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·Review·

Signaling pathways that regulate axon regeneration

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Neurons in the mammalian central nervous system (CNS) cannot regenerate axons after injury. In contrast, neurons in the mammalian peripheral nervous system and in some non-mammalian models, such as *C. elegans* and *Drosophila*, are able to regrow axons. Understanding the molecular mechanisms by which these neurons support axon regeneration will help us find ways to enhance mammalian CNS axon regeneration. Here, recent studies in which signaling pathways regulating naturally-occurring axon regeneration that have been identified are reviewed, focusing on how these pathways control gene expression and growth-cone function during axon regeneration.

Keywords: axon regeneration; axonal growth; signal transduction

Introduction

Damage to the central nervous system (CNS), such as spinal cord injury and traumatic brain injury, often results in damage to axons. Unfortunately, mature neurons in the mammalian CNS cannot regenerate axons after injury, leading to irreversible motor, sensory, or cognitive dysfunctions. Therefore, promoting axon regeneration is a major approach to treating these symptoms. One important reason for CNS regeneration failure is the irreversible loss of the intrinsic axon growth capacity of CNS neurons after maturation. To address this issue, recent attempts to augment the growth potential by regulating transcriptional or translational machinery have provided promising results[1-5]. However, the degree of axon regeneration promoted by these molecular interventions is still rudimentary compared with the regeneration that occurs after injury to the mammalian peripheral nervous system (PNS) or in non-mammals. This fact indicates that understanding the molecular mechanisms by which naturally-occurring axon regeneration is regulated should be a major focus of investigation. The first step would be to elucidate the underlying signaling pathways that regulate naturally-occurring axon regeneration.

Many mature CNS neurons in non-mammals, such as *C. elegans*, *Drosophila*, and zebrafish, are able to regenerate after injuries. The powerful genetic approaches available in these species have made them attractive model systems for dissecting the signaling pathways controlling axon regeneration. Indeed, recent studies using these systems, in particular *C. elegans*, have revealed several regeneration signaling pathways *in vivo*[6-8]. The mammalian PNS also mounts a robust regenerative response after nerve injury. However, in mice it is difficult to perform similar genetic analysis, which often involves genetic targeting of multiple signaling molecules at the same time, specifically in adult neurons. As a result, we know much less about the signaling pathways that regulate naturally-occurring mammalian axon regeneration *in vivo*.

Axon extension, during either development or regeneration, comprises several highly-coordinated processes, including gene transcription/expression in the soma, transport of synthesized molecules along the axon, and axon assembly at the growth cone (Fig. 1). Each process is regulated by spatially-controlled signaling pathways that target distinct effectors. For instance, pathways regulating axon assembly at the growth cone

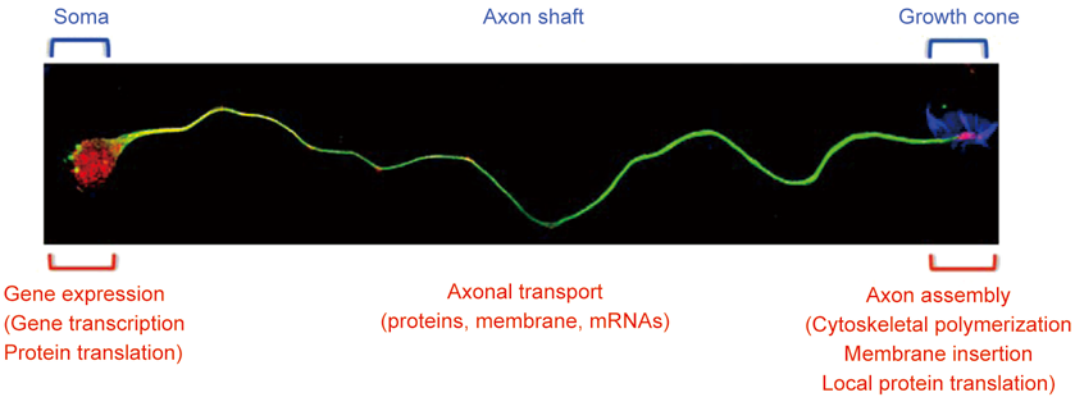

Fig. 1. Axon growth is achieved by coordinated regulation of gene expression in the soma, axonal transport along the shaft, and axon assembly at the growth cone.

