Deregulated expression of HDAC9 in B cells promotes development of lymphoproliferative disease and lymphoma in mice

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ABSTRACT

Histone deacetylase 9 (HDAC9) is expressed in B cells, and its overexpression has been observed in B-lymphoproliferative disorders, including B-cell non-Hodgkin lymphoma (B-NHL). We examined HDAC9 protein expression and copy number alterations in primary B-NHL samples, identifying high HDAC9 expression among various lymphoma entities and HDAC9 copy number gains in 50% of diffuse large B-cell lymphoma (DLBCL). To study the role of HDAC9 in lymphomagenesis, we generated a genetically engineered mouse (GEM) model that constitutively expressed an HDAC9 transgene throughout B-cell development under the control of the immunoglobulin heavy chain (IgH) enhancer (Eμ). Here, we report that the Eμ-HDAC9 GEM model develops splenic marginal zone lymphoma and lymphoproliferative disease (LPD) with progression towards aggressive DLBCL, with gene expression profiling supporting a germinal center cell origin, as is also seen in human B-NHL tumors. Analysis of Eμ-HDAC9 tumors suggested that HDAC9 might contribute to lymphomagenesis by altering pathways involved in growth and survival, as well as modulating BCL6 activity and p53 tumor suppressor function. Epigenetic modifications play an important role in the germinal center response, and deregulation of the B-cell epigenome as a consequence of mutations and other genomic aberrations are being increasingly recognized as important steps in the pathogenesis of a variety of B-cell lymphomas. A thorough mechanistic understanding of these alterations will inform the use of targeted therapies for these malignancies. These findings strongly suggest a role for HDAC9 in B-NHL and establish a novel GEM model for the study of lymphomagenesis and, potentially, preclinical testing of therapeutic approaches based on histone deacetylase inhibitors.

KEY WORDS: HDAC9, Lymphoma, Transgenic mouse

INTRODUCTION

Non-Hodgkin lymphoma (NHL) are a heterogeneous group of cancers of B, T or natural-killer cells, and constitute 4-5% of all cancers (Ferlay et al., 2013; Siegel et al., 2015), with diffuse large B-cell lymphoma (DLBCL) being the most common subtype, accounting for 31% of all adult NHLs (Martelli et al., 2013). Overall, approximately five cases of NHL per 100,000 individuals are identified annually (rising to 12 per 100,000 in North America), with incidence increasing, especially in developed countries. Similarly, NHL is the 11th most common cause of cancer death worldwide, resulting in around 200,000 deaths in 2012. Despite improvements in 5-year relative survival rates to 70% over the last four decades (largely due to the use of antibodies and antibody–drug conjugates directed against cell-surface antigens), patients with relapsed or refractory disease continue to have poor outcomes (Ansell, 2015; Grover and Park, 2015). New approaches are therefore required in NHLs, and therapeutic targeting of epigenetic modifiers, including histone deacetylases (HDACs), holds great promise (Hassler et al., 2013).

HDACs catalyze deacetylation of acetylated lysine residues on histones, and are also being found to act on a growing number of non-histone proteins (Haberland et al., 2009; Yang and Seto, 2008). As such, the functional interaction networks of HDACs encompass many biological and cellular processes beyond chromatin modification and gene regulation. In humans, there are 11 canonical HDACs grouped into three major classes: class I comprises HDACs 1, 2, 3 and 8; class II comprises HDACs 4, 5, 6, 7 and 9 and; and class IV is represented by HDAC11. HDAC9, alongside HDACs 4, 5 and 7, forms the class IIa subfamily, and these proteins are key transcriptional co-regulators in development and differentiation (Martin et al., 2009). Mutation or aberrant expression of HDAC9 has been implicated in diverse conditions, including ischemic stroke, schizophrenia and obesity (Bellenguez et al., 2012; Chatterjee et al., 2014; Lang et al., 2012), and also as a maker of poor outcome in cancer (Milde et al., 2010; Moreno et al., 2010). HDAC9, which is subject to complex regulation via differential promoter usage and alternative splicing, is preferentially expressed in the lymphoid lineage within the hematopoietic system (Petrie et al., 2003). HDAC9 is highly expressed in B-lymphoproliferative disorders, including in B-cell non-Hodgkin lymphoma (B-NHL) cell lines and patient samples, suggesting that its deregulation might lead to abnormal B-cell proliferation (Petrie et al., 2003; Sun et al., 2011). These findings are supported by the recurrent amplification of the HDAC9 locus (chr. 7p21.1) in B-NHL (Bea et al., 2005; Bentz et al., 1999, 1996; Monni et al., 1996; Rubio-Moscardo et al., 2005; Tagawa et al., 2005). Additionally, a number of HDAC inhibitors have been shown to induce cell death in B-NHL cells (Haery et al., 2015; Lemoine and Younes, 2010). Although several in vivo mouse models examining...
the biological functions of the class I and II HDACs are available (Witt et al., 2009), a role for HDAC9 or other family members in B-NHL has not been examined in vivo. The study of Hdac9
c−knockout mice has, however, highlighted HDAC9 as an important factor in inhibiting the generation and function of regulatory T (Treg) cells (Tao et al., 2007; Yan et al., 2011).

Underlining a potential role in B-NHL, HDAC9 interacts with BCL6 (Basso et al., 2010; Miles et al., 2005; Petrie et al., 2003), a transcriptional repressor that is crucial for germinal center (GC) formation (Basso and Dalla-Favera, 2012). Transgenic mice that constitutively express BCL6 in B cells develop a lymphoproliferative syndrome that culminates with the development of B-NHL (Cattoretti et al., 2005). BCL6 directly recruits class-II HDACs through its zinc-finger domain (Lemercier et al., 2002), and its transcriptional targets in GC B cells include TP53, thus modulating DNA-damage-induced apoptotic responses (Phan and Dalla-Favera, 2004). Evidence for a major role for defective acetylation in the pathogenesis of B-NHL is supported by the frequent occurrence of structural alterations inactivating CREBBP and EP300, genes encoding two highly related histone acetyltransferases (HATs) and non-HATs (Pasqualucci and Dalla-Favera, 2015; Pasqualucci et al., 2011). These mutations lead to aberrant activation and deactivation, respectively, of BCL6 and p53 (Pasqualucci et al., 2011), and we hypothesized that aberrant HDAC9 expression could also interfere with p53 and/or BCL6. We therefore sought to characterize HDAC9 expression in human B-cell lymphomas and establish whether aberrant expression can drive B-cell lymphoma in a genetically engineered mouse (GEM) model. Here, we report the development of a GEM in which an HDAC9 transgene was constitutively expressed in B cells under the control of the immunoglobulin heavy chain (Eμ) enhancer (Eμ-HDAC9). Eμ-HDAC9 mice developed B-cell lymphoproliferative disorders with progression towards B-NHL. This is consistent with the hypothesis that deregulated protein acetylation plays a pathological role in B-NHL, and provides a model for preclinical evaluation of HDAC inhibitors (HDACIs).

RESULTS

Within the immune system, a role for HDAC9 in the control of Treg cell function has previously been described (Beier et al., 2012; de Zoeten et al., 2010; Parra, 2015; Tao et al., 2007), and we found that, in normal human mature B cells, HDAC9 mRNA expression is significantly upregulated in the GC (Petrie et al., 2003) (Fig. 1A). HDAC9 protein is detected in a subset of GC cells, where it is co-expressed with BCL6 (Fig. 1A), as well as in a subset of lymphoid cells in the mantle zone and paracortex (Klein et al., 2003) (Fig. 1B). High HDAC9 gene expression in B-lymphoproliferative disorders, including B-NHL cell lines and patient samples, has pointed to a potential role in these diseases (Petrie et al., 2003; Sun et al., 2011). In line with these findings, we detected high HDAC9 protein levels among various lymphoma entities, including DLBCL (n=34), marginal zone lymphoma (MZL) (n=5), follicular lymphoma (FL) (n=9), classical Hodgkin lymphoma (CHL) (n=3) and mantle cell lymphoma (MCL) (n=6). Highest levels of HDAC9 expression were observed in the most aggressive lymphomas, such as DLBCL (both GC and non-GC subtypes) and MCL (77% and 83%, respectively, P=1.0, Fisher’s exact test). In contrast, low-grade B-cell lymphomas, as well as CHL, showed low HDAC9 expression in tumor cells when nuclear intensity was compared with that of adenocarcinoma cells as a positive control (P=0.004, Fisher’s exact test) (Fig. 1C). In addition to high HDAC9 expression, frequent amplification of the HDAC9 locus (chr. 7p21.1) has been observed in B-NHL (Bea et al., 2005) and, consistent with these results, we found copy number gains of HDAC9, including high-level amplifications, in 46.3% (25/54) of DLBCL patients (Fig. S1). A total of 46% (13/28) of samples with HDAC9 copy number gains presented trisomy 7 (Fig. S1A), whereas 43% (12/28) of cases reported with smaller regions of amplification within the chromosome that contained the HDAC9 gene (Fig. S1B). Here, one case displayed a specific amplification of HDAC9 (18,409,840-18,605,177 bp) (Fig. S1C, Table S1).

