The postinjury regenerative capacity of neurons is known to be mediated by a complex interaction of intrinsic regenerative pathways and external cues. In Caenorhabditis elegans, the initiation of axon regeneration is regulated by the nonmuscle myosin light chain (MLC)-4 phosphorylation signaling pathway. In this study, we have identified svh-16/cdk-14, a mammalian CDK14 homolog, as a positive regulator of axon regeneration in motor neurons. We then isolated the CDK-14-binding protein MIG-5/Disheveled (Dsh) and found that EGL-20/Wnt and the MIG-1/Frizzled receptor (Fz) are required for efficient axon regeneration. Further, we demonstrate that CDK-14 activates EPHX-1, the C. elegans homolog of the mammalian ephxin Rho-type GTPase guanine nucleotide exchange factor (GEF), in a kinase-independent manner. EPHX-1 functions as a GEF for the CDC-42 GTPase, inhibiting myosin phosphatase, which maintains MLC-4 phosphorylation. These results suggest that CDK14 activates the RhoGEF–CDC42–MLC phosphorylation axis in a noncanonical Wnt signaling pathway that promotes axon regeneration.

Key words: axon regeneration; C. elegans; cdk14; noncanonical Wnt signaling pathway

Significance Statement

Noncanonical Wnt signaling is mediated by Frizzled receptor (Fz), Disheveled (Dsh), Rho-type GTPase, and nonmuscle myosin light chain (MLC) phosphorylation. This study identified svh-16/cdk-14, which encodes a mammalian CDK14 homolog, as a regulator of axon regeneration in Caenorhabditis elegans motor neurons. We show that CDK-14 binds to MIG-5/Dsh, and that EGL-20/Wnt, MIG-1/Fz, and EPHX-1/RhoGEF are required for axon regeneration. The phosphorylation-mimetic MLC-4 suppressed axon regeneration defects in mig-1, cdk-14, and ephx-1 mutants. CDK-14 mediates kinase-independent activation of EPHX-1, which functions as a guanine nucleotide exchange factor for CDC-42 GTPase. Activated CDC-42 inactivates myosin phosphatase and thereby maintains MLC phosphorylation. Thus, the noncanonical Wnt signaling pathway controls axon regeneration via the CDK-14–EPHX-1–CDC-42–MLC phosphorylation axis.

Introduction

The ability of axons to regenerate after an injury is a fundamental and conserved property of neurons, governed by interactions between the intrinsic growth state and the extracellular microenvironment (Kaplan et al., 2015). Neurons in the mammalian peripheral nervous system, like neurons in most invertebrates, retain the ability to regenerate. In contrast, neurons in the mammalian CNS have a limited regenerative capacity (Case and Tessier-Lavigne, 2005). This difference in regenerative potential reflects alterations in the intrinsic capacity for axon growth. Accordingly, understanding the intrinsic mechanisms that regulate axon regeneration provides insights into possible neurologic injuries and disease treatments. However, these intrinsic signaling mechanisms operating in the adult nervous system are not yet fully understood.

Axon regeneration of injured neurons involves reorganization of the cytoskeleton (Bradke et al., 2012). After injury, neurons...
transform the damaged axon ends into new growth cone-like structures, driven by a dynamic reorganization of the actin network (Coles and Bradke, 2015). The Rhotype GTPase family is central to the regulation of cytoskeletal dynamics, including actin polymerization, F-actin stabilization, and actomyosin assembly in neuronal and non-neuronal cells (Hall, 1998). Rho GTPase functions as a bimolecular switch, with inactive GDP-bound and active GTP-bound forms. Activated Rho GTPases interact with effector proteins that trigger various cellular responses (Bishop and Hall, 2000). Among the Rho GTPase effectors, Rho-associated coiled-coil kinase (ROCK) plays an essential role in actin organization via myosin activation (Amano et al., 1996). Several Rho GTPases, including Rho, Cdc42, and Rac, have been demonstrated to function in the noncanonical Wnt pathway (Schlesinger et al., 2009). This pathway uses universal Wnt signaling components, such as Frizzled receptor (Fz) and Disheveled (Dsh). Although several Wnts have been shown to bind to Wnt signaling components, such as Frizzled receptor (Fz) and Disheveled (Dsh). Although several Wnts have been shown to function in the nervous system, including neuron migration, polarity, neurite extension, and neurite pruning (Whangbo and Kenyon, 1999; Lu et al., 2004; Montcouquiol et al., 2006; Hayashi et al., 2009), the Wnt pathway contribution to axon regeneration is poorly understood.

