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Role of the histone demethylase LSD1 in the regulation of differentiation of acute promyelocytic leukemia cells

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LIST OF ABBREVIATIONS

aa	amino acids
ac	(ex. H3K9ac) acetylated residues
AML	Acute Myeloid Leukemia
APL	Acute Promyelocytic Leukemia
ATP	Adenosine Triphosphate
ATRA/RA	All trans retinoic acid
bp	Base pairs
BSA	Bovine serum albumin
CBP/KAT3A	CREB binding protein
ChIP	Chromatin Immuno Precipitation
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CREB	camp response element binding protein
DMEM	Dulbecco's modified Eagle medium
DNMT	DNA methyltransferase(s)
DNMT	DNA methyltransferase
E2F	E2 Factor
ECL	Enhanced chemoluminescence
EDTA	Ethylene diamine tetra-acetate
FAB	French-American-British
FBS	Fetal bovine serum
FCS	Fetal Calf serum
h	Human
H	(ex.H3) histone H3
H2A, H3, H4	Histones 2A, 3, 4
HAT	Histone/lysine acetyltransferase
HDAC	Histone deactyltransferase
HMT	Histone/lysine methyltransferase
HRP	Horseradish peroxidase
HSC	Hematopoietic Stem cell
ING	inhibitor of growth
JMJD	histone/lysine demethylase
K	lysine
kb	Kilobase
KDa	Kilodalton
LSD1	lysine specific demethylase
me	(ex.H3K4me3) methylated residues
MC	LSD1 inhibitor
MLL1/2	myeloid/lymphoid or mixed-lineage leukemia 1/2
mRNA	Messenger RNA
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PML	Promyelocytic Leukemia protein
PML-RAR	Acute Promyelocytic Leukemia fusion protein
PMTs	post translational modification
Pol II	RNA Polymerase II

PR	PML-RAR
PR9	A clone of the cell line U937 with inducible PML/RARa
RA LOW	Physiological concentration of RA (0.01μM)
RA HIGH	Pharmacological concentration of RA (1μM)
RARα	Retinoic Acid Receptor
RNAi	RNA interference
RPM	Revolutions per minute
RT	Room temperature
S	Serine
SDS	Sodium dodecylsulfate
SPI1	PU.1 transcription factor
TF	Transcription factor(s)
TFBS	Transcription factor binding site
TRANSFAC	database of transcription factors matrices

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Introduction

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ABSTRACT

Acute promyelocitic leukemia (APL) is a subtype of acute myeloid leukemia characterized in all cases by translocations involving retinoic acid receptors (RAR). The most common translocation leads to the fusion of the promyelocytic leukemia (PML) and RAR alpha genes. The generated oncofusion protein, PML-RAR (PR), corresponds to the initiating event of the transformation. PR expression leads to secondary events and causes expansion of immature myeloid progenitors and a differentiation block at the promyelocytic stage. The oncogenic potential of PR is at least in part due to its ability to function as an aberrant counterpart of RAR. Unlike RAR, PR constitutively represses its target genes and is insensitive to physiological retinoic acid (RA) stimulation. Pharmacological RA doses, instead, lead to the transcriptional induction of its target genes, proteasomal degradation of the fusion protein and release of the differentiation block. Indeed RA (in combination with chemotherapy and/or arsenic) currently represents the first line treatment for APL patients. The proposed model by which PR represses its target genes involves the recruitment of several histone modifiers that help to create a chromatin environment less permissive for transcription.

Some of these same chromatin modifiers have been shown to interact with LSD1 in many other cellular contexts. LSD1 is a histone demethylase, which catalyzes the removal of H3K4me2/me1, whose activity is mainly associated with transcriptional repression in several physiological and pathological contexts. Recently, LSD1 depletion or inactivation has been demonstrated to induce differentiation and impairment in the clonogenic activity in models of acute myeloid leukemias (AML). The mechanism by which LSD1 leads to increased apoptosis and/or differentiation in

leukemia models has yet to be revealed. We found that LSD1 inhibition leads to the sensitization of APL cells to physiological concentrations of RA and subsequently to differentiation and growth arrest. These effects were even stronger than the ones caused by pharmacological doses of RA. To have mechanistic insights into LSD1's role in APL maintenance we took advantage of a potent and specific LSD1 inhibitor previously developed by Antonello Mai (University of Roma) and Andrea Mattevi (University of Pavia) in collaboration with our lab. First, we assessed whether a pulse of LSD1 inhibition was sufficient to prime differentiation. Then we generated data sets from several genome wide assays in order to dissect the specific contribution of LSD1 in the sensitization of APL cells to physiological concentrations of RA. We characterized for the first time the LSD1's genomic distribution in acute myeloid leukemia by performing ChIP-Seq experiments. In APL cells LSD1 binds both promoters and candidate enhancer regions, and potentially interplays with several TFs important for the myeloid lineages functions. Moreover, we found that LSD1 binds highly expressed genes and modulates their expression, working mainly as a transcriptional co-repressor. Bona fide LSD1 repressed genes are involved in differentiation and cell growth control as assessed by ontology pathway analysis. LSD1-dependent modulation of gene expression during APL differentiation is in part achieved by its ability to control H3K4 methylation. We demonstrated that the H3K4me2 regions showing the highest regulation were preferentially distributed in TSS distal regions and correspond to the ones presenting the largest overlap with LSD1. Of note, these regions were also enriched with TFbs of master regulators of the myeloid/monocytic lineage, suggesting their regulatory potential. We also described a previously unknown large fraction of common genomic regions bound by LSD1 and PR, and a possible role for LSD1 in favouring PR recruitment in the initial phases of

disease. Furthermore, PR/LSD1 common binding sites showed peculiar levels of H3K4me2 and a subset of them were found to be dynamically regulated, suggesting a functional interplay between the two proteins in reshaping the local chromatin environment.

Overall, our findings contribute towards understanding the mechanistic role played by LSD1 and of the control of histone methylation during differentiation of hematopoietic cells, thereby suggesting new therapeutic strategies for intervention in APL and potentially other leukemias.

INTRODUCTION

Epigenetics

All the cells within a human body are provided with the same DNA sequence, but during development they acquire distinct functions and morphologies leading to the formation of more than 200 different cell types. The specific cellular phenotype clearly does not depend solely upon the genomic sequences that remain unchanged during development, but instead mainly depends upon the expression of a specific pattern of genes, which must be preserved, strictly controlled and passed down to daughter cells in order to maintain the cell identity or to regulate their proper differentiation. The mechanism by which these processes (and many others) are controlled involves Epigenetics. A number of scientists have provided the community with some interpretation of what Epigenetics represents. For example, Conrad Waddington coined the term and described Epigenetics as the way by which from the genome and its products, the phenotype arises during development (Minucci and Pelicci 2006; Goldberg, Allis et al. 2007; Ernst, Kheradpour et al. 2011) he also envisaged the differentiation process as a path where the cell faces a combination of hills and valleys of energy (representing epigenetic influences) to achieve a more specialized phenotype (Figure intro 1). To Artur Riggs and colleagues, the hereditability is one of the main features of what should be considered as an epigenetic phenomenon. In fact, they define epigenetics as: "The study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by DNA sequence" (Bird 2002). Nowadays a less strict view of epigenetics has evolved to also include modifications that result in gene expression alterations that are not transmitted throughout the cell cycle and can be relatively short-lived. For example,

Bird provides a more general definition of epigenetics that does not necessarily include heritability across generations: “the structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states” (Bird 2007). In this view, it becomes essential to understand how the epigenetic platform, shaped by a variety of external stimuli, influences cell fate and cellular responses.

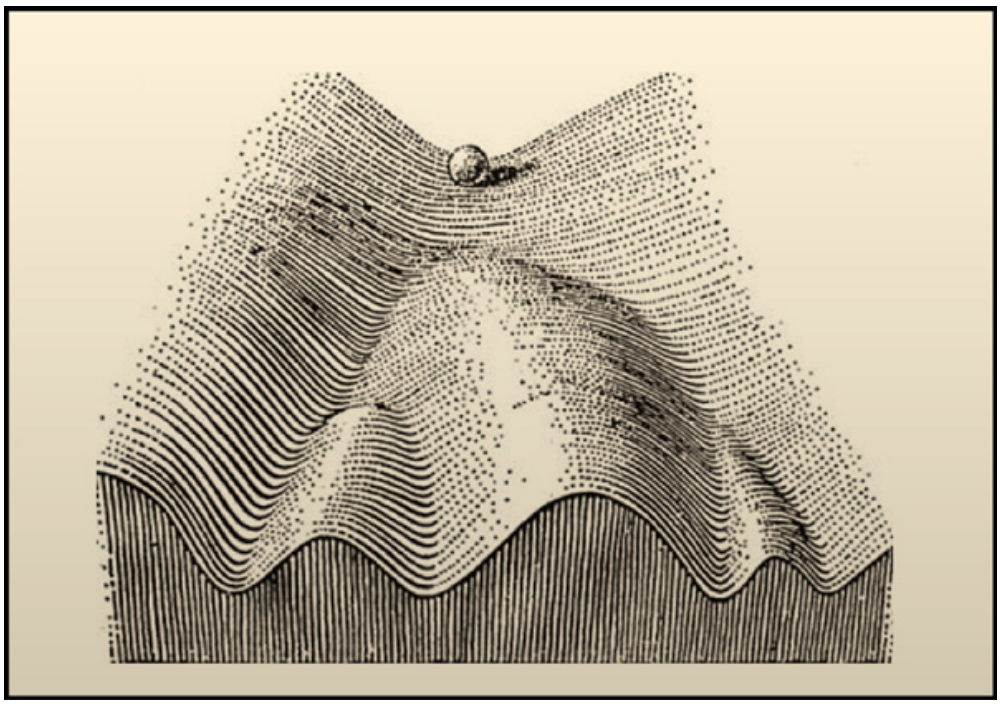


Figure intro 1: Graphical interpretation of epigenetic control of differentiation. The epigenetics landscape includes non genetic variations that influence the cell fate during development. In particular, the ball represents the cell and the hills and the valleys the epigenetic variables influencing the route of the ball and guiding it through to the proper fate.

Chromatin components

The first level of the epigenetic control of cellular processes falls to chromatin. Chromatin is formed by several proteins, which, by dynamic interactions, are

responsible for the proper wrapping of the DNA filament. The way in which DNA is organized within the nucleus has a strong impact on gene expression and thus on the majority of the cellular functions. The basic structural unit of chromatin is the nucleosome, which includes approximately 147 base pairs (bp) of DNA wrapped around the core particles constituted by histones which are organized in octamers. In turn, core histones are formed by a tetramer of H3-H4 histone and a couple of H2A-H2B histone dimers. The first level of compaction, called "beads on a string," is reached by the assembly of core nucleosomes thanks to fragments of linker DNA. Histone H1 binds the DNA linker regions and helps the structure to achieve a higher order of compaction, the so called "30nm fiber". The degree of chromatin wrapping is cell type-, differentiation stage- and region- specific. Chromatin within a cell is divided in two subtypes according to cytological and functional studies: euchromatin and heterochromatin. Euchromatin includes regions rich in genes, more accessible and generally more transcribed. Heterochromatin instead, represents highly condensed regions and consists mainly of repetitive DNA sequences with a relatively low number of genes that are transcriptionally repressed. Functionally, there are two types of heterochromatin: the one that remains inactive and condensed during the life of the cell, called constitutive, and the one which is inactive and condensed only in particular stages of the differentiation process, called facultative. Since the proper chromatin organization gives an indispensable contribution to the regulation of the cell life cycle, determining the evolution of the cell and its response to external stimuli. During evolution eukaryotes have acquired several tools to shape the chromatin landscape. Among them there are post-translational modifications of histones and DNA (PTMs), and enzymes endowed with chromatin remodelling activities.

DNA methylation

DNA methylation represents probably the best characterized epigenetic modification and has been described in several organisms (Goldberg, Allis et al. 2007); (Colot and Rossignol 1999). DNA methylation is involved in many cellular functions including gametogenesis, embryogenesis, X inactivation, imprinting and transcriptional control (Bird 2002), (Jones and Takai 2001). This modification shows a peculiar distribution in mammals, in fact cytosine residues found within CpGs dinucleotides are preferentially methylated. CpG sites are usually clustered in CpGs rich regions and defined as CpG islands. CpG islands are mainly distributed around promoters, the first exon and the 5'untraslated regions of genes (Esteller 2007) and normally protected from methylation. This unmethylated status makes the genes associated with CpGs islands prone to be transcriptionally activated, while on the contrary the methylation of CpG islands strongly correlates with the silencing of the corresponding gene (Esteller 2007). For instance, DNA methylation occurs at alleles which must be silenced on the X chromosome (Bird 2002). DNA methyltransferases (DNMTs) are the enzymes responsible for the methylation of DNA strands. Mammals have three different DNMTs: DNMT1, DNMT3a DNMT3b. DNMT1 regulates the maintenance of the methylation status by recognizing hemimethylated DNA (Okano, Bell et al. 1999); (Bestor 1988). It is involved in heritability of DNA methylation and is ubiquitously expressed; DNMT3a and 3b, on the other hand, were initially considered as de novo methyltransferases (Okano, Bell et al. 1999), and their expression is normally confined to the early stages of development (Meissner, Mikkelsen et al. 2008) when they are indispensable for the creation of the proper DNA methylation status. DNA methylation is indeed one of the major epigenetic players in the

differentiation process, in fact the levels of CpG methylation within differentiated cells are higher than in stem cells (Mikkelsen et al. 2008; (Mohn, Weber et al. 2008). Moreover, the genes mainly subjected to CpG methylation are the ones known to take part in the maintenance of pluripotency (Mohn, Weber et al. 2008), (Farthing, Ficz et al. 2008). Precisely how the transcriptional silencing depends upon CpGs methylation is still not completely understood (Li 2002). It has been shown that DNMTs recruit histone modifiers, like histone deacetylases (HDACs) and histone methyltransferases (HMTs), together with other co-repressors, to the promoter owing to the methylated CpG (Fuks, Burgers et al. 2000); (Burgers, Fuks et al. 2002); (Fuks, Burgers et al. 2001). Similarly, repressive complexes can also be recruited by methyl-binding proteins (Fuks, Burgers et al. 2001); (Magdinier and Wolffe 2001) ; (Fuks, Hurd et al. 2003). It is possible that the presence of these complexes on the methylated DNA, sterically impairs the binding of activator complexes as well as creates a closed chromatin conformation thus preventing transcription.

Quite recently, a new DNA modification has been better characterized, the 5-hydroxymethylation of cytosine (5hmc) (Branco, Ficz et al. 2012). The enzymes responsible for the deposition of this mark belong to the TET family (Tahiliani, Koh et al. 2009). The precise role of 5hmc is not fully understood, nevertheless it has been hypothesized that this modification represents an intermediate of the demethylation process and/or as a proper epigenetic modification recruiting specific transcription modulator complexes. (Branco, Ficz et al. 2012).