Although several in vivo mouse models examining the biological functions of the class I and II HDACs are available (Parra, 2015), a role for HDAC9 or other class-IIa family members in B-NHL has never been examined in vivo. We therefore expressed a human HDAC9 transgene (HDAC9TG) in the B-cell compartment from an early stage of B-cell development under the control of the immunoglobulin heavy chain (Igh) enhancer (Eμ) (Fig. S2A). We generated three independent transgenic lines (designated as 1468, 1469 and 1839) (Fig. S2B) and monitored a total of 124 mice (78 Eμ-HDAC9 and 46 wild type) for tumor formation and overall survival. We found expression of the HDAC9TG in the bone marrow and spleen but not in the liver (Fig. 2A). We detected expression of HDAC9TG throughout all B-cell stages in the bone marrow (pro-B, pre-B and naive-B) and spleen (transitional, marginal zone and follicular), with greatest expression of HDAC9TG found in the splenic marginal zone (Fig. 2B,C). When analyzed between 6 and 12 months of age, a fraction (3/17, 18%) of Eμ-HDAC9 mice exhibited splenomegaly (Fig. S3A,B), compared to 0/10 wild-type littersmates. Histopathology and fluorescence-activated cell sorting (FACS) analysis revealed evidence of abnormal B-cell expansion in the spleen, compatible with the development of lymphoproliferative disorder (LPD) (n=1), and splenic MZL (SMZL) (n=2); no abnormalities were observed in control mice (0/10) (P<0.0001) (Fig. S3A-C). Analysis of immunoglobulin (Ig) gene rearrangements in these mice confirmed monoclonal expansions of B-cell populations in 2/3 young-adult Eμ-HDAC9 mice at 8 months of age (Table 1). These results closely mirror those for a GEM model constitutively expressing BCL6 in B cells under the control of the immunoglobulin heavy chain (Igh) Iμ promoter (Cattoretti et al., 2005). A remarkably similar fraction of these mice (4/24, 17%) also displayed LPD at 6 months of age, representing early stages of lymphomagenesis before the evolution and onset of a B-cell neoplasm later in life (Cattoretti et al., 2005).

With age (i.e. past 14 months), Eμ-HDAC9 mice developed a significantly higher frequency of lymphoproliferations compared to control animals, such that, by 21 months of age, 48% of Eμ-HDAC9 mice survived compared with 95% of wild-type control mice (P=0.0010, Fig. 3A). Phenotypic analysis in a subset of animals revealed that approximately 40% (line 1469), 50% (line 1468) and 0% (line 1467) of Eμ-HDAC9 mice carried at least one copy of the Eμ-HDAC9 transgene (Fig. S2A). We monitored a total of 124 mice (78 Eμ-HDAC9 and 46 wild type) for tumor formation and overall survival. We found expression of the HDAC9TG in the bone marrow and spleen but not in the liver (Fig. 2A). We detected expression of HDAC9TG throughout all B-cell stages in the bone marrow (pro-B, pre-B and naive-B) and spleen (transitional, marginal zone and follicular), with greatest expression of HDAC9TG found in the splenic marginal zone (Fig. 2B,C). When analyzed between 6 and 12 months of age, a fraction (3/17, 18%) of Eμ-HDAC9 mice exhibited splenomegaly (Fig. S3A,B), compared to 0/10 wild-type littersmates. Histopathology and fluorescence-activated cell sorting (FACS) analysis revealed evidence of abnormal B-cell expansion in the spleen, compatible with the development of lymphoproliferative disorder (LPD) (n=1), and splenic MZL (SMZL) (n=2); no abnormalities were observed in control mice (0/10) (P<0.0001) (Fig. S3A-C). Analysis of immunoglobulin (Ig) gene rearrangements in these mice confirmed monoclonal expansions of B-cell populations in 2/3 young-adult Eμ-HDAC9 mice at 8 months of age (Table 1). These results closely mirror those for a GEM model constitutively expressing BCL6 in B cells under the control of the immunoglobulin heavy chain (Igh) Iμ promoter (Cattoretti et al., 2005). A remarkably similar fraction of these mice (4/24, 17%) also displayed LPD at 6 months of age, representing early stages of lymphomagenesis before the evolution and onset of a B-cell neoplasm later in life (Cattoretti et al., 2005).

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Molecular analysis of the rearranged IgH genes from Eμ-HDAC9 B-NHL samples confirmed their monoclonal origin (Fig. 3E) with
evidence of somatic hypermutation (SHM) of IgV genes in 4/7 (57%) of tumors (Fig. S5). Thus, we concluded that Eµ-HDAC9 lymphomas and LPDs were derived from B-cell precursors, with evidence of transit through the GC or having experienced the GC reaction. In mice, the lymphomas and LPDs exhibited both GC and post-/non-GC immunophenotypes (Table 1). Immunohistochemical analysis revealed heterogeneous expression of BCL6, including cases that were below the detection level by immunohistochemistry (IHC), as previously observed in Iμ-BCL6-derived lymphomas (Cattoretti et al., 2005). Eμ-HDAC9 tumors were also found to express variable levels of IRF4 (MUM1), a marker for GC B cells and plasma cells, and typically found in non-GC-type DLBCL (Fig. 4 and Table 1) (Falini et al., 2000).

We next performed gene expression analysis, and a comparison of three representative DLBCLs from Eμ-HDAC9 mice versus normal B-cell populations indicated that Eμ-HDAC9 B-cell tumors cluster with GC B cells and separately from non-GC B cells, further supporting a GC origin, comparable with human tumors (Fig. 5A). We also compared the expression pattern of DLBCLs from Eμ-HDAC9 mice with three age- and gender-matched wild-type controls, identifying a total of 1469 upregulated and 307 downregulated transcripts (Table S2). Among the upregulated genes, pathway analysis using KEGG and gene ontology (GO) annotated databases revealed enrichment for genes involved in the cell cycle, cell division and response to DNA damage (Fig. 5B, Table 2, and Tables S3 and S4). Pathway-based hierarchical clustering (Good, 2000) for genes differentially expressed in Eμ-HDAC9 mice (Tables S5, S6 and S7) confirmed regulation of cell-cycle-related genes and pointed to modulation of MAPK/ERK pathways. Furthermore, analysis of predicted protein–protein interactions between KEGG-classified genes from HDAC9-TG B-cell tumors (Table S2) showed high network connectivity (Fig. 5C).
A number of differentially expressed genes from the Eμ-HDAC9-driven B-cell tumors were identified as direct targets for BCL6 and p53 that have roles in apoptosis, the cell cycle and B-cell receptor (BCR) signaling (Table S2). Additionally, signal transduction pathways revealed substantial enrichment for genes involved in G1/S and G2/M transition followed by the Polo-like kinase 1 (PLK1) pathway (Fig. 5D). Among the most upregulated genes found in HDAC9TG B-cell tumors were those encoding factors such as Plk1, Birc5, Cdk1, Aurka, Aurkb and Chek1, which are involved in proliferation and survival, G1/S and G2/M transitions, mitosis G1/S and G2/M transition followed by the Polo-like kinase 1 (PLK1) pathway (Fig. 5D). Among the most upregulated genes found in HDAC9TG B-cell tumors were those encoding factors such as Plk1, Birc5, Cdk1, Aurka, Aurkb and Chek1, which are involved in proliferation and survival, G1/S and G2/M transitions, mitosis.

