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Neuronal intrinsic barriers for axon regeneration in the adult CNS

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Abstract

A major reason for the devastating and permanent disabilities after spinal cord and other types of CNS injury is the failure of injured axons to regenerate and to re-build the functional circuits. Thus, a long-standing goal has been to develop strategies that could promote axon regeneration and restore functions. Recent studies revealed that simply removing extracellular inhibitory activities is insufficient for successful axon regeneration in the adult CNS. On the other side, evidence from different species and different models is accumulating to support the notion that diminished intrinsic regenerative ability of mature neurons is a major contributor to regeneration failure. This review will summarize the molecular mechanisms regulating intrinsic axon growth capacity in the adult CNS and discuss potential implications for therapeutic strategies.

Introduction

Understanding why injured axons cannot regenerate after injury in the adult mammals has been a major challenge for both basic and clinical neuroscientists. Previous elegant studies by Aguayo and his colleagues showing that some injured CNS axons were able to grow into a permissive graft transplanted to the lesion site suggested that inhibitory activities in the lesion sites might be primarily responsible for preventing axon regeneration [1]. Thus, extensive studies in the past decades have been aimed at characterizing the molecular identities and functional mechanisms of these inhibitors. As a result, multiple molecules highly inhibitory to axon growth have been identified. They are associated with either myelin debris (eg, MAG, Nogo-A, and Omgp), or with glial scar formation (eg. CSPG and tenasin) [2–6]. Signaling pathways for these inhibitors have also been discovered. For example, a recent study suggests that a receptor tyrosine phosphatase acts as a functional receptor for CSPGs [7*]. However, removing these molecules by genetic deletions or pharmacological inhibitions only allows limited sprouting, but is not sufficient for long-distance axon regeneration [5,8]. These observations demand re-consideration of the intrinsic regenerative ability of mature neurons.

It is known that axon re-growth involves expressions of regeneration-associated genes (RAGs) such as *GAP-43, Cap23, Arg1, and Sprr1a*. In order to initiate the transcription program for axon regeneration, an injury signal is first generated by the lesioned axon and relayed to the neuronal soma (Figure 1a). However, not all neurons respond to injury signals in the same way. Whether successful axon regeneration could occur depends on the intrinsic competence of injured neurons in launching a growth program (Figure 1b). Recent studies from c. elegans,

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zebrafish and rodents revealed the possible molecular identities of injury signals and competence determinants.

Injury signals

Upon axotomy, different changes could occur at the injured axonal terminal, along the axon shaft, as well as in the neuronal soma. For example, the lesion site may have rapid ion influxes. In cultured neurons, axotomy leads to a significant increase in local calcium concentrations that rapidly trigger various responses in the soma [9]. At least in Aplysia, such calcium increase is important for initiating axonal regrowth program [10,11]. In addition to these acute changes, extensive evidence also suggest a set of slower propagating injury-induced signals propagating slower than calcium waves which comprise a decrease in the trafficking of trophic factors to the soma and an increase in the transport of new injury-induced molecular signals from the lesion site to the soma [12–14].

Nuclear transport proteins: Importins, RanGTP and JIP

Early studies in Aplysia and recent findings in the PNS neurons of rodents suggested that injuries triggered certain signaling molecules with nuclear localization signal (NLS) to be transported to the nucleus and initiate transcriptional program for axon regeneration. At least three classes of nuclear transport systems have been implicated and these include importins [15], RanGTP [16] and JNK-interacting proteins (JIP) [17]. Some components of importins and RanGTP are constitutively expressed in the axons of intact PNS neurons, but are insufficient to be functional. Axotomy triggers the local synthesis of other critical components such as importin-b [15], and Ran-binding protein RanBP1 [16], which allow the activation of these nuclear import systems. Similarly, injury also results in the activation of JNK3 in the axon, which will be relayed to the nucleus in activating the expression of c-Jun and other molecules [18,19].

