

# Autistic-Like Traits and Cerebellar Dysfunction in Purkinje Cell *PTEN* Knock-Out Mice

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Autism spectrum disorders (ASDs) are neurodevelopmental disorders characterized by impaired social interaction, isolated areas of interest, and insistence on sameness. Mutations in Phosphatase and tensin homolog on chromosome 10 (*PTEN*) have been reported in individuals with ASDs. Recent evidence highlights a crucial role of the cerebellum in the etiopathogenesis of ASDs. In the present study we analyzed the specific contribution of cerebellar Purkinje cell (PC) *PTEN* loss to these disorders. Using the Cre-loxP recombination system, we generated conditional knockout mice in which *PTEN* inactivation was induced specifically in PCs. We investigated PC morphology and physiology as well as sociability, repetitive behavior, motor learning, and cognitive inflexibility of adult PC *PTEN*-mutant mice. Loss of *PTEN* in PCs results in autistic-like traits, including impaired sociability, repetitive behavior and deficits in motor learning. Mutant PCs appear hypertrophic and show structural abnormalities in dendrites and axons, decreased excitability, disrupted parallel fiber and climbing fiber synapses and late-onset cell death. Our results unveil new roles of *PTEN* in PC function and provide the first evidence of a link between the loss of *PTEN* in PCs and the genesis of ASD-like traits.

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## INTRODUCTION

Autism spectrum disorders (ASDs) are complex neurodevelopmental disorders affecting around 1% of the population worldwide. ASDs are characterized by abnormal social interaction, deficits in verbal and nonverbal communication, repetitive behaviors and restricted interests (Lai *et al*, 2014). Mutation of phosphatase and tensin homolog missing on chromosome 10 (*PTEN*) is a causative factor in 5–10% of autism patients (Li *et al*, 1997; McBride *et al*, 2010). *PTEN* is a lipid and protein phosphatase, which dephosphorylates phosphatidylinositol3,4,5trisphosphate, negatively regulating the phosphoinositol-3 kinase (PI3K)-AKT-mammalian target of rapamycin (mTOR) signaling pathway, which stimulates protein synthesis and survival, and the AKT/glycogen synthase kinase 3 (GSK3) pathway, which drives cell survival, proliferation, and cellular metabolism (Song *et al*, 2012). Mouse models with *PTEN* germline haploinsufficiency show deficits in sociability and increased repetitive behavior, along with brain overgrowth (Clipperton-Allen and Page, 2014, 2015). Conditional knock-out mice with *PTEN* deletion in neurons of the cerebral

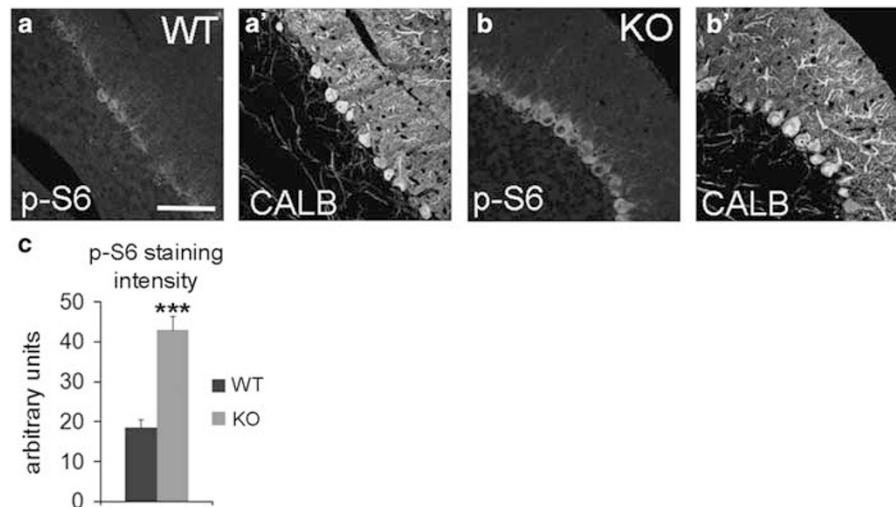
cortex and hippocampus develop macrocephaly due to neuronal hypertrophy and show behavioral abnormalities reminiscent of ASDs (Kwon *et al*, 2006).

Traditionally, the neurological basis of ASD has been thought to lie in the cerebral cortex. However, recent evidence has highlighted a crucial role of the cerebellum in this pathology (Courchesne *et al*, 2001; Fatemi *et al*, 2012; Wang *et al*, 2014). The cerebellum is involved not only in the control of balance, motor coordination, and learning, but also in higher order emotional, social, and cognitive functions, mainly through its projections (via the thalamus) to various cortical regions including the prefrontal, parietal, and limbic cortex (Fatemi *et al*, 2012; Reeber *et al*, 2013; Strick *et al*, 2009). Notably, Purkinje cell (PC) loss is one of the most common anatomical abnormalities seen in autopsy studies of autistic patients (Palmen *et al*, 2004; Whitney *et al*, 2008; Skefos *et al*, 2014; Wegiel *et al*, 2014). Cerebellar injury has been associated with a high incidence of ASDs (Wang *et al*, 2014), and patients with diseases confined to the cerebellum often demonstrate abnormalities seen in ASDs (Schmahmann, 1998). Recent works have established a strong link between abnormal PC function, caused by the loss of tuberous sclerosis complex (TSC), and behavioral deficits that are relevant to ASDs (Tsai *et al*, 2012; Reith *et al*, 2013). However, to date it is not known whether selective *PTEN* loss-of-function in PCs contributes to the development of ASD traits. To address this issue, we employed conditional knockout mice lacking *PTEN* specifically in PCs. In those mice we observed ASD-like features, including

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**Figure 1** Expression levels of p-S6 are increased in PCs of PTEN-KO mice. PTEN-KO mice of 1 month of age demonstrate increased p-S6 staining in PCs (b) when compared with WT mice (a), consistent with increased mTOR signaling from *PTEN* deletion. In (a') and (b'), PCs are stained by anti-calbindin antibodies. In (c) quantification of p-S6 staining intensity in WT and KO PC soma (WT: 45 PCs from 3 animals; KO: 77 PCs from 5 animals). Data shown are means  $\pm$  SEM. Scale bar: 80  $\mu$ m in a (also applies to a'–b'). \*\*\* $P < 0.001$ , Student's unpaired *t*-test. KO, knockout; mTOR, mammalian target of rapamycin; PC, Purkinje cell; PTEN, Phosphatase and tensin homolog missing on chromosome 10; WT, wild type.

impaired sociability and repetitive behavior, associated with substantial alterations in PC electrophysiological properties and morphological features, ending with PC loss.