**Table 1. Tumor type and IgH rearrangements in Eμ-HDAC9 mice**

| Mouse | Histopathology | Clonal statusa | IgHb | JH | B220 | CD3 | BCL6 | IRF4 | GC |
|-------|----------------|---------------|------|----|-----|-----|------|------|----|
| 1452c | SMZL           | Mono          | ND   | JH3 | ND  | ND  | ND   | ND   | U   |
| 1714  | SMZL           | Mono, UM      | –    | JH2 | +   | –   | –    | –    | –   |
| 1839  | SMZL           | Mono, UM      | –    | JH3 | +   | –   | –    | +/−  | −   |
| 1851  | DLBCL          | Mono, M(21)   | IgHV5| JH4 | +   | –   | +    | +/−  | +   |
| 1759  | DLBCL          | Mono          | ND   | JH3 | ND  | ND  | ND   | ND   | −   |
| 1454c | SMZL           | Oligo, M(28)  | IgHV1,2,5| JH4 | ND  | ND  | ND   | ND   | +   |
| 1469  | LPD            | Mono          | ND   | JH3 | ND  | ND  | ND   | ND   | U   |
| 1546c | SBCL/L, NOS    | Mono          | IgHV1| JH2 | ND  | ND  | ND   | ND   | U   |
| 1543  | SBCL/L, NOS    | Mono          | ND   | JH4 | ND  | ND  | ND   | ND   | U   |
| 1578  | HS             | ND            | ND   | ND  | ND  | ND  | ND   | ND   | U   |
| 4259  | HS             | ND            | ND   | ND  | −   | –   | –    | –    | U   |
| 4698c | LPD            | ND            | ND   | ND  | ND  | ND  | ND   | ND   | D   |

Classification based on H&E analysis. Italics: histopathology diagnosis only.

SMZL, splenic marginal zone lymphoma; DLBCL, diffuse large B-cell lymphoma; LPD, lymphoproliferative disease; SBCL/L, NOS, small B-cell lymphoma/leukemia not otherwise specified; HS, histiocytic sarcoma; U, unknown; Mono, monoclonal; Oligo, oligoclonal; ND, not determined; M(n), mutated (number of mutations); UM, unmaturated; GC, germinal center phenotype; +, expression of protein; −, no expression of protein; +/−, variable expression of protein.

aPCR products from tumors were compared to wild-type B cells to detect allele rearrangements.

bMouse IMGT IgHV subgroup.

cAnalyzed at 8 months of age.
and DNA-repair/checkpoint-mediated arrest (Table 2). A role for HDAC9 in proliferation and control of the cell cycle was confirmed by zinc-finger nuclease (ZFN)-mediated gene editing of HDAC9 in proliferation and control of the cell cycle was confirmed and DNA-repair/checkpoint-mediated arrest (Table 2). A role for HDAC9 in proliferation and control of the cell cycle was confirmed and DNA-repair/checkpoint-mediated arrest (Table 2).

A potential role for HDAC9 in the pathogenesis of B-NHL is strengthened by its interaction with BCL6 (Petrie et al., 2003), a transcriptional repressor crucial for GC formation (Basso and Dalla-Favera, 2004). BCL6 and p53 function in a negative-feedback loop whereby p53 promotes BCL6 expression, which in turn suppresses the expression of TP53 (Margalit et al., 2006; Phan and Dalla-Favera, 2004). BCL6 and p53 function in a negative-feedback loop whereby p53 promotes BCL6 expression, which in turn suppresses the expression of TP53 (Margalit et al., 2006; Phan and Dalla-Favera, 2004).

The BCL6–p53 axis is further modulated by post-translational acetylation of BCL6, which leads to its inactivation. Indicative of a potential role for HDAC9 in the acetylation of BCL6 in vivo, acetylated BCL6 was found to be abundant in normal mouse spleens but undetectable in HDAC9TG tumors (Fig. 7A). p53 is also post-translationally modified by acetylation, which is indispensable for its transcriptional activity in response to DNA damage and stress (Tang et al., 2008).

Highlighting the loss of p53 tumor suppressor function as a contributory factor in the development of B-cell lymphomas, B-cell-specific disruption of TP53 leads to the development of B-NHL (Chiang et al., 2012). Consistent with this, as well as with recent research demonstrating that HDAC9 binds to the TP53 promoter to repress gene expression (Zhao et al., 2015), we found diminished levels of total p53 as well as Lys379 acetyl-p53 in Eµ-HDAC9 (Fig. 7B,C).

**DISCUSSION**

**HDAC9** is highly expressed in B-NHL cell lines and patient samples (Petrie et al., 2003; Sun et al., 2011), and its locus, chromosome 7p21.1, is frequently amplified in B-NHL (Bea et al., 2005; Bentz et al., 1999; Monni et al., 1996; Rubio-Moscardo et al., 2005; Tagawa et al., 2005). In order to establish whether deregulated expression of HDAC9 in the lymphoid compartment could generate a disease phenotype, we designed a GEM model in which a human HDAC9 transgene was expressed under the control of the Eµ promoter. Our hypothesis was based on evidence of deregulation of HDAC9 expression in B-NHL cell lines and patient samples. The occurrence of B-NHL in these transgenic mice strongly indicates a link between deregulated HDAC9 expression and lymphoid tumors (Fig. 7A).
neoplasia, the first time that overexpression of a histone deacetylase in mice has resulted in a cancer phenotype. This is also, to our knowledge, the first time that expression of a single epigenetic transgene led to lymphoma—expression of mutated polycomb-group gene $\text{EZH2}$ fails to drive lymphomagenesis unless in a background of overexpressed $\text{BCL2}$ or $\text{Myc}$ (Béguelin et al., 2013; Berg et al., 2014). This does not, however, rule out a requirement for the acquisition of additional mutations in order for progression to lymphoma in $\text{Eµ-HDAC9}$ mice. Our results are also in agreement with a recent study utilizing an $\text{MRL/lpr}$ GEM model of systemic lupus erythematosus with $\text{HDAC9}$ deficiency (Yan et al., 2011). Here, $\text{MRL/lpr}$ transgenic mice lacking $\text{HDAC9}$ displayed decreased lymphoproliferation and expression of $\text{BCL6}$. Although the SMZL cases studied in $\text{Eµ-HDAC9}$ mice were $\text{BCL6}$-negative, it is generally accepted that MZL in humans is derived from a post-GC B cell, with associated somatic mutations in the IgVH gene (Dunn-Walters et al., 1998; Miranda et al., 1999; Zhu et al., 1995). Moreover, progression of indolent lymphoma (SMZL) to a more aggressive lymphoma (DLBCL) is a frequent occurrence for many subtypes of indolent B-cell lymphomas and could explain the late onset of aggressive lymphomas observed in our transgenic model (Camacho et al., 2001; Freedman, 2005). The development of SMZL in $\text{Eµ-HDAC9}$ mice is also consistent with results obtained in mice in which $\text{TP53}$ was disrupted specifically in B cells, leading to the development of highly penetrant SMZL (Chiang et al., 2012). Our results indicated that aberrant expression of $\text{HDAC9}$ in B cells leads to the upregulation of pathways that promote cell growth and survival, as well as impacting the activity and expression of key factors in lymphoma $\text{BCL6}$ and $\text{p53}$. The notion that transgenic expression of $\text{HDAC9}$ can promote lymphomagenesis, in part through deregulation of the activities of $\text{p53}$, is strengthened by studies suggesting that $\text{p53}$ might directly bind to the $\text{HDAC9}$ promoter and repress its expression (Akdemir et al., 2014; Wei et al., 2006). Of note, the $\text{p53}$-binding site in the $\text{HDAC9}$ promoter overlaps with a myocyte enhancer factor 2 (MEF2)-binding site, which, when bound by MEF2 family members, activates $\text{HDAC9}$ gene expression (Haberland et al., 2007). Activating mutations of $\text{MEF2B}$ (which occur in 11% of DLBCL and 12% of FL) have been reported to directly upregulate expression of $\text{BCL6}$ (the promoter of which also contains a MEF2-binding site) in GC B cells and drives DLBCL proliferation (Ying et al., 2013). It remains to be established whether mutant $\text{MEF2B}$ can drive $\text{HDAC9}$ expression in B-cell lymphomas, but recent research has identified a novel $\text{MEF2D–BCL9}$ fusion protein associated with high-risk acute B-cell precursor lymphoblastic leukemia (ALL) that directly upregulates $\text{HDAC9}$ (Suzuki et al., 2016). High expression of $\text{HDAC9}$ has been independently linked to poor prognosis in ALL (Moreno et al., 2010).

