Viewpoints

The Ubiquitinated Axon: Local Control of Axon Development and Function by Ubiquitin

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Ubiquitin tagging sets protein fate. With a wide range of possible patterns and reversibility, ubiquitination can assume many shapes to meet specific demands of a particular cell across time and space. In neurons, unique cells with functionally distinct axons and dendrites harboring dynamic synapses, the ubiquitin code is exploited at the height of its power. Indeed, wide expression of ubiquitination and proteasome machinery at synapses, a diverse brain ubiquitome, and the existence of ubiquitin-related neurodevelopmental diseases support a fundamental role of ubiquitin signaling in the developing and mature brain. While special attention has been given to dendritic ubiquitin-dependent control, how axonal biology is governed by this small but versatile molecule has been considerably less discussed. Herein, we set out to explore the ubiquitin-mediated spatiotemporal control of an axon's lifetime: from its differentiation and growth through presynaptic formation, function, and pruning.

Key words: axons; presynaptic terminal; ubiquitin; ubiquitin-proteasome system; neuronal disorders

Introduction

Ubiquitin functions in cells as a small tag bound in an extremely selective manner to target proteins. Cells use ubiquitination as a method to modify proteins: either transiently, by altering localization, activity, and signaling of the tagged protein, or permanently, by dispatching the protein for degradation in the so-called ubiquitin-proteasome system (UPS) or in lysosomes. Ubiquitination is therefore a strategy cells use for regulation at the local level in a temporal and target-specific manner. Such regulation is particularly suitable for cells, such as neurons, which have highly compartmentalized functions. Indeed, axons and dendrites could not be more distinct in their function and protein composition, despite being intimately and functionally connected by synapses. This necessitates the ability of different, but highly regulated, local mechanisms to change protein content and hence function, ultimately securing proper synaptic connectivity. In addition, the unique polarized morphology of neurons, with long axons and plastic synapses undergoing constant changes in their proteome, impose a challenge on the local mechanisms governing proteostasis. It is therefore unsurprising that neuronal ubiquitin signaling is of utmost importance for

proper brain function. This importance is underscored by the fact that ubiquitin-positive aggregates are present in nearly all neurodegenerative diseases (Perry et al., 1987; Lowe et al., 1988; Davies et al., 1997) and mutations in ubiquitin-related machinery underlie the pathogenesis of several neurodevelopmental diseases (Matsuura et al., 1997; Leroy et al., 1998; Morrow et al., 2008; Ramser et al., 2008; Paemka et al., 2015; Nguyen et al., 2016).

At the postsynaptic level, the UPS contributes to synaptic remodeling by providing activity-dependent control of key postsynaptic proteins, such as scaffolds and receptors (Colledge et al., 2003; Ehlers, 2003; Kato et al., 2005; Saliba et al., 2007; Hamilton et al., 2012; Shin et al., 2012; Widagdo et al., 2015). Indeed, proteasomes are recruited to dendritic spines on depolarization (Bingol and Schuman, 2006) to sculpt the content of a synapse consonantly to its activity status (Ehlers, 2003). As a result, the UPS is crucial for the establishment of synaptic plasticity, making it essential for memory formation and retrieval (Artinian et al., 2008; Lee et al., 2008; Jarome et al., 2011; Jarome and Helmstetter, 2013; Ferrara et al., 2019). In addition to postsynaptic proteins, the presynaptic proteome is regulated by ubiquitin-dependent mechanisms. Several axonal proteins have been identified as ubiquitin substrates (Franco et al., 2011; Na et al., 2012), suggesting an involvement of the UPS in axonal development and presynaptic function. Although knowledge about ubiquitin signaling in axons has accumulated in recent years, a comprehensive compilation of these studies is still lacking. In this review, we propose to unravel how, when, and where ubiquitin signaling is exploited throughout the early and mature life of axons.

Ubiquitin signaling

Ubiquitin is a highly conserved small protein with 76 amino acids that has the unusual property of being covalently attached

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Figure 1. Ubiquitin signaling. **A**, Attachment of ubiquitin moieties to naked substrates is promoted by a cascade of enzymes (E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme, and E3 ubiquitin-ligase) and counteracted by deubiquitinases (DUB) that trim bound ubiquitin. Several rounds of ubiquitination can lead to formation of polyubiquitin chains. **B**, Different topologies of ubiquitin signals on substrates. Substrates can be found in a monoubiquitinated state [single ubiquitin (monoUb) or multiple single ubiquitins (multi-monoUb)] or harboring polyubiquitin chains (polyUb). In homotypic chains, linkage between ubiquitin molecules occurs in the same lysine (K) residue, whereas heterotypic chains contain mixed linkages. For monoubiquitination and polyubiquitination, blue represents ubiquitin signals that may function as a tag for proteasome clearance. **C**, Proteins harboring a degradation tag are directed to the proteasome by shuttles, deubiquitinated, unfolded, and degraded within the 20S catalytic core.

to other proteins. The attachment of ubiquitin to a protein, known as ubiquitination, constitutes a type of post-translational modification that can alter several properties of the target protein, such as its stability, structure, function, localization, and interaction with partners (Komander and Rape, 2012). Ubiquitin is a highly stable protein that adopts a compact β -grasp fold with an exposed carboxy terminal tail that forms an isopeptide bond with lysine (K) residues of a protein substrate. In addition, ubiquitin itself has seven lysine residues that can all serve as attachment sites for additional ubiquitin molecules. As a consequence, cellular proteins can be found in a monoubiquitinated or polyubiquitinated form, the latter resulting from the polymerization of ubiquitin chains on the first substrate-conjugated ubiquitin (Fig. 1A,B). Because in ubiquitin all seven lysine residues, as well as the first methionine, are able to accept another ubiquitin, different homotypic chains linked via K6, K11, K27, K29, K33, K48, K63, and Met1 can be attached to substrates. In the rat brain, polyubiquitin chains are expressed in the following ascending order: K29, K27, K33, K6, K11, K63 and K48 (~0.05%, 0.5%, 8%, 9%, 15%, 29%, and 37%, respectively) (Na et al., 2012). Ubiquitin signaling is further diversified by the attachment of single ubiquitin molecules to multiple sites of a protein (multi-monoubiquitination) and formation of heterotypic chains containing mixed linkage types (Peng et al., 2003; Xu et al., 2009; Komander and Rape, 2012) (Fig. 1B). In the mouse brain, the majority of ubiquitin is in the free, unconjugated form, whereas only 35% and 5% of total ubiquitin is present, respectively, as monoubiquitin and polyubiquitin modifications on substrates (Kaiser et al., 2011). Thus, cells keep a considerably large pool of readily available free ubiquitin, suggesting that substrates can be rapidly ubiquitinated on need. The small pool of conjugated polyubiquitin further suggests that cells can rapidly respond to new intracellular substrate-conjugated ubiquitin signals, rather than requiring substrates to keep their ubiquitinated state for long periods of time.

Assembly of ubiquitin chains is performed by a three-step enzymatic cascade involving an E1 ubiquitin-activating enzyme, an E2 ubiquitin-conjugating enzyme, and an E3 ubiquitin-ligase. The first step comprises activation of ubiquitin by the E1 enzyme in an ATP-dependent manner. Activated ubiquitin is transferred to the E2 enzyme, which forms a complex with a substrate-carrying E3, ultimately leading to the covalent binding of ubiquitin to a lysine residue of the target protein (Pickart, 2001) (Fig. 1A). Importantly, E3 ligases are the key specifiers of protein ubiquitination, mostly because of their recognition of substrates by specific protein-protein interactions.

In general, E3 ligases are classified based on their E2 interaction domain: an interesting new gene (RING) finger domain, a U-box motif, or a homologous to E6-AP carboxyterminus (HECT) domain. While RING and U-box E3 ligases [e.g., RING finger protein (RNF) family and C-terminus of Hsp70-interacting protein, respectively] catalyze the direct transfer of ubiquitin from the E2 enzyme to the substrate, HECT E3 ligases [e.g., neuronal precursor cell expressed developmentally downregulated 4 (Nedd4)] produce an additional HECT-ubiquitin intermediate before forwarding ubiquitin to the substrate (Zheng and Shabek, 2017). In the recently discovered hybrid system of RING-between-RING E3 ligases (e.g., Parkin), the first RING transfers ubiquitin to the second RING domain before its relocation to the final substrate (Wenzel et al., 2011; Walden and Rittinger, 2018). RING E3 ligases can either function as a single protein or as a multisubunit complex. Examples of the latter are the Skp1-Cullin-F-box (SCF) and the anaphase promoting (APC) complex. These are multisubunit E3 ligases that rely on a substrate-recognizing protein, an F-box protein in the SCF complex, and Cdh1 or Cdc20 in the APC complex.

The reversibility of ubiquitin signaling is accomplished by deubiquitinases capable of removing ubiquitin molecules from substrates (Fig. 1A). Deubiquitinases can either fully deubiquitinate a substrate or edit polyubiquitin chains, thus redirecting a substrate's fate after the initial ubiquitination. They also have a prime role in disassembling unanchored ubiquitin chains and removing ubiquitin from proteasome substrates (Komander et al., 2009). Thus, in addition to their role in ensuring correct ubiquitination of substrates, they are essential for the maintenance of the cellular free ubiquitin pool, a prerequisite for proper cell function and viability (Swaminathan et al., 1999; Kimura et al., 2009), in particular nervous system development (Osaka et al., 2003; Chen et al., 2009; Ryu et al., 2014).

