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PHRs: bridging axon guidance, outgrowth and synapse development

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Axon guidance, outgrowth, and synapse formation are interrelated developmental events during the maturation of the nervous system. Establishing proper synaptic connectivity requires precise axon navigation and a coordinated switch between axon outgrowth and synaptogenesis. The PHR (human Pam, mouse Phr1, zebrafish Esrom, *Drosophila* Highwire, and *C. elegans* RPM-1) protein family regulates both axon and synapse development through their biochemical and functional interactions with multiple signaling pathways. Recent studies have begun to elucidate a common underlying mechanism for PHR functions: Consisting of motifs that affect intracellular signaling, selective protein degradation, and cytoskeleton organization, PHR proteins probably mediate the transition between axon outgrowth and synaptogenesis through integrating intracellular signaling and microtubule remodeling.

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Introduction

The establishment of proper synaptic connectivity in the nervous system requires the coordination of intimately coupled developmental processes, axon guidance, outgrowth, and synaptogenesis. Neurons and their projections have the capacity to migrate over long distances in response to guidance cues. Once navigated near their synaptic partners, neurons must transition from a state of outgrowth to that of synaptogenesis, during which a stable connection between the presynaptic and postsynaptic cell forms. Similar transitions are probably recapitulated during the developmental remodeling of the nervous system, as well as repair following nerve injury. In the past decade, the PHR (human *P*am, mouse Phr1, zebrafish Esrom, *Drosophila H*ighwire, and *C. elegans R*PM-1) family proteins have emerged as key regulators for axon guidance, outgrowth, regeneration, and synapse development. Averaging above 4000 amino acids, PHR proteins consist of multiple motifs that suggest their involvement in signaling, scaffolding, as well as ubiquitin-mediated protein degradation. Here we review studies that reveal the complex roles and mechanisms through which PHR proteins regulate axon guidance, synaptogenesis, and regeneration. A common theme emerges that PHR proteins mediate the transition between axon growth and synaptogenesis through promoting the remodeling of microtubule dynamics. We further summarize other functions of PHR proteins, which, in large, remain to be further explored.

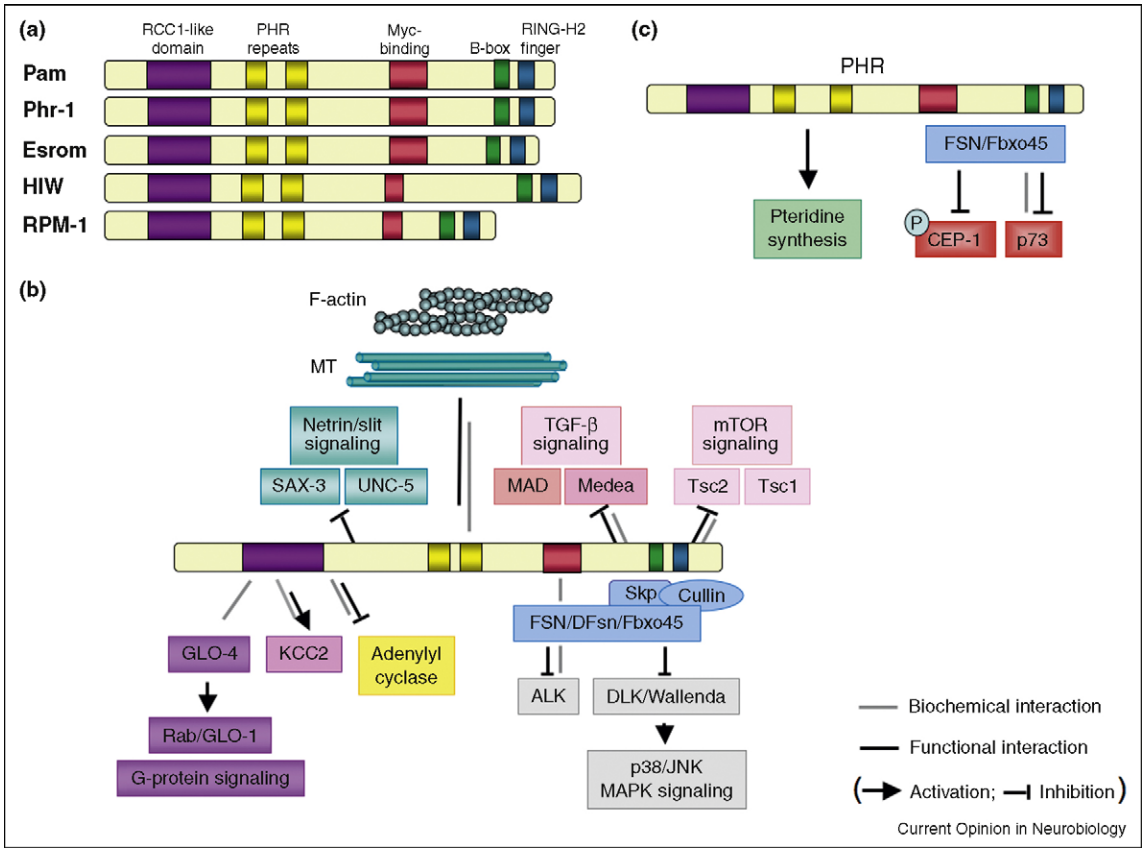
A brief history on the discovery of PHRs

