Huwe1 ubiquitin ligase is essential to synchronize neuronal and glial differentiation in the developing cerebellum

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We have generated a knockout mouse strain in which the gene coding for the ubiquitin ligase Huwe1 has been inactivated in cerebellar granule neuron precursors (CGNPs) and radial glia. These mice have a high rate of postnatal lethality and profound cerebellar abnormalities. The external granule layer of the cerebellum, which contains CGNPs, is expanded and displays aberrant proliferation and impaired differentiation of the progenitor cell population. The uncontrolled proliferation of the CGNPs is associated with accumulation of the N-Myc oncoprotein, a substrate of Huwe1, and consequent activation of the signaling events downstream to N-Myc. Furthermore, loss of Huwe1 in Bergmann glia leads to extensive disorganization of this cell population with layering aberrations, severe granule neuron migration defects, and persistence of ectopic clusters of granule neurons in the external granule layer. Our findings uncover an unexpected role for Huwe1 in regulating Bergmann glia differentiation and indicate that this ubiquitin ligase orchestrates the programming of the neural progenitors that give rise to neurons and glia in the cerebellum.

Results

Huwe1 Deletion Results in Postnatal Lethality and Severe Defects in Cerebellar Development. To study the role of the ubiquitin ligase Huwe1 in cerebellar development, we crossed conditional knockout mice for the Huwe1 gene (Huwe1fl/fl) (3) with mice carrying a human GFAP-Cre transgene (4). When driven by the GFAP promoter, expression of the Cre recombinase begins at embryonic day 13 and is detected in cerebellar granule neurons and Bergmann glia but not Purkinje cells (4). Because the Huwe1 gene is X-linked, we performed all our analyses on male offspring in which inactivation of the single Huwe1 allele results in the Huwe1-null genotype. Crossings between Huwe1fl/fl females and GFAP-Cre transgenic males generated Huwe1fl/flGFAP-Cre animals (hereafter referred to as Huwe1−/−GFAP). We observed efficient deletion of Huwe1 in the cerebellum (Fig. 1A). Western blot analysis of Huwe1 protein using two different antibodies, one recognizing the N terminus (N-Ter) and the other the HECT domain at the C terminus (HECT), confirmed that the HECT domain was absent in the cerebellum of Huwe1−/−GFAP mice. Moreover, the mutant Huwe1 protein was markedly reduced, confirming our previous findings in the cortex (Fig. 1B) (3). In the cerebellum at postnatal day 8 (P8), Huwe1 was highly expressed in Purkinje cells (Fig. 1C, yellow arrowheads). In the granule neuron layers, Huwe1 was low in the EGL and increased markedly as neurons migrate into the IGL (Fig. 1C Left). At P8, the Bergmann glia provide essential scaffold functions for migration of postmitotic neurons from the EGL to the IGL (1, 2). Double immunostaining for GLAST, a Bergmann glia specific marker (5), or the Bergmann glia and astrocytic marker GFAP and Huwe1 showed that Bergmann glia cells stain positive for nuclear Huwe1 (Fig. 1C Left, white arrowheads). White matter astrocytes were also positive for Huwe1 (Fig. 1C Left, white arrows). Consistent with the reported pattern of expression of the Cre

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recombinase in the GFAP-Cre transgenic mouse, Huwe1 immunostaining with antibodies targeting the HECT domain revealed that the intact Huwe1 protein was lost in the EGL, IGL, Bergmann glia, and astrocytes but not Purkinje cells of Huwe1F/YGFAP mice (Fig. 1 C and D Right).

