The C terminus of p73 is essential for hippocampal development

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The p53 family member p73 has a complex gene structure, including alternative promoters and alternative splicing of the 3′ UTR. This results in a complex range of isoforms whose biological relevance largely remains to be determined. By deleting exon 13 (which encodes a sterile α motif) from the Trp73 gene, we selectively engineered mice to replace the most abundantly expressed C-terminal isoform, p73α, with a shorter product of alternative splicing, p73β. These mice (Trp73Δ13/−) display severe neurodevelopmental defects with significant functional and morphological abnormalities. Replacement of p73α with p73β results in the deletion of Cajal–Retzius (CR) cells in embryonic stages, thus depriving the developing hippocampus of the pool of neurons necessary for correct hippocampal architecture. Consequently, Trp73Δ13/− mice display severe hippocampal dysgenesis, reduced synaptic functionality and impaired learning and memory capabilities. Our data shed light on the relevance of p73 alternative splicing and show that the full-length C terminus of p73 is essential for hippocampal development.

p73 is a member of the p53 family, has a complex gene organization. Alternative promoters give rise to the N-terminal isoforms TAp73 (which includes the transactivation domain) and ΔNp73 (which is N-terminal truncated); however, a larger complexity emerges at the 3′ UTR of p73 mRNA, where alternative splicing can give rise to seven different isoforms: α, β, γ, σ, ε, ζ, and η (1, 2). p73 plays major roles in development, as genetically modified mice lacking p73 expression (Trp73−/−) display severe neurodevelopmental defects with hippocampal dysgenesis, characterized by unusual arrangements of the cornus ammonis (CA) and dentate gyrus (DG) cell layers (2, 3). Expression of p73 is detected in Cajal–Retzius (CR) cells of developing mouse brain; Trp73−/− mice display severe depauverization of this pool of neurons and consequent reduced expression of Reelin, the key factor secreted by CR cells to direct the architecture of developing hippocampus (4). The Trp73−/− postnatal brain displays also severely disrupted architecture of the posterior telencephalon and mild hypoplasia of the rostral cortex, which could also be ascribed to defective CR function (4).

Coexpression of p73 and Reelin is also conserved in fetal human hippocampus. Here p73/Reelin-expressing cells occupy the marginal zone overlying the ammonic and dentate primordia and the cortico-choroid border in the temporal horn ventral cortical hem from 10 gestational weeks (GW). p73/Reelin positivity is also detected from 14 GW in the neuroepithelium near the dentate-fimbrial boundary, while reelin-positive, p73-negative cells are prominent from 14 GW in the prospective strata lacunosa molecular and radiatum of the cornus ammonis and around midgestation in the dentate molecular layer (ML) and hilus (5). These descriptive data are suggestive of a role of CR (p73/Reelin-positive) cells also in the development of the human brain.

Mice selectively lacking in TAp73 isoforms (TAp73−/−) exhibit a similar phenotype as the Trp73−/− mice, although less severe (6), while mice lacking the ΔNp73 isoforms show minimal signs of neurodevelopmental impairment (7). The TAp73−/− mouse model has also been used to help determine the contribution of this group of isoforms to different phenotypes, including male and female fertility, multiciliogenesis, and cancer (6, 8–10), but the individual contributions of the C-terminal isoforms remain elusive.

Cell culture overexpression studies have shown differential transactivation potential for TAp73α compared to that of the shorter isoforms TAp73β and TAp73γ in relation to their proapoptotic transcription targets (11–14). The global genomic binding profile for overexpressed TAp73α and TAp73β by chromatin immunoprecipitation sequencing showed a strong enrichment of AP1 motifs in close proximity to TAp73α-binding sites. Intriguingly, the AP1 motif-containing p73 target genes were found to be selectively up-regulated by TAp73α and down-regulated by TAp73β (15). However, expression profile analysis of C-terminal p73 isoforms shows that p73α is the major isoform physiologically expressed in mouse tissues, with all others below the limit of detection (16), suggesting that this isoform might be biologically relevant.

To address the relevance of p73 3′-alternative splicing, we generated a genetically modified mouse model in which we deleted exon 13 from the Trp73 gene. The result of this modification is an ectopic switch of expression from p73α to p73β under control of neurodevelopmental impairment (7).

