Supplementary Information

CHMP1A encodes an essential regulator of BMI1-INK4A in cerebellar development

Ganeshwaran H. Mochida[1-5,18], Vijay S. Ganesh[1-3,6,18], Maria I. de Michelena[7], Hugo Dias[8], Kutay D. Atabay[1-3], Katie L. Kathrein[3,9-11], Hsuan-Ting Huang[3,9-11], R. Sean Hill[1-3], Jillian M. Felie[1-3], Daniel Rakiec[1-3], Danielle Gleason[1-3], Anthony D. Hill[12], Athar N. Malik[6], Brenda J. Barry[1-3], Jennifer N. Partlow[1-3], Wen-Hann Tan[1,4], Laurie J. Glader[4,13], A. James Barkovich[14], William B. Dobyns[15], Leonard I. Zon[3,4,9-11,16], Christopher A. Walsh[1-4,17]

[1]Division of Genetics, Department of Medicine, Boston Children's Hospital, Boston, MA, USA
[2]Manton Center for Orphan Disease Research, Boston Children's Hospital, Boston, MA, USA
[3]Howard Hughes Medical Institute, Boston Children's Hospital, Boston, MA, USA
[4]Departments of Pediatrics, Harvard Medical School, Boston, MA, USA
[5]Pediatric Neurology Unit, Department of Neurology, Massachusetts General Hospital, Boston, MA, USA
[6]Harvard-MIT Division of Health Sciences and Technology, Cambridge, MA, USA
[7]Department of Morphologic Sciences, Cayetano Heredia University, Lima, Perú
[8]Institute for Child Development-ARIE, Lima, Perú
[9]Stem Cell Program, Boston Children's Hospital, Boston, MA, USA
[10]Division of Hematology/Oncology, Boston Children's Hospital, Boston, MA, USA
[11]Dana-Farber Cancer Institute, Boston, MA, USA
[12]Department of Neurology, Boston Children's Hospital, Boston, MA, USA
[13]Complex Care Service, Division of General Pediatrics, Boston Children's Hospital, Boston, MA, USA
[14]Department of Radiology and Biomedical Imaging, University of California, San Francisco, San Francisco, CA, USA
[15]Center for Integrative Brain Research, University of Washington, Seattle, WA, USA
[16]Harvard Stem Cell Institute, Cambridge, MA, USA
[17]Department of Neurology, Harvard Medical School, Boston, MA, USA
[18]These authors contributed equally to the work.

Corresponding author:
Dr. Christopher A. Walsh
Division of Genetics, Boston Children's Hospital
300 Longwood Ave, CLS 15062.2
Boston, MA 02115
Email: christopher.walsh@childrens.harvard.edu
Phone: 617-919-2923, Fax: 617-919-2010

