Asymmetric Wnt Pathway Signaling Facilitates Stem Cell-Like Divisions via the Nonreceptor Tyrosine Kinase FRK-1 in *Caenorhabditis elegans*

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ABSTRACT Asymmetric cell division is critical during development, as it influences processes such as cell fate specification and cell migration. We have characterized *FRK-1*, a homolog of the mammalian Fer nonreceptor tyrosine kinase, and found it to be required for differentiation and maintenance of epithelial cell types, including the stem cell-like seam cells of the hypodermis. A genomic knockout of *frk-1*, allele *ok760*, results in severely uncoordinated larvae that arrest at the L1 stage and have an excess number of lateral hypodermal cells that appear to have lost asymmetry in the stem cell-like divisions of the seam cell lineage. *frk-1(ok760)* mutants show that there are excess lateral hypodermal cells that are abnormally shaped and smaller in size compared to wild type, a defect that could be rescued only in a manner dependent on the kinase activity of *FRK-1*. Additionally, we observed a significant change in the expression of heterochronic regulators in *frk-1(ok760)* mutants. However, *frk-1(ok760)* mutants do not express late, nonseam hypodermal GFP markers, suggesting the seam cells do not precociously differentiate as adult-hyp7 cells. Finally, our data also demonstrate a clear role for *FRK-1* in seam cell proliferation, as eliminating *FRK-1* during the L3–L4 transition results in supernumerary seam cell nuclei that are dependent on asymmetric Wnt signaling. Specifically, we observe aberrant *POP-1* and *WRM-1* localization that is dependent on the presence of *FRK-1* and *APR-1*. Overall, our data suggest a requirement for *FRK-1* in maintaining the identity and proliferation of seam cells primarily through an interaction with the asymmetric Wnt pathway.

KEYWORDS Wnt; fer; kinase; asymmetry; development

ASYMMETRIC division is a critical component of stem cell populations, ranging from organismal development to tissue maintenance during adulthood. Without asymmetric divisions early embryos would not progress toward cellular specification and adult cell types, such as blood, would lose the ability to maintain numbers through self-renewal. Particularly important to stem cell division is the capability to maintain the stem cell population via asymmetric division where one daughter cell is specified to become a certain cell type, while the other daughter cell becomes another stem cell.

In the nematode *Caenorhabditis elegans* a stem cell-like population, called seam cells, exists in the hypodermis and undergoes a series of asymmetric divisions after each larval molt, thus facilitating postembryonic development (Sulston and Horvitz 1977). Seam cells are critical for proper formation of the hypodermis, the secreted cuticle, and other cell types derived from seam cells such as neuroblasts and glial cells. The seam cells consist of three anterior sets, H0, H1, and H2, followed by six V cells and one T cell in the posterior (Figure 1). The V cells undergo unique stem cell-like divisions during postembryonic development that lead to one anterior daughter that fuses with the hypodermal syncytial cell, hyp7, and one posterior daughter that goes on to divide asymmetrically again at the next larval molt. Interestingly, within these V cells, there is one symmetric division during the early L2 larval stage that precedes the asymmetric division later in L2. After the L4 molt, progressing to adulthood, the remaining seam cells then differentiate and exit the cell cycle (Joshi *et al.* 2010).

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doi: 10.1534/genetics.115.181412

Manuscript received July 31, 2015; accepted for publication September 5, 2015; published Early Online September 9, 2015.

Supporting information is available online at www.genetics.org/lookup/suppl/doi:10.1534/genetics.115.181412/-/DC1.

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Figure 1 Seam cell asymmetric divisions during postembryonic development in *C. elegans*. During postembryonic development, *C. elegans* larvae undergo a series of molts, each of which is associated with carefully timed seam cell divisions. In the V1–4, 6 cells the divisions are nearly all symmetrical where the anterior daughter becomes part of the hyp7 syncytium. The posterior daughter then self-renews as another stem cell-like seam cell. The exception to the asymmetric divisions is in early L2 where the V1–4, 6 cells undergo one symmetrical division prior to dividing again in an asymmetric manner. The V5 cell divides asymmetrically as well, but

gives rise to a series of neuronal postdeirid cells in the L2 division, whereas the remaining cell divisions parallel V1–4, 6. Thus, excluding the V5 postdeirid cells, the remaining cells differentiate at adulthood into hypodermal cells (as hyp7 or seam).

Seam cell asymmetry has been well studied and shown to be regulated primarily by two interacting pathways: (1) heterochronic genes, which regulate the timing of seam cell division (Rougvie and Moss 2013), and (2) asymmetric Wnt pathway components, which function to specify the anterior and posterior daughter during each round of division (Phillips and Kimble 2009; Sawa 2012). The Wnt pathway has been closely associated with asymmetric cell division and cell polarity in cellular contexts ranging from embryonic development (Maduro 2009; Sawa 2012) to maintenance of the stem cell niche (Clevers *et al.* 2014). The *C. elegans* asymmetric Wnt pathway, termed the Wnt/ β -catenin asymmetry (W β A) pathway, shares some attributes with the “canonical” Wnt/ β -catenin pathway involved in cellular proliferation (among other functions). In the canonical pathway the presence of a Wnt signal binding the Frizzled receptor stabilizes cytoplasmic β -catenin, which then localizes to the nucleus to interact with TCF/LEF factors to activate target gene expression (Clevers 2006). Similarly, the *C. elegans* W β A pathway increases the levels of a β -catenin transcriptional activator, called *SYS-1*, which then binds the TCF homolog *POP-1* and activates expression of Wnt target genes. To negatively regulate β -catenin in the absence of Wnt, both systems require a kinase, casein kinase I α (CKI α , *KIN-19* in *C. elegans*) and the protein scaffold, adenomatous polyposis coli (APC, *APR-1* in *C. elegans*). However, in W β A the transcriptional activity of *SYS-1* and *POP-1* is asymmetric and coupled to cell division, such that only one of two daughter cells (the “signaled” daughter) displays elevated *SYS-1* levels and target gene expression, compared to the “unsigned” daughter, thereby asymmetrically changing cellular identity (Herman 2001; Kidd *et al.* 2005; Huang *et al.* 2007; Phillips *et al.* 2007).

