Heterogeneous nuclear ribonucleoprotein U (HNRNPU) safeguards the developing mouse cortex

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HNRNPU encodes the heterogeneous nuclear ribonucleoprotein U, which participates in RNA splicing and chromatin organization. Microdeletions in the 1q44 locus encompassing HNRNPU and other genes and point mutations in HNRNPU cause brain disorders, including early-onset seizures and severe intellectual disability. We aimed to understand HNRNPU’s roles in the developing brain. Our work revealed that HNRNPU loss of function leads to rapid cell death of both postmitotic neurons and neural progenitors, with an apparent higher sensitivity of the latter. Further, expression and alternative splicing of multiple genes involved in cell survival, cell motility, and synapse formation are affected following Hnrnpu’s conditional truncation. Finally, we identified pharmaceutical and genetic agents that can partially reverse the loss of cortical structures in Hnrnpu mutated embryonic brains, ameliorate radial neuronal migration defects and rescue cultured neural progenitors’ cell death.
Elimination of cells is critical to maintaining tissue homeostasis and is vital for proper development. Cell death can result from various mechanisms of which TP53 is a crucial player via canonical and non-canonical pathways (reviewed by refs.1,2). Programmed cell death during mouse corticogenesis occurs during embryogenesis and postnatal development3. In the developing mouse, brain progenitors are mostly eliminated from the pallium. Mosaic analysis studies revealed asymmetric cell death after the first division that involved approximately 70% of the intermediate progenitors derived clones4. Increased cell death in the developing brain can result in pathologies, as have been documented in cases of mutations associated with autosomal recessive primary microcephaly5. These mutations delay mitotic progression and activate a TP53-dependent mitotic surveillance pathway6. The second wave of programmed cell death occurs during the first two weeks of life. It eliminates most of the transient populations, including Cajal-Retzius cells7,8 and subplate neurons, and fine-tunes the density of other cortical populations9. The extent of cell death in the cortex is cell type and region-specific and negatively correlates with electrical activity10. It is essential for shaping cortical connectivity, maintaining a proper excitation/inhibition balance10, and for functional myelination of interneurons11.

The regulation of cell death-mediated pathways occurs at multiple stages, including transcriptional, post-transcriptional, and post-translational levels. RNA splicing is a post-transcriptional event that allows the inclusion or exclusion of sequences depending on specific cellular settings. The spliceosome mediates splicing, a complex containing RNA (e.g., small nuclear ribonucleoproteins and heterogeneous nuclear ribonucleoproteins (HNRNPUs)) and other proteins. HNRNPU encodes for the heterogeneous nuclear ribonucleoprotein U12, also known as scaffold attachment factor A, a nuclear chromatin organizer13. HNRNPU is critical in mammalian development, and knockout mice exhibit early lethality14. Microdeletions in the 1q44 locus encompassing HNRNPU and other genes, as well as point mutations in HNRNPU, result in brain disorders, including early-onset seizures, severe intellectual disability, and lower penetrance microcephaly, a thin corpus callosum, dysmorphic facial features, and hypotonia15–21 (reviewed by ref. 22). However, understanding of HNRNPU’s functions in the developing brain is lacking. The HNRNPU protein binds to RNA, DNA, and other proteins, and these interactions facilitate its functions in organizing and stabilizing nuclear chromatin, regulating gene transcription23–32, RNA splicing33–35, and RNA stability36–39. HNRNPU displays a dynamic expression pattern in cultured cells and contributes to spindle assembly and stabilizing kinetochores-MT attachments40,41.

Our work revealed that HNRNPU is required for the survival of cultured neural progenitors in vitro and of the entire mouse cortex in vivo. Removal of HNRNPU activates death programs that were under check. We identified genes whose expression levels vary and alternatively spliced genes when Hnrnpu is mutated. These genes participate in cell viability, cell motility, and other functions, causing the death of neural progenitors and abnormalities in neuronal migration. The p53, TGFβ1, REST, and different critical pathways were affected. We suppressed cell death by pan-Caspase inhibitors, p53 inhibitors, and necroptosis inhibitors, demonstrating the involvement of p53-dependent canonical and noncanonical death mechanisms in neural progenitors. We showed that reducing the levels of the splicing factor SRSF3 opposes HNRNPU’s loss of function effects and improves neural progenitors’ viability and neuronal migration.

Results

HNRNPU is essential for cerebral cortex development. The mouse developing brain expresses abundant levels of HNRNPU, most prominent in the apical aspect of the ventricular zone (VZ) and the cortical plate (Fig. 1A–C). Within the VZ, we detected a high HNRNPU signal in metaphase cells. In those dividing cells, HNRNPU occupies the space between the condensed chromosomes and the poles (Fig. 1A–A’). HNRNPU is also localized to centrosomes and cilia (colocalized with the cilia marker, acetylated tubulin (Fig. 1B–B’), and the centriole component CENP-B (Fig. 1C–C’). HNRNPU in dissociated neurospheres was analyzed using an ImageStream flow cytometer (Fig. 1D, E). The protein expression is dynamic, and while mitotic cells express high levels of HNRNPU, the protein is not attached to chromatin. Conversely, cells in G1/S display reduced HNRNPU signal intensity, which is better colocalized with chromatin.