Recent studies on axon regeneration in genetic model organisms, such as Caenorhabditis elegans, have identified common biological pathways that use conserved molecules to control regeneration (Byrne and Hammarlund, 2017). Axon regeneration in C. elegans is regulated by the p38 MAP kinase (MAPK), JNK MAPK, and Rho GTPase signaling pathways (Hammarlund and Jin, 2014; Hisamoto and Matsumoto, 2017; Shimizu et al., 2018). The JNK pathway consists of MLK-1 MAPKKK, MEK-1 MAPKK, and KGB-1 JNK; it is inactivated at the KGB-1 activation step by VHP-1, which is a member of the MAPK phosphatase family (Mizuno et al., 2004). The vhp-1 loss-of-function mutation causes hyperactivation of the JNK pathway, resulting in developmental arrest at an early larval stage. We have previously undertaken a genome-wide RNAi screen for suppressors of vhp-1 lethality and isolated 92 svh genes (Li et al., 2012; Shimizu et al., 2021). Analysis of these svh genes was able to shed new light on the regulation of axon regeneration. Moreover, we demonstrated that in C. elegans, the RHO-1/RhoA–LET-502/ROCK signaling pathway promotes axon regeneration via phosphorylation of the regulatory nonmuscle myosin light chain [MLC (MLC-4); Shimizu et al., 2018]. We have also found that the svh-15 gene was previously identified as brc-2, which encodes a homolog of the mammalian BRCA2 tumor suppressor (Martin et al., 2005; Shimizu et al., 2018). We have demonstrated that SVH-15/BRC-2 is required for the activation of LET-502 by GTP-bound rho-1. MLC phosphorylation activates the Mg ATPase activity of nonmuscle myosin II and stimulates the interaction between actin and myosin (Somlyo and Somlyo, 2003). A growth cone contains a specific actin structure, which generates a mechanical force at the leading edge of the lamellipodium (Bradke et al., 2012). A previous study has revealed that myosin II functions in actin-bundle turnover in the growth cones of cultured neurons (Medeiros et al., 2006). These results suggest that MLC-4 phosphorylation regulates the dynamics of regenerating growth cones via the alteration of actin structures. Indeed, in C. elegans, MLC-4 is phosphorylated at the ends of the injured axons (Shimizu et al., 2018). The altered phosphorylation state of MLC is attributed to direct ROCK-mediated phosphorylation and inhibition of the myosin-binding subunit of myosin phosphatase (MYPT), which is a part of the MLC phosphatase complex (Totsukawa et al., 2000). Therefore, MYPT is expected to be involved in regulating C. elegans axon regeneration.

This study investigated the svh-16/cdk-14 and svh-21/mig-1 genes, which encode homologs of mammalian cyclin-dependent protein kinase-14 (CDK-14) and Fz, respectively. We found that CDK-14 binds to MIG-5/Dsh and EPHX-1/RhoGEF, and showed that D-type motor axones use EGL-20/Wnt and MIG-1/Fz as regulators of axon regeneration. This signal is transduced by the noncanonical Wnt pathway consisting of the CDK-14–EPHX-1–CDC-42–MLC-4 phosphorylation axis.

### Materials and Methods

C. elegans strains. The C. elegans strains used in this study are listed in Table 1. All strains were maintained on nematode growth media and fed with OP50, as described previously (Brenner, 1974).

**Plasmids.** The Punc-25:csrc-1 plasmid was generated by inserting the cdk-14 cDNA isolated from the pACT cDNA library (Sakamoto et al., 2005) into the pSC325 vector. The Punc-25:ephs-1 plasmid was generated by inserting the ephx-1 cDNA (isoform a) isolated from a cDNA library into the pSC325 vector. The Punc-25:csrc-2(G12V) plasmid was generated by inserting the cdc-42(G12V) cDNA (gift from Kozo Kaibuchi, Nagoya University, Japan) and the cdc-42 3’UTR into the pSC325. The pDBD-cdk-14 plasmid was generated by inserting the cdk-14 cDNA into pGDBD-C. The pAD-EPHX-1, pAD-EPHX-1N, and pAD-EPHX-1(SH3) plasmids were generated by inserting each cDNA into pACTII. T7-EPHX-1 (full length), and T7-EPHX-1AN(1-467) plasmids were generated by inserting the full-length cDNA or the N-terminal truncated forms of mig-5, brc-2 cDNAs isolated from a cDNA library into the pCMV-GFP vector. The GFP-DSH-1 and

### Table 1. Strains used in this study

| Strain | Genotype |
|--------|----------|
| KU91   | mig-5(km91) dsh-1(ok1445) jals-6 / mln1(mls14 dpy-10(e128)) II |
| KU501  | juls-6 II |
| KU1600 | cdk-14(tm4328) jals-6 II |
| KU1601 | cdk-14(tm4328) jals-6 II kmEx1601[Punc-25:cdk-14] |
| KU1602 | cdk-14(ok898) jals-6 II |
| KU1603 | cdk-14(ok898) jals-6 II |
| KU1604 | mig-5(h147) jals-6 II |
| KU1605 | mig-1(e1787) jals-6 II |
| KU1606 | juls-6 II; egl-20(n585) IV |
| KU1607 | mig-1(e1787) jals-6 II; egl-20(n585) IV |
| KU1608 | cwn-1(ok456) jals-6 II; cwn-2(20898) IV |
| KU1609 | cdk-14(tm4328) jals-6 II; kmEx1406[Punc-25:mkl-4(D)] |
| KU1610 | mig-1(e1787) jals-6 II; kmEx1610[Punc-25:mkl-4(D)] |
| KU1611 | cdk-14(tm4328) jals-6 II; kmEx1611[Punc-25:rho-1(GTP)] |
| KU1612 | cdk-14(tm4328) jals-6 II; kmEx1645[Punc-25:let-502(A)] |
| KU1613 | cdk-14(tm4328) jals-6 II; kmEx1613[Punc-25:cdk-42(GTP)] |
| KU1614 | cdk-14(tm4328) jals-6 II; mel-11(s565) juls-6/mmlc1(dpy-10(e128) amn-52(e444)) II |
| KU1615 | ephx-1(ok494) jals-6 II |
| KU1616 | cdk-14(tm4328) ephx-1(ok494) jals-6 II |
| KU1617 | ephx-1(ok494) jals-6 II kmEx1405[Punc-25:let-502(A)] |
| KU1618 | ephx-1(ok494) jals-6 II kmEx1613[Punc-25:cdk-42(GTP)] |
| KU1619 | cdk-14(tm4328) jals-6 II kmEx1619[Punc-25:ephs-1] |
| KU1620 | ephx-1(ok1319) jals-6 II |
| KU1621 | cdk-14(tm4328) ephx-1(ok1319) jals-6 II |
| KU1623 | ephx-1(ok1319) jals-6 II |
| KU1624 | mig-1(e1787) jals-6 II kmEx1613[Punc-25:cdk-42(GTP)] |
| KU1625 | juls-6 II kmEx1613[Punc-25:cdk-42(GTP)] |
| KU1626 | juls-6 II kmEx1619[Punc-25:ephs-1] |
CDK-14 (K89R) mutant using CRISPR–Cas9. The cdk-14(K89R) point mutation was generated using the CRISPR–Cas9 system (Sakai et al., 2021a). The CRISPR guide RNA (5 ’-GAUCUCUUUCAGGAGCAGUAGGCUAUCCG-3’) and single-stranded donor template DNA (5 ’-ACTAATTATATTTCAGACTTGACGGA TCTATAGTCGCTTGCGAGAGATCAAACTTCAATTTCAAGAA-3’) were synthesized (Integrated DNA Technologies, IDT), and co-injected with the transactivating CRISPR RNA (IDT), Streptococcus pyogenes Cas9 3NL5 (IDT) protein, and the Pmyo-2::dsred-monomer plasmid into the KU501 strain. Each of the F1 animals carrying the transgene was transferred onto a new dish and used for single-worm PCR, followed by DNA sequencing to detect the mutations.

