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Loss of Ahi1 Affects Early Development by Impairing BM88/Cend1-Mediated Neuronal Differentiation

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Mutations in the Abelson helper integration site-1 (*AHI1*) gene result in N-terminal Ahi1 fragments and cause Joubert syndrome, an autosomal recessive brain malformation disorder associated with delayed development. How *AHI1* mutations lead to delayed development remains unclear. Here we report that full-length, but not N-terminal, Ahi1 binds Hap1, a huntingtin-associated protein that is essential for the postnatal survival of mice and that this binding is regulated during neuronal differentiation by nerve growth factor. Nerve growth factor induces dephosphorylation of Hap1A and decreases its association with Ahi1, correlating with increased Hap1A distribution in neurite tips. Consistently, Ahi1 associates with phosphorylated Hap1A in cytosolic, but not in synaptosomal, fractions isolated from mouse brain, suggesting that Ahi1 functions mainly in the soma of neurons. Mass spectrometry analysis of cytosolic Ahi1 immunoprecipitates reveals that Ahi1 also binds Cend1 (cell cycle exit and neuronal differentiation protein 1)/BM88, a neuronal protein that mediates neuronal differentiation and is highly expressed in postnatal mouse brain. Loss of Ahi1 reduces the levels of Cend1 in the hypothalamus of Ahi1 KO mice, which show retarded growth during postnatal days. Overexpressed Ahi1 can stabilize Cend1 in cultured cells. Furthermore, overexpression of Cend1 can rescue the neurite extension defects of hypothalamic neurons from Ahi1 KO mice. Our findings suggest that Cend1 is involved in Ahi1-associated hypothalamic neuronal differentiation in early development, giving us fresh insight into the mechanism behind the delayed development in Joubert syndrome.

Introduction

Ahi1, also known as Jouberin (Dixon-Salazar et al., 2004), is a protein encoded by the Abelson helper integration-1 (*AHI1*) gene, mutations in which lead to Joubert syndrome (Dixon-Salazar et al., 2004; Ferland et al., 2004), a rare autosomal recessive disorder characterized by abnormal brain structure and delayed development (Poirier et al., 1989; Gitten et al., 1998; Maria et al., 1999; Koshy et al., 2010). The *AHI1* gene is also known as a susceptibility gene for schizophrenia and autism (Holroyd et al., 1991; Amann-Zalcenstein et al., 2006; Ingason et al., 2007, 2010; Alvarez Retuerto et al., 2008; Torri et al., 2010), two common neuropsychological disorders associated with abnormal brain development, as well (Hamlyn et al., 2013). The link between the *AHI1* gene and brain developmental disorders suggests that AHI1 plays an important role in early brain development. Consistently, mouse Ahi1 is expressed abundantly in neurons, and its expression is developmentally regulated (Doering et al., 2008; Sheng et al., 2008). However, how *AHI1* mutations retard early development remains to be investigated.

The function of Ahi1 is related to intracellular signaling and trafficking, as it contains seven Trp-Asp (WD) repeats, a Src homology 3 (SH3) domain, and a coiled-coil domain, which have been found in adaptor and signaling molecules (Jiang et al., 2002). Indeed, mouse Ahi1 is involved in Rab8a intracellular transport (Hsiao et al., 2009) and associates with huntingtin-associated protein-1 (Hap1), a neuronal protein that binds huntingtin to participate in microtubule-dependent transport (Li and Li, 2005; Borrell-Pagès et al., 2006; Caviston and Holzbaur, 2009). Hap1 is essential for the survival of postnatal mice (Chan et al., 2002; Li et al., 2003) and consists of two isoforms, Hap1A and Hap1B, which differ at their C-terminal regions (Li et al., 1995). The C-terminal region of Hap1A can be dephosphorylated by nerve growth factor (NGF), and this dephosphorylation can lead Hap1A to localize in neurite tips during neurite outgrowth (Rong et al., 2006). We do not yet know how the Hap1-Ahi1

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complex participates in the neuronal differentiation relevant to early brain development, but addressing this issue would help us understand how *AHI1* mutations affect neuronal development in Joubert syndrome.

Here we report that NGF, which induces neuronal differentiation, can decrease the association of mouse Ahi1 with Hap1 and that Ahi1 also associates with Cend1 (cell cycle exit and neuronal differentiation protein 1)/BM88, a protein that mediates neuronal differentiation and is highly expressed in postnatal mouse brain (Mamalaki et al., 1995; Politis et al., 2007; Katsimpardi et al., 2008). In the hypothalamus of Ahi1 knock-out (KO) mice, which show slow growth during postnatal days, we see a reduction of Cend1. Ahi1 binds Cend1 and stabilizes its level in cultured cells, and overexpression of Cend1 can rescue the neurite outgrowth defects of Ahi1 KO hypothalamic neurons. Our findings suggest that the Ahi1-Cend1 interaction is involved in hypothalamic neuronal differentiation during early brain development, providing new mechanistic insight into the delayed development in Joubert syndrome.

Materials and Methods

Antibodies and plasmids. Rabbit polyclonal antibodies against Hap1A, Hap1B, pHap1A (Rong et al., 2006), and Ahi1 (Xu et al., 2010) and guinea pig antibody (EM78) to Hap1 (Li et al., 2000) were generated in our previous studies. Mouse antibody to C38, equivalent to Cend1, was generated and described previously (Wakabayashi et al., 2010). Other antibodies used in the study were obtained from commercial sources as follows: mouse anti-γ-tubulin (Sigma-Aldrich), anti-GAPDH (Millipore), anti-PSD95 (Thermo), anti-SNAP25 (Millipore); anti-PI3K (BD Transduction Laboratory); rabbit anti-Akt, anti-p-Akt, anti-p-Erk (Cell Signaling Technology), anti-Erk (Santa Cruz Biotechnology), and anti-Cend1 (Cell Signaling Technology). All secondary antibodies were purchased from Jackson ImmunoResearch Laboratories.

Full-length Ahi1 (fAhi1) and truncated Ahi1 (tAhi1) plasmid were generated as described previously (Sheng et al., 2008). pCI-C38 (Cend1) plasmid was described in an earlier study (Wakabayashi et al., 2010). Hap1 siRNA and T598A plasmid were described in our previous study (Rong et al., 2006).

Mouse line. All animal procedures were approved by the Institutional Animal Care and Use Committee of Emory University. Ahi1$^{loxp/loxp}$ mice on a 129vEV/C57BL/6N background were produced as described previously (Xu et al., 2010). Mice homozygous for the floxed Ahi1 allele were crossed with mice carrying an EIIa promoter-driven Cre transgene [(The Jackson Laboratory, B6.FVB-Tg (EIIa-cre) C5379Lmgd/J)]. The EIIa adeno-viral promoter drives the expression of Cre recombinase in the early mouse embryo. Cre-mediated recombination occurs in a wide range of tissues, including the germ cells that transmit the genetic alteration to progeny. The resulting heterozygous mice were used to generate male homozygous KO (EIIa -Ahi1$^{-/-}$) mice, which were crossed with female wild-type (WT) mice (C57BL/6J) to produce heterozygous (Ahi1$^{+/-}$) mice that deplete one of the Ahi1 alleles via germline transmission. Such heterozygous Ahi1(+/−) mice were used to generate homozygous Ahi1 KO mice, which we call Ahi1 KO, on the mixed 129vEV/C57BL/6N background. Expression analysis of Ahi1 and behavioral tests of heterozygous mice (Ahi1$^{+/-}$) and WT mice revealed no differences. Because homozygous (Ahi1$^{-/-}$) and heterozygous (Ahi1$^{+/-}$) mice share the same mixed genetic background, mice of these two genotypes were mainly used to reveal behavioral differences related to Ahi1 deficiency.

Mouse behavior tests. Mouse body weight and growth were measured daily for the first month and were followed up monthly. Spontaneous locomotion was examined using an open field paradigm and a Versamax Animal Activity Monitoring System with infrared beams (AccuScan Instruments) for 36 h. Horizontal and vertical activities were automatically recorded. These values were averaged and chartered as total activity.

The forced swim test, which is widely used to examine depressive behavior in rodents, was performed as described previously (Xu et al., 2010). Mice were placed individually into a round plastic cylinder (18 cm height, 15 cm diameter) filled with water (25°C) at a depth of 10 cm. An investigator who was kept blind to the genotypes of the mice measured the immobility time for 6 min. Floating or the absence of active behaviors, such as swimming or struggling to escape, was measured as immobility time. No pretest training of mice was performed.

Immobility was rated in the tail suspension test according to an earlier description (Xu et al., 2010). Mice were suspended by taping the tail (~1 cm from tip of tail) to a horizontal bar at a height of 40 cm from the table surface for 6 min. The immobility time, defined as hanging passively and motionlessly without escape-oriented behaviors, was recorded.