To study HNRNPU’s role in the developing brain, we used a conditional Hnrnpu allele35 crossed the mice with Emx1Cre mice. The observed phenotype in the mutated brains (Emx1Cre/+; Hnrnpufl/fl) was dramatic. The brain regions in which Emx1Cre is expressed and Hnrnpu is mutated, were progressively omitted, whereas E18 cortices were smaller, and P8 brains lacked the telencephalon (Fig. 1F – I). At E18, the medial areas of the cortex are missing, but remnants of the cortex are still visible laterally (G, M). These areas express Tbr1, yet its typical localization at deeper cortical layers is lost (Fig. 1K, N). Astrocyte lineage can be detected in the mutant cortices; however, GFAP expression at the cortical plate is reduced by ~40% (Fig. 1L, O, P). At 21 days, the mutated animals survive, despite a smaller size and a substantial loss of all cortical structures. We could not anatomically annotate the “appendix” that is visible at this age (Fig. 1T, V); however, this region expresses an unorganized mixture of neuronal and glial markers (Fig. 1Q – V). Lineage tracing (E14-P21) of the progeny of electroporated neural progenitors with reduced HNRNPU levels revealed no bias toward reduction in the number of astrocytes at postnatal ages (P21, Supplementary Fig. 1).

We followed the survival of ex utero electroporated cultured cortical cells using time-lapse microscopy (Fig. 2A–M) to better understand this phenotype. The cultured cells were imaged for three hours (Fig. 2A–M) and fixed after two days in vitro (Fig. 2N–V). Hnrnpufl/fl embryonic brains were electroporated with a dual-color Cre reporter, CAG:spotlight (CAG::SL), in combination with either no Cre, a constitutive Cre (CAG::NLS-Cre-GFP), intermediate progenitors Cre (Tbr2::CRE), or a postmitotic neuronal Cre (Ta1::Cre). The CAG spotlight constitutively expresses a green fluorescent protein which following Cre activity is excised, allowing a red fluorescent protein to be expressed42. During the imaging period, no cell death was observed in the control cells (Fig. 2A–C, M). In contrast, cell death was evident soon after excision of the Cre reporter in progenitors (driven by either CAG or Tbr2 promoters) (Fig. 2D–I, M, P–S, V). In these treatments, cell death occurred shortly (1–5 h) after Cre was active (Fig. 2 and Supplementary movies 1–4). After two days in culture, the percentage of viable cells expressing an excised Cre reporter in the progeny of both treatments was similar. A higher proportion of viable cells expressing excised Cre reporter was seen when the Ta1 promoter was used (Fig. 2V). Despite attenuated death dynamics, postmitotic cells were sensitive to Hnrnpu loss of function. These results were recapitulated in organotypic slices obtained from Hnrnpufl/fl co-electroporated with a Cre::ERT2 expressing plasmid and the CAG::SL Cre-reporter. Organotypic slices were treated with tamofoxifen and subjected to time-lapse imaging. Whereas only a few red nuclei (indicating an excised Cre reporter) were observed when tamofoxifen was added, multiple red nuclei were noted following Cre activation by tamofoxifen treatment (Supplementary Fig. 2B, D). Cre-expressing cells developed nuclear blebbing indicative of apoptosis less than 2 h after Cre-dependent excision of the reporter was noticeable.
The tamoxifen-induced deletion was effective and visible by Western blot. Only the truncated HNRNPU protein (HNRNPU 1-276) was visible one day after tamoxifen induction of Cre in Hnrnpu<sup>fl/fl</sup> / Ubc::Cre-ERT2 derived neurospheres (Supplementary Fig. 2F). Immunostaining of E14 cortices taken from control and mutant embryos with anti-SOX2 and TBR2 antibodies conferred the loss of proliferating cells in the neocortex of mutant animals consistent with the observed reduction in the cortex volume (Supplementary Fig. 3).

