPI filter set was used with excitation through a 405 nm diode laser and emission detected between 420 and 490 nm. For the fluorescence intensity analysis, the X-Cite 120i fiber optic lamp was used. Deconvolution was performed using Slidebook 5.0 software (Intelligent Imaging Innovations, Tucson, Arizona) and labeled images using Adobe Photoshop 6.0 (Adobe Systems, Mountain View, CA). Table 1. Strains used

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
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<tbody>
<tr>
<td>JR667</td>
<td>unc-119(e2498::Tc1) III; wls51[scm::gfp; unc-119(+)]</td>
</tr>
<tr>
<td>SV875</td>
<td>unc-119(e2498::Tc1) III; alg-1(he210) X; wls51s</td>
</tr>
<tr>
<td>SV1009</td>
<td>hels63[Pwrt-2::GFP::PH; Pwrt-2::GFP::H2B; Plin-48::mCherry]</td>
</tr>
<tr>
<td>SV1050</td>
<td>unc-119(e2498::Tc1) III; alg-1(he210) X; wls51; heEx257[alg-1(+); Plin-48::mcherry]</td>
</tr>
<tr>
<td>SV1045</td>
<td>alg-1(he210) X; hels63[may contain (unc-119(e2498::Tc1) III; wls51s]</td>
</tr>
<tr>
<td>CB185</td>
<td>lan-1(e185) III</td>
</tr>
<tr>
<td>VT1145</td>
<td>lin-46(ma164) lin-58 &amp; mir-241(nDf51) V; mir-84(n4037) X</td>
</tr>
<tr>
<td>VT581</td>
<td>dpy-5(e61) lin-28(n719 I; lin-46(ma164) unc-76(e911) V</td>
</tr>
<tr>
<td>VT1101</td>
<td>lin-28(n719 I; lin-46(ma164) lin-58 &amp; mir-241(nDf51) V; mir-84(n4037) X</td>
</tr>
<tr>
<td>MT1524</td>
<td>lin-28(n719 I</td>
</tr>
<tr>
<td>MT2306</td>
<td>lin-28(n1119) I</td>
</tr>
<tr>
<td>HS1486</td>
<td>unc-76(e911) V; osls13[appr-1::appr-1::venus; unc-76 (+)]</td>
</tr>
<tr>
<td>WM53</td>
<td>alg-2(ok304) II</td>
</tr>
<tr>
<td>JI136</td>
<td>unc-119(e2498::Tc1) III; zuEx2[unc-119 (+) hmp-1::GFP]</td>
</tr>
<tr>
<td>OLB19</td>
<td>dusts[let-413::gfp; rol-6[ju1006]]</td>
</tr>
<tr>
<td>JK3437</td>
<td>him-5[1490] V; qbs74[pop-1::pop-1::gfp]</td>
</tr>
<tr>
<td>SV1176</td>
<td>rol-1(e911) miz-14(778) II; miz-35[pmec-7::gfp; lin-15(+)]; hels63; heEx119 [myo-2::GFP; R0689]</td>
</tr>
<tr>
<td>SV1124</td>
<td>dpy-11(e224); hels63</td>
</tr>
<tr>
<td>SV1125</td>
<td>lin-46(ma164) lin-58 &amp; mir-241(nDf51) V; mir-84(n4037) X; heEx421([Pwrt-2::GFP::PH; Pwrt-2::GFP::H2B; Plin-48::mCherry]</td>
</tr>
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</table>
RESULTS

The alg-1 Argonaute gene is required for normal epithelial stem cell-like division

To identify regulators of the cell division axis, we performed a genetic screen for mutants with abnormally positioned seam cell nuclei, based on seam-specific expression of the green fluorescent protein (scm::GFP; see Materials and methods). Of several identified mutants, he210 larvae displayed the most dramatically disorganized seam cell phenotype (Fig. 1). Starting at the L3 developmental stage, he210 mutant larvae showed an increased number of seam nuclei [average 22±4.6 after fusion in L3 versus 16±0.3 in wild type (±s.d.)] and an accumulation of nuclei outside the normally linear row (Fig. 1D). Some of the nuclei outside the row lost expression of the seam cell marker (data not shown), the normally linear row (Fig. 1D). Some of the nuclei outside the row, became more severe during the L4 stage (see below). Adult he210 animals displayed a normal overall morphology, but showed defects in the formation of adult aalae, frequently contained a protruding vulva or had burst at the vulva, and produced few progeny. Thus, the he210 mutation severely affects the seam lineage and a limited number of other cells.

