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Dissecting the role of histone deacetylase 3 (HDAC3) in leukemogenesis

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This dissertation is dedicated to my lovely mom & dad, my husband, my sister & my brother. Even though I am not in my country and at home to share these moments with you but I have all your supports every minute of my life. Thank you for your unconditional love.

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List of abbreviations

5mC	5-methylcytosine
AML	Acute Myeloid Leukemia
APL	Acute Promyelocytic Leukemia
ATO	Arsenic Trioxide
ATRA	All-Trans Retinoic Acid
BCL-6	B-Cell Leukemia-6
BCoR	Bcl6- interacting Co-Repressor
BM	Bone Marrow
Bp	Base Pair
CaMK	Ca ²⁺ /calmodulin-dependent kinase
CFU	Colony Forming Unit
CLP	Common lymphoid Progenitor
CML	Chronic Myelogenous Leukemia
CMP	Common Myeloid Progenitor
CoREST	Corepressor of Repressor Element 1 Silencing Transcription
CTCL	Cutaneous T-Cell Lymphoma
DAD	Deacetylase Activating Domain

DLBCL	Diffuse Large B-Cell Lymphoma
DMSO	Dimethyl Sulfoxide
DNMT	DNA Methyltransferase
eGFP	Enhanced- Green- Fluorescent- Protein
ESC	Embryonic Stem Cell
EST	Expressed Sequence Tag
EDTA	Ethylene Diamine Tetra Acetic acid
FAB	French-American-British
FACS	Fluorescence Activated Cell Sorting
FDA	Food and Drug Administration
GAPDH	Glyceraldehyde 3-Phosphate dehydrogenase
GMP	Granulocyte/Macrophage progenitor
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
Hdac1 ^{-/-}	Hdac1- null
HDAC3	Histone Deacetylase 3
HDAC3i	Histone deacetylase 3 inhibitor
HDACi	Histone deacetylase inhibitor
HIF	Hypoxia Inducible Factor
HMT	Histone methyltransferase
HR	Homologous recombination
HRP	Horseradish Proxidase

HSC	Hematopoietic Stem cell
HSPC	Hematopoietic Stem and Progenitor Cells
IMDM	Iscove's Modified Dulbecco's Medium
IP ₄	Inositol tetrphosphate
KD	Knock-Down
KO	Knockout
LIC	Leukemia Initiating Cell
Lin ⁻	Lineage negative
LT-HSC	Long-term hematopoietic stem cell
Luc	Luciferase
MEP	Megakaryotic/Erythroid progenitor
MPP	Multipotent Progenitor
MYH	Myosin Heavy chain
NHJ	Non-Homologous Recombination
NAD	Nicotinamide Adenine Dinucleotide
NCoR-SMRT	Nuclear receptor Corepressor- Silencing Mediator of Retinoid and Thyroid
NES	Nuclear Export Signal
NK	Natural Killer cells
NLS	Nuclear Localization Signal
NPM	Nucleophosmin
NT	Non- treated cells

NuMA	Nuclear Mitotic Apparatus
NuRD	Nucleosome Remodeling Deacetylase
PB	Peripheral blood
PBS	Phosphate-Buffered Saline
PI	Propodium-Iodide
PGK	Phosphoglycerate Kinase
PLZF	Promyelocytic- leukemia zinc factor
PML-NB	Promyelocytic Leukemia- Nuclear Body
PML-RAR α	Promyelocytic Leukemia- Retinoic acid receptor α
PMN	Polymorphonuclear
PT	Post-treatment
PTM	Post-translational modification
RbAp	Retinoblastoma- Associated protein
ROS	Reactive Oxygene Species
SAC	Spindle Assembly Checkpoint
SAHA	SuberoylAnilidehydroxamic acid
SD	Standard- deviation
SDS-PAGE	Sodium dodecyl sulfate- polyacrylamide gel electrophoresis
ST-HSC	Short-term hematopoietic stem cell
STAT5b	Signal transducer and activator of transcription 5b
T- ALL	T-cell acute lymphoblastic Leukemia
TAE	Tris-Acetate-EDTA

TBS	Tris-Buffered Saline
TF	Transcription Factor
TRE	Tetracyclin-Responsive Element
TSA	Trichostatin A
TSS	Transcription Start Site
VPA	Valproic Acid
WT	Wild-type
Zn ²⁺	Zinc Ion

Abstract

Histone deacetylases (HDACs) are epigenetic enzymes that modulate chromatin structure through the deacetylation of lysine residues in histones, playing a crucial role in cell viability, cell cycle progression and tumorigenesis. Yet the role of individual HDACs in these biological processes remains enigmatic. Inappropriate recruitment of HDACs is involved in the pathogenesis of several forms of leukemia and several lines of evidence point to a role for HDACs in tumor progression, consistent with the anti-proliferative and apoptotic effects of HDAC inhibitors (HDACi). In this regard, HDACs are considered promising targets for development of new molecules for cancer therapy. To date, some HDACi which have a broad antitumor activity and low toxicity towards normal cells, such as Romidepsin (Depsipeptide or FK228) and SAHA have been approved by U.S. food and drug administration (FDA) for the treatment of cutaneous T-cell lymphoma (CTCL). Moreover, HDACi are undergoing clinical trials for the treatment of hematological malignancies as well as for solid tumors. Most of the HDACi available at the moment are not isoform specific, being active on more than one HDAC. Thus, to design more selective HDACi for cancer therapy it is important to elucidate the role of individual HDACs.

Acute Promyelocytic Leukemia (APL) is the first model disease in which the involvement of HDACs has been documented. APL is a subtype of Acute Myeloid Leukemia (AML), a cancer of blood and bone marrow, which is characterized by hyperproliferation of immature

granulocytes blocked at the promyelocytic stage. It is genetically associated with a chromosomal translocation t(15;17)(q22;q21), which encodes the oncogenic fusion protein PML-RAR α found in more than 90% of APL patients. In murine models of APL that recapitulate the human disease, PML-RAR α induces a “pre-leukemic” stage with long latency and without an overtly dramatic phenotype before full leukemic transformation. In fact, for this reason it is assumed that in addition to this oncogenic fusion protein, other genetic hits are required for clonal expansion of leukemic blasts. This fusion protein recruits a number of chromatin modifier enzymes such as HDACs and DNA methyltransferases (DNMTs) to the promoter of retinoic acid (RA) target genes and transcriptionally silence them, leading to the myeloid differentiation block. Furthermore, PML-RAR α causes the impairment of p53 pathway by deacetylation and degradation of p53 through the recruitment of HDAC-containing complexes. HDACs from class I (HDACs 1, 2 and 3) have been found associated with PML-RAR α paving the way for the use of HDACi for APL treatment. Recently, a study on APL, which has been conducted by Santoro et al., showed that among class I HDACs, HDAC1 and to a lesser extent HDAC2 have a dual role in APL development and maintenance. In fact, while they behave as oncosuppressors at the early stages, they function as oncogenes in established tumor cells. Since inhibition of HDAC1 and HDAC2 in pre-leukemic stage leads to the acceleration of the disease in murine models of leukemia, it suggests caution in the clinical utility of epidrugs that target any of these two HDAC isoforms. Moreover, it has been shown that the expression of HDAC3, which associates with nuclear hormone corepressor and silencing mediator of retinoid hormone (NCoR/SMRT) complex, is frequently increased in tumors, while *Hdac3* downregulation results in reduced proliferation and survival of tumor cells. In view of these observations, in this study we functionally assessed the role of HDAC3 in the development and maintenance of APL. To achieve our goal, we have dissected the role of HDAC3 in two different phases of the disease: pre-leukemic phase and full-established

leukemia. The murine model of APL, which we used, is the mCG^{PR/PR} mouse model in which PML-RAR α is expressed under the control of the cathepsin G promoter. The mice show a very long latency (the pre-leukemic phase) associated with high penetrance (more than 90% of the mice develop APL).

We characterized the role of HDAC3 through a functional knock-down approach, assessing its impact on cellular differentiation, proliferation and the ability to influence the transplantation of HSCs and APL cells. Indeed, *Hdac3*-KD *in vitro* reduced the proliferative potential of both pre-leukemic and full leukemic cells and boosted their differentiation, suggesting that HDAC3 plays the role of an oncogene in APL initiation and progression. These results were not restricted to APL, because lymphoma driven by *c-myc* overexpression and leukemia driven by MLL-AF9, were both impaired in cell growth upon *Hdac3*-KD. *In vivo*, inoculation of *Hdac3*-KD pre-leukemic cells into lethally irradiated recipient mice or inoculation of *Hdac3*-KD APL cells into the recipient mice did not result in leukemia development or progression, respectively. These results suggest that HDAC3 can be considered as a target for epidrugs in the treatment of hematological malignancies. Thus, we assessed this hypothesis with the treatment of pre-leukemic and leukemic cells with the HDAC3 selective inhibitor, RGFP966. Indeed, inhibition of HDAC3 enzymatic activity with RGFP966, phenocopied *Hdac3*-KD phenotypes in pre-leukemic and leukemic cells confirming the putative oncogenic role of HDAC3.

In conclusion, my PhD project has expanded our comprehension about the role of HDAC3 in hematological malignancies and is beginning to unravel alternative views on the targets of epidrugs for the treatment of leukemic patients.

Introduction

1. Hematopoiesis:

Hematopoiesis serves as a paradigm for studying the role of stem cells in differentiation and development. Establishment and maintenance of the blood system relies on self-renewal ability of hematopoietic stem cells (HSCs), rare cells which reside in specific niches in the bone marrow (BM) of adult mammals. A wide variety of cellular morphologies corresponding to cells of various blood lineages and stages of differentiation have been found in the BM anatomic examination (1). To explain this diversity, A. Maximow in 1909 postulated that hematopoiesis is hierarchically organized. In this picture, BM cells derive from a common precursor (i.e. HSCs) that sits atop a hierarchy of progenitors that progressively commit to several or single lineages. The first evidence for the existence of HSCs came from the study conducted by Lorenz and colleagues in 1951, which showed that injection of syngenic spleen or BM cells from a donor could reconstitute the hematopoietic system of an irradiated recipient (2). HSCs are enriched for a population that are negative for lineage markers (Lin⁻) and positive for Sca.1 and c.Kit (LSK cells). The LSK cells include long-term self-renewing subset (LT-HSC), short-term HSC (ST-HSC) and multipotent progenitors (MPPs). The LT-HSC undergoes an asymmetric cell division and generates another LT-HSC that maintains the stem cell pool and a transient self-renewing ST-HSC that self-renews within a limited time window (3, 4). Further differentiation of ST-HSC generate MPPs and then oligopotent

progenitors, notably common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs), which respectively give rise to different myeloid and lymphoid lineages. Downstream of CMPs, there are megakaryotic/erythroid progenitors (MEPs), which differentiate to megakaryocytes/platelets or erythrocytes and myelo-monocytic progenitors, and granulocyte/macrophage progenitors (GMPs) that produce macrophages, monocytes and granulocytes. On the other hand, CLPs can differentiate into B and T lymphocytes or natural killer cells (NK) (5-7) (Figure 1).

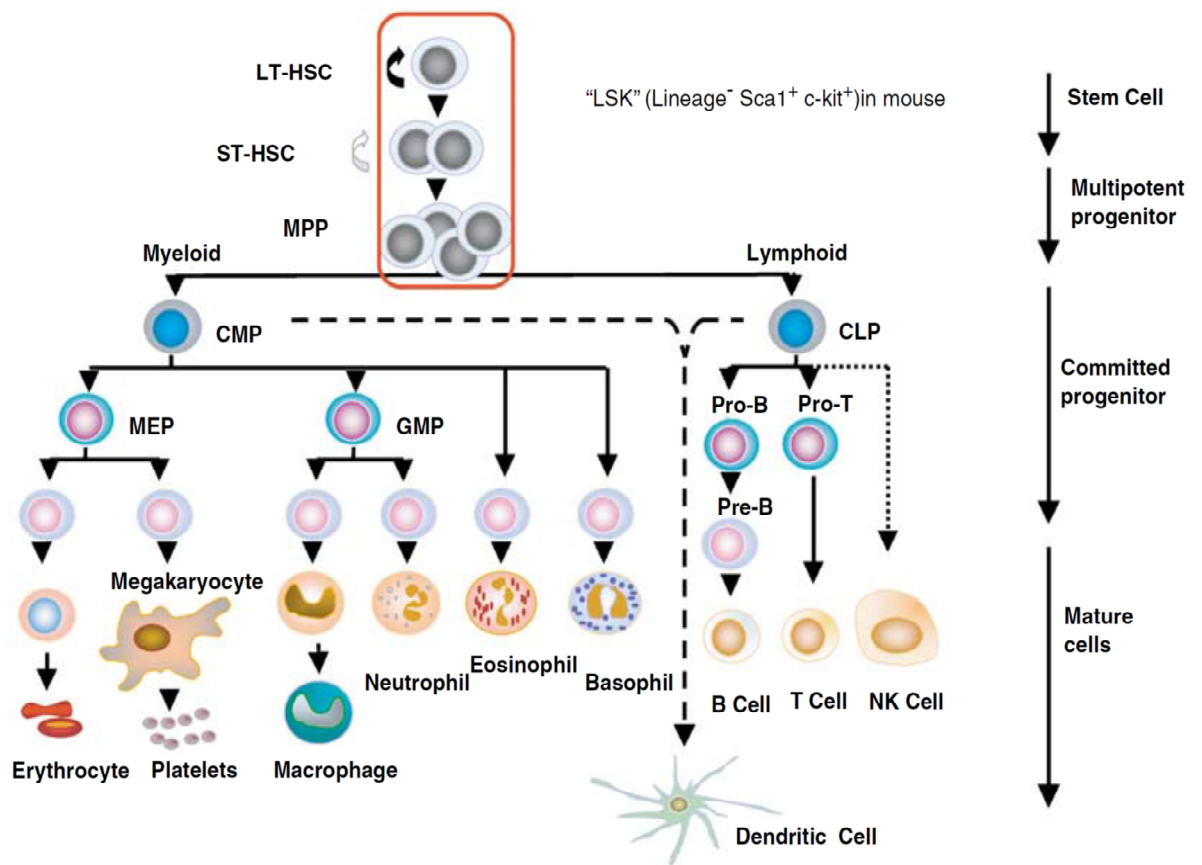


Figure 1. Schematic representation of hematopoietic hierarchy. At the apex of the hierarchy LT-HSC, long-term repopulating HSC; ST-HSC, short-term repopulating HSC; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; MEP, megakaryocyte/erythroid progenitor; GMP, Granulocyte/macrophage progenitor. $\text{Lin}^- \text{Sca}^+ \text{c.Kit}^+$ cells refer to encircled pluripotent population (i.e. LT-HSC, ST-HSC and MPP). Adapted from Larsson and Karlsson ,Oncogene, 2005 (8).

2. Deregulation of hematopoiesis:

Hematopoiesis as a developmental program is orchestrated by changes in the pattern of gene expression, which is directed by transcription factors (TFs). When the action of TFs is deregulated due to chromosomal aberrations (chromosomal translocations, deletions and inversions), gene mutations or deregulation of epigenetic modifiers, hematopoiesis fails leading to the differentiation block and abnormal cell proliferation. Eventually, these events cause development of leukemia (9). The most common acute leukemia affecting adults is acute myeloid leukemia (AML), which is known as a transcriptional disease. The main characteristic of AML is a severe block in myeloid differentiation characterized by rapid clonal proliferation of immature myeloid cells in the BM, that infiltrate other organs of the body (10-13). AML is a heterogeneous disease for which the standard treatment with cytotoxic chemotherapy has remained largely unchanged for over four decades, with unfavorable clinical results (14-16). Recently, the advent of massive parallel sequencing has revealed that >70% of AML cases have mutations in DNA methylation-related genes or mutations in histone modifiers. These data indicate that epigenetic alterations are key players in the development of most, if not all AMLs, and point to the concept that epigenetic alterations are as relevant as genetic mutations in leukemia development, paving the way for the use of drugs targeting epigenetic modifier enzymes (17).

3. Epigenetics:

3.1 Concept of epigenetics:

The basic functional and structural unit of all living organisms except viruses is known as the cell, which is often called “the building block of life”. Multi-cellular organisms arise from a

single cell and generate multi-cellular organisms through the cell division. There are hundred types of cells in human body, and their functions and morphologies vary from one to the other. Based on the human genome project approximately around 20,000 to 25,000 genes exist in the human genome but not all of these genes are expressed in each cell. In more details, while the majority of cells in multi-cellular organisms have an identical genotype, through development they will generate a diversity of cells with different functions and stable profile of gene expression. Regulation of gene expression patterns, which is involved in cell fate determination, is processed by epigenetic changes. Therefore, cellular differentiation can be considered as an epigenetic phenomenon, which results from the interplay between genes and environment, what Conrad Waddington defined as “ epigenetic landscape” (**Figure 2**) (**18**). Historically the term epigenetics stands for: “Any meiotically or mitotically heritable changes on gene expression without changes in DNA sequence” (**19, 20**). This definition nowadays is a bit controversial, because the word epigenetics is also used for describing any dynamic alterations that may or may not be heritable, still resulting in gene expression changes (**21**). The altered epigenetic pattern is central to many human diseases; the best-known example so far is cancer. Comparison of the genome of tumor cells with normal cells of the same individual shows a massive loss of DNA methylation, accompanied by aberrant activity of chromatin modifier enzymes and pattern of histone modification, resulting in a distorted epigenetic landscape of cancer. To appreciate the mechanisms behind epigenetic regulation, it is important to find the core molecular actors, which have key roles in epigenetic alterations.

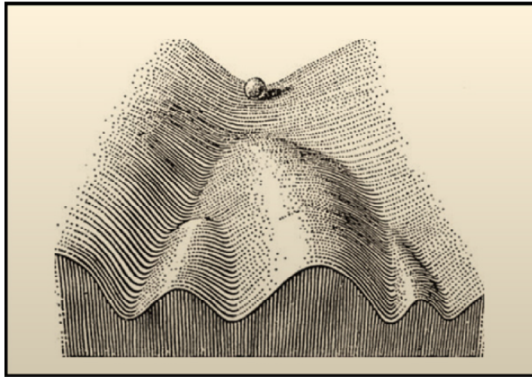


Figure 2. C.H. Waddington's epigenetic landscape model. Waddington first proposed the concept of epigenetic landscape in 1957; this figure represents the process of cellular differentiation and different trajectories that the cell (demonstrated by a ball) can undertake toward differentiation. Actually in this dynamic visual metaphor, the cell can go through specific paths that lead to different cell fates. Adapted from Goldberg et al, Cell, 2007 (18).

3.2 Nucleosome structure and stability:

When a cell undergoes proliferation, DNA replicates through the cell cycle. In higher organisms the nuclear DNA is tightly compacted and wrapped around nuclear proteins (termed histones) thereby forming the chromatin, which can be easily housed in the eukaryotic nucleus. Chromatin modifications have profound implications in the regulation of key biological processes such as DNA repair, replication and transcription. It is becoming clear that due to the important role of chromatin modifications in the above mentioned vital cell processes, aberrations in chromatin modifier enzymes also known as epigenetic modifiers can lead to abnormal cell functions and consequently cause the development of various diseases or/and cancer. The repeating building unit of chromatin is the nucleosome, which is composed of around 145-147 base pairs (bp) of DNA spooled two rounds around an octamer of histones consisting of two copies of H2A, H2B, H3 and H4. H1, known as linker histone, provides further structural stability through binding to DNA, which enters and exits from the nucleosome.

Nucleosomes are connected to each other through a linker DNA or a short DNA segment; these linked nucleosomes undergo short-range interactions with neighboring nucleosomes and form chromatin fibers. Higher order chromatin structure will be formed by fiber-fiber interactions (22) (Figure 3). Based on the state of chromatin condensation, there are two major regions: euchromatin and heterochromatin. Euchromatin is known as “relaxed” state or open configuration and is more prevalent in cells which are active in the transcription of many of their genes; in other word it comprises the most active portion of the genome (23). Euchromatin is characterized by high levels of histones acetylation and H3K4, H3K36 and H3K79 trimethylation (24). On the other hand, heterochromatin is considered as “closed” state, and is correlated with transcriptional repression, gene silencing (20, 22, 25), low levels of histones acetylation and high levels of H3K9, H3K27 and H4K29 methylation.

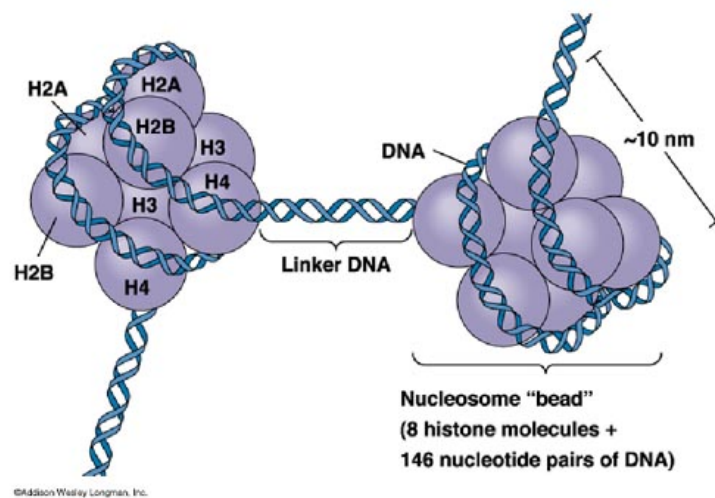


Figure 3. Schematic depiction of nucleosome structure. Adapted from www.zoology.ubc.ca/~bio463/lecture_3.htm

3.3 Epigenetic modifications:

Epigenetic modifications can be grouped into three main categories: DNA methylation, nucleosome positioning and histone post-translational modifications (PTMs).

3.3.1 DNA methylation:

The process of adding a methyl group into C5 of cytidine ring of DNA in the context of CpG dinucleotides (5mC) is generally associated with heterochromatin formation and transcriptional repression. This modification is catalyzed by a family of enzymes known as DNA methyltransferases (DNMTs). DNMT1 is required for the maintenance of methylation during DNA replication and targets hemi-methylated DNA, while DNMT3A and DNMT3B are required for *de novo* methylation (26). Regulation of gene expression by DNA methylation of target gene promoters is important for control of several developmental processes including hematopoiesis, X chromosome inactivation (27) and genomic imprinting (28). DNA methylation patterns are perturbed in many human cancers and DNA hypermethylation is usually observed at genes with tumor suppressor actions (29, 30).

3.3.2 Nucleosome positioning:

As mentioned previously, condensation of DNA into nucleosomes can affect all stages of transcription. It acts as a barrier for transcription initiation by blocking the access of transcriptional machinery to their binding sites on DNA, particularly in transcription start sites (TSSs), or inhibits the transcription elongation by engaged polymerases; all of these events regulate gene expression. Therefore, nucleosome positioning determines the accessibility of transcription factors to their target DNA and it is also involved in shaping the methylation landscape (31, 32). In addition to transcription regulation, nucleosome positioning directs mitotic recombination events. Nucleosome positioning and gene expression are regulated through the activity of histone variants. For instance, the genes will be protected against DNA methylation by the incorporation of histone variant H2A.Z. Histone variants are the key

modulators of nucleosomes' function. There are some important differences between histone variants and core histones. Specially, histone variants are expressed outside of S phase and are incorporated into chromatin independently from DNA replication, and furthermore their histone tails also differ from core histones (9). In addition to DNA methylation and specific histone methylation, nucleosome remodeling and spacing are influenced by the activity of chromatin remodeling complexes. These complexes are classified into four families: i) SWI/SNF family that is the master regulator of gene expression and modulates alternative splicing. ii) ISWI family, which promotes chromatin assembly and represses transcription. iii) CHD family that participates in nucleosome sliding and transcription promotion. iv) INO80 family, which is involved in DNA repair, transcriptional activation and DNA replication (31).

3.3.3 PTM of histone proteins:

All histones are subjected to post-translational modifications and these modifications serve as signaling mechanisms and as binding platforms for the recruitment of other proteins. Several post-translational modifications occur in histone tails (N-terminal of histone residues) as well as within globular domain of histones, such as acetylation, methylation, phosphorylation, ubiquitination, sumoylation and biotinylation (32). Epigenetic writers and erasers respectively govern addition or removal of these PTMs, and then epigenetic readers recognize and bind to certain histone modifications.

3.3.3.1 Histone acetylation and deacetylation:

Acetylation and deacetylation of histones (**Figure 4**) have emerged as critical modulators of chromatin status, respectively act on transcriptionally active and inactive chromatin domains.