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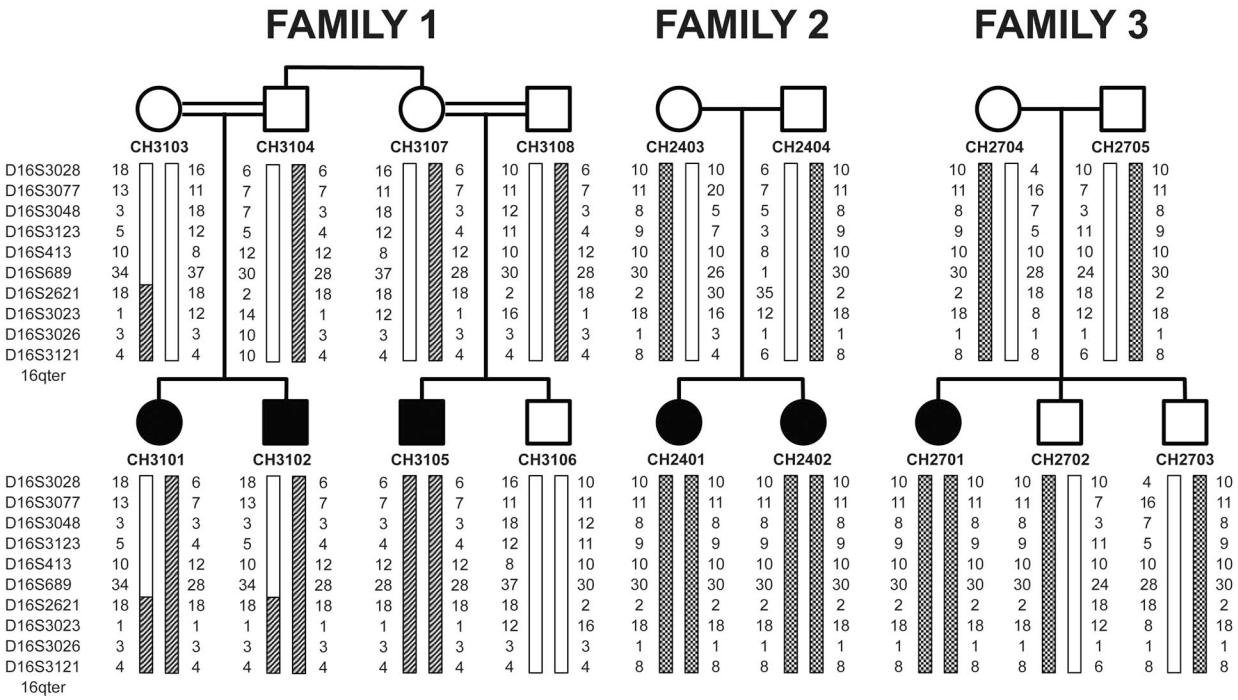
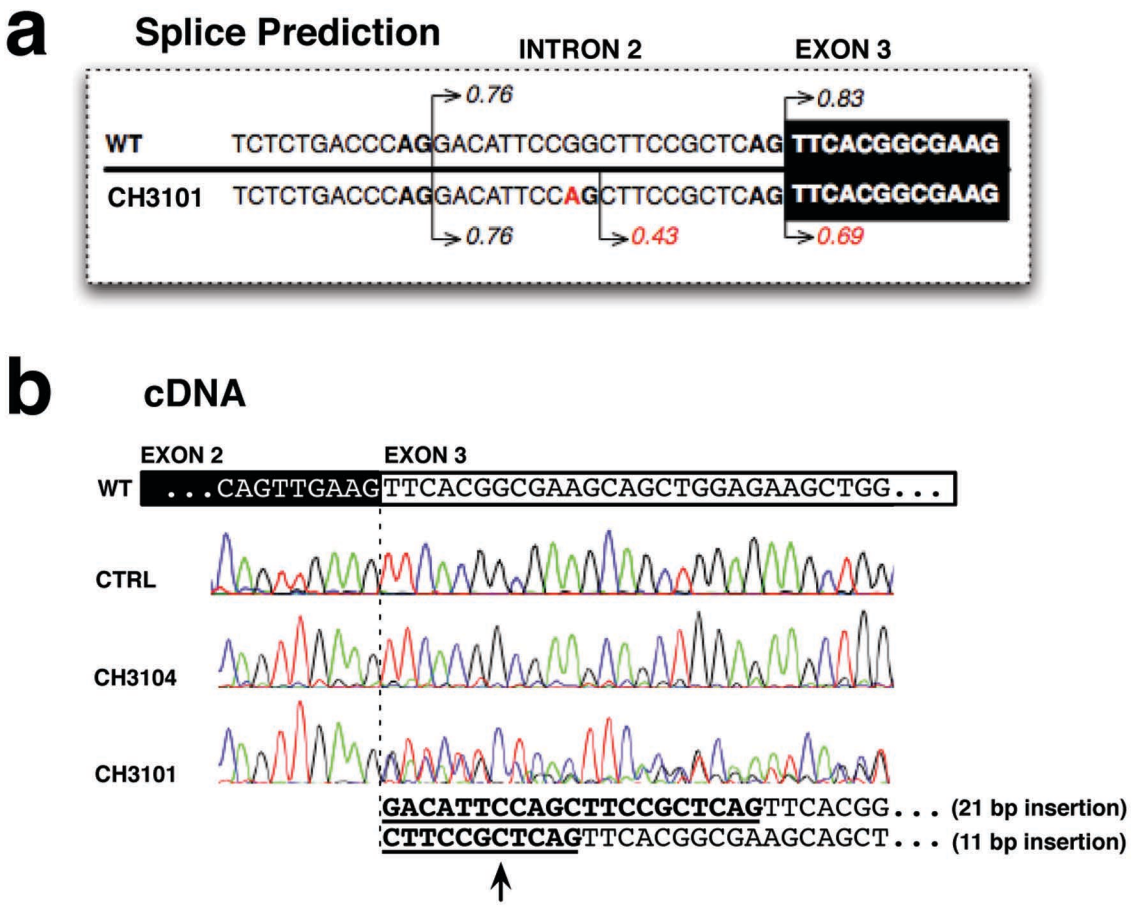


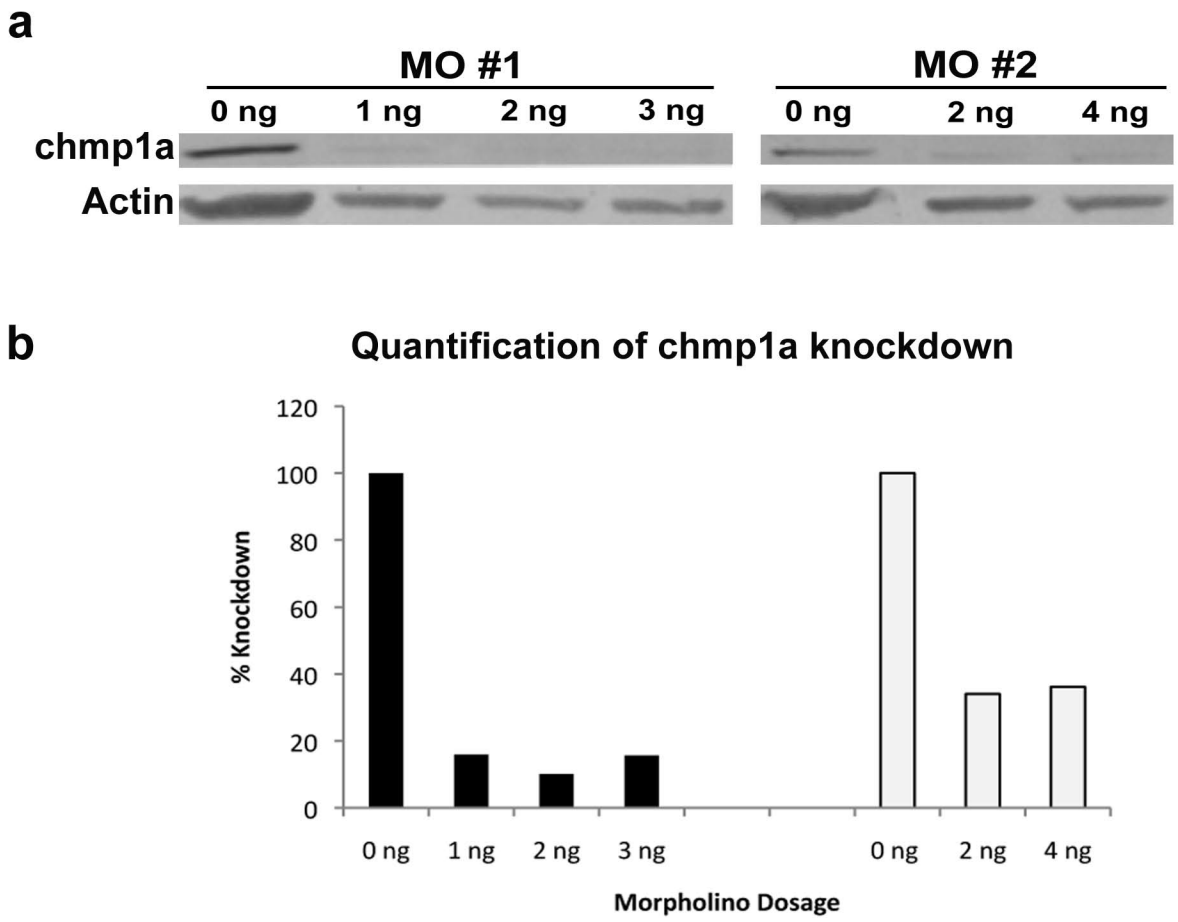
Supplementary Figure 1. Microsatellite marker haplotypes in the region of linkage on chromosome 16 in Families 1, 2, and 3.

