During neural development, growing axons express specific surface receptors in response to various environmental guidance cues. These axon guidance receptors are regulated through intracellular trafficking and degradation to enable navigating axons to reach their targets. In Caenorhabditis elegans, the UNC-5 receptor is necessary for dorsal migration of developing motor axons. We previously found that MAX-1 is required for UNC-5-mediated axon repulsion, but its mechanism of action remained unclear. Here, we demonstrate that UNC-5-mediated axon repulsion in C. elegans motor axons requires both MAX-1 SUMOylation and the AP-3 complex β subunit gene, apb-3. Genetic interaction studies show that max-1 is SUMOylated by gei-17/PIAS1 and acts upstream of apb-3. Biochemical analysis suggests that constitutive interaction of MAX-1 and UNC-5 receptor is weakened by MAX-1 SUMOylation and by the presence of APB-3, a competitive interactors with UNC-5. Overexpression of APB-3 reroutes the trafficking of UNC-5 receptor into the lysosome for protein degradation. In vivo fluorescence recovery after photobleaching experiments shows that MAX-1 SUMOylation and APB-3 are required for proper trafficking of UNC-5 receptor in the axon. Our results demonstrate that SUMOylation of MAX-1 plays an important role in regulating AP-3-mediated trafficking and degradation of UNC-5 receptors during axon guidance.

Axon guidance | MAX-1 SUMOylation | AP-3 complex | UNC-5 receptor | C. elegans

A functional nervous system requires proper formation of neuronal connections, which begins with axon guidance (1). During this process, the growing axon is directed by various attractive and repulsive environmental cues until its tip, called the growth cone, reaches its final target. The guidance information is received by receptors on the growth cone, triggering a series of intracellular signals that move the axon in the correct direction. The diversity of developing axonal connections is established through dynamic regulation of environmental cues, surface receptors, and intracellular signaling networks (1, 2).

During dorso lateral growth of developing motor axons in Caenorhabditis elegans, the UNC-5 receptor is expressed on axonal growth cones, which are then repelled by a gradient of UNC-6/Nrtn in the environment (3–5). Accumulating evidence suggests that axon guidance requires proper trafficking and distribution of the UNC-5 receptors and their coreceptors UNC-40/DCC (6–9).

We previously isolated max-1 (motor axon guidance-1) in a forward genetic screen and showed that max-1 works with unc-5 to regulate repulsion of motor axons in C. elegans (10). A subsequent genetic study in zebrafish also suggested that max-1 plays a role in regulating membrane localization of Ephrin-3b proteins, which provide guidance cues for the migration of intersegmental venous endothelial cells during embryogenesis (11). However, how max-1 functions in the growth cone during unc-5-mediated axon repulsion is not clear. To address this issue, we used yeast two-hybrid screens to identify molecules that interact with MAX-1 and UNC-5.

The MAX-1–interacting protein GEI-17/PIAS1 is a SUMOylation E3 ligase (12). Protein SUMOylation is a posttranslational modification process that alters the activity, stability, and subcellular localization of the substrate protein (13). The dynamic and reversible features of SUMOylation make it an ideal biochemical switch to modulate diverse cellular processes, including segregation of chromosomes, repair of damaged DNA, regulation of transcription and enzyme activities, and control of intracellular trafficking (14, 15). SUMOylation is also involved in various aspects of neural development, which include proliferation, differentiation, apoptosis, target selection, synaptogenesis, and synaptic plasticity (16–21). Here we show that SUMOylation also plays a role in axon guidance by demonstrating that MAX-1 is a substrate of GEI-17/PIAS1 and that SUMOylation of MAX-1 is essential for UNC-5-mediated axon repulsion.

Our screen also identified an UNC-5–interacting protein, APB-3, the β subunit of the AP-3 complex, which is located in the plasma membrane, Golgi complex, and endolysosomal compartments. The AP-3 complex functions as an adaptor for trafficking cargo proteins and mediating protein degradation (22, 23). Studies in cultured neurons and in AP-3-deficient animals showed that APB-3 is involved in synaptic vesicle formation from tubular endosomes (24, 25) and may play a role in trafficking proteins within neuronal processes (26, 27). A recent

Significance

During neural development, growing axons navigate over long distances to reach their targets. A critical step in this process is the regulation of its surface receptors on the axon’s growth cone in response to environmental cues. We focus on how the UNC-5 receptor in Caenorhabditis elegans motor axons is regulated during axon repulsion. By combining C. elegans genetics, biochemistry, and imaging, we found that MAX-1 SUMOylation and AP-3 complex have significant roles in UNC-5-mediated axon repulsion. Our findings reveal how SUMOylation and AP-3–mediated trafficking and degradation interact to help the growing axon find its final target.


The authors declare no conflict of interest.

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1To whom correspondence may be addressed. Email: phhuang@ntu.edu.tw or hjcheng@ucdavis.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1804373115/-/DCSupplemental.

