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Unveiling novel functions of the trascriptional repressor HDAC7 in B lymphocyte development

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UNVEILING NOVEL FUNCTIONS OF THE TRANSCRIPTIONAL REPRESSOR HDAC7 IN B LYMPHOCYTE DEVELOPMENT

Memòria presentada per Alba Azagra Rodríguez per optar al grau de Doctor en Biomedicina per la Universitat de Barcelona

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AGRAÏMENTS

Agraïments

"I will discover the cure of cancer". Aquesta frase era el meu recurs més usat a la ESO per exemplificar el futur simple a anglès, una frase que amb el pas dels anys s'ha tornat massa simple i innocentment ambiciosa. De vegades va bé recordar per què ens vam ficar en aquest món i quins eren els nostres primers pensaments, desitjos i objectius, fossin més o menys realistes, perquè segurament eren els més entregats, purs i desinteressats. El meu primer desig de curar el càncer contenia una visió utòpica del món de la ciència, essent els pacients la principal prioritat, i involucrava la col·laboració de tots els membres del camp per poder arribar abans al coneixement i la cerca de teràpies. Per sort, més d'una persona durant aquests anys de tesi m'ha portat a recordar les bases d'aquest primer desig.

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ABBREVIATIONS

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3D: three-dimensional

5-caC: 5-carboxylcytosine

5-fC: 5-formylcytosine

5-hmC: 5-hydroxymethylcytosine

5-mC: 5-methylcytosine

AGO: argonaute protein

AID: activation-induced cytidine deaminase

ALL: acute lymphoblastic leukaemia

AML: acute myeloid leukaemia

AZA: 5-azacitidine

BCL2: B- cell lymphoma 2

BCR: B-cell receptor

BER: base excision repair

BLIMP1/*PRDM1:* PR domain zinc finger protein 1

CAM: cell adhesion molcules

CaMK: calcium/calmodulin-dependent protein kinase

CD4/8/11b/19/28/40/43/44/69: cluster of differentiation 4/8/11b/19/28/40/43/44/69

CD40L: ligand of cluster of differentiation 40

CDK1: cyclin dependent kinase 1

cDNA: complementary deoxyribonucleic acid

C/EBP: CCAAT-enhancer-binding proteins

CFP1: CxxC finger protein 1

CGI: CpG island

ChIP: chromatin immunoprecipitation

ChIP-seq: chromatin immunoprecipitation and sequencing

CK2: casein kinase 2

CLPs: common lymphoid progenitors

CLL: chronic lymphoblastic leukaemia

CMML: chronic myelomonocytic leukaemia

CMP: common myeloid progenitors

CNS: central nervous system

CoREST: co-repressor for element-1-silencing transcription factor

CRISPR: clustered regularly interspaced short palindromic repeats

CSR: class switch recombination

CtBP: C-terminal binding protein

CTL: cytotoxic T cells

C-terminal: carboxy-terminal

Cys: cysteine

DAC: 5-Aza-2'-deoxycytidine

DDX21: DExD-Box Helicase 21

DGCR8: DiGeorge syndrome chromosomal region 8

Dicer: endoribonuclease Dicer/helicase with RNase motif

DLBCL: diffuse large B cell lymphoma

DNA: deoxyribonucleic acid

DNMT: DNA methyltransferase

Doxy: doxycycline

Drosha: double-stranded RNA-specific endoribonucleas

DSB: double strand break

DSBH: double stranded B helix

E2A: Basic Helix–Loop–Helix Factor E2A

EBF1: early B cell factor 1

EHT: endothelial-to-hematopoietic transition

EMT: epithelial-to-mesenchymal-transition

ESCs: embryonic stem cells (hECSs: human; mESCs: murine)

ETS: E26 transformation-specific or E-twenty-six

ETV6: ETS variant 6

FC: fold change

FDA: Food and Drug Administration

FDR: false discovery rate

FLT3: FMS-like tyrosine kinase 3

FOXO1: Forkhead box protein O1

FOXP1/3: Forkhead Box P1/3

G2: gap 2 phase

GATA-1/3: GATA binding factor 1/3

GC: germinal center

GEO: gene expression omnibus

GMPs: granulocyte/macrophage progenitors

GO: gene ontology

H: histidine

H1/2A2B/3: histone 1/2A/2B/3

γH2AX: histone variant H2AX phosphorylated on serine 139

H3K27ac: acetylation at the 27th lysine residue of the histone 3

H3K27me3: trimethylation at the 27th lysine residue of the histone 3

H3K4me1: monomethylation at the 4th lysine residue of the histone 3

H3K9K14ac: acetylation at the 9th and 14th lysine residue of the histone 3

H3K9me3: trimethylation at the 9th lysine residue of the histone 3

H4K12: 12th lysine residue of the histone 4

H4K16: 16th lysine residue of the histone 4

H4K5: 5th lysine residue of the histone 4

HATs: histone acetyltransferases

HDACs: histone deacetylases

HDI: HDAC inhibitor

HEB: helix-loop-helix protein

HmeDIP: hydroxymethylated DNA immunoprecipitation

HmeDIP-seq: hydroxymethylated DNA immunoprecipitation and sequencing

HP1: heterochromatin protein 1

HSCs: hematopoietic stem cells

ID2: inhibitor of differentiation 2

IDAX: CXXC-type zinc finger protein 4

IFN: interferon

Ig: immunoglobulin

IKZF1: IKAROS family zinc finger 1

IL10: interleukin 10

IL7: interleukin 7

IL7R: interleukin 7 receptor

Immgen: Immunological Genome Project Database

iNKT: invariant Natural Killer T cells

KD: knockdown

KDM2: lysine demethylase 2

KEGG: Kyoto Encyclopaedia of Genes and Genomes

KFGG: lysine-phenyalanine-glycine-glycine

KO: knockout

Lin28A: Lin-28 homolog A protein

LINE (L1): long interspersed nuclear element

LMPPs: lymphoid-primed multipotent progenitors

LTR: long terminal repeat

MBDs: Methyl-CpG-binding domain

MADS: MCM1 AGAMOUS DEFICIENC SRF box

MDS: myelodysplastic syndromes

MeCPs: methyl-CpG-binding proteins

MEF2C: myocyte enhancer factor C

MEPs: megakaryocyte/erythrocyte progenitors

miRNA: micro-RNA

MLL: mixed-lineage leukemia

MMP10: Matrix Metallopeptidase 10

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

MYTP1: myosin phosphatase targeting protein

NAD: nicotinamide adenine dinucleotide

N-CoR: nuclear receptor co-repressor 1

NFϰβ: nuclear factor kappa-light-chain-enhancer of activated B cells

NGIB/Nur77: nerve growth factor IB

NuRD: nucleosome remodelling and deacetylation complex

NK: natural killers

N-terminal: amino-terminal

OCT4: octamer-binding transcription factor 4

OR: odds ratio

ORF: open reading frame

p53/TP53: tumor protein 53

p73/TP73: tumoral protein 73

PAX5: paired box protein 5

PC: plasma cells

PD-L1: programmed death ligand 1

PKD: protein kinase D

piRNA: piwi-interacting RNA

PP2A: protein phosphatase 2

PP1β: protein phosphatase 1β

Pre-B: precursor B cells

Pre-miRNA: precursor micro-RNA

Pro-B: progenitor B cells

PTM: post-translational modifications

RAG: recombination activating genes

RIP-seq: RNA immunoprecipietation and sequencing

RISC: RNA-induced silencing complex

RNA: ribonucleic acid

RUNX1: runt-related factor 1

SAHA: suberoylanilide hydroxamic acid

SHM: somatic hypermutation

SINE: short interspersed nuclear element

SIRT: sirtuin

SMC3: structural maintenance of chromosomes 3

SMRT: silencing mediator f retinoid and thyroid hormone receptors

STAT3: signal transducer and activator of transcription 3

SUV39h1: histone-lysine N-methyltransferase

SVA: SINE-VNTR-Alu elements

TCF7: transcription factor 7

TDG: thymine DNA glycosylase

TE: transposable elements

TET: ten-eleven translocation

TGF-β: transforming growth factor beta

TF: transcription factor

Tfh: T follicular helper cells

TIRs: terminal inverted repeats

TMB: 3,3’,5,5’,-tetramethylbenzidine

TPRT: target-primed reverse transcription

Treg: T regulatory cells

TSA: trichostatin A

TSS: transcription start site

Tyr: tyrosine

UHRF2: E3 ubiquitin-protein ligase

UTR: untranslated region

WT: wild-type

Y: tyrosine

YY1: Ying Yang 1

ZNF423: zinc finger protein 423

THESIS ABSTRACT

Thesis abstract

B lymphopoiesis is the result of several cell lineage choices and differentiation steps whose perturbation leads to B cell malignancies. Cellular transitions for B cell generation have been associated with gene activation and silencing by networks of B cell specific transcription factors (TFs) and dynamic changes in DNA methylation. How gene repression is established and which lineage-specific transcriptional repressors are involved during B cell lymphopoiesis is still not totally understood. The Cellular Differentiaion group had previously reported that the transcriptional repressor HDAC7 is highly expressed in B cell progenitors (pro-B cells) and B cell precursors (pre-B cells) but not in myeloid cells such as macrophages.

Here, we have demonstrated that HDAC7 is essential for early B cell development and the acquisition of proper B cell identity. There is a block of pro-B to pre-B cell stages transition and a significant increase of cell death rate upon HDAC7 deletion in these populations. We found that HDAC7 represses myeloid and T lymphocyte genes in pro-B cells through specific interaction with the TF MEF2C. Chromatin immunoprecipitation (ChIP) experiments revealed that HDAC7 is recruited to the promoters and enhancers of lineage inappropriate genes in normal pro-B, leading to their transcriptional silencing.

Notably, by using *in vivo* and *in vitro* experimental approaches, we found that HDAC7 represses *Tet2* in pro-B. On one hand, microarray and RT-qPCR analysis showed that *Tet2* expression is up-regulated in pro-B cells from HDAC7-deficient mice. On the other hand, we found that HDAC7 is down-regulated during the conversion of pre-B cells into macrophages and its exogenous expression blocks the up-regulation of *Tet2*. Similarly to the case of other lineage inappropriate lineage genes, HDAC7 is recruited to the promoter and enhancer of the *Tet2* gene in pro-B cells and its absence leads to an increase and a decrease in active and repressive histone marks, respectively. Additionally, we observed that the absence of HDAC7 from pro-B cells results in a significant increase in the percentage of global 5-hydroxymethylation (5-hmC). To definitively prove the role of HDAC7 in 5-hmC, we performed a genome-wide experimental approach. hMeDIP-sequencing experiments revealed an increase in the enrichment of this epigenetic modification at many loci related to lineage inappropriate genes in the absence of HDAC7. Interestingly, we observed 5-hmC enrichment at retrotransposon elements (LINE-1) in HDAC7 deficient pro-B cells, suggesting a potential protector function of HDAC7 against chromatin instability and DNA damage. Additonal results revealed that 5-hmC enrichment at microRNAs and their expression was also regulated by HDAC7. Several miRNAs

involved in normal and aberrant hematopoiesis changed their expression levels depending on the presence of HDAC7 in pro-B cells.

Finally, we found that HDAC7 is expressed at very low levels in certain hematological malignancies, such as Burkitt lymphoma and B cell acute lymphoblastic leukemia (B-ALL) cell lines. In fact, induction of HDAC7 expression in these tumoral cells led to the activation of apoptotic processes, reducing significantly their viability, and to the reduction of oncogene c-MYC expression. Importantly, those effects were observed by interaction with the TF MEF2C and independently of the class I HDAC3 function. These results suggest an anti-oncogenic role for HDAC7 in some types of B cell malignancies.

Altogether, our results demonstrate that HDAC7 is an essential transcriptional repressor during early B cell development that silences lineage or functionally inappropriate genes at multiple levels. It exerts its function by direct recruitment to target genes through specific TF, by regulating LINE-1 and miRNA expression and by controlling the expression levels of a critical epigenetic regulator such as TET2 demethylase enzyme.

INTRODUCTION

1. B lymphocyte development

1.1 Overview

B lymphocyte generation is the result of several cell lineage choices and differentiation steps which are tightly regulated at the transcriptional level. During embryo development, first hematopoietic stem cells (HSCs) arise from endothelial cells that undergo endothelial-to-hematopoietic transition (EHT). This is a very dynamic process in which there is a progressive silencing of endothelial specific genes and activation of HSC genes. EHT is regulated by a huge set of epigenetic and post-transcriptional factors, considering that this transition is essential for the future generation and preservation of all blood cell types (Guibentif et al., 2017; Kasper and Nicoli, 2018).

Once in the bone marrow, HSCs differentiate into lymphoid-primed multipotent progenitors (LMPPs), which can give rise to both myeloid and lymphoid lineages (Adolfsson et al., 2005; Yoshida et al., 2006; Nimmo et al., 2015). LMPPs generate common lymphoid progenitors (CLPs), which can differentiate into T cells, B cells and Natural Killer cells (NK). LMPPs can also give rise to common myeloid progenitors (CMPs), which can either differentiate into granulocyte/macrophage progenitors (GMP) or either megakaryocyte/erythrocyte progenitors (MEPs) (Molawi and Sieweke, 2013; Shortman et al., 2013). During early B cell development in the bone marrow, CLPs differentiate into B cell progenitors (pro-B cells). pro-B cells will further differentiate into B cell precursors (pre-B cells), which in turn will give rise to immature B cells (Kondo et al., 1997; Cobaleda and Busslinger, 2008). Then immature B cells leave the bone marrow and migrate to secondary lymphoid organs such as the spleen and lymph nodes in order to complete B cell differentiation and, consequently, the acquisition of a proper humoral immune response. Briefly, resting naïve B cells are activated and become germinal center (GC) cells by generating high-affinity antibodies in response to antigens presented by T-helper cells. Finally, B cells exit the GC and differentiate into memory B cells or antibody-secreting plasma cells (PC) (Victora and Nussenzweig, 2012).

During early B cell development, every cellular transition and differentiation step is characterized by the activation of a new lineage-specific genetic program and the repression of the previous one, both regulated by complex networks of transcription factors (TFs) in association with dynamic changes in DNA methylation, histone modifications and chromatin conformation changes (Cobaleda & Busslinger, 2008; Kieffer-Kwon et al., 2017; Méndez & Mendoza, 2016; Parra M, 2009). Notably, deregulation of these specific transcriptional programs underlying B lymphopoiesis may

lead to the development of B cell malignancies such as leukemia and lymphoma. In the last years, many groups have focused their research on the study of the hematopoietic system and its regulation. Several studies, including single cell approaches and transplantation assays, have established that HSC are an heterogeneous population with different lineage priming at the same time and that lymphoid genes become expressed once cells arise LMPP stage (Figure 1A) (Adolfsson et al., 2005; Nimmo et al., 2015). Strikingly, recent single cell RNA sequencing studies have proposed a model for hematopoiesis in which cellular transitions are diffused in dynamic expression pattern changes, creating then a landscape of continuous differentiation (Watcham et al., 2019; Laurenti and Göttgens, 2018). These results support the representation of the hematopoietic development with continuum lines or channels in which cells flow until they reach their differentiated state (Figure 1B), questioning the validity of more classical representations. However, the understanding of the molecular mechanisms involved in such dynamic gene expression and functional changes during cellular transitions has not been completed yet.

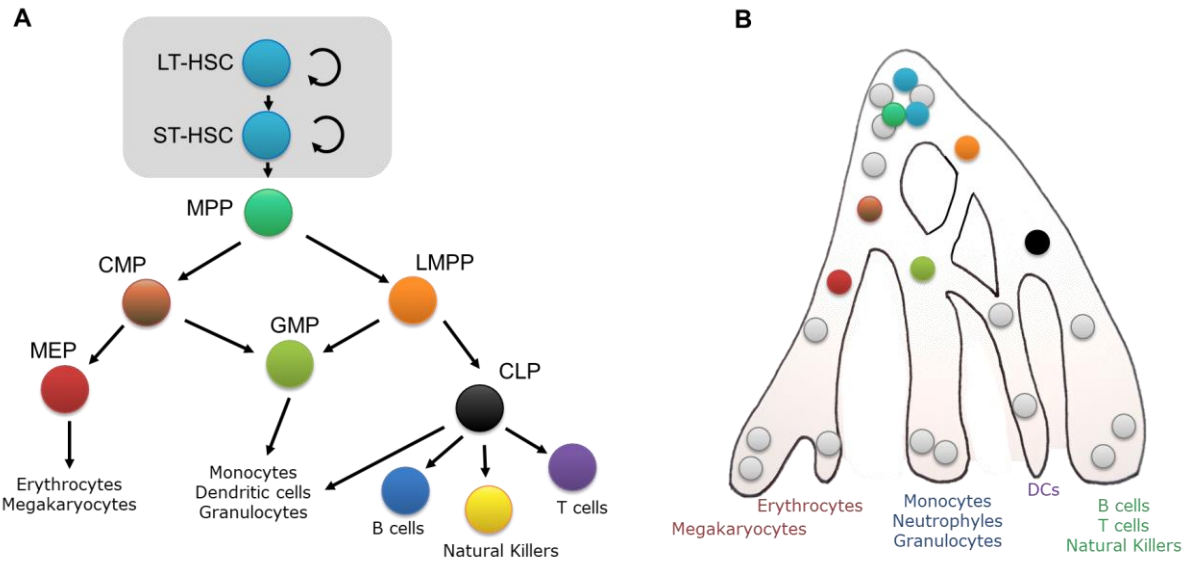

Figure 1. Representation of the hematopoietic system. (A). "Classical" representation of hematopoietic cell differentiation based on circles and narrows to define cell stages and differentiation decisions, respectively. HSC niche is represented in grey. MPP (multipotent progenitors), LMPP (lymphoid-primed progenitors), CMP (common myeloid progenitors), MEP (megakaryocyte-erythroid progenitors), GMP (granulocyte-monocyte progenitors), CLP (common lymphoid progenitors). (B) Representation based on single cell transcriptomic studies, in which cells undergo continuum differentiation. Grey circles represent cells, and colored circles represent the same cellular stage as in A. (Figure modified from Nimmo R et al 2015 (A) and Laurenti & Göttgens, 2018 (B)).

1.2 Transcriptional regulation in early B cell development

1.2.1. Transcription factors (TFs)

Early B cell development comprises all those developmental stages that take place in the bone marrow. At the LMPP stage, there are three main TFs involved in the choice towards lymphocyte development instead of the myeloid lineage: IKAROS, PU.1 and MEF2C (Parra M, 2009; Ramírez, Lukin, & Hagman, 2010). Later stages of B cell lineage development in the bone marrow depend on additional TFs, including E2A, EBF1 and PAX5, which act together to activate some early B cell specific genes such as *mb-1* (Figure 2 and Figure 3) (Parra M, 2009; Ramírez et al., 2010; Sigvardsson et al., 2002).

IKAROS

IKAROS is encoded by *Ikfz1* gene and is characterized by two highly conserved domains that allow them to form dimers and multimers with other IKAROS family members (Georgopoulos et al., 1992). IKAROS is involved in the generation of different cell lineages (GMPs, CMPs and CLPs) and its absence in *Ikaros*-null mice results in the alteration of HSCs, prevents the generation of several hematopoietic cell types such as B and T lymphocytes and erythrocytes, among others, and leads to the generation of lymphocytic leukemia (Heizmann et al., 2013; Kastner et al., 2013; T. Yoshida et al., 2006).

PU.1

PU.1 belongs to the Ets family of TFs encoded by the *Sfpi* gene. It is required for erythroid, lymphoid and myeloid lineages, and its expression levels dictate its influence in generating one cell type or another (Mak et al., 2011; Scott et al., 1994). High levels of PU.1 leads to macrophage generation, while lower concentrations direct lymphocyte or granulocyte differentiation (DeKoter and Singh, 2000; Dahl and Simon, 2003; Mak et al., 2011). In the case of T cells, PU.1 is required for initial T cell development but practically undetectable in later stages (Rothenberg et al., 2019). Notably, embryonic disruption of PU.1 expression affects the maintenance of HSCs, the generation of T cells and neutrophils, and prevents completely the differentiation of B cells and macrophages (Iwasaki et al., 2005; McKercher et al., 1996). Mice lacking PU.1 die approximately 2 days after birth due to serious septicemia. Mutations of *Sfpi* gene that do not annul completely but reduces its expression give rise to myeloid leukemia development (Rosenbauer et al., 2004; Kastner and Chan, 2008). Recent studies supported that PU.1 is crucial for B lymphoid differentiation in the earliest stages

(transition of LMPPs to CLPs), but further differentiation steps do not require its activity (Pang et al., 2018).

MEF2C

Myocyte enhancer factor 2C (MEF2C) was discovered in 1989 in skeletal muscle, but it is also expressed in other cell types such as neural, endothelial or immune (especially in B lymphocytes) cells (Gossett et al., 1989). The N-terminal domain of MEF2 factors has a highly conserved MADS-box and a MEF2 domain, which allow them to form homo- or heterodimers between MEF2 family members, DNA binding and interaction with co-factors such as GATA proteins or EBF1 (Swanson et al., 1998; Black and Olson, 1998; Morin et al., 2000; Kong et al., 2016). In lymphoid cells, *Mef2c* is a transcriptional target of PU.1 during B lymphocyte development. MEF2C is not only involved in the activation of several lymphoid-lineage genes such as *Foxo1*, *Myb*, *Ets1* and *IL7r*, it also mediates the silencing of myeloid characteristic genes. Conditional deletion of MEF2C in pro-B cells causes a significant decrease in B cell numbers and B cell development is blocked at the pre-B cell stage (Stehling-Sun et al., 2009; Gerstein, 2009; Debnath et al., 2013; Herglotz et al., 2016; Kong et al., 2016).

E2A

The TF E2A is encoded by the *Tcfe2a* gene that can give rise to two different loop-helix proteins equally important for B cell commitment, E12 and E47, through alternative splicing mechanisms (Bain et al., 1997, 1994). *In vitro*, E2A controls the initiation of early B cell development but it is not essential for its maintenance, as E2A-deficient pre-B cells do not suffer a complete loss of its target genes expression (Zhuang et al., 1994; Lazorchak et al., 2006). However, *in vivo* experiments revealed that E2A is continuously necessary for commitment into the B cell lineage, pro-B cell generation and further differentiation, including BCR formation and GC reaction in secondary lymphoid organs. In fact, loss of E2A leads to an arrest in development at the pre-pro-B cell stage, impairs the expression of PAX5, EBF1 and their target genes and also promotes the expression of alternative lineage genes in lymphoid progenitors (Dias et al., 2008; Kwon et al., 2008). E2A in cooperation with EBF1 and PAX5 regulates *Cd19*, *Iu, λ5, mb-1*, *Il7r*, and *Vpreb* gene expression. Additionally, *Foxo1* is regulated by E2A and HEB. Finally, coordinated action of FOXO1, FOXP1 and E2A mediate *RAG* gene expression, essential genes for V(D)J recombination and consequent immunoglobulin (Ig) assembly in B cells (Borghesi et al., 2005; Welinder et al., 2011; Chen et al., 2011).

EBF1

Early B cell factor 1 (EBF1) is another TF crucial for early B cell development, which was discovered in 1991 as a DNA-binding regulator of *mb-1* expression in the B cell lineage (Hagman et al., 1991, 1993). Similarly to E2A, loss of EBF1 expression leads to complete pre-pro-B cell stage blockage and a failure in pro-B cell proliferation. However, forced expression of EBF1 in HSCs favors cellular commitment to B lymphocytes and, consequently, detriments the generation of other hematopoietic lineage cells (Lin and Grosschedl, 1995; Zhang et al., 2003; Györy et al., 2012; Åhsberg et al., 2013). EBF1 expression is initiated by the coordinated action between E2A and FOXO1, and its activity is subsequently maintained by IL7 signaling and positive feedback loops that include other TFs such as PAX5 and FOXO1. Importantly, reciprocal activation between FOXO1 and EBF1 at the CLP stage is essential for further differentiation. In addition, recent studies showed that EBF1 and MEF2C interact and co-occupy a set of genes at the pre-B cell stage. EBF1 targets several components of the pre-BCR complex, such as *Vpreb1-3* and *mb-1* (Sigvardsson et al., 1997; Tsapogas et al., 2011; Mansson et al., 2012; Kong et al., 2016). Recent studies demonstrated that EBF1 possess a “pioneer” function through its C-terminal domain. In fact, EBF1 is able to induce changes in chromatin architecture in order to restrict B cell lineage and its binding to inaccessible chromatin regions is independent of the activity of other TFs. In order to achieve this dynamism in chromatin positioning, EBF1 recruits multiple chromatin modifiers (Treiber et al., 2010; Boller et al., 2016; Li et al., 2018b).

FOXO1

The relevance of FOXO1 in early B cell development was discovered few years later than the above described TFs. This protein plays an important role in many stages of B lymphopoiesis, as it is involved in the preservation of HSC pool, the development of B cell progenitors and precursors, DNA rearrangement and pre-BCR signaling, cellular tolerance, cell cycle and terminal differentiation (Inoue et al., 2017b; Szydłowski et al., 2014; Schmidt et al., 2002). B cells present different functional or genetic alterations depending on the stage in which FOXO1 has been deleted. In fact, complete loss of FOXO1 expression involves embryonic lethality (Furuyama et al., 2004). Knockout of FOXO1 at earliest stages produce a block in the pro-pre B cell stage and these cells can produce neither IgM nor IgD. In later stages such as late pro-B cells, FOXO1 absence lead to a block in pre-B cell stage due to a decrease in RAG proteins expression, impairing DNA rearrangement (Amin and Schlissel, 2008; Dengler et al., 2008). *Foxo1* is activated by E2A and both, FOXO1 and E2A together promote EBF1 expression. FOXO1 and EBF1 mediate a reciprocal positive activation. Computational

studies discovered that these three TFs generate a global network that is essential to orchestrate B cell development. In coordination with other TFs such as PU.1 they activate *Pax5*, another crucial factor implicated in preserving B cell identity (Boller and Grosschedl, 2014; Decker et al., 2009; Lin et al., 2010a; Welinder et al., 2011).

PAX5

PAX5 is considered “the guardian of B cell identity” for continuous maintaining B cell identity and establishment of B cell commitment (Cobaleda et al., 2007b). It is expressed at the pro-B cell stage in early B cell development and its activity is maintained during the following B cell stages until cells differentiate into plasma cells (Delogu et al., 2006; Horcher et al., 2001). The specific set of PAX5 target genes changes at every stage, which reflects how much dynamic is the genetic landscape reorganization across cellular differentiation (Revilla-i-Domingo et al., 2012). PAX5 regulates several chromatin and epigenetic modulators, in addition to key factors involved in B cell signaling, structural proteins implicated in cell migration and adhesion, and relevant TFs of later stages that mediate antigen presentation and germinal center formation (Schebesta et al., 2007; Cobaleda et al., 2007b). *Pax5*-null mice present a blockage of differentiation at the pro-B cells, which can differentiate into other hematopoietic lineages. Strikingly, PAX5-deficient pro-B cells do not progress in B cell differentiation until the expression of this TF is recovered, as it is essential to determine their cellular fate (Nutt et al., 1997; Rolink et al., 1999; Schaniel et al., 2002). These results suggest that PAX5 is not only involved in the activation of B lineage genes, but also in the repression of lineage inappropriate genes. In addition, many studies have defined *Pax5* as a tumor suppressor gene and have observed evidences that correlate PAX5 alterations (e.g. translocations or fusion proteins) to lymphomagenesis and leukemia development (Cobaleda et al., 2007b; Pridans et al., 2008; Liu et al., 2014; Smeenk et al., 2017; Bastian et al., 2019).

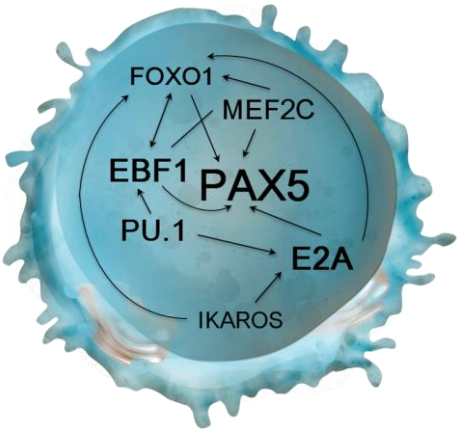

Figure 2. Transcription factors networks involved in early B cell development. Activating pathways are represented in a lymphocyte-like drawing.

1.2.2. Transcriptional repression

As mentioned above, lymphocyte-specific TFs do not only induce the expression of B cell specific genes. TFs are also involved in the silencing of lineage or functionally inappropriate genes ensuring the proper acquisition of the identity of B lymphocytes. E2A, EBF1, PAX5, MEF2C are clear examples of this “double” function. These TFs are implicated in both the activation of B cell specific genes and in the repression of alternative-lineage genes (Ikawa et al., 2004; Nutt and Kee, 2007; Pongubala et al., 2008; Stehling-Sun et al., 2009; Boller and Grosschedl, 2014; Ramírez et al., 2010). In particular, E2A is involved in the repression of alternative hematopoietic genes such as *Tcf* and *Gata-1*. E2A-deficient pro-B cells maintain a pluripotency status and are unable to undergo further B cell differentiation (Ikawa et al., 2004). In addition, many non-B cell specific genes, including *Tcf7, Id2, Flt3* and *Gata-3*, are occupied and repressed by EBF1 at progenitor and precursor cell stages in order to preserve B cell fate and avoid promiscuous transcription (Pongubala et al., 2008; Treiber et al., 2010; Lukin et al., 2010; Nechanitzky et al., 2013; Banerjee et al., 2013). In fact, EBF1-mediated repressive function is associated to its transient occupancy at target chromatin regions, preceding transcriptional silencing. The collaboration of chromatin modifiers, epigenetic factors and other TFs after EBF1 occupancy, by either factor replacement or competitive displacement, is supported by the fact that some silenced genes are co-occupied by EBF1 and other TFs such as PAX5. However, detailed mechanisms of chromatin regulation are still under study (Revilla-i-Domingo et al., 2012; Boller et al., 2016; Li et al., 2018b).

On the other hand, PAX5 deficient pro-B cells undergo down-regulation of B cell genes and up-regulation of lineage inappropriate genes. In fact, these cells are able to transdifferentiate *in vitro* into cells from other lineages, such as macrophages, osteoclasts and granulocytes (Delogu et al., 2006; Medvedovic et al., 2011; Pridans et al., 2008; Schebesta et al., 2007; Nutt et al., 1999). Similarly, conditional deletion of PAX5 in mature and peripheral B cells results in the de-differentiation and conversion of B into functional T cells in spite of the advanced differentiation stage of these cells (Cobaleda et al., 2007a).

MEF2C plays a crucial role in earliest stages of B lymphopoiesis (LMPP) and, as the other mentioned TFs, it is also involved in both repressor and activator functions. In fact, its deficiency in multipotent stage leads to myeloid lineage “drifting”. Specifically, MEF2C deficiency produces C/EBPα up-regulation, a key regulator of myeloid genes (Stehling-Sun et al., 2009; Debnath et al., 2013).

All these findings support the fact that transcriptional silencing is as relevant as transcriptional activation for correct B lymphocyte generation. However, detailed mechanisms of gene repression and the identification of specific transcriptional repressors that take part in this process are still under research.

1.3 Transcriptional regulation in late B cell differentiation

Terminal B cell differentiation is a critical process for the humoral immune response in vertebrates, which is based on the production of high affinity antibodies that recognize limitless antigens. It is mainly achieved by coordinated action of several TFs in response to antigen recognition and extracellular signals produced by T CD4+ cells (Janeway, 2001). This process takes place in germinal centers (GCs), leading to the generation of higher affinity memory B cells and plasma cells. The GC reaction involves somatic hypermutation (SHM) and class switch recombination (CSR) processes, which consist on the clonal expansion of antigen-specific B lymphocytes and the generation of B-cell sub-clones with related antigen specificities. The cells expressing immunoglobulins with improved affinity for the antigen are then positively selected (Victora and Nussenzweig, 2012). BCL6 is the main regulator of the process and is also essential for T follicular helper (Tfh) cells function. After GC reaction, activated B cells differentiate into antibody-secreting plasmatic cells (PC) and memory cells. B mature cell TFs such as PAX5 that define plasma cell origins must be silenced to initiate its differentiation (Shaffer et al., 2002). BLIMP-1 is considered the master regulator of plasma cell differentiation (Figure 3) (Angelin-Duclos et al., 2000).

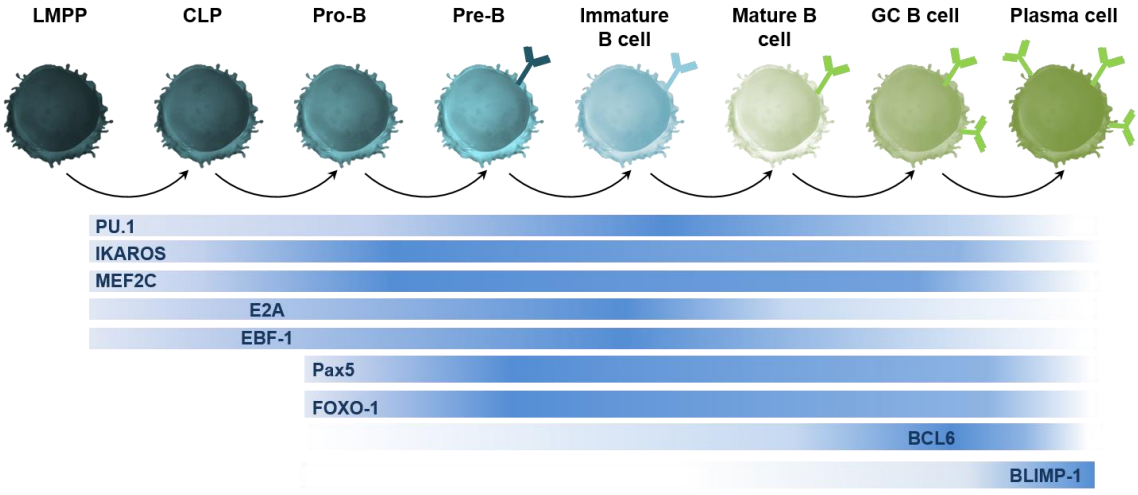

Figure3. **Transcription factors expression during B lymphopoiesis**. Representation of B cell early and terminal differentiation. Degraded bars represent the expression levels of transcription factors at each developmental stage. Darker color indicates higher level of expression. Initial step of stem cells is not represented. LMPPs (lymphoid-primed MPPs), CLPs (common lymphoid progenitors). Figure adapted from Ramirez J et al. 2010.

2. Epigenetic mechanisms

Epigenetic modifications comprise those inherited reversible changes in gene expression that do not depend on the DNA sequence, including DNA methylation, histone modifications, microRNA expression, nucleosome positioning and 3D chromatin architecture. The dsDNA is packaged by histone proteins into generally compact chromatin that prevents accessibility of DNA-binding proteins. The basic unit of chromatin is called nucleosome. This is basically composed of DNA and four core histones, which present specific characteristics depending on the context and cell type in higher eukaryotes and change its position and structure dynamically during regulation of genes expression. The regulation of genomic DNA depending on nucleosomes establish the base of epigenetic regulation (Koyama and Kurumizaka, 2018). In this section we describe the epigenetic mechanisms central to the studies performed during my doctoral thesis

2.1. DNA methylation

2.1.1. Overview

DNA methylation is one the best characterized epigenetic modification and, in the last years, it has been defined as essential for transcriptional regulation and critical for many developmental and pathological processes in mammals (Jin et al., 2011; Smith and Meissner, 2013). DNA methylation consists of the transfer of a methyl group by a DNA methyltransferase enzyme (DNMT) to the 5^{th} position of a cytosine that mainly belongs to a CG dinucleotide. There are three principal enzymes which catalyze these reactions (DNMT3A, DNMT3B and DNMT1). DNMT3A and DNMT3B are mainly involved in *de novo* methylation during embryonic development and in germ cells. In fact, their expression becomes down-regulated after differentiation in somatic cells. DNMT1 maintains the methylation patterns during DNA replication that takes place during cell division (Okano et al., 1999; Bestor, 2000; Edwards et al., 2017; Zeng and Chen, 2019).

CG dinucleotides are mainly located in regions called CpG islands (CGIs) (200bp genomic regions with higher GC percentage that are mainly located in DNA repetitive regions and GC-poor promoter regions of coding mammalian genes). Methylation of these dinucleotides has been associated to transcriptional repression (Weber et al., 2007; Deaton and Bird, 2011). The transcriptional silencing mediated by CGIs methylation can be explained by two molecular mechanisms. First, methylation of DNA can block the accessibility of chromatin and prevent the recruitment of TFs to their DNA-binding sites (DBS). Second, DNA methylation leads to the recruitment of 5-

methyl-CpG-binding proteins (MeCPs and MBDs) such as MeCP2, which associates with a co-repressor complex containing histone deacetylases (HDACs). Polycomb repressive complexes are also linked with DNA methylation by direct association with DNMTs (Nan et al., 1998; Viré et al., 2006; Jin et al., 2011).

2.1.2. CGIs and DNA methylation across the genome

In the mammalian genome, methylated regions are correlated to their higher CG dinucleotide frequency, since somatic cells possess methylation marks in approximately 80% of CpG sites. One relevant exception is CGIs that are mostly located in un-methylated regions. In fact, they are not methylated in germ cells, the early stages of embryo and in most somatic cell types. Most of them mark a gene promoter or its 5' region, as 70% of promoters contain CGIs. Their methylation leads to stable gene silencing in either physiological conditions such as X-chromosome inactivation or in pathological conditions such as cancer (Lokk et al., 2016; Deaton and Bird, 2011; Li and Zhang, 2014).

Histone modifications are closely related to DNA methylation patterns in the mammalian genome. Regarding promoter regions, acetylated histones have been linked to active transcription since first studies of chromatin. However, genome-wide analyses have also associated the histone mark H3K4me3 to active CGI promoters (Mikkelsen et al., 2007; Struhl K, 1998). CGIs from promoters are generally protected from methylation by different mechanisms. First, TFs prevent methylation of CGIs in a sequence depending manner. Second, demethylase enzymes (TET proteins) bind to CpG rich regions of DNA, preventing their methylation. Third, DNA binding proteins containing a CXXC domain, such as CFP1 and KDM2, recognize un-methylated CGIs, recruit additional proteins in order to keep cytosines in an unmodified status and prevent the binding of DNMT enzymes (Jeltsch et al., 2018; Blackledge et al., 2013).

Isolated CGIs that are not associated to TSS are called "orphans". It has been revealed that these CGIs regulate expression of far promoters and act under strict regulation (Sarda et al., 2017; Deaton and Bird, 2011).

Strikingly, methylation can also occur in non-CpG sites, such as CpA, CpT and CpC in a tissue specific manner. Given that this process is asymmetrical, *de novo* methylation is required after each cell replication. Further studies are needed in order to elucidate in detail the mechanisms involved in non-CpG methylation and its biological relevance in mammalian development. Experimental approaches based on disruption of DNMT activity cannot distinguish the biological consequences of one epigenetic modification,

since DNMT enzymes catalyze both CpG and non-CpG methylation (Patil V et al. 2014; Jang HS et al. 2017).

Despite there are still gaps of knowledge regarding the influence of DNA methylation in mammalian biology, recent state-of-the-art epigenetic techniques that analyze the detailed methylome of multiple cell types at the single cell level and at single base resolution allow researchers to investigate specific DNA methylation patterns in normal development and pathological situations (Li and Zhang, 2014).

2.1.3. DNA Methyltransferase enzymes

As mention above, there are three main DNA methytransferases (DNMTs) in mammals which are classified into two families; DNMT1 and DNMT3, which contains members DNMT3A and DNMT3B. DNMT3L will not be explained, as it has no catalytic function by itself. It acts as cofactor of DNMT3A in germ cells (Jurkowska et al., 2011; Edwards et al., 2017).

Methylation maintenance

DNMT1 was the first identified DNA methyltransferase in mammals. It is involved in maintaining DNA methylation, as this enzyme have preference for hemimethylated CGs containing regions that results from DNA replication rather than un-methylated regions (Figure 4). Thus, DNMT1 is critical for preserving DNA methylation patterns during cellular divisions (Bestor et al., 1988; Goyal et al., 2006). DNMT1 is ubiquitously expressed in cells under division, especially in somatic tissues, but its expression decreases in non-proliferating cells (Robertson et al., 1999). It is also essential in early development, as DNMT1 deficiency in mice leads to lethality at embryonic stages, specifically after gastrulation stage (Jurkowska et al., 2011).

De novo methylation

De novo methylation is critical in both early and late mammalian development. DNMT3 A and B have no-overlapping functions, as defect of one of the two enzymes produce lethal disorders during mouse embryonic development. DNMT3B appears to be more relevant in early embryonic development, while DNMT3A has target genes that are crucial for late development and after birth (Okano et al., 1999). DNMT3A and B are highly expressed in mammalian embryonic tissues and embryonic stem cells (ESCs), while they become down-regulated in differentiated somatic cells. Both enzymes have also substrate preference for CpGs (Figure 4), as these enzymes do not show enzymatic activity on DNA regions containing already methylated CpGs and other non-methylated cytosines (Okano et al., 1998; Chen et al., 2002; Baubec et al., 2015).

Structure

The structure of DNMTs basically consists of a large N-terminal domain with regulatory functions and a smaller C-terminal domain which possesses catalytic activity. The first domain regulates nuclear localization of the enzymes and their interaction with other proteins, chromatin and DNA, and the second domain contains the catalytic core (which is conserved among eukaryotes and prokaryotes) and a DNA specific recognition region (Cheng and Blumenthal, 2008).

Clinical relevance

Epigenetic dynamics in pathological contexts adds another layer of complexity and their understanding is essential to unveil affected mechanisms and design effective therapies. De-regulation of DNA methylation pattern in cells can be caused by several factors, including alterations in DNMTs expression or function, and this is strongly associated to several pathologies including cancer. Loss-of-function of DNMTs can lead to a DNA hypomethylation pattern and overexpression can produce hypermethylation. In particular, hypomethylation of DNA repetitive regions can lead to genome instability and increased transposon activity, while hypermethylation in CG sites from promoter of tumor suppressor genes give rise to tumorigenic development and worse prognosis in some types of cancer (Esteller, 2008; Feinberg & Vogelstein, 1983; Herman & Baylin, 2003; Zhang W and Xu J, 2017).

Loss of function

In particular, DNMT1 deficiency can produce alterations during murine mitotic division, e.g. *Dnmt1*-null mouse fibroblasts undergo p53-dependent apoptosis after several cell divisions (Jackson-Grusby et al., 2001), and is also associated to human neurological disorders (Baets et al., 2015). Additionally, DNMT1 deficiency is associated to chromosome instability and tumorigenic development such as T-cell lymphoma (Peters et al., 2013). Regarding DNMT3 enzymes, combined loss of A/B proteins increases invasive properties and aggressiveness of squamous carcinomas and mutations of DNMT3A is associated with worse prognosis in patients with acute myeloid leukemia (Rinaldi et al., 2017; Hou et al., 2012).

Overexpression

DNMT1 overexpression can also lead to aberrant DNA methylation patterns and contribute to worse prognostic in some malignancies (Saito et al., 2003). DNMT1 and DNMT3B have been found to be overexpressed in a MYC oncogene-depending manner in some T-cell leukemia and Burkitt's lymphoma, in order to maintain the tumorigenesis (Poole et al., 2017).

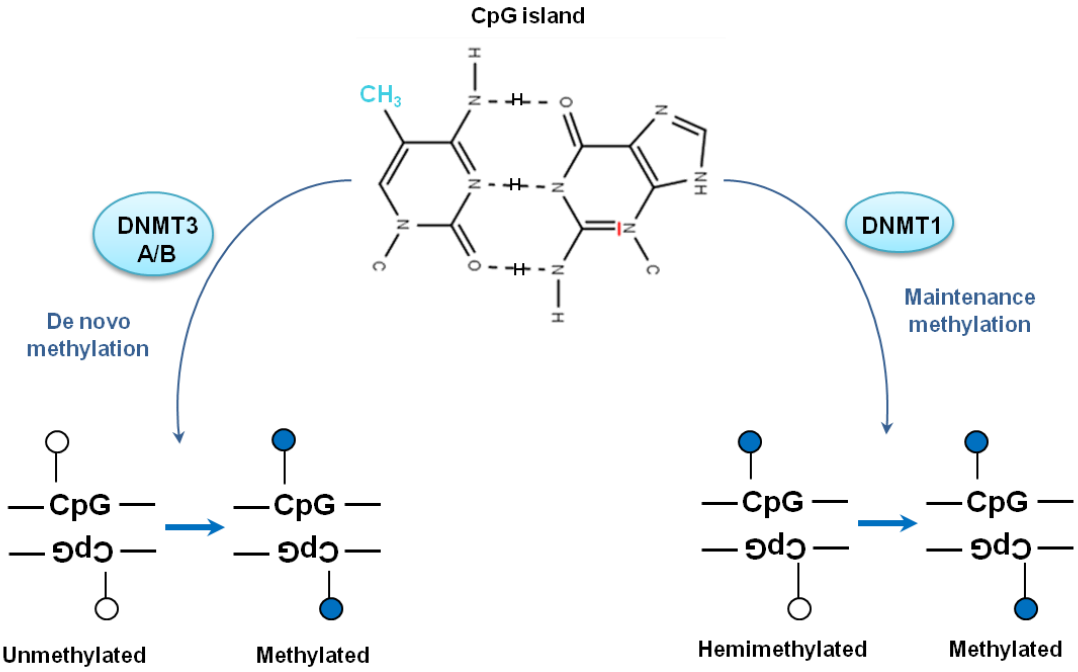

Figure 4 De novo and maintaining methylation of CpGs. Figure adapted from Laisné M et al. 2018. DNMT3A/B catalyzes de novo methylation in unmethylated DNA, whereas maintaining methylation of hemimethylated DNA resulted from DNA replication is catalyzed by DNMT1.

2.1.4. DNA methylation in the immune system

The differentiation of HSCs into cells of the different lineages implies multiple fate choices that are dictated by several coordinated regulatory events including DNA methylation, which plays a critical role in those decisions. Thus, the DNA methylome "tattle" the cell identity and its differentiation stage. Cellular commitment of multiple progenitors (MPPs) towards the myeloid or lymphoid lineage is influenced by the degree of methylation in DNA regulatory regions. In fact, GMPs possess lower levels of methylation on regulatory regions close to CpG islands than lymphoid progenitors in physiological and pathological conditions, as alteration of DNMT1 function results in disruption of lymphoid specification but myeloid lineage cells are not affected. DNA methylation differences, especially at gene promoters, are closely related to differential enrichment of histone modifications, lineage-specific TFs expression and chromatin architecture (Ji et al., 2010; Bock et al., 2012). For instance, Bock and colleagues observed higher levels of the enhancer associated histone mark H3K27ac in lymphoid cells compared to myeloid cells (Farlik et al., 2016). Additionally, Corces et al. reported that cells from each hematopoietic stage are characterized by a unique profile of chromatin accessibility regions and, therefore, a specific chromatin positioning in the nucleus (Corces et al., 2016b). Regarding B cell lineage, whole genome bisulfite sequencing experiments reported that approximately 30% of the DNA undergoes methylation changes during B cell differentiation, being the cellular lineage with the

most dynamic methylome of the organism (Kulis et al., 2015). Most of methylation changes during B cell differentiation are principally located in polycomb repressed regions, DNA repetitive regions and nuclear lamina associated domains (Martin-Subero and Oakes, 2018).

2.2. DNA demethylation

2.2.1. Passive and active DNA demethylation

DNA methylation is a reversible epigenetic mark, since it is established and maintained by DNMT enzymes but modified cytosines can be demethylated by either passive or active mechanisms. Passive demethylation is normally associated to a functional failure in DNMTs involved in methylation maintenance, either by inhibition or by loss-of-function mutations. Thus, 5'-methylcytosine (5-mC) can be progressively lost during cell replication. For example, pharmacological treatment with demethylating agents such as 5-Aza-2′-deoxycytidine (DAC) or 5-azacitidine (AZA) leads to DNMT1 inhibition, loss of methylation maintenance and consequent genetic instability and aneuploidy (Costa et al., 2016; Wu and Zhang, 2017). Additionally, in several tumorigenic processes in which DNMTs present mutations, DNA repetitive regions undergo hypomethylation that leads to sever chromosomal and genetic instability (Esteller, 2008; Sheaffer et al., 2016). Conversely, active demethylation is mediated by ten-eleven translocation (TET) enzymes. TETs proteins are dyoxigenase proteins that catalyze the oxidation of 5-mC into 5-hydroxymethylcytodine (5-hmC), 5-formylcytosine (5-fC) and 5-carboxylcytosine (5-caC). Then, thymine DNA glycosylase (TDG) mediates excision of 5-fC and 5-caC by base excision repair (BER), which will finally result in DNA demethylation and gene expression. Researchers have proposed other possible mechanisms of TET-mediated demethylation independent of TDG activity but the described model is the most accepted. Curiously, given that 5-hmC mark is less stable for DNMT1, methylation maintenance catalyzed by this enzyme is hindered and tend to be diluted during replication (Hashimoto et al., 2012; Weber et al., 2016; Wu and Zhang, 2017).

2.2.2. TET proteins

DNA demethylation is catalyzed by the TET protein family, which comprises TET1, TET2 and TET3 (Wu and Zhang, 2017).

Structure

TET proteins contain two major domains: the C-terminal domain possesses catalytic activity and the large N-terminal domain contains a CXXC DNA binding domain (Figure

5). TET2 suffered a chromosomal inversion during evolution and the CXXC domain became a new separated protein called IDAX. DNA binding through CXXC domain regulates TET enzymatic activity, so IDAX modulates *Tet2* expression and its catalytic function (Ko et al., 2013; Wu and Zhang, 2017). It is reported that CXXC DNA binding domain, including IDAX, is preferentially recruited at CpG rich regions, especially at gene promoters. Un-methylated cytosines are slighter preferred than methylated cytosines, preventing *de novo* methylation (Rasmussen and Helin, 2016; Wu et al., 2011b). Notably, TET enzymes have preference for methylated cytosines in a CpG context, as they present lower activity when methylated cytosines are not followed by a guanine base. In addition, it is reported that 5-mCs are preferable substrates than 5-hmC or 5-fC. Then, 5-hmC marks become more stable and less predisposed to suffer further oxidative reactions, suggesting that they may be relevant for regulatory functions (Hu et al., 2015; Wu and Zhang, 2017).

