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Repression of Wnt signaling by a Fer-type non-receptor tyrosine kinase

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Abstract

The Wnt signaling pathway must be properly modulated to ensure an appropriate output: pathological conditions result from either insufficient or excessive levels of Wnt signal. For example, hyperactivation of the Wnt pathway is associated with various cancers and subnormal Wnt signaling can lead to increased invasiveness of tumor cells. We found that the *C. elegans* orthologue of the Fer non-receptor tyrosine kinase, FRK-1, limits Wnt signaling by preventing the adhesion complex-associated β-catenin, HMP-2, from participating in Wnt-dependent specification of the endoderm during embryogenesis. Removal of FRK-1 function results in relocalization of HMP-2 to the nucleus of epidermal cells, and allows it to substitute for WRM-1, the nuclear β-catenin that normally transduces the Wnt signal during endoderm development. APR-1, the *C. elegans* APC orthologue, is similarly required to prevent HMP-2 relocalization and keeps it from participating in Wnt signal transduction; this finding partially explains the paradoxical observation that APR-1 acts either negatively or positively in Wnt signaling depending on context. The apparent hyperactivation of the Wnt response in the absence of FRK-1 leads to hyperproliferation in the endoderm, as is also seen when WRM-1 is overexpressed in wildtype embryos. The specification and proliferation activities of Wnt signaling are separable: while the Tcf/Lef factor POP-1 acts in Wnt-dependent endoderm specification, it is not apparently required for hyperproliferation resulting from excessive Wnt signaling. These findings highlight a role for a Fer-type kinase in setting the proper levels of Wnt signaling and demonstrate the importance of this modulation in ensuring appropriate cell division.

\body Introduction

The architecture of the Wnt signaling pathway, which is widely used during animal development to control many cellular processes, including adhesion, specification, and proliferation (1), is comprised of both negative and positive regulatory components that modulate the appropriate output. In canonical Wnt signaling, transduction through a Frizzled-type receptor results in relocalization of β-catenin from the cytoplasm to the nucleus, where it functions as a transcriptional coactivator in a complex with a Lef-1/Tcf transcription factor (reviewed in (2)). β-catenin levels are attenuated in the cytoplasm through interactions with a number of proteins, including APC, Axin, and GSK3-β which, in the absence of Wnt signal, promote phosphorylation of β-catenin and target it for degradation via ubiquitination (3).

β-catenin functions dually as a transcriptional coactivator in Wnt signaling (reviewed in (4)) and as a component of plasma membrane adhesion complexes, where it interacts with cadherin (5) and other adhesion components. While single β-catenin isoforms can perform both of these disparate processes in many animals, these functions appear to have been relegated to distinct molecules in *C. elegans*. The WRM-1, BAR-1, and SYS-1 β-catenin analogues, all function in transduction of the Wnt signal in different developmental contexts (6-8). In contrast, the HMP-2 β-catenin is localized to the plasma membrane, where it participates exclusively in cadherin-mediated cell adhesion (9).

Wnt signaling in *C. elegans*, known to regulate many developmental decisions (10), was first identified based on its essential role in endoderm induction. In the four-cell embryo, the MOM-2 Wnt ligand from the germline precursor, P_2, is received by the mesendoderm precursor, EMS, through the MOM-5 Wnt receptor (11), resulting in polarization of EMS, such that endoderm is specified in its posterior daughter, the E cell. Wnt-activated WRM-1 results in

conversion of the Lef-1/Tcf orthologue, POP-1, from a repressor to an activator of the *end-1* and *-3* genes, initiating endoderm differentiation (reviewed in (12)). In the absence of WRM-1, all embryos arrest without endoderm and the E cell adopts the fate of its sister, MS (6). This endoderm-inducing Wnt pathway is non-canonical in that the GSK3-β and APC homologues, SGG-1 and APR-1 respectively, activate, instead of inhibit, WRM-1 β–catenin function (6, 13).

We previously reported that FRK-1, the *C. elegans* orthologue of mammalian Fer non-receptor tyrosine kinase, which is known to interact with β-catenin (14), is required during embryogenesis for enclosure and elongation of the epidermis (15). Here we present evidence that FRK-1 excludes HMP-2 from the nucleus in epidermal cells, and that it prevents hyperactivation of the Wnt pathway during endoderm development. While it was previously thought that WRM-1 was the only β-catenin homologue capable of inducing endoderm *in vivo* (16), we found that when FRK-1 function is eliminated, HMP-2 is able to activate endoderm development even in the absence of WRM-1. We show that elevated Wnt signal transduction resulting from the absence of FRK-1 leads to hyperproliferation in the endoderm lineage, similar to when WRM-1 is overexpressed. Further, we report that the roles for the Wnt pathway in specification and proliferation are separable: POP-1 is required for the former but not the latter. These findings reveal a novel role for a Fer-type homologue in limiting Wnt pathway activity and suggest a mechanism by which Fer orthologues might contribute to tumorigenesis.

Results

HMP-2 relocalizes to the nucleus in the absence of the FRK-1 non-receptor tyrosine kinase

Fer is a member of a family of non-receptor tyrosine kinases, including the Fes/Fps proteins, that is known to interact with plasma membrane-associated β-catenin (14). Fer kinase is ubiquitously expressed (17) and localizes to the nucleus in a cell-cycle dependent manner (18). Forced expression of Fer can transform cells in culture (19) and altered levels of Fer are associated with proliferation of prostate cells (20). We showed in previous studies that, as with their mammalian orthologues, *C. elegans* FRK-1 and HMP-2 β-catenin interact *in vitro* (15). While HMP-2 localizes exclusively to the cadherin adhesion complex at the plasma membrane (9), it has been shown to be capable of functioning in Wnt-dependent signaling when overexpressed (21). Based on these observations, we sought to determine whether FRK-1 function might modulate Wnt signaling.

