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Vps18 deficiency inhibits dendritogenesis in Purkinje cells by blocking the lysosomal degradation of Lysyl Oxidase

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ABSTRACT

Dendrite development occupies a central position in the formation of nervous system. However, whether lysosomal degradative function is required for dendritogenesis of neurons remains unknown. We have recently demonstrated the critical role of *Vps18* in the lysosomal degradation pathway in mice. Here, we report that *Vps18* deficiency severely blocks the dendrite development of Pukinje cells but not cerebral cortical neurons. Furthermore, we also demonstrate that the lysyl oxidase (Lox) protein is degraded through lysosome and accumulated in the *Vps18* deficient cerebellum but not in cerebral cortices. Our results suggest that lysosome regulates dendritogenesis of Purkinje cells though degrading Lox.

1. Introduction

The development of dendrites occupies a central position in the formation of nervous system. Purkinje cells, an important group of neurons in the cerebellum, have very large, planar, and highly branched dendritic trees. This unique morphology makes it an ideal model system to study dendrite growth and differentiation [1,2]. In rodents, the development of Purkinje cell dendrites begins shortly before birth and completes at about 4 weeks after birth [3]. Extensive studies of Purkinje cells have made great contributions to our understanding of the dendrite development process. Recently, the accumulation of lysyl oxidase (Lox) was found to block Purkinje cell dendrite development through inhibiting the NF-κB signaling pathway [4].

Previous studies illustrate the critical role of endocytosis system in dendrite development [5]. However, despite the report that synaptotagmin VII-regulated exocytosis of lysosomes was critical for neurite outgrowth [6–9], little is known about the function of lysosomes in dendritogenesis. In a mouse model of Sandhoff disease, a kind of lysosome storage disease, the neurite outgrowth of the dorsal root ganglion neurons is normal [10]. Therefore, one fundamental question that remains unanswered is whether the degradative function of lysosomes is required for dendrite development.

The Vps18 protein is a subunit of class C Vps complex, which also includes Vps11, Vps16, and Vps33 [11]. Extensive researches in lower eukaryotic organisms have shown that Vps-C complex plays essential roles in late endosome and lysosome-related vesicle transport pathways. Vps-C complex dysfunction results in the loss of vacuole structure and accumulation of autophagosomes and late endosomes in yeast [11,12]. Deletion of *Vps18* in yeast leads to the breakdown of the whole Vps-C complex, thus putting *Vps18* in a central position [13]. Mutating *Vps18* homolog, *dor*, in *Drosophila* causes the accumulation of exaggerated multivesicular structure in retinal cells, blockage of autophagosome-lysosme fusion in larval fat body, and promotion of tumor metastasis [14–16]. In mammalian cells, knockdown of *Vps18* or treatment of anti-Vps18 antibody blocks autophagosome–lysosome and early endosome fusion [17,18]. Neural-specific deletion of *Vps18* leads to severer neurodegeneration and disturbed neuronal migration in mice due to the blockage of multiple vesicle transport pathways to lysosome, including endocytosis, autophage, and biosynthetic pathways [19]. However, the function of the *Vps18* gene in dendrite development of neural cells is still unknown.

Here we report the critical function of *Vps18* in the dendrite development of Purkinje cells. Dendritogenesis of Purkinje cells in $Vps18^{F/F}$; Nestin-*Cre* mice is severely inhibited. However, the dendrites of cerebral cortical neurons are largely unaffected. Interestingly, despite server Purkinje cell loss in $Vps18^{F/F}$; Pcp2-*Cre* mice, the dendrite of remaining Purkinje cells developed normally. Finally, we demonstrate that the Lox protein is degraded through lysosome pathway and accumulates in the cerebellum of $Vps18^{F/F}$;

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Nestin-*Cre* mice, which may cause the dendrite defect of Purkinje cells. Our results suggest for the first time a critical function of lysosome degradation pathway in dendrite development.

2. Materials and methods

2.1. Animals

Vps18$^{F/F}$; Nestin-*Cre* mice, *Vps18*$^{+/galeo}$ mice and *Vps18*$^{F/F}$; Pcp2-*Cre* mice were described by C. Peng et al. [19]. All animal experiments were performed according to protocols approved by the Animal Care and Use Committee of the Institute of Developmental Biology and Molecular Medicine at Fudan University.