TET1 CXXC domain binds to CpG sequences independently of their epigenetic mark, maybe due to its lack of lysine-phenylalanine-glycine-glycine (KFGG) motif, which allow TET1 to bind easier to methylated cytosines that the other family members (Pastor et al., 2013). TET1 presents a full-length isoform in early ESCs and in primordial germ cells, whereas a shorter truncated form that does not possess the CXXC DNA binding domain is expressed in more differentiated cells. The truncated form has weaker demethylase activity but it still targets CGIs and is overexpressed in several types of cancer (Good et al., 2017; Zhang et al., 2016b). Regarding other relevant domain of TET proteins, the C-terminal domain contains a catalytic core that adopts a double stranded B helix (DSBH) fold and possesses a cysteine (cys)-rich region which helps to target recognition (Figure 5) (Pastor et al., 2013). In fact, there are two zinc fingers that put the Cys-rich region and DSBH fold together in order to form a stable catalytic domain (Hu et al., 2013).

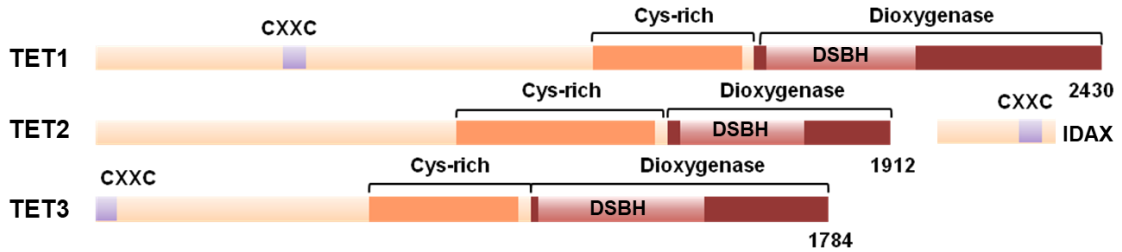

Figure 5. Representation of the structure of TET proteins. The N-terminal domain of TET1 and TET3 contains a CXXC DNA binding domain that recognizes un-methylated CpGs. In the case of TET2, the IDAX protein is required to bind DNA through its CXXC domain. The C-terminal domain contains the catalytic core of the protein that displays oxygenase activity and is positioned as double stranded beta helix (DSBH) and a Cys-rich region that is characteristic of this family of proteins. Figure adapted from Li & Zhang, 2014.

TET-TDG mediated demethylation

As mentioned above, several mechanisms have been proposed for TET-mediated DNA demethylation. However, the most accepted one consists of gradual TET-TDG-mediated demethylation in which 5-methylated cytosines are 5-hydroxymethylated (5-hmC), further oxidized (5-fC and 5-caC) and finally excised by base excision repair (BER) in order to preserve genome integrity (Figure 6) (Wu and Zhang, 2017; Jacobs and Schär, 2012). A recent study reported that this mechanism requires a direct physical interaction between TET proteins and TDG through both the N- and C-terminal domains of TET enzymes and the catalytic activities of both proteins are also equally needed during this process (Weber et al., 2016). Indeed, knockout mouse models for TDG results in epigenetic instability due to aberrancies in DNA methylation, compromising viability of mice at embryonic stages (Cortázar et al., 2011; Shen et al., 2013). Interestingly, despite TET proteins can target two symmetrically 5-mC in the dsDNA, the base excision process occurs in a sequential manner in order to avoid the generation of double strand breaks (DSB) (Weber et al., 2016).

Crystal structures of TDG interacting with artificial and stable substrates that mimic 5-fC and 5-caC with the enzyme revealed that Tyr-152 from TDG establishes tight polar bonds with oxygen from oxidized cytosines that do not take place with 5-mC or 5-hmC (Pidugu et al., 2016; Zhang et al., 2012).

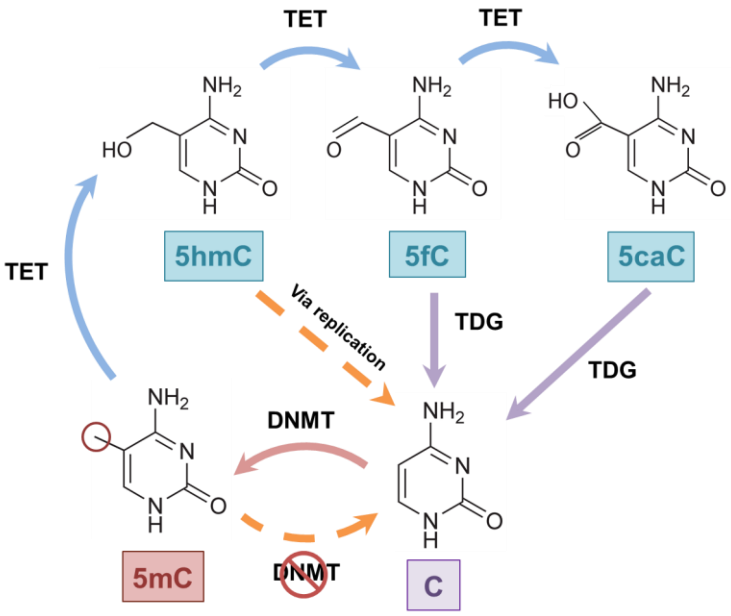

Figure 6. DNA demethylation mediated by TET proteins. Cytosine (C) methylation is calatyzed by DNMT proteins. 5-mC is then oxidized to 5-hmC, a stable modified state. Passive demethylation is represented in orange narrows, as 5-hmC is less accessible for DNMT1-mediated methylation, leading to progressive dilution of methylation mark during replication steps. DNMT block also leads to progressive demethylation. Alternatively, 5-hmC can be further oxidized to 5-formylcytosine (5-fC) and 5-carboxylcytosine (5-caC). These modified cytosines are eliminated by TDG by base excision repair (BER), resulting in un-methylated cytosine. Figure adapted from Tsagaratou et al. 2017.

Expression pattern

The three TET enzymes show specific expression patterns among different cell types and undergo dynamic changes during development under strict regulation. In particular, TET1 is mainly expressed in ESCs, either human or murine, and then becomes dow-nregulated in more differentiated cells. Conversely, despite TET2 is also expressed in murine stem cells, TET2 and TET3 are low expressed in human and mouse ESCs, respectively (Melamed, Yosefzon, David, Tsukerman, & Pnueli, 2018; Wu, Li, & Xie, 2018).

TET1 presents a full length isoform in early ESCs and in primordial germ cells, whereas a shorter truncated form is expressed in more differentiated cells and is overexpressed in several malignancies (Good et al., 2017; Zhang et al., 2016b). TET2 is highly expressed in specific somatic tissues, especially in the hematopoietic system and neuronal lineages. TET3 show expression oscillations, since it is highly expressed in zygotes and oocytes, fall sharply at two-cell embryonic stages and is up-regulated again in neuroectoderm-derived embryonic cells and primary neurons (Li et al., 2015; Shen et al., 2014; Li et al., 2011). Interestingly, Zhang and colleagues recently demonstrated that oxidation induced by TET3, in coordination with neuronal TFs, directs the conversion of mouse fibroblasts into functional neurons avoiding multipotent cell states (Zhang et al., 2016a).

TET's function can be regulated by several post-translational modifications (PTM), including ubiquitination and acetylation. In fact, the activity of TET2 becomes increased when acetylated by histone acetyl transferases (HATs) such as p300 in order to preserve protein stability in oxidative stress conditions. In addition of PTMs, TET enzymes are also regulated by microRNAs (miRNAs). For instance, miR-29 family members regulate TET1 in ESC and its repression increases progressively during differentiation of embryonic cells (Cui et al., 2016; Zhang et al., 2017). *TET2* is regulated by several of miRNAs, including miR-125b and miR-29b/c, and the de-regulation of these miRNAs leads to hematological malignancies (Cheng et al., 2013). Finally, *Tet3* is regulated in neural progenitor cells by miR-15b, promoting neuronal differentiation. Equilibrated balance between mir-15b and TET3 expression is essential for correct neurogenesis, maintaining the pool of neural progenitor cells (Lv et al., 2014).

TET-loss related disorders

TET proteins have an essential role in gene transcription during development and cellular lineage specification. Thus, their deficiency (either by silenced expression or

impaired activity) lead to a blockage in cell differentiation in several lineages, such as those included in brain and hematopoietic system (Lio and Rao, 2019; Rasmussen and Helin, 2016).

For instance, *TET2* is a common mutated gene in hematological malignancies in humans, including cases with lymphoid and myeloid origins such as diffuse large B cell lymphoma (DLBCL) and acute myeloid leukemia (AML). In adult mice, the deletion of both TET2 and TET3 in hematopoietic precursor cells results in aggressive AML with impairment to produce several hematopoietic lineages, failure in DNA damage repair and severe disruption of the spleen architecture and function (An et al., 2015). In fact, alterations in the 5-hmC profile at gene bodies correlate with multiple AML models (Han et al., 2016b). Regarding neural development, TET2 loss in neural progenitor cells leads to a sever impairment in cognitive function, affecting hippocampal-dependent learning and memory acquisition (Gontier et al., 2018).

2.2.3. 5-hmC distribution across the genome

The genomic pattern of 5-hmC, as well as TET proteins activity, changes dynamically during cell differentiation and has been correlated with active transcription. 5-hmC levels can oscillate from lower than 0.1% to approximately 0.7% in different cell types, reaching the highest levels in those from the central nervous system (CNS) and ESCs. 5-hmC is mainly found in gene bodies and enhancers of active genes, promoters and TF binding regions (Bachman et al., 2014; Kriaucionis and Heintz, 2009; Globisch et al., 2010; Wu et al., 2010; Khare et al., 2012; Iurlaro et al., 2013).

Genome-wide analysis of 5-hmC genome distribution in murine ESCs revealed that approximately 60% of the 5-hmC peaks were located in gene bodies, whose regions contained a CG medium density. As expected, 5-hmC enrichment correlated with TET1 bound genes. However, 5-hmC modification is found in both activated and repressed genes depending on the context. Wu et al. reported the presence of 5-hmC at promoters of TET1-silenced genes and in gene bodies of TET1-actively transcribed genes. Thus, DNA hydroxymethylation is a complex epigenetic mark that requires a tight regulation independent of 5-mC and its regulatory function depends on its location and the specific target gene (Neri et al., 2013; Wu et al., 2011a). However, later studies on human ESCs attributed dual functions of 5-hmC enrichment in gene promoters during neural differentiation, rather than exclusive repressive activity. Additionally, it has been reported recently 5-hmC enrichment is correlated with promoter activation, as it overlaps with H3K4me3 peaks during differentiation of human ESCs to pancreatic

cells. These results corroborate the complexity of epigenetic regulation underlying this cytosine modification (Kim et al., 2014; Li et al., 2018a).

Several studies are consistent with the correlation between 5-hmC enrichment in gene bodies and active transcription, independently of the cell type or developmental stage. For instance, 5-hmC enrichment in gene bodies correlates with progressive loss of the repressive histone mark H3K27me3 during neuronal differentiation. Thus, 5-hmC in gene bodies may be a relevant mark for active transcription by recruiting specific transcriptional regulators and cell-cycle mediators such as UHRF2 (Hahn et al., 2013; Wu et al., 2018, 2010; Spruijt et al., 2013; Kim et al., 2014). In fact, multiple TFs involved in differentiation processes are recruited by TET proteins, such as PU.1, E2A and EBF1 in hematopoietic cells (Han et al., 2016b). Additionally, 5-hmC is also enriched in other protein-DNA interaction sites, such as OCT4 and NANOG binding motifs (Stroud et al., 2011).

While TET1 principally facilitates promoter demethylation, TET2 and TET3 preferentially catalyze enhancer demethylation. In fact, TET2/3 proteins facilitate chromatin accessibility at enhancer regions by promoting TF binding and 5-hmC mark location is associated to specific enhancer-associated histone modifications, such as H3K27ac and H3K4me1 (C.-W. J. Lio & Rao, 2019; C.-W. Lio et al., 2016; Hong GC et al. 2014; Mostoslavsky et al., 2006; Stroud et al., 2011). This modification is principally enriched at tissue-specific enhancers in cells from the hematopoietic system and cardiac muscle. For instance, Tsagaratou et al. found that 5-hmC is enriched in enhancers of most active specific T cell genes that exert a key regulatory function, as 5-hmC location is strongly correlated with enhancer activity (Greco et al., 2016; Tsagaratou et al., 2014; Han et al., 2016a).

2.2.4. TETs role in the immune system

Considering that TET2 is the most relevant member of TET protein's family in the hematopoietic system, the following section will be principally focused on the role of this enzyme.

Lymphoid lineage

B cells

DNA demethylation, especially in enhancer regions, is an important event in the commitment of CLPs towards the B lymphoid lineage that continues progressively during the entire differentiation process. Indeed, almost half of the enhancers of genes involved in B cell differentiation undergo methylation changes. These regions also

contain B cell specific TF binding sites, such as PAX5, EBF1 or E2A. For instance, EBF1 contributes to chromatin accessibility and DNA demethylation through its C-terminal domain at the pre-pro-B cell stage (Kulis et al., 2015; Boller et al., 2016; Martin-Subero and Oakes, 2018). Interestingly, Beck and colleagues postulated that TET2 activity in such tissue-specific manner could be explained by interaction with tissue-specific TFs. In fact, their study obtained a potential interaction between EBF1 and TET2 in some cancer types such as AML (Guilhamon et al., 2013). Then, TET2 recruitment via EBF1 cannot be discarded in the context of physiological B lymphocytes development (Martin-Subero and Oakes, 2018).

PU.1, another pioneer factor involved in B cell differentiation, binds to Igk enhancers in pro-B cells and recruits TET2 and TET3 proteins in order to facilitate accessibility of the chromatin and the recruitment of additional TFs (Lio et al., 2016). TET2 has also been reported to be involved in CSR and plasma cell generation, as TET2 deficiency lead to decreased AID and BLIMP1 expression (Dominguez et al., 2018). However, depending on the cell stage in which TET2 is deleted, cells can differentiate normally. Thus, several studies support that TET proteins, especially TET2, are relevant in the regulation of early B cell development and late B cell differentiation. Their specific role depending on the cellular stage, the cellular environment and the physiological context add one additional step of complexity in regulation of DNA methylation in the immune system (Lio and Rao, 2019).

T cells

Given that TET2 and TET3 are expressed at higher levels than TET1 in somatic tissues, they are responsible for most of 5-hmC modifications in thymocytes and peripheral T cells. In fact, Tsagaratou and colleagues reported that double knockout of TET2 and TET3 in T cells produce an aberrant and excessive proliferaton that results in mice death at 8 weeks of age. During the pathological process, mice suffered a dramatic decrease in double positive T cells, failure in T regulatory cells (Treg) function and uncontrolled response to stress and inflammation (Tsagaratou et al., 2014, 2017). Thus, TET proteins are also essential for Treg cells function, as TET2 and TET3 are required for Foxp3 expression, a specific TF of this cell type (Yue et al., 2016). 5-hmC epigenetic mark in gene bodies correlates with active expression of specific T cell genes, supporting that TET proteins play an important role in regulating differentiation of naïve T cells into multiple types of peripheral T cells (Tsagaratou et al., 2014).

Myeloid lineage

The role of TET2 in the myeloid lineage has generated some controversy depending on the effect of TET deficiency towards different hematopoietic populations. On one hand,

it has been reported that disruption of TET2 at HSC or progenitor stages results in a dramatic fall of 5-hmC in HSC and an expansion of granulocyte and monocyte populations at the expense of erythrocytes, T and B lymphocytes generation. TET2 is also essential for the correct differentiation, proliferation and function of mast cells, and its deficiency leads to impaired differentiation and an up-regulation of C/EBP TFs family members. Given that C/EBPα is an essential TF for myeloid genes expression that represses other cell lineage genes, these results agree with myeloid expansion in TET2 deficiency conditions (Avellino and Delwel, 2017; Lio and Rao, 2019; Montagner et al., 2016). However, the Graf laboratory reported few years ago that TET2 contributes to the de-repression of myeloid genes in pre-B cells during a trans-differentiation system into macrophage-like functional cells depending on C/EBPα expression (Bussmann et al., 2009; Kallin et al., 2012). This process does not imply notable changes in DNA methylation, which correlates with the fact that myeloid differentiation requires less epigenetic reorganization than other lineages, but remarks the relevance of C/EBPα and TET2 in acquiring macrophage cell identity (Rodríguez-Ubreva et al., 2012; Lio and Rao, 2019). Additionally, PU.1, a TF that cooperates with C/EBPα in the activation of myeloid genes, recruits TET2 to the promoter of genes during the differentiation of monocytes to osteoclasts (de la Rica et al., 2013; Laiosa et al., 2006). In any case, myeloid abnormalities caused by TET2 deletion or loss-of-function mutations at hematopoietic progenitor stages confirm that this enzyme plays an important role in myeloid lineage specification. For instance, *in vivo* approaches showed that TET2 loss in progenitors give rise to progressive blockage of myeloid proliferation and acquisition of phenotypes similar to human chronic myelomonocytic leukemia (CMML) (Moran-Crusio et al., 2011; Rasmussen et al., 2015).

Erythroid lineage

The role of TET proteins in erythropoiesis has been practically unknown until very recently. A study based on zebrafish model reported that TET2 regulates the correct generation of erythroid progenitors by demethylating lineage-specific genes and that its deletion gives rise to erythrocyte dysplasia and anemia (Ge et al., 2014). Recent studies reported that TET2 and TET3, but not TET1, are expressed in human erythrocytes. Deletion of both enzymes results in an impaired differentiation of erythrocyte progenitors but they exert different functions. TET3 deletion increases cell death by apoptosis whereas TET2 deletion produces hyper-proliferation of progenitors. This second case with uncontrolled cell proliferation is closely related to hematological malignancies such as myelodysplastic syndromes (MDS) (Yan et al., 2017). These recent evidences establish another link between TET proteins and cancer and provide relevant information in order to study potential targets for tumor therapies.

2.2.5. Retrotransposons and DNA methylation

Transposable elements (TE) consist of DNA sequences that may change its location within a genome either by transposition or retrotransposition. They comprise a large fraction of eukaryotic genomes, reaching 50% of occupancy in human genomes, and contribute to the diversity in genome structure and function along evolution (Parisod et al., 2010; Sotero-Caio et al., 2017).

When discovered, these elements were thought to have no function and were defined as simple “parasites” able to jump within the genome. However, their prevalence in vertebrate genomes and their specific non-random conservation along evolution contributed to their study in the last years. Recent studies have elucidated some of their contributions to the host genome structure and function, such as their participation in the regulation of the transcriptional network involved in interferon (IFN)-response (Orgel and Crick, 1980; Chuong et al., 2017; Lynch et al., 2015).

TEs can be divided into two major classes based on their mechanism of transposition: retrotransposons and DNA transposons. Retrotransposons amplify themselves through a ‘copy-and-paste’ mechanism, in which they are transcribed to RNA, this intermediate is reverse-transcribed to cDNA as a copy and is inserted again in the host genome (Finnegan, 2012). On the other hand, the amplification of DNA transposons does not require an RNA intermediate. Instead of “copy”, their mechanism of action is mainly based on “cut-and-paste”, avoiding replication (Bourque et al., 2018). Retrotransposons are further subdivided into two groups depending on their mechanism of chromosome insertion. One group of retrotransposons is flanked by long terminal repeats (LTR) and its mechanism of activation is similar to retroviruses. The other group of TEs contains long and short interspersed elements (LINEs and SINEs) and SINE/VNTR/Alu (SVA) elements.

LINEs are the most active and abundant autonomous TEs in the mammalian genome and LINE-1 (L1) elements are the most abundant in humans, comprising approximately a 17% of the genome. They mobilize by a mechanism called target-primed reverse transcription (TPRT). Briefly, L1 endonuclease usually split host DNA sequence in an A-T rich specific region, setting a hydroxyl group free that is used as a primer for reverse transcription of L1 RNA (Luan et al., 1993; Luning Prak and Kazazian, 2000; Morrish et al., 2002; Han, 2010). Despite L1 elements preferentially target A-T rich non-coding DNA regions, they can also be inserted within genes, causing damaging insertions for the host organism. Most of L1-related diseases are caused by aberrant

splicing or mutagenesis insertion, in which gene function is completely nulled or altered (Hancks et al., 2016).

In order to preserve host genomic stability, multiple silencing mechanisms are known to enforce the transcriptional silencing of the huge amount of transposons and retroviruses that have accumulated in the mammalian genome, including genomic CpG methylation.

In the earliest stages of mammalian embryo, a global DNA demethylation is needed in order to activate the genetic program of embryonic development. This event could give transposons a break to amplify themselves and move across the genome, but additional mechanisms contribute to the control of retrotransposition. In fact, the enrichment of 5-hmC in L1 from mouse ESCs attribute to TET enzymes a role in regulating L1 activity in pluripotent cells, both the adapted (or exapted) and mutagenic elements (de la Rica et al., 2016; Gerdes et al., 2016).

Genome alterations caused by transposons are especially critical in germ cells, as these modifications will be inherited. DNA methylation, in combination with non-coding Piwi-interacting RNA (piRNA) system and repressive histone modifications like H3K9me3, mediates the retrotransposon silencing in male germ cell development, especially in the later stages. Importantly, DNA methylation only represses the expression of selected types of retrotransposon such as L1 elements. Hence, the coordination between transcriptional and post-transcriptional regulators during development of male germ cells reveal that retrotransposon silencing is essential for both genomic and transcriptomic integrity, enabling a correct meiotic recombination in these cells (Pezic et al., 2014; Zamudio et al., 2015; Yang and Wang, 2016; Inoue et al., 2017a)

Additionally, it has been recently reported that DNA methylation represses L1 insertions during human neuronal differentiation by differing methylation patterns dynamically in order to protect host genome from L1 accumulation (Rohrback et al., 2018; Salvador-Palomeque et al., 2019).

Thus, DNA methylation is crucial for TE regulation in pluripotent, germ and some somatic cells, but the detailed mechanisms in coordination with other epigenetic events are still unknown.

2.3. microRNAS

2.3.1. Brief introduction

microRNAs (miRNAs) integrate an extensive family of approximately 22nt RNAs that regulate gene expression post-transcriptionally. They were discovered 20 years ago and researchers observed that were highly conserved among vertebrates and invertebrates, suggesting that regulatory mechanisms through small RNAs were more general than expected (Lagos-Quintana et al., 2001; Lee and Ambros, 2001). The number of characterized functional miRNAs and their potential targets has dramatically increased in the last years due to up-to-date cloning and computational technologies.

2.3.2. Biogenesis and regulation

miRNA sequences can be located in different genomic contexts. In humans, most of miRNAs are encoded by introns of non-coding or coding genes, but some of them are encoded by exonic regions. In addition, some miRNA loci that are in close proximity to each other are transcribed as a polycistronic unit (Hutvagner et al., 2001; Lee et al., 2002). miRNA transcription is performed by RNA Polymerase II and is controlled by RNA Pol II-associated TFs and other regulatory elements (Lee et al., 2004).

In mammals, miRNAs are first transcribed as 1kb-long primary transcripts called pri-miRNAs. Depending on the proximal position of miRNA sequences in the genome, the primary transcript can contain multiple miRNA stem loops. The nuclear RNAse III Drosha, in association with its Microprocessor complex partner DGCR8, is recruited to split the pri-miRNA transcript into a 70nt hairpin-shaped RNA called precursor-miRNA (pre-miRNA) (Denli et al., 2004; Han et al., 2004; Gregory et al., 2004; Ha and Kim, 2014). This precursor is then exported out of the nucleus by Exportin-5 for further processing (Yi et al., 2003; Lund et al., 2004). Once in the cytoplasm, the terminal loop of pre-miRNA is cleaved by the RNAse III Dicer, leading to a mature miRNA duplex. The directionality of the miRNA strand determines the name of the mature miRNA form. Each mature miRNA strand is called depending on its original position in pre-miRNA, as 5p miRNAs belong to 5' premiRNA fragment and 3p miRNAs belong to 3' pre-miRNA fragment. In order to exert its repressive function, one selected strand of mature miRNAs (guide or active strand) need to be loaded into an Argonaute protein (AGO), generating this way the RNA-induced silencing complex (RISC). Notably, the strand selection and the assembly of this complex is not a simple binding. Several and tightly regulated steps are required for proper RISC function and strand selection depends on the cell type, the developmental stage and the pathological context. Briefly, miRNA guides AGO proteins to target sites in 3' or 5' regions from mRNAs. If the

complementarity of miRNA with its target is imperfect, translation of the target is inhibited, deadenylation machinery cleaves poly-A tail and, subsequently, the mRNA is degraded by exonucleases. Alternatively, if the complementarity is perfect, the mRNA target is destroyed by endonuclease AGO (Meijer et al., 2014; O'Brien et al., 2018; Nakanishi, 2016; Kobayashi and Tomari, 2016; Dueck et al., 2012). All the steps involved in the processing and the maturation of miRNAs are tightly regulated in order to prevent diseases such as cancer or developmental disorders associated to deregulation of miRNAs expression. Interestingly, some miRNAs regulate themselves indirectly by targeting TFs that regulate them in order to prevent their excessive accumulation (Inukai et al., 2018; Davis and Hata, 2009).

Once miRNAs are included in miRISC complex in association with Argonaute proteins, cellular mechanisms can dissolve the complex and expose miRNAs to exonuclease-mediated degradation (Wang et al., 2008b). This coordinated and balanced expression between targets and miRNAs might serve to prevent under-expression or overexpression of cell-type-specific miRNAs and to guarantee correct gene expression programs during development and to prevent disease (Gulyaeva and Kushlinskiy, 2016).

2.3.3. miRNAs and the immune system

miRNAs, in coordination with TFs and epigenetic modifications, play an important role in the regulation of the cellular differentiation of HSCs into all hematopoietic lineages, preserving HSC pool maintenance, directing cell commitment and preventing hematological disorders. It is well established that miRNAs can act as downstream and upstream effectors of TFs functions. Several studies have determined their expression profiles in specific immune cell types in order to elucidate different miRNA roles during hematopoiesis (Kim et al., 2019).

At the top of the hematopoietic hierarchy, miR-146 regulates HSC maintenance. Its deficiency in mouse models lead to cellular stress in HSCs and loss of HSC population (Zhao et al., 2013).

In B lymphopoiesis, the miRNAs repertoire is lineage-specific and changes dynamically at each cellular stage of differentiation. In fact, the aberrant expression of miRNAs involved in the differentiation of other cell lineages or the premature expression of some miRNAs can block B cell development, e.g. miR-34a and miR-150 respectively (Rao et al., 2010; Zhou et al., 2007). The de-regulation of miRNA expression either by negative feedback loops or by TFs and other regulators can lead to the development of

hematological malignancies, such as leukemia (Chaudhuri et al., 2012). The relevance of miRNAs function in the proper development and differentiation of hematopoietic cells from all lineages and the aberrant phenotypes upon their de-regulation point them as critical therapeutic targets in blood cancers (Stavast et al., 2018; Weiss and Ito, 2017). Finally, during murine T cell differentiation and maturation, there is an increased expression of miR-150 and miR-146, especially in Th1cells. In fact, miR146 target genes involved in activation of naïve T cell, such as CD40L and STAT1 (Torri et al., 2017).

2.4. Histone/ Protein Deacetylases

2.4.1. Overview

Histone post-translational modifications (PTMs) are one of the main types of epigenetic events and determine the structural conformation of chromatin. They include acetylation, methylation, phosphorylation, ubiquination and rybosilation, among others. More recently described modifications are crotonylation, propyonilation and butyrylation (Chen et al., 2017; Tan et al., 2011; Chen et al., 2007; Portela and Esteller, 2010). Histone acetylation, which is carried out by histone acetyltransferases (HATs), is generally associated to gene activation. HATs add an acetyl group to the ε-amino of lysines on the histone tails leading to the relaxation of chromatin and increasing the number of reachable binding sites for transcriptional activators (Marmorstein and Zhou, 2014). On the other hand, histone deacetylation catalyzed by histone deacetylases (HDACs) promotes chromatin compaction and is related to transcriptional repression (Gregoretti et al., 2004; Li and Seto, 2016a) (Figure 7).

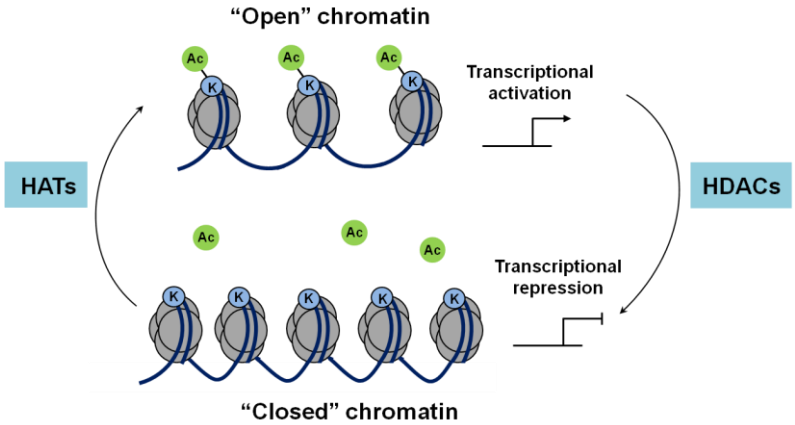

Figure 7. Representation of chromatin status depending HAT and HDAC enzymes mediation. In most cases, acetylation of lysine residues leads to open chromatin, TF recruitment and consequent gene activation, while deaceylation of lysine produce gene repression.

HDACs have emerged as critical transcriptional repressors in several physiological and pathological systems, such as oligodendrocyte differentiation, cardiac growth and morphogenesis, cancer, interstitial fibrosis, autoimmunity, aging, and metabolism disorders (Heideman et al., 2014). The first HDAC was discovered approximately 50 years ago (Inoue and Fujimoto, 1969) and, since then, a huge number of studies have been focused on the elucidation of the role of HDACs in gene regulation.

2.4.2. Family's classification

To date, 18 human HDACs have been identified and classified into four different classes according to their sequence homology to yeast proteins. There are numbered according to their date of discovery (Figure 8).

Class I HDACs

Class I HDACs (HDAC1, 2, 3 and 8) are enzymes ubiquitously expressed that play critical roles in differentiation, proliferation, and cancer. They have sequence similarity to the yeast transcriptional regulator Rpd3 (Taunton et al., 1996). They are predominantly localized in the nuclear compartment of the cell as components of multiple chromatin remodeling complexes, which are crucial for the regulation of gene expression (Hayakawa and Nakayama, 2011). Class I HDACs are basically composed by a conserved deacetylase domain flanked by short N- and C-terminal domains (Haberland et al., 2009).

HDAC1 and 2 highly are similar proteins that present overlapping functions in different cell types, except for few cases such as early embryogenesis, brain development and erythroblast differentiation. In fact, the deletion of both proteins *in vivo* is required to observe a severe biological effect, as in most cases, the specific deletion of one of the proteins is compensated by the up-regulation of the other. In mice, the double conditional knockout of HDAC1/2 in the hematopoietic system leads to a failure in megakaryocytes and erythrocytes development, generating a severe thrombocytopenia (Wilting et al., 2010; Heideman et al., 2014). Both HDACs act often together within three multiprotein repressor complexes: Sin3 complex, NuRD (nucleosome remodeling and deacetylation) and CoREST (co-repressor for element-1-silencing transcription factor) (Hassig et al., 1997; Feng and Zhang, 2003; Kelly and Cowley, 2013). HDAC1/2 activity and complex formation is regulated by several PTMs, which have a critical role in coordinating redundant and specific functions of HDACs depending on the context and the cell type (David et al., 2002; Segré and Chiocca, 2011).

HDAC3 is also expressed in several cell types and, despite possessing both nuclear and export signals, it is mainly located in the nucleus (Ruijter et al., 2003). HDAC3 requires the interaction with SMRT or N-CoR co-repressors and phosphorylation by protein kinase CK2 in order to form an active repressor-complex with deacetylase activity that is directed to promoters of their target genes *in vitro*. *In vivo,* the knockdown of catalytic subunits of CK2 results in a reduction of HDAC3 activity during mitosis. In fact, the role of HDAC3 over stability of G2-M phase-related protein cyclin dependent kinase 1(CDK1) propose a feedback loop in which HDAC3 activates CDK1, which in turn activates CK2 and it phosphorylates HDAC3 (Guenther et al., 2001; Zhang, 2005; Patil et al., 2016). HDAC3 knockout mice are embryonic lethal (Bhaskara et al., 2008). Finally, HDAC8 has not been linked to a repressor complex yet, but it is involved in the recycling of SMC3, a subunit of cohesin, and required for optimal murine fertility (Deardorff et al., 2012; Singh et al., 2019).

Class II HDACs

Class II HDACs are divided into two subclasses, class IIa (HDAC4, 5, 7, 9) and class IIb (HDAC6 and 10), according to sequence homology and domain organization.

Class IIa HDACs

Class IIa HDACs have unique features that do not share with other HDACs, which will be explained in more detail in the net section. Briefly, they are expressed in a tissue-specific manner and play important roles in differentiation and developmental processes. HDAC4, 5 and 9 are abundantly expressed in heart, skeletal muscle and brain. HDAC5 is also highly expressed in vascular endothelium acting as a specific negative regulator of angiogenesis (Urbich et al., 2009; Verdin et al., 2003; Parra, 2015). In the case of HDAC9, its expression in brain has generated some controversy. To date, studies support the fact that this protein is not expressed in neuronal stem cells and other brain cell types but it is required in mature neurons to exert its function properly (Lang et al., 2012). Furthermore, HDAC9 is also expressed in Treg cells and control their suppressive function (de Zoeten et al., 2010).

HDAC7 is mainly expressed in B lymphocytes, T lymphocytes, NK cells, heart, lung and vascular endothelium. HDAC7 deficiency in mice results in embryonic lethality due to a rupture of blood vessels (Kasler et al., 2011; Parra, 2015; Chang et al., 2006b; Lei et al., 2017). Additionally, class IIa HDACs possesses a long N-terminal that contains binding sites for tissue-restricted TFs such as MEF2 family members (Fig.8). This N-terminal region also determines their subcellular localization (nucleus or cytoplasm), which is crucial for their function (Parra and Verdin, 2010; Yang and Seto, 2008).

Class IIb HDACs

Class IIb HDACs have two catalytic domains, in N- and C-terminal regions, a unique feature that does not possess other HDACs. However, the leucine-rich catalytic domain of HDAC10 is not functional and HDAC6 harbors a C-terminal zinc finger domain. HDAC6 and HDAC10 can be localized in both cytoplasm and nucleus but mainly found in the cytoplasm (Kao et al., 2002; Zhang et al., 2008; Delcuve et al., 2012; Seto and Yoshida, 2014). Due to its cytoplasmic localization, HDAC6 known targets comprise cytoskeletal proteins such as α-tubulin and cortactin, transmembrane proteins such as the interferon receptor IFNαR, and chaperones (Hubbert et al., 2002; Haberland et al., 2009; Kaluza et al., 2011) . HDAC10 is known to interact with other HDACs from classes I and II such as HDAC3 and SMRT co-repressor, but its main function had been classified as a simple recruiter rather than a deacetylase enzyme (Tong et al., 2002). Interestingly, recent studies defined HDAC10 as a polyamine deacetylase enzyme (Hai et al., 2017). Finally, HDAC10 (together with HDAC9) has been implicated in DNA repair by homologues combination (Kotian et al., 2011).

Class III HDACs or Sirtuins

Class III HDACs or Sirtuins comprise seven proteins (SIRT1, 2, 3, 4, 5, 6 and 7) and show no homology to class I and II proteins. Sirtuins are classified as Nicotinamide-Adenine Dinucleotide (NAD^+) dependent protein decatylases. They possess three domains: a large domain that contains a Rossman-fold and a small zinc-binding domain are flanking a “gap” containing the deacetylase catalytic domain in which the substrate interacts with NAD. Unlike other HDACs, the catalytic domain of sirtuins has, in addition, mono-ADP-ribosyltransferase activity (Min et al., 2001; Avalos et al., 2004; Yoshida et al., 2017). Remarkably, the larger space in SIRT5 substrate binding region confers to this protein additional functions such as desuccinylase and demalonylase activities (Du et al., 2011).

Sirtuins have an especial pattern of cellular sub-localization. SIRT1 and 2 can be in both nucleus and cytoplasm; SIRT6 and SIRT7 are only localized in the nucleus. Finally, SIRT3, SIRT4, and SIRT5 are located in the mitochondria (Huang JY et al., 2010; Kiran et al., 2013; Liszt, Ford, Kurtev, & Guarente, 2005).

These enzymes are important for cell homeostasis through mediation between cell and its environment, as well as they play a relevant role in DNA damage repair and in preserving genomic stability under stressing conditions. Indeed, sirtuins deficiency in different cell types and in the whole organism leads to an increased stress during

replication, accumulation of DNA damage (e.g. R-loops), an accelerated organism aging and, consequently, its compromised viability (Kobayashi et al., 2005; Mostoslavsky et al., 2006; Serrano et al., 2013; Song, Hotz-Wagenblatt, Voit, & Grummt, 2017; Vazquez et al., 2016; Wang RH et al., 2008). Sirtuins reduce cellular stress and avoid cellular death by interacting with proteins involved in stress resistance such as FOXO family members (Kobayashi et al., 2005), helicase DDX21 (Song et al., 2017), helicase ku70 (Zhang L, Bai, Ren, Sun, & Si, 2018), among others, and by repressing proteins involved in cellular apoptosis, such as p53-related protein p73 (Dai et al., 2007; Kiran et al., 2015).

Class IV HDACs

Class IV HDACs only includes HDAC11, which is homologous to class I and II enzymes, and is involved in the regulation of development of different immune cells including neutrophils, myeloid derived suppressor cells and T-cells. T-cells from HDAC11 knockout mice undergo increased proliferation, production of pro-inflamatory cytokines and expressions of molecules involved in effector function. Considering its role on anti-inflammatory cytokine IL-10 suppression, HDAC11 activity is important for the balance between immune activation and immune tolerance (Seto and Yoshida, 2014; Sahakian et al., 2017; Yanginlar and Logie, 2018; Woods et al., 2017).

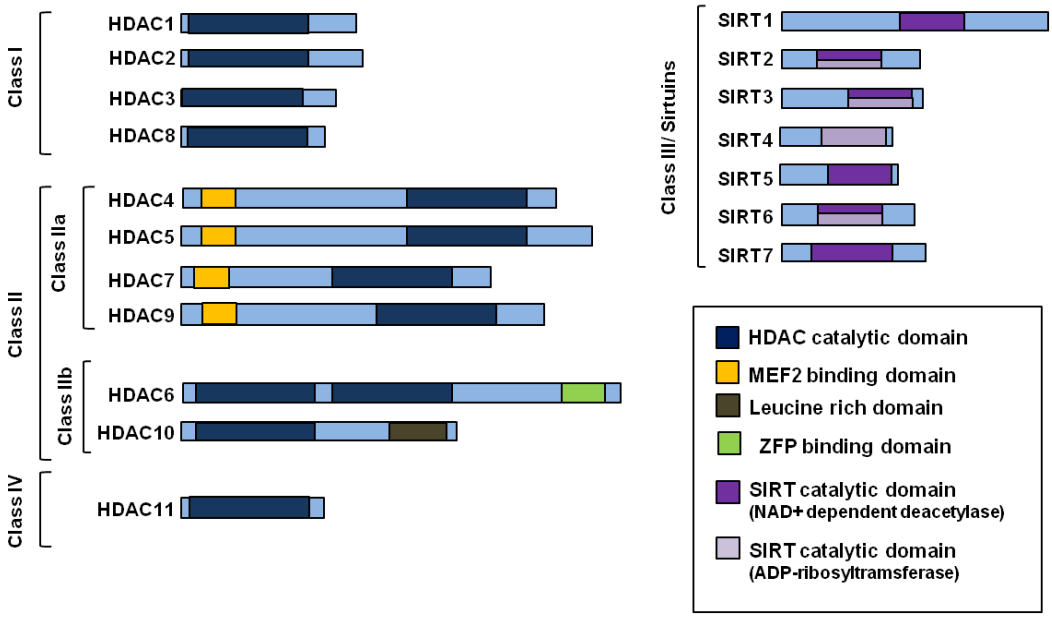

Figure 8. Classification of HDAC's family in four different classes. Most relevant domains are colored and defined. Figure modified from Barneda-Zahonero et al. 2012 and Heideman MR et al. 2014.

2.4.3. Mechanism of action

As mentioned above, HDACs are often located in multiprotein complexes, either with other HDACs (e.g. HDAC1 and HDAC2) or with other types of proteins. While some HDACs such as HDAC6 exert its function in a TF-independent manner, most of HDACs are recruited to their target genes by interaction with specific TFs. The best characterized TFs partners of class IIa HDACs are MEF2 proteins, which are involved in cell differentiation, cell growth, morphogenesis, tissue maintenance and survival (McKinsey et al., 2000; Grégoire et al., 2007; Pon and Marra, 2016; Seigneurin-Berny et al., 2001).

The ability of HDACs to interact with different binding partners forming macromolecular structures has an important role in the correct chromatin positioning and modification. In this regard, class I HDACs can form different multiprotein repressor complexes depending on their associated partners and class IIa HDACs associate with HDAC3 in order to exert their repressive activity (Fischle et al., 2002; Ruijter, 2003; Kelly and Cowley, 2013). Protein complexes formed by HDAC1/2 have been recently connected to Repo-man phosphatase in order to maintain gene repression (de Castro et al., 2017). HDAC4 and 5 are also associated to heterochromatin protein 1 (HP1), which produces the recruitment of histone methyltransferases such as SUV39h1 and mediates the transcriptional repression (Zhang et al., 2002b).

Importantly, the identification of substrate specificity in HDACs is a relevant factor in the elucidation of their mechanism of action. In contrast to HATs, the study of HDACs is much more difficult due to three main obstacles. First, some HDACs possess almost undetectable enzymatic activity when purified. Second, functional redundancy of some HDACs does not allow to unravel their specific targets, as the lack of one HDAC is compensated by the action of another. Finally, many HDACs are included in more than one complex, which have different substrate preferences (Marmorstein and Zhou, 2014; Seto and Yoshida, 2014).

Histone substrates

Histones are characterized by a basic charged that is caused by the high proportion of positively charged lysine and arginine residues. DNA, which is negatively charged, interacts with histone regions that contain those residues. Thus, histone modifications at multiple lysine residues regulate DNA-protein interactions by changing aminoacids charges and acts as indicators of chromatin states and gene expression (Kouzarides, 2007; Bannister and Kouzarides, 2011).

In neurons, HDAC1 deacetylates H3K9, leading to an increase in memory formation, and HDAC2 is involved in the deacetylation of H3K56, protecting mouse brain from DNA damage (Bahari-Javan et al., 2012; Miller et al., 2010).

However, the search for specific histone substrates for Class I, II and IV HDACs has been challenging for the researchers. The best example is the case of HDAC3. On one hand, HDAC3 shows specificity for H4K5 and H4K12, since *Hdac3*-null hepatocytes show high levels of acetylation marks on these lysines and, consequently, there is a loss of heterochromatin, an increase in DNA double strand breaks and less proliferation (Bhaskara et al., 2010). On the other hand, HDAC3 just partially deacetylates H3, H2B, H4K8, and H4K16. In addition, HDAC3 is included in multiprotein co-repressor complexes that contain other HDACs (e.g. class IIa HDACs), causing difficulties to distinguish between HDAC3-specific substrate and other complex-member HDAC target (Johnson et al., 2002).

Class IV HDAC11 specifically deacetylases H3K9/K14, which is related to oligodendrocyte development and specific gene expression (Liu et al., 2009).

HDAC6, SIRT4 and SIRT5 do not deacetylase histones because of their exclusive cytoplasmic and mitochondrial localizations, respectively. Sirtuins have easier detectable substrates than classical HDACs. SIRT1, 2 and 3 have preference for H4K16 (Vaquero et al., 2006). SIRT6 was initially restricted to exclusive mono-ADP-ribosyltransferase activity, but later studied revealed that it also deacetylates H3K9 and H3K56, modulating correct telomere metabolism and function (Liszt et al., 2005; Michishita et al., 2008). SIRT7 is a nuclear highly selective H3K18 deacetylase that plays a crucial role in oncogenic transformation (Barber et al., 2012).

Non-histone substrates

Interestingly, HDACs deacetylation is not only restricted to lysine residues from histones, as several articles in the literature have established that there are also non-histone substrates, such as TFs, structural proteins or viral proteins. Upon deacetylation, these proteins undergo alteration in activation, subcellular localization, complex formation, among others effects (Glozak et al., 2005; Singh et al., 2010). One of the first discovered non-histone substrates was p53, a DNA binding transcription factor that have tumor suppressor activity. Its acetylation by p300 facilitates its stabilization, but its deacetylation by HDAC1 or SIRT1 produces a decrease in its activity (followed by protein degradation) and subsequent reestablishment of the cell cycle and DNA repair (Gu and Roeder, 1997; Luo et al., 2001; Vaziri et al., 2001). SIRT1-p53 has been extensively studied for its close relation with tumorigenesis

processes. Deacetylation of a tumor repressor defines SIRT1 as a potential oncogene, but other studies support its function as a tumor suppressor in specific malignancies. Thus, this protein can exert both roles depending on the specific cell type and pathology (Deng, 2009; Lee and Gu, 2013).

Other TFs targeted by HDACs are GATA proteins by HDAC3 (Ozawa et al., 2001), STAT3 by HDAC3 and SIRT1 (Yuan et al., 2005; Gupta et al., 2012a), members of FOXO family by SIRT1-3 (Wang et al., 2007; Motta et al., 2004; Jacobs et al., 2008), and YY1 by HDAC2 (Glenn et al., 2009). STAT3 dimerization, nuclear translocation and consequent activity depend on the acetylation of Ly685. HDAC3 and SIRT1 deacetylation blocks STAT3 activity and represses activation of its targets genes. The relation between SIRT1/HDAC3, STAT3 and NFKB signaling has been studied in different types of cancer such as lymphoma, leukemia and gastric cancer (Bernier et al., 2011; Gupta et al., 2012b; Hu et al., 2014).

HDAC6 is considered the major cytoplasmic deacetylase with non-histones substrates. It is involved in the regulation of microtubule-dependent cell motility, vessel formation, cellular migration and ciliary disassembly by deacetylating α-tubulin and cortactin (Hubbert et al., 2002; Kaluza et al., 2011; Mihaylova and Shaw, 2013; Ran et al., 2015).

Finally, as mentioned above, some viral proteins are also regulated by acetylation and deacetylation reactions. For instance, acetylation of lysine 239 from adenoviral E1A protein, on one hand, blocks its interaction with C-terminal binding protein (CtBP) and produces the disruption of repressor complexes leading to gene activation, and, on the other hand, arrests E1A in the cytoplasm due to a blockage of nuclear import (Zhang et al., 2000; Madison et al., 2002).

Novel PTMs

Recent studies have revealed novel PTMs on lysine residues in addition to acetylation, such as propionylation, butyrylation, succinylation, manolynation, crotonylation and glutarylation. (Chen et al., 2007; Tan et al., 2011; Xu et al., 2016; Yoshida et al., 2017). Most of studies have focused on histone crotonylation due to its relation with gene activation and its shared effector with acetylation, p300. This highly evolutionary conserved mark is specifically enriched in active promoters and predicted enhancers regions, and is proposed to function as a specific mark of male specific gene expression after meiosis. HDAC1/2 complexes are proposed as main responsible of de-crotonylation reactions (Kelly RWD et al., 2018; Tan et al., 2011). Manolytaion in H2A has been recently related to chromosome segregation during mitosis and meiosis,

but the roles of histone propionylation, butyrylation, succynilation, malonylation in mammalian epigenetic gene regulation are still poorly understood (Ishiguro et al., 2018; Yoshida M et al., 2017).

2.4.4. Clinical Relevance

Abnormal expression of several HDACs has been observed in human disease, in particular several types of cancer. Despite Sirtuins activity has been associated to stress and aging “fighting” and HDACs loss in some cell types can lead to severe disorders such as bone marrow failure (Heideman et al., 2014; Grabowska et al., 2017), there is a well-established correlation between HDACs and acquisition of pathological and malignant phenotypes (Barneda-Zahonero and Parra, 2012; Li and Seto, 2016b; Yoon and Eom, 2016).

Regarding class I HDACs, the recruitment of the Sin3 repressor complex in order to repress the expression of adhesion proteins such as E-cadherin and the relation between HDAC1 and TGF-β during Epithelial-to-Mesenchymal-Transition (EMT) connect HDAC1 and HDAC2 to tumor invasiveness (Peinado et al., 2004; Lei et al., 2010; Serrano-Gomez et al., 2016). In addition, HDAC3 inhibition leads to PD-L1 up-regulation and, consequently, increases immunotherapy success in several cancers (Deng et al., 2019; Hu et al., 2019). HDAC8 has also been overexpressed in a variety of human cancers. Interestingly, HDAC8 inhibitors offer beneficial effects in the treatment of both solid and hematological tumors (Adhikari et al., 2018) .

Recent studies demonstrated that class IIa HDACs inhibition results in breast tumor reduction and metastasis prevention by modulating the innate response (Guerriero et al., 2017). HDAC6 has been principally linked to autophagy processes due to its cytoplasmic localization, this protein has also been related to expansion of malignant cells in pancreatic and breast cancers (Lee JY et al., 2010; Seo et al., 2014).

Sirtuins, especially SIRT1, are also related to metastasis, but their role in suppressing or promoting invasiveness and cancer cells motility depends on the cancer type (Li and Seto, 2016b).

