Dosage-dependent tumor suppression by histone deacetylases 1 and 2 through regulation of c-Myc collaborating genes and p53 function

Marinus R. Heideman, Roel H. Wilting, Eva Yanover, Arno Velds3, Johann de Jong1, Ron M. Kerkhoven3, Heinz Jacobs2, Lodewyk F.Wessels1, Jan-Hermen Dannenberg*

Division of Gene Regulation
1Division of Molecular Carcinogenesis
2Division of Immunology
3Genomics Core Facility
Netherlands Cancer Institute
Plesmanlaan 121
1066 CX Amsterdam
The Netherlands

*Corresponding author
Tel: +31 20 5121924  Fax: +31 20 5121998
e-mail: j.dannenberg@nki.nl

Running title: Hdac1 and Hdac2 dosage-dependent tumor suppression

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Key Points

1. Hdac1 and Hdac2 are dosage-dependent tumor suppressor

2. Hdac1 and Hdac2 regulate p53-modulating genes as a barrier to prevent Myc-driven tumorigenesis
Abstract
Histone deacetylases (HDACs) are epigenetic erasers of lysine-acetyl marks. Inhibition of HDACs using small molecule inhibitors (HDACi) is a potential strategy in the treatment of various diseases and is approved for treating hematological malignancies. Harnessing the therapeutic potential of HDACi requires knowledge of HDAC-function in vivo. Here, we generated a thymocyte-specific gradient of HDAC-activity using compound conditional knockout mice for Hdac1 and Hdac2. Unexpectedly, gradual loss of HDAC-activity engendered a dosage-dependent accumulation of immature thymocytes and correlated with the incidence and latency of monoclonal lymphoblastic thymic lymphomas. Strikingly, complete ablation of Hdac1 and Hdac2 abrogated lymphomagenesis due to a block in early thymic development. Genomic, biochemical and functional analyses of pre-leukemic thymocytes and tumors revealed a critical role for Hdac1/Hdac2-governed HDAC-activity in regulating a p53-dependent barrier to constrain Myc-overexpressing thymocytes from progressing into lymphomas by regulating Myc-collaborating genes. One Myc-collaborating and p53-suppressing gene, Jdp2, was derepressed in an Hdac1/2-dependent manner and critical for the survival of Jdp2-overexpressing lymphoma cells. Although reduced HDAC-activity facilitates oncogenic transformation in normal cells, resulting tumor cells remain highly dependent on HDAC-activity, indicating that a critical level of Hdac1 and Hdac2 governed HDAC-activity is required for tumor maintenance.
Introduction

Cancer develops and persists as a result of accumulating genetic and epigenetic changes\(^1\). The reversibility of epigenetic changes has generated increasing interest in the development of agents targeting epigenetic regulators such as histone deacetylases (HDACs)\(^2\).

HDACs are critical epigenetic erasers of lysine-acetyl marks of histones and non-histone substrates\(^3\). HDACs can be classified on the basis of their homology to yeast counterparts. Class I HDACs (HDAC1, -2, -3 and -8) are highly homologous to *Saccharomyces cerevisiae Rpd3*. Class IIa HDACs (HDAC4, -5, -7 and -9) and class IIb HDACs (HDAC6 and -10) consist of *S. cerevisiae* Hda1 homologues. HDAC11 is the sole member of the class IV HDACs, based on homology to both class I and class II HDACs\(^4\). While class I, II and IV HDACs are Zn\(^{2+}\)-dependent hydrolases, class III histone deacetylases, consisting of yeast Sir2 homologs (Sirtuins 1-7) form a structurally and mechanistically distinct class of NAD\(^{+}\)-dependent hydrolases.

A classic function of HDACs relates to their role as transcriptional co-repressors through deacetylation of lysine residues in histone tails. This results in a closed chromatin structure and diminished accessibility for the basal transcription machinery. Class I HDACs are present in repressor complexes such as SIN3A, NuRD, REST and N-CoR/SMRT, which acquire their regional activities in part by interacting with sequence-specific transcription factors.

In agreement with a high sequence similarity between class I HDAC1 and HDAC2, genetic studies in mice revealed redundant functions of these enzymes in many cell types\(^5\)-\(^10\). In addition, many biological processes are collectively regulated by HDAC1 and HDAC2 such as DNA damage repair, autophagy, hematopoiesis and cell cycle regulation\(^5,6,11,12\).

Nevertheless, Hdac1- and Hdac2-specific functions have been identified. Deletion of Hdac1 results in embryonic lethality as early as E9.5 of development\(^13\). In effector T-cells, Hdac1 regulates the inflammatory response in an *in vivo* allergic airway inflammation model suggesting that Hdac1 has a specific function in controlling an inflammatory response by modulating...
cytokine expression\textsuperscript{14}. In contrast to Hdac1 deficiency, Hdac2 loss of function results in viable mice with reduced body weight\textsuperscript{15-17}. Others have reported that Hdac2 deficiency is not compatible with life due to cardiac myopathy\textsuperscript{7}. In addition, Hdac2 plays a specific role in repression of genes involved in synaptogenesis, as evidenced by enhanced synapse formation, learning and memory in Hdac2-deficient mice\textsuperscript{17}.

The use of pharmacological HDAC inhibition in cancer treatment is rationalized by observations showing high expression of individual HDACs and recruitment of these proteins by oncogenic fusion proteins such as PML-RAR and AML-ETO in various cancer types\textsuperscript{18}. In contrast, class I HDACs, such as HDAC1 and HDAC2 have been identified in complexes harboring tumor suppressors, such as the retinoblastoma protein (RB1)\textsuperscript{19}, p53\textsuperscript{20}, BCL11B\textsuperscript{21} and RUNX1\textsuperscript{22}. Hence, inhibition of HDACs may have tumor-promoting and tumor-suppressive consequences. The growing interest in the use of HDACi and other epigenetic drugs as therapeutic agents in the treatment of cancer, acquired drug resistance, HIV, diabetes and neurological disorders such as Alzheimer\textsuperscript{23-26} necessitate full knowledge of HDACs in normal development to harness the therapeutic potential and future development of novel HDACi.
Methods

Mice

The Hdac1 and Hdac2 cKO alleles as well as MxCre;Hdac1<sup>L/L</sup>;Hdac2<sup>L/L</sup> mice have been described elsewhere. Thymocyte specific deletion of Hdac1 and Hdac2 was obtained using LckCre transgenic mice in combination with Hdac1 and/or Hdac2 cKO alleles. All cohorts were in a mixed FVB/n, C57BL/6 and 129/Sv background. All experiments were approved by a local ethical committee and performed according to national guidelines.