2.2. Western blot

Proteins were extracted with radioimmune precipitation assay (RIPA) buffer and resolved on SDS–PAGE followed by Western blotting with antibodies against Lox (sc-66948; Santa Cruz), c-Myc (sc-40; Santa Cruz) or GAPDH (KC-5G4; KangChen) as loading control.

2.3. Immunofluorescent staining analyses

The treatment of mouse brains for frozen section was described by C. Peng et al. [19]. Sections were then collected at 30 μm and stained with calbindin antibody (C9848; Sigma). Fluorescence micrographs were acquired using a Zeiss LSM710 confocal microscope.

2.4. In utero electroporation

In utero electroporation was carried out following standard protocols [20]. Plasmids expressing EYFP were injected into the lateral ventricles of mouse embryos at E14. Five days after electroporation, embryos were collected for analysis.

2.5. Quantification of dendrites

To calculate the total dendrite length, node or end number, and dendrite density (sholl analysis) of neural cells, 4–12 fields of Purkinje cells in the cerebellum (lobules V–VIII) or cerebral cortical neurons in layer II/III were randomly imaged by confocal microscopy (Zeiss Axiovert 100 M). Total dendrites were traced and analyzed by the Image J software with Neuron J plugin. Sholl analysis was performed as described by [4,21].

2.6. Behavioral tests

Behavioral tests were carried out on 6–11 months old *Vps18*$^{F/F}$; Pcp2-*Cre* mice and littermate controls, which were backcrossed C57BL6 for six generations.

2.6.1. Footprint test

Footprint analysis was carried out according to the protocol described [22]. Briefly, mouse hind paws were dipped in nontoxic ink, and mice were allowed to walk down a tunnel lined with white paper. The footprint patterns were analyzed and measured for gait width and stride length.

2.6.2. Dowel test

Mice were place on a horizontal wooden rod (9 mm diameter) and the latency time to fall was recorded up to a maximum of 3 min.

2.6.3. Accelerating rotating rod test

The accelerating rotating rod test was carried out as described [23]. Briefly, mice were placed on the rod of a rotating rod apparatus (Tianhuan Instruments), which accelerated from 4 to 40 rpm in 5 min linearly, and the time the mice stayed on the rod was measured.

2.7. Lox cDNA

Plasmid expressing Myc tagged Lox is a kind gift from Y. Eugene Chin (Institute of Health Sciences, Chinese Academy of Sciences).

2.8. Statistical analysis

Unpaired *t*-test was used. A value of $^{*}P < 0.05$, $^{**}P < 0.01$ or $^{***}P < 0.001$ denoted statistical significance.

3. Results

3.1. Suppression of the development of Purkinje cell dendrites in *Vps18*$^{F/F}$; Nestin-Cre mice

We have generated *Vps18*$^{F/F}$; Nestin-*Cre* mice [referred to as *Vps18* CKO (conditional knock out)], in which exon 3 and 4 of the *Vps18* gene were specifically deleted in neural cells [19]. To understand the function of the lysosome degradation pathway in the dendrite development of neural cells, we evaluated the effects of *Vps18* deficiency on dendrite development of Purkinje cells in *Vps18* CKO and *Vps18*$^{F/F}$ (referred to as Ctrl) mice by immunofluorecent staining with Calbindin antibody. Our results revealed that the dendrite of *Vps18* deficient Purkinje cells was shorter and less branched (Fig. 1A and B). Quantitative analysis showed that Purkinje cell dendrites in *Vps18* CKO mice had a shorter total length and significantly reduced numbers of nodes and ends (Fig. 1C). Sholl analysis [24], which determines the number of crossings between dendrite and concentric circles, showed that the dendrite density of Purkinje cells in *Vps18* CKO mice was greatly reduced compared with that of the control (Fig. 1D). These results indicate that *Vps18* plays a critical role in the development of Purkinje cell dendrites.