The pathologies mentioned above are few examples of malignant situations related to HDACs overexpression. Thus, HDAC’s specific inhibitors (HDI) have arised as crucial therapeutic agents and allow the study of biological relevance of these enzymes. In basic research, the first used inhibitor was discovered 40 years ago: n-butyrate (Rubenstein et al., 1979). TSA emerged few years later and became a key chemical agent for studying the role of histone acetylation in several biological contexts

(Yoshida, Kijima, Akita, & Beppu, 1990). Despite the list of used HDI in basic research have been increasing during the last years, only four inhibitors have been approved by FDA for tumoral therapies: vorinostat (SAHA, Zolinza) (Richon et al. 1998), romidepsin (FK228, Istodax) (Nakajima et al., 1998), belinostat (PXD101) and panobinostat (LBH589) (Chan et al., 2013). However, in basic research and clinical trials, treatments with HDAC inhibitors are still not specific enough and may produce biological side effects because off unrestrained action on other enzymes (Barneda-Zahonero & Parra, 2012; Yoshida et al., 2017).

2.5. Class IIa HDACs: a peculiar family

2.5.1. Unique features

As previously stated, class IIa HDACs present unique features. First, they are expressed in a tissue-specific manner and exert their transcriptional repressive functions in skeletal and cardiac muscle, the bone, the immune system and the vascular system (Parra, 2015). Second**,** in contrast to other HDACs, class IIa enzymes possess an N-terminal domain that mediates their interactions with specific TFs, such as MEF2 family members. Class IIa HDACs are recruited to their target genes through interaction with MEF2 proteins and other TFs on specific genomic regions to perform transcriptional repression (McKinsey et al., 2000; Di Giorgio and Brancolini, 2016). Third**,** the signaling-dependent phosphorylation of conserved serine residues in their N-terminal regulatory domain (Figure 9) mediates their shuttle between nucleus and cytoplasm and, therefore, their transcriptional repressive activity (Grozinger and Schreiber, 2000; Parra, 2015; Parra et al., 2007; Parra and Verdin, 2010). Last, although class IIa HDACs have a highly conserved catalytic domain, they have minimal deacetylase activity on acetylated histones. Strikingly, class I HDACs possess a tyrosine (Y) residue present in the catalytic domain crucial for the deacetylation of histones that is substituted by a histidine (H) residue in class IIa HDACs. Mutation of the H residue into Y confers class IIa HDACs with a high deacetylase activity on histones (Clocchiatti, Florean, & Brancolini, 2011; Lahm et al., 2007). These findings give rise to speculate that class IIa HDACs substrates may are no histones, but, to date, no histone or other protein substrates for class IIa HDACs have been identified. It was established that their enzymatic activity depends on their recruitment into a multiprotein repressor complex including HDAC3 and SMRT/N-CoR, categorizing them as simple co-factors but not real enzymes (Fischle et al., 2002). However, recent studies have focused on unveiling catalytic potential of these proteins and their implication in other novel lysine post-translational modifications such as crotonylation,

explained above (Lahm et al., 2007; Schuetz et al., 2008; Bottomley et al., 2008). Thus, further studies are needed to explore whether class IIa HDACs are real enzymes in their physiological context.

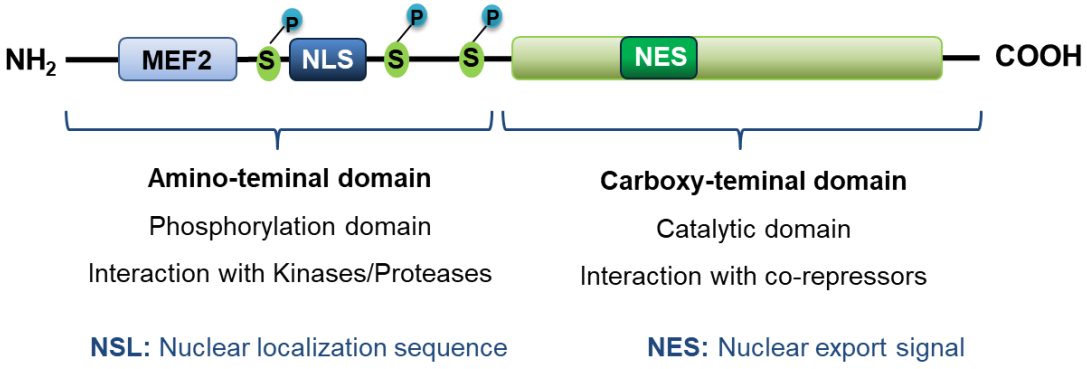

Figure 9. Schematic representation of Class IIa HDACs domains. From left to right, N-terminal domain containing the MEF2 binding region is represented in red, NSL in black and the serine residues subject to phosphorylation reaction in orange and C terminal catalytic domain containing binding regions to co-repressors. Figure extracted from (Parra, 2015) .

2.5.2 Role of class IIa HDACs in physiology and pathology

Null mice of each class IIa HDAC help to decipher their role in both physiology and pathology. All these mouse models present important defects in differentiation and developmental processes, since some of them die at embryonic stages.

HDAC4-deficient mice display a failure in osteogenesis and control of bone hypertrophy, resulting in premature calcification (Bradley et al., 2015; Vega et al., 2004). This phenotype was initially attributed to altered regulation of RUNX2 expression, but later studies demonstrated that a proper balance of expression between HDAC4 and MEF2C is essential for correct bone formation and development (Arnold et al., 2007). HDAC4, alternatively, is also implicated in regulating neuronal homeostasis, as it is involved in genetic response to denervation, synaptic plasticity and memory impairment (Fitzsimons, 2015). Abnormal nuclear accumulation of HDAC4 can trigger neurotoxicity, damage in dendritic architecture an affectation maturation of neural precursor cells (Litke et al., 2018; Trazzi et al., 2016; Q. Wu Q et al., 2017).

HDAC5 and/or HDAC9 null mice exhibit cardiomegaly in response to stress produced by artificially induced aortic stenosis, suggesting that HDAC5 and HDAC9 are modulators of cardiac response in stress conditions via MEF2 repression (Zhang et al., 2002a; Haberland et al., 2009; Chang et al., 2004). Notably, recent studies reported that HDAC5 is essential for HDAC1/Sin3a co-repressor complex recruitment, since

cardiac cells from HDAC5 knockout mice results present impaired interaction and subsequent de-regulation of cardiac genes such as *Ncx1* and *Bnp* (Harris et al., 2016). In addition, HDAC5 is also involved in the regulation of myogenesis in skeletal muscle. In this case, HDAC5 interacts with MEF2 and represses it, preventing the differentiation of myoblasts into myotubes. Its regulation through phosphorylation is a dynamic process in order to preserve a correct stress response (Lu et al.,2000; McGee & Hargreaves, 2004).

Mice lacking HDAC7 show embryonic lethality at day 11 resulting from a failure to form cell-to-cell junctions in the developing circulatory system and rupture of blood vessels. HDAC7 represses MMP10 (a critical regulator for blood vessel formation and extracellular matrix degradation) through MEF2 interaction. Thus, this HDAC is essential for maintaining vascular integrity (Chang et al., 2006a). In the hematopoietic system, HDAC7 overexpression inhibits apoptosis of T cells in response to activation signals, while conditional knockout mice in thymocytes have defective generation of $CD4^+$ T cells (Kasler et al., 2011; Dequiedt et al., 2003b).

HDAC9 has been related to the neuronal system in addition to cardiac muscle, by regulating gene expression and dendritic growth in cortical neurons in a dynamic signal-dependent manner. HDAC9-deficient neurons have altered dendritic branches and an increased sensitivity to denervation. Additionally, recent studies demonstrated that the nucleocytoplasmic shuttling of HDAC9 plays a critical role in axon branching (Méjat et al., 2005; Sugo and Yamamoto, 2016; Sugo et al., 2010; Alchini et al., 2017). Finally, HDAC9 overexpression in B cells is related to some Non-Hodgkin lymphomas such as diffuse large B cell lymphoma (DLBCL) (Gil et al., 2016).

2.5.3 The class IIa HDACs-MEF2 axis

The N-terminal domain of class IIa HDACs is a distinctive mark for this group of proteins. It consists of 450-600 aa and just 40% of them present homology with the rest of HDACs (Martin et al., 2007). This domain contains motifs involved in subcellular localization, several residues implicated in post-translational modifications such as ubiquitination, and conserved binding motifs that mediate interaction with tissue-specific transcription factors e.g. MEF2 (Han, He, Wu, Liu, & Chen, 2005; Lu et al., 2000; Martin M, Kettmann, & Dequiedt, 2009).

It is well-established that class IIa HDACs mediate transcriptional silencing of their target genes when located in the nucleus through recruitment of specific DNA binding proteins (Martin M et al., 2009). MEF2 contains a MADS box and MEF2 DNA binding domain that modulates dimerization, sequence-specific DNA binding and interaction

with different transcription factors such as EBF1 (Wu et al., 2010; Kong et al., 2016). In this case, the balance between gene activation produced by MEF2 and gene silencing caused by HDACs is critical for correct development and differentiation of diverse tissues, e.g. neurons, muscular cells and lymphocytes. Class IIa HDAC-MEF2 interaction by forming and dissociating complexes depending on phosphorylation of HDACs is highly dynamic (Chang et al., 2006a; Arnold et al., 2007; Jayathilaka et al., 2012; Parra, 2015). Many researchers are focused on studying in detail the interaction class IIa HDAC-MEF2 and their downstream targets and effects in order to increase knowledge about developmental biology and improve therapies for pathologies, especially cancer (Clocchiatti et al., 2015; Di Giorgio, Hancock, & Brancolini, 2018).

2.5.4 Subcellular shuttling

The N-terminal domain of class IIa HDACs also contains several highly conserved serine residues that are subject to phosphorylation in a specific signal-dependent manner. This reaction is crucial for their subcellular localization (Martin M et al., 2007; McKinsey, Zhang, & Olson, 2001; Parra & Verdin, 2010).

The phosphorylation status of class IIa HDACs determines whether these proteins are localized in the nucleus or in the cytoplasm and, consequently, the exertion of their repression activity on their target genes (Parra and Verdin, 2010).

In the nuclear compartment, class IIa HDACs specifically interact and form complex with MEF2 family members, the serum response factor (SRF), the calmodulin-binding transcription activator and the TF RUNX2. These interactions lead to DNA recruitment and subsequent silencing of target genes (Clocchiatti et al., 2011; Haberland et al., 2009). Conversely, in response to different extracellular physiological signals, class IIa HDACs become phosphorylated at serine residues from the N-terminal domain and then interact with 14-3-3 proteins. This results in their cytoplasmic sequestration, the dissociation of bound specific TFs and the de-repression of their target genes (Grozinger & Schreiber, 2000; Martin M et al., 2009; Parra & Verdin, 2010; A. H. Wang et al., 2000). The presence of some class IIa HDACs in both the nucleus and the cytoplasm give rise to additional mechanisms involved in shuttling regulation that are still unknown (Parra, 2015). The action of specific phosphatases produce the de-phosphorylation of class IIa HDACs, resulting in their nuclear import and the repression of their target genes again (Clocchiatti, Florean, & Brancolini, 2011; Maud Martin et al., 2009; Parra & Verdin, 2010).

To date, there are four families of serine/threonine kinases and two families of phosphatases implicated in this mechanism. The most relevant members are CaMK

and PKD, and PP2A and PP1β (included in myosin phosphatase complex) respectively. The coordinated activity between kinases and phosphatases constitutes an essential regulatory mechanism that allow correct, rapid, and reversible expression of class IIa HDAC target genes in response to specific external signals (Figure 10) (Clocchiatti et al., 2011; Martin M et al., 2009; Parra et al., 2007).

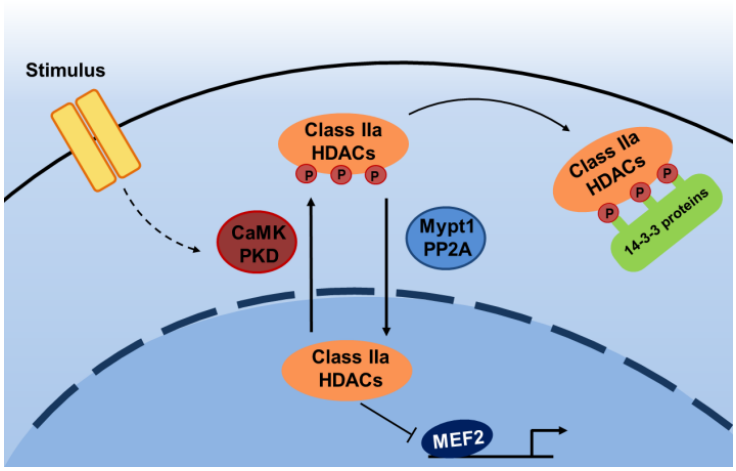

Figure10. **Subcellular shuttling of class IIa HDACs.** Illustration showing the modulation of nuclear-cytoplasmic shuttling of class IIa HDACs depending on phosphorylation. Serine phosphorylation by CaMK/PKD lead to nuclear export and 14-3-3 binding results in cytoplasmic arrest. Conversely, de-phosphorylation by PP2A/MYTP1 complex give rise to nuclear import and HDAC target genes repression. (Figure adapted from Clocchiatti A et al. 2011).

2.5.5 Class IIa HDACs in the immune system

HDAC7 and HDAC9 in T cell development

Interestingly, Eric Verdin's laboratory showed that particularly HDAC7 was the most class IIa HDAC expressed in double-positive thymocytes ($CD4^+CD8^+$) (Dequiedt et al., 2003a). It is localized in the nucleus of resting thymocytes, where it represses the expression of a long list of genes involved in T-cell positive and negative selection (Kasler and Verdin, 2007). Among these genes, HDAC7 represses the orphan nuclear receptor called *Nur77,* which is involved in the apoptosis of thymocytes and their negative selection (Dequiedt et al., 2003a; Parra et al., 2005; Kasler and Verdin, 2007). Upon T-cell activation, HDAC7 is phosphorylated and exported to the cytoplasm, resulting in Nur77 re-activation and induction of apoptosis (Parra et al., 2005; Dequiedt et al., 2005). Strikingly, *in vivo* conditional deletion of HDAC7 in double-positive thymocytes results in a significant defect in their ability to differentiate into $CD4^+$ single positive T cells and naïve CD4 thymocytes are unable to differentiate (Kasler et al., 2011; Myers et al., 2017). Conversely, nuclear accumulation of HDAC7 due to impaired

phosphorylation gave rise to a block of negative selection and consequent escape of autoreactive T cells, an impairment of the development of iNKT cells and the development of lethal tissue-specific autoimmune diseases (Kasler et al., 2018, 2012). All these evidences define HDAC7 as an essential regulator of T cell development with relevant signal-dependent repressive function. Regarding cytotoxic T cells (CTL), HDAC7 is constitutively phosphorylated and retained in the cytoplasm. In fact, a failure in phosphorylation of its serine residues and nuclear import of HDAC7 lead to impaired function of this cell type (Navarro et al., 2011).

HDAC7 and HDAC9 play an important role in various stages of T lymphopoyesis, likewise Foxp3 is an important transcriptional repressor and modulator for T cell development and function. In particular, HDAC9 is highly expressed in Treg cells compared to other T cell types. Strikingly, HDAC7, HDAC9 and HDAC3, are required for Foxp3-dependent transcriptional silencing activity. Upon Treg cell activation, HDAC9 is exported from the nucleus (Li B et al., 2007; Tao R et al., 2007; Wang L et al., 2015). Reduced expression of HDAC9 in mice induced Foxp3 expression, resulting in an increased Treg suppressor function and prevention of autoimmunity disorders (Wang et al., 2009; de Zoeten et al., 2010). In contrast to HDAC7, HDAC9 does not have a role in preventing autoimmunity disorders. Thus, HDAC9 becomes a relevant therapeutic target in order to modulate the balance between T effectors and T regulators function and, consequently, maintain cell tolerance and prevent immune-associated pathologies (Nijhuis et al., 2019).

HDAC7 in B lymphopoiesis

Our laboratory demonstrated that HDAC7 is highly expressed in pre-B cells but not in myeloid cells such as macrophages. Graf and colleagues developed an *in vitro* approach in which pre-B cells directly transdifferentiate into-macrophage-like cells in 48 hours after up-regulation of CEBP-α induced by β-estradiol treatment (Bussmann et al., 2009). During trans-differentiation, treated cells present a dramatic down-regulation of HDAC7 expression (Figure 11). Interestingly, treated cells expressing exogenous HDAC7 expression undergo a block in the up-regulation of myeloid transcriptional program during trans-differentiation. These results demonstrated that HDAC7 represses myeloid genes expression in B cells, becoming a potential and important protein for proper B cell development. Notably, in pre-B cells HDAC7 interacts with MEF2C and is recruited at MEF2 binding sites from promoters of macrophage genes (Barneda-Zahonero et al., 2013).

Preliminary results in our laboratory reported a block in B lymphocyte development, specifically at the pro-B cell to pre-B cell transition, upon deletion of HDAC7 by using a conditional knockout mouse model. However, the mechanisms by which HDAC7 regulates and control proper early B cell development and the role of HDAC7 in B cell malignancies were still unknown.

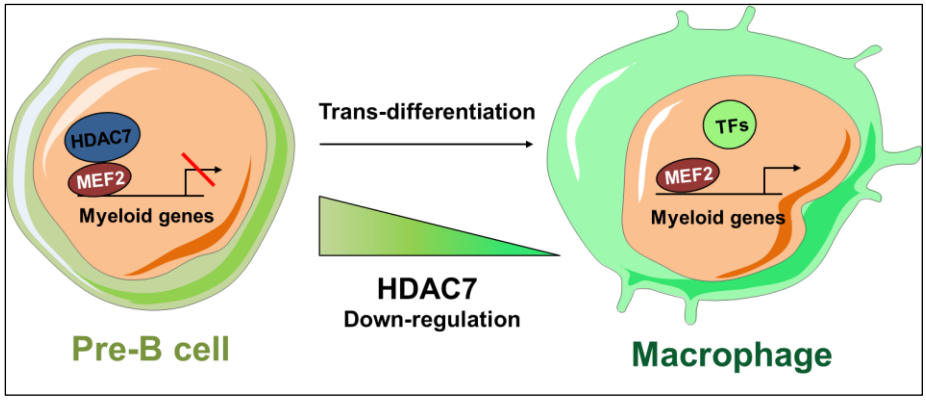

Figure 11. **Trans-differentiation of pre-B cells into macrophages**. This is an *established in vitro* model dependent on B-estradiol treatment and consequent upregulation of CEBPα expression. Figure adapted from Barneda-Zahonero et al. 2013. Cell illustrations were extracted and adapted from public webpage Smart servicer medical art.

HDAC7 and hematological malignancies

Acute lymphoblastic leukaemia (ALL) is the most frequent type of cancer in infants. Despite having achieved around 80-90% of remission from the total of ALL cases, it is still the main cause of leukaemia-associated death in children (Iacobucci and Mullighan, 2017). Most of the paediatric ALL cases are related to the cell lineage (B-ALL) and a great part of them are caused by the de-regulation of critical TFs involved in the regulation of correct B lymphopoiesis such as PAX5, EBF1, IKAROS and E2A. In parallel, multiple lymphoma types such as Burkitt or Diffuse Large B-cell Lymphoma (DLBCL) also present alterations in TFs expression. The activation of these TFs can be additionally affected by chromosomal translocations (e.g. ETV6-RUNX1 fusion protein), mutations, rearrangements, or deletions. The de-regulation of their activators or repressors, such as the upregulation of miR-125b that represses IRF4 activity or the over-expression of ZNF423 that represses EBF, also contributes to the ALL onset. In fact, modulation of the TF activity can be affected by multiple mechanisms, but the previously mentioned situations result in a reduced or loss of function of the corresponding TFs, leading to a developmental arrest and malignant cellular transformation (Mullighan et al., 2007; So et al., 2014; Somasundaram et al., 2015; Kuiper et al., 2007). Additionally, translocations and aberrant overexpression of *c-MYC*

was observed in T-cell acute leukemia (T-ALL), B-ALL, and some types of B-cell lymphoma such as Burkitt lymphoma or DLBCL (La Starza et al., 2014; Zhang and Amos Burke, 2018).

As mentioned above, HDACs deregulation has also been associated to the development of haematological malignancies, leading to the use of HDAC inhibitors as promising therapeutic agents in some blood cancers. In fact, some studies reported a correlation between HDAC7 and hematopoietic malignancies. For instance, by using a PiggyBac transposon mutagenesis screening in mice, HDAC7 was initially identified as a target gene in hematopoietic cancers (Rad et al., 2010). In the same line, several HDACs including HDAC7 were associated to a reduced viability in some hematological malignancies such as CLL (Wang et al., 2011). However, HDAC7 has been recently catalogued as a potential tumor suppressor gene in DLBCL after performing an exhaustive genetic study in a thousand of patients, despite the genetic heterogeneity of this pathology (Reddy et al., 2017). Thus, deregulation of HDACs in lymphoid malignancies cannot only be associated to their overexpression but also to their loss of function or expression.

Hence, the understanding of the regulatory networks that control proper B cell development is essential to study additional genetic alterations that can modulate these regulatory systems in each pathological condition.

OBJECTIVES

OBJECTIVES

The overall objectives of this PhD thesis are:

1. To dissect the HDAC7-mediated molecular silencing mechanisms during early B cell development.
    a. Based on the generation of a conditional knockout moue model, we will dissect the contribution of HDAC7 in regulating proper B cell development, especially at the B cell progenitors stage, as well as the biological consequences of HDAC7 deficiency in the hematopoietic system.
    b. Based on the unique structure of this class IIa HDAC, we will search for the details of its mechanism of action over its target genes.

2. To investigate the role of HDAC7 in DNA 5-hydroxymethylation status of B cells through repression of the demethylase enzyme *Tet2*
    a. To determine whether HDAC7 is recruited directly to regulatory regions of *Tet2* gene.
    b. To study the consequences of HDAC7 deficiency on DNA 5-hydroxymethylation in B cell progenitors.

3. To investigate the potential contribution of HDAC7 in the regulation of microRNAs expression in B cell progenitors.

4. To unveil the potential anti-oncogenic function of HDAC7 in B cell acute lymphocytic leukemia (B-ALL) and Burkitt Lymphoma

RESULTS

I hereby certify that the PhD student Alba Azagra Rodríguez will present her thesis as a compendium of three publications. Her contribution to each work is as follows:

ARTICLE 1: Alba Azagra*, Lídia Román-González*, Olga Collazo*, Javier Rodríguez-Ubreva, Virginia G. de Yébenes, Bruna Barneda-Zahonero, Jairo Rodríguez, Manuel Castro de Moura, Joaquim Grego-Bessa, Irene Fernández-Duran, Abul B.M.M.K. Islam, Manel Esteller, Almudena R. Ramiro, Esteban Ballestar and Maribel Parra

*contributed equally to this paper

TITLE: "In vivo conditional deletion of HDAC7 reveals its requirement to establish proper B lymphocyte identity and development".

JOURNAL: J Exp Med. 2016 Nov 14;213(12):2591-2601. Epub 2016 Oct 17. Impact factor (2016): 11.991

In this article, Alba Azagra participated in all the experiments reported. In particular, in spleen and bone marrow extraction and cell counting, cell sorting of bone marrow *Hdac7*$^{+/-}$ and *Hdac7*$^{fl/-}$ pro-B and pre-B cells, FACS analysis of cell cycle, cell death and Igμ, RT-qPCR of different genes, ChIP-qPCR validations of all ChIP-seq and validation of selected genes, ChIP-qPCR from microarray-selected genes, gain of function experiments, data collection and interpretation as well as edition of figures. Some of the data from this article has also been used for the thesis of Lídia Román González. In particular, the initial characterization of HDAC7 deficient mice and microarray experiments.

ARTICLE 2: Alba Azagra, Maria Vila-Casadesús*, Olga Collazo*, Ainara Meler, Oriol de Barrios, Thomas Graf and Maribel Parra

*contributed equally to this paper

TITLE: "Unveiling a novel function of HDAC7 in preserving the identity of B cell progenitors through TET2 regulation"

JOURNAL: Manuscript in preparation

In this article, Alba Azagra participated all the experiments reported. In particular, in cell sorting of *Hdac7*$^{+/-}$ and *Hdac7*$^{fl/-}$ pro-B and pre-B cells, RT-qPCR of different genes,

Western Blot experiments of CD19+ cells from *Hdac7*$^{+/-}$ and *Hdac7*$^{fl/-}$ mice, ELISA experiments, sample preparation and immunoprecipitation of hMeDIP-sequencing, hMeDIP-qPCR validations, ChIP-qPCR experiments, FACS analysis, miRCURY panel of microRNA expression and data analysis, data collection, figures edition and manuscript preparation.

ARTICLE 3: Bruna Barneda-Zahonero, Olga Collazo*, Alba Azagra*, Irene Fernández-Duran, Jordi Serra-Musach, Abul BMMK Islam, Nerea Vega-García, R Malatesta, Mireia Camós, Antonio Gómez, Lídia Román González, August Vidal, Núria López-Vigas, Alberto Villanueva, Manel Esteller and Maribel Parra.

*contributed equally to this paper

TITLE: "The transcriptional repressor HDAC7 promotes apoptosis and c-Myc downregulation in particular types of leukemia and lymphoma".

JOURNAL: Cell Death Dis. 2015 Feb 12;6:e1635. Impact factor (2015): 5.378

In this article, Alba Azagra participated in the Western blot experiments of c-Myc and MEF2C in all cell lines of ALL and Burkitt lymphoma, cell death, cell counting and cell viability (MTT) analyses, sh-HDAC3 cloning and infections of cancer cell lines with HDAC3 shRNA and data interpretation.

In witness whereof, I hereby sign this in Barcelona,

Barcelona, July 10th, 2019

Maribel Parra Bola, PhD

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ARTICLE 1

“In vivo conditional deletion of HDAC7 reveals its requirement to establish proper B lymphocyte identity and development”

Published October 17, 2016

In vivo conditional deletion of HDAC7 reveals its requirement to establish proper B lymphocyte identity and development

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Class IIa histone deacetylase (HDAC) subfamily members are tissue-specific gene repressors with crucial roles in development and differentiation processes. A prominent example is HDAC7, a class IIa HDAC that shows a lymphoid-specific expression pattern within the hematopoietic system. In this study, we explored its potential role in B cell development by generating a conditional knockout mouse model. Our study demonstrates for the first time that HDAC7 deletion dramatically blocks early B cell development and gives rise to a severe lymphopenia in peripheral organs, while also leading to pro–B cell lineage promiscuity. We find that HDAC7 represses myeloid and T lymphocyte genes in B cell progenitors through interaction with myocyte enhancer factor 2C (MEFC2). In B cell progenitors, HDAC7 is recruited to promoters and enhancers of target genes, and its absence leads to increased enrichment of histone active marks. Our results prove that HDAC7 is a bona fide transcriptional repressor essential for B cell development.

INTRODUCTION

Within the adult hematopoietic system, generation of various mature blood cell types is the result of several cell lineage choices and differentiation steps. At each particular branching or differentiation point, the silencing of genes from alternative lineages is crucial for acquiring the correct identity of the newly generated cell. In bone marrow, lymphoid-primed multipotent progenitors commit to the lymphoid branch by generating common lymphoid progenitors, which, in turn, have the ability to give rise to early B and T lymphocyte progenitors (Kondo et al., 1997; Cobaleda and Busslinger, 2008). Once generated, B cell progenitors (pro–B cells) undergo a series of differentiation steps that result in the generation of B cell precursors (pre–B cells) and immature B lymphocytes (Parra, 2009; Barneda-Zahonero et al., 2012). The latter migrate to the spleen to complete their maturation (Parra, 2009; Barneda-Zahonero et al., 2012). Intense research effort has revealed the identity and role of specific transcription factors responsible for the activation of B cell–specific genes (Parra, 2009; Barneda-Zahonero et al., 2012). The transcription factors E2A, EBF, and FOXO1 are involved in the early specification of common lymphoid progenitors into pro–B cells, whereas PAX5 is required to maintain B cell identity during differentiation into mature B cells (Urbánek et al., 1994; Zhuang et al., 1994; Lin and Grosschedl, 1995; Bain et al., 1997; Delogu et al., 2006; Dengler et al., 2008). Recently, Schwickert et al. (2014) reported that IKAROS is also required for early B cell development. In all cases, transcription factors induce the expression of genes characteristic of B cells. Notably, the transcription factor PAX5 not only induces the expression of a B cell–specific genetic program, but also suppresses inappropriate genes of alternative lineages, thereby ensuring proper B cell differentiation (Delogu et al., 2006; Pridans et al., 2008). Likewise, EBF and E2A are also involved in the repression of non–B cell genes (Ikawa et al., 2004; Pongubala et al., 2008; Lukin et al., 2011; Nechanitzky et al., 2013). The transcription factor myocyte enhancer factor 2C (MEF2C) is involved in the commitment of cells to

*A. Azagra, L. Román-González, and O. Collazo contributed equally to this paper.

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Abbreviations used: 7AAD, 7-aminoactinomycin D; ChIP, chromatin immunoprecipitation; HDAC, histone deacetylase; MEF2C, myocyte enhancer factor 2C.

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https://doi.org/10.1084/jem.20150821

the lymphoid lineage by activating lymphoid-specific genes and repressing myeloid genes (Stehling-Sun et al., 2009; Kong et al., 2016). How B cell transcription factors induce the silencing of genes that should not be expressed in B cells remains largely unknown.

The large superfamily of histone or protein deacetylases (HDACs) are crucial transcriptional repressors in many physiological and pathological processes. Among them, the class IIa HDAC subfamily, comprising HDAC4, HDAC5, HDAC7, and HDAC9, has specific features that differ from all other HDACs, such as tissue specificity and interaction with transcription factors (Parra and Verdin, 2010; Parra, 2015). Recently, we found that HDAC7 is down-regulated during the in vitro reprogramming of pre–B cells into macrophages, whereas exogenous expression of HDAC7 interfered with the acquisition of essential macrophage-specific cell functions in this in vitro system (Barneda-Zahonero et al., 2013). However, a role for HDAC7 in B cell development in vivo remains to be established. Of interest, Lin et al. (2010) identified *Hdac7* as a target of E2A, EBF, and Foxo1 in B lymphocyte progenitors, whereas Revilla-i-Domingo et al. (2012) showed that *Hdac7* may be a PAX5-activated gene. Whyte et al. (2013) showed that genes involved in cell lineage identity contain superenhancer regions that recruit specific transcription factors leading to their expression. Strikingly, they identified *Hdac7* as one of the lineage identity genes bearing a superenhancer region in pro–B cells (Whyte et al., 2013). These recent studies suggest that HDAC7 may regulate B cell development.

Here, we demonstrate that HDAC7 is essential for proper B cell development. Specifically, HDAC7 deficiency in pro–B cells in mice causes a block in early B cell development. This is accompanied by expression of genes from alternative lineages, such as myeloid cells and T lymphocytes. We propose that HDAC7-mediated repression occurs through MEF2C recruitment to crucial myeloid and T cell genes. These findings establish HDAC7 as a bona fide transcriptional repressor necessary for the acquisition of the correct gene cell identity during B cell generation.

RESULTS AND DISCUSSION

To investigate the potential role of HDAC7 in adult B lymphopoiesis, we generated a conditional mouse model for HDAC7 deficiency in pro–B cells. To this end, we crossed $Hdac7^{loxp/-}$ mice with the deleter line *mb1*-Cre, mb1-$Cre^{ki/+}$ mice (Hobeika et al., 2006). An initial analysis of bone marrow and spleen revealed a 30% and 45% reduction, respectively, in the total number of cells in $Hdac7^{loxp/-}$;mb1-$Cre^{ki/+}$ mice (referred to as $Hdac7^{fl/-}$) compared with their littermate controls $Hdac7^{+/-}$;mb1-$Cre^{ki/+}$ ($Hdac7^{+/-}$ mice; Fig. 1 A). Accordingly, we observed markedly smaller spleens in HDAC7 conditional knockout mice than in their littermate controls (Fig. 1 B). A detailed flow cytometry analysis of the various B cell subsets in the bone marrow showed that HDAC7 deficiency causes a block in B cell development at the pro–B to pre–B cell transition. In particular, we observed a significant decrease in the total number of B cells and an accumulation of pro–B cells in $Hdac7^{fl/-}$ compared with their littermate controls, $Hdac7^{+/-}$ (Fig. 1 C, and see Fig. S1, A–C for gating strategy). In contrast, the number of pre–B cells was significantly lower while immature and mature recirculating B cells were hardly detectable in the bone marrow of HDAC7 mutant mice (Fig. 1 C). Our staining strategy to distinguish between pro–B ($B220^+IgM^-CD43^+$) and pre–B ($B220^+IgM^-CD43^-$) lymphocytes revealed the presence of cells expressing intermediate CD43 levels in bone marrow from HDAC7-deficient mice (see Fig. S1 for gating strategy). A similar CD43 expression pattern was observed when the CD19 marker was included in the analysis (Fig. S1). Furthermore, an alternative staining strategy including CD25 as a marker corroborated that the absence of HDAC7 results in a block in B cell development at the pro–B to pre–B cell transition (Fig. S1). These findings collectively indicate that HDAC7 is necessary for early B cell development.

Next, we assessed the impact of HDAC7 deficiency in peripheral organs. We found that HDAC7 knockout mice had many fewer B cells in the spleen and blood than did control mice (Fig. 1 D). To assess the effect of HDAC7 deficiency at later B cell developmental stages, we next analyzed B cell maturation in the spleen of $Hdac7^{fl/-}$ mice and their littermate controls $Hdac7^{+/-}$. In particular, the analysis of immature B cells, marginal zone B cells, follicular B cells, and transitional B cells revealed that the absolute numbers of all B cell subtypes analyzed were significantly lower in HDAC7-deficient mice (Fig. 1 D, and see Fig. S2 for gating strategy). Hematoxylin and eosin staining revealed that the spleens of control mice had a normal structure with well-defined follicles represented by white pulp (hematoxylin staining in purple; Fig. 1 E) surrounded by red pulp (eosin staining in pink; Fig. 1 E). We observed that spleens from HDAC7-deficient mice had a highly abnormal and unstructured morphology, with smaller and poorly defined follicles (Fig. 1 E). We examined whether the proportions of macrophages and T cells were altered in the spleens of HDAC7 knockout mice. We found significantly higher proportions of Mac-1–positive cells in the spleens of HDAC7 mutant mice (Fig. 1 F). In addition, flow cytometry analysis revealed a higher percentage of T lymphocytes in HDAC7 knockout mice (Fig. 1 G). The data presented so far demonstrate that HDAC7 is essential for proper B lymphocyte development and its absence is associated with severe lymphopenia in the periphery and a higher density of macrophages and T cells in the spleen.

To get an insight into the mechanisms underlying the B lymphocyte developmental block observed, we first determined whether the absence of HDAC7 affected cell proliferation. Cell cycle analysis revealed that proliferation was not sensibly altered in either pro–B or pre–B cell subsets in the absence of HDAC7 (Fig. 2 A). Likewise, we did not observe differences in the expression of *Il7r* in pro–B or pre–B cells from wild-type and HDAC7-deficient mice, thus rein-

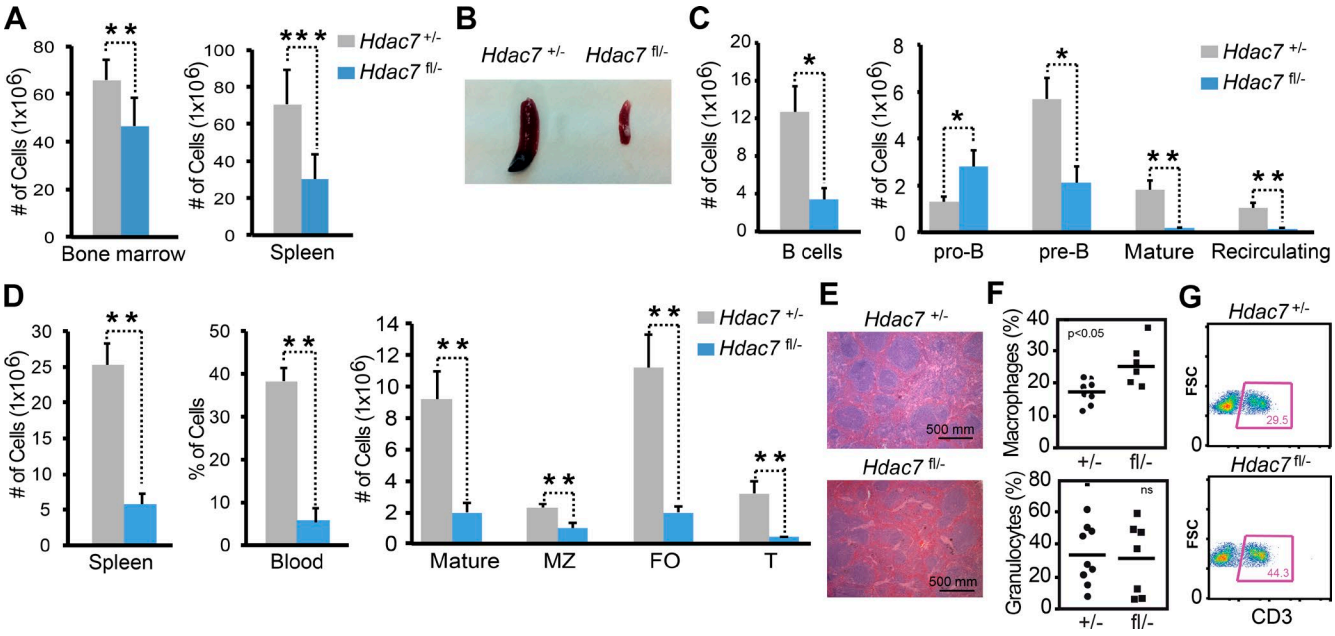

Figure 1. **HDAC7 is required for early B cell development.** (A) Absolute numbers of total bone marrow cells (*Hdac7*$^{fl/-}$ [*n* = 6] and *Hdac7*$^{+/-}$ [*n* = 8]) and total spleen cells (*Hdac7*$^{+/-}$ [*n* = 12] and *Hdac7*$^{fl/-}$ [*n* = 9]). (B) Representative photograph of the spleen from *Hdac7*$^{+/-}$ and *Hdac7*$^{fl/-}$ mice. (C) Absolute numbers of bone marrow B220^{+} B cells (*Hdac7*$^{fl/-}$ [*n* = 5] and *Hdac7*$^{+/-}$ [*n* = 8]), B220^{+}CD43^{+}IgM^{-} pro-B cells (*Hdac7*$^{fl/-}$ [*n* = 5] and *Hdac7*$^{+/-}$ [*n* = 8]), B220^{+}CD43^{-}IgM^{-} pre-B cells (*Hdac7*$^{fl/-}$ [*n* = 6] and *Hdac7*$^{+/-}$ [*n* = 6]), B220^{+}IgM^{+} immature B cells (*Hdac7*$^{fl/-}$ [*n* = 7] and *Hdac7*$^{+/-}$ [*n* = 9]), and B220^{+}IgM^{+} IgD^{+} mature recirculating B cells (*Hdac7*$^{fl/-}$ [*n* = 7] and *Hdac7*$^{+/-}$ [*n* = 9]). (D) Graph on the left shows the absolute numbers of B220^{+} B cells from spleen of *Hdac7*$^{+/-}$ (*n* = 12) and *Hdac7*$^{fl/-}$ (*n* = 9) mice. Graph in the middle shows the percentage of B220^{+} cells in the blood from *Hdac7*$^{+/-}$ (*n* = 4) and *Hdac7*$^{fl/-}$ (*n* = 4) mice. Graph on the right shows absolute numbers of spleen B cell subsets from wild-type and HDAC7-deficient mice: B220^{+}IgM^{+}IgD^{+} mature B cells (*Hdac7*$^{+/-}$ [*n* = 7] and *Hdac7*$^{fl/-}$ [*n* = 5]), CD21^{bright}CD23^{+} marginal zone (MZ) B cells (*Hdac7*$^{+/-}$ [*n* = 12] and *Hdac7*$^{fl/-}$ [*n* = 9]), and CD21^{+}CD23^{bright}CD93^{-} follicular (FO; *Hdac7*$^{+/-}$ [*n* = 9] and *Hdac7*$^{fl/-}$ [*n* = 7]) and CD21^{+}CD23^{bright}CD93^{+} transitional (T; *Hdac7*$^{+/-}$ [*n* = 8] and *Hdac7*$^{fl/-}$ [*n* = 7]) B cells. (E) Hematoxylin and eosin staining of the spleen from *Hdac7*$^{+/-}$ and *Hdac7*$^{fl/-}$ mice. (F) Percentages of granulocytes and macrophages from the spleen of *Hdac7*$^{+/-}$ (*n* = 9) and *Hdac7*$^{fl/-}$ (*n* = 7) mice. Mean values are shown as horizontal bars. ns, not significant. (G) Representative FACS analyses from three independent experiments are shown on T lymphocytes from the spleens of *Hdac7*$^{+/-}$ and *Hdac7*$^{fl/-}$ mice. FSC, forward scatter. Data are represented as the mean ± SEM. Statistical significances were identified using the unpaired two-tailed Student's *t* test. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

forcing the finding that the proliferation status is intact in HDAC7-deficient B cells (Fig. 2 B). Therefore, we wondered whether the developmental block at the pro–B to pre–B cell transition in mice lacking HDAC7 might be associated with cell death. HDAC7-deficient pre–B cells showed a significant increase of 7-aminoactinomycin D (7AAD)$^{+}$ cells compared with wild-type pre–B cells (Fig. 2 C). We also observed a trend to an increased cell death rate in HDAC7-deficient pro–B cells. Next, to test whether HDAC7 is required for V(D)J recombination in pro–B and pre–B cells, we examined the expression of intracellular IgHμ protein. We found that HDAC7-deficient pro–B and pre–B cells express IgHμ (although to a significantly lesser extent than control cells; Fig. 2 D), indicating that HDAC7 is not absolutely required for V(D)J recombination. Western blot experiments revealed that the Cre-Loxp system used in our study was highly efficient because HDAC7 was completely absent in B cells from bone marrow of knockout mice (Fig. 3 G). Therefore, V(D)J rearrangements in pro–B and pre–B cells from our knockout mouse model are not likely caused by the presence of residual HDAC7. Together, these data indicate that pro–B and pre–B cells lacking HDAC7 undergo V(D)J recombination but are more susceptible to apoptosis, which may explain the severely reduced numbers of pre–B and subsequent B cell subsets in the bone marrow and the periphery of HDAC7-deficient mice.

Because HDAC7 is a bona fide transcriptional repressor, we wondered whether HDAC7 is involved in silencing lineage-inappropriate genes, thereby ensuring the correct acquisition of B cell identity. To address this possibility, we performed global gene expression profiling in pro–B cells purified from *Hdac7*$^{fl/-}$ mice and their *Hdac7*$^{+/-}$ littermate control mice. Microarray analysis showed that 1,750 and 1,424 genes were up-regulated and down-regulated, respectively, in HDAC7-deficient pro–B cells (Dataset S1). To gain functional insights into the genes that were up-regulated in the absence of HDAC7, we performed gene set enrichment analysis based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and the gene ontology (GO) categories corresponding to Biological Processes (Fig. 3 A). The KEGG pathway analysis showed that the up-regulated genes were enriched in MAPK signaling, T cell receptor signaling, lysozyme, ubiquitin-mediated proteolysis, endo-

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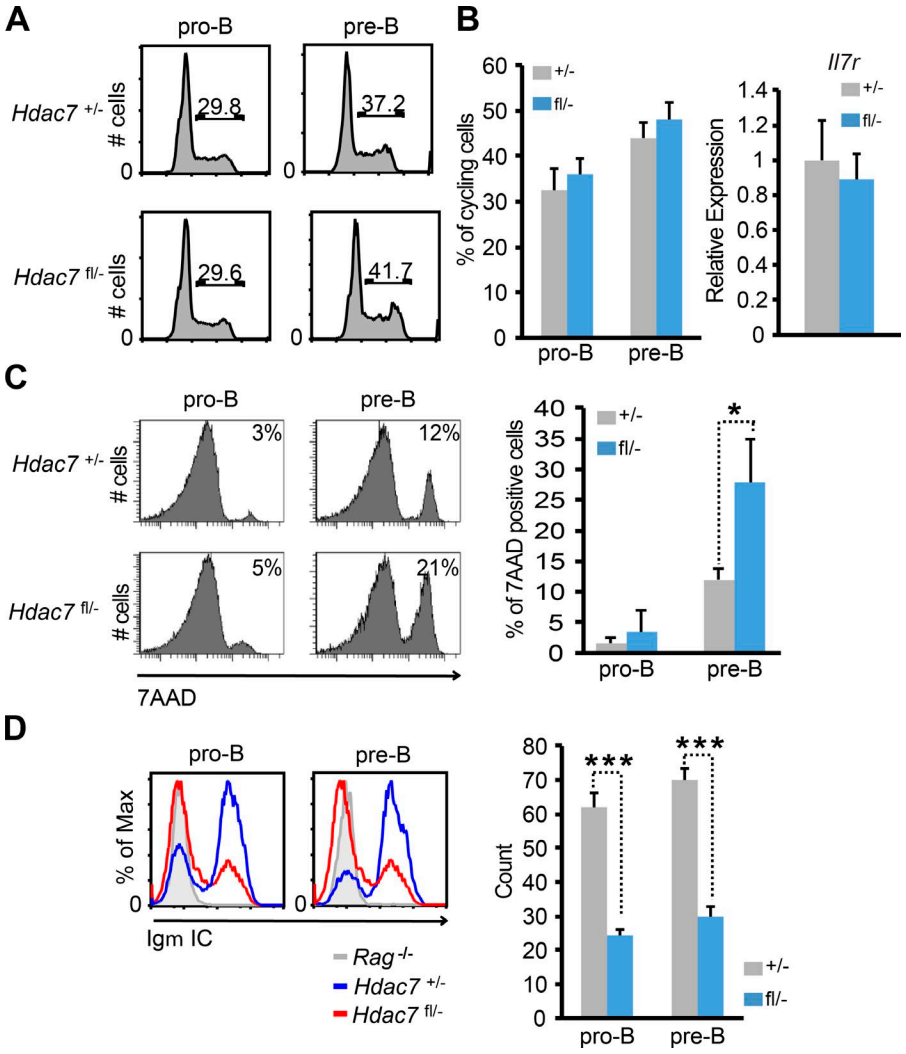

Figure 2. **HDAC7 deficiency results in increased cell death in pro–B and pre–B cells.** (A) Representative Hoechst staining of pro–B and pre–B cells from *Hdac7*$^{+/-}$ and *Hdac7*$^{fl/-}$ mice form three independent experiments. Bars represent the percentage of cycling cells. (B) RT-quantitative PCR experiments for gene expression of *Il7r* in pro–B cells from *Hdac7*$^{+/-}$ and *Hdac7*$^{fl/-}$ mice. (C) Increased cell death of pro–B and pre–B cells in the absence of HDAC7. Bar graph depicts the percentage of apoptotic-dead (7AAD^{+}) cells. (D) Representative histogram staining showing the expression of intracellular IgHμ (Igm IC) in pro–B and pre–B cells from *Hdac7*$^{+/-}$ and *Hdac7*$^{fl/-}$ mice. Bar graph depicts the cell count expressing IgHμ. All data are represented as the mean ± SEM of three independent experiments. Statistical significances were identified using the unpaired two-tailed Student's *t* test. *, $P < 0.05$; ***, $P < 0.001$.

cytosis, chemokine signaling, and Fc-γ–mediated phagocytosis pathways (Fig. 3 A). The Biological Processes analysis revealed that the up-regulated genes belong to the categories representing transcription, chromatin organization and modification, hemopoiesis, leukocyte activation, intracellular signaling cascade, immune system development, endocytosis, T cell activation and differentiation, and myeloid activation processes (Fig. 3 A). Strikingly, we observed that the absence of HDAC7 from pro–B cells was associated with the up-regulation of key genes for myeloid cell functions, such as *Itgam*, *Itgax*, *Ifi204*, *Ccl3*, *Ccl4*, and *Ccr2*, among others (Table S1). Indeed, HDAC7 deficiency prompted the expression of the key macrophage marker Mac-1 (encoded by *Itgam*) in gated bone marrow lymphocytes or purified CD19^{+} B cells (Fig. 3, B and C). In addition, loss of HDAC7 from pro–B cells led to the up-regulation of many transcription factors, several of which play a role in myeloid cell differentiation (*Fosb*, *Egr1*, *Crebzf*, *Cebpb*, and *Cebpd*; Table S1). Strikingly, analysis of data from the Immunological Genome Project to compare the expression pattern between HDAC7 and selected myeloid genes in purified B cells and macrophage populations revealed an inverse correlation with gene expression (Fig. 3 D). We also found that HDAC7 deficiency led to the up-regulation of T cell genes, such as *Cd28*, *Cd69*, *Il17*, and *Lck*, and the T cell transcription factors *Runx1*, *Runx3*, and *Nfat5* (Table S1). Changes in the expression of selected genes (*Itgam*, *Ccl3*, *Cd28*, and *Cd69*) were validated by quantitative RT-PCR (Fig. 3 E). Interestingly, analysis of the down-regulated genes in the absence of HDAC7 did not reveal any enrichment in either gene ontology or KEGG pathways related to biological aspects of B lymphocytes (not depicted). Of note, our microarray experiments did not reveal any changes either in the expression of key B cell transcription factors such as PAX5, E2A, and EBF or in other important B lymphocyte genes (CD19 and RAG2). The expression of *Cd19*, *Pax5*, and *Rag2* was determined by quantitative RT-PCR (Fig. 3 F). PAX5 and EBF protein levels were determined by Western blot assays (Fig. 3 G). The fact that HDAC7 deficiency imposes a strong block on B cell development, even in the presence of B cell master regulators, indicates that it is a potent regulator of

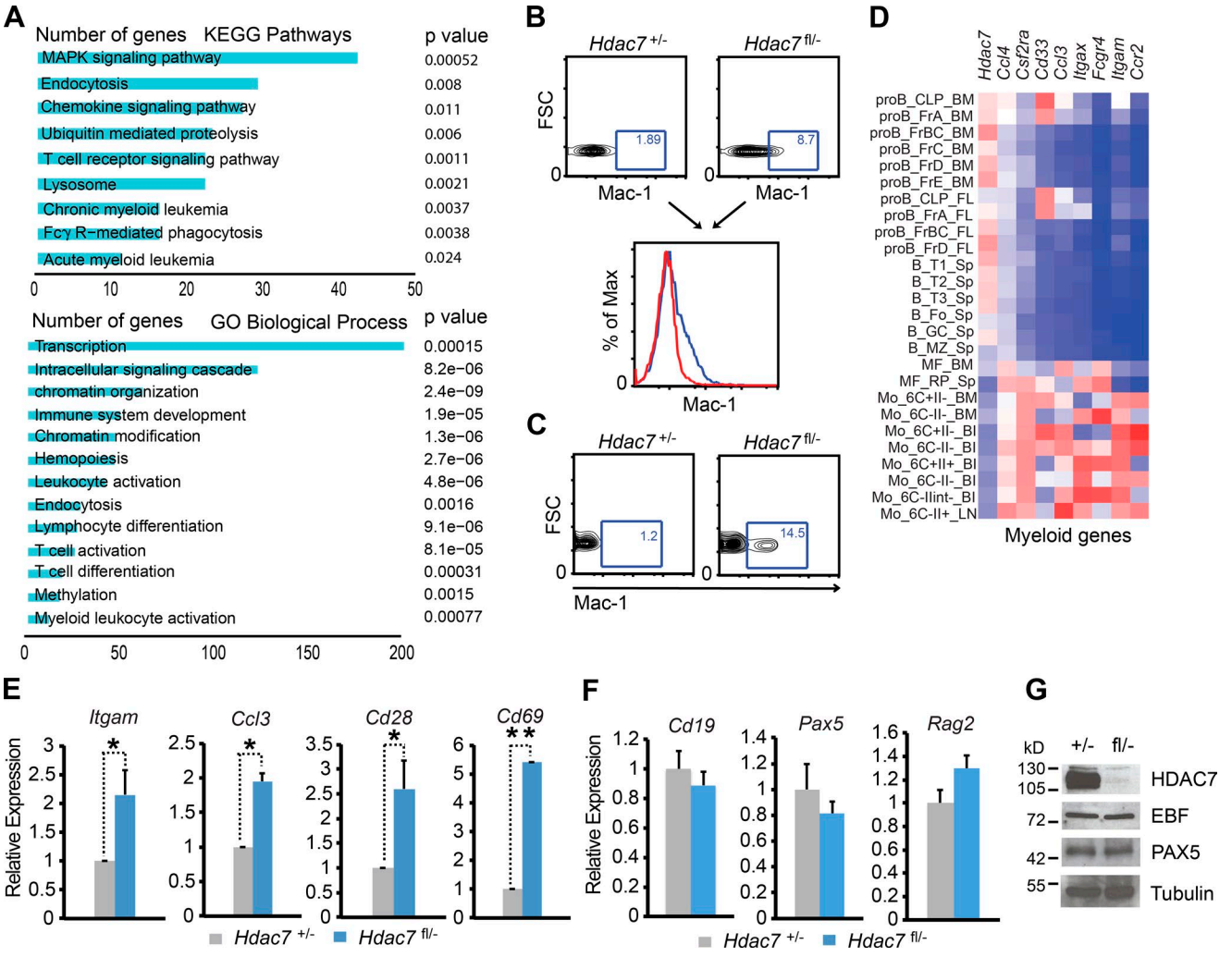

Figure 3. **Pro–B cells from HDAC7-deficient mice express genes from alternative lineages.** (A) KEGG pathway enrichment and Gene Ontology (GO) analysis of up-regulated genes in HDAC7-deficient pro–B cells. (B) Representative FACS analyses from three independent experiments showing Mac-1 expression in gated bone marrow lymphocytes from *Hdac7*$^{+/-}$ and *Hdac7*$^{fl/-}$ mice (top). Histogram showing Mac-1 expression in gated bone marrow lymphocytes from *Hdac7*$^{+/-}$ (red) and *Hdac7*$^{fl/-}$ (blue) mice (bottom). FSC, forward scatter. (C) Representative FACS analyses from three independent experiments showing Mac-1 expression in purified CD19^{+} bone marrow lymphocytes from *Hdac7*$^{+/-}$ and *Hdac7*$^{fl/-}$ mice. (D) Heatmap showing the expression pattern of HDAC7 and selected myeloid genes in different hematopoietic cell subsets. Data were obtained from the Immunological Genome Project. A description of the different cell subsets analyzed can be found at www.Immgen.org. (E) RT–quantitative PCR experiments for gene expression changes for selected up-regulated genes in the absence of HDAC7. *, $P < 0.05$; **, $P < 0.01$. (F) RT–quantitative PCR experiments for gene expression levels of B cell genes. (E and F) Data are represented as the mean ± SEM of three independent experiments. (G) Western blot analysis for the expression of HDAC7 and B cell transcription factors in wild-type and HDAC7-deficient B lymphocytes.

