Usp11 controls cortical neurogenesis and neuronal migration through Sox11 stabilization

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The role of protein stabilization in cortical development remains poorly understood. A recessive mutation in the Usp11 gene is found in a rare neurodevelopmental disorder with intellectual disability, but its pathogenicity and molecular mechanism are unknown. Here, we show that mouse Usp11 is expressed highly in embryonic cerebral cortex, and Usp11 deficiency impairs layer 6 neuron production, delays late-born neuronal migration, and disturbs cognition and anxiety behaviors. Mechanistically, these functions are mediated by a previously unidentified Usp11 substrate, Sox11. Usp11 ablation compromises Sox11 protein accumulation in the developing cortex, despite the induction of Sox17 mRNA. The disease-associated Usp11 mutant fails to stabilize Sox11 and is unable to support cortical neurogenesis and neuronal migration. Our findings define a critical function of Usp11 in cortical development and highlight the importance of orchestrating protein stabilization mechanisms into transcription regulatory programs for a robust induction of cell fate determinants during early brain development.

INTRODUCTION

The development of vertebrate cerebral cortex is a dynamic and precisely orchestrated process involving neurogenesis, neuronal migration, and postmigrational cortical organization and circuit formation. In the embryonic mouse cortex, radial glial cells (RGCs) function as neural progenitor cells (NPCs) to give rise to neurons either directly or indirectly via intermediate progenitor cells (IPCs). Newborn neurons migrate along radial fibers in an inside-out manner to form six highly organized layers (1). Neurons in each layer exhibit different morphologies, functions, and properties, and even subtle disturbances to neurogenesis or neuronal migration can lead to large defects in the assembly and connectivity of neural circuits. Since cerebral cortex is responsible for higher cognitive functions and emotional processing, malformations of early cortical development could result in a wide range of neurological disorders such as epilepsy, schizophrenia, intellectual disability, and autism (2, 3).

Not unexpectedly, the orderly process of neurogenesis and neuronal migration requires intricate and precise regulation. Previous studies have revealed a complex network of transcriptional programs mediated by transcription factors and chromatin modifiers that govern the dynamics of cortical neurogenesis (4, 5). In addition to transcription, another way to regulate gene expression is the post-translational control, with regulators affecting protein stability and degradation. Compared to transcriptional regulation, the role of protein degradation in cortical development is less well understood. Several ubiquitin ligases participate in cortical development by targeting key stem/progenitor factors for degradation. For example, Mib and Huw1 negatively regulate the Notch pathway to promote RGC differentiation (6, 7). Trim11 promotes Pax6 ubiquitination and degradation, and a negative feedback loop between Trim11 and Pax6 functions to maintain a balanced level of Pax6 during cortical neurogenesis (8). Ubiquitin ligases attach ubiquitin chains to proteins to mark them for degradation, whereas deubiquitinating enzymes (DUBs) counteract these effects by removing ubiquitin from proteins. The roles of DUBs in cortical development have been poorly understood, although several of them, such as Usp7, Usp9x, and Uchl1, have been shown to regulate neuronal differentiation and morphogenesis in vitro (9–11). In principle, DUBs could work hand-in-glove with transcriptional regulators, by promoting the stabilization of differentiation factors once their expression is induced by transcription factors. This could reinforce progenitor cells’ commitment to differentiation, by ensuring an irreversible cell fate transition. To test this hypothesis, it is important to define the role of protein deubiquitination events and their interaction with transcriptional networks to coordinate cortical development.

The SoxC family proteins Sox4 and Sox11 are high mobility group box–containing transcription factors that have multiple roles in cortical development (12). At early neurodevelopmental stages, Sox4 and Sox11 are critical for neural precursor survival since double knockout (KO) in mouse leads to massive cell death (13). Sox4 and Sox11 induce the expression of an overlapping set of neuronal genes (14, 15), but the two transcription factors seem to have non-redundant functions in cortical neurogenesis (16). Sox11 deficiency impairs the generation of early-born neurons by preventing the differentiation of RGCs without affecting IPCs. Sox4, however, is specifically required for the specification and maintenance of IPCs. These discrete functions are likely a result of their restricted expression patterns and distinct binding partners. In addition, Sox11 is required for the radial migration of late-born neurons by preventing precocious dendritic morphogenesis (17). In line with their functions in cortical neurogenesis, the expression of Sox4/11 is tightly controlled during this period. In neural precursor cells, their expression is suppressed by the transcriptional repressor RE1-silencing transcription factor (REST)/neuron restrictive silencing factor (NRSF) (18). Sox11 is also negatively regulated by transcription factor Lhx2, and this regulation is implicated in fate specification of deep
layer neurons (19). Whether Sox11 can be regulated by a transcription-independent mechanism during cortical development is currently unknown.

USP11, a USP-family DUB, is highly expressed in human brain and exerts an inhibitory role in the self-renewal of glioblastoma stem/initiating cells (20). Homozygous missense mutation of the USP11 gene has been found in a hereditary neurologic disorder patient, characterized by intellectual disability and multiple brain malformations, such as syntelencephaly and corpus callosum agenesis (21). However, the pathogenicity of this mutation remains elusive. Here, we show that Usp11 is highly expressed in the developing mouse cortex. Usp11 promotes layer 6 neurogenesis and late-born neuronal migration, and Usp11 deficiency leads to behavioral abnormalities. Mechanistically, we show that Usp11 ubiquitinates Sox11, which is critical for Sox11 stabilization during cortical development. We provide evidence that the mouse counterpart of human neurodevelopmental disorder–associated USP11 variant is defective in Sox11 stabilization and cortical development. Together, our findings reveal an integration of a protein ubiquitination event with a transcriptional network to control cortical development and provide mechanistic insights for how USP11 mutations cause a human neurologic disorder.

RESULTS

Usp11 is highly expressed in mouse embryonic brain including cerebral cortex

Our previous study revealed that USP11 mRNA is highly expressed in human brain, especially in the forebrain (20). This finding prompted us to explore the expression pattern of Usp11 in mouse. Using whole-mount in situ hybridization, we found that Usp11 mRNA expression at embryonic day 13.5 (E13.5) is high in the spinal cord and several brain regions, including neocortex (Fig. 1A). Within the cortex, Usp11 is expressed in both neurons (located in preplate and cortical plate) and NPCs (located in ventricular and subventricular zones) during the period of cortical neurogenesis, but the level is higher in neurons than in NPCs (Fig. 1B). These findings imply an up-regulation of Usp11 during neuronal differentiation. Since our previous study found that human USP11 transcription is repressed by the Notch pathway (20), the up-regulation of Usp11 is likely a consequence of Notch inactivation upon the induction of neuronal differentiation. In line with this notion, we showed that mouse Usp11 was repressed by the Notch/He1 axis, as overexpression of an active form of Notch (Notch intracellular domain, referred to as NIC) or He1 in a neuroblastoma cell line N2a diminished Usp11 expression and the promoter activity of Usp11 gene (fig. S1, A and B). Thus, our data suggest a role of Usp11 in cortical neurogenesis.

