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CWN-1 functions with DSH-2 to regulate *C. elegans* asymmetric neuroblast division in a β-catenin independent Wnt pathway

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ABSTRACT

In *Caenorhabditis elegans*, Wnt signaling regulates many asymmetric cell divisions. During embryogenesis, the *C. elegans* Dishevelled (Dsh) homolog, DSH-2, regulates asymmetric neuroblast division of the ABpl/rpppa blast cell. Dsh is a key intracellular component of both β-catenin dependent and β-catenin independent Wnt pathways. In *C. elegans*, most of the well-characterized asymmetric cell divisions regulated by Wnts are dependent on β-catenin. In the ABpl/rpppa neuroblast division, however, we determined that DSH-2 regulates cell polarity through a β-catenin independent Wnt pathway. We also established that the *C. elegans* Wnt homolog, *cwn-1*, functions to regulate asymmetric division of the ABpl/rpppa blast cell. Our results indicated that *cwn-1* does not act alone in this process, and it functions with another redundant ligand that appears not to be a Wnt. Finally, we show widespread requirements for DSH-2 during embryogenesis in the generation of many other neurons. In particular, DSH-2 function is necessary for the correct production of the embryonic ventral cord motor neurons. This study demonstrates a role for DSH-2 and Wnt signaling in neuronal specification during *C. elegans* embryogenesis.

Introduction

Wnts are a large family of conserved secreted signaling molecules that regulate a wide variety of developmental processes in organisms ranging from cnidarians to humans (reviewed in Cadigan and Nusse, 1997; Logan and Nusse, 2004; Korswagen, 2007). Upon binding to the Frizzled (Fz) family of seven transmembrane receptors, Wnts can activate several distinct downstream signaling pathways. The first Wnt-responsive intracellular signaling pathway identified was the β-catenin dependent pathway. The central feature of the Wnt/β-catenin-dependent pathway is the regulation of β-catenin stability. In the absence of a Wnt signal, β-catenin is phosphorylated and targeted for degradation by a multiprotein destruction complex. Upon reception of a Wnt signal, Fz signals to the cytoplasmic scaffolding protein Dishevelled (Dsh), that inhibits the activity of the destruction complex. This leads to an increase in the cytoplasmic levels of β-catenin, which then translocates to the nucleus and associates with the LEF/TCF family of transcription factors to regulate Wnt responsive genes. The transcriptional response mediated by β-catenin represents an important effect of Wnt signaling, but it is by no means the only intracellular response to Wnt ligands.

In addition to the β-catenin-dependent response, it has been shown that Wnts can also signal via a β-catenin independent pathways, the Wnt/Planar Cell Polarity (PCP) (Fanto and McNeill, 2004; Kohn and Moon, 2005; Montcouquiol et al., 2006). The Wnt/PCP pathway regulates cell polarity and controls cell movements. This pathway was originally characterized in *Drosophila* where it controls epithelial polarity within the plane of the epithelium, and can be seen in the ordered orientation of wing hairs, bristles and ommatidia. Two core genes in the PCP pathway, Fz and Dsh, also function in the Wnt/β-catenin pathway whereas others, including Flamingo (Fmi), Strabismus/Van Gogh (Stbm/Vang), Prickle (Pk) and Diego (Dgo) appear to be PCP specific (Gubb et al., 1999; Tree et al., 2002; Chae et al., 1999; Usui et al., 1999; Feiguin et al., 2001). During PCP establishment in the wing epithelia, Fz and Dsh proteins are asymmetrically localized along the proximal distal axis of the cell (reviewed in Strutt, 2002; Fanto and McNeill, 2004; Zallen, 2007). In vertebrates, Fz and Dsh homologs appear to play a similar role in the regulation of cell polarity during convergent extension movements, a process in which cells intercalate along one axis causing the tissue to elongate in the perpendicular axis (Mlodzik, 2002; Strutt, 2003; Klein and Mlodzik, 2005). In both flies and vertebrates, downstream effectors of PCP signaling include the small GTPases Rho and Rac, which activate Rho-associated kinase and the Jun-N-terminal kinase (JNK) pathways. These different Wnt-responsive

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pathways results in the activation of distinct effectors that in turn have specific effects on developmental decisions.

Dsh is the last common intracellular signaling component before the different Wnt pathways diverge. All Dsh proteins contain three highly conserved domains: an N-terminal DIX domain, a central PDZ domain, and a C-terminal DEP domain. The DIX domain is essential for Wnt/β-catenin signaling but it is dispensable for β-catenin independent Wnt pathways. Overexpression of the DIX domain causes a strong dominant negative phenotype, inhibiting Wnt/β-catenin signaling in *Drosophila* and *Xenopus* (Axelrod et al., 1998; Tamai et al., 2000). In tissue culture cells, Dsh constructs lacking the DIX domain fail to stabilize β-catenin or activate the LEF/TCF transcription factor, but can still activate the JNK pathway (Yanagawa et al., 1995; Li et al., 1999). *In vivo*, Dsh constructs specifically lacking the DIX domain cannot rescue Wnt/β-catenin phenotypes but still function in PCP signaling and convergent extension (Boutros et al., 1998; Wallingford et al., 2000). In contrast, the DEP domain is primarily required for PCP signaling. In *Drosophila*, a Dsh construct lacking the DEP domain cannot rescue a *dsh* mutant specifically defective in PCP signaling and, in tissue culture cells, could no longer activate JNK (Boutros et al., 1998; Moriguchi et al., 1999). In *Xenopus*, a Dsh construct lacking the DEP domain was still functional for Wnt/β-catenin dependent signaling (Wallingford et al., 2000). A variety of experiments implicate the PDZ domain in both β-catenin-dependent and -independent pathways (Wharton, 2003; Habas and Dawid, 2005).

In *Caenorhabditis elegans*, Wnt signaling regulates many developmental events including asymmetric cell division. Asymmetric cell division, the process by which a mother cell divides to produce two daughter cells that adopt distinct cell fates, is essential for the generation of cellular diversity in multicellular organisms. In *C. elegans*, the polarization of asymmetrically dividing cells by Wnt signaling pathways has been well studied in the asymmetric division of the EMS blastomere at the 4 cell stage of embryogenesis, the T cell in the larval tail, the B cell in the male tail, and the somatic gonadal precursor (SGP) cells Z1 and Z4 (reviewed in Korswagen, 2007; Mizumoto and Sawa, 2007a). Wnt signaling leads to the asymmetric levels of POP-1, the LEF/TCF transcription factor, in the nuclei of the two daughter cells (Lin et al., 1998; Herman, 2001). Nuclear POP-1 levels are higher in the anterior daughter cells (proximal daughter in the SGP division) and lower in the posterior daughter cells (distal daughter cell in the SGP division). Asymmetric division of the EMS blastomere, the T cell, and the SGP cells all require the function of two *C. elegans* β-catenin homologs, WRM-1 and SYS-1 (Rocheleau et al., 1997; Kidd et al., 2005; Takeshita and Sawa, 2005). Upon division, both WRM-1 and SYS-1 show higher levels of nuclear localization in the posterior (distal) daughter (Mizumoto and Sawa, 2007b; Nakamura et al., 2005; Phillips et al., 2007; Takeshita and Sawa, 2005). WRM-1 functions to regulate POP-1 asymmetry, while SYS-1 functions as a transcriptional co-activator for POP-1 in the posterior (distal) daughter cells. Because of the asymmetric localization of several pathway components, this pathway is now referred to as the Wnt/β-catenin asymmetry pathway (Mizumoto and Sawa, 2007a). Unlike the asymmetric divisions of the EMS, T and SGP cells, the asymmetric division of the B cell appears to be regulated by a β-catenin-independent Wnt pathway (Wu and Herman, 2006). Although polarity of this division requires LIN-44/Wnt and LIN-17/Fz there is little or no requirement for any of the *C. elegans* β-catenin homologs. Instead, the function of RHO-1/RhoA and LET-502/Rock, which mediate Wnt/PCP pathway signaling, are required for the asymmetric B cell division.

In *C. elegans*, two of the three Dsh homologs, *dsh-2* and *mig-5*, have been implicated in the regulation of asymmetric cell divisions. At the four-cell stage of embryogenesis the P2 blastomere secretes the Wnt MOM-2 to polarize the neighboring blastomere EMS (Rocheleau et al., 1997; Thorpe et al., 1997). EMS then divides asymmetrically to generate an anterior daughter MS, which produces mainly mesoderm, and a posterior daughter E, which produces endoderm. In the absence of MOM-2 function, EMS divides symmetrically to produce two MS daughters, resulting in embryos that lack endoderm. The DSH-2 protein is expressed at the four-cell stage of embryogenesis and is enriched at the P2/EMS boundary (Walston et al., 2004). In the specification of the E cell, *dsh-2* and *mig-5* function redundantly along with the tyrosine kinase related gene *src-1*(Bei et al., 2002; Walston et al., 2004). In the T cell, both DSH-2 and MIG-5 are asymmetrically localized to the posterior cortex prior to division in response to LIN-44/Wnt (Mizumoto and Sawa, 2007a). Although loss of *mig-5* or *dsh-2* function does not affect T cell division, this could be due to functional redundancy between *mig-5* and *dsh-2*. Both DSH-2 and MIG-5 are also required for asymmetric division of the SGP cells and DSH-2 is required for the normal POP-1 nuclear asymmetry in the SGP daughter cells (Chang et al., 2005; Walston et al., 2006). Asymmetric division of the B cell requires MIG-5 but not DSH-2 (Wu and Herman, 2006). Consistent with the hypothesis that asymmetric B cell division is regulated by a β-catenin independent Wnt pathway, a *mig-5* construct lacking the DEP domain fails to rescue B cell polarity defects of *mig-5* mutants while a construct lacking the DIX domain is capable of partial rescue. These findings underscore that in *C. elegans* Dsh homologs play an important role in many asymmetric cell divisions regulated by Wnt signaling.