Generation of the mig-5(km91) dsh-1(ok1445) mutant using CRISPR–Cas9. The mig-5(km91) mutation was generated using the CRISPR–Cas9 system (Sakai et al., 2021a). The CRISPR guide RNAs (5 ’-UCGUGUUAAUAGCCAGAGAGAGAGAGCAUAGCG-3’) were synthesized (IDT), and co-injected with the transactivating CRISPR RNA (IDT), Streptococcus pyogenes Cas9 3NL5 (IDT) protein, and the Pmyo-2::dsred-monomer plasmid into the dsh-1(ok1445) jtd76/mln1/mls14 dpy-10 (e128) II strain. Then, each F1 animal carrying the transgene was transferred onto a new dish and used for single-worm PCR, followed by DNA sequencing. The km91 mutation is a 28 bp insertion that generates a termination codon within the second exon of the mig-5 gene.

Transgenic animals. Transgenic animals were obtained using the standard C. elegans microinjection method (Mello et al., 1991). Pmyo-2::dsred-monomer, Punc-25::cdk-14, Punc-25::venus::mlc-4(DD), Punc-25::punc-25::cdk-14(25 ng/μl), Punc-25::cdk-14(25 ng/μl) were used in kmEx1601 [Punc-25::cdk-14 (25 ng/μl) + Pmyo-2::dsred-monomer (5 ng/μl)], kmEx1610 [Punc-25::venus::mlc-4(DD) (25 ng/μl) + Pmyo-2::dsred-monomer (5 ng/μl)], kmEx1611 [Punc-25::rha-1(G14V) (25 ng/μl) + Pmyo-2::dsred-monomer (5 ng/μl)], kmEx1613 [Punc-25::calc-4(G12V) (25 ng/μl) + Pmyo-2::dsred-monomer (5 ng/μl)], and kmEx1619 [Punc-25::epox-1(25 ng/μl) + Pmyo-2::dsred-monomer (5 ng/μl)], respectively. The kmEx1405 and kmEx1406 extrachromosomal arrays have been described previously (Shimizu et al., 2018).

Microscopy. Fluorescence imaging of transgenic animals was performed under a 100× objective lens on a fluorescent microscope (model ECLIPSE E800, Nikon) and photographed using a Zyla CCD camera (Oxford Instruments).

Axotomy. Axotomies were performed as described previously (Li et al., 2012). Animals were subjected to axotomy at the young adult stage. Commissures that displayed growth cones or small branches on the proximal fragment were counted as "regenerated." Proximal fragments that showed no change after 24 h were counted as "no regeneration." At least 20 individuals with one to three axotomized commissures were observed for most experiments.

Measurements of regenerating axons. The length of regenerating axons for D-type motor neurons was measured using the segmented line tool of ImageJ. Measurements were made from the site of injury to the tip of the longest branch of the regenerating axon. Axons that did not regenerate were excluded. Data were plotted using R (version 4.0.1) and R studio (version 1.3.959).

Yeast two-hybrid assays. The yeast two-hybrid analysis was performed as described previously (Sakamoto et al., 2005). In brief, plasmids were introduced into the Saccharomyces cerevisiae reporter strain PJ69-4A (MATa trp1-901 ura3-52 leu2-3112 his3-200 gal4Δ gal80Δ Met2::GAL7-lacZ lys2::GAL1-HIS3 Ade2::GAL2-ADE2) and cultured on SC-Ura-Leu plates. Transformants were streaked onto fresh SC-Ura-Leu-His plates with 5 mM 5-aminotriazole and incubated at 30°C for 3 d. Biochemical experiments using mammalian cells. For immunoprecipitation, transfected COS-7 cells were lysed in RIPA buffer [50 mM Tris-HCl, pH 7.4, 0.15 mM NaCl, 0.25% deoxycholic acid, 1% NP-40, 1 mM
**Results**

**SVH-16/CDK-14 is involved in axon regeneration**

So far, we have isolated 92 svh genes involved in axon regeneration (Li et al., 2012; Shimizu et al., 2018, 2021). The svh-16 gene corresponds to Wormbase ORF ZC123.4, which encodes a protein with high homology to mammalian CDK14, a member of the CDK family (Fig. 1A; Malumbres, 2014). We, therefore, renamed svh-16 as cdk-14. To clarify the role of cdk-14 in axon regeneration, we performed laser axotomy and assessed the regeneration of GABA-releasing D-type motor neurons (Fig. 1B;
in subdomain II, which is critical for ATP binding (Fig. 2C; Shu et al., 2007). The cdk-14(K89R) mutation had no effect on axon regeneration (Fig. 1C, Table 2), suggesting that CDK-14 regulates axon regeneration independent of its kinase activity.