Huwe1F/YGFAP mice were born at the expected ratio. However, more than 50% Huwe1F/YGFAP mice died within 4 weeks of postnatal age (Fig. 1E). Before the weaning age, the Huwe1F/YGFAP mice became significantly smaller than control littermates and began to show ataxic symptoms such as abnormal posture and hyperextension of the hind limbs (Fig. 1F). When suspended by the tail, Huwe1F/YGFAP mice displayed limb withdrawal and digit clasping. Histological analysis revealed that the Huwe1F/YGFAP cerebellum was similar to control at P0 (Fig. 24). However, a markedly smaller mutant cerebellum was observed at P8. At this age, Huwe1 mutant cerebellum had a conserved pattern of foliation with relatively enlarged EGL and underdeveloped IGL (Fig. 2B). The Purkinje cell layer was also abnormal with crowding and misalignment of Purkinje cells in mutant mice as shown by immunostaining for calbindin, a Purkinje cell-specific marker (Fig. 2C). As Huwe1 is not deleted in Purkinje neurons, the described defects are likely to be secondary to abnormalities of granule neurons or Bergmann glia. Although attenuated, the defect in the EGL persisted at P15, suggesting a delay in the development of the cerebellar cortex in the mutant mouse (Fig. 2D).

Severe Defects of Cell Cycle Withdrawal and Differentiation of Granule Neuron Precursors in Huwe1F/YGFAP Mice. During postnatal development, the EGL is divided into two sublayers: the granule cell progenitors continue to proliferate in the outer sublayer, whereas postmitotic granule cells extend bipolar axons and move horizontally in the inner part of the EGL (IEGL), prior to extending a third process perpendicular to the bipolar axon and initiate the migration into the IGL (6). To assess how loss of Huwe1 activity affected these crucial steps of granule cell development, we evaluated proliferation and differentiation of granule cells. To study cell proliferation, we performed quantitative analysis of the proliferation marker Ki67 at P8, the time when proliferative expansion of granule precursors is at peak, and P15, the time when the EGL is no longer evident, as postmitotic granule cells have completed migration into the IGL. When compared with littermate controls, we found a higher number of proliferating cells both

Fig. 1. Ataxic phenotype and postnatal lethality in Huwe1F/YGFAP mice. (A) The upper three panels are results from tail DNA genotyping for Huwe1 loxP allele, GFAP-Cre, and Sex-determining Region Y, SRY in representative P5 mice from heterozygous floxed Huwe1 females bred onto GFAP-Cre heterozygous males (lanes 4 and 5). Bottom panel is genomic PCR for Huwe1 using DNA from the cerebella of mice in the Upper panels. Lane 1 is PCR control for the Huwe1 WT allele. Lane 2 is PCR control for the recombined Huwe1 allele. Lane 3 is PCR control for Huwe1 loxP plus Huwe1 recombined alleles. (B) Western blot analysis of cerebellum lysates from mice in lanes 4 and 5 of A using an antibody against the HECT domain of the Huwe1 protein (HECT) or the N-terminal region (N-Ter). α-Tubulin is shown as control for loading. (C) Expression of Huwe1 protein in the cerebellum of Huwe1F/YGFAP and control mice at P8. Sagittal sections were immunostained using Huwe1 N-Ter antibody and antibodies against GLAST (Upper), or GFAP (Lower). Besides granule neurons, Bergmann glia, GLAST/GFAP-positive cells (white arrowhead), and GFAP-positive astrocytes in the IGL (arrows) were also positive for Huwe1 in control but not mutant cerebellum. Purkinje cells (yellow arrowheads) expressed high levels of Huwe1 in wild-type and mutant cerebella. (D) Immunofluorescence using the Huwe1-HECT antibody confirmed that the HECT domain of Huwe1 was efficiently deleted in the Huwe1F/YGFAP granule neurons, Bergmann glia, and astrocytes but not Purkinje cells (P15). (E) Kaplan-Meier curves of wild-type and Huwe1F/YGFAP mice. (F) Growth retardation and ataxia in Huwe1F/YGFAP mice. EGL, external granule layer; ML, molecular layer; IGL, internal granule layer.