Histone modifications

Nucleosomes, and in particular single histones show a large number and type of modified residues owing to PTMs (Kouzarides 2007). PTMs are predominantly localized on the N-terminal tail and include lysine acetylation, methylation, sumoylation and ubiquitination; arginine methylation and deamination; serine/threonine phosphorylation; ADP ribosylation (Kouzarides 2007). Functionally, all of these PTMs contribute to regulating transcription, but the high level of complexity makes it difficult to have a global understanding of the related mechanism. The complexity is intuitively due to the elevated number of possible combination of PTMs, also considering the time of appearance within the cell upon a particular stimulus (Kouzarides 2007). Those responsible for the deposition of these modifications are several classes of enzymes called "writers". The "readers" instead, are proteins and/or enzymes that can recognize some histone PTMs and induce secondary events leading to chromatin alteration and modulation of the transcriptional status (Chi, Allis et al. 2010). PTMs can be also dynamically removed by "erasers" depending on the functional outcome required by the cell (Chi, Allis et al. 2010). "Writers" and "eraser" correspond to several classes of enzymes endowed with specialized chromatin modifying activity; the "readers", instead, are proteins provided with a particular domain able to recognize specific modifications (see below) (Figure Intro 2). All the proteins cited above can interact directly or be included in the same protein complex supporting the idea that the chromatin reshaping derives from an interplay of sequential events. (Ruthenburg, Allis et al. 2007); (Yun, Wu et al. 2011);

Lysine acetyltransferases, lysine deacetylases and histone acetylation

Acetylation has been identified more than 40 years ago in eukaryotic cells (Allfrey, Faulkner et al. 1964) and nowadays is known to be broadly distributed across species. It is respectively deposited and removed on the e-amino group by lysine acetyl-transferases (KATs) and histone deacetylases (HDACs) (Shahbazian and Grunstein 2007). HAT and HDAC activities are important also for the control of a

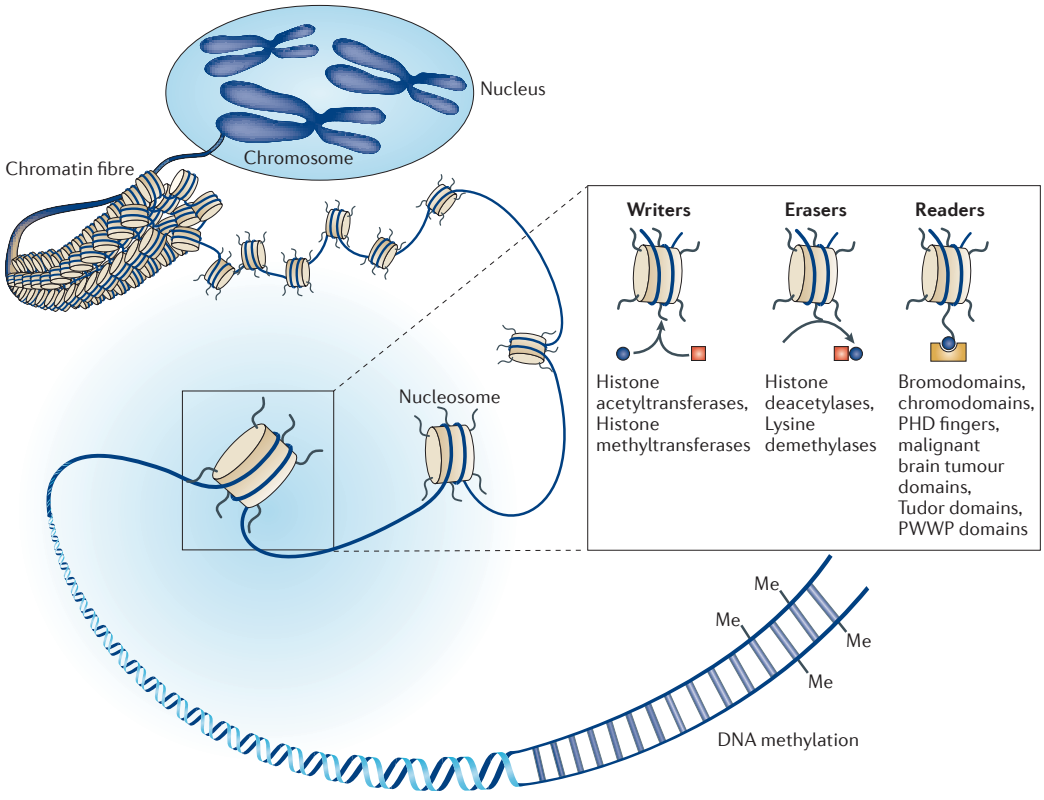


Figure intro 2. **Schematic representation of Writers, Erasers and Readers.** Chromatin related proteins responsible for the deposition, removal of the histone PTMs and, in general, the reshaping of the histone code (Arrowsmith, Bountra et al. 2012).

plethora of non-histone proteins and are involved in different cellular processes, such as transcription factor assembly, protein stability and the shuttling between nucleus and cytoplasm (Minucci and Pelicci 2006) In particular, two types of KATs have been characterized according to their cellular localization: A KATs and B KATs. The first group is nuclear, includes the GNAT, MYST and CBP/p300 families and is able to acetylate histones and other nuclear proteins. The second group works mainly in the cytoplasm and acetylates newly synthesized histones (Rea, Eisenhaber et al. 2000); (Nakayama, Rice et al. 2001). Instead, HDACs are subdivided into four classes including the following enzymes: Class 1: HDAC 1-3 and HDAC 8; Class II: HDAC 4-7 and HDAC 9-10; Class III: sirtuin 1-7 and Class IV: HDAC11. Both KATs and HDACs principally function within multiprotein complexes (Minucci and Pelicci 2006), (Rea, Eisenhaber et al. 2000), (Nakayama, Rice et al. 2001) and their catalytic activity is influenced by the protein with which they are interacting, by determining substrate specificity (Minucci and Pelicci 2006). From the biochemical point of view, histone acetylation changes the charge of the substrate, and in particular, in vitro studies have shown that acetylation of histones causes an increased distance between the two nucleosomes to which they belong. Histone tail-acetylation also contributes to chromatin reorganization and is generally associated with transcriptional activation. Its action can be exploited indirectly, in fact acetylated histones function as recruitment platforms for proteins containing a tandem plant homeodomain (PHD) fingers and bromodomains (Marmorstein 2001) (Dhalluin, Carlson et al. 1999). Moreover, acetylation cross-talks with other histone PTMs and they reciprocally influence their own deposition. An example is given by H3K4methylation, which induces H3 acetylation through the recruitment of SAGA and CBP. In turn, H3 acetylation can promote methylation on the lysine 4 of histone H3 (Murr 2010).

Methylation and acetylation can also compete for the binding of the same lysine, in particular methylation of H3K9 opposes the transcriptional activation function of the H3K9 acetylation (Rea, Eisenhaber et al. 2000), (Nakayama, Rice et al. 2001).

Lysine methyltransferases, demethylases and histone methylation

Methylation marks can be deposited both on arginine and lysine residues, and, importantly, the number of methyl groups that can be added to histones is variable. Arginines can be methylated with one or two groups, and the dimethylated form can occur in a symmetrical and asymmetrical conformation (Greer and Shi 2012). Lysines, on the other hand, can undergo mono, di- or tri- methylation (Martin and Zhang 2005). There are several residues where methylation has been observed, H3K4, H3K9 and H3K27 among others. Each methylation status, including the unmodified form, has been proposed to have a different correlation with the region specific transcriptional outcome (Martin and Zhang 2005). The enzymes responsible for the establishment of the histone methylation are the histone methyl transferases (HMTs). There are three protein families of HMTs, two acting on lysines residues and one working on arginines. Lysines methyltransferases group include both SET [Su(var)3-9, Enhancer of Zeste, Tritorax] domain containing proteins and the DOT1 family; enzymes acting on arginines belong to the protein arginine methyl transferases PRMTs family. The methylated residues are instead, actively removed by histone demethylases. LSD1, a lysine-specific demethylase acting specifically on the mono and di-methylated forms of H3K4, was the first demethylase discovered (Shi, Lan et

al. 2004). Several years later Karitinos and colleague characterized a homologue of LSD1, LSD2, showing similar biochemical activities on H3K4me1 and H3K4me2 (Karytinos, Forneris et al. 2009). LSD proteins catalyze demethylation by an amino oxidation reaction, using a molecule of flavin adenine dinucleotides (FAD) as a cofactor. LSD1 and LSD2 cannot work on trimethylated histones since they need a free electron pair to be present upon the lysine residue (Kooistra and Helin 2012). Jumanji (JMJ) proteins represent the second family of demethylases. The JMJ family consists of 30 members, 18 of which own a JMJ catalytic domain. They can act on trimethylated lysines (Mosammaparast and Shi 2010) through a dioxygenase reaction dependent upon Fe(II) and a-ketoglutarate. The ability to recognize specific substrates for all the enzymes listed above depends on the protein complexes in which they are included. In fact, LSD1 forms a stable complex with several other proteins and is involved in different functional outcomes. For instance, LSD1 when bound with the corepressor RE1-silencing transcription factor (Co-REST) mediates transcriptional repression, but, when recruited by the androgen receptor protein complex, positively influence gene activation by demethylating H3K9me2 (Cloos, Christensen et al. 2008). The methylation and the demethylation are evidently highly regulated and histone methylation has an enormous impact on the chromatin landscape assembly and, consequently, upon the regulation of gene expression. The methylated forms of the histones are recognized by several effector proteins endowed with particular domains contributing to both transcriptional activation and repression. Lysine four of histone H3, when trimethylated, serves as a mark for the recruitment of the PHD doamin-containing BPTF proteins. BPTFs work as subunits of the nucleosome remodelling factor (NURF) complex which plays a role in trancriptional activation of its target genes (Wysocka, Swigut et al. 2006) HP1 is a chromodomain endowed

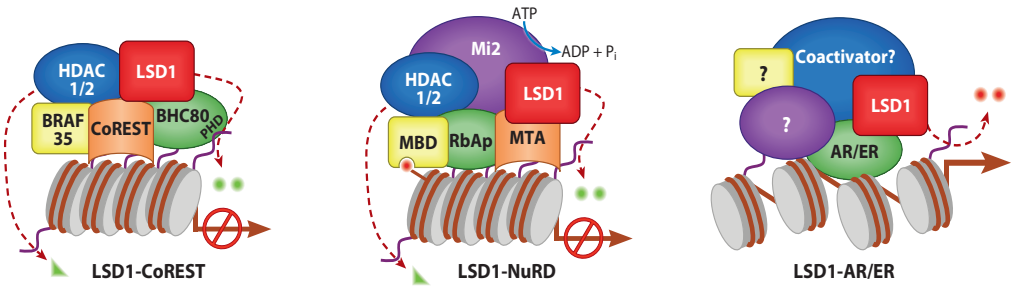
protein that when bound to methylated H3K9, leads to the formation of heterochromatin and is instead, associated with gene silencing (Bannister, Zegerman et al. 2001); (Lachner, O'Carroll et al. 2001). Moreover, as previously described in the Acetylation section, acetylation and methylation also contribute to shape chromatin by influencing each other's deposition and this extensive cross-talk has a strong impact on the gene expression.

The first demethylase discovered: LSD1

Histone methylation was thought to be a stable mark and that its removal could be obtained by several mechanisms including DNA replication-dependent dilution or by active histone replacement. The first experimental evidence of the existence of direct enzymatic demethylation, came with the discovery of LSD1, initially identified as a component of the HDAC-BRAF, CoREST complex (You, Tong et al. 2001); (Shi, Lan et al. 2004). This demonstration paved the way for the isolation of other demethylases: an entire family of demethylases, the JMJ proteins, was discovered (Kooistra and Helin 2012). More recently in humans, a homolog of LSD1 was characterized enlarging the lysine specific demethylase family. LSD2 exhibits poor sequence identity (30%) but similar domain homology with LSD1 (Fang, Barbera et al. 2010). The structures of both LSD proteins include a N-terminal SWIRM domain and an amino oxidase domain containing two binding sites, one for the substrate and another for the FAD molecule. These two pockets together form a globular domain responsible for the catalytic activity. LSD proteins remove the monomethyl and dimethyl, but not trimethylated form of H3K4 and cause the production of formaldehyde and a reduced form of FAD (Fang, Barbera et al. 2010). In addition to LSD1, LSD2 contains a zinc-finger domain in its N-terminal region, and vice-versa

LSD1 has a TOWER domain that protrudes from its globular portion and is essential for its interaction with other proteins. For instance, the TOWER domain mediates the binding of LSD1 with Co-REST. Interestingly, this interaction confers nucleosome demethylation ability to the purified LSD1 protein and enhances LSD1 stability in vivo. On the contrary, LSD1 demethylase ability was found to be impaired when it is bound to BHC80 (Shi, Matson et al. 2005). Thus the interaction with co-factors is extremely important for the regulation of the LSD1 demethylase activity (Shi, Matson et al. 2005). Several other proteins have been characterized as LSD1 interactors and, in particular, it is known to be involved in chromatin regulation mediated by a number of complexes (CoREST complex, the NURD complex and androgen/oestrogen receptor (AR/ER) complex) (see Figure 4).