Published online August 13, 2018.
C. elegans C. elegans C. elegans C. elegans max-1 subunit of AP-3 complex. Binding un-5 demonstrates that AP-3 is re-

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to Regulate gei-17 and Functions Upstream of

Isolation of GEI-17/PIAS1 and APB-3 as MAX-1

no. 35

vol. 115

PNAS

Results

play important roles in regulating the trafficking and degradation

results suggest that MAX-1 SUMOylation and the AP-3 complex

axons requires SUMOylated MAX-1 and APB-3. Together, our

lected sorting of vesicles mainly in the endolysosomal pathway

essential subunit of the tetrameric AP-3 complex, which regu-

UNC-5

mediated

PIAS1 suggests MAX-1 could be a SUMOylation substrate

The cytoplasmic region of UNC-5 contains a zona pellucida

UNC-5 (ZUS) domain, a DCC-binding (DB) domain, and a

defeated mainly in the endolysosomal

gei-17

gene; serine- and threonine-rich; SAP, SAF-A/B, acinus and PIAS. (E) Schematic diagrams of the cytoplasmic region of UNC-5 and the ZUS bait for the cytoplasmic region of UNC-5 contains a zona pellucida

effectors, including chromosome congression and telomere

report in C. elegans further demonstrates that AP-3 is re-

required for differential targeting of transmembrane proteins

Here we report that UNC-5 interacts with APB-3 and that

SUMOylated MAX-1 requires APB-3 to affect UNC-5-mediated

axon repulsion. UNC-5 is degraded mainly in the endolysosomal

UNC-5 could be a cargo regulated by AP-3 for some sort of

(27, 29), the interaction of UNC-5 with APB-3 suggests that

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protein interaction sites further revealed that a potential AP-

broad complex is confirmed by coimmunoprecipitation and subcellular colocalization in
cotransfected COS cells (Fig. 1 F, G, and H) and by in vitro GST pull-down assay (Fig. 1 G). Mapping for protein–protein interaction sites further revealed that a potential AP-binding dileucine motif of the ZUS domain is responsible for

UNC-5’s binding with APB-3 (Fig. 1 H). Given that APB-3 is an essential subunit of the tetrameric AP-3 complex, which regu-
lates the sorting of vesicles mainly in the endolysosomal pathway

(27, 29), the interaction of UNC-5 with APB-3 suggests that

UNC-5 could be a cargo regulated by AP-3 for some sort of intracellular vesicular trafficking.

gei-17 Functions Upstream of max-1 to Regulate unc-5-Mediated

Axon Repulsion. In C. elegans, gei-17 is involved in various cellular processes, including chromosome congression and telomere

Fig. 1. Isolation of GEI-17/PIAS1 and APB-3 as MAX-1 and UNC-5-interacting proteins, respectively. (A) Schematic diagrams of the full-length mouse MAX-1 and the bait for yeast two-hybrid screen, MAX-1 contains two PH domains, an MyTH4 domain, and a FERM domain. The bait consists of LexA fused to the C terminus of MAX-1, FERM, band 4.1/ezrin/radixin/moesin; MyTH4, myosin tail homology 4; PH, pleckstrin homology. (B) Immunoblots show that GFP-MAX-1 coimmunoprecipitates with PIAS1. In comparison, GFP-mock did not form protein complex with PIAS1. (C) The C terminus of MAX-1, but not the N terminus, binds directly to GEI-17 in the GST pull-down assay. Note that the loading input is 5% of the total protein lysate. (D) Domain mapping by yeast two-hybrid assay indicates that the N-terminal half of PIAS1, which includes SAP and RING finger domains, is required for its interaction with MAX-1. Shown is a strip of representative X-gal reactions for yeast cells transformed with the indicated construct prey plus the MAX-1 C terminus bait. RING, really interesting new gene; S/T, serine- and threonine-rich; SAP, SAF-A/B, acinus and PIAS. (E) Schematic diagrams of the cytoplasmic region of UNC-5 and the ZUS bait for two hybrid screen. DB, DCC-binding; DD, death domain; ZUS, zona pellucida UNC-5. (F) APB-3, not the mock-control, is detected in the protein complex immunoprecipitated by MYC-tagged UNC-5. Asterisks indicate the mCherry-mock and mCherry-APB-3 bands in the lysates. (G) The cytoplasmic region of UNC-5 binds directly to the purified APB-3 in the in vitro GST pull-down assay. The loading input is 5% of the total protein lysate and the GST-UNC-5 cytoplasmic band in the input is indicated by an asterisk. (H) Domain mapping by yeast two-hybrid assay shows the UNC-5 ZUS domain is sufficient for its interaction with APB-3. Mutations of both amino acids L(524)I(525) to A(524)A(525) in the ZU5 domain (asterisk) disrupt the interaction. A representative strip of X-gal reactions is shown for yeast cells transformed with indicated UNC-5 cytoplasmic construct tagged with LexA plus APB-3 full-length fused to GAL4AD.
Fig. 2. C. elegans gei-17 plays a role in the dorsal guidance of motor commissural axons. (A) gei-17 is expressed in developing ventral cord motor neurons. At L1 stage, monomeric RFP driven by unc-25 promoter is expressed strongly in DD neurons. GFP expression driven by the gei-17 promoter is observed in the same neurons. Anterior is to the left and dorsal is up. (Scale bar: 5 μm.) (B) Some motor commissural axons are misruided in gei-17(tm2723) mutants (arrow) in the jil676(punc-25::GFP) background. (Scale bars: 20 μm.) (C) Quantification of axon guidance defects in gei-17, max-1, and max-2 mutants. gei-17 mutants exhibit mild guidance defects, and the mutation does not enhance effects of the max-1 mutation. However, the defects of max-2 mutants are significantly enhanced by the gei-17 mutation. (D) Quantification of genetic interactions between gei-17 and max-1 with or without SUMOylation. SUMOylation mimic WT max-1 cDNA (Ex Punc-25::MAX-1;SMO-1) is able to rescue the axon guidance defects enhanced by RNAi knockdown of gei-17 in a max-2(cy2)-sensitized background. Mammalian PIAS1 (Ex Punc-25::PIAS1) cell-autonomously rescues the defects enhanced by RNAi knockdown of C. elegans gei-17. RNAi knockdown of NIL or col-86 served as controls. eri-1(mg366) enhances RNAi effect in neurons. (E) RNAi knockdown of each known gene involved in the SUMOylation pathway in C. elegans by soaking. Knocking-down of any of these genes significantly enhances the defects caused by max-2 mutant. For C–E, n = 21–64. Error bars indicate SEMs. n.s., no significant difference by Student’s t test; *P < 0.05; **P < 0.01; ***P < 0.001.