The founding member of the PHR family, human Pam (*p*rotein *a*ssociated with *M*yc), was identified from the Akata Burkitt's lymphoma cell line through its ability to interact with a proto-oncogene Myc [1]. The first insight into the physiological function of PHR proteins came from parallel studies on *Caenorhabditis elegans* (*C. elegans*) and *Drosophila* PHRs, RPM-1 [2••,3••], and Highwire (HIW) [4••], respectively. Both were identified as regulators of synapse development in genetic screens for abnormal axon and synapse morphology. Studies on the vertebrate PHRs, the zebrafish protein Esrom [5••,6], and mouse protein Phr1 [7], however, revealed a more prevalent role in axon navigation and outgrowth in early neural development. An involvement of RPM-1 in axon guidance, outgrowth, and regeneration was recently reported [8•,9•].

The multi-faceted roles of the PHR proteins are probably attributed to their unique multi-modal composition. Averaging about 4000 amino acids, PHRs are large proteins with multiple conserved motifs: an RCC1-like domain with inferred guanine exchange activity, two PHR protein-specific repeats, a Myc-binding region, a B-box zinc finger, and a RING-H2 zinc finger, a hallmark motif for E3 ubiquitin ligase activity (Figure 1A) [1]. Multiple binding partners of conserved domains or regions have been identified (Figure 1B, C), and their involvement in PHR functions are discussed in later sections. Many loss-of-function alleles in both vertebrate and invertebrate *PHR* mutants encode premature termination codons or missense mutations that affect the RCC1-like and the RING-H2 zinc finger motifs [2••,4••,5••], highlighting their functional importance.

PHR proteins are expressed in the developing and mature nervous systems. *C. elegans* RPM-1 localizes in a punctate

Figure 1



PHR family proteins and their interactions in neural and non-neural pathways. **(A)** Conserved domains in the PHR family protein Pam, Phr1, Esrom, HIW, and RPM-1; **(B)** PHRs interact with, and regulate multiple signaling pathways to regulate axon growth and synaptogenesis. The RCC1 domain binds GLO-4 (RPM-1), KCC2, and adenylyl cyclases (Phr1/PAM), which either promotes or inhibits their activities. The RING-H2 finger motif binds TSC2, E2 conjugating enzymes (Phr1), and Medea (HIW). PHRs regulate mTOR (Phr1/PAM/Esrom), BMP (HIW), and Netrin/Slit (RPM-1) signaling. PHRs interact with FSN-1-family F-box proteins to downregulate DLK level and activity (Phr1/HIW/RPM-1). PHR proteins also associate with microtubule-enriched and F-actin-enriched cytoskeletal complexes (Phr1/PAM) and affect their dynamics (Phr1/Esrom); **(C)** non-neural roles of PHR proteins. Esrom is required for pteridine synthesis. RPM-1 and FSN-1 are required to downregulate phosphorylated CEP-1/p53, whereas p73 can directly bind and be ubiquitinated by Fbxo45. Black lines implicate genetic/functional interactions; gray lines indicate direct or indirect biochemical interactions.

