Dissecting the roles of histone deacetylase 1 and 2 in the hematopoietic system

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Abstract

Reversible histone acetylation on lysine residues has been intensively studied as an epigenetic mark for gene activation in a variety of eukaryotes. Histone deacetylases (HDACs) act to repress gene expression by removing acetyl groups from histones and possibly other proteins. Based on their expression pattern, distribution and biochemical activity, HDAC1 and 2 have been considered to be the major HDACs to regulate gene expression in a wide range of tissues in mammals. Experiments using transformed cells and HDAC inhibitors suggest that HDACs are important for cell cycle progression, apoptosis regulation and induction of differentiation; however their *in vivo* roles in a higher organism as well as their molecular mechanism of actions remain largely unexplored.

I have generated mice conditionally targeted at the *HDAC1* or *HDAC2* locus and crossed these to different deleter mice in order to dissect the function of these enzymes *in vivo*; I focused on B cell specific inactivation in the first part of the thesis and on hematopoietic-lineage specific inactivation in the second part. Mice completely lacking HDAC1 die during embryogenesis while animals lacking HDAC2 show partial perinatal mortality. In contrast, ablation of HDAC1 or 2 in the B cell lineage led to no obvious developmental defect. Simultaneous deletion of both *HDAC1* and *2* in the B cell lineage causes a very severe blockade in the early B cell development accompanied by severe defects in cell cycle progression, apoptosis regulation and recombination of immunoglobulin heavy chain (IgH) variable (V_H) segments. Using three-dimensional DNA fluorescence *in situ* hybridization, we show that pre-BI cells lacking HDAC1 and 2 are severely impaired in the contraction of the *IgH* locus, a mechanism whereby distantly located variable genes are brought in close proximity to the rest of the locus. Analysis of histone modifications revealed that these pre-BI cells lshow global histone hyperacetylation as well as increased histone lysine 9 methylation. Taken together, we conclude that HDAC1 and 2 are crucial factors for B cell development, survival and IgH recombination by modulating histone modifications which may affect gene expression and the higher order of chromatin structure.

In the second part I have analyzed the roles of HDAC1 in the hematopoietic lineage. Loss of HDAC1 in the hematopoietic lineage has only minor impacts on the myeloid lineage development, but impaired IgG secretion and reduced germinal center formation were observed upon induction of a T cell dependent immune response. Using transcriptome analysis, we found that these germinal center B cells lacking HDAC1 exhibit higher expression of genes involved in cell cycle regulation as well as apoptosis. These results suggest that HDAC1 has important roles to regulate the adaptive immune response by controlling the expansion of B cells upon antigen stimulation.

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1. Introduction

1.1 Basics of transcription

1.1.1 Basal transcriptional machinery

Genome is often referred as the **"blueprint of life"** since it contains almost the complete, if not all, set of hereditary information needed for the next generation. The life of a living organism starts by following the plan encoded in its own genome.

In order to extract the genetic information from the genome, the DNA has to be transcribed to generate RNA that is processed and translated into proteins. In humans, there are 20,000-25,000 genes encoding proteins (2004). Despite such a large number of genes, there are only three types of RNA polymerases (RNAPs) to transcribe genes in eukaryotic nucleus and each of them has specific targets for transcription. RNAP I is responsible for synthesizing most of ribosomal RNAs, RNAP II is for messenger RNAs, most of small nuclear RNAs and micro RNAs, and RNAP III is for a variety of small stable RNAs including transfer RNA, 5S ribosomal RNA and U6 small nuclear RNA. Since protein-cording transcripts are synthesized by RNAP II, I will focus here on reviewing what is currently known about transcriptional control by this enzyme.

RNAP II is the holoezyme having 12 subunits with a total mass of about 500 kDa (Davey et al. 2002). Two major types of core DNA promoters are known for transcription initiation by RNAP II; focused and dispersed promoters. Focused promoters are composed of a single transcriptional start site. On the other hand, dispersed promoters contain multiple start sites over 50-100 nucleotides under the control of same regulatory mechanisms, which are often found in CpG islands in vertebrates (Juven-Gershon et al. 2008). Despite the fact that majority of core promoters in simple eukaryotes are focused promoters, more than two-third of core promoters in vertebrates are dispersed promoters. To date, major efforts to understand the mechanisms of core promoter transcription have been dedicated to the study of focused core promoters. Therefore our current knowledge about core promoters is mainly obtained from focused promoters, which I discuss here.

There are at least 7 conserved motifs found in focused core promoters (Fig. 1). The TATA box is a most conserved and utilized element in the core promoter with the sequence 5'-TATAa/tAAa/g-3', where the capital letters represent the most conserved and the small letters represent less conserved bases. The TATA box is located 28-33 bp upstream of the transcription start site in at least half of all RNAP II promoters (Carninci et al. 2006). The initiator (Inr) is usually found at the transcription start site and its sequence contains preferentially pyrimidines. Both a TATA and Inr sequences are recognized and bound by TFIID that contains TBP (TATA-binding protein) (discussed later). Most of RNAP II promoters contain a TATA box, an initiator or both thereby ensure efficient RNAP II recruitment and binding of TFIID, leading to accurate initiation and transcription. The BRE was first identified as a TFIIB-binding sequence that is located in upstream of a TATA box (Lagrange et al. 1998) and later on it has been shown that there are two BRE sites both upstream and downstream of a TATA (BRE^u and BRE^d). The DPE (downstream core promoter element) and MTE (motif ten element) sequences synergistically enhance basal transcriptional activity with the Inr and TATA box. The DPE is recognized by TFIID and the MTE appears to affect the interaction of TFIID with the core promoter (Burke and Kadonaga 1996; Lim et al. 2004). The DCE (downstream core element) frequently overlaps with the TATA sequences, spreads into three segments and is recognized by TAF1/TAF(II)250 of TFIID components (Lewis et al. 2000; Lee et al. 2005). The XCPE1 (X core promoter element 1) motif is often found in a few bases upstream of the initiation site (Tokusumi et al. 2007). XCPE1 alone

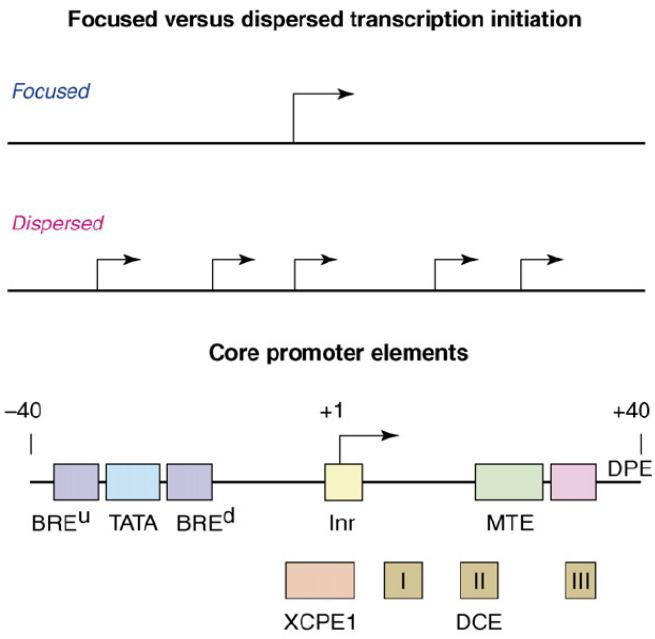

Figure 1. Transcriptional initiation at focused and dispersed promoters and core promoter elements (taken from (Juven-Gershon et al. 2008)). Focused promoters have a single initiation site whereas dispersed promoters contain multiple sites (the upper drawing). The known core promoter elements for transcription by RNAPII (the bottom diagram). Each of these elements is estimated to be found in 1 - 50% of core promoters, depending on the motif.

can potent transcription weakly however it can enhance transcription robustly in concert with sequence specific transcription factors such as NF-1 and Sp1.

A large body of biochemical studies has identified essential protein complexes to initiate transcription *in vitro* using focused promoters containing core promoter elements. RNAP II itself is not enough to recognize the transcription start site and initiate transcription but it requires assembly of additional factors to form a preinitiation complex (PIC). The PIC is composed of RNAP II and five general transcription factors (GTFs) that are TFIIB, TFIID, TFIIE, TFIIF and TFIIH (Woychik and Hampsey 2002). TFIID consists of the TBP and 8-14 TBP-associated factors (TAFs) (Liu et al. 2008). The TBP in a TFIID complex binds to the TATA box, TFIID binds to core promoter elements such as the DPE and DCE, and thereafter induces a sharp bend in the DNA. Such binding of TFIID is the first step to initiate the subsequent binding of the PIC. TFIIA, a member of GTFs, binds directly to the TBP and stabilizes an association of the TFIID and the TATA box. This binding is especially important for the promoter which contains a non-consensus TATA box even though TFIIA is dispensable in a highly purified *in vitro* transcription system. TFIIB enters the PIC after the binding of the TEIID on the TATA box, by which a novel nucleo-protein structure provides a binding site for TFIIB. TFIIB binds both side of the promoter DNA including the BRE^u and BRE^d; in the major groove of DNA at upstream and in the minor groove of DNA at downstream of the TATA box. Such an asymmetric contact encompasses the unidirectional recruitment of RNAP II which requires TFIIB as its binding platform. TFIIF is tightly associated with RNAP II, even

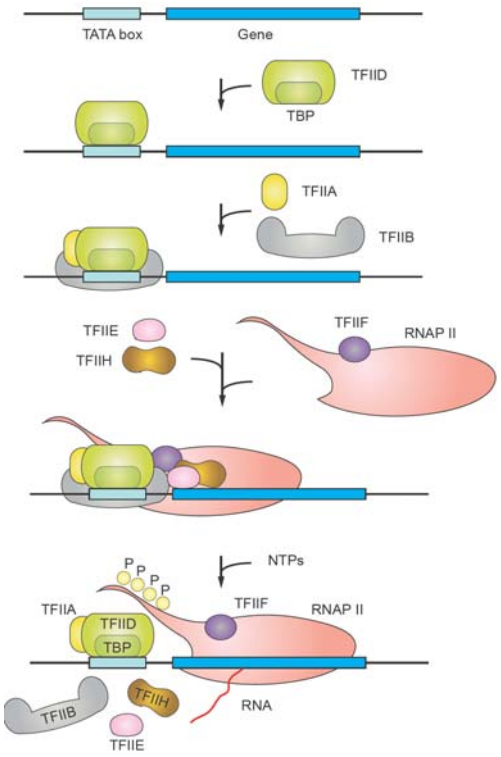

Figure 2. Ordered assembly of general transcription factors and RNAP II. Recognition of a promoter is achieved by the binding of TBP on the TATA sequence. After stepwise recruitment of factors, RNAP II gets phosphorylated in the C-terminus domain (P), which allows the initiation of the RNA synthesis using nucleoside triphosphates (NTPs).

in the absence of DNA. Both TFIIF and RNAP II can bind to TFIIB so that TFIIF association with RNAP II enhances the binding affinity to TFIIB on the promoter DNA. Two more basal factors are assembled before the initiation of transcription; TFIIE and TFIIH. TFIIE comes to bind to RNAP II and increase the binding capacity of the following factor, TFIIH, to RNAP II. TFIIH is the largest and most complicated complex in the PIC. TFIIH contains subunits harboring several distinct enzymatic activities. Subunits called XPB and XPD are ATP-dependent DNA helicases which function to unwind the DNA duplex during transcription. Cyclin H and cyclin-dependent protein kinase (cdk) 7 are also part of TFIIH and are important for the phosphorylation of the C-terminus domain of RNAP II (Fig. 2).

Once the PIC is assembled, transcription can begin with 3 modes of action as follows. First, TFIIE activates the kinase activity of TFIIH at the same time inhibits the helicase activity. Activated kinase activity of TFIIH leads to the phosphorylation of RNAP II C-terminal domain, which causes the dissociation of TFIIE from the PIC. Second, release of TFIIE let helicase subunits of TFIIH function to unwind the DNA duplex, which results in the initiation of RNA synthesis. Third, TFIIB and TFIIH are released from RNAP II by which RNAP II can be physically separated from TFIID at the TATA box and continue to transcribe through whole body of gene.

1.1.2 Activator, repressor, coactivator and corepressor

RNAP II and GTFs are enough to initiate transcription in *in vitro* highly purified system; however transcriptional initiation *in vivo* is regulated by far more sophisticated and complex manners. Higher eukaryotes often have additional regulatory elements rather than simple core promoters such as a promoter and an enhancer element. The promoter sequences are typically found within 1,000 bases upstream of the transcription start site, whereas the enhancers can be found many kilo bases away. These regulatory elements are recognized and occupied by sequence specific transcription factors (TFs), which can influence transcription positively (activators) or negatively (repressors). TFs contain typically 2 domains; one is a DNA binding domain and the other is a activator/represssor domain. TFs bind to a specific DNA sequence on the promoter or the enhancer element via DNA binding domain and modulate transcription by many different ways. The best-characterized

mechanism to enhance transcription by the activator is the recruitment of GTFs and coactivators. The activator domain of the protein is often able to interact directly with TFIID and then recruits and/or stabilizes its binding to the TATA box. In addition, the activator also can associate with and recruite a coactivator to the promoter region. The coactivator lacks a DNA binding capacity but can enhance a transcriptional activity. In the case of a repressor, it binds to the regulatory sequences and recruits a corepressor, which results in the suppression of gene expression. A corepressor as well unable to bind to DNA but has the capacity to repress gene expression, thereby it requires specific TFs for its recruitment to the promoter/enhancer element.

Both coactivators and corepressors often harbor an enzymatic activity to modulate histone posttranslational modifications, which I will intensively discuss later. In summary, an additional layer of mechanism, which is governed by sequence specific TFs and coregulatory factors, regulates eukaryotic gene expression with more sophisticated and complex manners (Fig. 3).

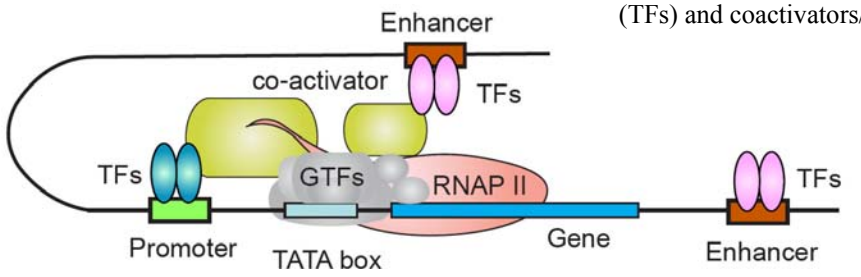

Figure 3. Transcriptional activation by sequence specific transcription factors (TFs) and coactivators/corepressor.

1.2 Epigenetic regulation of gene expression

1.2.1 Chromatin

The human genome, comprising of over three billion base pairs in haploid, would span about 2 meters if it was laid end to end (2004). This large amount of DNA must fit in a cellular nucleus whose diameter is about 10-20 μm. In eukaryotes, DNA is packaged into a highly compacted and organized structure called chromatin. Three billion base pairs of the human haploid genome are organized into 22 pairs of autosomes and a pair of sex chromosomes. Eukaryotic chromosomes have been historically defined in two forms; euchromatin and heterochromatin. Euchromatin is

the chromatin region mainly containing transcriptionally active genes and its structure is more relaxed or decondensed. On the other hand, heterochromatin is more condensed and tightly packed structure, which is able to prevent a transcriptional activation. Originally these structures are described based on the cytological staining. Heterochromatin regions are able to be visualized easily under the microscope, however molecular mechanisms affecting for these two distinct chromatin structures were not explored until recently.

The nucleosome is the fundamental unit of the chromatin structure; it contains a histone octamer complex, including two dimers of H2A/H2B and a tetramer of H3/H4, wrapped around 146 bp of DNA (Luger et al. 1997; Davey et al. 2002) (Fig. 4). Histone molecules are some of the most conserved proteins in evolution, with a molecular mass 10-14 kDa. Histone comprises a globular domain and a flexible tail, which is relatively unstructured; however the amino acid sequences are very well conserved, suggesting a crucial function throughout evolution. Details about functions of histone tails will be discussed in the later topic. Adjacent nucleosomes are connected via linker DNA and form an 11 nm fibre conformation called “beads on a string” which is believed to represent the active and relaxed interphase euchromatin. This 11 nm fibre can be organized in a further compacted form, the 30 nm fibre, via association with Histone H1. This 30 nm fibre structure is considered as a conformation for a transcriptionally repressive or silenced state of chromatin. In mitotic and meiotic cells, chromatin structure becomes the most condensed formation and a whole chromosome gets compacted to a length of a few μm. In summary, chromatin structure changes dynamically during cell cycle progression and distinct structural organization of chromatin is well correlated with transcriptional activity, suggesting that this conformational change may influence gene expression or may be the reflection of on going transcription.

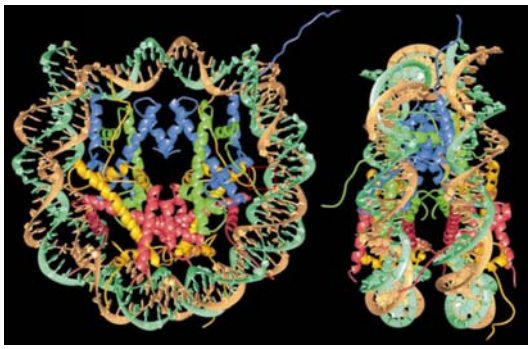

Figure 4. Crystal structure of the nucleosome core particle (Luger et al. 1997). Ribbon traces for the 146 bp DNA phosphodiester backbones (brown and turquoise) and eight histone protein main chains (blue: H3; green: H4; yellow: H2A; red: H2B. The views are down the DNA superhelix axis for the left particle and perpendicular to it for the right particle. For both particles, the pseudo-twofold axis is aligned vertically with the DNA centre at the top.

1.2.2 Nucleosome remodeling

Alteration of the chromatin structure is a significant step in transcriptional regulation of many eukaryotic genes, by which ATP-dependent nucleosome remodeling complexes are responsible for the process. The action of ATP-dependent nucleosome remodelling machineries influence for a variety of biological process, from the complete clearance of nucleosomes at regulatory sites to shifting nucleosome positions, increasing the accessibility of DNA on the surface of positioned nucleosomes and exchange of H2A variants (Eberharter and Becker 2004). This remodeling of nucleosomes increases the accessibility of DNA sequence elements to regulatory proteins that scan the genome for target sites, which results in the dynamic changes of transcription. A number of nucleosome remodeling complexes have been identified that modulate the arrangement and stability of nucleosomes without inducing covalent modifications. Generally, these ATP-dependent remodeling machines are divided into four major subfamilies including SWI/SNF, ISWI, NuRD and INO80, characterized by the identity of their central catalytic subunit comprising BRG1 (or hBrm), ISWI, Mi-2 and Ino80, respectively (Eberharter and Becker 2004). These nucleosome remodeling complexes can alter the chromatin structure and influence gene expression.

1.2.3 Histone post-translational modifications and modifiers

As discussed in the previous topic, dynamic alterations in chromatin structure are well associated with many aspects of biological processes. What makes chromatin structure so dynamic and what is the effect for transcription? One of the most important mechanisms is histone post-translational modifications (Fig. 5). Histone acetylation and methylation on H3 and H4 were identified in 1964, since then it was proposed that these covalent modifications correlate with transcriptional activity (Allfrey et al. 1964). Several decades later, the "**histone code**" hypothesis was proposed which postulates that different combinations of modifications in histones could be translated into functionally distinct effects on nuclear processes such as transcription, DNA repair and DNA replication (Strahl and Allis 2000; Jenuwein and Allis 2001). Recent accelerated progresses in the field brought us many evidences supporting this hypothesis and, in turn, the fact that the situation could be much more

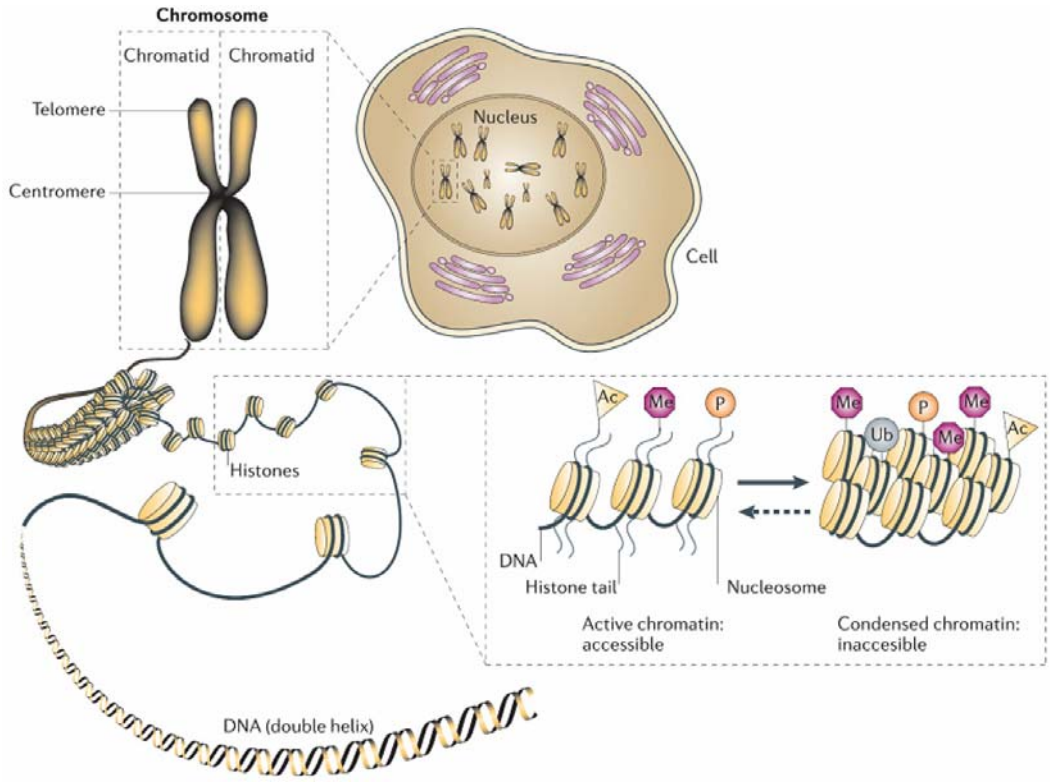

Figure 5. Chromosome organization and post-translational modifications (Sparmann and van Lohuizen 2006). Eukaryotic DNA is organized into a chromosome structure composed of nuclesomes. Highly condensed mitotic chromosome is shown in the top-left. A chromatid is one of two identical copies of DNA making up a chromosome. A centromere is the region where two of chromatids fuse together and a telomere is the each end of chromosome. Both centromere and telomere contain repetitive sequences. Histone tails are able to be modified by different types of post-translational modifications: Ac, acetylation; Me, methylation; P, phosphorylation; Ub, Ubiquitination; respectively. Many of them are known to be catalyzed reversibly.

complex than a simple “code”. At the moment, we know the following types of posttranslational modifications on core histones; acetylation, methylation, phosphorylation, ubiquitination, sumoylation, ADP ribosylation, biotinylation and proline isomerization. These modifications take place at specific residues of each histone and the specific patterns of them are very well correlated with distinct biological processes. In the following section, I will summarize the function of several modifications and enzymes responsible for modifications focusing on the aspect of transcriptional regulation.

In general, two ways of action are believed to alter the transcriptional activity through histone modifications. First histone modifications may directly affect for the higher order of the chromatin structure through the conformational changes in each nucleosome and controlling the association of the nucleosome arrays. Second histone

modifications may provide the binding sites for proteins harboring an activity altering gene expression; at the same time these histone modifications are also able to function to prevent proteins from binding.

1.2.4 Histone methylation and modifying enzymes

1.2.4.1 Histone methylation

Lysine and arginine residues on histone H3 and H4 can be methylated to different degrees: mono-, di- and, in some cases, tri-methylations are possible. Each type of modification could have different biological outcomes. In several cases, differences in outcome are very well characterized. For example, methylation at lysine 4 in histone H3 is well correlated with gene active regions. However distributions of mono-, di-, and tri-methylated H3K4 in the active gene loci are totally different, which may indicate their differential implication in transcription. Tri-methylation of H3K4 occurs at the 5' ends of ORFs as genes become induced, whereas di-methyl modification of H3K4 is peaked at the middle part of transcribing gene. In addition, mono-methylated H3K4 is enriched at the termination site of gene (Ruthenburg et al. 2007). Similar observations have been made in the case of H3K9 methylation. H3K9 methylation is the best characterized modification associated to the gene silencing and repression. In higher eukaryotes, tri-methylated H3K9 is typically connected to constitutive heterochromatin, while mono- and di-methylated H3K9 are mainly located in euchromatin and generally linked to repressed promoter regions (Berger 2007).

H3K27 tri-methylation at transcriptional start sites is generally correlated with the repression of gene expression. This repression is mediated by the protein complex called Polycomb repressive complex (PRC). H3K27 trimethylation contributes to the recruitment and/or stabilization of PRC on chromatin, which in turn mediates gene repression (Schuettengruber et al. 2007). Recent genome wide analyses identified chromatin regions containing both H3K4 and H3K27 tri-methylation, termed “bivalent domains”, which are enriched in embryonic stem (ES) cells at promoters of genes encoding key developmental TFs and components of critical signaling pathways (Bernstein et al. 2006). This enrichment of tri-methylation at both active (H3K4) and repressive (H3K27) position is interpreted as repression of gene expression by recruitment of PRC. Upon ES cell differentiation, many bivalent

domains, in turn, resolve into transcriptionally active H3K4 tri-methyl or transcriptionally silent H3K27 tri-methyl chromatin regions, depending on the specific cell fate and the lineage commitment, and some remain bivalent in terminally differentiated cells (Mikkelsen et al. 2007).

1.2.4.2 Histone methyltransferases

To date, it has been demonstrated that histone methylation can be reversibly catalyzed by site specific histone methyltransferases and histone demethylases. During the past few years, remarkable progresses have been made in the identification of the enzymatic machineries involved in histone methylation. These enzymes have been grouped into several classes, including the lysine-specific SET domain-containing histone methyltransferases involved in methylation of lysines 4, 9, 27, and 36 of histone H3 and lysine 20 of histone H4; non-SET domain-containing lysine methyltransferases involved in methylating lysine 79 of histone H3; and arginine methyltransferases involved in methylating arginine 2, 17, and 26 of histone H3 as well as arginine 3 of histone H4 (Shilatifard 2006) (Fig. 6).

The conserved lysine methyltransferases motif, SET domain, is named from the *Drosophila* proteins **S**u(var)3–9, **E**nhancer of zeste [E(z)], and **T**rithorax. All proteins are conserved in mammals and human homologous proteins are SUV39, EZH2, and MLL family, respectively. H3K4 methylation has been shown to be catalyzed by more than 10 methyltransferases. *Drosophila* trithorax-related proteins, **M**ixed **L**ineage **L**eukemia (MLL) protein family, are the main member of H3K4 methyltransferases, which consist of MLL1, MLL2, MLL3, MLL4, MLL5, SET1A and SET1B in human (Emerling et al. 2002; Ruthenburg et al. 2007). This multiplicity of H3K4 methyltransferase activity provides a wide range of functional diversities based on their tissue and temporal restricted expression patterns and target-specificities (Yu et al. 1995; Glaser et al. 2006; Lee et al. 2006).

H3K9 is known to be methylated by several enzymes, however three enzymes are the best characterized: G9a, SUV39h1 and SUV39h2. Mouse mutagenesis experiment revealed that *in vivo* G9a dominantly regulates H3K9 mono- and di-methylation at euchromatic regions, whereas both Suv39 proteins contribute to H3K9 tri-methylation on pericentric heterochromatin (Peters et al. 2003). Combined loss of Suv39h1 and

Suv39h2 impairs heterochromatin formation and genome stability, which results in the embryonic lethality around E12.5 (Peters et al. 2001).

H3K27 methylation is governed by mainly EZH2 protein *in vivo*. EZH2 is responsible for the key enzymatic activity of the Polycomb repressive complex PRC2. Polycomb group proteins were genetically identified in *Drosophila* as factors required for maintaining the lineage specifications during embryogenesis by repressing *Hox* genes in a body-segment-specific manner (Kanno et al. 2008). Therefore mouse Ezh2 also has been reported to contribute to regulate development in various biological systems (O'Carroll et al. 2001; Su et al. 2003; Puschendorf et al. 2008).

1.2.4.3 Histone demethylases

Until recently, it was assumed that histone methylation is an irreversible modification and that removal of methyl groups can only be achieved by turn-over and/or exchange of histones. However, in 2004, Shi and colleagues made a break through and identified the factor, **L**ysine **S**pecific **D**emethylase 1 (LSD1) (Shi et al. 2004). LSD1 is also called BHC110, a nuclear amine oxidase homologue previously found in several histone deacetylase corepressor complexes (You et al. 2001; Hakimi et al. 2002; Shi et al. 2003). LSD1 homologs are well conserved in eukaryotes except for *S. cerevisiae*. LSD1 demethylase activity is restricted toward H3K4me1/2 both *in vitro* and *in vivo*, and presence of the CoREST complex is required for the demethylase activity towards *in vitro* nucleosome substrates (Shi et al. 2004; Lee et al. 2005).

The discovery of LSD1 raised the question whether are there any other demethylases? A novel JmjC domain-containing protein, JHDM1 (JmjC domain-containing histone demethylase 1), was identified as a histone demethylase by biochemical purification in 2006 (Tsukada et al. 2006). The JmjC domain was originally found in the jumonji (jmj) protein which was identified to be essential in the mouse for the development of multiple tissues by a forward genetic approach (Takeuchi et al. 1995). Many jumonji family proteins have domains responsible for DNA and chromatin association and some of them have been shown to repress gene expression, however the molecular mechanisms underlying had not been explored until the discovery of their demethylase activity (Toyoda et al. 2003; Kim et al. 2004; Kim et al. 2005). JHDM1 preferentially demethylates H3K36me2 and to a lesser extent H3K36me1, which are

usually enriched in actively trnanscribed loci (Tsukada et al. 2006). In the mammalian genome, over 100 jmjC domain-containing genes are encoded; thereby these enzymes may have diverse- and specific- roles in different tissues during the mammalian development, which is largely unexplored yet.

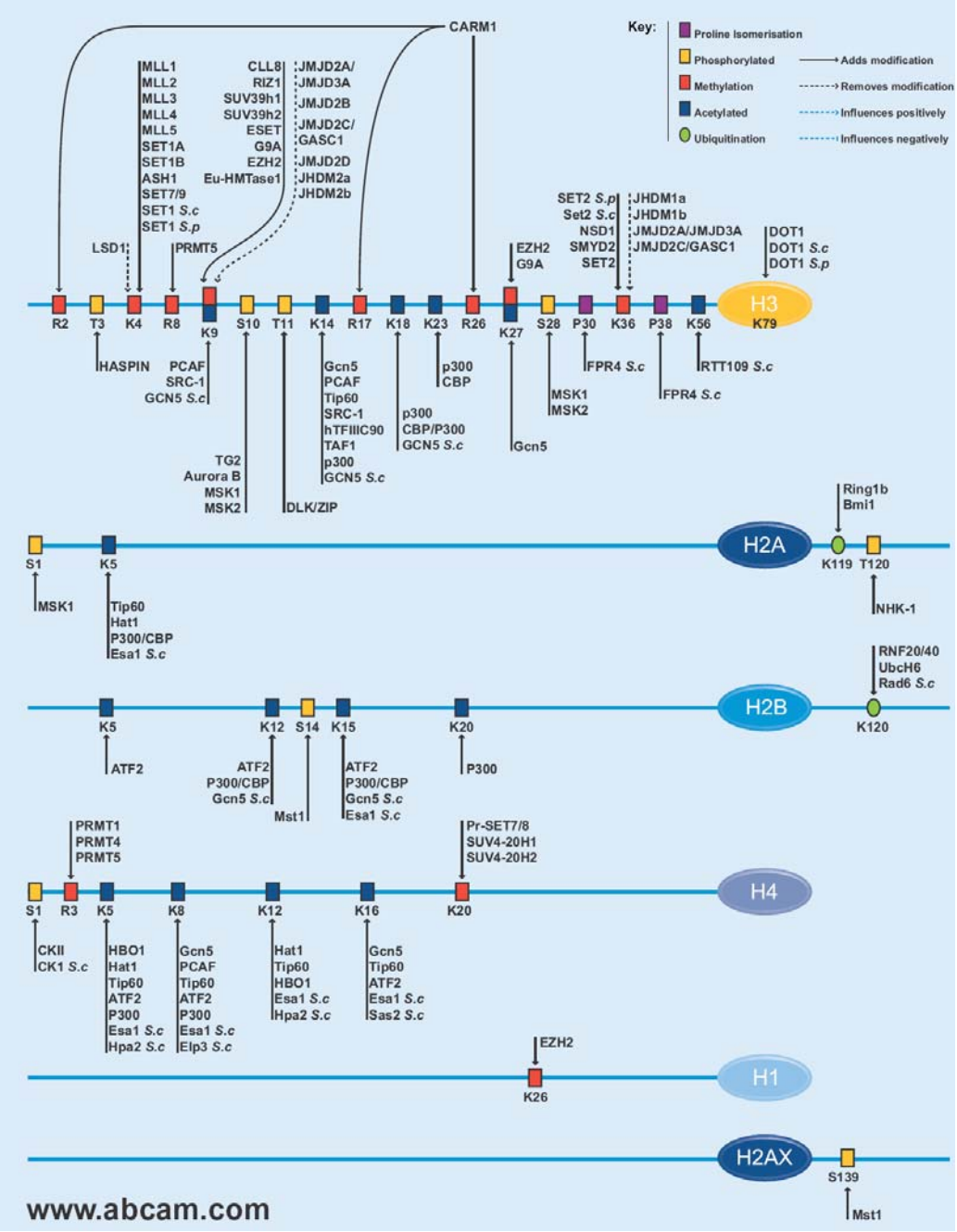

Figure 6. Human and yeast histone modifying enzymes. Currently known enzymes are drawn in the Figure. HDACs are not indicated since their specificity in higher eukaryotes has not been clear yet. *S.c* indicates Saccaromyses *Saccharomyces cerevisiae* and *S.p* represents *Schizosaccharomyces pombe*. The figure is taken from Abcam web site. (http://www.abcam.co.jp/ps/pdf/chromatin/a4_chip_card.pdf)

1.2.5 Histone acetylation and modifying enzymes

1.2.5.1 Histone acetylation

Histone acetylation is one of the best characterized modifications which positively correlate with gene transcription. A large number of genome-wide studies has shown that the bulk of histone H3 and H4 acetylation is enriched in gene active loci such as euchromatic regions, whereas hypo-acetylation is detected at inactive loci and in heterochromatic regions. Histone acetylation occurs reversively at the ε-amino group of lysine residues in all of the core histones, mainly at the tails but also at a few positions in the globular domain. Histone acetylation is proposed to regulate gene expression by making the chromatin structure "open" or "accessible", so that GTFs and RNAP II can bind onto DNA to initiate transcription. Originally the open structure of chromatin has been identified by the DNase I hyper sensitivity since DNase I is able to digest DNA if it is free from the nucleosome structure. These traditionally defined hyper sensitive sites are, in tern, shown to be associated with histone hyper acetylation, proving that open conformation of chromatin contains histone hyper acetylation. In addition, *in vitro* studies have proven that acetylation of H4K16 could inhibit an association of nucleosome arrays which are likely a luminescent situation of 30 nm fibre chromatin conformation *in vivo* (Dorigo et al. 2003; Shogren-Knaak et al. 2006). Taken together, histone acetylation can be a causal

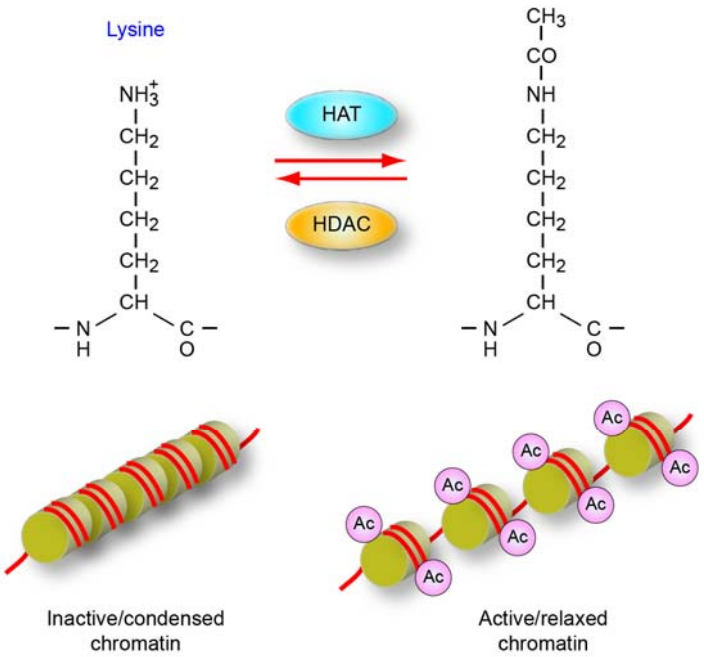

Figure 7. Histone acetylation and chromatin structure. Histone acetylation on a lysine residue is catalyzed by HAT and deacetylation by HDAC. Acetylated nucleosomes form more relaxed structure, which is transcriptionally active, whereas hypo-acetylated nucleosomes are condensed, which prevent the gene expression.

effect of opening up chromatin and has strong positive correlation with open conformation of chromatin (Fig. 7).

Another mechanism by which histone acetylation affects for gene expression is that acetyl-lysine can be recognized by proteins containing a bromodomain motif. Bromodomain motifs are often found in proteins acting as a transcriptional machinery (TAF1/TAFII250; a subunit of TFIID), coactivetors (p300, CBP, GCN5 and PCAF) and nucleosome remodeling proteins (BRG1 and SWI/SNF). In details of these bromodomain containing proteins are discussed in later. Thus histone acetylation facilitates the binding of these factors and promotes gene expression in a positive manner.

1.2.5.2 Histone acetyltransferases

This reversible acetylation on lysine residues is mediated by two opposing enzymatic activities; Histone acetyltransferases (HATs) and Histone deacetylases (HDACs). HATs are enzymes that acetylate lysine residues by transferring an acetyl group from acetyl-CoA to lysine to form ε-acetylated lysine. On the other hand, HDACs remove acetyl groups from ε-acetylated lysines by hydrolysis. HATs are classified into five families including the Gcn5-related acetyltransferases (GNATs); the MYST-related HATs (for **M**OZ, **Y**bf2/Sas3, **S**as2 and **T**ip60 named from yeast genes); p300/CBP HATs; the general TF HATs, TAF1/TAFII250; and the nuclear hormone-related HATs SRC1 and ACTR (SRC3) (Carrozza et al. 2003).

The GNAT superfamily in human includes GCN5 and PCAF, which are known to be a major source of nuclear type HATs. Both proteins share 73% identical amino acids and possess similar domains (Nagy and Tora 2007). In the N terminus, they contain a catalytically essential domain called the PCAF homology domain and a bromodomain in the C terminus. Similar to other superfamilies of HATs, they are found in several large protein complexes with a total mass of either 2 MDa or 700 kDa. In yeast, the SAGA and the ADA complexes have been identified to contain the Gcn5 protein (Baker and Grant 2007). In human, there are 3 large complexes; TFTC, STAGA and PCAF complexes and 1 small complex; ATAC. All of them catalyze acetylation preferentially on histone H3, in which they may have an additional preference for

specific lysines based on the results observed with complexes. All three large complexes contain several ADA proteins involved in the nucleosomal HAT activity, SPT proteins and TAFs for the interaction with TBP and GTFs. Yeast SAGA complex is known to contain Ubp8 protein which is the enzyme that deubiquitylate histone H2B at Lys 123. H2B ubiquitylation is transient, increasing early during gene activation, and then decreasing coincident with significant RNA accumulation. It might well be that H2B monoubiquitination is important for the initiation of transcription and/or association with the transcriptional machinery, however it will be deubiquitinated by SAGA complex during the transcriptional process. Based on these facts that coactivator HAT complexes might enhance transcription by following mechanisms;,(1) binding to the acetylated histone in the promoter regions via its own bromodomain, (2) directly catalyzing acetylation on histone, (3) stabilizing the binding to GTFs via its complex components, and (4) deubiquitination of H2B.

The MYST family currently comprises five human HATs: Tip60, MOZ, MORF, HBO1 and MOF. Their defining feature is the presence of the highly conserved MYST domain composed of an acetyl-CoA binding motif and a zinc finger. Some family members also have in common additional structural features such as chromodomains, plant homeodomain-linked (PHD) zinc fingers, and others (Avvakumov and Cote 2007). Tip60 is the catalytic subunit of the coactivator complex called NuA4 (Nucleosome acetyltransferase of histone H4), which can catalyze the acetyltransferase activity toward hisotne H4 and H2A. The function of Tip60 in transcription is well described. One example is that human NuA4 was linked to the function of transcription activators required for cell-cycle progression by which c-Myc recruits NuA4 to its target genes (McMahon et al. 1998). Another member of the MYST family, MOF was identified by *Drosophila* genetic screening to search for genes involved in dosage compensation of male X chromosome. MOF stands for *males-absent-on-the-first*. MOF is a HAT that specifically acetylates lysine 16 of histone H4 (H4K16ac), a modification found highly enriched on the male X chromosome in *Drosophila*. Recent genome-wide study has shown that *Drosophila* MOF complex is not only critical for the dosage compensation but also regulating gene expression in autosomes by affecting the level of H4K16 acetylation (Kind et al. 2008). The MOF complex is also highly conserved in human as well as its substrate specificity. Two studies have discribed that depletion of human MOF in cell lines and

the targeted inactivation of mouse MOF lead to genomic instability, spontaneous chromosomal aberrations, cell cycle defects, altered nuclear morphology, and reduced transcription of certain genes (Gupta et al. 2005; Gupta et al. 2008). Taken together, MOF functions are conserved in evolution and regulate essential gene expression as well as H4K16 acetylation in the whole genome.