We first asked whether disruption of the FRK-1/HMP-2 interaction results in altered localization of HMP-2. Similar to HMP-2 protein (9), we found that FLAG epitope-tagged HMP-2, when expressed in the epidermis, is nuclear-excluded throughout embryogenesis (Fig. 1A, B). In striking contrast, elimination of immunoreactive FRK-1 by RNAi (Fig. S1) results in partial nuclear localization of FLAG-HMP-2 throughout these cells (Fig. 1C, D). This mislocalization of HMP-2 may account for the adhesion defects in *frk-1(-)* embryos, as we described previously (15). This finding demonstrates that, although it normally functions exclusively in adhesion complexes, HMP-2 retains the capacity to move to the nucleus when not associated with cadherin complexes, and that FRK-1 normally prevents it from doing so.

Loss of FRK-1 allows HMP-2 β-catenin to activate endoderm development in the absence of WRM-1 β-catenin

The observation that FLAG-HMP-2 translocates to the nucleus in epidermal cells when FRK-1 is absent led us to speculate that HMP-2 β-catenin might be capable of substituting for WRM-1 β-catenin in transduction of the endoderm-inducing Wnt signal when liberated from cadherin complexes at the earlier 4-cell stage. FRK-1 is not required for endoderm specification: 100% of embryos (n=126) in which FRK-1 is eliminated by RNAi contain a well-differentiated intestine (Fig. 2A, S2 A). Confirming earlier reports (6), we observed that 0% (n = 310) of terminally arrested embryos in which WRM-1 is removed by RNAi contain a differentiated intestine, which is normally conspicuous under polarized light microscopy (Fig. 2A, S2 B). In contrast, elimination of FRK-1 in these *wrm-1(-)* animals largely restores gut differentiation: we found that 39% (n = 138) of such embryos contained a differentiated gut (Fig. 2A, S2 C).

These results suggest that FRK-1 prevents another factor, perhaps the HMP-2 β-catenin, from participating in Wnt signal transduction. Elimination of HMP-2 function in otherwise normal animals does not affect endoderm specification: 100% of *hmp-2(zu364)* homozygous mutant embryos undergo gut differentiation (Fig. 2A). In contrast, we found that this mutation abolishes endoderm in *wrm-1(-)* embryos that also lack FRK-1: similar to *wrm-1(RNAi)* knockdown animals alone, fewer than 1% of such embryos undergo gut differentiation (Fig. 2A, Fig. S2 D). The finding that the *hmp-2(zu3645)* mutation prevents gut formation under these conditions strongly suggests that not only is HMP-2 capable of transducing the Wnt signal, but in normal embryos FRK-1 prevents HMP-2 from doing so during endoderm specification, thus abrogating hyperactivation of Wnt signal transduction. Although both HMP-2 and WRM-1 are able to rescue the vulval development defects in *bar-1(-)* mutants when overexpressed from the

bar-1 promoter (21), our results provide the first demonstration that endogenous HMP-2 and WRM-1 are functionally interchangeable in transducing the Wnt signal.

We confirmed the requirement for FRK-1 in preventing HMP-2-mediated Wnt signal transduction by using a genomic deficiency, *mDf7*, that deletes the entire *frk-1* gene. While this deficiency also removes a number of additional genes, we previously showed that the lethality of this deficiency is fully rescued by a transgene containing only a wild-type *frk-1(+)* gene (15). Such rescued animals can develop to adulthood, demonstrating that *frk-1* is the only gene essential for viability in the deleted region. The same experiments described above with *frk-1(RNAi)* were performed in the *mDf7* deficiency strain and in every case the results were comparable (e.g., Fig. S2 E), confirming that FRK-1 prevents HMP-2 from participating in Wnt signal transduction.

If HMP-2 substitutes for WRM-1 by acting as a conventional β-catenin that transduces the Wnt signal, then its ability to function in the Wnt pathway in the absence of FRK-1 should be abrogated when components that normally function upstream of WRM-1 in the signaling pathway are eliminated. Indeed, we found that removal of FRK-1 in a chromosomal mutant defective for MOM-2, the Wnt ligand secreted from the P_2 cell that acts upstream of WRM-1 in endoderm induction, does not suppress the endoderm specification defect of this mutant (Fig. 2A). Moreover, elimination of FRK-1 does not rescue the absence of gut development seen in embryos in which the MAP kinase cascade, which acts in a separate branch of the pathway immediately upstream of WRM-1, is inactivated as a result of removing either the LIT-1 Nemo-like kinase or the MOM-4 MAP kinase (Fig. S2 F). These observations imply that the inhibitory function of FRK-1 on Wnt signal transduction acts downstream of the early events in the pathway, i.e, at the level of β-catenin action.

APR-1 collaborates with FRK-1 to prevent Wnt signal transduction by HMP-2

Based on our finding that removal of FRK-1 results in relocalization of HMP-2 into the nucleus and allows it to participate in Wnt signal transduction, we sought to determine whether removal of APR-1, the *C. elegans* orthologue of mammalian APC, might result in similar effects. APR-1 has been implicated in cadherin complex interactions at the plasma membrane (22) and has been shown to interact directly with membrane-associated HMP-2 β-catenin (21). Mammalian APC acts as a negative regulator of Wnt signaling by interacting with β-catenin and sequestering it from the nucleus (23). The function of *C. elegans* APR-1 in Wnt signaling is more complex: while APR-1 is known to be a negative regulator of Wnt signaling in vulval development (24), it shows a weak positive requirement in Wnt signaling during endoderm development (6). How can the discrepant action of APR-1 in endoderm-inducing Wnt signaling be explained? These considerations led us to postulate that APR-1 might repress Wnt signal transduction both by destabilizing the Wnt-involved β-catenin, in the case of BAR-1-mediated signaling, and by interacting with the cadherin-associated β-catenin (HMP-2) outside the nucleus, where it would be unavailable for Wnt signal transduction. We investigated this hypothesis by examining whether HMP-2 functions in Wnt signaling when APR-1 is removed.