pattern along the axon, but does not overlap with synaptic vesicle or active zone proteins [2••]. Its axonal localization depends on the PHR domains [10]. *Drosophila* HIW localizes to neuronal cell bodies, axon bundles, and neuromuscular junctions (NMJs), but does not overlap with synaptic markers [4••,11]. Human Pam, mouse Phr1, and zebrafish Esrom are present at the neuron soma and along axons as well [5••,7,12]. Unlike their invertebrate homologs, however, vertebrate PHRs are not always restricted to the nervous system. *PAM* transcripts are detected across tissues, but are most abundant in the brain and thymus [1]. While Phr1 protein is largely restricted to the nervous system, it may also be transiently expressed in non-neuronal tissues in embryos [7,13••]. *esrom* is ubiquitously expressed throughout development, further suggesting expanded roles of vertebrate PHR proteins [5••]. Another marked difference is that vertebrate PHRs, Phr1, and Esrom are essential for viability [6,7], whereas *hiw* and *rpm-1* mutants are viable and healthy, albeit exhibiting mild morphological or behavioral defects, for example, imbalanced walk in *hiw* and a shorter body size and slight delay in egg-laying for *rpm-1* [2••,3••,4••,8•].

PHR proteins as regulators for synapse development

C. elegans rpm-1 and *Drosophila hiw* mutants were identified from three independent genetic screens for abnormal axon and/or synapse morphology. In *rpm-1* mutants, the PLM mechanosensory neurons often extend their anterior longitudinal processes past synaptic targets, and fail to accumulate vesicles at their axonal branches [3••]. In GABAergic motoneurons however, both overgrown presynaptic terminals, associated with multiple active zones, and axonal regions with underdifferentiated

synaptic structures are present [2••]. *Drosophila hiw* mutants exhibit excessive axon branching and more, albeit smaller, synaptic boutons at glutamatergic NMJs [4••]. Although these boutons appear ultrastructurally normal, decreased quantal size and evoked response were observed at glutamatergic NMJs, indicating defective synaptic transmission in *hiw* mutants [4••]. Despite the difference in their phenotypic expression, both HIW and RPM-1 function cell-autonomously to regulate synaptic development and/or function [2••,3••,4••]. Reminiscent of the synaptic overgrowth defects in *hiw* mutants, in *Phr1* knockout mice, axons of the phrenic nerve that reach the diaphragm extend more extrasynaptic sprouting and develop more NMJs [7,14••]. The lack of *PHR1* expression in diaphragm indicates that it also functions in a cell-autonomous manner to regulate presynaptic differentiation [7].

A postsynaptic role for PHRs was recently discovered at the *C. elegans* central nervous system (CNS). While *rpm-1* mutants do not exhibit obvious presynaptic morphological defects in interneurons, there is an aberrant accumulation of an AMPA receptor GLR-1 along dendritic processes. RPM-1 is proposed to regulate the localization of postsynaptic receptors at CNS synapses through endosomal trafficking [15•]. Another potential postsynaptic function for PHR proteins is implicated by an *in vitro* interaction between the RCC1-like domain of Phr1 and the C-terminal region of KCC2 [16], a neuronal K^+-Cl^- co-transporter that regulates the migration and dendritic spine maturation of cortical interneurons [17].

PHR proteins as regulators for axon guidance and outgrowth

Although the earlier studies on invertebrate PHR proteins emphasized their roles in synapse formation and growth, defective axonal morphology, notably, the excessive branching of motoneuron axons in *hiw* mutants [4••], and the aberrant branching, retraction, overgrowth, and target passing in some sensory and motoneuron axons of *rpm-1* mutants have been noted [2••,3••]. These defects were attributed to defective axon termination, which may either contribute to, or be triggered by, a failure in target recognition or stabilization of synaptic contacts. This hypothesis is supported by the temporal requirement of RPM-1 coinciding with the period of synaptogenesis [2••,3••].

