In vivo characterization of the lysine-methyltransferases Set7/9 and G9a by conditional mutagenesis in the mouse

by

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Abstract

Increasing evidence suggests that site-specific lysine methylation of histone and non-histone proteins is fundamentally involved in epigenetic regulation of gene expression during cellular differentiation and tumorigenesis. To study the in vivo relevance of the lysine methyltransferases Set7/9 and G9a, I have generated and characterized two conditional knockout mouse strains.

Despite its widely proposed role in transcriptional activation through the methylation of histone H3 lysine 4 (H3K4) and a number of transcription factors, I found that Set7/9 knockout mice develop normally and do not display any overt phenotypic alteration. Since Set7/9 was shown to methylate the tumor suppressor protein p53 and was suggested to be important for its activity, I mainly focused my characterization of the Set7/9 knockout strain towards the proposed impairment of p53 function in these mice. Contrary to all reports, I found that in the absence of Set7/9, the p53 target genes p21\textsuperscript{WAF1/CIP1}, Mdm2, Puma and Bax are normally expressed under basal and stressed conditions in different cell types. As a consequence, no functional p53 impairment was detectable upon DNA damage or in response to ectopic oncogene expression in Set7/9\textsuperscript{-/-} cells. Hence, my data demonstrates that Set7/9-mediated methylation of p53 represents, if at all, only a minor event in its regulation and does not appreciably control p53 activity in vivo.

In the generated conditional G9a knockout strain, I primarily focused my efforts towards describing its role in the hematopoietic system. Mice that conditionally lack G9a expression in the blood, develop normally and can sustain the development of all hematopoietic cell types under homeostatic conditions. Interestingly however, when performing competitive bone marrow transplantation assays, I detected a marked impairment in G9a knockout bone marrow cells in the reconstitution of the hematopoietic system. Consistently, G9a-deficient myeloid and erythroid progenitors are dramatically reduced in their proliferation capacity. My experiments indicate for the first time, that G9a is specifically important for the biology of hematopoietic stem and progenitor cells under stress conditions and its inactivation might represent a promising way to interfere with blood development in pathological and regenerative settings.
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1. General Introduction

1.1 Chromatin biology

1.1.1 Chromatin structure

In eukaryotes, DNA is physiologically organized into a tightly compacted DNA-protein complex called chromatin (Kornberg, 1974). The regulation of this compaction is accomplished by highly evolutionarily conserved mechanisms.

The fundamental unit of chromatin is the nucleosome, comprised of 147 bp of the DNA double strand wrapped around two of each of the core histones H2A, H2B, H3 and H4 (Luger et al., 1997). Beyond its nucleosomal organization, chromatin can be found in different degrees of higher-order structures (Zlatanova et al., 1999). Microscopically, it is possible to differentiate between densely packed heterochromatin and more lightly packed euchromatin within the cell nucleus (Heitz, 1928).

Constitutive heterochromatin occupies centromeric and telomeric chromosomal regions (Blasco, 2007). Its structure is important for chromosome function and facilitates the faithful perpetuation of the genetic information between cellular and organismic generations (Peng and Karpen, 2008). It is transcriptionally mostly inactive and devoid of protein coding genes (Huisinga et al., 2006). The profile of constitutive heterochromatin varies little between different cell-types.

On the contrary, facultative heterochromatin is created in a cell-type-specific manner and involves the epigenetic silencing of single genes or whole chromosomal regions (Grewal and Jia, 2007; Trojer and Reinberg, 2007). The most extreme case of facultative heterochromatin formation is the inactivation of one of the two X-chromosomes in female mammals (Payer and Lee, 2008). Paradigms of domain-wide facultative heterochromatin formation are the mating type locus in yeast (Grewal and Moazed, 2003) and the β-globin locus in vertebrates (Litt et al., 2001).

Unlike heterochromatin, euchromatin comprises more loosely organized areas in the genome and harbors (potentially) transcribed genes. Current models suggest that the open configuration of euchromatin is caused by the concerted recruitment of
transcriptional activators to gene regulatory elements and thus favors transcriptionally poised or active chromatin states (Li et al., 2007).

1.1.2 Chromatin modifications and their cross-regulation

The dynamics involved in DNA-associated processes such as transcription (Li et al., 2007), replication and repair (Groth et al., 2007) are regulated by post-translational modifications deposited on histones or the DNA itself. Histone modifications are biochemically very diverse and can decorate serine, lysine and arginine residues in the N-terminal tails of histones H3 and H4 and in the core regions of histone H2A and H2B. They include serine or threonine phosphorylation, acetylation, methylation, ubiquitination, sumoylation of lysines and methylation of arginines (Kouzarides, 2007). Eukaryotic DNA methylation occurs exclusively on cytosine bases (Bestor, 2000). Illustration 1 gives an overview about post-translational modifications that occur on histones H3 and H4.

The site-specific nature and diversity of histone modifications have provoked the postulation of a “histone code hypothesis” by Turner and Jenuwein/Allis in 2000 (Jenuwein and Allis, 2001; Turner, 2000). This hypothesis states that “a ‘histone code’ exists, that may considerably extend the information potential of the genetic (DNA) code” (Jenuwein and Allis, 2001). However, the use of the word ‘code’ in the context of histone modifications should not be equated to the genetic DNA code. Rather, it appears that chromatin modifications follow general rules of cellular signaling also found in non-chromatin associated signaling pathways (Sims and Reinberg, 2008).

Among all chromatin modifications that are deposited synergistically or mutually exclusively to each other, the site-specific methylation of histone lysines has emerged as a fundamental framework for this abundant regulatory crosstalk. A wide variety of enzymes can catalyze the deposition (Jenuwein, 2001) or removal (Klose et al., 2006) of methyl groups on lysines. Moreover, several functional protein domains have been shown to harbor specific binding properties that depend on the methylation status of lysines (Taverna et al., 2007). Under the additional consideration that lysines can be mono, di or trimethylated, a great number of possible combinations equips the cell for
the establishment of numerous specialized chromatin states (Illustration 2). One example for the cross-regulation of histone lysine methylation states is that repressive DNA methylation can be locally directed by the presence of histone H3 lysine 9 trimethylation (H3K9me3) in an evolutionarily conserved pathway (Lehnertz et al., 2003). Conversely, H3K9me2/3 exclude H3K9 acetylation on the same or on adjacent nucleosomes (Peters et al., 2001). Furthermore, ubiquitination of H2A at lysine 119 (H2A119ub) is an upstream signaling event for H3 lysine 27 trimethylation (H3K27me3) (Wang et al., 2004) and H3K4 methylation antagonizes DNA methylation by interfering with the recruitment of DNA methyltransferases to the N-terminus of histone H3 (Ooi et al., 2007).

Another overall concept that transpired from our increasing knowledge of histone methylation is that there appears to be a general association of specific methylation states to either active or inactive chromatin configurations. This concept is currently growing with the additional insight that is gained by the use of novel high-throughput methods which enable us to determine and compare patterns of histone methylation states across entire genomes (Bernstein et al., 2007).

On a genomic scale, it appears that constitutive heterochromatin at centromeres and telomeres is uniformly marked with H3K9me3 and H4K20me3 (Benetti et al., 2007; Garcia-Cao et al., 2004; Peters et al., 2003; Schotta et al., 2004) whereas histone acetylation is underrepresented. Outside centromeric and telomeric regions, areas that harbor repeat regions such as endogenous retroviral elements are similarly marked with H3K9me2/3 and H4K20me3 (Barski et al., 2007). Gene poor regions exhibit low and uniform levels of H3K9me2/3. By contrast, euchromatic regions of regulated transcription display a far more complex distribution of histone methylation marks. Actively transcribed genes are typically demarcated with promoter associated peaks of H3K4me3 and to some extent also H3K4me1 and H3K4me2. H3K79me3, H3K36me3 and H3K9me1 usually appear in their active gene bodies, whereas silent genes are generally embedded in large domains of H3K9me2, H3K27me2 or H3K27me3 (Barski et al., 2007) (summarized in Illustration 3).
**Illustration 1: Histone Modifications.** Overview of histone modifications occurring on histones H3 and H4. The N-terminal tails of histone H3 and H4 protrude from the nucleosomal core and are subject to arginine methylation, lysine methylation, serine or threonine phosphorylation and lysine acetylation. To convey a special focus on histone lysine methylation, methylated lysines are numbered and include H3K4, H3K9, H3K27, H3K36, H3K79, and H4K20. Lysine residues can be mono-, di- or trimethylated.

**Illustration 2: Biochemistry of posttranslational lysine methylation.** The ε-amino group of lysine residues can accommodate one, two or three methyl groups, always retaining a positive charge. The modification can be enzymatically deposited by different lysine methyltransferases (KMTs) or removed by lysine demethylases (KDMs) in a manner that is dependent on the sequence context and the degree of methylation.
**Illustration 3: The localized association of different histone methylation states.** (a) On a chromosomal level, H3K9me3 and H4K20me3 demarcate constitutive heterochromatin at centromeres and telomeres. Within euchromatic regions repeat regions are enriched in H3K9me2/3 and H4K20me3 whereas gene poor regions are marked with low levels of H3K9me2/3 (b). Protein coding genes within genic regions are differentially marked with H3K4me3, H3K9me1, H3K36me3, H3K79me2, H3K27me1 for active and with H3K27me2/3 and H3K9me2 for inactive genes.
1.1.3 Enzymes of lysine methylation

The enzymatic activities that are responsible for the deposition and removal of histone lysine methylation are known to a great extent. The catalytic domain of all histone lysine methyltransferases (with one known exception so far, Dot1L (van Leeuwen et al., 2002)) is the SET domain, a highly conserved motif originally found in the modifier of position effect variegation (PEV) SU(VAR)3-9, the polycomb-group protein enhancer of zeste \( E(z) \) and the trithorax-group protein trithorax \( TRX \) (Jenuwein, 2001). On the other hand, lysine demethylases can fall into either the amino oxidase family (represented by Lsd1) or are characterized by the JmjC domain. Importantly, members of the amino oxidase family are at most capable to revert dimethylation states, whereas JmjC proteins can often but not always catalyze the removal of all three methylation states (Klose and Zhang, 2007).

It is commonly observed that two or more histone methylation activities occupy the same protein complexes and fulfill complementary roles. For example, the JmjC group H3K4me2/3-specific demethylase RBP2/Jarid1a interacts with the H3K27 trimethylase Ezh2 and the polycomb repressive complex 2 (PRC2) to facilitate polycomb repression by the concerted removal of the active H3K4me2/3 mark and the deposition of repressive H3K27me3 mark (Pasini et al., 2008).

Multiple sequence alignment analysis of SET domain-containing genes reveals the presence of approximately 50 of those genes in mammalian genomes (Sun et al., 2008). Many of these SET domain genes are evolutionarily conserved and can be categorized into 10 subclasses based on the sequence similarities in their SET domains. Several SET domain genes have been characterized biochemically and/or genetically. Table 1 summarizes the evolutionary relationships between all known SET domain genes from yeast to mammals based on their SET domain homology (Sun et al., 2008). Interestingly, a few SET domain genes such as \( Set1 \) and \( Set2 \) have very ancient ancestors that can be found in yeast, worm and the fly. Others, like \( Set7/9 \) seem to have emerged relatively late in evolution and can only be found in vertebrate genomes. A common feature in SET domain gene evolution is also the duplication of
specific genes during the transition to vertebrates as is the case with Suv39h1/2, Ezh1/2 and Set1a/Set1b.

Table 1 (next page): SET domain gene overview. The evolution of SET domain genes and their classification into 10 subfamilies based on sequence similarities of SET domains. Orthologous relationships are indicated from yeast to mouse. Note the presence of highly evolutionary conserved groups such as Set1 and Set2 in groups V and III, respectively. In contrast, Set7/9 is only mildly conserved and has no close homologue in mammals and only a single orthologue in other vertebrate genomes (here D. rerio). G9a and its close homologue Glp fall into the first subclass and are closely related to the Suv39h and Setdb groups.

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- **H3K9**: Histone 3 lysine 9 acetylation
- **H3K4**: Histone 3 lysine 4 acetylation

Note: The table represents different sets of genes and their acetylation patterns across various species, with MT activity indicating the type of acetylation.
1.1.4 Chromatin regulation and its relevance for development

Different cell types in our body are defined by distinct gene expression profiles. Contemporary models of development suggest that during the differentiation of stem cells into more specialized cell types, gene expression becomes increasingly restricted and stabilized (Miyamoto et al., 2002; Ye et al., 2003). Recent advances in the understanding of chromatin regulation have strengthened the hypothesis that histone modifying activities are fundamentally involved in the generation of transcription patterns among different cell types (Bracken et al., 2006; Mikkelsen et al., 2007). On a functional level however, it remains largely unclear to which extent the chromatin modifying activities per se are responsible for developmental gene expression programming.

The best studied chromatin regulators that have functional implications for development are the activities of trithorax and polycomb complexes (Schwartz and Pirrotta, 2008). Whereas trithorax-group proteins are generally involved in gene activation, polycomb-group proteins are responsible for gene repression of developmental patterning genes. It has become clear that both activities significantly rely on their methyltransferase activities towards H3K4 (Milne et al., 2002) and H3K27 (Cao et al., 2002; Czermin et al., 2002; Kuzmichev et al., 2002), respectively to exert their biological functions. When chromatin dynamics in differentiating ES cells were studied, it was found that a subset of genes is dually occupied by H3K4me3 and H3K27me3 and are kept poised for activation in stem cells (Bernstein et al., 2006). Upon differentiation, these bivalent chromatin domains are resolved into either H3K4me3 and active or H3K27me3 and repressed genes. Consistent with this notion, ES cells that are deficient in the H3K27 tri methyltransferase Ezh2 are impaired in their mesoendodermal differentiation potential (Shen et al., 2008). This implies a profound requirement of H3K4me3 and H3K27me3 methylation in the transcriptional patterning during cellular differentiation in mammals.

The role of H3K9me2 methylation in developmental gene repression appears different from that H3K27me3 methylation. The genome wide analysis of H3K9me2 revealed that in contrast to ES cells, differentiated cells exhibit large genomic blocks of
this modification that are mostly mediated by the methyltransferase G9a (Wen et al., 2009). Interestingly, there seems to be an overlap between H3K9me2 and H3K27me2 but not H3K27me3 enriched regions (Barski et al., 2007). G9a/− ES cells are deficient in the sustained silencing of pluripotency genes and as a result fail to undergo complete differentiation in vitro (Feldman et al., 2006; Epsztejn-Litman et al., 2008), indicating a physiological relevance for H3K9me2 in the regulation of developmental genes.

1.1.5 Phenotypes of SET domain knockout mouse models

In the last nine years, several studies have reported the targeted deletion of SET domain genes in the mouse. Interestingly, a wide variety of phenotypes were observed, suggesting the involvement of SET domain mediated lysine methylation in different aspects of chromatin biology. Based on the current status of research, the correlation between the evolutionary age of SET domain genes and their phenotypes in the mouse remains mostly obscure. Only in relatively few cases, the predictions of phenotypes in the mouse based on their known function on the cellular level were confirmed. For example, Suv39h1/2 plays a role in the regulation of constitutive heterochromatin, and mice lacking those enzymes display genomic instabilities. Further, since the mammalian trx homologue Mll is involved in Hox gene regulation, Mll/− animals exhibit multiple developmental perturbations and die during embryogenesis. Moreover, Setdb1 and Set8 are both required for cell viability and proliferation, as a result their homozygous knockouts display very early embryonic phenotypes. Although in many cases embryonic phenotypes were observed, they could not always be attributed to specific developmental mechanisms. It is also important to consider that, apart from those published reports, SET domain knockouts without an overt phenotype are likely underrepresented in the literature. Table 2 gives an overview about the so far published targeted mutations of murine SET domain genes and their observed phenotypes.
Table 2: Phenotypes of targeted SET domain mutations in the mouse. The table summarizes the SET domain genes with reported phenotypes in the mouse. The altered phenotypes occur at different developmental stages and affect different aspects of chromatin biology like replication (Set8), DNA repair and genomic rearrangements (Suv4-20h1/2), chromosome function (Suv39h1/2), development (Ezh2 and Mll) and tumor suppression (Riz1 and Set7/9). In many cases, SET domain deficiencies preclude embryonic development (grey fields). In cases where tissue-specific Cre-transgenics were used to study phenotypes in the adult, the respective transgenics are included in brackets.

<table>
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<th>HMT activity</th>
<th>embryonic phenotype?</th>
<th>adult phenotype?</th>
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<td><em>Suv39h1/2</em></td>
<td>H3K9me3</td>
<td>no</td>
<td>genomic instability</td>
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<td><em>G9a</em></td>
<td>H3K9me1/2, G9a, mAM</td>
<td>8.5 dpc</td>
<td>slight impairment in Igλ use in B-cells (CD19-Cre), male infertility (TNAP-Cre)</td>
<td>(Tachibana et al., 2007; Tachibana et al., 2002; Thomas et al., 2008)</td>
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<td><em>Glp</em></td>
<td>H3K9me1/2</td>
<td>8.5 dpc</td>
<td>?</td>
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<td><em>Setdb1</em></td>
<td>H3K9me2/3</td>
<td>at implantation</td>
<td>cells not viable</td>
<td>(Dodge et al., 2004)</td>
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<td><em>Ezh2</em></td>
<td>H3K27me1/2/3, H1</td>
<td>post implantation</td>
<td>multiple developmental</td>
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<td><em>Mll</em></td>
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<td>no</td>
<td>HSC defect</td>
<td>(Heuser et al., 2009; Madan et al., 2009; Zhang et al., 2009)</td>
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<td><em>Set8</em></td>
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<td>DNA repair, Igh rearrangement (Vav-Cre)</td>
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<td><em>Riz1</em></td>
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<td><em>Set7/9</em></td>
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<td>impairment in p53 function</td>
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1.2 Hematopoiesis

To assess potential roles for Set7/9 and G9a in cellular development, I primarily focused my efforts to the hematopoietic system. The exact reasons for this choice are discussed in more detail in my rationale section. Briefly however, the most relevant advantages of the hematopoietic system for this study are its relatively easy manipulatibility and the vast existing literature that has described the roles of transcription factors in hematopoietic lineage determination and stem cell biology.

1.2.1 General mechanisms of mammalian hematopoiesis

In vertebrates, hematopoiesis is organized as a hierarchical system in which self-renewing stem cells give rise to a number of transiently amplifying progenitor populations and terminally differentiated blood cell types (Orkin and Zon, 2008). The most referred-to model of hematopoietic development relies on the prospective isolation of hematopoietic stem cells and lineage restricted progenitor populations by Akashi, Kondo and Weissman (Akashi et al., 2000; Kondo et al., 1997). According to the “Akashi-Kondo-Weissman Scheme”, hematopoietic differentiation takes place at several binary decision making points (Illustration 4). In brief, multipotent progenitors can differentiate into either common myeloid or lymphoid progenitors, which in turn give rise to granulo-monocytic or megakaryocytic/erythrocyte progenitors in the myeloid and T, NK and B-cell progenitor in the lymphoid compartments. Although this model is oversimplified and controversial in some details, it is still an appropriate conceptualization for most hematopoietic differentiation mechanisms.
Illustration 4: Hematopoietic differentiation scheme according to Akashi and Weissman. During hematopoiesis, self-renewing and long-term reconstituting HSCs (LT-HSCs) give rise to transient short-term HSCs (ST-HSCs). ST-HSCs produce common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs). CLPs are the source of committed precursors of B and T lymphocytes, whereas CMPs give rise to megakaryocyte/erythroid progenitors (MEPs) and granulocyte/macrophage progenitors (GMPs). GMPs give rise to the committed precursors of mast cells, eosinophils, neutrophils, and macrophages.
1.2.2 Lineage specification in the hematopoietic system

Lineage specification in the hematopoietic system relies on the complex interplay between environmental and cell-autonomous mechanisms (Orkin, 2000). Since sites of hematopoiesis are physically compartmentalized, it is undisputed that different hematopoietic organs offer very distinct environments that can drive the differentiation of specific lineages. For example, the release of interleukin 7 (IL-7) by thymic epithelial cells is a critical requirement for the development of T-cells (von Freeden-Jeffry et al., 1995). Conversely, deletion of the IL-7 receptor in the mouse prevents the survival of lymphoid progenitors and prevents thymopoiesis (Peschon et al., 1994).

To our current knowledge, most cell intrinsic mechanisms of lineage specification can be attributed to the combinatorial expression of a number of critical transcription factors (Laiosa et al., 2006). It has become apparent that some transcription factors possess very discrete expression profiles among hematopoietic lineages. With this insight and the advent of techniques that allow the targeted genetic deletion or ectopic expression of transcription factors in the mouse (Rajewsky et al., 1996), an ample number of studies have established the instructive roles of several transcription factors in hematopoiesis (Table 3). Common themes that emerge from those studies are that (a) genes that are important for the function of distinct cell types (e.g. homing or chemokine receptors) are activated by these transcription factors, (b) lineage promiscuous gene expression is prevented by transcription factor mediated gene repression (Schebesta et al., 2002), (c) they are functionally engaged in transcriptional networks with abundant positive and negative feedback mechanisms (Cantor and Orkin, 2001), (d) their genetic deletion or over-expression profoundly impacts the development, survival or maturation of one or more hematopoietic lineages.
**Table 3: Transcriptional regulators of hematopoietic lineage specification.** Overview of transcription factors that are crucial for the development of specific hematopoietic lineages. Indicated are the observed phenotypes after the germ-line or lineage-specific disruption of the respective transcription factor. The altered phenotypes were either a complete loss of a lineage (lack), a maturational block (matur), a functional defect (func) or decreased numbers of lineage cells (decr). Lineage abbreviations are: hematopoietic stem cells (HSC), B-cells (B), T-cells (T), natural killer cells (NK), granulo-monocytic cells (GM), megakaryocyte and erythroid cells (MegE).

*Adapted from Laiosa CV, Stadtfeld M, Graf T: Determinants of lymphoid-myeloid lineage diversification, Annual review of immunology, 2006 vol. 24 pp. 705-38 (Laiosa et al., 2006).*

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<td>lack/matur</td>
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1.2.3 The biology of hematopoietic stem cells

To safeguard the sustained production of hematopoietic cells during development and throughout adulthood, hematopoietic stem cells must retain their multipotency and ability to self-renew under various physiological conditions (Akala and Clarke, 2006). This is accomplished by numerous mechanisms that, similar to the events that regulate lineage specification, can be categorized into environmental and cell-intrinsic activities.

Sites that harbor the appropriate combinations of environmental factors to support the emergence and maintenance of HSCs are referred to as stem cell niches. The locations of HSC niches are specific to different developmental stages and encompass the yolk sac (8.5-11.5 dpc), aorta-gonad-mesonephros region (AGM, 9.5-11 dpc), placenta (10.5-12.5 dpc), the fetal liver (10.5 dpc until birth) and eventually the bone marrow in the adult (Mikkola and Orkin, 2006). It is thought but not undisputed that in the adult bone marrow, self-renewing HSCs reside in close proximity to osteoblasts and receive crucial signaling stimuli from these cells and their extra cellular matrix (Adams and Scadden, 2006). Defined molecular components that initiate signaling pathways important for the self-renewal of HSCs are Wnt, Notch (Duncan et al., 2005) and Shh factors.

Most of the currently known intrinsic factors of HSC function are represented by classical transcription factors or chromatin regulators which are involved in the orchestration of stem cell specific transcription programmes. While the exact nature of an HSC specific transcription network (in analogy to that of a pluripotency network in ES cells (Kim et al., 2008a)) remains unidentified, it has emerged that fundamental requirements for HSC activity, that is cell cycle regulation, survival and the expression of developmentally relevant genes such as Hox transcription factors are integrated by the activities of such factors. Table 4 gives an overview on intrinsic regulators of HSC function whose activities were largely determined by the use of conventional or tissue-specific knockout mouse models.
Table 4: Intrinsic regulators of HSC activity. Overview of factors that are crucial for the biology of hematopoietic stem cells. Indicated are the observed phenotypes after the germ-line or lineage-specific disruption of the respective transcription factor. The altered phenotypes occur at different developmental stages and affect different aspects of HSCs.