mainly target cytoskeletal proteins, especially microtubules, to control axon extension, whereas pathways regulating gene expression in the soma mainly target transcription factors. Recently, several signaling pathways that regulate naturally-occurring axon regeneration have been identified in non-mammalian and mammalian PNS models. Here, we review these pathways, focusing on where they act (soma or growth cone) to regulate axon regeneration. Pathways that are ectopically manipulated to enhance axon regeneration are not discussed.

Signaling Pathways That Regulate Axon Regeneration in Non-mammalian Models

Non-mammalian organisms, including *C. elegans*, *Drosophila*, and zebrafish, are invaluable model systems for studying neuronal morphogenesis during development. The powerful genetic tools available in *C. elegans* and *Drosophila* have made them particularly useful for delineating upstream/downstream signaling pathways *via* epistatic analysis. Recent efforts have been made to induce axotomy and assess axon regeneration in adult neurons of *C. elegans* and *Drosophila*[6], thus making them attractive new model systems for dissecting axon regeneration pathways. To induce axotomy in *C. elegans*, Hammarlund *et al.* used a loss-of-function mutant of the unc-70 gene (homolog of β-spectrin). In such mutants, the adult axons become fragile and break when the animals move, which initiates a regenerative response[9]. Another approach for injury in live *C. elegans* is to use a femtosecond laser to perform acute axotomy of fluorescence-labeled neurons[10,11]. Similar laser axotomy has also been established in *Drosophila*[12] and zebrafish[13]. Using such *in vivo* axotomy approaches combined with large-scale genetic screening, recent studies have identified several signaling pathways that control axon regeneration *in vivo* either at the growth cone or at the soma (Fig. 2).

Signaling at the Growth Cone

Growth cones are highly specialized structures at the distal tips of axons that control the rate and direction of axon growth during development. Assembly of the cytoskeletal elements and membrane components at the growth cone drives its advancement and the subsequent axon extension. Formation of a growth cone after axotomy, which is achieved *via* reorganization of the cytoskeleton at the proximal axonal stump, is the first step for successful regeneration. Both growth-cone formation and advancement are tightly regulated by extracellular factors and intracellular signaling molecules[14,15].

Currently, there is no experimental approach in *C. elegans* to spatially distinguish signaling events during axon growth. However, based on downstream effectors and the time-courses in observed phenotypes, it seems that the regeneration pathways identified in *C. elegans* to date act mostly in the axon to control growth-cone formation after axotomy (Fig. 2). A large-scale RNAi-based genetic screen has identified dual leucine zipper-bearing kinase 1 (DLK-1) as a key regulator of axon regeneration[9]. Genetic interaction experiments have identified MKK-4 (MAP kinase kinase 4, MAP2K4) and PMK-3 (p38 MAP

