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DOI
10.1016/j.mcn.2020.103502

Publication date
2020

Document Version
Final published version

Published in
Molecular and cellular neuroscience

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Citation for published version (APA):
https://doi.org/10.1016/j.mcn.2020.103502
**Tcf4 is required for correct brain development during embryogenesis**

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**A R T I C L E   I N F O**

Keywords:
- Tcf4
- Brain development
- Cortical development
- Corpus callosum defect
- Hippocampal hypoplasia
- Major tract formation
- E-box factors
- Pitt-Hopkins syndrome

**A B S T R A C T**

Tcf4 has been linked to autism, schizophrenia, and Pitt-Hopkins Syndrome (PTHS) in humans, suggesting a role for Tcf4 in brain development and importantly cortical development. However, the mechanisms behind its role in disease and brain development are still elusive. We provide evidence that Tcf4 has a critical function in the differentiation of cortical regions, corpus callosum and anterior commissure formation, and development of the hippocampus during murine embryonic development.

In the present study, we show that Tcf4 is expressed throughout the developing brain at the peak of neurogenesis. Deletion of Tcf4 results in mis-specification of the cortical neurons, malformation of the corpus callosum and anterior commissure, and hypoplasia of the hippocampus. Furthermore, the Tcf4 mutant shows an absence of midline remodeling, underlined by the loss of GFAP-expressing midline glia in the indusium griseum and callosal wedge and midline zipper glia in the telencephalic midline. RNA-sequencing on E14.5 cortex material shows that Tcf4 functions as a transcriptional activator and loss of Tcf4 results in downregulation of genes linked to neurogenesis and neuronal maturation. Furthermore, many genes that are differentially expressed after Tcf4 ablation are linked to other neurodevelopmental disorders. Taken together, we show that correct brain development and neuronal differentiation are severely affected in Tcf4 mutants, phenocopying morphological brain defects detected in PTHS patients. The presented data identifies new leads to understand the mechanisms behind brain and specifically cortical development and can provide novel insights in developmental mechanisms underlying human brain defects.

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**1. Introduction**

Correct brain development depends on a complex genetic program executed through strongly regulated spatio-temporal expression of transcription factors. A specific group of transcription factors linked to brain development is the basic helix-loop-helix (bHLH) protein family (Powell and Jarman, 2008). The E-box protein sub-family of bHLH proteins consists out of three members; Tcf3 (E2A), Tcf12 (HEB), and Tcf4 (E2-2), which functions are dependent on specific bHLH binding partners through homo- or heterodimerization (Powell and Jarman, 2008; Massari and Murre, 2000; Murre et al., 1989). Mutations in the bHLH-domain, comprising the DNA-binding interface, inhibit such dimerization processes and proper DNA-binding, which directly interferes with their function in gene regulatory events (Sweatt, 2013). E-box proteins have mainly been studied in relation to their role in immune system development. They are found to be critical for the transition from CD4⁺CD8⁺ double-positive T-cells to CD4⁺ or CD8⁺ single-positive T-cells (Wojciechowski et al., 2007) and loss of either Tcf12 or Tcf3 leads to an early depletion of T-cell progenitors and a decrease in mature T-cells (Wojciechowski et al., 2007). Tcf4, on the other hand, has only a minor role during early thymocyte development and it has been suggested that compensatory mechanisms through Tcf3 and/or Tcf12 exist (Wikström et al., 2008).

In the brain, E-box proteins are thought to regulate neurogenesis and neuronal differentiation based on their spatio-temporal presence and binding partners (Powell and Jarman, 2008). In humans, haplo-insufficiency of Tcf4 has been found to be determinative for Pitt-Hopkins Syndrome (PTHS) (Sweatt, 2013; Brockschmidt et al., 2007; Marangi et al., 2011; Marangi et al., 2012), a rare mental disorder, hallmarked by severe intellectual disability (ID), typical facial gestalt, and additional features like breathing problems (Blake et al., 2010; Hasi et al., 2011; Peippo and Ignatius, 2012; Zollino et al., 2019). Imaging of PTHS patients shows major neurodevelopmental defects, ranging from a smaller corpus callosum, underdeveloped hippocampi, and defective cortical development, to microcephaly, enlarged ventricles, and bulging caudate nuclei (Blake et al., 2010; Hasi et al., 2011; Peippo and Ignatius, 2012; Zollino et al., 2019). Finally, Tcf4 has been implicated in other developmental brain disorders, like schizophrenia and autism (Brzózka and Rossner, 2013; Cousins et al., 2014; Wirgenes et al., 2012).
Until now several studies have described part of the role of Tcf4 in neurodevelopment, although a complete overview of the brain-wide defects detected in the Tcf4 mutant is lacking. Previous studies on a Tcf4 mouse mutant describe a loss of the pontine nucleus during development, but no other gross brain abnormalities (Flora et al., 2007). Furthermore, Fischer et al. (2014) reported that Tcf4 promotes differentiation of neural stem cells to neurons in the adult forebrain and may thus be involved in postnatal neurogenesis by orchestrating neural stem cell lineage progression, and in mammalian cell-lines Tcf4 has been found to interact with Mash1 and NeuroD2, important regulators of neuronal differentiation (Persson et al., 2000). Studies by Jung et al. (2018) and (Grubišić et al., 2015) showed that the phenotype detected in the brain and in the gut of patients with PTHS could also (partially) be detected in heterozygous mouse mutants of Tcf4. However, more recently a study by Li et al. (2019) described a role for Tcf4 in neuronal migration and correct spinal and synapse formation in the developing cortex (Li et al., 2019). Taken together, the available data point to a function of Tcf4 in neuronal development and links the developmental defects detected in heterozygous mouse mutants to the phenotype in PTHS patients.

In this study we aim to further specify the function of Tcf4 in the developing murine brain. We demonstrate the presence of Tcf4 throughout the developing brain and in specific cortical layers during development. Ablation of Tcf4 (Zhuang et al., 1996) induces major defects in brain and cortical development, as cortical architecture and specification of cortical neurons is severely affected. Affected specification of cortical neurons is clearly apparent by the loss of CUX1 expression in upper layer neurons in full Tcf4 mutants, and by the aberrant co-localization of Ctip2-positive neurons (lower layer neurons) with SATB2. Besides these specification defects in cortical neurons, the corpus callosum, anterior commissure, and both hippocampi are severely underdeveloped in these mutants. Transcriptome analyses through RNA-sequencing on E14.5 cortical material indicating that Tcf4 is an activator of gene expression in the developing cortex and activates genes that are linked to neurogenesis and neuronal differentiation. Importantly, Tcf4 regulates genes that are linked to clinical syndromes related to ID. Finally, the observed brain malformations phenocopy clinical features observed in PTHS patients, indicating that the full mutant can be used to gain mechanistic insight to PTHS and assist in the identification of novel factors for genetic screening and genetic counseling for human patients with neurodevelopmental disorders.