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<thead>
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<th>Factor</th>
<th>hematopoietic function</th>
<th>references</th>
</tr>
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<tbody>
<tr>
<td>Scl/tal1</td>
<td>HSC specification during embryogenesis, no requirement in the adult</td>
<td>(Porcher et al., 1996; Robb et al., 1996; Robb et al., 1995)</td>
</tr>
<tr>
<td>Sox17</td>
<td></td>
<td>(Kim et al., 2007)</td>
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<tr>
<td>Runx1/AML1</td>
<td></td>
<td>(Chen et al., 2009)</td>
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<tr>
<td>Tel/Etv6</td>
<td></td>
<td>(Hock et al., 2004b)</td>
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<tr>
<td>MCL-1</td>
<td></td>
<td>(Opferman et al., 2005)</td>
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<tr>
<td>Mll</td>
<td>HSC self-renewal, required for development</td>
<td>(Ernst et al., 2004a; Hess et al., 1997; Yagi et al., 1998)</td>
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<td>Evi-1/PRDM3</td>
<td></td>
<td>(Goyama et al., 2008)</td>
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<td>PRDM16</td>
<td></td>
<td>(Seale et al., 2008)</td>
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<tr>
<td>Gfi-1</td>
<td>HSC quiescence, not required for development</td>
<td>(Hock et al., 2004a)</td>
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<td>p53</td>
<td></td>
<td>(Liu et al., 2009)</td>
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<td>p21WAF1/CIP1</td>
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<td>(Cheng et al., 2000)</td>
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<tr>
<td>Bmi-1</td>
<td></td>
<td>(Lessard and Sauvageau, 2003; Park et al., 2003)</td>
</tr>
<tr>
<td>Mll5</td>
<td>HSC proliferation, maintenance, not required for development but has a role in the adult</td>
<td>(Heuser et al., 2009; Madan et al., 2008; Zhang et al., 2009)</td>
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<td>HoxB4</td>
<td></td>
<td>(Antonchuk et al., 2002; Thorsteinsdottir et al., 1999)</td>
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<td>ATM</td>
<td>HSC protection against oxidative stress in the adult, not required for development</td>
<td>(Ito et al., 2004)</td>
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<tr>
<td>FoxO1,3,4</td>
<td></td>
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1.2.4 The role of chromatin regulation in hematopoiesis

As discussed before, chromatin regulation has been increasingly implicated in the regulation of developmental processes. However, most of our current knowledge in this context originates from studies that focus on developmental mechanisms during ES cell differentiation. It is currently assumed that many of the chromatin dynamics that happen upon ES cell differentiation, can be transferred to the differentiation of adult stem cells such as hematopoietic stem cells. For example, it was observed that during erythroid differentiation, a number of developmental genes undergo a transition from inactive and H3K4 unmethylated over transcriptionally poised H3K4me2 to active H3K4me3 at their transcription start sites (Orford et al., 2008a). Some evidence also exists that bivalent, H3K4me3 and H3K27me3 marked genes are poised in HSCs and are either repressed or activated at later developmental stages (Cui et al., 2009). Furthermore, the hematopoietic transcription factors Gfi-1 and Gfi-1b partially exert their instructive roles during development by the recruitment and activities of the lysine demethylase Lsd1, HDACs and possibly G9a suggesting an involvement of repressive chromatin mechanisms in the specification of developmental transcription programmes (Saleque et al., 2007).

In the biology of hematopoietic stem cells, the two respective members of the trithorax and polycomb group genes, \textit{Mll} and \textit{Bmi-1}, were shown to be involved in leukemogenesis and HSC function. \textit{Mll} (\textit{multi lineage leukemia}) was originally identified as a common chromosomal translocation partner found in some subtypes of acute lymphocytic leukemia (ALL) and acute myelogenous leukemia (AML) (Ernst et al., 2002). \textit{Mll} is the mammalian homologue of the Drosophila trithorax gene and bears a SET domain with an H3K4 specific methyltransferase activity (Milne et al., 2002). Disruption of the \textit{Mll} gene in the mouse has revealed its role in the positive regulation of class I \textit{Hox} genes (Ernst et al., 2004b). On a functional level, Mll was shown to be important for HSC and progenitor function at different hematopoietic stages in the developing embryo (Hess et al., 1997; Yagi et al., 1998).

Another important paradigm in the modulation of HSC activity is the polycomb group member Bmi-1 which is part of the polycomb repressive complex 1 (PRC1) and
regulates the proliferative potential of adult HSCs (Lessard and Sauvageau, 2003; Park et al., 2003) and neural stem cells (Molofsky et al., 2003). Bmi1−/− mice are viable at birth but display a number of developmental abnormalities (van der Lugt et al., 1994). The most profound phenotype in Bmi1−/− mice is their marked impairment in maintaining adult hematopoiesis. This leads to their premature death at around 3-20 weeks of age due to apparent anemias and immunological defects. It was shown that although the number of HSCs in Bmi−/− animals was normal, their proliferative potential and ability to reconstitute transplanted recipients was greatly reduced. Interestingly, Bmi-1 was also necessary for the proliferation of leukemic stem cells and its abrogation resulted in the protection from the disease (Lessard and Sauvageau, 2003). On a molecular level, it was shown that Bmi-1, along with other polycomb activities occupies the INK4A-ARF locus, and thereby prevents its transcription and activation of pRB and p53 pathways (Bracken et al., 2007). This finding provided a model for the explanation of increased senescence and apoptosis as a consequence of the derepressed expression of p16INK4A and p19ARF in Bmi−/− HSCs.

Furthermore, two members of the PR-domain family, a subfamily of SET domain genes (see Table 1) have recently been shown to be crucial for the biology of hematopoietic stem cells. PRDM3, better known as Evi-1 (ecotropic viral integration site-1), has been identified as a common integration-site in retrovirus-induced leukemogenesis (Mucenski et al., 1988), suggesting its overexpression might confer growth activation in early hematopoietic cells. Evi-1 knockout HSCs are severely impaired in their repopulation ability and fail to maintain hematopoiesis during multiple developmental stages (Goyama et al., 2008). A similar stem cell phenotype has recently been reported for PRDM16 (Sean Morrison, Keystone meeting 2009), (Deneault et al., 2009). In both cases though, no catalytic activity for either PRDM3 or PRDM16 have been described yet, allowing no further speculation on their molecular modes of action at this point.

Importantly, although much has been hypothesized about the interplay of chromatin modifiers with hematopoietic transcription factors, no example of a chromatin regulator being specifically involved in the developmental segregation of different blood lineages (in analogy to those summarized in Table 2) is currently known.
1.3 General rationale

The identification of the first histone lysine methyltransferase in 2000 (Rea et al., 2000), has enabled us to biochemically characterize the large variety of different chromatin states and as a result has attracted enormous attention to the field of chromatin biology. At the same time, the SET domain became a signature for numerous, until then only marginally recognized chromatin regulators and predicted their enzymatic activities as lysine-methyltransferases. As a result, a large body of literature describing the catalytic and cell-biological properties of SET domain proteins became available in the years after 2000. As expected, many of these early publications were purely based on cell-free and *in vitro* studies and could only hypothesize about the relevance of histone lysine methylation in the context of the complex gene regulation that is basis of multi-cellular development.

In 2003, when I started my work for this thesis, our knowledge of SET domain biology was defined by a relatively small number of crucial points of information: The original founding members of the SET domain family in the fly, Su(var)3-9, E(z) and trx, and their mammalian homologues were assigned to repressive H3K9 methylation at constitutive heterochromatin regions (Peters et al., 2001) and the polycomb/trithorax-dependent regulation of repressive H3K27 (Cao et al., 2002; Czermin et al., 2002; Kuzmichev et al., 2002) and H3K4 methylation states (Milne et al., 2002), respectively. Traditionally and owing to extensive work in Drosophila, it was known that the two latter activities had profound implications for the regulation of gene expression during development. However, at the time it was also suggested that H3K9-methylation, known to be catalyzed by the Suv39h homologues (Rea et al., 2000), G9a (Tachibana et al., 2002) and Setdb1 (Schultz et al., 2002) was important for gene repression in many cellular contexts.

A long-standing discussion in the field of development in general but in hematopoiesis specifically is based on the notion that upon lineage-commitment, the gene expression profiles of different emerging cell-types become increasingly specialized. This process had been found to entail the activation of a lineage-specific genes and concomitantly, the permanent repression of lineage-promiscuous genes.
(Busslinger, 2004). The functions of different instructive transcription factors in hematopoietic lineage determination had been mapped to a great extent (see Table 2). By contrast, little was known about the chromatin dynamics that accompany the epigenetic diversification of transcription programmes during development.

Being fascinated by the mechanisms underlying lineage-specification and chromatin regulation, I decided to explore the role of novel epigenetic regulators in the well-defined system of hematopoiesis. My decision to study Set7/9 and G9a was influenced by a number of considerations. First, the primary candidates for SET domain genes involved in development, namely Ezh1 and Ezh2, crucial for H3K27 methylation and polycomb repression as well as the activating trithorax H3K4 methylase Mll had already been knocked out in the mouse (O’Carroll et al., 2001; Su et al., 2003; Yu et al., 1995). Second, the Suv39h1/2 H3K9-methylases had also been knocked out but did not yield a bona fide developmental phenotype (Peters et al., 2001a). Third, G9a was shown to be important for euchromatic gene repression, had been knocked out in a conventional targeting approach and revealed a role for development in early embryogenesis (Tachibana et al., 2002). This finding was a strong indication that G9a could be involved in lineage-promiscuous gene repression at later stages of development, in particular hematopoiesis, and inspired me to study its role during hematopoietic development. Since at the time, Set7/9 was known to be the only other H3K4-specific methyltransferase apart from Mll (Nishioka et al., 2002; Wang et al., 2001), I was inspired to additionally generate a conditional knockout for Set7/9. It was my overall aim to investigate two complementary aspects of global gene regulation by histone methylation, namely transcriptional repression and activation in an organismal context. To accomplish this task, I started a genetic approach to functionally assess the suggested activities of Set7/9 and G9a in vivo.
2. Set7/9

2.1 Introduction

2.1.1 Set7/9 is a lysine methyltransferase with a broad enzymatic specificity and targets p53

Set7/9 was first identified in a biochemical purification protocol to isolate histone methyltransferase activities towards a 20meric N-terminal H3 peptide in HeLa cells (Nishioka et al., 2002; Wang et al., 2001). Based on its observed activity towards H3K4, a methylation that precludes the binding of repressive chromatin regulators, it was suggested that Set7/9 plays a role during transcriptional activation (Nishioka et al., 2002). Interestingly however, upon its further enzymatic characterization it was found that the initially observed H3K4 methylation activity was significantly weakened or not detectable towards more native substrates such as recombinant histone octamers or nucleosomes (Chuikov et al., 2004). This either suggested that Set7/9 is dependent on essential cofactors that stimulate its activity in vivo, that Set7/9 can only methylate histone H3 before its incorporation into nucleosomes or that the H3K4 methyltransferase activity does not reflect its genuine physiological function.

Given that H3K4 appears not to be methylated prior to its incorporation (Loyola et al., 2006), the latter notion seems most likely and was supported by several studies which found that Set7/9 possesses strong enzymatic mono-methyltransferase activities towards a number of different transcription-associated proteins (Chuikov et al., 2004; Couture et al., 2006; Kouskouti et al., 2004; Subramanian et al., 2008). A general theme that transpired from these studies is that Set7/9-mediated methylation of transcription factors regulates their stability and transcriptionally activating properties by modulating protein-protein interactions and other downstream post-translational modifications (Illustration 5).

For example, it was found that Set7/9 can methylate the basic transcription factor Taf10, a component of the TFIID complex, at lysine 189 (K189) (Kouskouti et al.,
Although the biological activity of Taf10 was not entirely dependent on the methylation of K189, it was suggested that K189me potentiates the transcription rates of a subset of genes by increasing the recruitment of RNA polymerase II (PolII). In particular, genes that were activated in the presence of retinoic acid (RA) and cyclic AMP (cAMP) were responsive to K189me1. A potentially similar functional scenario was implied after the Set7/9-mediated methylation of Taf7, another component of TFIIID was found (Couture et al., 2006). In a similar study it was reported that Set7/9 also methylates Estrogen Receptor alpha (ERα), a nuclear receptor that exhibits a ligand-induced transcriptional activity and is involved in the execution of a female-specific gene expression program (Subramanian et al., 2008). Its methylation at lysine 302 was implicated in the ERα protein stability and the transactivation of ERα target genes upon exposure to estradiol in vitro. A further recently reported methylation substrate of Set7/9 is DNA methyltransferase 1 (DNMT1), important for the maintenance of DNA methylation in embryonic and somatic cells (Jackson-Grusby et al., 2001; Li et al., 1992). While it was the actual focus of the study to characterize the observed protein instability of DNMT1 and the concomitant genomic DNA demethylation in the absence of the lysine demethylase Lsd1, it was shown that Lsd1 counteracts the Set7/9-mediated methylation of DNMT1 at lysine 1096 and thereby negatively affects DNMT1 stability (Wang et al., 2009). It can therefore be reasoned that Set7/9 should also have a regulatory effect on the stability or activity of DNMT1. However, no such functional interrelationship has been described yet.

One of the most discussed roles of Set7/9 was sparked by the finding that Set7/9 methylates the tumor suppressor p53 at lysine 372 (K372) and that this methylation was suggested to be substantially involved in its biological activity (Chuikov et al., 2004). Whereas none of the other aforementioned roles of Set7/9 mediated protein regulations have been addressed in vivo, it was recently reported that Set7/9−/− mice display a phenotype that supports this finding of p53 regulation (Kurash et al., 2008). Due to my special focus on the functional relationship between Set7/9 and p53, the next introductory section in this thesis is devoted to p53 regulation.

In summary, Set7/9 can be regarded as a general lysine methyltransferase with a minimal substrate consensus of [R/K][S/T]K* (Illustration 5a) and a relatively broad
specificity (Couture et al., 2006). Most if not all of the substrates of Set7/9 are nuclear proteins involved in the regulation of transcriptional processes. Interestingly, Set7/9 is evolutionarily very isolated (Table 1) and does not possess any close homologue. It rather appears to be a newly emerged member of the SET domain family with a wide radius of action. Generating a Set7/9 knockout mouse strain represented a promising tool to investigate its proposed role in a variety of biological processes in vivo.

Illustration 5: Enzymatic specificity and substrates of Set7/9. (a) Based on described substrates of Set7/9, a minimal consensus sequence of [R/K][S/T]K* can be deduced. (b) Schematic overview of Set7/9 substrates and the proposed biological consequences of their respective methylation.

2.1.2 The function and activation of p53

The transcription factor p53 responds to various cellular stresses (including DNA damage, expression of oncogenes, oxidative stress and metabolic limitations) and is mutated in the majority of human cancers (Vousden and Lane, 2007). In normally proliferating cells, protein levels of p53 are kept at a minimum due to its perpetual proteasomal degradation. The E3 ligase Mdm2 (and to some extent also Mdm4) represent regulatory subunits that are responsible for the proteasomal degradation of p53 through protein ubiquitination. In turn, Mdm2 expression is also transcriptionally
activated by p53, assembling a direct feedback loop that is very effective in dynamically regulating p53 protein levels (Barak et al., 1993). Upon activation, the p53-Mdm2 interaction can be interrupted by a number of alternative mechanisms which eventually results in the accumulation of p53. Well described pathways that trigger p53 stabilization are either mediated by the N-terminal phosphorylation of p53 in response to genotoxic stress or by Mdm2 sequestration through the oncogene-activated tumor suppressor p19\textsuperscript{ARF} (Illustration 6).

Illustration 6: Regulation of the p53 pathway. In normal cells, p53 is perpetually degraded by the action of Mdm2. The interruption of this degradation cycle is triggered in response to various stresses. p53 stabilization can be mediated by the kinase activities of ATM, ATR and Chk2 or by the p19\textsuperscript{ARF}-mediated sequestration of Mdm2. Upon stabilization, p53 acts mainly as a transcription factor to activate the expression of a myriad of target genes that transduce the p53-activation into a physiological outcome such as cell cycle arrest, apoptosis or DNA repair. The transcriptional activity of p53 is additionally proposed to be modulated by a number of regulators such as the indicated acetyltransferases (p300, PCAF) (Barlev et al., 2001), deacetylases (Sirt1) (Cheng et al., 2003), methyltransferases (Set7/9 (Chuikov et al., 2004), Smyd2 (Huang et al., 2006), Set8 (Shi et al., 2007)) and demethylases (Lsd1) (Huang et al., 2007).
2.1.3 The tumor-suppressive function of p53 relies on its transactivation activity

Upon stress-induced activation of p53, its physical interaction between with Mdm2 gets interrupted, p53 becomes stabilized and engages in a number of activities that, dependent on the extent and context of stress, orchestrate physiological outcomes such as cell cycle arrest, DNA repair, cellular senescence or apoptosis (Vousden and Lu, 2002).

Naturally occurring somatic mutations have provided us with extensive insight into structure-function relationships in the p53 protein. For example, the systematic mutational analysis within the N-terminal transactivation domain of p53 revealed that the conversion of the codons for leucine 22 and tryptophan 23 into codons for glutamine and serine causes the severe impairment of p53 transactivation and its ability to bind Mdm2 (Lin et al., 1994). Consistent with this observation, it was reported that the same mutation in an engineered p53 transactivation-deficient mouse strain (p53<sup>QS</sup>) results in a phenotype that has profound consequences for a selection of p53 activities. For example, p53<sup>QS</sup> mice display a broad impairment in the induction of p53 target genes with the exception of Bax (Johnson et al., 2005). Interestingly, p53<sup>QS</sup> MEFs fail to induce apoptosis in response to DNA damage, but are only partially unable to do so when subjected to serum deprivation and upon exposure to hypoxia. Whereas our contemporary view is that a substantial amount of the tumor suppressive function of p53 can be attributed to its transcriptional activity, the exact regulation of p53-dependent transcription remains controversial (Toledo and Wahl, 2006).

2.1.4 The proposed modulatory role of C-terminal lysine modifications of p53

Like all transcription factors, p53 does not work on its own but in concert with numerous transcriptional co-regulators to co-ordinate a complex gene expression network which implies a context-specific physiological response to diverse stresses (Brooks and Gu, 2003). Since p53 is such a master regulator over life and death, it requires exquisite regulation of its transcriptional activity. In this context, it has been proposed that combinatorial post-translational modifications of lysines within the regulatory C-
terminus of p53 are responsible for the modulation of its transcriptional output (Carter and Vousden, 2009) **(Illustration 7).**

Reminiscent of signaling events on histone tails, it has been suggested that site-specific acetylation and methylation in the C-terminus of p53 can either promote or counteract each other, avoiding aberrant activation of target genes in unstressed cells (Huang and Berger, 2008). Specifically, monomethylation of lysine 372 in human p53 by Set7/9 is currently considered a prerequisite for efficient p53-dependent gene induction, cell cycle arrest and apoptosis (Chuikov et al., 2004). Methylation of K372 has been proposed to facilitate acetylation of K373 and K382 (Ivanov et al., 2007) and to inhibit methylation of K370 by Smyd2 (Huang et al., 2006). Thus, Set7/9-dependent p53 methylation may be a key regulatory event in the modulation of its transactivation activity.

Importantly however, most of the data in support of this hypothesis were obtained from studies in human cancer cell lines with compromised p53 activity and thus the biological relevance of lysine modifications on p53 remains highly controversial. A number of reports that were based on the investigation of p53 mutants engineered to lack the C-terminal lysine residues and that resulted in, if at all, only very subtle phenotypes are fueling this controversy.

For example, the *in vitro* analysis of a 6KR mutant of human p53, involving the mutational conversion all six lysine residues in the C-terminus of human p53 into arginines, yielded no detectable functional difference compared to the wildtype version of p53. Only the additional mutation of K120 and K164 into arginine residues resulted in a marked impairment of p53 transactivation, suggesting a highly redundant regulation of p53 activity through lysine modifications (Tang et al., 2008). Likewise, it was shown that knock-in mice that bear arginine residues in lieu of the seven C-terminal lysine residues exhibit only minimal phenotypes (Feng et al., 2005; Krummel et al., 2005).

Despite these controversial findings, it is still conceivable that the simultaneous elimination of all lysine residues in the C-terminus of p53 could affect both positive and negative activities at the same time and hence would not be consequential for the net activity of p53. Therefore, the physiological roles of individual methylation events on p53 in primary cells are still important investigation subjects.
**Illustration 7: Domain structure of the p53 protein.** The cluster of lysine residues in the C-terminal regulatory domain is subject to methylation and acetylation and was proposed to be important for the transcriptional activity of p53. K372 in human p53 is analogous to K369 in the mouse and can be methylated by Set7/9. Methylated K372 counteracts Smyd2-mediated methylation at K370 and positively influences acetylation at K373 and K382.

**2.1.5 Hypothesis.**

Since its identification in 2002, Set7/9 has been implicated in numerous nuclear signaling events that regulate the transcriptional activation of genes in their chromatin contexts. Because of its remarkably diverse substrate specificity, it could be inferred that Set7/9 is a very important co-activator that regulates gene induction by the synchronized methylation of several transcription-associated proteins and H3K4. Intriguingly, a number of publications also reported that the Set7/9-dependent transcriptional potentiation was acting in a very gene-specific manner. This notion was compatible with the hypothesis that discrete gene expression profiles in different cell-types are not only a result of the differential expression of classical transcription factors.
but are also determined by gene-specific signaling events on the epigenetic level of
gene expression. In my early experiments in this project, I developed a lentivirus that
expressed a short hairpin RNA (shRNA) efficiently knocking down Set7/9 expression in
embryonic stem cells. Interestingly, Set7/9\textsuperscript{kd} ES cells were normal in their self-renewal
properties but exhibited very high levels of apoptosis upon differentiation (data not
shown). This pilot experiment suggested a profound requirement for Set7/9 in cellular
differentiation and was compatible with the described role of Set7/9 in modulating Taf10
activity (Kouskouti et al., 2004). In a complementary study, Taf10 knockout embryos
had been shown to die shortly after implantation (Mohan et al., 2003), a result that
prompted me to derive a conditional Set7/9 mouse strain and investigate its expected
developmental phenotype during embryogenesis and in the hematopoietic system.

It is important to note, that in the course of my studies Set7/9\textsuperscript{-/-} mice did not
display the initially expected embryonic phenotype. I therefore concluded that my initial
RNAi approach was unspecific and affected the expression of one or more off-target
genes that yielded the above described phenotype in differentiating ES cells. Instead, I
redirected my efforts in this project to the suggested role of Set7/9 in regulating p53-
dependent transcription (Chuikov et al., 2004). Since p53 is a central sensor of cellular
stress and the most significant tumor suppressor in mammals, I hypothesized that the
loss of Set7/9 would in many respects recapitulate well described phenotypes in p53\textsuperscript{-/-}
mice. More specifically, I expected that cells lacking Set7/9 would display severely
impaired responses to DNA damage and other cellular stresses. Most importantly, I
anticipated the distinct possibility that Set7/9\textsuperscript{-/-} mice would be prone to tumor formation
in some of the classical cancer models of compound or oncogene-induced
tumorigenesis.
2.2 Results

2.2.1 Strategy for a conditional knockout allele for Set7/9 in the mouse

Due to its widely suggested role in the epigenetic regulation of gene expression, I expected a severe phenotype in Set7/9-deficient mice during development. Given our interest and expertise in adult stem cell models, I decided to generate a conditional knockout allele of Set7/9 in the mouse. In the potential case of a defective embryonic development, this approach would enable me to study the role of Set7/9 during adulthood in a cell-type specific manner.

The mouse Set7/9 gene is located on chromosome 3 and spans eight exons over a genomic distance of 45.5 kb. Although absent in current genomic databases (http://www.ensembl.org), the Celera database at the time indicated the presence of a single alternative splice variant encoding a truncated version of the protein, lacking the SET domain. This splice variant involves an alternative exon 5a upstream of exon 5 and is supported by a single EST (IMAGE #1383271).

To eliminate Set7/9 expression in the mouse, the conditional targeting approach included that the entire C-terminus encoding the SET domain, was flanked with loxP sites. The resulting deletion eliminates the coding of approximately half of the full-length protein (179 of 366 amino acids) including the catalytic SET domain and the polyadenylation signal (Figure 1). Similar approaches of directly eliminating the catalytic domain rather than inducing frameshift mutations in early exons have been undertaken in the targeting of other SET domain genes and proven to be successful (Sampath et al., 2007; Schotta et al., 2008; Su et al., 2003; Tachibana et al., 2007).

I performed the Set7/9 targeting in R1 ES cells, an ESC line of an F1 (129X1/SvJ x 129S1) background. All experiments involving the targeted Set7/9 locus in this thesis were performed with mice that were back-crossed five times into the C57B/6 strain.
Figure 1: Conditional targeting approach of the murine Set7/9 locus. (a) Genomic representation of Set7/9 and conditional targeting strategy. The mouse gene encodes two different isoforms of which one is lacking the C-terminal catalytic SET domain. A conditional knockout allele was generated by introducing loxP sites upstream of alternative exon 5a and and downstream of the terminal exon 8 including its polyA signal. The FRT-flanked Neo selection cassette was removed by crossing to an actin-Fipe deleter strain. Cre excision deleted a 17 kb fragment encompassing the catalytic domain. The position of the PCR primers used for genotyping is indicated. (b) Southern analysis with 5’ and 3’ DNA probes confirms the correct integration of the targeting vector and the deletion of the Neo selection cassette. Multiplex three-primer PCR was used to genotype wildtype, floxed and excised alleles.
2.2.2 Generation of the targeting vector

The process of gene targeting in mice initially requires the generation of a targeting construct. The targeting construct includes the conditionally targeted Set7/9 locus and, based on homologous recombination integrates into the endogenous locus in embryonic stem (ES) cells at low frequency (Capecchi and Capecchi, 2005). The Set7/9 targeting construct encompassed a region defined by an XhoI site downstream of exon 3 and an XhoI site downstream of exon 8 (Figure 2). I generated three sub-clones of the targeting region from BAC RP24-338C1 in the pBluescript vector: pBS-XE (XhoI in intron 3 to EagI in intron), pBS-SC (SalI in intron 6 to ClaI downstream of exon 8) and pBS-CX (ClaI to XhoI downstream of exon 8). An frt.Neo.frt.loxP-cassette was placed into a SmaI site between exon 4 and 5a within pBS-XE, resulting in pBS-XE (Neo.loxP). A double-stranded oligonucleotide containing a second loxP site, a PstI, BstBI, EcoRI and an XhoI site was inserted into the ClaI site in pBS-SC, resulting in pBS-SC(loxP). Next, pBS-SC(loxP) and pBS-SX were combined by ClaI to BstBI and XhoI to XhoI ligation, resulting in pBS-SX(loxP). The final targeting construct pBS-X (Neo.loxP)(loxP)X was assembled by recombination of pBS-XE(Neo.loxP) and pBS-SX (loxP) through the ligation of unique BstBI and SacII sites.
Figure 2: Cloning of the Set7/9 targeting construct. The Set7/9 locus was sub-cloned from a genomic BAC clone and modified by conventional restriction enzyme mediated recombination. The resulting plasmid was used for the transfection of R1 ES cells.
2.2.3 Introduction of the targeted Set7/9 mutation into ES cells and the derivation of a conditionally mutated mouse strain

The gene targeting of Set7/9 in R1 cells was performed according to standard ES cell electroporation protocols followed by a G418 selection period of 10 days. The screening strategy for correct integrations into the targeted locus relied on genomic Southern analysis on both ends of the targeting construct. I obtained 2 of 96 clones with correct integration into the Set7/9 locus. We injected clone D4 into wildtype C57B/6 blastocysts and obtained one chimera with 40% ES cell derived tissue contribution judged by the extent of agouti coat color. This chimera produced about 40% agouti-colored pups when crossed to wildtype C57B/6J females. (detailed summary in Materials and Methods, Section 5.6)

Among the agouti pups derived from the D4 chimera, approximately 50% carried the targeted Set7/9 allele including the frt-flanked Neomycin resistance cassette based on PCR-based genotyping. The frt-flanked Pgk-Neo cassette used for selection was deleted by crossing to the actin-Flpe mouse strain. Crossing through the ubiquitous Cre-expressing transgenic strain Pcx-Cre subsequently generated the knockout allele. We routinely genotyped the Set7/9 mice by PCR with three primers allowing the discrimination between wildtype, floxed and null alleles (Figure 1b).

