Regulation of axon repulsion by MAX-1 SUMOylation and AP-3

Shih-Yu Chen, Chun-Ta Ho, Wei-Wen Liu, Mark Lucanic, Hsiu-Ming Shih, Pei-Hsin Huang, and Hwai-Jong Cheng

*Center for Neuroscience, University of California, Davis, CA 95618; ‡Graduate Institute of Pathology, College of Medicine, National Taiwan University, Taipei 10048, Taiwan; †Institute of Biomedical Sciences, Academia Sinica, Taipei 11529, Taiwan; and ‡Graduate Institute of Mind and Brain Sciences, College of Medicine, National Taiwan University, Taipei 10048, Taiwan

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 interactor with UNC-5. Overexpression of APB-3 reorients 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.

Max-1 and SUMOylation

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 dorsal guidance 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/Natrin 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 Ephrin3b 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.

Author contributions: P.-H.H. and H.-J.C. designed research; S.-Y.C., C.-T.H., W.-W.L., M.L., H.-M.S., P.-H.H., and H.-J.C. performed research; S.-Y.C., C.-T.H., W.-W.L., M.L., H.-M.S., P.-H.H., and H.-J.C. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission. K.S. is a guest editor invited by the Editorial Board.

This open access article is distributed under Creative Commons Attribution-NonCommercial-NoDerivatives License 4.0 (CC BY-NC-ND).

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.
report in *C. elegans* further demonstrates that AP-3 is required for differential targeting of transmembrane proteins into axons (28).

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 compartment when APB-3 is overexpressed, and the interaction of UNC-5 and MAX-1 is significantly reduced in the presence of APB-3. We also show that the trafficking of UNC-5 receptors in axons requires SUMOylated MAX-1 and APB-3. Together, our results suggest that MAX-1 SUMOylation and the AP-3 complex play important roles in regulating the trafficking and degradation of UNC-5 receptors during axon guidance.

**Results**

**GEI-17/PIAS1 and APB-3 Interact with MAX-1 and UNC-5, Respectively.**

In a yeast two-hybrid screen using the C terminus of mouse MAX-1 ortholog as bait (Fig. 1A), we isolated 16 independent positive colonies. Among these, two encoded fragments of mouse protein inhibitor of activated STAT-1 (PIAS1). The interaction of MAX-1 and PIAS1 was confirmed by coimmunoprecipitation and subcellular colocalization in mammalian cells. The cytoplasmic region of UNC-5 contains a zona pellucida–mediated death domain (DD) (Fig. 1E). Because bait consisting of the entire UNC-5 cytoplasmic domain was self-activating, only the N-terminal half, which includes SAP and RING finger domains, is necessary and sufficient for PIAS1’s interaction with MAX-1 (Fig. 1D). The direct binding of MAX-1 to GEI-17/PIAS1 suggests MAX-1 could be a SUMOylation substrate regulated by GEI-17/PIAS1.

The cytoplasmic region of UNC-5 contains a zona pellucida UNC-5 (ZUS) domain, a DCC-binding (DB) domain, and a death domain (DD) (Fig. 1E). Because bait consisting of the entire UNC-5 cytoplasmic domain was self-activating, only the ZUS domain was used as bait (labeled as UNC-5-ZUS) in the yeast two-hybrid screen for UNC-5 interacting molecules, which identified APB-3, the β subunit of AP-3 complex. Binding of UNC-5 with APB-3 into a protein complex was confirmed by communoprecipitation and subcellular colocalization in cotransfected COS cells (Fig. 1F and SI Appendix, Fig. S1A) and by in vitro GST pull-down assay (Fig. 1G). Mapping for protein–protein interaction sites further revealed that a potential AP-binding dileucine motif of the ZU5 domain is responsible for UNC-5’s binding with APB-3 (Fig. 1H). Given that APB-3 is an essential subunit of the tetrameric AP-3 complex, which regulates the sorting of vesicles mainly in the endosomal 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...
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 motor 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

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. (B) gei-17 promoter GFP was expressed in the developing and adult C. elegans nervous system. At adult 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. (C) Failure to reach dorsal cord was measured. Scale bars: 20 μm. (D) 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. (E) Quantification of genetic interactions between gei-17 and max-1 with or without SUMOylation. SUMOylation mimetic 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. gei-17 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.
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 mimic 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 mimic 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 MAX-1 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
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-2, 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 SERNP-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(K476R) peptide was undetectable (Fig. 3E), but SUMOylated MAX-1