The affected individuals in Family 1, 2 and 3 share a region of homozygosity on chromosome 16q24.3. Family 2 and 3 have the same homozygous haplotype. The minimal critical interval is defined by D16S689 and 16qter.

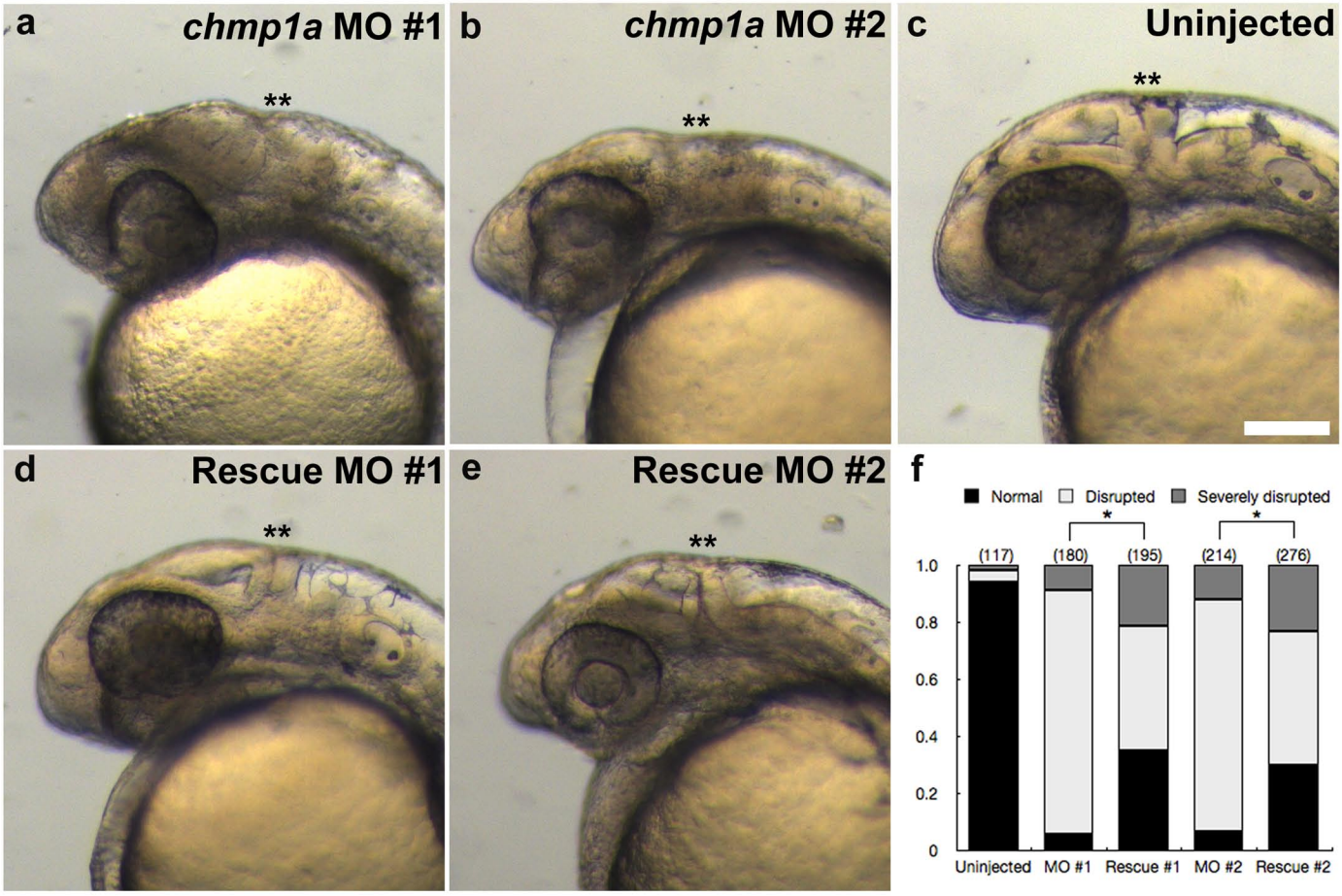


Supplementary Figure 2. Analysis of aberrant splicing due to the intronic mutation in Family 1.