Histone acetyltransferase enzymes (HATs), known as epigenetic writers, add the acetyl group from acetyl-coA to the ϵ -amino site of lysine residues in histones and non-histone proteins neutralize their positive charges, leading to the relaxed chromatin structure and makes it accessible for the transcription machinery. Importantly, acetylation of proteins can affect protein stability, protein-protein interaction, protein localization and DNA binding of transcription factors (33). HATs are divided into 2 types based on their cellular localization, type A; which are localized in the nucleus and type B; which are found in the cytoplasm. Furthermore, type A HATs are classified into 3 main families based on their homology and acetylation mechanisms: 1) the GNAT family (such as PCAF and Gnc5); 2) CBP/p300 family and; 3) MYST family (including Tip60, MOZ, MORF, HMOF and HBO1) (34). ϵ -N-acetylation of lysine residues are recognized and read by bromodomain-family proteins. The human proteome encodes 61 bromodomains, which are present in 46 diverse nuclear and cytoplasmic proteins (35). The antagonist role is imposed by Histone deacetylase enzymes (HDACs), which modulate chromatin structure through the deacetylation of lysine residues in histones and non-histone proteins, and playing crucial roles in cell viability, cell cycle progression and tumorigenesis. However, the role of individual HDACs in these biological processes remains enigmatic (36). The balance between the activity of these two types of enzymes acts as a key regulator of gene expression and developmental processes (37). Initially, it was thought the key substrates for HDACs were histones, but further phylogenetic studies showed that they evolved earlier than histones and they can also deacetylate non-histone proteins such as transcription factors, polyamines and metabolic intermediates (33, 38). Deacetylation of non-histone proteins mainly results in their degradation through ubiquitin-proteasome pathway (39). HDACs do not contain DNA binding domains, but they can be aberrantly recruited to the promoter of target genes through the association with transcription factors, nuclear receptors and oncogenic fusion proteins that result from

chromosomal translocations. In recent years, these types of enzymes, particularly HDACs arose as key targets in therapeutic arena for reversing aberrant changes associated with different types of cancers and non-cancer diseases (40). Inappropriate recruitment of HDACs is involved in the pathogenesis of several forms of leukemia (41) and several lines of evidence point to a role for HDACs in tumor progression, consistent with the anti-proliferative and apoptotic effects of HDAC inhibitors (HDACi) (42). In this regard, HDACs are considered promising targets for developing new molecules for cancer therapy (33, 36). For example it has been shown PML-RAR α which is involved in induction of Acute promyelocytic leukemia (APL) recruits HDAC-containing complexes to the promoter of specific target genes and consequently represses their expression (43).

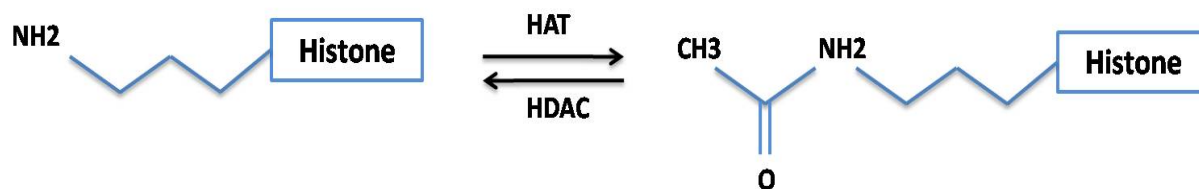


Figure 4. HAT and HDAC enzymatic activity. Acetylation of histones, catalyzed by histone acetyltransferase (HAT) and deacetylation of histones catalyzed by histone deacetylases (HDACs). Modified from Kouraklis and Theocharis, *Oncology reports*, 2006 (44).

4. HDAC superfamily:

There are 18 HDACs in mammals, which can be classified into 4 classes, based on their enzymatic activity and their homology to yeast HDACs: class I (HDAC1, 2, 3 and 8), class IIa and IIb (HDAC 4, 5, 6, 7, 9, 10), class III (Sirtuins1 to 7) and class IV (HDAC11) (45) (Figure 5). With the exception of class III, which has a Nicotinamide adenine dinucleotide

(NAD⁺)-dependent action, all other classes are zinc (Zn²⁺) dependent HDACs, and use the zinc ion as the cofactor for the deacetylation activity through a charge-relay-system. Class III sirtuins have widely distinct functions and subcellular localization compare to the other HDACs and while the roles of sirtuins in cancer are still debatable, their involvement in age associated diseases, including Alzheimer's and Parkinson's is well characterized (**46, 47**). The activities of HDAC enzymes are vital for cell proliferation, differentiation and normal cell development, as demonstrated by the embryonic lethality phenotype observed upon knockout (KO) of different *Hdacs* (e.g. *Hdac1*, 2, 3 and 4) (**42, 46, 48**).

4.1 Class I HDACs

Class I HDACs comprises HDAC1, 2, 3 and 8, which display higher enzymatic activity against histones. This class of HDACs is the most abundant and ubiquitously expressed HDACs, all contain a nuclear localization signal (NLS) thus predominantly are found in the nucleus. They have homology to the yeast HDAC, Rpd3. Based on phylogenetic studies this class of HDACs can be sub-divided into 3 classes, Class IA (HDAC1 and 2), Class IB (HDAC3) and Class IC (HDAC8) (**38**). These HDACs do not bind directly to DNA but instead are usually the catalytic component of multi-protein complexes. Repressive complexes such as Sin3 (Swi independent 3), Mi-2/NuRD (Nucleosome remodeling deacetylase), and CoREST (corepressor of repressor element 1 silencing transcription) mainly recruit HDAC1 and HDAC2 which share 82% identity and are the most similar members of class I, while HDAC3 is found in distinct complexes such as NCoR-SMRT (nuclear receptor corepressor-silencing mediator of retinoid and thyroid). Till now, no complex that recruits HDAC8 has been described, therefore it seems that HDAC8 can carry out its function independent of multi-protein complexes. HDAC3 was first identified in 1997 in a study which has been

conducted by Wen-ming yang et al. (49). HDAC3 is composed of 428 amino acids with a molecular mass of 49 kDa. It shares approximately 53% and 52% identity with HDAC1 and HDAC2, respectively (50).

Generation of mouse models harboring deletions of class I HDACs made it feasible to study the biological functions of these HDAC isoforms. Deletion of *Hdac1* led to embryonic lethality (E10.5), which established a crucial role for HDAC1 in embryogenesis (51). *Hdac1*-null (*Hdac1*^{-/-}) embryonic stem cells (ESCs), due to the increased expression of cell cycle inhibitors p21 and p27, showed proliferative defects (52). Deletion of *Hdac2* did not result in embryonic lethality but led to severe cardiac malformations that caused the death of most of mice within 24 hours after birth (53). Dovey et al. generated conditional knockout embryonic stem (ESCs) in which *Hdac1* or *Hdac2* genes could be inactivated. Deletion of *Hdac1*, but not *Hdac2* in ESCs, caused a significant reduction in the HDAC activity of Sin3A, NuRD, and CoREST corepressor complexes accompanied with a 1.6 fold increase in the acetylation of H3K56. Proliferation potential of ESCs was unaffected by loss of either *Hdac1* or *Hdac2*. However, loss of *Hdac1* impaired ESC differentiation (54). *Hdac3*-KO mice had an embryonic lethal phenotype before embryonic day 9.5 (E9.5), which showed a vital role for HDAC3 in embryogenesis and normal development (36). Upon removal of *Hdac3* in a mouse embryonic fibroblast (MEF) model, they underwent apoptosis and showed delay in cell cycle progression (55). Furthermore, tissue specific deletion of *Hdac3* in the liver was generated by using Cre recombinase under the control of albumin promoter (Cre-Alb). Removal of *Hdac3* upon administration of Tamoxifen, led to hypertrophy of liver and disrupted metabolism (56). In order to study the role of HDAC3 in hematopoiesis, another mouse model, *Hdac3*:Vav-Cre was generated. These mice showed a remarkable loss of lymphoid cells, stem and progenitor cells proliferation, and DNA replication defects (57). There are some evidence which shows

that global *Hdac8* deletion led to perinatal lethality due to skull instability, revealing an essential role for HDAC8 in patterning of the skull (58).

4.1.1 Class I HDAC-containing complexes:

Sin3: Sin3 was first identified as a global transcription regulator. 11 subunits make up Sin3 complex and are conserved from yeast to mammals (59). There are two Sin3 homologs in mammals; Sin3A and Sin3B. The Sin3A complex is composed of HDAC1 and HDAC2 as catalytic components and 9 other subunits as regulatory components including; binding proteins, Retinoblastoma associate protein 46 (RbAp46) and RbAp48 (60). Since Sin3/HDAC complexes lack DNA-binding activity they must be targeted to the gene promoters through the interaction with DNA-binding proteins (61).

Mi-2/NuRD: Mi-2/NuRD is a multi-subunit protein complex comprising HDAC1/2, ATP-dependent remodeling enzymes CHD3/4, histone chaperones RbAp46/48, CpG-binding proteins MBD2/3, the GATAD2a (p66 α) and/or GATAD2b (p66 β) and specific DNA-binding proteins MTA1/2/3 and Mi-2 (62, 63).

CoREST: CoREST was originally described as corepressor of REST (RE1-silencing transcription factor) found in complex with HDAC1 and HDAC2 and with additional subunits including sox-like proteins and the histone demethylase LSD1 (H3K4 demethylase) (64).

NCoR-SMRT: NCoR-SMRT are essential factors for HDAC3 activity, and both of them have a conserved deacetylase-activating domain (DAD) for HDAC3 activation (65). Studies which conducted on HDAC3 structure in 2012, showed that N-terminal helix of the DAD undergoes conformational changes upon forming complex with HDAC3 resulting in the standing of DAD on the surface of HDAC3. Further analysis led to discovery of inositol tetraphosphates

molecule (Ins (1, 4, 5, 6) P4 or IP4) as an essential requirement for the interaction between DAD of SMRT and N-terminal of HDAC3. All these discoveries on HDAC3 structure and its interaction with co-repressor complexes provide novel opportunities for therapeutic intervention through HDAC3 targeting (66). In addition to HDAC3, the members of class IIa HDACs interact with NCoR-SMRT complex through RD3 domain of NCoR. It has been shown that while HDAC3 is catalytically activated after binding to NCoR-SMRT complex, class IIa HDACs are not, and NCoR-SMRT actually serves as a bridge between HDAC3 and class II HDACs (67).

4.2 Class IIa HDACs

This class of HDACs is composed of HDAC4, 5, 7 and 9. They have similarity with the yeast HDA1 deacetylase. HDAC4 and HDAC5 have an overall similarity of 70%. Interestingly, HDAC4, 5 and 7 associates with HDAC3 through the recruitment into the NCoR/SMRT complex and disruption of this association leads to the inactivation of the enzymatic activity of these three HDACs (67). Class IIa HDACs bind to 14.3.3-chaperon protein following the phosphorylation by kinases and then shuttle from nucleus to the cytoplasm. For instance studies conducted in muscle cells have shown that upon a pre-differentiation signal HDAC4 is phosphorylated by Ca²⁺/calmodulin-dependent kinase (CaMK) and then is exported to the cytoplasm remaining there through the interaction with 14.3.3 protein. After receiving the post-differentiation signals, i.e. after fusion of muscle cells, its phosphorylation level is reduced leading to the release from the 14.3.3 protein and allowing it to shuttle back to the nucleus (65). These HDACs can also recruit class I HDACs through their C-terminal domain and among these HDACs, HDAC4, 5 and 7 are able to interact with NcoR/SMRT and Bcl6-interacting co-repressor (BCoR) (65, 67). In contrast to other HDACs the members of this

class are expressed in a tissue-specific manner. For example HDAC4 is highly expressed in brain (68, 69), HDAC5 and HDAC9 are enriched in muscles and HDAC7 in endothelial cells and thymocytes (37).

4.3 Class Iib HDACs












HDAC6 and HDAC10 belong to class Iib family. HDAC10 is involved in angiogenesis (70), modulation of HSP90 acetylation in cooperation with HDAC6 and regulation of homologous recombination in cooperation with HDAC9 (71). Two Rb-binding domains have been found on HDAC10, suggesting it may have a role in cell cycle regulation. HDAC6 is the main cytoplasmic deacetylase in mammalian cells and structurally different from all other HDACs because it contains two deacetylase domains and a C-terminal zinc finger domain: this domain acts as a signal for ubiquitination. HDAC6 targets and deacetylates transmembrane proteins such as interferon receptor and cytoskeletal proteins including α -tubulin and cortactin (37). Reversible acetylation of α -tubulin at Lys40 by HDAC6 has been implicated in regulating microtubule stability and function. *In vivo*, the overexpression of *Hdac6* leads to a global deacetylation of α -tubulin, whereas downregulation of *Hdac6* results in α -tubulin hyperacetylation. *In vitro*, purified HDAC6 potently deacetylates α -tubulin in assembled microtubules. Furthermore, overexpression of *Hdac6* promotes chemotactic cell movement supporting the idea that HDAC6-mediated deacetylation, regulates microtubule-dependent cell motility. HDAC6 has also an important role in aggresome formation and directing the misfolded proteins to the aggresome. Thus, loss of HDAC6 activity impacts on cell viability in response to misfolded proteins (72, 73).

4.4 Class III HDACs

This class is composed of 7 mammalian sirtuins (SIRT1-7); they are NAD⁺-dependent protein deacetylases. Sirtuins have homology to the yeast silencing information regulator 2 (Sir2), which has been identified as a transcriptional repressor of mating type loci and telomeres, which prolongs survival in yeast. The members of this family have gained attention in recent years due to their regulatory role in metabolic diseases, such as obesity and type 2 diabetes and in ageing. According to their subcellular localization they can be found in nucleus (SIRT1, SIRT6 and SIRT7), in mitochondria (SIRT3, SIRT4 and SIRT5) and cytoplasm (SIRT2) (74). Based on phylogenetic analysis, they can be divided into 4 subgroups; class I (SIRT1, SIRT2 and SIRT3), class II (SIRT4), class III (SIRT5) and class IV (SIRT6 and SIRT7) (75). The members of this class have a dual role as oncogenes and oncosuppressors (76, 77).

4.5 Class IV HDACs

The sole member of this class is HDAC11, which is mainly expressed in the brain, heart kidney and muscles. Based on phylogenetic studies, it is mainly related to HDAC3 and HDAC8. HDAC11 enzymatic activity which is driven by a catalytic domain on its N-terminus, can be inhibited by trapoxin (an analogue of TSA) (65).

	Chromosomal location	Cellular localization
Class I HDAC1  482 a.a	1p34	Nucleus HDAC3 nucleus & cytoplasm
HDAC2  488 a.a	6q21	
HDAC3  428 a.a	5q31	
HDAC8  377 a.a	Xq13	
Class IIa HDAC4  1084 a.a	2q37	Nucleus & cytoplasm
HDAC5  1122 a.a	17q21	
HDAC7  855 a.a	12q13	
HDAC9  1011a.a	7p15-p21	
Class IIb HDAC6  1215 a.a	Xp11	
HDAC10  669 a.a	22q13	
Class IV HDAC11  347 a.a	3p25	Nucleus

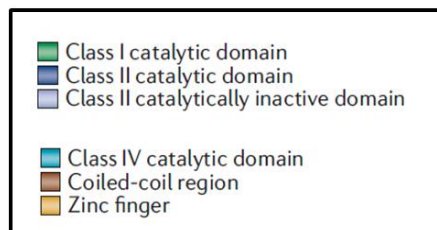


Figure 5. Structure of HDACs superfamily. This figure indicates the structure of different members of HDACs superfamily, their subcellular localization, length and their chromosomal location in Homo sapiens. Adapted from Bolden et al, Nature reviews. Drug discoveries, 2006 and Kim et al, American journal of translational research, 2011 (43, 74, 78).

5. HDACs and hematological malignancies:

APL is the first and the best-known example of hematological malignancies in which the involvement of HDACs has been demonstrated. This leukemia, being the focus of this

dissertation, will be discussed in details in sections 9, 10 and 11. Another example of HDAC involvement in the molecular mechanism of leukemogenesis is the leukemia subtype M2 derived from t(8;21) AML1-ETO chromosomal translocation, expresses in 12% of AML cases. AML1 is an important protein in hematopoietic development, and ETO is a corepressor. In AML1-ETO fusion protein, AML1 moiety retains its ability to bind to DNA and ETO moiety confers new docking sites for HDAC-containing complexes. Thus, this fusion protein acts as a transcriptional repressor of AML1-regulated genes (79). The differentiation block that is mediated by AML1-ETO is partially reversed by HDACi, that supports the involvement of HDACs in this type of leukemia (80). Inv(16) which makes up 8% of AML cases, results in fusion of the CBF β transcription factor, a master transcription factor for myeloid differentiation and granulocytic maturation, with the smooth muscle myosin heavy chain gene MYH11. In this chromosomal aberration the resultant fusion protein recruits the HDAC containing complexes and consequently represses AML1-transactivated genes (80, 81). Similarly to the hematological malignancies reported above, HDAC6 has been identified as the HDAC involved in the pathogenesis of Chronic Myelogenous Leukemia (CML) that is driven by t(9;22) (Bcr-Abl). Indeed, HDAC6 deacetylates HSP90, the cellular chaperone whose overexpression correlates with poor prognosis in tumor cells. HSP90 binds to a variety of client proteins such as Bcr-Abl, Flt3, Erb B2 and c-Raf and prevents their ubiquitination and proteosomal degradation. Thus, inhibition of HDAC6 enzymatic activity with inhibitors such as Tubacin, leads to hyperacetylation of HSP90 and degradation of Bcr-Abl (82).

Among distinct forms of hematopoietic malignancies in which HDAC-dependent transcriptional repression has been implicated in the pathogenesis of the disease, non-Hodgkin's lymphomas, diffuse large B-cell lymphoma (DLBCL) and the Follicular lymphoma can be named, in which B-cell lymphoma 6 (BCL-6) is the involved transcriptional repressor that is associated with class I and class II HDACs (83). BCL-6 acetylation by HAT p300

disrupts its ability to recruit HDACs and transform cells (80). One type of cancer that has shown tremendous improvement upon treatment with HDACi is CTCL, a heterogeneous group of non-Hodgkin's lymphoma that is characterized by an initial accumulation of malignant, mature T cells in the skin (84). Furthermore, development of T-cell acute lymphoblastic leukemia (T-ALL) in which SCL-TAL1 transcription factor is overexpressed, is due to the repression of genes involved in thymocytes differentiation and survival through the recruitment of HDAC1 containing complexes by the SCL-TAL1 (85). Thus, according to the described role of HDACs in different hematological malignancies, HDACi can be considered effective compounds for the treatment of these types of diseases. Far away from all the above-mentioned features, currently the role of individual HDACs in leukemogenesis remains largely unexplored with the exception of a few recent studies (86, 87).

In addition to aberrant recruitment of HDACs by oncogenic fusion proteins to specific loci in hematological malignancies, the altered expression of *Hdac*s have been demonstrated in non-hematological malignancies. Increased expression of *Hdac1* has been shown in gastric, prostate and colon cancer, *Hdac2* is overexpressed in colorectal and cervical cancer, *Hdac3* is overexpressed in colon cancer (88). Moreover, HDAC8 has an important role in the pathogenesis of neuroblastoma (79). All these studies highlight the importance and widespread therapeutic potential of HDACi and introduce HDACs as potential targets for therapeutic intervention.

6. HDAC inhibitors (HDACi):

The anti-tumor potential of small molecules, which interfere with the activity of HDACs, has led to development of a variety of HDACi. Difference in therapeutic potential of HDACi

stems from their active concentrations, distinct structures and also their broad anti-tumor activity and low toxicity towards normal cells (79). One of the proposed mechanisms to explain the selectivity of these compounds against malignant cells is the upregulation of the expression of death receptors and their ligands specifically in leukemic cells, leading to activation of the extrinsic and intrinsic apoptotic pathway, not observed in normal hematopoietic cells (89). Another possibility for the selectivity of these compounds against tumor cells is upregulation of the cell cycle inhibitor gene *p21* in transformed cells, that leads to cell cycle arrest (74). Therefore, HDACi which have anti-angiogenic potential (72) and may lead to induction of growth arrest, apoptosis, autophagy and differentiation of transformed cells (42) can be used as cancer therapeutics. To date, several HDACi, due to their proved anti-cancer potential, have been introduced into clinical trials for the treatment of hematological malignancies and solid tumors (90).

7. Classes of HDACi:

HDACi based on their chemical structures are divided into four groups: short chain fatty acids (or aliphatic acids), benzamides, cyclic peptides (the most structurally complex group) and hydroxamic acids (74) (Figure 6). In parallel, based on the specificity, these compounds are divided into pan-HDACi which inhibit the activity of both class I and II HDACs such as Vorinostat, Belinostat, Panobinostat and Trichostatin A (TSA) or selective HDACi, as Entinostat which exclusively inhibits class I HDACs (80). Due to the diverse cellular functions of HDACs, it is unclear which of the two broad groups of HDACi provide the best clinical benefit and optimum toxicity profile (72).

7.1 Short-chain fatty acids (aliphatic acids):

The short chain fatty acids or aliphatic acids, which are the smallest and simplest types of HDACi, include n-butyrate, Phenylbutyrate and Valproic acid (VPA) with the activity seen at milimolar concentrations. Owing to the high concentrations (mM) required to inhibit HDAC activity, these agents are not ideal for clinical use. Butyrate is a product of fermentation by intestinal micro flora in the colon. It was actually identified as the first compound, with HDACi activity (**91, 92**).

7.2 Benzamides:

The compounds belonging to this class contain a benzamide moiety. MS-275 is a member of this class, in which the HDACi activity is accompanied by induction of *p21* expression and cell cycle arrest in G₁ phase and induction of differentiation in association with the blockage of mTOR signaling (**93**). In a study conducted in 1999, MS-275 was orally administered to nude mice xenografted with 8 human cell lines. Seven out of 8 tumor cells, including gastric, cervix, ovary, pancreatic, lung, colorectal cancer and leukemia showed growth inhibition (**94**). This HDACi has isoform-specific inhibition activity (acts more on HDAC1/2); however, it is less potent than other HDACi. Approximately 9 years later, in 2008 a new family of benzamide derivatives HDACi was identified, which is less toxic in comparison with hydroximates and possesses class I specific inhibition. These HDACi are known as pimelic diphenylamide. Compound 106 was one of the first HDACi belonging to pimelic diphenylamides, with the inhibitory activity against class I HDACs, with the preference toward HDAC3 (**95**). Kinetic studies indicated that 106, is a slow, tight-binding compound with slow On/Off inhibitory mechanism, while hydroximates are fast On/Off HDACi.

Most recently, a new HDAC3 selective inhibitor (known as RGFP966) has been identified that is a derivative of pimelic diphenylamide family. Actually it is an *N*-(*o*-amino phenyl) carboxamide HDAC inhibitor. In a biochemical assay, it has been demonstrated that this compound has an inhibitory effect against HDAC3 with an IC₅₀ of 0.08 μM and no other inhibition toward any other HDACs at concentrations up to 15 μM (96). This class of HDACi provides the possibility to study the role of HDAC3 in cancer. In chapter III the effects of this HDACi (RGFP966) on APL cells will be explained.

7.3 Cyclic peptides:

This class which is the most complex class of HDACi includes Apicidin and Depsipeptide. In November 2009, Depsipeptide or FK228, also known as Romidepsin, was FDA approved for the treatment of CTCL (74). This compound works in nanomolar ranges as a potent inhibitor of class I and class II HDACs. During the screening for identifying compounds that can selectively reverse the phenotype of *ras* transformed cells, Romidepsin was isolated from the fermentation broth of a gram-negative, coccobacillus *Chromobacterium violaceum* strain by a pharmaceutical company in Japan. In a study which was conducted by Nakajima et al, it has been shown that Romidepsin caused the cell cycle arrest at both G₁ and G₂/M phases and induced the apoptosis in CTCL patients. They also demonstrated that this compound led to accumulation of acetylated histones (H1, H2A, H2B, H3, and H4) in the cell, and established its role as a novel HDACi (97). Another HDACi belonging to cyclic peptide, is Apicidin with a broad spectrum of antiproliferative activity against a variety of cancer cell lines, and it has been demonstrated that HL60 cells treated with Apicidin, showed decreased number of viable cells, concomitant with progressive accumulation of hyperacetylated histone H4 (98).

7.4 Hydroxamic acids:

In 1976, around 10 years after discovery of n-butyrate as an HDACi, the anti-fungal compound TSA was isolated from metabolites of strains of *Streptomyces hygroscopicus*. TSA was the first hydroxamic HDACi found to inhibit HDACs (**99**). This compound could induce differentiation of Friend cells leading to cell cycle arrest of rat fibroblasts. Since administration of TSA caused accumulation of acetylated histones in a variety of cell lines, it was determined as a potent HDACi (**99**). TSA has activity in nano molar concentrations but has also undesirable toxic side effects. In 1996, a second generation of hybrid polar compound was made and was called Suberoyl Amino Hydroxamic acid (SAHA), which was a potent inducer of differentiation of murine erythroleukemic cells (**100**). Less than two years later, the same group of researchers, reported the SAHA as a potent HDACi, which could lead to hyperacetylation of H4 (**101**). Indeed SAHA was the first HDACi that has been approved by U.S. FDA for the treatment of relapsed and refractory cutaneous T-Cell lymphoma (CTCL) (**102**). SAHA is also known as Vorinostat in the clinics or Zolinza, which appears to be very potent at micro molar concentrations and is a pan-HDACi which targets both HDAC class I and class II (**103**). There are other HDACi which belong to hydroxamic acid group such as Belinostat (PXD101) Panobinostat (LBH589), and Givinostat (ITF2375) which are all pan-HDACi (**80**).

Almost all of the HDACi which have been discovered or synthesized till now, are acting in a reversible manner, with the exception of Trapoxin and Depudesin, two HDACi belonging to cyclic tetrapeptides family that bind covalently to epoxy ketone group and irreversibly inhibit the enzymatic activity of HDACs (**65**).