2.2.4 Set7/9 is dispensable for mouse development and global H3K4 methylation levels

Despite its suggested role in many transcriptional processes, viable Set7/9−/− offspring was obtained at normal Mendelian ratios from Set7/9+−intercrosses, and appeared indistinguishable from their wildtype and heterozygous littermates (Figure 3a). Further, Set7/9−/− mice exhibited normal fertility and yielded normal litter sizes when interbred (Figure 3b).

Western analysis of embryonic fibroblasts with antibodies raised against either the full-length protein or an N-terminal peptide confirmed that our knockout strategy completely
eliminated expression of Set7/9 protein from the excised but not the floxed locus (Figure 4).

As discussed earlier, Set7/9 was initially identified as an H3K4 specific histone methyltransferase (Nishioka et al., 2002; Wang et al., 2001) but its catalytic activity towards nucleosomal H3 was later suggested to be considerably lower than towards monomeric histone H3 (Chuikov et al., 2004). Under consideration of the initial reports, I reasoned that loss of Set7/9 may result in a noticeable reduction of H3K4me1. Such observations have previously been made in other knockout strains of SET domain proteins (Peters et al., 2001a; Schotta et al., 2008; Tachibana et al., 2002). I therefore examined the contribution of Set7/9 towards global levels of the three H3K4 methylation states. Consistent with the observation in Chuikov et al. (2004), western analysis of wildtype and Set7/9−/− MEFs using methyl-specific H3K4 antibodies did not detect any change in global methylation levels (Figure 4). Although the presence of Set7/9-dependent H3K4 monomethylation in very selective regions of the genome cannot be excluded, my results support the notion that the catalytic activity of Set7/9 towards nucleosomal H3 is negligible compared to its other reported substrates.

**Figure 3: Set7/9 is dispensable for embryonic development and fertility.** (a) Set7/9−/− animals are born at expected mendelian ratios. (b) Litter sizes are unaffected by the absence of Set7/9. Error bars indicate standard deviations.
Figure 4: Western analysis confirms the loss of Set7/9 expression. Two antibodies raised against the whole protein or against an N-terminal peptide were used to confirm the complete absence of Set7/9 from knockout MEFs as well as its continued expression form the floxed allele. Heterozygous cells were found to express decreased Set7/9 protein levels. Global H3K4 methylation levels are unaffected in Set7/9−/− cells.
2.2.5 Set7/9 deficient MEFs display normal senescence behaviour in culture

Given the apparent dispensability for Set7/9 for global H3K4 methylation, I focused my attention on the hypothesized role of Set7/9 in regulating p53-dependent transcription by the selective monomethylation at K369. Primary cells undergo replicative senescence after prolonged culture, triggered by shortened telomeres and accumulation of genomic lesions resulting from oxidative stress (Collado et al., 2007). As p53 plays a key role in sensing such damage and implementing the cellular senescence program, cells deficient in p53 function escape senescence-induced cell cycle arrest and are easily immortalized.

Based on this, it could be reasoned that Set7/9-deficient cells might at least partially phenocopy p53-deficient cells and display an impaired senescence behaviour in vitro. To test this hypothesis, I investigated the growth kinetics of cultured MEFs from wildtype, Set7/9−/− and p53−/− mice according to the commonly used 3T3 protocol (Todaro and Green, 1963) (Figure 5a). Under 3T3 conditions, p53 null MEFs grew exponentially throughout the culture period, as expected. In contrast, both Set7/9−/− and wildtype MEFs underwent a replicative crisis after passage 9. This result was surprising and indicated that, Set7/9 is not required for culture-induced senescence mediated by p53 (Figure 5b).
2.2.6 No significant monomethylation at p53K369 is detectable in MEFs following Adriamycin-treatment

It was reported that Set7/9 methylates human p53 at lysine K372 under conditions of stress. I hypothesized that this methylation event should be detectable in MEFs that were treated with the DNA damage-inducing agent Adriamycin, as described before. I also reasoned that, if the Set7/9-dependent methylation of p53 indeed occurred under those conditions, it should be absent in my generated Set7/9 knockout strain. This result would be very informative with respect to the significance the methylation event and the role of Set7/9 being directly involved in p53-activation.

To test the methylation status on lysine 369 of mouse p53 (p53K369) in wildtype and Set7/9−/− cells, I used a methylation-sensitive antibody raised against human p53K372me1 in Adriamycin treated MEFs. This antibody had been reported to cross-react with mouse p53K369me1 regardless of a slight divergence in amino acid sequence in the vicinity of the methylation epitope. To my surprise though, I was never able to detect a signal for methylated p53 with this antibody in Adriamycin-treated
MEFs, despite numerous attempts and the exploration of different conditions in western and immunoprecipitation experiments (Figure 6a and data not shown).

Interestingly however, the α-p53K372me1 consistently yielded a very strong cross-reacting signal at 23 kD, which was present in all tested wildtype and p53<sup>−/−</sup> cell types but completely absent in Set7/9<sup>−/−</sup> cells (Figure 6a). Together with the observation that this cross-reaction signal was restored in Set7/9<sup>−/−</sup> cells upon reintroduction of myc-tagged Set7/9, I reason that the α-p53K372me1 antibody recognizes a p53-independent and uncharacterized substrate of Set7/9 in its methylated form and therefore conclude that the catalytic activity of Set7/9 is indeed absent from my knockout strain.

To alternatively address the methylation status of p53K369 as a function of Set7/9 expression and p53 activation, I performed mass-spectrometry analysis of endogenous p53 purified from Adriamycin treated wildtype and Set7/9<sup>−/−</sup> MEFs (Figure 6b). As a proof for the efficient activation of p53 under the applied conditions, I could readily detect elevated protein levels and the occurrence of serine 15 phosphorylation of p53 (Figure 6c). Next, I performed a large-scale affinity purification of p53 from roughly 100 mg protein lysate of wildtype and Set7/9<sup>−/−</sup> cells using an immobilized p53 antibody. Following my purification scheme, which quantitatively bound p53 from the input material (Figure 6d), we generated a 16mer peptide carrying lysines 367 and 369 by Arg-C mediated proteolysis and examined its modification status by liquid chromatography coupled mass-spectrometry (Figure 6e). We consistently observed a strong series of peaks corresponding to the quadruply charged unmodified 16mer peptide and a dimethylated derivative (Figure 6e, insert) of the same peptide as indicated by a mass shift of seven m/z units. To our surprise however, no signal corresponding to a monomethylated form was detectable in both wildtype and Set7/9<sup>−/−</sup> samples.

Since two previous reports have described the damage-associated dimethylation of lysine 370 on human p53 (Huang et al., 2007; Kachirskaja et al., 2008) (corresponding to mouse p53K367me2) by an unknown methylase, it can be inferred that the dimethylated species of the examined peptide reflects the same dimethylation event. Interestingly though, because of its low intensity, the observed dimethylation
event cannot be assigned to a specific lysine by MS/MS analysis. Given the unequivocal demonstration of Set7/9 being a monomethylase (Wilson et al., 2002) and that I observe no qualitative difference in the dimethylated species between wildtype and Set7/9\(-/-\) cells, I exclude the possibility that this modification is laid down by Set7/9. Hence, I conclude that Set7/9-mediated p53K369me1 is a far less prevalent modification than previously suggested. At the same time, I cannot completely exclude its occurrence under other physiological circumstances or at different time-points. It is important to note that current analyses are still underway to explore this possibility.
**Figure 6: Methylation status of p53K369 in Set7/9−/− mice.** (a) α-p53K372me1 does not recognize K369me1 of mouse p53 but displays a strong cross-reactivity with a protein of 23 kD size in wildtype and p53−/− but not Set7/9−/− MEFs. The cross-reactivity can be rescued by reintroduction of myc-Set7/9. (b) Experimental scheme to purify endogenous p53 and perform mass-spectrometric analysis of the methylation status of lysine 369. (c) Confirmation of p53-activation by Adriamycin detecting elevated levels of p53 protein and serine 15 phosphorylation (p53S15P). (d) p53 immuno-precipitation binds p53 quantitatively from wildtype and Set7/9−/− input. (e) Mass-spec analysis reveals the absence of p53K369me1 but the presence of low-level p53K367me2 in both wildtype and Set7/9−/− MEFs after Adriamycin-treatment.
2.2.7 Set7/9 is dispensable for p53-dependent transcription and cell cycle arrest in MEFs

In the absence of activating signals, p53 protein is targeted for degradation through ubiquitination by the E3 ligase Mdm2. Genotoxic stress causes the phosphorylation of serines within the N-terminus of p53, blocking the interaction with Mdm2 and leading to the accumulation of the protein (Prives, 1998). Over-expression and RNAi experiments suggest that p53, once activated, depends on SET7/9 to induce expression of its target genes \( p21 \) and \( MDM2 \) (Chuikov et al., 2004). To investigate this requirement for endogenous Set7/9 in primary mouse cells, I compared low passage MEFs from wildtype and \( \text{Set7/9}^{-/-} \) littermates, along with \( \text{p53}^{-/-} \) controls, in their ability to stabilize p53 and induce p53-dependent transcription and cell cycle arrest.

To this end, I exposed MEFs to gamma irradiation, Adriamycin or the Mdm2 antagonist Nutlin 3 and compared protein levels of p53, p21, Mdm2 and Puma after these treatments (Figure 7a). Under all conditions tested, I could not detect any expression of \( p21 \) and \( Mdm2 \) and only weak expression of \( \text{Puma} \) in \( \text{p53}^{-/-} \) cells, as expected. By contrast, expression of these p53 target genes was readily detectable in untreated wildtype and \( \text{Set7/9}^{-/-} \) MEFs and their expression was efficiently up-regulated in response to all treatments in both wildtype and \( \text{Set7/9}^{-/-} \) MEFs. Importantly, the extent of p53 stabilization, its acetylation at lysine 379 as well as the expression of \( p21 \), \( Mdm2 \) and \( \text{Puma} \) appeared comparable in \( \text{Set7/9}^{-/-} \) MEFs and wildtype controls. This suggests that Set7/9 is not required to control acetylation of K379 on p53 and the expression of its target genes in MEFs.

Next, I analysed the cell cycle profiles of the same MEFs by BrdU incorporation and PI staining (Figure 7b and c). Whereas as expected, treated p53 null MEFs were unaffected by the treatments as indicated by their continued BrdU incorporation, wildtype and \( \text{Set7/9}^{-/-} \) cells responded equally to all p53 activating agents by efficiently arresting DNA synthesis. Taken together, my results suggest that Set7/9 is dispensable for the p21-mediated G1/S-phase arrest induced by p53 in MEFs.
Figure 7: Cell cycle arrest and apoptosis induction is not compromised in $Set7/9^{-/-}$ MEFs. (a) Asynchronous populations of MEFs (< passage 4) were treated with different p53 stabilizing agents and subjected to western analysis. Under all conditions tested, the extent of p53K379 acetylation as well as the expression of $Mdm2$, $p21^{WAF1/CIP}$ or $Puma$ were comparable in $Set7/9^{-/-}$ and wildtype MEFs. (b) The cell cycle status of the same MEF populations were analyzed by BrdU incorporation (1h pulse) and PI staining. The percentages of cells in the G1, S and G2/M stages are indicated. Wildtype and $Set7/9^{-/-}$ MEFs respond to all treatments by withdrawing from the cell cycle. p53 null MEFs are characterized by an increased arrest in late S- and G2-phase in response to Adriamycin. (c) Cell cycle analysis summary. G1/S ratios were calculated from 3 replicate experiments as illustrated in (b). Error bars represent standard deviations.
2.2.8 Set7/9 is dispensable for γ-radiation-induced apoptosis in thymocytes

To investigate whether cell-type specific defects in the p53 pathway may be present in Set7/9−/− animals, I expanded my analysis to hematopoietic progenitors in the thymus. Thymocytes are characterized by high proliferation rates and are very sensitive to apoptotic stimuli, making them an ideal model to study p53-mediated cell death. I harvested thymocytes from wildtype and Set7/9−/− mice, subjected them to increasing dosages of ionizing radiation ranging from 0 to 2 Gy and quantified apoptosis within these samples by Annexin V staining (Figure 8a).

Regardless of the radiation dosage, I could not detect any significant difference in the rate with which Set7/9−/− and wildtype thymocytes entered apoptosis. Additionally, I was unable to score any significant differences in the expression of p53 target genes p21WAF1/CIP, Mdm2 and Bax by quantitative real-time PCR, or in the induction of p53 by western blot analysis (Figure 8b and c).
Figure 8: Normal irradiation-induced apoptosis in Set7/9-deficient thymocytes. (a) Thymocytes were prepared from 4 mice of each genotype and exposed to increasing γ-radiation dosages. Relative viability was determined by Annexin V staining and 7AAD exclusion. Error bars represent standard deviations. (b) Western analysis of irradiated thymocytes. Set7/9 is expressed in wildtype but not in knockout thymocytes. p53 and Bax induction is comparable in both samples. (c) Quantitative RT-PCR analysis of p53 target genes in irradiated thymocytes. Wildtype and Set7/9−/− mice (n=4) were exposed to whole body radiation at the indicated dosages. Two hours later, thymocytes were harvested for quantitative RT-PCR analysis. No significant difference in the ability of wildtype and Set7/9−/− cells to induce the expression of p21^{WAF1/CIP1}, Mdm2 and Bax was detected. Each bar in these graphs reflects the expression analysis of the respective p53 target gene from one mouse normalized to GAPDH expression. The expression levels of untreated controls were set to one.
2.2.9 Set7/9-/- MEFs are fully capable of initiating oncogene-induced senescence, apoptosis and are not predisposed to transformation

Failsafe mechanisms have evolved to sense and counteract uncontrolled growth signals caused by viral or cellular oncogenes. One of these pathways acts through the p19ARF-mediated stabilization of p53, eventually resulting in cell cycle arrest also known as premature senescence (Serrano et al., 1997) or apoptosis (de Stanchina et al., 1998).

A necessity for Set7/9 in p53-dependent transcription should attenuate the induction of senescence or apoptosis in Set7/9-/- cells in response to ectopic oncogene expression. To assess this hypothesis, early passage wildtype, Set7/9-/- and p53-/- MEFs were infected with H-RasV12 or E1A expressing retroviruses. After 48 hours of puromycin selection, 10^3 cells were plated into 10 cm tissue culture dishes and the resulting colonies were scored 18 days later (Figure 9a). Unlike p53-/- MEFs, which yielded a dramatically increased number of colonies under all conditions, Set7/9-/- MEFs were indistinguishable from wildtype controls in both colony numbers and size (Figures 9b and c).

To determine the ability of anchorage-independent growth as a hallmark of cellular transformation in H-RasV12 or E1A expressing MEFs, I also plated 2x10^4 infected MEFs in soft-agar based culture medium and incubated the cells for 14 days. Again and as expected, p53-/- MEFs gave rise to readily detectable multicellular colonies when expressing H-RasV12 or E1A. However, RasV12 or E1A expressing wildtype controls and Set7/9-/- MEFs were not able to support anchorage-independent growth under the same conditions (Figure 9d). This finding contradicted Kurash et al. (2008), who reported elevated frequencies of oncogenic foci as a sign of cellular transformation in high-density cultures of E1A or Ras infected Set7/9-/- MEFs.
Figure 9: *Set7/9* MEFs are not predisposed to oncogenic transformation. (a) Passage 1 wildtype, *Set7/9* or *p53* MEFs were infected with control, activated H-RasV12 or E1A 12S containing retroviruses. Puromycin selection was applied for 48 hours and 1000 cells were plated in duplicate on 10 cm dishes. Colonies were scored by crystal violet staining after 18 days of culture. (b) Visualization of the colonies reveals a comparable propensity of wildtype and *Set7/9* MEFs to be transformed by these oncogenes. (c) Summary of transformation assays. Average number of colonies per 1000 cells calculated from four wildtype, four *Set7/9* and one *p53 null* independent MEF preparations. Error bars represent standard deviations.
2.2.10 Transformed Set7/9\/- MEFs do not exhibit increased tumor growth in vivo

MEFs transformed by combined E1A and RasV12 expression acquire the ability to form tumors when injected subcutaneously in immuno-compromised mice (Jimenez et al., 2000). The growth of these tumors is strongly limited by p53, making it a significant way to measure p53 activity. To test whether the loss of Set7/9 in MEFs, as a result of its proposed importance for p53 activity, would promote an increased tumor growth in this assay, I double-infected wildtype, Set7/9\/- and p53\/- MEFs with E1A and Ras, and subcutaneously injected constant numbers of cells into NOD/SCID mice (Figure 10a). After 15 days, the resulting tumors were harvested and weighed. As expected, transformed p53 null MEFs yielded very large tumors while tumors from both wildtype and Set7/9\/- MEFs were at least one order of magnitude smaller in size. Consistent with my previous observations, I found that wildtype and Set7/9\/- tumors did not display any significant weight difference (Figure 10b). Together, my results indicate that loss of Set7/9 is not sufficient to bypass oncogene-induced senescence and does not attenuate apoptosis in MEFs.

Figure 10: Unchanged tumorigenicity of E1A/RasV12-infected Set7/9\/- MEFs. (a) Primary wt, Set7/9\/- and p53\/- MEFs were doubly infected with E1A and RasV12, expanded and injected subcutaneously into NOD/SCID mice. (b) Tumors were harvested after 14 days and weighed. Set7/9\/- cells yielded no increased tumor growth compared to wildtype cells and remain much smaller than tumors from p53 null cells. The data was obtained from multiple injections of three wildtype, three Set7/9\/- and two p53 null MEF lines. The indicated n-values indicate the number of tumors analyzed. Similar results were obtained when tumors were harvested after 20 days. (data not shown)
2.2.11 Set7/9 deficiency leads to no accelerated lymphomagenesis in a \textit{Eμ-Myc} tumor model

To further determine whether Set7/9 deficiency might influence tumorigenesis \textit{in vivo}, I took advantage of a transgenic mouse strain that was engineered to express c-Myc under the control of an immunoglobulin heavy chain enhancer (\textit{Eμ-Myc}) (Adams et al., 1985). This strain is well characterized and prone to the formation of B cell lymphomas \textit{in vivo}, modelling the involvement of c-Myc overexpression in a large fraction of naturally occurring Burkitt’s lymphoma (Harris et al., 1988).

Importantly, whereas \textit{Eμ-Myc} mice typically develop B-cell lymphomas within 4-6 months in a wildtype background, a number of co-operating mutations are known to accelerate lymphoma formation in this mouse model. For example, lymphomas in a \textit{p53} knockout background arise much earlier and display a characteristic amplified pathology (Schmitt et al., 1999), indicating a crucial role for \textit{p53} in sensing oncogenic activities and inducing cellular senescence and apoptosis. Furthermore, knockout mice deficient in the apoptosis-associated \textit{p53} target genes \textit{Bax} (Eischen et al., 2001), \textit{Bim} (Egle et al., 2004) and \textit{Puma} (Garrison et al., 2008) also co-operate with the \textit{Eμ-Myc} transgene and promote the onset of lymphomas at earlier time-points. Based on these reports and the proposed role for Set7/9 in \textit{p53} regulation, it could be expected that loss of Set7/9 may result in the potential acceleration of lymphomagenesis in \textit{Eμ-Myc} mice. To test this possibility, I bred the \textit{Eμ-Myc} transgene into \textit{Set7/9} floxed and knockout as well as the \textit{p53} knockout background. Since hematopoietic stem cells but no B-cells (and therefore potential lymphoma cells) reside in the fetal liver, I transferred fetal liver HSCs from these mice into lethally irradiated recipients and monitored their health over a time of 180 days. Mice that displayed palpable lymph node tumors were immediately sacrificed and examined to verify lymphoma incidence (Figure 11a).

When plotting the survival rates of \textit{Eμ-Myc}\(^+\), \textit{Set7/9}\(^{+/+}\) (control), \textit{Set7/9}\(^{-/-}\) and \textit{p53}\(^{+/+}\) recipients over time, it became apparent that in comparison to control mice, the \textit{Set7/9}\(^{-/-}\) recipients did not exhibit any significantly accelerated lymphoma onset (Figure 11b). On the contrary, in both the control group and among the recipients that received \textit{Set7/9}\(^{-/-}\) transplants, only 3 out of 12 mice developed lymphomas within the 180 days of...
the experiment. By stark contrast, 50% of the recipients of $E\mu$-Myc+/p53+/− fetal liver developed lymphoma by day 42 post-transplant and all mice had developed lymphomas by day 78 of the experiment.

Since all lymphomas that arise from heterozygous p53 cells are characterized by the loss of their wildtype p53 allele in this experimental setting (Schmitt et al., 1999), the observed lymphomas in this group consistently exhibited a dramatic increase in tumor size and increased invasiveness into non-hematopoietic tissues like liver or lung. Importantly, no such overt differences in the severity of pathology were observed between control and Set7/9−/− groups (data not shown). Due to the fact that I did not observe any lymphoma acceleration in Set7/9−/− mice, I conclude that Set7/9 has no discernible influence on the regulation and in vivo activity of p53 in counteracting c-Myc induced lymphomagenesis.

Figure 11: c-Myc-driven lymphomagenesis is not accelerated in Set7/9-deficient mice. (a) $E\mu$-Myc positive, Set7/9fl/+ (control), Set7/9−/− or p53+/− fetal livers were harvested at 14.5 dpc and transferred into lethally irradiated CD45.1 wildtype recipients. Mice were monitored until they became moribund or palpable tumors occurred. (b) p53+/− recipients displayed a marked acceleration of lymphomagenesis, as expected. Set7/9−/− mice did not exhibit any propensity to form tumors earlier than control mice.
2.2.12 Set7/9-deficiency results in no detectable disadvantage in the hematopoietic system

Bone marrow borne stem cells and a series of lineage restricted progenitors constantly regenerate all blood cell-types. The rate at which hematopoietic stem cells and progenitors proliferate requires tight regulation to ensure the life-long supply of blood cells (Orford et al., 2008b). Competitive bone marrow transplantation assays provide an unbiased and very sensitive way to reveal otherwise undetectable alterations in proliferation and survival of specific hematopoietic intermediates. Although Set7/9 has been proposed to act as a co-activator during the induction of numerous genes, its inactivation lead to no overt phenotype in mice. Nonetheless, I considered that in the absence of Set7/9, some of the cell-intrinsic mechanisms that contribute to blood formation might be delayed or accelerated. In this case, it should be possible to detect such differences in internally controlled competitive bone marrow transplantation experiments.

To measure the efficiency of hematopoietic regeneration and lineage-specification in the absence of Set7/9, I transplanted bone marrow from CD45.2+ Set7+/+ or Set7/9−/− litter mates in equal competition with congenic CD45.1+/2+ wildtype bone marrow cells into lethally irradiated CD45.1+ recipients (Figure 12a). FACS analysis with antibodies against CD45.1+, CD45.2+ and against lineage-specific antigens allowed me to determine the relative contribution of CD45.2+ labelled wildtype or knockout bone marrow in comparison to the CD45.1+/2+ labelled competitor bone marrow. In this internally controlled approach, any net difference in either the engraftment, expansion or maintenance of stem cells and progenitors and/or in the development of blood cell types will account for a noticeable deviation in the ratio of wildtype versus competitor bone marrow in one or more given cell-types.