We located the he210 mutation to a single base pair alteration in the alg-1 Argonaute gene. This mutation is predicted to change glycine 716 to arginine in the conserved PIWI domain (Fig. 1G). Introduction of PCR-amplified genomic alg-1 DNA rescued the he210 mutant phenotype to wild type (20/28 animals). Moreover, feeding RNAi of alg-1 during larval development phenocopied he210 (see below; Fig. 1H). Thus, downregulation of ALG-1 leads to the overproliferation and mislocalization of seam cells.

ALG-1 controls seam organization independently of the developmental timing pathway

ALG-1 is the core component of an RNA-induced silencing complex (RISC), which, in association with miRNAs, represses specific mRNAs (Grishok et al., 2001). The best-characterized miRNAs are key regulators of the heterochronic developmental timing pathway, including the pioneering lin-4 and let-7 miRNAs and the let-7s miRNAs mir-48, mir-84 and mir-241 (Abbott et al., 2005; Lee et al., 1993; Reinhart et al., 2000). These miRNAs require ALG-1, or the closely related ALG-2, for their function (Grishok et al., 2001; Tops et al., 2006). In agreement, alg-1 has also been associated with a heterochronic phenotype and increased seam cell numbers (Cai et al., 2008; Grishok et al., 2001).

We observed that alg-1 feeding RNAi in the alg-2(ok304) mutant reduced the seam cell number to ~10 (Table 2). This indicates that ALG-1/ALG-2 double inhibition blocks lin-4 miRNA function and prevents the L2-specific proliferative divisions. By contrast, homozygous alg-1(he210) mutants and larvae exposed to alg-1 RNAi by feeding from the L1 stage onward invariably formed more than 16 seam cells. In such alg-1 feeding RNAI larvae, most seam cells in the V1-V4 and V6 lineages completed two rounds of division during the L3 stage, which increased the seam cell number in late L3 larvae from 16 to 24±4.9 (±s.d.). Thus, alg-1 feeding RNAI and alg-1(he210) mutation cause a heterochronic phenotype that resembles let-7s miRNA inhibition, with repeated proliferative divisions and extra seam nuclei.

Fig. 1. Identification of ALG-1 Argonaute as a regulator of seam epithelium organization. (A) Seam cells (green) are aligned in a linear row on each lateral side of C. elegans larvae. (B) Postembryonic seam cell lineages. y-axis indicates time (hours) of development. Circle color indicates fate: green, seam cells; blue, hyp7 fusion fate; red, neuronal fate; cross, apoptosis. V1-V4 and V6 undergo a proliferative division in early L2. (C,D) Wild-type (WT) and he210 larvae after seam cell divisions in L4. Expression of scm::GFP (green) marks seam cell nuclei. The he210 mutant shows additional and abnormally positioned seam nuclei (D, arrowhead). (E,F) Seam cells visualized by apical junction staining (red, AJM-1) and DNA (blue, DAPI) of wild-type (E) and he210 (F) animals prior to L4 stage divisions. Arrowheads in F marks the disorganized seam. (G) he210 contains a G-to-A transition in alg-1, which is predicted to change glycine 716 to arginine in the conserved PIWI domain. (H) alg-1 RNAI results in seam cell defects similar to he210. L4 stage animal with disorganized seam cells (arrowhead). Seam-specific GFP::H2B and GFP::PH mark the DNA and cell membranes, respectively. Scale bars: 10 μm.
We examined whether the abnormal positions and gaps between seam cells are also normal aspects of the retarded heterochronic phenotype. Retarded daf-12(rh285) larvae showed a moderately increased number of seam cells, but these cells invariably remained present in a single linear row (Table 2). To obtain stronger reiterative L2 divisions, we combined mutation of the three let-7s miRNAs and lin-46, which act in parallel to promote the L2-to-L3 transition (Abbott et al., 2005). These quadruple mutants showed substantially increased numbers of seam cells [average 26±0.8 (±s.d.); Table 2], which again invariably remained aligned in a single row (Fig. 2C; 100%, n=160 cells observed in 16 animals). However, when these mutants were exposed to alg-1 feeding RNAi, the seam cell pattern became disorganized, with gaps and cells located outside the row, similar to the alg-1 RNAi phenotype (Fig. 2D; 100%, n=6 animals). Thus, disorganization of the seam epithelium does not automatically result from increased cell numbers, but indicates an alg-1 function that is independent of the let-7s miRNAs.