We previously established that DSH-2 along with the MOM-5/Fz is required for asymmetric neuroblast division in the lineage that generates PHA, a chemosensory neuron in the tail (Hawkins et al., 2005). Herein we show a widespread requirement for *dsh-2* in the production of many neurons during embryogenesis, including the production of embryonic ventral cord motor neurons. Through the functional analysis of DSH-2 domains, we determined that DSH-2 functions in a β-catenin-independent Wnt pathway to regulate asymmetric division in the PHA neuron lineage. We also identified a role for *cwn-1* in asymmetric neuroblast division. These findings establish a role for Wnt signaling in *C. elegans* neuronal specification in which a β-catenin-independent Wnt pathway is required for asymmetric cell divisions that govern neuronal cell fates.

Materials and methods

Strains

The growth and culture of *C. elegans* strains was carried out as described (Brenner, 1974). All strains were grown at 20 °C unless otherwise noted. The following mutations, GFP arrays and chromosomal rearrangements were used for this study: *Linkage Group (LG) I*: *lin-44(n2111)* (Herman and Horvitz, 1994); *LG II*: *cwn-1(ok546)* (Zinovyeva and Forrester, 2005), *dsh-2(or302)* (Hawkins et al., 2005), *juIs76[Punc-25::gfp] I* (Huang et al., 2002), *mIn1[dpy-109e128) mIs14]* (Edgley and Riddle, 2001); *LG III*: *gmIs12[srb-6::gfp]* (Hawkins et al., 2005); *LG IV: cwn-2(ok895)* (Zinovyeva and Forrester, 2005), *egl-20(n585)*, *ced-3 (n717)* (Ellis and Horvitz, 1986), *mgIs18[ttx-3::gfp]* (Altun-Gultekin et al., 2001); *LG V: mom-2(ne834)* (Nakamura et al., 2005); *LG X*: *gmIs18 [ceh-23::gfp]* (Withee et al., 2004), *otIs138[ser-2prom-3::gfp]* (Tsalik et al., 2000), *oyIs14[sra-6::gfp]* (Aurelio et al., 2002); *kyIs104[str-1::gfp]; Unknown LG: kyIs170[srh-220::gfp, lin-15(+)]* (Chang et al., 2006), *juIs14[acr-2::gfp; lin-15(+)]* (Hallam et al., 2000), *akIs3[nmr-1::gfp]* (Brockie et al., 2001), *bwIs2[flp-1::gfp]* (Much et al., 2000).

Strain confirmation

Both *cwn-1(ok546)* and *cwn-2(ok895)* are deletions alleles and their presence was confirmed by PCR amplification from worm lysates using the following primers:

cwn-1- cwn-1OL (5′-TCGTTTCTGACATGGCTCAC-3′), cwn-1OR (5′-ACCCATCCTTTCCCAATCTC-3′), and cwn-1P (5′-ACTTTGATCGATCCG-

GTGAC-3′); *cwn-2*- cwn-2OL (5′-CGGAGAAGTTGTTGGCTCAT-3′) and cwn-2OR (5′-ATTATGTGGTCGCAAGGAGG-3′). The presence of the *ced-3(n717)* mutation was confirmed by PCR followed by restriction enzyme digestion. The mutation eliminates an Fnu4 H1 restriction site. The primers n717-F (5′-TCGGATTGGTTTGAAAGTGG-3′) and n717-R (5′-ACAGACGGCTTGAATGAACC-3′) were used to amplify a 320 bp genomic fragment from the *ced-3* gene, and subsequent digestion with Fnu4 H1 was used to determine the genotype.

RNA interference

RNAi by injection was carried out as described (Fire et al., 1998). The cDNAs used were: yk236a10 *(cwn-1)*, yk343h8 *(cwn-2)*, yk1183a10 *(egl-20)*, yk120c7 *(lin-44)*, and yk446d6 *(mom-2)*. The cDNAs were subcloned into L4440, and inserts were PCR amplified using the T7 containing primer TAATACGACTCACTATGG. PCR products were then digested with appropriate restriction sites ensuring that only a single T7 site was available for binding of the T7 polymerase during synthesis of single stranded DNA. RNA was produced using the RiboMax Large scale RNA production system (Promega) following the manufactures instructions. Equal molar ratios of sense and antisense RNA strands were annealed in the presence of injection buffer to produce dsRNA.

Molecular biology

The *dsh-2* minigene containing the endogenous promoter and regulatory regions fused with GFP (pNH77) was used as a template for the deletion constructs (Hawkins et al., 2003). The deletion constructs removed the following bases: ΔDIX, bases 301–555 of the gene (aa 101–185) (pTW1), Δ*EP, bases 1672–1827 of the gene (aa 558–609) (pTW4). Deletion constructs were generated essentially as previously described (Wang and Wilkinson, 2001). The deletion constructs were sequenced to verify the lack of secondary mutations.

Generation of transgenic animals

The *dsh-2* deletion constructs were injected into *dsh-2/mIn1* hermaphrodites using standard techniques (Mello et al., 1991). The ΔDIX-DSH-2::GFP construct, pTW1, was injected at 10 ng/μl with the plasmids *Pdpy-30::NLS::dsRed2* (40 ng/μl) (Cordes et al., 2006) and pRF4 *(rol-6(su1006))* (30 ng/μl) as coinjection markers. The Δ*EP DSH-2::GFP construct, pTW4, was injected at 2.5 ng/μl with the plasmids *srb-6::gfp* (25 ng/μl) and pRF4 *(rol-6(su1006))* (30 ng/μl) as coinjection markers. We were unable to obtain lines injecting Δ*EP DSH-2::GFP at higher concentrations.

Antibody staining and microscopy

Antibody staining of embryos was done as previously described (Hawkins et al., 2005). Primary antibodies used were: affinity purified rat anti-DSH-2 (1:200) (Hawkins et al., 2005) and chicken anti-GFP antibody (Upstate Technologies; 1:200). Cy3- and FITC-coupled secondary antibodies were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). For imaging endogenous GFP fluorescence, worms were mounted in 100 mM sodium azide on a 5% agar pad and examined on a Leica DMRXA fluorescence microscope. Images were captured with a Hamamatsu ORCA AG digital camera using Openlab software (Improvision).

Results

Loss of dsh-2 affects the production of embryonic ventral cord motor neurons

Loss of *dsh-2* function results in defective asymmetric cell division of ABpl/rpppa. In wild-type embryos, the posterior daughter cell of the ABpl/rpppa neuroblast produces a hyp8/9 cell, a phasmid sheath cell, and a cell that undergoes apoptosis (Fig. 1A). The anterior daughter cell produces five neurons; PHA, PVC, LUA, DA7 and PDA on the right side and PHA, PVC, LUA, DA6 and DA9 on the left side. In *dsh-2* mutants, we have previously shown that the cells normally descended from the posterior daughter cell are often lost, while PHA and PVC are often duplicated (Hawkins et al., 2005). This led to the model that in the absence of *dsh-2* function, the posterior daughter cell of ABpl/rpppa is transformed into a second anterior like daughter cell (Fig. 1A).

Based on our model we predicted that the DA6, DA7 and DA9 embryonic motor neurons derived from the ABpl/rpppa neuroblast lineage would also be duplicated in a *dsh-2* (M−, Z−) mutant. To test this hypothesis we analyzed the expression of *juIs14(acr-2::gfp)* in *dsh-2* mutant animals. In L1 animals, the *acr-2* promoter drives GFP expression in a total of 11 cholinergic neurons in the ventral nerve cord; six DA motor neurons (DA2–7) and five DB motor neurons (DB3–7) (Fig. 1B). To determine whether *dsh-2* affected motor neurons, we counted the total number of DA and DB motor neurons in the ventral nerve cord. We observed both neuronal losses as well as the presence of additional *acr-2::gfp* expressing neurons in *dsh-2* mutants. In 31% ($N=67$) of larvae lacking both maternal and zygotic *dsh-2* function, one or two extra neurons were observed. Extra neurons were seen in positions expected for extra DA6 or DA7 neurons in the posterior region of the ventral nerve cord, which provides further support for our model (Fig. 1C). However, the morphogenesis defects associated with the complete loss of *dsh-2* function hindered our ability to unambiguously determine neuronal identity based solely on position. Of the additional neurons observed, 35% were located in the anterior regions of the ventral nerve cord (Fig. 1D). Finally, 27% of *dsh-2* mutant worms were missing either one or two neurons (Fig. 1E). These findings indicated that *dsh-2* is required for the specification of DA and/or DB motor neurons.