HNRNPU affects gene expression and splicing. We performed transcriptomic analyses to gain insights into the molecular mechanisms underlying the observed pathology of Hnrnpu deletion. We generated RNA-seq libraries from cortices derived from E13 Hnrnpu<sup>fl/fl</sup> Emx1<sup>Cre/+</sup> (Homo), Hnrnpu<sup>fl/+</sup> Emx1<sup>Cre/+</sup> (Het) embryos, and control littermates (WT) (Fig. 3). Our findings suggest that HNRNPU strongly affects gene expression. We detected 1556 differentially expressed (DE) genes between the Homo and the control cortices, and 346 DE genes were detected in control versus Het comparison (pAdj<0.05, log2FC > |1|, BM > 20, Fig. 3A and Supplementary Data 1). We noted a difference in the GO terms related to the upregulated and downregulated genes. The upregulated genes were related to synaptic activity, whereas the downregulated ones included several terms
Fig. 1 HNRNPU is strongly expressed in mitotic mouse neural progenitors and is essential for brain development. A–C Expression pattern of HNRNPU (Red) in coronal sections of E13 embryos cortices. A EdU (Green), 1 h post-injection highlights cells in the S-phase at the basal border of the ventricular zone. B Acetylated tubulin (red) marks stabilized tubulin at the radial progenitor primary cilium. C CENP-B (green) marks centrosomes tethered to the apical surface. D, E Flow Cytometry of dissociated neurospheres (E13, 2 days in vitro) at four stages of the cell cycle, classified by EdU incorporation and DAPI stain. Red: HNRNPU, blue:DAPI, E Distribution of the levels of HNRNPU and co-localization with DAPI (Similarity) in a population of dissociated neurospheres classified as G2/M n = 165 or G1/S n = 1225. Images of whole brains and corresponding 5 μM thick coronal sections (Nissl staining) of embryonic day 18 (E18, F, G) and postnatal day 8 (P8), of Emx1CreERT2 littermates carrying WT (F, H) or floxed hnrnpu alleles (G, I). J–O Expression of GFAP (Red) and Tbr1 (Green) in section of E18 obtained from control (Hnrnpu WT/+) and mutant (Hnrnpu fl/+ Emx1 Cre/+ ) littermates. Insert indicate region magnified in L, O. Tbr1 images (K, N) were captured from equivalent locations. P Average GFAP intensity histograms in arbitrary units after background subtraction along the upper 300 μM of coronal sections of E18 cortices (n = 3, each measured in triplicates). Q–V P21 brain sections of mutant and control (Hnrnpu fl/+ Emx1 Cre/+ ) littermates stained with cortical plate layers markers (Tbr1, Cux1 in Q, T) and glia markers, 2′,3′-cyclic nucleotide-3′-phosphodiesterase (CNPase, R, U) and GFAP (S, V). Schematic representation of the slice morphology (pink) showing images location (black rectangle). LV-lateral ventricle, CPu-caudate putamen, CTX- Cortex. Size bars are in μM.

HNRNPU loss of function activates TP53 mediated cell death. Since Hnrnpu’s mutation causes cell death, we examined whether culturing cortical embryonic neural progenitors as primary neurospheres can recapitulate this phenotype. We generated neurospheres from E13 mouse cortices, electroporated them with CRISPR/Cas9 Hnrnpu gRNA plasmids, and conducted a single-cell RNA-seq experiment using the 10× Genomics© platform (Supplementary Fig. 5). We found that cells with low Hnrnpu expression had increased TP53 pathway activity, whereas, within cells, with high Hnrnpu levels in the same culture, this pathway was suppressed. To further validate this finding, we followed the dynamic stabilization of TP53 in cultured neurospheres subjected to the same treatment, as well as in mutant brain sections (Fig. 4A–F). In treated neurospheres, cell numbers decreased as early as 12 h after introducing Hnrnpu sgRNA, coinciding with TP53 accumulation. Neither TP53 accumulation nor reduced cell numbers were evident in control cells (Fig. 4A, B). Accumulation of TP53 was detected in vivo in areas where the progressive loss of cortical structure was apparent (Fig. 4C–F). Using RNA derived from mutant and wildtype cortices, we verified the elevated expression of several P53 target genes Arpp21, Perp, Gas6, Sat1, Dcx, Anxa, Apoe, Atf3, Bbc3, P21, and Puma (Fig. 4G). These are a few genes among the 272 genes identified as TP53-regulated using Ingenuity analysis (Fig. 4G). Further, we confirmed the aberrant splicing of several Tp53 pathway genes (Fig. 4G, Supplementary Fig. 6). A schematic representation of the possible network connections between most verified genes related to the pathway is shown in Fig. 4H. Small molecule compounds were used to gain more insights regarding the death mechanisms driven by P53 accumulation. We followed their effect on neurospheres treated with Hnrnpu sgRNA (Fig. 4I, I′). The pan-Caspase inhibitor, N-benzoyloxycarbonyl-Val-Ala-Asp-fluoro-methyl ketone (Z-VAD-fmk), increased cell viability and proliferation indicated by neurosphere size and the normalized number of cells in the S-phase that incorporated EdU added to the media thirty minutes prefixation. The difluorophenoxymethylketone-based broad-spectrum Caspase inhibitor Q-VD-OPH was more effective than Z-VAD-fmk and has been previously reported to be less cytotoxic62 (Fig. 4I, I′). Furthermore, treatments with either Nec-1 or pifithrin-μ (pFT-μ) promoted NPC viability suggesting the involvement of both canonical and non-canonical TP53 dependent death, including necroptosis1,2.

Genetic means to rescue Hnrnpu deficiency. Taking into consideration that TP53 is a direct trans-activator of numerous proapoptotic genes, we postulated that Tp53 deletion could rescue the observed cell death in the developing brains. To that end, we crossed Hnrnpufl/+ Emx1Cre/+ Tp53loxP/+ with Hnrnpufl/+ Tp53loxP/loxP/+ (Fig. 5A). We observed that only when both alleles of Tp53 were deleted (Hnrnpufl/+ Emx1Cre/+ Tp53loxP/loxP) the associated with DNA (Supplementary Fig. 4A). The majority of the DE genes in the Homo had elevated expression. The DE genes contained, on average more exons. They were longer than the average length of the genome-wide gene (averages 7.4 exons vs. 5.4 and 3178 bp vs. 2299, Homo vs. control, respectively, significantly by Wilcoxon test, p < 2.2e-16). Ingenuity pathway analysis of the DE genes identified multiple affected upstream regulators, including TP53, REST, and ASCL1 (Fig. 3B). Affected cellular activities included cell movement, migration, synaptogenesis, and synapse formation (Fig. 3B). Multiple signaling pathways, including synaptogenesis, neuroinflammation, reelin signaling, and cell cycle control, were also affected (Fig. 3B and Supplementary Fig. 4). The DE genes related to synaptogenesis and glutamate receptor signaling changed in Homo and Het brains, including calcium and potassium channel subunits, glutamate receptors, etc. Their differential expression was verified by qPCR (Fig. 3C).

