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Caenorhabditis elegans F-box protein promotes axon regeneration by inducing degradation of the Mad transcription factor

Abbreviated Title: Regulation of axon regeneration by F-box protein

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Abstract

In Caenorhabditis elegans, axon regeneration is activated by a signaling cascade through the receptor tyrosine kinase (RTK) SVH-2. Axonal injury induces svh-2 gene expression by degradation of the Mad-like transcription factor MDL-1. In this study, we identify the svh-24/sdz-33 gene encoding a protein containing F-box and F-box associated domains as a regulator of axon regeneration in motor neurons. We find that sdz-33 is required for axon injury-induced svh-2 expression. SDZ-33 targets MDL-1 for poly-ubiquitylation and degradation. Furthermore, we demonstrate that SDZ-33 promotes axotomy-induced nuclear degradation of MDL-1, resulting in the activation of svh-2 expression in animals. These results suggest that the F-box protein is required for RTK signaling in the control of axon regeneration.
Significance Statement

In C. elegans, axon regeneration is positively regulated by the growth factor SVH-1 and its receptor tyrosine kinase SVH-2. Expression of the svh-2 gene is induced by axonal injury via the Ets-like transcription factor ETS-4, whose transcriptional activity is inhibited by the Mad-like transcription factor MDL-1. Axon injury leads to the degradation of MDL-1, and this is linked to the activation of ETS-4 transcriptional activity. In this study, we identify the sdz-33 gene encoding a protein containing an F-box domain as a regulator of axon regeneration. We demonstrate that MDL-1 is poly-ubiquitylated and degraded through the SDZ-33-mediated 26S proteasome pathway. These results reveal that an F-box protein promotes axon regeneration by degrading the Mad transcription factor.
Introduction

The ability of axons to regenerate after damage is a fundamental and conserved property of neurons, modulated by intrinsic processes that regulate axon growth potential. Upon axon severance, regenerative signals are transmitted from the sites of damage to the nucleus, whereupon several transcription factors are upregulated along with the synthesis of proteins participating in neurite outgrowth (Tedeschi and Bradke, 2018). Since manipulation of these signaling processes can improve the likelihood of successful axon regeneration, these processes are potential targets for regenerative therapies. However, these intrinsic signaling mechanisms have yet to be fully elucidated.

The nematode *Caenorhabditis elegans* has recently emerged as an attractive model to dissect the mechanisms of axon regeneration in the mature nervous system (Yanik et al., 2004). In *C. elegans*, the JNK MAP kinase (MAPK) cascade plays a crucial role in the initiation of axon regeneration (Nix et al., 2011; Hisamoto and Matsumoto, 2017). This pathway consists of MLK-1 MAPKKK, MEK-1 MAPKK, and KGB-1 JNK, and is inactivated at the KGB-1 activation step by VHP-1, a member of the MAPK phosphatase family (Mizuno et al., 2004).

The *vhp-1* null mutation causes hyper-activation of the JNK pathway, resulting in developmental arrest at an early larval stage. We recently have identified new components functioning in JNK-mediated signaling by employing a genome-wide RNAi screen for suppressors of *vhp-1* lethality (*svh* genes) (Li et al., 2012). The *svh-1* gene encodes a growth factor-like protein homologous to mammalian HGF and the *svh-2* gene encodes a homolog of mammalian Met, a receptor for HGF (Li et al., 2012). SVH-2 is a receptor tyrosine kinase (RTK) that activates the JNK pathway via the tyrosine phosphorylation of MLK-1 MAPKKK. SVH-1–SVH-2 signaling specifically regulates axon regeneration, and this
specificity is determined by induction of svh-2 gene expression following axon injury (Li et al., 2012). This up-regulation critically involves the physical interaction of the Ets-like transcription factor ETS-4 and the CCAAT/enhancer-binding protein (C/EBP)-like transcription factor CEBP-1 (Li et al., 2015). Upon axon injury, cyclic adenosine monophosphate (cAMP) levels increase in severed neurons, resulting in the activation of cAMP-dependent protein kinase (PKA), which in turn phosphorylates ETS-4. Phosphorylated ETS-4 is able to form a complex with CEBP-1, which then activates svh-2 transcription (Li et al., 2015). Furthermore, we recently identified the Mad-like transcription factor MDL-1, the Max-like transcription factor MXL-1, and TDP2 (Tyrosyl-DNA Phosphodiesterase 2)-like TDPT-1 as components involved in the regulation of ETS-4 transcriptional activity for induction of svh-2 gene expression (Figure 1A) (Sakai et al., 2019). TDPT-1 interacts with and induces SUMOylation of ETS-4, which interferes with PKA-mediated phosphorylation of ETS-4. As a result, formation of the ETS-4–CEBP-1 complex is inhibited, and its transcriptional activity thereby repressed. MXL-1 activates svh-2 transcription by interacting with TDPT-1 and relieving inhibition of ETS-4 activity. MDL-1 forms a complex with MXL-1 and this interaction induces the dissociation of TDPT-1 from MXL-1, enabling free TDPT-1 to inhibit ETS-4 transcriptional activity. Thus, TDPT-1 and MDL-1 negatively regulate axonal injury-induced expression of the svh-2 gene via modulation of ETS-4 (Figure 1A). Axon injury leads to the degradation of MDL-1, which is linked to the activation of ETS-4 transcriptional activity. Thus, MDL-1 protein stability is important in the regulation of axon regeneration, but the details of how this stability is modulated are at present unknown.

