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A cell-autonomous role for JSAP1 and JLP in mouse cerebellar Purkinje cell survival

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Abstract We previously reported that the structurally related JNK scaffolding proteins JSAP1 and JLP play functionally redundant and key roles in maintaining mouse cerebellar Purkinje cell (PC) survival, which may be related to their regulation of axonal transport. The JSAP1 and JLP proteins are also widely expressed in other cell types throughout the brain. Notably, in the cerebellum, JSAP1 is abundantly expressed in the pinceau, a cluster of basket cell (BC) axons and termini that surround the PC axon initial segment. Thus, it is possible that BC-expressed JSAP1 plays a role in PC survival. To investigate this possibility, we generated and analyzed mice containing a PC/BC-specific double knockout (DKO) of *Jsap1* and *Jlp* (*Jsap1*:*Jlp* cDKO mice). These mice exhibited PC axonal dystrophy, followed by progressive PC loss, consistent with the phenotypes previously reported for PC-specific *Jsap1* and *Jlp* DKO mice. Furthermore, we found that the phenotypes of *Jsap1*:*Jlp* cDKO PCs were rescued by PC-specific transgenic expression of JSAP1. Taken together, these results indicate that BC-expressed JSAP1 plays little or no role in PC survival, and that PC-expressed JSAP1 and JLP play cell-autonomous roles in preventing PC degeneration.

Keywords: Axonal swelling; Knockout mice; Neuronal degeneration

1 Introduction

The mitogen-activated protein kinase (MAPK) intracellular signaling pathway, consisting of MAPK, MAPK kinase (MAPKK), and MAPKK kinase, is well conserved from yeast to humans (Cano and Mahadevan, 1995; Herskowitz, 1995; Waskiewicz

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and Cooper, 1995). Scaffolding proteins associated with MAPK modules contribute, at least in part, to the specificity and efficiency of these signaling cascades (Morrison and Davis, 2003; Yoshioka, 2004; Dhanasekaran et al., 2007). JNK/stress-activated protein kinase-associated protein 1 (JSAP1, also known as JIP3) and JNK-associated leucine zipper protein (JLP, also known as JIP4) are structurally related scaffolding proteins associated with mammalian JNK and p38 MAPK signaling modules (Ito et al., 1999; Kelkar et al., 2000; Lee et al., 2002; Kelkar et al, 2005) that can also interact with kinesin-1 light and heavy chains (KLC and KHC, respectively) through specific binding domains. JSAP1 and JLP are reported to function as adaptor proteins that link cell cargoes to kinesin-1 (Verhey et al., 2001; Nguyen et al., 2005), and regulate kinesin-1 activity and motility (Sun et al., 2011; Watt et al., 2015). Studies of JSAP1 loss-of-function mice indicate that JSAP1 is required during early development of the corpus callosum (Kelkar et al., 2003; Ha et al., 2005), and *Jsap1* knockout (KO) mice die shortly after birth due to neuronal defects (Iwanaga et al., 2007). In contrast, *Jlp* KO mice are viable and grow normally (Iwanaga et al., 2008). Recently, however, JSAP1 and JLP were reported to have crucial and functionally redundant roles in the developing mouse brain and in the survival of Purkinje cells (PCs), both of which are associated with their regulation of kinesin-1-dependent axonal transport (Sato et al, 2015a and 2015b).

JSAP1 and JLP are widely expressed in the mouse brain. Notably, in the cerebellum, JSAP1 is highly expressed in the pinceau, a cluster of basket cell (BC) axons and termini that surround the PC axon initial segment (Miura et al., 2006). Thus, although we concluded from our previous study that PC-expressed JSAP1 and JLP play key, functionally redundant roles in PC survival (Sato et al, 2015b), it remains possible that BC-expressed JSAP1 and JLP are also involved in maintaining PC survival. To investigate this possibility, we generated and analyzed mice containing a PC/BC-specific double KO (DKO) in *Jsap1* and *Jlp* (*Jsap1*:*Jlp* cDKO), and then evaluated the effect of transgenic rescue with PC-expressed JSAP1.

