The Secretory Pathway Calcium ATPase PMR-1/SPCA1 Has Essential Roles in Cell Migration during Caenorhabditis elegans Embryonic Development

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Abstract

Maintaining levels of calcium in the cytosol is important for many cellular events, including cell migration, where localized regions of high calcium are required to regulate cytoskeletal dynamics, contractility, and adhesion. Studies show inositol-trisphosphate receptors (IP3R) and ryanodine receptors (RyR), which release calcium into the cytosol, are important regulators of cell migration. Similarly, proteins that return calcium to secretory stores are likely to be important for cell migration. The secretory protein calcium ATPase (SPCA) is a Golgi-localized protein that transports calcium from the cytosol into secretory stores. SPCA has established roles in protein processing, metal homeostasis, and inositol-trisphosphate signaling. Defects in the human SPCA1/ATP2C1 gene cause Hailey-Hailey disease (MIM #169600), a genodermatosis characterized by cutaneous blisters and fissures as well as keratinocyte cell adhesion defects. We have determined that PMR-1, the Caenorhabditis elegans ortholog of SPCA1, plays an essential role in embryogenesis. Pmr-1 strains isolated from genetic screens show terminal phenotypes, such as ventral and anterior enclosure failures, body morphogenesis defects, and an unattached pharynx, which are caused by earlier defects during gastrulation. In Pmr-1 embryos, migration rates are significantly reduced for cells moving along the embryo surface, such as ventral neuroblasts, C-derived, and anterior-most blastomeres. Gene interaction experiments show changing the activity of itr-1/IP3R and unc-68/RyR modulates levels of embryonic lethality in Pmr-1 strains, indicating pmr-1 acts with these calcium channels to regulate cell migration. This analysis reveals novel genes involved in C. elegans cell migration, as well as a new role in cell migration for the highly conserved SPCA gene family.

Introduction

Calcium is one of the most versatile and important small molecules in the cell: it is involved in cell processes starting at the beginning of new cell life during fertilization and ending with a role in cell death during apoptosis [1]. One developmental role for calcium is in cell migration. Studies have determined that migrating cells establish a relatively stable calcium gradient, with higher levels in the rear of the cell [2]. The increased calcium levels at the rear of the cell are likely important for regulating cell detachment and contraction [3,4]. More recently, studies identified transient flickers of high calcium enriched at the leading edge of migrating cells and preceding directional changes. These calcium flickers may provide localized cues that help direct migration [4–6].

Calcium-sensitive molecules important for cell migration can be divided into two broad categories: those that sense and respond to differences in calcium levels and those that control levels of calcium by transporting it across membranes [1]. In this second class, channels that release calcium into the cytosol, such as the plasma-membrane-associated transient receptor potential (TRP) channels, partnering with the secretory pathway inositol-trisphosphate channels (IP3R) and ryanodine receptors (RyR), have been shown to play a key role in producing calcium flickers and in cell migration [1,4,7,8]. The localized movement of calcium out of the cytosol must also be important for producing calcium flickers and gradients. Calcium uptake from the cytosol requires Ca2+ ATPase transporters found at three distinct subcellular locations: the plasma membrane (PMCA), the Sarco(endo)plasmic reticulum (SERCA) and the secretory pathway/Golgi (SPCA) [1,9,10]. SERCA, which localizes to both the endoplasmic reticulum (ER) and Golgi, helps to control cytosolic calcium levels in a variety of cells and is integral for proper IP3 signaling [1]. The Golgi-localized SPCA, which has not been as extensively characterized, transports both Ca2+ and Mn2+, playing an important role in homeostasis for both molecules. SPCA is also critical for protein...
Author Summary

During growth or regeneration after damage, skin cells migrate from basal to superficial layers, forming tight attachments that protect an individual from environmental assaults. Proteins that remove calcium from the cell cytosol into secretory stores, where it is available for future release, play a key role in skin cell integrity. Defects in these secretory pathway calcium ATPase (SPCA) channels in humans cause Hailey-Hailey disease, a chronic disorder marked by skin lesions in areas of high-stress. Our study of the SPCA gene pmr-1 in Caenorhabditis elegans indicates the gene is essential for viability. Embryos with defective PMR-1 die with cell attachment defects superficially similar to those of Hailey-Hailey disease patients. To better understand this phenotype, we tracked the position of individual cells during development of pmr-1 mutant embryos. This analysis revealed that the cell attachment defects are caused by primary failures in cell migration. We also identified other calcium channel proteins involved in this process, indicating proper regulation of calcium is crucial for cell migration in C. elegans. If SPCA proteins act similarly in humans, this research will lead to better understanding of the molecules important for skin cell regeneration, as well as help to explain the defects observed in Hailey-Hailey disease patients.

Processing in the Golgi, Ca$^{2+}$ release in response to IP3 signaling, and stress tolerance [1,9,11]. While at least one of these Ca$^{2+}$ ATPase proteins is likely to be involved in maintaining subcellular calcium gradients important for cell migration, this link has yet to be firmly established.

Genetic studies show the highly conserved SPCA1 protein has essential roles in embryonic development and disease. Loss of a single copy of the SPCA1 gene in humans causes Hailey-Hailey disease (MIM #169600), a skin disorder characterized by recurrent lesions or blisters of the skin in areas subject to high stress [12–16]. SPCA1 is likely essential in humans, as more severe phenotypes are found in patients who suffer clonal loss of both copies of the gene [17,18]. Mice embryos homozygous for null mutations in SPCA1 die with defects in neural tube closure, while heterozygotes show susceptibility to squamous cell tumors, a phenotype observed occasionally in humans with Hailey-Hailey [19–22]. The primary cellular defect in Hailey-Hailey disease patients is keratinocyte acantholysis or loss of cell adhesion, marked by aggregation of keratin intermediate filaments that have retracted from desmosomes [23,24]. In mice, cell adhesion appears normal, but cells show signs of Golgi dysfunction, which likely induces the high levels of apoptosis observed in the neural tube and mesenchyme [19]. Based on this genetic analysis, it is not evident that SPCA, which has a conserved role at the molecular level, has a similarly conserved role in development, although each phenotype may be the result of secretory pathway stress [25].

Caenorhabditis elegans has served as a good model system for studying calcium signaling because it has the full complement of membrane-associated calcium channels. Research has revealed a role for the calcium channel ITR-1/IP3R in oocyte maturation, gastrulation, pharyngeal pumping, defecation and other developmental processes [26]. It is expressed in the embryo and is the only channel shown to play a direct role in cell migration, as itr-1 mutants fail to complete ventral closure during C. elegans development [26–28]. The UNC-68/RyR localizes to both ER and Golgi and plays a key role in muscle function [7,29–32]. The single SPCA1 gene, pmr-1, encodes a protein that, like its vertebrate counterpart, co-localizes with Golgi markers, when expressed in COS cells or in C. elegans [33–35]. Based on RNA in situ hybridization and microarray analysis, pmr-1 is expressed in the adult germ line and at moderate levels throughout embryogenesis in most blastomeres [36–38]. pmr-1 transcriptional and translational fusion constructs show expression starting from the 3-fold embryo and continuing until the adult stage in the nervous system, intestine, intestinal valve, hypodermis, spermatheca, and gonad [35,39]. The absence of germ line and early embryo expression for these fusion constructs may indicate there are silenced [40] or contain incomplete regulatory sequences. PMR-1 protein also shows functional conservation, with important roles in Ca$^{2+}$ spiking in response to IP3 signaling, stress response, thermotolerance, pathogen resistance, and metal homeostasis [26,33–35,41,42].

Using alleles of pmr-1 obtained in forward genetic and deletion screens, we have identified a new, essential role for pmr-1 in embryonic development in C. elegans. Strains homozygous for mutations in pmr-1 show temperature-sensitive embryonic lethality caused by defects in ventral enclosure, apical enclosure, and morphogenesis. By identifying the temperature-sensitive period we have learned that pmr-1 plays an important role during the period preceding ventral enclosure. While pmr-1 mutants show normal cell fate specification, lineage, and cell ingressions during gastrulation, we see defects in the migration of cells along the surface of embryo following ingestion in the C-lineage, ventral neuroblasts, and in anterior lineages, a cell migration process that appears similar to migrations during neurulation in vertebrates. Phenotypes in pmr-1 mutants can be suppressed or enhanced by changes in the activity of the RyR unc-68 and IP3R itr-1 genes, indicating that these cell migrations are sensitive to calcium levels. This study identifies new molecules important for embryonic cell migration in C. elegans and reveals a new role in cell migration for the calcium channel PMR-1/SPCA protein family.

Results

Isolation and genetic analysis of pmr-1 alleles

Using two independent forward genetic screens designed to identify conditional mutants essential for embryonic enclosure and morphogenesis, we obtained two alleles of the C. elegans SPCA1 homolog pmr-1, jc10 and ru5. Strains homozygous for either the jc10 or ru5 allele produce inviable progeny when grown at 25°C, although these strains show increased embryonic viability at lower temperatures (Table 1). The tm1840 and tm1750 alleles [43] show similar temperature sensitivity, although overall embryonic viability is significantly lower at both 15°C and 20°C. For all four alleles examined, pmr-1 embryos that die during mid-embryogenesis display a range of terminal phenotypes, including ventral closure defects, head ruptures, morphogenesis defects, and pharynx unattached (Panp) phenotypes (Figure 1; Table 2).

We established that the embryonic lethal phenotypes observed in jc10 and ru5 strains are due to mutations in the pmr-1 gene by a series of criteria. Using conventional and snp-snp mapping strategies, the jc10 and ru5 alleles were mapped to a small interval on L.G. I (Figure S1A). Whole genome and conventional sequencing revealed that pmr-1 was the only gene in the mapped region that carried a mutation in both the jc10 and ru5 strains. Complementation analysis showed that jc10 and ru5 failed to complement each other and also failed to complement the pmr-1 deletions tm1750 and tm1840, consistent with the interpretation that all four mutations are allelic (Table 1; data not shown; Figure S1B). Transformation of ru5 strains with fosmids that contain the pmr-1 gene (Figure S1B, S1C) rescued the high temperature embryonic lethality. In contrast, we did not observe rescue in
strains transformed with any other fosmids or cosmids from the mapped region (Figure S1B, S1C). Taken together, these experiments confirm that the defects in embryogenesis that we observe are due to disruption of pmr-1 gene activity.