position in early embryos, DNA damage response, and development of pharyngeal muscle (30–33). However, whether GEI-17 functions in the development of the C. elegans nervous system has not been investigated. We showed that transgenic gei-17 promoter GFP was expressed in the developing and adult C. elegans motor neurons, which started as early as the threefold stage (Fig. 2A and SI Appendix, Fig. S1B). In addition, mild axon guidance defects were observed in a gei-17 mutant (tm2723) or a worm with gei-17 RNAi knockdown (Fig. 2B and C), indicating that gei-17 plays a role in axon guidance.

We previously showed that max-1 and max-2 acted via parallel rac-independent and -dependent genetic pathways in UNC-5-mediated axon repulsion (34–36). Genetic interaction analysis revealed that gei-17 did not enhance max-1’s axon guidance defect in max-1/gei-17 double mutants, but max-2’s defect was dramatically enhanced by gei-17 in max-2/gei-17 double mutants (Fig. 2C). This finding suggests gei-17 is likely to act in the max-1-mediated pathway, but in parallel to the rac-dependent pathway involving genes like max-2 and ced-10 (34). Taking advantage of this result, we performed several rescue and enhancement experiments in a sensitized background using rac pathway mutants such as max-2 or ced-10 to significantly enhance the axon guidance defects of gei-17, which was relatively weak by itself (SI Appendix, Fig. S1C). In the sensitized max-2 mutant background, the defects caused by gei-17 RNAi knockdown were significantly rescued by expressing a gei-17/PIAS1 cDNA specifically in motor axons under the unc-25 promoter (Fig. 2D). This result indicates...
that gei-17/PIAS1 is involved in axon repulsion in a cell-autonomous manner.

Because GEI-17 is a SUMOylation E3 ligase, we next asked if MAX-1 was its substrate by testing whether the defects caused by gei-17 knockdown were rescued by SUMOylated MAX-1. The function of a SUMOylated protein can be mimicked by fusing SUMO protein to its C terminus (37-39). We generated a SUMOylation mimetic max-1 construct by fusing the C. elegans SUMO gene smo-1 to max-1 (max-1::smo-1). In the max-2 mutant background, expressing the SUMOylation mimetic max-1, but not the WT max-1, significantly suppressed the axon guidance defect caused by gei-17 RNAi knockdown (Fig. 2D), suggesting that SUMOylated MAX-1 can bypass the requirement for gei-17 in axon repulsion. Accordingly, we conclude that gei-17 acts upstream of max-1 in axon guidance by facilitating SUMOylation.

