Chapter 4

Ring1b is required for brain development and neural stem cell proliferation

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DNA neither cares nor knows. DNA just *is*. And we dance to its music.
Richard Dawkins, 'River out of Eden'

Ring1b is required for brain development and neural stem cell proliferation

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Polycomb group proteins are epigenetic chromatin modifiers involved in gene repression. They function in two distinct multimeric protein complexes: the PRC2 complex initiates the silencing that is maintained by the PRC1 complex. Whereas loss of PRC2 genes unequivocally leads to early embryonic lethality, removal of PRC1 genes causes less severe phenotypes. The exception to this is PRC1 member *Ring1b*, which is also essential for mice to survive through embryonic development. We now demonstrate that mice deficient for *Ring1b* exclusively in the central nervous system can survive until birth. However, these animals suffer from neurological malfunctions and die prematurely within the first month of postnatal life. They exhibit defects in neural stem cell proliferation and cerebellar development, possibly due to the deregulation of the *Ink4a/Arf* and *Engrailed* genes.

Introduction

The Polycomb Group (PcG) of proteins constitutes a highly conserved family of gene silencers. Originally, they were discovered in *Drosophila melanogaster* as repressors of the *Homeotic* genes, which are required for the establishment of the body plan and proper segmentation. Accordingly, the vertebrate PcG homologues were found to be involved in *Homeobox* (*Hox*) gene regulation as well. PcG proteins function in distinct multimeric complexes designated Polycomb repressive complexes 1 and 2 (PRC1 and PRC2) (reviewed in Lund and van Lohuizen, 2004). The PRC2 complex initiates gene silencing and contains histone deacetylases and methyltransferases that adjust the epigenetic histone marks associated with repressive chromatin. Subsequently, PRC1 recognizes the K27 methyl mark placed on histone H3 by PRC2 component EZH2 and associates with the chromatin to maintain the repressive state (reviewed in Valk-Lingbeek et al., 2004). Deletion of PRC2 genes causes early embryonic lethality in mice indicating essential functions in development (Faust et al., 1998; Donohoe et al., 1999; O'Carroll et al., 2001; Pasini et al., 2004). In contrast, removal of PRC1 genes is compatible with postnatal life, although these animals usually have a shortened lifespan and display signs of *Hox* transformations such as aberrant segmentation of the axial skeleton (van der Lugt et al., 1994; Akasaka et al., 1996; Takihara et al., 1997; Core et al., 1997; Mar Lorente et al., 2000). However, the exception is PRC1 member *Ring1b* (*Rnf2*). Deletion of this gene results in embryonic death, suggesting that Ring1b has a special role in the PRC1 complex that cannot be compensated for (Voncken et al., 2003). Intriguingly, recent evidence showed that Ring1b has an enzymatic function as it acts as an E3 ubiquitin ligase for histone H2A (de Napoles et al., 2004; Wang et al., 2004). Monoubiquitinated H2A (uH2A) represents approximately one tenth of all H2A and is enriched on the inactivated X chromosome (X_i) of female cells, suggestive of a function in repression. Both PRC2 and PRC1 are involved in X chromosome silencing and removal of *Ring1b* leads to loss of global uH2A levels (de Napoles et al., 2004; Fang et al., 2004; Wang et al., 2004; Hernandez-Munoz et al., 2005). Interestingly, the E3 ligase activity of

Ring1b may be enhanced by binding to PRC1 protein Bmi1 through interactions between their N-terminal Ring fingers (Cao et al., 2005; Buchwald et al., 2006; Li et al., 2006). Bmi1 itself does not appear to have E3 ligase activity. Instead, it stabilizes Ring1b and modulates Ring1b self-ubiquitination required for H2A monoubiquitination (Ben Saadon et al., 2006).

Even though the *in vivo* relevance of H2A ubiquitination by the Bmi1/Ring1b heterodimer remains to be elucidated, it is clear that these PcG genes interact genetically. *Bmi1* deficient mice suffer from a range of developmental defects (van der Lugt et al., 1994). One striking feature is a severe reduction in size and cellularity of the cerebellum, which is aggravated in a *Ring1b* heterozygous background (Voncken et al., 2003). As mentioned, homozygous deletion of *Ring1b* is early embryonic lethal and no *Ring1b*$^{-/-}$ embryos can be found after embryonic day 10.5 (E10.5). Close inspection of the null embryos revealed an overall delay in development and abnormal gastrulation (Voncken et al., 2003). Notably, a *Ring1b* hypomorphic mouse was viable and exhibited only mild posterior *Hox* transformations, underlining the importance of gene dosage (Suzuki et al., 2002). One possible explanation for the gastrulation defect may be that Ring1b is required for the proper execution of differentiation programs. *Ring1b* conditional knockout embryonic stem (ES) cells show a strong reduction in proliferation and increase in cell death (Leeb and Wutz, 2007; Van der Stoop and Boutsma et al., 2008). The remaining cells are extremely sensitive to stress and prone to undergo differentiation, as illustrated by the untimely derepression of developmental genes. Concomitantly, they are impaired in the formation and differentiation of embryoid bodies (Leeb and Wutz, 2007).