Given the roles of HNRNPU in RNA splicing, we analyzed the RNA-seq data to identify differences in isoforms detected in the Hnrnpu mutant mice compared with wildtype using MAJIQ43. We saw 850 differentially spliced genes comparing the homoyogute mutants and the wildtype samples and 165 differentially spliced genes comparing the Het and the wildtype (Supplementary Data 2). When comparing the homoyogute mutants versus the wildtype, 1187 local splicing variations (LSV) were detected, and 1054 (88%) involved exon skipping. In 331 of the LSV events, an alternative 3′ or 5′ splice site was detected. In only 117 LSV events, intron retention was detected. Accordingly, we detected 164 LSVs when comparing the heteroyogute to the wildtype, of which 140 LSV involved exon skipping (85%) and 54 LSVs with alternative 3′ or 5′ splice sites. Twenty-five LSV events included intron retention. The list of genes was subjected to over-representation analysis44 using a gene ontology database (Fig. 3D)45,46. The most enriched cellular components amongst the mis-spliced mRNAs included the synapse and the cytoskeleton (see DE gene analysis, Fig. 3B). We verified several alternatively spliced gene events (Dcc, Siva1, and Mdm2, Fig. 3E). The alternative splicing of Dcc by NOVA has been implicated in neuronal migration and axonal guidance47,48. Siva1 is an apoptotic regulatory protein involved in synaptic function and is a downstream target of p5349–51. Mdm2, a critical negative regulator of the P53 protein, was identified as an alternatively spliced gene. Interestingly, the major MDM2 isoform expressed in Hnrnpufl/+ Emx1Cre/+ mutant cortices represented a higher abundance of the MDM2 isoform that lacked exon three. This exon encodes for a sequence required for Tp53 binding, and its exclusion is less frequent in the wild type. The physical interaction and activity of the E3 ubiquitin ligase MDM2 is a central regulatory mechanism that keeps in check the low levels of Tp53. Therefore, the absence of the binding domain facilitates Tp53 activation52–61.
immunoreactivity of Caspase 3 cleavage in the VZ was markedly reduced, and telencephalic derived structures were visible, indicating a clear reduction in cell death (Fig. 5B–D). The deletion of Tp53 did not affect the splicing of Mdm2 (Supplementary Fig. 7A). Some of the rescued cells were cycling cells. The number of cells that incorporated EdU within 30 min or that were labeled by anti-phospho-Histone H3 antibodies, or KI67 antibodies, increased. However, their distribution was usually ectopic, with proliferating cells appearing outside the VZ/sVZ (Fig. 5E–G, Y-AA). The number of cells incorporating EdU (30 min pulse) in the mutant brain was reduced to 31.7% ± 3.07 of control values (Average ±SE, of n = 3 brain). These numbers were partially restored to 56.9% ± 2.65 compared to the control in the rescued brains (Fig. 5AE). It was possible to visualize an increase in the number of intermediate progenitors (TBR2+) that appeared in the expected location in the rescued sVZ (Fig. 5V–X). To better understand the transcriptional profile of the rescue phenotype, we conducted 3′-RNA-seq (MARSeq) from the cortices of three
groups representing mutant, control, and rescue (Fig. 5A, H and Supplementary Data 3). Whereas the number of DE genes in control versus mutant comparison was 640, in the rescue versus mutant comparison, a mild increase in the number of DE genes was observed (737). The DE genes detected in control versus mutant comparison were subjected to GO-term analysis. The same terms found in the RNA-seq mentioned previously were detected. Only 148 DE genes were detected in the control versus
rescue comparison, suggesting that the expression of many genes was restored. Notably, the expression of p53 responsive genes was restored in the rescue cortices.

We observed that the expression of genes in different pathways was more similar to control levels and not only those related to the Tp53 pathway. This is due to the high level of crosstalk exhibited in the network of pathways. Pathway analysis revealed that the rescue differed from the control in several signaling pathways, including BDNF, synaptogenesis, CDK5, and cytoskeletal organization (Fig. 5). Non-rescued genes with a differential expression included those involved in RNA splicing (e.g., Hnrnpu, Hnrnpd, Rbfox1, and Rbfox3). Unlike the mutants, the rescued embryos developed a cortical plate with the laminar organization of TBR1 and CTIP2 positive cells, yet its width was not fully restored (Fig. 5J–R). The Reln mRNA and its protein product were upregulated in the mutants. The dramatic increase in protein levels was shown in mutated cortices (Fig. 5J, K). This phenotype was corrected in the rescued embryos (Fig. 5L). We observed a restoration of the apical F-actin belt, as well as a significant improvement in the overall actin organization following Tp53 loss (Fig. 5AB–AD).

