The chromodomain helicase Chd4 is required for Polycomb-mediated inhibition of astroglial differentiation

Since Advance Online Publication, Supplementary Table 1 and new Source Data files for Figures 2 and 4 have been added.

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Polycomb group (PcG) proteins form transcriptional repressor complexes with well-established functions during cell-fate determination. Yet, the mechanisms underlying their regulation remain poorly understood. Here, we extend the role of Polycomb complexes in the temporal control of neural progenitor cell (NPC) commitment by demonstrating that the PcG protein Ezh2 is necessary to prevent the premature onset of gliogenesis. In addition, we identify the chromodomain helicase DNA-binding protein 4 (Chd4) as a critical interaction partner of Ezh2 required specifically for PcG-mediated suppression of the key astrogenic marker gene *GFAP*. Accordingly, *in vivo* depletion of Chd4 in the developing neocortex promotes astrogenesis. Collectively, these results demonstrate that PcG proteins operate in a highly dynamic, developmental stage-dependent fashion during neural differentiation and suggest that target gene-specific mechanisms regulate Polycomb function during sequential cell-fate decisions.

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Introduction

During the development of an organism, pluripotent stem cells generate all the distinct cell types that form the various

organs and tissues of the body. While undergoing the required fate transitions, stem cells need to adapt alternative gene expression programs that specify the new identity of the differentiated cell. Epigenetic mechanisms such as DNA methylation as well as chromatin modifications play an essential role during this process. Therefore, proteins that control chromatin organization have emerged as critical coordinators of cellular reprogramming events.

Polycomb group (PcG) proteins constitute such a class of epigenetic regulators that establish and maintain cellular identity through heritable gene silencing. They exist in at least two biochemically distinct multiprotein complexes, termed Polycomb-repressive complexes (PRCs) (Levine *et al*, 2004; Lund and van Lohuizen, 2004). Polycomb-repressive complex 2 (PRC2), comprising the core components Ezh2, Suz12 and Eed, is thought to initiate transcriptional repression through the trimethylation of lysine 27 of histone H3 (H3K27^{me3}) (Cao *et al*, 2002). This mark provides a platform for the recruitment of the more diverse PRC1 complex, which is implicated in the maintenance of gene silencing (Fischle *et al*, 2003; Min *et al*, 2003; Wang *et al*, 2004b). Although the precise molecular mechanisms of Polycomb-mediated repression are still poorly understood, they are proposed to entail direct inhibition of the transcriptional machinery, PRC1-mediated ubiquitylation of lysine 119 of histone H2A as well as chromatin compaction (Dellino *et al*, 2004; Francis *et al*, 2004; Wang *et al*, 2004a; Cao *et al*, 2005; Eskeland *et al*, 2010). The importance of PcG proteins during embryogenesis is evidenced by the fact that targeted disruption of either the PRC2 members Ezh2 and Eed or the PRC1 component Rnf2 results in early embryonic lethality (Shumacher *et al*, 1996; O'Carroll *et al*, 2001; Voncken *et al*, 2003). Studies in mouse and human embryonic stem cells (ESCs) have demonstrated that PcGs directly repress a large group of transcription factors that control specific developmental lineages (Boyer *et al*, 2006; Lee *et al*, 2006). Importantly, genes silenced by PcG proteins in ESCs maintain the potential to become activated at the time of differentiation (Bracken *et al*, 2006). Furthermore, during cell-fate transitions, PcG proteins are re-recruited to distinct progenitor-specific targets that in turn tend to be regulators of subsequent differentiation steps (Mohn *et al*, 2008). Thus, PcG proteins can be viewed as a reversible repression system for genes poised for activation upon lineage commitment, ensuring that lasting fate decisions are postponed until a strong and specific stimulus is received (Pietersen and van Lohuizen, 2008). The context-dependent and transient repression exerted by PcG proteins implies a dynamic control of PRC function during differentiation, possibly through the cooperation with auxiliary proteins, such as sequence-specific transcription

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factors or enzymes modifying PRC core components. However, how Polycomb complexes become recruited to a changing set of target genes and how their activity is regulated remain open questions.

The developing central nervous system constitutes a particularly well-suited model to investigate the temporal regulation of cell-fate commitment. Here, multipotent progenitor cells produce different types of derivatives consecutively, that is, neurons are generated first, followed by the supporting glia (Temple, 2001). The mechanisms driving this sequential differentiation process involve both environmental signals and cell-intrinsic programs. Cytokine-induced activation of the Janus kinase (JAK)–signal transducer and activator of transcription (STAT) pathway, as well as changes in the DNA methylation of astrocyte-specific gene promoters, were shown to play a pivotal role in the promotion of the neurogenic-to-astrogenic switch (Takizawa *et al*, 2001; Fan *et al*, 2005; He *et al*, 2005). In addition, PcG proteins restrict the neurogenic competence of neural progenitor cells (NPCs) via repression of pro-neuronal genes (Hirabayashi *et al*, 2009), thereby triggering the onset of gliogenesis.

Here, we demonstrate that Ezh2, the enzymatic subunit of PRC2, has a disparate role during the early phase of neocortical development when it actually inhibits gliogenesis. Our data thus reveal an unappreciated switch in PRC2 function that ensures proper coordination of the neurogenic as well as the glial phase of NPC commitment. This emphasizes that PcG proteins act in a developmental stage-specific fashion to control diverse, even opposing, lineage choices. In addition, we identify the chromodomain helicase DNA-binding protein 4 (Chd4) as a sub-stoichiometric interaction partner of PRC2 complexes in NPCs. Chd4 is essential for the localization of Ezh2 at the promoter of the key astrogenic marker gene glial fibrillary acidic protein (*GFAP*) and is required for its transcriptional suppression. Consequently, depletion of Chd4 in the developing neocortex using *in utero* electroporation of short hairpins promotes gliogenesis. In contrast, Chd4 does not affect Polycomb-mediated repression of neuronal differentiation. These observations suggest that the temporary association of PRCs with distinct cofactors might play a role in the sequential regulation of Polycomb target genes.