Given these dramatic effects of *Ring1b* loss on early development, we wanted to address whether Ring1b is required for the formation of tissue and cells at later stages. It has been demonstrated that ectopic expression of *Ring1b* in the chick embryo causes neural tubes defects (Suzuki et al., 2002). Since heterozygosity for *Ring1b* had an additive effect on the cerebellar phenotype of *Bmi1* null mice, we hypothesized that Ring1b plays a role in the central nervous system (CNS) and set out to investigate *Ring1b* function in this tissue. Hereto, we took advantage of the possibility to ablate *Ring1b* specifically from the CNS by crossing our conditional *Ring1b* knockout mouse (Van der Stoop and Boutsma et al., 2008) with a transgenic mouse expressing the Cre recombinase under the control of the Nestin promoter and enhancer (Tronche et al., 1999). Surprisingly, although born at submendelian ratios, mice lacking *Ring1b* expression in the CNS are viable. They exhibit severe progressive growth retardation, have problems in balance-keeping and die within one month after birth. Similar to *Bmi1* deficiency, *Ring1b* knockout neural stem cell proliferation is impaired and the *Ring1b* null cerebellum has a smaller size. However, the cerebellum also has a unique defect in foliation not described for other PcG mutants. It has been shown that loss of the *Ink4a/Arf* tumor suppressor locus can partially rescue the early arrest in embryonic development of *Ring1b* null embryos (Voncken et al., 2003). Here we demonstrate that deletion of *Ink4a/Arf* rescues the neural stem cell proliferation defect. But in line with recent findings that PcG genes control numerous genes involved in cell fate determination (Boyer et al., 2006; Bracken et al., 2006; Lee et al., 2006; Negre et al., 2006; Tolhuis et al., 2006; Pasini et al., 2007), we also provide evidence that the *Engrailed* genes, which are implicated in cerebellar development and foliation, are deregulated in the absence of *Ring1b*.

Results

Generation of nervous system specific Ring1b knockout mice

We first analyzed Ring1b protein expression in the wild type brain. In the adult, Ring1b is

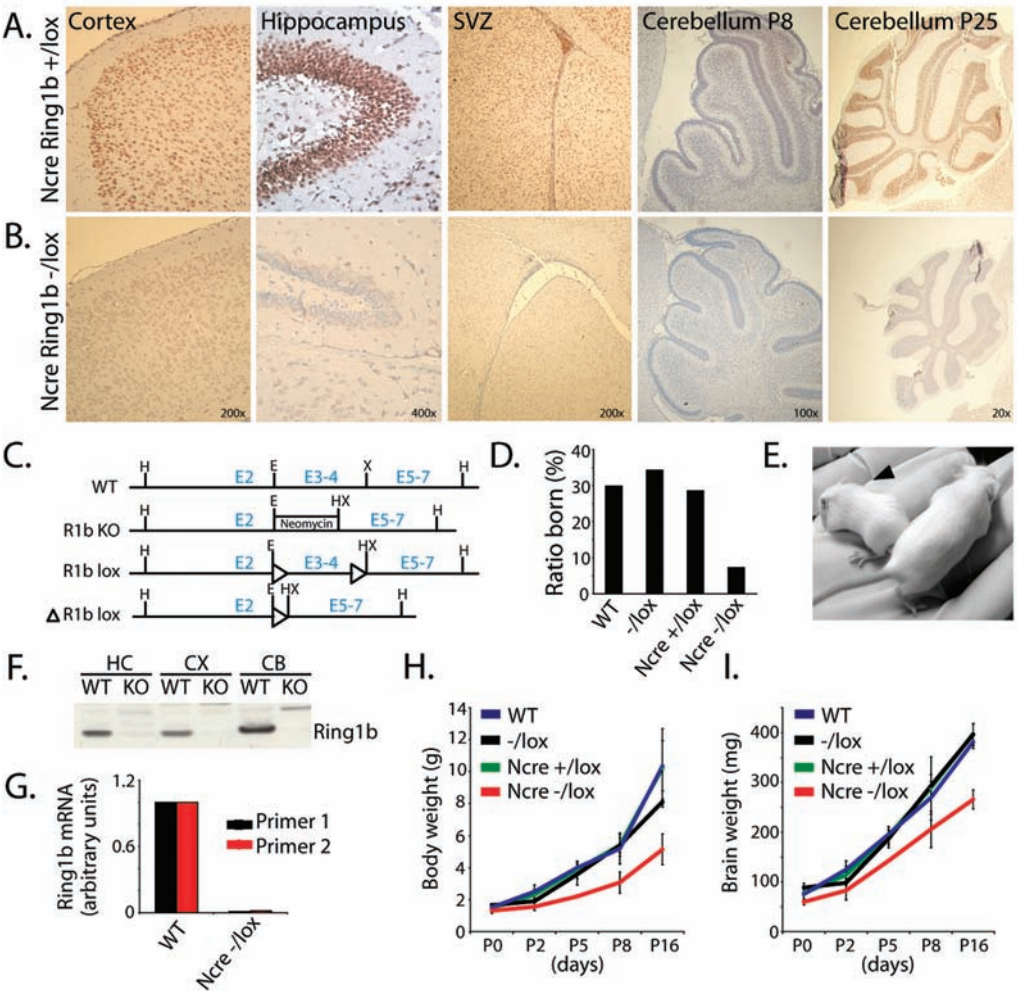

Figure 1. Generation of nervous system specific *Ring1b* deficient mice

(**A**) Ring1b protein immunolabeling in different areas of the adult and neonatal wild type brain (SVZ=subventricular zone, P=postnatal day).

(**B**) Immunolabeling for Ring1b in the *NCre;Ring1b*$^{-/lox}$ brain demonstrates that Ring1b protein is absent.

(**C**) Targeting constructs used to generate the Ring1b knockout (R1b KO) and the Ring1b conditional knockout allele (R1b lox). Targeted for deletion are exons 3 and 4 containing the Ring finger domain (H=HindIII restriction site, E=EcoRI, X=XhoI, Blue E=exon, WT=wild type).

(**D**) Ratios of pups born with specific genotypes; according to Mendelian inheritance, equal ratios were expected.

(**E**) Photographs of three weeks old control and *NCre;Ring1b*$^{-/lox}$ (black arrowhead) littermates.

(**F**) Western blot analysis demonstrating absence of Ring1b protein in different regions of the brain (HC=hippocampus, CX=cortex, CB=cerebellum, WT= wild type, KO=*Ring1b* knockout).

(**G**) qRT-PCR demonstrating absence of *Ring1b* mRNA in the *NCre;Ring1b*$^{-/lox}$ brain.

(**H**) Chart representing the total body weight of *Ring1b* knockout and control littermates.

