Mutations in Spliceosomal Genes PPIL1 and PRP17 Cause Neurodegenerative Pontocerebellar Hypoplasia with Microcephaly

Highlights

- Loss of spliceosomal components PPIL1 and PRP17 occurs in pontocerebellar hypoplasia
- Modeling shows neural apoptosis and disrupted global RNA splicing integrity
- PPIL1 and PRP17 form an enzyme-substrate pair within the active spliceosome
- PPIL1 and PRP17 control splicing independent of enzyme activity

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In Brief

Chai et al. discover that loss of splicing factors PPIL1 and PRP17 leads to a neurodegenerative brain disease, and even though they are an enzyme-substrate pair, they function instead to scaffold the spliceosome.
Mutations in Spliceosomal Genes PPIL1 and PRP17 Cause Neurodegenerative Pontocerebellar Hypoplasia with Microcephaly

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SUMMARY

Autosomal-recessive cerebellar hypoplasia and ataxia constitute a group of heterogeneous brain disorders caused by disruption of several fundamental cellular processes. Here, we identified 10 families showing a neurodegenerative condition involving pontocerebellar hypoplasia with microcephaly (PCHM). Patients harbored biallelic mutations in genes encoding the spliceosome components Peptidyl-Prolyl Isomerase Like-1 (PPIL1) or Pre-RNA Processing-17 (PRP17). Mouse knockouts of either gene were lethal in early embryogenesis, whereas PPIL1 patient mutation knockin mice showed neuron-specific apoptosis. Loss of either protein affected splicing integrity, predominantly affecting short and high GC-content introns and genes involved in brain disorders. PPIL1 and PRP17 form an active isomerase-substrate interaction, but we found that isomerase activity is not critical for function. Thus, we establish disrupted splicing integrity and “major spliceosome-opathies” as a new mechanism underlying PCHM and neurodegeneration and uncover a non-enzymatic function of a spliceosomal proline isomerase.

INTRODUCTION

Pontocerebellar hypoplasia (PCH) comprises a group of severe pediatric-onset neurodegenerative disorders affecting cellular survival in the brainstem and cerebellum, resulting in impaired neurological function and early death (Cassandrini et al., 2010). Humans with PCH show near normal early embryologic development, followed by mid-gestational slowing or cessation and later regression in select neuroanatomical regions (Joseph et al., 2014). Most genes implicated in PCH are involved in tRNA splicing or GTP availability, suggesting a potential effect on protein translation (Breuss et al., 2016; Budde et al., 2008; Karaca et al., 2014; Schaffer et al., 2014). Although postnatal progressive microcephaly can be part of the clinical spectrum, children are mostly born with normal or only mildly reduced head circumference (HC) (van Dijk et al., 2018).

Pre-mRNA splicing, mediated by the spliceosome complex, is essential for gene expression and regulation in higher organisms.
Families with biallelic mutations in PPIL1

Family 1
p.A99T

Family 2
p.A99T

Family 3
p.T107A

Family 4
p.Y78C

Family 5
p.R131Q

Family 6
p.[A101_D106dup; G109C]

Family 7
p.[F82S]; [R131Q]

Family 8
c.[379A>G]; [280+1G>A]
p.[T127A]; [?] p.[R45*]

Family 9
p.[T127A]; [R45*]

Control

Family 1-III:1

Family 4-III:3

Family 5-III:1

Family 5-III:2

Family 7-II:1

B

C

Homozygous mutations

p.A99T

Family 1

Family 4
p.A101_D106dup

Family 6
p.A99T

Family 3
p.T107A

Family 6
p.[F82S]

Family 6
p.[R131Q]

Family 7
p.[T127A]

Family 8
p.[R45*]

Compound heterozygous mutations

p.R45*

Family 9

Family 7
p.[R131Q]

Family 8
p.[T127A]

Family 7
p.[R45*]

D

(opposite to the enzymatic surface)

R131

T127

A99

T107

G109

Y78

(legend on next page)
Incorporated within the MSC are eight cyclophilin peptidyl-prolyl isomerases (PPIases), enzymes initially found as targets of immunosuppressants but later found to promote conformational changes of substrates by catalyzing cis-trans isomerization of Xaa-Proline peptide bonds (Agafonov et al., 2011; Bessonov et al., 2010; Davis et al., 2010; Evans et al., 1987; Rajiv and Davis, 2018; Teigelkamp et al., 1998; Zhou et al., 2002). Functions and substrates of most PPIases remain unknown. Here, we report that biallelic, hypomorphic mutations in two spliceosomal genes, PPIL1 and PRP17, encoding an active PPIase-substrate pair, disrupt RNA splicing integrity and cause converging neurodegenerative phenotypes in human and mouse. Although both proteins are required for splicing integrity and neuronal survival, surprisingly, mutations preventing PRP17 isomerization catalyzed by PPIL1 are tolerated, thus revealing a predominant non-enzymatic function of a spliceosomal PPlase.

RESULTS

Identification of Biallelic Mutations in PPIL1 in PCHM Families

From our cohort of 7,288 patients with recessive congenital neurological disorders, we identified rare damaging homozygous missense variants in PPIL1 among eight index patients from five families (Figures 1A–1C). All patients showed both features of PCH as well as congenital microcephaly (−3 to −6 SD HC); the latter phenotype progressed postnatally for all. Patients did not show features of known syndromic PCH subtypes (Akizu et al., 2013; Namavar et al., 2011; Table 1; Table S1). All subjects were enrolled with institutional review board (IRB)-approved protocols at referral institutions and provided signed consent. Through GeneMatcher (Sobreira et al., 2015), we identified four additional families with eight affected patients in whom PPIL1 mutations were independently identified as the likely cause (Tables S1 and S2). As all individuals exhibited PCH with microcephaly, we defined this presentation as a unique clinical entity, which we termed PCHM (PCH plus microcephaly). Further common phenotypes included hypotonia, difficulty swallowing, failure to control the airway, seizures, and delayed motor and language development. Brain MRI showed cortical changes in most affected patients (Figure 1B; Table S1), notably simplified gyri pattern, which was rarely reported for other subtypes of PCH.

The mutations in each family segregated with the phenotype according to recessive inheritance. Families 1–5 showed homozygous missense variants, and family 6 showed two separate homozygous variants predicting a 6 aa duplication and a p.G109C substitution. Families 7–9 showed compound heterozygous variants: families 5 and 7 and families 8 and 9 shared an identical variant. All substituted residues were highly conserved (Figure S1A), predicted “damaging” by MutationTaster (Schwarz et al., 2014), and clustered within PPIL1’s cyclophilin PPlase domain, suggesting deleterious functions (Figure 1C).

Patient Mutations Affect PPIL1 Function

PPIL1 joins the MSC together with its interacting protein SKIP, two of the NineTeen complex-related proteins, during B complex formation, and remains until splicing is complete (Rajiv and Davis, 2018; Wang et al., 2010). We mapped human mutations onto the ordered globular PPIL1 structure (Xu et al., 2006) and found that all the affected residues except p.R131 were located on the enzymatic face (Figure 1D). To test the impacts of patient variants, we expressed FLAG-tagged mutant protein in HEK293T cells and found that most variants led to unstable proteins (Figures S1B and S1C). Specifically, p.Y78C, p.A99T, p.[A101_D106dup;G109C], p.F82S, and to a lesser degree p.T127A were barely detectable. Likewise, reduced endogenous p.A99T PPIL1 protein was also observed in patient fibroblasts, which showed a slightly higher RNA level, possibly due to compensatory upregulation (Figures S1D–S1F).

Two variants, p.T107A and p.R131Q, did not show altered protein levels in HEK293T cells. However, we found that both purified mutant proteins showed reduced thermal stability and increased aggregation propensity (Figures S1G–S1I). PPIL1 associates with SKIP prior to its incorporation into the spliceosome (Wang et al., 2010; Xu et al., 2006). The SKIP binding interface is located on the non-enzyme face (Wang et al., 2010; Xu et al., 2006), and previous studies suggested that PPIL1 p.R131 was involved in binding to SKIP (Wang et al., 2010; Xu et al., 2006). As expected, we found that purified p.R131Q PPIL1 failed to associate with SKIP in both surface plasmon resonance and

Figure 1. Biallelic Mutations in PPIL1 Lead to Neurodegenerative Pontocerebellar Hypoplasia with Microcephaly (PCHM) in Human

(A) The families with predicted effects of PPIL1 variants listed above pedigree. All variants are homozygous in affected individuals, except families 7–9, which are compound heterozygous. All pathogenic variants segregated as a recessive trait. Filled symbols, affected; p.[?], splice donor site mutation, c.280+1G > A; square, male; circle, female; double bar, consanguinity; diagonal line, deceased. wt, reference allele; mut, patient variant allele.