**EGL-20/Wnt–MIG-1/Fz–Dsh signaling pathway regulates axon regeneration**

To understand how CDK-14 regulates axon regeneration, we attempted to identify proteins that interact with CDK-14. We performed a yeast two-hybrid screen using the kinase-negative CDK-14(K89R) as bait and isolated the mig-5 gene, which encodes a paralog of the Dsh protein (Fig. 3A; Walston et al., 2006). This gene product contains a fragment (311–666 aa) of MIG-5 isoform b (Fig. 3B). We confirmed the interaction between MIG-5b(311–666) and CDK-14 by immunoprecipitation assays using mammalian COS-7 cells. FLAG-tagged CDK-14 and GFP-tagged MIG-5b(311–666) were coexpressed in COS-7 cells. We immunoprecipitated GFP-MIG-5b(311–666) with anti-GFP antibody and probed for FLAG-CDK-14 by immunoblotting with anti-FLAG antibody. We found that CDK-14 was coimmunoprecipitated with MIG-5b(311–666; Fig. 3B) and confirmed that CDK-14 interacts with the full-length MIG-5b (Fig. 3B). However, the mig-5(km91) mutation (Fig. 3A) did not affect axon regeneration (Fig. 3C, Table 2). C. elegans has three Dsh paralogs: MIG-5, DSH-1, and DSH-2 (Fig. 3A; Walston et al., 2004). Although CDK-14 interacts with DSH-1 and DSH-2 (Fig. 3D), the dsh-2 (ok2162) and mig-5(km91) dsh-1(ok1445) dsh-2(or302) mutations did not cause impaired axon regeneration (Fig. 3C, Table 2). Since the dsh-1(ok1445) dsh-2(or302) double mutations affected the development of D-type motor neurons, we failed to examine axon regeneration in dsh-1(ok1445) dsh-2(or302) mutants. These results suggest that these three Dsh proteins are redundant regulators of axon regeneration.

As Dsh proteins link Fz receptors to the Wnt signaling pathway (Cadigan and Liu, 2006), we examined the possibility that Wnt regulates axon regeneration. In this pathway, a Wnt ligand binds to the Fz receptor and activates Dsh (Fig. 3A). To determine whether the Wnt pathway regulates axon regeneration, we further examined whether there are other gene functions that are related to the Wnt pathway. We then identified svh-21, which is identical to mig-1, encoding an Fz family member of C. elegans (Fig. 4A; Pan et al., 2006). Next, we examined whether the mig-1 gene was also involved in axon regeneration, wherein we found that the mig-1(e1787) mutation (Fig. 4A) significantly reduced axon regeneration after the laser injury (Fig. 4B, Table 2). Like cdk-14 mutants, the mig-1(e1787) mutation did not affect axon guidance or nerve development.

C. elegans has five Wnts (CWN-1, CWN-2, EGL-20, LIN-44, and MOM-2; Pan et al., 2006), and MIG-1/Fz mediates CWN-2 and EGL-20 signaling to regulate synapse formation and neurite outgrowth, respectively (Fig. 4A; Song et al., 2010; Mizumoto and Shen, 2013). We analyzed axon regeneration in cwn-1 (ok546); cwn-2(ok895) double deletion mutants (Fig. 4A). Loss of cwn-1 and cwn-2 did not affect axon regeneration (Fig. 4B, Table 2). The egl-20 n585 allele contains a missense mutation that replaces a cysteine at 99 with a serine (Fig. 4A); thereafter, a strong reduction-of-function mutation causes severe cell migration defects (Harris et al., 1996; Maloof et al., 1999). We found that the egl-20 mutant was defective in axon regeneration (Fig. 4B, Table 2). Furthermore, the regeneration defect in mig-1 (e1787); egl-20(n585) double mutants was found to be no more significant than the regeneration defect in single mig-1(e1787) or egl-20(n585) mutants (Fig. 4B, Table 2). Thus, MIG-1 and EGL-
20 act in the same pathway. As with the mig-1 mutant, the egl-20(n585) mutation did not affect axon guidance or nerve development. Interestingly, in glutamatergic touch sensory PLM (posterior lateral microtubule) neurons, egl-20 and mig-1 have been reported to be involved in axonal regeneration (Chen et al., 2011; Gokce et al., 2017). Thus, these results suggest that the EGL-20/Wnt–MIG-1/Fz pathway is generally required by neurons for axon regeneration.

**CDK-14 acts on the noncanonical Wnt pathway to promote axon regeneration**

Wnt signaling mediates canonical and noncanonical pathways (Fig. 5A; Cadigan and Liu, 2006). The latter pathway requires the activation of Rho GTPases, which regulate cytoskeletal dynamics (Schlessinger et al., 2009). We recently demonstrated that the C. elegans RhoA homolog RHO-1 promotes axon regeneration via MLC-4 phosphorylation (Shimizu et al., 2018). To address whether CDK-14 is involved in this pathway, we examined the effect of the phosphomimetic form of MLC-4, MLC-4(T17D; S18D) [termed MLC-4(DD)], on cdk-14 deficiency in axon regeneration. We found that MLC-4(DD) expression from the unc-25 promoter suppressed the cdk-14(tm4238) mutant phenotype (Fig. 5B, Table 2). Similarly, MLC-4(DD) expression suppressed the axon regeneration defect observed in mig-1(e1787) mutants (Fig. 5B, Table 2). Expression of MLC-4(DD) in wild-type animals does not affect the frequency of axon regeneration (Shimizu et al., 2018). These results suggest that the MIG-1–CDK-14-mediated noncanonical Wnt signaling pathway promotes axon regeneration by inducing MLC-4 phosphorylation.