In the present study, we investigate the role of the svh-24/sdz-33 gene in the regulation of axon regeneration. The sdz-33 gene encodes a protein containing
an F-box domain (Robertson et al., 2004), which confers substrate recognition by the SCF [S-phase kinase-associated protein 1 (Skp1)–Cullin1–F-box] E3-ubiquitin (Ub) ligases (Craig and Tyers, 1999). Here, we show that MDL-1 is recognized by SDZ-33, which directs its degradation via the 26S proteasome. Induction of thus SDZ-33-mediated MDL-1 degradation pathway following neuron injury is essential for axon regeneration.
**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 medium plates and fed with bacteria of the OP50 strain, as described previously (Brenner, 1974).

**Plasmids**

The *Psdz-33::sdz-33::sdz-33 3'UTR* clone was generated by PCR amplification of approximately 1.7 kb of the *sdz-33* gene from genomic DNA (using the primers `5'-tgcaattaagcaagatgagttc-3'` and `5'-cgctcaccgtatttcctgtgc-3'`) and inserted into the TOPO vector (Invitrogen). *Psdz-33::nls::gfp* was constructed by Gibson assembly of the PCR-amplified *Psdz-33* promoter sequence (using the above described *Psdz-33::sdz-33* sequence as a template) and the *nls::gfp* containing vector pPD95.67. The *Punc-25::sdz-33::sdz-33 3'UTR* (3' untranslated region) plasmid was constructed by replacing the *sdz-33* promoter of the *Psdz-33::sdz-33::sdz-33 3'UTR* with the *unc-25* promoter, which was amplified from the pSC325 vector. The *Punc-25::sdz-33* (cDNA) plasmid was constructed by inserting a DNA sequence corresponding to the *sdz-33* cDNA (synthesized by Eurofins) into the pSC325 vector. The *Pmec-7::sdz-33::sdz-33 3'UTR* plasmid was generated by PCR amplification of the *sdz-33* sequence from the *Punc-25::sdz-33::sdz-33 3'UTR* construct (using the primers `5'-atagctagcatggctactgtcccttttcattc-3'` and `5'-tttggtacccgctcaccgtatttcctgtgc-3'`), digestion with NheI/KpnI and ligation with NheI/KpnI-digested *Pmec-7* vector pPD52.102. To construct Flag-SDZ-33, the *sdz-33* cDNA was subcloned into the pCMV-Flag vector. Myc-MDL-1 was constructed by inserting the *mdl-1* cDNA into the pCMV-Myc-N vector (Clontech).
Punc-25::svh-2, Punc-25::cfp, Psvh-2::nls::venus, Punc-25::mdl-1::gfp, Pmyo-2::dsred-monomer, and HA-Ub plasmids were described previously (Hanafusa et al., 2011; Li et al., 2012, 2015; Sakai et al., 2019).

Transgenic animals

Transgenic animals were obtained by the standard C. elegans microinjection method (Mello et al., 1991). Psdz-33::sdz-33::sdz-33 3’UTR, Psdz-33::nls::gfp, Punc-25::sdz-33::sdz-33 3’UTR, Punc-25::sdz-33 (cDNA)::unc-54 3’UTR, Pmec-7::sdz-33::sdz-33 3’UTR, Punc-25::svh-2, Punc-25::mdl-1::gfp, and Pmyo-2::dsred-monomer plasmids were used in kmEx1534 [Psdz-33::sdz-33::sdz-33 3’UTR (25 ng) + Pmyo-2::dsred-monomer (25 ng)], kmEx1545 [Psdz-33::nls::gfp (25 ng) + Pmyo-2::dsred-monomer (25 ng)], kmEx1535 [Punc-25::sdz-33::sdz-33 3’UTR (12.5 ng) + Pmyo-2::dsred-monomer (5 ng)], kmEx1546 [Punc-25::sdz-33 (cDNA)::unc-54 3’UTR (12.5 ng) + Pmyo-2::dsred-monomer (5 ng)], kmEx1547 [Punc-25::svh-2 (25 ng) + Pmyo-2::dsred-monomer (5 ng)], kmEx1536 [Punc-25::mdl-1::gfp (25 ng) + Pmyo-2::dsred-monomer (5 ng)], and kmEx1543 [Punc-25::mdl-1::gfp (25 ng) + Pmyo-2::dsred-monomer (5 ng)]. The extrachromosomal array and wpl36 integrated array were described previously (Firnhaber and Hammarlund, 2013; Sakai et al., 2019).

Microscopy

Standard fluorescent images of transgenic animals were obtained under a x100 objective of a Nikon ECLIPSE E800 fluorescent microscope and photographed with a Zyla CCD camera. Confocal fluorescent images were taken on a Zeiss LSM-800 confocal laser-scanning microscope with a x63 objective.
Axotomy

Axotomy and microscopy were performed as described previously (Pastuhov et al., 2012). All animals were subjected to axotomy at the young adult stage. Imaged commissures that had growth cones or small branches present on the proximal fragment were counted as “regenerated”. Proximal fragments that showed no change after 24 h were counted as “no regeneration”. A minimum of 20 individuals with 1–3 axotomized commissures were observed for most experiments.

Measuring the length 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 from the measurements.