The pmr-1(tm1840) deletion allele most likely represents a null, as the deletion eliminates an early exon, found in all of the major identified isoforms (Figure S1B) [33]. Based on strong sequence homology between PMR-1 and SERCA, whose crystal structure has been solved [44], the deletion would eliminate a transmembrane domain and a portion of the E1/E2 ATPase domain, thus rendering the expressed protein non-functional. However, we were unable to confirm whether the pmr-1(tm1840) allele is a null. Genetic tests with the dxDf2 deficiency strain were uninformative because of background activity of the antisera (data not shown).

Injection of dsRNA did not enhance or diminish the defects in the pmr-1(tm1840) strain (Table 1), which is consistent with expectations for a null mutation. However, injection of dsRNA into controls did not phenocopy the pmr-1(tm1840) allele, a result consistent with those reported by others (Table 1); [33,45]. Alternative experiments to test for the null, such as use of the antisera generated against PMR-1 [34], were also ambiguous because of background activity of the antisera (data not shown).

While the tm1840 allele has the highest mortality at low temperatures, other alleles of pmr-1 show a range of phenotype severity. The pmr-1(tm1750) allele contains a deletion of coding sequence from just one isoform, but the severity of the phenotype, which is similar to pmr-1(tm1840), suggests either the isoform is critical or other isoforms are also disrupted (Table 1 and Table 2). The pmr-1(jc10) allele has a premature stop in an exon found in all isoforms (exon 4 of the pmr-1b isoform) that would eliminate transmembrane helices and the E1/E2 ATPase domain (Figure S1). The pmr-1(ru5) allele is a G142D substitution in a residue conserved from yeast to humans in PMR-1, but also conserved in SERCA (Figure S1) [46]. Based on homology with SERCA, this residue, in a beta strand that forms a portion of the A domain, would likely result in localized disruption of this domain, interfering with key interactions between the A and N domains of the E1/E2 ATPase [44]. Because all four alleles show the same embryonic lethal defects, varying only in the frequency of inviable embryos observed at a given temperature, the simplest interpretation is that they represent reduced or complete loss of function of pmr-1 gene activity, and are indicative of a key role for pmr-1 in embryogenesis.

In humans, Hailey-Hailey disease has a dominant inheritance pattern, likely due to haploinsufficiency of the pmr-1 homolog SPCA1 [10]. In C. elegans, analysis of dominance is complicated because we observe both maternal and zygotic rescue for pmr-1 alleles. pmr-1(ru5) hermaphrodites crossed to control males produce live progeny at 25°C, showing zygotic activity is sufficient to rescue the mutant phenotype. F1 hermaphrodites from a cross between pmr-1 and control strains produce between 96%–100% viable F2 embryos at 25°C, depending on the allele (Table 1).

pmr-1 is required for embryonic development

During C. elegans embryonic development the epidermal (hypodermal) cells that are born on the dorsal surface migrate to the ventral midline, enclosing the body of the embryo. The anterior-most epidermal cells also migrate to enclose the head, creating the buccal cavity (Figure 1A) [47–49]. pmr-1 mutant embryos die with variable terminal phenotypes at the temperatures tested. (A) Control embryos elongate into the vermiform shape just prior to hatching. In contrast, all four mutant alleles of pmr-1 produce the same range of terminal phenotypes that include B) and C) enclosure defects resulting in full body ruptures (arrow), D) and E) head ruptures (arrow), F) and G) a detached pharynx and body morphogenesis defects (arrow) or H) and I) cells outside the embryo (arrow). Embryos shown include deletion alleles pmr-1(tm1840) (B, D, F) and pmr-1(tm1750) (H), as well as hypomorphic alleles pmr-1(jc10) (C, G, I) and pmr-1(ru5) (E). See Table 2 for specific terminal phenotype frequencies for each allele. In all images, anterior is to the left. doi:10.1371/journal.pgen.1003506.g001

Figure 1. pmr-1 mutant embryos show a range of terminal phenotypes at the temperatures tested. (A) Control embryos elongate into the vermiform shape just prior to hatching. In contrast, all four mutant alleles of pmr-1 produce the same range of terminal phenotypes that include B) and C) enclosure defects resulting in full body ruptures (arrow), D) and E) head ruptures (arrow), F) and G) a detached pharynx and body morphogenesis defects (arrow) or H) and I) cells outside the embryo (arrow). Embryos shown include deletion alleles pmr-1(tm1840) (B, D, F) and pmr-1(tm1750) (H), as well as hypomorphic alleles pmr-1(jc10) (C, G, I) and pmr-1(ru5) (E). See Table 2 for specific terminal phenotype frequencies for each allele. In all images, anterior is to the left. doi:10.1371/journal.pgen.1003506.g001

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During C. elegans embryonic development the epidermal (hypodermal) cells that are born on the dorsal surface migrate to the ventral midline, enclosing the body of the embryo. The anterior-most epidermal cells also migrate to enclose the head, ultimately connecting epidermal tissue with the anterior foregut, creating the buccal cavity (Figure 1A) [47–49]. pmr-1 mutant embryos die with variable terminal phenotypes that include failure to enclose the body (Figure 1B, 1C) and head Figure 1D, 1E), as well as a detached pharynx (Pun) (Figure 1F, 1G). pmr-1 embryos...
that elongate also show defects in epidermal cell organization (1H, I). Additional phenotypes include reduced brood size and some larval lethality (Table 1). The terminal phenotypes due to pmr-1 disruption, such as ventral enclosure defects and head enclosure failures, are at least superficially similar to the neural tube closure failures in mice lacking the PMR-1 homolog SPCA, as well as the cell adhesion defects observed in Hailey-Hailey disease patients [19,23,24].

When pmr-1 is disrupted, the frequency of embryonic lethality varies depending on both allele and temperature. Given that all four alleles carry different types of genetic lesions, from point mutation to deletion, it is unlikely that they are temperature sensitive, per se, but rather there is a developmental process in which normal pmr-1 activity is increasingly important as temperature is increased. All alleles are also pleiotropic, showing a range of embryonic lethal phenotypes (Table 2). The frequency with which a specific terminal phenotype is observed is similar for all alleles, at all temperatures, and independent of whether the temperature shifts occur early or close to the temperature-sensitive period in development, as described below (Table 2). These data are consistent with the hypothesis that pmr-1 is playing a role in developmental events that affect cells positioned throughout the embryo, resulting in the variable effects.

PMR-1 is required during gastrulation

To better understand the defects leading to the Pmr-1 terminal phenotypes, we observed embryos during gastrulation using Nomarski optics. Using this approach, the first observable differences between control and pmr-1(ru5) embryos are in the position of the C blastomeres. In control embryos, the C lineage blastomeres are positioned along the ventral surface at the posterior end of the embryo just prior to ingression (Figure 2A). In pmr-1(ru5) mutant embryos at the same developmental stage, these cells occupy a more dorsal position (Figure 2E). At the end of gastrulation, the gastrulation cleft closes in control embryos (Figure 2B). However, in pmr-1(ru5) embryos, the cleft remains open (Figure 2F), reflecting mispositioned ventral neuroblasts. We also saw later differences in cell position, with anterior blastomeres properly positioned in controls (Figure 2G), but altered in pmr-1(ru5) embryos (Figure 2G). Finally, we observed that the basement membrane, which initially surrounds the pharynx but is lost along the anterior most cells of the pharynx in control

Table 1. The severity of the phenotypes in pmr-1 mutant strains is dependent on allele and temperature.

| Parental Genotype: | Average % Viability of Progeny1,2 | Average Brood Size1,2 |
|-------------------|----------------------------------|-----------------------|
|                   | 15°C | 20°C | 25°C | 15°C | 25°C |
| N2                | 100  | a    | 99   | a    | 96   | a    | 210  | a    | 182  | a    |
| tm1840            | 61   | b    | 2.9  | b    | 0.0  | b    | 155  | b    | 68   | b    |
| tm1750            | 67   | b    | 1.9  | b    | 0.1  | b    | 180  | c    | 119  | c    |
| JC10              | 87   | c    | 18   | c    | 0.2  | b    | 152  | b    | 63   | b    |
| ru5               | 94   | c    | 12   | c    | 0.2  | b    | 199  | c    | 113  | c    |
| ru5/+             | 100  | a    |      |      |      |      |      |      |      |      |
| tm1840/+          | 97   | a    |      |      |      |      |      |      |      |      |
| tm1750/+          | 95   | a    |      |      |      |      |      |      |      |      |
| JC10/+            | 97   | a    |      |      |      |      |      |      |      |      |
| tm1750/ru5        | 0.0  | b    |      |      |      |      |      |      |      |      |
| JC10/ru5          | 0.6  | b    |      |      |      |      |      |      |      |      |
| N2(RNAi)3         | 92   | c    |      |      |      |      |      |      |      |      |
| tm1840(RNAi)3     | 74   | b    |      |      |      |      |      |      |      |      |
| ru5(RNAi)3        | 93   | c    |      |      |      |      |      |      |      |      |

1 Each strain showed significant differences in brood size or viability when compared at each temperature except N2, which showed no differences in brood size between 15°C and 25°C or in viability between 15°C and 20°C.
2 Letters indicate strains that are not significantly different from each other, but have significant differences from the other strains tested at the indicated temperature. T-test; significant values are p < 0.05; n = 7 to 12 for all strains tested. The number of total embryos examined in viability experiments ranged from 341 to 2738.
3 RNAi indicates pmr-1(dsRNA) construct. See Materials and methods.