3.2. Vps18 deficiency does not affect dendritic morphogenesis in the cerebral cortex

Given the morphological defects observed in Purkinje cells, we questioned whether the effect of *Vps18* deficiency on dendritogenesis was Purkinje cell-specific or ubiquitous. We evaluated the dendritic development of cerebral cortical neurons by performing in utero electroporation with the plasmid pEYFP at E14 and analyzing dendritogenesis of EYFP-labeled neurons at E19. The dendrite morphology of cortical neurons in layer II/III of *Vps18* CKO mice looked similar to that of control mice (Fig. 2A and B). Quantitative analyses showed that total node or end numbers and total dendrite lengths per cell in *Vps18* CKO and control cerebral cortices were comparable (Fig. 2C), suggesting that *Vps18* is not essential for dendrite development in cerebral cortex at least during embryonic stage.

3.3. Purkinje cell dendrite developed normally in *Vps18*$^{F/F}$; Pcp2-Cre mice

Development of Purkinje cell dendrites begins shortly before birth and completes at about 4 weeks after birth [3]. Severe impairment of the development of Purkinje cell dendrites in *Vps18* CKO mice at P10 suggests that *Vps18* is required for early stage den-

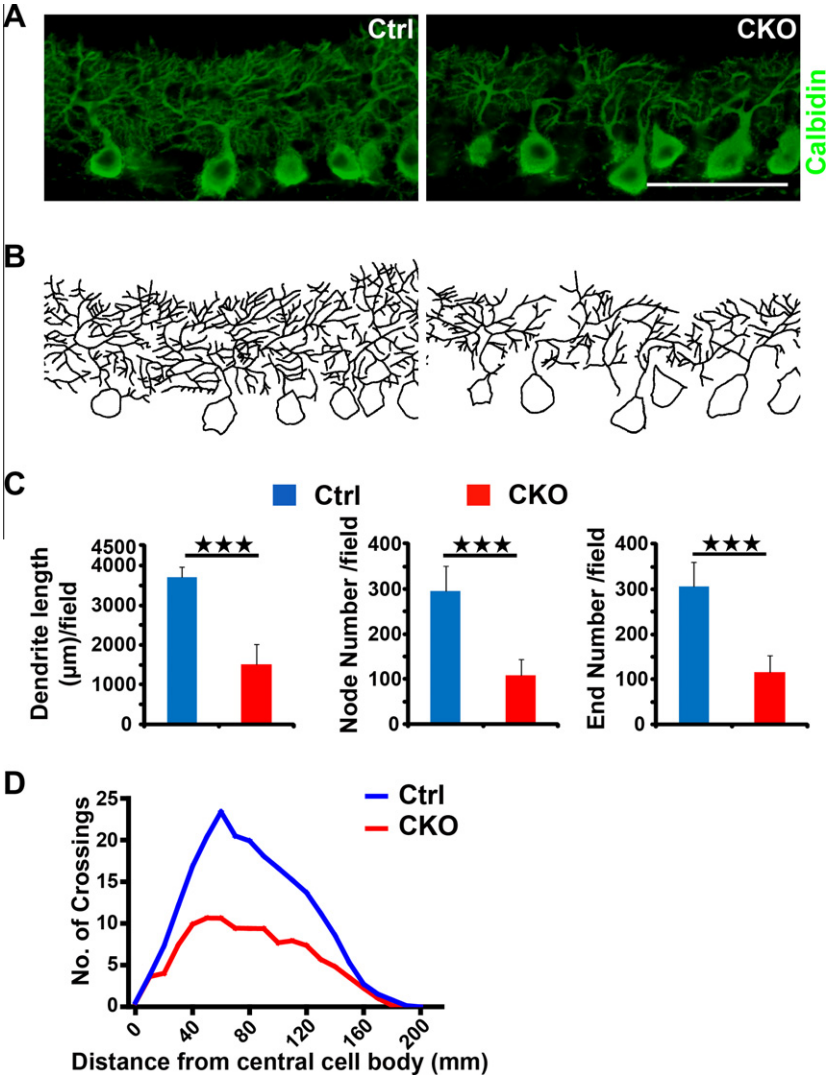

Fig. 1. *Vps18* deficiency impairs Purkinje cell dendritogenesis. (A and B) Representative images of P10 Purkinje cells in cryosections of cerebellum stained with anti-calbindin (A) and traced with Image J (B). (C) Quantitative analysis of total node or end number and total dendrite length per field in P10 *Vps18* CKO cerebellar cortices. (D) Sholl analysis showed total intersection numbers of Purkinje cell dendrites with each concentric circle (10 μm, 20 μm, . . .200 μm) and peak values representing areas with high dendritic density. The result illustrates a substantial reduction in dendritic density of P10 *Vps18* CKO Purkinje cells. ***P value < 0.001. Values represent the means ± SEM. (n = 12; Scale bar: 50 μm.).