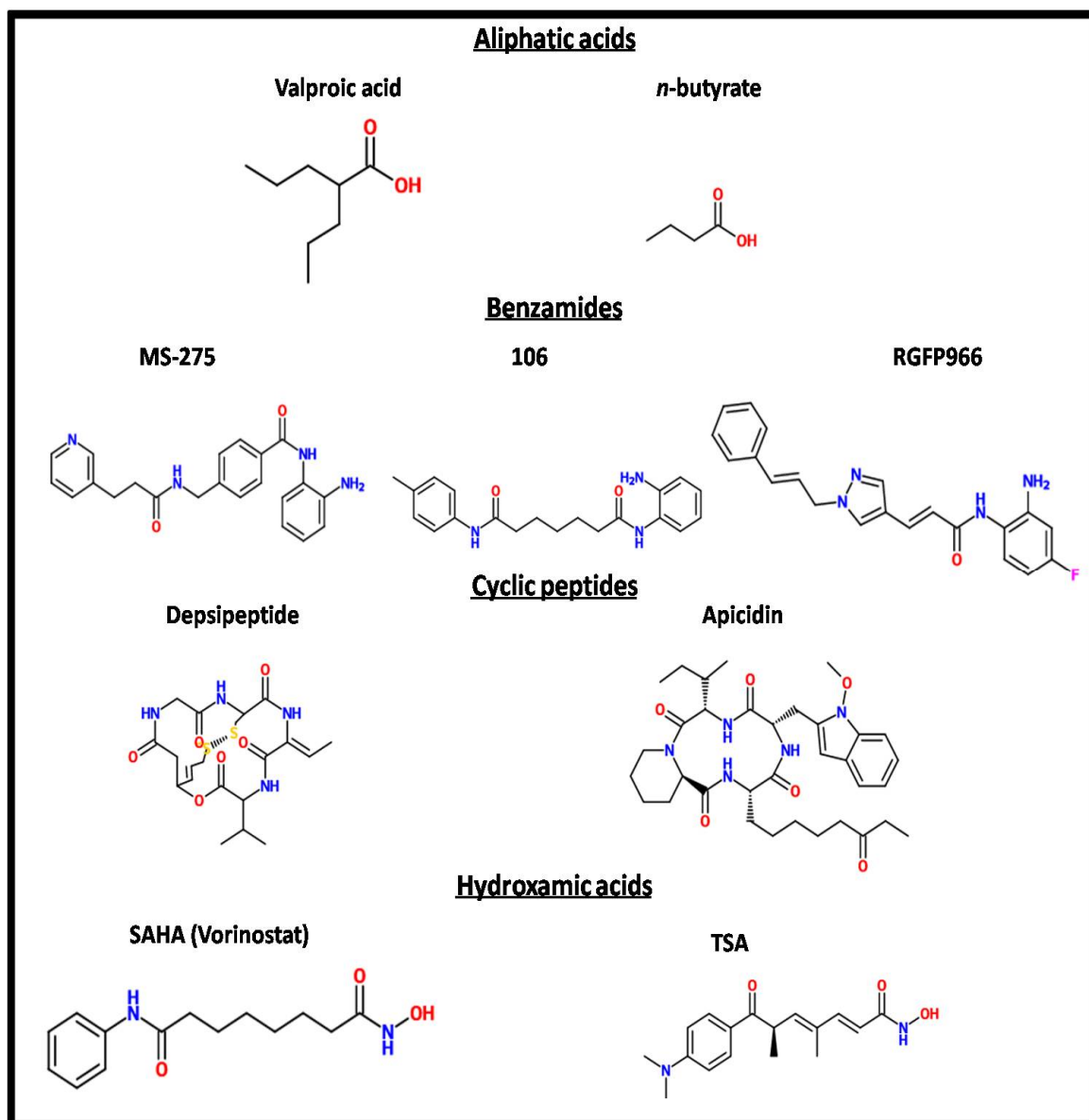


Figure 6. Structure of histone deacetylase inhibitors.

8. Mechanisms of action of HDAC inhibitors:

HDACi mediate cell death, cell cycle arrest or differentiation, through different pathways.

8.1 Effects on the cell cycle:

Low concentrations of HDACi predominantly induce G₁ arrest, while high concentrations induce both G₁ and G₂/M arrests (104). Several HDACi induce the cell cycle arrest at G₁ by

the upregulation of cyclin-dependent kinase inhibitor p21 (**105**). HDACi in normal and transformed cells can induce arrest at G₂/M stage of the cell cycle. Due to the lack of a functional G₂ checkpoint in transformed cells they undergo apoptosis, while the normal cells pass through DNA repair and enter M phase, perhaps explaining the ability of HDACi to cause selective cell death of tumor cells. HDACi induce G₁ arrest even in cells without p21, which is feasible through induction of other CDK inhibitors that cause cell cycle arrest, such as p15 and p27. HDACi can also lead to Rb dephosphorylation. Dephosphorylation of Rb blocks E2F transcriptional activity (target genes required for G₁ progression and G₁/S transition) through the downregulation of cyclins (**106**). In 2000, Kim et al, investigated the mechanism of cell cycle arrest at G₁ in human cervix carcinoma HeLa cells. They demonstrated that treatment of HeLa cells with TSA mechanistically led to histone hyperacetylation followed by growth arrest in G₁, as well as hypophosphorylation of Rb. Upon TSA treatment, amount of cyclin E and the CDK inhibitor p21 increased strikingly, while that of cyclin A decreased. Induction of p21 and reduction of cyclin A correlated well with the decreased CDK2 activity and cell cycle arrest (**107**).

HDACi can inhibit the spindle assembly checkpoint through disruption of spindle checkpoint proteins such as BUBR1 and CENP-F, leading to transient arrest of cells at prometaphase, followed by aberrant mitosis such as missegregation and loss of chromosomes, resulting in cell death by either apoptosis or mitotic cell death/catastrophe (**72**).

HDACi can induce mitotic defects through the aberrant acetylation of histones in heterochromatin domains. Newly replicated chromatin contains acetylated histones, which are deacetylated during or after DNA replication. Upon administration of TSA, histones in newly synthesized chromatin remain acetylated, and cause disruption of the structure and function of the centromere and the pericentric heterochromatin, with loss of binding to heterochromatin binding proteins (**106**).

8.2 Effects on DNA repair:

When HDACi inhibit the activity of HDACs, the hyper acetylated DNA cannot be protected in the form of condensed chromatin and will be exposed to radiation, cytotoxic drugs, reactive oxygen species (ROS) or UV. Therefore, these events promote the generation of DNA double-strand breaks (**108**). In a study conducted by Robert et al, it has been shown that inhibition of class I and II HDACs by VPA in yeast influences on the DNA damage response through: i) counteracting the checkpoint activation (Mec1 in yeast, orthologue of human ATR); ii) double-strand break processing in G₂/M and iii) acetylation and degradation of enzymes involved in DNA-double strand break repairs, through the autophagy (**109**). Accumulation of ROS after the treatment with HDACi leads to oxidative stress and subsequent apoptosis (**102**).

8.3 Effects on extrinsic and intrinsic apoptotic pathways:

The extrinsic apoptotic pathway is initiated by the binding of death receptors, such as Fas, tumor-necrosis factor receptor 1 (TNFR1) and TNF-related apoptosis-inducing ligand receptor (TRAIL-R) to their ligands, including FasL, TNF and TNF-related apoptosis-inducing ligand (TRAIL), which leads to activation of caspase 8 and 10. HDACi induce the extrinsic pathway of apoptosis through the induction of death receptors and their ligands in transformed cells. For instance, Insinga et al. demonstrated that VPA induced TRAIL and DR5 in the mouse model of APL, and this induction was limited to transformed cells (**110**).

The intrinsic apoptotic pathway, which is mediated by mitochondria, is induced by HDACi in transformed cells by releasing cytochrome c from mitochondria and activation of Caspase 9. HDACi induce the intrinsic apoptotic pathway by upregulation of pro-apoptotic proteins of Bcl-2 family or/and through reduction of anti-apoptotic proteins (**106**).

In addition, HDACi through inhibition of Hypoxia inducible factor (HIF) have anti-angiogenic effects and they impact on ubiquitin-proteasome system. The summary of pathways in which HDACi have an impact is shown in **Figure 7 (72)**.

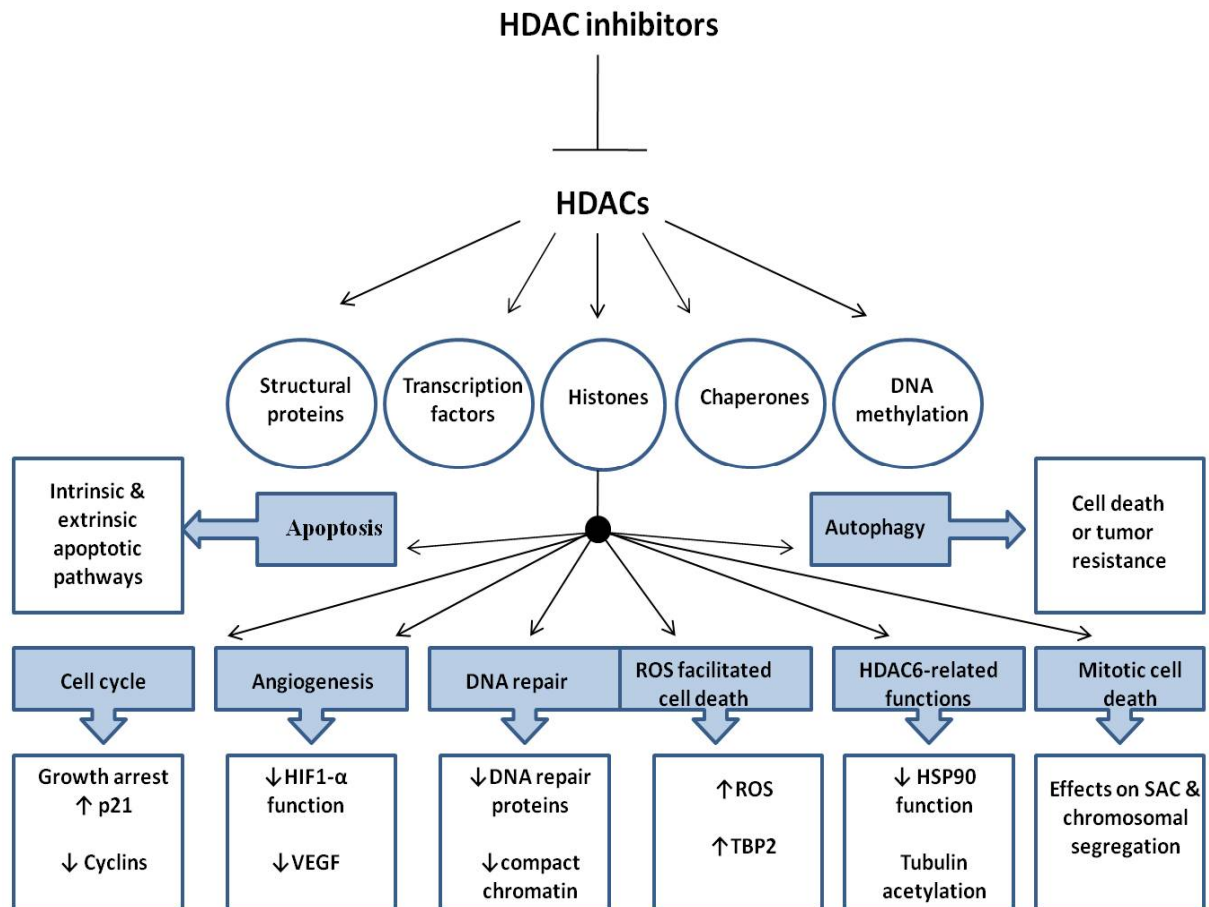


Figure 7. Impact of HDAC inhibitors on malignant cells through different pathways. HDAC inhibitors inhibit the deacetylation activity of HDACs. Thus, hyperacetylation of histones alter chromatin activity and deregulations of deacetylation of several non-histone proteins have effects on cell cycle, angiogenesis, apoptosis and so on. Taken together, as most of these pathways are impaired in tumor cells they can be targeted by HDAC inhibitors. Modified from Khan et al, Immunology and cell biology, 2012 (72).

9. Molecular pathogenesis of Acute Promyelocyte Leukemia:

APL is characterized by hyperproliferation of immature granulocytes, known as promyelocytes. According to the French-American-British (FAB) classification, APL belongs to the M3 subtype of AML and accounts for around 5-10% of all AML cases (*III*). APL is often associated with chromosomal translocations which lead to the fusion of the gene encoding for the retinoic acid receptor alpha ($RAR\alpha$), a master regulator of myeloid differentiation, located on chromosome 17, and a number of alternative genes, for instance; promyelocytic leukemia (PML), promyelocytic leukemia zinc factor (PLZF), nucleophosmin (NPM), nuclear mitotic apparatus (NuMA), signal transducer and activator of transcription 5b (STAT5b) (**Figure 8**).

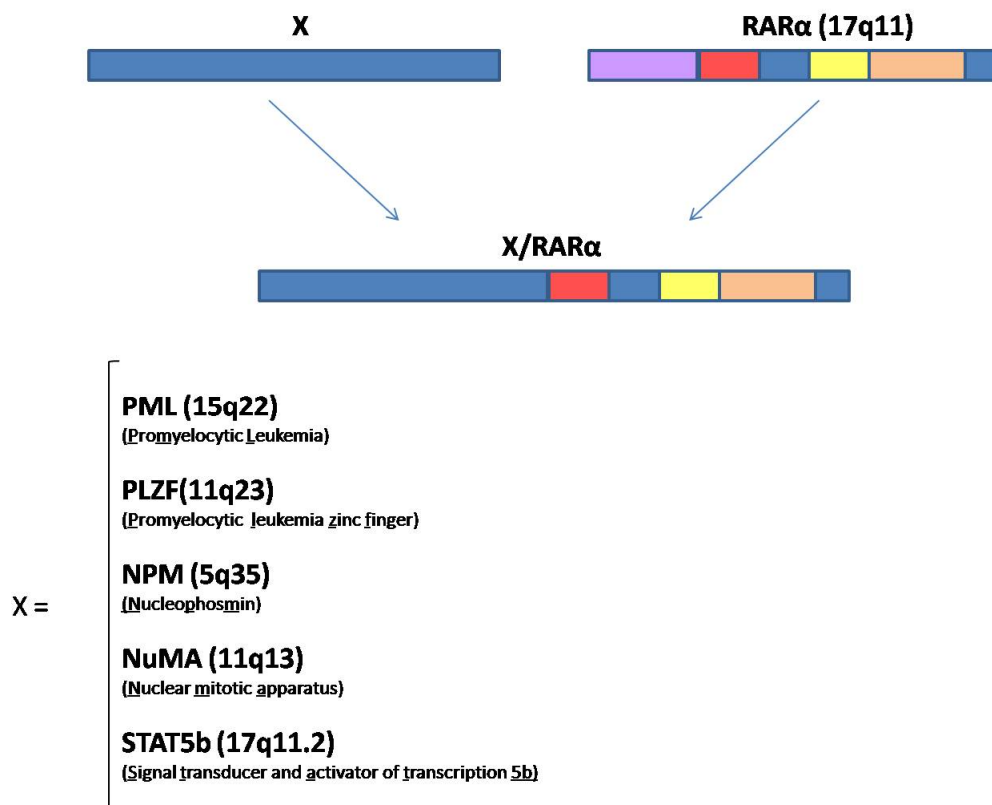


Figure 8. Chromosomal translocations associated with APL result in fusion proteins.

Modified from Melnick et al, Blood, 1999 (*112*).

In more than 95% of the cases, APL is caused by a balanced chromosomal translocation t(15;17)(q22;q21), which juxtaposes the genes encoding for the promyelocytic leukemia (PML) on chromosome 15 to the RAR α (**113**, **114**). PML-RAR α recruits different chromatin modifier factors, such as HDAC complexes, like NCoR (**115**) and SMRT (**116**) and DNA methyltransferases (DNMTs) (**117**) to the promoter of RAR α target genes and transcriptionally silence them, leading to alteration in chromatin structure and the myeloid differentiation block (**118**, **119**).

In 5% of APL cases, there is a chromosomal translocation involving RAR α on chromosome 17 that translocates to PLZF on chromosome 11. PLZF encodes a transcription repressor that is a member of the POK family of proteins. While APLs with PML-RAR α fusion protein are responsive to pharmacological doses of RA, APLs with t(11;17) do not respond or respond poorly to RA (**120**). In several studies, researchers made an attempt to discover the reason behind this phenomenon (resistance of PLZF-RAR α to RA). Results of these studies suggest that similar to PML-RAR α , PLZF-RAR α recruits corepressor complexes containing HDACs. PML-RAR α has a binding site for NCoR repressor complex in its RAR α moiety that is called CoR box, while PLZF-RAR α contains two NCoR binding sites: the first is RA sensitive and maps within the CoR box of RAR α , the second is RA resistant and is found at the amino terminal of PLZF, acting as a stronger repressor compared to PML-RAR α (**115**).

In contrast to most cancers, mutations of *p53* are rare in APL patients, but dysfunctionality of *p53* is imposed by the cooperation of PML-RAR α with class I HDACs, which deacetylate *p53* and induce its degradation through the MDM2-proteasome mediated pathway (**121**). HDACs from class I (HDAC1, 2 and 3) have been found associated with PML-RAR α paving the way for the use of HDACi in APL treatment. Disruption of PML nuclear bodies (PML-NBs), which have been implicated in a variety of cell functions is mediated by PML-RAR α (**122**). In

the mouse model of APL, the disease can be initiated by the expression of oncogenic fusion protein in hematopoietic stem cells or progenitors. This stage of the disease is known as pre-leukemic stage which leads to the onset of the disease after a long latency (full leukemic stage), suggesting the monoclonality or oligoclonality of APL and it faithfully recapitulates clinical and morphological symptoms of human APL (123). Analysis of different transgenic mice expressing AML-fusion proteins indicates that even though these fusion proteins have oncogenic potential, additional genetic and/or epigenetic lesions are required for progression to full leukemic stage. In APL the first hit which confers the oncogenic potential to cells is PML-RAR α and HDACs are among presumptive second hits (79). There are emerging evidences showing that alterations causing global chromatin changes such as mutations in genes encoding: DNA methylation and histone modification enzymes occur at the pre-leukemic stage, and the pre-leukemic HSCs may survive upon chemotherapy and by gaining additional mutations they will play the role of putative reservoir for relapsed disease, while mutations in proliferative genes occur later on (124).

10. APL and targeted therapy:

APL is the first malignancy cured by targeted therapy. All-trans retinoic acid (ATRA), also known as retinoic acid (RA), leads to the terminal differentiation of leukemic cells by reversing the PML-RAR α mediated transcriptional inhibition, through the degradation of PML-RAR α and substitution of co-repressor complexes with co-activators through conformational changes. Further studies have shown a prerequisite for chromatin repression by PML-RAR α through its binding to the PU.1 (a master regulator of hematopoietic differentiation process) (125). The first studies for the treatment of APL with ATRA began in

1980s. Following treatment with ATRA alone, patients experienced induction of an ATRA-resistance relapsed APL and for this reason for the previous 3 decades was used in combination with chemotherapy (126, 127). Arsenic trioxide (ATO or As₂O₃), which was first, approved in relapsed APL prompts the apoptosis of APL cells and as single agent cures approximately 70% of *de novo* APL. ATRA and ATO, which are known as the two most important drugs for early diagnosed APL, both target the stability of PML-RAR α , ATRA through the RAR α moiety and ATO via PML moiety (128). However, a recent study demonstrated that differentiation of leukemic blasts stimulated by ATRA is neither sufficient nor necessary for the treatment of APL, since the administration of ATRA in PLZF-RAR α driven APL that is known as ATRA-resistant variant of APL leads to the differentiation of leukemic blasts but not to the remission of the disease or in the relapsed PML-RAR α driven APL patients which have been become resistant to ATRA due to the suboptimal concentrations of drug, the cell proliferation and tumor growth will be sustained even if differentiation is ongoing. Therefore instead of therapy aimed at inducing differentiation, looking for therapies which target self-renewal ability of cancer cells can be more efficient (129). Genome-wide epigenetic studies revealed that treatment of APL with pharmacological doses of ATRA induces an increase in histone H3 acetylation at PML-RAR α binding sites or at nearby target genes. It has been suggested that this results in the release of PML-RAR α from the genes which are important for normal hematopoietic differentiation (17).

11. APL as a paradigm for epigenetic therapy:

Since APL is the best-known and most studied example of a hematological malignancy in which a causative role for HDACs has been clearly demonstrated, it represents a perfect model to validate the concept of epigenetic therapy. As it was described before, APL treatment with

retinoic acid (RA) represents the first example of: i) targeted therapy, ii) transcription therapy and, iii) differentiation therapy in which the drug restores the normal program of cellular differentiation rather than killing leukemic cells as conventional chemotherapy would do. Working as an altered transcription factor, PML-RAR α is able to alter the epigenetic landscape of its target cells, causing a differentiation block at the promyelocytic stage. RA reverts the action of the fusion protein and allows terminal differentiation of the leukemic cells, and in combination with other agents (chemotherapy, arsenic) leads to definitive cure of the majority of APL patients. Although PML-RAR α recruits HDACs for its action at target genes, the interplay of PML-RAR α with epigenetic modifiers is more complex than previously thought (17). Experimental studies aimed at dissecting the role of individual HDACs in APL development and maintenance have shown that transplantation of mice with lineage negative (Lin⁻) PML-RAR α expressing cells knocked down for *Hdac1* or *Hdac2*, caused an accelerated leukemia development by shortening the pre-leukemic phase. Importantly, a dramatic expansion of a subpopulation of pre-leukemic cells with the features of leukemia initiating/stem cells (LICs) could be detected as soon as 40 days post-transplantation of Lin⁻ cells expressing PML-RAR α and knocked down for *Hdac1*, suggesting that HDAC1 is required to control the self-renewal of LICs. Strikingly, in frank leukemic cells knockdown of the same *Hdacs* slows down leukemia growth, demonstrating that HDAC1/HDAC2 play different roles in APL initiation and maintenance. Complementing these studies, the HDAC inhibitor Valproic acid or VPA (mainly acting on class I HDACs) was shown to behave differently at the pre-leukemic vs. the leukemic stage, mimicking the *Hdac1* knockdown phenotype (87). Moreover, in established leukemia, VPA showed a different effect on the bulk of tumor cells vs. LICs, being mostly effective on the bulk of APL blasts and having only a mild effect on LICs (130). From these studies, it therefore appears that HDACs can not only play different roles at different stages of tumorigenesis, but also have different biological

effects in different tumor cell subtypes (LICs vs. bulk). Since epigenetic changes are important and required for normal cellular differentiation, it is not surprising that the effect of epigenetic drugs depends on the targeted cellular population.

Aims of the project

As epigenetic aberrations are found in almost all AML subtypes and these modifications are reversible, it is not surprising that drugs targeting epigenetic marks and the chromatin modifier enzymes are being developed and used to treat the disease. Thus, targeting of epigenetic aberrations is becoming an established approach for the treatment of AML patients. Several epigenetic drugs have been approved for clinical use, and others are in various stages of development. As an example of epigenetic therapy, HDACi that target multiple HDACs are effective for the treatment of CTCL.

Acute promyelocytic leukemia (APL), a subtype of acute myeloid leukemia (AML), has been deeply characterized. In particular PML-RAR α , the most frequent chimeric protein (approximately covers 95% of APL cases) responsible for the occurrence of APL, has been extensively studied and it has been identified as the first disease in which the involvement of HDACs in the pathogenesis of the disease has been implicated.

Since epigenetic drugs have single agent efficacy only in selected hematological malignancies, further studies aimed at better understanding of the epigenetic alterations and of epigenetic enzymes in different cancers are needed. Our group is studying the role of HDACs in the pathogenesis of APL. The group has previously shown that HDAC1 and to a lesser extent HDAC2 have a dual role in APL: oncosuppressor in establishment, oncogene in maintenance of the disease, and that HDACi can have different effects on bulk *vs.* leukemic stem cells.

Actually we believe that a better understanding of the role of individual HDACs in leukemia paves the way for developing more efficacious treatments with selective epigenetic drugs.

Taken together, my project has focused at studying the role of HDAC3 in the development of APL, and in the maintenance of the disease. To achieve our goals, we used APL as a model system, using a selective knock-down of *Hdac3* in primary murine hematopoietic progenitors to study the biological effects of the knock-down on APL initiation *in vitro* and *in vivo*. Then we took advantage of using the selective knockdown of *Hdac3* in primary APL cells to investigate the biological effects of the knockdown in APL maintenance. In this study HDACi (RGFP966) with selectivity against HDAC3 has been used, to investigate the feasibility of targeting HDAC3 for clinical use. To further investigate the role of HDAC3 in more hematological malignancies, its role has been systematically studied in AML driven by MLL-AF9 in collaboration with Prof. Johannes Zuber's group and in lymphoma driven by E μ -myc model in collaboration with Prof. Ricky Johnstone's group.

Materials & methods

1. Plasmids:

pRETRO-SUPER (pRS): a modified retroviral vector in which the cDNA for puromycin selection marker had been replaced with the one encoding for enhanced green fluorescence protein (eGFP). The shRNA-based plasmids were generated by cloning the synthetic oligonucleotides targeting the mRNAs for firefly luciferase (*Luc*) as the control and *Hdac3* into the modified pRS. The shRNA sequences (**Table 1**) are in the format: target sequence sense (underlined), loop-target sequence antisense (underlined).

shRNA name	Sequence
CTRL (<i>Luc</i>)	GATCCCC <u>CGTACGCGGAATACTTCGATTCAAGAGATCGAAGTATTCCG</u> <u>CGTACGTTTTTGGAAA</u>
<i>Hdac3</i>-KD	GATCCCCGTGGCTCTCTGAAACCTT <u>ATTCAAGAGATAAAGGTTTCAGAG</u> <u>AGCCACTTTTTGGAAA</u>

Table 1. shRNA sequences used in the experiments with pRetroSuper vector.

