Role of the *Ink4a/Arf* tumor suppressors in cerebellar development, stem cells and cancer

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Rol van de Ink4a/Arf tumor suppressors in ontwikkeling van het cerebellum, stam cellen en kanker

(met een samenvatting in het Nederlands)

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Stem Cells and Cancer: The Polycomb Connection

Review

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Proteins from the Polycomb group (PcG) are epigenetic chromatin modifiers involved in cancer development and also in the maintenance of embryonic and adult stem cells. The therapeutic potential of stem cells and the growing conviction that tumors contain stem cells highlights the importance of understanding the extrinsic and intrinsic circuitry controlling stem cell fate and their connections to cancer.

The Polycomb Group (PcG) gene family is highly conserved throughout evolution. Originally, PcG genes were discovered in *Drosophila* as repressors of *Homeotic* genes, which are necessary for establishment of the body plan and segmentation. Also in mammals, PcG genes are implicated in *Homeobox* (*Hox*) gene regulation. Their biological activity lies in stable silencing of specific sets of genes through chromatin modifications. This capacity makes them interesting subjects for stem cell research, since it is conceivable that dynamic reprogramming of cells, for instance during differentiation, requires alterations in the epigenetic state of gene expression programs.

Two distinct multiprotein PcG complexes have been identified (reviewed in Lund and van Lohuizen, 2004; and see Table 1 for names and corresponding *Drosophila* counterparts). Polycomb repressive complex 2 (PRC2) is involved in the initiation of silencing and contains histone deacetylases and histone methyltransferases, that can methylate histone H3 lysine 9 and 27, marks of silenced chromatin, and histone H1 lysine 26 (van der Vlag and Otte, 1999; Cao et al., 2002; Czermin et al., 2002; Muller et al., 2002; Kuzmichev et al., 2004). Deletion of PRC2 genes in mice results in early embryonic lethality, underscoring their importance in development (Schumacher et al., 1996; O'Carroll et al., 2001). Polycomb repressive complex 1 (PRC1) is implicated in stable maintenance of gene repression and recognizes, by means of a chromodomain, the H3 lysine 27 mark set by PRC2 (Czermin et al., 2002). Its precise in vivo mode of action is not completely understood, but in vitro it is found to interact with histone methyltransferases, histones, and to counteract SWI/SNF-chromatin-remodeling complexes (Breiling et al., 1999; Levine et al., 2002; Ogawa et al., 2002; Sewalt et al., 2002). Recent evidence in *Drosophila* suggests that PcG inhibits the transcription initiation machinery (Dellino et al., 2004; Wang et al., 2004). Mice mutant for most PRC1 members survive until birth as a result of partial functional redundancy provided by homologs (van der Lugt et al., 1994; Akasaka et al., 2001; Core et al., 1997; Takihara et al., 1997). An exception to this rule is *Rnf2* deficiency, resulting in an early lethal phenotype similar to PRC2-deficient mice (Voncken et al., 2003).

However, the existence of only two PcG complexes is an oversimplification, as recent evidence in flies and mammals indicates that heterogeneous protein complexes of varying composition can be formed even within one cell (reviewed in Lund and van Lohuizen, 2004). For instance, Ezh2 can associate with different isoforms of Eed thereby determining the specificity of the histone methyltransferase activity, i.e., toward histone H3 lysine 27 or histone H1 lysine 26 (Kuzmichev et al., 2004). This recent finding is intriguing given the important role of H1-linker histones in mediating higher order chromatin folding. Furthermore, PcG complexes are regulated in a cell cycle-dependent manner, necessary to ensure that chromatin marks are correctly reset upon DNA replication (Voncken et al., 1999; Akasaka et al., 2002). Lastly, posttranslational modifications influence localization and activity of PcG (Voncken et al., 1999; Akasaka et al., 2002). Clearly, PcG proteins and PcG complex composition are highly regulated in a dynamic and complicated manner allowing for gene, tissue, and differentiation stage-specific function.

Setting the Stage: Role for PcG Genes in Stem Cells

Stem cells are defined as cells able to both extensively self-renew and differentiate into progenitors. Embryonic stem (ES) cells and embryonic germ cells are said to be pluripotent because they can give rise to all cell types of the embryo proper. Adult or somatic stem cells (SSCs) often are multi- or oligopotent, indicating they can give rise to a subset of cell lineages, or unipotent, when they only contribute to one type of mature cells. Stem cells are believed to reside in many, if not all, adult tissues, and have been well described for intestine, skin, muscle, blood, and nervous system. Notably, it is not only intrinsic properties that determine stem cell fate: extrinsic cues given by the stem cell "niche" are at least of equal importance.

Recent results highlight that stem cell fate is in part governed by the PcG genes. First indications came from *Bmi1*-deficient mice, which suffer from progressive loss of hematopoietic cells and cerebellar neurons (van der Lugt et al., 1994). In addition, Mph1/Rae28, which directly interacts with Bmi1 in the PRC1 complex, is required for sustaining activity of hematopoietic stem cells (Ohta et al., 2002). Over the last year, direct evidence implicated *Bmi1* in the self-renewal of multiple stem cells as well as the proliferation of early cerebellar progenitors (Lessard and Sauvageau, 2003; Molofsky et al., 2003; Park et al., 2003; Leung et al., 2004). Importantly, in the cerebellum, *Bmi1* is regulated by an extracellular-signaling molecule, the morphogen Sonic hedgehog

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Table 1. Polycomb Nomenclature

Drosophila proteins		Human proteins	Mouse proteins
PRC2/Initiation complex			
Esc	Extra sex combs	EED	Eed
E(z)	Enhancer of Zeste	EZH1	Ezh1/Enx2
		EZH2	Ezh2/Enx1
Su(z)12	Suppressor of Zeste 12	SUZ12	
PRC1/Maintenance complex			
Pc	Polycomb	CBX2/HPC1	Cbx2/M33
		CBX4/HPC2	Cbx4/Mpc2
		CBX8/HPC3	
Ph	Polyhomeotic	EDR1/HPH1	Edr1/Mph1/Rae28
		EDR2/HPH2	Edr2/Mph2
		EDR3/HPH3	
dRING		RING1/RNF1/RING1A	Ring1/Ring1a
		RNF2/RING1B	Rnf2/Ring1b
Psc	Posterior sex combs	BMI1	Bmi1
		RNF110/ZFP144	Rnf110/Zfp144/Mel-18
		ZNF134	Znf134/Mblr
Pho	Pleiohomeotic	YY1	Yy1
Pho-like	Pleiohomeotic-like		
Scm	Sex combs on midleg	SCML1	Scmh1
		SCML2	Scmh2
Pcl	Polycomb-like	PHF1	

(Shh) (Leung et al., 2004), providing for the first time a connection between PcG and a major developmental-signaling pathway. This connection may only be the beginning of our understanding of how complex mechanisms, required for embryogenesis and stem cell behavior, are organized.

PcG and Embryonic Stem Cells

ES cells can be viewed as the "mother" of all stem cells. They possess the unique capacity to undergo efficient and remarkably robust self-renewal in cell culture, and even upon prolonged culturing they retain the ability to undergo multiple differentiation pathways. When placed back in their own "niche" upon injection into blastocysts, ES cells resume normal behavior and contribute faithfully to all cell lineages. Under carefully controlled tissue culture conditions, it was found that cell-extrinsic signals, such as LIF and BMP4, regulate the self-renewal of ES cells. Cell-intrinsic signaling involves the concomitant receptors and signals down to transcription factors, such as Oct4 and Nanog (Niwa et al., 2000; Chambers et al., 2003; Ying et al., 2003). These pathways are not yet fully understood, but PcG proteins might participate at some level considering that *Ezh2, Eed*, *YY1*, and *Rnf2* all are essential for embryonic development (Schumacher et al., 1996; Donohoe et al., 1999; O'Carroll et al., 2001; Voncken et al., 2003). *Ezh2* and *Rnf2* expression can be detected early in development at preimplantation stages, whereas robust *Eed* expression commences after implantation (Schumacher et al., 1996; O'Carroll et al., 2001; Voncken et al., 2003). In vitro, *Ezh2*-deficient blastocysts fail to grow out and *Ezh2*-deficient ES cell lines cannot be established (O'Carroll et al., 2001). In contrast, *Eed* mutant ES cells are viable and can in the context of embryoid bodies differentiate into multiple cell types. Interestingly, *Eed* mutant ES cells can also contribute to more advanced embryonic stages in chimeras, indicating partial noncell autonomous rescue of *Eed* deficiency in vivo (Morin-Kensicki et al., 2001). It is formally possible that early in development, *Ezh2* functions independently of *Eed* in the PRC2 complex. Alternatively, the *Eed* mutant may not represent a null allele, especially since *Eed* appears to be required for the ability of *Ezh2* to methylate histone H3 (Cao et al., 2002). Furthermore, both Eed and Ezh2 become transiently localized to the inactive X during ES cell differentiation, and most importantly, in *Eed*-deficient cells X inactivation is not maintained (Plath et al., 2003; Silva et al., 2003).

The crucial role of PRC1 member *Rnf2* in development, for which *Ring1a* cannot compensate, suggests either a central role in complex formation for Rnf2, or the requirement of a transient contact between PRC1 and PRC2 via Rnf2 as described for *Drosophila* (Francis et al., 2001; Poux et al., 2001). Another PRC1 protein, *Mph1/Rae28*, is highly expressed in ES cells but becomes rapidly downregulated upon differentiation (Loring et al., 2001; Fortunel et al., 2003). It is clear that Polycomb silencing early in development needs both complexes, however, the precise mode of action needs more investigation.

PRC1 PcG Genes *Bmi1*, *Mph1/Rae28*, and *Mel-18* Regulate the Self-Renewal of Hematopoietic Stem Cells

Hematopoiesis in mammals occurs in distinct temporal waves shifting from the extraembryonic yolk sac and fetal liver in embryos to bone marrow in adults. Definitive hematopoietic stem cells (HSCs) replenish the pool of blood cells both by maintaining the stem cells and by allowing daughter cells to differentiate into the lymphoid, myeloid, and erythroid lineages. The stem cell niche in the bone marrow provides the cells with a specialized extracellular matrix secreted by a number of

different cell types. An array of extracellular signaling pathways, such as *Notch*, *BMP*, *JAK-STAT*, and *Wnt* control hematopoietic stem cells (reviewed in Fuchs et al., 2004). However, relatively little is known about cell-intrinsic genetic and epigenetic mechanisms.

Expression of most PcG genes is upregulated in differentiating hematopoietic cells (Raaphorst et al., 2001), but *Bmi1* and *Mph1/Rae28* are highly expressed in primitive hematopoietic cells (Ohta et al., 2002; Park et al., 2003). Most compelling, *Bmi1*, *Mel-18*, *Mph1/Rae28*, and *M33* mutant mice suffer from various defects in the hematopoietic system, such as hypoplasia in spleen and thymus, reduction in overall T cell numbers, defects in B cell development, and an impaired proliferative response of lymphoid precursors to cytokines, in particular to interleukin 7 (IL-7) (van der Lugt et al., 1994; Akasaka et al., 1997, 2001; Coré et al., 1997; Takihara et al., 1997).

Close inspection of fetal HSC pools revealed interesting differences and similarities between *Bmi1-, Mel-18-*, and *Mph1/Rae28*-deficient mice. Whereas fetal liver-derived HSCs (FL-HSCs) are present in normal numbers in *Bmi1*-deficient mice, the number of FL-HSCs from *Mph1/Rae28*-deficient mice progressively decreases from E14.5 onward (Ohta et al., 2002; Park et al., 2003). However, both *Bmi1*- and *Mph1/Rae28*-deficient FL-HSCs are impaired in their proliferative and self-renewing capacity as was assessed in vitro and in vivo. (Ohta et al., 2002; Lessard and Sauvageau, 2003; Park et al., 2003). In the adult mouse, *Bmi1*-deficient HSCs are found less frequent and display strong defects in proliferation and self-renewal. However, they do give rise to a normal number of multipotent progenitors, suggesting that in vivo, the loss of stem cells is compensated by an increased formation of their immediate descendants (Park et al., 2003). Curiously, loss of *Mel-18*, a homolog of *Bmi1*, appears to enhance HSC self-renewal suggesting that the relative levels of a constituent in the complex dictate HSC self-renewal capacity (Kajiume et al., 2004). Analogous to B cell defects in PcG mutants resulting in part from unresponsiveness to IL-7, *Bmi1*, and *Mph1/Rae28* self-renewal defects might reflect a poor response of mutant HSCs to stem cell growth factors. As to the differences between *Bmi1*- and *Mph1/Rae28*-deficient HSCs, it is likely that fetal and adult HSCs are regulated by different signals and mechanisms, only the latter of which requires *Bmi1*.

Unexpected Functions for PRC2 PcG Genes in Hematopoietic Cells

Another question is whether PRC2 is also involved in HSC function. Panhematopoietic ablation of Ezh2 revealed a block in early B cell differentiation, but no obvious effects on other lineages (Su et al., 2003). Perhaps *Ezh2* is not required for normal HSC function, which would be remarkable since Ezh2 function is indispensable for ES cells. A more trivial explanation though is functional redundancy by *Ezh1* (Su et al., 2003).

Surprisingly, *Eed* heterozygous or hypomorphic animals display a myeloid and lymphoid overproliferation phenotype (Lessard et al., 1999). Furthermore, they have an increased incidence and decreased latency of chemical-induced thymic lymphoma (Richie et al., 2002). These results suggest differential functions for PcG proteins in the control of hematopoietic cell proliferation: a negative function for PRC2-Eed containing complexes and a positive function for PRC1-Bmi1-Mph1/Rae28-containing complexes. Since Eed and Bmi1 are involved in distinct complexes and at least in *Drosophila* autoregulation loops exist, loss of *Eed* might lead to an overrepresentation of the Bmi1-Mph1/Rae28-containing stimulatory complexes, ultimately resulting in hematopoietic overproliferation.

Bmi1 Is Necessary for Neural Stem Cell Renewal and Early Neural Progenitors

Throughout adult life, two major neurogenic regions persist: the subventricular zone (SVZ) of the lateral ventricle wall and the subgranular zone of the hippocampus (Doetsch, 2003). With appropriate growth factors, cultured neural stem cells (NSCs) grow as adherent colonies or as "neurospheres," floating clusters of stem cells and their progenitors. The precise cellular origin of neural stem cells and the nature of neurospheres in vitro is a subject of controversy. Accumulating evidence supports that in vivo, NSCs are astrocyte-like, glial fibrillary acidic protein (GFAP)-positive cells (Doetsch et al., 1999, Seri et al., 2001; Imura et al., 2003). However, in vitro neurospheres can also be derived from more mature transit-amplifying cells (Doetsch et al., 2002).

Prospective defects in the stem cell compartment of the nervous system of *Bmi1*-deficient mice could be deduced from their neurological phenotype (van der Lugt et al., 1994). The main problem appears to lie in the cerebellum, which is reduced in size due to severe loss of both molecular and granular layer neurons. Occasionally, degenerated neurons can be observed in the hippocampus, as well as extensive gliosis of the major white matter tracts. Strikingly, *Bmi1*-deficient mice become depleted of cerebral NSCs postnatally, indicating an in vivo requirement for *Bmi1* in NSC renewal (Molofsky et al., 2003). In contrast to the cerebellum, cerebral development is largely completed around time of birth. An intriguing possibility therefore is that similar to fetal versus adult hematopoietic stem cells, neurogenesis during embryogenesis might be under separate control from that of adult NSCs and cerebellar progenitor cells, allowing "normal" development prebirth. Another possibility is that in response to stem cell depletion, progenitors receive signals from the niche that instruct them to "reprogram" their gene expression profile and become committed to an unfamiliar compensatory task. In line with this, *Bmi1*-deficient committed progenitors are present at normal frequencies and proliferate at a similar rate as wild-type progenitor colonies (Molofsky et al., 2003).

A clue as to which cell-extrinsic signals modulate PcG function in the nervous system came from a study of cerebellar granule neuron progenitors (CGNPs). Development of the cerebellum is guided by Purkinje cell excreted Shh, which drives a postnatal wave of proliferation of CGNPs in the external granular layer (EGL). In time, these cells become postmitotic, migrate inward, and differentiate into cerebellar granule neurons (Goldowitz and Hamre, 1998). At the molecular level, Shh acts by binding to its receptor, Patched (Ptch), releasing

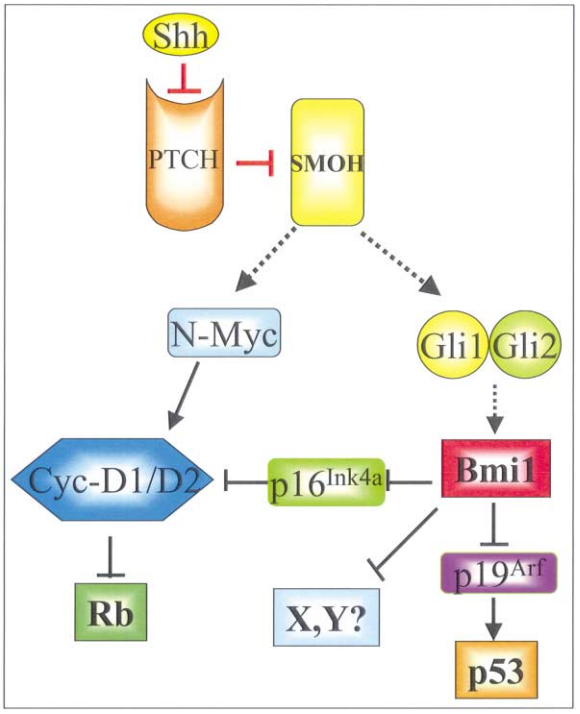

Figure 1. Working Model of *Bmi1* in the *Shh* Pathway in Cerebellar Progenitors

Bmi1 acts as a downstream effector of *Shh* signaling, required for full proliferation/self-renewal of cerebellar progenitor cells, in combination with activation of *N-myc*/*CyclinD2*. Thus, *Shh* is able to modulate both p*Rb* (via *N-myc* and *Bmi1*/*p16*^{Ink4a}) and *p53* (via *Bmi1*/*p19*^{Arf}). In addition, the involvement of other targets important in cerebellar biology remains a possibility (*Shh*, Sonic Hedgehog; *PTCH*, Patched; *SMOH*, Smoothened).

the inhibition of Ptch on the transmembrane protein Smoothened (Figure 1). This ultimately results in downstream signaling in the nucleus by Gli transcription factors (Rubin and Rowitch, 2002; Pasca di Magliano and Hebrok, 2003). Proliferation of CGNPs in vitro can be induced by Shh and is accompanied by induction of *N-myc, cyclin D1* and *D2*, in agreement with the observed defects in EGL of mice deficient for these genes (Ciemerych et al., 2002; Knoepfler et al., 2002; Kenney et al., 2003). It was found that *Bmi1*-deficient CGNPs have an impaired proliferative response upon Shh stimulation. Importantly, in the same cells, *Bmi1* expression can be induced by both Shh and Gli1 (Leung et al., 2004). These findings explain the reduced number of cerebellar granule neurons in *Bmi1*-deficient mice as a result of an attenuated Shh response due to lack of one of its downstream targets, *Bmi1* (Figure 1). Notably, in both hippocampal progenitors and SVZ neural stem cells, *Shh* is important for proliferation and renewal (Lai et al., 2003; Machold et al., 2003). Moreover, hematopoietic stem cells are regulated by this signaling route as well. *Indian hedgehog*, another member of the *Hh* family, activates hematopoiesis whereas *Shh* influences the proliferation of HSCs (Bhardwaj et al., 2001; Dyer et al., 2001). Whether *Bmi1* is an *Hh* target in other cell types and tissues awaits further investigation.

Other PcG Genes in the Nervous System

Studying the effects of PRC2 members on neurogenesis is complicated due to early embryonic lethality of knockout mice (Schumacher et al., 1996; Donohoe et al., 1999; O'Carroll et al., 2001). However, a subset of embryos heterozygous for *YY1* displays neural tube defects resembling exencephaly, pointing to a role for *YY1* in nervous system development (Donohoe et al., 1999). Interestingly, in chimeras with *Eed*-deficient cells, contribution to the forebrain appears to be specifically reduced (Morin-Kensicki et al., 2001). For other PRC1 members, a function in NSC renewal has not yet been reported despite high expression of several PcG genes in the developing nervous system (Akasaka et al., 2001; Schoorlemmer et al., 1997). This is most remarkable for *Mel-18*, which can compensate for *Bmi1* deficiency in many other organs, suggesting a high degree of functional specification between these recently diverged PcG genes. (Akasaka et al., 2001). The *Bmi1* cerebellar phenotype becomes more aggravated in *Bmi1*; *Rnf2* doubly deficient compound mice (Voncken et al., 2003), showing synergistic interactions between these two PRC1 proteins in the nervous system. Notably, deficiency of *Mph1/Rae28* leads to ocular abnormalities and malformations of neural-crest-derived tissues (Takihara et al., 1997). This is intriguing, since *Mph1/Rae28* is essential for renewal of HSCs and is highly expressed in ES cells (Loring et al., 2001; Ohta et al., 2002; Fortunel et al., 2003). It is plausible that, analogous to the situation with *Bmi1*, *Mph1/Rae28* functions in NSC renewal as well.

The Tumor Suppressor Locus *Ink4a/Arf* Is an Important PcG Target in Stem Cells

Bmi1 is a potent negative regulator of the *Ink4a/Arf* locus in mouse embryonic fibroblasts (Jacobs et al., 1999). This locus encodes the cell cycle regulators and tumor suppressors *p16*^{Ink4a} and *p19*^{Arf} (*p14*^{ARF} in humans). p16^{Ink4a} affects the retinoblastoma protein pRb by inhibiting the cyclin D-Cdk4/6 kinase complexes. Hypophosphorylated pRb will sequester E2F transcription factors and actively repress their target genes, ultimately leading to cell cycle arrest, senescence, or apoptosis depending on context (reviewed in Sharpless and DePinho, 1999). p19^{Arf} binds MDM2 and thereby inhibits degradation of the p53 transcription factor. This results in activation of p53 target genes, leading to cell cycle arrest and apoptosis (reviewed in Lowe and Sherr, 2003). Both *p16*^{Ink4a} and *p19*^{Arf} expression can be induced by aberrant mitogenic or oncogenic signaling, as well as upon tissue culture stress, thus functioning as a potent fail-safe mechanism preventing cells from engaging in uncontrolled proliferation.

Analogous to the situation in fibroblasts, in neural and hematopoietic stem cells lacking *Bmi1,* p16^{Ink4a}, and p19^{Arf} are upregulated (Molofsky et al., 2003; Park et al., 2003). Conversely, *Bmi1*; *Ink4a/Arf* doubly deficient animals display a substantial rescue of the stem cell defects, as indicated by restored lymphocyte counts and normal cerebellar size (Jacobs et al., 1999). In neurosphere assays, loss of *p16*^{Ink4a} alone in *Bmi1*-deficient NSCs partially rescues self-renewal capacity, suggesting that also p19^{Arf} may additionally help to limit the self-

renewal of these cells (Molofsky et al., 2003). Since the *Ink4a/Arf* locus strongly responds to tissue culture stress, careful in vivo analysis is needed to firmly establish the relative contribution of these proteins to the *Bmi1*-deficient phenotype. Nevertheless, the emerging role for this locus in restricting the potentially dangerous self-renewal divisions of stem cells through control of PcG signaling is an exciting connection. In line with PcG proteins acting in multiprotein complexes, additional PcG members regulate *Ink4a/Arf*, such as *Mel-18* and *Cbx7* (Jacobs et al., 1999; Gil et al., 2004). Further, the developmental arrest of *Rnf2*-deficient embryos is accompanied by an upregulation of $p16^{Ink4a}$ and is partially bypassed in an *Ink4a/Arf*-deficient background (Voncken et al., 2003).

There is additional evidence supporting a more general function for *Ink4a/Arf* in stem cells. First, enforced expression of $p16^{Ink4a}$ or $p19^{Arf}$ in normal HSCs results in proliferative arrest or p53-dependent cell death, respectively (Park et al., 2003). Second, *Ink4a/Arf*-deficient bone marrow cells proliferate better than wild-type cells in tissue culture assays (Lewis et al., 2001), although in vivo reconstitution assays have not been reported as of yet. Still, it is clear that stem cell-cycle control does not entirely depend on this locus, as the *Ink4a/Arf*-deficient mouse appears relatively normal. This suggests the existence of multiple additional levels of regulation. Indeed, the cell cycle inhibitors $p21^{Cip1}$ and $p18^{Ink4c}$ have already been reported to control stem cell proliferation (Cheng et al., 2000a; Yuan et al., 2004). Another cell cycle inhibitor, $p27^{Kip1}$, affects proliferation of progenitors both in the hematopoietic system and the cerebellum (Cheng et al., 2000b; Miyazawa et al., 2000). An important future goal will be to unravel the relative contribution of the different cell cycle inhibitors to stem cell proliferation and their respective regulatory cascades.

It is important to mention the profound differences in cell cycle regulation between stem cells early in development versus adult stem cells or differentiated cells. This is best exemplified in ES cells, which have a very short G1 phase with almost undetectable levels of hypophosphorylated pRb (reviewed in Burdon et al., 2002). In addition, ES cells do not arrest upon $p16^{Ink4a}$ overexpression. Furthermore, p53 remains largely cytoplasmic and appears not to participate in DNA damage responses in ES cells. *Ezh2* was postulated to regulate rapid cell proliferation during the transition from pre- to postimplantation stages (O'Carroll et al., 2001). Since it is unlikely that defects in *Ezh2*-deficient blastocysts result from *Ink4a/Arf* deregulation, there must be other as yet unknown PcG targets important in ES cells.

The best argument that not all PcG defects are associated with *Ink4a/Arf* deregulation is probably the incomplete rescue of multiple defects in *Bmi1*-deficient mice upon loss of the *Ink4a/Arf* locus (Jacobs et al., 1999). Also, *EZH2* knockdown in fibroblasts does not upregulate $p14^{ARF}$ levels (Bracken et al., 2003). Furthermore, *Ink4a/Arf* expression is not altered in the hematopoietic system of *Eed* mutant mice (Lessard et al., 1999), and moreover, $p16^{Ink4a}$ or $p19^{Arf}$ expression is not altered in E14.5 *Mph1/Rae28*-deficient FL-HSCs (Ohta et al., 2002). The stoichiometry of early acting PcG complexes, which are relatively abundant in Mph1/Rae28, likely differs from the complexes found later in development, which contain more Bmi1 and are therefore capable of keeping *Ink4a/Arf* levels in check.

Are *Hox* Genes Implicated as PcG Targets in Stem Cells?

The skeletal defects of PcG mutant mice revealed PcG genes as *Hox* gene regulators (van der Lugt et al., 1994). *Hox* genes also determine cell fate in several other tissues. *Hoxa5*, *Hoxa9*, *Hoxa10*, *Hoxb3*, *Hoxb4*, and *Hoxb6* are important in HSCs (Owens and Hawley, 2002). For instance, *Hoxb4* overexpression enhances the self-renewal of HSCs (Antonchuk et al., 2002). Reciprocally, *Hoxb4*-deficient mice have disturbed hematopoiesis (Bjornsson et al., 2003). Extrapolating from their classical role in axial patterning, a simple view could be that PcG proteins repress *Hox* genes in differentiated hematopoietic cells. Remarkable in that respect, expression of a panel of relevant *Hox* genes was not altered in the hematopoietic organs of *Eed*, *Bmi1*, or *Mph1/Rae28* mutant mice (Lessard et al., 1999; Ohta et al., 2002; Lessard and Sauvageau, 2003). In contrast, *Bmi1* regulates three *Hox* genes in NSCs: *Hoxd8*, *Hoxd9*, and *Hoxc9* (Molofsky et al., 2003). In vivo, *Hox* genes coordinate the patterning of the nervous system by influencing motor neuron diversification in the hindbrain and spinal cord (reviewed in Guthrie, 2004), though no robust expression has been described in NSCs (Santa-Olalla et al., 2003). Possibly, loss of *Bmi1* induces loss of the undifferentiated nature of NSCs, as reflected by reactivated *Hox* gene expression.

Self-Renewal Capacity of Cancer Stem Cells Is Regulated by *Bmi1*

The hypothesis that cells with stem cell characteristics or "cancer stem cells" (CSCs) "drive" cancer proliferation and progression is receiving increasing support. This hypothesis offers an explanation for the extensive proliferative capacity of tumor cells, resembling self-renewal of stem cells. In addition, it explains at least in part why tumors often consist of heterogeneous cell populations: a small proportion of proliferating stem cells and a majority representing differentiated daughter cells. Stem cells also form attractive candidates as the origin of cancers, as in their long lifespan mutations and epigenetic changes can accumulate allowing increasing evolution toward malignancy. Indeed, only a small percentage of leukemic cells in patients have strong proliferative capacity. Moreover, a small subpopulation of acute myeloid leukemia (AML) cells was able to give rise to leukemia in secondary recipients. This subpopulation could be identified using surface markers and these leukemic stem cells turned out to closely resemble human hematopoietic stem cells (Lapidot et al., 1994; Bonnet and Dick, 1997). Recently, cancer stem cells were also found to reside within solid tumors including several types of brain cancers (Hemmati et al., 2003; Singh et al., 2003) and breast carcinomas (Al-Hajj et al., 2003).

To date, it remains difficult to determine whether a cancer stem cell indeed is derived from a somatic stem cell, from a (de)differentiated progenitor or even a terminally differentiated cell. Notably, in vitro mature *Ink4a/Arf*-deficient astrocytes can regain neural stem cell-like characteristics upon activated epithelial growth factor

(EGF) signaling (Bachoo et al., 2002). Moreover, these cells cause glioblastoma multiforme when injected into the brains of recipient mice, illustrating their functional dedifferentiation. Additionally, somatic cell nuclear transfer experiments highlight a remarkable reprogramming capacity, not only of "normal" cell nuclei but also of cancer cell-derived nuclei, such as from medulloblastoma or melanoma (Li et al., 2003; Eggan et al., 2004). This illustrates that apart from genetic lesions, epigenetic processes by and large dictate (de)differentiation processes, which opens the challenge to identify the relevant players.

The *Bmi1* PcG gene was originally identified as an oncogene inducing B or T cell leukemia (Haupt et al., 1991; van Lohuizen et al., 1991). *BMI1* is overexpressed in several human cancers, including mantle cell lymphoma, colorectal carcinoma, liver carcinomas, and nonsmall cell lung cancer (Beá et al., 2001; Vonlanthen et al., 2001; Kim et al., 2004; Neo et al., 2004). Recent studies showed elegantly that *Hoxa9-Meis1*-transduced cells of *Bmi1*-deficient mice were able to generate AML in primary recipients, but unlike wild-type-derived AML cells, failed to reform AML in secondary recipients (Lessard and Sauvageau, 2003). Additionally, *Bmi1*-deficient hematopoietic progenitors are resistant to transformation by the chimeric oncogene *E2a-Pbx1*, a translocation frequently found in acute human pre-B lymphoblastic leukemias (Smith et al., 2003). Interestingly, overexpression of *E2a-Pbx1* induces *Bmi1,* providing an alternative way of *Ink4a/Arf* suppression. This may explain the selective absence of loss-of-function mutations of the *Ink4a/Arf* locus in leukemias with *E2a-Pbx1* translocations. Furthermore, cancer stem cells cultured from a panel of pediatric brain tumors showed high expression of *Bmi1* among other stem cells markers (Hemmati et al., 2003). Finally, *Bmi1* is overexpressed in a majority of medulloblastomas, tumors believed to arise from uncontrolled proliferating cerebellar granule cell precursors (Leung et al., 2004). It is possible that overexpression of *Bmi1* not only stimulates rapid proliferation through repression of the *Ink4a/Arf* locus, but, reflecting its function in neural stem cells, also allows the CGNPs to "return to" or maintain a more stem cell-like state. Interestingly, in line with the role of *Bmi1* in proliferation of CGNPs downstream of *Shh*, overexpression was found in those medulloblastomas harboring activated *Shh* signaling (Leung et al., 2004). In addition to contributing to medulloblastoma, misregulation of *Shh* signaling plays a role in multiple types of cancers with presumed cancer stem cell characteristics, including basal cell carcinoma, pancreatic adenocarcinoma, and small-cell lung carcinoma (reviewed in Pasca di Magliano and Hebrok, 2004). It will be important to assess if also in these cancers a connection between Bmi1 and Shh exists.

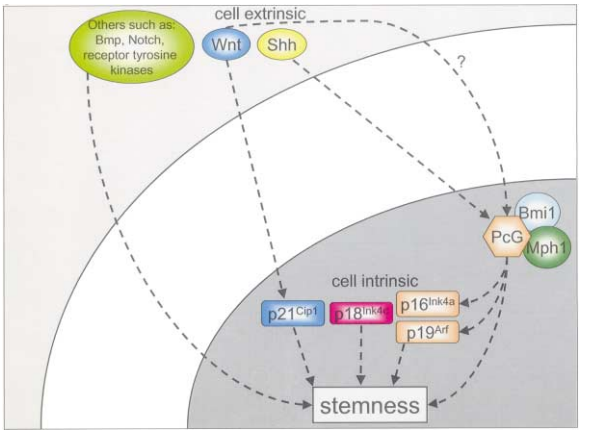

Figure 2. Speculation of How the Niche or Extrinsic Signals Can at Least in Part Regulate "Stemness" by Governing Intrinsic Cell Cycle Regulators

Wnt is in intestine known to regulate $p21^{Cip1}$ via *c-Myc*, however possible links with PcG proteins have also been suggested (see text). *Shh* regulates the PcG complex by increasing *Bmi1* levels, thereby possibly influencing the $p16^{Ink4a}$ and $p19^{Arf}$ cell cycle inhibitors. For other niche signals (such as *Notch* or *BMP*) and cell cycle inhibitors (such as $p18^{Ink4c}$), no such relationships have been described as yet.

Prospective Roles for Other PcG Genes in Cancer Stem Cells

PRC2 members are also associated with cancer. *EZH2* is upregulated in many cancers such as leukemia, prostate cancer, and breast cancer (Raaphorst et al., 2001; Varambally et al., 2002; Bracken et al., 2003; Kleer et al., 2003). Interestingly, high *EZH2* expression localizes to more primitive malignant cell types, often in combination with high *BMI1* expression (Raaphorst et al., 2001). Whether high PcG expression reflects the acquirement of stem cell-like properties and/or influences the self-renewal of cancer stem cells remains to be elucidated. In prostate cancer, high *EZH2* expression is indicative of a metastatic character of the disease and knockdown of *EZH2* in prostate cancer cell lines causes a marked inhibition of cell growth (Varambally et al., 2002). In vitro, *Ezh2* is capable of acting as an oncogene and can be induced by the E2F transcription factors. Curiously, *Ezh2* expression is not cell cycle regulated (Bracken et al., 2003). Furthermore, the marked growth arrest of U2OS tumor cell line upon EZH2 depletion suggests a far more drastic effect than cell cycle deregulation via *INK4a/ARF* or *p53* only.

Another component of PRC2, SU(Z)12, is upregulated in human colon and breast tumors (Kirmizis et al., 2003). Interestingly, the promoter of *SU(Z)12* can be bound by β-catenin/TCF complexes, downstream targets of Wnt signaling. Wnt signaling is essential for stem cell activity in various tissues, such as the hematopoietic system, skin, and intestine (Fuchs et al., 2004). Although highly speculative, it is possible that in skin and intestine and in cancers derived therefrom, PcG could be linked to another important developmental signaling pathway, such as Wnt, through PRC2.

Concluding Remarks

It seems reasonable to designate at least the PRC1 gene *Bmi1,* and probably also *Mph1/Rae28*, as genes intrinsically conferring stem cell characteristics to a cell. However, there is some debate whether such uniformly acting "stemness genes" exist at all, since the search for a stem cell "molecular signature" by comparing the transcriptional profiles of several stem cells and progen-

itors only identified one gene as a common outlier: *integrin α 6* (Ivanova et al., 2002; Ramalho-Santos et al., 2002; Iwashita et al., 2003; Fortunel et al., 2003). Several commentaries have highlighted the technical difficulties associated with these types of assays. Additionally, as cell-extrinsic signals are such important factors in the maintenance of the stem cell pool, it may be crucial to analyze stem cell expression profiles in the appropriate context of the stem cell niche.

It is evident though that *Bmi1* is essential for the self-renewal of hematopoietic, neural, and cancer stem cells, and proliferation of cerebellar granule neuron progenitors (Lessard and Sauvageau, 2003; Molofsky et al., 2003; Park et al., 2003; Leung et al., 2004). This is well in line with the specific defects observed in mice that have lost *Bmi1* expression in all cells from fertilization onward, which can all be traced back to malfunctioning stem cell compartments. Notably, the fact that *Bmi1* is indispensable for the self-renewal of cancer stem cells further stresses the importance of (de)regulation of developmental genes, such as the *PcG* genes, in cancer and stem cell biology.

The *Ink4a/Arf* tumor suppressor locus is one of the targets via which PcG exerts its control over stem cell proliferation (Figure 2). Thus, like other cyclin-dependent kinase inhibitors, *Ink4a/Arf* is also implicated in the developmental control of stem cells. However, other relevant PcG targets must exist. Possibly these are involved in alternative aspects of stem cell identity, such as the prevention of differentiation programs. As with the stemness profiles mentioned above, common PcG targets have not yet been revealed by expression array analysis. Apart from technical difficulties, stoichiometrical differences between the PcG complexes during development might account for altered sets of target genes, providing multiple levels of control.

We propose a model in which a distinct PcG complex confers stemness to cells, as opposed to other flavors of PcG protein complexes, which are required to maintain differentiated cell fates. The composition of this complex might vary between embryonic and adult stem cells. We assume that *Mph1/Rae28* is more important in embryonic stem cells relative to *Bmi1*, which seems more required for adult stem cells. The histone methyltransferase activity of Ezh2 in the PRC2 complex is essential for the self-renewal of ES cells, but the exact role of PRC2 in other stem cells remains to be investigated. The link between Shh signaling and PcG through *Bmi1* provides a first glimpse of connections between external signaling morphogens and cell-intrinsic epigenetic mechanisms controlling cell fate programs (Figure 2). The emerging complexity of PcG silencers, subdivided into at least two functionally different complexes and harboring many homologs, provides excellent opportunities for fine-tuning the output resulting in appropriate gene expression patterns and cell fate maintenance. Moreover, PcG signaling might also influence the characteristics of the niche cells, providing yet another layer of control. Future research is likely to reveal further connections between developmental morphogens that regulate cell fate such as Shh, Wnt, and Notch, and cell-intrinsic relays, such as PcG, which are able to mediate stable epigenetic regulation of gene expression programs.