PMR-1/SPCA Has Essential Roles in Cell Migration

Table 2. pmr-1 alleles are pleiotropic at all temperatures.

| genotype | T (°C) | Mild | Moderate | Severe | n   |
|----------|--------|------|----------|--------|-----|
| tm1840   | 15     | 39   | 23       | 38     | 44  |
| tm1840   | 20     | 64   | 9        | 27     | 20  |
| tm1750   | 20     | 50   | 15       | 35     | 26  |
| JC10     | 20     | 57   | 0        | 43     | 23  |
| ru5      | 20     | 62   | 15       | 23     | 32  |
| tm1840   | 25     | 55   | 21       | 24     | 30  |
| tm1750   | 25     | 53   | 28       | 19     | 32  |
| JC10     | 25     | 58   | 15       | 27     | 41  |
| ru5      | 25     | 51   | 26       | 23     | 37  |
| tm1750/ru5 | 25   | 66   | 29       | 5      | 41  |
| JC10/ru5 | 25     | 60   | 27       | 13     | 32  |

1 No significant differences in frequencies of terminal embryonic lethal phenotypes except for tm1840 at 15°C, which showed a reduced number of mild phenotypes; X² test p = 0.05. Severe phenotypes are enclosure failures, moderate phenotypes include head and body ruptures, and mild phenotypes include PUN and body morphogenesis defects.

PMR-1 is required during gastrulation

To better understand the defects leading to the Pmr-1 terminal phenotypes, we observed embryos during gastrulation using Nomarski optics. Using this approach, the first observable differences between control and pmr-1(ru5) embryos are in the position of the C blastomeres. In control embryos, the C lineage blastomeres are positioned along the ventral surface at the posterior end of the embryo just prior to ingression (Figure 2A). In pmr-1(ru5) mutant embryos at the same developmental stage, these cells occupy a more dorsal position (Figure 2E). At the end of gastrulation, the gastrulation cleft closes in control embryos (Figure 2B). However, in pmr-1(ru5) embryos, the cleft remains open (Figure 2F), reflecting mispositioned ventral neuroblasts. We also saw later differences in cell position, with anterior blastomeres properly positioned in controls (Figure 2G), but altered in pmr-1(ru5) embryos (Figure 2G). Finally, we observed that the basement membrane, which initially surrounds the pharynx but is lost along the anterior most cells of the pharynx in control
embryos (Figure 2D) [49], remains in place in pmr-1(ru5) embryos (Figure 2H). This analysis suggests the terminal phenotypes we observe are actually caused by problems in cell positioning earlier in embryogenesis.

To better pinpoint the developmental time point when pmr-1 activity is required, we took advantage of the temperature sensitive phenotypes of pmr-1 alleles. Using the pmr-1(ru5) allele, which has viable progeny at 15°C but not at 25°C and is less fragile than the pmr-1 deletion alleles, we performed temperature shift experiments. In reciprocal temperature shifts, we found that embryos grown at the restrictive temperature 2 to 3 hours after the two-cell stage are not viable, while those grown at the permissive temperature during this time period survive (Figure 3). Temperature pulse experiments show a one-hour pulse from the restrictive to permissive temperature can significantly rescue viability only if the pulse occurs between 2 and 3 hours after two-cell stage, consistent with the hypothesis that pmr-1 is required only during this specific time period (Figure S2). In contrast, one-hour pulses from 15°C to 25°C are insufficient to cause high lethality (Figure S2). The temperature-sensitive period for pmr-1 is during gastrulation, during a period when anterior, c-lineage, and ventral blastomeres migrate (Figure 3; Figure S2), and significantly earlier than the events directly associated with the enclosure failure, Pun, and morphogenetic terminal phenotypes.

pmr-1 mutants do not have lineage or cell fate defects

Given that the temperature-sensitive period is during a stage of development when cells are rapidly dividing and differentiating, we wanted to determine if the defects observed in pmr-1 embryos were due to changes in cell lineage. In C. elegans, the lineage pattern is essentially invariant in control embryos [47]. We took advantage of this to compare the lineage of pmr-1(ru5) embryos to controls, from the 2-cell to ∼300-cell stage, using StarryNite software [50–53]. Our analysis of pmr-1(ru5) embryos indicates that the cell division timing and pattern for all lineages is indistinguishable from controls (Figure 4). We also found that the correct number of cells underwent programmed cell death with normal timing, beginning one to two divisions after the temperature-sensitive period (data not shown). These data indicate that pmr-1 embryos are not dying because of premature cell death or general defects in cell division timing or patterns.

Figure 2. pmr-1 mutant embryos show differences in the positioning of cells during and after gastrulation. A) In control embryos, the C blastomeres are positioned at the posterior end of the embryo, on the ventral surface, surrounding the gastrulation cleft (arrow). E) In pmr-1 mutant embryos at the same developmental stage, the C blastomeres are shifted to a more dorsal position, out of the ventral focal plane (arrow). B) In control embryos, the gastrulation cleft closes as the last ventral cell ingresses (arrow). F) In pmr-1 mutant embryos at the same stage, the gastrulation cleft remains open (arrow). C) In control embryos, anterior cells form a smooth continuum (arrow), but are displaced in pmr-1 mutant embryos (G, arrow). D) In control embryos, the basement membrane that surrounds the anterior pharynx disappears as these cells migrate to the anterior (arrow, [49]). However, disruption in cell positioning disrupts this process in pmr-1 mutant embryos (H, arrow), preventing pharyngeal attachment. Genotypes are N2 control (A–E), pmr-1(tm1840) (F, H), or pmr-1(ru5) (E, G), all grown at 25°C. In all images, anterior is to the left. A–C and E–G are ventral views; D and H are lateral views.

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Figure 3. The temperature-sensitive period for pmr-1(ru5) embryos is during anterior, C-lineage, and ventral cell migrations. In the upshift experiments, embryos, extracted from gravid adults grown at the permissive temperature (15°C) from the mid-L4 stage, were switched to the restrictive temperature (25°C) at the indicated time. Embryos were then maintained at the restrictive temperature for the duration of embryogenesis and scored “viable” if they thrived past the L1 stage. In the downshift experiments, the same protocol was followed except that the embryos were switched from restrictive temperatures (25°C) to permissive temperatures (15°C) at the indicated times. The lines below the graph correspond to the times when anterior, C-derived, and ventral lineage cell migrations were assayed. All times were normalized to correspond to development at 25°C; n = 5 to 36 embryos at each time point.

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Although lineages appeared normal in pmr-1(ru5) embryos, we wanted to ascertain whether the cell fate was also normal. To address this question, we examined the expression patterns of GFP reporter fusion constructs that are expressed with specific timing, in a position-dependent manner within the embryo, or in specific tissues. Reporters included those expressed in the nervous system, hypodermis, muscle, intestine, and pharynx, some of which are derived from multiple lineages. We also included reporters, such as ceh-13, ceh-16 and sab-7, which are expressed in a position-dependent manner in multiple tissues and lineages [54–57]. In comparisons between control and pmr-1(ru5) embryos, the expression patterns of all of these reporter constructs were identical with only a few exceptions (Table 3; Figure S3, S4). These exceptions included differences in the position, but not in the number of cells expressing epidermal, neuroblast, and muscle markers. The other exception involved the expression of sab-7/even-skipped [54], which showed modest, but statistically significant delays in gene expression in C-lineage muscle and epidermal cells (Table 3; Figure S3). While we cannot rule out subtle effects on cell fate or changes in expression in C-lineage muscle and epidermal cells (Table 3; Figure S3), suggested that pmr-1 mutants may have defects in cell migration. Since cells that express sab-7 are derived from the C lineage [54], we looked more closely at cell migration in C-derived blastomeres. In control embryos, the C-lineage blastomeres migrate from a dorsal position to a more ventral one along the posterior surface of the embryo. Muscle precursors, which have a more ventral starting position and migrate ahead of the hypodermal precursors, eventually ingress into the embryo at a posterior/ventral position (Figure 5) [56,59]. When we compared control and pmr-1(ru5) embryos, we saw no differences in the timing of C-blastomere ingestions. Similarly, the timing of E, P4, MS, and D-derived blastomere ingestions were similar in control and pmr-1(ru5ts) embryos (n = 6 for each strain; t-test; p > 0.05). This analysis shows that pmr-1 does not appear to be playing an essential role in cell ingestion during C. elegans gastrulation.

In contrast, pmr-1 does have a critical role in the migration of cells along the surface of the embryo. Using 4-dimensional representations of embryos created with Acetree, we measured the migration rates of C blastomeres along the posterior surface of the embryo (Materials and methods). In pmr-1(ru5) embryos, the rates of migration of both muscle and hypodermal cell precursors were significantly slower, at 55% and 40% of controls, respectively (Figure 5; t-test; p < 0.05). Given that these cell migrations occur during the temperature-sensitive period for pmr-1 gene activity, this analysis is consistent with the interpretation that pmr-1 plays an important role in cell migration during C. elegans embryonic development.

Given the variability of the pmr-1 terminal phenotypes, we examined several other lineages to determine if other cells exhibited cell migration defects. Terminal phenotype and gene expression analysis suggested that many cells are properly positioned in the embryo, so we focused our attention on cells that migrate during the temperature-sensitive period and whose migration defects might lead to enclosure failures and pharyngeal defects. In control embryos, the ventral neuroblasts derived from ABprp and ABplp lineages migrate from lateral to central positions along the ventral surface of the embryo, closing the gastrulation cleft (Figure 6A; [47,58]). Hypodermal cells then crawl over these cells during enclosure [47,48]. Analysis of pmr-1(ru5) embryos showed that these ventral cells are properly positioned at the beginning of the temperature-sensitive period, but many move a shorter distance than controls (Figure 6A), in some cases failing to close the gastrulation cleft, leading to later enclosure failures. The migration distances of lineages examined varied widely, and some cells migrated distances similar to controls. However, the majority of cells examined showed significant reduction in cell migration, on average 44% of controls in pmr-1(ru5) embryos. The migration distances of lineages examined varied widely, and some cells migrated distances similar to controls. However, the majority of cells examined showed significant reduction in cell migration, on average 44% of controls in pmr-1(ru5) embryos (Figure 6B; T-test; p < 0.05). These results show that pmr-1 is playing a key role in cell migration in cells that give rise to ventral neuroblasts, as well as epidermal cells.