2 Materials and Methods

2.1 Animals

All experimental procedures involving animals were approved by the Institutional Animal Care and Use Committees of Kanazawa University and RIKEN Kobe, respectively. *GluRδ2-Cre* mice (Yamasaki et al., 2011), in which *Cre* is driven by *GluRδ2* promoter and thus is expressed specifically in BC and PC, were provided by Dr. Kenji Sakimura (Niigata University), and were crossed with $Jsap1^{f/f}$ (Iwanaga et al., 2007) and $Jlp^{f/f}$ (Sato et al., 2015a) mice. The green fluorescent protein (GFP) coding region in pBS-L7-GFP, a gift from Dr. Hirokazu Hirai (Gunma University), and a

derivative of the pL7ΔAUG vector containing the PS-specific *L7* promoter but disrupting the translational start site (ATG) of the *L7* gene (Oberdick et al, 1990; Tomomura et al., 2001), was replaced with the entire coding region of the mouse wild-type *Jsap1* gene. The *L7-Jsap1* transgene was then injected into fertilized eggs to generate *L7-Jsap1* transgenic mice (Accession No. CDB0513T: http://www2.clst.riken.jp/arg/TG%20mutant%20mice%20list.html). Genotyping of *L7-Jsap1* transgenic mice was performed using the following two primer sets:
5'-TGTTTGGAGGCACTTCTGACTTGC-3' and
5'-CTCGTCCATCTGGATCTCCATCAT-3' (primer set 1) and
5'-CGGAGAGGATGATGAAACTGAGGA-3' and
5'-CACTCAACTCTTTGTTGCTAGTGCC-3' (primer set 2) giving PCR products of 156-bp and 261-bp, respectively.

2.2 Antibodies (Abs)

The following primary Abs were used in this study: anti-JSAP1 (Miura et al, 2006), anti-calbindin D-28K (C9848, Sigma-Aldrich or AB1778, Millipore), anti-synaptotagmin 1 (SYT1) (105002, Synaptic Systems), anti-SNAP25 (S5187, Sigma-Aldrich), anti-glial fibrillary acidic protein (GFAP) (G3893, Sigma-Aldrich), anti-phospho-JNK (9251, Cell Signaling), and anti-α-tubulin (T5168, Sigma-Aldrich) Abs. The following secondary Abs were used: Alexa 488- and 568-conjugated anti-mouse or anti-rabbit IgG (Invitrogen) and horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit IgG (GE Healthcare) Abs.

2.3 Western blotting (WB), immunohistochemistry (IHC), and PC quantification

WB, IHC, and PC quantification were performed as described previously (Sato et al., 2008 and 2015b). IHC images were acquired with a confocal laser scanning microscope (LSM510 META, Carl Zeiss) using a 20x or 40x objective lens.

2.4 Reverse transcription (RT)-polymerase chain reaction (PCR)

RT-PCR was carried out as previously described (Sato et al, 2004), using the following primer pairs: *L7-Jsap1*, 5'-CGTGTAACAGTTAATTCCCTGCCTG-3' (*L7*) and
5'-CTCGTCCATCTGGATCTCCATCAT-3' (*Jsap1*); *Gapdh*,
5'-AAATGGTGAAGGTCGGTGTG-3' and 5'-TGAAGGGGTCGTTGATGG-3'.

2.5 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 and Discussion

3.1 PC/BC-specific deletions in *Jsap1* and *Jlp* cause PC axonal swelling and progressive cell loss

To investigate the roles of JSAP1 and JLP in BCs, we generated mice with PC/BC-targeted conditional deletions in *Jsap1*, *Jlp*, or both, using loxP-flanked (floxed) alleles of these genes (Iwanaga et al., 2007; Sato et al., 2015a), in combination with the *GluRδ2-Cre* transgene (Yamasaki et al., 2011). We first performed IHC of the mid-sagittal brain sections of 24-week-old control ($Jsap1^{f/f}$:$Jlp^{f/f}$), single *Jsap1* or *Jlp* cKO ($Jsap1^{f/f}$:$Jlp^{f/+}$:*GluRδ2-Cre* or $Jsap1^{f/+}$:$Jlp^{f/f}$:*GluRδ2-Cre*), and *Jsap1:Jlp* cDKO ($Jsap1^{f/f}$:$Jlp^{f/f}$:*GluRδ2-Cre*) mice using an Ab against calbindin, a PC marker. The results showed the presence of swollen PC axons associated with PC loss in the *Jsap1:Jlp* cDKO mice, but not in the single *Jsap1* or *Jlp* cKO mice (Fig. 1A). We quantified the PCs by Niss1 staining of the sections and found that the PC numbers in the cDKO mice were significantly reduced compared to those in the cKO or control mice (Fig. 1B). Taken together, these results indicated that JSAP1 and JLP play functionally redundant roles in PC survival, which may be related to the regulation of axonal transport in PCs.