To determine whether *dsh-2* was required in other neuroblast cell lineages, we examined *dsh-2* mutant animals for defects in the production of the six GABAergic DD motor neurons, the second class of embryonically derived ventral cord motor neurons. In wild-type L1 animals, the 6 DD motor neurons were visualized using the GFP reporter *juIs76(Punc25::gfp)*. The DD neurons are evenly spaced along the length of the animal, with DD2–5 located in the ventral nerve cord, DD1 in the retrovesicular ganglia in the head, and DD6 in the preanal ganglia (Fig. 2A). In *dsh-2* (M−, Z−) larvae, we observed a striking phenotype. In 30% of animals examined, we observed only 3 DD motor neurons ($N=67$) (Fig. 2C). Although these animals often displayed morphogenesis defects, the spacing of these neurons are consistent with the absence of either DD1, 3 and 5, or DD2, 4 and 6. Finally, a small percentage of *dsh-2* mutant animals (6%) were lacking all six DD motor neurons. In the DD motor neuron lineage, the DD1, 3, and 5 cells are descended from the posterior daughter of ABplppap, while DD2, 4, and 6 are descended from the posterior daughter of ABprppap (Fig. 2E). The consistent lack of exactly 3 DD motor neurons could be due to a cell fate transformation in which the posterior daughter of ABp(l/r)ppap is transformed into a second anterior like daughter cell. Alternatively, the posterior daughter of ABp(l/r)ppap may undergo cell death resulting in the absence of DD1, 3, 5, or DD2, 4, 6. However, the latter appears unlikely since the DD neuronal losses were not suppressed by a *ced-3* mutation (data not shown), which prevents all programmed cell deaths during embryogenesis (Ellis and Horvitz, 1986). Regardless, our analysis clearly indicates that *dsh-2* is required for the correct production of embryonic ventral cord motor neurons derived from several lineages.

dsh-2 mutants display widespread defects in the production of embryonically derived neurons

The additional defects in embryonic ventral cord motor neurons suggested that DSH-2 is likely required for the production of other

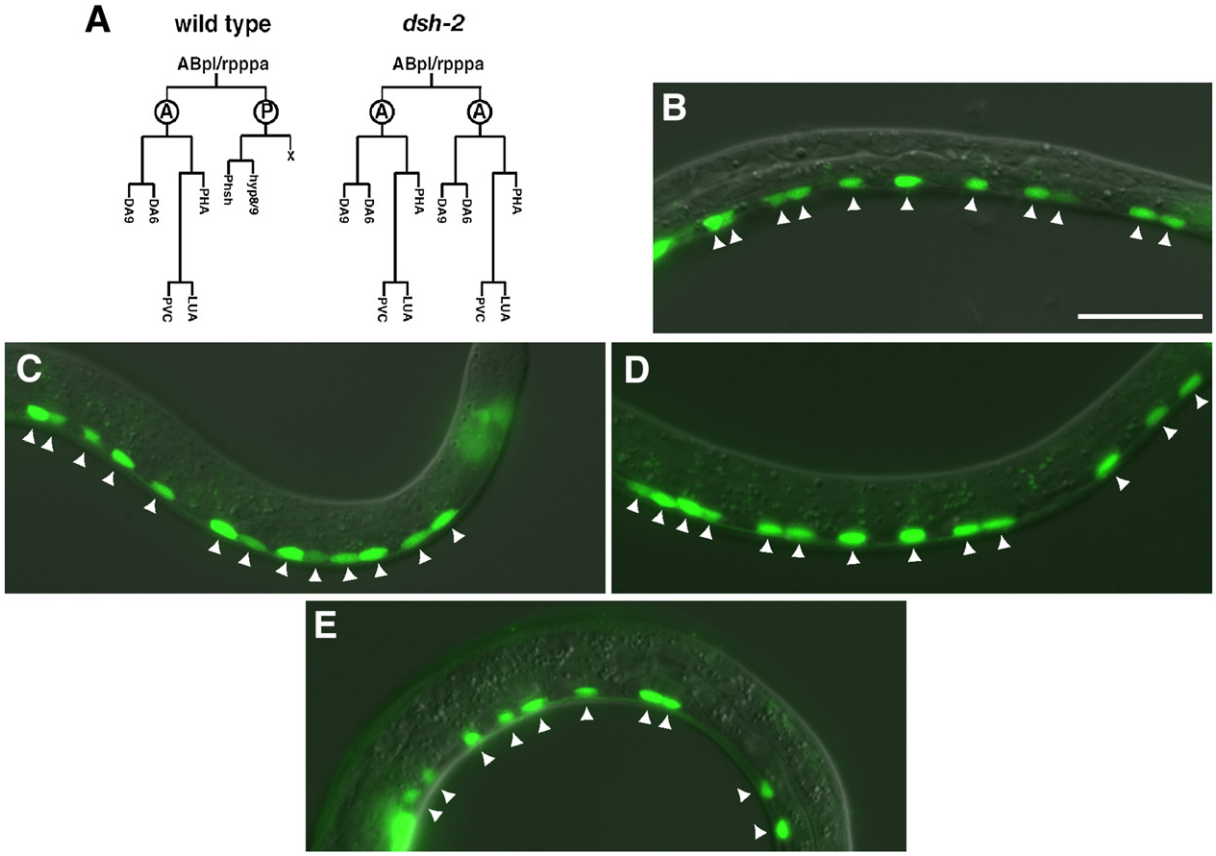

Fig. 1. Loss of *dsh-2* function affects the production of the DA/DB motor neurons. (A) Wild-type and proposed *dsh-2* mutant ABpl/rpppa lineages. In wild type embryos, the ABpl/rpppa neuroblast divides asymmetrically to generate distinct anterior (A) and posterior (P) daughter cells. The anterior daughter divides further to generate five neurons including PHA. The posterior daughter cell divides to produce only non-neuronal cells; a hypodermal cell (hyp8/9), a phasmid sheath cell (Phsh) and a cell that undergoes apoptosis (x). In *dsh-2* mutant embryos, we propose that the ABpl/rpppa neuroblast can divide symmetrically to generate two anterior-like daughter cells. (B–E) Expression of *juIs14(acr-2::gfp)* in (B) wild type and (C–E) *dsh-2(or302)* (M−, Z−) L1 larvae. All epi-fluorescence images are superimposed on the corresponding DIC image with anterior to the right and ventral down. (B) In wild type L1s, GFP expression is observed in 11 cholinergic embryonic motor neurons, DA2–7 and DB3–7 along the ventral nerve cord. (C) Two extra GFP expressing neurons are observed in the posterior region of the ventral nerve cord. (D) Two extra GFP expressing neurons are observed in the anterior region of the ventral nerve cord. (E) Two neurons are missing along the nerve cord. Scale bar: 20 μm.

neurons during embryogenesis. A broader requirement during embryogenesis is also supported by the observation that the DSH-2 protein is detected in most cells from the late 4-cell stage until morphogenesis (Hawkins et al., 2005). To assay for further neuronal defects we utilized neural specific GFP reporters. Because of the severe morphogenesis defects associated with *dsh-2* (M−, Z−) larval escapers, we preferentially chose GFP reporters that were specifically expressed in single neurons within L1 animals. We restricted our analysis to integrated GFP reporters so that the absence of GFP expression could not be the consequence of mosaicism. From this analysis we identified many additional neurons that were either lost and/or duplicated in *dsh-2* mutants.

In *dsh-2* mutant animals, neuronal duplications were observed for the CAN, PVQ, ADL, OLL and AIY neurons, whereas losses of the AWB and AVK neurons were seen (Tables 1 and 2). The CANs are bilaterally symmetric neurons that are born in the head and migrate posteriorly to the middle of the animal during embryogenesis and can be visualized using *gmIs18(ceh-23::gfp)* (Fig. 3A). In *dsh-2* (M−, Z−) animals, an extra CAN neuron was observed in 14% of sides scored (Fig. 3B). These extra CAN-like neurons were located along the normal CAN migratory route. In approximately 19% of sides scored, we were unable to detect any CANs. While the CAN might be lost in these animals, a migratory defect could also account for its apparent absence. Since *gmIs18* also expresses GFP in many additional neurons in the head, we are unable to distinguish a migratory defect from a loss of the CAN. PVQ neuron duplications were assayed by the expression of the GFP reporter *oyIs14(Psra-6::GFP)* (Fig. 3C). PVQs are bilaterally symmetric interneurons that reside in the tail in the lumbar ganglia. Rare PVQ duplications are observed in *dsh-2* mutant animals lacking only zygotic function (M+, Z−), but this frequency increased to 10% in *dsh-2* (M−, Z−) animals (Table 2) (Fig. 3D). The ADL neuron, an amphid neuron in the head, was also duplicated in a *dsh-2* mutant when observed using the GFP reporter *kyIs170(srh-220::gfp)*. In wild-type animals, there is a single ADL neuron on each side of the head (Fig. 3E) but in 8% of animals lacking zygotic *dsh-2* function (M+, Z−), extra ADL neurons were observed (Table 2) (Fig. 3F). This frequency of duplication increased to 13% in animals lacking all *dsh-2* function (M−, Z−) (Table 2). Similar to the PVQ and ADL neurons, low penetrance duplications were also observed for AIY, an interneuron located in the head (Table 1). In contrast, a higher frequency of duplications was observed for the OLL neurons. The OLL neurons are a pair of bilaterally symmetric sensory neurons whose cell bodies are located near the anterior bulb of the pharynx. These neurons were visualized with the *ser-2prom3::gfp* reporter *otIs138*, and in wild-type animals a single GFP expressing OLL neuron is observed on each side of the animal (Fig. 3G). In animals lacking only zygotic *dsh-2* function (M+, Z−), two adjacent GFP expressing cells on the same side of the animal were frequently observed (Fig. 3H, Table 1). Removal of maternal *dsh-2* function did not dramatically enhance the penetrance of this phenotype. However, three *ser-2prom3::gfp* expressing cells were occasionally observed and there were occasional OLL losses (Table 1; data not shown). For the AWB sensory neuron and the AVK interneuron, cell losses but no duplications were observed in animals lacking both

