REVIEW

Rabs and axonal regeneration

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Funding: *The work was supported by the National University of Singapore Graduate School for Integrative Sciences and Engineering (to BLT).*

Abstract
Membrane trafficking processes are presumably vital for axonal regeneration after injury, but mechanistic understanding in this regard has been sparse. A recent loss-of-function screen had been carried out for factors important for axonal regeneration by cultured cortical neurons and the results suggested that the activity of a number of Rab GTPases might act to restrict axonal regeneration. A loss of Rab27b, in particular, is shown to enhance axonal regeneration *in vitro*, as well as in *C. elegans* and mouse central nervous system injury models *in vivo*. Possible mechanisms underlying this new finding, which has important academic and translational implication, are discussed.

Key Words: *Arf; Rab; Rab27b; axon regeneration; axonal transport; cortical neurons; central nervous system injury; membrane trafficking; Myosin-V*

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doi: *10.4103/1673-5374.247422*

Received: *September 10, 2018*
Accepted: *October 23, 2018*

Introduction
Membrane trafficking in eukaryotes, which complexity is compounded in a polarized and morphologically extended cell type like neurons, is regulated by the small GTPases of the Rab and Arf families. The membrane transport processes mediated by Rab and Arf GTPases are important for neurite outgrowth and axon-dendrite polarization during development and neuronal differentiation. Fundamentally similar processes are presumably important for axonal regeneration upon injury to postmitotic neurons. This notion would appear to be intuitive, as directed membrane transport to the axonal plasma membrane domain underlies neurite outgrowth, and if nothing else this process forms the basis for growth cone formation and extension of the proximal end of severed axons. Despite a large number of Rabs and their regulatory factors or effectors having been implicated in neurite outgrowth and axon specification, only a few have confirmed roles in axonal regeneration after injury. Prominent amongst these are Rab11 and its effector (Koseki et al., 2017), as well as Arf6 (Eva et al., 2012) and its guanine-nucleotide-exchange factor EFA6 (exchange factor for Arf6) (Chen et al., 2015; Eva et al., 2017). Rab11 and its effector, the Rab coupling protein, are important for the axonal trafficking of molecules important for axonal growth, such as β1-integrin (Eva et al., 2010). Thus, exclusion of Rab11-bearing transport intermediates from axons and their preferred targeting to the somatodendritic domain may underlie difficulties in axonal outgrowth of mature neurons (Koseki et al., 2017). Arf6 also plays a role in integrin trafficking (Eva et al., 2012). Its guanine-nucleotide-exchange factor and activator EFA6 has been shown to modulate axonal microtubule dynamics (Chen et al., 2015) and polarized axonal transport. In addition, EFA6 expression also inhibits axonal regeneration (Eva et al., 2017).

A recent loss-of-function screen using cultured mouse cortical neurons for cellular factors important for axonal regeneration have identified a large number of genes when silenced will result in an axonal regeneration phenotype (Sekine et al., 2018). Interestingly, Rab GTPases are prominently represented among these genes.

Neuron Enriched Rab27b Restricts Axon Regeneration after Axotomy
Loss of function screens for genes involved in axonal regeneration have been previously performed with sensory and motor neuron injury model in *C. elegans* based on null mutations and RNA interference, respectively (Chen et al., 2011; Nix et al., 2014). EFA6 was indeed identified as a negative regulator of axonal regeneration by such a screen (Chen et al., 2011), but components of the membrane trafficking machinery were not prominently represented in the 214 genes associated with a significant regeneration phenotype. Sekine and colleagues have recently performed another screen with a short hairpin RNA (shRNA) library on a scrape injury model of mouse cortical neurons in culture (Sekine et al., 2018). The authors identified close to 500 genes whose silencing conferred a significant axonal regeneration phenotype. Interestingly, this cohort bears little overlap

with orthologues identified in the previous screens, as well as those found earlier to change significantly in terms of expression upon axotomy. A particularly intriguing set of genes identified by the author that significantly affects regeneration are the Rabs and Arfs. In fact, shRNA-mediated silencing of 15 out of 19 Rab/Arf and related genes resulted in statistically significant increases in axon regeneration compared to control. Of note, silencing of three Rab3 isoforms Rab3b, c and d; as well as the suppression of Arf6 and Rab11b expressions, appear to enhance axon regeneration. This regeneration inhibitory activity is corroborated for Rab3b, Rab3c and Rab27b, as the over-expression of the respective GTP-restricted, constitutively active mutants of these GTPases have similar effects.