In recent years, numerous structurally diverse HDAC inhibitors (HDACi) have emerged as clinical candidate therapeutic agents (West and Johnstone, 2014), including the recent development of class-Ila-specific HDACi (Lobera et al., 2013). Even in the case of cutaneous T-cell lymphoma (CTCL) where HDACi have shown efficacy as single-agent targeted therapies and been approved for use in the clinic (Prince and Dickinson, 2012), the use of HDACi in rational combinations will likely maximize their therapeutic potential. It is,
therefore, of interest that genes upregulated in Eµ-HDAC9 tumors, such as Cdk1, Chek1, Aurka and Aurkb, represent important clinical targets for which late-phase clinical trials are ongoing (Garrett and Collins, 2011; Lapenna and Giordano, 2009; Micel et al., 2013). The upregulation of Plk1 is also of potential clinical interest given that its high expression is a negative prognostic indicator in B-NHL (Liu et al., 2007; Xu et al., 2013; Yim et al., 2013). PLK1 plays a crucial role at checkpoint controls during G2/M transition of the mitotic cell cycle (Barr et al., 2004) and inhibits p53 function directly by phosphorylation (Ando et al., 2004). Therefore, the Eµ-HDAC9 GEM model could serve as a valuable tool both to better understand the molecular mechanisms involved in lymphomagenesis in humans and facilitate preclinical studies of new drugs and combination therapies.
Table 2. Gene expression profiling of Eμ-HDAC9 tumors

| Gene   | Fold change | Accession #                  |
|--------|-------------|------------------------------|
| Mki67  | 3.9         | NM_001081117                 |
| Aans   | 3.4         | NM_012055                    |
| Ccna2  | 3.1         | NM_009828                    |
| Cdc6   | 3.1         | NM_011799                    |
| Ube2c  | 3.1         | NM_026785                    |
| Pik1   | 2.6         | NM_011121                    |
| Top2a  | 2.6         | NM_011623                    |
| Kif11  | 2.6         | NM_010615                    |
| H2afx  | 2.6         | NM_010436                    |
| Prc1   | 2.5         | NM_145100                    |
| Ccnb2  | 2.4         | NM_007630                    |
| E2B   | 2.4         | NM_10101368                  |
| Cdkl   | 2.3         | NM_007669                    |
| Sgol1  | 2.3         | NM_028232                    |
| Itlb   | 2.3         | NM_008361                    |
| Kif18b | 2.3         | AK013867                     |
| Birc5  | 2.2         | NM_009689                    |
| Cdkn3  | 2.2         | NM_028222                    |
| Anin   | 2.2         | NM_028390                    |
| Apsm   | 2.2         | NM_009791                    |
| EzH2  | 2.1         | NM_007971                    |
| Dlgap5 | 2.1         | NM_144553                    |
| Mcm10  | 2.1         | NM_027290                    |
| Bub1   | 2.1         | NM_009773                    |
| Ect2   | 2.1         | NM_014700                    |
| Chek1  | 2.1         | ENSMUST0000173796            |
| Nusap1 | 2.1         | NM_10042652                  |
| Tpx2   | 2.1         | NM_028109                    |
| Ncapg2 | 2.1         | NM_133762                    |
| Slt1   | 2.1         | NM_011407                    |
| Cenpf  | 2.1         | AK029617                     |
| Casc5  | 2.1         | NM_029617                    |
| Sept   | 2.1         | AK144178                     |
| Gpsmn2 | 2.1         | Trans_XM_002762048           |
| Esco2  | 2.1         | NM_028039                    |
| Ncapg  | 2.1         | NM_019438                    |
| Ptgs2  | 2.1         | NM_011198                    |
| F630043A04Rik | 2 | AK_158142 |
| Cks1b  | 2           | NM_016904                    |
| Cdc3a  | 2           | NM_013538                    |
| Bcl2l1 | 2           | NM_009743                    |
| Aurkb  | 2           | NM_011496                    |
| Cd28   | 2           | NM_007642                    |
| Aurka  | 2           | ENSMUST00000128004           |
| Sgol2  | 2           | AK079371                     |
| Dit    | 2           | NM_029766                    |
| Ccne2  | 2           | NM_009830                    |
| Cdc2a  | 2           | ENSMUST00000124045           |
| Bub1   | 2           | NM_009772                    |
| Cld   | 2           | AK048096                     |
| Nuf2   | 2           | NM_023284                    |
| Bcat1  | 2           | ENSMUST00000145911           |
| Cdc20  | 2           | AK029424                     |
| Kif2c  | 2           | AK015046                     |
| Fign1  | 2           | NM_001163360                 |
| Steap3 | 2           | NM_133186                    |
| Trp53inp1 | 2 | AK054214 |

MATERIALS AND METHODS

Generation of Eμ-HDAC9 transgenic mice

FLAG-epitope-tagged full-length human HDAC9 cDNA was cloned into the pEμSR vector, placing the HDAC9-sequence-containing oligonucleotide cassette downstream of the immunoglobulin heavy chain (IgH) enhancer (Eμ) and the SV40 potent promoter (Bodrug et al., 1994). The Eμ-HDAC9 transgenic fragment was generated from the vector by enzymatic digestion using the Ncol restriction sites and injected into B6CBAF1 pronuclei. Mice were backcrossed and maintained in a C57BL/6 background to generate three transgenic lines: 1468, 1469 and 1839. PCR genotyping was performed using SV40 primers: F: 5’-GGAACTGATGAACTGGGAGCACA-3’ and R: 5’-GCAGTCAGCCTTTTCTTCTT-3’. Mice were housed and maintained in accordance with UK Home Office regulations. Animals were monitored and analyzed from birth to 23 months of age and sacrificed if showing signs of illness. Statistical analysis was performed using Prism (GraphPad Software). Kaplan–Meier cumulative survival and the log-rank (Mantel–Cox) test were used to determine tumor-free survival and the log-rank test was used to compare B-NHL incidence in Eμ-HDAC9 mice versus wild-type controls. P<0.05 was considered statistically significant. All experimental protocols were monitored and approved by The Institute of Cancer Research Animal Welfare and Ethical Review Body, in compliance with guidelines specified by the UK Home Office Animals (Scientific Procedures) Act 1986 and the United Kingdom National Cancer Research Institute guidelines for the welfare of animals in cancer research (Workman et al., 2010). ARRIVE guidelines were applied when reporting in vivo experiments (Kilkenny et al., 2010).

Ig gene rearrangements analysis

Genomic DNA was isolated from tumor specimens and prepared using All Prep Kit (Qiagen). Primers for detection of Ig rearrangements were described previously (Ehlich et al., 1994). D-JH rearrangements of the heavy chain (JH) locus were amplified and detected in a multiplex PCR reaction using two upstream primers, DFL/DSP and DQ52, together with one reverse primer positioned downstream of JH4 (Mårtensson et al., 1997). For mutational analysis, the rearranged Ig variable heavy chain genes were amplified from genomic DNA as previously described (Cattoretti et al., 2005). V-D-JH rearrangements were analyzed in separate PCR reactions using primers V HJ558a (5’-CAGGCACAGCGACAGCTGTGG-3’), V HJ7183b (5’-GTTAGGACTGGGAGGCTCC-3’), V HJQ52 (5’-CAGGTGCACTGGGACACATCGA-3’) and reverse JH4 primer (5’-TGGAGGAGGAGGACAGCTGGTGC-3’). The primers amplified all rearrangement products between JH4 and V HJ558, V HJ7183 or V HJQ52 where four different bands would be expected by the combination of each primer set. PCR reaction cycling conditions were: 95°C for 5 min, 95°C for 30 s, 63°C for 30 s and 72°C for 2 min, for 35 cycles. PCR products were gel-purified using the QIAQuick method (Qiagen) and directly sequenced using the ABI PRISM 310xl Genetic Analyzer (Applied Biosystems). Sequences were aligned to those in the international ImMunoGeneTics information system (IMGT) database.

Flow cytometry and cell sorting

Single-cell suspensions were obtained from dissected tissues, washed in phosphate buffered saline (PBS) supplemented with 0.2% FBS, filtered through a 45 μm cell strainer and red blood cells were lysed using ammonium chloride solution (STEMCELL Technologies). The antibody combination used for tumor analysis included: CD23, CD19, IgM, IgD, CD21 and B220. Anti-mouse conjugated antibodies were obtained from eBioscience and BioLegend. Cell sorting of splenic CD19-positive mouse B cells was carried out using immunomagnetic isolation with CD19-labeled beads (EasySep Mouse CD19 Positive Selection Kit, STEMCELL Technologies) and purity assessed using anti-mouse PE-conjugated CD19 antibody (eBioscience). Data were acquired on a FACS LSRII analyzer (BD Biosciences) and analyzed using FlowJo software (Tree Star).