Usp11 deficiency reduces cortex thickness

To analyze the function of Usp11 in brain development, we obtained a Usp11 KO mouse from the European Mouse Mutant Archive (EMMA) Mouse Repository (www.infrachainrue/search). This strain harbors a deletion of exons 3 to 10 of the Usp11 gene (Fig. 1C, top), leading to frameshift and translation stop at exon 11. We performed immunoblot analysis of lysate taken from Usp11 KO cortices to confirm the absence of Usp11 expression (Fig. 1C, bottom). Usp11 deletion did not significantly affect body weight and brain weight and only modestly reduced the brain width at postnatal day 7 (P7) (fig. S1, C to F). However, close examination identified a significant reduction of cortex thickness in the Usp11 KO mice at P7 (Fig. 1, D and E). We stained for layer-specific markers (Tbr1, Ctip2, and Cux1 for layers 6, 5, and 2 to 4, respectively) and found that Usp11 deletion did not alter the relative position of cortical layers. However, the thickness of layer 6 and the number of layer 6 neurons were reduced in Usp11 KO animals, whereas other layers were not significantly affected (Fig. 1, F to H). These findings support a role of Usp11 in regulating the generation of deep layer neurons during cortical development.

Usp11 deficiency impairs the generation of layer 6 neurons

Next, we investigated the role of Usp11 in NPC differentiation into deep layer neurons. Immunostaining for RGC marker Pax6 and IPC marker Tbr2 revealed no significant difference in their gross numbers between wild-type and Usp11 KO embryos throughout early neurogenesis from E12.5 to E15.5 (fig. S1, G to J). To investigate the reason for fewer Tbr1+ layer 6 neurons in the Usp11 KO cortices, we examined the ratio of cells that exit the cell cycle during early embryonic neurogenesis. We labeled proliferating cells by a pulse of 5-ethyl-2′-deoxyuridine (EdU) and collected the embryos 24 hours later. We then used Ki67 staining to detect proliferating progenitors when the cortex was collected. Cells labeled by EdU, but not Ki67, are the progenitors that have exited the cell cycle. We observed that Usp11-deficient NPCs showed a decreased tendency to exit the cell cycle at E12.5 (Fig. 2, A and B). Since most of the layer 6 neurons are generated from NPCs born at E12.5 (22), this finding is consistent with the observation of fewer layer 6 neurons generated in Usp11 KO. No difference was observed at other time points, consistent with the fact that layers 2 to 5 were not significantly affected in Usp11 KO. We thus focused on the time point of E12.5 for the following studies. To determine the underlying mechanism for the decreased tendency of cell cycle exit observed in Usp11 KO NPCs, we performed EdU–5-bromo-2′-deoxyuridine (BrdU) double labeling assay for measuring cell cycle length (23). Our data indicated that the Usp11 deficiency increased cell cycle length of NPCs at E12.5 (Fig. 2C). Furthermore, we did not observe significant differences in cell apoptosis in wild-type and Usp11 KO cortices during the period of E12.5 to E18.5 (fig. S1, K and L). Thus, our findings uncover a role of Usp11 in regulating NPC cell cycle and the dynamics of NPC differentiation, with the most obvious effect observed from NPCs at E12.5.

To investigate what kinds of cortical progenitor subtypes are affected by Usp11 ablation, we stained Pax6 for RGC and Tbr2 for IPC 1 day after EdU labeling at E12.5 and observed that there were more EdU/Pax6 double-positive cells in Usp11 KO embryos, compared to control embryos (Fig. 2, D and E). In contrast, Usp11 deletion did not significantly affect the IPC population (Fig. 2, F and G). These data suggest that Usp11 deficiency specifically delays RGC cell cycle exit at E12.5, which influences their differentiation into layer 6 neurons. To substantiate this idea, we monitored the generation and distribution of neurons derived from EdU-labeled E12.5 NPCs at E15.5. Usp11 deletion resulted in a significant reduction in the proportion of Tbr1+ EdU+ cells and in the distribution of EdU+ cells to the cortical plate (Fig. 3, A to C) but did not affect the percentage of Ctip2+ EdU+ cells (fig. S1, M and N). Since the generation of deep layer neurons is completed at E15.5 (22), we also monitored the total Tbr1+ cells in E15.5 cortices. Our result indicates that the total Tbr1+ cells were diminished in Usp11 KO compared to wild-type littermates (Fig. 3D). Together, these data are consistent with the findings from gross analysis of the Usp11 KO cortices at P7 and support a role of Usp11 in RGC differentiation into layer 6 neurons.
Usp11 acts in progenitor cells to promote layer 6 neurogenesis

To define whether the role of Usp11 in layer 6 neurogenesis is derived from its direct action in RGCs, we generated Usp11 floxed mice using CRISPR technology (fig. S2A). We then bred Usp11 floxed mice with Emx1-Cre transgenic line (24) to generate Emx1:Usp11 f/f or Empty Spiracles Homeobox 1 (Emx1):Usp11 f/y mice (collectively referred to as Usp11 E-cKO), in which Usp11 deletion occurs in Emx1-expressing cortical progenitors. In addition, we deleted Usp11 specifically in the postmitotic neurons by breeding Usp11 floxed mice with Nex-Cre (25) to generate Nex:Usp11 f/y and Nex:Usp11 f/f mice (collectively referred to as Usp11 N-cKO) (fig. S2A). Immunofluorescence staining revealed that Usp11 staining intensities in cortical progenitors and neurons were reduced to background levels in Usp11 E-cKO cortices (fig. S2C). For Usp11 N-cKO, Usp11 expression remained in the cortical progenitors but was virtually lost in cortical neurons (fig. S2D). We found that the cortices of Usp11 E-cKO mice at P7 were thinner than the control mice, with a specific reduction in the thickness of layer 6, but not other layers (fig. S2, E to G). Consistently, the number of Tbr1+ (layer 6) neurons was reduced in Usp11 E-cKO cortices at P7, whereas Ctip2+ (layer 5) and Cux1+ neurons (layers 2 to 4) were not affected (fig. S2H). However, the cortex thickness and layer 6 thickness did not differ between control and Usp11 N-cKO mice (fig. S2, I to K), indicating that the defects seen in Usp11 E-cKO resulted from Usp11 ablation in cortical progenitors.

In line with this idea, we found that Usp11 E-cKO increased the number of E12.5-born progenitor cells retained in the RGC state at E13.5 (Fig. 3, E and F). Consistently, the total number of Tbr1+ (layer 6) neurons was reduced in Usp11 E-cKO cortices at P7, whereas Ctip2+ (layer 5) and Cux1+ neurons (layers 2 to 4) were not affected (fig. S2H). However, the cortex thickness and layer 6 thickness did not differ between control and Usp11 N-cKO mice (fig. S2, I to K), indicating that the defects seen in Usp11 E-cKO resulted from Usp11 ablation in cortical progenitors.
distributed to the cortical plate (Fig. 3, H to J). These findings provide evidence that Usp11 acts directly in neural progenitors to promote the generation of layer 6 neurons.