Fig. 4. 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 Pabp-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 max-2 mutant background. RNAi knockdown of each of these genes, which include genes encoding β (apb-3), δ (apd-3), μ (apm-3), and ε (aps-3) 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)(K784R) and SUMOylation mimic 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.
We found that and mutants (subunit gene), RNAi knockdown apb-3;max-1;ced-10 but not in apb-3 δ max-1 double mutants, but the SUMOylation-mimetic MAX-1 is cell-autonomously involved in triple mutant, the SUMOylation-mimetic mutant was rescued by the expression mutation did not enhance the defect of aps-3 max-1;ced-10 phenotype en-

C. elegans apb-3(ok429) cDNA driven by the motor neuron-specific max-1 Functions Downstream of subunit δ subunits mutant, suggesting that max-2 motor neurons (Fig. 4 and and is required for SUMOylated MAX-1 to be accelerated. This effect depended on APB-3 concentration, mediated SUMOylation of MAX-1 is required for the function of a WT AP-3 complex is involved in the motor axon guidance (Fig. 4D).

To determine the genetic epistasis between apb-3 and max-1, we crossed apb-3(ok429) into max-1;ced-10 double mutants expressing either MAX-1 SUMOylation mutant MAX-1(K746R) (K784R) or SUMOylation-mimetic MAX-1(K476R)(K784R): SMO-1 (Fig. 4E). As shown earlier (Fig. 3C), MAX-1(K746R) (K784R) could only partially rescue the defects in the max-1;ced-10 double mutants, but the SUMOylation-mimetic MAX-1 (K746R)(K784R):SMO-1 significantly suppressed the defects, producing a similar phenotype to WT MAX-1. By contrast, in the apb-3(max-1;ced-10) triple mutant, the SUMOylation-mimetic MAX-1(K746R)(K784R):SMO-1 was unable to further suppress the defects compared with MAX-1(K476R)(K784R), indicating that apb-3 is required for SUMOylation MAX-1 to function properly. This effect was not due to the pleiotropic effect of apb-3, since apb-3 mutation did not enhance the defect of max-1;ced-10 double mutant and transgenic animals expressing MAX-1(K746R)(K784R) showed similar axon guidance defect with or without apb-3 mutation (Fig. 4E). Collectively, these data demonstrate that the APB-3-containing AP-3 complex acts downstream of SUMOylated MAX-1 to regulate motor axon repulsion.

The UNC-5 Receptor Is Routed to Lysosomes by Overexpressing APB-3. In cultured cortical neurons, UNC-5 was partially colocalized with a trans-Golgi marker (TGN p230) and lysosome-associated membrane protein 1 (LAMP-1). The AP-3 complex has previously been shown to reside in these organelles for cargo sorting and degradation (SI Appendix, Fig. S2 A and B) (22, 27), suggesting that UNC-5 might be degraded in the lysosome via AP-3 complex. To explore this possibility, we turned to COS cells where no endogenous MAX-1 or UNC-5 is expressed. The halflife of the turnover of UNC-5 overexpressed in COS cells was ~60 min, as determined by a cycloheximide-chase experiment (Fig. 5A). Either the proteasome inhibitor MG132 or the lysosome acidifier blocker bafilomycin A1 partially inhibited the degradation of UNC-5, suggesting that both the ubiquitin-proteasome system and the lysosomal degradation pathway are involved in the degradation of UNC-5 (Fig. 5B). Intriguingly, when UNC-5 was coexpressed with APB-3 in COS cells, UNC-5 degradation was accelerated. This effect depended on APB-3 concentration, with UNC-5 degradation seeming to saturate at higher APB-3 concentrations (Fig. 5C). In addition, when UNC-5 was coexpressed with APB-3, MG132 treatment no longer protected UNC-5 from degradation. By contrast, lysosome blockers, either BafA1 or NH4Cl, still inhibited UNC-5 degradation (Fig. 5D). These data suggest that UNC-5 is preferentially routed to the lysosomal degradation pathway in the presence of APB-3.

Interaction Between UNC-5 and MAX-1 Is Modulated by MAX-1 SUMOylation and APB-3. SUMOylation modifies protein’s surface 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 did interact with UNC-5, as they reciprocally coprecipitated each other in a protein complex (Fig. 6A). The previous negative

(K784R) peptide was still present (Fig. 3F), suggesting that K476 amino acid is the primary SUMO acceptor site of MAX-1 protein. Taken together, these data suggest that GEI-17 can SUMOylate MAX-1 at specific lysine sites and such GEI-17-mediated SUMOylation of MAX-1 is required for the function of MAX-1 in regulating dorsal guidance of motor axons.

