

# Critical role of JSAP1 and JLP in axonal transport in the cerebellar Purkinje cells of mice

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**Critical role of JSAP1 and JLP in axonal transport in the cerebellar  
Purkinje cells of mice**

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## **Abstract**

JSAP1 and JLP are structurally related scaffolding proteins that are highly expressed in the brain. Here, we found that JSAP1 and JLP play functionally redundant and essential roles in mouse cerebellar Purkinje cell (PC) survival. Mice containing PCs with deletions in both JSAP1 and JLP exhibited PC axonal dystrophy, followed by gradual, progressive neuronal loss. Kinesin-1 cargoes accumulated selectively in the swollen axons of *Jsap1/Jlp*-deficient PCs. In addition, autophagy inactivation in these mice markedly accelerated PC degeneration. These findings suggest that JSAP1 and JLP play critical roles in kinesin-1-dependent axonal transport, which prevents brain neuronal degeneration.

## **Keywords**

Autophagy; Axonal swelling; Kinesin-1; Neurodegeneration; Ubiquitin

## **Abbreviations**

APP, amyloid precursor protein; cDKO, conditional double KO; cKO, conditional KO; cTKO, conditional triple KO; Cyt c, cytochrome c; DAPI, 4,6-diamidino-2-phenylindole; DCN, deep cerebellar nuclei; floxed, *loxP*-flanked; GFAP, glial fibrillary acidic protein; HRP, horseradish peroxidase; IHC, immunohistochemistry; JIP, JNK-interacting protein; JLP, JNK-associated leucine zipper protein; JNK, c-Jun NH<sub>2</sub>-terminal kinase; JSAP1, JNK/stress-activated protein kinase-associated protein 1; KHC, kinesin heavy chain; KLC, kinesin light chain; KO, knockout; MAPK, mitogen-activated protein kinase; PC, Purkinje cell; Phospho, phosphorylated; SYP, synaptophysin; SYT1, synaptotagmin 1

## 1. Introduction

Mitogen-activated protein kinase (MAPK) cascades transmit signals through protein-phosphorylation relays. The specificity of MAPK signaling is mediated, at least in part, by scaffolding proteins, such as c-Jun NH<sub>2</sub>-terminal kinase (JNK)-interacting protein 1 (JIP1) and JNK/stress-activated protein kinase-associated protein 1 (JSAP1, also known as JIP3 or Sunday Driver) [1-6]. Recent *in vitro* studies have suggested that JSAP1 and the structurally related JNK-associated leucine zipper protein (JLP, also known as JIP4 or SPAG9) [7-9] may be multifunctional proteins involved in diverse cellular processes, such as cell signaling, intracellular transport, and cytokinesis. JSAP1 and JLP were first identified as scaffolding proteins for mammalian JNK and p38 MAPK signaling modules [4,5,7,8]. They were subsequently shown to interact with the kinesin light chain (KLC) subunit of kinesin-1 (which contains two KLCs and two kinesin heavy chains [KHCs]), and were suggested to function as adaptor proteins that link cargoes and kinesin-1 [10,11]. JSAP1 also plays important roles in neuronal morphogenesis, including axonal elongation (through its interaction with KHC) [12,13], and branching [14,15]. Furthermore, in cycling cells, JSAP1 and JLP have been suggested to function as either effectors [16] or regulators of the subcellular localization [17] of the small GTPase, ADP-ribosylation factor 6, during cytokinesis. In some cases, such as axonal transport [18] and cytokinesis [17], JSAP1 and JLP have overlapping functions.

JSAP1 and JLP are highly expressed in the brain, and recent studies involving mouse knockouts have begun to uncover their physiological roles. *Jsap1* knockout (KO) mice die shortly after birth, most likely due to neural defects [19-21]. These mice exhibit various developmental brain defects, including an axon guidance defect of the corpus callosum, although abnormal vesicle accumulation is not observed in the axons [19,20]. In contrast, *Jlp* KO mice are viable and grow normally, exhibiting a lightened

coat color and pale skin [22,23]. These deleterious and nearly normal phenotypes of *Jsap1* and *Jlp* KO mice, respectively, may hinder the functional analysis of JSAP1 and JLP *in vivo*. Recently, however, the conditional double KO (cDKO) of both *Jsap1* and *Jlp* revealed that they play crucial and functionally redundant roles in the developing mouse brain [18].