Inducible Tet-On shRNAmiR30 dual vector system: the vectors have been generated in collaboration with R.W Johnstone's and J. Zuber's lab from (The Sir Peter MacCallum Department of Oncology, University of Melbourne and Institute of Molecular Pathology, Vienna, Austria), this system represents a better experimental set-up allowing a more thorough functional analysis of the role of HDAC3 in APL and precise tracking of both retrovirally

transduced cells and shRNA induction, through two fluorescent reporters; dsRed which shows the expression of shRNA and Venus which shows the transduction of vector into cells (**Figure 9**).

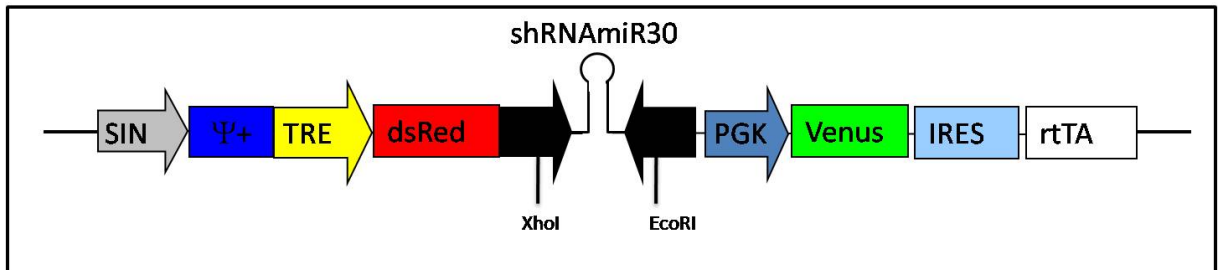


Figure 9. Schematic representation of an inducible Tet-On miR30 dual color vector (All-in-one system). An inducible shRNA expression system that enables precise tracking of retroviral transduction and shRNA induction through two fluorescent reporters. In TRE-dsRed-miR30/shRNA-PGK-Venus-IRES-rtTA (TRMPV), upon induction by doxycycline the TRE drives expression of a dsRed fluorescent protein and shRNA, whereas the phosphoglycerate kinase (PGK) promoter drives constitutive expression of the yellow-green fluorescent protein Venus. The vector is constructed in the pQCXIX self-inactivating (SIN) retroviral backbone.

In TRE-dsRed-miR30/shRNA-PGK-Venus-IRES-rtTA or TRMPV vector, while phosphoglycerate kinase (PGK) promoter drives constitutive expression of Venus fluorescent protein, Tetracyclin-responsive element (TRE) drives expression of dsRed fluorescent protein and microRNA (miR30)-embedded shRNA, upon administration of doxycycline. In fact we had two types of inducible vectors, the “All in one system” depicted in **Figure 9** and the “Double system” (**Figure 10**). In the double system, cells were first infected with reverse-tetracyclin transactivator (rtTA) and transduced cells were selected by hygromycin and then they were infected with TRMPV vector, which contains miR30-embedded shRNA. The shRNA sequences used in these types of vectors can be found in **Table 2**.

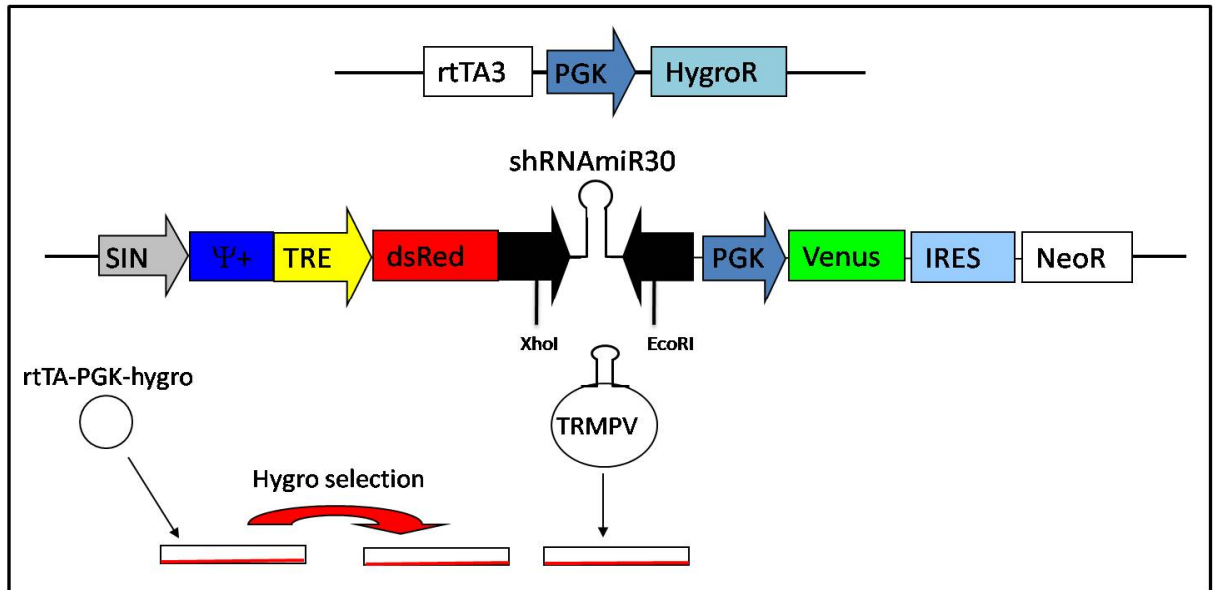


Figure 10. Map of inducible TRMPVIN vector or double system. In this system rtTA expression is enforced in a vector which contains hygromycin selection marker and miR30-embedded shRNA expression is enforced in the second vector by administration of doxycyclin.

Targeting sequences for short hairpin RNA (shRNA)-miR30 constructs against murine HDAC3 (RefSeq NM_010411) were identified using Designer of Small Interfering RNA (DSIR) algorithm. The top-ranked shRNAs were used to create 10 miR30 sequences (97mer, Sigma-Aldrich) and these were cloned into the XhoI/EcoRI sites of TRMPVIN plasmid following the generation of approximately 110-bp shRNA-miR30s by amplification of 97 mers using 5'miR30-XhoI (CAGAAGGCTCGAGAAGGTATATTGCTGTTGACAGTGAGCG) and 5'miR30-EcoRI (CTAAAGTAGCCCCTGAATTCCGAGGCAGTAGGCA) primers. The miRNA oligomer sequences are in the format: miR-30 context-sense ()-loop-antisense ().

shRNA name	sequence
Tet-On-Scrambled shRNAmiR30	<u>TGCTGTTGACAGTGAGCGATCTCGCTTGGGCGAGAGTAA</u> <u>GTAGTGAAGCCACAGATGTACTTACTCTCGCCCAAGCGA</u> <u>GAGTGCCTACTGCCTCGGA</u>
Tet-On-Hdac3 shRNAmiR30	<u>TGCTGTTGACAGTGAGCGCTAAGAAGATGATCGTCTTCAA</u> <u>TAGTGAAGCCACAGATGTATTGAAGACGATCATCTTCTTA</u> <u>TTGCCTACTGCCTCGGA</u>
Tet-On-Renilla shRNAmiR30	<u>TGCTGTTGACAGTGAGCGCAGGAATTATAATGCTTATCTA</u> <u>TAGTGAAGCCACAGATGTATAGATAAGCATTATAATTCCT</u> <u>ATGCCTACTGCCTCGGA</u>

Table 2. Sequence of shRNAs used in the experiments with Tet-On-inducible vector. 97 mer

XhoI/EcoRI miR30-shRNA fragments are provided.

2. Calcium Phosphate transfection:

This technique can be used for both transient and stable introduction of exogenous DNA into mammalian cells through the formation of calcium phosphate-DNA precipitates.

The day before transfection the packaging cells (in this study packaging ecotropic phoenix cells which were purchased from ATCC) were seeded at the density such that the day of transfection they were 50% to 60% confluent. Phoenix cells were maintained in Dulbecco modified eagle medium supplemented with 10% fetal bovine serum (FBS), 2 mM of L-glutamine and antibiotics. For the transfection two solutions were prepared, the first one contained DNA of pRS (10µg/ 100mm plate) and DNA of packaging plasmid, pCL-Eco (5µg/100mm plate), 61µL of CaCl₂ and H₂O in the final volume of 500µL; the second solution contained (500µL/100mm plate) of 2X Heps-buffer (HBS). The first solution was slowly added drop wise to the 2X HBS while bubbling air through it with a Pasteur pipette. After 10-20 minutes the formed DNA-calcium precipitates were added to the media of the cells. The cells were incubated at 37°C in a humidified CO₂ incubator for 12 hours, and then the media

was removed and replenished with fresh one. Forty-eight and 72 hours post-transfection the first and second viral supernatant were collected.

3. Concentrating viral supernatant:

In order to concentrate retroviral-based particles, 48 hours and 72 hours post-transfection the first and second viral supernatant respectively were filtrated through 0.45 µm Millipore syringe filters and transferred into sterile 50mL falcons, and 1 volume of cold PEG-itTM virus precipitation solution was added to every 4 volumes of retrovector-containing supernatant. Then it was refrigerated overnight at 4°C, and the next day it was centrifuged at 1500x g for 30 minutes at 4°C. After centrifugation the retrovector particles appeared as a white pellet at the bottom of the falcon all traces of fluid was removed by aspiration and the retroviral pellet was resuspended 1:100 of original volume using cold, sterile PBS, aliquoted into cryogenic vials and stored at -80°C.

4. Immunoblots and antibodies

Whole cell extracts were obtained by using 100µL to 200µL of sodium-dodecyl-sulfate (SDS) lysis buffer (2% SDS, 10% glycerol, 50mM of Tris HCL). Protein was quantified by a Bio-rad Bradford assay. 50µg total protein was boiled for 5 minutes at 95°C. Protein was electrophorased and separated on 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a nitrocellulose membrane (Whatman, Schleicher & Schuell) in a 1X transfer buffer containing 20% methanol, at 100V for 1 hour at 4°C. Ponceau S was used for staining of membrane, which allows the visualizing of proteins and thus the quality of the transfer.

After a brief wash in water and 1% Tris-buffered Saline-Tween (TBS-T), the membrane was blocked in 5% milk dissolved in 1% TBS-T for 1 hour at room temperature and then probed with the primary antibody diluted in 5% milk 4°C overnight. The next day, after 3 washes with 1% TBS-T (each wash 10 minutes), membrane was incubated with the proper horseradish peroxidase (HRP)-conjugated secondary antibody diluted in 5% milk, for 30-60 minute at room temperature. After 3 washes in 1% TBS-T, the bound secondary antibody was revealed by using the ECL (Enhanced Chemi luminescence) method. The antibodies used in this study were anti-HDAC3 (ab7030) and anti-Vinculin (Sigma Aldrich).

5. Primary cells:

Lin⁻ (lineage negative) cells which are enriched for hematopoietic stem and progenitor cells obtained from transgenic mCG^{PR/PR} mice, 6 to 12 weeks old (pre-leukemic cells) or from C57BL/6J wild-type mice (wild-type cells). The purified Lin⁻ cells were cultured in RPMI supplemented with 10% FBS Stem, antibiotics, 2mM of L-glutamine and cytokines. All cytokines were purchased from Peprotech. Cells were maintained between 500×10^3 - 1×10^6 cells/ mL.

APL cells were produced by inoculation of transduced Lin⁻ cells derived from 129SvEv mice, with PML-RAR α expressing PINCO vector into syngenic irradiated mice and then the APL blasts were obtained from leukemic animals, as previously described (**131**) and cultured in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with FBS (12.5%), horse serum (12.5%), 2mM L-glutamine, penicillin/streptomycin (1%), hydrocortisone (260ng/mL), β -mercapthoethanol (50 μ M), Amphotericine B (1%), recombinant IL-3 (2 ng/mL), IL-6 (2 ng/mL) and stem cell factor (SCF; 10 ng/mL).

6. Purification of Lin⁻ cells:

To purify murine hematopoietic stem and progenitor cells, the negative selection system (StemSep[®] Hematopoietic Progenitor Enrichment) was used. In this system the unwanted cells which are the vast majority of the cells in a start cell suspension, are immunomagnetically labeled and bound to a magnetic column, while the desired cells which do not have the antibody bound to their surface will be collected in the column flow through. Usually 1-2% of Lin⁻ cells will be recovered from the start mononuclear cell population. Specifically, bone marrow was collected from femurs and tibias of 6- to 12-week old mCG^{PR/PR} mice, and to harvest the total bone marrow cells, the bones were crashed and the whole marrows were washed in phosphate-buffered saline (PBS); red cells were removed by incubation in red cell lysis buffer. Sequentially, cells were briefly blocked with rat serum, which prevents the non-specific binding of rat antibodies to the mouse cells. This procedure takes advantage of a cocktail of biotinylated monoclonal antibodies directed against mouse lineage specific surface markers (CD5, CD11b, CD45R/B220, Gr-1, TER119 and neutrophils) and a tetrameric antibody complex directed against both biotin and dextran iron magnetic particles. In the last step of the purification procedure, the cells which were labeled with the colloid were loaded into a magnetic column, and therefore the lineage negative cells pool were eluted and collected for further analysis, while differentiated cells were sustained in the column (**Figure 11**).

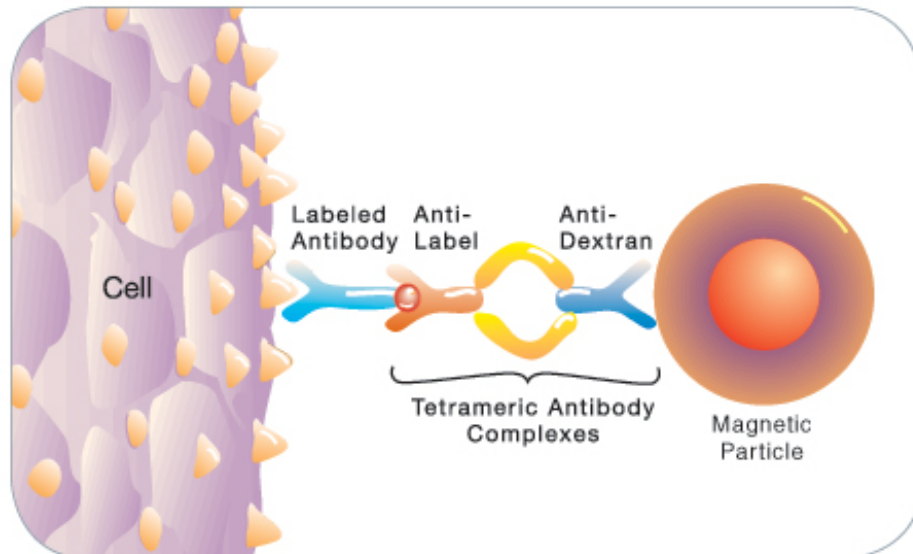


Figure 11. Schematic representation of the negative selection procedure for Lin⁻ purification.

Adapted from www.stemcell.com/en/Products/Popular-Product-Lines/EasySep.aspx

7. Preparing retronectin coated plates:

Recombinant human fibronectin fragment, called retronectin (Takara) favors the co-localization of target cells and retroviruses, therefore enhancing the retroviral mediated gene transduction. Retronectin was diluted with 9 volume of PBS then 500µL of diluted retronectin was added to each well. The plate was incubated at room temperature (RT) for 2 hours, retronectin was then aspirated and the wells were washed once with BSA 2%, then BSA 2% was added for the second time to each well (500µL/well), and the plate was kept at RT for 30 minutes. The last step was washing the wells twice with sterile PBS and then the plate was wrapped in parafilm in order to avoid evaporation and kept at 4°C for the subsequent infection.

8. Infection of Lin⁻ cells:

Enriched hematopoietic stem and progenitor cells (Lin⁻) which were purified as described above, were maintained (500×10^3 to 10^6 cells/mL) in a medium composed of RPMI, 1% glutamine, 1% antibiotics, 10% FBS stem (StemSep), recombinant murine interleukine-3 (rm IL-3) (20ng/mL), rm IL-6 (20ng/mL) and stem cell factor (rm SCF, 100ng/mL). The day after purification, Lin⁻ cells were plated (200×10^3 to 300×10^3 cells/mL) into a 24-well plates which were already coated with retronectin. Cells were infected simply by adding the concentrated retroviral supernatant (20-35 μ L/well) onto the cells; the plate was centrifuged for 15 minutes at 4°C in 3000rpm and then incubated at 37°C overnight. The second round of infection was performed the day after by adding (20-35 μ L/well) of concentrated retroviral supernatant to the cells and centrifugation for 15 minutes at 4°C in 3000rpm. Infected cells expressed shRNA against the mRNA of the protein of interest and GFP as the selection marker. Therefore the selection of cells expressing the shRNA was done by sorting of GFP positive cells by fluorescence-activated cell sorting (FACS) instrument (FACSVantage instrument, BC).

9. Cell proliferative potential: serial replating on methylcellulose:

Colony forming unit assay (CFU assay) is an *in vitro* functional assay for enumerating multipotential and lineage-committed hematopoietic progenitor cells (HPCs) in bone marrow. Based on a study conducted by Occhionorelli et al, it has been shown that PML-RAR α expression results in an enhanced proliferative ability of Lin⁻ cells. While PML-RAR α expression increased the colony-forming potential, which lasted at least eight platings, Lin⁻ cells derived from wild-type mice exhausted their proliferative potential after a few platings (132). A total of 5,000 sorted or unsorted (for the study with inhibitor) Lin⁻ cells or 10,000

sorted APL blast were seeded in methylcellulose medium which is a semisolid medium (MethoCult SF M3434, Stem Cell Technology, Vancouver, BC, Canada) containing 15% fetal bovin serum (StemSep), 1% BSA- 10 μ g/mL Insulin- 200 μ g/mL transferrin- 50ng/mL rmSCF, 10ng/mL rm IL-3, 10ng/mL rm IL-6 and 3U/mL rh EPO, 2-Mercaptoethanol and 2mM L-glutamine. After 7 to 10 days of culture, colonies were scored and the cells were used for immunolabeling, morphologic analysis and serial replating.

10. Cell differentiation: analysis of cell surface markers:

Five thousands GFP⁺ sorted pre-leukemic cells or 10,000 GFP⁺ sorted APL cells were plated in methylcellulose medium. After 7 to 10 days, cells were collected and resuspended in PBS and blocked for 30 minutes with BSA 5%, then they were immunolabeled with Ly-6A/E (SCA1) PE-Cy5.5 conjugated, CD117 (C.Kit) (APC conjugated), CD11b (MAC1) and Ly-6G (Gr-1) both PE-Cy7 conjugated, surface markers.

SCA1 and c.Kit had emerged as a phenotypic marker of choice for identifying and isolating hematopoietic stem and progenitor cells. Hematopoietic stem cells (HSC), multipotent progenitors (MPP), and common myeloid progenitors (CMP) express high levels of CD117. CD11b is expressed at variable levels on monocytes/macrophages, dendritic cells, granulocytes and their precursors and on Natural killer (NK) cells. This marker constitutes a differentiation marker expressed relatively late in hematopoiesis, and is almost undetectable on more primitive stem cells and precursors. The Ly-6G previously named Gr-1, is a myeloid differentiation marker as well; it is briefly found on monocytes during their differentiate pattern and mainly expressed on mature granulocytes.

11. Mice strains and transplantation experiments:

Transgenic mCG^{PR/PR} mice were generously provided by T.J. Ley (Washington University, St Louis, MO) and backcrossed into the C57BL/6J strain. 129*SvEv* mice were bought from the Jackson laboratory.

For lineage-negative (Lin⁻) transplantation experiments, lethally irradiated (9Gy) C57BL/6J mice, 12 to 14 weeks old, were inoculated intravenously with 300,000 GFP⁺-transduced Lin⁻ cells, together with 500,000 spleen cells obtained from a wild type (WT) mouse (to transiently support the new hematopoiesis).

For leukemia transplantation, GFP⁺-transduced leukemic cells were injected intravenously (200,000 cells/mouse) into 129*SvEv* recipient mice. Generation of APL leukemias used for investigating the role of HDAC3 in full leukemic stage has been described previously (*131*).

12. Monitoring of transplanted mice:

Mice that were inoculated with the above mentioned cells were checked periodically for clinical signs of disease (by inspection and periodic blood analysis using the AcTTM 5diff Beckman Coulter, Brea, CA). Long-term reconstitution of the hematopoietic compartment was assessed by scoring the percentage of GFP⁺ cells in peripheral blood at least 4 months after transplantation. Blood samples were obtained from mouse tail vein prepared for flow cytometric analysis by red cell lysis and then FACS analysis was performed using BD, FACSCantoII BD, FACSDiva Software V6.1.1.). Mice were euthanized by CO₂ inhalation when they became detectably ill. The survival rate was calculated using the Kaplan-Meier

method. All these procedures involving animals were done in accordance with national and international laws and policies.

13. Quantitative polymerase chain reaction (Q-PCR):

In order to quantify the HDAC3 mRNA relative levels upon functional knockdown, RNA was extracted from desired transduced-sorted Lin⁻ or APL cells, isolated with RNeasy Mini kit (QIAGEN, Valenica, CA) eluted RNA was quantified by spectrophotometer (ND1000 NanoDrop) and reverse transcribed to cDNA. Ten to 20ng of cDNA were used to perform QPCR using SYBER green reaction mix (Perkin Elmer, Boston, MA). mRNA levels were normalized against GAPDH mRNA. The sequences of the primers used in this study are listed in the table 3.

Primers name	Sequence (5'-3')
mHDAC3fw	AGCTGGACACCCAATGAAAC
mHDAC3rw	TATTGGTGGGGCTGACTCTC
GAPDH fw	AAC TTTGGCATTGTGGAAGG
GAPDH rw	CACATTGGGGGTAGGAACAC

Table 3. Primers for quantitative PCR used in this study.

14. Histone deacetylase inhibitors (HDACi):

The HDACi RGFP966 was kindly given to us by Prof. R.W. Johnstone's lab taken from Repligen Corporation. This compound is an analog of previously published compounds (**133**) but has different HDAC inhibition selectivity. In purified enzyme assays, RGFP966 had the following HDAC inhibition $IC_{50} = 0.08$ mM for HDAC3: and $IC_{50} > 15$ mM for HDAC1 and

HDAC2. RGFP966 was diluted in DMSO (10mM) prior to treatment of Lin⁻ or APL cells at concentrations depicted in Figure 27 and Figure 30 respectively.

15. Treatment with HDACi :

Lin⁻ cells derived from wild type or mCG^{PR/PR} mice and APL cells were treated *in vitro* with different concentrations of the HDAC3-selective inhibitor, RGFP966 (Repligen), then plated in methylcellulose for C.F.U assay and mature and immature populations were defined based on cells morphology

16. May-Grünwald-Giemsa staining:

The cells collected from methylcellulose plates were spun onto a cytological slide by using a cytopsin centrifuge (Cytospin™ 4 Cyto centrifuge). Cytospins were stained using the May-Grünwald-Giemsa staining method. The fixed cells were stained for 8 minutes in May-Grünwald stain, then slides were washed sequentially 3 times in distilled water, kept for 40 minutes in Giemsa stain and diluted with 19 volumes of distilled water. After this step the cytological slides were rinsed again 3 times in distilled water and air-dried. For long time storage, a cover slip was attached to the slides by Eukitt ® mounting medium, which is an adhesive and specimen preservative that can be used manually and in automated cover slipping equipment. Mature and immature populations were defined based on their morphology, by scoring the percentage of mature and immature cells in each condition. In particular, the prevalence of mature and immature cells was analyzed morphologically in

cytological slides in each case and the absolute percentage of mature cells was reported. At least 300 cells were scanned for each case.

17. Mouse tail vein sampling:

Prior to take the sample to dilate the blood vessel, the mouse tail was warmed for some minutes. Then the lateral tail vein was used and 80 μ L of blood obtained per sample. Before collecting the samples 20 μ L of 1mM EDTA had been added into 1.5mL tube in order to prevent blood coagulation. Applying finger pressure on the soft tissue stopped blood flow. A finger was placed at the blood-sampling site for approximately 30 seconds before the animal was returned to its cage.

18. Blood cell staining for the FACS analysis:

1mL of red cell lysis buffer was added to each 100 μ L of blood sample, and then incubated at room temperature for 10 minutes. After incubation it was centrifuged for 5 minutes at 5,000rpm, and the white pellet resuspended in the blocking serum (BSA 5%). After 30 minutes incubation at 4°C, the cells already resuspended in blocking serum were stained with the desired antibody and kept at 4°C for 30 minutes, after washing the unbound antibody by PBS, the cells were fixed with formaldehyde 2% and were used for the FACS analysis (BD, FACSCantoII BD, FACSDiva Software V6.1.1.)

19. Statistical analysis:

Statistical analyses were performed using the Fisher exact test (GraphPad software) unless otherwise specified. Statistical analysis of the Kaplan-Meier survival curves were done using the log-rank test (Prism 4.0 software).

20. Contents of general buffers used in this study:

20.1. Phosphate-Buffered Saline (PBS), pH 7.4:

137 mM NaCl

2.7 mM KCl

8.1 mM Na₂HPO₄

1.76 mM KH₂PO₄

20.2. Tris-Buffered Saline (TBS)-Tween:

150 mM NaCl

2.7 mM KCl

25 mM Tris base

0.1% Tween 20

20.3. Tris-Acetate-EDTA (TAE):

40 mM Tris acetate

1 mM EDTA pH 8.