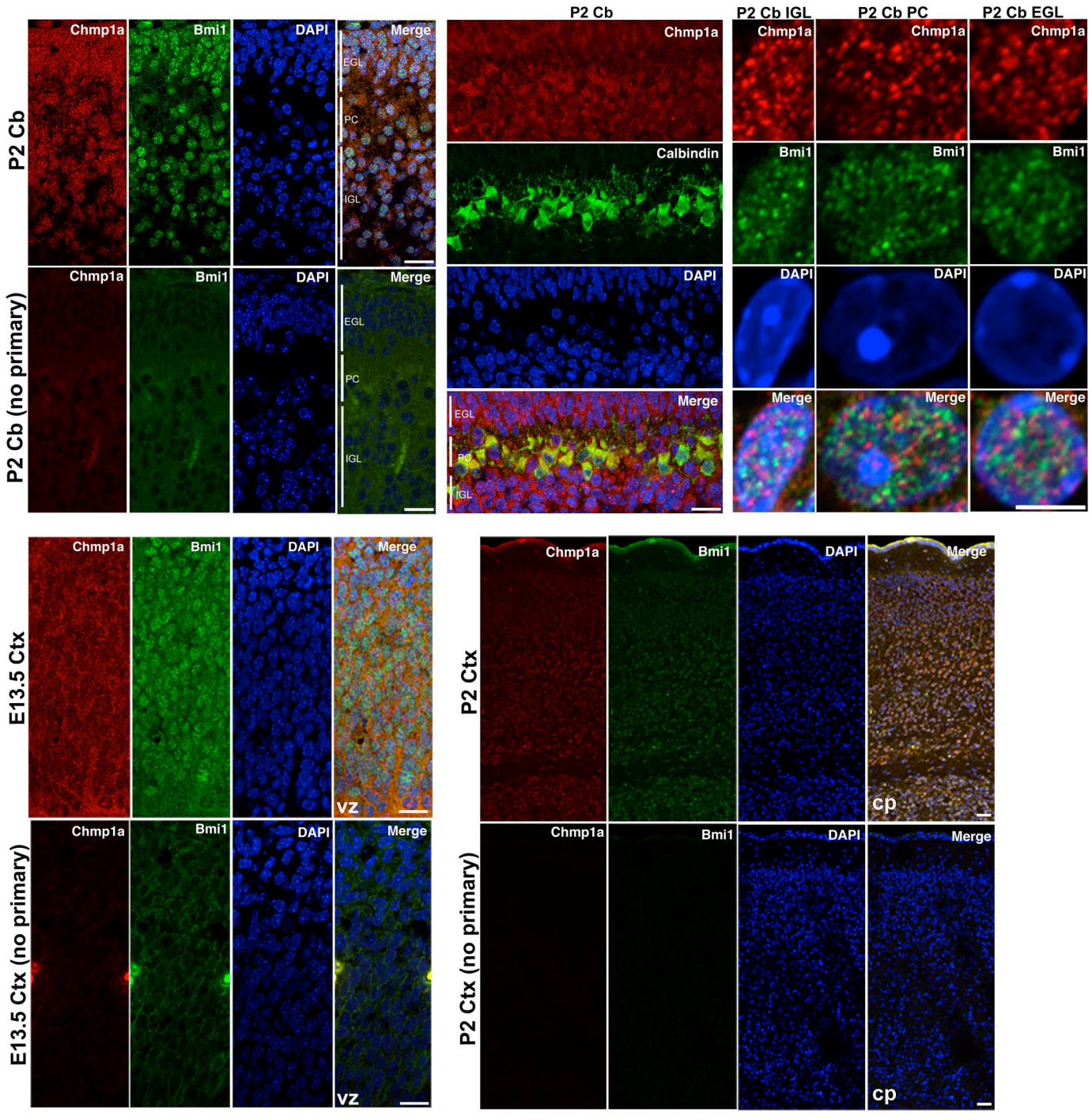
(a) NetGene2[1] web-based prediction is performed on the region surrounding the intron 2-exon 3 junction of *CHMP1A*, with and without the presence of the nucleotide change found in Family 1 (c.28-13G>A). Confidence values for predicted splice acceptors are scaled from zero to one, with a higher number indicating a greater probability of being a splice acceptor. In the wildtype sequence (WT), the annotated acceptor is predicted with a high confidence value (0.83). A cryptic splice acceptor 21 bp upstream of the annotated acceptor is also predicted (0.76). In the presence of the mutation (CH3101), the confidence value for the annotated acceptor decreases (0.69), and a novel acceptor 11 bp upstream is predicted (0.43). The confidence value for the cryptic acceptor 21 bp upstream is not affected by the presence of the mutation. (b) *CHMP1A* cDNA from lymphoblastoid cell lines were sequenced. A control sample (CTRL) reveals a single transcript, which utilizes the annotated splice acceptor. An affected individual (CH3101) does not show any wildtype transcript (most conspicuously demonstrated at the nucleotide position indicated by the arrow), and instead reveals a transcript that utilizes the novel acceptor 11 bp upstream of the annotated acceptor (resulting in a 11 bp insertion), as well as a transcript that utilizes the cryptic acceptor 21 bp upstream of the annotated acceptor (resulting in a 21 bp insertion). CH3104, the father of CH3101, shows a similar tracing to control, suggesting that the abnormal transcripts are quite unstable.



Supplementary Figure 3. Western blot analysis of zebrafish *chmp1a* morpholinos.
(a) Western blot analysis of lysates from zebrafish embryos injected with two different *chmp1a* morpholinos (MO #1 and MO #2) shows reduced chmp1a protein expression. (b) Quantification of the western blot results shows approximately 85% and 65% knockdown with MO #1 and MO #2, respectively.