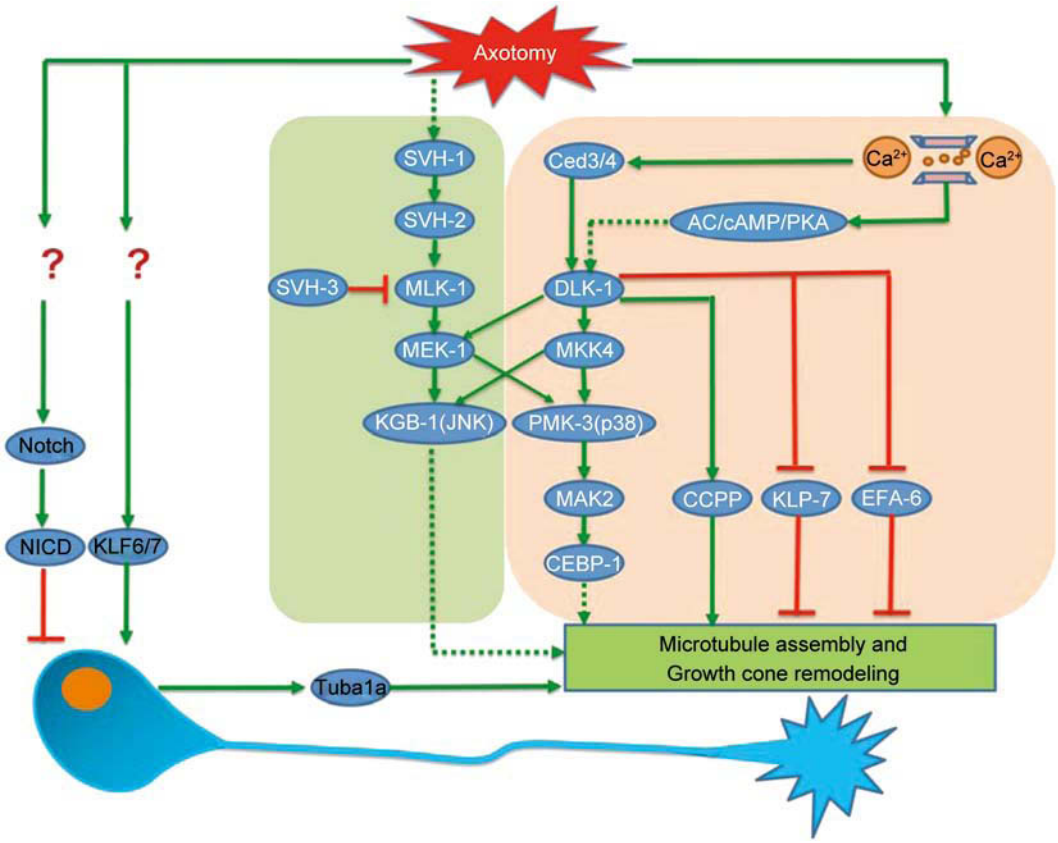

Fig. 2. Signaling pathways regulating axon regeneration in non-mammalian models. In the soma, axotomy activates the transcription factors KLF6/7 to control the expression of genes supporting axon regeneration, whereas Notch signaling acts as a negative regulator. At the growth cone, axotomy triggers Ca^{2+} influx, which subsequently activates the apoptotic Ced3/4 pathway and/or the second messenger AC/cAMP/PKA pathway, both of which converge onto the DLK-1 pathway. Downstream, DLK-1 regulates local protein translation through the MKK4/PMK-3/MAK2 pathway and/or promotes axonal microtubule assembly by controlling multiple microtubule binding proteins, including CCPP, KLP-7, and EFA-6. Specifically, DLK-1 activates CCPP, which deglutamylates tubulin and functions to promote microtubule stabilization. Conversely, both KLP-7, which depolymerizes microtubules, and EFA-6, which destabilizes them, are inhibited by DLK-1. Another MAP kinase pathway, MLK-1/MEK-1/KGB-1, acts in parallel with the DLK-1 pathway to control growth-cone formation after axotomy. SVH-1, a homolog of the mammalian hepatocyte growth factor and its receptor SVH-2 function upstream of the MLK-1 pathway to promote axon regeneration, whereas SVH-3, a homolog of mammalian fatty-acid amide hydrolase, inhibits axon regeneration by antagonizing the MLK-1 pathway. Lastly, the expression of the tubulin isoform Tuba1a regulated by KLF6/7 may contribute to the control of growth-cone remodeling during axon regeneration.