The major difference between the W β A and the canonical pathways is that, in *C. elegans*, the asymmetric Wnt pathway also regulates *POP-1*/TCF nuclear localization, which is normally asymmetric between the two nuclei of recently divided cells affected by the presence of a Wnt signal (Lin *et al.* 1995; Rocheleau *et al.* 1997; Wildwater *et al.* 2011). Somewhat counterintuitively, Wnt signaling *lowers* nuclear *POP-1* levels in the same cell that has elevated *SYS-1*/ β -catenin, resulting in reciprocal β -catenin and TCF asymmetry (Thorpe *et al.* 1997; Lin *et al.* 1998; Huang *et al.* 2007; Phillips *et al.* 2007; Phillips and Kimble 2009; Sawa 2012). Current models suggest this

lowering results in an optimal β -catenin:TCF ratio where, in the signaled daughter, most of the *POP-1* is complexed with its *SYS-1* coactivator, while in the unsigned daughter, most of the *POP-1* is unbound by β -catenin and represses target gene expression. Like *SYS-1* asymmetry, *POP-1* nuclear asymmetry has been shown to be required in myriad cell types, including the intestinal and gonadal precursors as well as the hypodermal seam. The mechanism of *POP-1* lowering is dependent on a distinct branch of W β A pathway that utilizes a second β -catenin, *WRM-1*. *WRM-1* is elevated in the signaled nucleus where it binds and phosphorylates *POP-1* with the Nemo-like kinase, *LIT-1*, to induce *POP-1* nuclear export rather than transcriptional activation (Rocheleau *et al.* 1999; Shin *et al.* 1999; Lo *et al.* 2004; Yang *et al.* 2011). Thus the asymmetric regulation of the two β -catenins is critical to the asymmetric cell division and the subsequent cell fate decision. Interestingly, both *WRM-1* and *SYS-1* are kept low in the nucleus of the unsigned daughter by *APR-1*/APC. *APR-1* negatively regulates total cellular levels of *SYS-1* in a manner consistent with its degradation (Baldwin and Phillips 2014), while also lowering nuclear *WRM-1* by stabilizing its cortical localization (Mizumoto and Sawa 2007a; Sugioka *et al.* 2011). Although much has been reported on the importance of the Wnt signaling influence on seam cell asymmetric division, certain aspects remain unclear, such as the mechanism by which *POP-1* asymmetry is established in the two daughter cells.

An important aspect of asymmetric signaling is the presence of kinases that facilitate the progression of the signal from the plasma membrane to the nucleus. Kinases such as *LIT-1* and *KIN-19* have both been shown to be critical for proper asymmetric division within the seam cell lineage. However, these two kinases have been reported to function in separate manners, with the heterochronic genes regulating the *LIT-1* influence on seam cell division in addition to regulating *WRM-1* and *POP-1* (Takeshita and Sawa 2005; Mizumoto and Sawa 2007a; Harandi and Ambros 2015). *KIN-19* negatively regulates *SYS-1* in the unsigned daughter along with *APR-1*, which promotes the hypodermal fate (Banerjee *et al.* 2010; Gleason and Eisenmann 2010; Baldwin and Phillips 2014). We report here the requirement for a nonreceptor tyrosine kinase, *FRK-1*, that facilitates the proper asymmetric localization of *POP-1*, and thus identity, of each seam cell daughter. *FRK-1* is a homolog of the vertebrate Fer kinase, which is activated downstream of

pathways such as PDGF and EGFR, and has been shown to be involved in adhesion, proliferation, and activation of NFκB (Guo and Stark 2011; Lennartsson *et al.* 2013). We have previously shown that the absence of **FRK-1** during embryogenesis prevents proper enclosure in a kinase-independent manner (Putzke *et al.* 2005). Furthermore, we reported a role for **FRK-1** in regulating proliferation of *C. elegans* intestine, during embryogenesis, by preventing hyperactivation of a Wnt response in a kinase-dependent manner (Putzke and Rothman 2010). Importantly, in each case we showed that **FRK-1** interacts with a third *C. elegans* β-catenin, **HMP-2**, involved in cell adhesion but not WβA signaling, as well as **APR-1/APC**.

Related to asymmetric cell division is the heterochronic gene regulation pathway that controls the timing of progression during postembryonic development in *C. elegans*. More specifically, expression of microRNAs is activated and suppressed in a synchronized manner to regulate translation of messenger RNA (mRNA) and thus modulate protein activity, based on the stage of development (reviewed in Resnick *et al.* 2010). Importantly, heterochronic regulation has been linked to asymmetric cell division within the seam cell lineage of *C. elegans* in a manner that generally causes arrested or precocious seam cell division in the absence of specific microRNAs (miRNAs) such as occurs with the function of *let-7* and *lin-4* (Feinbaum and Ambros 1999; Reinhart *et al.* 2000). More recently, Harandi and Ambros (2015) demonstrated that there is a distinct interconnection between heterochronic genes and the WβA pathway, in that heterochronic genes regulate the ability of the WβA pathway to achieve asymmetric seam cell division.

Here we characterize that loss of zygotic **FRK-1** (via genomic knockout) results in loss of seam cell regulation, such that, although the animal arrests at the L1 stage of postembryonic development, the seam cells within the V-cell lineage divide symmetrically, similar to the first division within the L2 stage of development. Mutant larvae show a marked decrease in heterochronic gene targets, due to aberrant expression of the heterochronic regulator miRNA, *lin-4*. In later larvae (L3–L4) we show that the absence of **FRK-1** or **APR-1** results in excess seam cell division that is sufficient to compensate for the loss of **WRM-1** in a manner that is independent of **SYS-1**. Finally, we show that **FRK-1** is asymmetrically localized in seam cell daughter nuclei parallel to that of **POP-1**. Loss of **FRK-1** results in loss of **POP-1** asymmetry, due to the loss of **WRM-1** asymmetry and a shift in **APR-1** nuclear/cytoplasmic ratio. Finally, we present a novel mechanism by which **FRK-1** functions to regulate **POP-1** nuclear accumulation in dividing seam cells through **APR-1** and **WRM-1**, in a **SYS-1**-independent fashion, the loss of which results in compromised seam cell identity and premature arrest.

Materials and Methods

Strains and alleles

The wild-type strain used was the Bristol **N2** strain, described by Brenner (1974). To examine the loss of *frk-1* phenotype we used

line **VC558** containing allele (*ok760*). To examine the tissues in which the *frk-1* gene region was active an extrachromosomal array of $P_{frk-1}::GFP$ consisting of 1.8 kb of the *frk-1* promoter region was fused to a nuclear localized transcriptional GFP, using **pPD96.62** (gift from A. Fire, University of California, Santa Barbara, CA) plus the *rol-6(su1006)* marker, on plasmid **pRF4**, to create **JR2355**. Other strains used: **JR667** ($P_{scm}::GFP$), **HS1417** (**WRM-1::YFP**), **BTP118** [*unc-76* V; *osl13* ($P_{apr-1}::APR-1::VNS$; *unc-76* (+))], **uiwIs4** ($p_{sys-1}::mCherry::SYS-1$). Strains **AP110** and **AP112** contain extrachromosomal arrays consisting of the *frk-1* coding region [or ($P_{scm}::FRK-1(D308R)$), respectively] fused to the *scm* promoter region contained in plasmid **PMF1** (a generous gift from J. Rothman).

RNA-mediated interference

Primer pairs were made to the coding regions of *frk-1*, *worm-1*, *apr-1*, *hmp-2*, *sys-1*, *pop-1* and *unc-22*, and tagged with a T7 RNA polymerase promoter sequence. Each fragment was PCR-amplified from genomic DNA or cDNA. dsRNA was generated using a T7 reverse transcriptase kit (Ambion). Newly transitioned L3 larvae were soaked with dsRNA or fed in IPTG-containing plates with **OP50** expressing *frk-1* dsRNA (**HT115**; **PLT61**; **T04B2.2**) for 10 hr to ensure the L3–L4 seam cell divisions were captured. Larvae were analyzed using by fluorescence microscopy for nuclear GFP detection in seam cells. RNA-mediated interference (RNAi) knockdown efficiency was determined via quantitative PCR with knockdown percentages ranging from 57% to 75% (i.e., gene expression ranged from 25% to 43% after knockdown depending on the target) when performed from the L3–L4 transition (see [Supporting Information, Figure S3](#)).