dritogenesis, but it provides no clue whether *Vps18* also functions in late stage development and maintenance of Purkinje cell dendrites. Because *Vps18* CKO mice died by postnatal day 12 [19], we addressed the question using $Vps18^{F/F}$; Pcp2-*Cre* mice.

Unlike *Vps18* CKO mice, $Vps18^{F/F}$; Pcp2-*Cre* mice can live up to at least 12 months (unpublished observation). However, the mice displayed abnormal behavior as early as 3 months old. We carried out behavior tests on 6 to 11 month old $Vps18^{F/F}$; Pcp2-*Cre* mice and littermate controls. Our results showed that $Vps18^{F/F}$; Pcp2-*Cre* mice displayed a severe impairment of performance on the rotarod apparatus and static rod, compared to littermate controls (Fig. 3A and B). Analysis of the footprint pattern showed that both the stride length and gait width were also shorter for $Vps18^{F/F}$; Pcp2-*Cre* mice (Fig. 3C and D). These results demonstrate an impairment of the balance and coordination ability of $Vps18^{F/F}$; Pcp2-*Cre* mice, indicating the disturbance of Purkinje cell functions, which may result from loss and/or dendrite defects of Purkinje cells in $Vps18^{F/F}$; Pcp2-*Cre* mice.

We next assessed the dendrite development of Purkinje cells in $Vps18^{F/F}$; *Pcp2-Cre* mice at the age of 3 months by immunofluorescent staining with anti-calbindin. Surprisingly, we found that the dendrite of the remaining Purkinje cells looked quite normal (Fig. 3E and F), although most Purkinje cells were lost in the mutant mice [19]. Quantitative analyses showed that the total note and end numbers and total length of Purkinje cell dendrites in $Vps18^{F/F}$; Pcp2-*Cre* mice were all similar to that in littermate controls, although sholl analysis illustrated a slight decrease of dendrite density in mutant mice (Fig. 3G and H). These results indicate that *Vps18* has little if any function in late stage development or maintenance of Purkinje cell dendrites. However, we cannot completely rule out other possibilities currently (see Discussion).

3.4. Lox protein accumulates in the Vps18 CKO cerebellum

Since *Vps18* plays a critical role in the lysosome degradation pathway [11,19], the defects of Purkinje cell dendrite in *Vps18* CKO mice may be caused by disturbed degradation of proteins that are important for dendrite development. Recent research has demonstrated that the accumulation of Lox in Purkinje cells impedes