(**I**) Chart representing the total brain weight of *Ring1b* knockout and control littermates.

widely expressed throughout the brain and is detected in the vast majority of cells (Figure 1A). Based on morphology and location, these cells represent both neurons and glia. In the P8 (postnatal day 8) cerebellum, Ring1b seems more strongly expressed in specific areas.

To achieve the nervous system specific removal of *Ring1b*, we made use of a transgenic mouse line (NCre) that expresses the Cre recombinase under the control of the Nestin promoter and second intron enhancer (Tronche et al., 1999). This transgene has been reported to be highly specific for the CNS and is expressed from E10.5 onwards (Graus-Porta et al., 2001; Knoepfler et al., 2002). We crossed NCre transgenic mice heterozygous for the *Ring1b* knockout allele (Voncken et al., 2003), or NCre mice heterozygous for the conditional *Ring1b* allele (referred to as *Ring1b*^{lox}), with mice carrying two copies of the *Ring1b*^{lox} allele. The floxed region in the *Ring1b* conditional allele is similar to the deleted region of the knockout allele, such that Cre expression causes not only the excision of the Ring finger domain, which is located in exons 3 and 4 (Figure 1C), but also introduces several in-frame stop codons. Exact details of the targeting strategy of the conditional *Ring1b* allele is described in chapter 3 of this thesis.

NCre;Ring1b$^{-/lox}$ (or *NCre;Ring1b*$^{lox/lox}$) mice were born at submendelian ratios, indicating the occurrence of some pre- or perinatal death (Figure 1D). We could not detect any Ring1b protein in the brain of *NCre;Ring1b*$^{-/lox}$ mice by immunohistochemical staining, demonstrating that recombination of the floxed allele was very efficient (Figure 1B). Western blot (Figure 1F) or quantitative real-time PCR (qRT-PCR) analysis (Figure 1G) confirmed the absence of Ring1b protein or mRNA in the knockout brain. Of note, inspection of other organs revealed no differences in *Ring1b* expression corroborating the nervous system specificity of the transgene (data not shown). Interestingly, *Ring1b* CNS knockout mice were significantly smaller than wild type littermates, a phenotype that became progressively stronger in time (Figure 1E and 1H). They also had a smaller brain than control animals (Figure 1I). Notably, all pups we dissected (aged between P0 and P16) had milk in their stomachs, making the growth retardation probably not the result of poor suckling. Another striking feature of the *Ring1b* knockout mice was their lack of control of movement. They displayed severe problems in balance-keeping and an ataxic gait, which is suggestive of defects in the cerebellum. Because of as yet unknown reasons, *Ring1b* knockout mice died prematurely. Some survived for approximately one month whereas others already died at earlier time points.

The Ring1b knockout cerebellum has foliation defects

The total size of the entire *Ring1b* knockout brain is greatly reduced, yet the cerebrum did not show any obvious gross abnormalities. The cerebellum on the other hand appeared abnormal macroscopically as some (sub) lobules, which are also called folia, of the vermis and hemispheres were missing (Figure 2A). To decipher which (sub)lobules were absent, we prepared sagittal sections of adult control and *Ring1b* null cerebella. To ensure that a complete overview was obtained, we analyzed haematoxylin and eosin (H&E) stained slides at 100 micron intervals along the entire medial-lateral axis. In few knockout animals complete separation of the hemispherical ansiform sublobules into Crus I and II had not occurred, but this was not reproducibly observed. At the level of the anterior vermis, we found with a high penetrance (5/6 animals) that the precentral fissure had not formed resulting in a fusion between the ventral (II) and dorsal lobules (III) of the central vermian lobe (Figure 2B).

The cerebellum is a favored model for studying cortical development due to its relatively simple histology, but the precise genetic mechanisms underlying the formation of its numerous folia