Next, we used anti-calbindin Ab IHC to examine the PC axons of *Jsap1:Jlp* cDKO mice at different ages (4, 8, 16, and 24 weeks of age; Fig. 2A). Many swollen axons were observed in regions near the PC bodies of the cDKO mice at each of the ages examined. We also quantified the PCs in *Jsap1:Jlp* cDKO mice at those ages, and at 40 weeks of age. The PC numbers were comparable between the control and *Jsap1:Jlp* cDKO mice at 4 and 8 weeks of age (Fig. 2B). However, the cDKO mice at16 weeks of age showed significantly decreased PC numbers compared to control mice, and the degree of PC loss increased with age (Fig. 2B). In addition, intense immunosignals for GFAP, a hallmark of reactive gliosis, were detected in association with PC death in the *Jsap1:Jlp* cDKO mice at 24 weeks of age (Fig. 2C). These results are consistent with those previously obtained using PC-specific *Jsap1:Jlp* DKO mice (Sato et al., 2015b), suggesting that JSAP1 and JLP expressed in BCs have little or no effect on PC survival.

3.2 Selective accumulation of the kinesin-1 cargo SNAP25 in the swollen axons of JSAP1- and JLP-deficient PCs

Axonal dystrophy is thought to reflect defective axonal transport. Since JSAP1 and JLP are known to interact with kinesin-1, a motor protein involved in axonal transport, the axonal dystrophy phenotype of *Jsap1:Jlp* cDKO may be due to defects in kinesin-1-dependent axonal transport. To examine this possibility, we analyzed the swollen axons of PCs in 16-week-old *Jsap1:Jlp* cDKO mice by double-immunostaining, using