Notably, alg-1 RNAi induced very few abnormally positioned seam cells in lin-28 mutants (Fig. 2F; 2 of 11 animals showed a single mispositioned seam cell). As lin-28 is a target for inhibition by lin-4 miRNA, we examined whether enhanced lin-28 activity contributes to the alg-1 disorganized seam phenotype. Mammalian LIN28 is highly expressed in stem cells and can contribute to the conversion of differentiated somatic cells to induced pluripotent stem (iPS) cells (Yu et al., 2007). C. elegans lin-28 mutants skip the L2 stage proliferative seam cell divisions and contained only ~10 seam cells (Table 2). However, larvae with combined lin-28, lin-46 and let-7s mutations reiterated L2 divisions during the L3 and subsequent stages (Abbott et al., 2005) (Table 2). These quintuple mutants showed a substantial increase in the number of seam cell nuclei [average 29±0.7 after fusion (±s.d.)], which remained localized in a continuous linear row (Fig. 2G; 100%, n=609 cells in 21 animals), unless alg-1 was also inhibited (Fig. 2H; 100%, n=27 animals). Thus, alg-1 RNAi causes disorganization of the seam epithelium independently of lin-28. In summary, ALG-1 promotes the developmental timing of seam cell divisions through the heterochronic pathway and has an additional role in organizing the seam epithelium.

**ALG-1 controls the orientation of the cell division axis and motility of the cell membranes**

To examine whether alg-1 inhibition causes an abnormal seam cell division axis we generated seam cell-specific GFP reporters that mark the cell membrane (Pwrt-2::GFP::PHPLC1) and DNA (Pwrt-2::GFP::H2B), and established a protocol for time-lapse recording.
of the developing larvae. Animals with integrated reporter transgenes were synchronized at the early L1 stage, transferred to control bacteria or exposed to alg-1 feeding RNAi, and followed for various time periods of larval development by time-lapse fluorescence microscopy (see Materials and methods and Movies 1-4 in the supplementary material).

This approach revealed abnormally oriented seam cell divisions in alg-1(RNAi) larvae from the early L3 stage onward. In the wild type, chromosomes invariably segregated along the anterior-posterior axis, with a maximal deviation of 10 degrees (Fig. 3A-A'/H11033,C,D). After alg-1 RNAi, the division axis was less stringent during the reiterated proliferative divisions in L3 (Fig. 3E), and even more abnormal during the subsequent second round of division in the L3 stage (Fig. 3B-B'/F). We even observed cell divisions perpendicular to the normal anterior-posterior axis (a deviation of 90 degrees) (see Movie 2 in the supplementary material). Thus, ALG-1 is required for normal orientation of seam cell division.

Our time-lapse recordings also revealed an additional mechanism for abnormal seam organization. In contrast to wild-type animals, alg-1 RNAi-treated L3 larvae showed highly motile seam cell membranes with apparent ruffles and extensions (11 out of 12 animals; see Fig. S2 in the supplementary material). As a potential consequence of this high motility, we observed two initially neighboring cells extending next to each other (Fig. 3G-G'/H11033; see Movie 3 in the supplementary material). Thus, seam cells

**Fig. 3. ALG-1 regulates the seam cell division axis and cell motility.** (A-B') Still images from time-lapse recordings of asymmetric divisions in wild-type and alg-1(RNAi) L3 C. elegans larvae. (A-A') Normal seam cells divide along the anterior-posterior axis. (B-B') alg-1 inhibition changes the seam cell division axis. Metaphase (A, arrowhead B), anaphase (A’, arrowhead B’) and telophase (A”, arrowhead B”). Time is indicated from metaphase onward. (C-F) Quantification of cell division angles (C) in wild-type (D) and alg-1(RNAi) (E,F) L3 larvae. (G-G”) A sequential series of time-lapse microscopy images of seam cells migrating after division (arrowheads) in an alg-1(RNAi) L3 larva. The migrating cells end up outside the row. Cell migration time is indicated. (H,I) Late alg-1(RNAi) larvae show disconnected seam cell clusters (H) and occasional networks of aberrantly connected seam cells (I). Seam-specific GFP::H2B and GFP::PH mark DNA and cell membrane, respectively. Scale bars: 10 μm.

