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Roles of the ubiquitin-proteosome system in neurogenesis

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The ubiquitin-proteosome system (UPS) is a non-lysosomal proteolysis system involved in the degradation of irrelevant/misfolded intracellular proteins. The protein substrates of this system are tagged by ubiquitin in sequential reactions that target them for proteasome-dependent destruction. In developing central nervous system, ubiquitin-mediated proteolysis has recently emerged as an important player in the regulation of neural progenitor proliferation, cell specification, neuronal differentiation, maturation and migration. E3 ubiquitin ligases are crucial components in the UPS because they provide the specificity that determines which substrates are targeted for ubiquitin-dependent proteolysis. In this review, we discuss the molecular mechanisms of the UPS, focusing primarily on the roles of E3 ligases and their substrates in sequential steps of neurogenesis.

Key words: neurogenesis, ubiquitin-proteosome system (UPS), E3 ligases

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Introduction

The complexity of the adult vertebrate central nervous system (CNS) is established throughout development, during which progenitors in germinative zones first generate multiple types of neurons, followed by generation of oligodendrocytes and astrocytes.[1] Neurogenesis, the generation of new neurons, is a complex process that involves several tightly coordinated steps, including commitment of neural stem cells (NSCs) toward neuronal fates, specification of neuronal subtype identities, neuronal differentiation and neuronal migration.[2] Accumulating evidence indicates that the proper progression of neurogenesis depends on the strict control of key molecular players in these processes during development.

Targeted proteolysis via the ubiquitin proteasome system (UPS) plays an essential role in strictly controlling temporal and spatial gene expression. Ubiquitin-dependent degradation relies on the covalent attachment of the small protein ubiquitin to target substrates, a modification that triggers the transfer of targeted proteins to and subsequent proteolysis by, the 26S proteasome complex. Ubiquitin molecules are attached to the substrate through the coordinated action of three enzymes: E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugation enzyme; and E3, ubiquitin protein ligase. E3 ligases are crucial factors in this pathway because they confer substrate specificity.[3] There are at least three distinct classes of E3 ubiquitin ligases: the HECT domain family, the RING finger family and the U-box family.[3] Here, we summarize recent progress in understanding the function of the UPS in the regulation of events underlying the generation of neurons in the developing vertebrate CNS. The roles of the UPS in synaptic plasticity, neuronal function and dysfunction are not considered here owing to space limitations; readers are referred to an excellent recent review on the subject.[4]

Renewal of Neural Stem Cells

In the embryonic mouse telencephalon, neurons are generated by three populations of progenitors: neuroepithelial (NE) cells (stem cells); radial glial progenitor (RGP) cells; and basal progenitor (BP) cells, also termed intermediate progenitors (IPs). Prior to initiation of neurogenesis, the germinative NE forms a single layer of neural stem cells (NSCs). These stem cells

have the capacity to self-renew and generate all the different cell types that make up the brain. Recent findings have implicated a number of signaling pathways controlled by different factors, including Notch, Wnt, Fgf, Numb, Numb-like and Bmi-1, as essential contributors to this process.[2,5,6]

The best-characterized role of the Notch pathway is in the maintenance of NSC renewal.[7] In fact, signaling through the Notch pathway has two distinct roles during neurogenesis of the mammalian brain: inhibiting neurogenesis and promoting the maintenance of neural RGPs.[8] When activated by binding its ligands (Delta-like 1, 3 and 4; Jagged 1 and 2), the Notch-receptor is cleaved and its intracellular domain (Notch-ICD) is translocated to the nucleus, where it interacts with the factors CBF1 and MAML to form a transcriptionally active complex. This causes inhibition of neurogenesis by activating Hes-genes, the products of which suppress the activity of neurogenic basic helix-loop-helix (bHLH) transcription factors. Later, during the period in which gliogenesis occurs, Notch directly promotes differentiation of astrocytes while inhibiting oligodendrocyte differentiation.[9]