Western blotting, subcellular fractionation, and coimmunoprecipitation. For Western blots, cultured cells or brain tissues were homogenized in RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA pH 8.0, 1 mM EGTA, pH 8.0, 0.1% SDS, 0.5% DOC, and 1% Triton X-100) with 1 × protease inhibitor from Sigma (P8340). The cell or tissue lysates were diluted in 1 × SDS sample buffer (62.6 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, and 0.01% bromophenol blue) and sonicated for 10 s after incubation at 100°C for 5 min. The total lysates were resolved in a Tris-glycine gel (Invitrogen) and blotted to a nitrocellulose membrane. The Western blots were developed using the ECL prime kit (GE Healthcare). Subcellular fractionation was performed as described previously (Gu et al., 2009). For immunoprecipitation, protein samples were precleared with a 50 μl volume of protein A agarose beads (Sigma), and the resulting supernatant was incubated with the primary antibody overnight, followed by the addition of 30 μl of protein A agarose beads for 1 more hour at 4° with constant agitation. The immunoprecipitates were washed three times in ice-cold lysis buffer and then eluted by boiling in 1 × SDS sample buffer for Western blotting.

Immunofluorescent staining. For immunofluorescent staining of cultured cells, cultured PC12 cells grown in 6-well plates were fixed in 4% paraformaldehyde in PBS for 10 min, permeabilized with 0.2% Triton X-100 in PBS for 30 min, blocked with 5% normal goat serum in PBS for 1 h, and incubated with primary antibodies in 2% normal goat serum/3% BSA/PBS overnight at 4°C. After several washes, the cells were incubated with secondary antibodies conjugated with either Alexa-488 or rhodamine Red-X (Jackson ImmunoResearch Laboratories). Hoechst dye (1 μg/ml) was used to label the nuclei.

For immunofluorescent staining of mouse brains, mice were anesthetized with 5% chloral hydrate and perfused with 10 ml 0.9% NaCl in PBS through the left cardiac ventricle. Mouse brains were then cut into sections with a cryostat (Leica) at −20°C. Mouse brain sections were fixed with 4% paraformaldehyde in PBS for 30 min and permeabilized with 0.5% Triton/PBS for 30 min. The sections were then stained with antibodies to Ahi1 or Cend1 for immunofluorescent labeling. Light micrographs were taken using a Zeiss microscope (Axiovert 200 MOT) and a 63× lens (LD-Achroplan 63×/0.75) with a digital camera (Hamamatsu Orca-100) and Openlab software.

Mass spectrometry analysis. The hypothalamic tissues from WT or Ahi1 KO mice were lysed and immunoprecipitated with anti-Ahi1. The immunoprecipitates were analyzed by reverse-phase liquid chromatography tandem mass spectrometry at the Emory Core facility. All analyses were performed on an LTQ-Orbitrap ion trap mass spectrometer (Thermo Finnigan). Acquired MS/MS spectra were searched against the mouse reference database of the National Center for Biotechnology Information, uncovering 379 proteins in our mass spectrometry analysis. Proteins that were more abundant in WT than Ahi1 KO immunoprecipitates were further characterized.

Cell culture. Human embryonic kidney (HEK) 293 cells were cultured in DMEM/F12 supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B. The rat pheochromocytoma (PC12) cell line was obtained from ATCC, and cells were grown in DMEM/F12 medium supplemented with 5% FBS, 10% horse serum, 100 μg/ml penicillin, and 100 μg/ml streptomycin, and incubated at 37°C in a humidified 5% CO_2 atmosphere chamber. Mouse neuroblastoma (N2A) cells were maintained in growth medium consisting of DMEM supplemented with 10% FBS and 1% penicillin/streptomycin solution. For Cend1 half-life assay, N2A cells were transfected with fAhi1 or PRK-vector plasmid for 24 h. On the second day, cells were treated with cycloheximide (100 μg/ml) for different times and then subjected to Western blotting analysis.

To evaluate neurite outgrowth, PC12 cells treated with NGF (100 ng/ml) or IGF (20 ng/ml) for 48 h were fixed with 4% paraformaldehyde in PBS for 15 min. After several washes with PBS, cells with neurites exceed-

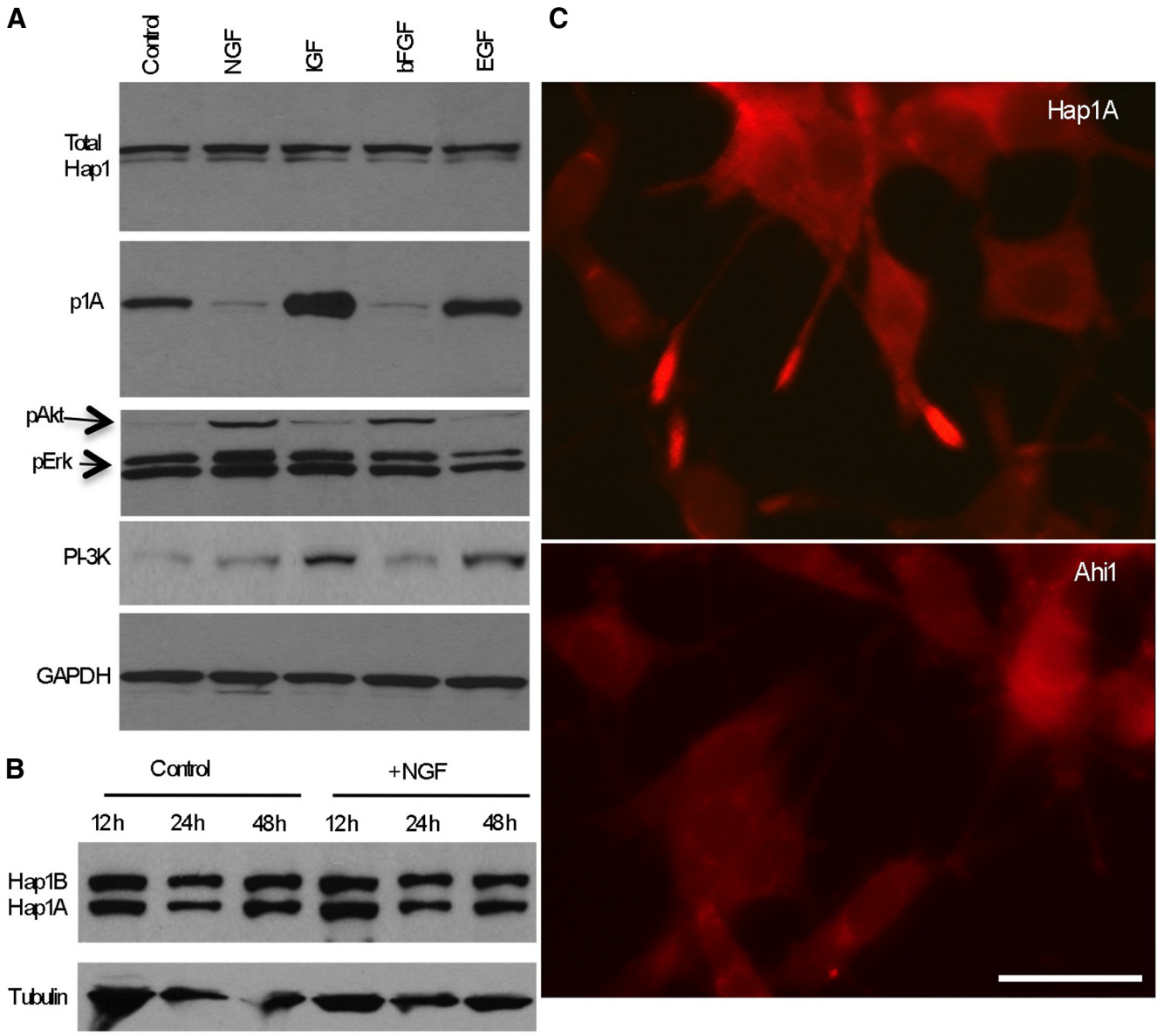


Figure 1. NGF treatment alters Hap1A phosphorylation and causes different distributions of Hap1A and Ahi1. ***A***, PC12 cells were treated with different neurotrophic factors (NGF, IGF, bFGF, and EGF) for 48 h and then subjected to Western blotting with antibodies to pAkt, pErk, and PI3K. NGF and bFGF increased pAkt and reduced phosphorylation of Hap1A (p1A). ***B***, NGF (100 ng/ml) treatment for 12– 48 h does not alter the levels of total Hap1 in PC12 cells. ***C***, Hap1A is enriched in neurite tips, whereas Ahi1 remains in the cell body after NGF treatment for 48 h. NGF-treated PC12 cells were stained by rabbit antibodies to Hap1A and Ahi1. Scale bar, 10 μm.

ing the cell body diameter were counted using an inverted microscope (Axiovert 200M) to obtain the percentage of total cells in the field (20×).

To culture primary neurons, hypothalamic neurons were isolated from the hypothalamus of postnatal day 1 mice. Dissected tissue was treated with 0.0625 mg/ml trypsin and 0.0625 mg/ml DNase (Invitrogen) in 1 × HBSS buffer without calcium and magnesium for 10 min at 37°C, followed by triturating with a 1 ml pipette tip 20 times. Cells were then washed once with the tissue culture medium and spun down at 1500 × g for 3 min. Cells were plated on top of a layer of astrocytes and grown initially in a 50% glial-conditioned medium [DMEM containing 0.25% glucose, 2 mM glutamate, 10% FCS, 500 nM insulin, 1 × vitamin mixture (M6895; Sigma), and 1% antibiotic–antimycotic (Invitrogen)]. The cells were then cultured in neurobasal/B27 medium following the method used in our previous study (Xu et al., 2010).