(B) Sagittal (top) and axial (bottom) T1-weighted brain magnetic resonance images show reduced cerebellar volume (yellow arrowhead), atrophic pons (white arrowhead), and posterior fossa fluid accumulation (yellow arrows) indicative of cerebellar atrophy in affected individuals. Simplified gyri pattern is most apparent in the affected from families 1 and 4.

(C) Identified PPIL1 mutations. Top: homozygous variants. Bottom: compound heterozygous mutations.

(D) En face view of enzymatic surface with labeled variant residues in nuclear magnetic resonance (NMR)-resolved PPIL1 structure (PDB: 2K7N). All except R131 (blue) localized to the enzymatic surface. Red, duplicated region in family 6 (A101-D106).

See also Figure S1.
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<th>Mutation cDNA</th>
<th>Mutation Protein</th>
<th>Gender</th>
<th>Ethnic origin</th>
<th>Parental consanguinity</th>
<th>Head circumference (HC) at birth</th>
<th>HC at last examination</th>
<th>Pontocerebellar hypoplasia</th>
<th>Simplified cortical gyral patterning</th>
<th>Agenesis of corpus callosum</th>
<th>Cerebellar hypoplasia</th>
<th>Brainstem hypoplasia</th>
<th>Hydrocephalus</th>
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<td>+</td>
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<td>+</td>
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See also Tables S1 and S2.
immunoprecipitation assays (Figures S1J and S1K). PPIL1 p.T107A, although localized to the enzymatic face, showed reduced SKIP interaction (Figure S1J). Thus, all assessed PPIL1 patient mutations either affect protein stability or interaction with SKIP.

**Defective Brain Development and Neuron-Specific Apoptosis in Knockin Mice**

To reveal functions of PPIL1 in brain development, we studied expression during development. RNA in situ hybridization showed ubiquitous Ppil1 expression in the developing cortex (Figures 2A and 2B). Because of a lack of specific PPIL1 antibodies, we generated a CRISPR knockin (KI) mouse introducing an N-terminal HA epitope in Ppil1, which confirmed ubiquitous protein expression (Figures 2C, 2D, and S2A–S2D). We next generated a Ppil1 frameshift mouse line (fs, c.302delC, p.N102Tfs*13) but found no viable homozygous embryos any time after embryonic day (E) 12.5 (0 in 41 embryos; p < 0.00001, binomial test), while Ppil1/−/− pups were indistinguishable from wild-type (WT) littermates. However, at E9.5 we recovered several partially resorbed embryos, all of which were genotyped as Ppil1fs/− (13 mutants in 46 embryos, with the expected 25%; Figures 2E and S2E). We conclude that Ppil1 is essential for embryogenesis.

We next generated a patient p.A99T KI mouse line, chosen because it was the first allele we identified. Ppil1A99T/− mice were born at the expected Mendelian ratios (22 Ppil1A99T/−, 42 Ppil1A99T/−, and 23 WT) but died within 24 h. Newborns showed smaller head size, severely reduced cerebral and cerebellar size, and reduced cortical surface area and thickness (Figures 2F–2M), matching human PCH4 phenotypes. Although the cortex showed normal lamination, neuronal numbers were severely reduced, with CUX1+ upper layer neurons decreased by ~25% and CTIP2+ deep layer neurons by ~60% (Figures 2N and 2O). Like patient fibroblast, Ppil1 protein was also severely reduced by ~80% in Ppil1A99T/− embryso lysates (Figures S2F and S2G). Additionally, compound heterozygous Ppil1A99Tfs/− mutant embryos showed much more severe phenotypes across the body at E18.5 (Figure S2H), suggesting that Ppil1 p.A99T is a hypomorphic mutation.

On the basis of the severe reduction in cortical thickness and neuronal numbers, we hypothesized that this could be caused by cell death during embryonic development. We examined apoptosis by assessing cleaved caspase-3 (CC3) and p53 expression in the embryonic brains. Indeed, the apoptosis showed a striking accumulation in TUJ1+ neurons within deep layers of the lateral cortical margin in the mutant brains starting at E12.5 and dramatic at E14.5 (Figures 3A–3F), and some apoptotic cells were GAD65/67+ interneurons (Figures S2I and S2J). The apoptosis in the rest of the cortex appeared at E14.5 and became massive at E16.5 (Figures 3A–3N). Apoptotic cells predominantly localized in the cortical plate and did not overlap with SOX2+ neural stem cells (NSCs) and TBR2+ intermediate neural progenitors (INPs) (Figures 3G–3N and S2K–S2M). About 70% of p53-positive cells were positive for CTIP2 and ~30% for CUX1, consistent with a more severe reduction of deep layer neurons. CC3 upregulation was also observed in the cerebellum and pons at E18.5 (Figures S2N and S2O). Consistent with the predominant impact of the brain in the patients and KI mutant mice, no significant upregulation of apoptosis was observed in other major organs (Figures S2P and S2Q).

Recent studies proposed MDD defects result in genome instability, due partially to accumulated R-loops, transient RNA:DNA hybrid structures that displace the non-templated strand and generate susceptibility to DNA damage (Jang et al., 2017; Paulsen et al., 2009), evidenced by γ-H2AX and p53 accumulation (Denis et al., 2005; Sorrells et al., 2018). Like p53, we indeed observed a dramatic upregulation of γ-H2AX in the mutant brains (Figures 3M, 3N, and S2L), suggesting similar mechanisms.

In contrast to other genetic models of microcephaly (Gruber et al., 2011; Insolera et al., 2014; Silver et al., 2010), no premature neurogenesis was observed at E12.5 (Figures S3A and S3B), and apoptosis initiation through p53 was predominantly localized to postmitotic neurons but not neural progenitors; consequently, the numbers of both NSCs and INPs only showed a slight reduction (Figures 3O–3X). The cell cycle of the mutant progenitors seems not to undergo dramatic alterations, as we observed only a slightly increased percentage of INPs at the G2/M phase (Figures S3C–S3F). Together, our results suggest that genotoxic stress, neuronal apoptosis, and perturbations of progenitors lead to brain volume loss in Ppil1 KI mutants.

**Ppil1 Is Required for AS Integrity**

Despite its discovery in the spliceosome many years ago (Rapspiber et al., 2002), the function of PPIL1 in RNA splicing remains mostly unknown. Because Ppil1fs/− embryos survived until E9.5, we suspected that some cells might tolerate a complete loss of PPIL1. Thus, we generated Ppil1 knockout (KO) human HAP1 cells (Figure S4A), which were viable and subjected to RNA sequencing (RNA-seq). Five different forms of AS, including skipped exons (SE), mutually exclusive exons (MXE), alternative 5′ and 3′ splice sites selection (A5SS and A3SS), and retained introns (RI), were evaluated using rMATS software (Shen et al., 2018). We benchmarked rMATS by comparing variation within groups of three controls versus three controls, where a baseline of 4,045 significant differential splicing events (SDSEs) (1.7%) were identified from 231,850 total AS events (Table S3). In contrast, 8,602 SDSEs (3.4%) were identified comparing three KOs versus three controls, with the number of SDSEs scaling with number replicates in each group (Figures S4B and S4C).

We repeated the computational analysis using LeafCutter (Li et al., 2018), which identified 951 SDSEs between three KOs and three controls, compared with 8 and 0 SDSEs for three controls versus three controls and three KOs versus three KOs (Table S3), respectively. All these revealed a dramatic disruption of global AS integrity upon loss of Ppil1.

We also confirmed altered AS by assessing “sashimi-plot pile-ups” of RNA-seq and performing RT-PCR validation on selected RI and SE events (Figures S4D–S4H). Loss of Ppil1 predominantly affected the splicing of short and high GC-content introns, without significant bias toward splice site strength (Figures S4I–S4M). The most severe changes were present in introns ≤75 bp in length and with >70% GC content (Figures S4N and S4O), where 12.6% of such introns were retained at higher levels in KO cells, compared with only 0.66% higher in controls.
Finally, we compared the list of SDSE genes in KO with Online Mendelian Inheritance in Man (OMIM) disease categories on the basis of organ system involved and found overrepresentation for neurodevelopmental disease (p = 3.45 × 10^{-20}, Bonferroni-corrected chi-square test; Figure S4P), but not for cancer, cardiac disease, or immune disease (p > 0.05). We also found that SDSE genes were significantly enriched in genes known to undergo AS in the brain (p = 1.12 × 10^{-28}; Figure S3L). All these findings are consistent with its predominant impact on the brain.