In this study, we show that JSAP1 and JLP play essential and functionally redundant roles in cerebellar Purkinje cell (PC) survival in mice, and that their deletion causes axonal degeneration followed by gradual, progressive neuronal loss. Our findings also indicated that JSAP1 and JLP regulate kinesin-1-dependent axonal transport in PCs, and that disruption of this process leads to neurodegeneration. Furthermore, we found that the deletion of JSAP1 and JLP in PCs resulted in the activation of macroautophagy (hereafter referred to as autophagy), which appeared to be a mechanism for controlling the extent of neurodegeneration in these mice.

## 2. Materials and Methods

### 2.1. Animals

*Jsap1<sup>ff</sup>* mice [21], *Jlp<sup>ff</sup>* mice [18], and *Atg5<sup>ff</sup>* mice [24] were generated previously. To generate PC-specific conditional knockout (cKO) mice, *Pcp2-Cre* transgenic mice [25] were obtained from the Jackson Laboratory and crossed with *Jsap1<sup>ff</sup>*, *Jlp<sup>ff</sup>*, and/or *Atg5<sup>ff</sup>* mice. The animal experiments were conducted according to the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology of Japan, and approved by the Committee on Animal Experimentation of Kanazawa University.

### 2.2. Antibodies

The following primary antibodies were used for this study: anti-JSAP1 (1 µg/ml) [26], anti-JLP (2 µg/ml) [22], anti-calbindin D-28K (1:2,000; C9848, Sigma-Aldrich, St. Louis, MO, USA or 1:1,000; AB1778, Millipore, Bedford, MA, USA), anti-amyloid precursor protein (APP) (1:50; MAB348, Millipore), anti-cytochrome c (Cyt c) (1:500; #556432, BD Biosciences, San Jose, CA, USA), anti-synaptophysin (SYP) (1:200; S5768, Sigma-Aldrich), anti-synaptotagmin 1 (SYT1) (1:100; #105002, Synaptic Systems, Göttingen, Germany), anti-ubiquitin (1:100; Z0458, Dako, Glostrup, Denmark), anti-glial fibrillary acidic protein (GFAP) (1:500; G3893, Sigma-Aldrich), anti-LC3 (1:5,000; #010-22841, Wako, Osaka, Japan), anti-phosphorylated (Phospho) JNK (1:100; #9251, Cell Signaling, Boston, MA, USA), and anti- $\alpha$ -tubulin (1:3,000; T5168, Sigma-Aldrich) antibodies. The following secondary antibodies were used: Alexa 488- and 568-conjugated anti-mouse or anti-rabbit IgG (1:1,000; Invitrogen,

Rockville, MD, USA) and horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit IgG (1:5,000; GE Healthcare, Buckinghamshire, UK) antibodies.

### *2.3. Immunohistochemistry*

Mice were deeply anesthetized and fixed by transcardial perfusion with 4% paraformaldehyde in 0.1 M sodium phosphate buffer pH 7.4. Then, brain samples were collected and post-fixed overnight at 4°C. The samples were cryoprotected in 30% sucrose and embedded in Tissue-Tek OCT compound (Sakura Finetek, Kyoto, Japan). Immunohistochemistry (IHC) was performed as previously described [27]. The nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich). IHC images were acquired with a confocal laser scanning microscope (LSM510 META, Carl Zeiss, Oberkochen, Germany) with a 20× or 40× objective lens. To generate Z-stacking images, ten single-plane images (2-μm optical thickness) were acquired at 2-μm intervals. To capture overviews of the cerebellum, a fluorescent microscope with a 10× objective lens (BZ-9000, Keyence, Osaka, Japan) was used, and the images were combined using the image-joiner function in the BZ-Analyzer software (Keyence). All images were captured below saturation level.

### *2.4. Quantification of PC numbers*

Two midsagittal 25-μm-thick frozen serial sections were prepared from control, single *Jsap1* or *Jlp* cKO, and *Jsap1:Jlp* cDKO mice as described above. The sections were then stained with the Nissl stain, cresyl violet. Optical images of Nissl-stained lobules (II to IX) were captured using an inverted microscope with a 20× objective lens (IX71, Olympus, Tokyo, Japan), attached to a CCD camera (DP50, Olympus). The Nissl-stained PCs in the lobules were then counted. Segments connecting the center of

the soma of each PC along the entire length of the PC layer were created, and the sum of their lengths was determined. Using this length, the average number of PCs per  $\mu\text{m}$  was calculated from two serial sections per mouse.

### *2.5. Immunoblotting*

Cerebellar total cell lysates were prepared and analyzed by immunoblotting as described previously [27]. Protein bands were detected with the Immobilon Western Chemiluminescence HRP Substrate (Millipore). Image J software was used to quantify the intensity of the protein bands.