Transgenic transcript detection

Total RNA from mouse bone marrow and spleen was extracted using TRIzol reagent (15596-026, Ambion) following the manufacturer’s instructions. Reverse transcription was performed following standard protocol by using AccessQuick Master Mix (A1701, Promega). Real-time quantitative PCR was carried out using Fast SYBR Green Master Mix (436802, Applied Biosystems). Primer sets used to detect mouse and human HDAC9 transcripts in B-cell subsets: F: 5’-TCTGGGATGTTCACCATGAGGA-3’ and R: 5’-CAGTCCAGGGAAGAGTCTC-3’. Primer sets to detect mouse Hdad9: F: 5’-GGTTAGTATGCTGGAGGTATTC-3’ and R: 5’-AAAGCAGCAGCTCAATGACACA-3’. Abill mRNA expression was used as normalization control, F: 5’-CAGGGCCAGTTCAGCTACTGACTT-3’ and R: 5’-GCTTCACACATCCCCCATT-3’.
Purification of B-cell subsets

Single-cell suspensions from spleen (n=3) and bone marrow (n=5) from 8- to 12-week-old mice (transgenic and wild type) were prepared as previously described (Green et al., 2011). Bone marrow and spleen B-cell subpopulations were identified and sorted by three-color FACS method (Green et al., 2011). Anti-mouse antibodies CD43, IgM and B220 were used to obtain Pro-B, Pre-B and Mature-B-cell fractions from bone marrow. Splenic B-cell subtypes, follicular and marginal zone, were purified using anti-mouse CD19, CD21 and CD23 antibodies, whereas a combination of anti-mouse B220, IgM and CD93 was used to sort for transitional B cells. Each subset was above 90% purity. Purified cells were re-suspended in TRIzol reagent (Life Technologies) for further RNA extraction.

Histopathology and immunohistochemistry (IHC)

Tissues were freshly collected and fixed in 10% formalin for 24 h, embedded in paraffin and sectioned. Following deparaffinization and rehydration, samples were pre-treated with antigen-unmasking solution (Vector Laboratories, H-3300). After pre-treatment, tissue sections were blocked in Tris-NaCl buffer, washed in PBS and permeabilized in 0.5% Triton X-100. Slides were washed in Tris-NaCl-Tween buffer and reacted with primary antibody overnight at 4°C. Preliminary experiments were performed to determine optimal dilutions for each primary antibody used. Detection methods included: standard IHC, Vectastain Elite ABC system, Vector Laboratories or EnVision system (Dako) DAB staining and ABC-tyramide signal amplification (TSA Plus Fluorescence System, Perkin Elmer) (see Fig. 7).

Fig. 6. HDAC9 regulates cell cycle progression. (A) Immunoblot analysis of HDAC9 expression in untreated wild-type Raji B-cell non-Hodgkin lymphoma (B-NHL) cells (control, lane 1), and the mutants GFP ZFN-derived control (lane 2) and HDAC9 ZFN-derived cells prior to single cell sorting (lane 3). Immunoblot analysis of GAPDH expression was used as loading control. (B) Cell growth curve of wild-type Raji control and representative ZFN-generated HDAC9 mutant clones 3B2 and 3G9 obtained by single-cell sorting. (C) 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay of proliferation of HDAC9 ZFN-derived clones. Shown are values for HDAC9 ZFN-derived mutant clones 3B2 and 3G9, a pool of unsorted HDAC9 ZFN mutants, and wild-type cells and untreated Raji cells. (D) Effects of HDAC9 depletion on cell cycle progression are depicted in histogram plots generated by propidium-iodide uptake.

Fig. 7. Eµ-HDAC9 tumors display deregulated acetylation of BCL6 and p53. (A) IHC triple-immunostaining using the ABC-TSA method in mouse spleens from Eµ-HDAC9 and wild-type tumors, showing HDAC9 (red) expression in conjunction with levels of acetylated (Ac)-BCL6 (green). (B) Immunoblot analysis of Ac-p53 in Eµ-HDAC9 and wild-type spleen. GAPDH was used as a loading control. (C) ABC-TSA immunofluorescence analysis of Ac-p53 (green) and HDAC9 (red) in spleen in Eµ-HDAC9 and wild-type controls.
below). Histopathological evaluation of HDAC9TG tumors was performed using hematoxylin and eosin (H&E)-stained sections. Immunophenotypic characterization of the HDAC9TG lymphomas was performed using antibodies against B220, PAX5, CD3, BCL6 and IRF4 as previously described (Mandelbaum et al., 2010). Human HDAC9 expression was assessed by IHC using tissue microarrays and/or individual tumor sections of 59 B-NHLs and three CHLs using a specific antibody generated against the C-terminal region of HDAC9 (Petrie et al., 2003). The B-NHL group consisted of 34 DLBCLs, nine FLs, five MZLs, six MCLs and two chronic lymphocytic leukemias/small lymphocytic lymphomas (CLLs/SLLs). Nuclear expression intensity of lymphomas was compared with that of rectal adenocarcinoma cells (positive control). If equal or higher intensity than control, HDAC9 expression was considered high (score 2+), and, if low, scored 1+. 

Immunoblotting and antibody validation

Cell lysates were prepared using RIPA buffer supplemented with protease inhibitors (Roche). Rat monoclonal (IgM) antibody specific to the C-terminus of human HDAC9 (clone 45a7b5b) was developed using a synthetic peptide (DVEQPFAQEDSRTAG) conjugated to Diphtheria toxoid (Mimotopes), corresponding to unique amino acids 1046-1060. Validation was performed using Mini-PROTEAN II Multiscreen Apparatus (Bio-Rad). Other antibodies used for immunoblots included mouse monoclonal anti-FLAG M2 antibody (F1804, Sigma), β-actin loading-control antibody (BA3R) (MA5-15739, Thermo Scientific) and acetyl-p53 (Lys379) (PA5-17287, Thermo Scientific).

IHC and immunofluorescence detection methods

Single immunolabeling using ABC-TSA

Formalin-fixed paraffin-embedded tissue sections were dried for 45 min at 58°C, followed by deparaffinization and hydration in HistoSeqelar (National Diagnostics) and a graded series of ethanol, respectively. Samples were pretreated by microwave incubation in a pH 6.0 citrate-based antigen-unmasking solution (Vector Laboratories, H-3300) followed by 2×5 min washes in PBS and permeabilization in 0.5% Triton X-100 (in PBS) for 20 min at room temperature (RT). Samples were washed in PBS prior to blocking for 30 min at RT in Triton-X11 (TNT) blocking buffer with subsequent incubation in the monoclonal anti-HDAC9 antibody (clone 45a7b5b, 1/100 dilution) overnight at 4°C. Primary antibody was followed by biotinylated rabbit anti-rabbit secondary antibody for 30 min at RT. Endogenous peroxidase activity was inactivated by incubation with 3% hydrogen peroxide in methanol for 15 min at RT. After washing in Triton-X11–TWEEN-20 (TNT) buffer, samples were incubated in streptavidin (SA)-horseradish peroxidase (HRP) (Vectastain ABC Elite Kit, Vector Laboratories, PK-6104) for 45 min at RT. Following this, FITC fluorophore tyramide (Perkin Elmer, NEL741) was added for 10 min at RT, which results in the deposition of numerous fluorophore labels adjacent to the HRP. Slides were washed 3×5 min in TNT buffer, any remaining HRP was deactivated by incubating in 3% hydrogen peroxide (in methanol) for 15 min at RT. A second primary antibody was added sequentially [anti-acetylated p53 (Lys379) Thermo Scientific, cat. # 17287, 1/100 dilution] and the protocol was repeated with the FITC fluorophore tyramide (Perkin Elmer, NEL741) utilized to detect acetylated p53. Slides were counterstained with To-pro 3 iodide (Life Technologies, T-3605) and mounted in Vectashield (Vector Laboratories, cat. # H-1000) mounting medium. Negative control slides were prepared by excluding the primary antibody, by excluding the conjugated secondary antibody-fluorophore and by excluding TSA reagents; negative controls showed no immunoreactivity. Single-labeling experiments carried out to observe the patterns of staining of each primary antibody validated the double-immunostaining results.