Figure 2. Patient PPIL1 Mutation Knockin Mice Exhibit PCHM-like Phenotype
(A and B) Fluorescent in situ hybridization (FISH) on coronal sections of E14.5 brain cortex hybridized with Ppil1 (A) and Pax6 (B) probes using RNAscope. Scale bar: 50 μm.
(C and D) Coronal sections of E14.5 embryos from Ppil1^{f/f} (C) and WT (D) embryos immunostained with an anti-HA antibody showing ubiquitous expression of Ppil1. CP, cortical plate. Scale bar, 50 μm.
(E) Ppil1^{f/f} mouse embryos showed reabsorption at E9.5. Scale bar, 2 mm.
(F and G) Homozygous patient variant p.A99T knockin mouse with microcephaly at E18.5. (H–M) Nissl-stained sagittal sections of E18.5 Ppil1^{A99T/A99T} brains, magnified for dashed regions in the cerebral cortex and cerebellum.
(N) E18.5 Ppil1^{A99T/A99T} cortex (coronal) shows reduced thickness but with intact lamination on the basis of immunostaining against CUX1 (upper layer neurons) and CTIP2 (lower layer neurons).
(O) Reduced density of cortical CUX1* and CTIP2* neurons in E18.5 Ppil1^{A99T/A99T} cortex. n = 4 mice/genotype. Mean ± SD; p = 0.0003, CUX1* cells; p < 0.0001, CTIP2* cells; two-tailed unpaired t test.
Scale bars: 1 mm in (H) and (K) and 50 μm in (I), (J), (L), and (M). See also Figure S2.
Disrupted AS Integrity in Ppi1A99T/A99T Brains

To test the impact of its loss on AS integrity in the brain tissue, we performed RNA-seq on three WT and three Ppi1A99T/A99T KI E14.5 brains before the accumulation of apoptotic cells. Using rMATS, we detected 3,797 SDSEs among 236,870 total AS events (1.6%; Figures 4 A and S5A–S5C). Splicing alterations were also confirmed with LeafCutter, which identified 115 SDSEs of 16,528 total AS events (0.7%).

Figure 3. Ppi1-Knockin Mice Show Increased Neuron-Specific Apoptosis and Depletion of Neural Progenitor Cells

(A–F) Embryonic Ppi1A99T/A99T brains (coronal) shows increased cleaved caspase-3 (CC3; green).

(G–N) Coronal sections of E16.5 brain cortex from WT and Ppi1A99T/A99T embryos stained for TUNEL and TBR2 (G and H), CC3 and CTIP2 (I and J), p53 and CTIP2 (K and L), and γ-H2AX and CTIP2 (M and N).

(O–V) Embryonic Ppi1A99T/A99T cortex (coronal) shows reduced PAX6 (neural stem cells [NSCs]) and TBR2 (intermediate neural progenitor [INP]) positive cells.

Scale bar: 50 μm. See also Figures S2 and S3.

Disrupted AS Integrity in Ppi1A99T/A99T Brains

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Figure 4. Global Splicing Integrity Defects in Ppil1A99T/A99T Developing Brain

(A) Impact of p.A99T mutation on five major types of AS events detected with rMATs in E14.5 brain hemispheres (three KI versus three WT). A3SS was most affected, followed by SE, RI, Mxe, and A5SS.

(B) Minigene splicing reporter assays in transfected Ppil1A99T/A99T mouse embryonic fibroblasts show higher intron retention levels in mutant cells for both Atg4d and Evi5l.

(C and D) Distribution of differential splicing identified by rMATs in KI or control, on the basis of intron length and GC content. Introns with short length or high GC content show significantly retained higher in Ppil1-KI brains. All introns represent all identified introns from mouse reference genome. p value from Wilcoxon test.

(E–H) Splice-site strength analysis of 5’SS and 3’SS in all introns (All; gray), non-significant A5SS or A3SS events (Non-sig; green), and significant A5SS or A3SS events (Sig; red) identified using rMATs. The 5’SS and 3’SS strength show lower maximum entropy for choice points that were significantly different in KI compared with control, p value from Wilcoxon test.

(I) Metascape visualization of enriched networks and pathways among all misspliced genes in E14.5 Ppil1A99T/A99T brains (n = 2,134 misspliced genes), showing several key modules represented, including “mRNA metabolic process” among others.

See also Figures S4 and S5 and Table S3.
group of significant RI and SE events were verified in semi-quantitative RT-PCR (Figures S5D and S5E). Two “minigene” constructs transfected into MEFs for the SDSE introns of Atg4d and EvsI confirmed that the splicing defects were not secondary to non-specific effects (Figures 4B and S5F). Similar to HAP1 cells, KI brains showed significant RI events for short and high GC-content introns (Figures 4C and 4D). However, unlike HAP1 KO cells, we also observed a preference for weak 5’ and 3’ splicing sites among significant events (Figures 4E–4H and S5G), which may reflect competition for reduced PP1L protein between strong and weak splicing sites. Profiling of misspliced genes revealed protein translation, RNA processing, and DNA damage response as the most significantly disturbed modules (Figures 4I, S5H, and S5I), whose disruptions are major causes of cerebellar ataxia (Synofzik et al., 2019). Moreover, genes involved in axon development and cell cycle were also significantly affected (Figure 4I), reinforcing the phenotypes observed in KI brains.

**PP1L Catalyzes the Isomerization of PRP17 Gly94-Pro95 In Vitro**

Recent cryoelectron microscopy (cryo-EM) structures allowed detailed analysis of proline isomerases within the MSC (Bertram et al., 2017; Fica et al., 2019; Haselbach et al., 2018; Yan et al., 2015a; Zhan et al., 2018; Zhang et al., 2017, 2018, 2019), six of which were evident in one or more MSC complexes (Table S4). However, only for PP1L was it possible to identify a Prp from an adjacent protein within the enzymatic pocket, where we identified Gly94-Pro95 of PRP17 within the B^C^C aromatic core, C, C^*, P, and ILS complexes (Fica et al., 2019; Zhan et al., 2018; Zhang et al., 2017, 2018, 2019), conserved from *S. pombe* (Yan et al., 2015a) to human (Figures 5A, 5B, and S6A–S6E). This finding suggests that PP1L may be a substrate of PP1L in the MSC.

Gly94-Pro95 in PRP17 occurs within an intrinsically unstructured region, between two *α*-helical domains, conserved from *S. pombe* to human (Figure 5C). There is an adjacent residue (human Phe93 or *S. pombe* Lys67) within the S2 pocket, which likely determines substrate specificity (Davis et al., 2010; Teigelkamp et al., 1998). To test their interaction, we performed heteronuclear single-quantum coherence (HSQC) spectral analysis with 15N-labeled PP1L and a 13-mer PRP17 peptide (aa 89–101) containing Pro95. We confirmed that PP1L peptide interacts with PRP17 along its enzymatic surface (Figures 5D–5F). Using isothermal titration calorimetry assay, we defined a dissociation constant (Kd) of 111.9 ± 4.0 μM (Figure 5F). To investigate whether PP1L catalyzes PRP17 isomerization, we used a PRP17 peptide (aa 89–101) with 15N and 13C double-labeled P95 and demonstrated the Gly94-Pro95 bond was present in both cis and trans conformations (Figures S6G and S6H). Addition of catalytic concentrations of PP1L accelerated the rate of proline isomerization in PRP17 peptide, evidenced by the appearance of exchange peaks in 1H 15N H(Ca)N ZZ exchange spectra (Figures 5G–5I). This was also confirmed using two-dimensional (2D) 1H-15N ZZ exchange spectra of a uniformly 15N-labeled PRP17 peptide (aa 84–101) with catalytic concentrations of PP1L WT (Figure S6I). We conclude that PP1L is capable of catalyzing PRP17 isomerization in vitro.

**PRP17 Loss Associates with PCH**

Only a few of the >100 MSC components are associated with human disease (Lines et al., 2012; Pellagatti and Boulwood, 2017; Růžičková and Staněk, 2017; Xu et al., 2017), so we considered genes encoding PP1L-associated proteins as candidates for PCHM. In addition to PRP17, we found that SKIP and RBM22 bound to PP1L (Figures S6A–S6C; Video S1). We thus searched our unsolved pediatric brain disease cases and identified a multi-plex consanguineous family with PCHM (family 10), also with chronic anemia and thrombocytopenia, and with a homozygous PRP17 variant, predicting a damaging p.F502C protein change (Figures 6A and 6B; Tables S1 and S2). No further families were identified in GeneMatcher.

PRP17 contains a C-terminal WD40 domain, where F502 resides, which is fully evolutionarily conserved (Figures 6C and 6D). In cryo-EM MSC structure, the WD40 assumes a classical “7-propeller” architecture and associates with U2 small nuclear RNA (snRNA) and the U2/branchpoint sequence (BPS) duplex, stabilizing the catalytic site (Video S1; Bertram et al., 2017; Haselbach et al., 2018; Zhan et al., 2018; Zhang et al., 2017). We assessed the impact of p.F502C on protein using HA-tagged cDNA expressed in HEK293T cells and found dramatic protein destabilization, which was also confirmed in patient fibroblasts (Figures 6E–6G and S7A).