### *2.6. Rotarod analysis*

To investigate motor coordination and balance, an accelerating rotarod analysis was performed using 8- to 9-week-old mice. The mice were placed on a horizontal rubber-coated rod (30-mm diameter) and tested in three trials with a 300-sec accelerating program (from 5 to 40 rpm) using a rotarod (RRAC-3002; O'Hara & Co. Ltd., Tokyo, Japan). The length of time each mouse remained on the rod before falling (retention time) was measured. The average retention time for each mouse was calculated from the results of three trials.

### *2.7. Statistical analysis*

Significance was determined using the two-tailed unpaired Student's *t-test*. *P* values < 0.05 were considered to be statistically significant.



### 3. Results

#### 3.1. Targeted deletion of *Jsap1* and *Jlp* in PCs

To investigate the role of JSAP1 and JLP in cerebellar PCs, we generated mice with PC-targeted conditional deletions in *Jsap1*, *Jlp*, or both, using *loxP*-flanked (floxed) alleles of these genes, in combination with the *Pcp2-Cre* transgene. PC-specific deletion of *Jsap1* and *Jlp* was confirmed by the immunohistochemical analysis of 4-week-old control (*Jsap1<sup>ff</sup>:Jlp<sup>ff</sup>*) and cDKO (*Jsap1<sup>ff</sup>:Jlp<sup>ff</sup>:Pcp2-Cre*) mice (Fig. 1A). Notably, both the control and cDKO mice exhibited intense JSAP1-positive immunosignals in the cerebellar pinceau (see Fig. 1A) [26], clustered axons and terminals of basket cells, indicating the specificity of the *Pcp2* promoter-driven Cre expression. Histological analysis of 24-week-old control, single *Jsap1* or *Jlp* cKO, and *Jsap1:Jlp* cDKO mice revealed a specific loss of PCs in the *Jsap1:Jlp* cDKO mice (Fig. 1B). Quantitative analysis showed that the cDKO mice, but not the single *Jsap1* or *Jlp* cKO mice, exhibited a significantly reduced number of PCs compared with control mice (Fig. 1C). In addition, swollen PC axons were observed in the cDKO mice, but not in the single *Jsap1* or *Jlp* cKO mice (Fig. 1B). Taken together, these results indicated that JSAP1 and JLP play functionally redundant and crucial roles in PC survival, and are likely to play important roles in PC axonal transport.

#### 3.2. Deletion of JSAP1 and JLP in PCs causes axonal dystrophy and progressive neuronal loss

Next, we analyzed control and *Jsap1:Jlp* cDKO mice at different ages (Fig. 2). Midsagittal sections from mice at 8, 12, 16, 24, and 40 weeks of age were stained with Nissl, and the PCs were counted. At 8 weeks of age, the PC numbers were comparable

between the control and *Jsap1:Jlp* cDKO mice (Fig. 2A). However, the cDKO mice older than 8 weeks exhibited significantly decreased PC numbers compared to control mice, and the degree of neuronal loss increased with increasing age (Fig. 2A). In addition, immunostaining for GFAP revealed the presence of reactive gliosis, a hallmark of central nervous system injury, in association with PC death in the *Jsap1:Jlp* cDKO mice (Supplementary Fig. 1). We also examined the PC axons of the cDKO mice at different ages by IHC, using an antibody against calbindin (a PC marker). Many dilated axons were observed in regions near the PC bodies of the cDKO mice at all ages examined, including 8 weeks of age, when no PC loss was detected (Fig. 2B). Furthermore, hypertrophic PC axons were found in the deep cerebellar nuclei (DCN) of *Jsap1:Jlp* cDKO mice at all ages examined, and the PC axon numbers in the DCN were noticeably decreased at 16 and 24 weeks of age (Fig. 2C). Taken together, these data indicated that the loss of JSAP1 and JLP in PCs causes axonal dystrophy followed by progressive PC degeneration.

### *3.3. Selective accumulation of kinesin-1 cargoes in the swollen axons of Jsap1- and Jlp-deficient PCs*

Since JSAP1 and JLP are known to interact with kinesin-1, the axonal dystrophy in *Jsap1:Jlp* cDKO mice might have been due to defects in kinesin-1-dependent axonal transport. To examine this possibility, we analyzed the PC swollen axons in 8-week-old *Jsap1:Jlp* cDKO mice by double-immunostaining, using antibodies against calbindin and either APP, Cyt c (a mitochondrial protein), SYP, or SYT1 (Fig. 3). APP and mitochondria are kinesin-1 cargoes, and SYP and SYT1 are kinesin-3 cargoes [28]. Intense kinesin-1 cargo signals were present throughout almost all of the swollen axons, whereas the kinesin-3 cargo intensities were moderate or very low with limited distribution. These results suggested that JSAP1 and JLP play important roles in

kinesin-1-dependent axonal transport in PCs, and that their absence causes PC axonal dystrophy.