The ability of the Notch signaling pathway to exert these opposite functions is made possible by tight regulation by a variety of molecular mechanisms, including the UPS. The multiple E3 ubiquitin ligases involved in regulating Notch signaling are divided into two groups: one ubiquitinates Notch receptors and the other regulates Notch ligands.[10] One E3 ligase in the latter category is Mib1 (Mind bomb), which serves as the primary upstream regulator of Notch signaling. Evidence for the centrality of this E3 ligase is provided by the observation that *Mib1* knockout mice exhibit severe Notch signaling defects.[11] Mib1 regulates the ubiquitination-dependent endocytosis of all canonical Notch ligands. It is not yet clear how endocytosis of Notch ligands acts as a key event in the activation of Notch signaling. However, it is known that Mib1-mediated endocytosis of Notch ligands in signal-sending cells results in co-endocytosis of bound Notch receptors, which facilitates cleavage of the Notch extracellular domain and release of the activated intracellular domain of Notch into adjacent signal-receiving cells.[11,12]

In the developing mouse cortex, expression of the E3 ligase Mib1 is restricted to IPs in the subventricular zone and postmitotic neurons in the intermediate zone and cortical plate. Interestingly, conditional inactivation of the *Mib1* gene in neural progenitors results in a complete loss of Notch activation in the ventricular zone and elimination of RGP pools. Conversely, over-activation of Notch signaling rescues the phenotypes of *Mib1*-deficient mice, indicating that IPs and post-mitotic neurons that express Mib1 are the main cellular sources for activation of Notch signaling in RGPs.[13]

Commitment and Specification

Proneural bHLH transcription factors play a major and evolutionarily conserved role in neurogenesis. These genes have been implicated not only in the commitment and specification of neuronal fate, but also in neuronal differentiation and migration.[14]

A recent study in Drosophila demonstrated that cell cycle progression from G_2 to M phase in progenitors is largely controlled by UPS-dependent degradation of the proneural achaete-scute complex (AS-C), which comprises four bHLH transcription factors.[15] An E3 ubiquitin ligase complex containing the RING protein Sina (an E3 ligase) and the conjugation protein Phyl has an important role in this process. Phyl physically interacts with and promotes Sina-dependent degradation of the AS-C complex. Mutation of Phyl increases AS-C levels in a Sina-dependent manner, leading to cell cycle arrest of progenitors at the G_2-M phase. Furthermore, the AS-C directly controls the expression of Phyl. Thus, the interplay between the Phyl-Sina E3 ubiquitin ligase complex and proneural AS-C proteins plays an essential role in cell cycle progression during neurogenesis in Drosophila.

Although the molecular mechanisms that regulate the stability of proneural proteins in mammalian neurogenesis are not yet fully defined, the available data indicate that UPS-mediated control of mammalian proneural proteins is important for neurogenesis. Similar to Notch, the bone morphogenetic proteins (BMPs) act as potent inhibitors of neurogenesis. During neurogenesis, the expression of the proneural bHLH transcription factor Mash1 is downregulated at the protein level by BMP signaling, resulting in premature differentiation of neuronal progenitors.[16] Interestingly, pharmacological inhibition of the proteasome system abolishes BMP-stimulated degradation of Mash1,[16] although it is not yet known whether a specific E3 ligase mediates ubiquitination of Mash1.

Expression of the bHLH transcription factor Ngn2 is restricted to cortical progenitors, suggesting that the Ngn2 protein may be rapidly degraded in immature neurons. Indeed, proteosome inhibitory assays have shown that Ngn2 protein level in vitro is significantly increased in the presence of a proteosome inhibitor.[17] Whereas overexpression of the cyclin-dependent kinase inhibitor $p27^{Kip1}$ in the developing cortex in vivo results in a marked increase in the number of cells expressing Ngn2, loss of $p27^{Kip1}$ function leads to a significant reduction in the levels of Ngn2p protein without affecting Ngn2p mRNA level.[17] In addition, co-expression of Ngn2 rescues the defect in neuronal differentiation induced by $p27^{Kip1}$ loss of function. These observations suggest that $p27^{Kip1}$ promotes the differentiation of cortical progenitors by upregulating Ngn2 expression in a proteasome-dependent manner. Although the molecular mechanism by which $p27^{Kip1}$ modulates the stability of Ngn2 requires further investigation, the available data indicate that this process might be dependent on the ability of $p27^{Kip1}$ to interact with and sequester a specific ubiquitin E3 ligase that may target Ngn2 to the proteasome for degradation.[17]