## MATERIALS AND METHODS

### Experimental Mice

*PTEN* conditional knock-out mice (L7/Cre; *PTEN*<sup>fl/fl</sup>, named PTEN-KO) were obtained by crossing L7/Cre transgenic mice, in which Cre expression is driven by the PC-specific promoter L7, with mice bearing LoxP sites flanking *PTEN* exon 5. As ASDs show a higher incidence in males than females (Lai *et al*, 2014), the experiments were conducted on male mice. Further details can be found in Supplementary Materials and Methods.

### Genotyping

The presence of Cre and *PTEN*<sup>fl/fl</sup> sequences was assessed by PCR. Primer sequences and PCR conditions are reported in Supplementary Table S1.

### Histological Examination

Methods used for immunostaining and quantification analysis can be found in Supplementary Materials and Methods.

### Electrophysiological Recordings

Male PTEN-KO and WT mice of 12–18 weeks of age were used. Detailed information can be found in Supplementary Materials and Methods.

### Behavioral Testing

All behavioral experiments were conducted on male mice of 3–4 months of age (if not specified otherwise) between 0900 hours and 1600 hours. Detailed information can be found in Supplementary Materials and Methods.

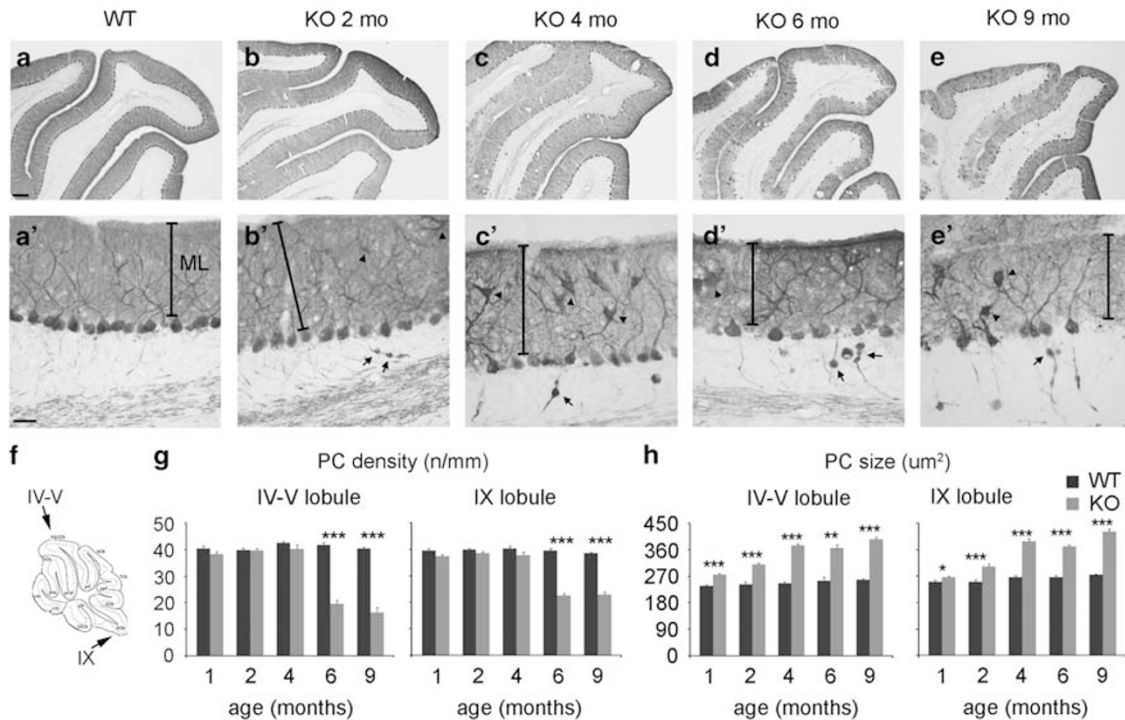
### Statistical Analysis

Detailed information can be found in Supplementary Materials and Methods.

## RESULTS

### PTEN-KO PCs Undergo Substantial Morphological Alterations

To evaluate whether the lack of *PTEN* in PCs contribute to the development of ASD-like behavior, we generated conditional knockout mice in which *PTEN* is specifically deleted in PCs (PTEN-KO mice). Although the L7 promoter is also expressed in retinal bipolar neurons (Oberdick *et al*, 1990), the L7/CRE mouse line we used show only a few L7-driven  $\beta$ -galactosidase-positive cells in the latter neurons (Barski *et al*, 2000). Cre expression is evident in PCs by postnatal day 6 and it is fully established by 2–3 weeks after birth (Barski *et al*, 2000). We found that PTEN-KO mice did not show reduced survival or fertility and displayed basic cerebellar architecture. A reliable marker for impaired *PTEN* function is the upregulation of phosphorylated ribosomal protein S6 (p-S6) (Park *et al*, 2008), a downstream effector of mTOR signaling, which is inhibited by *PTEN* activity (Song *et al*, 2012). We found increased p-S6 staining in PCs of PTEN-KO mice at 1 month of age (Student's *t*-test  $t_{(122)} = 5.22$ ,  $P < 0.001$ ; Figure 1a–c), as well as at 4 months of age (Supplementary Figure S1), while no difference



**Figure 2** PTEN-KO mice display abnormal PC morphology and reduced PC number. (a–e′) Representative pictures from a 2-month-old WT mouse and KO mice of various ages show calbindin-stained PCs. Mutant PCs, starting from 1 month of age, are hypertrophic. PC dendrites display several enlargements (arrowheads in b′–e′). Numerous swellings are evident along the proximal segment of PC axons (arrows in b′–e′). The thickness of the molecular layer (ML) was increased in KO cerebella at 2 and 4 months of age, whereas it decreased afterwards. PC loss was apparent in 6- and 9-month-old KO animals (d–e′). (g) The quantification of PC density (3 slices/animal from at least 3 animals/each time point), performed in the IV–V and IX lobule (f). (h) The quantification of PC soma area (3 slices/animal from at least 3 animals/each time point). Data shown are means  $\pm$  SEM. Scale bars: 200  $\mu$ m in (a–e), 50  $\mu$ m in (a′–e′). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , Student's unpaired *t*-test. PC, Purkinje cell; PTEN-KO, Phosphatase and tensin homolog missing on chromosome 10-knockout; WT, wild type.