Biochemical experiments using mammalian cells

Transfected HEK293 or COS-7 cells were incubated with or without MG132 (10 μM; Sigma) for 4.5 h. Cells were lysed in RIPA buffer [50 mM Tris–HCl, pH 7.4, 0.15 M NaCl, 0.25% deoxycholic acid, 1% NP-40, 1 mM EDTA, 1 mM dithiothreitol, phosphatase inhibitor cocktail 2 (Sigma), and protease inhibitor cocktail (Sigma)], followed by centrifugation at 15,000 g for 12 min. The supernatant was added to 50 μl (1.5 mg) of Dynabeads Protein G (Invitrogen) with the indicated antibodies (each antibody was used at 5 μg/sample) and rotated for 2 h at 4°C. The beads were then washed three times with ice-cold phosphate-buffered saline and subjected to immunoblotting. Antibodies and their suppliers were as follows: anti-Flag (M2; Sigma), anti-Myc (9E10 or A14; Santa
Quantification of VENUS expression

Expression of VENUS fluorescence was quantified using the ImageJ program (NIH). Cell bodies of severed or unsevered D neurons were outlined with closed polygons and the mean fluorescent intensities of VENUS and CFP were measured to obtain $I_{\text{VENUS}}$ and $I_{\text{CFP}}$, respectively. As a control, the cell body next to the cell body of interest was similarly analyzed [$I_{\text{VENUS}(c)}$ and $I_{\text{CFP}(c)}$]. To determine the background intensity of each cell, the same polygon was placed in the area neighboring the cell body and fluorescence was measured [$I_{\text{VENUS(BG)}}$, $I_{\text{VENUS(c)(BG)}}$, $I_{\text{CFP(BG)}}$, and $I_{\text{CFP(c)(BG)}}$, respectively]. The ratio of background-subtracted VENUS to CFP intensity was calculated as \[
\frac{I_{\text{VENUS}} - I_{\text{VENUS(BG)}}}{I_{\text{CFP}} - I_{\text{CFP(BG)}}}
\] and \[
\frac{I_{\text{VENUS(c)}} - I_{\text{VENUS(c)(BG)}}}{I_{\text{CFP(c)}} - I_{\text{CFP(c)(BG)}}}
\], respectively. The normalized relative intensity ($I_r$) was calculated as \[
\frac{I_{\text{VENUS}} - I_{\text{VENUS(BG)}}}{I_{\text{CFP}} - I_{\text{CFP(BG)}}}
\] for each cell.

Time-lapse imaging of MDL-1::GFP after axotomy

Animals expressing mCherry and MDL-1::GFP in their D-type motor neurons were imaged for 6 h at 15 min intervals beginning shortly after axotomy of selected motor neuron axons.

Quantitative measures of fluorescence intensity for MDL-1 degradation

Animals expressing mCherry and MDL-1::GFP in their D-type motor neurons were imaged shortly after (0 h) and 6 h after axotomy of selected motor neuron axons. An LSM800 (Zeiss) confocal microscope was used to obtain z-stacks of fluorescent images for mCherry and MDL-1::GFP. The mean intensities of...
MDL-1::GFP and mCherry in the nuclei of neurons with severed axons were measured by drawing a circular ROI in the middle of the cell and utilizing the measure function of ImageJ. Background intensities were measured near the measured cells. Relative MDL-1::GFP intensity (RIMDL-1) was calculated by dividing the background-subtracted value for GFP by the corresponding background-corrected value for mCherry at 6 h post axotomy divided by the corresponding value at 0 h post axotomy. The RIMDL-1 values for the wild type and sdz-33 mutant were plotted and checked for significant differences (Wilcoxon rank sum exact test) using RStudio.

**Statistical analysis**

Statistical analyses were carried out as described previously (Pastuhov et al., 2012). Briefly, confidence intervals (95%) were calculated using the modified Wald method and two-tailed P-values were calculated using Fisher’s exact test (http://www.graphpad.com/quickcalcs/contingency1/). Welch’s t-test and the Wilcoxon rank sum test (two-tailed) were performed using a t-test calculator (http://www.graphpad.com/quickcalcs/ttest1/) and the R function wilcox.test, respectively.

**Homology search, phylogenetic analysis, identification of domains, and alignment of amino acids**

A homology search, identification of conserved domains, and alignment of amino acids were performed using the NCBI DELTA-BLAST, NCBI CD-search, and GENETYX-MAC programs, respectively.
Results

SVH-24/SDZ-33 is required for efficient axon regeneration

To identify additional components functioning in the JNK pathway regulation of axon regeneration, we previously undertook a genome-wide RNAi screen for suppressors of *vhp-1* lethality and isolated 92 *svh* genes (Table 2). In order to identify components involved in axonal injury-induced degradation of MDL-1, we asked whether any of the *svh* genes encode factors that participate in protein degradation. Among these *svh* genes, we focused on *svh-24*, which encodes a protein containing an F-box domain.