In the anterior of control embryos, cells derived from the ABala and ABalp lineages, many of which ultimately differentiate into epithelial or nervous system cells, undergo dynamic migration patterns, with individual blastomeres crossing the dorsal-ventral or left-right midlines during embryogenesis. Some anterior blastomeres also make specific turns and ingress to form the anterior pharynx (Figure 7; [47,58,60]). In pmr-1(ru5) embryos, many anterior cells show migration defects. While some cells migrated normally in a subset of the pmr-1(ru5) embryos examined, many cells migrated shorter distances, in the wrong direction, or failed to make key turns, resulting in cells that were improperly positioned (Figure 7; t-test; p < 0.05). Since these cell divisions can give rise to arc, cells, hypodermis, and ring ganglia, their failure to migrate properly during the temperature-sensitive period can readily account for the terminal phenotypes we observe in pmr-1(ru5) embryos. Taken

Figure 4. Cell division patterns and timing are normal in pmr-1(ru5) embryos. We determined the cell division patterns in control and pmr-1(ru5) embryos at 25°C to ~300-cell stage using 4-D microscopy and cell lineage analysis software (Materials and Methods; n = 6 for each strain). Two representative lineages for control (left) and pmr-1(ru5) (right) embryos are shown. We did not observe any differences in cell lineage, cell deaths, or cell division timing in pmr-1(ru5) embryos throughout the temperature-sensitive period. Apoptotic cell deaths, which begin at least one cell division after the temperature sensitive period, occurred normally in all lineages examined (n = 2 controls; n = 4 pmr-1(ru5) strains). doi:10.1371/journal.pgen.1003506.g004
together, these results show that pmr-1 plays a key role in cell migration and positioning during gastrulation.

**pmr-1 mutants do not show disruptions in cell polarity, adhesion, or basement membrane structures**

In Hailey-Hailey disease, cells show defects in cell attachment structures [23,24]. The defects in cell migration we observe in pmr-1 mutants could be due to changes in cell adhesion, as well as in other cellular processes including cell polarity or establishment of the basement membrane. Our analysis of gene expression suggests that each of these processes is relatively normal in pmr-1 mutants [23]. pmr-1 embryos (Table 3; KR514; [103]). These data show that the pmr-1 allele specific, as we observed similar suppression of phenotypes of these receptors would suppress or enhance the pmr-1 embryonic lethal phenotypes. We made strains carrying pmr-1 mutations and itr-1(sy327), a gain-of-function allele that is thought to increase the affinity for IP3 [28,71]. At 20°C, the itr-1(sy327) strain has slightly reduced embryonic viability (91%) compared to controls while the pmr-1(tm1840) strain has 9% embryonic viability. The pmr-1(tm1840);itr-1(sy327) double mutant shows significant improvements in viability compared to the pmr-1(tm1840) single mutant (42%; Table 4). This affect is not allele specific, as we observed similar suppression of phenotypes of the pmr-1(g10), pmr-1(ra5), and pmr-1(tm1750) lines when placed in an itr-1(sy327) background (Table 4). These data show itr-1 phenotypes are epistatic to pmr-1. We also made double mutants carrying pmr-1 alleles and itr-1(g53), a loss-of-function allele [27]. While we were unable to generate double mutants with pmr-1(tm1840) or pmr-1(tm1750), suggesting the combination is lethal (data not shown, V.P., R.W. and R.M.), the pmr-1(ra5);itr-1(g53) strain showed enhanced lethality compared to the pmr-1(ra5) strain alone (Table 4), suggesting the two genes act in parallel pathways during this stage of development. In contrast, depletion of unc-68 using RNAi significantly suppressed the embryonic lethality of pmr-1(ra5) strains (Table 4), indicating

### Table 3. pmr-1(mu5ts) strains properly express cell fate and cell polarity markers.

| Marker | Expression pattern | Stages examined | Observed Pattern in pmr-1(mu5) embryos | Parental Strain |
|--------|--------------------|----------------|----------------------------------------|----------------|
| AJM-1  | Adhesion junctions of hypodermis, pharynx, gut | Enclosure and comma | Normal timing, cell number and localization; changes in position of V3-VS | SU93; [99] |
| vab-7  | C-derived hypodermis and body wall muscles. | 3 hours after 2-cell | Delayed expression; altered organization | This study. [54] |
| pha-4  | Pharynx | Enclosure and comma | Normal timing, cell number and position | SM467 and SM469; [49] |
| hth-1  | Body wall muscle | 3 hours after 2-cell | Normal timing and cell number; cells mispositioned | PD7963; [100,101] |
| ceh-16 | Seam hypodermis | Gastrulation, enclosure comma | Normal timing and cell number, Change in position of V3–V5 seam cells | SU324; [56,102] |
| ceh-13 | A, D, E and M5 lineages; anterior body wall muscle and hypodermis | 3 hours after 2-cell | Normal timing and cell number | FR317; [103] |
| KAL-1  | AB-derived neuroblasts | Enclosure | Normal timing of expression; cells mispositioned | OH904; [104] |
| PLX-2  | Ventral neuroblasts | Enclosure | Normal timing of expression; cells mispositioned | SU272; [105] |
| SMA-1  | Apical membrane of hypodermis, pharynx, gut | Enclosure and comma | Normal expression and localization pattern. | Antisera; [62] |
| NID-1  | Basement membrane of muscle; around pharynx and gut | Comma and 1.5× stage | Normal localization pattern. | Antisera; [63] |
| MEL-11 | Apical membrane of hypodermis | Comma and 1.5× stage | Normal localization pattern. | Antisera; [68] |
| VAB-9  | Apical membrane of hypodermis | Comma and 1.5× stage | Normal localization pattern. | Antisera; [65] |
| PAR-6  | Apical membranes of pharynx and intestine | Comma and 1.5× stage | Normal localization pattern. | JJ1579; [61,106] |

1 A normal number of cells expressing marker. P>0.05.
2 Expression of vab-7::GFP 3 hours after 2-cell stage was significantly reduced. P<0.05; n = 24 (control) and 18 (mu5).
3 See Figure S3 and Figure S4 for images of expression patterns.

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**Altering calcium channel activity can modulate pmr-1 mutant phenotypes**

Given the conserved molecular roles of PMR-1/SPCA, we asked whether perturbations in Ca2+ signaling were chiefly responsible for the pmr-1 mutant phenotypes. To address this question, we altered the activity of either the ITR-1/IP3R or UNC-68/RyR calcium channels, reasoning that if calcium levels were critical for normal cell migration, changes in the activities of these receptors would suppress or enhance the pmr-1 embryonic lethal phenotypes. We made strains carrying pmr-1 mutations and itr-1(sy327), a gain-of-function allele that is thought to increase the affinity for IP3 [28,71]. At 20°C, the itr-1(sy327) strain has slightly reduced embryonic viability (91%) compared to controls while the pmr-1(tm1840) strain has 9% embryonic viability. The pmr-1(tm1840);itr-1(sy327) double mutant shows significant improvements in viability compared to the pmr-1(tm1840) single mutant (42%; Table 4). This affect is not allele specific, as we observed similar suppression of phenotypes of the pmr-1(g10), pmr-1(ra5), and pmr-1(tm1750) lines when placed in an itr-1(sy327) background (Table 4). These data show itr-1 phenotypes are epistatic to pmr-1. We also made double mutants carrying pmr-1 alleles and itr-1(g53), a loss-of-function allele [27]. While we were unable to generate double mutants with pmr-1(tm1840) or pmr-1(tm1750), suggesting the combination is lethal (data not shown, V.P., R.W. and R.M.), the pmr-1(ra5);itr-1(g53) strain showed enhanced lethality compared to the pmr-1(ra5) strain alone (Table 4), suggesting the two genes act in parallel pathways during this stage of development. In contrast, depletion of unc-68 using RNAi significantly suppressed the embryonic lethality of pmr-1(ra5) strains (Table 4), indicating
PMR-1/SPCA Has Essential Roles in Cell Migration

Discussion

Our analysis of PMR-1/SPCA1 in *C. elegans* has revealed a novel and essential role in cell migration for this highly conserved gene [1,9]. Using a forward genetic screen designed to look for genes required for normal epidermal cell migration and adhesion, we identified mutations in the *C. elegans* SPCA1 gene *pmr-1*. Strains homozygous for *pmr-1* show temperature-sensitive embryonic lethality, with terminal phenotypes that include ventral enclosure failures, head ruptures, and morphogenetic phenotypes including a detached pharynx (Pun) phenotype. Phenotype and temperature shift analysis indicates that the *pmr-1* gene plays a crucial role in a specific period of embryonic development, during gastrulation. Cell fate specification, cell division patterns, apoptosis, and cell ingression during gastrulation are all similar to control embryos. However, the migrations of specific subsets of cells, along the posterior, anterior, and ventral surfaces of the embryo, are defective in *pmr-1* mutant embryos. The specificity of the phenotype, coupled with the temperature shift analysis, indicate that PMR-1/SPCA plays an essential role in a specific set of cell migrations during gastrulation in the developing *C. elegans* embryo.

Given the broad expression pattern for *pmr-1* in all cells, coupled with the proposed housekeeping functions one would expect for a calcium transporter [1,9], the specific timing and proposed role for PMR-1 in ectodermal cell migration is unexpected. Studies examining the expression of *pmr-1* indicate that PMR-1 protein localizes to the Golgi and that the gene product is present in the germ line and widely expressed in cells throughout embryogenesis, including during gastrulation [33–38]. This expression pattern, which is consistent with our genetic analysis that maternally or zygotically provided gene product is sufficient to rescue the Pmr-1 mutant phenotype, would suggest a broad role for the gene product. Yet our temperature shift and pulse analysis indicates requirements for PMR-1 can be bypassed during other stages of embryogenesis. The phenotype analysis identifies a very specific role in the migration of anterior, C-lineage, and ventral cells along the surface of the embryo during gastrulation, but with no discernable effects on other cell migration events such as ingestion or ventral closure, nor on any other developmental pathway that might alter cell fate, division patterns, or cell polarity. The best explanation for these data is that PMR-1 may act redundantly in some aspects of embryogenesis, but plays a more critical or specific role in the cell migrations along the embryo surface that follow cell ingression, a markedly different developmental process [58,60].