To quantify neuritic length of cultured hypothalamic neurons, we examined WT neurons and Ahi1 KO neurons. Neurites were quantified from images of neurons stained with antibodies to β-tubulin. Neurites that were fasciculated or could not be accurately assigned to specific cells were omitted from analysis. Total neurite length per cell was quantified by measuring the length of all neurites and branches on each cell with IMARIS software (Bitplane). Imaged cells were selected at random, and all visible processes of selected neurons were imaged.

Statistical analysis. Each experiment was repeated at least three times. For animal behavioral tests, 15–18 mice per group were examined. All data were expressed as mean ± SE. Statistical results were analyzed by GraphPad Prism (version 5) software, and statistical significance ($p < 0.05$) was assessed using Student's *t* test or one-way ANOVA, followed when appropriate by a *post hoc* analysis using Dunnett's test.

Results

Association of Ahi1 with Hap1A and regulation by phosphorylation

Because Ahi1 forms a stable protein complex with Hap1 (Sheng et al., 2008) and Hap1A is phosphorylated at its C-terminal region (Rong et al., 2006), we wanted to investigate how Hap1A is

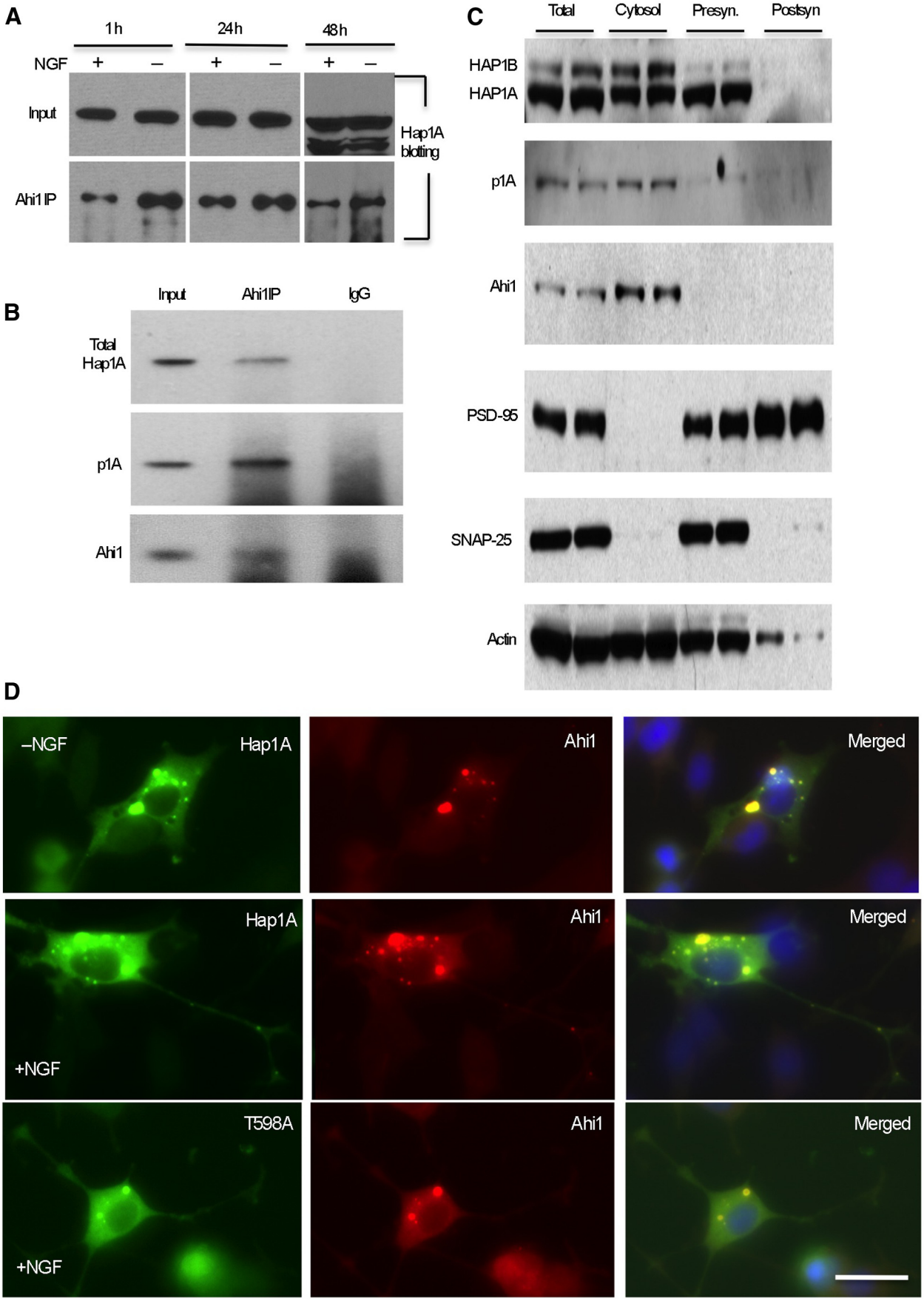


Figure 2. Dephosphorylation of Hap1A reduces its association with Ahi1. ***A***, PC12 cells were treated with NGF (100 ng/ml) for 1, 24, and 48 h, and then subjected to Ahi1 immunoprecipitation with anti-Ahi1. The immunoprecipitates were analyzed via Western blotting with antibody to Hap1A. More Hap1A was precipitated without NGF (−) than with NGF (+) (*Figure legend continues.*)

phosphorylated and whether this phosphorylation regulates the association of Ahi1 with Hap1. PC12 cells differentiate and grow long neurites when treated with NGF and bFGF, but not IGF and EGF (Vaudry et al., 2002; Itakura et al., 2005). Importantly, NGF (100 ng/ml) and bFGF (10 ng/ml) reduced Hap1A phosphorylation, whereas IGF (20 ng/ml) and EGF (20 ng/ml) increased Hap1A phosphorylation (Fig. 1*A*), suggesting that Hap1A phosphorylation is regulated by different signaling pathways for neuronal differentiation and proliferation. Indeed, knocking down Hap1 suppresses NGF-induced neurite outgrowth (Rong et al., 2006). These findings support the idea that Hap1-associated intracellular transport is critical for neuronal differentiation and growth.

PC12 cells treated with NGF, bFGF, IGF, or EGF for 48 h were also collected for Western blot analysis with antibodies to signaling molecules, such as phosphorylated Hap1A (p1A), Akt (pAkt), Erk (pErk), and phosphatidylinositide 3-kinases (PI3K). We saw a reduction in p1A, which was mediated not only by NGF, but also by bFGF (Fig. 1*A*). It is known that NGF and bFGF activate cAMP- and PKC-dependent signaling (Damon et al., 1990) in PC12 cells. Consistently, phosphorylation of Akt was increased by NGF and bFGF (Fig. 1*A*). In contrast, IGF and EGF, which did not increase pAkt and pErk but increased PI3K level (Vaudry et al., 2002; Itakura et al., 2005), also increased the phosphorylation of Hap1A (Fig. 1*A*). Examination of PC12 cells treated with NGF for different lengths of time revealed no significant difference in the levels of either Hap1A or Hap1B versus non–NGF-treated cells (Fig. 1*B*). These results suggest that various neurotrophic factors regulate Hap1A phosphorylation via different signaling pathways.

Because the action of NGF on the neurite outgrowth of PC12 cells has been well characterized, we next focused our study on the effect of NGF on the association of Ahi1 with Hap1. Because Hap1A plays a critical role in promoting neurite outgrowth (Li et al., 2000; Rong et al., 2006), we first examined the distribution of Hap1A and Ahi1 in PC12 cells treated with NGF and found that Hap1A is distributed in the neurite tips, whereas Ahi1 remains in the cytoplasm (Fig. 1*C*). This result suggests that NGF-mediated dephosphorylation may dissociate Ahi1 from Hap1A, or p1A may bind more Ahi1. To test this idea, we immunoprecipitated Ahi1 in PC12 cells treated with NGF for 1, 24, and 48 h and found that NGF treatment, which reduces Hap1A phosphorylation, indeed reduced the association of the total amount of Hap1A coprecipitated with Ahi1 (Fig. 2*A*). Compared with the relative amount of total Hap1A in the Ahi1 immunoprecipitates (precipitated/input = 0.78), more p1A (precipitated/input = 1.51) could be coprecipitated with Ahi1 by anti-Ahi1 (Fig. 2*B*). To more rigorously examine whether p1A shows an increased association with Ahi1, we isolated presynaptic and postsynaptic fractions from the mouse brain. We found that Hap1A is more enriched in the presynaptic fraction than Hap1B. Importantly, both Ahi1 and p1A are less abundant in the presynaptic fraction, but they are enriched in the cytosolic fraction (Fig. 2*C*). Overexpression of transfected Hap1A and its mutant (T598A) that eliminates the phosphorylated threonine at amino acid residue 598 could lead to cytoplasmic puncta in PC12 cells. These cytoplasmic puncta are also colocalized with transfected Ahi1. However, after NGF treatment, which can reduce Hap1A phosphorylation, Hap1A and T598A distributed in the neurites whereas transfected Ahi1 still largely remained in the cytoplasm (Fig. 2*D*). These findings from transfected cells also support the idea that Ahi1 associates with p1A in the soma of neuronal cells.