In the noncanonical pathway, Rho and CDC42 activate ROCK, myonic dystrophy kinase-related CDC42 binding kinase (MRCK), and p21-activated kinase (PAK; Fig. 5C; Gally et al., 2018). These kinases induce myosin II activation by directly phosphorylating MLC (Totsukawa et al., 2000). They also inhibit myosin phosphatase activity by phosphorylating the myosin-binding subunit of MYPT, causing phosphorylation of MLC (Wilkinson et al., 2005). Next, we examined which Rho GTPase participates in the CDK-14-mediated axon regeneration pathway. When active GTP-bound RHO-1(G14V) was expressed from the unc-25 promoter in cdk-14(tm4238) mutants, it failed to suppress the defect in axon regeneration (Fig. 5D, Table 2). The C. elegans ROCK homolog LET-502 is an effector for RHO-1 (Fig. 5C; Piekny et al., 2000). The C-terminal segment of ROCK contains the autoinhibitory Rho-binding domain, the function of which is released via interaction with Rho-GTP (Bishop and Hall, 2000). Accordingly, a LET-502AC variant that lacks this C-terminal domain is constitutively active (Shimizu et al., 2018). As with RHO-1(G14V), the expression of LET-502AC from the unc-25 promoter in D-type motor neurons could not suppress the cdk-14(tm4238) mutant phenotype (Fig. 5D, Table 2). In contrast, the expression of active GTP-bound CDC42(G12V) from the unc-25 promoter and the mel-11(sb56) mutation defective in MYPT were able to suppress the axon regeneration defect observed in cdk-14(tm4238) mutants, respectively (Fig. 5E, Table 2). Expression of CDC42(G12V) did not affect axon regeneration in wild-type animals (Fig. 5E, Table 2). Furthermore, we found that expression of CDC42(G12V) could suppress the axon regeneration defect in mig-1(e1787) mutants (Fig. 5E, Table 2). Thus, CDK-14 and MIG-1 function upstream of CDC42, leading to myosin phosphatase inactivation, thereby sustaining MLC-4 phosphorylation.
Hiramoto et al. • CDK14 Signaling Regulates Axon Regeneration

J. Neurosci., October 6, 2021 - 41(40):8309-8320 • 8315

Figure 4. EGL-20/Wnt and Mig-1/Fz are required for axon regeneration. A. Mig-1/Fz-mediated signaling pathways. Mig-1 mediates CWN-2 and EGL-20 signaling to regulate synapse formation and neurite outgrowth, respectively. Schematic diagrams of Mig-1, CWN-1, CWN-2, and EGL-20 are shown. The transmembrane region and signal sequence are indicated by gray and black boxes, respectively. The e1787 and n585 mutation sites are indicated. The regions deleted in ok546 and ok895 are indicated by black bars. B. Percentages of axons that initiated regeneration 24 h after laser surgery in young adults. The number of axons examined is shown. Error bars indicate 95% confidence intervals. *p < 0.05, **p < 0.01, as determined by Fisher’s exact test. NS, Not significant.

**EPHX-1/RhoGEF functions between CDK-14 and CDC-42**

GTPase in the axon regeneration pathway

Activation of Rho GTPase depends on GEF family members that catalyze the GDP–GTP exchange reaction (Rossman et al., 2005). Therefore, a GEF for the CDC-42 GTPase should function in the noncanonical Wnt pathway to regulate axon regeneration. Human weak-similarity GEF (WGEF) directly interacts with Dsh and functions within the noncanonical Wnt pathway (Fig. 6A; Tanegashima et al., 2008). WGEF belongs to the Dbl family of RhoGEF proteins that usually possess tandem Db1 homology (DH), pleckstrin homology (PH), and Src homology 3 (SH3) domains (Fig. 6A; Tanegashima et al., 2008). The C. elegans ephx-1 gene encodes a Dbl family protein homologous to WGEF and ephexin (Fig. 6A). We, therefore, examined whether EPHX-1 acts upstream of CDC-42 to regulate axonal regeneration. We found that the ephx-1(ok494) mutant (Fig. 6B) was impaired in axon regeneration (Fig. 6C, Table 2). Like the cdk-14 mutant, the ephx-1(ok494) mutant did not affect the length of the regenerated axons (Fig. 6D), axon guidance, or nerve development. Furthermore, the ephx-1 phenotype defective in axon regeneration was suppressed by the expression of GTP-bound CDC-42(G12V), but not by that of LET-502AC (Fig. 6E, Table 2). These results support the hypothesis that EPHX-1 functions as a GEF for the CDC-42 GTPase in the noncanonical Wnt pathway to regulate axon regeneration.

Next, we investigated the genetic interaction between ephx-1 and cdk-14 in the regulation of axon regeneration. Animals that harbor the ephx-1(ok494); cdk-14(tm4238) double mutations exhibited a similar regeneration frequency as the ephx-1(ok494) single mutant (Fig. 6C, Table 2), indicating that EPHX-1 and CDK-14 act in the same pathway. We then tested the epistatic relationship between ephx-1 and cdk-14. The ephx-1 gene produces four transcripts from different initiation exons that encode EPHX-1a, EPHX-1b, EPHX-1c, and EPHX-1d (Fig. 6B). We constructed the Punc-25:ephx-1 fusion gene, in which the ephx-1a cDNA was fused with the unc-25 promoter and found that introducing the Punc-25:ephx-1a transgene rescued the defect associated with the cdk-14(tm4238) mutation (Fig. 6C, Table 2). Overexpression of ephx-1a did not affect axon regeneration in wild-type animals (Fig. 6C, Table 2). Therefore, overexpression of EPHX-1a from the unc-25 promoter suppresses the cdk-14(tm4238) mutant phenotype. We can conclude that CDK-14 is an upstream component in the EPHX-1–CDC-42 pathway regulating axon regeneration.