Glutamatergic pyramidal cortical neurons are generated predominantly by the pluripotent RGPs. Our previous results indicated that the transcription factor Pax6 is an intrinsic determinant of RGPs,[18] endowing these cells with neurogenic properties.[19] Expressed at much higher levels at the onset of neurogenesis,[20] Pax6 seems to act exclusively in the early RGPs to control mitotic cell cycle parameters, thus maintaining pools of NSCs/progenitors.[21,22] Functional studies from our and other laboratories have shown that normal development requires that the level of Pax6

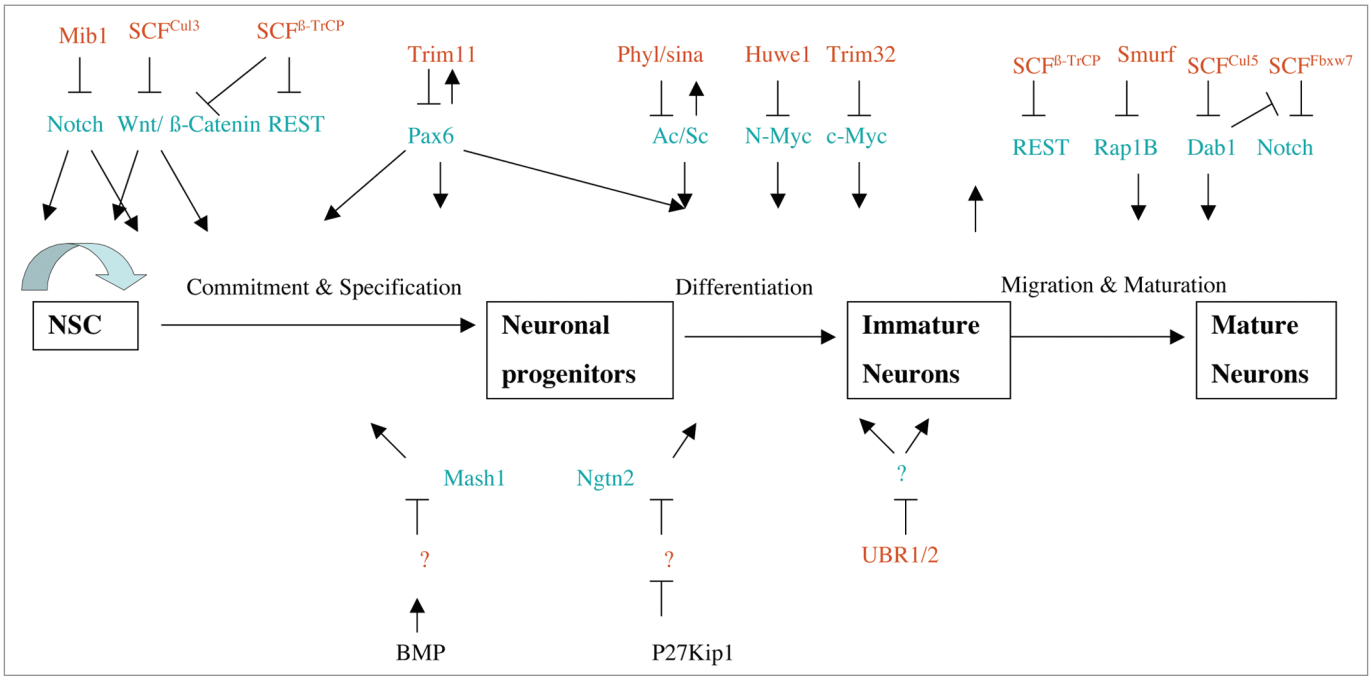

Figure 1. Schematic summarizing the functions of reported E3 ligases (red) and their substrates (green) in the sequential steps of neurogenesis. Question marks represent unidentified E3 ligases or substrates involved in neurogenesis.

protein be maintained within a specific physiological range. Both reduced and increased Pax6 gene dosages cause similar developmental defects in the eye[23,24] and developing cortex,[20,25-27] highlighting the importance of Pax6 levels. Accordingly, maintenance of normal Pax6 dosage in RGPs is a prerequisite for the specification of these cells as neuron generators.