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Chapter 2

Introduction

Ink4a/Arf in cancer, aging and development

Ink4a/Arf in cancer, aging and development

Cell proliferation is a very critical process, which if not executed timely can lead to a multitude of defects. On the one hand, correct cell cycling is needed to ensure proper development. Therefore a tight balance between differentiation and proliferation is maintained. On the other hand, hyperproliferation leads to cancer, a disease threatening almost one third of our population. Cancer research has identified numerous genes deregulated in cancer, however inactivation of the retinoblastoma (Rb) and p53 pathways is central to all cancers both *in vivo* in human and mouse tumors and *in vitro* in tissue culture cells. Interestingly, the gene second most mutated next to *p53* in cancer is *Ink4a/Arf*. This introduction highlights recent research on *Ink4a/Arf*, implicating it not only in cancer but also in aging and development.

The Ink4a/Arf locus and role of its gene products in the p53 and Rb pathways

The genomic organization of the *Ink4a/Arf* locus is extraordinary (figure 1). It encodes for two proteins, $p16^{Ink4a}$ and $p19^{Arf}$ ($p14^{ARF}$ in human). Both genes use an alternative first exon, exon 1α and exon 1β for *Ink4a* and *Arf* respectively (Serrano et al. 1993; Quelle et al. 1995). Exons 2 and 3 are shared by both genes, be it in a different reading frame. These alternative reading frames lead to production of two totally unrelated proteins. Another member of the *INK4* family, *Ink4b*, is located upstream of *Ink4a/Arf*. Corroborating an important role for these genes, numerous studies have described genetic alterations at 9p21, the chromosomal location of these genes, in a variety of human cancers (reviewed in Ruas and Peters, 1998).

Both *Ink4a* and *Arf* are important tumor suppressors and cell cycle regulators (figure 2). $p16^{Ink4a}$ was identified initially as a CDK4-associated protein capable of inhibiting CDK4/6-mediated phosphorylation of Rb (Serrano et al., 1993). $p16^{Ink4a}$ expression results in cell cycle arrest because hypophosphorylated Rb binds to and represses E2F transcriptional activity, resulting in G1 arrest (reviewed in Sherr, 2001). $p19^{Arf}$ associates directly with Mdm2 and blocks its ability to interact productively with p53, both by localizing Mdm2 within the nucleolus and by inhibiting Mdm2's E3 ubiquitin protein ligase activity (Honda and Yasuda, 1999; Weber et al., 1999). Thus $p19^{Arf}$ expression leads to stabilized and activated p53, which initiates a transcriptional programme that can trigger either cell-cycle arrest or apoptosis. However, cell cycle exit in response to $p19^{Arf}$-p53 activation is also dependent on active repression by E2F/Rb-complexes (Rowland et al., 2002). This is because overexpression of a mutant of E2F, that is defective in Rb binding and transactivation but can bind DNA, immortalizes MEFs despite *Arf* induction. Moreover, the latter cells are resistant to overexpression of *Arf* or *p53*. Recent studies suggest a $p19^{Arf}$ function that is seemingly independent of Mdm2 and p53 (Qi et al., 2004). That is, $p19^{Arf}$ can negatively regulate Myc's transcriptional activity through a direct physical interaction, impacting only on Myc's transactivating but not repressing functions. This has been suggested as a form of feedback control whereby $p19^{Arf}$ blocks the proliferation induced by c-Myc.

As both proteins are important in cell cycle regulation, a major effort of the past decade has been to unravel their individual contribution to different

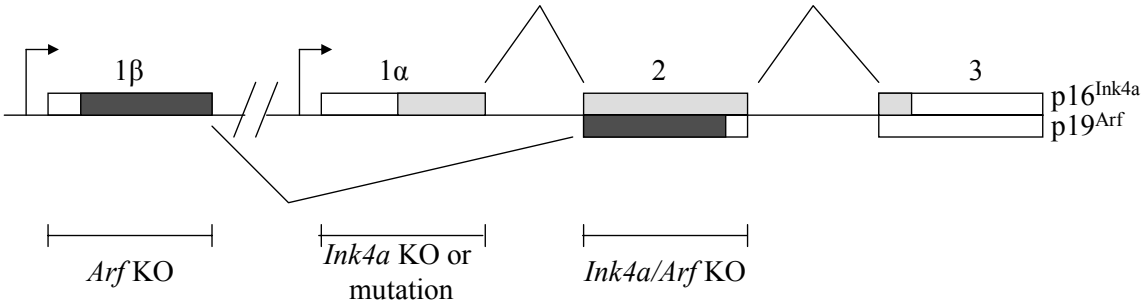

Fig. 1 Structure of the *Ink4a/Arf* locus and knockout strategies in mice as indicated below. Two different promoters (arrows) drive expression from two alternative first exons (1α and 1β). These exons are spliced to the same acceptor site in exon 2, which is translated in alternative frames (light shading represent *Ink4a* coding sequences whereas dark shading represent *Arf* coding sequences). Note that drawing is schematic and not to scale. Exon 1α and 1β are separated by more than 15 kb (adapted from Quelle et al., 1995).

cancers. However, as they are genetically interlinked, careful knockout strategies had to be developed (figure 1). In essence there are three different mouse strains described. First, the *Ink4a/Arf* knockout mouse, where exon 2 of the locus is ablated, disrupts both the *Ink4a* and *Arf* reading frames (Serrano, 1996). Second, disruption of exon 1β was used to generate mice lacking *Arf* alone (Kamijo, 1997; Sharpless et al., 2004). Third, *Ink4a* knockout animals have been obtained by two different strategies. One group has 'knocked in' a point mutated *Ink4a* gene, thereby replacing the wild-type gene with an altered form containing a translational termination codon that does not affect the $p19^{Arf}$ protein (Krimpenfort, 2001). The other group specifically deleted exon 1α, taking care to leave at least all known splice sites and cis-regulatory sequences intact (Sharpless, 2001). The relevant phenotypes of these mice and cells derived thereof will be described below.

Regulation of Ink4a/Arf expression

As *Ink4a/Arf* is such an important and central locus in cell cycle regulation and cancer, an important aspect is regulation of the locus. First of all, a number of oncogenes (*E2F1*, *Myc*, *E1A*, *Abelson*, and constitutively active *Ras*, *Raf*, *MEK* or *β-catenin*) induce $p16^{Ink4a}$ and/or $p19^{Arf}$(Palmero et al., 1997; Bates et al., 1998; de Stanchina et al., 1998; Zindy et al., 1998; Cong et al., 1999; Damalas et al., 2001). Generally this is thought of as a cancer protection mechanism: induction of cell cycle inhibitors by hyperproliferative signals will prevent the outgrowth of oncogene expressing cells. Second, *Dmp1* and *DAP kinase* were found to be activators of $p19^{Arf}$ expression and itself to be tumor suppressor genes (Inoue et al., 1999; Inoue et al., 2000; Raveh et al., 2001). Furthermore, a few repressors of $p19^{Arf}$ have been identified that are also oncogenes, such as: *Bmi1*, *TBX2*, *TBX3*, *Twist*, *JunD*, and *p53* itself (Robertson and Jones, 1998; Jacobs et al., 1999; Maestro et al., 1999; Jacobs et al., 2000; Weitzman et al., 2000; Brummelkamp et al., 2001). Interestingly, the Polycomb group gene and chromatin modifier *Bmi1* is capable of both repressing *Ink4a* and *Arf*. TBX2 is a transcriptional regulator of *Arf* however through an as yet unknown mechanism it also negatively regulates *Ink4a* and *Ink4b* RNA expression. Whereas Bmi1 is a stronger regulator of $p16^{Ink4a}$ and to a lesser extent $p19^{Arf}$ protein, TBX2 is strongly repressing $p19^{Arf}$ and only moderately affecting $p16^{Ink4a}$ protein.

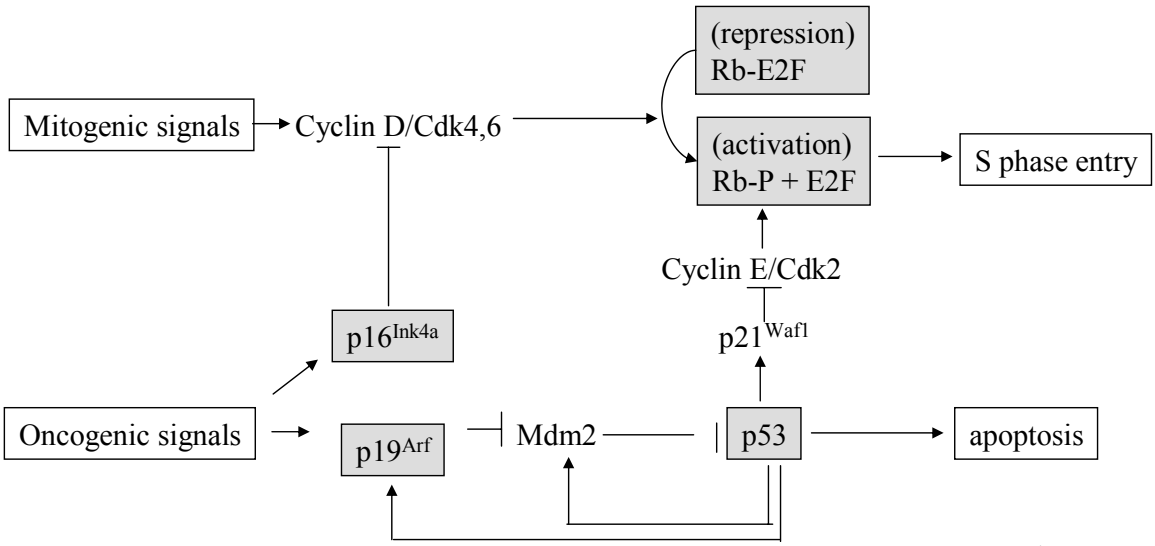

Fig. 2 Classical model of the *Ink4a/Arf* network. Oncogenic signals can stimulate both $p16^{Ink4a}$ and $p19^{Arf}$ expression. $p16^{Ink4a}$ is an inhibitor of the cyclin D/Cdk4,6 complexes that are normally activated by mitogenic signals. These cyclin dependent kinases phosphorylate Rb, releasing Rb from E2F transcription factors, resulting in S phase entry. $P19^{Arf}$ expression negatively regulates Mdm2, thereby releasing the inhibition of Mdm2 on p53. This results in activation of p53 target genes. P53 induces apoptosis or contributes to cell cycle arrest, via induction of $p21^{Waf1}$, a concomitant inhibition of cyclin E/Cdk2 resulting in Rb hypophosporylation. Note that p53 also induces feedback loops by inducing $p19^{Arf}$ and Mdm2. This scheme is an oversimplification as for instance *Arf* expression has been shown to be under the control of both E2F-activating and E2F-repressing complexes.

Although these aspects are not fully understood, dual regulation of both *Ink4a* and *Arf* by Bmi1 and TBX2 suggest there is a locus control region involved in keeping both genes in check. Moreover, these findings may hint at a role for the unusual intimate relationship of *Ink4a* and *Arf*, as their genomic organization may have evolved to allow co-regulation. Another considerable notion is that several factors are able to govern *Ink4a/Arf* expression. Possibly, this enables regulation in different tissues and at different developmental stages. A downside is the multiple possibilities to encounter aberrancies or mutations in this regulation, leading to cancerous outgrowth.

Ink4a/Arf deregulation in cancer

The *Ink4a/Arf* locus is found mutated or deregulated in a wide variety of cancers and a major effort has been to unravel the relative contributions of both proteins to different types of cancer. Although attempts have been made to model these in the various mouse models, detailed studies of human cancers are still needed as it turns out that there are profound differences between the effects of mouse and human *Ink4a/Arf* mutations in tumorigenesis.

In humans, $p16^{Ink4a}$ is thought of as the predominant tumor suppressor. Although most mutations of the locus disrupt both *INK4A* and *ARF* function, mutations are described that specifically target *INK4A* yet spare *ARF* (Rozenblum et al. 1997; Liu et al. 1999; Lal et al. 2000; Lynch et al. 2002). With the development of RNAi a tool was generated to study contribution of both proteins to growth of human cells. Although $p14^{ARF}$ suppression does enhance proliferation of human fibroblasts, it has little tumorigenic effect

(Voorhoeve and Agami, 2003). Underscoring its importance as a tumor suppressor, $p16^{INK4A}$ suppression synergizes with p53 suppression in growth and transformation of human fibroblasts. Nevertheless, these studies were performed in fibroblasts and as differences between cell types are expected, a future goal should be to address these points in different human cell types.

In mice, $p19^{Arf}$ is generally believed to be the more potent tumor suppressor. In mouse embryonic fibroblasts, loss of *Arf* but not *Ink4a* leads to immortalization (Kamijo et al, 1997). *In vivo*, this is supported by the rapid and spontaneous development of lymphomas in both *Ink4a/Arf*-/- and *Arf*-/- mice (Serrano et al., 1996; Kamijo et al., 1997). In contrast, *Ink4a*-/- mice are less tumor prone (Krimpenfort et al., 2001; Sharpless et al., 2001). Interestingly, there are a few notable differences between the two *Ink4a* mutant mice. Whereas no spontaneous tumors and no transformation by oncogenic Ras were observed in mice and MEFs with the mutant *Ink4a* allele (Krimpenfort et al., 2001), *in vivo* and *in vitro* cells with a deletion of exon 1α undergo spontaneous immortalization at low frequency (Sharpless et al., 2001). Although these differences could be attributed to multiple differences between the two experimental set-ups (reviewed in Sherr, 2001), a bit underexposed topic is the effect of deletions in the *Ink4a/Arf* locus on expression of either protein. It was known that the original *Arf* knockout mice have elevated $p16^{Ink4a}$ expression (Kamijo et al., 1997). Therefore, a more careful deletion approach was undertaken, however although less severe still a small $p16^{Ink4a}$ induction could be observed in the *Arf* knockout cells (Sharpless, 2004). Although in *Ink4a* MEFs there is no evidence for deregulated *Arf* expression, this does not exclude that in other cells this is the case.

Despite low spontaneous tumor development in *Ink4a* mutant mice, no doubt exists that in mice *Ink4a* contributes to tumorigenesis too. For instance, spontaneous tumor development in both *Ink4a/Arf*-/- and *Ink4a*-/- mice gives rise to histiocytic (B cell) lymphomas whereas *Arf*-loss leads to lymphocytic (T cell) lymphomas and epithelial tumors (Serrano et al., 1996; Kamijo et al., 1997; Sharpless et al., 2004). Also, loss of either *Ink4a/Arf* product can cooperate with RAS activation to produce clinically indistinguishable melanomas (Sharpless et al., 2003). Analysis of those melanomas provided evidence that loss of both genes is required and cooperates in these tumors. As the above described mice develop tumors rapidly, contribution of *Ink4a/Arf* loss to other tumors is difficult to assess. Therefore, conditional knockout approaches have been undertaken to validate the relevance of *Ink4a/Arf* loss in tissues other than lymphocytes. An important advantage of such an experimental system is that *Ink4a/Arf* loss can be combined with other relevant genetic lesions, which are required for tumorigenesis. For instance, pancreatic specific targeting has shown that activation of Kras combined with *Ink4a/Arf* deficiency potently induces ductal pancreatic adenocarcinoma (Aguirre, 2003).

In conclusion, the studies mentioned above already point to a different requirement of *Ink4a* versus *Arf* silencing in different cancers. As many regulators of the locus have been identified, a challenging future task will be to unravel cancer-specific aberrancies in the regulatory cascades as well.

Senescence and aging

The *Ink4a/Arf* locus has long been recognized as the mediator of a special form of growth arrest, termed senescence. Under stressful conditions, such as *in vitro* culture or oncogenic signalling, mouse embryonic fibroblasts upregulate $p16^{Ink4a}$ and $p19^{Arf}$ expression, thereby halting cell cycle progression. Sharpless and DePinho have postulated that the protection against oncogenesis via upregulation of *Ink4a/Arf* comes at the expense of aging (Sharpless and DePinho, 2004). Stem cells are under the constant surveillance of growth inhibitory actions of $p16^{Ink4a}$ and $p19^{Arf}$, reflecting the tight protection against cancerous outgrowth. Indeed, loss of either *Ink4a* or *Arf* or both significantly enhances neural stem cell self-renewal, illustrating tight stem cell control mechanisms (Molofsky et al., 2003; chapter 5). Analogous to the aging of fibroblasts that is accompanied by decreased Bmi1 expression and accumulating $p16^{Ink4a}$ and $p19^{Arf}$ levels (Itahana et al, 2003), similar expression changes might also occur in stem cells. As a result of this, stem cells might senesce contributing to aging of the involved organism. Indeed *Bmi1*-/- mice, which have highly elevated $p16^{Ink4a}$ and $p19^{Arf}$ levels, do suffer from stem cells that have impaired self-renewal and this phenotype was shown to be critically dependent on *Ink4a/Arf* deregulation (Molofsky et al., 2003; this thesis).
In addition, several studies have made correlations between (stem cell) aging and increased *Ink4a/Arf* expression (Ito et al., 2004; Krishnamurthy et al., 2004; Sun et al., 2004).

With the establishment of *Ink4a/Arf* as a biomarker of aging across multiple tissues, focus of the aging research area will probably move to the relevant transcriptional control of the locus. However, no correlation could be found between overall increased *Ink4a/Arf* expression and lowered Bmi1 expression (Krishnamurthy et al., 2004). As Krihnamurthy et al. studied expression in whole organs and only on RNA level, it is possible that they missed such a connection as effects on Bmi1 levels may only be occurring on protein levels or in stem cells of those tissues. In addition, these results do not exclude that in specific tissues a functional connection between *Bmi-Ink4a/Arf* and aging exists, reflecting tissue-specific regulation of the locus. Nevertheless, caution should still be taken in drawing conclusions about stem cells and senescence as functional data to support that *in vivo* stem cells do undergo aging are still controversial (reviewed in Park et al., 2004). Moreover, increased *Ink4a/Arf* dosage in an *Ink4a/Arf* transgenic mouse did protect from transformation, but did not enhance aging (Matheu et al., 2004). The subtle increase in dosage in this *Ink4a/Arf* transgenic mouse might simply not be enough to cross the threshold needed to inflict an aberrant cell cycle stop. Lastly, indicators of aging are still rather ill-defined and are therefore difficult to compare between different studies.

Ink4a/Arf in development?

The function of *Ink4a/Arf*, to guard against tumorigenesis at the cost of aging, is unlikely to be its only function especially in short-lived species such as the mouse. Nevertheless, all knockout mice described for the locus develop without any major aberrations, suggesting dispensable roles for development. Measurement of any possible aging effects is prohibited by fast tumor development and concomitant death of animals. In line with this, expression of $p16^{Ink4a}$ and $p19^{ARF}$ is only detected postnatally, although subtle patterns may have escaped detection (Zindy et al., 1997; Zindy et al., 2003). A role for $p19^{Arf}$ expression in the vitreous

of the eye has recently been shown (McKeller et al, 2002). More insight in developmental roles for *Arf* is expected by more widespread surveillance of expression in a mouse strain harboring *GFP* under the control of the *Arf* promoter (Zindy et al, 2003). Indeed, several lines of recent evidence suggest roles for *Ink4a/Arf* in development, as $p19^{Arf}$ has been linked to ribosome biogenesis (Bertwistle et al, 2004). Possibly this implicates $p19^{Arf}$ as a kind of metabolic sensor, coupling cell growth to proliferation. For $p16^{Ink4a}$ a role in thymic development and T-cell proliferation has been suggested as *Ink4a* deficient mice suffer from thymic hyperplasia (Sharpless, 2001).

In addition, several developmental genes such as *Bmi1* and *TBX2*/*TBX3* regulate *Ink4a/Arf* expression. Importantly, the defects observed in *Bmi1*-/- mice are relieved upon *Ink4a/Arf* loss (Jacobs et al., 1999). In this thesis we describe tissue specific requirements for *Ink4a/Arf* repression in the context of *Bmi1* deficiency (chapter 5). Our recent analysis of MEFs, thymocytes, splenocytes, cerebellar granule neuron progenitors and cerebral neural stem cells revealed a general role for $p19^{Arf}$ repression whereas $p16^{Ink4a}$ mediated cell cycle control seemed more implicated in undifferentiated cell types (chapter 5). Crossing the *Arf-GFP* transgene into the *Bmi1*-/- background could potentially reveal more sites of Bmi1 mediated $p19^{Arf}$ repression. Although we described a potent and direct repression of *Arf* by TBX2 in induction of proliferation (Jacobs et al., 2000; chapter 2), the recent report of the *Tbx2*-/- mouse did not reveal as yet a developmental role of *Ink4a/Arf* repression by Tbx2 (Harrelson et al., 2004). However, as the *Tbx2*-/- mice is early embryonic lethal, this does not preclude a potential role of Tbx2 in suppressing $p19^{Arf}$ later in development. If so, the *Arf-GFP* transgenic mouse would be a convenient tool to assess whether Bmi1 and Tbx2 are repressing *Ink4a/Arf* in a tissue-specific manner.

Concluding remarks

In conclusion, studies on the *Ink4a/Arf* locus have undoubtedly linked it with cancer, underscoring its dual role in regulating both the p53 and pRb pathways. Notably, more recent research has implicated the locus in cell cycle control of stem cells, thereby connecting it to aging and possibly also tissue repair. But research in this thesis also underscores the importance of correct *Ink4a/Arf* expression in differentiated cells and progenitors (chapter 5). As stem cells are central to at least a subset of cancers but also might be relevant for therapeutic intervention, either by reactivating stem cells *in vivo* or by applying *in vitro* engineered stem cells to patients, further understanding of *Ink4a/Arf* function will also be of critical importance in the coming decades.

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Chapter 3

The T-box repressors *TBX2* and *TBX3* specifically regulate the tumor suppressor gene $p14^{ARF}$ via a variant T-site in the initiator

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The T-box Repressors *TBX2* and *TBX3* Specifically Regulate the Tumor Suppressor Gene $p14^{ARF}$ via a Variant T-site in the Initiator*

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The murine tumor suppressor $p19^{ARF}$ ($p14^{ARF}$ in humans) is thought to fulfill an important protective role in preventing primary cells from oncogenic transformation via its action in the p53 pathway. Several disease-implicated regulators of $p19^{ARF}$ are known to date, among which are the T-box genes *TBX2*, which resides on an amplicon in primary breast tumors, and *TBX3*, which is mutated in the human developmental disorder Ulnar-Mammary syndrome. Here we identify a variant T-site, matching 13 of 20 nucleotides of a consensus T-site, as the essential TBX2/TBX3-binding element in the human $p14^{ARF}$ promoter. Mutant analysis indicates that both the consensus T-box and a C-terminal conserved repression domain are essential for $p14^{ARF}$ repression. Whereas the core nucleotides required for interaction of the archetypal T-box protein Brachyury with a consensus T-site are conserved in the variant site, additional flanking nucleotides contribute to the specificity of TBX2 binding. This is illustrated by the inability of TBX1A or Xbra to activate via the variant $p14^{ARF}$ T-site. Importantly, this suggests a hitherto unsuspected level of specificity associated with T-box factors and corresponding recognition sites in regulating their target genes *in vivo*.

T-box genes are a relatively recently discovered gene family characterized by a 180–200-amino acid DNA-binding T-box domain (reviewed in Refs. 1–3). Genetic studies in species ranging from *Xenopus* to human point to a crucial role for T-box genes in controlling development in a gene dose-dependent manner. This is clearly illustrated by two human syndromes that lead to multiple developmental abnormalities and that are characterized by both patterning defects and hypoplasia: Holt-Oram syndrome and Ulnar-Mammary syndrome. These syndromes are associated with haplo-insufficiency of *TBX5* and *TBX3*, respectively (4, 5). Although data on the functions of the steadily growing family of T-box genes are now emerging, relatively little is known about T-box gene targets and the molecular mechanism underlying specific gene regulation by individual T-box genes. *In vitro* site selection experiments with *Brachyury*, the founder of the T-box gene family, revealed the preference for a palindromic sequence (6) and the crystal structure showed that the Brachyury T-box binds this so-called T-site as a dimer (7). Of all T-box genes only three, *TBX2*, *TBX3* and *Xenopus ET*, are currently known to be transcriptional repressors, probably acting via a putative repression domain (8, 9). *TBX2* was shown to act on the melanocyte-specific *TRP-1* promoter, not by a regular T-site but by two T-half-sites located 200 bp apart (8). Also for other *in vivo* T-box targets a picture is now emerging in which they are controlled by separate T-half-sites.

We recently found TBX2 in a genetic senescence bypass screen to potently down-regulate the $p19^{ARF}$ tumor suppressor, thereby causing efficient immortalization of primary fibroblasts (10). Additionally, in an independent screen, TBX3 was found to have that same activity (11). This placed T-box gene family members for the first time in the regulation of cell proliferation. $P19^{ARF}$ in mice or $p14^{ARF}$ in humans is the alternative transcript encoded by the unusual *INK4a/ARF* locus coding also for $p16^{INK4a}$ (reviewed in Ref. 12). Both of these cell cycle inhibitors and tumor suppressors are implicated in cancer-relevant pathways; $p16^{INK4a}$ acts to inhibit CDK4 and CDK6, thereby preventing inactivating phosphorylation on the Rb tumor suppressor protein (13, 14), whereas $p19^{ARF}$ acts in the p53 pathway (reviewed in Refs. 15 and 16). Induction of $p19^{ARF}$ upon serial passaging of rodent cells leads via MDM2 to a stable and transcriptionally active p53, which by activating its targets can lead to a growth arrest called replicative senescence. $P19^{ARF}$ is also induced upon hyperproliferative signaling by oncogenes such as *c-myc* and adenoviruses *E1A*, *E2F-1*, and Ras^{V12} (17–20). Therefore, $p19^{ARF}$ activation is thought to work as an important fail-safe mechanism, because efficient oncogenic transformation by these factors can occur only when the ARF-Mdm2-p53 pathway is inactivated (15, 16).

Several regulators of the $p19^{ARF}$ promoter are known to date. Of these a number are most likely indirect regulators, such as c-Myc (17), Twist (21), JunD (22), DAP kinase (23), c-Abl (24), E1A (18), and Ras^{V12} (20). However, some of them potentially are direct regulators, for example DMP-1 (25, 26), BMI-1 (27), p53 (28), and E2F1 (19). In accordance with the important cancer-preventing role of $p19^{ARF}$, deregulation of many of these $p19^{ARF}$ transcriptional regulators has been shown to play a role in tumorigenesis. For instance overexpression of Bmi-1, a member of the Polycomb-group of repressor proteins, results in lymphomagenesis and likely contributes to several human malignancies (29, 30). For *TBX2* a potential role in breast cancer development has been suggested because the locus resides on an amplicon present in a subset of primary breast cancers (10). Here we have studied the mechanism and *cis*-requirements for repression of $p14^{ARF}/p19^{ARF}$ as a *bona fide* TBX2 and TBX3 target and found that TBX2 and TBX3 are direct and specific regulators acting via a variant T-site that is present close to the $p14^{ARF}$ transcriptional start site.

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EXPERIMENTAL PROCEDURES

Cell Culture, Growth Curves, and Retroviral Infection—We performed cell culturing of primary mouse embryo fibroblasts (MEFs), retroviral infections, growth curves, and 3T3 schedules as described (10, 27, 31) using the following retroviruses: LZRS-iresGFP (control), LZRS-TBX2-iresGFP, LZRS-TBX2RD-iresGFP, MSCV (control), MSCV-TBX2, MSCV-TBX2RD, MSCV-TBX2TB(R122E,R123E), and MSCV-TBX2TB(A272E). The latter two constructs, the T-box mutants, were generated by PCR and verified by sequencing.

Western Blot Analysis—For protein expression analysis, cell lysates were separated on 9% (for TBX2 levels) or 13% (for $p19^{ARF}$ levels) SDS-PAGE and blotted on nitrocellulose or Immobilon-P membranes (Amersham Biosciences). Analysis was done according to standard methods using enhanced chemiluminesence (Amersham Biosciences). Primary antibodies were R562 (Abcam) for $p19^{ARF}$, 12CA5 for HA-tagged TBX1A, and rabbit polyclonal or mouse monoclonal α-TBX2 antibodies (10).

Repression Assays—COS-7 cells were co-transfected at 40% confluency by calcium phosphate precipitation with 20 μg of reporter plasmid and 0–1 μg of expression plasmid. The TBX2 constructs, TBX2, TBX2RD, TBX2TB(R122E, R123E), and TBX2TB(A272E), were expressed from pCDNA3.1 (Invitrogen), TBX1A-HA from pCMV, Xbra from pEGFP-C1 (32), and E2F1 from pCMV. For the $p14^{ARF}$ promoter, the original CAT reporter constructs described by Robertson and Jones (28) were used. In addition we used the pGL3-basic vector (Promega) in which we recloned a *Hin*dIII/*Sal*I fragment containing the wild-type (−19/+54) $p14^{ARF}$ promoter insert. To circumvent problems with low reporter activity, $p14^{ARF}$ reporters with double (−19/+54) inserts were used because these constructs are more active than those with single inserts (data not shown). Mutants of the T-site were generated by PCR and sequence-verified. The HSV tk reporter was constructed as described (32). Into the *Sma*I site of this construct the same double-stranded oligos as used for electrophoretic mobility shift assays (EMSAs)[1] (see below) were cloned, resulting in a construct with two Brachyury consensus binding sites (B), two variant T-sites (+9/+29), two mutant variant T-sites (+9/+29mut), or one variant T-site including flanking sequences (−14/+35). All transfections contained equal amounts of pCDNA3.1 and included 1 μg of pSV β-galactosidase plasmid (Promega) as an internal transfection control. CAT, luciferase, and β-galactosidase activities were measured 48 h after transfection by standard methods. All transfections were performed in duplicate, and at least two independent experiments were done to confirm reproducibility.

Electrophoretic Mobility Shift Assays—Whole cell extracts were prepared from COS-7 cells transfected with expression vectors (pCDNA3.1) for TBX2, TBX2RD, TBX2TB(R122E, R123E), TBX2TB(A272E), TBX3, L143P TBX3, or Y149S TBX3 or from ARF−/− MEFs infected with LZRS-iresGFP, LZRS-TBX2-iresGFP, or LZRS-TBX2RD-iresGFP retroviruses as described (33). Binding reactions were performed with double-stranded ^{32}P-labeled oligonucleotide probes, cell extracts, antibodies, and competitor oligos as described (8) and resolved on a 4% native polyacrylamide gel. Oligonucleotides used (complementary strand not shown) were: +9/+29, 5′-CTGCTCACCTCTGGTGCCAAA-3′; +9/+29mut, 5′-CTGCTTGACTCTAAGTCCAAA-3′; −14/+35, 5′-AGTTAAGGGGGCAGGAGTGGCGCTGCTCACCTCTGGTGCCAAAGGGCGG-3′; B, 5′-GGGAATTTCACACCTAGGTGTGAAATTCCCT-3′; and E2F, 5′-AATTTAAGTTTCGCGCCCTTTCTCAA-3′. T-site mutations for probe –14/+35 are described in Fig. 2*B*. Probe −19/+54 was generated by PCR with primers 5′-GAGCTCGGCAGCCGCTGCGCCGCCCTTTGGCACCA-3′ and 5′-TCTGCAGTTAAGGGGGCAGG-3′.

RESULTS

Both T-box Mutations and Deletion of the Repression Domain of TBX2 Abrogate $p19^{ARF}$ Repression—To investigate the role of the T-box domain of TBX2 in mediating $p19^{ARF}$ repression, we made two point mutants, TBX2TB(R122E,R123E) and TBX2TB(A272E). These mutations were designed based on the crystal structure of the T-box domain of Brachyury in complex with a consensus T-site (7) and were predicted to disrupt DNA binding. In previous work we showed that TBX2 rescues MEFs from senescence and leads to their immortalization (10). In addition, the high $p19^{ARF}$ levels in *Bmi*−/− MEFs due to abrogation of Polycomb repression of the *INK4a/ARF* locus is efficiently reduced by even low levels of TBX2, resulting in immortalization of these prematurely senescent fibroblasts (27). In contrast, mock-infected wild-type and *Bmi*−/− MEFs as well as cells infected with either one of the T-box mutants enter senescence at the same passage (Fig. 1*A*). Western blot analysis revealed that both T-box mutants are expressed but show the same high levels of $p19^{ARF}$ as senescent cells, in sharp contrast to the severely down-regulated $p19^{ARF}$ levels in TBX2-overexpressing cells (Fig. 1*B*). Mutants of the T-box of TBX3 were also incapable of rescuing MEFs from senescence, as opposed to the readily immortalizing wild-type protein (11). This indicates an essential role for the conserved T-box domain in $p19^{ARF}$ repression.

In our previous study a deletion mutant of TBX2 lacking the repression domain (TBX2RD) proved to be impaired in repressing $p19^{ARF}$, although it still can to some extent down-regulate $p19^{ARF}$, probably because of relatively higher expression levels (Ref. 10 and Fig. 1*D*; also see discussion below). Here we show that this residual activity also induces a prolonged life span of *Bmi*−/− MEFs, which have high $p19^{ARF}$ levels. However these cells still arrest at later passages. This indicates that TBX2RD is not sufficient to fully bypass senescence (Fig. 1*C*) and demonstrates that the repression domain also is a critical contributor to $p19^{ARF}$ repression.

Deletion constructs of the human $p14^{ARF}$ promoter transfected to COS-7 cells revealed that TBX2 and TBX3 act as strong dose-dependent repressors through an element located in the region of –19 to +54 (10, 11). This repression was found to be dependent on the repression domain of TBX2 (10). In agreement with the data described above, TBX2TB(R122E,R123E) is incapable of and TBX2TBA272E is severely impaired in repressing $p14^{ARF}$, whereas both proteins could be detected readily by Western blot (Fig. 1, *E* and *F*). Although both the repression domain and the T-box domain are involved in mediating $p19^{ARF}$ or $p14^{ARF}$ repression, neither of the mutants appears to work as a dominant-negative in transient repression assays, *i.e.* we observed no competition with wild-type TBX2 or TBX3 for $p14^{ARF}$ promoter repression (data not shown).

A Newly Identified Variant T-site in the $p14^{ARF}$ Promoter Is Bound by TBX2 and TBX3—To test whether the inactivating mutations of the T-box reflect impaired binding to the $p14^{ARF}$ promoter we performed electrophoretic mobility shift assays. Incubation of probe −14/+35, spanning part of the repressed region, with either TBX2- or TBX3-overexpressing COS-7 cell extracts resulted in a shifted complex that was not observed using mock-transfected extracts (Fig. 2*A*, *lanes 1*, *2*, 15, and *16*). This complex does contain TBX2 because it is not affected by pre-immune serum but can be supershifted by rabbit polyclonal α-TBX2 antibody to a complex barely capable of entering the gel (Fig. 2*A*, *lanes 3* and *4*). The same supershifts were observed with an independently generated mouse monoclonal α-TBX2 antibody (data not shown and Fig. 2*E*). The presence of such large aggregates upon antibody addition was noted before in case of TBX2 (32). The complex is specific because cold –14/+35 probe is able to compete the complex, whereas an unrelated E2F binding site oligo cannot do so (Fig. 2*A*, *lanes 5* and *7*). A probe containing a Brachyury consensus binding site that has been described to bind TBX2 (Ref. 8 and our own unpublished observations), is also able to compete the complex (Fig. 2*A*, *lane 6*). When overexpressing the repression domain mutant the complex with probe –14/+35 could still be formed and supershifted with α-TBX2 antibody, although less efficiently (Fig. 2*A*, *lanes 8–10*). In contrast, both T-box mutants of

[1] The abbreviations used are: EMSA, electrophoretic mobility shift assay; MEF, mouse embryo fibroblast; HA, hemagglutinin; CAT, chloramphenicol acetyltransferase; HSV, herpes simplex virus; tk, thymidine kinase; GFP, green fluorescent protein; MSCV, mouse stem cell virus; Luc, luciferase; oligo, oligonucleotide.

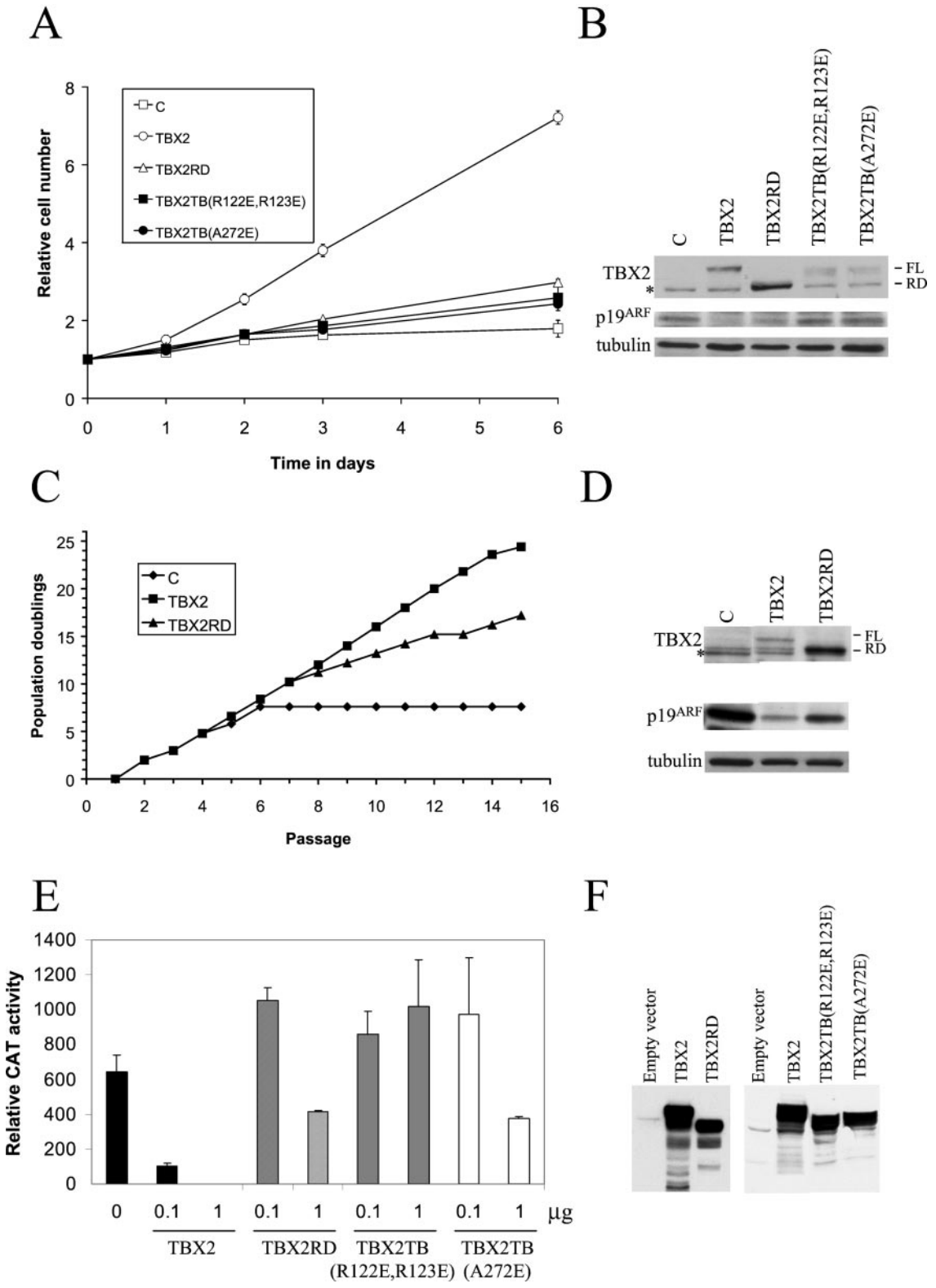

FIG. 1. **Both T-box point mutants and a repression domain mutant of TBX2 are impaired in repressing *p19^{ARF}/p14^{ARF}* and incapable of rescuing MEFs from senescence.** *A*, growth curve of passage 7 wild-type MEFs infected at passage 1 with empty control, TBX2, TBX2RD, TBX2TB(R122E, R123E), or TBX2TB(A272E) MSCV retroviruses. *B*, Western blot analysis of TBX2 (*FL*, full-length protein; *RD*, repression domain deletion mutant) and p19^{ARF} levels of the cells mentioned in *panel A* at passage 7. Tubulin levels serve as loading control. The *asterisk* indicates a band that appears because of non-specific antibody recognition. *C*, proliferation of *Bmi*−/− MEFs infected with control, TBX2, and TBX2RD LZRS-iresGFP retroviruses on a 3T3 schedule. Note that TBX2RD is capable of extending MEF life span although it will not immortalize cells as TBX2 can do. *D*, Western blot analysis of TBX2 and p19^{ARF} levels of *Bmi*−/− MEFs at passage 4. Note that TBX2RD is less effective in down-regulating p19^{ARF} than TBX2, despite its higher expression levels. *E*, dose-dependent repression of the (−19/+54) *p14^{ARF}* promoter CAT reporter by TBX2 is impaired in the case of TBX2RD, TBX2TB(R122E,R123E), and TBX2TB(A272E) mutants. *F*, expression of TBX2 wild-type and mutant proteins in COS-7 cells.