Significance

Alteration of splicing is emerging as a relevant cause of human disease. The C-terminal region of p73 is subject to complex alternative splicing that can give rise to seven different isoforms. Here, using a newly generated mouse model, we determine the functional consequence of in vivo ectopic switch from the physiologically expressed and most abundant isoform p73α to the shorter p73β isoform. Expression of p73β leads to neurodevelopmental defects with functional and morphological abnormalities in the mouse hippocampus. The ectopic isoform switch results in deletion of Cajal–Retzius (CR) neurons in embryonic stages, leading to aberrant hippocampal architecture. Our study indicates that deregulation in p73 alternative splicing might underlie neurodevelopmental human conditions.

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of the endogenous promoters. This mouse model (Trp73Δ13/Δ13) displays severe morphological and functional neurodevelopmental defects ascribed to the aberrant direction of brain architecture during development. Replacement of p73α with p73β resulted in depletion of the CR cells during embryonic development, resulting in a reduction in Reelin and aberrant hippocampal development (17–22). Thus, our data demonstrate that p73α is essential for correct hippocampal morphogenesis and functionality.

Results

Deletion of Exon 13 in the Trp73 Gene Promotes the C-Terminal Isoform Switch from p73α to p73β. Alternative splicing of the Trp73 gene can give rise to a wide range of C-terminal isoforms (Fig. 1A and SI Appendix, Fig. S1A), with p73α the most abundantly expressed in the developing hippocampal DG and in layer I of the cortex of the mouse brain (at embryonic day [E] 18.5) (Fig. 1A and SI Appendix, Fig. S1B) (16). Notably, the expression pattern of TAP73 shows stable high protein levels in the developing hippocampus (from E16.5 to postnatal day [P] 2), with quickly declining expression during postnatal development (from P10) (Fig. 1B). This highlights the relevance of p73 in hippocampal embryonic and perinatal development.

To determine the role of splicing of the C terminus of p73 during mouse development, we crossed mice carrying Trp73 floxed alleles with a cytomegalovirus (CMV)-driven CRE recombinase-expressing mouse to specifically delete exon 13 and delete p73- expressing tissues of the α isoform (SI Appendix, Fig. S1C). As predicted, in Trp73Δ13/Δ13 mice, the mRNA of the predominant p73 isoform, α, was replaced by p73β, as the deletion of exon 13 results in the generation of an exon 12 to 14 junction, which recapitulates the alternative splicing required to produce p73β (Fig. 1A and SI Appendix, Fig. S1A). Immunohistochemistry (IHC) analysis with a TAP73-selective antibody showed expression of TAP73 in the E11.5 mouse striatal neuromere, the early-developing hippocampal and cortical structure, in both Trp73Δ13/Δ13 and Trp73Δ12/Δ13 mice (Fig. 1C). However, in subsequent stages of development (from E16.5 onward), including when the DG appears in the hippocampal fissure, expression of TAP73β was strongly reduced in Trp73Δ12/Δ13 mice compared to the levels of TAP73 observed in wild-type mice, and this change was associated with failure of normal DG development (Fig. 1D and E and SI Appendix, Fig. S1D). In particular, the expression pattern of TAP73 was substantially altered. TAP73-positive cells were observed in the hippocampal fissure (HF) of Trp73Δ12/Δ13 mice, but not in that of Trp73Δ13/Δ13 mice (Fig. 1D, HF dotted line region); however, Trp73Δ13/Δ13 mice retained a population of TAP73-positive cells outside the HF (Fig. 1D, green arrows). After birth, in the ML surrounding the DG, the Trp73Δ12/Δ13 mice had a specific population of TAP73-positive cells that decreased during the postnatal stages of hippocampal development (P1, P5, P10, and P60). In contrast, the developing hippocampus of Trp73Δ12/Δ13 mice from P1 onward is already completely depleted of TAP73-positive cells, reflecting the reduction of TAP73 in the HF at E16.5 (Fig. 1D).

At the gross phenotypic level, replacement of p73α with p73β significantly affected the normal growth pattern of mice. Trp73Δ13/Δ13 mice were significantly smaller than control mice beginning at P7, and 40% of them died within the first 3 wk of life (Fig. 1 F–H and SI Appendix, Fig. S1 E and F).

Overall, these data indicate that an artificial constitutive switch of the C-terminal isoforms from α to β impairs gross phenotypic development in mice.

p73α Is Required for Correct Hippocampal Development. As p73 shows a specific pattern of expression in the hippocampus, we analyzed hippocampal morphology and functionality to gain a better understanding of the basis of the developmental impairment in the Trp73Δ17Δ13 mice. The hippocampal architecture of Trp73Δ17Δ13 mice is completely misshapen. Hippocampal neurons were able to form CA and DG cell layers, but these regions were morphologically misassembled and displayed a severely altered architecture (Fig. 2A).