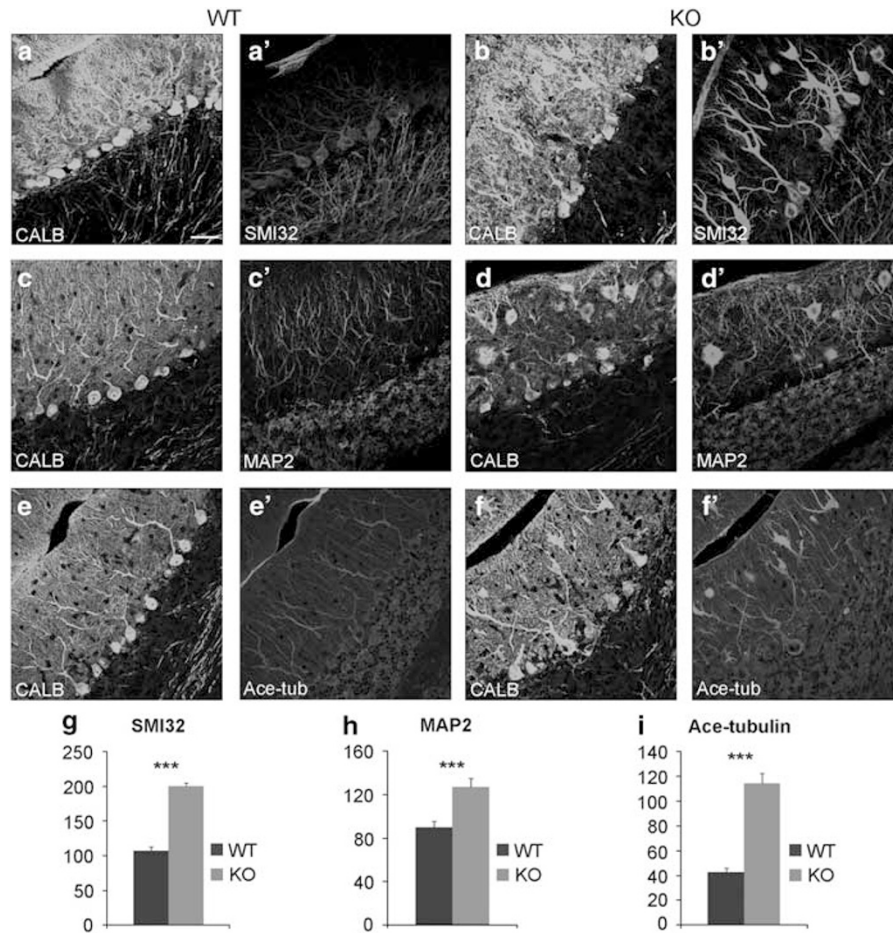
between mutants and wild-type (WT) was detected in other brain regions (Supplementary Figure S1).

In all cerebellar lobules PTEN-KO PCs showed enlarged soma. Quantification of the area of PC soma in the IV–V and the IX lobule (Figure 2f) showed that PC soma size was increased by 25% in the IV–V lobule (Student's *t*-test  $t_{(15)} = 6.57$ ,  $P < 0.001$ ) and 15% in the IX lobule at 2 months of age (Student's *t*-test  $t_{(15)} = 4.42$ ,  $P < 0.001$ ), and reached a 50% increase at 4 months (IV–V lobule: Student's *t*-test  $t_{(17)} = 16.66$ ,  $P < 0.001$ ; IX lobule:  $t_{(17)} = 11.86$ ,  $P < 0.001$ ) and a 60% increase at 9 months in both lobules (IV–V lobule: Student's *t*-test  $t_{(16)} = 16.67$ ,  $P < 0.001$ ; IX lobule:  $t_{(14)} = 14.64$ ,  $P < 0.001$ ; Figure 2a–e′ and h, Supplementary Figure S2A and B). Moreover, starting from 2 months of age, PCs of mutant mice showed thicker dendrites and axons when compared with WT PCs, and developed focal dendritic swellings and axonal rounded varicosities (called torpedoes) (Figure 2a–e′, Supplementary Figure S2A–D), which became more widespread with age (Supplementary Table S2). Dendritic swellings often displayed abnormal radial outgrowth of dendritic branches (Supplementary Figure S2B and C), and hypertrophic spines were observed on thicker dendrites (Supplementary Figure S2E–F′). Torpedoes, which were mainly located along the axon proximal segment and in many instances were in close proximity to one another in the same axon, displayed a smooth surface, although occasional sprouts could be detected (Supplementary Figure S2B and D). Consistent with PC soma and dendritic enlargement, the

thickness of the molecular layer was increased in 2 and 4 months old PTEN-KO (Figure 2a–c′; Supplementary Table S3). Overgrowth of PCs also concerned their axon terminals. PC boutons, which were visualized by double staining for calbindin and VGAT, showed a progressive increase in size with time, which was particularly remarkable after 6 months of age (1.5-fold-increase at 4 months (Student's *t*-test  $t_{(396)} = 10.57$ ,  $P < 0.001$ ), 3-fold increase at 6 months (Student's *t*-test  $t_{(241)} = 7.31$ ,  $P < 0.001$ ), and 9 months (Student's *t*-test  $t_{(192)} = 6.91$ ,  $P < 0.001$ ); Supplementary Figure S3A–F).