**SUMOylation of MAX-1 Is Required in UNC-5-Mediated Axon Repulsion.** In addition to the specific substrate-recognition E3 ligases, the common components of SUMOylation pathway in *C. elegans* include the SUMO gene smo-1, the E1-activating enzymes uba-2 and aos-1, and the E2 conjugating enzyme ubc-9. Using RNAi to eliminate any of these SUMOylation pathway component genes results in embryonic lethality (37, 40). To address whether the SUMOylation pathway is involved in motor axon guidance, we performed a weak RNAi knockdown

**Fig. 3.** SUMOylation of MAX-1 is required for its function in the guidance of motor commissural axons in *C. elegans*. (A) A schematic diagram of candidate SUMOylation sites of *C. elegans* MAX-1 protein predicted by SUMOplot. FERM, band 4.1/ezrin/radixin/moesin; MyTH4, myosin tail homology 4; PH, pleckstrin homology. (B and C) Quantification of the ability of MAX-1 SUMOylation site mutant variants to rescue the axon guidance defects in sensitized max-1;ced-10 mutant background. Amino acid K-to-R mutations of the predicted SUMOylation sites were introduced into max-1 cDNA under unc-25 promoter. These mutant max-1 cDNA constructs were injected into max-1;ced-10 double-mutant worms to assess their ability to rescue the axon-guidance defects. Among all single SUMOylation site mutations, only max-1(K476R) and max-1(K784R) are unable to fully rescue the defects as WT max-1 (B). The failure-to-rescue effects of max-1(K476R) or max-1(K784R) can be restored by SUMOylation mimetic max-1(K476R)::smo-1 or max-1(K784R)::smo-1 but not by ubiquitination mimetic max-1(K476R)::ubq-1 or max-1(K784R)::ubq-1 (B). Multiple K-to-R mutations (up to all six) of the predicted SUMOylation sites do not change the effects caused by max-1(K476R) or max-1(K784R) single mutation (C). The SUMOylation mimetic max-1(K476R)(K784R)::smo-1, but not the ubiquitination mimetic max-1(K476R)(K784R)::ubq-1, can rescue the defects as does the WT max-1 (C). Shown here are combined data from at least three independent generated transgenic lines. The defects were quantified and compared using transgenic animals and their nontransgenic siblings. n = 26–56. Error bars indicate SEMs. n.s., no significant difference by Student’s t test; ***P < 0.001. (D–F) Immunoblots of in vitro SUMOylation reactions of the recombinant proteins GST-MAX-1(330–1099), GST-MAX-1(330–616), and GST-MAX-1(550–1099). MAX-1 is SUMOylated in vitro in a GEI-17 concentration-dependent manner (D). The SUMOylated GST-MAX-1(330–1099) proteins (indicated by a bracket) are not present without E1 and E2 ligase enzyme or with a SUMO-specific protease SENP-1 (D). K476R mutation in GST-MAX-1(330–616) attenuates SUMOylation of GST-MAX-1 fusion proteins; the bracket indicates SUMOylated GST-MAX-1(330–616) proteins (E). K784R mutation in GST-MAX-1(550–1099) does not attenuate SUMOylation of GST-MAX-1 fusion proteins; the bracket indicates SUMOylated GST-MAX-1(550–1099) proteins (F). Arrowheads in all panels indicate un-SUMOylated GST-MAX-1 fusion proteins. rxn, reaction.

**Legend:**

- **A:** MAX-1 full-length
- **B:** SUMOylation site (predicted)
- **C:** Transgenic animals
- **D:** SUMOylation rxn mixture
- **E:** SUMOylation rxn mixture
- **F:** SUMOylation rxn mixture
- **WB:** GST

**Supplemental Figure 2D:** SUMOylation of MAX-1 by SUMO-specific E3 ligase GEI-17. SUMOylation rxn mixture

**Supplemental Figure 3C:** SUMOylation of MAX-1 by SUMO-specific E3 ligase GEI-17. SUMOylation rxn mixture

**Supplemental Figure 3D:** SUMOylation of MAX-1 by SUMO-specific E3 ligase GEI-17. SUMOylation rxn mixture

**Supplemental Figure 3F:** SUMOylation of MAX-1 by SUMO-specific E3 ligase GEI-17. SUMOylation rxn mixture

**Supplemental Figure 3G:** SUMOylation of MAX-1 by SUMO-specific E3 ligase GEI-17. SUMOylation rxn mixture

**Supplemental Figure 3H:** SUMOylation of MAX-1 by SUMO-specific E3 ligase GEI-17. SUMOylation rxn mixture
The axon guidance mediated by SUMOylated MAX-1 requires apb-3. (A) apb-3 is expressed in developing ventral cord motor neurons. At L1 stage, the Pab-3::GFP (green) is coexpressed with monomeric RFP driven by unc-25 promoter (red) in DD neurons. (Scale bar: 5 μm.) Anterior is to the left and dorsal is up. (B) Quantification of axon guidance defects in apb-3, max-1, and max-2 mutants. apb-3 mutant by itself does not show axon-guidance defects, but it significantly enhances the defects of the max-2, but not the max-1, mutant. The apb-3;max-2 defects can be cell-autonomously rescued by Punc-25::APB-3::GFP. (C) Representative images of motor commissural axon-guidance defects observed in apb-3;max-2 (Upper images) and apb-3;max-1 (Lower image) double mutants. Defects are indicated by arrowheads. (Scale bars: 20 μm.) (D) Quantification of axon-guidance defects caused by RNAi knockdown of each component of C. elegans AP-3 complex in the sensitized promoter. The transgenic animals C promoter (red) in DD neurons. (Scale bar: 5 μm) mutants to generate enhanced RNAi effect in were expressed in sensitized mutant background. RNAi knockdown of each of these genes, which include genes encoding subunits, significantly enhances the defects caused by max-2 mutant alone. eri-1(mg366) enhances RNAi effect in neurons; col-86 is a control. (E) Quantification of genetic interactions between apb-3 and SUMOylated MAX-1. max-1(K476R) and SUMOylation mimetic max-1(K476R)(K784R)::smo-1 were expressed in sensitized max-1;ced-10 double-mutant background under unc-25 promoter. The transgenic animals were then crossed into apb-3 mutants to generate max-1;ced-10;apb-3 triple mutants. The defects were quantified and compared using the transgenic animals and their nontransgenic siblings. For B, D, and E, n = 36–64. Error bars indicate SEMs. n.s., no significant difference by Student’s t test; *P < 0.05; **P < 0.01; ***P < 0.001.