TBX2 and TBX3 were unable to form a complex with probe –14/+35 either in the presence or absence of antibody (Fig. 2*A*, *lanes 11–14* and *17–18*). For TBX2TB(R122E, R123E) this is in agreement with the inability of a TBX2R122A mutant to bind a consensus T-site (32).

The only elements present in the small promoter fragment of $p14^{ARF}$ that is still repressed by TBX2 are an initiator element and an inverse E2F site (28). Although no consensus T-site is present, after a closer inspection of region –19 to +54 we identified a variant T-site matching 13 of 20 of the nucleotides of a consensus palindromic T-site (Fig. 2*B*). In this variant T-site the T-half-sites are spaced one nucleotide apart but are orientated similarly as in the original consensus site. The possibility that this site is a functional T-site is strengthened by the fact that all four nucleotides marked as important DNA specificity determinants in the crystal structure of Brachyury in complex with a T-site are retained (Ref. 7, Fig. 2*B*), as are most of the nucleotides (9 of 12) that are selected in 85% of the cases of *in vitro* binding site selection experiments with Brachyury (Ref. 6, Fig. 2*B*).

Using EMSA, a TBX2-specific complex could be seen with probe +9/+29 (spanning this variant T-site) that could be supershifted with the rabbit polyclonal α-TBX2 antibody but was absent from mock-transfected lysates (Fig. 2*C*, *lanes 1–4*). The complex with probe +9/+29 appears to be formed less efficiently than with probe –14/+35, indicating that sequences outside of the +9/+29 probe might also contribute to DNA binding. The addition of α-TBX2 antibody appears to stabilize the complex (Fig. 2*C*, *lane 4*). Others have seen such a T-protein-DNA complex stabilization by antibodies as well in the case of Brachyury and Xbra (6, 32). In contrast to probe –14/+35, the supershifted complex with probe +9/+29 migrates faster and yields a sharper band. The complex could be competed with cold +9/+29 oligo and a Brachyury consensus binding site but not with +9/+29mut in which critical nucleotides were mutated (Fig. 2*C*, *lanes 5–7*). Moreover, when +9/+29mut was used (as probe), no complex in TBX2 overexpression extracts could be seen either in the presence or absence of α-TBX2 antibody, indicating that the mutated nucleotides are indeed involved in DNA binding (data not shown). The repression domain mutant was able to bind +9/+29 in the presence of α-TBX2 antibody, again indicating that DNA binding ability of this mutant apparently is weaker and that α-TBX2 antibody stabilizes this complex (Fig. 2*C*, *lanes 8* and *9*). As expected, the T-box mutant proteins are incapable of binding the variant T-site (Fig. 2*C*, *lanes 10–13*).

To get a better insight into the requirements for DNA binding by TBX2, we performed a more detailed mutagenesis of the variant T-site. We made a set of four mutants: two (14/+35CC and –14/+35GG) in which we mutated two nucleotides in either T-half-site, thereby leaving the other T-half-site intact; and two (−14/+35CCGG and –14/+35mut) in which we mutated both T-half-sites, respectively, by a four- or seven-nucleotide change (Fig. 2*B*). When introduced into probe +9/+29 all mutations including those that disrupt only one half-site disrupted DNA binding (data not shown). However when introduced into the larger –14/+35 probe, only mutations disrupting both T-half-sites completely abolished DNA binding (Fig. 2*D*). In agreement with TBX2 being able to bind consensus T-half-sites (32), we could still detect binding of TBX2 to mutants of either T-half-site, albeit with a lower efficiency. Moreover mutating the left T-half-site appears to disrupt DNA binding more severely than mutating the right T-half-site. Together, these results indicate that the core structure of T-sites is functionally conserved in the variant T-site and that additional sequences in probe –14/+35 contribute to the stability of the complex.

In extracts of MEFs infected with TBX2 we were unable to detect a complex with $p14^{ARF}$ promoter probes (Fig. 2*E*, *lanes 1* and *2*, *4* and *5*). Similar TBX2 binding detection problems have been observed previously (Ref. 8). The detection problems are most likely due to masking by the multiple complexes that apparently are formed with the probe in these extracts, as in strong support of direct binding we could detect a large supershifted complex in TBX2-infected MEF cell extracts with both the rabbit polyclonal and mouse monoclonal α-TBX2 antibody (Fig. 2*E*, *lane 3*). Likewise, a somewhat faster migrating complex was observed with the repression domain mutant (Fig. 2*E*, *lane 6*). The complex was absent when a probe containing the incomplete T-site was used (probe –12/+23, data not shown) or when non-TBX2-overexpressing MEFs were used (Fig. 2*E*, *lanes 9* and *10*). In conclusion these EMSA experiments prove that TBX2 and TBX3 can directly bind $p14^{ARF}$ promoter oligos that span a variant T-site.

The Variant T-site of $p14^{ARF}$ Is Required for $p14^{ARF}$ Promoter Repression—To prove that the variant T-site is the relevant site via which TBX2 and TBX3 mediate repression we mutated the site in (−19/+54) $p14^{ARF}$ promoter-reporter constructs at exactly the same positions as those used in EMSAs. Because these mutations reside in the core promoter region, it was not surprising that substantial and progressive activity loss was observed. However, in agreement with the binding data obtained in EMSA, T-half-site mutants (−19/+54CC and −19/+54GG) could still be repressed by TBX2, whereas mutants in which both T-half-sites were disrupted (−19/+54CCGG and –19/+54mut) were insensitive to TBX2 even at a very high dose (Fig. 3*A*). From this finding we not only conclude that mutating the variant T-site abolishes the repressive effect of TBX2, thereby proving that this is the relevant site, but also that TBX2 is able to bind and repress when only one half-site is intact.

To further test whether TBX2 can mediate repression through a variant T-site, we made chimeric promoters consisting of the HSV tk promoter driving the luciferase gene, upstream, of which we cloned both the consensus and variant T-sites (Fig. 3*B*). These constructs have been described previously as only being responsive to TBX2 when four T-half-sites are present (32). However, in our hands the sole HSV tk promoter was repressed 4-fold by TBX2 (Fig. 3*C*). This might be explained by the cell type used (COS-7 and Phoenix cells by us *versus* 293 cells in the other study). Nevertheless, when either two consensus T-sites, two variant T-sites of the $p14^{ARF}$ promoter (sequence +9/+29) or a somewhat larger part of the $p14^{ARF}$ promoter (sequence –14/+35), are upstream of the HSV tk promoter, these constructs were repressed up to 10-fold by TBX2 (Fig. 3*C*). In contrast, when the mutant T-site (+9/+29mut), which was incapable of binding TBX2 in EMSAs, was cloned upstream, the level of repression was comparable with that of HSV tk alone (~4-fold, Fig. 3*C*). In conclusion, these results demonstrate that TBX2 is able to mediate repression through the variant T-site in the $p14^{ARF}$ promoter.

TBX1A and Xbra Are Not Able to Act on the $p14^{ARF}$ Promoter—To test whether the variant T-site in the $p14^{ARF}$ promoter can also potentially be regulated by other T-box factors or might be specific to TBX2 and TBX3, we performed co-transfection assays in COS-7 cells with the $p14^{ARF}$ reporter constructs and two T-box-containing transcriptional activators, TBX1A and Xbra. Xbra had no effect on the small (−19/+54) $p14^{ARF}$ promoter (Fig. 4*A*) and could only activate the long (−2465/+54) $p14^{ARF}$ promoter 2-fold at high dose (Fig. 4*B*). Expression of the protein could clearly be detected by its eGFP tag (data not shown). In agreement with previous results, we could potently activate the HSV tk promoter with two up-

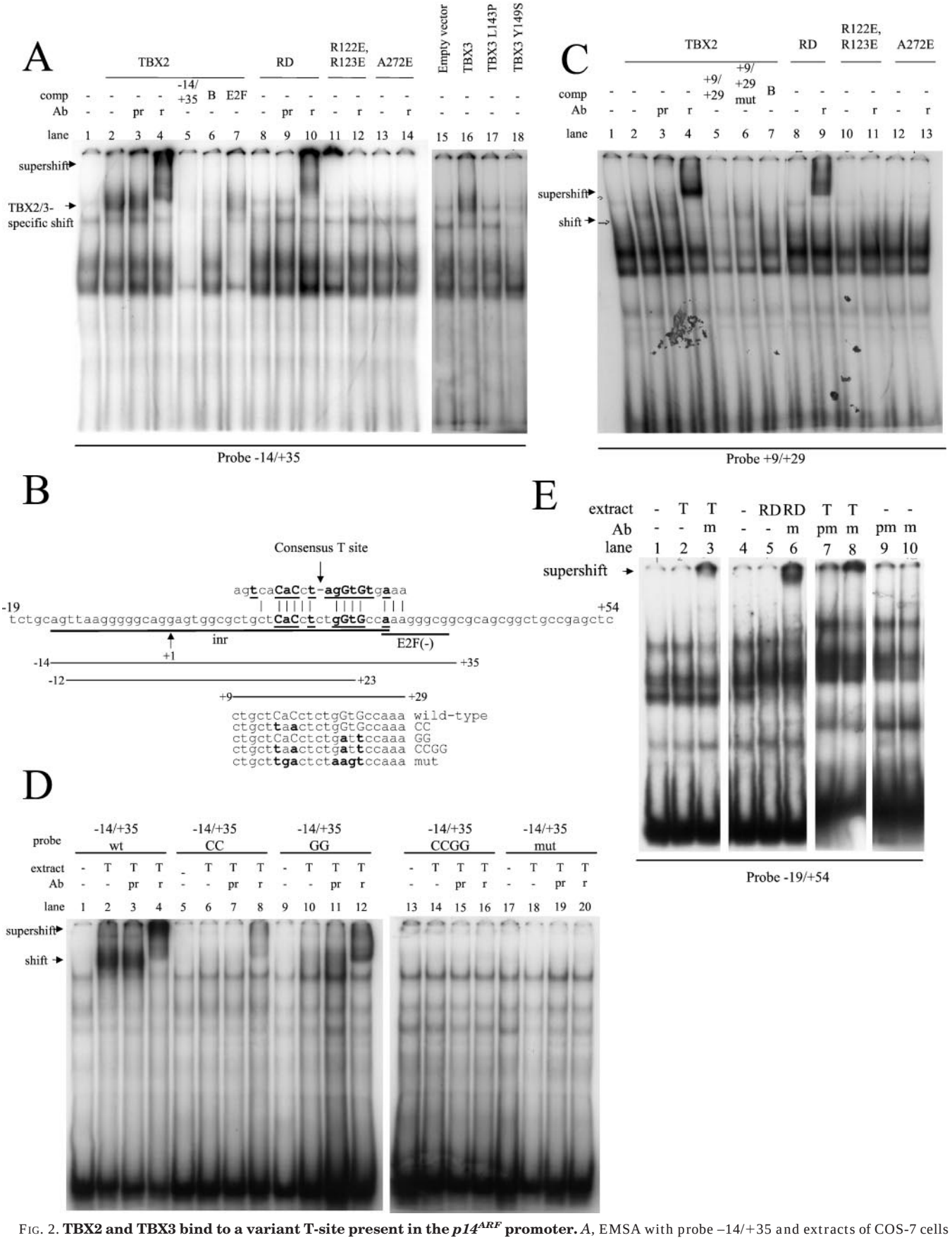

FIG. 2. **TBX2 and TBX3 bind to a variant T-site present in the *p14*^{ARF} promoter.** *A*, EMSA with probe –14/+35 and extracts of COS-7 cells overexpressing TBX2, TBX2RD, TBX2TB(R122E, R123E), TBX2TB(A272E), TBX3, TBX3 L143P, or TBX3 Y149S. Competitor oligos (−14/+35, Brachyury consensus binding site B, and E2F binding site) and antibodies (*pr*, pre-immune serum; *r*, rabbit polyclonal α-TBX2 antibody) are added as indicated. Note that TBX2, TBX2RD, and TBX3 can bind probe −14/+35 in both the absence and presence of α-TBX2 antibody as opposed to the DNA binding inability of T-box mutants of TBX2 and TBX3. *B*, schematic outline of sequences –19 to +54 of the *p14*^{ARF} promoter with the

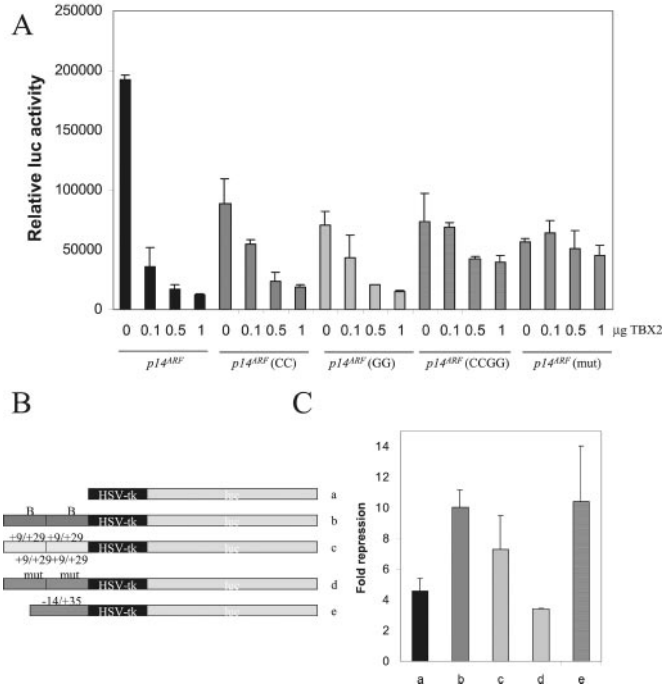

FIG. 3. **The variant T-site is necessary for the repression of the *p14*^{ARF} promoter by TBX2.** *A*, dose-dependent repression of wild-type and T-half-site mutant (−19/+54) *p14*^{ARF} promoter-Luc reporters (*p14*^{ARF} wt, *p14*^{ARF} (*CC*), and *p14*^{ARF} (*GG*)) by TBX2 but not of (−19/+54) *p14*^{ARF} promoter-Luc reporter constructs in which both T-half-sites are disrupted (*p14*^{ARF} (*CCGG*) and *p14*^{ARF} (*mut*)). *B*, schematic representation of the chimeric HSV tk constructs (labeled *a–e*) made with the upstream consensus T-site (*B*), the variant T-site (+9/+29 and –14/+35), and the mutated variant T-site (+9/+29 mut). *C*, fold repression of the constructs depicted in *panel B* by the presence of 1 µg of TBX2. Note that the constructs harboring consensus or variant T-sites (*columns b, c*, and *e*) are relatively more strongly repressed (up to 10-fold) than the sole HSV tk promoter (*column a*) or the construct harboring the mutant variant T-site (*column d*) (both are repressed 4-fold).

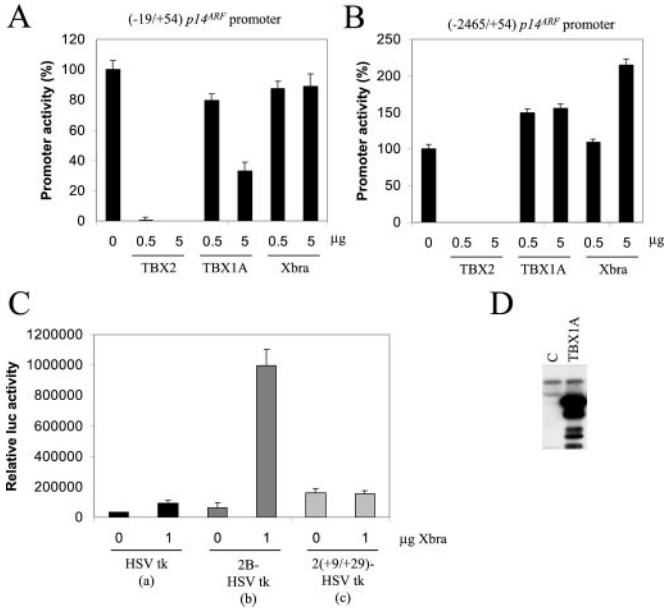

FIG. 4. **The *p14*^{ARF} promoter is not regulated by TBX1A and Xbra.** *A*, dose-dependent repression of the (−19/+54) *p14*^{ARF} promoter CAT reporter by TBX2 but not by TBX1A and Xbra. The activity level of the promoter in the absence of TBX2 is set at 100%. *B*, dose-dependent repression of the (−2465/+54) *p14*^{ARF} promoter-CAT reporter by TBX2 but not by TBX1A and Xbra. The activity level of the promoter in absence of TBX2 is set at 100%. *C*, activity of Xbra on the HSV tk Luc constructs depicted in Fig. 3*B*. Note that Xbra is able to activate via consensus T-sites but not via the variant T-sites. *D*, Western blot analysis of HA-tagged TBX1A in COS-7 cells.

stream consensus T-sites with Xbra (Fig. 4*C* and Ref. 32). In contrast, Xbra did not activate the HSV tk promoter with two upstream variant T-sites (Fig. 4*C*). Both experiments show that Xbra is not able to regulate via the *p14*^{ARF} variant T-site.

TBX1A can reduce *p14*^{ARF} promoter activity of the short (−19/+54) construct when added at very high concentrations, but in comparison, TBX2 is much more active in repressing, also at much lower concentrations (Fig. 4*A*). Moreover, on the long and more active (−2465/+54) *p14*^{ARF} promoter, we did not see an effect of TBX1A (Fig. 4*B*). Although we could clearly detect expression of TBX1A (Fig. 4*D*), we could not check the activity of the protein because TBX1A is not active on the HSV tk promoter with the two upstream T-sites (Ref. 32) and no other TBX1A-responsive promoters have been described to our knowledge. In conclusion, we show that at least the T-box factors TBX1A and Xbra are not able to act via the variant T-site located in the *p14*^{ARF} promoter, suggesting a level of specificity for T-box family proteins in target gene recognition.

DISCUSSION

In this report we demonstrate that TBX2 and TBX3 bind a variant T-site in the *p14*^{ARF} promoter, thereby down-regulating its gene expression. TBX2/TBX3 can bind this variant T-site, whereas point mutants of the DNA-binding T-box domain, both at the C-terminal and N-terminal parts, can not do so. The point mutants are to a variable extent impaired in repressing *p14*^{ARF} in transient repression assays, *i.e.* TBX2TB(R122E,R123E) is completely inactive and TBX2TB(A272E) weakly active at high concentrations. Nevertheless, both mutants are incapable of down-regulating endogenous p19^{ARF} levels in MEFs and because of this are incapable of bypassing senescence arrest. Although *in vitro* site selection experiments with Brachyury in the past (6) and with Xbra, VegT, and eomesodermin more recently (34) appear to select for a repeat (palindrome) of T-half-sites, thus far described *in vivo* T-box targets seem to be regulated by T-half-sites. Some of these promoters contain multiple, separate T-half-sites, such as in the case of *Ci-trop* regulation by Ci-Bra in *Ciona intestinalis* (35), *Bix4* by VegT (36) or *eFGF* by Xbra in *Xenopus* (37), *orthopedia* regulation by Brachyenteron (Byn) in *Drosophila* (38), and the melanocyte-specific *TRP-1* promoter by Tbx2 (8). Interestingly, for *Bix4* and *TRP-1*, the T-half-sites map in the vicinity of the transcriptional start site or even within the initiator element (*TRP-1*), in analogy to the here described variant T-site in the *p14*^{ARF}

initiator (*inr*) element and the inverse E2F site *underlined*. An alignment is made with a consensus T-site. Nucleotides marked as important DNA specificity determinants in the crystal structure of Brachyury in complex with a T-site are shown in *capital letters* (7), and nucleotides that are selected in 85% of the cases of *in vitro* binding site selection experiments with Brachyury are *underlined* in *bold* (6). Underneath the *p14*^{ARF} promoter sequence, the probes used in the EMSAs are depicted. For variant T-site mutations the differences with wild-type are indicated in *bold*. *C*, EMSA with probe +9/+29 and extracts of COS-7 cells. In analogy to *panel A*, TBX2 and TBX2RD are able to bind probe +9/+29, spanning the variant T-site in *p14*^{ARF}. *D*, EMSA with both wild-type and mutant –14/+35 probes demonstrating the TBX2 is able to bind when one of the T-half-sites is disrupted but not when both T-half-sites are disrupted. Extracts used are from mock-transfected (−) or TBX2-overexpressing (*T*) COS-7 cells. *E*, EMSA with probe –19/+54 and extracts of ARF−/− MEFs noninfected (−), TBX2-infected (*T*), or TBX2RD-infected (*RD*). Pre-immune serum (*pm*) and mouse polyclonal α-TBX2 antibody (*m*) were added as indicated. In strong support of direct binding, a supershifted complex is detected, upon addition of antibody, that migrates slightly faster for TBX2RD compared with wild-type TBX2.

initiator. Other T-box targets are regulated by cooperation of a T-box protein and a cofactor that bind to contiguous sites within the same regulatory element. Good examples are Tbx5 and Nkx2–5, which synergistically activate the cardiac-specific *Nppa*, *ANF,* and *cx40* promoters. Moreover, these two proteins bind these promoters in tandem, on an element containing both binding sites, but can also interact in the absence of DNA (39, 40). In addition, TBX2 and Nkx2–5 simultaneously interact on a double site of the *ANF* promoter, leading to its repression (41). In pituitary cells, Tpit or TBX19 and Pitx cooperate to activate *POMC* (pro-opiomelanocortin) gene transcription also by binding such a double site (42, 43). Notwithstanding these recent discoveries, relatively little is known about the sequence requirements that determine individual target gene specificity for the large family of T-box domain-containing transcription factors.

Here, we demonstrate for the first time that an important T-box target, the $p14^{ARF}$ tumor suppressor gene, is regulated by a variant palindromic T-site. Whereas the core CACC-*NNN*GGTG nucleotides of the variant site, which form an imperfect palindrome, are well conserved in the "consensus" T-site, flanking nucleotides are divergent (Fig. 2*B*). The imperfect palindromic site is reminiscent of the preference for a repeat of T-half-sites for Brachyury, Xbra, VegT, and eomesodermin found in *in vitro* binding studies (6, 34). In addition, our mutational analysis of the conserved "core" sequence clearly demonstrates its requirement for the binding of TBX2 and TBX3 to the variant site, likely reflecting the highly conserved structure of the T-box domain (7). Although TBX2 *in vitro* has previously been shown able to bind to T-half-sites, a preference for (palindromic) T-sites appears to exist (32). In addition, it was shown that TBX2 can bind variants of a consensus T-half-site that were generated by mutagenesis of the consensus T-site (8). However such sequences are not present in the small –19/+54 $p14^{ARF}$ promoter fragment, and their biological relevance for *in vivo* target gene regulation remains to be demonstrated. Interestingly, it has just been reported that *orthopedia* is regulated by Byn via 15 binding sites, which all differ in at least two nucleotides from a consensus T-half-site (38). Therefore, in agreement with our data, Byn can regulate a promoter through variants of a T-half-site. However, the *orthopedia* promoter does not contain a palindromic T-site as seen for the $p14^{ARF}$ promoter. Close inspection of more upstream sequence of the $p14^{ARF}$ promoter revealed no other variant T-(half) sites. Presumably the $p14^{ARF}$ palindromic variant T-site is a high affinity site *in vivo* and might therefore be capable of conferring repression on its own. Importantly, the divergent flanking sequences of the variant $p14^{ARF}$ T-site contribute to determining the specificity for binding to TBX2 and TBX3, as the related T-box-containing proteins TBX1A and Xbra are not able to activate transcription of reporter constructs harboring this variant site. In contrast, the *orthopedia* promoter could also be activated by mouse and *Xenopus* Brachyury, suggesting that these proteins can also recognize Byn sites (38). However the activation by Brachyury via the multiple Byn sites is much lower than in case of Byn, which could reflect intrinsic Byn preference/specificity associated with these sites. The occurrence of variant T-sites in relevant target genes clearly illustrates the *in vivo* selected specificity in target gene recognition associated with individual T-box protein family members.

A further indication of such divergence in target gene recognition is illustrated by the fact that TBX2 alone can potently repress $p14^{ARF}$, in contrast to the situation for TBX5 and its co-factor Nkx2–5, which need to bind and act in cooperation to strongly activate their target genes (39, 40). This fact and the apparent inability of the C-terminally deleted TBX2 mutant to compete with wild type TBX2 for $p14^{ARF}$ binding suggest that the existence of a co-factor for TBX2 may not be a prerequisite. However, our EMSAs do point to a role for flanking sequences outside of the variant T-site to promote stable complex formation, which in turn could point to the requirement for binding of such a co-factor, although the sequences of the $p14^{ARF}$ promoter, aside from an imperfect E2F site, do not indicate the presence of consensus binding sites for established or potential co-factors such as Pitx or Nkx2–5.

In addition to the T-box we also found a C-terminal conserved domain of TBX2 to be essential for repression. A deletion mutant of this repression domain could still bind the variant T-site, although somewhat less efficiently. As the affinity of the repression domain mutant for the variant T-site is impaired, this might explain why this mutant could not compete with wild-type TBX2 for repression. In contrast, previously others (32) have demonstrated comparable DNA binding activity of wild-type and repression domain mutant proteins; however, in these studies a probe was used that contained four T-half-sites, which may well explain the different outcome. The repression domain was first mapped within *Xenopus* ET and its human ortholog, TBX3, and has subsequently been found to be highly conserved in TBX2 (amino acids 535–629 in TBX2, Ref. 9). Others (32) claim the existence of an additional repression domain in TBX2 at position 407–561, although they do not acknowledge the small overlap between these two regions. Our inactivating deletion encompassed amino acids 501–618, thus affecting both proposed domains. Although the repression domain deletion mutant is incapable of fully rescuing MEFs from senescence, the protein does appear to have some residual ability to down-regulate $p19^{ARF}$, contributing to an extended MEF life span. Likewise, others (44) have recently noticed a low efficiency of MEF immortalization by TBX3 repression domain deletion mutants as well. This may well be explained by the fact that the repression domain mutants retain the ability to bind to the variant T-site, which is embedded in the initiator sequence. Conceivably, such binding may interfere to some extent with promoter function by obstruction. Nevertheless, our results clearly indicate that active repression by TBX2 via the repression domain is required for $p14^{ARF}$ down-regulation. We hypothesize that the repression domain is involved in recruiting other proteins, such as co-repressors, to the $p14^{ARF}$ promoter, thereby mediating repression and creating the observed slow migrating EMSA complexes. Remarkably, so far only one protein, in addition to the two above described transcription co-factors, has been shown to bind a T-box protein family member. This factor is CASK (calcium/calmodulin-dependent serine protein kinase), a membrane-associated guanylate kinase and component of cell junctions, which binds Tbr-1 and then translocates to the nucleus (45). *In vitro* this complex can bind T-sites and activate transcription of the T-site-containing *reelin* promoter. Whether this reflects a brain-specific or more general mechanism remains to be seen.

In conclusion we identified a variant T-site, composed of two inverted, imperfect T-half-sites, as the essential TBX2/TBX3 binding element in an important *in vivo* relevant TBX2/TBX3 target, the $p14^{ARF}$ tumor suppressor promoter. The core structure of T-box/T-site is conserved, as disrupting mutations could be made in either the binding site DNA or in critical T-box amino acids, based on knowledge of the complex of Brachyury and a consensus T-site. The existence of a variant palindromic T-site described herein is, to our knowledge, unprecedented and offers a possible explanation for the selection of a repeat of T-half-sites in the *in vitro* binding site selection experiments performed with T-box proteins. Importantly, our study points to a hitherto unsuspected level of specificity for individual

T-box factors in recognizing their respective target genes, which opens new avenues of research in the further exploration of T-box target gene regulation.

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Chapter 4

Bmi1 is essential for cerebellar development and is overexpressed in human medulloblastomas

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Bmi1 is essential for cerebellar development and is overexpressed in human medulloblastomas

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Overexpression of the polycomb group gene *Bmi1* promotes cell proliferation and induces leukaemia through repression of *Cdkn2a* (also known as *ink4a/Arf*) tumour suppressors[1,2]. Conversely, loss of Bmi1 leads to haematological defects and severe progressive neurological abnormalities in which de-repression of the *ink4a/Arf* locus is critically implicated[1,3]. Here, we show that Bmi1 is strongly expressed in proliferating cerebellar precursor cells in mice and humans. Using *Bmi1*-null mice we demonstrate a crucial role for Bmi1 in clonal expansion of granule cell precursors both *in vivo* and *in vitro*. Deregulated proliferation of these progenitor cells, by activation of the sonic hedgehog (Shh) pathway, leads to medulloblastoma development[4]. We also demonstrate linked overexpression of BMI1 and patched (PTCH), suggestive of SHH pathway activation, in a substantial fraction of primary human medulloblastomas. Together with the rapid induction of Bmi1 expression on addition of Shh or on overexpression of the Shh target Gli1 in cerebellar granule cell cultures, these findings implicate BMI1 overexpression as an alternative or additive mechanism in the pathogenesis of medulloblastomas, and highlight a role for Bmi1-containing polycomb complexes in proliferation of cerebellar precursor cells.

We characterized the expression of Bmi1 during mouse cerebellar development (at embryonic day (E)14.5 and E16.5 and at postnatal day 1, 5, 8, 15 and 30). Immunohistochemical analysis showed strong nuclear staining for Bmi1 in the cells of the external granular layer (EGL) (Fig. 1, upper row; see also Supplementary Fig. S1) and weaker staining in neural precursor cells of the cerebellar neuroepithelium (Fig. 1, upper row) at all embryonic time points analysed. During postnatal development the strongest Bmi1 staining was observed between days 5 and 8 in actively proliferating granule cell precursors located in the EGL (Fig. 1, middle panel of left column). Immunoblotting of extracts obtained from EGL precursor cells and/or granule cells of newborn mice and mice at postnatal day 5, 8 and 15 confirmed high Bmi1 expression in proliferating granule cell precursors and showed its downregulation in postmitotic and terminally differentiated granule cells (Fig. 1, middle panel of right column). Notably, the time course of this expression paralleled the expression of N-Myc and its target gene cyclin D2 (Fig. 1, middle panel of right column), which are involved in the rapid expansion of progenitor cells during neurogenesis[5–7]. Immunohistochemical staining on autoptic cerebellar sections of human fetuses at gestational week 17, 19, 21, 26 and 32, and of a 2-month-old child and an adult, demonstrate a comparable expression pattern (Fig. 1, bottom panels).

In addition to haematopoietic and skeletal defects, Bmi1-deficient mice develop progressive ataxic gait, balance disorders, tremors and behavioural abnormalities at the age of 2–4 weeks[3]. Although the mice survive to adulthood, they exhibit severe reduction in total postnatal brain mass with particularly severe

phenotypic changes in the cerebellum, as expected from the clinical signs (ref. 3; see also Fig. 2a).

Histological analysis revealed normal cerebellar architecture but a markedly reduced cellularity in the granular and molecular layers (Fig. 2a, NeuN and parvalbumin, and c, area/cell density measurements), in addition to an almost complete lack of stellate neurons and striking morphological abnormalities of the dendritic arborizations of the few basket neurons present (Fig. 2a, NF200). Immunohistochemical staining for NeuN (granule cells), calbindin and parvalbumin (Purkinje cells), as well as messenger RNA *in situ* hybridization for metabotropic glutamate receptor 2 (mGluR-2; Golgi cells, data not shown), revealed normal morphology and terminal differentiation of these cerebellar neurons (Fig. 2a).

To track down the defects in cerebellar development in Bmi1-deficient mice, we analysed E14.5 and E16.5 mouse embryos as well as mice at postnatal day 1, 8 and 15. The overall size of the cerebellum is reduced from E16.5 onwards, and the Bmi1-null EGL and neuroepithelium appear 1–2 cells thinner (Fig. 2b). In line with a critical role of Bmi1 in proliferation of EGL progenitor cells, we found a significant reduction of MATH-1-expressing mitotically active granule cell progenitors in the outer EGL of newborn *Bmi1* $^{-/-}$ mice. Widespread expression of p27 throughout the whole width of the EGL confirmed the postmitotic state of most of the *Bmi1* $^{-/-}$ EGL cells (Fig. 2b). At the peak of EGL proliferation at postnatal day 8, *Bmi1* $^{-/-}$ mice showed a significant reduction of 5-bromodeoxyuridine (BrdU)-positive EGL cells (Fig. 2c) as well as a marked increase in apoptotic cells (Fig. 2c), which might imply that failure to respond to proliferation induction shunts EGL cells into cell death.

Together these results demonstrate a crucial role of Bmi1 in the maintenance and expansion of immature granule cell precursors. This extends the recently reported essential role of Bmi1 in renewal of adult haematopoietic stem cells[8,9] and cortical neural stem cells (ref. 10 and D. Zencak *et al.*, unpublished work) to include more

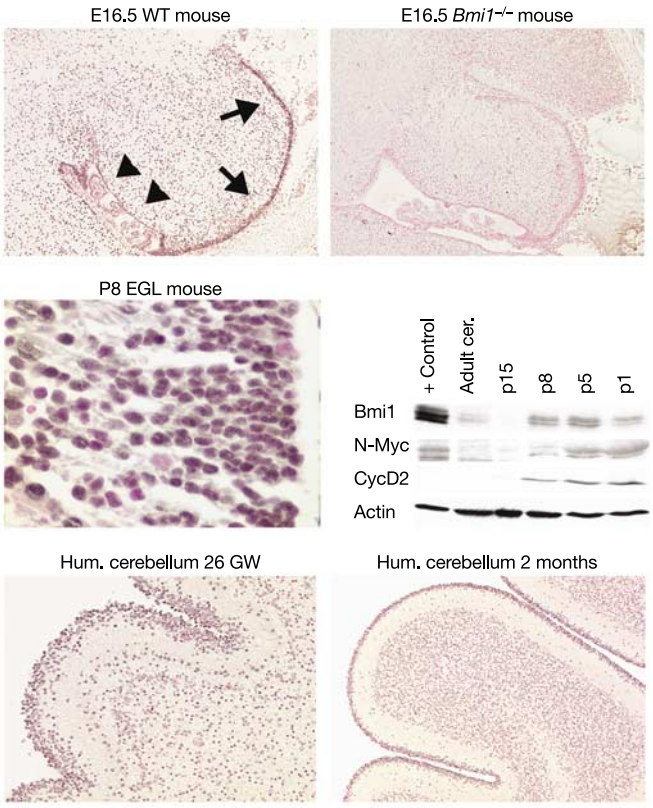

Figure 1 Bmi1 expression in cerebellar precursor cells during development. Bmi1 is expressed in EGL precursors (arrows) during embryonic (E16.5 and gestational week (GW) 26) and early postnatal (P) (8 days and 2 months) cerebellar development, most notably during clonal expansion, in mouse (top and middle rows) and human (bottom row). Weaker expression is found in neuroepithelium precursors (arrowheads) and in terminally differentiated neurons (immunohistochemical analysis, Bmi1 F6 antibody). Final magnification, ×28, ×56 and ×38. Western blot analysis of granule cell precursors/granule cells isolated at postnatal day 1, 5, 8 and 15 (middle panel of right column) confirmed the expression of Bmi1 in proliferating granule cell precursors.

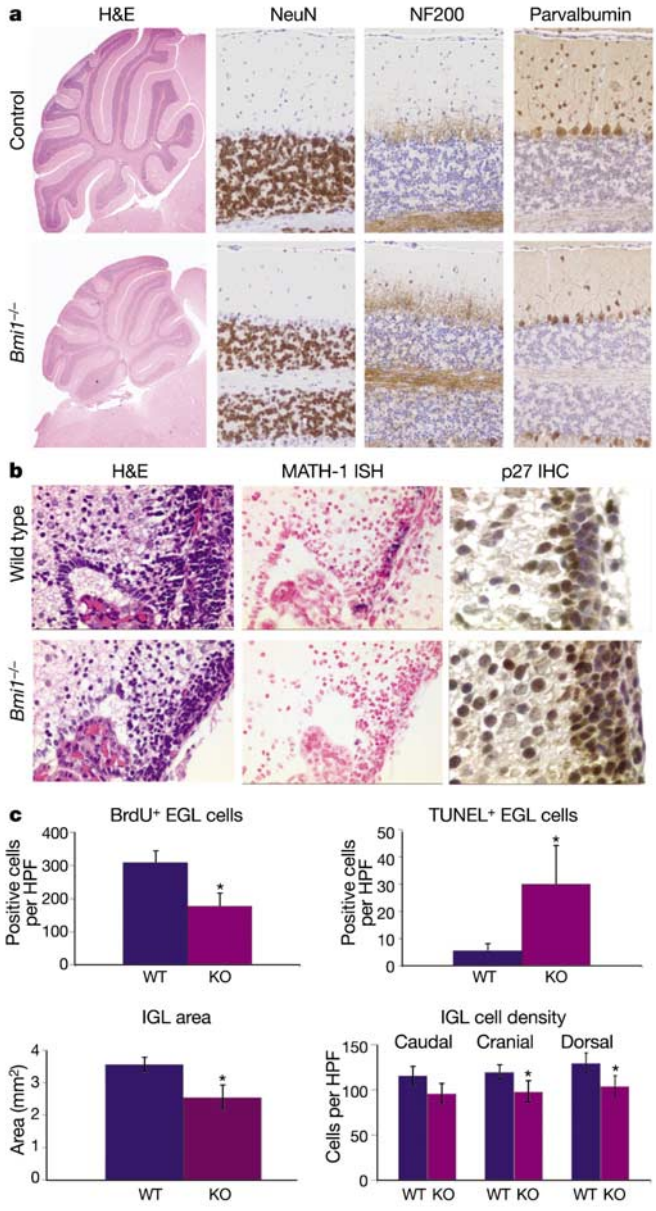

Figure 2 Cerebellar phenotype of *Bmi1* $^{-/-}$ mice and analysis of proliferation and apoptosis. **a**, Histopathological analysis of *Bmi1* $^{-/-}$ (bottom) versus wild-type cerebellum (top) in 2-month-old mice shows reduced cerebellar size, reduced thickness of molecular and granular layers and lack of stellate cells in the *Bmi1* $^{-/-}$ mouse. H&E, haematoxylin and eosin. Final magnification, ×5, ×60. **b**, Reduction of MATH-1-positive EGL precursors and wider expression of the postmitotic marker p27 in *Bmi1* $^{-/-}$ mice at postnatal day 1. IHC, immunohistochemistry; ISH, *in situ* hybridization. Final magnification, ×80, ×160. **c**, Significant reduction of BrdU incorporation (asterisk, $P < 0.001$) and increased apoptosis (asterisk, $P < 0.006$) are observed in the EGL of *Bmi1* $^{-/-}$ mice at postnatal day 8. Area measurements and cell density counts show significant (asterisk, $P < 0.008$ and $P < 0.05$) reduction of size and cell density of the internal granular layer (IGL) as early as postnatal day 15. HPF, high-power field; KO, knockout (*Bmi1* $^{-/-}$); WT, wild type.

committed cerebellar precursor cells. No gross impairment of fate determination and terminal differentiation of neural precursor cells was observed in the absence of Bmi1.