Similar to our observations in *frk-1(-)* knockdown embryos, we found that FLAG-HMP-2 partially mislocalizes to the nucleus in the early epidermal lineage in *apr-1(-)* knockdown embryos (Fig. 1 E, F). Thus, HMP-2 may become available to transduce the Wnt signal in the absence of APR-1. We found that gut differentiation occurs in 76% (n = 165) of embryos depleted of APR-1 by RNAi (Fig. 2B), confirming earlier reports that APR-1 is partially required for endoderm specification. In support of our hypothesis, we found that this impenetrance is

explained in part by HMP-2-dependent rescue of endoderm specification: depletion of APR-1 in a *hmp-2(zu364)* mutant resulted in a marked decrease in the frequency of embryos with differentiated gut (43%; n = 510). Thus, the ability of embryos to make gut in the absence of APR-1 is attributable in part to the participation of HMP-2 in Wnt signal transduction. Earlier studies suggested that APR-1 performs only an activating role in endoderm specification, in contrast to its repressive activity in Wnt-dependent vulval development (24) and the inhibitory role of APC in mammalian Wnt signaling. Our findings reveal that this APC family member, presumably through direct protein-protein interactions (21), does in fact repress Wnt signal transduction during endoderm induction by preventing HMP-2 from participating in the Wnt pathway.

APR-1 and FRK-1 collaborate to repress HMP-2-dependent Wnt signaling. When the functions of both *apr-1* and *frk-1* are simultaneously eliminated, the fraction of embryos with differentiated gut increases (to 88%; n = 175; $p<0.001$) compared to those lacking *apr-1* function alone (76%). Eliminating the function of both genes in the *hmp-2(zu364)* mutant decreases the proportion of embryos with gut, again showing that HMP-2 functions to promote endoderm specification when FRK-1 and APR-1 are absent (Fig. 2B).

Excessive Wnt signaling results in hyperproliferation in the endoderm

The foregoing results suggest that FRK-1 and APC-1 prevent inappropriately high levels of Wnt signal transduction in the endoderm by excluding HMP-2 β-catenin from acting in the pathway. What effect might elevated Wnt signaling have as a consequence of removal of these factors? Excessive Wnt signaling has been associated with increased cell proliferation and can promote tumor formation (25, 26). The known mitogenic action of Wnt signaling led us to

hypothesize that hyperactivation of Wnt signal transduction might lead to increased cell division in the endoderm.

As with all other somatic cell lineages in *C. elegans*, the number of rounds of cell division occurring in the embryonic endoderm lineage is highly reproducible: the E progenitor cell undergoes a precisely determined number of cell division rounds by an invariant pattern, culminating in production of exactly 20 intestinal cells in virtually every animal by the end of embryogenesis (27). This highly reproducible cell division pattern makes the endoderm lineage a sensitive system for analyzing changes in mitotic proliferation (e.g., (28)). We therefore sought to investigate the consequence of removing FRK-1 and APR-1 function on the number of cells generated by the endoderm lineage. A chromosomal deficiency that removes *frk-1*, or RNAi of the gene, causes embryos to arrest without undergoing epidermal enclosure, leading to clustering of the epidermal cells on the dorsal side, while cells that normally become covered by epidermis remain exposed on the ventral surface (15). We analyzed the number of gut nuclei in such *frk-1(-)* arrested embryos using an *elt-2::GFP* reporter that marks all differentiated intestinal nuclei (29) and found that many contain extra gut nuclei (Fig. 3A and 4). In contrast to the 20 gut nuclei observed in wild-type animals at the end of embryogenesis (with rare variants containing as few as 19 or as many as 21), or those in which an *unc-22* negative control gene is inactivated by RNAi, we found that 58% of the *frk-1(RNAi)* arrested embryos contained >20 gut nuclei (n = 45). *frk-1(RNAi)* embryos contained an average of 25.5 gut nuclei and some embryos contained nearly double the normal number (as many as 35 nuclei). A similar elevation in the number of gut nuclei was observed in *apr-1(RNAi)* embryos (Fig. 4). Moreover, we found that, as with rescue of endoderm specification described above, simultaneous elimination of both *frk-1* and *apr-1* resulted in a stronger effect than in either single knockdown experiment: the number of

embryos containing >20 gut nuclei increased to 71.4% and as many as 37 gut nuclei were observed when the function of both genes was eliminated.

The supernumerary gut nuclei observed in *frk-1(RNAi)* embryos might result from increased cell division in the endoderm or instead from inappropriate endoderm differentiation arising in non-E lineage progenitors as occurs, for example, in *pop-1(-)* mutants, in which the sister of E, the MS cell, is respecified from a mesodermal to an endodermal progenitor. However, unlike in *pop-1(-)* mutant embryos (30), in which MS-derived tissues are absent, we did not observe an absence of MS-derived mesodermal tissues by Nomarski microscopy or marker analysis (e.g., all embryos contained apparently normal amounts of pharyngeal tissue based on expression of *ceh-22::GFP*; n = 23). Furthermore, we found that laser ablation of the E cell in early (6-7-cell stage) embryos eliminated all differentiated gut nuclei in both wildtype (9/9) and *frk-1(RNAi)* (5/5) embryos (Fig. 3B). These results imply that the endoderm undergoes extra cell division in embryos lacking FRK-1.