The diverse ubiquitin messages on proteins are decoded by proteins that recognize ubiquitin signals, interpret them, and generate a biochemical output in the cell. These proteins are generally termed ubiquitin-binding proteins, and they contain specialized ubiquitin-binding domains that bind transiently and noncovalently to either monoubiquitin or polyubiquitin. They may exhibit relative or absolute selectivity for different types of ubiquitin chains (Husnjak and Dikic, 2012). A quick and self-explanatory example are proteasome shuttle factors that predominantly bind to proteins bearing a K48 polyubiquitin chain and deliver them to the proteasome for degradation (Verma et al., 2004; Elsasser and Finley, 2005) (see The UPS).

In summary, cells possess all the required molecular machinery for coordinated assembly and editing of ubiquitin signals on proteins, as well as for their decoding and translation into different cell responses. As we will discuss later, axons possess all these elements, and thus can use ubiquitin signaling in an autonomous and local manner.

Functions of ubiquitination

The diverse patterns of ubiquitination found on different proteins (Fig. 1B) suggests that ubiquitination has a wide range of functions. The best documented of these is the role of K48 ubiquitin chains in tagging proteins for disposal by the proteasome. Although a few other roles have been suggested, the functional significance of most forms of conjugated ubiquitin species is still unknown.

The UPS. Proteasome-mediated degradation is the most well-known outcome of ubiquitin signaling, classically associated with K48 ubiquitin chains, more precisely to substrates harboring a ubiquitin chain with four or more ubiquitin moieties (Thrower et al., 2000). The UPS is the major degradative pathway of soluble short-lived proteins in cells; and, as a result, it is involved in essentially every cellular event. In neurons, it is of utmost importance for neuronal development, function, and plasticity (Bingol and Sheng, 2011).

The macromolecular structure that degrades polyubiquitinated proteins is the 26S proteasome, which is formed by a barrel-shaped 20S catalytic core particle and the 19S regulatory particle (Fig. 1C). In brief, the 19S specifically selects and guides target proteins to the 20S proteolytic chamber. Equipped with an array of functionally distinct subunits, the 19S traps polyubiquitinated substrates, detaches ubiquitin chains, and generates ATP to unfold the protein and open the 20S. Once inside, substrates are cleaved by the inwardly facing catalytic subunits of the 20S particle (Kish-Trier and Hill, 2013; Tanaka, 2013). Instrumental for effective function of the UPS are proteasome shuttle factors, such as Rad23, Dsk2, and Ddi1, that collect ubiquitinated substrates and deliver them to the proteasome for their final breakdown (Verma et al., 2004; Elsasser and Finley, 2005).

Recently, substantial data have been gathered demonstrating that K48 ubiquitin is not the sole tag for proteasome clearance. Remarkably, all polyubiquitin chains, except for K63, accumulate in cells shortly after proteasome inhibition (Xu et al., 2009), as well as in the brain of a 26S conditional KO mouse (Bedford et al., 2011), thus indirectly suggesting the involvement of these polyubiquitin chains in the UPS. Indeed, the E3 complex APC is a master supplier of K11 ubiquitin chains to mitotic regulators, thereby sentencing them to degradation (Jin et al., 2008). The repertoire of proteolytic signals also includes substrates harboring heterotypic chains, one or multiple single ubiquitins, and nonubiquitinated substrates (Murakami et al., 1992; Boutet et al., 2007; Kravtsova-Ivantsiv et al., 2009; C. Liu et al., 2017) (Fig. 1B). This broad range of degradation tags may confer on the UPS higher specificity and plasticity in its selection of targets to eliminate. For example, it is conceivable that K48 tags function in cells as constitutive signals for degradation, while noncanonical ubiquitin signals are exploited to fulfill specific cellular needs.

Proteasome-unrelated outcomes of the ubiquitin code. Protein ubiquitination is not always interpreted in cells as a proteasome tag. Indeed, it may alter the targeted protein and the concomitant cellular pathway in myriad ways. For instance, ubiquitination has been shown to regulate the endocytic pathway and lysosome degradation (Piper et al., 2014), DNA repair (Jackson and

Durocher, 2013), and the NF- κ B regulatory pathway (Deng et al., 2000; Wang et al., 2001; Windheim et al., 2008).

In the endocytic pathway, membrane proteins are internalized into endosomes, from which they can be sorted into late endosomes. Subsequent fusion of late endosomes with lysosomes leads to proteolysis. Both internalization at the plasma membrane and sorting to late endosomes are governed by ubiquitination, mostly by K63 ubiquitin chains, single or multiple monoubiquitin moieties on substrates (Piper et al., 2014). In this manner, ubiquitin can spatiotemporally control the signaling downstream of activated surface receptors, such as members of the receptor tyrosine kinase family (see Table 2).

Ubiquitin's mode of action in DNA repair and the NF- κ B signaling are slightly different. Herein, ubiquitin chains act as recruiters of molecular machinery to specific cellular locations. For instance, DNA lesions are first detected by proteins that, in part through K63 ubiquitin chains or monoubiquitination, recruit and activate additional components of the DNA repair machinery (Jackson and Durocher, 2013).

Ubiquitination can also alter, either directly or indirectly, several properties of the tagged protein itself, including activity, location, or propensity to interact with partners (Laub et al., 1998; Li et al., 2003; DuPont et al., 2009; Sasaki et al., 2011; Baker et al., 2013; Wang et al., 2018). A good example of the latter is the K29 ubiquitination of axin, a scaffold for Wnt signaling, by E3 Smad ubiquitination regulatory factor 1 (Smurf1) (Fei et al., 2013). Axin interacts with the coreceptor LRP5/6 of the synaptogenic protein Wnt; however, on addition of a K29 ubiquitin tag, this interaction is disrupted and Wnt signaling is repressed (Fei et al., 2013).

Overall, the diverse world of possible ubiquitin tags (Fig. 1B), along with the ever-broadening list of possible functions (Swatek and Komander, 2016), suggests that ubiquitin is involved in a horde of cellular events that have so far not been investigated. Thus, there is a great unexplored potential of ubiquitin signaling. In the coming sections, we propose to unveil the role of ubiquitin signaling in axons, the neuronal compartment that functions as the main dispatcher of neurotransmission.

Ubiquitin and the proteasome in axons

Brain functioning relies on effective connectivity between neurons, exquisitely complex cells that display a high degree of morphologic and functional polarity. During nervous system development, an ordered sequence of elaborate events leads to the establishment of short- or long-range axonal projections with numerous presynaptic terminals that are precisely connected to postsynaptic cells. Soon after a growing neurite acquires initial axonal characteristics (Yogev and Shen, 2017), the growing axon is guided by extracellular cues (Polleux and Snider, 2010; Stoeckli, 2018) to a precise spatial location where it encounters the receptive dendrites of its partner. Formation of a presynaptic terminal is then triggered and specified by soluble and cell adhesion factors (Pinto and Almeida, 2016). Later, synapses are strengthened and enlarged or pruned, mostly depending on their activity level (Riccomagno and Kolodkin, 2015). When mature and functional, presynaptic terminals mediate rapid and controlled release of neurotransmitters (Südhof, 2013).

In this section, we provide a chronological overview of ubiquitin-related events taking place in axons throughout the course of their development (sections Axon specification, Axon outgrowth, Axon guidance, and Presynapse formation) and in their mature form (sections Presynaptic function and Axonal self-destructive events). We hope this will provide a deep understanding

Figure 2. Molecular players in ubiquitin control of axon development. Local ubiquitin-mediated regulation occurs and supports all steps of axon morphogenesis, starting with the initial phase in which an axon gains its identity (**A**, axon specification), and continuing as the axon grows (**B,C**, axon outgrowth) and navigates its way to its target (**D**, axon guidance). Ubiquitin-targeted proteins (blue) and respective E3 ubiquitin ligases (orange) or deubiquitinases (yellow) are illustrated at their site of action. The type of ubiquitin chains involved is indicated. For complementary information, see Table 1. CHIP, C-terminus of Hsp70-interacting protein; DCC, deleted in colorectal cancer; EBAX-CRL, EBAX-type Cullin-RING; LIMK1, LIM kinase 1; RhoGEF, Rho guanine nucleotide exchange factor.

of ubiquitin-dependent mechanisms acting locally to govern axon development and function (Figs. 2, 3). To aid this endeavor, we provide a thorough description of the ubiquitination machinery and axonal targets involved (Table 1). We also provide a list of additional axonal proteins whose modulation by ubiquitin has been described, but not yet assigned a functional role in the axon (Table 2).