Cerebellar granule cell (CGC) cultures, which can be induced to proliferate on addition of Shh[4,11], are a well-established method to study neural precursor cell proliferation and differentiation *in vitro*. We show here that *Bmi1* mRNA is upregulated in response to Shh treatment as early as 4 h after incubation (Fig. 3a). Expression of Bmi1 consistently remains high for as long as 72 h after incubation with Shh (Fig. 3a, b), and parallels Gli1, suppressor of fused (Sufu) and cyclin D2 expression, indicative of activation of the Shh pathway and induction of proliferation. Moreover, overexpression of Gli1 in CGCs and in rat kidney cells induces Bmi1 expression (Fig. 3c), implying that Bmi1 is a downstream target in the Shh pathway. As confirmation of this, we found a significant reduction in proliferation, as measured by BrdU incorporation, after Shh treatment in CGCs lacking Bmi1 when compared with wild-type CGCs (Fig. 3d).

Next, we overexpressed Bmi1 in *Bmi1* $^{-/-}$ CGCs in the absence of Shh. As is shown in Fig. 3e, f, Bmi1 overexpression induces proliferation, as measured by induction of the DNA replication marker PCNA and by increased BrdU incorporation in *Bmi1* $^{-/-}$ CGCs. In contrast to massive induction of cyclin D2 expression by Shh, overexpression of Bmi1 in *Bmi1* $^{-/-}$ CGCs does not significantly increase cyclin D2 levels, and the effects on BrdU incorporation are reduced when compared with Shh stimulation. This finding is most compatible with a dual and separate action of the Shh pathway on both Bmi1 and N-Myc/cyclin D2 (see below). The similar expression kinetics of Bmi1 and known downstream targets of the Shh pathway during granule cell development *in vivo*; their critical role in granule precursor cell proliferation; and the similar cerebellar phenotype of mice lacking N-Myc[6], cyclin D2 (ref. 7) or Bmi1 (ref. 3), strongly support a critical role of Bmi1 in Shh-dependent proliferation of granule cell progenitors.

Medulloblastomas—highly malignant brain tumours of childhood—originate from an uncontrolled proliferation of EGL precursor cells and represent an intriguing example of neoplastic transformation of undifferentiated progenitor cells[12]. To assess whether BMI1 might be involved in the pathogenesis of human medulloblastomas, we analysed three medulloblastoma cell lines (DAOY, D-341 and D-458) and 12 primary human medulloblastoma samples for BMI1 expression. Western blot analysis revealed strong overexpression of BMI1 in all cell lines (Fig. 4b) and in eight out of 12 primary tumours tested (Fig. 4c) when compared with normal cerebellum. CDK2 protein levels, a marker for proliferation, served as a control to exclude significant contamination of normal tissue, and was found to be equally expressed in all

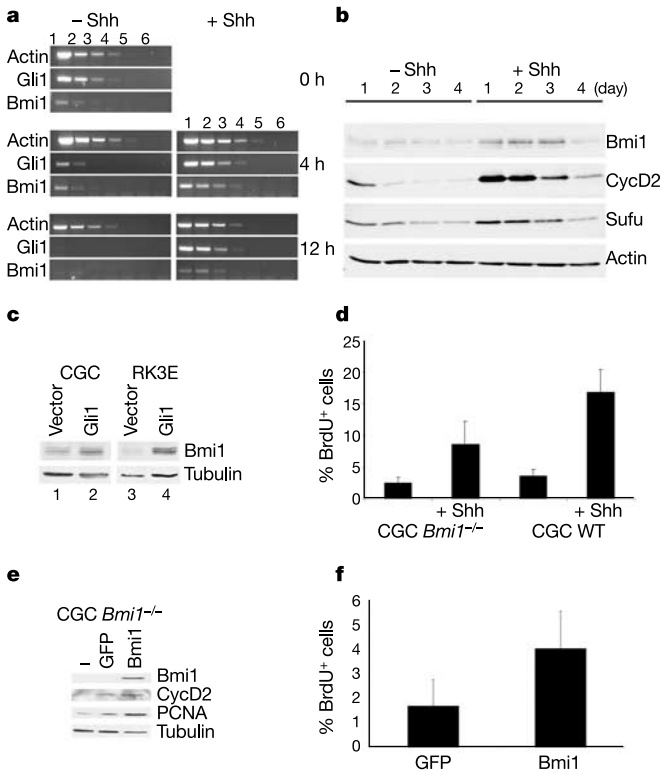

Figure 3 Bmi1 is essential for efficient CGC proliferation on Shh treatment. **a**, Upregulation of *Bmi1* expression parallels upregulation of Gli1 on Shh treatment. Semi-quantitative RT–PCR analysis at 0, 4 and 12 h with and without Shh treatment. Lanes 1–5, serial cDNA dilutions; lane 6, control. **b**, Time course of Bmi1, cyclin D2 and Sufu expression at later time points after Shh treatment (western blot). **c**, Gli1 overexpression induces upregulation of Bmi1 in wild-type CGCs and in RK3E cells. **d**, Reduced BrdU incorporation in *Bmi1* $^{-/-}$ CGCs versus wild-type CGCs on Shh treatment ($P < 0.003$). **e**, Lentiviral overexpression of Bmi1 in *Bmi1* $^{-/-}$ CGCs induces the proliferation marker PCNA. No induction of cyclin D2 is observed. **f**, Overexpression of Bmi1 in *Bmi1* $^{-/-}$ CGCs increases BrdU incorporation ($P < 0.02$). The weak induction of proliferation observed in the control infection with GFP (**e**, **f**) is probably due to serum components co-precipitating during the ultracentrifugation step during lentivirus preparation.

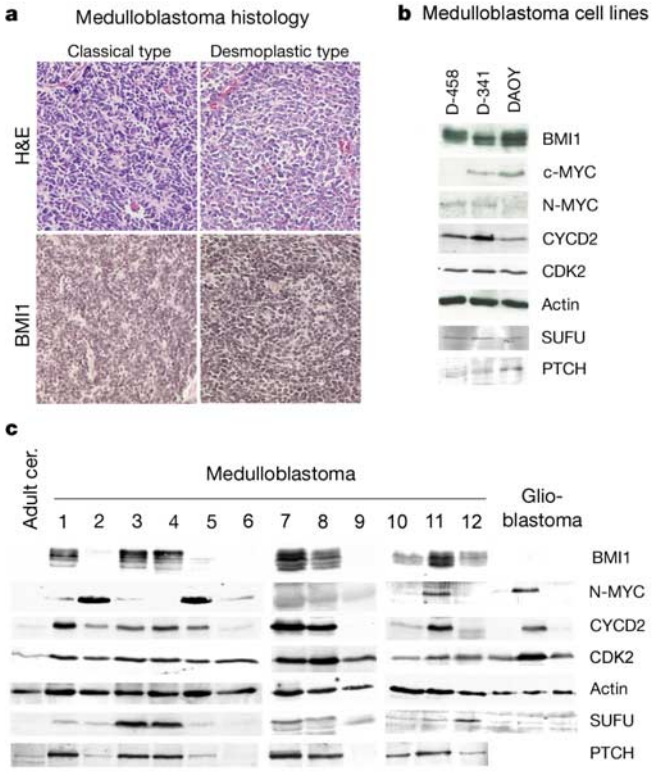

Figure 4 Overexpression of BMI1 in 67% of analysed medulloblastomas correlates with SHH pathway activation. **a**, Both classical and desmoplastic medulloblastomas overexpress BMI1. Final magnification, ×50. **b**, Overexpression of BMI1 correlates with overexpression of cyclin D2 and c-MYC in medulloblastoma cell lines. No overexpression of N-MYC is observed. Equal expression of CDK2 (proliferation marker) and actin is shown. **c**, Overexpression of BMI1 shows a marked correlation with overexpression of PTCH and cyclin D2 in medulloblastomas (samples 1, 3, 4, 7, 8, 10, 11 and 12). Co-overexpression of N-MYC and BMI1 is observed in two tumour samples. Equal expression of CDK2 and actin is shown. Note that tumours without BMI1 overexpression (2, 5, 6 and 9) have low or absent levels of PTCH. Overexpression of BMI1 is specific to medulloblastomas, as three analysed glioblastomas do not show high BMI1 levels (see also Supplementary Fig. S2).

samples (Fig. 4c). In contrast, ten glioblastomas—highly proliferating brain tumours of glial origin that are not related to medulloblastomas—showed no overexpression of BMI1 (Fig. 4c; see also Supplementary Fig. S2).

Immunohistochemical analysis of primary medulloblastomas revealed no significant correlation between high BMI1 expression and the histological variant of the tumours (classical or desmoplastic; Fig. 4a).

Mutation analysis in human medulloblastomas, as well as analysis of the development of medulloblastomas in a fraction of mice heterozygous for a null mutation of *Ptch*, suggests that inappropriate maintenance of Shh signalling in proliferative EGL precursor cells promotes medulloblastoma formation. Indeed mutations of PTCH, smoothened (SMOH) and SUFU, leading to activation of the SHH pathway, have been found in 25% of sporadic human medulloblastomas[13–15].

Overexpression of BMI1 correlated well with overexpression of PTCH and SUFU (Fig. 4c), suggesting at least a partial activation of the hedgehog pathway in BMI1-overexpressing tumours. Notably, all tumours with low levels of PTCH expression did not overexpress BMI1, further implying that BMI1 is a possible downstream effector of SHH signalling and that BMI1 overexpression is an alternative or additive mechanism to PTCH mutation in medulloblastoma pathogenesis. The observed frequent overexpression of PTCH protein is in agreement with previous analyses of mRNA levels[16,17] but it contrasts with mRNA expression profiling demonstrating *PTCH* activation mainly in desmoplastic medulloblastomas[18]. Although the reason for this remains to be clarified, it may in part be attributable to different detection methods (protein versus mRNA profile analysis).

Bmi1 actively collaborates with the *c-Myc* oncogene in murine lymphomagenesis through inhibition of Myc-induced apoptosis via downregulation of *ink4a/ARF* (ref. 2). Moreover, N-Myc has recently been shown to be essential for the rapid expansion of progenitor cell populations during neurogenesis[6], and to be regulated by hedgehog signalling. Amplification of *c-MYC*, and more often of *N-MYC*, occurs in human medulloblastomas and correlates with poor clinical outcome[19,20]. As shown in Fig. 4b, c-MYC is overexpressed in two out of three medulloblastoma cell lines but not in the human tumour samples; however, we found overexpression of N-MYC in four out of 12 medulloblastomas, of which two also demonstrated BMI1 overexpression (Fig. 4c). These data suggest that BMI1 is not a target of N-MYC.

It is possible that the SHH pathway branches upstream of N-MYC, and that both Bmi1 induction and N-Myc upregulation (leading to cyclin D1 and D2 induction[5]) by Gli transcription factors are required for full Shh-mediated proliferation response of CGCs. Bmi1 affects the activity of cyclin D, Cdk4/Cdk6 and p53 by repressing $p16^{ink4a}$ and $p19^{arf}$ tumour suppressors[1]. As such, BMI1 overexpression in a majority of human medulloblastomas can potentially lead to repression of both the RB and p53 pathways, thus disturbing the balance between differentiation and proliferation of cerebellar precursors. Although we cannot exclude the involvement of other Bmi1 targets, it is intriguing in this respect that medulloblastomas develop with high frequency in mice selectively lacking Rb and p53 in EGL precursors[21], whereas loss of these two tumour suppressors are rare events in the pathogenesis of human medulloblastomas[22].

The recently established requirement for Bmi1 in controlling proliferation of both normal and leukaemic adult stem cells[8,9,23], together with the essential role of Bmi1 in the proliferation of EGL progenitor cells and its overexpression in a majority of medulloblastomas (this paper), indicate a common conserved role for Bmi1-containing polycomb complexes in the maintenance and expansion of stem cells or committed progenitors, and in the pathogenesis of tumours originating from the neoplastic transformation of these cells. □

Methods

Isolation of CGCs and culturing conditions

CGC cultures were isolated from 7–8-day-old FVB mice according to established protocols[11]. After isolation, 1.6×10^6 cells ml^{-1} were seeded onto plates pre-coated with 100 μg ml^{-1} poly-L-lysine (Sigma) and allowed to adhere for 30 min at 37 °C, 5% CO_2. Non-adherent cells were removed after 30 min and replaced with fresh media.

For proliferation experiments, CGCs were cultured in neural basal medium (Gibco) containing 2 mM L-glutamine, 4 μg ml^{-1} insulin, 1 mM sodium pyruvate, 1 × Penicillin/Streptomycin (Gibco), 0.062 μg ml^{-1} progesterone, 16 μg ml^{-1} putrescine, 100 μg ml^{-1} BSA, 0.04 μg ml^{-1} sodium selenite, 100 μg ml^{-1} *apo*-transferrin and 25 mM KCl. All chemicals were obtained from Sigma. For Shh induction of proliferation, cells were incubated with 3 μg ml^{-1} of Shh (R&D), added immediately after media change and harvested at 4, 12, 24, 48,72 and 96 h for RNA and protein analysis.

For BrdU incorporation assays, CGCs were pulsed with 25 μg ml^{-1} BrdU for 2 h before fixation in 4% paraformaldehyde.

E1A-immortalized rat kidney RK3E cells are susceptible to transformation by Gli1 (ref. 24). Gli1 complementary DNA was cloned into a MCSV retrovirus and used to infect RK3E cells according to standard protocols[1].

Immunoblotting and RT–PCR experiments

Protein analysis was performed according to standard protocols. A list of the antibodies and dilutions used is available as Supplementary Information.

Total RNA was isolated using the RNeasy Mini kit (Qiagen). One microgram of total RNA was reverse transcribed using Superscript III RT and oligo-dT primers (Invitrogen). cDNA was diluted 5, 25, 125, 625 and 3,125 times and 2 μl was used as template for further polymerase chain reaction (PCR) amplification. Amplification was performed as follows: 94 °C for 3 min followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min. All primer sequences are listed in the Supplementary Information.

CGC infection with lentivirus

Gli1 cDNA was cloned in the HIV-CS-CG transfer vector, replacing the green fluorescent protein (GFP) marker gene. Lentivirus for Bmi or Gli1 overexpression was produced as previously described[25] and was added to freshly established CGC cultures 12 h after seeding. Infected CGCs were harvested 72 h after infection.

Histology, immunohistochemistry and *in situ* hybridization

For routine sections, brains were removed, immersion-fixed in 4% buffered paraformaldehyde for at least 4 h, cut in coronal or sagittal planes, paraffin embedded, cut in 3-μm-thick sections, and stained with haematoxylin and eosin. A Ventana Nexes Machine was used for immunohistochemical staining. A list of the antibodies and dilutions used is available as Supplementary Information. TdT-mediated dUTP nick end labelling (TUNEL) staining was performed with the *in situ* cell death kit (Roche).

For BrdU detection in cells, coverslips with fixed CGCs were treated for 2 min with 2 M HCl and incubated with mouse anti-BrdU antibody (DAKO) and with fluorescein-isothiocyanate-conjugated anti-mouse antibody. DAPI (4,6-diamidino-2-phenylindole) staining was used to label nuclei. The percentage of BrdU-positive cells on the total amount of DAPI-stained cells was calculated. Four 400-cell fields were counted per condition, using separate litters of animals and two coverslips per isolate.

In situ hybridizations for proteolipid protein, mGluR-2 and MATH-1 were carried out as described in ref. 21. The TSA amplification kit was used for mGluR-2.

Cell counts

Area measurement of the internal granular layer was carried out with AnalySiS imaging software. Cell density was determined in cranial, dorsal and caudal cerebellar regions (total number of internal granular layer neurons in three high-power fields each) for three wild-type and three $Bmi1^{-/-}$ mice (postnatal day 15), respectively. BrdU-positive or TUNEL-positive cells were counted in ten high-power fields centred on the EGL in four wild-type and four $Bmi1^{-/-}$ mice (postnatal day 8), respectively.

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Chapter 5

Oncogenic transformation of RK3E by Gli1 and Gli2 proteins involve L-myc induction and selection for N-myc upregulation

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manuscript in preparation

Oncogenic transformation of RK3E by Gli1 and Gli2 proteins involve L-myc induction and selection for N-myc upregulation

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Hedgehog signaling is suggested to be a major oncogenic pathway in a subset of tumors. Subverted Hedgehog signalling in normal tissue stem cells may lead to the inappropriate maintenance of stem cell characteristics and subsequent tumorigenesis of those stem cells. The Shh response is downstream at least partially mediated by the Gli transcription factors. Here, we describe that in cultured rat kidney epithelial cells, immortalized by E1A, can be transformed by Gli1 and Gli2 but not Gli3. Known Gli targets, such as Bmi1, cyclin D1 and D2, were found upregulated in Gli-transformed cells. Furthermore we observe an interesting difference in morphology between Gli1 and Gli2 overexpressing cells, possibly reflecting intrinsic differences in gene regulation potentially resulting in differential functional outcomes. Differences observed between Gli1 and Gli2 are in concert with the reported different skin tumor spectra induced by Gli1 and Gli2 as well as the recent report of a more important role for Gli2 in cerebellar development. Finally, we observe in parallel to what expression studies have been reported for Shh pathway activated tumors, a selection for N-myc upregulation as well as a novel induction of L-myc expression. Our results suggest an active selection for N-myc/L-myc induction, but not c-Myc, in Shh pathway activated tumors.

Introduction

Sonic hedgehog (Shh) is an excreted morphogen involved in major developmental pathways as well as stem cell biology. During embryogenesis Shh signaling is mainly implicated in differentiation programs and organ formation (e.g. specification of ventral fates in early neural tube)(reviewed in Ruiz I Altaba et al., 2003; Pasca di Magliano and Hebrok, 2003). In the adult, Shh is required for stem-cell maintenance and proliferation of a number of cell types (e.g. hematopoietic and neural stem cells, cerebellar granule neuron progenitors, pancreas precursors). Furthermore, (aberrant) Shh signalling has been linked to tumor development; specifically medulloblastoma, basal cell carcinoma (BCC), prostate cancer, pancreas cancer and small cell lung cancer (SCLC) (reviewed in Pasca di Magliano and Hebrok, 2003). These types of cancers show stem cell characteristics, fuelling the hypothesis that aberrant proliferation of stem cells and/or dedifferentiation of precursors 'drives' tumor cell proliferation and cancer progression. Additionally, this hints to a possible role for Hedgehog signalling in the normal environment or 'niche' of these cells. Such a role is firmly established for cerebellum and hair follicle but also suggested for pancreas and lung.

The Shh pathway is intricate as multiple homologs of the proteins in the pathway exist and several levels of

regulation have been described (reviewed in Ruiz I Altaba et al., 2003). Excreted Shh acts by binding to its receptor, Patched (Ptch), thereby releasing the inhibition of Ptch on the transmembrane protein Smoothened. Smoothened then signals downstream to the Gli transcription factors. Three Gli family members are known in mammals: Gli1, Gli2 and Gli3. Both Gli2 and Gli3 contain repressor and activator domains whereas Gli1 only harbors a strong activator domain. Proteolytic cleavage is thought to regulate the activator/repressor functions of Gli2 and Gli3. Feedback mechanisms involve Gli2 and Gli3 proteins that are transcriptionally regulating *Gli1* expression levels (Sasaki et al., 1999; Dai et al., 1999). Furthermore, *Patched* itself is a Gli target, providing a feedback inhibition loop (Dahmane et al., 1997; Agren et al., 2004). Gli proteins are functionally redundant as *Gli1* loss in mice affects viability only in a *Gli2*-heterozygote background (Park et al., 2000). Considering these data, a current model suggests combinatorial Gli activity with activator functions for Gli1, activating and repressing functions for Gli2 and repressing functions for Gli3 (Ruiz I Altaba, 1997). However such a model might be too simplistic, as for instance activator functions for Gli3 have been suggested (Bai et al., 2004; Lei et al., 2004). The different roles of the Gli proteins in each biological process have to be carefully analyzed since their action can be context dependent.

Although the developmental role of the different Gli proteins on dorsal-ventral patterning is well established and it has been shown that Gli1 and Gli2 are able to induce skin tumors in transgenic mouse models, data about the role of individual Gli proteins in adult tissues or other Shh dependent cancers is lacking (Ruiz I Altaba et al., 2003, Nilsson et al., 2000, Grachtchouk et al., 2000). Apart from the upregulation of *cyclin D1* and *D2*, which appear to be important for *Gli2*-dependent embryonic hair follicle development, little is known about Gli targets in other tissues (Mill et al., 2003). A complication in dissecting the relative importance of the Gli transcription factors is that few *in vitro* cell systems exist that show a proliferative response to *Gli* overexpression. Furthermore, to date no direct comparison between the three Gli proteins in one *in vitro* system has been made. We took advantage of the fact that RK3E cells, an adenovirus E1A immortalized epithelial cell line derived from neonatal rat kidney, were reported to transform upon overexpression of *Gli1* (Ruppert et al., 1991). We show that Gli2, but not Gli3, transforms RK3E cells as measured by their ability to grow in soft agar. Next to known downstream Gli targets such as *cyclin D1*, *D2* and *Bmi1*, we find that *L-myc* is upregulated upon *Gli1* or *Gli2* overexpression. In multiple independent *Gli* overexpressing clones, high expression of *N-myc* was selected for. Together with *N-myc* being induced by Shh in developing cerebellum (Kenney et al., 2003) and overexpression of *N-myc* and *L-myc* in Hedgehog-dependent cancers (reviewed in Nesbit et al., 1999) this suggests a strong link between Gli transcription factors and myc family member expression.

Materials and methods

Cell culture, retroviral infection, and soft agar assays - RK3E cells were maintained in DMEM supplemented with 10% fetal bovine serum. *Gli1* (human), *Gli2* (mouse), *Gli2ΔN2* (human) and *Gli3* (human) cDNAs were recloned to MSCV retroviruses and retroviral infections were performed as described (Jacobs et al., 1999a; Jacobs et al., 1999b). For analysis of growth in semisolid medium, 3 x 10^4 cell were plated per well

of a six-well dish in DMEM containing 10% serum and 0.4% low gelling temperature agarose (Sigma).

Western Blot Analysis- For protein expression analysis, RIPA lysates were separated on 9% SDS-PAGE and blotted on Immobilon-P membranes (Amersham Biosciences). Analysis was done according to standard methods using enhanced chemiluminesence (Amersham Biosciences). Primary antibodies were Bmi1 F6 mAb, C-19 for N-myc (SantaCruz), H-295 for Cyclin D1 (SantaCruz), M-20 for Cyclin D2 (SantaCruz), N-16 for Gli1 (SantaCruz), M2 for FLAG (Sigma) and 9E10 for myc-tagged protein.

Quantitative PCR- RNA of a six-well dish with RK3E cells was extracted using TRIZOL reagent (Invitrogen). Reverse transcription was performed with 1 ug RNA using Superscript II (Invitrogen) and oligod$(T)_n$ primers. qPCR was performed with 50 ng cDNA on an ABI7000 using a SYBR Green PCR Mastermix (Applied Biosystems). Primers were designed with Primer Express software. The amount of target, normalized to an endogenous reference and relative to a calibrator, was calculated by: $2^{-\Delta\Delta C}T$ (http://docs.appliedbiosystems.com/pebiodocs/04303859.pdf). Primer sequences used are: *L-myc* sense 5'-CGAGAGCCCCAGCGATT-3', *L-myc* antisense 5'-GATGTCCAGGGATCGTCTCTTC-3', *c-Myc* sense 5'-GCCCCTAGTGCTGCATGAA-3', *c-Myc* antisense 5'-AATTTCTTCCTCATCATCTTGTTCTTC-3', *Bmi1* sense 5'-TTACACCTGGAGACGGAATGG-3', *Bmi1* antisense 5'-TTGCAAGTTGGCCGAACTC-3', *N-myc* sense 5'-GCCCGAGCTGGTGAAGAA-3', *N-myc* antisense 5'-ACTCGGTGGCCTTTTTTAAGATG-3', *β-actin* sense 5'-CCTCATGAAGATCCTGACCGA-3', *β-actin* antisense 5'-TTTAATGTCACGCACGATTTCC-3', *Gli3* sense 5'-CTCTCATAGGAAATGGCACACAGT, *Gli3* antisense 5'-CCCGGGAGAAGCGTCAT-3'.

Results

RK3E cells transform upon Gli1 and Gli2 but not Gli3 overexpression- The epithelial cell line RK3E cells provides an effective *in vitro* model for oncogenic transformation. Many human tumors originate from epithelium whereas the oncogenic or tumor suppressive capacity of genes *in vitro* is often only tested in mesenchymal cells (fibroblasts). Constitutive activation of major developmental signal transduction pathways such as Wnt and Notch will transform *E1A* overexpressing RK3E cells (Kolligs et al., 1999; Kolligs et al., 2000; Foster et al., 1999; Kolligs et al., 2002; Capobianco et al., 1997; Dumont et al., 2000). Notably, some of these factors such as mutant β-catenin (Kolligs et al., 1999) and Gli1 (our unpublished observations) fail to transform fibroblasts. Whether this difference in transforming capacity of fibroblasts as opposed to RK3E cells is due to cell-type specific effects or due to *E1A* overexpression or any other genetic defect in the RK3E cell line is currently unknown. Of importance to mention here is that RK3E cells are diploid and show a remarkably low spontaneous immortalization upon prolonged tissue culture in monolayer or in soft agar. Hence, they provide a sensitive and convenient model system to assay Shh-mediated transforming capacity.

We assayed the effects of overexpression of *Gli1*, *Gli2* or *Gli3* by using replication-defective retroviruses in RK3E cells. Western blot analysis for Gli1 and Gli2 and qPCR on cDNA for *Gli3* confirmed that all constructs were overexpressed (Fig. 1A and 1B). A generally accepted hallmark of transformation is the ability of cells to grow anchorage-independently in semi-solid medium or soft agar. Overexpression of *Gli1* or *Gli2* enables RK3E cells to grow in soft agar whereas overexpression of *Gli3* does not (Fig. 1D). Interestingly, Gli2 induces a much more potent transformation as Gli1 as measured by the higher number of colonies in *Gli2*-overexpressing cultures (Fig. 1C) Moreover, there is also a clear difference in morphological phenotype

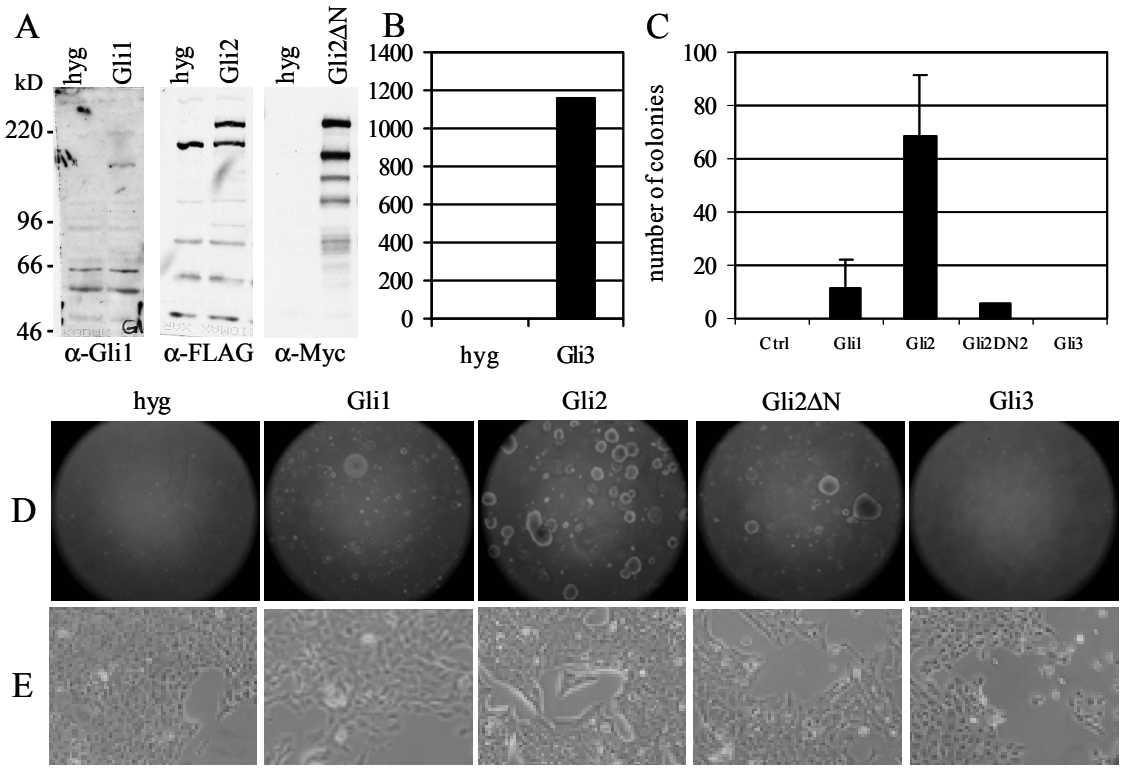

Fig. 1 **Transformation of RK3E cells by overexpression of Gli1, Gli2, and Gli2ΔN2 but not Gli3.** A). Western blot analysis of Gli1, Gli2 and Gli2ΔN2 overexpression in retrovirally infected RK3E cells with the indicated antibodies. B). qPCR on cDNA showing *Gli3* overexpression in retrovirally infected RK3E cells C). Quantification of growth of different Gli clones in soft agar as depicted in D). by counting the number of colonies in multiple experiments from a defined surface. D). Growth of RK3E cells in soft agar for 10 days. Note that *Gli2* overexpressing cells are most transformed, *Gli1* and *Gli2ΔN2* show a less transformed phenotype whereas *Gli3* and control hygromycin-infected cells were not transformed at all. E). Morphology of RK3E cells grown in monolayer. Note that only wild-type *Gli2* overexpressing cells show a remarkably different phenotype from the other cells.

between *Gli1* and *Gli2* overexpressing cells (Fig. 1E). Whereas *Gli1* overexpressing cells look similar to control cells at subconfluent density, *Gli2* overexpressing cells grow in discrete colonies. As Gli2 has already been reported to repress epidermal differentiation markers, this morphology might simply reflect a less differentiated cell type as compared to control or *Gli1* overexpressing cells (Regl et al., 2004). We also analyzed effects of *Gli2ΔN2*, a mutant of *Gli2* lacking the N-terminal repressor domain. In spite of high overexpression of *Gli2ΔN2* (Fig. 1A) both the morphological phenotype in monolayer culture as well as the relative transforming capacity was more similar to that of Gli1 (Fig. 1C, D and E). In conclusion, our results show a strong effect of Gli1 and Gli2, but not Gli3, on transformation of RK3E cells. Moreover, we establish a role for the repressor domain of Gli2 in enhancing the transforming capacity of the protein and influencing the morphology of the cells.

Bmi1, cyclin D1 and D2 are induced by Gli1 and Gli2 in RK3E cells- In order to analyze the mechanism of Gli-mediated transformation of RK3E cells, we set out to examine expression of Gli target genes. We recently published the regulation of the Polycomb group gene and epigenetic chromatin modifier *Bmi1* by Shh signaling (Leung et al., 2004). Gli1, Gli2 and Gli2ΔN2

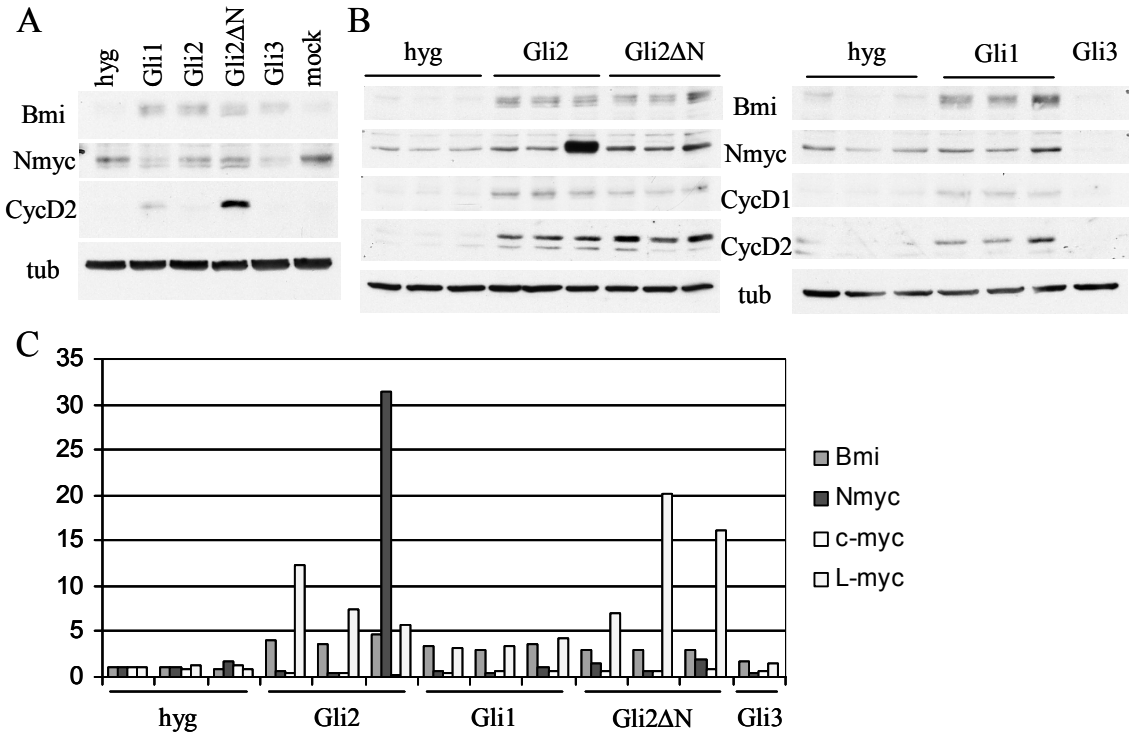

Fig. 2 **Expression analysis of Gli target genes.**
A). Western blot analysis of polyclonal pools of retrovirally infected RK3E cells 2 weeks after infection. Note that although Bmi1 and cyclin D2 are upregulated in *Gli1* and *Gli2* overexpressing cells, no effect is seen on N-myc expression. B). Western blot analysis another 3 weeks later of the same RK3E cells as in A). but then maintained as three individual pools. Note that one *Gli2*-overexpressing clone has upregulated N-myc to a high extent. C). qPCR analysis of cDNA of the same cells at the same time as in panel B). Note that Gli1, Gli2 and Gli2ΔN2 clones all show upregulation of *Bmi1*, and *L-myc* and one Gli2 overexpressing clone shows elevated *N-myc* transcription.

induce *Bmi1* expression whereas Gli3 does not (Fig 2A and 2B). Quantitative PCR analysis indicates *Bmi1* is transcriptionally regulated by Gli1 and Gli2 (Fig. 2C). As we found no Gli consensus binding sites in the *Bmi1* promoter, we speculate that the transcriptional regulation of *Bmi1* by Gli is indirect. In addition to *Bmi1*, the known Gli target genes *cyclin D1* and *D2* are also upregulated in *Gli1*, *Gli2* and *GliΔN2* overexpressing cells (Fig. 2A and 2B).