Results

The chromodomain helicase Chd4 associates with PRC2 in NPCs

Although the core components of PRC2 are well established, knowledge of auxiliary cell type-specific cofactors that control Polycomb function during cell-fate transitions is lacking. Here, we take advantage of *in vitro* derived NPCs in order to identify novel regulators of Polycomb-mediated gene silencing that function during neural differentiation. These cells can be propagated as homogeneous cultures without spontaneous differentiation and uniformly express morphological and cell biological features of radial glia, developmental precursors of neurons and glia (Conti *et al*, 2005). We analysed proteins associated with the catalytic component of PRC2, the histone methyltransferase Ezh2, using a single-step purification technique based on specific biotinylation of a tagged transgene followed by streptavidin-mediated purification (de Boer *et al*, 2003). NPCs expressing the *Escherichia coli* biotin-ligase BirA from the *Rosa26* locus

were retrovirally transduced with Ezh2 complementary DNA bearing an amino-terminal peptide tag that serves as substrate for the bacterial biotin ligase. The Ezh2 transgene is expressed at ~20% of the endogenous level, thus reducing the likelihood that the stoichiometry of endogenous protein complexes is perturbed and that nonphysiological interactions are identified (Figure 1A). Furthermore, cells coexpressing BirA and transgenic Ezh2 are functionally equivalent to either wild-type NPCs or NPCs expressing BirA alone in both marker gene expression and growth characteristics (Figure 1A and B and data not shown). Biotinylated Ezh2, as well as other known PRC2 members, are efficiently recovered from NPC nuclear extracts using streptavidin-coupled beads (Figure 1C). Mass spectrometric analysis of Ezh2 complexes using a label-free quantitation algorithm implemented in the MaxQuant software (Hubner *et al*, 2010; Luber *et al*, 2010) confirms robust interactions with the two PRC2 core components Suz12 and Eed, as well as with other known interaction partners. All other identified proteins interact in sub-stoichiometric ratios, indicating that they only associate with a subset of PRC2 complexes (Figure 1D and Supplementary Table S1).

We focussed our subsequent analysis on the interaction between Ezh2 and Chd4. Chd4 has previously been implicated in Polycomb-mediated gene silencing using genetic approaches in invertebrates (Kehle *et al*, 1998; Unhavaithaya *et al*, 2002), as well as through biochemical experiments in mammalian cell lines (Morey *et al*, 2008; Stielow *et al*, 2008; Reynolds *et al*, 2011). However, a collaboration between both chromatin modifiers during neurogenesis has not been explored thus far. To rule out the possibility that interaction between both proteins is mediated by interceding DNA, we treated NPC nuclear extracts with the endonuclease benzonase before Ezh2 purification. Despite effective digestion of nucleic acids, Chd4 associates with Ezh2 (Supplementary Figure S1). Co-immunoprecipitation experiments of endogenous Chd4 with Ezh2 and vice versa on nuclear extracts of wild-type NPCs confirm the mass spectrometry data (Figure 1E). The low efficiency of Chd4 co-precipitation compared with PRC2 core components reiterates that this protein does not stably associate with all cellular Ezh2 complexes. Nevertheless, the interaction between both proteins takes place in the context of PRC2, because we detect Suz12 in the α -Chd4 immunoprecipitation (Figure 1E).

Chd4 is an established component of the nucleosome remodelling and histone deacetylation complex (NuRD), which is linked to transcriptional repression (Tong *et al*, 1998; Xue *et al*, 1998; Zhang *et al*, 1998). Therefore, we sought to determine if additional NuRD complex members interact with Ezh2. Whereas common interaction partners of NuRD and PRC2, like RbAp46/48 and HDAC2, associate with both Chd4 and Ezh2, MBD3 and MTA-2, proteins unique to the NuRD complex, are found only in α -Chd4 precipitates (Figure 1E). These results suggest that the interaction between Chd4 and PRC2 does not encompass all NuRD components.

Chd4 is essential for Ezh2-mediated inhibition of astroglial differentiation

To establish the significance of the Chd4–PRC2 interaction during NPC commitment, we first analysed the effect of exogenous Ezh2 on neurogenic and gliogenic fate decisions

Figure 1 Purification of Ezh2 complexes in *in vitro* derived neural progenitor cells (NPCs) identifies Chd4 as a sub-stoichiometric PRC2 interaction partner. (A–C) *In vitro* derived NPCs constitutively expressing the biotin ligase BirA are retrovirally transduced with Ezh2 complementary DNA fused to a target sequence for BirA-mediated biotinylation (bioEzh2). (A) Total cell extracts are used to detect both exogenous and endogenous Ezh2, as well as the neural stem cell marker Nestin by western blot analysis. Tubulin serves as loading control. (B) Cells are immunocytochemically stained with either a Nestin or Sox2 antibody as indicated (green) and counterstained with DAPI to visualize the nuclei. Scale bar = 20 μ m. (C) Immunoblot analysis for biotinylated Ezh2 (left). Streptavidin-coupled beads are used to precipitate bioEzh2 (right). Endogenously biotinylated proteins are indicated by asterisk (*). (D) Mass spectrometric analysis and label-free quantification of a bioEzh2 purification from NPC nuclear extracts. Plotted in the figure is the ratio of all proteins identified by more than one unique peptide in the specific versus BirA control sample determined by label-free quantification (X axis) against the total intensity of each protein measured in the mass spectrometer (Y axis). See also Supplementary Table 1. (E) NPC nuclear extracts are used to immunoprecipitate endogenous Chd4 or Ezh2. An IgG isotype-matched antibody serves as control. The precipitates and 2.5% of the input material are immunoblotted with the indicated antibodies. See also Supplementary Figure S1. Source data for this figure is available on the online supplementary information page.

in these cells. Therefore, we cultured *in vitro* derived NPCs expressing BirA alone or in combination with Ezh2 under either neuronal or glial differentiation conditions as specified in the Materials and methods section. Transgenic Ezh2 has no effect on marker gene expression in the stem cell state, presumably due to near-physiological expression levels (Figure 1A and B). However, during the differentiation process, endogenous Ezh2 is downregulated (Figure 2A). Although endogenous Ezh2 decreases more substantially in the transgenic line, total Ezh2 protein levels remain

slightly higher (Figure 2A). Despite the apparently marginal effect, exogenous Ezh2 nevertheless inhibits neuronal differentiation, as indicated by a reduction in the number of cells positive for the neuronal marker β -III Tubulin (Tuj1) (Figure 2B and C). This observation is in agreement with a recent study that demonstrates that Polycomb proteins limit the neurogenic competence of NPCs (Hirabayashi *et al*, 2009). Transgenic Ezh2 also causes a decrease in GFAP-expressing cells, revealing an additional role for Polycomb proteins during astrogenic cell-fate determination (Figure 2B and C).