### Table 2. Summary of seam cell phenotypes in several genetic backgrounds

<table>
<thead>
<tr>
<th>Genetic background</th>
<th>Seam number (as.d.)</th>
<th>Orientation defects</th>
<th>Loss of contacts</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Changes in cell shape and Wnt signaling affect the division axis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT (N2)</td>
<td>16±0.3</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>alg-1(he210)</td>
<td>22±4.6</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>alg-1(RNAi)</td>
<td>24±4.9</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>let-7s + lin-46(ma164)</td>
<td>26±0.8</td>
<td>–*</td>
<td>–</td>
</tr>
<tr>
<td>let-7s + lin-46(ma164) + alg-1(RNAi)</td>
<td>25±2.1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>let-7s + lin-46(ma164) + mig-14(RNAi)</td>
<td>ND</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>lin-28(n719)</td>
<td>10±0.5</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>lin-28(n719) + or(n947) + alg-1(RNAi)</td>
<td>ND</td>
<td>+/–</td>
<td>+</td>
</tr>
<tr>
<td>lon-1(e185)</td>
<td>16±0.7</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>lon-1(e185) + alg-1(RNAi)</td>
<td>22±3.4</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>dpy-11(RNAi)</td>
<td>16±0.3</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>mig-14(or87)</td>
<td>16±0.7</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>mig-14(or87) + dpy-11(RNAi)</td>
<td>17±1.3</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>hmr-1(RNAi)</td>
<td>16±1.3</td>
<td>–</td>
<td>+/-</td>
</tr>
<tr>
<td>hmr-1(RNAi) + dpy-11(e224)</td>
<td>16±0.9</td>
<td>–</td>
<td>+</td>
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<tr>
<td>B. alg-1 and alg-2 are partially redundant</td>
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<tr>
<td>alg-2(ok304)</td>
<td>15.9±0.74</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>alg-1(he210) + alg-1(RNAi)</td>
<td>16.1±0.75</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>alg-2(ok304) + alg-1(RNAi)</td>
<td>10.8±1.9</td>
<td>–</td>
<td>+</td>
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</tbody>
</table>

Seam cells were counted after L3 divisions, fusion with hyp7 and reconnection of seam cells (column 2 in A). Division orientation defects were scored during L3 and L4 divisions. The plus sign indicates defects, whereas minus indicates normal. Note that let-7s indicates triple inactivation of let-7 sister miRNAs through mir-48 mir-241(nDf51); mir-84(n4037) mutation.

*Defects were seen in late L4 division: 8 of 23 cells showed a division orientation defect.

ND, not determined.
in alg-1(RNAi) larvae show aberrant cell division axes and hypermotile cell membranes, which lead to a highly disorganized seam epithelium.

**ALG-1 affects seam cell fate and localization of Wnt/β-catenin asymmetry pathway components**

To determine how alg-1 affects the axis of seam cell division, we searched for abnormalities that precede the abnormal division orientation. Using time-lapse fluorescence microscopy, we detected abnormalities as early as the L2 division program. At the L2 stage, the V1-4 and V6 seam cells undergo a proliferative symmetric division followed by an asymmetric division in which the anterior daughter fuses with hyp7 (Fig. 1B, Fig. 4A–A″) (Sulston and Horvitz, 1977). In alg-1(RNAi) larvae, we occasionally observed pairs of cells of which both the posterior and anterior daughter fused with the hyp7 syncytium (Fig. 4B-B″, 2 of 30 pairs in three animals). Moreover, we observed that some anterior daughters failed to fuse, and that some seam-derived hyp7 nuclei were missing (4 of 49 cells; data not shown). All seam cells recombine after fusion to form a continuous row in wild-type animals, whereas some gaps remained in alg-1(RNAi) larvae (data not shown). A few other seam cells appeared hyperextended, suggesting that they closed gaps generated by aberrant hyp7 fusion events (data not shown). These results illustrate that alg-1 RNAi leads to occasional defects in the segregation of the seam versus non-seam (hyp7) fate.