In addition, we examined whether the double KO affected the activation of JNK, by IHC using an antibody against Phospho-JNK. Substantial JNK activation was observed in the PC swollen axons of 8-week-old *Jsap1:Jlp* cDKO mice (Supplementary Fig. 2), similar to the observation in *Jsap1:Jlp* double KO primary cultured neurons [18].

### *3.4. Jsap1 and Jlp deficiency in PCs causes increased autophagy, which controls the extent of PC degeneration*

Defective axonal transport in *Jsap1:Jlp* cDKO mice may induce protein aggregation. Indeed, IHC with an antibody against ubiquitin, a marker of misfolded proteins, revealed the presence of protein aggregates in the PC cell bodies of cDKO mice (Fig. 4A). We then asked whether *Jsap1*- and *Jlp*-deficient PCs undergo increased autophagy. Immunoblotting analysis of the cerebellar cell lysates from *Jsap1:Jlp* cDKO mice showed significant increases in the LC3-II level and LC3-II/LC3-I ratio, compared with those of control mice, indicating the increased presence of cells undergoing autophagy (Fig. 4B and C). Finally, we assessed the effect of autophagy on PC degeneration. To this end, we generated mice with PC-specific triple deletions in *Jsap1*, *Jlp*, and *Atg5*, an essential autophagy gene, as well as mice with a PC-specific single deletion in *Atg5*, using the *Pcp2-Cre* transgene. We noticed that the conditional triple KO (cTKO) mice (*Jsap1<sup>fl/fl</sup>:Jlp<sup>fl/fl</sup>:Atg5<sup>fl/fl</sup>:Pcp2-Cre*), but not single *Atg5* cKO or *Jsap1:Jlp* cDKO mice, exhibited abnormal gait at 8 weeks of age (Supplementary Videos 1-4). In addition, rotarod analysis showed that *Jsap1:Jlp:Atg5* cTKO mice exhibited reduced motor coordination and balance compared to control, single *Atg5* cKO, and *Jsap1:Jlp* cDKO mice (Supplementary Fig. 3). Furthermore, calbindin immunostaining demonstrated

severe PC loss in *Jsap1:Jlp:Atg5* cTKO mice, but not in the single cKO or the cDKO mice, at 8 weeks of age (Fig. 4D). These data indicated that *Jsap1* and *Jlp* deficiency in PCs leads to increased autophagy, which suppresses the JSAP1 and JLP depletion-induced PC degeneration.

#### 4. Discussion

In the present study, we demonstrated that JSAP1 and JLP play crucial and functionally redundant roles in mouse cerebellar PC survival. These findings are consistent with our previous observation that mice with dorsal telencephalon-specific double deletions in *Jsap1* and *Jlp* exhibit neurodegeneration in the developing brain [18]. Thus, JSAP1 and JLP may play overlapping, essential roles in neurons throughout the brain. Although *in vitro* studies have indicated that JSAP1 and JLP are multifunctional proteins involved in cellular signaling, intracellular transport, and cytokinesis, their physiological roles *in vivo* have remained unclear. Here we propose that JSAP1 and JLP function as regulators of kinesin-1-dependent intracellular transport in PCs and that their absence leads to axonal degeneration and neuronal death. The following lines of evidence support this proposal: 1) dystrophic PC axons were detected in *Jsap1:Jlp* cDKO mice at 8 weeks of age or older (Fig. 2B), 2) kinesin-1 cargoes accumulated selectively in the swollen axons of PCs in the cDKO mice (Fig. 3), 3) ubiquitin-positive aggregates were present in PC bodies in the cDKO mice (Fig. 4A), 4) fewer PC terminals impinging on DCN neurons were found in the cDKO mice (Fig. 2C), and 5) reduced PC numbers were detected at 12 weeks of age in the cDKO mice, and progressively decreased with age (Fig. 2A).