Because nonsense mutations in the human *AHI1* gene generate N-terminal truncated AHI1 shorter than the first 435 amino acids (Dixon-Salazar et al., 2004; Ferland et al., 2004), we thought it would be interesting to examine whether N-terminal Ahi1 (nAhi1) has any function or is toxic. We therefore generated a series of GST-Ahi1 fusion proteins to examine their association with Hap1 (Fig. 3*A*), of which mouse nAhi1 (1–284 amino acids) corresponds to the nonsense mutation at amino acid 435 of human AHI1. fAhi1 was found to strongly bind Hap1A expressed in HEK293 cells. Although the amount of nAhi1 used in GST-pulldown is much greater than fAhi1, this truncated Ahi1 showed a weak Hap1 binding signal. Other truncated Ahi1 proteins (mAhi1 or cAhi1) that contain only a WD40 or SH3 domain also showed a reduction in binding to Hap1 (Fig. 3*B*). All these results suggest that nAhi1 is unable to bind or binds weakly to Hap1 and that both the WD40 and SH3 domains are required for a strong association between Ahi1 and Hap1.

We then transfected nAhi1 into PC12 cells to compare its effect with transfected fAhi1. Although nAhi1 does not bind Hap1, it inhibited neurite outgrowth compared with transfected fAhi1 in PC12 cells that were treated with NGF (Fig. 3*C*). Consistent with our previous studies that tAhi1 was unable to increase both Hap1A and Hap1B compared with fAhi1 (Sheng et al., 2008), Western blot analysis showed that nAhi1 could also reduce the phosphorylation of Erk induced by NGF treatment (Fig. 3*D*). Thus, nAhi1 does not seem to maintain the normal function but could mediate a gain of toxic function.

Loss of Ahi1 retards early development in mice and reduces the level of Cend1

To rigorously examine the *in vivo* effects of *AHI1* mutations, we created the Ahi1-null mouse model, as most *AHI1* mutations cause the loss of fAhi1 (Dixon-Salazar et al., 2004; Ferland et al., 2004). We previously established floxed Ahi1 mice that deplete Ahi1 expression in neuronal cells by Cre expression under the control of the nestin promoter (Xu et al., 2010). To genetically recapitulate the condition in Joubert syndrome, we crossed floxed Ahi1 mice with transgenic mice expressing Cre during early development. The crossed male mice were then mated with WT female mice to select offspring mice that have depleted the *Ahi1* gene in the germline and can be used to produce homozygous Ahi1 KO mice in a manner inherited the same as in Joubert syndrome patients (Fig. 4*A*). Western blotting results confirmed the deletion of Ahi1 in brain regions, such as the hypothalamus and brainstem of Ahi1 KO mice. Consistent with our previous findings (Sheng et al., 2008), depletion of Ahi1 also reduced the levels of Hap1 (Fig. 4*B*). It was also interesting to see that Ahi1 could not be detected in the kidney and lung under our experimental conditions, underscoring the importance of studying the neuronal function of Ahi1.

As reported previously, some homozygous Ahi1 KO mice die at birth, and the death appears to depend on the genetic background (Hsiao et al., 2009; Louie et al., 2010). We generated Ahi1

←

(*Figure legend continued.*) treatment. ***B***, Ahi1 immunoprecipitates from PC12 cells showing more phosphorylated Hap1A (p1A or pHap1A) coprecipitated with Ahi1 compared with total Hap1A. ***C***, Mouse brain lysates were fractionated to separate presynaptic (Presyn.) from postsynaptic (Postsyn) fractions. Hap1A is enriched in the presynaptic fraction, whereas phosphorylated Hap1A (p1A) and Ahi1 remain in the cytosolic fraction. The blots were also probed with antibodies to the presynaptic protein SNAP25 and postsynaptic protein PSD95. ***D***, PC12 cells were transfected with Ahi1 with Hap1A or HAP1A mutant T598A. NGF treatment led to the distribution of transfected Hap1A or T598A in neuritis, whereas transfected Ahi1 remained in the cytoplasm. Scale bar, 10 μm.

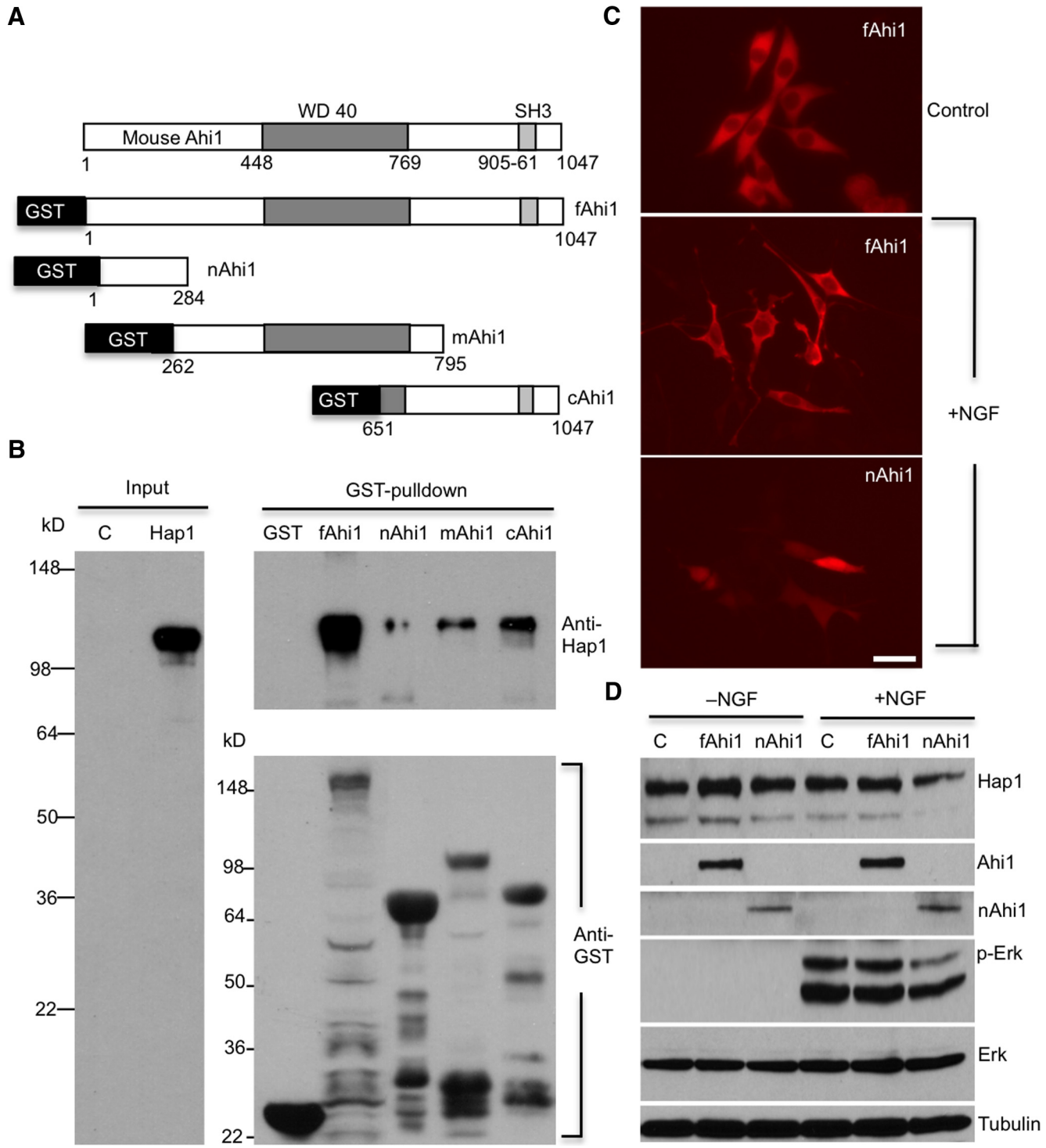


Figure 3. nAhi1 does not bind Hap1. ***A***, Structures of GST-fusion proteins used for *in vitro* binding to Hap1. ***B***, HEK293 cells were transfected with Hap1A, and cell lysates were pulled down by GST or GST-Ahi1 proteins. Only fAhi1 binds strongly to Hap1 (top). The amounts of GST-Ahi1 used for binding were revealed by probing the blot with anti-GST (bottom). ***C***, Transfection of fAhi1 and nAhi1 into PC12 cells showing reduced neurite length in nAhi1-transfected cells after NGF (100 ng/ml) treatment for 48 h. Control in fAhi1 transfected cells without NGF treatment. Scale bar, 10 μm. ***D***, Western blotting showing that NGF can increase pErk signaling, whereas expression of nAhi1 can inhibit this signaling or phosphorylation of Erk (pErk).