The defects in daughter cell fate could reflect defects in the Wnt/β-catenin asymmetry pathway (Herman, 2001; Mizumoto and Sawa, 2007a; Takeshita and Sawa, 2005a). This pathway promotes the seam fate through export of the POP-1 TCF transcriptional repressor and activation of POP-1–SYS-1 β-catenin in the posterior nucleus (Kidd et al., 2005; Shetty et al., 2005). After asymmetric division, the anterior non-seam daughter cell contains high levels of nuclear POP-1 and the posterior seam daughter cell contains low levels of nuclear POP-1 (Fig. 4D) (Phillips and Kimble, 2009). The upstream component APR-1 APC localizes asymmetrically to the anterior cortex of seam cells before division, and becomes enriched in the anterior daughter cell after division (Fig. 4G) (Mizumoto and Sawa, 2007b). Surprisingly, we observed that POP-1 and APR-1 localize asymmetrically not only during the asymmetric L2 divisions, but also during the proliferative symmetric divisions in wild-type L2 stage larvae (Fig. 4C,F). Thus, the symmetric L2 divisions seem to uncouple high nuclear POP-1 levels from the hyp7 fate. Immunostaining of POP-1 in alg-1(RNAi) larvae showed occasionally reversed POP-1 asymmetry after asymmetric L2 divisions (2 of 60 cell pairs in nine alg-1(RNAi) L2 larvae versus

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Fig. 4. ALG-1 regulates the localization of Wnt pathway components and cell fate. (A-B″) Time-lapse microscopy shows fusing seam daughter cells after division in wild-type (A–A″) and alg-1(RNAi) (B–B″) L2 stage *C. elegans* larvae. alg-1 RNAi occasionally leads to the fusion of neighboring daughter cells (arrowheads in B–B″). Time is measured from completion of cytokinesis onward. Seam-specific GFP::H2B and GFP::PH mark the DNA and cell membrane, respectively. (C-E) POP-1 TCF/LEF asymmetry. In wild-type L2 larvae, POP-1 levels are high (arrowheads) in anterior daughter nuclei after symmetric (C) and asymmetric (D) cell divisions. After alg-1 RNAi, localization of POP-1 is occasionally reversed (E, arrowheads point to high-level nuclear POP-1). Green, POP-1; red, AJM-1. (F-L) APR-1::Venus localization in wild-type and alg-1(RNAi) larvae. APR-1 APC (green) is enriched at the anterior cortex during symmetric (F) and asymmetric (G) cell division in wild-type (arrowheads) and remains enriched at the cortex of the anterior daughter cell after division (the left-hand cells within the brackets in F,G,I). After alg-1 RNAi, APR-1 can be posteriorly enriched or fails to localize (arrowhead, H), and an abnormally localized APR-1 crescent has been observed (compare K with L; dotted lines indicate the metaphase plate). pd, neuronal postdereid cells. Scale bars: 10 μm.
0 of 62 cell pairs in 13 wild-type animals] (Fig. 4E). By the L3 stage, abnormal POP-1 localization was much more prominent [34 of 183 cell pairs (19%) in 14 animals]. Live imaging of APR-1::Venus localization showed occasional enrichment of APR-1 at the posterior cortex prior to L3 division in alg-1(RNAi) larvae (3 of 240 cells in 30 animals) (Fig. 4H), or high APR-1 localization all around the cell membrane (6 of 240 cells) as compared with no abnormal APR-1 localization in wild-type animals (n=300 cells in 30 animals). By the L3 divisions, abnormal APR-1 localization was detected in 38 of 168 (23%) seam cells (or cell pairs after division) (Fig. 4J,L). These observations indicate that ALG-1 contributes to the proper localization of Wnt/β-catenin asymmetry pathway components and to cell fate acquisition of the seam cell daughters.

The upstream components of the Wnt/β-catenin asymmetry pathway not only control cell fate, but also the cell division axis of specific embryonic blastomeres (Cabello et al., 2010). As a possible explanation for the abnormally oriented divisions in alg-1 larvae, we examined whether Wnt signaling controls the seam cell division axis in wild-type animals. As C. elegans expresses five different Wnt ligands, we focused on the transmembrane protein MIG-14 Wntless, which is thought to be generally required for Wnt secretion (Bänziger et al., 2006; Pan et al., 2008; Yang et al., 2008). We used the lethal mutation mig-14(or87), rescued with an extrachromosomal array with wild-type MIG-14 Wntless, which is thought to be generally required for Wnt secretion (Bänziger et al., 2006). Larvae that do not inherit the extrachromosomal array from the mother complete embryogenesis but lack zygotic mig-14 function. To reduce the risk of residual maternal function, we studied seam cell divisions at the L3 and L4 larval stages. Interestingly, 48% of the seam cell divisions in mig-14 larvae (n=35 cell divisions in five animals) showed a reversed fate, such that the anterior daughter remained present as a seam cell and the posterior daughter cell fused with the hyp7 syncytium (Fig. 6E). However, we never observed cells outside the seam cell row and seam-seam cell contacts were invariably retained. Thus, whereas alg-1 affects the Wnt/β-catenin pathway and seam cell fate determination, loss of Wnt signaling alone does not lead to seam cell division with an abnormal axis.

