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Profiling of methylation and demethylation pathways during brain development and ageing

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Abstract Numerous signal pathways are epigenetically controlled during brain development and ageing. Thereby, both 5-methylcytosine (5mC) and the newly described 5-hydroxymethylcytosine (5hmC) are highly exhibited in the brain. As there is an uneven distribution of 5hmC in the brain depending on age and region, there is the need to investigate the underlying mechanisms being responsible for 5hmC generation and decline. The aim of this study was to quantify expression levels of genes that are associated with DNA methylation/demethylation in different brain regions and at different ages. Therefore, we investigated frontal cortex and cerebellum of 40 mice (strain C57BL/6), each eight mice sacrificed at day 0, 7, 15, 30 and 120 after birth. We performed expression profiling of methylation/demethylation genes depending on age and brain region. Interestingly, we see significant expression differences of genes being responsible for methylation/demethylation with a significant reduction of expression levels during ageing. Validating selected expression data on protein level using immunohistochemistry verified the expression data. In conclusion, our findings demonstrate that the regulation of methylation/demethylation pathways is highly controlled depending on brain region and age. Thus our data will help to better understand the complexity and plasticity of the brain epigenome.

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Introduction

During brain development and ageing numerous signal pathways are epigenetically controlled. Thereby, DNA methylation, i.e. the methylation of cytosine-bases by the DNA methyltransferases (DNMT) within the promoter region of genes by addition of a methyl-group at position 5 of cytosine (C), is one of the most common epigenetic effects; the resulting 5-methylcytosine (5mC) is responsible for regulation of gene transcription (Branco et al. 2012; Kraus et al. 2012; Pfaffeneder et al. 2011; Tahiliani et al. 2009).

But 5mC is not the only epigenetically important cytosine modification. In 2009 Kriaucionis and Heintz described a hydroxymethylation of cytosine in Purkinje cells of the cerebellum (Kriaucionis and Heintz 2009). This new base modification, 5-hydroxymethylcytosine (5hmC) is generated by the 2-oxoglutarate/Fe(II)-dependent methylcytosine dioxygenases TET (ten-eleven-translocation) that are capable to convert 5mC to 5hmC (Branco et al. 2012; He et al. 2011; Ito et al. 2011; Pfaffeneder et al. 2011). Interestingly, 5hmC is not equally distributed across different tissues: whereas there are very low amounts of 5hmC in many tissue types, such as in liver tissue (Munzel et al. 2011), there are very high amounts in the brain (Jin et al. 2011; Kraus et al. 2012, 2014; Munzel et al. 2011). Interestingly, the amount of 5hmC in the brain is region and age dependant: there are low amounts of 5hmC in early developmental stages but during brain development there are increasing amounts of 5hmC that reach their maximum values at early adulthood and then remain at high levels

during ageing (Kraus et al. 2014; Wagner et al. 2015). But 5hmC is also important for controlling pluripotency and stem cell development (Branco et al. 2012). Ficz et al. found that 5hmC is mostly associated with euchromatin and increased gene transcription levels (Ficz et al. 2011).

Up to date, there are three potential causes for this increase of 5hmC: (1) highly active methylation and demethylation processes; (2) passive accumulation of 5hmC due to methylation without significant demethylation; (3) generation of 5hmC independently of passive methylation/demethylation processes (Branco et al. 2012). However, there are so far no systematic studies analysing expression profiles of methylation/demethylation pathways in the brain. Thus, it is of fundamental importance to analyse the activity of methylation and demethylation pathways during ageing in order to understand the biology beneath.

This problem is now addressed by us. In the current study, we investigated the expression patterns of enzymes that are responsible for methylation and demethylation (*Dnmt1*, *Dnmt3a*, *Dnmt3b*, *Tet1*, *Tet2*, *Tet3*, *Apobec1*, *Apobec2*, *Apobec3*, *Mbd4*, *Smug1*, *Tdg*) in distinct developmental stages (day of birth, day 7, 15, 30 and 120 after birth) in the frontal cortex and the cerebellum of murine brains. Thereby, we found distinct regulatory patterns depending on brain region and age and validated expression data by immunohistochemistry. Our data will help to better understand the biological role of 5hmC and will contribute to recognize epigenomic processes occurring during brain development and ageing.

Materials and methods

Sample data

In this study, brain tissue samples of 40 mice (strain C57BL/6) were investigated. Both female and male mice were used. Each eight mice were sacrificed at day 0, 7, 15, 30 and 120 after birth. Mice were housed together with the mother till the age of 3 weeks, then they were removed from the mother and each three were housed in standard cages (30 × 15 × 20 cm). The brains were removed and each five brains were snap-frozen and stored at −80 °C for molecular genetic analysis. Each three brains were formalin-fixed for 24 h and finally paraffin embedded. We selected frontal and cerebellar cortex as target regions. In order to determine the possible influence of sex on the brain epigenome, we performed an analysis on previously published data of 22 brain samples (Kraus et al. 2012). However, we did not find statistically significant effects of sex on the global brain epigenome (Supplementary Fig. 1). All procedures performed in this study involving mice

were in accordance with the ethical standards of the institute at which the experiments were conducted.

Extraction of RNA and reverse transcription reaction

RNA extraction was performed using the RNeasy micro kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol including a DNAse digestion step. We diluted the extracted RNA in 15 µL RNAse-free water. Reverse transcription reaction of RNA into cDNA was performed using the Superscript Vilo cDNA synthesis kit (life technologies, Darmstadt, Germany) and up to 2.5 µg of extracted RNA (500 ng/µL) according to the manufacturer's instructions.

Quantification of gene expression using real-time PCR

For real-time PCR, equal amounts of cDNA were used with Power SYBR green PCR master mix (Life Technologies, Darmstadt, Germany) on a LightCycler 480 II System (Roche, Basel, Switzerland) according to the manufacturer's protocols. The gene expression levels were calculated according to the comparative C_T-method ($\Delta\Delta C_T$-method) (Abdel Nour et al. 2014). Expression levels were normalized to *Gapdh*, *Hprt*, *Ipo8* and *Tbp*. Primer sequences can be found in Supplementary Table 1.

Immunohistochemistry and quantification

Immunohistochemistry was performed following standard protocols. We used 4 µm FFPE sections that were deparaffinized. Therefore, sections were first incubated in xylene for 30 min, then in 100 % ethanol for 10 min, in 96 % ethanol for 5 min and finally in 70 % ethanol for 5 min. Visualization of 5hmC was performed using the polyclonal anti-5hmC antibody (1:1.000, Active Motif, Rixensart, Belgium) as described previously (Kraus et al. 2015a, b). Detection of 5mC was performed using the monoclonal anti-5mC antibody (clone 33D3, 1:3.000, Diagenode, Seraign, Belgium) on a Ventana Benchmark system (Roche, Basel, Switzerland) using standard protocols. In order to validate expression data, we used polyclonal anti-Tdg (1:200, Active Motif, Rixensart, Belgium), polyclonal anti-Dnmt3a (1:400, Abcam, Cambridge, UK) and monoclonal anti-Ki67 (1:100, Abcam, Cambridge, UK) antibodies. Staining was performed on a Ventana Benchmark system (Roche, Basel, Switzerland) using standard protocols. Sections were counterstained using hemalum.

Quantification of positive nuclei was performed on digital pictures of the stained regions of interest (ROI).

Pictures were taken using an Olympus BX50 microscope (Olympus, Tokyo, Japan) and a Color View III camera system (Soft Imaging System, Münster, Germany) with 10× and 20× objectives (Olympus, Tokyo, Japan). Representative areas without any technical artefacts were selected for quantification. We quantified the amount of 5hmC- and 5mC-positive cells as well as the amount of Tdg-, Dnmt3a- and Ki67-positive cells in the frontal and cerebellar cortex, thereby cells were considered positive even when the immunohistochemical signal was weak or dot-like. In the cerebellum, counting was performed separately for the external granular cell layer (EGCL, only in immature cerebellum), the molecular cell layer (MCL), for Purkinje cells (PC) and for the internal granular cell layer (IGCL). Quantifications were performed using ImageJ software.

Statistical analysis

Statistical analysis was performed using SPSS Statistics 22 (IBM) and Prism 5 (GraphPad) software. In this study, we applied t test and one-way ANOVA with Newman–Keuls post hoc test. Correlation analysis was performed using Prism 5 (GraphPad). Statistical significance was assumed for p values <0.05. Heat maps were generated using the Gene-E analysis software suite (http://www.broadinstitute.org/cancer/software/GENE-E/). We performed an un-supervised hierarchical clustering based on pair-wise distance measures applying the One minus Pearson correlation.