Cargos of the injury-activated nuclear transport systems are also being revealed. Perlson et al suggested that vimentin might be also a candidate of cargo for injury-induced retrograde transport [20]. Zou et al showed that peripheral axon injury could activate the nuclear import of Smad1, a critical signaling mediator of BMPs, which promote axon growth in adult sensory neurons [21]. Recent interesting genetic studies in C. elegans identified the DLK (dual leucine zipper-bearing kinase 1) MAP kinase pathway as a positive regulator of growth cone formation and axon regeneration [22**,23**]. DLK-1 is a component of a conserved MAPK cascade that includes the MAP kinase kinase MKK-4 and the p38 MAP kinase PMK-3. Loss-of-function mutations of the dlk-1, mkk-4 or pmk-3 gene result in axon regeneration defects, suggesting that this entire signaling pathway is required for axon regeneration. Furthermore, activated PMK-3 is likely to be transported to the nucleus for its biological function [22**]. It is less clear though, whether these pathways are important for neuronal survival after injury, or directly involved in regulating axon re-growth, or both in the adult CNS. For example, Erk pathway was shown to be critical for BDNF-induced corticospinal regeneration after a subcortical injury model [24]. Similarly, Mammalian sterile 20-like kinase-3b (Mst3b, encoded by Stk24), an Erk downstream signaling molecule, has also been implicated in promoting axon growth and regrowth [25]. However, over-expression of Erk1/2 promoted neuronal survival, but not axon regeneration, after optic nerve injury model [26].

Injury-triggered expression of cytokines

Several studies suggested that axotomy triggers the expression of cytokines such as interleukin-6 (IL-6), cilliary neurotrophic factor (CNTF) in the lesion sites after peripheral nerve injury [27–29]. These cytokines are known to act through their receptor complexes with a shared protein gp130 [30]. Downstream signaling mediators of this pathway are JAK-STAT

cascade. Injury at the peripheral, but not central, axon branch of dorsal root ganglion (DRG) sensory neurons results in accumulation of phospho-STAT3 in the nucleus [31,32], which correlates with the activation of axon regeneration program [33,34]. In vitro, while inhibitors of ERK or PI3K could block neurite growth in embryonic DRG neurons, a JAK2 inhibitor could efficiently abolish outgrowth from adult DRG neurons with a peripheral lesion [35]. These findings suggest a critical role of this pathway in mediating the effects of a peripheral lesion on enhancing the regenerative ability. Furthermore, cytokines such as IL-6 could mimic the growth-promoting effects of a conditioning lesion and cAMP [36,37]. However, IL6 knockout mice showed normal axon regeneration with or without a conditioning lesion [37].

Several reports have shown that cytokines such as CNTF and LIF are up-regulated in the retina, likely in astrocytes, after optic nerve injury [38,39], although how axotomy leads to their up-regulation remains unknown. The importance of injury-induced cytokines in axon regeneration was shown in studies of lens injury-triggered enhancement of axon regeneration of adult retinal ganglion cells (RGCs) [40*]. Lens injury or intravitreal application of zymosan could switch RGCs into an active regenerative state, enabling these neurons to survive axotomy and to regenerate axons into the injured optic nerve [41,42]. Molecules such as oncomodulin were previously shown to be involved in lens injury enhanced axon regeneration [43–45]. More recently, Leibinger et al showed that the effect of lens injury is reduced in CNTF−/− mice and completely blocked in CNTF−/− LIF−/− double mutant mice [40*].