The *svh-24* gene is identical to the *sdz-33* gene, which has been identified as a ubiquitously expressed early zygotic transcript (Robertson et al., 2004). The SDZ-33 protein contains F-box and type 2 F-box associated (FBA) domains (Figure 1B) (Thomas, 2006), and has weak homology with the human F-box protein UG063H01 (Figures 1C and 1D). The *sdz-33(tm1210)* deletion is a null allele (Figure 1B). To characterize the role of *sdz-33* in axon regeneration, we performed laser axotomy and assayed regeneration in γ-aminobutyric acid (GABA)-releasing D-type motor neurons. In young adult wild-type animals, 62% of the severed axons initiated regeneration within 24 h after axon injury, whereas in *sdz-33(tm1210)* mutant animals this frequency was reduced (Figures 2A and 2B; Table 3). Although the morphology of D-type motor neurons was normal in *sdz-33* mutants, the size of bulb-like structures in the non-regenerating axons of *sdz-33* mutants was larger than that in wild-type animals (Figure 2A). We found that the length of regenerating axons in *sdz-33* mutants was shorter than that observed in wild-type animals (Figure 2C). However, *sdz-33* mutants had no other detectable developmental or behavioral phenotypes. To verify that the *sdz-33* mutation was responsible for this defect in axon regeneration, we
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generated the transgene *Psdz-33::sdz-33::sdz-33 3’UTR*, which contains the entire genomic *sdz-33* region (Figure 2B). Introduction of *Psdz-33::sdz-33::sdz-33 3’UTR* into *sdz-33(tm1210)* mutants rescued the defect in axon regeneration (Figure 2B; Table 3).

To investigate whether SDZ-33 functions in D-type motor neurons, we examined the expression pattern of the *sdz-33* gene. We constructed a transgene *Psdz-33::nls::gfp*, which expresses the fluorescent protein GFP fused to a nuclear localization signal (NLS) under the control of the *sdz-33* promoter. In the young adult stage, animals carrying *Psdz-33::nls::gfp* exhibited weak expression of GFP in vulval muscles but not in D-type motor neurons. GFP expression was still not observed in D neurons after axon injury (Figure 2D).

Therefore, we examined whether SDZ-33 acts in D-type motor neurons. In *sdz-33(tm1210)* mutants, expression of the *sdz-33* cDNA driven by the *unc-25* promoter with the *unc-54 3’UTR* could not rescue the *sdz-33 (tm1210)* defect in axon regeneration (Figure 2B; Table 3). On the other hand, when *sdz-33::sdz-33 3’UTR* was expressed by the *unc-25* promoter in *sdz-33(tm1210)* mutants, the regeneration defect was rescued (Figure 2B; Table 3). These results suggest that the intron or 3’UTR region of the *sdz-33* gene is required for *sdz-33* expression. It is known that axon injury promotes stability and local translation of the mRNA encoding CEBP-1, a homolog of mammalian C/EBP (CCAAT/enhancer-binding protein), via its 3’UTR (Yan et al., 2009). This suggests that the 3’UTR region of the *sdz-33* gene may determine the stability and proper localization of the *sdz-33* mRNA. In contrast to expression of *sdz-33::sdz-33 3’UTR* from the *unc-25* promoter, expression of the *sdz-33::sdz-33 3’UTR* DNA from the *mec-7* promoter in sensory neurons failed to rescue the *sdz-33(tm1210)* defect (Figure 2B; Table 3). Thus, SDZ-33 regulates axon regeneration in injured D-type motor neurons after laser axotomy.
in a cell-autonomous manner.

**SDZ-33 is involved in axotomy-induced svh-2 expression in injured D-type motor neurons**

Our RNAi screen for svh genes was originally designed to identify components functioning in the JNK pathway (Li et al., 2012; Pastuhov et al., 2015). We next investigated where in this pathway SDZ-33 functions during axon regeneration. Activation of the JNK cascade following axonal injury is mediated by the SVH-2 Met-like RTK (Li et al., 2012). We confirmed that SDZ-33 functions in the same pathway as SVH-2 because the phenotype of *sdz-33(tm1210); svh-2(tm737)* double mutants was indistinguishable from that of either single mutant (Figure 3A; Table 3). We examined whether overexpression of *svh-2* might reverse the defect in axon regeneration observed in *sdz-33* mutants. We found that this was indeed the case (Figure 3A; Table 3). These results suggest that SDZ-33 functions at or upstream of SVH-2 in the SVH-2–JNK pathway.

Since expression of *svh-2* is induced by axonal injury (Li et al., 2015), we examined whether SDZ-33 is involved in this induction using a reporter construct, *Psvh-2::nls::venus*, which consists of the *svh-2* promoter driving the fluorescent protein VENUS fused to NLS (Li et al., 2015). In wild-type animals, axon injury induced expression of *Psvh-2::nls::venus* in D-type motor neurons (Figures 3B and 3C), as reported previously (Li et al., 2015; Sakai et al., 2019). However, in *sdz-33(tm1210)* mutants, no induction was observed (Figures 3B and 3C). These results suggest that SDZ-33 is required for axotomy-induced *svh-2* expression.

**SDZ-33 targets MDL-1 for Ub-mediated degradation**

What is the target for SDZ-33 in the regulation of axon regeneration? F-box
proteins are involved in protein degradation mediated by SCF E3-Ub ligase complexes (Craig and Tyers, 1999). Since SDZ-33 is required for axon regeneration, its target should be a negative regulator of axon regeneration. We recently demonstrated that axon injury-induced svh-2 expression is negatively regulated by Mad-like MDL-1 and TDP2-like TDPT-1 (Figure 4A) (Sakai et al., 2019). We examined the effects of mdl-1 and tdpt-1 loss-of-function mutations on axon regeneration in sdz-33 mutants. We found that both mdl-1(tm311) and tdpt-1(km68) mutations could suppress the defect in regeneration in sdz-33(tm1210) mutants (Figure 4B; Table 3). As MDL-1 acts upstream of TDPT-1 in the axon regeneration pathway (Figure 4A), these results suggest that SDZ-33 acts upstream of MDL-1 to induce svh-2 expression in response to axon injury. Indeed, we confirmed that expression of Psvh-2::nls::venus in D-type neurons was induced by laser surgery in sdz-33(tm1210); mdl-1(tm311) mutants (Figures 3B and 3C).