An elegant combination of genetic and time-lapse imaging studies on vertebrate PHRs demonstrates their prominent and prevalent role in growth cone navigation during axon guidance and outgrowth. *Magellan* mutants, isolated from a genetic screen for morphological defects in mouse embryonic motoneurons, harbor premature termination codons in *PHR1* [13••]. In *Magellan* mutants, motor axons often wander or stall at major choice points, resulting in their failure to exit the ventral root or crural plexus, misprojection to the dorsal root ganglion, and fewer axons in the intercostal nerve and other distal limb projections. As *Magellan* explants respond normally to various guidance cues, and the level of several guidance receptors is unaltered, these axon defects cannot be attributed solely to a defect in the intrinsic guidance response. In *Magellan* motor and sensory neuron cultures, axons fail to coordinate stalk extension and growth cone positioning: axon stalks often grow past growth cones, causing axon bending. Moreover, disorganized microtubules invade and sometimes split growth cones. These defects are suppressed by taxol, a microtubule stabilizer. Aberrant microtubule dynamics in the developing axon is thus proposed to compromise the growth cone's ability to react properly to spatial guidance cues at choice points [13••].

Prominent axon guidance/outgrowth defects are also observed in targeted *Phr1* knockout mice, in which the phrenic nerve contains fewer axons and fails to fully innervate the diaphragm. *Phr1* knockouts also exhibit a severe reduction of major axon tracts in the CNS, with reduced thickness of the corpus callosum, the absence of the anterior commissure, and an overall reduction of neurites in the cerebral cortex. Similar to the pausing of motor axons at choice points, cortical neurons fail to extend axons beyond the corticostriatal boundary. Intriguingly, the cortex-specific knockout of Phr1 does not affect the axonal projection of cortical neurons, suggesting that Phr1 functions cell non-autonomously to generate a permissive environment for axon passing [14••].

The primary neuronal function of the zebrafish PHR protein Esrom also appears to be axon navigation. In *esrom* mutants, retinal axons fail to project to the posterior tectum. Instead, they project to, and defasciculate at the anterior tectum [5••]. Moreover, in a phenotype analogous to axon pausing in the *Magellan*/*Phr1* mice, habenular commissural axons stall at the roof plate boundary and thus fail to form the habenular commissure in *esrom* mutants [18]. These navigation defects also result from aberrant microtubule dynamics. In *esrom* mutants, forebrain axons exhibit complex, retracting microtubule loops in growth cones and along the axon shaft. Opposite to the mouse motoneurons, however, stabilizing microtubules by taxol leads to microtubule looping similar to those observed in *esrom* explants, whereas promoting microtubule disassembly by nocodazole reduces the microtubule abnormality in *esrom* explants, and restores commissural axon crossing in *esrom* mutants [19••]. Despite their differences in response to microtubule-perturbing drugs, both studies clearly demonstrate that microtubule remodeling is crucial for Phr1/Esrom-mediated axon navigation. Indeed, microtubule organization may underlie both synapse and axon defects in vertebrate and invertebrate *PHR* mutants, a model that we discuss later in this review.

Invertebrate PHR affects axon guidance, outgrowth and regeneration

Although invertebrate *PHR* mutants do not exhibit systemic defects in axon guidance and outgrowth, recent studies suggest that RPM-1 activity can modify the phenotype of axon guidance mutants, and vice versa. For example, *rpm-1* mutations partially suppress the ventral–dorsal guidance defects in motoneurons when the guidance cue (Netrin/UNC-6) is reduced. Reducing the activity of UNC-5/UNC-5 or Robo/SAX-3 guidance receptors partially suppresses the overextension of mechanosensory axons in *rpm-1* mutants. The localization and expression of guidance receptors are moderately affected in *rpm-1* mutants; RPM-1 therefore may modulate axon guidance and outgrowth, at least partly, through affecting guidance receptors [8•].