PMR-1/SPCA is part of a network of proteins that regulate calcium levels in the cell cytoplasm and calcium stores in the secretory pathway. Models for modulating cellular calcium levels start with a signal transduced through PLC and IP3 triggering release of Ca^{2+} into the cytoplasm from secretory stores through ITR-1/IP3 and UNC-68/RyR. The calcium ATPases SCA-1/SECA and PMR-1/SPCA, located predominantly in the ER and Golgi, respectively, then pump Ca^{2+} back out of the cytoplasm into these organelles [7,26]. A simple model would predict that reduced activity of a transporter acting in one direction is suppressed by reduced activity of channel acting in the opposite direction. This is the result we observe for interactions between *pmr-1* and *unc-68*, where *pmr-1(ru5)* strains showed reduced lethality in an *unc-68(RM40)* background (Table 4). In contrast, strains carrying *pmr-1* and *itr-1(jc5)* loss-of-function alleles showed enhanced lethality at semi-permissive conditions while strains carrying the *itr-1(tg327)* gain-of-function allele showed significant suppression of *pmr-1(ru5)* embryonic lethality. This gene interaction analysis suggests that *itr-1* and *pmr-1* act in parallel pathways during embryonic development and that enhanced sensitivity of

unc-68 acts in opposition to *pmr-1* in a role very different from that of *itr-1*. Taken together, these data show that the embryonic lethality we observe in *pmr-1* mutant embryos can be affected by changes in the activity of calcium channels. While this type of analysis does not allow us to rule out other possibilities, it provides strong support for the hypothesis that *pmr-1* is acting through its role in Ca^{2+} signaling to affect cell migration.

![Cell migration defects in the C lineage cells of *pmr-1(ru5)* embryos.](image-url)
Figure 6. Cell migration defects in ventral cells of pmr-1(ru5) embryos. A. Ventral ABp-derived blastomeres migrate from the left (ABplp; blue) or right (ABprp; red) side of the embryo toward the ventral mid-line in control embryos (left panels). In pmr-1(ru5) embryos (right panels), the migration of these blastomeres is significantly reduced. For example, the rectal and interneuron precursor AB.plp appp (Blue; *) and the ring interneuron and ventral cord neuron precursor AB.prp appa (Red; *) migrate further in control than in pmr-1(ru5) embryos. Each panel represents a 15-minute interval, starting at 2 hours (~162 cells) after the 2-cell stage at 25°C, which corresponds to the temperature-sensitive period. Ventral view is shown, with anterior to the left. B. In comparisons of average migration distance for ABprp (red) and ABplp (blue) blastomeres, cells migrated significantly farther in controls (dark boxes) than in pmr-1(ru5) (light boxes), with significant reductions in migration distance in at least one daughter in 8 of the 12 cells examined. The migration rates in pmr-1(ru5) embryos were 44% of those in controls, when all ventral blastomere migration values were considered.
itr-1 to IP3 signaling in the itr-1(sy32y7) mutant [72] can help bypass the requirement for pmr-1. While these interpretations reflect the genetic analysis, they are puzzling because ITR-1 and PMR-1 act in opposite directions with respect to cytosolic calcium. Similarly, UNC-68 does not appear to have any developmental roles nor does it appear to be expressed at high levels in the embryo until later stages [7,29–32].

A speculative model to explain the genetic interaction data we observe is that these proteins may play some redundant and some non-redundant roles in regulating calcium levels in the cell, due in part to the polarity of the secretory pathway. One would predict that when PMR-1 activity is reduced, the pool of calcium in secretory stores is also modestly reduced and the calcium levels in the cytoplasm might be elevated [34]. Reduction of UNC-68 or ITR-1 calcium release would, in theory, restore overall secretory pool levels. However, the calcium flicker model for cell migration predicts that calcium must be supplied in localized bursts at the leading edge of the cell [5,6]. In a pmr-1 mutant, the pool of available calcium would be disproportionately reduced in the Golgi, the secretory organelle closest to the leading edge. If release is, as predicted, strongly dependent on ITR-1 [5,6], an itr-1 loss-of-function mutant would further diminish calcium release, making the phenotype worse, while the itr-1(sy327) gain-of-function mutation would release calcium at lower levels of signal [28,71], suppressing the phenotype. A second possibility to explain the suppressor data reflects the distinct mechanisms by which the channels are activated. While both the UNC-68/RyR and ITR-1/IP3R channels are sensitive to changes in cytosolic calcium, only the ITR-1/IP3R channels respond to an IP3 ligand [73,74]. Reduced activity of the ITR-1/IP3R channel would make the secretory pathway insensitive to IP3 signaling, which when coupled with reduced stores would make the phenotype worse. Increasing IP3R sensitivity might compensate for altered cytosolic calcium, making the situation better, as observed. Finally, since the regulation of calcium signaling and transport is highly interdependent, with channels and targets acutely sensitive to minor changes in calcium concentration [1,72–74], single loss-of-function mutations may result in some compensatory changes in the signaling system that cannot occur if two systems are defective.

While it is tempting to assume that disruption of pmr-1 has a direct effect on calcium-mediated cell signaling, disruptions in the gene might instead affect other calcium-dependent processes required for cell migration. The embryonic lethal phenotypes in Pmr-1 strains were observed at all the temperatures assayed, but they were more severe at high temperatures for all four alleles tested. Although our experiments were in the normal temperature range for C. elegans growth [75] and we saw no evidence of necrosis in pmr-1 mutant embryos, recent research indicates that pmr-1 is important for survival of heat shock, acting to prevent necrosis [42]. It is possible that the migrating cells of the embryo are particularly sensitive to changes in the activity of this stress resistance pathway. Alternatively, the cytoskeletal and membrane dynamics of migrating cells may simply be more sensitive to temperature than other developmental processes, or the timing is more precise, such that changes in pmr-1 activity have a stronger impact. Another possibility is that the changes in pmr-1 activity alter calcium-dependent processing of specific proteins in the Golgi. No matter the precise mechanism by which changes in calcium dynamics alter cell migration, our identification of a role for PMR-1, acting with ITR-1/IP3R and UNC-68/RyR, confirms earlier work that ITR-1 plays an important role in cell migration [27,28] and identifies new genes important for normal cell migration in the C. elegans embryo.

The phenotypes we observe in C. elegans pmr-1 mutants have at least some superficial similarities to those observed in vertebrates with defects in the SPCA1 gene, including humans with Hailey-Hailey disease. In both C. elegans and in vertebrates, we observe semi-dominant phenotypes and the severity of the observed phenotypes are influenced by environmental stresses. The terminal phenotypes, including apparent loss of cell adhesion in the epidermis of humans with Hailey-Hailey or failures to close the rostral neural tube in mice embryos homozygous for SPCA loss-of-function alleles [12–16,19,23,24] also look similar to the enclosure failures and head ruptures we observe in C. elegans embryos.

One major difference between the worm pmr-1 mutant phenotype and Hailey-Hailey disease patients is the nature of defects in cell adhesion. In Hailey-Hailey disease, symptoms include acantholysis of keratinocytes, marked by keratin dissociation from desmosomes [23,24]. Our analysis of pmr-1 mutants suggests that the gene does not play a direct role in cell adhesion. Proteins that localize to adhesion complexes, such as MEL-1, AJM-1, and VAB-9, look normal in pmr-1 mutant embryos and epistasis analysis indicates that vab-9 and pmr-1 are in different pathways (unpublished data, J. Simske). While intermediate filament and desmosomal structures have not yet been carefully examined in pmr-1 mutants, we do not suspect an association. While pmr-1 is essential during gastrulation, analysis by others indicates genes that encode intermediate filament and attachment structures in C. elegans, such as IFA-1, IFA-2/MUA-6, IFA-3, IFA-4, IFB-1, VAB-9, MUP-4, and LET-805, assemble later in development, playing key roles in the embryonic elongation that follows ventral closure [76–83]. This analysis strongly suggests intermediate filaments are unlikely to be directly involved in the PMR-1-mediated cell migration, although it is possible PMR-1 may play a later, non-essential role in maintaining these attachment structures. Similarly, we did not see elevated levels of cell death in pmr-1 mutants, as reported for the mice knockouts [19]. These data suggest that there may be differences between SPCA function in C. elegans and in vertebrate systems, perhaps due to fundamental differences in ectodermal cell structure. Alternatively, the terminal phenotypes observed in vertebrates could reflect earlier defects.

Given our results in C. elegans, as well as the strong sequence conservation of the gene, it is tempting to speculate that the SPCA’s have cell migration roles in vertebrates. The relative simplicity of C. elegans as model system, as well as powerful new lineageing tools [50–53], has allowed us to distinguish between the terminal phenotype and the actual defects in cell migration found in pmr-1 mutants. In humans, keratinocytes differentiate in response to changes in extracellular calcium, migrating from the stratum basale into upper layers of the epidermis as they differentiate [84]. It is conceivable that in Hailey-Hailey patients, stress-damaged cells are replaced inefficiently because of a defect in cell migration, caused by altered calcium levels, leading to the observed lesions and keratinocyte defects [23,24]. Similarly, the rostral neural tube closure failures in SPCA mouse knockouts [19] could be due to defects in migration of specific subsets of ectodermal cells during this stage of development.

In summary, we have shown that the PMR-1/SPCA1 Ca2+/Mn2+ ATPase plays a key role in cell migration during C. elegans.
Figure 7. Cell migration defects in the anterior-most cells of pmr-1(ru5) embryos at 25°C. The anterior cells of control embryos (left panels) undergo distinct and reproducible cell rearrangements during gastrulation. CANL, labial, and ring ganglia precursors ABalapaa (magenta) and ABalappa (blue) cross the left/right mid-line, labial and ring ganglia precursors ABalpppa (yellow) and ABalppp (gray) cross the dorsal/ventral mid-line, and pharyngeal precursor ABalppaa (cyan) migrates from the left to a ventral position. Other cells, such as ring ganglia precursor ABalaaa (red), migrate from peripheral to central positions or vice versa, as with pharyngeal precursor ABalapaa (green) and labial/ring ganglia/CANL precursor ABalappa (pink). In a pmr-1(ru5) embryo (right panels), many of these anterior cells migrate shorter distances, migrate in the wrong direction, or fail to change directions, resulting in cells that are mis-positioned. While not every cell showed a migration defect in each embryo, measurements of the Cartesian coordinates for all of these 8 cells except ABalpppa (yellow), as well as three others not labeled above (labial and ring ganglia precursors ABalaaap, ABalpppp, and ABalpppa) showed significant positional differences in pmr-1(ru5) embryos (n = 6) compared to control embryos (n = 6; p < 0.05). Each panel represents a 15-minute interval, starting at 2-cell stage at 25°C, which corresponds to the temperature-sensitive period. Anterior view is shown, with dorsal (top) and embryo right (left). Cell fate information from [97,98].