20.4. Urea lysis buffer:

8 M Urea

25 mM Tris-HCL pH 6.8

1 mM EDTA

10% glycerol

20.5. Resolving gel mix (1 gel at 12%):

Acrylamide/Bis-acrylamide solution 30 %: 2.52 mL for 12% gel

Tris-HCl, pH 8.8 (1.5 mL)

ddH₂O (1.7 mL)

SDS 10% (60 µL)

APS 10% (50 µL)

TEMED (2 µL)

20.6. Stacking gel mix (1 gel):

Acrylamide/Bis-acrylamide stock solution – 30%: 0.8 ratio at 30% (0.52 mL)

Tris-HCl, pH 6.8 (1.25 mL)

ddH₂O (3.10 mL)

SDS 10% (50 µL)

APS 10% (50 µL)

TEMED (5 µL)

20.7. Laemmli loading buffer (5X):

100mM Tris-HCl pH 6.8

20% Glycerol

2% SDS

5% β -mercaptoethanol

20.8. Western blot transfer buffer:

25 mM Tris base

192 mM Glycine

20% Methanol

20.9. Western blot Stripping buffer:

62.5 mM Tris-HCl, pH 7.6

2% SDS

100 mM β -mercaptoethanol

20.10. Red cell lysis buffer:

150 mM NH_4Cl

10 mM KHCO_3

0.1 mM EDTA

Results

Previous studies in our laboratory have shown that the interplay of PML-RAR α with epigenetic modifiers is more complex than previously thought. Indeed, knock-down (KD) of *Hdac1/Hdac2* in PML-RAR α transgenic mice accelerates leukemia development, accompanied by a dramatic expansion of a subpopulation of pre-leukemic cells with the property of leukemia initiating/stem cells (LICs), suggesting that HDACs are required to control the self-renewal of LICs. This event was not restricted only to APL because lymphomagenesis driven by deletion of *p53* or, to a lesser extent, by *c-myc* overexpression, was also accelerated by *Hdac1* knock-down. Strikingly, in established leukemic cells knock-down of the same HDACs slows down leukemia growth, demonstrating that HDAC1/HDAC2 play different roles in APL initiation and maintenance (87). Overall, our data suggest that different HDACs have different roles in different stages of the disease, and imply that more studies are needed to systematically dissect the role of individual HDACs, at different stages of tumorigenesis (initiation vs. maintenance), in different tumor compartments (bulk vs. cancer stem cells) and in different tumor cell types. Thus, these results prompted us to further investigate the role of other class I HDACs in APL development, and this study addresses the role of HDAC3 in APL. APL is characterized by induction of cell proliferation and a block in myeloid differentiation. Direct action of PML-RAR α is apparently involved in both events and this is in part due to its association with HDACs, although HDACs can affect both biological processes independently of PML-RAR α . Therefore, we investigated the role of HDAC3 in

these processes in the pre-leukemic and leukemic stages of APL through a selective knock-down approach. The murine model of APL we used is the mCG^{PR/PR} mouse model in which PML-RAR α is expressed under the control of the cathepsin G promoter (**123**). To study the biological roles of HDAC3 in APL we used the functional knock-down approach. To achieve this aim, shRNA-based plasmids were generated as described in chapter II of this dissertation, by ligating synthetic oligonucleotides targeting HDAC3 and the control firefly luciferase (LUC) mRNAs into a modified pRetroSuper vector in which the cDNA for puromycin selection was replaced with the one encoding enhanced GFP (eGFP).

1. Effect of *Hdac3* knock-down at the pre-leukemic stage:

The well-characterized mCG^{PR/PR} mouse model (**123**) was used as a model for PML-RAR α driven APL. Lin⁻ (lineage negative) cells, which are enriched in hematopoietic stem and progenitor cells (HSPCs) from mCG^{PR/PR} mice, were purified and transduced with GFP-tagged retroviral vectors (pRetroSuper) targeting the control firefly luciferase (LUC) or HDAC3 transcripts. FACS-purified GFP⁺/Lin⁻ cells, were then analyzed *in vitro* and *in vivo*, as summarized in **Figure 12**.

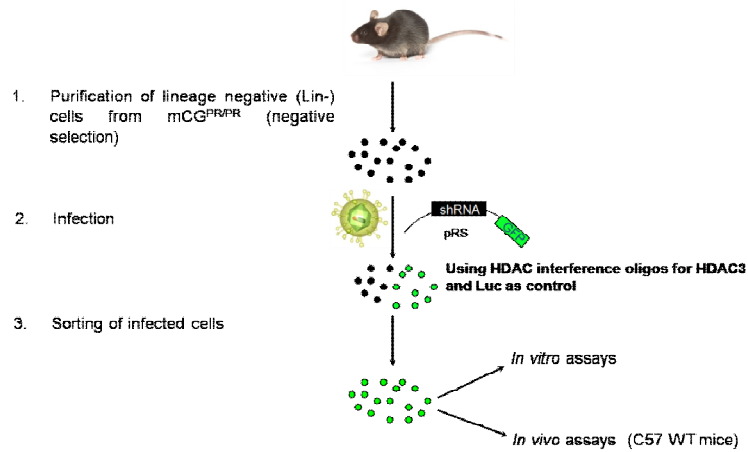


Figure 12. Schematic depiction of experimental procedure employed to assess the role of HDAC3 at the pre-leukemic stage.

1.1 *In vitro* assays:

RNA and protein analysis of GFP⁺/Lin⁻ sorted cells, confirmed reduction of HDAC3 by the targeting shRNA constructs (**Figure 13**). Furthermore, Knockdown of *Hdac3* had no effect on the expression of PML-RAR α .

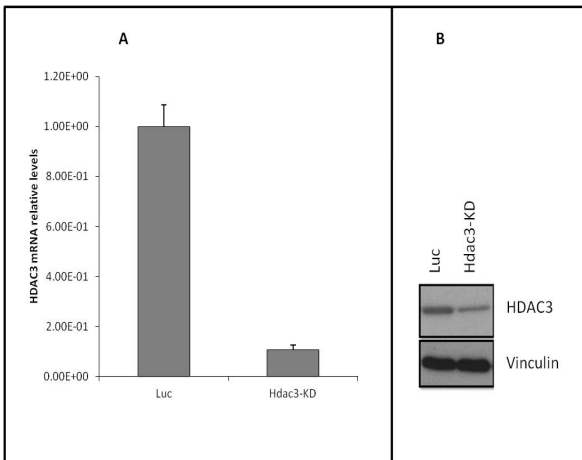


Figure 13. Constitutive depletion of *Hdac3* at pre-leukemic stage. A) Analysis of mRNA relative levels of HDAC3 in GFP⁺/Lin⁻ cells knocked down for *Hdac3*. Values are normalized against GAPDH and referred to *Luc*. B) Immunoblot analysis of HDAC3 expression in GFP⁺/Lin⁻ cells derived from mCG^{PR/PR} mice and transduced with the indicated retroviral vector. Vinculin was used as a loading control.

To evaluate the effect of *Hdac3*-KD on colony-forming ability of PML-RAR α expressing cells, 5,000 GFP⁺/Lin⁻ cells from mCG^{PR/PR} mice were seeded in a semi-solid methylcellulose. After 7 days the colonies were scored, harvested, pooled and the cells were used for the subsequent platings. As shown in **Figure 14**, *Hdac3*-KD led to a decrease in proliferative potential of PML-RAR α expressing Lin⁻ cells, as demonstrated by lower number of colonies recovered in serial replating experiments compared to the control (*Luc*). The colonies depleted for *Hdac3* were more dispersed resembling mature cells, while *Luc* colonies were more compact (**Fig. 14 A-B**).

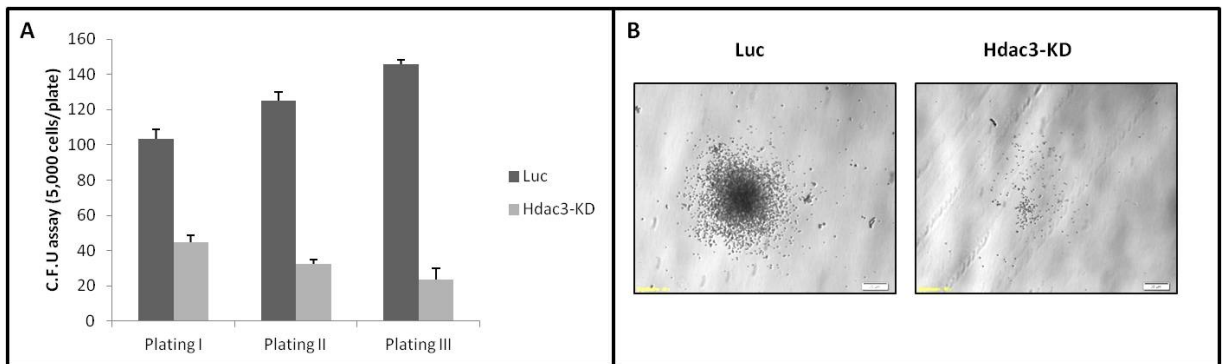


Figure 14. Analysis of the proliferative potential of pre-leukemic *Luc* and *Hdac3* knock-down cells. A) The graph indicates the number of colonies observed per 5,000 cells plated in serial replating assays. B) Colony morphology (Original magnification X1000) of individual colonies picked from first methylcellulose plates of either *Luc* or *Hdac3*-KD PML/RAR α expressing Lin⁻ cells.

We next analyzed the effect of *Hdac3*-KD on the differentiation state of PML-RAR α expressing Lin⁻ cells, through Q-PCR and FACS analysis. As described previously (**131, 132**), PML-RAR α expression hampers differentiation at myeloid stage (decrease in the number of cells expressing MAC.1/ GR.1 differentiation markers), which is accompanied with enhanced proliferative potential of Lin⁻ cells. Downregulation of *Hdac3* led to an increase in the mRNA relative levels of MAC.1 in PML-RAR α expressing Lin⁻ cells. MAC.1 or (cd11b/CD18 is a

surface integrin receptor, predominantly found on the surface of polymorphonuclear leukocytes (PMNs), macrophages and natural killer cells (**Figure 15**).

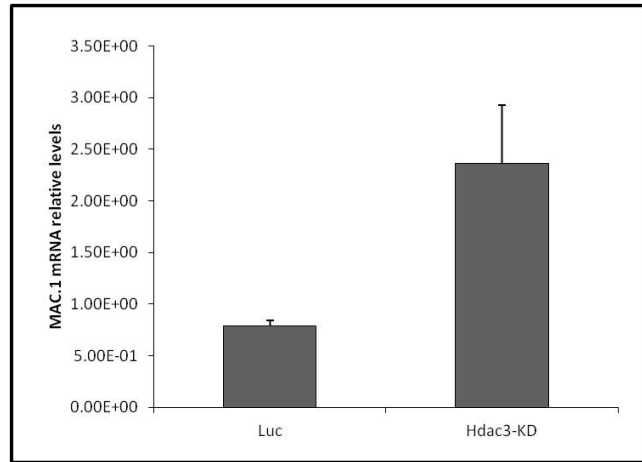


Figure 15. Q-PCR analysis of MAC.1 mRNA relative levels in GFP⁺/Lin⁻ cells. The graph demonstrates the higher expression level of MAC.1 myeloid surface marker in GFP⁺/Lin⁻ cells depleted for *Hdac3*, values are normalized against GAPDH and referred to *Luc*.

The induction of differentiation of pre-leukemic cells upon *Hdac3*-KD was further confirmed by immunophenotypic analysis, which revealed a greater percentage of MAC.1⁺ cells in *Hdac3*-KD population in comparison with *Luc* (**Figure 16**).

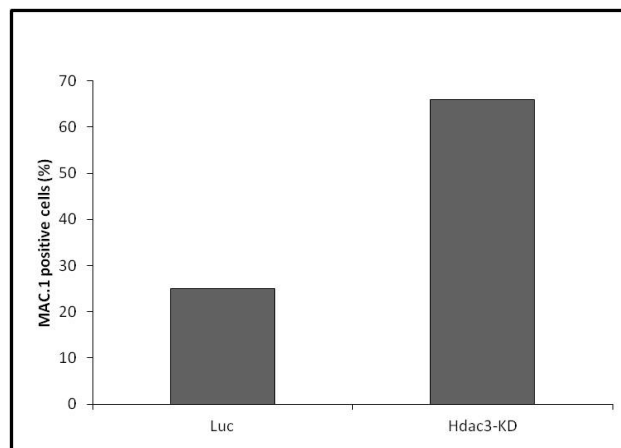


Figure 16. Immunophenotypic analysis of GFP⁺/Lin⁻ cells depleted for *Hdac3* and *Luc*. The plot indicates the percentage of MAC.1 positive cells in the GFP⁺/Lin⁻ cells harvested from the first methylcellulose plating of sorted cells.

1.2 *In vivo* analysis of GFP⁺/Lin⁻ cells knocked down for *Hdac3*:

As mentioned above, our *in vitro* results showed that depletion of *Hdac3* in the pre-leukemic myeloid cells, greatly impaired the differentiation block by PML-RAR α leading to the activation of the differentiation program. Equally important, PML-RAR α expression results in an enhanced proliferative ability of Lin⁻ cells (132), and consistent with the observed differentiation induction upon *Hdac3*-KD, the proliferative potential of PML-RAR α expressing Lin⁻ cells was reduced. To test *in vivo* the effect of *Hdac3* downregulation on the leukemogenic potential of PML-RAR α expressing cells, lethally irradiated C57BL/6J mice, 12 to 14 weeks old, were inoculated intravenously with 300,000 GFP⁺ transduced Lin⁻ cells, mixed with 500,000 splenocytes obtained from a wild type (WT) mouse to support the short-term reconstitution of hematopoiesis. Mice were observed periodically, to control survival and the appearance of clinical signs of leukemia. GFP⁺/Lin⁻/*Hdac3*-KD cells were unable to reconstitute the lethally irradiated syngenic recipient mice efficiently and we never observed the leukemia onset in the recipient mice in comparison with controls (*Luc*) (Figure 17). According to our results, *Hdac3*-KD impaired APL development in the recipient mice.

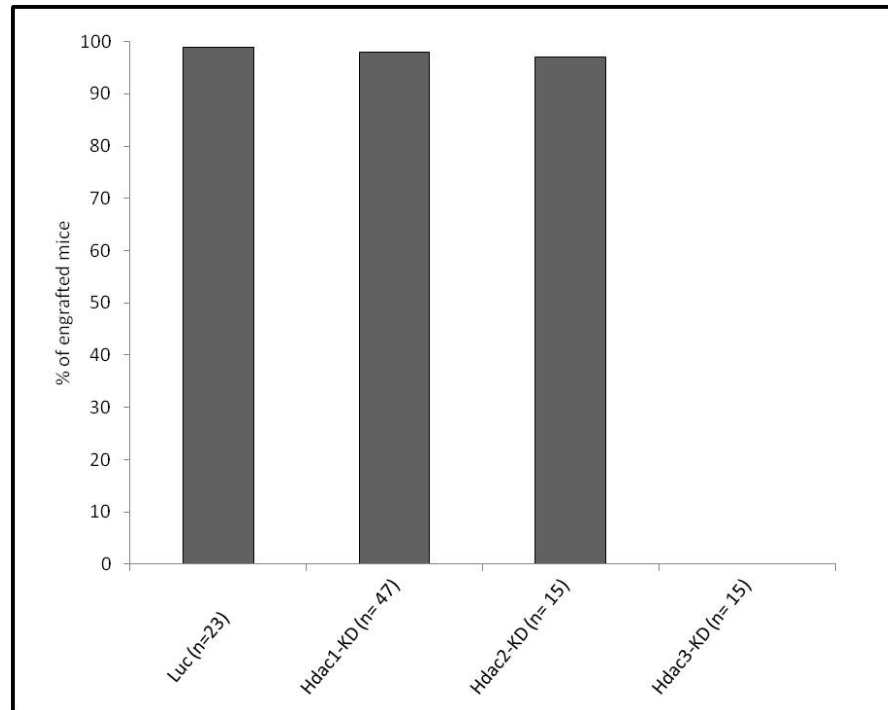


Figure 17. Percentage of mice engrafted with GFP⁺/Lin⁻ cells. GFP⁺/Lin⁻ cells derived from mCG^{PR/PR} mice were transduced respectively, from left to right, with firefly luciferase (*Luc*), *Hdac1*, *Hdac2* and *Hdac3* shRNA retroviral vectors and after sorting they were inoculated into lethally irradiated C57BL/6J recipient mice.

2. Role of HDAC3 at the full-leukemic stage:

2.1 *in vitro* assays:

Based on our data at the pre-leukemic stage of APL, it seems that *Hdac3*-KD is detrimental to pre-leukemic cells and it impairs APL development. To investigate the role of HDAC3 at the leukemic stage of APL, we knocked down *Hdac3* (or firefly luciferase as the control: *Luc*) in frankly leukemic APL cells. Depletion of *Hdac3* on APL cells was confirmed at mRNA and protein levels (**Fig. 18A-B**).

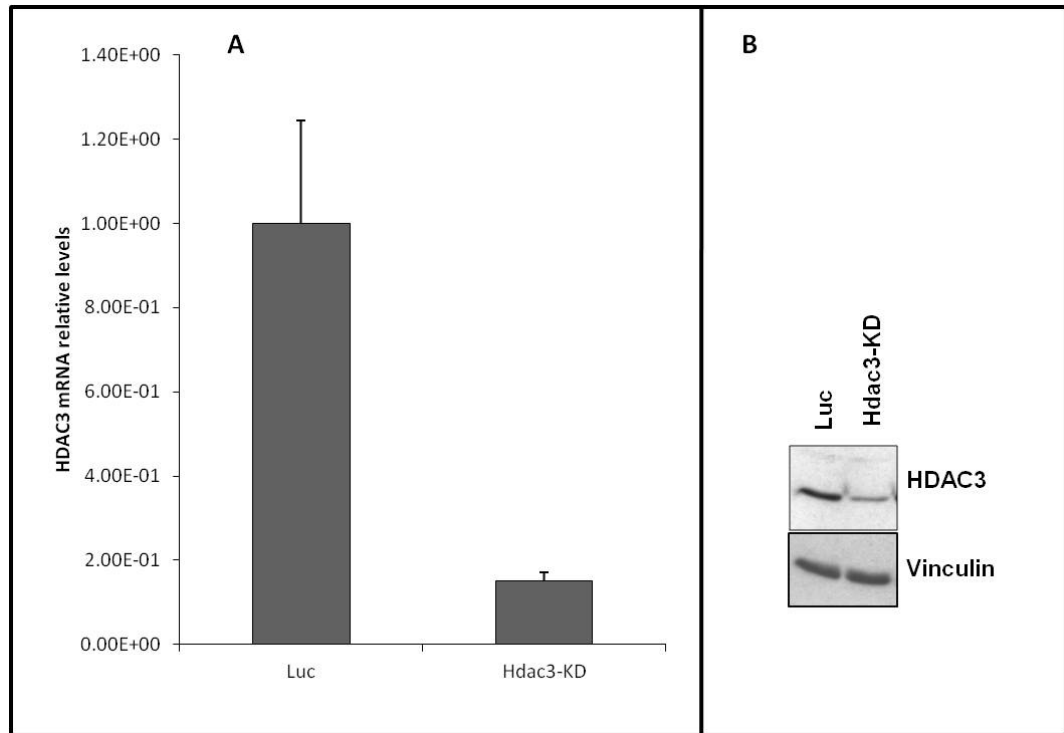


Figure 18. *Hdac3* depletion in APL cells. A) Analysis of HDAC3 mRNA relative levels in APL cells derived from 129SvEv mice, transduced with the indicated retroviral vectors. Values are normalized against GAPDH and referred to *Luc*. B) Immunoblot analysis of HDAC3 expression in GFP⁺/APL cells. Vinculin was used as a loading control.

To investigate the effect of *Hdac3* depletion on the proliferative potential of APL cells, 10,000 FACS sorted GFP⁺/APL cells were cultured on methylcellulose. After 7 to 10 days the colonies were scored, pooled, harvested and the cells were used for replating, immunophenotyping and morphologic analysis. Colony forming unit (C.F.U) assay showed reduction of proliferative potential of *Hdac3*-KD APL cells in serial replating (**Figure 19**).

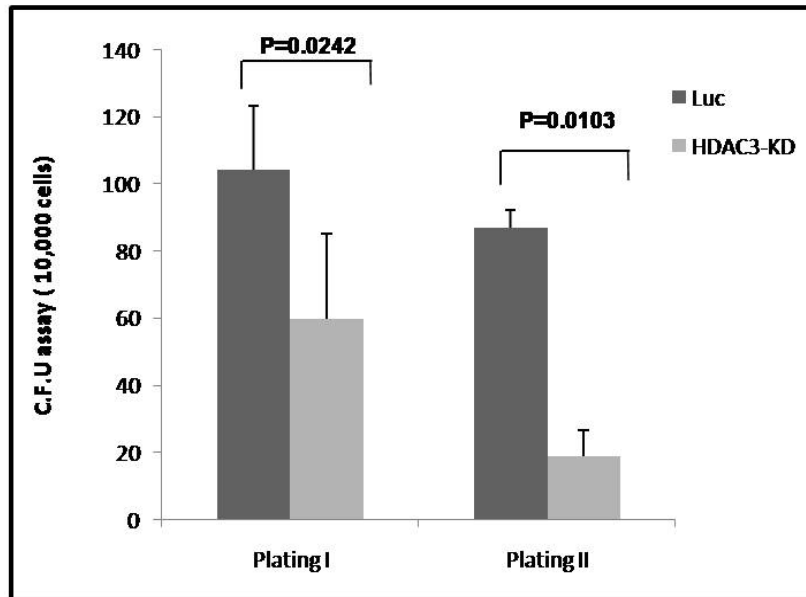


Figure 19. Analysis of the proliferative potential of *Luc* and *Hdac3*-KD APL cells in a serial replating assay. 10,000 GFP⁺/APL cells were plated in methylcellulose medium and were scored for colony-forming ability 10 days after plating. Error bars demonstrate standard deviation (SD) of biological replicates (n=3).

To assess the effect of *Hdac3*-KD on differentiation of APL cells, cytopins were prepared from cells harvested from the first methylcellulose (10 days after plating) and subsequently they stained with May-Grünwald-Giemsa method. Interestingly, morphologic analysis of APL cells demonstrated that *Hdac3* depletion induces a more mature cell phenotype (**Fig. 20A-B**). Mature and immature populations were defined based on their morphology, by scoring the percentage of mature and immature cells in each condition. In particular, the prevalence of mature and immature cells was analyzed morphologically in cytological slides in each case and the absolute percentage of mature and immature cells was reported. At least 300 cells were scanned for each case.

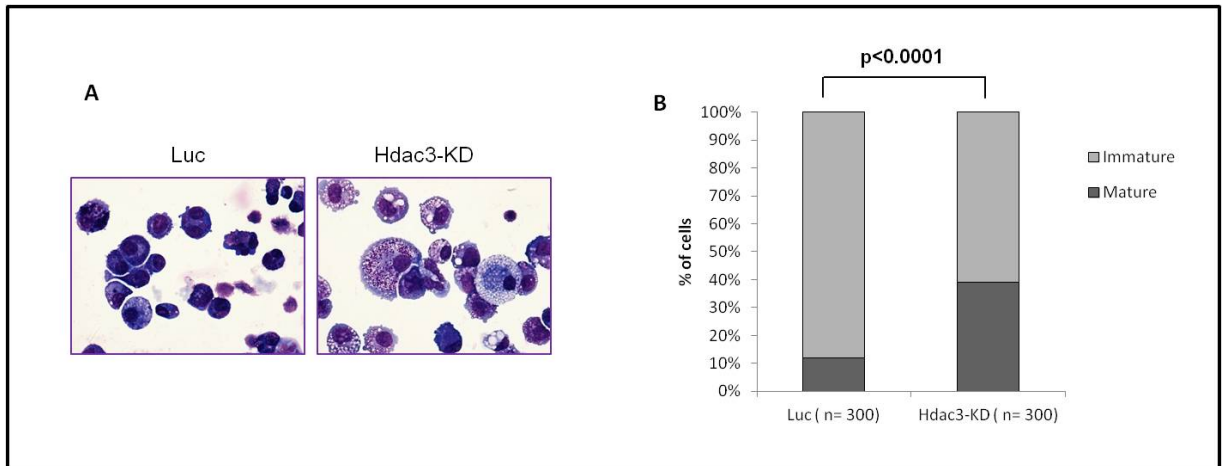


Figure 20. Morphological analysis of APL cells. *Luc* and *Hdac3*-KD APL cells cultured in methylcellulose medium were harvested after 10 days, A) representative cytopins. (Original magnification X1000, Olympus BX51); B) percentage of mature and immature cells. Statistical analysis was performed with the Fisher exact test.

In summary, our results showed, *Hdac3* knock-down has an antitumor activity on APL cells at both pre-leukemic and leukemic stages, demonstrated by reduction of clonogenicity of pre-leukemic and leukemic cells, and enhancement of their differentiation.

2.2 *in vivo* assays:

Sorted GFP⁺/APL cells depleted for *Luc* and *Hdac3* were transplanted into 129SvEv recipient mice (200,000 cells/mouse) and engraftment was followed via FACS analysis by detection of GFP⁺ cells in peripheral blood (PB). Inoculation of GFP⁺/sh*Hdac3* APL cells resulted in none, or very few GFP⁺ cells in PB samples, while control GFP⁺ cells grew exponentially (**Figure 21**).