The authors went on to further characterize the restriction potential of axonal regeneration of Rab27b, a Rab27 isoform enriched in cortical neurons. Multiple shRNAs targeting Rab27b showed consistent enhancement of axonal regeneration capacity *in vitro*. Furthermore, axonal regeneration by cortical neurons cultured from *Rab27*$^{-/-}$ mice is significantly enhanced compared to those from wild-type mice, and this enhancement is abolished by exogenous expression of Rab27b. The axonal regeneration restrictive nature of Rab27 was confirmed in separate *in vivo* settings with both an invertebrate and a vertebrate model. In the invertebrate model, γ-aminobutyric acid (GABA)ergic neurons in *C. elegans* harboring two different hypomorphic alleles of *rab-27* exhibited a significantly increased regeneration compared to wild-type neurons after laser axotomy. Selective over-expression of Rab27 in these GABAergic neurons reversed the increased regeneration competence phenotype, attesting to the cell autonomous nature of Rab27 activity in this regard. In the vertebrate model, crushed optic nerves of *Rab27*$^{-/-}$mice showed more robust regeneration, with a significant increase in tracer-marked axons regenerating beyond the injury site. Interestingly, this regeneration enhancement due to loss of Rab27b is even more pronounced in the presence of zymosan-induced inflammation (which promotes axonal regeneration).

Could the loss of Rab27b actually enhance functional recovery after central nervous system injury in adult animals? The authors noted that Rab27b is expressed in the adult mouse motor cortex. Rab27$^{-/-}$ mice which received a midthoracic spinal cord dorsal hemisection showed a significant enhanced recovery of hindlimb function compared to wild-type mice as measured by the Basso Mouse Scale score. Consistent with this, the mutant mice also do better in the gridwalk and rotarod tests. Furthermore, there is morphological evidence in support of a more robust axonal regeneration in the Rab27$^{-/-}$ mice. When the projections of the raphespinal serotonergic axonal tract in these mice were traced, 5-hydroxytryptamine-positive fibers caudal to the lesion site showed a higher density. Also, a greater number of corticospinal axons could be found immediately rostral to the injury site, as compared to wild-type mice. These findings collectively attest to a role for Rab27b in restricting axonal regeneration upon injury (schematically summarized in **Figure 1A**).

How Does Rab27b Restrict Axonal Regeneration?

An interesting general finding made by the authors is that most of the Rab/Arf examined by the loss-of-function screen appeared to have an axonal regeneration restrictive or inhibitory activity. This may be expected to be the case for Arf6, as Arf6 inactivation is known previously to increase recycling of β1-integrins to the neuronal surface and enhanced anterograde axonal transport (Eva et al., 2012). However, Rab11 over-expression has, on the contrary been previously shown to enhance neurite growth (Eva et al., 2010) and axonal regeneration after axotomy (Koseki et al., 2017). As Rabs are expected to function in axonal plasma membrane transport, one would expect that the loss of some of these would have an adverse effect on the axonal regeneration. Therefore, the generally inhibitory effect of Rab/Arf in the Sekine et al. screen is somewhat surprising. Of course, more targeted loss-of-function assays complemented by over-expression studies may uncover Rabs that positively contribute to axonal regeneration. Another possible explanation for Rabs/Arfs' general restriction of axonal regeneration could also be a contingent need to drastically alter the axonal membrane traffic upon axotomy. When massive membrane transport is required for more urgent and critical processes such as resealing of the lesion site (Bloom and Morgan, 2011), the trafficking involved under energetically-deprived conditions may become fairly independent of the GTPases and could instead be hindered by the normal activities of Rabs and their effectors. This notion would require further investigation.