Our genetic analysis of mdl-1 and sdz-33 above raised the possibility that SDZ-33 could act as a specific subunit in an SCF complex to mediate ubiquitylation of MDL-1, leading to its degradation. Since an F-box protein functions to target substrate proteins, we first examined whether SDZ-33 interacts with MDL-1 by co-transfecting mammalian HEK293 cells with Flag-tagged SDZ-33 and Myc-tagged MDL-1. Co-immunoprecipitation experiments revealed that Flag-SDZ-33 associated with Myc-MDL-1 (Figure 4C). We next determined whether MDL-1 can be degraded by the SDZ-33-mediated 26S proteasome pathway in mammalian COS-7 cells. For this purpose, we co-expressed Myc-MDL-1 with HA-tagged Ub in COS-7 cells. Cell lysates were immunoprecipitated with anti-Myc antibody and immunoblotted with anti-HA antibody. We found that MDL-1 was poly-ubiquitylated (Figure 4D), suggesting that endogenous mammalian E3-Ub ligases are capable of ubiquitylating MDL-1.
We then asked whether SDZ-33 expression could cause ubiquitylation of MDL-1. COS-7 cells were co-transfected with Myc-MDL-1, HA-Ub, and Flag-SDZ-33. This assay assumes that *C. elegans* SDZ-33 interacts with the endogenous COS-7 SCF complex and provides substrate specificity for MDL-1. We found that co-expression of Flag-SDZ-33 decreased the levels of poly-ubiquitylated MDL-1 protein (Figure 4D), suggesting that SDZ-33 promotes the degradation of ubiquitylated MDL-1. Consistent with this possibility, when cells were treated with MG132, a specific inhibitor of the 26S proteasome, the amounts of poly-ubiquitylated MDL-1 protein clearly increased (Figure 4D). Thus, proteasome-mediated degradation contributes to the control of MDL-1 protein levels by SDZ-33. Together, the above results indicate that SDZ-33 is an F-box protein that directly targets MDL-1 for Ub-mediated degradation.

We next investigated whether SDZ-33 regulates MDL-1 protein levels in animals expressing GFP-tagged MDL-1 from the *unc-25* promoter. As observed previously (Sakai et al., 2019), in wild-type animals MDL-1::GFP was found predominantly in the nucleus of D neurons and its fluorescence intensity significantly decreased following axon injury (Figures 5A and 5B). In contrast, we found that the *sdz-33(tm1210)* mutation resulted in significant stabilization of nuclear MDL-1::GFP levels, which persisted even 6 h after axotomy (Figures 5A and 5B). These results provide evidence that SDZ-33 is involved in axon injury-induced degradation of MDL-1.
Discussion

In *C. elegans*, *svh-2* expression is upregulated in neurons following axon injury. This gene induction is mediated by a transcription factor complex composed of ETS-4 and CEBP-1 (Li et al., 2015). We have recently found that TDPT-1 inhibits axon injury-induced *svh-2* expression by inducing the SUMOylation of ETS-4, which inhibits formation of an ETS-4–CEBP-1 complex (Sakai et al., 2019). We have additionally identified a transcription factor, MXL-1, that acts as a positive regulator of *svh-2* expression in response to axon injury. However, in contrast to MXL-1, its partner transcription factor MDL-1 negatively regulates *svh-2* induction. Based on these findings, we propose the following molecular mechanism of how axon injury activates *svh-2* expression (Figure 6). In the absence of axon damage, TDPT-1 maintains the SUMOylation of ETS-4, resulting in the repression of its transcriptional activity. Axon injury leads to the degradation of MDL-1, allowing free MXL-1 to interact with TDPT-1. This causes the release of ETS-4 from TDPT-1, resulting in ETS-4 deSUMOylation and consequent complex formation with CEBP-1. This ETS-4–CEBP-1 complex then induces *svh-2* expression.

By this model, there must be some mechanism by which axon injury induces the degradation of MDL-1. Here, we show that the *sdz-33* gene encoding an F-box protein is required for induction of *svh-2* gene expression following axon injury. F-box proteins serve as substrate recruitment factors for the SCF E3-Ub ligase (Craig and Tyers, 1999). SDZ-33 promotes poly-ubiquitylation of MDL-1, leading to its subsequent destruction by the 26S proteasome (Figure 6). Deletion of *sdz-33* stabilizes MDL-1, which accumulates and is able to repress *svh-2* gene expression in response to axon injury. The ubiquitylation of target proteins is orchestrated by a Ub-activating enzyme (E1), a Ub-conjugating enzyme (E2),
and an E3-Ub ligase, that latter of which is responsible for substrate recognition
and Ub transfer. One E3-Ub ligase is the SCF complex, composed of Skp1,
Cullin1, Rbx1, and an F-box protein (Craig and Tyers, 1999). Cullin functions as
a scaffold that interacts with Skp1 and Rbx1 to form a complex generating the
core E3-Ub ligase activity. F-box proteins selectively recruit target proteins and
thereby mediate Ub interactions with their substrates. Thus, the F-box protein
determines substrate specificity. F-box proteins contain an N-terminal F-box
domain that binds to Skp1 to enable ubiquitylation of the target. F-box proteins
often contain other motifs such as a WD-40 repeat and a leucine-rich repeat,
both of which are involved in substrate binding (Craig and Tyers, 1999).
Identifying target substrates for F-box proteins would enhance our
understanding of how SCF E3-Ub ligase mediates multiple biological functions.
The *C. elegans* genome contains about 700 genes encoding F-box proteins
(Thomas, 2006), but only a few have been successfully assigned a function
(Kiperos et al., 2000; Clifford et al., 2000; Liao et al., 2004; Fielenbach et al.,
2007; Bounoutas et al., 2009; Wu et al., 2017). Here, we identify SDZ-33 as an
F-box E3-Ub ligase that promotes the degradation of MDL-1. SDZ-33 lacks
WD40 or leucine-rich repeats, but it does contain an FBA domain, which may be
involved in the interaction with MDL-1. The *C. elegans* genome encodes more
than 20 Skp1-related proteins and six Cullin proteins (Nayak et al., 2002). It
would be interesting to determine which Skp1-related proteins or Cullins are
involved in SDZ-33-mediated degradation of MDL-1.