We sought to assess whether the increased number of gut nuclei in the absence of *frk-1* function is likely to be the result of excessive activation of the Wnt pathway by examining the involvement of β-catenin activity. Indeed, elimination of HMP-2 in *frk-1(-)* embryos restored gut nuclear number to that of wildtype (n = 31 ; Fig. 4). Similarly, the increased number of gut nuclei seen in *apr-1(-)* animals is partially reversed in the *hmp-2(-)* mutant (Fig. 4). Thus, HMP-2, presumably as a result of its release from cadherin complexes, is correlated with the production of supernumerary gut cells.

As a more direct test of whether elevated signaling through the Wnt pathway results in extra gut cells, we tested the effect of overexpressing WRM-1 under control of a heat-shock promoter and found that 90.4% of arrested embryos contained >20 gut nuclei, with an average of

28.5 (n = 52)(Fig. 4). Remarkably, some of these embryos contained over twice the normal number of gut cells. To assess whether the supernumerary gut nuclei observed in embryos overexpressing WRM-1 arose exclusively from the E lineage or might be the result of ectopic endoderm development, we laser-ablated the E cell, or both E and its sister, MS, following heat-shock-induced overexpression of WRM-1 and found that no gut nuclei were made in such embryos (6/6 for E ablations and 6/6 for E + MS ablations; Fig. 3B), again demonstrating that the extra gut nuclei did not appear to arise as a result of a transformation in the fate of cells outside the normal E or EMS lineage.

We found that simultaneously overexpressing WRM-1 and allowing HMP-2 to participate in Wnt signal transduction by eliminating FRK-1 resulted in an even further increase in the number of gut nuclei, to an average of 29.7 nuclei per embryo ($p<0.05$) (Fig. 4). We conclude that hyperactivation of the Wnt signaling pathway results in excessive cell proliferation in the endoderm lineage.

POP-1 controls Wnt-mediated specification but not proliferation in the endoderm

The transcriptional output from canonical Wnt signaling is mediated through Lef/Tcf-type transcription factors (31). In *C. elegans*, a single such factor, POP-1, functions in Wnt signaling, generally by the canonical mechanism. While POP-1 transduces the endoderm-inducing signal and is essential for endoderm development in the closely related nematode *C. briggsae* (32), in *C. elegans* its endoderm-activating function acts redundantly with a parallel transcriptional regulatory cascade, the SKN-1→ MED pathway, to activate the endoderm-specifying *end* genes; it is therefore not required for endoderm formation (33, 34). As with other Lef/Tcf factors, POP-1 acts both as a Wnt-dependent activator of its targets and a repressor of

the same genes in the absence of signaling; hence, elimination of POP-1 in *C. elegans* leads to derepression of endoderm in the sister of E, the MS cell, where it normally functions to repress transcription of the *end* genes owing to the absence of Wnt signal in MS (30).

As POP-1 is the only transcription factor known to transmit the Wnt endoderm-inducing signal, we asked whether it is required to mediate the hyperproliferation effect resulting from excessive Wnt signaling. As expected, *pop-1(RNAi)* embryos contain approximately twice the average number of gut nuclei as in wildtype owing to transformation in the fate of MS into an E-like cell (Fig. 4). (Note that POP-1 also functions in establishing differences between daughter cells born from anteroposterior asymmetric cell divisions throughout *C. elegans* development (35). As a result, posterior →anterior transformations would be expected to occur throughout the endoderm lineage in *pop-1(-)* embryos, and hence the number of gut cells produced would not necessarily be a simple doubling of the normal number. Regardless, we found that the average number of gut nuclei across such embryos was 39, i.e., approximately twice that of wildtype.) If POP-1 were required to transmit the elevated Wnt signal resulting from the absence of FRK-1, then one would expect this number to remain essentially unchanged in *frk-1(-); pop-1(-)* double knockdown embryos. On the contrary, we found that *frk-1(-); pop-1(-)* embryos contained many more gut nuclei (average of 75.3 nuclei; n = 32) than in the *pop-1(-)* single knockdown embryos (39.0; n = 46). Thus, there is an apparently synergistic effect resulting from the combined duplication of an E-like fate as a result of POP-1 removal and the excessive proliferation resulting from the absence of FRK-1. Moreover, we found that *pop-1(RNAi)* embryos in which the Wnt pathway is hyperactivated by heat-shock-driven overexpression of WRM-1 also contain approximately twice as many gut nuclei (78.5 nuclei; n = 39) as similarly heat-shocked *pop-1(-)* embryos lacking the WRM-1 overexpression construct (Fig. 4). In extreme cases, such embryos

can contain up to 90 gut cells, more than four times as many as in normal embryos. These findings imply that, in contrast to Wnt-dependent endoderm specification, the mitogenic effect of increased Wnt signaling, resulting from elevated WRM-1 or HMP-2 β-catenin levels, is mediated through a mechanism that appears to be entirely independent of POP-1, suggesting a novel, non-canonical, mechanism of action of Wnt signal transduction in its proliferation-inducing effect.

Discussion

We report four advances regarding the mechanisms and importance of modulating Wnt signaling during *C. elegans* endoderm development. First, we found that FRK-1 and APR-1 are required to exclude HMP-2 from the nucleus in epidermal cells. Second, in the absence of FRK-1 or APR-1, HMP-2 can functionally substitute for WRM-1 in Wnt-mediated induction of endoderm. Third, under elevated Wnt signaling conditions, resulting from excess β-catenin activity, the endoderm undergoes hyperproliferation, resulting in supernumerary gut cells. This finding provides the first demonstration that the normally fixed cell division number in the endoderm lineage can be overridden by inappropriately high levels of Wnt signaling. Finally, we found that excess cell division occurs even when POP-1 is depleted, demonstrating a non-canonical Wnt requirement beyond the stage at which β-catenin functions. These relationships are summarized in Fig. S3.