Emerging evidence indicates that autophagy has two opposing roles in the nervous system [29,30]. In this study, we demonstrated that autophagy played a neuroprotective role in *Jsap1:Jlp* cDKO mice, although the mechanism by which autophagy is activated

in these mice is currently unclear. Our results indicated that autophagy was increased in *Jsap1:Jlp* cDKO mice at 8 weeks of age (Fig. 4B and C), at a time when PC neuronal loss was not detected. Although JNK is reported to function as a negative regulator of autophagy in neurons [31], we observed substantial JNK activation in the PC swollen axons of 8-week-old *Jsap1:Jlp* cDKO mice (Supplementary Fig. 2). Therefore, it is unlikely that reduced JNK activity in the cDKO mice leads to autophagy activation. It is possible that the defective mitochondrial transport in *Jsap1:Jlp* cDKO PC axons results in decreased levels of local intra-axonal ATP [32], which induces autophagy activation through the AMP-activated protein kinase signaling pathway [33].

In summary, the results of our *in vivo* study suggest that JSAP1 and JLP regulate kinesin-1-dependent axonal transport in the brain, which prevents axonal degeneration and neuronal death. *Jsap1:Jlp* cDKO mice may serve as a useful animal model for studying the molecular mechanisms of neurodegenerative disorders and for developing therapeutic drugs for these diseases.

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## Figure legends

### Fig. 1. Targeted deletion of *Jsap1* and *Jlp* in PCs.

(A) IHC analysis of *Jsap1<sup>ff</sup>:Jlp<sup>ff</sup>* (control) and *Jsap1:Jlp* cDKO mice. Midsagittal sections from 4-week-old control and cDKO mice were stained with anti-JSAP1 and anti-JLP antibodies. Higher magnifications of the boxed areas are shown in the panels to the right. PC bodies are outlined by dotted lines. (B) Axonal swelling and PC loss in 24-week-old *Jsap1:Jlp* cDKO mice. Midsagittal sections of control, single *Jsap1* or *Jlp* cKO, and *Jsap1:Jlp* cDKO mice were stained with an anti-calbindin antibody. (C) Quantification of PCs. Midsagittal sections from the indicated mice at 24 weeks of age were stained with Nissl and the PCs per  $\mu\text{m}$  in lobules II to IX were counted (n = 4 mice per group). Scale bars, 25  $\mu\text{m}$ . \*\* $P < 0.01$ ; n.s., not significant.