by soaking worms in diluted double-stranded RNA to avoid lethality. In the sensitized max-2 mutant background, weak RNAi knockdown of any of the SUMOylation pathway component genes significantly enhanced the axon-guidance defect caused by max-2 mutation alone (Fig. 2E). While further cell-autonomous experiments are necessary to demonstrate direct regulations of these SUMOylation pathway genes, these data together are consistent with the idea that the SUMOylation pathway is involved in motor axon guidance.

Six lysine residues in the MAX-1 protein are predicted to be SUMOylation sites (www.abgent.com/sumoplot) (Fig. 3A). To determine which of these lysine residues are important for its function, we generated various max-1 cDNA mutant constructs with lysine (K) mutated to arginine (R) at these candidate sites. Each mutant’s function was then evaluated in a sensitized max-1;ced-10 double-mutant background (35). WT max-1 rescued the axon guidance defect of the max-1;ced-10 double mutant by reducing the 70% failure rate to 20%. Among the six max-1 constructs with a single K-to-R mutation, only max-1(K476R) or max-1(K784R) was unable to significantly rescue the defects, compared with WT or other mutants (Fig. 3B). However, the SUMOylation mimic max-1(K476R)::smo-1 or max-1(K784R)::smo-1 regained the ability to rescue the defects as the WT max-1 did, indicating that K476R or K784R is essential for MAX-1 SUMOylation (Fig. 3B). As a control, the max-1(K476R) or max-1(K784R) fused with the C. elegans ubiquitin gene ubq-1 did not have the same effects (Fig. 3B). Interestingly, multiple point mutation constructs from double up to sextuple did not further change the effects observed in either K476R or K784R alone (Fig. 3C). These results together indicate that K476 and K784 are required for MAX-1-mediated axon guidance, and that these two sites are in the same SUMOylation genetic pathway.

An in vitro SUMOylation assay on GST-fused MAX-1 peptide (amino acid residues 330–1099; labeled as GST-MAX-1(330–1099)) confirmed that MAX-1 was SUMOylated in the presence of GEI-17 in a concentration-dependent manner and this SUMOylation effect was eliminated by adding the SUMO-specific protease SENP-1 (Fig. 3D). When GST-MAX-1(330–1099) was further split into two peptides—GST-MAX-1(330–616), which contains K476, and GST-MAX-1(550–1099), which contains K784—both could be SUMOylated in vitro (Fig. 3 E and F). However, when the MAX-1 peptide with either K476R or K784R mutation was tested, SUMOylated MAX-1(330–K476R) peptide was undetectable (Fig. 3E), but SUMOylated MAX-1(550–K784R) peptide was detected (Fig. 3E).
SUMOylation modifies protein structure and can change its ability to interact with other proteins (41). We previously reported that MAX-1 did not seem to interact with UNC-5 (10). However, by adjusting the coimmunoprecipitation condition, we were able to demonstrate that MAX-1 interacted with UNC-5, as they reciprocally coprecipitated in the precipitation condition, we were able to demonstrate that MAX-1 interacted with UNC-5, as they reciprocally coprecipitated.
result might have occurred because overexpressed MAX-1 is modified by SUMOylation in COS cells, so that the interaction was too unstable to detect. We tested this hypothesis by using SUMOylation-mimetic MAX-1 and found that SUMOylated MAX-1 significantly lost its binding affinity to UNC-5 (Fig. 6B).

Domain mapping analysis revealed that the ZU5 domain of UNC-5, which is responsible for its interaction with APB-3 (Fig. 1H), also interacted with MAX-1 (SI Appendix, Fig. S3A), suggesting that APB-3 might compete with MAX-1 for interaction with UNC-5. Indeed, overexpressing APB-3 significantly interfered with the binding of MAX-1 to UNC-5 (Fig. 6C). APB-3 seemed to have higher affinity for UNC-5 than did MAX-1, as a small amount of APB-3 expression is sufficient to achieve maximal interference with UNC-5’s binding to MAX-1 (Fig. 6C).