More severe than *Ppil1Δfs/fs*, *Ppil1* homozygous frameshift mice were lethal prior to E9.5 (c.277_287del11, E9.5, 0 mutant in 47 embryos; p < 0.00001, binomial test). We further examined RNA splicing and cell survival in HEK293T cells after CRISPRi-induced repression of PRP17 followed by expression of rescue PRP17 cDNAs (Gilbert et al., 2013). Both Atg4d and EvsI minigene reporters showed higher intron retention levels after PRP17 knockdown, rescued by WT but not p.F502C PRP17 (Figures 6H, 6I, S7B, and S7C). We also observed that cell viability was significantly decreased after PRP17 repression, which was almost fully rescued by WT but only slightly by p.F502C cDNA (Figure 6J). These results suggest that p.F502C impairs PRP17’s function within the MSC in a fashion similar to *Ppil1* patient variants.

**PP1L-Mediated Proline Isomerization of PRP17 Is Not Required for Function**

Given that PP1L and PRP17 are both essential for embryonic development and form an active PPIase-substrate pair, we hypothesized that catalyzed isomerization of PRP17 Gly94-Pro95 by PP1L is required for function. First, to rule out an effect of PP1L outside the spliceosome in the brain, we knocked in *Ppil1R131Q/R131Q* in *Ppil1−/−* mice, as our results showed that this substitution selectively prevents its binding to SKIP, which recruits PP1L to the spliceosome (Wang et al., 2010). Homozygous *Ppil1R131Q/R131Q* mice showed perinatal lethality, microcephaly, and evidence of neurodegeneration in a fashion similar to *Ppil1A99T/A99T* (Figures 6K–6N and S7D–S7F). Selected splicing defects identified in *Ppil1A99T/A99T* brains were also confirmed in *Ppil1R131Q/R131Q* brains (Figures 6O and 6P). Although we observed a slightly reduced PP1L protein (~30%) in *Ppil1A99T/A99T* embryos (Figures S7D and S7E), this is not sufficient to cause PCHM, as heterozygous *Ppil1A99T/+* showed even a higher reduction (~40%) but did not show any phenotype (Figures S2F and S2G). Together, we conclude that PP1L mediates its effect in the brain by subserving its role in the spliceosome.
Second, we generated two additional KImouse lines: isomerase-inactive PPIL1 p.Arg55Ala (Davis et al., 2010; Zhang et al., 2013; Zydowsky et al., 1992), and non-isomerizable PRP17 p.Pro95Ala. We reasoned that if isomerization catalysis is crucial, these mutations should phenocopy patient mutations. Surprisingly, we observed no phenotype in either homozygous mutant: both Ppil1R55A/R55A and Prp17P95A/P95A were viable, were fertile, and showed no microcephaly, cell death, or defective cortical lamination.

Figure 5. PPIL1 P95 Is Positioned in the Enzymatic Pocket of PRP17 in the Activated Spliceosome
(A and B) Cryo-EM structure of human spliceosome C* complex (PDB: 5XJC) shows an N-terminal loop of PRP17 (cartoon in purple) bound to PPIL1 (teal) enzymatic surface with Pro95 buried inside the S1 enzymatic pocket.
(C) Protein sequence alignment of PRP17 from six species shows an evolutionarily conserved Gly-Pro (G-P) motif in PRP17. Asterisk denotes identical residues; colon indicates similar residues.
(D) Overlaid 1H-15N HSQC spectra of PPIL1 with PRP17 peptide titrations (0–5 molar equivalents, aa 89–101: FAPEFG|PENPFRT). Specific resonance shifts or broadening beyond detection indicates specific binding of PRP17 to PPIL1. Inset: Examples of PPIL1 resonance changes that shift (arrowhead), broaden beyond detection (open arrowhead), or were unchanged (arrow) as a result of PRP17 titration.
(E) Average chemical shift perturbations of PPIL1 residues upon titration with PRP17. Gray, shifted resonances; red, broadened beyond detection resonances. Solid or dashed lines, shifts >1 or >2 SDs above mean, respectively.
(F) Space-filling model of PPIL1 (PDB: 2X7K) showed significantly perturbed residues upon PRP17 peptide binding. Red, residues broadened beyond detection; orange, residues >2 SDs above mean; yellow, residues >1 SD above mean. Residues affected in patients are labeled in red.
(G) Schematic of cis-trans Xaa-Pro peptide bond isomerization catalyzed by PPiase. IS, intermediate state.
(H and I) Two-dimensional 1H, 15N-H(Cα)N ZZ exchange spectra of PRP17 peptide in the absence (H) or presence (I) of sub-stoichiometric concentrations (1% molar ratio) of PPIL1, with appearance of “exchange signals” (dashed circles) (i.e., significant interconversion between cis-trans states).
See also Figures S5 and S6, Table S4, and Video S1.
Quantification of cortical CUX1+ and CTIP2+ neurons in E18.5 cortex of or possible environmental contributions. vere phenotypes, suggesting a potential unexplored mechanism. Although T107A reduced SKIP binding only moderately, the ment of protein stability or function by different patient variants. Thus, our study highlighted the essential role of global splicing integrity in brain development and neurodegeneration and uncovered a new pathway and mechanism underlying this heterogeneous group of degenerative brain disorders.

In general, clinical severity correlated with degree of impairment of protein stability or function by different patient variants. Although T107A reduced SKIP binding only moderately, the affected children carrying this mutation exhibited even more severe phenotypes, suggesting a potential unexplored mechanism or possible environmental contributions.

PPIL1 and PRP17 form an active enzyme-substrate pair in the spliceosome, and both are required for RNA splicing and neuronal survival. Surprisingly, our results showed PPIL1-mediated isomerization of PRP17 was not critical for their functions in splicing and neuronal survival, thus revealing a predominant non-enzymatic function of a spliceosome isomerase. Previous in vitro work also suggested that the enzymatic activity of PPIL1 is not required for splicing of selective pre-mRNA substrates (Adams et al., 2015). In line with this, only trans PRP17 G94-P95 was observed in MSC structures reported to date (Figures S7K and S7L). In the spliceosome, PPIL1 interacts with PRP17, RBM22, and SKIP. RBM22 is an RNA-interacting protein, grasping the 5’ intron right after the 5’sS region where U6 snRNA hybridizes (Bertram et al., 2017; Haselbach et al., 2018; Zhan et al., 2018; Zhang et al., 2017), whereas the PRP17 WD40 domain binds to the intron branching point/U2 duplex (Video S1). Both N-terminal SKIP and PRP17 are intrinsically disordered and undergo disorder-order transition upon PPIL1 binding (Wang et al., 2010; Zhang et al., 2017, 2018). We propose that PPIL1 stabilizes these structures, allowing the MSC to function as a “torque wrench” to bend “challenged” introns to bring the 5’sS and the branching point into proximity. Loss of PPIL1 may reduce the ability to “torque” the more rigid introns, which would explain the primary impact on short and high GC-content introns.

Although mutations in MSC genes had been linked to several types of diseases, such as cancer and autosomal-dominant retinitis pigmentosa (Nik and Bowman, 2019; Scotti and Swanson, 2016; Singh and Cooper, 2012), our study is, to our knowledge, the first to connect MSC gene mutations and global splicing integrity to neuronal survival and neurodegeneration. Why do mutations in these ubiquitously expressed spliceosomal genes lead to brain-specific disease? In our mouse model, apoptosis was limited to postmitotic neurons in homozygous KI mice, strikingly different from other microcephaly models that affect mitosis and survival of neural progenitors or show premature neurogenesis (Gruber et al., 2011; Insoleira et al., 2014; (Figures 6K–6N). Likewise, splicing defects were not observed in Prp17P95A/P95A or Ppil1R55A/R55A mice (Figures 6O and 6P). To confirm these results in vitro, we performed rescue assays in cultured cells by re-expressing either p.R55A PPL1 or p.P95A PRP17. We found that both mutants rescued proliferation and splicing defects as well as WT (Figures 6H–6J and S7G–S7J). Thus, although PPIL1 is capable of catalyzing PRP17 isomerization, and both are required for RNA splicing and mutated in human brain disease, their enzymatic interaction is not an essential part of the function of either protein. These data suggest that the two proteins maintain a scaffolding rather than an enzyme-substrate interaction.

**DISCUSSION**

Here, we report that biallelic, partial loss-of-function mutations in PPIL1 and PRP17, encoding two core spliceosomal compo- nents, disrupt RNA splicing integrity and lead to neurodegenerative PCH with microcephaly in human and mouse. The reported patients showed severe congenital microcephaly and simplified cortical gyri, which are rarely observed in other reported PCH types. We described this unique form of PCH as a new clinical entity PCHM, wherein spliceosome genes are the major contrib- utors. Thus, our study highlighted the essential role of global splicing integrity in brain development and neurodegeneration and uncovered a new pathway and mechanism underlying this heterogeneous group of degenerative brain disorders.