B cell lymphopoiesis. Collectively, these findings demonstrate that HDAC7 acts as a transcriptional repressor of lineage-inappropriate genes in B cell progenitors.

Because we previously showed that HDAC7 specifically interacts with the transcription factor MEF2C but not with other B cell–specific transcription factors in B cell precursors (Barneda-Zahonero et al., 2013), we examined whether HDAC7 is recruited to MEF2 binding sites located at the promoter of nonlymphoid genes in pro–B cells, leading to their transcriptional silencing, by performing chromatin immunoprecipitation (ChIP) experiments. First, using the TFconsite bioinformatic tool, we found that promoters of *Itgam*, *Cd69*, *Cd28*, and *Ccl3* contain putative MEF2 binding sites. Chromatin prepared from bone marrow wild-type B cells was subjected to ChIP assays with an antibody specific to HDAC7. Quantitative PCR analysis of the immunoprecipitated material with specific primers for the *Itgam*, *Ccl3*, *Cd28*, and *Cd69* promoters indicated that HDAC7 was enriched at their putative binding sites in these loci in pro–B cells (Fig. 4 A). Interestingly, we did not observe an

enrichment of HDAC7 at the *Pax5* promoter (Fig. 4 A). We also found that MEF2C was enriched at the promoters of HDAC7 target genes (Fig. 4 A). To definitively prove that HDAC7 represses its target genes through interaction with MEF2C, we performed a rescue or gain of function experimental approach. We transduced purified B cells from the bone marrow and spleens of wild-type and HDAC7-deficient mice with retroviral vectors for either normal HDAC7 or a mutant form carrying a deletion of the entire 17–amino acid stretch that mediates the interaction with MEF2 transcription factors (MIG-HDAC7-ΔMEF; Dequiedt et al., 2003), fused to GFP, and GFP-positive cells were sorted. We tested the HDAC7 mutant for its ability to repress *Itgam* and *Cd69* in bone marrow B cells lacking HDAC7. As experimental controls, we transduced wild-type and HDAC7-deficient cells with empty retroviral vector (MIG). Expression of wild-type HDAC7 in knockout cells resulted in a significant decrease in the expression of *Itgam* and *Cd69* mRNA levels (Fig. 4 B), whereas expression of the HDAC7 mutant defective for MEF2C binding had no significant effect. We also observed that the expression of wild-type, but not mutant, HDAC7 reduced the expression of Mac-1 protein in purified B cells in the spleens of knockout mice (Fig. 4 C). These experiments demonstrate that the HDAC7–MEF2C interaction is necessary for HDAC7 to repress its target genes.

Next, we analyzed the enrichment of active and repressive histone marks at HDAC7 target genes in the absence or presence of HDAC7. Interestingly, we observed that H3Ac(K9/K14) and H4K16Ac were already present at the promoters of HDAC7 target genes in wild-type B cells. The absence of HDAC7 from B cells was associated with a moderate but significant increase of both histone marks and a decrease of the repressive mark H3K27me3 in the promoters of its target genes (Fig. 4 D). To test whether, in addition to target promoters, H3Ac(K9/14) are also enriched at other genomic regions, we performed ChIP-seq assays using purified pro–B cells from wild-type and HDAC7-deficient mice. Both histone marks were also found at other genomic locations such as introns, exons, and intergenic regions (Fig. 5 A and Dataset S2). Interestingly, many of the genes up-regulated in HDAC7-deficient pro–B cells showed an enrichment in H3Ac(K9/K14) in wild-type and HDAC7-deficient pro–B cells (Dataset S3). At the global level, only slight changes in enrichment of H3Ac(K9/K14) were detectable in the absence of HDAC7 (Fig. 5 B). However, individual analysis by ChIP coupled with quantitative PCR confirmed the significance of such changes observed for HDAC7-deficient pro–B cells (Fig. 4 D). Karmodiya et al. (2012) recently reported that H3K9Ac and H3K14Ac marks not only associate with promoters of actively transcribed genes, but also with developmentally regulated bivalent promoters, as well as with enhancers in mouse embryonic stem cells. Similarly, pro–B cells in the bone marrow are largely undifferentiated lymphocyte progenitors, and the presence of histone active marks may indicate that genes from alternative lineages are poised, resulting in a certain degree of cell plasticity toward other hematopoietic cell types. In fact, genetic ablation of key B cell factors such as PAX5, E2A, and EBF1 in mice results in the inappropriate expression of genes from other cell types within the hematopoietic system. Accordingly, here we demonstrate that HDAC7 deficiency from pro–B cells leads to expression of myeloid and T cell genes. Recently, van Oevelen et al. (2015) determined the presence of enhancers at macrophage genes in pre–B cells. The authors showed that during trans-differentiation of pre–B cells into macrophages by exogenous expression of the myeloid transcription factor C/EBP-α, this transcription factor binds to two types of myeloid enhancers in B cells: preexisting enhancers that are bound by PU.1, providing a platform for incoming C/EBP-α; and de novo enhancers that are targeted by C/EBP-α (van Oevelen et al., 2015). This prompted us to analyze whether these macrophage gene enhancers were also enriched in H3K9/K14Ac in our ChIP-seq experiment. Interestingly, preexisting enhancers that are bound by PU.1 were indeed occupied by both histone marks in wild-type and HDAC7-deficient pro–B cells (Fig. 5 B and Dataset S4). This finding further supports the notion that lineage-inappropriate genes may be epigenetically poised in B cell progenitors. To test the possibility that HDAC7 could be also recruited at enhancers of its target genes, we performed ChIP experiments. Strikingly, we found that HDAC7 is also bound to enhancers of *Itgam* and *Cd69* (Fig. 5 C), in addition to their promoters. The absence of HDAC7 from pro–B cells was associated with a significant enrichment of the activating histone marks, H3Ac(K9/K14) and H4K16Ac, in the enhancers of its target genes. Interestingly, enrichment of the enhancer mark H3K27Ac was also increased in the same enhancer loci in HDAC7-deficient pro–B cells (Fig. 5 D). Collectively, our results demonstrate that HDAC7 is recruited to the promoters and enhancers of lineage-inappropriate genes in pro–B cells, resulting in their transcriptional silencing. The finding that HDAC7 is also recruited at enhancers of its target genes represents a novel mechanism by which this HDAC may control gene expression in B lymphocytes.

Our results represent the first evidence that HDAC7 acts in vivo as a master regulator of B cell identity and development. We demonstrate that HDAC7 is an essential transcriptional repressor of genes from alternative lineages that ensures proper B cell development. The fact that B cell transcription factors, such as E2A and PAX5, may induce the expression of HDAC7 in pro–B cells indicates the potential existence of an alternative mechanism involved in gene repression. B cell transcription factors not only recruit corepressors to silence their lineage-inappropriate target genes, but also may act through an indirect mechanism that induces the expression of a transcriptional repressor, such as HDAC7, which, in turn, through the interaction with MEF2C, directly represses genes from alternative lineages. In conclusion, we have identified HDAC7 as a lymphoid-specific and bona fide transcriptional repressor that is essential for proper B lymphocyte development and for ensuring the acquisition of the correct gene

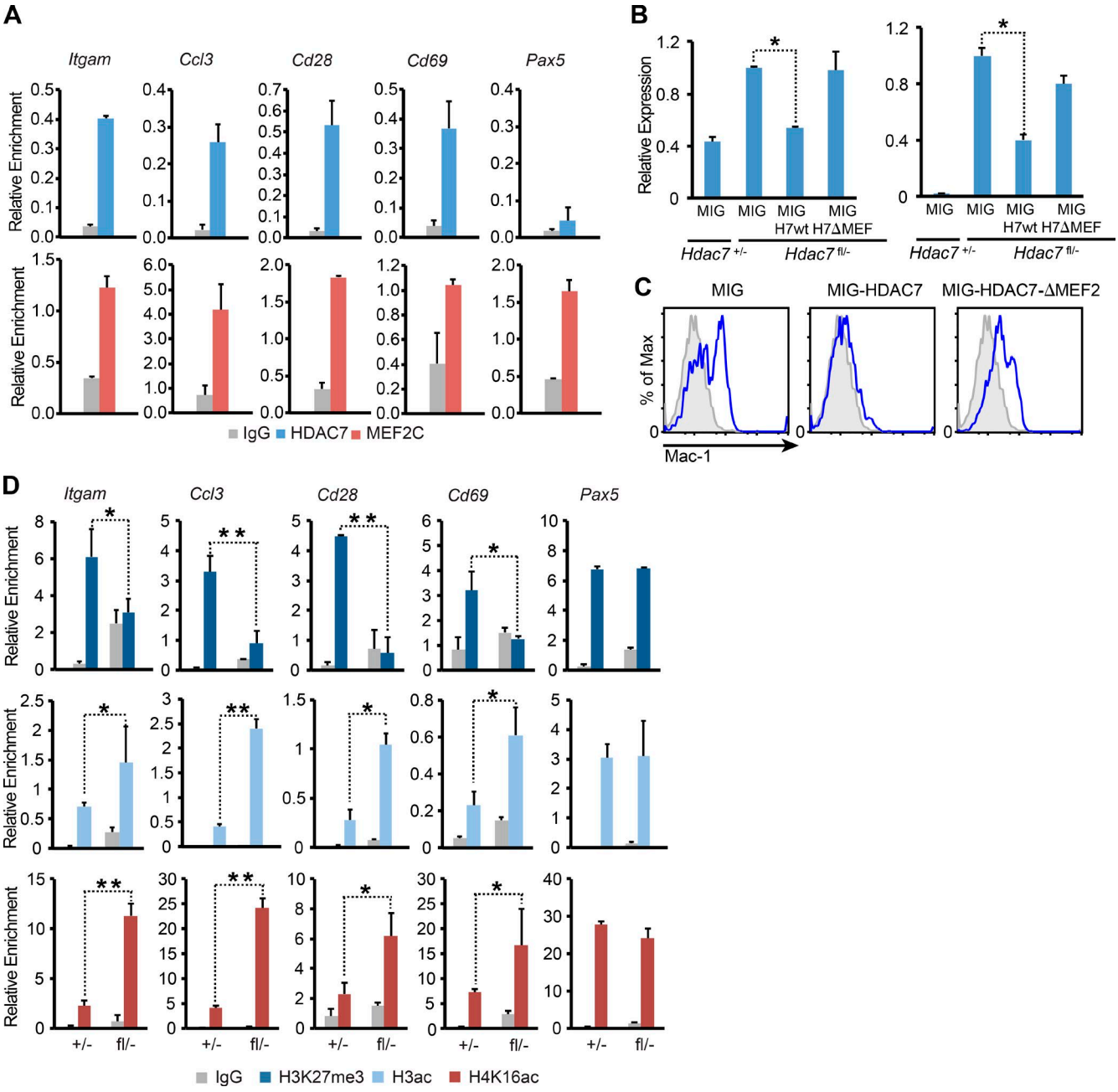

Figure 4. **HDAC7 and MEF2C are recruited to the promoters of lineage-inappropriate genes in bone marrow B lymphocytes.** (A) ChIP experiments showing the enrichment of HDAC7 and MEF2C to putative MEF2 binding sites on the *Itgam*, *Ccl3*, *Cd28*, *Cd69*, and *Pax5* gene loci in bone marrow CD19$^+$ B cells. Results are presented as the relative enrichment over input and are based on the results of three independent experiments. (B and C) Purified HDAC7-deficient B cells from bone marrow (B) and spleen (C) were infected with MSCV-GFP, MSCV-GFP-HDAC7, and MSCV-GFP-HDAC7(ΔMEF) viruses. As an experimental control, wild-type B cells were transduced with MSCV-GFP. (B) GFP$^+$ cells were sorted and mRNA extracted. RT–quantitative PCR experiments for *Itgam* (left) and *Cd69* (right) gene expression changes in HDAC7-deficient B cells. Data are given as mean ± SEM of values obtained in three independent experiments. (C) Cells were stained with a Mac-1 antibody, and the GFP-positive fractions were gated and the results plotted. (D) ChIP experiments showing the enrichment of H3Ac(K9/K14), H3K27me3, and H4K16Ac to the *Itgam*, *Ccl3*, *Cd28*, *Cd69*, and *Pax5* gene loci in bone marrow CD19$^+$ B cells from *Hdac7*$^{+/-}$ and *Hdac7*$^{fl/-}$ mice. Results are presented as the relative enrichment over input and are based on the results of three independent experiments. Data are given as mean ± SEM of values obtained in three independent experiments. Statistical significances were identified using the unpaired two-tailed Student's *t* test. *, $P < 0.05$; **, $P < 0.01$.

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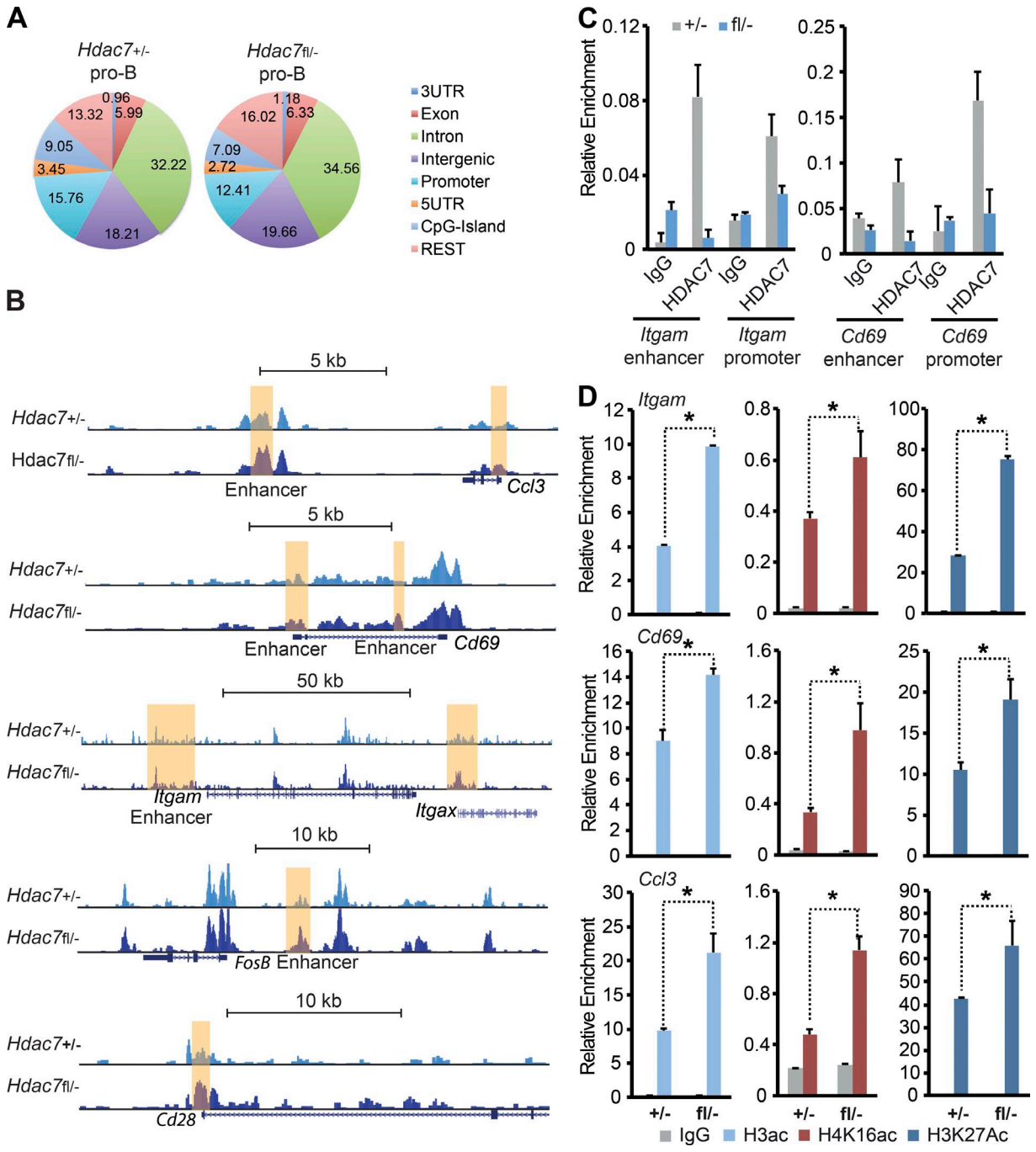

Figure 5. **HDAC7 is recruited at enhancers of its target genes, and its deficiency results in increased enrichment of histone active marks.** (A) Genomic distribution of H3Ac(K9/K14) enrichment in *Hdac7*$^{+/-}$ and *Hdac7*$^{fl/-}$ pro-B cells. (B) Examples for H3Ac(K9/K14) enrichment at enhancers and promoters of selected genes. (C) ChIP experiments showing the enrichment of HDAC7 to *Itgam* and *Cd69* promoters and enhancers in pro-B cells from *Hdac7*$^{+/-}$ and *Hdac7*$^{fl/-}$ mice. (D) H3Ac(K9/K14), H4K16Ac, and H3K27Ac enrichment to the *Itgam*, *Ccl3*, and *Cd69* enhancers in pro-B cells from *Hdac7*$^{+/-}$ and *Hdac7*$^{fl/-}$ mice. Results are presented as the relative enrichment over input and are based on the results of three independent experiments. (C and D) Data are given as mean ± SEM of values obtained in three (C) and four (D) independent experiments. Statistical significances were identified using the unpaired two-tailed Student's *t* test. *, $P < 0.05$.

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identity of B cells. Our findings represent a significant advance in our understanding of the transcriptional complexity underlying B cell generation.

MATERIALS AND METHODS

Mouse and animal care

Hdac7$^{fl/-}$ mice were previously described (Chang et al., 2006) and were provided by E. Olson (University of Texas Southwestern Medical Center, Dallas, TX). mb1-Cre$^{ki/+}$ mice were provided by M. Reth (Max Planck Institute of Immunology and Epigenetics, Freiburg, Germany). Experiments were performed with mice that were 4–6 wk of age. Animal housing and handling, and all procedures involving mice, were approved by the Bellvitge Biomedical Research Institute ethics committee, in accordance with Spanish national guidelines and regulations.

Flow cytometry and cell-sorting experiments

Cells were extracted from bone marrow and spleen. Isolated cells were incubated with Fc receptor–blocking antibody (BD) for 10 min at 4°C to reduce nonspecific staining. Cells were then stained with anti-B220 (PerCP-Cy5.5), anti-CD43 (APC), anti-IgM (FITC), anti-CD25 (APC), anti-IgD (PE), anti-Gr1 (PE), anti-CD11b (APC), anti-CD21 (FITC), anti-CD23 (PE), anti-CD93 (APC), and anti-CD3 (FITC; BD) for 30 min at 4°C in the dark. For intracellular IgHμ staining, cells were first stained with surface markers, permeabilized and fixed with buffer Perm/Wash (BD), and stained with anti-IgM mu-biotin antibody (Jackson ImmunoResearch Laboratories, Inc.) followed by incubation with streptavidin-PE. Cells were processed in a Gallios flow cytometer (Beckman Coulter), and the data were analyzed using FlowJo software (Tree Star). For cell-sorting experiments, bone marrow cells were incubated with Fc receptor–binding antibody and then stained with anti-B220 (PerCP-Cy5.5), anti-CD43 (APC), and anti-IgM (FITC; BD), under the same conditions. B220^{+}CD43^{+}IgM^{-} cells were isolated on a MoFlo sorter (Beckman Coulter).

Retroviral supernatant generation and cellular transduction

For retrovirus generation, the MSCV-GFP, MSCV-GFP-HDAC7, and MSCV-GFP-HDAC7(ΔMEF) plasmids were transfected into the packaged cell line Platinum-E, and supernatants were collected at 48–72 h after transfection. Purified B cells were spin infected, and 48 h later, GFP^{+} cells were either sorted or analyzed by flow cytometry.

Spleen section histology

Spleens from *Hdac7*$^{fl/-}$ mice and *Hdac7*$^{+/-}$ control mice were fixed in 4% formaldehyde, embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin. Samples were imaged under a Scope.A1 microscope (ZEISS).

Western blot

Western blot analysis was performed according to standard procedures. Western blots were developed with the Enhanced Chemiluminescence detection kit (GE Healthcare).

Proliferation and cell cycle assays

Cell proliferation and cell death were assessed by Hoechst and 7AAD staining, respectively. Cells were analyzed by flow cytometry using a Gallios flow cytometer.

Microarray experiments

Total RNA from sorted pro–B cells of *Hdac7*$^{+/-}$ and *Hdac7*$^{fl/-}$ mice was extracted by TRIzol and then purified. PCR-amplified RNAs were hybridized against mouse array chips (Mouse Genome 430 p.m. strip; Affymetrix) at the Institute for Research in Biomedicine Genomics Facility (Barcelona, Spain). Microarray analysis (GEO accession no. GSE66163) was performed as previously described (Barneda-Zahonero et al., 2013).

RT–quantitative PCR assays

Pro–B cells were purified by cell sorting. RNA was extracted by TRIzol extraction (QIAGEN), and cDNA was synthesized using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). RT–quantitative PCR was performed in triplicate using SYBR Green I Master (Roche). PCR reactions were run and analyzed using the LightCycler 480 Detection System (Roche).

ChIP assays

For ChIP assays, purified CD19^{+} B or pro–B cells from the bone marrow of *Hdac7*$^{+/-}$ and *Hdac7*$^{fl/-}$ mice were cross-linked with 1% formaldehyde and subjected to immunoprecipitation after sonication. ChIP experiments were performed using the LowCell# ChIP kit (Diagenode) according to the manufacturer's instructions. The following antibodies were used: anti-HDAC7 (Abcam), anti-MEF2C (Cell Signaling Technology), anti-H3Ac(K9/K14) (EMD Millipore), anti-H4K16Ac (Active Motif), anti-H3K27me3 (EMD Millipore), and anti-H3K27Ac (Abcam). Analyses were performed by real-time quantitative PCR. Data are represented as the ratio between the bound fraction of the HDAC7, MEF2C, and histone modification antibody relative to the input control.

ChIP-seq experiments

Sorted pro–B cells from the bone marrow of wild-type and *Hdac7*-deficient mice were cross-linked with 1% formaldehyde and sonicated using an S220 Focused ultrasonicator (Covaris). An anti-H3Ac(K9/K14) antibody (EMD Millipore) was used to perform ChIP from 100,000 pro–B cells using the True MicroChIP kit (Diagenode) according to the manufacturer's instructions. ChIP library construction and sequencing were performed according to standard procedures at the Centre for Genomic Regulation Genomics Core Facility (Barcelona, Spain). The quality of raw reads was checked using FastQC, and adapters were trimmed with a Skewer trimming tool (Bioinformatics) before mapping. Trimmed reads were mapped against the mouse genome (*Mus musculus*, genome version mm9) with Burrows-Wheeler Aligner–MEM, and duplicates were removed with the MarkDuplicates

tool in the Picard software. Data analysis, involving peak calling and annotation, was performed by qGenomics Laboratories using the Hypergeometric Optimization of Motif EnRichment v.4.8 suite. Variable-width peaks were called in the immunoprecipitated sample, using the input sample as a control, with the findPeaks function in the histone mode and a false discovery rate set to 0.001. Peaks were annotated using the annotatePeaks (Integrative Genomics) tool (SRA accession no. SRP076788).

Statistics

Statistical significance was determined by the two-tailed unpaired Student's *t* test.

Online supplemental material

Figs. S1 and S2 show the gating strategy used in the phenotypic characterization of wild-type and HDAC7-deficient mice. Table S1 shows a selection of up-regulated genes in HDAC7-deficient pro–B cells. Dataset S1 shows the list of genes up-regulated and down-regulated in HDAC7-deficient pro–B cells. Datasets S2, S3, and S4 show the ChIP-seq analysis of H3Ac(K9/K14) enrichment in wild-type and HDAC7-deficient pro–B cells (genomic distribution, annotated peaks, and enrichment at macrophage gene enhancers).

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SUPPLEMENTAL MATERIAL

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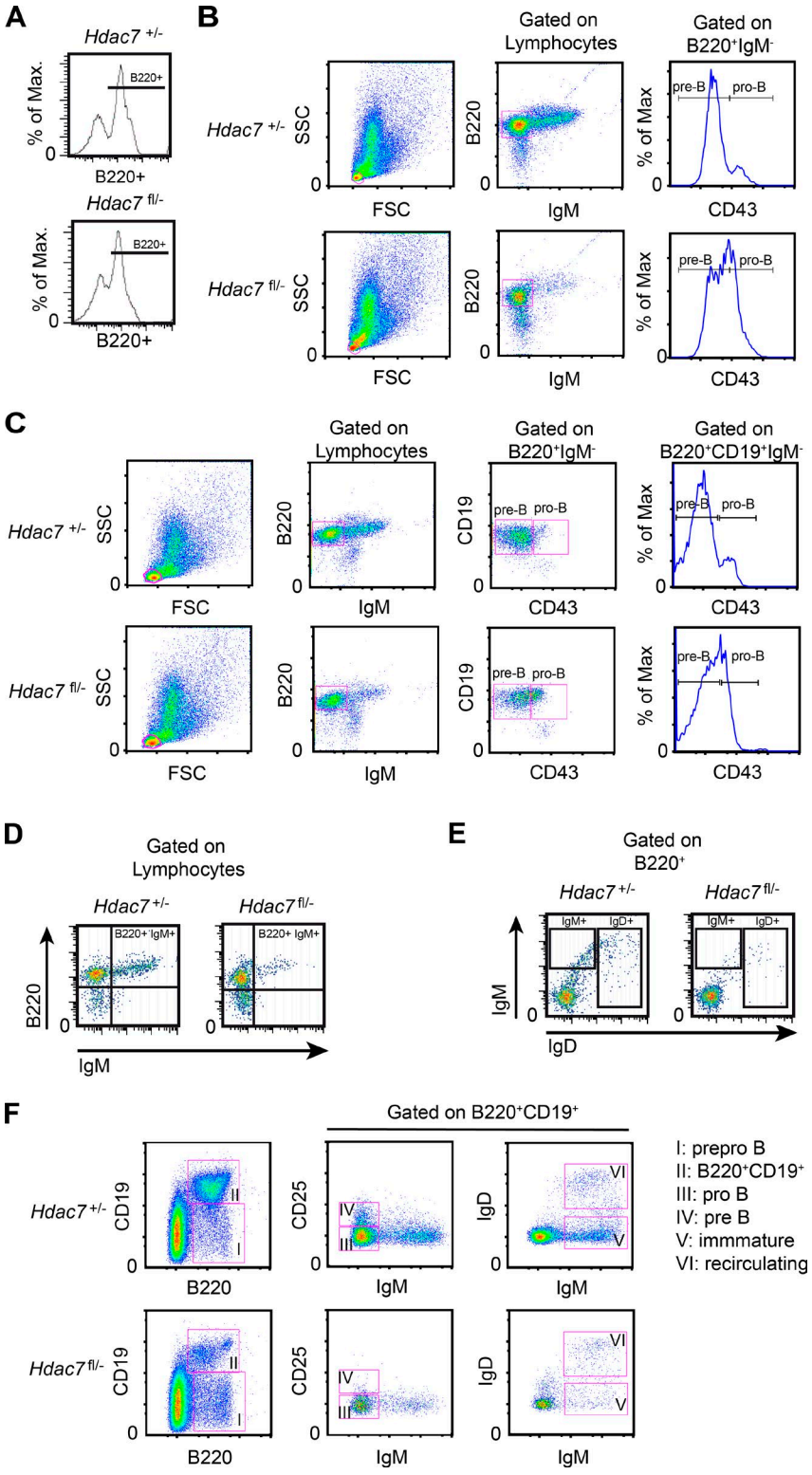

Figure S1. **Cell population and gating strategy of B cell subsets from bone marrow of wild-type and HDAC7-deficient mice.** (A) Representative FACS analyses are shown for $B220^+$ cells. (B) Gating strategy to distinguish pro-B ($B220^+IgM^-CD43^+$) and pre-B ($B220^+IgM^-CD43^-$) cells. (C) Gating strategy to distinguish pro-B ($B220^+IgM^-CD19^+CD43^+$) and pre-B ($B220^+IgM^-CD19^+CD43^-$) cells. FSC, forward scatter; SSC, side scatter. (D and E) Representative FACS analyses are shown for $B220^+IgM^+$ immature B cells (D) and $B220^+IgM^+IgD^+$ mature recirculating B cells (E). (F) Gating strategy to distinguish pro-B ($B220^+IgM^-CD19^+CD25^-$) and pre-B ($B220^+IgM^-CD19^+CD25^+$) cells.

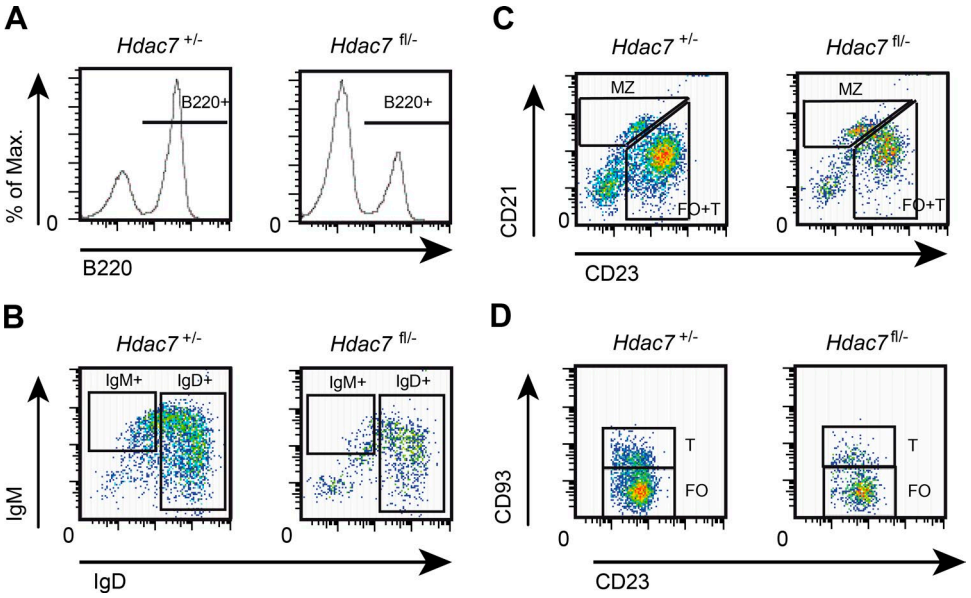

Figure S2. **B cell subsets from the spleen of wild-type and HDAC7-deficient mice.** Representative FACS analyses are shown for $B220^+$ cells (A), $B220^+IgM^+IgD^+$ mature B cells (B), $CD21^{bright}CD23^+$ marginal zone (MZ) B cells (C), and $CD21^+CD23^{bright}CD93^-$ follicular (FO) and $CD21^+CD23^{bright}CD93^+$ transitional (T) B cells (D).

Table S1. **Selection of up-regulated genes in HDAC7-deficient pro–B cells**

Transcription factors	Gene	Log2 fold change
AP-1 family	*FosB*	4.6
	Fos	3.2
	Jun	2.7
	JunB	1.8
	Jund	1.0
C/EBP family	*Cebpb*	0.7
	Cebpz	0.6
	Cebpd	0.7
Runx family	*Runx1*	0.5
	Runx2	1.2
	Runx3	0.7
Notch family	*Notch2*	0.7
	Notch3	0.7
Klf family	*Klf2*	1.6
	Klf4	2.0
	Klf6	2.18
	Klf7	0.84
Zeb family	*Zeb1*	1.7
	Zeb2	1.3
Others	*Foxp1*	1.4
	Egr1	3.5
	Nfat5	0.6
	Mef2a	0.7
	Crebzf	1.0
	Stat3	1.6
	Ikzf2	0.5
Myeloid-related genes		
	Ccl3	1.2
	Ccl4	1.2
	Ccr2	0.63
	Ccr6	0.52
	Ccr9	1.2
	Ccrl1	0.52
	Ccrl2	0.6
	Cd33	0.92
	Itgam	1.3
	Itgax	0.7
	Fcgr4	0.8
	Csf2ra	1.4
	Csf3r	0.8
	Cr1l	0.7
T cell–related genes		
	Cd28	0.77
	Nfat5	0.85
	Lck	0.8
	Sla2	0.84
Chromatin-associated factors		
	Brd1	0.65
	Brd8	1.0
	Cbx4	0.6
	Chd2	1.0
	Chd6	0.7
	Chd8	1.0
	Ezh1	0.6
	Kdm2a	1.1
	Kdm5a	0.6
	Kdm5c	1.42
	Kdm6a	1.66
	Kdm6b	1.8
	Mll1	0.51
	Mll2	1.0
	Mll3	0.6
	Mll5	0.72
	Mllt10	0.6
	Mllt3	0.55
	Setd2	0.74
	Setd3	1.0
	Setd6	0.6
	Setd8	1.0
	Smarca2	0.71
	Smarce1	1.22
	Suv39h2	0.56
	Suv420h1	0.8
	Tet2	1.0
	Jhdm1d	1.0
	Jmjd1c	1.52
	Jmjd6	0.8
	Hdac9	0.82
	Mbd1	0.92
	Mbd6	0.82
	Hdac9	0.82
Inflammatory response genes		
	Il10ra	0.75
	Il12rb2	0.85
	Il17rb	0.56
	Il1f9	0.76
	Il1r2	1.1
	Il1rn	0.55
	Ifi203	0.55
	Ifi204	1.13
	Ifng	1.6
	Tnfaip2	1.0
	Tnfaip3	1.4
Intracellular signal cascade		
MAPK pathway	*Map3k2*	0.85
	Map3k8	1.34
	Map4k4	0.95
	Map4k5	1.0
	Mapk14	0.6
	Mapk1ip1	0.72
	Mapk1ip1l	1.6
	Mapk6	1.6
	Mapk8ip3	0.80
NF-κB family	*Nfkbia*	0.92
	Nfkbiz	2.3
	Nfrkb	0.66
	Ikbkb	0.72
	Ikbkg	0.7
PKC family	*Prkca*	0.7
	Prkcb	0.7
	Prkcc	0.9
	Prkcd	0.8
	Prkd3	0.6
Protein ubiquitination		
	Cblb	1.2
	Birc3	1.2
	Birc6	1.8
	Ube2j2	1.3
	Ube2b	1.4
	Ubr2	0.6
	Ube2i	0.8
	Mdm2	1.4
	Malt1	1.1
	Cbl	0.8
	Cnot1	0.93
	Cnot2	0.8
	Cnot4	1.0
	Cnot7	0.83
Cell cycle–related genes		
	Bcl2	1.1
	E2f5	1.0
	Gadd45a	1.39
	Gadd45b	0.51
	Gadd45g	0.7
	Pten	1.0
	Trp53	1.1
	Cdc40	0.5
	Cdc42	1.23
	Cdk13	0.7
	Cdk16	0.73
	Ccnc	0.62
	Ccnd1	0.9
	Ccni	0.7
	Ccnl2	0.61
	Ccnt2	0.74

Dataset S1 is an Excel file showing the list of genes up-regulated and down-regulated in HDAC7-deficient pro–B cells.

Dataset S2 is an Excel file showing the genomic distribution of H3Ac(K9/K14) in wild-type and HDAC7-deficient pro–B cells.

Dataset S3 is an Excel file showing annotated significant peaks for H3Ac(K9/K14) enrichment in wild-type and HDAC7-deficient pro–B cells.

Dataset S4 is an Excel file showing significant peaks for H3Ac(K9/K14) enrichment at enhancers of macrophage genes in wild-type and HDAC7-deficient pro–B cells.

ARTICLE 2

“Unveiling a novel function of HDAC7 in preserving the identity of B cell progenitors through TET2 regulation”

“Unveiling a novel function of HDAC7 in preserving the identity of B cell progenitors through TET2 regulation"

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Class IIa histone deacetylases (HDAC4, 5, 7 and 9) are transcriptional repressors that play crucial roles in differentiation and developmental processes. In the hematopoietic system, HDAC7 is expressed in B and T lymphocytes as well as in NK cells. In B cells, HDAC7 is involved in the repression of genes from alternative cell types such as macrophages and T cells [1–6]. Here we found that HDAC7 is necessary to maintain physiologically low levels of TET2 in B cell progenitors. TET2 is a methylcytosine dioxygenase that converts 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5hmC), resulting in DNA demethylation and directing gene activation [7]. Our results indicate that HDAC7 induces gene silencing not only by direct recruitment to target genes, but also by regulating the expression of additional epigenetic players. Our data reveal an unexpected role for a class IIa HDAC, HDAC7, in controlling gene expression through DNA methylation-related mechanisms.

B lymphocyte generation is a complex process that requires tight regulation. Early B cell development takes place in the bone marrow where common lymphoid progenitors (CLPs) commit to the B cell lineage through the generation of B cell progenitors (pro-B cells). These pro-B cells further differentiate into B cell precursors (pre-B cells), which in turn give rise to immature B cells that migrate to secondary lymphoid organs such as the spleen and the lymph nodes, where they terminally differentiate into plasma cells and memory B cells. Every cellular transition and differentiation step is characterized by the activation of a new lineage-specific genetic program and the repression of the previous one [8]. This dynamic process, in which cells progressively change their genetic "identity", is guided by networks of transcription factors (TFs) which, in addition to being responsible for the activation of B cell-specific genes, are also involved in the repression of alternative lineage genes [8,9]. There is also a close relationship between transcriptional regulators and dynamic changes in DNA methylation during B cell development [9,10]. Early differentiation stages generally experience enhancer demethylation, which is associated with up-regulation of important B lymphocyte TFs, and affects multiple genes involved in B cell biology [9]. In mammals, DNA demethylation depends on Ten-Eleven

Translocation (TET) enzyme action. This protein family comprises TET1, TET2 and TET3, which convert 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC), leading to DNA demethylation and consequent gene expression [11].

TET2 appears to play crucial roles during hematopoiesis [12–14]. Although broadly expressed within the hematopoietic system, myeloid cells express higher levels of TET2 compared with lymphoid cell populations [15,16]. Accordingly, TET2 has been shown to be a critical enzyme for correct myelopoiesis [15,16]. However, despite lower levels being present than in myeloid cell types, TET2, with the necessary cooperation with TET3, has also been reported to play a role in B cell development [17–19]. Examination of microarray data from the Immunological Genome Project Database (Immgen) (http://www.immgen.org/) confirmed that *Tet2* is expressed at much higher levels in myeloid cells than in lymphocyte populations (Fig. 1a). In addition, analysis of RNA-seq data from Immgen confirmed that *Tet2* is more strongly expressed in macrophages than in $CD19^+$ B cells (Supplementary Fig. 1a). As expected, *Pax5* and *Itgam* are specifically expressed in B lymphocytes and macrophages, respectively (Supplementary Fig. 1a), which suggests that different physiological levels of TET2 protein may have a function in the hematopoietic system. How and why is TET2 expressed at different levels in myeloid cells than in lymphocytes? We have recently shown that HDAC7 is a crucial transcriptional repressor during early B cell development [5]. HDAC7-deficient pro-B cells present lineage promiscuity and express many genes from alternative cell types, such as macrophages and T cells [5]. We observed up-regulation of *Tet2* in our published microarray data set for wild-type ($Hdac7^{+/}$) mice and their HDAC7-deficient ($Hdac7^{fl/-}$) counterparts. Here, using our mouse model for HDAC7 deficiency in pro-B cells, we confirmed by RT-qPCR experiments that the absence of HDAC7 from pro-B cells leads to a significant increase in the expression of *Tet2* (Fig. 1b), reaching similar levels to those in macrophage cells (Supplementary Fig. 1b). Graf and colleagues previously demonstrated that *Tet2* is up-regulated during the transdifferentiation of pre-B cells into macrophages facilitating the de-repression of myeloid genes [16], confirming its higher level of expression in myeloid cell types. Using

this system, we have previously reported that HDAC7 is down-regulated during the conversion of pre-B cells into macrophages and that its ectopic expression interferes with cellular transdifferentiation [4]. HDAC7 exogenous expression blocks the up-regulation of *Tet2* during cellular conversion (Fig. 1c). Forced expression of HDAC7 does not interfere with the down-regulation of *Pax5* during cellular conversion (Fig. 1c). To determine whether HDAC7 is involved in *Tet2* gene silencing, we performed western blot experiments with purified bone marrow $CD19^+$ B cells from wild-type and HDAC7-deficient mice. We used the $CD19^-$ cell population, which includes myeloid cells such as macrophages, as an experimental control for high levels of TET2. We confirmed that the absence of HDAC7 from B cells leads to an increase in TET2 protein levels (Fig. 1d). To corroborate the potential repressive action of HDAC7 on *Tet2* gene expression, we took advantage of the "modules and regulators" interactive tool in the Immgen database to search for putative positive and negative *Tet2* regulators. Remarkably, the analysis revealed that HDAC7 might be a unique and potential negative regulator of *Tet2* expression (Figure 1d). Together, these findings indicate that HDAC7 is involved in *Tet2* gene silencing and that it may be essential for maintaining its physiologically low levels in B cell progenitors compared with myeloid cells.

We had previously demonstrated that the HDAC7-MEF2C interaction is necessary for HDAC7 to repress its target genes in pro-B cells [5], and *Tet2* has recently been reported to be an MEF2C direct target gene in pro-B cells [20]. To test whether *Tet2* is also a direct HDAC7 target gene in pro-B cells we next performed chromatin immunoprecipitation (ChIP) assays, in which HDAC7 proved to be recruited to a putative MEF2 binding site located at the promoter of the *Tet2* gene (Fig. 2a). We also observed HDAC7 recruitment at a previously described *Tet2* enhancer [21] (Fig. 2a). Our published ChIP-seq analysis revealed that the absence of HDAC7 from pro-B cells was associated with a moderate but significant increase of the active histone marks, H3K9/K14ac, at the promoter and enhancers of its target genes [5]. As further evidence that *Tet2* may be a repressed HDAC7 gene, ChIP-seq analysis revealed an increase in H3K9/K14ac enrichment at both the *Tet2* promoter and enhancer

in HDAC7-deficient pro-B cells (Fig. 2b). We next determined by ChIP-qPCR that HDAC7 absence from pro-B cells causes a significant increase in the enrichment of the active histone marks H3K9/K14ac and H3K27ac and decreases the enrichment of the repressive histone marks H3K27me3 and H3K9me3 at both *Tet2* gene loci (Fig. 2c). Collectively, these data demonstrate that *Tet2* is an HDAC7 direct target gene in pro-B cells.

Our data suggest that HDAC7 may have a role in 5-hmC DNA, which would represent a novel and unprecedented mechanism by which a class IIa HDAC mediates gene silencing to establish proper B cell identity and function. To investigate this hypothesis, we first determined the global levels of hydroxymethylated DNA in wild-type and HDAC7-deficient pro-B cells. ELISA assays revealed that the absence of HDAC7 from pro-B cells led to a significant increase in the global levels of 5-hmC (Fig. 3a). Next, we performed 5-hmC DNA immunoprecipitation (hMeDIP) followed by next-generation sequencing (hMeDIP-seq) in pro-B cells purified from *Hdac7*$^{fl/-}$ mice and their *Hdac7*$^{+/-}$ control littermates. The total frequencies of 5-hmC peaks in wild-type and HDAC7-deficient pro-B cells were similar, but their global intensity was higher in pro-B cells from *Hdac7*$^{fl/-}$ mice, in concordance with the results obtained from ELISA experiments (Fig. 3b and Supplementary Fig. 3a,b). The genomic distribution of the peaks did not differ significantly between the two conditions, and most peaks were located in intergenic regions, introns and within LINE-1 elements (Fig. 3c). Next, we looked for differential peaks in 5-hmC DNA enrichment between wild-type and HDAC7-deficient pro-B cells. We found that HDAC7 deficiency from pro-B cells resulted in an increase of 10,000 and a decrease in 12,000 5-hmC peaks. To gain functional insights into the genes associated with 5-hmC peaks that were more highly enriched in the absence of HDAC7, we performed gene set enrichment analysis based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and the gene ontology (GO) categories corresponding to Biological Processes. We found an increase in the enrichment of 5-hmC in lineage-inappropriate genes, such as *Jun* and *Fosl2*, in HDAC7-deficient pro-B cells compared with wild-type cells (Fig. 3d and Supplementary Fig. 3c). These results were

confirmed by hmeDIP-qPCR experiments (Fig. 3e). Accordingly, the increased enrichment at both genes that was due to 5-hmC was correlated with increased expression levels. A heatmap derived from public data from Immgen and RT-qPCR experiments confirmed that myeloid and T cell genes were upregulated in HDAC7-deficient pro-B cells and macrophages relative to wild-type pro-B cells (Fig. 3f,g). Finally, we performed a motif enrichment analysis to determine whether HDAC7 deficiency could have produced an alteration in chromatin positioning and could change the occupancy of different TFs in the genome. Although we found no differences associated with HDAC7, we did note enrichment of relevant TFs in the hematopoietic system such as PU.1 (Fig. 3h). The occupancy of PU.1 under both conditions is consistent with it being essential for lymphoid and myeloid lineages. These results highlight the importance of HDAC7's role in silencing inappropriate lineage genes and reveal its influence on 5-hmC DNA in B cells by regulating an important epigenetic modulator such as TET2.