Results

Quantification of the amount of 5hmC and 5mC during brain development and ageing

In order to generate a complete picture of methylation and demethylation during brain development and ageing, we integrated our previously published 5hmC data (Kraus et al. 2014) with newly generated 5mC data and gene expression levels of enzymes that are responsible for methylation and demethylation. Detection of 5hmC and 5mC during brain development and ageing was performed using immunohistochemical stains of FFPE tissue on murine frontal cortex (Fig. 1a–o) and cerebellum (Fig. 1p–δ). Thereby, we found uneven distribution of immunopositive nuclei depending on brain region and age. While 5hmC-positive cells increased during ageing in the frontal cortex (Fig. 1a–e), almost all cells were 5mC positive in all investigated ages with an interesting uneven intranuclear patter (Fig. 1f–o): whilst only some nuclei were completely 5mC positively stained, most nuclei showed small 5mC positive dots (Fig. 1k–o). In the

cerebellum we found a layer-dependent increase of 5hmC during ageing (Fig. 1p–t), whereas 5mC was positive in all nuclei regardless of the age but there was also an uneven distribution of 5mC in most nuclei indicating 5mC-positive dots in many nuclei (Fig. 1u–δ).

Quantification of 5hmC- and 5mC-positive cells demonstrated significant differences in immunopositive cells depending on brain region and age (Fig. 2). As reported previously (Kraus et al. 2014), there was a significant increase of 5hmC-positive cells in the murine frontal cortex from the day of birth (day 0) till the age of 120 days ($p < 0.01$, ANOVA with Newman–Keuls post hoc, Fig. 2a). Almost all nuclei stained positive for 5mC regardless of the age (Fig. 2b). Detailed quantification data on 5hmC can be found in Supplementary Table 2 and on 5mC in Supplementary Table 3.

Quantification of 5hmC and 5mC in the murine cerebellar cortex using immunohistochemistry revealed that the amount of 5hmC-positive nuclei increased in the external granular cell layer (EGCL) from the day of birth till day 15 when the EGCL fades out ($p < 0.01$, ANOVA with Newman–Keuls post hoc, Fig. 2c) as reported previously (Kraus et al. 2014). Almost all nuclei stained positive for 5mC from day 0 to 15 (Fig. 2d). The internal granular cell layer (IGCL) showed a significant increase of 5hmC-positive cells from the day of birth till the age of 30 days ($p < 0.01$, ANOVA with Newman–Keuls post hoc) that was followed by a slight decrease till the age of 150 days ($p < 0.01$, ANOVA with Newman–Keuls post hoc, Fig. 2e). Almost all nuclei stained positive for 5mC regardless of the age (Fig. 2f). In the molecular cell layer (MCL), we found a significant increase of 5hmC-positive cells from the day of birth till the age of 30 days ($p < 0.01$, ANOVA with Newman–Keuls post hoc) that was followed by a slight decrease till the age of 120 days (ANOVA with Newman–Keuls post hoc, Fig. 2g). Almost all nuclei stained positive for 5mC regardless of the age (Fig. 2h). Detailed quantification data on 5hmC can be found in Supplementary Table 2 and on 5mC in Supplementary Table 3.

Expression differences of methylation/demethylation genes during brain development and ageing

Investigating the expression levels of enzymes being responsible for methylation and demethylation (*Dnmt1*, *Dnmt2a*, *Dnmt2b*, *Tet1*, *Tet2*, *Tet3*, *Apobec1*, *Apobec2*, *Apobec3*, *Tdg*, *Smug1*, *Tdg*) in murine frontal cortex and cerebellum from the day of birth till the age of 120 days demonstrated, we found distinct differences of gene expressions (Fig. 3). An overview on expression data can be found in Supplementary Table 4.
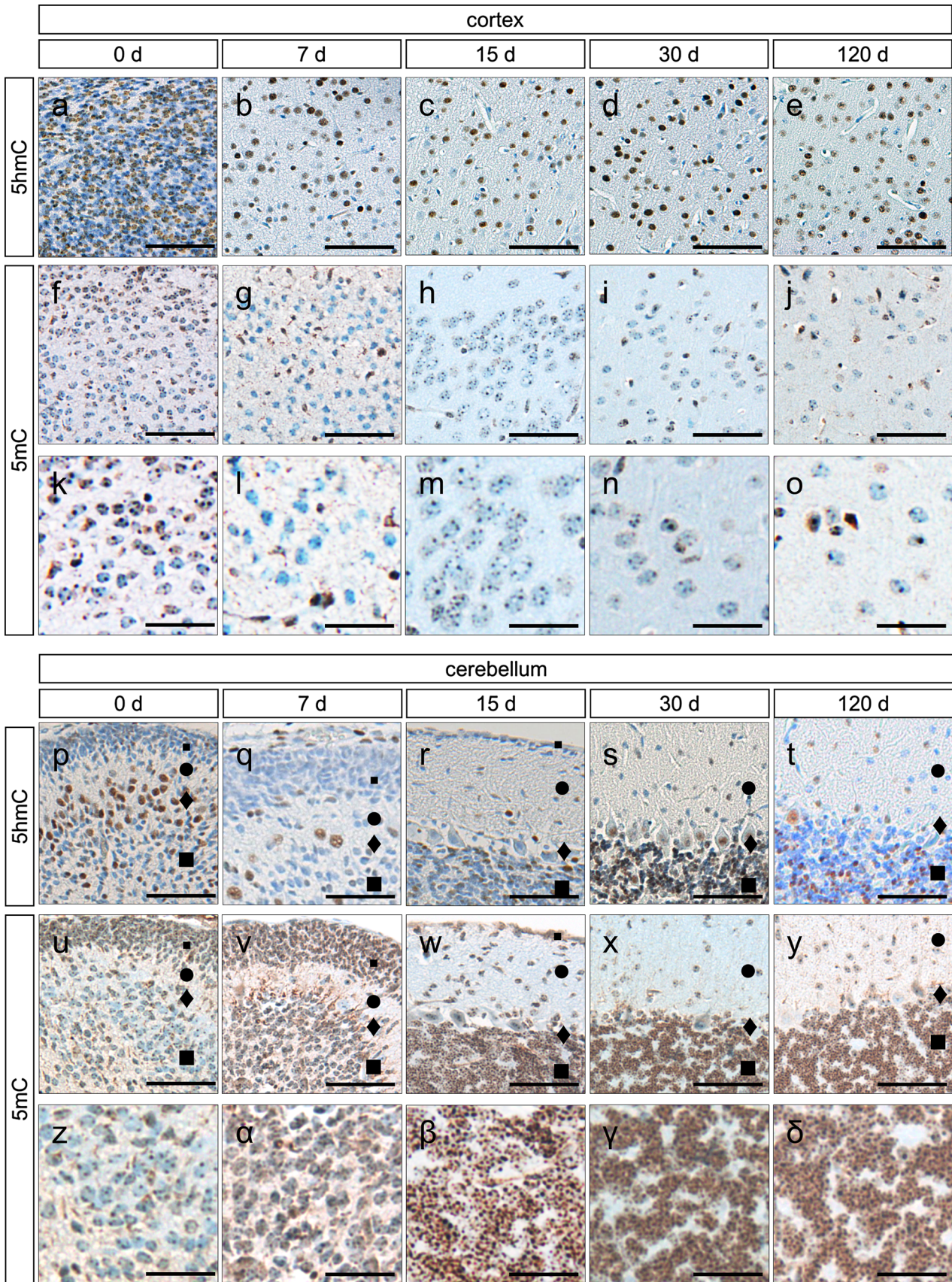
◄**Fig. 1** Immunohistochemical detection of 5hmC and 5mC in frontal cortex (**a–o**) and cerebellum (**p–δ**). In the frontal cortex of mice sacrificed at day 0, 7, 15, 30 and 120 after birth we see age-dependent amounts of immunopositive (*brown colour*) 5hmC (**a–e**) and 5mC (**f–o**) cells. Higher-resolution images of 5mC show that whilst only some nuclei are completely 5mC positive, most nuclei show small 5mC-positive dots (**k–o**). In the cerebellum of mice sacrificed at day 0, 7, 15, 30 and 120 after birth we see layer- and age-dependent amounts of 5hmC (**p–t**) and 5mC (**u–δ**) positive nuclei. Exemplary higher-resolution images of the internal granular cell layer of 5mC-stained slides show that only some nuclei are completely 5mC positive, but most nuclei show small 5mC-positive dots (**z–δ**). 5hmC data used in this study were adapted from previously published work (Kraus et al. 2014). *Small square* external granular cell layer, *circle* molecular cell layer, *diamond* Purkinje cell layer, *large square* internal granular cell layer. *Scale bar* 100 µm (**a–j, p–y**); 50 µm (**k–o, z–δ**)