**The elongated seam cell shape is an important cleavage plane determinant**

We considered other factors that might contribute to the abnormally oriented divisions in alg-1 larvae. The disrupted contacts between seam cells could be important, as cell junctional contacts contribute to cell polarity, proliferation control, tissue organization and cell division orientation (Lu et al., 2001). However, immunostaining of AJM-1 showed that the localization of apical junctions is maintained in seam cells that are detached (see Fig. S1 in the supplementary material). Based on the localization of LET-413 Scribble and HMP-1 α-catenin, alg-1 RNAi did not prevent the establishment of apical-basal polarity (see Fig. S1 in the supplementary material). Moreover, most seam cells in alg-1(RNAi) larvae remained connected to at least one neighbor. A comparison of seam cells connected on both sides with seam cells neighboring a gap did not reveal a significant difference in the frequency or angle of aberrant divisions (see Fig. S1E,F in the supplementary material). Finally, partial reduction of hmr-1 E-cadherin in wild-type animals with hmr-1 feeding RNAi occasionally disrupted seam cell contacts [Fig. 6C; 13 of 400 cells (3.25%) in 40 animals], but these cells did not change their orientation of division. We conclude that partial loss of seam cell contacts does not lead to abnormally oriented seam cell divisions.

We noticed that seam cells that were relatively round after alg-1 RNAi frequently showed an aberrant division axis. Indeed, measurements of the seam cell length (X) and width (Y) showed a significant correlation between increased roundness (X/Y closer to 1) and abnormal axis of division (P=0.009) (Fig. 5C). Examination of z-stack images obtained by confocal microscopy showed that cells round up in all three dimensions. The normally relatively flat

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**Fig. 5. The seam cell division axis follows geometric constraints.** (A,B) z-stacks of seam cells in wild-type and alg-1(RNAi) L4 C. elegans larvae. Normal seam cells are flat and elongated (A),alg-1 RNAi results in rounded seam cells (B). Red and green lines indicate the position of the cross-sections shown at the top and left, respectively. The star marks a cell fusing with hyp7. GFP::H2B and GFP::PH mark the DNA and cell membrane, respectively. (C) Elongation factor [cell length (X) divided by width (Y)] comparison between cells with normal versus abnormal division axes in alg-1(RNAi) larvae (second division in L3). Division angles were measured at anaphase onset. Bars indicate the average X/Y ratio±s.d. (D) Cell elongation factor during interphase in several mutant/RNAi backgrounds. Dev timing refers to lin-28(n719); lin-46(ma164) triple let-7s mutant. Error bars indicate s.d. (E-G) Seam cell elongation suppresses aberrant cell division orientation. Seam cells are disorganized in alg-1(RNAi) larvae (E, arrow), whereas they are stretched in lon-1 larvae (F), alg-1 RNAi-treated lon-1(e185) mutant shows disconnected seam-cell-cell contacts (arrow, G), but no seam cells outside the row (G). Red, AJM-1; blue, DNA (DAPI). Scale bars: 10 μm.
seam cells became progressively more spherical during the L3 to L4 stage of development in alg-1(RNAi) larvae (compare Fig. 5A with 5B).

To further investigate whether the rounded shape influences the division axis, we used the abnormally long (Lon) mutant lon-1(e185). These mutants display, on average, a 25% increased body length (Maduzia et al., 2002). Examination of lon-1 mutants showed that seam cells are present in normal numbers, form a single row, and are more stretched compared with those in the wild type (Fig. 5F). Inhibition of alg-1 in lon-1(e185) mutants resulted in gaps between the seam cells, but the seam cells almost invariably remained present in a single linear row (Fig. 5G) (only 1 of 16 lon-1(e185), alg-1(RNAi) animals formed a single small seam cell cluster; 7 of 12 control alg-1(RNAi) animals formed one or more groups of misplaced seam cells). These data indicate that the elongated cell shape contributes an important cue for the orientation of seam cell divisions. The rounding of seam cells after alg-1 loss of function eliminates this cue.