Establishment, culturing and treatment of mouse thymic lymphoma tumor cell lines

Tumors were dissected from the thorax of LckCre;Hdac1<sup>Δ/Δ</sup>, LckCre;Hdac1<sup>+/Δ</sup>;Hdac2<sup>Δ/Δ</sup>, LckCre;Hdac1<sup>Δ/Δ</sup>;Hdac2<sup>+/Δ</sup>, Eμ-Myc, or p53<sup>−/−</sup> mice. Single cell suspensions were cultured in DMEM or IMDM medium containing 10% FBS, glutamine, penicillin/streptomycin supplemented with 20% Methocult (3434, Stem Cell Technologies). CD4 and CD8 flow-cytometry analysis was used to confirm the T-cell identity of the cell lines. To determine HDACi sensitivity, tumor cell lines were treated with different concentrations of SAHA (Selleck) for 72 hours. Cell viability was measured using Cell Titer Blue assay (Promega). To infect lymphoma cell lines with lentiviral shRNA constructs 5 x 10<sup>5</sup> cells were infected twice with 30 μl of concentrated lentiviral supernatants containing 4μg/ml polybrene in a total volume of 530 μl for 24 hours and subsequently selected with 2.0 μg/ml puromycin for at least 48 hours. pLKO.1 Jdp2 and non-targeting (NT) shRNA vectors were obtained from the NKI Robotics and Screening facility. Jdp2 mRNA levels were analyzed by qPCR using the following primers: Jdp2_F 5’-CGCTGACATCCGCAACATTG-3’, Jdp2_R 5’-CATCTGGCTGCAGCGACTTT-3’.
In vivo BrdU labeling

Mice were injected intraperitoneal with 200 μl BrdU solution (10mg/ml). After 1.5 hours thymocytes were intracellular labeled with Bride antibodies (α-BrdU-APC) and with the DNA binding fluorescent dye 7-AAD (BrdU Flow Kit, BD Pharmingen). Subsequently, stained thymocytes were analyzed on a multi-color CyAn flow-cytometer (Beckman Coulter). Data was analyzed with FlowJo software (Treestar).

Histology

Tissues were fixed in ethanol-acetic acid-formol saline for 24 hours and subsequently embedded in paraffin. For immunohistochemistry, sections were pre-incubated with goat serum (Sanquin) for 30 min and subsequently incubated o/n with an Hdac1 antibody (Abcam), Hdac2 (Invitrogen), or p53 (VectorLabs), and secondary poly-HRP-anti-Rabbit IgG (Immunologic) for 30 min. The slides were washed with PBS, incubated with DAB substrate chromogen system (Dako) and counterstained with hematoxylin (Merck).

Flow cytometry

Thymocytes and thymic lymphoma cells were stained with Thy1-PE, CD4-PacificBlue, CD8-FITC, CD25-PerCP-Cy5.5, CD44-APC, TCRβ-APC and CD24-PE (BD Biosciences). Apoptotic thymocytes were determined using an antibody against AnnexinV (AnnexinV apoptosis kit BD Biosciences) and propidium-iodide (PI) counterstain. All experiments were performed using a multi-color CyAn flow-cytometer (Beckman Coulter). Data was analyzed with FlowJo software (Treestar).
**Western blot analysis**

For western blot analysis, tissues and cells were lysed in RIPA buffer (20 mM Tris, pH 7.5, 150 mM sodium chloride, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EDTA, 0.1% SDS), protease inhibitors (Roche), phosphatase inhibitors, 5 μM trichostatin A and 1 mM nicotinamide). 20 μg of total protein was used for western blotting and incubated with antibodies against Hdac1 (IMG-337, Imgenex), Hdac2 (SC-7899, Santa Cruz), Hdac3 (Cell Signaling), Hdac8 (Santa Cruz) and γ-tubulin (T6557, Sigma). Dr. A. Aronheim generously provided the Jdp2 antibody. Protein levels were quantified using IRDye 680/800CW secondary antibodies (Li-Cor). Nitrocellulose membranes were stained and imaged with the LI-COR Odyssey Infrared Imaging System. Other antibodies used are against p19Arf (Ab80; Abcam), p53 (IMX25, Novocastra), c-Myc (N-262, Santa Cruz), GFP (11814460001, Roche) acetylated H3 (06599, Millipore), acetylated H4 (06866, Millipore), histone H3 (Ab1791, Abcam), histone H4 (kindly provided by dr. F. van Leeuwen) and horse radish peroxidase (HRP) coupled secondary antibodies (DAKO). Western blots were stained with ECL (Pierce), imaged and quantified with ChemiDoc software (BioRad).

**Southern blot analysis**

Genomic DNA (10 μg) of tumor samples or primary thymocytes was digested with EcoRI overnight at 37 ºC. The DNA fragments were separated on a 0.8% agarose gel and transferred to a nitrocellulose membrane. The blots were hybridized with a 32P labeled probe harboring the Jβ2 region of the TCRβ locus.

**HDAC activity assay**

Lysates from fresh thymocytes were assayed for HDAC activity using the HDAC Fluorimetric Activity Assay kit (Enzo life Sciences)
Comparative genomic hybridization

Genomic DNA was isolated from tumor samples using the Puregene purification kit (Qiagen). As a reference we used genomic tail DNA from the same mouse. Tumor and tail DNA were Cy3 and Cy5 labeled using the Dual Color labeling kit (Nimblegen) according to the manufacturers’ instructions. Labeled DNAs were hybridized onto Mouse CGH 12x135K Whole-Genome Tiling Arrays. The arrays were scanned on an Agilent Scanner (Model G2505B) at a resolution of 2 micron double pass at 100% PMT for both channels. The data was analyzed with NimbleScan software (Nimblegen). aCGH data were deposited at the GEO database: accession number: GSE43407

Chromosome spreads

Cells were incubated for 90 min. in medium with 0.05 μg/ml colcemid (Gibco). Hereafter, the cells were washed with PBS and resuspended in 75 mM KCl and incubated at 37°C for 10 min. Subsequently the cells were fixed in methanol/acetic (3:1) and dropped on microscope slides. These slides were dried and cells were mounted with Vectashield/DAPI (Vector Laboratories).

Methylation genomic DNA tumors

Genomic DNA was isolated and digested with methylation-sensitive (HpaII) or -insensitive restriction enzymes (MspI) and were analyzed on DNA Southern blots. As a probe for methylation analysis of minor satellites we used pMR150 (gift from dr. Antoine Peters).

Transcriptome analysis and bioinformatics

For gene expression analysis total RNA from thymi or tumor tissue was extracted, labeled and hybridized onto Illumina MouseWG-6 v2.0 Expression BeadChip Arrays by the Netherlands
Cancer Institute Central Microarray and Deep Sequencing Core Facility according to the manufacturer’s protocol. 20312 MuLV integration sites from 1020 tumors in mice with various genetic backgrounds were mapped to their putative target genes using a kernel-convolved rule-based mapping approach (KC-RBM) and the Ensembl reference genome (build 61). Commonly targeted genes (CTGs) were called based on a threshold of at least one insertion per target gene. A set of 547 genes consistently deregulated in \( \text{Hdac1}^{\Delta/\Delta}, \text{Hdac2}^{\Delta/+} \) thymocytes and lymphomas were mapped to mouse Ensembl gene identifiers. Overlap between the resulting 413 mouse Ensembl identifiers and the CTGs was then assessed by performing a Fisher’s exact test, using the complete set of Ensembl gene identifiers as a reference. Gene expression data were deposited at the ArrayExpress database: accession number: E-MTAB-1448

**Tissue Micro Array**

A tissue micro-array (TMA) was generated by collecting 3 cores of tumor or normal tissue, as determined by hematoxylin-eosin stained paraffin-embedded lymphomas as well as 3-week old thymi. For control and orientation purposes cores of paraffin embedded wild-type liver were included in the TMA. Cores were embedded in a paraffin block and subsequently sectioned in 3 \( \mu \)m slides. Hdac1, Hdac2 and p53 staining were performed as described above. Hdac1 and Hdac2 staining were used to confirm the genotype of the lymphoma. Scoring for p53 staining intensity was performed by using a p53-mutant lymphoma as a positive control and wild-type thymus as a negative control. Only in case all three cores showed a consistent staining pattern, a score was assigned.