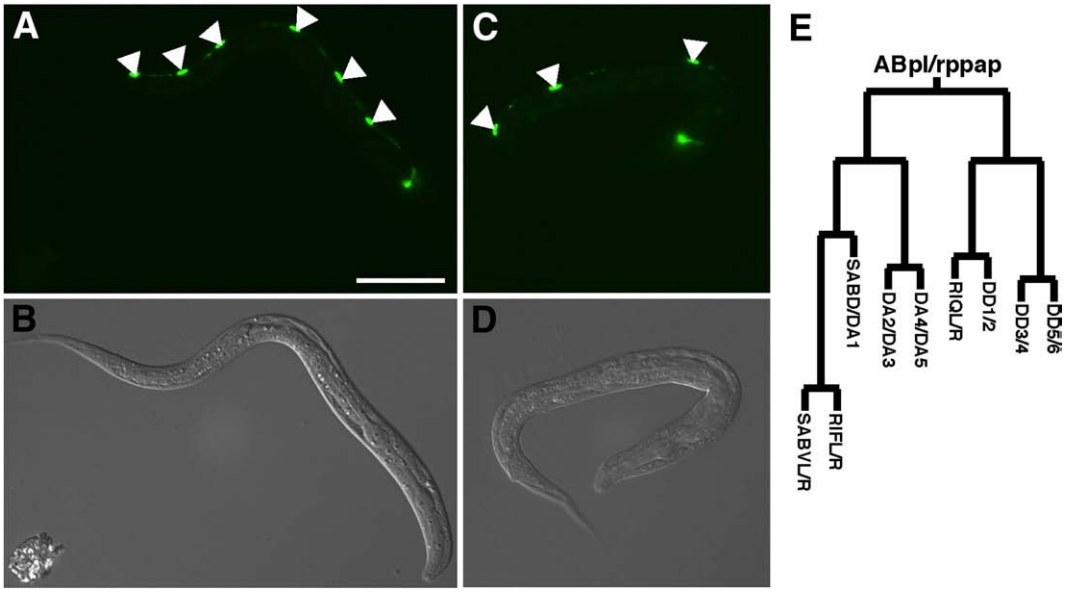

Fig. 2. Loss of *dsh-2* function results in the specific loss of 3 DD motor neurons. (A, C) Expression of *juIs76(unc-25::gfp)* in the DD embryonic motor neurons in L1 animals. (A) In wild type, 6 DD motor neurons are observed long the ventral nerve cord. (C) A *dsh-2(or302)* (M−, Z−) mutant larvae with only 3 DD motor neurons. (B, D) Corresponding DIC images. For all images anterior is to the right and ventral is down. (E) Embryonic lineage for the DD motor neurons. In wild type embryos, the ABpl/rppap neuroblasts divide asymmetrically to generate distinct anterior and posterior daughter cells. The left and right posterior daughter cells divide further to generate DD1, 3 and 5 and DD2, 4 and 6 respectively. Scale bar: 30 μm.

maternal and zygotic *dsh-2* function (M−, Z−) (Table 1). These findings confirmed that *dsh-2* plays an important role in either the asymmetric cell divisions and/or cell fate specifications that generate a variety of different neuron subtypes derived from many different cell lineages along the length of the animal.

Lineage analysis revealed that for three of the duplicated neurons observed in *dsh-2* mutant animals, PVQ, OLL and ADL, their sister cell normally undergoes apoptosis (Figs. 3I, J). In wild type, a total of 131 cells undergo apoptosis. Mutations in *ced-3*, a *C. elegans* caspase homolog, prevent all cells deaths from occurring. Therefore, we constructed a *dsh-2; ced-3* double mutants carrying specific neuronal GFP reporters and determined if the frequency of duplications were enhanced. This would suggest that the source of the extra neuron is likely the sister cell that normally undergoes apoptosis. We observed that the frequency of both PVQ and ADL neuronal duplications were significantly enhanced in a *ced-3* mutant background (Table 2), while the frequency of OLL duplications was not enhanced (data not shown). In *dsh-2* (M+, Z−); *ced-3* animals, the penetrance of PVQ duplications increased to 33% compared to only 3% in *dsh-2* (M+, Z−) animals while the penetrance of ADL duplications increased from 8% to 41%. The absence of *ced-3* function also enhanced both PVQ and ADL duplications in *dsh-2* (M−, Z−) nearly five fold, clearly indicating a synergistic effect between *dsh-2* and *ced-3*. Thus, this data indicates that the duplicated PVQ and ADL neurons observed in *dsh-2* mutant animals are likely due to a cell fate transformation of the PVQ/ADL sister cell.

DSH-2 domain analysis indicates that it regulates asymmetric neuroblast division in a β-catenin-independent Wnt pathway

In the lineage that generates the PHA neuron, preliminary evidence suggested that *dsh-2* was regulating asymmetric cell division of the ABpl/rpppa neuroblast via a β-catenin independent pathway (Hawkins et al., 2005). This conclusion was based on finding no requirement for the single LEF/TCF transcription factor *pop-1*. These experiments utilized knock down by RNAi and analysis of a *pop-1* hypomorphic mutation since we were unable to assay the consequences of complete loss of *pop-1* function due to lethality. Thus, one concern is that low levels of residual *pop-1* activity may be sufficient for asymmetric division. We are also unable to assess a complete lack of *wrm-1* or *sys-1* activity because of their early embryonic requirements. However, nuclear POP-1 asymmetry is still observed in *dsh-2* mutant embryos (King et al., 2009). This suggested

Table 1
Additional neuronal losses and duplications in *dsh-2* mutants

Genotype	Neuron	%		*N*
		loss	duplication	
dsh-2(or302) (M+ Z−)[a]; *gmIs18(ceh-23::gfp)*	CAN	0	0	86
dsh-2(or302) (M− Z−)[b]; *gmIs18(ceh-23::gfp)*	CAN	19	14	104
dsh-2(or302) (M+ Z−); *otIs138(ser-2prom3::gfp)*	OLL	2	27	94
dsh-2(or302) (M− Z−); *otIs138(ser-2prom3::gfp)*	OLL	10	33	108
dsh-2(or302) (M+ Z−); *kyIs170(srh-220::gfp)*	ADL	2	8	106
dsh-2(or302) (M− Z−); *kyIs170(srh-220::gfp)*	ADL	7	13	118
dsh-2(or302) (M+ Z−); *mgIs18(ttx-3::gfp)*	AIY	0	0	98
dsh-2(or302) (M− Z−); *mgIs18(ttx-3::gfp)*	AIY	0	9	100
dsh-2(or302) (M+ Z−); *kyIs104(str-1::gfp)*	AWB	2	0	102
dsh-2(or302) (M− Z−); *kyIs104(str-1::gfp)*	AWB	19	0	119
dsh-2(or302) (M+ Z−); *bwIs2(flp-1::gfp)*	AVK	4	0	100
dsh-2(or302) (M− Z−); *bwIs2(flp-1::gfp)*	AVK	8	0	102

[a] M+ Z− are animals with maternal *dsh-2* function but lacking zygotic *dsh-2* function.
[b] M− Z− are animals lacking both maternal and zygotic *dsh-2* function.

Table 2
Preventing cell death enhances PVQ and ADL neuronal duplications in a *dsh-2* mutant

Genotype	% duplication (number of sides scored)	
	PVQ[a]	ADL[b]
dsh-2(or302)/mIn1[c]	1 (116)	0 (100)
dsh-2(or302) (M+ Z−)[d]	3 (88)	8 (106)
dsh-2(or302) (M− Z−)[e]	10 (98)	13 (118)
ced-3(n717)	5 (198)	2 (104)
dsh-2(or302) (M+ Z−); *ced-3(n717)*	33 (79)	41 (104)
dsh-2(or302) (M− Z−); *ced-3(n717)*	50 (102)	54 (98)

[a] *oyIs14(sra-6::gfp)* expressing PVQ-like neurons.
[b] *kyIs170(srh-220::gfp)* expressing ADL-like neurons.
[c] mIn1 = *mIn1[dpy-10(e128) mIs14]*.
[d] M+ Z− are animals with maternal *dsh-2* function but lacking zygotic *dsh-2* function.
[e] M− Z− are animals lacking both maternal and zygotic *dsh-2* function.