Interestingly, both *Bmi1* and *cyclin D1* and *D2* induction correlate with the transformed phenotype of *Gli*-overexpressing RK3E cells. Elevated *Bmi1* expression does not merely reflect the transformed status of the *Gli* overexpressing RK3E cells as RK3E cells transformed by mutant β-catenin do not show upregulation of *Bmi1* (data not shown). This places *Bmi1* specifically in the Shh signaling pathway and suggests it is not regulated by Wnt signaling. Bmi1 is a regulator of *Ink4a/Arf*, the locus encoding for $p16^{Ink4a}$ and $p19^{Arf}$ tumor suppressors that are regulating the Rb and p53 pathways respectively (Jacobs et al., 1999a). Therefore, one could easily envision a role for Gli mediated cell cycle regulation via Bmi1 and cyclin D1 and D2. However, knockdown of either *Bmi1* or *cyclin D2* levels by RNAi did not affect the growth capacity of Gli transformed cells. Successful targeting of both proteins by siRNA constructs was confirmed by

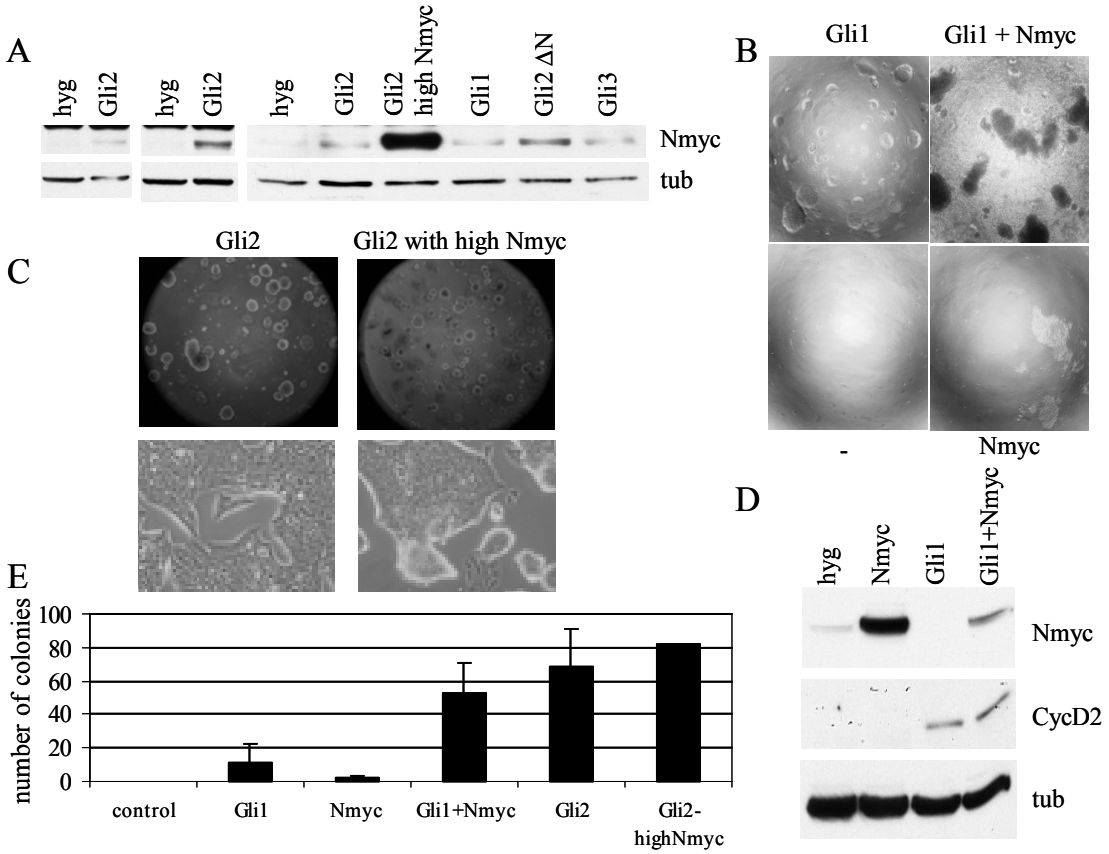

Fig. 3 **Overexpression of N-myc is selected for and collaborates with Gli1 and Gli2 in transformation.**
A). Western blot analysis of high N-myc expression in multiple independent RK3E cells overexpressing *Gli1*, *Gli2* or *Gli2ΔN2* upon prolonged culture. B). Gli1 and N-myc overexpression collaborate in transformation: indicated RK3E cells were grown for 10 days in soft agar. C). Gli2 and N-myc overxpression collaborate in transformation: indicated RK3E cells were grown for 10 days in soft agar. D). Western blot analysis of Cyclin D2 levels in N-myc and Gli1 overexpressing RK3E cells. E). Quantification of growth of different clones in soft agar as depicted in B). and C). by counting the number of colonies in multiple experiments from a defined surface.

Western blot analysis (data not shown). In addition, we know at least for the *Bmi1* siRNA that it is functional, as it induces premature senescence in fibroblasts similar to what has been reported for *Bmi1*-/- fibroblasts (Jacobs et al., 1999a; data not shown). Moreover, *Bmi1* overexpression alone did not transform RK3E cells whereas we have shown that in fibroblasts Bmi1 can cooperate with c-Myc or Ras in the transformation of fibroblasts (Jacobs et al., 1999a, Jacobs et al., 1999b). *In vivo* both *Bmi1* and *cyclin D1/D2* regulation are relevant for the mitogenic response to Shh stimulation as evidenced by the decreased proliferation in cerebellum of *Bmi1* and *cyclin D1/D2* knockout mice (Leung et al., 2004; Ciemerych et al., 2002). RK3E cells overexpress *E1A*, a protein that influences cell cycle via Rb and p53 (reviewed in Frisch and Mymryk, 2002). Therefore it is possible that there is no mitogenic effect of the depletion of these Gli targets as their downstream targets are already affected by *E1A* overexpression.

Gli overexpressing RK3E cells show elevated levels of L-myc and upon prolonged culture select for increased N-myc expression- Since *N-myc* is an

essential downstream target of Shh in cerebellar development and proliferation (Kenney et al., 2003), we analyzed *N-myc* expression levels in *Gli* overexpressing RK3E cells. Although in initial cultures we do not see any robust effect on *N-myc* expression (Fig. 2A), after prolonged culturing *N-myc*, but not *c-Myc*, overexpression in several independent *Gli*-overexpression cell lines is selected for (Fig. 2B and 3A). In addition, when a polyclonal pool of *Gli2*-infected RK3E cells were split and maintained as three independent cultures, one clone exhibited substantial overexpression of *N-myc*, suggestive of gene amplification (Fig. 2B). Therefore, we conclude that in RK3E cells, in contrast to what has been suggested for cerebellar granule neuron progenitors (Kenney et al., 2003), *N-myc* is not directly regulated by Gli transcription factors. However, high *N-myc* expression is selected for in *Gli*-overexpressing clones. Interestingly, in independent clones N-myc levels are elevated to different levels suggesting a certain threshold level of N-myc sufficient for transformation of the cells. Although N-myc protein levels are slightly elevated in all Gli overexpressing cell lines, *N-myc* mRNA is only upregulated in the case where there is also highest protein expression. This suggests N-myc levels are also regulated post-transcriptionally. N-myc cooperates with Gli1 and Gli2 in transformation. First, the *Gli2*-overexpressing clone with high N-myc levels distinguishes itself morphologically from *Gli2* overexpressing cells in the sense that these cells even more than *Gli2* overexpressing cells grow in distinct colonies (Fig. 3C). Second, forced overexpression of *N-myc* with *Gli1* will enhance the ability of *Gli1* overexpressing cells to grow in soft agar (Fig. 3B, E). Unfortunately, we were not able to show that Gli transformation is dependent on *N-myc* expression as we experienced extreme toxicity of *N-myc* RNAi in RK3E cells (data not shown). Interestingly however, we observed that Gli induction of *cyclin D2* is independent of *N-myc*. First *N-myc* overexpression is not able to induce *cyclin D2* expression (Fig. 3D). Second, the cells with combined *Gli1* or *Gli2* and *N-myc* overexpression do not show an enhanced *cyclin D2* induction (Fig. 2B and 3D). Third, the *cyclin D2* induction by Gli1 and Gli2 can already be observed in the absence of effects on *N-myc* expression (Fig. 2A). The data are in contrast to results obtained in cerebellar granule neuron progenitors where Shh has been suggested to induce *cyclin D1* and *D2* via *N-myc* (Kenney et al., 2003).

However, forced *N-myc* overexpression is not as potent as *Gli* overexpression in inducing RK3E transformation suggesting the existence of other Gli targets that influence Gli-mediated transformation (Fig. 3B). In this respect, it is interesting that in *Gli1* and *Gli2* overexpressing cell lines we see an upregulation of *L-myc* (Fig. 2C). L-myc is the third family member of the myc oncoprotein family and is found amplified and/or overexpressed in SCLC (reviewed in Nesbit et al., 1999). A connection between high *L-myc* expression and Hedgehog signalling is to our knowledge unprecedented and might be very relevant for *in vivo* tumorigenesis.

Discussion

In this report we compare the oncogenic potential of the transcription factors that are downstream of the Shh pathway: Gli1, Gli2 and Gli3. We show that wild-type Gli3 is unable to transform RK3E cells, rat kidney epithelial cells overexpressing *E1A*. Although *Gli1* overexpression is able to transform RK3E cells, *Gli2* has the most potent

oncogenic capacity in these cells accompanied by a striking morphological change upon overexpression. *Gli* overexpressing RK3E cells did show features of an active Shh pathway as known Gli target genes such as *Bmi1* and *cyclin D1* and D2 were upregulated. Nevertheless, we were unable to show any biological relevance of these inductions in RK3E cells, which might be due to *E1A* overexpression. This does not disprove a role for these targets in normal development as well as in tumorigenesis. Indeed, we recently reported *Bmi1* as a critical Shh target in cerebellar development as well as *Bmi1* overexpression in medulloblastoma (Leung et al., 2004). Moreover, others have shown Shh signalling to induce *cyclin D1* and *D2* expression in cerebellum, in good agreement with the cerebellar defects in *cyclin D* deficient mice (Ciemerych et al., 2001; Kenney et al., 2000).

Interestingly, our data correlates very well with data obtained for skin tumorigenesis in *K5-Gli* transgenic mice. Whereas *K5-Gli2* transgenic mice only develop basal cell carcinomas (BCCs), *K5-Gli1* and *K5-Gli2ΔN2* transgenic mice develop multiple different types of skin tumors and only a minority of these are BCCs (Sheng et al., 2002; Nilsson et al., 2000, Grachtchouk et al., 2000). This is in line with the more potent transformation and different phenotype of *Gli2* overexpressing RK3E cells as opposed to *Gli1* and *Gli2ΔN2*. Apparently, the repressor domain of Gli2 is regulating other targets involved in transformation. As microarray studies have suggested targets of Gli1 in epithelial to mesenchymal transition (EMT) (Louro et al., 2002), the repressor domain of Gli2 might be implicated in preventing such a transition or alternatively in preventing cells from differentiating. Thereby, it could contribute to a more stem cell like tumor phenotype, such as BCCs, whereas overexpression of *Gli1* and *Gli2ΔN2* could give rise to the more differentiated tumor types.

As Gli3 has been mainly thought of as a repressor of Shh signalling, it might not be surprising that the full length protein will not transform RK3E cells. Nevertheless, Gli3 also contains both a repressor and activator domain. Recently, Gli3 was implicated as an activator in developing spinal cord (Bai et al., 2004; Lei et al., 2004). In rare cases of hereditary Greig syndrome, a disease caused by mutations in *GLI3*, medulloblastomas have been reported to develop. Importantly, these *GLI3* mutations cause the occurrence of a truncated protein lacking the GLI3 repressor domain (Erez et al., 2002). This suggests, along with the tumorigenic potential of Gli1 and the activator domain of Gli2, an important function for Gli activation domains in tumorigenesis as well.

In the past, RK3E cells have mainly been used to evaluate the effect of Wnt signalling and it was found that both mutant *β-catenin* and wild-type *γ-catenin* overexpression transforms these cells (Kolligs et al., 1999; Kolligs et al., 2000). Although it has been proposed that in neoplastic tissues *c-Myc* is a critical target of the APC/β-catenin/TCF pathway (He et al., 1998), these authors claim that in RK3E cells *c-Myc* expression is only elevated in half of the mutant *β-catenin* overexpressing cells. Therefore, they conclude that *c-Myc* is not a direct transcriptional target of β-catenin in RK3E cells. Interestingly, we observe a similar phenomenon by studying overexpressing of *Gli* transcription factors in RK3E cells. It is known that Shh stimulation in cerebellar granule neuron progenitors leads to induced *N-myc* expression, although direct regulation of *N-myc* by Gli has so

far not been reported (Kenney et al., 2003). Our results show that although *N-myc* expression is not directly regulated by Gli transcription factors in RK3E cells, in *Gli*-expressing clones elevated expression of *N-myc* is selected for. Interestingly, this selection seems specific for *N-myc* upregulation as *c-Myc* is not elevated on the transcriptional level in these clones. As in these same RK3E cells high *c-Myc* expression is selected for in combination with mutant *β-catenin* overexpression, this suggests an *in vivo* relevance. We propose that high *c-Myc* expression is more associated with Wnt-pathway-activated tumors whereas high *N-myc* expression is actively selected for in Shh-pathway-activated tumors. In gastrointestinal tumors, a causal relationship between activated APC/β-catenin and elevated *c-Myc* expression has already been established (He et al., 1998). In neuroblastoma, *N-myc* is frequently found amplified (Brodeur et al., 1984). However, in those cases were there is allelic imbalance of the *APC* locus, specifically *N-myc* amplification is lacking (Meltzer et al., 1996). Intriguingly, in a panel of mouse medulloblastomas that developed in different genetic backgrounds, all tumors invariably show active Shh signalling as well as high *N-myc* expression (Lee et al., 2003). Notably, in human desmoplastic medulloblastoma *PTCH* levels (*PTCH* itself being a transcriptional target of SHH) and *GLI1* expression correlate best with *N-MYC* (Pomeroy et al., 2002).

In *Gli*-overexpressing RK3E clones, with the exception of one clone showing highly elevated *N-myc* transcription, most clones showed moderately elevated N-myc protein but not mRNA levels. Recently it was reported that in cerebellar granule neuron progenitors PI3K signalling regulates the phosphorylation status of N-myc thereby stabilizing the protein (Kenney et al., 2004). This suggests elevated *PI3K* levels could help to stabilize N-myc in RK3E cells. Interestingly, IGF signalling regulates cell survival through PI3K-AKT-TOR network (reviewed in Pollak et al., 2004) and an IGF pathway component (*Igf2*) has already been placed downstream of Shh pathway (Hahn et al., 2000; Louro et al., 2002; Yoon et al., 2001). Importantly, IGF signalling and Shh synergize in medulloblastoma formation suggesting this stabilization of N-myc protein via Igf2-PI3K signalling is of relevance in tumorigenesis as well (Rao et al., 2004).

In addition to upregulation of *N-myc* levels, we observed elevated *L-myc* expression in *Gli1* and *Gli2* overexpressing cell lines. Although *L-myc* is not upregulated in Shh-treated cerebellar granule cells (Kenney et al., 2003), several findings argue in favor of a role of L-myc in Hedgehog signaling. First, treatment of a medulloblastoma cell line with the Shh-pathway inhibitor cyclopamine decreased expression of all *myc* genes including *L-myc* (Berman et al., 2002). Second, *L-myc* is found amplified and/or overexpressed in SCLC (reviewed in Nesbit et al., 1999). Recently, a crucial role for the Shh pathway was shown in these tumors with primitive neuroendocrine features (Watkins et al., 2003). Therefore, we suggest that a causal relationship between activated Shh signaling and elevated *L-myc* expression in SCLC might exist alike in RK3E cells. It will therefore be of interest to test whether in the airway epithelium progenitors *L-myc* is a critical target of Shh during development and/or in repair. Another parallel between our results and previous data is the observed co-amplification or co-overexpression of (almost always) two *Myc* family members (reviewed in Nesbit et al., 1999). We suggest expression analysis for the *Myc* family

members, *c-Myc*, *N-myc* and *L-myc*, should be undertaken in several Hedgehog and Wnt dependent tumors to prove a correlation between overexpression of specific *Myc* members with either Shh or Wnt pathway components in these tumors. We show that both Gli1 and Gli2 collaborate with N-myc in the transformation of RK3E cells. Others have shown a small increase in medulloblastoma occurrence by targeting both expression of *Shh* and *c-Myc* as opposed to *Shh* alone to neural progenitor cells (Rao et al., 2003). Intriguingly however, in *Ptc*+/- mice, that spontaneously develop medulloblastoma at a low frequency, targeting *c-Myc* expression to *Ptc*+/- neural progenitor cells did not enhance tumorigenesis (Fults et al., 2002). Our results suggest *N-myc* and/or *L-myc* expression might be more relevant in these Hedgehog dependent cancers.

In conclusion, we show in a cancer relevant and developmental pathway sensitive cell type, rat kidney cells overexpressing *E1A*, a very potent transforming activity of mainly Gli2 and to a lesser extent Gli1. Importantly, we observed selection for high expression of *N-myc*, similar to what happens *in vivo* during tumorigenesis. Moreover, we show for the first time a collaboration between *N-myc* and *Gli* overexpression in transformation. In addition, we show an unexpected and unprecedented upregulation of *L-myc* expression in these Shh-pathway activated cell lines. Our results call for a more in depth analysis of *N-myc* and *L-myc* expression in and contribution to Hedgehog dependent cancers.

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Chapter 6

Ink4a and *Arf* differentially contribute to *Bmi1* deficient phenotypes in the hematopoietic and central nervous system, and in neural progenitors

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Ink4a and *Arf* differentially contribute to *Bmi1* deficient phenotypes in the hematopoietic and central nervous system, and in neural progenitors

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The Polycomb group (PcG) gene *Bmi1* functions as a strong repressor of the *Ink4a/Arf* tumor suppressor locus. Deregulation of this locus is implicated in *Bmi1* induced tumorigenesis and in developmental defects of *Bmi1* deficient mice. Notably, neurological and hematopoietic abnormalities of these animals can be traced back to aberrations in the respective stem cell compartments. Here, we assess the relative importance of *Ink4a* versus *Arf* in mediating the proliferative defects and premature senescence caused by *Bmi1* deficiency. We analyzed the rescue of *Bmi1* loss-of-function phenotypes on proliferation and renewal of neural stem cells, cerebellar development, axial skeleton identity, overall body growth, B and T cell compartments and embryonic fibroblasts in the context of *Ink4a, Arf,* or *Ink4a/Arf* deficiency. We demonstrate significant rescue of lymphoid and cerebellar cell proliferation by *Arf* or *Ink4a/Arf* heterozygosity, highlighting specific threshold levels for this locus *in vivo.* Furthermore, we show that *Arf* loss enhances proliferation or self-renewal in all cell systems analyzed, indicating a general role for the *Arf/p53* pathway. In contrast, *Ink4a* de-repression is profoundly affecting more undifferentiated cells. This suggests differential cell type specific roles for *Ink4a* and *Arf* in cell cycle control of precursor- versus terminally differentiated cells by PcG mediated repression.

Introduction

The *Bmi1* gene was identified as a collaborating oncogene in *c-Myc* induced lymphomagenesis and subsequently found to be overexpressed in several human cancers (Haupt et al. 1991; Van Lohuizen et al. 1991; Bea et al. 2001; Van Kemenade et al. 2001; Vonlanthen et al. 2001; Kim et al. 2004; Leung et al. 2004; Neo et al. 2004). *Bmi1* is a member of the Polycomb group (PcG), a conserved gene family of chromatin modifiers and transcriptional repressors (reviewed in Lund and Van Lohuizen, 2004). Originally, PcG proteins were discovered in *Drosophila* as repressors of the *Homeotic* genes. In mammals, loss of a PcG gene is accompanied by alterations in *Homeobox (Hox)* gene expression that commonly manifests in skeletal malformations (Van der Lugt et al. 1994; Akasaka et al. 1996; Core et al. 1997; Del Mar Lorente et al. 2000). However, *Hox* genes are not the only targets of PcG mediated repression, as the tumor suppressor locus *CDKN2A* (hereafter

Ink4a/Arf locus) also is negatively regulated by *Bmi1* and other PcG members (Jacobs et al. 1999a; Voncken et al. 2003; Core et al. 2004; Gil et al. 2004).

The *Ink4a/Arf* locus is unusual as it codes for two proteins, $p16^{ink4a}$ and $p19^{arf}$ (*Ink4a* and *Arf*), by use of alternative reading frames (Serrano et al. 1993; Quelle et al. 1995). *Ink4a* and *Arf* are important players in the *retinoblastoma* (*pRB*) and *p53* pathways, respectively, and their activation results in growth arrest, senescence or apoptosis thereby safeguarding against neoplastic transformation (reviewed in Sharpless and DePinho, 1999; and in Lowe and Sherr, 2003). After mutations in the *p53* gene, inactivation of the *Ink4a/Arf* locus is the most frequently found alteration in a wide variety of cancers underlining its importance as a tumor suppressor (Lowe and Sherr, 2003).

It is conceivable that the *Ink4a/Arf* locus has other biological functions, especially in short-lived species that are not cancer prone. However, apart from a specific function for *Arf* in the vitreous of the eye and *Ink4a* in the thymus, no obvious role for *Ink4a/Arf* expression in development has so far been reported (Serrano et al. 1996; Kamijo et al. 1997; Krimpenfort et al. 2001; Sharpless et al. 2001; McKeller et al. 2002; Sharpless et al. 2004). It has been suggested that the locus is involved in aging as both *Ink4a* and *Arf* were found to be elevated in aging mice (Zindy et al. 1997; Krishnamurthy et al. 2004; Sun et al. 2004). Indeed, one can imagine that in contrast to the situation in less complex organisms such as *Drosophila*, lifelong homeostatic maintenance of tissues in mammals critically depends on proper regulation and longevity of adult stem cells or progenitors. Stress signals received during life may eventually exhaust the long-lived progenitor or stem cell populations.

Interestingly, loss of *Bmi1* has revealed a putative role for *Ink4a/Arf* in stem cells. In addition to skeletal malformations and overall growth retardation, *Bmi1* deficient mice suffer from severe abnormalities of the hematopoietic and nervous systems (Van der Lugt et al, 1994). Generation of *Bmi1;Ink4a/Arf* compound mutant mice provided genetic evidence that at least part of these defects are due to deregulation of the *Ink4a/Arf* locus (Jacobs et al. 1999a). Importantly, we and others have recently shown that *Bmi1* is essential for the renewal of hematopoietic and neural stem cells of the central and peripheral nervous systems as well as for proliferation of cerebellar granule neuron progenitors (Molofsky et al. 2003; Park et al. 2003; Iwama et al. 2004; Leung et al. 2004). Loss of *Ink4a* could partially alleviate the defect in self-renewal of *Bmi1* deficient neural stem cells, implicating that, eventhough complete loss or mild overexpression does not seem to grossly influence development, deregulated activation of the *Ink4a/Arf* locus negatively regulates stem cell renewal (Serrano et al. 1996; Kamijo et al. 1997; Krimpenfort et al. 2001; Sharpless et al. 2001; Matheu et al. 2004; Sharpless et al. 2004).

However, as *Bmi1* is a potent repressor of both *Ink4a* and *Arf* it is important to discriminate which one of the *Ink4a/Arf* products is more crucial for relaying *Bmi1* function. Here we took advantage of the various specific knockout mouse models made for the locus. We addressed the relative contribution of *Ink4a* and *Arf* deregulation upon loss of *Bmi1* to overall body growth and development, to proliferation and senescence of MEFs,

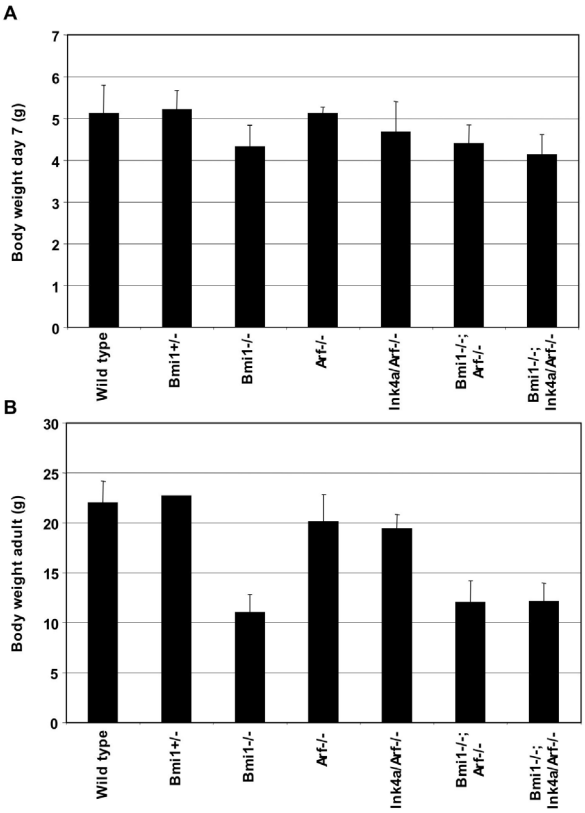

Figure 1. Loss of *Ink4a/Arf* does not rescue overall development of *Bmi1* deficient mice. (A) At postnatal day 7 *Bmi1*$^{-/-}$ mice display a modest but significant 15% reduction in body weight ($p<0.05$). (B) The growth retardation phenotype of *Bmi1*$^{-/-}$ mice progressively persists into adulthood. The 50% reduction in their body weight is not significantly rescued by loss of either *Arf* or *Ink4a/Arf*.

lymphoid cells and cerebellar granule neuron progenitors, and to the self-renewal capacity of neural stem cells. We demonstrate that in some tissues, repression of *Arf* is the main function of *Bmi1*, however, particularly in less differentiated cells, deregulated expression of *Ink4a* significantly contributes to the *Bmi1* deficient phenotype. Furthermore, we reveal specific dosage effects of *Ink4a* and *Arf* both in *vitro* and *in vivo*, and show that the *Ink4a/Arf* locus restricts neural stem cell self-renewal.

Results

Loss of the Ink4a/Arf locus does not significantly rescue overall growth or skeletal malformations

As *Bmi1* deficient mice suffer from growth retardation and an overall reduction in body mass, we sought to determine to what extent loss of *Ink4a* and *Arf* are capable of rescuing this phenotype. At 7 days after birth, *Bmi1* deficient but not heterozygous pups have an approximate reduced body mass of 15% ($p=0.05$)(fig.1a). This trend perseveres progressively into adulthood (1-2 months old mice) as adult *Bmi1* knockout mice display a 50% reduced body mass when compared to wild types (fig.1b). Deficiency for *Ink4a/Arf* or *Arf* alone does not influence body mass in agreement with earlier publications. Loss of either *Arf* or *Ink4a/Arf* in the *Bmi1* knockout background during adult life results in a mild but not significant trend for an increase in body mass. However, at postnatal day 7 such a partial rescue cannot (yet) be observed. Considering its proposed role in aging, it is possible that *Ink4a* effects are relevant only during adult life. However, we were not able to further monitor this phenotype as *Bmi1* deficient mice have to be sacrificed due to increased sickness, usually around 8-12 weeks.

The previous analysis of skeletal whole mounts of newborn *Bmi1* deficient mice revealed several malformations along the anteroposterior axis accompanied by subtle de-repression of *Hox* genes, in line with *Bmi1* functioning as a repressor of the *Hox* genes (Van der Lugt et al, 1994; Van der Lugt et al, 1996). We tried to establish whether de-repression of the *Ink4a/Arf* locus is implicated in these posterior axial skeletal transformations. As is shown in table 1, no clear improvements of these malformations were observed in

Skeletal phenotype (%)			
	WT	Bmi1 -/- (n=8)	Bmi1 -/-; Ink4a/Arf-/- (n=7)
Cervical region			
Evidence for proatlas:			
Rostral piece of bone/cartilage (C0)	-	87.5	42.9
Atlas (C1):			
Split	-	75	100
(C1→C2)	-	100	100
Tuberculum anterior:			
C6	100	100	100
C5	-	25	-
Ribanlage:			
C7 rudimentary rib	-	93.7	92.9
C7 small rib	-	6.3	7.1
Thoracic region			
# of vertebrosternal ribs:			
7	100	31.2	28.6
6	-	68.8	71.4
# of sternebrae:			
6	100	-	-
5	-	100	100
Lumbar region			
Last rib on:			
T12 (T13→L1)	-	37.5	-
T13 rudimentary (partial T13→L1)	-	18.8	42.9
T13	100	43.7	57.1
# of lumbal vertebrae:			
5	-	-	21.4
6	100	93.7	78.6
7	-	6.3	-

Table 1. Genetic removal of the *Ink4a/Arf* locus does not lead to improvement of the *Bmi1* deficient axial skeleton phenotypes.

Bmi1;Ink4a/Arf knockout mice. Since the *Bmi1*deficient skeletal phenotype is not fully penetrant, we think the minor differences observed are not significant. Together, these results indicate that *Bmi1* exerts its function on segmentation and overall body growth via other target genes.

Arf is the main target of Bmi1 repression in proliferating mouse embryonic fibroblasts

Upon explantation, mouse embryonic fibroblasts (MEFs) upregulate *Ink4a* and *Arf* and this accumulation leads to a special form of growth arrest termed senescence (reviewed in Sherr and DePinho, 2000). When encountering cellular stress such as oncogene overexpression or aberrant cell culture conditions, cells can senesce prematurely through activation of the *pRB* and *p53* pathways (reviewed in Lundberg et al. 2000). *Bmi1* deficient MEFs also undergo premature senescence due to de-repression of the *Ink4a/Arf* locus (Jacobs et al. 1999a). This *Bmi1* mediated suppression of *Ink4a* and *Arf* is dose dependent (fig.2a). Eventhough $p16^{ink4a}$ protein levels are upregulated to a large extent, absence of $p16^{ink4a}$ does not overcome premature senescence in *Bmi1* deficient primary MEFs (fig.2b). In contrast, *Bmi1/Arf* doubly deficient cells proliferate as fast as *Arf* deficient cells and are immortal (fig.2c). This is in agreement with the earlier observation that *Bmi1;Ink4a/Arf* knockout MEFs are rescued from premature senescence, and demonstrates that *Arf* is the main target of *Bmi1* in proliferating MEFs.

Arf controls the activity of p53 by sequestering Mdm2, an E3 ubiquitin ligase for p53, thereby stabilizing p53 which leads to transcription of p53 target genes (reviewed in Sherr and Weber, 2000). However, recent evidence showed that *Arf*

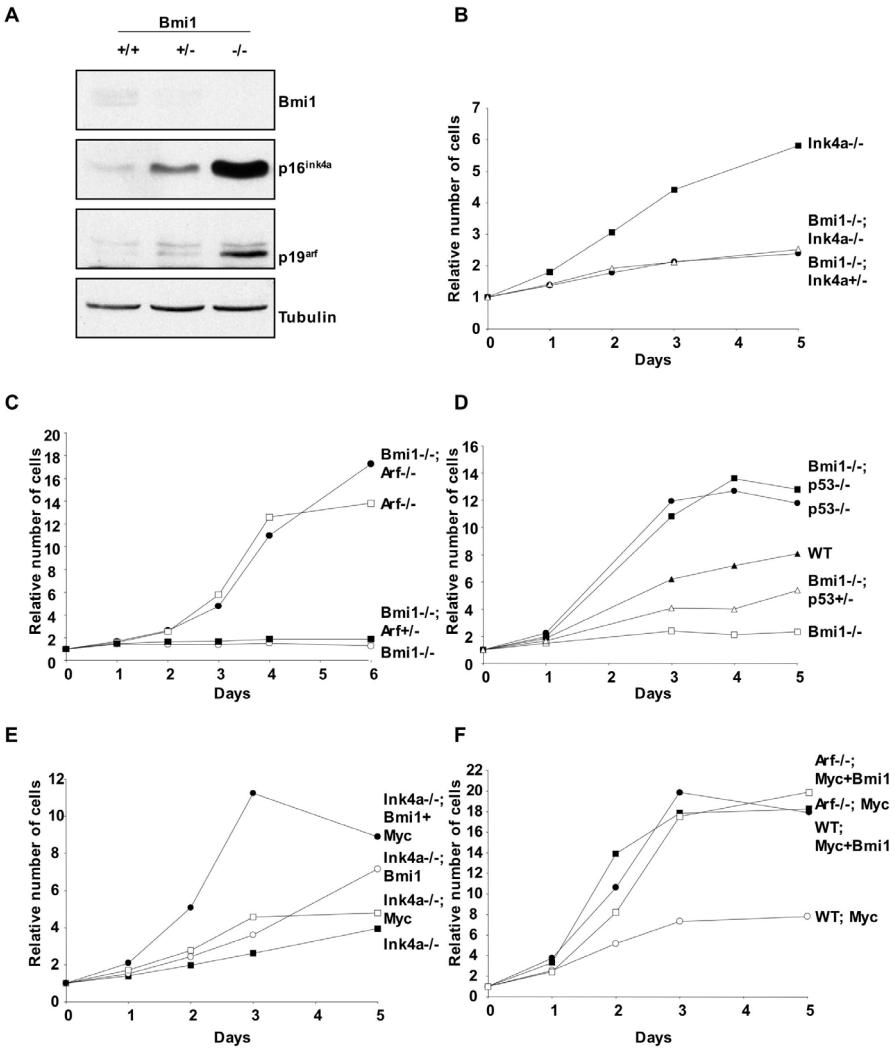

Figure 2. The *Arf/p53* pathway is the main target of repression by *Bmi1* in mouse embryo fibroblasts. (A) Dose dependent regulation of $p16^{ink4a}$ and $p19^{arf}$ by *Bmi1* as shown by Western blot analysis on lysates from wild type, $Bmi1^{+/-}$ and $Bmi1^{-/-}$ MEFs. (B) Growth curves of passage 3 MEFs show that $Bmi1^{-/-};Ink4a^{+/-}$ and $Bmi1^{-/-};Ink4a^{-/-}$ MEFs both enter senescence at an equally early timepoint, indicating that loss of *Ink4a* cannot rescue the premature senescence of *Bmi1* knockout MEFs (C) Growth curves of passage 4 MEFs demonstrate that $Bmi1^{-/-};Arf^{-/-}$ MEFs proliferate as well as $Arf^{-/-}$ MEFs, whereas at the same passage, $Bmi1^{-/-}$ or $Bmi1^{-/-};Arf^{+/-}$ MEFs undergo premature growth arrest and cease proliferation. (D) Growth curves showing that passage 4 $Bmi1^{-/-};p53^{-/-}$ MEFs proliferate to a similar extent as $p53^{-/-}$ MEFs, in contrast to $Bmi1^{-/-}$ MEFs which undergo premature senescence. (E) Growth curves showing that *Bmi1* overexpression strongly enhances the proliferation rate of *c-Myc* overexpressing $Ink4a^{-/-}$ MEFs. (F) The oncogenic collaboration between *Bmi1* and *c-Myc* is solely dependent on *Arf* repression as *Bmi1* overexpression does not further enhance *c-Myc* induced proliferation of $Arf^{-/-}$ MEFs. Each chart is representative for 3 independent experiments.

also has *p53* independent functions as an *Myc* antagonist (Weber et al. 2000; Eymin et al. 2003; Qi et al. 2004). This prompted us to question if *p53* loss can substitute for *Arf* loss in rescuing *Bmi1* deficient MEFs. As is represented in fig.2d, *Bmi1/p53* doubly deficient MEFs are immortal as are the *p53* deficient controls. This suggests that *p53* activation by *Arf* is responsible for the impaired proliferation of $Bmi1^{-/-}$ fibroblasts. In agreement with this, *Mdm2* overexpression rescues the impaired proliferation of $Bmi1^{-/-}$ MEFs as well (supplementary fig.1).

c-Myc is a bHLH/LZ transcription factor that is both a growth promoting

oncogene and a potent inducer of apoptosis (Reviewed in Nilsson and Cleveland, 2003). Part of *c-Myc*-induced apoptosis depends on activation of *p53* through *Arf* (Zindy et al. 1998). Previously, we demonstrated that *Bmi1* through inhibition of *Arf* activation desensitizes cells to *c-Myc*-induced apoptosis both *in vitro* and *in vivo* (Jacobs et al. 1999b). Consistent with this is the strong collaboration in lymphomagenesis between the Eμ-*Myc* transgene and heterozygosity of the *Ink4a/Arf* locus or *Arf* alone (Eischen et al. 1999; Jacobs et al. 1999b; Schmitt et al. 1999). However, our previous experiments did not exclude that downregulation of *Ink4a* by *Bmi1* also contributes to the oncogenic collaboration between *Bmi1* and *c-Myc*. In fact, a possible role for *Ink4a* is supported by the slower rate of tumor formation in Eμ-*Myc;Arf* heterozygous mice compared to Eμ-*Myc;Ink4a/Arf* heterozygous mice (Eischen et al. 1999; Jacobs et al. 1999b). *c-Myc* overexpressing *Ink4a*$^{-/-}$ MEFs proliferate at a similar rate as *Ink4a*$^{-/-}$ MEFs indicating that loss of *Ink4a* does not overtly stimulate *c-Myc* induced proliferation (fig. 2e). Importantly, *Bmi1* overexpression in *Ink4a* knockout MEFs transduced with *c-Myc* strongly enhances proliferation, suggesting a role for *Bmi1* in repressing *Arf* (fig.2e). Indeed, failure of *Bmi1* overexpression to further increase the proliferation rate of *c-Myc* overexpressing *Arf* knockout MEFs shows that repression of *Arf* is the main function of *Bmi1* in collaboration with *c-Myc,* and argues against a major role for *Ink4a* in this process (fig.2f).

Differential effects of Ink4a and Arf de-repression in Bmi1 deficient spleen and thymus

Adult *Bmi1* knockout mice suffer from a marked progressive hypoplasia of the spleen and thymus, which appears to be due to an impaired proliferative capacity or a decreased survival rate of lymphocytes, rather than to a complete block in differentiation. This is supported by the presence of all B- and T-cell maturation states in young *Bmi1* deficient mice, albeit in altered ratios. (Van der Lugt et al. 1994; Jacobs et al. 1999b).

When dissecting the relative contribution of loss of *Ink4a* and *Arf* to restoration of nucleated cell counts in *Bmi1* deficient spleen and thymus, a number of observations stand out (fig.3). Both in spleen and in thymus, none of the compound mutant mice showed a complete rescue suggesting that other yet unidentified *Bmi1* targets are involved (fig.3a-3d). In thymus but not in spleen, loss of *Arf* gives a significant smaller rescue than deletion of the complete locus, indicating that *Ink4a* plays a more prominent role in thymocyte growth ($p<0.01$)(fig.3c). Surprisingly however, our preliminary results show that loss of *Ink4a* alone does not lead to a significant rescue of the phenotype (fig.3b and 3d). In line with *Bmi1* signaling to *p53* through *Arf*, loss of *p53* can also partially rescue the reduced cellularity in both organs (fig.3a and 3c). Furthermore, in the *Bmi1* deficient spleen but not in thymus, heterozygosity for either *Arf* or *Ink4a/Arf* alleviates the reduced cell counts to a small yet significant extent ($p<0.001$) (fig.3a). Apparently, the levels of both *Ink4a* and *Arf* differentially affect these lymphoid organs.