RPM-1 activity also affects axon regeneration. After axotomy, the regeneration of motoneuron axons is enhanced in *rpm-1* mutants, whereas an overexpression of RPM-1 severely inhibits axon regeneration [9•]. Therefore, while RPM-1 activity is not essential for axon guidance and outgrowth, it affects the sensitivity or responsiveness of developing axons to guidance cues and ability of injured axons to reinitiate outgrowth.

PHRs as E3 ubiquitin ligases

The RING-H2 zinc finger motif is a hallmark of E3 ubiquitin ligases. Moreover, many vertebrate and invertebrate *PHR* mutations affect the RING-H2 domain. *hiw* mutants exhibit genetic interactions with *fat facets*, a gene encoding a deubiquitinating enzyme in a dosage-dependent manner, providing the first evidence that HIW functions through ubiquitin-mediated proteasome degradation [20]. The identification of FSN-1 family F-box proteins as the functional partners for PHRs provides further evidence for their E3 ligase activity [21••,22••,23••]. F-box proteins are target recognition modules for SCF (Skp/Cullin/F-box) E3 ubiquitin ligase complexes [24,25]. *C. elegans fsn-1* (*F*-box protein at the *sy*napse) mutants exhibit similar synaptic defects as *rpm-1*, and *fsn-1* loss-of-function mutations do not enhance *rpm-1* defects. Also expressed in the nervous system, FSN-1 forms a divergent SCF-like complex with RPM-1, scaffolding proteins Skp1, and Cullin, but not Rbx1 [21••]. RPM-1 and FSN-1 therefore regulate synapse development as an E3 ligase complex to downregulate selective targets. Such an association may also be important for their stability or subcellular localization [10,23••].

The physical and functional interactions between the FSN-1 family proteins and PHRs are evolutionarily conserved. The *Drosophila* F-box protein DFsn is present in the same protein complex with HIW [22••]. Furthermore, *DFsn* mutants exhibit synaptic overgrowth and defective synaptic transmission at glutamatergic NMJs, phenocopying *hiw* mutants. Knockout mice for the FSN-1 vertebrate homolog, Fbxo45, exhibit phenotypes reminiscent of the *Phr1*/*Magellan* mice, such as respiratory failure at birth and a reduction of axon tracks in the CNS [7,13••,14••,23••]. Phr1 and Fbxo45 also form an E3 ligase complex that includes Skp1. However, instead of interacting through Cullin, Phr1 and Fbxo45 interact directly through their Myc-binding and SPRY domains [23••], respectively, diverging further from the classic SCF E3 ligase complexes.

Convergence on the MAP kinase signaling pathway

What are the targets of this E3 complex during axon or synapse development? In the absence of the ligase activity, the increased level and/or activity of their targets are expected to contribute to defects exhibited by *PHR* mutants. Genetic and biochemical interactions between *PHR* mutants and multiple signaling pathways have provided a list of candidates; one conserved target of PHRs is the *d*ual-*l*eucine zipper *k*inase, DLK-1/Wallenda/DLK, and the respective MAP kinase signaling cascades they activate (Figure 1B) [13••,22••,26••].

In *C. elegans*, RPM-1 and FSN-1 negatively regulate the activity of a MAP kinase cascade consisting MAPKKK/DLK-1, MAPKK/MKK-4, and p38/PMK-3 [9•,26••,27••]. Loss-of-function mutations in any component of this cascade result in the suppression of *rpm-1* and *fsn-1* defects. DLK-1 levels are increased in *rpm-1* mutants, and DLK-1 can be ubiquitinated by RPM-1 *in vitro* [26••]. The overexpression or constitutive activation of the p38 MAPK cascade induces *rpm-1*-like defects. RPM-1, therefore, affects synapse development by targeting DLK-1 for degradation and negatively regulating p38 signaling. A recent study further revealed that p38 signaling regulates synapse development and axon regeneration through a basic-leucine-zipper (bZip) transcription factor (CEBP-1) and local protein translation [28].