Figure 2 Chd4 is required for Ezh2-mediated inhibition of astroglial, but not neuronal differentiation. (A) Western blot analysis of whole-cell extracts of *in vitro* derived NPCs expressing BirA alone or in combination with the *bio*Ezh2 transgene grown in stem cell (non-diff) or astroglial differentiation (astro-diff) conditions as specified in the Materials and methods section. Immunoreactive bands are quantified using the ImageJ software. The intensity of the band detected in the nondifferentiated BirA control sample is used as reference. (B) *In vitro* derived NPCs, cultured under either neuronal or astroglial differentiation conditions as indicated, are immunocytochemically stained with antibodies against either the neuronal-specific protein β -III Tubulin (TuJ1, red) or the astrocyte marker glial fibrillary acidic protein (GFAP, green) and counterstained with DAPI to visualize the nuclei. Representative examples of three independent experiments are depicted. Scale bar = 20 μ m. (C) Results are quantified as the percentage of cells positive for either TuJ1 or GFAP in 15 high-magnification fields per slide. Values represent the mean $\pm$ s.d. of three independent experiments (* P < 0.01). (D–F) *In vitro* derived NPCs harbouring BirA alone or in combination with the *bio*Ezh2 transgene are infected with a lentivirus expressing a short hairpin targeting Chd4 (shChd4-I) under a doxycycline-responsive promoter. Cells are treated with vehicle control or 100 ng/ml doxycycline 48 h before induction of either astroglial or neuronal differentiation. (D) Formaldehyde-fixed cells were immunocytochemically stained with antibodies against GFAP, S100 β or TuJ1 as indicated and counterstained with DAPI to visualize the nuclei. Representative examples of three independent experiments are depicted. Scale bar = 20 μ m. (E) Results are quantified as described in (C). (F) Western blots of total cell lysates are probed with the indicated antibodies. See also Supplementary Figure S2. Source data for this figure is available on the online supplementary information page.

Next, we sought to determine whether these effects of Ezh2 are dependent on Chd4. To this end, we infected NPCs harbouring the BirA transgene alone or in combination with

Ezh2 with lentiviruses engineered to express a short hairpin targeting Chd4 under a doxycycline-responsive promoter (Herold *et al*, 2008). In order to rule out nonspecific

Figure 3 Reduction of Ezh2 levels in acutely isolated NPCs has opposing effects on NPC differentiation depending on developmental stage. NPCs isolated from the developing neocortex of either E16.5 (A–C) or E11.5 (D–F) ArtEzh2tetKD mouse embryos or wild-type littermates are cultured for 3 days in suspension in the absence or presence of 100 ng/ml doxycycline as indicated. Subsequently, floating spheres are dissociated, seeded onto poly-ornithine/laminin-coated dishes and kept under differentiation conditions following the neuronal differentiation protocol. (A, D) Formaldehyde-fixed cells are immunocytochemically stained with antibodies against Tuj1 (red) and GFAP (green). Representative examples of three independent NPC isolations are shown in the photomicrographs. Scale bar = 20 μ m. (B, E) Results are quantified as the percentage of cells positive for either Tuj1 or GFAP in 5 high-magnification fields per slide. Each data point is derived from five different embryos and corresponds to the mean $\pm$ s.d. (* P < 0.01). (C, F) Whole-cell extracts of NPCs isolated from two ArtEzh2tetKD embryos and a wild-type littermate are immunoblotted with the indicated antibodies. See also Supplementary Figures S3 and S4. Source data for this figure is available on the online supplementary information page.

off-target effects, we employed two alternative hairpins directed against Chd4, as well as a nontargeting random control. Knockdown of Chd4 48 h before induction of differentiation alleviates the suppression of GFAP induction caused by exogenous Ezh2, whereas expression of the control hairpin has no consequences (Figure 2D–F and Supplementary Figure S2). We observe analogous effects on an additional astrocytic marker gene, *S100 β* (Figure 2D–F and Supplementary Figure S2). Strikingly, reduction of Chd4 levels has no influence on the ability of Ezh2 to inhibit neuronal differentiation as indicated by persistent repression of Tuj1 (Figure 2D–F). Together, these findings imply that Chd4 is specifically required for Ezh2-mediated inhibition of astroglial differentiation.

Ezh2 acts in a developmental stage-specific fashion to regulate NPC fate

During embryonic brain formation, multipotent NPCs first pass through a phase of neurogenesis, which in rodents lasts approximately from embryonic day 12 (E12) to E18. At the end of the neurogenic period, at $\sim$ E18.5, glial cells start to appear, with their numbers peaking in the neonatal period (Qian *et al*, 2000; Kriegstein and Alvarez-Buylla, 2009). To investigate the role of Ezh2 during these consecutive

stages of neurogenesis, we acutely isolated neuroepithelial cells from the neocortex of either E11.5 or E16.5 mouse embryos using a strain that carries a doxycycline-inducible short hairpin targeting Ezh2 integrated in the *Rosa26* locus (ArtEzh2tetKD). These cells are cultured for 3 days under nonadherent conditions in the absence or presence of 100 ng/ml doxycycline. Subsequently, floating spheres are dissociated, seeded onto poly-O/laminin-coated plates and either maintained in stem cell media or induced to differentiate following the neuronal differentiation protocol specified in the Materials and methods section. Knockdown of Ezh2 in primary NPCs grown under stem cell conditions has little effect. We do observe a minor impact on cell proliferation and apoptosis, which correlate with a slight derepression of the *Ink4a/Arf* tumor-suppressor locus, a *bona fide* PcG target (Supplementary Figure S3A–F). However, reduction in Ezh2 levels does not affect uniform expression of the neural stem cell marker nestin, indicating that it does not cause precocious differentiation *per se* (Supplementary Figure S3G). This is most likely due to the absence of the required activating stimuli in the defined culture medium.

It has previously been demonstrated that Polycomb proteins promote astrogenic fate transitions during the neurogenic-to-gliogenic switch (Hirabayashi *et al*, 2009).