Our genome-wide approach revealed increased enrichment in 5-hmC DNA in a significant number of repetitive genome regions (LINE-1 elements) in the absence of HDAC7. According to the average signal from all the peaks obtained, the signal intensity of 5-hmC peaks associated with LINE-1 elements in HDAC7-deficient pro-B cells was higher than in their wild-type counterparts (Fig. 4a). Previous published data revealed that TET1 and TET2 are recruited at the 5'UTR of young LINE-1 elements in embryonic stem cells [22]. Regions with differential peaks were validated by hMeDIP-qPCR assays, confirming that the absence of HDAC7 from pro-B cells results in higher levels of 5-hmC in several LINE-1 elements (Fig. 4 b,c). Although 5-hmC does not always involve an increase in gene expression [23], we observed a clear tendency towards stronger expression of L1 transcripts in HDAC7-deficient pro-B cells (Fig. 4e). Given that aberrant expression of LINE-1 elements has been associated with chromatin instability, these results suggest that HDAC7 might be required to preserve chromatin integrity by mediating the silenced status of LINE-1 elements. This is consistent with our previous findings demonstrating that HDAC7-deficient pro-B and pre-B cells have a higher cell death rate than

do wild-type B cell populations [5,24]. It is of note that recent studies have shown that the tight regulation of TET2 activity to be essential for correct maintenance of genome stability, since TET2 deficiency produces defects in DNA damage response and its overexpression produces chromosome instability and aneuploidy due to a collapse in BER activity [25,26]. Thus, HDAC7 might have a role in preserving genome stability and integrity in B cells by restricting the levels of *Tet2* expression and, consequently, maintaining physiological levels of 5-hmC at lineage-inappropriate genes and LINE-1 retrotransposons.

Further examination of our hMeDIP-seq data revealed differential 5-hmC enrichment at miRNA-associated loci. Coincident with peaks located in LINE-1 associated regions, the coverage depth of 5-hmC peaks at miRNAs in HDAC7-deficient pro-B cells was higher than in control pro-B cells (Fig. 5a). We found that several miRNAs involved in leukemia and lymphoma as well as in myeloid differentiation were more enriched in 5-hmC in pro-B cells from *Hdac7*$^{fl/-}$ mice, such as miR-125b and miR-148a (Fig. 5b). We also observed miRNAs involved in B cell differentiation with an increased 5-hmC mark in wild-type pro-B cells, such as miR-28a (Fig. 5a). We performed RT-qPCR experiments to establish whether 5-hmC and miRNA expression were correlated. Interestingly, mir-125b and miR-28a were up-regulated and down-regulated in HDAC7-deficient pro-B cells, respectively (Fig. 5c). MiR-125b-5p is known to be more abundant in macrophages than in other immune cell types and up-regulated in several types of leukemia such as acute myeloid leukemia (AML) [27,28]. MiR-28a regulates proliferation of B cells and its down-regulation is involved in lymphomagenesis. To examine the potential role of HDAC7 in regulating the expression of miRNAs, we performed miRNA profiling using a quantitative PCR-based panel containing over 375 miRNAs (miRCURY LNA^{TM} microRNA Array (Exiqon)) in wild-type and HDAC7-deficient pro-B cells. We found 25 miRNAs whose levels of expression differed significantly between wild-type and HDAC7-deficient pro-B cells (Fig. 5d). MiRNAs represented in the heatmap, and others involved in the hematopoietic system and related disorders, were validated by RT-qPCR (Fig. 5e). Few miRNAs are highly specifically expressed in an

individual tissue or cell type [29], such as miR-142 and miR-181, in the case of hematopoietic cells. Although they are not represented in the heatmap due to statistical constraints in the analysis, we observed that both miRNAs, whose role is essential for B maturation and differentiation, were down-regulated in HDAC7-deficient pro-B cells (Fig. 5e). Additionally, miR-150, which is down-regulated in pro-B cells from *Hdac*7-null mice, is related to B-cell development and performs tumor-suppressor functions in AML cells [30]. Among the miRNAs that are up-regulated under HDAC7-deficient conditions, we found miR-125b-5p, miR-126, miR-29b, miR-34a and miR-99a. Previous studies indicated that miR-126 is down-regulated in lymphoid cells, which is consistent with previous results [31,32]. Moreover, miR-29b is known to be activated by C/EBPα and to repress *Tet2* expression, which concords with C/EBPα and *Tet2* up-regulation when HDAC7 is deficient [33]. Furthermore, miR-34a has been shown to be strongly expressed in myeloid cells, and its constitutive expression in B cells blocks the pro-B to pre-B cell transition [34]. Finally, some members of the miR-99 family, such as miR-99b, have been reported to be abundant in macrophages, neutrophils and monocytes. Here, we observed that another member of the family related to leukemic stem cells, miR-99a, was up-regulated in HDAC7-deficient pro-B cells [35]. Considered as a whole, our data indicate that, through the regulation of *Tet2*, HDAC7 controls the expression levels of crucial miRNAs of the immune system.

Our findings represent a significant step forward in our understanding of how B cells acquire their genetic identity, from two different perspectives. First, we have identified HDAC7 as the specific transcriptional repressor controlling TET2 activity, which it achieves by fine-tuning its physiological expression levels in pro-B cells. This may be the mechanistic explanation for the different TET2 expression levels observed in myeloid and lymphoid cells. Second, our results reveal an unexpected role for HDAC7 in controlling proper DNA 5-hydroxymethylation status and expression of lineage-inappropriate or functionally inappropriate genes, microRNAs and LINE-1 elements in pro-B cells. We suspect that other members of the class IIa HDAC subfamily may have similar functions in the

cellular differentiation and developmental processes in which they participate (e.g., skeletal and cardiac muscle, bone formation and brain).

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Author contributions

A.A., O.C., A.M., O.d.B. conducted experiments and analyzed data, M.V-C. performed bioinformatics analyses, T.G. provided crucial conceptual input, A.A. and M.P. designed the study and wrote the manuscript. M.P. supervised the entire project.

Competing interests statement

The authors declare no competing financial interests.

Materials and Methods

Mouse and animal care

Hdac7$^{fl/-}$ mice have been previously described and were kindly provided by Dr. Eric Olson (UT Southwestern Medical Center, Dallas, TX, USA). *mb1*-Cre$^{ki/+}$ mice were kindly provided by Dr. Michael Reth (Max Planck Institute of Immunology and Epigenetics, Freiburg, Germany). Experiments were performed with 4-6-week-old mice. Animal housing and handling, and all procedures involving mice, were approved by the Bellvitge Biomedical Research Institute (IDIBELL) ethics committee, in accordance with Spanish national guidelines and regulations.

Cell culture and β-estradiol treatment

C10 cells (transduced with a MSCVGFP- C/EBPα retroviral vector) were cultured and treated as previously described [4].

Flow cytometry and cell-sorting experiments

Cells were extracted from bone marrow and spleen. Isolated cells were incubated with Fc-receptor-blocking antibody (BD Bioscience) for 10 min at 4°C to reduce non-specific staining. Cells were then stained with anti-B220 (PerCP-Cy5.5), anti-CD43 (APC), anti-IgM (FITC), anti-CD19 (PE) and anti-Cd11b (V450) (BD Bioscience) for 30 min at 4°C in the dark. Cells were processed in a Gallios flow cytometer (Beckman-Coulter, Inc.) and the data analyzed using FlowJo software (Tree Star, Inc.). For cell-sorting experiments, bone marrow cells were incubated with Fc-receptor binding antibody and then stained with anti-CD19 (PE), anti-B220 (PerCP-Cy5.5), anti-CD43 (APC) and anti-IgM (FITC) (BD Bioscience) under the same conditions. Pro-B cells ($CD19^+B220^+CD43^+IgM^-$) were isolated in a BD FACSAria™ Fusion cell sorter (BD Biosciences). The gating strategy used is illustrated in Supplementary Fig. 2.

Western blot

$CD19^+$ and $CD19^-$ cell populations were obtained by magnetic cell isolation using Miltenyi MACS manual separators. Western blot analysis was performed according to standard procedures. Blots were developed with the ECL detection kit (Amersham Biosciences). The antibodies used were anti-HDAC7 (Santa Cruz), anti-TET2 (Abcam) and anti-β-actin (Abcam).

RT-qPCR assays

Pro-B cells were purified by cell sorting. RNA was extracted with an RNeasy Mini kit (Qiagen) and cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (AB Applied Biosystems). RT-qPCR was performed in triplicate using SYBR Green I Master (Roche). PCR reactions were run and analyzed using the LightCycler 480 Detection System (Roche). Primer sequences are available from the authors upon request.

Chromatin immunoprecipitation assays

For chromatin immunoprecipitation (ChIP) assays, purified pro-B cells from the bone marrow of *Hdac7*$^{+/-}$ and *Hdac7*$^{fl/-}$ mice were crosslinked with 1% formaldehyde and subjected to immunoprecipitation after sonication. ChIP experiments were performed using the LowCell# ChIP kit (Diagenode) according to the manufacturer's instructions. The antibodies used were anti-HDAC7 (Abcam), anti-H3(K9/K14)Ac (Millipore), anti-K27H3K27me3 (Millipore), anti-H3K27Ac (Abcam) and anti-H3k9me3 (Abcam). Real-time quantitative PCR was performed and the results analyzed. Data are presented as the ratio between the bound fraction of the HDAC7 and histone modification antibody relative to the input control.

ChIP-sequencing experiments

Data were extracted from ChIP-sequencing experiments, as described elsewhere [5]. Data are available under accession code: GEO: ChIP-seq data, SRA submission SUB1614653.

Quantification of global 5-hydroxymethylation levels

To quantify 5-hmC, a Quest 5-hmC DNA ELISA kit (Zymo Research) was used according to the manufacturer's protocol. Briefly, the bottom of the well was coated with anti-5-hmC polyclonal antibody (pAb), and 100 ng of denatured genomic DNA was added. Anti-DNA HRP antibody and HRP developer (3, 3', 5, 5'-tetramethylbenzidine (TMB)) were applied to detect the DNA bound to the anti-5-hmC pAb. After 20-30 min of incubation, the color reaction was stopped by the addition of sulfuric acid. The resulting color was analyzed at 450 nm. The percentage of 5-hmC DNA was estimated from linear regression.

hMeDIP-qPCR experiments

Purified genomic DNA (1 µg) from wild-type and HDAC7-deficient pro-B cells was sonicated to obtain fragments of 300-400 bp. Fragmented DNA was incubated with 1 µg anti-5-hmC (Active Motif, 39769) and 20 µl of Dynabeads G (Life Technologies) for 16 h at 4ºC. Real-time quantitative PCR was

performed and the results analyzed. Data are presented as the ratio of the enrichment of 5-hmC relative to the input control.

hMeDIP sequencing experiments

Purified genomic DNA (1 µg) from wild-type and HDAC7-deficient pro-B cells was sonicated to obtain fragments of 300-400 bp. Adaptor ligations were performed and libraries constructed by qGenomics Laboratories (Barcelona, Spain). 1 µg anti-5-hmC (Active Motif, 39769) was incubated with 20 µl of Dynabeads G (Life Technologies) for 2 h at 4ºC. Fragmented DNA was incubated with Dynabeads and antibody for 16 h at 4ºC. DNA from immunocomplexes was purified with the QIAquick MinElute kit (Qiagen). Amplified libraries were constructed and sequenced at qGenomics Laboratories (Barcelona, Spain). Fastq data were obtained with Trim Galore-0.4.2 and Cutadapt-1.6. Reads were mapped with bwa-0.7.12. Sorting Sam to Bam was carried out with Picard-2.8.0 SortSam and duplicates were removed with Picard-2.8.0 MarkDuplicates. Peak calling was performed using MACS2 software with the default narrow option and a threshold of p=0.001. DESEQ analysis was then used to define peaks and perform quantitative analyses. The Diffbind-2.6.6 R package was used for differential binding analysis. Differential enrichment was defined by a threshold value of p=0.005 and >1FC difference in KO relative to WT samples. Motif enrichment was analyzed and peak depth quantified with HOMER software.

Expression profiling of microRNAs

We used miRCURY LNA™ Universal RT microRNA PCR System (Exiqon) to determine miRNA expression profiles. The miRNA annotation of mirBase version 20.0 was used. Single-stranded cDNA was synthesized by reverse transcription of 8 µL of RNA, using the universal cDNA Synthesis Kit II (Exiqon). Diluted cDNA was mixed with ExiLENT SYBR® Green master mix (Exiqon), and quantitative PCR was performed using the Roche LightCycler® 480 RealTime PCR system (Roche).

Statistical analysis

Data were analyzed by Student's two-tailed unpaired t-test using GraphPad Prism.

Figure legends

Fig. 1: HDAC7 regulates the expression levels of *Tet2* in pro-B cells. a, *Tet2* expression profile in hematopoietic cells subsets. Data were obtained from the Immunological Genome Project (Immgen) database. **b**, RT-qPCR experiments for gene expression changes for *Tet2* gene in wild-type and HDAC7-deficient pro-B cells. **c**, RT-qPCR experiments for gene expression changes for *Tet2* and *Pax5* in the absence or presence of HDAC7 during the conversion of pre-B cells into macrophage-like cells. Data from **b,c** are represented as the mean ± standard error of the mean (SEM) of three independent experiments. Statistical significance was identified using the unpaired two-tailed Student's t-test. *p<0.05. **d**, Western blot analysis of the expression of TET2 and HDAC7 in wild-type and HDAC7-deficient B lymphocytes ($CD19^+$). Non-B lymphoid cells ($CD19^-$) were used as a control of TET2 expression. **e**, Heatmap showing the expression of potential *Tet2* gene regulators in hematopoietic cell subsets. Data were obtained from the Immgen database.

Fig. 2: *Tet2* is an HDAC7 direct target gene in pro-B cells. a, Chromatin immunoprecipitation (ChIP) experiments showing the recruitment of HDAC7 to *Tet2* promoter and enhancer. Results are presented as the relative enrichment over input and are based on the results of three independent experiments. Statistical significance was identified using Student's unpaired two-tailed t-test. *p<0.05. **b**, ChIP-seq peaks for H3(K9/K14)ac enrichment at the enhancer and promoter of the *Tet2* gene in wild-type and HDAC7-deficient pro-B cells. Data were taken from SRA accession no. SRP07. **c,** ChIP-qPCR experiments showing the enrichment of active histone marks (H3K9/k14ac and H3K27ac) and repressive histone marks (H3K27me3 and H3K9me3) to the promoter and enhancer of *Tet2* in pro-B cells from $Hdac7^{+/-}$ and $Hdac7^{fl/-}$ mice. Results are presented and analyzed as in **a**, and are based on the results of four independent experiments. *p<0.05, **p<0.01.

Fig. 3: HDAC7 regulates the levels of DNA 5-hydroxymethylation in pro-B cells. a, Global levels of DNA 5-hydroxymethylation (5-hmC) in pro-B cells from wild-type and HDAC7-deficient mice were tested by ELISA assays. Data are presented as the mean ± SEM of four independent experiments

and statistical significance was identified using Student's unpaired two-tailed t-test. *p<0.05. **b**, 5-hmC coverage depth (per base pair per peak per 10 million mapped reads) of 5-hmC peaks (−2 kb to +2 kb) in wild-type and HDAC7-deficient pro-B cells. **c**, Genomic distribution of 5-hmC enrichment in *Hdac7*$^{+/-}$ and *Hdac7*$^{fl/-}$ pro-B cells. **d**, Genome browser snapshot of the *Jun* gene showing signal for 5-hmC in wild-type and HDAC7-deficient pro-B cells. Genome browser snapshot of RNA-seq and ATAC-seq data of B cells and macrophages (MF) were obtained from the Immgen database. **e,** hmeDIP-qPCR experiments showing the enrichment of 5-hmC in wild-type and HDAC7-deficient pro-B cells in myeloid and T cell genes such as *Fosl2* and *Jun*. Data are presented as the mean ± SEM of three independent experiments. The statistical analysis was performed as in **a**. *p<0.05, **p<0.01. **f**, RT-qPCR experiments for myeloid genes expression in wild-type and HDAC7-deficient pro-B cells. Data are presented and analyzed as in **a,c. g**, Heatmap of color-coded expression levels of selected genes between pro-B cells and macrophages. Data were obtained from RNA-seq experiments available in MyGeneSet browser of the Immgen database. **h**, Most relevant TF binding motifs in control (grey) and HDAC7-deficient (blue) pro-B cells based on their enrichment in both genomes using the HOMER database of known motifs.

Fig. 4: HDAC7 regulates DNA 5-hydroxymethylation at LINE-1 elements in pro-B cells. a, Example of 5-hmC enrichment in two young retrotransposons (L1) from peaks detected in hMeDIP-seq experiments. The peak location found in the hMeDIP-seq analysis is located in the yellow-shaded rectangle **b**, hMeDIP-qPCR experiments showing the enrichment of 5-hmC in wild-type and HDAC7-deficient pro-B cells in L1 retrotransposable elements. Data are presented as the mean ± SEM of three independent experiments and statistical significance was identified using Student's unpaired two-tailed t-test. *p<0.05, **p<0.01. **c**, 5-hmC coverage depth (per base pair per peak per 10 million mapped reads) of 5-hmC peaks located in L1 elements (−2 kb to +2 kb) in wild-type and HDAC7-deficient pro-B cells. **d**, Gene ontology (GO) analysis of 5-hmC-enriched regions in HDAC7-deficient pro-B

cells associated with LINE-1 elements. **e**, RT-qPCR experiments to examine the expression of L1 elements in pro-B cells from *Hdac7*$^{+/-}$ and *Hdac7*$^{fl/-}$ mice. Data are presented and analyzed as in **b**.

Fig. 5: HDAC7 regulates microRNA expression in pro-B cells

a, Example of 5-hmC enrichment peaks at miR-125b from hMeDIP-seq experiments. **b**, RT-qPCR experiments of 5-hmC enriched miRNAs in HDAC7-deficient pro-B cells compared with wild-type cells. The levels of U6 RNA were used for normalization. Data are presented as the mean ± SEM of three independent experiments and statistical significance was identified using Student's unpaired two-tailed t-test. *p<0.05. **c**, 5-hmC coverage depth (per base pair per peak per 10 million mapped reads) of 5-hmC peaks located in microRNAs (−2 kb to +2 kb) in wild-type and HDAC7-deficient pro-B cells **d**, Heatmap of the differential expression of miRNAs for two *Hdac7*$^{+/-}$ vs. *Hdac7*$^{fl/-}$ replicates. Only those with a greater or less than two-fold difference between samples are included. **e**, RT-qPCR analysis of selected microRNAs from miRCURY LNA™ Universal RT panel in wild-type and HDAC7-deficient pro-B cells. Data are presented and analyzed as in **b.**

Supplementary Fig. 1:

a) RNA-seq data obtained from Immgen showing the expression peaks of *Tet2* in pro-B cells and macrophages. *Pax5* and *Itgam* expression peaks are shown as the control of specific genes of lymphoid and myeloid lineages, respectively. **b**) RT-qPCR experiments showing expression of Tet family members (1-3) in bone marrow wild-type and HDAC7-deficient pro-B cells and Cd11b^{+} cells. Data are presented as the mean ± SEM of three independent experiments and statistical significance was identified using Student's unpaired two-tailed t-test. *p<0.05

Supplementary Fig. 2:

Flow cytometry plots showing the gating strategy to sort pro-B (IgM^{-}, CD19^{+}, B220^{-}, CD43^{+}) cells from the bone marrow of control and *Hdac7* $^{fl/-}$ conditional mice.

Supplementary Fig. 3:

a) Heatmaps of replicates from hMeDIP sequencing experiments. **b**), Coverage depth of 5-hmC peaks signal in intergenic and promoter (TSS) regions from hMeDIP-sequencing experiments. (**c**), Genome browser snapshot of 5-hmC peaks at *FosL2* promoter in wild-type and HDAC7-deficient pro-B cells.

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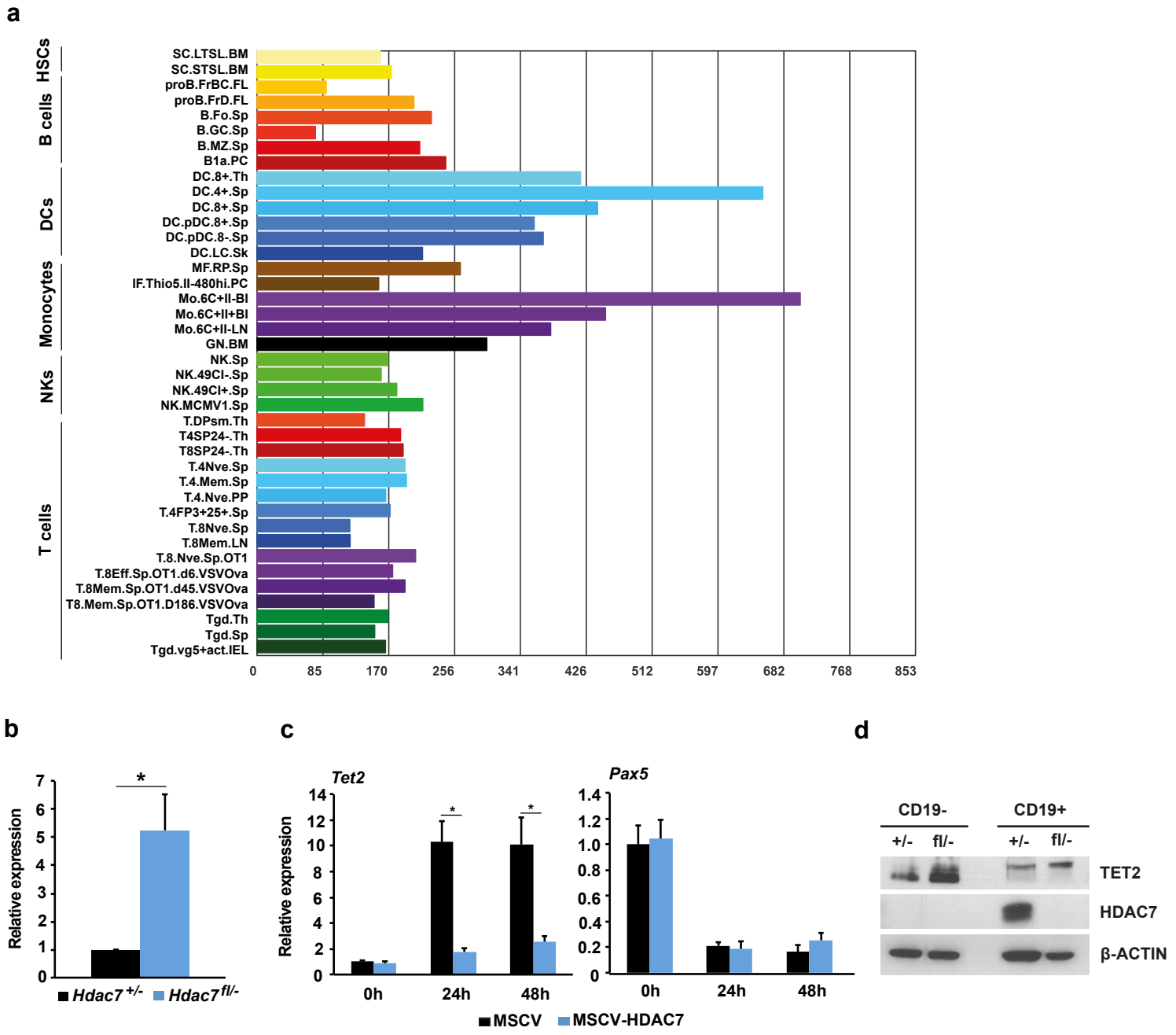
Figure 1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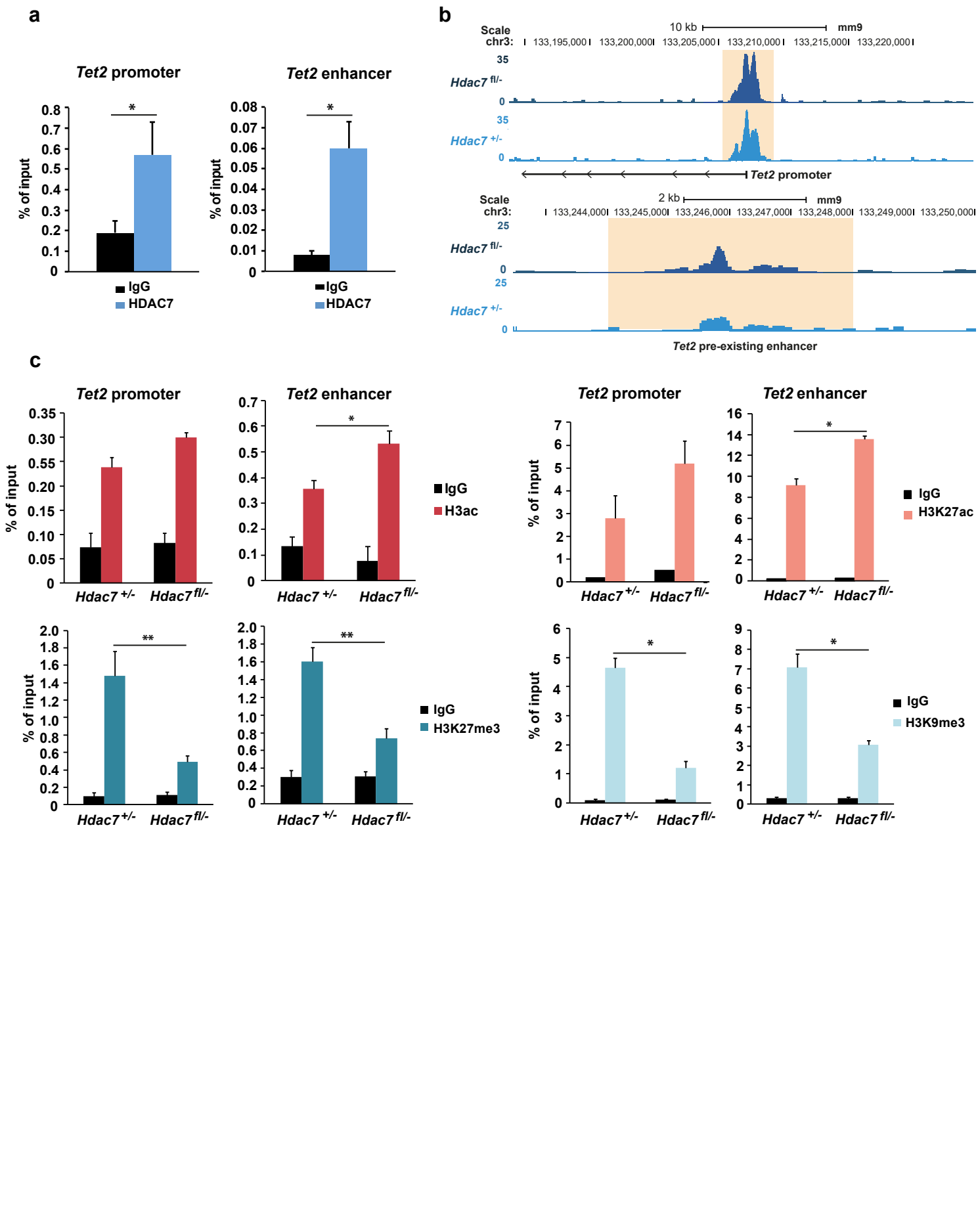
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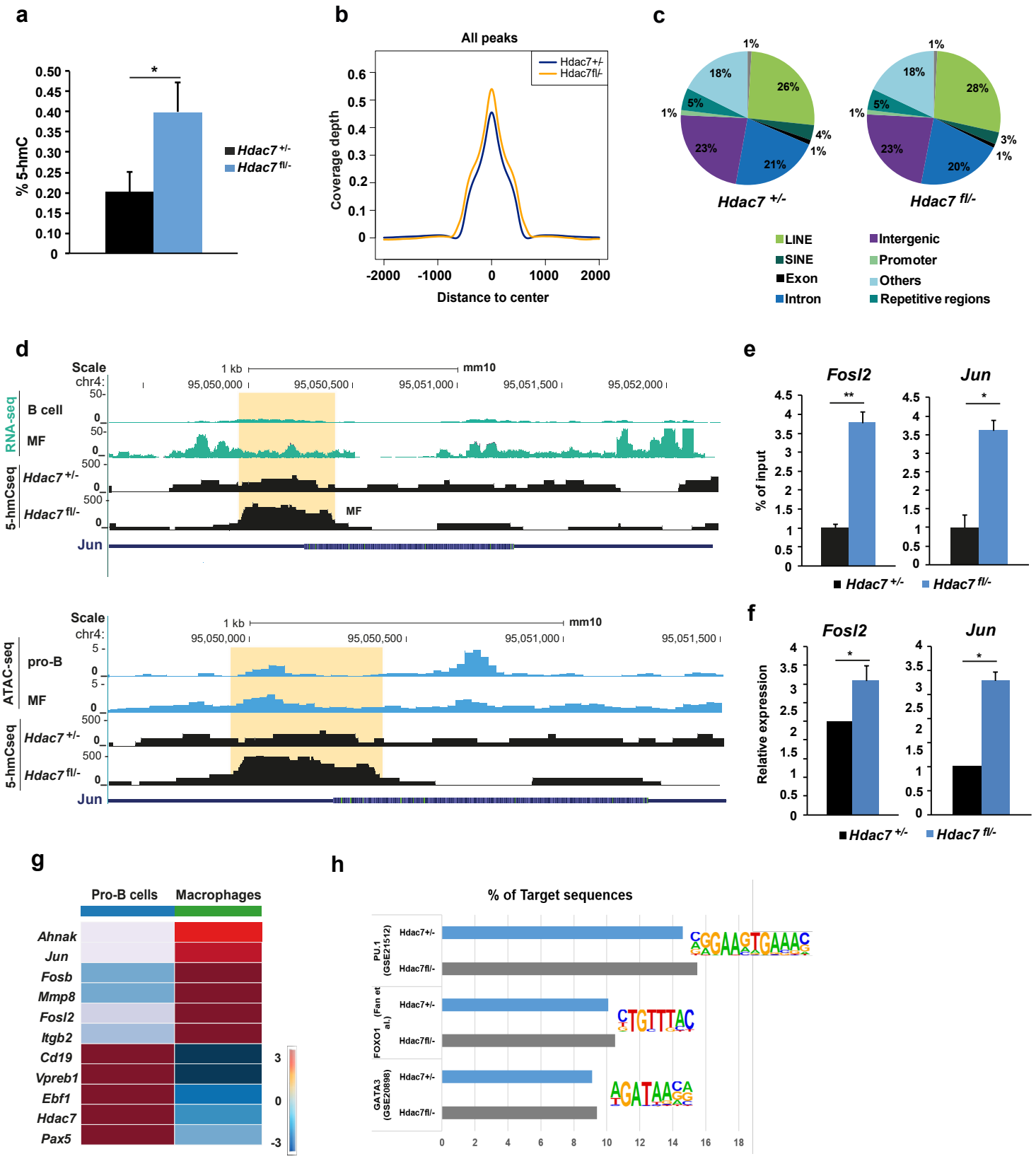
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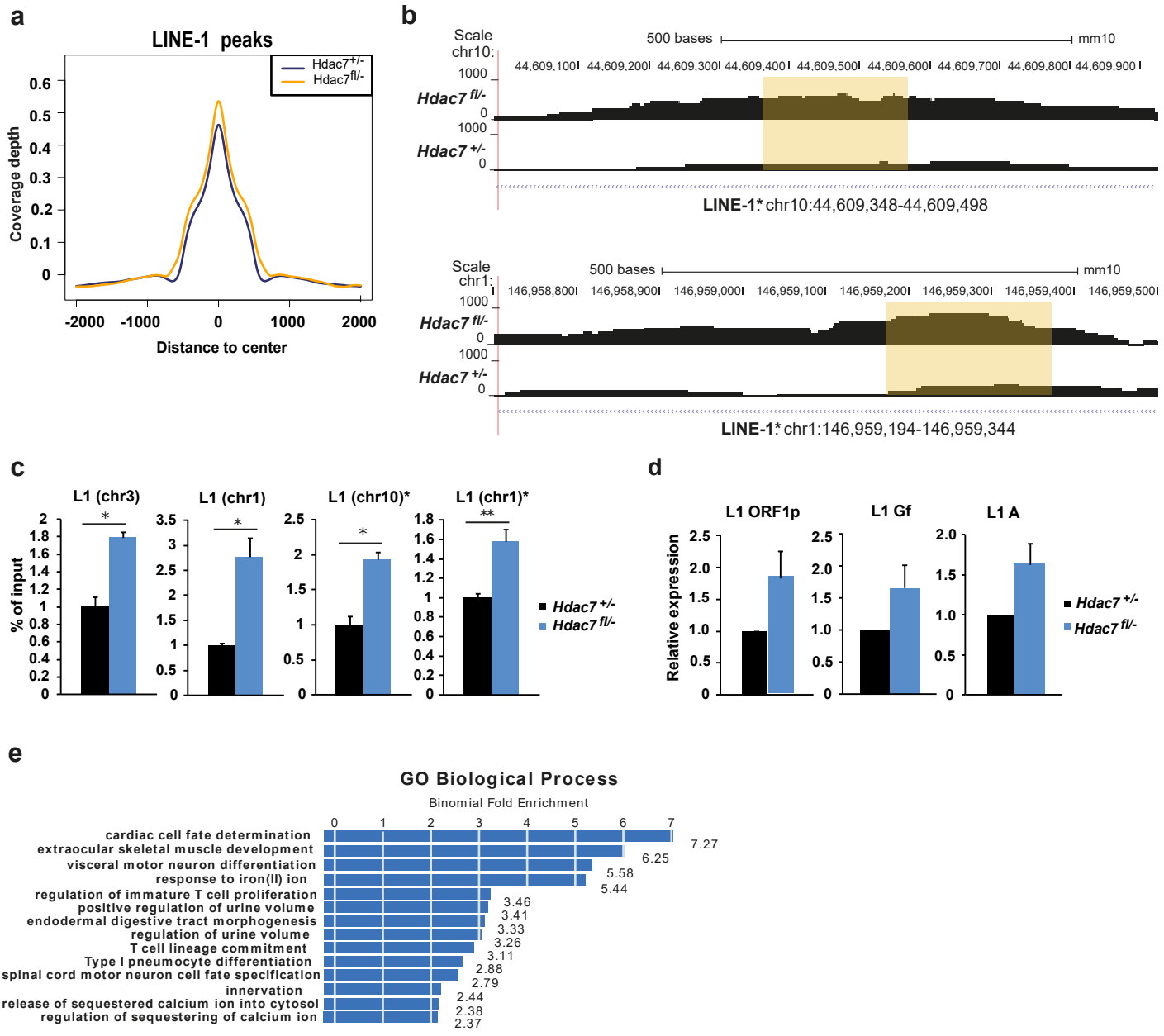
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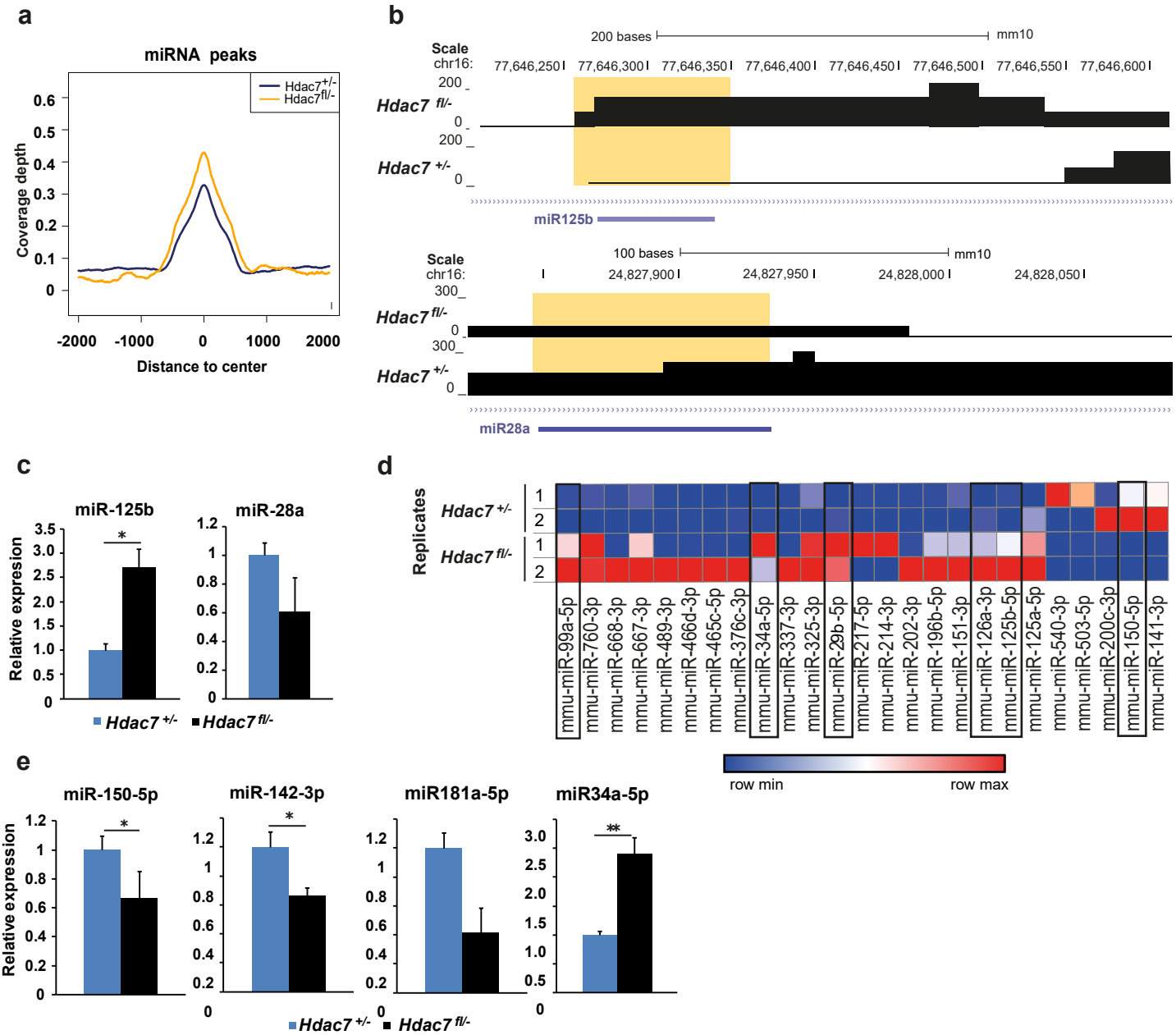
Figure 5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Relative expression
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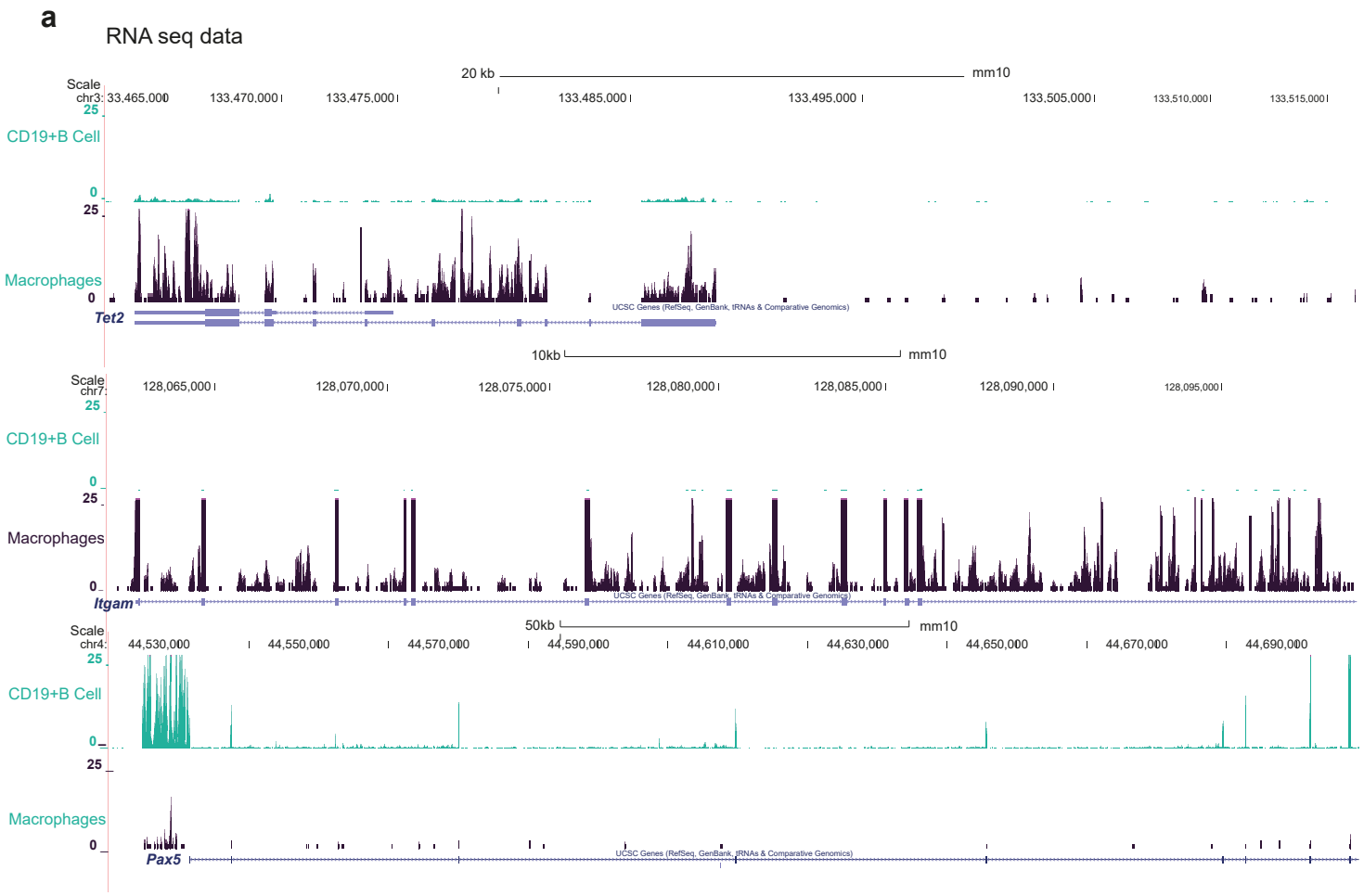
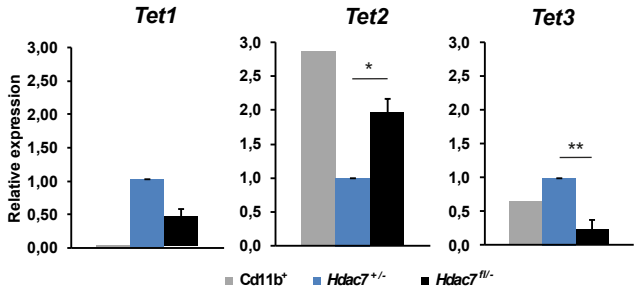
Hdac7 fl/-

Supplementary Figure 1





Supplementary Figure 2

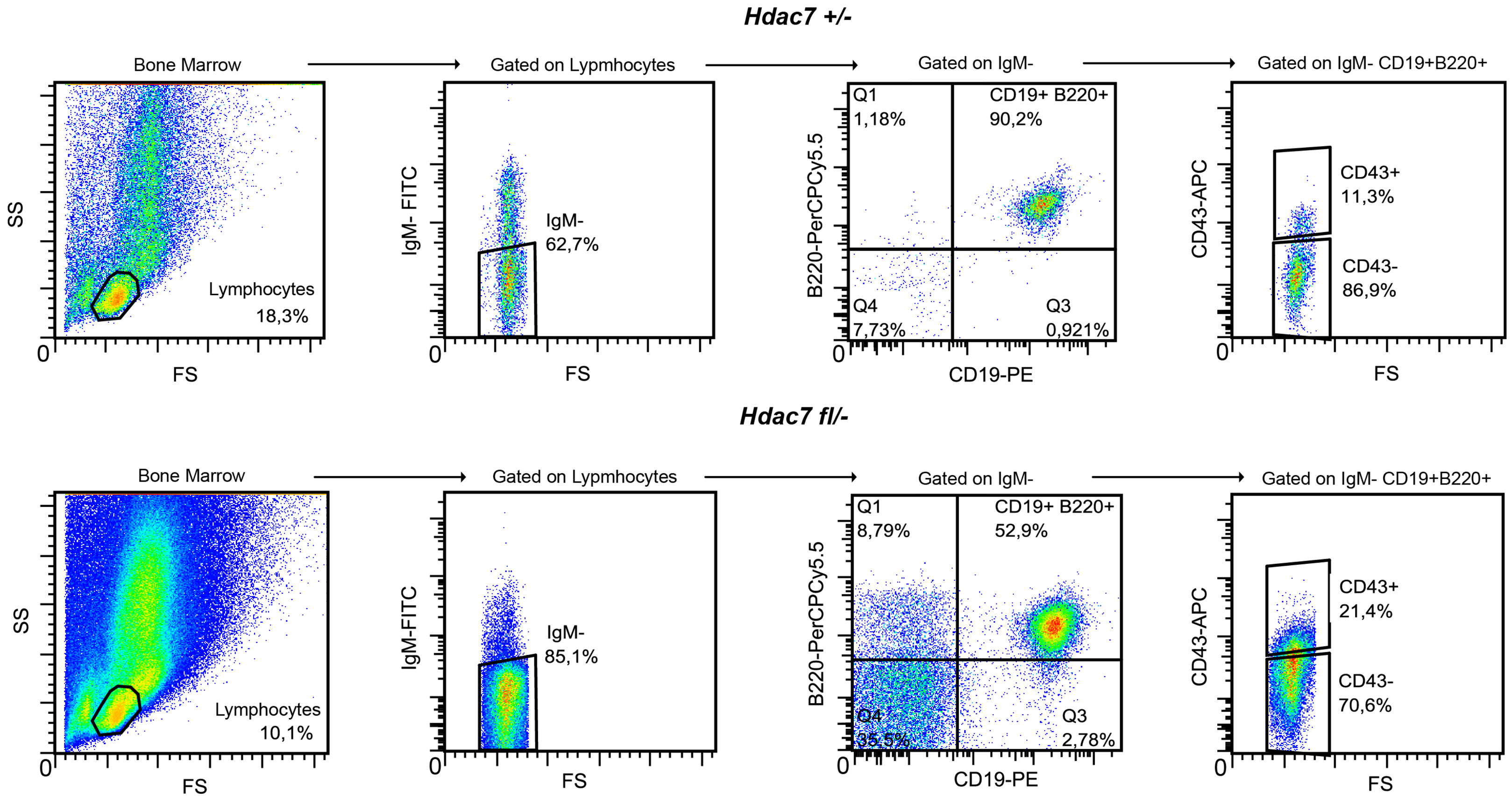


Figure Supplementary 3

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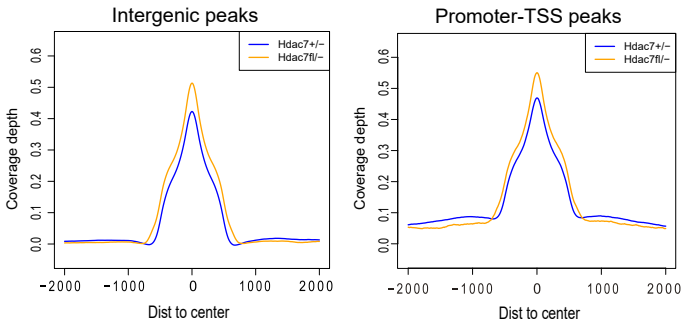

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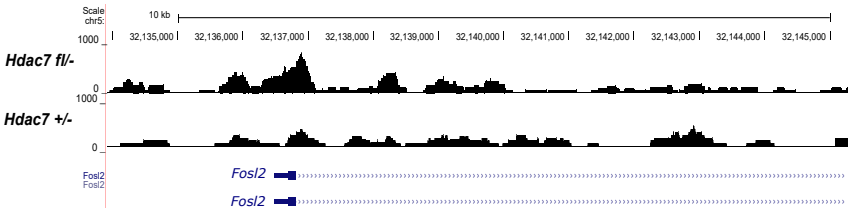

ARTICLE 3

"The transcriptional repressor HDAC7 promotes apoptosis and c-Myc downregulation in particular types of leukemia and lymphoma"

OPEN

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The transcriptional repressor HDAC7 promotes apoptosis and c-Myc downregulation in particular types of leukemia and lymphoma

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The generation of B cells is a complex process requiring several cellular transitions, including cell commitment and differentiation. Proper transcriptional control to establish the genetic programs characteristic of each cellular stage is essential for the correct development of B lymphocytes. Deregulation of these particular transcriptional programs may result in a block in B-cell maturation, contributing to the development of hematological malignancies such as leukemia and lymphoma. However, very little is currently known about the role of transcriptional repressors in normal and aberrant B lymphopoiesis. Here we report that histone deacetylase 7 (HDAC7) is underexpressed in pro-B acute lymphoblastic leukemia (pro-B-ALL) and Burkitt lymphoma. Ectopic expression of HDAC7 induces apoptosis, leads to the downregulation of c-Myc and inhibits the oncogenic potential of cells *in vivo*, in a xenograft model. Most significantly, we have observed low levels of HDAC7 expression in B-ALL patient samples, which is correlated with the increased levels of c-Myc. From a mechanistic angle, we show that ectopically expressed HDAC7 localizes to the nucleus and interacts with the transcription factor myocyte enhancer factor C (MEF2C) and the corepressors HDAC3 and SMRT. Accordingly, both the HDAC7–MEF2C interaction domain as well as its catalytic domain are involved in the reduced cell viability induced by HDAC7. We conclude that HDAC7 has a potent anti-oncogenic effect on specific B-cell malignancies, indicating that its deregulation may contribute to the pathogenesis of the disease.
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Proper generation of mature B lymphocytes is the result of complex cell lineage commitment and differentiation processes. Each cellular transition is tightly regulated at the transcriptional level by the action of linage-specific transcription factors (TFs), such as PU.1, Ikaros, myocyte enhancer factor C (MEF2C), E2A and PAX5 among others.[1–9] The deregulation of these particular transcriptional programs may result in a block in the differentiation and a hyperproliferative cellular state, thereby contributing to the development of hematological malignancies such as leukemia and lymphoma. Aberrant expression or mutation of many of the lineage-specific TFs involved in B lymphocyte development have been linked to the outcome of hematopoietic malignancies.[10,11] In addition, the overexpression of c-Myc has been found in T-cell acute lymphoblastic leukemia (T-ALL) and B-ALL, and some types of B-cell lymphoma, such as Burkitt lymphoma, present translocations in the *MYC* gene (*c-MYC-IgH*).[12] The deregulation of B-cell TFs in combination with chromosomal aberrations, such as gene translocations (*ETV6-RUNX1* and *BCR-ABL1*) and rearrangements in the MLL gene are key events in aberrant B lymphopoiesis and considered as primary lesions.[11,13]

In recent years, the idea has begun to emerge that, in addition to the activation of gene expression, transcriptional repression is a fundamental mechanism to ensure proper B lymphopoiesis.[3,14,15] Among the different types of transcriptional repressors, histone deacetylases (HDACs) are thought to be crucial enzymes in many physiological and pathological processes.[16,17] Mutation and/or aberrant expression of HDACs have often been observed in human disease, in particular cancer, making them important therapeutic targets.[18,19] In pathological situations where classic HDACs are overexpressed, HDAC inhibitors (HDIs) have emerged as promising therapeutic agents.[19] However, it is worth mentioning that the contribution of HDACs to cancer could be due to mechanisms other than overexpression. In fact, HDACs may

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Abbreviations: HDAC7, histone deacetylase 7; B-ALL, B-cell acute lymphoblastic leukemia; MEF2C, myocyte enhancer factor C; HDIs, histone deacetylase inhibitors

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also present truncating or inactivating mutations.[18] Therefore, we are far from fully understanding the contribution of individual HDACs to cancer.