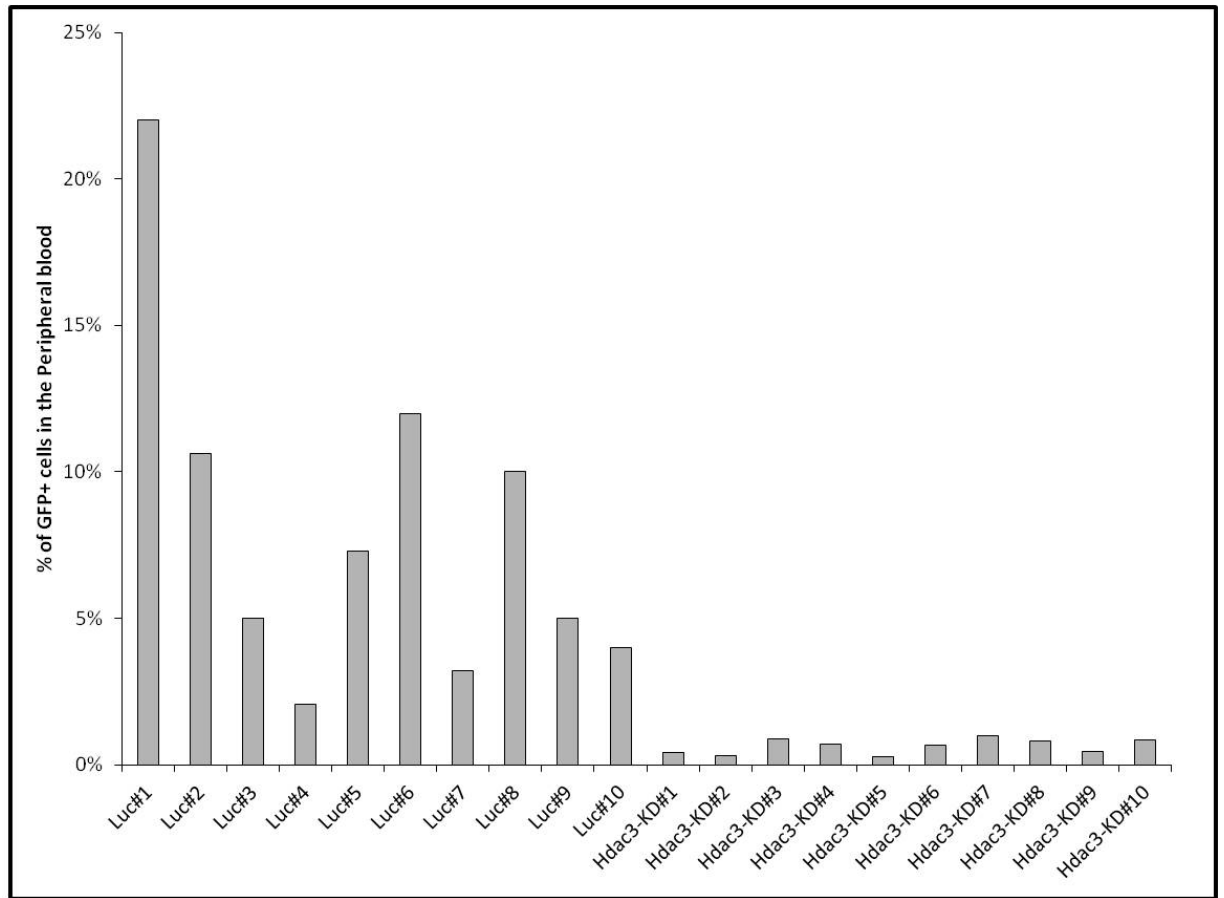


Figure 21. Percentage of GFP⁺ cells in the peripheral blood of mice inoculated with GFP⁺/APL cells. GFP⁺ was determined, through FACS analysis, 4 weeks post-transplantation on the peripheral blood of recipient mice (the plot shows the data for 10 mice inoculated with 200,000 GFP⁺/APL/ *shLuc* and 10 mice inoculated with 200,000 GFP⁺/APL/*shHdac3* cells).

Strikingly, while all *Luc* mice (n = 10) developed APL and were sacrificed within 50 days post-transplantation, as much as 7 out of 10 GFP⁺/*shHdac3* transplanted mice remained disease-free for more than 300 days (**Figure 22**). According to this experiment it can be concluded that *Hdac3* knock-down impairs leukemia transplantation.

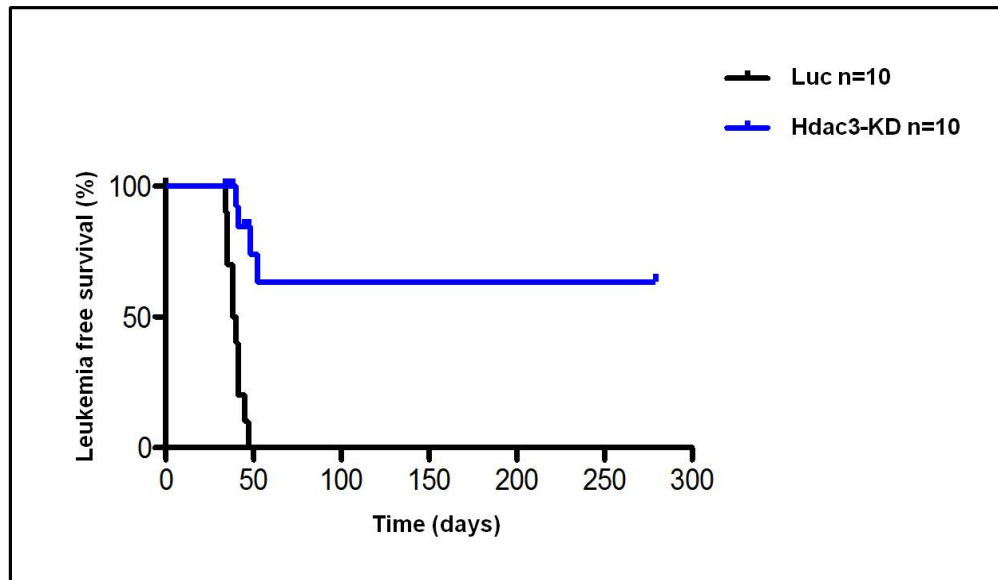


Figure 22. Leukemia-free survival curves of 129SvEv recipient mice transplanted with APL cells. GFP⁺/APL cells constitutively depleted of *Hdac3* or control sh*Luc* were transplanted into 129SvEv mice and leukemia-free survival assessed by Kaplan-Meier curve. Data are represented as time since tumor inoculation (days). *Luc* vs. *Hdac3*: $P < .0001$.

The GFP negativity in the peripheral blood of recipient mice inoculated with GFP⁺/sh*Hdac3* prompted us to further investigate the nature of developed leukemia in 3 out of 10 GFP⁺/sh*Hdac3* mice which died due to the development of APL. To determine the mRNA expression of HDAC3 in leukemic cells, the Q-PCR was undertaken in APL cells, collected from spleen of 4 control (sh*Luc*) mice and 2 out of 3 sh*Hdac3* mice that developed APL. APL cells resident at the end of the *in vivo* experiment showed wild-type HDAC3 expression and there was no down modulation at the mRNA level of *Hdac3*-KD leukemic mice, suggesting a selection against *Hdac3* depletion (**Figure 23**).

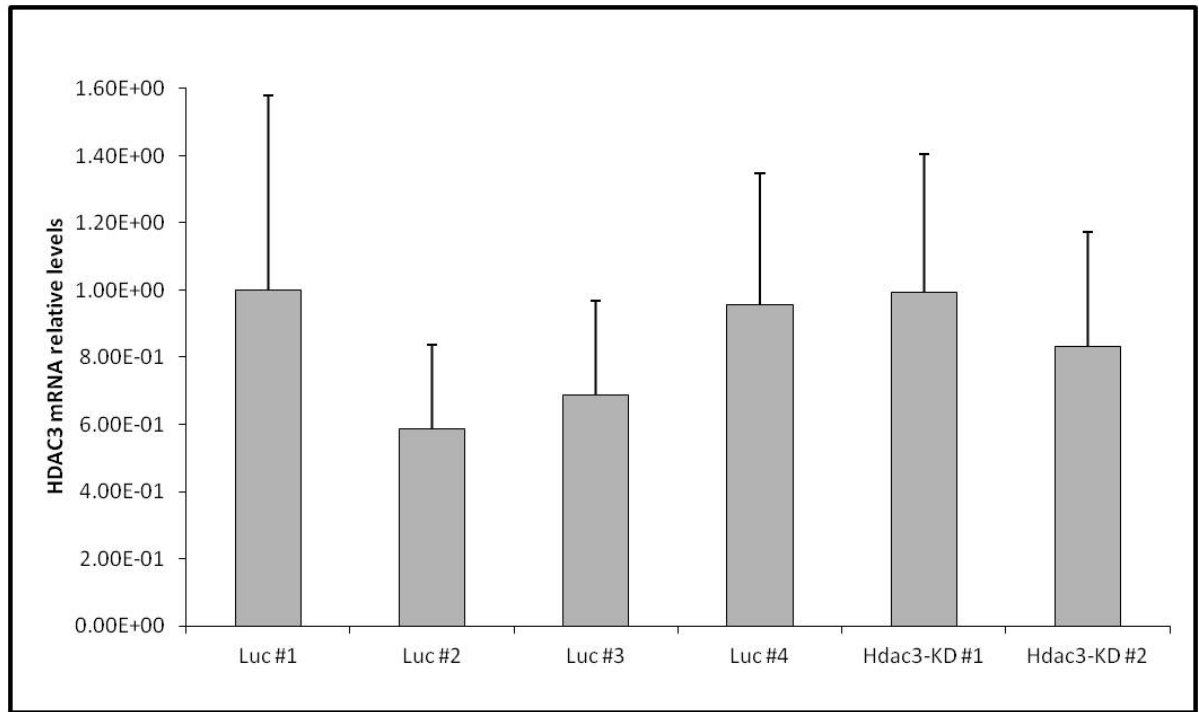


Figure 23. Analysis of HDAC3 mRNA relative levels in APL cells. Q-PCR was undertaken in APL cells collected from spleen of 4 control (sh*Luc*) mice and 2/3 sh*Hdac3* mice that developed APL to determine the mRNA expression of HDAC3 in leukemic cells. Individual bars represent HDAC3 expression in leukemic cells from individual mice taken at end point. Values are normalized against GAPDH and referred to control (*Luc*). The error bars indicate SD of technical replicates.

Since we did not detect any down modulation at HDAC3 mRNA level, we wondered if the leukemic cells were indeed transduced with the described vector (pRetroSuper). As showed in **Figure 24**, the qualitative PCR analysis of the APL cells derived from the spleen of leukemic mice were negative for the proviral integration confirming that the leukemia developed in shHDAC3 mice, originated from the cells not transduced with the desired vector. Thus, APL development, in these mice, is due to the outgrowth of GFP⁻ cells.

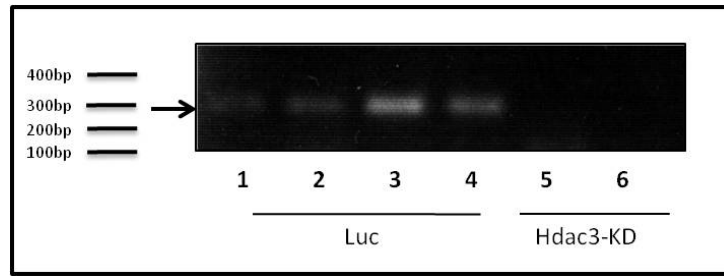


Figure 24. PCR on genomic DNA extracted from spleen of leukemic mice. Qualitative PCR was undertaken on genomic DNA extracted from spleen of leukemic mice (using pRetroSuper primers). While the band of pRetroSuper (pRS) is present in the leukemic cells derived from control mice (*Luc*) the absence of pRS in leukemic cells from the mice inoculated with APL/GFP⁺/*Hdac3*-KD cells (the arrow shows the positive band), confirmed the leukemic cells are originated from cells which are not transduced with *Hdac3* shRNA expressing retroviral construct.

In addition to the above-mentioned experiments, counter selection against *Hdac3* depletion was further confirmed by the absence of GFP⁺ cells in spleen of 2 sh*Hdac3* leukemic mice, compared to the 4 sh*Luc* leukemic mice (**Figure 25**). The third leukemia, derived from *Hdac3*-KD inoculated mouse, could not be analyzed due to the advanced necrosis of the spleen.

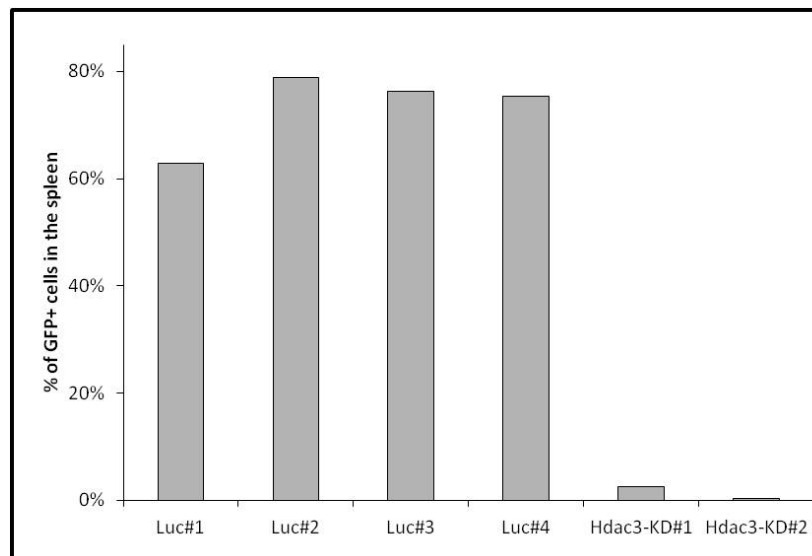


Figure 25. FACS analysis on the spleen of leukemic mice. Leukemic cells derived from spleen of 4 leukemic sh*Luc* and 2 leukemic sh*Hdac3* mice were analyzed for GFP expression through the FACS analysis.

3. Using an inducible vector for depletion of *Hdac3*:

Taken together, HDAC3 seems to be involved and required for the transformation mediated by PML-RAR α , and for the maintenance of APL blasts. Since knock-down of *Hdac3* was detrimental to pre-leukemic and APL cells, to further the role of HDAC3, we set up an inducible Tet-On shRNAmiR30 dual color vector system, TRMPV vector. The vectors were generated in collaboration with Prof. R.W Johnstone's and Prof. J. Zuber's lab. This inducible vector allows the precise tracking of both retroviral transduced cells and shRNA induction through two fluorescent reporters: dsRed which shows the induction of the expression of shRNA and Venus which shows the transduction of vector into the cells (134). In order to achieve our aim, APL cells were infected with the inducible vectors, the one that is called "double-system" described in details in the materials and methods chapter of this dissertation. In this system APL cells were infected with the first vector, which contained rtTA, and then transduced cells were selected by adding hygromycin (30 μ L/mL) to the cell culture for 3 days. Subsequently, APL cells transduced with rtTA were infected with TRMPV vector, containing miR30-embedded shRNA (sh*Scrambled* and sh*Hdac3*) cells transduced with the second vector were sorted based on the expression of Venus fluorescent protein. Unfortunately the infection efficiency was very low (Fig. 26A) and the APL/Venus⁺ sorted cells never engrafted into the recipient mice, although the shRNA expression was not induced by doxycycline yet. According to the limiting dilution experiments on APL cells the frequency of LICs has been estimated to be around 1 in 10,000 cells. Consequently the possibility to get transduced LICs with this low percentage of infection efficiency, is very low and almost infeasible. To solve this technical problem, we used the "all-in-one system" inducible vector described in materials and methods. Even this vector infected APL cells with a very low efficiency and Venus⁺/APL cells did not engraft into the recipient mice. The experiment was repeated more than once, but

we never observed the engraftment of APL cells into the recipients, although the HDAC3 depletion was not induced by doxycycline. Therefore, due to the described technical reasons, we decided not to further pursue this method.

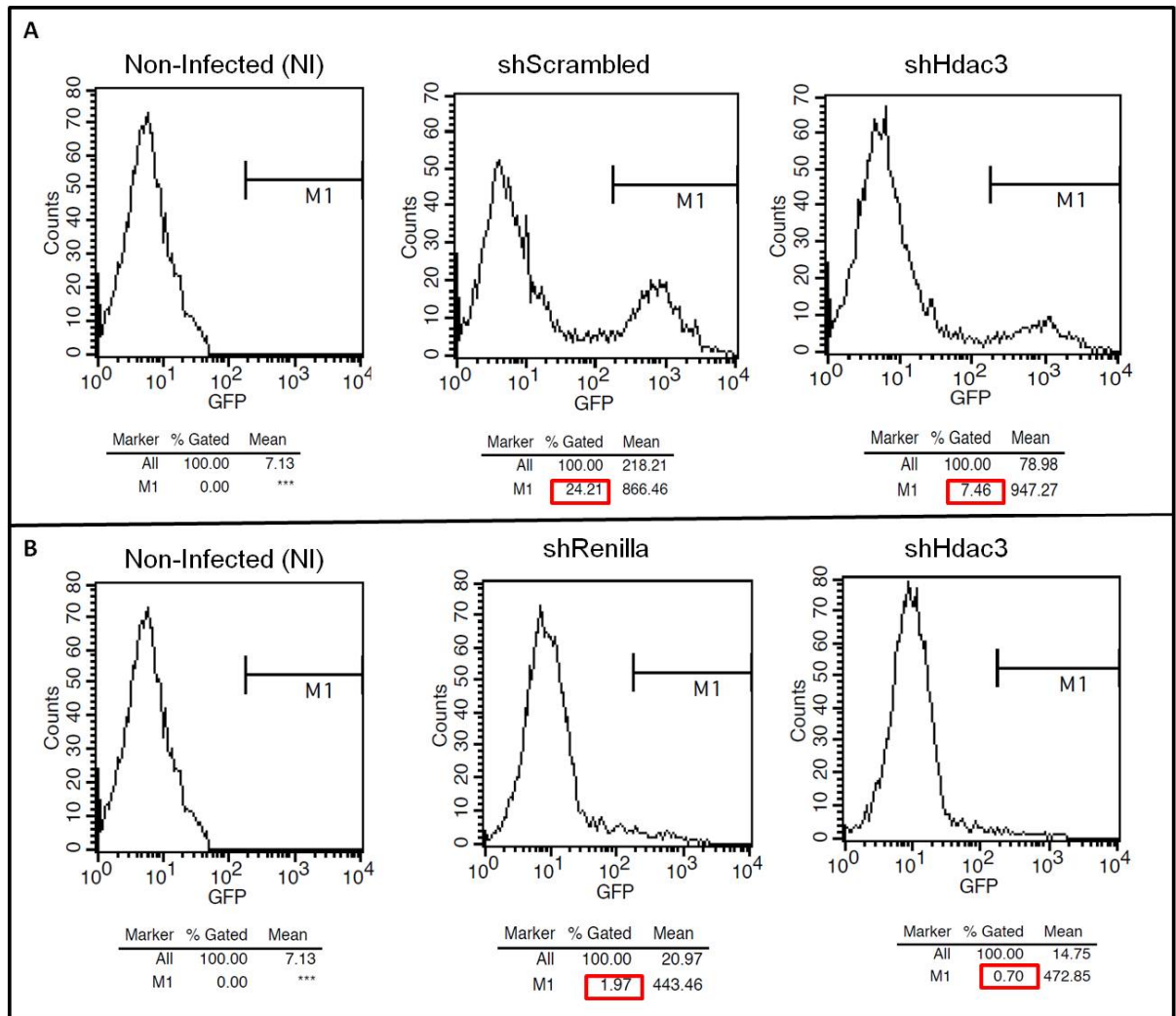


Figure 26. Percentage of APL/Venus⁺ cells, transduced with Tet-On inducible vector. A) Percentage of APL/Venus⁺ cells transduced with Double-System *shScrambled* and *shHdac3*. B) Percentage of APL/Venus⁺ cells transduced with All-in one system *shRenilla* and *ShHdac3*. Non-Infected APL cells have been used as a negative control for Venus fluorescent expression.

4. The *in vitro* pharmacological inhibition of HDAC3 in pre-leukemic cells recapitulates the observed *Hdac3*-KD phenotypes.

Taken together, our *Hdac3*-knockdown results in pre-leukemia and full-blown leukemia support the notion that HDAC3 could play an active role in APL onset and progression and pinpoint HDAC3 as an attractive target for therapeutic intervention. To confirm this notion, translate these results to pre-clinical models of APL and to determine if *Hdac3*-KD phenotypes could be phenocopied with HDAC3 pharmacologically, we used the small-molecule HDAC3 inhibitor, with the capacity to interfere with HDAC3 activity. We used the selective HDAC3 inhibitor (HDAC3i) known as RGFP966 (Repligen). 5,000 Lin⁻ cells derived from mCG^{PR/PR} mice and C57BL/6J WT mice were treated with different concentrations of HDAC3i (2.5 μM - 5μM and 10 μM) and plated on methylcellulose plates. 7 days later, the number of colonies arose from the first plating of pre-leukemic cells treated with HDAC3i was mildly lower than the colonies formed from the controls (Non-treated pre-leukemic cells), while in the 2nd and 3rd replating of pre-leukemic cells treated with the same concentrations of RGFP966, there was a dramatic reduction in the number of colonies (**Figure 27**).

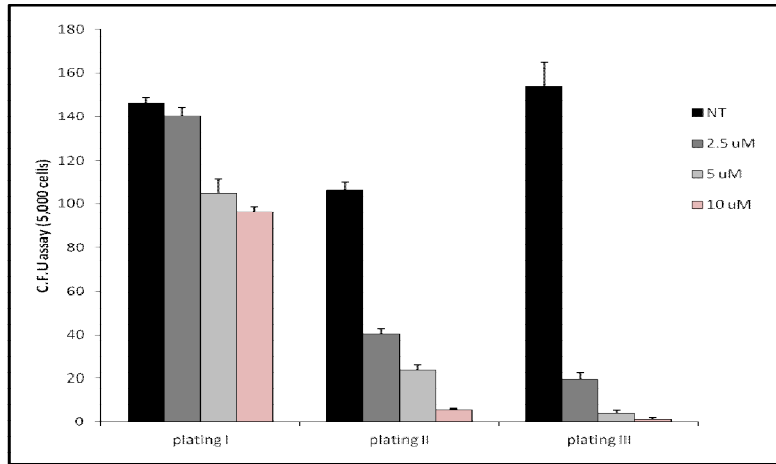


Figure 27. Colony forming ability of PML-RAR α expressing Lin⁻ cells upon administration of HDAC3i. Colony forming unit assay was done on PML-RAR α expressing Lin⁻ cells treated with 3 different concentrations of RGP966. After 7 days the colonies were scored, pooled and cells were harvested and used for subsequent platings. The error bars represent standard deviation of the technical replicates. NT stands for non-treated PML-RAR α expressing Lin⁻ cells.

In addition, the selectivity of HDAC3 inhibitor for tumorigenic cells against normal ones was observed by the mild effect of RGF966 on the colony-forming ability of Lin⁻ cells derived from WT mice in comparison with Lin⁻ cells derived from PML-RAR α expressing cells and APL cells (**Figure 28**).

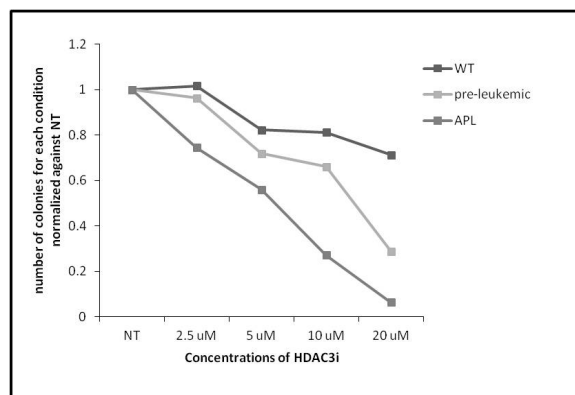


Figure 28. Comparative proliferative potential of wild type, PML-RAR α expressing Lin⁻ and APL cells. While the colony forming ability of WT Lin⁻ cells were reduced mildly after the treatment with HDAC3i, the clonogenicity of PML-RAR α expressing Lin⁻ and APL were reduced dramatically.

Furthermore, treatment of PML-RAR α expressing Lin⁻ cells with HDAC3 selective inhibitor (RGFP966) caused induction of differentiation of pre-leukemic myeloid cells. After scoring the colonies from the 1st methylcellulose, the cells were harvested and used for serial replating and in parallel for morphologic analysis through the May-Grünwald-Giemsa method (**Figure 29**).