2. Materials and methods

2.1. Ethics statement

All animal studies are performed in accordance with local animal welfare regulations, as this project has been approved by the animal experimental committee (Dier ethische commissie, Universiteit van Amsterdam; DEC-UvA), and international (ARRIVE) guidelines.

2.2. Animals

The transgenic mouse line B6;129-Tcf4<sup>tm1Zhu</sup>/J originates from the Jackson Laboratory. B6;129-Tcf4<sup>tm1Zhu</sup>/J mouse line was back-crossed with the C57BL/6 line and knock-out embryos were generated by crossing heterozygous Tcf4 mice. WT embryos, to examine WT expression patterns of Tcf4, were generated by crossing C57BL/6 mice. Pregnant mice [embryonic day 0.5 (E0.5) is defined as the morning of plug formation] were sacrificed by cervical dislocation. Embryos were collected in 1× PBS and immediately frozen on dry-ice, or fixed by immersion for 3–12 h in 4% paraformaldehyde (PFA) at 4 °C. After PFA incubation samples were washed in 1× PBS and cryoprotected overnight (O/N) at 4 °C in 30% sucrose. Embryos were frozen on dry-ice and stored at −80 °C. Cryosections were cut at 16 μm, mounted on Superfrost Plus slides (Thermo Fisher Scientific), air-dried, and stored at −80 °C until further use.

2.3. Genotyping

Genotyping of B6;129-Tcf4<sup>tm1Zhu</sup>/J transgenic embryos and mice was performed according to the protocol of the Jackson Laboratory. Briefly, 100 ng of genomic DNA was used together with primer pair: FP 5′-AGCCGAGAAGGAACGGAGGAGGA-3′, RP1 5′-GGCAATTCCTCGGAG GGTGCTT-3′, and RP2 5′-CCAGAAAGCGAAGGAGCA-3′ resulting in a product at 229 bp for the WT allele and a product at 400 bp for the KO allele.

2.4. In situ hybridization

In situ hybridization with digoxigenin (DIG)-labeled probes was performed as described previously (Smidt et al., 2004). Fresh frozen sections were fixed in 4% PFA for 30 min and acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min. Probe hybridization was carried out at 68 °C O/N with a probe concentration of 0.4 ng/μl in a hybridization solution containing 50% deionized formamide, 5× saline-sodium citrate (SSC), 5× Denhardt’s solution, 250 μg/ml RNA Baker’s yeast, and 500 μg/ml sonificated salmon sperm DNA. The following day slides were washed in 0.2× SSC for 2 h at 68 °C followed by blocking with 10% heat-inactivated fetal calf serum (HIFCS) in buffer 1 (100 mM Tris Hcl, pH 7.4 and 150 mM NaCl) for 1 h at RT. DIG-labeled probes were detected by incubating with an alkaline-phosphatase-labeled anti-DIG antibody (Roche, Mannheim, 1:5000), using NBT-BCIP (ThermoFisher) as a substrate. Slides were washed 2× 5 min in T<sub>10</sub>E<sub>5</sub>, dehydrated with ethanol and embedded in Entellan.

DIG in situ hybridization was performed with a 918 bp Tcf4 fragment between 1101–2018 of mouse cDNA.

2.5. Immunohistochemistry

Fluorescence immunohistochemistry (IHC) was carried out as described previously (Penstemaker et al., 2010; Kolk et al., 2009). Cryosections were blocked with 4% HIFCS in 1× THZT (50 mM Tris, 0.5 M NaCl, 0.5% Triton, pH 7.6) and incubated with a primary antibody ([Rb-Gap43 (ab16053 Abcam 1:1000), Ms-Tcf4 (H00006925-M03 Abnova 1:1000), Antibody specificity has been shown by Forrest et al. (2013) and Tanaka et al. (2010), Rb-Ctip2 (ab28488 Abcam, 1:1000), Ms-Satb2 (ab51502 Abcam, 1:1000), Rb-Tbr1 (ab31940 Abcam, 1:1000), Rb-Tbr2 (ab23345 Abcam, 1:1000), Rb-Cux1 (sc-13024 Santa Cruz, 1:1000), Rb-GFAP (Z0334 Dako, 1:1000), Rb-Ki67 (ab15580 Abcam 1:500)] diluted in 1× THZT O/N at RT. The next day sections were incubated with a secondary Alexafluor antibody (anti-rabbit, anti-mouse) diluted 1:1000 in 1× Tris Buffered Saline (TBS) for 2 h at RT. This procedure was repeated for double-labeling with a different primary antibody. After immunostaining nuclei were stained with DAPI (1:3000) and washed extensively in 1× PBS. Slides were embedded in FluorSave (Calbiogen) and analyzed with the use of a fluorescent microscope (Leica).

Antibodies against TCF4, CTP2, SATB2, TBR1, and TBR2 required antigen retrieval as follows. Slides were incubated with 0.1 M citrate buffer pH 6 for 3 min at 800 W and 9 min a 400 W, cooled down to RT in a water bath, after which the protocol was followed as usual. Quantification of CTP2- and SATB2-expressing cells was performed in 4–7 (matching) coronal sections (WT n = 3–4;KO n = 3–4;Het n = 3–4). To determine the cellular position of CTP2-expressing cells, a grid containing 10 bins was placed over coronal sections before quantification. Cells were counted as CTP2<sup>+</sup> when staining co-localized with the nuclear DAPI staining, and as double positive when staining of CTP2 and SATB2 co-localized. Cortical thickness, cortical plate, and GAP43 staining were analyzed by measuring the cortex (from ventricle to pia mater) or between the borders of the different compartments. Volume of the hippocampal folding was measured with the use of ImageJ. The scale was set to the amount of pixels per mm and the...
surface of the affected area was measured and multiplied by 16 μm (thickness of the slices). The volumes of each slice were added to determine the total volume of the measured hippocampi. To determine whether there are statistically significant differences between the 3 groups a one-way ANOVA was performed. Post-hoc analysis was performed via a one or two-tailed t-test with Bonferroni correction for multiple comparisons. One-tailed t-tests were performed when the direction of the effect was clear, whereas two-tailed t-tests were performed when the direction of the effect was unclear.

2.6. RNA-sequencing

RNA was isolated from dissected E14.5 cortices of 6 Tcf4 WT and 6 full Tcf4 mutant embryos. RNA was isolated with Trizol (ThermoFisher) according to manufacturer's protocol. After isolation RNA clean-up was performed with an RNA mini kit from Qiagen according to manufacturer's protocol. Isolated RNA of the dissected cortex of two WT or full Tcf4 mutant embryos were pooled to eventually form 3 pools of WT and 3 pools of Tcf4 mutant samples (n = 3 per genotype). Pair-end RNA-sequencing (minimal 2*10^6 reads per sample), mapping on the mouse genome and statistical analysis (Deseq2) on read counts was performed commercially (Service XS, Leiden, The Netherlands).