Importantly, the MYST family of HATs is involved in the process of DNA repair (Avvakumov and Cote 2007). This effect may be partly due to acetyltransferase activity toward histones, however Tip60 and MOF are also shown to acetylate p53 directly on lysine 120 (Sykes et al. 2006; Tang et al. 2006). As a result of this acetylation on DNA binding module, acetylated p53 appears to bind preferentially to the promoter of pro-apoptotic genes. Acetylation of p53 is the important switch to decide the cell fate toward either cell cycle arrest or apoptosis.

Another family of HATs comprises the cAMP response element-binding protein-binding protein (CBP) and p300 which were originally identified as factors binding to the cAMP response element-binding protein (CREB) and the adenoviral E1A transcription factor, respectively (Karamouzis et al. 2007). The preferred *in vitro* sites of acetylation on the N-terminal histone tails are K12 and K15 in histone H2B, K14 and K18 in histone H3 and K5 and K8 in histone H4 (Schiltz et al. 1999). Importantly both p300 and CBP are the acetyltransferases not only for core histones but also a number of non-histone proteins such as p53, FoxO1, STAT1, NF-κB and type I interferon (IFN) receptor 2 (Goodman and Smolik 2000; Chen et al. 2001; Motta et al. 2004; Matsuzaki et al. 2005; Kramer et al. 2006). Following DNA damage, p53 is activated by kinase-mediated phosphorylation as well as by acetylation at specific residues by CBP/p300, resulting in increased stability of the p53-CBP/p300-DNA complex. Furthermore, CBP/p300 is required for p53-mediated transactivation of target genes through their co-activator function and through local histone acetylation. Interestingly, CBP and p300 also control p53 stability by regulating its ubiquitination and degradation, through both Mdm2-dependent and independent mechanisms. Degradation of p53 has been shown to be mediated by a complex comprising p53, Mdm2 and CBP/p300 (Grossman et al. 1998). Recently, the CH1 domain of CBP/p300 was found to exhibit ubiquitin ligase activity towards p53; this indicates

that CBP/p300 can play a direct role in regulating the level of p53 protein (Grossman et al. 2003).

In summary, HATs family proteins are the catalytic core of various large protein coactivator complexes, which function to acetylate lysine residues of core histones at the promoter regions of genes. As a result of this, HATs are able to activate gene expression. Each HAT displays preferred lysine sites on core histones, which determines their *in vivo* specific roles. In addition, an increasing number of non-histone proteins are found to be a substrate of "histone acetyltransferases", by which HATs can modulate various properties of the target protein such as protein stability, intra-cellular localization or DNA binding capacity.

1.2.6 Histone deacetylases

1.2.6.1 Yeast HDACs

Yeast, *S. cerevisiae, Rpd3* was the first HDAC gene identified genetically as a factor that represses the expression of potassium transporters in yeast (*S. cerevisia*) (Vidal et al. 1990; Vidal and Gaber 1991), subsequently, later on it was found that it has an activity to deacetylate histones and repress gene expression (Rundlett et al.). Since then, using genetic, biochemical and bioinfomatic approaches, many HDAC genes have been identified from yeast to human. So far 10 yeast genes are known to encode proteins harboring the deacetylase activity: *Rpd3*, *Hda1*, *Hos1*, *Hos2*, *Hos3*, *Sir2*, *Hst1*, *Hst2*, *Hst3* and *Hst4* (Brachmann et al. 1995; Kurdistani and Grunstein 2003). According to the sequence and functional similarity, they are grouped into 3 classes. *Rpd3*, *Hos1* and *Hos2* are the members of class I HDACs. Rpd3 and Hos1 are necessary for the deacetylation of H4K5, 8 and 12, and only Hos2 is required for H4K16 (Wang et al. 2002). Rpd3 forms two distinct large protein complexes; Rpd3L and Rpd3S which are apparent mass of 1.3 MDa and 0.8 MDa respectively (Carrozza et al. 2005). Both protein complexes share the core subunits including Rpd3, Sin3 and Ume1. In addition, the Rpd3L complex contains different proteins such as Ume6, Sds3, Sap30 and others, whereas Rpd3S contains Rco1 and Eaf3. The mechanisms targeting these distinct complexes to specific genomic loci appears to be different. Genome-wide studies have shown that Rpd3 proteins are predominantly recruited to promoter regions of genes via sequence specific DNA binding factors, and at the same time a significant amount of proteins is detected globally on the genome (Kurdistani et al. 2002). Altough many publications described the correlation between gene expression and promoter histone acetylation, recent reports suggested that recruitments of Rpd3S complexes to gene cording regions are also an important function of HDACs acting to repress the aberrant transcription initiation from cryptic start sites (Carrozza et al. 2005; Keogh et al. 2005).

Hos3 belongs to class II HDACs, whereas *Sir2* (Silent information regulator) and its homologous genes *Hst1*, *Hst2*, *Hst3* and *Hst4* belong to class III. *Sir2* gene was first identified as a yeast mutation required for repressing the expression of mating type genes (Buck et al. 2004). Class III HDACs need a nicotinamide adenine dinucleotide^{+} (NAD^{+}) for their enzymatic activity and their protein structures are unrelated to any of class I and II enzymes.

1.2.6.2 Mammalian HDACs

In mouse and human, 18 HDACs have been identified that can be grouped into four classes based on their protein homology to yeast HDACs (Fig. 8). Class I enzymes comprise HDAC1, 2, 3 and 8, class II includes HDAC4, 5, 6, 7 and 9, class IV contains only HDAC11, and class III, NAD^+ dependent-Sir2 homolog, contains SIRT1-7 (Yang and Seto 2008). To date, a tremendous amount of researches has been carried out to understand the function of these enzymes since it has became clear that HDACs are intimately involved in many different types of diseases, and that HDACs might be potential targets of drug therapies. It has been known for long time that HDAC inibitors (HDACis), such as trichostatin A (TSA) and suberoylanilide hydroxamic acid (SAHA), function as anti-cancer drugs, which can inhibit cell cycle progression and/or induce apoptosis and differentiation in a cell type- and context-dependent manner (Yoshida et al. 1987; Richon et al. 1996; Richon et al. 1998; Minucci and Pelicci 2006). Recent progresses in the field have pointed out that the targeting of HDACs could potentially be applied for very diverse diseases including inflammatory diseases, heart diseases, neurodegenerative diseases, and others (Saha and Pahan 2006; Adcock 2007; Berry et al. 2008). Inhibition of HDACs has such a great impact on a diverse set of diseases that it is not surprising that functions of HDACs are not only restricted to the regulation of gene expression through modulating histone acetylation but also controlling the many aspect of biological processes such as a protein stability, protein translocation, enzymatic activity, and DNA binding affinity via acetylation of non-histone proteins (Minucci and Pelicci 2006).

To date, at least ten different HDACis are currently under c.a. 80 clinical trials as anti-cancer drugs, tested in everything from rare leukemias and lymphomas to breast, prostate and ovarian cancer either alone or in combination with other drugs. Importantly SAHA, also called Vorinostat (*Zolinza*™, Merck & Co., Whitehouse Station, NJ, USA), has been approved by the U.S. Food and Drug Administration for treatment of cutaneous T-cell lymphoma (CTCL) (Duvic and Vu 2007; Mottet and Castronovo 2008). However there are several hurdles to overcome in the future of the HDACi field. First many of the HDACis work very well *in vitro* in tumor cell lines

including hematological and solid tumors, but in animal trials the anti-cancer effects are not as striking as seen in *in vitro* experiments, especially on solid tumors, although most trials are in early stages. In addition, there are several cases reported that HDACis show heart-related side effects. Most of these effects are thought to be medically insignificant; yet one HDACi, depsipeptide, was dropped from the trial because of that (Garber 2007). Why HDACis is not working well on solid tumors *in vivo* and how do HDACis affect the heart? Answers for these questions have not been addressed yet mainly due to the lack of knowledge showing molecular mechanisms of HDAC function *in vivo*, key targets of HDACs, and subtype-specific functions of HDACs. Given the complexity of mammalian HDACs (18 homologs), non-selective inhibitory activity of HDACis (the majority of HDACis inhibits both class I and II HDACs), and a growing number of non-histone substrates for HDACs, evaluating the effects by HDACis has been facing a huge problem. Therefore, research to dissect the role of specific HDACs *in vivo* using a genetic approach will help to understand how

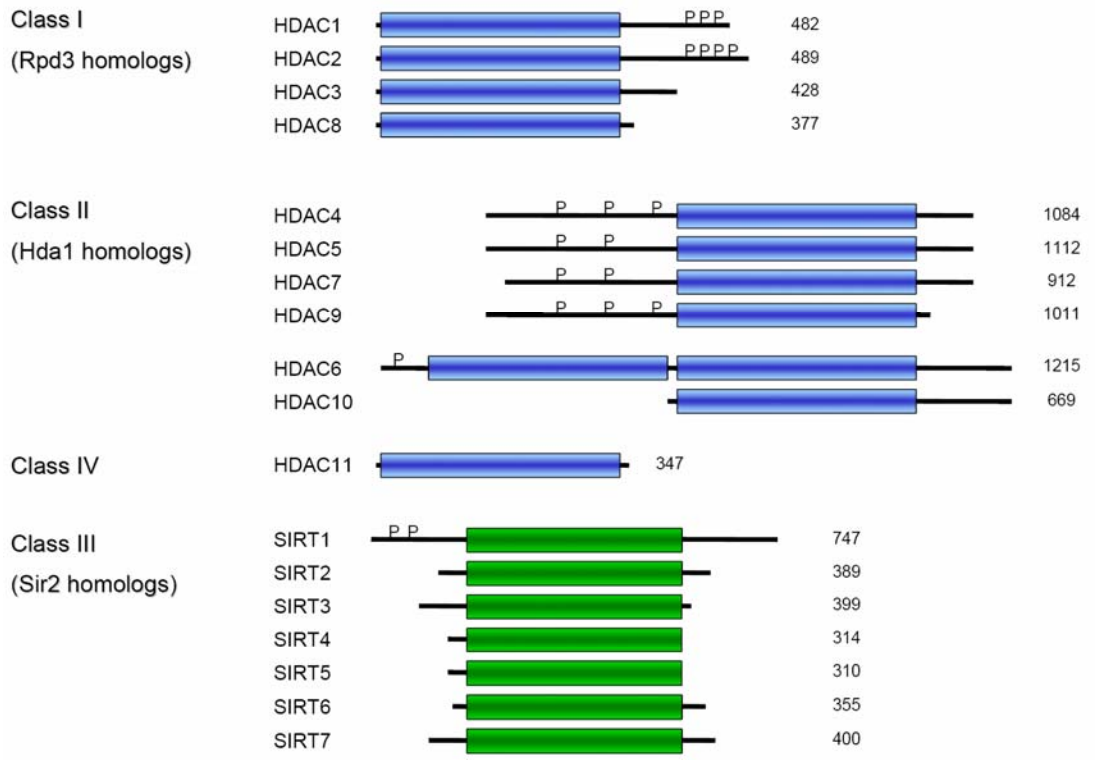

Figure 8. Schematic representation of mammalian HDACs. HDACs are grouped into 4 subfamily based on their amino acids homology. Blue or green boxes represent a conserved HDAC domain for class I, II and IV enzymes, and Sir2 homologs. Numbers of amino acids in each protein are shown in the right side. P in each HDAC indicates the identified phosphorylation site.

individual HDAC functions and which HDAC is implicated in specific biological processes. Here I summarize reports describing the *in vivo* functions of the class I and II HDACs, which are the main targets of HDACis, as revealed by genetic approaches to remove specifically individual enzymes.

1.2.6.3 Class I HDACs in matazoans

As mentioned above, mammalian class I HDACs (HDAC1, 2, 3 and 8) are closely related to yeast Rpd3, which is predominantly found on the promoter region of genes as well as globally on the genome (Kurdistani et al. 2002). Thus class I HDACs in mammals are also thought to be regulating gene expression by removing the acetyl group from core histones in the promoter regions, however this remain to be demonstrated experimantaly in higher eukaryotes by the genome-wide manner. There are at least 4 protein complexes identified in mammals containing at least one of class I HDACs; Sin3, Mi-2b/NuRD, CoREST, and N-CoR/SMRT. The Sin3, Mi-2b/NuRD and CoREST complexes include HDAC1 and 2 as catalytically core enzymes, whereas N-CoR/SMRT harbors HDAC3 in the complex (Karagianni and Wong 2007; Yang and Seto 2008) (Fig. 9). All complexes are known to function as a co-repressor to shut down gene expression, by which sequence specific TFs recruit them on the promoter regions. In other metazoans such as *C. elegans* (worm), *D. melanogaster* (fly), and *D. rerio* (zebrafish), orthologs of mammalian HDAC1 and 3 are found, and,

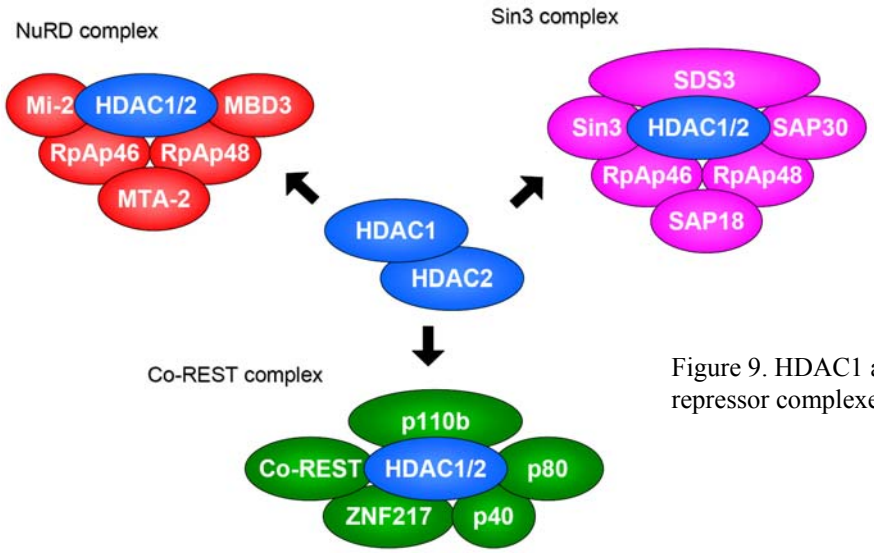

Figure 9. HDAC1 and 2 containing co-repressor complexes.

in addition, the zebrafish genome encodes an ortholog of HDAC8. Several members of three co-repressor complexes containing HDAC1 and 2 are encoded in the genome of *C. elegans*, *D. melanogaster*, and *D. rerio*, whereas N-CoR/SMRT are not found in *C. elegans*, although it has HDAC3 homolog as a functional protein (Shi and Mello 1998; Yang and Seto 2008).

Since all of HDAC1/2 complexes lack the ability to bind to DNA, their target genes are determined by interaction partners having a sequence specific DNA binding capacity. To date a number of proteins has been identified to associate directly with HDAC1 and/or 2 or co-repressor complexes in different types of transformed cells as well as primary cells. One of the first identified interaction partner in mammalian cells is Retinoblastoma (Rb) protein which represses specific genes that are active in the S phase of the cell cycle. The Rb protein masks the trans-activation domain of E2F transcription factor and, at the same time, brings HDAC1 and/or 2 to the promoter region of genes involved in proper control of the G1/S transition, which results in repression of genes inhibiting this step (Brehm et al. 1998; Ferreira et al. 1998; Luo et al. 1998; Magnaghi-Jaulin et al. 1998).

The multisubunit transcription factor NF-κB plays an essential role in the regulation of gene expression involved in immune and inflammatory responses and cell survival. The most abundant form of NF-κB is a heterodimer of p50 and p65 (RelA). The subunit p65 is shown to associate with both HDAC1/2 and repress the interleukin-8 (*Il8*) gene induced by the TNF-dependent manner (Ashburner et al. 2001).

In addition to these factors, HDAC1/2 interaction was confirmed with other TFs such as Blimp-1 (Yu et al. 2000), YY1 (Coull et al. 2000; Yang et al. 2001), Sp1 and Sp3 (Choi et al. 2002; Won et al. 2002), BCL6 (Bereshchenko et al. 2002), Nanog and Oct4 (Liang et al. 2008).

It is worthy to note again in this chapter that an increasing number of reports have demonstrated that acetylation of lysine residues on non-histone proteins are also important to regulate biological features. Acetylation of proteins can modulate their function at multiple levels such as: the transcriptional activity, DNA binding affinity, protein stability, protein localization and protein-protein interaction (Glozak et al. 2005).

Among the large number of proteins identified, p53 is one of the most and best characterized proteins for the impact of acetyl modification. The activity of the p53 tumor suppressor is modulated by protein stability and post-translational

modifications in the response to various forms of cellular stress (Gu and Roeder 1997; Lavin and Gueven 2006). Acetyl modification of p53 at lysine 120 and 164 within the DNA binding domain and 6 additional sites in the C-terminus of p53 occurs following genotoxic stress and enhances its transcriptional activity towards *p21* gene expression, which leads to a blockade of the cell cycle and promotes the DNA repair pathway. This acetylation also stabilizes p53 itself, preventing the binding to Mdm2 which ubiquitinates p53 for degradation. It has been confirmed that the HDAC1/2 complex is responsible to deacetylate several sites, therefore HDAC1/2 complex is crucial to reduce the activity of p53 via deacetylation, which results in repression of genes inhibiting the cell cycle progression (Luo et al. 2000; Ito et al. 2002; Tang et al. 2008).

1.2.6.4 Developmental roles of class I HDACs in organisms

With the exception of HDAC8, all class I HDACs and several subunits of HDAC complexes have been inactivated either in the entire mouse or in a tissue-specific manner (Table 1). *HDAC1* knock out mice were the first reported HDAC deficient mice, which results in embryonic lethality before E9.5 (Lagger et al. 2002; Montgomery et al. 2007). *HDAC1*$^{-/-}$ ES cells show proliferation defects associated with upregulation of cyclin-dependent kinase inhibitors; p21 and p27, later on it was shown that acetylation of the *p21* promoter is directly regulated by HDAC1 in these cells (Lagger et al. 2003). A tissue-specific role of HDAC1 was reported together in a comparison with the effect of HDAC2 inactivation (Montgomery et al. 2007). *HDAC2*$^{-/-}$ mice show the complete lethality within 24 hours after birth, although they are born at near expected Mendelian ratios. Histological analysis of *HDAC2*$^{-/-}$ neonates revealed unusual morphological abnormalities of the right ventricular chamber in the heart. In sharp contrast to the lethality in the absence of HDAC1 or 2, tissue specific abrogation of one of both enzymes in the neural crest, skeletal muscle, central nervous system, endothelium, smooth muscle, secondary heart field and B lymphocytes does not have apparent defects (Montgomery et al. 2007; Yamaguchi et al. submitted). Combined cardiac inactivation of both HDAC1 and 2 leads to postnatal death with severe cardiac defects by day 14 accompanied by misregulation of genes encoding skeletal muscle-specific contractile proteins and calcium channels (Montgomery et al. 2007). These results have demonstrated that essential functions

for both proteins during embryogenesis and dispensable functions in the adult mice. It is worth mentioning that other modified alleles of *HDAC2* mutant mice generated by either a gene-trap method or removal of exon 6 show only partial perinatal lethality (Trivedi et al. 2007; Ymaguchi et al. submitted). In these mutant mice, about 50% of them die within a month, and the rest has a smaller body size as well as cardiac defects. The heart of these mutant mice are protected from cardiac hypertrophy by upregulating the gene encoding inositol polyphosphate-5-phosphatase f (*Inpp5f*), which results in constitutive activation of glycogen synthase kinase 3β (Gsk3β).

HDAC3$^{-/-}$ mice were recently reported to show embryonic lethality prior to E9.5 (Bhaskara et al. 2008). Further analysis was conducted using mouse embryonic fibroblasts (MEFs) to investigate the requirement of HDAC3 for the cell-cycle progression, which had previously been observced in cancer cell lines (Li et al. 2006). Although primary MEFs lacking HDAC3 show a delay in cell-cycle progression, they do not exhibit decreased H3S10 phosphorylation, which leads to the dissociation of the heterochromatin protein 1 (HP1) from chromatin during mitosis, whereas cancer cell lines as well as immortalized MEFs show mitotic catastrophe accompanied with reduced H3S10 phosphorylation. In addition, an accumulation of DNA damage was observed in *HDAC3*$^{-/-}$ interphase MEF cells, which appears to be associated with a defective DNA double-strand break repair. However *HDAC3*$^{-/-}$ MEFs were protected from DNA damage when they are not cycling or quiescent. These results might partially explain the observation that HDACi affects selectivity to the cancer cells or highly proliferating cells rather than primary or quiescent cells.

1.2.6.5 Class II HDACs in matazoans

HDAC4, 5, 6, 7, 9, and 10 belong to the class II family, and they are further divided into 2 subfamilies based on their sequence homology; class IIa as HDAC4, 5, 7 and 9, and class IIb as HDAC6 and 10. Interestingly *C. elegans*, *D. melanogaster*, and *D. rerio* encode only one gene in each subfamily, so that only mammalians show multiple enzymes in both class II families. The class IIa enzymes consist of a large N-terminal extension and a conserved HDAC domain in the C-terminus; this structure is

conserved in other metazoans. The N-terminal domain contains multiple phosphorylation sites as well as a binding site for myocyte enhancer factor-2 (MEF2). Phosphorylation on the conserved serine residues by calcium/calmodulin-dependent kinase (CaMK) and other kinases in response to specific stimuli creates binding sites for the 14-3-3 proteins which can mediate shuttling of proteins from the nucleus to the cytoplasm. In fact all class IIa enzymes are found in both the cytoplasm and the nucleus, and mutations in these phosphorylation sites prevent binding of 14-3-3 proteins, which results in the lack of nuclear export (Kao et al. 2001). Class IIb enzymes, HDAC6 and 10, are able to be clustered from class IIa because of a longer C-terminal extension; furthermore HDAC6 is unique in that it contains two HDAC domains. The subcellular localization of class IIb enzymes is predominantly, if not exclusive, cytosolic, and no shuttling activity has been detected.

Whereas class I HDACs are detected in a wide range of culture cell line, tissues and organs, class IIa HDACs are expressed in a more restricted type of cells. The highest expression of HDAC4, 5 and 9, is detected in heart, skeletal muscle and brain, and HDAC7 expression is found predominantly in thymocytes, heart and lung (Fischle et al. 1999; Grozinger et al. 1999; Miska et al. 1999; Verdel and Khochbin 1999; Wang et al. 1999; Dequiedt et al. 2003). Class IIb family enzymes are also abundant in many cells and tissues however HDAC6 expression is the highest in testis and HDAC10 has the highest level in liver, kidney and spleen (Verdel and Khochbin 1999; Seigneurin-Berny et al. 2001; Fischer et al. 2002; Guardiola and Yao 2002; Kao et al. 2002; Tong et al. 2002; Zhang et al. 2008).

1.2.6.6 Developmental roles of class II HDACs in organisms

Class II HDACs have also been genetically ablated in mice, and their contributions to mouse development were definitely more restricted than those of class I enzymes, in agreement with their more limited expression patterns (Table 1). In sharp contrast to the early embryonic death of HDAC1 and 3 mutant mice, many of class II HDAC mutants reported to date show tissue-specific defects, which implies that class I

HDACs function as a general HDACs to regulate expression of key and essential genes whereas class II enzymes have selected targets in specific tissues and situations.

The *HDAC9* locus was the first genetically modified class II *HDAC* gene in a mouse (Zhang et al. 2002). *HDAC9* locus encodes two forms of proteins which are a full length HDAC9 protein and MEF2-interacting transcriptional repressor (MITR), a splice variant of HDAC9 lacking a HDAC domain and expressed predominantly in the heart (Sparrow et al. 1999). Both forms of *HDAC9* gene products can bind to the MEF2 transcription factor and inactivate its transcriptional activity (McKinsey et al. 2002). This association of HDAC9 in the nucleus can be revoked by phosphorylation in the N-terminus, which results in the export of HDAC9 by 14-3-3 proteins (Kao et al. 2001). The upstream kinase CaMK is activated by cardiac hypertrophic stimuli and induces dissociation of HDAC9 and MEF2, which results in the de-repression of genes involved in the prevention of hypertrophy. *HDAC9* mutant mice appear to be viable and show a morphologically and functionally normal heart at young age, however at old age or under stress conditions by overloaded pressure or calcineurin activation, they show severe cardiomyocyte hypertrophy (Zhang et al. 2002).

HDAC5, another class IIa HDAC highly expressed in the heart, mutant mice show age- and stress-dependent cardiac hypertrophy which is similar defects observed in the heart of *HDAC9* mutant (Chang et al. 2004). Combined loss of both HDAC5 and 9 leads to a propensity for lethal ventricular septal defects and thin-walled myocardium, which suggest redundant functions in the control of cardiac development. In addition to the response to cardio-hypertrophic stress, chronic cocaine and stress exposure in brain was also examined in *HDAC5* mutant mice (Renthal et al. 2007). Chronic exposure to cocaine or stress enhances the nuclear export of HDAC5 in the nucleus accumbens (NAc), a set of neurons within the forebrain thought to be a major brain reward region. Reduction of HDAC5 activity in the nucleus of NAc causes hypersensitive responses to chronic cocaine or stress.

Mice lacking HDAC7 appear to have the most severe phenotype among reported class II HDAC mutants, as they die during embryogenesis,; this lethality is solely due to a failure in endothelial cell-cell adhesion and consequent dilatation and rupture of blood vessels since *HDAC7* specific deletion in endothelial cells recapitulates this defect

(Chang et al. 2006). Phosphorylation of HDAC7 in endothelial cells is regulated by protein kinase D under the control of VEGF signaling, which disrupts the binding of HDAC7 and MEF2 and de-represses expression of genes involved in endothelial cell proliferation and migration (Wang et al. 2008).

Inactivation of HDAC4 in mice shows chondrocyte hypertrophy, which results in ectopic bone formation leading to complete lethality by the time of weaning (Vega et al. 2004). Defects observed in *HDAC4*$^{-/-}$ mice resemble the phenotype of mice continuously expressing Runx2 (Takeda et al. 2001; Ueta et al. 2001). In fact, HDAC4 can bind to Runx2 and function to repress the target genes. Loss of HDAC4 is partially rescued by the reduced amount of MEF2C transcription factor (Arnold et al. 2007). Chondrocyte hypertrophy in *HDAC4*$^{-/-}$ mice can be diminished by a heterozygous MEF2C mutation, which clearly demonstrated that HDAC4 is genetically upstream of MEF2C and inhibiting the expression of genes normally activated by MEF2C depend on the stimuli.

Genetic analysis of class IIa HDACs revealed that the functional importance of each HDAC activity in different tissues was all associated, at least to a certain degree, with the binding to MEF2 transcription factor, which is regulated by the sub-cellular localization of HDAC. Export of class IIa HDACs is mediated not only by phosphorylation but also the redox status in the cells that is involved in the pathogenesis of cardiac hypertrophy and heart failure (Ago et al. 2008). Therefore extensive extracellular stimulation such as VEGF signaling as well as stressed conditions such as cardiac hypertrophic stimuli and the redox status may dominantly determine the export of class IIa HDACs and the dissociation from MEF2 transcription factor, which results in misregulation of gene expression and provokes the abnormal development observed in each HDAC mutant mouse. Thus class IIa HDACs are integrated in a cellular signaling cascade as a both mediator (transduces cytosolic signaling to nucleus) and director of gene expression (represses gene expression directly).

The class IIb family of HDAC6 has unique features distinguished from other HDACs in the protein structure and in its function. HDAC6 is composed of two catalytic domains and a C-terminal zinc finger domain (ZnF-UBP) binding with very high-

affinity free ubiquitin as well as mono- and polyubiquitinated proteins (Seigneurin-Berny et al. 2001; Hook et al. 2002; Boyault et al. 2006). The double catalytic domains are conserved during evolution, which strongly suggests a critical and possibly unique role for this domain duplication in HDAC6 functions. An obvious question is whether both catalytic domains are required for the intact deacetylase activity. An unambiguous answer is so far not avairable since there are steep discrepancies in three independent reports which have shown that (1) each domain functions independently, (2) both domains are required for the catalytic activity, and (3) the catalytic activity resides in the C-terminal second domain (Grozinger et al. 1999; Zhang et al. 2006; Zou et al. 2006).

Another remarkable feature of HDAC6 is its function in the cytoplasm. Despite the fact that the roles of class IIa HDACs in cytoplasm remain obscure, a growing number of evidences have demonstrated the functional importance of HDAC6 in the cytoplasm. The first important achievement uncovering the cytosolic functions of HDAC6 was the identification of α-tublin as a substrate of deacetylase activity (Hubbert et al. 2002; Matsuyama et al. 2002; Zhang et al. 2003). In addition, the chaperone HSP90 was found to be a substrate of HDAC6, which regulates its activity (Bali et al. 2005; Kovacs et al. 2005).

In addition to its deacetylase activity, the C-terminal ZnF-UBP domain has critical roles in the fate of ubiquitinated proteins. HDAC6 interacts with the chaperone-like AAA ATPase p97/VCP, a protein that is critical for proteasomal degradation of misfolded proteins, and this association controls the levels of polyubiquitinated cellular proteins, mostly consisting of misfolded proteins (Boyault et al. 2006). HDAC6 also facilitates the clearance of misfolded ubiquitinated proteins by promoting their accumulation in an aggresome, thereby HDAC6 protects cells from apoptosis following stress induced by misfolded proteins (Kawaguchi et al. 2003). HDAC6 senses ubiquitinated cellular aggregates comprising polyubiquitinated proteins and consequently induces the expression of major cellular chaperones by triggering the dissociation of a repressive HDAC6/HSF1 (heat-shock factor 1)/HSP90 complex and a subsequent HSF1 activation, which leads to the activation of the heat-shock protein-encoding genes (Boyault et al. 2007). Such an implication of HDAC6 in the ubiquitin-proteasome system was examined in *Drosophila* neurodegenerative disease model, which showed crucial functions of HDAC6 to remove misfolded

proteins that is an early onset of neurodegenerative diseases (Pandey et al. 2007). HDAC6 is also essential for formation of stress granules which are dynamic cytoplasmic structures containing mRNAs prevented from translation reversiblely (Kwon et al. 2007).

HDAC6 deficient mice appear to be viable and no obvious defects in most of tissue examined, despite they show hyperacetylated tubulin (Zhang et al. 2008). There is a minor effect in the adaptive immune response as well as a small increase in cancellous bone mineral density.

Table 1. Summary of HDAC mutant mice. Exons represents the number of exons encoding mRNAs, which is taken from the UCSC Genome Browser (http://genome.ucsc.edu/)

	Class	Mutant viability	Exons	Modified allele	Phenotypes
HDAC1	I	Embryonic lethal by E9.5	15	*lacZ*/*neo* replaces exon 5-7 (Lagger et al. 2002) Exon 5-7 is floxed (Montgomery et al. 2007) Exon 6 is floxed (Zhang et al. unpublished)	Proliferation defect in ES cells and primary MEF cells Dispensable in the brain, liver, lung, heart or skeletal muscle Slight reduction of splenic myeloid cells and impaired adaptive immune reaction
HDAC2	I	Complete (Montgomery et al. 2007) or partial (Trivedi et al. 2007; Zimmermann et al. 2007) perinatal lethality	14	Exon 2-4 is floxed (Montgomery et al. 2007) Insertion of *lacZ* after exon 8 (Trivedi et al. 2007; Zimmermann et al. 2007) Exon 6 is floxed (Yamaguchi et al. submitted)	Cardiac defects (complete KO) Dispensable in the brain, liver, lung, heart or skeletal muscle
HDAC3	I	Embryonic lethal by E9.5	15	Exon 7 is floxed (Bhaskara et al. 2008)	Proliferation defect, apoptosis induction and DNA damage sensitivity in MEF cells Defects in G2/M transition in immortalized and transformed cells
HDAC4	IIa	Postnatal lethality by weaning	26	*lacZ*/*neo* replaces exon 6 (Vega et al. 2004)	Chondrocyte hypertrophy
HDAC5	IIa	Viable and fertile	26	*lacZ*/*neo* replaces exon 3-7 (Chang et al. 2004)	Age- and stress-dependent cardiac hypertrophy Hypersensitive responses to chronic cocaine or stress
HDAC6	IIb	Viable and fertile	28	Exon 8-10 is floxed (Zhang et al. 2008)	Increased acetylation of α-tublin and HSP90 Modest impairment in the immune response
HDAC7	IIa	Embryonic lethal around E12.5	24	*lacZ*/*neo* replaces exon 2-5 (Chang et al. 2006)	Failure in the formation of blood vessels
HDAC8	I	N.D.	11		
HDAC9	IIa	Viable and fertile	12	*lacZ*/*neo* replaces exon 4 and 5 (Zhang et al. 2002)	Age- and stress-dependent cardiac hypertrophy
HDAC10	IIb	N.D.	20		
HDAC11	IV	N.D.	10		

1.3. Differentiation

Differentiation is the process which cells become specialized to a particular cell lineage and execute specific functions. The cells capable to self-renew and also to generate progenitors of differented cells are defined stem cells, which are further separated based on their differentiation potential (Table 2). In mammals, all terminally differentiated cells such as red blood cells, fibroblasts and neurons are originated from one totipotent stem cell, the zygote. During the differentiation process, a totipotent stem cell loses its "potency" or "plasticity" to generate all kinds of lineages and is restricted into one lineage (Fig. 10). This lineage restriction process is tightly controlled by the specific pattern of gene expression, which decides the cellular fate toward terminal differentiation. Specific patterns of gene expression are governed by mainly two mechanisms; lineage- and temporally-restricted expression of sequence specific TFs, and epigenetic regulation of gene expression.

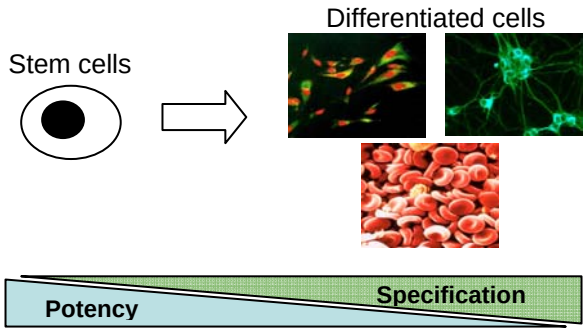

Figure 10. Cellular differentiation. A stem cell loses its potency and gains a lineage/cell-type specificity

Table 2. Classification of different stem cells (modified from (Jaenisch and Young 2008; Roobrouck et al. 2008))

	Developmental potency	Example
Totipotent stem cell	All lineages of organisms	Zygote Cells in the morula at the early stage
Pluripotent stem cell	All lineages of body (the three germ layers: endoderm, mesoderm and ectoderm but not for the extraembryonic trophoblast)	Embryonic stem cells (ES cells) induced pluripotent stem cells (iPS cells) the embryonic inner cell mass
Multipotent stem cell	Multiple cell types	Hematopoietic stem cells (HSCs) Neural stem cells Mesenchymal stem cells
Unipotent stem cell	One cell type	Spermatogonial stem cells Epithelial stem cells Corneal stem cells

1.3.1 Hematopoietic cell development

Hematopoietic cell development (i.e. hematopoiesis) takes place in different manners and places during the entire life of higher eukaryotes. During embryogenesis, multipotent stem cells, hematopoietic stem cells (HSCs), are generated from ventral mesoderm and the yolk sac in mammals becomes the source of hematopoiesis around embryonic day (E) 7.5-9, which is mainly providing red blood cells allowing embryos to grow rapidly without suffering from low oxygen availability. Hematopoiesis in mammals occurs subsequently in the aorta-gonad-mesonephros (E9.5-11.5), the fetal liver (E10.5- birth) and the bone marrow (E15.5-). In an adult mouse, HSCs reside in the bone marrow, where hematopoiesis continues throughout life (Fig. 11). HSCs can be isolated exclusively within the lineage marker negative (Lin^-) Sca-1^+ c-kit^{hi} (LSK), which are all cell surface molecules, population that constitutes ca. 0.1% of bone marrow cells in the adult mouse (Morrison and Weissman 1994). The lineage marker is the mixture of the antibodies and can identify T lymphocytes, B lymphocytes, monocytes/macrophages, NK cells, erythrocytes, and granulocytes. Based on the length of its life span and expression

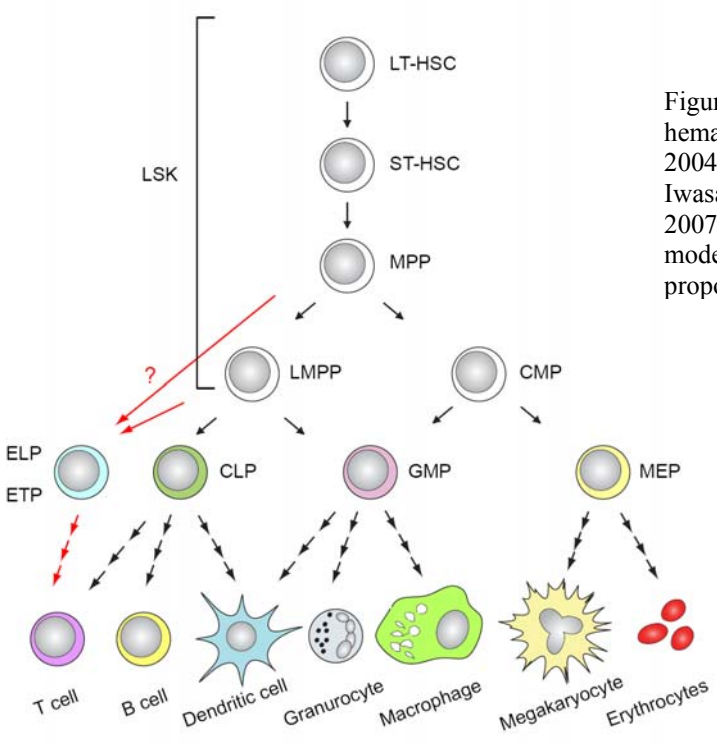

Figure 11. A pathway for adult hematopoiesis (Modified from (Johnson et al. 2004; Nagasawa 2006; Dakic et al. 2007; Iwasaki and Akashi 2007; Nutt and Kee 2007)). Red arrows show the alternative model of T cell development recently proposed.

of cell surface molecules, the LSK population can be divided into long-term HSCs (LT-HSCs), short-term HSCs (ST-HSCs), multipotential progenitors (MPPs) and lymphoid-primed multipotent progenitors (LMPPs). The cell-cell adhesion factor CD34 and the tyrosine kinase receptor Flt3 are used to distinguish these four populations as follows: LT-HSCs are $Flt3^-$ $CD34^-$, ST-HSCs/MPPs are $Flt3^-$ $CD34^+$, and LMPPs are $Flt3^+$ $CD34^+$ (Nagasawa 2006; Dakic et al. 2007; Iwasaki and Akashi 2007; Nutt and Kee 2007). In cell transfer experiments into leathaly irradiated mice, LT-HSCs give rise to a long-term reconstitution, while ST-HSCs only allow a short-term reconstitution. This reflects the different self-renewal capacity of LT-HSCs and ST-HSCs. MPPs lack the self-renewal ability although they are able to give rise to all hematopoietic lineages (Morrison and Weissman 1994). It has not been identified yet that a cell surface molecule can distinguish clearly the populations between ST-HSCs and MPPs. LMPPs have been recently proposed as a first step towards commitment to the lymphoid-myeloid lineages since these cells do not have the capacity to self-renew and give rise to the erythrocyte and megakaryocyte lineages (Adolfsson et al. 2001; Adolfsson et al. 2005). This result indicates that the first lineage restriction from the multipotent state is the separation of the erythrocyte and megakaryocyte lineages from the other hematopoietic lineages. This finding proposed a new view on the lineage restriction of myeloid lineage development. In the old model, all cells of myeloid lineages including granulocytes, macrophages, erythrocytes and megakaryocytes are thought to be generated from a common myeloid progenitor (CMP), and then further restricted to a megakaryocyte-erythrocyte progenitor (MEP) and a granulocyte-macrophage progenitor (GMP). It implies the additional or alternative pathway for the myeloid lineage commitment that LMPP unable to produce megakaryocytes and erythrocytes.

The first and major branch to separate the lymphoid lineage from the myeloid lineage is the step giving rise to a common lymphoid progenitor (CLP) which gives rise to the B, T, NK and lymphoid dendritic cell lineages. Several publications have shown that there are also other pathways leading to the T cell lineage, originating from an early T lineage progenitor (ETP), early lymphoid progenitor (ELP) or from a LSK $CD62L^+$ population (Medina et al. 2001; Allman et al. 2003; Perry et al. 2004). All these populations were

found within the LSK population, suggesting that the branch between the B and T cell lineages could be a very early step in hematopoiesis (Fig. 11; red arrows).

The intrinsic determination of cellular fate during hematopoietic cell development is controlled by the sequential expression of genes that is conducted by coordinated regulation by TFs and epigenetic modifications. Studies describing the fundamental requirement of TFs as well as an increasing number of epigenetic modifiers have been intensively done by using mouse genetic approachs to inactivate or enforce expression of molecules in hematopoietic cells and identify affected subpopulations. This allowed establishing a very complex hierarchy and ordered expression of these factors during hematopoiesis have been defined.