Fig. 4 Activation of TP53-mediated apoptotic pathway following Hnrnpu KO. A Time-dependent accumulation of TP53 (CM5 antibody) in neurospheres treated with control CRISPR/CAS9 plasmid (px330) or Hnrnpu sgRNA’s and plated at indicated time. Acquisition of the intensity of the CM5 signal was done in identical imaging parameters for all images. B Measurements of the dynamic accumulation of TP53. Cells were identified DAPI, and the average intensity in each spot was normalized to a non-specific background (upper panel). The accumulative number of cells in all fields of view is plotted against time (lower panel). C–F Low magnification images (Dapi, blue) of coronal sections (E18) of control (Hnrnpu+/−Emx1Cre/+/−) and mutant (Hnrnpufl/flEmx1Cre/+/−) brains. Inserts indicate the location of high magnification images (D, F). Anti-TP53 (CM5, Red) fails to react with sections from control cortices. D Reveals stabilization of TP53 in cells throughout mutant cortices (F). G Normalized expression levels (left panel) and alternative splicing (right panel) in the mutant (Hnrnpufl/flEmx1Cre) and control (Hnrnpu+/−Emx1Cre+) E13 cortices, showing elevated expression of Tp53 targets and misrepresentation of alternative splice variants of genes in the Tp53 pathway n = 3 biological repeats (each measured in triplicates). Error bars ±SEM. H Schematic representation of functional connections between differentially expressed (ellipsoid) and abnormal spliced (star) gene products presented in C. color code: Red-Strong activation, Pink-overexpression, Blue- downregulation, White- no change in expression levels. n = 4 biological repeats I–I’ Effective protection from apoptotic cell death of CRISPR/CAS9 sgRNA (Hnrnpu sgRNA, Red) Non-electroporated neurospheres (gray) and control (px330, blue) Electroporated neurospheres were treated with solvent only (DMSO), Q-VD-OPh (50 μM), Z-VAD-fmk (50 μM), Necrostatin-1 (Nec1, 1 μM) and Pifithrin-μ (pFT-μ, 5 μM). Bars indicate the number of EdU+ cells per 100 μM² of the neurospheres surface area, following 30 min exposure to EdU. Ordinary one-way ANOVA with Tukey’s multiple comparison test was used for data analysis. P values: **p < 0.01, ***p < 0.001, ****p < 0.0001. Error bars (+SEM) are indicated F Images of neurospheres treated with either DMSO or indicated small molecules. EdU incorporation (Click chemistry, magenta) and Cleaved Caspase, Asp175 (CC3, Green), are presented. Size bars units are μM. Source data are provided as a Source Data file.
Nevertheless, the rescue was partial. The F-Actin alignment in the apical aspect of the VZ was partially reestablished, but the overall actin levels were lower than those in control littermate cortices (Fig. 5AB–AD). The organization of the centrosomes of the radial glia cells at the apical surface was disrupted in the mutant brains and was significantly improved in the rescue sections (Supplementary Fig. 7B–G). In our mouse model, Hnrnpu is mutated in Emx1 positive cells only. To verify that the viable cells in the cortex are deleted, we immunostained the sections with antibodies that recognize the N-terminus of the truncated HNRNPU protein (Fig. 5S–U). The majority of the cortical-plate cells in the sections of the rescued embryos exhibited reduced expression of HNRNPU similar to those seen in the mutant brains, suggesting that the truncated protein is unstable.

We next examined genetic means to lessen the observed cell death in neurosphere cultures. In addition to manipulating the levels of P53 either by CRISPR-mediated deletion of Tp53 or by ectopic expression of exon 3-containing Mdm2, we also tested CRISPR-mediated deletion of Srsf3 (Fig. 6A–D). SR (serine/
arginine-rich) splicing factors (SRSF) generally oppose HNRNPs mediated deletion outcomes. The reduced SRSF3 protein level in the absence of HNRNPU restored the ratio of MDM2 exon 3 inclusion to wildtype levels and reverted the size of the neurospheres (Fig. 6A–D). Further, Hnrnpu’s deletion affected genes implicated in cell migration and movement as well as the cytoskeleton, all of which are crucial for proper neuronal migration (Fig. 3B). We tested the effect of Hnrnpu deletion by
in utero electroporation on neuronal migration. In utero delivery of \textit{Hnrnpu} sgRNA effectively reduced HNRNPU protein levels as estimated by staining plated cortical neurons. A small yet significant reduction of Srsf3 was also noted (Supplementary Fig. 8). Four days post electroporation, the treated cells were primarily negative for Cleaved Caspase 3 (Supplementary Fig. 9). The radial migration of pyramidal neurons born at E14 to layers II–IV was mildly impaired following CRISPR/CAS9 sgRNA targeting \textit{Hnrnpu} and more severely affected when Srsf3 was targeted (Fig. 6E, F). In utero delivery of a combination of sgRNAs targeting both Srsf3 and \textit{Hnrnpu} resulted in a comparable reduction of both proteins (Supplementary Fig. 10). The combined deletion of both RNA-binding proteins completely rescued the migration to the cortical plate in four out of five bins (Fig. 6E, F). Collectively, our data indicate that modulation of RNA splicing can reverse both impaired cell viability and neuronal migration resulting from HNRNPU loss of function.