The relocalization of HMP-2 to the nucleus in the absence of FRK-1, combined with our earlier findings that FRK-1 and HMP-2 physically interact (15), strongly supports the hypothesis that FRK-1 helps to stabilize adhesion complexes, and provides a possible explanation for why HMP-2 does not normally participate in Wnt signaling. Indeed, it is conceivable that the primary difference between HMP-2 and the other *C. elegans* β-catenins is the ability to bind FRK-1, thus differentiating between adhesion and Wnt signal transduction. While it was previously shown that overproduction of HMP-2 can rescue a *bar-1* mutation (21), our findings reveal that endogenous levels of HMP-2 can function in Wnt signaling when it is not bound to FRK-1.

Studies in other systems have shown that β-catenin function in Wnt signaling can be attenuated by its retention at the plasma membrane. For example, when β-catenin is tethered to the membrane, preventing it from entering the nucleus, the Wnt response is blocked (36).

Overexpression of cadherin (37) or its β-catenin binding domain (38) similarly prevents β-catenin from participating in the Wnt response. It seems likely that the stability of the cadherin complex may be a key factor in modulating Wnt response, and that its destabilization, resulting in release of β-catenin from the membrane, may generally activate Wnt signal transduction.

APR-1 has previously been reported to function positively in Wnt signal transduction during *C. elegans* endoderm development (6), and has also been suggested to be part of the cadherin complex at the plasma membrane (22). We have found that, while APR-1 may function as an activator of nuclear WRM-1, it also contributes to sequestering of HMP-2 from the nucleus, thereby preventing HMP-2 from participating in Wnt signal transduction (Fig. S3 A). Thus APR-1 functions dually in the endoderm-inducing Wnt signal: it not only provides a non-canonical positive function in Wnt-induced endoderm (6) but also attenuates Wnt signaling, fulfilling its function as a negative regulator in the Wnt pathway.

Although it is reasonable to suggest that removal of FRK-1 might cause HMP-2 to relocalize to the EMS nucleus in a 4-cell embryo, we have not ruled out the possibility that the hyperactivated Wnt response we observe might result from increased HMP-2 levels. However, when FRK-1 is absent, FLAG-HMP-2 in epidermal cells relocalizes to the nucleus without apparently increasing its expression (Fig. 1). HMP-2 and FRK-1, which physically interact, are expressed in early embryos and in the epidermis and endoderm (9, 15) and localize to the plasma membrane in all blastomeres at the 4-cell stage, when the endoderm Wnt signal acts. Thus, it is not unreasonable to suppose that the striking mislocalization of HMP-2 in epidermal cells observed when FRK-1 is absent would extend to other cells in which they show identical localization patterns, including the EMS lineage. Moreover, HMP-2, acting genetically

downstream of the Wnt ligand and MAPK cascade, can substitute for WRM-1, which is known to function in the nucleus at the same position in the signaling pathway (6, 39-41).

In addition to its action in regulating cell specification, Wnt signaling has been associated with cellular proliferation in many contexts, including embryonic development (42), gut stem cell renewal in humans (43), and a variety of cancers (e.g., (44, 45)). Excess cell proliferation is often associated with mutations in the Wnt response pathway (reviewed in (46)). No association has previously been made between Wnt signaling and proliferation during embryonic development in *C. elegans*. However, Wnt signaling has been shown to be required for normal post-embryonic divisions in the lateral seam cells (47) and during vulva development (48, 49). Our findings establish that hyperactivation of Wnt signaling results in excessive proliferation in the endoderm lineage, which normally undergoes a rigidly fixed number of cell divisions.

While canonical Wnt signaling exerts its effects through Lef-1/Tcf-type transcription factors, our results point to a possible divergent mechanism in Wnt-activated cell proliferation, as proposed in Figure S3 B. Hyperproliferation resulting from excess WRM-1 or HMP-2 occurs even in the absence of POP-1, the only known Lef1/Tcf factor in *C. elegans*, suggesting involvement of another transcription factor, or perhaps a transcription-independent mechanism.

The Fer class of proteins has been shown to promote tumorigenesis in a number of contexts. While disruption of Fer function results in cell adhesion defects, our results raise the possibility that altered expression of Fer proteins in humans might cause inappropriate elevation of Wnt signaling, perhaps explaining part of their action in promoting tumorigenesis.

Materials and Methods

Strains

C. elegans was grown according to standard procedures (50). Knockout strains for *apr-1(zh10)* (AH75) and *lit-1(t1512)* (GE2244) were obtained from the Caenorhabditis Genetics Center. *hmp-2(zu364)* (JJ1068) and *mom-2(or42)* (EU384) were gifts from J. Priess and B. Bowerman, respectively. Gut nuclear counts and ablations were performed on strain JR1838, which contains an integrated extrachromosomal array that expresses GFP under control of the *elt-2* promoter (29). The *mDf7* chromosomal deficiency strain JR2482 (*dpy-13(e184) mDf7 IV / unc-24(e1172) dpy-4(e1166) IV*) was rebalanced from existing strain, DR793 (*dpy-13(e184) mDf7 IV/nT1[let-?(m435)](IV;V)*).