KO mice on the mixed 129vEV/C57BL/6N background; these mice could survive but showed a reduction in body weight during the postnatal stage. Ahi1 KO mice were no different from WT or heterozygous mice on postnatal day 2 (P2) but had a significantly retarded growth or gained less weight on postnatal day 15 (P15) (Fig. 4*C*). Statistical analysis of the body weight of control (heterozygous) and KO mice ($n = 15$ each group) revealed that the slower increase in body weight of Ahi1 KO mice became insignificant when these mice were older than 26 d (Fig. 4*D*), suggesting that loss of Ahi1 selectively retards mouse growth during the postnatal period. We also found that adult Ahi1 KO mice at 3–5 months of age displayed abnormal locomotor activity (Fig. 4*E*). Like nestin-Ahi1 KO mice (Xu et al., 2010), Ahi1-null mice also showed increased immobility in the forced swim test and tail

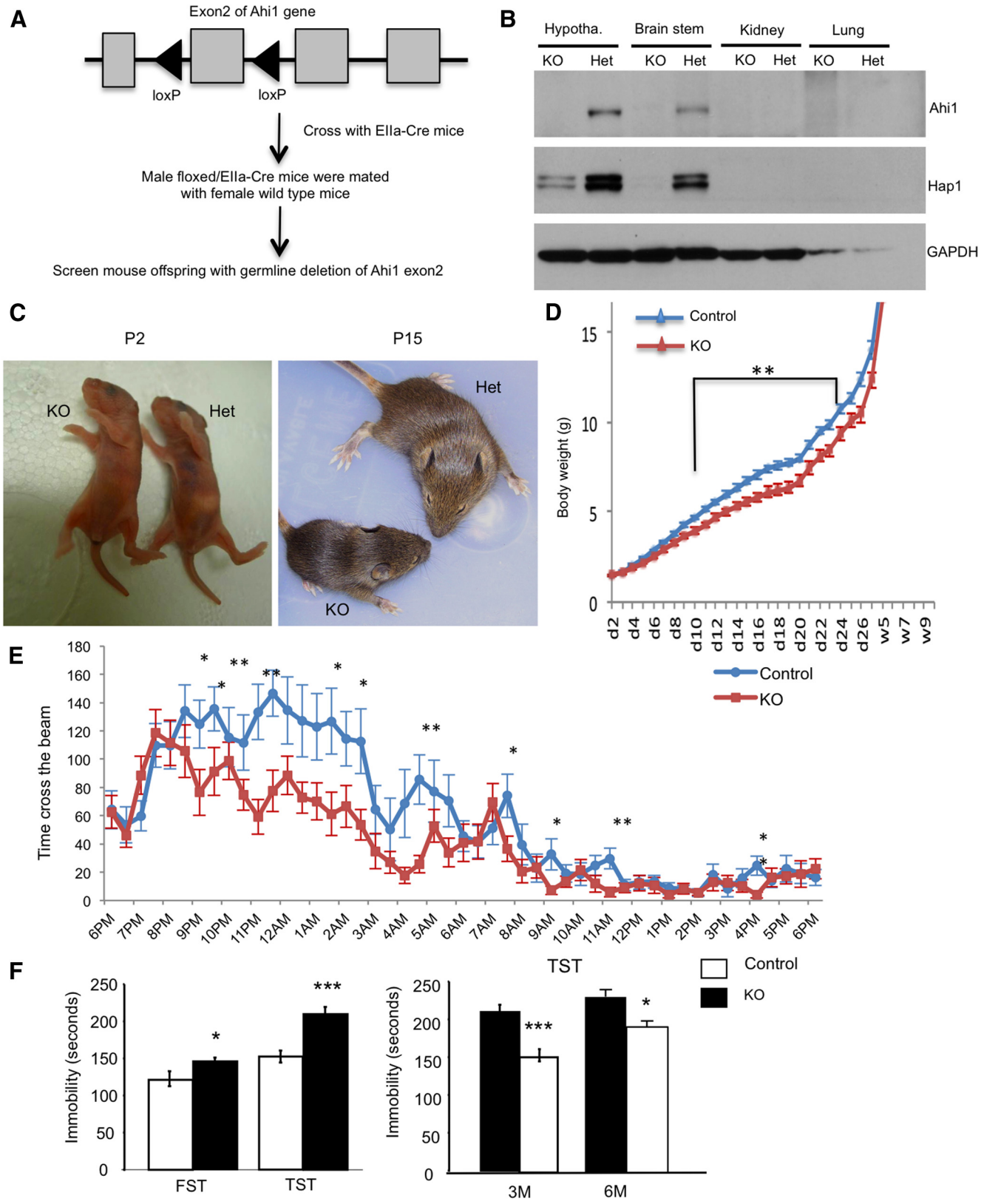
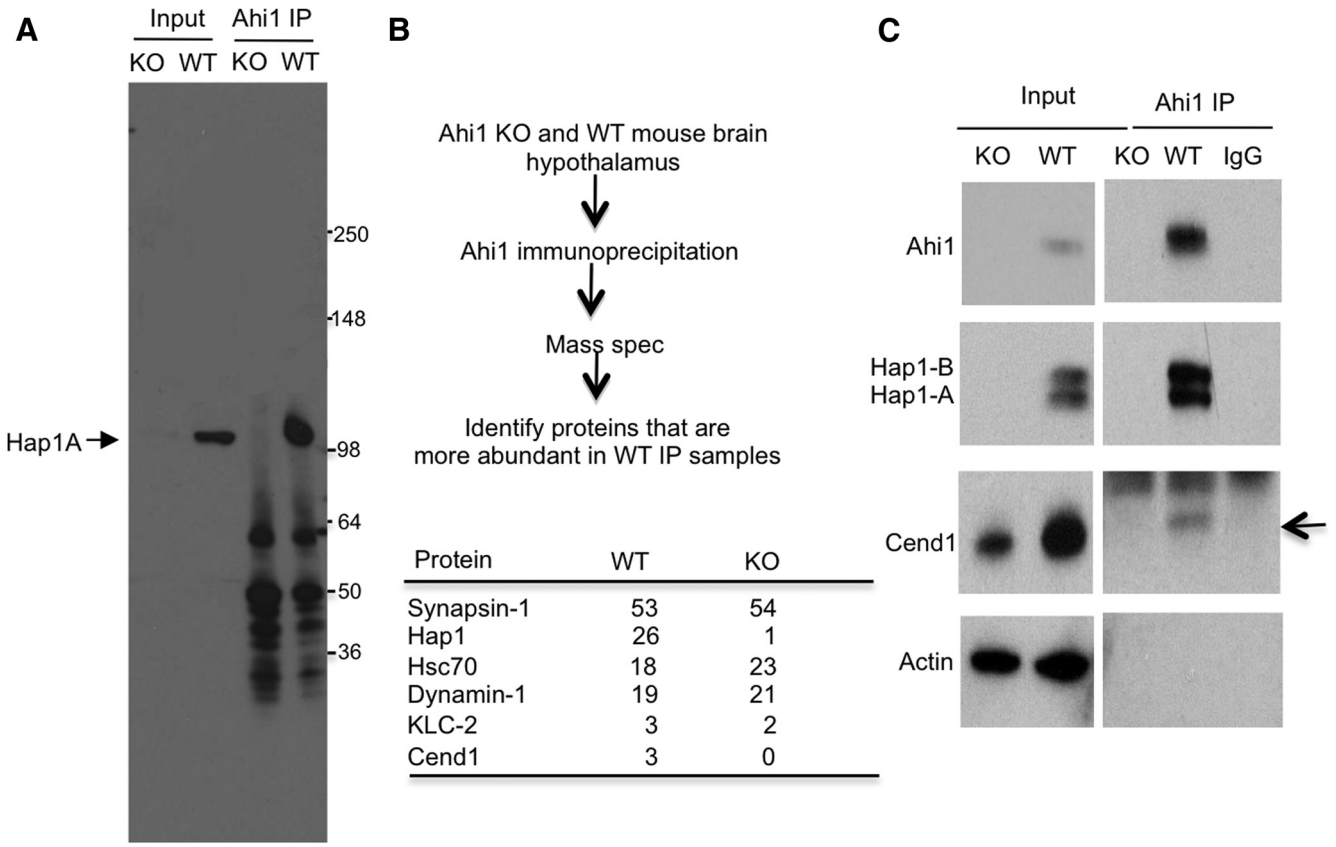


Figure 4. Loss of Ahi1 retards the early growth of mice. ***A***, The generation of Ahi1 KO mice. Floxed Ahi1 mice were mated with Ella-Cre transgenic mice. The crossed male mice were then mated with WT female mice to produce heterozygous and then homozygous germline Ahi1 KO offspring. ***B***, Western blotting verified the depletion of Ahi1 in the hypothalamus (Hypotha.) and brainstem. In control (heterozygous) mice, Ahi1 is not detected in kidney and lung. ***C***, P2 and P15 pups of heterozygous and Ahi1 KO mice. ***D***, The body weight of control (heterozygous) and Ahi1 KO mice also showing the retarded growth of Ahi1 KO mice from P10-P26; $n = 15$ in each group. $^{**}p < 0.01$. ***E***, Locomotor activity of control and Ahi1 KO mice showing reduced activity of KO mice. ***F***, Forced swim test (FST) and tail suspension test (TST) showing increased immobility or depressive phenotypes of Ahi1 KO mice at 3 and 6 months of age. $^{*}p < 0.05$, compared with control ($n = 15$–18 mice each group). $^{**}p < 0.01$, compared with control ($n = 15$–18 mice each group). $^{***}p < 0.001$ ($n = 15$–18 mice in each group).