**Discussion**

\textit{Hnrnpu}’s truncation in the developing telencephalon using Emx1-driven Cre recombinase resulted in protein loss, massive cell death, and elimination of the cerebral cortex at an early postnatal stage. Neural progenitors and postmitotic neurons were vulnerable to loss of function of the HNRNPU protein. Nevertheless, our data do not rule out the possibility that a broader range of cell types contributes to the dramatic cortical loss following Cre activation in Emx1 expressing cells. Based on the different cell death dynamics that we observed in vitro using CAG, Trb2, or Ta1 promoter-driven HNRNPU truncation, we postulate cell-specific sensitivity to HNRNPU loss of function may exist. This could partly reflect the relatively high expression of several anti-apoptotic genes in postmitotic neurons. Bcl2l1, Mcl1, and Bcl2l2 were highly expressed in the RNA-seq data. The consequences of crossing the same mouse model with other Cre recombinase lines also demonstrated cell-type-specific sensitivity. A constitutive deletion using a germ-line-expressing Sox2-Cre line did not result in viable offspring, confirming previous findings. The genomic deletion of \textit{Hnrnpu} in cardiomyocytes and skeletal muscle using muscle creatine kinase-Cre (\textit{Ckmm-Cre}) resulted in lethal dilated cardiomyopathy at P14. In the same study, muscle lethality at P10 was observed using a cardiomyocyte-specific Cre line, cardiac myosin heavy chain-α Cre (\textit{Myh6-Cre}) compared to our findings, apoptosis was only slightly increased in the \textit{Hnrnpu} mutant hearts. Gene expression analysis from P14 hearts detected 734 DE genes, and similar to our results, more genes were upregulated (438) than downregulated (296). Comparison analysis of both data sets detected the highest scoring gene signature, “Organismal death”. Several common DE genes included ephrinB3, and its receptor EphB3, which have been implicated in cell survival, axonal pathfinding, and neuronal migration and were also involved in heart development. Other common DE genes included subunits of glutamate receptors, channels, and transporters, which may contribute to neuronal hyperexcitability.

The expression of several genes was abnormal in our heterozygous mice, consistent with the epilepsy phenotypes detected in patients. The common DE genes in these two mouse models included a signature of the \textit{Tp53} pathway activation and negative regulation of G1/S transition. Using the human skeletal actin promoter (HSA-Cre), a skeletal muscle-specific deletion did not affect the development and early postnatal growth. Still, it resulted in severe adult-onset myopathy. Changes in gene expression were also noted in this study.

Our mouse model expressed reduced levels of a truncated protein, which was hardly detectable in mutant and rescued brains, suggesting protein instability. This truncated protein was designed to include chromatin/DNA binding domains (through the N-terminal SAP domain) but lacked the RNA binding motifs. Notably, RNA-binding proteins can also regulate gene expression. Although HNRNPU’s role in splicing is well known, its targets in the developing brain are poorly defined. The role of HNRNPU in RNA splicing depends on the spatial and temporal context. We found that most LSV involved skipped exons (88%), similar to the adult muscle (74%) and neuron. However, in the developing heart, the fraction of retained introns was higher (40%) than that of skipped exons (35%). Pathway analysis of the differentially spliced genes indicated the involvement of HNRNPU in regulating the cytoskeleton and synapse formation. One alternatively spliced gene is \textit{Dynactin-1}, which co-regulates the activity of the molecular motor cytoplasmic dynein with LIS1 and is critical in neuronal migration. The alternatively spliced exons regulate the interaction between Dynactin-1 and microtubules.

We then focused on the role of \textit{Tp53} in the death of cultured progenitors and on the complete loss of cortical structures in vivo; both events of transcriptional regulation and alternative splicing resulted in further activation of the pathway. Applying different cell-death inhibitors (e.g., Caspase, \textit{Tp53}, necroptosis inhibitors) rescued neurospheres’ growth. In vivo, we inactivated the \textit{Tp53}-dependent cell death by crossing the mice with a floxed allele of \textit{Tp53}. This genetic manipulation enabled cortical formation, increased progenitors proliferating, and partially restored the expression of layer markers. Overall, the cortices displayed mended organization and considerably improved centrosomal localization. Gene expression revealed a higher similarity between the rescued mice and the control ones, although the rescued cortices were abnormal. Ectopic positions of mitotic cells, actin organization, and cortical plate width were not restored to normal parameters. Of interest, it is possible to note that mutations in \textit{Tp73}, a member of the \textit{Tp53} protein family, affect airway motile cilia and cause lissencephaly.

HNRNPU is a centrosomal protein, and apoptosis has also been observed in case of mutations in other centrosomal proteins, yet not to the extent of the loss of the telencephalon. Cell death occurred after conditional \textit{Sax4} knockout or either \textit{Cep63} or \textit{Cpap} knockout. When these mice were crossed with \textit{Tp53-/-}, microcephaly was eliminated, yet, the mitotic cells were displaced. Crossing \textit{Tp53-/-} with mice overexpressing PLK4 resulted in a partial rescue of brain size. \textit{Hnrnpu}’s deletion in mitotic cells caused changes in upstream regulators of \textit{Tp53} leading to the pathway’s activation. However, the partial rescue observed suggests that in addition to \textit{Tp53}, other death pathways are activated.