Recently, we demonstrated an important role for the E3 ligase Trim11 in the regulation of Pax6 protein levels in cortical progenitors.[28] To date, the only Trim11 substrates that have been identified are Humanin and ARC105 proteins.[29,30] Our analysis indicated that Trim11 physically interacts with Pax6 in cortical progenitors and catalyzes the generation of ubiquitin-conjugated Pax6, which is subsequently transferred to the proteosome system for degradation. Trim11 contains a Ring domain, a coiled-coil domain, a B-box and a B30.2 domain (**Fig. 1**). Molecular dissection of the Trim11 functional domains showed that B-box and coiled-coil subdomains play a role in Pax6 binding and together with the Ring domain mediate UPS-dependent Pax6 proteolysis. Notably, the B30.2 subdomain of Trim11, which binds to both the proteosomes and ubiquitin,[31] was found to mediate proteosome-dependent degradation of misfolded forms of the Pax6 protein (inclusion bodies). In addition to regulating Pax6 degradation, the B30.2 domain recognizes and mediates the degradation of misfolded proteins known to pathologically accumulate, such as Huntingtin and Arx, indicating that Trim11 is involved in global quality control of cell proteins. Similar to Trim11, other proteins with E3 ligase activity, such as Hsc70-interacting protein (CHIP), Parkin and Dorfin, which contain carboxy-terminal E3 ligase domains, are also known to perform proteosome clearance of targeted proteins.[32-34] Interestingly, the Hsc70 chaperone inhibits its degradation of insoluble Pax6 protein, mediated by B30.2 domain of Trim11, suggesting that the interplay between Hsc70 and Trim11 may determine whether nascent or misfolded Pax6 proteins are salvaged by chaperone-mediated repair or targeted for elimination by the UPS.[28]

Transcription factors and elements of the UPS have been shown to react to each other to establish homeostatic feedback loops that maintain their respective activities at equilibrium levels.[35] Our analysis indicated that, in addition to mediating Pax6 degradation via the UPS, the E3 ligase Trim11 is capable of repressing Pax6-dependent neurogenesis by inhibiting the transcriptional activity of Pax6 toward its downstream target genes, including those for the bHLH transcription factors Ngn2,[36] and Er81.[37] Conversely, Pax6 binds to a consensus binding sequence in intron 3 of the *Trim11* gene, thus exerting transcriptional control over the synthesis of Trim11 E3 ligase. Provided the established basal Pax6-Trim11 regulatory loop is fully closed, the balance tends to favor maintenance of Pax6 levels within physiological limits that ensure normal neurogenesis. Any disruption that shifts this equilibrium, such as Pax6 overexpression or a reduction in the level of Trim11, results in apoptosis or a change in cellular adhesive properties.[25,28] To prevent such pathological outcomes, the Pax6-Trim11 autoregulatory feedback mechanism responds to abnormally increased Pax6 levels by promoting the expression of Trim11, which decreases Pax6 protein levels and transcriptional activity, restoring the system to its physiological range.