Therefore, we first focussed on this developmental period by analysing the differentiation potential of neocortical progenitors isolated on E16.5. In accordance with the aforementioned report, reduction of Ezh2 levels in primary NPCs that have gained gliogenic competence inhibits astrocyte differentiation (Figure 3A–C). This might be due to an inability of these cells to repress the pro-neuronal gene *neurogenin 1* (*Ngn1*) in the absence of Ezh2 as previously established (Supplementary Figure S4; Hirabayashi *et al*, 2009).

Next, in order to address the consequences of reduced Ezh2 levels in the earlier neurogenic period, we prepared neocortical NPCs from E11.5 mouse embryos. In contrast to our observations with gliogenic NPCs, expression of the short hairpin targeting Ezh2 at a time when NPCs isolated from wild-type littermates or noninduced ArtEzh2tetKD cells are not yet capable of astroglial differentiation leads to a marked increase in GFAP-positive cells (Figure 3D–F). Consistent with our results, deletion of Ezh2 before onset of neurogenesis has been shown to cause precocious astrocyte differentiation *in vivo* (Pereira *et al*, 2010).

Collectively, these results demonstrate that PcG proteins function in a highly dynamic manner throughout neocortical development. During the neurogenic phase, Ezh2 inhibits precocious gliogenesis, whereas at the time of the neurogenic-to-gliogenic switch, it restricts neuronal differentiation, thereby triggering the onset of astrogenesis.

Chd4 inhibits gliogenesis without affecting neuronal differentiation *in vivo*

Our data generated in *in vitro* derived NPCs indicate that Chd4 is specifically required for Ezh2-mediated inhibition of astrogenesis. To evaluate the physiological relevance of this observation, we first analysed Chd4 expression patterns in mouse embryonic forebrain. Shortly after the start of neurogenesis at E12.5, Chd4 protein can be detected broadly throughout the developing neocortex, including the cortical ventricular zone (VZ), where it is expressed in Pax6-positive NPCs (Figure 4A). Notably, Chd4 levels in these cells gradually decline as demonstrated by western blot analysis of cell lysates from NPCs isolated at different embryonic stages (Figure 4B). Nevertheless, Chd4 retains a ubiquitous expression pattern also at later developmental time points (Supplementary Figure S5A).

To investigate whether Chd4 influences the differentiation potential of NPCs, we infected primary NPCs with GFP-marked lentiviruses expressing a doxycycline-inducible short hairpin targeting Chd4 or a nontargeting control sequence immediately after isolation on E11.5. Cells were grown for 3 days in suspension in the absence or presence of 100 ng/ml doxycycline, followed by monolayer culture under neuronal differentiation conditions. Chd4 knockdown triggers a gain in gliogenic competence as indicated by the premature emergence of GFAP-positive astrocytes, whereas it does not affect neuronal differentiation (Figures 4C–F). In NPCs isolated on E16.5, reduction of Chd4 levels does not influence either glial or neuronal differentiation (Supplementary Figures S5B and C and data not shown).

In order to determine whether Chd4 suppresses the glial differentiation potential of NPCs *in vivo*, we used *in utero* electroporation to introduce a GFP reporter construct in conjunction with a short hairpin targeting Chd4 or a non-

targeting control sequence into NPCs lining the lateral ventricle at E13.5. At this stage, the VZ progenitors are primarily neurogenic. When examined 5 days later, the majority of GFP-expressing cells in control embryos are indeed negative for GFAP immunohistochemistry (Figure 5A). In contrast, electroporation of the short hairpin targeting Chd4 resulted in a nearly two-fold increase in GFAP-positive glial cells in the VZ and SVZ (Figure 5A and B). We do not detect astrocytes in the cortical plate, possibly due to the fact that the cells have not yet migrated into the upper layers at the time of analysis. Alternatively, reduction of Chd4 levels *in vivo* might be insufficient to enable complete maturation of astrocytes. Nevertheless, the moderate but significant increase in GFAP-positive cells suggests that Chd4 depletion from neocortical progenitors promotes astroglial differentiation. In contrast, Chd4 knockdown does not appear to affect neurogenesis. NPCs depleted for Chd4 are capable of generating neurons that migrate properly to the appropriate location within the cortical plate, where they express the superficial layer marker *Satb2* and the deep layer marker *Tbr1*, respectively (Figure 5C and D). Together, these data indicate that Chd4 specifically affects gliogenesis in the developing mouse brain.

Ezh2 directly represses the GFAP promoter

The transcriptional control of *GFAP*, a key astrocyte differentiation marker, is essential in the timely induction of gliogenesis (Miller and Gauthier, 2007). Specific CpG methylation of a STAT3 binding element within the *GFAP* promoter determines the responsiveness of *GFAP* transcription to gp130 cytokines, thereby regulating the cell-fate switch of neural precursors (Takizawa *et al*, 2001; Fan *et al*, 2005). Interestingly, the *GFAP* promoter region was also found to be H3K27 trimethylated in NPCs in a study that analysed genome-wide epigenetic modification by Polycomb proteins during lineage commitment of ESCs (Mohn *et al*, 2008). We therefore investigated if the inhibitory effect of exogenous Ezh2 on astroglial differentiation might be due to prolonged H3K27 trimethylation of the *GFAP* promoter. Chromatin immunoprecipitation (ChIP) experiments in undifferentiated *in vitro* derived NPCs confirm H3K27 trimethylation at the promoter and gene body of *GFAP* compared with an unrelated intergenic control region (Figure 6A and B). Expression of exogenous Ezh2 in the progenitor cell state does not lead to an increase in this modification, consistent with an unaltered physiological status of these cells (Figure 6C). Upon differentiation towards the astrocytic fate, the H3K27 trimethylation mark is lost concurrent with the substantial transcriptional induction of *GFAP* (Figure 6C and E). Interestingly, NPCs expressing transgenic Ezh2 maintain H3K27 trimethylation at the *GFAP* promoter in conjunction with sustained repression of this gene (Figure 6C and E). Differentiation also induces a slight decrease in total histone H3 levels, but this reduction is not sufficient to account for the observed effects on H3K27 trimethylation (Figure 6D).