Antibodies and immunofluorescence

MH27 is a mouse monoclonal antibody that recognizes epithelial apical junctions (Priess and Hirsh 1986). **NE2-1B4** is a mouse monoclonal antibody that recognizes an antigen expressed only in seam cells (Schnabel 1991). **P4G4** is a monoclonal mouse antibody recognizing **POP-1** (Lin *et al.* 1998). Immunofluorescence was imaged via a Nikon (Garden City, NY) **A1** confocal microscope and a Zeiss (Thornwood, NY) **Axio Imager D2** compound fluorescent microscope equipped with Zeiss Zen software. Raw image data were exported into TIFF format and analyzed using ImageJ software. Nuclei of seam cells were identified via DIC images and then mean protein fluorescence in fluorescent images was quantified using ImageJ. Background fluorescence was normalized for each experiment by using the same channels and exposures to image seam cells in **N2** wild-type worms ($N = 24$ for each exposure setting). All statistical comparisons were performed with either Student's *t*-test in Microsoft Excel or the Mann–Whitney test and VassarStats software (Lowry 2010). Bar graphs show error bars that are standard error of the mean.

Data availability

Strains from APP and BTP available upon request. Other strains available as noted by source.

Results

A fusion between the *frk-1* promoter region and nGFP shows the *frk-1* promoter to be active primarily in epithelial cell types, including the hypodermis, intestine, and germline uterine and spermathecal lining. Specifically, *frk-1* expression is observed in the developing seam cells during embryogenesis and larval development (Figure 2, A and B). We previously reported that FRK-1 protein localizes primarily to the plasma membrane in these cell types, with the exception of mitotically active cells, in which case FRK-1 is also observed localized to the nucleus (Putzke *et al.* 2005).

To address the mechanism by which FRK-1 functions during development in *C. elegans*, we obtained a genomic knockout of *frk-1*, allele *ok760*. The *frk-1(ok760)* allele disrupts *frk-1* expression by deleting 1500 bp of genomic region that correspond to 910 bp of promoter region as well as the first two exons and the second intron of the *frk-1* gene (Figure 2C). The *ok760* deletion results in no detectable transcription of *frk-1*, when compared to wild-type larvae (not shown), thus eliminating the possibility of expression from *frk-1* due to transposition to another location in the genome as a result of the mutation process.

Homozygous *frk-1(ok760)* mutants exhibit lethal larval arrest at the L1 stage with no progression to L2. The mutants remain alive for 3–4 days prior to death. In addition to L1 arrest, homozygous mutants are severely uncoordinated, being able to move their head region with very little body movement posterior to the pharynx. Death is likely not due to an inability to feed after hatching as we have observed bacteria in the intestine of *frk-1(ok760)* mutant larvae after being exposed to plates growing GFP expressing OP50 (not shown). Although the nature of the phenotype in these mutants is complex, there are likely multiple tissues affected by the mutation, thus resulting in the uncoordinated nature and early larval arrest, in addition to the seam cell division defect upon which this study focused.

Based on our previous studies showing that FRK-1 is required for proper patterning of the hypodermal seam cells (Putzke *et al.* 2005), we investigated whether the seam cells in *frk-1(ok760)* mutants were morphologically normal. In the absence of *frk-1*, seam cells appear to have lost their asymmetry, as both daughter cells are small, similar in size to the anterior seam cell in a wild-type larva, suggesting that these cells may have both taken on an anterior identity (Figure 2D, small box, and Figure 3C).

With the observed phenotype of the *frk-1(ok760)* homozygous mutants, we then tested the hypothesis that larval progression was halted in the mutants primarily due to improperly functioning seam cells. We first observed seam cell function by looking at seam cell numbers and expression of seam cell markers, normally observed in wild-type embryos. When newly hatched *frk-1(ok760)* mutant larvae are stained with MH27, which marks plasma membrane in epithelial cell types, the seam cells, especially the V-seam cells, appear misshapen [Figure 3, A (wild type) and C]. When the *frk-1(ok760)* allele

Figure 2 The *frk-1* gene is active in seam cells throughout development and results in early larval arrest when deleted from the genome. (A and B) *frk-1*:nGFP fusion shows *frk-1* promoter activity in early seam cells at 1.5-fold (black arrows) and persists in seam cells through adulthood (seam cells, white arrows; other nuclei shown are nonseam hypodermal nuclei, as *frk-1* is expressed in multiple epithelial cell types). (C) PCR of the *ok760* allele in *frk-1* resulting in ~1500 bp of DNA deleted corresponding to 910 bp of promoter and the first two exons of the *frk-1* coding region. Presence of a truncated transcript expressed from the remaining *frk-1* sequence was not detected (data not shown). (D) *frk-1(ok760)* mutants arrest at the L1 stage with an apparent loss of asymmetric division of the seam cell lineage. Wild-type seam cells are asymmetric postdivision with a larger posterior daughter (inset, top), while *frk-1(ok760)* mutants contain two similar sized daughters (smaller like the anterior daughter in a wild-type individual) (inset, bottom).

is crossed with a transgenic line expressing GFP exclusively in seam cells, homozygous mutants show an increase in seam cell proliferation that occurs primarily within the V1–V4 seam cells

Figure 3 The absence of FRK-1 results in aberrant seam cell function and inappropriate division. (A–D) *frk-1(ok760)* mutants do not have regularly shaped seam cells (A and C, MH27) and lack alae (B and D, NE21B4) at the L1 stage, suggesting nonfunctional seam cells. (E and F) Additionally, there are supernumerary seam cells, especially in V1–V4 (*scm::GFP*). Wild-type larvae (E, lateral view) show single cells post-division (when anterior daughter GFP has mostly subsided, white arrowhead; posterior nucleus, orange arrowhead), while *frk-1(ok760)* mutants (F, lateral view) show arrested daughter pairs with GFP persistence in the anterior daughter. (G) *frk-1* mutants exhibit significant loss of heterochronic gene markers as well as seam-specific markers.

[Figure 3, E (wild type) and F]. In these cases, the V1–V4 cells divide without division of the other seam cells and remain as a pair without the anterior cell fusing with the hypodermal syncytium, as is normally observed in wild-type larvae [average in wild type (WT) = 4.3, average in *ok760* mutants = 7.7, $P = 2.6 \times 10^{-8}$, $n = 25$ animals per condition]. The seam cell GFP expression in the anterior daughter persists until fusion with the hypodermis in wild type (Figure 3E, white arrowheads) while it persists indefinitely in the *ok760* mutant (Figure 3F, white arrowheads).