GO-term analysis was performed via the PANTHER over-representation test (release 20150430) with Bonferroni correction for multiple comparisons.

3. Results

3.1. Tcf4 is expressed throughout the brain at different stages of development

In order to determine the role of Tcf4 in brain development we first assessed its spatio-temporal expression pattern (Fig. 1A). Since Tcf4 transcript shows a very weak expression at E12.5 (Suppl. Fig. 1), we analyzed the expression pattern of Tcf4 from E14.5 onwards. At E14.5 Tcf4 transcript is detected throughout the developing brain, although it is most abundantly expressed in the neocortex, with a strong expression in the cortical plate (CP). At E16.5 the expression of Tcf4 is still present throughout the neocortex, however the intensity of Tcf4 transcript levels appears to be layer specific at this stage, with a high expression in the intermediate zone (IZ) and CP. These data are consistent with single-cell RNA-sequencing, as shown by Telley et al. (2019) (http://genebrowser.unige.ch/telagirdon), which shows that Tcf4 transcript is expressed throughout development in apical progenitors, basal progenitors, 1 day-old neurons, and 4-day-old neurons, suggesting that Tcf4 transcript is expressed throughout neuronal development (Telley et al., 2019).

To confirm these data and to determine the cortical layer-specific expression of the TCF4 protein, we examined TCF4 protein expression in the neocortex during development. As TCF4 protein was not detected in the neocortex until E14.5 and becomes more apparent at later stages (Suppl. Fig. 1), we focused on the expression pattern of TCF4 and layer-specific marks at E16.5 when most neurons of the developing neocortex are specified (Molyneaux et al., 2007). At E16.5, similar to Tcf4 transcript, TCF4 is found throughout the developing neocortex. It co-localizes with CTIP2 (Fig. 1B-1 white arrowheads), a marker for layer V and VI, and TBR1 (Fig. 1B-2 white arrowheads), a marker for layer VI. However, not all CTIP2+ or TBR1+ cells express TCF4 (Fig. 1B-1, and -2 white arrows). Although Tcf4 transcript was detected in the ventricular- and subventricular zone (V(Z/SVZ) at this stage, TCF4 protein is only marginally present in the V(Z/SVZ), which is only marginally present in this area, suggesting that protein expression is activated after stem cells in the V(Z/SVZ) acquire a neuronal cell-fate.

3.2. Loss of Tcf4 results in an affected cortical architecture

As shown above, Tcf4 is present in themurine developing neocortex at the peak of neurogenesis. In order to determine the consequence of heterozygous or full deletion of Tcf4 on development of the neocortex, we first examined the general cortical architecture by means of DAPI-staining. At E17.5 the WT neocortex shows a clear distinction in different layers. Based on cellular density we were able to divide the neocortex into a CP, Layer VI, IZ, and the V(Z/SVZ) (Molyneaux et al., 2007; Laporte et al., 2017; Paap et al., 2016). Interestingly, the clear distinction between the IZ and layer VI was not present in Tcf4 heterozygous and full mutant embryos (Fig. 2A white asterisk). Furthermore, the V(Z/SVZ) appears to be decreased in the Tcf4 mutant compared to WT and heterozygous animals, which could indicate a decrease in proliferation. However, expression of the proliferation marker Ki67, did not show a statistically significant difference in the overall cortical thickness between the WT, Tcf4 heterozygous, and full mutant embryos (one-way ANOVA F(2,9)=2.8, p = 0.12) (Fig. 2B). However, we did find a statistically significant difference in CP thickness between the groups (one-way ANOVA, F(2,9)=16, p = 0.0011). Post-hoc testing (two-tailed t-test with Bonferroni correction for multiple testing) showed that the thickness of the CP was significantly reduced with ~29% compared to the WT (WT was set at 100%, n = 4, p = 0.002) and ~20% compared to heterozygous embryos (n = 4, p = 0.026) (Fig. 2C). This change is reflected in the ratio of the CP compared to the CT, which shows a statistically significant difference between the groups (one-way ANOVA, F(2,9)=10.17, p = 0.0049), but is only apparent between the WT and full mutant embryos, since it is ~6% smaller in full mutants compared to WT (WT was set to 100%, n = 4; p = 0.027, post-hoc two-tailed with Bonferroni correction for multiple testing) (Fig. 2D). No significant difference was detected between heterozygous and full mutant, and WT and heterozygous embryos.

At P0 we were able to distinguish several cortical layers based on cell-density; CP, Layer V-VI, sub-plate (SP), IZ, and V(Z/SVZ) (Molyneaux et al., 2007). Similar as observed at E17.5, at P0 the layers of the neocortex are less distinctive in both the heterozygous and full mutant animals (Fig. 2E white asterisks). Although the CT is unchanged between the groups (Fig. 2F), CP thickness is statistically significantly different between the groups (one-way ANOVA F(2,6)=30.9, p = 0.00069), CP thickness is ~19% smaller in full mutants compared to WT (WT was set at 100%, n = 3; p = 0.027, post-hoc two-tailed t-test with Bonferroni correction for multiple testing), and ~27% smaller compared to heterozygous animals (n = 3; p = 0.002, post-hoc two-tailed t-test with Bonferroni correction for multiple testing) (Fig. 2G). The ratio between the CP and the CT is more severely affected at P0 (one-way ANOVA F(2,6)=25.3, p = 0.0012) (Fig. 2H). The ratio of the CP to the CT is ~54% in WT and ~58% in the heterozygous animals, which is decreased to ~44% in full mutant animals compared to WT (n = 3; p = 0.02, post-hoc two-tailed t-test with Bonferroni correction for multiple testing) and heterozygous (n = 3; p = 0.0095, post-hoc two-tailed t-test with Bonferroni correction for multiple testing) animals. No significant difference was detected between WT and heterozygous animals.

Taken together, these data indicate that the correct developmental process of the murine neocortex is affected upon loss of one or two alleles of Tcf4.

3.3. Loss of Tcf4 results in mis-specification of CTIP2- and SATB2-expressing neurons at E17.5

Above we have shown that loss of one or two alleles of Tcf4 results
in a difference in cortical architecture, which could point to defective neuronal differentiation at E17.5 and P0. To determine whether expression of layer-specific differentiation markers in the neocortex is affected accordingly, we performed immunohistochemistry for SATB2 for layer II-VI and CTIP2 for layer V and VI at E17.5 in Tcf4 mutants (+/− and −/−) compared to WT. These two proteins mark two different neuronal populations, as SATB2+ neurons are callosal projection neurons (Alcamo et al., 2008; Leone et al., 2015; Srinivasan et al., 2012; Srivasta et al., 2014) and CTIP2+ neurons form corticospinal projections (Srinivasan et al., 2012; Srivasta et al., 2014; Leid et al., 2004). SATB2 is present throughout the differentiated layers of the neocortex and not in the VZ and SVZ in Tcf4 WT, heterozygous, and full mutant cortices at E17.5 (Fig. 3A). CTIP2, a marker for neurons in layer V and VI of the neocortex, is also present in Tcf4 heterozygous and full mutant cortices (Fig. 3B). Quantification of CTIP2+ neurons at E17.5 shows a statistically significant difference between the groups (one-way ANOVA F(2,6)=6.2, p = 0.034), which is reflected in a decrease of ~17% in the amount of CTIP2+ cells between the heterozygous and full mutant embryos (WT was set to 100%, n = 3; p = 0.04, post-hoc two-tailed t-test with Bonferroni correction for multiple testing) embryos (Fig. 3D). No significant difference was detected between WT and full mutant and WT and heterozygous embryos.