The transcription factor stem cell leukaemia *SCL/tal-1* gene product was identified as essential for the HSC generation and proper development of the erythrocyte and megakaryocytic lineages, however dispensable for the maintenance of HSC function in long term (Mikkola et al. 2003). The Lim-domain containing protein LMO2, an interaction partner of SCL, is also critical for the yolk sac hematopoiesis (Warren et al. 1994). *Runx1* (previously known as *AML-1*) is also not required for adult HSC function, but critical for the fetal liver HSC generation as well as for megakaryocytic maturation and lymphocytic differentiation in adult hematopoiesis (Okuda et al. 1996; Ichikawa et al. 2004). With the importance of epigenetic regulation for the cellular differentiation, it is not surprising that epigenetic modifiers are essential for the hematopoiesis. A recent study has shown that the SNF2-like ATPase Mi-2β of the NuRD complex is required for maintenance of and multi-lineage hematopoietic differentiation (Yoshida et al. 2008). The Mi-2β/NuRD complex is implicated in the regulation of gene repression through its activity to remodel nucleosomes and deacetylate core histones. In addition, Mi-2β is highly expressed in HSCs and can physically associate with TFs required for early stages of hematopoiesis such as Ikaros and GATA-1 (Kim et al. 1999; O'Neill et al. 2000; Rodriguez et al. 2005; Sridharan and Smale 2007). Loss of Mi-2 β increases activity of cell cycling in HSCs and expression of genes crucial for commitment to the different lineages; however, the committed cells toward each lineage are severely blocked during their development at early progenitor stages.

1.3.2 B lymphocyte development

The development of B lymphocytes from multipotent progenitor cells is a tightly controlled, yet flexible, process under the control of a number of lineage-specific or ubiquitous transcription factors which initiate, maintain and restrict specific sets of gene expression patterns required for B cell identity (Busslinger 2004; Matthias and Rolink 2005; Cobaleda et al. 2007; Nutt and Kee 2007). Recent development of gene targeting studies in mice enabled to identify intrinsically required factors in B lymphocyte development and loss of these essential factors has uncovered several checkpoints for B cell development which are associated with key biological events. Distinct B cell subpopulations have been identified by the expression of cell surface molecules, the state of immunoglobulin recombination, cell size, and proliferation ability as shown in Fig 12. Several TFs are identified as essential components to commit progenitor cells to the B cell lineage: PU.1, E2A, EBF and Pax5. The regulatory network exerted by these TFs is

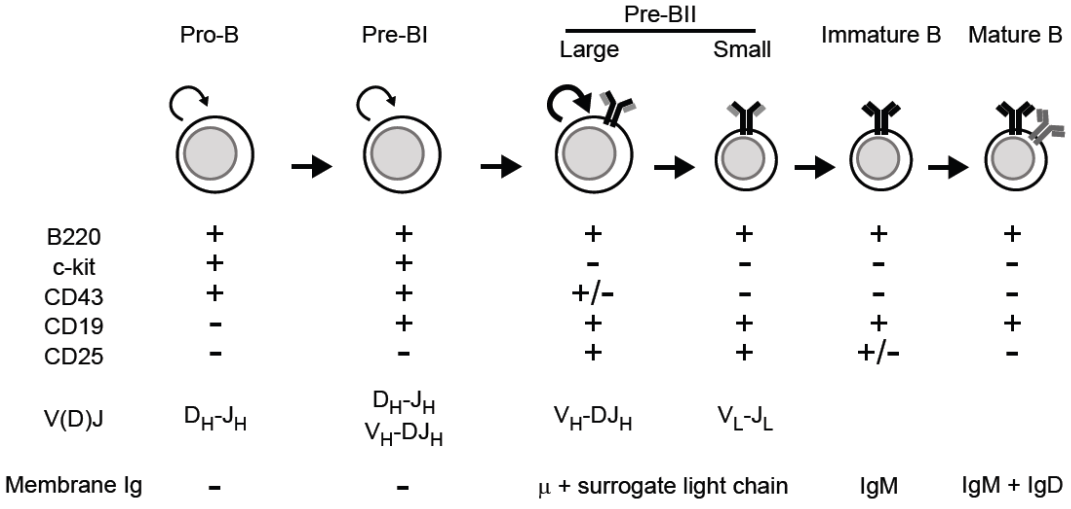

Figure 12. Scheme of early B cell differentiation, depicting the different developmental stages and the expression of markers used to discriminate them (marker expression is indicated by +, lack of expression by -). Curved arrows indicate relative cellular proliferation activities: very high in large pre-BII, intermediate in pro- and pre-BI, low or absent in small pre-BII, immature and mature B cells. V(D)J shows the rearrangement status of the different Ig gene alleles during B cell differentiation. The kind of immunoglobulin present at the membrane is also indicated at the bottom.

controlling the early stage of B cell development (Busslinger 2004; Matthias and Rolink 2005; Cobaleda et al. 2007; Nutt and Kee 2007).

PU.1

PU.1 is an Ets family transcription factor which is essential for both myeloid and lymphoid progenitors (Scott et al. 1994; McKercher et al. 1996). *PU.1*$^{-/-}$ MPPs, which are reduced in mutant mice, show reduced expression of *Flt3* and *Il7ra* (encodes the IL-7 receptor α-chain) genes, both of which encode cell surface cytokine receptors required for B cell differentiation and survival (DeKoter et al. 2002). In fact, *PU.1*$^{-/-}$ MPPs fail to respond to IL-7 and enforced expression of *Il7ra* can partially rescue the blockade of PU.1$^{-/-}$ cells (DeKoter et al. 2002). Thus PU.1 is one of the earliest TF required for B cell fate determination.

E2A

The *E2A* gene encodes the E12 and E47 proteins which are two helix-loop-helix transcription factors generated by alternative splicing. Loss of E2A shows marked defects in B cell differentiation at the pro-B cell stage (Bain et al. 1994; Sun 1994; Zhuang et al. 1994). In E2A inactivated pro-B cells, expression of genes encoding EBF, Pax5, Igα and germline transcription from the IgH locus, which are all discussed later, is absent and no IgH recombination takes place. Over-expression of EBF1 in E2A deficient HSCs can rescue B cell differentiation *in vitro*, although these pro B cells fail to proliferate in response to IL-7 (Bain et al. 1994; Seet et al. 2004).

EBF1

EBF1 is a TF that binds DNA through an N-terminal domain containing a novel zinc-coordination motif (Hagman et al. 1995). EBF1 contains a putative helix-loop-helix domain which is required for its homodimerization and functions (Hagman and Lukin 2005). Ablation of EBF1 in mice blocks B cell differentiation at the pro-B cell stage, similar to the defect in E2A mutant mice; *EBF1*$^{-/-}$ pro-B cells fail to express genes essential for the B cell commitment including *mb-1* (encoding Igα), *B29* (Igβ), *Igll1* (λ5), *VpreB1* and *Pax5* although *E2a* is normally expressed (Lin and Grosschedl 1995).

Importantly, enforced expression of neither the upstream factor *E2A* nor the downstream target *Pax5* (discussed later) can restore the defects in *EBF1*$^{-/-}$ pro-B cells; this indicates that EBF1 is essential to activate the B cell gene program independently of Pax5 (Seet et al. 2004). Similar to the defect observed in *E2A* mutant mice, *Ebf1*$^{-/-}$ pro-B cells lack IgH recombination (Lin and Grosschedl 1995). Ectopic expression of either *EBF1* or *E2A* together with *RAG1* or *RAG2* in non-lymphoid cells can induce D-J rearrangement of the *IgH* gene but no V-DJ recombination (Romanow et al. 2000).

Pax5

Pax5 is a paired homeodomain protein that is expressed throughout B cell differentiation (Fuxa et al. 2004). Pax5 was first identified as a transcription factor binding to the *IgH* and *Igk* gene loci (Weaver and Baltimore 1987). Independently, the B cell–specific activator protein (BSAP), which turned out to be encoded by *Pax5*, was shown to activate the expression of *CD19*, an absolutely required factor to commit and maintain the B cell identity (Kozmik et al. 1992; Liao et al. 1994). *Pax5* expression is under the control of EBF1, observed throughout B cell development and repressed when B cells terminally differentiate to plasma cells (Cobaleda et al. 2007). Loss of Pax5 completely blocks B cell development at the pro-B cell stage (B220^{+} c-kit^{+}), when B cells are not yet fully committed; thereby *Pax5*$^{-/-}$ pro-B cells maintain multipotency and can give rise to several other lineages such as T, NK and myeloid cells (Nutt et al. 1999; Rolink et al. 1999). Moreover, conditional *Pax5* deletion in mature B cells allows committed B cells to dedifferentiate *in vivo* back to early uncommitted progenitors, which can give rise to T cells in the thymus (Cobaleda et al. 2007). These results indicate that, although PU.1, E2A and EBF1 are crucial for the formation of pro-B cells and their specification, Pax5 is essential to fully commit to the B cell lineage and maintain cellular identity throughout B-cell ontogeny.

1.3.2.1 Mechanisms to create an antibody repertoire

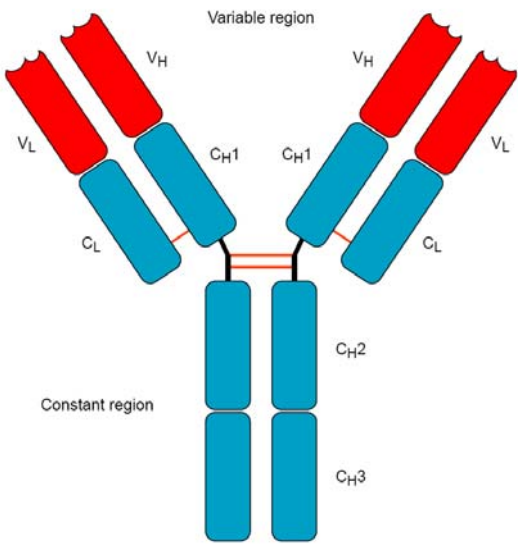

Figure 13. A schematic representation of an immunoglobulin molecule. The constant regions are depicted in blue, and the variable regions are shown in red. The two antigen binding sites are at the top of the variable regions. Two of heavy chains and light chains are connected disulfide bonds.

The principal role of B cell in the immune system is to generate antibodies against a wide range of pathogens encountered during the lifetime of the organism (Fig. 13). In order to achieve this goal, B cells must undergo the process rearranging the large parts of chromosome encoding the immunoglobulin heavy and light chain genes (*IgH* and *IgL*, respectively), whose translated products associate and function as an antibody. This assembly of gene segments in *IgH* and *IgL* includes the cleavage of specific DNA sequences by Recombination Activating Gene 1 and 2 (RAG1/2), which introduces DNA double-strand breaks (DSBs) and re-ligation of breaks by the DNA DSBs repair, which is mainly done by non-homologous end joining pathway (NHEJ). *IgH* and *IgL* recombination takes place independently and sequentially, allowing a combinational assembly of antibodies. Mature B cells harboring successfully recombined *IgH* and *IgL* express IgM, a tetramer structure of two heavy chain and two light chain molecules, and its splice variant IgD on their cell surface. Together with their associated partners, two heterodimers of Igα and Igβ, these cell surface antibodies form the B cell receptor (BCR).

Once B cells encounter a foreign antigen, they expand their antibody repertoire by two processes called **C**lass **S**witch **R**ecombination (CSR) and **S**omatic **H**yper-**M**utation (SHM). CSR is a secondary DNA rearrangement in the constant region of the *IgH* locus whereby the μ constant region can be replaced by other C regions with other effector functions. SHM is the process by which mutations are introduced in the DNA both in the *IgH* and *IgL* variable regions allowing the generation of higher affinity antibodies.

The adaptive immunity using diverse specificity of antibodies is extremely important and efficient in vertebrates to fight infections; yet, at the same time, the process of reassembling chromosome segments and mutating the DNA can potentially be very harmful to cells if it eacapes the proper control. In fact, c.a. 95% of lymphomas are of B cell origin and a majority of them contain reciprocal chromosomal translocations involving one of the *Ig* loci and a proto-oncogene such as *BCL6* and *ABL*.

1.3.2.2. Check points of Ig recombination during B cell maturation

The murine *IgH* locus spans approximately 3 Mb and comprises 150 or more variable (V_H), 12 diversity (D_H) and 4 joining (J_H) gene segments followed by 8 genes encoding the different constant (C_H) regions (Fig. 14) (Jung and Alt 2004; Jung et al. 2006). The first recombination event in immunoglobulin genes takes place in the *IgH* locus from the CLP cell up to the pro-B cell stage before full commitment to the B cell lineage (these cells do not yet express the transcription factor *Pax5*, which ensures and maintains the B cell identity (Cobaleda et al. 2007)), and starts with D_H to J_H (DJ) rearrangement. At the subsequent pre-BI developmental stage, the locus undergoes V_H to DJ_H rearrangement. While DJ_H recombination takes place on both alleles concomitantly, V_H to DJ_H recombination occurs on one allele at a time with the second allele not being processed once a locus has been successfully rearranged. B cells harboring a rearranged allele start to express the *IgH* gene at the large pre-BII cell stage, whose translated product is called μ heavy chain. μ heavy chain forms the protein complex, pre-BCR, on a cell surface that contains two of μ heavy chains, surrogate light chains and two heterodimers of Igα and β proteins. A surrogate light chain comprises a pair of VpreB and λ5 proteins that are close similarity to IgL protein. Once large pre-BII cells expose pre-BCR on their cell surface, it receive signal that leads to enforce cessation of further recombination of the second IgH allele, which is the process called allelic exclusion, enhance proliferation of cells, repress gene expression of surrogate light chains and *RAG1/2*, and move to next developmental stage small pre-BII where the *IgL* rearrangement takes place. Two gene loci encode the immunoglobulin light chain; κ and λ genes. The murine *Igκ* locus spans over 3 Mb

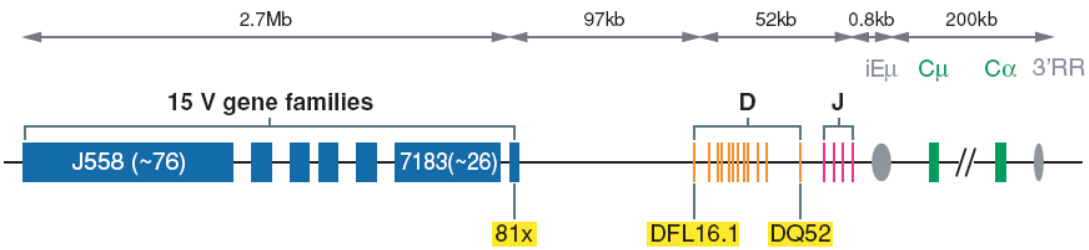

Figure 14. Genomic organization of the IgH locus (not drawn to scale) (taken from (Jung et al. 2006)). The VH81X, DFL16.1, and DQ52 gene segments are highlighted in yellow. 15 V gene families are depicted in blue. The most distal V_H genes are the V_HJ558 gene family, whereas the most proximal is the V_H7183 family. D, J and C indicate each gene segments. iEμ is the intronic enhancer and 3'RR is the 3' regulatory region. Distances shown are from the murine 129 strain and vary between strains

including about 120 Vκ gene segments and five Jκ gene segments, one of which is a pseudogene, whereas the V_λ and J_λ segments of the λ gene are spread over approximately 230 kb. The rearrangement of light chain loci takes place only between V to J segments; therefore it is referred as VJ_L recombination. In human and mouse, one allele of κ gene tends to be rearranged prior to λ gene rearrangement. As is the case for *IgH* recombination, a further rearrangement is prevented once B cell completes the *IgL* rearrangement and generates a functional light chain. After successful recombination on *IgH* and *IgL* loci, B cell starts to express IgM on their cell surface. Since each B cell contains and expresses only one allele of rearranged *IgH* and *IgL*, each B cell produces one form of IgM and exposed on a cell surface. This B cell clonal specificity of antibody production ensures that all the antibodies secreted by the activated B cells are specific for the antigen that stimulates its activation. This is a cardinal feature of adaptive immunity by B cells that prevents secretion of non-specific antibodies, which might potentially be harmful to the organism.

The completion of *Ig* recombination in the bone marrow allows B cells to further develop to the immature B cell stage where they face to an additional challenge to pass the quality control of antibodies. Immature B cells express IgM on their cell surface and these antibodies are tested whether they can recognize self-cell surface molecules and soluble self molecules. When developing immature B cells express IgM that recognize self-cell surface molecules such as those of MHC molecules, they are deleted by apoptosis through BCR stimulation, a process called clonal deletion. On the other hand, when cells

encounter and bind to soluble self antigens, they are rendered unresponsive to the antigen (anergic), move to the periphery, and express more IgD molecules than IgM on their cell surface. Since these cells are not able to respond to the antigens, in the periphery they can not proliferate following BCR stimulation, therefore this anegic B cell population is rapidly lost after its maturation. Once B cells pass through this quality control process at the immature B cell stage in the bone marrow, they move to a peripheral lymphoid organ, spleen, to further maturate, and then they start to express IgD on their cell surface. There is the process called the receptor editing that occurs after generation of both an unproductive rearrangement during V(D)J recombination and self-reactive IgM. These non-functional or self-reactive *Ig* genes can be further rearranged using a different V gene segments. In this way, B cells can change their antibodiy specificities wich are functional/productive and non-self reavctive.

The last step of immunoglobulin gene rearrangement, the CSR and the SHM, takes place in cells that meet the specific foreign antigen recognized by their own cell surface antibodies (Fig. 15). These two processes are linked to the formation of specialized microenvironment, the germinal center (GC), in secondary lymphoid organs. B cells encountering antigens are activated in the GC as a result of their interaction with $CD4^+$ T cells and antigen-presenting cells (MacLennan 1994). Activated B cells undergo the clonal expansion step which is one of the fastest cell division process in a mammalian body (c.a. 6 hour / division). These cells differentiate into centroblasts and start the process of SHM that introduces base-pair changes into the rearranged V(D)J region of *IgH* and *IgL* genes (Klein and Dalla-Favera 2008). Both V(D)J regions of *IgH* and *IgL* encode the immunoglobulin variable region (IgV); therefore some of these base-pair mutations lead to a change in the amino-acid sequence and may increase the affinity of the antibody against its antigen. Centroblasts then give rise to centrocytes and move to the light zone in the GC, where the mutated/modified antibody is selected for improved binding to the antigen. Newly generated centrocytes that produce high affinity antibodies can escape from undergoing apoptosis, whereas centrocytes with low affinity antibodies are removed. A portion of centrocytes undergoes immunoglobulin heavy chain CSR, by which B cells can switch their immunoglobulin expression from IgM and IgD to other classes that have distinct effector functions such as IgG, IgA and IgE (Chaudhuri et al.

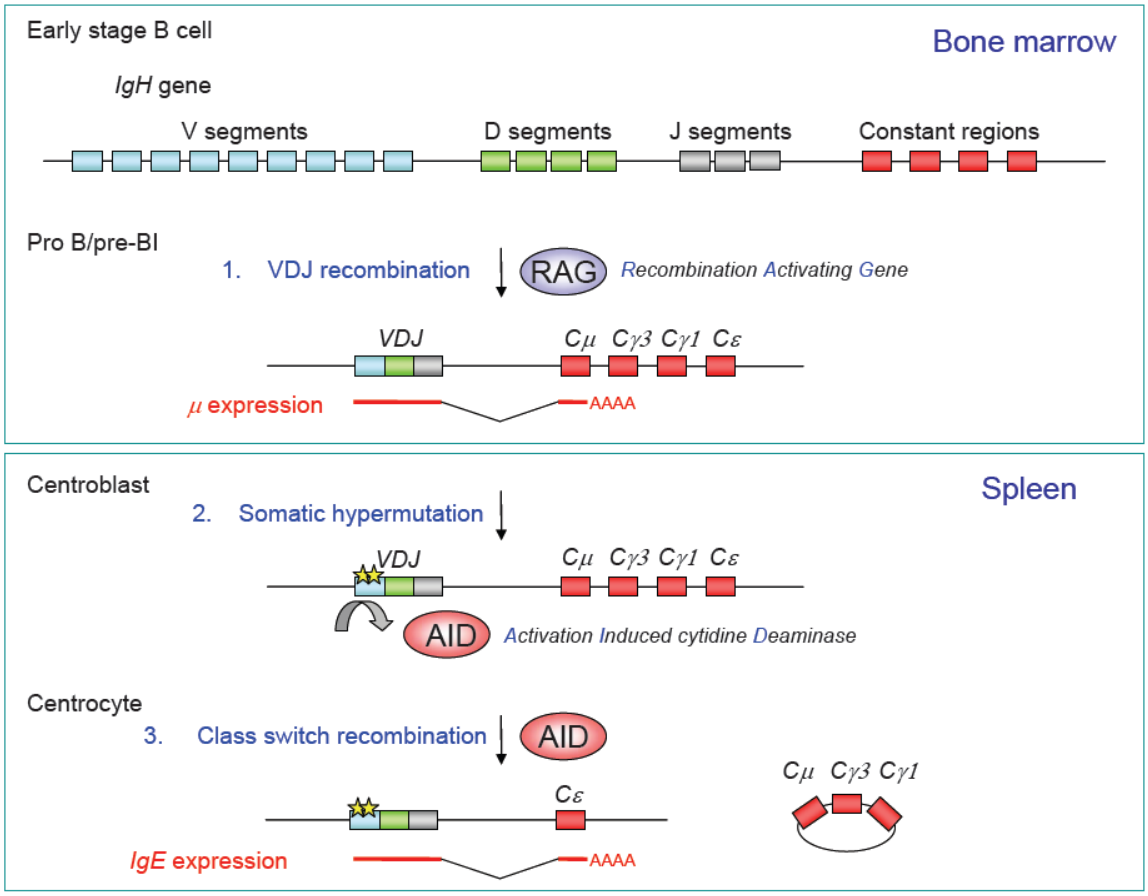

Figure 15. A simplified schematic drawing of the 3 main steps of immunoglobulin heavy chain modifications. The variable (V), diversity (D), joining (J) and constant (C) gene segments are represented as boxes. Yellow stars indicate the DNA mutation introduced by the somatic hypermutation. Class switching to the Cε, which encodes the exons of IgE, is shown as an example. RAG enzymes are absolutely required for V(D)J recombination, whereas somatic hypermutation and class switch recombination are initiated by AID.

2007; Muramatsu et al. 2007). There are 8 different constant gene segments in the mouse genome, which encode the constant regions of the different immunoglobulin isotypes. CSR recombines between the Cμ sequence to one of the downstream C genes, and then the intervening DNA segment is looped-out as a circular DNA. Successful CSR allows centrocytes to terminally differentiate towards antigen-secreting cells, plasma cells. Both SHM and CSR are initiated by the enzyme called AID encoded by the ***A**ctivation **I**nduced cytidine **D**eaminase* gene. AID can initiate both processes by deamination of cytosine to uracil in the target DNA which results in both the DNA mutations and the cleavage of DNA (Peled et al. 2008).

1.3.3 Regulation of V(D)J recombination in the *IgH* locus

1.3.3.1 *cis*-DNA elements

Recombination of the *Ig* loci is tightly controlled by different layers of regulatory mechanisms that ensure the Ig recombination takes place specifically in the lymphocyte lineage, at appropriate developmental stages, and on the specific loci/regions that must be recombined. Here I discuss the several different types of regulation occurring during *IgH* recombination.

The first line of regulation is governed by *cis*-regulatory DNA elements on the *Ig* loci, the recombination signal sequences (RSSs). RSSs include one pair of conserved heptamer and nonamer sequences, spaced by a relatively non-conserved sequence of either 12 or 23 bp, called 12 RSS and 23 RSS, respectively. Both types of sequences can be recognized by RAG proteins, which initiate V(D)J recombination by cleaving the DNA to introduce DNA DSBs. V_H and J_H gene segments are flanked by 23 RSSs, whereas D_H segments are flanked by 12 RSSs (Fig. 16). V(D)J recombination occurs almost exclusively between two signals with spacers of different lengths. This restriction, called the 12/23 rule, is mediated at the level of RAG1/2 recognition and DNA cutting (Eastman et al. 1996; van Gent et al. 1996), therefore the reassembly of *Ig* genes takes place in a sequential manner (D-J first and V-DJ later) and unidirectionally.

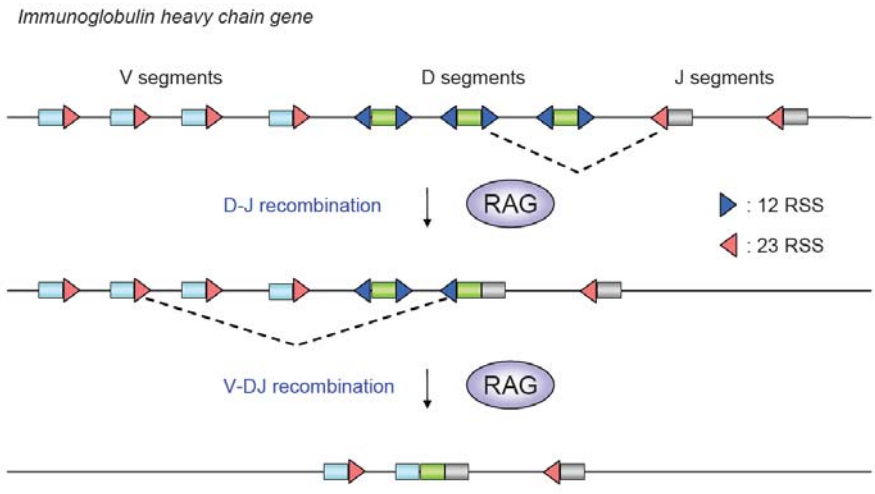

Figure 16. The 12/23 rules at the immunoglobulin heavy chain. The variable (V), diversity (D) and joining (J) segments are indicated as boxes together with associated RSSs.

1.3.3.2 Tissue- and temporally- restricted expression of required factors

The Second regulatory process is governed by the tissue- and temporally- restricted expression of essential factors, *RAG1* and *RAG2*. *RAG1/2* are co-expressed in distinct specific developmental stages of lymphoid cells: first in early progenitor- to pre-BI cells, and later again in small pre-BII and immature stages (Oettinger 2004). In the first stage, early progenitor cells such as CLP carry out D-to-J and, at pre-BI cell stage, V-to-DJ rearrangement, respectively. Successful assembly of pre-BCR transmits the signal to shut down *RAG* expression. Later on B cells re-express *RAG* genes at the small pre-BII stage to initiate recombination of the *IgL* locus. In addition to transcriptional regulation, the level of RAG2 proteins is controlled by ubiquitylation-mediated degradation coupled with cell cycle progression. V(D)J recombination occurs in the G1 phase of the cell cycle since in mammalian cells the NHEJ pathway predominates during the G1 and early S phases (Jiang et al. 2005). Thus the level of transcription and the stability of RAG proteins ensures the initiation of V(D)J recombination only at the specific time and in the lymphocyte lineage.

1.3.3.2 Accessibility control of the *Ig* loci

The tissue- and temporally- restricted expression of RAG1/2 are not enough to account for the restrictions of V(D)J recombination in lymphocytes. During normal development, recombination takes place only in lymphoid cells due to the absence of the RAG proteins in other tissues. However, when *RAG* genes are ectopically expressed in other cell types such as fibroblasts, the endogenous *Ig* loci remain in germline configuration/unrearranged, even though artificial recombination substrates that are either episomal or integrated into the genome can be recombined (Schatz et al. 1992). Furthermore, the temporally regulated order of recombination at *Ig* loci is strictly maintained during normal B cell development as *IgL* recombination follows successful recombination of the *IgH* locus. With regards to the 12/23 rule, it could potentially happen that V(D)J recombination starts rearranging the D_H segments to V_H segments,

however this does not take place during normal B cell development. Therefore an additional layer of regulation is clearly required to explain these observations. Pioneer studies described a correlation between transcription of unrearranged V, D and J gene segments "germline transcription" and the onset of rearrangement at those same sites (Yancopoulos and Alt 1985). Analysis of the regulatory elements of the *IgH* locus identified multiple control elements: V segments all contain a promoter and a strong B-cell specific enhancer, iEμ, is found in the large intron upstream of Cμ (see Fig. 14). Furthermore the D and J segments also carry promoters that control expression of the germline transcripts. Experiments with transgenic substrates and targeted deletions have revealed that the iEμ and promoter elements control the locus accessibility of the different regions of the *Ig* locus during B cell development, accompanied with germline transcription. Thus germline transcripts might be the by-products of local open/accessible chromatin conformation (Oettinger 2004). However it may also be that the germline transcripts are necessary to open up the chromatin. Recent analysis of chromatin modifications and its structural changes have further elucidated the molecular mechanisms of the locus accessibility control. Germline transcripts are well associated with local histone acetylation and methylation on H3K4, which are the hallmark of open conformational chromatin structures (Kwon et al. 2000; Chowdhury and Sen 2001; Johnson et al. 2003; Sen and Oltz 2006). In early B cell stage during which D_H-J_H recombination occurs, histones associated with D_H and J_H segments become acetylated, thereby transcriptional machinery can access the locus to start gene expression and at the same time the recombinase machinery can also initiate the rearrangement process. On the other hand, V_H segments as well as the whole *IgL* genes remain hypoacetylated (i.e. are not transcriptionally active), preventing access by the recombinase machinery and precocious rearrangement. Activation and hyper acetylation of V_H segments is observed during the subsequent V_H-DJ_H recombination at the pre-BI cell stage, which is under the control of IL-7 signaling via the transcription factors, STAT5a/b (Bertolino et al. 2005). Thus regulation by the level of chromatin modifications in the *Ig* loci ensures an additional layer of mechanisms that controls the accessibility of the recombinase machinery on the *Ig* loci (Fig. 17).

1.3.3.3 Nuclear positioning and contraction of the *Ig* loci

In non-B lineage cells, the *IgH* and *IgL* loci are positioned at close proximity to the nuclear periphery and have a highly condensed form of chromatin with high H3K9 methylation. In the B cell lineage, during the process of IgH recombination concomitant with B cell development, the *IgH* loci become accessible as evidenced by local increases in histone acetylation and coincidental relocation to the center of the nucleus (Kosak et al. 2002; Bertolino et al. 2005). This deassociation from the nuclear periphery occurs coincidentaly with V-(D)J recombination as well as an increased accessibility in the V_H segments, namely at distal V_H segments, thereby it is proposed that this relocalization to the center of the nucleus might be important for the control of distal V segments accessibility. At the same time, the IgH locus undergoes a large scale conformational change of chromatin, IgH locus contraction. “Contraction” of the IgH locus brings distal V_H segments closer to the already recombined DJ_H segments, thereby enabling V(D)J recombination of distal V_H segments such as V_HJ558 family genes (Fuxa et al. 2004; Roldan et al. 2005; Sayegh et al. 2005). Once an allele has successfully completed its V(D)J recombination, the second allele, which has not proceeded to V-DJ recombination yet, reverses the contraction (decontraction) and moves to the pericentromeric heterochromatin. This re-localization of the second unrearranged *IgH* locus is proposed to induce the heterochromatinization in the locus by the association with pericentromeric heterochromatin, based on the fact that the allelic exclusion in *Igk* locus utilizes this mechanism (Goldmit et al. 2005). Both re-localization and decontraction processes are thought to ensure the allelic exclusion as well as preventing further recombination of the *IgH* locus (Fig. 17).

With the similar degree to the locus accessibility control, all these processes, which change the higher order of chromatin structure and the sub-nuclear localization of the *IgH* locus, are closely linked to the modifications of histones that are proposed to have a critical impact on them (Luger and Hansen 2005; Hansen 2006). In fact, *Ig* locus contraction is observed at the time which B cells show histone hyper-acetylation on the

distal V_H segments. However in details of mechanisms, functions of acetylation and required factors for Ig locus contraction remain to be explored.

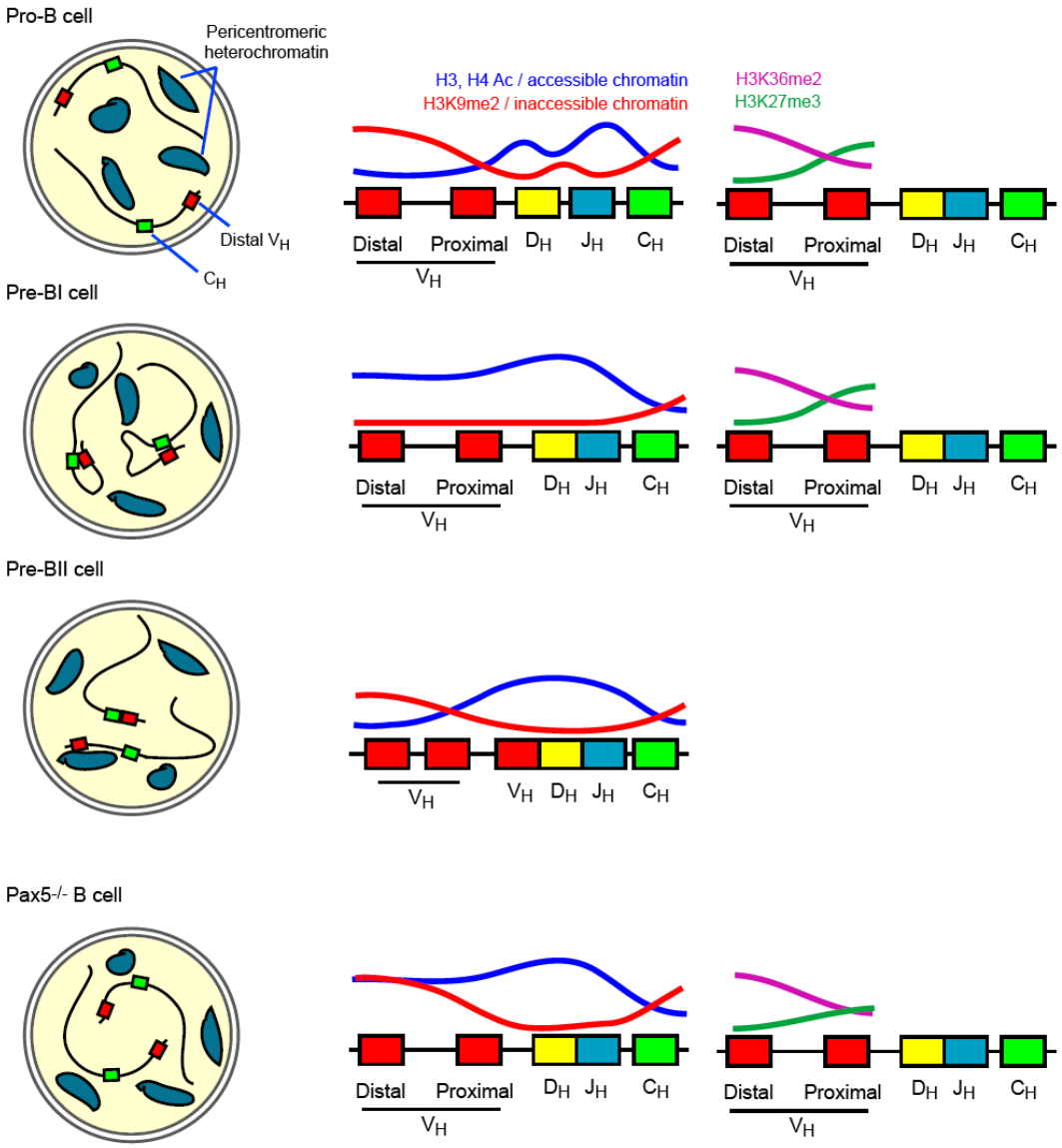

Figure 17. Simplified scheme of IgH nuclear positioning and histone modifications (modified from (Bergman and Cedar 2004; Sen 2005)). Typical positioning of two alleles of *IgH* genes in the B cell nucleus are drawn in the left side. Patterns of histone acetylation and H3K9me2 in the left, and H3K36me2 and H3K27me3 in the right are shown. Note that histone acetylation and H3K9me2 modifications in the *IgH* locus have not yet been revealed in the high resolution such as a ChIP-seq or ChIP-on-chip experiment, so that the relative enrichments of them were examined by the sequence specific primers in particular regions. H3K36me2 and H3K27me3 are examined by ChIP-on-chip experiments (Xu et al. 2008). *Pax5*$^{-/-}$ B cells lack IgH contraction, enrich H3K9me2 at distal V_H segments, and reduce H3K36me3 at proximal V_H segments whereas levels of acetylation and H3K36me2 are largely unchanged (Fuxa et al. 2004).

1.3.3.4 Factors required for IgH locus contraction

Many factors have been identified to be required for early B cell development, however so far only two of them are known to be involved in the process of IgH contraction: Pax5 and YY1 (Busslinger 2004; Matthias and Rolink 2005; Cobaleda et al. 2007; Nutt and Kee 2007).

Pax5$^{-/-}$ pro-B cells (B220^{+} c-kit^{+}) exhibit a profound defect of IgH recombination on distal V_H segments, whereas almost no defect is seen on D-J_H or proximal V_H recombination. Three-dimensional DNA fluorescence in situ hybridization (3D-FISH) experiments revealed that *Pax5*$^{-/-}$ pro-B cells can not contract the *IgH* locus to facilitate the recombination of distal V_H segments (Fuxa et al. 2004). Since Pax5 functions both as a gene activator and repressor, many genes crucial for B cell development are under control of Pax5 proteins. However, none of the genes known to control V(D)J recombination were deregulated in the *Pax5* mutant cells such as *RAG1/2*, *EBF1* and *E2A* (Fuxa et al. 2004). Recent reports have shed some light on the molecular mechanisms of Pax5's roles in IgH contraction. In the absence of Pax5, distal V_H segments are accessible and hyper-acetylated, however at the same time an accumulation of the inactive/condensed histone mark H3K9 methylation is observced. Ectopic expression of Pax5 in non-B cell lineage induces the removal of H3K9 methylation on the *IgH* locus even though normally this locus is highly enriched with H3K9 methylation in non-B cells (Johnson et al. 2004). Another report suggested that Pax5 binds to the coding regions of human and mouse both distal and proximal, with the higher affinity, V_H gene segments *in vivo* and *in vitro*, and then interacts with the RAG protein complex to enhance the RAG mediated cleavage of V gene segments (Zhang et al. 2006).

Ying Yang 1 (YY1), a zinc finger containing transcription factor expressed ubiquitously, functions as a transcriptional activator, repressor, transcription-initiator element-binding protein, and is also a part of the polycomb Group protein complex depending on the promoter context. Loss of YY1 in the B cell lineage shows severe defects in B cell development and distal V_H recombination without changing the expression of known genes important for V(D)J recombination (Liu et al. 2007). 3D-FISH analysis showed

that B cells lacking YY1 exhibit a similar degree of IgH locus contraction defect as described in *Pax5* mutant mice. YY1 was shown to bind to the *IgH* intronic enhancer (iEμ) whose function is known to be crucial for V(D)J recombination (Sakai et al. 1999; Perlot et al. 2005).

So far only a very limited knowledge is available about the IgH locus contraction phenomenon so that the molecular mechanisms as well as additional factors involved remain largely unexplored.

2. Results

2.1 Research publication (Submitted)

Histone deacetylases 1 and 2 are essential for early B cell development, V(D)J recombination and contraction of the immunoglobulin heavy chain locus

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Title for a running head: HDAC1/2 are crucial for IgH contraction

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Abstract

The development of mature B lymphocytes from multipotent progenitors is a tightly regulated process whose integrity is ensured at several checkpoints. One of these is the recombination of the immunoglobulin heavy chain (IgH) locus leading to the production of the mu heavy chain. This site-specific DNA recombination is controlled by multiple regulatory components including cis-acting DNA elements, specific expression of the required factors, accessibility of the locus, chromosome sub-nuclear positioning and locus contraction. Here we show that the histone modifying enzymes, histone deacetylase (HDAC) 1 and 2, are essential for early B cell development and IgH recombination. Combined loss of both proteins causes a very strong block in early B cell development accompanied by severe defects in the recombination of IgH variable (V_H) segments. Using three-dimensional DNA fluorescence *in situ* hybridization, we show that this defect is due to the lack of IgH locus contraction. Analysis of histone modification revealed that pre-BI cells lacking HDAC1 and 2 show histone hyperacetylation as well as increased histone lysine 9 methylation. Taken together, we conclude that HDAC1 and 2 are crucial factors for B cell development and IgH recombination by modulating histone modifications which may affect the higher order of chromatin structure.

Introduction

The development of B lymphocytes from progenitor cells is a tightly controlled, yet flexible, process under the control of a number of lineage-specific or ubiquitous transcription factors which initiate, maintain and restrict specific sets of gene expression patterns required for B cell identity (Matthias and Rolink 2005; Cobaleda et al. 2007; Fuxa and Skok 2007). Recent gene targeting studies in mice have identified several factors that are essential for B cell development and whose loss leads to a developmental block at specific stages thought to be checkpoints for B cell development. During development, B cells must undergo the sequential rearrangement of immunoglobulin heavy (*IgH*) and light (*IgL*) chain gene loci to produce an almost infinite diversity of antigen receptors. Only cells harboring productively rearranged antigen receptors are able to proliferate and complete their maturation.

The murine *IgH* locus spans approximately 3 Mb and comprises 150 or more variable (V_H), 12 diversity (D_H) and 4 joining (J_H) gene segments followed by 8 genes encoding the different constant (C_H) regions (Jung and Alt 2004; Jung et al. 2006). The first recombination event takes place at the pro-B cell stage, before full commitment to the B cell lineage (these cells do not yet express the surface marker CD19), and starts with D_H to J_H (DJ) rearrangement. At the subsequent pre-BI developmental stage, the locus undergoes V_H to DJ_H rearrangement. While DJ_H recombination takes place on both alleles, V_H to DJ_H recombination occurs on one allele at a time with the second allele not being processed once a locus has been successfully rearranged. At a later developmental stage, the Ig light chain is similarly rearranged by bringing V_L and J_L segments together in order to ultimately produce a functional Ig molecule. Because of the multiple Ig loci involved and the stochastic nature of the process, V(D)J recombination needs to be under a sophisticated control mechanism. This includes a multitude of cis-acting DNA elements, lineage-specific and temporally-restricted expression of the required factors such as the recombinases, control of locus accessibility at the level of chromatin and intranuclear locus repositioning and contraction (Kosak et al. 2002; Jung et al. 2006; Sen and Oltz 2006).