The *Drosophila* DLK homolog, *wallenda*, was also identified as a genetic suppressor for *hiw*. Wallenda is negatively regulated by HIW and DFsn in a similar manner: the level of Wallenda is increased in *hiw* and *DFsn* mutants, and an overexpression of Wallenda phenocopies *hiw* and *DFsn* mutants at NMJs. Instead of functioning through p38 signaling, however, Wallenda regulates synaptic growth through JNK/Bsk and the Fos transcription factor (D-fos). In addition, mutations in *wallenda* suppress the excessive branching without restoring defective synaptic transmission of *hiw* mutants, suggesting that HIW regulates synaptic function through other unidentified targets [29••].

Mouse Phr1 also downregulates DLK in developing motoneurons. In embryonic motoneuron cultures, Phr1

localizes along the axon shaft, but is excluded from growth cones, where DLK localization is restricted. In *Phr1/Magellan* neuronal cultures, DLK spreads along the axon shaft, and treatment with a p38 inhibitor rescues microtubule morphology [13••]. This is consistent with Phr1 regulating microtubule dynamics through inhibiting DLK and p38 signaling at the growth cone.

However, as for HIW, there are additional unidentified targets for Phr1. In *Phr1* and *Fbxo45* knockout mice, no gross change in DLK levels is detected [14••,23••], and the CNS axon defects are not suppressed in *Phr1;Dlk* double mutants [14••], consistent with Phr1 regulating other targets at the CNS. Similarly, axon targeting and outgrowth defects in zebrafish *esrom* mutants are not associated with aberrant p38 signaling [19••]. Therefore, in addition to DLK, PHR proteins regulate other cell-type or developmental stage-specific targets and signaling pathways during development.

Interactions with other signaling pathways

Consistent with PHR proteins regulating additional targets, *PHR* mutants exhibit genetic and/or biochemical interactions with components of multiple signaling pathways (Figure 1B). *In vivo* and *in vitro* interactions between PHRs and a tumor suppressor protein TSC2/tuberin were noted in several systems. The RING-H2 finger motif of PAM can bind and ubiquitinate TSC2 *in vitro* [30••]. Phr1 co-immunoprecipitates with the TSC1–TSC2 complex in PC12 cell lines and rat brain, and RNAi-mediated knockdown of Phr1 in primary rat cortical and hippocampal neuron cultures result in an increased level of TSC2 [31]. Esrom affects TSC2/tuberin as well: in *esrom* mutants, there is an elevated level of phosphorylated TSC2 at the growth cone and axon tip and a subsequent reduction of TSC2 activity [5••]. Given that *Drosophila* TSC1–TSC2 affects axon guidance and synaptic growth [30••], TSC2/Tuberin makes another plausible candidate target for PHRs.

The RING-H2 domain of HIW binds Medea, a co-SMAD in the bone morphogenetic protein (BMP) signaling pathway [32••]. Reduced activity in components of the BMP signaling, the Type II receptor (Wishful thinking), its ligand (Glass bottom boat), and Nemo, a kinase that phosphorylates the transcription factor Mad, results in fewer and smaller boutons at *Drosophila* glutamatergic NMJs. Reduction of BMP signaling also reduces the excessive bouton number of *Hiw* mutants [33], consistent with its synaptic overgrowth associated with elevated BMP signaling in motoneurons. Similarly, *C. elegans rpm-1* defects are partially suppressed by reducing axon guidance receptors UNC-5 or Robo/SAX-3 [8•]. Reducing the activity of an anaplastic lymphoma receptor kinase ALK/SCD-2 partially suppresses the synaptic defects of *fsn-1* and SCD-2 levels are increased in *fsn-1* mutants [21••]. However, *scd-2* exhibits a weak interaction with RPM-1; *scd-2* does not rescue *rpm-1*, and the protein level is only moderately affected by the *rpm-1* mutation [21••]. It is important to test whether these proteins could be directly targeted by PHRs for ubiquitination and degradation.