**Usp11 deficiency impairs radial migration of cortical neurons**

In Usp11 KO and Usp11 E-cKO cortices, we observed a reduction of E12.5-labeled cells to arrive at cortical plates at E15.5 (Fig. 3, C to J). Although these findings are consistent with a differentiation defect of E12.5-born progenitors caused by Usp11 deficiency, Usp11 may elicit an additional effect to directly regulate the radial migration of cortical neurons. To more specifically evaluate the role of Usp11 in the migration of cortical neurons, we thought to focus on the late-born neurons, as the finding that Usp11 KO did not affect the thickness of the superficial layer (layers 2 to 4) at P7 suggests its dispensable role in the neurogenesis of superficial layers. To substantiate this hypothesis, we performed EdU injection at E15.5, since cortical progenitors at this time point mainly give rise to layers 2 to 4 neurons (22). We harvested embryos at E18.5 and analyzed the fate of EdU+ cells by staining for Pax6, Tbr2, or Satb2 (a marker of layers 2 to 4 neurons). We found that Usp11 KO mice and wild-type littermates showed no difference in total number of EdU+ cells and the percentages of Pax6+EdU+, Tbr2+EdU+, and Satb2+EdU+ cells (Fig. 4A and fig. S3, A to F), indicating that Usp11 ablation does not affect the differentiation of E15.5-born progenitor cells. However, compared to wild-type cortices, Usp11 KO cortices showed a higher percentage of EdU+ cells in IZ and a lower percentage in layers 2 to 4 (Fig. 4, A and B). In line with this finding, fewer Satb2+EdU+ neurons in Usp11 KO cortex migrated to their final destination (layers 2 to 4) (Fig. 4C). By P7, however, most EdU+ cells and virtually all EdU+Cux1+ cells had migrated to the superficial layers in both Usp11 KO and wild-type animals (fig. S3, G to I). These findings indicate that late-born neuronal migration was delayed by Usp11 deletion.

Next, we investigated whether the neuronal migration defect seen in the Usp11 KO mice is due to a cell-autonomous effect. This is important since the migration of upper layer neurons is dependent on radial fibers and can also be regulated by factors secreted from other cell lineages (26). To address this question, we analyzed Usp11 N-cKO and control embryos. Usp11 N-cKO cortices showed a similar delay in the migration of late-born neurons to Usp11 KO (Fig. 4, D to F). Thus, Usp11 has a cell-autonomous role in promoting the radial migration of late-born neurons.

**Usp11 KO mice exhibit learning/memory and anxiety abnormalities**

The defects in neurogenesis and neuronal migration we observed in Usp11 KO mice suggest that Usp11 deficiency could impair neuron number, maturation, and connectivity, which might compromise cortical functions. Therefore, we investigated whether Usp11 KO causes behavior abnormalities related to neurodevelopmental disorders, such as deficits in cognitive function and anxiety. To assess cognitive function, we used Morris water maze (MWM), contextual fear conditioning (CFC), and novel object recognition (NOR) tests for spatial learning/memory, associative learning/memory, and non-spatial memory, respectively. In MWM, we did not observe differences in performance between wild-type and Usp11 KO mice with a visible platform, demonstrating that their swimming ability and visual acuity were not impaired (fig. S4A). However, when the platform was hidden from the mice during training, the Usp11 KO mice exhibited deficits in spatial learning by taking more time to locate the platform at day 2 and day 3 (fig. S4B). After they showed no significant difference at day 4, we tested them for memory retention at day 5 by removing the platform and measuring the amount of...
time they spent in the area where the platform was previously located (probe trial). The Usp11 KO mice spent less time in the target quadrant than wild-type littermates (fig. S4C), indicating a deficit in spatial memory. In the CFC test, mice were trained to express a fear response (freezing) by linking a conditioned stimulus with an aversive unconditioned stimulus (an electronic shock). The wild-type and Usp11 KO mice did not show differences in habituation and exhibited a similar increase in freezing during the training period at day 1 (fig. S4D, left). While exposed to the context stimulus alone at day 2, they were initially indistinguishable, suggesting no difference in memory retention. However, Usp11 KO mice showed a slower decline in freezing than wild-type littersmates during the extinction tests (fig. S4D, right), suggesting a deficit in memory extinction. Last, in the NOR test, the two groups of mice showed no difference in exploring a novel object, suggesting normal nonspatial memory (fig. S4E). However, when we used the elevated plus maze (EPM) and open field tests to assess anxiety-related behaviors, the Usp11 KO mice showed increased duration and distance in the open arms of EPM and the center zone of the open field compared to wild-type littersmates (fig. S4, F and G), suggesting anxiety and/or attention deficit. Of note, they performed similarly in the rotarod test (fig. S4H). Together, Usp11 KO disrupts learning, memory, and anxiety behaviors, consistent with defects in cortical development.

### Sox11 is identified as a Usp11 substrate

Having demonstrated the effects of Usp11 deficiency on cortical development, we next sought to define its molecular mechanism. Since Usp11 encodes a DUB, we reasoned that Usp11 deficiency may lead to increased ubiquitination and degradation of a protein that is critical for neuronal differentiation and migration. We first performed a label-free quantitative liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis to detect the protein expression profiles in E17.5 wild-type and Usp11 KO cortices and identified 3871 proteins in four biological repeats. Proteins down-regulated in Usp11 KO were defined with the following criteria: wild type/Usp11 KO ratio >1.5, P < 0.05, and a total of 122 proteins were recovered (Fig. 5A and table S1). Gene Ontology (GO) analysis with these 122 proteins revealed that neurogenesis and nervous system development are among the enriched terms (fig. S5A), and 7 proteins were found in these two categories (Fig. 5A, marked in red). Since a direct target...
of Usp11 should exhibit not only a decreased protein level but also an increased ubiquitination level in response to Usp11 ablation. We sought to conduct a ubiquitylome analysis to globally assess ubiquitination changes by Usp11. To obtain enough cells for analysis, we used NPCs/neurons induced from mouse embryonic stem (ES) cells via an established protocol (fig. S5B). Neuron formation started at day 8, and Usp11 up-regulation increased the number of cells expressing the neuron marker Tuj1 (fig. S5, C and D), indicating the ability of this ex vivo model to recapitulate the in vivo neurogenesis-promoting activity of Usp11. We performed an MS-based protocol for ubiquitylome changes (27) on day 8 culture with some modifications (fig. S5E). For ubiquitylome analysis, trypsin-digested proteins from cell lysates were subjected to immunoprecipitation with a K-ε-GG antibody, which recognizes the di-glycine remnants of ubiquitinated lysine. Peptides before and after enrichment (for proteome and ubiquitylome analyses, respectively) were labeled with isotopically distinct tandem mass tags (TMTs), thus enabling the quantification of relative abundances of each peptide across samples (28). To increase the number of identified peptides, peptides for proteome analysis were fractionated before LC-MS/MS analysis. Among the 1004 proteins uncovered from both proteome and ubiquitylome analyses, 29 displayed [protein-normalized K-ε-GG pep-