Of the various HDACs, HDAC7 appears to be a lymphoid-specific transcriptional repressor.[20–26] In addition to its critical role in T lymphocyte biology, we have recently reported that HDAC7 is critical in maintaining the genetic identity of B lymphocytes.[20] Interestingly, HDAC7 has been identified as a target gene in hematopoietic cancers in a PiggyBac transposon mutagenesis screening in mice.[27] On the basis of our recent findings, we postulated that HDAC7 might be deregulated in B-cell malignancies. Here we report the loss of HDAC7 expression in cell lines established from B-ALL and Burkitt lymphoma as well as in pro-B-ALL samples from patients. Forced expression of HDAC7 induces the apoptosis of the cells. Strikingly, the presence of HDAC7 results in the downregulation of the oncogene c-Myc. HDAC7 expression interferes with the oncogenic potential of the cells in a xenograft model. Most significantly, we have found low levels of HDAC7 expression in B-ALL samples from patients, which are associated with high levels of c-Myc. Taken together, our findings suggest that HDAC7 expression may exert an anti-oncogenic activity in particular types of B-cell malignancies and that its deregulation may contribute to the pathogenesis of B-ALL and B-cell lymphoma.

Results

HDAC7 is underexpressed in pro-B-ALL and B-cell lymphoma. First, to further validate the notion that HDAC7 is a lymphoid-specific transcriptional repressor within the hematopoietic system we examined the Immunological Genome Project Database (Immgen) (http://www.immgen.org/). Using this database we confirmed that HDAC7 is specifically expressed in lymphoid cells but not in cells from the myeloid lineage (Supplementary Figure 1). This finding led us to speculate that HDAC7 expression could be deregulated in B-cell malignancies. To test this hypothesis, we first examined HDAC7 expression levels in a publicly available microarray GEO data set (GSE34861), which consists of the gene expression profile of 191 samples of adult B-ALL and 3 normal samples. The B-ALL samples comprised 28 corresponding to pro-B-ALL, 125 to early pre-B-ALL, 23 to pre-B-ALL, 5 to mature B-ALL, 5 to CD56+ B-ALL and 5 to transitional-pre-B-ALL immunophenotypes. Analysis using the Fisher test showed that HDAC7 was significantly associated with underexpression in pro-B-ALL samples (Figure 1a). We found no significant association with deregulation of HDAC7 expression in samples from the other immunophenotypes analyzed (Figure 1a). To further confirm our findings, we next assessed the HDAC7 protein levels in established cell lines from six B-ALLs. We observed that SD-1 and JVM-2 cells presented low or undetectable HDAC7 protein levels (Figure 1b). Next we tested whether HDAC7 expression could also be deregulated in lymphomas. We found that HDAC7 was underexpressed in the Burkitt lymphoma-derived Namalwa cell line (Figure 1c). Altogether, these data indicate that HDAC7 is deregulated in particular types of B-ALL and B-cell lymphoma.

HDAC7 expression induces apoptosis in SD-1 and Namalwa cells. To assess whether the absence of HDAC7 is associated with the oncogenic features of SD-1 and Namalwa cells we adopted a gain-of-function experimental approach. We generated a doxycycline-inducible system to express HDAC7 exogenously in both the cell lines (Figures 2a and b). Expression of HDAC7 after the addition of doxycycline to SD-1 cells resulted in complete cell growth arrest over a course of 3 days (Figure 2c). In contrast, addition of doxycycline to the parental SD-1 cell line had no significant effect on cell growth, demonstrating that HDAC7 specifically mediates the growth arrest of the cells (Figure 2c). We then tested whether the reduction in the total number of cells was the result of a block in cell

a

B-ALL phenotype	Total	underexpressed	%	Cds1 OR	Cds1 q-value	Cds2 OR	Cds2 q-value
Pro-B	28	21	75	19.76	3.14x1e-10	19.76	5.23x1e-11
Early Pre-B	125	10	8	0.09	1.18x1e-9	0.09	3.94x1e-10
Pre-B	23	8	34.5	2.09	3.52x1e-1	2.09	1.76x1e-1
Mature B cell	5	1	20	0.88	1	0.88	1
CD56+ B	5	1	20	0.88	1	0.88	1
Transitional Pre-B	5	1	20	0.88	1	0.88	1

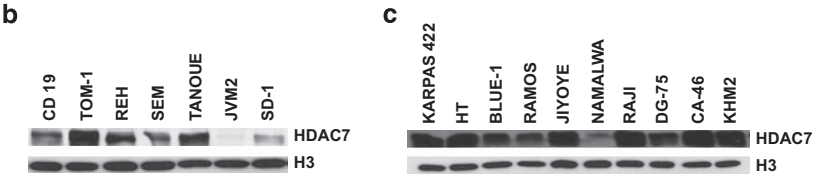

Figure 1 HDAC7 is underexpressed in pro-B acute lymphoblastic leukemia (ALL) and B-cell lymphoma. (**a**) Table shows the results of the Fisher test. The *q*-values result from multiple correction by FDR. The OR (odds ratio) shows the association between an ALL type and HDAC7 underexpression. OR > 1 indicates that ALL type is positively associated with underexpression, whereas OR < 1 indicates that it is negatively associated with underexpression. An OR = 1 indicates no association between ALL type and regulation. (**b**) and (**c**) represent the western blots for HDAC7 in B-ALL and lymphoma cell lines

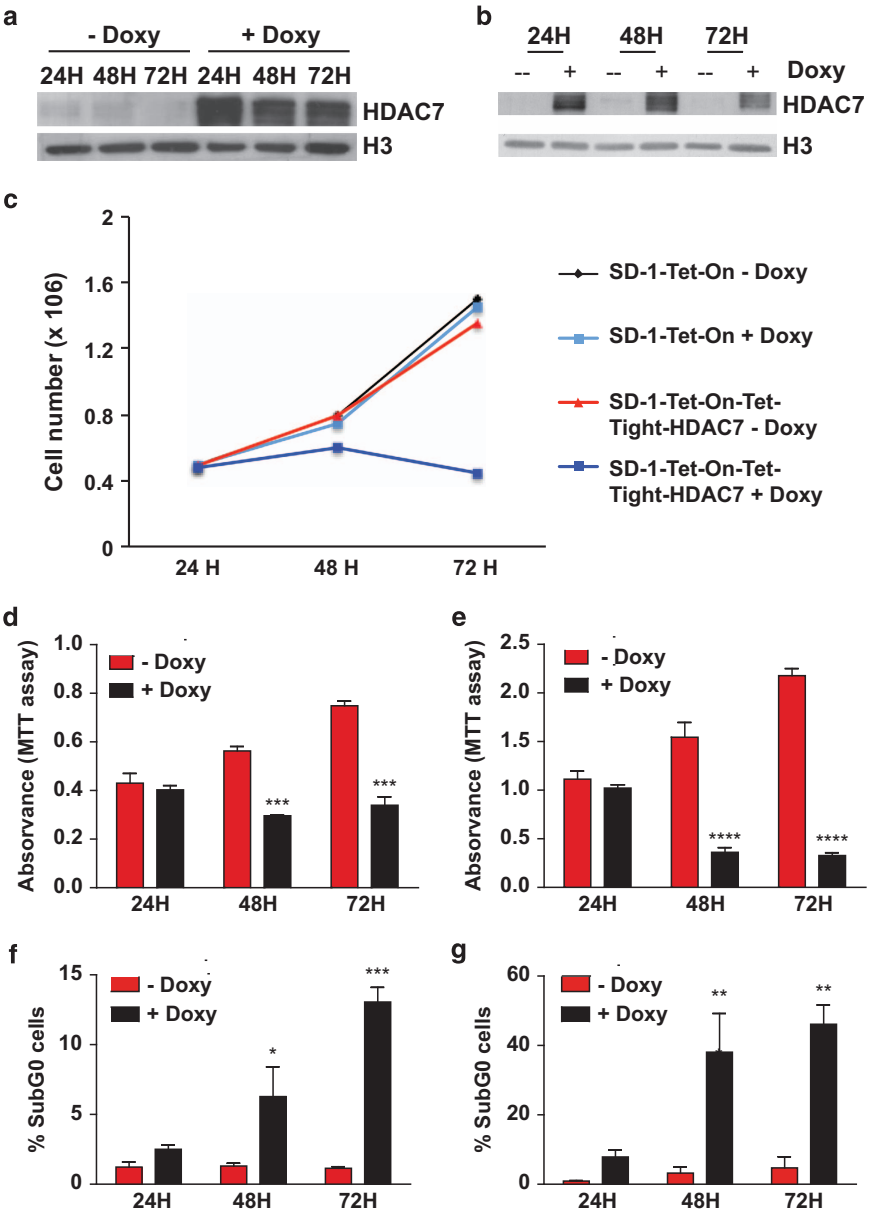

Figure 2 Forced expression of HDAC7 in SD-1 and Namalwa cell lines blocks their proliferation capacity and induces apoptosis. (**a**) and (**b**) SD-1 and Namalwa cells were transduced to express HDAC7 in a doxycycline-inducible manner (SD-1-Tet-On-Tight-HDAC7 and Namalwa-Tet-On-Tight-HDAC7 cells). Representative western blots showing HDAC7 protein levels after cell treatment with doxycycline. (**c**) SD-1-Tet-On and SD-1-Tet-On-Tight-HDAC7 cells were cultured and treated, or not, with doxycycline. At the indicated times after treatment, the cell number was assessed by cell counting. Trypan blue-dyed cells were omitted from the cell counts. Means ± S.D. of the four independent experiments performed in triplicate. (**d**) and (**e**) Mean ± S.E.M. of the absorbance units from five independent MTT assays performed in triplicate, of SD-1-Tet-On-Tight-HDAC7 (left) and Namalwa-Tet-On-Tight-HDAC7 (right) treated, or not, with doxycycline. ***$P<0.001$; ****$P<0.0001$. (**f**) and (**g**) show the percentage of cells in SubG0 from three independent experiments in SD-1-Tet-On-Tight-HDAC7 (left) and Namalwa-Tet-On-Tight-HDAC7 (right) cells treated, or not, with doxycycline for the indicated times. *$P<0.05$; **$P<0.01$; ***$P<0.001$

proliferation by performing an MTT assay in the absence and presence of HDAC7. HDAC7 expression significantly reduced the cell viability of both SD-1 and Namalwa cell lines (Figures 2d and e). To rule out the possibility that the effect observed on cell viability is due to the toxicity of ectopic overexpression of HDAC7, we generated HDAC7-inducible lines in cells that express normal HDAC7 levels. We found that ectopic expression of HDAC7 in RAJI and TOM-1 cell lines did not affect their viability (Supplementary Figure 2a and b). Moreover, the class IIa HDACs, HDAC4 and HDAC9 were found to be expressed in both SD-1 and Namalwa cells in the absence and in the presence of doxycycline indicating that the effect observed was specific to the expression of HDAC7 (Supplementary Figure 2c). Next we examined whether HDAC7 could induce apoptosis and assessed the cell cycle status. We observed a significant accumulation of cells in subG0 at 48 and 72 h after doxycycline treatment of both SD-1 and Namalwa cell lines, demonstrating that HDAC7 induced apoptosis (Figures 2d and e and Supplementary Figure 3). These findings indicate that the

absence of HDAC7 could be associated with the survival of both the cancer cell lines and suggest that its re-expression may exert a therapeutic anti-oncogenic effect.

HDAC7 expression induces apoptosis and inhibits tumor growth in a xenograft model. To determine the physiological consequences of HDAC7 expression in SD-1 and Namalwa cells we performed *in vivo* functional experiments using a xenograft model in athymic mice. First, SD-1 cells were injected subcutaneously into the back of several athymic mice. When tumors reached a homogeneous size they were randomly allocated into two treatment groups: (i) mice drank glucose; and (ii) drank glucose plus doxycycline in water. Tumors in mice taking glucose-treated water that did not express HDAC7 continued to grow. Strikingly, tumors in mice treated with doxycycline showed a marked decrease in their size (Figures 3a and b). Next to test the effect of HDAC7 expression on the lymphomagenic capacity of the Namalwa cell line, 1.5×10^6 cells were injected orthotopically into the spleen of 19 athymic mice and they were randomly allocated into two treatment groups. (i) mice drank glucose; and (ii) drank glucose plus doxycycline in water. Notably, 15 days later, the tumors of mice treated with doxycycline were almost undetectable at palpation. At that point, all mice were killed and their spleens surgically removed. Similar to the results obtained with leukemic cells, the expression of HDAC7 in Namalwa cells markedly interfered with the growth of lymphomas (Figures 3c and d). Immunofluorescence assays revealed a significant reduction of proliferation, as revealed by Ki67 staining, and increased apoptosis in tumor cells expressing HDAC7 (Figures 3e and f and Supplementary Figure 4). Thus, our *in vivo* data confirm that HDAC7 induces apoptosis and exerts a potent anti-oncogenic effect suggesting that its absence may be involved in the pathogenesis of specific types of B-ALL and B-cell lymphoma.

HDAC7 expression induces the apoptotic gene program of leukemic cells. As HDAC7 is a transcriptional regulator, we decided to investigate the impact of HDAC7 expression on the global gene expression profile of SD-1 cells. Microarray analysis revealed that 660 genes were differentially expressed when HDAC7 was ectopically expressed in SD-1 cells. Of these, 410 genes were upregulated and 250 were downregulated (Supplementary Figure 5, Supplementary Data Sets 1 and 2). Next we examined the list of upregulated genes after HDAC7 expression and looked for the presence of apoptosis-related genes. We observed that HDAC7 induced the expression of several genes, such as *CD44*, *FAS*, *ATM*, *TP53BP2*, *CD40* and *BIRC3*, with known apoptotic functions (Supplementary Table 1). In addition, we also found that the presence of HDAC7 led to the upregulation of genes related to immune processes (*IL16*, *FCGR2A*, *IRAK2*, *CD86* and *CD40*, among others) and cancer (*RASSF4*, *RAB31*, *NEDD9* and *RASSF2,* among others; Supplementary Table 1). To further investigate if HDAC7 expression leads to the activation of the apoptotic genetic program in SD-1 cells, we performed a gene set enrichment analysis based on the gene ontology (GO) categories corresponding to the biological processes and on the KEGG pathways. The biological processes analysis revealed that the set of genes upregulated upon HDAC7 expression belong to GO categories representing immune system processes, regulation of cell death and regulation of cell proliferation, among others (Figure 4a). KEGG pathway enrichment analysis confirmed that the genes whose expression was induced by HDAC7 were significantly enriched in the apoptosis pathway (Figure 4b). A selected number of genes were validated by RT-qPCR (Figure 4c). To better understand the mechanism associated with the HDAC7-induced apoptotic pathway in SD-1 cells, we investigated the possible enrichment of TF motifs from the TRANSFAC database in the set of upregulated genes. We observed a significant enrichment of a set of TFs. Remarkably, we found the enrichment of the binding motif for p53, suggesting that the induction of apoptosis may occur in a p53-mediated manner (Figure 4d). To test this possibility we assessed the status of p53 activation after the expression of HDAC7 in both SD-1 and Namalwa cells and found that the presence of HDAC7 in both the cell lines resulted in the phosphorylation and acetylation of p53 at serine 392 and lysine 382, respectively, two post-translational modifications indicative of p53 activation (Figure 4e). Overall, our data demonstrate that HDAC7 induces apoptosis presumably via the activation of the p53 pathway.

HDAC7 represses the expression of c-Myc. Our findings strongly indicate that HDAC7 exerts a strong anti-oncogenic effect in pro-B-ALL and B-cell lymphoma. Given that HDAC7 is a transcriptional repressor, we wondered whether its expression could lead to the repression of key oncogenes in leukemia and lymphoma. We examined our microarray data and looked for the presence of potential oncogenes in the list of downregulated genes after HDAC7 expression. Strikingly, we observed that the presence of HDAC7 resulted in the downregulation of crucial genes with known oncogenic potential, such as *MYC*, *TERT* and *AICDA* (Supplementary Table 2). This finding was validated by RT-qPCR in both SD-1 and Namalwa cells (Figures 5a and b). Using the TRANSFAC database, we found a significant enrichment of the binding site motifs for MYC factors in the HDAC7-induced downregulated genes (Figure 5c). Moreover, we also found that HDAC7 expression resulted in the reduction of c-Myc protein levels (Figure 5d). Next we tested whether the ectopic expression of c-Myc could prevent the cell growth arrest induced by HDAC7 in both SD-1 and Namalwa cells. We found that exogenous expression of c-Myc induced a significant rescue of cell growth in cells treated with doxycycline to express HDAC7. This finding further corroborates that the anti-oncogenic capacity of HDAC7 is mediated, at least in part, by the downregulation of c-Myc in SD-1 and Namalwa cells (Figure 5e). To confirm the relevance of our finding we further analyzed the published microarray GEO data set (GSE34861) and examined whether there was an association between HDAC7 and c-Myc expression levels. Strikingly, we found that a low level of expression of HDAC7 was significantly associated with high levels of c-Myc in B-ALL patients (Figure 5f). These data strongly support the hypothesis that HDAC7 posses an anti-oncogenic potential on the B-cell malignancies studied.

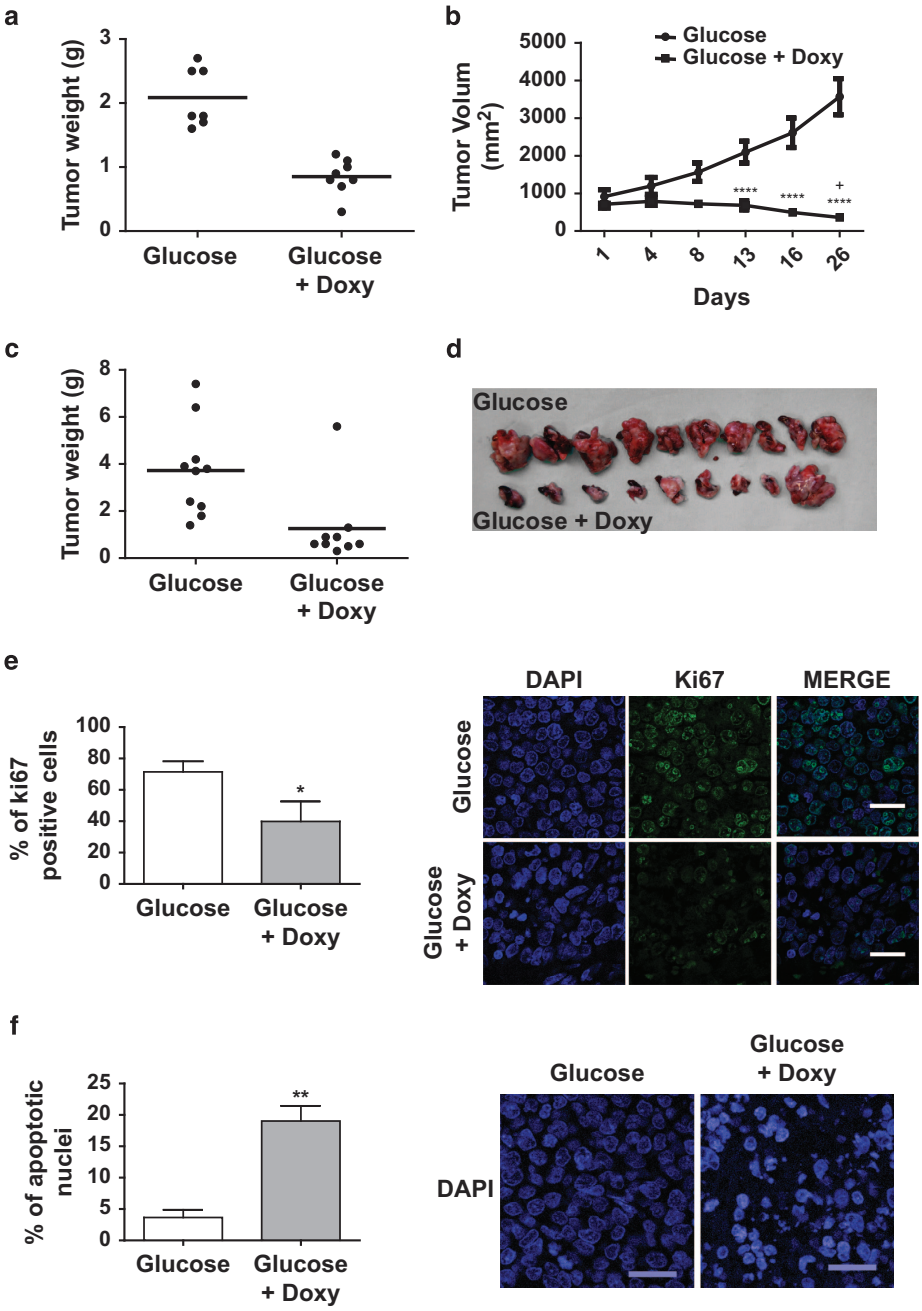

Figure 3 HDAC7 impairs the oncogenic capacity of SD-1 and Namalwa cells. Xenographic assays were performed with SD-1-Tet-On-Tight-HDAC7 and Namalwa-Tet-On-Tight-HDAC7 cells. SD-1 cells (5×10^{6}) were injected subcutaneously and 1.5×10^{6} Namalwa cells were orthotopically injected into the spleen. Treatment with doxycycline was started 2 weeks after injection. (**a**) and (**b**) Tumor weight at the end point of the experiment and tumor growth during the experiment of SD-1-Tet-On-Tight HDAC7 cell xenographic assays. (**c**) and (**d**) Graph of tumor weight and pictures of the tumors of Namalwa Tet-On-Tight-HDAC7 cell xenographic assays. (**e**) HDAC7 expression reduced the number of KI67-positive cells and promoted an increased in the number of apoptotic nuclei. Panel (**e**) shows the frequency of Ki67-positive cells in the SD-1-Tet-On-Tight-HDAC7 xenographic assay. More than 2400 cells per animal (three glucose; three glucose+Doxy) were analyzed. $^{*}P < 0.05$. (**f**) Percentage of condensed or fragmented nuclei of all nuclei from >2400 cells per animal (three glucose; three glucose+Doxy). $^{**}P < 0.01$

HDAC7 interacts with MEF2C, HDAC3 and SMRT and is localized in the nucleus. Class IIa HDACs posses a highly conserved C-terminal catalytic domain that mediates their recruitment to a corepressor complex containing HDAC3 and SRMT/N-CoR. In addition, class IIa HDACs contain a long N-terminal region that has been shown to mediate their interaction with tissue-specific TFs and their phosphorylation-dependent subcellular localization. To gain an insight into the mechanism of action of HDAC7, we first assessed its subcellular distribution in Namalwa cells treated, or not, with doxycycline. As expected, we found that ectopically expressed HDAC7 was mainly localized in the nuclear

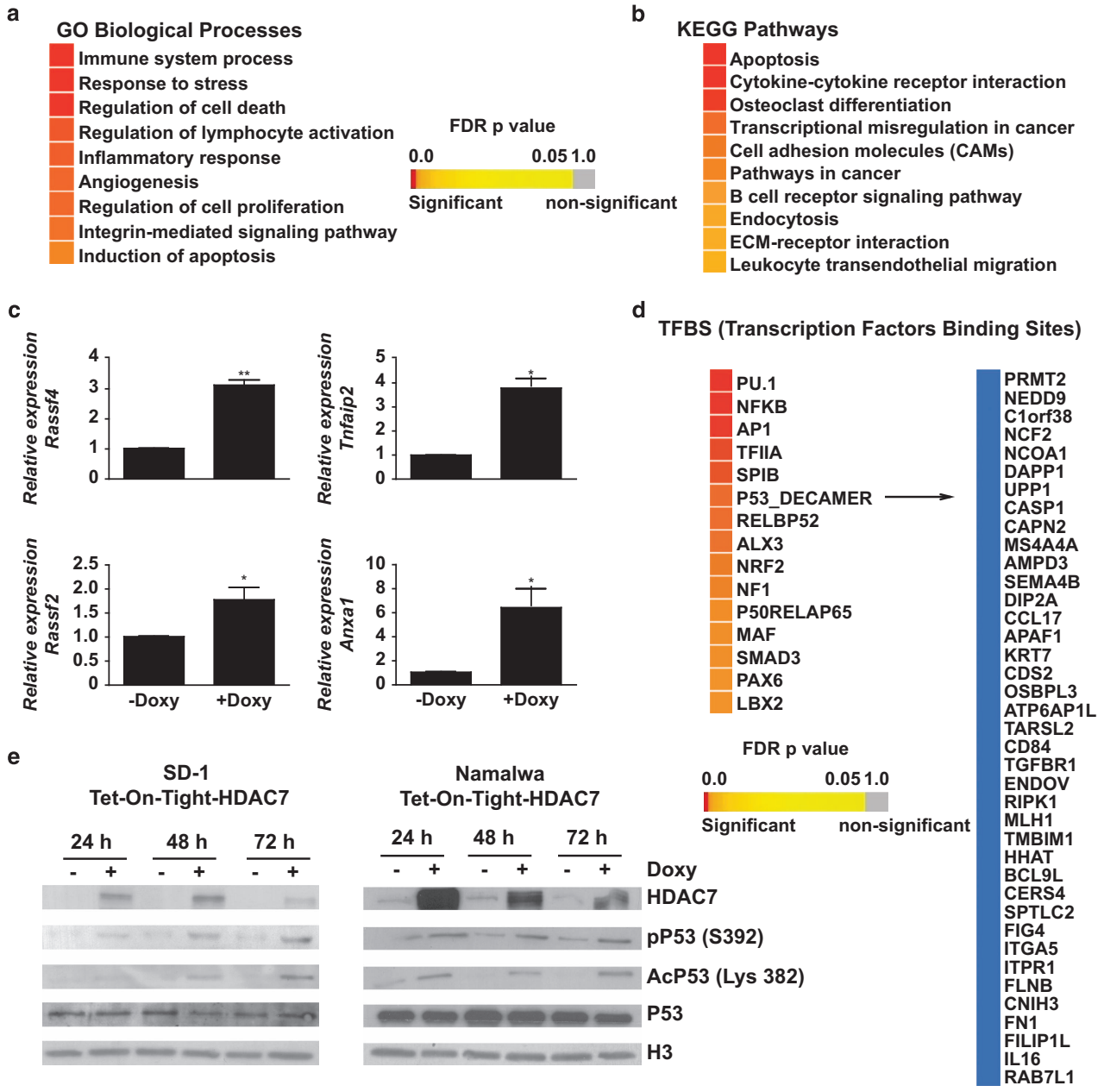

Figure 4 HDAC7 expression results in the enrichment of apoptotic and immune system programs and p53 activation in SD-1 cells. (**a**) and (**b**) Heatmaps showing significantly (adjusted $P<0.05$) enriched GO biological processes and KEGG pathways among the upregulated genes after HDAC7 expression. (**c**) RT-qPCR validation for the selected upregulated genes. $^{*}P<0.05$; $^{**}P<0.01$. (**d**) TF-binding sites enriched in the upregulated genes after HDAC7 expression. p53 target genes are shown. (**e**) Representative western blot of SD-1-1-Tet-On-Tight HDAC7 and Namalwa-1-Tet-On-Tight HDAC7 cells treated, or not, with doxycycline for the indicated times showing p53 phosphorylation and acetylation status

compartment (Figure 6a). Next we tested the potential requirement of both the TF-binding domain and the catalytic domain in HDAC7-decreased cell viability. We generated retroviral vectors carrying a C-terminal truncated construct HDAC7 (1–487) that completely lacks the HDAC catalytic domain but contains the MEF2 interacting motif, and an N-terminal truncated construct HDAC7 (438–915) bearing the enzymatic motif but lacking the MEF2 domain. Expression of wild-type HDAC7 resulted in a significant decrease in the viability of Namalwa cells, whereas the expression of HDAC7 (1–487) and HDAC7 (438–915) constructs did not have a significant effect (Figure 6b). We have recently reported that HDAC7 interacts with the TF MEF2C in B lymphocytes. To address whether ectopically expressed HDAC7 specifically interacts with MEF2C in Namalwa cells, we performed co-immunoprecipitation experiments. We found that HDAC7 associated with MEF2C and not with other B-cell-specific TFs in Namalwa cells (Figure 6c). We also observed that HDAC7 interacted with HDAC3 and SMRT (Figure 6c). It has been proposed that HDAC7 lacks any enzymatic activity and that it

exerts its repressive function via the interaction with HDAC3. However, the function of HDAC7 in the absence of HDAC3 has not been properly studied. To address whether HDAC7 induces cell growth arrest through the interaction with HDAC3 in SD-1 and Namalwa cells, we performed a loss-of-function experimental approach (Figure 6d).

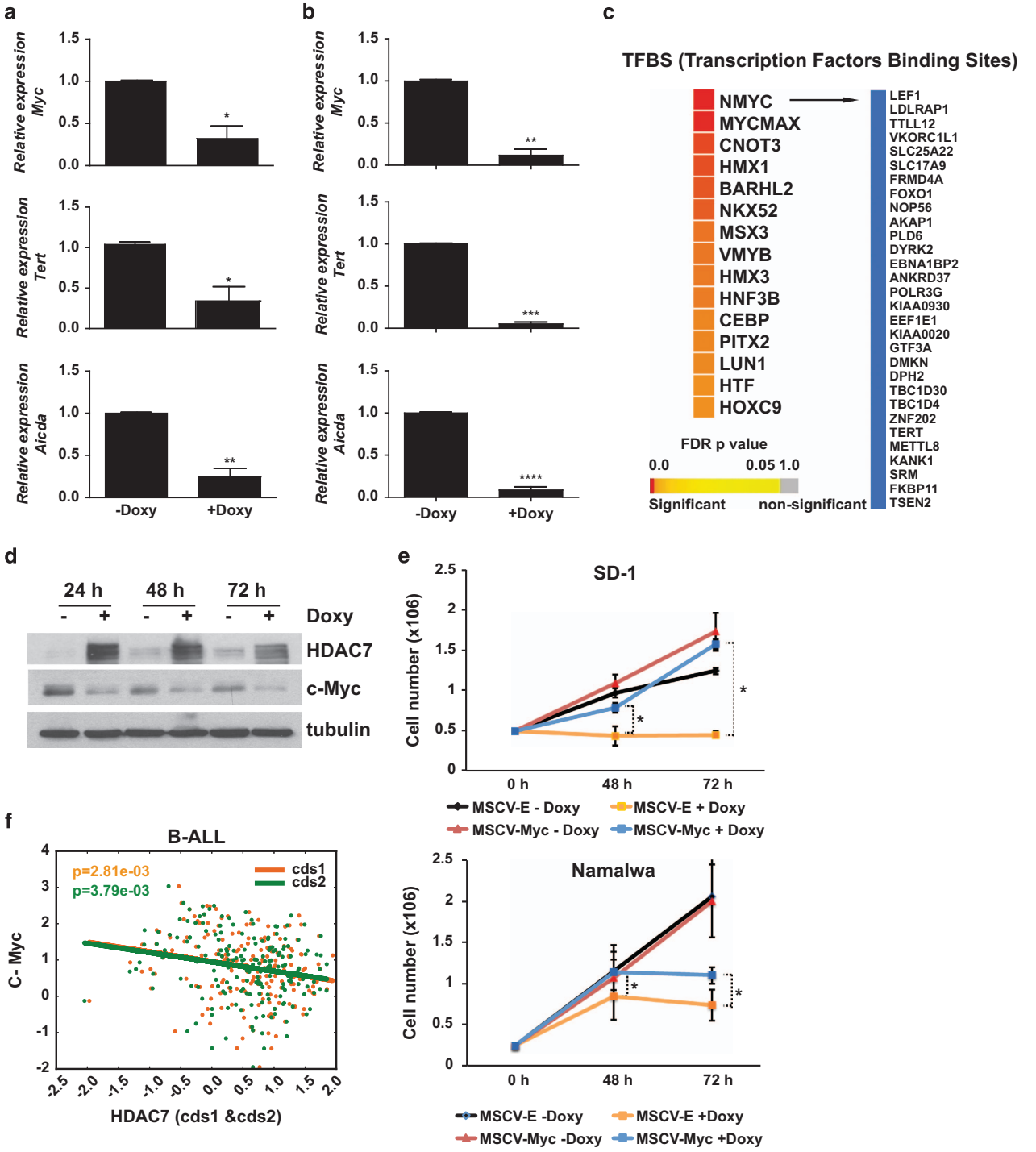

Figure 5 HDAC7 leads to the repression of c-Myc in SD-1 and Namalwa cells. (**a**) and (**b**) RT-qPCR validation for selected downregulated genes in the presence of HDAC7 are shown in SD-1-1-Tet-On-Tight HDAC7 (**a**) SD-1 and Namalwa-Tet-On-Tight HDAC7 cells (**b**). *$P<0.05$; **$P<0.01$; ***$P<0.001$; ****$P<0.0001$. (**c**) TF-binding sites enriched in the downregulated genes after HDAC7 expression in SD-1 cells. Myc target genes are shown. (**d**) Western blot showing the downregulation of c-Myc after HDAC7 expression in SD-1 cells. (**e**) SD-1-Tet-On-Tight-HDAC7 and Namalwa-Tet-On-Tight-HDAC7 cells transduced with either MSCV-Empty or MSCV-c-Myc retroviral vectors were cultured and treated, or not, with doxycycline. At the indicated times after treatment, the cell number was assessed by cell counting. Trypan blue-dyed cells were omitted from the cell counts. Means ± S.D. of the three independent experiments performed in triplicate. *$P<0.05$ (**f**) GSE34861 data were analyzed to determine any correlation between *HDAC7* and *c-MYC* expression. c-MYC and HDAC7 probes were normalized with respect to values of healthy patients. The graph shows the negative correlation between c-MYC and HDAC7 (cds1 $\rho = -2.15e\text{-}01$; cds2 $\rho = -2.09e\text{-}01$) expression in the B-ALL patients

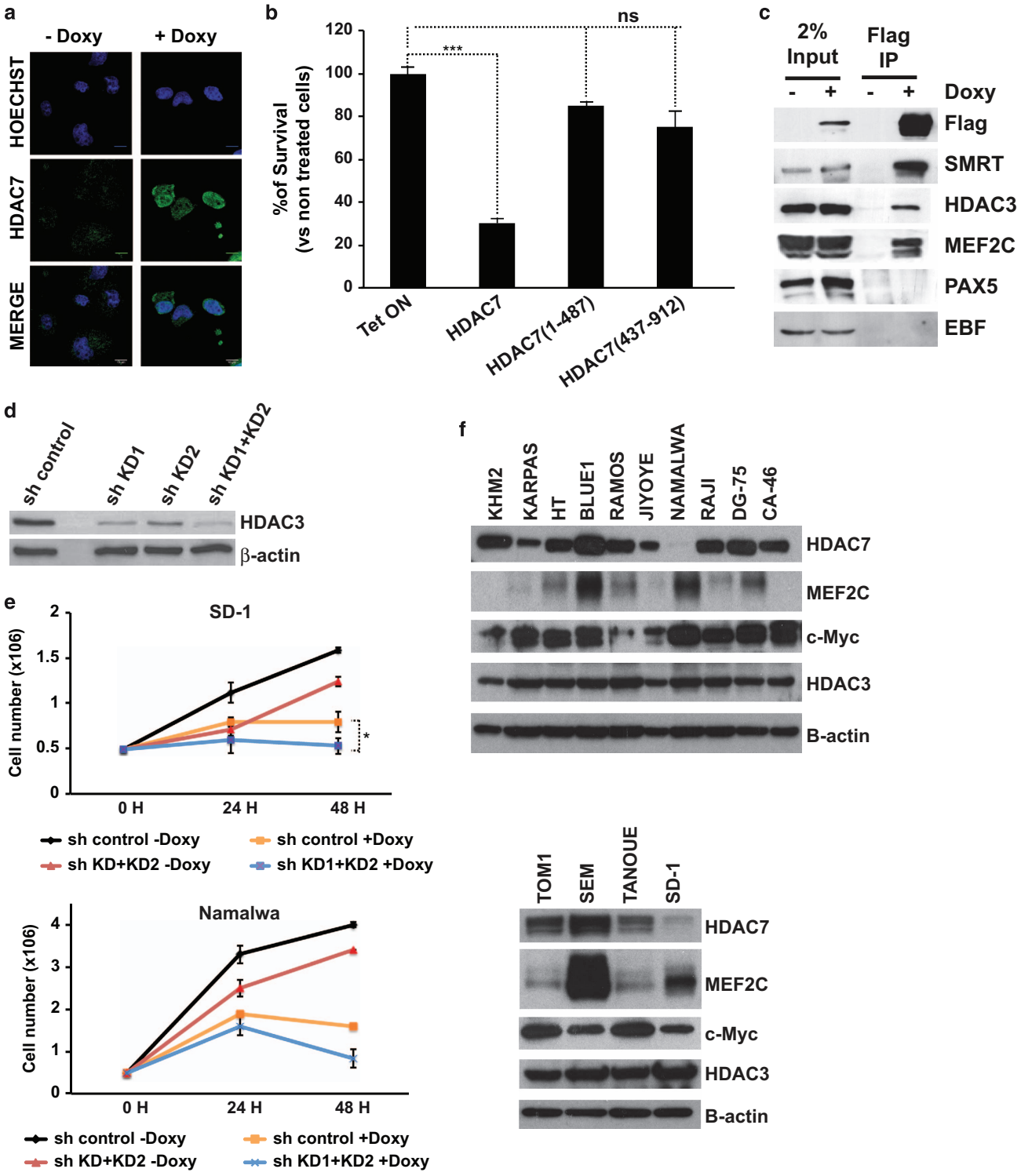

Figure 6 HDAC7 interacts with MEF2C, HDAC3 and SMRT and is localized in the nucleus. (**a**) Namalwa-Tet-On-Tight-HDAC7 cells were treated, or not, with doxycycline for 24 h. The subcellular localization of HDAC7 was determined by immunofluorescence. (**b**) Mean ± S.E.M. of the percentage of survival cells from three independent MTT assays performed in triplicate of Namalwa cells expressing empty vector, wild-type HDAC7, and HDAC7 (1–487) and HDAC7 (438–915) deleted forms treated, or not, with doxycycline. ***$P<0.001$. (**c**) Cell lysates from Namalwa-Tet-On-Tight-HDAC7 treated, or not, with doxycycline were immunoprecipitated with anti-M2 agarose beads and analyzed by western blotting with the indicated antibodies. (**d**) Namalwa-Tet-On-Tight-HDAC7 cells were transduced with lentiviral vectors for the expression of the control shRNA or the shRNAs targeting HDAC3, and GFP-positive cells were purified by flow cytometry. HDAC3 protein levels were assessed by western blot. (**e**) SD-1-Tet-On-Tight-HDAC7 and Namalwa-Tet-On-Tight-HDAC7 cells transduced with either pLKO.1-shRNA control or pLKO.1-shHDAC3KD1+KD2 lentiviral vectors were cultured and treated, or not, with doxycycline. At the indicated times after treatment, the cell number was assessed by cell counting. Trypan blue-dyed cells were omitted from the cell counts. Means ± S.D. of the three independent experiments performed in triplicate. (**f**) Western blots for HDAC7, MEF2C, c-Myc and HDAC3 in B-ALL and lymphoma cell lines

Strikingly, we observed that HDAC3 knockdown in both cell lines reduced cell growth in the absence of HDAC7, indicating that targeting HDAC3 would also have an anti-oncogenic effect. However, in the presence of HDAC7, lowering HDAC3 levels did not lead to a rescue in the cell growth block induced by HDAC7 (Figure 6e). This result indicates that HDAC7 may possess an intrinsic enzymatic activity independent of HDAC3. Next we wondered whether the expression of HDAC7 partners could be deregulated in leukemia and lymphoma cells expressing HDAC7. To test this possibility, we determined the protein levels of HDAC3, MEF2C and c-Myc in leukemia and lymphoma cell lines. As expected, most of the cell lines express high levels of c-Myc (Figure 6f). However, we did not observed differences in the expression of HDAC3 between the cell lines tested (Figure 6f). This supports the finding that HDAC7 exerts its anti-oncogenic effect in an HDAC3-independent manner. Strikingly, we observed that in contrast to SD-1 and Namalwa cells that express the TF MEF2C, many of the other cell lines either lack or express very low levels of this TF. Therefore, the absence of MEF2C may explain why those cell lines can tolerate normal HDAC7 levels. Altogether, these experiments demonstrate that both the HDAC7–MEF2C interaction and its catalytic domain, are necessary for HDAC7 to reduce the viability of SD-1 and Namalwa cells.

Discussion

We present data demonstrating that the transcriptional repressor HDAC7 has a potent anti-oncogenic effect in particular types of B-ALL and B-cell lymphoma. First, we report the loss of HDAC7 expression in pro-B-ALL patients and in established B-ALL and B lymphoma cell lines. Second, we show that HDAC7 expression induces apoptosis and inhibits the oncogenic potential of the cell lines tested *in vitro* and *in vivo*. Third, using genome-wide transcriptome profiling we show that ectopically expressed HDAC7 induces the expression of apoptotic genes and leads to the downregulation of key oncogenes such as c-Myc. And fourth, we report the key finding that samples from pro-B-ALL patients present low levels of HDAC7, which are associated with high levels of c-Myc expression.

The idea that HDACs are aberrantly overexpressed in cancer has been prevalent for some time, to the point where it is stated as dogma. In fact, inhibition of HDACs has been reported to have promising effects in cancer treatment.[18] However, most HDIs are disadvantaged by their lack of enzyme specificity and have a broad range of potential side effects.[28] Our findings from this study reveal an unexpected anti-oncogenic function for an HDAC in pro-B-ALL and B-cell lymphoma. We demonstrated that the expression of a crucial HDAC, HDAC7, for B lymphocyte biology is lost in pro-B-ALL patients and in the B-ALL and B-cell lymphoma cell lines, and that its re-expression has a potent anti-oncogenic effect. In this regard, it is important to note that under-expression levels, truncating or inactivating mutations in some HDACs in cancer have also been reported.[18] Recently, Heideman *et al*.[29] demonstrated that the reduction in HDAC1 and HDAC2 expression levels *in vivo* brings about T-cell lymphomagenesis owing to a block in the early thymocyte development. A different study demonstrated that the lack of HDAC3 specifically in the liver leads to the development of hepatocellular carcinomas.[30] Therefore, our understanding of the contribution of specific HDACs to a given cancer type continues to be incomplete. Efforts are needed to establish definitively the role of specific HDACs and whether they are overexpressed, underexpressed or mutated in a particular cancer. This will allow for the design and development of HDAC isoform-specific HDIs or other molecules that can modulate the expression of a particular HDAC.

Several reports have described a potential role for HDAC7 in hematological malignancies.[27,31–34] However, the functional contribution of HDAC7 to B-ALL remains to be elucidated. In an elegant study using a PiggyBac transposon screening in mice, Rad *et al*.[27] revealed that HDAC7 is a target gene in hematopoietic cancers. In addition, HDAC7 has been shown to be overexpressed in childhood ALL.[34] This discrepancy with our data could be explained by the different analytical methods used in the two studies. Tone and colleagues analyzed 94 samples from childhood ALL patients, of which 78 corresponded to B-ALL and only 4 had a pro-B-ALL immunophenotype.[34] In the present work, we took advantage of a data set obtained in an integrative epigenomic study where they analyzed adult B-ALL patients distinguishing different immunophenotypes.[35] Performing an accurate analysis of the expression of HDAC7, we found that HDAC7 was significantly underexpressed in pro-B-ALL patients. Therefore, it is possible that Gonzaga and colleagues did not find low levels of HDAC7 because pro-B-ALL was underrepresented in their study.

Why is HDAC7 underexpressed in specific types of B-ALL and B-cell lymphoma? Leukemogenesis and lymphomagenesis are complex malignant processes that may comprise a broad number of driver mutations, rearrangements and translocations in crucial genes, which are considered as primary lesions. We speculate that the loss of HDAC7 expression in particular types of leukemia and lymphoma may be the result of the transcriptome changes induced by a specific primary lesion. Several mechanisms could account for the loss of HDAC7 expression in leukemia and lymphoma. First, it is possible that the HDAC7 gene suffers from DNA methylation leading to its epigenetic silencing. A second potential mechanism responsible for the deregulation of HDAC7 is the action of microRNAs. In fact, another class IIa HDAC, HDAC4, has been reported to be a target of miR-155 in a Eu-miR-155 transgenic mouse model.[36] Eu-miR-155 mice exhibit high proliferation rates of pre-B cells and develop lymphoma/leukemia. Croce and colleagues have shown that miR-155 targets HDAC4, leading to its underexpression, and that the ectopic expression of HDAC4 in diffuse large B-cell lymphoma cells inhibits miR-155-induced proliferation and increases the apoptosis of the cells.[36] The elucidation of the molecular mechanisms involved in the repression of HDAC7 in pro-B-ALL and B-cell lymphoma is a current focus of study in our laboratory. The modulation of HDAC7 expression in specific types of B-ALL and B-cell lymphoma leading to the induction of apoptosis and the downregulation of the c-Myc oncogene may be a promising therapeutic pathway in future. On the basis of this study, HDAC7 appears to be a promising therapeutic target in these particular types of hematological

disease. Our data will help to generate new, highly specific and personalized therapies for the treatment of pro-B-ALL and B-cell lymphoma.

Materials and methods

Plasmids and retroviral supernatant generation. pRetro-X-Tight-Pur-HDAC7 constructs were generated by cloning full length or deleted HDAC7 cDNAs obtained by EcoRI digestion of the pcDNA3.1-HDAC7 plasmids into the pRetro-X-Tight-Pur vector (Takara Bio, Otsu, Japan). MSCV-c-Myc-GFP retroviral vector was obtained from Addgene (Cambridge, MA, USA). pLKO.1-shHDAC3KD1-GFP and pLKO.1-shHDAC3KD2-GFP constructs were generated by cloning two validated shRNAs sequences that target HDAC3 (SIGMA, St. Louis, MO, USA), into the pLKO.1-GFP lentiviral vector. For retrovirus generation, pRetro-X-Tight-Pur-HDAC7 and pRetro-X-Tet-On-Advanced (Takara Bio) plasmids were transfected into the packaged cell line Platinum-E and the supernatant was collected 48 h post transfection. For lentivirus generation, the generated constructs were transfected into 293 T cells together with enveloped and packaging plasmids and supernatant was collected 48 h post transfection.

Retroviral transduction and doxycycline treatment. Inducible HDAC7 expression in SD-1 and Namalwa cell lines was achieved by the generation of the SD-1 and Namalwa Tet-On-Tight-HDAC7 cell lines. In brief, SD-1 and Namalwa cells were first infected with the supernatant containing the pRetro-X-Tet-On-Advanced viral particles overnight and 72 h later selected with 1.5 μg/ml geneticin (GIBCO, Carlsbad, CA, USA). Next the selected cells were infected with the pRetro-X-Tight-Pur-HDAC7 viral particles overnight and after 72 h selected with 3 μg/ml puromycin. For HDAC7 expression, cells were treated with 500 ng/ml of doxycycline for the indicated periods. For c-Myc expression or HDAC3 knockdown, SD-1 and Namalwa Tet-On-Tight-HDAC7 cell lines were transduced with MSCV-Empty-GFP, MSCV-c-Myc-GFP, pLKO.1-GFP and pLKO.1-shHDAC3KD1+KD2-GFP and GFP-positive cells were sorted by flow cytometry.

Proliferation and cell cycle assays. For the MTT assays, 5×10^4 cells were plated onto 24-well plates. At different times, MTT was added at a final concentration of 5 mg/ml. After incubation for 3 h (37 °C, 5% CO_2), the blue formazan derivative was solubilized in dimethyl sulfoxide and the absorbance was measured at 570 nm. Cell proliferation was also assessed by cell counting. Cell cycle and apoptosis were assessed by propidium iodide staining (distribution of cells in G_0/G_1, S and G_2/M phase, and in $SudG_0$) followed by flow cytometry analysis using a Gallios flow cytometer (Gallios, Beckman-Coulter, Brea, CA, USA).

