

● PERSPECTIVE

The necessary role of mTORC1 in central nervous system axon regeneration

Permanent loss of vital functions after central nervous system (CNS) injury, *e.g.*, blindness in traumatic optic nerve (ON) injury or paralysis in spinal cord injury, occurs in part because axons in the adult mammalian CNS do not regenerate after injury. Growth failure is due to the diminished intrinsic regenerative capacity of mature neurons and the inhibitory environment of the adult CNS. Neutralizing extracellular inhibitory molecules genetically or pharmacologically yields only limited regeneration and functional recovery, highlighting the critical importance of neuron-intrinsic factors. To explore the intrinsic regenerative signaling molecules, a relatively simple but robust *in vivo* model that replicates CNS traumatic injury and permits straightforward interpretation is required. Mouse retinal ganglion cell (RGC) and ON provide a valuable *in vivo* neural injury system to study intrinsic growth mechanisms in adult CNS neurons (**Figure 1**). Retina and ON are CNS structures that are essential for visual functions. RGC is the only neuronal type to relay visual information from retina to brain. The ON is formed by the projection axons sent exclusively from RGCs; it has the simplicity of a unidirectional axon pathway, which insures that any nerve fibers observed distal to a complete crush injury have regenerated and do not represent spared axons that underwent collateral sprouting or efferent axons from the brain to the retina. Its easy access allows adeno-associated viruses to be injected directly into the vitreous chamber of the eye and express transgenes specifically and efficiently in adult RGCs. This spatially and temporally controlled genetic manipulation allows us to overcome developmental issues associated with germ line manipulation and to test interventions that can potentially be translated to therapies. Exploiting the anatomical and technical advantages of the RGC/ON crush model to understand the intrinsic mechanisms of regenerative failure led us to the finding that deletion of phosphatase and tensin homolog (PTEN) promoted significant ON regeneration (Park et al., 2008). PTEN, a lipid phosphatase, is a major negative regulator of the phosphatidylinositol 3-kinase (PI3K)-mammalian target of rapamycin complex 1 (mTORC1) pathway. Similar axon regeneration phenotypes after PTEN deletion have been reported for mouse cortical motor neurons (Liu et al., 2010), drosophila sensory neurons (Song et al., 2012) and *C. elegans* motor neurons (Byrne et al., 2014), presumably through activating PI3K-mTORC1-controlled cell growth (**Figure 2**). Direct activation of mTORC1 also promotes axon regeneration in dopaminergic neurons (Kim et al., 2011) and peripheral nerves (Abe et al., 2010), further support

for the critical role of mTORC1 in axon regeneration.

PI3K is a lipid kinase which can be activated by growth factors, such as insulin and insulin-like growth factor-1 (IGF1), through receptor tyrosine kinase (RTK). PI3K subsequently phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP₂) in the lipid membrane to produce phosphatidylinositol (3,4,5)-triphosphate (PIP₃). PIP₃ in turn recruits AKT, a member of the AGC family of serine/threonine kinases, to the membrane to be activated by phosphorylation *via* phosphoinositide-dependent kinase-1 (PDK1). One of the multiple AKT downstream effectors is the complex formed by tuberous sclerosis 1 and 2 (TSC1/TSC2) heterodimer, the negative regulator of mTORC1. Thus AKT activation removes the inhibition of TSC and activates mTORC1. mTOR is a serine/threonine protein kinase that interacts with other proteins to form a functional complex, mTORC1, which acts as a key downstream signal of the PI3K-AKT pathway to regulate cell metabolism, cell growth and cell survival by promoting translation and protein synthesis (Laplante and Sabatini, 2012).

PTEN converts PIP₃ to PIP₂ and thus inhibits the activation of downstream effectors of PI3K. Deletion of PTEN therefore results in constitutive activation of the PI3K-AKT-mTORC1 pathway, suggesting that the control of translation and protein synthesis through mTORC1 plays an important role in determining the intrinsic regenerative ability of injured adult CNS neurons. Thus mTORC1 and its downstream effectors are logical therapeutic targets for enhancing axon regeneration and functional recovery after neural injury. Unfortunately, over-activation of mTORC1 signaling can also result in tumor formation, metabolic diseases, and neurological disorders due to uncontrolled protein synthesis and cell proliferation. Clearly the clinical usefulness of mTORC1 activation is limited by the threat of deleterious side effects such as malignancy and cognitive deficits. Understanding of how mTORC1 regulates cellular functions is surprisingly limited, however, because very few of its direct substrates have been identified. It is extremely intriguing scientifically and critical clinically to elucidate the mechanisms by which mTORC1 regulates neuron-intrinsic growth ability. This information has the potential to identify the targets by which mTORC1 promotes axon regeneration, and to isolate them from targets that mediate mTORC1's deleterious effects.