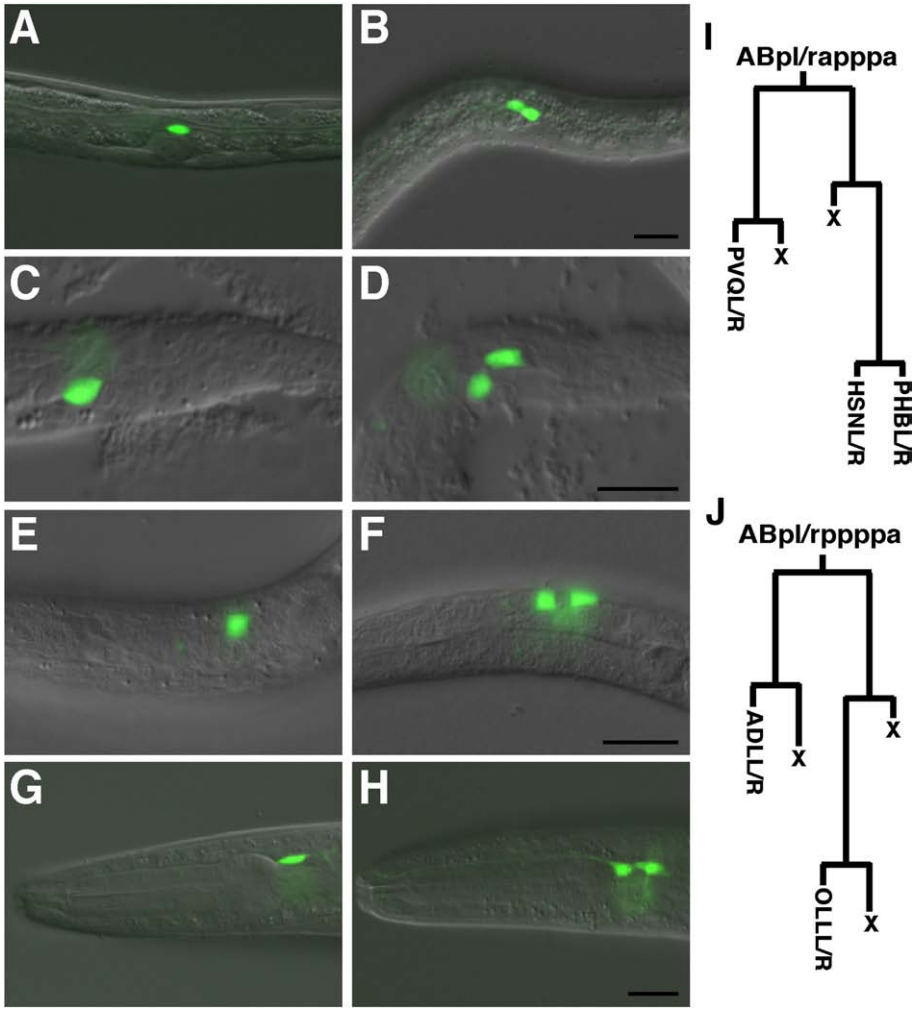

Fig. 3. CAN, PVQ, ADL and OLL neuron defects in *dsh-2* mutants. (A) GFP expression in a *gmIs18[ceh-23::gfp]* larva. A single CAN neuron expressing GFP is observed in the middle of the animal. (B) In a *dsh-2(or302)* (M−, Z−); *gmIs18[ceh-23::gfp]*, two CAN like neurons are observed expressing GFP. (C) GFP expression in a *oyIs14[sra-6::gfp]* larva. A single PVQ neuron expressing GFP is observed in the tail of the animal. (D) In a *dsh-2(or302)* (M−, Z−); *oyIs14[sra-6::gfp]*, two PVQ like neurons are observed expressing GFP. (E) GFP expression in a *kyIs170[srh-220::gfp]* larva. A single ADL neuron expressing GFP is observed in the head of the animal. (F) In a *dsh-2(or302)* (M+, Z−); *kyIs170[srh-220::gfp]*, two ADL like neurons are observed expressing GFP. (G) GFP expression in *otIs138[ser-2prom-3::gfp]* larva. A single OLL neuron expressing GFP is observed in the head of the animal on one side of the animal. (H) In a *dsh-2(or302)* (M+, Z) *otIs138[ser-2prom-3::gfp]* GFP expressing neurons are observed on both the left and right sides of the animal. On the right side is a single OLL neuron, while on the left side two OLL like neurons are observed expressing GFP. (I) Embryonic lineage for the PVQ neurons. The sister cell to the PVQ neurons undergo apoptosis (x). (J) Embryonic lineage for the ADL and OLL neurons. The sister cells to both the ADL and OLL neurons undergo apoptosis (x). Scale bar: 10 μm.

that the primary effect of *dsh-2* loss of function is not on POP-1 nuclear asymmetry, but rather on a β-catenin independent pathway.

Because different DSH-2 domains are associated with different Wnt signaling pathways, we undertook a domain analysis to identify the pertinent pathway involved in DSH-2 function during neuronal asymmetric cell division. Two *dsh-2* deletion constructs were generated. The first construct was missing amino acids 101 to 185 and lacked the entire DIX domain (ΔDIX), whereas the second construct contained a deletion of amino acids 535–609, thus lacking all but 23 amino acids of the DEP domain (Δ*EP). In both constructs, GFP was fused to the C-terminus and expression was under the control of the endogenous *dsh-2* promoter. These constructs were injected into *dsh-2* mutants to generate transgenic animals, which were then assayed for both rescue of PHA neuronal duplications and localization of the DSH-2::GFP fusion protein. Because the DIX domain confers β-catenin-dependent Wnt signaling, and the DEP mediates β-catenin-independent signaling, the sufficiency of either DSH-2 deletion construct to function in PHA production is a direct reflection of which Wnt pathway is involved.

The construct lacking the DIX domain was capable of rescuing both the *dsh-2* maternal effect lethality and PHA neuronal duplications. Worms lacking both maternal and zygotic *dsh-2* function (M−, Z−) die as embryos or early larvae. All three independent transgenic lines expressing ΔDIX-DSH-2::GFP rescued the *dsh-2* maternal effect lethality as assayed by their ability to maintain *dsh-2*(*or302*) as a viable

Table 3
The DIX domain of DSH-2 is dispensable while the DEP domain is required for asymmetric division of the ABpl/rpppa neuroblast

Genotype	% phasmid neurons/side[a]			*N*
	3	2	1	
dsh-2(or302)/mIn1	0	100	0	134
dsh-2(or302) (M− Z−)	24	71	5	145
dsh-2(or302)/mIn1; hkEx84(ΔDIX DSH-2::GFP)	0	100	0	78
dsh-2(or302) (M− Z−); hkEx84(ΔDIX DSH-2::GFP)	0	100	0	83
dsh-2(or302)/Min1; hkEx86(ΔDIX DSH-2::GFP)	0	99	1	116
dsh-2(or302) (M− Z−); hkEx86(ΔDIX DSH-2::GFP)	1	97	2	126
*dsh-2(or302)/mIn1; hkIs36(Δ*EP DSH-2::GFP)*	0	100	0	100
*dsh-2(or302) (M− Z−); hkIs36(Δ*EP DSH-2::GFP)*	21	67	12	100
*dsh-2(or302)/mIn1; hkIs38(Δ*EP DSH-2::GFP)*	0	100	0	112
*dsh-2(or302) (M− Z−); hkIs38(Δ*EP DSH-2::GFP)*	16	75	9	86

[a] The number of phasmid neurons (PHA and PHB) were determined by counting the number of cells in the tail of first larval stage animals expressing GFP from the integrated array *gmIs12(srb-6::gfp)*. Loss of *dsh-2* function specifically affects the number of PHA neurons. N is the number of sides scored.

Table 4
DSH-2 constructs lacking either the DIX or DEP domain can both partially rescue *dsh-2* gonadogenesis defects

Genotype	Hermaphrodite gonadal morphology (%)			
	Two arms	One arm	No arms	*N*
Wild type	100	0	0	100
dsh-2(or302)	17	40	42	52
dsh-2(or302); hkEx214 (DSH-2::GFP)	42	33	25	104
dsh-2(or302); hkEx84(ΔDIX DSH-2::GFP)	57	25	18	104
*dsh-2(or302); hkIs35(Δ*EP DSH-2::GFP)*	63	19	18	100
*dsh-2(or302); hkIs38(Δ*EP DSH-2::GFP)*	57	27	17	90

homozygote. To assay for rescue of the PHA neuronal duplications, the GFP reporter *gmIs12(srb-6::GFP)* was introduced into two of these transgenic lines. *gmIs12* expresses GFP in the two pairs of phasmid neurons, PHA and PHB. In wild-type animals, there are two GFP expressing cells in the tail on each side of the animal whereas in *dsh-2* (M−, Z−) mutants, three GFP expressing cells are frequently observed due to the duplication of the PHA neuron (Hawkins et al., 2005) (Table 3). We previously showed that production of the lineally unrelated PHB neuron is unaffected in a *dsh-2* mutant (Hawkins et al., 2005). The *dsh-2* construct lacking the DIX domain resulted in nearly complete rescue of the PHA neuronal duplications (Table 3). Thus, the DIX domain is dispensable for the function of DSH-2 in the asymmetric division of the ABpl/rpppa neuroblast.

In contrast, the construct expressing the DSH-2 protein lacking the DEP domain was unable to rescue either the *dsh-2* maternal effect lethality or the asymmetric division defects of ABpl/rpppa. The ΔEP-DSH-2::GFP construct was directly co-injected with the *srb-6::gfp* reporter to assess for possible rescue of PHA neuronal duplications. Nine independent transgenic lines that express ΔEP-DSH-2::GFP were obtained, and none showed any appreciable rescue of the *dsh-2* (M−, Z−) maternal effect lethality as assayed by their inability to maintain *dsh-2(or302)* as a viable homozygote. Because all nine lines were highly mosaic, and the *srb-6*::*gfp* reporter was contained in the extra-chromosomal arrays, we could not reliably score for rescue of the PHA neuronal duplications. Therefore, we integrated one of the arrays (*hkEx55*) and established two integrated lines, *hkIs36* and *hkIs38*. Neither integrated line showed any appreciable rescue of the PHA neuronal duplications (Table 3) nor any rescue of the maternal effect lethality. These findings suggested that the DSH-2 DIX domain was not required for ABpl/rpppa asymmetric division but the DEP domain was essential.