Chd4 is specifically required for Ezh2-mediated GFAP silencing

To examine if Ezh2 and Chd4 cooperate at the chromatin level, we investigated Chd4 occupancy at the *GFAP* locus in undifferentiated *in vitro* derived NPCs. Consistent with our functional data, we detect Chd4 predominantly upstream of

Figure 4 Chd4 prevents premature astrocyte differentiation in neurogenic NPCs. (A) Coronal forebrain sections of E12.5 embryos are immunohistochemically stained with an antibody against Chd4 (red) and against the neural stem cell marker Pax6 (green). Nuclei are visualized with DAPI. The boxed region corresponds to the higher-magnification image (i). LV, lateral ventricle. Scale bar = 10 μ m. (B) Total cell lysates of NPCs isolated at different developmental time points are immunoblotted with the indicated antibodies. (C–F) NPCs isolated from the developing neocortex of E11.5 embryos are infected with a GFP-marked lentivirus expressing either a nontargeting random short hairpin (shCtrl) or a sequence targeting Chd4 (shChd4-I) under a doxycycline-responsive promoter. Cells are cultured for 3 days in suspension in the absence or presence of 100 ng/ml doxycycline as indicated and subsequently differentiated following the neuronal differentiation protocol. (C, E) Formaldehyde-fixed cells were immunocytochemically stained with antibodies against GFP (green) and GFAP (C) or Tuj1 (E) (red). Representative examples of three independent experiments are depicted in the photomicrographs. Scale bar = 20 μ m. (D) Results are quantified as the percentage of marker-positive cells among the cells expressing GFP in 15 high-magnification fields per slide. Each data point represents the mean $\pm$ s.d. of three independent experiments (* P < 0.01). See also Supplementary Figure S5. Source data for this figure is available on the online supplementary information page.

the transcriptional start site and at the 3' end of the gene (Figure 6F). In contrast, it is not present at the pro-neuronal gene *Ngn1*, although the locus is significantly enriched in H3K27 trimethylation as described earlier (Hirabayashi *et al*, 2009) (Supplementary Figure S6).

Therefore, we reasoned that Chd4 binding to the *GFAP* locus might be specifically involved in the repression of this gene. To test this hypothesis, we utilized the previously established *in vitro* derived NPC lines expressing doxycycline-inducible short hairpins targeting Chd4.

Figure 5 Chd4 inhibits gliogenesis without affecting neuronal differentiation *in vivo*. Control (shCtrl) or Chd4 shRNA (shChd4-I) constructs are electroporated into E13.5 embryonic mouse brains and mice are sacrificed at E18.5. (**A**, **C**, **D**) Images of coronal sections of E18.5 cortices electroporated with either shCtrl (left panels) or shChd4-I (right panels) immune-stained with GFAP (**A**), Tbr1 (**C**) or Satb2 (**D**) (all red), and counterstained with a DNA dye (blue). The asterisks in the boxed regions (i and ii) indicate GFP and GFAP double-positive cells (**A**), GFP and Tbr1 double-positive cells (**C**) and GFP and Satb2 double-positive cells (**D**). VZ/SVZ, ventricular zone/subventricular zone. Scale bar = 10 μ m. (**B**) Quantification of the percentage of GFP-positive cells that express GFAP ($n = 3$, $P < 0.001$, $\pm$ s.e.m.).

Figure 6 Exogenous Ezh2 causes sustained H3K27 trimethylation of the *GFAP* promoter. (A) Schematic representation of the *GFAP* genomic locus. Positions of primer pairs are indicated by letters. IGR, intergenic region. (B) ChIP analysis using an antibody specific for trimethylated H3K27 in *in vitro* derived undifferentiated NPCs. The precipitated DNA is amplified by real-time qPCR using primers indicated in (A). An IgG isotype-matched antibody serves as control in all ChIP experiments. (C, D) ChIP analysis using an antibody specific for trimethylated H3K27 (C) or Histone H3 (D) in *in vitro* derived NPCs expressing BirA alone or in combination with *bio*Ezh2 grown in stem cell (non-diff) or astroglial differentiation (astro-diff) conditions as specified in the Material and methods section. For qPCR analysis, primer pair 'D' recognizing a region from -237 bp to -300 bp upstream the transcriptional start site is used. (E) qPCR analysis of *GFAP* mRNA levels in *in vitro* derived NPCs expressing BirA alone or in combination with *bio*Ezh2 grown in stem cell (non-diff) or astroglial differentiation (astro-diff) conditions. Data are normalized to *GAPDH* expression. Values represent the mean $\pm$ s.d. of three independent experiments performed in duplicate. (F) ChIP analysis performed as described above using an antibody specific for Chd4. For all ChIP experiments, enrichments are presented as percentages of total input and reported as the mean $\pm$ s.d. of a representative example of three independent experiments performed in duplicate. See also Supplementary Figure S6.

Reduction of Chd4 protein levels in undifferentiated NPCs causes a loss of H3K27 trimethylation at the *GFAP* locus in both BirA control cells and cells expressing exogenous Ezh2, indicating that Chd4 is required for the deposition of this mark (Figure 7A and C). Moreover, ChIP analysis using streptavidin-coupled beads demonstrates that the presence of biotinylated Ezh2 at the *GFAP* promoter is dependent on Chd4, whereas there are no effects on Ezh2 protein levels (Figures 7B and C). Notably, Chd4 ablation leads to precocious expression of GFAP, although at significantly lower levels than after the onset of differentiation (Figure 7D). This could be due to the presence of additional repressive marks at the *GFAP* promoter, as well as to the lack of activating stimuli owing to the chemically defined culture

conditions. Together, these observations demonstrate that Chd4 is required for Ezh2-mediated repression of the *GFAP* promoter in *in vitro* derived NPCs.