We found a reduction of PC number in PTEN-KO mice at 6 months of age, when PCs in both IV–V and IX lobule decreased by  $\sim 50\%$  when compared with WT mice (IV–V lobule: Student's *t*-test  $t_{(10)} = 9.42$ ,  $P < 0.001$ ; IX lobule:  $t_{(12)} = 14.27$ ,  $P < 0.001$ ) and PTEN-KO mice of younger age (Figure 2a–e′ and g). In 9-month-old PTEN-KO mice, PC number was similar to that of 6-month-old PTEN-KO mice (Student's *t*-test  $t_{(16)} = 1.46$ ,  $P = 0.16$  and Student's *t*-test  $t_{(16)} = 0.52$ ,  $P = 0.61$  in IV–V and IX lobule, respectively; Figure 2d–e′ and g), whereas PCs were further decreased (60%) at 12 months (not shown). In parallel, a reduction in the number of PC axon boutons was found. A 20% decrease was already detected at 4 months of age (Student's *t*-test  $t_{(59)} = 4.66$ ,  $P < 0.001$ ; Supplementary Figure S3A, C and G), even without apparent PC loss, suggestive of retraction of the terminal arborization. At later time points, in parallel with massive PC death, considerable decrease in





**Figure 3** Increased levels of cytoskeletal elements in PTEN-KO PCs. In (a–b'), PCs of KO mice display stronger SMI32 staining than WT PCs, including in dendritic enlargements (compare b' with a'). (c' and d') Representative pictures of WT and KO cerebellum labeled with anti-MAP2 antibodies, which reveal high staining intensity in KO PC dendrites, particularly in their enlargements. In (e') and (f'), increased levels of acetylated alpha-tubulin are apparent in dendrites of KO PCs when compared with WT ones. (a, b, c, d, e and f) Anti-calbindin staining. (g, h and i) Quantification of SMI32, MAP2, and ace-tubulin staining intensity, respectively (arbitrary units on the y axis). Scale bar: 50  $\mu$ m in a (also applies to a'–f'). \*\*\* $P < 0.001$ , Student's unpaired *t*-test. PC, Purkinje cell; PTEN-KO, Phosphatase and tensin homolog missing on chromosome 10-knockout; WT, wild type.

the number of PC axon terminals was evident (70% decrease at 6 and 9 months of age, Student's *t*-test  $t_{(38)} = 10.04$ ,  $P < 0.05$ , Student's *t*-test  $t_{(42)} = 18.12$ ,  $P < 0.001$ , respectively; Supplementary Figure S3D, E and G). Consistent with PC death, the thickness of the mutant molecular layer was decreased from 6 months of age when compared with the thickness of 4-month-old PTEN-KO mice (Figure 2c–e'; Supplementary Table S3).

To assess whether PC death was due to apoptosis, we analyzed PTEN-KO cerebella from 9-month-old mice for the expression of the apoptosis marker cleaved caspase-3. A few PCs positive for cleaved caspase-3-positive (approximately one cell/slice) were detected in mutant animals (Supplementary Figure S4A and B), whereas no cleaved caspase-3-positive PCs were found in WT mice, indicating that apoptosis is one mechanism of PC death in mutant mice.

Abnormal focal swellings of dendritic shafts (accompanied by abnormally high levels of the microtubule-associate protein MAP1B and tubulins), axonal torpedoes and neuronal degeneration have been observed in PCs of mutant mice lacking MAP1A (Liu *et al*, 2015). The presence of

similar dendritic and axonal features in PTEN mutant PCs prompted us to investigate the organization of cytoskeletal components, such as microtubules and neurofilaments. We analyzed MAP2, acetylated  $\alpha$ -tubulin and neurofilaments. We found a remarkable increase of neurofilaments content in all PC compartments of PTEN-KO PCs (Student's *t*-test  $t_{(115)} = 12.23$ ,  $P < 0.001$ ; Figure 3a–b' and g), in accordance with previous evidence in the same mouse line (Nayeem *et al*, 2007). MAP2 and acetylated  $\alpha$ -tubulin were expressed at higher levels in mutant PC dendrites (MAP2: Student's *t*-test  $t_{(48)} = 3.89$ ,  $P < 0.001$ , acetylated  $\alpha$ -tubulin: Student's *t*-test  $t_{(66)} = 9.11$ ,  $P < 0.001$ ) and were particularly accumulated in dendritic swellings (Figure 3c–f'). Altogether, these data suggest that PTEN is important for the maintenance of microtubule and neurofilament structures that are pivotal for the normal morphology of PCs.

#### Deficits of Spontaneous and Evoked Firing Activity of PTEN-KO PCs

By means of cell-attached patch-clamp recordings we measured the action potential firing of single PCs during

block of the main excitatory and inhibitory ionotropic receptors. In the spontaneous firing activity, the mean interspike interval (ISI) in PTEN-KO PCs was significantly increased with respect to WT mice ((KO cells ( $n=18$ ):  $65.61 \pm 8.01$  ms; WT cells ( $n=19$ ):  $37.85 \pm 4.38$  ms, Student's  $t$ -test  $t_{(35)}=3.08$ ,  $P<0.05$ ) without significant changes in the regularity of firing (coefficient of variation, KO:  $0.13 \pm 0.03$ ; WT:  $0.09 \pm 0.03$ ; Student's  $t$ -test  $t_{(34)}=1.16$ ,  $P>0.05$ ; Figure 4a–d and Supplementary Table S4). In order to study the mechanisms responsible for the reduced spontaneous firing, we studied evoked action potentials. In response to hyperpolarizing stimuli, PTEN-KO PCs showed a lower input resistance ( $-54.5 \pm 6.5$  M $\Omega$ ,  $n=13$ ) relative to WT neurons ( $-70.9 \pm 4.0$  M $\Omega$ ,  $n=12$ ; Student's  $t$ -test  $t_{(29)}=2.18$ ,  $P<0.05$ ; Figure 4e and f). On the contrary, no significant changes were observed for the bump due to the  $I_H$  current (Student's  $t$ -test  $t_{(29)}=1.15$ ,  $P>0.05$ ; Figure 4g and Supplementary Table S4). In line with the reduction of spontaneous firing, also in the evoked discharge PTEN-KO PCs fired action potentials with a lower frequency at all stimulus intensities (Figure 4h and i). In accordance with this result, PTEN-KO PCs displayed a more pronounced frequency adaptation with significantly prolonged second, third and tenth ISI (Student's  $t$ -test,  $t_{(13)}=2.78$ ,  $P<0.05$ ;  $t_{(12)}=3.58$ ,  $P<0.01$ , and  $t_{(12)}=3.46$ ,  $P<0.01$ , respectively) (Figure 4j and k and Supplementary Table S4). The latency of the first spike was highly variable and displayed a tendency to longer values (Supplementary Table S4). The other parameters of action potential were comparable between PTEN-KO and WT PCs (Supplementary Table S4). These data, taken together, corroborate the finding of a reduced excitability of PTEN-KO PCs.