The two best-characterized downstream signaling molecules of mTORC1 are ribosomal protein S6 kinase (S6K) and eukaryotic translation initiation factor 4E (eIF4E)-binding protein (4E-BP). Phosphorylation of 4E-BP by mTORC1 releases its binding from eIF4E, enabling incorporation of eIF4E into eIF4F complex to initiate cap-dependent translation. S6K is phosphorylated and activated by mTORC1 to promote mRNA biogenesis, translation initiation/elongation and lipid synthesis (Laplante and Sabatini, 2012). Although 4E-BP inhibition and S6K activation are both downstream of mTORC1 activation,

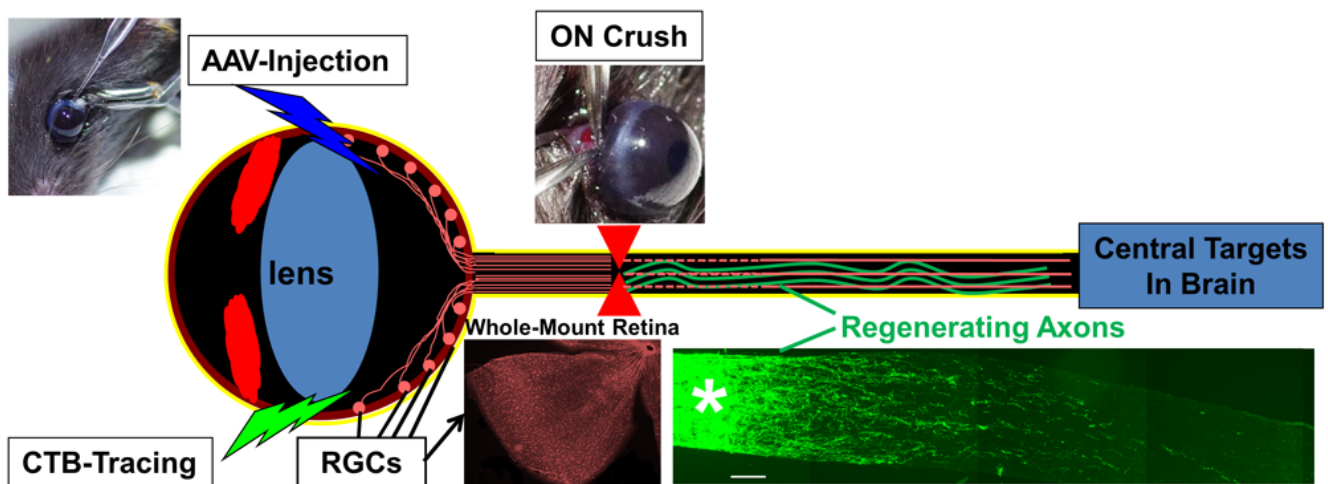


Figure 1 RGC/ON crush provides an *in vivo* CNS axon injury/regeneration model with advantages that include: straightforward anatomy, clear readout, easy access, amenable to genetic manipulation and relevant diseases. Mouse eye can be injected with AAV for genetic manipulation of RGCs and fluorescence-labeled CTB for axon tracing. Whole-mount retina is for RGC detection and ON longitudinal cryostat section is for visualizing regenerating axons. RGC: Retinal ganglion cell; ON: optic nerve; CNS: central nervous system; AAV: adeno-associated viruses; CTB: cholera toxin subunit B.

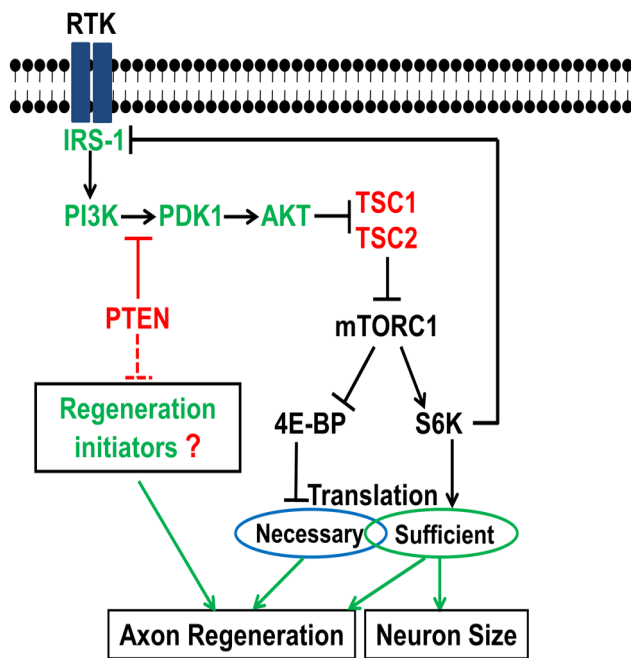


Figure 2 Schematic summary of the PTEN/mTORC1 signaling pathways in CNS axon regeneration.