In general, clinical severity correlated with degree of impairment of protein stability or function by different patient variants. Although T107A reduced SKIP binding only moderately, the affected children carrying this mutation exhibited even more severe phenotypes, suggesting a potential unexplored mechanism or possible environmental contributions.

**Figure 6. PPIL1 and PRP17 Control Neuronal Survival Independent of Catalysis**

(A) Pedigrees of PCHM family 10 with homozygous PRP17 p.F502C variant segregating as a recessive trait. Filled symbols, affected; square, male; circle, female; double bar, consanguinity; diagonal line, deceased.

(B) T2-weighted brain MRI shows reduced cerebellar volume (yellow arrowhead), atrophic pons (white arrowhead), and posterior fossa fluid accumulation (yellow arrows) indicative of cerebellar atrophy in the living affected.

(C) The structure of PRP17 resolved from the cryo-EM structure of spliceosomal C complex (PDB: 5XJC) showing mutated residue F502 within the C-terminal WD40 domain.

(D) Protein sequence alignment of PRP17 showing mutated F502 residue highly conserved across eukaryotes. Asterisk, identical.

(E) Western blot of overexpressed HA-tagged PRP17 shows that the p.F502C substitution destabilized the protein.

(F) Western blot of endogenous PRP17 from dermal fibroblasts demonstrating reduced protein levels from affected (A) compared with mother (M) and unaffected control (U).

(G) Quantification of exogenous and endogenous PRP17 protein in transfected HEK293T cells and human dermal fibroblasts, respectively. n = 3.

(H) RT-PCR-based minigene splicing assay following PRP17 repression in HEK293T cells, showing full rescue by WT or p.P95A PRP17 but only partial rescue by p.F502C PRP17.

(I) Quantification of percentage splicing inclusion (PSI) for minigene splicing reporters. PSI was calculated as percentage of inclusion form transcripts among all transcripts (inclusion and exclusion forms). n = 3.

(J) Reduced cell viability following PRP17 repression was fully rescued by WT or p.P95A PRP17 but only partially by p.F502C PRP17. n = 4.

(K) Konal sections of E16.5 (top) and E18.5 (bottom) mouse brains with upregulated cleaved caspase-3 (CC3) and reduced cortical thickness in Prp17P95A/P95A and Ppil1R55A/R55A, but not in Prp17R55A/R55A or Prp17R55A/R55A, CUX1 and CTIP2 label upper and deep layer cortical neurons, respectively. Scale bar: 50 μm.

(L–N) Quantification of cortical CUX1* and CTIP2* neurons in E18.5 cortex of Prp17R55A/R55A (L), Prp17R55A/R55A (M), Prp17P95A/P95A (N), and littermate controls. n = 3 mice/genotype.

(O) Semiquantitative RT-PCR analysis of significant RI events in Prp17R55A/R55A among E14.5 brains of Prp17R55A/R55A (red), Prp17R55A/R55A (blue), Prp17P95A/P95A (green), and littermate controls. GAPDH as loading control.

(P) Quantification of percentage splicing inclusion (PSI) for RI events. n = 3 for each genotype.

Mean ± SD; ns, p > 0.05; *p < 0.05, **p < 0.005, ***p < 0.001, and ****p < 0.0001; one-way ANOVA for all panels. See also Figure S7.
Silver et al., 2010). Moreover, defects were observed across the body of compound heterozygous Ppil1A99T/fs mice. These suggest a distinct mechanism in PCHM and also reveal a higher susceptibility of neurons to global splicing perturbation. Alternatively, the developing brain expresses longer genes with more AS (Lipscombe and Lopez Soto, 2019; Raj and Blencowe, 2015; Yeo and Burge, 2004), which might render neurons more susceptible. Patient mutations may affect some neural-specific splicing events critical for neuronal survival (Lin et al., 2020). Postmitotic neurons might accumulate higher levels of mis-spliced mRNA, affecting protein production or accumulating toxic or unfolded proteins. Loss of PPIL1 predominantly affected AS of brain-expressed or brain disease genes, involved in protein translation, DNA repair, and noncoding RNA processing, whose disruptions are the main causes for degeneration in cerebellar ataxia. Moreover, we further found evidence for DNA double-strand breaks with upregulated p53 and CC3 expression, consistent with accumulated transcriptional R-loops (Costantino and Koshland, 2018), although the exact mechanism remains to be explored. All these suggest a potentially shared mechanism of neurodegeneration underlying several genetic forms of PCH.

STAR METHODS

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SUPPLEMENTAL INFORMATION

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AUTHOR CONTRIBUTIONS


DECLARATION OF INTERESTS

The authors declare no competing interests.

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REFERENCES


**STAR METHODS**

**KEY RESOURCES TABLE**

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### Oligonucleotides

- **Ppil1 frameshift mice, gRNA:** GTCTGTCCTGCGGTGGCCA
  - For the generation of patient mutation Ppil1<sup>A99T</sup> knockin mice, gRNA: GTCTGGTTCGCGGTGGCCA; single-strand repair DNA oligo: TGCCCTCTATGCTTCTCCTTATATGTCGCCGCGCAGCCAGACACCCCGC ACAAGCTTCGCTGAACGCTGGCCAG.
  - This paper: N/A
- **Ppil1 N-terminal HA epitope knockin mice, gRNA:** GATAACCTGCCT CAGCAGGG: single-strand repair DNA: CGGGGTTAACCTCGCGGAAGTAGTAGT GATTGCTAGCGGGGGGATACCTTC GTCTGTCCTGCGGTGGCCA; single-strand repair DNA oligo: TGCCCTCTATGCTTCTCCTTATATGTCGCCGCGCAGCCAGACACCCCGC ACAAGCTTCGCTGAACGCTGGCCAG.
  - This paper: N/A
- **Ppil1<sup>R131Q</sup> knockin mice, gRNA:** TCCC TATACCCTGGCACACT; single-strand repair DNA oligo: GTCTGTCCTGCGGTGGCCA; single-strand repair DNA oligo: TGCCCTCTATGCTTCTCCTTATATGTCGCCGCGCAGCCAGACACCCCGC ACAAGCTTCGCTGAACGCTGGCCAG.
  - This paper: N/A
- **To produce Prp17<sup>P95A</sup> knockin and knockout mice, gRNA:** TTCCTTATGCT GCGGTGGCCA; Single-strand repair DNA: GAGGATATAGGTTAAATACATTGAATT TCGAGATCTCCTCTCTCTCTCTCTCTATAT GTGTCAGTTGCGAGAGAAAAATCC CTTTCGAAACAGCAAATGCGCTGCCC CATAAAATATGCTTTCTGGGTAGTC AGAGCCAGG.
  - This paper: N/A

(Continued on next page)
To generate of *Ppil1p*. R55A knockin mice, gRNA: TGAAGTCCTTGATGATCCTG; single-strand repair DNA oligo: GTCATTGTCCTGGAGCTATACTGGAAGCATGCGCCCAAGACCTGCAAGAACTTCCCGGAGCTGGCTCGGCGGGGCTACATCAATGGCACCAAGTTTCACGCGATCATCAAGGACTTCATGATCCAAGGCGGCGACCCGACAGGCACAGGTACACTTAAGCACCATTGGGGAGGAACTGGGTGGTAAGGCAGCCACAGCT.

CRISPRi scramble gRNA: GCACCTACgagaGCTAACTCA

CRISPRi *PRP17* gRNA 01: CTCACTGTCCGAGTCCGATT

CRISPRi *PRP17* gRNA 02: GGACCCTGAACCCGAACCAT

CRISPRi *PRP17* gRNA 03: GCGATTTAGTCAAGTGCATG

CRISPRi *PRP17* gRNA 04: GGCAGTGGACTCGGCTCCGG

Recombinant DNA

pET22b-PPIL1

cDNA3.1-PPIL1-FLAG

pET28b-His-SUMO-PRP17(18aa)

pGEX-6P1-GST-SKIP(aa59-129)

PX330-U6-2XBsmBI-gRNA-CBh-dCas9-KRAB-T2a-Puro

pLV-hU6-sgRNA hUbC-dCas9-KRAB-T2a-Puro

pMD2.G envelope plasmid

psPAX2 packaging plasmid

pSPLICEEXPRESS-Atg4d

pSPLICEEXPRESS-Evi5l

pcDNA3-HA-PRP17-IRES-GFP

pINDUCER20-PPIL1

Software and Algorithms

Dynamic NMR (DNMR) module of the TopSpin 3.2 acquisition and processing software

Origin 7

Graphpad Prism 7

Adobe Illustrator

Adobe Photoshosh

Inkscape

Image Lab

STAR

DESeq2

rMATS 3.2.5

Leafcutter

WebLogo

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**RESOURCE AVAILABILITY**

**Lead Contact**
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Joseph G. Gleeon (jogleeson@ucsd.edu).

**Materials Availability**
This study did not generate new unique reagents.

**Data and Code Availability**
The accession number for the raw RNA_seq data reported in this paper, including human HAP1 cells and mouse E14.5 brain samples, is SRA: PRJNA669300 (https://www.ncbi.nlm.nih.gov/sra).