I performed FACS analysis after a long-term engraftment period of 16 weeks and scored the relative chimerism among all donor-derived cells within four major blood lineages by comparing the ratios of CD45.2+ to CD45.1+/2+ inside gated populations of granulocytes (Mac1/GR1+), monocytes (Mac1+), B-cells (B220+) and T-cells (CD3+) (Figure 12b). Against my hope to detect a Set7/9-dependent phenotype in this
serendipitous approach, all experiments indicated that, in comparison to wildtype littermate controls, Set7/9⁻/⁻ bone marrow is not appreciably compromised during hematopoietic regeneration (Figure 12c). Hence, Set7/9 does not appear to play a role in the development or homeostasis of the hematopoietic system, similar to its apparent expendability in mouse development.
**Figure 12: Normal hematopoiesis in the absence of Set7/9.** (a) CD45.2+ bone marrow from Set7/9−/− mice or Set7/9+/− littermates was transplanted in 1:1 competition with CD45.1/45.2+ wildtype bone marrow into lethally irradiated CD45.1+ recipients. (b) The resulting chimeraism after long-term engraftment was determined by flow-cytometry using antibodies against CD45.1+, CD45.2+, Mac1+, Gr1+, B220+ and CD3+ as indicated. (c) No significant difference in the relative contribution to peripheral blood leukocytes could be determined between Set7/9−/− mice or Set7/9+/− controls. The shown data is representative for two independent experiments. Error bars indicate standard deviations from three control and eight knockout recipients. No statistical significance could be detected.
2.3 Discussion

2.3.1 Validity of the generated conditional Set7/9 knockout strain

Given that most of the performed experiments with Set7/9\(^{-/-}\) cells derived from my targeted mouse strain did not reveal any phenotypic alteration compared to the wildtype controls, it is of particular importance to evaluate the used knockout strategy. The key strategy in the generated conditional Set7/9 knockout strain was to eliminate the coding for the catalytic activity associated with the SET domain. Two experiments routinely confirmed the functional elimination of the Set7/9 gene. First, the applied PCR genotyping strategy relied on the amplification of a fragment that is generated only upon loxP recombination and indicated that the resulting null allele indeed lacks the complete floxed region. Second and most importantly, western analysis of whole cell lysates of wildtype and Set7/9\(^{-/-}\) origin with specific antibodies raised against either the full-length or against a peptide in the N-terminus of the protein indicated the complete absence of any form of the Set7/9 protein. This unambiguously proved that the generated mouse strain lacks the expression of the targeted gene and does not generate a truncated version of Set7/9.

Contrary to my results, Kurash at al. (2008) did indeed observe a severe impairment of p53-function in their generated knockout mouse strain. To a neutral observer, one explanation for this discrepancy may lie in the fact that significant differences between my and Kurash’s knockout strategy exist. Kurash et al. (2008) used a very common way to eliminate Set7/9 expression. Their strategy relied on the replacement of the constitutive second exon of the Set7/9 gene with a splice acceptor-coupled lacZ-neo cassette. Since both knockout strategies appear convincing, another possibility for the inconsistency in the results that were derived may lie in a difference in the genetic background that both mutations were maintained. However, both strains were derived in 129-based ES cell lines and then back-crossed to the C57B/6 strain for five generations, suggesting that a significant difference in the genetic background of the two mouse lines is unlikely.
2.3.2 The proposed *in vivo* role of Set7/9 in regulating p53 function

In the course of my studies, Kurash et al. (2008) published a phenotypic characterization of a Set7/9 knockout mouse line. They showed that in Set7/9\(^{-/-}\) cells, p53 is not methylated on lysine 369 (K369), corresponding to lysine 372 (K372) in human p53, and that its acetylation on several other lysines is greatly reduced. They also showed that the binding of Tip60, an acetyltransferase that had previously been shown to acetylate p53 (Sykes et al., 2006), is K369me1-dependent. Under conditions that activate p53, they observed a severely impaired induction of \( p21^{WAF1/CIP1} \) and \( PUMA \), two well-characterized p53 target genes. Additionally, they showed that mouse embryonic fibroblasts (MEFs) lacking Set7/9 are easily transformed by ectopic expression of the oncogenes \( E1A \) and \( RasV12 \). Based on this data, it was concluded that Set7/9 is indeed critically required for the transcriptional and tumor-suppressive function of p53 *in vivo* (Illustration 8).

**Illustration 8: The proposed model of Set7/9-dependent p53 regulation.** (a) Upon stabilization, chromatin-associated p53 becomes methylated by Set7/9 at K369 (K372 in human). This methylation is recognized by the acetyltransferase Tip60, which in turn acetylates p53 and ensures the efficient transcriptional activation of p53 target genes such as \( p21^{WAF1/CIP1} \), Puma and others. (b) In the absence of Set7/9 (by gene disruption or knockdown), chromatin-associated p53 is not sufficiently activated and largely fails to induce the transcription of its target genes leading to multiple phenotypes that resemble the ones of p53 knockout cells.
2.3.3 Little evidence for a role of Set7/9 in regulating p53 activity in vivo

Set7/9 was shown to methylate p53 and suggested to be critically required for its tumor-suppressive function (Chuikov et al., 2004; Kurash et al., 2008). Since p53 knockout mice (like Set7/9 knockout mice) are developmentally normal, this hypothesis appeared very plausible. Interestingly, the results I obtained investigating the integrity of different p53 functions in my Set7/9 knockout mouse strain were not compatible with those reported by Kurash et al. (2008), despite the use of very similar assays.

More specifically, Kurash et al. (2008) were successful in showing that K369 methylation is absent in Set7/9-/- cells using an immunoprecipitation approach with the commercially available α-p53K372me1 antibody. However, I was unable to directly assess the p53K369 methylation status in mouse cells using the same antibody, probably due to two critical amino acid conversions in the direct vicinity of the methylated epitope. Given the importance of this unanswered question, I used an elaborate approach to assess the methylation status of p53 in Set7/9 knockout cells in response to Adriamycin-treatment by a mass-spectrometry approach in analogy to what was done before (Kachirskaya et al., 2008). As a proof for the activation of p53 following the Adriamycin treatment, I could show that protein levels of p53 and its phosphorylation at serine 15 where dramatically increased in the applied conditions. To my surprise however, I was unable to detect the proposed Set7/9-dependent methylation of p53K369 in the mass-spectrometric analysis even in wildtype cells. However, low levels of a dimethylation event, presumably of p53K367 (Huang et al., 2007) where detectable in both wildtype and Set7/9 knockout cells. Importantly, p53K369me1 has been shown to be present at high levels ranging from 1.5 hours until 18 hours after Adriamycin administration, it is possible that at the chosen time-point of six hours post treatment p53K369me1 is not yet present. The fact that other lysine modifications in the C-terminus of p53 are also not yet detectable at this stage supports this hypothesis. Further experiments to detect p53K369me1 at later time-points after Adriamycin treatment are therefore currently underway.

Notably, western analyses and immunoprecipitation experiments in which I applied the α-p53K372me1 antibody indicated that at least two methylation-dependent
epitopes exist which are recognized by the antibody and that depend on the expression of Set7/9. I conclude from these findings and from the fact that the catalytic SET domain is deleted in my knockout strain that all Set7/9-dependent lysine methylation activity is indeed gone in the generated knockout mice.

Kurash et al. (2008) observed that Set7/9-- cells do not undergo cell cycle arrest in the presence of high concentrations (1 µM) of the genotoxic drug Adriamycin. In conflict with this result, I found that wildtype and Set7/9-- MEFs equally responded to a number of p53-activating agents including Adriamycin by arresting in G1 or G2/M phase. As Adriamycin causes DNA double strand breaks during replication, it triggers both p53-dependent G1 arrest and p53-independent G2 arrest (Attardi et al., 2004). Indeed, I observed the previously reported pronounced accumulation of cells in late S and G2/M phases in Adriamycin treated p53 null controls. In contrast, Kurash et al. (2008) reported that Adriamycin-treated Set7/9-- cells displayed a normal cell-cycle profile with the majority of cells residing in G1, a finding that they interpreted as a result of diminished p53 activity. It is arguable that because of this finding, the Adriamycin-treatment in Kurash et al. (2008) for some reason is not working properly. In their experiment, a p53-- control MEF line would have revealed this discrepancy, but interestingly was not included in their experiment.

An additional discrepancy between my findings and those in Kurash et al. (2008) lies in my inability to detect significant differences between Set7/9-- and wildtype MEFs or thymocytes in the induction of p21, Mdm2, Puma or Bax expression upon p53 activation using quantitative techniques, whereas Kurash et al. (2008) reported a profound defect in the induction of p21 and Puma in Set7/9-- cells. Moreover, I found that acetylation of p53 at lysine 379 was readily detectable in Set7/9-- cells, unlike what was reported by Kurash et al. (2008) Interestingly, I also observed strong p53 target gene expression upon treatment with Nutlin 3 without any detectable induction of K379 acetylation, indicating that this modification by itself also may not be essential for the transactivation activity of p53.

Kurash et al. (2008) also report that, Set7/9-- cells are easily transformed by oncogenes. However, despite using the same oncogenes as Kurash et al. (2008), I cannot observe any increase in colony formation or the support of anchorage-
independent growth by Set7/9⁻/⁻ MEFs. Furthermore, in MEFs that were transformed by the combined expression of two complimentary oncogenes, E1A and RasV12, I detected no difference in the size of tumors that arose from sub-cutaneously transplanted wildtype and Set7/9⁻/⁻ cells. By contrast, the growth that was supported by p53 null MEFs was at least tenfold bigger, indicating that Set7/9 if at all only contributes to a small fraction of p53 activity in vivo.

In a complementary and more physiologically significant assay, I investigated the efficiency of Set7/9 knockout cells in suppressing c-Myc induced lymphomagenesis. The used mouse strain Eµ-Myc is one of the most commonly used cancer models in biology (Hanahan et al., 2007) and efficiently mimics the frequent occurrence of translocations that place the endogenous c-myc gene in proximity to enhancer elements of the immunoglobulin heavy chain. Its significance in this context is indicated by the fact that mutations in p53 itself, but also in the p53 target genes Bax (Eischen et al., 2001), Puma (Hemann, 2004) and Bim (Egle et al., 2004) significantly accelerate the onset of lymphomas in these mice.

A general hallmark of Kurash et al. (2008) is that the authors discuss their results in a way that suggests that Set7/9⁻/⁻ cells behave like p53⁻/⁻ cells, without having included those as positive controls in their experiments. In light of my results, the role of Set7/9 in p53 regulation requires a considerable re-evaluation by the field. Furthermore, it can be argued that if Set7/9 indeed had a tumor suppressor activity that is comparable to the one of p53, earlier studies on the genetic events underlying tumorigenesis should have already identified Set7/9 as a reoccurring lesion in at least a subset of cases. Similarly, recent studies have used genetic screens to identify modulators of p53-activity. Curiously however, although very powerful RNAi libraries have been applied, Set7/9 (to my knowledge) was never identified as a target in such an unbiased approach (Berns et al., 2004).

Conversely, a number of studies have addressed the roles of post-translational modifications occurring on C-terminal lysines by targeted mutation of the p53 gene in the mouse. For example, mutational conversion of seven C-terminal lysines, including K369, into arginines, yielded only minor changes in p53 activity (Krummel et al., 2005), (Feng et al., 2005). In a human cell line, only the combined mutation of eight lysines,
including the mutation of two lysines in the DNA binding domain of p53, yielded a transcriptionally inert form of p53, a result that argues for the overall importance of lysine modifications but also suggests their highly redundant activities (Tang et al., 2008). My results are consistent with those findings and suggest that in healthy primary cells, the transcriptional activity of p53 is regulated by a myriad of functionally equal mechanisms, ensuring that several fail-safe arrangements protect cells from unregulated growth and tumor development.

In summary, I cannot confirm Kurash et al’s conclusion that Set7/9, on its own, is indispensable for p53 activity in vivo by virtue of my own data. Importantly, several published studies argue significantly against or at least not for Kurash’s finding. At the same time, I cannot rule out the possibility that Set7/9 may have a minor role in preventing tumor progression, as its role in transformed cell lines seems very significant. It is widely accepted that cancer formation is a multistep process (Hanahan and Weinberg, 2000), in which cells with compromised tumor suppressor activities increasingly acquire genetic or epigenetic alteration allowing further growth advantages. In this context, it is conceivable that loss of Set7/9 in combination with the loss of several p53-acetylating activities might become a critical lesion in cells that are already deficient in other p53-regulating pathways.

2.3.4 Set7/9 is dispensable for global H3K4 methylation

Set7/9 was identified as a mono-methyltransferase activity towards H3K4 (Wilson et al., 2002; Zhang et al., 2003). However, this result was acquired in vitro by using 20mer peptides and monomeric recombinant histone H3 as substrates. Later studies suggested that the catalytic activity towards nucleosomal histone H3 is much less profound in comparison (Chuikov et al., 2004). While it remains formally possible that Set7/9 depends on additional factors to exert its H3K4 specific activity in vivo, it was also shown that in cells where Set7/9 expression was knocked down, the H3K4me1 status stayed unchanged on promoters of Set7/9-regulated genes (Ivanov et al., 2007).

To assess Set7/9-dependent H3K4 methylation in my knockout strain, I tested all three H3K4 methylation states in MEFs derived from Set7/9−/− embryos, and found no
change in global H3K4 methylation levels in comparison to the corresponding wildtype controls. Given that I conducted my analysis on bulk histones, it is conceivable that in the absence of Set7/9, very specific genomic regions do in fact exhibit minor changes in H3K4me1 levels. Interestingly, the presence of dense regions marked with H3K4me1 has been mapped to enhancer elements of active genes (Heintzman et al., 2007). Despite the fact that Set7/9 is the only H3K4 specific monomethylase currently identified in mammals, I did not test whether Set7/9 is responsible for enhancer-associated H3K4me1. In order to pursue this task best, it would preferable to apply a comparative Chromatin-IP with H3K4me1-specific antibody and the subsequent Solexa-based sequencing (ChIP-seq) of the enriched genomic DNA from wildtype versus Set7/9 knockout cells. Although feasible, we could not justify to pursue this experiment because of the lack of a phenotypic alteration in Set7/9 knockout cells.

2.3.5 The role of Set7/9 in lysine methylation of non-histone proteins

A number of seminal publications suggest that Set7/9 acts as a more general lysine methyltransferase that can modulate the functions of several, mainly transcription-associated proteins by direct lysine methylation.

For example, Set7/9 was shown to methylate Taf10, a component of the general transcription factor complex TFIID (Kouskouti et al., 2004). Set7/9-dependent methylation of Taf10 was proposed to enhance the recruitment of the RNA Polymerase II holoenzyme to a subset of active promoters resulting in the potentiation of their transcription rate. Such gene-specific modulation of transcriptional activity appears very plausible and could help to set up the specification of transcription programmes during development. Indicating a requirement for Taf10 in development, Taf10<sup>−/−</sup> mice die during early embryogenesis although the gene is not generally required for cell viability (Mohan et al., 2003). The absence of an overt developmental phenotype in the Set7/9 knockout strain has caused me to not directly pursue the investigation of Taf10 methylation in mouse development.
2.3.6 Set7/9 function in the activity of Estrogen Receptor α (ERα)

Another, recently published example of Set7/9 methylation involves the well-described estrogen receptor alpha (ERα). In female mammals, ERα binds to its natural ligand estradiol and exhibits a ligand-dependent transcription factor activity responsible for the execution of female-specific transcription programmes. Subramanian et al. (Subramanian et al., 2008) claim that Set7/9 methylates ERα at lysine 302 (ERαK302) in human cells and that this methylation is necessary for protein stability, efficient recruitment to ERα target genes and its transcriptional output. Importantly, female mice lacking the ERα gene develop normally but are infertile due to ovarian dysfunction in the adult (Lubahn et al., 1993). A functional requirement of Set7/9-dependent ERα methylation at K302 should thus lead to a comparable phenotype in adult Set7/9−/− females. However, Set7/9−/− females do not exhibit compromised fertility indicated by their normal litter sizes.

Due to the poorly conserved sequence context of ERαK302 between human and mouse, I did not attempt to investigate the methylation status of ERα in Set7/9−/− mice by using a specific antibody raised by Subramanian et al. Hence, I am not able to hypothesize whether an alternative lysine methyltransferase exists that can compensate for the loss of Set7/9 or whether ERαK302me1 per se is dispensable for ERα function in vivo.

2.3.7 Set7/9 function in the regeneration of the hematopoietic system

The original aim of this part of my studies was to generate a Set7/9-deficient mouse line which would enable us to investigate complex biological processes such as the functionality of the Set7/9-deficient hematopoietic system as an in vivo model for cellular development. Hence, I took advantage of the commonly used approach of a competitive bone marrow transplantation assay. The potential Set7/9-dependent deregulation of any process that factors into the net engraftment efficiency of donor-derived HSCs in the recipients, should be reflected in the relative PBL contribution of the competitively transplanted bone marrow. Interestingly and despite repeated
attempts, I was not able to detect any impaired contribution of *Set7/9*−/− bone marrow during the regeneration of the hematopoietic system.

This result led me to conclude that Set7/9, although widely expressed in the blood, has no overt role in the survival rate, expansion or developmental specification of any of the examined blood lineages. In light of the fact that *Set7/9*−/− mice develop normally throughout embryogenesis and their adult life, this result was not very surprising. I therefore further conclude that Set7/9 does not play a role in cellular development *per se* and that future studies aiming to determine such a role in other developmental model systems such as muscle regeneration or neural development seem not very promising.

However, a recent report suggests that Set7/9 co-regulates NF-κB-dependent transcription in a human macrophages cell-line (Li et al., 2008). This connection should result in the impairment of the innate immunological response to a variety of pathogens, and has not been studied *in vivo*.
3. G9a

3.1 Introduction

3.1.1 G9a is a euchromatic H3K9me2-specific methyltransferase

G9a was originally described as a gene that maps to a human major histocompatibility complex locus (Milner and Campbell, 1993). Due to its conserved SET domain (Illustration 9), it was later characterized as a histone methyltransferase with mono and dimethylation activities towards H3K9 and H3K27 (Tachibana et al., 2002). Its genetic locus can produce two different isoforms that differ in the N-terminus. However, the functional difference of these two isoforms is currently unknown.

Although substrate specificities towards histone H1 (Trojer et al., 2009), mAM as well as its automethylation have been observed for G9a (Sampath et al., 2007), recent studies have primarily focused on its H3K9 methyltransferase activity and role in transcriptional repression. G9a and its closest homologue G9a-like-protein (Glp/Ehmt1) possess similar expression profiles and operate as a heteromeric complex in a wide variety of cell types (Tachibana et al., 2005). Their individual genetic deletions result in comparable phenotypes and a significant drop in global H3K9me1/2 levels. Interestingly, the H3K9 methyltransferase activities of both proteins are thought to rely on the presence of the respective heteromeric interaction partner indicating that G9a and Glp cooperatively exert their H3K9 methyltransferase functions in vivo. In contrast to the Suv39h homologues, which are primarily responsible for H3K9me3 at sites of constitutive heterochromatin, G9a and Glp target H3K9me2 mostly to euchromatic regions (Peters et al., 2003; Tachibana et al., 2002).

Biochemical analysis of the G9a interaction partners revealed that G9a and Glp stability depends on their interaction with the zinc finger protein Wiz in various kinds of human and murine cells (Ueda et al., 2006) (Illustration 10). In addition, Wiz links the G9a/GLP heteromeric complex to the CtBP co-repressor machinery which includes additional chromatin modifying activities such as HDACs and the lysine demethylase Lsd1 (Shi et al., 2003). Moreover, Wiz itself exhibits single- and double-stranded DNA
binding activities in vitro, suggesting that it may also contribute to the recruitment of the G9a/GLP complex to their target chromatin loci. In addition, G9a can bind multiple other zinc-finger molecules including Prdm1/Blimp-1 (Gyory et al., 2004), NRSF/REST (Roopra et al., 2004), Gfi1 (Duan et al., 2005), Gif1b (Vassen et al., 2006) and PRIZM/PRDM6 (Davis et al., 2006). A general theme that has emerged from a number of studies is that G9a plays a role in the epigenetic silencing of cell-type inappropriate genes (Gyory et al., 2004; Roopra et al., 2004) or genes that regulate proliferation (Davis et al., 2006; Kim et al., 2008b; Nishio, 2004; Ogawa et al., 2002).

**Illustration 9: Domain structure of G9a and its homologue Glp.** The G9a gene is expressed in two different isoforms caused by the alternative splicing involving an alternative exon 1a and the skipping of exon 9. The homology of G9a to Ehmt1/Glp is given over the length of the whole protein with the exception of a ~200 amino acid (aa) stretch in the N-terminus.
3.1.2 G9a and its role in development

A number of independent studies have demonstrated that G9a is crucially involved in cellular differentiation and development. For example, G9a knockout mice do not develop beyond day 8.5 of gestation and exhibit a severe growth retardation phenotype (Tachibana et al., 2002). Furthermore, G9a-/- ES cells are characterized by widespread alterations in DNA methylation patterns and the concomitant misexpression of numerous genes (Dong et al., 2008; Tachibana et al., 2008). Most interesting in this context is that the sustained silencing of pluripotency associated genes in G9a-/- ES cells is impaired and results in the reversal of the differentiated into a pluripotent state in a significant fraction of cells (Feldman et al., 2006; Epsztejn-Litman et al., 2008). Related to this finding, the induction of the pluripotent state in mouse embryonic

Illustration 10: Protein interactions of G9a and their functional implications. G9a interacts with its close homologue Glp and the zinc finger protein Wiz. This trimeric complex interacts with CtBP corepressor complex and integrates several complimentary histone modifying activities such as histone deacetylation, H3K4 demethylation by Lsd1 and H3K9 methylation by G9a/Glp. This corepressor complex can be recruited by a number of transcription factors to exert their biological functions such as in hematopoietic stem cell (HSC) activity (Gfi-1), B-cell function (Blimp-1), neuronal function (REST) and smooth muscle function (PRDM6).
fibroblasts (MEFs) can be increased in the presence of BIX01294, a G9a-specific methyltransferase inhibitor (Kubicek et al., 2007; Shi et al., 2008).

A genomic approach to map the localization of H3K9me2 in ES versus differentiated cells detected the presence of thousands of surprisingly large domains (up to 4.9 Mb) that are decorated with G9a-dependent H3K9me2 (Wen et al., 2009). Although the colocalization of H3K9me2 and H3K27me2 has been described independently (Barski et al., 2007), it remains unclear whether H3K27me2 consistently falls in the same genomic distribution pattern and is also G9a-dependent. Interestingly, the H3K9me2 domains were transcriptionally silent but did not overlap with H3K27me3 regions suggesting that apart from the well documented polycomb-dependent repression that occurs during development, G9a seems to be at the core of a second silencing system that integrates histone deacetylation, H3K4 demethylation (by Lsd1) and DNA methylation in a developmentally relevant manner (Shi et al., 2003).

In contrast to our growing knowledge of G9a function in ES cells, its role during adulthood remains largely elusive. However, many of the aforementioned interaction partners of G9a have been linked to various developmental processes in the adult and it is therefore conceivable that G9a is at least partially responsible for their underlying modes of action.

For example, due to the recruitment of G9a by the global regulator of B-cell maturation Blimp-1 (Gyory et al., 2004), it was suggested that G9a might have a role during the genome-wide transcriptional silencing that happens after the antigenic stimulation of B-cells and their differentiation into antibody producing plasma cells (Martins and Calame, 2008). Interestingly though, G9a deficiency in the B-cell lineage lead to no overt developmental phenotype and only resulted in a modest growth and survival disadvantage upon IL-4 and LPS stimulation (Thomas et al., 2008). It was solely found that G9a−/− B-cells fail to use the Ig λ light chain indicating that G9a-dependent H3K9me2 is dispensable for most but a few very specific processes during B-cell development and maturation. It can be hypothesized that the local reduction of H3K9me2 is often compensated for by alternative chromatin modifications. In such cases, only the compounded deletion of G9a and some of its interaction partners (e.g. Lsd1) might result in a noticeable developmental phenotype.
As noted before, G9a also interacts with Gfi-1 and Gfi-1b, two homologous zinc-finger transcription factors that have profound roles in the hematopoietic system and in particular in the maintenance of hematopoietic stem cell quiescence (Hock et al., 2004a; Saleque et al., 2002). One role for Gfi-1 in HSCs has been suggested to be the regulation of \( p21^{WAF1/CIP1} \) expression. Interestingly, G9a has also been linked to \( p21^{WAF1/CIP1} \) repression, albeit by recruitment through the transcription factor CDP/cut (Nishio, 2004). This relationship might be of particular relevance in my effort to describe a putative role for G9a in hematopoietic stem cell function.

### 3.1.3 Hypothesis

G9a has been suggested to be an abundant co-regulator of transcriptional repression through its H3K9 methyltransferase activity. It is thought that its targeting to localized regions of the genome is directed by physical interactions with numerous transcription factors. Importantly, most if not all of the studies reporting protein interactions of G9a are entirely based on cell lines. Hence, it is likely that these reports only represent snapshots in development or might be peculiar to transformed cell lines. Under the consideration that G9a is widely expressed throughout the body and transcription factors often exhibit tissue-specific expression profiles, it was conceivable that G9a is recruited in a cell-type specific manner to facilitate developmentally-relevant gene expression programmes. Importantly, it was shown just before I started this project, that G9a knockout mice fail to develop through embryogenesis. This indeed seemed to confirm the often suggested involvement of G9a and H3K9me2 in the determination of cellular identities. Interestingly, the embryonic lethality in the reported G9a knockout strain precluded the field from studying its very anticipated role in other developmental models. As a result, the contemporary understanding of the cell-type specific regulation of G9a-dependent target gene repression in a developmental context was (and still is) very limited.

Motivated by this lack of knowledge, it was my aim in this project to take a genetic approach and study the loss-of-function phenotype of \( G9a \) in a powerful and easily manipulatable developmental system. The hematopoietic system had been an
an extremely fruitful paradigm to study transcriptional control of differentiation and has provided us with insight on numerous transcriptional regulators of stem cell biology, tumorigenesis and lineage specification. It thus became my model of choice but required me to generate a conditional knockout allele for G9a in the mouse and breed this allele to blood-specific Cre deleter lines. Considering that G9a had been widely implicated in transcriptional repression, I was expecting that the deletion of G9a in the blood would lead to a number of phenotypes that could be attributed to this suggested role in global gene repression. Without being able to predict very discrete outcomes of the G9a deletion in the blood, I anticipated several possibilities:

1. *Severe and unspecific phenotype.* G9a might be essentially involved in the repression of a significant fraction of the genome, its inactivation might therefore cause global derepression of genes, which would activate cellular failsafe mechanisms and severely impair proliferation and survival. If this response was relatively unspecific and would affect most cell-types, a severe impairment in hematopoiesis should be expected. Given that Dnmt1 (Jackson-Grusby et al., 2001), Setdb1 (Dodge et al., 2004) and Set8 (Oda et al., 2009) are required for cellular survival in this extent, this scenario was not completely unthinkable, especially since G9a−/− embryos and differentiating ES cells had been reported to show signs of elevated apoptosis (Tachibana et al., 2002).