**CDK-14 interacts with EPHX-1**

The GEF potential of Dbl family members is autoinhibited by an intramolecular interaction between the N-terminal poly-proline region and the C-terminal SH3 domain (Fig. 7A; Schiller et al., 2006). Indeed, the SH3-containing domain of EPHX-1 was associated with the N-terminal domain in the yeast two-hybrid assay (Fig. 7B,C), and the EPHX-1 N-terminus contains poly-proline regions (Fig. 7B). Since CDK-14 regulation of axon regeneration is kinase independent (Fig. 1C), we hypothesized that CDK-14 interacts with EPHX-1 to activate EPHX-1 activity by inhibiting intramolecular binding. To investigate this possibility, we asked whether CDK-14 physically interacts with EPHX-1. Yeast two-hybrid analysis revealed that CDK-14 is associated with the SH3-containing (amino acids 1033–1159) domain of EPHX-1 (Fig. 7D). These results suggest that the interaction between CDK-14 and the SH3 domain of EPHX-1 disrupts intramolecular binding and activates EPHX-1 in axon regeneration.

**EPHX-1 N-terminal domain inhibits interaction with CDC-42**

Since the SH3 domain of EPHX-1 interacted with the N-terminal region (Fig. 7C), we expected the N-terminal region of EPHX-1 to exert GEF autoinhibitory activity. The ok494 allele of the ephx-1 gene encodes a protein with a frameshift deletion between amino acids 719 and 977 of the DH domain, essentially a truncation mutation (Fig. 6A; Tanegashima et al., 2006). Therefore, a GEF for the CDC-42 GTPase should function in the noncanonical Wnt pathway to regulate axon regeneration. We then tested the epistatic relationship between ephx-1 and cdk-14. The ephx-1 gene produces four transcripts from different initiation exons that encode EPHX-1a, EPHX-1b, EPHX-1c, and EPHX-1d (Fig. 6B). We constructed the Punc-25:ephx-1 fusion gene, in which the ephx-1a cDNA was fused with the unc-25 promoter and found that introducing the Punc-25:ephx-1a transgene rescued the defect associated with the cdk-14(tm4238) mutation (Fig. 6C, Table 2). Overexpression of ephx-1a did not affect axon regeneration in wild-type animals (Fig. 6C, Table 2). Therefore, overexpression of EPHX-1a from the unc-25 promoter suppresses the cdk-14(tm4238) mutant phenotype. We can conclude that CDK-14 is an upstream component in the EPHX-1–CDC-42 pathway regulating axon regeneration.

The GEF potential of Dbl family members is autoinhibited by an intramolecular interaction between the N-terminal poly-proline region and the C-terminal SH3 domain (Fig. 7A; Schiller et al., 2006). Indeed, the SH3-containing domain of EPHX-1 was associated with the N-terminal domain in the yeast two-hybrid assay (Fig. 7B,C), and the EPHX-1 N-terminus contains poly-proline regions (Fig. 7B). Since CDK-14 regulation of axon regeneration is kinase independent (Fig. 1C), we hypothesized that CDK-14 interacts with EPHX-1 to activate EPHX-1 activity by inhibiting intramolecular binding. To investigate this possibility, we asked whether CDK-14 physically interacts with EPHX-1. Yeast two-hybrid analysis revealed that CDK-14 is associated with the SH3-containing (amino acids 1033–1159) domain of EPHX-1 (Fig. 7D). These results suggest that the interaction between CDK-14 and the SH3 domain of EPHX-1 disrupts intramolecular binding and activates EPHX-1 in axon regeneration.

**EPHX-1 N-terminal domain inhibits interaction with CDC-42**

Since the SH3 domain of EPHX-1 interacted with the N-terminal region (Fig. 7C), we expected the N-terminal region of EPHX-1 to exert GEF autoinhibitory activity. The ok494 allele of the ephx-1 gene encodes a protein with a frameshift deletion between amino acids 719 and 977 of the DH domain, essentially a truncation at amino acid 723 (Fig. 6B). The tm11319 allele has a deletion of amino acids 719 and 977 of the DH domain, essentially a truncation at amino acid 723 (Fig. 6B). The tm11319 allele has a deletion of the N-terminal domain, resulting in a truncated EPHX-1 (EPHX-1d), with the N-terminal region of EPHX-1 containing poly-proline regions (Fig. 7B). Since CDK-14 regulation of axon regeneration is kinase independent (Fig. 1C), we hypothesized that CDK-14 interacts with EPHX-1 to activate EPHX-1 activity by inhibiting intramolecular binding. To investigate this possibility, we asked whether CDK-14 physically interacts with EPHX-1. Yeast two-hybrid analysis revealed that CDK-14 is associated with the SH3-containing (amino acids 1033–1159) domain of EPHX-1 (Fig. 7D). These results suggest that the interaction between CDK-14 and the SH3 domain of EPHX-1 disrupts intramolecular binding and activates EPHX-1 in axon regeneration.
Figure 5. CDK-14 acts on the noncanonical Wnt pathway to promote axon regeneration. A, Canonical and noncanonical Wnt signaling pathways. In the canonical pathway, Wnt stimulation stabilizes β-catenin and induces the expression of TCF target genes. The noncanonical Wnt/pathway activates Rho GTPases and induces reorganization of the actin cytoskeleton. B, D, E, Percentages of axons that initiated regeneration 24 h after laser surgery in young adults. The number of axons examined is shown. Error bars indicate 95% confidence intervals. *p < 0.05, **p < 0.001, as determined by Fisher’s exact test. NS, Not significant. C, Noncanonical Wnt pathways. RHO-1 activates LET-502, which phosphorylates MLC-4. CDK-42 activates MRCK and PAK, which phosphorylate MEL-11 to inhibit myosin phosphatase and induce MLC-4 phosphorylation.