Fig. 2. Histological analysis of Huwe1F/YGFAP cerebellum. (A) Hematoxylin and eosin staining of sagittal sections from P0 Huwe1F/YGFAP and Huwe1F/YGFAP cerebella. (B) Hematoxylin and eosin staining from P8 Huwe1F/YGFAP and Huwe1F/YGFAP cerebella. Higher magnification view (Middle and Lower) of framed area in the Upper panels shows an enlarged EGL, decreased thickness of the ML, and reduced cell density in the IGL in Huwe1F/YGFAP compared to Huwe1F/YGFAP. (C) Immunofluorescence for the Purkinje cell marker calbindin in Huwe1F/YGFAP and Huwe1F/YGFAP mice. (D) Hematoxylin and eosin staining from P15 Huwe1F/YGFAP and Huwe1F/YGFAP cerebella. (Lower) Higher magnification view of framed area in Upper. P15 Huwe1F/YGFAP mice exhibit a residual EGL and migrating granule cells are visible in the ML (arrows).
in P8 and P15 mutant mice (Fig. 3 A and B and Fig. S1A Upper; P8, $P = 1.85293E-07$; P15, $P = 0.000975$). Consistently, mutant granule cell precursors exhibited a 2-fold increase in phosphorylated histone H3 (pH3), a marker of mitosis (Fig. 3 C and D and Fig. S1A Lower). Ki67-positive granule cells forming heterotopic clusters at the pial surface were identified until P25 in Huwe1F/YGFAP mice (Fig. S2A). In the surviving adult Huwe1F/YGFAP mice (12 months) the ectopic granule cell clusters persisted, although they had lost Ki67 immunoreactivity and expressed the neuronal differentiation marker NeuN (Fig. S2 B and C).

Previous studies have shown that Huwe1 targets N-Myc for ubiquitination and degradation in neural cells (3, 7). N-Myc activity is necessary for proliferation of granule cell precursors (8, 9) and down-regulation of N-Myc is required for their terminal cell cycle exit and differentiation (10). When compared with controls, P8 Huwe1F/YGFAP EGL exhibited an enlarged N-Myc-positive compartment and concordant increased expression of cyclin D2, a transcriptional target of N-Myc in CGNPs (11) (Fig. 3 E and F). A similar pattern of expression of N-Myc and cyclin D2 was observed in P15 cerebella and paralleled the histological defects at this age (Fig. S1B). Conversely, the cyclin-dependent kinase (cdk) inhibitor p27Kip1, whose expression is derepressed in the N-myc-null cerebellum (11), was markedly reduced in Huwe1F/YGFAP cerebella (Fig. 4A). Furthermore, the expression of the cdk inhibitor p27Kip1, which marks the post-mitotic granule cells in the premigratory EGL and the IGL, is also reduced in mutant mice (12), was also reduced in mutant mice, being restricted to a limited strip of the IGL and displaying a heterogeneous pattern in the IGL (Fig. 4B). Accordingly, the ratio of p27Kip1-positive to total nuclei was significantly reduced in Huwe1F/YGFAP mice (Fig. 4B; $P = 4.85935E-05$).

Besides proliferation, Huwe1 was proposed as a direct regulator of programmed cell death (13–15). However, the deregulated activity of Myc oncoproteins is generally sufficient to trigger secondary apoptosis (16). To establish the exact relationship between the two phenotypic events, we conducted a parallel and quantitative analysis of proliferation (Ki67) and apoptosis (cleaved caspase3) in Huwe1F/YGFAP mice at two stages of cerebellar development (P0 and P8, Fig. S3). At P0, proliferation was already elevated in the Huwe1 mutant cerebellum but apoptosis was unaffected (Fig. S3 A and B). However, the hyperproliferative activity in the mutant cerebellum at P8 (Fig. 3 A–D) was also accompanied by elevated numbers of apoptotic cells (Fig. S3C). Together, these findings indicate that the primary consequence of deleting Huwe1 in the mouse cerebellum is enhancement of cell proliferation, and persistent proliferative stress eventually triggers secondary apoptosis.