Figure intro 3. LSD1 works in chromatin modifying complexes to regulate transcription. LSD1 binds CoREST, NURD and AR/ER complexes and contributes to chromatin and transcriptional regulation by demethylating H3K4me2 or H3K9me2. (Adapted from Mosammaparast and Shi Ann Rev Biochem 2010)



The CoREST complex is composed of HDAC1, BRAF35, BHC80, all of which are thought to play specific roles in the complex. As an example, it has been shown that

LSD1 preferentially demethylates deacetylated histones. In keeping with this view, HDACs could help to prime chromatin for the LSD1 action (Shi, Matson et al. 2005) while CoREST, BRAF35 and BHC80 could instead guide the recruitment of the complex to specific genomic loci (Mosammaparast and Shi 2010). Recently, LSD1 has also been found to cooperate with the NURD complex to control H3K4me2/me1 levels (Wang, Zhang et al. 2009); (Whyte, Bilodeau et al. 2012). NURD complex also contains HDAC activity and shows chromatin remodelling activity specifically thanks to the presence of proteins belonging to the SWI/SNF family (Ho and Crabtree 2010). In NURD complexes MTA proteins regulate the LSD1 demethylases activity (Wang, Zhang et al. 2009). When interacting both with CoREST and NURD, LSD1 demethylates the lysine 4 of the histone H3, when instead involved in the AR/ER transcriptional regulation its action shifts towards H3K9 (Metzger, Wissmann et al. 2005); (Garcia-Bassets, Kwon et al. 2007). Until now the ability of LSD1 to likewise act upon H3K9 is not supported by biochemical data the human form (Yang, Culhane et al. 2007). While H3K4me2 demethylation by LSD1 has been associated with transcriptional repression, the activity performed on H3K9me2 seems to be linked to gene induction (Cloos, Christensen et al. 2008). LSD1-dependent transcriptional control is required for many biological processes with Several reports describing the role of LSD1 in differentiation of a great variety of tissues, such as pituitary cells (Wang, Scully et al. 2007) and adipogenesis (Musri, Carmona et al. 2010). Moreover, Orkin and colleagues used in vitro experiments to uncover the role of LSD1 in the multi-lineage hematopoietic differentiation (Saleque, Kim et al. 2007). This role has been further explored by conditional KO/KD murine systems confirming a clear involvement of LSD1 in several steps of the physiological haematopoietic development (Sprussel, Schulte et al. 2012) (Kerenyi, Shao et al 2013) LSD1

conditional KD leads to an improper expansion of immature progenitors and an impairment in most lineage terminal differentiation. Interestingly, these defects can be reverted by restoring LSD1 expression (Sprussel, Schulte et al. 2012). In accordance with this finding, complete deletion of the gene in the hematopoietic compartment leads to alteration of HSC self renewal and impairment of terminal granulocytic and erythroid maturation. The use of conditional KD systems was necessary since LSD1 KO mice are embryonic lethal (Wang, Scully et al. 2007). Subsequent studies both in mouse and human KO ESC showed that LSD1 protein depletion was associated with proliferation and differentiation defects (Wang, Zhang et al. 2009); (Adamo, Sese et al. 2011), (Whyte, Bilodeau et al. 2012). In particular in LSD1 KO mESC, an impairment in the proper maintenance of global DNA methylation was noted. In this case, LSD1 was shown to regulate the removal of a methyl group on K1096 critical for DNMT1 stability and thus for the establishment of DNA methylation (Wang, Hevi et al. 2009). LSD1 had been previously shown to act upon non-histone substrates in 2007, when Huang and colleagues demonstrated its ability to regulate P53 activation by removing K370me2 (Huang, Sengupta et al. 2007). Later, E2F1 was added to the list of LSD1 substrates, as it was discovered that the LSD1 dependent removal of the methyl group from lysine-185 leads to protein stabilization and apoptosis (Kontaki and Talianidis 2010). These data highlight the wide repertoire of crucial cellular functions in which LSD1 is involved, such as cell cycle, differentiation, stem cell regulation, and further support the interest in studying how the alterations of its expression/activity could contribute to disease formation or maintenance (see paragraph above LSD1 as Drug target).

Histone modifications and transcriptional regulation

As anticipated in the first paragraph describing epigenetics, all chromatin features can contribute to regulate the expression pattern and indeed the identity of cells and many other processes. In particular, it has been theorized that the plethora of histone modifications can influence the expression modulation by a strict interplay. In this view the possible combination of PTMs constitutes the "histone code" which must be decrypted by chromatin binding proteins to properly regulate the transcriptional outcome. (Jenuwein and Allis 2001); (Strahl and Allis 2000). Histone modifications can induce the recruitment of transcription elongation factors as show for H3S10p-dependent H4K16acetylation, in particular during gene activation (Wysocka, Swigut et al. 2006). A great step forward in the decryption of the complex matrix of the epigenome has been accomplished thanks to recent advances in high-throughput sequencing technology. The possibility to map histone modifications at a chromosomal or genomic scale and to generate gene expression information, has lead to the identification of chromatin features indicative of both transcribed and repressed genes (Figure intro 4). From the regulatory point of view, clear patterns of PTMs have been shown to associate with enhancers, promoters and intragenic regions (Ernst, Kheradpour et al. 2011). Promoters correspond to cis-regulatory regions spanning the transcriptional start site (TSS), which are necessary to guide transcription activation. Merging gene expression data and chromatin maps has uncovered a strong association between H3K4me3 and H3-H4 acetylation for expressed gene promoters; repressed genes, instead show an enrichment of H3K27 and H3K9 trimethylated marks within their promoter regions. (Barski, Cuddapah et al. 2007); (Heintzman, Stuart et al. 2007). Even if H3K4me3 and H3K27me3 seem to be alternatively distributed, in early development they coexist in promoters of genes called "bivalent". These genes

are “poised” and later in the differentiation program, could either undergo activation, by the loss of H3K27me3 and the acquistion of H3K4me3 or repression via the contrary process (Sharov and Ko 2007).This switch to one or another modification status and to a particular transcriptional outcome is strictly regulated by HMTs and HDMs, and seems to play a fundamental role in the cell differentiation process. The pattern of PTMs associated with enhancers instead, has just started to be understood. It includes a combination of high levels of H3K4me1 and low levels of H3K4me3 and the binding of P300 associated with these genomic traits (Heintzman, Stuart et al. 2007). Interestingly, during the early stage of development two additional chromatin marks can sub-divide enhancers as being either “poised” when presenting H3K27me3, or “active“ when associated with H3K27ac (Rada-Iglesias, Bajpai et al. 2011); A number of PTMs were also found to associate with other particular genomic locations, for example H3k36me3 was found to span the gene body of transcribed genes and to be enriched in the 3’ end, suggesting a role in elongation or termination (Bannister, Schneider et al. 2005). Naturally, there are also some exceptions to these correlations between transcriptional activity and histone PTMs. In fact, when inhibitor of growth complex 2 (ING2) binds H3K4me2 and H3K4me3, classically associated with gene poising or activation, it stabilizes a histone deacetylase complex and in this particular case H3K4me2 and H3K4me3 mediates gene repression (Greer and Shi 2012). These studies have highlighted some clues about the epigenetic influence on gene transcription even if precise and global mechanistic bases still remain to be fully elucidated. What is emerging is the very fine regulation needed by the cell in controlling chromatin and expression, in fact the epigenetic alteration even of a single gene could predispose to diseases, including cancer.

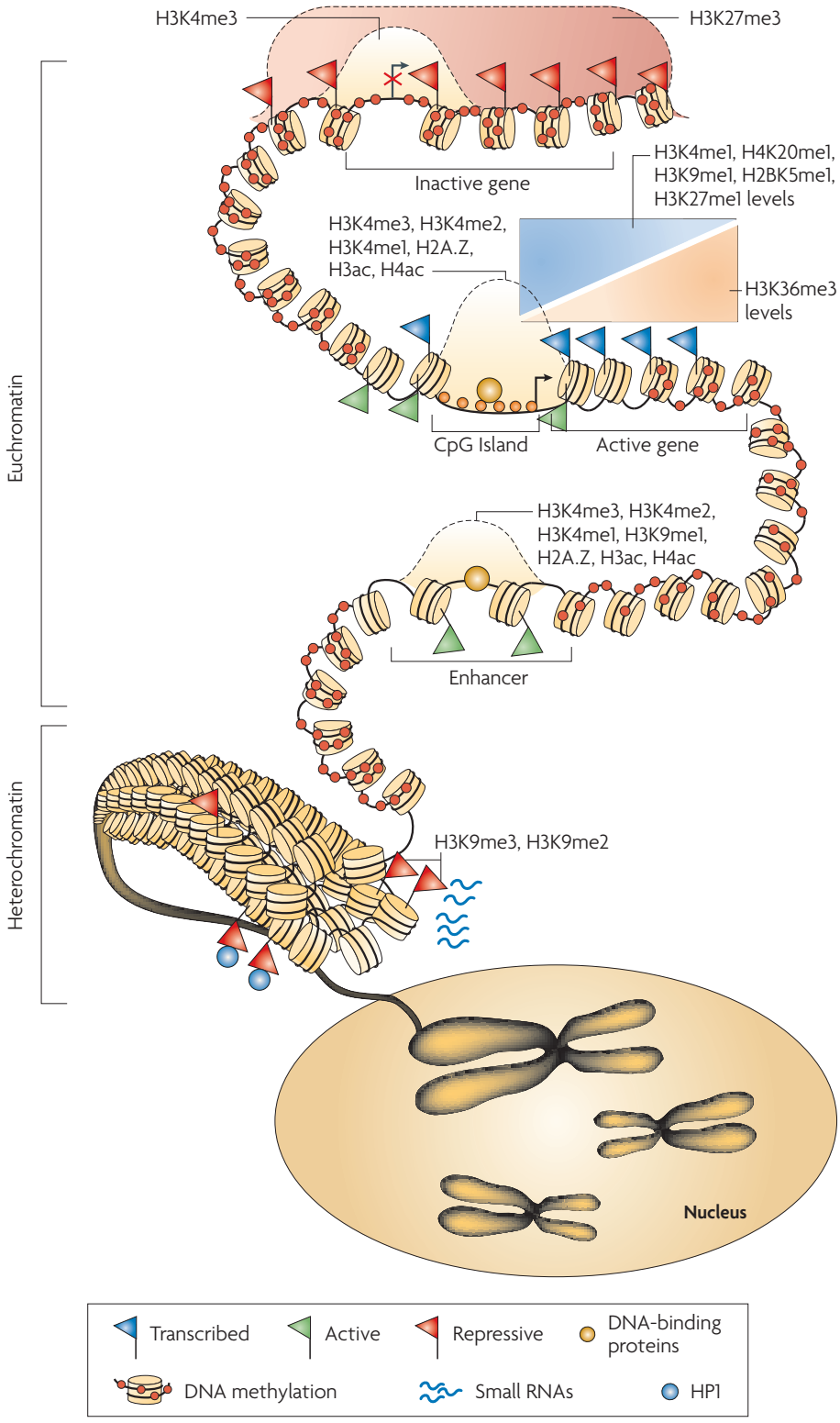


Figure intro 4: **Chromatin modifications are associated with specific functional genomic regions**. In general chromatin is divided in heterochromatin (more compacted and enriched with silenced genes) and in euchromatin, (more accessible and associated with expressed or poised genes). Heterochromatin

is mainly associated with H3K9me while the lysine 4, 27 and 36 of histone H3 plus acetylated forms of histones are generally enriched in euchromatin. Thanks to genome wide studies it has been possible to appreciate such specific distribution of histone/DNA modifications (Schones and Zhao 2008).

Epigenetic alterations in cancer

DNA methylation and histone modifications in cancer

The epigenetic machinery contributes to regulate most cellular functions and its perturbation could lead to improper activation/inhibition of several transcriptional pathways, poising or even causing cancer. Cancer cells exhibit profound epigenetic abnormalities, including altered methylation. The first suggestion of a role for epigenetics in cancer came from an observation by Stephen Baylin. He noticed that region specific methylation levels were relatively higher in cancer cells in respect to normal cells. He supposed that methylation of a tumor suppressor gene promoter would reduce or even stop its transcription. The proof came almost 10 years later via a collaboration between Baylin and James Herman studying renal cell carcinomas (RCC). In 60% of the cases, RCC is due to inherited silencing mutation of the Von Hippen Lindau tumor suppressor gene (VHL). They saw that in the 20% of the cases which did not present the mutation, the gene silencing was achieved by hypermethylation (Brower 2011). The epigenetic-induced loss of function of tumor suppressors caused by hypermethylation, has been found in many other cases such as p16, BRCA and Rarβ (Esteller and Herman 2002), (Kulis and Esteller 2010). Additionally, many genes involved in DNA repair are subject to this phenomenon, for instance MGMT. In response to carcinogen exposure, MGMT catalyzes the removal

of O6-methylguanine adducts from DNA. Interestingly, cancers which have MGMT promoter-hypermethylation, are susceptible to genetic mutations in specific genes, such as p53 or KRAS (Esteller 2007); (Baylin and Jones 2011). Another gene undergoing to loss of function by promoter hypermethylation is MLH1. Since it plays a role in genomic stability maintenance, its epigenetic depletion can induce several cancers, including colorectal and endometrial carcinomas (Krivtsov and Armstrong 2007). Not only is DNA hypermethylation associated with cancer cell physiology, in fact in the mid eighties Feinberg and colleagues, uncovered a global and progressive loss of CpG methylation across the genome (Feinberg and Vogelstein 1983). Subsequently, it was also shown that the global hypomethylation in gastrointestinal cancer correlates with chromosomal instability and DNA damage (Rodriguez, Frigola et al. 2006). Another consequence of genome-hypomethylation could be the loss of imprinting, as exemplified in the case of insulin-like growth factor 2 gene (IGF2) in colorectal cancer (Cui, Horon et al. 1998). Therefore, in tumors, the hypomethylation seems to occur in CpG island-negative promoters, introns, repetitive regions and transposons (Gama-Sosa, Wang et al. 1983); (Rodriguez, Frigola et al. 2006), while DNA hypermethylation is restricted to CpG islands of tumor suppressor gene promoters. Moreover, regions located up to 2kb from the nearest gene, called CpG-shores, were shown to undergo methylation changes in cancer. They show conserved methylation profiles between human and mice suggesting a role in the tissue specificity (Irizarry, Ladd-Acosta et al. 2009); (Doi, Park et al. 2009). (Baylin and Jones 2011); (Esteller 2007). Aberrant histone modification patterns have also been found to be associated with several cancer types. In 2005 Fraga and coworkers, unveiled a loss of H4K16 acetylation and H4K20me3 in lymphomas and colorectal cancer by comparing normal tissues, primary tumors and cancer cell lines, (Fraga,

Ballestar et al. 2005). Subsequently, it has been shown that the combination of 5 histone modifications in prostate cancer biopsies correlates with the patient's clinical outcome (Seligson, Horvath et al. 2005). Moreover, an alteration of H3K9me3 levels in core promoters was observed in leukemia when compared to normal samples (Muller-Tidow, Klein et al. 2010) and this H3K9me3 pattern strongly contributes, along with othner clinical parameters, to determine a more precise prognosis (Muller-Tidow, Klein et al. 2010)

Genetic alterations of chromatin modifiers

Thanks to the improvement in sequencing technology the opportunity to extensively map cancer genomes has rendered it possible to highlight mutations of several chromatin regulators (Dawson and Kouzarides 2012). These mutations can be hypothesized to cause a profound alteration of the normal epigenome of the cell at several levels. For instance, given the important role of DNMTs proteins in the maintenance and deposition of the proper methylation profile, their genetic loci have been found to be broadly mutated in many tumors. While DNMT1 mutations have been associated with colorectal cancer (Kanai, Ushijima et al. 2003), DNMT3a loss of function mutations were found in myelodysplastic syndromes (MDS) and in about 22% of acute myeloid leukemia (AML) (Ley, Ding et al. 2010); (Yamashita, Yuan et al. 2010); (Yan, Xu et al. 2011). In a DNMT3B-depleted cellular context, the Myc-induced lymphomagenesis was accelerated and SNPs in DNMT3B seem to correlate with a higher risk of lung adenocarcinoma (Shen, Wang et al. 2002). On the other hand, DNMT3B is also found to be overexpressed in a number of other cancer types:

glioblastomas, retinoblastomas, prostate, gastric, colorectal and hepatocellular carcinoma (Gros, Fahy et al. 2012).