A detailed analysis of gene expression levels in the frontal cortex showed that the expression levels of DNA methylation maintaining *DNMT1* (*DNA methyltransferase*, Fig. 4a) and the de novo methyltransferase *Dnmt3a* (Fig. 4b) decreased from the day of birth till day 120, in adulthood (days 30 and 120) there were no more changes detectable. *DNMT3b* did not show significant expression differences (Fig. 4c). The methylcytosine dioxygenases *Tet1* (*ten-eleven-translocation*, Fig. 4d), *Tet2* (Fig. 4e) and *Tet3* (Fig. 4f) showed significantly decreased expressions during ageing; however, the expression levels were stable in adulthood. *Apobec1* (*apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like*) showed a significant reduction of expression from day 7 to 120 (Fig. 4g), *Apobec2* showed a significant decrease of expression from day 0 to 120 (Fig. 4h) with stable levels in adulthood and *Apobec3* showed a significant decrease of expression from day 7 to 120 (Fig. 4i) with stable levels in adulthood. *Mbd4* (*methyl-CpG binding domain protein 4*, Fig. 4j). *Smug1* (*single-strand-selective monofunctional uracil-DNA glycosylase 1*, Fig. 4k) and *TDG* (*thymine-DNA glycosylase,* Fig. 4l) showed a significant decrease of expression from the day of birth till the age of 120 days with stable levels in adulthood. In the cerebellar cortex we found a significantly decreased expression of *DNMT1* (Fig. 5a) and *Dnmt3a* (Fig. 5b) during ageing with stable levels in adulthood. *Dnmt3b* did not show differences in gene expression (Fig. 5c). *Tet1* (Fig. 5d)*, Tet2* (Fig. 5e) and *Tet3* (Fig. 5f) showed decreased expressions during ageing with increased expressions in adult cerebellum. *Apobec1* showed a significant decrease of expression from day 15 to 120 (Fig. 5g). *Apobec2* showed decreased expression levels from day 0 to 7 followed by a subsequent increase of expression (Fig. 5h). *Apobec3* showed a decrease of expression during ageing (Fig. 5i). *Mbd4* did not show significant expression differences during ageing (Fig. 5j). *Smug1* showed a decreased

expression from day 7 to 15 (Fig. 5k). *Tdg* showed a significant expression decrease from day 7 to 30 (Fig. 5l).

An analysis of expression levels of different anatomical regions (frontal cortex and cerebellar cortex) at distinct time points (day 0, 7, 15, 30, 120) revealed that gene expression levels are not only significantly changing during ageing, but that they are also brain region specific and that the regional expression differences are changing during ageing (Supplementary Table 5).

Differences in expression levels are confirmed using immunohistochemistry

As we found significant differences in expression levels of enzymes being responsible of methylation and demethylation during brain development and ageing, we next validated distinct gene expressions on protein level. Therefore, we selected the DNA methyltransferase DNMT3a and the thymine-DNA glycosylase TDG to performed immunohistochemical stains on FFPE sections of murine brains of mice sacrificed at the day of birth (day 0), 7 days after birth, at the age of 15 days, at 30 days and at 120 days of age. As for expression profiling, we selected frontal cortex and cerebellar cortex as target regions to validate the expression profiles on protein level. Additionally, we performed immunohistochemistry using antibodies against the proliferation-associated protein Ki67 to check for immature, proliferating cells as there are many proliferating cells at early developmental stages with a decrease of positive cells during development and ageing.

Immunohistochemical stains of frontal cortex (Fig. 6a–o) as well as of cerebellar cortex (Fig. 6p–δ) at day 0, 7, 15, 30 and 120 showed uneven distributions of Dnmt3a, Tdg and Ki67 protein levels depending on brain region, layer and age. In the frontal cortex (Fig. 6a–o) we found a decrease of immunohistochemically positive cells for Dnmt3a (Fig. 6a–e), Tdg (Fig. 6f–j) and Ki67 (Fig. 6k–o) from day 0 to 120.

In the cerebellar cortex (Fig. 6p–δ), we found uneven distributions of Dnmt3a (Fig. 6p–t), Tdg (Fig. 6u–y) and Ki67 (Fig. 6z–δ) depending on age. Additionally, this approach allowed us to distinguish between protein levels in the external granular cell layer (EGCL), the molecular cell layer (MCL), the Purkinje cells (PC) and the internal granular cell layer (IGCL). We found a decrease of Dnmt3a (Fig. 6p–t) especially in the EGCL and in the MCL and only minor variations of protein amount in the IGCL during development and ageing from day 0 to 120. PC demonstrated positive signals only in early stages till day 7. Tdg (Fig. 6u–y) showed numerous positive cells at days 0 and 7 in the MCL and the IGCL with a decrease at day 15. Cells of the EGCL stained positive especially in the lower part of the layer indicating positivity of cells that