The morphology and behavior of CGNPs in the mutant cerebellum raised the possibility that differentiation might be disturbed. To test this hypothesis, we examined the pattern of expression of the molecular marker DCX. DCX is a molecular marker of migrating and differentiating neuroblasts in the central and peripheral nervous system (17, 18). In control cerebella at P8, DCX was highly expressed in the premigratory region of the EGL (IEGL) and identified T-shaped axons. DCX was also detected in granule cells migrating radially in the ML (Fig. 4C, white arrowheads, trailing fibers), in postmitotic granule cells migrating tangentially (Fig. 4C Left; white arrows, spindle-shaped cells; yellow arrowheads, parallel fibers), and in several neurons in the IGL (Fig. 4C Lower Left). The premigratory region of Huwe1F/YGFAP cerebella expressed lower amounts of DCX and contained only round-shaped cells, which lacked parallel fibers and the perpendicular process (Fig. 4C Upper Right, arrows). The population of DCX-positive neurons in the IGL was also markedly reduced in Huwe1F/YGFAP cerebella (Fig. 4C Lower Right). Immunostaining for the mature neuronal marker NeuN confirmed that a reduced number of cells in the IGL had acquired differentiation features and fewer mature granule cells had reached the IGL in the mutant cerebella compared with controls (Fig. 4D). Together, these results indicate a delay in granule cell differentiation and suggest that Huwe1 functions as an intrinsic factor necessary for executing terminal cell cycle exit and initiating the differentiation programs in the external granule precursor cells.

**Severe Bergmann Glia Abnormalities in Huwe1F/YGFAP Mice.** Analysis of Huwe1 expression indicated that Huwe1 was efficiently deleted in cerebellar glial cells in Huwe1F/YGFAP cerebella (Fig. 1C). Therefore, we asked whether defects of the cerebellar glia are also implicated in the phenotype of Huwe1F/YGFAP mice. The Bergmann glia are the major glial cell type in the cerebellar cortex. During postnatal life Bergmann glia undergo significant morphological changes to support granule neuron differentiation and migration (1, 2). The protein BLBP is not only a useful marker for radial glia but it is also an important protein for granule cell migration, given the observation that anti-BLBP antibodies block the migration of granule cells along radial glia fibers (19). Bergmann glia cell bodies align nearly in a single layer adjacent to Purkinje cells and exhibit unipolar fibers, which extend and contact the pial surface. In P8 wild-type cerebella, immunostaining with the anti-BLBP antibody identified the classical palisade of Bergmann glia extensions in the ML. Scattered BLBP-positive cells were also present in the IGL, marking astrocytes (Fig. 5A Left). In mutant mice, the pattern of BLBP...
immunostaining demonstrated the disorganized microarchitecture of the cerebellar folia. Bergmann glia cell bodies were not aligned but BLBP-positive cells were irregularly located in both the EGL and IGL (Fig. 5A Right). Immunostaining for the Bergmann glia marker GLAST confirmed that most mutant Bergmann glia cells had rudimentary or irregular fibers that lacked contact with the pial surface and were completely misaligned (Fig. 5B). Moreover, GFAP-positive, mature astrocytes were reduced in the deep white matter of mutant cerebella (Fig. 5C).