A second level of cancer-associated misregulation is represented by the presence of aberrant histone modification patterns. The altered balance in the deposition and removal of histone marks can be due to improper activity or dosage of the assigned modifying enzymes. Truncating mutations associated with loss of heterozygosity of P300 have been observed in a number of tumors including glioblastomas and colorectal cancer (Phillips and Vousden 2000). Class I HDACs show enhanced expression in many solid and hematological malignancies, and in some cases overexpression correlates with poor prognosis (Weichert 2009). The expression of Class II HDACs, has been reported to be reduced in cancer while their high expression levels are associated with better prognosis (Weichert 2009). Among the HMTs, mixed lineage leukemia (MLL) is frequently involved in chromosomal translocations in acute leukemia (You and Jones 2012). Translocations of MLL can cause the formation of fusion proteins that in turn can lead to altered H3K4me3 deposition. The MLL fusion proteins interact also with other histone modifiers, such as DOT1L. This specific interplay induces an aberrant H3K79 methylation pattern required for the maintenance of the oncogenic transcriptional program (Bernt, Zhu et al. 2011).

EZH2, an H3K27 methyltransferase, has been observed to be overexpressed in a large number of tumor types, which is often associated with tumors in advanced stages (Chang and Hung 2012). In particular, EZH2 overexpression in prostate, breast lung and bladder tumors leads to an increase of H3K27 methylation. While no association between EZH2 and H3K27me3 levels has been found in ovarian and pancreatic cancers (You and Jones 2012), EZH2 mutations have been reported in

lymphoma and myeloid neoplasms (Chase and Cross 2011). In lymphoma a missense mutation within its catalytic domain causes a gain of function, however in myeloid malignancies the mutations result in loss of function.

Similarly, histone demethylase-expression and function has been found to be altered in many types of cancer. It was shown that in SUV39 I-II and SUV39H2 (H3K9 methyltransferases) deficient mice was associated with an increase of B cell lymphomas incidence (Peters, O'Carroll et al. 2001). Furthermore, the genomic loci corresponding to the JMJD3 and JMJD1 genes are often often lost in tumors (Cloos, Christensen et al. 2008). Importantly, JMJD5 and FBXL10 were identified in mice as tumor suppressors of leukemia. (Suzuki, Minehata et al. 2006). On the contrary, overexpression of JMJD2A,B,C has been found in prostate cancer (Cloos, Christensen et al. 2008). LSD1 also exhibits high expression levels in prostate cancer as well as neuroblastoma, bladder cancer, colorectal cancer (Amente, Lania et al. 2013). Chromatin remodelers can also contribute to tumor phenotypes as can the many irregular transcription factors generated by chromosomal translocations which often lead to the alteration of target cell epigenetics via the aberrant recruitment of histone modifying proteins. In particular, Acute promyelocytic leukemia (APL) represents the first disease in which HDAC involvement in cancer pathology has been demonstrated. In APL, the PML-RAR (PR) fusion protein works as a dominant negative counterpart of retinoic acid receptor (RAR) and induces a block of myeloid differentiation (see paragraphs below). In particular, the PR oncogenic potential is primarily mediated by its interaction with the N-COR/HDAC repressor complex (Minucci and Pelicci 2006). The PR/HDAC1 interplay likely leads to a general hypoacetylated chromatin environment around PR target genes and induces their transcriptional silencing (Mikesch, Gronemeyer et al. 2010). The presence of epigenetic enzymes that are

overxpressed and seem to function as tumor promoters has made them interesting candidates as drug targets. Indeed, the possibility to revert the epigenetic alterations thought to be necessary for tumor maintenance represents an intriguing opportunity for cancer therapy, which in recent years has been extensively explored.

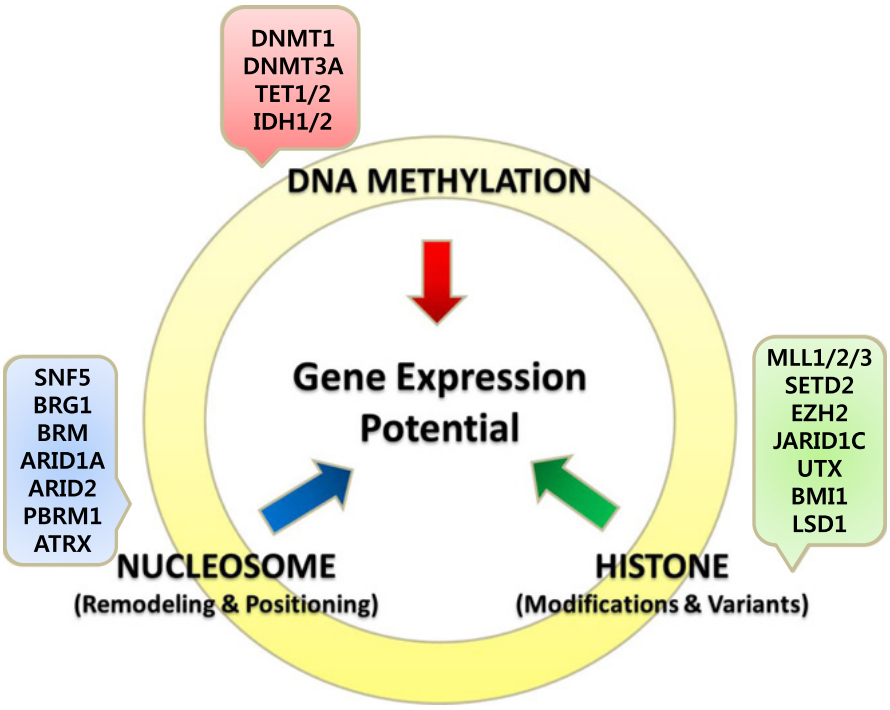


Figure intro 5. Some examples of chromatin modifying proteins found mutated in cancer. Whole exome sequencing of several types of tumors have highlighted a close relationship between genetics and epigenetics. Many chromatin modifiers have been found mutated in cancer. These mutations probably induce severe alterations in the epigenetic landscape of the cells. Epigenetics aberrations, as opposed to the genetics aberrations, can likely be more easily reverted underscoring new opportunities for drug design and epigenetic therapies in a wider range of cancers (You and Jones 2012).

Epigenetic inhibitors in cancer therapy

A number of the previously described genetic alterations within chromatin modifier genes are likely "driver mutations" as they are found to be highly represented in specific cancer types or in a variety of tumors (Dawson and Kouzarides 2012). The resulting epigenetic lesions associated with cancer, as opposed genetic lesions, can be potentially reverted and for this reason several drugs targeting chromatin modifiers have been developed. Azacitidine (AZA) and decitabine (DAC) are the first approved epigenetic drugs. They target DNMT1 and DNMT3 proteins and are currently used as standard combined therapies for myelodysplastic syndrome (MDS). What has emerged from several phase II and phase III clinical trials in MDS is that, in comparison to chemotherapy, the clinical responses occur after multiple therapeutic cycles and improve over time with minimal side effects (mainly neutropenia). DAC and AZA often in association with standard chemotherapy, have also been tested in refractory/relapsed leukemias as well as late stage solid tumors with the most promising results seen with previously untreated AML patients (Issa and Kantarjian 2009). Even if global hypomethylation has been observed upon treatment with DNMT inhibitors, there is no correlation with clinical response. Indeed the current limit of these two drugs is both the absence of markers to predict patient sensitivity and the incomplete understanding of their underlying molecular mechanisms (Issa and Kantarjian 2009); (Helin and Dhanak 2013). In the case of HDACi, their use anticipated the identification of their molecular target. In fact inhibitors were used to isolate the HDAC proteins (Minucci and Pelicci 2006). Nowadays, two HDAC inhibitors, suberoylanilide hidroyamic acid (SAHA) and romidepsin (FK228) were approved for refracted or relapsed T-cell lymphoma treatment. Other HDAC inhibitors have been tested in clinical trials for lymphoma and solid malignancies

(Botrugno, Santoro et al. 2009). They showed had shown promising results when associated with DNMT inhibitors, but only a limited efficacy in early phases clinical trials, both as single therapiesy or in combination with retinoic acid (RA). It is possible that the not somewhat unexciting clinical results for HDAC inhibitors are duecan be attributed to the lack of the rightan ideal target population (Altucci and Minucci 2009), due to the absence of a clear indicator to predictive of clinical activityoutcome. , or additionally, to the poor selectivity of the inhibitors, sinceas individual HDACs show specific biological functions (Botrugno, Santoro et al. 2009); (Helin and Dhanak 2013). Recently, other classes of small molecule inhibitors have been developed, including those targeting protein methyltransferases and demethylases. Methyltransferases, as described previously, includes PRMTs and KMTs. EZH2 belongs to the KMT family and higher expression and somatic mutations of EZH2 have been found in lymphomas, and, in solid tumors where they correlate with poor prognosis (Helin and Dhanak 2013), (Morin, Johnson et al. 2010), (Pasqualucci, Trifonov et al. 2011), (Ryan, Hoff et al. 2010). EZH2 inhibition strategies are mainly based on the design of competitive inhibitors. EZH2, in fact, shows high affinity for s-adenosyilhomocysteine (SAH) and substrates containing lysines. A competitive inhibitor, 3-deazaneplanocin (DZNep), is able to induce degradation of the PRC2 protein complex and reactivate PRC2 repressed genes. A significant limit of this compound lies in the impossibility to discriminate if the associated cellular phenotypes resultant upon treatment are specifically due to EZH2 inhibition since all the other functions of the PRC2 complex are impaired (such as scaffolding and microRNA binding). Recently, highly potent and specific EZH2 inhibitors have been derived from high throughput screening and hits optimization processes. Some of them have shown also increased bioavailability and in vivo

antitumor activity in preclinical studies, and in particular, E7438 has been advanced to human clinical trials. (Helin and Dhanak 2013). In the past few years, among the lysine demethylases, LSD1 is considered as a really promising target for cancer therapy due to advances in understanding its role in tumor progression (Amente, Lania et al. 2013).

LSD1 as a drug target

As previously described, LSD1 is a FAD-dependent oxidoreductase, which demethylates lysine 4 of histone H3. Additionally, LSD1 can act upon lysine 9 of histone H3 when associated with the androgen receptor transcriptional regulation (Garcia-Bassets, Kwon et al. 2007) (Metzger, Wissmann et al. 2005). From the biological point of view, LSD1-depletion causes embryonic lethality in mice (Wang, Hevi et al. 2009). LSD1 involvement in cancer has been elucidated recently, with expression analyses and immunohistochemistry stainings demonstrating increased LSD1 protein/mRNA levels in different types of solid tumors (Amente, Lania et al. 2013). In particular, in prostate cancer LSD1 expression serves as a marker for cancer recurrence (Kahl, Gullotti et al. 2006), in poorly differentiated neuroblastoma tumors it is often found overexpressed (Schulte, Lim et al. 2009) and in non-small cell lung cancer its overexpression promotes invasion, progression and proliferation and is associated with poor prognosis (Amente, Lania et al. 2013). In these cases LSD1 inhibition impairs cell growth and tackle its oncogenicity. Consistent with its role as a tumor promoter, LSD1 activity was proposed to be involved in the epigenetic

reprogramming associated with early phases of EMT transition (McDonald, Wu et al. 2011). Other studies, on the contrary, have proposed LSD1 as a tumor suppressor. Wang and colleagues demonstrated that LSD1 regulates breast cancer cells migration and its expression is down regulated in breast carcinomas (Wang, Zhang et al. 2009). A number of studies have highlighted the involvement of LSD1 in cellular differentiation (Wang, Scully et al. 2007);(Musri, Carmona et al. 2010) and in particular in 2007 Saleque and colleagues proposed that LSD1 can repress GFI1 targets, thus contributing to proper hematopoietic differentiation (Saleque, Kim et al. 2007). Two recent articles clearly proposed LSD1 as an interesting target for AML therapy. By using knock-down and pharmacological approaches, LSD1 targeting, alone or in combination with retinoic acid (RA), resulted in the induction of differentiation and impairment of clonogenic activity in both murine and human primary non-APL AML cells (Schenk, Chen et al. 2012) (Harris, Huang et al. 2012). Structurally, LSD1 has a N-terminal SWIRM domain and an enzymatic domain including both a FAD- and a substrate- binding pocket (Mosammaparast and Shi 2010). The most effective compounds developed as LSD1 inhibitors include those fitting within the catalytic pocket of the enzyme. They derive from a more general monoaminooxidase inhibitor, the tranylcypromine (TCP), already approved for depression-treatment (Lee, Wynder et al. 2006), (Binda, Valente et al. 2010), (Harris, Huang et al. 2012). We have recently developed a novel LSD1 inhibitor, more specific and acting at relatively low concentrations: MC (Binda, Valente et al. 2010. We found that LSD1-inhibition enhances sensitivity of a human cell line derived from an acute promyelocitic leukemia (APL) patient (NB4 cells) to retinoic acid induced growth arrest/differentiation. The mechanism by which LSD1 leads to increased apoptosis and/or differentiation in leukemia models still remains to be discovered.

Previous studies demonstrated that LSD1 associates with several protein complexes among which NURD and CO-REST that are mainly associated with transcriptional repression (Amente, Lania et al. 2013). LSD1 inhibition leads to induction of specific target genes and is associated with an increase of H3K4me2 at respective promoters, but, when associated with androgen- and estrogen- receptor LSD1 seems to act as a coactivator via the demethylation of H3K9me2 a modification generally associated with stable gene repression (Cloos, Christensen et al. 2008). Other studies have demonstrated that LSD1 is able to control transcription by acting as a coactivator. Harris and colleagues (Harris, Huang et al. 2012) link the LSD1 inhibition-dependent H3K4me2 increase to gene repression, thus pointing out a more intricate mechanism of action of LSD1 in the transcription regulation in cancer cells (Harris, Huang et al. 2012).

Hematopoiesis

The hematopoietic system shows a strictly regulated hierarchy, where all blood cell lineages derive from the hematopoietic stem cells (HSC) (Orkin and Zon 2008). The entire process of hematopoietic differentiation consists in the progressive formation, starting from hematopoietic stem cell, of several intermediate progenitor cells (such as the common lymphoid progenitor (CLP) and the common myeloid progenitor (CMP) that in turn will generate mature hematopoietic populations including B, T and natural killer (NK) cells (lymphoid lineage), granulocytes, megakaryocytes, platelets, macrophages and erythrocytes (myeloid lineage) (Orkin and Zon 2008); (Rice, Hormaeche et al. 2007). Hematopoietic stem cells are able to self renew in order to guarantee the appropriate replenishment of the relatively short living mature cells

throughout the organism's entire life (Orkin and Zon 2008). The decision between differentiation and self renewal of stem- and- progenitor cells is dependent upon the outcome of the highly regulated interplay of several molecular determinants, such as external stimuli and transcription factors (TFs) activity. Some TFs are essential for maintenance of the stem cell compartment such as SCL/TAL1, LMO2, SET-domain-containing proteins and the RUNX1 family (Kim and Bresnick 2007); (Orkin 2000) while others are considered to be more "lineage specific" including PU.1, C/EBPa and GFI-1. GATA1 is necessary for erythroid lineage specification, while PU.1 is expressed in HSC, multipotent progenitors, differentiated B cells and is required for myeloid differentiation (Rice, Hormaeche et al. 2007). The PU.1 expression pattern suggests that the distinction between "stem cell- and lineage-restricted" TFs is not so strict. Indeed, the same TF can play a role both in early and in late phases of hematopoietic development as well as in multiple lineages (Orkin and Zon 2008); (Rice, Hormaeche et al. 2007); (Scott, Simon et al. 1994). TFs often cooperate with one another and with chromatin modifiers in order to control epigenetic patterns within gene regulatory regions and thus fine-tune the transcription of their target genes. For example, during erythroid maturation GATA1 directly recruits protein complexes containing HAT activity to the b-globin locus thus facilitating globin transcription (Scott, Simon et al. 1994). The majority of TFs and chromatin modifiers having a role in hematopoiesis are involved with somatic mutations and chromosomal translocations in hematopoietic diseases, including several types of leukemia (Rice, Hormaeche et al. 2007).