Fig. 2 Quantification of 5hmC- and 5mC-positive cells. In the frontal cortex (**a**–**b**) the number of 5hmC-positive cells increases from the day of birth till the age of 120 days (**a**). Almost all nuclei stain positive for 5mC regardless of the age (**b**). In the cerebellum, there is a significant increase of 5hmC-positive cells in the EGCL from the day of birth to 15 when the EGCL fades out (**c**). All nuclei stain positive for 5mC regardless of the age (**d**). In the IGCL there is a significant increase of 5hmC-positive cells from the day of birth till the age of 30 days that is followed by a slight decrease (**e**). Almost all nuclei stain positive for 5mC regardless of the age (**f**). In the MCL there is a significant increase of 5hmC positive nuclei from the day of birth till the age of 30 that is followed by a slight decrease (**g**). Almost all nuclei stain positive for 5mC regardless of the age (**h**). 5hmC data used in this study were adapted from previously published work (Kraus et al. 2014). Indicated are mean and SEM. *EGCL* external granular cell layer, *MCL* molecular cell layer, *IGCL* internal granular cell layer. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$
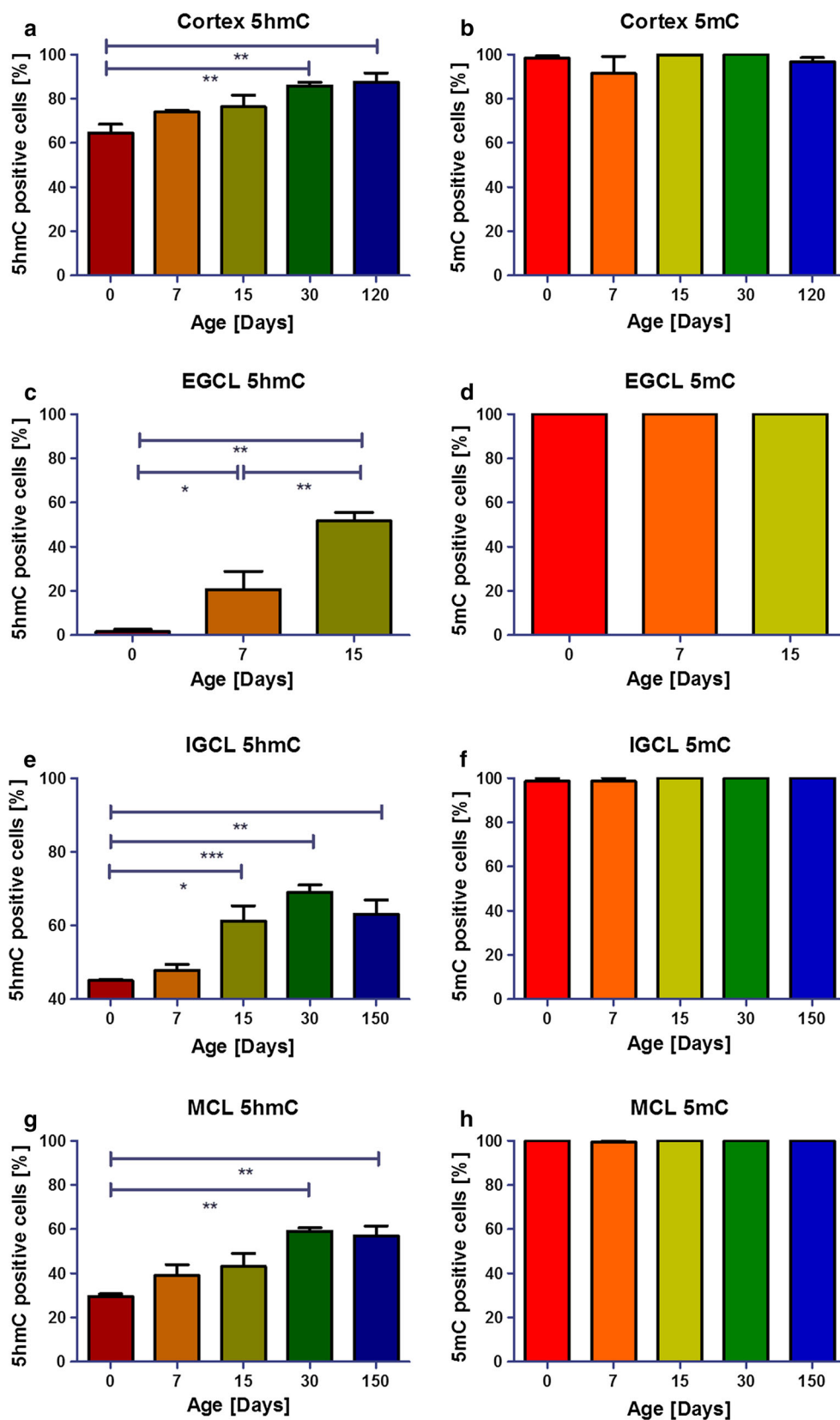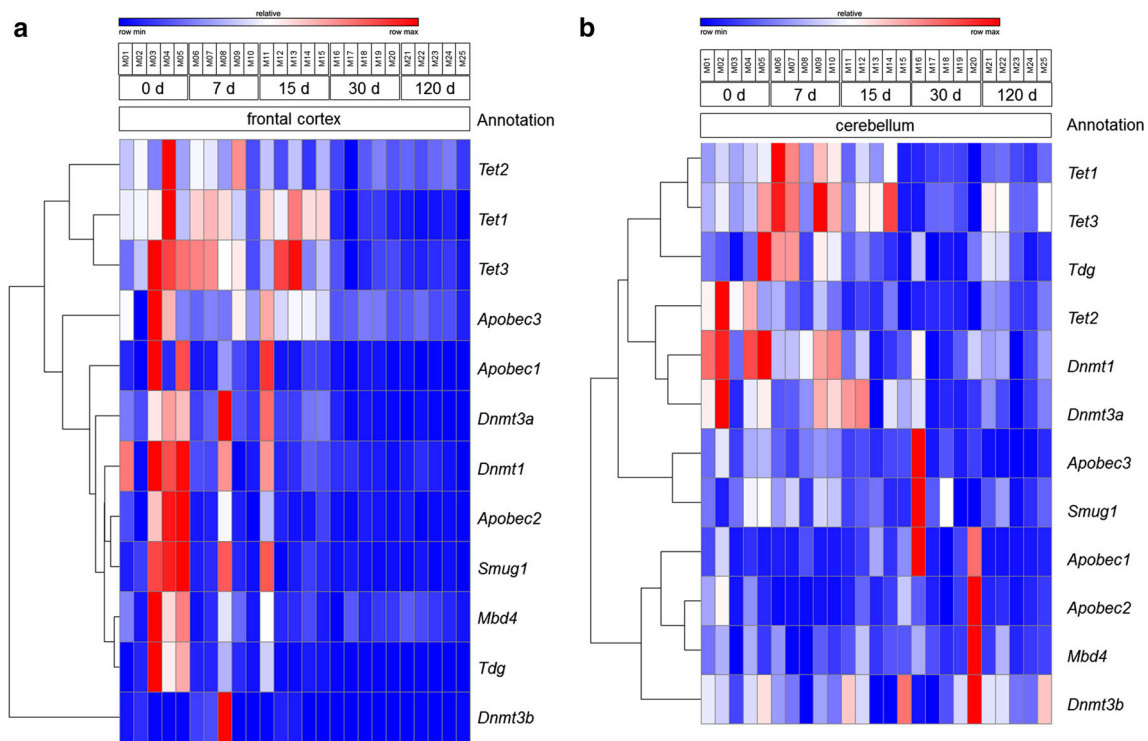
Fig. 3 Heat maps indicating gene expression levels. In frontal cortex (**a**) and cerebellar cortex (**b**) there are expression differences of methylation and demethylation genes during brain development and ageing. *Blue marks* indicate low expression levels, *red marks* indicate high expression levels. *M* identification number of mouse

migrate towards the deeper cortical layers. PC demonstrated constant positive signals during ageing but signal intensity is reduced in higher age. Ki67 (Fig. 6z–δ) showed numerous positive cells especially in early developmental stages and in the EGCL with a decrease at day 15 after birth. PC stained negative using Ki67 antibodies.

A graphical heat map presentation of protein amount quantifications in the frontal cortex and the different cerebellar cortical layers showed that especially in early developmental stages, there were high amounts of Dnmt3a and Tdg as well as Ki67 proteins indicating high methylation and demethylation activities followed by reduced protein levels at the ages of 15–120 days (Fig. 7). Detailed cell counts are listed in Supplementary Table 6.