In non-B lineage cells, the *IgH* and *IgL* loci are positioned at close proximity to the nuclear periphery and preserved from recombination. In the B cell lineage, during the process of IgH recombination concomitant with B cell development, the IgH locus becomes accessible as evidenced by local increases in histone acetylation and relocation to the center of the nucleus (Kosak et al. 2002). “Contraction” of the *IgH* locus brings distal V_H segments closer to the already recombined DJ_H segments, thereby facilitating V(D)J recombination (Fuxa et al. 2004; Roldan et al. 2005; Sayegh et al. 2005). All these processes, which change the higher order of chromatin structure and the sub-nuclear localization of the IgH locus, are closely linked to the post-translational modifications of histones that are proposed to have a critical impact on them (Luger and Hansen 2005; Hansen 2006). For example, increased accessibility of the *IgH* locus is well correlated to hyperacetylation of histones and inversely to histone lysine 9 methylation (Kwon et al. 2000; Chowdhury and Sen 2001; Johnson et al. 2003; Johnson et al. 2004).

Histone deacetylases (HDACs) are enzymes that catalyze the deacetylation of acetylated lysine residues of histones as well as of an increasing number of other proteins. In mammals, there are 18 HDACs identified that can be grouped into three classes (Yang and Seto 2008). The class I enzymes HDAC1 and 2 are the closest homologs of yeast Rpd3 which is the main HDAC regulating the global level of histone acetylation in yeast (Kurdistani et al. 2002). In mammals, HDAC1 and 2 are highly related proteins, predominantly localized in the nucleus and expressed in a wide range of tissues and cell types. HDAC1 and 2 have been identified as components of three different repressive complexes; the Sin3, NuRD and Co-REST complexes which are recruited to various genes (Yang and Seto 2008). Gene targeting studies of *HDAC1* have demonstrated that this protein is essential for early embryogenesis, possibly due to its role in cell proliferation (Lagger et al. 2002). Furthermore, *HDAC1* null ES cells exhibit a proliferation defect, in part caused by upregulation of the cell cycle inhibitor p21 which was found to be a direct target of HDAC1 (Lagger et al. 2002; Lagger et al. 2003). In contrast, recently published *HDAC2* deficient mice are viable during embryogenesis and show partial or complete perinatal lethality depending on the allele (Montgomery et al. 2007; Trivedi et al. 2007; Zimmermann et al. 2007), demonstrating that these two proteins are only partly redundant. A conditional knockout study of *HDAC1* and *2* in cardiomyocytes revealed the importance of these two proteins for myocardial growth, morphogenesis, and contractility (Montgomery et al. 2007). In spite of these recent studies, the physiological function of HDAC1 and 2 remains largely unexplored and their role in the lymphoid system is not known.

Here we have examined the role of HDAC1 and 2 in B cell development. For this we have generated mice conditionally targeted at the *HDAC1* and *2* gene and eliminated these enzymes specifically in the B cell lineage by intercrossing with *mb1-cre* mice to induce the deletion from the pre-BI stage onwards. We find that in the absence of either of these two proteins B cell development proceeds normally. In contrast, elimination of both enzymes simultaneously leads to a complete absence of B cells from the periphery. We show that this is the result of a severe developmental block in the bone marrow that comprises two steps. While pre-BII cells exhibit massive apoptosis, pre-BI cells are fully

viable. However, these cells show a severe impairment in V(D)J recombination, associated with a failure of the *IgH* locus to contract. Furthermore, we show that these cells have elevated acetylation of histone H4, but also aberrant other histone modifications such as an increased level of methylated H3K9. These data identify HDAC1 and 2 as critical novel regulators of B cell development and suggest that histone modifications control IgH recombination by altering the higher order of chromatin structure.

Results

Generation of HDAC1 and 2 conditional knockout mice

To explore the functions of HDAC1 and HDAC2 in B cell development, we generated conditional *HDAC1* and *2* alleles in which exon 6 of either gene is flanked by *loxP* sites (Supplemental Fig. 1). This exon is identical in the two genes and encodes three amino acids which have been shown to be essential for the deacetylase activity of HDAC1 (Hassig et al. 1998). *Flox* alleles of HDAC1 and HDAC2 produce normal amounts of each protein, and both homozygous mice appear to be indistinguishable from their wild type (WT) littermates (data not shown). Before analyzing the function of HDAC1 and 2 in B lymphocytes, we first generated complete knockout (KO) mice by crossing *HDAC1* or *2 flox* mice with mice expressing Cre recombinase under the control of a germline specific promoter. The offspring obtained allowed us to derive homozygous knockout mice by intercrossing. We analyzed these $HDAC1^{-/-}$ mice and confirmed a previous report that HDAC1 inactivation leads to embryonic lethality before E10.5 (Lagger et al. 2002) (data not shown). In contrast, $HDAC2^{-/-}$ mice show partial (ca. 50%) perinatal lethality, with the rest of the mice being smaller than their WT littermates (Supplemental Fig. 2). Thus, the phenotype of our $HDAC2^{-/-}$ strain resembles that of another recently reported HDAC2 deficient mouse allele (Trivedi et al. 2007; Zimmermann et al. 2007).

B cell-specific elimination of HDAC1 or 2 does not impair B cell development

We first examined the expression of *HDAC1* and *2* during B cell development using a quantitative real time PCR assay and RNA isolated from different stages of B cell development. Cell surface markers and developmental events defining each B cell stages are drawn in Figure 18A. As shown in Figure 18B both enzymes are expressed throughout B cell development, but *HDAC1* expression moderately increases during B cell maturation; in contrast, *HDAC2* shows the highest expression in the pro-B/pre-BI fraction and then exhibits a gradual decrease up to the mature B cell stage. To inactivate the function of HDAC1 and 2 in the B cell lineage, we used the *mb1-cre* transgenic mouse line (Hobeika et al. 2006) which starts to express *cre* in pre-BI cells, at the beginning of B cell commitment. First, we analyzed the efficiency of *flox* allele deletion by a genotyping PCR. For this, DNA was isolated from mice heterozygous for both the

HDAC1 and *2 flox* alleles and also carrying the *mb1-cre* gene (*HDAC1/2*$^{F/+}$ + *mb1-cre*). With this assay we did not detect deleted alleles (Δ) of either *HDAC1* or *2* in a sorted bone marrow progenitor population lacking committed B cells (B220^{+} CD19^{-}), whereas no *flox* (i.e. unrecombined) alleles of either gene were seen in the B cell-committed pre-BI cell population (B220^{+} CD19^{+} CD43^{+} CD25^{-}, Fig. 18C). This indicates that the recombination of the *HDAC1* and *2* loci is highly efficient and takes place already at the pre-BI stage. We also analyzed by western blotting the levels of HDAC1 and 2 proteins in splenic mature B cells using samples from mice homozygous for the *flox* allele (*HDAC1*$^{F/F}$ or *HDAC2*$^{F/F}$) and carrying *mb1-cre*. As presented in Figure 18D, both proteins were below detection in these mice, and no truncated form of HDAC1 or 2 was observed (not shown). Together, these results confirm the successful inactivation of HDAC1 and 2 in the B cell lineage. These mice were next analyzed by flow cytometry (FACS) to determine the effect of ablating HDAC1 or 2 in B cells. For this, a series of antibodies were used to distinguish specific stages of B cell development. *HDAC1*$^{F/F}$ + *mb1-cre* as well as *HDAC2*$^{F/F}$ + *mb1-cre* mice have all the B cell developmental stages normally represented in the bone marrow and also normal numbers of B cells in the spleen (see Supplemental Fig. 3). Thus, individually HDAC1 and 2 appear to be dispensable for normal B cell development.

Ablation of both HDAC1 and 2 blocks early B cell development

The observations presented above suggest that HDAC1 and 2 might be redundant or compensate for each other in B cells; therefore, we set out to eliminate both enzymes simultaneously. Mice were generated that are homozygously *floxed* at both genes and also carry the *mb1-cre* transgene (*HDAC1/2*$^{F/F}$ + *mb1-cre*); for the analyses, littermates lacking the *cre* transgene were usually used as controls. As shown in Figure 19A, the spleen of mice lacking both HDAC1 and 2 is virtually empty of mature B cells that were identified here by expression of the marker B220 in combination with IgM. In the bone marrow, where developing B cells are generated, the stages of B cell differentiation were detected by staining cells with B220 in combination with c-kit or CD43 (pro-B / pre-BI cells), CD19 (committed B cells), CD25 (pre-BII cells) or IgM (immature and mature B cells). As is evident from the dot plots presented, absence of HDAC1 and 2 leads to a

severe developmental block, as early as the pre-BI stage. Accordingly, the number of committed B cells (i.e. positive for CD19) is dramatically reduced, as well as all subsequent stages. The absolute numbers, obtained from several similar experiments, are presented in Figure 19B and C: $B220^+$ $CD19^-$ cells, representing the progenitor compartment lacking committed B cells and which have not yet recombined the *flox* allele, are present in normal numbers. In contrast, pre-BI cells are reduced about 5 fold in the absence of HDAC1 and 2, and pre-BII cells are reduced more than 100 fold. Thus, normal progression through early B cell development strictly requires the presence of HDAC1 or 2.

Pre-BI cells lacking HDAC1 and 2 show normal cell cycle and apoptosis

Work done largely in cancer cell lines with HDAC inhibitors has implicated HDACs in the control of cell cycle as well as apoptosis (Yoshida et al. 1987; Minucci and Pelicci 2006). We therefore examined these aspects in the pre-B cells of *HDAC1/2*$^{F/F}$ + *mb1-cre* mice by *in vivo* 5-bromo-2-deoxyuridine (BrdU) incorporation assays. For this, BrdU was injected intraperitonealy to label cycling cells and pre-BI cells were purified. Total DNA and incorporated BrdU were stained and the cell cycle profile was determined by FACS analysis. As shown in Figure 20A, at the pre-BI cell stage, the cell cycle profile of *HDAC1/2*$^{F/F}$ + *mb1-cre* mice was largely unchanged compared to that of control mice, although the G2/M and sub G1 populations were slightly, but significantly, altered (Fig. 3A). In contrast, in pre-BII cells a cell cycle block was evident (data not shown). Next, apoptosis induction of pre-BI or pre-BII cells was measured by staining with AnnexinV which can bind to exposed phosphatidylserines of cells undergoing apoptosis. While no significant apoptosis was observed in the mutant pre-BI cells, a dramatic apoptosis increase was seen in cells of the next developmental stages, the large or small pre-BII cells (Fig. 20B).

V_H-DJ_H recombination is severely impaired in pre-BI cells lacking HDAC1 and 2

Pre-BI cells correspond to the stage at which V_H to DJ_H recombination takes place and we therefore considered the possibility that this process might be impaired in the absence of HDAC1 and 2. To analyze DNA recombination at the IgH locus we used a semi

quantitative PCR assay to amplifiy DNA fragments corresponding to alleles rearranged between the proximal V_H7183, or the distal V_HJ558, and the DJ_H segments (Fuxa et al. 2004). As controls, a Cμ fragment, which does not rearrange, or a D_H–J_H segment, which rearranges prior to the deletion of *HDAC1* and *2*, were amplified (Fig. 21A). Using DNA from WT pre-BI cells, we were able to detect fragments from proximal or distal recombined alleles, as expected. In contrast, in DNA from mutant cells, distal V_HJ558 rearrangements could not be detected and proximal V_H7183 rearrangements were reduced about 4 fold, while D_H–J_H amplification was not affected (Fig. 21B). The strong reduction in V_H–DJ_H rearrangements was confirmed by measuring spliced μ mRNA transcripts initiating at the V_HJ558 or V_H7183 locus by RT-PCR and also by staining pre-BI cells for the presence of intracellular μ protein. In each case a strong reduction of the signal was observed, indicating that the HDAC1/2 deficiency in pre-BI cells abrogates the distal V_H-DJ_H and also has an impact on proximal V_H-DJ_H recombination (Fig. 21C and D). These data suggest that the strong block of early B cell development in the absence of HDAC1 and 2 is due, at least in part, to the reduced number of cells harboring a successfully recombined IgH locus, which are therefore unable to present the pre-BCR on their cell surface for clonal expansion at the pre-BII stage.

Combined loss of HDAC1 and 2 in pre-BI cells does not affect distal V_HJ558 germline transcripts

Previous work has shown that V(D)J recombination correlates positively with accessibility of the Ig V_H genes, which can be inferred from the presence of corresponding germline transcripts (Yancopoulos and Alt 1985). Distal V_HJ558 germline transcripts are known to be regulated at the pre-BI cell stage by the transcription factor STAT5 under the control of IL-7R signaling, which leads to enhanced accessibility of distal V_H regions and local histone hyperacetylation (Bertolino et al. 2005). To examine whether the defect caused by loss of HDAC1 and 2 may impinge on that process, we monitored the expression of distal V_H germline transcripts. As shown in Figure 21E, the level of V_HJ558 germline transcripts is not significantly reduced in mutant pre-BI cells compared to wild-type pre-BI cells (Fig. 21E). Thus, loss of HDAC1 and 2 is not crucial for the proper expression of V_H germline transcripts, suggesting that IL-7R signaling is

normal in these mice. Moreover, other germline transcripts such as those originating in the Cμ locus are also normal in the absence of HDAC1 and 2.

HDAC1 and 2 are essential for IgH contraction

IgH recombination first takes place in non committed early B cell progenitors, starting with D_H to J_H recombination. At this stage, the distal V_H segments are separated from the recombined DJ_H locus by a large distance, as the locus is extended. During the subsequent V_H to DJ_H recombination taking place in committed pre-BI cells, the distal V_H segments are brought in proximity of the DJ segment, a phenomenon called locus contraction (Kosak et al. 2002; Fuxa et al. 2004; Roldan et al. 2005; Sayegh et al. 2005). Importantly, mice lacking the transcription factor Pax5 or YY1 exhibit defects in distal V_H recombination and also in IgH locus contraction (Fuxa et al. 2004; Liu et al. 2007).

To assess whether lack of distal V_H recombination in HDAC1 and 2 deficient pre-BI cells is due to impaired locus contraction, we performed three-color three-dimensional DNA fluorescence in situ hybridization (3D FISH) with DNA probes located in the distal V_HJ558 family and in the IgH constant region, together with a γ-satellite probe marking pericentromeric heterochromatin (Fuxa et al. 2004; Liu et al. 2007; Hewitt et al. 2008). Pre-BI cells were sorted from *HDAC1/2*$^{F/F}$ + *mb1-cre* or control mice and used for 3D FISH experiments (Fig. 22A). IgH locus contraction was scored by measuring the distance separating the distal V_H segments from the C_H region; in addition, the proportion of cells showing mono- or bi-allelic recruitment of the IgH locus to pericentromeric heterochromatin was determined. As expected, in WT pre-BI cells the vast majority (88%) of the IgH alleles were fully contracted. In contrast, pre-BI cells lacking HDAC1 and 2 only had 37% of the IgH alleles contracted, with another ca. 47% of the alleles in an intermediate configuration (Fig. 22B and Supplemental Table 1A). Furthermore, in WT pre-BI cells 37% of the cells showed monoallelic- and almost none biallelic-recruitment of the IgH locus to pericentromeric regions, in good agreement with previous results (Roldan et al. 2005). Strikingly, in the absence of HDAC1 and 2 pre-BI cells exhibited aberrant recruitment of the IgH locus: 48% of the cells have monoallelic and 14% biallelic recruitment (Figure 22C and Supplemental Table 1B). Taken together, the

results of Figure 21 and 22 identify HDAC1 and 2 as novel essential factors for IgH contraction and recombination.

Combined loss of HDAC1 and 2 does not affect mRNA accumulation of genes essential for early B cell development and IgH recombination

To investigate the molecular mechanism by which HDAC1 and 2 regulate IgH recombination and contraction, we first examined the expression of transcription factors critical for early B cell development. E2A, EBF and Ikaros are known to regulate gene expression of factors essential for early B cell development (Bain et al. 1994; Georgopoulos et al. 1994; Sun 1994; Zhuang et al. 1994; Lin and Grosschedl 1995). As shown in Figure 23A, we detected comparable amount of transcripts of all these genes in *HDAC1/2*$^{F/F}$ + *mb1-cre* pre-BI cells compared to WT pre-BI cells. Pax5 and YY1 are transcription factors and Ezh2 is a histone methyltransferase part of the Polycomb complex, which have all been reported to be crucial for recombination of distal V_H genes (Su et al. 2003; Fuxa et al. 2004; Liu et al. 2007). We found unchanged levels of Ezh2 and YY1 transcripts and a slight, but reproducible, upregulation of Pax5 transcripts (2-4 fold). In these samples, the mRNA encoding HDAC1 and 2 were also greatly reduced, confirming the efficient deletion of both alleles in *HDAC1/2*$^{F/F}$ + *mb1-cre* pre-BI cells. We next examined the expression of genes involved in the process of IgH recombination including Rag-1, Rag-2, terminal deoxynucleotidyl transferase (TdT), DNA-dependent protein kinase catalytic subunit (DNA-PKc), Ku70 and Ku80 (Jung and Alt 2004). Except for a slight reduction of Rag-2 transcripts (2-4 fold), all other transcripts were as abundant in HDAC1 and 2 deficient pre-BI cells as in WT cells (Fig. 23B). Furthermore, transcripts from cell surface receptor molecules such as IL-7R, Igα (*mb1*), Igβ (*B29*), lambda5 and VpreB were also unaffected. We conclude from these data that HDAC1 and 2 regulate IgH recombination and contraction without significantly altering expression of known genes involved in early B cell development and the process of V(D)J recombination.

Global histone modifications are altered in pre-BI cells lacking HDAC1 and 2

Histone post-translational modifications are known to be important for regulating chromatin higher order structure, flexibility and accessibility (Hansen 2006; Sen and Oltz 2006; Berger 2007), therefore we investigated the possibility that HDAC1 and 2 regulate IgH recombination and contraction by modulating histone modifications. Because of the limitation on number of HDAC1 and 2 deficient pre-BI cells for experiments such as western blot and chromatin immunoprecipitation, we carried out quantitative measurements of histone modifications in single cells by FACS analysis. For this we first established the conditions for intracellular staining of histone modifications using a B cell lymphoma line treated with HDAC inhibitors. As shown in Supplemental Figure 4, strong increases in histone H3 and H4 acetylation are detected in cells that have been treated with the pan-HDAC inhibitors. Next, mouse pre-BI cells were identified using a combination of cell surface markers and, after fixation and permeabilization, stained with specific antibodies against different histone modifications. As expected, the total amount of histone H3 in WT and HDAC1 and 2 deficient pre-BI cells was not significantly different (Fig. 24). In contrast, the level of acetylated histone H4 was greatly elevated in mutant pre-BI cells, whereas acetylated H3K9 and H3K14 were unchanged. This observation suggests that in pre-BI cells HDAC1 and 2 participate in maintaining global histone acetylation, preferentially on histone H4. Histone acetylation, as well as di- and tri-methylation of histone H3 lysine 4 (H3K4me2, H3K4me3), are associated with active genes within open chromatin, whereas histone H3 lysine 9 methylation is a marker for repressed loci associated with a more condensed and tightly packed chromatin (Berger 2007). As shown in Figure 7, the level of H3K4me2 and H3K4me3 were unchanged in HDAC1 and 2 deficient pre-BI cells, whereas a robust enrichment of H3K9me2 and H3K9me3 was detected. Taken together, these findings suggest that loss of HDAC1 and 2 dramatically alters the global pattern of histone modifications in pre-BI cells whose chromatin, although being more acetylated, does not appear to be globally open, but rather shows hallmarks of a repressive conformation.

DISCUSSION

We have shown here that HDAC1 and 2 are essential factors for early B cell development as well as V(D)J recombination by regulating the contraction and pericentromeric recruitment of the IgH locus. In the absence of these two enzymes, B cells are virtually absent from the periphery and a severe developmental defect is apparent at the pre-BI and pre-BII stage. The number of pre-BII cells is more than 100 fold reduced and the rare cells that can be detected are highly apoptotic. In contrast, the number of pre-BI cells is reduced about five fold, but these cells cycle normally and do not show apoptosis. However, in these cells a strong block in V(D)J recombination can be seen, which abrogates rearrangement of distal V_H genes and moderately reduces rearrangement of proximal V_H genes. By 3D FISH analysis we found that in these cells the contraction of the IgH locus is impaired, thus providing an explanation for the rearrangement defect. Our expression analysis demonstrated that this defect is not due to misregulated expression of known genes important for early B cell development, or V(D)J recombination and DNA repair. Moreover our results show that combined loss of HDAC1 and 2 leads to an increase in global histone H4 acetylation, as anticipated, but also to an increase in histone H3K9 methylation which may influence the higher order of chromatin structure. Taken these data together, we hypothesize that HDAC1 and 2 control IgH recombination directly or indirectly by regulating the level of histone modifications. This conclusion, however, is paradoxical since many reports have pointed out that histone hyperacetylation is positively correlated with IgH recombination (Kwon et al. 2000; Chowdhury and Sen 2001; Johnson et al. 2003). In the case of the distal V_H segments, histone hyperacetylation correlates with the presence of germline transcripts and is under the control of IL-7R signaling via STAT5 a/b activation at the pre-BI cell stage (Bertolino et al. 2005). We did not detect any significant changes in the level of germline transcripts from distal V_H segments, which suggests that in the absence of HDAC1 and 2 the locus around the distal V_H segments is accessible and the IL-7R signaling pathway is unaffected.

How do HDAC1 and 2 participate in the process of IgH contraction and pericentromeric recruitment? Our global histone modification analysis has shown that combined loss of HDAC1 and 2 leads to hyperacetylation of histone H4, as anticipated. Surprisingly

however, we observed an increase in repressive histone modifications, such as H3K9 di- and tri-methylation, whereas no significant alteration was seen in the active modifications marks H3K9 and K14 acetylation, and H3K4 di- and tri-methylation. Previous reports have shown that in Pax5 deficient mice, which were the first mutant mice found to be impaired in IgH locus contraction, there is an accumulation of H3K9 di-methylation in distal V_H regions, yet the chromatin conformation of distal V regions is accessible, as evidenced by their hyperacetylation and the normal presence of germline transcripts (Fuxa et al. 2004; Johnson et al. 2004). Interestingly, this phenotype is similar to that of the HDAC1 and 2 B cell deficient mutant mice described here. This similarity raises the question whether these different factors might all work in the same pathway to regulate IgH recombination. According to our expression analysis, Pax5 is still expressed in the absence of HDAC1 and 2, yet IgH contraction was abrogated. In this context, HDAC1 and 2 and Pax5 may function in parallel pathways independently required for IgH contraction. However, Pax5 is known to regulate gene expression by both active and repressive mechanisms, and it has been reported that Pax5 interacts with Grg4/TLE4, one of the four members of the mammalian Groucho copressor family (Eberhard et al. 2000). In addition, Drosophila Rpd3, which is the closest homolog of mammalian HDAC1 and 2 in Drosophila, has been identified as an interaction partner of Drosophila Groucho (Chen et al. 1999). Thus, it appears plausible that Pax5 might interact with HDAC1 and 2 and that this could contribute to the regulation of IgH locus contraction.

How could histone hyperacetylation negatively affect DNA rearrangement of the IgH locus? As mentioned above, a positive correlation between histone hyperacetylation and IgH recombination has been well documented. Based on these previous studies, one might have expected increased recombination efficiency in the absence of HDAC1 and 2, but the opposite was observed. A few possibilities can be considered to help explain this paradox. First, excessive histone acetylation of the IgH locus may directly influence its contraction and sub-nuclear localization through dynamic alterations of the higher order structure of chromatin. *In vitro* studies using recombinant nucleosomal arrays have demonstrated that acetylation of histones can affect chromatin organization (Tse et al. 1998; Dorigo et al. 2003; Shogren-Knaak et al. 2006). Several reports pointed out that *in*

vitro acetylation of histone H4, especially on lysine 16, could inhibit the formation of compact 30-nanometer-like fibers and the ability of chromatin to form cross-fiber interactions (Shogren-Knaak et al. 2006; Wang and Hayes 2008). A very recent report from Murre and colleagues provided the first complete picture of the 3D structural organization of the IgH locus (Jhunjhunwala et al. 2008). In cells undergoing IgH contraction the topology of the IgH locus is more condensed as compared to non-committed B cells (*E2A*$^{-/-}$ pre-pro-B cells): not only are distal V_H and D_H segments in close proximity, but the entire IgH locus is also within a relatively close distance, implying that there might be a long range interchromosomal association within the entire IgH locus. Therefore, based on these different *in vitro* and *in vivo* results, one could imagine that highly acetylated histone H4 in HDAC1 and 2 deficient pre-BI cells might alter the higher order of chromatin structure to a more decondensed form, which impairs IgH contraction.

Another possibility is that increased histone acetylation is indirectly important for IgH contraction. Our global analysis of histone modifications clearly demonstrated that loss of HDAC1 and 2 in pre-BI cells also led to accumulation of repressive marks such as histone H3K9 di- and tri-methylation. Interestingly, pre-BI cells lacking Pax5 were shown to have a higher level of H3K9 dimethylation at distal V_H regions (Johnson et al. 2004). Thus, it could be that HDAC1 and 2 are important either for the removal of or for preventing H3K9 dimethylation, which in turn may prevent contraction of the IgH locus through alteration of higher order of chromatin structure. Increased histone H3K9 methylation at the IgH locus may for example lead to increased recruitment of chromodomain-containing factors that bind methylated lysine residues and might prevent IgH contraction.

Another possibility might be that HDAC1 and 2 impact IgH recombination indirectly through regulating gene expression or even the acetylation of non-histone proteins. Our analysis showed that a number of transcription factors that are critical for early B cell development, such as Pax-5, E2A or EBF, are well expressed in cells lacking HDAC1 and 2. Likewise, genes essential for Ig recombination, such as *Rag-1* and *Rag-2*, are also normally expressed in mutant cells. Yet it is possible that the expression of other

unknown genes essential for IgH contraction and recombination requires HDAC1 and 2. Given the complexity of the process, it is likely that many such genes remain to be identified. Analysis of the transcriptome of HDAC1 and 2 double deficient pre-BI cells may be useful to that end. Finally, it is also possible that some of the phenotypes described here are not due to a primary defect at the level of chromatin (i.e. changes in histone modifications), but rather reflect the lack of deacetylation of an essential factor implicated in contraction and rearrangement of the IgH locus. Given the increasingly recognized importance of acetylation for a wide range of cellular processes (Minucci and Pelicci 2006) this appears a plausible possibility. While it is not known whether acetylation/deacetylation of Rag proteins may be important for their function, this scenario appears unlikely since the IgH locus, although not rearranged, is contracted normally in Rag-2 deficient pro-B cells (Jhunjhunwala et al. 2008). Yet, another protein implicated in this process might be regulated by acetylation.

METHODS

Generation of HDAC1 and 2 conditional knockout mice and animal experiment

Each exon 6 of mouse *HDAC1* and *2* was flanked by a single *loxP* site and a cassette expressing the neomycin resistance gene and thymidine kinase gene flanked by two *loxP* sites. E14 ES cells (129/Ola strain) were electroporated with either the targeting vector for mouse *HDAC1* or *2* and correctly targeted ES clones were identified by PCR, sequencing and Southern blot analysis. One targeted ES cell clone for *HDAC1* and *2*, number 149 and 233 respectively, was used for aggregation to generate chimeric mice. Chimeric mice were crossed to C57/Bl6 animals to obtain germ line transmission of the *targeted* allele. To delete the neomycin cassette (*flox* allele) and generate *delta* allele, *targeted* mice were crossed with either *EIIa-Cre* mice or *Meox-Cre* mice. The chimeras that contain both *Cre* and *targeted* alleles were genotyped and we identified mice having recombined *delta* or *flox* alleles by PCR and verified by sequencing. For the generation of B cell specific KO mouse, we used *mb1-cre* transgenic mice kindly provided by Dr. Michael Reth.

For all analyses, *flox* allele littermates of the same sex were used as controls. All experiments were done using mixed background (129/Ola and C57/Bl6 mixed strain) transgenic mice from 6 to 12 weeks olds. Animal experiments were carried out according to regulations effective in the Kanton of Basel-Stadt, Switzerland. The mice were housed in groups of one to eight animals at 25°C with a 12:12 h light-dark cycle. They were fed a standard laboratory diet containing 0.8% phosphorus and 1.1% calcium (NAFAG 890, Kliba, Basel, Switzerland). Food and water was provided *ad libitum*.

Flow cytometry (FACS)

Single cell suspensions were prepared from indicated lymphoid tissues. Cell staining was done according to standard procedures. FACS analysis was performed on a FACSCalibur (BD Biosciences, San Jose, CA) by gating on live cells. Samples were analyzed by using CellQuest (Becton Dickinson) or Flow-Jo (Tree star) software. Cell sorting was performed on a MoFlo (DakoCytomation). The purity of sorted cells was checked by reanalysis.

The following mAbs were from BD Biosciences; RA3-6B2 (anti-B220) conjugated to allophycocyanin (APC), ACK45 (anti-c-kit) conjugated to phycoerythrin (PE) or biotinylated, S7 (anti-CD43) conjugated to fluorescein isothiocyanate (FITC), 1D3 (anti-CD19) conjugated to APC-Cy7 and 7D4 (anti-CD25) biotinylated. 1B4B1 (anti-IgM) conjugated to FITC was from Southern Biotech. 6D5 (anti-CD19) conjugated to PE was from Biolegend. Streptavidin conjugated to PE-Cy5.5 was from CALTAG. For the Annexin V staining, we followed the manufacturer's protocol from BD Biosciences.

Quantitative RT-PCR and semi quantitative RT-PCR

RNA was purified from sorted cell population mentioned in each figure with the RNeasy Microkit (Qiagen) according to the manufacturer's protocol. cDNA was synthesized by using the Thermoscript Reverse Transcriptase Kit (Invitrogen). Oligo dT primers provided in the kit were used for the first strand synthesis.

Quantitative PCR was performed on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) using a MESA GREEN qPCR MasterMix Plus for SYBR® Assay (Eurogentec). The amount of *HDAC1* and *2* transcripts was normalized by *Gapdh*.

For semi quantitative RT-PCR, cDNA was serially diluted 4 fold for PCR reactions. As a control reaction for contamination by genomic DNA, we prepared samples without reverse transcriptase (-RT). Primers used for these experiments are provided in Supplementary Table 3. Primers for semi quantitative RT-PCR were previously reported (DeKoter et al. 2002; Fuxa et al. 2004; Delogu et al. 2006; Liu et al. 2007). For the amplification of μ transcripts, combinations of a forward primer used for V(D)J recombination analysis (V_HJ558 and V_H7183) and a reverse primer, Cμ transcript, were used.

PCR analysis of deleted *HDAC1* and *2* alleles and V(D)J recombination

DNA was extracted from sorted cell populations mentioned in each figure by overnight proteinase K digestion, phenol/chloroform extraction and ethanol precipitation. DNA was serially diluted at 1:4 ratios and used for each PCR reaction. Primers used for the PCR detection of *HDAC1* and *2* genotyping and V(D)J recombination are shown in

Supplementary Table 2 (Fuxa et al. 2004; Liu et al. 2007). PCR analysis of V(D)J recombination was described previously (Fuxa et al. 2004). Combinations of a forward primer for different parts of the IgH locus (V_HJ558, V_H7183 and D_H) and a reverse J_H3 primer were used. Amplicons from PCR reactions were separated on 1.5% agarose gel and visualized by ethidium bromide.

Western blot analysis of mature B cells

Naïve mature B cells from spleen were isolated by negative selection with CD43 microbeads (Miltenyi Biotec) according to the manufacturer's protocol. Sample preparation, SDS-PAGE and blotting were done as described previously (Zhang et al. 2008). Mouse monoclonal anti-HDAC1 and anti-HDAC2 were described in (Zupkovitz et al. 2006).

***In vivo* BrdU incorporation assay**

Mice were injected intraperitneally with 20 μg of BrdU and sacrificed 30 minutes later. Bone marrow single cell suspensions were prepared, stained and sorted with the combination of antibodies against the following cell surface molecules; $CD19^+$ $CD43^+$ $CD25^-$. BrdU staining using sorted cells was performed according to the manufacturer's protocol of BrdU flow kit (BD bioscience). Analysis was performed on a FACSCalibur and analyzed by Flow-Jo software.

Three-dimensional DNA FISH and confocal microscopy

Cells sorted by flow cytometry were washed in PBS and then were fixed on poly-L-lysine-coated slides for three-color and three-dimensional DNA-FISH analysis as described (Fuxa et al. 2004). Probes were directly labeled by nick translation with ChromaTide Alexa Fluor 488-5-dUTP, ChromaTide Alexa Fluor 594-5-dUTP (Molecular Probes) or dUTP-indodicarbocyanine (GE Healthcare). The γ-satellite probe was prepared from a plasmid containing eight copies of the γ-satellite repeat sequence (Hewitt et al. 2008) and was directly labeled with dUTP-fluorescein isothiocyanate (Roche; Enzo Biochem) or dUTP-indodicarbocyanine. Cells were analyzed by confocal microscopy on a Leica SP5 AOBS system (Acousto-Optical Beam Splitter). Optical

sections separated by 0.3 μm were collected, and only cells with signals from both alleles (typically 90%) were analyzed. Details of sample sizes and statistical analysis are provided in Supplementary Table 1.

Intracellular FACS for histone modifications

Cells were stained with fluorochrome coupled antibodies; B220-APC, CD19-PE and c-kit-biotinylated. Streptavidin-PE-Cy5.5 was used for the staining of c-kit-biotinylated antibody. After staining, cells were fixed with 3% paraformaldehyde for 15 minutes at room temperature, permeabilized with 0.1% saponin, 3% bovine serum albumin (BSA) in PBS for 30 minutes at room temperature, and then subjected to the intracellular staining with different histone modification antibodies as follows; anti-Histone H3 (ab1791; Abcam), anti-acetyl-Histone H3K9 and K14 (06-599; Millipore), anti-acetyl-Histone H4 (06-866; Millipore), anti-Histone H3 di-methyl lysine 4 (07-030; Millipore), anti-Histone H3 tri-methyl lysine 4 (ab8580; Abcam), anti-Histone H3 di- and tri-methyl lysine 9 antibodies were kindly provided by Dr. Antoine Peters (Peters et al. 2003). After washing of cells to remove nonspecific antibody binding, cells were stained with the secondary antibody recognizing rabbit IgG coupled to alexa Fluor® 488 (A11034; Invitrogen). FACS analysis was performed after extensive washing. The buffer containing 0.1% saponin in PBS was used for all staining, washing and FACS analysis after permeabilization.

Statistical analysis

Significance was assessed by analysis of normal distribution, variances and two-tail T test.

Author contributions

T.Y. designed and carried out the experiments, analyzed data and wrote the manuscript; Y.Z. isolated HDAC1 conditional ES cells; S.L.H. and J.A.S did 3D-FISH experiments and data analysis; H.K. helped with FACS sorting; C.S. constructed the HDAC1 targeting vector; P.M. supervised the research and wrote the manuscript.

Acknowledgments

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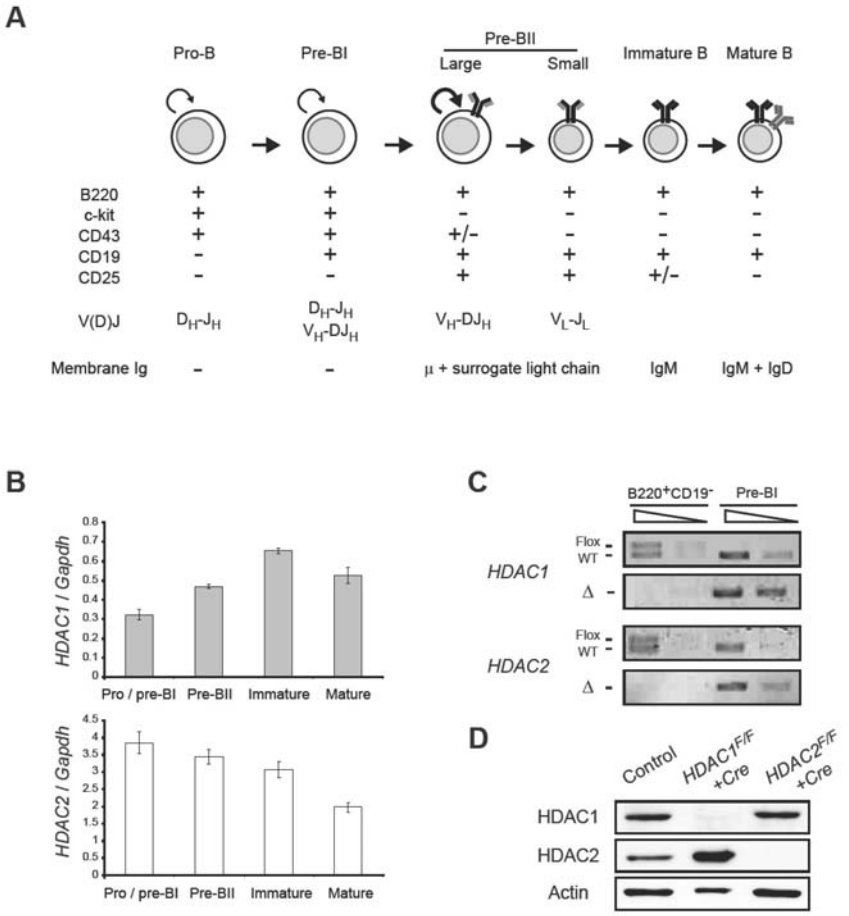


Figure 18

Ablation of HDAC1 or 2 in B cells. (A) Scheme of early B cell differentiation, depicting the different developmental stages and the expression of markers used to discriminate them (marker expression is indicated by +, lack of expression by -). Curved arrows indicate relative cellular proliferation activities: very high in large pre-BII, intermediate in pro- and pre-BI, low or absent in small pre-BII, immature and mature B cells. V(D)J shows the rearrangement status of the different Ig gene alleles during B cell differentiation. The kind of immunoglobulin present at the membrane is also indicated at the bottom. (*B*) *HDAC1* and *2* transcripts were analyzed by quantitative RT-PCR and normalized to *Gapdh* expression. B cell populations were purified from three wild type mice and RNA was extracted. Pro/pre-BI cells: $B220^{+}$ $c\text{-}kit^{+}$ $CD25^{-}$ IgM^{-}; pre-BII cells: $B220^{+}$ $c\text{-}kit^{-}$ $CD25^{+}$ IgM^{-}; immature B cells: $B220^{+}$ IgM^{+}; mature B cells: $B220^{High}$ IgM^{+}. (*C*) PCR detection of recombination at HDAC1 and HDAC2 alleles. Cells were purified from a mouse (*HDAC1/2*$^{F/+}$ *+mb1-Cre*) and DNA was extracted from two sorted cell populations: $B220^{+}CD19^{-}$ and pre-BI cells ($B220^{+}$ $CD19^{+}$ $CD43^{+}$ $CD25^{-}$). Specific sets of primers were used to discriminate *flox*, wild type and KO alleles. (*D*) Western blot analysis of HDAC1 or HDAC2 single KO mouse. Protein lysates from splenic mature B cells were detected by anti-HDAC1, HDAC2 and Actin antibodies, as indicated.