2. *Severe and lineage-specific phenotype.* G9a might be a specific and essential cofactor for one or several transcription factors that are instructing lineage commitment. In this case, a number of hematopoietic lineages could be blocked or delayed in their development. An example for such a phenotype is the transcription factor Ikaros, which is required to develop all lymphoid lineages (Georgopoulos et al., 1994). In a setting, where G9a would be critical for the repression of lineage promiscuous gene expression, an increased developmental plasticity might occur, similar to Pax5−/− B-cells (Cobaleda et al., 2007).

3. *Subtle phenotype at selective stages of hematopoiesis.* In the likely case of widely abundant functional redundancy, G9a expression might be limiting only during very
discrete aspects of hematopoiesis. For example, the patterning and specification of most hematopoietic lineages would remain mostly unaffected but transcription programmes that regulate proliferation, apoptosis or cytokine expression might become deregulated.

Given the difficulty to predict specific outcomes upon G9a abrogation in the blood, it was my aim to take an unbiased genetic approach. It was therefore important to generate a pure C57B/6 conditional G9a knockout strain that would facilitate the use of competitive transplantation assays. With this approach, I would be prepared to study severe or very subtle phenotypes extremely efficiently.
3.2 Results

3.2.1 A conditional knockout allele for G9a in the mouse

Mice homozygous for a null mutation of G9a are stalled in their growth and embryonic development at 8.5 dpc. Hence, in order to be able to study the role of G9a in the adult, I performed a conditional targeting approach of the G9a locus. G9a is a large protein encoded by a gene encompassing 27 exons over a genomic distance of 15.07 Kb.

The G9a gene is located on chromosome 17 in the mouse, a region corresponding to human chromosome 6. Both human and mouse G9a genes are located in close proximity of a cluster of MHC class I genes. In order to avoid adverse immunologic effects with transplanted cells obtained from the resulting conditionally targeted G9a mice, the gene targeting was performed in the pure C57B/6 ES cell line C2. This eliminated the need for extensive backcrossing into a C57B/6 background and simplified the use of C57B/6 congenic mouse strains commonly used in transplantation assays.

To cause a conditional inactivation of the G9a gene, exons 4-20 were to be flanked by unidirectional loxP-sites to allow their deletion upon Cre expression. The targeted region was analogous to the one used in the conventional knockout strategy by Tachibana et al. (2002). Exons 4-20 code for a central part of the G9a protein, spanning amino acids 249 to 976 of the total 1263 aa. The conditionally targeted region includes six ankyrin repeats (aa 737-935), motives that are involved in protein-protein interactions such as the interaction of G9a with Dnmt3a (Epsztejn-Litman et al., 2008) or H3K9me1/2 (Collins et al., 2008). Importantly, the coding of the catalytic SET domain is prevented by a resulting frameshift after the deletion ensuring the functional abrogation of all G9a activities (Figure 13a).
Figure 13: Conditional targeting approach of the murine G9a locus. (a) The targeting was designed to flank exons 4-20 by loxP sites and delete amino acids 249-976 of the long isoform of the G9a protein (G9a-L). Downstream coding was prevented by a resulting frameshift. Correct integration events in C2 ES cells were detected by PCR analysis at the 5’ end of the targeting region and by genomic Southern analysis using a 3’ probe to detect an enlarged HindIII fragment resulting from the insertion of the Neo selection cassette. Genotyping was routinely carried out by a three primer PCR reaction (b).

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**Diagram:**

- **targeted deletion**
  - 249
  - 976
  - Ank
  - SET

- **frameshift**
  - 1
  - 1263

- **G9a-L protein**
  - wildtype (+)
  - neo-floxed
  - floxed (fl)
  - null (-)

- **PCR targeting analysis**
  - primers ① and ②
  - BAC wt H3 H6

- **Southern targeting analysis**
  - HindIII/probe 3'
  - NdeI, BamHI

- **Genotyping PCR**
  - primers ③, ④ and ⑤
  - wildtype (+)
  - floxed (fl)
  - null (-)
3.2.2 Generation of the knockout construct

Similar to my cloning of the Set7/9 targeting construct, all cloning steps in the generation of the targeting construct were performed using a combination of conventional restriction enzyme-mediated recombination of BAC and plasmid vectors and the use of chemically synthesized and annealed DNA oligonucleotides to insert ectopic loxP and restriction sites. The use of any strategy involving PCR amplification was avoided to minimize the risk of point mutations (Figure 14).

Specifically, a genomic fragment of the C57B/6-derived G9a locus was obtained as BAC #RP28-349B4. From this BAC, I sub-cloned two adjacent DNA fragments that together are defined by an EcoRI site 5’ of exon 4 and a KpnI site 3’ of Exon 27 and divided by a ClaI site in exon 18 into pBluescript. The resulting clones were called pBS-EC and pBS-CK, respectively. An oligonucleotide containing a loxP site was inserted into the BamHI site of pBS-EC, resulting in pBS-EC(loxP). In parallel, an oligonucleotide supplying an AgeI and SalI site was inserted into the Ndel site 5’ of exon 21 in pBS-CK was inserted, resulting in pBS-CK(AS). A PGK-Neo expression cassette flanked by two frt-sites and a 3’-loxP site was cloned into the AgeI and SalI sites of pBS-CK(AS), resulting in pBS-CK(Neo.loxP). Finally, the ClaI-KpnI fragment of this plasmid was inserted into the ClaI and KpnI sites of pBS-EC(loxP), yielding the complete targeting vector pBS-E(loxP)(Neo.loxP)K. During the course of the cloning procedure, all introduced loxP and frt sites were sequence-verified to ensure their functionality. The unique KpnI-site at the 3’-end of the targeting region was cut to linearize the targeting vector prior to transfection into ES cells.
**Figure 14: Cloning of the conditional G9a knockout construct.** The G9a locus was sub-cloned from a genomic BAC clone and modified by conventional restriction enzyme mediated recombination. The resulting plasmid was used for the transfection of C2 ES cells.
3.2.3 Introduction of the targeted G9a mutation into ES cells and the derivation of a conditionally mutated mouse strain

The gene targeting of G9a in C2 cells was performed according to standard ES cell electroporation protocols followed by a G418 selection period of 10 days. The screening strategy for correct integrations into the endogenous G9a locus relied on a PCR analysis using one primer annealing external to the 5’ end of the homologous region and one primer annealing to the 5’ loxP site. Subsequently, PCR-positive clones (6/384 = 1.5%) were expanded, frozen and tested for correct integration of the 3’ loxP site by genomic Southern analysis (Figure 13b). Of six PCR-positive clones five clones displayed correct integration at the 3’ end of the targeting construct based on Southern analysis. We injected three different PCR and Southern verified clones into albino C57B/6-Tyr<sup>c-2J</sup>-derived blastocysts. The ES cell derived tissue contribution in the resulting chimeras could therefore be estimated based on the extent of black coat color. As expected, all chimeras with promising ES cell derived tissue contribution were of male gender and were bred to albino C57B/6 females. This provided for the quick assessment of germline transmission based on the presence of black eyes and coat color among the resulting offspring. (The mutation Tyr<sup>c-2J</sup> in the Tyrosinase gene is responsible for the albino phenotype and is recessive to the wildtype allele.) We obtained about 50% black pups from two chimeras (termed 3H3-4 and 3H3-5), derived from the second round of blastocyst injections with ES cell clone # 3H3. (detailed summary in Materials and Methods, section 5.7)

Among the black pups derived from the 3H3-4 and 3H3-5 chimeras, approximately 50% carried the targeted G9a allele including the frt-flanked Neomycin resistance cassette based on PCR genotyping. Further breeding to actb-Flpe transgenic mice caused the deletion of the Neo cassette and generated a functional floxed G9a allele in the subsequent generation. Alternatively, breeding to Pcx-Cre transgenic mice resulted in the deletion of exons 4-20 and the Neo cassette, resulting in a G9a null allele. A three primer genotyping reaction allowed for the efficient discrimination between wildtype, floxed and null alleles. As expected, intercrosses of G9a<sup>+/−</sup> mice did not yield any G9a<sup>/−</sup> offspring (data not shown), indicating the complete
functional abrogation of the G9a gene in the generated null allele and the recapitulation of the previously observed G9a knockout phenotype in Tachibana et al. (2002).

3.2.4 Functional confirmation of the conditional G9a allele in immortalized MEFs

To functionally characterize the conditional knockout allele, I derived MEFs from G9afl/fl embryos with the aim to generate G9a-/− MEFs by expression of the Cre recombinase in those cells. Since Cre expression is toxic in primary MEFs, I decided to first immortalize G9afl/fl MEFs by retroviral transduction with the SV40 large T antigen and take advantage of an inducible Cre recombinase fused to the ligand binding domain of the estrogen receptor (CreERT2) (Figure 15a). Cells that were transduced with the retrovirus expressing the CreERT2 protein, showed no sign of cell cycle arrest or toxicity. However, upon addition of 4-hydroxy-tamoxifen (4OHT) to the medium, a large percentage of the SV40T and CreERT2 positive G9afl/fl MEFs appeared to stall their replication and reached a senescence-like state (data not shown). Subsequent PCR-genotyping revealed an almost complete excision of the floxed G9a allele immediately after 4-OHT administration (Figure 15b) but the increased presence of the unexcised allele after an expansion period of one week. This indicated the inherent leakiness in the Cre-mediated excision in combination with the growth advantage of G9a-retaining cells over G9a-/− MEFs. Since the leakiness most likely resulted from the variegated expression of the CreERT2 transgene, I derived clones of cells from the bulk SV40T and CreERT2 positive G9afl/fl MEFs and tested them for their ability to quantitatively excise all floxed alleles upon 4OHT administration. I obtained two clones, #7 and #9, that met this criterion. From each of those two clones, I could derive five further sub-clones that completely consisted of G9a-/− cells. Western analysis of the G9a-/− cells in comparison to their parental G9afl/fl clones indicated the complete absence of G9a protein and a marked drop in the global levels of H3K9me2, confirming the published findings with previous G9a knockouts (Figure 15c).
Figure 15: Inducible G9a deletion in immortalized MEFs. (a) Primary MEFs were derived from 13.5 dpc embryos and infected with retroviruses encoding SV40T/Neo and CreERT2/puro, respectively. After selection, single cells were seeded in 96 well plates, expanded and duplicated. Excision efficiency upon 4-hydroxy-tamoxifen (4OHT) was tested (b) and two clones (#7 and #9) were used for further analyses. (c) Western analysis of clones #7 and #9 before and after excision indicated the successful abrogation of G9a expression in knockout MEFs. A characteristic drop in global H3K9me2 levels to a remaining 30% was observed in knockout MEFs.
3.2.5 G9a directs the local deposition of DNA methylation in ES cells but not in MEFs

H3K9 methylation and DNA methylation are functionally linked modifications of repressive chromatin structures in mammals (Cedar et al., 2009). Their interrelationship has been widely studied, but is still poorly understood. For example, DNA methylation of centromeric major satellite repeats relies on H3K9me3 catalyzed by the Suv39h1/2 enzymes (Lehnertz et al., 2003). In a related pathway, G9a targets DNA methylation to endogenous retro-elements and a subset of genes in ES cells (Dong et al., 2008; Tachibana et al., 2008). Dynamics in DNA methylation patterns occur predominantly during embryonic and germ cell development. Under normal circumstances, global DNA methylation profiles remain mostly unchanged in the adult but have been connected to epigenetic changes underlying tumorigenesis (Jones and Baylin, 2002). The intriguing observation that G9a is such a prominent regulator of DNA methylation in ES cells, prompted me to investigate whether the same role for G9a in directing global DNA methylation existed in somatic cells. A confirmation of this result could point towards a profound propensity for genomic instabilities and the tendency to develop neoplastic transformations in G9a-/- cells as is the case in mice expressing a hypomorphic version of the maintenance DNA methyltransferase Dnmt1 (Gaudet et al., 2003).

To test whether DNA methylation was changed in somatic cells lacking G9a, HpaII-digested genomic DNA from G9afl/fl and G9a-/- MEFs in comparison to digested DNA from control ES cell lines was subjected to Southern analysis probing for IAP repeats. Interestingly, whereas IAP repeats were substantially hypo-methylated in G9a-deficient ES cells, the degree of methylation on the same loci in MEFs appeared unchanged upon conditional deletion of G9a (Figure 16). In agreement with this finding, Tachibana et al. (2008) state that the DNA methylation status in G9a-/- embryos at a post-implantation stage at 9.5 dpc is unchanged to wildtype controls. Likewise, Suv39h-deficient MEFs do not (unlike ES cells of the same genotype) exhibit any hypomethylation of major satellite DNA repeats (Bernhard Lehnertz, En Li, Thomas Jenuwein, unpublished). It can therefore be concluded that DNA methylation in somatic
tissues is regulated in a manner that is independent of or not entirely dependent on G9a and H3K9 methylation.

**Figure 16: DNA methylation analysis of G9a-deficient ES cells and MEFs.** Genomic DNA from the indicated ES cells and MEFs was digested with MspI (methylation insensitive) or HpaII (methylation sensitive), transferred to nitrocellulose and probed for IAP elements. G9a<sup>−/−</sup> ES cells display a marked reduction of cytosine methylation of IAP elements reminiscent of Dnmt1<sup>−/−</sup> ES cells. In MEFs, no reduction in DNA methylation on IAP elements is detectable.
3.2.6 *G9a* is dynamically expressed in the hematopoietic system

Given my special interest in the hematopoietic system, I set out to determine the relative expression levels of *G9a* in stem cells, progenitors and differentiated cell populations of the blood system. A dynamic expression of *G9a* could potentially point towards its pronounced requirement in the cell-types where the highest expression levels were observed. Since *G9a* had been mostly investigated in ES cells, we compared its expression in the investigated hematopoietic populations to R1 ES cells. To obtain purified hematopoietic cell-types, we used previously defined marker profiles of hematopoietic stem cells, myeloid and lymphoid progenitors as well as mature lineage marker positive cells from different hematopoietic organs to prospectively FACS-sort these cell populations (Table 4).

**Table 4: Summary of sorted hematopoietic populations used for G9a expression profiling.** All sorts were resorted and purity-controlled.

<table>
<thead>
<tr>
<th>source</th>
<th>population</th>
<th>surface phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>BM</td>
<td>hematopoietic stem cells, <strong>HSC</strong></td>
<td>(Ter119, B220, CD3, Gr1, Mac1)^−;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sca-1^+, c-kit^+</td>
</tr>
<tr>
<td>BM</td>
<td>common myeloid progenitor, <strong>CMP</strong></td>
<td>(Ter119, B220, CD3, Gr1, Mac1)^−;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sca-1^−, c-kit^+, FcyR^low, CD34^+</td>
</tr>
<tr>
<td>BM</td>
<td>granulo-monocytic progenitor, <strong>GMP</strong></td>
<td>(Ter119, B220, CD3, Gr1, Mac1)^−;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sca-1^−, c-kit^+, FcyR^high, CD34^+</td>
</tr>
<tr>
<td>BM</td>
<td>megakaryocyte-erythrocyte progenitor, <strong>MEP</strong></td>
<td>(Ter119, B220, CD3, Gr1, Mac1)^−;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sca-1^−, c-kit^+, FcyR^low, CD34^+</td>
</tr>
<tr>
<td>thymus</td>
<td>double-negative (CD4/CD8)^−/^+, <strong>DN1/2</strong></td>
<td>(Ter119, B220, NK1.1, Gr1, Mac1,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TCRα, TCRγδ)^−, c-kit^+</td>
</tr>
<tr>
<td>thymus</td>
<td>double-negative (CD4/CD8)^−/^+, <strong>DN3/4</strong></td>
<td>(Ter119, B220, NK1.1, Gr1, Mac1,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TCRα, TCRγδ)^−, CD4^−, CD8^+</td>
</tr>
<tr>
<td>thymus</td>
<td>double-positive (CD4/CD8)^+, <strong>DP</strong></td>
<td>(Ter119, B220, NK1.1, Gr1, Mac1,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TCRα, TCRγδ)^−, CD4^+, CD8^+</td>
</tr>
<tr>
<td>source</td>
<td>population</td>
<td>surface phenotype</td>
</tr>
<tr>
<td>--------------</td>
<td>---------------------</td>
<td>----------------------------------------</td>
</tr>
<tr>
<td>spleen</td>
<td>helper T cells, CD4</td>
<td>(Ter119, Gr1, Mac1)^-; CD4^+, CD8^-, B220^+</td>
</tr>
<tr>
<td></td>
<td>cytotoxic T-cells, CD8</td>
<td>(Ter119, Gr1, Mac1)^-; CD4^-, CD8^+, B220^-</td>
</tr>
<tr>
<td></td>
<td>B-cells, B220</td>
<td>(Ter119, Gr1, Mac1)^-; CD4^-, CD8^-, B220^+</td>
</tr>
<tr>
<td>peripheral</td>
<td>granulocytes, Gr1</td>
<td>(CD3, B220)^-; Gr1^+, Mac1^{all}</td>
</tr>
<tr>
<td>blood</td>
<td>macrophages, Mac1</td>
<td>(CD3, B220)^-; Gr1^-, Mac1^+</td>
</tr>
</tbody>
</table>

After RNA extraction and cDNA synthesis from the isolated material, we quantitatively determined G9a expression levels normalized to GAPDH expression in the same samples using RT-PCR analysis. Interestingly, we found that G9a expression is dynamically regulated between many of the investigated populations (Figure 17). In particular, we found that its expression levels are the highest in early hematopoietic cells, such as HSCs or myeloid progenitors. Of note, expression in these cell types is comparable to the one observed in ES cells, where G9a function has been widely explored. We interpreted this finding as an indication that G9a might be particularly important during early stages of hematopoiesis.
3.2.7 Hematopoietic G9a-deficiency is dispensable for mouse development and survival

Considering its severe embryonic phenotype, it has been proposed that G9a acts as an epigenetic regulator of gene expression during development. To expand our knowledge of G9a in other developmental systems and in an in vivo context, I set out to study the hypothesized role for G9a during hematopoiesis. To accomplish the abrogation of G9a expression in the blood, I crossed the conditional G9α knockout allele to the inducible and constitutive hematopoiesis-specific Cre deleter strains Mx-Cre and Vav-Cre, respectively. (Our laboratory obtained the Vav-Cre strain at a much later time-point during this project. Therefore, most of the presented data in this chapter was obtained from G9αfl/−, Mx-Cre mice and their appropriate controls unless otherwise stated. It should be noted however, that both Cre strains yielded the same results in all thus far performed experiments.) Induction of Cre in Mx-Cre mice is dependent on the administration of pIpC, a double-stranded RNA analogon that mimics viral RNA and triggers an interferon response and the transient activation of the Mx-promoter in all

Figure 17: Relative expression levels of G9a in the hematopoietic system. Bone marrow, thymus and spleens of 8 week old wildtype mice were harvested and prepared for FACSorting (see table 4). Quantitative RT-PCR was performed in triplicate for G9a and GAPDH. The chart shows mean values and standard deviations of G9a mRNA relative to GAPDH and normalized to G9a expression in R1 ES cells. Error bars indicate standard deviations.
blood cells (Kühn et al., 1995). Since it was not known whether G9a-deficiency in the blood would permit the development or survival of the animals, the crucial advantage in this approach was that $G9a^{fl/-}$, $Mx$-$Cre$ mice would develop normally and the gene knockout could be induced under homeostatic conditions.

To induce Cre expression and the $G9a$ knockout, $G9a^{fl/-}$, $Mx$-$Cre$ and control $G9a^{fl/+}$, $Mx$-$Cre$ mice were injected three times intra-peritoneally with 400 µg of plpC at an age of 4-8 weeks (Figure 18a). Interestingly, Cre induction in these mice did not result in death of the animals. In order to assess the efficiency of the $G9a$ knockout induction, the plpC-treated $G9a^{fl/-}$, $Mx$-$Cre$ and $G9a^{fl/+}$, $Mx$-$Cre$ control mice were sacrificed and their bone marrow and thymi were harvested. To accumulate sufficient material for western analysis of G9a and H3K9me2 protein levels, I derived macrophages by bone marrow culture in L-cell conditioned medium. When performing genotyping-PCR analysis on genomic DNA originating from these bone marrow-derived macrophages, I found that the Mx-Cre-mediated disruption of the $G9a$ gene occurred at a very high efficiency (Figure 18b).

Consistent with this observation, western analysis of thymocytes and macrophages revealed that upon $Mx$-$Cre$ induction, $G9a$ expression was quantitatively abrogated (Figure 18c). When analyzing the levels of H3K9me2 in the same samples, I consistently observed the characteristic drop of this modification in macrophages but not as overtly in thymocytes indicating that the relative contribution of G9a towards global H3K9me2 levels varies among different cell types.

As indicated above and despite my initial expectation, the induced knockout mice showed no sign of sickness for up to 6 months post plpC administration, indicating that G9a is not vitally required in the adult hematopoietic system under homeostatic conditions. Since conventional $G9a$ knockout mice arrest in their development before the first hematopoietic cells emerge in the embryo, it was still conceivable that G9a might be essential for hematopoiesis at earlier stages of development. I addressed this hypothesis by crossing the $G9a$ allele to the blood-specific and constitutively active $Vav$-$Cre$ strain. Complementing the initial result, $G9a^{fl/-}$, $Vav$-$Cre$ mice were born at normal Mendelian ratios and without any overt developmental retardation, suggesting that G9a is not essential for hematopoietic development and homeostasis. Importantly, it was
previously shown that the Vav-Cre deleter is efficient enough to preclude development in conditionally targeted Rac1 mice (Ghiaur et al., 2008), excluding the possibility of leakiness in this strain.

Figure 18: Inducible G9a deletion in hematopoietic lineages. (a) Cre expression was induced in G9a<sup>fl/+</sup>, Mx-Cre<sup>+</sup> controls and G9a<sup>fl/-</sup>, Mx-Cre<sup>+</sup> mice by intra-peritoneal pIpC administration. (b) The efficiency of Cre-mediated excision was assessed by genotyping PCR of the targeted G9a locus in cultured macrophages (MØ). (c) Protein levels of G9a and Glp in control and knockout macrophages and thymocytes were assessed by western analysis. Although G9a and Glp are not detectable in macrophages, global H3K9me2 levels drop significantly in G9a<sup>-/-</sup> samples. G9a expression is efficiently abrogated in knockout thymocytes.
3.2.8 G9a is not appreciably involved in hematopoiesis under homeostatic conditions

Despite the absence of an overt phenotypic alteration in mice that lack G9a expression in the hematopoietic system, it appeared still plausible to me that G9a-deficient HSCs and progenitors might display subtle impairments in the production of blood cells. As discussed earlier, competitive bone marrow transplantation assays represent the most sensitive approach to detect relative disadvantages in knockout versus wildtype hematopoiesis. I therefore performed competitive transplants with G9a\textsuperscript{fl/-}, Mx-Cre and G9a\textsuperscript{fl/+}, Mx-Cre mice and, similar to the previously described competitive bone marrow transplants involving the Set7/9 knockout mice, I used CD45.1\textsuperscript{+} and CD45.2\textsuperscript{+} congenic mouse strains to trace hematopoietic cells originating from different competitor bone marrow. More specifically, I competitively transplanted CD45.2\textsuperscript{+} G9a\textsuperscript{fl/+}, MxCre control or G9a\textsuperscript{fl/-}, MxCre bone marrow at 50:50 ratios with CD45.1/2 positive wildtype bone marrow into CD45.1 positive recipients (Figure 19a). To generate the G9a knockout in the targeted cells, Cre was induced in the recipient mice six weeks after the competitive BM transplant. This strategy allowed (a) for a direct comparison between lineage-specific pre- and post-induction ratios of competitor BM in the same recipients and (b) assured that both CD45.2 test and CD45.1/2 competitor cells were exposed to the exact same dosages of pIpC in the competitively reconstituted animals.

I used multi-color FACS analysis to trace the relative contribution of CD45.2\textsuperscript{+} targeted cells and CD45.1/45.2\textsuperscript{+} competitor cells among the donor derived cells in the recipient exactly as described in the competitive Set7/9 bone marrow transplant experiments (Figure 20a). As expected, my analysis of peripheral blood leukocytes at six weeks post-transplant indicated that both G9a\textsuperscript{fl/+}, Mx-Cre and G9a\textsuperscript{fl/-}, Mx-Cre bone marrow in relation to their respective CD45.1/2 wildtype competitor bone marrow contributed equally to around 30-50% across all tested hematopoietic lineages (Figure 20b). After the six week analysis time-point, all recipients received three injections of pIpC to induce the knockout of G9a in the G9a\textsuperscript{fl/-}, Mx-Cre cells and the generation of heterozygous blood and BM cells in the G9a\textsuperscript{fl/+}, Mx-Cre control cells. Genotyping analysis of bulk bone marrow from the competitively reconstituted recipients indicated that upon
Cre induction most of the floxed alleles were indeed excised, assuring the validity of the experimental results (Figure 19b).

Since most hematopoietic progenitors have a lifespan of less than 6 weeks, I waited for 8 weeks after Cre induction to re-assess the relative lineage contribution of test and competitor cells after the knockout-induction in G9a\(^{-/-}\) cells. To my surprise, I could not detect any significant change in the relative contribution of G9a\(^{-/-}\) bone marrow compared to either pre-excision or G9a\(^{-/-}\) controls at eight or 32 weeks post induction (Figure 20b). This indicated that G9a is not appreciably required for hematopoietic development under homeostatic conditions.