Because *dsh-2* is also involved in the asymmetric division of the SGP cells Z1 and Z4, we analyzed the requirements for the DSH-2 DIX and DEP domains in this division. In wild-type hermaphrodites, Z1 and Z4 divide along the proximal–distal axis to produce daughter cells with distinct fates. The distal daughters each generate a distal tip cell (DTC), which are migratory cells that lead the migration of the posterior and anterior gonadal arms. The proximal daughters generate either an anchor cell (AC) or a ventral uterine (VU) cell. In animals mutant for *lin-17* (Fz), *pop-1*, or the β-catenins *wrm-1* or *sys-1*, Z1 and Z4 divide symmetrically to generate two proximal daughters (Siegfried and Kimble, 2002; Kidd et al., 2005). Loss of zygotic *dsh-2* (M+, Z−) function produces a similar phenotype (Chang et al., 2005). In the absence of the distal daughter cells, one or both DTCs are often missing, resulting in *dsh-2* animals lacking one or both gonadal arms. In *dsh-2* (M+, Z−) hermaphrodites, 42% lacked both gonadal arms, while 40% of animals were missing a single arm (Table 4). Unlike the requirements for the DEP and DIX domains in asymmetric neuroblast divisions, both ΔDIX-DSH-2::GFP and Δ*EP-DSH-2::GFP were able to partially rescue the *dsh-2* gonadogenesis defects (Table 4). In both ΔDIX-DSH-2::GFP and Δ*EP-DSH-2::GFP transgenic animals, the percentage of *dsh-2* (M+, Z−) hermaphrodites lacking both gonad arms decreased to 18%. We also observed that the frequency of animals missing one gonad arm was reduced in transgenic animals carrying either construct (Table 4). These results suggested that while the DIX domain is dispensable for asymmetric division of the ABpl/rpppa neuroblast, both domains are required for division of the SGP cells.

In wild-type embryos, DSH-2 is detected at the cell periphery from the four-cell stage until mid-embryogenesis (Hawkins et al., 2005) (Fig. 4A). To examine the localization of the ΔDIX-DSH-2::GFP and Δ*EP-DSH-2::GFP fusion proteins, transgenic embryos were stained with anti-GFP antibodies. The ΔDIX-DSH-2::GFP protein was detected at the cell periphery in a manner indistinguishable from the localization of endogenous DSH-2 (Figs. 4C, D). In contrast, the DEP

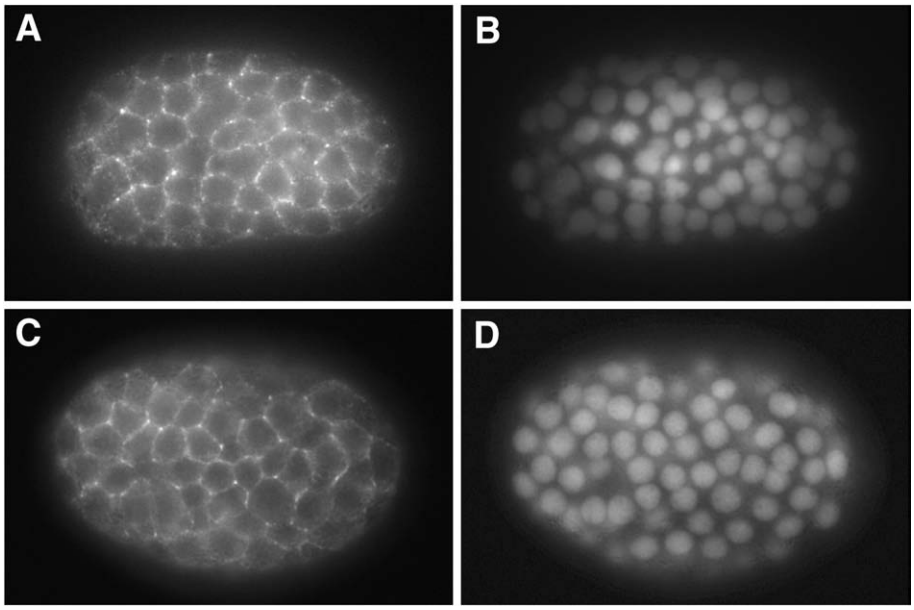

Fig. 4. ΔDIX-DSH-2::GFP is localized to the cell cortex in embryos. (A, B) A wild type embryo stained with anti-DSH-2 antibodies (A) and the corresponding DAPI stained image (B). The endogenous protein is localized to the cell cortex. (C, D) A transgenic embryo expressing ΔDIX-DSH-2::GFP (*hkEx84*) stained with anti-GFP antibodies to detect the fusion protein (C) and the corresponding DAPI stained image. The DSH-2 protein lacking the DIX domain is still detected at the cell cortex similar to the wild type protein.

domain is essential for localization of DSH-2 to the cell periphery. For the Δ*EP-DSH-2::GFP fusion, no protein localization at the cell periphery was observed. Levels were too low and diffuse in the cytoplasm for detection above background even though the fusion protein was expressed, as assayed by Western blot (data not shown). This is consistent with previous results showing that the DEP domain is required to localize Dsh proteins to the cell periphery. Our results demonstrated that the DEP domain, but not the DIX domain, plays an important role in conferring DSH-2 localization to the cell periphery.

CWN-1 functions in asymmetric neuroblast division

We next wanted to determine if a Wnt (or Wnts) function upstream of DSH-2 to regulate asymmetric cell division. In *C. elegans*, there are five Wnt genes; *cwn-1*, *cwn-2*, *lin-44*, *egl-20* and *mom-2*; and they regulate a variety of processes during development, including cell polarization and cell migration. Although a Wnt (or Wnts) have not been implicated in the asymmetric division of the SGP cells, Z1 and Z4, Wnts are required for the other asymmetric divisions (Korswagen, 2007).

Initially, to determine whether a Wnt functions upstream of DSH-2 in asymmetric division in the PHA lineage, we crossed *gmIs12(srb-6::GFP)* into null or strong loss of function mutants for *egl-20*, *cwn-1*, *cwn-2* and *lin-44*. The number of phasmid neurons was wild type in all four Wnt mutant backgrounds (Table 5; data not shown). Because mutations in *mom-2* are maternal-effect embryonic lethal, we used RNAi to knockdown gene function and then assayed the number of phasmid neurons in the few viable L1 animals that survived. Again, no change in the number of phasmid neurons was observed (data not shown). These results suggested that either Wnts are not required for asymmetric division in the PHA lineage, or more than one Wnt is involved.

To test the possibility that there is functional redundancy among the Wnt ligands, we constructed two strains mutant for multiple Wnts; a *lin-44; cwn-1; cwn-2* triple mutant, and a *lin-44; egl-20; cwn-1* triple mutant. However, no defects in the production of phasmid neurons were observed in either strain (Table 5). As a further test of functional redundancy, we used RNAi to reduce the function of *egl-20* and *mom-2* in the *lin-44; cwn-1; cwn-2* triple mutant. While there was a high degree of embryonic lethality no phasmid neuron defects were observed in surviving L1 animals.

As another approach to uncover a role for a Wnt (or Wnts) in asymmetric neuroblast division, we analyzed the requirements for Wnts in a sensitized genetic background. In *dsh-2* mutants lacking both zygotic and maternal function (M−, Z−) we observe 22% duplication of PHA (Hawkins et al., 2005; Table 5). However, in animals lacking only zygotic function (M+, Z−) we observe a modest 3–4% PHA duplication (Hawkins et al., 2005; Table 5). This indicates that maternal product is usually, but not always, sufficient for proper asymmetric neuroblast division in the PHA lineage. Thus, we reasoned that in *dsh-2* (M+, Z−) animals the pathway is already partially compromised and might be sensitized to further perturbations. Therefore, we performed a series of RNAi experiments looking for an enhancement of the weak *dsh-2* (M+, Z−) phenotype. dsRNAs for each of the five Wnts were injected in pair-wise combinations into a *dsh-2(or302)/mIn1; gmIs12(srb-6::GFP)* strain, and then the number of phasmid neurons in the resulting *dsh-2 (or302)* (M+, Z−) F1 progeny were scored. In the uninjected control animals, a duplicated phasmid neuron was observed at a frequency of 4%. For any Wnt RNAi combination that contained *cwn-1* dsRNA, we consistently observed an enhancement of the PHA neuronal duplications in the range of 11–14% (Table 5). There was no enhancement observed for any of the other double RNAi combinations tested. Because the common Wnt in these RNAi experiments