Figure 6. EPHX-1/RhoGEF functions between CDK-14 and CDC-42 GTPase in the axon regeneration pathway. A, Structures of Dbl family proteins. Human WGEF interacts with Dsh in the Wnt signaling pathway. Schematic diagrams of C. elegans EPHX-1, human WGEF, and human ephexin-1 are shown. B, Structure of the ephx-1 gene. Schematic diagrams of EPHX-1 isoforms are shown. The ephx-1 gene encodes four isoforms, EPHX-1a, EPHX-1b, EPHX-1c, and EPHX-1d. Boxes indicate exons, and lines indicate introns and untranslated regions. The yellow, green, and red boxes represent the DH, PH, and SH3 domains, respectively. The regions deleted in tm11319 and ok494 alleles are indicated by black bars. C, E, Percentages of axons that initiated regeneration 24 h after laser surgery in young adults. The number of axons examined is shown. Error bars indicate 95% confidence intervals. *p < 0.05, **p < 0.01, ***p < 0.001, as determined by Fisher’s exact test. NS, Not significant. D, Length of regenerating axons 24 h after laser surgery. Data are presented as a boxplot representing the median (thick line within the box) and interquartile range (edge of box) with individual data points. The number (n) of axons examined is shown. Statistical significance was determined by the Wilcoxon rank-sum test.
Increased activity of the N-terminal EPHX-1 deletion mutant could be explained by a conformational change in the remaining part of the molecule, affecting its binding to CDC-42. We tested this possibility by analyzing the binding activity of EPHX-1 (full-length) and EPHX-1ΔN(1–467) with CDC-42. EPHX-1ΔN(1–467) corresponds to the EPHX-1d isoform (Figs. 6B, 8B). We cotransfected FLAG-tagged GDP-bound CDC-42(T17N) and T7-tagged EPHX-1 into mammalian COS-7 cells. Coimmunoprecipitation experiments revealed that EPHX-1ΔN(1–467) had significantly higher CDC-42-binding activity than full-length EPHX-1 (Fig. 8B), suggesting that the N-terminal domain of EPHX-1 has an autoinhibitory function.

Discussion

The Wnt signaling pathway plays multiple roles in the nervous system, including axon guidance, polarity establishment, axon regeneration, and synaptic specificity (He et al., 2018). The importance of Wnt signaling is demonstrated by the conservation of its molecular components across organisms from C. elegans to humans. The Wnt pathway is classified into canonical or noncanonical pathways. In the canonical Wnt signaling pathway, Wnt binds to the Fz receptor to activate the transcription of target genes through the regulation of β-catenin and T-cell factor (TCF). The noncanonical Wnt pathway is independent of β-catenin and TCF. This pathway uses universal Wnt signaling components such as Fz and Dsh, but, unlike the canonical Wnt pathway, Dsh functions through Rho family GTPases, subsequently activating downstream JNK to induce cytoskeletal rearrangements (Montcouquiol et al., 2006). Recent studies in mammalian and fish model systems have demonstrated that Wnt signaling promotes axon regeneration after injury in the adult optic nerve and spinal cord (Osakada et al., 2007; Herman et al., 2018), increasing the potential therapeutic value of Wnt. However, our understanding of its downstream signaling pathways remains limited. Our genetic analysis identified a set of Wnt components and demonstrated that EGL-20/Wnt functions in axon regeneration via the Fz receptor MIG-1. Our study reveals that the noncanonical Wnt pathway triggers downstream events that eventually act on the cytoskeleton to initiate axon regeneration (Fig. 9).

How is Wnt signaling transduced from Dsh to Rho GTPase activation in axon regeneration? Our results reveal a mechanistic link between Wnt–Dsh signaling and CDK14. Mammalian CDK14, also called PFTK1, is a member of the CDKs and is activated by a specific interaction with cyclin Y (Jiang et al., 2009). The CDK14/PFTK1–cyclin Y complex activates the noncanonical Wnt pathway, which subsequently activates Rho GTPases, such as Rho, Cdc42, and Rac, resulting in actin polymerization that underlies cell motility and migration (Sun et al., 2014). Analogous to mammalian CDK14/PFTK1, we showed that C. elegans CDK-14 functions as an essential regulator of the noncanonical Wnt pathway via activation of CDC-42 in axon regeneration. However, in contrast to mammalian CDK14, the C. elegans cyclin Y homolog is not involved in axon regeneration (Nix et al., 2014). Therefore, the kinase activity of CDK-14 is not required for axon regeneration. Consistent with this, a kinase-negative cdk-14(K89R) mutation had no effect on axon regeneration, indicating that CDK-14 regulation of axon regeneration is kinase independent.

How does CDK-14 control axon regeneration in a kinase-independent manner? Several studies have shown that the noncanonical Wnt pathway depends on the activation of Rho GTPases (Strutt et al., 1997; Choi and Han, 2002; Habas et al., 2003), which is mediated by RhoGEFs that catalyze the GDP–GTP exchange.
exchange reaction (Piekny et al., 2000). Indeed, WGEF has been identified in *Xenopus* as a link between Dsh and Rho activation in the noncanonical Wnt signaling pathway (Tanegashima et al., 2008). We also found that *C. elegans* EPHX-1/ephexin RhoGEF mediates noncanonical Wnt signaling in axon regeneration and demonstrated that CDK-14 promotes axon regeneration by activating EPHX-1, which acts on CDC-42. We propose that EPHX-1 is a necessary component in the pathway linking CDK-14 to CDC-42 activation (Fig. 9).

EPHX-1 belongs to the Dbl family of GEFs for Rho GTPases, whose domain structure consists of the DH–PH cassette followed by the SH3 domain (Rossman et al., 2005). The GEF potential of Dbl family members, such as WGEF and ephexin, is autoinhibited by intramolecular and intermolecular interactions (Schiller et al., 2006). The C-terminal SH3 domain binds to the N-terminal region, stabilizing the autoinhibitory helix on the DH domain. This interaction tightly suppresses Rho GTPase access to the DH domain required for GDP–GTP exchange. Indeed, truncation of the N-terminal region of Dbl results in its activation (Eva and Aaronson, 1985). We also showed that the N-terminal region of EPHX-1 inhibits binding to CDC-42. Based on these results, we propose the following mechanism by which CDK-14 activates the GEF activity of EPHX-1. EPHX-1 exchange potential is autoinhibited by an intramolecular interaction between the SH3 domain and the N-terminal region. The binding of CDK-14 to the SH3 domain of EPHX-1 removes the SH3 domain from the N-terminal region, activating the exchange potential of EPHX-1 (Fig. 9).