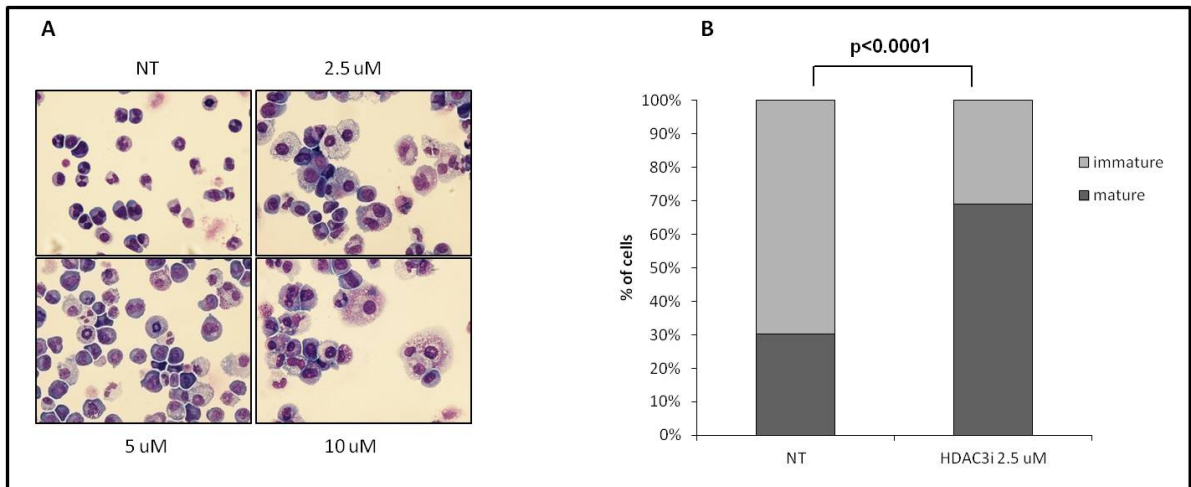


Figure 29. Morphologic analysis of PML-RAR α expressing Lin⁻ cells, after treatment with RGF966. A) Representative cytopins related to the cells harvested after plating in the 1st methylcellulose medium. (Original magnification X1000, May-Grünwald-giemsa staining, Olympus BX51). B) Percentage of mature and immature cells. Statistical analysis was performed with the Fisher exact test.

These results demonstrate that pharmacologic inhibition of HDAC3 enzymatic activity in pre-leukemic cells mimics the *Hdac3* knock-down phenotype.

5. Pharmacological inhibition of HDAC3 enzymatic activity in APL cells mimics the phenotype of *Hdac3*-KD.

Based on our data in APL cells, we showed that *Hdac3*-KD results in reduction of clonogenicity of APL cells and increases their differentiation. Thus, we tested the effect of

RGFP966, the HDAC3 selective inhibitor on frankly leukemic APL cells. To achieve our goal, 10,000 APL cells were treated with different concentrations of RGFP966 (0.05 μ M, 0.1 μ M, 0.5 μ M and 1 μ M), and seeded on methylcellulose medium. 10 days after plating, the colonies were scored, harvested, pooled and used for further analysis by cytopins and serial replating experiments. Consistent with our results on pre-leukemic cells and with *Hdac3*-KD effect on proliferative potential of APL cells, RGFP966 reduced the clonogenicity of APL cells in C.F.U assay (**Figure 30**).

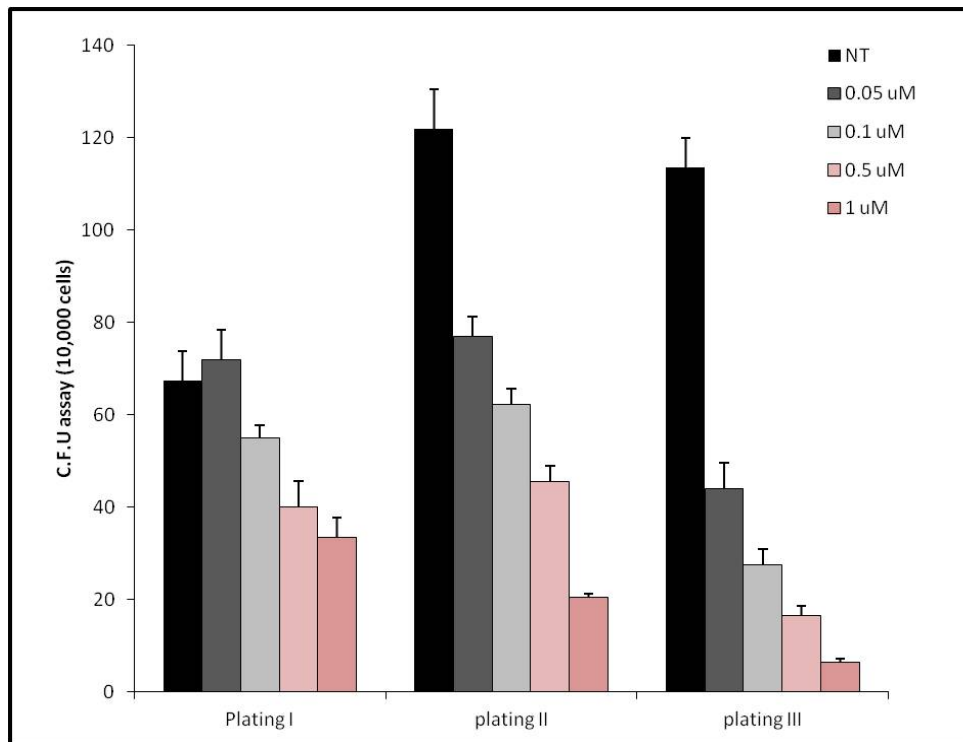


Figure 30. C.F.U assay on APL cells, treated with RGFP966. The APL cells were treated with RGFP966 at 4 different concentrations (0.05 μ M, 0.1 μ M, 0.5 μ M and 1 μ M), NT stands for non-treated sample (DMSO was used as vehicle).

Cytopins were also prepared from the harvested cells and observed microscopically for morphologic analysis and consistent with *Hdac3*-KD phenotype there was an induction of differentiation upon treatment of APL cells with RGFP966 (**Figure 31**).

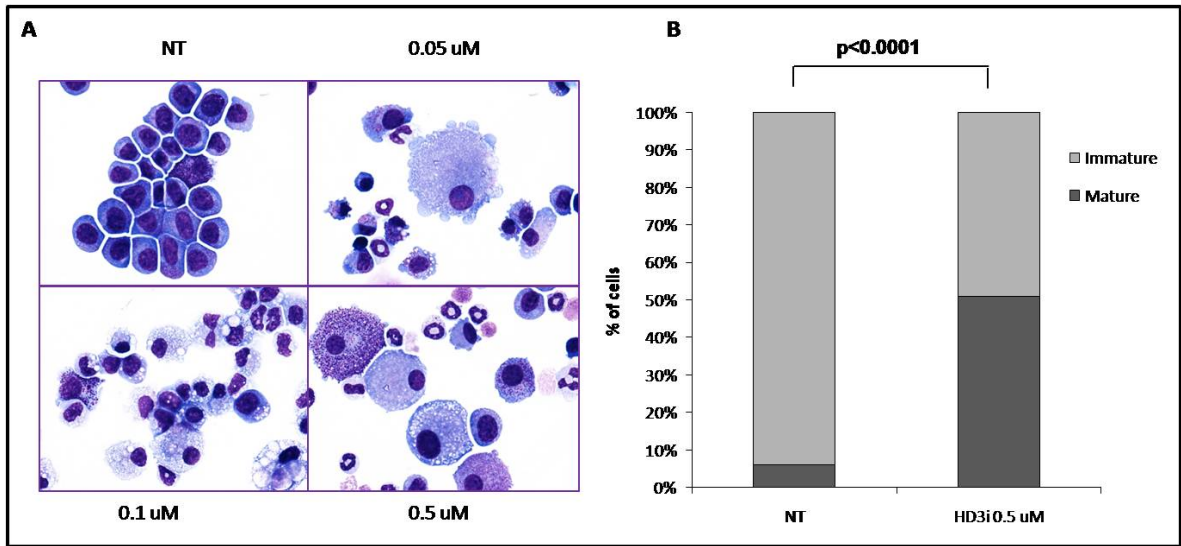


Figure 31. Morphological analysis of APL cells treated with different concentrations of RGFP966. A) representative cytopins (original magnification X1000, Olympus BX51), while Non-treated sample shows mainly the morphology of promyelocytes, the APL treated samples shows mainly the morphology of more mature cells such as; macrophages B) Plot of percentage of mature and immature cells. The statistical analysis was performed with the Fisher exact test.

The induction of differentiation in APL cells treated with low concentrations of RGFP966, was further confirmed by FACS analysis carried out on the cells harvested from the 1st methylcellulose (**Figure 32**). In this experimental condition, consistent with the phenotypes obtained upon *Hdac3*-KD in frankly leukemic cells, we observed reduction of clonogenicity of APL cells accompanied with an induction of myeloid differentiation of APL cells.

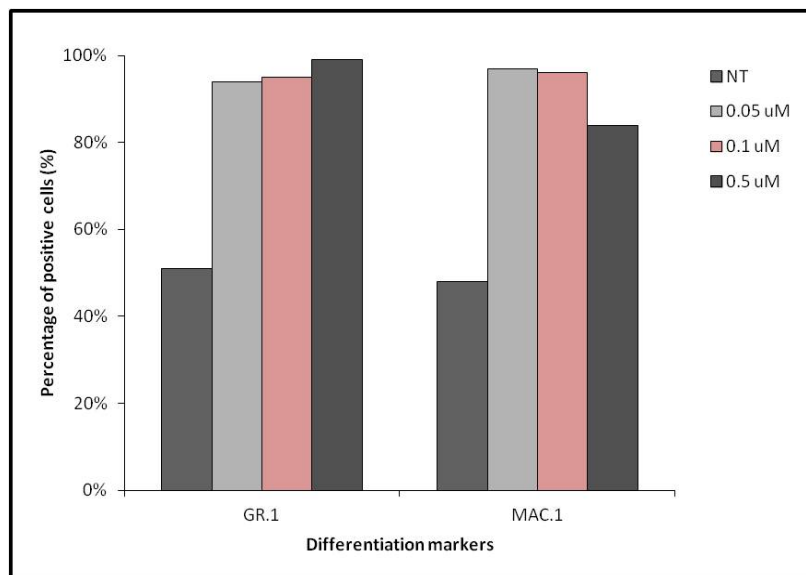


Figure 32. GR.1 and MAC.1 positive cells increase in APL cells upon the treatment with RFP966. An increased percentage of cells positive for GR-1(Ly-6G) or MAC-1 (CD11b) confirms that low concentrations of RFP966 trigger differentiation in APL cells similar to *Hdac3* depletion.

5.1 HDAC3 inhibition cooperates with ATRA in reduction of clonogenicity of APL cells

There are some studies on APL patients in whom a combination of ATRA and an HDACi has been used to assess whether there was an improvement in response to these compounds and consequently a complete remission. In one of those studies a combination of HDACi phenylbutyrate and ATRA led to induced histone hyperacetylation and complete remission in a case of highly resistant promyelocytic leukemia even though the disease was relapsed after 7 months (135). We thus asked whether there is a cooperative activity between RFP966 and ATRA. We treated 10,000 frankly leukemic APL cells either with RFP966 as single agent, or with ATRA, and with both compounds at the concentrations indicated in the **Figure 33**. Then the cells were seeded on methylcellulose medium. After 10 days, the colonies were scored. As it can be seen in C.F.U assay, there is a synergistic effect between ATRA and

RGFP966 in reducing the clonogenicity of APL cells. This suggests that these two compounds can be used as a potential combination therapy for the treatment of APL.

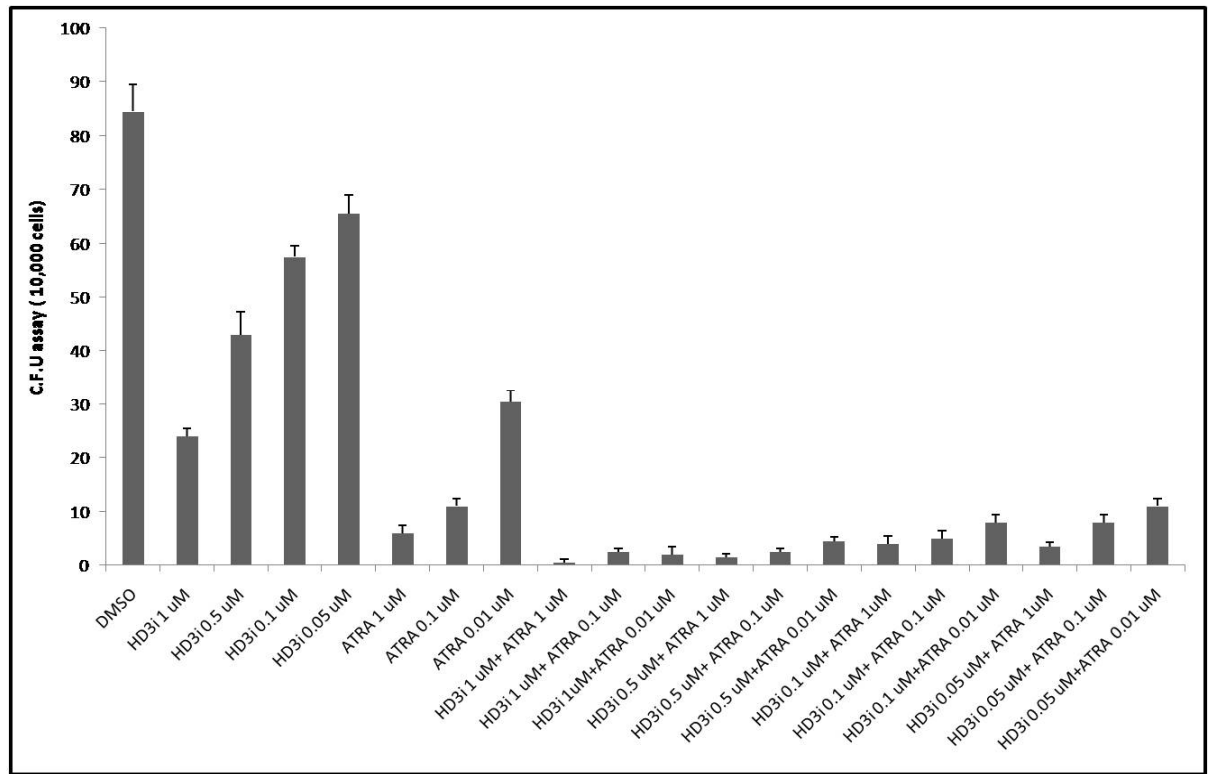


Figure 33. Synergistic effect of ATRA and RGFP966 on reducing the colony forming ability of APL cells. RGFP966 (HDAC3 inhibitor) was used at 4 different concentrations; 1 μM, 0.5 μM, 0.1 μM and 0.05 μM. ATRA has been used at 3 different concentrations; 1 μM (High), 0.1 μM (intermediate) and 0.01 μM (Low). The cooperative activity of two compounds in reducing the colony forming ability of the cells can be seen at all concentrations in combination therapy.

This project has been done in collaboration with two other groups: Prof. R.W Johnstone's lab from The Sir Peter MacCallum Department of Oncology and Prof. J. Zuber's lab from University of Melbourne and Institute of Molecular Pathology, Vienna, Austria. The data obtained in APL model have been generated by our group and further the role of HDAC3 in Eμ-myc lymphoma cells has been investigated by the lab of R.W. Johnstone and its role also been assessed in AML model driven by MLL-AF9 cells by the lab of J. Zuber. Thus, here the data generated on the role of HDAC3 in two other models, are summarized.

6. Investigating the role of HDAC3 in AML driven by MLL-AF9:Nras^{G12D}

In a published pooled shRNA screen from Zuber's lab the potential sensitivities of aggressive MLL-AF9;Nras^{G12D} AML to HDAC inhibition, had been observed (**136**). In particular, they have evaluated this phenotype by depleting *Hdacs*1-11 using independently derived shRNAs and utilized competitive assays to monitor their effects on representation of AML cells *in vitro*. Strikingly, in contrast to any other single HDAC, cells depleted of *Hdac3* exhibited reduced representation over 12 days of serial passaging (49 shRNAs in total, 3 to 5 shRNAs per each individual HDAC have been used) (**Figure 34**).

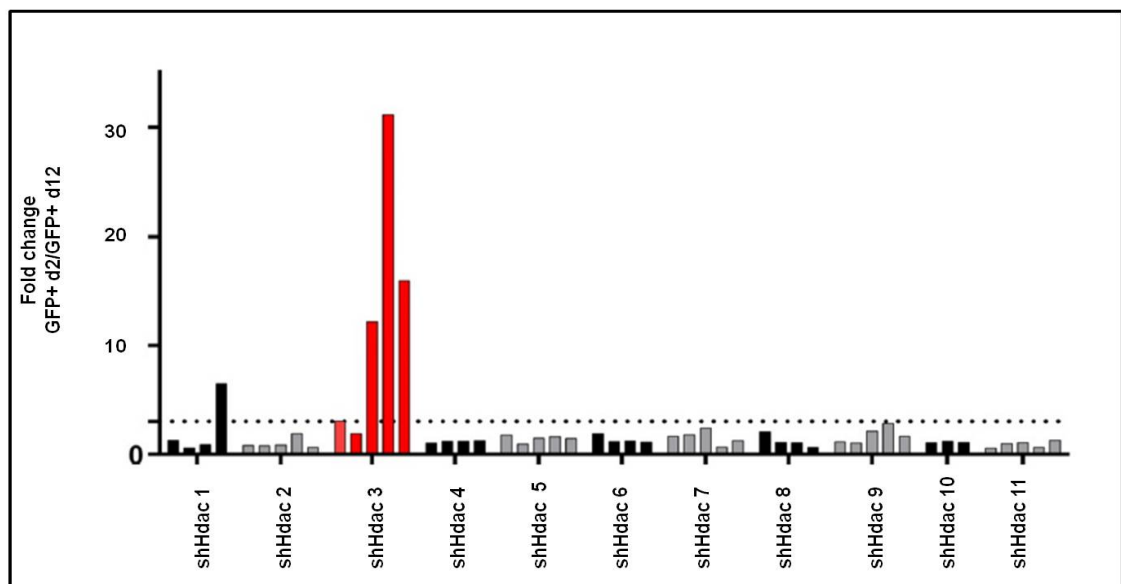


Figure 34. AML cells (MLL-AF9/Nras^{G12D}) have a unique sensitivity to *Hdac3* depletion. RNAi mediated screen of all 11 classical HDAC isoforms in AML cells driven by MLL-AF9/Nras^{G12D} demonstrates dependency on HDAC3 expression. The bar chart demonstrates the fold change in GFP expression as a ratio between day 2 post-infection and day 12. Between 3 to 5 shRNAs have been used for each HDAC isoform.

6.1 *In vivo* assays:

The *in vitro* study reproducibly demonstrates that *Hdac3* depletion reduces the proliferation of AML cells. Prof. Zuber's Lab sought to confirm this result *in vivo* by using the Tet-On inducible vectors. Luciferase –expressing MLL-AF9;Nras^{G12D} AML cells transduced with dox-inducible sh*Hdac3* (n=14) or non-targeting sh*Renilla* (n=12) constructs (pTRMPV-Neo) were transplanted into CD^{45.1+} mice and shRNA expression was induced two days after tumor inoculation by addition of doxycycline to food and drinking water, (shRNA expression can be followed by dsRed expression). Remarkably, *in vivo* depletion of *Hdac3* significantly reduced tumor burden (**Figure 35A**) and although sh*Hdac3* mice finally died due to the development of leukemia, but showed survival benefit in comparison with sh*Ren* inoculated mice (**Figure 35B**).

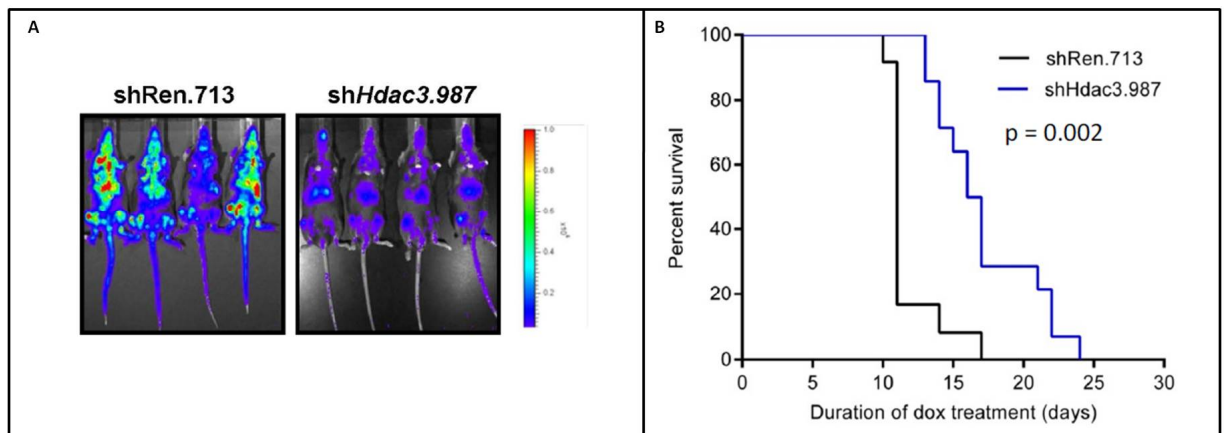


Figure 35. *Hdac3* depletion reduces tumor burden and/or significantly extends the survival of mice bearing AML. **A)** Tumor burden was assessed by bioluminescent imaging following 8 days of doxycycline treatment. **B)** Kaplan-Meier curve for survival analysis of mice bearing transplanted AML tumor with indicated pTRMPV-Neo constructs. Day 0 indicates the day of doxycycline treatment.

At terminal disease stage, bone marrow of control mice predominantly showed sh*Ren*-expressing cells (dsRed⁺/Venus⁺), while recipients of sh*Hdac3*-expressing cells showed an

outgrowth of AML cells that had evaded shRNA expression ($CD^{45.2+}/dsRed^{-}/Venus^{+}$) (**Fig. 36 C-D**), indicating a strong selection against effective *Hdac3* suppression.

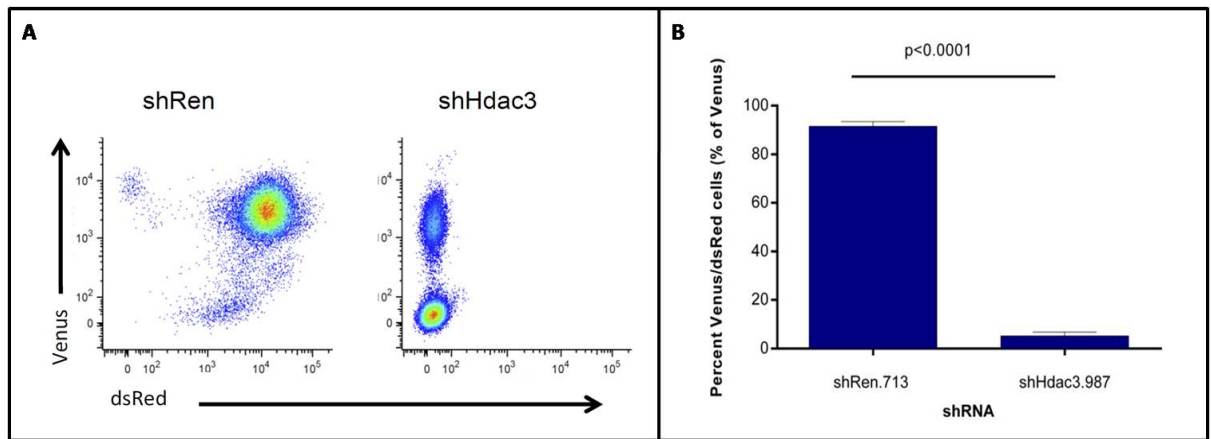


Figure 36. Strong selection against *Hdac3* depletion, results in AML development in the recipient mice. A) FACS analysis of Venus⁺/dsRed⁺ cells at terminal disease stage; (while control mice inoculated with sh*Ren* are Venus⁺/dsRed⁺, sh*Hdac3* recipient mice are Venus⁺/dsRed⁻). B) Quantitation of the percentage of Venus/dsRed expressing tumor cells remaining at terminal disease stage.

7. HDAC3 has a crucial role in the survival of lymphoma cells driven by E μ -myc:

7.1 *In vitro* assays:

Prof. Ricky's lab focused on HDACs inhibited by currently approved HDACi (HDAC1, 2, 3, and 6) in E μ -Myc lymphoma. shRNAs were validated in NIH-3T3 cells using pLMS constitutive retroviral vector by western blot (**Figure 37**). At least 2 shRNAs effectively depleted each individual HDAC were selected and utilized in subsequent experiments.