Triple immunolabeling

First, the primary antibodies monoclonal rat anti-HDAC9 (clone 7b5b, 1/100 dilution) and rabbit polyclonal anti-acetylated lysine (Millipore 06-933, 1/500 dilution) were added together and left overnight at 4°C. Following this, secondary anti-rat biotinylated antibody (1/200 dilution) was added and the TSA protocol was carried out using Cy3 fluorophore tyramide (Perkin Elmer, NEL753). Next, HRP was inactivated before the secondary antibody against acetylated lysine was added (anti-rabbit biotinylated) and the TSA protocol followed using FITC fluorophore tyramide (Perkin Elmer, NEL753). The HRP was once again inactivated with 3% hydrogen peroxide (in methanol) for 15 min at RT and rabbit polyclonal anti-BCL6 antibody (ab19011) (1/1000 dilution) was added (overnight at 4°C). Following this, the TSA protocol was applied using Cy5 fluorophore tyramide (Perkin Elmer, NEL745). Secondary-antibody controls were performed to detect any non-specific background staining. Single-staining controls were carried out as in the same-species double-labeling experiments.

IHC of human tumors

The EnVision detection system HRP/DAB+ (Dako) was used as previously described (Kim et al., 2009). Briefly, tissue sections were deparaffinized in xylene and rehydrated in ethanol following treatment in pre-heated target retrieval solution. Following washes, serum-free blocking solution was applied for 40 min at RT. In-house anti-HDAC9 monoclonal antibody was used overnight at 4°C then treated with polymer/HRP and DAB. After washes, the slides were counterstained with hematoxylin, dried and mounted with Permount. Photomicrographs were captured using an Olympus BX41 dual head light microscope equipped with an Olympus Q-Color 5 digital camera (Olympus America).

Antibodies

Primary antibodies included polyclonal rabbit anti-Ac-p53 (Lys379) (Thermo Scientific), monoclonal rat anti-HDAC9 antibody (clone 45a7b5b), monoclonal mouse anti-FLAG M2 (Sigma), polyclonal rabbit anti-acetylated lysine (Millipore 06-933), polyclonal rabbit anti-BCL6 (ab19011), anti-CD45R(B220) (ab64100) and anti-CD3 (ab5690). Secondary antibodies included: biotinylated rabbit anti-rat secondary antibody (Vector Laboratories, PK-6104), biotinylated horse anti-mouse secondary (Vector Laboratories, PK6102) and biotinylated goat anti-rabbit.
secondary (Vector Laboratories, PK6101), fluorochromes and chromogens included SA-HRP (Vector Laboratories, PK-6102), FITC fluorochrome tyramide (Perkin Elmer, NEL741, NEL753), FITC-HRP (Perkin Elmer, NEL710), Cy3 fluorochrome tyramide (Perkin Elmer, NEL741, NEL753), Cy5 fluorochrome tyramide (Perkin Elmer, NEL745) and AEC (AbD Serotec, BUF019B).

**High-density SNP array analysis**

Genome-wide DNA profiles were obtained from high-molecular-weight genomic DNA of DLBCL patients using the Affymetrix Genome-Wide Human SNP Array 6.0 (Affymetrix) following the manufacturer’s instructions. Image data analysis and quality control for the hybridized samples were performed using the Affymetrix Genotyping Console 3.0 software, and only samples passing the Affymetrix recommended contrast QC and SNP call rates threshold (in the Birdseed v2.0 algorithm) were considered for analysis. Affymetrix CEL files and corresponding SNP genotype call files generated by the Affymetrix Genotyping Console tool were then analyzed using the dChip software. Model-based expression was performed using the perfect-match/mismatch (PM/MM) model to summarize signal intensities for each probe set. Probe intensity data for each array were normalized using a diploid reference set of three normal (non-tumor) DNA samples that had been processed and hybridized in the same experiment as the tumor samples. The standard invariant-set normalization approach in dChip was implemented by a karyotype-guided normalization method as previously described (Mullighan et al., 2007; Pounds et al., 2009). To identify regions of amplification and deletion, the circular binary segmentation algorithm was applied to the SNP array data as described (Mullighan et al., 2007). The following criteria were used to obtain candidate genomic regions (gains or losses): (1) mean log2 ratios of ≥0.2 or ≤−0.2; (2) ≥8 SNP markers within a segment. The results of the CBS algorithm were then compared to those of dChip. To exclude calls of genomic gains or loss arising from inherent genomic copy number variants (CNVs), the dCHIPSNP algorithm was also applied to 130 normal DNAs from an independent study as well as to 230 normal DNAs from the HapMap project; alterations identified in the pool of reference samples were excluded. In addition, CNVs were excluded if present in the Database of Genomic Variants (http://projects.tcag.ca/vari-bin/variation/gbrowse/hg18/).

**Gene expression analysis**

Affymetrix GeneChip Mouse Gene 1.0 ST hybridizations were performed using biotin end-labeled cDNA prepared from CD19-positive B cells isolated from tumors. Unsupervised hierarchical clustering was performed on gene expression data from representative HDCA*9/G*9-derived lymphomas versus normal murine mature B-cell subpopulations, including GC and non-GC (folicular and marginal zone) B cells (GeneChip Mouse Gene 1.0 ST Arrays). Data from normal B-cell subsets were obtained from the Immunological Genome Project (GSE15907) (www.impactgen.org). Only probes with minimal expression level equal to 10 and minimal standard deviation of 1.5 (log2 transformed) were considered. The hierarchical clustering algorithm is based on the average-linkage, Pearson correlation.

Gene data sets were also analyzed for interactions and pathways using: GGA (Genomatix Genome Analyzer, https://mygga.genomatix.de), the Search Tool for the Retrieval of Interacting Genes/Proteins STRING v9.1 (http://string-db.org) to develop interactomes or networks (Franceschini et al., 2013) and the KEGG pathway database (Kanehisa et al., 2004). GO (Ashburner et al., 2000) clustering was performed with cslb.go (Ovaska et al., 2008). Partek Genomics Suite 6.6 was additionally used for data set analysis and comparisons.

**Zinc-finger nuclease (ZFN) knockout of HDAC9**

ZFNs targeting human HDAC9 sequence were obtained from Sigma-Aldrich (CompoZr Knockout Zinc Finger Nucleases, KCOZFD9H51-1KT). The ZFN binding-cutting site (in lowercase) was 5’-CTCTGGTCCAGGTTACCAAAATGGGCCACTGAGGTG3’. Delivery of ZFN was performed following the manufacturer’s protocol (Sigma-Aldrich). The human Burkitt’s lymphoma cell line Raji (ATCC CCL-86) was used for the study. Cells were maintained in RPMI medium 1640 (1+5) (Gibco) supplemented with 10% FBS (Sigma-Aldrich) and grown in a 5% CO2 incubator at 37°C. Cells were transfected by nucleoeption (electroporation) using Amaza Cell Line Nucleofector Kit V (Lonza), and grown for 48 h followed by single-cell sorting in a 96-well format using BD FACS Aria (BD Biosciences). After 3-4 weeks, single-cell-derived clones were screened and analyzed using CEL-I assay (SURVEYOR mutation detection assay) following the manufacturer’s instructions (Transgenomic). Genomic DNA was obtained by high-throughput HotSHOT DNA preparation method in 96-well plates. ZFN mutant clones were confirmed by sequencing.

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**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**

V.S.G. conceived the study, performed all experiments unless otherwise indicated, analyzed the data, performed statistical analyses, and co-wrote and co-edited the manuscript. G.B., L.H. and C.H.K. performed immunohistochemistry and immunofluorescence experiments. G.B. and F.V. carried out histopathological analysis of tumor samples. J.Z. and S.S. performed genetic and expression microarray analysis of tumor samples. A.Z. conceived the study and analyzed the data. K.P. conceived the study, analyzed the data, and co-wrote, co-edited and submitted the final version of the manuscript.

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**Data availability**

Microarray expression profiles of HDAC9/G*9 mouse tumors and wild-type counterparts have been deposited The Gene Expression Omnibus (GEO) (https://www.ncbi.nlm.nih.gov/geo) under accession numbers GSE89954 and GSE89955. The GEO Series accession number for this study is GSE89956.