To determine whether the affected architecture of the neocortex, especially in regard to layer V and VI, is reflected in the distribution of CTIP2-expressing cells, we divided the neocortex in 10 bins (bin 1 positioned in the upper part of the neocortex to bin 10 at the VZ of the neocortex) and quantified the CTIP2-expressing cells per bin (Fig. 3B and E). We detected a slight shift of CTIP2+ cells to lower bins in the full Tcf4 mutant compared to the WT (one-way ANOVA was performed per bin). In bin 3 (one-way ANOVA F(2,9)=4.4, p = 0.046) less CTIP2+ cells were observed (~29% of total in the WT compared to ~24.8% of total in the full mutant) (n = 4, p = 0.03, post-hoc two-tailed t-test with Bonferroni correction for multiple testing), whereas more CTIP2+ cells were detected in bin 6 (one-way ANOVA F(2,9) = 4.8, p = 0.037) (WT ~6.7%, full mutant ~11.3%; n = 4; p = 0.022, post-hoc two-tailed t-test with Bonferroni correction for multiple testing). No significant difference in distribution was detected between WT and heterozygous embryos at E17.5.

Since the distribution of CTIP2+ cells is altered in full Tcf4 mutants, we set out to determine whether the identity of these neurons is changed accordingly. In WT animals ~5% of the total amount of CTIP2+ cells in layer VI co-localizes with SATB2+ cells (Srinivasan et al., 2012). Since we described a shift of CTIP2+ cells towards lower parts, we determined whether the SATB2/CTIP2 co-localizing population may be over-represented in full Tcf4 mutants (Fig. 3C). The amount of SATB2/CTIP2 co-localizing neurons shows a statistically significant difference between the groups (one-way ANOVA F(2,6)=8.9, p = 0.016). In the neocortex of WT embryos at E17.5 we found that ~7.3% of the total CTIP2+ population co-localizes with SATB2-expressing neurons (Fig. 3C-1 and F), similar to what has been described earlier (Srinivasan et al., 2012). In heterozygous embryos (Fig. 3C-2) the amount of SATB2/CTIP2 co-localizing neurons is not altered compared to the WT (~10.8%). Interestingly, the amount of SATB2/CTIP2 double-positive cells became significantly higher in full Tcf4 mutants (~24.7%, Fig. 3C-3) compared to WT (n = 4; p = 0.04, post-hoc one-tailed t-test with Bonferroni correction for multiple testing).

3.4. Deletion of Tcf4 results in a mis-specification of CTIP2- and SATB2-expressing neurons and loss of Cux1+ expression at P0

Above we have shown that loss of one or two alleles of Tcf4 results in a defective segregation of CTIP2 and SATB2 neurons in the cerebral neocortex. To determine whether this phenotype is persistent after birth, we performed similar experiments on P0 animals. Expression of SATB2 is still relatively normal at P0 in the heterozygous and full mutant neocortex compared to the WT (Fig. 4A). Whereas the decrease in the amount of CTIP2-expressing cells as detected at E17.5 is no longer detected at this stage (Fig. 4B and D), which could be due to a delayed differentiation of these neurons. Interestingly, the distribution of the CTIP2+ cells shows a stronger shift to lower parts of the neocortex between the WT and full Tcf4 mutant animals, and a similar shift was detected for heterozygous compared to WT animals (one-way ANOVA was performed per bin) (Fig. 4E). Bin 4 (one-way ANOVA F(2,6)=92.5, p = 3.1×10−5) (WT ~21.6%, heterozygous ~13.9%, full mutant ~14.7%; n = 3; p = 0.001 (WT vs full mutant, and WT vs heterozygous), post-hoc two-tailed t-test with Bonferroni correction for multiple testing) and 5 (one-way ANOVA F(2,6)=17.9, p = 0.0029) (WT ~22%, heterozygous ~18%, full mutant ~15.3%; n = 3; p = 0.026 (WT vs full mutant) and p = 0.018 (WT vs heterozygous), post-hoc two-tailed t-test with Bonferroni correction for multiple testing) showed a decrease in CTIP2+ neurons for both full mutant and heterozygous compared to WT animals, whereas bin 7 (one-way ANOVA F(2,6)=23.6, p = 0.0015) (WT ~11%, heterozygous ~18%, full mutant ~21.5%; n = 3; p = 0.00095 (WT vs full mutant) and, p = 0.016 (WT vs heterozygous), post-hoc two-tailed t-test with Bonferroni correction for multiple testing) and bin 8 (one-way ANOVA F(2,6)=0.032, p = 0.0071) (WT ~2.6%, heterozygous ~7.8%, full mutant ~9.3%; n = 3; p = 0.032 (WT vs full mutant), post-hoc two-tailed t-test with Bonferroni correction for multiple testing) showed an increase in the amount of CTIP2-expressing cells compared to WT animals (Fig. 4E).

Quantification of the population CTIP2+/SATB2+ neurons showed that the amount of double-positive cells is statistically significantly different between the groups (one-way ANOVA F(2,6)=45.4, p = .00024). Confirming the E17.5 data, the amount of double-positive cells was ~8% of the CTIP2+ population in the WT neocortex (Fig. 4C-1 and F). The amount of CTIP2+/SATB2+ neurons showed a small but non-significant increase to ~12.7% in heterozygous animals (Fig. 4C-2) compared to WT animals. Finally, the amount of double-positive neurons in the full mutant neocortex (Fig. 4C-3) increased to ~39% of the total CTIP2-expressing population, which was significant compared to both WT (n = 3; p = 0.0012, post-hoc one-tailed t-test with Bonferroni correction for multiple testing) and heterozygous (n = 3; p = 0.0053, post-hoc one-tailed t-test with Bonferroni correction for multiple testing) animals (Fig. 4F).