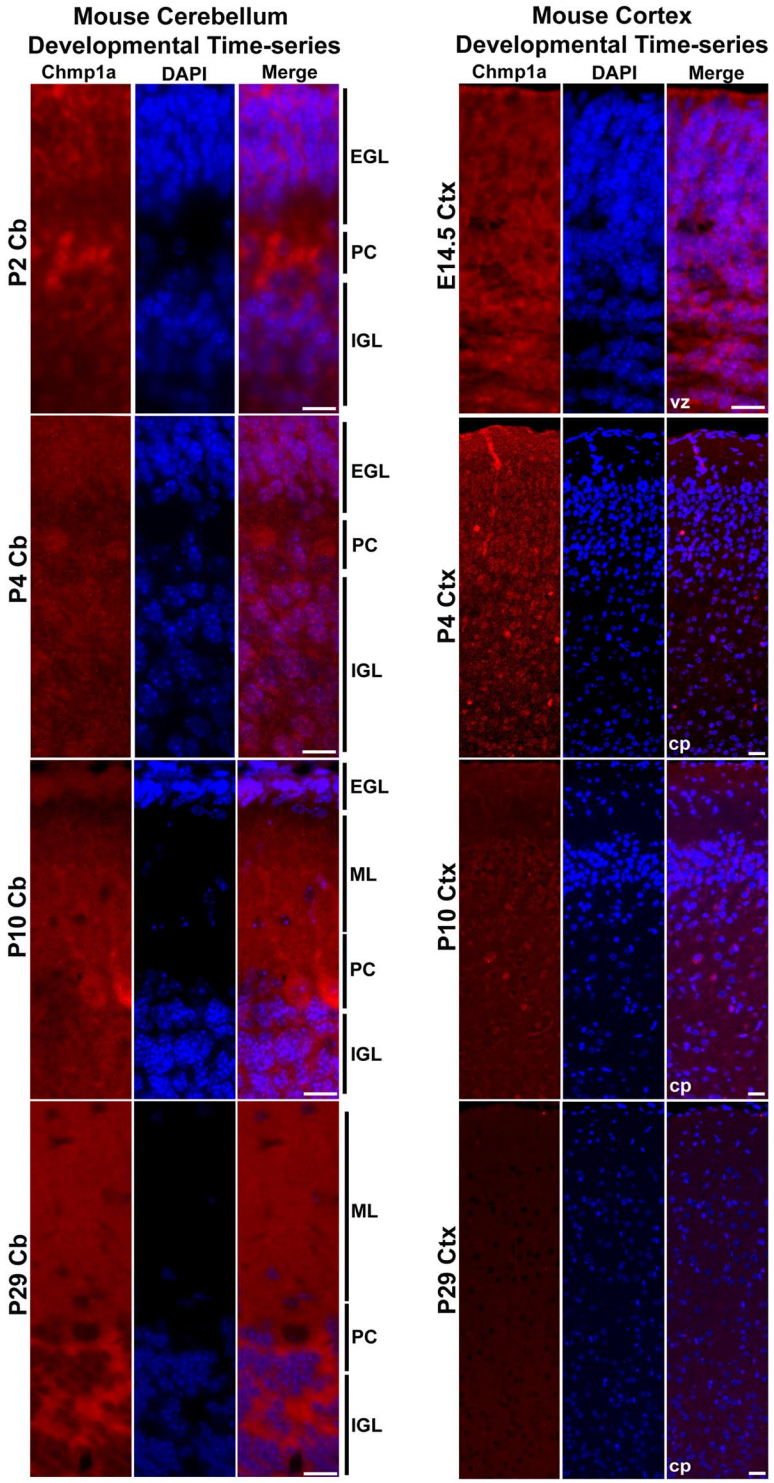


Supplementary Figure 4. Identical brain phenotypes of two zebrafish morpholinos.
(a-f) Zebrafish injected with two different *chmp1a* morpholinos (MO #1, MO #2) (a, b) show a reduction in head size, with the hindbrain more markedly reduced in thickness (asterisks), compared to control (c). The phenotypes are partially rescued when human *CHMP1A* mRNA is coinjected with MO #1 (Rescue MO #1) (d) or MO #2 (Rescue MO #2) (e), with restoration of hindbrain structure (asterisks). Embryos are classified at 28 hours post fertilization as either normal, disrupted, or severely disrupted (see Online Methods) for each of uninjected controls, *chmp1a* MO #1, *chmp1a* MO #2, and human *CHMP1A* mRNA with *chmp1a* MO #1 (Rescue #1) or chmp1a MO #2 (Rescue #2) (f). The number above each bar is the total number of embryos examined. Scale bar = 200μm (a-e). * $p < 0.001$, two-tailed Pearson's chi-squared test (f).



Supplementary Figure 5. Localization of Chmp1a and Bmi1 expression in the developing mouse cerebellum and cerebral cortex.

In the developing cerebellum at postnatal day 2 (P2 Cb), Chmp1a is expressed in the nucleus and cytoplasm of cells in the external germinal layer (EGL), Purkinje cells (PC) and cells in the internal granule layer (IGL). Bmi1 is expressed in these cells that are positive for Chmp1a, and it primarily localizes to the nucleus. Co-staining of Chmp1a and calbindin confirms expression of Chmp1a in all three layers. In the nucleus of P2 EGL, Purkinje and IGL cells, speckled Chmp1a and Bmi1 staining is seen. Chmp1a and Bmi1 signals in the nucleus are sometimes seen adjacent to each other, but they rarely colocalize. In the developing cerebral cortex at E13.5, Chmp1a is expressed in both ventricular zone progenitors and cortical plate cells, and at P2 cerebral cortex, cortical plate cells continue to express Chmp1a. Bmi1 is expressed in the same population of cells in both cerebellum and cerebral cortex. Control staining with no primary antibodies confirms specificity of Chmp1a and Bmi1 staining. vz = ventricular zone. cp= cortical plate. Scale bars = 20μm, except on the high-magnification images of nuclei (5μm).