Axon specification

The characteristic morphology of the nerve cell stems partly from the polarization of its neurites (i.e., the formation of a single axon and multiple dendrites). A newborn neuron becomes polarized at an early stage of development, when one of several unspecified neurites acquires axonal characteristics. This is generally triggered by an external cue that creates asymmetric domains in the developing neuron by changing local protein content. This ultimately elicits major cytoskeletal rearrangements selectively

favoring growth of the prospective axon (Yogev and Shen, 2017). The UPS contributes to the generation of this asymmetry by degrading specific proteins in a regulated spatiotemporal fashion (Fig. 2A; Table 1) (Yan et al., 2006; Cheng et al., 2011a; Lin et al., 2011). Axon growth-promoting and -inhibiting proteins will be, respectively, enriched and eliminated in nascent axons by differential proteasome degradation. In addition, proteasomal removal of axon-promoting proteins in nascent dendrites ensures formation of a single axon. For example, axon differentiation is initiated in part by phosphatidylinositol-3-kinase (PI3K) activation of Akt (Jiang et al., 2005), which is restricted to the future axon as a result of its proteasomal degradation in the prospective dendritic branches (Yan et al., 2006). In concert with PI3K, localized axonal accumulation of portioning-defective proteins (Par) contributes to axonal specification (Shi et al., 2003). Interestingly, the E3 ligase Smurf1 can induce degradation of either portioning-defective protein 6 (Par6) and the growth-disrupting small GTPase RhoA

depending on its phosphorylation status (Cheng et al., 2011a). Specifically, BDNF, identified as a symmetry-breaking cue (Cheng et al., 2011b; Nakamuta et al., 2011), stimulates Smurf1 phosphorylation, which then loses affinity for Par6 and preferentially ubiquitinates RhoA. This increases the spatial gradient of Par6 versus RhoA, which is required for axon formation (Cheng et al., 2011a). Smurf1 is itself sent for degradation by the E3 complex Cdh1-APC, which ultimately controls axon growth (Kannan et al., 2012). The spatiotemporal enrichment of axonal-promoting proteins at unspecified neurites, created by differential local proteolysis (Fig. 2A), is further reinforced by axonal degradation of the RhoA activator Rho guanine nucleotide exchange factor by the KLHL20-based E3 ligase complex (Lin et al., 2011) and by the joint action of another proteolytic system, the calpains [beyond the scope of this review but nicely reviewed elsewhere (Bórquez and González-Billault, 2011)]. Despite the plausibility of this UPS-facilitated asymmetry model, differences in the behavior of nascent axons in culture and in intact tissue indicate that *in vivo* validation of UPS involvement should be sought.

Axon outgrowth

After a neurite has been specified as the axon, it must grow until it reaches its target. Axon outgrowth is primarily driven by dynamic cytoskeleton rearrangements that propel the axon forward, bolstered by changes in gene expression that favor growth (Polleux and Snider, 2010). Accumulating evidence suggests that the UPS modulates axon growth by acting at both of these levels (Fig. 2B,C; Table 1), as discussed below.

In the nucleus, the E3 complex Cdh1-APC acts as a negative regulator of genes involved in axon growth and patterning (Fig. 2B) (Yang et al., 2010). Indeed, mice lacking Cdh1 have longer axons (Konishi et al., 2004). Activation of Cdh1-APC in the nucleus enhances ubiquitination and proteasomal degradation of the transcriptional regulators SnoN (Stegmüller et al., 2006) and inhibitor of DNA binding 2 (Id2) (Lasorella et al., 2006). Whereas SnoN upregulates the expression of axonally enriched growth-promoting proteins (e.g., the scaffold protein Cdc1) (Ikeuchi et al., 2009), Id2 leads to downregulation of growth-inhibiting proteins (e.g., the Nogo receptor) (Lasorella et al., 2006). By inducing degradation of SnoN and Id2, Cdh1-APC leads to inhibition of positive axon growth modulators and disinhibition of negative growth modulators, thus repressing axonal growth. Interestingly, Akt phosphorylation of Id2 prevents its association with Cdh1-APC, thereby preventing proteasomal degradation and enhancing Id2 protein stability. Subsequently, Id2 is translocated to the growth cone, where it contributes to growth cone formation and axon outgrowth via interaction with radixin, a protein known to regulate growth cone dynamics by linking F-actin to the plasma membrane (Ko et al., 2016). How activity of nuclear Cdh1-APC is adjusted to fit the growing needs of a developing or regenerating axon remains elusive. Given that axonal growth is generally triggered by external cues, a yet unknown retrograde signal is likely to be involved.

Outside the nucleus, the UPS regulates axon growth by locally interfering with the cytoskeleton elements engaged in motion, such as actin microfilaments in the growth cone and tubulin microtubules along the axon shaft (Fig. 2C). LIM kinase 1 enhances polymerization of F-actin and thus accelerates axon extension (Rosso et al., 2004). Its levels in the growth cone are negatively regulated by the E3 ligase RING finger protein 6, which thus shortens axons (Tursun et al., 2005). In addition, the axonal actin cytoskeleton is indirectly regulated by the UPS through the control of phosphatase and tensin homolog (PTEN) levels, a negative regulator of PI3K control of cytoskeleton dynamics (Jiménez

et al., 2000). Both in rat and *Xenopus*, PTEN is targeted for proteasome degradation by the E3 ligase Nedd4, and this enhances axons' capacity to grow and branch (Christie et al., 2012). Together, these studies reveal that the UPS controls actin dynamics by modulating the levels of actin cytoskeleton regulators. Nevertheless, the highly dynamic behavior of actin filaments would benefit from a faster mode of regulation, which ubiquitin could potentially offer by affecting the activity and interaction properties of actin regulators directly, rather than by targeting proteins for degradation. Identification of such modulation awaits future study.

Ubiquitin has also been shown to limit axon growth by interfering with remodeling of microtubules, dynamic cytoskeletal elements that are essential for axon extension and stabilization. For instance, levels of katanin-p60, a subunit of a microtubule-disassembling enzyme (McNally and Vale, 1993), are balanced by the concerted action of the E3 ligase C-terminus of Hsp70-interacting protein and the deubiquitinase ubiquitin-specific protease (USP) 47 (Yang et al., 2013). In a context favoring stability of katanin-p60, such as FGF-induced USP47 upregulation, microtubules dynamics are sustained, thus promoting axon growth (Yang et al., 2013).

In addition, modulation of microtubule dynamics by E3 ligases is likely to occur through effects on Wnt/ β -catenin signaling. This signaling pathway alters microtubule organization (Hall et al., 2000; Packard et al., 2002) and maintains the balance between axonal outgrowth and growth cone enlargement by regulating microtubule dynamics (Purro et al., 2008). In *Caenorhabditis elegans* and *Drosophila*, PLR-1 and HUWE1 are E3 ligases that help regulate axon outgrowth and branching (Vandewalle et al., 2013; Bhat et al., 2015), likely by adjusting the availability of components of the Wnt/ β -catenin pathway (Vandewalle et al., 2013; Moffat et al., 2014; Bhat et al., 2015), such as disheveled (Vandewalle et al., 2013), which is required for Wnt-mediated microtubule reorganization and hence changes in axon behavior (Krylova et al., 2000; Ciani et al., 2004; Purro et al., 2008). Notably, other components of Wnt signaling (Table 2) are liable to undergo ubiquitin regulation, and therefore are also potentially involved in ubiquitin-dependent modulation of microtubule dynamics. Another E3 likely involved is the E3 ligase protein family PHR (human PAM or Mycbp2, mouse Phr1 or Mycbp2, zebrafish Esrom, *Drosophila* Highwire and *C. elegans* RPM-1), which has roles in both axon and synapse development (for a comprehensive review, see Po et al., 2010). Deletion of PHR has deleterious effects on axon navigation, with axons failing to reach their correct destination (Bloom et al., 2007; Lewcock et al., 2007; Hendricks and Jesuthasan, 2009). The authors concluded that these phenotypes resulted from aberrant microtubule dynamics that could be corrected by pharmacological manipulation of microtubule assembly (Lewcock et al., 2007; Hendricks and Jesuthasan, 2009). Interestingly, growth cones from *phr* mutant axons (Lewcock et al., 2007; Hendricks and Jesuthasan, 2009) are strikingly similar to those obtained after Wnt stimulation or dishevelled overexpression (Purro et al., 2008), thus suggesting the likely involvement of Wnt/ β -catenin signaling in PHR-mediated axon outgrowth via modulation of microtubule dynamics. It is, however, imperative to validate this correlation in future investigations and precisely define how axon-enriched E3 ligases locally control canonical and atypical Wnt/ β -catenin pathways. Nevertheless, these studies highlight the role of UPS in modulating axon development primarily through dynamic changes in microtubule organization.