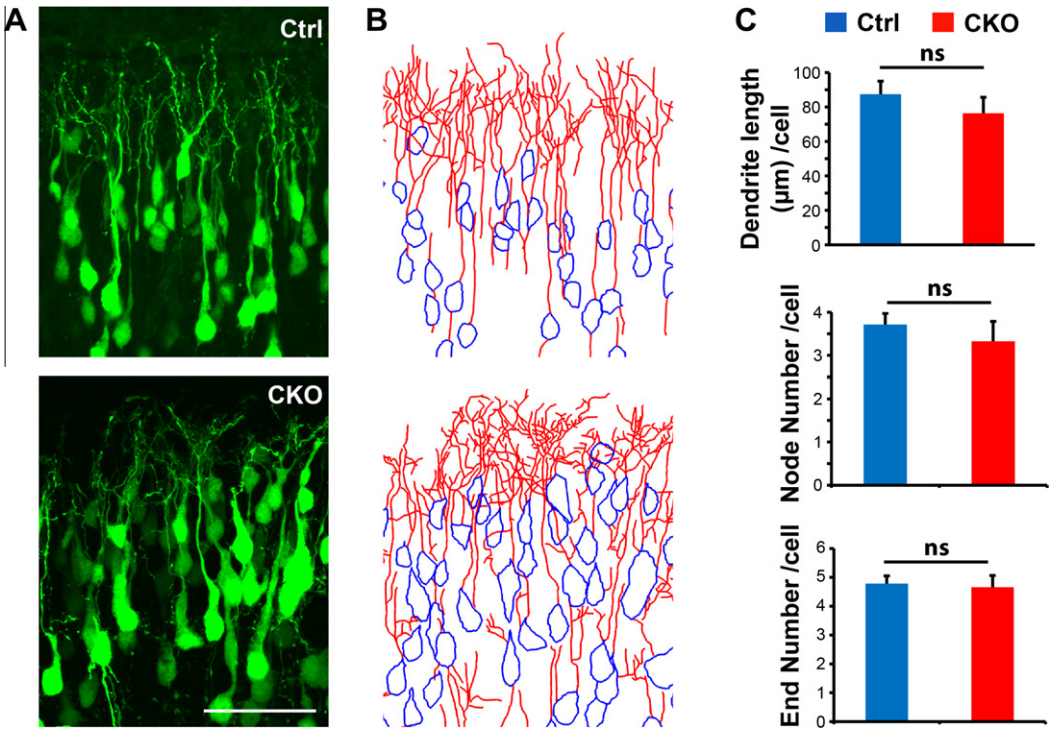

Fig. 2. *Vps18* deficiency dose not inhibit dendritogenesis of the cerebral cortical neurons. (A and B) Representative images of control or *Vps18* deficient cortical neurons in layer II/III transfected with plasmid expressing EYFP (A) and traced with Image J (B). (C) Quantitative analysis of total node or end number and total dendrite length per cell in the cerebral cortices of E19 *Vps18* CKO and control mice. ns, no significance. Values represent the means ± SEM. ($n = 4$; Scale bar: 50 μm).