Protein	WT	KO
Synapsin-1	53	54
Hap1	26	1
Hsc70	18	23
Dynamin-1	19	21
KLC-2	3	2
Cend1	3	0

Figure 5. Ahi1 associates with Cend1. ***A***, The hypothalamic tissue lysates of WT and Ahi1 KO mice were precipitated by anti-Ahi1 and subjected to Western blotting with anti-Hap1A. Hap1A is coprecipitated with Ahi1. ***B***, The strategy to identify Ahi1-interacting proteins. Ahi1 KO and WT hypothalamic tissue lysates were precipitated with anti-Ahi1. The immunoprecipitates were analyzed by mass spectrometry, which revealed some proteins that were more frequently identified in WT than in KO immunoprecipitates. ***C***, Western blotting verified the *in vivo* association of Cend1 (arrow) with Ahi1 in the mouse hypothalamic immunoprecipitates by anti-Ah1.

suspension test (Fig. 4*F*), which have been used to reflect depressive phenotypes in rodents (Cryan et al., 2005; Overstreet et al., 2007).

Despite these phenotypes, we were not able to identify any significant morphologic defects in the brains of surviving Ahi1 KO mice. The normal appearance of the overall brain morphology in other Ahi1-null mice has also been reported in earlier studies (Hsiao et al., 2009; Lancaster et al., 2011). Thus, loss of Ahi1 in mice is more likely to alter cellular function rather than cause an overt neuronal loss. To identify targets that are involved in Ahi1 depletion-related pathology, we performed Ahi1 immunoprecipitation of hypothalamic tissues from WT and Ahi1 KO mice to uncover Ahi1-interacting proteins, which should be more abundant in WT samples than KO samples (Fig. 5*A*). Mass spectrometry analysis revealed a total of 379 proteins that were present in the immunoprecipitates (Fig. 5*B*). As expected, we saw more Hap1 in WT immunoprecipitates than in KO immunoprecipitates. In addition, Cend1, also known as BM88, was only present in WT immunoprecipitates and was not detected in KO immunoprecipitates (Fig. 5*B*). As Cend1 is critical for neuronal differentiation (Mamalaki et al., 1995; Katsimpardi et al., 2008), we further performed Ahi1 immunoprecipitation of WT and KO hypothalamic lysates. Western blotting and quantitation of the ratio of precipitated to input on the same Western blot verified that Cend1 was coprecipitated with Ahi1 from WT (precipitated/input = 0.226) mouse hypothalamic tissue compare with Ahi1 KO immunoprecipitates (precipitated/input = 0.048) (Fig. 5*C*). Because both Hap1A and Hap1B form heterodimers and associate with Ahi1 (Sheng et al., 2008), the presence of both Hap1A and Hap1B in the immunoprecipitates also validates the association of Cend1 with Ahi1–Hap1 complex.

Western blot analysis shows that Cend1 is expressed at a high level in 1-month-old mouse brain and then declines in mature mouse brains at the age of 3 and 7 months (Fig. 6*A*), which is consistent with the important role of Cend1 in early brain development. Importantly, we saw that the Cend1 level was decreased in Ahi1 KO mouse brain hypothalamus. Further comparison of the relative levels of Cend1 in the hypothalamus, brainstem, hippocampus, and whole brain in mice at the age of 3 months revealed that Cend1 was selectively reduced in Ahi1 KO hypothalamus and brainstem (Fig. 6*B*), two brain regions normally enriched in Ahi1. Immunofluorescent staining of the mouse hypothalamic region also showed a reduction of Cend1 level in Ahi1 KO neurons (Fig. 6*C*). In addition, our results show that Cend1 is not colocalized with Ahi1-positive puncta in the cytoplasm (Fig. 6*C*), suggesting that Cend1 interacts mainly with soluble Ahi1.

Ahi1 stabilizes Cend1 to promote neurite differentiation

Because Ahi1 can stabilize neurotrophic factor receptors (Sheng et al., 2008; Xu et al., 2010), we wanted to examine whether Ahi1 also stabilizes Cend1, such that a loss of Ahi1 reduces the level of Cend1. We first expressed fAhi1 in cultured N2A cells, and then examined the endogenous level of Cend1 that could be recognized by the available rabbit anti-Cend1. Two controls were used in this experiment: vector-transfected cells and transfected cells expressing nAhi1, which does not bind Hap1. We found that only

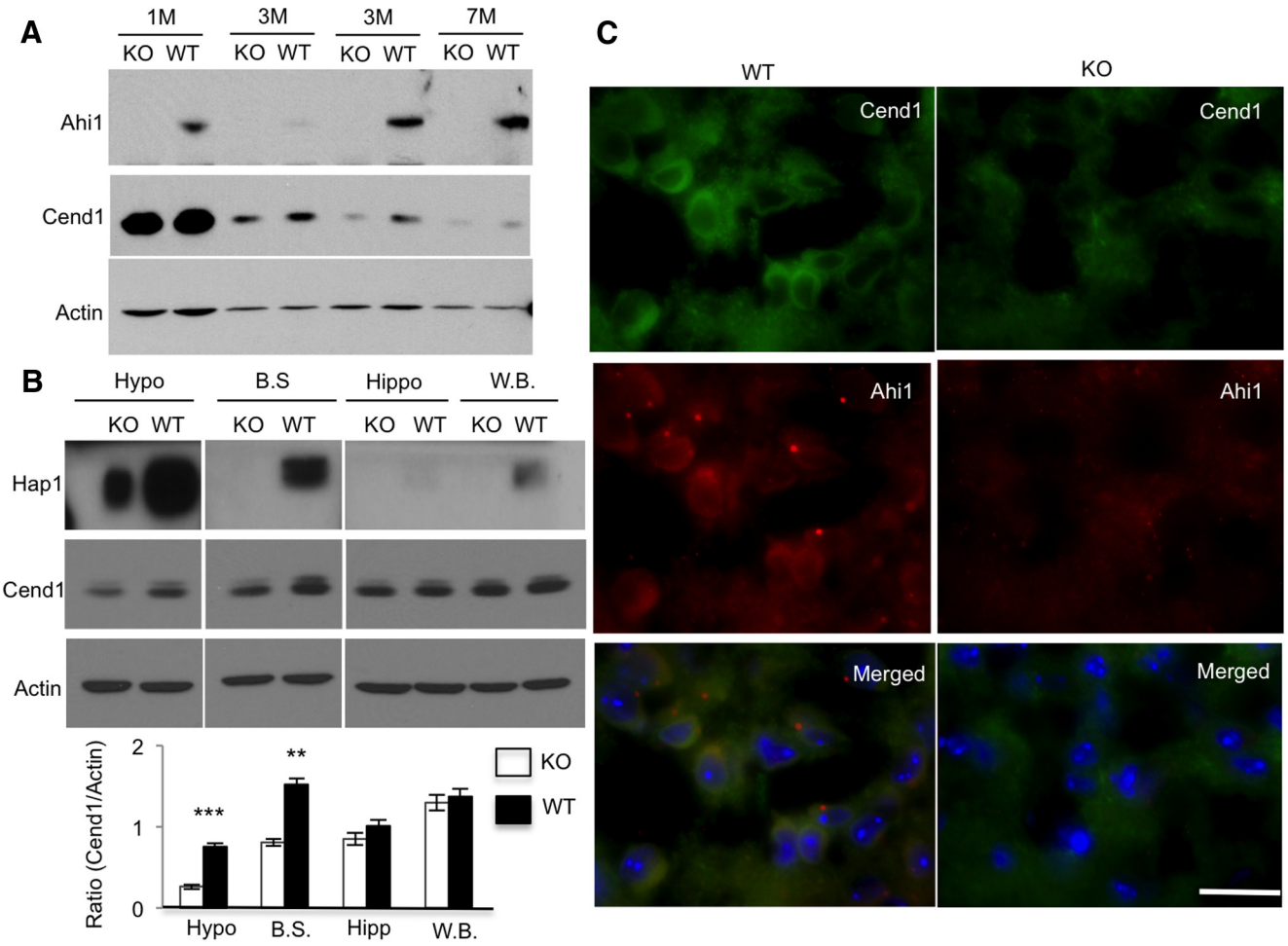


Figure 6. ***A***, Western blot analysis of Cend1 in the whole brain of mice at 1, 3, and 7 months of age showing that Cend1 is highly expressed in 1-month-old mouse brain. There is a decrease in Cend1 level in Ahi1 KO mouse brain. ***B***, Western blot analysis of Cend1 in the hypothalamus (Hypo), brainstem (B.S.), hippocampus (Hipp), and whole brain (W.B.) showing that Cend1 is selectively reduced in the hypothalamus and brainstem, in which Ahi1/Hap1 are normally enriched in WT mice. The relative amounts of Cend1 (ratio of Cend1 to actin) in different brain regions are also presented (bottom). $^{**}p < 0.01$, compared with WT ($n = 5$). $^{***}p < 0.001$, compared with WT ($n = 5$). ***C***, Immunofluorescent staining of WT and Ahi1 KO mouse hypothalamus also showing the reduced level of Cend1 in Ahi1 KO neurons. Cend1 is not colocalized with Ahi1 in the cytoplasmic puncta in WT hypothalamic neurons. Scale bar, 10 μm.

fAhi1 could increase the level of endogenous Cend1 (Fig. 7*A*,*B*). We then assessed the half-life of Cend1 in cultured N2A cells transfected with fAhi1. Compared with the vector control, the half-life of endogenous Cend1 is significantly prolonged in the presence of fAhi1 (Fig. 7*C*,*D*).