Surprisingly, exogenously delivered cytokines have limited effects on promoting survival and regeneration following either optic nerve [46,47] or spinal cord injury [48]. These findings have puzzled the field until a recent study provided a plausible explanation. Park et al developed a highly efficient method to conditionally delete genes in adult retinal ganglion neurons (RGCs) by injecting Cre-expressing AAV into the vitreous body of floxed mice [49**]. This allows one to assess the effects of RGC-specific gene knockout on axon regeneration after optic nerve injury in adult mice. Among different floxed alleles tested, Smith et al found extensive axon regeneration upon conditional deletion of SOCS3, a negative regulator of the JAK-STAT pathway [50**]. By contrast, no RGC axon regeneration was observed when SOCS3 and gp130 were both deleted, indicating that the regeneration of SOCS3 mutant axons is dependent on cytokine ligands of gp130. Consistently, exogenous application of CNTF to SOCS3 deleted mice could dramatically increase the extent of axon regeneration [50**]. Another interesting observation is that cAMP-induced outgrowth-promoting effects may be partially mediated by down-regulation of SOCS3 [38]. Since SOCS3 is a well-known transcription target of the JAK-STAT pathway [51], in wildtype neurons, despite injury-induced induction of cytokines, the presence and induced expression of SOCS3 put a strong negative brake to prevent axon regeneration (Figure 2). In addition to confirming the role of the JAK/STAT pathway in promoting axon regeneration, these new observations highlight the dominant role of negative regulators of signaling pathways in restricting axon regeneration in adult neurons. Thus, it will be interesting to find out what other negative regulators of JAK-STAT and other growth factor signaling pathways (for example BDNF as show in [52]) also act as intrinsic barriers of axon regeneration.

Neuronal competence of axon regeneration

Obviously, CNS neurons differ in their responses to injuries and injury-induced signals. While some axotomized neurons undergo cell death, those that survive the injuries differ in their abilities to initiate axon regeneration. Thus, an important question is what determines the intrinsic competence of neurons to regenerate injured axons.

Regeneration-associated transcription factors

In order to identify possible master control gene(s) for axon regeneration, microarray based experiments have been performed to compare gene expression differences in neurons with or without enhanced axon regeneration. Several models have been used and these include DRG neurons with or without pre-conditioning lesions [53–56], adult RGCs with or without lens injuries [57], and adult RGCs from regeneration-competent species such as zebrafish [58*]. In addition, Goldberg et al found a development-dependent decline of axon growth ability of rodent RGCs [59] and thus RGCs of different developmental stages were also used in searching for genes critical for axon regeneration [60].

Studies from DRG neurons with a conditioning lesion identified several transcription factors such as c-Jun [61,62], c-EBP [63], CREB [64], STAT3 [32,65], ATF3 [19,62,66,67], SOX11 [68,56], and Smad1 [21], as positively associated with axon regeneration. Indeed, constitutive expression of ATF3 in neurons of Thy-1.2 ATF3 transgenic mice enhanced PNS regeneration [69]. C/EBPβ is also up-regulated and phosphorylated after peripheral injury in rodent, which is needed for injury-induced up-regulation of α-tubulin and GAP-43 [63]. On the flip side, transcription factor such as NFIL3 was implicated as an injury-induced transcription suppressor of axon regeneration, likely by antagonizing the positive effects of CREB family members [70].

Recent studies from several groups pointed to a critical role of Kruppel-like factors (KLFs), which are zinc-finger transcription factors, in axon growth control. These genes were initially implicated in regulating cell cycle exit and terminal differentiation in non-neuronal cells [71]. KLF4 is one of the four transcription factors sufficient to transform fibroblasts into pluripotent stem cells [72]. In zebrafish, KLF6 and KLF7 were identified among the group of up-regulated genes in regenerating RGCs [58*]. Importantly, knockdown of these molecules reduces axon growth [58*,73]. In an independent study, KLF4 was found to be a potent inhibitory molecule for axon growth in embryonic hippocampal neurons and RGCs [60**]. Interestingly, these different KLFs differ in their expression levels over the course of development: while KLF6/7 are down-regulated, KLF4/9 are up-regulated in adult RGCs [60**]. Consistently, over-expression of different KLFs results in opposite effects on neurite growth in cortical neurons [60**]. These studies provide an example of how complicated transcriptional factor networks regulate the process of axon growth and regeneration. Thus, differences in the ability to express growth-promoting versus growth-inhibitory transcription factors may result in different intrinsic regenerative competence in adult neurons.