Acute promyelocytic leukemia

Acute myeloid leukemias (AML) correspond to a group of malignancies characterized by impaired hematopoietic differentiation associated with an expansion of abnormal progenitors (blasts). Blast infiltration is mainly localized to the bone marrow and accompanied often by thrombocytopenia, and anemia. Acute myeloid leukemias very frequently are associated with structural chromosomal alterations, such as deletions, inversions and translocations. Transformed cells within a patient show the same cytogenetic abnormalities supporting the idea that leukemias are indeed clonal diseases and that these lesions correspond to early or initiating events in the pathogenesis. Translocations can induce TFs misregulation by mislocating the gene coding regions in the proximity of regulatory regions normally associated with other genes, e.g., c-myc which is placed under the control of immunoglobulin (Igs) gene enhancers in Burkitt lymphoma and B-cell leukemia. In myeloid leukemias, it is frequently observed that translocations generate chimeric proteins which involve transcription factors and chromatin modifiers (Look 1997). Leukemias are classified according to two systems: the WHO system that includes morphological, immunological, genetic, biologic and clinical information; and the French-American-British (FAB) classification, based mainly on cytological features of the expanding population. Acute promyelocytic leukemia (APL), according to FAB, is included within the M3 subtype (Bennett, Young et al. 1997). APL is characterized by accumulation of progenitors blocked at the promyelocytic state and by the presence of chromosomal translocations involving retinoic acid receptor RAR (Minucci and Pelicci 2007). RAR is fused with genes on different chromosomes such as PLZF,

NPM and STAT5b (Pandolfi 2001). About 95% of APL patient present the translocation t(15:17)(q22;q12). The fusion of these chromosomal branches causes the creation of a chimeric gene formed by the promyelocytic leukemia (PML) gene and retinoic receptor alpha (RARa) gene, resulting in the PML-RAR (PR) protein (de The, Chomienne et al. 1990); (Kakizuka, Miller et al. 1991). The onset of the disease shows constant incidence with age supporting the idea that APL is caused by a single rate limiting genetic alteration (Vickers, Jackson et al. 2000). The cell of origin of APL has not yet been identified, however in humans it has been proposed to be either a T – or myeloid progenitor cell (de The and Chen 2010). PR, in this case, should be able to induce lineage switching or induce self renewal properties within progenitor cells. In vitro studies have demonstrated that PR confers self renewal abilities to murine CMP and GMP progenitors enhancing their colony forming capacity (Welch, Yuan et al. 2011). In mice, the expression of PR leads to APL development, albeit with variable penetrance and after a "pre-leukemia" stage, suggesting other mutations are required for the onset of the disease. For example, a recent work in our lab characterized the dual role of HDAC in APL development. HDAC depletion in the pre-leukemic phase, works as a tumor suppressor by accelerating the disease onset, while HDAC KD post-transformation establishment instead leads to increased survival in APL mice (Santoro, Botrugno et al. 2013)

PML-RAR fusion protein

PML

PML co-localizes with several proteins such as p53, pRb and CREB-binding protein (CBP) (Zhong, Salomoni et al. 2000) in nuclear macromolecular structures called nuclear bodies (NBs). PML, thanks to RING-, zinc finger- and

coiled coil- domains shows a homo-oligomerization ability which is responsible for the formation NBs (Bernardi and Pandolfi 2007). NBs have been suggested to play a role in the cell cycle, the control of apoptosis, senescence, and surveillance of genomic stability (Bernardi and Pandolfi 2007). Studies on the PML cellular function, highlighted its role as a tumor suppressor protein (Salomoni and Pandolfi 2002). PML deficiency in thymocytes, and mouse embryonic fibroblasts induces a proliferative advantage (Salomoni and Pandolfi 2002). In various cell types deriving from PML Knock-out mice it has been observed that there is a protection from FAS ligand-, TNF-, and INFs- dependent cell death. Additionally, PML-deficient mice are more resistant to anti-FAS antibody and to lethal exposure of ionizing radiation (Wang, Ruggero et al. 1998). PML activity is necessary to control p53 dependent and independent apoptotic pathways and RAS-induced senescence (Piazza, Gurrieri et al. 2001).

RARa

Retinoic acid receptors (RARs) belong to the steroid/thyroid nuclear receptor family. There are three isoforms of RARs (RARa, RARb and RARy), RARa alone is involved in APL pathogenesis. RARa functions as a nuclear transcription factor, it recognizes retinoic acid response elements (RARE) constituted by direct repeats of a hexameric sequence spaced by 1 to 5 bp. *In vivo* studies revealed that the normal hematopoietic process is not impaired upon RARs depletion, but that these receptors play a role as regulators at different steps of myelopoiesis (Collins 2002). For instance, RARa and RARy knock out mice present a block in the terminal granulocytic differentiation (Labrecque, Allan et al. 1998). In the absence of retinoic acid (RA), RARa represses its target genes while RARb and RARy, both in the

absence or presence of RA, function as transcriptional activators (Hauksdottir, Farboud et al. 2003). RARa heterodimerizes with retinoid x receptor (RXR) and interacts with co-repressor protein complexes such as N-COR and SMRT thus imparting HDAC activity. When RA binds RARa, it causes the displacement of co-repressors and the recruitment of chromatin modifying complexes that act as co-activators (such as HATs) thereby leading to the transcriptional induction of RAR targets (Drumea, Yang et al. 2008); (Hauksdottir, Farboud et al. 2003)

PML-RAR oncogenic potential

The fusion protein PR contains all the functional domains of both of the parental proteins: it retains the ability to bind DNA and to interact with RXR of RAR and the RING, zinc-finger and coil-coiled domain of PML. PR leads to cell transformation in part by working as a dominant negative counterpart of RARa. The repression of RAR target genes in the presence of PR becomes constitutive since PR is insensitive to physiological concentration of RA. Moreover PR is able to recruit several chromatin modifier proteins (such as, EZH2, HDAC1, SUV39H1, MBD1, DNMTs) that help to repress target gene expression (de The and Chen 2010). Pharmacological doses of RA are needed to release co-repressors and to induce recruitment of co activators, the transcriptional reactivation of the target genes and the proteosomal-dependent degradation of PR. PR oligomerization through the PML coil-coiled domain was demonstrated to be a crucial event for PR mediated transformation. (Minucci, Maccarana et al. 2000). Later, it was discovered that RAR fusion proteins also have oligomerization domains (Kwok, Zeisig et al. 2006) (Licht 2006). The oligomerization of the fusion protein leads to increased affinity for corepressors, partially explaining the resistance to a

physiological RA stimulus. This oligomerization model has been further modified by the discovery of high order interactions between PR and retinoid X receptor (RXR) (Zeisig, Kwok et al. 2007). Two studies in different APL subtypes (one in PR-expressing and the other in STAT5-RAR translocation-positive tumors) demonstrated that x-RAR/RXR heterooligomerization was required for X-RAR fusion's oncogenic activity (Zhu, Nasr et al. 2007) (Zeisig, Kwok et al. 2007). In particular, Zhu and colleague generated PR mutants not able to bind RXR which were able to recapitulate most of the original PR oncogenic features in vitro, but they failed to develop APL in vivo (Minucci and Pelicci Cancer Cell 2007). Recently, our lab has demonstrated that only a PML coiled-coil domain dependent oligomerization of RAR leads to *in vivo* leukomogenesis in a similar extent to PR (Occhionorelli, Santoro et al. 2011). Interestingly, Martens and colleagues performed ChIP-sequencing for PR and RXR in primary APL cells and consequently provided the evidence that PML-RAR/RXR oligomers do interact on a large portion of genomic regions (Martens, Brinkman et al. 2010). Another report characterizing the genome wide distribution of PR, found that PR is recruited to non canonical RAR binding motif thus showing an enlarged pattern of target genes (relatively to RAR) and in particular has been suggested to affect PU.1 dependent transcriptional control (Mikesch, Gronemeyer et al. 2010). PR bindings seem to occur in specific genomic regions characterized by P300 binding, low levels of acetylated histones and chromatin accessibility (Saeed, Logie et al. 2012) and to influence their local epigenetic pattern, mainly acting by reducing acetylation (Martens, Brinkman et al. 2010). PR oncogenic activity is further regulated by a number of PTMs occurring within its different moieties. While the PML part may be sumoylated, the RAR moiety undergoes

phosphorylation. Sumoylation leads to SUMO dependent recruitment of death domain associated protein (DAXX) which modulates apoptosis (de The and Chen 2010). Instead, Serine 369 phosphorylation instead, is important for the recruitment of TFIIH, which indirectly participates in the RA-dependent transcriptional activation of PR targets. PR can also interfere with the physiological PML molecular functions. For instance, in primary APL blasts, PR associates with PML endogenous proteins and leads to the disruption of NBs and delocalization of PML to microspeckles (Rego, Wang et al. 2001; Salomoni and Pandolfi 2002). Indirectly, PML hemizygosity, as a consequence of the translocation, could also contribute to the APL phenotype together with the PR fusion. In support of this hypothesis, it has been shown that in a mouse model of APL, the progressive reduction of PML dosage leads to an increase in the incidence and to an acceleration in the onset of PR induced leukemia. (Rego, Wang et al. 2001)

APL treatment

APL is one of the best-characterized malignancies, and represents the best example of successful treatment with a targeted therapy. Chemotherapy based on anthracycline administration was the first treatment leading to the recovery of some patients. The subsequent prognosis of APL patients greatly improved when RA and the pure form of arsenic trioxide were introduced as APL treatments in 1985 and 1994, respectively. The standard approach for newly diagnosed patients nowadays is represented by RA treatment in combination with anthracycline, and the rate of patients experiencing a complete remission reaches 95% (Sanz and Lo-Coco 2011). RA treatment, in

particular, was introduced before the cloning of the PR fusion protein. The translocation generating PR, as described above, is the only genetic lesion always present in APL blasts (de The and Chen 2010). Pharmacological concentrations of RA induce terminal differentiation of APL cells, PR proteosomal-dependent degradation and re-assembly of NBs (de The and Chen 2010). In some cases, disease relapses and RA alone can successfully cure only a few patients. Indeed, while the standard cure can reach 70% of effectiveness on relapsed patients, combined treatments with both RA and arsenic trioxide, even without chemotherapy, made most patients disease-free (de The and Chen 2010). Molecularly, Arsenic trioxide induces the sumoylation of the PML moiety of PR leading to the proteosomal-dependent degradation of the oncoprotein (Zhang, Yan et al. 2010) and for this reason, it is only effective on PR-positive APL and not in other x-RAR promyelocytic leukemias. A number of APL mouse models were developed to understand the mechanistic basis of the therapeutic response and to possibly generate new therapeutic approaches. PR expression is put under the control of a gene promoter specific for the myeloid compartment (cathepsin G or S100A8) (de Thè and Zu, 2010). There is a now well-established hierarchical organization among the cellular constituents of myeloid leukemia. Thanks to transplantation experiments it has become evident that only particular cell subpopulations are able to reconstitute the disease in secondary recipient mice. These subpopulations harbour leukemia initiating cells (LIC) endowed with self-renewal ability. LICs clearance has been proposed as the final goal to accomplish in order to completely eradicate leukemia. RA has been shown to induce differentiation and, to a different extent, to impair the self-renewal ability of LICs. RA, from the molecular point of view, is able to both activate transcriptional responses and to induce PR-degradation while arsenic trioxide induces PR

degradation and a partial differentiation (de Thè and Zu, 2010). Recent works suggested that the transcriptional activation of PR targets and the PR degradation are uncoupled. The first seems to be mainly associated with the differentiation capacity while the second seems to instead be responsible for the LIC clearance (Nasr, Guillemin et al. 2008) (Ablain, Leiva et al. 2013). Currently, evaluation of the combined therapy RA/arsenic tyroxide as a first line treatment is underway. A recent report compared classical RA/chemotherapy combination with RA/arsenic trioxide treatment in low/to intermediate risk patients and the two combinations seem to show comparable results, when comparing two year event free survival rates (Lo-Coco, Avvisati et al. 2013). Other alternative therapies are also under investigation, since in leukemogenesis, epigenetic enzymes and histone modifications importantly contribute to the malignant phenotype (Chen, Odenike et al. 2010), epigenetic targets are being used to develop new compounds that, alone or in combination, can be added to the standard therapy of APL (Mercurio, Minucci et al. 2010). Preclinical data have demonstrated that HDACis induce differentiation of both APL RA-sensitive and -insensitive cell lines and effectively yielded *in vivo* remission in RA-resistant APL mouse models (Minucci and Pelicci 2006). In the clinics, RA resistance emerges upon first line treatment and relapsing patient were then effectively treated with sodium phenylbutyrate which was able to restore RA responsiveness (Chen, Odenike et al. 2010). Although, Phenylbutyrate behaves as a mild HDAC inhibitors, more potent compounds, such as hydroxamic acid, cyclic tetrapeptides and benzamides, were developed and have been tested in clinical settings (Chen, Odenike et al. 2010).

Materials and Methods

Cell culture

NB4 cells, isolated from an APL patient by Lanotte and colleagues, have characteristics similar to APL blasts (Lanotte et al., Blood 1999). NB4 cells were grown in RPMI plus 10% of (FCS) fetal calf serum, 2mM glutamine and 1% Penicillin/Streptomycin.

U937(PR9 clone) was previously generated in our laboratory by stable transfection of PML/RARα cDNA cloned in the Zinc2+-inducible PINCO plasmid vector as already described (Grignani et al., 1996). PR9 cells were grown in RPMI plus 10% of (FCS) fetal calf serum, 2mM glutamine and 1% Penicillin/Streptomycin.

Phoenix-AMPHO are human kidney cells used for the generation of helper-free amphotropic retroviruses. Phoenix-AMPHO were grown in DMEM plus FBS 10% 2mM glutamine and 1% Penicillin/Streptomycin. These cells were used to generate LMP retroviral vectors carrying LSD1 interfering sequences.