Supplementary Figure 6. Developmental time-series of Chmp1a expression in the developing mouse cerebellum and cerebral cortex.
In the cerebellum (Cb) at P2, P4 and P10, Chmp1a is expressed in the nucleus and cytoplasm of external germinal layer (EGL) cells, Purkinje cells (PC), and internal granule layer (IGL) cells. At P29, Chmp1a expression remains in the molecular layer (ML), Purkinje cells and internal granule layer cells. In the cerebral cortex (Ctx) at E14.5, Chmp1a shows ubiquitous nuclear and cytoplasmic expression in the neuroepithelial cells. At P4, P10 and P29, Chmp1a expression in postmitotic neurons of the cortical plate gradually decreases, and becomes almost undetectable by P29. vz = ventricular zone, cp = cortical plate. Scale bars = 20μm.

Supplementary Table 1. Primer and morpholino sequences.

Human *CHMP1A* sequencing

Exon 1-Forward	GCCTCCTTTACCTTTCCTGG
Exon 1-Reverse	GCCCGAGCTCTCCTGAGT
Exon 2-Forward	CCCTTTGAGATTGGCTGGTA
Exon 2-Reverse	AATCTGCCCGACTGTTTCTG
Exon 3-Forward	AAAACCCAGACAGAGCCTGA
Exon 3-Reverse	CCCAAAAGGCATCAAAACAA
Exon 4-Forward	CAAAACAAAAACTGGGCTCC
Exon 4-Reverse	CTGATCCAGCTTCCCTCAAC
Exon 5-Forward	CGAGTTGGTGGTCTTTCCAT
Exon 5-Reverse	CAGGGAGCAACCACAGGTAT
Exon 6-Forward	GTGCAGTCGTACCCTTGTGA
Exon 6-Reverse	AGCCTCTAACCCACAACCCT
Exon 7-Forward	GGAGGAAAAGGGTCACCTGT
Exon 7-Reverse	ACAGTGCTGGGTGAAAGTCC

Human *CHMP1A* RT-PCR

Forward (5' UTR)	CAGCTTGGTCGGTTCGAT
Reverse (exon 6)	CAGCTGGCTGAGCTGGTC

Quantitative PCR

INK4A-Forward	CCCCTTGCCTGGAAAGATAC
INK4A-Reverse	AGCCCCTCCTCTTTCTTCC
ARF-Forward	AACATGGTGCGCAGGTTC
ARF-Reverse	GGGATGTGAACCACGAAAAC
GAPDH-Forward	CCAAGGTCATCCATGACAAC
GAPDH-Reverse	GGCCATCCACAGTCTTCTG

Chromatin immunoprecipitation-quantitative PCR

INK4A-Forward	CAACGCACCGAATAGTTACG
INK4A-Reverse	CCAATTCCCCTGCAAACTTC
ARF-Forward	TCCTTGCCCTGCTTTTACTG
ARF-Reverse	ACATCACCGATCCTTTCTGG
7kb upstream-Forward	ATGTTGTTGGGGCTACTTCC
7kb upstream-Reverse	AGGTTTTAGCCAAGGGGATG

Human *CHMP1A* cDNA cloning

Forward (5' UTR)	CCGGAAGTGGGGCGGCGACCCCG
Reverse (3' UTR)	TGTTTATTTACAACAGCATTCTA

Zebrafish morpholinos

chmp1a MO #1	CAGTGTGTCGTCCATGTCGCTGTCT
chmp1a MO #2	TCGGGTTATTCGCTGCTCTTCTGCT
bmi1a	GCATCGTCATGGGATAGAAAAGAAC
bmi1b	CATTGTCATGGGAGTACGCCGACCT
cdkn2a	TAAAGCGCGTCTAAACCTACCTGTA

Supplemental Note

Clinical Information

There are three affected individuals in Family 1 (CH3101, CH3102, and CH3105). CH3101 (female) was born at 33 weeks of gestation via Caesarean section. The pregnancy was complicated by hyperemesis gravidarum. At birth, her head circumference was 33cm (91st percentile), and weight was 2.2kg (57th percentile). She was found to have a ventricular septal defect during the neonatal period, which later resolved. Her early motor and speech development were delayed. She started to walk independently at 8 years of age. She had bilateral equinocavovarus and claw foot deformities, which were worse on the left, and underwent orthopedic procedures for them. On examination at 16 years of age, her head circumference was 50cm (<3rd percentile; -4.0 SD), height was 138cm (<3rd percentile; -3.7 SD) and weight was 32kg (<3rd percentile; -3.2 SD). No dysmorphic features were noted. She spoke only in single words, and had poor social interaction. There were no oral motor difficulties. She had generalized hypotonia and walked with an ataxic gait. She had normal deep tendon reflexes. Her visual examination was remarkable for myopia, astigmatism and convergent strabismus. She had negative TORCH titers and her karyotype was normal. She underwent MRI of the brain at 12 years of age, which revealed a very small cerebellum (vermis and hemispheres). The brain stem was thin with a short pons and long medulla. There was no cerebral cortical malformation but the cerebral white matter was moderately reduced in volume with a fully formed but thin corpus callosum. Myelination of the white matter was age-appropriate.