**Figure 19:** Experimental strategy to competitively assess G9a-deficient hematopoiesis. (a) Bone marrow from CD45.2\(^+\), G9a\(^{+/+}\), Mx-Cre\(^+\) controls or G9a\(^{+/-}\), MxCre\(^+\) mice was mixed with wildtype CD45.1/45.2\(^+\) at a 50:50 ratio and transplanted into lethally irradiated CD45.1\(^+\) recipients. At six weeks post-transplant, all recipient mice received three injections of pIpC to induce Cre expression. Peripheral blood leukocytes (PBL) were analyzed 8 and 32 weeks later. The bone marrow from two control and two knockout BM recipients was serially transplanted into secondary CD45.1\(^+\) recipients and the relative contribution of control G9a\(^{+/-}\) and G9a\(^{-/-}\) was assessed by PBL analysis at the indicated time-points. (b) Bone marrow from primary and secondary recipients was subjected to PCR genotyping of the excised G9a locus. All examined recipients exhibited efficient excision of the targeted allele as indicated by the overall absence of the floxed PCR products. The wildtype band originates from competitor and recipient cells.
Figure 20: Continuing myelopoiesis and lymphopoiesis in the induced absence of G9a. (a) The relative contribution of donor cells before and after plpC administration was monitored by the expression of CD45.1 and CD45.2 antigens on PBL. Donor-derived CD45.2+ granulocytes, macrophages, B and T cells were analyzed for the respective contribution of CD45.1- test and CD45.1+ competitor cells. (b) The knockout induction of G9a resulted in no difference in the development of lineage marker positive cells over as long as 32 weeks post plpC administration. Error bars indicate standard deviation, no statistical differences were observed.
3.2.9 G9a plays a role during the engraftment of hematopoietic stem cells following bone marrow transplantation

The functionality of hematopoietic stem cells following a bone marrow transplantation is limited by a number of factors that have effects on their engraftment efficiency. In order to address the performance of G9a-deficient HSCs during their engraftment or expansion, I serially transferred whole bone marrow from two primary recipients of both the control G9a+/+ and the G9a−/− groups into lethally irradiated secondary recipients.

In analogy to the primary transplant analysis, I performed FACS analysis of peripheral blood leukocytes after a long-term engraftment period at six and twelve weeks post transplant. Surprisingly, I observed a significant reduction in CD45.2+ G9a−/− peripheral leukocytes in comparison to their G9a+/+ controls which was reflected in all four major hematopoietic lineages (Figure 21). Given the lack of any noticeable disadvantage in the primary recipients after the knockout induction, I conclude that HSC’s specifically require G9a for the process of hematopoietic engraftment following a bone marrow transplantation.

Several knockout strains have been described to display competitive disadvantages in serial transplants experiments. For example, mice deficient in p21CIP1/WAF1 (Cheng et al., 2000) or Foxo3a (Miyamoto et al., 2007) display HSC defects that do not become noticeable before secondary bone marrow transplantations.

To test whether the observed reconstitution defect in G9a knockout HSCs is also detectable in primary transplants, I treated G9afl/+, Mx-Cre control and G9afl/−, Mx-Cre mice with pIpC as before and transplanted equal numbers of lineage−, Sca-1+, c-kit+ (LSK, 1000-fold enriched HSCs) from these mice in competition with wildtype CD45.1/45.2 competitor bone marrow in a 1:1000 ratio into lethally irradiated recipient mice. Exactly like in my previous experiments, I performed FACS analysis on peripheral blood leukocytes to determine the relative lineage contribution of the competitively transplanted donor cells after long-term reconstitution. Interestingly, I observed a relative reconstitution disadvantage in G9afl/−, Mx-Cre mice compared to the G9afl/+, Mx-Cre control group, resembling the result from the previous post induction secondary
transplant experiment (Figure 22). This result confirmed that HSCs require G9a during the hematopoietic reconstitution process that follows a bone marrow transplantation.

**Figure 21:** Impaired HSC function in serially transplanted G9a-deficient bone marrow. The effect of G9a loss on the function of HSC’s was studied by serial bone marrow transplantation. Contribution of the gene targeted cells to lineage marker positive PBL was assessed by FACS analysis exactly as before. In comparison to G9a+/- controls, G9a-/- bone marrow was highly significantly compromised in the contribution to all major hematopoietic lineages. Error bars indicate standard deviations, statistical significances were calculated with two-tailed student’s t-test and are indicated by asterisks. * is p<0.05, ** is p<0.01.

**Figure 22:** Impaired function in competitively transplanted G9a-deficient HSCs. The effect of G9a loss on the function of HSCs was studied by the transplantation of Lin-, c-Kit+, Sca-1+ (LSK) bone marrow cells (a) in competition with CD45.1/45.2+ unfractionated BM cells (2000 KLS: 2 million WBM competitor cells per recipient). Contribution of the gene targeted cells to Lin+ PBL was assessed by FACS analysis exactly as before. Compared to G9a+/- controls, G9a-/- bone marrow was compromised in the contribution to all major hematopoietic lineages. (b) shows PBL contribution at 36 weeks post-transplant and is representative of earlier time-points. Error bars indicate standard deviations, statistical significances were calculated with unpaired two-tailed student’s t-test: * is p<0.05, ** is p<0.01.
3.2.10 G9a-deficient HSCs are underrepresented in the bone marrow after transplantation

Like many other somatic stem cells, hematopoietic stem cells are mostly quiescent and primarily go into cell cycle and expansion in response to injury-like circumstances like viral infections or bone marrow transplantation. In the absence of such insults, it is thought that HSC quiescence minimizes the risk of accumulating mutations that can be tumorigenic or otherwise detrimental to the hematopoietic system.

To complement my investigation of the intrinsic requirement of G9a in HSCs during transplantation, I decided to analyze and quantify the HSC pool in the reconstituted recipients from my previous experiments. To this end, I stained whole bone marrow of these mice with a combination of antibodies that allowed me to phenotypically quantify HSC and myeloid progenitor containing populations by their combinatorial expression of Sca-1 and c-kit within the lineage negative fraction of the G9a-targeted CD45.2+ control and knockout cells.

When comparing the Sca-1 and c-kit profiles within the lineage negative population, I detected very significant differences between G9a-/− and control bone marrow in the single Sca-1 or c-kit populations (Figure 23). Less obvious differences could be found in the HSC containing Lin−, Sca-1+, c-kit+ (LSK) population. However, it appeared that, compared to the controls, phenotypic HSCs had a tendency to be less frequent in G9a-/− bone marrow. It can be suggested from this data that G9a-deficient HSCs are impaired in their ability to engraft or expand after transplantation. It appears likely that the relative reduced contribution of G9a-/− bone marrow is due to this defect in HSC engraftment or expansion in the competitively transplanted animals. However, more conclusive experiments are necessary to address this hypothesis more accurately.

It is well established that all myeloid and erythroid progenitor activities can be found in the Lin− c-kit+ population (Akashi et al., 2000). Interestingly, this Lin− c-kit+ population was markedly increased among G9a-/− derived bone marrow cells, whereas the Lin− Sca-1+ population, a far less characterized fraction, displayed a dramatic decrease in cell numbers. Importantly, it is known that upon transplantation, both the
Sca-1 and c-kit antigen distribution drifts substantially (Yilmaz et al., 2006), making the interpretation about the differences between \( G9a^{-/-} \) and the control bone marrow cells difficult. Given that knockout and test cells were transplanted in competition with wildtype cells and into a wildtype environment, it appears however, that G9a-deficiency results in a cell-autonomous difference in either the expression levels of the c-kit and Sca-1 antigens or in the selective expansion and loss of the respective Lin-; single positive sub-populations.
Figure 23: Altered expression of stem cell markers in transplanted G9α−/− bone marrow. FACS analysis of stem cell and progenitor populations in competitively reconstituted G9α+/− and G9α−/− bone marrow. Whole bone marrow was stained with CD45.1-PE, CD45.2-FITC, lineage-PECy7 (Mac1, GR1, B220, CD3, Ter119), Sca1-APC and c-kit-PECy5. (a) Donor-derived CD45.2+ cells, G9α-targeted CD45.1− and lineage neg. cells were gated and analyzed for the presence of Sca1 and c-kit antigens. (b) Summary of the above FACS analysis. Compared to G9α+/− controls, G9α−/− BM cells are greatly reduced and contain increased percentages of c-kit+ but decreased percentages of Sca1+ cells within the lineage neg. compartment. The HSC containing Lin−, Sca1+, c-kit+ population is mildly reduced in G9α−/− cells. Error bars indicate standard deviations, statistical significances were calculated with two-tailed student’s t-test and are indicated by asterisks. * is p<0.05, ** is p<0.01, *** is p<0.001.
3.2.11 G9a-deficient progenitors display a dramatic impairment in their proliferative potential

To ensure the constant and sufficient generation of new blood cells, transient amplifying progenitors exhibit the biggest proliferation capacity in the hematopoietic system. These progenitors respond to cytokines and can be expanded under appropriate conditions in vitro.

Since the transplantation assays that I presented above, provide (at least in short term) a combined functional readout of stem cells and progenitors, I used a complementary assay to test the developmental and proliferative capacity of bone marrow residing progenitors of the myeloid system. In the shown experiment, I sorted CD45.2+ G9a+/− and G9a−/− or bone marrow from the competitively reconstituted recipients in the same FACS analysis of the previous experiment shown in Figure 23.

As shown in Figure 24a, I plated the sorted whole bone marrow in triplicate into a methylcellulose-based medium containing the cytokines SCF, IL-3, IL-6 and Epo. All myeloid and erythroid progenitors respond to these cytokines, and over a time-period of 8-10 days, give rise to colonies that, based on their morphologies, can be traced back to a particular progenitor type.

When comparing the number and size of the obtained colonies from G9a+/− or G9a−/− bone marrow, it became very clear that G9a deficiency resulted in a dramatic reduction in the number of colonies (Figure 24b), the total cell-number obtained per plate (Figure 24c) and importantly also in the mean number of cells per colony (Figure 24d). This suggests that in G9a−/− bone marrow, a reduced number of progenitors is present and that these fewer progenitors also are severely compromised in their proliferative capacity.

Given the small size of the obtained colonies in the absence of G9a, it was not possible to clearly categorize these colonies. Instead, I used FACS analysis to characterize the identity of emerging differentiation markers on the progenitor cultures at day 10. In both G9a+/− and G9a−/− samples the predominant fraction of the cells were Gr1-positive. Interestingly, a trend in G9a−/− cells indicted a slight reduction of the Gr1+−-fraction.
To confirm whether the same impairment in myeloid progenitors was present upon Vav-Cre-mediated deletion and in non-transplanted animals, I repeated the same experiment with bone marrow harvested from a \(G9a^{fl/+}, \text{Vav-Cre}\) and a \(G9a^{fl/fl}, \text{Vav-Cre}\) yielding \(G9a^{+/+}\) and \(G9a^{-/-}\) bone marrow cells, respectively. Figure 24f shows representative mixed colonies derived from \(\text{Vav-Cre}^{+}, G9a^{+/+}\) and \(G9a^{-/-}\) bone marrow, respectively. Importantly, all above discussed findings could be confirmed, indicating the functionality of the \(\text{Vav-Cre}\) strain and that the impairment in progenitor function in the absence of G9a also occurs in non-transplanted cells.
Figure 24: Impaired progenitor function in G9a-deficient bone marrow. (a) CD45.2+ bone marrow cells were sorted from competitively reconstituted mice and plated into SCF, IL-3, IL-6 and Epo-containing progenitor cultures. After ten days, G9a+/−,Mx-Cre bone marrow yielded a severe reduction in (b) colony numbers, (c) total cell number, (d) cells per colony. A slight reduction in the percentage of Gr1+ cells was observed (e). Similar results were obtained from non-transplanted G9a−/−,Vav-Cre bone marrow (f). Error bars indicate standard deviations, statistical significances were calculated with two-tailed student’s t-test and are indicated by asterisks. * is p<0.05, ** is p<0.01.

(a) FACS sort G9a +/- or G9a +/- CD45.1/-/CD45.2+

(b) colony number

(c) cell number

(d) cells per colony

(e) % of GR1 positive

(f) G9afl/+, Vav-Cre+ or G9afl/fl, Vav-Cre+

bone marrow

day 8, mixed colony

G9a+/- (Vav-Cre) 5x

G9a-/-(Vav-Cre) 5x
3.3 Discussion

3.3.1 The generation of a conditional G9a knockout strain

To directly investigate the role of G9a in the adult hematopoietic system, generating a conditional G9a knockout strain represented the most promising approach. Importantly, the functionality of the generated G9a allele was confirmed by the fact that, when interbreeding G9a\textsuperscript{flo} or G9a\textsuperscript{+/+} mice, I never observed the emergence of G9a\textsuperscript{−/−} pups, confirming the reported inability of G9a\textsuperscript{−/−} mice to develop beyond mid-gestation. Furthermore, western analysis of G9a-deficient MEFs in comparison to their parental G9a\textsuperscript{flo/flo} controls demonstrated that the G9a gene product was indeed absent upon Cre-mediated deletion and that global H3K9me2 dropped to about 30% of its original degree.

A significant advantage but also complication when using conditional mouse models is the necessity to select one or a few specific Cre-deleter strains. This determines and restricts the tissue in which the analyses is undertaken. If the role of the gene of interest is to be analyzed in different tissues, it is necessary to obtain additional deleter lines and cross the targeted allele with these strains. This poses a significant logistic effort. Importantly though, my derivation of a conditional G9a allele in the mouse has paved the way for many future studies that aim to investigate the biological significance of G9a in different tissues. In light of the fact, that our laboratory is very well equipped to analyze hematopoietic development and my personal interest lies in this system for reasons that were discussed earlier, my work using the conditional G9a allele has so far mostly focused on the blood system. It is noteworthy that currently, a number of additional projects are underway to characterize the roles of G9a in other developmental but also immunological paradigms. Whereas some of these projects are being worked on in our own lab, others are the focus of collaborators to whom we sent the conditional G9a strain. My discussion will therefore focus primarily on the role of G9a in the blood system, but will also set my findings in context with roles of G9a that seem to emerge from other systems.
3.3.2 A role for G9a in the hematopoietic system

My analyses of the induced or constitutive G9a-deficiency in the blood system has revealed a remarkably specific role for G9a during hematopoiesis. I found that G9a is not essential for the life-sustaining function of the hematopoietic system through development and adulthood. Moreover, blood-specific G9a knockout mice were not distinguishable by any gross phenotype, and G9a-deficient hematopoiesis was not appreciably compromised under homeostatic conditions. Strikingly however, during conditions that mimic stress situations in the hematopoietic system, G9a-dependent phenotypes became very evident. This finding shows that G9a is not involved in hematopoietic development per se, but plays an important role under very discrete circumstances during hematopoietic regeneration. Importantly, this result was not predictable from the existing literature and thus will serve as important information to delineate the roles of G9a in other regenerating tissues.

While my general conclusion of G9a-involvement in hematopoiesis appears reasonable, it is important to note that the characterization of G9a-deficient hematopoiesis still requires more attention to depict the exact mechanisms underlying the specific HSC and progenitor defects. For example, although a proliferative defect in hematopoietic progenitors seemed very obvious ex vivo, my current understanding of the relative defect in the ability of G9a-/- HSCs to repopulate the hematopoietic system is still incomplete. In theory, all functional stages that comprise the process of hematopoietic reconstitution could be affected and thus still need to be addressed. These stages include homing to the HSC niche, the expansion of the stem cell pool after engraftment and the differentiation capability of the stem cells. In future experiments, it will be necessary to delineate in which of these stages the G9a-deficiency becomes noticeable. Short-term bone marrow homing assays or better, parabiosis experiments should be able to detect potential disadvantages in G9a-/- HSCs to migrate efficiently into their physiological niches.

However, considering that G9a-/- progenitors display such a marked reduction in their proliferation potential, it seems convincing that G9a-/- HSCs are similarly impaired. This could explain why under homeostatic conditions, where HSCs are mostly dormant,
no competitive disadvantage in the absence of G9a is evident. Following injury and
during the process of repair however, in my experiments modeled by bone marrow
transplantation, HSCs are required to enter the cell cycle and through symmetric
divisions, replenish the HSC pool in the recipient animals. It is therefore plausible that
G9a-deficiency is specifically important during the HSC expansion that follows a bone
marrow transplantation.

Interestingly, I observed a significant change in the expression of Sca-1 and c-kit
in lineage negative cells in \( G9a^{-/-} \) bone marrow. In future experiments, it will be
interesting to address this observation more specifically. It appears that in the absence
of G9a, immature cells in the hematopoietic system undergo intrinsic compensatory
mechanisms that are reflected in the expression of these stem cell markers. In
particular, since c-kit has a role in sensing and transmitting external growth-stimulatory
signals like steel factor (SCF), it appears plausible that the increased expression of c-kit
is occurring to compensate for the intrinsic proliferative impairment of \( G9a^{-/-} \) cells.
Overall, it is of note that the change of Sca-1 and c-kit expression is detrimental to my
ability to phenotypically assess HSC numbers in \( G9a^{-/-} \) bone marrow. Future
experiments are thus aimed to use SLAM molecule based stem cell markers (Kiel et al.,
2005) to determine the abundance of HSCs in un-transplanted and transplanted \( G9a^{-/-} \)
bone marrow more reliably.

Moreover, to confirm that \( G9a^{-/-} \) HSCs are impaired in their proliferation upon
injury, I will attempt to expand this notion by causing stem cell mobilization by
alternative means. For example, treatment with the chemotherapeutic 5-fluoro-uracil (5-
FU) depletes cycling hematopoietic cells and in turn forces the HSCs to enter the cell
cycle and help in the regeneration process. Serial 5-FU treatment in mice applies
constant stress to the hematopoietic system and is an efficient way to measure its
regenerative capacity (Wilson et al., 2008). If G9a-deficiency indeed results in the
impairment of HSCs expansion, \( G9a^{-/-} \) mice should display an increased sensitivity in
this experiment and succumb to the sequential treatment earlier than their controls. In a
related experiment, a one-time administration of 5-FU might be sufficient to lead to the
imbalance of blood cell contribution in competitively transplanted \( G9a^{-/-}, Mx-Cre \) bone
marrow where the knockout has been induced after transplantation.
It is known that in reconstituted mice, the HSC pool never reaches its original size, indicating that wildtype HSCs have a limited expansion capacity \textit{in vivo}. Interestingly though, a number of factors have been found to promote the expansion of HSCs \textit{in vivo}. The best described example of such a factor is HoxB4. When ectopically expressed in bone marrow cells, HoxB4 causes the increased proliferation of HSCs and results in a faster and more efficient hematopoietic engraftment (Antonchuk et al., 2001). If G9a is indeed required for HSC and progenitor expansion, it is possible that ectopic expression of HoxB4 can revert this phenotype in $G9a^{-/-}$ bone marrow transplants.

Moreover, it has been shown in a number of instances, that genes that have important roles in the proliferation and survival of HSCs also have similar roles in maintaining the self-renewing properties of leukemic stem cells. For a therapeutic application, it would be extremely valuable to identify drug targets that are more profoundly required by leukemia-initiating cells than by normal HSCs. Given that G9a is seemingly only required for activated and proliferating HSCs, it will be extremely interesting to investigate whether constitutively proliferating leukemic stem cells rely G9a to sustain the disease. Our future experiments are aimed to use leukemia-inducing retroviruses to generate wildtype and $G9a^{-/-}$ neoplasms from normal HSCs and test those in their ability to give rise to leukemia in mice. If G9a is required for the transplantability of leukemia-initiating cells, its pharmacological inhibition might represent a way to specifically target leukemic stem cells without depleting the normal HSC pool in leukemia patients. In summary, a number of experiments are still necessary to determine the exact mechanisms underlying the G9a-dependent regulation of HSC proliferation.

Moreover, under the consideration that G9a is responsible for such an abundant chromatin mark and the fact that deficiency in the B-cell lineage results in a mild phenotype in immunoglobulin rearrangements, it appears plausible that a number of thus far undetected hematopoietic phenotypes will be demonstrated in future studies using the generated allele. In particular, it is conceivable that loss of G9a may lead to aberrant expression of immunologically relevant genes such as cytokines, resulting in a
deregulated immune response under specific circumstances. To explore this possibility, we are currently collaborating with immunologists in our institute.

Furthermore, it can be hypothesized that even after the global reduction of H3K9me2 levels in G9a-deficient cells, other chromatin-modifying activities may act as compensatory mechanisms that are largely sufficient to maintain G9a-dependent gene repression. In a similar observation, although the polycomb-dependent contraction of an imprinted gene locus is lost in Ezh2−/− embryos, its repression is still maintained by H3K9me, possibly catalyzed by G9a (Terranova et al., 2008). This suggests that partial redundancy or synergy of different silencing systems are widespread mechanisms in mammalian chromatin. The observation that DNA methylation on endogenous retro-elements and G9a-repressed genes is absent in embryonic stem cells but is later established in an alternative way also supports this hypothesis and is proof for a very interwoven network of compensatory players that regulate chromatin structure. Given its physical association with DNA methyltransferases (Esteve et al., 2006; Epsztejn-Litman et al., 2008), histone deacetylases and the H3K4me-specific histone demethylase Lsd1 (Shi et al., 2003), possible redundant activities for G9a are likely among but not restricted to these factors. Therefore, the compounded pharmacological or genetic inactivation of one or more repressive chromatin regulators in combination with G9a are promising avenues of future research.

Furthermore, it is often suggested that hematopoietic stem cells, in comparison to their more differentiated counterparts are characterized by a more promiscuous gene expression profile. The basis for this expression promiscuity may lie in a more plastic chromatin structure and the overall underrepresentation of repressive chromatin signatures similar to what has been found in ES cells. Since H3K9me2 appears to be one of these repressive marks in mammalian euchromatin, it will be interesting to investigate whether G9a is involved in the silencing of HSC specific genes and whether HSCs that lack G9a can be more easily maintained in their undifferentiated state. Indeed, the combined treatment of early human HSCs with the HDAC inhibitor Trichostatin A (TSA) and 5-aza-deoxycytidine has been shown to help maintain their stemness in vitro (Milhem et al., 2004). It is conceivable that G9a inactivation on its own or in combination with either one of these drugs may cause the same differentiation
block *in vitro*. Under the consideration, that a specific G9a inhibitor, BIX01294 is available (Kubicek et al., 2007), this result would be extremely promising for the improvement of *in vitro* culture conditions of early human bone marrow cells before bone marrow transplantation.

### 3.3.3 What alterations in gene expression can be observed in G9a<sup>-/-</sup> cells?

Given its suggested role in gene repression that is mainly described in ES cells, it can be assumed that the observed phenotype of G9a-deficient HSC engraftment and progenitor function can be attributed to the misexpression of a number of genes in these cells. Interestingly, *G9a<sup>-/-</sup>* MEFs are also characterized by the deregulation of a number of genes (Sampath et al., 2007). Importantly however, the set of aberrantly expressed genes in ES cells differs significantly from that in MEFs, indicating that G9a-dependent gene repression, and its derepression upon G9a-deletion are mostly cell-type specific. This notion can be explained by a model in which G9a (like other chromatin modifying activities) are recruited to their target sites by specific transcription factors. These transcription might exhibit tissue-specific expression patterns and thus be crucial in the determination of cell-type specific gene expression. It cannot be excluded however, that G9a is involved in the regulation of specific genes across different cell-types.

With this conclusion, it should be very interesting and informative to assess the alteration of gene expression in G9a-deficient hematopoietic cells on their own and in comparison to other cell-types. To pursue this aim, future experiments are planned to perform genome-wide expression profiling in purified HSC and progenitor populations of G9a<sup>-/-</sup>, Vav-Cre mice in comparison to their wildtype controls. A global analysis of G9a-dependent gene expression in these cells should be very helpful in the interpretation and further characterization of the phenotype in G9a<sup>-/-</sup> HSCs and progenitors.
3.3.4 G9a phenotypes in other developmental paradigms

Recent experiments in our lab have expanded our focus on additional developmental models to investigate the role of G9a. One of these systems relies on the conditional deletion of the floxed G9a alleles in tissues and cells that are derived from the neural crest by virtue of the Wnt1-Cre deleter strain (Danielian et al., 1997). The neural crest is a cell population that emerges from the neural plate border during embryogenesis and is characterized by its multi-potentiality and migratory properties. It gives rise to diverse cell-types and tissues ranging from facial bone structures to the peripheral nervous system and melanocytes (Sauka-Spengler and Bronner-Fraser, 2008). Our preliminary results indicate that in the absence of G9a, all tissues that are derived from the neural crest are still specified. This confirms the notion from my experiments in the hematopoietic system in that G9a seems to be dispensable for the determination of different cell-types from common precursor cells. As a result, G9a^fl/fl, Wnt1-Cre animals are viable but interestingly, display a marked growth retardation in neural crest derived tissues after birth. This result is remarkably reminiscent of the situation in the blood (and perhaps in the developing embryo) and suggests that G9a is critical for the expansion of stem cells and progenitors in different tissues. It is intriguing to speculate that G9a regulates the same gene expression programmes that coordinate cellular proliferation in developmental contexts across different tissues. As noted before, G9a has been shown to be a regulator of p21\(^{WAF1/CIP1}\) expression in several cell lines (Duan et al., 2005; Kim et al., 2008b; Nishio, 2004). Ectopic expression of p21\(^{WAF1/CIP1}\) resulting from the absence of G9a under specific circumstances might therefore play an important role for the observed phenotype in the blood and in cells derived from the neural crest. It is tempting to predict that this general role for G9a will also become evident after its deletion in other somatic stem cell systems that ensure the constant or injury-induced tissue regeneration. I therefore propose that the G9a deletion during muscle regeneration, in intestinal stem cells or in the skin are highly promising directions of future research.
3.3.5 The functional connection between G9a and DNA methylation

My results in this chapter also indicated that G9a does not regulate DNA methylation in somatic cells, similar to what has been reported before. One important functional consequence of the connection between G9a and DNA methylation is that during ESC differentiation, embryonic-specific genes are silenced by the coordination of DNA methylation through recruitment of DNA methyltransferases by G9a. Therefore, in differentiating G9a knockout ES cells, this silencing is inefficient and reversible. Intriguingly, a catalytically dead mutant but not a truncated version of G9a that lacks the Dnmt3a/b interaction domain can rescue this phenotype, indicating that it is DNA methylation that irreversibly locks the silencing of Oct4 and Nanog (Epsztejn-Litman et al., 2008). In contradiction with this conclusion, the G9a-inhibitor BIX01294 was shown to increase the induction of pluripotency (iPS cells) from neural precursor cells (Shi et al., 2008), a result that suggests that depletion of H3K9me2 in itself can reverse DNA methylation and the silencing of embryonic genes. It remains to be determined, whether a similar derepression of developmentally restricted genes can occur in different cell-types derived from the generated G9a knockout mice. It is therefore promising to investigate, whether G9a knockout MEFs or even more interestingly, somatic stem cells and progenitors display a propensity to reprogram more efficiently into a pluripotent state than their wildtype counterparts.
4. General discussion

The in vivo roles of two members of the SET domain family were addressed in this thesis. While most studies in the chromatin field use cell-line based assays, I generated conditional mouse models of Set7/9 and G9a. This approach in itself allowed me to address whether (a) these genes are required for mouse development, and (b) if so, whether this requirement was general to all developmental processes or specific to discrete developmental systems or stages.