Neuronal Differentiation

In vertebrates, the transition of stem cells from committed neuronal progenitors

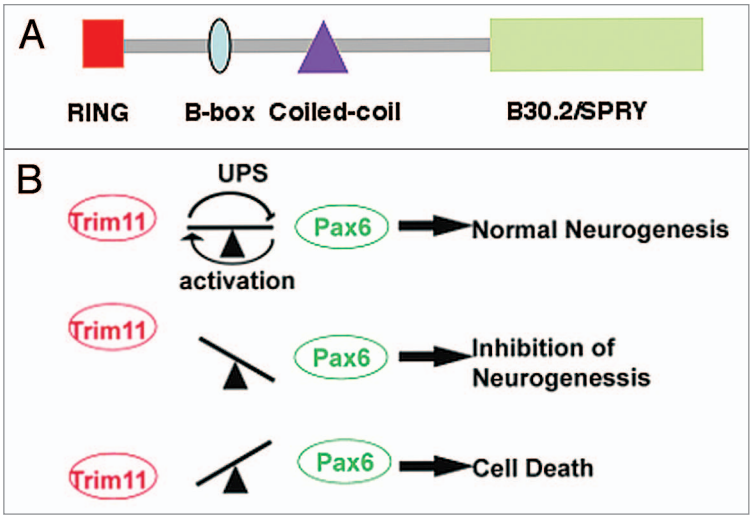

Figure 2. Interplay between the neurogenic factor Pax6 and the E3 ligase Trim11. (A) Diagram presenting the functional domains of the Trim11 protein. The RING domain posses E3 ligase activity and the B-Box and coiled-coil domains mediate protein-protein interactions with specific protein substrates, such as Humanin[29] and Pax6.[28] The B30.2/SPRY domain binds both ubiquitin and elements of the proteosome system, and is responsible for the degradation of ubiquitinated misfolded proteins. Thus Trim11 has dual roles: controlling the stability of specific protein substrates via RING, B-Box and coiled-coil domains and global protein quality control via the B30.2/SPRY domain. (B) Proposed model for the feedback loop between the neurogenic factor Pax6 and the E3 ubiquitin ligase Trim11. Severe deregulation of Trim11 levels affects the fate of the cortical progenitors in two ways: (i) an excessive elevation of Trim11 in progenitors leads to a drastic reduction of Pax6 level and downregulation of Pax6 downstream neurogenic target genes, resulting in impaired neurogenesis; and (ii) a pronounced downregulation of Trim11 levels in progenitors leads to extensive progenitor apoptosis due, at least in part, to an elevation of endogenous Pax6 levels far beyond its physiological range.

toward differentiated neurons involves a highly coordinated switch in the transcriptional program. This switch is regulated not only by transcriptional activators, such as the proneural bHLH proteins or Pax6, but also by transcriptional repressors. In fact, REST/NRSF, a factor known to repress neuronal gene expression in non-neuronal cells, was recently shown to be an important modulator of neurogenesis.[38] This important study revealed that a progressive reduction in the level of REST protein is crucial for the transition from pluripotent stem cells to neural stem cells (RGPs) and then to neurons.

Proteosome-mediated degradation has been implicated in downregulating REST during neuronal differentiation.[38] Recently, Westbrook, et al. (2008)[39] identified a Skp1-Cul1-F-box (SCF) protein complex containing the F-box protein β-TRCP ($SCF^{\beta\text{-}TRCP}$) as the E3 ubiquitin ligase responsible for mediating REST degradation. The SCF complex is a large subfamily of E3 ubiquitin ligases that are key factors in protein regulation. The F-box subunits of SCF ubiquitin ligases mediate substrate-recognition, thus providing the specificity of the ubiquitin conjugation reaction. Each SCF subfamily contains a unique Cullin scaffold, a cofactor that is required for ligase function. Skip proteins (Skp) act as adaptors to link substrate receptors, F-box and E3 ligase Cullins.[3] Using gain- and loss-of-function experiments in embryonic stem cell models, Westbrook, et al. (2008)[39] demonstrated that increased expression of $SCF^{\beta\text{-}TRCP}$ leads to decreases in REST protein levels and REST-dependent neuronal differentiation. Inhibition of neuronal differentiation by $SCF^{\beta\text{-}TRCP}$ knockdown was fully suppressed by simultaneous REST knockdown. In addition, REST and $SCF^{\beta\text{-}TRCP}$ show complementary expression patterns during the progression of neuronal differentiation from embryonic stem cells in vitro. Induction of neuronal differentiation in embryonic stem cells causes progressive upregulation of $SCF^{\beta\text{-}TRCP}$ expression in association with reduced REST stability. These findings indicate that $SCF^{\beta\text{-}TRCP}$-mediated degradation of REST facilities neuronal differentiation.