**Supplementary information**

Supplementary information available online at http://dmm.biologists.org/lookup/doi/10.1242/dmm.023366 supplemental

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Supplementary Fig. 1. High-density SNP array analysis of HDAC9 copy number. (A) dChip SNP inferred log2 ratio heatmap of chromosome 7 in 54 DLBCL samples and 3 normal diploid DNAs. The chromosome 7 ideogram is aligned on the left side and the arrow points to band 7p21.1, where the HDAC9 gene is located. (B) dChip SNP inferred log2 ratio heatmap showing chromosomal region 7p21.1 in 25 DLBCL samples carrying copy number gains encompassing the HDAC9 gene, as compared to 3 normal DNA samples. The right panel depicts median smoothed log2 ratio copy number plot for patient 2184 (denoted by an asterisk in the heatmap), carrying a focal amplification of HDAC9. (C) The figure shows chromosomal region 7p21.1, encompassing the HDAC9 gene (boxed area in the ideogram); red lines aligned below the gene indicate the extent of genomic gains affecting HDAC9 in individual DLBCL cases, as obtained from SNP array data. A majority of these samples harbor chromosome 7 trisomy (see supplemental Table 2).
**Supplementary Fig. 2.** Generation of Eµ-HDAC9 mice. (A) Schematic representation of the Eµ-HDAC9 transgene (5.88Kb) containing FLAG-epitope tagged human HDAC9 cDNA cloned between SmaI–SpeI sites within the modified polylinker of original vector pEµSR. The fragment also contains the mouse immunoglobulin heavy chain (IgH) enhancer (Eµ), SRα promoter, rabbit globin poly(A) addition region (pA) and SV40, a simian virus sequence for identification of transgenic mice. (B) Estimation of transgene copy number by Southern blot analysis. Genomic DNA from from indicated mice was analyzed by restriction enzyme (RE) digestion with SapI. Genomic DNA from indicated mice was digested with PstI. Restriction fragments were visualized with a BglI-digested probe from the HDAC9 transgene. Copy number was calculated approximately based on the intensity signal of the bands for standard controls 0, 1, 5, 25 and 50 copies used in the same experiment.

| Line  | Transgene copy number | Number of integration sites |
|-------|------------------------|-----------------------------|
| 1468  | 10-20                  | 1                           |
| 1469  | ~50                    | 1                           |
| 1839  | 25-50                  | >1                          |
Supplementary Fig. 3. Early onset of disease in Eμ-HDAC9 mice. (A) Three mice were diagnosed with malignant B-cell disorders up to 12 months of age. (B) Representative picture of spleen showing mild splenomegaly at young age. (C) Histopahology of spleens for 2/3 TG mice diagnosed as SMZL (left) as compared with wild type controls (right).
Supplementary Fig. 4. Immunophenotype of B-cell tumors in Eµ-HDAC9 mice. Flow cytometric analysis of Eµ-HDAC9 splenic B-cell populations based on expression of mature B-cell surface markers reveals an immunophenotype representative of lymphoproliferative disease (LPD). All tumors arising in Eµ-HDAC9 mice were analysed by flow cytometry and a representative dataset is shown. Plots corresponding to wild type controls are also shown. Splenic cells were first gated based on FSC (forward scatter) versus SSC (side scatter) followed by 7-AAD negative selection (A, E). Cells were analysed for expression of CD23 (B, F), IgM and IgD (C, G) and CD21 (C, H) surface markers. Also shown are histogram profiles for cells gated by FSC versus SSC followed by 7-AAD negative selection, and examined for expression of B220 (I, M), CD23 (J, N), IgD (K, O) and IgM (L, P). Negative control or unstained (red). Surface marker expression (grey).
Supplementary Fig. 5. IgV rearrangements and SHM analysis. (A) IgV rearrangement products between JH4 and VH7183 (top) and VHJ558a (bottom) were amplified by PCR from genomic DNA isolated from spleen. PCR products generated by specific primers (VDJH1, VDJH2, VDJH3 and VDJH4) were separated by agarose gel electrophoresis and the presence of indicated bands denotes polyclonal rearrangements. Single PCR products were amplified for tumor samples #2 (VH7183) and #4 (VH7183 and VHJ558a). Bands at VDJH4 (0.46Kb) were confirmed to be monoclonal by sequencing analysis. Tumor sample #1 displays a polyclonal rearrangement pattern of bands for V-regions. Tumor sample #3, no bands observed, likely due to low number of tumor cells and extensive non-tumor reactive infiltrates (Supplemental Table S1). Tumor lanes as numbered: #1, SMZL; #2, DLBCL; #3, SBCL/L; #4, SMZL. Germline, Mouse -tail DNA. Wild type littermate control DNA from CD19+ B-cells (WT1) and total spleen cells (WT2). M: 1Kb Plus DNA Ladder. SMZL, splenic marginal zone lymphoma; DLBCL, diffuse large B-cell lymphoma; SBCL/L, small B-cell lymphoma/leukemia. (B) A monoclonal VH sequence of DLBCL tumor sample #2 (#1851) aligned to IGHV5-17*01 gene sequence (AC079273). Alignment and analysis of mutations was performed using IMGT/V-QUEST (http://www.imgt.org). Correspondence between nomenclatures for Mouse (Mus musculus) IGHV: previous IGHV subgroup Vh7183 or 7183 corresponds to IMGT IGHV subgroup IGHV5. VH mutated nucleotides are highlighted (yellow). CDR3 region corresponds to joining V to DJ sequence. Dots (.) indicate gaps. Hyphens (-) indicate nucleotide identity.
Supplemental Table 1

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Supplemental Table 2

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## Supplemental Table 3: KEGG-classified genes in Eμ-HDAC9 B-cell tumors

| Gene-Function                          | Fold-change |
|----------------------------------------|-------------|
| **DNA repair/replication/maintenance** |             |
| Cdca3                                  | +2.1        |
| Cdca2                                  | +1.9        |
| Cdc6                                   | +1.8        |
| Chaf1b                                 | +1.7        |
| Rad51                                  | +1.7        |
| Dlgap5                                 | +2.1        |
| Mcm10                                  | +2.1        |
| **Cell cycle/cell division**           |             |
| Ccna2                                  | +3.9        |
| Ccnb1                                  | +3.1        |
| Cdk1                                   | +2.7        |
| Ccnb2                                  | +2.6        |
| Plk1**                                 | +3.0        |
| Cdc6                                   | +1.9        |
| Cdkn3                                  | +2.2        |
| Bub1b                                  | +2.1        |
| Rrm2*                                  | +2.9        |
| Esco2                                  | +2.1        |
| Aurka                                  | +2.0        |
| Chek1^                                 | +2.1        |
| Aurkb                                  | +2.0        |
| Cdc20                                  | +2.0        |
| Prc1                                   | +2.6        |
| Ccne2                                  | +2.0        |
| Sgo1                                   | +2.3        |
| Sgo2                                   | +2.0        |
| Nuf2                                   | +2.1        |
| Casc5                                  | +2.1        |
| Birc5**                                | +2.6        |
| Bub1                                   | +2.0        |
| Hspa8                                  | -2.2        |
| **Transcriptional regulation**         |             |
| E2f8 (cell proliferation, antagonizes p53-mediated apoptosis via repression of E2f1) | +2.3        |
| **Cell proliferation**                 |             |
| Mki67                                  | +3.9        |
| **GC biology**                         |             |
| Ezh2                                   | +2.1        |
| Prdm1**                                | +2.2        |
| Aicda                                  | +2.0        |
| **Apoptosis**                          |             |
| Bcl211 (Bcl-x)^                        | +2.1        |
| Cd5I                                   | +2.3        |
| Birc5                                  | +2.6        |
| Chp                                    | +2.3        |
| Il11b                                  | +2.3        |
| **p53 signalling pathways**            |             |
| Ccne2                                  | +2.0        |
| Chek1^                                 | +2.2        |
| Ccnb2                                  | +2.4        |
| Rrm2                                   | +3.1        |
| Cdk1                                   | +2.3        |
| Steap3                                 | +2.0        |