RNA interference

Primer pairs were made to the coding regions of *frk-1*, *wrm-1*, *apr-1*, *hmp-2*, 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). L4/young adult hermaphrodites were either injected or soaked overnight with dsRNA. Arrested embryos were analyzed after a minimum of 12 hours. Embryos were analyzed using either polarized light microscopy to score for the presence of birefringent gut granules (51) or by fluorescence microscopy for immunolocalization and GFP detection.

Immunofluorescence Analysis

FLAG-HMP-2 was generated using a pCMV-FLAG plasmid. The FLAG-HMP-2 fusion was inserted into a plasmid containing the *elt-1* promoter region and injected into the N2 wildtype strain. FLAG-HMP-2 localization was visualized using anti-FLAG antibodies (Sigma).

Laser ablations

Laser ablations were performed using a VSL-337 (Laser Science) nitrogen-pumped dye laser mounted to a Nikon microscope equipped with Nomarski optics. Embryos were mounted on agar pads in M9 buffer and EMS cells at the four-cell stage, or individual E cells immediately after division of EMS, were ablated. Only embryos in which the ablated cell underwent no more than one division following the ablation were counted in the results.

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References

1. Cadigan KM & Nusse R (1997) Wnt signaling: a common theme in animal development. *Genes Dev* 11(24):3286-3305.
2. Seidensticker MJ & Behrens J (2000) Biochemical interactions in the wnt pathway. *Biochim Biophys Acta* 1495(2):168-182.
3. Peifer M, Sweeton D, Casey M, & Wieschaus E (1994) wingless signal and Zeste-white 3 kinase trigger opposing changes in the intracellular distribution of Armadillo. *Development* 120(2):369-380.
4. Mosimann C, Hausmann G, & Basler K (2009) Beta-catenin hits chromatin: regulation of Wnt target gene activation. *Nat Rev Mol Cell Biol* 10(4):276-286.
5. Pai LM*, et al.* (1996) Drosophila alpha-catenin and E-cadherin bind to distinct regions of Drosophila Armadillo. *J Biol Chem* 271(50):32411-32420.
6. Rocheleau CE*, et al.* (1997) Wnt signaling and an APC-related gene specify endoderm in early C. elegans embryos. *Cell* 90(4):707-716.
7. Eisenmann DM, Maloof JN, Simske JS, Kenyon C, & Kim SK (1998) The beta-catenin homolog BAR-1 and LET-60 Ras coordinately regulate the Hox gene lin-39 during Caenorhabditis elegans vulval development. *Development* 125(18):3667-3680.
8. Kidd AR, 3rd, Miskowski JA, Siegfried KR, Sawa H, & Kimble J (2005) A beta-catenin identified by functional rather than sequence criteria and its role in Wnt/MAPK signaling. *Cell* 121(5):761-772.
9. Costa M*, et al.* (1998) A putative catenin-cadherin system mediates morphogenesis of the Caenorhabditis elegans embryo. *J Cell Biol* 141(1):297-308.
10. Korswagen HC (2002) Canonical and non-canonical Wnt signaling pathways in Caenorhabditis elegans: variations on a common signaling theme. *Bioessays* 24(9):801-810.
11. Thorpe CJ, Schlesinger A, Carter JC, & Bowerman B (1997) Wnt signaling polarizes an early C. elegans blastomere to distinguish endoderm from mesoderm. *Cell* 90(4):695-705.
12. Maduro MF & Rothman JH (2002) Making worm guts: the gene regulatory network of the Caenorhabditis elegans endoderm. *Dev Biol* 246(1):68-85.
13. Schlesinger A, Shelton CA, Maloof JN, Meneghini M, & Bowerman B (1999) Wnt pathway components orient a mitotic spindle in the early Caenorhabditis elegans embryo without requiring gene transcription in the responding cell. *Genes Dev* 13(15):2028-2038.
14. Rosato R, Veltmaat JM, Groffen J, & Heisterkamp N (1998) Involvement of the tyrosine kinase fer in cell adhesion. *Mol Cell Biol* 18(10):5762-5770.
15. Putzke AP, Hikita ST, Clegg DO, & Rothman JH (2005) Essential kinase-independent role of a Fer-like non-receptor tyrosine kinase in Caenorhabditis elegans morphogenesis. *Development* 132(14):3185-3195.
16. Rocheleau CE*, et al.* (1999) WRM-1 activates the LIT-1 protein kinase to transduce anterior/posterior polarity signals in C. elegans. *Cell* 97(6):717-726.
17. Letwin K, Yee SP, & Pawson T (1988) Novel protein-tyrosine kinase cDNAs related to fps/fes and eph cloned using anti-phosphotyrosine antibody. *Oncogene* 3(6):621-627.
18. Ben-Dor I, Bern O, Tennenbaum T, & Nir U (1999) Cell cycle-dependent nuclear accumulation of the p94fer tyrosine kinase is regulated by its NH2 terminus and is affected by kinase domain integrity and ATP binding. *Cell Growth Differ* 10(2):113-129.