One remarkable feature of the vertebrate neocortex is the inside-out architecture of the cortical laminae, in which the oldest neurons are located at the lowest positions and younger neurons are arranged in progressively more upward positions. Most of these neocortical neurons are generated during a specific developmental time window, either through direct neurogenesis by Pax6-positive RGPs in the ventricular zone or indirectly through IPs in the subventricular zone. A crucial element in the initiation of neuronal differentiation and establishment of neuronal subtype diversity and normal architecture of the cortex is control of progenitor exit from the mitotic cycle.[40] The helix-loop-helix leucine zipper-type transcription factors of the Myc family, comprising C-Myc, N-Myc and L-Myc, are among the best-characterized proto-oncoproteins with vital roles in cell cycle control. Two members, N-Myc and C-Myc, are expressed in the developing nervous system and play important roles in NSC proliferation, cell cycle exit and neuronal differentiation. High levels of C-Myc are important for the ability of NSCs to self-renew and Myc overexpression has been shown to promote neural progenitor proliferation in the mouse CNS.[41] In addition, C-Myc overexpression in glial fibrillary acidic protein (GFAP)-positive astrocytes promotes formation of less differentiated Nestin-positive progenitor-like cells.[42] Recently Schwamborn, et al. (2009),[43] demonstrated that another member of the TRIM protein family, the E3 ligase Trim32, interacts with C-Myc and mediates C-Myc degradation, leading to suppression of progenitor proliferation and induction of premature neuronal differentiation.

Like C-Myc, N-Myc is also expressed in NSCs and progenitors. Analyses of mice carrying a targeted deletion of the *N-myc* gene have revealed a premature differentiation of cortical progenitors that is associated with increased expression of Cdk inhibitors and downregulation of cyclin D2, an N-Myc target gene.[44] The cellular N-Myc content is primarily regulated through control of its stability.

During neuronal differentiation, N-Myc degradation is progressively induced in a process that requires the HECT-domain ubiquitin ligase Huwe1, which directly interacts with N-Myc and mediates its ubiquitination and proteosomal-dependent degradation. Whereas overexpression of Huwe1 in vivo causes diminished expression of N-Myc in association with suppression of proliferation, genetic inactivation of Huwe1 leads to increased levels of N-Myc protein, resulting in impaired neuronal differentiation.[45] Notably, Huwe1 gain-of-function and loss-of-function phenotypes are rescued by activation and inactivation of N-Myc, respectively, indicating that correct levels of Huwe1 and N-Myc regulate the balance between progenitor proliferation and neuronal differentiation.[45]

The SCF/skp2 complex, another member of the SCF E3 ubiquitin ligase family, targets a number of cell cycle regulators, including the transcription factor E2F1 and the cyclin-dependent kinase inhibitors $p21^{Cip1}$ and $p27^{Kip1}$.[46-48] Gain- and loss-of-function studies in a Xenopus model system have revealed an essential role for SCF/skp2 in neurogenesis. Although the physiological relevance of SCF/skp2 and its substrates has not yet been established, the available data strongly indicate that SCF/skp2 is involved in coordinating cell proliferation, differentiation and cell cycle progression.[49]

Neuronal Migration and Maturation

Development of the CNS requires precise positioning of many neuronal types. In the developing cortex, neurons, generated by RGPs and IPs in germinative zones migrate radially outward to their final locations in the six cortical layers. The migration and laminated distribution of cortical neurons are largely regulated by Reelin/Dab1 signaling.[50] Any failure in Reelin/Dab1 signaling results in an inversion of cortical plate layering that is associated with various neurological and psychiatric disorders. Reelin is produced mostly by cells of the marginal zone located in the uppermost position of the cortex. By increasing the activity of two Src tyrosinase kinases, Reelin induces phosphorylation of Dab1, which leads to polyubiquitination and proteosome-dependent degradation of Dab1.[51] As recently shown by Feng, et al. (2007),[52] Dab1 degradation is dependent on Cullin 5 (Cul5), a component of SCF complex E3 ligase. According to this mechanism, Cul5 interacts with phosphorylated Dab1 via the adaptor protein SOCS1. Cul5 knockdown in the embryonic mouse cortex results in the accumulation of active (phosphorylated) Dab1 in migrating neurons, markedly affecting on neuronal migration and resulting in accumulation of most neurons at the uppermost region of the cortex. These results provide the first evidence that ubiquitin-mediated proteolysis plays an important role in coordinating neuronal migration and cortical lamination.