### Altered Excitatory Postsynaptic Currents in PTEN-KO PCs

Parallel fibers (PF) were stimulated with intensities ranging from 3 to 15  $\mu$ A. The PF-EPSC amplitudes in PTEN-KO mice were significantly higher than in WT from stimulus intensity 6 to 15  $\mu$ A (WT:  $n=20$ ; KO:  $n=20$ ; two-way repeated measures ANOVA  $F_{(1,152)}=6.15$ ,  $P<0.05$ ); Figure 4l and m). This result indicates that the basal synaptic transmission is altered. To better understand whether the changes in this synapse are pre- or post-synaptic we performed paired-pulse stimulation with variable inter-stimulus interval. The time course of paired-pulse facilitation was similar in WT and PTEN-KO PCs at all inter-stimulus intervals (from 50 to 200 ms; two-way repeated measures ANOVA  $F_{(1,34)}=0.38$ ,  $P>0.05$ ; Figure 4n). The equal values of paired pulse facilitation across the genotypes indicate that the changes in PF-PC synapse are postsynaptic, in accordance with the severe dysmorphisms of PC dendrites.

The balance between PF and climbing fiber (CF) inputs is disrupted in other murine models of autism (Baudouin *et al*, 2012). Therefore, we decided to investigate the other excitatory input to PCs, the CF synapse. The amplitude of CF-EPSCs was reduced in PTEN-KO PCs (Figure 4o and p; KO:  $0.41 \pm 0.12$  nA,  $n=11$ ; WT:  $0.77 \pm 0.12$  nA,  $n=9$ ; Mann-Whitney test  $P<0.05$ ). To test whether such a decreased synaptic size of the CF-PC synapse was due to pre- vs post-synaptic mechanisms, we analyzed its short-term depression, which is known to be affected selectively by

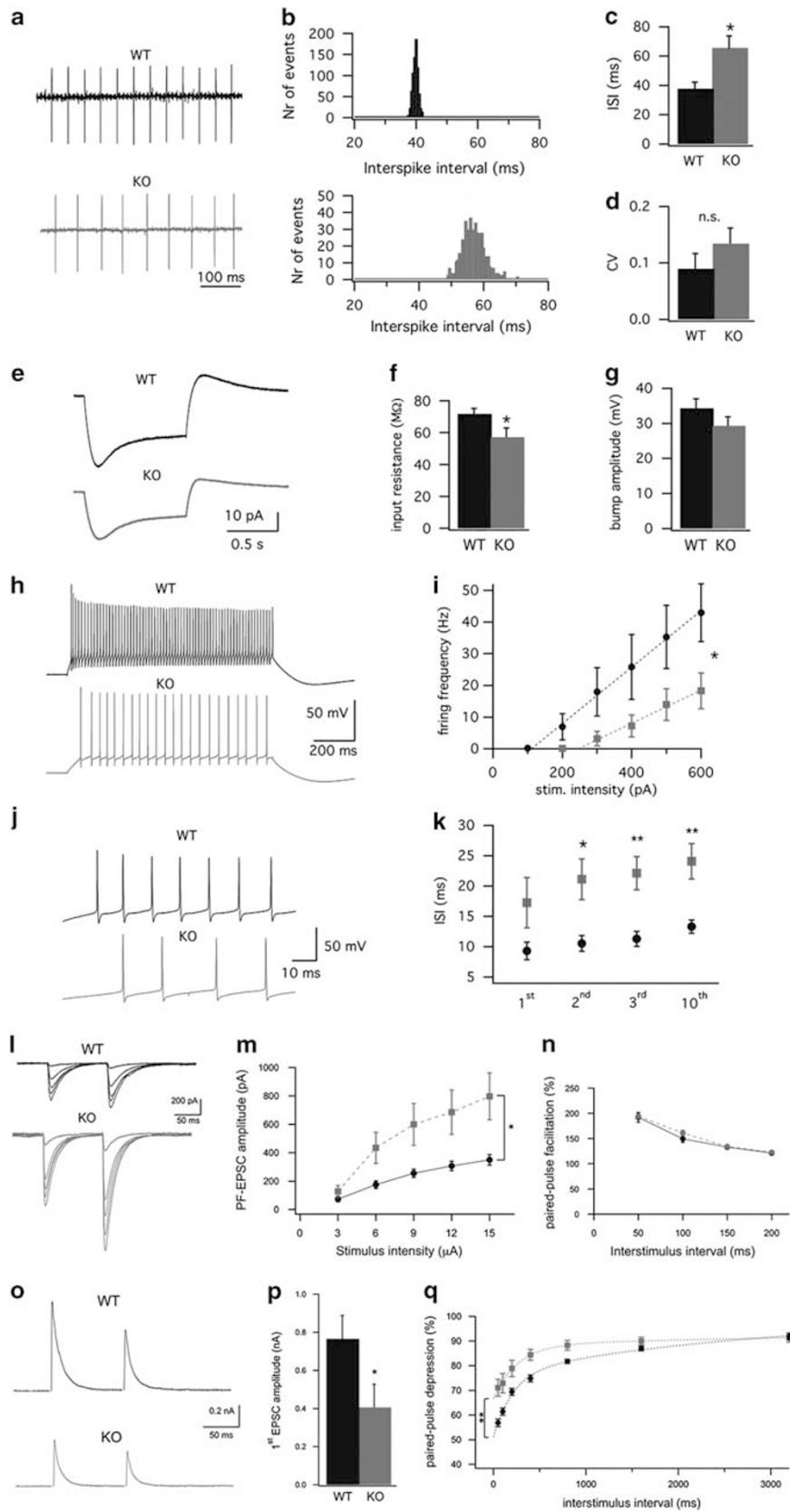
presynaptic changes (Hashimoto and Kano, 1998). At short and intermediate inter-stimulus intervals, between 50 and 400 ms, PTEN-KO PCs displayed a reduced short-term depression relative to WT (Figure 4q; two-way repeated measures ANOVA  $F_{(1,16)}=8.99$ ,  $P<0.01$ ). The time course of short-term depression was described by double exponential functions with the following fast and slow time constants and relative amplitudes: WT,  $\tau_f=208.2$  ms, 46.7%;  $\tau_s=2.93$  s, 53.3%; KO,  $\tau_f=260.2$  ms, 61.4%;  $\tau_s=11.31$  s, 38.6%. These results on the CF synapse are consistent with a presynaptic deficiency.