CH3102 (male), younger brother of CH3101, was born after an unremarkable pregnancy via Caesarean section at 38 weeks of gestation. At birth, his head circumference was 36cm (86th percentile), and weight was 2.8kg (8th percentile). He had delay in motor and speech development, and also had feeding difficulties. He started to walk independently at 7 years of age. He had surgery for bilateral talipes valgus. On examination at 11 years of age, his head

circumference was 50cm (<3rd percentile; -2.4 SD), height was 127cm (<3rd percentile; -2.4 SD), and weight was 26kg (<3rd percentile; -2.0 SD). No dysmorphic features were noted. He spoke only in single words, and had poor social interaction. He had low muscle tone, except in the lower extremities, which were hypertonic and showed dystonic posturing. He also showed involuntary, repetitive vertical head movements. He had increased deep tendon reflexes. Ophthalmologic examination was remarkable for myopia, astigmatism, convergent strabismus of the right eye, bilateral pseudoptosis, and limitation of extraocular movements. He had normal TORCH titers and a normal karyotype. He had MRI of the brain initially at 2 years and 5 months of age. The findings were similar to those of his older sister (CH3101), including a very small cerebellum (vermis and hemispheres) and pons. There was no cerebral cortical malformation but the cerebral white matter was moderately diminished in volume with a fully formed but thin corpus callosum. He had a repeat brain MRI at 11 years of age, which showed similar findings and no progression from the first study.

CH3105 (male) is a cousin of CH3101 and CH3102, and was born after 38 weeks of gestation via Caesarean section with a birth head circumference of 36cm (86th percentile) and weight of 2.97kg (17th percentile). There were no perinatal complications. He had delay in motor and speech development, but no feeding difficulties. On examination at 7 years of age, his head circumference was 50cm (7th percentile), height was 115cm (10th percentile), and weight was 27kg (79th percentile). No dysmorphic features were noted. He spoke only in single words, and had poor social interaction, similar to his affected cousins. He had esotropia. He had low muscle tone, and showed involuntary, repetitive vertical movement of his head, similar to CH3102. He had normal deep tendon reflexes, and had not achieved independent walking. He had negative TORCH titers, Fragile X syndrome DNA testing, and ataxia gene sequencing panel. He had normal plasma amino acid, very long-chain fatty acid, and urine organic acid profiles. Serum transferrin electrophoresis was also normal. He underwent MRI of the brain at the age of 3 months. The findings were very similar to those of his cousins (CH3101 and CH3102) and

included a very small cerebellum (vermis and hemispheres) and pons, a normal cerebral cortex, mild to moderate decrease in the cerebral white matter volume, and a thin but fully formed corpus callosum. Myelination of the cerebral white matter was thought to be slightly delayed for his age. None of the affected individuals in Family 1 has history of seizures.

There are two affected individuals in Family 2 (CH2401 and CH2402). CH2401 (female) was born after uncomplicated pregnancy at 36 weeks of gestation via Caesarean section due to breech presentation. Measurements at birth are not available. Joint stiffness was noted soon after birth, and was worse in the legs with hip dislocation. She had gastrointestinal issues including constipation and gastroesophageal reflux. Her development was severely delayed from early on, and she was found to have impaired ability to control swallowing. Examination at 2 years and 7 months of age revealed head circumference of 44cm (<3rd percentile; -2.8 SD), length of 82.5cm (<3rd percentile; -2.4 SD) and weight of 11.25kg (7th percentile). She was found to have frequent babbling, but no communication with words or signs. She did not interact with her environment, and did not follow commands. She had mildly increased tone in the arms and moderately increased tone in the legs. She showed frequent involuntary movements including side-to-side head shaking and slow kicking movements of the legs. Ophthalmological examination at 2 years and 10 months of age revealed no visual tracking. She had esotropia, hyperopia and astigmatism. Her pupils were normal, and there was no nystagmus. She was felt to have predominantly cortical visual impairment. On examination at 5 years of age, her head circumference was 44.8cm (<3rd percentile; -4.0 SD), length was 97cm (<3rd percentile; -2.3 SD), and weight was 14.5kg (4th percentile). She had a mild scoliosis. She still did not show visual tracking, but responded to visual threat by blinking. Side-to-side head shaking was again noted. She was diffusely spastic, with brisk deep tendon reflexes. She had normal serum lactate and pyruvate, serum amino acids and urine organic acids. Carbohydrate-deficient transferrin analysis and testing for *MECP2* were also normal. She had MRI of the brain at 4 months of age and again at 4 years of age. They were remarkable for severe reduction in size of the

cerebellum (vermis and hemispheres) and pons, decreased cerebral white matter volume, a thin corpus callosum, and a grossly normal cerebral cortex. There was no progression of the findings between the two studies.

CH2402 (female) is a younger sister of CH2401. The course of the pregnancy was uncomplicated, but at 37 weeks of gestation, fetal decelerations were noted, and delivery was precipitous. Her Apgar scores were 8 and 9, and her birth weight was 2.9 kg (38th percentile). She was noted to be microcephalic during the newborn period, and had a right club foot and multiple joint contractures, most prominent in the hands. At 3 months of age, her head circumference was 37.4cm (<3rd percentile; -2.0 SD), length was 53cm (<3rd percentile; -2.6 SD), and weight was 6.4kg (89th percentile). She was noted to have mild asymmetry of the forehead and hollow temples. She had upturned nares, slightly tented upper lip, and mildly low set and posteriorly rotated ears. She showed no visual tracking or social smile. She had diminished spontaneous movements, but had frequent irregular small jerky movements, suggestive of chorea. On examination at 11 months of age, her head circumference was 41.8cm (<3rd percentile; -2.4 SD), length was 68.3cm (6th percentile), and weight was 9.95kg (72nd percentile). She was noted to have brief visual tracking, but otherwise had gained few developmental milestones. Choreiform movements involving all body regions were again noted. She had diffusely increased muscle tone and deep tendon reflexes. She had a normal karyotype and negative urine culture for cytomegalovirus. Echocardiogram was remarkable for patent foramen ovale. Renal ultrasound was normal. There was no history of seizure, and video EEG monitoring showed no seizures or epileptiform activities. She had MRI of the brain at 2 days of age and again at 10 months of age. They showed similar findings to those of her older sister (CH2401), including a very small cerebellum (vermis and hemispheres) and pons, no cerebral cortical malformation, and severely reduced cerebral white matter volume with a fully formed but thin corpus callosum. Her MRI also revealed abnormal T2 hyperintensity diffusely in the cerebral white matter, in addition to the reduced volume. There was no clear evidence of

progression between the two studies.