kinase) as downstream mediators of DLK-1 to control axon regeneration. How does the DLK-1/MKK-4/PMK-3 MAP kinase pathway regulate axon regeneration? Because the major phenotype of the DLK-1 mutant is a defect in growth-cone formation after axotomy[9] and the temporal requirement of DLK-1 function for regeneration is very soon after the axotomy, it has been suggested that DLK-1 mainly functions locally at the growth cone to control axon regeneration. In support, a different study has shown that MAK-2 (MAP kinase activated kinase, MAPKAP) acts downstream of DLK-1 to control axon regeneration *via* controlling the local translation of CEBP-1 in the axon[16]. To further support the role of DLK-1 in the growth cone, a later study showed that KLP-7 (kinesin-13) and CCPP-6 (cytosolic carboxipeptidase), two proteins directly involved in the regulation of microtubule dynamics, act downstream of DLK-1 to control axon regeneration[17]. Specifically, DLK-1 inhibits KLP-7, which has microtubule depolymerase activity and functions to restrict microtubule assembly. Conversely, DLK-1 activates CCPP-6, which deglutamylates tubulin

and promotes microtubule stabilization. Moreover, another genetic screen study has identified EFA-6, an Arf guanine nucleotide exchange factor, as a negative regulator of axon regeneration *via* destabilizing axonal microtubules[18]. Because the loss-of-function mutant of EFA-6 partially bypasses the requirement of DLK-1 for axon regeneration, it is likely that EFA-6 is also a downstream mediator of DLK-1. What are the upstream regulators of the DLK-1 pathway? First, an axotomy-induced Ca^{2+} increase through voltage-gated Ca^{2+} channels or internal Ca^{2+} stores is necessary for axon regeneration in *C. elegans*[19]. The promoting effect of Ca^{2+} signaling on axon regeneration is mediated by activation of adenyl cyclase (AC) and the subsequent elevation of cAMP, which in turn activates PKA[19]. Importantly, the Ca^{2+}/AC/cAMP/PKA pathway acts upstream of DLK-1 to promote axon regeneration. Second, a very recent study[20] has shown that the core apoptotic executioner protein CED-3 (caspase) and its activator CED-4 (Apaf-1) play surprising roles in controlling axon regeneration by acting upstream of DLK-1. Interestingly, in the same study, the CED-4/CED-3 pathway was also placed downstream of axotomy-induced local Ca^{2+} influx.

In the same RNAi screen using a β-spectrin mutant that identified DLK-1[9], another MAPKKK, MLK-1, was shown to control axon regeneration in parallel with the DLK-1 pathway[21] (Fig. 2). Downstream mediators of MLK-1 are MEK-1 and KGB-1 (JNK). JNK signaling is also upregulated in *Drosophila* upon axonal injury in a brain needle-injury model[22] and a crushed larval ventral nerve cord model[23]. In a *Drosophila* whole-brain culture model, expression of dominant-negative JNK blocks axon regeneration, whereas expression of the constitutively active form of *Drosophila* JNK kinase induces robust regrowth of the severed axons, one-third of which even extend to re-enter the target area[24]. Because JNK is well known to regulate axonal microtubules in mammalian neurons during axon growth[25-28], it is likely that the MLK-1/MEK-1/KGB-1 pathway in *C. elegans* also targets microtubules for controlling axon regeneration. Additional studies in *C. elegans* have shown that SVH-1, a homolog of mammalian hepatocyte growth factor and its receptor SVH-2 function upstream of the MLK-1 pathway to promote axon regeneration[29], whereas SVH-3, a homolog of mammalian fatty acid amide hydrolase, inhibits axon regeneration through antagonizing the MLK-1 pathway[30].

In summary, two MAP kinase pathways function in *C. elegans* to regulate naturally-occurring axon regeneration, and both pathways mainly act locally at the axon to control microtubule reorganization and growth-cone remodeling upon axotomy (Fig. 2).