Usp11 stabilizes Sox11 during cortical neurogenesis

We next analyzed the functional consequence of Sox11 deubiquitination by Usp11. Knockdown of Usp11 in N2a cells using three independent short hairpin–mediated RNAs (shRNAs) markedly reduced Sox11 protein level without affecting its mRNA level (Fig. 6A and fig. S6C). Conversely, overexpression of Usp11, but not a catalytically dead mutant, elevated Sox11 protein abundance (Fig. 6B). Human USP11 also up-regulated SOX11 expression (fig. S6D), demonstrating the evolutionary conservation of the Usp11/Sox11 axis. In Usp11 knockdown cells, treatment with the proteasome inhibitor MG132 mitigated the effect of Usp11 knockdown on Sox11 levels (Fig. 6C). Last, using a cycloheximide-chase assay, we found that Usp11 knockdown decreased the stability of Sox11 (Fig. 6D). These data indicate that Usp11 stabilizes Sox11 by preventing its proteasomal degradation. Sox11 is regulated at the transcriptional level during cortical neurogenesis (18). Our identification of an additional level of regulation (i.e., at the protein stability level) by Usp11 prompted us to investigate the kinetics of Sox11 mRNA level changes and protein level changes (i.e., at the protein stability level) by Usp11 prompted us to investigate the kinetics of Sox11 mRNA level changes and protein level
changes during neurogenesis. We performed ex vivo and in vivo experiments. For the ex vivo experiment, we induced neuronal differentiation of NPCs isolated from E12.5 mouse cortex. Successful neuron induction was confirmed by a gradual increase in Tuj1 expression and a decrease in Pax6 expression (Fig. 6E). During this period, Sox11 mRNA was only modestly elevated (~1.4-fold for day 3 versus day 0). Nevertheless, we found a sevenfold elevation to its protein level (Fig. 6F). Similarly, although Sox11 mRNA and protein expression in mouse cortex were both elevated during E10.5 to E14.5, the fold induction of protein was higher than mRNA (Fig. 6, G to I). In Usp11 KO cortices, although Sox11 mRNA was induced at levels similar to that in wild-type cortices, Sox11 protein induction was greatly impaired. The Sox11 protein induction folds detected by Western blot seem to be a little higher than that from the proteomics analysis, presumably due to different methods used. Together, our data collectively indicate that the induction of Sox11 mRNA during cortical neurogenesis is insufficient to lead to a robust protein elevation and that Usp11-dependent Sox11 stabilization accounts for a major role in Sox11 up-regulation during cortical neurogenesis.

Among the Sox family proteins, Sox4 is the most closely related member to Sox11. However, no interaction between Sox4 and Usp11 could be detected using E18.5 cortical lysates (fig. S6E). Consistently, wild-type and Usp11 KO cortices during E12.5 to E18.5 expressed comparable amounts of Sox4 protein (fig. S6F). In addition, the
expression of Pml, a Usp11 substrate previously identified in brain tumors (20), was comparable in wild-type and Usp11 KO cortices (fig. S6F). Thus, our findings indicate that the function of Usp11 in cortical neurogenesis is unlikely mediated by Sox4 and Pml.

**Usp11 stabilization of Sox11 is required for cortical development**

To define the contribution of Sox11 stabilization to the effects of Usp11 on cortical development, we performed rescue experiments. In particular, we intended to test whether Sox11 overexpression rescues the phenotypes of Usp11 deficiency. To do so, we first tested whether Usp11 knockdown causes the same cortical developmental defects observed in Usp11 KO, and second, we examined whether these defects are rescued by Sox11 overexpression. Using in utero electroporation (IUE) to transfect control or Usp11 shRNA expression constructs at E12.5, we found that Usp11 knockdown by two independent shRNAs greatly reduced the number of Tbr1+ neurons at E15.5 (Fig. 7, A and B). Consequently, most Usp11 shRNA-expressing cells (marked by mCherry) did not arrive at cortical plates at this time point, in sharp contrast to cells expressing control shRNA (Fig. 7, A and C). Thus, by up-regulating Sox11, we could rescue the defects in early cortical neurogenesis caused by Usp11 knockdown.

To analyze the migration of late-born neurons, we performed IUE at E15.5 and analyzed cortices at E18.5. Compared to cells carrying control shRNA, more Usp11 shRNA-expressing cells stayed at IZ and less migrated to cortical plate (Fig. 7, D and E). Furthermore, Usp11 knockdown decreased the arrival of Satb2+ neurons to the superficial layers, an indication of a migration defect (Fig. 7F). Sox11 overexpression was sufficient to partially rescue this migration defect of late-born neurons. Together, our findings provide evidence for the contribution of Sox11 stabilization to the functions of Usp11 in cortical development.

**Disease-associated Usp11 mutant is defective in Sox11 stabilization and cortical development**

A recent study reported the presence of a homozygous Usp11 mutation (R241Q) in a patient with malformations of several brain regions and intellectual disability (21). This residue is evolutionarily conserved and located near the ubiquitin-like (UBL) domain (fig. S7), which is implicated in the binding of substrates or modulators (29). To test the potential pathogenicity of this mutation, we investigated its impact on Sox11 regulation. Whereas expression of wild-type Usp11 increased Sox11 abundance, this mutant did not up-regulate Sox11 ubiquitination level when overexpressed in cells (Fig. 8B). To test the potential pathogenicity of this mutation, we investigated its impact on Sox11 regulation. Whereas expression of wild-type Usp11 increased Sox11 abundance, this mutant did not up-regulate Sox11 ubiquitination level when overexpressed in cells (Fig. 8B). Moreover, the R241Q mutant exhibited a reduced ability in binding Sox11 (Fig. 8C). Thus, the disease-associated Usp11 mutant is defective in targeting Sox11 for deubiquitination and stabilization.

To assess the in vivo functions of Usp11 R241Q mutant during cortical development, we used IUE experiments to introduce wild-type and Usp11 R241Q in Usp11-depleted conditions. As expected, Usp11 knockdown decreased the expression of Sox11. This Sox11 down-regulation was rescued by re-expression of wild-type Usp11, but not Usp11 R241Q (Fig. 8, D and E). Functionally, while wild-type Usp11 could completely rescue the defects of Usp11 knockdown in generation of Tbr1+ neurons and migration of upper layer neurons, the Usp11 R241Q mutant failed to do so (Fig. 9, A to F). These data provide evidence that the disease-associated Usp11 mutant is defective in Sox11 stabilization, neurogenesis, and neuronal migration and support the pathogenicity of this mutation.