As the mis-specification of the cortical layers was more severe at P0 and the CP displayed a strong decrease in thickness, we set out to determine the presence of CUX1, a marker for the CP and future layer I-IV (Cubelo et al., 2008). CUX1 cells can be detected in the upper layers of the WT and Tcf4 heterozygous neocortex, but is completely absent in the upper layers of the full Tcf4 mutant neocortex (Fig. 4G CP). Interestingly, CUX1 expression in the VZ/SVZ, as shown by Nieto et al. (2004) and Cipriani et al. (2016), is still present in all genotypes.
Fig. 2. Tcf4 deletion leads to a general effect on the cortical architecture at E17.5 and P0. (A) Compartmentalization of the neocortex is affected in the Tcf4 heterozygous and full mutant embryos at E17.5. As DAPI staining (white) shows a less prominent distinction of the cortical layers in the neocortex (white asterisk). (B) Cortical thickness (CT) is not statistically significantly different between the full mutant (white bars), compared to the WT (black bars) and heterozygous embryos (gray bars). (C) The cortical plate (CP) is about 29% smaller in the full mutant compared to the WT (n = 4; *p = 0.002, two-tailed t-test with Bonferroni correction) and about 21% smaller compared to heterozygous embryos (n = 4; *p = 0.026, two-tailed t-test with Bonferroni correction). (D) A similar shift was seen in the ratio of CP and CT, which is about 6% smaller in the full mutant compared to the WT (n = 4; *p = 0.026, two-tailed t-test with Bonferroni correction) and (E) Compartmentalization of the neocortex remains unaffected at P0 in the heterozygous and full mutant animals. DAPI staining (white) shows a less prominent distinction of the cortical layers in the heterozygous and full mutant neocortex (white asterisks). (F) Cortical thickness (CT) is not significantly different at P0 between the WT (black bars), heterozygous (gray bars), and full mutant (white bars) animals. (G) The thickness of the cortical plate (CP) is decreased with about 19% in the full mutant compared to the WT (n = 3; *p = 0.027, two-tailed t-test with Bonferroni correction) and with about 28% compared to heterozygous animals (n = 3; **p = 0.002, two-tailed t-test with Bonferroni). (H) A similar shift was seen in the ratio between the CP and total CT, which is about 10% smaller in the full mutant compared to the WT (n = 3; *p = 0.02, two-tailed t-test with Bonferroni correction) and about 15% smaller compared to heterozygous animals (n = 3; **p = 0.0095, two-tailed t-test with Bonferroni correction).

(Fig. 4G V2). Taken together, these data further substantiate that cortical differentiation is affected upon loss of Tcf4 and that this effect aggravates during development, leading to an absence of CUX1 expression in upper layers of the neocortex in full Tcf4 mutants.

3.5. Loss of Tcf4 leads to defective development of the corpus callosum, anterior commissure, and hippocampus at E17.5

As shown above, deletion of Tcf4 has a measurable effect on the neuronal differentiation within the developing cerebral neocortex. To determine whether this effect is represented by axonal projections and development of cortex-derived structures, we performed GAP43 immunohistochemistry in combination with DAPI to visualize the axonal tracts in the cortex, hippocampus and the corpus callosum at E17.5 (Benowitz and Ruttenberg, 1997; Grasselli and Strata, 2013; Strittmatter et al., 1995a). GAP43 expression in the neocortex can be divided at this stage in three compartments, an upper (projections), middle (cell bodies), and lower (axonal tracts) part, based on neuronal and axonal density. Quantification of the thickness of the compartments compared to the total thickness, shows a small but significant change in thickness of the middle compartment (one-way ANOVA, F(2,9) = 13.4, p = 0.002) between the full mutant and both the WT (n = 4; p = 0.028, post-hoc two-tailed t-test with Bonferroni correction for multiple testing) and heterozygous (n = 4; p = 0.011, post-hoc two-tailed t-test with Bonferroni correction for multiple testing) embryos of ~6% (Fig. 5A). The upper and lower compartments did not show a statistically significant difference between the groups (one-way ANOVA, F(2,9) = 2.7, p = 0.12, and F(2,9) = 2.99, p = 0.10 respectively).

As described above, we have detected a significant higher proportion of SATB2-CTIP2 double-positive neurons in full Tcf4 mutants. Since SATB2-expressing cells are known to project through the corpus callosum (Alcama et al., 2008; Leone et al., 2015; Srinivasan et al., 2012; Srivatsa et al., 2014), we set out to determine whether this potential mis-specification influences the development of the corpus callosum (Fig. 5B). One-way ANOVA showed that there is a statistically significant difference between the groups (F(2,9) = 50.4, p = 1.29*10^{-5}). In both WT (Fig. 5B-1) and heterozygous animals (Fig. 5B-2) the corpus callosum seems unaffected (n = 3), with the callosal bundle visualized through GAP43 immunohistochemistry and the callosal wedge (Fig. 5B white arrowheads) by DAPI staining (Chinn et al., 2015; Shu and Richards, 2001).

Importantly, in the full Tcf4 mutant (Fig. 5B-3) the callosal bundle is completely absent (n = 3; p = 1.66*10^{-5} (WT vs full mutant) and p = 0.00033 (heterozygous vs full mutant), post-hoc one-tailed t-test with Bonferroni correction for multiple testing), although the callosal wedge (Fig. 5B white arrowheads) seems unaffected. Interestingly, although other acallosal mutants, like the Satb2 mutant (Alcama et al., 2008), show an enlarged anterior commissure due to rewiring of the axonal tracts, the anterior commissure is not present in the Tcf4 mutant, indicating that there is a general defect in axonal tract formation upon loss of Tcf4 (Fig. 5C, white arrowheads, Suppl. Fig. 3A).

Examination of the hippocampus in the full Tcf4 mutant showed an absence of the dentate gyrus (Fig. 5D-1 to 3 for magnifications), next to an incomplete development in heterozygous animals (see Suppl. Fig. 4A for rostral-caudal overview of the hippocampal defect). Volume measurements of the affected folding of the hippocampus at E17.5 show that heterozygous and full mutant embryos have a ~21% (n = 3; p = 0.33, post-hoc one-tailed t-test with Bonferroni correction for multiple testing) and ~59% (n = 3; p = 0.054, post-hoc one-tailed t-test with Bonferroni correction for multiple testing) smaller hippocampal volume than WT embryos, respectively. However, these data are not statistically significant due to the large variation in hippocampal development between embryos (Suppl. Fig. 4A). Together, these data indicate that not only the development of the cerebral neocortex is disrupted by the loss of Tcf4, also the development of other brain structures, like the corpus callosum, anterior commissure, and hippocampus, is affected at this stage of development.

3.6. Corpus callosum, anterior commissure and hippocampal defects persists towards birth in Tcf4 mutants

As shown above, the corpus callosum and hippocampus show an aberrant development upon loss of Tcf4. To determine whether this effect persists towards birth, we tracked the development of these structures by GAP43 immunohistochemistry at P0, similar as shown for E17.5 above (Fig. 6). Whereas at E17.5 distribution of GAP43 only showed a significant change in the middle compartment, this change is no longer apparent at P0 (one-way ANOVA F(2,6) = 8.6 p = 0.017, post-hoc two-tailed t-test with Bonferroni correction for multiple testing) showed no statistically significant changes. At P0 GAP43 distribution in the cerebral neocortex only shows statistically significant changes in the upper (projection) compartment (one-way ANOVA F(2,6) = 72.89, p = 6.3*10^{-5} (Fig. 6A). The upper (projections) compartment of GAP43 is increased in the full mutant with ~9% (n = 3 p = 0.0028, post-hoc two-tailed t-test with Bonferroni correction for multiple testing) compared to WT. The difference of ~6% between the heterozygous and full mutant animals is similarly significant (n = 3 p = 0.0059, post-hoc two-tailed t-test with Bonferroni correction for multiple testing). The difference in GAP43 between E17.5 and P0 indicates that axonal tract formation in the cortex is affected even at late stages of development.