Statistical analysis of protein levels in the frontal cortex showed a significant decrease of Dnmt3a protein levels during ageing (Fig. 8a). Interestingly protein levels remained stable from day 0 to 15 followed by a decreased level at day 30 and 120. These data confirmed that the expression differences as described above are followed by decreased protein levels. In case of Tdg we found a significant decrease of protein levels during development and ageing (Fig. 8b). These data also confirmed that the expression differences are followed by decreased protein levels. The proliferation-associated protein Ki67 showed stable amounts of positive cells from day 0 to 15 followed

by a significant decrease at day 30 and 120 (Fig. 8c). In summary, the comparison of expression data and protein levels showed that expression differences can be confirmed on protein level of selected proteins in the frontal cortex. In the cerebellar cortex immunohistochemical quantification of Dnmt3a, Tdg and Ki67 amount was performed on each layer separately to get a detailed view of molecular mechanisms occurring during development and ageing. As the EGCL consists of immature cells that migrate downwards to the deeper layers during early development, the EGCL fades at around day 15. In the EGCL we detected a significant decrease of Dnmt3a amount from day 0 to 15 (Fig. 8d). In case of Tdg we found no significant decrease during brain development (Fig. 8e). The proliferation marker Ki67 showed constantly high amounts in the EGCL with a slight decrease at day 7 (Fig. 8f). In the IGCL we detected a significant increase of Dnmt3a protein levels from day 0 to 30 that is followed by a significant decrease till day 120 (Fig. 8g). Tdg showed a significant decrease of protein levels from day 0 to 120 in the IGCL (Fig. 8h). The Ki67 protein showed a significant decrease in the IGCL from day 0 to 120 (Fig. 8i). In the MCL we detected decreasing amounts of Dnmt3a from day 0 to 120 (Fig. 8j). In case of Tdg we observed a decrease of protein amount from day 0 till the age of 120 days in the MCL (Fig. 8k). Ki67 protein showed a decrease in the IGCL from day 0 to
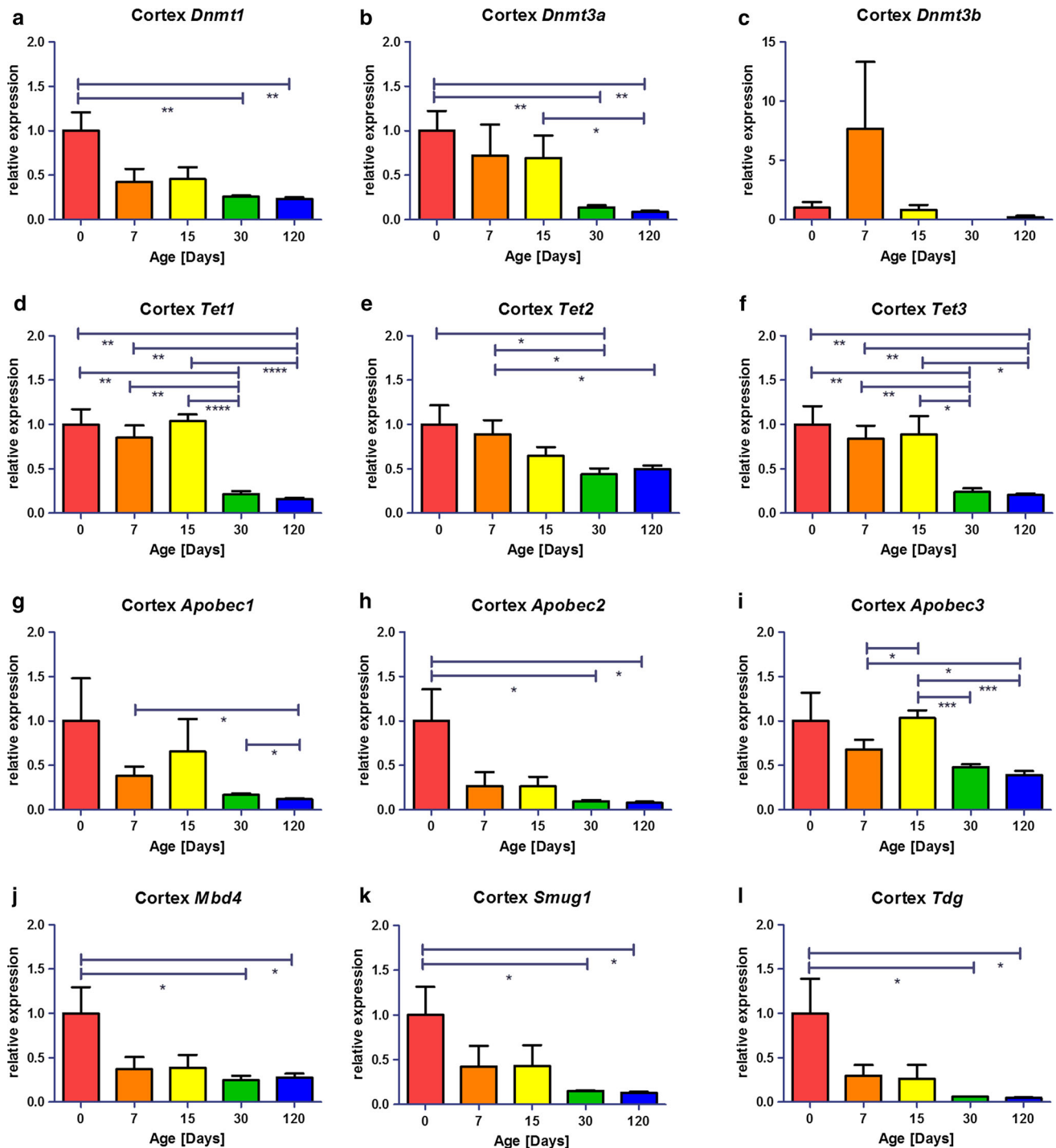
Fig. 4 Quantification of relative expression levels in frontal cortex. We see a decrease of the expression levels of *Dnmt1* (**a**) and *Dnmt3a* (**b**). There is no significant difference in case of *Dnmt3b* (**c**). In case of *Tet1* (**d**), *Tet2* (**e**) and *Tet3* (**f**) there is a significant decrease. *Apobec1* (**g**), *Apobec2* (**h**) and *Apobec3* (**i**) show a significant decrease in expression during ageing. *Mbd4* (**j**), *Smug1* (**k**) and *Tdg* (**l**) show a significantly decreased expression during ageing. Indicated are mean and SEM. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$