To test the functionality of the Trp73Δ17Δ13 hippocampus, we analyzed learning and memory capacities in the genetically modified mice by performing a set of behavioral tests and assessed functionality in electrophysiological studies. The burrowing test showed a significantly reduced (~30%) capacity for food burrowing in the Trp73Δ17Δ13 mice compared to the control mice (Fig. 2B). We next performed a T-maze test, in which the ability of the mice to reach the goal within the maze was evaluated after 3 consecutive days of training. Both the time to reach the goal and the percentage of correct choices were different in the Trp73Δ17Δ13 group compared to the control group. Trp73Δ17Δ13 mice showed an increased time to reach the goal (3 s in the control mice vs. 16 s in the Trp73Δ17Δ13 mice) and a reduced frequency of making correct choices (70% correct in the control mice vs. 20% in the Trp73Δ17Δ13 mice) (Fig. 2C). We also analyzed the synapse ultrastructure in the hippocampal CA regions by transmission electron microscopy (TEM). Although the synapse density (number of synapses per area) was similar in the control group and the Trp73Δ17Δ13 group, we observed a significant reduction in synaptic area in the Trp73Δ17Δ13 mice (Fig. 2 D and E).

We next measured the synaptic activity in CA regions by recording both the miniature excitatory postsynaptic current (mEPSC) and the evoked excitatory postsynaptic current (eEPSC) in acute brain slices. In our analysis of the spontaneous release, we did not observe differences in mEPSC amplitude (readout of postsynaptic function; Fig. 2 F and G); however, we did observe a significant reduction in mEPSC frequency, from ~5 Hz in the Trp73Δ17Δ13 mice to ~3 Hz in the Trp73Δ17Δ13 mice (Fig. 2H), as a readout of presynaptic neurotransmitter release (23, 24). We also evaluated the eEPSC in the same mice by recording the synaptic activity of CA pyramidal neurons following Schaffer collateral stimulation with 300-Hz trains for 900 ms (Fig. 2I). Evoked responses were strongly suppressed in mutant hippocampi compared with control hippocampi (Fig. 2I). We also estimated the vesicle pool size by linear back-extrapolation of the cumulative eEPSC amplitudes and noted a significant decrease in the size of the available vesicle pool in Trp73Δ17Δ13 mice compared with the Trp73Δ17Δ13 controls (Fig. 2K), which is consistent with the reduced size of the synaptic area.

These data demonstrate that p73α is required for proper hippocampal development. Expression of an inappropriate p73 C-terminal isoform during mouse development resulted in alteration of physiological hippocampal morphogenesis, with severe disruption of its functionality, memory, and learning and reduction of synaptic transmission. Thus, proper control of p73 alternative splicing is crucial for neurodevelopment.

Alteration in p73 C-Terminal Isomers Influences the Postnatal Neuronal Progenitor Pool. During adult neurogenesis, the mature neuronal repertoire is generated from a pool of neuronal progenitors (25, 26). This process primarily involves the subventricular zone and the DG and is essential for developing learning and memory in young mice. In the hippocampus, adult neurogenesis occurs in the granule cell layer of the DG. Here neuronal precursors (GFAP-, Nestin-, and Sox2-positive neurons) differentiate into mature and postmitotic neurons expressing specific differentiation markers (Calbindin, NeuN, and NeuroD) (27). To evaluate the impact of switching from p73α to p73β on the neuronal progenitor pool in the postnatal hippocampus, we analyzed the GFAP- and Nestin-positive progenitor neurons in P10 and P20 mice. As expected, the wild-type mice showed a progressive decline in the neuronal progenitor pool throughout aging (compare Fig. 3 A and B with Fig. 3 C and D). Compared to the control mice, mice with a Trp73Δ17Δ13 genotype displayed a more substantial reduction in

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Nestin-positive cells at P10 and P20 (Fig. 3 A–D, green dots), in GFAP-positive cells at P20 only (Fig. 3 A–D, GFAP+ red dots), and in double-positive cells at P10 and P20 (Fig. 3 A–D, Nestin/GFAP+, yellow dots). At P10, Trp73+/+ A13 mice still displayed a significant proportion of neuronal progenitors (160 Nestin/GFAP+ cells per field; Fig. 3 A and B, yellow dots, Trp73+/+ A13), indicating that neurogenesis was still occurring; however, at a later postnatal time point (P20), the number of progenitors was dramatically reduced (15 Nestin/GFAP+ cells per field; Fig. 3 C and D, yellow dots, Trp73+/+ A13). Consistently, there were significantly fewer actively proliferating neurons in Trp73+/+ A13 mice than in their wild-type littermates, as measured by Ki67 positivity in the DG granule cell layer (Fig. 3 E and F). Thus, this analysis appears compatible with reduced self-renewal capacity in Trp73+/+ A13, which is possibly secondary to the important developmental defects accumulated embryonically and perinatally.