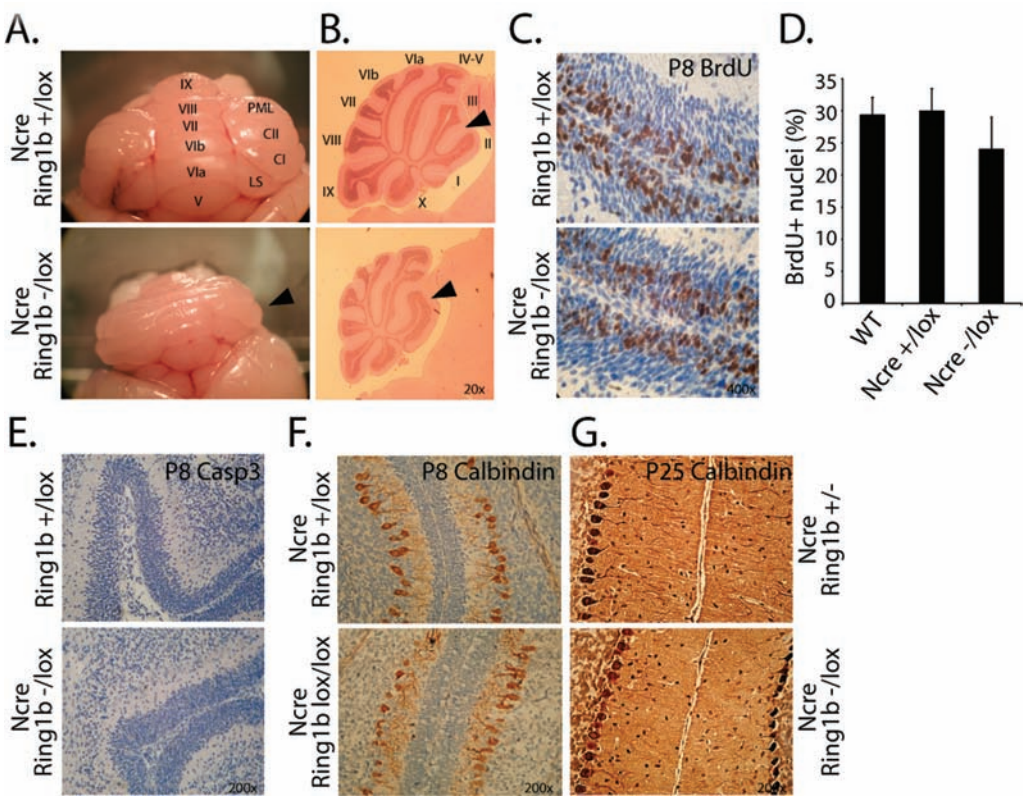

Figure 2. *Ring1b* deficient mice display anterior defects in cerebellar development
(**A**) Photographs of *NCre Ring1b*$^{+/lox}$ control and *NCre;Ring1b*$^{-/lox}$ cerebella showing altered foliation of the hemispheres (arrowhead) and reduced cerebellar size. Roman numerals refer to the vermian (sub)lobules (LS=lobulus simplex, C=Crus I and II, PML=paramedial lobule).
(**B**) H&E stained midsagittal sections through the vermis reveal fusion of lobules II and III due to lack of the precentral fissure (indicated by arrowheads).
(**C**) BrdU staining of the P8 cerebellum of *Ring1b*$^{+/lox}$ control and *NCre;Ring1b*$^{-/lox}$ mice.
(**D**) Quantification of the BrdU positive nuclei in the P8 cerebellum shows a minor, insignificant reduction in proliferation in the *Ring1b* deficient cerebellum.
(**E**) Activated Caspase-3 staining in sagittal cerebellar sections containing the EGL and developing IGL reveals no differences in apoptosis.
(**F**) Calbindin staining marking Purkinje neurons in the P8 cerebellum.
(**G**) Calbindin staining marking Purkinje and molecular neurons in the P25 cerebellum.

remains unclear. A number of mutations leading to foliation defects is known, but a phenotype identical to the *Ring1b* null cerebella has not yet been reported. The onset of the five cardinal lobes of the cerebellum is present from E18.5, while the extensive formation of (sub)lobules does not occur until after birth and is therefore generally believed to be the result of the expansion of the granule neuron population (Sillitoe and Joyner, 2007). Therefore, we histologically analyzed the granule neurons in control and *Ring1b* knockout cerebella. In the P8 cerebellum, when cerebellar granule neuron progenitor (CGNP) amplification is at its peak, we measured proliferation by

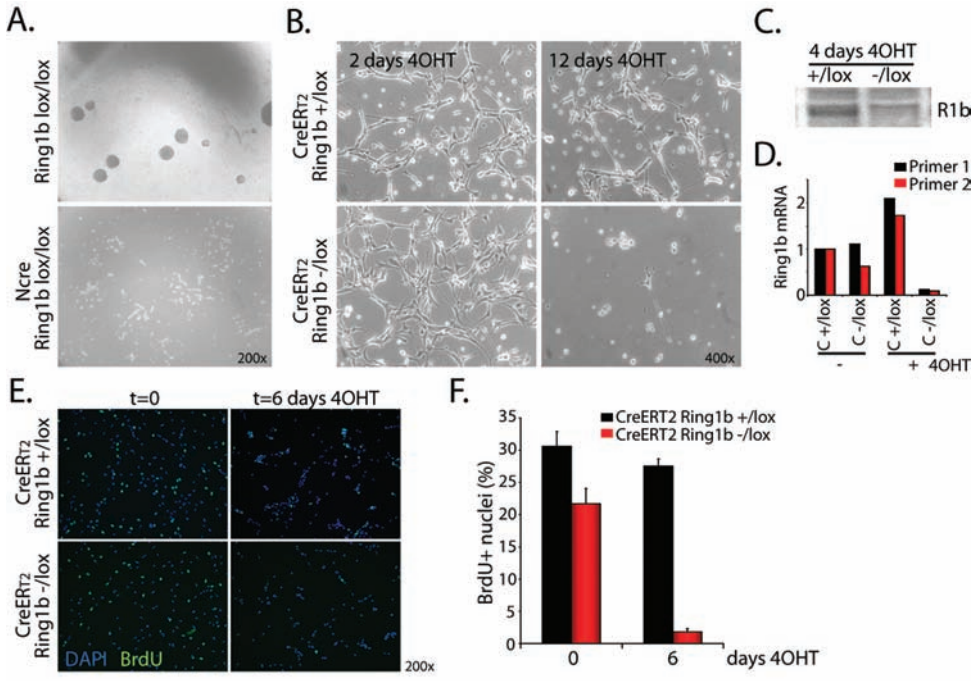

Figure 3. *Ring1b* deficient neural stem cells have proliferation defects
(**A**) Microphotographs showing primary neurosphere cultures from *Ring1b*$^{lox/lox}$ control and *NCre;Ring1b*$^{lox/lox}$ knockout SVZ.
(**B**) Treatment of inducible *Ring1b* knockout NSCs (*CreER*^{T2}*;Ring1b*$^{-/lox}$) with 4-hydroxytamoxifen (4-OHT) for 12 days induces a growth arrest.
(**C**) Western blot analysis demonstrating the absence of Ring1b in 4-OHT treated inducible knockout NSCs (+/lox=CreER^{T2};Ring1b$^{+/lox}$, -/lox=CreER^{T2};Ring1b$^{-/lox}$).
(**D**) qRT-PCR analysis confirming reduction of *Ring1b* mRNA levels in *CreER*^{T2}*;Ring1b*$^{-/lox}$ NSCs treated with 4-OHT (C=CreER^{T2}, +/lox=Ring1b$^{+/lox}$, -/lox=Ring1b$^{-/lox}$).
(**E**) Treatment of inducible *Ring1b* knockout NSCs with 4-OHT leads to a reduction in BrdU incorporation (green signal). Nuclei counterstained with DAPI (blue).
(**F**) Quantification of BrdU positive nuclei demonstrates a strong reduction in proliferation in inducible *Ring1b* knockout NSCs upon 4-OHT treatment.