*p53 status assessment by sequencing, \( \gamma \)-irradiation and Nutlin-3 treatment*
To determine p53 sequence genomic DNA of primary thymocytes and tumor cell lines was isolated with DNeasy blood&tissue kit (Qiagen). p53 exon 2-11 were PCR-amplified and sequenced on a 3730 DNA analyzer (Applied Biosystems). To determine DNA-damage mediated p53 induction, thymocytes were irradiated with 6 Gy using the Gammacell 40 EXACTOR and tumor cell lines were treated with 8 μM Nutlin-3 (Cayman Chemical). Irradiated thymocytes were cultured for 16 hours and Nutlin-3 treated cells for 6 hours and subsequently analyzed for p53 protein expression. For the apoptosis assay 2x10^6 fresh thymocytes were irradiated with 0, 2, 4, 6, 8 and 10 Gy and cultured for 16 hours. Apoptosis was assayed by staining with AnnexinV and PI and subsequent analysis by flow cytometry (FITC-AnnexinV apoptosis kit, BD-Biosciences).

**Chromatin immunoprecipitation**

Chromatin immunoprecipitation was performed by cross-linking chromatin from 5 x 10^7 p53^-/- T-cell lymphoma cells expressing GFP, Hdac1-GFP, or Hdac2-GFP (Wilting et al., unpublished results) using 1% formaldehyde for 10 minutes at room temperature. Cross-linking was stopped in 1.25 M glycine for 5 minutes on ice. Chromatin was subsequently sonicated in lysis buffer (50 mM Tris-HCl pH8.0, 10 mM EDTA, 1% SDS, protease inhibitors (Roche)) using a probe sonicator (Bandelin, cycle 90%, output 8, 15 seconds on/off) and subsequently diluted in dilution buffer (10 mM Tris-HCl pH 8.0, 160 mM NaCl, 5 mM EDTA, 1% Triton X-100, protease inhibitors). Chromatin was incubated overnight with 10 μl of 10% BSA-blocked GFP-Trap beads (ChromoTek) in dilution buffer at 4 °C. Chromatin bound beads were washed with RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% NaDOC, 1% NP-40, protease inhibitors) and with TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). To elute chromatin the beads were incubated in TE plus 2% SDS at 65 °C for 15 minutes while shaking. Crosslinks were reverted by incubating chromatin at 68 °C overnight.
with proteinase K. DNA was purified using MinElute PCR purification columns (Qiagen) and subjected to quantitative PCR using the Roche LightCycler system. Primers used for qPCR are \textit{Jdp2} 5’ UTR: 5’-TGTGAGCTGTCACCCATCAT-3’, 5’-CCACCCAGATAGAGAAGCA-3’; \textit{Jdp2} intron 1-2: 5’-ATGCTATGGCTCTGTTCT-3’, 5’-TGACCCCTCAAGACCACTGC-3’.
Results

Spontaneous lymphomagenesis in MxCre+;Hdac1L/L and MxCre+;Hdac1L/L;Hdac2L/L mice

To study HDAC function in vivo we previously generated conditional knockout alleles for Hdac1\(^5\) and Hdac2\(^7\). While interferon-inducible MxCre-recombinase mediated deletion of Hdac1 and Hdac2 in the hematopoietic system resulted in anemia and thrombocytopenia related death\(^5\), we noted that aging, uninduced, MxCre+;Hdac1L/L and MxCre+;Hdac1L/L;Hdac2L/L mice, became lethargic and presented in all cases CD4\(^+\)CD8\(^+\) thymic lymphomas (Fig. 1A,B(top panel)). Intriguingly, MxCre+;Hdac1L/L;Hdac2L/L mice developed tumors with a higher incidence (85% vs 25%) and shorter latency (15 weeks vs 25 weeks) compared to MxCre+;Hdac1L/L mice. While Hdac1 expression was absent in lymphomas derived from MxCre+;Hdac1L/L and MxCre+;Hdac1L/L;Hdac2L/L mice, lymphomas derived from both genotypes still expressed Hdac2 (Fig. 1B(bottom panel); Supplemental Fig. S1A). Interestingly, genetic analysis of MxCre+;Hdac1L/L;Hdac2L/L lymphoma cell lines derived from primary tumors exclusively displayed complete loss of Hdac1 whereas only one conditional Hdac2 allele was deleted (Supplemental Fig. S1B). These results indicate that leaky Cre expression from the Mx promoter, resulted in sporadic deletion of Hdac1 and Hdac2. Apparently, loss of Hdac1 or mono-allelic expression of Hdac2 in the absence of Hdac1 conferred a selective advantage in thymocytes resulting in lymphomagenesis, which uncovers a previously unknown tumor suppressor function for Hdac1 and Hdac2. In addition, the differential lymphoma incidence in MxCre+;Hdac1L/L and MxCre+;Hdac1L/L;Hdac2L/L mice suggests a dosage-dependency in tumor suppression by Hdac1 and Hdac2.

Thymocyte specific deletion of Hdac1 and Hdac2 results in an HDAC-activity gradient

To test whether Hdac1 and Hdac2 suppress tumorigenesis in a dosage-dependent manner, we generated a thymocyte specific series of inactivated Hdac1 and Hdac2 alleles using Lck-promoter
driven Cre-recombinase expression. Western blot analysis of isolated thymocytes from these mice indicated efficient deletion of Hdac1 and Hdac2 (Supplemental Fig. S2A,B). Ablation of Hdac1 resulted in elevated Hdac2 protein levels while class I Hdac3 and Hdac8 proteins levels were unaffected (Supplemental Fig. S2B). While Hdac2 deficiency did not result in increased Hdac1 proteins levels, loss of one allele of Hdac1 in the absence of Hdac2 resulted in elevated Hdac1 protein levels. These results suggest compensatory regulation of Hdac1 and Hdac2 protein levels in thymocytes (Supplemental Fig. S2B).

Deletion of combinations of *Hdac1* and *Hdac2* alleles resulted in differential effects on global HDAC-activity, as HDAC-activity measurements in one-week-old thymocytes revealed a progressive loss of global HDAC-activity in wild-type > *Hdac1*^{+/Δ};*Hdac2*^{1/Δ} ≥ *Hdac1*^{1/Δ};*Hdac2*^{+/Δ} thymocytes (Fig. 1C). Although ablation of Hdac1 resulted specifically in elevated Hdac2 protein levels in *Hdac1*^{Δ/Δ} thymocytes (Supplemental Fig. S2B), it could only partially compensate for the loss of global HDAC-activity (Fig. 1C). Indeed, mono-allelic expression of Hdac2 in Hdac1 deficient (*Hdac1*^{Δ/Δ};*Hdac2*^{+/Δ}) thymocytes resulted in a further reduction of HDAC-activity (Fig. 1C). These results indicate a dynamic interplay between Hdac1 and Hdac2 in contributing to global HDAC-activity in thymocytes in which Hdac1 seems to act as dominant histone deacetylase. In conclusion, we have established an *in vivo* gradient of Hdac1 and Hdac2 activity, which provides a unique model to study HDAC-activity dosage in tumor suppression.