Treatments: NB4 cells were plated at 100.000/ml, treated with dimethyl sulfoxide (DMSO) 1/1000; with 2μM of LSD1 inhibitor (MC) for 6, 12, 24 or 96h; with RA 0.01μM (RA LOW) 24 or 96h; with RA 1μM (RA HIGH) for 24 or 96h. RA was supplied by Sigma.

PR9 cells were induced with Zn [100 μM] for 8h and the induction of the PR expression was tested by western blot

Morphological characterization

Cytospin preparations obtained from 200.000 cells per sample were stained for 8 minutes with May-grunwald solution, washed 6 times in deionized water and then incubated for 30 min with Giemsa. After three more washes, samples were air dried and evaluated. This method of coloration represents the most common way to stain blood cells. The two solutions contain a basic dye (methylene blue) and an acid dye (eosin). The first carrying a basic net positive charges stain nuclei because of the negative charges of phosphate groups of DNA and RNA molecules, basophil granules and RNA molecules within the cytoplasm. The eosin carries a net negative charge and stains red blood cells and granules of eosinophil granulocytes. We used this kind of coloration to assess morphological changes associated with differentiation.

Interfering vectors

To interfere with LSD1 we tested 4 shRNAmir sequences. We inserted interfering sequences onto the LMP vector by XHO-ECORI double digestion. shRNAmir transcription in these vectors is RNA Polymerase II (Pol II) mediated and is under the control of an LTR promoter and expresses puromycine resistance cassette. LTR is a strong promoter able to drive highly effective knockdown of a target gene, even when integrated in single copy. The obtained plasmids were used to produce retroviruses and to infect NB4 cells

Calcium phosphate transfection

Phoenix-AMPHO Cells are plated at 50% confluence the day before transfection. The next day two solutions were prepared, mixed by bubbling and added to the cell plates. Solution A: PCAT plasmid (5 **µ**g/10 cm plate), DNA (10 µg/10 cm plate), 61 µl CaCl2 and water in a final Volume of 500 µl; Solution B: 500 µl of 2x Hepes-buffered solution (HBS) 15-20 minutes after mixing they are distributed on the cells. Cells are left at 37°C and after 12-16 hours are replenished with fresh medium. 24 hours and 48h later medium containing the viral supernatant was collected/substituted, filtered and stored at 4°C until PEG viral concentration (1 day later).

Viral concentration

Viral concentration was performed by using a 5x PEG solution (System Bioscience).
1/5 of volume was added to supernatants containing retroviral particles and centrifuged at 1500 x g at 4°C for 30min. The pellet was stored in PBS at -80°C .

Spin Infection of NB4 cells

Cells were put in 24 multiwell plates and plated at a density 500.000 cells in 500 µl of medium per well. Virus was diluted in RPMI 10% serum pen/strep in order to add 500 µl to the cells. Three cycles of infection were performed. Each cycle included a 45min centrifuge at 1800 rpm and replacement of the old medium with fresh medium + virus particles. At the end of the three cycles, the medium with viral particles was removed and substituted with virus-free medium. 24h after infection puromycin

selection (2 µg/ml) was performed and resulting in the killing of non-infected cells within 48 hours. At this time, cells were then treated with different concentration of RA.

Western blot analysis

Cells were counted and directly lysed in 2X SDS Laemmli buffer (50mM Tris HCl, 10% glycerol, 2% SDS and water) plus protease inhibitor cocktail. Cell lysate was then sonicated by Bioruptor (Diagenode) for 10min. After sonication, samples were centrifuged for 15 min at 4°C, 13000 rpm. Lysates were quantified by colorimetric methods and protein concentration was assessed as direct function of the 595nm absorbance. 80 µg of proteins were mixed with Laemmli (b-mercaptoethanol and bromophenol blue) and denaturated for 8 min at 95°C. Cell lysates were loaded onto an 7.5% polyacrylamide gel and run in SDS Running Buffer. Transfer to nitrocellulose membranes was performed at 100V for 1 hour at 4°C or over night at 30V in Transfer Buffer containing 20% methanol, After a brief wash in water, membranes were blocked in 10% milk/ TBS-Tween for 1 hour at room temperature or over night at 4°C and then probed with primary antibodies diluted in TBS-Tween + 5% milk at 4°C for 2h or over night. After three washes with TBS-Tween (5 minutes each), membranes were incubated with the proper secondary antibody in TBS-Tween+5 % milk for 30 min at room temperature. After 3 more washes, signals were revealed using the ECL (Enhanced Chemiluminescence) method.

Antibodies used for WB:

LSD1 Antibody Cell signalling #2139, RAR santa cruz sc-551

Tubulin T8328 Sigma

ChIP- qPCR/Seq

Cells were cross-linked in culture medium with 1% formaldehyde in PBS and the reaction was stopped after 10 min at RT by adding 0.125 M glycine for 5 min at 4°C. The cells were washed twice with PBS and collected by centrifugation. Pellets were stored at -80° in SDS buffer (50 mM Tris•HCl pH 8.1, 0.33% SDS, 150mM NaCl, 5 mM EDTA, and protease inhibitor cocktail) or directly processed. Fixed cells were resuspended in IP buffer (100mM tris ph 8.6 0.3% SDS 1.7% TRITON x-100 and 5mM EDTA). Chromatin was then fragmented to obtain ~300 bp in length by using a Branson Sonifier 250. Chromatin pre-clearing was obtained with protein A-sepharose beads (Amersham). Then, the supernatant was immunoprecipitated o.n in the presence of 30-50 microL of protein G magnetic beads. For histone modification 1 ml corresponding to $3x10^6$ cells per each IP and 4ug/ml primary antibody were used; for LSD1/PML Chip-Seq $40x10^6$ cells per each IP; 10ug/ml. Before IP 2.5% of input was stored at 4% prior to the decrosslinking procedure. Decrosslinking was performed for all the IP samples and corresponding inputs, o.n in 0.1%SDS and 0.1% NaCOH3. The day after, the enriched DNA was treated with proteinase K at 56°C for 40 min and purified with a DNA purification kit (Qiagen). The obtained DNA was then quantified by picogreen (see below) and 5-10ng were processed for ChIP-Seq library preparation (as described for the Illumina protocol) or used for quantitative real-time PCR (qPCR) as follows.

For the validation of specific regions, ChIP-qPCR were performed as follows. Immunoprecipitated DNA was diluted in 9,6**µ**l of H_2O per reaction, plus 400 nM primers in a final volume of 20µl in SYBR Green. Each ChIP experiment was performed at least three times with biological replicates. For ChiP-Seq the immuno

precipitated DNA was quantified by picogreen (see below).

Ab used for ChIP qPCR/Seq: LSD1 17721 abcam; H3K4me2 32356 abcam; PML sc-5621; Santacruz, IgG sc-2027

PicoGreen(pcg) quantification of ChIP DNA.

This protocol was established with a Glomax fluorometer. This allows for the quantification of as few as 25pg/mL of dsDNA. PicoGreen 2x solution was prepared by diluting 200x stock in TE (final volume 200μL). A standard DNA curve was performed by using genomic DNA as a reference. ChIP DNA generally has a low concentration, therefore standard DNA dilutions should range from 25pg/ml up to 25ng/ml. We prepared a reference sample of 2μg/ml of genomic DNA, which was then diluted 40x in TE this stock to get a final 50ng/ml solution. This is further diluted ½ and then by serial dilution you may obtain 1/10-1/100-1/1000 the last of which corresponds to a 25pg/ml concentration. Briefly, we routinely took 2μl from ChIP samples, brought the volume to 200μL TE - to have a replica - including total control and mock. We then added 100 μL/ml of pcg to each well and incubated 2-5 minutes, mixed and further incubated for 2 to 5 minutes at RT, protected from light. After reading, we plot a low-range standard curve corrected againist the reagent blank fluorescence value.

ChIP-western

In order to clearly assess if the LSD1 antibody worked properly in our ChIP conditions we performed ChIP western keeping similar experimental conditions for

our Chromatin IP- qPCR/Seq protocol. IP was performed as for the ChiP-qPCR and ChIP-Seq protocol. Briefly, the 60 x 10^6 crosslinked cells were lysed in SDS buffer (2ml) resuspended in IP buffer (3-3.5 ml), sonicated to reach 300bp in length bulk. Samples were precleared with protein A-sepharose beads (Amersham). IP was performed with the chromatin recovered from 20 x 10^6 cells and two different quantities of Ab were used (5μg and 10 μg) while 5% of the input was kept at 4°C. The day after, the same percentage of Input was recovered for the unbound fraction /5%). IP samples were washed and beads with enriched chromatin fractions, IPs, unbound fractions and inputs were decrossilinked directly in Laemmli buffer (2X) and loaded onto an SDS page 7.5%. The membrane was then processed as described above (see Western blot)

Ab used: LSD1 17721 abcam; IgG sc-2027 Santacruz

RNA extraction and qPCR

Total RNA was extracted from NB4 cells with Trizol reagent (Invitrogen) and then purified using the RNeasy kit (Quiagen). To remove residual genomic DNA, on column DNAseI digestion was performed (Quiagen Kit). Reverse transcription was performed with Superscript II Kit (Invitrogen), according to the manufacturer's protocol. qPCR were performed in triplicates in 20 μL of final reaction volume containing SYBR green buffer (Applied Biosystems), 20 ng of cDNA retrotrancribed from the RNA, and 0.4μM of each primer mix. All the qPCR amplifications were performed in the AB-7000 sequence detection system (Applied Biosystem) at 50°C for 2 minutes 95°C for 10 min followed by 40 cycles at 95°C.

RNA-seq protocol

mRNA-seq was performed according to the True-seq Low sample protocol selecting only polyadenylated transcripts. In brief, before starting mRNA isolation and library preparations the integrity of the total RNA was evaluated by running samples on a Bioanalyzer. Then, starting from 1μg of RNA isolated as described above (see RNA extraction), poly-T oligos attached to magnetic beads were used to purify mRNAs. Two rounds of isolation were performed by denaturing and letting the RNA bind to poly-T oligos conjugated to the beads. After the second round, RNA was fragmented and primed with random hexamers. Immediately after, we proceeded with the first strand synthesis by using Superscript II reverse transcriptase and random primers. The subsequent second strand synthesis led to the elimination of RNA molecules and the formation of cDNA. The subsequent isolation of the cDNA was achieved by using AMPure XP beads (depending on the concentration used, these beads can efficiently recover PCR products of different sizes). The product recovered contained overhanging strands of various length due to the fragmentation procedure. Thanks to the 3'-5' exonuclease activity and the polymerase activity the cDNA ends were efficiently converted into blunt ends. The following steps follow the classical protocol for Illumina library preparations: adenylation of the 3' ends plus adapter ligation was then followed by a short amplification step to enrich the DNA fragments.

ChIP- Seq analysis

Raw data corresponding to reads coming from Illumina Genome Analyizer II were analyzed according to the Fish the ChIPs pipeline (Barozzi et al., 2011). The automated procedure includes alignment to Human NCBI36/hg18. To exclude PCR

reads coming from library production, only sequences showing unique alignment were used for peak calling, allowing for a maximum of two mismatches. MACS (Zhang et 2008) was used for peaks detection. For LSD1 ChiP-Seq in NB4 cells according to our validation analysis we set a stringent threshold; pvalue ≤ 0.017, while for the all other ChIP-seq p-value ≤ 0.05. The reads from each sample were normalized to the input of the corresponding cell line. For the detection of IMRs or for the changes in the LSD1 recruitment the signals were normalized ChIP vs ChIP and only regions that were significantly called versus the input were retained. Peaks were assigned to Refseq annotated genes according to GIN (Cesaroni et al., 2008). Intergenic regions were considered as having more than 22kb of distance from the nearest gene. Genome tracks were generated using MACS and normalized to the same sequencing depth.

Overlapping peaks were calculated with Galaxy software and were considered as regions sharing at least 1 bp between the two datasets.

Heatmaps for IMRs PR/LSD1 specific were obtained comparing -10*LOG10(pvalue) for each region significantly enriched vs the DMSO.

TFBS enrichment analysis

We used PSCAN for the analysis of gene promoters bound by LSD1. This tool has been developed to scan promoters of a given set of genes to find over-represented and under-represented TFBS. CLOVER instead calculates p-value of enriched TFBS within input sequences generating empirical controls. In detail, Clover scans for similarities between the input sequence and a list of Matrix from the Jaspar database and experimentally determines the relevant score.

Gene expression Analysis

RNA-seq analysis was performed by using the Cufflinks algorithm (Trapnell et al. 2010), Cufflinks is able to re-assemble transcripts to give a quantitative estimation of their presence and to calculate differential gene regulation among several samples. We decide to adopt a 36 bp paired end sequencing strategy, in order to have a high resolution and to also detect relatively low expressed genes. The number of reads obtained were comparable among the 5 samples.

Cufflinks does not restrict by prior gene annotation and is able to also detect new splice variants or alternative TSS. In detail, the first step of the alignment process consists in creating a set of candidate alternative (splice or TSS) transcripts (mutually incompatible transcripts) by a series of sequential overlaps among all the reads coming from the sequencing. Once basal transcripts were defined, reads were realigned to quantify the relative abundance of each splice or TSS variants. The value that we considered to quantify the relative expression of a given gene corresponds to the number of reads aligned per kilobases of the transcript per million mappable fragments detected, namely FPKM (fragments per kilobase of exon per million fragments mapped). This value has also been used for comparative analysis: "same gene different samples", "same sample different genes". The heatmap includes all the genes that were found to be modulated at least in one sample. The threshold set to consider a gene as being regulated was set at FDR ≤ 0.05 Fold Change ≥ 2 fold if not otherwise indicated.

AIMS

We have characterized a novel LSD1 inhibitor showing high specificity and acting at relatively low concentrations (we will refer to this as ”MC”) (Binda, Valente et al. 2010). By taking advantage of this inhibitor we have been able to investigate the mechanistic role of LSD1 in APL. As a model system we used NB4 cells, which are derived from an APL patient and recapitulate numerous characteristics of APL blasts (Lanotte, Martin-Thouvenin et al. 1991) (Saeed et al., 2012). NB4 cells express PML-RAR (PR) and they are insensitive to physiological RA concentrations (0.01μM, defined as “RA LOW”). Instead, treatment with pharmacological concentrations of RA (1μM, defined as “RA HIGH”) causes cell growth arrest and terminal differentiation. The rational behind the RA based therapy is that pharmacological doses of RA can overcome the cellular differentiation block, induce PR degradation and reactivate PR repressed genes (Chen, Odenike et al. 2010). My thesis started from the initial observation that LSD1 inhibition induces sensitivity of NB4 cells to physiological concentration of RA and impairs cell growth driving cells through differentiation (Binda, Valente et al. 2010). LSD1 is mainly involved in transcriptional regulation, in part by modifying the chromatin surrounding regulatory regions (Cloos, Christensen et al. 2008) (Whyte, Bilodeau et al. 2012). Therefore, to investigate the molecular mechanisms and the underlying dynamics of APL cell sensitization we performed a genome wide profiling of both transcriptional and epigenetic changes accompanying LSD1 inhibition-primed cell differentiation. Moreover, since the current model by which PR represses its target genes involves the cooperation with several chromatin modifiers, some of which were also shown to

interact with LSD1 in other systems, (Minucci and Pelicci 2006) (Mosammaparast and Shi 2010) we investigated the possible direct interplay between LSD1 and PR.