Next, we applied flow cytometry using standard T- and B-cell differentiation markers for a global survey of the T- and

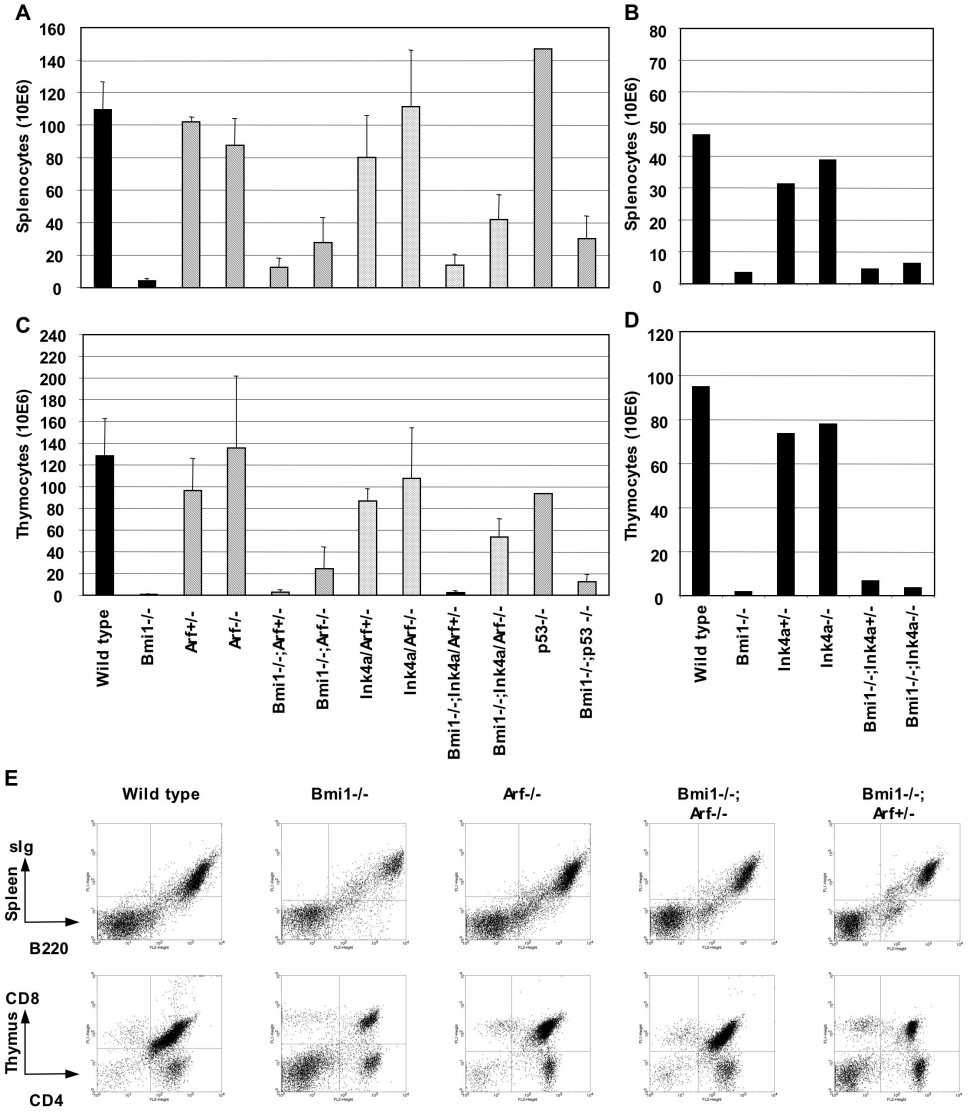

Figure 3. Differential effects of *Ink4a* and *Arf* dosage and de-repression in *Bmi1* deficient lymphoid organs. (A) Splenic nucleated cell counts are dramatically reduced in *Bmi1* knockout mice. Heterozygosity for *Arf* or *Ink4a/Arf* partially rescues the *Bmi1* deficient phenotype ($p<0.01$) but complete loss of *Arf* or *Ink4a/Arf* results in a better rescue. Loss of *p53* alone also induces a partial rescue. (B,D) Preliminary data show that neither *Bmi1*$^{-/-}$ thymocyte nor splenocyte counts are significantly restored by loss of *Ink4a* as shown for two individual animals. (C) Thymocyte counts are dramatically reduced in *Bmi1* deficient mice. Loss of the complete *Ink4a/Arf* locus gives a substantially better rescue of the *Bmi1* deficient phenotype than loss of *Arf* alone ($p<0.05$). Note that heterozygosity does not lead to a rescue. Loss of *p53* induces a minor rescue in lymphocyte counts. (E) FACS plots show that in spleen, the relative population frequency of mature *Bmi1*$^{-/-}$ sIg^{+}B220^{+} B-cells is normalized by both heterozygosity or complete loss of *Arf*. In thymus, only complete loss of *Arf,* but not heterozygosity, is capable of restoring the relative distribution of *Bmi1* deficient CD4^{-}CD8^{-} immature T-cell populations to control levels.

B-cell populations in spleen and thymus. As described before, *Bmi1* deficient mice show increased thymic populations of CD4^{-}CD8^{-} immature T-cells, and a decreased number of splenic mature B220^{+}sIg^{+} B-cells (Van der Lugt et al. 1994; Jacobs et al 1999a). Interestingly in spleen, heterozygosity for either *Arf* or *Ink4a/Arf* can completely restore the relative population frequency of mature B-cells to wild type levels, which is in

concert with the results we obtained for total nucleated cells counts in spleen (fig.3e and supplementary fig.2a). In agreement with our results for thymic cell counts, only complete loss of *Arf* or *Ink4a/Arf* but not heterozygosity fully contributes to restoration of wild type levels of immature $CD8^-CD4^-$ T-cells (fig.3e and supplementary fig.2b). Of note, though their relative distribution is restored, absolute cell numbers in spleen and thymus are not fully rescued.

Altogether, these data clearly show that the *Arf-p53* pathway is the main target of *Bmi1* repression in spleen and thymus, and that *Ink4a* only contributes to a lesser extent in thymus. In addition to this, haploinsufficiency of the locus differentially influences the lymphoid organs. This points at dosage effects of *Arf* in agreement to our previous results in fibroblast transformation assays (Jacobs et al. 1999b). Furthermore, our findings stress that loss of *Arf* alone is sufficient to normalize *Bmi1* deficient T- and B-cell population frequencies to control levels. This might suggest that proliferation regulated by the *Ink4a/Arf* locus is in fact also affecting lymphocyte differentiation, although it cannot be excluded that these results are confounded by the inherent coupling of proliferation and differentiation.

Proliferation defects in Bmi1 deficient cerebellum reflects both Ink4a and Arf deregulation

When investigating the neurological defects of *Bmi1* knockout mice, we recently pinpointed a reduced number of poorly proliferating cells of the cerebellar external granular layer (EGL) to be at least partially responsible (Leung et al. 2004). During normal early postnatal development, a wave of proliferation of cerebellar granule neuron progenitors (CGNPs) in the EGL is induced by Purkinje neuron secreted Sonic Hedgehog (Shh) (Dahmane and Ruiz-I-Altaba, 1999; Wechsler-Reya and Scott, 1999). In time, CGNPs differentiate and migrate inwards past the molecular layer (ML) until they reach the internal granule layer (IGL) where they reside as mature granule neurons (reviewed in Wang and Zoghbi, 2001). We have previously shown that *Bmi1* is a target of the *Shh* pathway, thereby explaining the impaired proliferative response of *Bmi1* deficient CGNPs upon Shh stimulation (Leung et al. 2004). As *Bmi1* deficient CGNPs are still partially responsive to Shh, we proposed a model where Shh controls proliferation via at least two routes, *i.e.* through induction of *N-Myc*/*Cyclin D2* (Kenney et al. 2003) and through induction of *Bmi1*. Here, we evaluated to what extent de-repression of *Ink4a* and *Arf* affects cerebellar proliferation and development in *Bmi1* deficient mice.

First, we sought to determine whether *Ink4a* and *Arf* expression is deregulated in primary *Bmi1* deficient CGNPs isolated at postnatal day 7. Analysis by quantitative real-time PCR (qRT-PCR) shows that *Ink4a* expression is about 10-fold increased, whereas *Arf* expression is 3-fold increased (fig.4a). As *Ink4a* and *Arf* can be induced by tissue culture stress, we also analyzed their expression in cerebella isolated at postnatal day 7. In line with the results for CGNPs and MEFs, we found a profound increase in *Ink4a* expression in $Bmi1^{-/-}$ cerebella and a moderate increase in *Arf* mRNA levels (fig.4a). Together this indicates that the *Ink4a/Arf* locus is a relevant *in vivo* target of *Bmi1* in cerebellum. Moreover, we demonstrate

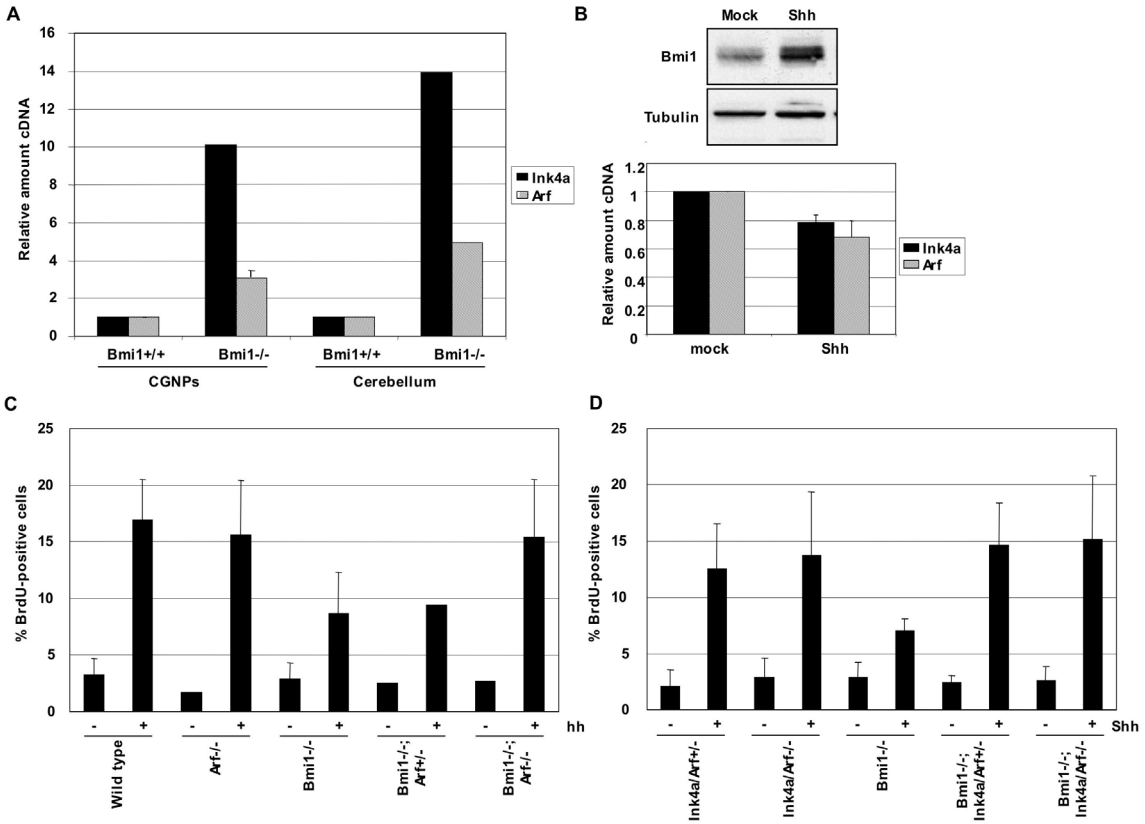

Figure 4. *Ink4a/Arf* de-repression upon loss of *Bmi1* prevents efficient Shh induced CGNP proliferation. (A) qRT-PCR shows an approximate 10-fold upregulation of *Ink4a* and 3-fold upregulation of *Arf* mRNA expression in both 24 hrs cultured *Bmi1*$^{-/-}$ CGNPs and uncultured *Bmi1*$^{-/-}$ cerebella. (B) In wild type CGNPs, Shh treatment induces an increase in Bmi1 protein levels and a concomitant small yet significant decrease in *Ink4a* ($p<0.005$) and *Arf* ($p<0.02$) mRNA expression. (C) Reduced BrdU incorporation in *Bmi1*$^{-/-}$ CGNPs versus wild type CGNPs upon Shh treatment ($p=0.0001$) is rescued upon subsequent *Arf* loss ($p<0.01$) but not by *Arf* heterozygosity. Multiple wells per animal were analyzed. (D) Reduced BrdU incorporation in *Bmi1*$^{-/-}$ CGNPs versus wild type CGNPs upon Shh treatment is rescued by *Ink4a/Arf* loss ($p<0.05$) and by *Ink4a/Arf* haploinsufficiency ($p<0.03$).

that stimulation of wild type CGNPs with Shh leads to an induction of Bmi1 expression and a modest, significant downregulation of both *Ink4a* and *Arf* transcript levels, consistent with the idea of Shh regulating *Ink4a/Arf* through *Bmi1* (fig.4b).

Next, to assess to what extent *Ink4a* and *Arf* deregulation underlies the proliferative defects of *Bmi1*$^{-/-}$ CGNPs, we investigated the Shh response of *Bmi1;Arf* and *Bmi1;Ink4a/Arf* doubly deficient CGNPs and their respective controls. Notably, neither *Arf* nor *Ink4a/Arf* loss causes enhanced proliferation in absence of Shh, in line with the multiple levels of regulation downstream of Shh as suggested above (fig.4c and 4d). However in the context of *Bmi1* deficiency, both *Arf* loss alone and complete loss of the *Ink4a/Arf* locus rescues Shh induced proliferation of *Bmi1* knockout CGNPs to control levels ($p<0.05$)(fig.4c and 4d). Similarly to the hematopoietic system, in CGNPs we observe a clear effect of heterozygosity for *Ink4a/Arf*, which is able to restore proliferation of *Bmi1* deficient CGNPs to wild type levels (fig.4d). Intriguingly, our preliminary results indicate that loss of only *Ink4a* also gives a partial rescue in *Bmi1* knockout CGNPs (data not shown). This highlights further the dual importance of both *Ink4a* and *Arf* in these precursor

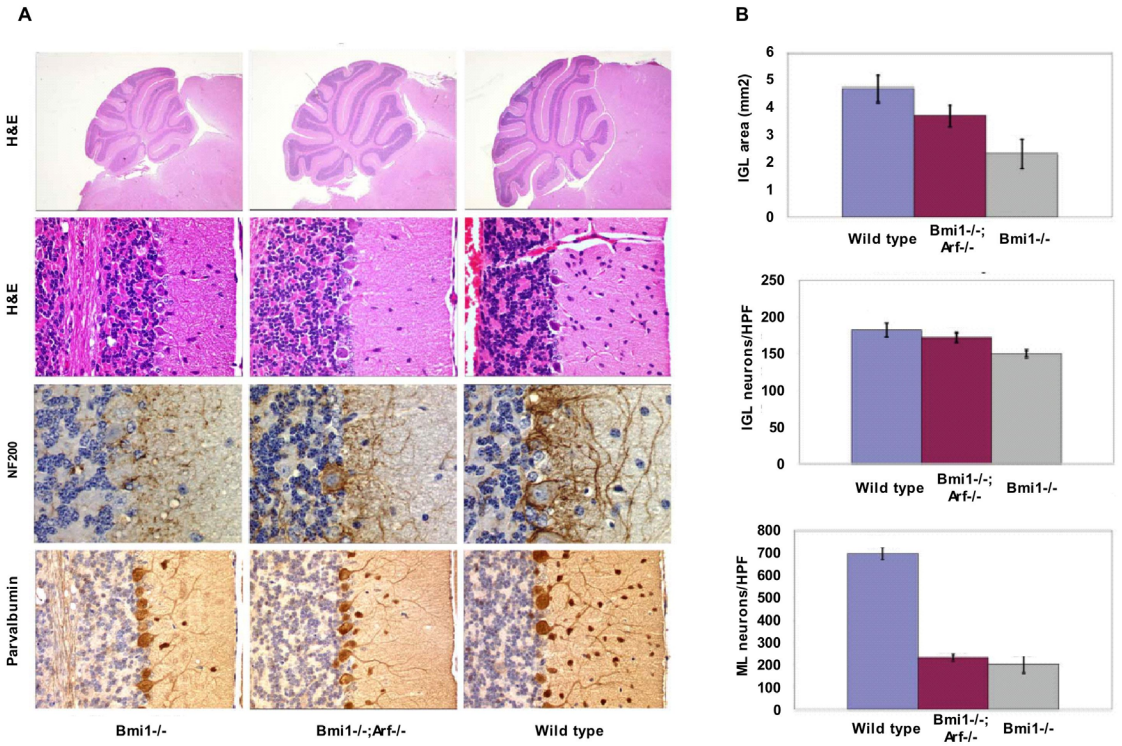

Figure 5. *Arf* loss partially rescues the histopathological abnormalities of *Bmi1* deficient cerebella. (A) Morphological analysis of *Bmi1*$^{-/-}$ (left panels), *Bmi1*$^{-/-}$;*Arf*$^{-/-}$ (middle panels), and wild type cerebellum (right panels) in adult mice. Haematoxylin and eosin (H&E, top) staining shows partial rescue of overall cerebellar size in *Bmi1*$^{-/-}$;*Arf*$^{-/-}$ as compared to wild type cerebellum. The aberrant arborization of basket neurons (NF200 immunohistochemical staining, middle) and the reduced number of stellate neurons (parvalbumin staining, bottom) is observed in both *Bmi1*$^{-/-}$ and *Bmi1*$^{-/-}$;*Arf* cerebella. Final magnification 5X and 60X. (B) IGL area measurements (top) and cell density counts (middle) show significant rescue in *Bmi1*$^{-/-}$;*Arf*$^{-/-}$ mice ($p<0.05$ and $p<0.01$, respectively). However, in agreement with results in (A), the number of molecular layer (ML, bottom) neurons is not rescued upon *Arf* loss. HPF, high power field.

cells. To test whether other PcG members are also implicated in Shh induced proliferation, we isolated CGNPs from 7 days old *M33, Ring1a* or *Mel18* deficient mice. We observed a slight decrease in proliferative capacity in *Mel18*$^{-/-}$ mice, but not in *Ring1a*$^{-/-}$ mice (supplementary fig.3a).

We have previously shown that loss of *Ink4a/Arf* gives a qualitative rescue of the histological cerebellar defects (Jacobs et al. 1999a). As *Arf* loss could restore *Bmi1* deficient CGNP proliferation, we questioned to what extent this affects *in vivo* proliferation of cerebellar progenitor cells as well as the histological abnormalities. We found that the reduced cellularity of the *Bmi1* deficient granular layer is partially restored in *Bmi1*$^{-/-}$;*Arf*$^{-/-}$ mice as assessed by area and cell density measurements (fig.5b, $p<0.05$ and $p<0.01$, respectively). But we still observed aberrations such as reduced cellularity of the molecular layer (fig.5b), abnormal arborization of the basket neurons and absence of stellate neurons (fig.5a). Disturbed EGL proliferation can explain multiple abnormalities of the cerebellum. Particularly later during development, granule neurons signal to Purkinje and Basket cells to create the appropriate amount of arborization (Baptista et al. 1994). However, despite proliferation being restored in *Bmi1;Arf* deficient CGNPs *in vitro*, the mice still display neurological symptoms such as an ataxic gait, tremors and seizures. This implies that at least part of the *Bmi1* knockout cerebellar phenotype is due to

defects in cell types other than the CGNPs. The cerebellum originates from two germinal layers, namely the rhombic lip from which the CGNPs are derived, and the ventricular zone which gives birth to the molecular neurons (reviewed in Wang and Zoghbi, 2001). It is conceivable that *Bmi1* also plays a specific role independent of *Ink4a/Arf* in cells derived from the ventricular zone, especially since *Bmi1* is required for subventricular zone derived neural stem cells (see below).

Neural stem cell self-renewal critically depends on repression of Ink4a and Arf by Bmi1

A stem cell is defined as a multipotent cell capable of extensive self-renewal. For neural stem cells, multipotency means the cell can give rise to neurons and glial cells. In the adult cerebrum, there are two major neurogenic regions harboring stem cells: the subventricular zone (SVZ) of the lateral ventricle wall and the subgranular zone of the hippocampus (Reviewed in Doetsch, 2003). Progenitors or neural stem cells, isolated from both regions (Reynolds and Weiss, 1992; Morshead et al. 1994; Gage et al. 1995, Seaberg and Van der Kooy 2002), can conveniently be grown as either adherent colonies or as 'neurospheres', floating clusters of stem cells and progeny.

Recently, it was shown that *Bmi1* is essential for the self-renewal of SVZ derived neurospheres and that *Ink4a* repression is partially mediating this effect (Molofsky et al. 2003). Here we set out to determine whether *Arf* is required as well. First, we tested whether *Ink4a/Arf* is an *in vivo* target of *Bmi1* in the cerebrum. Western blot analysis on wild type and *Bmi1* knockout SVZ, hippocampus, neocortex, and cerebellum (isolated from postnatal day 30 mice) revealed upregulated *Ink4a* expression in all regions of the *Bmi1* deficient brain (fig.6b). Unfortunately, we were not able to detect an increase in $p19^{arf}$ expression, probably due to low expression of the protein or a technical difficulty in detecting this unusually charged small protein.

Bmi1 deficient adult primary neurospheres not only form less frequently (Molofsky et al. 2003 and data not shown), but also are much smaller in size than control neurospheres (fig.6a and 6c). This suggests that proliferation within a *Bmi1* deficient neurosphere, reflecting the sum of cell divisions from self-renewing stem cells and their progeny, is impaired. Indeed, *Bmi1* deficient colonies incorporate far less BrdU than control colonies (fig.6e). Notably, this defect in proliferation can be rescued by both loss of *Arf* and by complete deletion of the *Ink4a/Arf* locus implicating *Arf* as an important downstream effector of *Bmi1* for stem cell and progenitor proliferation (fig.6e). This finding is further supported by the significant restoration of neurosphere size upon loss of either *Arf* or the whole locus (fig.6a and 6c).

Next, we performed a neurosphere self-renewal assay with primary *Bmi1* deficient, *Bmi1;Arf* and *Bmi1;Ink4a/Arf* doubly deficient and control neurospheres to specifically study self-renewal capacity. In this assay, the capacity of a neurosphere derived from a single stem cell to form new multipotent neurospheres, is measured. In this way, the number of new neurospheres is directly proportional to the number of self-renewing cell divisions the founding stem cell has made. We find a dramatic decrease in the self-renewing capacity of both adult and postnatal day 7 derived *Bmi1* deficient neurospheres in agreement with previous findings (fig.6d

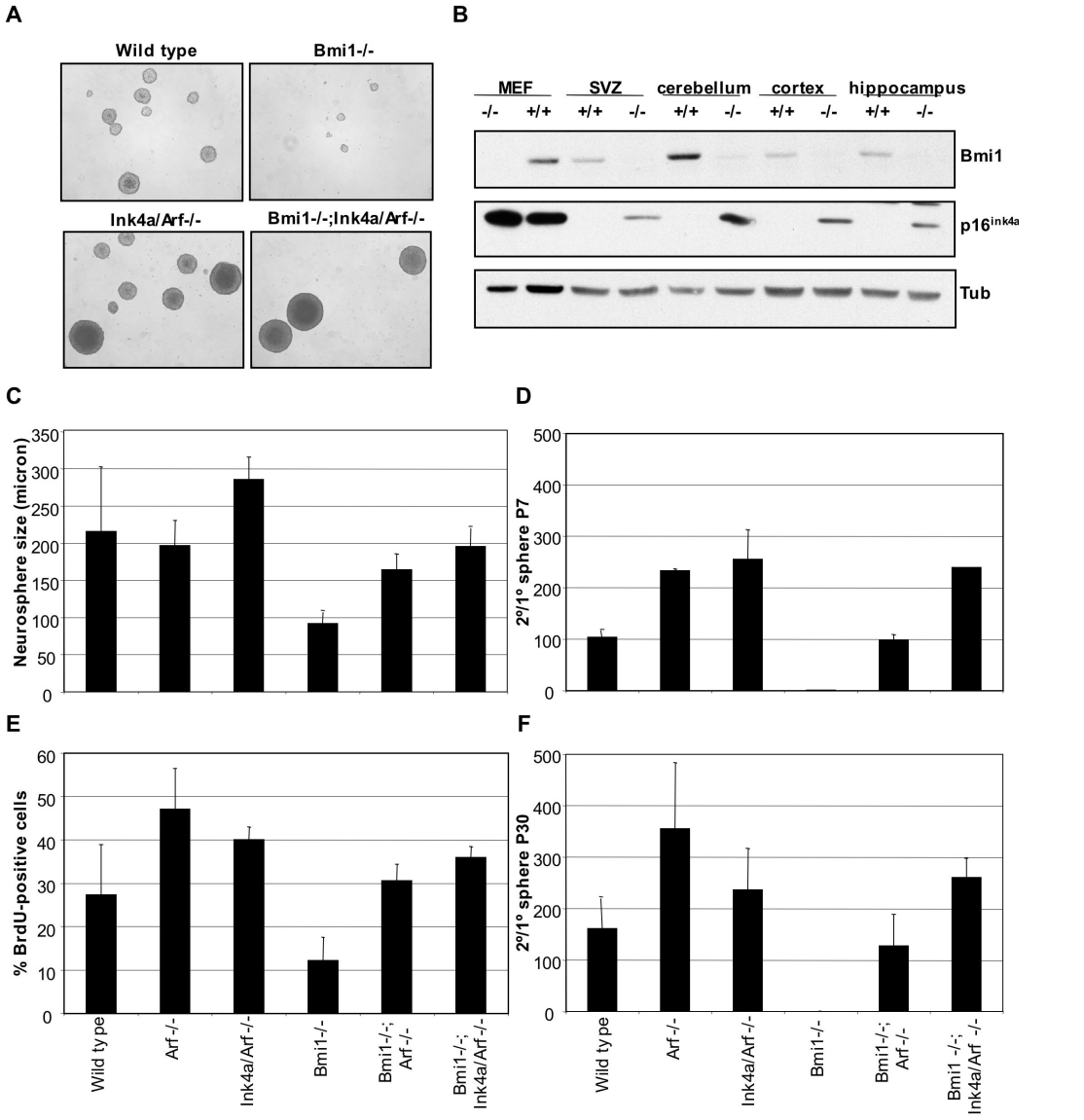

Figure 6. Accurate repression of both *Ink4a* and *Arf* is required for neurosphere proliferation and self-renewal. (A,C). Phase-contrast pictures and diameter measurements show that *Bmi1*$^{-/-}$ neurospheres are much smaller than wild type neurospheres. Loss of either *Arf* or *Ink4a/Arf* completely rescues this *Bmi1* deficient phenotype. Multiple neurospheres were analyzed per mouse. Final magnification, 10X. (B). Protein analysis by Western blot reveals increased p16^{ink4a} expression in brain and in MEFs. (E) *Bmi1*$^{-/-}$ SVZ adherent colonies incorporate far less BrdU than control colonies, which is completely rescued upon loss of either *Arf* or *Ink4a/Arf*. Multiple colonies per mouse were analyzed. (D,F) *Bmi1* deficient neurospheres derived from both postnatal day 7 and postnatal day 30 SVZ are severely impaired in their self-renewal capacity. Loss of *Arf* alone gives a partial rescue of the *Bmi1* deficient phenotype ($p=0.03$). However, removal of the complete *Ink4a/Arf* locus fully restores the self-renewing ability of *Bmi1*$^{-/-}$ neurospheres. Note that loss of *Arf* or *Ink4a/Arf* in a *Bmi1*$^{+/+}$ background enhances self-renewal ($p<0.01$).

and 6f). In contrast, deletion of the *Ink4a/Arf* locus in *Bmi1* deficient neurospheres completely rescues this defect. Importantly, loss of *Arf* alone gives a partial rescue ($p<0.05$), thus reinforcing the earlier observation that proper repression of *Ink4a* is also required for neurosphere self-renewal. Upon induction of differentiation, all newly formed neurospheres of the tested genotypes stained positive for neuron and

astrocyte specific markers revealing their multipotency (data not shown). Strikingly, there is a clear increase in both proliferation and self-renewing capacity of *Arf* and *Ink4a/Arf* deficient neurospheres compared to wild types (fig.6d-f). This strongly suggests that the *Ink4a/Arf* locus actively restricts self-renewing cell divisions thus playing a role in controlling the stem cell compartment in culture. Alike for the cerebellar progenitor cells, we found a modest yet not significant decrease in the self-renewal capacity of *Mel18*$^{-/-}$ but not *Ring1a*$^{-/-}$ or *M33*$^{-/-}$ neurospheres, indicating that a specific subset of PcG members affects stem cell behaviour (supplementary fig.3b).

Lastly, we tested these neurospheres for long-term self-renewal. Our neurosphere assays showed that *Bmi1;Arf* and *Bmi1;Ink4a/Arf* double mutant neurospheres can self-renew once, however, it did not exclude the possibility that they eventually may loose this capacity. For instance, exhaustion of hematopoietic stem cells sometimes occurs after a prolonged period of time (Park et al. 2003; Hock et al. 2004). However, we were able to keep these neurospheres in culture for at least five weekly passages, indicating that the *Bmi1* knockout phenotype is fully reversed.

Taken together, we find that both products of the *Ink4a/Arf* locus need to be accurately repressed by *Bmi1* in order to allow appropriate neural stem cell self-renewal. In addition, we show that also in wild type neurospheres there is a requirement for *Ink4a/Arf* repression since removal of either *Arf* or *Ink4a/Arf* enhances neural stem cell renewal and proliferation.

Discussion

Control of proliferation is an important cellular feature as erroneous cell divisions may have adverse consequences for an organism. Therefore, it is not surprising that several molecular pathways have evolved via which proliferation, in particular that of the long-lasting stem cells, can be tightly regulated. The complexity of the Polycomb Group offers wide prospects for sophisticated fine-tuning of gene expression profiles necessary to preserve the balance between self-renewal and differentiation of stem cells. An increase in the relative amount of a critical PcG member like *Bmi1*, may alter the affinity of a PcG complex towards chromatin in such a way that for instance self-renewing divisions are favored (reviewed in Valk-Lingbeek et al. 2004). Underscoring the special role for *Bmi1* in PcG complexes, we found that besides loss of *Bmi1,* only loss of *Mel18*, a close homologue of *Bmi1,* but not that of other PcG family members, has a modest negative influence on stem cell and progenitor proliferation.

Dosage effects for Ink4a and Arf downstream of Bmi1 in controlling proliferation

The PcG exerts its control over proliferation through repression of the *Ink4a/Arf* tumor suppressor locus, an important integrator of mitogenic signals (Jacobs et al. 1999a; Voncken et al. 2003; Core et al. 2004; Gil et al. 2004). We set out to determine the relative importance of *Ink4a* and *Arf* repression by *Bmi1* and show that correct *Arf* regulation is crucial for the proliferation of MEFs, lymphocytes, cerebellar progenitors in vivo and in vitro as well as for SVZ neural stem cells. However between these cell types, the extent of *Arf* repression required for proliferation varies. In *Bmi1* deficient spleen, loss of only one allele of *Arf* is sufficient to give a small but significant rescue of *Bmi1* deficient cell counts. Correspondingly, the decreased percentage of mature *Bmi1*$^{-}$

$^{-/-}$ B-cells is also rescued by *Arf* heterozygosity. We have previously observed dosage effects for the *Ink4a/Arf* locus, as a 50% reduction in *Ink4a/Arf* expression gave a selective growth advantage to MEFs overexpressing *c-Myc* and *Ras* (Jacobs et al. 1999b). Here we show that similar effects occur *in vivo* but selectively for certain tissues. Conversely, the super *Ink4a/Arf* mouse, carrying a transgenic copy of the entire *Ink4a/Arf* locus, is better protected against oncogenic transformation, and mice heterozygous for *Bmi1* are less susceptible to Eμ-Myc induced lymphomagenesis (Jacobs et al. 1999b; Matheu et al. 2004).

However, it is not only *Arf* which mediates *Bmi1's* controlling function. A significant contribution of *Ink4a* in various though not all cell types is evident from our results. Interestingly in several cases, this *Ink4a* effect can only be observed in a background of reduced *Arf* levels. For instance in the thymus, loss of *Ink4a* only leads to a better rescue in cell counts in a *Bmi1;Arf* deficient background. In the cerebellum, even a partial loss of *Ink4a* in an *Arf* $^{+/-}$ background induces a full rescue of the proliferative defect of *Bmi1* deficient CGNPs. The reason for this may be that *Ink4a* has a more pronounced role in CGNPs than in thymocytes as our preliminary data indicate that in contrast to the thymus, loss of *Ink4a* can substantially rescue the lack of Shh induced proliferation of *Bmi1* deficient CGNPs.

It remains striking that lymphoid cell counts, cerebellar development, skeletal transformations and overall body growth in *Bmi1;Ink4a/Arf* doubly deficient mice are only partially, if at all, rescued. In line with *Drosophila* not possessing genes resembling the *Ink4a/Arf* locus, the acquisition of PcG mediated proliferation control likely evolved later, perhaps reflecting a demand for protection of stem cells in higher organisms. In this way, PcG-mediated repression contributes to the protection of stem cell integrity, but when defective may elicit adverse side effects such as premature senescence and aging. It is clear though that other *Bmi1* regulated genes exist. Good candidates for such additional targets are the *Hox* genes, homologues of *Drosophila Homeotic* genes. Interestingly, a subset of *Hox* genes has been implicated in mammalian brain development and in the control of hematopoietic stem cells. Moreover, several *Hox* genes are differentially expressed in *Bmi1* deficient SVZ neurospheres (Molofsky et al. 2003). Testing the functional implications of these *Hox* genes will be an important focus for future studies.

Arf and Ink4a are differentially required in curtailing proliferation of differentiated cells versus stem/progenitor cells

Why would the requirement for proper *Ink4a* repression differ throughout tissues? The answer may lie in the nature of the cell type in which it is expressed. For example, MEFs are readily immortalized upon loss of *Arf* alone, however, astrocytes only become immortal upon removal of the complete *Ink4a/Arf* locus (Kamijo et al. 1997; Holland et al.1998; Bachoo et al. 2002). Intriguingly, astrocytes can alter their cellular identity in response to extracellular cues upon loss of *Ink4a* and *Arf,* but not *Arf* alone, indicative of a degree of plasticity akin to that of progenitor cells (Bachoo et al. 2002). We speculate that perhaps *Ink4a* has the special ability to govern the differentiation status of cells in addition to (or through) controlling their cell cycle. This hypothesis fits well with our results, since we observe a prevalence for *Ink4a* de-repression contributing to the *Bmi1* loss-of-function phenotype in more

undifferentiated cell systems. We find *Arf* to be the primary target of *Bmi1* in MEF proliferation, in total splenocyte counts and in mature B-cells, compartments consisting of relatively well-differentiated cells. In contrast in thymus, the site of T-cell renewal and differentiation, *Ink4a* loss significantly contributes to restoration of thymocyte counts and importantly, to restoration of the relative distribution of wild type mature and immature T-cell population frequencies in *Bmi1* knockout mice. In addition, we revealed a role for *Ink4a* regulation in CGNPs from the cerebellum, cells which have not yet terminally differentiated into granule neurons at this stage. The most compelling evidence for *Ink4a* acting downstream of *Bmi1* and contributing to maintenance of undifferentiated cells comes from our study of adult neural stem cells. We find that *Arf* de-repression contributes to both the attenuation of proliferation within a *Bmi1* deficient neurosphere, and to the self-renewal capacity of the stem cells themselves. Importantly, loss of *Arf* in a wild type background also enhances neural stem cell self-renewal significantly. But only upon genetic removal of both *Ink4a* and *Arf*, *Bmi1* deficient neurospheres self-renew to an extent equivalent to control wild type spheres, highlighting the important role of both *Arf* and *Ink4a* in keeping this stem cell compartment in check. These results fit well with the earlier study by Molofsky et al. showing that *Ink4a* de-repression affects *Bmi1*$^{-/-}$ neural stem cell but not progenitor proliferation (Molofsky et al. 2003). Their recent finding that *Ink4a* loss partially restores *Bmi1*$^{-/-}$ stem cell frequency and neurogenesis in the peripheral nervous system *in vivo* highlights that this is not simply a tissue culture phenomenon (Molofsky et al. co-submitted).

As mentioned before, proliferation and differentiation are likely to be coupled processes. It is conceivable that a stem or progenitor cell, on the verge of commitment to differentiation, needs to temporarily cease proliferation to allow proper execution of differentiation programs. Interestingly, *Ink4a* is not the only cell cycle inhibitor implicated in controlling the stem cell compartment. Roles for p18^{ink4c}, the pocket proteins p107 and pRB, which notably are controlled by *Ink4a/Cyclin D*, and the CKIs p21 and p27^{kip1} have been described in the proliferation of hematopoietic stem cells, neural stem cells, and progenitors (Cheng et al. 2000a; Cheng et al. 2000b; Ferguson et al. 2000; Miyazawa et al. 2000; Doetsch et al. 2002; Vanderluit et al. 2004; Yuan et al. 2004). With the emerging conviction that at least certain cancers harbor subsets of cells with stem cell characteristics, it is intriguing to observe that commonly affected tumor suppressors of the *Arf/p53* and *Ink4a/pRB* pathways in cancer also are implicated in curtailing normal stem cell proliferation and renewal (Lapidot et al. 1994; Bonnet and Dick 1997; Al-Hajj 2003; Hemmati et al. 2003; Singh et al. 2003). In this respect, it is of special interest that a major regulator of *Ink4a/Arf* expression, *Bmi1,* is essential for the renewal of leukemic stem cells, is highly expressed in brain tumor-derived cancer stem cells, and is overexpressed in a specific subset of primitive medulloblastomas (Hemmati et al. 2003; Lessard and Sauvageau, 2003; Leung et al. 2004). We speculate there are intrinsic differences between self-renewing cell cycle control and control of normal cell divisions, which are at least partially governed by PcG mediated repression.

Altogether, we have shown profound tissue and cell type specific differences in the effects of *Ink4a* versus *Arf* de-

repression in *Bmi1* deficient mice. In addition, we demonstrated that *Ink4a* or *Arf* haploinsufficiency can contribute to the *Bmi1* loss-of-function phenotype, suggesting that appropriate threshold levels of these two proteins are required to ensure proper proliferation. Overall, our study using *Arf*-specific knockouts suggests a general role for *Arf/p53* pathway repression in the rescue of proliferative defects in most cell types. However, *Ink4a* repression, perhaps due to its capacity to modulate pRB, is emphatically implicated in controlling the cell cycle of undifferentiated cell types, as loss of this gene has a strong influence on neural stem cells and cerebellar precursors. These observations implicate PcG proteins not only in embryonic developmental fate decisions, but also in discriminative processes between cell cycle control of primitive cells and terminally differentiated cells. Such different patterns of cell cycle regulation are of high relevance for diseases such as cancer, as understanding the biology specific for those rare cancer-initiating cells might lead to better targeting the source of the disease.

Materials and methods

Mice breeding and monitoring

Bmi1$^{+/-}$ FVB mice (Van der Lugt et al. 1994) were crossed with *Ink4a/Arf*$^{+/-}$ FVB mice (Serrano et al. 1996), with *Arf*$^{+/-}$ FVB mice (Kamijo et al. 1997), or *p53*$^{+/-}$ FVB mice (Donehower et al. 1992) to generate *Bmi1*$^{+/-}$ mice with mutant *Ink4a/Arf, Arf or p53* alleles. These mice were subsequently intercrossed to obtain the genotypes required for our experiments together with control littermates. For analysis of *Ink4a* downstream of *Bmi1, Bmi1*$^{+/-}$ C57BL/6 mice were crossed with *Ink4a*$^{+/-}$ C57BL/6 mice (Krimpenfort et al. 2001), and their offspring was intercrossed to obtain the required genotypes. All mice were genotyped routinely by PCR (list of primers available upon request). *Ring1a* (Del Mar Lorente et al. 2000) and *M33* (Core et al. 1997) mutant mice were in an FVB background. *Mel18* (Akasaka et al. 1996) knockout mice were in a mixed background. Multiple independent animals of the respective genotypes were assayed and all results were statistically analyzed using Student-T tests and subjected to Bonferroni correction when appropriate.

Skeletal whole mount analysis and mouse embryonic fibroblast assays

Skeletal whole mounts of newborn mice were prepared as described before with some modifications (Van der Lugt et al. 1994). In short, eviscerated animals with the skin removed were fixed overnight in 96% ethanol, followed by incubation for 24-48 hrs at 37ºC in glacial acetic acid: 96% ethanol (1:4), 0.015% alcian blue 8GS. Skeletons were rinsed for 1 hr in 96% ethanol, incubated for 6-24 hrs in 2% KOH, for 12-24 hrs in 1% KOH with 75 µg/ml alizirin red S, and cleared for 1 week in 1% KOH, 20% glycerol with daily changes. Skeletons were stored in 50% glycerol, 50% ethanol and scored for malformations.