There is one affected individual in Family 3 (CH2701). CH2701 (female) was born after a full term, uncomplicated pregnancy. She reportedly did not cry at birth and had low Apgar scores. At birth, her head circumference was 34.3cm (39th percentile) and weight was 3.3kg (42nd percentile). She was noted to have arthrogryposis multiplex congenita. At 3 weeks of age, her head circumference was 36cm (39th percentile), length was 48cm (3rd percentile), and weight was 3.4kg (17th percentile). She had severe delay in her development. She never sat or stood independently. She developed some babbling, but no meaningful words. She was noted to have mild swallowing incoordination and esophageal dysmotility. At around 1 year of age, she started to have episodes of upward gaze deviation with head extension for a few seconds. She also developed episodes of clonic head movements to the left. These episodes were felt to be seizures and she was placed on an anticonvulsant medication. She began to have vomiting with eating and started to lose weight after 1 year of age, requiring G-tube placement and Nissen fundoplication. She started to show developmental regression at around 18 months of age. On examination at 2 years 8 months of age, she was noted to be cachectic with severe wasting. Her head circumference was 41.5cm (<3rd percentile; -4.4 SD), length was 64cm (<3rd percentile; -7.4 SD), and weight was 5.3kg (<3rd percentile; -6.3 SD). She was noted to have long eyelashes, bushy eyebrows and synophrys. She had generalized hypertrichosis. By 5 years of age, her nutritional status significantly improved, and she started to show slow progress in her development again. On examination at 7 years of age, her head circumference was 48.0cm (<3rd percentile; -2.8 SD), length was 86.1cm (<3rd percentile; -6.8 SD), and weight was 13.0kg (<3rd percentile; -3.6 SD). She did not show visual fixation or tracking, but blinked in response to light. Her eyes intermittently showed conjugate upward deviation for a few seconds. She had low muscle tone in the trunk and increased tone in the hip and lower extremities. She was noted to have multiple joint contractures of the lower extremities. She was unable to roll over or sit independently, but she was able to reach for objects. Her ophthalmological

examination was remarkable for no measurable vision, but normal ocular structures and no cataracts. She had a normal karyotype and chromosomal microarray. Plasma amino acid and urine organic acid analyses did not show a recognizable pattern for a disorder of amino acid or organic acid metabolism. Complete blood count, serum immunoglobulin profile and T- and B-cell subsets were unremarkable. Work-up for congenital disorders of glycosylation included carbohydrate-deficient transferrin analysis, as well as phosphomannomutase and phosphomannose isomerase activity on skin fibroblast, which were all unremarkable. She had MRI of the brain at 6 months of age, which revealed a very small cerebellum (vermis and hemispheres) and brainstem, especially the pons, no cerebral cortical malformation, and markedly diminished cerebral white matter volume with a very thin corpus callosum. A follow-up MRI of the brain at 22 months of age showed no obvious change from the initial study. Another MRI of the brain at 7 years of age was unchanged compared to the prior studies, except for slightly more prominent subarachnoid spaces in the anterior middle cranial fossa. Overall there was no clear evidence of progression over the three studies.

In summary, no clear evidence of progression of the radiological findings was noted in any of the individuals who had more than one MRI study. This suggests that the condition is developmental rather than degenerative.

CHMP1A isoforms

Though another isoform has been annotated for the human *CHMP1A* locus on the UCSC Human Genome Browser, the evidence in support of this alternative isoform is weak, and it is not detectable in any other species. The putative alternative isoform (NM_001083314) is identical to *CHMP1A*, except it lacks exon 2. Exon 2 is 20 nucleotides in length, and therefore the majority of the alternative isoform is in a different reading frame from *CHMP1A*. The alternative isoform is predicted to encode a protein of 240 amino acids. The mutation identified in Families 2 and 3 is predicted to result in a conservative missense change in this alternative

isoform (p.A24V), whereas the mutation identified in Family 1 creates a frameshift and premature stop codon for both isoforms. While western blot analysis shows complete absence of CHMP1A in affected individuals from both families, similar western blot analysis using an antiserum against the predicted alternative isoform shows low or undetectable expression of the alternative isoform in both affected and unaffected individuals from Family 2 (data not shown). In mouse, only the transcript orthologous to *CHMP1A* has been annotated, and it has 97% amino acid identity to human CHMP1A. When exon 2 was removed from the mouse *Chmp1a* sequence, the resulting amino acid sequence would be dissimilar to the human alternative isoform, and only 21 amino acids in length. In the chimpanzee genome, the *CHMP1A* orthologue has not been annotated, but N-SCAN gene prediction software [2-3] predicts the presence of a *CHMP1A* homologue, with 95% amino acid identity to human CHMP1A. Again, removing exon 2 results in a highly dissimilar amino acid sequence from the human alternative isoform, with the resulting protein length being only 28 amino acids. Furthermore, the NHLBI Exome Sequencing Project Exome Variant Server lists 5 nonsense variants for the alternative isoform, but none for *CHMP1A*. Taken together, these findings strongly suggest that *CHMP1A* is the essential form.

Supplementary References

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