FBXW7 (F-box and WD-40 domain protein 7), another evolutionarily conserved component of the SCF ubiquitin ligase complex, is an additional factor that acts as a negative regulator of Notch signaling, thereby affecting cell proliferation. SCF/FBXW7 binds and ubiquitinates the cleaved (nuclear) Notch1 intracellular domain (Notch-ICD), targeting it for proteosome-mediated proteolysis.[53] Recent evidence indicates that neuronal migration in the developing cortex is dependent on the interaction between Reelin and Notch signaling pathways.[54] In the absence of functional Reelin (Reelin-deficient mice), Notch-ICD levels are severely reduced, causing abrogation of Notch signaling in migrating neurons and migrational and morphological defects. Interestingly, degradation of Notch-ICD through the FBXW7-mediated proteosome pathway was found to be inhibited by activated DAB1, suggesting that Reelin-Dab1 signaling may facilitate the sequestration of Notch-ICD.[54]

During maturation, neurons normally produce multiple neurites, only one of which ultimately becomes the axon of a mature neuron. Therefore, generation of a polarized axon is an essential step in the neuronal maturation process. Rap1B, a member of the RAS protein super-family, is required for this process. Rap1B is expressed in all neurites of immature neurons, but eventually is restricted to a single neurite, which will become the axon. Data reported by Schwamborn, et al. (2007),[55] show that the restricted expression of Rap1B is controlled by UPS-dependent degradation. Accordingly, chemical inhibition of the UPS in cultured neurons leads to increased levels of Rap1B protein, causing formation of more than one axon. Additionally, in vitro ubiquitination and proteasome inhibition assays have shown that the ubiquitin ligase Smurf2 mediates the proteosome-dependent degradation of Rap1B. Consistent with this, depletion of Smurf2 also results in the formation of extra axons in cultured neurons. Collectively, these observations indicate that degradation of Rap1B by the UPS is important for specification of the single neurite that becomes an axon.

In addition to the well-studied examples described above, a number of additional factors involved in controlling neurogenesis have been shown to be targets of ubiquitin-mediated proteolysis in vitro. These include p21, p35, E2F1, Nodal, Smad2 and 3 (in TGFβ signaling) and Dishevelled (in Wnt/β-catenin signaling). However, functional studies have yet to identify the E3 ligases that target these important substrates.[51,56-60] Likewise, mutagenesis studies have shown that some E3 ligases, such as UBR1 and UBR2, the substrates of which have not yet been identified, cause severe impairment of neurogenesis.[61] Further investigation is needed to understand the downstream effectors of these E3 ligases and their possible roles in neurogenesis.

Conclusions and Perspectives

Neurogenesis comprises sequential steps that must be tightly coordinated, a process that involves temporally regulated extracellular signals, crosstalk among different signaling pathways and activation or inactivation of neurogenic factors. An intrinsic feature of protein proteolysis is its irreversibility; thus, it is not surprising that ubiquitin-dependent degradation has emerged as an important mechanism for regulating neurogenesis. Accurate timing of proteolysis and tight control of the expression levels of key regulators during neurogenesis are achieved through the dynamic interplay of the action of E3 ligases on their substrates and by feedback

regulation of E3 ligases by their substrates, as exemplified by the case of Pax6 and proneural proteins.[15,28]

Recently, in situ hybridization was used to map the expression of more than 1,000 transcription factors genes in the brains of developing mice. Among the genes expressed in the developing mouse brain, more than 600 members encode for proteins of the RING finger family, the largest class of transcription factor proteins.[62] Biochemical studies of this protein family suggest that all RING finger domains posses E3 ubiquitin ligase activity.[63,64] This diversity of RING finger domain-containing proteins and the complexity of the neurogenic process raises a number of questions: How many of these 600 E3 ligases might regulate neurogenesis in addition to those already identified? What is the specific composition of the resulting ubiquitin ligase complexes? And how do these factors cooperate in regulating the degradation of multiple substrates during neurogenesis? Clearly, studying the emerging roles of the UPS in the regulation of neurogenesis is an important challenge for the future.

Acknowledgements

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