The development of the corpus callosum (Fig. 6B) and the anterior commissure (Fig. 6D white arrowheads, Suppl. Fig. 3B) is severely affected at P0. Quantification of corpus callosum crossing (one-way ANOVA F(2,6) = 78.7, p = 4.96*10^{-5}) showed a severe decrease in corpus callosum thickness in full mutants (Fig. 6B-3) compared to both WT (Fig. 6B-1) (n = 3 p = 4.53*10^{-7}, post-hoc one-tailed t-test with Bonferroni correction for multiple testing) and heterozygous (Fig. 6B-2) (n = 3 p = 0.0092, post-hoc one-tailed t-test with Bonferroni correction for multiple testing) animals at P0, confirming the initial defects observed at E17.5 including the apparent absence of a defective callosal wedge (Fig. 6B-1 to 3 white arrowheads). Interestingly, in the most caudal sections of the mutant some callosal fibers were detected to cross the midline (Suppl. Fig. 5), indicating that some crossing is present at

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late developmental stages in the mutant. Since midline crossing of callosal fibers is severely affected in the full Tcf4 mutant at E17.5 and P0, although the callosal bundle appears to reach the midline, we set out to determine which factors, besides the mis-specification of CTIP2/SATB2 expressing neurons, could contribute to the loss of the corpus callosum. Previously, research by Shu et al. (2003) has suggested that midline glia in the indusium griseum and callosal wedge are essential in the development of the corpus callosum and in correct crossing of the callosal bundle (Shu et al., 2003; Richards, 2002; Lindvall et al., 2007). In order to determine whether TCF4 expression overlaps with GFAP+ midline glia, we performed immunohistochemistry on P0 WT brains (Fig. 6E). Strong TCF4 expression is detected surrounding GFAP-expressing glia in the indusium griseum (Fig. 6E-1), whereas weaker, but apparent, TCF4 expression is detected in the callosal wedge, overlapping with the expression area of GFAP+ midline glia (Fig. 6E-2). The indusium griseum and callosal wedge of the full Tcf4 mutant animals is devoid of GFAP+ midline glia, whereas these cells were normally present in WT and heterozygous animals (Fig. 6F). The presence of midline zipper glia (MZG) in the telencephalic midline is similarly affected (Fig. 6G), consistent with the lack of fusion of the midline in the mutant (Gobius et al., 2016). However, some MZG were detected in the Tcf4 full mutant, indicating that a subpopulation of these cells arise in Tcf4 depleted animals (Gobius et al., 2016). This effect was detected in 3 animals of each genotype in 2 different litters. These data indicate that,
besides the mis-specification of CTIP2/SATB2 neurons, loss of midline (zipper) glia may (at least in part, if not mostly) be responsible to the corpus callosum defect detected in full Tcf4 mutant animals. Confirming the data observed at E17.5, the development of the hippocampus, and specifically the dentategyrus, is affected in full Tcf4 mutants, although at this stage some initial cortical folding could be detected (Fig. 6C-1 to 3) (see Suppl. Fig. 4B for rostral-caudal overview of the hippocampal defect). Quantification of the hippocampal volume at P0 (one-way ANOVA F(2,6) = 19.18, p = 0.0025) showed that there is a decrease in hippocampal volume of ~21% between WT and heterozygous animals (n = 3, p = 0.0094, post-hoc one-tailed t-test with Bonferroni correction), ~51% between WT and full mutant animals (n = 3, p = 0.032, two-tailed t-test with Bonferroni correction), and ~30% between heterozygous and full mutant animals (n = 3, p = 0.045, post-hoc one-tailed t-test with Bonferroni correction for multiple testing) (Suppl. Fig. 4B). Taken together, the initial aberrations in cortical and brain development, as identified at E17.5, persisted towards birth.

3.7. Tcf4 acts as a transcriptional activator during cortical development and regulates genes involved in neuronal differentiation and maturation

As shown above, ablation of Tcf4 during development results in a defective development of the neocortex and other brain structures. In order to gain a better insight in the molecular mechanism of TCF4 action during cortical development we aimed to determine the early and therefore possible direct effects of Tcf4 ablation on the transcriptome in the developing cortex. Since TCF4 expression is detected from E14.5 onwards we performed RNA-sequencing on E14.5 dissected cortices of WT and full mutant embryos (n = 3; 2 pooled embryos per biological replicate). These analyzes showed that Tcf4 mainly acts as a transcriptional activator since we observed 131 downregulated and 6 upregulated genes. Analysis of the top 25 downregulated (Table 1) and the top 5 upregulated genes (Table 1) (genes were only included if their coverage reached at least 100 transcripts in the WT samples), based on the log2 fold-change of the transcript compared to WT samples, indicates that Tcf4 may regulate genes that are involved in the regulation...
...of neuronal differentiation and neuronal migration (e.g. NeuroD1, Mash1, Nos1, and Id2) (Casarosa et al., 1999; Kim, 2013; Park et al., 2013; Strittmatter et al., 1995b) (Table 1).

Confirming the data previously shown and described above, GO-term analysis (PANTHER Over-representation Test, Table 2) shows that Tcf4 is involved in the regulation of (neuronal) differentiation, cell signaling, synaptic plasticity, and development of the telencephalon and hippocampus (see Suppl. Table 1 for a list of the Tcf4-regulated genes associated to the different GO-terms). Further analysis of the genes regulated by Tcf4 shows that 26 of the 137 genes regulated by Tcf4 (19%) have previously been shown to be mutated in cases of ID (Table 3) (Brockschmidt et al., 2007; Srivatsa et al., 2014; Alders et al., 2014; Backx et al., 2010; Cocchella et al., 2010; Ehmke et al., 2017; Gerber et al., 2016; Gillentine et al., 2017; Guo et al., 2016; Labonne et al., 2016; Magoulas and El-Hattab, 2012; Merla et al., 2002; Matsu et al., 2014; Mikhail et al., 2011; Montesinos, 2014; Moore et al., 2016; Mulatinho et al., 2012; Myers et al., 2012; Nesbitt et al., 2015; Poot et al., 2010; Schoonjans et al., 2016; Schuurs-Hoeijmakers et al., 2013; Tassano et al., 2015; Thevenon et al., 2014; Tutar–Cunia et al., 2012). Characteristics of these cases range from moderate to severe ID, autistic phenotypes, brain malformations, speech impairments, and epilepsy. These traits can similarly be detected in patients with PTHS (Blake et al., 2010; Has et al., 2011; Peippo and Ignatius, 2012). Taken together, the transcriptome analyzes shows that Tcf4 acts mainly as a transcriptional activator of the neurogenic profile and the genetic program relates to neurodevelopmental disorders as PTHS and ID.