determining BrdU incorporation (Figure 2C). In the *Ring1b* null cerebellum, BrdU positive cells were located in the outer external granular layer (EGL) like the control cells, indicating that they properly cease proliferation when they migrate into the inner EGL. We did observe a minor, reproducible reduction in the amount of BrdU positive cells, however this difference was not significant (Figure 4D). In the P25 cerebellum, all EGL cells had disappeared and an internal granular layer (IGL) revealing no abnormalities had developed. We also questioned whether there was increased cell death in the *Ring1b* deficient EGL. We stained P8 sagittal cerebellar sections for activated Caspase-3, but found no evidence for increased apoptosis in the absence of *Ring1b* (Figure 2E). Since the granule neurons appeared unaffected, we next focused on the other cellular layers: the Purkinje neuron and the molecular layer. As revealed by Calbindin staining, there were no defects in Purkinje neuron deposition or arborization either at P8

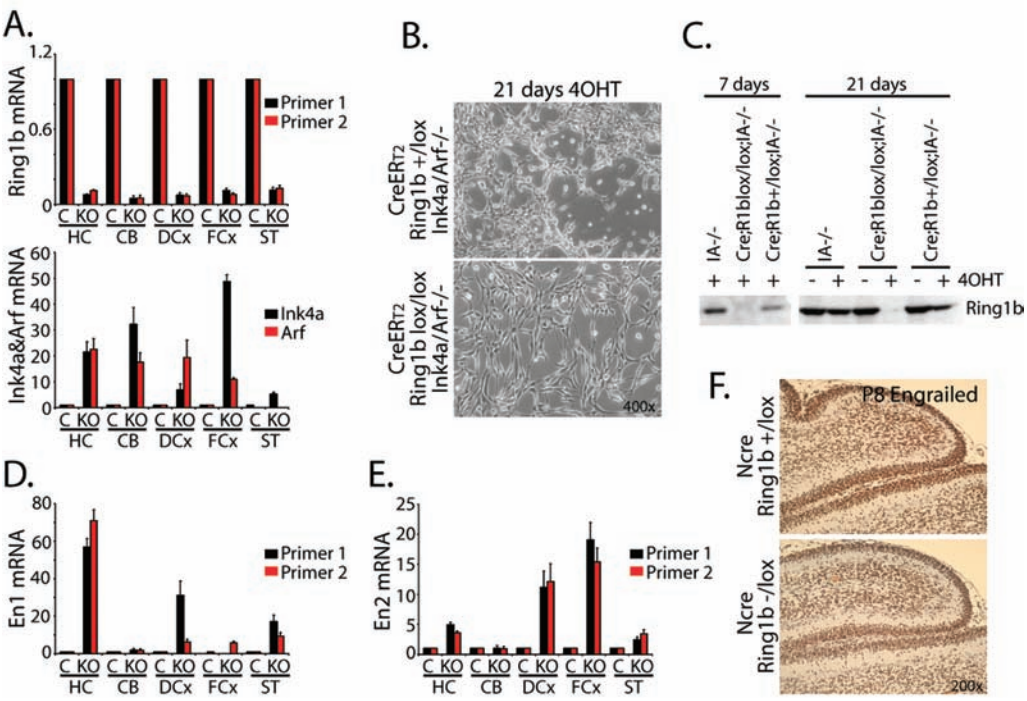

Figure 4. Deregulation of genes in the absence of *Ring1b*

(**A**) qRT-PCR analysis demonstrating reduced *Ring1b* mRNA (upper panel) and increased *Ink4a* and *Arf* mRNA levels (lower panel) in various regions of the knockout brain (C=control, KO=NCre;Ring1b$^{-/lox}$, HC=hippocampus, CB=cerebellum, DCx=dorsal cortex, FCx=frontal cortex, ST=striatum).

(**B**) Treatment of *CreER^{T2};Ring1b$^{+/lox}$;Ink4a/Arf$^{-/-}$* and *CreER^{T2};Ring1b$^{-/lox}$;Ink4a/Arf$^{-/-}$* NSCs with 4-OHT reveals equal proliferative capacity in the presence and absence of *Ring1b*.

(**C**) Western blot analysis showing that no Ring1b protein is present in floxed *Ring1b* null NSCs deficient for *Ink4a/Arf* up to 21 days of 4-OHT treatment (IA=Ink4a/Arf, Cre=CreER^{T2}, R1b=*Ring1b*).

(**D**) qRT-PCR showing strong transcriptional upregulation of the *Engrailed-1* gene (En-1) throughout the *Ring1b* knockout brain.

(**E**) qRT-PCR showing transcriptional upregulation of *Engrailed-2* (En-2) throughout the *Ring1b* knockout brain.

(**F**) Immunolabeling for Engrailed-1 and -2 protein in P8 sagittal *NCre;Ring1b$^{+/lox}$* and *NCre;Ring1b$^{-/lox}$* cerebellar sections.

(Figure 2F) or at P25 (Figure 2G). Also the *Ring1b* deficient molecular layer contained normal cell numbers (Figure 2G).

Ring1b deficient neural stem cells have impaired proliferation

To gain further insight into the *Ring1b* null phenotype, we made use of a culture system that provides the opportunity to study proliferation and differentiation *in vitro*. Hereto, we isolated adult neural stem cells (NSCs) from the subventricular zone (SVZ) of the lateral ventricles of the cerebrum, a region known to have continuous neurogenesis throughout adult life (Alvarez-Buylla and Lim, 2004). These cells can be propagated as floating clusters of stem cells and more differentiated progeny termed neurospheres (Reynolds and Weiss, 1996), or as homogeneous adherent monolayers (Conti et al., 2005). However, as we were readily able