Gene datasets grouped into functional categories by KEGG pathways and GGA

* p53 targets; ^ BCL6 targets; ** regulation by p53
### Supplemental Table 5: KEGG pathways for differentially expressed genes

| Cluster | Gene                  | KEGG pathways                                                                 |
|---------|-----------------------|-------------------------------------------------------------------------------|
| GO1     | Steap4, Cacna1h       | p53 signalling, MAPK signalling                                               |
|         |                       |                                                                               |
| GO2     | Cd28, Pdcd1g2, Fyn,   | T-cell receptor signalling                                                   |
|         | Clidn13, Ititm1, Il13ra1, Il10ra, Il5ra, Cldn13                               | Cell adhesion, Adhesion, T-cell receptor signalling                           |
|         | Ifitm1, Il13ra1, Il10ra, Il5ra, Csfr3, Cxcr2                                  | Cell adhesion, BCR signalling                                                |
|         |                       | Cytokines interaction, Jak-STAT signalling, PI3K-Akt, Jak-STAT, Hematopoiesis |
|         |                       | Cytokines, Chemokine signalling, Endocytosis                                  |
|         |                       |                                                                               |
| GO3     | Dusp2, Dusp5          | MAPK signalling                                                               |
|         |                       |                                                                               |
| GO4     | Diap3, Ank1, Chek1, Ccnb2, Bub1b, Bub1, PLK1, Cdc6, Cdk1                       | Regulation of actin cytoskeleton                                               |
|         |                       | Proteoglycans in cancer                                                       |
|         |                       | Cell cycle, p53 pathway                                                       |
|         |                       | Cell cycle, thyroid cancer                                                    |
|         |                       | Cell cycle, p53 pathway                                                       |
|         |                       | EBV infection, Oocyte meiosis                                                  |
|         |                       | Cell cycle, viral carcinogenesis, EBV infection                               |
|         | Dusp6, Bcl21, Ski, Nr4a1, Ezh2                                              | MAPK signalling                                                               |
|         |                       | PI3K-Akt, Apoptosis, NK-kappa B signalling                                    |
|         |                       | Carcinoma, melanoma                                                           |
|         |                       | MAPK signalling, PI3K-Akt signalling                                         |
|         |                       | miRNAs in cancer                                                              |
|         |                       |                                                                               |
| GO5     | Rasgrf2, Ccne2, Arfgap3                                                | MAPK signalling, PI3K-Akt signalling, mTOR signalling, Apoptosis, T-cell      |
|         |                       | signaling (TCR NF-AT immune response)                                         |
|         |                       | Cell cycle, p53 pathway                                                       |
|         |                       | Endocytosis                                                                   |
|         |                       |                                                                               |
| GO6     | Hmmr                  | Extracellular matrix receptor-receptor interaction                           |
|         |                       |                                                                               |
| GO7     | Wars                  | Aminoacyl t-RNA synthesis                                                     |
|         |                       |                                                                               |
| GO8     | Ddt                   | Metabolic pathways                                                           |
|         |                       |                                                                               |
| GO9     | Sgo1                  | Oocyte meiosis                                                                |
|         |                       |                                                                               |
| GO10    | Alas2, Hk2, Bcat1, Pycr1, Fut1, Pklr, Chst1, Rtm2                           | Metabolic pathways                                                           |
|         |                       |                                                                               |
|         |                       | Metabolic pathways, p53 pathway                                               |
| GO11    | -                     |                                                                               |
| GO12    | Chp                   | MAPK signalling, Oocyte meiosis, BCR signalling                               |
| GO13    | Ptges                 | Metabolic pathways                                                           |
| GO14    | Spint1, Birc5, Fn1     | Transcriptional misregulation in cancer                                       |
|         |                       | Hippo signalling pathway                                                      |
|         |                       | ECM Rc interaction, Regulation of actin cytoskeleton, PI3K signalling         |

Genes not shown in table do not have KEGG pathway annotation. Other genes not shown in table are not cancer-related genes but do have a KEGG pathway annotation. Genes in italic are BCL6- and p53-direct targets. Blue denotes down-regulated genes.
### Supplemental Table 6: Most informative GO terms for the clusters obtained from microarray data

| Cluster | Size | P-value | IC       | GO term                                                      |
|---------|------|---------|----------|--------------------------------------------------------------|
| GO1     | 23   | 0.093   | 3.496    | Transport                                                   |
| GO2     | 42   | 0.288   | 1.792    | Membrane                                                   |
| GO3     | 4    | 0.0046  | 7.738    | D Dephosphorylation                                        |
|         |      |         | 7.712    | Phosphoprotein phosphatase activity                         |
|         |      |         | 1.26     | Intracellular part                                          |
| GO4     | 118  | 0.437   | 1.193    | Intracellular                                              |
| GO5     | 8    | 0.017   | 5.823    | Enzyme regulator activity                                  |
|         |      |         |          | Regulation of catalytic activity                            |
|         |      |         |          | Regulation of cellular process                              |
| GO6     | 2    | 0.0057  | 7.430    | Carbohydrate binding                                       |
|         |      |         | 1.260    | Intracellular part                                          |
| GO7     | 4    | 0.165   | 2.591    | Developmental binding                                      |
|         |      |         | 0.813    | Cellular process                                            |
| GO8     | 2    | 0.0004  | 11.116   | Pigment biosynthetic process                               |
|         |      | 0.0048  | 7.695    | Lyase activity                                              |
|         |      |         | 3.380    | Cellular biosynthetic process                               |
| GO9     | 7    | 0.0049  | 7.663    | Cell division                                               |
|         |      | 0.0070  | 7.146    | M phase                                                    |
|         |      |         | 4.952    | Organelle organization                                     |
| GO10    | 46   | 0.233   | 2.095    | Catalytic activity                                         |
| GO11    | 5    | 0.0073  | 7.096    | Calcium ion binding                                        |
|         |      |         | 2.099    | Cytoplasm                                                  |
| GO12    | 6    | 0.0744  | 3.747    | Metal ion binding                                          |
|         |      |         | 0.813    | Cellular process                                           |
| GO13    | 6    | 0.155   | 2.688    | Cytoplasmic part                                           |
|         |      |         | 2.134    | Intracellular membrane organelle                           |
| GO14    | 18   | -       | -        | -                                                           |

**IC** = information content (-log2 of priori). **P-values** are derived from a priori probability for a given GO term to be associated with a gene in a background data set of genes. More general GO terms show higher priori values (**P-values**) and more specific GO terms show lower values. GO14: analysis yielded no groups.
## Supplemental Table 7: Down-regulated genes in Eμ-HDAC9 B-cell tumors.

| GO Function                  | Gene      | Fold-change | KEGG pathway                                                                 |
|------------------------------|-----------|-------------|------------------------------------------------------------------------------|
| **GO2-membrane**             |           |             |                                                                              |
| Fyn                          |           | -2.1        | T-cell receptor signalling, Viral myocarditis, Adhesion and more             |
| Il5ra                        |           | -2.2        | Jak-STAT signalling pathway, cytokine R<sub>c</sub> interaction, Haematopoietic cell lineage |
| Il10ra                       |           | -2.4        | Jak-STAT signalling pathway, cytokine R<sub>c</sub> interaction             |
| Cd69                         |           | -2.7        | Wnt signalling pathway                                                       |
| Fzd6                         |           | -2.2        | Cell adhesion                                                                |
| Pdcd1lg2                     |           | -2.3        |                                                                              |
| **GO3-dephosphorylation**    |           |             |                                                                              |
| Dusp2                        |           | -2.1        | MAPK signalling                                                              |
| Dusp5                        |           | -2.4        | MAPK signalling                                                              |
| **GO4-intracellular**        |           |             |                                                                              |
| Trio                         |           | -2.1        | -                                                                           |
| Dusp6                        |           | -2.0        | MAPK signalling pathway                                                      |
| Dmd                          |           | -2.3        | Cardiomyopathy, Viral myocarditis                                           |
| Rps13                        |           | -2.1        | Ribosome                                                                     |
| Rps3                         |           | -2.2        | Ribosome                                                                     |
| Rpl4                         |           | -2.2        | Ribosome                                                                     |
| Ski                          |           | -2.1        | Carcinoma, melanoma                                                          |
| Nr3c2                        |           | -2.1        | Aldosterone                                                                  |
| Nr4a1                        |           | -2.8        | MAPK and PI3K-Akt signalling pathway                                         |
| Fosb                         |           | -4.5        | Cell proliferation, differentiation, drug addiction, Transcriptional regulation, Viral carcinogenesis |
| Egr3                         |           | -2.1        | MAPK signalling pathway, bladder cancer, neurotrophin signalling pathway     |
| Rps6ka5                      |           | -2.0        | MAPK signalling pathway, Ag processing/presentation, endocytosis, spliceosome |
| Hspa8                        |           | -2.2        |                                                                              |
| **GO5-enzymatic activity**   |           |             |                                                                              |
| Rasgrf2                      |           | -2.1        | MAPK/ERK pathway, TCR+NF-AT mediated immune response                         |
| Rgs18                        |           | -2.5        | -                                                                           |