Table 4. Genetic interactions with pmr-1.

| Genotype                      | Temp | % viable | n  |
|-------------------------------|------|----------|----|
| pmr-1(tm1840)                 | 20C  | 9        | a  | 743 |
| pmr-1(tm750)                  | 20C  | 7        | a  | 665 |
| itr-1(y3327)                  | 20C  | 91       | b  | 886 |
| pmr-1(tm1840); itr-1(y3327)   | 20C  | 42       | c  | 487 |
| pmr-1(tm750); itr-1(y3327)    | 20C  | 34       | c  | 517 |
| pmr-1(ru5)                    | 24C  | 8        | a  | 565 |
| pmr-1(ru5); itr-1(y3327)      | 24C  | 4        | a  | 289 |
| pmr-1(ru5); itr-1(y3327)      | 24C  | 90       | b  | 297 |
| pmr-1(ru5); itr-1(y3327)      | 24C  | 22       | c  | 1434 |
| pmr-1(ru5); itr-1(y3327)      | 24C  | 21       | c  | 261 |
| pmr-1(ru5)                    | 20C  | 26       | a  | 603 |
| pmr-1(ru5); itr-1(y3327)      | 20C  | 60       | b  | 567 |
| pmr-1(ru5); itr-1(y3327)      | 20C  | 14       | c  | 1000 |
| pmr-1(ru5); itr-1(y3327)      | 20C  | 97       | a  | 1067 |
| pmr-1(ru5); itr-1(y3327)      | 20C  | 99       | a  | 1249 |
| pmr-1(ru5); itr-1(y3327)      | 20C  | 10       | b  | 2027 |
| pmr-1(ru5); itr-1(y3327)      | 20C  | 59       | c  | 1755 |

1Letters indicate strains that are not significantly different from each other, but have significant differences from the other strains tested in each cluster. T-test; significant values are p < 0.05; n = 10 to 49 hermaphrodites produced progeny for all strains tested.

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embryonic development. Strains carrying mutant pmr-1 show defects in migration of cells along the surface of the embryo following the ingestion of cells during gastrulation. The cell migration defects are likely caused by changes in intracellular calcium levels, as they can be enhanced or suppressed by changes in activity of the ITR-1/IP3R or UNC-68/RyR calcium channels. This analysis reveals a new role for calcium signaling in the migration of specific blastomeres during C. elegans development. It also reveals a new role in cell migration for the SPCA1 family of genes.

Materials and Methods

Growth conditions, strains, and alleles

Strains were grown and maintained under standard conditions [85]. Wild type strain N2 was used as a control. The ru5 and j(c10) alleles of pmr-1 were identified in two independent embryonic conditional embryonic lethal screens using the mutagen EMS [85]; J. Simske). The tm1840 and tm1750 alleles were produced in the National Bioresource Project (kindly provided by S. Mitani; [43]. All pmr-1 mutant strains, which were maintained at 15°C, were backcrossed to the N2 strain at least 3 times. The deletions in tm1840 and tm1750 were confirmed using PCR analysis. PCR mapping lines CB4856, RW700, deficiency lines CB2775 eDf9/eDf24 I, CB2770 eDf4/eDf24 I, CB2769 eDf5/eDf24 I, KR2838 hDf17/hDf11, [unc-4(h1040)]; JK1542 ces-1(n703) qD77/dpy-3(e61) sf-2(g262) unc-75(e950) I, SL356 dxD2/q(e19) unc-101(n1) I, and conventional mapping lines MT1363 dpy-5(e61) I, rh-2(e768) II, unc-32(I1); III, MT464 unc-3(e53) IV, dpy-11(e224) V, lon-2(e674) X, DR210 dpy-5(e61), daf-16(m26), unc-75(e950) I, CB2010 unc-54(e675), dpy-5(e61) I, JK228 glp-4(bn2), unc-54(e675) I were used in mapping experiments. SU180 itr-1(y3327) jcI5 IV, PS2368 itr-1(y3327) unc-24(e138) IV and QQ101 vab-9(yu6) II strains were used for double mutant analysis. For analysis using
GFP fusions, F2 progeny exhibiting the Pmr-1 phenotype and carrying the appropriate markers were selected from crosses between pnr-1(ra5) males and the following strains: RW10026 unc-119(ed3); slt10026, SM1467 ph4; rol-6(sa1606), SM1469 ph6, SU956 jcl; PD7965 al57963, SU324 jk526, FR317 sax; OH904 oh5; SU252 jeB1, esB136, and J11579 sax. Strains expressing vab-7:GFP were generated by microparticle bombardment of pJAx6 and pDPMM016b into a SM467 analysis, ru5 for embryonic lethality at 25°C, or tm1840. In complementation analysis, ru5 regions of these candidate genes were then sequenced in and Fred Hutchinson DNA sequencing facilities. The codingational sequencing (U. of Iowa Carver Center, U of Wisconsin, subcloning (Strataclone, Agile nt Technologies) and conven-tional sequencing (U. of Iowa Carver Center, U of Wisconsin, and Fred Hutchinson DNA sequencing facilities). The coding regions of these candidate genes were then sequenced in je10. In complementation analysis, ru5 males were crossed to je10, tm1840, or tm1750 hermaphrodites and the F1 progeny scored for embryonic lethality at 25°C. For complementation rescue analysis, ru5 young adults were transformed by injection of 25–100 μg/ml of a fosmid or cosmid covering each of the candidate genes, along with the co-transformation marker pRF4 [92,93]. Rol Progeny were assayed for survivorship at 25°C for each injected construct.

Mutant and RNAi viability and brood size analysis

To determine rates of viability, L4 hermaphrodites were shifted from 15°C to the indicated temperature. Eggs from single hermaphrodites were counted and the number of resultant late larva or adult progeny scored. In brood size experiments, single L4 hermaphrodites were shifted to the indicated temperature and total broods quantified as previously described [94]. Both brood size and viability data were compared using t-tests. Terminal phenotypes were scored during mid-to-late embryogenesis. Embryos that failed to enclose were classified as “severe”, those with head or later body ruptures as “moderate”, and those with cell position or pharynx unattached (Pun) phenotypes as “mild”. We used chi-square analysis to compare the frequency of each class for a given strain or condition. For RNA interference experiments with pnr-1, young adult hermaphrodites were injected with a pnr-1 dsRNA construct mV ZK256.1a at 0.9 mg/ml using standard methods [95]. Injected worms were allowed to recover and lay eggs. The embryos of injected progeny were examined for levels of embryonic lethality and terminal phenotypes. For RNAi with unc-68, we used an RNAi feeding protocol [95].

Temperature shift experiments

Embryos were extracted from gravid pnr-1(ra5) adults grown at the indicated temperature from the early-to-mid-L4 stage, shifted to the second temperature at the indicated time point, and maintained at that second temperature for the duration of embryogenesis. Each embryo was scored for hatching and terminal phenotypes. Experiments at the two temperatures were calibrated based on comparisons of the timing of key development-mental events.

Mapping, complementation, and genome-sequencing experiments

The ru5 and je10 alleles were mapped using conventional and deficiency mapping strategies [87]. The position of the ru5 allele was further refined using PCR-based and snp-snp mapping techniques [88–90]. Genomic DNA was extracted from the ru5 strain using Illumina sequencing and MAQGene, as described [91]. Alterations in candidate genes were confirmed in ru5 using PCR amplification followed by subcloning (Stratagene, Agilent Technologies) and conventional sequencing (U. of Iowa Carver Center, U of Wisconsin, and Fred Hutchinson DNA sequencing facilities). The coding regions of these candidate genes were then sequenced in. In complementation analysis, ru5 males were crossed to je10, tm1840, or tm1750 hermaphrodites and the F1 progeny scored for embryonic lethality at 25°C. For complementation rescue analysis, ru5 young adults were transformed by injection of 25–100 μg/ml of a fosmid or cosmid covering each of the candidate genes, along with the co-transformation marker pRF4 [92,93]. Rol Progeny were assayed for survivorship at 25°C for each injected construct.

Lineaging and cell migration analysis

For lineaging and cell migration analysis, pnr-1(ra5) embryos carrying nuclear-localized GFP were imaged at 25°C according to the protocol outlined in [50,52,53]. Four-dimensional representations of embryos were generated using the Acetree program [51]. pnr-1(ra5) lineages (n = 6) were compared to controls (n = 6 to 8) (kindly provided by M. Boeck, P. Weisdepp, and R. Waterston). Files for each embryo were synchronized to the same developmental time points based on cell division patterns in several lineages. Because of the different migration patterns for the lineages tested, several techniques were used for measuring cell movement. To analyze ingestion, the timing of the ingestions of P4, D, MS, and C-lineage daughters in N2 was compared to pnr-1(ra5) embryos. C-lineage cell migration was calculated by measuring the distance of the migrating cell nucleus relative to the ventral-most cell in the embryo. The average slopes of distance over time were used for comparisons. Ventral cell migration was calculated by measuring the distance of the migrating cell nucleus relative to the left or right edge of the embryo and total migration distances over time were compared. The position of the anterior-most cells was measured using polar or Cartesian coordinates, the positions plotted over time, and positions compared in each dimension. In all cases, t-tests were used to determine if the differences between the controls and pnr-1(ra5) embryos were statistically significant.

Gene expression analysis

For analysis of the expression patterns of GFP-fusion proteins, control and pnr-1(ra5) embryos were grown at 25°C and compared at specific developmental stages. In some experiments, the position and number of nuclei expressing markers were analyzed using t-tests. For analysis using in situ immunolocalization, control and pnr-1 strains were collected and fixed using a freeze cracking technique and stained with anti-SMA-1, anti-NID-1, anti-AJM-1, anti-SQV-8, or anti-PMR-1 antisera [34,62,63,66,96]. Nomarski and fluorescent images of embryos were collected using an Olympus spinning disc microscope using Slidebook, Nikon E600 microscopes using a cool snap CCD camera controlled by Metaview software, and Olympus DeltaVisi-on deconvolution microscope (Applied Precision, Issaquah, WA); projected images were created using 4D macros within NIH Image and Image J.