**DISCUSSION**

Using conventional and conditional KO models, we have provided evidence for dual roles of Usp11 in cortical development—promoting the differentiation of RGCs into layer 6 neurons and then the migration of late-born neurons, through a cell-autonomous manner. We show that these effects of Usp11 are mediated in part by Sox11, which was previously shown to contribute to neurogenesis and neuronal migration in the developing cortex (16, 17). Even though Sox11 is transcriptionally up-regulated during cortical development, our findings reveal an additional layer of regulation—protein stability.
These findings highlight the importance of protein deubiquitination/stabilization for the robust induction of molecular determinants of cell fate and morphogenesis to ensure an irreversible developmental process. We reason that this posttranslational mechanism is particularly needed to extend the half-life of a labile protein, such as Sox11. Notably, since Usp11 is itself regulated at the transcriptional level by Notch signaling, our study indicates that the Usp11-mediated posttranslational regulation of Sox11 is integrated into the transcriptional circuits governed by Notch and Sox11, thus facilitating the proper development of mouse cortex.

One intriguing question is whether the functions of Usp11 in layer 6 neurogenesis and upper layer neuronal migration are interconnected. On the basis of our findings with Usp11 N-cKO and Usp11 E-cKO mice, we believe that they most likely represent separate functions of Usp11 occurring in different cell populations and at different developmental periods. While the layer 6 neurogenesis function represents a cell-autonomous action of Usp11 in E12.5-born NPCs, the late-born neuronal migration function is attributed to Usp11’s function in the postmitotic neurons.

In addition to the cortical developmental defects observed from Usp11 KO embryos, adult Usp11 KO mice exhibited a number of behavior abnormalities, including anxiety and learning/memory deficits. It is currently unclear whether these behavior abnormalities originated from defects in the cerebral cortex development. Nevertheless, impairment of layer 6 neurogenesis has been linked to learning/memory abnormality (30). In addition, the layer 6 marker Tbr1 is essential for specifying layer 6 identity (31), and its heterozygous variants have been found in patients with autism spectrum disorder (32). Despite these linkages of layer 6 neurogenesis defect to abnormal behaviors, we cannot rule out the possibility that the behavior abnormalities...
of Usp11 KO mice are caused by migration defect of upper layer neurons or an uncharacterized developmental defect in other brain regions.

Notably, the learning/memory deficit seen in Usp11 KO mice resembles the intellectual disability observed from a neurodevelopmental disorder patient carrying a homozygous missense mutation in the Usp11 gene (21). The patient also exhibits malformations of cortical development (MCD) such as syntelencephaly and corpus callosum agenesis. Since this is the only reported mutation in USP11 in human disease and because this and other variants are present in publicly available exome and genome databases, we sought to test the functional consequences of this variant. We provide evidence that the mouse counterpart of this USP11 mutant not only is defective in binding and deubiquitinating Sox11, leading to its persistent degradation, but also fails to rescue cortical developmental defects caused by Usp11 depletion. Thus, by comparing the information gained from the patient with the experimental data derived from this study, including in vivo characterization, behavior tests, and biochemical analyses, we hypothesize that the inability to stabilize Sox11 likely represents a major mechanism for the behavioral and brain architectural abnormalities seen in this patient. Consistent with this hypothesis, heterozygous deletion, truncation, and missense mutations in the Sox11 gene have been linked to Coffin-Siris syndrome, a congenital disorder characterized by intellectual disability, MCD, and other developmental defects (33).

We show that Sox11 overexpression rescues the defects in RGC differentiation and neuronal migration caused by Usp11 deficiency, which supports the contribution of Sox11 stabilization to the effects of Usp11 on cortical development. Nevertheless, we do not exclude the possibility for the involvement of other Usp11 substrates in these functions. Our previous study identified a role of Usp11 in the deubiquitination and stabilization of promyelocytic leukemia protein (PML) in glioblastoma (20). Notably, Pml deficiency leads to an increase in NPC cycling and an impairment of the transition from RGCs to IPCs, thereby resulting in reduced neurogenesis and cortex thickness (34). These phenotypes are not entirely consistent with that of Usp11 deficiency. Accordingly, we could not detect an obvious difference in Pml abundance between wild-type and Usp11 KO cortices. This finding suggests a context-dependent effect of Usp11 on Pml and a dispensable role of Pml in Usp11’s function during cortical development.

It is intriguing that Usp11 targets Sox11, but not its paralog Sox4, in the developing cortex. The inability of Usp11 to regulate Sox4 may be due to an intrinsic difference in the sequences of the two proteins. Notably, although the two proteins share many redundant functions in cortical development (13, 35, 36), KO mouse studies have revealed the unique/noncompensatory functions of Sox11 in RGC differentiation in early neurogenesis and migration of late-born neurons (16, 17), consistent with the phenotypes of Usp11 KO mice. Similarly, SOX11 haploinsufficiency cannot be compensated for by SOX4 in human and results in neurodevelopmental disorders (33, 37, 38). These nonredundant functions could be partly explained by the overlapping but nonidentical expression patterns of the two proteins in developing cortex (16). We postulate that the selectivity of Usp11 for Sox11 may result in a specific impairment of layer 6 neurogenesis seen in Usp11 KO mice. Perhaps Sox11 is uniquely required for the generation of this layer of neurons, while Sox11 and Sox4 are functionally redundant in the neurogenesis of other layers. Accordingly, despite the fact that Usp11 is capable of regulating Sox11 abundance in the cortex during the period of E12.5 to E18.5, Usp11 ablation elicits a transient effect on NPC differentiation in cerebral cortex, with E12.5-born NPCs showing the most prominent defects. In summary, our study identifies the functions of Usp11-mediated Sox11 stabilization in cortical development, provides an explanation for the association of Usp11 mutation with neurological disorder, and highlights the importance of deubiquitination-triggered protein stabilization in the developmental process.

Fig. 9. Disease-associated Usp11 mutant is defective in supporting cortical neurogenesis and neuronal migration. (A) Immunostaining for Tbr1 and DAPI staining using cortical sections of E15.5 embryos subjected to IUE of indicated constructs and mCherry expressing construct at E12.5. The transfected cells are marked by mCherry. Scale bar, 100 μm. (B) Quantification of results from (A) showing the location of transfected cells (B) and the percentage of transfected cells expressing Tbr1 (C). Data are means ± SD; **P < 0.01 and ***P < 0.001 by one-way ANOVA with Tukey’s post hoc test, n = 4. (D) Immunostaining for Satb2 and DAPI staining using cortical sections of E18.5 embryos electroporated in utero with indicated constructs and mCherry expressing construct at E15.5. The transfected cells are marked by mCherry. Scale bar, 100 μm. (E) and (F) Quantification of results from (D) showing the location of mCherry+ cells (E) or mCherry+Satb2+ cells (F). Data are means ± SD; **P < 0.05, ***P < 0.01, and ****P < 0.001 by one-way ANOVA with Tukey’s post hoc test, n = 4.
**MATERIALS AND METHODS**

**Plasmids**

Plasmids encoding human USP11, His-ubiquitin, and myc-NIC were described previously (20). Complementary DNA (cDNA) for mouse Usp11 was amplified by reverse transcription polymerase chain reaction (RT-PCR) from N2a cells and then subcloned to pRK5-Flag and pBybe-myc. Usp11 C275/283S (CS) and R241Q mutants and human USP11 R241Q mutant were generated by Pfu site-directed mutagenesis (Thermo Fisher Scientific). Hes1 and Sox11 cDNAs were purchased from Origene and subcloned to pRK5-Flag and/or pUS2. To clone Usp11 promoter, a DNA fragment corresponding to nucleotides from −1667 to +343 of the mouse Usp11 gene was amplified from the genomic DNA of N2a cells and inserted to pGL3-based vector.