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

**Human subjects**
We studied patients from 10 unrelated families (Figures 1A and 6A). Information about gender, age, and health status are listed in Table S1. All work with patients was approved by the UCSD IRB protocol 140028 or local protocols, and performed according to accepted guidelines. All patients and/or parents/guardians signed a consent form for participation. All mutations were confirmed with Sanger sequencing according to the base change and inheritance within the family.

**Animals**
All mice used were maintained on a C57BL/6 background and bred under standard group housing laboratory conditions with 12 hours light/dark cycle and free access to food and water. Age and number of mice used for each experiment are detailed in the figure legends. Sex of embryos used was not tested. All work with mice was performed in accordance with UCSD IACUC protocol S15113. To generate mutations in mice, the mixture of gRNA (0.6 μM), Cas9 protein (0.6 μM, NEB, #M0646T), and single-strand DNA oligo (10 ng/μl, only for knockin) was injected to mouse zygotes at UCSD Transgenic Mouse Core, offspring genotyped by PCR Sanger sequencing. Mice with correct genotypes were backcrossed with C57BL/6J WT mice for at least two generations before breeding to generate homozygous mice for phenotypic analyses.

**Ppil1** knock-out mice (1 bp deletion, c.302delC; NM_026845.4), gRNA targeting GTCTGGTCTGGTGGCCA was transcribed and purified as described previously (Ran et al., 2013). For the generation of patient mutation knockin mice in **Ppil1**p.A99T, single-strand DNA oligo (TGCCCTTCATGCTCTTCTCTTCTTATGTCCCCAGGGGCTGGGATTCTCACGATGGCCAACGCA GGACCCACCACCAATGGCAGCCAGCTTTCTTTTGACC) was co-injected with gRNA and Cas9 protein.

To generate **Ppil1** N-terminal HA epitope knockin mice, synthesized crRNA (0.6 μM) targeting TCCCTATACCCTGCCACACT was co-injected with tracrRNA, Cas9 protein, and single-strand repair DNA (GGATTAGAGTTGAAAATACATTGTAATTTCAGGATCCTTCTTTTCCTTTATATCG TTGACATTATTGCCAGCGACAAATCTCTTCCCCGACAGCAGCAATACGCTTGGGCTTGGAGACTAGGTGAG).

**Prp17**P.P95A knockin mice, synthesized crRNA targeting TGAAGTCCTTGATGATCCTG was co-injected with tracrRNA, Cas9 protein, and single-strand repair DNA (GTCATTGTCCTGGAGCTATACTGGAAGCATGCGCCAA GACCTGCAAGACACACAGCTTGGGCTTGGAGACTAGGTGAG).

**Prp17**P.R55A knockin mice, we also got a **Prp17** frameshift mouse (11 bp deletion, c.277_287delTTTGGACCAGA; NM_027879.2) and was bred to homozygosity after backcrossing with WT mice.
Mammalian Cells
Dermal punch biopsy was obtained under UCSD IRB protocol 171094, patients underwent sterile 0.5cm biopsy, which was mechanically dissociated and then cultured in 20% FBS in DMEM and 100U/ml penicillin-streptomycin until confluent as described (Vangipuram et al., 2013). Cells at low passage were used for protein expression analysis. HEK293T were cultured in DMEM supplemented with 10% FBS, 2mM L-glutamine and 100 units/ml penicillin-streptomycin. Mouse embryonic fibroblast (MEF) cells were isolated from E13.5 embryos using mouse embryonic fibroblast isolation kit (ThermoFisher, #88279) and cultured in DMEM supplemented with 10% FBS, 2mM L-glutamine and 100 units/ml penicillin-streptomycin on gelatin-coated dishes. PPL1 knockout HAP1 cells was generated in Horizon using the CRISPR/Cas9 system, and carries a 22 bp deletion in the first exon (22 bp deletion, c.28_49del, NM_016059). HAP1 cells were grown in Iscove’s Modified Dulbecco’s Medium (IMDM) with 10% FBS and 100 units/ml Pen/Strep. All cells used were tested negative for mycoplasma.

METHOD DETAILS

Analysis of patient phenotype
Patients were recruited as part of a multiyear effort to identify pedigrees showing multiple affected children with neurodegenerative or neurodevelopmental phenotypes, in the presence of parental consanguinity, in order to identify causes of recessive pediatric brain disease. Families were recruited at several locations around the world including the US, UK, Egypt, Pakistan, and Turkey. Subjects underwent detailed phenotyping analysis including standard medical, genetic, and neurological evaluations, serial evaluations over the course of months to year to characterize the natural history of disease progression, pedigree analysis, exclusion of previously identified genetic syndromes through the use of the London Dysmorphology Database and OMIM, followed by brain MRI or CT scan to evaluate for structural defects, and candidate gene sequencing where appropriate to exclude previously reported syndromes. Phlebotomy was performed on the entire family including all genetically informative members, for segregation analysis and linkage.

Human Brain MRI
Imaging was performed on standard clinical radiology equipment (0.5-1.5T) GE instruments, using standard T1, T2, and FLAIR settings. Hard copies of brain images were available in all cases, whereas digital files were available on a minority. This precluded quantitative analysis of brain morphology but allowed for comparison of images in the sagittal, axial and coronal orientations.

DNA extraction, whole-exome, and whole-genome sequencing
Patient DNA extraction and whole-exome sequencing libraries were performed using the Agilent SureSelect Human All Exon v2.0 (44Mb baited target) and sequenced on an Illumina HiSeq 2500 with v2 chemistry (Read Length: 151). Variant calling and filtering were performed using in-house software with Annovar, Variant Effect Prediction software to define population-specific allele frequencies from 1000 Genomes, the Greater Middle East Variome, dbsNP, and gnomAD. Variants were prioritized according to allele frequency, conservation, and predicted effects on protein function.

Variant prioritization
Variant calling and filtering were performed following an established exome sequencing pipeline (Lee et al., 2019). Identified variants were filtered out if not consistent with recessive monogenic inheritance, if the minor allele frequency (MAF) of gnomAD was > 1:10,000, if MAF of local cohort was > 1:1,000, if not moderate or high impact, if CADD PHRED score ≤ 20, or if not predicted as damaging by either SIFT, PolyPhen, or MutationTaster.

Sanger sequencing
Primers for Sanger sequencing were designed using the Primer3 program (U. Massachusetts) and tested for specificity using the Alamut Visual 2.7.1 software. PCR products were treated with Exonuclease I (Fermentas) and Shrimp Alkaline Phosphatase (USB Corp) and sequenced using the Big Dye terminator cycle sequencing kit v.3.1 (Applied Biosystems) on an ABI DNA analyzer (Applied Biosystems). Sequence data were analyzed using Snapgene software.

PPIP1 protein expression and purification
Full-length PPIP1 (GenBank NM_016059.4) open reading frame was cloned into NdeI/ Xhol sites in pET22b expression vector. Plasmids were transformed into BL21(DE3) E. coli cells, grown at 37°C in 2YT media (5 g/l NaCl, 10 g/l yeast extract, 16 g/l tryptone), or M9 minimal media supplemented with 0.05% w/v 15N NH₄Cl (for 15N labeled protein), with 100 µg/ml ampicillin. Protein expression was induced using 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at OD₆₀₀ 0.5-0.8 and subsequently incubated at 18°C overnight. PPIP1 protein was purified from cell lysate using a 1 mL Ni-NTA column (GE Healthcare) and eluted using an imidazole gradient (10 mM to 500 mM imidazole, 0.5 M NaCl, 20 mM sodium phosphate). Purity and identity of purified proteins were confirmed using SDS-PAGE Western analysis with an anti-PPIP1 antibody, and mass spectrometry.
Protein aggregation assay
Protein unfolding and aggregation were measured over a temperature gradient using an Optim machine (Unchained Labs). Protein unfolding was assessed using the barycentric mean wavelength of intrinsic protein fluorescence. Fluorescence was excited using a laser at 266 nm and emission monitored from 280 nm to 450 nm. Protein aggregation was detected by measuring static light scattering at 266 nm. Assays were carried out in PBS buffer (pH 7.4).