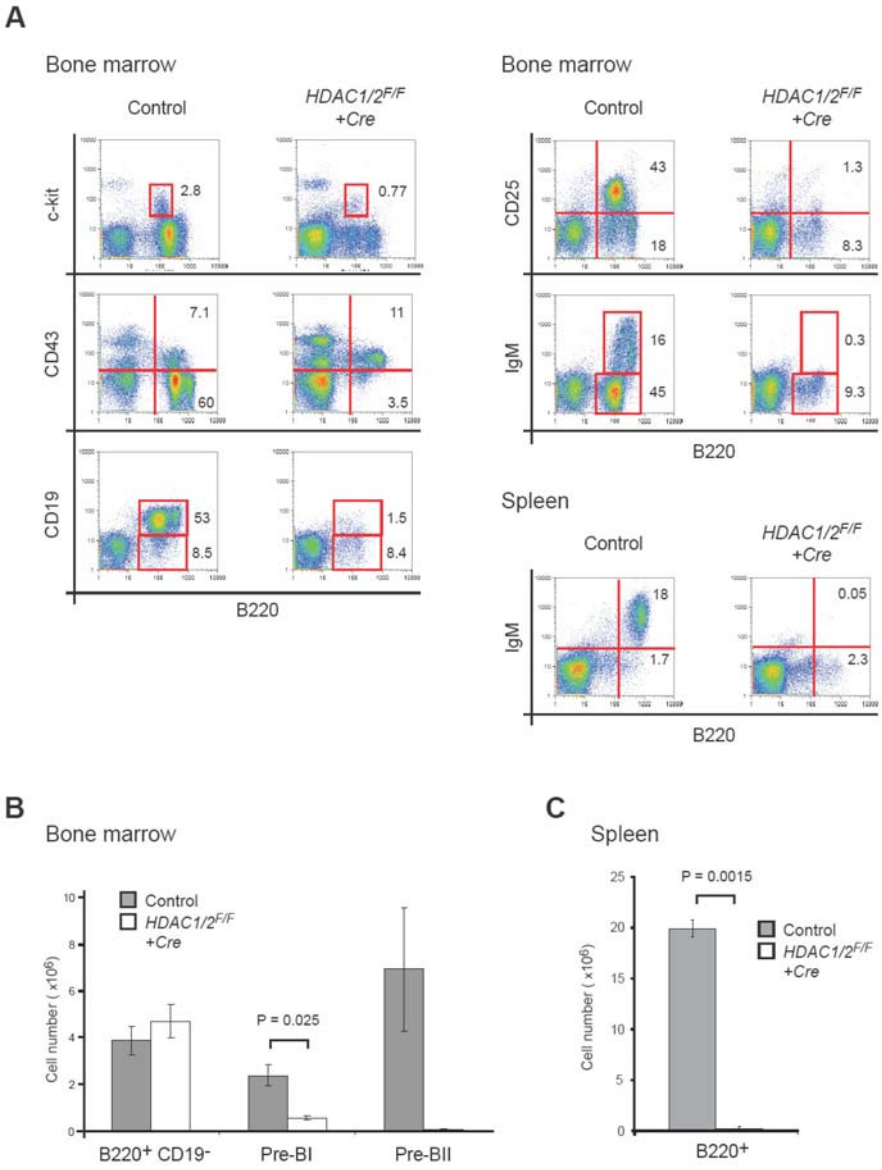


Figure 19
Early block in B cell development after combined loss of HDAC1 and 2. (*A*) Flow cytometric analysis of *HDAC1/2$^{F/F}$* + *mb1-Cre* mice. Single cell suspensions from bone marrow or spleen were stained with the indicated antibody combinations and representative data are shown. Numbers in plots represent the percentage of cells in the respective gate or quadrant. (*B*) Total cell numbers present in different B cell populations. The absolute numbers were determined based on FACS analysis and cell counting. Pro-B cells were identified as B220$^+$ CD19$^-$, pre-BI cells as B220$^+$ CD19$^+$ CD43$^+$ CD25$^-$ and pre-BII cells as B220$^+$ CD19$^+$ CD25$^+$. (*C*) Absolute numbers of B220$^+$ splenic B cells. The histograms represent the mean ± SE based on the analysis of at least four mice per genotype.

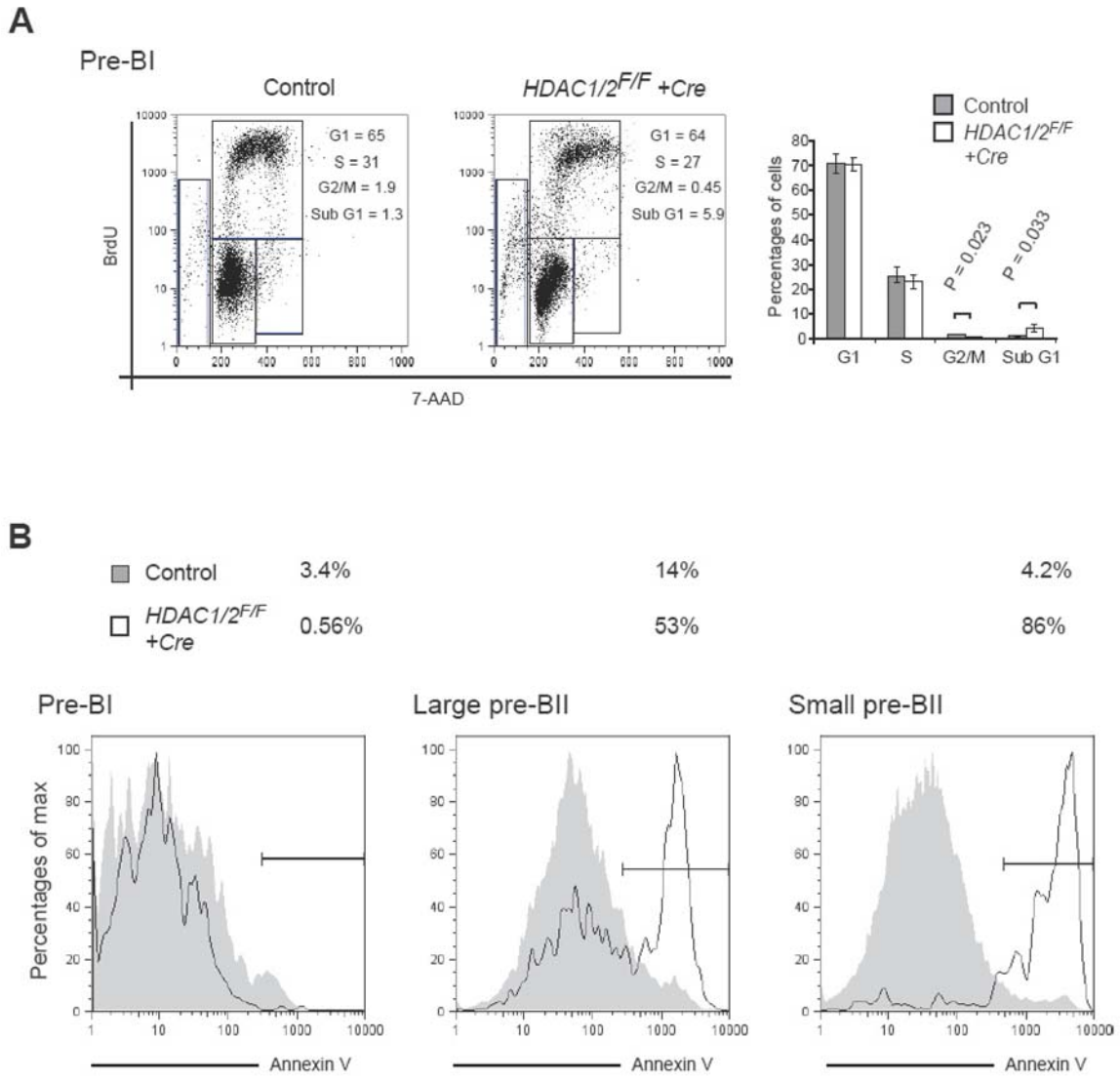


Figure 20
HDAC1/2 deficient pre-BI cells have a normal cell cycle profile and apoptosis while pre-BII cells are highly apoptotic. (*A*) *In vivo* BrdU incorporation assay. Pre-BI cells ($CD19^+ CD43^+ CD25^-$) were sorted and after fixation, permeabilization and DNA digestion, their DNA was stained with an anti-BrdU antibody and 7-AAD. Numbers in plots indicate the percentage of cells in each cell cycle phase. The histogram on the right presents the results of cell cycle profiling from four mice (mean ± SE). (*B*) Analysis of apoptosis by annexin V staining. Cells were stained with combinations of cell surface markers (as in Figure 20A for pre-BI cells and $CD19^+ CD25^+$ for pre-BII cells) and annexin V. Data is representative of more than three mice of each genotype.

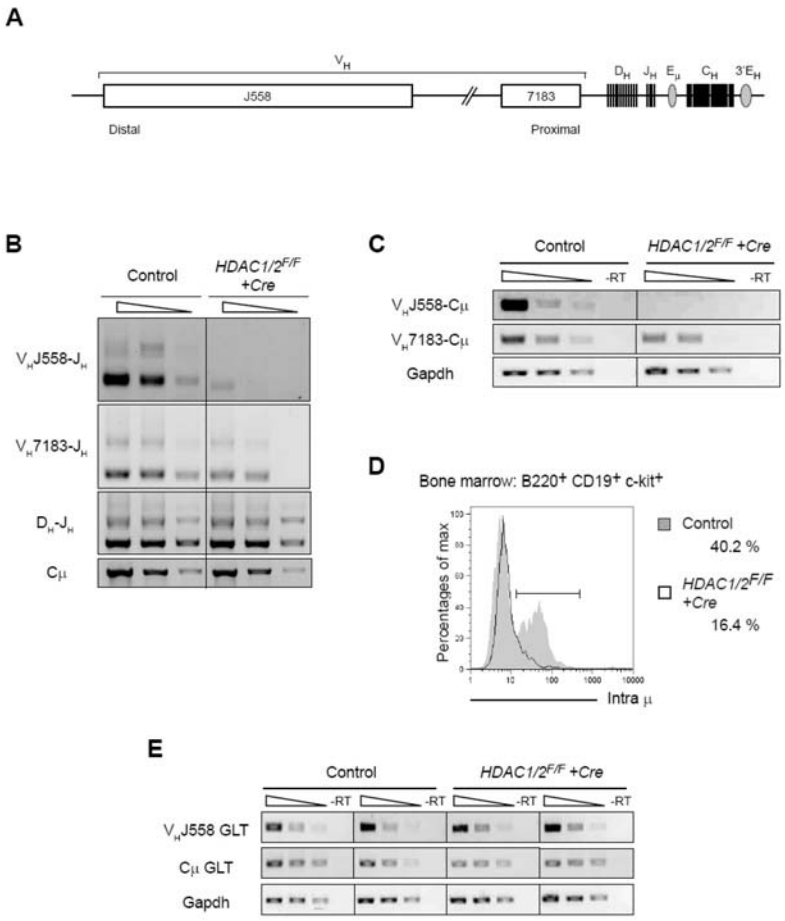


Figure 21

Impaired V_H-DJ_H recombination but normal V_H germline transcript expression in the absence of HDAC1 and 2. (*A*) Schematic of the *IgH* locus in germline configuration with relative positions of distal V_H (J558) and proximal V_H (7183) gene families (not drawn to scale). (*B*) Semi quantitative PCR analysis of V(D)J rearrangements. DNA was isolated from pre-BI cells ($B220^+$ $CD19^+$ $c\text{-}kit^+$ $CD25^-$ IgM^-) and used in four fold serial dilutions for PCR with specific primers. The *Cμ* locus was amplified as a loading control. (*C*) RT-PCR detection of spliced *Igμ* transcripts. RNA was isolated from pre-BI cells (as above), reverse transcribed to cDNA and decreasing amounts (4 fold steps) were used for PCR reactions. Equal loading of cDNA was shown by the *Gapdh* transcripts. (*D*) Intracellular μ staining. Pre-BI cells were identified as $B220^+$ $CD19^+$ $c\text{-}kit^+$ and intracellular staining was done by an anti-IgM antibody. Gray shadow represents staining of control cells and the black line shows staining of HDAC1/2 double deficient cells. Percentages of μ positive cells are shown on the right. Data is representative of three mice of each genotype. (*E*) *V_H* germline transcript (GLT) is normally expressed in double KO pre-BI cells. RNA as in Figure 4C was used for the detection of *V_H* GLT and *Cμ* GLT. *Gapdh* was amplified as a loading control. Two samples are presented for each genotype. All PCR experiments shown in Figure 21 are representatives of at least three experiments with independent samples.

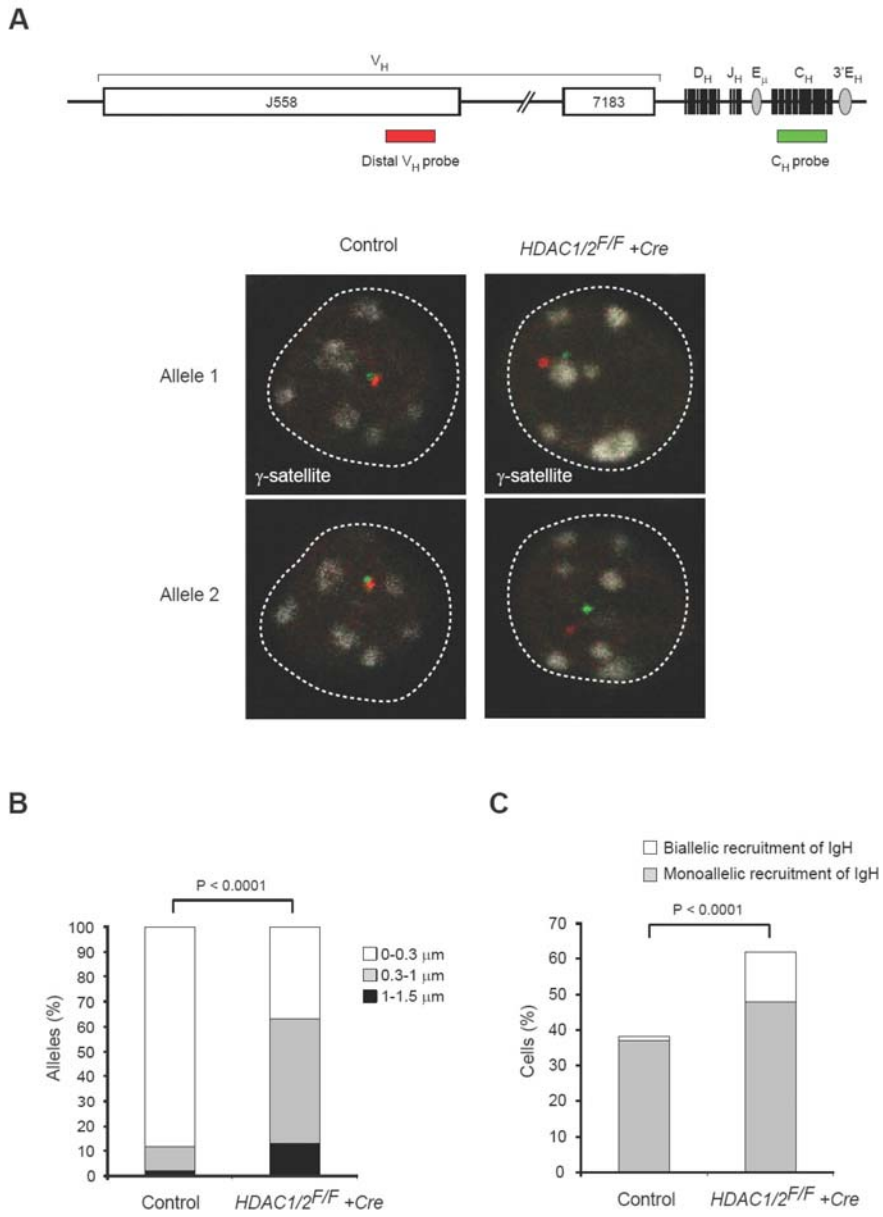


Figure 22
Impaired *IgH* locus contraction in HDAC1/2 deficient pre-BI cells. (*A*) Schematic of the mouse *IgH* locus, indicating the positions of probes used for three-color three-dimensional DNA FISH analysis. The BAC probes used were CT7-526A21 for the IgH 5' J558 segment and CT7-34H6 for the 3' IgH constant region (green) plus a gamma-satellite probe (white). Cells were FACS-sorted as in Figure 21B and used for the experiment. A representative confocal picture is presented; individual alleles from the same cell are shown in different z sections. (*B*) Distances separating the V_HJ558 and C_H gene segments in pre-BI cells of wild type and HDAC1/2 double deficient mice (for percentages, sample sizes and statistic analysis, see Supplemental Table 1A). (*C*) Percentage of cells showing mono- or biallelic pericentromeric recruitment of the *IgH* locus (percentages, sample sizes and statistical analysis, Supplemental Table 1B).

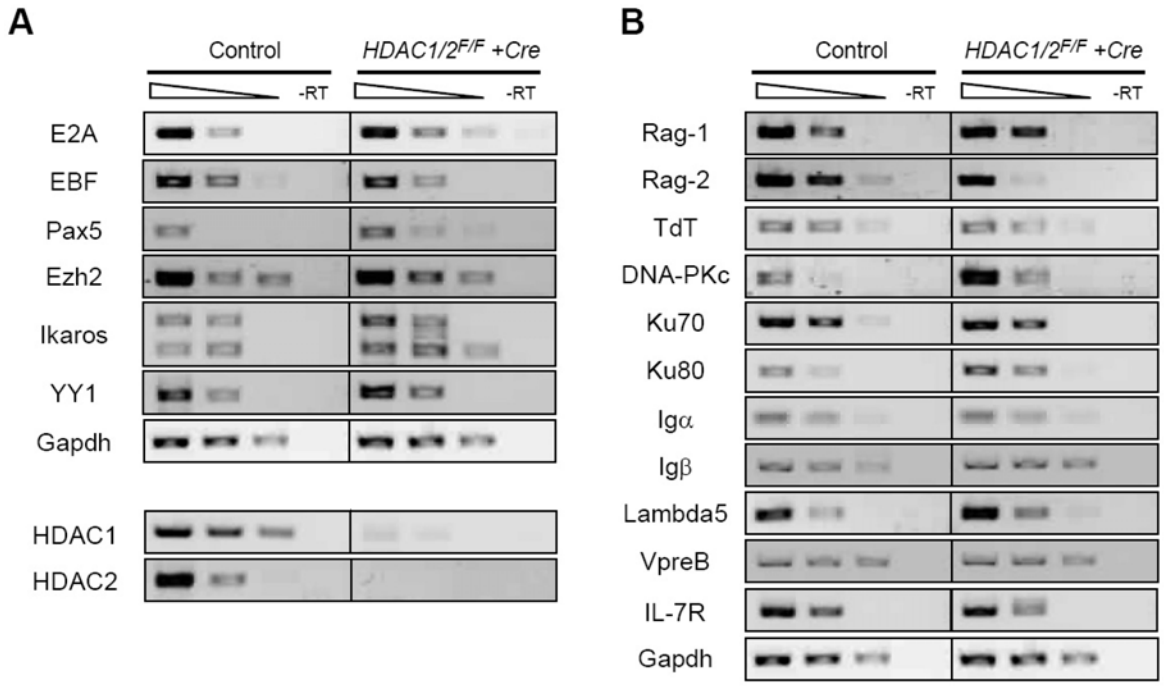


Figure 23
Cells lacking HDAC1 and 2 have a mostly normal expression of genes required for early B cell development and V(D)J recombination. RNA from pre-BI cells (as in Fig. 21C) was used to measure gene expression by RT-PCR using decreasing amounts (4 fold steps) of cDNA. (*A*) Transcription factors and histone modifiers essential for early B cell development. (*B*) Genes involved in IgH recombination and cell surface or signaling molecules. All PCR experiments in Figre 23 are representative of at least three experiments with independent samples.

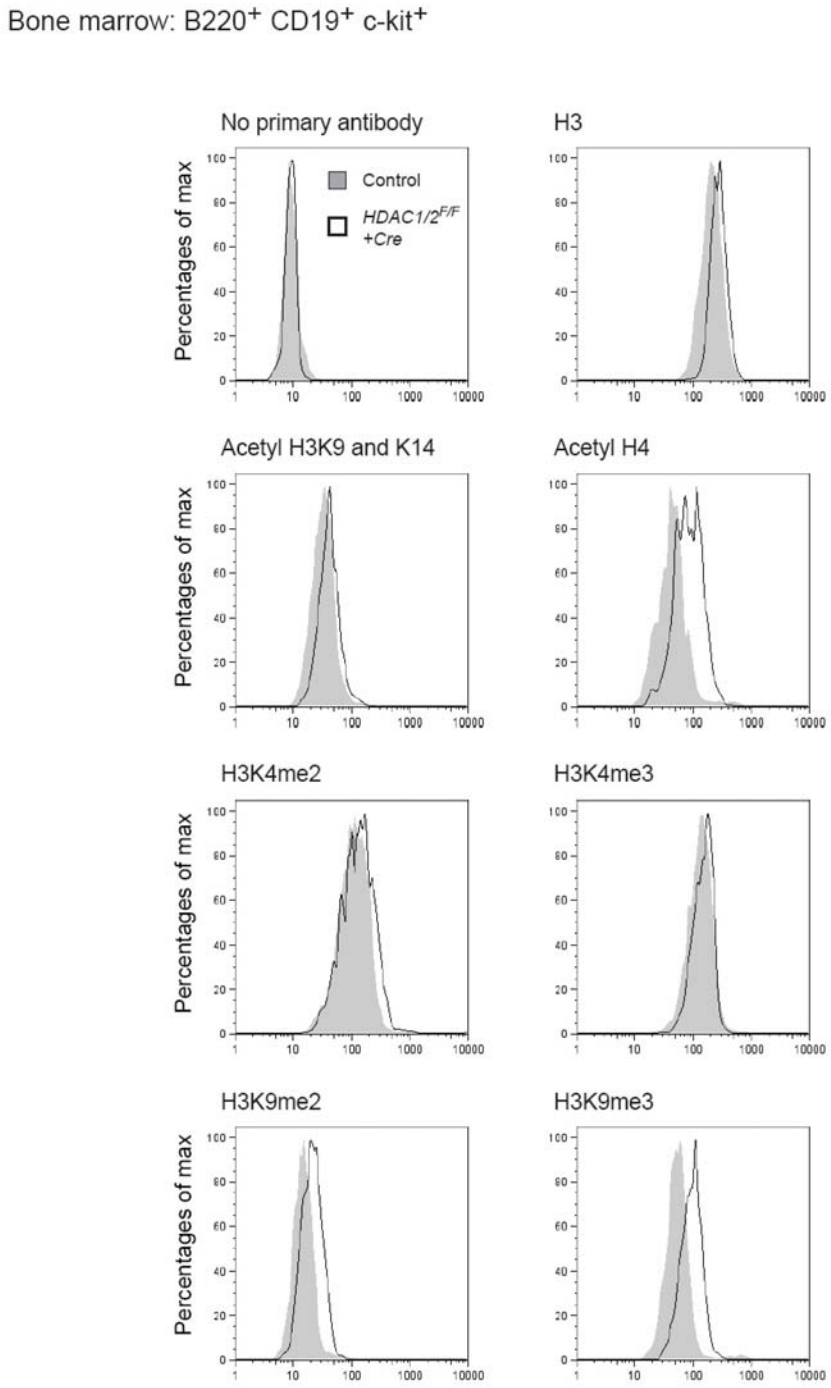
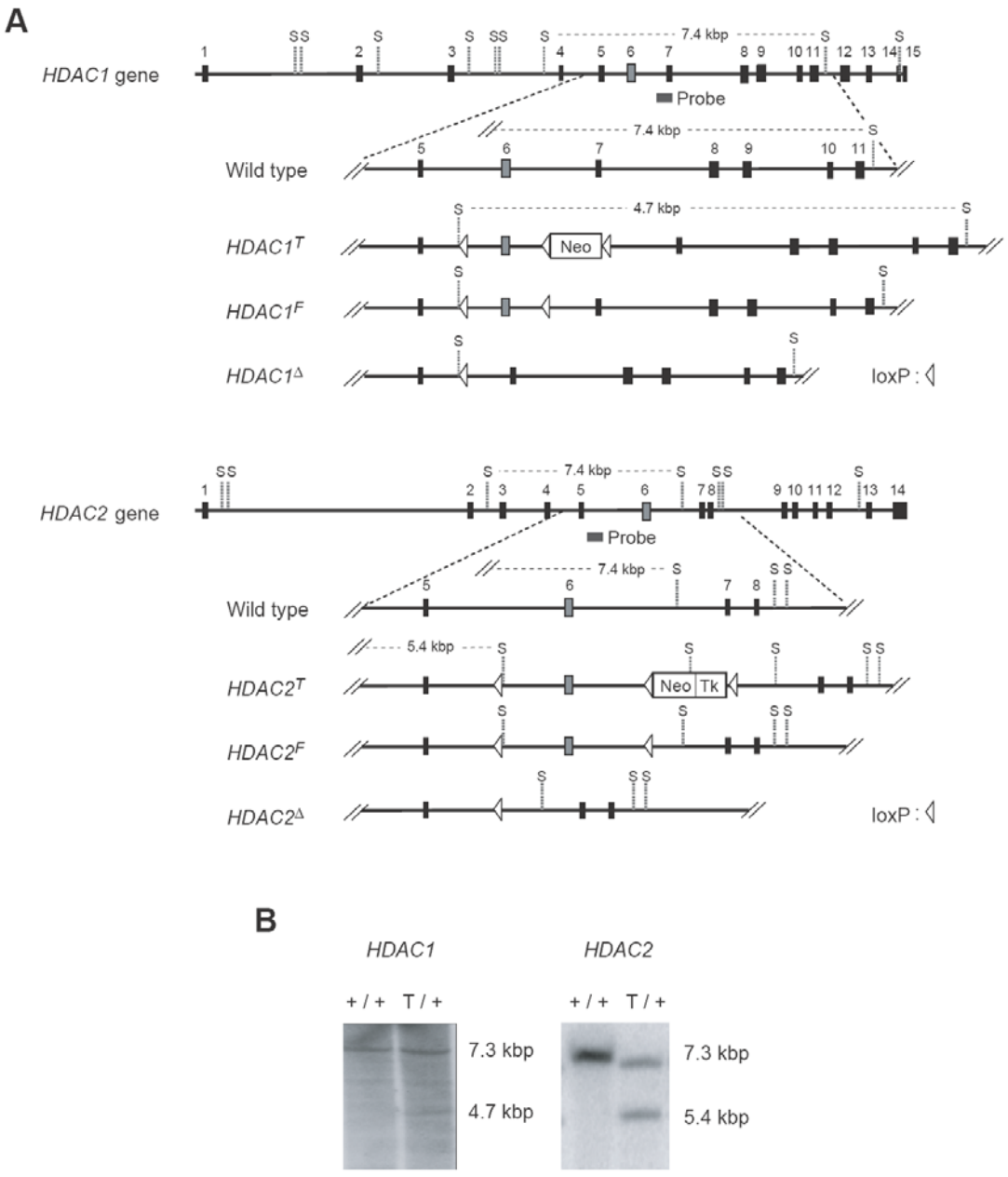


Figure 24
Combined loss of HDAC1 and 2 leads to altered global histone modification patterns. Cells were stained with $B220^+$ $CD19^+$ $c\text{-}kit^+$ and subjected to intracellular staining with an antibody recognizing different histone modifications. Gray shadows represent staining of control cells and black lines show staining of HDAC1/2 double deficient cells. All data are representatives of more than three independent experiments.

Supplemental Figure 1 Yamaguchi et al.



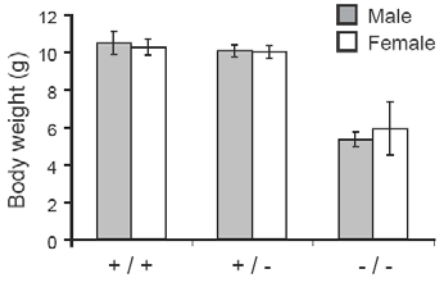
Supplemental Figure 1. Generation of *HDAC1* and *2* conditional mice. (*A*) Wild type and modified alleles of *HDAC1* (upper) and *HDAC2* (lower) are shown. The structure of each gene is depicted at the top. Modified alleles are represented as follows: *T*, targeted allele; *F*, *flox* allele; Δ, deleted (KO) allele. Black and gray boxes represent the coding exons, white rectangular boxes show the neomycin gene cassette, white triangles symbolize the *loxP* sequence and S represents SacI restriction sites. (*B*) Southern blot analysis of genomic DNA isolated from mouse embryonic stem cells. The probes used for hybridization are indicated by the small black rectangles in 1A.

Supplemental Figure 2 Yamaguchi et al.

A

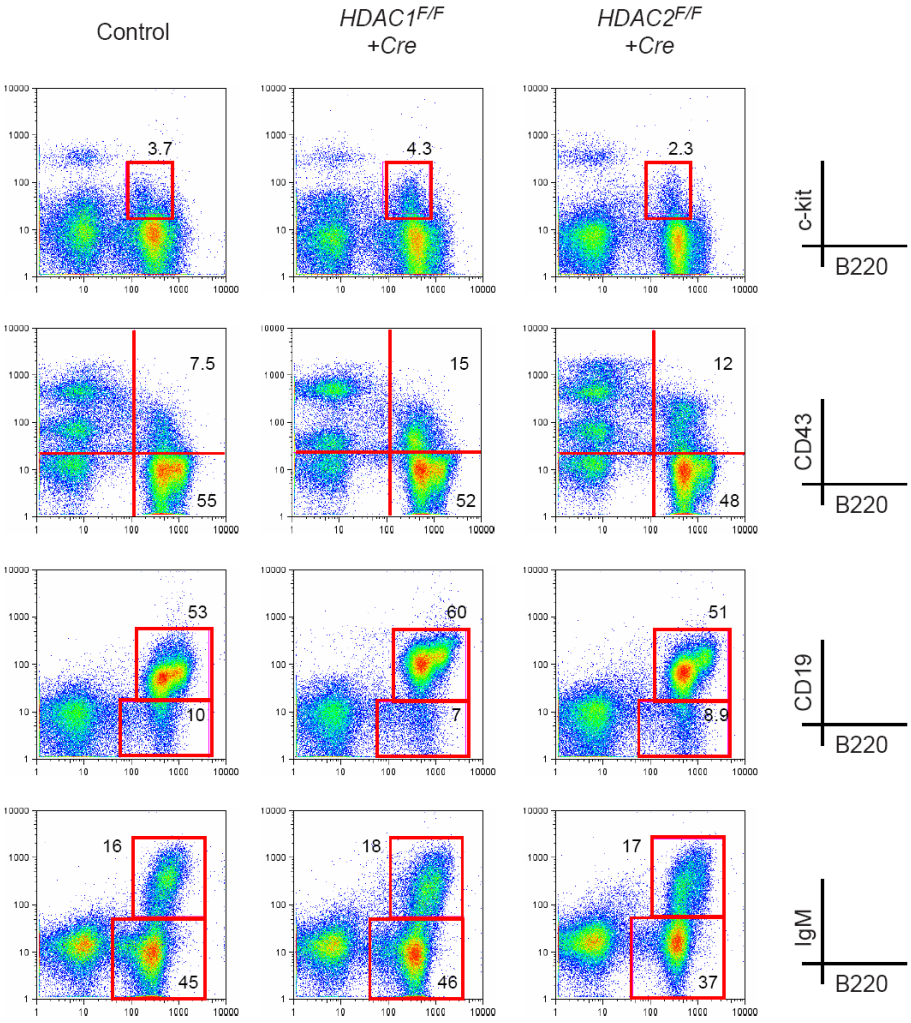
HDAC2	+ / +	+ / -	- / -
P21	27	35	10
Expected	18	36	18

B



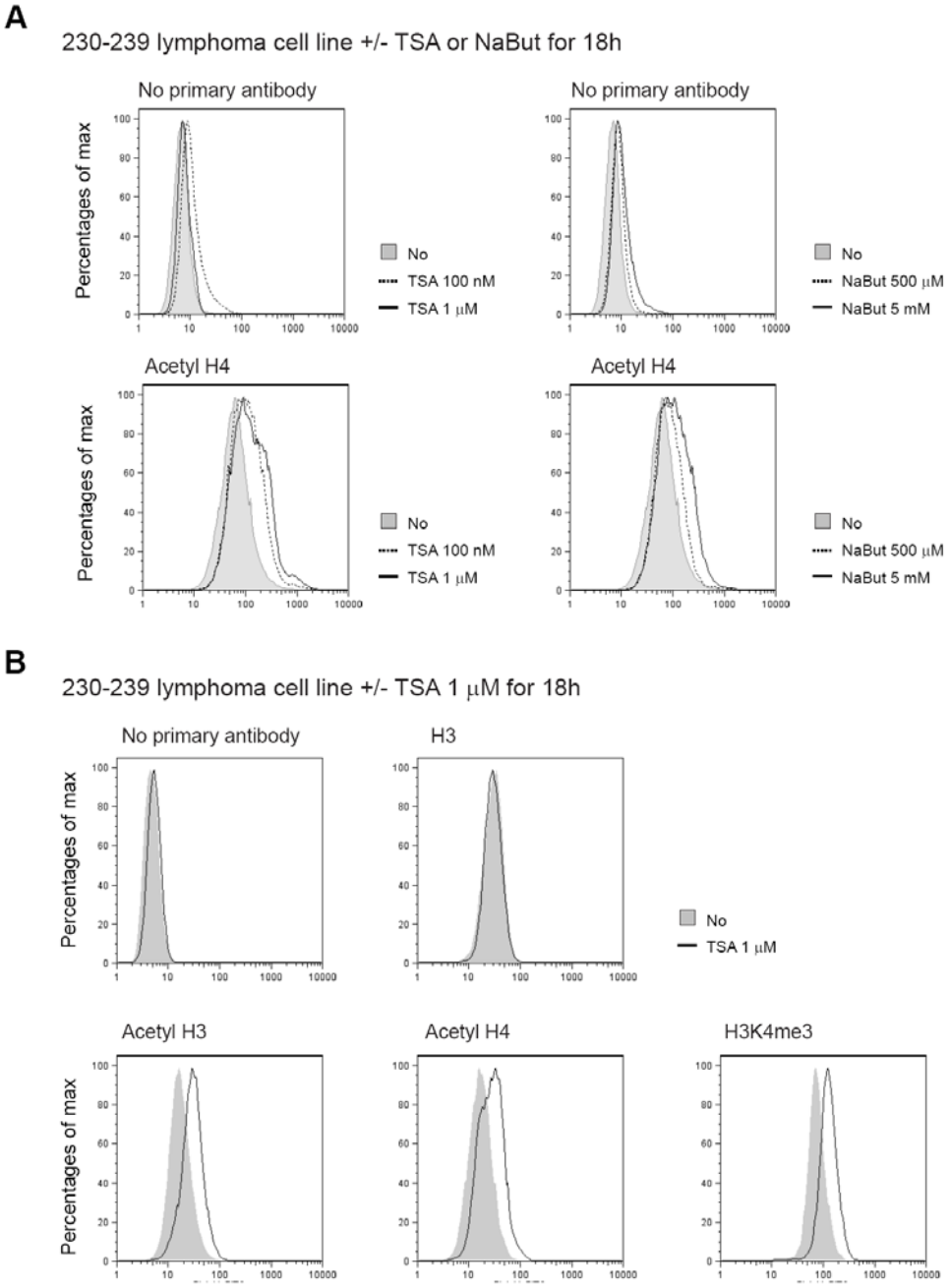
Supplemental Figure 2. Partial perinatal lethality and reduced body weight of *HDAC2 -/-* mice. (*A*) Total numbers of mice at 21 days after birth. Expected numbers based on Mendelian ratios are written in the bottom. (*B*) Body weight of each mouse genotype was measured at 21 days after birth. The data were taken from more than four mice for each sex (mean ± SE).

Supplemental Figure 3 Yamaguchi et al.



Supplemental Figure 3. Ablation of HDAC1 or 2 in B cells is not critical for B cell development. Single cell suspensions from bone marrow were stained with the indicated antibody combinations and representative data are shown. Numbers in plots represent the percentage of cells in the respective gate or quadrant.

Supplemental Figure 4 Yamaguchi et al.



Supplemental Figure 4. Effect on histone modifications in cells treated with HDAC inhibitors. (*A*) Mouse lymphoma cell line 230-238 was cultured for 18 hours +/- TSA or sodium butylate (NaBut) at the indicated concentration. The level of acetyl H4 was measured as described in **Fig. 24.** A peak filled with gray color represents non treated cells, dashed line for cells treated with lower concentration of the inhibitor and solid line for higher concentration. (*B*) TSA treated cells (1 μM) for 18 hours were analyzed with antibodies against different histone modifications.

A Separation of IgH V_HJ558 and C_H gene segments

	% contraction				actual numbers			
Genotype	1-1.5 µm	0.3-1 µm	0-0.3 µm	sample size	1-1.5 µm	0.3-1 µm	0-0.3 µm	sample size
Control	2	10	88	194	3.88	19.4	170.72	194
HDAC1/2 f/f +mb-1 Cre	13	50	37	232	30.16	116	85.84	232

	Observed frequencies (actual numbers)			Expected frequencies				significance	
Genotype	1-1.5 µm	0.3-1 µm	0-0.3 µm	1-1.5 µm	0.3-1 µm	0-0.3 µm	Chi test	p value	level
Control	3.88	19.4	170.72	15.50	61.66	116.84	1.171E-25	1.17E-25	***
HDAC1/2 f/f +mb-1 Cre	30.16	116	85.84	18.54	73.74	139.72			

Genotype		**Genotype**	**probability**	**significance**
Control	———	*HDAC1/2 f/f + mb-1 Cre*	1.17E-25	***

B Pericentromeric recruitment

	% recruitment				actual numbers			
Genotype	none	monoallelic	biallelic	sample size	none	monoallelic	biallelic	sample size
Control	62	37	1	130	80.6	48.1	1.3	130
HDAC1/2 f/f +mb-1 Cre	38	48	14	195	74.1	93.6	27.3	195

	Observed frequencies (actual numbers)			Expected frequencies				significance	
Genotype	none	monoallelic	biallelic	none	monoallelic	biallelic	Chi test	p value	level
Control	80.6	48.1	1.3	61.88	56.68	11.44	1.6889E-06	1.69E-06	***
HDAC1/2 f/f +mb-1 Cre	74.1	93.6	27.3	92.82	85.02	17.16			

Genotype		**Genotype**	**probability**	**significance**
Control	———	*HDAC1/2 f/f + mb-1 Cre*	1.69E-06	***

Supplemental Table 1. Sample sizes and statistical analysis of three-dimensional DNA FISH. The table details the percentages of cells counted in each category, the sample sizes and the χ^2 statistical significance in a pairwise analysis for the separation of IgH V_HJ588 and C_H gene segments (*A*) and pericentromeric recruitment (*B*). χ^2 statistical analysis was applied to observed and expected frequencies, based on the original data of cell numbers rather than percentages. Expected frequencies were calculated according to standard methods[1, 2] and χ^2 probabilities were calculated in Microsoft Excel.

1. Campbell, R. Statistics for Biologists 3rd edn. 107-117 (Cambridge University Press, 1989).
2. Hewitt, S.L. et al. Association between the *Igk* and *Igh* immunoglobulin loci mediated by the 3' *Igk* enhancer induces 'decontraction' of the *Igh* locus in pre-B cells. Nat Immunol 9, 396-404 (2008).

Supplemental Table 2 Yamaguchi et al.