**Antibodies and reagents**

Antibodies used in this study are listed in table S3. Cycloheximide was purchased from Sigma-Aldrich. MG132 was obtained from Calbiochem.

**Cell culture and transfection**

293T cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (PS; 100 U/ml). N2a mouse neuroblastoma cells were maintained in high-glucose minimal essential medium (MEM) supplemented with 10% FBS, 1 mM GlutaMax, and PS (100 U/ml). D3 mouse ES cells were maintained in undifferentiated state on a feeder layer of mitomycin C–inactivated mouse embryonic fibroblasts in ES medium [DMEM supplemented with 15% FBS, 55 μM β-mercaptoethanol, 1 mM GlutaMax, 1% nonessential amino acid, PS (100 U/ml), and recombinant mouse leukemia inhibitory factor (LIF; 1000 U/ml)]. Transfection was performed using calcium phosphate method or Lipofectamine 3000 reagent.

**Neuron induction**

Neuron induction from mES cells was followed by an established protocol with modifications (39). Briefly, 2 days before induction, ES cells were seeded on a plate precoated with 0.1% gelatin (Millipore) and Synthenmax (1 mg/ml; Corning) without feeder cells and cultured in N2B27 medium (1:1 mix of N2 medium and B27 medium) supplemented with 3 μM GSK3β inhibitor CHIR99021 (Stemgent), 1 μM MEK1/2 inhibitor PD0325901 (Stemgent), and LIF (1000 U/ml). They were then hang-dropped in LIF-free ES medium for 2 days to form embryoid bodies (EBs). EBs were transferred to ultralow attachment plate (Corning) and cultivated in LIF-free ES medium for 2 days, followed by N2B27 medium with 5 μM retinoic acid (RA) for 2 days. To induce neuron differentiation, EBs were dissociated by Accutase (Millipore), and the dissociated cells were seeded on a plate precoated with laminin (5 μg/ml) and poly-d-lysine (40 μg/ml) and cultured in N2B27 medium with 5 μM RA.

For neuron induction from NPC, the neocortex was dissected from E13.5 C57BL/6J mouse and incubated with Accutase solution (Millipore) for 15 min at 37°C. The dissociated cells were seeded on ultralow attachment plate for suspension culture at a density of 100,000 cells/ml in culture medium composed of DMEM/F12 (Invitrogen), N2 supplement (Invitrogen), 1% PSA (penicillin-streptomycin-amphotericin; Invitrogen), epidermal growth factor (20 ng/ml; Millipore), and fibroblast growth factor (20 ng/ml; BD Biosciences). To induce neuron differentiation, the suspended NPC aggregates were dissociated by Accutase, and cells were seeded on a plate precoated with poly-d-lysine (40 μg/ml) and cultured in N2B27 medium.

**In situ hybridization**

Whole embryos were perfused with 4% phosphate-buffered paraformaldehyde (PFA), washed with phosphate-buffered saline (PBS), and cryo-protected with 30% sucrose in PBS. They were then embedded in Tissue-Tec optimal cutting temperature (OCT) (Sakura Finite) and sectioned at 20 μm on a Cryostat (Leica). Sections were secondarily fixed with 4% PFA, treated with acetic anhydride, and dehydrated in a series of ethanol baths. Sense and antisense RNA probes for Usp11 were labeled using a Digoxigenin (DIG)–RNA labeling kit (Roche). Hybridization was performed overnight at 55°C. Next, the sections were treated with ribonuclease A (20 mg/ml) at 37°C for 30 min and washed with 0.2× saline sodium citrate (SSC) and then 0.1× SSC at 55°C for 30 min each. DIG-labeled RNA probes were immunodetected with antidigoxigenin conjugated to alkaline phosphatase. The antibody conjugate was visualized with chemiluminescent substrates Disodium 3-(4-methoxyspiro {1,2-dioxetane-3,2′-(5′-chloro)tricyclo [3.3.1.13,7]decan}-4-yl)phenyl phosphate (CSPD). Then, the sections were dehydrated in a series of alcohol and xylene, air-dried, and mounted.

**Immunoprecipitation and Western blotting**

Immunoprecipitation and Western blotting using cell lysates containing equal amounts of proteins were performed as described previously (20).

**Mouse husbandry**

Mice were housed in a specific pathogen–free animal facility under temperature-controlled (22° ± 2°C) and humidity-controlled (55 ± 5%) conditions with a 12-hour light/12-hour dark circadian cycle and access to food and water. When mice were mated, the morning that vaginal plug was identified was designated as E0.5. During behavioral testing, mice were group-housed with two to five animals of the same sex per cage. All mouse experiments were conducted with approval from the Institutional Animal Care and Use Committee, Academia Sinica, and followed the guidelines of ethical regulations.

**Behavioral analyses**

All assays were conducted during 1:00 p.m. to 6:00 p.m. light phase by observers who did not know the genotype of the mice until the test had been completed and were performed with 3- to 4-month-old male littermates. For MWM test, mice were trained in a circular pool with a diameter of 1.54 m, filled with milky water and maintained at 20°C. A circular platform (13 cm in diameter and 12.5 cm in height) was placed in the center of one quadrant (target quadrant) and hidden 1 cm beneath the water surface. Training for the hidden platform consisted of four trials each day for four consecutive days. During this period, mice were randomly released to three nontarget quadrants and were allowed to stand on the platform for 15 s before being transferred back to cages. The probe trial was performed on the 5th day in the MWM without a platform. The percentage of time spent by the mouse in each quadrant was recorded. Last, the visible platform, which was marked by a flag, was placed to ensure the swimming ability and visual acuity of the mice. For all of the trials, the maximal swimming duration was 1 min, and the intertrial interval was 1 min. The trajectories of the mice...
were recorded and analyzed using TrackMot (Singa Technology Corporation, Taiwan).

For the CFC test, a chamber with an electrified floor grid and a video camera (CleverSys FreezeScan) was used to measure the freezing response. Mice were placed in the chamber, and a 2-s, 0.5-mA foot shock was given every 2 min for four times. The mice were placed back into their cages 2 min after the final shock. Extinction trials were performed at 24 hours later in the same chamber. Each trial duration was 6 min, and the percentage of freezing was analyzed.