### PTEN-KO Mice Show Repetitive Behavior

All behavioral tests were performed before the onset of PC death, namely at 3–4 months of age. Among stereotyped/repetitive behaviors, we analyzed grooming and upright scurrying/jumping. Upright scurrying consists in a fast, rhythmic movement of the forepaws, and sometimes of one hind paw as well, against the cage walls while the mouse is standing in an upright position (Supplementary Video S1). This behavior, which sometimes was mixed with jumping, has been previously observed in ASD-mouse models (Ryan *et al*, 2010; Muehlmann and Lewis, 2012; Won *et al*, 2012). In PTEN KO mice, grooming was decreased ( $88.56 \pm 16.83$  s in WT vs  $35.00 \pm 11.62$  s in KOs, Student's  $t$ -test  $t_{(19)}=2.69$ ,  $P<0.05$ ; Figure 5b), whereas upright scurrying/jumping was greatly enhanced ( $7.50 \pm 3.67$  s in WT vs  $129.09 \pm 20.15$  s in KOs, Student's  $t$ -test  $t_{(19)}=5.66$ ,  $P<0.001$ ; Figure 5a). This behavior was present in all analyzed PTEN-KO mice (in which it lasted  $>60$  s), whereas shorter bouts of this behavior were only seen in 2 out of 10 WT mice (lasting 21 and 35 s, respectively; Supplementary Figure S5).

### PTEN-KO Mice Show Abnormal Social Behavior

In the three-chambered assay of sociability, WT mice spent significantly more time in the chamber with the stranger mouse than in the chamber with the object ( $284.66 \pm 15.32$  s vs  $154.60 \pm 9.11$  s, Student's paired  $t$ -test  $t_{(19)}=6.22$ ,  $P<0.001$ ; Figure 5c). In contrast, PTEN-KO mice showed no preference for the stranger mouse ( $260.30 \pm 14.64$  s vs  $236.00 \pm 11.79$  s, Student's paired  $t$ -test  $t_{(23)}=1.06$ ,  $P=0.30$ ; Figure 5c). The social behavior was further tested by assessing the social interaction with a female conspecific. We observed a significant reduction in the time spent by male PTEN-KO mice sniffing, allogrooming, mounting, or following the female with respect to WT ( $59.70 \pm 12.13$  s in KO vs  $111.30 \pm 8.24$  s in WT mice; Student's  $t$ -test  $t_{(18)}=3.52$ ,  $P<0.01$ ; Figure 5d). To exclude that the impaired sociability score was due to locomotor deficits or bouts of repetitive behavior, we measured the distance travelled by the mice during the social approach test and in the open field. In the three-chamber apparatus, in both the habituation and test phase, locomotion was not significantly different between genotypes (distance travelled in the habituation phase, WT:  $4318 \pm 283$  cm; KOs:  $4391 \pm 218$  cm, Student's  $t$ -test  $t_{(42)}=0.20$ ,  $P=0.84$ ; in presence of stimuli, WT:  $3358 \pm 222$  cm, KO:  $3463 \pm 120$  cm; Student's  $t$ -test  $t_{(42)}=0.43$ ,  $P=0.68$ ). Also in the open field, the distance travelled by the mice was comparable in WT and





mutants (WT:  $3827.73 \pm 213.11$  cm, KO:  $4153.58 \pm 327.62$  cm; Student's *t*-test  $t_{(19)} = 0.85$ ,  $P = 0.41$ ).

### Spatial Learning and Cognitive Flexibility are Preserved in PTEN-KO Mice

To model cognitive inflexibility we tested animals in a reversal learning paradigm using a Morris water maze or a water T-maze. In both the acquisition phase and the probe trial of Morris water maze, WT and PTEN-KO mice showed similar results (acquisition phase: two-way repeated measures ANOVA  $F_{(1,23)} = 0.24$ ,  $P > 0.05$ ; probe trial: Student's *t*-test for target area  $t_{(23)} = 0.53$ ,  $P = 0.60$ ; Supplementary Figure S6A and B). In the reversal phase, the latency to find the platform of PTEN-KO mice was comparable to that of WT mice over 2 days of training (two-way repeated measures ANOVA  $F_{(1,23)} = 1.94$ ,  $P > 0.05$ ; Supplementary Figure S6C), and the percentage of time spent in the target zone in the probe trial was similar to that of WT (Student's *t*-test  $t_{(23)} = 0.05$ ,  $P = 0.96$ ; Supplementary Figure S6D). We also tested PTEN-KO mice in the reversal water T-maze test. On the I acquisition day, mutant animals had similar acquisition learning compared with WT mice (number of correct trials: Student's *t*-test  $t_{(18)} = 1.34$ ,  $P = 0.20$ ; number of trials prior to five consecutive correct trials: Student's *t*-test  $t_{(18)} = 1.02$ ,  $P = 0.32$ ; Supplementary Figure S6E and F). In the reversal phase, mutant mice did not show any impairment in learning of the new platform location