In addition, NE21B4, a marker of functional seam cells, is primarily absent in *frk-1(ok760)* homozygotes, suggesting that the seam cells are not functioning properly even when present [Figure 3, B (wild type) and D]. We then examined a series of genes that are normally expressed in functioning seam cells and the surrounding hypodermis and have been

shown to facilitate proper asymmetry and division during embryonic and larval progression: *lin-14*, *lin-28*, *lin-41*, *elt-3*, *egl-18*, *nhr-72*, and *nhr-81* (Ambros and Horvitz 1987; Moss *et al.* 1997; Gilleard *et al.* 1999; Koh and Rothman 2001; Yamamoto *et al.* 2011). In the case of markers such as *lin-14* and *lin-28*, we observed a near complete loss of expression in the *frk-1* mutants, with other seam-determining genes, such as *lin-41*, *egl-18*, *nhr-72*, and *nhr-81*, being significantly reduced (or nearly absent) when compared to wild type (Figure 3G). The marker, *elt-3*, is expressed in nonseam hypodermal cells, suggesting that regulation of hypodermal development in mutant larvae is disrupted beyond the seam cells. Interestingly, *lin-14* and *lin-28* are heterochronic genes, suggesting that timing of division may be inappropriately altered in *frk-1* mutants; however, it is more likely that the aberrant expression of heterochronic genes is due to upstream

gene expression changes and not genuine defects in the heterochronic pathway.

We then tested the hypothesis that the excess number of seam cells, combined with the apparent loss of asymmetry with the seam cells, was due to precocious differentiation of the seam cells, which would be supported by the premature loss of heterochronic gene expression (*lin-14* and *lin-28*). When we performed quantitative RT-PCR with *lin-4* and *let-7*, two miRNAs known to regulate developmental progression (Moss 2007), on newly hatched L1s, we observed an increase of *lin-4* expression in excess of 16-fold compared to wild type (Figure S2), further suggesting that the heterochronic gene regulation was initiated prematurely in the absence of FRK-1. We did not, however, observe *let-7* expression in newly hatched *frk-1(ok760)* mutants (Figure S2), implying that although early heterochronic regulation may be precocious, later regulation, directed by *let-7* expression beginning in L4, is not contributing to the misregulation of seam cells. Indeed, when we crossed the *frk-1(ok760)* mutation into a variety of transgenic lines expressing GFP under the regulation of genes that are active in late larval to adult seam cells (*col-19*, *col-49*, *col-38*, and *bli-1*) (Jackson *et al.* 2014), we found that there was no GFP expression in the *frk-1* mutants ($n = 50$ per transgenic line) (Table S1). The only instances in which we observed expression were in two constructs that include expression in seam cells ranging from embryonic to L4 or adulthood (*grd-10* and *egl-18*). Together these data suggest that the seam cells in *frk-1* mutants may be misregulated, but are not precociously differentiating. With these observations we pursued the idea that seam cells were not functional due to lack of asymmetry and thus, loss of specific identity, in the absence of FRK-1.

Since it has been previously reported that there is kinase-independent activity of the Fer-related kinases, and we were able to demonstrate this specifically in *C. elegans* intestinal proliferation (Putzke and Rothman 2010), we investigated whether the larval arrest and seam cell proliferation defects might be rescued in the presence of a kinase inactive FRK-1. In the presence of wild-type FRK-1, we observed a significant rescue of the larval lethality, with ~90.1% of larvae progressing beyond the L3 stage ($n = 237/263$), compared to *frk-1(ok760)* homozygous larvae containing the empty vector, in which all individuals arrested at the L1 stage ($n = 126/126$) (Figure 4A). The majority of *frk-1(ok760); FRK-1* (WT) rescued animals died as L4 or adults, with none viable enough to produce progeny, likely due to mosaicism of the rescuing construct. Larvae expressing the kinase inactive [kinase dead (KD)] version of FRK-1 were able to progress beyond the L1 stage 34.9% of the time but none progressed beyond L2, strongly suggesting that the kinase activity of FRK-1 is required during postembryonic development ($n = 65/186$) (Figure 4A). We then examined whether seam cell proliferation was dependent on FRK-1 kinase activity by performing the same rescue experiment as mentioned above, but in the presence of the seam cell marker GFP (used in Figure 3). Similar to viability rescue, in the presence of

wild-type FRK-1, 88.1% of larvae exhibited normal seam cell numbers, when compared to the *ok760* allele alone (Figure 4B). In the presence of the kinase inactive FRK-1 (KD), only 16.4% of embryos showed normal seam cell proliferation, compared to 94.4% in wild-type embryos. These data demonstrate that the kinase activity is required not only for viability, but also for proper proliferation within the seam cell lineage, during postembryonic development.

Additionally, to determine whether the seam cell defect could be rescued by expressing *frk-1* exclusively in the seam cells, we fused the *frk-1* coding region to the seam cell promoter used in the $P_{scm}::GFP$ construct (see *Materials and Methods*). Indeed, when wild-type *frk-1* was expressed in the seam cells of the *frk-1(ok760)* homozygous mutant, the larval lethality was abrogated such that most larvae progressed beyond the L1 stage (61.2%, $n = 134$), with over half of the larvae progressing to L3 or beyond when compared to either the *frk-1(ok760)* alone or *frk-1(ok760); P_{scm}::GFP* (negative control), in which $\geq 97\%$ of the larvae arrested at the L1 stage ($n = 147$ and 163 , respectively) (Figure 4C). This occurred in a kinase-dependent manner such that expressing the kinase inactive (KD) version of FRK-1 was unable to compensate for the loss of endogenous *frk-1*, with 90.9% of *ok760* larvae arresting at the L1 stage ($n = 177$). Similar to the rescue from the *frk-1* promoter the construct was not able to stably rescue any *frk-1(ok760)* homozygous individuals to viable, reproducing adults (again likely due to the mosaic nature of the extrachromosomal array). Importantly, we were able to observe rescue of the precocious proliferation in the seam cell lineage, observed in *frk-1* mutants, with the seam cell-exclusive expression of *frk-1* (Figure 4D). As with viability, the wild-type *frk-1* expressed solely in seam cells was able to return the number of V1–4 seam cells to normal levels (avg = 4.35), while the kinase inactive version was unable to do so (avg = 7.70), with seam cell numbers being similar to those in *frk-1(ok760)* alone or with the $P_{scm}::GFP$ control (avg = 8.15) ($n = 50$ for each rescue condition).

With an established connection between FRK-1 and seam cell proliferation, we examined whether FRK-1 might be participating in the asymmetric seam cell division regulatory pathway. Using the $P_{scm}::GFP$ transgenic line, we performed RNAi on L3 larvae and observed seam cell proliferation during progression to the L4 stage. We chose this stage as it is a stage in which to consistently monitor seam cell divisions with ease of manipulation compared to earlier stages. Additionally, we suspect FRK-1 functions in the developmental decisions of many tissues, so waiting until these nonseam lineages have terminally differentiated bypasses this role and instead allows us to focus on the tissue under study. However, because of this we needed to use RNAi instead of the *frk-1(ok760)* allele as the homozygous mutants do not progress beyond the L1 stage. In *frk-1(RNAi)* individuals there are an excess number of seam cells, with the average being 29.2 ($n = 84$) (Figure 5, A and B), when compared to animals exposed to *unc-22(RNAi)*, which does not affect

Figure 4 FRK-1 activity in postembryonic development is kinase dependent. (A and B) *frk-1(ok760)* mutants are rescued by wild-type *frk-1* in a kinase activity-dependent manner. (A) *frk-1(ok760)* mutants are rescued to adulthood with wild-type *frk-1* but not kinase inactive *frk-1*. (B) Similarly, wild-type, but not kinase inactive, *frk-1* is sufficient to rescue proliferation defects within the seam cells of *frk-1(ok760)* mutants. (C) Rescue to adulthood is sufficient with *frk-1* activity confined exclusively to seam cells, via expression of *frk-1* from the *scm* promoter. (D) As with the expression from the *frk-1* promoter, expression from the *scm* promoter rescues seam cell proliferation in a kinase-dependent manner. (V1–4 cells are shown at 30 hr postfertilization. At this stage, in wild type, the asymmetric L2 divisions are complete and the cells exhibit asymmetric GFP expression.)

seam cell numbers (14.5, $n = 72$). The Wnt asymmetry pathway components affect seam cell proliferation such that loss of *POP-1*, via RNAi, results in excess numbers averaging 45.3 cells per larva ($n = 73$) (Figure 5, A and B), whereas loss of *WRM-1* causes loss of seam cells, with <10 per larva (average = 8.6 per larva, $n = 62$) (Figure 5B).