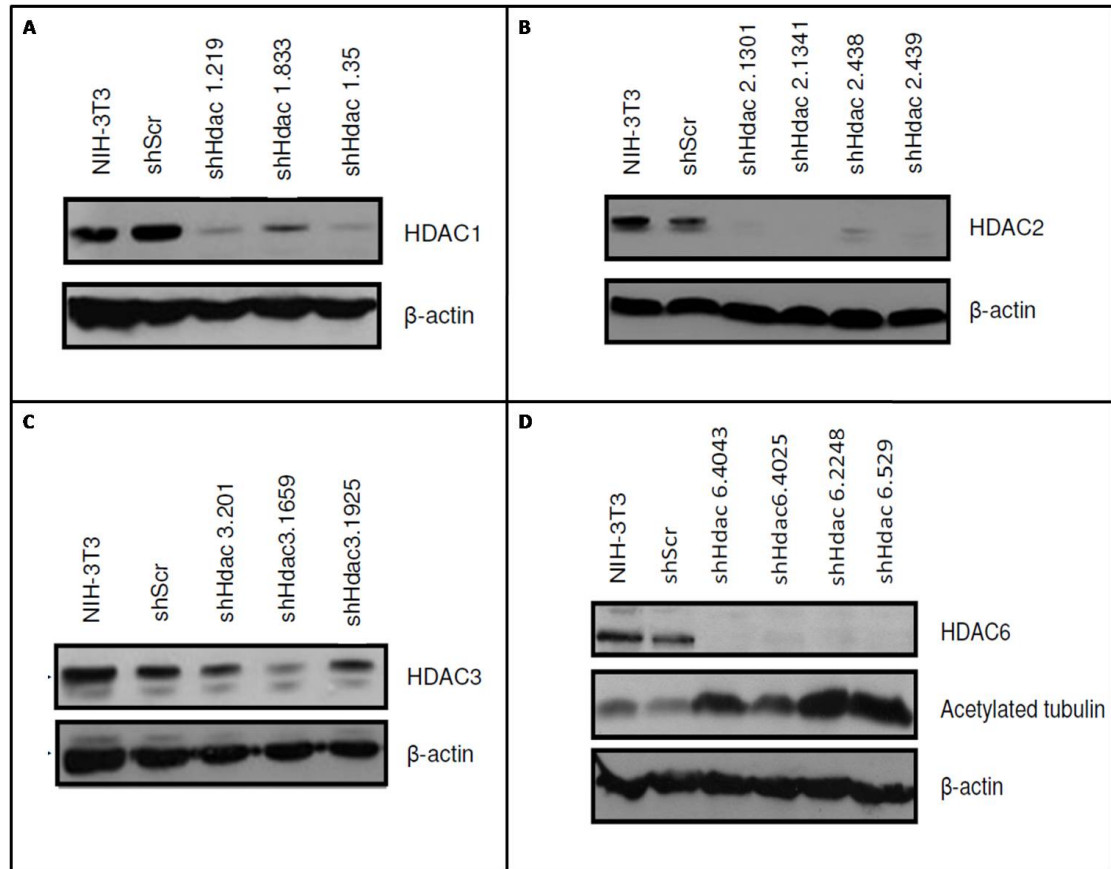


Figure 37. Validation of shRNAs against *Hdac1*, *Hdac2*, *Hdac3* and *Hdac6* in NIH3T3 cells. NIH-3T3 cells were transduced with retroviral pLMS vectors containing various shRNAs against: **A)** *Hdac1*; **B)** *Hdac2*; **C)** *Hdac3*; and **D)** *Hdac6* (3-4 per HDAC isoform), FACS sorted GFP⁺ cells, expanded in culture and then assessed for on-target Hdac depletion by western blot. The depletion of *Hdac6* was also confirmed by probing for hyperacetylated tubulin. Individual shRNAs (n=2) that demonstrated effective knockdown for each HDAC isoform were chosen for subsequent experiments in E μ -Myc lymphoma cells.

E μ -myc tumor cells were transduced with constitutive (pLMS) or dox-inducible (pTRMPV-Neo) retroviral vectors. FACS sorted to approximately 50% GFP or Venus positive cells and mixed with 50% of non-transduced cells. Then they were serially passaged (\pm dox) for up to 13 days (for constitutive system) or 15 days (for inducible system). The percentage of GFP (pLMS) or Venus/dsRed double positive (pTRMPV-Neo) cells were assessed every second day (days 1-13 or 1-15) and data were normalized to day 0 or day 1. The exquisite sensitivity of E μ -Myc lymphoma cells to constitutive depletion of *Hdac3* is depicted in **Fig. 38A**.

Moreover, E μ -myc cells transduced with dox-inducible pTRMPV-Neo retroviral vectors, indicated that depletion of *Hdac3* reproducibly led to the loss of representation of E μ -Myc lymphoma. Importantly, suppression of no other individual HDAC isoform reproducibly altered the growth/survival of E μ -Myc lymphoma (**Fig. 38B**). Knock-down of individual *Hdacs* in E μ -Myc tumors were validated using Q-PCR and western blot analysis.

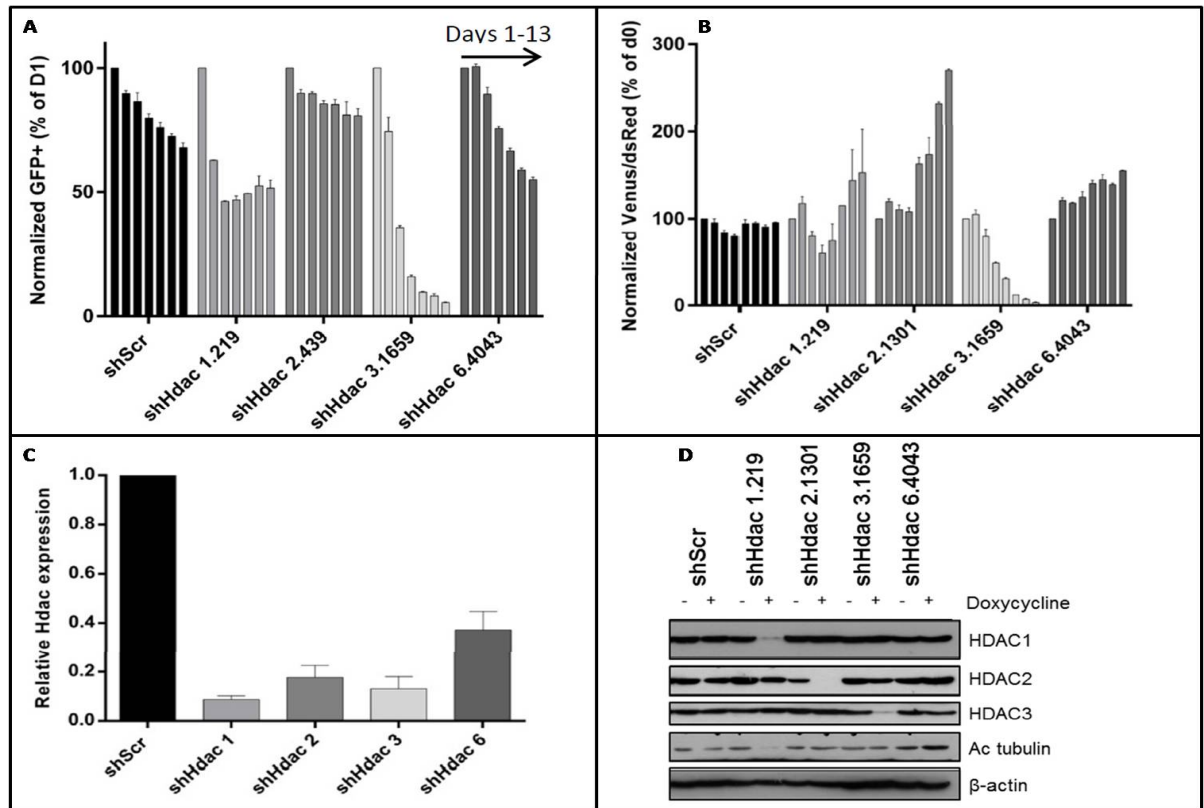


Figure 38. E μ -myc lymphoma cells show sensitivity to *Hdac3*-KD. Competitive proliferation assay has been utilized to investigate the proliferative effects of depleting *Hdac1*, 2, 3 and 6 in E μ -Myc lymphoma cells. A) Following constitutive depletion of *Hdacs1*, 2, 3 or 6 in E μ -Myc lymphoma cells, the complete loss of representation of cells expressing sh*Hdac3* is demonstrated, while acute effects are observed in cells depleted of *Hdac1*. B) Schematic representation of the dox-inducible vector system (pTRMPV-Neo) utilized to inducibly deplete HDAC isoforms and bar charts demonstrating the expression of Venus⁺/dsRed⁺ cells following serial passaging in dox-containing culture media. C) Q-PCR demonstrates depletion of individual HDAC isoform expression. D) Western blots demonstrating the efficiency of inducible *Hdac* depletion following doxycycline treatment (3 days) of E μ -Myc tumor cells. Hyper-acetylated tubulin (Ac tubulin) was employed as surrogate readout for *Hdac6* depletion. Note the variability of background levels of hyper-acetylated tubulin (for example sh*Hdac1*). β -actin was used as a loading control.

7.2 *In vivo* assays:

Next, researchers from Prof. Ricky's lab transplanted E μ -Myc lymphoma transduced with FACS sorted Venus⁺/dox-inducible sh*Scrambled*, sh*Hdac3.1659* or sh*Hdac3.201* into CD^{45.1+} mice (n=36). On day 3 post-inoculation, mice were fed doxycycline in food and water to initiate expression of shRNAs *in vivo*. On day 10, mice were bled and then sacrificed. *In vivo* depletion of *Hdac3* significantly reduced white-blood cell count, percentages of Venus⁺ tumor cells in PB and spleen weight. As per AML studies, outgrowth of non-transduced E μ -Myc cells (Venus⁻) (**Figure 39**) in mice at ethical endpoints prevented any survival advantage (data not shown).

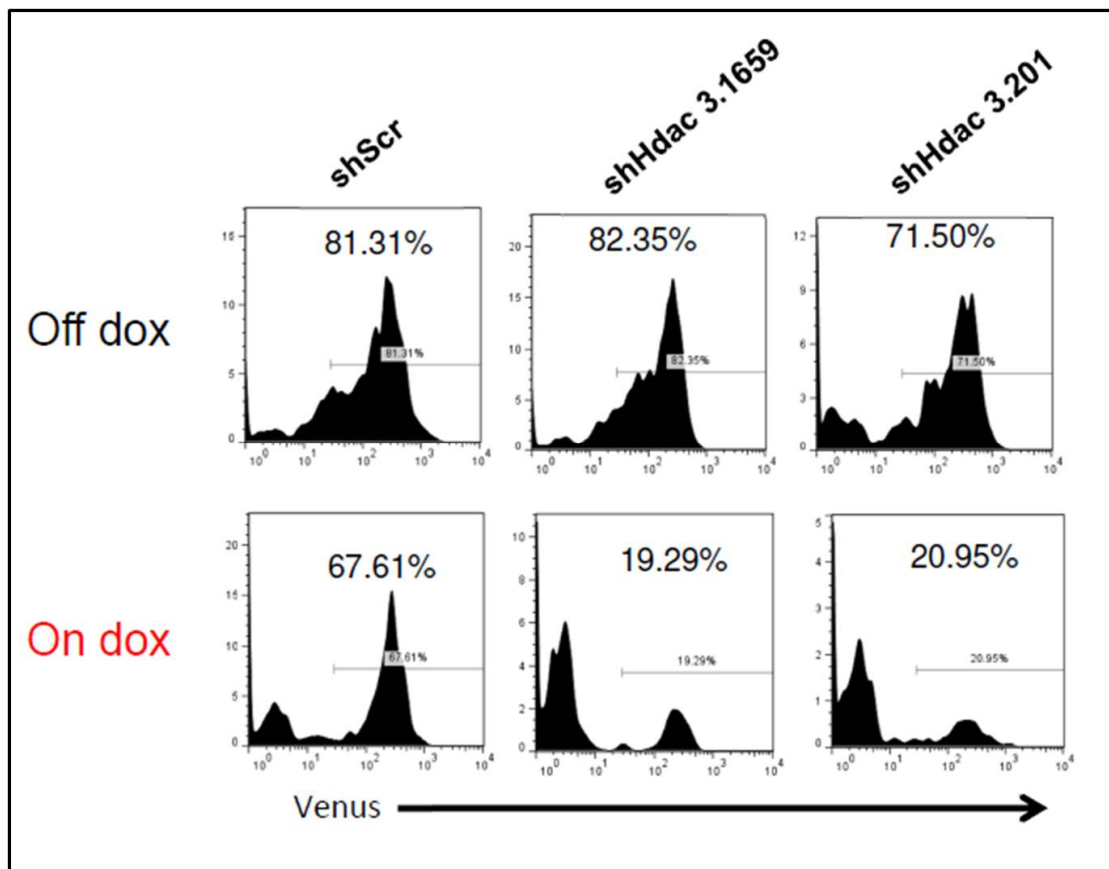


Figure 39. Outgrowth of Venus⁻ cells, led to lymphomagenesis in the recipient mice. Representative histograms of donor-derived (CD^{45.2+}) E μ -Myc lymphoma cells (day 10) demonstrating the outgrowth of non-transduced (Venus negative) cells.

While all three leukemias/lymphomas demonstrated significant anti-proliferative effects following *Hdac3* depletion *in vitro* and *in vivo*, *Hdac3* depletion in APL cells also triggered differentiation and led to a more mature phenotype, suggesting that HDAC3 can represent a good molecular target for developing new drugs. Furthermore, RGFP966 in APL cells was able to mimic *Hdac3* depletion by reducing clonogenicity and upregulating the myeloid differentiation markers Gr.1 and Cd11b (MAC.1) concomitant with morphological changes reminiscent of differentiated myeloid cells. This suggests that low dose HDAC3-selective inhibition in patients with APL may promote tumor cell differentiation and enable tumor remissions without the need for toxic chemotherapy.

Discussion

Epigenetic abnormalities observed in cancer can be targeted pharmacologically. As an example of epigenetic therapy HDACi, that target multiple HDACs, are effective for the treatment of CTCL. Epigenetic drugs, however, have single agent efficacy only in selected hematological malignancies, underlying the need for a better understanding of epigenetic alterations and of epigenetic enzymes in cancer. Therefore, my PhD project has been based on the hypothesis that epigenetic therapies have to be directed against specific functional epigenetic alterations present in cancer cells to achieve maximal effects.

Currently due to this fact that most of available HDACi are pan-HDACi and behave in a non-selective manner, the clinical utility of these agents is limited. Thus, to better use HDACi we need to understand the role of individual HDACs in tumorigenesis. In the last two decades there has been a considerable effort in understanding the biological roles and functions of HDACs. Conclusively, identification of the HDAC isoforms most critical for tumor promotion could lead to the design of more targeted therapies and reduced toxicities.

Based on these premises, my project has been planned to study the role of HDAC3 in the development of APL, and in maintenance of the disease. As mentioned previously, the study has been done in collaboration with two other groups: Prof. R.W Johnstone's Lab (from The Sir Peter MacCallum Department of Oncology, University of Melbourne), which provided data generated on E μ -myc lymphoma model and Prof. J. Zuber's lab (from Institute of

Molecular Pathology, Vienna, Austria), which provided data generated on the MLL-AF9/Nras^{G12D} model.

The rationale for assessing the role of HDAC3 in these 3 specific hematological malignancies (APL, AML driven by MLL-AF9 and E μ -myc lymphoma) is as mentioned before; APL is the first model disease in which the involvement of HDACs has been demonstrated. Thus, our group is studying the role of individual HDACs in the pathogenesis of APL caused by the fusion protein PML-RAR α . The group has previously shown that HDAC1 and to a lesser extent HDAC2 has a dual role in APL: oncosuppressor in establishment (pre-leukemic stage), oncogene in maintenance (leukemic stage) of the disease, and that HDACi can have different effects on bulk *vs.* leukemic stem cells (87). From the previous work of my group, this general statement can be further refined as follows: different HDACs may play distinct roles at different stages of tumor development, and within the tumor mass they play different roles in distinct cell compartments (bulk *vs.* cancer stem cells). Taken together, through a better understanding of the role of HDACs in leukemia, we can identify better treatments with epigenetic drugs.

In this study, the effects of *Hdac3* depletion has been assessed also in murine MLL-AF9 Nras^{G12D} driven AML. MLL is one of the mammalian homologs of the *Drosophila* trithorax genes, and is highly expressed in progenitor cells and downregulated during cellular differentiation and maturation. Chromosomal translocations leading to the formation of MLL fusion proteins are involved in 5–10% of AML. MLL interacts with HATs, and possesses histone methyltransferase activity towards Lys4 of histone H3 through its Su(var)3-9enhancer of zeste trithorax domain; it is therefore considered to be a positive regulator of gene expression. MLL rearrangements are subdivided into reciprocal chromosomal translocations, deletions, and inversions. Among the major fusion partners of MLL are the transcriptional

activators AF4, AF9, AF10, and ENL. These fusion proteins usually lead to the overexpression of MLL target genes, including HOXA9 and MEIS1, which are required for HSC self-renewal. The leukemogenic transformation mediated by MLL fusion proteins is abolished *in vivo* by point mutations in its CXXC motif (the DNA-binding motif), which is retained in all MLL fusion proteins. This motif interacts with corepressor proteins such as HDAC1 and the polycomb group protein BMI-1. MLL fusion partners activate the transcriptional elongation of MLL target genes through epigenetic mechanisms. Whereas MLL loses its own transcriptional activation capacity through the translocation process, the fusion proteins form the superelongation complex via interactions with positive transcription elongation factor b, cyclin-dependent kinase 9, and cyclin T1. The superelongation complex phosphorylates RNA polymerase II. Then, DOT1L is recruited to the MLL fusion complex by interaction with ENL, AF9, and AF10. DOT1L, the only known histone H3 Lys79 methyltransferase, has been shown to have a broad role in transformation mediated by MLL fusion proteins through interactions with multiple MLL fusion partners, and its gene disruption in mice has pointed to a pivotal role of DOT1L in hematopoiesis, cardiac function, and the development of leukemia. Mistargeting of DOT1L to HOXA9 and MEIS1 through its interaction with MLL fusion partners leads to the constitutive transcriptional activation of these genes through aberrant hypermethylation of histone H3 Lys79, which in turn results in leukemic transformation (17).

The complexity of chromatin-remodeling enzymes recruited by MLL fusion proteins has been supported by the involvement of additional epigenetic mechanisms in its molecular pathogenesis: MLL-AF9 interacts with TIP60 histone acetyltransferase, histone deacetylases and polycomb group proteins. An initial hint for the involvement of HDACs in the pathogenesis of MLL-rearranged leukemia stems from the study conducted by Xia et al, which showed the binding of class I HDACs to the repression domain of MLL protein that results in

silencing of the respective genes (**137**). Based on our study, it seems that HDAC3 is one of the critical HDACs involved in the leukemogenic potential of MLL-AF9 and it can be considered as an epitarget for the treatment of MLL-rearranged leukemias.

The effects of *Hdac3* depletion in E μ -myc lymphoma have been demonstrated by this study. Indeed overexpression of the proto-oncogene MYC is one of the most frequent genetic alterations found in human cancers. In E μ -myc mice, the intronic enhancer of the μ -immunoglobulin heavy chain drives the expression of *Myc* (**138**). Recently a study conducted by Dovey et al. demonstrated that deletion of *Hdac1* and *Hdac2* in T cells caused neoplastic transformation of immature T cells concomitant with elevated levels of MYC and decreased tumour suppressor p53 activity (**139**). Then consistent with a putative genetic interaction between MYC and HDAC1 or HDAC2, Prof. Johnstone's lab showed that knockdown of these *Hdacs* in hematopoietic progenitor cells accelerated MYC-driven lymphomagenesis (**87**). Respecting the results of the current study, one of the possible mechanisms of HDAC3 involvement in the pathogenesis of E μ -myc lymphoma is the association of HDAC3 activity with MYC oncogene. Possibly, HDAC3 cooperates with MYC in p53 dysfunctionality and therefore the tumorigenic cells escape from apoptosis.

Experimentally, we have decided to use a selective knock-down of *Hdac3* in primary murine hematopoietic progenitors to study the biological effects of the KD *in vitro* and *in vivo* and to better understand the response of tumor cells to novel treatments with epigenetic drugs.

In this study we have shown that HDAC3 has a cooperative activity with PML-RAR α fusion protein in full leukemic transformation. Knock-down of *Hdac3* resulted in loss of proliferative potential and induction of myeloid differentiation of pre-leukemic and frankly leukemic APL cells. A possible mechanism for the induction of differentiation in APL cells upon *Hdac3*-KD

can be inferred from a previous study from Villa et al, (**140**). Indeed, HDAC3 appeared to have an important role in the PML-RAR α associated repressor complex. Actually MBD1, a conserved protein that binds to methylated DNA, cooperates with PML-RAR α in repressing the transcription of target genes and HDAC3 mediates this cooperation. In this study *Hdac3*-KD in NB4 leukemic cells led to a release of RAR β 2 repression (the canonical target of PML-RAR α). This could happen also for the differentiation genes, which are repressed upon PML-RAR α expression. Thus, when *Hdac3* is downregulated, it could lead to loss of transcriptional repression of differentiation promoting genes. Moreover, in another study from Martens et al, (**141**) it has been showed that PML-RAR α /RXR functions as a local chromatin modulator and that specific recruitment of HDAC activities to genes important for hematopoietic differentiation (such as SPI1, GFI1 and RUNX1), RAR signaling and epigenetic control, is essential to its transforming potential.

Additionally, we uncovered that depletion of *Hdac3* in PML-RAR α expressing pre-leukemic and leukemic cells caused the impairment of APL development upon transplantation into recipient mice, supporting the notion that HDAC3 has an oncogenic activity in tumor initiation and maintenance. Additionally and potentially linked to these results, Prof. Zuber's lab has observed the sensitivity of AML cells (driven by MLL-AF9/Nras^{G12D}) to depletion of *Hdac3* and beneficial enhanced survival of mice inoculated with *Hdac3* depleted cells compared to the controls. Furthermore, anti-proliferative effect of *Hdac3* knock-down on E μ -myc lymphomas has been shown by Prof. Johnstone's Lab. However, in contrast to a recent report that *Hdac3* suppression induces apoptosis in multiple myeloma cells (**142**) researchers from Prof. Johnstone's were unable to detect any significant level of apoptosis following *Hdac3* depletion in E μ -Myc lymphoma. Indeed, loss of *Hdac3* in E μ -Myc tumor cells led to an anti-proliferative response that was not attenuated by over-expression of prosurvival BCL-2, or pharmacological inhibition of pro-apoptotic caspases.

Moreover, anti-proliferative effects of HDACi point to a role for HDACs in tumorigenesis. In this regard, HDACs have been widely considered to have oncogenic roles, especially given the impressive anti-proliferative and apoptotic responses mediated by HDACi in certain hematological malignancies (88). Strikingly, we have demonstrated that depletion of *Hdac3*, through a functional knock-down approach consistently attenuated tumor cell differentiation in APL.

Furthermore, we took advantage of a HDAC3 selective inhibitor RGFP966, to assess whether pharmacological inhibition of HDAC3 activity can reproduce the same phenotypes obtained upon genetic depletion of *Hdac3* and sequentially the drug can be used in pre-clinical models of cancer. In agreement with knock-down experiments, treatment of pre-leukemic cells with HDAC3-selective inhibitor RGFP966 reproduced the same biological effects as *Hdac3*-KD, and treatment of APL cells with RGFP966 induced differentiation at low micro molar concentrations. This suggests that low dose HDAC3-selective inhibition in patients with APL may promote tumor cell differentiation and enable tumor remissions without the need for toxic chemotherapy, similar to that observed with ATRA or arsenic trioxide (143).

Interestingly, treatment of wild-type cells and pre-leukemic and full-leukemic cells with RGFP966 showed the higher sensitivity of PML-RAR α expressing cells compared to the WT cells, which is in agreement with the notion that HDACi are more effective on transformed cells rather than normal cells. Notably, this study suggests that HDAC3 could be a good target for the treatment of hematological malignancies, such as APL and lymphoma.

Besides our study, there are other studies, which showed the oncogenic role of HDAC3 in a subset of cancer, such as colon cancer. In colon cancer *Hdac3*-KD led to reduction of β -catenin levels, which downregulated *c-myc* and eventually caused the reduced cancer cell proliferation (144).

It has been shown that HDAC3 is also required for maintenance of chromatin structure and genome stability through the maintenance of an efficient DNA repair. Indeed, HDAC3 activity is required for an appropriate Non-homologous end joining (NHEJ) and homologous-recombination (HR), the repair mechanisms involved in double-strand breaks. These HDAC3 functions may impact on its usefulness as a therapeutic target in cancer and other diseases **(36)**.

There are some HDACi, such as SAHA, that are well tolerated *in vivo*. SAHA may cause S-phase associated DNA damage for cycling cancer cells during the therapeutic window (3-6 hours) in which SAHA is active, whereas it has mild impact on non-cycling normal cells. Actually, in normal proliferating cells SAHA-mediated DNA damage is repaired by the proper activity of DNA damage response. On the other hand, one of the drawbacks in using these agents is that when they are used at too high-doses or for too long time, they cause genomic instability in normal cells and consequently secondary cancers arise as side effects. Similar to almost all cancer therapeutics, HDAC3 inhibitors can develop a therapeutic window and, although continuous inhibition of HDAC3 is detrimental, transient inhibition by HDAC3 selective inhibitor that can be frequently repeated could be safe.

In summary, this is the first study to provide genetic and pharmacological information regarding sensitivities of three distinct hematological tumor types to suppression of *Hdac3 in vitro* and *in vivo*. Using advanced genetic techniques and pharmacological inhibitors we demonstrate that depletion of *Hdac3* reduces the proliferation and/or triggers the differentiation of tumor cells.

Taken together, our novel genetic and pharmacological approach suggests that HDAC3-selective inhibitors may be effective for the treatment of hematological malignancies and that newly developed agent should prioritize HDAC3 as the molecular target to induce effective

anti-tumor responses. However, complete understanding of the mechanisms mediated by HDAC3 in tumor cells *vs.* normal cells, has obvious therapeutic importance. Whether the inhibition of HDACs additional to those examined to date in leukemia, including the class I HDAC8 and the class II HDACs 4, 5, 6, 7, 9 and 10, are also required for the HDACi response remains to be determined.

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