to generate neurosphere cultures from control mice, the few neurospheres that formed from *NCre;Ring1b$^{lox/lox}$* animals could not self-renew, but instead attached to the plastic and entered a growth arrest (Figure 3A). Since we were not able to retrieve sufficient material from the *NCre Ring1b* null mice, we had to change to a different system that would allow us to conditionally induce *Ring1b* deletion *in vitro*. We did this by crossing the *Ring1b* (conditional) knockout mouse with the CreER^{T2} transgenic knockin mouse (Feil et al., 1997; Ate Loonstra and Anton Berns, unpublished). This transgene consists of a Cre recombinase fused to the mutated ligand-binding domain of the Estrogen receptor (ER). Upon addition of 4-hydroxytamoxifen (4-OHT), the CreER^{T2} fusion protein translocates to the nucleus and gets access to the genome. We established adherent neural stem cell cultures from *CreER^{T2};Ring1b$^{+/lox}$* and *CreER^{T2};Ring1b$^{-/lox}$* mice, plated these cells at sub-confluency and treated them with 4-OHT for 12 days (Figure 3B). Again we noticed that NSCs deficient for *Ring1b* ceased proliferation and appeared to undergo cell death. Western blot analysis (Figure 3C) and qRT-PCR (Figure 3D) revealed that Ring1b protein and mRNA was completely absent in the *CreER^{T2};Ring1b$^{-/lox}$* cells after 4 days of 4-OHT treatment. The reduction in proliferation was confirmed by a strong decline in BrdU incorporation (Figure 3E and 3F).

Deletion of Ring1b causes derepression of the Ink4a/Arf and Engrailed genes

It had been demonstrated that the *Ink4a/Arf* tumor suppressor locus was upregulated in *Ring1b* deficient embryos, and its removal partially alleviated the gastrulation defect (Voncken et al., 2003). We wanted to test if *Ink4a/Arf* derepression was responsible for the *Ring1b* null phenotypes we observed. As determined by qRT-PCR, both *p16^{ink4a}* and *p19^{arf}* mRNA levels in all regions of the *Ring1b* knockout brain were elevated (Figure 4A). Subsequently, we performed additional mice breedings and isolated NSCs from *CreER^{T2};Ring1b$^{-/lox}$;Ink4a/Arf$^{-/-}$* mice. Remarkably, NSCs lacking *Ring1b* and *Ink4a/Arf* continue to proliferate upon 4-OHT treatment for at least 21 days (Figure 4B), even though Ring1b protein was already undetectable after 7 days and remained absent for the entire length of the experiment (Figure 4C). This suggests that at least the defect in neural stem cell proliferation is indeed caused by deregulation of *Ink4a/Arf*.

But since PcG genes are known to bind and repress a wide variety of developmental genes (Boyer et al., 2006; Lee et al., 2006), we speculated that more genes ought to be deregulated in the absence of *Ring1b*. We took a candidate approach and started to verify expression of genes which have a demonstrable function in cerebellar foliation. Surprisingly, we found strong increases in the expression of the *Engrailed-1* and *-2* genes in many regions of the *Ring1b* knockout brain, except for the cerebellum, where only *Engrailed-1* showed a minor induction (Figure 4D,E). However, when we stained sagittal sections of the *Ring1b* knockout cerebellum with antibodies that recognize both Engrailed-1 and -2 (Figure 4F), or only Engrailed-1 (not shown), we did not detect higher Engrailed expression, nor did we find ectopic expression in for instance the Purkinje neurons.

Discussion

The complete removal of the PcG gene *Ring1b* from mice is not compatible with life as *Ring1b* knockout embryos only develop until the second half of embryogenesis (Voncken et al., 2003). These embryos exhibit a general delay in development and gastrulation is abnormal. In line with this, embryonic stem cells and fibroblasts deficient for *Ring1b* are extremely sensitive to stress and prone to differentiate or undergo apoptosis (Leeb and Wutz, 2007; Van der Stoop and Boutsma et al., 2008). Therefore, it is somewhat surprising that mice specifically lacking *Ring1b* expression in the central nervous system can survive until birth

and the first weeks of postnatal life. Although these animals clearly suffer from neurological malfunctions, their smaller yet relatively normal brain does not immediately suggest a myriad of developmental problems nor does it directly expose the origin of the neurological defects. Interestingly, these mice exhibit progressive growth retardation which is also observed in animals deficient for a related PcG gene, *Bmi1*. It has been assumed that *Bmi1* knockout mice are smaller due to systemic *Bmi1* absence. But the nervous system-specific *Ring1b* knockout in fact argues that the sole activity of PcG in brain could determine body size. Such phenomenon is not without precedent. Conditional ablation of *Gli2*, a downstream effector of the developmental morphogen Sonic Hedgehog (Shh), in the cerebellum evokes a reduction in the total body weight (Corrales et al., 2006). Additionally, these mice suffer from problems in locomotion like the *Bmi1* and *Ring1b* deficient mice. Notably, *Bmi1* has previously been implicated in Sonic Hedgehog signaling in the cerebellum and in medulloblastoma, a type of brain cancer believed to originate from the granule neuron progenitors (Leung et al., 2004). It is believed that Bmi1 is induced by Shh to prevent expression of the *Ink4a/Arf* genes, which will allow the CGNPs to proliferate (Leung et al., 2004; Bruggeman et al., 2005). A link between *Ring1b* and *Shh* has not yet been demonstrated. However, it is tempting to speculate that such connection exists since there is evidence that cerebellar foliation, which has occurred improperly in *Ring1b* null cerebella, is governed by *Shh* signaling.