Table 1. Ubiquitination machinery and targeted axonal substrates working locally to regulate axon development and function^a

E3	Axonal target	Ub chain	Outcome of ubiquitination	Neuronal compartment	Role in axon	Model	Reference
KLHL20	AKT ^b	polyUb	Prot deg	dendrites	Axon specification	Rat	Yan et al., 2006
	RhoGEF	polyUb	Prot deg; RhoA inactivation	axon		Rat	Lin et al., 2011
Smurf1	Par6	polyUb	Prot deg	axon		Rat	Cheng et al., 2011a; Deglincerti et al., 2015
	RhoA	polyUb			Axon specification/growth cone collapse		
Praja2	Nogo-A	polyUb	Prot deg	axon	Axon outgrowth	Rat; mouse	Sepe et al., 2014
RNF6	LIMK1	–	Prot deg; reduced actin dynamics	growth cone	Inhibition of axon outgrowth	Mouse	Tursun et al., 2005
CHIP	katanin-p60	polyUb	Prot deg; altered microtubule dynamics	axon	Inhibition of axon outgrowth	Rat	Yang et al., 2013
PLR-1	–	–	Disruption of Wnt/ β -catenin pathway	axon	Axon outgrowth/axon guidance	Cel	Bhat et al., 2015
HUWE1	dishevelled	–		axon	Axon branching	Dro	Vandewalle et al., 2013
	–	–		presynaptic terminal	GABAergic transmission	Cel	Opperman et al., 2017
EBAX-CRL	MBNL1	K63	Cytoplasmic localization	axon	Axon outgrowth	Mouse	Wang et al., 2018
	Roundabout	–	Degradation of misfolded protein	axon	Accuracy of guidance signaling	Dro	Wang et al., 2013
TRIM9	VASP	–	Growth cone localization	growth cone	Axon guidance	Mouse	Menon et al., 2015
Nedd4	PTEN	–	Prot deg	growth cone	Axon branching/axon outgrowth	Xen; Rat	Drinjakovic et al., 2010; Christie et al., 2012
Cdh1-APC	Commissureless	monoUb	Endocytosis	growth cone; muscle	Axon guidance/synaptic formation	Dro	Myat et al., 2002; Ing et al., 2007
	Smurf1	polyUb	Prot deg	axon	Inhibition of axonal outgrowth	Rat	Kannan et al., 2012
	SnoN	polyUb	Prot deg	nucleus		Rat	Stegmüller et al., 2006
	Id2	polyUb	Prot deg	nucleus		Rat	Lasorella et al., 2006
Cdc20-APC	Liprin α	–	–	presynaptic terminal	Synaptic size/GABAergic transmission	Dro; Cel	van Roessel et al., 2004; Kowalski et al., 2014
	NEUROD2	polyUb	Prot deg	nucleus	Promotion of presynaptic differentiation	Rat	Yang et al., 2009
	UBE3A	K48	Prot deg	presynaptic terminal	Suppression of presynaptic formation	Dro	Li et al., 2016
	RNF8	–	–	axon	Suppression of presynaptic formation	Mouse	Valnegri et al., 2017
PHR ^c	–	–	Altered microtubule dynamics	axon	Axon outgrowth/pathfinding	Zeb; mouse	Bloom et al., 2007; Lewcock et al., 2007; Hendricks and Jesuthasan, 2009
	ALK	–	Prot deg?	presynaptic terminal	Presynaptic formation	Cel	Liao et al., 2004
	DLK	polyUb	Prot deg?	presynaptic terminal	Presynaptic formation	Cel	Nakata et al., 2005; Collins et al., 2006; Wu et al., 2007; Shin and DiAntonio, 2011; Opperman and Grill, 2014
	Munc13/Dunc13	–	Prot deg	presynaptic terminal	Presynaptic release	Mouse; Dro	Speese et al., 2003; Tada et al., 2010
ZNF1	AKT	polyUb	Prot deg	axon	Axon degeneration	Mouse	Wakatsuki et al., 2011
	MCL1	polyUb	Prot deg	axon	Axon degeneration	Mouse	Wakatsuki et al., 2017
SCF ^{SEL-10}	–	–	–	axon	Synapse elimination	Cel	Ding et al., 2007
SCF ^{SCRAPER}	RIM1	polyUb	Prot deg	presynaptic terminal	Presynaptic release	Mouse	Yao et al., 2007
RNF13	snarin	K29	Higher association with SNAP25	presynaptic terminal	SNARE complex assembly	Mouse	Zhang et al., 2013
SCF ^{MEC-15}	VAMP	–	Synaptic abundance	axon	GABAergic transmission	Cel	Sun et al., 2013
E2	Role in axon					Model	Reference
Bendless	Initial stages of presynaptic formation					Dro	Uthaman et al., 2008
UEV-3	Control of RPM-1/DLK-1 cascade in presynaptic formation					Cel	Trujillo et al., 2010
UBC13	Suppression of presynaptic formation					Mouse	Valnegri et al., 2017
Deubiquitinase	Target	Role in axon				Model	Reference
USP47	katanin-p60	Axon outgrowth				Rat	Yang et al., 2013
USP4/USP20	–	Axon outgrowth				Rat	Anckar and Bonni, 2015
USP33	Roundabout	Axon guidance				Mouse	Yuasa-Kawada et al., 2009
Fat facets	Liquid facets	Promotion of presynaptic formation				Dro	DiAntonio et al., 2001; Bao et al., 2008
USP14	–	Presynaptic formation and function				Mouse	Chen et al., 2009; Bhattacharyya et al., 2012

^aALK, Anaplastic lymphoma kinase; Cel, *C. elegans*; CHIP, C-terminus of Hsp70-interacting protein; Dro, *Drosophila*; EBAX-CRL, EBAX-type Cullin-RING; Id2, inhibitor of DNA binding 2; LIMK1, LIM kinase 1; monoUb, monoubiquitin; Par, partitioning-defective proteins; polyUb, polyubiquitin; Prot deg, proteasomal degradation; RIM1, Rab3-interacting molecule 1; RhoGEF, Rho guanine nucleotide exchange factor; UBC13, ubiquitin-conjugating enzyme 13; VAMP, vesicle-associated membrane protein; Xen, *Xenopus*; Zeb, zebrafish; ZNRF1, zinc and ring finger 1.

^bIn HeLa cells, Akt was shown to be negatively regulated in a UPS manner by the E3 ligase mitochondrial ubiquitin ligase activator of NF- κ B (MULAN) (Bae et al., 2012).

^cPHR proteins form atypical cullin-free SCF complexes containing Skp and an F-box protein: FSN1-RPM1 (Cel) (Liao et al., 2004), Dfsn-Highwire (Dro) (Brace et al., 2014), and Fbxo45-PAM (mouse) (Saiga et al., 2009).

Ubiquitination can also regulate axon elongation in a nonproteolytic manner by influencing the intracellular trafficking of growth regulators. This is the case of muscleblind-like protein 1 (MBNL1), an RNA metabolism regulator whose cytoplasmic localization in neurons is secured by its K63 polyubiquitination (Wang et al., 2018). While in the cytoplasm, MBNL1 promotes axonal outgrowth, but this effect halts when MBNL1 undergoes deubiquitination and subsequent nuclear translocation (Wang et al., 2018). Of note, several other proteins involved in axon formation are targets of ubiquitin modulation (Table 2), hinting at a much wider impact of axonal ubiquitination than currently

appreciated. Of particular interest is the involvement of ubiquitin in controlling the surface levels of several members of the receptor tyrosine kinase family (e.g., FGF receptor, Trk, insulin-like growth factor receptor, and epidermal growth factor receptor), which are abundantly expressed at the axonal membrane and known to coordinate axon formation (Williams et al., 1994; Koprivica et al., 2005; Cheng et al., 2011b; Dupraz et al., 2013).

Axon guidance

Axon outgrowth per se would be pointless without mechanisms securing accurate navigation through the tissue, a process known

Table 2. Additional axonal proteins whose ubiquitin regulation had hitherto not been implicated in axon development and function^a

Axonal protein	Known function in the axon	Axonal localization	E3	Ub chain	Outcome of ubiquitination	Reference
K _v 7 channels	Neuronal excitability	AIS	Nedd4	polyUb	Endocytosis	Jespersen et al., 2007
FGFR ^b	Receptor tyrosine kinase signaling/axon formation	axon			Endocytosis; Lys deg	Haugsten et al., 2008; Persaud et al., 2011
Trk ^b	and regeneration	axon		multimonoUb	Endocytosis; Lys deg	Arévalo et al., 2006; Georgieva et al., 2011; Murray et al., 2019
IGF-1R ^b		axon		multimonoUb	Endocytosis; Prot and Lys deg	Vecchione et al., 2003; Monami et al., 2008
			Mdm2/Cbl	K63/K48	Endocytosis	Sehat et al., 2008
EGFR ^b		>axon	Cbl	multimonoUb	Endocytosis; Lys deg	Haglund et al., 2003; Mossessan et al., 2003
			HUWE1	polyUb	Prot deg	Zhu et al., 2020
tau ^b	Microtubule stabilization	axon	axotrophin	multimonoUb	Reduced microtubule binding	Flach et al., 2014
Frizzled ^b	Wnt signaling component/axon and synapse formation	axon	RNF43 PLR-1 (C.e.l)	multimonoUb	Endocytosis; Lys deg	Hao et al., 2012; Koo et al., 2012; Moffat et al., 2014
axin ^b	Wnt signaling component/axon formation	axon growth cone	Smurf1	K29	No interaction with LRP6; Repression of Wnt/ β cat	Fei et al., 2013
β -catenin ^b	Wnt signaling component/axon formation/presynaptic formation and release	axon	EDD	K11/29	Enhanced stability	Hay-Koren et al., 2011
			Siah ^c	1	Prot deg	Dimitrova et al., 2010
DCC	Netrin receptor/axon guidance	axon growth cone		polyUb	Prot deg	Hu et al., 1997; Kim et al., 2005
Synaptophysin ^c	Synaptic vesicles protein/presynaptic release	presynaptic terminal		polyUb	Prot deg	Wheeler et al., 2002
Synphilin-1 ^c	Synaptic vesicles protein			polyUb	Prot deg	Nagano et al., 2003; Liani et al., 2004
α -synuclein ^c	Synaptic vesicles clustering and exocytosis/endocytosis			monoUb	Prot deg	Liani et al., 2004; Rott et al., 2011
mGlu ^{7c}	Presynaptic release		Nedd4	K48/63	Endocytosis; Prot and Lys deg	Lee et al., 2019
Syntaxin ^c	SNARE complex component/presynaptic release		Starling	polyUb	Prot deg	Chin et al., 2002
Synaptotagmin ^c	Calcium sensor/presynaptic release		Parkin	polyUb	Prot deg	Kabayama et al., 2017
CDCrel-1 ^c	Synaptic vesicles GTPase/presynaptic release			polyUb	Prot deg	Zhang et al., 2000
Eps15 ^c	Endocytic adaptor protein/synaptic vesicles endocytosis			monoUb	No interaction with partners (e.g., EGFR)	Fallon et al., 2006
Ca _v 2.2 ^c	Presynaptic release		–	polyUb	Prot deg	Marangoudakis et al., 2012; Ferron et al., 2014
CASK ^c	Active zone scaffolding protein		–	polyUb	Prot deg	Sun and Kelly, 2010
Bruchpilot ^c			–	polyUb	Prot deg	Zang et al., 2013
Syntaxin ^c			–	polyUb		Okumura et al., 2011
Additional axonal proteins candidates for ubiquitin regulation						
Identified in <i>Drosophila</i> embryos ubiquitome		Fax, Akap200, Hsp83, Hsp26, Eps15, flotillin1/2, 14–3–3, fasciclin 2/3 ^d				Franco et al., 2011
		Hsc70–4, neurotactin, TER94, Hsp27, ArgK, Enolase				
Identified in the rat brain ubiquitome		Bassoon, NSF, SNAP25, synapsin, SV2, VGLUT, APP, neurexin ^c				Na et al., 2012
Identified in mouse APP pulldown ubiquitome		Scamp1, SV2, VAMP2, VGLUT, SNAP25, synapsin ^c				Del Prete et al., 2016
Proteasome-dependent expression in rat		Bassoon, liprin- α , synapsin, syntaxin, CAST/ELKS, α / β -SNAP, SNAP25, neurexin ^c				Lazarevic et al., 2011; Hakim et al., 2016