In the mammalian Myc/Mad/Max family of transcription factors, Myc forms a
heterodimer with its partner Max. This Myc–Max complex then binds to the
promoter region of target genes to activate their transcription. Mad1 suppresses
Myc transcriptional activity by interacting with Max and inhibiting Myc–Max
complex formation (Amati and Land, 1994). The *C. elegans* *mxi-1* and *mdl-1*
genes encode homologs of vertebrate Max and Mad, respectively, but *C. elegans* lacks an obvious homolog of Myc (Pickett et al., 2007). MXL-1 has been shown to form a complex with MDL-1 and TDPT-1, and MDL-1 protein levels are tightly regulated in response to axon injury (Sakai et al., 2019). Therefore, the availability of MXL-1 to TDPT-1 SUMOylation is dependent on MDL-1 protein expression levels. Association of MXL-1 with TDPT-1 redirects the MXL-1–MDL-1 complex to regulate ETS-4. The results presented here suggest that Ub-mediated degradation regulates the Max–Mad network by promoting Mad degradation (Figure 6). This work thus reveals an important module of this transcriptional regulatory system and illustrates the complex nature of the Max transcription factor network.
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Figure legends

Figure 1. Identification of SDZ-33.

A, Regulation of svh-2 expression in response to axon injury. MXL-1 forms a complex with MDL-1, and TDPT-1 interacts with ETS-4 to induce its SUMOylation, resulting in the repression of ETS-4 transcriptional activity. Axon injury leads to the degradation of MDL-1, allowing free MXL-1 to interact with TDPT-1. ETS-4 is then de-SUMOylated, and subsequently induces svh-2 expression.

B, Structures of SDZ-33 and human UG063H01. The domains shown are the F-box domain (blue) and type 2 F-box associated domain (FBA; yellow). Percentages of similarities are indicated. The bold line underneath indicates the extent of the deleted region in the tm1210 deletion mutant.

C, D, Amino acid alignments of the N- (C) and C-terminal (D) domains. Identical and similar residues are highlighted with dark and pale gray shading, respectively.

Figure 2. SDZ-33 is involved in axon regeneration.

A, Representative D-type motor neurons in wild-type and sdz-33 mutant animals 24 h after laser surgery. In wild-type animals, a severed axon has regenerated a growth cone (arrow). In mutants, the proximal end of axon failed to regenerate (arrowhead). Scale bar = 10 μm.

B, Percentages of axons that initiated regeneration 24 h after laser surgery. The numbers (n) of axons examined are shown. Error bars indicate 95% confidence intervals (CI). *P < 0.05, **P < 0.01 as determined by Fisher’s exact test. NS, not significant. Schematic diagrams for Psdz-33::sdz-33::sdz-33 3'UTR, Punc-25::sdz-33 (cDNA)::unc-54 3'UTR, Punc-25::sdz-33::sdz-33 3'UTR, and...
Pmec-7::sdz-33::sdz-33 3'UTR are shown in the upper part.

C, Length of regenerating axons 24 h after laser surgery. The data are presented as box-blot graph with median (thick line within the box) and interquartile range (edge of box). A white circle indicates an outlier. The numbers (n) of axons examined are shown. Statistical significance was determined using the Wilcoxon rank sum test; \( P = 0.0036 \).

D, Expression of the Psdz-33::nls::gfp gene. Fluorescent and DIC images of animals carrying Psdz-33::nls::gfp and Punc-47::mcherry 6h after cutting are shown. D-type motor neurons are visualized by mCherry under control of the unc-47 promoter. White arrow indicates vulval muscles. Yellow arrowheads indicate severed axons. The fluorescent signal in the pharynx (asterisk) is from the injection marker Pmyo-2::DsRed monomer. V, ventral side; D, dorsal side. Scale bar = 20 \( \mu \)m.

Figure 3. SDZ-33 is required for transcriptional induction of the svh-2 gene in response to axon injury.

A, Percentages of axons that initiated regeneration 24 h after laser surgery. The numbers (n) of axons examined are shown. Error bars indicate 95% confidence intervals (CI). **\( P < 0.01 \), ***\( P < 0.001 \) as determined by Fisher's exact test. NS, not significant.