If Cend1 is involved in the neuropathology caused by the loss of Ahi1, its overexpression should rescue or alleviate this neuropathology. To test this hypothesis, we performed two sets of experiments. In the first, we expressed fAhi1 or nAhi1 in PC12 cells. As expected, nAhi1 inhibited NGF-induced neurite outgrowth in PC12 cells. However, when Cend1 was expressed in the same transfected cells, neurite outgrowth was restored (Fig. 8*A*). Counting PC12 cells that showed neurites longer than two cell bodies also verified that overexpression of Cend1 can protect against the adverse effect of nAhi1 on neurite outgrowth (Fig. 8*B*). To further test the rescue effect of Cend1 on primary neurons, we cultured hypothalamic neurons from WT and Ahi1 KO mice and performed immunofluorescent double staining. Using a rabbit anti-β-tubulin III, which specifically labels neuronal cells, and guinea pig anti-Hap1, we confirmed that the cultured hypothalamic neurons labeled by anti-β-tubulin III also express Hap1 (Fig. 8*C*, top). We then used mouse anti-Cend1 and rabbit anti-β-tubulin III to examine cultured hypothalamic neurons from Ahi1 KO mice. Anti-β-tubulin III staining showed that many Ahi1 KO hypothalamic neurons had interrupted and short neurites (Fig. 8*C*, middle), indicating that lack of Ahi1 leads to impaired neuronal differentiation. Cend1 was then transfected into cultured Ahi1 KO hypothalamic neurons. Ahi1 KO neurons expressing Cend1 showed long and intact neurites, in contrast to the fragmented and short neurites of Ahi1 KO neurons not expressing Cend1 (Fig. 8*C*, bottom). Quantitative analysis of the neurite length verified that expression of Cend1 did significantly increase the neurite length of Ahi1 KO hypothalamic neurons (Fig. 8*D*), supporting the idea that Cend1 dysfunction is involved in Ahi1 deficiency-mediated neuropathology.

Discussion

Our findings here suggest that Cend1 is involved in *Ahi1* mutation-induced neuropathology. Because Cend1 is a neuron-specific protein implicated in neuronal differentiation and is highly expressed in postnatal mouse brain (Mamalaki et al., 1995; Politis et al., 2007; Katsimpardi et al., 2008), our results yield fresh insights into the brain malformation and delayed development of Joubert syndrome, as well as revealing a potential therapeutic target for treating abnormal early brain development that can lead to Joubert syndrome and other psychiatric disorders.

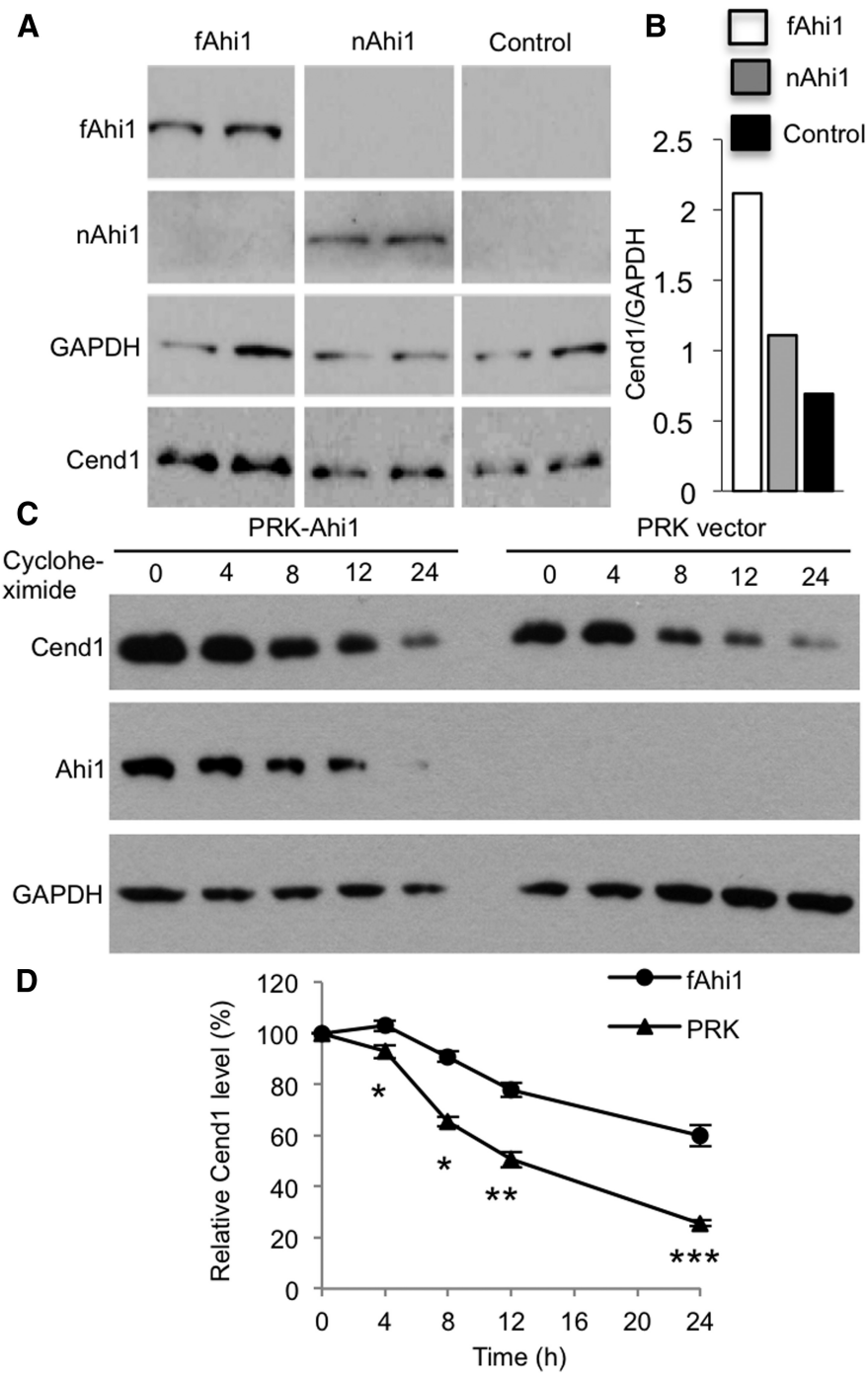


Figure 7. Ahi1 stabilizes the level of Cend1. ***A***, N2A cells were transfected with fAhi1 or nAhi1 and subjected to Western blotting. Expressing fAhi1 could increase the level of Cend1 compared with nontransfected control and nAhi1-transfected cells. ***B***, The ratio of Cend1 to GAPDH is presented in the right panel. ***C***, Western blot analysis of the half-life of Cend1 in fAhi1 or PRK vector-transfected cells after cycloheximide treatment for different times (4–24 h). The Cend1 degraded faster in the vector-transfected cells. ***D***, Statistical analysis of the half-life of Cend1 also showing a significant increase in the half-life of Cend1 in the presence of Ahi1 (fAhi1). *$p < 0.05$, compared with PRK vector. **$p < 0.01$, compared with PRK vector. ***$p < 0.001$, compared with PRK vector.

Recent studies have shown that Ahi1 regulates primary cilium formation and that loss of Ahi1 causes retinal and renal degeneration, which may account for the cerebellar hypoplasia, retinal dystrophy, and nephronophthisis in Joubert syndrome (Hsiao et al., 2009; Louie et al., 2010; Westfall et al., 2010; Lancaster et al., 2011). However, unlike patients with Joubert syndrome, Ahi1-null mice do not develop severe cerebellar vermis hypoplasia or ataxia associated with cerebellar dysfunction. Differences in early brain development between primates and rodents may account for the different phenotypes seen in Ahi1 KO mice. Despite these differences, Ahi1 KO mice show retarded growth during the postnatal period, a phenomenon that is similar to the delayed development of children with Joubert syndrome (Gitten et al., 1998; Maria et al., 1999; Koshy et al., 2010). The developmental delay in Joubert syndrome cannot be fully explained by the cerebellar malformation. Because Ahi1 is abundant in the hypothalamus (Sheng et al., 2008) and the hypothalamus functions as a control center for growth and metabolism by regulating hormonal secretion and signaling (Szarek et al., 2010), it is important to know whether loss of Ahi1 can affect hypothalamic neurons.