Supporting Information

Figure S1 The genomic location of pnr-1 rescuing constructs and mutations. A. Genomic location of pnr-1 on L.G. I. B. Position of the pnr-1 gene, including the position of rescuing cosmid WR0626dG10 and WR066cH12, as well as cosmid C47B2 and CC4. WR0622dA01 (not shown) is to the right of the region shown on this map. The dashed lines indicate the position of pnr-1, with pnr-1 intron and exon structure for each of three major isoforms [33]. The tm1750 allele has a 253 bp deletion upstream of exon 1 for pnr-1a/b but does alter pnr-1c. The tm1840 allele removes the first exon and flanking sequencing of pnr-1a/b. The je10 allele has a premature stop, in exon 4 (Q351stop) of pnr-1a/b; ru5 has an amino acid change (G142D) in exon 2 of pnr-1b (sequences of tm1750 and tm1840 alleles available at [46]). C. Complementation rescue data showing the cosmid and fosmid used for transformation into pnr-1(ra5). After selecting for lines that stably expressed the co-transformation marker Rol6, lines were tested for significant growth at 25°C, demonstrating rescue of the pnr-1(ra5) mutant phenotype.
Figure S2  A one-hour pulse to the permissive temperature can rescue pmr-1 mutant embryonic lethal phenotypes during anterior, C-lineage and ventral cell migrations. In temperature pulse experiments, embryos, extracted from gravid adults grown at the first temperature from the mid-L4 stage, were moved to the second temperature at the indicated time. At the end of a one-hour pulse, the embryos were returned to the original temperature for the remainder of embryogenesis and scored “viable” if they thrived past the L1 stage. Lines below the graph correspond to time of cell migration assays for anterior, C-derived, and ventral lineages. All times were normalized to correspond to development at 25°C; n = 4 to 23 embryos at each time point.

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Figure S3  pmr-1 mutant embryos show normal expression of cell fate markers. Control (A, C, E, I, K) and pmr-1(ra5) mutant embryos (B, D, F, H, J) express cell fate markers AJM-1 (A, B), pha-4 (C, D), vab-7 (E, F), htd-1 (G, H), plk-2 (I, J), and kal-1 (K, L). Note that the position and number of cells expressing markers in pmr-1(ra5) mutant embryos is similar to that observed in controls, indicating normal acquisition of cell fates during development. However, some cells show positioning defects in pmr-1(ra5) mutant embryos, as in the anterior-most cells expressing AJM-1 (B, arrow) or in the C-lineage-derived muscles cells expressing htd-1 (H, arrows). A–D are comma stage embryos, E–H are gastrulation stage embryos. I–L are enclosure stage embryos. Strains, reagents, and references are as indicated in Table 3. Anterior is to the left; A–D are lateral views, E–L are ventral views.

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Figure S4  pmr-1 mutant embryos have normal cell polarity in polarized epithelial cells. The localization of proteins with distinctive polarized expression patterns is similar in control and pmr-1(ra5) mutant embryos, indicating the mutation does not cause a visible loss of cell polarity. PAR-6 is localized to apical membrane in the pharynx (A, B, arrow), AJM-1 is localized to apical adhesion junctions in the gut (C, D arrow) and SMA-1 is apically localized in the hypodermis (E, F, arrow) in both control (A, C, E) and pmr-1(ra5) mutant embryos (B, D, F), although we do see some altered expression due to mis-positioned anterior blastomeres in pmr-1(ra5) mutant embryos (arrowheads in B, D, F). The localization of AJM-1 to apical adhesion complexes (G, H, arrow) and the basal localization of NID-1 (I, J, arrow) in the pharynx are similar in control (G, I K) and pmr-1(ra5) mutant embryos (H, J, L). VAB-9 (Q) and MEL-11 (P, R) show normal co-localization with AJM-1 (M, N, Q, R) in the lateral hypodermis in pmr-1(ra5) mutant embryos (M–R). Even in ventral (M, arrowhead) or lateral (N, arrow) hypodermal cells with positioning defects, AJM-1, VAB-9 and MEL-11 (M–R) are properly localized to the apical adhesion junction. Strains, reagents, and references are as indicated in Table 3; all strains grown at 25°C. Anterior is the left in all figures; lateral views. Embryos are comma stage (A–F) or elongation stage (G–R).

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Author Contributions

Conceived and designed the experiments: VP JS SK RM LI CF MBM JM WL RW AC D-GH ASF AS-W TS. Analyzed the data: VP JS SK RM LI CF MBM JM WL RW AC D-GH ASF AS-W TS. Contributed reagents/materials/analysis tools: VP JS. Wrote the paper: VP.

References

1. Brini, M. and Carafoli, E. (2009) Calcium pumps in health and disease. Phys Rev 89: 1341–1378.
2. Brundage, R.A., Fogarty, K.E., Tuft, R.A. and Fay, F.S. (1991) Calcium gradients underlying polarization and chemotaxis of eosinophils. Science 253: 703–706.
3. Ridley, A.J., Schwartz, M.A., Burridge, K., Firtel, R.A., Ginsberg, M.H., et al. (2003) Cell Migration: Integrating signals from front to back. Science 302: 1704–1709.
4. Wei, C., Wang, X., Zheng, M. and Cheng, H. (2012) Calcium gradients underlying cell migration. Curr Opin Cell Biol 24: 254–261.
5. Wei, C., Wang, X., Chen, M., Ouyang, K., Zheng, M., et al. (2010) Flickering calcium microdomains signal turning of migrating cells. Can J Physiol Pharmacol 88: 105–110.
6. Wei, C., Wang, X., Chen, M., Ouyang, K., Song, L.S., et al. (2009) Calcium flickers steer cell migration. Nature 457: 901–906.
7. Zampese, E. and Pizzo, P. (2012) Intracellular organelles in the saga of Ca2+ homeostasis: different molecules for different purposes? Cell Mol Life Sci 69: 1077–1104.
8. Howes, A.K. (2011) Cross-talk between calcium and protein kinase A in the regulation of cell migration. Curr Opin Cell Biol 23: 545–561.
9. Vandenaeveck, I., Vangheluwe, P., Raeymaekers, L., Wuytack, F. and Vanoeveren, J. (2011) The Ca2+-pumps of the endoplasmic reticulum and Golgi apparatus. Cold Spring Harbor Perspectives in Biology 3: a004184.
10. Missiaen, L., Dode, L., Vanoeveren, J., Raeymaekers, L. and Wuytack, F. (2007) Calcium pumps in the Golgi apparatus. Cell Calcium 41: 403–416.
11. He, W. and Hu, Z. (2012) The role of the Golgi-resident SPCA Ca2+-Mn2+ pump in ionic homeostasis and neural function. Neurochem Res 37: 435–440.
12. Hailey, H. and Hailey, H. (1939) Familial benign chronic purpura. Arch Dermatol Syphilol 39: 679–683.
13. Hu, Z., Bonifas, J.M., Breech, J., Bench, G., Shigihara, T., et al. (2000) Mutations in ATP2C1, encoding a calcium pump, cause Hailey-Hailey disease. Nature Genet 24: 61–65.
14. Kellermayer, R. (2005) Hailey-Hailey disease is an orthodiscine of PMR-1 deficiency in Sarcophaga cecereusar. FEBS Letters 579: 2021–2025.
15. Missiaen, v., Raeymaekers, L., Dode, L., Vanoeveren, J., Van Baelen, k., et al. (2004) SPCA1 pumps and Hailey-Hailey disease. Biochim Biophys Res Comm 322: 1204–1213.
16. Sudbrak, R., Brown, J., Dobson-Stone, C., Carter, S., Ramser, j. and et al. (2000) Hailey-Hailey disease is caused by mutations in ATP2C1 encoding a novel Ca2+ pump. Hum Mol Genet 11: 1131–1140.
17. Hwang, L., Lee, J., Richard, G., Utto, J. and Hsu, S. (2004) Type 1 segmental manifestation of Hailey-Hailey disease. J Am Acad Dermatol 49: 712–714.
18. Poblete-Gutierrez, P., Wiedholt, T., Konig, A., Jüger, F., Marquardt, Y., et al. (2004) Allelic loss underlies type 2 segmental Hailey-Hailey disease, providing molecular confirmation of a novel genetic concept. J Clin Invest 114: 1867–1869.
19. Okunade, G.W., Miller, M.L., Ashar, M., Andringa, A., Sanford, L., et al. (2007) Loss of the Atp2c1 Secretory Pathway Ca2+-ATPase (SPCA1) in mice causes Golgi stress, apoptosis, and midgestational death in homozygous embryos and squamous cell tumors in adult heterozygotes. J Biol Chem 282: 26517–26527.
20. Chun, S.I., Whang, K.C. and Su, W.P. (1988) Squamous cell carcinoma arising in Hailey-Hailey disease. J Cutan Pathol 15: 234–237.
21. Cockayne, S.E., Rassl, D.M. and Thomas, S.E. (2000) Squamous cell carcinoma arising in Hailey-Hailey disease of the vulva. Br J Dermatol 142: 540–542.
22. Holst, V.A., Fair, K.P., Wilson, B.B. and Patterson, J.W. (2000) Squamous cell carcinoma arising in Hailey-Hailey disease. J Am Acad Dermatol 43: 368–371.
23. Metze, D., Hamann, H., Schorat, A. and Luger, T. (1995) Involvement of the NHE1 gene in Hailey-Hailey disease. A histological, ultrastructural, and histochemical study of lesional and non-lesional skin. J Cutan Pathol 23: 211–222.
24. Hashimoto, K., Fujishara, K., Tada, J., Harada, M., Setoyama, M., et al. (1995) Desmosomal dissolution in Grover’s disease, Hailey-Hailey disease and Darier’s disease. J Cutan Pathol 1995: 488–501.
25. Shull, Y. and Saam, S. (2011) Secretory pathway stress responses as possible mechanisms of disease in involving Golgi Ca2+ pump dysfunction. Biofactor 37: 150–158.
26. Baylis, H. and Varzaneh-Marinique, R. (2012) Genetic analysis of IP3 and calcium signaling pathways in C. elegans. Biochimica et Biophysica Acta General Subjects 1280: 1253–1268.
44. Toyoshima, C and Mizutani, T (2004) Crystal structure of the calcium pump.