Our previous studies showed *FRK-1* interacting with asymmetric Wnt pathway elements by preventing excess β -catenin activity during intestine proliferation within the E lineage (Putzke and Rothman 2010). It has also been demonstrated that the asymmetric Wnt pathway, functioning through factors such as *WRM-1*, *APR-1*, *PRY-1*, and *SYS-1*, affects asymmetry and proliferation of seam cells (Phillips *et al.* 2007; Gleason and Eisenmann 2010; Baldwin and Phillips 2014). Thus, we tested the hypothesis that *FRK-1* may be functioning in a similar manner in lateral seam cells to that in the intestine. Eliminating *APR-1*, *FRK-1*, or *POP-1* via RNAi resulted in a significant increase in the number of seam cells (22.2, 29.8, and 45.3, respectively) ($n = 53, 58, 49$) (Figure 5B). When eliminated in tandem, *apr-1*, *pop-1* (double RNAi) yielded an average of 52.9, whereas *frk-1*, *pop-1* (double RNAi) resulted in 61.3 seam cells ($n = 45$ for each case). Although the increase is significant (*pop-1* RNAi alone

vs. *frk-1*, *pop-1* or *apr-1*, *pop-1* double RNAi), it points to an additive rather than a synergistic effect, suggesting they are acting in the same pathway as opposed to separate signaling mechanisms.

Having observed a genetic interaction with *POP-1*, we then investigated whether *FRK-1* and *WRM-1* might be functioning together to regulate seam cell proliferation. When *FRK-1* and *WRM-1* are knocked down simultaneously, a modest, but significant, change was observed with seam cell numbers increasing to 18.7 ($n = 41$), which is consistent with a rescue of *wrm-1*(RNAi) to wild-type seam cell numbers, compared to loss of *WRM-1* alone (8.6, $n = 55$) (Figure 5B). Interestingly, when the expression of *FRK-1* and *APR-1* is knocked down, the number of seam cells increases to 34.3 ($n = 47$). However, the increase due to the loss of either *FRK-1* or *APR-1* (or both *FRK-1* and *APR-1*) was abrogated by loss of *WRM-1*, strongly suggesting that regulation of *POP-1*-mediated seam cell asymmetry and proliferation functions through *FRK-1*, *APR-1*, and *WRM-1*.

To determine whether the supernumerary seam cells in the absence of *FRK-1* were due to participation of other β -catenin homologs, we tested the loss of both *SYS-1*, a major contributor to seam cell asymmetry, and *HMP-2*, a β -catenin

Figure 5 (A) Elimination of *frk-1* by RNAi at the L3–L4 transition results in excess seam cell divisions (top), approximately half as much as *pop-1* elimination (middle), when compared to control RNAi (*unc-22*, bottom). Removal of *frk-1* or *apr-1* results in supernumerary seam cell proliferation that is additive with *pop-1* elimination. (B) The defects in seam cell proliferation observed with loss of *frk-1* and *apr-1* are *wrm-1* dependent but independent of *sys-1*.

homolog not previously associated with seam cell identity or proliferation. Loss of either *hmp-2* or *sys-1* (both via RNAi) did not result in a significant change in seam cell number, with their averages being 16.3 and 15.1, respectively ($n = 42, 38$) (Figure 5B). When either β -catenin homolog was eliminated along with *frk-1*, the subsequent increase in seam cell number was not significantly different from elimination for *frk-1* alone [*frk-1, sys-1* (double RNAi) avg = 25.4, $n = 45$; *frk-1, hmp-2* (double RNAi) avg = 28.2, $n = 52$]. These results suggest that the increase we observed was specific for an interaction between *frk-1*, *apr-1*, and the β -catenin homolog, *wrm-1*.

With an established connection between seam cell proliferation and the asymmetric Wnt pathway, we next tested the hypothesis that *POP-1* asymmetry in the anterior and posterior nucleus of the newly dividing seam cells was perturbed in the absence of *FRK-1*. It has been long established that *POP-1* is localized asymmetrically in seam cell nuclei, such that the anterior nucleus has a higher level of *POP-1* than the posterior nucleus (Lin *et al.* 1998). Although we previously reported that *FRK-1* is nuclear in mitotically active cells in embryos (Putzke *et al.* 2005), the subcellular localization of *FRK-1* in mitotically active seam cells is not known. Interestingly, we found that, under wild-type conditions, in actively dividing seam cells (L1–L2), *FRK-1* mimics *POP-1* asymmetric localization such that both proteins exhibit higher levels in the anterior nucleus and low levels in the corresponding posterior nucleus (Figure 6A). In the absence of *FRK-1*

[*frk-1(ok760)* mutants], *POP-1* asymmetry is lost, with both seam cell nuclei exhibiting low levels of *POP-1* ($n = 20$). Quantitation of the signal intensity for each protein shows the asymmetric localization for *POP-1* and *FRK-1* is significant between the two nuclei (Figure 6B). In stark contrast, the loss of *FRK-1* results in no quantitative difference between levels of *POP-1* between the anterior and posterior nuclei in all 20 cell divisions that were analyzed.

After establishing that *FRK-1* is involved in regulation of seam cell division through the asymmetric Wnt pathway, we then tested the hypothesis that subcellular localization of pathway components was perturbed, resulting in the loss of *POP-1* asymmetry in seam cell nuclei. We first examined localization of *APR-1* (APR-1::YFP) and *SYS-1* (mCherry::SYS-1) in *frk-1(ok760)*, observing the initial seam cell division at the L1 stage. In wild-type larvae *APR-1* localizes to the cytoplasm with punctae on the plasma membrane, as well as some nuclear localization, with no quantitative difference between the anterior and posterior nuclei (Figure S1, A, B, E, and F). This *APR-1* asymmetry can be seen as an anterior crescent in sections that bisect the cell (Figure S1A, left column), or as asymmetric punctae that localize to the basal surface of cortical sections (Figure S1B, left column). However, *SYS-1* is normally nuclear localized with an asymmetry that favors stronger localization in the posterior nucleus (Figure S1, A and B, center column). In the absence of *FRK-1*, there is no change in the nuclear localization or the anterior–posterior

Figure 6 FRK-1 nuclear localization parallels that of POP-1 in seam cells nuclei postdivision. (A) FRK-1 is nuclear localized and asymmetric in an anterior–posterior manner in dividing seam cells within the V1–4 population (middle) similar to POP-1 (top), with higher levels in the anterior daughter. POP-1 asymmetry is lost in the absence of FRK-1, with both daughters containing low levels of POP-1 (bottom). (B) Quantification of FRK-1 and POP-1 asymmetry in seam cell daughters is similar in wild type compared to POP-1 in *frk-1(ok760)* mutants (FITC signal in A is normalized to DAPI for quantitation). (C) WRM-1 is normally asymmetric in dividing seam cell nuclei (top), but becomes more symmetric in the absence of *frk-1* (bottom). (D) Quantitation of WRM-1::YFP localization in the anterior and posterior daughter nuclei shows a significant loss of asymmetry immediately postdivision (* $P < 0.05$, with all other comparisons $P > 0.05$).

asymmetry of *SYS-1* in dividing seam cells (Figure S1, C and D, center column). Additionally, we did not observe a significant shift in *APR-1* localization that occurs in the absence of *FRK-1*, when compared to that in wild-type larvae (Figure S1, E and F).