**Apb-3 Functions Downstream of Max-1 to Regulate Unc-5-Mediated Axon Repulsion.** We found that apb-3 was also expressed in *C. elegans* motor neurons (Fig. 4A). Although the apb-3(ok429) mutant did not exhibit an obvious axon guidance defect, it significantly enhanced the defect in max-2 but not in max-1 mutants (Fig. 4 B and C), suggesting that apb-3, like gei-17, functions in the max-1-mediated signaling pathway. This max-2 phenotype enhancement caused by apb-3 mutant was rescued by the expression of a WT apb-3 cDNA driven by the motor neuron-specific unc-25 promoter (Fig. 4B). Thus, apb-3 is cell-autonomously involved in the max-1-mediated signaling pathway in motor neurons. AP-3 is a heterotetrameric complex composed of β, δ, μ, and σ subunits (27). In addition to apb-3 (the β subunit gene), RNAi knockdown of δ subunit gene apd-3, μ subunit gene aprm-3, or σ subunit aprs-3 similarly enhanced the defect of max-2 mutant, suggesting that AP-3 complex is involved in the motor axon guidance (Fig. 4D).

![Fig. 5. Overexpression of APB-3 regulates UNC-5 degradation.](image-url)
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. S2A).
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(K476R)(K784R)::SMO-1], but not by SUMOylation-mutated max-1 (MAX-1(K476R)(K784R); E8243). 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.

**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 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)(K784R) or max-1(K476R) or max-1(K476R)(K784R)::SMO-1. As shown in Fig. 7B and C, max-1(K476R) (K784R)::SMO-1 rescued the recovery rate of fluorescent intensity significantly better than max-1(K476R)(K784R) (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.
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):smoo1 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 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-5 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.

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)XXXL(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)XXXL(L/I) mimics the dileucine sorting motif (D/E)XXXL(L/I) for binding adaptor protein complexes (45–48). An (S/T)XXXL(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)I(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)XXXL(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 AP-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 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 growing 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-5 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 microscopy, immunoblotting, pulse-chase experiments, coimmunoprecipitation, and statistical analysis. The information about C. elegans strains and the generation of plasmids and transgene 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 Ogura, Yoshiro Goshima, Gary Ruvkun, Andy Fire, and Lindsey Hinck for materials. We thank Li-Ting Jang and Fang-Jen Lee for their contributions in the initial stage of this project. We thank Dr. Chao Min for technical help and Noelle L’Etoile, Ting-Wen Cheng, Damien O’Halloran, Scott Hamilton, Bi-Tszeng Juan, the members of the H.-J.C. and H.-L. 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.).