120 (Fig. 8l). These data demonstrated that the expression levels correspond with protein amounts in the cerebellar cortex and that the use of immunohistochemistry adds a further level to the analysis by enabling to distinguish between different cortical layers.

As the analysis of 5hmC- and 5mC-positive cells was performed not only in frontal cortex and cerebellar cortex but in distinct cortical layers, we next performed correlation analysis of 5mC- and 5hmC-positive cells with immunohistochemically validated proteins being
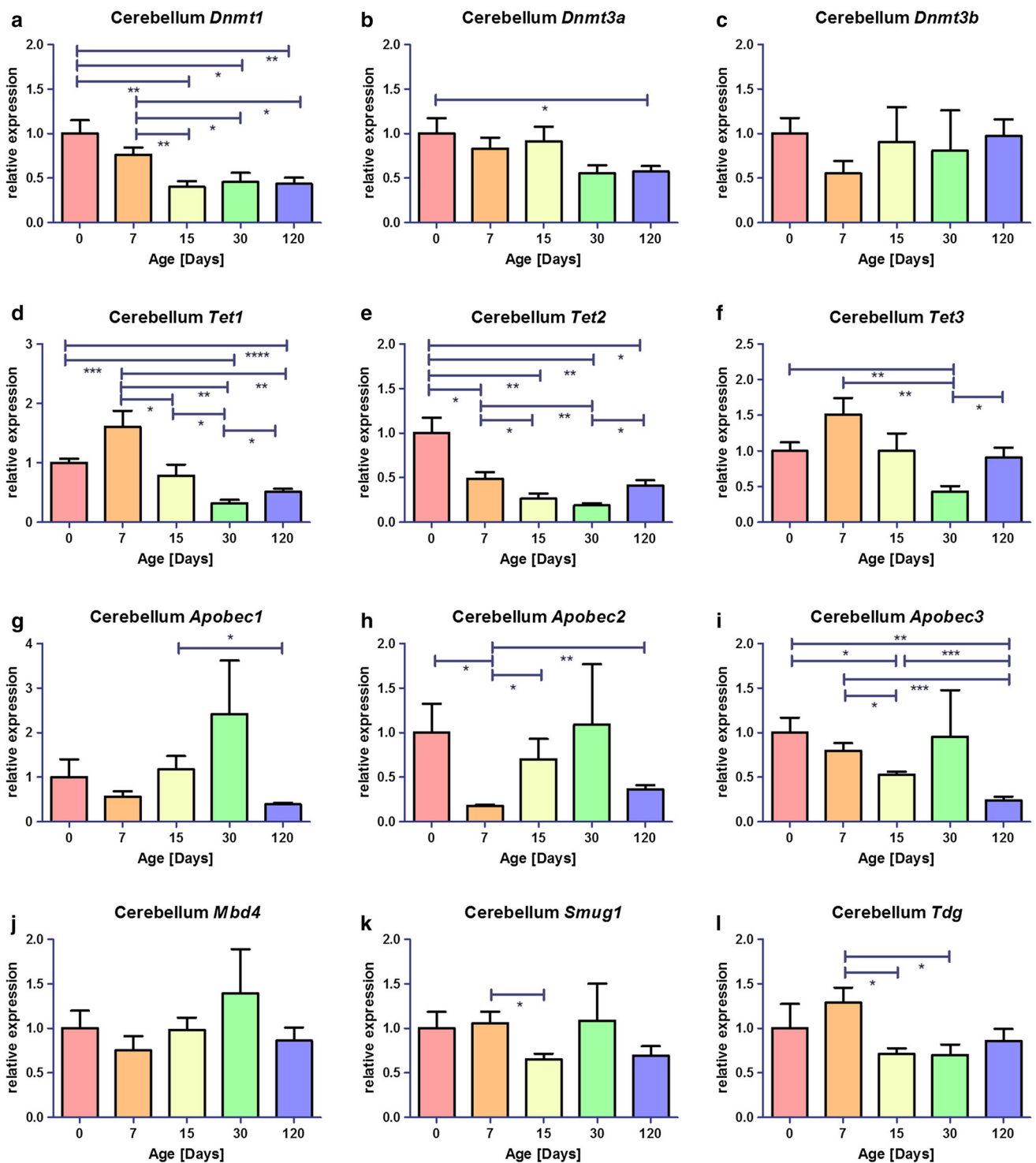
Fig. 5 Quantification of relative expression levels in cerebellar cortex. We see a decrease of expression levels of *Dnmt1* (**a**) and *Dnmt3a* (**b**). There is no significant difference in case of *Dnmt3b* (**c**). *Tet1* (**d**), *Tet2* (**e**) and *Tet3* (**f**) show significantly decreased expressions during ageing. *Apobec1* (**g**) shows a decreased expression from day 15 to 120. *Apobec2* (**h**) shows a decrease from day 0 to 7 that is followed by an increase till day 120. *Apobec3* (**i**) shows a decreased expression during ageing. *Mbd4* (**j**) does not show significant expression differences during ageing. *Smug1* (**k**) shows a decrease from day 7 to 15. *Tdg* (**l**) shows a decreased expression from day 7 to 30. Indicated are mean and SEM. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$
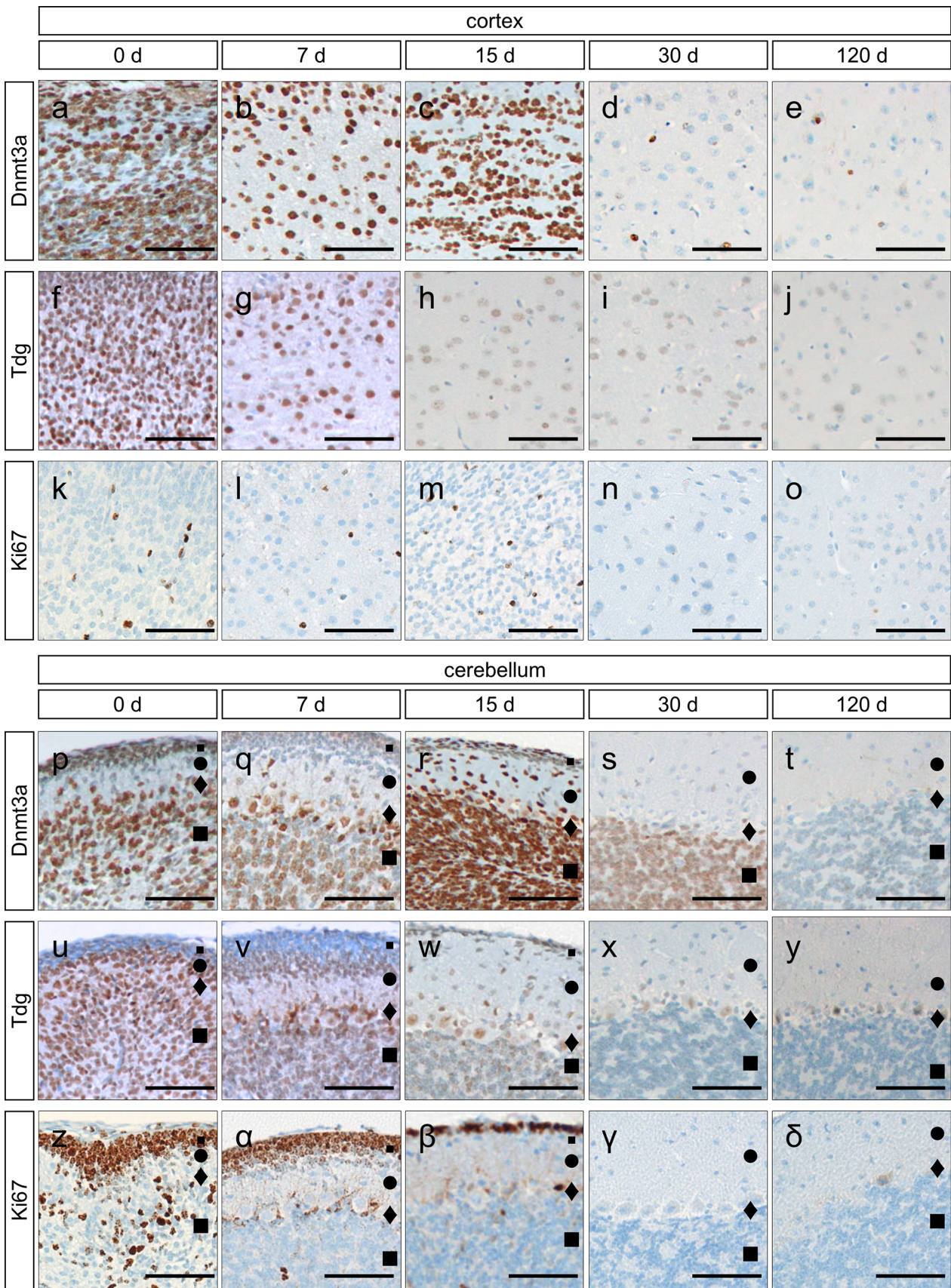
Fig. 6 Immunohistochemical detection of Dnmt3a, Tdg and Ki67 in frontal cortex (**a–o**) and cerebellum (**p–δ**). In the frontal cortex of mice sacrificed at day 0, 7, 15, 30 and 120 after birth we see age-dependent amounts of immunopositive (*brown colour*) Dnmt3a (**a–e**), Tdg (**f–j**) and Ki67 (**k–o**) cells. In the cerebellum of mice sacrificed at day 0, 7, 15, 30 and 120 after birth we see layer- and age-dependent amounts of Dnmt3a (**p–t**), Tdg (**u–y**) and Ki67 (**z–δ**) positive nuclei. *Small square* external granular cell layer, *circle* molecular cell layer, *diamond* Purkinje cell layer, *large square* internal granular cell layer. *Scale bar* 100 μm (**a–δ**)
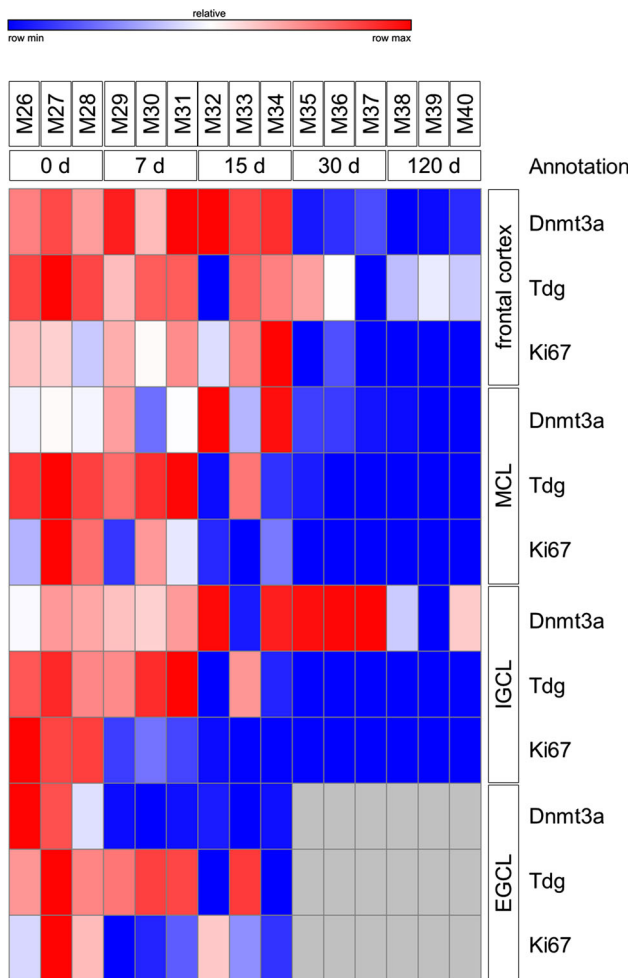

Fig. 7 Heat map generation to visualize immunohistochemically detected protein levels of Dnmt3a, Tdg and Ki67 in the frontal cortex and the cerebellum of mice aged 0, 7, 15, 30 and 120 days. *Blue marks* indicate low protein levels, *red marks* indicate high protein levels. As the EGCL fades at around day 15 there are no quantification data at day 30 and 120 (represented by *grey marks*). *EGCL* external granular cell layer of the cerebellum, *MCL* molecular cell layer of the cerebellum, *IGCL* internal granular cell layer of the cerebellum, *M* identification number of mouse

responsible for the generation and depletion of modified cytosine. Correlation analysis revealed that, indeed, there are brain region and layer-specific correlations. The amount of 5hmC significantly correlated with the protein

levels of Dnmt3a (Fig. 9a) and Tdg (Fig. 9b) in the frontal cortex: in early age we found high amounts of Dnmt3a and Tdg, enzymes that are responsible for methylation and demethylation, and only low amount of 5hmC. This observation mirrors that in early developmental stages there is a highly active methylation/demethylation that is associated with a highly plastic epigenomic control of gene expression. In higher age, the epigenome is more stable with constantly high amounts of 5hmC. This observation was also well in line with quantifications of the proliferation marker Ki67: there was a significant correlation of the amount of 5hmC and Ki67-positive cells (Fig. 9c). High proliferating activity was thus accompanied by low 5hmC amounts. In the cerebellum, we found significant correlations of 5hmC amount and protein levels of Dnmt3a (Fig. 9d), but we did not find significant correlations of 5hmC amount and Tdg (Fig. 9e) and Ki67 levels (Fig. 9f) in the EGCL. In the IGCL of the cerebellum, there was no correlation of 5hmC amount and Dnmt3a (Fig. 9g), but there was a highly significant correlation of 5hmC and Tdg (Fig. 9h) and Ki67 (Fig. 9i). In the MCL of the cerebellum we found that there was no correlation of 5hmC amount and Dnmt3a (Fig. 9j), but there was a significant correlation of 5hmC amount and Tdg (Fig. 9k) as well as Ki67 (Fig. 9l). Analysis of the amount of 5mC and protein levels of Dnmt3a, Tdg and Ki67 revealed no significant correlations (Supplementary Fig. 2). Analysing the amount of 5mC and 5hmC (Supplementary Fig. 3) did not show significant correlations. As almost all nuclei already exhibited 5mC at the day of birth both in the frontal cortex and in the cerebellum, it is likely that the molecular mechanisms that are responsible for primary generation of 5mC occur in very early brain development and were already finished at the day of birth. As opposed to this, the generation of 5hmC is a very flexible process during brain development that lasts till early adulthood.