Signaling at the Neuronal Soma

The large-scale genetic screen in *C. elegans*[18] has also identified several transcription factors that may regulate axon regeneration by modulating gene expression in the soma. However, no detailed study of these molecules has been reported yet. One unexpected player that may function to control gene expression during *C. elegans* axon regeneration is the protein Notch[31]. Specifically, Notch acts as a negative regulator of axon regeneration independent of extracellular Notch ligands. Downstream of Notch, ADAM metalloprotease and γ-secretase in the canonical pathway cleave the Notch to generate the Notch intracellular domain (NCID), which translocates into the nucleus to control gene expression. Importantly, direct ectopic expression of GFP-NCID is sufficient to allow entry into the nucleus and inhibit axon regeneration, confirming the role of Notch signaling in the regulation of gene expression. Axons in the zebrafish optic nerve are able to regenerate after injury. By comparing gene expression in injured and uninjured neurons, Krüeppel-like factors (KLFs) 6/7 have been shown to regulate the axon regeneration of zebrafish retinal ganglion cells[32]. A subsequent study showed that KLF6/7 regulate axon regeneration by controlling the transcription of the tubulin isoform Tuba1a[33]. How Notch signaling and KLF6/7 are regulated by upstream signaling pathways is not known.

In sum, very few signaling pathways have been shown to control gene expression during axon regeneration. Future studies using these non-mammalian models should focus on somatic signaling to expand our knowledge of how gene expression is regulated during successful axon regeneration.

Signaling Pathways That Regulate Mammalian Axon Regeneration

Adult Dorsal Root Ganglion (DRG) as a Model System for Mammalian Axon Regeneration

Among adult PNS neurons, DRG neurons provide

a favorable model for studying mammalian axon regeneration. Each DRG neuron extends a unipolar axon that splits into two branches: one innervates peripheral targets (peripheral branch) and the other enters the spinal cord (central branch). The peripheral branch regenerates robustly after peripheral nerve injury, whereas the central branch in the spinal cord fails to regenerate after spinal cord injury. However, if axotomy of the peripheral branch occurs prior to central branch axotomy (a pre-conditioning lesion), regeneration of the central axons is greatly enhanced[34]. Recent studies found that this effect could be achieved if the peripheral axotomy was performed at the same time[35] or even after the central axotomy[36]. These results indicate that peripheral axotomy increases the intrinsic ability of DRG neurons to support axon regeneration by activation of a transcription-dependent regeneration program and the subsequent expression of a group of regeneration-associated genes in the soma[37]. Because the central branches of the DRG neurons share the same environment as corticospinal axons, it is believed that activation of a similar regeneration program in adult CNS neurons would enhance CNS axon regeneration. Unfortunately, the signaling pathways underlying this peripheral axotomy-induced transcriptional program remain elusive. As noted, it is very difficult to dissect signaling pathways *in vivo* in the adult mouse model because the genetic modulation of multiple genes specifically in adult tissues is required. Therefore, to date no signaling pathways (consisting of multiple upstream/downstream signaling molecules) that regulate naturally-occurring mammalian axon regeneration have been delineated *in vivo*. A novel *in vivo* electroporation technique has recently been developed that allows specific manipulation of multiple gene expression in adult DRG neurons simultaneously[38], providing a potentially useful tool for the *in vivo* dissection of pathways regulating mammalian axon regeneration.

In contrast to the *in vivo* model, adult DRG neurons can readily be cultured and therefore have been widely used to study axon growth from mature neurons *in vitro*[39]. Depending on the experimental procedure or the culture conditions, *in vitro* axon growth from adult DRG neurons can be stimulated and regulated by distinct mechanisms and signaling pathways[40, 41]. Therefore, to study regeneration pathways, it is important to use an *in vitro* model that mimics *in vivo* peripheral axotomy-induced axon regeneration. If an *in vivo* peripheral nerve injury is performed prior to culture, the cultured DRG neurons are able to extend long axons overnight. Such peripheral axotomy-induced regenerative axon growth often starts within a few hours after cell plating and the axons extend with few branches (elongation mode)[41]. In contrast, naïve (uninjured) DRG neurons cultured at low density grow few axons in the first 24 h in the absence of growth factors. Addition of growth factors (e.g. NGF or GDNF) induces robust axon growth. However, such NGF-induced growth is regulated by a different pathway from that of peripheral axotomy-induced axon growth (see below) and the processes show a highly-branched morphology (sprouting mode)[39, 41]. Together, this evidence suggests that peripheral axotomy-induced regenerative axon growth and a culture condition without axon-promoting growth factors should be the model of choice for studying regeneration signaling pathways *in vitro*. Signaling pathways regulating mammalian axon regeneration in the PNS are summarized in Fig. 3.