Isolation and culturing of MEFs, retroviral transductions and growth curves were performed as described previously (Jacobs et al. 1999a,b). Retroviruses used were LZRS-iresGFP, LZRS-MDM2-iresGFP, LZRS-MycHA-iresGFP, and LZRS-Bmi1-iresGFP. To verify infection efficiency, virus infected MEFs were analyzed for GFP expression. To investigate bypass of premature senescence, *Bmi1*$^{-/-}$, compound mutant, and control cells were transduced at the first passage after isolation. Their proliferative capacity was assessed at passage 3-4 when control *Bmi1* deficient MEFs cease proliferation. Experiments were done with multiple independently isolated batches of MEFs.

Western blot analysis and quantitative real-time PCR

Cleared lysates from cells and tissue were assayed for protein concentration using the DC protein assay kit (Biorad). Equal amounts of protein were separated on 13% SDS-PAGE or pre-cast gels (Invitrogen) and blotted onto Immobilon-P membranes (Amersham Biosciences). Bands were visualized according to standard methods using enhanced chemiluminescence (Amersham). Primary antibodies used were F6 mouse monoclonal for Bmi1 (Alkema et al. 1997), M156 for p16^{ink4a} (Santa Cruz), or R562 for p19^{arf} (Abcam). Secondary antibodies were goat-anti-mouse (ZyMed) or goat-anti-rabbit (BioSource), both HRP conjugated.

RNA of cells and tissue was extracted using TRIZOL reagent (Invitrogen). Reverse transcription was performed with 1 ug RNA using Superscript II (Invitrogen) and oligod(T)$_n$ primers. qPCR was performed with 50 ng cDNA on an ABI7000 using a SYBR Green PCR Mastermix (Applied Biosystems). Primers were

designed with Primer Express software. The amount of target, normalized to an endogenous reference and relative to a calibrator, was calculated by: $2^{-\Delta\Delta C}T$ (http://docs.appliedbiosystems.com/pebiodocs/04303859.pdf). Primer sequences used are: *β-actin* sense 5'-CCTCATGAAGATCCTGACTGA-3', *β-actin* antisense 5'-TTTATGTCACGAACAATTTCC-3', *Arf* sense 5'-GCCGCACCGGAATCCT-3', *Arf* antisense 5'-TGGAGCAGAAGAGCTGCTACGT-3', *Arf* sense 5'-CGCTCTGGCTTTCGTGAAC-3', *Arf* antisense 5'-GTGCGGCCCTCTTCTCAA-3', *Ink4a* sense 5'-CGTACCCCGATTCAGGTGAT-3', *Ink4a* antisense 5'-TTGAGCAGAAGAGCTGCTACGT-3', *Ink4a* sense 5'-CATCTGGAGCAGCATGGAGTC-3', *Ink4a* antisense 5'-GGGTACGACCGAAAGAGTTCG-3'.

Lymphocyte counts and flow cytometry

Cell suspensions of lymphoid organs from 8 weeks old mice were prepared by mincing the tissue through an open filter chamber. Erythrocytes were removed and the number of nucleated cells were was determined with a Casy-1 TT automated cell counter (Schäfe). Flow cytometry on splenic and thymic cell suspensions using standard B- and T-cell markers was done as described previously (Jacobs et al. 1999b).

CGNP isolation and histology of cerebellar tissue

CGNP cultures were isolated from 7 days old mice and cultured according to established protocols (Wechsler-Reya and Scott, 1999; Leung et al. 2004). For protein and RNA isolation, $1.2 \cdot 10^6$ cells were plated on poly-L-lysine (Sigma) coated 12 well plates. Quantification of Shh induced proliferation was performed as described before (Leung et al. 2004). Briefly, CGNPs were seeded at a density of $3.2 \cdot 10^5$ cells per 48 well. Cells were incubated with 3 µg/ml Shh (R&D Systems) in defined medium for 24 hrs and pulsed with 25 µg/ml BrdU for 2 hrs before fixation in 4% formaldehyde. Immunocytochemistry was performed using mouse monoclonal anti-BrdU (DAKO) as a primary antibody and Alexa Fluor m488 goat-anti-mouse (Molecular Probes) as secondary antibody. DAPI (Molecular Probes) was used to visualize nuclei. The percentage of cells in S-phase was determined by calculating the ratio of BrdU labeled cells. Immunohistochemistry, histological analysis, area and density measurements as well as cell counting of *Bmi1, Arf and Bmi1;Arf* deficient cerebella (three animals per genotype) was performed as described before (Leung et al. 2004).

Neural stem cell isolation, neurosphere proliferation and self-renewal assays

Mice were sacrificed at postnatal day 7 or 30. Brains were removed and placed in ice-cold HBSS. The lateral wall of the SVZ was dissected, minced and washed with HBSS. SVZ from P7 mice was resuspended in neurosphere medium and subsequently triturated mechanically using a fire-polished Pasteur pipette. SVZ from P30 mice was incubated with 0.025%Trypsin/0.5mM EDTA (Calbiochem) and 0.001% Dnase1 (Roche) in HBSS for 20 minutes at 37ºC, and quenched in neurosphere medium containing 0.015 % soy bean trypsin inhibitor (Roche) and 0.001% Dnase1, before mechanical dissociation. SVZ cells were filtered through a 40 µm cell strainer (Becton Dickinson) and live cells were counted using trypan blue (Sigma). To generate neurospheres, SVZ cells were seeded in ultra-low binding 6 well plates (Corning) at a density of 3000 cells/well in neurosphere medium (DMEM-F12 supplemented with modified N2, 2 mM glutamine (Gibco), 6 mg/ml glucose, 14 mM $NaHCO_3$, 5 mM HEPES (Sigma)) containing 20 ng/ml EGF and bFGF (R&D Systems). For adherent cultures, 1000 SVZ cells were seeded onto poly-ornithine (Sigma) and fibronectin (Biomedical Technologies Inc.) coated 6 well plates.

To quantify the number of proliferating cells within a colony, 7 days after isolation primary adherent cells were pulsed for 1 hour with 1 µM BrdU and subsequently fixed with 4% formaldehyde. Immunocytochemistry was performed as described for CGNPs. For assessment of neurosphere self-renewal capacity, 10 days after isolation individual primary neurospheres were dissociated and replated in 6 well ultra-low binding plates. After another 10 days, the number of newly generated neurospheres derived from an individual neurospheres was determined. To reveal multipotency, primary and secondary neurospheres were plated onto poly-ornithine and fibronectin or laminin (Sigma) coated plates and allowed to differentiate for 4 days in neurosphere medium supplemented with 2% FBS (ICN Biochemicals). Neurospheres were fixed for 15 minutes in 4% formaldehyde and permeabilized for 5 minutes with 0.03% triton/PBS. They were labeled for 2 hrs with antibodies against GFAP (DAKO) and ß-tubulin-III (Sigma) in 10% normal goat serum containing PBS. Secondary antibodies were FITC conjugated goat-anti-rabbit and Cy3 conjugated goat-anti-mouse (both obtained from Jackson ImmunoResearch). Nuclei were stained with DAPI.

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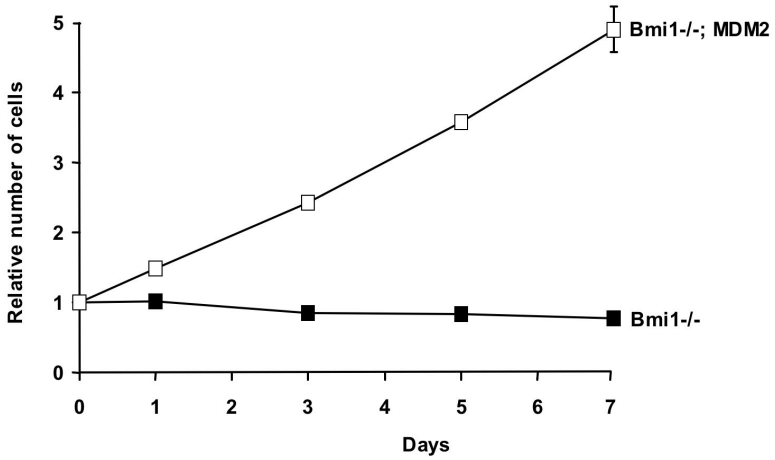
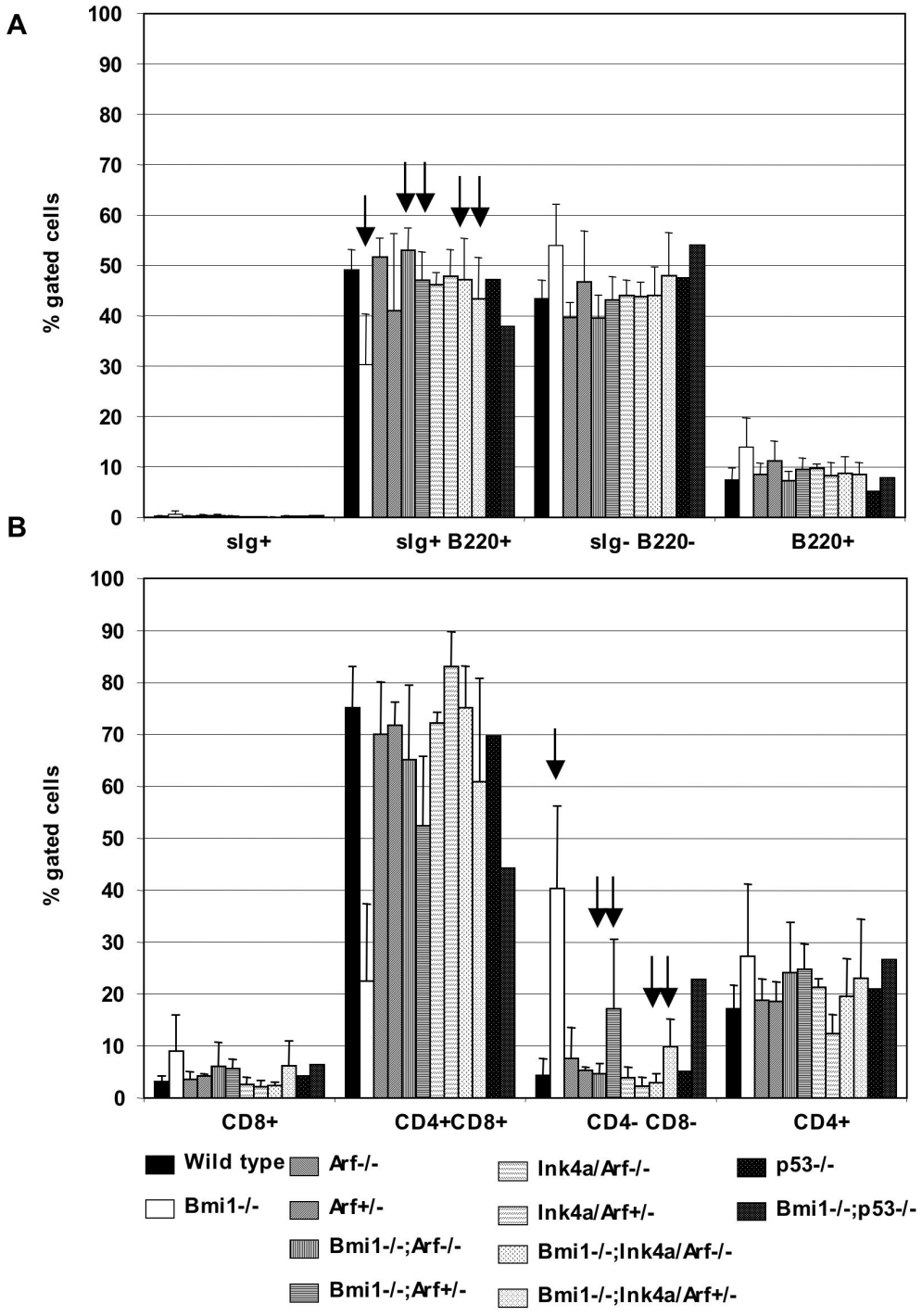
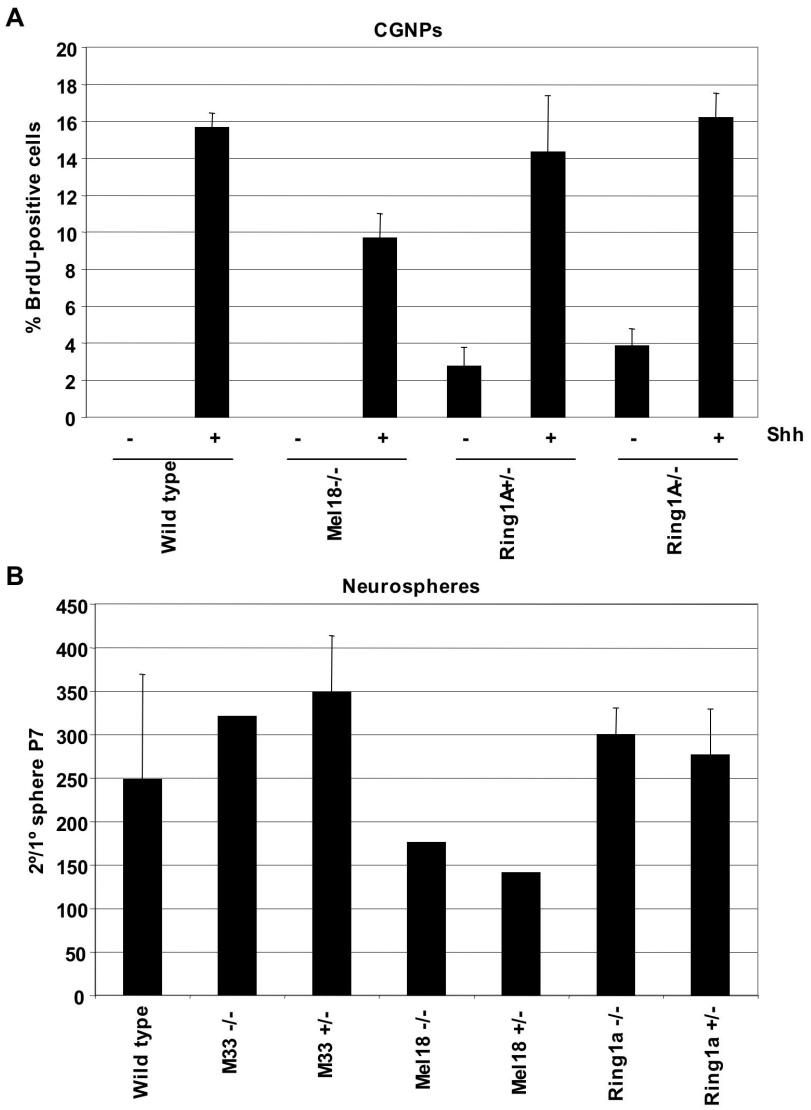


Supplementary figure 1. *Bmi1* mediates MEF proliferation through the *Arf/p53* pathway. Premature senescence of *Bmi1*$^{-/-}$ MEFs can be rescued by overexpression of *Mdm2*.



Supplementary figure 2. De-repression of *Ink4a* and *Arf* differentially affects the relative distribution of *Bmi1* deficient lymphocytes in spleen and thymus. (A) Quantitative analysis of flow cytometry data shows that the decrease in relative levels of splenic *Bmi1*$^{-/-}$ sIg^{+}B220^{+} B-cells is fully rescued by both heterozygosity and complete loss of *Arf* or *Ink4a/Arf* (indicated by arrows). (B) In thymus, the increase in *Bmi1*$^{-/-}$ deficient CD4^{-}CD8^{-} double negative T-cells is only fully rescued upon complete genetic removal of *Arf* or *Ink4a/Arf*.



Supplementary figure 3. Analysis of the role of other PcG genes in CGNP proliferation and SVZ neurosphere self-renewal. (A) Subtle decrease in the proliferative response of Shh stimulated *Mel18*$^{-/-}$ CGNPs, whereas loss of *Ring1a* does not affect CGNP proliferation. (B) Minor decrease in the self-renewal capacity of *Mel18*$^{-/-}$ postnatal day 7 derived neurospheres. Note that loss of either *Polycomb* homolog *M33,* or *Ring1a* does not lead to impaired self-renewal.

Chapter 7

General discussion

General discussion

In essence, all biological processes are guided by epigenetic gene regulatory cascades. In this thesis, we further discussed the control of the *Ink4a/Arf* tumor suppressors by the regulatory proteins Bmi1 and Tbx2 in processes ranging from normal development, to stem cells and cancer. *Ink4a/Arf* encodes for two proteins, $p16^{Ink4a}$ and $p19^{Arf}$, that act functionally upstream of the Rb and p53 pathway respectively. Furthermore, we identified Shh as an upstream regulator of Bmi1. Here I want to discuss unpublished results and findings of others that reinforce the research presented in this thesis. As the chromatin modifier Bmi1 and the transcription factor Tbx2 are not likely to exclusively regulate *Ink4a/Arf*, some suggestions for additional targets and/or future directions will be made.

TBX2 and TBX3-mediated Arf regulation: possible connection to development and cancer

TBX2 and TBX3 have both been established as direct regulators of *Arf* (Jacobs et al., 2000; Brummelkamp et al., 2002; chapter 2). Our studies shed further light on the mechanism of transcriptional regulation by the repressor *TBX* family members, by identifying a novel and *TBX2/TBX3*-specific DNA binding T site in the *Arf* promoter. However, much remains to be learned about the control of TBX factors on the *Arf* gene. First of all, it is likely that TBX2 or TBX3 act in a complex to establish repression. Unfortunately, we did not succeed in Y2H experiments with TBX2 nor did we identify new binding partners by immunoprecipitating with TBX2 antibodies in TBX2-overexpressing, ^{35}S-pulsed cell extracts. To our knowledge, also no binding partners have been reported in literature as yet. Given the palindromic nature of the T site, it is likely that a dimer of TBX proteins is regulating transcription. This proposed regulation could involve either homodimers or heterodimers or both. However, in transient transfection assays we never observed any dominant negative effect of the T box domain mutant of TBX2 on TBX3 repression or vice versa (data not shown). Nevertheless, these results do not exclude that *in vivo* regulation by heterodimers is relevant. With the development of the chromatin immunoprecipitation (ChIP) technique, assessment of the true *in vivo* occupancy of the *Arf* promoter could be performed. As dimers or other proteins may only associate with TBX2/TBX3 on the DNA, subsequent ChIP experiments with two antibodies on the same lysate will be necessary to unravel such regulation.

TBX2 or TBX3 overexpression readily immortalizes MEFs. Although we always attributed this effect of TBX2/3 to its capacity to downregulate *Arf* in MEFs, others have now shown that endogenous TBX2 is directly regulating $p21^{WAF1}$, a p53-reponsive cdk inhibitor, in melanoma cells (Prince et al., 2004). Both TBX2-mediated downregulation of $p14^{ARF}$ and $p21^{WAF1}$ could be instrumental in driving proliferation of human fibroblasts, as both $p14^{ARF}$and $p21^{WAF1}$ siRNA were shown to induce proliferation in human primary BJ fibroblasts (Voorhoeve and Agami, 2003; Berns et al., 2004). In concordance with their role in inducing proliferation, several studies have described overexpression or amplification of *TBX2* and *TBX3* in breast cancer, pancreatic cancer and melanoma (Sinclair et al., 2002, Mahlamaki et al., 2002; van 't Veer et al., 2002; Adem et al., 2004; Hansel et al., 2004; Hoek et al., 2004). Interestingly, *TBX2* is amplified more frequently in breast cancers with *BRCA1*

and *BRCA2* mutations, suggesting an active selection for *TBX2* amplification and a possible role for TBX2 in tumor initiation (Sinclair et al., 2002, Adem et al., 2004). In mouse models it was shown that *p53* deficiency is highly cooperative with both *Brca1* and *Brca2* in promoting tumorigenesis (reviewed in Moynahan, 2002). As *TBX2* impinges on the p53 pathway both by regulating $p19^{Arf}$ and $p21^{WAF1}$, this may explain the selective amplification in *BRCA1* and *BRCA2* deficient tumors. Nevertheless, although several arguments for a role of both TBX2 and TBX3 in tumorigenesis exist, no transgenic mouse has been reported as yet. Given the observed amplification of the *TBX2* gene in breast cancer, we constructed a *MMTV-TBX2* transgenic mouse model. Unfortunately, we did not obtain transgenics with clear overexpression of TBX2, possibly due to the toxicity upon TBX2 overexpression which we also have experienced in multiple cell types in tissue culture.

On the other end of the spectrum, TBX2-mediated *Arf* regulation has so far yielded no developmental implications. In line with Tbx2's expression pattern and ability to repress the heart-relevant *ANF* gene, the recently reported *Tbx2*-/- mouse shows developmental cardiac defects (Habets et al., 2002; Harrelson et al., 2004). A role in cardiac development is in line with the implication of both its family members *TBX1* and *TBX5* in heart development (Bruneau et al., 2001; Jerome et al., 2001). However, defects in *Tbx2*-/- mice can not be attributed to deregulated $p19^{Arf}$ or $p21^{WAF1}$ expression, as their expression is not induced in *Tbx2*-/- embryos. Moreover, *Tbx2* associated defects could not be alleviated in a *p53*-/- background. There are several possible explanations for these findings. A first possibility is redundancy with Tbx3. Second, Tbx2-mediated *Arf* regulation might be only relevant later in development and can thus, due to the early embryonic lethality of *Tbx2*-/- mice, not be observed. Third, Tbx2 suppression of *Arf* is not implicated in cardiac tissue but in other tissues that due to the early embryonic lethality can not be examined. Clearly, unraveling these possibilities asks for a conditional transgenic approach.

Repression of Ink4a/Arf by Bmi1

Analysis of the *Bmi1*-/- mouse revealed a crucial role for Bmi1-mediated suppression of *Ink4a/Arf* during development (Jacobs et al., 1999). Bmi1 is unique in its capacity to regulate both $p16^{Ink4a}$ and $p19^{Arf}$ expression. Careful analysis of *Bmi1*-/-;*Ink4a*-/-, *Bmi1*-/-;*Arf*-/- and *Bmi1*-/-;*Ink4a/Arf*-/- mice revealed functional contribution for both $p16^{Ink4a}$ and $p19^{Arf}$ in different tissues (chapter 5). Interestingly, both $p16^{Ink4a}$ and $p19^{Arf}$ contribute to the *Bmi1*-/- phenotype. However, our results suggest that Bmi1-mediated suppression of *Ink4a* might be more relevant in relatively undifferentiated cells, whereas *Arf* suppression seems important in all cell types tested. This suggests a previously unanticipated specificity in cell cycle regulation of differentiated versus undifferentiated cells.

Although *Ink4a/Arf* clearly is a Bmi1 target in multiple tissues, a mechanism for Bmi1-mediated *Ink4a/Arf* repression is still lacking. Knowledge about the biochemical functions of PcG proteins is accumulating. PRC2 harbors methyltransferase activity and associates with HDACs (see chapter 1). In *Drosophila*, regulation of targets by PcG is well characterized and involves both PcG proteins binding at the Polycomb Responsive Element (PRE) as well as at the promoter (Cao et al., 2002). Interestingly, at exactly those sequences histone H3 is methylated at K27, dependent on the E(Z) protein. In mammals, analysis of PcG regulation has been hampered by the absence of a

known PRE. However, recent studies in mammals too show colocalization by ChIP of PRC2 proteins and histone H3 methylated at lysine 27 mostly at promoters (Kirzimis et al., 2004; Cao et al., 2004; Caretti et al., 2004). In addition, recent evidence in *Drosophila* suggests that PcG inhibits the transcription initiation machinery (Dellino et al., 2004, Wang et al., 2004). The mode of action of PRC1 is less well understood. *In vitro* experiments with nucleosomal arrays showed that a *Drosophila* PRC1 complex is able to induce physical compaction suggesting this is the chromatin structure that is repressive to gene transcription (Francis et al., 2004). Remarkably, this new activity of the complex is independent of histone tails. Other lines of evidence do suggest histone modifying capacity in or associated with the PRC1 complex. Although PRC1 members associate with histone methyltransferase activity, no results were shown to confirm colocalization of methylated histones and PRC1 proteins on endogenous loci (Ogawa et al., 2002). Recent studies have implicated PRC1 complex in ubiquitination of histone H2A (de Napoles et al., 2004; Fang et al., 2004; Wang et al., 2004; E.Boutsma et al. in preparation). Functionally, ubiquitinated H2A has been suggested to be instrumental in PcG-mediated X chromosome silencing in mammals and PcG target gene *Ubx* silencing in *Drosophila*. Together, these results imply that PcG is able to provide multiple histone modification marks offering multiple layers of silencing.

We set out to analyze whether we could establish the mode of regulation of *Ink4a/Arf* by Bmi1 and whether this regulation is direct. In line with the increased expression of *Ink4a* and to a lesser extent *Arf* in *Bmi1*-/- MEFs, we reproducibly observed by ChIP relatively more acetylated histone H3 and H4 at the promoters of *Ink4a* and *Arf* in *Bmi1*-/- cells (data not shown, collaboration with M. Prudenziati). This is in good agreement with the more active status of those loci in *Bmi1*-/- MEFs. However, we were never able to show any difference in lysine 27 methylation status, suggestive of PcG regulation, on the *Ink4a/Arf* gene between wildtype and *Bmi1*-/- MEFs. With the recent observation of histone ubiquitination capacity of the PRC1 complex, it might be more relevant to look into histone ubiquitination on the *Ink4a/Arf* locus. Another approach we took is to determine whether Bmi1 itself associates with the locus. We did not detect any binding of endogenous Bmi1 to *Ink4a/Arf* or *Hoxc8*. *Hoxc8* was chosen as a putative Bmi1 target, as its Trithorax counterpart protein MLL was reported to associate with specific elements of the *Hoxc8* gene (Milne et al., 2002). Moreover, Hoxc8 was found deregulated in *Bmi1*-/- embryo's (van der Lugt et al., 1996). In sharp contrast, upon overexpression of Bmi1 we could detect Bmi1 associated with chromatin. However, this association never appeared to be specific, as it could also be detected on every genomic locus we tested, even the ones we anticipated to be negative for Bmi1 binding. Upon overexpression, Psc, the *Drosophila* Bmi1 homolog, also aberrantly covers whole polytene chromosomes suggesting our results might reflect a true biological capacity of overexpressed Bmi1 protein (Sharp et al., 1994). Nevertheless, such a-specific binding does not allow us to screen for PREs either in the *Ink4a/Arf* locus or on other putative target genes. Technically, it is possible that mammalian PREs do not exist at all and that PcG proteins bind to repetitive elements. Such repeat binding is indeed reflected in the nuclear heterochromatic staining of Bmi1 in tumor cell lines and the association of Bmi1 with a region of chromosome 1

that is rich in α-satellite repeats (Voncken et al., 1999).

Important to mention here, is that we encountered many problems with the ChIP technique. First of all, it is somewhat counterintuitive to understand how this technique is able to work at all. The experiment relies on cross-linking of proteins to the DNA, which might be more difficult for bulky protein complexes such as PcG, that are further away from the DNA, as for instance for histones. We experienced that shearing the DNA by sonication is difficult to control and yields different fragment sizes in independent experiments. Of special interest, a recent study in *Drosophila* has shown that in a density gradient PREs and their associated PcG complexes are not found in the chromatin fractions but rather in the fractions previously thought to contain free protein (Schwartz et al., 2005). Moreover, the PRE-PcG complexes seemed more sensitive to sonication. For the IP, the quality of the antibody is a critical aspect, to be able to recognize the epitope buried away in cross-linked protein-DNA structures. For the modified histones, we and others encountered specificity problems, as the antibodies were cross-reactive for histone H3 methylated at K9 and K27. Recently, these problems have been solved by the development of highly specific antibodies for methylated histone H3 at K27, even distinguishing between the mono-, di-, or trimethylated state (Peters et al., 2003). For detection, ChIP relies on quantification of the immunoprecipitated DNA by PCR. Although others mainly show a PCR product on agarose gels, we always analyzed our ChIP results by quantitative real-time PCR. This enables us to make sure that the PCR efficiencies of control and target PCRs are comparable, a prerequisite for quantitative comparison between different PCRs and something that is almost impossible to assess with regular agarose gels. This is especially important in promoter areas, such as for example the *Arf* promoter, which can be quite GC-rich and therefore difficult to PCR. Thus although ChIP seems a very powerful technique, technically it is still demanding and prone to artifacts. Therefore, our lab has currently engaged on unraveling PcG binding elements exploiting a new technique: the use of PcG protein-Dam methyltransferase fusions (van Steensel and Henikoff, 2000; I. Muijrers and B.Tolhuis).

Bmi1 in cancer

In addition to the recent implication of Bmi1 in the renewal of multiple adult stem cells, Bmi1 also functions in the self-renewal of cancer stem cells (Lessard and Sauvageau, 2003; Molofsky et al., 2003; Smith et al., 2003; Park et al., 2003). In essence, both Smith et al. and Lessard et al. showed a critical dependence on Bmi1 of the transformation of hematopoietic progenitors by E2a-Pbx1 or self-renewal capability of hematopoietic stem cells transformed by Hoxa9-Meis1. Moreover, we demonstrated overexpression of Bmi1 in a different type of cancer with stem cell characteristics, medulloblastoma (chapter 3).

In humans, mutations in the Shh pathway were found to account for approximately 25 percent of all medulloblastoma cases. Several mouse models have shed light on the additional genetic requirements for medulloblastoma formation. Analogous to the human situation where *PTCH* mutations lead to Gorlins syndrome, *Ptch* heterozygous mice spontaneously develop medulloblastomas (Goodrich et al, 1997). Also, inherited *p53* mutations in human Li-Fraumeni patients and *p53* loss in mice contribute to medulloblastoma formation. Tumorigenesis becomes accelerated when *p53* loss occurs simultaneously

with either *Ptch* mutations, or in a DNA repair deficient background, or upon loss of the tumor suppressors *pRb* or *p15Ink4c* (Marino et al, 2000; Wetmore et al, 2001; Lee and McKinnon, 2003; Tong et al, 2003; Zindy et al, 2003). Expression profiling suggested Shh pathway activation in the majority of these tumors (Lee et al, 2003). This not only further emphasizes the central and critical role of the Shh pathway in medulloblastoma but also implicates alternative routes in addition to the currently known mutations to activate the pathway. Intriguingly, *p53* mutations in human medulloblastoma are rare whereas in mice *p53* loss readily contributes to the formation of medulloblastoma in a *Ptch*+/- background (Wetmore et al, 2001). *INK4a/ARF* mutations are reported only in a minority of cases, and surprisingly *Ptch*+/-;*Arf*-/- mice do not show an increased medulloblastoma frequency (Wetmore et al, 2001). Apparently in this setting loss of Rb function, possibly via *Ink4a* inhibition, is essential for medulloblastoma formation, as is also illustrated by the development of medulloblastomas in mice conditionally deficient in cerebellum for *Rb* and *p53* (Marino et al., 2000). Therefore, the observed overexpression of Bmi1 in human medulloblastoma could be instrumental in downregulating both *Ink4a* and *Arf*, thereby alleviating the need for p53 and Rb mutations. A similar scenario has been suggested for pre-B cell leukemias induced by the chimeric oncoprotein E2a-Pbx1. Induction of Bmi1 by E2a-Pbx1 alleviates the need for mutation of the *Ink4a/Arf* locus, explaining the absence of such mutations in this type of cancers. These results further stress the powerful dual oncogenic capacity of Bmi1, as a repressor of both *Ink4a* and *Arf*. It might be also possible that overexpression of *Bmi1* not only stimulates rapid proliferation through repression of the *Ink4a/Arf* locus, but also allows the cells to 'return' to a more stem cell-like state. Or alternatively, in cancers with stem cell characteristics it is critically required to ensure aberrant maintenance of self-renewal cell divisions via activation of Bmi1.

In order to show the relevance of Bmi1 overexpression to medulloblastoma formation, we constructed cerebellum-specific *Bmi1* transgenics. To accelerate tumorigenesis, we want to cross *Bmi1* transgenics to *Ptch*+/- mice. Although we demonstrated Bmi1 to be downstream of Shh signalling (chapter 3), we have reasons to believe that Bmi1 overexpression alone will not be enough to induce medulloblastoma. First, Lee et al. showed Shh pathway activation to be a prerequisite for medulloblastoma formation (Lee et al., 2003). Second, we have shown that *Bmi1*-/- cerebellar granule neuron progenitors have an impaired response to Shh, but nevertheless still respond to Shh treatment. Therefore, we proposed that Shh is regulating multiple pathways, potentially Bmi1 and N-myc/CyclinD, which together are necessary to induce proliferation and perhaps to block differentiation. In a first attempt we crossed *Eμ-Bmi1* transgenics to *Ptch*+/- mice. Whereas originally this transgenic was generated to overexpress Bmi1 in the hematopoietic compartment, we observed overexpression of Bmi1 as well in postnatal day 7 cerebellar granule neuron progenitors, exactly the cells that medulloblastomas are thought to derive from (Alkema et al., 1997; unpublished observation). A small cohort of *Eμ-Bmi1*;*Ptch*+/- animals so far did not show any brain tumors and succumbed from lymphomas as was reported for *Bmi1* transgenics. Our second strategy was to make use of *GFAP*-driven transgene expression. Although *GFAP* is best-known for its expression in astrocytes, medulloblastomas could be generated by

expressing a *GFAP-Cre* transgene in a *Rb;p53* conditional mouse, showing that *GFAP* is expressed in granule neuron progenitors (Marino et al., 2000). We generated 8 *GFAP-Bmi1* transgenic mice with substantial copy numbers of the transgene, however in none of these mice we saw elevated expression of Bmi1, which might be due to relatively low expression from the *GFAP* promoter. Such a problem could be circumvented by using a conditional *Bmi1* transgene driven by a strong promoter in combination with the mentioned *GFAP-Cre* construct. Our final strategy is an *alpha6-Bmi1* transgenic mouse (generated in collaboration with S. Marino), a promoter reported to be expressed in adult cerebellar granule cells (Aller et al., 2003). Bmi1 expression from this promoter therefore will probably also need to induce some degree of dedifferentiation to induce tumorigenesis. In a different model for medulloblastoma, it has already been shown that whereas IFN-γ overexpression at postnatal day 12 will induce medulloblastoma, cerebella are resistant to IFN-γ induction at postnatal day 21 (Lin et al., 2004). So this *alpha6-Bmi1* transgenic mouse will be the ultimate test whether Bmi1 overexpression, provided it is substantial, is able to confer dedifferentiation and self-renewal as well as proliferative capacities on adult cerebellar granule cells.

On the other end of the spectrum, we could perform experiments to show a genetic requirement for *Bmi1* in the transformation as well as self-renewal. To accomplish this, we could compare *Bmi1*-/-;*Ptch*+/- mice to *Bmi1*-/- mice to evaluate whether some of the defects of the latter mice can be rescued by activated Shh signalling. If so, this would be in line with our results in cerebellar granule neuron progenitors where Shh is able to induce proliferation, be it impaired, in *Bmi1*-/- cells via other pathways. On the other hand we could assess whether medulloblastomas develop with a lower frequency in *Bmi1*-/-;*Ptch*+/- as compared to *Ptch*+/-. Nevertheless, as medulloblastoma appear with a very low frequency and late in the lifespan of the *Ptch*+/- mouse, whereas *Bmi1*-/- mice have to be sacrificed between 8 and 12 weeks of age due to increased sickness, some mode of accelerating tumorigenesis has to be employed. Since inhibiting p53 activity is probably part of Bmi1's oncogenic function, using a *p53*-/- background is not preferable. Rather, one would need to use a non-prejudiced approach, such as X-ray irradiation, that has already been shown to induce high frequency of medulloblastoma when used in *Ptch*+/- newborn mice (Pazzaglia et al., 2002).

In vitro models for Shh pathway; relevance to tumorigenesis

Recently, a lot of attention has been drawn towards the hypothesis that tumors harbour cancers stem cells that drive tumor proliferation and progression (see also chapter 1). This hypothesis would not only explain tumor heterogeneity but also explains why so many tumors harbour mutations or deregulation of developmental pathways such as Shh. Normal stem cells or precursors might aberrantly switch on or keep on their developmental expression programmes, enabling them to proliferate extensively. In practice, this could explain the childhood brain tumor medulloblastoma, which derives from cerebellar granule neuron progenitors that by mistake have acquired Shh pathway activation and thus extended proliferation capacity. Also many other tumors may derive from aberrant Shh pathway activation in precursor cells that were responsive to this morphogen during development and/or tissue repair, such as pancreas, prostate, lung and skin

tumors (Pasca di Magliano and Hebrok, 2003; Karhadkar et al., 2004; Sanchez et al., 2004).

An extremely important finding in the Shh field was the identification of specific pathway inhibitors that could inhibit *in vivo* the growth of both medulloblastoma xenografts but more importantly also of true medulloblastomas in *Ptch*+/-*p53*-/- mice (Berman et al., 2002; Romer et al., 2004). Subsequently, many studies used this drug, cyclopamine, to show that cancer cell lines are responsive. In combination with Shh pathway expression data obtained in tumors, this argues in favour of a central role of Shh pathway activation in tumorigenesis (Berman et al., 2002; Berman et al., 2003; Thayer et al., 2003; Watkins et al., 2003; Karhadkar et al., 2004; Sanchez et al., 2004). However, *in vitro* experiments investigating Shh pathway activity using cyclopamine have some drawbacks. We and others have experienced that some cell lines with apparent activation of Hh signaling remained resistant to cyclopamine. The reasons for this are still unclear but will be important to understand as efforts proceed to target the Hh pathway with pathway-specific drugs. Moreover, Romer et al. have shown that drug concentrations required to block *in vitro* cell growth were 100 fold higher than those required to inhibit Shh pathway activity, as measured by Gli reporter constructs (Romer et al., 2004). These results raise the concern that growth inhibition in cell culture may not be related to inhibition of the pathway. Surprisingly, it is still not completely understood how cyclopamine acts molecularly, although the dogma is that it acts at the level of Smoothened. Part of the confusion may stem from incomplete understanding of how Ptch signals to Smoothened and the nature of the link between Smoothened and the Gli transcription factors (Lum and Beachy, 2004). A crucial experiment will be to test the response of medulloblastomas to cyclopamine in of mice transgenically expressing a constitutively active form of *Smoothened* (Hallahan et al., 2004). Somewhat unrelated, *in vivo* cyclopamine treatment in mice is surprisingly non-toxic. This is counterintuitive as Shh signaling has been implicated in stem cell maintenance and proliferation in many tissues of the adult. On the positive side, this is beneficial for the patient, as it allows for treatment without any dramatic side-effects.