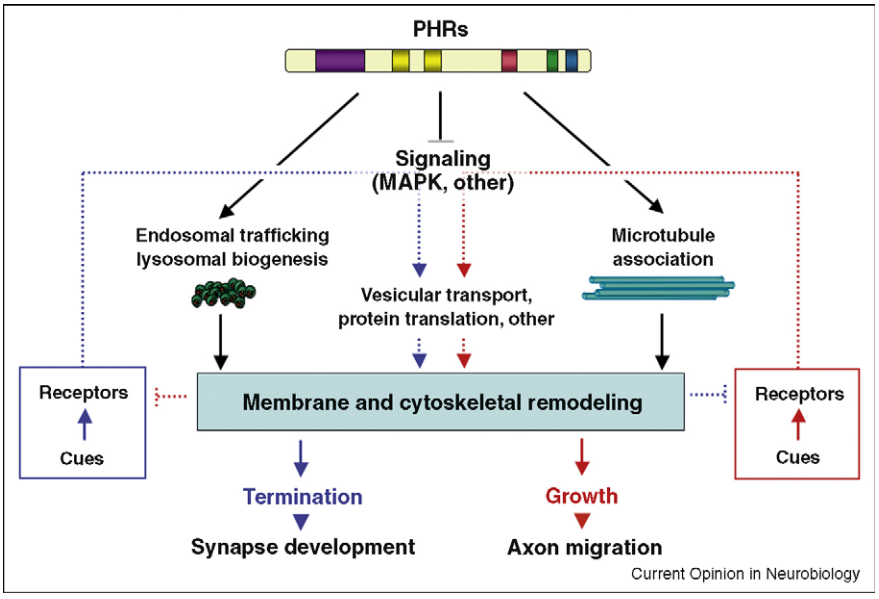
Not all biochemical interactions with PHRs are involved in, or result in protein degradation. A guanine nucleotide exchange factor GLO-4 binds RPM-1 (Figure 1), and this interaction does not affect their respective stability or subcellular localization. Given its requirement for lysosomal biogenesis in the gut and the normal expression of late endosomal marker in neurons, GLO-4 probably facilitates RPM-1's involvement in endosomal/lysosomal trafficking through mechanisms independent of protein degradation [27••]. The RCC1-like domain of PAM/Phr1 associates with a K^+-/Cl^- co-transporter KCC2, and increases the transcription and activity of KCC2 when co-transfected in HEK293T cells [16]. The RCC1-like motif can also associate with, and potently inhibit the enzymatic activity of adenylyl cyclases [34]. Further examination of degradation-independent functions of PHR proteins, and the contribution of these interactors in PHR-mediated axon guidance, synapse development, or other functions is crucial.

A switch between axon guidance, outgrowth and synapse formation

Axon guidance, outgrowth, branching, and synaptogenesis are interconnected developmental events during the maturation of the nervous system. The coordination of these events depends on the spatial regulation of extrinsic cues as well as the temporal regulation of intrinsic programming that dictates an axon's response to cues. *PHR* mutants exhibit defects in varied combinations of the above events; a likely unifying theme is the failure in transitions between the initiation and termination of axon growth. Failure in activating this switch, therefore 'locking' the axon in either phase, could cause premature or prolonged axon stalling, axon oversprouting or overshooting, underdevelopment or overgrowth of synapses, depending on its cell-type and environment (Figure 2).