^aAIS, Axon initial segment; APP, amyloid precursor protein; ArgK, arginine kinase; CASK, calcium/calmodulin-dependent serine kinase; CAST/ELKS, CAZ-associated structural protein/ELKS; DCC, deleted in colorectal cancer; EDD, E3 ubiquitin ligase identified by differential display; EGFR, epidermal growth factor receptor; Eps15, endocytic adaptor epidermal growth factor receptor substrate 15; Fax, failed axon connection; FGFR1, FGF receptor 1; Hsp, heat-shock protein; Lys deg, lysosomal degradation; mGlu7, metabotropic glutamate receptor 7; multimonoUb, multi-monoubiquitin; NSF, ATPase N-ethylmaleimide-sensitive fusion protein; polyUb, polyubiquitin; Prot deg, proteasomal degradation; RNF, RING finger protein; SNAP25, synaptosomal-associated protein 25; SV2, synaptic vesicle glycoprotein 2; VAMP, vesicle-associated membrane protein; VGLUT, vesicular glutamate transporter.

^bProtein with known role in axon formation.

^cProtein with known role in presynaptic release.

^dProtein with known role in synaptogenesis.

^eSiah activity was proposed to be regulated by Bassoon and Piccolo (Waites et al., 2013).

as axon guidance. The directed growth of axons in the complex embryonic environment relies on the ability of axonal growth cones to respond to multiple sources of spatial information in the form of guidance cues. This is achieved through the spatio-temporally regulated expression of specific receptors that integrate information from various guidance cues to steer the axon in the right direction. The dynamic changes in growth cone behavior rely on many cellular mechanisms, including transcription, local translation, protein trafficking, and post-translational processing of guidance receptors (Stoeckli, 2018). In addition, ubiquitin signaling is actively involved, as highlighted by the severe defects in axon pathfinding observed on deletion of E3 ligases. PHR loss leads to impaired segregation of axons to different lobes in *Drosophila* (Shin and DiAntonio, 2011); and in mice, it leads to loss of retinal innervation and sensory innervation of the dorsal olfactory bulb (Bloom et al., 2007; James et al., 2014). Moreover, downregulation of Nedd4 suppresses midline crossing by commissural axons (Myat et al., 2002); and mice lacking TRIM9, a member of the tripartite motif (TRIM) family of E3 ligases, show defects in cortical projections (Menon et al., 2015).

Mechanistically, ubiquitin control of axon guidance occurs at the growth cone level. Early findings suggested that guidance cues steer axons by triggering rapid local changes in growth cone protein content, in part through ubiquitination and proteasome

activity (Campbell and Holt, 2001). Although the full impact of such local activation of ubiquitination and proteolytic pathways remains elusive, evidence supports cue-mediated control of axonal responsiveness by altered expression of guidance receptors (Fig. 2D; Table 1). Indeed, levels of the netrin receptor deleted in colorectal cancer are decreased by netrin itself through the UPS (Kim et al., 2005).

A prime example of ubiquitin-mediated control of guidance receptors occurs as commissural axons cross the midline. During this time, axons' responsiveness to the midline repulsive cue Slit is kept low by preventing membrane localization of the Slit receptor Roundabout (Kidd et al., 1998, 1999). In flies, the mechanism goes as follows: the transmembrane protein Commissureless is a Roundabout binding partner whose surface levels are downregulated by Nedd4-mediated ubiquitination and internalization into late endosomes. Roundabout is internalized together with Commissureless, thus keeping minimal levels of the receptor at the axonal surface (Myat et al., 2002). After the growth cone has crossed the midline, Commissureless/Roundabout internalization is halted, leading to increased surface levels of Roundabout and gained sensitivity to Slit; this prevents midline recrossing, thus ensuring accurate pathfinding (Myat et al., 2002). In mammals, the Nedd4-family interacting proteins Ndfip1 and Ndfip2 function analogously to Commissureless in

removing Roundabout from the axonal membrane through ubiquitin-dependent degradation (Gorla et al., 2019). Downregulation of Roundabout is counteracted by the deubiquitinase USP33, which interacts with Roundabout and favors its stability in a Slit1-enriched environment (Yuasa-Kawada et al., 2009).

The UPS also maintains accuracy of axon guidance by performing protein quality control and degrading misfolded Roundabout proteins (Wang et al., 2013). Briefly, the EBAX-type Cullin-RING E3 ligase interacts with the cytosolic chaperone heat shock protein 90 and can regulate axon guidance by tagging for proteasome degradation damaged Roundabout receptors that accumulate in newborn *C. elegans* grown in higher temperature conditions (Wang et al., 2013). Whether similar quality control mechanisms are present in more complex organisms awaits future studies.

In addition, ubiquitination may regulate axonal responses to guidance cues by locally interfering with cytoskeletal regulatory proteins. VASP is an actin polymerase that is present at filopodia tips and is required for netrin-1-dependent increases in growth cone filopodia number (Lebrand et al., 2004). VASP is ubiquitinated by the E3 TRIM9, which directly binds to the netrin receptor deleted in colorectal cancer. Once ubiquitinated, VASP localization and actin polymerization at filopodia tips are markedly reduced, causing filopodial instability (Menon et al., 2015). Netrin induces VASP deubiquitination by a yet unknown deubiquitinase that counteracts TRIM9 action and thus allows the axon to move toward netrin. Accordingly, TRIM9 deletion causes axon guidance defects by disrupting the attractive turning of cortical axons toward netrin both *in vitro* and *in vivo* (Menon et al., 2015).

Overall, ubiquitin signaling seems to contribute to the maintenance of axon pathfinding by tuning the surface levels of guidance cue receptors and actin regulators (Fig. 2D). The literature suggests that this level of control is geared by guidance cues, raising the exciting questions of whether and how extrinsic factors engage the axonal ubiquitination cascade and/or the proteasome. Chances are that signaling initiated by guidance receptors controls the activity of both E3 ligases and deubiquitinases, which might be anchored to the receptor. Such localization would create rapidly activatable (de)ubiquitination hubs in the steering growth cone, and these in turn could control downstream effectors. Future investigation will certainly shed light on the axon intrinsic pathways translating extrinsic signals into appropriate axonal behavior through modification of ubiquitination.

Presynapse formation

After reaching its target, the axon undergoes a series of modifications to give rise to presynaptic terminals juxtaposed to postsynaptic specializations. In broad terms, presynaptic material will be recruited to specified axonal sites and properly assembled into an active zone near which synaptic vesicles are packed (Pinto and Almeida, 2016).