For the open field test, mice were individually placed in the apparatus consisting of four transparent Plexiglas arenas (480 mm by 480 mm by 350 mm) in which the floor and walls were covered with black paper to prevent any interference from movements of the neighboring mouse. Each mouse was released into a corner of the box and allowed to explore for 30 min. Mouse behaviors were recorded and analyzed using the ANY-maze software.

For the EPM test, a maze consisting of two open arms (30 cm by 5 cm) with a 1-cm (in height) ledge and two enclosed arms with 15-cm (in height) walls was elevated to a height of 50 cm above the floor. Mice were placed in the central area of the maze, facing one of the open arms. Mouse behaviors were recorded in a 5-min testing period and analyzed using the ANY-maze software.

For the NOR test, mice were individually habituated in a 48 cm by 48 cm by 35 cm open field for 5 min and then tested individually with two identical objects placed in the field. Each mouse was allowed to explore for 10 min with the objects present. After 24 hours, the mouse was tested in the object novelty recognition in which a novel object was replaced with one of the familiar objects. Mouse behaviors were video-recorded and analyzed using the ANY-maze software. Contact is defined by touching the object or staying within 0.5 cm of the object.

For the rotarod test, mice were pretrained twice a day for four consecutive days. For the pretrained trial, the mice were habituated to stay on the rod at a constant speed of 4 rpm for 60 s. On the day of testing, mice were kept in their home cages and acclimated to the testing room for at least 2 hours. For the testing phase, mice were placed on the accelerating rod with the speed from 4 to 40 rpm over a 300-s period for three test trials with 15-min intertrial intervals. The latency to fall of each mouse was recorded. Rotarod data were collected by averaging three trials.

Generation of the Usp11-floxed mice

The two loxp sequences were inserted into introns 1 and 9 of the Usp11 gene using CRISPR-Cas9 to generate the conditional allele. The guide RNA (gRNA) sequences used were 5′-CCGCGATACTAGCCGAGTG and 5′-TTATGAGTTAAGGGAGTG. A T7 promoter sequence (5′-TTAATACGACTCACTATA) was added upstream of the loxp sequence (uppercase) and Xho I site (uppercase and underlined) to disrupt the promoter. Mice behaviors were video-recorded and analyzed using the ANY-maze software. Contact is defined by touching the object or staying within 0.5 cm of the object.

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Calculation of cell cycle length with EdU-BrdU double labeling

Cell cycle length analysis essentially followed a previously described method (23). Briefly, the pregnant female mice were injected intraperitoneally with EdU at a dose of 100 μg/g body weight. Incorporated EdU was detected with the Click-iT EdU Kit (Roche).
Terminals deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end labeling assay
Apoptotic cells in the sections of embryonic brains were labeled with the In Situ Cell Death Detection Kit Fluorescein (Roche, Basel, Switzerland).

Immunofluorescence
Frozen sections were washed twice with filtered PBS and permeabilized with cytoskeleton (CSK) buffer containing 50 mM NaCl, 3 mM MgCl₂, 300 mM sucrose, 0.5% Triton X-100, and 10 mM Pipes (pH 6.8) for 20 min and blocked with PBS supplemented with 10% goat serum and 1% bovine serum albumin for 1 hour. Cells were incubated with primary antibody at 4°C overnight. The slides were washed three times with 1% PBS at room temperature and then incubated with FITC-conjugated secondary antibody (Invitrogen) together with DAPI (1 μg/ml) for 1 hour.

Microscopy and image analysis
Macro-view of stained sections was examined by an Olympus SZX16 Stereo microscope equipped with a ×10 objective lens (Olympus), and fluorescent images were captured by a DP80 digital camera with controller software (Analysis LS Research, Olympus). For microview, the sections were examined by a Leica SP5 Confocal Microscope equipped with a 40× or 60× objective lens (Leica), and images were captured by a cooled charge-coupled device camera operated by the Leica Application Suite Advanced Fluorescence Software. Fluorescence intensity was quantified by the ImageJ software. For quantifying cells in a brain section, an appropriate image width was chosen (IUE experiment: 300 μm; terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick end labeling assay: 1600 μm for E18.5, and 800 μm for E12.5 to E15.5; all other experiments: 1000 μm; for P7, 450 μm for E18.5, and 350 μm for E11.5 to E15.5), and the numbers or distribution of labeled cells in the entire cortical column were quantified.

RNA extraction, RT-PCR, and real-time PCR
Total RNA was extracted using Trizol reagent (Invitrogen). One microgram of total RNA was used for cDNA synthesis with the iScript reverse transcriptase (Bio-Rad). Real-time PCR was performed using the Power SYBR Green PCR Master Kit (Applied Biosystems) on a LightCycler 480 (Roche). The conditions for PCR analysis were 95°C for 15 min, 35 cycles at 94°C for 15 s, 55°C for 30 s, and 70°C for 30 s. Expression data were normalized to the mean of housekeeping gene glyceraldehyde-3-phosphate dehydrogenase. The primer sequences are as follows: Usp11: Fw 5′-AACAACAT-ACCGGACGAGGAT, Rv 5′-CCTTCCATGCTAGGCTTCC; Sox11: Fw 5′-CGACGGACCTGATGTCGACC, Rv 5′-GACAGG-GATAGGTCCCAGC; Pax6: Fw 5′-TGCGACAACACCTGCGTATG, Rv 5′-TGCAAGATGATGAGGTTCC; Tuj1: Fw 5′-AGGTCGCGTGGAACCGATT, Rv 5′-TG-TAGACCATTAGTGAGGTCA.

RNA interference
Lentivirus-based shRNA constructs were obtained from National RNAi Core Facility in Taiwan. Lentivirus generation and transduction were described previously (40). The target sequences of various shRNAs are as follows: shUspl1#1: 5′-GGGAGACCATAGAGCAGGAAA; shUspl1#2: 5′-CCAGACCTCTACAAATATGT; and shUspl1#3: 5′-CCTACTAGTGCTGATACTTT.

Deubiquitination assays
For in vitro deubiquitination, ubiquitinated Sox11 was purified from 293T cells transfected with Sox11-Flag and His-ubiquitin with M2 agarose beads (Sigma-Aldrich) and then eluted by Flag peptide. Myc-Usp11 or its mutant was affinity-purified with c-Myc agarose beads from transfected 293T cells and incubated with ubiquitinylated Sox11 in 40-μl deubiquitination reaction mix containing 100 mM Hepes (pH 7.4), 500 mM NaCl, 100 mM MgCl₂, 100 mM dithiothreitol, and 10 mM adenosine 5′-triphosphate at 37°C for 2 hours. Reaction products were analyzed by Western blot.