Co-Immunoprecipitation assays. Co-immunoprecipitation assays were performed as previously described in.[26]

Mouse xenograft assay. Five-week-old male athymic *nu/nu* mice (Charles River, Wilmington, MA, USA), housed under specific pathogen-free conditions, were used in this study. To minimize tumor growth dispersion observed by subcutaneous injection of SD-1 cell line, SD-1-Tet-On-Tight-HDAC7 cells were developed in two steps: (i) 5×10^6 SD-1-Tet-On-Tight-HDAC7 cells were subcutaneously injected into the back of $n = 5$ animals. Once the tumors grew, they were harvested, cut into equal size small fragments and subcutaneous transplanted into the back of other nude mice. Mice bearing subcutaneous engrafted tumors (150–200 mm^3) were randomly allocated in the two treatment groups: (i) mice drank 1% glucose; and (ii) drank 1% glucose plus 2 mg/ml doxycycline in water. The tumor growth was recorded twice per week and tumor volume (in mm3) was estimated according to the formula $V = \pi/6 \times L \times W^2$.(W) width and (L) length. For Namalwa-Tet-On-Tight-HDAC7 cells (1.5×10^6) were injected orthotopically into the middle of the spleen and the tumors were monitored by palpation twice a week. Likewise, mice were randomly allocated in the two treatment groups: (i) mice drank 1% glucose; and (ii) drank 1% glucose plus 2 mg/ml doxycycline in water. At the time of killing (30 days after induction in SD-1 cells and 15 days for Namalwa cells) all the tumors were excised and weighed, analyzed macroscopically and by hematoxylin and eosin tissue staining for histological assessment. All experiments were approved by the IDIBELL animal care and use committee.

Immunofluorescence. Tumors were fixed in 4% formaldehyde overnight at 4 °C, embedded in paraffin wax and sectioned at 4 μm. For immunofluorescence staining, antigen retrieval was performed in 10 mM sodium citrate (pH 6.0). Tumor sections were blocked with 5% horse serum in phosphate-buffered saline for 1 h at room temperature and incubated with primary antibodies overnight at 4 °C. The primary antibody used was anti-Ki67 (Thermo Scientific, Alcobendas, Spain). Tumor sections were then incubated with secondary antibodies for 1 h at room temperature. Nuclei were stained using 4′,6-diamidino-2-phenylindole. Samples were imaged on a Leica TCS SP5 spectral confocal microscope (LEICA, Barcelona, Spain), with a 63XNA 1.4 objective and using the LASAF software version 7 (LEICA). Microphotographs were analyzed with Fiji software (http://fiji.sc/). In brief, images were analyzed in gray scale and median filtered. More than 2400 cells per animal (three glucose; three glucose+Doxy) where analyzed in Ki67 staining and apoptotic nuclei.

Western blot. Western blot analysis was performed according to standard procedures. Western blots were developed with the ECL detection kit (Amersham Biosciences, Pittsburgh, PA, USA).

RT-qPCR expression analyses. RT-qPCR analysis were performed as previously described in.[20]

GSE34861 analysis. Expression profiling (microarray) of 191 samples of adult B-lineage ALL and 3 normal pre-B samples were extracted from GEO database (GSE34861). Raw data were robust multichip average (RMA) normalized using the RMA algorithm in NimbleScan 2.5 software (Roche NimbleGen, Inc, Basel, Switzerland). For differential expression analysis, average Log2 expression of the normal samples were subtracted from each cancer patient expression data (Log2 value) for each gene using in house python script. The association between the immunophenotypes of the various B-ALL patients and the downregulated levels of HDAC7 was examined using the Fisher test. Two HDAC7 probes were analyzed: cds1 Homo sapiens histone deacetylase 7A (HDAC7A), transcript variant 1, mR and cds2 Homo sapiens histone deacetylase 7A, mRNA (cDNA_clone_MGC:74915_IMAGE:6179239), complete cds. Both the probes were normalized with respect to values of healthy patients. GSE34861 data were analyzed under R statistical language. The *q*-values where obtained from multiple correction by false discovery rate (FDR). The odds ratio (OR) shows the association between ALL immunophenotype and the downregulation of HDAC7. If an $OR > 1$ indicates that ALL immunophenotype is positively associated with having downregulated levels of HAC7, whereas $OR < 1$ indicates negative association between having the specific ALL immunophenotype and HDAC7 downregulation. An $OR = 1$ indicates no association between ALL immunophenotype and HDAC7 expression. To identify the associations between the characterized cytogenetic features of B-ALL and low levels of HDAC7 expression we applied the Wilcox test. Data were analyzed using the R statistical language. To assess the correlation between *c-MYC* and the two *HDAC7* probes in the 191 B-ALL patients the Pearson correlation coefficient was computed (rho) and linear regression was performed.

Microarray experiments. SD-1 Tet-On-Tight-HDAC7 or SD-1 cells were treated or not for 24 h with doxycycline and collected in Trizol reagent (Invitrogen, Carlsbad, CA, USA). RNA was extracted as described above. PCR-amplified RNAs were hybridized against an Affymetrix human array chip (Affymetrix Human Genome U219 Strip, Santa Clara, CA, USA) at the IRB genomics facility. Affymetrix raw CEL files have been deposited in the GEO database (GSE51895).

Microarray analysis. Expression data were analyzed using the R statistical program. The RMA method was applied to the raw data. This comprises three steps: convolution background adjustment, probe-level quantile normalization and median polish summarization. Linear model analysis (LIMMA package, Bioconductor) was used to identify the significant upregulated and downregulated genes. A FDR multiple test was applied to the *P*-values obtained from the LIMMA procedure; the upregulated and downregulated genes were considered to be significant for the values of adjusted $P \leq 0.05$. Values of $\log2FC \geq 0.5$ and $\log2FC \leq 0.5$, respectively, indicated the upregulated and downregulated genes.

Functional and pathway enrichment analysis. Functional annotation of differentially expressed genes was based on GO (Consortium, 2000; http://www.geneontology.org) as extracted from the EnsEMBL and the KEGG pathway databases. Accordingly, all genes were classified into ontology categories. We took only the GO/pathway categories that had at least 10 annotated genes. We used GiTools for enrichment analysis and heatmap generation [37](www.gitools.org). The resulting *P*-values were adjusted for multiple testing using Benjamin and Hochberg's FDR method.

Putative TF motif occurrence in promoter region. The possible occurrence of the TF motif in the promoter region (500-bp upstream and 200-bp downstream of the transcription start site) was predicted with the STORM algorithm,[38] applying a cutoff of $P = 0.0000125$ and position frequency matrices from the TRANSFAC database[39] (professional version release 2009.4).

Statistical analyses. All data, except those from the arrays, were analyzed using GraphPad Prism5 (GraphPad, San Diego, CA, USA). Student's *t*-test and one- or two-way ANOVA, incorporating Bonferroni multiple comparisons, were carried out to evaluate the differences between the groups.

Conflict of Interest

The authors declare no conflict of interest.

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Supplementary Information accompanies this paper on Cell Death and Disease website (http://www.nature.com/cddis)

Supplementary Figure legends

Supplementary Figure 1. HDAC7 is a specifically expressed in lymphoid cells. HDAC7 expression levels in murine hematopoietic cell populations obtained from the Immgen public database. Numbers in the x axis represent post-normalized gene expression values. The labels shown in the y axis represent different immune cellular subtypes. A precise description of each cell type and how they were purified is provided in Immgen.org.

Supplementary Figure 2. HDAC7 expression blocks SD-1 cell growth. (**a**) and (**b**) Tom-1 and Raji cells were transduced to express HDAC7 in a doxycycline-inducible manner. Representative western blots showing HDAC7 protein levels after cell treatment with doxycycline. Mean ± SEM of the absorbance units from 3 independent MTT assays performed in triplicate. (**c**) Western blots showing HDAC4 and HDAC9 protein levels in SD-1-Tet-On-Tight-HDAC7 and Namalwa-Tet-On-Tight-HDAC7 cells treated or not with doxycycline.

Supplementary Figure 3. Forced expression of HDAC7 in SD-1 and Namalwa cell lines blocks their proliferation capacity and induces apoptosis. (**a**) Cell cycle and apoptosis were assessed by PI-staining. Distribution of the cells in G0/G1, S, G2/M phases and subG0 was analyzed by flow cytometry. Representative histograms at the indicated times after doxycycline SD-1-Tet-On-Tight-HDAC7 cell treatment are shown. cells in G1, S, G2/M phases and subG0 was analyzed by flow cytometry. Representative histograms at the indicated times after doxycycline SD-1 cell treatment are shown. (**b**) and (**c**) show the percentage of cells in G0/G1, S, G2/M phases from 3 independent experiments with (**b**) SD-1 and (**c**) Namalwa cells treated or not with doxycycline for the indicated times. * $p < 0.05$; ** $p < 0.01$.

Supplementary Figure 4. HDAC7 expression reduced the number of KI67-positive cells and promoted an increase in the number of apoptotic nuclei. Panel (**a**) shows the percentage of cleaved caspase 3 positive cells and panel (**b**) shows the frequency of Ki67-positive cells in the Namalwa-Tet-On-Tight-HDAC7 xenographic assay. More than 2400 cells per animal (3 glucose; 3 glucose + Doxy) were analyzed. * $p < 0.05$. (**c**) Percentage of condensed or fragmented nuclei of all nuclei from > 2400 cells per animal (3 glucose; 3 glucose + Doxy). ** $p < 0.01$. Fluorescence photomicrographs of representative fields are shown. Scale bar: 25 μm.

Supplementary Figure 5. Heatmap and clustering analysis of the differentially expressed genes. Heatmap and clustering analysis of the differentially expressed genes is shown.

Supplementary Datasets

Supplementary Dataset 1. List of upregulated genes after HDAC7 expression in SD-1 cells.

Supplementary Dataset 2. List of downregulated genes after HDAC7 expression in SD-1 cells.

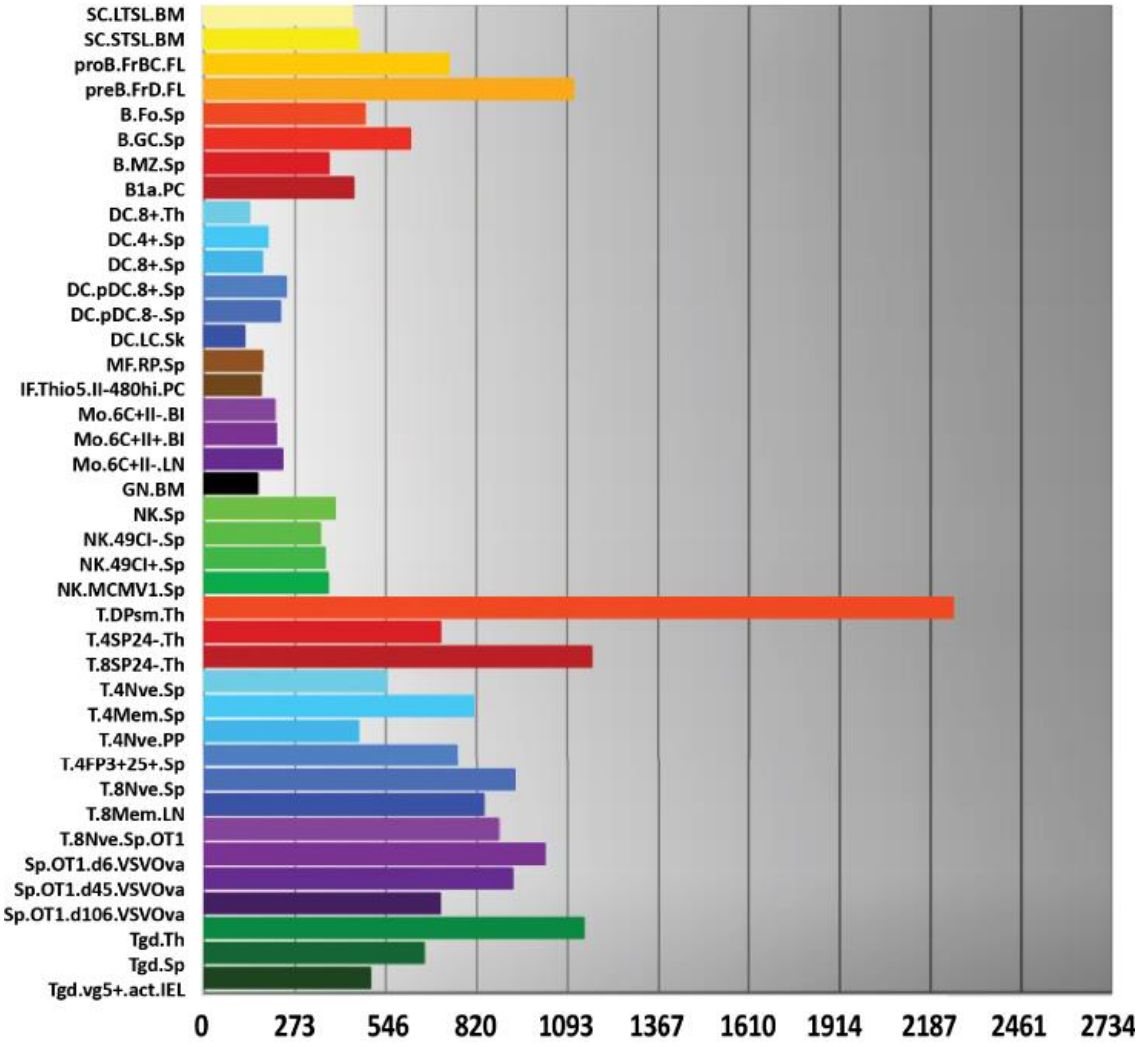
Supplementary Tables

Supplementary Table 1. HDAC7-induced genes belonging to apoptosis, immune processes and cancer categories. SD-1 Tet-On-Tight-HDAC7 or SD-1 cells were treated or not for 24 hours with doxycycline and RNA was collected. Samples were subjected to the Affymetrix Human Genome U219 Strip array. Expression data were analyzed using the R statistical language. The robust multichip average (RMA) method was applied to the raw data. The LIMMA package was used to identify informative

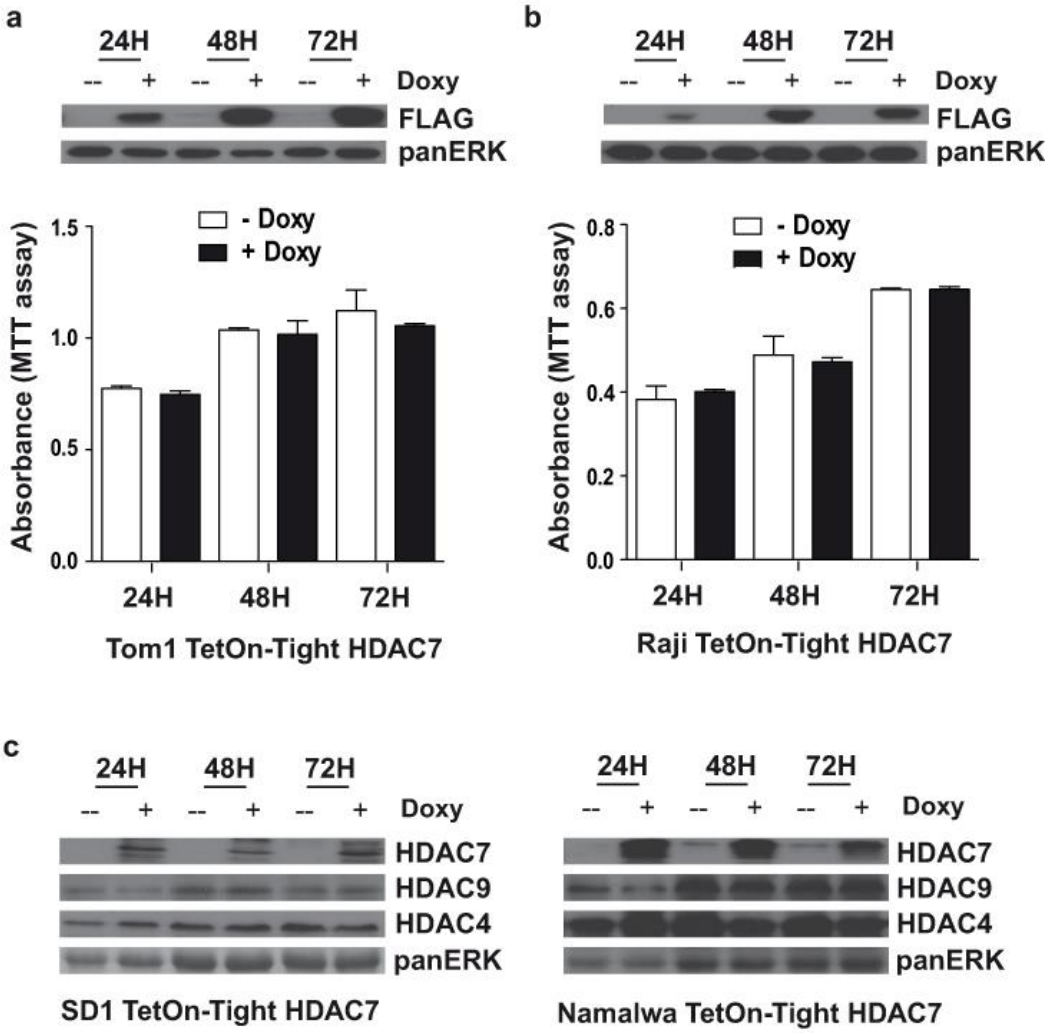
upregulated and downregulated genes, $p < 0.005$. An FDR multiple test was applied to the p-values obtained by the LIMMA procedure; upregulated and downregulated genes were considered to be informative for values of adjusted $p < 0.05$. Distribution of enriched genes upregulated in apoptosis, immune system and/or cancer biological categories (FC, n-fold change *vs.* control).

Supplementary Table 2. HDAC7-repressed genes belonging to immune processes and cancer categories. SD-1 Tet-On-Tight-HDAC7 or SD-1 cells were treated or not for 24 hours with doxycycline and RNA was collected. Samples were submitted to the Affymetrix Human Genome U219 Strip array. Expression data were analyzed using the R statistical language. The robust multichip average (RMA) method was applied to raw data. The LIMMA package was used to identify informative upregulated and downregulated genes. An FDR multiple test was applied to the p-values obtained using LIMMA procedure; upregulated and downregulated genes were considered to be informative for values of adjusted $p < 0.05$ Distribution of enriched genes downregulated in apoptosis, immune system and/or cancer biological categories (FC, n-fold change *vs.* control).

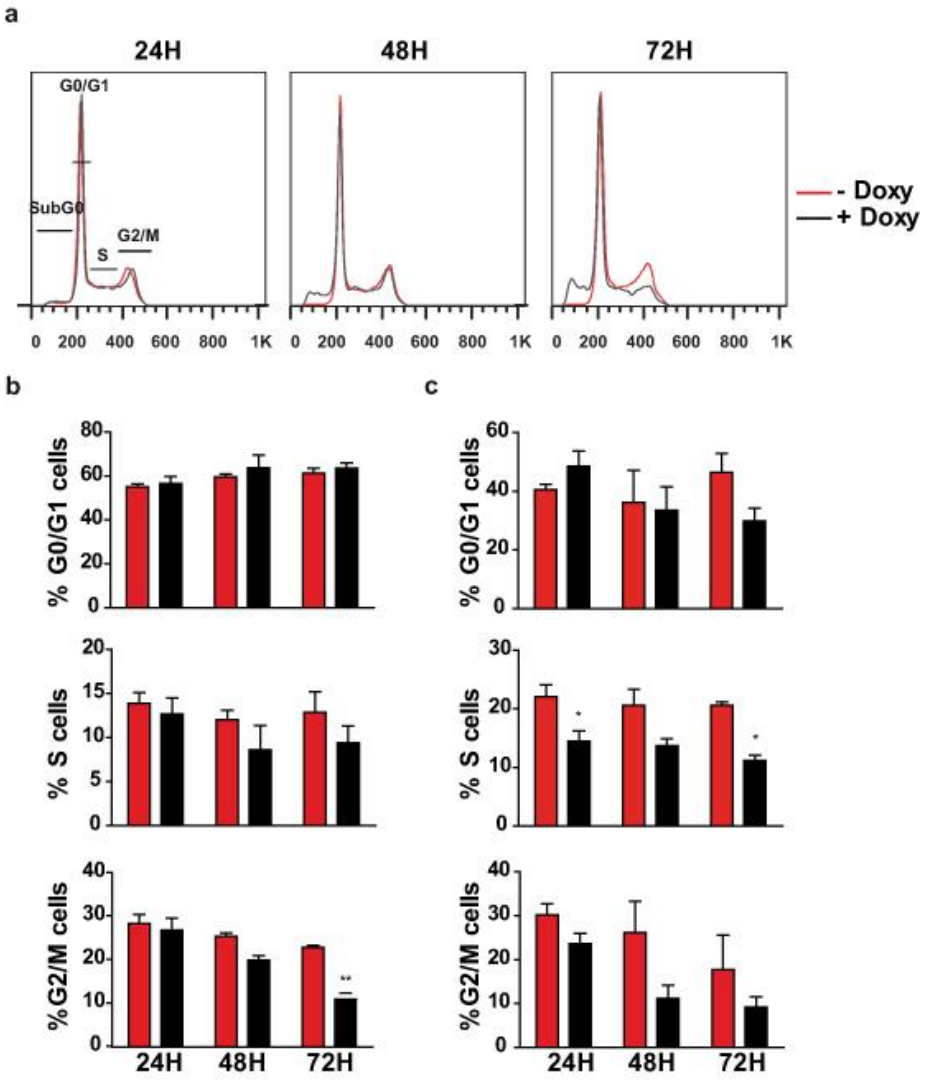
Supplementary Figure 1



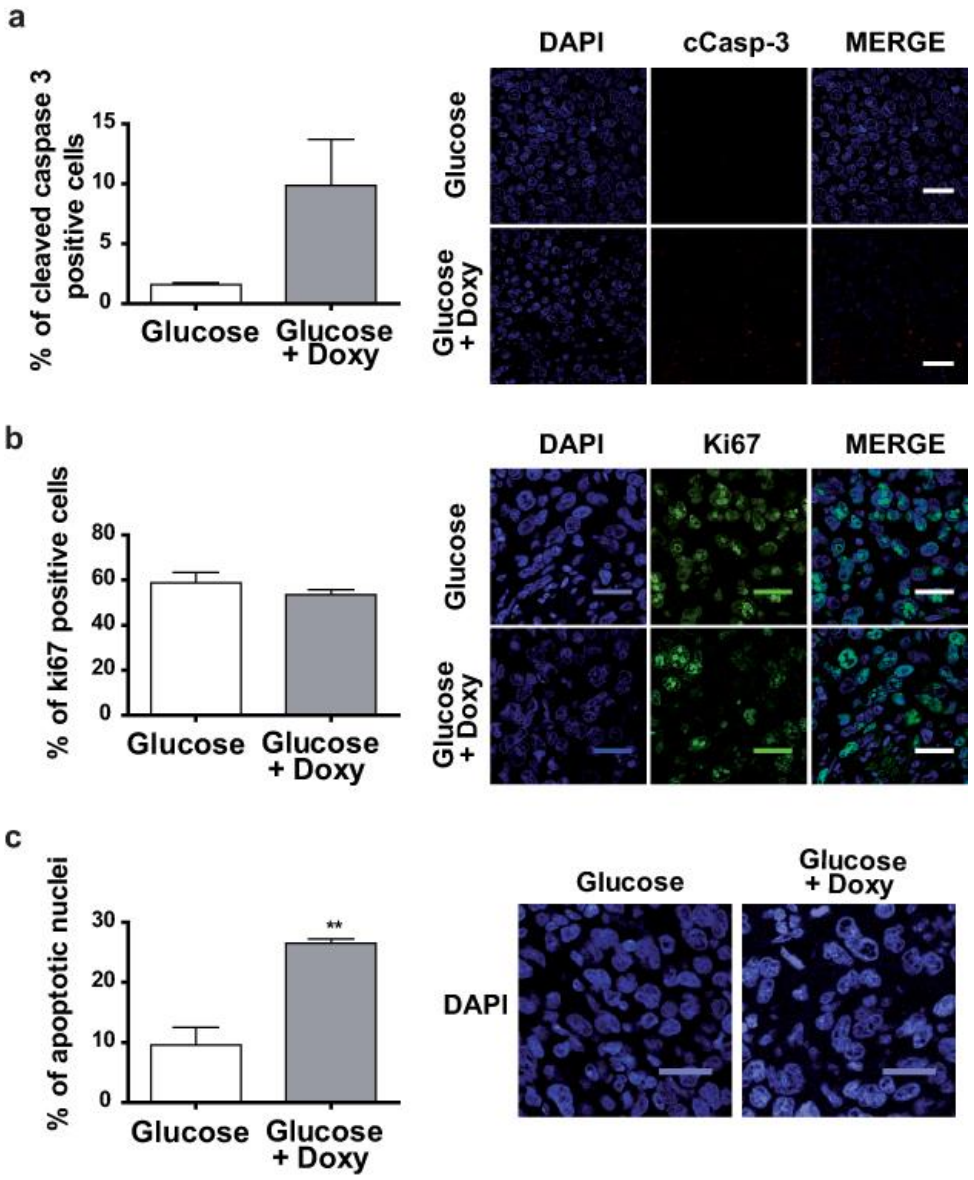
Supplementary Figure 2



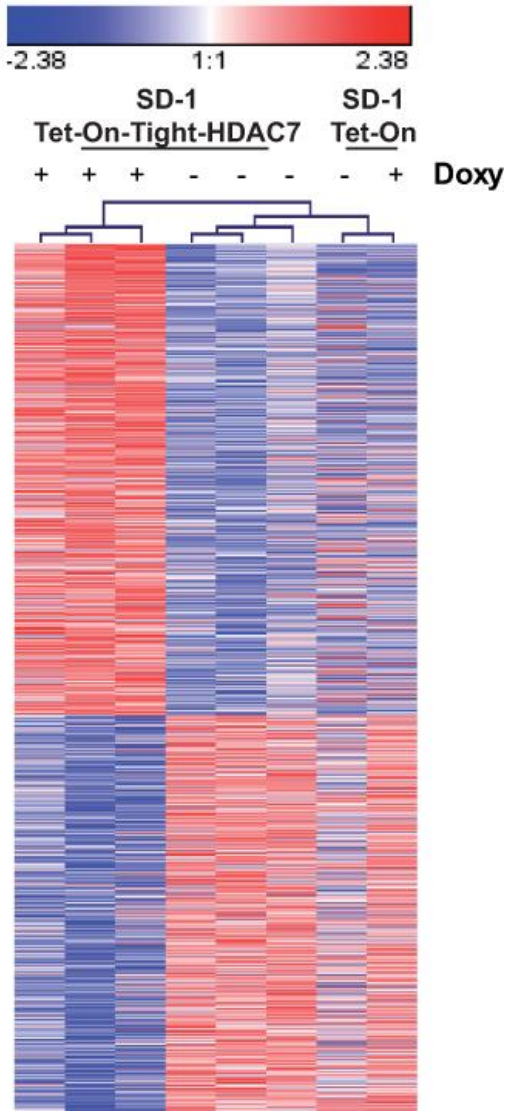
Supplementary Figure 3



Supplementary Figure 4



Supplementary Figure 5



Supplementary Table S2. HDAC7 repressed genes belonging to immune processes and cancer categories.

Log2FC	pValue	Gene Symbol	Apoptosis	Immune System	Cancer	Nature
-1,5	3,41E-05	TNFRSF19			*	TNF-receptor superfamily
-1,5	8,84E-07	IGFBP3			*	insulin-like growth factor binding protein (IGFBP) family
-1,5	7,42E-05	ARHGAP18			*	Rho GTPase-activating protein
-1,4	4,12E-03	AICDA		*	*	RNA-editing deaminase
-1,2	1,90E-04	VAV3			*	Guanine nucleotide exchange factor for RHO GTPASES
-1,1	4,04E-04	ZFAT		*		Transcription factor
-1,1	2,71E-05	CERKL	*			Ceramide kinase-like protein
-1,1	4,30E-05	TERT			*	Telomerase
-1,0	1,66E-04	ITGA4		*	*	Integrin alpha subunit
-1,0	1,23E-03	MYC			*	Multifunctional nuclear phosphoprotein
-0,9	2,50E-03	GNG7			*	Guanine nucleotide-binding protein γ-7
-0,9	2,47E-04	IRF4		*	*	Lymphoid-specific interferon regulatory factor
-0,9	5,16E-04	CTSC		*		A papain-like cysteine protease
-0,8	7,52E-05	DHX33			*	DEAD box protein
-0,7	4,48E-04	MTDH			*	Cell adhesion mollecule
-0,7	1,52E-03	MYBBP1A			*	Nucleo-cytoplasmic transporter protein
-0,7	1,39E-03	EEF1E1			*	Peptide elongation factor
-0,7	5,46E-04	PNO1		*		Rna binding protein
-0,7	1,12E-03	MGLL			*	Serine hydrolase of the AB hydrolase superfamily
-0,6	2,30E-03	TNFRSF13B		*		TNF-receptor superfamily member
-0,5	2,47E-03	TPD52			*	Neoplasm protein

SUMMARY OF RESULTS

SUMMARY OF RESULTS

Among the transcriptional regulators involved in the activation of B cell specific genes and the repression of lineage inappropriate genes, HDAC7 emerged as an important transcriptional repressor of myeloid genes in pre-B cells which becomes down-regulated upon trans-differentiation of pre-B cells into macrophage-like cells. Our main goal, englobed in this thesis, has consisted on studying the role of HDAC7 during early B lymphocyte development by using an *in vivo* conditional knockout mouse model. Additionally, the role of HDAC7 in B cell acute lymphoblastic leukemia (B-ALL) and Burkitt lymphoma cell lines has been studied by using *in vitro* and *in vivo* approaches.

1. HDAC7 is critical for early B cell development through repression of non-B cell genes

In order to elucidate the role of HDAC7 in B cell development *in vivo*, our group developed a conditional knockout mouse model in which HDAC7 is deleted at the pro-B cell stage. First analysis of cell numbers in bone marrow and spleen from wild-type and *Hdac7*$^{fl/-}$ mice revealed that the second group presented a significant reduction by 30% and 45% in the respective organs. Interestingly, analysis of separated cell stages by flow cytometry showed that HDAC7 deficiency lead to a block at the pro-B cell stage, as pro-B cell number increased significantly in HDAC7 deficient mice, whereas pre-B cells and immature B cells underwent a dramatic fall in their numbers. Cell death rate of HDAC7 deficient pro-B and pre-B cells was greater than control cells. Given that HDAC7 appears to be a transcriptional repressor specific for B cells, a gene expression profile was required in order to elucidate whether HDAC7 represses lineage or functionally inappropriate genes preserving B cell identity. Results from microarray experiments showed that HDAC7 deficiency led to up-regulation of >1700 genes and down-regulation of >1400 genes. Relevant up-regulated genes include myeloid and T-cell genes such as *Itgam* and *Cd28*, whereas expression of B lineage genes such as *Pax5* or *Cd19* did not change their expression depending on HDAC7**.** These results indicate that HDAC7 promotes proper early B cell development by repressing non-B cell genes.

2. HDAC7 exert its action through direct recruitment by MEF2C to the promoter and enhancer of inappropriate lineage genes

Our group had previously demonstrated that HDAC7 interacts specifically with the TF MEF2C in pre-B cells but not with other B cell-specific TFs (Barneda-Zahonero et al.,

2013). Thus, we investigated whether HDAC7 is directly recruited to MEF2 binding sites of non-B cell genes promoters in pro-B cells by chromatin immunoprecipitation (ChIP) experiments. Interestingly, HDAC7 was recruited at MEF2 sites located in the promoters and enhancers of lineage inappropriate genes such as *Itgam* and *Cd69*. Similarly, there was a significant increase in the enrichment of the active histone marks H3(K9K14)ac, H3K27ac and a decreased enrichment of the repressive histone mark H3k27me3 at promoters and enhancers of alternative lineage genes upon HDAC7 deletion in pro-B cells. As expected, enrichment at *Pax5* regulatory regions did not change depending on HDAC7 presence. These results agree with the gene expression profiles obtained in microarray experiments. Next, we performed a ChIP-seq experimental approach in order to map H3(K9K14)ac enrichment in all genomic regions in wild-type and $Hdac7^{fl/-}$ pro-B cells. Accordingly, myeloid genes such as *Itgam* or *Cd69* and T cell genes such as *Cd28* possess increased H3(K9K14)ac enrichment upon HDAC7 deficiency in promoter and enhancer regions. Finally, in order to definitively demonstrate the specific recruitment of HDAC7 by MEF2C, $Hdac7^{fl/-}$ B cells were transduced with retroviral vectors containing functional HDAC7 (H7WT) and truncated HDAC7 (H7ΔMEF), which is unable to interact with MEF2C. Wild-type pro-B cells were transduced with an empty vector (MIG) as an experimental control. Expression of normal HDAC7 in knockout cells lead to a significant reduction in expression of *Itgam* and *Cd69* genes, whereas expression of truncated HDAC7 produced the same effect on *Itgam* and *Cd69* as the empty vector. The same results were observed by performing flow cytometry experiments in which Mac-1 expression in splenic knockout B cells was reduced upon normal HDAC7 expression. Thus, these results confirm that the interaction between HDAC7 and MEF2C is crucial for HDAC7 repressive action

3. HDAC7 represses *Tet2* in pro-B cells

Notably, among the genes analyzed in microarray experiments, *Tet2* appeared in the group of up-regulated genes in HDAC7 deficient pro-B cells. RT-qPCR experiments then confirmed that the absence of HDAC7 from pro-B cells leads to a significant increase in the expression of *Tet2*. Using the reprogramming system developed by Graf lab in which our group reported that HDAC7 is progressively downregulated upon trans-differentiation of pre-B cells into macrophages (Barneda-Zahonero et al., 2013), we observed that *Tet2* becomes up-regulated during this process and that exogenous HDAC7 expression prevents the up-regulation of *Tet2*. As expected, forced expression of HDAC7 had no influence on the expression of *Pax5*. To further corroborate that HDAC7 is involved in *Tet2* gene silencing, western blot experiments showed Tet2 protein levels of purified

bone marrow B cells from wild-type and HDAC7-deficient mice. Tet2 levels in HDAC7-deficient B cells were higher than levels from control B cells and similar to non-B cells. Finally, public data from the Immgen database classify HDAC7 as a potential negative and unique regulator of *Tet2*. Together, these findings indicate that HDAC7 acts as a *Tet2* transcriptional repressor in B cell progenitors.

4. HDAC7 is recruited to the promoter and enhancer of the *Tet2* gene in pro-B cells

Further supporting the notion that *Tet2* is an HDAC7 repressed gene in B lymphocytes, data from ChIP-sequencing experiments showed an increase in H3k9k14ac enrichment at both *Tet2* promoter and enhancer in HDAC7-deficient pro-B cells. HDAC7 was recruited to both promoter and enhancer loci of the *Tet2* gene, indicating that it is a direct target. In addition, HDAC7 absence from pro-B cells lead to a significant increase in the enrichment of the active histone marks H3(K9K14)ac and H3K27ac, and a decreased enrichment in the repressive histone marks H3K27me3 and H3K9me3. These data demonstrate that HDAC7 is involved in maintaining low levels of *Tet2* in pro-B cells through interaction with MEF2C and recruitment to its promoter and enhancer.

5. HDAC7 deficiency leads to an increase in 5-hydroxymethylation in pro-B cells

The finding that HDAC7 is involved in maintaining low levels of *Tet2* in pro-B cells suggest that it may have a potential and unprecedented role in DNA 5-hydroxymethylation and methylation status. To investigate this hypothesis, the global levels of 5-hmC in wild-type and HDAC7-deficient pro-B cells were determined by ELISA assays. The absence of HDAC7 led to a significant increase in global 5-hydroxymethylation levels in pro-B cells. Next, we performed a hMeDIP-sequencing genome-wide experimental approach in order to map 5-hmC distribution and enrichment across genome depending on HDAC7. 5-hmC mark was increased in myeloid and other inappropriate lineage genes such as *Jun* in HDAC7 deficient conditions. In fact, RNA-sequencing and ATAC-sequencing data from Immgen database corroborates our results, as the increased 5-hmC mark in HDAC7 deficient pro-B cells coincide with increased expression and chromatin accessibility of *Jun* in macrophages compared to control B cells. HMeDIP-qPCR experiments validated the obtained results from sequencing experiment showing the increase in 5-hydroxymethylation at regulatory regions of inappropriate lineage genes such as *FosL2* and *Jun*. RT-qPCR experiments confirmed an up-regulation in the expression of selected myeloid genes. These results show that HDAC7 plays a role in the DNA hydroxymethylation status at both global and

individual levels through *Tet2* repression and that this hydroxymethylation is linked to demethylation and subsequent expression activation of target genes.

6. HDAC7 regulates expression of micro-RNAs and 5-hmC of L1 transposable elements

Strikingly, additional results revealed that there was an increased enrichment in 5-hydroxymethylation in some repetitive genome regions (LINE-1 retrotransposons) upon the absence of HDAC7. Previous published data revealed that TET proteins (especially TET1 and TET2) are recruited at the extreme 5'UTR of LINE-1 elements in embryonic stem cells (ESCs) (de la Rica et al., 2016). Given that deletion of HDAC7 leads to an up-regulation of *Tet2* expression, this can produce an increase in TET2 recruitment and 5-hmC enrichment at LINE-1 elements. In order to investigate whether 5-hydroxymethylation might be correlated to expression in these genomic regions, we performed RT-qPCR experiments for L1 transcripts including transcript of the chaperon-like protein (ORF1p). Notably, expression of 1 transcript increased in HDAC7 deficient pro-B cells. These results shed light on the potential role of HDAC7 in regulating somehow L1 transposon methylation and activity. Alternatively, selected microRNAs such as miR125-b also presented increased 5hmC enrichment in pro-B cell from *Hdac7*$^{fl/-}$ mice. We wondered whether HDAC7 might be silencing inadequate genes and preserving B cell identity through micro-RNA expression. Thus, we performed miRNA profiling by using a quantitative PCR-based panel containing over 375 different miRNAs (miRCURY LNA^{TM} microRNA Array (Exiqon)) in wild-type and HDAC7 deficient pro-B cells. Among them, 25 microRNAs presented significant differential expression between control and HDAC7 deficient pro-B cells. They are represented in a heat map and were validated with RT-qPCR experiments. Among up-regulated miRNAs in HDAC7 deficient conditions, we obtained miR125b-5p, a micro RNA more abundant in macrophage cells than other immune cell types and up-regulated in several types of leukemia, and miR-34a, a miRNA that blocks B cell development (Chaudhuri et al., 2012; Rao et al., 2010).

7. HDAC7 is down-regulated in some leukemia and lymphoma cell types

The de-regulation of specific transcriptional programs involved in B lymphocyte development might result in a block in B-cell proliferation and differentiation, promoting the development of hematological malignancies such as leukemia and lymphoma. The study of the role of transcriptional repressors in normal and aberrant B lymphopoiesis is still incomplete, as HDAC's contribution to tumor-suppressing and oncogenic functions and the use of HDAC inhibitors for cancer therapy have been

controversial during the last years. We report that HDAC7 is under-expressed in pro-B acute lymphoblastic leukemia (pro-B-ALL) and Burkitt lymphoma. In addition, *in vivo* (xenograft models) and *in vitro* approaches demonstrated that ectopic expression of HDAC7 blocks cell proliferation, induces apoptosis, give rise to the downregulation of c-Myc and inhibits the oncogenic potential of cells. Notably, we also observed that low levels of HDAC7 expression in B-ALL patient samples correlated with the increased levels of c-Myc. Remarkably, role of HDAC7 on apoptosis induction is independent of HDAC3 function and that requires MEF2C interaction to exert its function. Altogether, our findings report that HDAC7 has a potent anti-oncogenic effect on selected blood cancers and that its de-regulation may contribute to the tumorigenesis in B lymphocytes.

DISCUSSION

DISCUSSION

How do cells decide to acquire their final identity to generate specific tissues and organs? For many years, this has been a fundamental question for basic and clinical researches that work in the field of cell development. Hematopoiesis has been an extensively studied model to answer this question. Within the hematopoietic system, the generation and differentiation of B lymphocytes is a complex process that takes place in a step-wise manner and requires a tight regulation. Indeed, genetic and epigenetic programs are dynamically modified at each cellular transition. This is achieved by the action of specific networks of TFs (Bruna Barneda-Zahonero B et al., 2012, 2012; Parra, 2009; Recaldin & Fear, 2016). The concept that gene silencing is essential for proper cell differentiation and development has been considered for many years. Notably, aberrant establishment of inappropriate transcriptional programs may lead to the development of B cell malignancies. Critical TFs for B cell development and differentiation are not only involved in the activation of B cell specific genes, since they are also involved in the repression of undesirable genes, ensuring maintenance of proper B cell identity and differentiation (Delogu et al., 2006; Ikawa et al.,2004; Kong, et al., 2016; Nechanitzky et al., 2013; Pridans et al., 2008; Ramírez, Lukin, & Hagman, 2010; Stehling-Sun et al., 2009). However, how gene silencing is established and which lineage-specific transcriptional repressors are involved during hematopoiesis and B cell lymphopoiesis are still unsolved questions in the field.

1. The transcriptional repressor HDAC7 is a key regulator of early B cell development

The Cellular Differentiation group found that HDAC7 is highly expressed in pre-B cells and not in myeloid cells such as macrophages (Barneda-Zahonero et al., 2013), pointing to its potential role as a B cell-specific transcriptional repressor. Using a pre-B cell to macrophage reprogramming system, our laboratory observed that HDAC7 is dramatically downregulated during cellular conversion. Exogenous HDAC7 expression abrogates the functional capacities of the reprogrammed macrophages and the establishment of macrophage gene transcriptional program (Barneda-Zahonero et al., 2013). Interestingly, several studies reported that *Hdac7* is a potential target gene of the TFs PAX5, E2A, EBF1 and FOXO1 in pro-B cells (Revilla-i-Domingo et al., 2012; Lin et al., 2010b). Additionally, Young and colleagues showed that the *Hdac7* gene carries a super-enhancer occupied by PU.1 in pro-B cells (Whyte et al., 2013). These data support our hypothesis that HDAC7 may be a master transcriptional repressor during early B cell development.

During the execution of this PhD thesis, by using a mouse model for specific deletion of HDAC7 in pro-B cells, we have demonstrated that HDAC7 is a transcriptional repressor crucial for early B cell development (Azagra et al., 2016). In pro-B cells, HDAC7 represses lineage inappropriate genes such as *Itgam* and *Cd28* characteristics of macrophages and T cells, respectively.

Additional evidences supporting the relevance of HDAC7 for B cell development are based on the dramatic impairment in the transition from pro-B to pre-B cells in HDAC7-deficient mice, similarly to other critical TFs for B cell development such as PAX5 (Nutt et al., 1997). Increased cell death rate in pro-B and pre-B cells (statistically significant in the second case) and reduced total number of cells in spleen and bone marrow in *Hdac7*$^{fl/-}$ mice indicate that both B cell stages are more prone to apoptosis in the absence of HDAC7. Despite Annexin-V test did not present significant differences in apoptosis rates between control and HDAC7 deficient pro-B cells, we observed the upregulation of *Trp53* and other apoptotic-related genes. Thus, alternative cell programed death processes (such as necroptosis or pyroptosis) may be *a priori* discarded and we could speculate that the increased cell death of HDAC7 deficient pro-B cells could be due to a higher susceptibility to apoptotic processes (Tait et al., 2014).

Next, we aimed to decipher the mechanism of action of HDAC7 during early B cell development. Given that HDAC7 interacts with the TF MEF2C and that MEF2C binding motifs are found at promoters of HDAC7 targets genes in pre-B cells (Barneda-Zahonero et al., 2013), we wondered whether HDAC7 was be recruited by MEF2C to lineage inappropriate genes in wild-type pro-B cells. Chromatin immunoprecipitation (ChIP) experiments corroborated this hypothesis as both HDAC7 and MEF2C were recruited at the promoters of myeloid and T cell genes in pro-B cells. Furthermore, gain-of-function experiments revealed that the interaction between the two proteins is required for HDAC7 repressing function. The induction of a truncated form of HDAC7 in pro-B cells from *Hdac7*$^{fl/-}$ mice was unable to interact with MEF2C and, consequently, did not suppress the upregulation of *Itgam* and *Cd69* genes. Thus, these results demonstrate that HDAC7 is recruited at both the promoter and the enhancers of its targets genes through TF MEF2C in pro-B cell stage (Figure 1).

As an epigenetic regulator, HDAC7 is thought to modulate histone marks enrichment, in particular histone acetylation, in pro-B cells. During the analysis of histone marks enrichment at HDAC7 target genes, we observed that H3(K9/K14)ac and H4K16ac were already present at the promoters of target genes in wild-type pro-B cells.

However, the absence of HDAC7 in pro-B cells produced a significant increase of both histone marks and a decrease of the repressive mark H3K27me3 in the promoters of its target genes. The concept that pro-B and pre-B cells present some grade of cellular plasticity, in which alternative lineage genes may be poised and silenced in a reversible manner is supported by three evidences. First, active histone marks are slightly enriched at macrophage genes in wild-type pro-B cells. Second, the deletion of specific B cell regulators alters the lineage expression pattern. And third, variations in levels of selected TFs such as C/EBP family members can change initial cell fate decisions, leading to conversion of precursor B cells into functional myeloid cells such as macrophages or granulocytes (Cirovic et al., 2017; Cobaleda and Busslinger, 2008). This plasticity is guided by multiple factors, including the TFs PAX5, C/EBPα, Myc, chromatin regulators and three-dimensional (3D) chromatin conformation, which in coordination modulate lineage specific gene signatures, orchestrate cell commitment and fate decisions and determine the predisposition of a cell to transdifferentiate efficiently (Boya et al., 2017; Francesconi et al., 2019).

Given that the active histone marks H3(K9K14)ac are present in bivalent promoters and enhancers in addition to active promoters in murine ES cells (Karmodiya et al., 2012), we looked for other genomic regions with H3(K9/K14)ac enrichment in wild-type and HDAC7 deficient pro-B cells in our ChIP-seq experiment. Interestingly, Graf and colleagues determined the presence of enhancers at myeloid genes in pre-B cells. During the transdifferentiation of pre-B cells into macrophages, the myeloid TF C/EBPα can bind to pre-existing enhancers already bound by PU.1 or to *de novo* enhancers, both of them myeloid enhancers in pre-B cells (van Oevelen et al., 2015). Notably, by examining our ChIP-seq data for those enhancers in myeloid genes with H3(K9K14)ac enrichment in pro-B cells, we found that pre-existing enhancers bound by PU.1 were indeed occupied by H3(K9/K14)ac mark in wild-type pro-B cells and presented an increased enrichment in HDAC7 deficient pro-B cells. Some studies indicated that enhancer priming has a scarce contribution to lineage specification (either lymphoid or myeloid), since most of enhancer activity comes from *de novo* generation and repertoire of activated enhancers in progenitor cells differs almost completely from mature cells (Choukrallah et al., 2015; Luyten et al., 2014). However, our findings support the fact that epigenetic machinery presents a key function in conferring plasticity through enhancer priming or poising and, consequently, in preventing alternative lineage genes from being activated in pro-B cells in a reversible manner.

Consistent with our observation, we found that HDAC7 is also recruited to enhancers of *Itgam* and *Cd69 g*enes, and that H3(K9K14)ac, H4K16ac and H3K27ac are higher

enriched at these loci in HDAC7 deficient pro-B cells compared to wild-type cells. The increased enrichment of H3K27ac at enhancers of macrophage genes in *Hdac7*$^{fl/-}$ pro-B cells supports the evidence of close relationship existing between enhancer's activity and cell identity (Creyghton et al., 2010).

Several studies have correlated an increase in H3K27ac enrichment at global levels with some hematological malignancies, such as myeloid leukemia with MLL-AF9 fusion protein expression. In fact, the increase of enrichment of several active histone marks and the decrease of repressive marks such as H3K27me3 at regulatory regions of lineage inappropriate lineage might produce a histone methylation-to-acetylation change that may trigger hematological malignancies progression and, therefore, endow HDAC7 with a potential preventive role in tumor development (Sun et al., 2015). The connection between HDAC7 and hematological diseases will be discussed in the last part of the discussion.

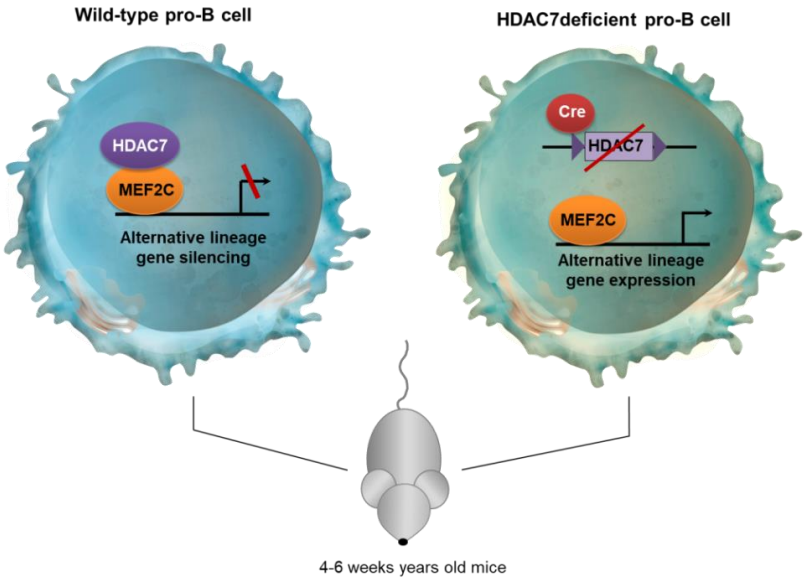


Figure1. HDAC7 represses the expression of lineage inappropriate genes in pro-B cells. In wild-type pro-B cells (left), HDAC7 is recruited to regulatory regions of lineage inappropriate genes through the interaction with the TF MEF2C in order to repress their expression. In pro-B cells where *Hdac7* gene is cleft by Cre recombinase activity (right), there is an aberrant activation of these alternative lineage genes.