Discussion

Since the discovery of 5hmC, the complexity of the brain epigenome has even increased. Despite intensive research, the distinct function of 5-hydroxymethylation in brain cell DNA still remains unclear. Szulwach et al. described that 5hmC may be of major importance for gene regulation (Szulwach et al. 2011a), regulation of neurodevelopment and control of cellular ageing (Szulwach et al. 2011b). Furthermore, 5hmC may be important for active demethylation (Branco et al. 2012; Mellen et al. 2012; Spruijt et al. 2013).

Thereby, the DNA methyltransferases DNMT1, DNMT3a and DNMT3b are responsible for the generation of 5mC by methylation of unmodified cytosine (C).
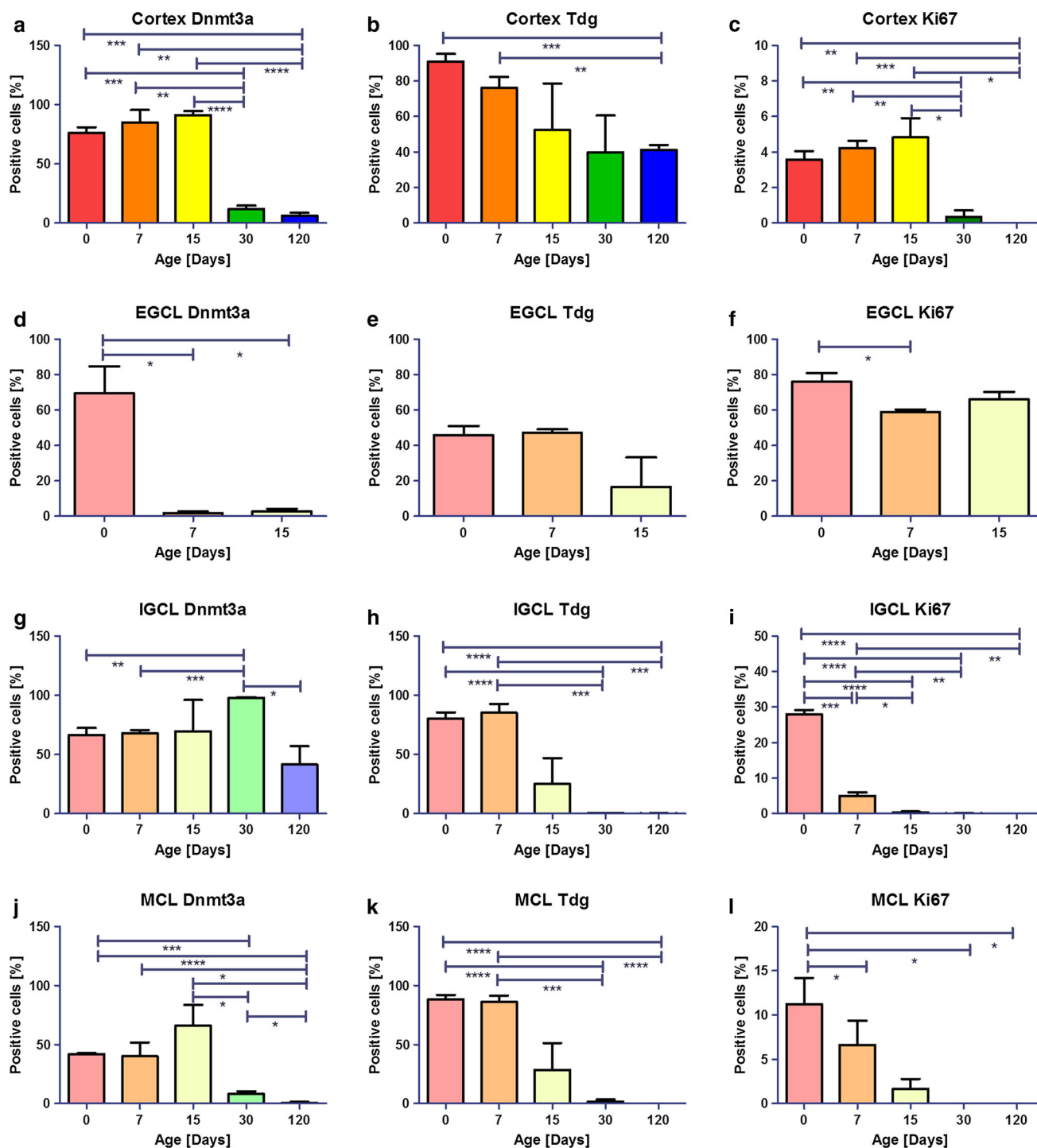
Fig. 8 Quantification of Dnmt3a, Tdg and Ki67-positive cells during brain development and ageing. In the frontal cortex (**a**–**c**) the number of Dnmt3a (**a**), Tdg (**b**) and Ki67 (**c**) positive cells decreases significantly during ageing. In the cerebellar cortex (**d**–**l**) we see layer-dependent differences during brain development and ageing. The EGCL (**d**–**f**) shows a decrease of Dnmt3a (**d**) from day 0 to 15 when the EGCL fades. Tdg (**e**) does not show significant differences from day 0 to 15. Ki67 (**f**) shows a significant decrease from day 0 to 7. In the IGCL (**g**–**i**) there is an increase of Dnmt3a (**g**) from day 0 to 30 followed by a decrease till day 120, a decrease of Tdg (**h**) from day 0 to 120 and a decrease of Ki67 (**i**) from day 0 to 120. In the MCL (**j**–**l**) there is a decrease of Dnmt3a (**j**), Tdg (**k**) and Ki67 (**l**) during ageing. Indicated are mean and SEM. *EGCL* external granular cell layer, *MCL* molecular cell layer, *IGCL* internal granular cell layer. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$
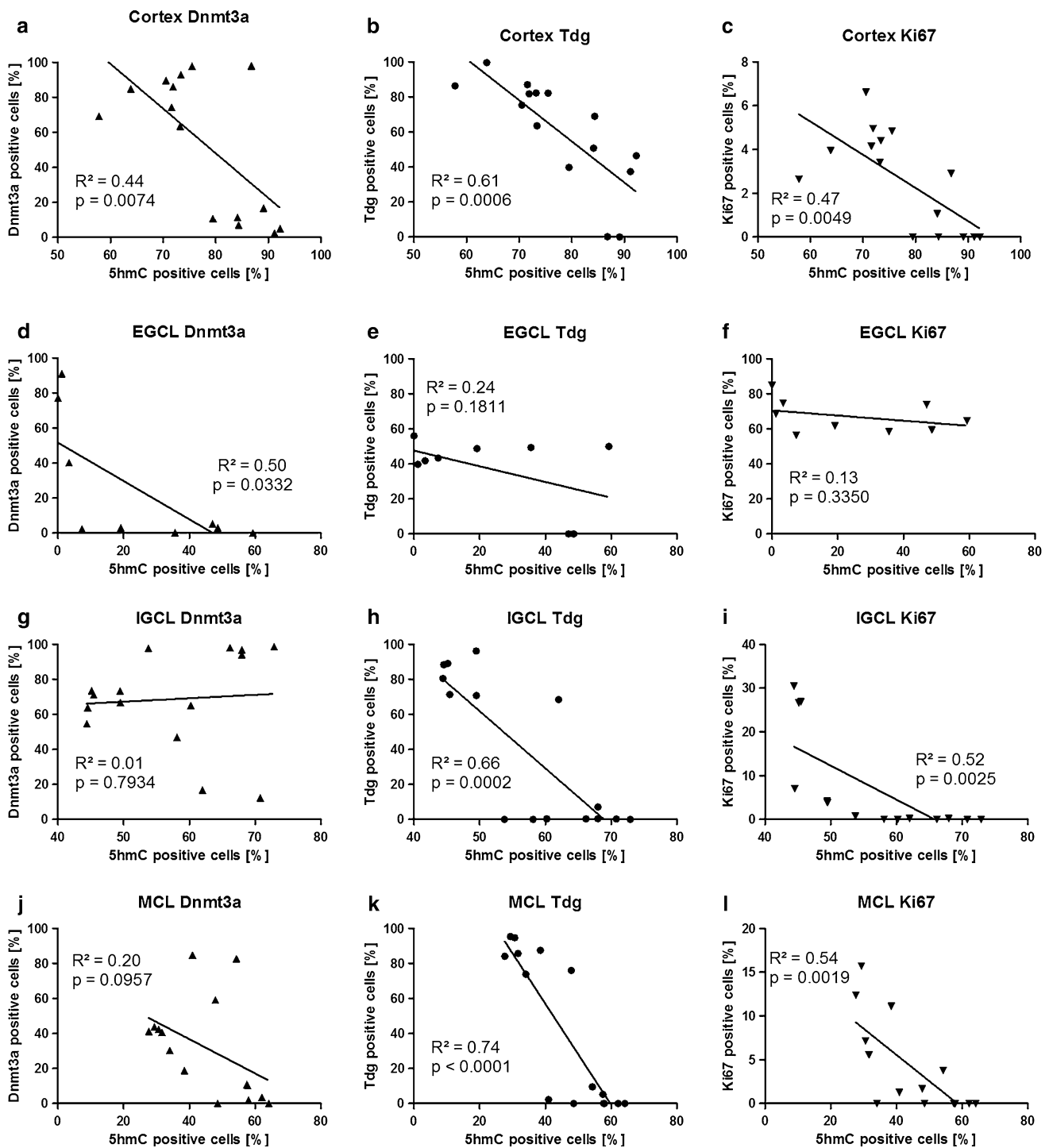
Fig. 9 Correlation analysis of 5hmC amount and Dnmt3a, Tdg and Ki67-positive cells. In the frontal cortex (**a–c**) there is a significant correlation of 5hmC amount and the number of Dnmt3a (**a**), Tdg (**b**) and Ki67 (**c**) positive cells. In the cerebellar cortex (**d–l**) there are layer-dependent correlations of 5hmC amount and Dnmt3a, Tdg and Ki67-positive cells during brain development and ageing. The EGCL (**d–f**) shows a significant correlation of 5hmC amount and the amount of Dnmt3a (**d**), but no significant correlation of 5hmC amount and