The lack of any shift in localization of *APR-1* from the anterior nucleus led us to speculate that *WRM-1* localization might be perturbed when *FRK-1* is not present. We then performed *frk-1(RNAi)* on early larvae, examining *WRM-1::YFP* localization during the L1–L2 seam cell divisions. Normally, although distributed in both the cytoplasm and the nucleus, *WRM-1* is asymmetrically localized between the seam cell nuclei, with highest levels being in the posterior nucleus of dividing seam cells ($n = 24$) (Figure 6C). However, loss of normal *FRK-1* activity causes a loss of *WRM-1* asymmetry such that the anterior and posterior nuclei are no longer significantly different with respect to *WRM-1* levels ($n = 22$) (Figure 6, C and D). These data suggest that *FRK-1* is working in conjunction with *WRM-1* (and likely *APR-1*), but independently of *SYS-1*, to alter *POP-1* levels and thus perturb seam cell divisions during postembryonic development.

Discussion

Asymmetric cell divisions, and the timing thereof, in the seam cells of the hypodermal lineage are critical during postem-

bryonic development. There are two major pathways in the seam cells that drive division: (1) the noncanonical Wnt pathway that initiates asymmetric division within the seam cells and (2) heterochronic gene expression that regulates the timing of the divisions during larval progression. Within the asymmetric Wnt pathway, it has been well established that the regulation of *POP-1* localization between the two daughter nuclei of a dividing seam cell, in an unequal manner, determines the identity of those daughters (Lin *et al.* 1998; Herman 2001; Kidd *et al.* 2005; Phillips *et al.* 2007). More recently, the interplay between the two pathways has been shown to be important in controlling the switch between symmetric and asymmetric divisions (Harandi and Ambros 2015), thus allowing for the proper number of hypodermal cells to form and organize during development.

In this study we have characterized the role of a nonreceptor tyrosine kinase, *FRK-1*, that is crucial for proper seam cell division during postembryonic development in *C. elegans*. We report that in the absence of *FRK-1*, beyond the general arrest at the L1 larval stage, the seam cell division that occurs during L1 has been inappropriately switched from asymmetric to symmetric. We further show that identities of the resulting daughter cells appear to be compromised, as genes regulating seam cell development are significantly altered.

Through investigating the aberrant divisions within the *frk-1* V1–4 seam cells we found that *POP-1* asymmetry is lost,

such that each daughter nucleus shows low levels of **POP-1**. Since **FRK-1** nuclear localization parallels **POP-1** in the seam cells, we investigated localization of **WRM-1** and found it too lost the asymmetric nuclear localization immediately following division, with an increase of **WRM-1** in the anterior nucleus when **FRK-1** is lost. Yang *et al.* (2011) demonstrated that **POP-1** is phosphorylated through **LIT-1** and bound by **WRM-1** for export from the nucleus. The same study found that **SYS-1**-bound **POP-1** inhibits **WRM-1** binding, thereby preventing nuclear export. Additionally, it has also been reported that **APR-1** is likely involved in exporting **WRM-1** from the seam cell nuclei (Takeshita and Sawa 2005; Mizumoto and Sawa 2007a; Gleason and Eisenmann 2010). **FRK-1** has been shown to interact with both **APR-1** and **WRM-1** previously in regulating cellular identity and proliferation (Putzke *et al.* 2005; Putzke and Rothman 2010). Our data contribute significantly to these previous studies by supporting a model whereby **FRK-1** facilitates **POP-1** export via **WRM-1**.

Interestingly, in the absence of **FRK-1** we observed no significant change in **SYS-1** or **APR-1** levels in seam cell nuclei, which is required for proper **POP-1** activity in asymmetric cell divisions (Kidd *et al.* 2005; Banerjee *et al.* 2010). It has been reported that another kinase, **KIN-19**, interacts with **SYS-1** in preventing seam cell hyperplasia (Banerjee *et al.* 2010) and that **KIN-19** also controls the asymmetric localization of an **APR-1** population that is capable of regulating the **WRM-1/POP-1** branch of the pathway (Baldwin and Phillips 2014). We also observed no combined effect on the number of seam cell divisions when eliminating **sys-1** and **frk-1** simultaneously via RNAi, further supporting a model in which the export of **POP-1** is uncoupled from **POP-1** nuclear transcriptional activation of target genes by **SYS-1**. It is clear that the increased seam cell proliferation we observed in the absence of **FRK-1** is due to aberrant signaling through the asymmetric Wnt pathway primarily involving **WRM-1** and **POP-1**, but not **SYS-1**, which regulates **POP-1** activity but not localization.

Our study shows that **FRK-1** acts in conjunction with components of the asymmetric pathway and may have an overall effect that confounds heterochronic gene expression. Heterochronic genes are also affected in the absence of **KIN-19**, suggesting a complex interplay between the uncoupled pathways leading to **POP-1** export and activity. Although our data show that there is altered expression of some heterochronic genes in the absence of **FRK-1**, we were not able to determine whether this was directly due to loss of **FRK-1** interacting with the transcription factors responsible for activating those genes or whether it was an indirect effect due to a compromised state of the seam cells (and possibly other hypodermal cells). Alternatively, it could be due to loss of **POP-1** asymmetry but maintenance of **SYS-1** asymmetry that leads to misregulation of heterochronic gene expression.

Harandi and Ambros (2015) recently hypothesized that weak **POP-1/APR-1** activity leads to the symmetric division in the early L2 stage. The fact that we observe that the L1 seam cell division appears symmetric, similar to early L2, led

us to hypothesize that there is an interruption in the binary fate decision of seam cells in the absence of **FRK-1**. Although our data imply a possible precocious L2 seam cell division in L1 due to loss of asymmetry, our gene expression data show that could be due to inappropriate expression of heterochronic regulators, such as **lin-4**, which Harandi and Ambros hypothesize is likely contributing to lowering **POP-1** activity. However, this is confounded by the fact that we also observe a loss of **lin-28** expression, which could release inhibition of **lin-46** and thus counteract the effects of **lin-4** activity on **POP-1**. Therefore, we submit that the conflicting gene expression patterns observed in **frk-1(ok760)** mutants are not due to a heterochronic phenotype but are an indicator of disrupted upstream Wnt signaling, which results in compromised seam cell identity.