Taken together, our studies aimed to:

- Further validate the role of LSD1 in differentiation of APL;
- Identify the transcriptional networks involved;
- Define the molecular mechanisms underlying the differentiation block mediated by the activity of LSD1, and the interplay of LSD1 with the oncoprotein PR.

Overall, our studies should provide several novel information regarding the role of LSD1 in APL, and hopefully these findings could be extended to other forms of cancer.

RESULTS

Inhibition of LSD1 mimics its depletion and has a similar effect on cell growth of APL cells

We demonstrated that LSD1 inhibition sensitizes NB4 cells to RA treatment and induces cell growth arrest and differentiation when combined with a physiological concentration of RA (RA LOW) (Binda, Valente et al. 2010). In order to assess whether the effects of the inhibitor were specifically due to its action on LSD1, we decided to deplete LSD1 protein levels by a retroviral based knock down strategy. We tested 4 sequences (Figure 1) in order to knock down LSD1, three of them effectively reduce the protein levels with LSD1 KD vector #5 showing the best interfering ability. Then, we verified whether LSD1 depletion affects the RA induced growth arrest. The untreated LSD1 KD cells do not show significant differences in cell growth in comparison with control cells (NI and scramble), while all these three LSD1 interfering sequences enhance the sensitivity of NB4 to RA treatment. In particular, NB4 cells become sensitive to a physiological concentration of retinoic acid (RA LOW) (Figure 1) suggesting that LSD1 depletion effectively mimics the effect of LSD1 inhibition, thus confirming a direct role of LSD1 in RA sensitization.

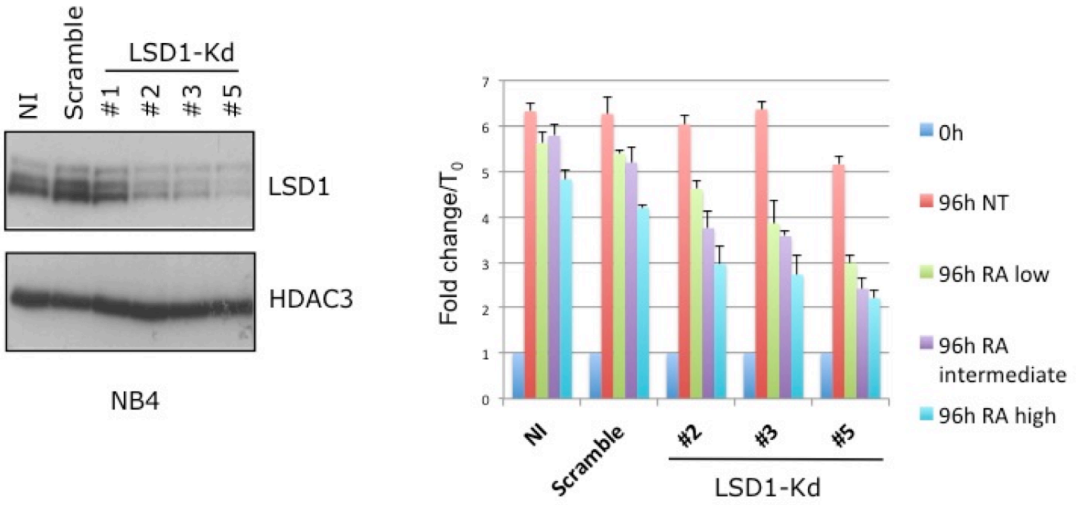


Figure 1: LSD1 KD recapitulates LSD1 inhibition induced effect on growth arrest in NB4 cells. Western blot on extracts derived from NB4 cells infected with scramble and four different LSD1-interfering retroviral vectors. NI: not infected cells. HDAC3 has been used as a loading control. On the right, Cell growth of NB4 cells infected with scramble and LSD1-interfering expressing vectors. Cells after puromycin selection were treated with RA at different concentrations: RA LOW (0.01 μM), RA intermediate (0.1μM), RA HIGH (1μM). Error bars represent SD of two independent experiments.

A transient wave of LSD1 inhibition is sufficient to induce APL cell sensitivity to physiological RA concentrations

We wanted to dissect the molecular mechanism underlying the LSD1 contribution to APL cell maintenance. In order to understand the temporal window in which LSD1 dependent regulation is determinant for APL cell growth arrest, we measured different durations of treatment of NB4 cells with MC. We performed wash out experiments, by removing the LSD1 inhibitor after different intervals of time, while the RA LOW was kept continuously in the medium. As observed previously, MC

alone and RA LOW treatment induces only a mild response. We confirmed that the co-treatment with MC and RA LOW induces cell growth arrest, and interestingly we found that 24h of MC treatment in the presence of RA LOW mimics the growth arrest observed upon 96h of continuous co-treatment (see Figure 2). Thus, inhibiting LSD1 activity for 24h is sufficient to sensitize NB4 cells to physiological RA concentrations (Figure 2), suggesting that most of the LSD1 dependent regulation occurs in the first 24h interval of time. For this reason, we selected the 24h timepoint as the treatment condition for the subsequent studies (see below).

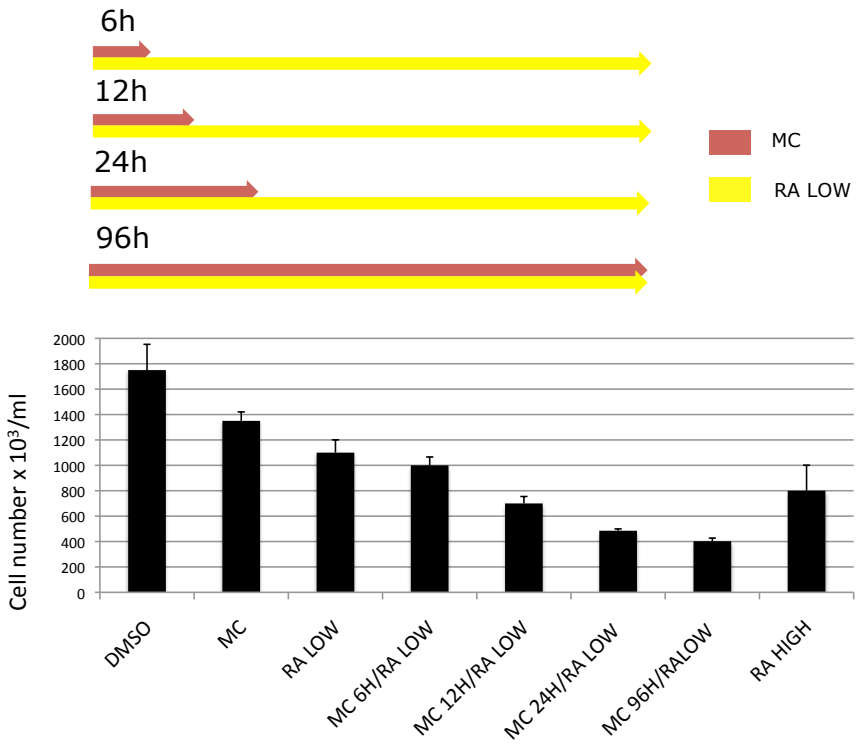


Figure 2: Schematic representation of the wash out experiments and NB4 cell growth evaluation upon LSD1 inhibition.

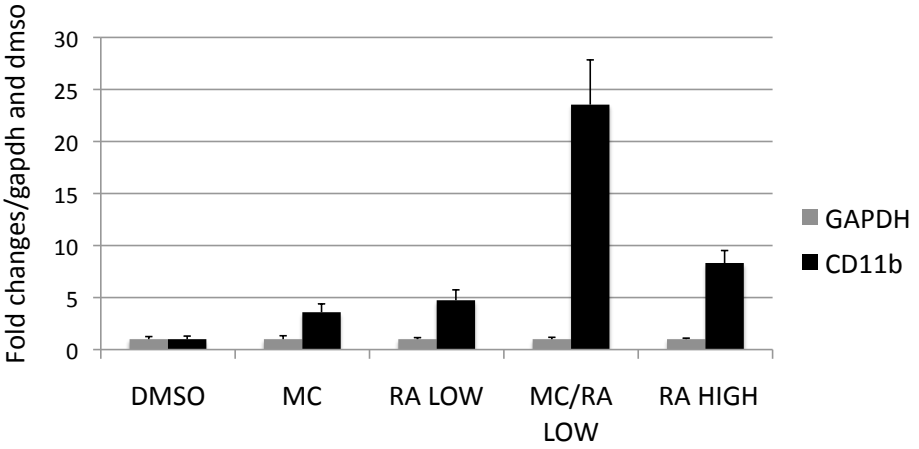
NB4 cells were co-treated with RA LOW (0.01µM) for 96h and MC 2µM for 6h, 12h and 24h, or as a positive control with RA HIGH (1µM) alone. MC was used at 2 µM, the concentration in which it has been shown to reach the maximum target modulation (Binda, Valente et al. 2010). NB4 cells were plated at 100.000/ml and counted at 96h. Error bars represent SD of four independent experiments.

Differentiation accompanies LSD1 inhibition primed growth arrest

Since prolonged co-treatment with MC and physiological doses of RA (96hours of continuous co-treatment) leads to differentiation, we wanted to assess if the LSD1 primed (24h of MC treatment plus 96h of RA LOW) growth arrest was associated with cell differentiation. Differentiation is accompanied by the expression of surface markers and morphological changes. In particular, APL blasts upon differentiating stimuli express neutrophil surface markers and acquire granulocyte-like morphology. Main features of granulocytic differentiation include: reduced nucleus/cytoplasm ratio, round cellular shape substituted by an irregular morphology and the formation of cytoplasmic granules. We decided to characterize morphological changes and expression of a differentiation marker on the following samples: cells treated with dimethyl sulfoxide (DMSO), cells treated with LSD1 inhibitor alone (MC), cells treated with RA 0.01μM alone (RA LOW), cells treated for 24h with MC in presence of RA LOW for 96h (from now on, MC/RA LOW), and cells treated for 96h with RA 1μM (RA HIGH).

We, therefore, first checked the induction of the expression of CD11b (Figure 3). As shown in the qPCR histograms, both RA LOW and MC exhibit a significant effect on the expression of this marker and the combination of the two agents causes a stronger induction of CD11b expression. We next assessed appearance of differentiation morphological features by eosine/methylene blue staining (Figure 3). LSD1 inhibition alone is not able to induce any strong morphological change, as well as only a modest increase of cytoplasmic fraction was observed in RA LOW treated cells. As expected,

cells treated with RA 1μM (RA HIGH) showed lower a ratio between nucleus and cytoplasm compared with control cells and some pink/white granule appeared. Interestingly, MC/RA LOW co-treated cells were even more differentiated. Indeed, MC/RA LOW co-treated cells present: irregular shapes, diffuse granules formation and a partial acquirement of a horseshoe shaped nucleus. All the above evidence demonstrated a clear cooperative effect between the LSD1 inhibition and the physiological RA stimulus in inducing differentiation and growth arrest of APL cells, even more pronounced than that induced by pharmacological RA doses.



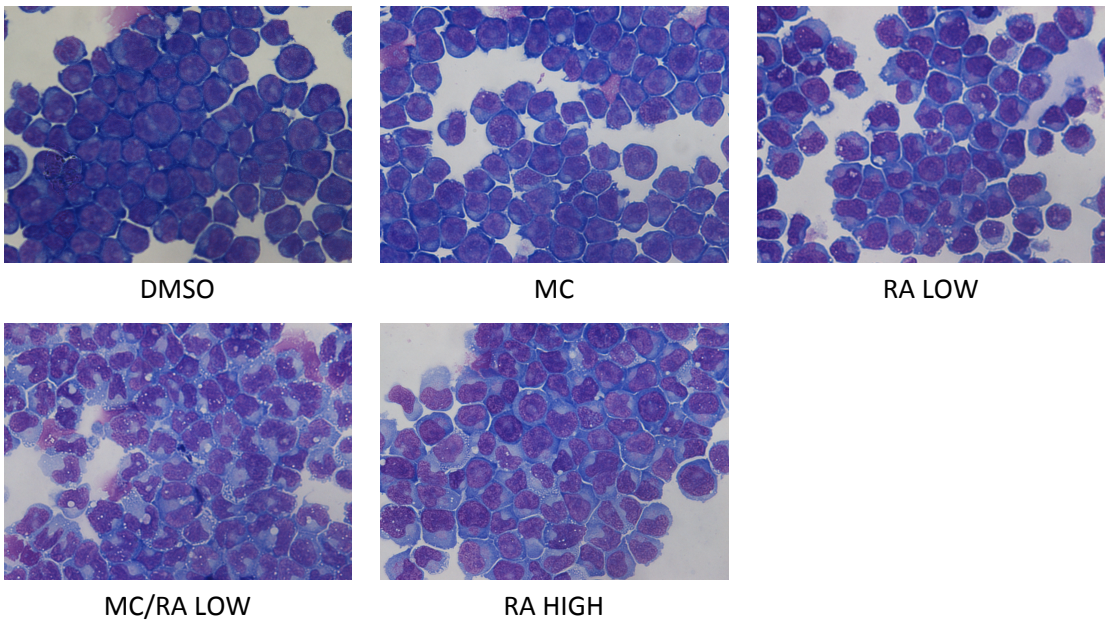


Figure 3: Effects on cellular differentiation upon LSD1 inhibition. RNA extracts from NB4 cells after 24h were retro-transcribed and analyzed by qPCR: fold changes normalized versus DMSO and *gapdh* (used as housekeeping) are showed. Error bars represent SD of two independent experiments. Cytospin of NB4 cells after 96h of each treatment were stained with May-Grunwald and Giemsa. RA LOW and MC treated cells showed modest differentiation while MC/RA LOW and RA HIGH (RA 1μM) treated cells exhibited stronger differentiation-associated morphological changes.

LSD1 genomic distribution

LSD1 binds both TSS-proximal and TSS-distal regions in NB4 cells

Since LSD1 mainly functions in multiprotein chromatin modifying complexes that regulate gene expression, we decided to assess the genomic distribution of LSD1 and to correlate the obtained results with the transcriptomic profiling and with the histone modifications associated with its enzymatic activity. Firstly, we characterized the specificity of the LSD1 antibody by ChIP-western to perform ChIP-Seq experiments. The selected antibody was able to immunoprecipitate LSD1 endogenous protein in a dosage-dependent manner, as shown in Figure 4. Thus, we performed ChIP-Seq for LSD1 in untreated NB4 cells. We performed q-PCR validation for several regions (see Figure 5) and set a threshold to consider a peak validated at –log10 p-value $\geq$ 169. LSD1 binds 15,188 regions and more than 45% of them are proximal to the TSS (+/- 2500bp) of annotated genes and about 35% of LSD1 peaks lay in intergenic and intronic regions, possibly representing enhancer regions (Figure 4), consistent with other findings in mESC (Whyte, Bilodeau et al. 2012).