Redundant control of the anterior-posterior division axis
To further investigate the influence of cell shape on the seam cell division plane, we examined whether Dpy (short and fat) animals have fewer elongated seam cells. As a severe Dpy mutant, we used dpy-11, which encodes a thioredoxin-like enzyme that probably modifies collagens. Feeding RNAi of dpy-11(e224) caused a weak seam cell fate phenotype in L4 larvae (only 1 of 56 cells showed a reversed fate). Nevertheless, the mig-14(RNAi); lin-46 let-7s mutant combination resulted in abnormally oriented seam cell divisions and a highly disorganized seam epithelium (15 of 23 cells showed abnormal division orientation) (Fig. 6F; see Movie 4 in the supplementary material). We conclude that multiple factors promote the anterior-posterior orientation of seam cell divisions, including the elongated cell shape, the Wnt/β-catenin asymmetry...
pathway and cell junctional contacts. Because of redundancy between these contributions, the orientation of seam cell division is robust and only becomes abnormal when two different levels of control are lost.

**DISCUSSION**

In this study, we examined the regulation of the cell division axis in a *C. elegans* epithelial stem cell lineage. Mutants with abnormally oriented seam cell divisions appeared relatively rare; in a moderately sized screen, only the *alg-1*(he210) mutant stood out for its remarkably disorganized seam epithelium. Detailed analysis of this phenotype uncovered novel roles for ALG-1 in contributing to proper asymmetry in the Wnt/β-catenin pathway, cell adhesion, cell shape, cortical dynamics and division orientation, in addition to its previously reported role in the heterochronous pathway. Cell rounding in *alg-1*(RNAi) larvae correlated with abnormally oriented divisions, and introduction of a lon-1 mutation suppressed both defects. Unexpectedly, we found that mig-14 Wntless, a transmembrane protein dedicated to Wnt ligand secretion (Bänziger et al., 2006; Pan et al., 2008; Yang et al., 2008), controls the anterior-posterior pattern of seam and non-seam cell fates. Moreover, when combined with reduced seam cell elongation, inactivation of mig-14 resulted in a highly disorganized seam epithelium. These data support redundant roles for Wnt signaling and cell shape in determining the orientation of seam cell division (see Fig. S3 in the supplementary material).

**alg-1 provides multiple developmental functions**

*C. elegans* alg-1 was known to contribute to the developmental timing of larval development (Cai et al., 2008; Grishok et al., 2001; Morita and Han, 2006), but not to the control of the cell division axis. Although ALG-1 probably acts with miRNAs to control the division axis, it remains unclear which miRNAs are involved. Recent studies identified large numbers of high-confidence miRNA-mRNA interactions, which are associated with various biological processes (Chan et al., 2008; Zhang et al., 2007; Zisoulis et al., 2010). Genetic confirmation of such interactions is hampered by redundancies between miRNAs and, in general, by the modest inhibition of target genes by individual miRNAs (Baek et al., 2008; Inui et al., 2010). Key targets should be upregulated after *alg-1* inhibition and thereby contribute to disrupted Wnt signaling, increased cortical dynamics, loss of adherence or cell rounding. The effects could be indirect, as indicated by *ipla-1*, which is the only gene previously found to act in seam division orientation (Kanamori et al., 2008). IPLA-1 has phospholipase A(1) activity and is needed for proper localization of WRM-1 β-catenin and membrane lipid composition (Imae et al., 2010; Kanamori et al., 2008). Future studies might focus on candidate genes that are present in the target lists, including Wnt pathway components and regulators of cell adhesion, membrane transport and cortical contractility.

**Wnt signaling depends on alg-1 and promotes tissue polarity**

Several observations implicate *alg-1* in the Wnt/β-catenin asymmetry pathway. During seam cell division, several Wnt signaling components become asymmetrically localized to the anterior (e.g. APR-1 APC) or the posterior (e.g. LIN-17 Frizzled) cortex (Mizumoto and Sawa, 2007a; Mizumoto and Sawa, 2007b; Sawa et al., 1996; Takeshita and Sawa, 2005b). This leads to activation of the POP-1–SYS-1 β-catenin transcriptional complex in the posterior nucleus (Kidd et al., 2005; Shetty et al., 2005), and establishment of the posterior seam fate. Interestingly, we observed that the L2-specific symmetric divisions also showed asymmetric APR-1 and POP-1 localizations. Hence, the seam cell fate might be established independently of Wnt/β-catenin signaling during the proliferative L2 divisions.