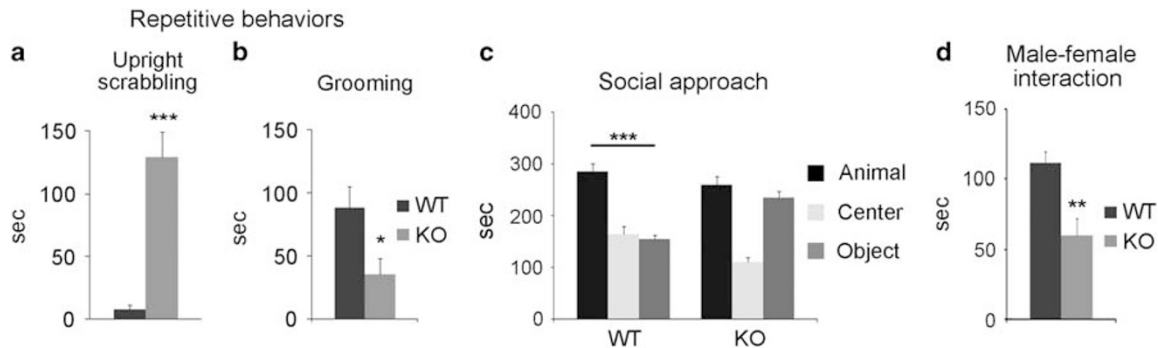
(I day, number of correct trials: Student's *t*-test  $t_{(17)} = 0.93$ ,  $P = 0.37$ ; number of trials prior to five consecutive correct trials: Student's *t*-test  $t_{(17)} = 0.19$ ,  $P = 0.85$ ; II day: number of correct trials: Student's *t*-test  $t_{(17)} = 0.46$ ,  $P = 0.65$ ; number of trials prior to five consecutive correct trials: Student's *t*-test  $t_{(17)} = 0.15$ ,  $P = 0.88$ ; Supplementary Figure S6E and F).

### Coordination and Motor Learning in PTEN-KO Mice

We investigated whether PTEN-KO mice showed motor deficits in the rotarod test. In 2–3 months old mice, the latency to fall was not significantly different between genotypes (two-way repeated measures ANOVA  $F_{(1,13)} = 1.28$ ,  $P > 0.05$ ; Supplementary Figure S7A). However, at 5–6 months of age, PTEN-KO mice tended to have a shorter latency than WT mice on the I day (two-way repeated measures ANOVA  $F_{(1,11)} = 12.94$ , Bonferroni *post hoc*  $P = 0.09$ ) and showed no improvement with time (Supplementary Figure S7B; WT vs KOs: Bonferroni *post hoc*  $P < 0.01$  on the II day,  $P < 0.01$  on the III day), indicating deficits in motor coordination and learning in association with ongoing PC loss.

### DISCUSSION

In this study we provide evidence that PTEN in cerebellar PCs has a key role in mechanisms involved in ASDs. We show that mice with a selective disruption of PTEN in PCs



**Figure 5** Repetitive behavior and impaired sociability in PTEN-KO mice. PTEN-KO mice spend more time doing upright scrabbling mixed with jumping than WT mice (a) and less time self-grooming (b) (WT:  $n = 10$ ; KOs:  $n = 11$ ). In the social approach test, WT mice spend more time in the chamber with the stranger animal than in the chamber with the inanimate object, whereas KO mice show no preference (WT:  $n = 20$ ; KOs:  $n = 24$ ; c). Reduced sociability of KO mice is also evident when measuring the time spent interacting with a female conspecific, which is significantly less than that of WT mice (WT:  $n = 10$ ; KO:  $n = 10$ ; d). Data shown are means  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , Student's unpaired *t*-test in (a), (b) and (d), Student's paired *t*-test in (c).

**Figure 4** PC spontaneous and evoked firing activity, PF-EPSCs and CF-EPSCs in PTEN-KO and WT PCs. (a) Representative traces of spontaneous firing in cell attached configuration in a WT (upper trace) and a KO PC (lower trace). (b) Distribution histograms of interspike intervals from the WT and KO PCs shown in (a). (c) Mean interspike interval (ISI) of WT ( $n = 19$ ) and KO ( $n = 18$ ) PCs. (d) Coefficient of variation (CV) of interspike intervals in WT and KO PCs. (e) Hyperpolarizations evoked by negative current injection ( $-400$  pA) in WT (black) and KO (grey) PCs. (f) Mean input resistance of WT and KO PCs. (g) Mean amplitude of the bump due to the inward rectifier current. (h) Representative traces of firing evoked by injection of depolarizing current. (i) Evoked firing frequency as a function of stimulation intensity. Dashed lines are linear fittings of points above threshold. (j) Representative traces of evoked firing showing the first spikes where the interspike intervals was measured. (k) Mean values of first, second, third and tenth interspike intervals. (l) Representative traces of PF-EPSCs recorded from WT and KO PCs. (m) Input-output curve of PF-EPSCs of WT ( $n = 20$ ) and KO ( $n = 20$ ) mice. (n) PF-EPSC short-term facilitation. The amplitude of the second response is expressed as a percentage of the first response (mean  $\pm$  SEM) and is plotted as a function of interstimulus intervals. (o) Representative traces of CF-EPSCs recorded from WT and KO PCs. (p) Mean CF-EPSC amplitude in WT ( $n = 11$ ) and KO ( $n = 9$ ) mice. (q) Time course of paired-pulse depression of CF-EPSC in WT (black circles) and KO mice (grey squares). The amplitude of the second response is expressed as a percentage of the first response (mean  $\pm$  SEM) and is plotted as a function of interstimulus intervals. The dashed lines are double exponential fittings of WT and KO data points. Stimulus artifacts have been omitted for clarity. In all panels, WT data are shown in black and KO ones in grey. \* $P < 0.05$ , \*\* $P < 0.01$ , Student's unpaired *t*-test in (c, f, k, p), two way-ANOVA followed by Bonferroni's *post hoc* analysis in (i, m, q). CF, climbing fiber; KO, knockout; PF, parallel fibers; WT, wild type.

display behavioral traits related to ASDs, associated with abnormal signaling and aberrant morphology of PCs.

### Behavioral Features

The three core symptoms of ASDs are impairment in social interaction, communication deficits, and the propensity for restricted, repetitive behaviors (Lai *et al*, 2014). Several mouse models of ASD are currently available, which reproduce one or more of these core symptoms (Pasciuto *et al*, 2015). PTEN-KO mice showed a severe impairment in social interaction, as well as a repetitive behavior, namely upright scrambling/jumping. Self-grooming, in contrast, was decreased. The longer time spent in futile, stereotyped jumping/scrabbling may have significantly decreased the time left for physiological grooming. In addition, our data are in line with the finding of a single type of repetitive behavior in other ASD-mouse models (Ryan *et al*, 2010; Muehlmann and Lewis, 2012; Won *et al*, 2012). Taken together, the deficits in sociability and the presence of a restricted repetitive behavior validate PTEN-KO mice as a model of ASDs. In order to distinguish ASD-related symptoms from mental retardation, it is necessary to exclude cognitive impairment. Indeed, PTEN-KO mice showed a normal performance in all cognitive tests, including cognitive flexibility.