Biochemical analysis in vertebrates suggests that MLC phosphorylation induces the motility activity of myosin II (Somlyo and Somlyo, 2003). This process involves several Rho GTPase effector kinases, including ROCK, MRCK, and PAK. These kinases phosphorylate MLC directly or inhibit myosin phosphatase activity by phosphorylating MYPT, both of which can induce activation of myosin II (Totsukawa et al., 2000; Somlyo and Somlyo, 2003). These biochemical data suggest two MLC-4 activation pathways. First, LET-502/ROCK directly phosphorylates MLC-4. Second, redundant activities of MRCK and PAK phosphatase, including ROCK, MRCK, and PAK. These kinases phosphorylate MLC directly or inhibit myosin phosphatase activity by phosphorylating MYPT, both of which can induce activation of myosin II (Totsukawa et al., 2000; Somlyo and Somlyo, 2003). This interaction tightly suppresses Rho GTPase access to the DH domain required for GDP–GTP exchange. Indeed, truncation of the N-terminal region of Dbl results in its activation (Eva and Aaronson, 1985). We also showed that the N-terminal region of EPHX-1 inhibits binding to CDC-42. Based on these results, we propose the following mechanism by which CDK-14 activates the GEF activity of EPHX-1. EPHX-1 exchange potential is autoinhibited by an intramolecular interaction between the SH3 domain and the N-terminal region. The binding of CDK-14 to the SH3 domain of EPHX-1 removes the SH3 domain from the N-terminal region, activating the exchange potential of EPHX-1 (Fig. 9).

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**Figure 8.** EPHX-1 N-terminal domain inhibits interaction with CDC-42. A, Percentages of axons that initiated regeneration 24 h after laser surgery in young adults. The number of axons examined is shown. Error bars indicate 95% confidence intervals. *p < 0.05, as determined by Fisher’s exact test. NS, Not significant. B, Interaction of EPHX-1 with CDC-42. COS-7 cells were cotransfected with T7-EPHX-1 (full length), T7-EPHX-1ΔN1–467, and FLAG–CDC-42(T17N; GDP) as indicated. Complex formation was detected by immunoprecipitation (IP) with anti-T7 antibody, followed by immunoblotting (IB) with anti-FLAG and anti-T7 antibodies. Total lysates were immunoblotted with anti-FLAG antibody. Schematic diagrams of EPHX-1 (full length) and EPHX-1ΔN1–467 are shown above.

**Figure 9.** Schematic model for the regulation of axon regeneration by the noncanonical Wnt signaling pathway. The EGL-20/Wnt–MIG-1/Fz signaling promotes axon regeneration by activating the noncanonical Wnt pathway consisting of the CDK-14–EPHX-1–CDC-42–MLC-4 phosphorylation axis. The exchange potential of EPHX-1 is autoinhibited by an intramolecular interaction between the SH3 domain of EPHX-1. CDC-42 binding to the SH3 domain of EPHX-1 removes the SH3 domain from the N-terminal region, activating the exchange potential of EPHX-1 toward CDC-42. Finally, GTP-bound CDC-42 causes the inactivation of MEL-11, leading to MLC-4 phosphorylation, which promotes axon regeneration.

EPHX-1 functions as a GEF for RHO-1 in axon regeneration regulated by integrin signaling (Sakai et al., 2021b). This indicates that EPHX-1 can act on both RHO-1 and CDC-42 GTPases. How is the specificity of EPHX-1 RhoGEF for cognate GTPases determined? Mammalian ephexin is tyrosine phosphorylated in an N-terminal motif, which then alters the specificity.
for cognate GTPases. When ephexin is not tyrosine phosphorylated, it activates RhoA, Cdc42, and Rac1; however, it activates RhoA exclusively when the ephexin is phosphorylated (Sahin et al., 2005). Similarly, in C. elegans, integrin activation directs the exchange activity of EPHX-1 toward RHO-1 via the SRC-1-mediated tyrosine phosphorylation of the EPHX-1 N-terminal region. These results suggest that tyrosine phosphorylation of EPHX-1 determines the specificity of its GEF activity for RHO-1. Consistent with this, activated LET-502AC suppressed the axon regeneration defect caused by the nonphosphorylated ephlx-1 (Y568F) mutation, but not by the ephlx-1 deletion mutation. In the noncanonical Wnt signaling pathway, however, the CDE-14 interaction determines the specificity of EPHX-1 GEF for CDC-42. In this case, the GTP-bound form of CDC-42 suppressed the ephlx-1 deletion defect. One explanation for this result is that the protein kinase activated by active CDC-42 may phosphorylate both MLC-4 and MEL-11. MRCK and PAK are possible kinases that function downstream of CDC-42 (Wilkinson et al., 2005; Gally et al., 2009), but independent mutations in mrck-1 or pak-1 did not affect axon regeneration (Chen et al., 2011; Nix et al., 2014). Therefore, MRCK-1 and PAK-1 appear to be redundant kinases that induce MLC-4 phosphorylation during axon regeneration. Although this is the simplest model, we cannot exclude the possibility that a third kinase distinct from MRCK-1 and PAK-1 may work in the axon regeneration pathway. Thus, this calls for further studies to focus on identifying kinases that regulate axon regeneration downstream of CDC-42 in the noncanonical Wnt pathway.

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