4. Discussion

Here, we have shown that Tcf4 is expressed throughout the brain, with specific expression in different layers of the murine neocortex during development, and that it has a central role in brain development. Complete loss of Tcf4 results in a disorganized neocortex, in which the clear distinction between the different layers is affected, and post-mitotic developing neurons are mis-specified. Furthermore, other brain structures, like the corpus callosum, anterior commissure, and hippocampus show clear developmental defects. Transcriptome analyzes through RNA-sequencing at E14.5 suggested that Tcf4 acts mainly as a transcriptional activator involved in neuronal differentiation and maturation, and that Tcf4 directly or indirectly regulates genes that are known to be mutated in cases of ID.

From the RNA-seq data we determined that many genes that are normally involved in regulating the switch from proliferating neural progenitors to differentiated neurons (e.g. Mash1 (Casarosa et al., 1999), NeuroD1 (Patakas et al., 2016), and Id2 (Jung et al., 2010)), are differentially expressed upon loss of Tcf4. This suggests that Tcf4 acts during the switch from proliferating neural progenitors to differentiating neurons and loss of Tcf4 leads to a non-determined cell-fate, resulting in neurons that are differentiating but are mis-specified. This hypothesis is further substantiated by the disorganized neocortex, resulting in neurons that are differentiating but are mis-specified. The decrease in CP thickness, the increase in SATB2+CTIP2+ expressing cells and affected distribution of CTIP2+ cells, and the loss of CUX1...
Tcf4 deletion affects neocortical GAP43 distribution, corpus callosum crossing, midline glia development, anterior commissure formation, and hippocampi development at P0.

(A) Distribution of GAP43 (green) in the neocortex is altered in both heterozygous and full mutant compared to WT animals at P0. Thickness of the upper (projections) compartment is increased by ~9% in the full mutant compared to WT (n = 3; ***p = 0.00028, two-tailed t-test with Bonferroni correction) and with ~6% compared to heterozygous animals (n = 3; **p = 0.0059, two-tailed t-test with Bonferroni correction). Thickness of the middle (cell bodies) compartment and the lower (axonal tracts) compartment is not significantly altered between full mutant and WT or heterozygous animals. Ratio to total thickness GAP43. (B) The callosal bundle, visualized by GAP43 (green), develops normally in the WT (1) and heterozygous (2) animals, and is lost in the full mutant (3) at P0. The callosal wedge, visualized by DAPI (white), appears to be normally present in WT, heterozygous, and full mutant animals (white arrowheads). Quantification of the callosal thickness shows a significant loss of ~89% between WT (black bar) and full mutant (white bar) (n = 3; ***p = 4.53*10^{-5}, one-tailed t-test with Bonferroni correction) and ~114% between heterozygous (gray bar) and full mutant (n = 3; ***p = 0.00092, one-tailed t-test with Bonferroni correction) animals. The increase of ~25% in corpus callosum thickness between the WT and heterozygous animals was not statistically significant. (C) The hippocampus is underdeveloped in the full Tcf4 mutant at P0 compared to WT and heterozygous animals, mainly in the dentate gyrus (1-3). In the heterozygous animals the hippocampus appears to show a slight underdevelopment compared to the WT. (D) The anterior commissure is absent in the Tcf4 mutant brain compared to WT and heterozygous animals. The anterior commissure is visualized by DAPI staining (E) Expression of TCF4 (red) surrounds the expression of GFAP-expressing (green) glia in the indusium griseum (1), and in the callosal wedge (2). (F) The full Tcf4 mutant is devoid of GFAP-expressing (green) midline glia in the indusium griseum and callosal wedge, whereas these are normally present in the WT and heterozygous animals at P0 (1-3 white arrowheads). (G) GFAP-expressing (green) midline zipper glia at the telencephalic midline are normally present in the brain of both WT and heterozygous animals, whereas there are less midline zipper glia present in the brain of the Tcf4 full mutant. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
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Table 1
Tcf4 top 25 up- and top 5 downregulated genes.

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<th>Gene</th>
<th>2log FC</th>
<th>p-Value</th>
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<tr>
<td>Dkg</td>
<td>−2.89</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Kcn1</td>
<td>−2.60</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>I2</td>
<td>−1.87</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Nos1</td>
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<td>&lt; 0.001</td>
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<tr>
<td>Cnn2</td>
<td>−1.85</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Adra2c</td>
<td>−1.60</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Necab1</td>
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<tr>
<td>Nphp3</td>
<td>−1.50</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Prkq</td>
<td>−1.45</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Olig1</td>
<td>−1.40</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Cdh8p1</td>
<td>−1.38</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Lpl</td>
<td>−1.35</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Tiam1</td>
<td>−1.31</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Mical1</td>
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</tr>
<tr>
<td>Nphp2</td>
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<td>&lt; 0.001</td>
</tr>
<tr>
<td>Snt3</td>
<td>−1.23</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Dusp14</td>
<td>−1.18</td>
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</tr>
<tr>
<td>Sst</td>
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<td>&lt; 0.001</td>
</tr>
<tr>
<td>Hs3at5</td>
<td>−1.17</td>
<td>&lt; 0.001</td>
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<tr>
<td>Npy</td>
<td>−1.16</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Nrl4a3</td>
<td>−1.16</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Tcf4</td>
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<td>&lt; 0.001</td>
</tr>
<tr>
<td>Rsag1f1</td>
<td>−1.13</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Cnmnap3</td>
<td>−1.13</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Mmp17</td>
<td>−1.11</td>
<td>&lt; 0.001</td>
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Table 2
PANTHER over-representation test of E14.5 full Tcf4 mutant RNA sequencing.

<table>
<thead>
<tr>
<th>GO-term</th>
<th>Count</th>
<th>%</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell differentiation</td>
<td>45</td>
<td>33</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Regulation of signaling</td>
<td>39</td>
<td>28</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Neurogenesis</td>
<td>35</td>
<td>25</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Neuron differentiation</td>
<td>21</td>
<td>15</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Telencephalon development</td>
<td>17</td>
<td>12</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Regulation of synaptic plasticity</td>
<td>10</td>
<td>7</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Hippocampus development</td>
<td>7</td>
<td>5</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

PANTHER over-representation tests show that Tcf4 mainly regulates genes involved in (neuronal) differentiation, synapse regulation and cellular maturation at E14.5. Genes involved in the development of the telencephalon and hippocampus are similarly over-represented.

expression in upper layers of the neocortex at P0. Further research should focus on how Tcf4 functions as a switch between un-differentiated committed neural progenitors and differentiated post-mitotic neurons.