We next analyzed the neurogenesis potential of Trp73+/+ A13 embryonic precursors. To this end, we measured the ex vivo self-renewal capacity of neurospheres isolated from the E14.5 mouse cortex. We stratified the neurospheres into three populations: small (<25 μm), medium (25 to 50 μm), and large (>50 μm) at passage 0 and small (<20 μm), medium (20 to 40 μm), and large (>40 μm) at passage 1. (The average diameter tends to naturally decrease during passage in culture.) The normal (Gaussian) distribution of the Trp73+/+ neurospheres was progressively different that of the Trp73+/+ A13 samples across the passages. A significantly
larger proportion of $\text{Trp73}^{\Delta 13/13}$ neurospheres was indeed classified as small (<20 μm) compared to that of the wild-type controls (27% vs. 10% at passage 0 and 46% vs. 21% at passage 1) (SI Appendix, Fig. S2 A–D).

In agreement with these data, K667 positivity was reduced in the $\text{Trp73}^{\Delta 13/13}$ cortex (SI Appendix, Fig. S2 F and G). These results are consistent with the previously identified role for p73 during ex vivo neurogenesis in TAp73+/- neurons (28, 29). Overall, a significantly impaired proliferation potential accompanied the morphological defects observed in the $\text{Trp73}^{\Delta 13/13}$ brain.

**p73α Regulates Function and Distribution of CR Cells.** To determine the mechanism by which p73 participates in brain development, we queried single-cell RNA-sequencing (scRNA-seq) data of the mouse DG during development (30). $\text{Trp73}$ expression was exclusively observed in CR neurons (Fig. 4A). CR cells are a population of Reelin-producing GABAergic neurons present in the developing cerebral cortex and hippocampus that are involved in correct brain organization, allowing cortical stratification and hippocampal development (31). The expression of p73 perfectly matched the expression pattern of the CR markers Reelin (Reelin) and Calb2 (Calretinin) (Fig. 4A).

To validate these scRNA-seq data, we costained mouse hippocampal regions for TAp73 and Reelin using an IHC multiplex approach. Clear colocalization of TAp73 and Reelin was observed in the HF at E16.5 and in the DG ML at different stages: P1, P4 (Fig. 4B), and P10 (SI Appendix, Fig. S3A). The reduction...
However, we did not observe any increased apoptosis in the potential of TAp73 CR cell depletion might be ascribed to a stronger transactivation and S4 associated with a consistent reduction in Reelin-expressing cells during development.

To further confirm the absence of CR neurons in Trp73Δ13 mice, we also evaluated the expression of an alternative CR marker, Calretinin, by IF at the same developmental stages. Consistently, we found a strong reduction in Calretinin-positive cells specifically in the DG ML during early postnatal development.

Notably, Calretinin was expressed in the granule cell layer of the DG in Trp73+/+, Trp73+/Δ13, and Trp73Δ13 mice (Fig. 4C, P1 and P4; SI Appendix, Fig. S3B), indicating that its expression is selectively lost in p73-expressing cells of the ML.

Our data indicate that the C-terminal isoform switch from α to β is associated with depletion of the CR cell population. This loss of CR cells leads to severe hippocampal dysgenesis in Trp73Δ13 mice. The lack of TAp73β expression in Trp73Δ13 mice beginning at E16.5 (Figs. 1 and 4D) could also be explained by this depletion of the CR cell population.