Based on my results, it can be inferred that Set7/9 is not involved in mouse development in any appreciable way. This result was, under consideration of many of its suggested activities, somewhat surprising. Furthermore, I could not confirm a number of suggested non-developmental roles for Set7/9, most notably its proposed regulation of p53. Given the previously reported requirement for G9a during embryonic development, it was perhaps even more surprising that G9a is not involved in the development of the blood system during embryonic and adult hematopoiesis. I therefore would like to speculate about other potential in vivo roles of Set7/9 and G9a.

4.1 The specialization of SET domain genes during evolution

My results, in combination with those from other reported SET domain gene knockout mouse models, support the argument that lysine methyltransferases have assumed specific roles in chromatin regulation early during their evolution but have sacrificed part of this specificity due to the development of redundant functionality. It can be hypothesized that the diversification of lysine methyltransferases was driven by constantly emerging challenges in the evolutionary transitions from unicellular to multicellular organisms and resulted in functionally very specialized activities. It is conceivable that early in evolution, histone lysine methylation served to regulate the dynamics of a small variety of different chromatin states that are essential for chromosome function, DNA replication, DNA repair and transcription. Given that these processes are fundamental to all eukaryotic species, it is not very surprising that those SET domain genes that appear to facilitate these processes are the most evolutionary
conserved representatives of this family of genes. Therefore, the most ancient SET
domain genes and their homologues can be found from yeast to to man. They
encompass the Suv39h homologue *Clr4* (*in S. pombe*), the H3K4-specific homologues
for the *Set1a/b* genes and the H3K36-specific orthologue for *Set2* (Table 1).

Consistent with their evolutionarily conserved role in regulating constitutive
heterochromatin and chromosome function, mice that are knockout for both Suv39h
enzymes exhibit their most profound phenotype in genomic instabilities (Peters et al.,
2001a). However, *Suv39h* double null mice appear normal in development display
normal patterning of body structures and the specification different cell-types.
Interestingly, the emergence of the trithorax and polycomb-associated SET domain
genes *trx* and *E(z)* accompanied the occurrence of multicellular organisms and was
likely driven by the additional need to specify different cell-types. Indeed, murine
knockout models for the different *Mll*-homologues and *Ezh2* arguably exhibit the most
apparent developmental phenotypes among all known SET domain mutations (Glaser
et al., 2006; Heuser et al., 2009; Madan et al., 2008; O'Carroll et al., 2001; Yu et al.,
1995; Zhang et al., 2009). Intriguingly however, most other members of the SET
domain family have also emerged during the transition to multicellularity. Yet, apart from
few exceptions, their specific roles in chromatin regulation *in vivo* remain mostly
obscure. One of these exceptions is the *PR-Set7/Set8* gene. Its extremely severe
phenotypes in fly and mouse have indicated that PR-Set7/8-dependent H4K20me1
plays an important role in the regulation of chromatin condensation during the G2/M
phase of the cell cycle (Karachentsev et al., 2005; Oda et al., 2009).

One complication in the interpretation of many SET domain phenotypes might be
that, over time, their radius of action has expanded and their function became less
selective. A number of general and specific observations support this hypothesis. First,
it is becoming increasingly evident that most if not all SET domain proteins possess
additional catalytic activities towards non-histone proteins. Notably, most of those non-
histone substrates still appear to be nuclear proteins, suggesting that the newly
invented activities remain chromatin-related and are not completely divergent from the
presumed original function of the respective SET domain protein. Apart from the
extensively discussed role for Set7/9 in p53 function, one example for an enzyme with
a heterogeneous substrate specificity is also G9a. G9a has been shown to methylate mAM (Sampath et al., 2007), a regulator of Setdb1-dependent H3K9 trimethylation (Wang et al., 2003). Yet, the significance of this methylation remains unclear. Furthermore, it seems to be a common theme that most SET domain genes have acquired additional functions over time that do not involve the methylation of alternative substrates. For example, Suv39h1, although predominantly involved in the compaction of constitutive heterochromatin, has a pivotal role in the pRb-dependent gene repression that accompanies cellular senescence in response to oncogenic stimuli (Braig et al., 2005).

A result of the functional divergence of SET domain genes is that, their activities started to overlap and/or compensate for each other. With the genome-wide analysis of chromatin marks, it has become obvious that virtually all regions of the genome are marked by multiple histone lysine methylation species. It has been widely suggested that the different lysine modifications are engaged in positive and negative cross-regulatory relationships. Indeed, this often seems to be the case. For example, pericentromeric H4K20me3 is dependent on the preexisting H3K9me3 mark (Schotta et al., 2004). Furthermore, in the absence of H3K9me3 at pericentromeric heterochromatin in Suv39h double null cells, H3K27me1 is instead deposited at those regions (Puschendorf et al., 2008). However, in many other instances, those essential functional interrelationships between histone methylation marks are not as pronounced. For example, G9a-dependent H3K9me2 and Setdb1-dependent H3K9me3 both occur at endogenous retro-elements. However, they seem to be deposited independently of each other, since H3K9me3 persists on these elements in the absence of G9a and H3K9me2 (Dong et al., 2008). A further complication of this notion is indicated by my finding that DNA methylation on retro-elements also persists in the absence of G9a in somatic cells, while it clearly depends on G9a in ES cells. This suggests that the overlapping functions of different chromatin modification systems can also be cell-type specific. Interestingly, this hypothesis may provide an explanation for the different severities of G9a-phenotypes in different tissues.

In summary, I conclude that the specific roles of SET domain genes in the complexity of multicellular organisms cannot be predicted solely based on in vitro
studies but should rather be approached in vivo, preferably by genetic means. I propose that in the future, a comprehensive combination of genetic and epigenomic methodology could provide valuable insight into the functionally overlapping and redundant interrelationships of different chromatin modifications in a variety of cell-types.

4.2 Assigning a specific role for G9a

As discussed above, it can be assumed that the activities of many SET domain genes are co-regulated by related pathways, which in their absence can act as compensatory mechanisms. G9a is perhaps the most dominant histone methyltransferase for the genomic H3K9me2 mark. This is indicated that by the fact that global levels of this modification drop significantly. It is commonly believed that G9a and Glp always act together. The extent of H3K9me2 loss varies among different cell types and thus may argue for the possibility that G9a and Glp also might also act redundantly in some instances. A double knockout for G9a and Glp should be able to address this possibility and might yield a stronger phenotype than G9a on its own.

As discussed before, my studies using the conditional knockout strain for G9a have so far only focused on developmental systems. Since the deletion of G9a in the blood selectively lead to a phenotype upon injury, it can be hypothesized that G9a might play a specific role in the relative repression of genes that need to be rapidly induced under any given situation. The necessity for the tight control of these genes is accepted, and it is possible that \( G9a^{-/-} \) cells hyper-respond and induce several genes at lower thresholds and/or to higher expression levels. Under consideration of this possibility, it would be promising to use well defined systems to investigate the induction of gene expression in response to intrinsic or extrinsic stimuli in the absence of G9a. While the induction of injury in the hematopoietic system can perhaps be considered as such a system, other more definitive experiments could reveal a specific role for G9a in fine-tuning inducible gene expression. Very suitable and highly significant paradigms for the induction of gene expression are the immunologically-relevant induction of cytokine expression upon exposure to antigens or the
transcriptional response of β-islet cells to different levels of blood glucose. Another, not investigated role of G9a is the response to oncogenic signals and the suppression of tumorigenesis. While my studies involving Set7/9 have made use of several assays to test for the propensity of cancer formation in these mice, my experiments with the G9a strain have not yet systematically addressed whether G9a has tumor suppressive functions in the mouse.

The perhaps most significant limitation in my studies involving G9a is the fact that only tissue-specific deletions of G9a were used for all of my experiments. This automatically meant that my focus was biased to detect mostly cell-autonomous phenotypes. Hence, an additional strategy in future experiments should use inducible and non-tissue specific ways to abrogate G9a expression in adult mice. To accomplish this task, a mouse strain expressing a ligand-dependent CreER-transgene is available. In G9aflox/lox, CreER mice, the administration of tamoxifen will lead to a complete G9a knockout in all tissues. It is conceivable that in these mice, very noticeable phenotypes might occur.

### 4.3 What is the in vivo function of Set7/9?

Since most of the described experiments in this thesis entailed no detectable phenotypic differences between wildtype and Set7/9-/- mice, it is necessary to hypothesize about potential but uncharacterized functions of Set7/9. While it is unclear whether future studies will be able to detect overt phenotypes in my Set7/9 knockout strain in non-developmental experiments, I would like to discuss two general characteristics about the Set7/9 gene.

First, it appears that if regarded from an evolutionary perspective, Set7/9 is a very “young” gene. As discussed above, the highly conserved SET domain can be found in a multitude of genes throughout the four eukaryotic kingdoms (plants, animals, fungi and protists). By contrast, Set7/9 orthologues can only be identified in vertebrate genomes, ranging from fish (Danio rerio, accession # NM_001002456) over amphibia (Xenopus laevis, accession # NM_001032339) to mammals (Homo sapiens, accession # NM_030648). Since most genes to which we can attribute fundamental roles in
organismal development, are highly evolutionarily conserved and have characterized orthologues in worms, flies and mammals (e.g. Hox genes, Pax genes, Polycomb factors), it can be hypothesized that Set7/9, perhaps as a result of its short evolutionary existence, has (a) roles in fine-tuning more ancient processes and thus stabilizes the regulation of nuclear signaling pathways and (b) rather represents an auxiliary factor whose activity, when deleted, is redundantly regulated by other fallback mechanisms. This theory is supported by the impression that Set7/9-dependent methylation of p53 is redundantly compensated for by numerous acetylation events in the same region of p53.

Second, from our current perspective, Set7/9 appears to possess an unusually high enzymatic promiscuity. This might explain why the initially reported specificity towards histone H3K4 may just be of a rudimentary nature and cannot be verified in enzymatic assays that rely on more native histone H3. Taken together with my results that Set7/9 deletion does not entail the expected drop in H3K4me1, it can be hypothesized that Set7/9 may be in transition to evolve into a more specific lysine methyltransferase with an essential biological function.

4.4 Promising avenues to study additional functions of Set7/9 and G9a

My phenotypical analyses in this thesis have predominantly focused on the hematopoietic system and, in the case of Set7/9 also on cancer-relevant pathways. Given my limited success in detecting overt phenotypes, (with the exception of the regenerative requirement for G9a in the blood) it can be hypothesized that more profound phenotypes for both genes are to be identified in areas that I have so far neglected. It is generally believed that at least some epigenetic mechanisms of gene expression are considerably influenced by environmental factors. Perhaps, Set7/9 and G9a have evolved to facilitate specific responses to environmental circumstances that do not apply to laboratory animals under controlled conditions. For example, the exposure to different pathogens like viruses or parasites are usually excluded under quarantine. Related to this issue is the extensively studied epigenetic regulation of cytokine expression (Wilson and Merkenschlager, 2006). It is possible that Set7/9 and
G9a are involved in the activation or repression of specific cytokine genes and therefore might reveal roles in the immunological responses to pathogens. For example, it would be promising to explore the helminth resistances of the knockout strains used in this thesis. Similarly, the induction of asthma and other allergy paradigms will potentially yield significant phenotypic alterations in Set7/9\textsuperscript{-/-} and G9a\textsuperscript{-/-} \textsuperscript{Vav-Cre} mice. Currently, some of these assays are being performed by collaborating immunologists in our institute.

Moreover, a recent report of a knockout strain of the H3K9me-specific demethylase Jhdm2a revealed its surprisingly specific role in regulating metabolic gene expression. When fed with a high-fat diet, Jhdm2a\textsuperscript{-/-} mice gained weight significantly faster than their controls, providing strong evidence for the epigenetic regulation of different metabolic programmes (Tateishi et al., 2009). It is well possible that metabolic phenotypes could be observed in my generated knockout strains when subjected to alternative food sources.

It can be expected that the generated mouse strains will pave the way for a large number of future studies which might have far-reaching implications for a wide variety of biological research areas. It will be promising to explore the possibilities of Set7/9 and G9a-dependent phenotypes in projects that focus on topics such as immunology, aging and degenerative diseases, metabolic and hormonal regulation, behaviour and memory formation.
4.5 Conclusion

In my thesis, I generated *in vivo* data for Set7/9 and G9a, that are changing our views about the biology of these SET domain genes. My studies question the importance of Set7/9 as a p53 regulator and its suggested tumor suppressor activity. Future *in vivo* analyses will be crucial to evaluate the importance of other Set7/9 functions, those that were recently proposed in the literature and those that are still unidentified. In addition, my analyses of G9a point towards a specific role in regulating the expansion of undifferentiated cells in the blood and possibly in other developmental systems. However, it will also be important to widen our horizon and initiate studies that test the involvement of G9a in other aspect of epigenetic regulation, independent of developmental processes.

In general, I propose that future studies of other epigenetic factors, in particular SET domain genes, should increasingly rely on genetic models in the mouse and explore the phenotypes of compounded knockouts of multiple, possibly redundant activities. Since targeted mutations of SET domain genes are very time-consuming and expensive, other alternative genetic methods like RNAi approaches should be optimized *in vivo*. This should accelerate the pace at which SET domain phenotypes can be analyzed, and minimize the cost of those studies. Importantly, the increased understanding of chromatin regulation made available by these studies will provide a new generation of therapeutic agents to enable us to interfere with the epigenetic regulation of gene expression underlying developmental and other medically relevant applications.
5. Materials and methods.

5.1 Restriction enzyme mediated recombination of plasmid vectors

1-3 µg of plasmid mini preparations (Fermentas K0503) were typically digested with 1 µl of each restriction enzyme (NEB or Fermentas) in single or double digests in 30 µl total volumes of the recommended dilution of reaction buffers. The reactions were incubated at 37°C for two hours or overnight. DNA fragments were separated on 0.8-1.5% TAE-based agarose gels at an applied voltage of 10 V/cm of gel width. DNA fragments were visualized by the presence of SYBRsafe (Invitrogen) under a UV source. The DNA fragments were cut out and purified using the QIAquick gel extraction kit (QIAGEN) according to the manufacturer’s instructions. Afterwards, the compatible DNA fragments were ligated at a vector/insert-ratio of < 1/2 using T4 DNA ligase (Invitrogen) for two hours at room temperature or 12-72 hours at 4°C in 10 µl the appropriate dilution of the supplied reaction buffer. Next, the ligation reaction was used to transform Dh5α E. coli (Invitrogen, sub-cloning efficiency) and plated on LB plates containing 100 µg/ml ampicillin under sterile conditions. The plates were usually incubated at 37°C overnight or in the case of very large plasmids (>20 Kb) at room temperature for 2-3 days. Colonies were picked and transferred into liquid LB media containing ampicillin and incubated at 37°C under agitation overnight.

5.2 Cloning of synthesized DNA oligonucleotides

Complementary 5'-phosphorylated DNA oligonucleotides of 20 to 100 nt lengths were designed to bear 5' or 3'-overhangs that were compatible with the used restriction enzyme mediated restriction interfaces and purchased from Invitrogen. Annealing of equimolar amounts of the complementary DNA oligonucleotides was performed in 1x T4 ligase buffer by heating to 95°C and the consecutive cooling by 1°C/ minute to 8°C in a Thermocycler. The annealed DNA fragments were subjected to T4 ligation as described above.
5.3 Amplification of Plasmid and BAC DNA

Transformed E. coli were picked from single colonies on culture plates and cultured in liquid LB media in the presence of antibiotics (usually ampicillin 100 µg/ml) at 30°C (for plasmids > 15 Kb and BAC’s) or 37°C overnight and under agitation (250 rpm). On the next day, the bacteria was pelleted at 4000 rpm/ 10’ in 50 ml Falcon tubes in an Eppendorf 5402R centrifuge. The plasmid DNA was prepared using QIAGEN midi or maxi kits according to the manufacturer’s instructions. DNA concentrations were determined on a Nanodrop 1000 (Thermo Scientific).

5.4 Electroporation of ES cells and the selection of clones

Early passage (< p12) R1 (used for Set7/9 targeting) or C2 (G9a) ES cells were obtained from Marina Gertsenstein and Andras Nagy, Samuel Lunenfeld Research Institute, Toronto, ON. The original vials were thawed and expanded on feeder containing 10 cm plates in ES media. Initially, several early passage cryopreservations were made. The differentiation status of the ES cells was assessed by colony morphology. To carry out the gene targeting, single frozen vials were broken out and plated on feeder coated dishes. After typically 2-3 days, at a confluency of ~75%, the cells were trypsinized, taken up in ES media and counted. the cells were washed with 50 ml PBS (1l: 8 g NaCl, 0.2 g KCl, 1.44 g Na2HPO4, 0.24 g KH2PO4, pH 7.4, autoclaved) and resuspended to 30M cells per ml of PBS, then put on ice. 30 µg of the purified linearized targeting construct was pipetted into a 0.4 cm gap electroporation cuvette (Biorad) and put on ice. 400-800 µl of the cell suspension was added and gently mixed with the targeting DNA. In a Gene Pulser II (Biorad), set to 240V and 500 µF (high capacitance), the cells were electroporated and put back on ice immediately. The Gene Pulser commonly recorded time constants and voltages of 13.5-14.5 ms and 235-240 V, respectively. After 10 min on ice, the cells were transferred into 50 ml of cold ES media and plated on gelatin coated 10 cm dishes (neo resistant feeder coated dishes for the C2 ESC line) at around 2.5M cells per dish. After 24 hours, G418 selection was applied (150 µg/ml) and the media was renewed every 36h for 8 days.
Next, the media was replaced with PBS and individual colonies were picked with a 20 µl pipette (Gilson) and transferred into feeder coated 96-well plates. The cells were cultured to confluency (usually 3 days) with daily media changes and duplicated with 8-channel pipette. In one of the two plates, the cells were frozen in freezing media (50% FBS, 40% ES media, 10% DMSO), sealed with parafilm and stored in a zip-lock bag at -80°C for up to four weeks. In the meantime, duplicate plates were cultured to confluency and the analysis of correct locus integration was carried out.

5.5 Genomic DNA preparation from ES cell clones and Southern analysis

After their duplication, ES cells clones were cultured in 96-well plates until the media of most wells turned yellow. The media was washed away with PBS. The cells were lysed with 50 µl of Sarkosyl-Lysis buffer (10 mM Tris pH 8.0, 10mM EDTA, 10 mM NaCl, 0.5% Na-Sarkosyl, 100µg/ml Proteinase K Fermentas EO0491) overnight in a humidified 37°C incubator. On the next day, the DNA was precipitated by addition of 100 µl of salt-saturated cold 96% Ethanol (1.5 ml of 5M NaCl/ 100 ml Ethanol). The 96 well plates were gently vortexed, and incubated at room temperature until DNA became visible (~30 min). The DNA was spun down at 4000 rpm/ 10 min in an Eppendorf 5402R centrifuge and washed twice with 70% Ethanol without centrifugation and then dried on paper towels. Next, 60 µl of TE buffer was added to each well the DNA was allowed to resolve overnight in a humidified tissue culture incubator. On the next day, 20 µl of DNA solution was transferred into a new 96 well V-bottom plate. Next, 10 µl of 3x concentrated restriction digest reaction (3 µl reaction buffer,.3 µl of 100 mM Spermidine,.3 µl of RNase A,.5 µl of the indicated restriction enzyme, 5.9 µl H2O) was added and the reaction was incubated overnight in a tissue culture incubator. On the next morning, the digested DNA was loaded on a TAE-based 0.8% agarose gel and run at a low voltage (~2-3V/cm gel width) for 8 hours. After sufficient separation, the gel was inspected under UV light. Distinct low molecular weight bands indicated complete restriction. The gel was rinsed twice with dH2O and then denatured in denaturation solution (1.5 NaCl, 0.5 M NaOH) for 30 min under gentle agitation. Neutralization was done in 1.5 NaCl / 0.5 M Tris pH 7.5 for 30 min under gentle agitation. The DNA was
transferred overnight to a nylon membrane (Roche 11 417 240 001) by capillary action in 20x SSC (3 M NaCl, 0.3 M Na-citrate, pH 7.0, autoclaved) in a set-up according to Maniatis. On the next morning or up to 2 days later, the transfer set-up was disassembled, the membrane was rinsed with dH2O, and baked dry at 80°C for 2 hours to cross-link the DNA to the membrane. The membrane was stored dry until further proceeding. Next, the membrane was blocked with 15 ml of reconstituted DIG Easy Hyb solution (Roche 1 796 895) in a roller bottle at 45°C for 2 hours. Next, 10 µl of the denatured DIG-11-dUTP (Roche 11 175 033 910, generated as recommended) PCR-labelled dsDNA probe (~ 1Kb in length) was added to the DIG Easy Hyb buffer and the hybridization was allowed to occur under rolling for > 8 hours at 45°C. Afterwards, the hybridization solution was recovered and stored at -20C for reuse (up to 4 times). The blot was washed with 30 ml of low stringency buffer (2x SSC, 0.1% SDS) at 68°C for 30 min. Next two 15 min high stringency (0.5x SSC, 0.1% SDS, preheated) washes were carried out at 68°C. The membrane was then removed from the hybridization oven and rinsed in 1x maleic acid buffer (from 5x concentrated 500mM maleic acid, 750 mM NaCl, pH 7.5, autoclaved) and blocked at room temperature in 2x DIG blocking reagent (Roche 11 585 762 001) in 1x maleic acid buffer for 2 hours under gentle agitation. The blot was developed according to the instructions in DIG Luminescent Detection Kit (Roche 11 363 514 910). Exposure time on Kodak X-ray film was usually between 1.5 and 6 hours.
5.6 Summary of the Set7/9 conditional knockout derivation

Electroporation parameters:
- 20 mio R1 cells of passage 13, cultured on irradiated MEF feeders,
- 25 µg of linearized targeting DNA
- plated on 10 gelatin coated 10 cm TC plates
- selection with 150 µg/ml G418, media changed very 36 hrs
- 96 clones were picked on day 10 of selection into 96 well plates

Analysis and targeting frequencies:
- Southern analysis over short 5’ arm (NcoI digest/ external probe)
  - 2/96 positive clones: D4, D7
- Southern analysis using PstI and a 3’ internal probe
  - 2/96 positive clones: D4, D7 verified

Injections into host wildtype C57B/6 blastocysts:

<table>
<thead>
<tr>
<th>clone</th>
<th># injections</th>
<th>#/ extent of chimeras</th>
<th>germline positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>D4</td>
<td>2</td>
<td>1/ 40%</td>
<td>yes, ~40%</td>
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</tbody>
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5.7 Summary of the G9a conditional knockout derivation

Electroporation parameters:
25 mio C2 cells of passage 10, cultured on irradiated MEF feeders,
25 µg of linearized targeting DNA
plated on 12 gelatin coated 10 cm TC plates
selection with 150 µg/ml G418, media changed very 36 hrs
384 clones were picked on day 10 of selection into four feeder-coated 96 well plates

Analysis and targeting frequencies:
PCR analysis over short 5’ arm (one primer on loxP site)
- 6/384 positive clones
Southern analysis using a 3’ external probe
- 5/6 clones were verified

Injections into host albino C57B/6 blastocysts:

<table>
<thead>
<tr>
<th>clone</th>
<th># injections</th>
<th>#/ extent of chimeras</th>
<th>germline positive?</th>
</tr>
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<tr>
<td>3H7</td>
<td>2</td>
<td>2/ 10% and 40%</td>
<td>no</td>
</tr>
<tr>
<td>1H6</td>
<td>2</td>
<td>2/ 10% each</td>
<td>not tested</td>
</tr>
<tr>
<td>3H3</td>
<td>2</td>
<td>3/ 2x 100% and 50%</td>
<td>both 100% died after 10d along with 3 non-chimeric animals, 50% chimera produced no germline transmission after four litters.</td>
</tr>
<tr>
<td>3H3</td>
<td>4 (2nd round)</td>
<td>6/ 10-50%</td>
<td>50% germline transmission from two 50% chimeras (3H3-4; 3H3-5), genotype verified.</td>
</tr>
</tbody>
</table>
5.8 Generation of ES cell derived chimeras and the establishment of conditionally targeted mouse lines

After Southern analysis, positive ES clones were thawed and transferred into feeder coated 24-well plates, expanded in ESC or RESGRO media (Chemicon, in the case of C2 ESC) until three to four vials could be frozen down again. For blastocyst injections of correctly integrated clones, the cells were thawed 2 days ahead. The injections were carried out by Amy Bennie and Danny Chui at the BCCancer Agency, Vancouver, BC. In short, ~ 25 day old albino B6(Cg)-TyrC2-J/J females were induced to super-ovulate by the injection of 2.5-10 UI of pregnant mare’s serum (PMS) and 42-48 hours later with 2.5-5.0 IU of human chorionic gonadotropin (hCG). Females were then mated with selected males and plug-checked on the next morning. At 3.5 dpc, blastocysts were collected and injected with 10 targeted ES cells and transferred into pseudo-pregnant ICR females. The extent of coat color was used to assess ES cell tissue contribution. Breeding was set-up as described in the results section.