Signaling at the Growth Cone

One way to spatially dissect signaling pathways in neurons is the two-compartmental culture system that separates somata from axons both physically and chemically, such as the traditional Campenot chamber[42, 43] and the recently-developed microfluidic-based chambers[44, 45]. Using such approaches, previous studies have shown that PI3K and ERK, two major signaling molecules mediating NGF-induced axon growth[46], are both required locally at the axon to control axon growth from developing sensory neurons[47]. To our knowledge, no similar study has been reported for regenerative axon growth from adult neurons. However, an interesting observation of peripheral axotomy-induced regenerative axon growth from adult DRG neurons is that active gene transcription is not required for axon growth in the first 24 h[41, 48]. Such a property therefore provides a unique opportunity for investigating transcription-independent signaling mechanisms that specifically regulate local axon assembly. In contrast to that of developing DRG neurons, regenerative axon growth from adult DRG neurons does not require PI3K and ERK activity for local axon assembly[40, 41], indicating growth factor-independent pathways for PNS axon regeneration. Indeed,

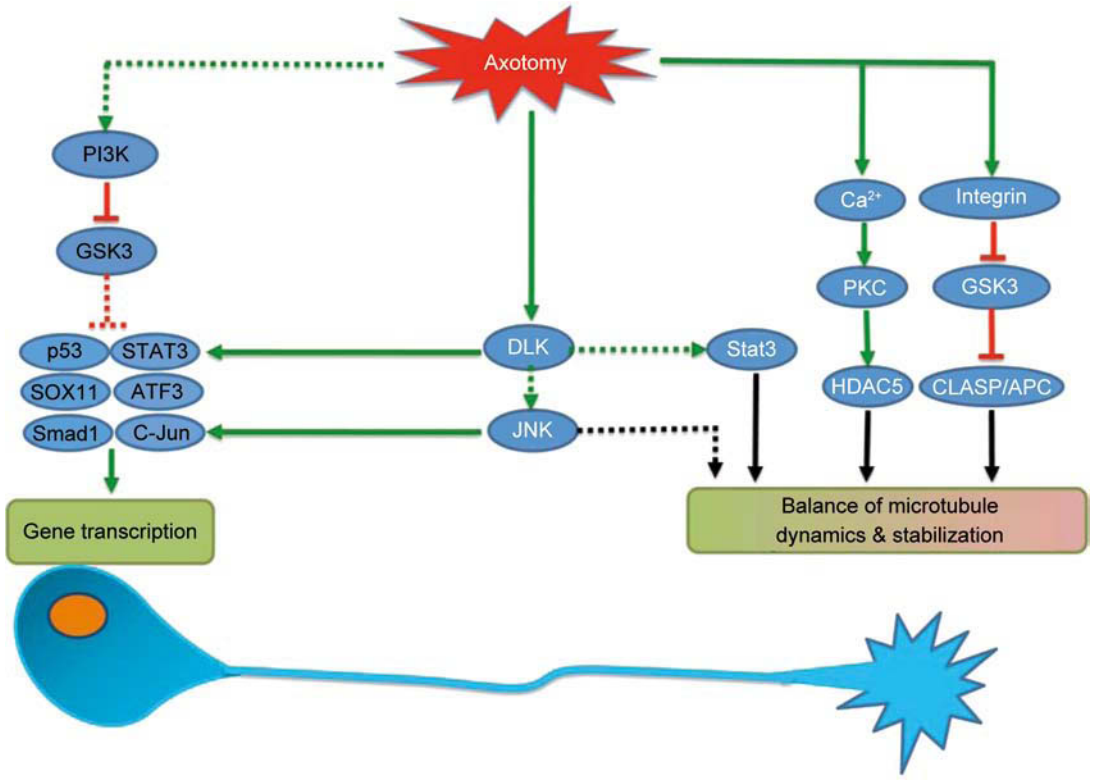

Fig. 3. Signaling pathways regulating naturally-occurring mammalian axon regeneration in the peripheral nervous system. In the soma, peripheral axotomy upregulates and activates many regeneration-associated transcription factors, such as p53, Stat3, Sox11, ATF3, Smad1, and c-Jun, which control gene expression that supports axon regeneration. The PI3K–GSK3 and DLK–JNK pathways are potential upstream regulators of these transcription factors. At the growth cone, the integrin–GSK3–CLASP/APC pathway stabilizes axonal microtubules, whereas the Ca^{2+}–PKC–HDAC5 pathway maintains microtubule dynamics. The coordination of the two pathways controls the balance of microtubule dynamics and stabilization to achieve optimal microtubule assembly and axon growth. The DLK–Stat3/JNK pathways may also be involved in the regulation of microtubule assembly during axon regeneration.

application of a function-blocking antibody to NGF is unable to inhibit peripheral nerve regeneration[49], providing solid evidence that *in vivo* peripheral axon regeneration does not require NGF. Consistent with this, adult DRG neurons undergoing peripheral axotomy are able to extend long axons in culture in the absence of growth factors[41].