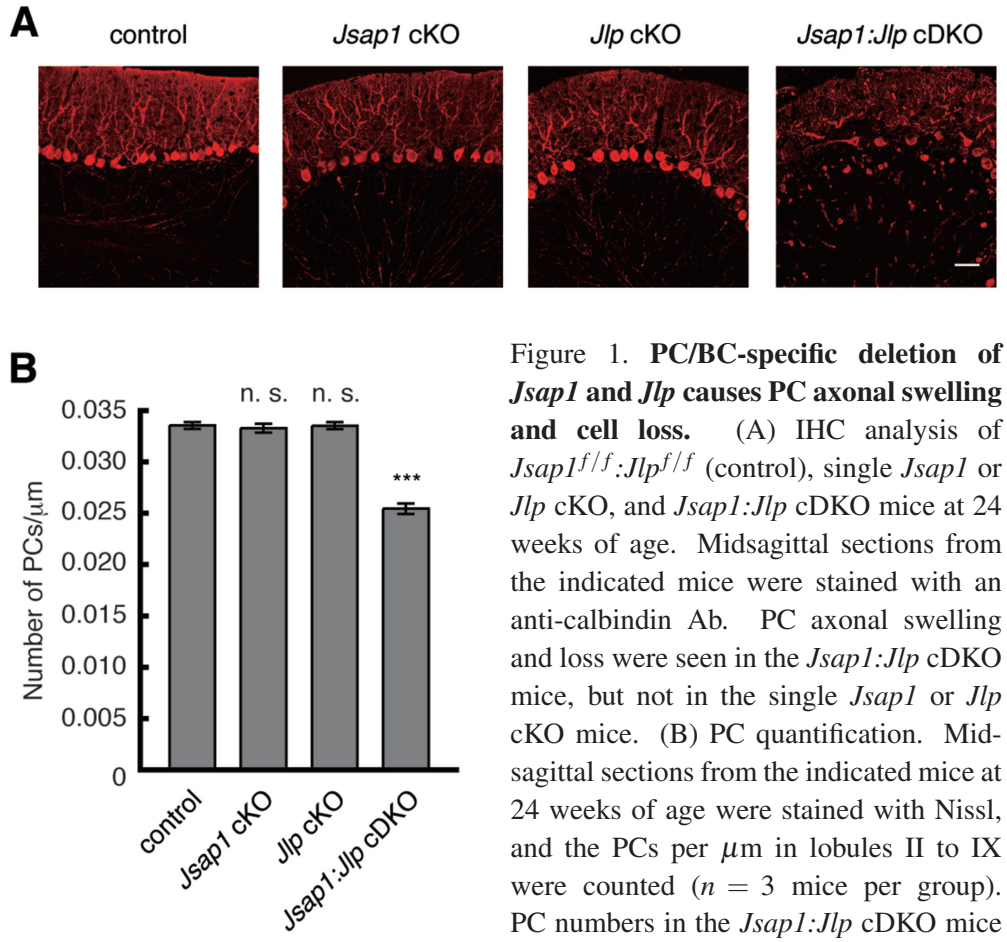

Figure 1. **PC/BC-specific deletion of *Jsap1* and *Jlp* causes PC axonal swelling and cell loss.** (A) IHC analysis of $Jsap1^{f/f}$:$Jlp^{f/f}$ (control), single *Jsap1* or *Jlp* cKO, and *Jsap1:Jlp* cDKO mice at 24 weeks of age. Midsagittal sections from the indicated mice were stained with an anti-calbindin Ab. PC axonal swelling and loss were seen in the *Jsap1:Jlp* cDKO mice, but not in the single *Jsap1* or *Jlp* cKO mice. (B) PC quantification. Midsagittal sections from the indicated mice at 24 weeks of age were stained with Nissl, and the PCs per μm in lobules II to IX were counted ($n = 3$ mice per group). PC numbers in the *Jsap1:Jlp* cDKO mice were significantly decreased compared to those in the control mice. Scale bar, 50 μm. $^{***}P < 0.001$; n.s., not significant.

Abs against calbindin and either SNAP25, a kinesin-1 cargo (Hirokawa et al., 2009), or SYT1, a kinesin-3 cargo (Hirokawa et al., 2009). Strong SNAP25 immunosignals were detected throughout almost all of the swollen PC axons, whereas the SYT1 immunosignals were very low and limited in distribution (Fig. 3A). These results, together with the earlier finding of progressive PC loss in these mice, suggested that JSAP1 and JLP play important roles in the kinesin-1-dependent axonal transport in PCs, and that their ablation causes PC axonal dystrophy, leading to neuronal loss.

In addition, we analyzed JNK activation in the axons using a phospho-JNK Ab. The results showed substantial JNK activation in the swollen axons of PCs in the *Jsap1:Jlp* cDKO mice (Fig. 3B), consistent with that observed in the PC-specific *Jsap1:Jlp* DKO