5.9 Transgenic and knockout mouse strains.

All additional mouse lines used in this study were back-crossed and maintained on the C57J/B6 background and were originally derived from the Jackson Laboratories, Bar Harbor, Maine, USA unless stated otherwise.

**B6SJL CD45.1+:** a C57J/B6 congenic mouse strain that is homozygous for CD45.1, a variant of the pan-hematopoietic marker CD45, as oppose to CD45.2 in C57J/B6.

**TgN(ACTFLPe)9205Dym:** a transgenic C57J/B6 congenic mouse strain that ubiquitously expresses an hyperactive version of the FLP1 recombinase under the control of the human ACTB promoter. Used as a deleter strain to eliminate the frt-flanked PGK-Neo cassette in the gene targeted founder mice.
B6-Tg(pCX-NLS-Cre): a transgenic C57J/B6 congenic mouse strain that ubiquitously expresses the Cre recombinase under the control of the CAGGS promoter. Used as a deleter strain to eliminate loxP site flanked (floxed) regions in the gene targeted founder mice to yield null alleles that transmit through the germ line. The strain was obtained from Corinne Lobe, University of Toronto, ON.

B6.Cg-Tg(Mx1-cre)1Cgn/J: a transgenic C57J/B6 congenic mouse strain that expresses the Cre recombinase under the control of the Mx1 promoter. This promoter is silent in healthy mice, but can be induced to high levels of transcription by administration of interferon α, interferon β, or synthetic double-stranded RNA (such as pIpC). This permits the induction of the knockout at any time during development. The deletion efficiency of the targeted gene varies depending on tissue type and its responsiveness to interferon. The highest efficiencies are observed in the hematopoietic system and the liver.

B6-Tg(HS21/45-Vav-Cre-ires-YFP): a transgenic C57J/B6 mouse strain that expresses the Cre recombinase under the control of the HS21/45 control region of the Vav promoter. The expression cassette is flanked by a 250 bp core region of the chicken β-globin insulator. The strain was obtained from the laboratory of Thomas Graf at AECOM, Bronx, NY. Cre expression occurs in all cells of hematopoietic origin throughout development, YFP expression is not detectable (Stadtfeld and Graf, 2005).

B6;129S2-Trp53tm1Tyj/J: The Trp53tm1Tyj mutant strain lacks complete expression of p53 and was developed in the laboratory of Dr. Tyler Jacks at the Center for Cancer Research at the Massachusetts Institute of Technology. The 129-derived D3 ES cell line was used and the mice have been back-crossed to C57J/B6 for more than eight generations.

B6.Cg-Tg(IghMyc)22Bri/J: A congenic C57B/6 mouse strain that carries a transgenic c-Myc gene under the B-cell specific activation of the immunoglobulin heavy chain enhancer element. This mouse was generated by Dr. Allan Harris, Walter and Eliza Hall
Institute of Medical Research, Melbourne, Australia. 50% of the hemizygous mice spontaneously develop pre B and B cell lymphomas within 15-20 weeks of age. The mouse strain is referred to as \( E\mu\)-Myc in the rest of the thesis.

## 5.10 PCR genotyping analysis

Genomic DNA from cells or mouse biopsies was prepared according to standard protocols using Sarkosyl-Lysis buffer (10 mM Tris pH 8.0, 10mM EDTA, 10 mM NaCl, 0.5% Na-Sarkosyl, 100 µg/ml Proteinase K Fermentas). The DNA was diluted 30 fold and used as template in gene-specific PCR reactions with Taq polymerase (Fermentas, EP0403) exactly as recommended. The following primers and thermocycler programmes were used:

**Set7/9:**
- 5‘Set7/9.fw: CCCTGAGCAGGCTTCTTTAATGGC
- 3‘Set7/9.fw: GGCTTGTGAGACAGACAGCTCATTG
- 3‘Set7/9.rv: AGGCCCTCTCGGTTGATGGACACCTT
- wt: 384 bp  floxed: 431 bp  null: 522 bp
- annealing temperature: 55°C  cycle #: 40

**G9a:**
- 5‘G9a.fw: GCTGAGGGGAACAGACGCCATAACTTT
- 5‘G9a.rv: GCATGTCATACACTATCGCAGGGAAATG
- 3‘G9a.rv: GCACCTTTATCTGACGACAGCAG
- wt: 324 bp  floxed: 376 bp  null: 574 bp
- annealing temperature: 55°C  cycles #: 40

**p53:**
- p53x6.5: CAGCGTGGTGGTGACCTTAT
- p53x7: TATACCTGAGGCCGGCCT
- Neo18.5: CTATCAGGACATAGCGTTG
- wt: 450 bp  null: 615 bp
- annealing temperature: 60°C  cycle #: 30
The PCR reactions were always controlled by H₂O, wildtype and targeted samples.

5.11 Mouse husbandry and breeding

All mice were maintained in the BRC animal unit under approved standards of the UBC animal care committee. G9a mice were kept on a pure C57J/B6 background and Set7/9 mice were kept on a mixed background after five back cross generations into C57J/B6.

5.12 General tissue culture and long-term cell storage

All tissue culture work was performed in a level II biosafety cabinet under sterile conditions. All primary cell preparations and cell line were cultured in tissue-culture grade plastic ware (Falcon or Nalge-Nunc) except bone marrow derived macrophages were cultured on non-treated sterile 10 cm petri-dishes (Fisher) and ES cells were
propagated on 10 cm TC dishes that had been coated with irradiated MEF’s as feeder cells. Growth media for ES cells consisted of high-glucose DMEM (Gibco), 15% ESC qualified FBS (StemCell Technologies, Vancouver BC), non-essential amino acids, L-glutamine, Na-Pyruvate (all Invitrogen), 100 µM β-mercaptoethanol (Sigma-Aldrich, M7522), LIF (ESGRO/Chemicon or an in house source). Growth media for bone marrow derived macrophages consisted of RPMI (Gibco), 10% FBS, non-essential amino acids, L-glutamine, Na-Pyruvate (all Invitrogen), 100µM β-mercaptoethanol and 10% conditioned L-cell supernatant. Phoenix E cells were grown in MEF media. For passaging, the media was aspirated, the cells were washed once with PBS, and were trypsinized with 10x trypsin-EDTA reagent (Sigma-Aldrich, 59418C) diluted tenfold in PBS until cells dissociated from the plates. The cells were resuspended in media and re-plated at appropriate dilutions. For long-term storage, cells were trypsinized and resuspended in freezing media (50% FBS, 40% growth media, 10% DMSO, Sigma-Aldrich D2650) and aliquoted at ~2 M cells/ml in Cryovials (Corning) on ice. The cells were transferred to styrofoam containers and kept at -80°C overnight. The next day, the cells were transferred and stored in liquid N2. For thawing, the cryovials were removed from liquid N2, quickly thawed in a 37°C water bath, washed once in growth media and plated on appropriate TC ware. The next day, media was replaced and dead cells were washed away with PBS.

5.13 MEF derivation and culture

Embryos were harvested at 13.5 dpc under sterile conditions. After the head and all red organs were removed, remaining tissues were minced using a 19-gauge needle and a syringe. The tissue was trypsinized for 10 minutes at 37°C and plated in a T75 flask (Falcon). Genotyping was performed on limb biopsies and MEFs were frozen at 2M cells/vial after reaching confluency within typically two days of culture. Thawed MEF preps were designated as passage 1 and were used until passage 4 unless stated otherwise. MEF culture medium consisted of DMEM (Gibco), 10% FBS, non-essential amino acids, L-glutamine, Pen/Strep (all Invitrogen) and 100µM β-mercaptoethanol (Sigma-Aldrich, M7522).
5.14 Mass spectrometry of p53 modifications

Primary or E1a-infected wildtype and Set7/9-/- MEFs were grown under standard conditions. For each genotype, 25 large tissue culture dishes were grown to near-confluency. Adriamycin was added to the medium at a final concentration of 5 µM and incubated for 6 hours. Next, the cells were harvested and lysed in 10 ml RIPA buffer (150 mM NaCl, 50 mM Tris-HCl pH7.4, 1 mM EDTA, 1% Triton X100, 1% deoxycholic acid, 0.1% SDS, Roche Complete protease inhibitors), sonicated at 9 watts for 30 seconds and centrifuged at 4000 rpm and 8°C. The supernatant was incubated with agarose-coupled p53 antibody (GAF-1355, R&D systems, 1/2 vial per IP) overnight at 4°C. Afterwards, the anti p53 beads were washed twice in 10 ml RIPA buffer for 20 min. and once in 5 ml IP buffer (50mM Tris pH 7.5, 150 NaCl, 1mM EDTA, 1% NP40, Roche Complete protease inhibitors). The immuno-precipitated material was separated on a non-reducing PAGE gel and stained with coomassie brilliant blue (Biorad). The p53 bands were cut out and digested with Arg-C (Protea Biosciences) in an in-gel digestion protocol according to the manufacturer’s instructions. The generated peptides were subjected to liquid chromatography coupled mass-spectrometry (LC-MS/MS). LC-MS/MS experiments were performed on either an Applied Biosystems QStar XL or a Thermo Electron LTQ-FT mass spectrometer, using self-packed 75µM x 150mm C-18 columns and nanoelectrospray ionization. MS/MS data was extracted and blasted against the ENSEMBL database using the X!Tandem algorithm in the Global Proteome Machine (www.thegpm.org). Modified forms of peptides were confirmed if peptide mass mapping discovered a species with the correct charge state, a signal-to-noise ratio >3, and a retention time similar to the unmodified form.

5.15 3T3 assay

Passage 1 MEFs were counted using latex beads normalized FACS analysis and plated in duplicate at 3x10^5 cells per 6 cm tissue culture dish (Nalge-Nunc). Every third day, cells were trypsinized and counted on a FACScan (BD). Cells re-plated at original densities over 15 passages.
5.16 Thymocyte apoptosis

For apoptosis assays, thymocytes were harvested, equally distributed into four parts in MEF medium and γ-irradiated in a $^{64}$Co source at 0.5, 1 and 2 Gy. Cells were cultured for 5 hours and their viability was determined by AnnexinV-APC (Molecular Probes/Invitrogen) and 7AAD staining on a FACScalibur (BD). For RT-PCR, thymi from sex-matched wt and Set7/9−/− littermates were harvested 3 hours after whole body irradiation. In total, 8 mice of each genotype were tested.

5.17 Reverse transcription and quantitative RT-PCR

RNA from thymocytes was prepared using RNeasy Mini Kit (QIAGEN) according to manufacturer’s instructions. cDNA was generated using the Invitrogen Superscript III RT protocol using random hexamer primers. Real-time PCR was carried out using a Roche Lightcycler 480 and gene specific Taqman reagents (Applied Biosystems, p21: Mm00432448_m1, Mdm2: Mm00487656_m1, Bax: Mm00432050_m1, GAPDH Hs02758991_g1) according to manufacturers instructions. Realtime PCR for G9a expression was performed using a gene specific RT primer (AGAAGGCAATGCCTGGGAAC) and PCR primers (TCATCTGCCGGACGTAGCC and CTTCACATCAGCCTCGGC).

5.18 Western analysis and antibodies

Whole cell lysates were done in IP buffer (50mM Tris pH 7.5, 150 NaCl, 1mM EDTA, 1% NP40, Roche Complete protease inhibitors), sonicated and spun down at 14,000 rpm/ 4°C in a micro-centrifuge (Eppendorf 5417R). The supernatant was measured for protein content using a Biorad RC/DC kit according to the manufacturer’s instructions. Typically, 50-100 µg of protein were run on 8-12% SDS-PAGE gels (Biorad Mini Gels) at 80 to 120 V and then transferred to nitro-cellulose (Biorad) using a semidry transfer apparatus (Hoefer/ Amersham). Blots were incubated face-down on primary antibodies at 4°C overnight without agitation and developed using either HRP-coupled secondary
antibodies (Jackson Immuno) and ECL reagent (Amersham) or alternatively with Alexa Fluor 680 (Molecular Probes) or IRDye 800 (Rockland Immunochemicals) on a LI-COR Odyssey analyzer. Primary antibodies were: α-Set9 (Upstate), α-SET7/9 (Cell Signaling clone C24B1), α-p53 (CM-5, Novocastra), α-p53 (PAb 240, BD), α-p53K379ac (Cell Signaling #2570) α-H3K4me (Abcam), α-p21 (C-19, Santa Cruz), α-p21 (Ab-6, Oncogene), α-Mdm2 (Calbiochem clone 2A10), α-Bax (Calbiochem Ab-1), α-PUMA (Cell Signaling #4976), α-G9a (Perseus Proteomics Inc., clone A8620A), α-Glp (Perseus Proteomics Inc., clone B0422), α-H3K9me2 (Abcam ab1220), α-actin (Abcam ab3280).

5.19 Retroviral gene transfer

The retroviral transfer vectors pWZL E1A hygro and pBabe RasV12 puro were obtained from Scott Lowe (CSHL), pBabe SV40T neo was obtained from Addgene (plasmid 1780). The pWZL CreERT2 hygro was constructed by replacing E1A with the CreERT2 gene (a gift from Daniel Metzger, Strasbourg, France) in pWZL E1A hygro. Virus production was performed by calcium phosphate transfection of Phoenix E (a gift from Gary Nolan, Stanford) cells. In short, the Phoenix E cells were thawed and grown to around 80% confluency in a 10 cm TC dish. Next, the cells were split 1:5. On the next day, the cells were inspected and the medium was changed. At least 1 hour later, the transfection of the retroviral transfer plasmid was performed as follows: Per 10 cm dish, 8 µg of plasmid DNA was mixed with 60 µl of 2M CaCl₂, then the reaction was filled up to 500 µl with H₂O. Next, 500 µl of 2xHBS (500 ml: 8 g NaCl, 6.5 g Hapes, NaH₂PO₄ 1.5 mM, pH 7.00) was added slowly. The reaction was vortexed to ensure complete mixing and added drop-wise to the Phoenix E cells. On the next morning the media was replaced and the virus containing supernatants were filtered through .45 µm syringe filters (PALL), supplemented with polybrene at 10 µg/ml and transferred to early passage MEFs at 24h, 36h and 48h post transfection. 48 hours after the last supernatant transfer, infected MEFs were selected with G418 (Invitrogen, 300 µg/ml), hygromycin (Invitrogen, 200 µg/ml) or puromycin (Calbiochem, 2 µg/ml) for at least 48h until non-infected control cells were completely killed.
5.20 Tumor graft assays

Early passage MEFs were sequentially infected with pWZL E1A hygro and pBabe RasV12 puro, selected as described above and expanded over one passage. Four million cells were suspended in 300 µl PBS and injected subcutaneously into the rear flanks of isofluorane anesthetized NOD/SCID mice. Mice were sacrificed after 15 day and the resulting tumors were dissected and weighed.

5.21 Cell cycle analysis

Early passage MEFs were plated at 1 million cells per 10 cm dish in the morning, and were treated with p53 activating agents over-night (Adriamycin, Calbiochem #324380, Nutlin 3, Sigma-Aldrich N6287). On the next day, the cells were pulsed with 10µM BrdU (Sigma-Aldrich, B5002) for 60 minutes. Cells were harvested and fixed overnight in 70% ethanol at 4°C. The DNA was denatured with 2N HCl for 20 minutes and neutralized by addition of Na-borate (100 mM). The cells were stained with FITC anti-BrdU (Invitrogen) in 0.5% Tween 20/ 1% FBS/ PBS for 45 minutes at room temperature in the dark, and washed once. After resuspension in FACS buffer (PBS, 2% FBS, 2 mM EDTA) containing 200 ng/ml of propidium iodide (Sigma-Aldrich, P4170), the cells were analyzed on a FACSCalibur (Becton Dickinson).

5.22 Fetal liver transplantation of \( E\mu\text{-}Myc^+ \) cells and the analysis of lymphomas

Hemizygous \( E\mu\text{-}Myc \) mice were bred to \( Set7/9 \) floxed and null backgrounds. To obtain fetal livers of the required genotypes, \( E\mu\text{-}Myc^+/Set7^{+/+} \) mice were bred to \( Set7^{-/-} \) mice, \( E\mu\text{-}Myc^+/Set7^{fl/+} \) mice were bred to \( Set7^{+/+} \) mice and \( E\mu\text{-}Myc^+ \) mice were bred to \( p53^{+/+} \) mice. The females were plug-checked, sacrificed at 14.5 dpc and the embryos were harvested. The intact embryos were stored for up to 8 hours in FACS buffer at 4°C, while limb biopsies were genotyped. Fetal livers of the desired genotypes were dissected, pushed through a 40 µm filter, washed once in PBS and transplanted into irradiated recipients exactly as described in the bone marrow transplantation protocol.
One fetal liver was used for six transplants. The reconstituted recipients were examined three times per week and sacrificed as soon as they became moribund or exhibited enlarged lymph nodes. Lymphomas were diagnosed based on markedly enlarged lymph nodes.

### 5.23 Cre induction in G9a<sub>fl/fl</sub> MEFs

Early passage G9a<sub>fl/fl</sub> MEFs were infected with pBabe SV40T neo and frozen down as described earlier. The MEFs did not change in their morphological appearance but acquired constant growth rates beyond passage 8, as a sign of their efficient immortalization. To introduce an inducible Cre construct, the SV40T MEFs were then infected with pWZL CreERT2 hygro and selected with hygromycin B (Invitrogen). To derive sub-clones, bulk populations of the resulting MEFs were sorted as single cells into several 96-well plates with a DiVa sorter (BD). Media was changed twice per week and after 3-4 weeks, individual wells showed confluent clonal MEF populations. 24 clones were further expanded in a 24-well plate. After confluency and duplication, one of each of the duplicates was frozen and kept at -80°C. The remaining clones were duplicated again and cultured in the absence or presence of 4-hydroxy-tamoxifen (4OHT, Sigma-Aldrich H7904, 200 ng/ml) for 5 days. Genomic DNA was prepared as described and subjected to G9a genotyping. Two of 24 clones (#7 and #9) showed efficient excision of the floxed allele and were expanded from the stocks. I derived five further sub-clones from clones #7 and #9 in the same fashion except for the fact that before sorting the cells were cultured in 4OHT for 5 days. The resulting G9a<sup>-/-</sup> sub-clones were expanded, frozen and used for the indicated western and DNA methylation analyses.

### 5.24 Cre induction in Mx-Cre transgenic mice and bone marrow recipients

To induce Cre in Mx-Cre transgenic bone marrow, mice were given three intra peritoneal (i.p.) injections of pIpC (Calbiochem, # 528906, 400 µg in 200 µl of sterile PBS per injection). The mice were left recovering for at least four weeks.
5.25 Bone marrow collection

Adult mice were sacrificed by CO$_2$-mediated asphyxiation, placed on their backs and the tibiae and femora were dissected and placed into a 6 cm petri dish containing ~ 5 ml of FACS buffer (PBS, 2% FBS, 2 mM EDTA). After de-capping of the bone ends, the marrow was flushed out with the help of a 5 ml syringe and a 26G needle. The bone marrow (BM) was dissociated into single cells by repeated aspiration and expelling from the syringe while its opening was held closely to the bottom of the petri dish. Next, the BM was pelleted in a 15 ml Falcon tube at 1,200 rpm in an Eppendorf 5810R centrifuge at 8°C for 5 min. The supernatant was aspirated and the BM was resuspended in 1 ml of red cell lysis buffer (150 mM NH$_4$Cl, 1 mM KHCO$_3$ pH 7.3, sterile filtered) and incubated for 15 min on ice. The tubes were filled up with FACS buffer and spun at 1,200 rpm again. For transplantation, the cells were resuspended in FACS buffer, counted and mixed to a resulting ratio of 50:50 with competitor BM. The cells were then pelleted once more and resuspended in PBS at a concentration of 2-4M cells/ 200 µl. For FACS analysis, the BM cells were stained with α-CD45.1 PE, α-CD45.2 FITC, α-GR1 PECy7, α-Mac1 PECy7, α-B220 PECy7, α-CD3 PECy7, α-Ter119 PECy7, α-c-kit PECy5 and α-Sca-1 APC and analyzed on a BD DiVa analyzer/sorter. One million events per sample were acquired and the analysis was carried out with Flowjo.

5.26 Bone marrow transplantation

BM cells were resuspended in sterile PBS at a concentration to accommodate the desired cell number in 200 µl of transplant volume per mouse. The cell suspension was kept on ice for no more than 2 hours and transferred into the quarantined BRC animal unit. CD45.1+ BSJL congenic recipient mice of 4-10 weeks age received a single γ-irradiation dosage of 9.5 Gy (7’30” on Jan. 15th 2008) in a lead-shielded $^{64}$CO source. Immediately thereafter, the mice were placed under a heat lamp and were injected with 200 µl volume of donor BM in PBS into their tail veins with the use of a 1 cc syringe and
a 26G needle. The mice were put back into their cages and peripheral blood leukocytes were first analyzed six weeks later.

5.27 Sampling of peripheral blood leukocytes (PBL) and their preparation for FACS analysis

In a laminar flow hood, transplanted mice were warmed up under a heat lamp and individually loaded into a restrainer device that allowed access to the tail. The tail vein was nicked with the use of a 21G needle and several drops of blood were collected into Eppendorf tubes containing 800 µl of FACS buffer. The samples were kept on ice. Next, the samples were spun at 4,500 rpm for 5 min in a microcentrifuge (Eppendorf 5417R) and the clear supernatant was aspirated. The pellet was resuspended in 1 ml of red cell lysis buffer, incubated for 15 min at room temperature and spun down again. The pellet was resuspended in FACS buffer and, split in two equal parts and transferred to v-bottom 96-well plates (Nalge-Nunc). The PBL were stained with two different cocktail of fluorescence-labelled antibodies at their appropriate dilutions for 45 min on ice and protected from light. The combinations of antibodies were:

**Cocktail A (GR1/Mac1):**
- α-CD45.1 Alexa 647 clone A20 1/150 dilution channel 4
- α-CD45.2 FITC clone 104 1/150 dilution channel 1
- α-GR1 PE clone PB6-8V5 1/400 dilution channel 2
- α-Mac1 PECy7 clone M1/70 1/100 dilution channel 3

**Cocktail B (B220/CD3):**
- α-CD45.1 Alexa 647 clone A20 1/150 dilution channel 4
- α-CD45.2 FITC clone 104 1/150 dilution channel 1
- α-B220 PE clone RA3-6B2 1/200 dilution channel 2
- α-CD3 PECy7 clone 145-2C11 1/100 dilution channel 3
Next, the cells were washed twice in 200 µl of FACS buffer, transferred to micro FACS tubes and read in an FACSCalibur after compensation set-up with unstained and single color labelled control samples. For each sample, 20,000 events were acquired. The statistical analysis was performed on Flowjo software.

### 5.28 Statistical analyses

Statistical analyses were performed in Microsoft Excel. All shown graphs indicate mean values and error bars indicate standard deviations. The number of samples used for each analysis is indicated in the figure itself or in the figure legend. To calculate statistical significances, the two-tailed, un-paired student’s t-test function in Excel was applied. Statistical significance was indicated by a resulting p<0.05. Different degrees of statistical significances are indicated by asterisks as follows: * is p<0.05, ** is p<0.01, *** is p<0.001.
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Genet 3, 415-428.
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progenitors in mouse bone marrow. Cell 91, 661-672.
868-871.


Appendices.

Animal protocols.
ANIMAL CARE CERTIFICATE

**Application Number:** A05-0351

**Investigator or Course Director:** Fabio Rossi

**Department:** Medicine, Faculty of

**Animals:**

- Mice multiple inbred and transgenic strains 1177

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The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.
THE UNIVERSITY OF BRITISH COLUMBIA

ANIMAL CARE CERTIFICATE
BREEDING PROGRAMS

Application Number: A05-0467

Investigator or Course Director: Fabio Rossi

Department: Medicine, Faculty of

Animals:

Mice C57/B6, wild type, KOs and transgenics 2000

Approval Date: April 9, 2008

Funding Sources:

Funding Agency: Muscular Dystrophy Association (US)
Funding Title: Identification and engineering of circulating myogenic progenitors

Funding Agency: Canadian Institutes of Health Research (CIHR)
Funding Title: Mesenchymal stem cells and biomaterials in bone regeneration: a team approach

Funding Agency: Canadian Institutes of Health Research (CIHR)
Funding Title: Mesenchymal stem cells and biomaterials in bone regeneration

Funding Agency: Stem Cell Network (SCN) - Networks of Centres of Excellence (NCE)
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102, 6190 Agronomy Road, Vancouver, BC V6T 1Z3
Phone: 604-827-5111 Fax: 604-822-5093