Immunoprecipitation assay and western blotting
HEK293T cells were seeded into 6-well plates and transfected with indicated plasmids using Lipofectamine 2000. 36 h after transfection, cells were lysed in RIPA buffer with protease inhibitor cocktail (Roche Applied Science, 11836170001). Cell lysates were centrifuged at 14,000 g for 15 min at 4°C. 45 μL supernatant was mixed with 15 μL 4 × SDS-loading buffer and further heated at 95°C for 2 min as total lysates. The remaining supernatant was incubated with 10 μL prewashed anti-FLAG M2 magnetic beads (Sigma-Aldrich, M8823) for 3 h at 4°C. The beads were washed four times with lysis buffer and eluted in 40 μL 2 × SDS-loading buffer. Total lysates and immunoprecipitates were further separated by SDS-PAGE and analyzed by immunoblotting. Primary antibodies used include mouse anti-Flag M2 (1:10,000, Sigma-Aldrich, F1804), mouse anti-SKIP (1:1,000, Santa Cruz, sc-393856), mouse anti-beta-actin (1:1,000, Santa Cruz, sc-47778), rabbit anti-CDC40/PRP17 (1:2,000, Abcam, ab175924) and rabbit anti-PPIL1 (1:2,000, Proteintech, 15144-1-AP).

SKIP protein expression and purification
SKIP 59-129 was cloned into pGEX-6P1 expression vector (linearized using Sall and Notl restriction enzymes) using In-Fusion HD cloning kit (Clontech). The vector was transformed into BL21(DE3) Escherichia coli cells and grown at 37°C in 2YT media (100 μg/ml ampicillin). SKIP 59-129 expression was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (at OD600 0.5-0.8) for three hours at 37°C. 1 mL glutathione Sepharose columns (GE Healthcare) were used to purify GST-SKIP 59-129 from cell lysates and protein was eluted with a 10 mL glutathione injection (10 mM glutathione, 50 mM Tris-HCl pH 7.4).

Surface plasmon resonance (SPR) assay
Anti-GST antibodies were immobilized on a CM5 chip (GE Healthcare) using an amine coupling reaction, in 100 mM sodium acetate buffer (pH 5.6). The chip surface was activated using 35 μL 0.05 M N-hydroxysuccinimide/ 0.2 M N-ethyl-N’-(dimethylaminopropyl) carbodiimide injections (GE Healthcare amine coupling kit). 20 μL anti-GST antibody (GE healthcare, 27457701) was injected at 30 μg/ml and unreacted material was subsequently eluted with 40 μL 1 M NaCl. Unreacted sites were capped using 35 μL 1 M ethanolamine·HCl pH 8.5. Amine coupling was performed in 100 mM sodium acetate running buffer (pH 5.6). N-terminal GST tagged SKIP 59-129 protein was injected across the chip surface to allow immobilization on anti-GST antibodies. In order to measure binding, PPIL1 proteins were injected across the chip surface (50 μl/min, 2.5 minutes) in PBS, 0.05% v/v IGEPAL running buffer. Experiments were carried out using Biacore 3000 system at 25°C and the data were analyzed using Biacore BiaEvaluation software.

Nissl staining
Dissected mouse brains were fixed overnight in Bouin’s solution and embedded in paraffin. Sagittal sections were collected at a thickness of 5 μm and stained with Cresyl violet after deparaffinization and rehydration. Images were taken using a Leica Aperio AT2 scanner and a Keyence BZX-700 microscope.

Immunofluorescent staining and Fluorescence in situ Hybridization
Embryos were fixed in 4% paraformaldehyde (PFA), cryoprotected in 30% sucrose and then cryosectioned for immunostaining. Primary antibodies and reagents used include: rabbit anti-CUX1 (1:100, Santa Cruz, sc-13024), rat anti-CTIP2 (1:300, Abcam, ab18465), rabbit anti-cleaved caspase3 (1:400, Cell Signaling, 9661), rabbit anti-HA (1:300, Cell Signaling, 3724), rabbit anti-P53 (1:500, Leica, P53-CM5P), rabbit anti-PAX6 (1:300, Biolegend, 901301), rabbit anti-TBR2 (1:1,000, Abcam, ab183991), Rabbit anti-γ-H2AX (1:400, Cell Signaling, 9718), Mouse anti-SATB2 (1:400, Abcam, ab51502), and Goat anti-SOX2 (1:400, R&D systems, AF2018).

Fluorescence in situ Hybridization (FISH) was performed following the manufacturer’s instructions (Advanced Cell Diagnostics) of RNAscope Multiplex Fluorescent Assays V2 kit. TUNEL staining was performed with Apoptag Fluorescein in situ apoptosis kit (Millipore, S7110) following the provided manual. Edu fluorescence staining was performed with Click-it Edu Alexa Fluor 488 Flow Cytometry Assay Kit (ThermoFisher, C10420). Images were taken using a Zeiss LSM 880 confocal microscope and Keyence BZX-700 fluorescence microscope and analyzed with Adobe Photoshop and Illustrator.

RNA-sequencing and data analysis
Total RNA was extracted from cultured cells or dissected mouse tissues following the manual of RNEasy Plus Mini Kit (QIAGEN), yield and quality of RNA assessed by NanoDrop (Thermo Fisher Scientific) and Agilent Bioanalyzer (Agilent Technologies), respectively, enriched by poly-A capture. Paired-end libraries were prepared according to the manufacturer’s protocols (TruSeq Stranded mRNA, Illumina) and sequenced using Illumina HiSeq2500 or 4000 system (paired-end 100, Illumina). 80-100 million reads were collected for each sample.
Splicing minigene constructs were generated as previously reported (Kishore et al., 2008). Briefly, DNA fragment containing 3' intron-exon-intron-exon-5' intron was amplified by PCR using mouse genomic DNA as a template, which was further cloned into pSpliceExpress reporter vector using gateway recombination cloning technique. For splicing analysis, cells were transfected with pSpliceExpress plasmids and cultured for additional 36-48 hours before the extraction of total RNA. RNA was then reversed transcribed and semiquantitative RT-PCR was performed to check the splicing of the introns. Primer sequences were: CTCTCTACCTGGTGTGTGGG (forward), and AGTGCCAAGGTCTGAAGGTC (reverse). GADPH was amplified as control for each experiment.

LeafCutter computational analysis
LeafCutter release 0.2.8 with STAR aligned bam files were performed according to published methods (Li et al., 2018). Intron clustering was performed by leafcutter_cluster.py with default options (maximum intron length as 100,000 bp, minimum reads in a cluster as 10, and the minimum fraction of reads in a cluster that support a junction as 0.001, respectively). Differential intron excision analysis was performed by leafcutter_ds.R with default options but–min_coverage = 30 to increase specificity and–min_samples_per_intron = 3 as recommended. Significance was determined by adjusted p-value (FDR) < 0.05 from the final output.

Sashimi plot
Sashimi plots were generated with python script rmats2sashimiplotlib, with sorted BAM files from STAR used as inputs. Event files contain selected events extracted from rMATS outputs. Plots for different splicing types were specified by -t argument.

Enrichment analysis for misspliced genes in diseases
rMATS output produced lists containing the genes of significant events (misspliced genes) and genes in non-significant events (un-misspliced genes). Brain AS genes (n = 16052) derived from (Yan et al., 2015b), OMIM genes associated with neurodevelopmental disorders (n = 2755), cancer (n = 589), cardiac/heart disease (n = 446), and immune disorders (n = 386) derived were requested from NCBI. A 2x2 contingency table was generated for each disease gene list and the number of genes was calculated for inclusion or exclusion in the misspliced and un-misspliced genes, respectively, or calculated with 95% confidence interval. p-values were generated using Fischer’s Exact test and corrected for multiple tests with the Bonferroni method.

Pathway and Process Enrichment Analysis
Enrichment of Gene Ontology (GO) Biological Processes and KEGG pathways was carried out with KOBAS 2.0 as previous described (Xie et al., 2011). The list of genes (n = 2134) with altered AS in the mutant mouse brains were created from all the significant AS events in rMATS analysis. All expressed genes (n = 15169) in E14.5 mouse brain hemisphere were obtained from RNA-seq data with RPKM > 1.0 and used as a background list. Networks of enriched pathways were generated by Metascape (Zhou et al., 2019), with ontology sources: GO Biological Processes, KEGG Pathway, Reactome Gene Sets, and CORUM. Terms with a p < 0.01, a minimum count of 3, and an enrichment factor > 1.5, grouped into clusters based on membership similarities.