A significant contributing factor to normal seam cell asymmetry and the reaction to the Wnt signal may be the level of **EGL-18**. Gorrepati *et al.* (2013) reported that **EGL-18** overexpression in a Wnt-sensitized background is capable of activating seam cell marker expression in head and ventral hypodermis and that **EGL-18** is required for seam cell fate specification. It has also been previously reported that **frk-1** acts upstream of **elt-18** and **elt-3** in regulating hypodermal cell specification during embryonic development (Putzke *et al.* 2005). Here, we demonstrate that, in addition to a decrease in expression of certain seam cell regulating genes, there was significantly less expression of both **egl-18** and **elt-3** in **frk-1(ok760)** mutants. Being that it is a nonseam, hypodermis marker, lower levels of **elt-3** suggest a broader hypodermal specification role for **FRK-1**. However, the lower levels of **elt-18** expression again point to a compromised identity of both seam cell daughters, likely prompting an arrest in subsequent divisions. Thus, consistent with both studies, our data show that **FRK-1** expression is required for proper seam cell maintenance and subsequent division during larval development.

Our data further support previous studies suggesting separate pools of active **APR-1** that are involved in exporting **WRM-1** and thus regulating **POP-1** activity via localization in seam cell nuclei from those regulating **SYS-1** levels through **KIN-19** (Baldwin and Phillips 2014). We present here a model in which nuclear **FRK-1**, present in dividing seam cells of the V lineage during postembryonic development, interacts with the **WRM-1** complex to regulate **POP-1** accumulation in the nucleus. Normally, when seam cells divide, there is a higher level of **FRK-1** in the nucleus of the anterior daughter compared to the posterior daughter nucleus shortly after division (Figure 7, top). It is reasonable to hypothesize that, based on our data, **FRK-1** interacts with the nuclear pool of **WRM-1** and/or **POP-1** to facilitate **POP-1** accumulation in the nucleus of the anterior daughter, while the low levels of **FRK-1** in the posterior daughter allow for **POP-1** export, via **APR-1/WRM-1**. This appears to be directed toward **WRM-1** and independent of **SYS-1**, such that in the absence of **FRK-1** we observe altered subcellular localization of both **WRM-1** and **POP-1** but not **SYS-1** or **APR-1** (Figure 5

“compromised” because it cannot be assumed that the posterior fate is the default setting in the absence of FRK-1, since normally there are low levels of FRK-1 in the posterior nucleus, as opposed to none.

and Figure 6). Importantly, our genetic data point to an interaction between **FRK-1**, **APR-1**, and **WRM-1** that facilitates nuclear export of **WRM-1** without significantly altering nucleocytoplasmic ratios of **APR-1** (Figure S1). Thus, in the absence of **FRK-1**, there is a compromised seam cell fate, due to the accumulation of **WRM-1** in both recently divided seam cell nuclei, without altering normal **SYS-1** asymmetry, resulting in loss of asymmetric division (Figure 7, bottom).

Current models of seam cell asymmetric division focus on the coordination of the asymmetric Wnt pathway for proper division and subsequent advancement to the next larval stage. Previous models consist of variations on cytoplasmic localization of the β -catenin homologs along with the APC homolog, **APR-1** (Mizumoto and Sawa 2007b; Baldwin and Phillips 2014; Gorrepati *et al.* 2015), and that it is the shuttling of these proteins, in the presence of a polarizing Wnt signal, that facilitates **POP-1** (TCF) export from the nucleus in the posterior daughter cell (Sugioka *et al.* 2011). However, a continuing question in this area remained: Is there a nuclear mechanism that sensitizes **WRM-1** to export from the nucleus in the anterior daughter? Our data clarify this question by supporting a role for **FRK-1**, due to the parallel localization between **FRK-1** and **POP-1** in seam cell daughter nuclei, in normally preventing **WRM-1** from exporting **POP-1** in the posterior daughter by functioning primarily in the nucleus and not in the cytoplasm of each seam cell daughter. However, whether **FRK-1** modifies the **WRM-1** complex or **POP-1** to prevent the export of **POP-1** in the anterior daughter remains to be determined. (Note that regarding the fate of each daughter cell in our model, we labeled each nucleus in the bottom of Figure 7 as “compromised” since we were not able to confirm that the posterior cell retains its posterior identity in the complete absence of **FRK-1**, as opposed to the low levels of **FRK-1** it normally contains in wild type.)

Figure 7 Model for FRK-1 regulation of Wnt asymmetry in dividing seam cells. During normal seam cell divisions, high levels of FRK-1 in the anterior nucleus influence POP-1 accumulation, while low levels of FRK-1 facilitate export from the posterior seam cell (top). FRK-1 activity is likely via modification of the WRM-1 complex or perhaps through POP-1 to prevent a WRM-1/POP-1 interaction. Additionally, FRK-1 appears to act in parallel with SYS-1 in either case. In the absence of FRK-1, the WRM-1 complex is improperly localized, with no change in SYS-1, compromising the fate of each daughter cell (bottom), resulting in a symmetric division with an anterior cell size but more posterior gene expression pattern. Thus, the cells in the bottom frame are labeled

Importantly, unlike the kinase-independent roles of **FRK-1** activity during embryonic development (Putzke *et al.* 2005), **FRK-1** regulation of seam cell division in larvae appears to be kinase dependent. This suggests that the Wnt pathway components, such as **WRM-1**, might be direct targets of **FRK-1** phosphorylation either in establishing export of the complex from the seam cell nuclei during **POP-1** export in the posterior daughter or, perhaps, to prevent anterior accumulation of the **WRM-1** in the anterior daughter. Our data are consistent with a prior study that demonstrated **FRK-1** regulation of cell division within the endodermal lineage; however, that study did not test whether the endodermal proliferation influence of **FRK-1** was kinase dependent (Putzke and Rothman 2010). In addition to **FRK-1** in *C. elegans*, kinase-independent activities of Fer kinase were shown by Craig *et al.* (2001) in which a kinase-inactivating knock-in resulted in viable mice. Furthermore, similar kinase inactive roles have been shown for the Fps/Fes kinase in mice (Senis *et al.* 2003), providing additional evidence of the potential for kinases to acquire functions within the cell beyond their phosphorylation capabilities.

Finally, our observations in *C. elegans*, consistent with our prior studies, suggest that normally **FRK-1** might phosphorylate a β -catenin homolog in *C. elegans* to stabilize it at the cortical membrane or adherens junctions (Putzke *et al.* 2005; Putzke and Rothman 2010). There have been conflicting reports with Fer kinase in mammalian systems, showing that Fer kinase activity leads to stabilization and/or destabilization of cadherin complexes. This is in part due to the fact that β -catenin phosphorylation on Tyr654 regulates association with E-cadherin, while phosphorylation on Tyr142 facilitates interactions with α -catenin. For instance, Piedra *et al.* (2003) showed that although the cadherin complex was disrupted with increased Fer activity, β -catenin was still associated with

E-cadherin, with the disruption of the complex being between β -catenin and α -catenin. In contrast, Xu *et al.* (2004) demonstrated that the presence of Fer phosphorylates PTP1B, which then dephosphorylates β -catenin, thus stabilizing the cadherin complex (Xu *et al.* 2004). The same study also showed that in the presence of a kinase inactive Fer the cadherin complex destabilizes, due to the subsequent phosphorylation of β -catenin. More recently, two studies have further supported the role of Fer directly phosphorylating β -catenin at Tyr142 (Chang *et al.* 2014) as well as causing indirect dephosphorylation of β -catenin at Tyr654 (Lee *et al.* 2008). Thus, the destabilization of cadherin complexes can occur either through dissociation of the β -catenin/ α -catenin interaction (when Fer phosphorylates Tyr142 on β -catenin) or via the lack of Fer activity on PTP1B (or SHP2 as shown by Lee *et al.* 2008), resulting in disruption of the E-cadherin/ β -catenin interaction (due to lack of dephosphorylation at Tyr654 on β -catenin).