A

HDAC1 qPCR	F: ACGGCATTGACGACGAATC	R: TAAGACCACTGCACTAGGCTGG
HDAC2 qPCR	F: CCAGAGGATGCTGTTCATGA	R: GCTATCCGTTTGTCTGATGCT
Gapdh qPCR	F: GAGGCCGGTGCTGAGTATGTCGTG	R: TCGGCAGAAGGGGCGGAGAT

B

HDAC1 flox or WT	F: CCTGTGTCATTAGAATCTACTT	R: GGTAGTTCACAGCATAGTACTT
HDAC1 delta	F: GTTACGTCAATGACATCGTCCT	R: GGTAGTTCACAGCATAGTACTT
HDAC2 flox or WT	F: CCCTTTAGGTGTGAGTACAT	R: AACCTGGAGAGGACAGCAAA
HDAC2 delta	F: CCACAGGGAAAAGGAAACAA	R: AACCTGGAGAGGACAGCAAA

C

E2A	F: GACGCCGAAGAGGACAAGAA	R: CAGGATGACCTGCACCGCCT
EBF	F: GCCCGTGGAGATTGAGAGGAC	R: GTGCTTGGAGTTATTGTGGAC
Ikaros	F: CACTACCTCTGGAGCACAGC	R: TCTGAGGCATAGAGCTCTTA
Pax5	F: CTGCGACATCTCCAGGCA	R: GACACTATGCTGTGACTG
Ezh2	F: GCCAGACTGGGAAGAAATCTG	R: TGTGCTGGAAAATCCAAGTCA
YY1	F: TCACCATGTGGTCCTCGGATGAAA	R: TGTCTCCGGTATGGATTCGCACAT
Gapdh	F: TGCACCACCAACTGCTTAG	R: TGGAAGAGTGGGAGTTGCTG
HDAC1	F: GTTACGTCAATGACATCGTCCT	R: GGTAGTTCACAGCATAGTACTT
HDAC2	F: TATTGTGCTTGCCATCCTCG	R: GCATCAGCAATGGCAAGTTA
Rag-1	F: TGCAGACATTCTAGCACTCTGGCC	R: ACATCTGCCTTCACGTCGATCCGG
Rag-2	F: CACATCCACAAGCAGGAAGTACAC	R: TCCCTCGACTATACACCACGTCAA
TdT	F: GAAGATGGGAACAACTCGAAGAG	R: CAGGTGCTGGAACATTCTGGGAG
DNA-PKc	F: AGCCGAGCTAACCGTACAGAAACA	R: AAGGCATCAACTCAGGGACTGGAA
Ku70	F: TCAAGCAAGCTGGAAGACCTGCTA	R: TGAACAAGGTTGAGCTCCCACTGA
Ku80	F: TCCTCCCTCGTTCATGCTTTGGAT	R: AGCAGACACTGGTACAATCGCTGA
Ig-α	F: TACCAAGAACCGCATCATCA	R: CATGTCCACCCCAAACTTCT
Ig-β	F: GCAGCCCCAGGAACTGGTCT	R: CCTCCATCCCAGCCTTGCCG
Lambda5	F: CTTGAGGGTCAATGAAGCTCAGAAGA	R: CTTGGGCTGACCTAGGATTG
VpreB	F: CGTCTGTCCTGCTCATGCTGC	R: ACGGCACAGTAATACACAGCC
IL-7R	F: TTACTTCAAAGGCTTCTGGAG	R: CTGGCTTCAACGCCTTTCACCTCA

D

V_HJ558	F: CGAGCTCTCCARCACAGCCTWCATGCARCTCARC	
V_H7183	F: CGGTACCAAGAASAMCCTGTWCCTGCAAATGASC	
D_H	F: TTCAAAGCACAATGCCTGGCT	
J_H3	R: GTCTAGATTCTCACAAGAGTCCGATAGACCCTGG	
Cμ	F: TGGCCATGGGCTGCCTAGCCCGGGACTT	R: GCCTGACTGAGCTCACACAAGGAGGA
Cμ transcript	R: ATGCAGATCTCTGTTTTTGCCTCC	
Cμ GLT	F: AAGGATGGGAAGCTCGTGGAATCT	R: TCAGGGTTTCATAGGTTGCCAGGT
V_HJ558 GLT	F: AAGGATGGGAAGCTCGTGGAATCT	R: TCAGGGTTTCATAGGTTGCCAGGT

Supplemental Table 2. List of primers used for quantitative PCR (*A*: **Fig. 18B**), genotyping (*B*: Fig. **18C**), RT-PCR (*C*: **Fig. 23**), VDJ recombination, μ transcripts and germline transcripts (*D*: **Fig. 21**)

2.2 Research publication (in preparation)

Histone deacetylase 1 is critical for the B cell terminal differentiation and adaptive immune reaction by regulating the proliferation of activated B cells

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Title for a running head: HDAC1 is critical for the immune reaction

Keywords: germinal center formation, B cell terminal differentiation, transcriptional regulation, histone deacetylase

Results

HDAC1 is dispensable for hematopoietic cell development

To explore the functions of HDAC1 in hematopoietic cell development, we generated HDAC1 conditional knock out mice in which exon 6 of the *HDAC1* gene is removed by Cre recombinase expressed specifically in hematopoietic cells. This exon has been shown to be essential for the deacetylase activity of HDAC1 (Hassig et al. 1998). Flox alleles of HDAC1 produce normal amounts of protein, and homozygous mice appear to be indistinguishable from their wild type (WT) littermates (data not shown). To inactivate the function of HDAC1 in hematopoietic cell lineages, we used the *Vav-iCre* transgenic mouse line which starts to express the codon-improved *Cre* (*iCre*) in the entire hematopoietic cell lineage (de Boer et al. 2003). First, we analyzed the efficiency of *flox* allele deletion by southern hybridization analysis. For this, DNA was isolated from bone marrow cells, splenocytes and thymocytes of mice heterozygous for the *HDAC1 flox* allele and either positive for *Vav-iCre* gene or negative, and mice homozygous for the *HDAC1 flox* allele and *Vav-iCre* gene ($HDAC1^{F/+}$, $HDAC1^{F/+}$::*Vav-iCre*, and $HDAC1^{F/F}$::*Vav-iCre*, respectively). After digestion by the restriction enzyme, DNA was blotted onto the membrane and hybridized by the radio-labeled probes which specifically recognize the DNA sequences on the *HDAC1* genomic locus. With this assay, we did not detect deleted alleles (Δ) of *HDAC1* in $HDAC1^{F/+}$ mouse DNA from all 3 cell types, whereas no *flox* (i.e. unrecombined) allele was seen in all 3 cell types of $HDAC1^{F/+}$::*Vav-iCre* and $HDAC1^{F/F}$::*Vav-iCre* mice (Fig. 25A). This result indicates that the recombination of the *HDAC1* locus is highly efficient and takes place at an early progenitor stage, resulting in the deletion of *HDAC1* in all hematopoietic lineages. We also analyzed by western blotting the level of HDAC1 proteins in same types of cells used in southern hybridization. As presented in Figure 25B, HDAC1 proteins were below detection in bone marrow, splenic and thymic cells from $HDAC1^{F/F}$::*Vav-iCre* mice, and no truncated form of HDAC1 protein was observed (data not shown). Interestingly, in the absence of HDAC1 protein, the amount of HDAC2 protein was found to be increased. Together, these results confirm the successful inactivation of HDAC1 in hematopoietic cell lineages and show that loss of HDAC1 leads to an increase in the level of HDAC2. These mice were next analyzed by flow cytometry (FACS) to determine the effect of ablating HDAC1 on hematopoietic cell development. For this,

a series of antibodies recognizing cell surface molecules were used to distinguish specific subsets of hematopoietic cell populations in bone marrow, spleen and thymus. B220 in combination with IgM were used to detect distinct B cell subpopulations, CD4 and CD8 were used for T cells, Mac1 and Gr-1 were used for myeloid cells, and TER119 and CD71 were used for erythrocytes. Apparently *HDAC1*$^{F/F}$::*Vav-iCre* mice have all hematopoietic cell populations normally represented in the bone marrow, spleen and thymus (Fig. 25C), although a slight reduction of Mac1^{+}Gr-1^{+} cells is reproducibly observed in the spleen. Thus, HDAC1 appears to be dispensable for the majority of hematopoietic cell development.

Impaired T cell dependent immune response in hematopoietic HDAC1 deficient mice

We next tested the functional implication of HDAC1 for the immune response. To check that, we measured the amount of immunoglobulin (Ig) secreted in the mouse serum. We collected sera from *HDAC1*$^{F/+}$::*Vav-iCre* and *HDAC1*$^{F/F}$::*Vav-iCre* mice and measured different isotypes of Ig by the Enzyme-Linked ImmunoSorbent Assay (ELISA) assay. Surprisingly we saw that all of class switched Ig isotypes examined; IgG1, IgG2a, IgG2b, IgG3 and IgA were reduced about 2-3 fold in the hematopoietic HDAC1 deficient mice, whereas no change was observed in the level of IgM (Fig. 26A). This result tempted us to consider the possibility that HDAC1 might be important for the immune response against exogenous antigens. To investigate this possibility, we carried out immunization experiments injecting intraperitneally the T cell dependent antigen, dinitrophenylated keyhole limpet haemocyanin (DNP-KLH). We collected sera weekly from immunized control and HDAC1 hematopoietic deficient mice until 3 weeks post-injection. At 21 day post-injection, mice were subjected again to an immunization to boost the immune reaction and sera were collected 6 days after the second injection. Thereafter we performed the ELISA experiments to measure the relative titers of specific antibodies against DNP (Fig. 26B). The level of specific IgM recognizing DNP-KLH was largely unchanged, whereas the level of IgG in sera of HDAC1 deficient mice was reduced. After the boost, this reduction of IgG antibody level was further enhanced. Histological analysis of HDAC1 deficient spleen showed an impaired formation of the germinal centers (GCs) (Fig. 26C). The defect in the GC formation was also confirmed by FACS analysis identifying cells harboring the GC-specific antigen, GL7. Collectively,

these data demonstrate that HDAC1 plays important roles to regulate the T-cell dependent immune response. Additionally, we observed that spleens from immunized HDAC1 deficient mice were enlarged, and this was observed at as early as 5 days post immunization. Splenic mass of hematopoietic HDAC1 deficient mice was c.a. 1.5 times heavier than the littermate control, and the number of splenocytes was as well increased c.a. 1.5 fold. Interestingly, the proportion of each hematopoietic cell subpopulation in the spleen was largely the same as in unimmunized mice, indicating that all populations were equally increased compared to the unimmunized condition (Supplementary Fig. 5). To investigate the proliferation rate of hematopoietic cells, we injected BrdU into immunized mice and investigated BrdU-incorporated cells (i.e. cells having replicated their DNA) in the spleen. To our surprise, the proportion of BrdU positive cells in splenic B and T cells of HDAC1 hematopoietic deficient mice was largely unchanged compared to WT littermates controls (Supplementary Fig. 5). This result suggests that the increased cell number in the peripheral hematopoietic cell compartments might be due to enhanced proliferation of progenitor cells such as common lymphoid progenitors (see discussion).

B cell intrinsic immune defect in HDAC1 deficient mice

To identify the cell lineage responsible for the defect observed in the T dependent immune response, we next used *HDAC1*$^{F/F}$::*mb1-Cre* mice, which specifically lack HDAC1 in the B cell lineage (Hobeika et al. 2006). As shown in Fig. 27A, the steady state level of Ig isotypes in mice lacking HDAC1 in B cells are reduced similarly as was seen in hematopoietic deficient mice. The DNP-KLH immunization experiments using B cell deficient mice showed a reduced titer of specific anti-DNP IgG, whereas there was no obvious change in the anti-DNP IgM titer (Fig. 27B). Moreover, we have carried out the same experiment using T cell specific HDAC1 inactivated mice, *HDAC1*$^{F/F}$::*lck-Cre*. However we did not observe any alteration of the T dependent immune response (Grausenburger *et al*. Manuscript in preparation). These results therefore strongly suggest that the impaired T dependent immune reaction described here is due to the lack of HDAC1 protein in the B cell lineage.

***In vitro* class switching and B cell proliferation are not abrogated in the absence of HDAC1**

Next we examined the possibility that the impaired adaptive immune response might be due to critical roles of HDAC1 for class switch recombination of the *Ig* gene. To assess that, we detected antibody secretion *in vitro* by culturing primary B cells under conditions allowing them to undergo class switching. Mature B cells were isolated from spleens of *HDAC1*$^{F/+}$::*Vav-iCre* and *HDAC1*$^{F/F}$::*Vav-iCre* mice, cultured for several days in the presence of bacterial lipopolysaccharide (LPS) with or without interleukin-4 (IL-4), and class switched antibody secretion in the medium was measured by ELISA. In the presence of LPS and IL-4, B cells undergo class switch recombination between the Cμ gene and the Cγ1 gene, which results in the production of IgG1 antibodies, whereas in the presence of LPS alone, B cells switch to Cγ3 and produce IgG3 antibodies. Surprisingly, as shown in Fig. 28A, the secreted IgG1 as well as IgG3 were more abundant in *HDAC1*$^{-/-}$ mature B cells than in control cells: IgG1 4 fold and IgG3 1.5 fold increased. This increased secretion was not due to a change in B cell proliferation in response to various mitogenic stimuli (Fig. 28B). These results thus suggest that HDAC1 functions as an inhibitory factor for class switch recombination, at least, *in vitro* situation.

Aberrant patterns of gene expression in the absence of HDAC1

To further investigate the cause of the impaired germinal center formation as well as the reduced immune response observed in the absence of HDAC1, we carried out microarray analysis using RNA isolated from mature B cells as well as germinal center cells of immunized mice. In the absence of HDAC1, the expression of 133 genes on the microarray was found to be up- or down-regulated more than 2 fold in HDAC1 KO mature B cells, whereas 1545 genes were deregulated in *HDAC1*$^{-/-}$ germinal center cells. Furthermore, 57 genes were commonly misregulated (Fig. 29A). Many transcription factors critical for GC formation, such as *Bcl6*, *Irf4*, *Irf8*, *Pax5*, *Nfkb1*, *Nfkb2*, *Prdm1* (encodes Blimp1) and *Xbp1*, were found to be unaffected (less than 1.7 fold changes) by the absence of HDAC1; however, *Irf4* was found to be up-regulated c.a. 2.1 fold. Moreover the essential gene for class switch recombination, *Aicda* encoding the AID protein, was shown to be c.a. 3.5 fold reduced in the *HDAC1*$^{-/-}$ GC cells (Fig. 29B). IRF4 plays a critical role in the noncycling centrocytes as well as in post–GC plasma cell development (Klein et al. 2006). IRF4 is present in mature B cells, absent from highly proliferating GC centroblasts and is then re-expressed in a

subpopulation of centrocytes as well as in plasma cells (Falini et al. 2000). Our expression analysis implies that in the absense of HDAC1, the *Irf4* gene fails to be repressed in the GC centroblasts. Next we clustered the expression patterns of misregulated genes from the microarray data to identify genes deregulated similary to the *Irf4* gene. We classified the expression profiles of the 1545 misregulated genes into 9 clusters based on relative expression in mature B cells and GC cells; the *Irf4* gene falls into cluster b. This cluster (307 genes) represents the set of genes that are highly expressed in WT mature B cells and are repressed in GC cells, but remain expressed in *HDAC1*$^{-/-}$ GC cells (Fig. 29C). This set of genes is particularly interesting, because these genes are actively down-regulated when mature B cells undergo differentiation into GC cells. Since HDAC1 may repress gene expression by modulating histone acetylation, these genes, therefore, might be potential direct targets of HDAC1. Cluster a and d contain genes that are also upregulated in *HDAC1*$^{-/-}$ GC cells, which therefore may be potential direct targets of HDAC1. Genes clustered in a, b and d were further analyzed through the use of Ingenuity Pathways Analysis (Ingenuity Systems®, www.ingenuity.com) and significantly relevant molecular and cellular functions associated with deregulated genes in each cluster are listed in Supplementary Figure 6A. Interestingly, all 3 clusters contain genes associated with cell cycle functions in a significantly high proportion. It implies that the potential targets of HDAC1 must be down-regulated in GC cells are highly represented in the category involved in the cell cycle regulation. In the Fig. 29D, The heat maps of transcriptional regulators, cell cycle associated genes and apoptosis/cell death associated genes from cluster b are shown. Importantly in cluster b, *E2f5* and *Cdkn1b* are found to be highly upregulated in *HDAC1*$^{-/-}$ GC cells. Both gene products, E2F5 and p27 are known to be important for the G1 cell cycle progression. Clusters c and e, containing genes failed to be activated in *HDAC1*$^{-/-}$ GC cells, show namely different functions such as lipid metabolism, molecular transport and small molecule biochemistry (Supplementary Fig. 6B). These facts indicate that the lack of gene repression in HDAC1 deficient GC cells may directly affect cell cycle regulation, which may trigger the failure of GC cell differentiation and the inappropriate expression of genes implicated in cellular metabolisms.

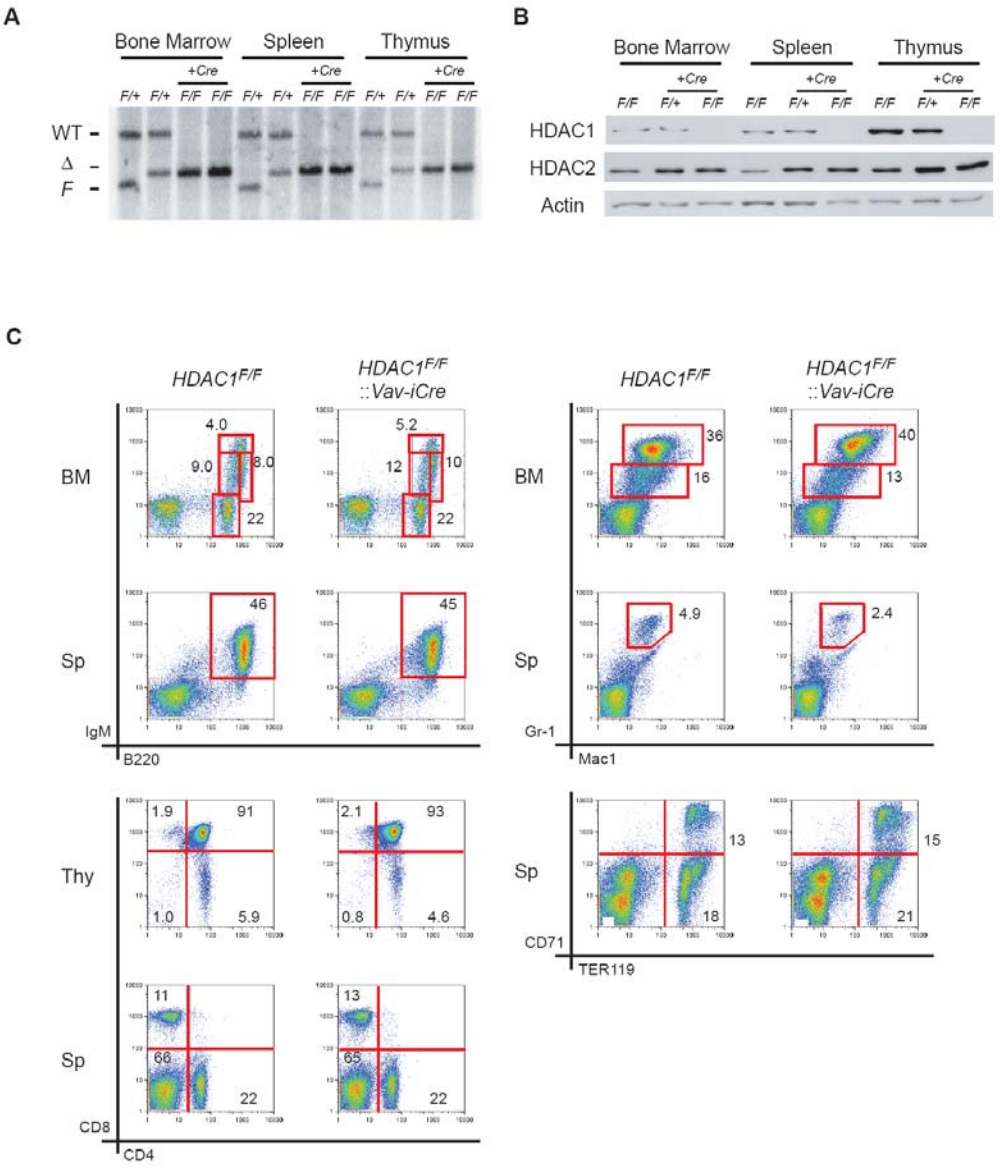


Figure 25. Conditional inactivation of HDAC1 in hematopoietic cell lineages. (A) Southern hybridization analysis of HDAC1 genomic alleles. DNA from mice of the indicated genotypes were digested with SacI, separated by gel electrophoresis, blotted on a membrane and detected by the specific probe. Modified alleles are represented as follows: *F*, *flox* allele; *Δ*, deleted (KO) allele and alleles corresponding to each genotype are indicated in the left. (B) Western blot analysis of HDAC1 KO mice. Protein lysates from cells from the bone marrow, spleen and thymus were detected by anti-HDAC1, HDAC2 and Actin antibodies, as indicated. (C) Flow cytometric analysis of *HDAC1$^{F/F}$::Vav-iCre* mice. Single cell suspensions from bone marrow, spleen or thymus were stained with the indicated antibody combinations and representative data are shown. Numbers in plots represent the percentage of cells in the respective gate or quadrant.

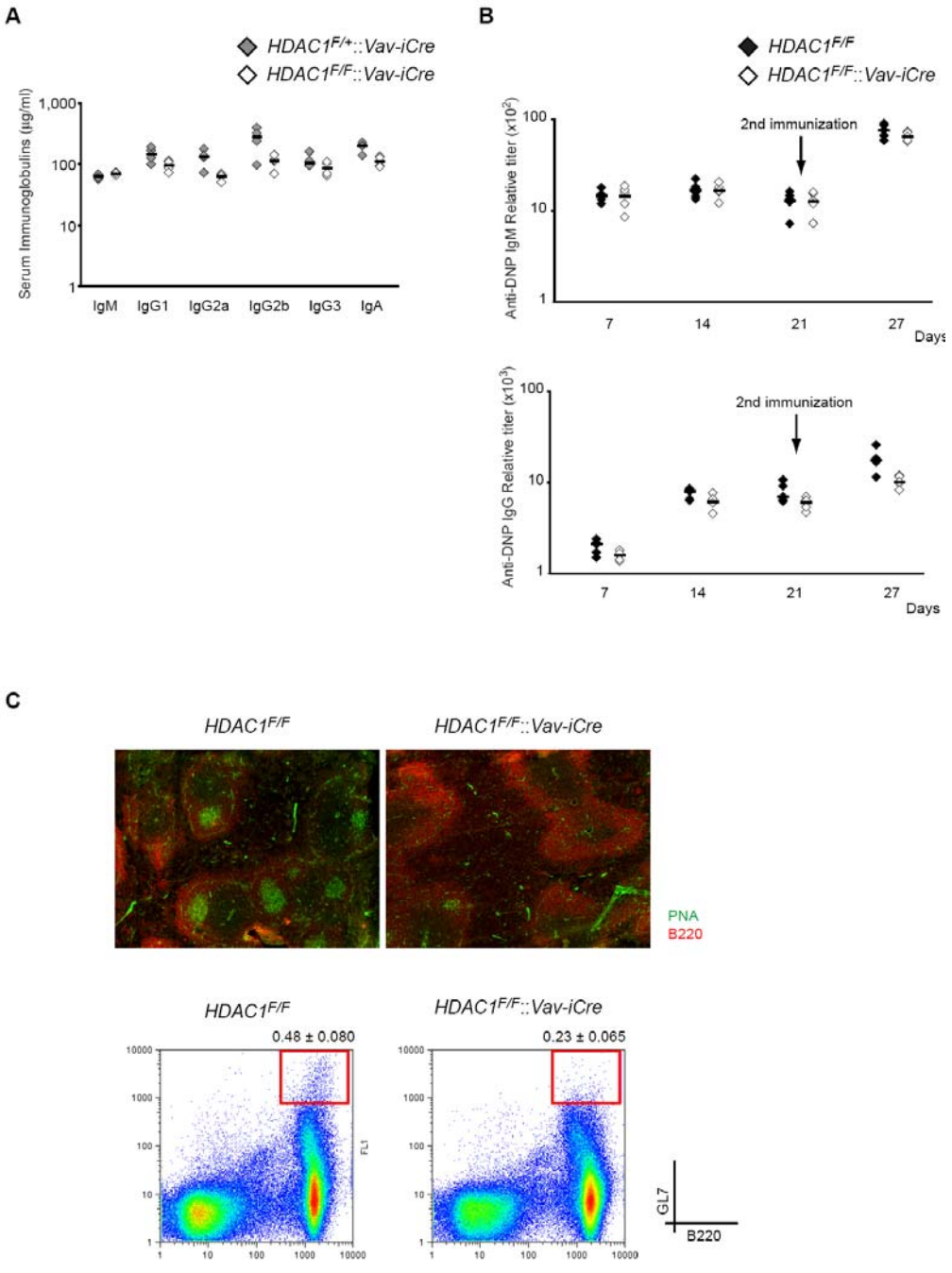


Figure 26. Impaired immune response and germinal center formation in HDAC1 hematopoietic deficient mice. (A) Serum immunoglobulin detection by ELISA. Sera were collected from 4 mice of each genotype and the concentration of each Ig isotypewas plotted. The median value of each genotype is indicated by black bars. (B) DNP-specific IgM and IgG titers. Five Mice of each genotype were immunized with DNP-KLH and sera were collected at the indicated days post injection. At 21 day, a second immunization was done. (C) Impaired germinal center formation. Frozen sections of spleens were stained with anti-B220-PE labeled antibody and biotinylated-peanut agglutinin (PNA) (Top). Streptavidin-FITC was used for the detection of PNA. FACS analysis of germinal center cells (bottom). Single cell suspensions from immunized mouse spleen were stained with anti-B220 and anti-GL7 antibodies. Numbers in plots represent the average percentage of cells and S.E. in the gate (N=3; P=0.016).

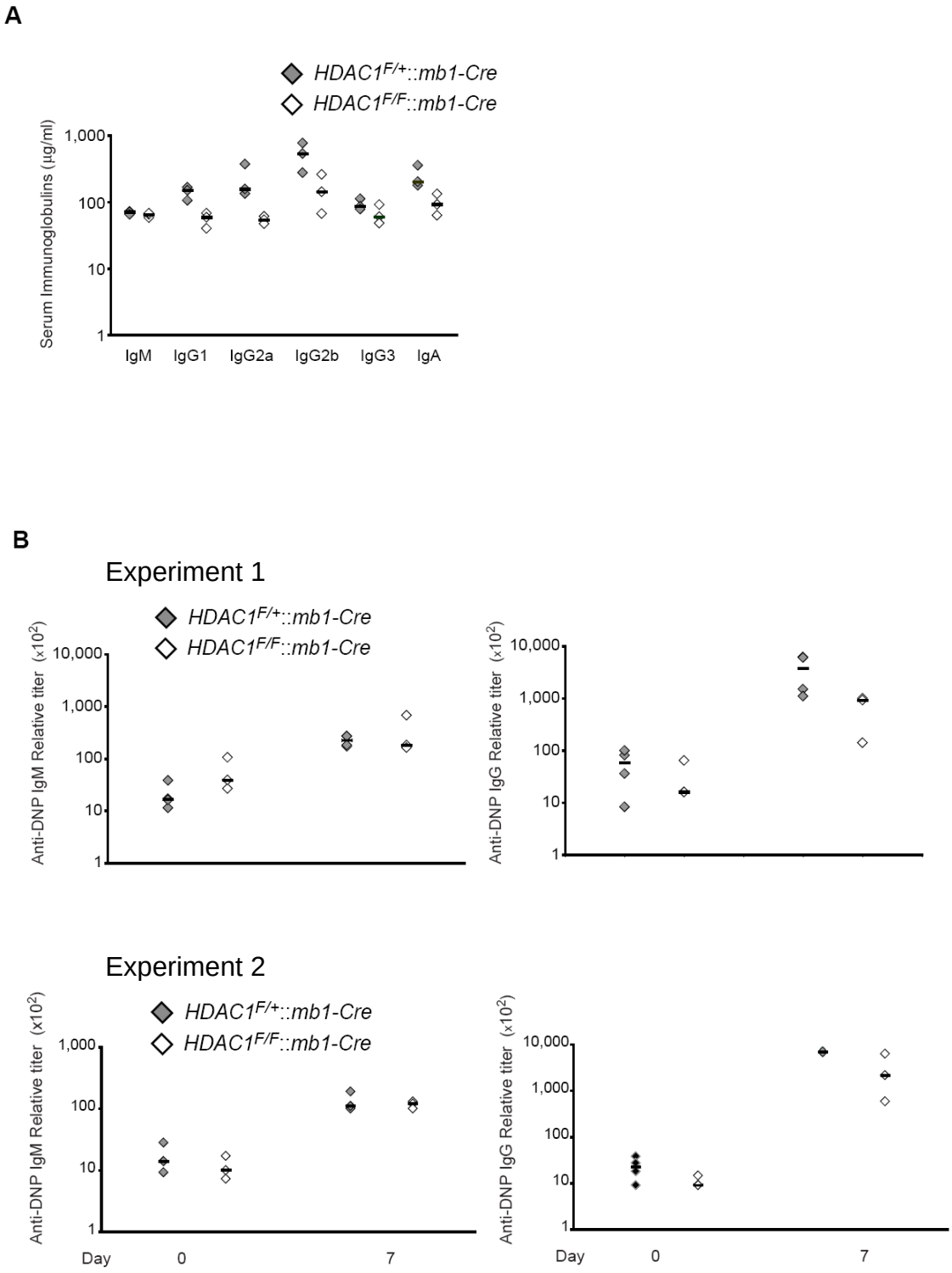


Figure 27. Impaired immune response in HDAC1 B cell deficient mice. (A) Serum immunoglobulin detection by ELISA. Sera were collected from 3 mice of each genotype and each isotype concentration was plotted. The median value of each genotype was indicated as a black bar. (B) DNP-specific IgM and IgG titers. More than 3 mice of each genotype were immunized by DNP-KLH and sera were collected at listed days post injection. The results from 2 independent experiments are shown.

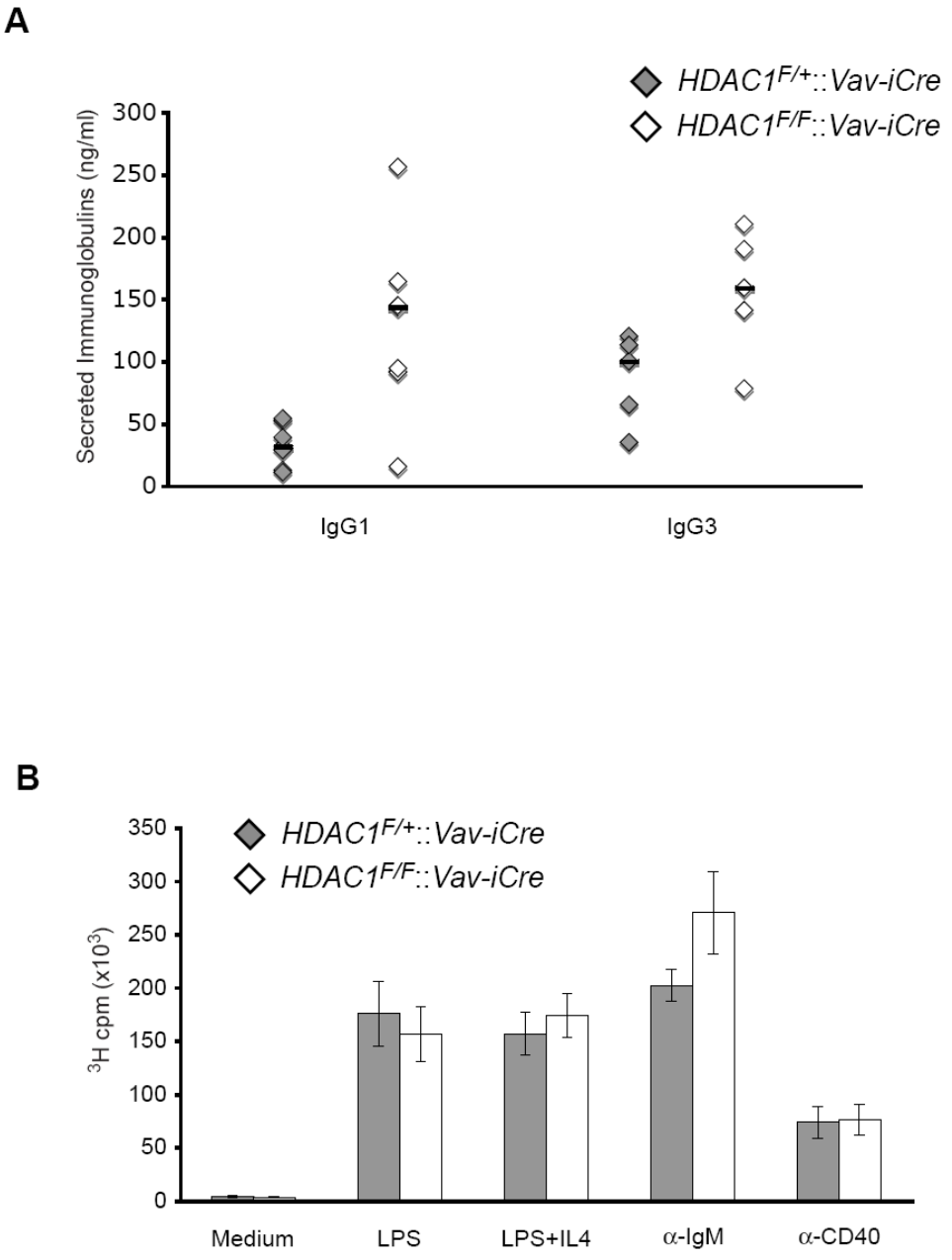


Figure 28. Enhanced secretion of IgG1 and IgG3 and normal proliferation of HDAC1 inactivated mature B cells *in vitro*. (A) Secreted immunoglobulin detection by ELISA. The supernatants from B cell cultures were collected and IgG1 and IgG3 concentration was measured. The median value of each genotype is indicated as black bars. (B) ^{3}H-thymidine incorporation assay after 3 days culture *in vitro*. Mature B cells were cultured with the indicated mitogens and ^{3}H-labeled thymidine was added in the medium 24h before harvesting.

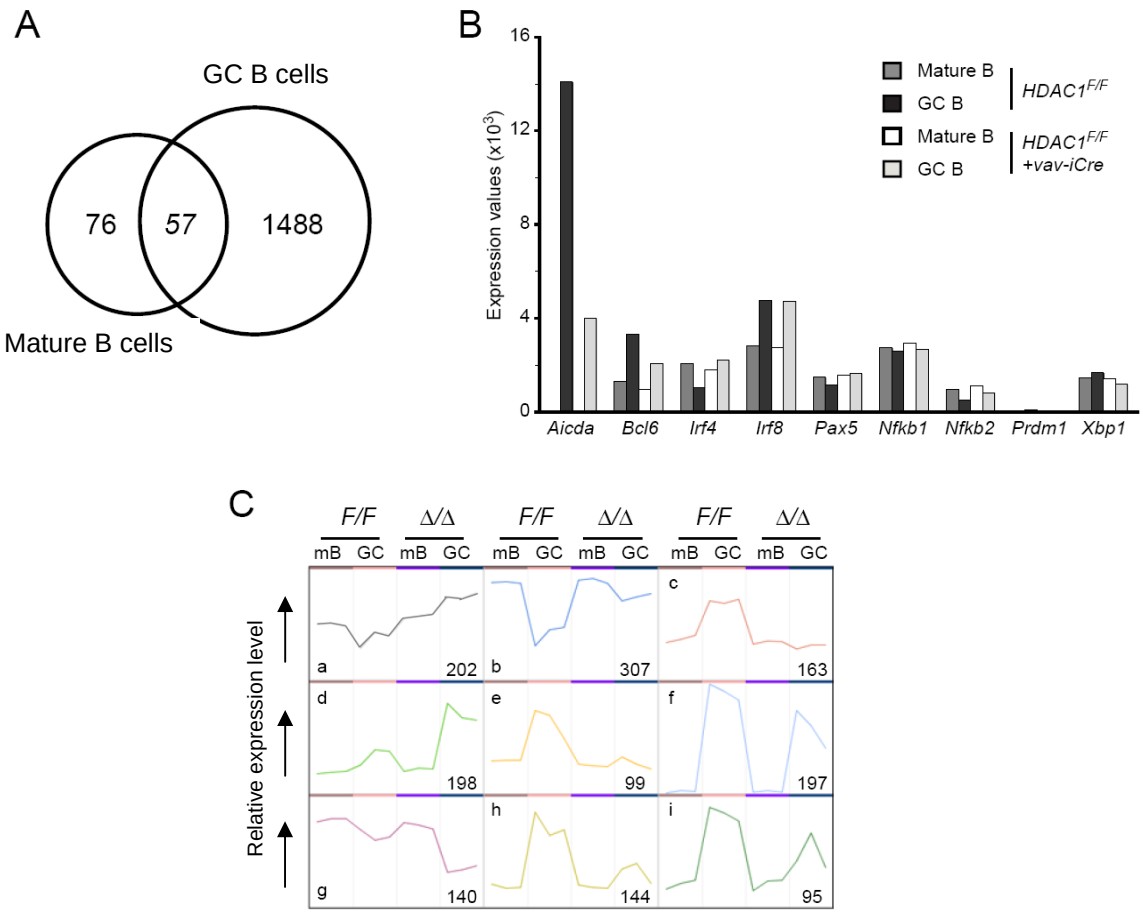


Figure 29. Microarray analysis of mature B cells and GC B cells. RNA was isolated from mature B cells and GC B cells from spleens of immunized mice at 10 days post injection (N=3 for each genotype). (A) The numbers of misregulated genes (more than 2 folds changes) in HDAC1 deficient mature B cells and GC B cells are shown. (B) Expression value for *Aicda* and key TFs in mature and GC B cells of each genotype. (C) Clustering of genes misregulated in HDAC1 KO GC B cells. All genes misregulated are grouped into 9 types of expression patterns represented in the panel (N=3 per genotype and condition). A high expression value is indicated in the upper side of each panel and a low expression value is indicated close to the bottom side. Numbers in the bottom-right of each panel indicate the number of genes in the corresponding cluster. For example, cluster b contains 307 misregulated genes. These genes are highly expressed in mature B cells and downregulated in GC cells from control mice, whereas the same genes are highly expressed in mature B cells and remain expressed in GC cells from HDAC1 KO mice.

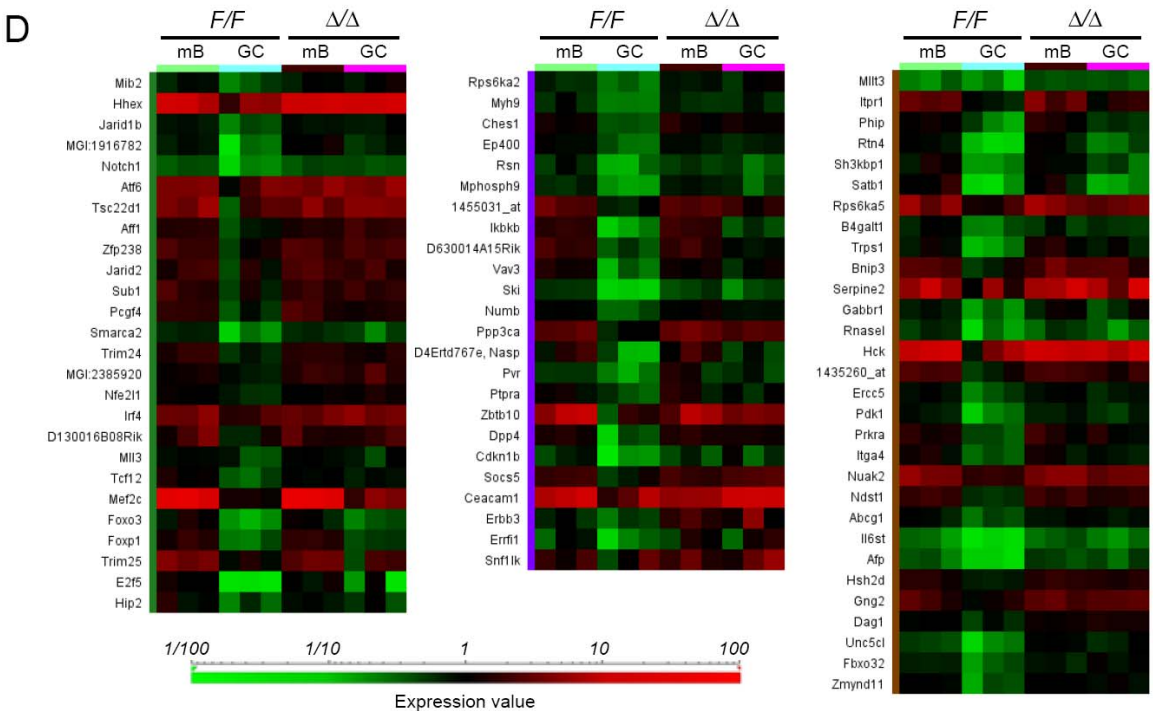
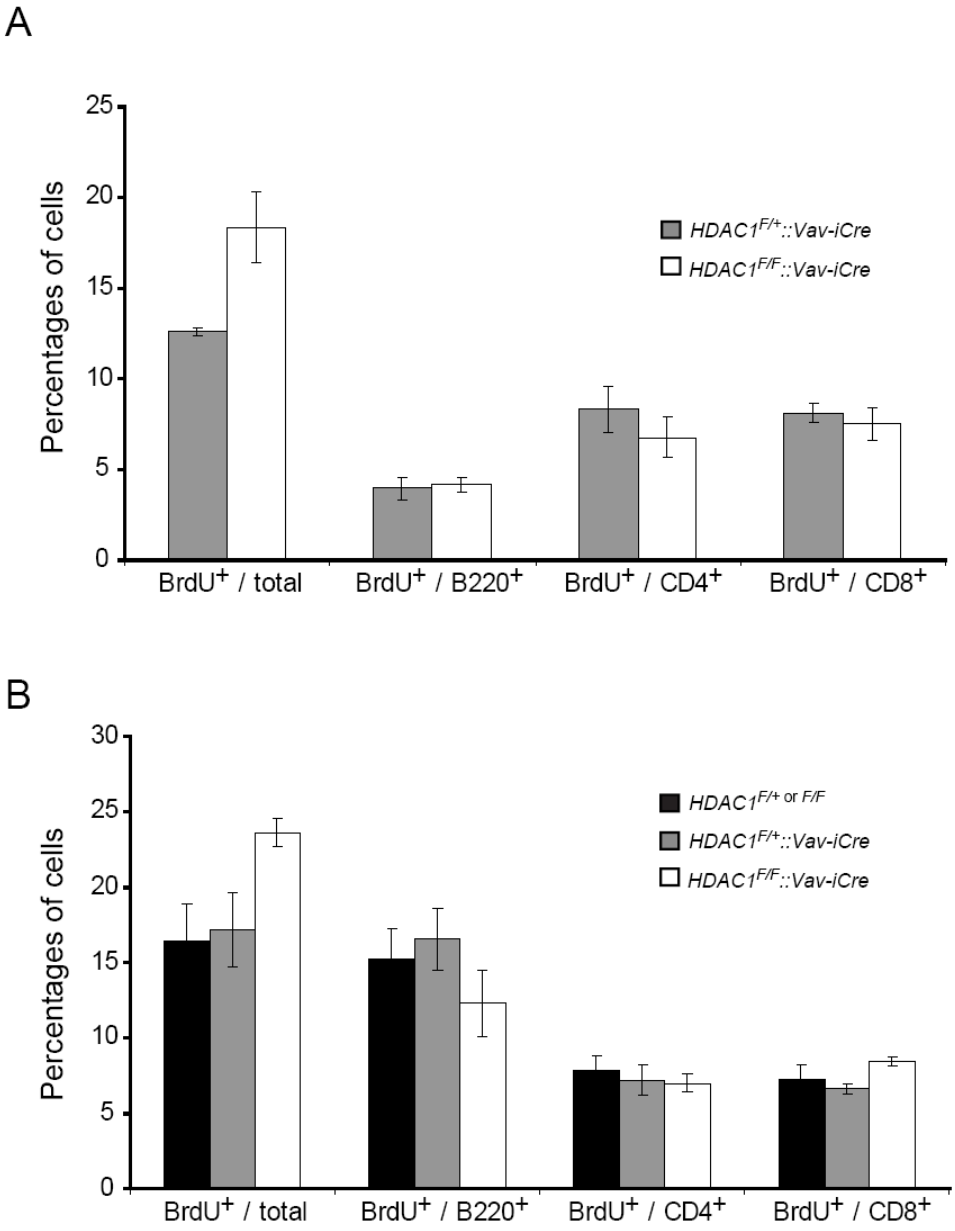


Figure 29. Microarray analysis of mature B and GC B cells. RNA was isolated from mature B cells and GC B cells from spleens of immunized mice at day 10 post injection (N=3 for each genotype). (D) Heat map analysis of particular genes in cluster b. Relative expression values are used, which are indicated by the colors in the bottom. The left heat map shows genes defined or predicted to be transcriptional regulators. The middle heat map present genes involved in cell cycle regulation. The right heat map shows genes involved in apoptosis and cell death processes.