Rab27b's restriction of axonal regeneration is studied in detail by Sekine et al., and confirmed both *in vitro* and *in vivo*. What is yet unclear is the mecha-

nism underlying this restriction. Rab27a and Rab27b share over 70% sequence identity and likely has large overlaps in terms of their function. These Rabs are more prominently known for their roles in melanocytes, platelets and cytotoxic T-cells. Rab27b is widely expressed in cells performing regulated exocytosis, including neurons, and is involved in secretory granule transport to the plasma membrane (Gomi et al., 2007). However, Rab27b's role in neurons is not well-defined. Based on its role in regulated secretion, as is the case for Rab18 as well as the Rab3 isoforms in synaptic vesicle exocytosis, the authors have postulated that "…a loss of these Rabs may shift membrane traffic from synaptic function to permit greater plasma membrane addition for axon extension…" (Sekine et al., 2018). This notion of Rab27b and Rab3 isoforms loss that contribute indirectly towards axonal extension appears reasonable, but the question is whether the traffic volume redirected by the loss of a single Rab (such as Rab27b) is significant enough to add to axonal transport. It would also be important to consider the role of Rabs at different phases of axonal regeneration. As postulated above, axonal transport in the acute phase post-lesion could well be Rab-independent and may indeed be somewhat hindered by Rab activities competing for membrane and cytoplasmic resources. Shutting down unnecessary exocytosis and endocytic processes, or active membrane protein targeting to the somatodendritic surface, perhaps triggered by the retrogradely propagating Ca2+ wave from the lesion, would in fact be a logical cellular response in this acute phase of injury. However, after the lesion has been resealed and short-term survival of the soma ensured, growth cone formation and subsequent axonal elongation after the acute phase may become dependent on the activity of certain Rabs, such as Rab11.

As Rab27b's restrictive action is cell autonomous, it could, in principle, act indirectly by diverting neuronal plasma membrane traffic elsewhere other than the regenerating axon, or it could actively block axon targeting or transport. A clue to Rab27b's restrictive effect on axonal regeneration may come from its axonal localization. Activated, GTP-bound Rab27b appears to be localized differently from its inactive, GDP-bound counterpart, as demonstrated by the distribution of the respective GTP or GDP-restricted mutants. The former being prominent in regenerating axon shafts, while the latter is enriched in the actin-enriched regenerating growth cones. The mechanism and role for this differential distribution is yet unclear. Whether Rab27b has any role in modulating growth cone actin dynamics or axonal shaft microtubule changes is not known. Also, any clear crosstalk between Rab27b and the actin filament/microtubule-modulating small GTPases of the Rho family has not been reported. However, active Rab27 is known to engage the actin motor Myosin Va in its better-known actions, such as melanosome transport. Interestingly, Myosin Va in neurons functions in dendritic targeting (Lewis et al., 2009). In fact, Myosin-V could oppose kinesin-mediated axonal transport. A recent study has shown that recruitment of Myosin-V to kinesin-propelled cargoes could stall their motility and cause their immobilization and the axonal initial segment (Janssen et al., 2017). Although Rab27b's role in dendritic transport is unclear, it is conceivably plausible that Rab27b's localized engagement of Myosin-V at the axon shaft could actively counter axonal growth during regeneration. These speculative possibilities are depicted in **Figure 1B**.

Therapeutic Implications

The finding that Rab27b restricts axonal regeneration may have future therapeutic implications. Strategies targeting the Rho GTPase and its effectors, which modulate both actin and microtubule dynamics, have been shown to enhance axonal regeneration both *in vitro* and *in vivo* (McKerracher et al., 2012). A global loss of Rab27b is well-tolerated in adult mice, and as such its selective silencing or specific inhibition at central nervous system lesion sites could possibly enhance the robustness of axonal regeneration in central nervous system injuries without undesirable side effects. Given the dearth of promising therapeutic strategies for central nervous system axon regeneration, Rab27b may well be novel therapeutic target worth further pursuit.