Throughout development, the amount of ubiquitin-related machinery and ubiquitinated conjugates fluctuates throughout development with a peak coincident with synaptogenesis (Petersen et al., 2010; Chen et al., 2011; Franco et al., 2011), suggesting that ubiquitination cascades are highly industrious and dynamic at this stage. Indeed, in *Drosophila* embryos, several proteins with known roles in synaptogenesis are substrates for ubiquitination, including structural and signaling proteins, such as adhesion molecules, presynaptic scaffolds, kinases, and chaperones (Franco et al., 2011) (Table 2). We and others observed enhancement of presynaptic formation on proteasome inhibition (Zhao et al., 2003; Pinto et al., 2016a),

suggesting that modulation of axonal proteasome activity may provide spatiotemporal control of synaptogenesis onset. We further concluded that, downstream of local UPS inhibition, on-site accumulation of proteins in a K11 and K48 polyubiquitinated state functions as a triggering signal for presynaptic clustering (Pinto et al., 2016a,b). This suggests that the ubiquitinated state of axonal proteins modulates presynaptic assembly. In agreement with this concept, downregulation of the deubiquitinases USP14 and Ubiquitin C-terminal hydrolase L1 (UCH-L1) leads to severe synaptic malformations as the result of a reduction in the synaptic ubiquitin pool and therefore in the ubiquitination competence of these synaptic terminals (Osaka et al., 2003; Cartier et al., 2009; Chen et al., 2009, 2011). Furthermore, in the *Drosophila* neuromuscular junction (NMJ), overexpression of the deubiquitinase Fat facets and loss-of-function mutations in Highwire, the PHR *Drosophila* homolog, both result in synaptic overgrowth (Wan et al., 2000; DiAntonio et al., 2001). The PHR loss-of-function phenotype is suppressed in the absence of Fat facets (DiAntonio et al., 2001), further indicating that dynamic (de)ubiquitination-dependent mechanisms act to regulate presynaptic differentiation. Future work will certainly help to fully characterize the molecular basis of such ubiquitin-dependent control, including the axonal regulators of ubiquitination, topology of chains and involved substrates, recognition and decoding machinery, and downstream cascades leading to presynaptic formation.

Modulation of presynapse assembly by ubiquitin occurs, at least partially, by controlling levels of specific proteins. Pioneer efforts to identify targets of ubiquitin modifications revealed that PHR, which forms a noncanonical SCF complex with Skp (Brace et al., 2014) and the F-box protein Dfsn (Wu et al., 2007), downregulates Wallenda, the *Drosophila* homolog of dual leucine zipper-bearing kinase (DLK) (Collins et al., 2006; Wu et al., 2007). This kinase activates a signaling pathway involving c-Jun N-terminal kinase activity and Fos-mediated transcription that confers synaptogenic capacity. Therefore, PHR restrains synaptic development by promoting DLK elimination (Collins et al., 2006). Synaptic overgrowth induced by the deubiquitinase Fat facets also converges on DLK (Collins et al., 2006), emphasizing that, in *Drosophila*, DLK is a key substrate whose ubiquitinated status determines the propensity to form presynaptic specializations. In addition, Fat facets acts via the epsin 1 *Drosophila* homolog Liquid facets, a protein involved in endocytosis (Bao et al., 2008). Together, these studies laid the groundwork for the identification of the molecular mechanisms underlying ubiquitin-driven control of presynaptic formation through its action at nascent terminals (Fig. 3A; Table 1).

Notably, the involvement of PHR in presynaptic formation is conserved between species. In *C. elegans*, the PHR protein RPM-1, rather than restricting presynaptic development, promotes it by negatively targeting DLK, which initiates a cell-autonomous kinase cascade that suppresses presynaptic development (Nakata et al., 2005). RPM-1 further controls the extent and positioning of presynaptic formation by locally modulating levels of anaplastic lymphoma kinase. This is accomplished by an SCF-like complex comprising the F-box protein FSN-1 and RPM-1 in the periaxonal zone (Liao et al., 2004). Of note, the fact that the F-box protein Fbxo45 (human ortholog of *C. elegans* FSN-1 and *Drosophila* Dfsn) associates with PAM (human PHR homolog) and that mice lacking both Phr1 and Fbxo45 display severe synaptic defects (Burgess et al., 2004; Saiga et al., 2009) makes it likely that similar modes of regulation underlie synapse formation in mammals.

Figure 3. Ubiquitin-dependent signaling in the lifetime of presynaptic terminals. Following correct pathfinding, an axon establishes synaptic contacts (**A**, presynaptic formation), which mature into release-competent synaptic terminals (**B**, presynaptic function). Trimming of the axonal arbor (**C**, presynaptic elimination; and **D**, axon degeneration) can occur during development or adulthood. All these axonal events are locally controlled by ubiquitin and/or the proteasome. Ubiquitin-targeted proteins (blue) and respective E3 ubiquitin ligases (orange) or deubiquitinases (yellow) are illustrated at their site of action. The type of ubiquitin chains involved is indicated. For complementary information, see Table 1. ALK, Anaplastic lymphoma kinase; RIM1, Rab3-interacting molecule 1; UBC13, ubiquitin-conjugating enzyme 13; VAMP, vesicle-associated membrane protein; ZNRF1, zinc and ring finger 1.

Additional axonal E3 ligases have emerged as important local players in orchestrating presynaptic formation (Fig. 3A). Presynaptically located Cdh1-APC downregulates the active zone scaffolding protein liprin- α , thereby restricting presynaptic size (van Roessel et al., 2004). The E3 UBE3A mediates UPS degradation of the presynaptic bone morphogenetic protein receptor thickveins, a function that is conserved in mammalian cells (Li et al., 2016). Loss of UBE3A increases the density of synaptic boutons at the *Drosophila* NMJ through excessive activation of signaling by bone morphogenetic protein (Li et al., 2016), a secreted polypeptide that retrogradely promotes NMJ growth through activation of thickveins (Aberle et al., 2002; McCabe et al., 2003).

Recently, a novel cytoplasmatic ubiquitin cascade comprising the E2 ubiquitin-conjugating enzyme 13 and the E3 RNF8 was identified in the mouse cerebellum and was shown to suppress

synapse formation between granule cells and Purkinje neurons (Valnegri et al., 2017). Efforts to identify the involved players downstream this ubiquitination cascade are expected to offer insights into the ubiquitin-related local pathways in vertebrates, which have so far evaded us. Caution should be exercised in the interpretation and generalization of future work, however, because synapse type- or region-specific modulations are highly likely.

Interestingly, ubiquitin-mediated protein removal controls synapse formation by acting not only at the site of nascent terminals but also at other locations (Fig. 3A). In the nucleus, the E3 complex Cdc20-APC ubiquitinates and sends for proteolysis the transcription factor NeuroD2 (Yang et al., 2009), thus precluding expression of complexin, a protein that normally acts in the axon to obstruct formation of presynaptic sites. Accordingly, Cdc20-APC upre-

gulation during synaptogenesis alleviates complexin-mediated constraint of presynaptic formation (Yang et al., 2009). Furthermore, in the postsynaptic muscle cell, ubiquitin can trigger presynapse formation by promoting endocytosis of the transmembrane protein Commissureless (Wolf et al., 1998; Ing et al., 2007). During the period of motoneuron-muscle interaction, Commissureless is highly expressed in the muscle cell, and its endocytosis is mandatory for synaptogenesis to be initiated (Wolf et al., 1998). This is promoted by monoubiquitination-induced Commissureless internalization via the E3 Nedd4 located in the muscle cell (Ing et al., 2007). Thus, in Nedd4 mutant mice, axons project to the muscle but are unable to establish synaptic contacts (Y. Liu et al., 2009).

From the examples above, it appears that, by controlling the levels of specific targets, ubiquitin modulates presynaptic formation through three main routes: changes in gene expression, synapse-promoting kinase cascades, and synaptic scaffolds. The timing and sequence of this control are unclear. It is however tempting to speculate that ubiquitin-induced changes in transcription contribute to the priming of a developing neuron into a synaptogenic state, likely followed by ubiquitin local control of signaling pathways that launch the recruitment of synaptic material and scaffolds. Finally, analogous to the engagement of ubiquitin control by guidance cues, presynaptogenic cues (Pinto and Almeida, 2016) are potential upstream triggers, a link that is yet to be established.

Presynaptic function

The prime function of a fully formed presynaptic terminal is the release of neurotransmitters in a controlled and accurate manner following depolarization-induced presynaptic calcium entry (Südhof, 2013). Can ubiquitin regulate presynaptic release? Two main findings support a strong link between presynaptic release and ubiquitination. First, brief proteasome inhibition boosts neurotransmitter release without changing presynaptic protein levels (Speese et al., 2003; Zhao et al., 2003; Rinetti and Schweizer, 2010). Second, depolarization-induced calcium elevation rapidly decreases the presynaptic pool of ubiquitinated protein in a non-proteolytic manner (Chen et al., 2003). Together, these observations suggest that dynamic ubiquitination, not necessarily associated with proteasome clearance, controls the events surrounding neurotransmitter release. As a proof of concept, mice harboring a loss-of-function mutation in the deubiquitinase USP14, with concomitant loss of free ubiquitin and presynaptic ubiquitination (Chen et al., 2009, 2011), show an inability to mobilize synaptic vesicles for fusion and thus have reduced quantal release (Bhattacharyya et al., 2012). Such deficits are rescued on restoration of ubiquitin levels (Chen et al., 2009, 2011), further suggesting that presynaptic function is governed by a subset of dynamically ubiquitinated proteins. One notable example of the requirement of dynamic ubiquitination to presynaptic release involves the E3 RNF13 that adds a K29 ubiquitin chain to snapin, thus fortifying its association with the SNARE protein SNAP25 (Zhang et al., 2013). Snapin binding to SNAP25 potentiates SNAP25's interaction with synaptobrevin (Ilardi et al., 1999), thus accelerating the zippering of the SNARE complex that mediates synaptic vesicle fusion. This example emphasizes the ability of ubiquitin to rapidly convert inactive presynaptic proteins into active release machinery. Many other proteins involved in presynaptic release, including synaptic vesicle machinery and active zone scaffolds, are substrates of ubiquitin-mediated regulation (Table 2). Moreover, if one considers the reversibility and versatility nature of ubiquitin signaling, it is

quite expectable that additional modes of local nonproteolytic ubiquitin modulation control presynaptic release efficacy. We therefore believe that a thorough analysis of the presynaptic ubiquitome, including identification of substrates, analysis of chain topologies, and depolarization- or calcium-induced ubiquitination dynamics, would allow for great advances in the field. Moreover, the attractive possibility of a calcium-sensitive modulation of E3s and deubiquitinases' activity (Wang et al., 2010; Shimamoto et al., 2013), which could regulate ubiquitination in the range of seconds, should be explored at the presynaptic terminal.