PHR mutants display diverse defects and genetic interactions with multiple signaling pathways, often in a cell-type, developmental stage, or organism-specific manner. Some differences are probably due to the regulation of different signaling events. The conservation of PHR proteins, however, makes it difficult to attribute all differences to contextual changes in targets. The simplest interpretation for the complex phenotypes and genetic interactions exhibited by *PHR* mutants is that PHR proteins regulate a few shared downstream effectors of signaling pathways, whose activities are responsible for switching on or off axon growth in response to cues. This allows neurons to initiate and commit to the next developmental phase, for example, migrating to the next

Figure 2



PHR proteins mediate transitions between axon growth, termination, and synapse formation. A tentative model on how PHR proteins integrate signaling and cytoskeletal remodeling to mediate transitions between axon growth, termination, and synapse formation. Associating with the cytoskeleton, PHRs initiate cytoskeletal remodeling in response to the intracellular signaling changes induced by extrinsic cues that signal growth (red) or termination (blue). PHRs also directly (via RCC1 and other domains) and indirectly (via E3 ligase activity) regulate intracellular signaling that alter microtubule-based vesicular transport, endosomal trafficking/lysosomal biogenesis, and local protein translation to enforce the transition between growth and termination. This allows axons to activate and commit to the next phase of development, such as guidance to subsequent targets or synapse development.

choice point or establishing synapses. When axons pause prematurely or grow past their targets, the transition fails owing to the lack of cues, and/or the activation of intrinsic programs to respond to cues.

PHR proteins regulate intracellular signaling; they also associate with microtubule [13••] and F-actin-enriched cytoskeletal complexes [35], making them ideal molecular scaffolds to integrate signaling changes and cytoskeletal reorganization (Figure 2). Signaling cascades regulated by PHRs affect axon regeneration [9•,28] or degeneration [36•] after axotomy (DLK-1/p38 and DLK/JNK) and axonal transport (Wallenda/JNK) [37]. Perturbation of microtubule assembly or disassembly augments, at least partially, the developmental defects in *Phr1/esrom* mutant neurons [13••,19••], further supporting this model.

More functions for PHRs?

PHR proteins have additional *in vivo* functions that are not obviously related to signaling or cytoskeletal dynamics of neurons (Figure 1C). In addition to axon defects, zebrafish *esrom* mutants exhibit a paler color owing to the decrease of specific pteridines in xanthophores. Esrom is required for the synthesis of tetrahydrobiopterin not only in xanthophores, but also probably in the retinal neurons through unknown mechanisms [38•,39]. PHR proteins also regulate apoptosis. *C. elegans* *rpm-1* and *fsn-1* mutants both exhibit hypersensitivity to ENU-induced apoptosis and exhibit an increased level of phosphorylated p53/CEP-1 in the germline [40•]. Fbxo45 binds and ubiquitinates p73 *in vitro* and in a neuroblastoma cell line; it also promotes p73 stability in a breast cancer cell line [41]. However, as endogenous RPM-1 or FSN-1 have not been detected in *C. elegans* germline, mechanisms for such a function remain unclear. Further morphological, biochemical, and genetic analyses of PHRs are likely to reveal novel functions and developmental processes regulated by PHRs.

Summary and future prospects

PHR proteins have diverse roles in axon guidance, outgrowth, termination, and synaptogenesis. Recent studies have further implicated a role for PHR proteins in axon regeneration. *PHR* mutants exhibit differences in phenotypes. Nonetheless, PHR proteins may function as regulators to integrate cell signaling and cytoskeletal dynamics through similar mechanisms. It is enticing to speculate that PHRs affect axon and synapse development by promoting cytoskeleton rearrangements in response to extracellular cues and intracellular signaling in a temporally and spatially regulated manner (Figure 2).

This leads to several key questions that remain unanswered. First, DLK and DLK-mediated signaling cannot account for all observed defects in *PHR* mutants. More

PHR targets remain to be confirmed or identified; second, this model predicts a precise temporal regulation of PHR subcellular localization crucial for their functions. Mechanisms that regulate the temporal and spatial localization of PHRs remain largely undiscovered; third, as *PHR* mutants exhibit cell-type specific phenotypes and organism-specific phenotypes, it is important to identify factors that dictate the phenotypic expression of PHR regulated signaling. Lastly, not all phenotypes of *PHR* mutants are neuronal or even cell-autonomous. Future studies are therefore expected to reveal more biological processes under PHR regulation.

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- •• of outstanding interest

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