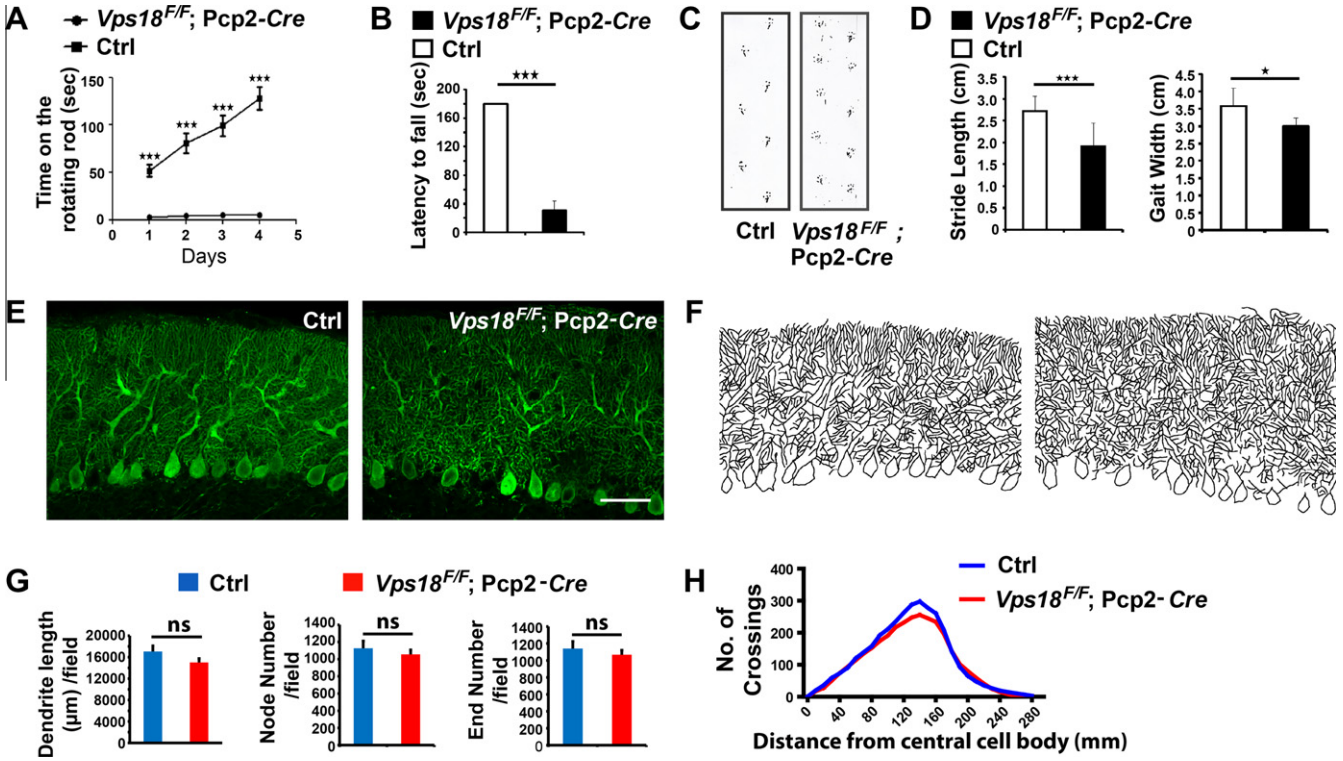

Fig. 3. The effects of *Vps18* deficiency on behavior and Purkinje cell dendrite development of $Vps18^{F/F}$; Pcp2-*Cre* mice. (A) Poor performance of $Vps18^{F/F}$; Pcp2-*Cre* mice on the rotating rod. $Vps18^{F/F}$; Pcp2-*Cre* mice and littermate controls were tested for endurance to stay on the rotating rod with four trials per day for four consecutive days. (B) Static rod performance of $Vps18^{F/F}$; Pcp2-*Cre* mice and littermate controls. (C and D) Abnormal gait in $Vps18^{F/F}$; Pcp2-*Cre* mice. Representative footprint patterns of the $Vps18^{F/F}$; Pcp2-*Cre* mice and littermate controls (C). Statistics of stride length and gait width (D). (E and F) Images of Purkinje cells from 3 months old $Vps18^{F/F}$; Pcp2-*Cre* and littermate control mice stained with anti-calbindin (E) and traced with Image J (F). (G) Quantitative analysis of total node or end number and total dendrite length per field of Purkinje cells in the $Vps18^{F/F}$; Pcp2-*Cre* and control mice. (H) Sholl analysis showed total intersection numbers of Purkinje cell dendrites with each concentric circle (10 μm, 20 μm,…280 μm) and peak values representing areas with high dendritic density. ns, no significance. $^{*}P$ value < 0.05, $^{***}P$ value < 0.001. Values represent the means ± SEM. (For all behavior tests $n = 8$; for quantitative analysis of dendrites $n = 6$; Scale bar: 50 μm.).

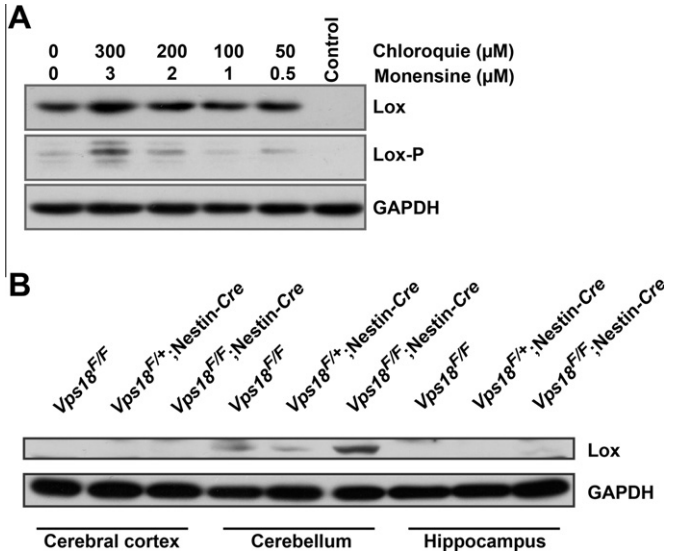

Fig. 4. Accumulation of Lox in the *Vps18* CKO cerebellum. (A) The Lox protein was degraded through the lysosome. 293T cells transfected with myc-tagged human *Lox* gene or *RFP* (control) were treated with lysome inhibitors, chloroquie and monensine, at indicated concentrations for 24 h and analyzed by Western blot with anti-myc antibody. (B) Western blot analysis showed the restricted expression of the Lox protein in P10 mouse cerebellum and the accumulation in *Vps18* CKO cerebellums.