**Hdac1 and Hdac2 dosage-dependent tumor suppression**

To test whether stepwise reduction of HDAC-activity resulted in tumorigenesis, we monitored cohorts of mice carrying combinations of inactivated *Hdac1* and *Hdac2* alleles over time. Intriguingly, a progressive reduction of HDAC-activity correlated with increased tumorigenesis in mice. Whereas *LckCre;Hdac1*^{+/Δ};*Hdac2*^{Δ/Δ} mice developed tumors with a 25% incidence and a
mean latency of over 52 weeks, tumor incidence increased significantly in LckCre;Hdac1Δ/Δ mice with an 75% incidence and a 23 weeks mean latency. Consistent with a further reduction of global HDAC-activity, tumorigenesis was drastically accelerated in LckCre;Hdac1Δ/Δ;Hdac2Δ/Δ mice, with a 100% tumor incidence and a mean latency of 10 weeks (Fig. 1D). Tumors across genotypes were identified as thymic lymphomas, which disseminated predominantly to lung, kidney, liver and lymph nodes (Supplemental Fig. S2C,D). Mono-allelic expression of Hdac1 and Hdac2 was maintained in Hdac1+/Δ;Hdac2Δ/Δ and Hdac1Δ/Δ;Hdac2+/Δ lymphomas as evidenced by Hdac1 and Hdac2 immunohistochemical staining on paraffin embedded primary tumors and genomic analysis of Hdac1 and Hdac2 alleles in lymphoma cell lines (Fig. 1E; Supplemental Fig. S2E). These findings were corroborated by the appearance of a thymic lymphoma in one out of 10 LckCre;Hdac1+/Δ;Hdac2Δ/Δ mice at 46 weeks, which lost Hdac1 and retained Hdac2 expression (Fig. 1D; Supplemental Fig. S2F). Genetic analysis of this tumor indicated loss of the wild-type Hdac1 allele and maintenance of one wild-type Hdac2 copy, thereby recapitulating Hdac1Δ/Δ;Hdac2+/Δ tumors (Supplemental Fig. S2G). This result suggests that reduced Hdac1 and Hdac2 levels generate a tumor prone condition allowing in vivo selection for Hdac1 loss-of-heterozygosity (LOH).

In summary, here we established for the first time a tumor suppressor function for Hdac1 and Hdac2, which act collectively in a dosage-dependent manner.

Complete loss of Hdac1 and Hdac2 abrogates lymphomagenesis

Surprisingly, further reduction of Hdac1 and Hdac2 in LckCre;Hdac1Δ/Δ;Hdac2Δ/Δ (DKO) mice never resulted in tumors lacking both Hdac1 and Hdac2, but resulted in selection for Hdac1Δ/Δ;Hdac2+/Δ thymocytes and consequently Hdac1Δ/Δ;Hdac2Δ/Δ lymphomas thereby phenocopying LckCre;Hdac1Δ/Δ;Hdac2Δ/Δ mice. In agreement with a selection for Hdac1Δ/Δ;Hdac2Δ/Δ thymocytes in DKO mice, the kinetics of tumor development in DKO mice are
significantly slower compared to *LckCre;Hdac1Δ/Δ;Hdac2Δ/+* mice (Supplemental Fig. S2H). In summary, while Hdac1 and Hdac2 suppress lymphomagenesis in a dosage-dependent manner; complete inactivation of Hdac1 and Hdac2 abrogates lymphomagenesis.

**A critical level of HDAC-activity is required for tumor maintenance**

Since complete inactivation of Hdac1 and Hdac2 abrogated lymphomagenesis, it suggested a requirement for critical HDAC-activity to initiate or maintain tumorigenesis. Indeed, pharmacological inhibition of remaining HDAC-activity in established *Hdac1Δ/Δ;Hdac2Δ/+* lymphoma cell lines resulted in a dose-dependent cell death, indicating that tumor maintenance is dependent on critical HDAC-activity levels (Fig. 1G). Interestingly, *Hdac1Δ/Δ;Hdac2Δ/+* lymphoma cell lines, which displayed relative low HDAC-activity compared to Hdac1/2 expressing *Eμ−Myc* lymphoma cell lines, were 2-10 fold more sensitive to HDACi compared to *Eμ−Myc* lymphoma cell lines (Fig. 1F, G). These data show that whilst reduced HDAC-activity facilitates oncogenic transformation in normal cells, the resulting tumor cells remain highly dependent on HDAC-activity, thereby exposing a cancer cell vulnerability.

**HDAC-activity dosage-dependent increase of ISP thymocytes in pre-leukemic thymi**

To investigate whether deregulated thymocyte development underlies lymphoma development, we analysed T-cell differentiation in wild-type, *LckCre;Hdac2Δ/Δ* *LckCre;Hdac1Δ/Δ;Hdac2Δ/Δ* *LckCre;Hdac1Δ/Δ* and *LckCre;Hdac1Δ/Δ;Hdac2Δ/+* mice. T-cell receptor β (Tcrβ) diversity analysis of *LckCre;Hdac1Δ/Δ;Hdac2Δ/+* thymi revealed a polyclonal population of thymocytes, indicative for pre-leukemic *Hdac1Δ/Δ;Hdac2Δ/+* thymocytes (Fig. 2A). *LckCre;Hdac1Δ/Δ* and *LckCre;Hdac1Δ/Δ;Hdac2Δ/+* mice showed reduced thymocyte numbers which was associated with increased apoptosis and a specific decrease in CD4;CD8 double positive (DP) thymocytes (Fig. 2B, C, D(top panel), E(left panel). Remarkably, *LckCre;Hdac1Δ/Δ;Hdac2Δ/+* mice displayed a
dramatic increase in CD8 single positive (CD8 SP) thymocytes, which predominantly consisted of immature CD8 SP (ISP) thymocytes, as determined by CD24 and TCRβ surface markers (Fig. 2D,E). Moreover, we observed a dosage-dependent expansion of the ISP thymocyte population in all lymphoma-prone genotypes (LckCre;Hdac1Δ/Δ;Hdac2Δ/Δ, LckCre;Hdac1Δ/Δ and LckCre;Hdac1Δ/Δ;Hdac2Δ/Δ) which correlated with global HDAC-activity levels and tumor incidence (Fig. 2D(bottom panel),E(right panel)). In vivo BrdU labeling revealed a 2-fold increase in the percentage of BrdU-positive cells in one-week-old LckCre;Hdac1Δ/Δ;Hdac2Δ/Δ thymi, which consisted primarily of CD8 SP cells (Fig. 2F), indicating the proliferative capacity of the expanded ISP population. These data indicate a crucial role for Hdac1 and Hdac2 in the regulation of pre-T cell development by controlling ISP thymocytes, a T-cell developmental stage that was previously implicated in lymphomagenesis. The correlation between global HDAC-activity, tumor-latency and -incidence, and ISP thymocyte numbers strongly suggests that Hdac1 and Hdac2 suppress lymphomagenesis by controlling ISP thymocytes in a dosage-dependent manner.

**Hdac1 and Hdac2 are required for early thymocyte development**

In order to provide a rationale for the absence of Hdac1- and Hdac2-deficient tumors in LckCre;Hdac1Δ/Δ;Hdac2Δ/Δ mice, we analyzed thymi in these mice at one week of age. Total thymocyte numbers were dramatically reduced compared to wild-type thymocytes and LckCre;Hdac1Δ/Δ;Hdac2Δ/Δ thymocytes (Fig. 3A; Fig. 2A) and associated with increased apoptosis (Fig. 3B). Surprisingly, flow cytometric analysis showed a 5-fold increase in CD4;CD8 double negative (DN) thymocytes specifically in LckCre;Hdac1Δ/Δ;Hdac2Δ/Δ thymi, which was accompanied by a severe reduction in CD4;CD8 DP thymocytes (Fig. 3C,D). Thymic analysis across genotypes revealed specifically in DKO thymi an early developmental block at the CD4-CD8- double negative stage 3 (DN3) (Fig. 3D, Supplemental Fig. S3A,B) demonstrating that complete ablation of Hdac1 and Hdac2 is not compatible with developmental progression of early
thymocytes and consequently prevents tumorigenesis. Collectively, our results show that a gradual
decrease in Hdac1 and Hdac2 governed HDAC-activity predisposes to tumorigenesis, whereas
complete loss of Hdac1 and Hdac2 prevents oncogenic transformation.