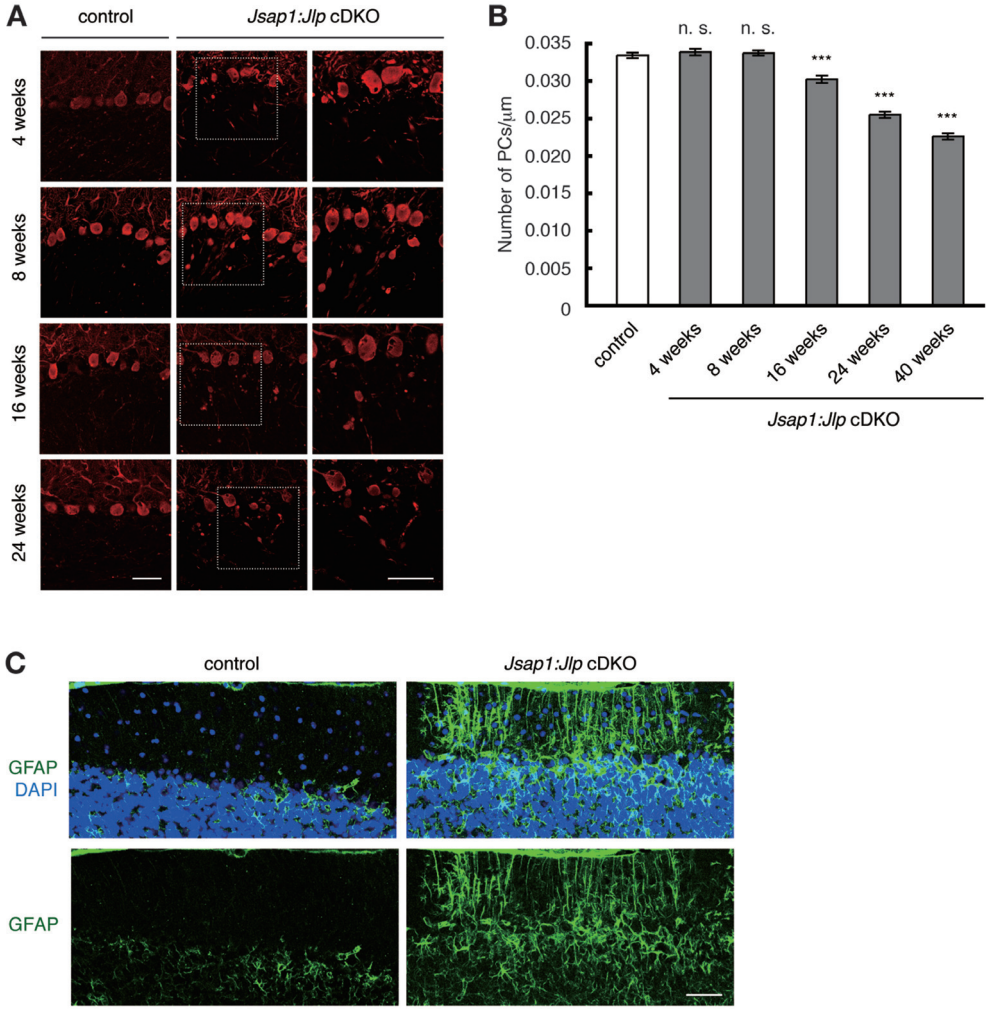

Figure 2. **PC axonal dystrophy, progressive PC loss, and reactive gliosis in *Jsap1:Jlp* cDKO mice.** (A) Midsagittal sections of control and *Jsap1:Jlp* cDKO mice at 4, 8, 16, and 24 weeks of age were subjected to IHC with an anti-calbindin Ab. Higher magnifications of the boxed areas are shown in the right-most panels. (B) Midsagittal sections from control and *Jsap1:Jlp* cDKO mice at the indicated ages were stained with Nissl, and the PCs were counted as in Fig. 1B ($n = 4$ mice per group). PC numbers were significantly decreased in *Jsap1:Jlp* cDKO mice at 16 weeks of age, compared to control mice, and the degree of PC loss increased with age. (C) Sections of the PC layer in control and *Jsap1:Jlp* cDKO mice at 24 weeks of age were examined by IHC with an anti-GFAP Ab and 4,6-diamidino-2-phenylindole (DAPI). Scale bars, 50 μm. ***$P < 0.001$; n.s., not significant.