We then considered ameliorating the observed phenotype by additional genetic means. In neurospheres, the co-deletion of \textit{Tp53} and \textit{Hnrnpu} significantly increased their size. Similarly, introducing an \textit{MDM2}-expressing plasmid containing exon 3 significantly increased the size of the neurospheres, yet not to control levels. Impressively, the co-deletion of \textit{Srsf3} and \textit{Hnrnpu} resulted in neurospheres that were similar to controls. This treatment also partially corrected the splicing defect of \textit{Mdm2}, reversing the ratio between the \textit{MDM2} splice variants to nearly control levels, namely, the higher relative abundance of the inclusion of E3 and possibly more efficient ubiquitination of P53. Using the same approach, we were able to reverse neuronal migration delays caused by HNRNPU loss of function. The expression and splicing of multiple genes are likely affected following the reduction of HNRNPU and SRSF3. At present, we do not have a comprehensive molecular analysis to explain the observed rescue phenotypes fully. Collectively, our data suggest that HNRNPU regulates splicing, which is critical for brain development.
Methods

Ethical regulations. All animal work included in the manuscript is covered by accepted IACUC protocols. Animal protocols were approved by the Weizmann Institute Institutional Animal Care and Use Committee.

Animal studies. The mice were kept in the animal facility at a temperature of 22°C ± 1°C, 50% ± 10% humidity, and a 12 h light/dark cycle.

In utero electroporation (IUE) and primary cultures: ICR mice at 14 d after gestation were anesthetized with ketamine (20 mg/kg) and xylazine (10 mg/kg). The DNA mix containing Cre expressing plasmid (1 μg) and a dual-color reporter (CAG-Stop, 1 μg). Electroporation parameters were identical to those used for in utero electroporation, described below, except for the former done bilaterally. Immediately after electroporation, the brains were mechanically dissociated and plated on PLL/laminin-coated glass. Time-lapse imaging was done 24 h post-plating, for 5 h, on a Leica DMi8 with an Andor Dragonfly 202, spinning disk confocal.

Construction of bulk MARS-seq libraries: Mouse embryonic fibroblasts (EC1) were carefully dissected snap-frozen in liquid nitrogen until RNA was simultaneously extracted from 3 to 4 embryos of each genotype. Bulk MARS-seq libraries were produced from 50 ng of total RNA as previously described. Libraries were then sequenced with a 75 bp single-end read on the Illumina NEXTSEQ500 platform.

Computational methods: RNAseq and MAReq samples were analyzed using the UTAP pipeline. Briefly, sequenced reads were trimmed with cutadapt and were mapped to GRCh37/hg38 reference genome with STAR v2.4.2a with the following parameters alignEnds=Type EndToEnd, outFilterMismatchNoverLmax 0.05, allowN=7, and ReferenceEnds=NESTED, GTF. The distributions of reads across the genome were computed for all samples, with the most 1000 bp of each gene was performed using HTSeq-count in union mode while marking UMI duplicates (in-house script and HTSeq-count). For the RNA-seq samples, we used the gene count of STAIR, the pipeline further applied DESeq2, with the parameters: betaPrior=False, cooksCutoff=False, and independentFiltering=False for normalization and testing for differential expression. Raw p values were adjusted for multiple testing using Benjamini and Hochberg procedure. Genes with a base mean > 10, log2FC > 1 and padj < 0.05 were considered as differentially expressed.

The differential splicing variations were analyzed using the default parameters with MAJIQ, using the default parameters. Events were considered significantly different using deltaPSI > =0.20, as recommended by the authors.

Functional enrichment was performed with QIAGEN Ingenuity Pathway Analysis IPA (QIAGEN Inc., https://digitalinsights.qiagen.com/IPA). Webgestalt and GeneAnalytics.

For the single-cell RNAseq data alignment, quantitation and aggregation of sample count matrices were performed using the 10x Genomics Cell Ranger 2.0.0. 10x Genomics Loupe software was used for data visualization. Libraries prepared from both sexes were included as our data indicated that sex-bias in gene expression was negligible compared to the genotype effect.

Organotypic slice cultures: Hnrnpufl/fl or ICR mice were mated with identical genotypes and electroporated in utero at E13. The DNA mix injected into the ventricles contained 1.5ug of CAG-Cre-ERT2 and 1 ug/ul of the dual Cre reporter (CAG Spotlight reporter, CAG-SL, carrying a constitutively expressed floxed ZsGreen whose removal allows the expression of a mCherry). One day later (E14), the brains were removed from the embryos and kept in oxygenated cold L15 supplemented with Glucose (0.6%), immersed in a 4% paraformaldehyde solution (4% PFA, 4% PFA), 30 min before dissociation and later placed on MEM:HBSS (1:1) supplemented with 25% horse serum, 4.5 mg/ml Glucose, and Penicillin-Streptomycin.

Organotypic slice cultures were cut at 300 μm thick slices. The slices were placed on membrane inserts (millipore, 0.4 μm, Millipore) and placed on MEM:HBSS (1:1) supplemented with 25% Hours Serum, 4.5 mg/ml Glucose, and Penicillin-Streptomycin. Tumorixen (1 mm) or DMSto were added to the media. Slices were embedded on a glass-bottom plate in 50% Matrigel: Media for imaging. Imaging was carried out in a spinning disk confocal microscope based on an OLYMPUS IX83 inverted microscope, VisiScope CSU-W1-T1 confocal system (VisiScope Systems, Germany), and an SCOMOS 4.2 MPixel camera.