Table 5
Genetic interactions between *dsh-2* and *cwn-1*

Genotype	# of phasmid neurons/side[a]			*N*
	3(%)	2(%)	1(%)	
wild type	0	100	0	200
dsh-2(or302) (M+Z−)[b,c]	3	94	3	122
lin-44(n2111); cwn-1(ok546); cwn-2(ok895)	0	100	0	110
lin-44(n2111); cwn-1(ok546); egl-20(n585)	0	100	0	116
lin-44(n2111); cwn-1(ok546); cwn-2(ok895); egl-20(RNAi); mom-2(RNAi)	0	99	1	74
dsh-2(or302)/mIn1[d]*; cwn-1(RNAi); mom-2(RNAi)*	0	100	0	92
dsh-2(or302) (M+Z−); *cwn-1(RNAi); mom-2(RNAi)*	11	87	2	102
dsh-2(or302)/mIn1; cwn-1(RNAi); lin-44(RNAi)	0	100	0	104
dsh-2(or302) (M+Z−); *cwn-1(RNAi); lin-44(RNAi)*	11	87	2	104
dsh-2(or302)/mIn1; cwn-1(RNAi); cwn-2(RNAi)	0	100	0	114
dsh-2(or302) (M+Z−); *cwn-1(RNAi); cwn-2(RNAi)*	14	85	1	162
dsh-2(or302)/mIn1; cwn-1(RNAi); egl-20(RNAi)	0	100	0	100
dsh-2(or302) (M+Z−); *cwn-1(RNAi); egl-20(RNAi)*	12	85	3	120
dsh-2(or302)/mIn1; cwn-2(RNAi); egl-20(RNAi)	0	100	0	116
dsh-2(or302) (M+Z−); *cwn-2(RNAi); egl-20(RNAi)*	6	92	2	108
dsh-2(or302)/mIn1; lin-44(RNAi); cwn-2(RNAi)	1	99	0	94
dsh-2(or302) (M+Z−); *lin-44(RNAi); cwn-2(RNAi)*	5	88	7	90
dsh-2(or302)/mIn1; lin-44(RNAi); egl-20(RNAi)	1	99	0	114
dsh-2(or302) (M+Z−); *lin-44(RNAi); egl-20(RNAi)*	5	92	3	118
dsh-2(or302)/mIn1; cwn-1(RNAi)	0	100	0	100
dsh-2(or302) (M+Z−); *cwn-1(RNAi)*	13	85	2	110
dsh-2(or302)/mIn1; cwn-1(ok546)	0	99	1	176
dsh-2(or302) (M+Z−); *cwn-1(ok546)*	19	78	3	218
dsh-2(or302) (M−Z−)[e]	22	75	4	331
dsh-2(or302) (M−Z−); *cwn-1(ok546)*	23	74	3	118
cwn-1(ok546)	0	100	0	160

[a] The number of phasmid neurons (PHA and PHB) were determined by counting the number of cells in the tail of first larval stage animals expressing GFP from the integrated array *gmIs12(srb-6::gfp)*. Loss of *dsh-2* function specifically affects the number of PHA neurons. *N* is the number of sides scored.
[b] M+Z− are animals with maternal *dsh-2* function but lacking zygotic *dsh-2* function.
[c] Hawkins et al. (2005).
[d] mIn1 = *mIn1[dpy-10(e128) mIs14]*.
[e] M−Z− are animals lacking both maternal and zygotic *dsh-2* function.

was *cwn-1*, we tested whether RNAi of *cwn-1* alone was responsible for the enhancement of phasmid neuron duplications in *dsh-2 (or302)* (M+, Z−) animals. A 13% phasmid neuron duplication was observed indicating that the effects observed in the pair wise RNAi experiment was primarily, if not exclusively, due to knockdown of *cwn-1*. To further investigate the requirement for *cwn-1*, we constructed a *dsh-2(or302) cwn-1(ok546)* double mutant. The *cwn-1* allele is a deletion that removes exons 2, 3 and the majority of exon 4, and is predicted to be a strong loss-of-function or a null allele (Zinovyeva and Forrester, 2005). In *dsh-2(or302)* (M+, Z−) *cwn-1 (ok546)* double-mutant animals, the frequency of phasmid neuron duplications was 19%. The penetrance of this defect is essentially identical to that observed in *dsh-2(or302)* (M−, Z−) animals. However, unlike the complete loss of *dsh-2* function, *dsh-2* (M+, Z−) *cwn-1* animals are viable. This suggested that if *cwn-1* functions synergistically with *dsh-2*, it only affects a subset of *dsh-2* requirements during embryogenesis. The loss of *dsh-2* maternal product (M−, Z−) did not further enhance *dsh-2* (M+, Z−) *cwn-1* defects. As a control, the PHA specific GFP reporter, *ynIs45(pflp-15::gfp)*, was used to confirm that duplicated phasmid neurons observed were indeed PHA (data not shown). Based on our model (Fig. 1A), we also predicted an increased frequency of PVC neuron duplications in *dsh-2* (M+, Z−) *cwn-1* mutant animals. We constructed a *dsh-2(or302) cwn-1(ok546)* double mutant carrying the PVC specific GFP reporter *akIs3*(*nmr-1::GFP*). In *dsh-2(or302)* (M+, Z−) animals, we observed 4.5% PVC neuronal duplications ($N=110$). This frequency increased to 23% in the *dsh-2 (or302)* (M+, Z−) *cwn-1(ok546)* double mutant ($N=106$). These results established a role for *cwn-1* in asymmetric division of the ABpl/rpppa neuroblast.

In wild-type animals, complete loss of *cwn-1* alone did not affect asymmetric division in the PHA lineage. This suggested that *cwn-1* is functionally redundant with at least one other ligand. Based on our triple Wnt mutant combinations, *cwn-1* does not appear to be redundant with *lin-44*, *egl-20* or *cwn-2*. Complete loss of *mom-2* function is maternal effect embryonic lethal. Because we used RNAi to knockdown *mom-2* function and scored surviving L1 animals, our RNAi reduced but did not eliminate *mom-2* function. It is possible that the residual *mom-2* function was sufficient for correct asymmetric cell division in the PHA lineage. Therefore, to further test a requirement for *mom-2*, we constructed a double mutant between *cwn-1* and the temperature sensitive *mom-2(ne834)* allele. When we shifted embryos to the non permissive temperature, we saw no division defects in the PHA lineage (data not shown). This raises the intriguing possibility that a non-Wnt ligand may participate in the asymmetric division of ABpl/rpppa.

Discussion

Using cell specific GFP reporters we identified several additional neuronal losses and duplications in a *dsh-2* mutant. These affected neurons are born at various positions along the anterior–posterior axis of the embryo, indicating a widespread requirement for *dsh-2* during embryogenesis. The neuronal duplications and losses observed could be the result of asymmetric division defects, defects in cell fate specification or misexpression of the GFP reporter. To distinguish among these possibilities requires the ability to assay for the presence or absence of lineally related cells. Previously, we had shown that loss of *dsh-2* function resulted in the duplication of the sensory neuron PHA and, because of the availability of lineally related markers, were able to show that this neuronal duplication was due to a defect in the asymmetric division of ABpl/rpppa (Hawkins et al., 2005).

For the additional neuronal defects observed, limited markers are available to allow us to distinguish between asymmetric cell division and cell fate specification defects. However, the duplication of the PVQ and ADL neurons and the specific loss of a subset of DD motor neurons are likely the result of asymmetric cell division defects. In the lineages that give rise to the PVQ and ADL neurons, the final division is asymmetric, generating a neuron (either PVQ or ADL) and a cell that undergoes apoptosis. Enhancement of the *dsh-2* PVQ and ADL neuronal duplications by a *ced-3* mutation suggests that the extra neurons are derived from their sister cell, which normally undergoes apoptosis. In the lineage that generates the DD motor neurons, the ABpl/rppap neuroblast divides asymmetrically to generate distinct anterior and posterior daughter cells. The posterior daughter cells generate either DD1, 3, and 5 or DD2, 4 and 6 while the anterior daughter cells generate several of the DA motor neurons. One anterior daughter divides to produce DA2 and DA3 while the other daughter produces DA1, 4 and 5. The specific loss of three DD motor neurons could be the result of a transformation of the posterior daughter cell into a second anterior-like daughter cell. Consistent with that hypothesis we observed extra cholinergic motor neurons in the ventral nerve cord. However, because the *juIs14(acr-2::gfp)* reporter expresses in both DA and DB motor neurons we were unable to unambiguously determine exactly which motor neurons were duplicated. Regardless, *dsh-2* is required for the proper production of embryonic ventral cord motor neurons.

DSH-2 functions in a β-catenin independent Wnt pathway to regulate asymmetric division of ABpl/rpppa

Our previous results suggested that DSH-2 and MOM-5 may function in a Wnt/PCP like pathway to regulate asymmetric neuroblast division (Hawkins et al., 2005). First, the membrane association of DSH-2 in *C. elegans* and its dependence on MOM-5 (Fz) is similar to Dsh localization in *Drosophila* PCP and in vertebrate CE. During establishment of PCP in the *Drosophila* wing, Fz and Dsh asymmetrically localize to the distal vertex of the wing epithelial cells (Axelrod, 2001; Shimada et al., 2001). In a *fz* mutant, Dsh fails to localize at the membrane and instead accumulates in the cytoplasm. In *Xenopus*, Xdsh is localized to the cortex of cells undergoing convergent extension movements (Wallingford et al., 2000). Second, loss or overexpression of *dsh-2* in *C. elegans* resulted in similar cell fate transformations, while in *Drosophila* overexpression of Dsh causes a dominant PCP phenotype that is similar to the phenotype caused by a *dsh* mutant that specifically disrupts PCP (Axelrod et al., 1998). Finally, we have not found a requirement for POP-1 in the asymmetric cell division of ABpl/rpppa. However, because of early embryonic requirements for POP-1, and the two β-catenins WRM-1 and SYS-1, we could not assess a complete loss of gene function. Therefore, we tested the function of *dsh-2* constructs lacking either the DIX or DEP domains for rescue of PHA neuronal duplications due to ABpl/rpppa division defects. We determined that the DIX domain was completely dispensable for rescue while the DEP domain was essential. Based on domain analysis in other organisms this provides further evidence that DSH-2 is functioning in a β-catenin independent Wnt pathway.