19. Paulson R, Jackson J, Immergluck K, & Bishop JM (1997) The DFer gene of Drosophila melanogaster encodes two membrane-associated proteins that can both transform vertebrate cells. *Oncogene* 14(6):641-652.
20. Allard P*, et al.* (2000) Links between Fer tyrosine kinase expression levels and prostate cell proliferation. *Mol Cell Endocrinol* 159(1-2):63-77.
21. Natarajan L, Witwer NE, & Eisenmann DM (2001) The divergent Caenorhabditis elegans beta-catenin proteins BAR-1, WRM-1 and HMP-2 make distinct protein interactions but retain functional redundancy in vivo. *Genetics* 159(1):159-172.
22. Hoier EF, Mohler WA, Kim SK, & Hajnal A (2000) The Caenorhabditis elegans APC-related gene apr-1 is required for epithelial cell migration and Hox gene expression. *Genes Dev* 14(7):874-886.
23. Hulsken J, Birchmeier W, & Behrens J (1994) E-cadherin and APC compete for the interaction with beta-catenin and the cytoskeleton. *J Cell Biol* 127(6 Pt 2):2061-2069.
24. Gleason JE, Korswagen HC, & Eisenmann DM (2002) Activation of Wnt signaling bypasses the requirement for RTK/Ras signaling during C. elegans vulval induction. *Genes Dev* 16(10):1281-1290.
25. Nhieu JT*, et al.* (1999) Nuclear accumulation of mutated beta-catenin in hepatocellular carcinoma is associated with increased cell proliferation. *Am J Pathol* 155(3):703-710.
26. Liu CC, Prior J, Piwnica-Worms D, & Bu G (2010) LRP6 overexpression defines a class of breast cancer subtype and is a target for therapy. *Proc Natl Acad Sci U S A* 107(11):5136-5141.
27. Sulston JE, Schierenberg E, White JG, & Thomson JN (1983) The embryonic cell lineage of the nematode Caenorhabditis elegans. *Dev Biol* 100(1):64-119.
28. Bao Z, Zhao Z, Boyle TJ, Murray JI, & Waterston RH (2008) Control of cell cycle timing during C. elegans embryogenesis. *Dev Biol* 318(1):65-72.
29. Fukushige T, Hendzel MJ, Bazett-Jones DP, & McGhee JD (1999) Direct visualization of the elt-2 gut-specific GATA factor binding to a target promoter inside the living Caenorhabditis elegans embryo. *Proc Natl Acad Sci U S A* 96(21):11883-11888.
30. Lin R, Thompson S, & Priess JR (1995) pop-1 encodes an HMG box protein required for the specification of a mesoderm precursor in early C. elegans embryos. *Cell* 83(4):599-609.
31. MacDonald BT, Tamai K, & He X (2009) Wnt/beta-catenin signaling: components, mechanisms, and diseases. *Dev Cell* 17(1):9-26.
32. Lin KT, Broitman-Maduro G, Hung WW, Cervantes S, & Maduro MF (2009) Knockdown of SKN-1 and the Wnt effector TCF/POP-1 reveals differences in endomesoderm specification in C. briggsae as compared with C. elegans. *Dev Biol* 325(1):296-306.
33. Maduro MF, Kasmir JJ, Zhu J, & Rothman JH (2005) The Wnt effector POP-1 and the PAL-1/Caudal homeoprotein collaborate with SKN-1 to activate C. elegans endoderm development. *Dev Biol* 285(2):510-523.
34. Shetty P, Lo MC, Robertson SM, & Lin R (2005) C. elegans TCF protein, POP-1, converts from repressor to activator as a result of Wnt-induced lowering of nuclear levels. *Dev Biol* 285(2):584-592.
35. Lin R, Hill RJ, & Priess JR (1998) POP-1 and anterior-posterior fate decisions in C. elegans embryos. *Cell* 92(2):229-239.

36. Cox RT, *et al.* (1999) Membrane-tethered Drosophila Armadillo cannot transduce Wingless signal on its own. *Development* 126(6):1327-1335.
37. Sanson B, White P, & Vincent JP (1996) Uncoupling cadherin-based adhesion from wingless signalling in Drosophila. *Nature* 383(6601):627-630.
38. Pierce M, Wang C, Stump M, & Kamb A (2003) Overexpression of the beta-catenin binding domain of cadherin selectively kills colorectal cancer cells. *Int J Cancer* 107(2):229-237.
39. Nakamura K, *et al.* (2005) Wnt signaling drives WRM-1/β-catenin asymmetries in early C. elegans embryos *Genes Dev* 19:1749-1754.
40. Miao-Chia Lo FG, Raanan Odom, Yang Shi and Rueyling Lin (2004) Phosphorylation by the β-Catenin/MAPK Complex Promotes 14-3-3-Mediated Nuclear Export of TCF/POP-1 in Signal-Responsive Cells in C. elegans *Cell* 117(1):95-106.
41. Tae Ho Shin JY, Christian E. Rocheleau, Rueyling Lin, Martha Soto, Yanxia Bei, Roger J. Davis and Craig C. Mello (1999) MOM-4, a MAP Kinase Kinase Kinase–Related Protein, Activates WRM-1/LIT-1 Kinase to Transduce Anterior/Posterior Polarity Signals in C. elegans *Mol Cell* 4(2):275-280.
42. Chesnutt C, Burrus LW, Brown AM, & Niswander L (2004) Coordinate regulation of neural tube patterning and proliferation by TGFbeta and WNT activity. *Dev Biol* 274(2):334-347.
43. Kuhnert F, *et al.* (2004) Essential requirement for Wnt signaling in proliferation of adult small intestine and colon revealed by adenoviral expression of Dickkopf-1. *Proc Natl Acad Sci U S A* 101(1):266-271.
44. Lee J, Yoon YS, & Chung JH (2008) Epigenetic silencing of the WNT antagonist DICKKOPF-1 in cervical cancer cell lines. *Gynecol Oncol* 109(2):270-274.
45. Wang L, *et al.* (2009) Oncogenic function of ATDC in pancreatic cancer through Wnt pathway activation and beta-catenin stabilization. *Cancer Cell* 15(3):207-219.
46. Kikuchi A (2003) Tumor formation by genetic mutations in the components of the Wnt signaling pathway. *Cancer Sci* 94(3):225-229.
47. Takeshita H & Sawa H (2005) Asymmetric cortical and nuclear localizations of WRM-1/beta-catenin during asymmetric cell division in C. elegans. *Genes Dev* 19(15):1743-1748.
48. Gleason JE, Szyleyko EA, & Eisenmann DM (2006) Multiple redundant Wnt signaling components function in two processes during C. elegans vulval development. *Dev Biol* 298(2):442-457.
49. Green JL, Inoue T, & Sternberg PW (2008) Opposing Wnt pathways orient cell polarity during organogenesis. *Cell* 134(4):646-656.
50. Brenner S (1974) The genetics of *Caenorhabditis elegans*. *Genetics* 77:71-94.
51. Clokey GV & Jacobson LA (1986) The autofluorescent "lipofuscin granules" in the intestinal cells of Caenorhabditis elegans are secondary lysosomes. *Mech Ageing Dev* 35(1):79-94.