**Loss of Huwe1 Impairs Migration of Granule Neurons.** Mature Bergmann glia provide the indispensable scaffold for cerebellar neuron migration and positioning into the IGL. Given the profound defect of the Bergmann glia scaffold in Huwe1<sup>YF</sup>GFAP cerebellum, we predicted that radial migration of granule cells might be perturbed in mutant mice. To test this hypothesis, we pulse labeled in vivo granule neurons born at P6 with bromodeoxyuridine (BrdU) and analyzed them after 20 h and 110 h, respectively. Fig. 6A shows immunostaining for BrdU to identify the labeled granule cells and calbindin to identify Purkinje cells, thus marking the ML/IGL boundary. After 20 hours from labeling, the number of BrdU-positive cells that entered the IGL was small in both controls and mutant cerebella (Fig. 6A Upper). However, evaluation of the ratio of BrdU-positive cells in the ML to BrdU-positive cells in the IGL suggested that a defective migration was already detectable in mutant mice at this early time (Fig. 6B). After 110 h, a vast majority of BrdU-positive granule cells had migrated into the IGL in control cerebella. Conversely, mutant cerebella retained more than 80% of the BrdU-positive cells in the ML and only a small number of BrdU-labeled cells reached the IGL (Fig. 6A Lower and Fig. 6B). The failure of granule cell migration reflects the dual defect of differentiation in granule and glial cells that is manifested by the collapse of the intricate neuron–glia interactions that regulate cerebellar development (Fig. 6C).

![Fig. 4. Defective differentiation of granule neurons in Huwe1<sup>YF</sup>GFAP mice.](image1)

![Fig. 5. Defects in Bergmann glia scaffold in Huwe1<sup>YF</sup>GFAP mice.](image2)
Discussion

In this study, we demonstrated that Huwe1 plays a key role in differentiation of CGNPs and maturation of Bergmann glia during the postnatal development of the cerebellum. Detailed analysis showed that the proliferative window of Huwe1-null granule neurons was prolonged and was associated with elevated N-Myc and cyclin D2 and reduced p18Ink4C and p27Kip1. The examination of several differentiation markers of granule neurons and Bergmann glia in the developing cerebellum revealed remarkable changes in these cell populations after Huwe1 deletion, supporting the notion that the interplay between the Bergmann glial scaffold and migrating granule neurons is vital for granule neuron migration and formation of the final structure of the IGL. Besides the phenotypes in granule neurons and Bergmann glia, Purkinje cells were misaligned and abnormally crowded. A recent study suggested that maturation and alignment of Purkinje cells requires support from extended Bergmann fibers (5, 20). Indeed, the lack of GFAP-Cre expression and Huwe1 deletion in Purkinje cells indicates that phenotypic alterations of Purkinje cells in Huwe1-null cerebella are likely secondary to the defective Bergmann glial scaffold. Together with our recent demonstration that Huwe1 is required for timely cell cycle withdrawal, differentiation, and patterning of the developing cortex, the present study establishes the essential role of the Huwe1 ubiquitin ligase in coordinating maturation of neurons and glia in the nervous system.

The development of laminar structure of the cerebellum is orchestrated by signals from diverse cell types. The intimate structural association between granule neurons and Bergmann glial fibers led to the long-standing notion that elongated and anchored radial Bergmann glial cells serve as a scaffold that is crucial for granule neuron migration (21). Genetic models have indicated that a number of cell intrinsic and extrinsic signals contribute to Bergmann glia maturation and cerebellar lamination (22–29). One of the intrinsic factors is the PTEN tumor suppressor. It has been reported that GFAP-Cre-mediated deletion of PTEN causes defects of the Bergmann glia similar to those we describe in the Huwe1 knockout cerebellum (30). However, an important difference among these two mouse strains (Huwe1 null and PTEN null) is the absence of neuronal abnormalities in the PTEN mutant cerebellum, whereas we found that granule neuron differentiation defects are present in the Huwe1 mutant cerebellum. This difference points to two important conclusions: (i) exclusive Bergmann glia defects (as seen in PTEN knockout) result in impaired neuronal migration but do not affect the intrinsic ability of neurons to progress through the programmed differentiation steps; and (ii) dual neuron–glia differentiation abnormalities (as in Huwe1 knockout) not only cause a defective neuronal migration but also enhance the basal proliferative capacity of granule precursors and delay cell cycle withdrawal leading to the formation of ectopic clusters of granule cells. Whether direct or indirect interactions exist between the Huwe1-N-Myc and the PTEN-AKT pathways in Bergmann glia will have to be elucidated by future studies. However, the similarity of the findings suggests that the physiological role of Huwe1 in cerebellar development may be linked to the prodifferentiation function of a tumor suppressor protein.