**Chromosome 15 trisomy is associated with c-Myc overexpression in monoclonal T-cell
lymphomas**

Analysis of T-cell receptor β (Tcrβ) diversity revealed a monoclonal origin of Hdac1<sup>Δ/Δ</sup>;Hdac2<sup>Δ/Δ</sup>
lymphomas, suggesting a requirement for additional genetic events driving full oncogenic
transformation upon reduced HDAC-activity (Fig. 4A). Comparative genomic hybridization
(CGH) of normal versus tumor DNA identified, independent of genotype and HDAC-activity,
trisomy of chromosome 15 as a common and early genetic alteration in all analyzed lymphomas
(Fig. 4B). Chromosome 15 trisomy was previously observed in murine thymic lymphomas and
invariably associated with overexpression of the c-Myc oncogene, a major driver of
lymphomagenesis located on chromosome 15<sup>31</sup>. Indeed, c-Myc expression levels were increased
in Hdac1<sup>Δ/Δ</sup>, Hdac1<sup>Δ/+</sup>;Hdac2<sup>Δ/Δ</sup> and Hdac1<sup>Δ/Δ</sup>;Hdac2<sup>Δ/+</sup> tumors (Fig. 4C,D), suggesting that
during lymphomagenesis reduced HDAC-activity allows the clonal outgrowth of c-Myc
overexpressing thymocytes.

Cytogenetic analysis of Hdac1<sup>Δ/Δ</sup>, Hdac1<sup>Δ/+</sup>;Hdac2<sup>Δ/Δ</sup> and Hdac1<sup>Δ/Δ</sup>;Hdac2<sup>Δ/+</sup> lymphoma cell
lines displayed mild aneuploidy and increased centromeric attachment of multiple chromosomes
(Supplemental Fig. S4A,B) suggesting that reduced HDAC-activity may result in chromosome
misseggregation and generate chromosomal instability. However, analysis of pre-leukemic
thymocytes from one-week-old Hdac1<sup>Δ/Δ</sup>;Hdac2<sup>Δ/+</sup> mice revealed no chromosomal abnormalities
nor altered DNA methylation of pericentric minor-satellite sequences (Supplemental Fig. S4C,D),
indicating that gradual loss of HDAC-activity does not drive tumorigenesis by inducing massive,
acute chromosomal instability.
Hdac1/2 dosage-dependent alleviation of p53 inactivation in lymphomagenesis

Myc-driven tumorigenesis requires inactivation of the p19Arf-Mdm2-p53 pathway either by activating Notch1 mutations, Mdm2 amplification, inactivating mutations in p53 or deletion of the Cdkn2a locus encoding p16Ink4a and p19Arf. Hdac1Δ/Δ;Hdac2+/Δ lymphomas did not harbor Notch1 PEST domain mutations and no transcriptional changes were found in Notch1 targets (data not shown, Supplemental Fig. S5A). Furthermore, no amplification or overexpression of the p53 antagonists Mdm2 and Mdmx was observed in these lymphomas (Fig. 4B; Supplemental Fig. S5A). Western blot analysis of Hdac1+/Δ;Hdac2+/Δ and Hdac1Δ/Δ lymphomas revealed elevated p53 in combination with high p19Arf expression, indicative for inactivating mutations in p53. Remarkably, this was not observed in Hdac1Δ/Δ;Hdac2+/Δ lymphomas (Fig. 5A). p53 immunohistochemical analysis of a tissue micro-array (TMA) containing 50 lymphomas revealed a statistically significant decreased number of Hdac1Δ/Δ;Hdac2+/Δ tumors with high p53 expression compared to Hdac1+/Δ;Hdac2+/Δ and Hdac1Δ/Δ tumors (p = 0.0038, Fishers’s test) (Fig. 5B; Supplemental Fig. S5B). Moreover, sequencing of p53 exons 2-11 revealed p53 wild-type sequences in Hdac1Δ/Δ;Hdac2+/Δ lymphoma cell lines while only missense mutations in the p53 DNA binding domain, a hotspot for tumor-relevant p53 mutations were found in Hdac1+/Δ;Hdac2+/Δ and Hdac1Δ/Δ lymphoma cell lines, which displayed p53 protein stabilization and p19Arf expression (Fig. 5C). Consistently, inhibition of Mdm2-p53 interaction using the small molecule inhibitor Nutlin-3, resulted in stabilization of p53 protein levels and cell death only in Hdac1Δ/Δ;Hdac2+/Δ lymphoma cell lines similar to a wild-type p53 carrying human lymphoma cell line (MOLT-3) (Fig. 5D). In addition, γ-irradiation of Hdac1Δ/Δ;Hdac2+/Δ lymphoma cell lines resulted in increased p53 protein levels, indicative for non-mutated p53 (Fig. 5E). These results indicate an Hdac1 and Hdac2 dosage-dependency for inactivation of the p19Arf-Mdm2-p53 tumor suppressor pathway by p53 inactivating mutations during Myc-driven lymphomagenesis.
Although $Hdac1^{Δ/Δ}:Hdac2^{+/+}$ lymphomas harbor wild-type $p53$ sequence, pre-leukemic $Hdac1^{Δ/Δ}:Hdac2^{+/+}$ thymocytes showed significantly reduced $p53$ levels upon $γ$-irradiation compared to wild-type thymocytes (Fig. 5F). In agreement with impaired $p53$ function, survival of $Hdac1^{Δ/Δ}:Hdac2^{+/+}$ thymocytes was increased upon differential doses of $γ$-irradiation (Fig. 5G). These findings suggest that reduced Hdac1 and Hdac2-governed HDAC-activity in thymocytes resulted in an impaired $p53$ pathway, which bypasses the requirement for $p53$ mutations in c-Myc induced lymphomagenesis. Consequently, impaired $p53$ function may allow clonal outgrowth of c-Myc overexpressing thymocytes but also tolerate mitotic slippage resulting in chromosome 15 trisomy.

**Hdac1 and Hdac2 regulate Myc-collaborating genes**

As transcriptional regulation is a prime function of Hdac1 and Hdac2, we used Hdac1 and Hdac2 transcriptomes in thymocytes and lymphomas to obtain insight into Hdac1/2 mediated suppression of lymphomagenesis. In agreement with reduced HDAC-activity (Fig. 1C), $Hdac1^{Δ/Δ}$ and $Hdac1^{Δ/Δ}:Hdac2^{+/+}$ thymocytes displayed increased levels of acetylated histone H4 and a dosage-dependent increase in acetylated histone H3, a hallmark of transcriptional activation (Fig. 6A). Transcriptomic analysis of pre-leukemic thymocytes from one and three weeks old $LckCre;Hdac1^{Δ/Δ}:Hdac2^{+/+}$ mice and $Hdac1^{Δ/Δ}:Hdac2^{+/+}$ tumors, revealed consistent transcriptional deregulation of 547 transcripts throughout lymphomagenesis of which 238 transcripts were also found deregulated in $Hdac1^{+/+}:Hdac2^{Δ/Δ}$ and $Hdac1^{Δ/Δ}$ tumors (Fig. 6B; Supplemental Table S1,S2). In correlation with HDAC-activity, tumor incidence and tumor latency we observed a dosage-dependent fold change in mRNA levels of many deregulated genes in $Hdac1^{Δ/Δ}$ and $Hdac1^{Δ/Δ}:Hdac2^{+/+}$ thymocytes, providing a rationale for the observed dosage-dependency in tumor suppression (Supplemental Fig. S6A; Fig. 1D). Since chromosome 15 trisomy and Myc overexpression was a common feature of Hdac1/2 lymphomas we investigated
whether genes deregulated in these lymphomas were enriched for genes able to collaborate with Myc in oncogenic transformation. Among the deregulated genes we identified a significant enrichment (164 out of 410 mapped transcripts; Fisher’s exact test: \( p = 6.13 \times 10^{-34} \)) of commonly targeted genes (CTGs) previously found in insertional mutagenesis screens aimed at identifying Myc collaborating genes in lymphomagenesis\(^{28,29}\) (Fig. 6C; Supplemental Table S3). These data indicate that Hdac1 and Hdac2, in a dosage-dependent manner, regulate the expression of genes able to synergize with c-Myc in oncogenic transformation of thymocytes.