1. Kolodkin AL, Tessier-Lavigne M (2011) Mechanisms and molecules of neuronal wiring: A primer. Cold Spring Harb Perspect Biol 3:a001727.
2. Dong Y-CH, Chance RK, Bashaw GJ (2009) Axon growth and guidance: Receptor regulation and signal transduction. Annu Rev Neurosci 32:383–412.
3. Araujo S, Tase G (2003) Axon guidance mechanisms and molecules: Lessons from invertebrates. Nat Rev Neurosci 4:910–922.
4. Dell’Angelica EC (2000) AP-3 proteins: minding the cargo in a worm: Netrin-UNC-6 and circumferential axon guidance in C. elegans. Trends Neurosci 25:423–429.
5. MacNeil LT, Hardy WR, Pawson T, Wrana JL, Culotti JG (2009) UNC-129 regulates the balance between UNC-40 dependent and independent UNC-5 signaling pathways. Nat Neurosci 12:1092–1101.
6. Bartoe JL, et al. (2006) Protein interacting with C-kinase 1/protein kinase Calpha-epsilon-25. Salem N, Faúndez V, Horng J-T, Kelly RB (1998) A v-SNARE participates in synaptic transmission. Neuron 22:274–287.
7. Craig TJ, Henley JM (2012) Protein SUMOylation in spine structure and function. J Cell Sci 125:3489–3499.
8. Huang X, Cheng HJ, Tessier-Lavigne M, Jin Y (2002) MAX-1, a novel PH/MyTH4/FERM domain cytoplasmic protein implicated in netrin-mediated axon repulsion. Neuron 34:536–573.
9. Zhang H, et al. (2006) Vertebrate MAX-1 is required for vascular patterning in zebrafish. Proc Natl Acad Sci USA 103:16800–16805.
10. Rytkönen MM, Kääkönen S, Pekkonen P, Jääskeläinen T, Palvimo JJ (2002) PIAS proteins: Pleiotropic interactors associated with SUMO. Cell Mol Life Sci 66:3029–3406.
11. Williams ME, Wu SC, McKenna WL, Hinck L (2003) Surface expression of the netrin receptor UNC5H1 is regulated through a protein kinase C interacting protein/protein kinase-dependent mechanism. J Neurosci 23:12172–12188.
12. Rytinki MM, Kaikkonen S, Pehkonen P, Jääskeläinen T, Palvimo JJ (2009) PIAS protein AP-3 in sorting to the regulated secretory pathway. EMBO J 28:1514–1528.
13. Huang CH, Jussila-Laguzen M, Y Jinn Y (2002) MAX-1, a novel PH/MyTH4/FERM domain cytoplasmic protein implicated in netrin-mediated axon repulsion. Neuron 34:536–573.
14. Zhang H, et al. (2006) Vertebrate MAX-1 is required for vascular patterning in zebrafish. Proc Natl Acad Sci USA 103:16800–16805.
15. Ross S, Best JL, Zon LI, Gill G (2002) SUMO-1 modification represses Sp3 transcriptional activity and modulates its subnuclear localization. Mol Cell 10:831–842.
16. Shalizi A, et al. (2006) A calcium-regulated MEF2 sumoylation switch controls post-translational differentiation. Science 311:1012–1017.
17. Zhang H, et al. (2004) SUMO modification is required for in vivo Hox gene regulation by the Caenorhabditis elegans Polycomb group protein SOP-2. Nat Genet 36:507–511.
18. Chang Y, et al. (2007) Global analysis of SUMO protein-binding identities SUMOylation as a key regulator of the INO80 chromatin remodeling complex. Mol Cell Proteomics 6:812–823.
19. Asensio CS, Sirkis DW, Edwards RH (2010) RNAi screen identifies a role for adaptor complex 1 in regulating 3T3-L1 adipocyte lipid accumulation. Cell 143:6549–6559.
20. Lu H, et al. (2005) A Ca2+-dependent SUMOylation regulates the subnuclear localization of the netrin receptor UNC5. Neuron 45:539–559.
21. Keleman K, Dickson BJ (2001) Short- and long-range repulsion by the Drosophila Unc5 and Dcc family receptors converts netrin-induced growth cone attraction to chemorepulsion. Neuron 26:2419–2423.
22. Dell’Angelica EC (2000) AP-3 proteins: minding the cargo in a worm: Netrin-UNC-6 and circumferential axon guidance in C. elegans. Trends Neurosci 25:423–429.
23. Ogasra K, Goshima Y (2006) The autosomal-related protein UNC-51 and its binding partner UNC-14 regulate the subcellular localization of the netrin receptor UNC-5 in Caenorhabditis elegans. Development 133:3441–3450.
24. Chen et al. (2008) Cell Rep 6:251:348–350.
25. Dietrich J, Hou X, Weis WI (1994) CD3 gamma contains a leucine-based receptor-sorting motif: Effects on expression and signaling. J Biol Chem 269:1325–1328.
26. Keleman K, Dickson BJ (2001) Short- and long-range repulsion by the Drosophila Unc5 and Dcc family receptors converts netrin-induced growth cone attraction to chemorepulsion. Neuron 26:2419–2423.
27. Dietrich J, Weis WI (1994) CD3 gamma contains a leucine-based receptor-sorting motif: Effects on expression and signaling. J Biol Chem 269:1325–1328.
28. Keleman K, Dickson BJ (2001) Short- and long-range repulsion by the Drosophila Unc5 and Dcc family receptors converts netrin-induced growth cone attraction to chemorepulsion. Neuron 26:2419–2423.
29. Dietrich J, Weis WI (1994) CD3 gamma contains a leucine-based receptor-sorting motif: Effects on expression and signaling. J Biol Chem 269:1325–1328.
30. Dietrich J, Hou X, Weis WI (1994) CD3 gamma contains a leucine-based receptor-sorting motif: Effects on expression and signaling. J Biol Chem 269:1325–1328.
31. Dietrich J, Weis WI (1994) CD3 gamma contains a leucine-based receptor-sorting motif: Effects on expression and signaling. J Biol Chem 269:1325–1328.