We further demonstrated that using Brefeldin A to deplete the insertion of AP-3 complex into intracellular vesicular membrane increased colocalization of MAX-1 with UNC-5 in the cytoplasmic vesicles of transfected COS cells (SI Appendix, Fig. S3B) and enhanced protein complex formation between MAX-1 and UNC-5 despite the presence of APB-3 (Fig. 6D). This result suggests that the AP-3 complex on the intracytoplasmic vesicles can specifically interfere with binding between MAX-1 and UNC-5. Together, these results indicate that the interaction between UNC-5 and MAX-1 is modulated by MAX-1 SUMOylation and by the level of AP-3 complex in the intracellular vesicles.

Genetic interaction analysis indicates max-1 is upstream of apb-3 in regulating unc-5–mediated axon repulsion, which raises the possibility that MAX-1 might mediate UNC-5 degradation through APB-3. We found that MAX-1 and APB-3 did not interact with each other biochemically (SI Appendix, Fig. S3D). MAX-1 alone was degraded mainly through the endolysosomal pathway, given that the lysosome blocker NH4Cl but not the proteasome inhibitor MG132 prevented MAX-1 from degradation (SI Appendix, Fig. S3C). In addition, the turnover time of UNC-5 was not affected by overexpressing MAX-1, SUMOylation mutant MAX-1(K476R)(K784R), or SUMOylation-mimetic MAX-1 (SI Appendix, Fig. S2C). Likewise, the degradation of MAX-1 was not affected by coexpressed UNC-5 either (SI Appendix, Fig. S3D).
Regulation of UNC-5 receptor trafficking in C. elegans axons in vivo requires APB-3 and SUMOylated MAX-1. (A) Schematic diagrams to show the punctated (red dots) expression pattern of UNC-5::mRFP in a C. elegans motor axon (Left) and the FRAP experimental procedures (Right). The approximate axon segment shown in images in B is indicated in the boxed area. (B) Representative FRAP time-course images showing that the recoveries of UNC-5::mRFP puncta after photobleaching (oval areas) are impaired in max-1 and apb-3 mutant backgrounds. The impaired recoveries of UNC-5::mRFP puncta in the max-1 mutant background are cell-autonomously rescued by SUMOylation mimetic max-1 [MAX-1(K476R)(K784R)::SMO-1], but not by SUMOylation-mutated max-1 [MAX-1(K476R)] alone, or max-1 [MAX-1(K476R)(K784R)]; SUMO-1; apb-3. (Scale bar: 5 μm.) (C) The average FRAP recovery profiles from each set of experiments are shown as scattered line plots. The P values shown at t = 30 min were calculated by one-way ANOVA with Holm–Sidak post hoc comparison. n ≥ 3. Error bars indicate SDs. *P < 0.05; **P < 0.01.

Appendix, Fig. S3E). These results suggest that, while UNC-5 forms a protein complex with MAX-1, the degradation of UNC-5 and MAX-1 in lysosomes is independently regulated.

Both SUMOylated MAX-1 and APB-3 Regulate UNC-5 Trafficking Along the Axons. The AP-3 complex is involved not only in regulating protein degradation but also in sorting and trafficking intracellular vesicles (22, 23, 28, 42). To address how MAX-1 and APB-3 regulate the trafficking of vesicles carrying UNC-5 receptor, we generated a transgenic line expressing monomeric RFP-tagged UNC-5 (UNC-5::mRFP) in C. elegans motor axons. As shown previously (8), UNC-5::mRFP accumulated as puncta along the motor axons (Fig. 7A). In max-1 or apb-3 mutants, punctated UNC-5::mRFP was still observed, indicating that UNC-5 receptors were transported along axons in these mutants. We then performed fluorescence recovery after photobleaching (FRAP) experiments to access the dynamics of UNC-5 receptor trafficking along axons. In unc-5 mutants that were rescued by specifically expressing UNC-5::mRFP in motor neurons (producing WT motor axons), 43% recovery of fluorescent intensity was observed at 30 min after photobleaching (Fig. 7B and C). However, when these transgenic worms were in the max-1 mutant background, the recovery was significantly reduced to 4%. Similarly, in the transgenics under apb-3 mutant background, only 9% recovery was observed (Fig. 7B and C). Therefore, both max-1 and apb-3 are involved in regulating the dynamics of UNC-5 transportation along the axon in vivo.

We next performed FRAP in these transgenics under max-1 mutant background but expressing either max-1 [K476R] or max-1 [K784R]; SMO-1. As shown in Fig. 7B and C, max-1 [K476R]; SMO-1 rescued the recovery rate of fluorescent intensity significantly better than max-1 [K476R] (32% vs. 6%; P = 0.029). Altogether, these data indicate that the trafficking of UNC-5 receptor in the axon is regulated by both SUMOylated MAX-1 and APB-3.