Author contributions: *CQY and BLT co-wrote the paper.*
Conflicts of interest: *Both authors declare no conflict of interest.*
Financial support: *The work was supported by the National University of Singapore Graduate School for Integrative Sciences and Engineering (to BLT).*

Plagiarism check: *Checked twice by iThenticate.*
Peer review: *Externally peer reviewed.*

Open peer reviewer: *Melissa Renee Andrews, University of St Andrews, UK.*

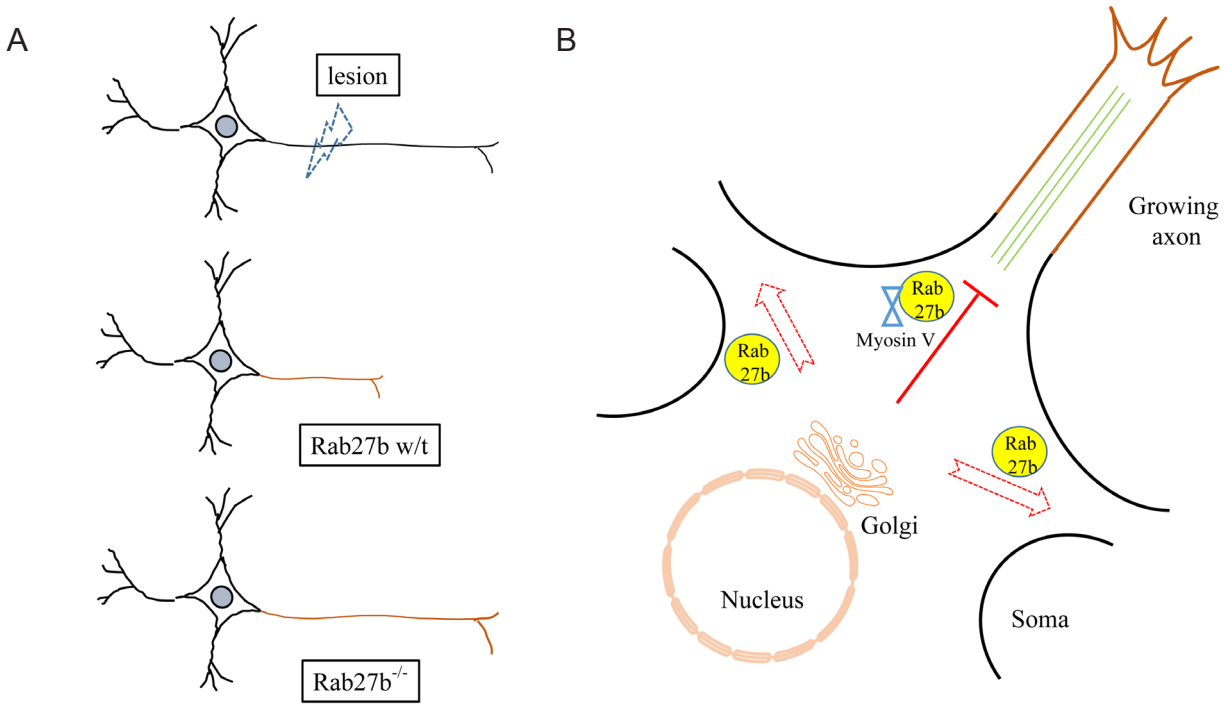

Figure 1 Schematic diagram illustrating the findings of neuronal Rab27b being axonal regeneration restrictive.
(A) Axonal regeneration is enhanced in a generalized Rab27b depleted or Rab27b$^{-/-}$ neuron (those examined include *C. elegans* γ-aminobutyric acid (GABA)ergic neurons, as well as mouse cortical, motor and optic nerves). (B) Rab27b's potential modes of action. Rab27b could possibly act in directing neuronal plasma membrane traffic elsewhere (such as secretory and dendritic transport) other than the regenerating axon, or by actively blocking axon targeting or transport (such as *via* its localized engagement of Myosin-V at the axon). w/t: Wild type.

Additional file: *Open peer review report 1.*

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P-Reviewer: Andrews MR; C-Editors: Zhao M, Yu J; T-Editor: Liu XL