Supplementary Figure 5. BrdU incorporation assay using DNP-KLH immunized mice. BrdU were injected into immunized mice (2x 1mg / a day) continuously post immunization day 1-4 (A) and 5-9 (B). $BrdU^+$ cells were stained together with cell surface markers: B220, CD4 and CD8. Percentages of $BrdU^+$ cells within each population were plotted. Three mice of each genotype were examined. The error bar represents S.E..

A

Cluster a

Relevant Function & Diseases	p-value	Numbers of genes (100 total)
Cancer	1.00E-07 - 1.95E-02	51
Gene Expression	2.10E-05 - 1.95E-02	30
Cell Mophology	2.34E-05 - 1.95E-02	30
Cellular Development	2.34E-05 - 1.95E-02	38
Cellular Growth and Proliferation	5.80E-05 - 1.90E-02	43

Cluster b

Relevant Function & Diseases	p-value	Numbers of genes (134 total)
Cell Death	4.79E-06 - 2.71E-02	58
Cell Cycle	6.23E-05 - 2.84E-02	32
Amino Acid Metabolism	7.93E-05 - 1.43E-02	21
Post-Translational Modification	7.93E-05 - 1.43E-02	34
Small Molecule Biochemistry	7.93E-05 - 2.13E-02	25

Cluster d

Relevant Function & Diseases	p-value	Numbers of genes (69 total)
Gene Expression	4.85E-05 - 2.09E-02	9
Cell Cycle	4.47E-04 - 2.09E-02	10
Cancer	4.79E-04 - 2.09E-02	29
Cell Mophology	4.79E-04 - 2.09E-02	17
Immnological Disease	4.79E-04 - 2.09E-02	4

B

Cluster c

Relevant Function & Diseases	p-value	Numbers of genes (69 total)
Cancer	1.02E-04 - 3.38E-02	24
Hematological Disease	1.02E-04 - 2.55E-02	19
Immnological Disease	1.27E-04 - 3.03E-02	19
Hematological System Development and Function	1.61E-05 - 3.38E-02	26
Immune and Lymphatic System Development and Function	1.61E-05 - 3.36E-02	26

Cluster e

Relevant Function & Diseases	p-value	Numbers of genes (47 total)
Lipid Metabolism	1.51E-05 - 2.65E-02	10
Small Molecule Biochemistry	1.51E-05 - 2.65E-02	13
Cellular Assembly and Organization	2.83E-05 - 2.13E-02	11
Cellular Compromise	4.84E-05 - 2.13E-02	6
Cellular Movement	4.19E-04 - 2.59E-02	6

Supplementary Figure 6. Ingenuity Pathways Analysis revealing significantly relevant molecular and cellular functions associated with disregulated genes in the absence of HDAC1. (A) The lists of relevant functions and diseases are associated with cluster a, b and d which are highly upregulated in HDAC1 KO GC cells. (B) The lists of relevant functions and diseases are associated with cluster c and e which are downregulated in HDAC1 KO GC cells.

DISCUSSION

3.1 Histone deacetylase 1/2 functions in B cells

3.1.1 Misregulated genes in HDAC1/2 inactivated pre-BI cells

We have shown here that HDAC1 and 2 are essential factors for early B cell development as well as V(D)J recombination by regulating the contraction and pericentromeric recruitment of the IgH locus. In the absence of HDAC1 and 2 at the pre-BI cell stage, cells show largely normal proliferation and apoptosis but are reduced about 4-5 fold in numbers. To find out other potential defects in B cell development other than the impairment of V(D)J recombination, we have carried out a microarray analysis of pre-BI cells. Pre-BI cells were isolated by FACS sorting using the following cell surface molecules; $B220^{+}CD19^{+}CD43^{+}CD25^{-}$. *HDAC1/2*$^{F/F}$ *+mb-1 Cre* mice and their littermate control (*Flox* allele mice without the *Cre* gene) were used for the experiment. The summary of the microarray analysis is shown in Appendix 8.4.

In total, 747 genes are deregulated in the absence of HDAC1/2 (P<0.05 and 2 fold change). Ingenuity Pathways Analysis (Ingenuity Systems®, www.ingenuity.com) was used to classify the relevant molecular and cellular functions associated with deregulated genes and a list is presented in Appendix 8.4. Many deregulated genes are classified into the functions such as Cell Death and Cellular Growth and Proliferation although HDAC1/2 inactivated pre-BI cells show normal cell viability. This suggests that these cells look phenotypically normal, however genes associated with cell death and proliferation start to be misregulated. It should be pointed out that this clustering of genes based on their function does not separate positive functions or negative functions; thus, for example, pro-apoptotic and anti-apoptotic factors are both categorized in the Cell Death group. In fact, the best characterized HDAC1 target, *p21*, which is crucial to inhibit the cell cycle, was not misregulated in HDAC1/2 abrogated pre-BI cells. This indicates that these cells are likely not yet blocked in the cell cycle progression.

In addition to the genes categorized to the cell viability group, a high proportion of deregulated genes is associated with lymphocyte development, as listed in Appendix 8.4. I summarize below several potential direct targets of HDAC1 and 2 at the pre-BI cell stage. Since HDACs are well characterized as corepressors, I mainly discuss genes highly upregulated in HDAC1/2 mutant cells.

Flt3

The tyrosine kinase receptor Flt3 (also called flk2 and CD135) is up-regulated during early hematopoietic cell differentiation, concomitantly to a reduction of self-renewal capacity of the cells (Adolfsson et al. 2001). The earliest $Flt3^+$ population described so far is the lymphoid-primed multipotent progenitor (LMPP) and its expression remains until cells fully commit to the B lineage ($CD19^+$ pre-BI cells) (Nagasawa 2006; Iwasaki and Akashi 2007). Even though these $Flt3^+$ progenitors have a lymphoid lineage short-term reconstitution potential, they can also give rise to, at the single-cell level, the myeloid lineage (Adolfsson et al. 2001). *In vivo,* the functions of Flt3 or the Flt3 ligand have been further elucidated in studies of gene inactivated mice; in these mice a reduction in early B and T cell progenitors as well as common lymphoid progenitors (CLPs) is observed (Veiby et al. 1996; McKenna et al. 2000; Sitnicka et al. 2002). Although Flt3 and its ligand are essential for early B cell progenitors, repression of Flt3 in pre-BI cells is crucial for B cell lineage commitment. In the absence of Pax5, the key transcription factor for B-cell lineage commitment, B cells are blocked at the pro-B cell stage with a high level of Flt3 expression (Holmes et al. 2006). These $Pax5^{-/-}$ pro-B cells maintain the potency to differentiate into multiple hematopoietic lineages (Nutt et al. 1999; Rolink et al. 1999). Furthermore, enforced expression of *Flt3* in bone marrow stem cells selectively inhibits B cell differentiation and biases commitment to the myeloid lineage (Holmes et al. 2006). Repression of *Flt3* is inversely correlated to the up-regulation of *CD19* gene expression, by which Pax5 regulates down- and up-regulation by directly binding to the promoters of both genes (Holmes et al. 2006). Thus several functions of Flt3 are essential for early lymphocyte progenitors and repression of *Flt3* in committed B cells, mediated by Pax5, has critical roles to specify the cell fate towards B cell lineage.

Tal1

Stem cell leukemia/T cell acute leukemia 1 (SCL/Tal1) is a member of the basic helix-loop-helix (bHLH) transcription factors. During hematopoietic cell differentiation, Tal1 is expressed mainly in erythroid cells, megakaryocytes, and hematopoietic stem cells (HSCs) and its expression decreases with differentiation in all lineages. Tal1 is essential for blood cell development in the yolk sac (Kallianpur et al. 1994; Robb et al. 1995; Shivdasani et al. 1995; Elefanty et al. 1998). In the B cell lineage, *Tal1* expression is the highest in pro-B cells, whereas its expression is more

than 5 fold reduced in committed pre-BI cells (Herblot et al. 2002). In addition ectopic expression of *Tal1* blocks B cell development at the pro-B cell stage and cell numbers in later stages from pre-BI (Hardy fraction B) to mature B (Hardy fraction F) are decreased. Thus sustained up-regulation of *Tal1* in committed B cells inhibits B cell development.

Cebpa

Cebpa encodes the transcription factor C/EBPα belonging to the CCAAT enhancer binding protein (C/EBP) family (Ramji and Foka 2002). In the hematopoietic system, *Cebpa* expression is detected in myeloid cells and C/EBPα activates myeloid specific genes such as granulocyte colony stimulating factor (G-CSF), macrophage CSF (M-CSF) and granulocyte–macrophage CSF (GM-CSF) (Hohaus et al. 1995; Smith et al. 1996; Zhang et al. 1996). In addition, in the absence of C/EBPα cells fail to undergo myeloid differentiation and generate mature neutrophils, whereas no defect has been shown in B cell development (Zhang et al. 1997). These studies strongly suggest that C/EBPα plays a role in myeloid cell development. Interestingly, enforced expression of C/EBPα and its homolog, C/EBPβ in differentiated B cells leads to their rapid and efficient reprogramming into macrophages. C/EBPs induce this trans-differentiation by inhibiting Pax5, which results in the down-regulation of the Pax5 target CD19 and up-regulation of Mac-1 and other myeloid markers (Xie et al. 2004). Moreover the full reprogramming of terminally differentiated mature B cells into induced pluripotent stem cells (iPS cells) requires introducing C/EBPα in addition to the "conventional" set of 4 transcription factors, Oct4, Sox2, Klf4, and c-Myc (Hanna et al. 2008). Thus C/EBPα, if expressed in the B cell lineage, antagonizes the functions of Pax5.

Csf2rb2

Colony stimulating factor 2 receptor, beta 2, low-affinity (granulocyte-macrophage), is the common subunit of GM-CSF, IL-3 and IL-5 receptors, which is critical for eosinophil differentiation but not essential for B cell differentiation (Nishinakamura et al. 1996).

Mlf1

Myeloid leukemia factor 1 (MLF1) was first discovered as the leukemic fusion protein generated by some chromosomal translocations (Yoneda-Kato et al. 1996). MLF1 expression is preferentially detected in non-committed progenitor cells and declines in more lineage-restricted cells during hematopoietic cell development (Matsumoto et al. 2000). Enforced *Mlf1* expression impairs the development of erythroid colonies in normal hematopoietic precursors and differentiation to erythrocytes in an erythropoietin-dependent cell line (Williams et al. 1999). Thus Mlf1 can have an influence on commitment to the myeloid lineage.

Meis1

The *Meis1* gene locus was first discovered as a common viral integration site in myeloid leukemic cells of BXH-2 mice and Meis1 is critical for the MLL protein-induced leukemogenesis (Moskow et al. 1995; Wong et al. 2007). Though Meis1 is clearly crucial for the MLL dependent leukemogenesis, the role of Meis1 in normal hematopoiesis remains unclear. *Meis1* is expressed in the most primitive HSCs and is down-regulated following differentiation (Pineault et al. 2002). Moreover *Meis1*-deficient mice show embryonic lethality by E14.5, presenting with extensive hemorrhaging due to the lack of megakaryocytes (Hisa et al. 2004).

Ndn

Necdin is a nuclear protein which promotes neuronal differentiation and survival through the regulation of p53 acetylation by facilitating the interaction between Sirt1 and p53 (Maruyama et al. 1991; Aizawa et al. 1992; Hasegawa and Yoshikawa 2008). In mammals, the *Ndn* gene locus is maternally imprinted and expressed in the wide range of tissues except in peripheral blood leukocytes (Jay et al. 1997; MacDonald and Wevrick 1997). Loss of necdin show abnormalities and apoptosis of central and peripheral neurons (Muscatelli et al. 2000; Ren et al. 2003).

Hmga2

The High Mobility Group A (HMGA) family of proteins are abundant non-histone chromatin associating factors that bind to DNA and alter the chromatin structure. HMGA2 is one of two members of the HMGA proteins which have three AT-hook domains that bind the minor groove of AT-rich DNA sequences (Reeves et al. 2001). HMGA proteins are involved in many essential biological processes, including

regulation of gene expression, cell cycle, and differentiation. HMGA proteins are often associated with gene active loci and have positive effects on cell proliferation (Reeves et al. 2001). Therefore, HMGA2 is abundant in highly proliferating cells, undifferentiated progenitor cells and its expression is gradually decreased during terminal differentiation (Zhou et al., 1995). A recent paper described that HMGA proteins facilitate senescence-associated heterochromatic foci formation in concert with the p16 tumor suppressor and stabilize senescence by repressing proliferation-associated genes (Narita et al. 2006).

In B lymphocytes, HMGA1 functions as a co-activator of the Ets family TFs required for the IgH μ enhancer (iEμ) activity. HMGA1 interacts with the Ets factor PU.1 and enhanced transcriptional synergy with Ets-1 on the iEμ (Andreucci et al. 2002; McCarthy et al. 2003). Thus it may be interesting to test the binding of HMGA2 on the *IgH* locus, since HMGA proteins are known to regulate the higher order of chromatin structure.

Tcf3

Tcf3 is the HMG box transcription factor 3, which encodes a protein with a DNA binding domain and a ß-catenin interaction domain. Through activation of the Wnt signaling pathway, β-catenin protein is stabilized and can associate with the Tcf/Lef1 family of DNA binding proteins to activate downstream target genes. WNT signaling is essential for various biological processes, such as progenitor cell survival, cell-fate determination, establishment of the dorsal axis and control of asymmetric cell division (Staal et al. 2008). In both T and B cells, WNT signaling is crucial for differentiation as well as TCRα gene expression mediated by Lef1 and Tcf1 (Okamura et al. 1998; Reya et al. 2000; Timm and Grosschedl 2005). In contrast, complete loss of Tcf3 results in gastrulation defects and impairment in hair stem cells; therefore Tcf3 is proposed to function to maintain an undifferentiated state in the absence of Wnt and, through Wnt activation, it directs stem cells to the hair lineage (Kim et al. 2000; Nguyen et al. 2006).

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3.1.2 Developmental defects in HDAC1/2 KO pre-BI cells

Three factors shown to be critical for the development of "Hematological System Development" are up-regulated in HDAC1/2 deficient pre-BI cells: *Flt3*, 15.8 fold; *Tal1*, 3.91 fold; *Cebpa*, 3.62 fold. Importantly these genes are normally detected at very low levels in B-lineage committed pre-BI cells and their primary functions are found in early progenitor stages and non-B cell lineages. Moreover, enforced expression of these genes leads to the blockade of B cell development. Thus, HDAC1/2 inactivated pre-BI cells might not fully commit to the B lineage, as HDAC1/2 are required to repress genes involved in the maintenance of the multi-potency and development into non-B cell lineages. Therefore these mutant pre-BI cells may not be fully equivalent to the pre-BI cells present in the WT situation. An alternative possibility is that loss of HDAC1/2 initiates *CD19* gene expression earlier than at the "usual" pre-BI cell stage, i.e. in pro-B cells. In pro-B cells, expression of the genes identified in the microarray experiment is higher than in pre-BI cells. This interpretation is, in theory, also possible, although unlikely; since we know that the deletion of *HDAC1/2* flox allele in pro-B cells is not as complete as in pre-BI cells. In addition, another important gene in the pre-BI cell stage, Pax5, is expressed as highly as in WT cells. Thus, we think that the first possibility which HDAC1/2 KO pre-BI cells fail to shut down expression of certain genes is more likely. So far we do not know whether these deregulated genes are the cause of the developmental blockade observed in HDAC1/2 B cell KO mice or affect recombination and contraction of the *IgH* locus. To address this, we need to knock down their expression to see whether this rescues some of the phenotype.

3.2 Histone deacetylase 1 functions in the hematopoietic system

We have shown that HDAC1 is largely redundant for normal hematopoiesis but has crucial roles in the T cell dependent immune response and GC cell differentiation by regulating genes involved in cell cycle regulation. In the absence of HDAC1 in the hematopoietic compartments, the amount of IgG is reduced which is in line with impaired formation of GCs. This defect is due to HDAC1 B cell-intrinsic functions, as was confirmed by the *in vivo* conditional inactivation of HDAC1 specifically in the B cell lineage. Using transcriptome analysis, we found that genes that must be repressed during GC cell differentiation are not properly controlled in the absence of HDAC1, These genes are enriched for genes involved in cell cycle control and apoptosis.

Several genes are highly upregulated in HDAC1 mutant GC cells such as *Irf4* or are important for the G1 cell cycle progression such as *E2f5* and *Cdkn1b*, encoding p27 (Fig. 30). Centroblasts in the GC dark zone undergo extremely fast proliferation; the time for one cell cycle ranges from 6 to 12 hours (Klein and Dalla-Favera 2008). Therefore the transition from resting/non-cycling mature B cells to highly proliferating centroblasts requires a dramatic change in gene expression ensuring the ability to complete cell cycle as fast as they should be (Klein et al. 2003).

3.2.1 Gene repression by Bcl6 during GC formation

Bcl6 is a key transcriptional repressor which control groups of genes that function in the cell cycle regulation and lymphocyte differentiation upon induction of the GC formation. Mice lacking Bcl6 contain normal proportions of B cell subpopulations, however show defects in T cell dependent immune responses, which is due to the failure of mature B cells to proliferate and form GCs (Ye et al. 1997). Basal serum levels of Ig isotypes as well as the antigen-specific antibody production induced by the T cell dependent response are modestly reduced in Bcl6 mutant mice. Interestingly the reduction of antigen-specific Ig isotypes ranges from a modest effect on IgM to more severe effects on all IgGs, which is similar to defects observed in HDAC1 mutant mice. This phenotypic similarity implies the possibility that Bcl6 and HDAC1 might work together to repress genes involved in the formation of GCs and in T-dependent antibody responses. Several reports demonstrated protein-protein interaction between HDAC1 and Bcl6, which contributes to sequence-specific transcriptional repression via the Bcl6 DNA binding activity (Dhordain et al. 1998; Bereshchenko et al. 2002). In addition, acetylation of Bcl6 inactivates its repressive activity by dissociating it from HDACs (Bereshchenko et al. 2002). Taken together, the loss of HDAC1 may alleviate the Bcl6 gene repressive activity in a two steps mechanism: Bcl6 itself is hyper-aectylated and looses its co-represssor partner, HDAC1. Several genes have been identified as repressed targets of Bcl6, which may account for severe defects in proliferation and GC formation of Bcl6 mutant mice (discussed in the next topic) (Shaffer et al. 2000; Phan et al. 2005).

3.2.2 Upregulated genes in HDAC1 mutant GC cells

Cdkn1b is one of the potential direct targets of Bcl6, whose expression was detected to inversely correlate to the presence of Bcl6 (Shaffer et al. 2000). *Cdkn1b* encodes a

cyclin dependent kinase (Cdk) inhibitor protein, $p27^{kip1}$, which inhibits the catalytic activity of Cdk4 and arrests the cell cycle at the G1 phase (Polyak et al. 1994). Therefore highly proliferative centroblasts express no detectable level of $p27^{kip1}$ (Quintanilla-Martinez et al. 1998). Our microarray analysis identified that *Cdkn1b* expression in WT mice is highly abundant in resting/non-cycling mature B cells and reduced in vigorously dividing GC cells. In sharp contrast, in HDAC1 inactivated GC cells *Cdkn1b* expression remains as high as in mature B cells. This upregulation of *Cdkn1b* may inhibit progression through the cell cycle, which results in the failure of proliferation and subsequent B cell terminal differentiation. In addition to known Bcl6 target genes, the E2F transcription factor 5, *E2f5* was also identified as a gene that is expressed at low level in WT GC cells (33 fold reduction in WT GC cells compared to WT mature B cells) but 13 fold higher expression in HDAC1 mutant GC cells (only 2.4 fold reduction in KO GC cells compared to KO mature B cells). E2F5 belongs to the transcriptional repressor group of the E2F family and inactivates genes required for the cell cycle progression whereas E2F1, 2 and 3a function as transcriptional activators to enhance expression of these genes and promote the G1

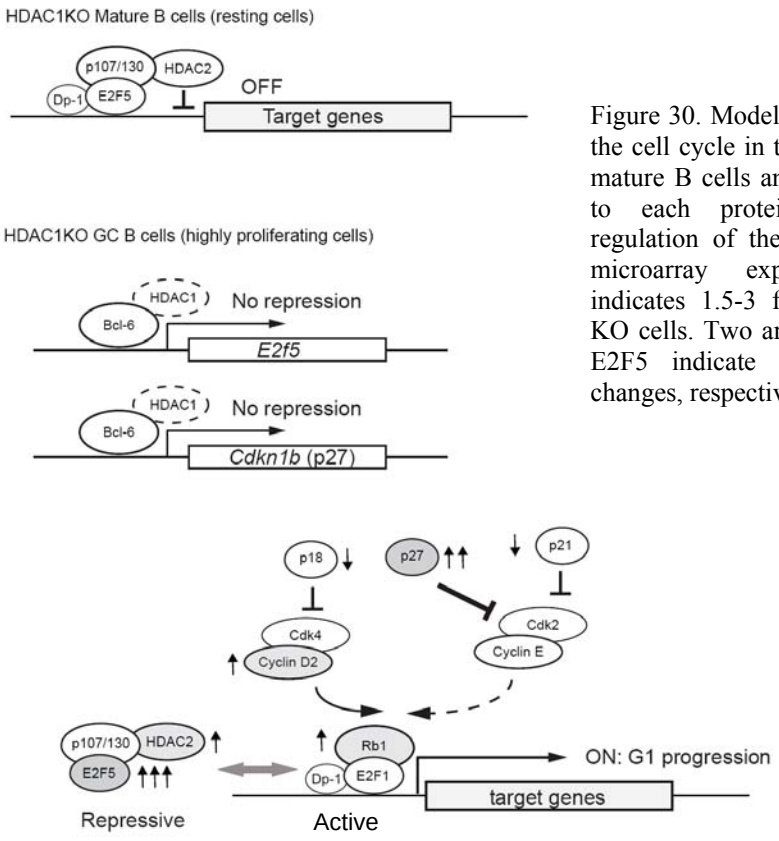

Figure 30. Model of the G1-S transition of the cell cycle in the absence of HDAC1 in mature B cells and GC cells. Arrows next to each protein represent up/down-regulation of the mRNA taken from the microarray experiment. One arrow indicates 1.5-3 fold changes in HDAC1 KO cells. Two arrows in p27, 3 arrows in E2F5 indicate 3.8 fold and 13 fold changes, respectively.

phase of the cell cycle (McClellan and Slack 2007; Sun et al. 2007). Both E2F4 and E2F5 are expressed throughout the cell cycle as well as in quiescent (G0) cells (Sardet et al. 1995; Moberg et al. 1996). E2F5 associates with tumor suppressor gene products; pRb2/p130 or pRb/p107, which facilitate the translocation of E2F5 into the nucleus and the binding to various promoter regions. This E2F5-p107/p130 complex recruits chromatin remodelers and modifiers such as SWI/SNF and HDAC1/2 to repress gene expression in G0 cells (Ferreira et al. 1998; Stiegler et al. 1998; Iavarone and Massague 1999). HDAC1 mutant GC cells contain abundant E2F5 which may inhibit the transition from the G0/G1 phase of the cell cycle to the S phase by repressing genes essential for the G1/S transition (Fig. 30).

3.2.3 Phenotypic difference between in vivo and *in vitro* activation of mature B cells

Surprisingly, HDAC1 inactivated mature B cells show largely normal proliferation and higher production of IgG1 and IgG3 antibodies *in vitro* under different mitogenic conditions. *In vitro* mature B cells divide c.a. 5-7 times within 96 hours in LPS containing culture medium (T.Y. unpublished data); thus, one cell cycle takes c.a. 14-19 hours. This relatively slow cell cycle progression compared to centroblasts *in vivo* may account for one possible explanation for this difference between *in vivo* and *in vitro*. Alternatively, loss of HDAC1 may affect gene expression of cell surface and signaling molecules involved in B cell activation, so that mutant GC cells fail to receive appropriate signals for the clonal expansion after the immunization *in vivo*. We also found that *in vitro* mature B cells lacking HDAC1 secrete more class switched antibodies (IgG1 and IgG3) in the medium. This result suggests that there may be an inhibitory effect of HDAC1 on class switch recombination, at least, *in vitro*. There is ample literature describing the positive correlation between histone acetylation and class switch recombination (Nambu et al. 2003; Li et al. 2004; Odegard et al. 2005). In general, histone acetylation is found in opened/accessible conformation of chromatin loci in the genome. It may well be that loss of HDAC1 leads to increased histone acetylation in the IgH locus, which will result in increased accessibility for the recombination machinery such as AID protein. HDAC1 also might regulate negatively expression of genes involved in class switch recombination and the DNA double strand repair pathway by deacetylating their promoter regions.

3.2.4 An increased number of splenocytes accompanied with the induction of the dependent immune response

The number of splenocytes in HDAC1 hematopoietic cell KO mice are increased about 1.5 fold in immunized mice. This is observed as early as 5 days post injection of DNP-KLH. In HDAC1 mutant spleens, B, T and NK cell numbers are all increased proportionally about 1.5 fold, whereas the number of $Mac1^{+}Gr\text{-}1^{+}$ (myeloid) cells stays the same as in unimmunized mice. In addition, the percentages of BrdU positive cells in the B and T cell compartments are largely similar to the WT control. Based on these observations, we could have the hypothesis explaining the increase of the peripheral B and T cell numbers without changing their intrinsic proliferation/survival activity. A number of early progenitor populations such as common lymphoid progenitors may be increased in association with the enhanced proliferation/survival upon immunization. If this is the case in HDAC1 hematopoietic KO mice, peripheral B and T cells will appear to be increased without changing their proliferation since early progenitor cells are increased. It is worth to note that loss of Mi-2β, a chromatin remodeler in part of the Nucleosome Remodeling Deacetylase (NuRD) complex, in hematopoietic lineages results in an increase of hematopoietic stem cell proliferation and an accumulation of lympho myeloid primed progenitors (LMPP) and erythrocyte progenitors whereas myeloid progenitors are reduced (Yoshida et al. 2008) (Fig. 31). HDAC1 is responsible for the catalytic activity of the NuRD complex to remove acetyl groups from proteins. Therefore our observation of HDAC1 KO mice in hematopoietic lineages might be explained, at least in part, by similar mechanisms as reported in Mi-2β mutant mice. Thus HDAC1 is potentially an important factor for the fate decision of hematopoietic lineages. Additional studies addressing the roles of HDAC1 in the early stages of cell lineage choice will yield more complete picture of epigenetic regulation for hematopoietic cell development.

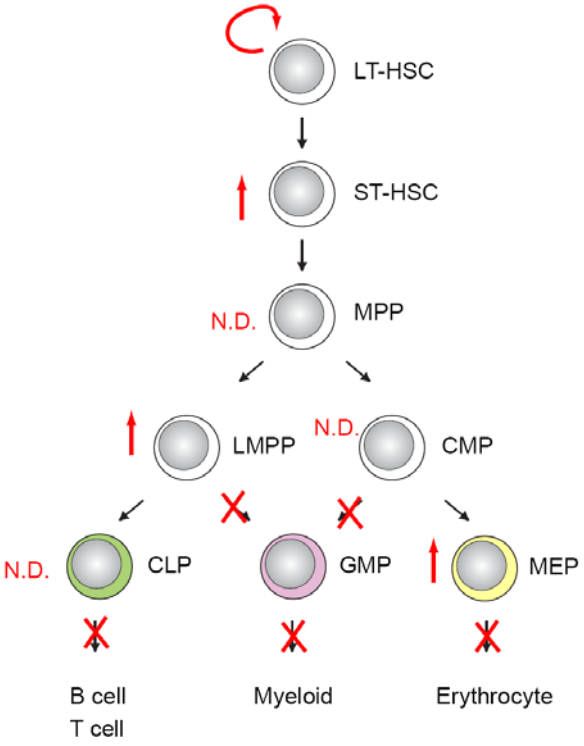

Figure 31. The effects of Mi-2β KO in hematopoietic cells (modified from (Yoshida et al. 2008)). In the absence of Mi-2 β, long term-hematopoietic stem cells (LT-HSC) proliferate more and accumulate down-stream subpopulations. Accumulated subpopulations are indicated by red arrows. Developmental blockades are depicted with red crosses. Subpopulations which were not explained are shown as N.D.

4. Material and Methods

Generation of HDAC1 and 2 conditional knockout mice and animal experiment

Each exon 6 of mouse *HDAC1* and *2* was flanked by a single *loxP* site and a cassette expressing the neomycin resistance gene and thymidine kinase gene flanked by two *loxP* sites. E14 ES cells (129/Ola strain) were electroporated with either the targeting vector for mouse *HDAC1* or *2* and correctly targeted ES clones were identified by PCR, sequencing and Southern blot analysis. One targeted ES cell clone for *HDAC1* and *2*, number 149 and 233 respectively, was used for aggregation to generate chimeric mice. Chimeric mice were crossed to C57/Bl6 animals to obtain germ line transmission of the *targeted* allele. To delete the neomycin cassette (*flox* allele) and generate *delta* allele, *targeted* mice were crossed with either *EIIa-Cre* mice or *Meox-Cre* mice. The chimeras that contain both *Cre* and *targeted* alleles were genotyped and we identified mice having recombined *delta* or *flox* alleles by PCR and verified by sequencing. For the generation of hematopoietic and B cell specific KO mice, we used *Vav-iCre* and *mb1-Cre* transgenic mice kindly provided by Dr. Dimitris Kioussis and Dr. Michael Reth, respectively.

For all analyses, *flox* allele littermates of the same sex were used as controls. All experiments were done using mixed background (129/Ola and C57/Bl6 mixed strain) transgenic mice from 6 to 12 weeks olds. Animal experiments were carried out according to regulations effective in the Kanton of Basel-Stadt, Switzerland. The mice were housed in groups of one to eight animals at 25°C with a 12:12 h light-dark cycle. They were fed a standard laboratory diet containing 0.8% phosphorus and 1.1% calcium (NAFAG 890, Kliba, Basel, Switzerland). Food and water was provided *ad libitum*.

Southern hybridization analysis

DNA was purified from bone marrow cells, splenocytes and thymocytes from indicated genotypes and digested by Sac I enzyme. After inactivation of restriction enzymes and purification, DNA was electropholated and blotted onto the membrane. The probe indicated in Supplementary Figure 1 was prepared by PCR, radio-labeled and incubated for over night at 65 °C. Hybridized DNA was visualized after 3 times intensive washing.

Western blot analysis

Bone marrow cells, splenocytes and thymocytes were isolated from indicated genotype of mice. Sample preparation, SDS-PAGE and blotting were done as described previously (Zhang et al., 2008). Mouse monoclonal anti-HDAC1 and anti-HDAC2 were kindly provided by Dr. Christian Seiser and described in (Zupkovitz et al., 2006).

Quantitative RT-PCR and semi quantitative RT-PCR

RNA was purified from sorted cell population mentioned in each figure with the RNeasy Microkit (Qiagen) according to the manufacturer's protocol. cDNA was synthesized by using the Thermoscript Reverse Transcriptase Kit (Invitrogen). Oligo dT primers provided in the kit were used for the first strand synthesis.

Quantitative PCR was performed on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) using a MESA GREEN qPCR MasterMix Plus for SYBR® Assay (Eurogentec). The amount of *HDAC1* and *2* transcripts was normalized by *Gapdh*.

For semi quantitative RT-PCR, cDNA was serially diluted 4 fold for PCR reactions. As a control reaction for contamination by genomic DNA, we prepared samples without reverse transcriptase (-RT). Primers used for these experiments are provided in Supplementary Table 3. Primers for semi quantitative RT-PCR were previously reported (DeKoter et al. 2002; Fuxa et al. 2004; Delogu et al. 2006; Liu et al. 2007). For the amplification of μ transcripts, combinations of a forward primer used for V(D)J recombination analysis (V_HJ558 and V_H7183) and a reverse primer, Cμ transcript, were used.

Flow cytometry (FACS)

Single cell suspensions were prepared from indicated lymphoid tissues. Cell staining was done according to standard procedures. FACS analysis was performed on a FACSCalibur (BD Biosciences, San Jose, CA) by gating on live cells. Samples were analyzed by using CellQuest (Becton Dickinson) or Flow-Jo (Tree star) software. Cell sorting was performed on a MoFlo (DakoCytomation). The purity of sorted cells was checked by reanalysis.

The following mAbs were from BD Biosciences; RA3-6B2 (anti-B220) conjugated to allophycocyanin (APC), ACK45 (anti-c-kit) conjugated to phycoerythrin (PE) or biotinylated, S7 (anti-CD43) conjugated to fluorescein isothiocyanate (FITC), 1D3 (anti-CD19) conjugated to APC-Cy7 and 7D4 (anti-CD25) biotinylated. 1B4B1 (anti-IgM) conjugated to FITC was from Southern Biotech. 6D5 (anti-CD19) conjugated to PE was from Biolegend. Streptavidin conjugated to PE-Cy5.5 was from CALTAG. For the Annexin V staining, we followed the manufacturer's protocol from BD Biosciences.

PCR analysis of deleted *HDAC1* and *2* alleles and V(D)J recombination

DNA was extracted from sorted cell populations mentioned in each figure by overnight proteinase K digestion, phenol/chloroform extraction and ethanol precipitation. DNA was serially diluted at 1:4 ratios and used for each PCR reaction. Primers used for the PCR detection of *HDAC1* and *2* genotyping and V(D)J recombination are shown in Supplementary Table 2 (Fuxa et al. 2004; Liu et al. 2007). PCR analysis of V(D)J recombination was described previously (Fuxa et al. 2004). Combinations of a forward primer for different parts of the IgH locus (V_HJ558, V_H7183 and D_H) and a reverse J_H3 primer were used. Amplicons from PCR reactions were separated on 1.5% agarose gel and visualized by ethidium bromide.

***In vivo* BrdU incorporation assay**

Mice were injected intraperitneally with 20 μg of BrdU and sacrificed 30 minutes later. Bone marrow single cell suspensions were prepared, stained and sorted with the combination of antibodies against the following cell surface molecules; $CD19^+$ $CD43^+$ $CD25^-$. BrdU staining using sorted cells was performed according to the manufacturer's protocol of BrdU flow kit (BD bioscience). Analysis was performed on a FACSCalibur and analyzed by Flow-Jo software.

Three-dimensional DNA FISH and confocal microscopy

Cells sorted by flow cytometry were washed in PBS and then were fixed on poly-L-lysine-coated slides for three-color and three-dimensional DNA-FISH analysis as described (Fuxa et al. 2004). Probes were directly labeled by nick translation with ChromaTide Alexa Fluor 488-5-dUTP, ChromaTide Alexa Fluor 594-5-dUTP

(Molecular Probes) or dUTP-indodicarbocyanine (GE Healthcare). The γ-satellite probe was prepared from a plasmid containing eight copies of the γ-satellite repeat sequence (Hewitt et al. 2008) and was directly labeled with dUTP-fluorescein isothiocyanate (Roche; Enzo Biochem) or dUTP-indodicarbocyanine. Cells were analyzed by confocal microscopy on a Leica SP5 AOBS system (Acousto-Optical Beam Splitter). Optical sections separated by 0.3 μm were collected, and only cells with signals from both alleles (typically 90%) were analyzed. Details of sample sizes and statistical analysis are provided in Supplementary Table 1.

Intracellular FACS for histone modifications

Cells were stained with fluorochrome coupled antibodies; B220-APC, CD19-PE and c-kit-biotinylated. Streptavidin-PE-Cy5.5 was used for the staining of c-kit-biotinylated antibody. After staining, cells were fixed with 3% paraformaldehyde for 15 minutes at room temperature, permeabilized with 0.1% saponin, 3% bovine serum albumin (BSA) in PBS for 30 minutes at room temperature, and then subjected to the intracellular staining with different histone modification antibodies as follows; anti-Histone H3 (ab1791; Abcam), anti-acetyl-Histone H3K9 and K14 (06-599; Millipore), anti-acetyl-Histone H4 (06-866; Millipore), anti-Histone H3 di-methyl lysine 4 (07-030; Millipore), anti-Histone H3 tri-methyl lysine 4 (ab8580; Abcam), anti-Histone H3 di- and tri-methyl lysine 9 antibodies were kindly provided by Dr. Antoine Peters (Peters et al. 2003). After washing of cells to remove nonspecific antibody binding, cells were stained with the secondary antibody recognizing rabbit IgG coupled to alexa Fluor® 488 (A11034; Invitrogen). FACS analysis was performed after extensive washing. The buffer containing 0.1% saponin in PBS was used for all staining, washing and FACS analysis after permeabilization.

Immunization

Mice were injected intraperitoneally with 100 μg of DNP-KLH. Sera were collected from tail bleeding at indicated dates in each figure. The second immunization was done by same way but using 50 μg of DNP-KLH at 21 days post first-injection.

ELISA

DNP-BSA (5 mg/ml in PBS) was coated into 96 well microplates over night at 4 °C. The coated plate was washed three times with PBS$^-$/0.05% Tween-20 and blocked with 10 mg/ml BSA in PBS$^-$/0.05% Tween-20 for 1 hour at 37 °C. After 3 times washing with PBS$^-$/0.05% Tween-20, serial diluted sera were added in the plate and incubated for 2 hours at 37 °C. The plate was washed three times with PBS$^-$/0.05% Tween-20 and secondary antibodies coupled with alkaline phosphatases were added. After 1 hour incubation at 37 °C, washing 3 times with PBS$^-$/0.05% Tween-20 and then substrates were added into the plate. The absorbance at 405 nm was read and relative titers were calculated.

RNA preparation and hybridization to Affymetrix Microarrays

Cells were FACS sorted according to the stage specific cell surface markers and RNA was purified with RNeasy Micro Kit from Qiagen. Total RNA (~50 ng) from each sample was reverse transcribed and labeled using the Affymetrix 2-cycles labeling kit according to manufacture's protocol. Mouse 430v2 GeneChips (Affymetrix, Santa Clara, CA) were used for the hybridization. Data were analyzed using Expressionist (Genedata AG). The normalized data were subjected to a Student *t*-test ($P < 0.05$) and were required to have a median fold change of more than 2.

Statistical analysis

Significance was assessed by analysis of normal distribution, variances and two-tail T test.

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Yu Zhang, SoHee Kwon, Teppei Yamaguchi, Fabien Cubizolles, Sophie Rousseaux, Michaela Kneissel, Chun Cao, Na Li, Hwei-Ling Cheng, Katrin Chua, David Lombard, Adam Mizeracki, Gabriele Matthias, Frederick W. Alt, Saadi Khochbin & Patrick Matthias *Mol Cell Biol. 2008 Mar;28(5):1688-1701.*

Histone deacetylases 1 and 2 are essential for early B cell development, V(D)J recombination and contraction of the immunoglobulin heavy chain locus
Teppei Yamaguchi, Yu Zhang, Susannah L Hewitt, Hubertus Kohler, Christian Seiser, Jane A Skok and Patrick Matthias (Submitted to Immunity)

Histone deacetylase 1 is critical for B cell terminal differentiation and the adaptive immune reaction by regulating the proliferation of activated B cells
Teppei Yamaguchi, Fabien Cubizolles and Patrick Matthias (Manuscript in preparation)

Histone deacetylases as general regulators of gene expression (review)
Teppei Yamaguchi and Patrick Matthias (Manuscript in preparation)

Histone deacetylases 1 and 2 act in concert to promote G1 cell cycle progression
Fabien Cubizolles, Teppei Yamaguchi and Patrick Matthias (Manuscript in preparation)

8. Appendix

8.1 HDAC2 inactivation in hematopoietic lineages

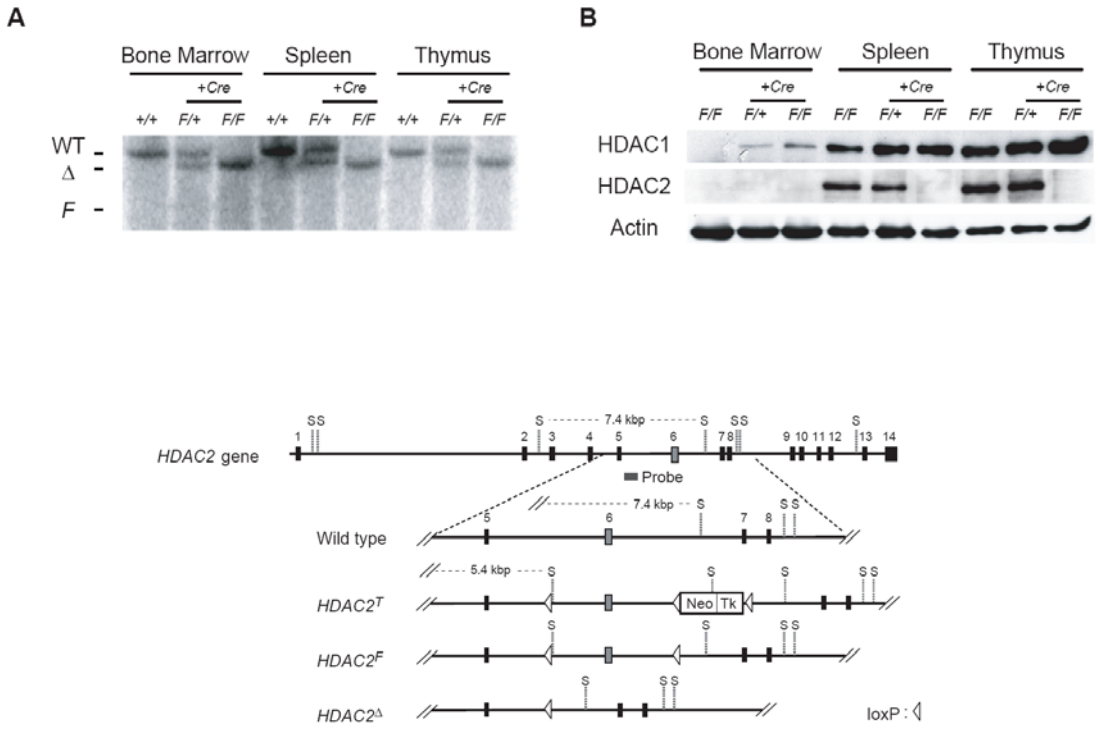
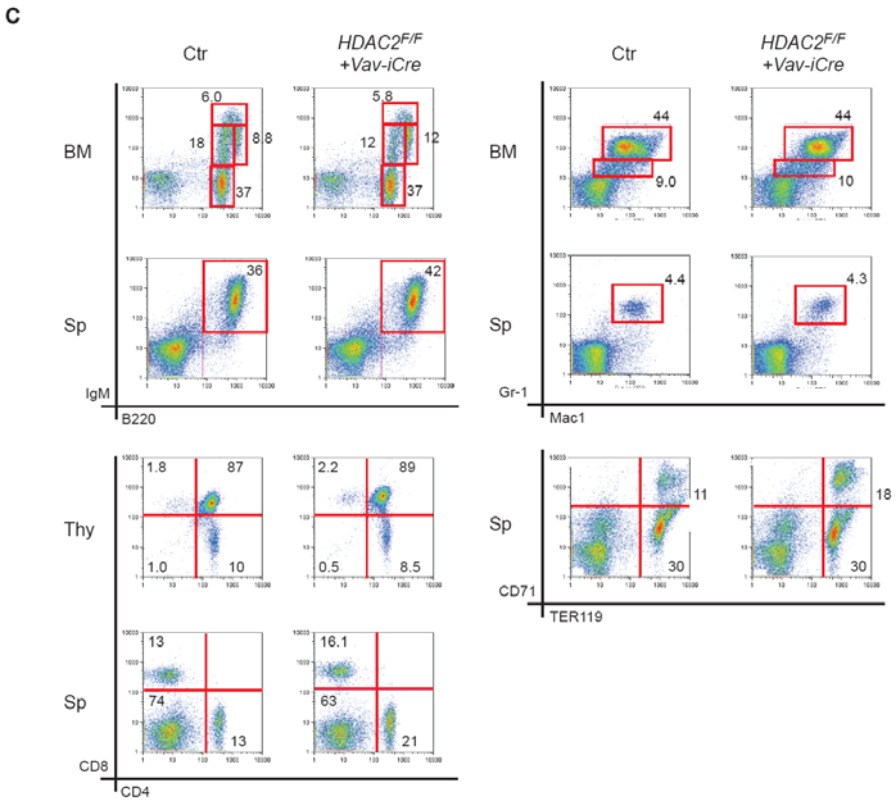


Figure 8.1 HDAC2 inactivation in hematopoietic cell lineages by iCre recombinase (*Vav-iCre* transgene). (A) Southern hybridization analysis of HDAC2 genomic alleles. Wild type and modified alleles of HDAC2 are shown. The structure of HDAC2 gene is depicted at the top. Modified alleles are represented as follows: *T*, *targeted* allele; *F*, *flox* allele; *Δ*, *deleted* (KO) allele. Black and gray boxes represent the coding exons, white rectangular boxes show the neomycin gene cassette, white triangles symbolize the *loxP* sequence and S represents SacI restriction sites. DNA from indicated genotypes of mice were digested by SacI, blotted on a membrane and detected by the specific probe indicated. (B) Western blot analysis of HDAC2 KO mice. Protein lysates from cells in bone marrow, spleen and thymus were detected by anti-HDAC1, HDAC2 and Actin antibodies, as indicated.