Ubiquitin also regulates presynaptic function by controlling levels of active zone and synaptic vesicle proteins (Fig. 3B; Table 1). To maintain synaptic activity within reasonable levels and thus avoiding excessive release, activity of the E3 ligases SCRAPER and the Fbxo45-containing PHR complex (Saiga et al., 2009) reduces levels of the active zone proteins Rab3-interacting molecule 1 and Munc13 (Yao et al., 2007; Tada et al., 2010), which function as coordinators of synaptic vesicle fusion. Accordingly, facilitation of excitatory transmission is observed on loss of SCRAPER or Fbxo45 (Tada et al., 2010; Koga et al., 2017). In contrast, in *Drosophila* the E3 PHR maintains evoked release by attenuating levels of presynaptic nicotinamide mononucleotide adenylyltransferase 2 (NMNAT2), a chaperone of active zone scaffolds which in excess dampens transmission by disturbing active zone structure (Russo et al., 2019).

E3s may also function in a synapse-type-specific manner to regulate the excitation/inhibition balance. In *C. elegans*, the F-box protein MEC-15 maintains levels of the presynaptic protein synaptobrevin (homolog of mammalian vesicle-associated membrane protein) at GABAergic terminals and, accordingly, its loss impairs inhibitory transmission (Sun et al., 2013). Moreover, despite broad expression of the ubiquitin ligases HUWE1 and APC in *C. elegans* motor system, these proteins specifically affect presynaptic release of GABAergic inputs (Kowalski et al., 2014; Opperman et al., 2017). Further investigating the synapse specificity underlying E3-mediated presynaptic control in vertebrates may prove useful for the understanding of neurologic conditions involving altered excitation to inhibition ratio stemming from mutations in E3 ligases (Rotaru et al., 2018).

Axonal self-destructive events

Throughout the lifetime of an axon, there are occasions in which reorganization of the axonal arbor involving selective self-destruction of axonal domains is necessary. Elimination of presynaptic terminals (Fig. 3C) and degeneration of axon segments (Fig. 3D) are examples of such self-destructive events in which, once more, the involvement of ubiquitin signaling is coming to light.

Following the initial boost in synapse formation, elimination of unwanted or unnecessary terminals refines the neuronal network. In *C. elegans*, elimination of extra presynaptic clusters in the hermaphrodite-specific motor neuron is mediated by an SCF complex (Ding et al., 2007). Terminals stabilized by the establishment of the SYG-SYG transsynaptic pair will be spared because of SYG-dependent inhibition of SCF assembly (Ding et al., 2007). Moreover, the active zone proteins Bassoon and Piccolo have been identified as important players in the maintenance of presynaptic integrity by regulating ubiquitination and degradation of presynaptic proteins. Through their zinc finger domains, Bassoon and Piccolo bind to and inhibit the E3 ligase Siah1. Their loss results in aberrant degradation of multiple presynaptic proteins, resulting in synapse elimination (Waites et al., 2013). These studies show that ubiquitination machinery can be

anchored to and negatively regulated by presynaptic elements, thus preventing widescale degradation of presynaptic proteins that might ultimately result in synaptic degeneration. This implies that well-established, mature synapses exert a stronger restraining effect on these specific E3 ligases than weak or immature synapses, which are more vulnerable to elimination. Hence, local control of UPS degradation is likely to contribute to the selectivity of synaptic elimination and therefore refinement of neuronal networks during development. Whether neuronal activity governs UPS-based synaptic elimination and how it is coordinated with other ongoing cellular events are unresolved issues that would be important to address.

Axon degeneration occurs not only during development to refine the axonal tree, but also in pathologic contexts, such as after injury and in neurologic disorders. A classical model of axon degeneration is Wallerian degeneration, in which the distal portion of a severed axon undergoes fragmentation and elimination in an active cellular process triggered by depletion of nicotinamide adenine dinucleotide (NAD^+) and activation of a kinase signaling cascade (Geden and Deshmukh, 2016). The broad-spectrum E3 PHR controls Wallerian degeneration in part through downregulation of the NAD^+ synthesizing and axon survival molecule NMNAT2 (Xiong et al., 2012). Consistent with this, depletion of PHR components increases axonal NMNAT2, thereby delaying axon degeneration after injury both *in vitro* and *in vivo* (Yamagishi and Tessier-Lavigne, 2016). Likewise, the E3 zinc and ring finger 1 promotes axon degeneration by targeting AKT to proteasomal degradation (Wakatsuki et al., 2011). Decline in AKT levels decreases glycogen synthase kinase-3 β phosphorylation, thus converting it into its active form, which in turn destabilizes microtubule assembly, with concomitant loss of cytoskeleton integrity and thereby axon degeneration (Wakatsuki et al., 2011). Axon degeneration occurring during development, in particular the pruning of γ neurons of *Drosophila* mushroom bodies, also relies on UPS machinery to modulate microtubule cytoskeleton (Watts et al., 2003; Wong et al., 2013). Despite this shared UPS effector, distinct molecular pathways underpin developmental axon degeneration and Wallerian degeneration (for an informative discussion of this issue, see Geden and Deshmukh, 2016), and hence the pending need to identify the ubiquitin-related players involved in developmental regulated pruning. Finally, proteasome clearance can trigger Wallerian degeneration by launching axonal autophagy. MCL1, a BCL2 family member, acts as a break on axonal autophagy because of the sequestering of the autophagy regulator BECLIN1. Glycogen synthase kinase-3 β phosphorylates MCL1, which is then ubiquitinated by the E3 ligase FBXW7 and targeted to proteolysis, thus relieving the constraint on BECLIN1 (Wakatsuki et al., 2017). Overall, ubiquitin seems to act on several fronts to coordinate axon degeneration: either at the triggering phase through modulation of NMNAT2 or at the executing phase through disintegration of microtubules and autophagy.

The prominent role of ubiquitin signaling in both the assembly (discussed in sections Axon specification, Axon outgrowth, Axon guidance, and Presynapse formation) and pruning of axonal domains strongly suggests its involvement in the refinement of neuronal circuit during development. Thus, the high prevalence of axonal ubiquitin dysfunction as a causative factor in neurodevelopmental diseases (discussed in the next section) is not surprising.

When ubiquitin signaling goes awry in axons

Dysfunction of the UPS is thought to underlie several neurologic disorders. In the brain, the proteasome and ubiquitin control

diverse functions, including serving as key regulators of neuronal protein homeostasis (Tai and Schuman, 2008; Bingol and Sheng, 2011). Therefore, not surprisingly, UPS dysfunction has been closely linked to neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, or Huntington's disease. Generally speaking, neurodegenerative diseases are characterized by aberrant accumulation of ubiquitin-rich intracellular protein aggregates (Perry et al., 1987; Lowe et al., 1988; Davies et al., 1997). This can stem from mutations or dysfunctions of UPS proteins (Kitada et al., 1998; Leroy et al., 1998; Van Leeuwen et al., 1998) or from saturation or deterioration of UPS clearance capacity by abnormal protein loads (Bence et al., 2001; Keck et al., 2003; Lindersson et al., 2004). In addition, impairments in the ubiquitination cascade can alter specific neuronal mechanisms into generating pathogenic phenotypes as it is the case of neurodevelopmental disorders. Because this review examines ubiquitin involvement in axon development and function, we will focus the remainder of this section on how aberrant ubiquitin signaling leads to axonal dysfunction and contributes to the pathogenesis of specific neurologic conditions.

Mutations in ubiquitination enzymes are linked to presynaptic dysfunctions in neurodevelopmental diseases. In X-linked infantile spinal-muscular atrophy (SMA), characterized by severe muscle atrophy and premature death, mutations in the gene encoding the E1 ubiquitin-activating enzyme UBA1 were reported (Ramser et al., 2008). Similarly, Type I-III SMA, caused by loss of survival motor neuron protein (Lefebvre et al., 1995), also has reduced levels of UBA1 and therefore disrupted ubiquitin homeostasis (Wishart et al., 2014). Importantly, UBA1 deficiency was shown to directly contribute to axonal and synaptic neuromuscular pathogenesis in zebrafish, *Drosophila*, and mouse models of SMA (Wishart et al., 2014), and these were rescued by restoration of UBA1 levels, with concomitant improvement in motor performance (Powis et al., 2016). This suggests that anomalous ubiquitin dynamics at the axonal level is likely to contribute to the onset of several forms of SMA.