Several lines of evidence in our study suggest that Ahi1 participates in hypothalamic neuronal differentiation. First, the association of Ahi1 with Hap1, another neuronal protein enriched in the hypothalamic neurons (Li et al., 2003), occurs mainly to the p1A in the soma of neuronal cells and is regulated by NGF, which promotes neuronal differentiation. Second, Ahi1 also interacts with Cend1, a neuronal protein that can promote neurite outgrowth by activating intracellular signaling pathways (Mamalaki et al., 1995; Politis et al., 2007; Katsimpardi et al., 2008). This interaction is important for stabilizing Cend1, as the loss of Ahi1 can reduce the level of Cend1 in Ahi1 KO mouse brain. More importantly, the loss of Ahi1 selectively reduces Cend1 in the hypothalamus and brainstem, which are normally enriched in Ahi1. Thus, a high level of Ahi1 is required for regulating the stability of Cend1 and specifies the role of Ahi1 in Cend1-mediated neuronal differentiation in the hypothalamus. Finally, loss of Ahi1 can cause a neurite extension defect in hypothalamic neurons, which can be alleviated by overexpressing Cend1.

The Ahi1/Hap1 complex is involved in intracellular trafficking of neurotrophic receptors, such as TrkB (Sheng et al., 2008; Xu et al., 2010). Hap1 participates in the endocytosis of channel receptor and transport of different growth factors (Li and Li 2005; Wu and Zhou, 2009). The diverse functions of Ahi1/Hap1 in the transport of various proteins can be specified in particular types of cells or during specific periods of brain development, depending on their interacting proteins. Cend1 is likely to associate with Ahi1-related function in hypothalamic neurons during the postnatal development. First, Cend1 is highly expressed in postnatal mouse brain and then declines in mature mouse brain. Ahi1 KO mice show retarded growth during the postnatal period, perhaps

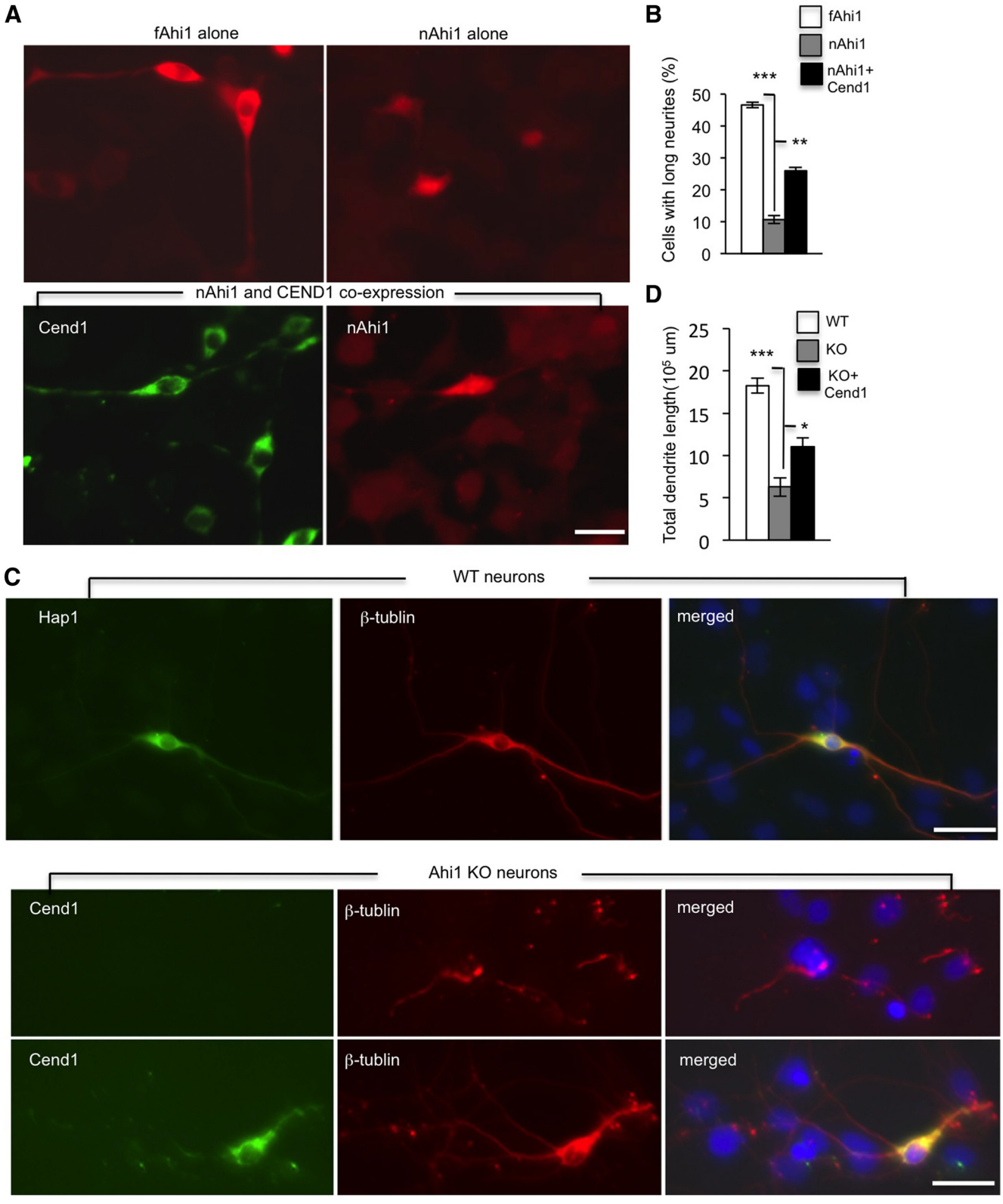


Figure 8. Rescue effect of Cend1 on defective neuronal differentiation of mutant Ahi1 neuronal cells. ***A***, PC12 cells were transfected with nAhi1 (red) showing reduced neurites compared with fAhi1-transfected cells. Overexpression of Cend1 (green) restored long neurites in nAhi1-transfected cells. Scale bar, 10 μm. ***B***, The numbers of cells with long neurites were assessed ($n = 300$). *$p < 0.05$, compared with nAhi1 alone. **$p < 0.01$, compared with nAhi1 alone. ***$p < 0.001$ compared with nAhi1 alone. ***C***, Immunofluorescent double staining of cultured hypothalamic neurons from WT mouse showing that a hypothalamic neuron expressing β-tubulin III is also positive for Hap1 immunostaining (top). Defective neurites are seen in cultured hypothalamic neurons from Ahi1 KO mouse brain (middle). Transfected Cend1 could increase neuritic length of Ahi1 KO hypothalamic neurons (bottom). Scale bar, 10 μm. ***D***, The lengths of neurites of β-tubulin III-positive neurons from WT and Ahi1 KO mice were measured ($n = 30$). Transfected Cend1 could significantly increase the length of neurites of Ahi1 KO hypothalamic neurons. *$p < 0.05$, compared with WT. ***$p < 0.001$, compared with WT.

because the interaction of Ahi1 with Cend1 in hypothalamic neurons is required for the normal function of hypothalamic neurons during early development. When mice mature, the level of Cend1 declines and may be less critical for regulating hypothalamic neuronal function. This could explain why the growth of adult Ahi1 KO mice appears to be normal. Second, because Ahi1 participates in trafficking and stabilizing membrane receptors, it may also function on Cend1 via a similar mechanism, as Cend1 is an integral membrane protein. It is possible that Ahi1 stabilizes the complex of Cend1 and its ligand during their endocytic process to maintain their signaling activity related to neuronal differentiation; however, whether Cend1 responds to neurotrophic factors remains to be explored. Thus, future experiments to identify how Cend1 triggers intracellular signaling would help us understand the mechanism by which Ahi1 stabilizes Cend1. Earlier studies have provided strong evidence that Cend1 plays a role in the coordination of cell cycle exit and differentiation of neuronal precursors (Koutmani et al., 2004; Georgopoulou et al., 2006; Politis et al., 2007; Katsimpardi et al., 2008) and that loss of Cend1 can impair differentiation of certain types of neurons, including cerebellar Purkinje cells (Sergaki et al., 2010). In Ahi1 KO mice, the Cend1 level is partially reduced in the hypothalamus, and this reduction is likely to cause hypothalamic dysfunction and the slow growth related to it in Ahi1-null mice. Because the hypothalamic dysfunction could also contribute to the retarded growth in patients with Joubert syndrome, the identification of Cend1 as an Ahi1-interacting protein provides us with a target to investigate the pathogenesis of Joubert syndrome.

Although *AHI1* mutations cause Joubert syndrome in a recessive manner, it is possible that, in homozygous Joubert syndrome patients, two copies of the mutated *AHI1* gene can increase the level of N-terminal AHI1 (nAHI1), leading to a possible gain of toxicity. Such a possibility is supported by our result that overexpression of nAhi1 in cultured PC12 cells inhibits their differentiation. Because nAhi1 does not bind Hap1, nAhi1 may be able to interact with other proteins that normally associate with fAhi1, resulting in a gain of toxic function. Our finding that the overexpression of Cend1 could alleviate nAhi1-mediated neurite defects also suggests that Cend1 is a potential therapeutic target in Ahi1 mutation-related neuropathology. Recent studies also reveal that the AHI1 gene is associated with the susceptibility to schizophrenia and autism (Holroyd et al., 1991; Amann-Zalcenstein et al., 2006; Ingason et al., 2007, 2010; Alvarez Retuerto et al., 2008; Torri et al., 2010). These important complex diseases are likely caused by problems with early brain development (Hamlyn et al., 2013), so identifying Cend1 as an Ahi1-interacting protein should be highly interesting to these fields as well.

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