The deregulated proliferation conferred by loss of Huwe1 ultimately leads to severe perturbation of CGNP differentiation and disorganization of the cerebellar architecture. Within the prolonged proliferative window of Huwe1-null CGNPs, the N-Myc oncoprotein, a Huwe1 substrate, accumulated aberrantly with consequent activation of cyclin D2 and repression of p18Ink4C and p27Kip1, downstream events triggered by N-Myc in CGNPs (9, 31–33). Although our work does not rule out the possibility that the accumulation of other, yet unknown Huwe1 substrates in granule neurons (and Bergmann glia) may also participate in the manifestation of the Huwe1-null phenotype, the importance of N-Myc inactivation for coordinated cell cycle withdrawal and differentiation of CGNPs has been firmly established in previous studies (8, 10, 34). One interesting finding of our study was the observation of ectopic clusters of granule neurons at the pial surface in adult mutant mice. These clusters originated from CGNPs with extended proliferation window, which were able eventually to
execute a normal program of differentiation and did not progress to a neoplastic or tumorigenic state. This is consistent with data obtained in many systems, which indicate that N-Myc overexpression alone is not sufficient to transform granule precursor cells but cooperates with different genetic lesions to promote medulloblastoma, the tumor derived from granule precursors. Among these lesions is loss of the Sonic hedgehog (Shh) antagonist Ptch (35, 36), which constitutively activates the signaling pathway that promotes proliferation of the CGNPs in the EGL during cerebellar development and cell cycle regulators such as the cyclin-dependent kinase inhibitor Ink4c and p27Kip1 (37–41). It remains to be tested whether Huwel deletion cooperates with such lesions to promote medulloblastoma in the mouse and whether, as found in human glioblastoma, Huwel is also inactivated in medulloblastoma (3).

Materials and Methods

**Generation of Huwe1Flox GFAP Mice.** To generate Huwe1Flox/GFAP-Cre animals heterozygous or homozygous floxed Huwe1 females (HuwelFlox or Huwe1Flox/Flox) were bred onto GFAP-Cre heterozygous males. Huwe1 mutants were generated by PCR of genomic DNA prepared from tail using primers described previously (3). All animal experiments were approved by and performed in accordance with the guidelines of the International Agency for Research on Cancer’s Animal Care and Use Committee.

**Tissue Preparation, Histology, and Immunostaining.** Histological analysis was carried out on 5-μm paraffin sections stained with hematoxylin and eosin (H&E). For granule neuron migration experiments, 50 μg/kg body weight bromodeoxyuridine (BrdU, Sigma-Aldrich) was injected intraperitoneally in P6 mice. Mice were killed after 20 h and 110 h. The primary antibodies used were: anti-Huwe1 HECT-domain (Lifespan Biosciences), anti-Huwe1-Lasu1 (Bethyl Laboratories), anti-BLBP (Abcam), anti-Calbindin (Chemicon), anti-p18Ink4c (Santa Cruz), anti-p27Kip1 (Thermo Scientific), anti-Doublecortin (Santa Cruz), anti-NeuN (Calbiochem), anti-Brdu (Roche), anti-Ki67 (Novocastra), anti-N-Myc (Calbiochem), anti-Cyclin D2 (NeoMarkers), and anti-cleaved caspase3 (Cell Signaling). Fluorescent detection was performed using Cy3 or FITC-labeled secondary antibodies (Jackson Immunoresearch Laboratories) or biotin-conjugated secondary antibodies (Vector Laboratories) followed by VECTASTAIN Elite ABC development and the Tyramide amplification system (Perkin-Elmer).