Discussion

The data presented here suggest that p73α is essential for the survival of CR cells and thus for the normal morphological and neurodevelopmental trajectory of the DG.
Fig. 4. p73α is essential for CR cells during hippocampal development. (A) Single-cell RNA sequencing from mouse DG showing that the expression of Trp73 is almost entirely confined to the CR neurons. Reln (Reelin) and Calb2 (Calbindin 2 or Calretinin) are well-known markers of CR cells. (Data are from ref. 30.) (B) IHC colabeling for p73 (purple) and Reelin (yellow) in E16.5 HF (Left), P1 (Middle), and P4 (Right) hippocampi from Trp73+/+ and Trp73Δ13/Δ13 mice. (Scale bars: at E16.5, 200 μm in the left images) and 50 μm in the magnifications; at P1 and P4, 500 μm in the left images and 50 μm in the magnifications. (C) Calretinin IF (green) of E16.5 HF (Left), P1 DG (Middle), and P4 DG (Right) from Trp73+/+ and Trp73Δ13/Δ13 mice performed using sections consecutive with those used in B. (Scale bars: 200 μm at E16.5, 400 μm at P1, and 500 μm at P4.) DAPI labels the nuclei; white dotted lines delimit the ML. GCL, granule cell layer. (D) Graphical representation of hippocampus morphogenesis during development along with the distribution of p73α-positive CR cells.
functional development of the DG. Mice lacking p73α that have the β isoform instead show abnormalities in both the development and function of the DG. In addition, mice lacking exon 13 also have other developmental abnormalities that may be secondary to this primary neurologic deficit.

Correct execution of alternative splicing is crucial for ensuring proper expression of functional proteins. Alteration of this process in vivo that the p73 α isoform is essential for p73 neurodevelopmental function. Our data indicate that the p73 C terminus plays a critical role in CR cell biology, directing proper Reelin-mediated hippocampal development and organization. By removing exon 13 from the Trp73 gene in mice, we generated a genetic model replacing the most abundant p73 C-terminal isoform, α, with the shorter β isoform. Mice expressing p73β displayed severe hippocampal dysgenesis, reduced learning and memory functions, and a reduced survival expectancy, which largely revere hippocampal dysgenesis, reduced learning and memory functions in mice. However, our analyses indicate that p73 is expressed exclusively in CR cells, suggesting that p73 does not seem to be directly implicated in maintaining the stem cell characteristics of neuronal precursors. Thus, disruption of proper hippocampal morphogenesis and organization, which appear to be directly regulated by p73 in CR cells, could be causative of a secondary result of inadequate neurogenesis, which further exacerbates the neurologic phenotype of the mice. Trp73Δ13/Δ13 mice were also smaller than their wild-type littermates, possibly reflecting their behavioral deficiencies in failing to suckle.

A direct implication of p73 in human neurodevelopmental conditions remains to be assessed. Nonetheless, it is remarkable that the chromosomal region including Trp73 is a target of genetic deletions in such human conditions as the lp36 deletion syndrome. Interestingly, clinical signs of this syndrome, such as developmental delay, intellectual disability, seizures, hearing loss, and short stature, are highly recapitulated in p73 genetically modified mice (35). The contribution of p73 defects to the spectrum of symptoms in the human syndrome remains unknown, however.

Although our Trp73Δ13/Δ13 mice largely recapitulated the phenotype of the Trp73Δ−/− mice, they did not display hydrocephalus. Hydrocephalus in Trp73Δ−/− mice is a result of ventricular enlargement and inappropriate functionality of the ependymal ciliated epithelium (36). Further studies are needed to assess the functionality of ciliated epithelium in Trp73Δ13/Δ13 mice and determine whether p73β can selectively replace p73α in a tissue/region-specific manner.

In conclusion, our newly developed mouse model has proved essential for interrogating the specific role of the C-terminal domains of the p73 protein during neuronal development, providing further molecular insight into the role of Trp73 during neurogenesis. Our work emphasizes the relevance of p73 in the function and biology of CR cells and leaves open the connection of these mechanisms with human neurodevelopmental conditions.

Materials and Methods

Animals. C57BL/6J mice were housed in the central research facility of the University of Leicester. All experimental work involving animals was approved by the local Ethics Committee and performed in accordance with United Kingdom regulations.

The experiments were performed on sex-matched, age-matched, and strain-matched mice. Trp73Δ13/Δ13 mice were generated using the Cre-LoxP system. First, floxed (Trp73Δ13/Δ13) mice were obtained by introducing a vector containing LoxP sites flanking exon 13 of the Trp73 gene, which was replaced by a Neo cassette to enable selection. Immediately upstream and downstream of these sites, long terminal repeats facilitated incorporation of the vector into embryonic stem cells by homologous recombination. Floxed mice were subsequently crossed with mice ubiquitously expressing Cre-recombinase under the human CMV promoter (CMV-CRE) to delete exon 13 in all tissues.

More details of the study methodology, and primers, sequences, and antibodies (Datasets 51 and 52), are provided in SI Appendix.

Data Availability. All other relevant data are provided in SI Appendix or are available on request.

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