Discussion

SUMOylation of MAX-1 Regulates UNC-5-Mediated Axon Repulsion. In this study, we identified the SUMOylation E3 ligase GEI-17/PIAS1 as a regulator of MAX-1 and demonstrated that MAX-1
SUMOylation is critical for UNC-5-mediated axon repulsion. Detailed molecular, genetic, and biochemical analysis revealed that MAX-1 acts as a dynamic regulator of the UNC-5 receptor: MAX-1 constitutively binds to UNC-5 receptor in axons but dissociates from UNC-5 when MAX-1 is SUMOylated. This dynamic switch regulates the trafficking and degradation of UNC-5 receptor.

Our results show that SUMOylation of the conserved K476 amino acid in MAX-1 is essential for its function. Another amino acid, K784, is also required for MAX-1’s function genetically, but biochemical evidence suggests it is not a primary SUMO acceptor site. Because SUMOylation-mimetic MAX-1(K784R):sumo-1 rescues the axon-guidance defect caused by max-1 mutation, K784 might have a modulatory role in MAX-1’s function. Post-translational modifications such as phosphorylation, ubiquitination, and acetylation can modify a potential SUMO acceptor site to change its SUMOylation status (13, 14). Such intramolecular “cross-talk” between posttranslational modifications has been reported for several SUMOylated proteins (13, 43, 44). It will therefore be interesting to know how the SUMO acceptor sites of MAX-1 are regulated by other posttranslational modifications.

Another important question to be addressed is how the SUMOylation of E3 ligase GEI-17 is activated. Our current data do not provide information on the activation of GEI-17, although we know that both the get-17 gene and SUMOylation of MAX-1 are required for normal function of UNC-5. It remains to be investigated whether the signaling of the UNC-5 receptor activated by UNC-6 triggers the activation of GEI-17 and the subsequent SUMOylation of MAX-1.

AP-3 Routes UNC-5 Degradation Through the Lysosomal Pathway and Modulates UNC Receptor Trafficking Along the Axon. The AP-3 complex is one of the intracellular coat protein complexes that function as adaptors for vesicular sorting (23, 27, 29). We found that AP-3 complex directs the UNC-5 receptor to the lysosomal pathway for accelerated turnover. In cultured mammalian neurons, UNC-5 receptors are internalized by endocytosis upon stimulation of UNC-6 (6). We therefore envision that an increase in UNC-5–containing vesicles sorted by AP-3 for degradation in lysosomes can provide a mechanism for shutting down the UNC-5 signaling activated by UNC-6 during axon guidance.

In our in vivo FRAP study suggests that the AP-3 complex is involved in regulating the trafficking of UNC-5 receptor in axons, which also requires SUMOylated MAX-1. However, this approach does not address the nature of UNC-5–associated vesicles and the directionality of UNC-5 transportation along the axon. Neither does it address whether the control of such trafficking is related to UNC-5 degradation. Nevertheless, given our genetic data showing apb-3 is required for max-1 to regulate UNC-5–mediated axon repulsion, we suspect that AP-3 acts via degradation and protein trafficking, as well as its coordination with MAX-1, in the dynamic control of UNC-5 receptors during axon guidance.

The regulation of UNC-5 receptor by AP-3 complex is likely to involve other molecular mechanisms. AP-3, like other AP adaptor complexes, recognizes a conserved sorting motif, the dileucine motif (D/E)XX(L/I), on vesicle cargo proteins and facilitates the assembly and sorting of vesicles by binding the vesicular trafficking machinery, such as BLOC-1 and HOPS (27). Phosphorylated (S/T)XXX(L/I) mimics the dileucine sorting motif (D/E)XX(L/I) for binding adaptor protein complexes (45–48). An (S/T)XX(L/I) motif can be identified in the ZU5 domain of UNC-5 (amino acid 520–525). We demonstrate here that the ZU5 domain binds APB-3 directly and that mutations of the amino acids L(524)l(525) to A(524)A(525) disrupt the interaction between UNC-5 and APB-3 (Fig. 1G). Thus, we reason that, after phosphorylation, the (S/T)XXX(L/I) motif on the ZU5 domain of UNC-5 can function as an AP-3 binding dileucine motif. As several protein kinases or phosphatases are involved in UNC-5–mediated axon guidance (8, 9, 35, 49, 50), it is likely that the interaction of AP-3 with UNC-5 is activated by regulated phosphorylation.

A Model for SUMOylated MAX-1 and APB-3 in the Regulation of UNC-5 Receptors During Axon Repulsion. Previous studies have clearly demonstrated that the ZU5 domain of UNC-5 receptor is crucial for axon guidance (51–53). Here we show that MAX-1 and APB-3 competitively bind the ZU5 domain of UNC-5 and together they regulate the guidance of C. elegans motor axons. Genetically, max-1 acts upstream of apb-3 in the unc-5–mediated axon repulsion. Both max-1 and apb-3 are required for UNC-5 receptor trafficking in axons. In addition, our biochemical analysis indicates that APB-3 has stronger binding affinity to UNC-5 than does MAX-1 and that SUMOylated MAX-1 weakens its binding with UNC-5. Although APB-3 facilitates UNC-5 degradation, MAX-1, with or without SUMOylation, does not affect the degradation of UNC-5 regulated by APB-3.