(C) Flow cytometric analysis of *HDAC2*$^{F/F}$::*Vav-iCre* mice. Single cell suspensions from bone marrow, spleen or thymus were stained with the indicated antibody combinations and representative data are shown. Numbers in plots represent the percentage of cells in the respective gate or quadrant.

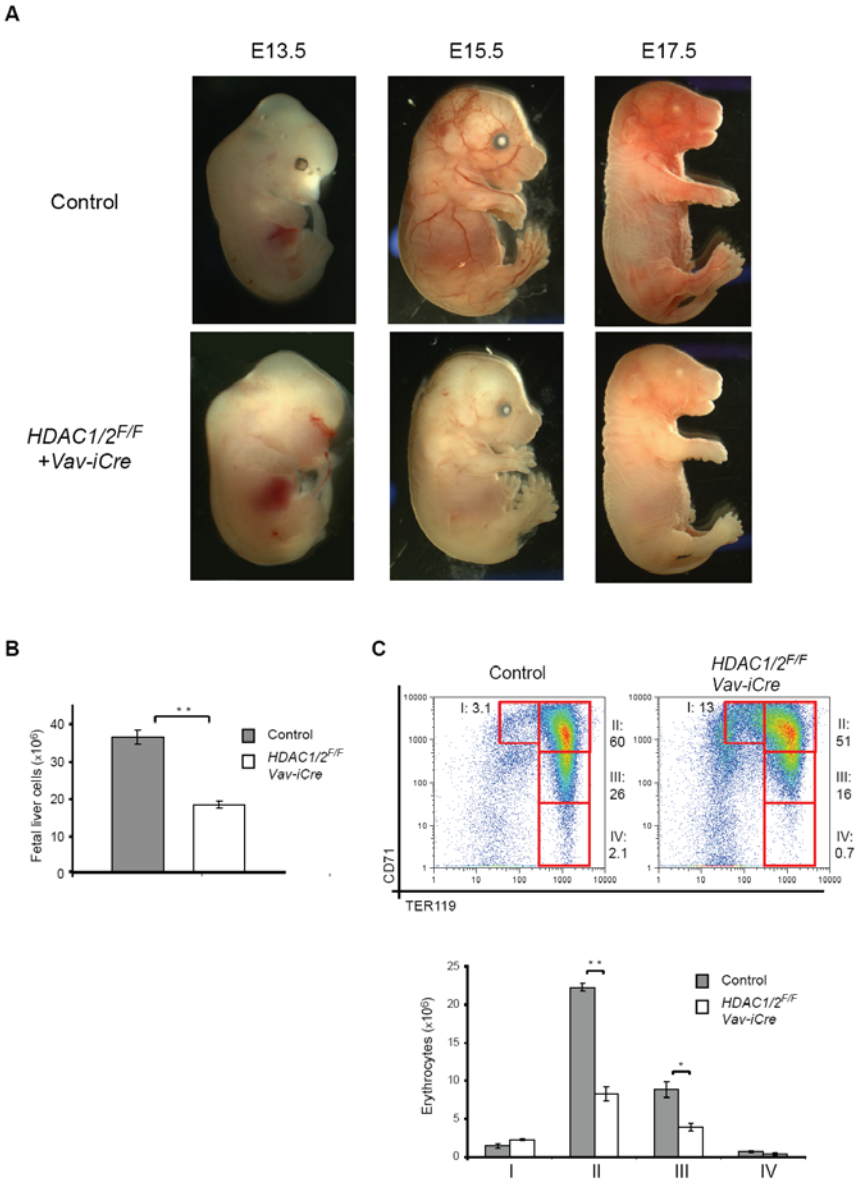


Figure 8.2 Combined inactivation of HDAC1/2 in hematopoietic cell lineages leads to the embryonic lethality due to anemia. (A) Pictures of embryos from control (without the *iCre* gene) and *HDAC2*$^{F/F}$::*Vav-iCre* at indicated embryonic days. The *Vav-iCre* gene starts to be active around E13.5 in the fetal liver. Therefore *HDAC2*$^{F/F}$::*Vav-iCre* embryos appear to be pale around E15.5. (B) A total number of fetal liver cells at E15.5. (N = 3 per each genotype) (C) FACS staining of fetal liver erythrocytes. TER119 and CD71 antibodies are used to distinguish different developmental stages of erythrocytes. Red windows indicated in the FACS profiling represent the different developmental stages (earlier progenitors; I – mature cells; IV). The bottom graph shows the absolute number of each stage of erythrocytes (N = 3). HDAC1/2 DKO embryos show the accumulation of stage I cells whereas much less cells in the stages II and III.

8.3 Phenotype of the intermediate genotype of HDAC1/2 B cell KO mice

A

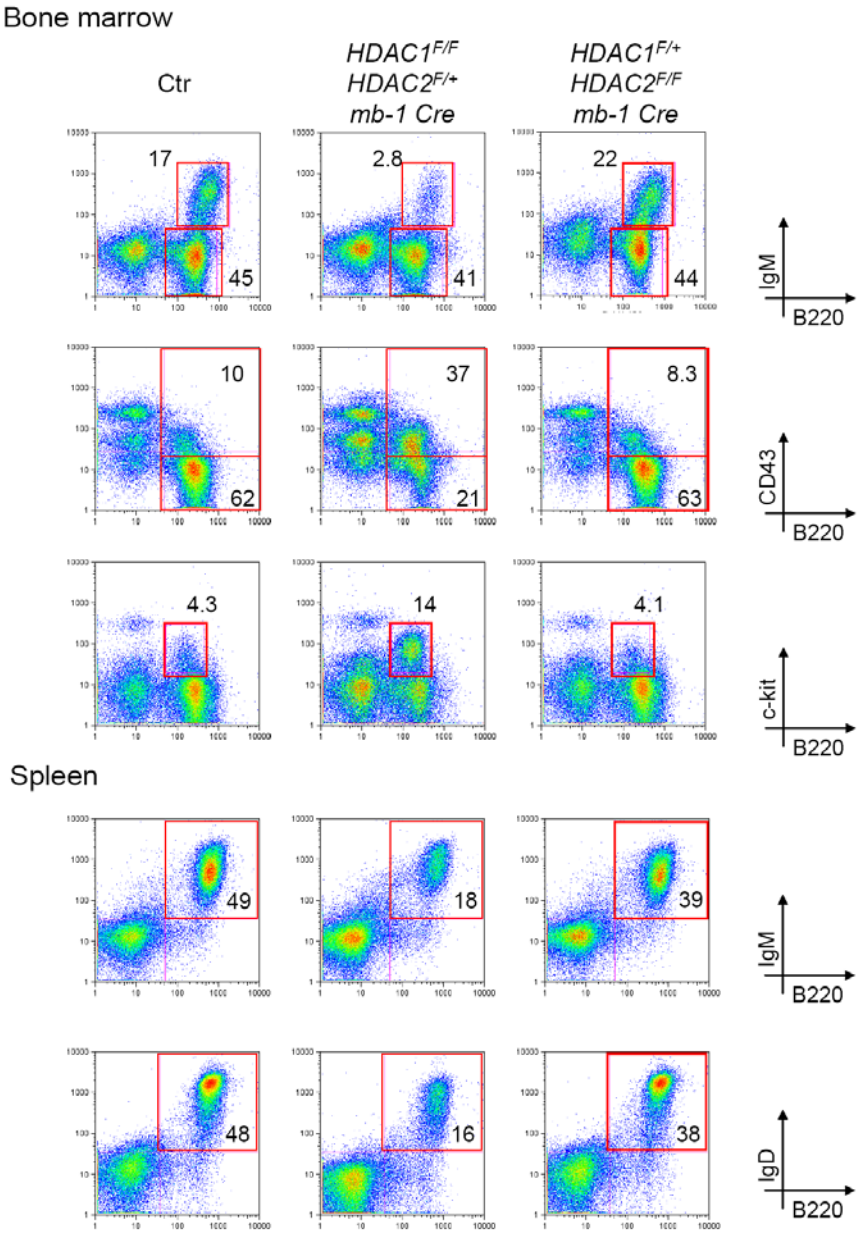


Figure 8.3 Phenotypic deferences in the intermediate genotype of HDAC1/2 B cell KO mice (A) FACS analysis of the intermediate genotype mice. Single cell suspensions from bone marrow and spleen were stained with the indicated antibody combinations and representative data are shown. Numbers in plots represent the percentage of cells in the respective gate or quadrant.

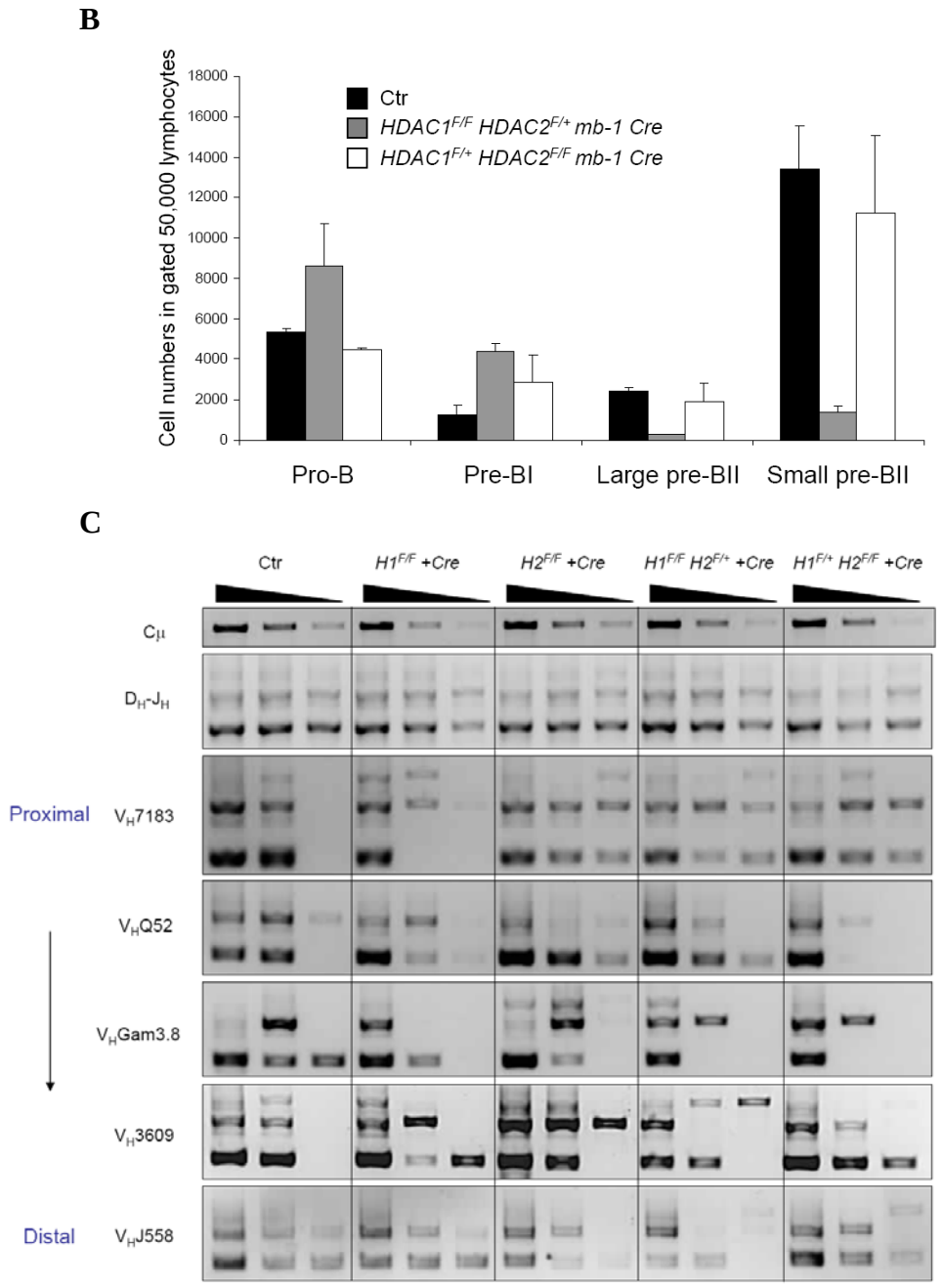
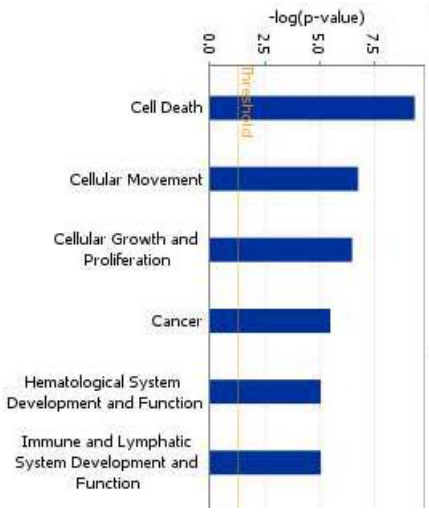


Figure 8.3. (B) Cell numbers of B cell subpopulations in each genotype. The numbers are calculated based on cell surface marker combinations and lymphocyte gated cells (50,000, N = 2). (C) VDJ recombination assay using different genotype of pre-BI cells. DNA was isolated from pre-BI cells ($B220^{+}CD19^{+}CD43^{+}CD25^{-}$). VDJ recombination assay by PCR was done as described in material and methods. The order of V segments PCR (top to bottom) follows the sequential order in the IgH locus (V_H7183 is the most proximal and V_HJ558 is the most distal from DJ segments).

8.4 The summary of the microarray analysis of HDAC1/2 deficient pre-BI cells

A. Significantly relevant molecular and cellular functions associated with disregulated genes (total 800 genes P<0.05 and more than 2 fold change). Top 6 categories are listed.



B. The list of genes found in the category of "Cell Death" (150 genes)

Molecule Name	Entrez Gene ID	Fold Change	
Edg3	13610	27.93	endothelial differentiation, sphingolipid G-protein-coupled receptor, 3
Ndn	17984	25.44	necdin
Gstm5	14867	20.52	glutathione S-transferase, mu 5
Hmga2	15364	18.73	high mobility group AT-hook 2
Flt3	14255	15.80	FMS-like tyrosine kinase 3
Agrn	11603	14.96	agrin
Ptk2	14083	12.37	PTK2 protein tyrosine kinase 2
Tmepai	65112	10.17	transmembrane, prostate androgen induced RNA
Tmem158	72309	9.82	transmembrane protein 158
Tcf3	21415	9.50	transcription factor 3
Itsn1	16443	7.63	intersectin 1 (SH3 domain protein 1A)
Fgfr1	14182	7.62	fibroblast growth factor receptor 1
Ptger3	19218	7.48	prostaglandin E receptor 3 (subtype EP3)
Ctsl	13039	6.76	cathepsin L
Jundm2	81703	6.13	Jun dimerization protein 2
Ppap2a	19012	5.98	phosphatidic acid phosphatase 2a
Maged1	94275	5.73	melanoma antigen, family D, 1
Cadm1	54725	5.39	cell adhesion molecule 1
Kif1b	16561	5.37	kinesin family member 1B
Lta	16992	5.26	lymphotoxin A
Runx2	12393	5.26	runt related transcription factor 2
P2rx7	18439	5.25	purinergic receptor P2X, ligand-gated ion channel, 7
App	11820	5.22	amyloid beta (A4) precursor protein
Trip10	106628	5.14	thyroid hormone receptor interactor 10
Itgb5	16419	4.99	integrin beta 5
Tgfbi	21810	4.90	transforming growth factor, beta induced
Timp2	21858	4.76	tissue inhibitor of metalloproteinase 2
Rras	20130	4.68	Harvey rat sarcoma oncogene, subgroup R
Ppp1r9a	243725	4.60	protein phosphatase 1, regulatory (inhibitor) subunit 9A

Fcgr3		14131	4.41	Fc receptor, IgG, low affinity III
Laptm4b		114128	4.35	lysosomal-associated protein transmembrane 4B
3222402P14Rik		235542	4.22	RIKEN cDNA 3222402P14 gene
Bag3		29810	4.05	Bcl2-associated athanogene 3
S100a4		20198	4.02	S100 calcium binding protein A4
Tnfrsf22		79202	3.95	tumor necrosis factor receptor superfamily, member 22
Tal1		21349	3.91	T-cell acute lymphocytic leukemia 1
Csf2rb2	)84|12983		3.84	colony stimulating factor 2 receptor, beta 2, low-affinity (granulocyte-macrophage)
LOC100045055 : Rbp1		19659	3.71	retinol binding protein 1, cellular : similar to cellular retinol binding protein I
Nek6		59126	3.66	NIMA (never in mitosis gene a)-related expressed kinase 6
Cebpa		12606	3.62	CCAAT/enhancer binding protein (C/EBP), alpha
Ldlr		16835	3.59	low density lipoprotein receptor
Nrtn		18188	3.55	neurturin
Pla2g4a		18783	3.54	phospholipase A2, group IVA (cytosolic, calcium-dependent)
Klrd1		16643	3.54	killer cell lectin-like receptor, subfamily D, member 1
Dmc1		13404	3.50	DMC1 dosage suppressor of mck1 homolog, meiosis-specific homologous recombination (yeast)disrupted meiotic cDNA 1
Ccnd1		12443	3.36	cyclin D1
Ppm2c		381511	3.32	protein phosphatase 2C, magnesium dependent, catalytic subunit
Apbb2		11787	3.29	amyloid beta (A4) precursor protein-binding, family B, member 2
Gsr		14782	3.28	glutathione reductase 1
Fbln1		14114	3.26	fibulin 1
Endog		13804	3.25	endonuclease G
Ern1		78943	3.14	Endoplasmic reticulum (ER) to nucleus signalling 1
Pak1		18479	3.13	p21 (CDKN1A)-activated kinase 1
LOC100047069 : Rxra		20181	3.13	retinoid X receptor alpha : similar to retinoid X receptor-alpha
Pvrl2		19294	3.13	poliovirus receptor-related 2
Pea15a		18611	3.12	phosphoprotein enriched in astrocytes 15A
1200009F10Rik		67454	3.01	RIKEN cDNA 1200009F10 gene
Abcc1		17250	2.89	ATP-binding cassette, sub-family C (CFTR/MRP), member 1
Lair1		52855	2.76	leukocyte-associated Ig-like receptor 1
Ms4a6c		23856	2.75	membrane-spanning 4-domains, subfamily A, member 6C
Trf		22041	2.73	transferrin
Igfbp4		16010	2.69	insulin-like growth factor binding protein 4
Plekhf1		72287	2.67	pleckstrin homology domain containing, family F (with FYVE domain) member 1
Acvr1		11477	2.66	activin A receptor, type 1
Smox		228608	2.65	spermine oxidase
Vcl		22330	2.59	vinculin
Kit		16590	2.37	kit oncogene
Ptprf		19268	2.37	protein tyrosine phosphatase, receptor type, F
Gabbr1		54393	2.36	gamma-aminobutyric acid (GABA-B) receptor, 1
Sod2		20656	2.32	superoxide dismutase 2, mitochondrial
Ece1		230857	2.30	endothelin converting enzyme 1
Lgals3		16854	2.29	lectin, galactose binding, soluble 3
Cdkn1c		12577	2.28	cyclin-dependent kinase inhibitor 1C (P57)
Meis1		17268	2.24	myeloid ecotropic viral integration site 1
Fosl2 : LOC634417		14284	2.19	fos-like antigen 2 : similar to fos-like antigen 2
Ms4a2		14126	2.16	membrane-spanning 4-domains, subfamily A, member 2
Gas6		14456	2.14	growth arrest specific 6
Prkra		23992	2.08	protein kinase, interferon inducible double stranded RNA dependent activator
Adk		11534	1.99	adenosine kinase
Ripk3		56532	1.95	receptor-interacting serine-threonine kinase 3
Smpd2		20598	1.94	sphingomyelin phosphodiesterase 2, neutral

Mfn1		67414	1.89	mitofusin 1
Ccbl1		70266	1.76	cysteine conjugate-beta lyase 1
Sdc4		20971	-1.87	syndecan 4
Cd40		21939	-1.94	CD40 antigen
Birc2		11797	-2.06	baculoviral IAP repeat-containing 2
Akt2		11652	-2.07	thymoma viral proto-oncogene 2
Cd79b		15985	-2.11	CD79B antigen
Pqbp1		54633	-2.11	polyglutamine binding protein 1
Gmeb1		56809	-2.13	glucocorticoid modulatory element binding protein 1
Smug1		71726	-2.15	single-strand selective monofunctional uracil DNA glycosylase
Herpud1		64209	-2.17	homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1
Trp53inp1		60599	-2.21	transformation related protein 53 inducible nuclear protein 1
Nme3		79059	-2.22	expressed in non-metastatic cells 3
Irf5		27056	-2.27	interferon regulatory factor 5
Traf2		22030	-2.28	Tnf receptor-associated factor 2
Gab2		14389	-2.29	Growth factor receptor bound protein 2-associated protein 2
Eme1		268465	-2.30	essential meiotic endonuclease 1 homolog 1 (S. pombe)
Birc3		11796	-2.31	baculoviral IAP repeat-containing 3
Pik3cg		30955	-2.33	phosphoinositide-3-kinase, catalytic, gamma polypeptide
Ercc1		13870	-2.34	excision repair cross-complementing rodent repair deficiency, complementation group 1
Dgka		13139	-2.39	diacylglycerol kinase, alpha
Cd1d1	ł80|12479		-2.43	CD1d1 antigen
Akap13		75547	-2.43	A kinase (PRKA) anchor protein 13
Traf3ip3		103213	-2.56	TRAF3 interacting protein 3
Icam2		15896	-2.57	intercellular adhesion molecule 2
Zfp36		22695	-2.63	zinc finger protein 36
Tnfaip3		21929	-2.64	tumor necrosis factor, alpha-induced protein 3
Gfi1		14581	-2.70	growth factor independent 1
Ndufs1		227197	-2.71	NADH dehydrogenase (ubiquinone) Fe-S protein 1
Dnajb6		23950	-2.74	DnaJ (Hsp40) homolog, subfamily B, member 6
Wapal		218914	-2.77	wings apart-like homolog (Drosophila)
Phlda1		21664	-2.81	pleckstrin homology-like domain, family A, member 1
Itpr2		16439	-2.86	inositol 1,4,5-triphosphate receptor 2
Tnfsf10		22035	-2.89	tumor necrosis factor (ligand) superfamily, member 10
Cerk : LOC676420		223753	-2.89	ceramide kinase : similar to Ceramide kinase (Acylsphingosine kinase) (mCERK)
Dnase1		13419	-2.90	deoxyribonuclease I
Lck		16818	-2.94	lymphocyte protein tyrosine kinase
Snn		20621	-2.96	stannin
Hdac5		15184	-2.96	histone deacetylase 5
2010001M09Rik		69816	-3.03	RIKEN cDNA 2010001M09 gene
Ets1		23871	-3.06	E26 avian leukemia oncogene 1, 5' domain
Sh2d2a		27371	-3.09	SH2 domain protein 2A
Lef1		16842	-3.12	lymphoid enhancer binding factor 1
Apbb1		11785	-3.13	amyloid beta (A4) precursor protein-binding, family B, member 1
Ppp1r16b		228852	-3.13	protein phosphatase 1, regulatory (inhibitor) subunit 16B
Egr3		13655	-3.14	early growth response 3
Ptk2b		19229	-3.18	PTK2 protein tyrosine kinase 2 beta
Tnfrsf13b		57916	-3.30	tumor necrosis factor receptor superfamily, member 13b
Ptprcap		19265	-3.35	protein tyrosine phosphatase, receptor type, C polypeptide-associated protein
Cd5		12507	-3.44	CD5 antigen
Rrm2b		382985	-3.90	ribonucleotide reductase M2 B (TP53 inducible)
Dpp4		13482	-4.01	dipeptidylpeptidase 4

Igh : Igh-6 : Igh-V7183 : Igh-VS107 : Ighg : Ighv14-2 :	16017	-4.12	immunoglobulin heavy chain 6 (heavy chain of IgM) : immunoglobulin heavy chain (V7183 family) : immunoglobulin heavy chain (S107 family) : immunoglobulin heavy chain complex : Immunoglobulin heavy chain (gamma polypeptide) : expressed sequence AI324046 : similar to Ig heavy chain V region M315 precursor : immunoglobulin heavy variable V14-2 : similar to Ig alpha-chain precursor (V139-D-J1)
Ube1l	74153	-4.13	ubiquitin-activating enzyme E1-like
Pecam1	18613	-4.18	platelet/endothelial cell adhesion molecule 1
Irs1	16367	-4.28	insulin receptor substrate 1
Cdc25b	12531	-4.61	cell division cycle 25 homolog B (S. pombe)
Msh5	17687	-5.03	mutS homolog 5 (E. coli)
Cd22	12483	-5.15	CD22 antigen
Heyl	56198	-5.24	hairy/enhancer-of-split related with YRPW motif-like
Lig4	319583	-6.22	ligase IV, DNA, ATP-dependent
Blk	12143	-6.34	B lymphoid kinase
Mbd4	17193	-6.44	methyl-CpG binding domain protein 4
Slamf7	75345	-7.06	SLAM family member 7
Cyfip2	76884	-10.38	cytoplasmic FMR1 interacting protein 2
Hdac2	15182	-11.16	histone deacetylase 2
Ikzf3	22780	-11.76	IKAROS family zinc finger 3
Rasgrp1	19419	-13.21	RAS guanyl releasing protein 1
Ms4a1	12482	-96.58	membrane-spanning 4-domains, subfamily A, member 1

C. The list of genes found in the category of "Immune and Lymphatic System Development and Function" (35 genes)

Molecule Name	Entrez Gene ID	Fold Change	
Flt3	14255	15.80	FMS-like tyrosine kinase 3
Runx2	12393	5.26	runt related transcription factor 2
App	11820	5.22	amyloid beta (A4) precursor protein
Fcgr3	14131	4.41	Fc receptor, IgG, low affinity III
Tal1	21349	3.91	T-cell acute lymphocytic leukemia 1
Csf2rb2	12984|12983	3.84	colony stimulating factor 2 receptor, beta 2, low-affinity (granulocyte-macrophage)
Cebpa	12606	3.62	CCAAT/enhancer binding protein (C/EBP), alpha
Ccnd1	12443	3.36	cyclin D1
Mlf1	17349	3.32	myeloid leukemia factor 1
Lair1	52855	2.76	leukocyte-associated Ig-like receptor 1
Kit	16590	2.37	kit oncogene
Meis1	17268	2.24	myeloid ecotropic viral integration site 1
Cd79b	15985	-2.11	CD79B antigen
Traf2	22030	-2.28	Tnf receptor-associated factor 2
Pik3cg	30955	-2.33	phosphoinositide-3-kinase, catalytic, gamma polypeptide
Cd1d1	12480|12479	-2.43	CD1d1 antigen
Zfp36	22695	-2.63	zinc finger protein 36
Gfi1	14581	-2.70	growth factor independent 1
Tec	21682	-2.70	cytoplasmic tyrosine kinase, Dscr28C related (Drosophila)
Hivep2	15273	-2.88	human immunodeficiency virus type I enhancer binding protein 2
Tnfsf10	22035	-2.89	tumor necrosis factor (ligand) superfamily, member 10
Lck	16818	-2.94	lymphocyte protein tyrosine kinase
Hdac5	15184	-2.96	histone deacetylase 5
Ets1	23871	-3.06	E26 avian leukemia oncogene 1, 5' domain
Lef1	16842	-3.12	lymphoid enhancer binding factor 1
Egr3	13655	-3.14	early growth response 3

Tnfrsf13b	57916	-3.30	tumor necrosis factor receptor superfamily, member 13b
Igh : Igh-6 : Igh-V7183 : Igh-VS107 : Ighg : Ighv14-2 :	16017	-4.12	immunoglobulin heavy chain 6 (heavy chain of IgM) : immunoglobulin heavy chain (V7183 family) : immunoglobulin heavy chain (S107 family) : immunoglobulin heavy chain complex : Immunoglobulin heavy chain (gamma polypeptide) : expressed sequence AI324046 : similar to Ig heavy chain V region M315 precursor : immunoglobulin heavy variable V14-2 : similar to Ig alpha-chain precursor (V139-D-J1)
Irs1	16367	-4.28	insulin receptor substrate 1
Cd22	12483	-5.15	CD22 antigen
Lig4	319583	-6.22	ligase IV, DNA, ATP-dependent
Blk	12143	-6.34	B lymphoid kinase
Il33	77125	-12.94	interleukin 33
Rasgrp1	19419	-13.21	RAS guanyl releasing protein 1

The list of genes found in HDAC1 KO GC cells (Cluster b).

A. The lists of TFs and transcriptional regulator found in the cluster b.

Molecule Name	Entrez GeneID	WTmB	WTGC	KOmB	KOGC	KOGC /WTGC	
E2f5	13559	29985.9	916.7	27856.3	11709.7	12.77	E2F transcription factor 5
MGI:1916782	74318	966.4	231.8	1386.7	823.0	3.55	homeobox only domain
Hhex	15242	2578.7	908.7	2775.1	3065.9	3.37	hematopoietically expressed homeobox
Aff1	17355	2070.3	844.7	1897.4	2527.7	2.99	AF4/FMR2 family, member 1
Mef2c	17260	932.4	39.2	1127.1	116.5	2.97	Myocyte enhancer factor 2C, mRNA
Tsc22d1	21807	373.6	128.6	290.2	369.0	2.87	TSC22 domain family, member 1
Mib2	76580	343.7	156.0	383.5	441.9	2.83	mindbomb homolog 2 (Drosophila)
MGI:2385920	64451	150.9	93.2	217.6	242.2	2.60	disco interacting protein 2 homolog (Drosophila)
Notch1	18128	710.6	291.3	783.5	753.9	2.59	Notch gene homolog 1 (Drosophila)
Jarid1b	75605	700.8	298.9	751.7	726.7	2.43	jumonji, AT rich interactive domain 1B (Rbp2 like)
Zfp238	30928	2120.2	983.3	2274.1	2350.6	2.39	zinc finger protein 238
Pcgf4	12151	886.2	292.8	1076.4	691.8	2.36	polycomb group ring finger 4
Smarca2	67155	627.6	160.2	571.6	365.7	2.28	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 2
Tcf12	21406	113.8	34.6	93.0	76.8	2.22	transcription factor 12
Sub1	20024	118.7	51.2	108.2	113.7	2.22	SUB1 homolog (S. cerevisia
D130016B08Rik	319524	65.8	20.7	47.9	45.9	2.22	RIKEN cDNA D130016B08 gene
Trim24	21848	1341.2	495.0	1262.9	1068.7	2.16	tripartite motif protein 24
Irf4	16364	2027.3	1011.8	1770.1	2171.7	2.15	interferon regulatory factor 4
Foxo3	56484	229.0	32.3	198.1	68.4	2.12	Forkhead box O3a (Foxo3a), mRNA

B. The lists of genes associated with the cell cycle regulation in the cluster b.

Molecule Name	Entrez GeneID	WTmB	WTGC	KOmB	KOGC	KOGC /WTGC	
Cdkn1b	12576	810.0	213.6	639.5	819.4	3.84	cyclin-dependent kinase inhibitor 1B (P27)
Erbb3	13867	39.4	25.6	81.9	97.1	3.79	v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian)
Errfi1	74155	130.0	49.1	171.7	181.5	3.70	ERBB receptor feedback inhibitor 1
Dpp4	13482	1575.6	458.7	1681.5	1480.7	3.23	dipeptidylpeptidase 4
Ski	20481	261.6	58.6	232.5	188.8	3.22	Sloan-Kettering viral oncogene homolog
Vav3	57257	386.4	102.8	358.6	285.6	2.78	vav 3 oncogene
Zbtb10	229055	188.9	43.4	147.2	108.7	2.51	zinc finger and BTB domain containing 10
Ches1	71375	2275.5	945.8	2464.9	2341.9	2.48	checkpoint supressor 1
Rps6ka2	20112	31.8	16.7	38.4	41.1	2.46	ribosomal protein S6 kinase, polypeptide 2
Socs5	56468	1036.7	512.5	1012.0	1240.3	2.42	suppressor of cytokine signaling 5
Ikbkb	16150	1234.8	197.6	1343.7	454.6	2.30	inhibitor of kappaB kinase beta
Pvr	52118	24.3	11.5	40.6	26.4	2.30	poliovirus receptor
Mphosph9	269702	250.6	85.8	286.6	194.5	2.27	M-phase phosphoprotein 9
Ppp3ca	19055	1292.6	519.1	1474.1	1167.9	2.25	protein phosphatase 3, catalytic subunit, alpha isoform
Myh9	17886	2332.0	1015.2	2191.2	2269.1	2.24	myosin, heavy polypeptide 9, non-muscle
Ceacam1	26365	601.4	286.2	365.2	618.1	2.16	CEA-related cell adhesion molecule 1
D4Ertd767e, Nasp	50927, 52413	131.8	30.3	140.9	63.2	2.08	nuclear autoantigenic sperm protein (histone-binding), DNA segment, Chr 4, ERATO Doi 767, expressed

C. The lists of genes associated with apoptosis in the cluster b.

Molecule Name	Entrez GeneID	WTmB	WTGC	KOmB	KOGC	KOGC /WTGC	
Afp	11576	245.3	52.7	335.2	304.4	5.77	alpha fetoprotein
Il6st	16195	205.7	43.2	276.9	199.9	4.62	interleukin 6 signal transducer
Trps1	83925	371.8	71.2	508.3	321.6	4.52	trichorhinophalangeal syndrome I (human)
Unc5cl	76589	259.0	96.2	531.1	415.2	4.31	unc-5 homolog C (C. elegans)-like
Rtn4	68585	529.3	48.4	545.2	191.5	3.96	RTN4 (Rtn4) mRNA, complete cds, alternatively spliced
Serpine2	20720	133.9	53.8	186.1	192.6	3.58	serine (or cysteine) peptidase inhibitor, clade E, member 2
Pdk1	228026	174.9	44.2	165.6	136.4	3.09	pyruvate dehydrogenase kinase, isoenzyme 1
Gabbr1	54393	221.7	69.1	289.1	211.7	3.06	gamma-aminobutyric acid (GABA-B) receptor, 1
Fbxo32	67731	552.9	245.0	597.0	744.6	3.04	F-box only protein 32
Prkra	23992	103.4	29.8	108.6	84.8	2.85	protein kinase, interferon inducible double stranded RNA dependent activator
Zmynd11	66505	1867.2	634.6	1927.0	1792.3	2.82	zinc finger, MYND domain containing 11
Bnip3	12176	463.2	151.6	509.0	414.6	2.73	BCL2/adenovirus E1B 19kDa-interacting protein 1, NIP3
Ndst1	15531	1544.7	486.9	1640.6	1300.1	2.67	N-deacetylase/N-sulfotransferase (heparan glucosaminyl) 1
Hck	15162	439.5	143.4	376.0	348.3	2.43	Hemopoietic cell kinase (Hck), mRNA
Itga4	16401	1851.5	547.3	1678.9	1263.6	2.31	integrin alpha 4
Abcg1	11307	1427.7	642.5	1436.3	1427.2	2.22	ATP-binding cassette, sub-family G (WHITE), member 1
Gng2	14702	134.5	74.7	165.5	165.9	2.22	guanine nucleotide binding protein (G protein), gamma 2 subunit
Sh3kbp1	58194	163.9	29.9	148.5	62.1	2.08	SH3-domain kinase binding protein 1 (Sh3kbp1), mRNA
		705.2	243.1	663.4	500.2	2.06	gb:BG695418 /DB_XREF=gi:13955314 /DB_XREF=NISC_iv17b11.w2
Hsh2d	209488	751.1	368.3	836.2	749.0	2.03	Hematopoietic SH2 domain containing, mRNA (cDNA clone MGC:106069 IMAGE:5316178)
Rps6ka5	73086	243.6	81.5	247.1	163.5	2.01	ribosomal protein S6 kinase, polypeptide 5
Dag1	13138	631.0	408.7	712.9	819.3	2.00	dystroglycan 1

8.5 Abbreviations

3D-FISH	three-dimensional DNA fluorescence *in situ* hybridization	iPS cell	induced pluripotent stem cell
AID	activation induced cytidine deaminase	KO	knock out
BCR	B cell receptor	LMPP	lymphoid-primed multipotent progenitor
BrdU	5-bromo-2-deoxyuridine	LPS	lipopolysaccharides
Cdk	cyclin-dependent protein kinase	LSK	Lin^{-} $Sca\text{-}1^{+}$ $c\text{-}kit^{hi}$ population
CLP	common lymphoid progenitor	LT-HSC	long-term HSC
CMP	common myeloid progenitor	MEF	mouse embryonic fibroblast
CoREST	corepressor for REST (RE1 silencing transcription factor 1)	MEF2	myocyte enhancer factor-2
CSR	class switch recombination	MEP	megakaryocyte-erythrocyte progenitor
DNP-KLH	dinitrophenylated keyhole limpet haemocyanin	MPP	multipotential progenitor
DSB	DNA double-strand break	NHEJ	non-homologous end joining pathway
ELISA	enzyme-linked immunosorbent assay	NuRD	nucleosomal remodeling and deacetylation
ELP	early lymphoid progenitor	PIC	preinitiation complex
ES cell	embryonic stem cell	PRC	Polycomb repressive complex
ETP	early T lineage progenitor	Rb	Retinoblastoma
FACS	fluorescence-activated cell sorting / flow cytometry	RNAP	RNA polymerase
GC	germinal center	Rpd3	reduced potassium dependency 3
GMP	granulocyte-macrophage progenitor	RSS	recombination signal sequence
GTF	general transcription factor	SAHA	suberoylanilide hydroxamic acid
HAT	histone acetyltransferase	SHM	somatic hyper-mutation
HDAC	histone deacetylase	Sir2	silent information regulator 2
HDACi	HDAC inibitor	SIRT	sirtuin
HP1	heterochromatin protein 1	ST-HSC	short-term HSC
HSC	Hematopoietic stem cell	TAF	TBP-associated factor
IgH	immunoglobulin heavy chain	TBP	TATA-binding protein
IgL	immunoglobulin light chain	TF	transcription factor
IL	interleukin	TSA	trichostatin A