Mutations in E3 ligases are also implicated in neurologic conditions. For instance, the E3 UBE3A has been implicated in the pathogenesis of autism, Rett syndrome (Makedonski et al., 2005), and schizophrenia (Kohlbrenner et al., 2018). UBE3A is also lost in Angelman syndrome (AS) (Kishino et al., 1997; Matsuura et al., 1997), which is characterized by developmental delay and intellectual and motor disability (Angelman, 1965). AS mouse models show deficits in learning and impairments in synaptic maturation, transmission, and plasticity (Jiang et al., 1998; Yashiro et al., 2009; Wallace et al., 2012). The fact that UBE3A is present at presynaptic terminals (Dindot et al., 2008) suggests that AS pathology may emerge from axonal dysfunction. Indeed, defects in axon guidance (Tonazzini et al., 2019), presynaptic development (Li et al., 2016), and presynaptic vesicle cycling (Wallace et al., 2012) are observed with AS-like UBE3A loss. In addition, the increased susceptibility of AS patients to seizures likely results from augmented intrinsic neuronal excitability because of impaired UBE3A-mediated degradation of calcium and voltage-dependent big potassium channels (Sun et al., 2019), which are located in axons and presynaptic terminals (Misonou et al., 2006). Interestingly, presynaptic formation is also regulated by the autism-linked E3 ligase RNF8 (Valnegri et al., 2017) (Fig. 3A), leading to the speculation that ubiquitin-driven presynaptic dysregulation is a common pathogenic mechanism in AS and autism. The involvement of E3 ligases in presynaptic function and subsequent disease progression is further reinforced by studies in the *tambaleante* mutant mouse. These animals carry a

spontaneous mutation in the E3 HERC1 (Mashimo et al., 2009), a protein that is also mutated in cases of intellectual disability (Nguyen et al., 2016; Utine et al., 2017). *Tambaleante* mice exhibit severe ataxia and neuronal degeneration (Mashimo et al., 2009), which is preceded by presynaptic dysfunction at the NMJ (Bachiller et al., 2015).

Dysfunction of ubiquitin decoding systems in axons may also contribute to disease expression. A point mutation in the proteasome shuttle UBQLN4, identified in familial amyotrophic lateral sclerosis, was found to cause aberrant axon branching because of inappropriate proteolysis of β -catenin (Edens et al., 2017). Moreover, mutations in the ubiquitin-binding protein phospholipase A2-activating protein were shown to cause a lethal infantile epileptic encephalopathy by disrupting the endolysosomal trafficking of K63-polyubiquitinated proteins at the presynapse, leading to impaired presynaptic release (Hall et al., 2017).

Finally, abnormal function of deubiquitinases has also been linked to the etiology of neurologic conditions. A spontaneously arising loss-of-function mutation in the deubiquitinase USP14 has been shown to underlie ataxia in $\alpha\alpha'$ mice. These animals exhibit severe resting tremor at 2–3 weeks old, followed by hindlimb paralysis and death early in development (Wilson et al., 2002). USP14 recycles ubiquitin from substrates before their proteasome removal, and therefore levels of monomeric ubiquitin are reduced in brain tissue of the mutant mice (Anderson et al., 2005). In addition, these mice display severe presynaptic structural and functional defects at the NMJ that are rescued by neuron-specific overexpression of ubiquitin (Chen et al., 2009, 2011). These findings indicate that fluctuations in synaptic ubiquitin levels may lead to the development of synaptic anomalies. This hypothesis is further fortified by analysis of the gracile axonal dystrophy mice, which have a spontaneous mutation in the deubiquitinase UCH-L1 (Saigoh et al., 1999) and exhibit severe sensory ataxia at early stages, as a result of axonal degeneration in the gracile tract (Kikuchi et al., 1990). Similarly to the ataxia $\alpha\alpha'$ mice, these animals have reduced levels of monomeric ubiquitin (Osaka et al., 2003). Moreover, alterations of synaptic structure by UCH-L1 inhibition are reversed by ubiquitin overexpression (Cartier et al., 2009). Interestingly, loss-of-function genetic modifications (Leroy et al., 1998), reduced levels (Choi et al., 2004; Barrachina et al., 2006), and post-translational modifications (Choi et al., 2004; Z. Liu et al., 2009) in UCH-L1 have been linked to Alzheimer's and Parkinson's disease, thus further reinforcing that the inability to maintain steady-state levels of neuronal ubiquitin is likely to underlie emergence of neurologic defects.

Mutations in another deubiquitinase, the ubiquitin-specific peptidase 9 X-linked (USP9X), and in its target protein PRICKLE (Paemka et al., 2015), have been identified in epileptic patients (Bassuk et al., 2008; Tao et al., 2011; Paemka et al., 2015). PRICKLE promotes axon outgrowth (Mrkusich et al., 2011) and regulates axonal vesicle transport (Ehaideb et al., 2014). In flies, a PRICKLE loss-of-function mutation increases predisposition for seizures, which are repressed by pharmacological or genetic inhibition of USP9X (Tao et al., 2011; Paemka et al., 2015). Importantly, such seizure-prone phenotype results from alterations in axonal transport (Ehaideb et al., 2014), supporting that PRICKLE-USP9X axonal interaction is an important pathway in controlling seizures.

Another example of pathogenic deficits in deubiquitination in the early stages of disease occurs in the muscle weakening disorder myotonic dystrophy Type 1. In diseased brains, exacerbated deubiquitination of MBNL1 resulting from expanded CUG RNA (a disease hallmark) results in translocation of the

protein from the cytoplasm to the nucleus (Wang et al., 2018). As a result, the axon growth-promoting function of cytoplasmic MBNL1 (previously discussed in Axon outgrowth; see Fig. 2C) is abrogated (Wang et al., 2018), which can partly explain the neurite degeneration observed at early disease stages (Wang et al., 2017). Together, these findings indicate that anomalous ubiquitin signaling at the axonal level is likely to contribute, at least partially, to the pathophysiology of diverse brain disorders, with a particular emphasis on neurodevelopmental diseases. This substantial prevalence as a causative factor for the pathogenesis of neurologic diseases highlights the need to fully understand and characterize the physiological function of the proteasome and ubiquitin in distinct neuronal compartments.

Remarkable insight into the diseased brain has been provided by the identification of causative mutations and follow-up studies unraveling the corresponding protein function. As discussed above, this approach has been particularly prolific to the neuronal ubiquitin field. It may, however, neglect the putative involvement of ubiquitin signaling to the etiology of diseases free of ubiquitin-related genetic abnormalities. A dysfunctional neuronal network may increase the burden on housekeeping mechanisms (e.g., the UPS, but also protein translation and quality control, mitochondrial ATP production, intracellular trafficking, etc.), thus affecting their local homeostasis and concomitantly worsening the disease phenotype and progression. We therefore predict that understanding how local ubiquitin signaling is altered in diseased neurons could reveal a broader impact of ubiquitin in pathology, particularly in conditions of unbalanced homeostasis, such as stress, depression, or aging.

Conclusion

In conclusion, overall, mounting data demonstrate that ubiquitin signaling, in most cases culminating in proteasome degradation of tagged proteins, operates in axons from the beginning of their differentiation to the establishment of proper presynaptic transmission (Figs. 2, 3). In addition to the list of ubiquitin and/or proteasome targets summarized in Table 1, whose selective (de) ubiquitination has been shown to exert direct roles in axonal events, many other axonal molecules, including signaling, synaptic vesicles, and active zone proteins, are known to be post-translationally regulated in such fashion (Table 2). It is thus possible that ubiquitin influences axon-related events in countless other ways that are yet to be identified.

A question remains as to whether and how ubiquitin pathways are locally modulated. In other words, do axons control ubiquitin signaling? It is fair to believe they do. Surely axonal control of ubiquitin and proteolytic machinery would guarantee effective exploitation of ubiquitin power to spatiotemporally meet the particular demands of an axon. First glimpses into this issue indeed show that presynaptic proteins can modulate the activity of E3 ligases (Ding et al., 2007; Waites et al., 2013; Del Prete et al., 2016). Similarly, in dendrites, proteasome activity and localization can be regulated by postsynaptic CaMKII α (Bingol et al., 2010). A broader picture of such bidirectional regulation awaits future efforts.

While information on the proteins involved in ubiquitination is solidly growing (Tables 1 and 2), the axonal machinery engaged in downstream decoding and translation of ubiquitin signals remains elusive. The same holds true for local proteasome regulators and shuttles. Of note, the fact that ubiquitin-binding domains were identified in presynaptic proteins (Polo et al., 2002; Stamenova et al., 2007; Okumura et al., 2011) suggests a

layer of presynaptic ubiquitin-related modulation that has so far evaded us. Last, but definitely not least, it would be of great value to better grasp whether and how axonal translation (Cioni et al., 2018) and ubiquitin-dependent degradation act jointly in the control of axonal biology. A high degree of interdependence is indeed expected: axonally translated proteins are the main targets of UPS (Deglincerti et al., 2015), which in turn can control the levels of ribosomes in axons (Costa et al., 2019). Together, future research in the field will undoubtedly improve our understanding of neurologic disorders featuring deficits in axonal ubiquitin signaling and hence abnormal neuronal function.

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