We therefore propose the following model (SI Appendix, Fig. S4): MAX-1 is a dynamic regulator of UNC-5 receptor in the axon. MAX-1 constitutively binds to UNC-5 receptor during axonal development. When the SUMOylation of E3 ligase PIAS1/GEI-17 is activated, either through ligand binding to UNC-5 receptor or via other unidentified mechanisms, MAX-1 is SUMOylated. As a consequence, UNC-5 receptor is dissociated from SUMOylated MAX-1, favoring more interaction between UNC-5 receptor and other molecules such as APB-3. Thus, MAX-1 acts as a modulatory molecular switch to regulate UNC-5 receptor’s intracellular interactions. In the presence of AP-3 complex, after dissociating from SUMOylated MAX-1, UNC-5 receptor can be sorted for trafficking in the axon and/or routed for endolysosomal degradation. Without MAX-1, as in the max-1 mutant, the trafficking and degradation of UNC-5 receptors are dysregulated, resulting in axon-guidance defects. Overexpressing unc-5, which presumably provides more available surface UNC-5 receptors, can thus partially rescue the axon guidance defects in max-1 mutants (10).

Guiding axons through concentration gradients of environmental cues is an essential mechanism for forming a proper neuronal connection network. During axon repulsion, the growing axons migrate by sensing concentration differences of the guidance cues. Because the concentration differences along the gradient are constantly changing, the navigating growth cone has to actively regulate its response so that it can move directly away from the guidance cue (54, 55). Previous studies have demonstrated regulated interactions of ligands (UNC-6 and UNC-129) and receptors (UNC-5 and UNC-40) are essential for dorsal repulsion of motor axons. The growth cone is initially repelled by a high concentration of UNC-6 through UNC-5 receptor alone, but when the growth cone moves dorsally and the concentration of UNC-6 in the environment becomes low, the UNC-5 receptor needs both UNC-129 and UNC-40 to properly guide the axons (5). However, these studies did not address an alternative mechanism by which the UNC-5 receptor itself is regulated (56).

Lysosomal degradation of proteins can occur in the growth cone locally (57). If UNC-6 binding to UNC-5 could trigger PIAS1/GEI-17 activation, our model (SI Appendix, Fig. S4) could provide a potential alternative for how UNC-5 receptor is regulated locally when the growth cone is moving away from the UNC-6 gradient. Regulated degradation of surface receptors in response to a morphogen concentration gradient would lead to a dampened response downstream of the receptors in the high-concentration morphogen field but a heightened response in the low-concentration field (58). Consistent with this effect, our model (SI Appendix, Fig. S4) predicts that the surface availability of UNC-5 is increased at low UNC-6 concentration due to less activation of PIAS1/GEI-17 and thus less UNC-5 degradation. In addition, in a model predicting the variability and the reliability of biological response toward a diffusible morphogen concentration gradient (59), changes in available receptors would result.
in a shift in amount of surface receptor occupancy, which reduces the accuracy of the response where the ligand concentration is low. Based on our findings that MAX-1 and AP-3 regulate the trafficking and degradation of UNC-5 receptor, we can predict that the guidance of axon is most likely to be affected where the concentration of UNC-6 is low. Consistent with this prediction, misguided DA and DB motor axons turn prematurely only in the dorsal half of max-1 mutants, where UNC-6 is low (10).

Materials and Methods
Details are provided in SI Appendix, SI Materials and Methods, including detailed methods for yeast two-hybrid screen, C. elegans RNAi experiments, C. elegans phenotypic analysis, FRAP experiments, in vitro SUMOylation assay, in vitro binding assay, cell culture and transfection, fluorescence microscope, immunoblotting, pulse-chase experiments, coimmunoprecipitation, and statistical analysis. The information about C. elegans strains and the generation of plasmids and transgenic constructs are also described in SI Appendix, SI Materials and Methods.

ACKNOWLEDGMENTS. We thank the International C. elegans Gene Knock-out Consortium for strains. We are grateful to Yuji Kohara, Ken-Ichi Oguira, Yoshiho Goshima, Gary Ruvkun, Andy Fire, and Lindsay Hinck for materials. We thank Li-Ting Jang and Fang-Jen Lee for their contributions in the initial stage of AP-3 studies, and we thank Jianhong Chen for technical help.

We also thank Abraham Noor-Bakhsh, Kimberly Zhou, and Chin-Min Ho for technical help and Noelle Etoile, Ting-Wen Cheng, Damien O’Halloran, Scott Hamilton, Bi-Tzeng Juang, the members of the H.-J.C. and P.-H.H. labs, and the members of the University of California, Davis (UC Davis) “Super Worm Group” for comments and advice. This work was supported in part by a Health System grant from UC Davis (to H.-J.C.) and a grant from National Taiwan University College of Medicine (to P.-H.H.).