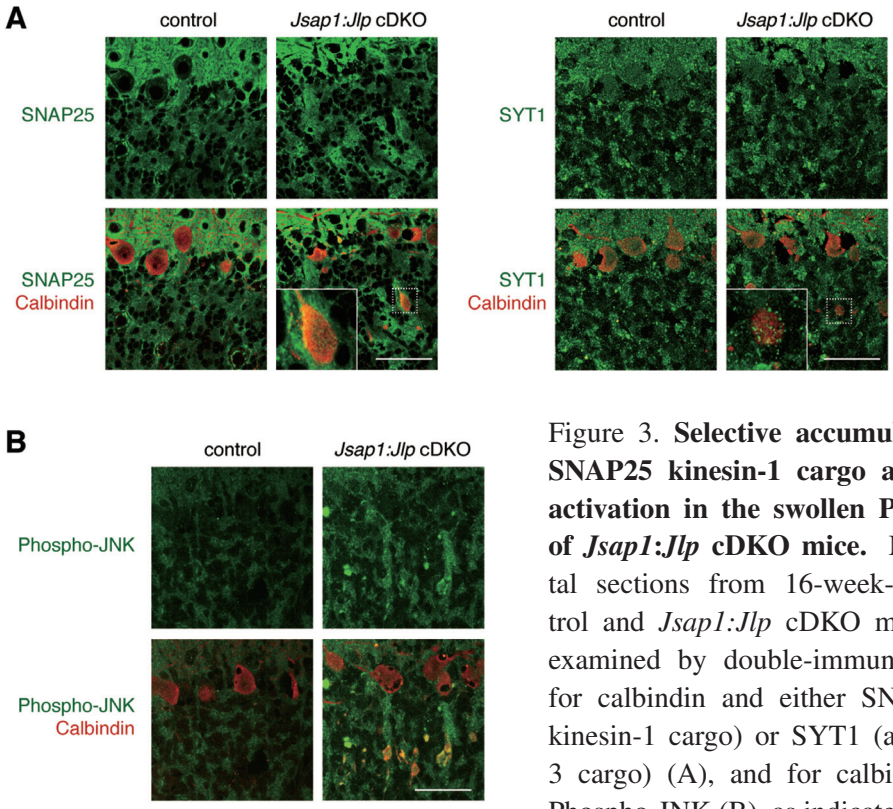

Figure 3. **Selective accumulation of SNAP25 kinesin-1 cargo and JNK activation in the swollen PC axons of *Jsap1*:*Jlp* cDKO mice.** Midsagittal sections from 16-week-old control and *Jsap1:Jlp* cDKO mice were examined by double-immunostaining for calbindin and either SNAP25 (a kinesin-1 cargo) or SYT1 (a kinesin-3 cargo) (A), and for calbindin and Phospho-JNK (B), as indicated. Boxed areas are magnified in the insets. Scale bars, 50 μm.

mice (Sato et al., 2015b). At present, the precise mechanism underlying the PC death induced by the ablation of JSAP1 and JLP remains unknown. However, these results, together with the results of our previous study using *Jsap1:Jlp* DKO primary cultured neurons (Sato et al., 2015a), suggest that JNK activation may be involved in mediating the neuronal death.

3.3 Transgenic expression of JSAP1 in PCs rescues the PC degeneration in *Jsap1:Jlp* cDKO mice

To examine whether the JSAP1 expressed in BCs affects PC survival, we generated transgenic mice expressing the PC *L7* promoter-driven *Jsap1* transgene (*L7-Jsap1*, Fig. 4A), and performed rescue experiments by crossing *Jsap1:Jlp* cDKO mice with the transgenic mice. We first confirmed that the exogenous *Jsap1* mRNA was specifically expressed in the cerebellum, and not the cerebral cortex (Fig. 4B). We then analyzed the compound mice (*Jsap1:Jlp* cDKO/*L7-Jsap1*) by WB with an anti-JSAP1