2. Role of HDAC7 in DNA 5-hydroxymethylation in B cell progenitors

Interestingly, the gene encoding for the TET2 enzyme was found among the set of upregulated genes upon HDAC7 deletion in pro-B cells. While HDAC7 has a specific lymphoid expression pattern, TET2 is an enzyme broadly expressed in the

hematopoietic system including B cells but reaches highest expression levels in myeloid cells (Ko et al., 2010; Kallin et al., 2012; Barneda-Zahonero et al., 2013). Despite several studies have reported a relevant role of TET2, in cooperation with TET3, during B cell development and differentiation, as well as its abundant presence through this lineage, others have observed that it becomes essential for proper myelopoiesis and its expression is higher in myeloid cells compared to lymphocytes (Lio et al., 2016; Orlanski et al., 2016; Ko et al., 2010). In fact, public data from the Immunological Genome project (Immgen) unveiled HDAC7 as a unique potential negative regulator of *Tet2* expression, considering the opposite expression patterns between the two proteins in the hematopoietic lineages. Given the apparent controversy about the levels of TET2 expression in hematopoietic cell lineages, our results combined with Immgen data highlight the importance of comparing cell lineages and reflect that further studies are required to unveil more detailed TET2 regulation mechanisms in the the hematopoietic system.

Using our *in vivo* HDAC7 conditional knockout mouse model and the *in vitro* reprogramming system developed by Thomas Graf's group (Bussmann et al., 2009), we observed that *Tet2* was upregulated in HDAC7 deficient pro-B cells and that HDAC7 exogenous expression lead to a block in the upregulation of *Tet2* during trans-differentiation of pre-B cells into macrophages (Azagra et al., 2016). Next, by performing ChIP experiments, we observed that HDAC7 is recruited to the promoter and the enhancer of the *Tet2* gene and that HDAC7 deficiency lead to an increased and decreased enrichment in active (H3(K9K14)ac, H3K27ac) and repressive (H3K27me3, H3K9me3) histone marks placed at those regulatory regions, respectively. Interestingly, H3K9me3 mark is not only related to retrotransposons repression in embryonic cells. Recent studies have reported that it is also involved in the silencing of lineage inappropriate genes, the prevention of cell reprogramming and maintaining oligodendrocytes and T-lymphocytes lineage stability (Allan et al., 2012; Becker et al., 2016; Bulut-Karslioglu et al., 2014). Thus, the significant decline in H3K9me3 enrichment upon HDAC7 deficiency may be associated with the loss of B lymphocyte identity.

Our findings corroborate that HDAC7 indeed represses *Tet2* expression in pro-B cells and pre-B cells by a direct silencing mechanism. Its classical partner MEF2C may recruit HDAC7 at the promoter as well as the enhancer of *Tet2*, similar as occurred with the myeloid genes *Itgam* and *Cd69* reported in the first article and mentioned in the first part of the discussion (Azagra et al., 2016).

TET2 functions as an epigenetic regulator involved in the conversion of 5-mC to 5-hmC. Next, we wondered whether HDAC7 could have a contribution in 5-hmC in pro-B cells, which would suppose a totally unexpected role for a class IIa HDAC. Despite the average percentage of 5-hmC levels in hematopoietic cells is quite low (~0,2%) compared to other cell types such as mouse Purkinje cells or embryonic stem cells (ESCs) (~5%) (Kriaucionis and Heintz, 2009; Ficz et al., 2011), we observed that global levels of 5-hmC in pro-B cells increased significantly by 2-fold upon HDAC7 deletion.

In order to analyze all those regions that presented differential enrichment in 5-hmC depending on HDAC7 function, we performed hMeDIP-seq experiments. We observed higher number of 5-hmC peaks were located at intergenic and distal TSS regions compared to promoter regions. These results support the dependence of distal regulatory regions with enhancer features to TET2-mediated DNA demethylation and correlate with the existence of additional mechanisms that control DNA methylation status at promoter-associated regions (Rasmussen et al., 2019; Rasmussen and Helin, 2016).

Next, we corroborated that *Tet2* upregulation in pro-B cells from *Hdac7*$^{fl/-}$ mice produced an increased enrichment of 5-hmC at promoters and enhancers of myeloid and T cell genes including *Jun*, *Fosl2* and *Cd28* (Figure 2). These results are in accordance with RNA-seq and ATAC-seq data from the Immgen database in which the expression and chromatin accessibility of these genes is higher in macrophages compared to pro-B cells. Furthermore, the 5-hmC enrichment in *Jun* gene in HDAC7 deficient pro-B cells correlates with *Jun* enhancer demethylation prior to B cell reprogramming through Tet2 recruitment (Sardina et al., 2018).Altogether, these findings consolidate DNA 5-hmC as an essential regulatory strategy in order to guide cell differentiation and maintain B cell identity, by reinforcing the repressive role of HDAC7 towards inappropriate lineage genes and raising it as an essential key regulator of B cell development, through the unexpected ability to modulate DNA 5-hmC levels. Our data indicate that HDAC7 not only induces gene silencing by direct recruitment to target genes, but also by regulating the expression of additional epigenetics players such as TET2.

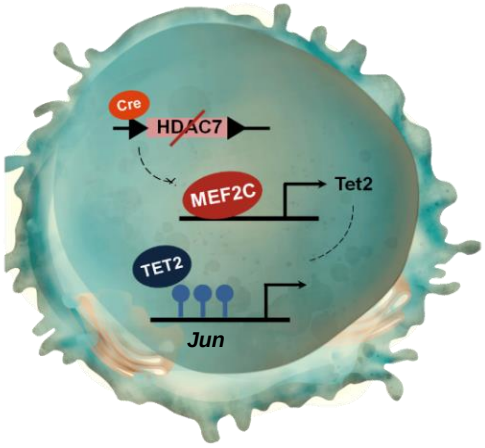


Figure2. HDAC7 conditional deletion leads to *Tet2* upregulation and increase 5-hmC in pro-B cells. HDAC7 deletion by Cre recombinase activity at the pro-B cell stage leads to *Tet2* upregulation and increase of 5-hmC, at global and specific levels (e.g. myeloid genes such as *Jun*).

3. Potential role of HDAC7 in preserving B lymphocyte chromatin stability

DNA methylation is one of the main epigenetic mechanisms involved in preserving chromatin stability, preventing aberrant transcriptional activation and maintaining genetic content. Strikingly, HDAC7 deficiency and consequent *Tet2* upregulation also gave rise to enrichment of 5-hmC in multiple non-LTR-retrotransposon elements (LINE-1) in pro-B cells. These results are consistent with previous studies which supported that TET enzymes are recruited at the 5'UTR of young L1 elements in murine embryonic stem cells (mESC) and that LINE-1 elements are regulated by DNA methylation in more differentiated cells. Despite H3K9me3 related to L1 repression is associated to embryonic cell stage, the decrease of its enrichment upon HDAC7 deletion in progenitor cells could be attributed to enhanced 5-hmC in L1 elements (Bulut-Karslioglu et al., 2014; de la Rica et al., 2016).

Recent studies indicated that 5-hmC does not necessarily involve an increased expression of L1 elements, since the deletion of TET enzymes in ESCs does not alter L1 expression levels. These results suggest that activation of L1 activity may require additional factors and mechanisms rather than TET-mediated demethylation (Gerdes et al., 2016). However, our results from RT-qPCR experiments showed that higher 5-hmC levels at L1 elements were accompanied by a tendency of increased L1 transcripts in HDAC7 deficient pro-B cells. Given that aberrant expression of LINE-1 elements is associated with chromatin instability and that uncontrolled retrotransposition can activate apoptotic pathways, these results suggest that HDAC7 might be required to maintaining the silenced status of LINE-1 elements in accordance to our previous

results in which HDAC7-deficient pro-B and pre-B cells showed an increased cell death rate compared to wild-type B cell populations (Azagra et al., 2016; Bourque et al., 2018; Haoudi et al., 2004).

Therefore, an apparent increase in L1 transposition in HDAC7 deficient conditions could give rise to higher rates of DNA damage and, consequently, higher signal of γH2AX, a relevant marker of double strand breaks (DSB). We obtained unexpected results, since γH2AX signal in pro-B cells from *Hdac7*$^{fl/-}$ mice was even weaker than in their wild-type counterparts. We wondered whether these results could be explained by different mitotic rates between two conditions, as multiple studies indicate that γH2AX signal gains intensity with the progression of cells through the cell cycle, reaching their maximum levels in metaphase, independently of DNA damaging (Ichijima et al., 2005; McManus and Hendzel, 2005; Turinetto and Giachino, 2015). However, cell cycle analyses performed in the first article of the results section showed that there were no significant differences in mitotic rates between control and HDAC7 deficient pro-B cells (Azagra et al., 2016).

Notably, B lymphocytes also undergo RAG-driven DSBs at multiple developing stages, including pro-B and pre-B cell stages, to produce rearrangements at the heavy chain of immunoglobulin (IgHμ) and pre-BCR assembly (Bednarski and Sleckman, 2019). Hence, the significant fall of IgH expression and the less efficient V(D)J rearrangement upon HDAC7 deficiency could explain the observed increase in γH2AX signal in control B cells, associating them to DSBs induced by RAG proteins. Then, is L1 enhanced transcription in *Hdac7*$^{fl/-}$ pro-B cells producing additional DNA damage? Is this additional damage related to increased cell death rates?

As mentioned above, it is well-known that de-regulated L1 expression and consequent transposition can lead to chromatin instability and mutagenesis. We could speculate that pro-B cells, upon HDAC7 deficiency, might undergo p53-driven apoptosis to silence the de-regulation of L1 elements, preventing mutagenesis and aberrant transformation. Notably, it has been reported that de-regulation of short retrotransposons (SINEs) in murine fibroblasts led to an apoptotic induction mediated by type I IFN response (Leonova et al., 2013; Levine et al., 2016; Tiwari et al., 2018). On the other hand, some studies reported a correlation between L1 mRNA expression and IFN expression in some autoimmune diseases (Mavragani et al., 2016). Regarding potential mutagenic effect of deregulated L1 activity, it has been reported that blood cancers have registered very few cases of retrotransposition compared to other cancer types such as epithelial tumors (Burns, 2017). This could be explained by the fact that

initiating cancer cells must restrict expression of TEs through H3K9me3-mediated silencing in order to avoid an immune response that could threaten their viability and activate programmed cell death (Chiappinelli et al., 2015). Thus, accumulation of mutagenic events produced by enhanced L1 activity which might promote transformation of immune cells could generate either a IFN-driven immune response or excessive genome instability that lead to apoptosis (Robbez-Masson et al., 2017; Cuellar et al., 2017). Additionally, apoptotic mechanisms induced by IFN in malignant cells involve the activation of genes such as *Bcl2* (Kotredes and Gamero, 2013).

Despite not all L1 elements produce mutagenic effects during their genomic movements (Bourque et al., 2018), L1 enhanced 5-hydroxymethylation and expression upon HDAC7 deletion in pro-B cells may be correlated to the upregulation of pro-apoptotic genes such as *Trp53 and Ifng.* This evidence suggests that these transposable elements might be compromising host cell viability. Thus, HDAC7 might contribute to the maintenance of chromatin stability by preventing aberrant DNA demethylation and subsequent activation of L1 elements through repression of TET2 demethylase enzyme, avoiding additional DNA damaging events.

Additional experiments should be performed, in order to elucidate whether L1 elements are disturbing severely the chromatin stability of HDAC7 deficient pro-B and pre-B cells. For instance, it would be necessary to evaluate whether these cells present aberrant loss of DNA content (e.g. aneuploidy) or impaired nucleosome integrity.

Accordingly, recent studies indicated that tight regulation of TET2 activity is essential for the correct maintenance of genome stability, as TET2 deficiency produce defects in DNA damage response whereas *Tet2* gene over-expression leads to chromosome instability and aneuploidy due to a collapse of BER activity (Kafer et al. 2016; Manfoudhie et al. 2016). These evidences correlate with our proposal about the potential role of HDAC7 in preserving genome stability and integrity in B cells by regulating proper levels of TET2 expression.

Despite the mechanisms of TEs regulation in mammals are still poorly understood, it has been established that an important part of the epigenetic machinery is dedicated to the regulation of TEs through complex regulatory systems that act in a very specific-manner (He et al., 2019)). Further studies are needed to define the contribution of HDAC7 in TE regulation. However, our findings place HDAC7 as a critical regulator of proper B cell identity, by preventing aberrant gene expression and preserving chromatin integrity. Furthermore, HDAC7 potential tumor-suppressive role in B cells cannot be discarded and will be discussed in the 5th section of the Discussion.

4. HDAC7 and regulation of micro-RNAs expression

As mentioned in the Introduction, B lymphocyte generation is a complex process tightly regulated by several mechanisms, given that every single cellular stage during development is characterized by a different epigenetic landscape, transcriptional program and function. Among these mechanisms, recent studies the crucial role of miRNAs in both normal and malignant B cell development, by modulating the expression of crucial regulatory genes across different cellular transitions such as *c-Myb* and *Foxp1* (Zhang et al., 2009; Zheng et al., 2018; Chen et al., 2004)**.**

miRNAs expression in the hematopoietic system must be also tightly regulated, since their de-regulation has been associated with the onset and progression of several types of cancers, including diffuse large B cell lymphoma (DLBCL) and chronic lymphocytic leukemia (CLL), as well as with their response to therapy. Remarkably, changes in methylation of the regulatory regions of miRNAs can alter their expression. For instance, the promoter hypermethylation of miR-34b and miR-34c is a common feature of CLL patients and are considered tumour suppressors (Deneberg et al., 2014; Craig et al., 2011; Gulyaeva and Kushlinskiy, 2016; Marques et al., 2015). Alternatively, miRNAs depend on the transcription of a host gene for being expressed, since some of them are included in cluster genes that depend on external stimuli from the immune system for their activation (Gulyaeva and Kushlinskiy, 2016). In addition, some TFs directly modulate the expression of specific miRNAs. For example, MEF2C activates the expression of a region encoding several miRNAs involved in muscle growth and differentiation via a muscle-specific enhancer (Liu et al., 2007).

Strikingly, an analysis of miRNA expression and 5-hmC enrichment in wild-type and HDAC7 deficient pro-B cells revealed different microRNA expression and hydroxymethylation profiles. Among the upregulated miRNAs in HDAC7 deficient conditions, which in addition present higher 5hmC enrichment, we could highlight miR-125b-5p and miR-34a. miR-125b is expressed more abundantly in macrophages and is upregulated in several types of leukemia such as acute myeloid leukemia (AML). Interestingly, de-regulation of miR-125b expression depends on C/EBPα, a critical TF in the myeloid lineage (Chaudhuri et al., 2012; Vargas Romero et al., 2015). In addition, miR-34b/c methylation is observed in leukemic cases whereas miR-34a is highly expressed in myeloid cells and its expression in B cells leads to *Foxp1* repression and, consequently, a blockage of pro-B to pre-B cell transition (Rao et al., 2010). Accordingly, given the oncogenic potential of the de-regulation of Foxp1 expression, miR-34a is upregulated upon DNA damage via p53 pathway in CLL cases,

which correlates with increased L1 upregulation and the increased cell death rate observed in HDAC7 deficient pro-B cells (Cerna et al., 2019). Finally, the analysis of our hMeDIP-seq experiment unveiled that 5-hmC enrichment at miR-34b/c is present in wild-type pro-B cells but totally absent in HDAC7 deficient pro-B cells. This might correlates with aberrant hypermethylation of these genes in tumoral cases.

Among the downregulated miRNAs in HDAC7-deficient pro-B cells, we focused on miR-29a/b, miR-28a, miR-142-3p, miR-150p and miR-181. miR-181 was the first reported miRNA to have a role in B cell lineage. Its expression is upregulated in bone marrow, thymus and spleen and its over-expression leads to an expansion in the number of B lymphocytes (Zheng et al., 2018; Chen et al., 2004). miR-29a/b represses *Tet2* expression and is probably activated by C/EBPα (Kriegel et al., 2012). miR-28a regulates proliferation of B cells and its downregulation is involved in lymphomagenesis, especially in the development of Burkitt lymphoma (Schneider et al., 2014). miR-142 is essential for the correct generation and homeostasis of lymphocytes, since miR-142 null mice present a disruption in B cell expansion and severe immunodeficiency (Kramer et al., 2015). miR-150 is required for mature B cell development. Its ectopic expression in early B cell stages produces a premature inhibition of the TF c-*Myb*, leading to a blockage of the pro-B to pre-B transition. In contrast, its inhibition enhances expression of *c-Myb* and produces an expansion of antibody-producing B cells (Zhou et al., 2007; Marques et al., 2015; Xiao et al., 2007). Such specific-stage action of miR-150 reinforces the required coordination between multiple regulators, ensuring the dynamic changes at every stage during B cell development.

In summary, our findings demonstrate that a class IIa HDAC, HDAC7, can also exert its gene silencing function during B cell development by regulating miRNAs expression, presumably trough interaction with its classical partner MEF2C that may be recruited to regulatory regions of these miRNAs as it does at other specific miRNAs in skeletal muscle (Liu et al., 2007). Consequently, these results shed more light on our understanding of the regulatory mechanisms that preserve B lymphocyte identity and function.

5. HDAC7 and hematological malignancies

The de-regulation of lineage-specific transcriptional programs can originate a block of B cell differentiation, an expansion of immature cells and the development of hematological malignancies such as leukemia and lymphoma.

Interestingly, as other key regulators of B cell development, we found that HDAC7 is under-expressed in pro-B-ALL and Burkitt lymphoma cell lines. The ectopic expression of HDAC7 leaded to a block of proliferation, *c-Myc* down-regulation and apoptosis induction in both *in vitro* and *in vivo* approaches. In consequence, the expression of HDAC7 caused a block of tumor growth in a murine xenograft model. In addition, analysis of B-ALL patient samples revealed that low expression of HDAC7 correlated with higher levels of the c-*MYC* oncogene. Although the aberrant expression of *c-MYC* in ALL patients has no impact on their prognosis (Allen et al., 2014), this potentially negative regulation exerted by HDAC7 on c-MYC confers it a potential anti-oncogenic activity. Apparent controversy of HDAC7 role in apoptosis of normal pro-B and pro-B-ALL cells reinforce the dual function of some key regulatory factors such as IKAROS, depending on the cellular context (either physiological or pathological status).

Following with the controversy, several studies indicated that aberrant over-expression of HDACs was related to malignant development and adverse prognosis, e.g. the association of HDAC1/2 with tumor invasiveness and the upregulation of HDAC8 in neuroblastoma, as explained in the Introduction chapter. Therefore, HDAC inhibitors (HDIs) have emerged as promising therapeutic agents against cancer. However, most HDIs are not specific enough to avoid side effects because of the alteration of additional enzymes (Li and Seto, 2016b; Barneda-Zahonero and Parra, 2012). On the other hand, it has been reported that some HDACs are under-expressed in some tumors. For instance, Heideman et al demonstrated that deletion of HDAC1/2 *in vivo* promoted T-cell lymphomagenesis due to a block in the early thymocyte development and *c-Myc* over-expression (Heideman et al., 2013). In fact, HDAC1 exerts a tumor-suppressor role in initial tumorigenesis, while it has an oncogenic-role in established tumors (Santoro et al., 2013). Thus, our understanding of the role and influence of specific HDACs in a given cancer type is still incomplete.

Additional results from recent studies support our findings that suggest an anti-oncogenic role to HDAC7 in B cells. First, results from miRNA expression analysis explained in the second part of the results revealed the upregulation of miR-125b in absence of HDAC7 in pro-B cells, potentially contributing to leukemia development. Regarding HDAC7 role in lymphoma development, our findings correlate with more

recent publications in which HDAC7 has been reported to be mutated in 6% of DLBCL patients and classified as a potential tumor suppressor gene in a CRISPR screening study in DBLCL cell lines (Reddy et al., 2017; Morin et al., 2011).

How is HDAC7 downregulated in hematological tumors? Is there a specific and unique mechanism driving its repression? There is no defined answer to these questions, but several possibilities should be considered. On one hand, analysis of the DNA methylation of two hundred leukemia pediatric patients revealed that CpG islands of *HDAC7* gene body were hypomethylated. Given that gene body hypomethylation could be associated with gene repression, it may represent an interesting mechanism of HDAC7 silencing in B-ALL (Lee et al., 2015). On the other hand, the deleterious alterations of key regulators that seems to act upstream of HDAC7 (e.g. PAX5) in such malignancies could affect HDAC7 expression. Finally, current studies investigate changes in chromatin architecture upon malignant development and try to link chromatin profiles to clinical outcomes, as single cell analysis from AML samples present unique epigenetic profiles (Corces et al., 2016a). 3D chromatin reorganization could also modify accessibility of the *HDAC7* gene, preventing its expression.

6. HDAC7–mediated regulation at multiple levels, ¿is it a real enzyme?

It has been reported that class IIa HDACs do not possess intrinsic enzymatic activity on acetylated histones, acting as simple cofactors of transcriptional repressor HDAC3 included in SMRT/N-CoR multiprotein complexes by using (Fischle et al., 2002). In fact, Lahm et al reported that the lack of catalytic activity in this subclass of enzymes lied in a residue change. A conserved tyrosine from the catalytic domain of class I HDACs is substituted for a histidine in class IIa HDACs. Strikingly, the conversion of the histidine into a tyrosine recovered the lost activity of these class IIa HDACs (Lahm et al., 2007). However, it is important to mention that the deacetylase activity of class IIa HDACs, in particular HDAC7, has not been determined in its physiological context, B cells. Additionally, the possibility that HDAC7 may catalyze the removal of other acyl-lysine modifications cannot be discarded.

Our obtained results during these years support the fact that HDAC7 may have intrinsic enzymatic activity. First, HDAC7 is under-expressed in some types of leukemia (pro-B ALL) and lymphoma (Burkitt lymphoma) and its ectopic expression lead to reduced proliferation and apoptosis activation independently of HDAC3 activity (Barneda-Zahonero et al., 2015). Second, HDAC7 deficiency in pro-B cells lead to a block in B lymphopoiesis, specifically in the transition from pro-B to pre-B cells, independently of

other key B cell-specific regulators such as PAX5, E2A or EBF1 (Azagra et al., 2016). Third, HDAC7 is classified as a potential negative regulator of *Tet2* gene in a public database. Other cofactors were not found as potential negative regulators indicating a specific and intrinsic function to HDAC7 over *Tet2* gene silencing.

Altogether, our findings demonstrate that HDAC7 is a master transcriptional repressor of early B cell development, which preserves proper development and maintains cellular identity and chromatin integrity at multiple levels: direct recruitment at promoters and enhancers of lineage inappropriate genes through recruitment of TF MEF2C, repression of *Tet2*, control of 5-hmC level in these genes and LINE-1 retrotransposons and regulation of miRNA expression (Figure 3). Additionally, our results provide a new perspective for other class IIa HDACs potential functions in the physiological context where they are expressed. However, further studies are required in order to elucidate whether HDAC7 catalyzes alternative histone modifications, if HDAC7 is involved in long-range promoter enhancer interactions and has a role in chromatin positioning, or whether HDAC7 have also a critical role in terminal B cell differentiation.

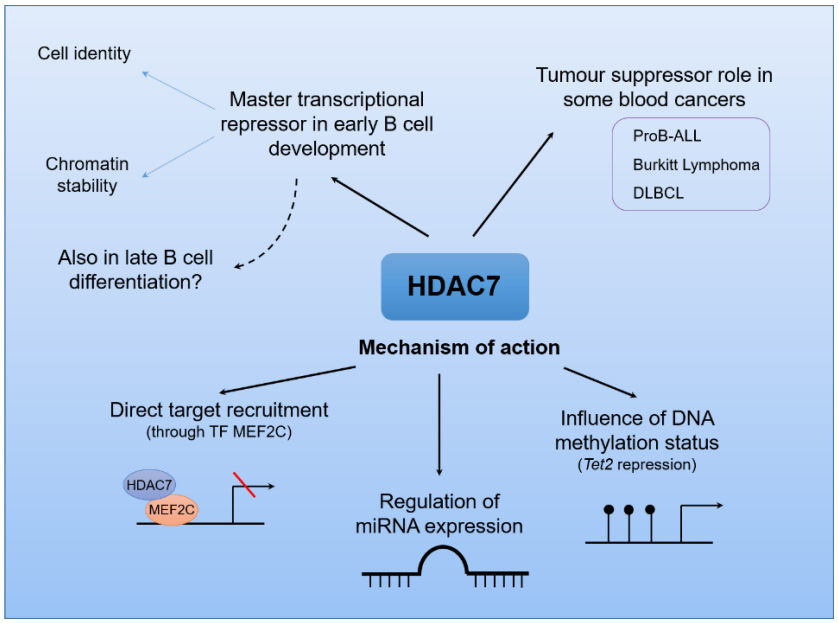


Figure3. Graphical summary of HDAC7 regulation mechanisms in pro-B cells. HDAC7 has been established as a master transcriptional repressor of early B cell development, but further studies are needed to evaluate whether it is also involved in the regulation of late B cell differentiation. Regarding role of HDAC7 in blood cancers, it has been associated t anti-oncogenic roles in several malignancies (see list in the image). Finally, three mechanisms have been defined for HDAC7-mediated repression: direct recruitment through specific TFs, indirect role on 5-hmC status and regulation of miRNAs expression.

CONCLUSIONS

CONCLUSIONS

1. HDAC7 deficiency leads to a block in B cell development, impairing the transition from pro-B cells to pre-B cells.

2. Pro-B and pre-B cells undergo increased cell death rates upon HDAC7 deficiency, being significant in the case of pre-B cells, which suggest that these cells are more susceptibility to apoptosis.

3. In normal pro-B cells, HDAC7 is recruited to the promoter and enhancers of lineage inappropriate genes such as *Itgam* or *Cd69* through interaction with tissue-specific TF MEF2C, preventing their expression and ensuring the maintenance of B cell identity.

4. The repressive function of HDAC7 is independent from other crucial B cell regulators such as TFs PAX5, E2A or EBF1.

5. HDAC7 represses *Tet2* gene in pro-B cells, controlling its physiological levels during early B cell development.

6. HDAC7 deficiency causes a significant increase in DNA 5-hydroxymethylation at both global levels and at specific genes loci, especially targeting lineage inappropriate genes such as *Jun* or *Fosl2*.

7. HDAC7 deficiency produces an increase in 5-hmC enrichment at LINE-1 elements and leads to their expression, suggesting a potential contribution of HDAC7 in maintaining B cell genome integrity.

8. HDAC7 regulates the expression microRNAs involved in the development of both lymphocyte and myeloid lineages, as well as in hematological malignancies in pro-B cells.

9. HDAC7 is under-expressed in some types of B cell malignancies, including ALL, DLBCL and Burkitt lymphoma.

10. HDAC7 ectopic expression in these blood cancers lead to *c-Myc* downregulation and induction of apoptosis independently of HDAC3 function.

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ANNEX 1

Review article: “From loops to looks: how transcription factors and chromatin organization shape terminal B cell differentiation?”

From loops to looks: how transcription factors and chromatin organization shape terminal B cell differentiation?

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Abstract

The generation of B lymphocytes is tightly regulated at the level of gene transcription. In recent years, investigators have shed light on the transcription factor networks and the epigenetic machinery involved at all differentiation steps of B cell development. During terminal differentiation, B cells undergo dramatic changes in their gene transcriptional programs to generate germinal center B cells, plasma cells and memory B cells. Recent evidence indicates that mature B cell formation involves an essential contribution from the three-dimensional chromatin conformation through its interplay with transcription factors and the epigenetic machinery. In this review, we provide an up-to-date overview of the coordination between transcription factors, epigenetic changes, and chromatin conformation during terminal B cell differentiation, with a particular focus on the most recent discoveries.

B lymphocyte development: a brief overview

B cell differentiation underlies the development of the vertebrate humoral immune response, which is based on the production of highly diverse antibodies that can recognize and eliminate virtually any antigen. Antibody diversity is achieved at two stages during B cell differentiation. The first is V(D)J recombination in early B cell precursors during bone marrow differentiation and involves the combinatorial rearrangement of variable (V), diversity (D), and joining (J) coding segments of immunoglobulins (Ig) genes [1,2]. Successful V(D)J recombination on the heavy-chain and light-chain Ig genes leads to B cell receptor (BCR) expression on naïve B cells, which can then exit the bone marrow and migrate to the peripheral lymphoid organs. In the periphery, antigens encountered by B cells in the context of a cognate T helper cell [3] can trigger the formation of microstructures called germinal centers (GC), where B cell Ig genes diversify through somatic hypermutation (SHM) and class switch recombination (CSR) [4,5]. CSR is a region-specific recombination reaction in the heavy-chain Ig locus that replaces the IgM or IgD constant regions with alternative downstream constant regions, generating the IgG, IgE or IgA isotypes, thus increasing the versatility of antibody-mediated antigen removal. SHM introduces nucleotide changes, mostly point mutations, into the variable, antigen-binding region of the Ig heavy and light chains. These mutations generate Ig variants from which those with higher affinity for antigen are selected in the context of T follicular helper (Tfh) cells and follicular dendritic cells (FDC) in a process called affinity maturation. Finally, B cells exit the GC and terminally differentiate into either long-lived antibody-producing plasma cells (PC) or memory B cells [3]. SHM and CSR are both initiated in a transcription-dependent manner by activation-induced cytidine deaminase (AID), which deaminates cytosine to uracil on single-stranded DNA [6].

Terminal B cell differentiation is controlled by a cohort of transcription factors (TFs) that integrate inputs from BCR signals, cytokine signals, and direct interactions with T cells. TFs such as Pax5, Bcl6 and Bach2 form complex regulatory networks during the GC reaction, whereas IRF4, Blimp-1 and XBP1 act in the generation of plasma cells (PC) [7]. The master transcriptional regulator in GC B cells is Bcl6 [8], while Pax5 and Bach2 are

expressed in naïve B cells and play important roles at earlier B cell activation stages. Conversely, memory B cell and PC development requires the downregulation of Bcl6 and the upregulation of IRF4 for memory B cells and Blimp-1 for PCs [9–11]. In addition, PC differentiation requires the extinction of the B cell gene program [12–14] (Figure 1, Key Figure).

Three-dimensional (3D) chromatin conformation and its impact on gene regulation

Every nucleated human cell contains about 2 meters of linear DNA encompassing all the genes that shape our being. This DNA, which is the same in almost every cell, is packed into a nucleus measuring only a few microns in diameter; this packaging is not random, and the specific folding of DNA plays a fundamental role in the regulation of gene expression. In some cases, the folded DNA conformation brings promoters of co-regulated genes or regulatory elements, such as enhancers, into physical contact with target gene promoters. This phenomenon partly explains how cells endowed with the same genetic information are phenotypically and functionally different. Accordingly, the ordered complexity of B cell differentiation is not only achieved by the action of the TF network, but also requires the coordination of epigenetic regulators and architectural proteins that establish a correct and permissive/non-permissive chromatin structure. Proper conformation of the chromatin architecture is essential for the correct gene expression and cell development and involves genome compartmentalization and the establishment of chromosome territories, gene clusters, and three-dimensional (3D) interactions between gene regulatory regions [15–17]. 3D genome organization is remodeled dynamically in each cell type, facilitating accessibility and interactions between regulatory regions of the specific gene cohort required at each developmental stage [18,19]. In B lymphocytes, the tightly regulated processes of V(D)J recombination and CSR are closely linked to transcription and involve the formation of DNA loops that facilitate interaction between distant regulatory regions [20–22]. The increasing ability to characterize these parameters provides an unprecedented vision of genome topology and allows a better understanding of basic developmental processes, misregulation in pathological conditions, and evolution [17]. In the following sections, we provide an overview of the experimental techniques to study 3D

chromatin conformation and summarize the most recent advances in our knowledge of how chromatin structure and TFs underlie the activation of naïve B cells, the GC reaction, and terminal B cell differentiation into PCs and memory B cells.

An overview of recent technical advances to study 3D chromatin conformation

No cell activity or function can be understood without considering the time-dependent 3D organization of the genome within the nucleus. The explosion of chromosome conformation capture (3C)-based methods over the last decade has complemented and enriched classical microscopy analysis, and has positioned nuclear genome organization as one of the hottest fields in molecular biology. In this section, we provide a brief overview of the 3C method and the most common C-based technologies, which have allowed investigators to reveal the general features of genome architecture, from compartments [23] to topologically associating domains (TADs) [24,25], sub-TADs [26], and chromatin loops [19,27]. More detailed information about these methods and other emerging technologies can be obtained in specialized reviews [16,28].

C-based technologies detect the frequency of pairwise contacts between distant genomic fragments. After chemical fixation to preserve the 3D genome architecture, chromatin is enzymatically fragmented and re-ligated, and the generated DNA concatemers are isolated. The hierarchical folding of eukaryotic genomes can bring distant genome regions, such as enhancers and target gene promoters, into physical proximity, allowing regulatory interactions between them. The closer two genomic fragments are within the nucleus, the more ligation products will be generated. The abundance and nature of these ligation products can be detected by a variety of C-based technologies.

The original 3C method is the earliest and the lowest throughput C-based technology. In this method, the ligation junctions are detected by semi-quantitative or quantitative PCR, allowing estimation of the 3D proximity of two preselected loci [29]. In contrast, circularized chromosome conformation capture (4C) is based on the design of a single primer targeting a specific locus; this approach allows the genome-wide detection of all possible interacting partners of the targeted locus by microarray hybridization [30] or high-throughput sequencing [31]. Although 3C and 4C provide insights into gene regulation, these methods

are not quantitative due to PCR amplification biases. This limitation has been resolved with the UMI-4C method, which combines 4C with the use of sonication-based unique molecular identifiers (UMIs) [32]. The best method for detecting the folding of a genomic region measuring several megabases is carbon copy chromosome conformation capture (5C). 5C uses a pool of primers for the multiplexed ligation-mediated amplification of a target region in a 3C library, which is then detected by microarray analysis [33] or high-throughput sequencing [34]. On the other hand, if the biological question is focused on the interactions of a given protein, the best choice is chromatin interaction analysis paired-end tag sequencing (ChIA-PET), combining ChIP, 3C, and sequencing. Although ChIA-PET can detect rare interactions specifically mediated by the protein of interest, the library quality is dependent on the antibody quality, and extremely large numbers of cells are needed due to the low efficiency of proximity ligation after chromatin sonication. The most powerful C-technology is Hi-C, which allows detection of the entire ensemble of chromosomal interactions within a cell population [23] or in a single cell [35]. The key point of Hi-C is the addition of biotinylated nucleotides to the sticky ends generated after restriction digestion. These molecular tags allow the use of magnetic streptavidin-coated beads to separate informative ligation junctions from non-informative products generated after sonication, greatly reducing the sequencing costs and increasing the resolution. Hi-C and 3C libraries generated from a mammalian genome are highly complex, and reproducible and reliable identification of significant interactions between individual restriction fragments requires ultra-deep sequencing, which is a significant additional cost. Capture Hi-C [36] and Capture-C [37] pull-down specific sequences of interest and their frequent interaction partners from Hi-C and 3C libraries, respectively. This requires complex computational analysis, but these capture methods also have advantages, including economically viable detection of significant interactions at fragment resolution and compatibility with custom capture systems targeting specific genome regions of interest. For example, Capture Hi-C has been successfully used to identify distal sequences, such as enhancers and other regulatory elements, that significantly interact with almost all promoters in the genome [19] and to demonstrate that actively transcribed genes cluster as a result of DNA double-strand

breaks [38]. The different techniques used to study 3D chromatin conformation are summarized in Figure 2, and more detailed technical information is available in recent reviews [16,28].

Chromatin conformation changes during the germinal center reaction

Antigen recognition by naïve B cells triggers dramatic phenotypic and gene expression changes and their differentiation into GC B cells. This developmental program involve intense proliferation, increase in nucleus size, and major genetic and epigenetic changes in GC B cells [3]. Using Hi-C technology, two recent studies revealed that B cells undergo progressive chromatin decondensation upon antigen recognition and during the GC reaction, leading to a loss of interactions between the longest chromosome arms [39,40]. This is an energy-dependent process, in which Myc activity leads to increases in ATP production, the number of DNA loops mediated by the architectural protein CTCF, and the proportion of nearby DNA interactions between promoters and enhancers of crucial genes in the GC transcriptional program, such as Bcl6, promoting their upregulation [39,40]. The recent study by Casellas and colleagues [39] defined the main chromatin spatial reorganization events during B cell activation: chromatin acetylation, chromatin decompaction and the spreading of mononucleosome fibers throughout the nucleus, and the unexpected requirement of Myc and energy input. Meanwhile, Melnick and colleagues [40] identified the stratified genome reorganization in naïve and GC B cells. Chromosome distribution in naïve B cells groups genes share the same regulatory program into regions called “gene neighborhoods”. After B cell activation, these separated units become interconnected in “gene cities”, so that the coordination and control of GC regulatory regions can be optimized independently of their physical distance in the genome [40,41]. These interactions are accompanied by the coordinated addition of the histone marks H3K27ac and H3K27me3 during the GC reaction, and these histone modifications are required for the changes in gene expression and genome architecture [40]. Studies have also identified an important role during B cell activation for the complex chromatin architectural protein CTCF. CTCF is a well characterized regulator of the 3D chromatin architecture and function that mediates interactions between distant interdomain and

intradomain regulatory sequences [42,43]. Several studies have shed light on the contribution of CTCF during V(D)J recombination and the GC reaction. First, CTCF contributes to antibody repertoire diversity by altering the balance of rearrangements between distal and proximal constant regions of the IgH locus through DNA looping and interaction with the intergenic control region (IGCR) [44,45]. Second, CTCF prevents CSR before B cell activation by generating a “closed” chromatin conformation that blocks activation of germline promoters in naïve B cells [46]. Third, as a consequence of its repression of premature CSR, CTCF contributes to the maintenance of the GC reaction and the prevention of premature PC differentiation by inhibiting Blimp-1 [46–48]. The dynamic role of CTCF is thus modulated at several steps during B cell differentiation.

Additional architectural proteins or regulators play important roles during CSR. Reina-San-Martin and colleagues reported that AID interacts with subunits of the cohesin complex [49]. The cohesin complex together with CTCF plays a crucial role in promoting long-range DNA interactions and loop structures [50]. Several components of the cohesin complex are recruited to the IgH locus during CSR, partially correlating with the recruitment of AID [49]. A more recent study from the same laboratory found that mediator, a multiprotein complex required for gene transcription, is involved in long-range interactions of the IgH locus and facilitates transcriptional activation during CSR [51]. Two laboratories have uncovered a novel function of 53BP1 in CSR. Using a 4C-seq experimental approach, Skok and colleages have reported that 53BP1 controls chromatin conformation of IgH independently of DNA damage during CSR [52]. Simultaneously, Kenter and colleagues also unveiled an architectural role for 53BP1 in the chromatin looping of IgH in mouse B cells [53]. Finally, the transcriptional regulator YY1, which is involved in early and terminal B cell differentiation, have also been reported to impact DNA loops during CSR. Using 3C, Atchison and colleagues demonstrated that YY1 is required to proper establish the Eμ-3′RR DNA loop of IgH [54].

Among the TFs involved in late B cell differentiation, IRF4 is a versatile regulator that drives B cell fate toward the GC reaction or PC differentiation, depending on its expression level. IRF4 is required for the initiation of the GC reaction but not for its maintenance; in GC

B cells, low levels of IRF4 co-occupy EICE motifs together with PU.1, leading to B cell gene transcriptional activation [11,55]. EICE motif binding by IRF4/PU.1/Ikaros complexes activates target genes such as *Ebf1*, which consequently promotes *Pax5* and *Bach2* expression [56]. Cooperation between these three TFs is important for GC differentiation, and the progressive reduction of *Pu.1* expression during differentiation results in repression of *Ikaros/Irf4* targets genes, thus ensuring the initiation of PC differentiation [11,56]. There is also emerging evidence that long non-coding RNAs (lncRNA) influence epigenomes and, consequently, 3D looping and interactomes, with recent publications reporting the co-expression of upstream lncRNAs and their interaction with the *Bcl6* promoter [40,57].

Chromatin conformation changes in plasma cells

The spatial conformation of PC chromatin has been studied since the early in the last century. Cajal drew faithful and characteristic chromatin patterns reminiscent of a cartwheel [58]. This distinctive feature is in part acquired as a result of the transformation of active chromatin (euchromatin) into inactive chromatin (heterochromatin), a process undergone by most terminally differentiating cells [59]. In general, the initiation of differentiation and the establishment of a specific gene expression program involve a complex interaction of TFs and the action of thousands of *cis*-regulatory elements (enhancers). This complexity is increased by histone modifications and changes in DNA methylation, which provide dynamic mechanisms for controlling gene expression changes over time [60–62]. To initiate differentiation, the B cell TFs that define the mature B cell gene expression program must be silenced [13]. The most important PC-specific TFs are XBP1, IRF4 and Blimp-1, which is the master regulator of PC differentiation. Blimp-1 is essential for full PC differentiation but not for the initiation of the process [63]. A critical step in the transition to PC commitment is *Pax5* repression [63,64]. The detailed molecular mechanisms regulating PC differentiation have not been fully defined; however, several mechanisms of B cell specific gene repression have been identified. One involves an important role for microRNAs in cell fate decisions, with miR-155 repressing PU.1 and thus leading to Pax5 repression [65]. GC B cells bearing BCRs with low antigen affinity decrease IRF4 expression and consequently maintain AID expression, allowing continued SHM and the opportunity to generate higher

affinity BCRs. In contrast, GC B cells with high affinity BCRs increase IRF4 expression, promoting Blimp-1 expression and repressing Pax5 [11,62,66,67]. The molecular mechanisms mediating repression of some other IRF4 target genes are still unknown. Nevertheless, Igarashi and colleagues described a correlation between PC differentiation and the high-density binding of IRF4 to interferon sequence response element (IRSE) motifs [56]. Moreover, differentiating PCs acquire several histone modifications that contribute to gene silencing. In particular, the acquisition of H3K9me3 and H3K27me3 repressive epigenetic marks correlates with heterochromatinization [68]. ATAC-seq experiments revealed that the restriction of chromatin accessibility in PCs involves H3K27me3 enrichment mediated by EZH2, the catalytic subunit of the Polycomb complex. In fact, EZH2 absence led to de-regulation of B cell-related TFs and genes repressed by Blimp-1, impairing PC function. CTCF binding motifs are more accessible in the absence of EZH2, and EZH2 deficiency also leads to early Blimp-1 expression, giving hints as to how cells optimize the establishment of different genetic programs by using rapid alternative steps [61,69]. In an elegant and comprehensive work, Busslinguer and colleagues used a broad range of experimental techniques to identify Blimp-1 target genes, associated chromatin regulators and epigenetic changes at the onset of plasmablast differentiation. The found that Blimp-1 regulates a plethora of events; it directly represses several TFs and Aicda leading to the silencing of the B cell gene program, promotes Ig transcription and induces the expression of IRF4 and proteins required for immunoglobulins secretion [70]. Finally, proper PC differentiation is determined by a unique chromatin conformation. Intriguingly, the Ig genes IgK, IgH and IgJ, which are located on different chromosomes, colocalize in the same cluster or *transcription factory.* FISH experiments demonstrated that these clusters are located at the nuclear periphery and show interchromosomal enhancer interaction. Although the nuclear periphery is commonly associated with gene silencing, DNA regions containing active genes are positioned close to the nuclear pores and the endoplasmic reticulum in PCs. This DNA distribution facilitates the rapid secretion of antibodies and an efficient humoral immune response [71]. A recent preprint published by the Murre laboratory describes the chromatin conformation changes underlying PC

differentiation [72]. Using DNA fish and chromosomal conformation capture techniques to study 3D chromatin status, they confirmed that PC possess a unique chromatin conformation compared to other B cell types. Chromosomes of PC show helical structures and generate inter-chromosomal interactions in the Prdm1 gene as well as in genes characteristics of the of the unfolded protein response (UPR) and the endoplasmic reticulum [72]. Allan and colleagues have determined by Hi-C the genome organization of B cells at several developmental stages [73]. Pax5 is involved in genome organization across B cell differentiation until the plasmablast stage correlating with Pax5 silencing and the loss of the B cell chromosome architecture that precedes PC generation [73].

Chromatin conformation changes in memory B cells

Memory B cells are long-lived cells that self-maintain in an antigen-independent fashion and exert a rapid and robust antibody response to subsequent antigen exposure [74,75]. Naïve and memory B cells share a similar transcriptional program, but differ in the expression of factors involved in mounting a rapid immune response and self-renewal [76]. As differentiated cells, PCs and memory B cells share a similar DNA methylome, but they differ in their gene transcriptional profile. Memory B cells thus have unique functional and molecular features [77,78]. It remains unknown how the chromatin architecture determines the final fate decision between becoming a memory B cell or a PC. However, several studies demonstrate tight coordination between chromatin 3D organization and transcription, and nuclear positioning of genes at naïve B cell stages may determine terminal B cell differentiation, in coordination with cytokines and other signals. Thus, in line with findings in memory T cells [62,79], gene promoters and enhancers associated with memory B cell differentiation may be positioned together and occupied by specific TF networks, priming them for a rapid response to the appropriate stimulus.

Concluding remarks

The recent explosive growth in techniques for studying chromatin conformation places the B cell field in a position to advance our understanding of how a proper humoral response is generated. However, further studies are needed to decipher the precise

mechanisms involved in regulating and enacting specific and dynamic genome reorganization during terminal B cell differentiation. Recent evidence establishes a strong relationship between nuclear architecture, the epigenetics machinery, and gene regulation, and understanding this relationship promises to reveal the complex coordination among all factors and signals involved in the humoral immune response. Nevertheless, B cell biology continues to present technical challenges to the determination of chromatin organization and DNA architecture in rare populations such as memory B cells.

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Figure legends

Figure 1 (key Figure). **Transcription factors interaction and chromatin remodeling during late B cell differentiation**. Germinal Centers (GC) are specialized structures located in secondary lymphoid organs upon antigen detection, either by infection or immunization. GCs are divided into to two compartments. The dark zone contains hyperproliferative B cells that undergo somatic hypermutation (SHM). In the light zone, follicular and T cells interact with these B cells in order to present them the antigen and start de selective process. Negative selected cells activate apoptotic process. B cells undergo class switch recombination (CSR) in order to improve antigen-specificity in their antibody production. Then, they can become antibody-producing plasma cells (PC) or memory cells, or re-renter to the dark zone for further SHM and affinity selection. Several TFs are involved during GC formation. The most relevant are included in the representation. Dynamic chromatin reorganization is represented in 3 terminal B cell stages: naïve B cell (compacted chromatin), GC B cell (deconsensed chromatin spread on the nucleus) and plasma cell (cartwheel-like open chromatin).

Figure 2. Chromosome Conformation Capture (3C) and derivative methods. Schematic representation of 3C-related techniques highlighting their main characteristics. The first three steps are shared by all methodologies: crosslinking of DNA with formaldehyde, fragmentation of DNA either by restricted digestion or by sonication, and reverse of crosslinking (very few variations between them, e.g. reverse crosslinking is followed by biotinylation in the case of Hi-C). Next, depending on their specific objective, 5 represented methods follow different technical approaches. 4C requires self-circularization of des-crosslinked DNA followed by reverse PCR. 5C requires multiplexed ligation-mediated amplification (LMA), in which tails (T7 and T3) of 5C primers allow amplification after primers ligation. Hi-C requires biotin labeling to detect all genome junctions (dark blue circle). ChiA-PET combines chromatin immunoprecipitation (ChIP) with 3C methods, limiting interactions between regions bound to a specific protein (green circle). Most of techniques require next generation sequencing approaches in order to detect 3D-interactions in the chromatin, except for 3C that study interactions at smaller scale.

Figure 1 (Key Figure)

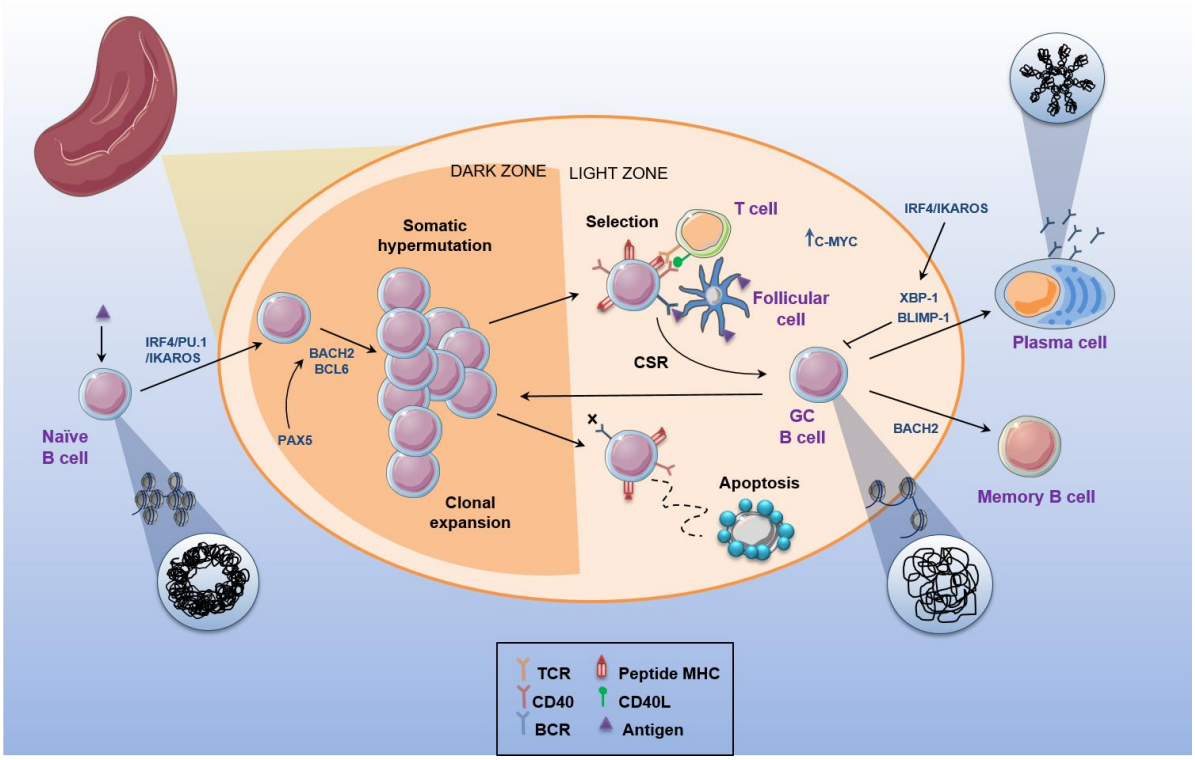

Figure 2