Although we do not know whether FRK-1 directly phosphorylates any β -catenin homologs in *C. elegans*, such as HMP-2 and WRM-1, we previously reported that they interact with FRK-1 (Putzke *et al.* 2005; Putzke and Rothman 2010). Furthermore, the possibility of a direct interaction is supported by a report from Sumiyoshi *et al.* (2011) that showed that Src, another nonreceptor tyrosine kinase, of which Fer is a subfamily member, is capable of phosphorylating HMP-2, thereby causing HMP-2 to dissociate from the cortical membrane.

The issue of Fer/FRK-1 phosphorylation of cortically associated β -catenin is further complicated by two major issues: (1) it has been shown that Fer phosphorylation of plakoglobin (a β -catenin homolog in vertebrates) has the opposite effect of a direct Fer phosphorylation of β -catenin in mammalian cells (*i.e.*, it stabilizes cadherin junctions but also upregulates β -catenin/Tcf4 transcriptional activity) (Miravet *et al.* 2003) and (2) *C. elegans* has four β -catenin homologs (with diverging functions) and no reported plakoglobin homologs. Thus, it is possible that the activity of FRK-1 in *C. elegans* modifies different β -catenin homologs, depending on the interactions at the cortical membrane. However, our data suggest a novel role in which FRK-1 is interacting with β -catenin (in this case WRM-1), not at the cellular cortex but in the nucleus. In this context FRK-1 may be phosphorylating WRM-1 (or the WRM-1 complex) in a way that facilitates WRM-1 export from the nucleus (Figure 7). Alternatively, FRK-1 may function to destabilize any interaction between WRM-1 and POP-1, thereby preventing WRM-1 accumulation in the nucleus. Although this model describes a novel mechanism for FRK-1, it is not completely unexpected in that prior studies have shown Fer/FRK-1 to translocate to the nucleus of actively dividing cells (Ben-Dor *et al.* 1999; Putzke *et al.* 2005).

Proper cell division within differentiating stem cell lineages is crucial for morphogenetic movements during development. When cell divisions occur in an unregulated manner, consequences ranging from loss of cellular identity to improper

tissue size and function arise as a detriment to the organism. The Fer kinase homolog in *C. elegans*, FRK-1, is a crucial component in regulating the asymmetric divisions within the stem cell-like divisions of the hypodermal seam cells during postembryonic development through the asymmetric Wnt pathway. It will be of interest in future studies to determine whether FRK-1 asymmetric localization in seam cell nuclei occurs in a Wnt-dependent manner. Furthermore, whether FRK-1 is interacting directly with the β -catenin homolog, WRM-1, remains to be seen, but investigating this interaction may further elucidate whether targets are merely shuttled by FRK-1 or modified as *bona fide* phosphorylation targets of this nonreceptor tyrosine kinase.

Acknowledgments

We thank members of the Putzke and Phillips laboratories for helpful discussions. We are grateful to Robert Waterston (University of Washington, Seattle, WA) for the MH27, Ralf Schnabel (Technische Universität, Braunschweig, Germany) for the NE2-1B4, and Rueyling Lin (University of Texas Southwestern, Dallas, TX) for P4G4 (POP-1) antibodies; Xantha Karp (Central Michigan University, Mount Pleasant, MI) and David Eisenmann (University of Maryland, Baltimore, MD) for late seam-expressing GFP strains; and Joel Rothman for the JR667 strain. Some nematode strains were provided by the *Caenorhabditis* Genetics Center, which is funded by the National Institutes of Health National Center for Research Resources. This work was supported by grants from the M. J. Murdock Charitable Trust (to A.P.P.) and by the American Cancer Society (grant RSG-11-140-01-DC) and the Roy J. Carver Charitable Trust (grant 13-4131) (to B.T.P.).

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Communicating editor: B. Goldstein

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Supporting Information

www.genetics.org/lookup/suppl/doi:10.1534/genetics.115.181412/-/DC1

Asymmetric Wnt Pathway Signaling Facilitates Stem Cell-Like Divisions via the Nonreceptor Tyrosine Kinase FRK-1 in *Caenorhabditis elegans*

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Table S1 Loss of *frk-1* does not result in functional, precociously differentiated seam cells. Although seam cells do express markers present from embryo to adult, the late-larval and adult specific GFP-reporters do not express in the absence of *frk-1*.

reporter gene	stage	SC expression
<i>col-19::GFP</i>	adult	no
<i>col-49::GFP</i>	L4-gravid	no
<i>col-38::GFP</i>	L4-gravid	no
<i>bli-1::GFP</i>	L4-gravid	no
<i>grd-10::GFP</i>	emb-L4	yes
<i>egl-18::GFP</i>	emb-adult	yes

Figure S1 Loss of FRK-1 does not significantly affect subcellular localization of APR-1 or SYS-1 in dividing seam cells. APR-1 is normally symmetric in dividing seam cells, while SYS-1 is asymmetric, with higher concentrations in the posterior daughter (A,B). In *frk-1(ok760)* mutants, the SYS-1 asymmetry is unaffected; however, APR-1 becomes slightly asymmetric with higher concentrations in the anterior cytoplasm compared to the posterior daughter (C,D). APR-1 whole cell, nucleus and whole-cell/nucleus ratios in WT versus in *frk-1(ok760)* mutants (E,F). Bar graphs: x-axis: white bar is the anterior nucleus [A], while the filled bars represent the posterior nucleus [P]; y-axis: relative fluorescence units of APR-1::Venus expression.

Figure S2 The absence of FRK-1 results in aberrant heterochronic miRNA expression. The heterochronic regulating miRNA, *lin-4*, is expressed at significantly higher levels in *frk-1* mutants, while *let-7* remains undetectable in arrested mutants, similar to wildtype larvae at the same stage.

Figure S3 RNAi knockdown efficiency for the asymmetric Wnt pathway genes examined in this study. Quantitative PCR was performed on gene targets after RNAi from the L3-L4 transition. Knockdown efficiency ranged from 57-75% depending on the gene target. The bar graph shows gene expression remaining after knockdown via RNAi, with the expression levels being normalized to wildtype (represented as 100%). There was a slight decrease in knockdown efficiency (increase in remaining expression) when RNAi was performed at the L3 stage (green bars) when compared to RNAi performed at the L1 stage (red). [The rationale for examining L3-L4 transition is due to the fact that when *frk-1*(RNAi) was performed on L1 animals there were more complications leading to arrest than when we performed RNAi at a later larval stage. Thus, since we wanted to observe the specific effect on seam cell proliferation, we chose to observe the L3-L4 seam cell division, just prior to the onset of adulthood when the seam cells differentiate.]