The necessary role of mTORC1 and its substrates 4E-BP and S6K is illustrated. The green coded molecules are positive regulators of mTORC1 and the red coded molecules are negative regulators. The question mark represents unknown effectors downstream of PTEN that are sufficient to initiate CNS axon regeneration, and which potentially interact with the translational targets of mTORC1 to promote more significant axon regeneration. PTEN: phosphatase and tensin homolog; mTORC1: mammalian target of rapamycin complex 1; CNS: central nervous system; 4E-BP: eukaryotic translation initiation factor 4E-binding protein; S6K: S6 kinase; PI3K: phosphatidylinositol 3-kinase; RTK: receptor tyrosine kinase; IRS-1: insulin receptor substrate 1; PDK1: phosphoinositide-dependent kinase-1; TSC: tuberous sclerosis.

and both promote protein synthesis, previous studies suggested that S6K and 4E-BP differentially control cell growth and proliferation: S6K controls cell size but not cell cycle progression, whereas 4E-BP controls cell proliferation but not cell size. Consistently, over-expression of the constitutively active mutant of S6K1, but not deletion of 4E-BP1 and 4E-BP2, significantly increases RGC cell size after ON crush (Yang et al., 2014). Moreover, both of these translational control pathways downstream of mTORC1 contribute to axon regeneration, albeit through distinct mechanisms: S6K1 activation, but not 4E-BP inhibition, is sufficient to promote axon regeneration to a small degree; 4E-BP1-4A mutant, however, which cannot be phosphorylated and inhibited by mTORC1, inhibits axon regeneration to a much larger degree than S6K1 dominant negative mutant in PTEN knockout (KO) mice (Yang et al., 2014). Additionally, the combination of S6K1 activation and 4E-BP KO in RGCs does not promote more axon regeneration than S6K1 activation alone. Thus the current evidence suggests that S6K1 is not the primary mediator of PTEN KO signaling and that 4E-BP inhibition by mTORC1 is more important than S6K1 for the PTEN KO effect on axon regeneration. We have not ruled out the possibility that other substrates of mTORC1 in addition to S6K1 and 4E-BP may contribute to axon regeneration in a more active way, however, we do favor the idea that the mTORC1 pathway essentially plays a necessary role in axon regeneration which requires a key permissive signal from unidentified effectors downstream of PTEN to trigger the neuron-intrinsic growth machinery. Our future efforts will focus on identifying these critical initiators, whose activation is sufficient to allow significant axon regeneration and which can be combined

with other necessary boosters, such as 4E-BP effectors, to achieve even more robust regeneration. Identification of the complete sets of translational targets of S6K and 4E-BP in CNS neurons will be required to better understand how they contribute to axon regeneration and to determine whether these effectors can be manipulated to avoid mTORC1's undesirable actions. Another intriguing idea is to explore the possible role and potential translational targets of 4E-BP and S6K1 in axons, given the increasing evidence for the important contribution of intra-axonal protein synthesis to axon regeneration in peripheral nervous system (Willis and Twiss, 2006).

Proper translational control is crucial for normal cell growth. The increased protein synthesis induced by PI3K-mTORC1 activation needs to be balanced by an antagonistic mechanism. It has previously been shown that S6K1 participates in a negative feedback loop that regulates Insulin/Insulin receptor/PI3K signaling by phosphorylating insulin receptor substrate 1 (IRS-1) to reduce activities of PI3K and its downstream effectors (Laplante and Sabatini, 2012). Indeed we detected decreased axon regeneration in PTEN KO mice after activation of S6K1, presumably through feedback inhibition of PI3K-dependent and S6K1-independent mechanisms. Possibly through a similar mechanism, S6K inhibits axon regeneration in *C. elegans* (Hubert et al., 2014). It would not be surprising if additional balancing mechanisms fine-tune the growth control loop.

Our studies provide evidence that differential translational control of mTORC1 and its substrates is an important determinant of the neuron's intrinsic growth ability, and reveal the more complicated cross-regulating mechanisms among PTEN, PI3K and mTORC1 signaling pathways. Future investigations will elucidate the interactions among S6K1-dependent signaling, the necessary targets of 4E-BP-dependent signaling, the uncharacterized substrates of mTORC1 and the PTEN/PI3K-dependent but S6K1-independent effectors that guide cohesive axon regeneration. Importantly, the readily available models for additional diseases that are associated with ON degeneration besides traumatic crush injury, such as autoimmune or virus induced optic neuritis (Shindler et al., 2006; Khan et al., 2014) and glaucoma, will help to determine whether therapies targeted to the PTEN-mTORC1 pathway also enhance neural repair in these neurodegenerative diseases.

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