To investigate to what extent other Polycomb-responsive loci are altered in their transcriptional status after Chd4 knock-down, we analysed expression of an arbitrary set of five genes, which have previously been found to be H3K27 trimethylated in NPCs (Mohn *et al*, 2008) and are induced during astrocyte differentiation (Meissner *et al*, 2008). These genes show an intriguing trend of derepression after a reduction in Chd4 levels (Figure 7E). Although the effects are mild, they correlate well with knockdown efficiency (Figure 7C). However, four H3K27-trimethylated *Hox* genes, *bona fide* Polycomb targets, are not transcriptionally responsive to Chd4 depletion

Figure 7 Chd4 depletion leads to a loss of H3K27 trimethylation and Ezh2 binding at the GFAP promoter. NPCs harbouring BirA alone or in combination with *bio*Ezh2 are infected with lentiviruses expressing a nontargeting random short hairpin (shCtrl) or two alternative short hairpin sequences targeting Chd4 (shChd4-I and shChd4-II) under a doxycycline-responsive promoter. Cells are cultured in the absence (–) or presence (+) of 100 ng/ml doxycycline for 48 h in stem cell conditions. (A, B) ChIP analysis of the GFAP promoter using an antibody specific for trimethylated H3K27 (A) or streptavidin-coupled beads (B) as described in Figure 6. For qPCR analysis, primer pair ‘D’ recognizing a region from –237 bp to –300 bp upstream the transcriptional start site is used in (A). For (B) the precipitated DNA is amplified using primers indicated in Figure 6A. Enrichments are presented as percentages of total input and reported as the mean $\pm$ s.d. of a representative example of three independent experiments performed in triplicate. (C) NPC whole-cell extracts are used for western blot analysis to detect Chd4 and Ezh2 protein levels. (D–F) qPCR analysis on RNA isolated from undifferentiated NPCs using primers to GFAP (D) or the indicated genes (E, F). Data are normalized to GAPDH expression. Values represent the mean $\pm$ s.d. of a representative example of three independent experiments performed in triplicate. Differentiated NPCs are used as positive control. See also Supplementary Figure S7. Source data for this figure is available on the online supplementary information page.

(Figure 7F). Thus, Chd4 might be involved in Polycomb-mediated repression of specific sets of genes, whereas other Polycomb targets are silenced independent of Chd4.

In principle, this effect of Chd4 could be dependent on its well-established association with the NuRD complex. However, stable knockdown of MBD3, an integral component

of NuRD necessary for complex stability (Hendrich *et al*, 2001), does not affect transcription of GFAP in *in vitro* derived undifferentiated NPCs, nor does it induce a derepression of other Chd4-dependent Polycomb targets (Supplementary Figures S7A–C). The same holds true for a reduction in the levels of HDAC-1, another NuRD-associated protein

(Supplementary Figures S7A–C). In addition, MBD3 depletion does not influence the levels of H3K27 trimethylation at the GFAP locus (Supplementary Figure S7D). Therefore, it seems likely that the effects of Chd4 on Polycomb-mediated gene silencing during NPC differentiation are distinct from its functions as part of the NuRD complex as suggested by our biochemical data.

Discussion

In the present study, we have conducted a comprehensive mass spectrometric analysis of PRC2 in NPCs. Our biochemical investigation of PRC composition was facilitated by the employment of a robust *in vitro* neurogenesis model (Conti *et al*, 2005). Using this differentiation protocol, we were able to derive a highly uniform, stable population of nestin-positive radial glia-like NPCs from ESC cultures. Although these cells lack temporal restriction of lineage commitment, they are capable of efficient differentiation into neurons and astrocytes even after prolonged expansion. This enabled the purification of progenitor cell-specific PRC interaction partners and the subsequent analysis of their functional relevance during differentiation processes.

Apart from the PRC2 core complex, we identified predominantly proteins that interacted with Ezh2, the catalytic subunit of PRC2, in sub-stoichiometric ratios. This might explain why they have escaped prior detection by more vigorous purification techniques. Furthermore, it suggests the existence of diverse Polycomb subcomplexes that vary in their association with distinct auxiliary factors. Notably, a similar heterogeneity has recently been demonstrated for PRC1 complexes, which can be categorized into several, functionally distinct groups based on their subunit composition (Gao *et al*, 2012). This diversity, although still poorly understood, may prove to be of great importance for the multifaceted functions of PRCs, particularly under circumstances in which Polycomb proteins perform different roles during subsequent developmental stages.

The temporal control of NPC commitment exemplifies such an event. Whereas ablation of Polycomb proteins at the time of the neurogenic-to-astrogenic fate switch has been shown to delay the onset of gliogenesis (Hirabayashi *et al*, 2009), early cortex-specific deletion of Ezh2 causes premature astrocyte differentiation instead (Pereira *et al*, 2010). Our study now consolidates the apparently contrasting findings by demonstrating that Ezh2 holds opposing functions in different phases of NPC differentiation (Figure 8).

This versatile behaviour of PRCs implies a context-dependent and target gene-specific regulation, possibly through the temporary binding to different cofactors that aid in the repression of a restricted set of targets. Here, we identify Chd4 as one such cofactor and establish the physiological significance of its interaction with Ezh2 in the context of neural differentiation. Our observation that Chd4 is essential for Ezh2-mediated repression of premature gliogenesis, but has no effect on its ability to inhibit neuronal differentiation, suggests that it might play a role in imparting specificity to PcG repression. Indeed, we find that Chd4 is specifically required for the silencing of glial marker genes.

Chd4 is an integral component of the repressive multiprotein complex NuRD, which combines ATP-dependent chromatin remodelling with histone deacetylase activity

(Tong *et al*, 1998; Xue *et al*, 1998; Zhang *et al*, 1998). Disruption of nucleosomes catalysed by the ATPase/helicase domain of Chd4 was suggested to facilitate access of the histone tails to deacetylation, thereby promoting transcriptional silencing (Tong *et al*, 1998; Xue *et al*, 1998; Zhang *et al*, 1998). Local chromatin changes initiated by the helicase activity of Chd4 could also ease Polycomb access to the nucleosomal template, which might be particularly relevant under circumstances in which the target locus is already compacted due to the presence of repressive chromatin marks. This is the case for the *GFAP* promoter, which is not only extensively DNA methylated in neurogenic NPCs (Takizawa *et al*, 2001), but also enriched in H3K9^{me3} (Song and Ghosh, 2004).

PcG proteins have recently been postulated to temporarily restrict expression of genes poised for activation during lineage commitment (Boyer *et al*, 2006; Lee *et al*, 2006; Mohn *et al*, 2008). DNA methylation, on the other hand, is generally regarded as a more permanent mark and might be incompatible with rapid transcriptional activation. Therefore, it is intriguing to speculate that Polycomb-mediated silencing might replace more stable epigenetic mechanisms at times of cell-fate choices in order to facilitate the appropriate induction of genes specifying terminal differentiation. Our present finding that Chd4 is essential for Ezh2-mediated silencing of the *GFAP* promoter before the onset of astroglial differentiation points to a setting in which chromatin remodelling might be necessary for the deposition of an alternative epigenetic mark.