mTOR and protein translation in axon regeneration

In an effort to analyze the effects of knockout of individual genes involved in cellular growth control on axon regeneration in an optic nerve injury model, Park et al discovered robust long-distance axon regenerations in adult mice with targeted deletion of the phosphatase and tensin homolog (PTEN) gene [49**]. PTEN is a well-established dual phosphatase which could convert phosphatidylinositol (3,4,5) trisphosphate (PIP_3) to phosphatidylinositol (4,5) bisphosphate (PIP_2), in antagonizing the activity of phosphoinositide 3-kinases (PI3K). PTEN deletion leads to the accumulation of PIP_3, which in turn recruits and activates phosphatidylinositol-dependent kinase 1/2 (PDK1/2), resulting in the activation of Akt [74, 75] (Figure 3). Among the multiple down-stream targets of this pathway, mTOR controls cap-dependent protein translation [76] and GSK-3 can regulate cytoskeleton assemble and axonal transport [77]. Rapamycin administration abolishes the regeneration effect of PTEN deletion, suggesting a requirement of mTOR in axon regeneration. In addition, targeted deletion of tuberous sclerosis protein 1 (TSC1), a specific negative regulator of mTOR (Figure 3), also promotes axon regeneration. However, the extent of axon regeneration after TSC1 is less than that after PTEN deletion. These results suggest a model in which mTOR activity may control

the protein synthesis for axon growth, while mTOR-independent pathways such as GSK-3 may promotes axonal cytoskeleton assembly and axonal transport [78].

In further support for the loss of mTOR activity as a major intrinsic barrier for axon regeneration, Park et al found that mTOR signaling is down-regulated in the CNS neurons over the course of development [49**]. The residual mTOR activity in adult RGCs are further diminished by axotomy-triggered stress response [49**]. By this two-step mTOR suppression, injured RGCs retain extremely low level of mTOR activity, and hence very limited ability to initiate new protein synthesis required for axon regeneration. It remains to be determined whether local protein synthesis in axons [79–81] or translation in the soma is crucial for axon regeneration.

The mechanisms that mediate the changes of mTOR activity during development and after injury are largely unknown. The localization and function of PTEN may be controlled by myosin V [82]. In addition, Nedd4, an E3 ligase, was shown to promote axonal branching by down-regulating PTEN [83]. Many other molecules and pathways such as REDD1 [84–86], sestrin [87], could down-regulate mTOR activity in the cells under stress conditions. At present, whether any of these above-mentioned pathways mediate axotomy-triggered mTOR suppression is unknown.

Other mechanisms

In addition to transcription and translation regulations, evidence also suggested the involvement of other protein post-translational modifications in the process of axon regeneration. The anaphase-promoting complex (APC), an E3 ligase complex that was extensively studied in cell cycle, is expressed in postmitotic neurons and may degrade molecules required for axon growth [88,89]. It will be interesting to test the axon regeneration effects of these molecular mechanisms on axon regeneration in vivo. Recently, an elegant study demonstrated a critical role of microRNA-206 in promoting regeneration of neuromuscular synapses in mice [90]. The roles of microRNA in axon growth and regeneration await to be explored. In addition, it will be interesting to find out how other established pathways such as cAMP/Arg1 [91,92] cross-talk with the mechanisms discussed above.

Taken together, these recent studies identified several critical intrinsic barriers preventing axon regeneration in adult CNS neurons. Thus, removing these negative brakes may allow mature neurons to regain regenerative ability after injury. As recently demonstrated [93–96], combinatorial strategies to deal with extrinsic and intrinsic mechanisms may represent a promising avenue to promoting axon regeneration and functional recovery after CNS injury.