Ring1b deficient mice have defects in cerebellar foliation, a Shh-guided process

Foliation patterns vary between species and even within different mouse strains (Inouye and Oda, 1980; Cooper et al., 1991; Wahlsten and Andison, 1991). Since our mice were in a mixed FVB/C57B6 background, the fusion of lobules II and III might have been the result of combining the foliation patterns of two different strains. However, the control animals never displayed such fusion nor has it been described for any mouse strain, making it highly unlikely that inter-strain differences account for the *Ring1b* phenotype. Presumably, foliation occurs in distinct phases (Corrales et al., 2006). The original smooth surfaced cerebellar anlage is divided by four fissures into the five cardinal lobes. After birth, these lobes separate further into (sub) lobules. In mice, cerebellar development is largely completed within the third postnatal week, but in humans development takes much longer and the EGL persists for approximately fifteen months. Notably, defects in cerebellar foliation in humans are associated with a number of clinical abnormalities underscoring the importance of proper foliation (Demaerel, 2002). The specification of the cardinal lobes and the position of the fissures are determined by an unknown mechanism. But the number and extent of folia that arise are dose-dependently orchestrated by Shh induced CGNP proliferation (Dahmane and Altaba, 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999; Corrales et al., 2004; Lewis et al., 2004; Corrales et al., 2006). Since Shh is secreted by Purkinje neurons, animals lacking these cells, like the *Lurcher* and *Staggerer* mutants, are impaired in EGL formation and concomitantly in foliation (Sidman et al., 1962; Caddy and Biscoe, 1979; Wetts and Herrup, 1982; Smeyne et al., 1995). Shh activates the *D1* and *D2 Cyclins* through the transcriptional induction of *N-Myc*, stimulating progression through the cell cycle (Ciemerych et al., 2002; Kenney et al., 2003; Kenney et al., 2004). Loss of *N-Myc* results in the induction of cell cycle inhibitors $p18^{ink4c}$ and $p27^{kip1}$ and reduced CGNP proliferation (Knoepfler et al., 2002; Zindy et al., 2006). Curiously, we found that in the absence of *Ring1b*, the tumor suppressor locus *Ink4a/Arf* is derepressed in all areas of the brain including the cerebellum. $p16^{ink4a}$

is a powerful inhibitor of the Cyclin D/Cyclin dependent kinase (CDK) complexes and its ectopic expression might compromise the mitogenic activity of Shh. Additionally, *p19^{arf}* controls proliferation through modulation of *p53* activity and directly regulates the activity of *c-Myc*, an *N-myc* homologue also implicated in cerebellar development, providing yet another path via which *Ring1b* could affect CGNP proliferation and foliation (Datta et al., 2004; Qi et al., 2004; Zindy et al., 2006). Resuming, it would be worthwhile to investigate if a link between Shh signaling and *Ring1b* exists. One could for instance analyze patterns of *Gli1* expression, which is commonly used as readout for active Shh signaling, in the *Ring1b* null cerebellum (Corrales et al., 2004; Corrales et al., 2006). But other mechanisms described to affect foliation and EGL development, like *HGF-Met*, *BDNF-Trk* or *BMP* signaling, and *Math1* or *Zic1* controlled transcription, should be studied as well (Ben Arie et al., 1997; Schwartz et al., 1997; Aruga et al., 1998; Helms et al., 2001; Ieraci et al., 2002; Qin et al., 2006). We recommend these investigations to be conducted in a defined spatial and temporal manner as cerebellar development is highly dynamic. Capturing just one single moment will inevitably lead to clues remaining unnoticed. This might also explain why we so far have only observed minor defects in histology.

Possible role for Engrailed genes in cerebellar proliferation

Position-dependent effects can be anticipated in PcG mutants. PcG proteins are known to repress genes to variable extents at different locations, which is illustrated by the occurrence of ectopic bones in mutant mice, or extremities in *Drosophila,* at fixed places. The most obvious place to expect abnormalities in the *Ring1b* null cerebellum is in the anterior vermis, around the time the precentral fissure normally separates lobules II and III. Intriguingly, in the young cerebellum, *Ring1b* expression may be locally restricted corroborating spatial specificity. It is interesting that expression of *Engrailed-1* and *2*, two novel *Ring1b* targets that are both implicated in foliation, is also highly localized. *Engrailed-1* (*En-1*) is induced in the region of the cerebellar anlage at the one somite stage whereas *Engrailed-2* (*En-2*) is found slightly later at the five somite stage. Their expression pattern is partially overlapping but after birth, *Engrailed-2* becomes exclusively expressed in the cerebellum. (Davidson et al., 1988; Davis et al., 1988; Davis and Joyner, 1988; McMahon et al., 1992). Mutation in either gene causes a strong reduction in cerebellar size. Specific deletion of *En-1* evokes severe or mild cerebellar defects depending on the mouse strain used (Wurst et al., 1994; Bilovocky et al., 2003). In the mild case, anterior defects in foliation occur together with abnormal projection of mossy fibers (Bilovocky et al., 2003). Removal of *En-2* is associated with defects in the foliation of the vermis and hemispheres and altered parasagittal banding patterns (Millen et al., 1994; Kuemerle et al., 1997; Herrup et al., 2005;). In contrast to *En-1*, the foliation defects of the *En-2* knockout are mostly posterior. Since *Ring1b* represses both *En* genes and the precise mode of action of these genes is not fully understood, it is difficult to predict of which gene the ectopic expression would be more likely to mediate the *Ring1b* foliation defect. Of note, an *En-2* transgenic mouse also has foliation defects and smaller cerebellar size, suggesting that *En-2* upregulation could induce *Ring1b*-like phenotypes (Baader et al., 1998; Baader et al., 1999). Generation of *Ring1b;En-1* or *Ring1b;En-2* compound mutants might unravel whether or not there is a genetic interaction. Furthermore, the *Engrailed* genes have been implicated in other areas of the brain such as in the mesencephalic dopaminergic neurons forming the substantia nigra, and in the serotonergic and noradrenergic neurons of the dorsal raphe nucleus and locus caeruleus,

respectively (Alberi et al., 2004; Simon et al., 2005). Therefore, it would also be interesting to investigate whether these areas are affected in the *Ring1b* knockout brain.