For in vivo deubiquitination, cells transfected with various constructs together with Sox11-Flag and His-ubiquitin were treated with 1 μM MG132 for 16 hours and then lysed with radiolabeled-precipitation assay lysis buffer containing 50 mM tris (pH 8.0), 0.15 M NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride (PMSF), aprotinin (1 μg/ml), and leupeptin (1 μg/ml). Lysates were used for immunoprecipitation with anti-Flag antibody, followed by Western blot with anti-His antibody. Alternatively, cells were lysed under denaturing conditions by buffer A [6 M guanidine-HCl, 0.1 M Na₂HPO₄/NaH₂PO₄ (pH 8.0), and 10 mM imidazole], and lysates were incubated with nickel-nitritoltriacetic acid (Ni-NTA) agarose for 2 hours at 4°C. The beads were washed twice with buffer A/TT [1 vol buffer A:3 vol buffer TI [25 mM tris-HCl (pH 6.8) and 20 mM imidazole]] and five times with buffer TI and then analyzed by Western blot. In all experiments, equal expression of His-ubiquitin was verified by Western blot.

Luciferase reporter assay
Cells were cotransfected with pGL3-based reporter construct and pCMV-Renilla. Forty hours after transfection, luciferase activity in cell lysate was assayed by the Dual Luciferase Reporter Assay System (Promega). The relative promoter activity was expressed as fold changes in firefly luciferase activity after normalization to the Renilla luciferase activity.

In utero electroporation
E12.5, E13.5, or E15.5 embryos were visualized through the uterus using a fiber-optic light source. DNA solution containing cDNA (1 μg/μl) and/or shRNA expression constructs together with CAG-mCherry plasmid (0.2 μg/μl) and 1% fast green (Sigma-Aldrich) was injected with a glass capillary into the ventricle of each embryo, and electroporation was performed with a CUY21 Electroporator (Nepa Gene) in a series of five square-wave current pulses (35 V, 100 ms × 5). Embryos were allowed to develop until E15.5 or E18.5 and were analyzed by direct visualization of the mCherry expression.

Sample preparation for ubiquitolytic or protease analyses
Cells treated with 5 μM MG132 for 1 hour or cortices dissected from E17.5 mouse embryos were lysed in buffer containing 8 M urea, 50 mM tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, aprotinin (2 μg/ml), leupeptin (10 μg/ml), and 1 mM PMSF. Lysates were reduced with 5 mM dithiothreitol (Sigma-Aldrich) at 37°C for 45 min followed by alkylation with 10 mM iodoacetamide (Sigma-Aldrich) for 30 min at room temperature in the dark. Lysates were diluted to 4 M urea with 50 mM tris-HCl (pH 8.0) and digested with Lys-C (WAKO) for 4 hours at 37°C. The peptide mixture was further diluted to 2 M urea and digested with trypsin (Promega) overnight at 37°C. The resulting peptides were acidified with trifluoroacetic acid,
desalted using a tC18 sep-Pak SPE cartridge or C18 Zip-Tip, and lyophilized.

**Quantitative ubiquitylome analysis using TMT labeling and LC-MS/MS**

For K-ε-GG peptide enrichment, lyophilized peptides were re-suspended in immunoaffinity purification (IAP) buffer (Cell Signaling Technology) and incubated with PTMScan Ubiquitin Remnant Motif (K-ε-GG) Antibody Bead Conjugate (Cell Signaling Technology) overnight at 4°C. The beads were washed twice with ice-cold IAP buffer, followed by washing three times with ice cold water. Ubiquitinated peptides were eluted with 80 µl of 0.15% trifluoroacetic acid twice. The eluent was cleaned up by C18 Stage Tips (Millipore) and then lyophilized.

For TMT labeling, peptides before and after K-ε-GG peptide enrichment were reconstituted in 100 mM triethyl ammonium bicarbonate and incubated with TMT-labeling reagent (0.8 mg TMT-129 or TMT-131 in 41 µl of anhydrous acetonitrile) for 1 hour at room temperature. The reaction was quenched by adding 8 µl of 4% hydroxyamine. The solution was cleaned up with C18 Zip-Tip and lyophilized.

After TMT labeling, peptides for proteome analysis were fractionated by strong cation exchange (SCX) chromatography. Briefly, peptides were resuspended in SCX buffer A [7 mM KH₂PO₄ (pH 2.65) and 30% acetonitrile] and separated on a PolySULFOETHYL A column (200 × 4.6 mm, 5 µm, 200 Å) using an Agilent 1100 system. A 160-min SCX gradient was used for separation at a flow rate of 1 ml/min.

The gradient consisted of a 20-min equilibration phase with 100% buffer A, a linear increase to 20% buffer B [7 mM KH₂PO₄ (pH 2.65), 350 mM KCl, and 30% acetonitrile] within 20 min, a second linear increase to 50% buffer B in 40 min, a third linear increase to 75% buffer B in 10 min, and 100% buffer B for 10 min.

NanoLC-MS/MS analysis was performed on a Thermo Fisher Scientific Ultimate 3000 RSLCnano system connected to a Thermo Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a PicoView nanospray interface (New Objective, Woburn, MA) and followed procedures as previously described (41). Briefly, peptide mixtures were loaded onto a 75-µm-ID, 25-cm-long PepMap C18 column (Thermo Fisher Scientific) packed with 2-µm particles with a pore width of 100 Å and were separated using a segmented gradient in 120 min from 5 to 35% solvent B (0.1% formic acid in acetonitrile) at a flow rate of 300 nl/min. Solvent A was 0.1% formic acid in water. The mass spectrometer was operated in the data-dependent mode. Briefly, survey scans of peptide precursors from 350 to 1600 m/z were performed at 240K resolution with a 2 × 10⁵ ion count target. Tandem MS was performed by isolation window at 1.6 Da with the quadrupole, HCD fragmentation with a normalized collision energy of 30, and rapid scan MS analysis in the ion trap. The MS² ion count target was set to 1 × 10⁵, and the maximum injection time was 50 ms. Only those precursors with charge states 2 to 6 were sampled for MS². The instrument was run in top speed mode with 3-s cycles, the dynamic exclusion duration was set to 15 s with a 10 ppm tolerance around the selected precursor and its isotopes, and monoisotopic precursor selection was turned on.

Label-free quantification was processed using the Progenesis QI for proteomics 4.1 (Nonlinear Dynamics Ltd., Newcastle, UK) with the default peak-picking settings. "Relative quantitation using non-conflicting peptides" setting was used, which calculates protein abundance in a run as the sum of all the unique peptide ion abundances corresponding to that protein. Peptide identification was performed using Mascot search engine (v.2.7.0; Matrix Science, Boston, MA, USA) against the Swiss-Prot Mouse database (17,046 entries total). Search criteria used were trypsin digestion, allowing up to two missed cleavages, and high confident peptides with a global false discovery rate (FDR) < 1% based on a target-decoy approach, and first-ranked peptides were included in the results. For quantification, the ratios of TMT reporter ion intensities in MS/MS spectra [mass/charge ratio (m/z) 129.14 and 131.14] from raw data sets were used to calculate fold changes between samples via the relative ratio to the reference pool. Only peptides unique for a given protein were considered for quantitation.

**Bioinformatics**

GO analysis was carried out using the online bioinformatics tool DAVID v6.8 (https://david.ncifcrf.gov/), and GO terms were filtered by P value <0.05.
Supplementary Materials

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