B, Induction of Psvh-2::nls::venus expression in D-type motor neurons by laser surgery. Expression of fluorescent proteins in D-type motor neurons of wild type, \( sdz-33 \), and \( sdz-33; mdl-1 \) mutants 3 h after laser surgery is shown. White arrows indicate cell bodies of D-type neurons. Yellow arrowheads indicate the sites of laser surgery. D neurons are visualized by CFP under control of the unc-25 promoter. Cell bodies of D-type neurons are magnified and shown within the red boxes. Most of the intestinal fluorescence in these photos is from...
endogenous and variable background autofluorescence. A schematic representation of D-type motor neurons is shown in the right panel. Scale bars = 10 μm.

**C**, Quantification of the relative fluorescent levels of VENUS in D neurons with (+) or without (−) laser surgery (see Materials and Methods). The data are presented as box-plot graph with median (thick line within the box) and interquartile range (edge of box). The numbers (n) of animals examined are shown. Statistical significance was determined using the Wilcoxon rank sum test.

**Figure 4. SDZ-33 targets MDL-1 for Ub-mediated degradation.**

**A**, Inhibition of axon regeneration by MDL-1 and TDPT-1.

**B**, Percentages of axons that initiated regeneration 24 h after laser surgery. The numbers (n) of axons examined are shown. Error bars indicate 95% confidence intervals (CI). *P < 0.05, **P < 0.01 as determined by Fisher’s exact test.

**C**, Interaction of SDZ-33 with MDL-1. HEK293 cells were co-transfected with Myc-MDL-1 and Flag-SDZ-33 as indicated. Complex formation was detected by immunoprecipitation (IP) with anti-Flag antibody, followed by immunoblotting (IB) with the anti-Myc antibody. Total lysates were immunoblotted with anti-Myc antibody.

**D**, SDZ-33 mediates poly-ubiquitylation of MDL-1. COS-7 cells were transfected with Myc-MDL-1, HA-Ub, and Flag-SDZ-33, as indicated. Cells were incubated with or without MG132. Cell lysates were immunoprecipitated (IP) with anti-Myc antibody and immunoblotted (IB) with anti-HA antibody. Whole-cell extracts (WCE) were analyzed by immunoblotting with anti-Myc and anti-Flag antibodies.

**Figure 5. SDZ-33 promotes axotomy-induced nuclear degradation of**
MDL-1.

A, Fluorescent images of wild type and sdz-33 mutant expressing Punc-47::mcherry (D-type motor neuron, top) and Punc-25:mdl-1::gfp (bottom) are shown. Images were taken at 0, 3, or 6 h after laser surgery. White arrowheads indicate the tips of the severed axons. Cell bodies corresponding to the severed axons are outlined in yellow and shown magnified in the insets. Scale bar = 10 μm.

B, Quantification of the fluorescent levels of MDL-1::GFP in the nucleus of D neurons. Relative MDL-1 intensity was calculated as a fraction of the relative MDL-1::GFP intensity 6 h after laser surgery divided by the corresponding value at 0 h post axotomy (see Materials and Methods). The data are presented as box-blot graph with median (thick line within the box) and interquartile range (edge of box). White circles indicate outliers. The numbers (n) of axons examined are shown. Statistical significance was determined using the Wilcoxon rank sum test; P = 0.0496.

Figure 6. Schematic diagram of the role of SDZ-33 in axon regeneration.

SDZ-33 recruits MDL-1 to the SCF E3-Ub ligase complex, leading to the subsequent destruction of MDL-1 by the 26S proteasome. MDL-1 degradation allows ETS-4 to activate svh-2 expression.
A

\[
\text{MDL-1} \quad \downarrow \\
\text{MXL-1} \quad \downarrow \\
\text{TDPT-1} \quad \downarrow \\
\text{ETS-4} \quad \downarrow \\
\text{Axon regeneration}
\]

B

![Graph showing regeneration percentages with error bars]

C

| Flag-SDZ-33 | Myc-MDL1 | IB: Myc | IB: Flag |
|-------------|----------|---------|----------|
| -           | -        | ![Myc band](#) | ![Flag band](#) |
| +           | +        | ![Myc band](#) | ![Flag band](#) |

D

| Myc-MDL-1 | HA-Ub   | Flag-SDZ-33 | MG132 |
|-----------|---------|-------------|-------|
| +         | +       | =           | +     |
| +         | +       | =           | +     |
| +         | +       | =           | +     |

(kDa)

![Western blot images]

IB: Myc

IB: Flag

WCE
A

| cut (h) | 0   | 3   | 6   |
|---------|-----|-----|-----|
| D-type motor neuron | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) |
| MDL-1 ::GFP | ![Image](image4.png) | ![Image](image5.png) | ![Image](image6.png) |

WT

sdz-33

| D-type motor neuron | ![Image](image7.png) | ![Image](image8.png) | ![Image](image9.png) |
| MDL-1 ::GFP | ![Image](image10.png) | ![Image](image11.png) | ![Image](image12.png) |

B

Relative MDL-1 intensity

![Box plot](image13.png)