**Expression of Jdp2 is critical for survival of Hdac1\(^{ΔΔ}\);Hdac2\(^{+/Δ}\) tumors**

As Myc collaborating genes have been shown to synergize with c-Myc by inactivating the tumor-protective p53 pathway\(^{35}\) and \(Hdac1^{ΔΔ}/Hdac2^{+/Δ}\) pre-leukemic thymocytes display reduced p53 protein levels upon \(γ\)-irradiation, we searched for deregulated genes in \(Hdac1^{ΔΔ}/Hdac2^{+/Δ}\) pre-leukemic thymocytes known to suppress p53 function. Indeed, several Myc collaborating genes were identified which have been implicated in suppression of p53 function such as Jun dimerization partner 2 (\(Jdp2\)), Juxtaposed with another zinc finger protein 1 (\(Jazf1\)), Dual specificity tyrosine-phosphorylation-regulated kinase 3 (\(Dyrk3\)), LPA receptor 2 (\(Lpar2\)), a transcript with unknown function 2010107G12RIK and Ephrin type-B receptor 2 (\(Ephb2\))\(^{36-38}\) (Fig. 6D). The \(Jdp2\) gene specifically drew our interest as \(Jdp2\) is targeted in insertional mutagenesis screens with high frequency in Myc overexpressing lymphomas\(^{28,29}\). In addition, \(Jdp2\) was preferentially activated by insertional mutagens in murine \(p53^{+/−}\) lymphomas which retained the \(p53\) wild-type allele and was shown to suppress \(p53\) expression\(^{37}\). Indeed, we observed an Hdac1/2 dosage-dependent derepression of \(Jdp2\) in \(Hdac1^{ΔΔ}/Hdac2^{+/Δ}\) pre-leukemic thymocytes, which increased further during lymphomagenesis. Moreover, Jdp2 levels inversely correlated with the presence of stabilizing p53 mutations (Supplemental Fig. S6A; Fig. 6D,E). These data suggest that Hdac1/2 levels directly regulate Jdp2, which subsequently in a dosage-
dependent manner represses p53. Indeed, chromatin immunoprecipitation (ChIP) experiments using T-cell lymphoma cell lines expressing GFP-tagged versions of Hdac1 and Hdac2 revealed a direct binding of Hdac1 and Hdac2 to a Jdp2 intron 1-2 promoter region in contrast to a more 5’ UTR region of the Jdp2 locus (Fig. 6F). Moreover, down regulation of Jdp2 using two independent Jdp2 shRNAs resulted in rapid cell death of Hdac1Δ/Δ,Hdac2Δ/Δ lymphoma cells but did not affect the survival of a p53−/− lymphoma cell line (Fig. 6G, Supplementary Fig. S6B), suggesting that Jdp2 is critical for the survival of lymphoma cells in a p53-dependent manner. Collectively, the data presented here demonstrate that Hdac1 and Hdac2 function, in a dosage-dependent manner, as a p53-dependent barrier to prevent oncogenic transformation of Myc overexpressing thymocytes by regulating transcription of p53 suppressors such as Jdp2.
Discussion

In summary, using a unique mouse model displaying a gradient of Hdac1 and Hdac2 governed HDAC-activity, we provide genetic evidence for a previously unknown dosage-dependent tumor suppressor function for Hdac1 and Hdac2.

In contrast to HDAC-activity independent chromosome 15 trisomy associated c-Myc upregulation, we observed HDAC-activity dependent histone H3 hyperacetylation and associated gene deregulation in pre-leukemic thymocytes. The significant enrichment of Myc-collaborating genes (MCGs) amongst the deregulated genes attest to the importance of Hdac1 and Hdac2 as critical regulators of genes that determine cellular fate upon oncogenic insults such as Myc overexpression. MCGs have been shown to synergize with c-Myc in tumorigenesis by inactivating the tumor-protective p19\textsuperscript{Arf}-Mdm2-p53 pathway\textsuperscript{35}. Indeed, pre-leukemic thymocytes harboring relative low HDAC-activity (\textit{Hdac1}$^{−/−}$;\textit{Hdac2}$^{+/−}$) displayed a dysfunctional p53 response and showed no inactivating mutations in \textit{p53}. Consistently, several deregulated MCGs in pre-leukemic \textit{Hdac1}$^{−/−}$;\textit{Hdac2}$^{+/−}$ thymocytes were shown to suppress p53 function. Although the pleiotropic effect caused by loss of HDAC-activity suggests that multiple MCGs contributed to lymphomagenesis, Jun dimerization partner 2 (Jdp2, also known as Jundm2) seemed to play a critical role. Jdp2 was upregulated in an HDAC-activity dependent manner in primary thymocytes as well as lymphomas and Jdp2 levels inversely correlated with p53 mutations (Fig. 6E). A rationale for the latter observation is provided by the known p53 suppressing activity of Jdp2\textsuperscript{37}, which alleviates the need to inactivate p53 upon an oncogenic insult and may also explain why activating retroviral insertions in Jdp2 strongly accelerated Myc-driven lymphomagenesis\textsuperscript{28,39}. This explanation is further supported by observations that \textit{p53}$^{+/−}$ tumors harboring \textit{Jdp2} activating transposon insertions retained wild-type p53\textsuperscript{37}. Finally, ablation of Jdp2 in \textit{Hdac1}$^{−/−}$;\textit{Hdac2}$^{+/−}$ lymphoma cells resulted in rapid cell death while \textit{p53}$^{+/−}$ lymphoma cells remained unaffected, attesting to the p53-dependent role of Jdp2 in maintenance of tumor cells harboring wild-type p53.
Next to Jdp2 we identified deregulated expression of other MCGs regulating p53 function. Dyrk3 was shown to impair p53 activity through phosphorylation of Sirt1\(^{36}\). In addition, Lpar2 was shown to collaborate with c-Myc oncogenic transformation and allowed by-pass of p53-induced senescence in the presence of wild-type p53, suggesting a role for Lpar2 in regulating the p53 fail-safe function\(^{38,40}\). Although we cannot exclude that these Hdac1/2 regulated genes also contributed to lymphomagenesis, the fact that these MCGs do not regulate p53 transcription suggests that Jdp2 is a crucial contributor to the tumor phenotype. Together these results indicate a role for Hdac1- and Hdac2 governed HDAC-activity in preventing “transcriptional instability”. Gradual loss of HDAC-activity will result in a gradual increased transcriptional instability providing the cell with a repertoire of mis-expressed proteins. Upon encountering an oncogenic insult, like c-Myc overexpression, selection from the miss-expressed protein repertoire for the most advantageous combination including proteins inactivating p53 function, will generate a volatile situation that facilitates tumorigenesis (Supplemental Fig. S6C).