### Fig. 2. Deletion of JSAP1 and JLP in PCs causes axonal dystrophy and progressive neuronal loss.

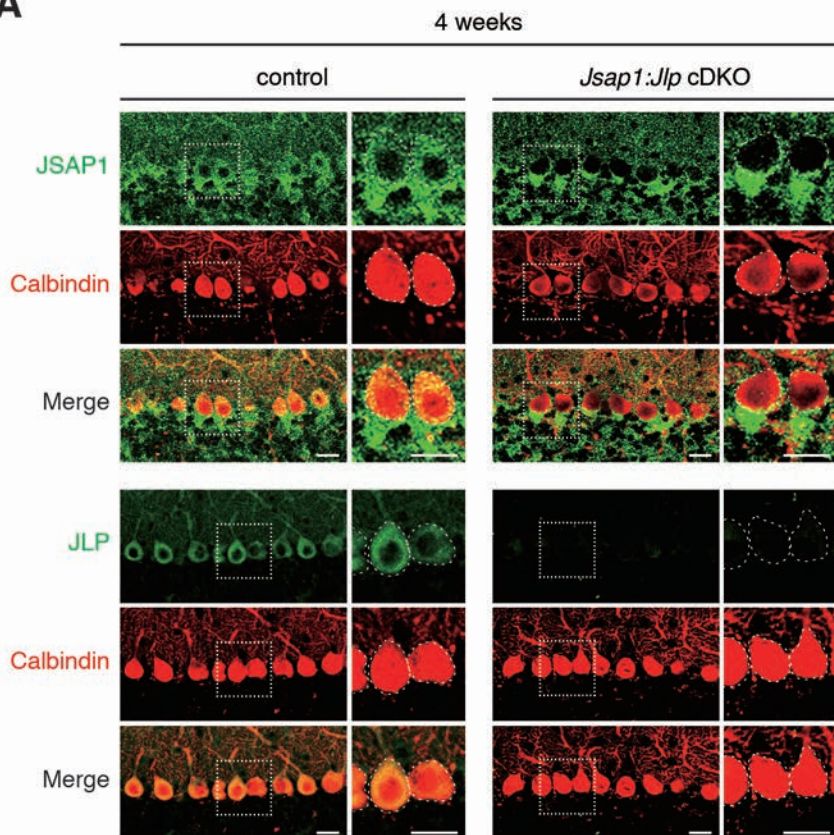
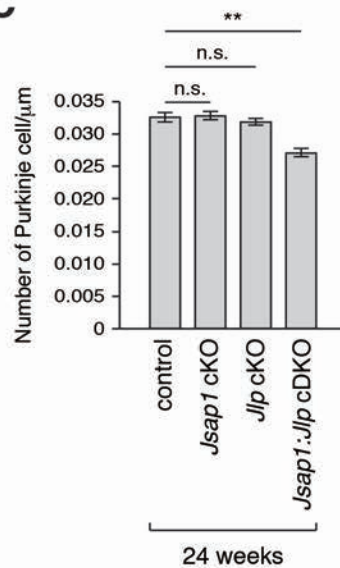
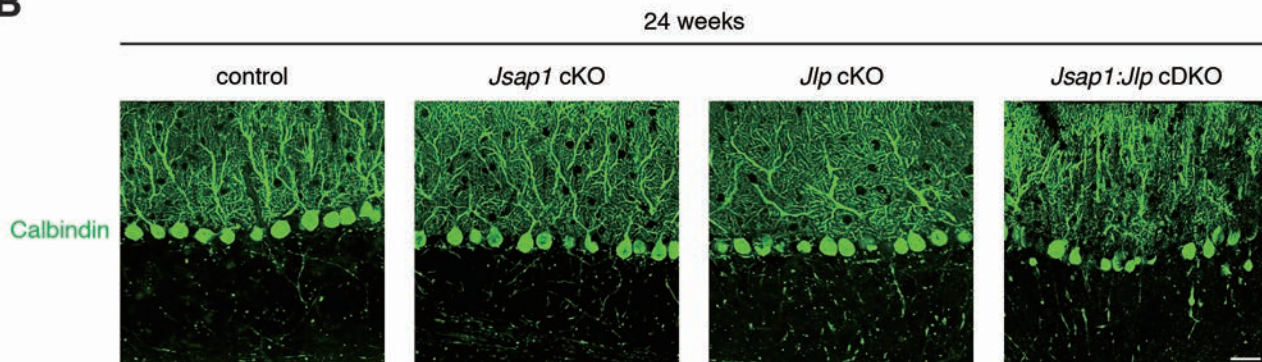
(A) PC quantification as a function of age. Midsagittal sections from control and *Jsap1:Jlp* cDKO mice at 8, 12, 16, 24, and 40 weeks of age were stained with Nissl and the PCs were counted as in Fig. 1C (n = 4 mice per group). (B) Midsagittal sections of control and *Jsap1:Jlp* cDKO mice at the indicated ages were subjected to IHC with an anti-calbindin antibody and DAPI, and the PC layers were examined. Higher magnifications of the boxed areas are shown in the right-most panels. Z-stack images of 10 optical sections, each 2- $\mu\text{m}$  thick, were acquired using a confocal microscope as described in Materials and Methods. Some of the PC axons in the *Jsap1:Jlp* cDKO mice exhibited a swollen appearance. (C) Midsagittal sections of control and *Jsap1:Jlp* cDKO mice at different ages were subjected to IHC as in B, without Z-stacking, and the DCN were examined. Scale bars, 25  $\mu\text{m}$ . \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; n.s., not significant.