Instead, activation of integrin signaling by laminin is sufficient to induce robust axon growth from these pre-axotomized DRG neurons, suggesting that integrin signaling plays a key role in peripheral nerve regeneration. Indeed, laminin isoforms are highly enriched in peripheral nerve pathways[50], and loss of integrin subtype alpha 7 impedes facial nerve regeneration *in vivo* after a lesion[51]. Downstream of integrin signaling, regenerative axon growth is mediated by the inactivation of glycogen synthase 3 (GSK3)[41], which in turn controls growth-cone microtubule assembly *via* the microtubule-binding protein adenomatous polyposis coli (APC) and CLIP-associating proteins (CLASPs)[52]. Both APC and CLASPs are microtubule plus-end tracking proteins that stabilize dynamic microtubules without interfering with microtubule polymerization[53]. Down-regulation of CLASPs in adult DRG neurons reduces tubulin acetylation/microtubule stability and inhibits sensory axon regeneration *in vivo*[52], providing strong evidence that regulation of axonal microtubule dynamics is important for successful axon regeneration.

In addition to microtubule-binding proteins, microtubule dynamics are also regulated by post-translational modification, in particular acetylation, which is associated with increased microtubule stabilization[54]. A recent study showed that

peripheral axotomy induces tubulin deacetylation and therefore increases microtubule dynamics in axons at the proximal injury site[55]. Inhibition of tubulin deacetylation with a pharmacological inhibitor of histone deacetylase (HDAC) blocks sensory axon regeneration *in vivo*, suggesting that maintaining microtubule dynamics is necessary for axon regeneration. Importantly, using the Campenot chamber culture system, it was shown that inhibition of HDAC locally at the axon is sufficient to block axon growth, confirming that HDAC acts locally on axonal microtubules to control axon growth rather than at the soma to control gene transcription. HDAC5 has been identified as the deacetylase that mediates peripheral axotomy-induced tubulin deacetylation. Further studies showed that axotomy-induced Ca^{2+} influx and the subsequent activation of PKC act upstream of HDAC5 to regulate tubulin acetylation and axon growth.

Together, these studies highlight the important role of axonal microtubule dynamics in the regulation of mammalian axon regeneration. Notably, either down-regulation of CLASPs which reduces tubulin acetylation[52], or inhibition of HDAC which increases tubulin acetylation[55], blocks sensory axon regeneration, suggesting that a balance of microtubule stabilization and dynamics is required for optimal axon regeneration.