Interestingly, in the context of the NuRD complex, Chd4 is directed to methylated DNA via its association with the methyl-CpG binding protein MBD2 (Wade *et al*, 1999; Zhang *et al*, 1999; Feng and Zhang, 2001). It is therefore conceivable that DNA methylation of the *GFAP* promoter might play a role in Chd4-dependent PRC2 recruitment to this locus. However, NPCs deficient for three different methyl-CpG binding proteins, including MBD2, show no apparent propensity for premature gliogenesis (Martin Caballero *et al*, 2009). In combination with our data suggesting that the effect of Chd4 on Polycomb-mediated gene silencing during NPC differentiation is NuRD independent, MBD-2-driven targeting seems unlikely to be a contributing factor in this context.

Recently, studies in ESCs have demonstrated that NuRD complex components are enriched at a subset of Polycomb target genes (Reynolds *et al*, 2011; Yildirim *et al*, 2012). Here, NuRD-mediated deacetylation of histone H3K27 enables PRC2 recruitment and subsequent H3K27 trimethylation of NuRD target promoters (Reynolds *et al*, 2011). Our finding that the interaction between Chd4 and Ezh2 in NPCs is direct and does not seem to encompass all NuRD members points to an additional mechanism through which both chromatin regulators cooperate. It will be important to investigate if a similar association exists during the specification of other tissue types. In particular, Chd4 has been shown to be required for the maintenance and multilineage differentiation of hematopoietic stem cells as well as for the self-renewal capacity of epidermal precursors (Kashiwagi *et al*, 2007; Yoshida *et al*, 2008). Interestingly, several PcG proteins have well-established functions during these processes (van der Lugt *et al*, 1994; Ohta *et al*, 2002; Lessard and Sauvageau, 2003; Park *et al*, 2003; Kamminga

Figure 8 Developmental stage-specific effects of Polycomb proteins during neural differentiation. Within the developing rodent neocortex, multipotent neuronal progenitor cells (NPCs) produce neurons from ~E12 to E18, followed by a period in which the supporting glial cells are generated. During the neurogenic phase, Polycomb group proteins (depicted here as PRC2) prevent the premature onset of gliogenesis by directly repressing the key astrogenic marker gene *GFAP*, a function that is dependent on the presence of the chromodomain helicase Chd4. At the time of the neurogenic-to-astrogenic switch at ~E18, Polycomb proteins shift tasks and start to inhibit expression of the pro-neuronal gene *neurogenin1* (*Ngn1*), thereby restricting the neurogenic competence of NPCs and allowing gliogenesis to proceed.

et al, 2006; Ezhkova *et al*, 2009). Future studies addressing the extent to which Chd4 contributes to Polycomb-mediated gene silencing in diverse tissues will be instrumental in expanding the global implications of our analysis.

Materials and methods

NPC derivation and differentiation

NPCs are derived from embryonic stem cell cultures as described (Conti *et al*, 2005). In brief, cells are plated in a 1:1 mixture of DMEM/

F12 (Gibco) and Neurobasal (Gibco) supplemented with B27 (Gibco) and modified N2 (Ying and Smith, 2003). After 7 days, cells are replated in EuroMed (EuroClone) supplemented with modified N2 and 20 ng/ml EGF and bFGF (R&D Systems) and passaged until the establishment of stable NPC lines. For astrocyte differentiation, NPCs are cultured in DMEM/F12 supplemented with modified N2 and 5% fetal bovine serum (FBS) for 48 h. For neuronal differentiation, NPCs are plated onto poly-ornithine/laminin-coated culture dishes in EuroMed supplemented with B27 and 20 ng/ml bFGF. After 5 days, the media are replaced by a 1:1 mixture of EuroMed and Neurobasal supplemented with B27 and modified N2 for 2 days.

Primary NPC isolation

Primary NPCs are prepared from the telencephalon of ArtEzh2tetKD (Artemis Pharmaceuticals GmbH) mouse embryos at either E11.5 or E16.5. Dissected tissue is incubated with 0.5 mg/ml DNase (Sigma) in Hank's balanced salt solution (HBSS; Gibco) and afterwards triturated by mild pipetting. Dissociated cells are cultured for 3 days in neurosphere media (DMEM/F12 supplemented with modified N2, 2 mM glutamine (Gibco), 6 mg/ml sucrose, 14 mM NaHCO₃, 5 mM HEPES (Sigma) containing 20 ng/ml EGF and bFGF) under ultralow binding conditions. Subsequently, spheres are disrupted and plated on poly-ornithine/laminin-coated culture dishes and differentiated following the neuronal differentiation protocol described above. Animals are kept according to the 'Guide for the Care and Use of Laboratory Animals' and all animal studies are approved by an independent Animal Ethics Committee (DEC).

Streptavidin affinity purification and co-immunoprecipitation

Nuclear extracts from NPCs expressing BirA alone or in combination with biotin-tagged Ezh2 are sonicated, cleared by centrifugation and subsequently incubated with paramagnetic streptavidin-coupled beads (Dynabeads M-280, Dynal) in a buffer containing 50 mM HEPES, pH 7, 5 mM EDTA, 150 mM NaCl and 0.1% Nonidet P40. Ethidium bromide (50 µg/ml) is added to disrupt interactions mediated by DNA. Binding is performed at 4°C for 1 h, followed by five washes in binding buffer. Bound material is eluted by boiling in LDS sample buffer (NuPAGE, Invitrogen). For co-immunoprecipitation, NPC nuclear extracts are precleared with Protein A-sepharose beads (GE Healthcare) and lysates are incubated with an IgG control antibody (ab17890, Abcam), a mouse monoclonal antibody against Ezh2 (AE25, kindly provided by Dr Kristian Helin, University of Copenhagen) or a mouse monoclonal antibody against Chd4 (ab70469, Abcam) for 1.5 h at 4°C. Immunoprecipitates are collected with Protein A-sepharose beads and treated as described above.