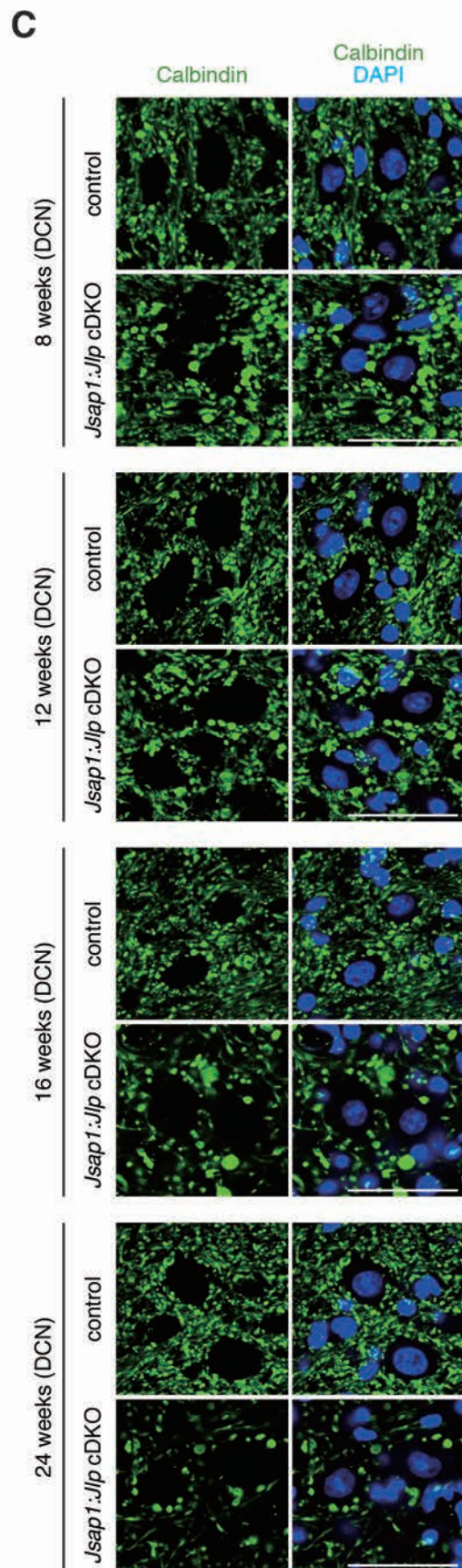
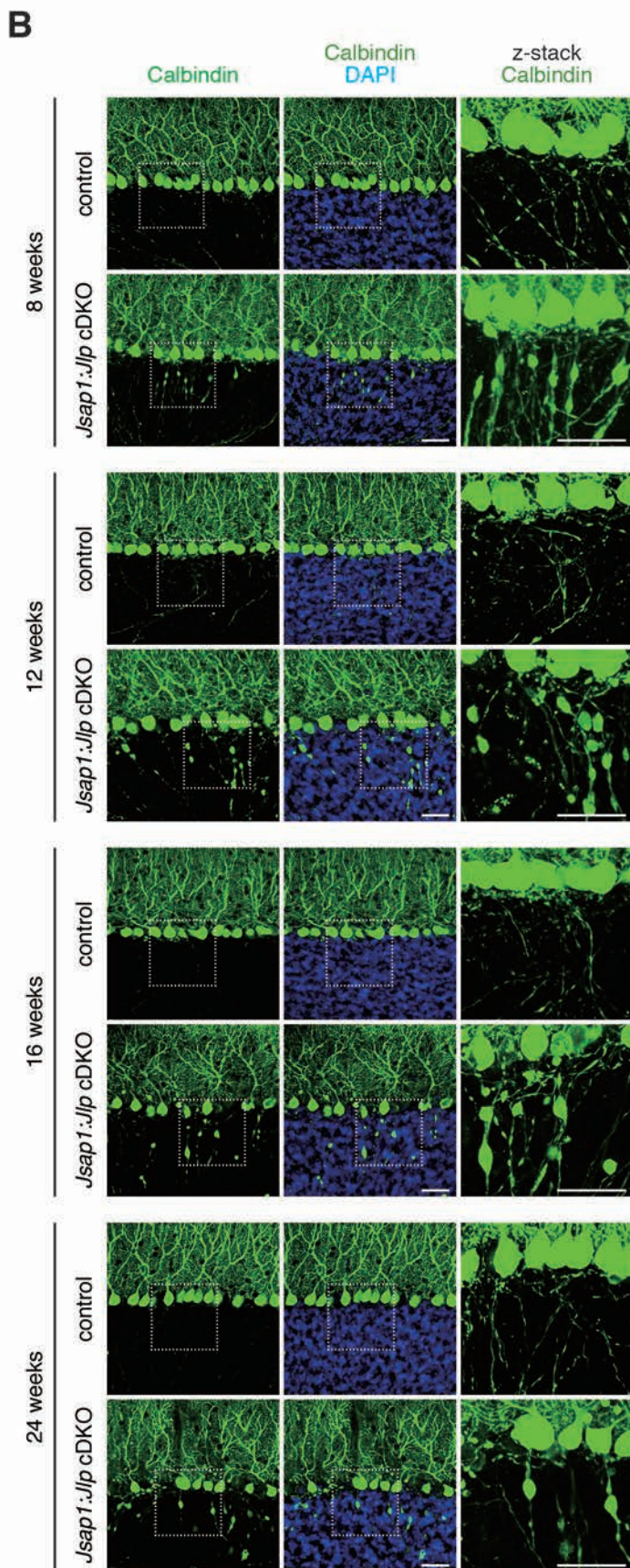
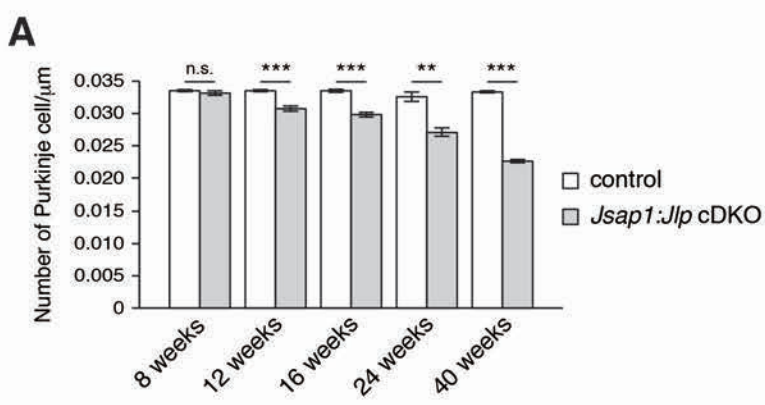
**Fig. 3. Selective accumulation of kinesin-1 cargoes in the swollen axons of *Jsap1*- and *Jlp*-deficient PCs.**