Further underlining the effects of Tcf4 ablation on neuronal differentiation as shown in this study, is a recent study by Li et al. (2019), which similarly showed that loss of Tcf4 results in abnormal neuronal migration, defective laminar organization, and a loss of upper layer markers like Cux2 at late developmental stages (Li et al., 2019). The transcriptome analysis at P0 described in the study of Li et al. showed some overlap with the RNA-sequencing results at E14.5 of the study presented here, indicating that Tcf4 regulates some key genes throughout neuronal development, like NeuroD1, Lpl, Kcn1a, and Id2 (Li et al., 2019). However, since RNA-sequencing was performed at P0 in the study of Li et al., they focus on the final stage of cortical development after Tcf4 ablation, resulting in a loss of information about early onset genes and genes that could be importantly involved in the start of neuronal differentiation. Our study fills this gap in knowledge and accurately shows early effects of Tcf4 ablation on gene expression in the murine cortex.

Furthermore, the loss of the corpus callosum and anterior commissure, two major axonal tracts, suggests that Tcf4 is importantly involved in axonal outgrowth and neuronal connectivity, further underlined by the fact that at E14.5 genes involved in axonal tract formation are downregulated upon Tcf4 ablation (e.g. Ctn2 (Kasriti et al., 2019), Mical2 (Terman et al., 2002), Sema7a (Pasterkamp et al., 2003)). However, the function of Tcf4 in axonal tract and specifically corpus callosum formation could be two-fold, as midline glia in the indusium griseum and callosal wedge and midline zipper glia, which are directly linked to the correct development of the corpus callosum and fusion of the telencephalic midline (Shu et al., 2003; Richards, 2002; Lindwall et al., 2007; Gobius et al., 2016), are severely depleted or even absent in the Tcf4 mutant brain. In order to fully understand the neuronal outgrowth defects upon Tcf4 ablation, future studies should focus on the trajectory of axonal tracts normally destined for the corpus callosum or anterior commissure, to determine whether axonal tract formation is completely absent, or these axons are guided to other areas of the brain. Furthermore, it would be interesting to examine neuronal differentiation in the cortical areas normally projecting through the anterior commissure (e.g. piriform cortex, anterior olfactory nucleus), to determine whether this is altered, as seen for the SATB2* neurons projecting through the corpus callosum, which could similarly contribute to the loss of the major axonal tracts.

In humans, mutations within the bHLH domain in one copy of the Tcf4 gene results in Pitt-Hopkins syndrome (PHS) (Sweatt, 2013; Brockschmidt et al., 2007; Marangi et al., 2011; Marangi et al., 2012; de
Pontual et al., 2009), a rare mental disorder that is characterized by severe mental retardation, breathing abnormalities, and distinctive facial features (Blake et al., 2010; Hasi et al., 2011; Peippo and Ignatius, 2012; Zollino et al., 2019). Brain defects seen in PTHS patients include a smaller corpus callosum, bulging caudate nuclei, underdeveloped hippocampi, enlarged ventricles, and in some cases microcephaly (Blake et al., 2010; Hasi et al., 2011; Peippo and Ignatius, 2012; Ghosh et al., 2012). Many of these traits were traced back in full Tcf4 mouse mutants, like the defects in the corpus callosum and hippocampi, and initial underdevelopment of the neocortex. In some embryos bulging caudate nuclei (Suppl. Fig. 6A) or enlarged ventricles (Suppl. Fig. 6B) were detected. Interestingly, although patients with PTHS only lack one functional allele of the Tcf4 gene, heterozygous animals show relatively mild developmental effects on cortical architecture and hippocampal folding. This suggests that there is a dose-dependency of the effects of Tcf4 in murine brain development and loss of one allele of Tcf4 may be compensated by the functional Tcf4 allele. The full Tcf4 mutant displays the most consistent phenotypic characteristics as observed in human PTHS patients. The smaller hippocampi, initial underdevelopment of the neocortex, and agenesis of the corpus callosum detected in this study match the data from Jung et al. (2018). However, when analyzing these mutants it is important to keep in mind that the study of Jung et al. focuses on the heterozygous mice of a mouse model, which lacks exon 4 of the Tcf4 gene, whereas in this study we examined full mutant embryos of a mouse model in which the bHLH domain of the Tcf4 gene is replaced by a Neo-cassette (Zhuang et al., 1996). Differences between the study of Jung et al. and the results presented in this study could be due to the different types of mutations and the use of Tcf4 heterozygous animals, as a more intermediate phenotype would be expected compared to the phenotype described for the full Tcf4 mutant in the present study.

As stated above, patients with PTHS mainly show missense mutations or deletions in the basic region of the bHLH domain, resulting in a defective gene and possible protein in the human brain, indicating that the model used in this study could be used to examine the underlying mechanisms of the main defects detected in PTHS patients. Importantly, studying the full Tcf4 mutant provides information about the function of Tcf4 during brain development since these animals are devoid of functional Tcf4. This information is necessary to truly comprehend the functional aspects of Tcf4 in brain development and is crucial when searching for novel leads in treatment of these patients.

Besides its role in PTHS and other ID syndromes, Tcf4 has been linked to the onset and progression of autism and schizophrenia (Brzózka and Rosner, 2013; Cousijn et al., 2014; de Munnik et al., 2019). Brain defects seen in PTHS patients include a smaller corpus callosum, bulging caudate nuclei, underdeveloped hippocampi, enlarged ventricles, and in some cases microcephaly (Blake et al., 2010; Hasi et al., 2011; Peippo and Ignatius, 2012; Ghosh et al., 2012). Many of these traits were traced back in full Tcf4 mouse mutants, like the defects in the corpus callosum and hippocampi, and initial underdevelopment of the neocortex. In some embryos bulging caudate nuclei (Suppl. Fig. 6A) or enlarged ventricles (Suppl. Fig. 6B) were detected. Interestingly, although patients with PTHS only lack one functional allele of the Tcf4 gene, heterozygous animals show relatively mild developmental effects on cortical architecture and hippocampal folding. This suggests that there is a dose-dependency of the effects of Tcf4 in murine brain development and loss of one allele of Tcf4 may be compensated by the functional Tcf4 allele. The full Tcf4 mutant displays the most consistent phenotypic characteristics as observed in human PTHS patients. The smaller hippocampi, initial underdevelopment of the neocortex, and agenesis of the corpus callosum detected in this study match the data from Jung et al. (2018). However, when analyzing these mutants it is important to keep in mind that the study of Jung et al. focuses on the heterozygous mice of a mouse model, which lacks exon 4 of the Tcf4 gene, whereas in this study we examined full mutant embryos of a mouse model in which the bHLH domain of the Tcf4 gene is replaced by a Neo-cassette (Zhuang et al., 1996). Differences between the study of Jung et al. and the results presented in this study could be due to the different types of mutations and the use of Tcf4 heterozygous animals, as a more intermediate phenotype would be expected compared to the phenotype described for the full Tcf4 mutant in the present study.
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