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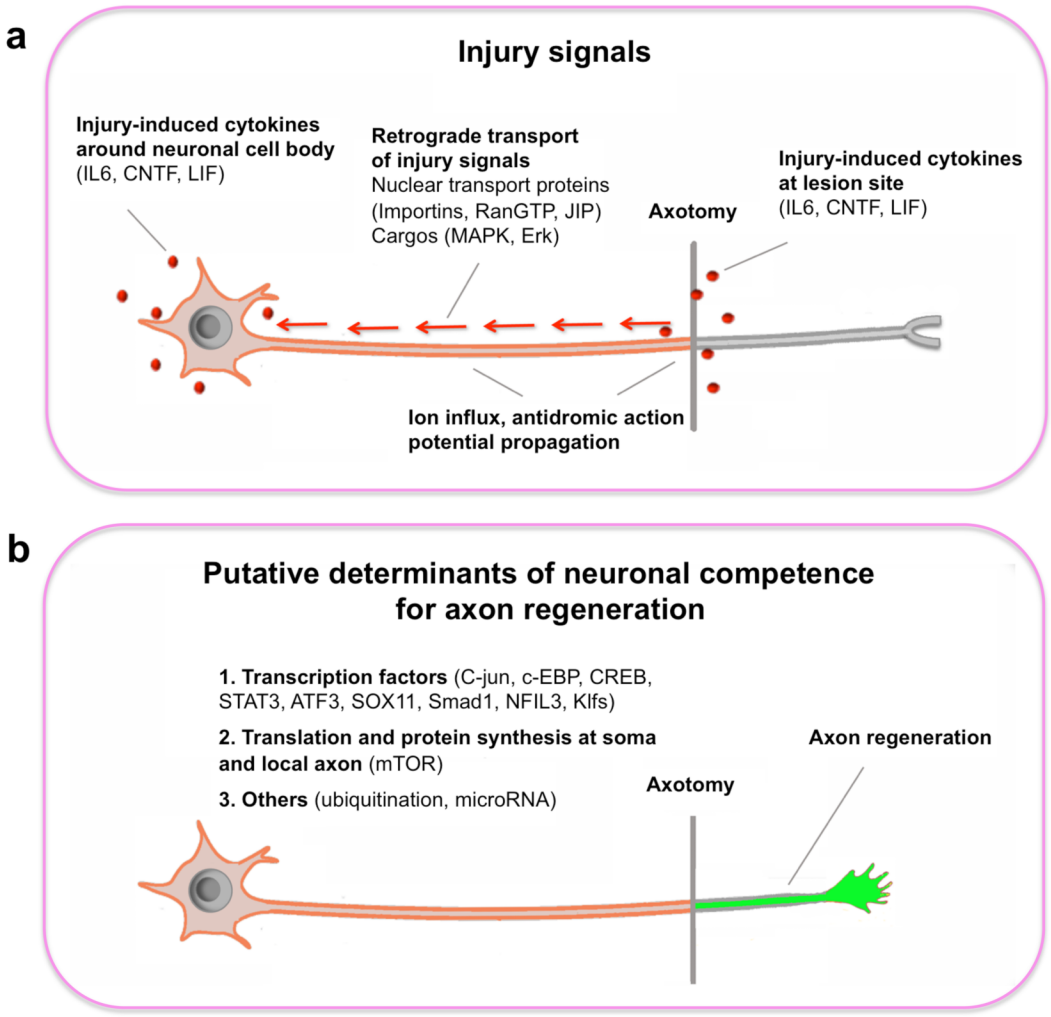

Figure 1.

Two aspects of neuronal intrinsic mechanisms for axon regeneration. **(a)** Schematic of axotomy-triggered retrograde signals. In addition to acute axotomy-induced changes such as ion influx and antidromic action potential propagation, cytokines such as IL-6, CNTF and LIF could be up-regulated at the lesion site and/or around the cell body. Activated signaling components in the axon or at the cell body could be transported to the nucleus by nuclear transport proteins such as importins, RanGTP and JIP. **(b)** Putative determinants of neuronal competence for regenerative responses. These include the steps required for synthesis and assembly of materials for axon extension: transcription, translation and other post-translational modifications.