Signaling at the Neuronal Soma

Gene expression at the soma, which provides the raw materials for axon assembly, is necessary for sustained axon growth. The regeneration-associated genes identified by genetic profiling include many transcription factors, such as c-Jun, Stat3, ATF3, Smad1, p53, and SOX11 (Fig. 3). It is well recognized that these factors control the transcription of genes that support peripheral axotomy-induced axon regeneration[56, 57]. How upstream signaling pathways regulate the expression and/or activation of these factors is less clear.

Genetic deletion of Pten and the subsequent activation of the PI3K–Akt pathway have recently been shown to drastically promote the intrinsic axon growth of mature mammalian CNS neurons by controlling the mTor pathway[1,2,4]. Does the PI3K pathway play any role in the regulation of intrinsic axon growth during naturally-occurring PNS axon regeneration? In a mutant mouse line, where p110δ, the endogenous catalytic subunit of PI3K, is replaced with an inactive mutation, sensory axon regeneration is greatly impaired[58]. Because the PI3K pathway is not required for local axon assembly during sensory axon regeneration[40,41], this result suggests that PI3K regulates mammalian sensory axon regeneration by controlling gene expression in the soma. Indeed, in such p110δ mutant mice, the expression level of Sprr1A, a well-known regeneration-associated gene, is significantly diminished. How PI3K signaling regulates the expression of Sprr1A is not known, but it is most likely through controlling transcription factors. For instance, many of the regeneration-associated transcription factors are regulated by GSK3, which is a major downstream signaling mediator of PI3K[57]. Future study using a compartmentalized culture system is required to provide a more definitive answer regarding the role of PI3K in the regulation of gene expression during mammalian axon regeneration.

As discussed earlier, in *C. elegans*, DLK-1 regulates axon regeneration mainly by acting at the growth cone (Fig. 2). In contrast, recent studies of DLK knockout mice have shown that mammalian DLK controls axon regeneration *via* the activation of c-Jun and Stat3[59, 60], suggesting a role of DLK in controlling gene expression in the soma (Fig. 3). Further experiments showed that DLK does not directly activate Stat3 but regulates the retrograde transport of activated Stat3 from the injury site[59]. Although not tested directly, it is likely that DLK regulates c-Jun activation through JNK. To date, however, there is no direct evidence showing that DLK regulates peripheral axotomy-induced gene expression, and future genetic-profiling studies of DLK knockout mice may provide an answer. It should be noted that besides regulating transcription factors, DLK–JNK has also been shown to regulate developmental axon growth by controlling microtubule stability at the growth cone[61]. Whether DLK–JNK signaling also regulates axonal microtubules during mammalian axon regeneration awaits further study. Moreover, a new study[62] found that Stat3 controls microtubule stability locally in the axon independent of its transcriptional activity, raising the possibility that DLK–Stat3 activated at the injury site after peripheral axotomy may also play a local role in the regulation of axon regeneration. Together, the evidence to date suggests that DLK signaling regulates mammalian axon regeneration by controlling gene expression in the

neuronal soma and/or microtubule dynamics in the growth cone.

Summary and Future Directions

Successful axonal regeneration in the injured nervous system is a complex process that involves coordinated regulation of gene expression in the soma and cytoskeleton assembly at the growth cone. The emergence of non-mammalian injury and regeneration models has provided new opportunities to identify signaling pathways that regulate naturally-occurring axon regeneration. Because the genetic approaches used in the non-mammalian models are not available for mammalian models, it is not practical to perform similar large-scale genetic screening in mice to identify mammalian axon regeneration pathways. Therefore, verifying if signaling pathways identified in the non-mammalian models also function in mammalian models will be the focus of future studies. The recently-developed technique of *in vivo* electroporation of adult sensory neurons[38] would make such studies more feasible and efficient.

Most of the axon regeneration pathways identified to date are located at the growth cone (see Figs. 2 and 3), and much less is known about signaling pathways functioning at the soma to control gene expression. Because the manipulation of gene expression (transcription or translation) has proven effective in promoting CNS axon regeneration[1-5], elucidating the signaling pathways that regulate gene expression during naturally-occurring axon regeneration in the mammalian PNS should become a major focus of axon regeneration research.

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