Besides these technical issues, the use of Shh pathway inhibitors is not instrumental in identifying Shh downstream pathway components essential for tumorigenesis. Although perhaps provocative, it is possible that we do not know all transcription factors downstream of Shh. Nevertheless, here I want to focus on the established Gli transcription factors and their targets. Only in skin, analysis of transgenic overexpression of the Gli transcription factors has firmly established their role in oncogenesis. Interestingly, a clear difference could be observed between tumorigenesis induced by Gli1 or Gli2: whereas *Gli1* transgenics develop a number of different skin tumors, *Gli2* transgenics only develop basal cell carcinomas (Grachtchouk et al., 2000; Nilsson et al., 2000). Transgenic models for *Gli* overexpression in cerebellum are until now lacking. It has been shown that medulloblastomas can be induced by Shh overexpression in cerebella of *Gli1*-/- mice (Weiner et al., 2002). In addition, whereas *Gli1* mutants have a normal cerebellum, *Gli2* mutants have a decrease in the number of cerebellar granule neuron progenitors (Corrales et al., 2004). This suggests a more prominent role for Gli2 in transducing Shh signals both during development as well as in tumorigenesis. In line with this, we also

observed a more potent transforming capacity of Gli2 overexpression in RK3E cells (chapter 4). As this is an *in vitro* system, it allows for dissection of the downstream requirements of Gli transcription factors for transformation. In agreement with a previous report, Gli1 and Gli2 are able to downregulate E-Cadherin expression, possibly via upregulation of Snail, providing a potential explanation for their ability to induce transformation (Louro et al., 2002; A. Sparmann and M. Lingbeek, unpublished observation).

We also linked overexpression of Gli transcription factors in RK3E cells to induction of the Myc oncogene family (chapter 4). Transformation by Gli is accompanied by a fast upregulation of *L-Myc* RNA, whereas during culturing Gli overexpressing clones acquire elevated N-myc protein levels. Gli overexpressing RK3E cells also upregulate Bmi1 expression (see later). Originally, *Bmi1* was identified as a c-Myc collaborating oncogene in insertional mutagenesis screens (Haupt et al., 1991; van Lohuizen et al., 1991). The ability of Bmi1 to down-regulate *Ink4a/ARF* was shown to be the basis of its cooperation with c-Myc in tumorigenesis (Jacobs et al., 1999), suggesting this might also be a function of Gli-induced Bmi1 in N-myc/L-myc overexpressing tumors. However, RK3E cells preclude analysis of such a hypothesis as these cells overexpress E1A, disturbing the $p16^{Ink4a}$/Rb and $p19^{Arf}$/p53 pathways that are also affected by Bmi1. In line with this we did not see collaboration between N-myc and Bmi1 overexpression in RK3E, whereas Bmi1 and c-Myc do collaborate in fibroblasts in soft agar assays (unpublished observation; Jacobs et al., 1999). Alternatively, Bmi1 might not collaborate with N-myc. Confounding the latter; Bmi1 was not found in an insertional mutagenesis screen with *N-myc* transgenic mice (Sheppard et al., 1998). Nevertheless, we think reiterating Myc and Bmi1 connections in lymphomas, fibroblasts and RK3E cells are highly suggestive of a functional link in multiple cancers. Possibly in different cancer types either c-Myc or conversely N-myc (combined with L-myc) overexpression is important and allowed in the context of Bmi1 overexpression. Thus, Myc family members are not interchangeable and although they share many related functions, at the same time they retain other, unique functions entailing them with the best oncogenic capacity in each specific tissue.

Regulation of Bmi1 expression

The notion that Bmi1 is overexpressed in exactly those medulloblastomas that show an active Shh pathway, prompted us to investigate Bmi1 expression in primary cerebellar granule neuron progenitors as these cells are thought to be the precursors of medulloblastoma (chapter 3). Interestingly, this revealed Bmi1 as a critical target of Shh, partly mediating the proliferative induction imposed on cells by Shh. Regulation of PcG genes is still a relative unexplored field. This finding immediately connected expression of a developmental gene involved in self-renewal, *Bmi1*, to one of the major developmental signalling pathways available, Shh.

In line with Shh-mediated regulation, Gli1 and Gli2 (but not Gli3) induce Bmi1 expression in RK3E cells accompanied by oncogenic transformation (chapter 3 and chapter 4). For some unknown reason, not all tissue culture cells are Shh-Gli pathway responsive, as we did not observe Bmi1 induction or transformation in other cell types. *Bmi1* upregulation in RK3E cells turned out to occur at the RNA expression level. Therefore, we characterized a 5.3 Kb *Bmi1* promoter in

combination with Gli proteins in transient transfection assays (J.Vissers, data not shown). In sharp contrast to endogenous Bmi1 being upregulated by Gli1 and Gli2 overexpression, in transient reporter assays consistently Gli2 and Gli3 induce *Bmi1* promoter activity. Although we tried to address the different results obtained in these two experiments, we do not completely understand it. Probably the seemingly conflicting data result from an incomplete promoter construct not encompassing all regulatory regions or the proper chromatin context and/or from indirect regulation of Bmi1 by the Gli transcription factors. The latter is in agreement with the absence of Gli consensus binding sites in the *Bmi1* promoter.

Analysis of the promoter of *Bmi1* revealed several other interesting regulatory elements. First, there is an E-box, that is conserved between human, mouse and zebrafish, suggesting potential important regulatory functions (J.Vissers). Transcriptional activation by Myc is mediated through dimerization with Max and binding to its DNA consensus sequence: the E-box. However, we never observed induction of the *Bmi1* promoter reporter by c-Myc or elevation of Bmi1 protein levels by c-Myc overexpression in RK3E cells. As Gli1 and Gli2 induce L-myc and Bmi1 expression in RK3E cells, an interesting option might be that downstream of Gli transcription factors L-myc is regulating Bmi1 expression via its E-box. Second, in agreement with the putative E2F binding site in the *Bmi1* promoter, we and others found E2F1 to induce our reporter construct (unpublished observations). Interestingly, E2F transcription factors control more PcG genes, as the oncogene *Ezh2* is also regulated by E2Fs (Bracken et al., 2003). However, for E2F1 regulation of both Bmi1 and Ezh2 no clear functional role has been described although one could easily envision a role in mediating proliferation.

Apart from transcriptional regulation, Bmi1 function can also be regulated at the protein level. We previously recognized that PcG complexes are phosphorylated *in vivo*, which regulates their association with chromatin (Voncken et al., 1999). More recently, it was found that Bmi1 protein is phosphorylated by the kinase MAPKAP kinase 3pK (Voncken et al., 2004). The exact function of phosphorylation to the activity of the Bmi1 protein or the PcG complex as a whole is not known. However, regulation of Bmi1 protein by phosphorylation might also be important in cerebellar granule neuron progenitors. Although we showed Shh treatment to transcriptionally upregulate *Bmi1* expression levels (chapter 3), under sub-optimal Shh concentrations we were able to see an induction of Bmi1 protein but not RNA levels (unpublished observations). Such results are in line with the absence of *Bmi1* induction on microarray analysis of cerebellar granule neuron progenitors treated with Shh (Oliver et al., 2003). Moreover, in those Shh treated cerebellar granule neuron progenitors the majority of Bmi1 protein seems to shift to a higher mobility species, alike to what has been described during cell cycle (unpublished observation, Voncken et al., 1999). Of course these results might merely reflect the different cell cycle status of non-treated resting cerebellar granule cells versus Shh-treated proliferating progenitors. However, it is also possible this reflects an intriguing feedback regulation of Bmi1 protein. At first, Shh induces Bmi1 expression thereby presumably negatively regulating the *Ink4a* and *Arf* cell cycle regulators leading to proliferation. Subsequently, Bmi1 protein is phosphorylated and

released from the chromatin and its targets, halting proliferation again. The *N-myc* oncogene is transcriptionally regulated by Shh in cerebellar granule neuron precursors and is essential for their expansion (Kenney et al, 2004). In addition, the PI3K pathway stabilizes N-myc protein via inhibition of GSK3-dependent N-myc phosphorylation and degradation. It would be interesting to check if the presumed Bmi1 phosphorylation is influenced by PI3K signaling pathway as well. Finally, results of our group suggest Bmi1 protein can both be poly- and monoubiquitinated (I.Hernandez-M., A.Lund, P. van der Stoop, E.Boutsma, P.Taghavi). As both the variant histone MACROH2A and Bmi1-containing PRC1 complexes are recruited to the inactive X chromosome, as well as both proteins are ubiquitinated by the same SPOP/CULLIN3 ligase, this is highly suggestive of a role of Bmi1 ubiquitination in X inactivation.

Bmi1 targets beyond Ink4a/Arf

There are several arguments for the presence of additional Bmi1 target genes besides the *Ink4a/Arf* locus. Probably the best argument is the incomplete rescue of multiple *Bmi1*-/- defects, such as reduced body weight, skeletal transformations and neurological phenotype, upon subsequent loss of the *Ink4a/Arf* locus (chapter 5). However, also in tumorigenesis, suppression of the *Ink4a/Arf* tumor suppressors appears to be not the only action of Bmi1 protein (Lessard and Sauvageau, 2003). High proliferative clones (HPCs) can be derived from *Bmi1*-/- leukemic cells by applying strong selection procedures, although this is more difficult than in wild-type cells. These *Bmi1*-/- HPCs invariably lose expression of several cyclin kinase inhibitors among which $p16^{Ink4a}$ and $p19^{Arf}$. Importantly, re-expression of Bmi1 in these *Bmi1*-/- clones, that are deficient for $p16^{Ink4a}$ and $p19^{Arf}$, rescues their weakly tumorigenic capacity. Together the results suggest additional targets for Bmi1.

More specifically, I want to discuss potential additional targets of Bmi1 in cerebellar development. In brief, cerebellar cells derive from two different embryonic germinal layers (reviewed in Wang and Zoghbi, 2001). The ventricular zone gives rise to the stellate, basket and Golgi neurons, that later will end up in the molecular layer (ML), and the Purkinje cells which form a separate layer. The rhombic lip, a germinal epithelium located between the fourth ventricle and the roof plate in the metencephalon, gives rise to the most abundant cells of the cerebellum, the granule cells. During postnatal development cerebellar granule neuron progenitors sit in the external granule layer (EGL) on top of the molecular layer, where their proliferation is driven by Purkinje cell excreted Shh. Progressively, cerebellar granule neuron progenitors differentiate and migrate inwards towards the IGL, to their final destination as neurons. In adult, the EGL ceases to exist. Apart from reduced IGL thickness, also a lot of other cerebellar defects have been suggested to derive from aberrant EGL proliferation. For instance, granule neurons signal to Purkinje cells in order to create the right amount of arborization on Purkinje cells (Baptista et al., 1994). Therefore we could easily explain the aberrant *Bmi1*-/- Purkinje trees by the fact that these cerebella also have less granule neurons. Although IGL area and numbers are partially rescued in *Bmi1*-/- upon *Arf* loss, surprisingly the drastic defect in ML neuron number in *Bmi1*-/- is not rescued at all by *Arf* loss. As ML neurons do not derive from the rhombic lip this suggests an additional defect in cerebellar development of *Bmi1*-/- independent of *Ink4a/Arf*, possibly in the cells deriving from the ventricular zone.

It is really difficult to uncouple all effects on cerebellar development in mutant mice especially since most of these mice have several cerebellar abnormalities that all seem to influence each other. Moreover, a lot of the focus has been on EGL proliferation and for instance Basket and stellate neuron specification/ development is an understudied aspect. Development of these cell types is interesting, as for instance almost no stellate neurons are found in *Bmi1*-/- animals and arborization of Basket neurons is aberrant. Moreover, these phenotypes are independent of *Ink4a/Arf* as they still persist in *Bmi1*-/-;*Ink4a/Arf*-/- cerebella. Therefore Bmi1 might have a more early role in cerebellar development, for instance at the time of patterning of the hindbrain. Potential targets could be *Hox* genes that have already been implicated in hindbrain patterning. *Hoxa2*-/- mice develop enlarged cerebella due to expanded rhombomere 1 and concomitant increase in granule neurons (Gavalas et al., 1997). In that line of reasoning *Bmi1*-/- mice would have elevated Hoxa2 expression and thus smaller cerebella. Moreover, Engrailed 1 and Engrailed 2 are important in cerebellar development and a connection between Engrailed and PcG has already been established in *Drosophila* (Moazed and O'Farrell, 1992; Millen et al., 1994; Wurst et al., 1994; Kuemerle et al., 1997). Preliminary results showed a slight downregulation of Engrailed 2 in *Bmi1*-/- cerebella of day 7 old mice, but more appropriate would be to look at expression during embryogenesis. Finally, one should keep in mind the strong phenotype of *Bmi1*-/- mice, suffering from increasing ataxic gait. In that sense, it would be interesting to assess what is more relevant for motor coordination through the cerebellum: the aberrant arborization of the Purkinje/Basket neurons (therefore not being able to properly signal and integrate signals necessary for motor systems) or the reduced number of granule neurons present in the *Bmi1*-/- brain.

Stem cell regulation; increasing attention towards niche context in terms of adhesion and signaling

Stem cell biology not only involves self-renewing division but also implicates a specialized and well equipped microenvironment in tissues for such divisions, the niche (reviewed in Fuchs et al., 2004). The location and cellular composition of stem cell niches in higher organisms is still ill-defined. The only well-studied example is the *Drosphila* ovary where a niche of somatic cells maintains germ-line stem cells via secretion of several signaling molecules as well as a critical attachment to the niche cells via DE-cadherin mediated adhesion. Importantly, PcG was recently implicated for the first time in regulating niche biology in *Drosophila*. Aberrant follicle formation in *ph* mutants, ph being a component of the PRC1 complex, results from defects in somatic cell proliferation and differentiation but not from defects in the germ line stem cells(Narbonne et al., 2004). If PcG complexes also functions in niche cells of higher organism, this would implicate PcG genes in multiple aspects of stem cell biology. First intrinsically in stem cells, controlling self-renewal and second in controlling the niche cells, which are crucial in regulating the flexibility of stem cells in response to disease and injury.

Both Bmi1 and Shh have been implicated in stem cell biology. In the adult, Shh signaling is required for stem-cell maintenance and proliferation of a number of precursors (reviewed in Pasca di Magliano and Hebrok, 2003). Of note, the transcription factors downstream of Shh seem to influence adhesion

phenotypes (A.Sparmann, unpublished observations). First, both Gli1 and Gli2 down-regulate E-cadherin expression (Louro et al., 2002). Second, Gli2 induces a striking morphological change, accompanied by a reorganization of the actin cytoskeleton from stress-fibers to cortical actin bundles. Whether this function of Gli2 is important in cerebellar development is speculative. Adhesion in cerebellum is probably important, taking into account that cerebellum is composed of multiple layers and the active cell migration occurs during development. Of particular interest, β1-integrin was recently shown to be critical for cerebellar granule cell precursor proliferation (Blaess et al., 2004). We have shown Bmi1 as a critical target of Shh in regulating cerebellar granule neuron progenitor proliferation (chapter 3). Upregulated *Ink4a/Arf* expression is part of the explanation for the deregulated proliferation of *Bmi1*-/- cerebellar granule neuron progenitors *in vitro* and *in vivo* (chapter 5). In addition to other targets of Bmi1 in cerebellar development as suggested above, PcG might also in cerebellum alike in *Drosophila* ovary function in adhesion. A possible functional connection between Shh and Bmi1 in regulation of adhesion is still highly speculative. Moreover, relevance to stem cell biology for both should still be investigated.

A very interesting recent study connected stem cell self-renewal, differentiation and adhesion in the niche (Wilson et al., 2004). These authors revealed an unexpected function for the *c-Myc* oncogene in HSC homeostasis. Whereas in commited progenitors c-Myc is required for cell cycle progression and expansion, in HSCs c-Myc is regulating the balance between stem cell self-renewal and differentiation. Both in gain-of-function and loss-of-function approaches they showed that c-Myc regulates E-cadherin expression and thereby E-cadherin mediated adhesion between stem cells and their niche. It is challenging to try to translate these results to cancer biology, as should be done considering the recent hype around cancer stem cells, especially in leukemia (see also chapter 1). Enforced c-Myc expression in HSCs leads to down-regulation of E-cadherin, release of the HSCs from the niche and thereby loss of self-renewal activity. Nevertheless, enforced expression of c-Myc in *BCL-2* transgenic HSCs did result in lymphomas. An outstanding question is whether in the presence of c-Myc overexpression and consequetive stem cell loss, it is then the more differentiated cells that actually cause the lymphoma. This would be somewhat in contradiction with the cancer stem cell hypothesis. Alternatively, these cells may have bypassed their self-renewal defect by upregulation of Bmi1, which also has been implicated in the self-renewal of HSCs (Park et al., 2003; Lessard and Sauvageau, 2003). Although highly speculative, this would then be a second level of collaboration between Bmi1 and c-Myc, in addition to inhibition of c-Myc induced apoptosis, (Jacobs et al., 1999).

Future research should aim at analyzing stem cell phenotypes *in vivo* in the niche, although this kind of experiments will be technically demanding. Especially, since differences between *in vitro* and *in vivo c-Myc* deficient HSC behaviour have been reported (Wilson et al., 2004). Moreover, identifying crucial niche signals will be instrumental in reconstructing the niche *in vitro*, providing attractive possibilities of stem cell manipulations *in vitro*, possibly even relevant for treating stem cell disease.

Concluding remarks

In conclusion, our results show a crucial regulation of *Ink4a/Arf* by Bmi1

and TBX2 in development, stem cells and cancer. In addition, we demonstrated for the first time transcriptional regulation of Bmi1 by the developmental Shh signal transduction pathway. Thus, in part Shh is mediating proliferation in cerebellar granule cells through regulation of *Ink4a/Arf* via Bmi1. *In vitro*, we demonstrated transforming capacity of Gli1 and Gli2, transcription factors downstream of Shh, together with induction of expression of N-myc and L-myc. Though speculative, a dual induction of Bmi1 and Myc's by Gli could be important as Bmi1 has been shown to collaborate with c-Myc via suppression of apoptosis. Finally, we have suggested an unanticipated role for Bmi1-mediated *Ink4a/Arf* suppression, as *Ink4a* downregulation appears to be relevant in undifferentiated cells, such as thymocytes, cerebellar granule neuron progenitors and neural stem cells, whereas *Arf* repression is needed in all cell types examined, that is the undifferentiated cells plus MEFs and splenocytes/mature B cells. Thus, our results stress the multiple levels of Ink4a/Arf regulation in different tissues, implicating PcG/Bmi1-*Ink4a/Arf* in the control of proliferation and self-renewal of differentiated cells, progenitors and stem cells. Clearly, these are attractive qualities for Bmi1 in mediating the adverse effects on proliferation/self-renewal resulting in cancer. In line with this, we reported Bmi1 overexpression in medulloblastoma, a cancer with stem cell characteristics. Bmi1's regulation by Shh and its implication in self-renewal is a preface to possibly a general role in normal stem cells as well as cancer stem cells.

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Summary

Summary

In order to take proper cell fate decisions, cells have to guide their biochemical machinery through the appropriate decisions in both differentiation and proliferation. Especially for stem cells such decisions are critical as they have the capacity to self-renew, i.e. give rise to new daughter stem cells and differentiated progeny. If gene regulatory cascades are deregulated, this can lead to serious defects in organisms. On the one hand, one can expect developmental defects, if the right amount or type of cells is not in place at the right time. On the other hand, cancer can develop, when cells start to proliferate in an uncontrolled manner. Important to mention here is at least a subset of cancers is now thought of as to derive from cancer stem cells. The current view is that either stem cells aberrantly keep on or more differentiated progenitors switch on a developmental expression programme that is enabling them with the dangerous capacity to self-renew. A locus that emerges as central in the regulation of both self-renewal and proliferation in stem cells, progenitors and cancer is the *Ink4a/Arf* locus.

The *Ink4a/Arf* locus is a unique locus in several aspects. It encodes for two proteins, $p16^{Ink4a}$ and $p19^{Arf}$, by the use of the alternative first exons, which splice to a shared second and third exon. Due to alternative reading frame usage this locus produces two completely unrelated proteins. Probably the most compelling aspect of *Ink4a/Arf* is the fact that both its gene products are upstream of the two most important and well-known tumor suppressors, i.e. $p16^{Ink4a}$ is regulating Rb and $p19^{Arf}$ ($p14^{ARF}$ in human) is upstream of p53. In that respect, *Ink4a/Arf* is important in regulating cell cycle progression and several lines of evidence both in mouse and in humans have shown that loss of *Ink4a/Arf* function leads to tumor development. In contrast, Ink4a/Arf accumulation, such as induced by tissue culture stress, can lead to a special form of cell cycle arrest, termed senescence. Some have suggested that such stress also occurs in stem cells, partly to prevent them from oncogenic transformation, and that possibly stem cells senescence contributes to aging of the organism. Such a role for *Ink4a/Arf* late in development is still speculative. Also, early in development observations are sparse to implicate a role for *Ink4a/Arf*, as *Ink4a/Arf* deficient mice apart from being tumor prone seem to develop normally. In this thesis, we discussed a role for *Inka4a/Arf* under the control of Bmi1 and TBX2/TBX3 in stem cells, progenitors and cancer.

TBX2 and *TBX3* are members of the T box gene family of DNA binding transcription factors, which have both been found as negative regulators of the *Ink4a/Arf* locus thereby leading to immortilization of mouse embryonic fibroblasts. In chapter 2 we further investigated the mode of *Ink4a/Arf* regulation by TBX2/TBX3 and firmly established that TBX2/3 are direct regulators of *Arf* expression. Interestingly, we identified a new DNA-binding T site in the *Arf* promoter, that is a variant of the archetypical and consensus T site and is essential for TBX2/3 mediated regulation. Furthermore, we found that the variant T site is specific for TBX2/3, potentially explaining how *in vivo* T box genes confer specificity in regulating target genes. Our analysis contributes to the connection between T box transcription factors and cell cycle regulation. Hypoproliferation in Ulnar-Mammary syndrome patients, which suffer from *TBX3* mutations, could result from an aberrant cell cycle stop due to deregulated *INK4A/ARF* expression. In addition, TBX2, that is found frequently amplified in human breast cancers, is suggested to be implicated in tumor development through regulation of *Ink4a/Arf*.

Another negative regulator of the *Ink4a/Arf* locus is Bmi1. Originally, *Bmi1* was found as an oncogene, collaborating with *c-Myc* in lymphomagenesis. *Bmi1* is a member of the Polycomb group gene family of epigenetic chromatin repressors, which have been implicated in cell fate decisions. *Bmi1* deficient mice suffer from defects in skeleton, which are typical PcG phenotypes as resulting from deregulation of their targets the *Hox* genes. Furthermore, *Bmi1* deficient

mice suffer from progressive neurological and hematopoietic defects that can all be traced back to an intrinsic defect in the self-renewal of stem cells in those compartments. In chapter 3 we analyze the defects of *Bmi1* deficient mice in cerebellar development. We demonstrate that the *Bmi1* deficient mice have significantly reduced cerebellar size resulting from hypoproliferation in the cerebellar granule neuron progenitors. Those cells are the precursors for granule neurons, the most abundant cells of the cerebellum, and their proliferation is guided by the important morphogen Sonic Hedgehog (Shh). Interestingly, we show that Bmi1 itself is a target of Shh signaling. We proposed that multiple pathways downstream of Shh are regulating cerebellar granule neuron progenitor proliferation, one of which is via Bmi1. We also show implications for our findings in tumor development. Aberrant Shh signaling has been implicated in medulloblastoma, a disease resulting from deregulated proliferation of cerebellar granule neuron progenitors. We demonstrate Bmi1 overexpression in a substantial amount of primary medulloblastomas. This not only further emphasizes Bmi1 as a Shh target but also reinforces Bmi1 as proliferation inducer downstream of Shh.

The morphogen Shh is known to signal, at least partially, via the Gli transcription factors. Corroborating Bmi1 as part of the Shh pathway, its expression is also induced by Gli transcription factors in cerebellar granule neuron progenitors and rat kidney epithelial cells. Three Gli transcription factors have been described, for which we describe different functions in chapter 4. Corroborating earlier studies in skin tumorigenesis, we demonstrate oncogenic transformation of rat kidney epithelial cells overexpressing E1A by Gli1 and Gli2, as opposed to Gli3, which has no tumorigenic potential. We suggest that the striking difference we observe in morphological phenotype between Gli1 and Gli2 overexpressing cells might reflect the differences in skin tumorigenesis, i.e. overexpression of Gli1 induces multiple types of skin tumors whereas Gli2 specifically induces basal cell carcinomas. In addition, we show that overexpression of Gli1 and Gli2 induces L-myc expression and selects for N-myc but not c-Myc overexpression. As *in vivo* tumors with activated Shh signaling almost always show N-myc overexpression, our results suggest a special preference for tumorigenesis induced by the combination of N-myc/L-myc and Shh pathway activation.

Finally, we demonstrated that in cerebellum Shh is regulating *Ink4a/Arf* through Bmi1. As *Ink4a/Arf* is coding for two genes, we determined in chapter 5 the different requirements for *Ink4a* versus *Arf* regulation in multiple defects of *Bmi1* deficient mice. We observed that Arf deregulation is affecting almost all defects of *Bmi1* deficient mice and also recognize that *Arf* levels in some cell types can be important suggesting threshold levels in cell cycle regulation. Furthermore, we suggest *Ink4a* deregulation is more profoundly affecting undifferentiated cells, as *Arf* loss is affecting proliferation of mouse embryonic fibroblasts and B cells, whereas both *Ink4a* and *Arf* loss are affecting T cells, cerebellar granule neuron progenitors and neural stem cells. This suggests differential self-renewal and cell cycle regulation in stem cells and progenitors versus differentiated cells under the control of PcG regulation.

In conclusion, research in this thesis extends the knowledge about the mode and function of *Ink4a/Arf* regulation by Bmi1 and TBX2. Our results have contributed to a further understanding of the regulation of stem cells and progenitors, by implicating Bmi1 as a downstream pathway component of the important Shh signalling pathway and by genetically demonstrating *Ink4a/Arf* as an important Bmi1 target in these cells. Our analysis extends the oncogenic potential of Bmi1 from lymphomagenesis to medulloblastoma, a cancer with stem cell characteristics. Understanding stem cells is of increasing importance, as some have suggested therapeutic potential in stem cells and benefits may be expected in the treatment of cancer by eradication of the cancer stem cells.

Samenvatting

Samenvatting

Om tot de juiste beslissing te komen over het lot van cellen, is er een biochemische machinerie of signaleringsroute waardoor cellen worden geleid om tot de juiste keuzes te komen ten aanzien van proliferatie en differentiatie. Zeker voor stamcellen zijn zulke beslissingen kritiek omdat zij in staat zijn tot zelfvernieuwing. Dat is het proces waarbij uit stamcellen nieuwe dochter stamcellen maar ook gedifferentieerde nakomelingen ontstaan. Als gen regulatie of signalerings cascades gedereguleerd zijn, kan dit leiden tot ernstige defecten in organismen. Aan de ene kant kan men ontwikkelingsstoornissen verwachten, wanneer de juiste hoeveelheid of het juiste type cellen niet op tijd op de juiste plek is. Aan de andere kant kan kanker ontstaan, wanneer cellen op een ongecontroleerde wijze gaan prolifereren. Belangrijk om hier te noemen is dat tenminste van een aantal kankers nu wordt gedacht dat ze voortkomen uit zogenaamde kanker stamcellen. De huidige opvatting is dat of stamcellen foutief een gen expressie programma aanhouden of dat meer gedifferentieerde voorloper cellen een gen expressie programma aanschakelen welke hun de gevaarlijke capaciteit verschaft om zelfvernieuwing te ondergaan. Een gen dat nu naar voren komt als centraal in de regulatie van zowel zelfvernieuwing als proliferatie in stamcellen, voorloper cellen en kanker is het *Ink4a/Arf* locus.

Verschillende aspecten aan het *Ink4a/Arf* locus zijn uniek. Het locus codeert voor twee eiwitten, $p16^{Ink4a}$ en $p19^{Arf}$, door gebruik te maken van verschillende eerste exons die splicen naar een gedeeld tweede en derde exon. Niettemin door het gebruik van alternatieve leesramen, codeert het voor twee totaal ongerelateerde eiwitten. Maar misschien wel het meest unieke aan *Ink4a/Arf* is dat beide gen producten de twee belangrijkste en bekendste tumor suppressoren reguleren, d.w.z. $p16^{Ink4a}$ reguleert Rb en $p19^{Arf}$ ($p14^{ARF}$ in mens) reguleert p53. Door deze functies zijn *Ink4a* en *Arf* belangrijk in het reguleren van cel cyclus vooruitgang. Inderdaad is er bewijs, zowel in muis als mens, dat verlies van het *Ink4a/Arf* locus kan leiden tot tumor ontwikkeling. Daarentegen, accumulatie van *Ink4a/Arf*, zoals kan worden geïnduceerd door stress veroorzaakt door weefselkweek, kan leiden tot een speciale vorm van het stoppen van de cel cyclus, ook wel genaamd ‘senescence’. Sommigen hebben de suggestie gewekt dat stress resulterende in *Ink4a/Arf* accumulatie ook voor komt in stamcellen. Dit zou dan deels zijn om cellen te beschermen tegen oncogene transformatie maar mogelijk deels ook resulteren in ‘senescence’ van stamcellen wat dan weer zou kunnen bijdragen aan de veroudering van het organisme. Zo’n soort rol voor *Ink4a/Arf* laat in de ontwikkeling is nog steeds speculatief. Ook vroeg in de ontwikkeling zijn er niet veel aanwijzingen voor een belangrijke rol van *Ink4a/Arf*, immers de *Ink4a/Arf* knock-out muizen ontwikkelen zich normaal, afgezonderd van het feit dat ze vele tumoren krijgen. In dit proefschrift hebben we de rol van *Ink4a/Arf* besproken onder de controle van de genen *Bmi1* en *TBX2/TBX3* in stamcellen, voorloper cellen en kanker.

TBX2 en *TBX3* zijn genen uit de T box gen familie van DNA bindende transcriptie factoren. Beide genen remmen *Ink4a/Arf* en kunnen daardoor leiden tot immortalizatie van embryonale fibroblasten van muizen. In hoofdstuk twee wordt verder onderzocht hoe TBX2/TBX3 *Ink4a/Arf* reguleren en daaruit blijkt dat TBX2/3 directe regulators van *Arf* expressie zijn. Wij hebben een nieuwe DNA-bindings ’T site’ gevonden in de *Arf* promoter, die een variant is van de oorspronkelijke consensus ’T site’ en die essentieel is voor TBX2/3 gemediëerde regulatie. Verder laten wij zien dat de variant van de ’T site’ specifiek is voor *TBX2/3*, wat een mogelijke verklaring zou kunnen zijn voor hoe *in vivo* T box genen specifiek hun target genen kunnen reguleren. Onze analyse draagt bij aan de connectie tussen T box transcriptie factoren en cel cyclus regulatie. Hypoproliferatie in Ulnar-Mammary syndroom patiënten, die een mutatie in het *TBX3* gen dragen, komt mogelijk van een foutieve cel cyclus stop door gedereguleerde *INK4A/ARF* expressie.

Ook *TBX2*, die frequent geamplificeerd is in humane borstkankercellen, is mogelijk betrokken bij tumor ontwikkeling door *INK4A/ARF*.

Een andere negatieve regulator van het *Ink4a/Arf* locus is *Bmi1*. *Bmi1* is ontdekt als een oncogen dat samen met *c-Myc* lymphomagenese geeft. *Bmi1* is een lid van de Polycomb groep gen familie met epigenetische chromatine remmers, die betrokken zijn bij beslissingen over het lot van cellen. Muizen die het *Bmi1* gen missen hebben defecten in hun skelet, wat een typisch PcG fenotype is door de deregulatie van hun targets, de *Hox* genen. Verder lijden *Bmi1* knock-out muizen aan progressieve neurologische en hematopoietische aandoeningen die allemaal terug te voeren zijn op een intrinsiek defect in de zelfvernieuwing van de stamcellen van deze compartimenten. In hoofdstuk 3 onderzoeken we de defecten van de *Bmi1* knock-out muis in de ontwikkeling van de kleine hersenen of cerebellum. Wij laten zien dat de hypoproliferatie van de granulaire neuronale voorloper cellen van het cerebellum de oorzaak is van de kleine cerebella in *Bmi1* knock-out muizen. De genoemde cellen zijn de voorlopers voor de granulaire neuronen, de meest voorkomende neuronen van het cerebellum, en hun proliferatie wordt aangedreven door de belangrijke groeifactor Sonic Hedgehog (Shh). Wij laten zien dat Shh signalering Bmi1 beïnvloedt. Gebaseerd daarop stellen wij voor dat Shh de proliferatie van granulaire neuronale voorloper cellen van het cerebellum op meerdere manieren stuurt, waarvan één manier gaat via regulatie van Bmi1. Wij laten ook implicaties van onze vindingen in tumor ontwikkeling zien. Afwijkende Shh signalering kan leiden tot medulloblastoma, de kanker die ontstaat wanneer de proliferatie van de cerebellaire granulaire neuronale voorloper cellen gedereguleerd is. Wij laten zien dat *Bmi1* te hoog tot expressie komt in een deel van de primaire medulloblastomen. Dit bevestigt niet alleen nogmaals dat Bmi1 wordt beïnvloedt door Shh signalering maar versterkt ook de hypothese dat Bmi1 proliferatie induceert onder invloed van Shh.

Het is bekend dat de groeifactor Shh, tenminste deels, signaleert via de Gli transcriptie factoren. Ter bevestiging van Bmi1 als deel van de Shh signalering, hebben wij ook gevonden dat de Gli transcriptie factoren *Bmi1* expressie stimuleren in granulaire neuronale voorloper cellen van het cerebellum en in rat nier epitheel cellen. Er bestaan drie Gli transcriptie factoren, voor welke we verschillende functies beschrijven in hoofdstuk 4. Wij laten zien dat Gli1 en Gli2, maar niet Gli3, oncogene transformatie veroorzaken in rat nier epitheel cellen die verhoogd E1A tot expressie brengen. Deze resultaten zijn in lijn met eerdere resultaten verkregen bij de bestudering van huidkanker. Ook zien we een opvallend verschil in morfologie tussen cellen met verhoogd *Gli1* of *Gli2* expressie. Dit zou de verklaring kunnen zijn voor de verschillende tumor spectra veroorzaakt door verhoogde *Gli1* dan wel *Gli2* expressie, welke respectievelijk allerhande huidtumoren induceert dan wel alleen basale cel carcinomen. Ook laten we zien dat overexpressie van *Gli1* en *Gli2 L-myc* expressie induceert en dat je onder die condities selecteert voor hoge *N-myc* maar niet *c-Myc* expressie. Aangezien analyse van *in vivo* tumoren al heeft laten zien dat geactiveerde Shh signalering altijd samengaat met verhoogde *N-myc* expressie, stellen wij voor dat er mogelijk een voorkeur/selectie is in tumoren voor de combinatie van N-myc/L-myc en Shh activatie.

Als laatste laten we zien dat in cerebellum Shh *Ink4a/Arf* reguleert door Bmi1. Omdat *Ink4a/Arf* codeert voor twee genen, hebben we in hoofdstuk 5 bepaald of veranderde expressie van *Ink4a* of *Arf* de onderliggende oorzaak is van de stoornissen van *Bmi1* knock-out muizen. Onze observatie is dat in alle celtypes deregulatie van *Arf* expressie bijdraagt aan het *Bmi1* knock-out fenotype maar ook dat *Arf* niveau's in sommige celtypes belangrijk zijn, suggererend dat drempelwaardes belangrijk zijn in cel cyclus regulatie. Verder suggereren we dat *Ink4a* deregulatie meer de ongedifferentieerde cellen aantast, omdat Arf verlies de proliferatie van embryonale fibroblasten van de muis en B cellen

beïnvloedt, terwijl zowel *Arf* als *Ink4a* verlies T cellen, granulaire neuronale voorloper cellen van het cerebellum en neurale stamcellen beïnvloedt. Dit wekt de suggestie dat zelfvernieuwing en proliferatie in stamcellen versus voorloper cellen zich onderscheiden en dit alles onder de controle van PcG regulatie.

In essentie heeft het onderzoek beschreven in dit proefschrift de kennis over de regulatie van *Ink4a/Arf* onder de controle van *Bmi1* en *TBX2/3* uitgebreid. Onze resultaten hebben bijgedragen aan het verder begrijpen van de regulatie van stamcellen en voorloper cellen, door *Bmi1* te identificeren als een deel van de belangrijke Shh signaleringsroute en door genetisch te laten zien dat *Bmi1 Ink4a/Arf* reguleert in deze cellen. Onze analyse laat zien dat *Bmi1* niet alleen een oncogen is in lymfoma maar ook in medulloblastoma, een kanker met stamcel karakteristieken. Stamcellen beter leren begrijpen lijkt steeds belangrijker te worden. Niet alleen omdat men verwacht stamcellen therapeutisch te kunnen inzetten maar ook omdat in de behandeling van kanker het elimineren van de kanker stamcellen essentieel lijkt.

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Curriculum Vitae

Merel Valk-Lingbeek werd op 16 juni 1978 geboren te Vilvoorde (België). In 1996 behaalde zij het Gymnasium diploma aan het Stedelijk Gymnasium te Apeldoorn. Vervolgens ging zij Medische Biologie studeren aan de Universiteit van Amsterdam. Tijdens haar studie liep zij stage bij Prof. Dr. Westerveld/ Dr. R. Versteeg op de afdeling Antropogenetica van het Academische Medisch Centrum te Amsterdam en bij Prof. Dr. Maarten van Lohuizen op de afdeling Moleculaire Carcinogenese van het Nederlands Kanker Instituut te Amsterdam. In oktober 2000 behaalde zij haar doctoraal examen Medische Biologie cum laude. Van september 2000 tot december 2004 was zij werkzaam als onderzoeker in opleiding binnen de afdelingen Moleculaire Carcinogenese en Moleculaire Genetica van het Nederlands Kanker Instituut bij Prof. Dr. M. Van Lohuizen op een door een Pioniersbeurs (NWO) gefinancierd project.

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Allereerst mijn promotor Maarten. Sinds ik begon op het NKI als student zijn er een hoop dingen veranderd. Ten eerste zijn we verhuisd van H2 naar H5, waar jij baas van de afdeling bent geworden. Ten tweede ben je in de tussentijd buitengewoon hoogleraar geworden aan de Universiteit Utrecht. Ik beschouw het als een eer dat ik jouw eerste promovenda mag zijn. Ik heb het altijd als bijzonder prettig ervaren dat je mij zelfstandig en naar eigen inzicht liet werken. Ook ben ik je dankbaar voor de vele samenwerkingsverbanden die je op touw hebt gezet, zowel binnen als buiten het lab. Uiteindelijk waren dat toch in mijn ogen de meest waardevolle ervaringen, zowel omdat ik nieuwe proeven leerde als omdat ik eens iemand anders had om mee van gedachten te wisselen. Ik wens jou heel veel succes toe met je diversiteit aan projecten. Ik hoop dat de ingeslagen weg richting het stamcelveld een succesvolle zal zijn.

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