In addition to asymmetric division defects, complete loss of *dsh-2* function is also maternal effect lethal. The majority of animals die as embryos while the low percentage of animals that hatch display morphogenesis defects and arrest as young larvae (Hawkins et al., 2005; King et al., 2009). The embryonic lethality is primarily due to defects in the morphogenetic process of ventral enclosure, where ventral hypodermal cells migrate to the ventral midline and fuse (King et al., 2009). A failure in ventral enclosure leads to rupture of the embryo during elongation. The construct lacking the DIX domain also rescued the maternal effect lethality, as assayed by the ability to maintain a *dsh-2(or302)* homozygous mutant strain with a ΔDIX-DSH-2::GFP extrachromosomal array. In contrast, we could not maintain a *dsh-2(or302)* homozygous mutant strain with a Δ*EP-DSH-2::GFP extrachromosomal array. However, weak rescue of hypodermal morphogenesis defects with this deletion construct has been observed (King et al., 2009). These results indicate that the essential requirement(s) for *dsh-2* during

embryogenesis may primarily be mediated by a Wnt/β-catenin independent pathway, but there also appears to be a contribution from a Wnt//β-catenin pathway (King et al.,2009).

In *C. elegans*, asymmetric division of the B cell in the male tail is also regulated by a β-catenin independent Wnt pathway. The B cell divides asymmetrically to generate a larger anterior–dorsal daughter cell and a smaller posterior–ventral daughter cell. In a *lin-44/Wnt* mutant the polarity of this division is reversed (Herman and Horvitz, 1994; Herman et al., 1995), while in a *lin-17/fz* this division becomes symmetric (Sternberg and Horvitz, 1988; Sawa et al., 1996). Of the three *C. elegans* Dsh homologs, only loss of *mig-5* function disrupts B cell polarity (Wu and Herman, 2006). Like the requirement for the DSH-2 domains in asymmetric division of ABpl/rpppa, the DEP domain of MIG-5 was essential to rescue the B cell polarity defects of *mig-5* mutants (Wu and Herman, 2007). No major requirement for any of the four β-catenin homologs was observed, though very weak division defects were seen when *sys-1* function was reduced (Wu and Herman, 2007). Instead, disrupting the function of *rho-1* (RhoA) or *let-502* (Rock), two Wnt/PCP pathway members in *Drosophila* and vertebrates, resulted in strong B cell division defects. Unlike the asymmetric division of ABpl/rpppa, asymmetric division of the B cell requires POP-1. Loss of *pop-1* function results in B cell polarity defects and higher levels of GFP::POP-1 are observed in the B.a daughter nucleus compared to the B.p daughter nucleus. Interestingly, a ΔDIXMIG-5::GFP construct only partially rescued *mig-5* B cell division defects. Thus, although DSH-2 and MIG-5 both function in a β-catenin independent Wnt pathway to regulate asymmetric division of ABpl/rpppa and the B cell respectively, the differences in requirements for the DIX domain and POP-1 suggest that the downstream signaling pathways are not identical.

DSH-2 is also required for the asymmetric division of the somatic gonadal precursor cells, Z1 and Z4. Since this division is also regulated by the β-catenins, WRM-1 and SYS-1, and requires the LEF/TCF transcription factor POP-1, we predicted that the DIX domain of DSH-2 would be essential for this division and the DEP domain would be dispensable. Instead, we discovered that constructs lacking either domain were both capable of partially rescuing the gonadogenesis defects. This raises the possibility that *dsh-2* signals to more than one downstream pathway to regulate Z1/Z4 division.

cwn-1 has a role in the asymmetric division of ABpl/rpppa

In *Drosophila*, there have been no Wnts implicated in PCP signaling (Klein and Mlodzik, 2005). In zebrafish however, loss of function mutants in two Wnt homologs, *wnt11/silberblick(slb)* and *wnt5/pipetail(ppt)* both result in CE defects during gastrulation (Heisenberg et al., 2000; Kilian et al., 2003). Injection of *wnt11* mRNA into the one cell stage embryo rescued the *slb* mutant phenotypes. Therefore, *wnt11* appears to act as a permissive cue for cell polarization during gastrulation, which suggests that an unidentified cue functions to specify polarity information.

Initially, to identify a Wnt that regulates asymmetric division of ABpl/rpppa, we analyzed individual Wnt mutants, or in the case of *mom-2*, reduced function by RNAi. Loss of any individual Wnt had no effect on the number of PHA neurons. However, loss of *cwn-1* function specifically, but none of the other four Wnts, could enhance the *dsh-2* (M+, Z−) PHA duplication. Animals lacking only zygotic *dsh-2* function (M+, Z−) display rare PHA duplications (3%). The phenotype was enhanced to 19% in a *dsh-2* (M+, Z−); *cwn-1* double mutant, essentially the same penetrance that is observed for a complete loss of both maternal and zygotic *dsh-2* function. This phenotype did not become any more severe in a *dsh-2* (M−, Z−); *cwn-1* double mutant. Normally, when a double mutant is constructed between two null alleles and the phenotype is no worse than either individual single mutant, this would provide a genetic argument that the two genes function in the same pathway. However, because loss of *cwn-1* function on its own had no phenotype, this does not eliminate the possibility that *cwn-1* may function in a parallel pathway that is redundant with *dsh-2*. In this case the requirement for this pathway is only revealed when the function of the *dsh-2* pathway is compromised.

Since the asymmetric division of ABpl/rpppa was unaffected in a *cwn-1* null mutant alone, this led us to test functional redundancy with one or more of the other Wnt genes. Functional redundancy among Wnt genes is an emerging theme in *C. elegans*. Specification of the vulval precursor cells (VPCs), migration of neurons and growth cones, and cell polarization all require multiple Wnts (Gleason et al., 2006; Hilliard and Bargmann, 2006; Inoue et al., 2004; Pan et al., 2006; Zinovyeva and Forrester, 2005). In an extreme example, all five Wnts contribute to the specification of the VPC cells (Gleason et al., 2006). Thus, we tested whether *cwn-1* functions redundantly with any of the remaining four *C. elegans* Wnt genes. Several lines of evidence suggest that the redundant polarizing signaling is unlikely to be another Wnt. In the triple Wnt mutant, *lin44; cwn-1; cwn-2* reduction of the two remaining Wnts, *egl-20* and *mom-2* by RNAi did not produce asymmetric division defects. However, since *mom-2* is required for embryonic viability we could not completely eliminate *mom-2* function. Therefore, in a second approach to test for a *mom-2* requirement we took advantage of *mom-2* temperature sensitive allele. We shifted *mom-2* and *mom-2; cwn-1* embryos to the non-permissive temperature after the 4 cell stage of embryogenesis but prior to 180 min of embryogenesis when division of the ABpl/rpppa occurs. Again, we did not observe any division defects. It cannot be ruled out that at the non-permissive temperature there is enough residual *mom-2* activity remaining to regulate the asymmetric division. However, the more intriguing possibility is that a non-Wnt signal functions redundantly with *cwn-1*.

Currently, the identity of a potentially redundant signal is unknown. In vertebrates, two additional ligands have been recently discovered that can activate Wnt/β-catenin signaling. One is the secreted cystine knot protein Norrin encoded by the Norrie disease gene (Xu et al., 2004). In mammals, Norrin binds specifically to Fz4 to control vascular development in the retina and inner ear (Smallwood et al., 2007; Xu et al., 2004). The second are the R-spondins, a family of secreted proteins characterized by two cysteine-rich furin like domains followed by a thrombospondin (TSP-1) motif that can also activate β-catenin signaling (Kim et al., 2005; Nam et al., 2006). However, neither R-spondins or Norrins have been identified in the *C. elegans* genome.

We specifically assayed for Wnt regulation in the asymmetric division of ABpl/rpppa. We discovered that while the *dsh-2* (M+, Z−); *cwn-1* double mutant is viable, the penetrance of PHA neuronal duplications is comparable to a complete loss of *dsh-2* function (M−, Z−). Since *dsh-2* (M−, Z−) animals are maternal effect lethal this suggests that *cwn-1* only functions in a subset of *dsh-2* related processes. This is consistent with the reported pattern of *cwn-1* expression. Both transcriptional and translational *cwn-1::gfp* fusions indicate that *cwn-1* expression is restricted to the posterior end of the embryo (Gleason et al., 2006; Pan et al., 2006). The asymmetrically dividing ABpl/rpppa blast cell is also located at the posterior end of the embryo. Both *mom-2* and *cwn-2* are expressed in early embryos (Gleason et al., 2006) and could function upstream of *dsh-2* in regulating the production of other embryonically derived neurons. Thus, the multiple requirements for *dsh-2* during development are regulated by different ligands.

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