In addition to the symptoms directly related to ASD, PTEN-KO mice also displayed impaired motor learning in the rotarod test in concomitance with PC loss. This motor deficit was expected, as motor performance requires a proper function of the cerebellar network. Indeed, it has also been observed in a number of other mutants displaying PC loss (see for review Porras-García *et al*, 2013). Nonetheless, gait abnormalities, anomalous motor learning or other types of motor symptoms are often present in ASD patients, and they have been related to cerebellar malformation (Marko *et al*, 2015).

### PC Functional Alterations

Alterations in the generation of PC action potentials have been classically considered responsible for cerebellar motor deficits and, more recently, for ASD-related symptoms (Tsai *et al*, 2012). In our study we found that PTEN-KO PCs have a lower frequency of spontaneous firing, are less excitable and have a smaller input resistance. The reduction in input resistance can be responsible, at least in part, for the lower excitability. A reduced firing frequency and input resistance were previously described also in PC-selective *Tsc1* knock-out mice (Tsai *et al*, 2012), which also display an autistic-like behavior, suggesting that these features could be common alterations related to this disorder.

The output signals from PCs are finely regulated by impinging synaptic inputs. An increase in excitatory transmission in PTEN-KO models is well documented in several studies in different types of neuronal cells (Luikart *et al*, 2011; Takeuchi *et al*, 2013). Here we report for the first time in PTEN-KO PCs an increased amplitude of EPSCs evoked by stimulation of the PF-PC synapse. This change seems to be postsynaptic because the paired-pulse facilitation is unchanged. On the contrary, the amplitude of EPSCs evoked by stimulation of the CF-PC synapse was reduced

and the paired-pulse depression was enhanced in PTEN-KO mice, suggesting a presynaptic mechanism (Hashimoto and Kano, 1998). At this synapse, presynaptic changes induced by postsynaptic signals have been described both during development (Bosman and Konnerth, 2009) and in the adult (Safo *et al*, 2006). Since CFs exert a powerful tonic inhibition on PCs (Montarolo *et al*, 1982), a weaker CF input and a stronger PF synapse have additive effects, resulting in a greatly enhanced excitatory drive. It is interesting to note that in a different model of autism, the neuroligin3 KO mouse, PF-PC and CF-PC synapses are altered, but in the opposite direction than in PTEN-KO PCs (Baudouin *et al*, 2012). This suggests that an incorrect balance between PF and CF synapses might underlie autistic-like deficiencies.

### PC Morphological Alterations

In this study we show that PTEN has a key role also in the regulation of PC morphology. The lack of *PTEN* in PCs induced remarkable structural changes, including overgrowth of all PC compartments, axonal and dendritic swellings, abnormal dendritic branching, accumulation of cytoskeletal components, ending with late-onset PC loss. Those findings add to preceding reports of hypertrophy and anomalous morphology in *PTEN*-deleted neurons (Kwon *et al*, 2006; Jaworski *et al*, 2005). PC hypertrophy was also observed in mice lacking *TSC1* or *TSC2* in PCs (Tsai *et al*, 2012; Reith *et al*, 2011), which are downstream targets of the *PTEN*/*PI3K* pathway. Intriguingly, in our model *PTEN* appears to have a critical role in modulating the organization/distribution of microtubule networks in the PC dendritic compartment, which are pivotal for normal dendritic morphology, intracellular transport and neuronal survival (Liu *et al*, 2015).

### PCs and Autism

PC loss has been observed in several autopsy studies of autistic patients (Palmen *et al*, 2004; Whitney *et al*, 2008; Skefes *et al*, 2014; Wegiel *et al*, 2014). Regarding animal models, Lurcher chimeric mice, which show cerebellar abnormalities including postnatal loss of PCs, display behavioral inflexibility and repetitive behavior (Martin *et al*, 2010). PC-selective p75 neurotrophin receptor KO mice exhibit less allogrooming and socialization/fighting, and more stereotyped jumping behavior (Lotta *et al*, 2014). Similar features were also strongly evident in our mutants, reinforcing the idea that PCs have a role in the regulation of specific autistic-like traits. In addition, in PC *Tsc1* mutant mice, social and cognitive deficits are associated with increased PC size, altered electrophysiological properties, and PC loss (Tsai *et al*, 2012), which remind some of the features observed in our model. As shown by p-S6 increase, *TSC*/*mTOR* signaling pathway is one of the downstream pathways, which may mediate the effects of *PTEN* loss in our model. However, several differences at the cellular and behavioral level occur between *Tsc1/Tsc2* mice (Tsai *et al*, 2012; Reith *et al*, 2011) and PTEN-KO mice, suggesting that *PTEN*, besides acting on *TSC*, may also operate through alternative mechanisms, such as *AKT/GSK-3* pathway (Nayeem *et al*, 2007; Marino *et al*, 2002), to induce specific morphological features and behavioral traits.



How the cerebellum modulates the abnormal behaviors of autism remains a topic of intense investigation. Autism has been proposed to be a disorder of multiple brain networks, including neocortical substrates (Muller, 2007). The cerebellum is connected, through the cerebellar nuclei, to these networks, and has been proposed to regulate them. For example, cerebellar output through the cerebellar nuclei modulates dopamine release in the medial prefrontal cortex via dopaminergic projections from the ventral tegmental area and glutamatergic projections from the mediodorsal and ventrolateral thalamus (Mittleman *et al*, 2008; Rogers *et al*, 2011). By affecting cerebellar nuclei activity, PTEN-KO PC dysfunction may thus alter downstream neuronal networks, thereby contributing to autistic-like behaviors.

On the whole, PTEN-KO mice represent a valuable experimental model to investigate the effects of PC dysfunction on ASD-related neuronal networks and to gain a deeper insight on mechanisms contributing to the pathogenesis of ASDs caused by *PTEN* mutation.

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