**Western Blot.** Cerebella were triturated using RIPA lysis buffer. Equal amounts of total protein were separated on SDS-PAGE gel. After blocking in 5% dry milk in PBS, blots were incubated with primary antibodies against Huwe1-Lasu1 N terminus (Bethyl Laboratories), Huwe1 HECT-domain (Lifespan Biosciences), or α-tubulin (Sigma) before exposure to peroxidase-conjugated secondary antibodies and detection using ECL enhanced chemiluminescence (Amersham).

**Statistical Analysis.** Each experiment was performed with samples from at least three animals from two independent litters. In histograms, values represent the mean values; error bars are standard deviations. Statistical significance was determined by t test (with Welch’s correction) using Prism 4.0 software (GraphPad).

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Fig. S1. P15 Huwe1<sup>F/Y</sup>GFAP cerebella display increased proliferation, accumulation of N-Myc and cyclin D2 in the EGL. (A) Sagittal sections of the cerebellum of wild-type and Huwe1<sup>F/Y</sup>GFAP mice at P15 were immunostained with anti-Ki67 antibody (red, Upper) and anti-pHH3 antibody (red, Lower). (B) Sagittal sections of cerebella from P15 Huwe1<sup>F/Y</sup> and Huwe1<sup>F/Y</sup>GFAP mice were immunostained for N-Myc antibody (red, Upper) and anti-cyclin D2 antibody (red, Lower).
**Fig. S2.** Analysis of cerebellum of adult *Huwe1<sup>ΔF/Y</sup>* GFAP mice. (A) Sagittal sections of the cerebellum of wild-type and *Huwe1<sup>ΔF/Y</sup>* GFAP mice at P25 were immunostained with anti-Ki67 antibody (red) and counterstained with DAPI (blue). Note the heterotopic clusters of granule cells containing Ki67 positive cells. (B) Hematoxylin and eosin staining shows clusters of granule cells at the pial surface in a representative 12-month old *Huwe1<sup>ΔF/Y</sup>* GFAP mouse (Right). (C) Immunofluorescence using NeuN (red), marker of terminally differentiated neurons, demonstrated that in 12-month old *Huwe1<sup>ΔF/Y</sup>* GFAP mice the ectopic cell clusters are composed of differentiated cells.
Fig. S3. Induction of apoptosis in Huwe1<sup>F/Y</sup>GFAP cerebella is secondary to aberrant proliferation. (A) Sagittal sections of the cerebellum of wild-type and Huwe1<sup>F/Y</sup>GFAP mice at P0 were immunostained with anti-Ki67 antibody (red) and counterstained with DAPI (blue). Histogram is quantification of Ki67<sup>+</sup> cells normalized by area (150 μm<sup>2</sup>). Bars indicate mean ± SD n = 3 for Huwe1<sup>F/Y</sup> and n = 3 for Huwe1<sup>F/Y</sup>GFAP; P = 0.0015832. (B) Sagittal sections of cerebella from P0 Huwe1<sup>F/Y</sup> and Huwe1<sup>F/Y</sup>GFAP mice were immunostained for the apoptotic marker cleaved caspase3 (red) and counterstained with DAPI (blue). Histogram is quantification of cleaved caspase3<sup>+</sup> cells in the entire cerebellum. Bars indicate mean ± SD n = 3 for Huwe1<sup>F/Y</sup> and n = 3 for Huwe1<sup>F/Y</sup>GFAP; P = 0.373861. (C) Immunofluorescence using the apoptotic marker cleaved caspase3 (red) in P8 Huwe1<sup>F/Y</sup> and Huwe1<sup>F/Y</sup>GFAP mice. Nuclei were counterstained with DAPI (blue). Histogram is quantification of cleaved caspase3<sup>+</sup> cells in each folium. Bars indicate mean ± SD n = 3 for Huwe1<sup>F/Y</sup> and n = 3 for Huwe1<sup>F/Y</sup>GFAP; P = 0.0004755.