(n) 26 30
### Table 1. Strains used in this study

| Strain   | Genotype                                                                 |
|----------|---------------------------------------------------------------------------|
| KU501    | juIs76 II                                                                 |
| KU503    | juIs76 II; svh-2(tm737) X                                                 |
| KU1533   | juIs76 II; sdz-33(tm1210) III                                            |
| KU1534   | juIs76 II; sdz-33(tm1210) III; kmEx1534 [Psdz-33::sdz-33::sdz-33 3'UTR]   |
| KU1535   | juIs76 II; sdz-33(tm1210) III; kmEx1535 [Punc-25::sdz-33::sdz-33 3'UTR]   |
| KU1536   | juIs76 II; sdz-33(tm1210) III; kmEx1536 [Punc-25::svh-2]                  |
| KU1537   | juIs76 II; sdz-33(tm1210) III; svh-2(tm737) X                             |
| KU1538   | tdpt-1(km68) I; juIs76 II; sdz-33(tm1210) III                             |
| KU1539   | juIs76 II; sdz-33(tm1210) III; mdl-1(tm311) X                             |
| KU1527   | kmEx1527 [Punc-25::cfp + Psvh-2::nls::venus]                             |
| KU1540   | sdz-33(tm1210) III; kmEx1527 [Punc-25::cfp + Psvh-2::nls::venus]          |
| KU1541   | sdz-33(tm1210) III; mdl-1(tm311) X; kmEx1527 [Punc-25::cfp + Psvh-2::nls::venus] |
| KU1542   | mdl-1(tm311) X; kmEx1527 [Punc-25::cfp + Psvh-2::nls::venus]              |
| KU1543   | wpl36 I; kmEx1543 [Punc-25::mdl-1::gfp]                                  |
| KU1544   | wpl36 I; sdz-33(tm1210) III; kmEx1543 [Punc-25::mdl-1::gfp]               |
| KU1545   | wpl36 I; kmEx1545 [Psdz-33::nls::gfp]                                    |
| KU1546   | juIs76 II; sdz-33(tm1210) III; kmEx1546 [Punc-25::sdz-33(cDNA)::unc-54 3'UTR] |
| KU1547   | juIs76 II; sdz-33(tm1210) III; kmEx1547 [Pmec-7::sdz-33::sdz-33 3'UTR]    |
Table 2. List of svh genes

| svh gene | Name        | Mammal homolog                | Ref.                |
|----------|-------------|------------------------------|---------------------|
| svh-1    | HGF/plasminogen | Growth factor               | Hisamoto et al., 2014 |
| svh-2    | c-Met       | RTK                         | Li et al., 2012     |
| svh-3    | faah-1      | FAAH                        | Pastuhov et al., 2012 |
| svh-4    | ddr-2       | DDR                         | Hisamoto et al., 2016 |
| svh-5    | Ets         | Transcription factor         | Li et al., 2015     |
| svh-6    | tns-1       | Tensin                      | Hisamoto et al., 2019 |
| svh-8    | cebp-1      | C/EBP                       | Li et al., 2015     |
| svh-9    | nstp-1      | SLC35B4                     | Shimizu et al., 2019 |
| svh-10   | sqv-3       | B4GALT7                     | Shimizu et al., 2019 |
| svh-11   | FUT         | Sugar transferase           | Shimizu et al., 2019 |
| svh-12   | egl-30      | Gqαx                        | Pastuhov et al., 2012 |
| svh-13   | trr-11      | Lipid-binding protein       | Hisamoto et al., 2018 |
| svh-14   | mxl-1       | Max                         | Sakai et al., 2019  |
| svh-15   | brc-2       | BRCA2                       | Shimizu et al., 2018 |
| svh-19   | let-502     | ROCK                        | Shimizu et al., 2018 |
| svh-20   | emb-9       | COL4A5                      | Hisamoto et al., 2016 |
Table 3. Raw data of genotypes tested by axotomy

| Strain         | Genotype (juIs76 background)            | No. of animals | No. of axons | No. of regenerations (% of total) | p-value       | Compared with |
|----------------|----------------------------------------|----------------|--------------|----------------------------------|---------------|---------------|
| KU501          | wild type                              | 49             | 87           | 54 (62%)                         | -             | -             |
| KU1533         | szd-33(tm1210)                         | 23             | 52           | 18 (35%)                         | 0.0027        | KU501         |
| KU1534         | szd-33(tm1210); Ex[P:sdz-33::sdz-33 3'UTR] | 22             | 50           | 32 (64%)                         | 0.0053        | KU1533        |
| KU1535         | szd-33(tm1210); Ex[P:unc-25::sdz-33::sdz-33 3'UTR] | 23             | 61           | 36 (59%)                         | 0.0138        | KU1533        |
| KU1546         | szd-33(tm1210); Ex[P:unc-25::sdz-33(cDNA)::unc-54 3'UTR] | 26             | 67           | 29 (43%)                         | 0.3523        | KU1533        |
| KU1547         | szd-33(tm1210); Ex[P:mec-7::sdz-33::sdz-33 3'UTR] | 14             | 40           | 14 (35%)                         | 1             | KU1533        |
| KU1536         | szd-33(tm1210); Ex[P:unc-25::svh-2]     | 21             | 54           | 35 (65%)                         | 0.0034        | KU1533        |
| KU503          | svh-2(tm737)                           | 30             | 54           | 13 (24%)                         | 0.0002        | KU501         |
| KU1537         | szd-33(tm1210); svh-2(tm737)           | 27             | 55           | 17 (31%)                         | 0.8369        | KU1533        |
| KU1538         | tdpt-1(km68); sdz-33(tm1210)           | 27             | 61           | 37 (61%)                         | 0.0081        | KU1533        |
| KU1539         | szd-33(tm1210); mdl-1(tm311)           | 24             | 58           | 33 (57%)                         | 0.0226        | KU1533        |