In utero electroporation (IUEx) and primary cultures: ICR mice at 14 d after gestation were anesthetized with 10% ketamine/20 mg xylazine (1/10 mixture, 0.11 μg of body weight, i.p.). The uterine horns were exposed, and plasmids mixed with Fast Green (2 μg/μl Sigma) were microinjected through the uterus of the lateral ventricles of embryos by pulled glass capillaries (Sutter Instrument). Electroporation was accomplished by discharging five 35 mV, 50-ms long pulses with 950 ms intervals generated by a NepaGene electroporator. The pulses were delivered using 10-mm-diameter platinum-plated tweezer electrodes (Protech International) situated at either side of the head of each embryo through the uterus. Animals were killed 24 h post-electroporation. After midline incision, the cranium was opened and the brain was removed. For migration assays, E14 embryos were intraventricularly injected with 6 μg px330 CRISP/CAS9 or px330 CRISP/CAS9—sgRNA’s (two sequences targeting each gene of...
Blanquie, O. et al. Electrical activity controls area-specific imaging on Leica DMi8 with an Andor Dragon. Cryo-sections or glass adherent NS, were treated with DNase (0.03 U/ml) and washed before further immunohistochemical analysis. Click reaction mix (PBS containing 100 mM Tris-HCl, pH 8.5, 1 mM CuSO4, 2.5 mM Cy3-Azide or Cy5-Azide, and 100 mM Ascorbic acid). Samples were washed before further immunohistochemical analysis. Additional information can be found in the Supplementary information tables. Supplementary information table 1: primary antibodies list. Supplementary information table 2: secondary antibodies list. Supplementary information table 3: chemical list. Supplementary information table 4: media. Supplementary information table 5: kits. Supplementary information table 6: oligonucleotides. Supplementary information table 7: recombinant DNA. Supplementary information table 8: softwares.

**Data availability** Source data are provided in this paper. The RNA-seq data generated in this study have been deposited in the NCBI GEO database under accession code GSE181527. The additional data generated in this study are provided in the Supplementary Information/Source Data file. Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Orly Reiner (orly.reiner@weizmann.ac.il). Source data are provided with this paper.

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Author contributions
T.S. led the research, conducted all animal-related experiments described in the manuscript, harvested material for library construction and prepared >10 Genomics libraries, prepared all the figures, and contributed to the manuscript writing. A.K. constructed MARS-seq libraries and assisted in pipeline analysis of MARS-seq data. A.G. performed all RT experiments designed and performed splice sensitive PCR and western blot analysis. T.O. lead all bioinformatics analyses. Z.P. conducted the ImageStream run and downstream analysis of the FACS data. I.E.S. acquired funds for research, contributed analysis. T.O. lead all bioinformatics analyses. Z.P. conducted the ImageStream run and all RT experiments designed and performed splice sensitive PCR and western blot MARS-seq libraries and assisted in pipeline analysis of MARS-seq data. A.G. performed

Competing interests
D.B.G. is the founder of Praxis Precision Medicines and Action Biosciences. O.D. receives grant support from NINDS, NIMH, MURI, CDC, and NSF. He has equity and/or compensation from the following companies: Tilray, Receptor Life Sciences, Qstate Biosciences, Hitch Biosciences, Tevard Biosciences, Regel Biosciences, Script Biosciences, Actio Biosciences, Empatica, SilverSpike, and California Cannabis Enterprises (CCE). He has received consulting fees from Zogenix, Ultragenyx, BridgeBio, and Marinus. He holds patents for cannabinoid in treating neurological disorders, but these are owned by GW Pharmaceuticals, and he has waived any financial interests. He holds other patents in molecular biology. He is the managing partner of the PhiFund Ventures. Ingrid Scheffer has served on scientific advisory boards for BioMarin, Chiesi, Eisai, Encoded Therapeutics, GlaxoSmithKline, Knopp Biosciences, Nutricia, Bogenon, Takeda Pharmaceuticals, UCB, Xenon Pharmaceuticals, has received speaker honoraria from GlaxoSmithKline, UCB, BioMarin, Biocodex, Chiesi, Liva Nova and Eisai; has received funding for travel from UCB, Biocodex, GlaxoSmithKline, Biomarin and Eisai; has served as an investigator for Anavex Life Sciences, Cerecin Inc, Cereval Therapeutics, Eisai, Encoded Therapeutics, Epimind Inc, Epogenyx, ES-Therapeutics, GW Pharma, Marinus, Neurocrine Biosciences, Ovid Therapeutics, Takeda Pharmaceuticals, UCB, Ultragenyx, Xenon Pharmaceutical, Zogenix and Zynebra; and has consulted for Athena Partners, Care Beyond Diagnosis, Epilepsy Consortium, Ovid Therapeutics, UCB and Zynebra Pharmaceuticals; and is a Non-Executive Director of Bellberry Ltd and a Director of the Australian Academy of Health and Medical Sciences and the Australian Council of Learned Academies Limited. The other authors declare no competing interests.

Additional information
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