Midsagittal sections from 8-week-old control and *Jsap1:Jlp* cDKO mice were examined by double-immunostaining for calbindin and either APP, Cyt c (a mitochondrial marker), SYP, or SYT1, as indicated. Boxed areas are magnified in the insets. APP and mitochondria are kinesin-1 cargoes; SYP and SYT1 are kinesin-3 cargoes. Scale bars, 25  $\mu\text{m}$ .

**Fig. 4. *Jsap1*- and *Jlp*-deficient PCs undergo increased autophagy, which controls the extent of PC degeneration.**

(A) Protein aggregates in 8-week-old *Jsap1:Jlp* cDKO mice. Midsagittal sections of control and *Jsap1:Jlp* cDKO mice were examined by double-immunostaining for calbindin and ubiquitin. Boxed areas are magnified in the insets. Scale bar, 25  $\mu\text{m}$ . (B) Cell lysates from control and 8-week-old *Jsap1:Jlp* cDKO mice were subjected to immunoblotting analysis with an anti-LC3 antibody. (C) Quantification of the results in B. (D) Midsagittal sections of control, *Jsap1:Jlp* cDKO, single *Atg5* cKO, and *Jsap1:Jlp:Atg5* cTKO mice at 8 weeks of age were examined by immunostaining for calbindin. Scale bar, 500  $\mu\text{m}$ . \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

**A****C****B**

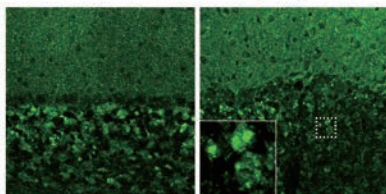
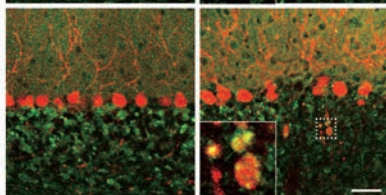


Sato *et al.* Figure 2

control

*Jsap1:Jlp* cDKO

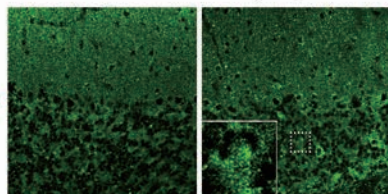
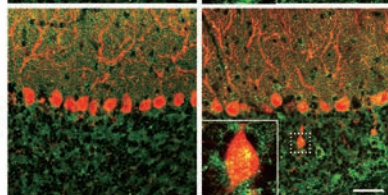
APP

APP  
Calbindin

control

*Jsap1:Jlp* cDKO

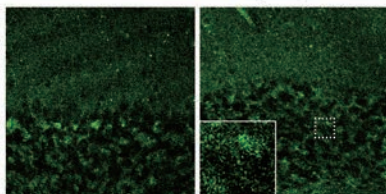
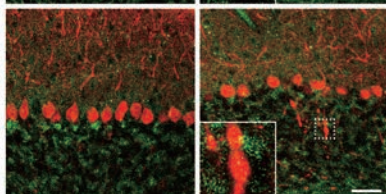
Cyt-c

Cyt-c  
Calbindin

control

*Jsap1:Jlp* cDKO

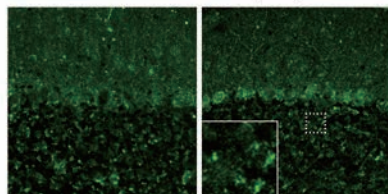
SYP

SYP  
Calbindin

control

*Jsap1:Jlp* cDKO

SYT1

SYT1  
Calbindin