Figure Legends

Figure 1. HMP-2 mislocalizes to the nucleus in the absence of FRK-1 or APR-1 function.

(A, B) In wildtype embryos, FLAG-HMP-2 (red) is excluded from nuclei, detected with DAPI (overlay in B). FLAG-HMP-2 translocates to the nucleus in *frk-1(RNAi)* (C, D) and *apr-1(RNAi)* (E, F) embryos.

Figure 2. Removal of FRK-1 or APR-1 function results in HMP-2-dependent rescue of endoderm development in the absence of WRM-1.

Fields of embryos viewed under polarized light microscopy reveal those embryos containing differentiated gut tissue, which is apparent by the bright polarization signal from the rhabditin granules. Embryos lacking gut show only a lower general level of signal. Percent of embryos of the indicated mutant genotype containing a differentiated gut is shown in (A) and (B). In (A) and (B), RNAi was used for *apr-1(-)*, *frk-1(-)*, and *wrm-1(-)* and the following chromosomal mutations were used for the other genes: *lit-1(t1512)*, *hmp-2(zu364)*, and *mom-2(or42)*.

Figure 3. Absence of FRK-1 and overexpression of WRM-1 lead to excessive cell proliferation in the endoderm.

(A) The number of endoderm nuclei was determined in embryos expressing an endoderm-specific (*elt-2*::GFP) construct. Wild-type embryos contain 20 nuclei (left), while *frk-1(RNAi)* and heat-shocked embryos overexpressing WRM-1 from a heat-shock promoter contain elevated numbers (middle and right, respectively), as shown in the micrographs. (B) Laser-ablation of the endoderm precursor (E cell) or E and the mesodermal precursor (MS) revealed that the supernumerary gut nuclei arise do not arise from outside the E or EMS lineages.

Figure 4. Supernumerary gut nuclei resulting from elevated levels of WRM-1 or nuclear HMP-2 β-catenins arise independently of POP-1 activity.

Histograms of gut nuclear counts in embryos of the genotype indicated at the left. Pie diagrams to the left of each histogram indicate the percent of embryos with normal (<21; white sectors) or supernumerary (21 or more; black sectors) gut nuclei. Average numbers of gut nuclei ± standard deviation are shown to the right of the histograms, with number of embryos analyzed in parentheses. *hs-wrm-1* indicates embryos containing the *hs-wrm-1* construct that were subjected to heat-shock. Owing to space limitations, the scaling of histograms varies; however, the shortest bar equals one embryo in each histogram.

Supplemental Figure 1. Efficacy of *frk-1(RNAi)*. FRK-1-specific polyclonal antibodies, whose preparation and specificity were described previously (15), were used to test the efficiency of RNAi in eliminating FRK-1 function. The antibodies show strong immunoreactivity in wildtype embryos as seen in (A). FRK-1 is undetectable in all *frk-1(RNAi)* embryos at any stage of development (e.g., B).

Supplemental Figure 2. Removal of FRK-1 function results in HMP-2-dependent rescue of endoderm development in the absence of WRM-1.

Fields of embryos viewed under polarized light microscopy reveal those embryos containing differentiated gut tissue in *wrm-1(RNAi)*. Each embryo is approximately 50 microns along the long axis and each field contains ~50-100 embryos. Gut cells are apparent by the bright polarization signal from the rhabditin granules. Embryos lacking gut show only a lower general

level of signal. (A) *frk-1(RNAi)*. Although the embryos have not elongated, owing to the morphogenetic defect caused by loss of FRK-1 (15), all contain a differentiated gut, as also shown in one embryo (inset). (B) *wrm-1 (RNAi)*, (C) *frk-1(RNAi)*; *wrm-1(RNAi)* double knockdown, and (D) *wrm-1(RNAi)*; *frk-1(RNAi)*; *hmp-2(zu364)* triple knockdown. (E) Using the chromosomal deficiency mutant, *mDf7*, to eliminate *frk-1* genomically, yields a similar demonstration of the HMP-2-dependent rescue of endoderm, supporting the data obtained for the knockdown of FRK-1 via RNAi. In these experiments HMP-2 and WRM-1 were knocked down using RNAi in the *mDf7* background. Embryos lacking FRK-1 were confirmed via immunofluorescence with a purified FRK-1 antibody. (F) FRK-1 knockdown demonstrating that the hyperactive endoderm Wnt signal observed in the absence of FRK-1 is at the level of β-catenin, downstream of the MAPK (MOM-4) and Nemo-like kinase (LIT-1). *frk-1(RNAi)* was performed in the *lit-1(t1512)* and *mom-4(or42)* chromosomal mutant backgrounds.

Supplemental Figure 3. Proposed pathways that modulate Wnt signaling in endoderm specification and cell proliferation.

(A) Schematic of the endoderm-specifying Wnt pathway showing regulatory relationships between the key components described here. (B) Apparent divergence of WRM-1 in specification (through POP-1) and proliferation (via an unknown factor, X) in the endoderm Wnt pathway.