Tdg (**e**) and Ki67-positive cells (**f**) shows a significant decrease from day 0 to 7. In the IGCL (**g–i**) there no significant correlation of 5hmC amount and Dnmt3a (**g**) but there are significant correlations of 5hmC amount and Tdg (**h**) and Ki67 (**i**). In the MCL (**j–l**) there is no significant correlation of 5hmC amount and Dnmt3a (**j**), but there are significant correlations of 5hmC amount and Tdg (**k**) and Ki67 (**l**). *EGCL* external granular cell layer, *MCL* molecular cell layer, *IGCL* internal granular cell layer

Whereas DNMT1 is responsible for the maintenance of DNA methylation, DNMT3a and DNMT3b are de novo methyltransferases. The methylcytosine dioxygenases TET1-3 (ten-eleven-translocation) convert 5mC to 5hmC. Potential active demethylation pathways include Apobec1-3 (apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like), Mbd4 (methyl-CpG binding domain protein 4), Smug1 (single-strand-selective monofunctional uracil-DNA glycosylase 1) and Tdg (thymine-DNA glycosylase) that are able to subsequently demethylate 5hmC by deamination of modified cytosine bases resulting in unmodified cytosine or by oxidation of 5hmC to 5fC and 5caC and a final decarboxylation reaction to unmodified cytosine. Almost all nuclei stained positive for 5mC regardless of the brain region and the age but whilst only some nuclei were completely 5mC positively stained most nuclei showed only small 5mC-positive dots (Figs. 1, 2). Interestingly, the hydroxymethylome is strongly depending on the investigated brain region and on age. As previously published (Kraus et al. 2014), there are only few 5hmC-positive cells in the cerebral cortex in very early developmental stages (day of birth) in frontal and cerebellar cortex (Figs. 1, 2). In adulthood, 5hmC amounts reach their plateau and show maximum amounts depending on the investigated brain region (Kraus et al. 2014). However, sex may also influence the global epigenome as a confounding factor. Zhang et al. showed that there are significant differences in global genomic DNA methylation in peripheral blood (Zhang et al. 2011). As mice used in this study were sacrificed at five different time points (day 0, 7, 15, 30, and 120 after birth), we do not have enough mice per time point to perform adequate analysis on the effect of sex on the brain epigenome. Therefore, a critical evaluation of possible sex-dependant confounding effects on the brain epigenome was performed using data of a previously published study investigating 22 different brain samples (Kraus et al. 2012). Thereby, we did not find any sex-dependent differences of the brain epigenome (Supplementary Fig. 1). However, further studies with more investigated subjects per age will be necessary to clarify if there are sex-dependent effects on the global brain epigenome and on expression profiling of methylation/demethylation enzymes. A further limitation of this study is a potential inter-mouse difference in gene expression levels, i.e. the question arises if the gene expression pattern at day 30 of a mouse actually sacrificed at day 120 was similar to the gene expression pattern of another mouse that was actually sacrificed at day 30. As all investigated mice were siblings and born at the same time and as they all were housed together, variations of the environment affecting epigenomic changes were reduced to a minimum. However, inter-mouse variations due to inter-individual modulations of the epigenome cannot be generally excluded. Thus, additional studies will be necessary to confirm the effects detected in this study in a larger cohort.

Till date, it was not clarified if the increase of 5hmC during ageing is accompanied only by 5hmC production that reaches a plateau or if there is an additional demethylation leading to rapid methylation/demethylation processes during development. The latter one would correlate with highly plastic methylation and hydroxymethylation patterns representing a rapid switch of methylation/demethylation on numerous distinct genomic loci.

The data presented in this study emphasize that there is not only methylation and hydroxymethylation during brain development and ageing, but also a rapid demethylation depending on age and brain region. Then, at adulthood, the intensity of methylation/demethylation decreases leading to a (more or less) stable state with only minor methylation/demethylation activity left. Whilst methylating (Dnmt) and hydroxymethylating (Tet) as well as demethylating (Apobec, Mbd4, Smug1, Tdg) enzymes are highly expressed in early developmental stages, they are significantly down-regulated at adulthood. To validate our expression data, we also quantified protein levels of selected methylating/demethylating proteins (Dnmt3a, Tdg) and found that expression levels correlate with protein levels. In addition, we found that, e.g. in the cerebellum, protein levels are even depending on cortical layer (EGCL, MCL, IGCL), i.e. each layer shows distinct protein levels of Dnmt3a and Tdg during development. Interestingly, high levels of methylating/demethylating proteins are also associated with high amount of the Ki67 protein, a proliferation-associated marker. Decrease of proliferation activity is coincident with a reduction of methylating/demethylating protein levels.

To further characterize methylation/demethylation it would be necessary to absolutely quantify amounts of the oxidative products of 5hmC, i.e. 5fC and 5caC, as well as 5hmU during brain development, e.g. using mass spectrometry. As high methylation/demethylation activities may be accompanied by increased amounts of 5fC and 5caC, we expect high amounts of 5fC and 5caC in early development and reduced amounts in aged brains showing (more or less) stable (hydroxy)methylomes. Inoue et al. analysed the amount of 5fC and 5caC in mouse preimplantation development (Inoue et al. 2011). During preimplantation and insemination an epigenomic reprogramming occurs that is associated with strongly increasing amounts of 5fC and 5caC during preimplantation and a decay of 5fC and 5caC during insemination (Inoue et al. 2011). Interestingly, these methylation/demethylation processes depend on the origin of DNA. The paternally and maternally originating DNAs undergo different epigenomic processes (Inoue et al. 2011). Although the epigenomic plasticity is not as high during early brain development as

during preimplantation and insemination, the high expression levels of methylation/demethylation proteins is still impressive. Thus, the amount of 5fC and 5caC should be determined in future studies using high sensitive, quantitative approaches, such as mass spectrometry (Kraus et al. 2012; Pfaffeneder et al. 2011) to get further insights into the epigenomic regulation of brain development and ageing.

In summary, the data presented in this study demonstrate that there is an age- and brain region-dependent regulation of methylation and demethylation pathways showing high methylation/demethylation activity in early developmental stages that is followed by decreased activities in mature brains. This underlines that there is not only a generation of 5mC and 5hmC in early development, but a highly active de- and remethylation throughout the genome representing high regulatory plasticity. This emphasizes that 5hmC is not only an intermediate in demethylation, but also of strong regulatory importance during brain development and ageing. Our data will boost further research on causal epigenomic changes underlying brain development and ageing.

Compliance with ethical standards

Conflict of interest There is no conflict of interest.

Ethical standard All procedures performed in this study were in accordance with the ethical standards of the institute at which the experiments were conducted.

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