dendrite development [4]. Therefore, we hypothesized that the blockage of lysosome function resulted from *Vps18* deficiency might lead to accumulation of Lox and then interfere with Purkinje cell arborization. To test this hypothesis, we first examined if Lox could be degraded through the lysosome. 293T cells were transfected with myc-tagged human *Lox*, which will yield two forms of Lox proteins (full length Lox and Lox propeptide) in cells, and treated with lysosome inhibitors. Our results showed that both full length Lox and Lox propeptide (Lox-P) were accumulated by co-treatment of chloroquine and monensine (Fig. 4A), two lysosome inhibitors [16], indicating that Lox is degraded through lysosome. Then, we further evaluated the expression of Lox *in vivo* by Western blot and found that the level of Lox proteins in cerebellum was much higher than those in hippocampus and cerebral cortex (Fig. 4B). More importantly, we found that the level of Lox protein was increased in the *Vps18* deficient cerebellum (Fig. 4B), suggesting that *Vps18* deficiency-mediated accumulation of Lox may contribute to the underdevelopment of dendrites of *Vps18* deficient Purkinje cells.

4. Discussion

Although the cellular and molecular mechanisms of dendrite development in neural cells have been extensively studied, the function of lysosomes in this process remains largely unknown. Particularly, it is still not clear whether the degradative function of lysosomes plays a role in dendrite development. Here, we report the characterization of the dendrite development in Purkinje and cerebral cortical neurons in the *Vps18* CKO mice. We found that dendritogenesis was impaired in the Purkinje cells of the *Vps18* CKO mice but appeared normal in cerebral cortical neurons at least during embryonic development. We also found that Purkinje cell dendrites were normal in $Vps18^{F/F}$; Pcp2-*Cre* mice. Furthermore, we demonstrated that Lox was degraded though lysosomes, and Lox protein accumulated in the cerebellum but not in the hippocampus or cerebral cortex of *Vps18* CKO mice. Our results provide the first link between lysosome degradative function and dendrite development, and indicate that lysosome may regulate the dendrite development of Purkinje cells though degradation of Lox.

The buff mutant mouse carrying a *Vps33a* point mutation displays reduced Purkinje cell number and smaller size of cerebellum in older age. But whether *Vps33a* mutation affects dendrite development of Purkinje cells or other neurons remains unknown [25,26]. Therefore, our research represents the first report about the function of Class C Vps complex in neuronal dendrite development.

In *Vps18* CKO mice, *Vps18* deficiency severely impairs dendrite outgrowth of Purkinje cells, but have no obvious effect on dendrite development of cerebral cortical neurons at least during the embryonic stage. This difference may be caused by the differential expression of *Lox* in cerebral cortex and cerebellum since our result showed that the expression level of Lox was much higher in the latter.

Although *Vps18* deletion in $Vps18^{F/F}$; Pcp2-*Cre* mice lead to severe loss of Purkinje cells at the age of 3 months [19] and complete loss at the age of 10 months (data not shown), we surprisingly found that it had no obvious effect on the dendrite morphology of Purkinje cells in $Vps18^{F/F}$; Pcp2-*Cre* mice. There are several possible explanations for this result. The first explanation is that *Vps18* is required for early stage dendritogenesis but not for the late stage development or maintenance of dendrite. The second explanation is that the protein level of Lox is not accumulated high enough to impair the dendrite development and maintenance in $Vps18^{F/F}$; Pcp2-*Cre* mice because (1) *Vps18* is deleted much later (starting from P6) in $Vps18^{F/F}$; Pcp2-*Cre* mice than what happens in the *Vps18* CKO mice (starting from E11); and (2) Lox expression decreases with CNS development and maturation [4]. The third explanation is that the *Vps18* gene is not completely deleted in the Purkinje cells of 3 months old mutant mice although a previous study has demonstrated that in Pcp2-*Cre* mice the *Cre*-mediated recombination is fully established 2–3 weeks after birth [27]. Further extensive experiments are needed to distinguish these possibilities.

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