A recent study suggested a role for Wnt/β-catenin asymmetry signaling upstream of the *alg-1*/miRNA heterochronous pathway (Ren and Zhang, 2010). Our results indicate that *alg-1*, independently of the heterochronous pathway, also affects Wnt signaling. The observed contribution of Wnt ligands was remarkable, as only the V5 and T seam cell lineages were previously shown to depend on Wnt ligands (Herman et al., 1995; Wangbo et al., 2000). Mutation of mig-14 Wntless was previously reported to have no effect on the seam cell number (Gleason and Eisenmann, 2010). However, we found that mig-14 mutation causes a randomized order of seam and non-seam cell fates. This indicates that seam cells can polarize independently of Wnt ligands, but coordinating this polarity with respect to the anterior-posterior body axes requires Wnt. The *mig-14* phenotype thus resembles a tissue polarity or planar cell polarity (PCP) phenotype, in that cells fail to coordinate their polarity with the plane of the epithelium. Similar observations have been made for a quintuple Wnt mutant (Yamamoto et al., 2011).

**The elongated seam cell shape depends on alg-1 and promotes anterior-posterior division**

Although *alg-1* RNAi caused seam cells to lose adhesive connections, this did not appear to cause abnormally oriented divisions. By contrast, reduced elongation of the seam cells clearly contributes to the aberrant anterior-posterior orientation of seam cell divisions. What causes the cell-shape change in *alg-1* mutants? Reiterated L2 proliferative divisions probably provide one mechanism; extra proliferative divisions add extra cells to the row and reduce elongation. Indeed, *alg-1* RNAi did not cause abnormal division orientation in *lin-28* mutants that skip L2 proliferative divisions. However, some of the seam cells in *alg-1*(RNAi) larvae were more rounded even before extra cells were formed, and cells rounded off in three dimensions (Fig. 5). This suggests that *alg-1* promotes seam cell elongation independently of cell number, possibly by affecting the actin cytoskeleton or adherence to the extracellular matrix.

It has long been recognized that cell shape affects the division axis through preferential orientation of the mitotic spindle along the longest axis of the cell (“Hertwig’s rule”) (Théry and Bornens, 2006). However, in a developmental context, cortical cues generally determine spindle orientation, as is well described for *C. elegans* early embryos and *Drosophila* neuroblasts and sensory organ precursor cells (Galli and van den Heuvel, 2008; Gonczy, 2008; Knoblich, 2008). Such cues can overrule the effect of geometric constraints. For example, PCP instructs a spindle position perpendicular to the long cell axis in dorsal tissue during zebrafish gastrulation (Gong et al., 2004).

Cells in culture round up during mitosis, yet orient the division axis according to their shape in interphase (Théry et al., 2007). During cell rounding, adhesive contacts with the extracellular matrix lead to the formation of retraction fibers (Théry et al., 2007; Toyoshima and Nishida, 2007). This creates cortical tension and provides anchor points in mitosis that presumably promote the stabilization of microtubule attachments and localization of force generators (Théry et al., 2007; Toyoshima and Nishida, 2007; Wen et al., 2004). Interestingly, although seam cells round off in mitosis, they remain attached at apical junctions through long thin extensions. Thus, tension and shape are partly maintained in
mitosis, which probably promotes spindle orientation. The cell rounding in alg-1 and dpy-11 larvae is likely to reduce the tension in the anterior-posterior direction and thereby the bias in spindle orientation.

Other factors, such as the stiffness and resistance of the surrounding tissue, are also important in creating tension (Erler and Weaver, 2009). The composition of the extracellular matrix and collagen cuticle contribute to these factors (Clark and Brugge, 1995; Erler and Weaver, 2009; Miranti and Brugge, 2002). As such, mutation of dpy-11 might affect seam cell division through reduced collagen rigidity. However, division orientation was affected by alg-1, lon-1, lin-28, dpy-11 and retarded heterochromatic mutations, which all change the seam cell elongation factor. Hence, cell shape is likely to be a major determinant.

Redundant control of the cell division axis

The redundant control of cell division orientation explains the low number of mutants identified in our screen. alg-1 is exceptional in that it affects cell shape as well as Wnt signaling. Redundant geometric and polarity contributions are probably used broadly to provide robust control over the cell division axis. Indeed, the elliptical egg shape contributes to longitudinal division of the C. elegans zygote even when PAR polarity is lost (Tsou et al., 2002). This insight can be used in future studies aimed at determining how each of the individual cues contributes to cell division orientation and how ALG-1 promotes seam cell elongation and Wnt signaling. Ultimately, these studies will improve our understanding of the formation and maintenance of normal epithelia and of the defects that contribute to carcinogenesis.

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Supplementary material

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Control of epithelial stem cell division

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