Ink4a/Arf derepression is implicated in Ring1b deficient phenotypes

Another obvious question is to what extent derepression of the *Ink4a/Arf* locus is involved in the *Ring1b* phenotypes. Cultured *Ring1b* knockout NSCs undergo a complete growth arrest, the nature of which still awaiting further investigation. This strong effect is rather surprising given the relatively mild *in vivo* phenotype. However, more careful inspection of sites with ongoing neurogenic activity like the SVZ, rostral migratory stream, olfactory bulbs and hippocampus might disclose subtle defects in *in vivo* NSC function. Remarkably, the *Ring1b* null NSC proliferation defect was completely rescued by co-deletion of the *Ink4a/Arf* locus and it would be interesting to test if removal of this locus can also bypass the reduced proliferation and increased differentiation seen in embryonic stem cells. However, it should be taken into consideration that the *Ink4a/Arf* locus is extremely sensitive to stress, especially when cells are transferred from hypoxic *in vivo* conditions to oxygen-rich environments. Hence, the effect of genes controlling *Ink4a/Arf* expression may be somewhat overstated in tissue culture experiments in general. Therefore, it will be essential to cross brain-specific *Ring1b* knockout mice with *Ink4a/Arf* deficient mice, and analyze its relative contribution to the *Ring1b* phenotypes under physiological conditions.

Altogether we have demonstrated that, while early ablation of *Ring1b* from mice is not compatible with embryonic development, nervous-system specific removal of *Ring1b* at later stages of embryogenesis leads to viable animals. However, these mice suffer from progressive growth retardation, a lack of locomotive control and premature death. These abnormalities may be the result of stem cell self-renewal or cerebellar foliation defects and involve deregulation of the *Ink4a/Arf* and *Engrailed* genes.

Materials and Methods

Mouse breedings and tissue isolation

Ring1b conventional and conditional knockout mice were in an FVB background (Voncken et al., 2003; Van der Stoop and Boutsma et al., 2008). Nestin-Cre (NCre) transgenic mice were originally in a C57/B6 background (Tronche et al., 1999), but they were backcrossed twice into the FVB background. CreER^{T2} transgenic mice (kindly provided by dr. A. Berns) and *Ink4a/Arf* knockout mice (Serrano et al., 1996) were in an FVB background. For timed breedings, presence of a vaginal plug in the morning was considered E0.5. Mice were decapitated or killed with an overdose of CO_2 and tissue was instantly removed. NSCs were isolated and cultured as described before (Bruggeman et al., 2005). They were maintained in defined serum-free NSC medium containing 20 ng/ml EGF and 10 ng/ml bFGF (R&D systems) as neurospheres, or as adherent cultures on poly-L-Ornithine (15 µg/ml) and Laminin (5 µg/ml, both Sigma) coated plates (Conti et al., 2005). All animal experiments were done in agreement with the ethical boards.

Cell culture experiments

For neurosphere cultures, SVZ-derived NSCs were seeded at a density of <1 cell per µl medium into tissue culture treated plastic dishes. For 4-hydroxytamoxifen (4-OHT) induced floxing experiments, 300,000 NSCs were plated onto poly-L-Ornithine and Laminin coated 6 wells plates in NSC medium supplemented with 20 ng/ml EGF and 10 ng/ml bFGF. The following day, 400 nM 4-OHT (Sigma) was added and refreshed every other day. BrdU incorporation experiments were

performed as described before (Bruggeman et al., 2005).

qRT-PCR and Western blot analysis
Quantitative real time PCR (qRT-PCR) and Western blotting were essentially performed as described previously (Bruggeman et al., 2005). Primer sequences were Ring1b-1 sense 5'-AAATGTCTCAGGCTGTGCAG -3', antisense 5'-TTTCCAAGCCATCTGTTATTGCC-3'; Ring1b-2 sense 5'-TCGGTTTTGCGCGGATT-3', antisense 5'-AGTTTTTTCCGACAGGTAGGACACT-3'; Ink4a sense 5'-CGTACCCCGATTCAGGTGAT-3', antisense 5'-TTGAGCAGAAGAGCTGCTACGT-3'; Arf sense 5'-GCCGCACCGGAATCCT-3', antisense 5'-TTGAGCAGAAGAGCTGCTACGT-3'; Engrailed1-1 sense 5'-TCCGTCCTCTGGTCC ACG-3', antisense 5'-CCTTCTCGTTCTTTTTCTTCT TTAGC-3'; Engrailed1-2 sense 5'-AGGCCAG ACTGGTGCAGGT-3', antisense 5'-AGCTCGTGT GCCCAGAGAGT-3'; Engrailed2-1 sense 5'-TGCA CGCGCTATTCTGACC-3', antisense 5'-CTTCTTTG GTTTTCGGGACCT-3'; Engrailed2-2 sense 5'-CTGCCCGAGGTCCTACAGG-3', antisense 5'-AAGTTGGTGATGCGATGTGG-3'. Loading controls were β-Actin sense 5'-CCTCA TGAAGATCCTGACTGA-3', antisense 5'-TTTAT GTCACGAACAATTTCC-3' and HPRT sense 5'-CTGGTGAAAAGGACCTCTCG-3', antisense 5'-TGAAGTACTCATTATAGTCAAGGGCA-3'.
For Western analysis of Ring1b expression, a mouse monoclonal antibody was used (1:50, a kind gift of dr. Koseki).

Histological analysis
Tissue was formalin fixed, paraffin embedded and processed into 4 µm thick slices. Antigen was retrieved by boiling in Citrate buffer (100 mM, pH 6.0), and endogenous peroxidase activity was removed by applying 3% H_2O_2 (Merck). Primary antibodies (mouse monoclonal Ring1b, 1:50; Engrail-1, DSHB, 1:500; BrdU, Dako, 1:50; Cleaved Caspase-2, Cell Signaling, 1:50; and rabbit polyclonal Enhb1, kindly provided by dr. A. Joyner, 1:200; Calbindin, Chemicon, 1:400) were added overnight in 5% normal goat serum. Proteins were visualized using secondary biotinylated antibodies in combination with the StreptABComplex/ HRP system according to the manufacturer's protocols (Dako). For BrdU measurements, mice were injected intraperitoneally with 50 mg/kg BrdU two hours prior to sacrifice. The ratio of BrdU positive nuclei per high power field was determined.

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