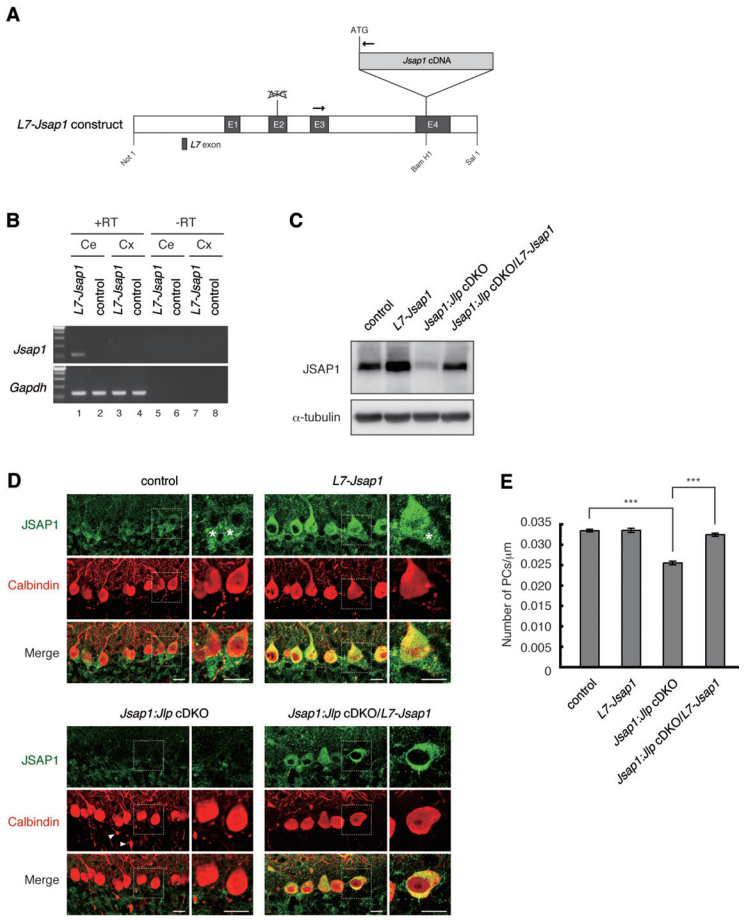

Figure 4. **Rescue of PC degeneration in *Jsap1*:*Jlp* cDKO mice by the PC-specific transgenic expression of JSAP1.** (A) Schematic illustration of DNA construct used to generate transgenic mice that express *L7* promoter-driven *Jsap1*. Arrows indicate primers for RT-PCR in **B**. (B) Total RNAs were extracted from the cerebellum (Ce) and cerebral cortex (Cx) of 8-week-old control and *L7-Jsap1* transgenic mice, as indicated, and RT-PCR was performed using specific primers (indicated as arrows in **A**) for the *L7-Jsap1* transgene, with (lanes 1-4), or without (lanes 5-8) reverse transcriptase (RT). *Gapdh* RT-PCR was used as a control. (C) WB analysis of JSAP1 in the cerebellum of the indicated mice at 8 weeks of age. α-tubulin was used as a loading control. (D) Midsagittal sections from the indicated mice at 8 weeks of age were examined by double-immunostaining for JSAP1 and calbindin. Asterisks and arrowheads denote JSAP1-labeled pinceaus and swollen PC axons, respectively. Higher magnifications of the boxed regions are shown in the panels at right. (E) Rescue of PC loss in *Jsap1:Jlp* cDKO mice by the transgenic expression of JSAP1. PC numbers in the indicated mice at 24 weeks of age were quantified as in Fig. 1B ($n = 4$ mice per group). Scale bars, 25 μm. $^{***}P < 0.001$.

Ab (Fig. 4C), and by IHC using Abs against calbindin and JSAP1 (Fig. 4D). The expression levels of JSAP1 in the cerebellum were comparable between the control and *Jsap1:Jlp* cDKO/*L7-Jsap1* mice (Fig. 4C). JSAP1 immunosignals were detected in the pinceau of the control and transgenic mice, but not in that of the *Jsap1:Jlp* cDKO and *Jsap1:Jlp* cDKO/*L7-Jsap1* mice (Fig. 4D), indicating that the exogenous JSAP1 was expressed specifically in the PCs. Although swollen PC axons were observed in the *Jsap1:Jlp* cDKO mice at 8 weeks of age (Fig. 4D lower left panels, see also Fig. 2A), no axonal dystrophy was detected in the *Jsap1:Jlp* cDKO/*L7-Jsap1* mice at the same age (Fig. 4D lower right panels). Furthermore, the decreased number of PCs in the *Jsap1:Jlp* cDKO mice at 24 weeks of age was rescued in the *Jsap1:Jlp* cDKO/*L7-Jsap1* mice of the same age (Fig. 4E). Taken together, these results indicated that the JSAP1expressed in BCs is not required for PC survival, and that intrinsically expressed JSAP1 and JLP play functionally redundant roles in PC survival.

In conclusion, our molecular genetic and immunohistological analyses highlighted the key roles of JSAP1 and JLP in mouse PC survival, and strongly suggested that JSAP1 and JLP function cell autonomously to prevent PC degeneration. At present, the role of JSAP1 in the pinceau remains unknown. Further studies are required to clarify this issue.

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