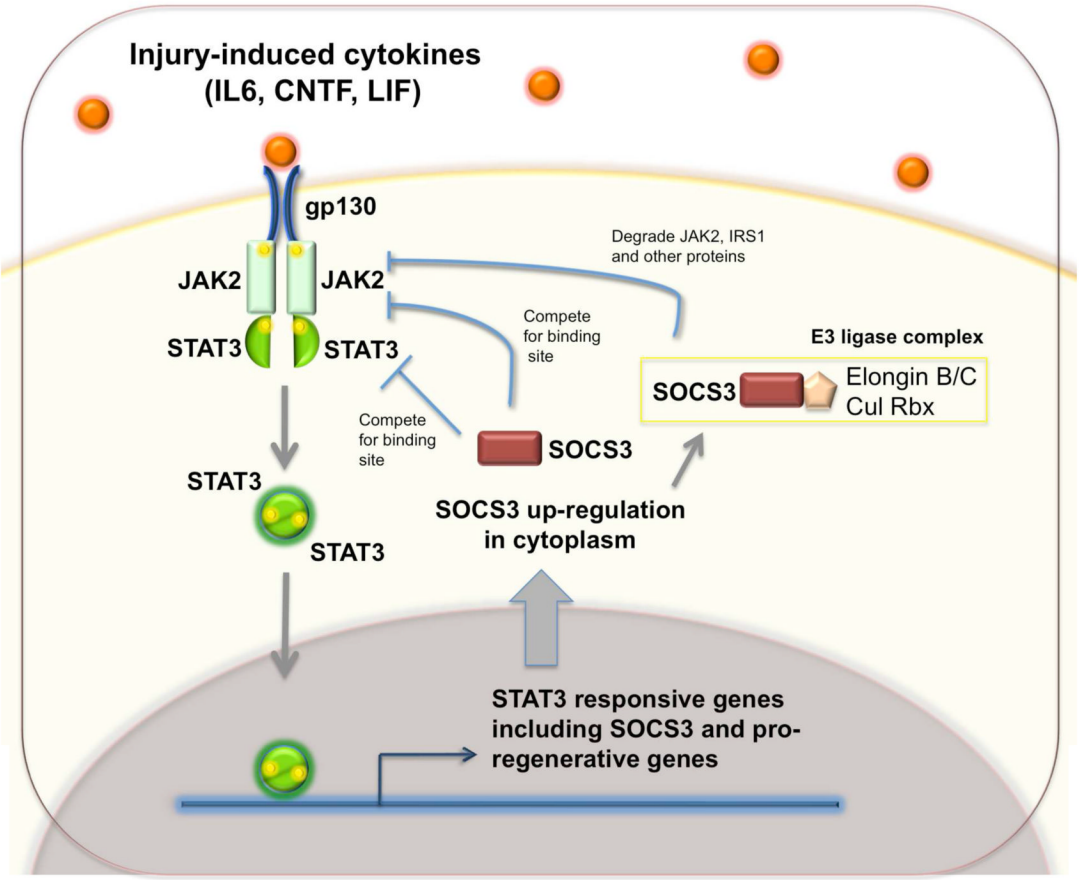

Figure 2.

gp130-dependent cytokines promote axon regeneration and SOCS3 act as a critical negative regulator of the signaling pathway. Injury-induced cytokines act on their receptor complexes with a shared component gp130 and activate the JAK-STAT cascade in both PNS and CNS neurons. Phosphorylated STAT-3 is translocated to the nucleus and initiate gene expression for axon regeneration. However, in the adult CNS, the activation of this pathway leads to the up-regulation of SOCS3 which will inhibit JAK2 and STAT3 and in turn inhibit this pathway. Thus, in SOCS3 deleted RGCs, both endogenous and exogenous cytokines such as CNTF promote significant axon regeneration [50**].