Previously, we have shown that loss of Hdac1 and Hdac2 in mouse embryonic fibroblasts (MEFs) resulted in a senescence-like growth arrest independent of p16\(^{INK} /\)p19\(^{ARF}\) and p53, suggesting that other pathways are involved in mediating the loss of Hdac1 and Hdac2\(^2\). Although the mechanism underlying the developmental block during T-cell differentiation in \(\textit{DKO}\) mice remains elusive, it is tempting to speculate that similar pathways are activated upon loss of Hdac1 and Hdac2 in thymocytes. Identification of these pathways may also provide insights in the early thymocyte block in \(\textit{DKO}\) mice.

Our findings provide a cautionary note on the use of HDACi in the clinic as these agents may enable therapy-induced tumorigenesis. Although constitutive genetic inactivation of HDACs is different from temporal, chemical inactivation of HDAC-activity using HDACi, the progression of \(\textit{Hdac1}^{+/\Delta};\textit{Hdac2}^{+/\Delta}\) thymocytes into a full-blown lymphoma suggests that prolonged subtle changes in HDAC-activity may generate a tumor-prone condition that can result in
lymphomagenesis. Prolonged treatment of patients with HDACi may recapitulate the partial HDAC-activity reduction as observed in LekCre:Hdac1Δ/Δ mice and LekCre:Hdac1+/+:Hdac2Δ/Δ mice. Most notably, somatic mutations in HDAC1 were identified in 8.3% of dedifferentiated liposarcoma (DLPS), providing support for a tumor suppressive function of HDAC1 in humans. Moreover, several studies have shown that inactivation of epigenetic regulators may result in adverse effects such as tumorigenesis. Genetic inactivation of the epigenetic regulators DNA methyltransferase 1 (Dnmt1), histone deacetylase 3 (Hdac3) and Sirtuin 2 (Sirt2) resulted in tumor formation in mouse models. Moreover, cancer genome sequencing projects have revealed recurrent mutations in methyltransferase EZH2 and DNA methyl transferase DNMT3A underscoring the potential adverse effects of small molecule inhibitors on these enzymes in the treatment of (cancer) patients.

Although genetic alterations affecting HDAC1 and/or HDAC2 in human T-cell acute lymphoblastic lymphomas (T-ALL) have not been identified, full genome sequencing identified mutations affecting known HDAC1- and HDAC2-interactors, such as BCL11B and RUNX1. The identification of HDAC1- and HDAC2-negative tumor specimen in various other tumor types may encourage to survey T-cell lymphomas for HDAC1 or HDAC2 expression.

Despite the concerns raised by our studies regarding HDACi treatment, they simultaneously revealed that reduced HDAC-activity generates a cancer cell vulnerability. Since critical HDAC activity levels are required to sustain tumor cells one can envision increased HDACi-sensitivity of tumors displaying reduced HDAC-activity since less remaining HDAC-activity needs to be inactivated to reach the HDAC-activity threshold that is critical for viability. Indeed, our data showed an increased responsiveness of tumors displaying relative low HDAC-activity towards pharmacological inhibition of HDACs. Therefore, identification of tumors with low HDAC levels, as has been observed in some tumor types, in contrast to high HDAC expression, may be used as a biomarker to predict HDACi-responsiveness and stratify cancer patient populations.
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Authorship contributions


Conflict of interest

The authors declare no conflict of interest
References

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Figure Legends

**Figure 1.** Hdac1 and Hdac2 dosage-dependent tumor suppression. (A) Kaplan-Meier tumor free survival plot of WT, MxCre⁺;Hdac1⁺⁺ and MxCre⁺;Hdac1⁺⁺;Hdac2⁺⁺ mice. P-value was calculated using a Chi-square test. (B) CD4/CD8 flow cytometry (top) and Hdac1 and Hdac2 immuno-histochemistry (bottom) of tumors from MxCre⁺;Hdac1⁺⁺ and MxCre⁺;Hdac1⁺⁺;Hdac2⁺⁺ mice. (C) Global HDAC-activity in thymocytes with indicated genotypes relative to WT thymocytes. (D) Kaplan-Meier tumor free survival plot of mice harboring thymocytes with indicated genotypes. P-values were calculated using a Chi-square test. (E) Representative pictures of Hdac1 and Hdac2 immuno-histochemical analysis of lymphomas with indicated genotypes. Magnification: 100x. (F) Global HDAC-activity in 4 independent Eμ−Myc and 4 independent Hdac1⁺⁺⁺;Hdac2⁺⁺ tumor cell lines. (G) Dose response curves of 4 independent Eμ−Myc and 4 independent Hdac1⁺⁺⁺;Hdac2⁺⁺ tumor cell lines treated with increasing concentrations of suberoylanilide hydroxamic acid (SAHA). Error bars indicate standard deviations of 3 independent experiments per tumor cell line.

**Figure 2.** Hdac1 and Hdac2 control pre-T cell development in a dosage-dependent manner. (A) Tcrβ repertoire determined in 3 weeks old WT and H dac1⁺⁺⁺;Hdac2⁺⁺ thymi by Southern blot analysis using a Jβ2 probe sequence (right panel). H dac1⁺⁺⁺;Hdac2⁺⁺ lymphoma DNA was used as a positive control, while ethidium bromide stained gel served as a loading control (left panel). (B) Quantification of thymocytes from 1 week old mice of the indicated genotypes (n = 3 per genotype). (C) Apoptosis in thymocytes of one-week-old WT, LckCre;Hdac1⁺⁺⁺ and LckCre;Hdac1⁺⁺⁺;Hdac2⁺⁺⁺ mice, as determined by Annexin V and propidium iodide staining (PI). Mean percentages of apoptotic (AnnexinV⁺PI⁻) cells are presented on top (n = 3 mice per genotype). (D) Representative dot plots of CD4/CD8 (top)
and CD24/TCRβ (bottom) flow cytometric analyses of thymi from one-week-old mice with indicated genotypes. (E) Quantification of thymic subsets of one-week-old mice with indicated genotypes. DN = CD4−CD8−, DP = CD4+CD8+, CD4 SP = CD4+CD8−, CD8 SP = CD4−CD8+, (left) ISP = CD4 CD8+CD24+Tcrβ+/-, mature CD8 = CD4 CD8+CD24+/-Tcrβ+ (right). (F) Dot plots representing BrdU-7-AAD flow cytometric analysis of thymocytes from WT and LckCre:Hdac1+/Δ;Hdac2+/Δ mice 1.5 hour after BrdU injection. Average and standard deviation (SD) of BrdU-positive thymocytes are indicated on top (n = 3 mice per group).

Figure 3. Hdac1 and Hdac2 collectively show obligate haploinsufficiency in tumor suppression. (A) Quantification of thymocytes in one-week-old WT and LckCre:Hdac1+/Δ;Hdac2+/Δ (DKO) mice (n = 3 mice per genotype). (B) Apoptosis in thymi of one-week-old WT and DKO mice, as determined by Annexin V and propidium iodide staining (PI). Mean percentages and standard deviations of apoptotic (AnnexinV+PI−) cells are presented on top (n= 3 mice per genotype). (C) Representative CD4/CD8 (top panel) and CD25/CD44 (bottom) flow cytometry dot plots of thymocytes from one-week-old WT and DKO mice. Scheme on the right indicates DN stages of thymocyte development. (D) Quantification of thymic subsets in one-week-old WT and DKO mice; DN=CD4−CD8−; DP=CD4+CD8+; CD4 SP=CD4+CD8−; CD8 SP=CD4−CD8+; DN1=CD25−CD44+; DN2=CD25+CD44+; DN3=CD25+CD44−; DN4=CD25−CD44−.