32. Zalk, R., Lehnart, S.E. and Marks, A.R. (2007) Modulation of the Ryanodine Receptor and intracellular calcium. Ann Rev Biochem 76: 367–385.

56. Cassata, G, Shemer, G, Morandi, P, Donhauser, R and Podbilewicz, B (2005) C. elegans reveals new functions in feeding and embryogenesis. Mol Cell Biol 15: 1108–1118.

34. Missiaen, L., Van Acker, K., Parys, J.B., De Smedt, H., Van Baelen, K., et al. (2003) Baseline cytosolic Ca2+ oscillations during early nematode gastrulation depend on common cytoskeletal mechanisms but different cell polarity and cell fate regulators. Dev Biol 253: 33–45.

37. Tabara, H., Motohashi, T. and Kohara, Y. (1996) A multi-well version of in vitro embryonic cell lineage of the nematode Caenorhabditis elegans reveals new functions in feeding and embryogenesis. Mol Biol Cell 124: 4193–4200.

53. Murray, J I, Bao, Z, Boyle, T and Waterston, R H (2006) The lineaging of C. elegans. WormBook, ed. The C. elegans Research Community, WormBook, doi:10.1895/wormbook.1.23.1, http://www.wormbook.org.

46. Wormbase (2012) http://www.wormbase.org/db/get?name = WBGene00004063; class = Gene.

43. Gengyo-Ando, K and Mitani, S (2000) Characterization of mutations induced by ethyl methanesulfonate, UV, and trimethylpsoralen in the nematode Caenorhabditis elegans. Proc Natl Acad Sci U S A 97: 7403–7408.

52. Murray, J.I., Bao, Z., Boyle, T.J., Boeck, M.E., Nicholas, T.J., et al. (2003) Coordinated regulation of cell and cell lineage in C. elegans. Curr Biol 13: 1529–1535.

40. Kelly, W G, Xu, S, Montgomery, M K and Fire, A (1997) Distinct patterns the embryonic epidermis of Caenorhabditis elegans. C. elegans Par-3 and PAR-6 are required for apico-basal polarity and cell adhesion and gastrulation. Development 130: 5393–5402.

45. Celniker, S.E., Dillon, L.A.L., Gerstein, M.B., Gunsalus, K.C., Henikoff, S., et al. (2004) The AceTree: a tool for visual analysis of embryonic cell lineage of the nematode C. elegans. C. elegans. Nat Genet 36: 2119–2124.

63. Nance, J. et al. Gastrulation in Caenorhabditis elegans. Curr Biol 14: 1317–1326.

60. Trzebiatowska, A, Topf, U, Saarler, U, Drabikowski, K and Chiquet-Ehrismann, R (2008) Interactions between the let-502/Rho-binding kinase and nmy-1, nmy-2, and nmy-4 is required for gonadal and pharyngeal basement membrane integrity and acts redundantly with integrin α-in-1 and dystroglycan dmy-1. Mol Cell Biol 19: 3908–3916.

57. Brunschwig, K, Wittman, C, Burglin, T, Tobler, H, et al. (1999) Anterior organization of the C. elegans embryo by the labial-like Hox gene ceh-13. Development 126: 1537–1546.

38. Nance, J. et al. Gastrulation in C. elegans. September 26, 2005, WormBook, ed. The C. elegans Research Community, WormBook, doi:10.1895/wormbook.1.23.1, http://www.wormbook.org.

59. Rossler, Schneider, and Nance, J (2009) Polarity and cell fate specification in the control of Caenorhabditis elegans gastrulation. Development 25: 759-767.

55. Harnett, J B and Goldstein, B (2011) Internalization of multiple cells during C. elegans gastrulation depends on common cytoskeletal mechanisms but different cell polarity and cell fate regulators. Dev Biol 350: 1–12.

51. Praxis, V, Cieczone, E and Austin, J (2005) SMA-1 spectrin has essential roles in epithelial cell sheet morphogenesis in C. elegans. Dev Biol 283: 157–170.

65. Nance, J., Murro, M E and Press, I (2003) C. elegans PAR-3 and PAR-6 are required for apico-basal polarity associated with cell adhesion and gastrulation. Development 130: 5393–5402.

29. Maryon, E.B., Saari, B. and Anderson, P. (1998) Muscle-specific functions of ryanodine receptor channels in C. elegans: Golgi pmr-1 receptor and intracellular calcium. Ann Rev Biochem 76: 367–385.

33. Misaki, D, Van Acker, K., Parys, J.B., De Smedt, H., Van Baelen, K., et al. (2003) Functional assessment of Nramp-like metal transporters and manganese in Caenorhabditis elegans reveals new functions in feeding and embryogenesis. Mol Biol Cell 14: 1108–1118.

62. Nance, J. et al. Gastrulation in Caenorhabditis elegans. Curr Biol 14: 1317–1326.

54. Trzebiatowska, A, Topf, U, Saarler, U, Drabikowski, K and Chiquet-Ehrismann, R (2008) Interactions between the let-502/Rho-binding kinase and nmy-1, nmy-2, and nmy-4 is required for gonadal and pharyngeal basement membrane integrity and acts redundantly with integrin α-in-1 and dystroglycan dmy-1. Mol Cell Biol 19: 3908–3916.

50. Praxis, V, Cieczone, E and Austin, J (2005) SMA-1 spectrin has essential roles in epithelial cell sheet morphogenesis in C. elegans. Dev Biol 283: 157–170.

58. Nance, J. et al. Gastrulation in Caenorhabditis elegans. Curr Biol 14: 1317–1326.
85. Brenner, S (1974) The genetics of Caenorhabditis elegans. Genetics 77: 71–94.
86. Praitis, V, Casey, E, Collar, D and Austin, J (2001) Creation of low-copy integrated transgenic lines in Caenorhabditis elegans. Genetics 77: 71–94.
87. Fay, D. Genetic mapping and manipulation: Chapter 5-SNPs: Three-point mapping (February 17, 2006), WormBook, ed. The C. elegans Research Community, WormBook, doi/10.1895/wormbook.1.94.1, http://www.wormbook.org.
88. Williams, B D, Schrank, B, Huynh, C, Shownkeen, R and Waterston, R H (1992) A genetic mapping system in C. elegans based on polymorphic sequence-tagged sites. Genetics 131: 609–624.
89. Wicks, S, Yeh, R, Gish, W, Waterston, R and Plasterk, R (2001) Rapid gene mapping in Caenorhabditis elegans using a high density polymorphism map. Nature Genetics 28: 160–164.
90. Bigelow, H, Doitsidou, M, Sarin, S and Hobert, O (2009) MAQGene: Software to facilitate C. elegans mutant genome sequence analysis. Nature Methods 6: 549.
91. Mello, C C, Kramer, J M, Stinchcomb, D and Ambros, V (1991) Efficient gene transfer in C. elegans: extrachromosomal maintenance and integration of transforming sequences. EMBO Journal 10: 3959–3970.
92. Evans, T C, Crittenden, S L, Koyoyianni, V and Kimble, J (1994) Translational control of maternal gfp-l mRNA establishes an asymmetry in the C. elegans embryo. Cell 77: 183–194.
93. McKewen, C, Prattis, V and Austin, J (1990) sma-1 encodes a BH-spectrin homolog required for Caenorhabditis elegans morphogenesis. Development 125: 2087–2098.
94. Ahringer, J., ed. Reverse genetics (April 6, 2006), WormBook, ed. The C. elegans Research Community, WormBook, doi/10.1895/wormbook.1.47.1, http://www.wormbook.org.
95. Hadwiger, G, Dour, S, Arrur, S, Fo, x P and Nonet, M (2010) A monoclonal antibody toolkit for C. elegans. PLoS ONE 5: e100161. doi:10.1371/journal.pone.0010161.
96. Sulston, J E and White, J G (1988) “Parts List”. In: Wood, W. B., Wood, W. B.s. The Nematode Caenorhabditis elegans. Cold Spring Harbor, New York, , USA. Cold Spring Harbor Laboratory Press. pp. 415–451.
97. Mohler, W A, Simske, J S, Williams-Masson, E M, Hardin, J D and White, J G (1998) Dynamics and ultrastructure of developmental cell fusions in the Caenorhabditis elegans hypodermis. Curr Biol 8: 1097–1099.
98. King, R S, Maiden, S L, Hawkins, N C, Kohara, Y, et al. (2009) The N- or C-terminal domains of DSH-2 can activate the C. elegans Wnt/beta-catenin asymmetry pathway. Developmental Biology 238: 234–244.
99. Altun-Gultekin, Z F, Andach, i Y, Tsalik, E L, Pilgrim, D, Kohara, Y, et al. (2001) A regulatory cascade of three homeobox genes, ceh-10, ttx-3 and ceh-23, controls cell fate specification of a defined interneuron class in C. elegans. Development 128: 1631–1646.
100. Chalfie, M, Tu, Y, Euskirchen, G, Ward, W W and Prasher, D C (1994) Green fluorescent protein as a marker for gene expression. Science 263: 802–805.
102. Chen, L, M, Krause, M, Sepanski, and Fire, A (1994) Elements regulating cell- and stage-specific expression of the C. elegans MyoD family homolog hlh-1. Developmental Biology 166: 133–148.
103. Ikegami, R, Simokat, K, Zheng, H, Brown, L, Garriga, G, et al. (2012) Semaphorin and Eph receptor signaling guide a series of cell movements for ventral enclosure in C. elegans. Current Biology 22: 1–11.
104. Grana, T M, Cox, E A, Lynch, A M and Hardin, J (2010) SAX-7/L1CAM and HMR-1/cadherin function redundantly in blastomere compaction and non-muscle myosin accumulation during Caenorhabditis elegans gastrulation. Developmental Biology 344: 731–744.