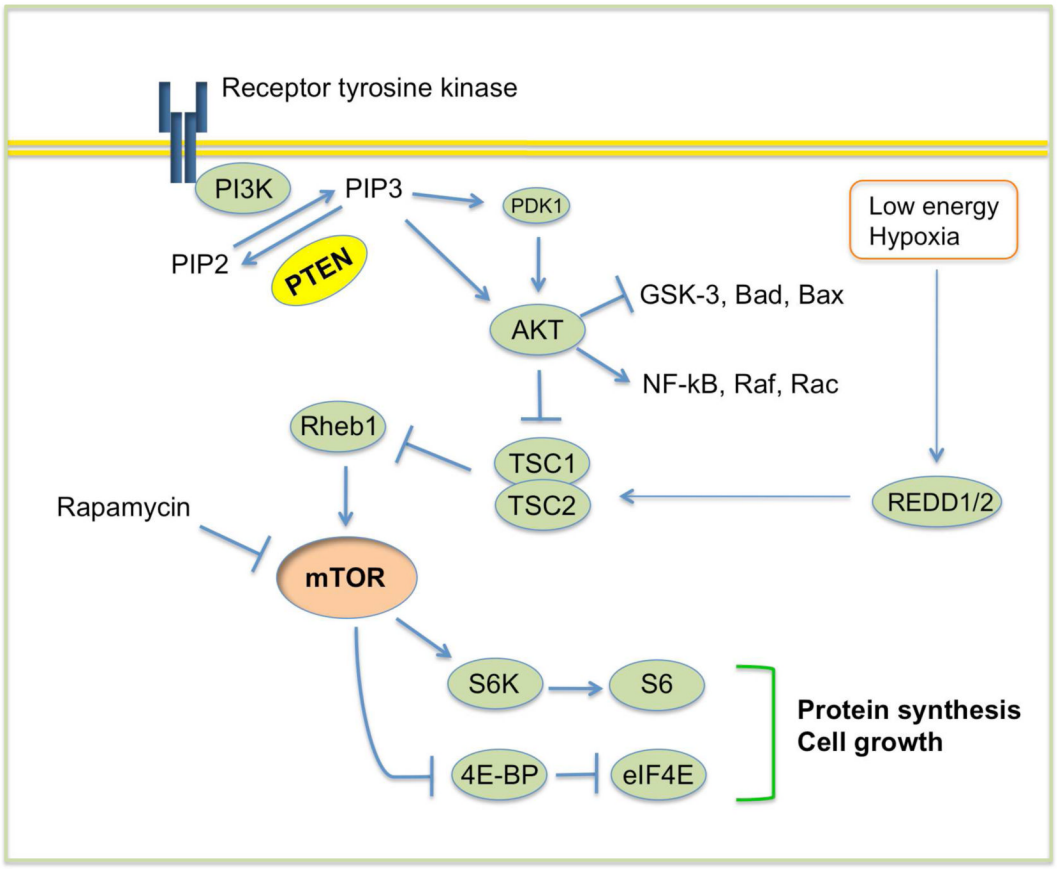

Figure 3.

PTEN-regulated signaling pathway. In response to receptor tyrosine kinase activation, PI3K phosphorylates and converts the lipid second messenger phosphatidylinositol (4,5) bisphosphate (PIP_2) into phosphatidylinositol (3,4,5) trisphosphate (PIP_3), which recruits and activates phosphatidylinositol-dependent kinase 1/2 (PDK1/2). PDK1/2, in turn, phosphorylates and activates Akt. PTEN catalyzes the conversion from PIP_3 to PIP_2. Thus, inactivation of PTEN results in the accumulation of PIP_3 and the activation of Akt. Akt controls a host of signaling molecules, including GSK-3 and TSC1/2. Inactivation of the TSC1/2 complex leads to activation of mTOR, which integrates various cellular signals including nutrient availability to control protein translation, cell growth, and other processes. The ribosomal protein S6 kinase (RP-S6) and the eukaryotic initiation factor 4E (eIF-4E) binding protein 1 (4E-BP1) are the mTOR effector molecules executing these functions. Cellular stresses such as hypoxia and low energy induce expression of Redd1/2, which augments TSC1/2 activity and in turn suppress the mTOR activity.