Semiquantitative RT-PCR
Total RNA was extracted from cultured cells or tissue with RNeasy Plus Mini Kit (QIAGEN), according to the manufacturer’s instructions. 1 µg RNA was reverse transcribed with cDNA Synthesis Kit (Maxima First Strand cDNA Synthesis Kit for RT-qPCR, with dsDNase, ThermoFisher) following the provided manual. Minus reverse transcription (RT) negative control was added for each sample to check for DNA contamination. Negative control for PCR was included for each experiment. Primers sequences used were listed in Table S5.
**Purification of uniformly $^{15}$N labeled PRP17 peptide**

His-SUMO-PRP17 (18-mer: T84-T101, TYETMFAPEGPENPFRT) was cloned into NcoI/XhoI sites in pET-28b expression vector, transformed into BL21(DE3) E. coli cells, grown in 5 mL LB medium at 37°C for 6 hours and 500 μL cultured LB medium was then added to 100 mL M9 minimal media supplemented with 0.05% w/v $^{15}$NH$_4$Cl for overnight culture at 37°C. Cells were further transferred to 4 L $^{15}$N minimal medium. Protein expression was induced using 1 mM IPTG at OD$_{600}$ 0.7-0.8 and cells were subsequently incubated at 37°C for 8 hours. The His-tagged recombinant protein was purified from cell lysates using Ni-NTA agarose (GE healthcare) and eluted protein was further dialyzed into SUMO cleavage buffer (25 mM Tris-HCl, 100 mM NaCl, PH = 8.0). Purified protein was cleaved by SUMO protease (MC-LAB, SP-100) at 30°C for 6 hours. After cleavage, His-SUMO and SUMO proteases were absorbed by Ni-NTA agarose and the collected flow-through containing PRP17 peptide was loaded to reverse-phase HPLC column (WATERS, C18) with 0.1% TFA (Trifluoroacetic acid). The peptide was eluted using an increased gradient of elution buffer (90% Acetonitrile, 0.1% TFA, and 10% H$_2$O). Purified $^{15}$N labeled PRP17 peptide was collected and further lyophilized. Purity and identity of purified peptide were confirmed using peptide SDS-PAGE with 16.5% Mini-PROTEAN Tris-Tricine Gel (BioRad, Inc), MALDI-TOFMS analysis for molecular weight, and mass spectrometry.

**$^1$H $^{15}$N HSQC of PPIL1**

$^1$H $^{15}$N heteronuclear single quantum coherence (HSQC) spectra of $^{15}$N labeled PPIL1 were recorded in the presence and absence of PRP17 peptide (Bio-FAPEFGPENPFRT-NH$_2$; purchased from Peptide Synthetics, Inc). PPIL1 concentration was 80 μM and PRP17 peptide was titrated in at 0-5 molar equivalents in 10 mM sodium phosphate, 100 mM NaCl, 5% D$_2$O at pH 6.5. Spectra were collected on a Bruker 600 MHz NMR spectrometer equipped with a quadruple-resonance QCI-P cryo-probe (QCI-P CP). Resonances were identified using the published assignment of PPIL1 (Stegmann et al., 2010; Xu et al., 2005). Average chemical shift perturbations were calculated as described elsewhere (Hewitt et al., 2017).

**$^1$H $^{15}$N H(C$_2$)N ZZ exchange spectra of PRP17 peptide**

$^1$H $^{15}$N-H(C$_2$)N ZZ exchange spectra (based on spectra used by previous study (Dujardin et al., 2015)) were acquired of 500 μM PRP17 (residues 89-101 PRP17, Ac-FAPEFGPENPFRT-NH$_2$, $^{15}$N, and $^{13}$C labeled Pro95) in the presence and absence of catalytic concentrations of PPIL1 (5 μM). The sample buffer used was PBS buffer (pH 7.4), 5% D$_2$O and spectra were acquired using a 950 MHz triple resonance spectrometer equipped with a TXO triple resonance cryo-probe (TXO-CP).

**$^1$H $^{15}$N ZZ exchange spectra of uniformly $^{15}$N labeled PRP17 peptide**

$^1$H $^{15}$N ZZ exchange spectra were acquired of uniformly $^{15}$N labeled PRP17 peptide (residues 84-101 PRP17; 400 μM) in the presence and absence of catalytic concentrations of PPIL1 (4 μM). Mixing times used were 10, 20, 40, 60, 80, 100, 120(x2), 150, 200, 250(x2), 350, 450, 600 and 750 ms. The sample buffer used was: 25 mM sodium phosphate buffer, 100 mM NaCl, 1 mM dithiothreitol, 5% D$_2$O at pH 7.0. Spectra were acquired on a Bruker 950 MHz NMR spectrometer with a TXO triple resonance cryo-probe (TXO-CP).

**Isothermal Titration Calorimetry (ITC) assay**

PRP17 peptide (2 mM) was titrated into PPIL1 WT protein (40 μM), in PBS buffer (pH 7.4). Experiments were carried out using Microcal ITC 200 calorimeter at 25°C. PRP17 titrations were carried out using 2 μl, 4 s injections spaced 2 minutes apart. Results were analyzed using Microcal Origin 7 software. Binding curves were fit to a one-site interaction model using a fixed stoichiometry (n = 1), which is recommended for low-affinity interactions (Tumbull and Daranis, 2003).

**Assignment of cis and trans resonances, PRP17 peptide**

A $^1$H-$^1^3$C heteronuclear single quantum coherence (HSQC) spectrum of PRP17 peptide (PRP17 89-101; $^1^3$C and $^{15}$N labeled pro95) were recorded with 500 μM peptide in PBS (pH 7.4), 5% v/v D$_2$O using a Bruker 750 MHz NMR spectrometer equipped with a triple-resonance TCI triple resonance cryo-probe (TCI-CP). Cis and trans PRP17 Gly94-Pro95 peptide bond assignment was based on the $^1^3$C chemical shift for C$_a$ and C$_b$ resonances, as reported (Shen and Bax, 2010).

**CRISPRi knockdown assay in HEK293T cells**

Empty CRISPRi plasmid (PX330-U6-2XBsmBI-gRNA-CBh-dCas9-KRAB-T2a-Puro) was generated on the modified PX330 with 2x BsmBI gRNA cloning sites. dCas9-KRAB-T2a-Puro was amplified from vector pLV-hU6-sgRNA-hUbC-dCas9-KRAB-T2a-Puro (Addgene #71236) and cloned inside PX330 to replace original WT Cas9. gRNAs targeting PRP17 or scramble gRNA was further cloned between 2XBsmBI sites. CRISPRi plasmids containing PRP17 or scramble gRNAs were co-transfected into HEK293T cells with either empty pcDNA3 or PRP17 cDNAs. 24 hours after transfection, cells were treated with 5 μg/ml puromycin for 36 hours to kill untransfected cells and then cultured in medium without puromycin for additional 24-36 hours before used for RNA extraction or Resazurin cell viability assay.
Resazurin (cell viability/proliferation) assay
Cultured cells were treated with 20% Resazurin (R&D, AR002) in the medium for 2 hours at 37 °C. 100 μL resazurin medium was further transferred to a well in 96-well plate. Fluorescence was read using Ex544nm/Em590 nm by Spectramax M5 microplate reader (Molecular Devices) and the final reading was subtracted from the background control (Resazurin in medium without cells).

Splicing rescue assay in HAP1 cells
WT and PPIase-inactive (p.R55A) PPIL1 CDS with a stop-codon was cloned into Doxycycline (DOX)-inducible pINDUCER20 expression vector, using Gateway cloning system (ThermoFisher). Lentivirus was generated by co-transfecting pINDUCER20 (empty, WT PPIL1, and R55A PPIL1, respectively), pMD2.G envelope plasmid (Addgene 12259) and psPAX2 packaging plasmid (Addgene 12260) into HEK293T cells, using Lipofectamine 2000 (ThermoFisher Scientific). The viral supernatant medium was collected at 48 and 72 hours, respectively, and then pooled and concentrated 100x using Lenti-X Concentrator (Clontech, # 631232). WT and PPIL1 knockout HAP1 cells were infected with concentrated lentivirus overnight in the presence of 5 μg/ml polybrene. 72 hours after infection, cells were selected with 1 mg/ml G418 for 2 weeks. Stable HAP1 cells were further treated with different concentrations of DOX (0, 0.1, and 0.5 μg/ml, respectively) for 72 hours and then lysed in RIPA buffer. Western blot was then applied to test the expression level of DOX-induced WT and R55A PPIL1. For splicing minigene assay, stable HAP1 cells were first cultured with 0.1 μg/ml DOX for 5 days and then transfected with the minigene splicing plasmid using lipofectamine 3000 (ThermoFisher Scientific) in the presence of DOX. Total RNA was extracted from transfected HAP1 cells 48 hours later and then reverse transcribed followed by semiquantitative RT-PCR. For checking the splicing of endogenous genes, stable HAP1 cells were cultured in the presence of 0.1 μg/ml DOX for 7 days and then lysed for total RNA extraction, reverse transcription and semiquantitative RT-PCR.

QUANTIFICATION AND STATISTICAL ANALYSIS
Unless specifically stated, each experiment was performed at least twice for each condition. Due to variabilities in the absolute values obtained in each experiment, for some assays, data from one experiment is presented and noted in the figure legend. For normally distributed data, unpaired t tests were performed using Graphpad Prism 7 and p values were labeled in the figure panels with ns (p > 0.05), *(p < 0.05), **(p < 0.01), *** (p < 0.001), and ****(p < 0.0001), and the exact values were shown in either panels or figure legends. Other statistics used include one-way ANOVA test, non-parametric Mann-Whitney test, Wilcox test, and Fischer’s Exact test, and were described in the figure legend and methods.