Figure 4. c-Myc overexpression associated chromosome 15 trisomy in monoclonal T-cell lymphoma. (A) Tcrβ diversity in 12 independent Hdac1+/Δ Hdac2+/Δ lymphomas analyzed by Southern blot, using a Jβ2 region specific probe, g.l.= germline Tcrβ. (B) Representative CGH plots of 6 independent lymphomas with indicated genotypes. (C) c-Myc levels in WT thymocytes and Hdac1+/Δ Hdac2+/Δ tumors analyzed by quantitative PCR. (D) c-Myc protein
levels in wild-type thymi as well as $Hdac1^{+/\Delta}$, $Hdac1^{+/\Delta};Hdac2^{+/\Delta}$ and $Hdac1^{+/\Delta};Hdac2^{+/\Delta}$ lymphoma cell lines. Bottom panel shows quantification of c-Myc protein levels relative to tubulin.

**Figure 5.** Hdac1 and Hdac2 dosage-dependent requirement for p53 inactivation. (A) Western blot of protein lysates from tumors with indicated genotypes using Hdac1, Hdac2, p19$^{\text{Arf}}$ and p53 antibodies. $\alpha$-tubulin served as loading control. (B) Summary of p53 staining intensity, indicative for stabilized/mutant p53, of a tissue micro-array containing $Hdac1^{+/\Delta};Hdac2^{+\Delta}$ (n=6) $Hdac1^{+/\Delta}$ (n=8) and $Hdac1^{+/\Delta};Hdac2^{+/\Delta}$ (n=36) tumors, revealed a significant decrease of mutant p53 in $Hdac1^{+/\Delta};Hdac2^{+/\Delta}$ tumors (Fisher’s test $p=0.0038$; bottom table). (C) Western blot analysis of $Hdac1^{+/\Delta};Hdac2^{+/\Delta}$, $Hdac1^{+\Delta}$ and $Hdac1^{+\Delta};Hdac2^{+/\Delta}$ lymphoma cell lines as well as wild-type thymocytes for expression of p53 and p19$^{\text{Arf}}$. Tubulin served as a loading control (top). p53 DNA binding domain missense mutations (F130S) in $Hdac1^{+/\Delta}$ and $Hdac1^{+/\Delta};Hdac2^{+/\Delta}$ tumor cell lines. p53 sequence of wild-type thymocytes served as reference (bottom). (D) Western blot analysis of Nutlin-3 treated $Hdac1^{+/\Delta};Hdac2^{+/\Delta}$ lymphoma cell line for expression of p53. Human MOLT-3 T-ALL cell line, harboring wild-type p53, and a murine $p53^{+/\Delta}$ T-cell lymphoma served as controls. Tubulin served as a loading control. Nutlin-3 treatment resulted only in wild-type p53 bearing lymphoma cell lines in loss of viability as determined by Cell-titer blue assay (lower panel) and visual inspection of cell cultures (right panel; magnification 200x). (E) Western blot analysis of protein lysates of $p53^{+/\Delta}$, $Hdac1^{+/\Delta}$ and $Hdac1^{+/\Delta};Hdac2^{+/\Delta}$ tumor cell lines treated with ionizing radiation (6 Gy) for p53 and p19$^{\text{Arf}}$. Hdac1$^{+/\Delta};Hdac2^{+\Delta}$ tumor cell lines showed radiation induced p53 in contrast to an $Hdac1^{+/\Delta}$ lymphoma cell line expressing mutant p53. (F) Western blot analysis (top panel) of p53 protein levels in one-week-old WT and $Hdac1^{+/\Delta};Hdac2^{+/\Delta}$ thymocytes, 6 hours after mock or 6 Gy $\gamma$-irradiation. Tubulin served as
loading control. Bottom panel shows quantification of p53 levels relative to tubulin. Error bars indicate standard deviations of thymocyte cultures isolated from 3 independent mice per genotype. (G) Percentage of viable cells in 3 independent WT and Hdac1Δ/Δ;Hdac2Δ/+ thymocyte cultures, 24 hours after indicated doses of γ-irradiation.

**Figure 6.** (A) Western blot analysis of histone H3 and H4 acetylation in nuclear lysates of pre-leukemic thymocytes from 3 independent one-week-old wild-type, LckCre⁺;Hdac1Δ/Δ and LckCre⁺;Hdac1Δ/Δ;Hdac2Δ/+ mice (left). Acetylated H3 and H4 signals were quantified over total H3 and H4 signal, respectively. While AcH3/H4 levels were significantly higher in LckCre⁺;Hdac1Δ/Δ and LckCre⁺;Hdac1Δ/Δ;Hdac2Δ/+ compared to wild-type thymocytes, only AcH3 levels were increased in LckCre⁺;Hdac1Δ/Δ;Hdac2Δ/+ thymocytes compared to LckCre⁺;Hdac1Δ/Δ counterparts. (B) Venn-diagram demonstrating the overlap between sets of differentially expressed genes (adjusted P-value < 0.05, fold change ≥ 1.5x, n ≥ 3 independent mice per group) in one and three week old Hdac1Δ/Δ;Hdac2Δ/+ thymocytes and lymphomas. (C) Schematic representation of the overlap between commonly targeted genes (CTG) identified in insertional mutagenesis screens and genes deregulated in Hdac1Δ/Δ;Hdac2Δ/+ thymocytes and lymphomas (“HDAC”) (Fisher's exact test: p = 6.36e⁻¹⁰). (D) Fold changes in mRNA of Myc-collaborating genes Jdp2, Jazf1, Dyrk3, Lpar2, 2010107G12RIK and Ephb2 in one and three-week-old Hdac1Δ/Δ;Hdac2Δ/+ thymocytes and Hdac1Δ/Δ;Hdac2Δ/+ lymphomas, relative to age matched wild-type thym. (E) Western blot analysis of protein lysates of lymphomas of indicated genotypes for Hdac1, Jdp2 (lower band) and p53. Tubulin served as a loading control. (F) Western blot analysis (left) and chromatin immunoprecipitation analysis (right) of GFP, Hdac1-GFP or Hdac2 GFP expressing p53Δ/+ T-cell lymphoma cell lines for the Jdp2 intron 1-2 and 5’ UTR. (G) Cell viability assessed by Cell titer blue assay (left) and representative images (right; magnification 100x) of
$Hdac1^{+/+};Hdac2^{+/-}$ and $p53^{-/-}$ lymphoma cell lines infected with independent lentiviral $Jdp2$ shRNA constructs ($Jdp2_{KD1}$ or $Jdp2_{KD2}$), a lentiviral non-targeting shRNA (NT) construct, or mock infection.
Heideman_Figure 2
Dosage-dependent tumor suppression by histone deacetylases 1 and 2 through regulation of c-Myc collaborating genes and p53 function

Marinus R. Heideman, Roel H. Wilting, Eva Yanover, Arno Velds, Johann de Jong, Ron M. Kerkhoven, Heinz Jacobs, Lodewyk F. Wessels and Jan-Hermen Dannenberg