Mass spectrometry of Polycomb complexes

Purified complexes are separated on 4–12% SDS-polyacrylamide gradient gels (NuPAGE, Invitrogen) and visualized using colloidal blue staining (Invitrogen). Gel lanes are divided into six parts and separately subjected to in-gel trypsin (Promega) digestion (Shevchenko *et al*, 2006). Tryptic peptides are analysed using an LTQ-Orbitrap mass spectrometer as described (Vermeulen *et al*, 2007). The raw data files are analysed using MaxQuant, version 1.0.12.33 (Cox and Mann, 2008) in combination with the Mascot search engine (Matrix Science). The data are searched against a decoy mouse IPI database version 3.37 including common contaminants. False discovery rates, both at the peptide and protein levels, are set to 1%. Minimal peptide length is set to 6 amino acids. False positive rates for peptides are calculated as described previously (Nesvizhskii *et al*, 2007). A recently developed label-free quantitation algorithm (Hubner *et al*, 2010; Lubner *et al*, 2010) is used to compare the relative abundance of proteins between the control and specific pull-down. Only proteins identified by two or more unique peptides in two independent experiments are included in the final data set.

Immunocytochemical staining

Cells are fixed with 3.7% formaldehyde in PBS, permeabilized with 0.3% Triton and stained with one of the following primary antibodies: nestin-specific mouse monoclonal (611658, BD Transduction), a Sox2-specific rabbit polyclonal (AB5603, Millipore), GFAP-specific rabbit polyclonal (Z0334, Dako), GFAP-specific mouse monoclonal (G3893, Sigma), Tuj1-specific mouse monoclonal (T8660, Sigma), S100β-specific mouse monoclonal (ab4066, Abcam) or a GFP-specific mouse monoclonal (ab6556, Abcam). The following secondary antibodies are used: Alexa Fluor 568-conjugated goat anti-mouse (Invitrogen) and Alexa Fluor 488-conjugated goat anti-rabbit (Invitrogen). Nuclei are counterstained with 200 ng/ml DAPI (Molecular Probes).

Immunohistochemistry

For immunohistochemical staining, formalin-fixed material is sectioned to 5 µm thickness. Paraffin-embedded sections are deparaffinized and rehydrated, followed by antigen retrieval in citrate buffer. Primary antibodies are added in the following dilutions: α-Ezh2

1:100 (612666, BD Biosciences), α-GFAP 1:200 (Z0334, DAKO), α-Chd4 1:200 (ab54603, Abcam), α-Pax6 1:200 (PRB-278P, Covance), α-Tbr1 1:250 (ab31940, Abcam), α-Satb2 1:300 (ab34735, Abcam) and α-GFP (ab13970, Abcam). The sections are incubated overnight at 4°C, after which the following secondary antibodies are added: Alexa Fluor 568-conjugated goat anti mouse or Alexa Fluor 488-conjugated goat anti-rabbit (Invitrogen). Nuclei are counterstained with 200 ng/ml DAPI (Molecular Probes).

In utero electroporation

The *in utero* electroporation was performed as previously described (Postiglione *et al*, 2011). Briefly, timed pregnant C57BL/6J mice are anaesthetized at E13.5, and the uterine horns are exposed. Plasmids expressing a short hairpin targeting Chd4 and a control vector are injected in conjunction with a plasmid expressing GFP into lateral ventricle at the final concentration of 1.5 and 0.15 µg/µl, respectively. Platinum electrodes (5 mm, BTX) are positioned on the either side of the embryonic head. Five 50 ms pulses of 33 mV with 950 ms interval are charged with an electroporator (BTX, ECM830). After electroporation, uterine horns are placed back into abdominal cavity and the wounds are sutured. After 5 days, electroporated brains are dissected for further analysis. All procedures are performed in accordance to approved protocols and institutional guidelines.

RNA isolation and quantitative reverse transcriptase (qRT)-PCR analysis

Total RNA is extracted using Trizol Reagent (Invitrogen). cDNA is synthesized from 1 µg of total RNA after treatment with DNase (Promega) using Superscript II RT and Oligo-dT primers (Invitrogen) and further analysed using SYBR Green (PCR Master Mix, Nalgene) on an ABI 7000 SDS (Applied Biosystems).

Chromatin immunoprecipitation

ChIP experiments are conducted as previously described (Gargiulo *et al*, 2009). In brief, cells are crosslinked with 1% formaldehyde, quenched in 125 mM glycine and subsequently sonicated in lysis buffer (0.5% SDS, 100 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 8.0) to generate chromatin fragments of ~500 bp in length. The material is cleared by centrifugation, diluted 10-fold in dilution buffer (1.5% Triton X-100, 5 mM EDTA, 100 mM NaCl, 100 mM Tris-HCl, pH 8.6) and precleared with protein A-sepharose beads (GE Healthcare). Chromatin lysates are used for immunoprecipitation reactions with either an IgG control antibody (ab17890, Abcam) or antibodies directed against H3K27^{me3} (07-449, Millipore), H3 (ab1791, Abcam), H3K9^{me3} (ab8898, Abcam) or Chd4 (PAB-10573, Orbigen). Immunoprecipitates are collected with paramagnetic Protein A-coupled beads (Dynabeads Protein A, Invitrogen). Alternatively, lysates are incubated with paramagnetic streptavidin-coupled beads (Dynabeads M-280, Invitrogen). After extensive washing, beads are resuspended in 10% chelex resin in water and protein/DNA crosslinks are reversed by heating. ChIP DNA is analysed by quantitative real-time PCR as described above. Sequences of primers used in this analysis are available under Supplementary Information.

Statistical analysis

To determine significance between two groups, comparisons are made using Student's *t*-test for unpaired data. In all analyses, *P* < 0.01 is considered statistically significant.

Additional experimental procedures as well as primer sequences and expression constructs used in this study are available in the Supplementary Information.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Author contributions: AS designed and performed experiments, interpreted the data and wrote the manuscript; YX conducted the *in utero* electroporation experiments and immunohistochemical stainings; EV carried out experiments; MV conducted all mass spectrometric analysis; CL performed neural progenitor cell isolations; GG contributed to ChIP experiments; DH contributed to qPCR analyses and tissue culture;

JAK supervised the *in utero* electroporation experiments; MM supervised the mass spectrometric analysis; MvL conceived and supervised the study. All authors critically commented on the manuscript.

Conflict of interest

AS is employed at *The EMBO Journal* as a scientific editor. AS was not involved in any way in the review process or the editorial evaluation of this manuscript and is not privy to the referee identities.

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