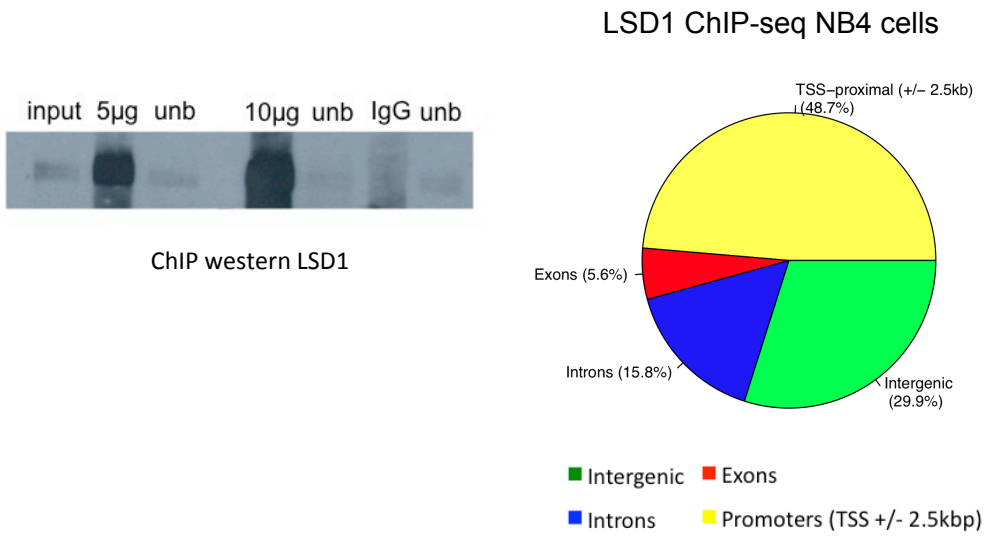
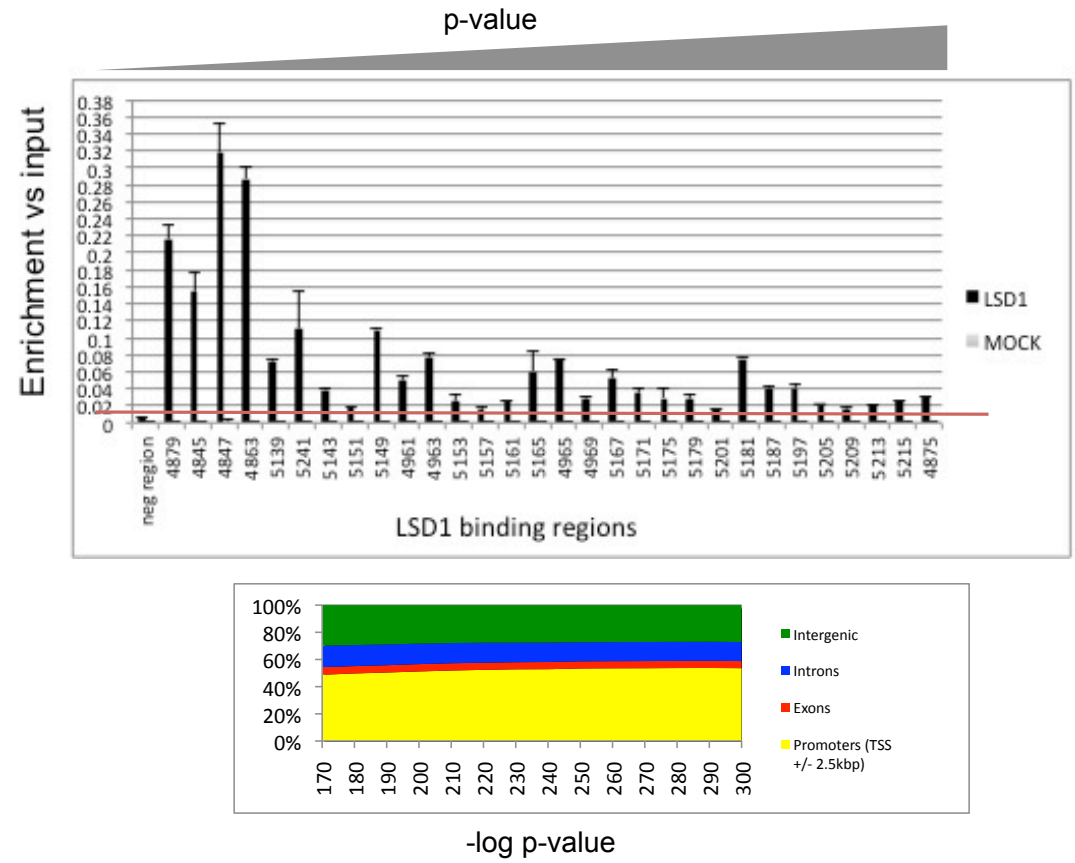


Figure 4: LSD1 ChIP western and LSD1 genomic distribution. A) ChIP western: 20 x 10^6 NB4 cells per each samples were crosslinked with 1% formaldeyde and lysed (see methods). Chromatin was sonicated, precleared and samples were immunoprecipitated o.n. at 4°c with 5 and 10 µg of an anti-LSD1 antibody. The input represent 5% of the total lysate. The tested antibody is able to immunoprecipitate the target protein in a dose-dependent manner. LSD1 ChIP-Seq was performed on NB4 untreated cells, Raw sequencing data were analyzed as described in Barozzi et al. (see methods) LSD1 binds mostly gene promoters and a significant portion of intergenic regions. Regions spanning around the TSS (+/- 2.5Kb) were considered as promoters. Intergenic regions correspond to regions more than 22kb distant from the nearest gene.

We validated several genomic regions for the binding of LSD1 at different p-values by q-PCR assays and 26/30 regions tested were considered validated, corresponding to 86%. We also highlighted that the proportion of the LSD1 distribution between promoters and putative enhancer does not change between highly enriched peaks and the rest of the binding sites. Interestingly, among the LSD1 binding regions we noticed *Cd11b* and other genes important for myeloid differentiation such as *Cebpe* (Figure 5).



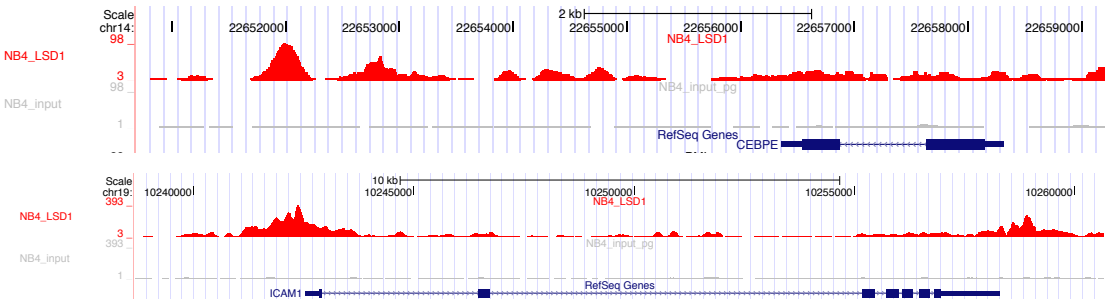


Figure 5: LSD1 ChIP-Seq validation. Validation by ChIP-qPCR assay of LSD1 positive regions at several p-values in three independent experiments. The first column represents an intergenic region negative for LSD1 binding; anti-IgG antibody was used as mock control. Enrichment of the negative region plus three times the relative SD was set as threshold: 26/30 regions were considered as validated. Genomic distribution of LSD1 peaks in NB4 cells. The two screenshots represent the LSD1 target genes, *Cebpe* and *Icam1*:

LSD1 binds at promoter regions of genes potentially regulated by transcription factors (TFs) required for hematopoietic differentiation

Given the potentiation of differentiation observed upon treatment with MC; one prediction would be that LSD1 is involved in controlling the expression of genes required for hematopoietic differentiation. In order to characterize the LSD1 bound genes, we performed TF binding sites enrichment analysis on the LSD1 bound regions in NB4 cells. LSD1 has been shown to cooperate with several TFs, such as GFI1/1B, TAL1, SALL4, to regulate transcription of some specific genes in the hematopoietic system (Saleque, Kim et al. 2007); (Li, Deng et al. 2012); Hu et al., PNAS 2009). We scanned promoter regions of LSD1 bound genes using the PSCAN algorithm (Zambelli, Pesole et al. 2009) in order to find over-represented transcription factor binding motifs. LSD1 bound promoters showed an enrichment of a large number of transcription factors, in particular, SPI1, EGR1, E2F1-3 (Figure 6). Interestingly, these TFs play a role in granulocytic/monocytic differentiation. Moreover, among the significant enriched position weight matrices we also found REST, a known LSD1 recruiter (Mosammaparast and Shi 2010) and the canonical PML-RAR binding sequences (RAR:RXR DR5), suggesting that LSD1 can also interact with the oncogenic fusion protein characteristic of APL (see below). This strongly suggests that LSD1 binds genes probably involved in terminal differentiation of APL cells, and that it can interplay with a number of hematopoietic related TFs.

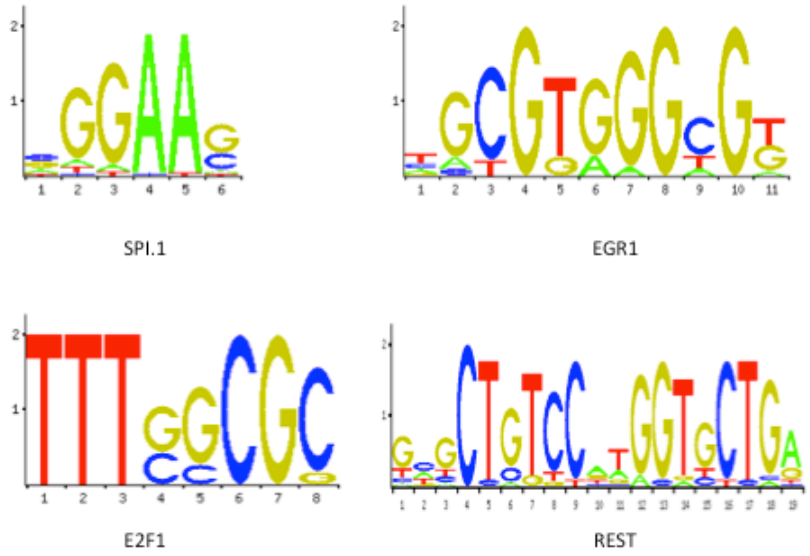


Figure 6: PSCAN analysis on LSD1 bound promoters reveals enrichment for hematopoietic-related TF. This bioinformatic tool scans TFs binding matrixes enriched within the promoter of a given gene list and each matrix is ranked according to its p-value. In particular, all the TFs cited were represented by more than one matrix. Here we report the best scoring matrix for each TF. A0080.1 SPI1 (p-value 3.74316E-68); EGR1 with BU0010 Egr1_primary (p-value 1.01E-206); BU0009 E2F3_primary (p-value 1.4124E-185); BU0008 E2F2_primary (p-value 4.82E-182); MA0024.1 E2F1 (p-value 3.98452E-37). MA0138.1 REST (p-value 7.08501E-46) MA0159.1 RXR::RAR_DR5 (p-value 5.12432E-07).

LSD1 dependent transcriptional regulation during APL cell differentiation upon treatment with physiological concentrations of RA

We then investigated the consequences of LSD1 inhibition and RA treatments on the transcription program of APL cells. We performed an RNA-Seq analysis on cells treated with MC alone, with RA LOW, cells co-treated with MC/RA LOW and cells treated with RA HIGH, compared to untreated control cells –DMSO- (see figure 2).

Given the results of the wash-out experiments, we selected 24h as the optimal time point to obtain transcriptional information.

LSD1 regulates highly expressed genes

LSD1 has been generally associated with gene repression, so we first assessed if LSD1 bound genes were relatively less expressed than all the others. Unexpectedly, we found that LSD1 bound genes are more expressed when compared with the LSD1 unbound ones. Moreover, we observed a strong overlap between LSD1 and POLII binding in NB4 cells (Figure 7).

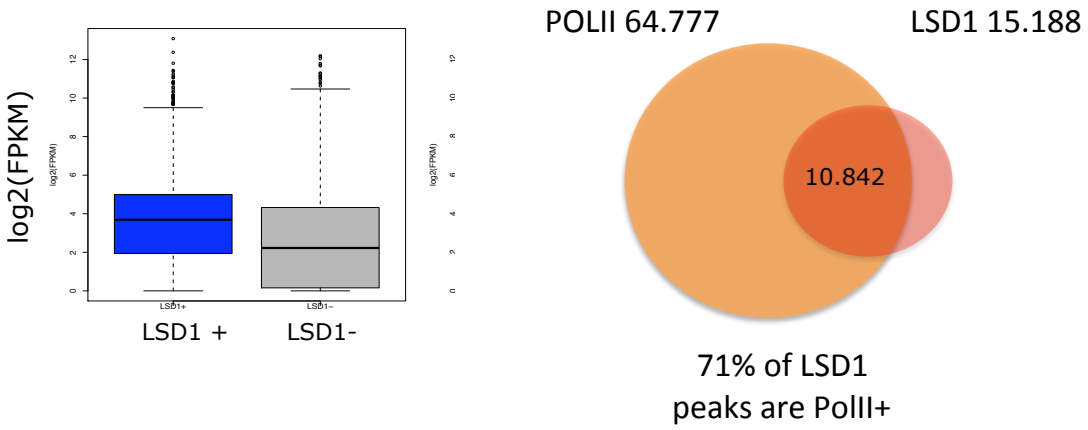


Figure 7: LSD1 bound genes are more expressed than LSD1 negative ones. The RNA was prepared from NB4 cell extracts and mRNA-seq was performed according to Illumina True-seq technology selecting poly-adenylated transcripts (see methods). The box plot shows the median of log2(FPKM), FPKM correspond to the relative number of tags of a given transcript obtained in paired end mRNA-seq (see methods for full explanation). Expression levels of LSD1 bound genes (LSD1 peak within 22 kb upstream or in all gene body). POLII binding coordinates in NB4 cells come from the ENCODE project (GSM935354).

We then evaluated the impact of LSD1 inhibition, to assess the specific activity of LSD1 in controlling the gene expression of regulated genes. We focused our attention on genes that were regulated (UP or DOWN) upon LSD1 inhibition (treatment with MC). Considering all the regulated genes, we saw that 58% were induced while 42% were down regulated (see table 1). In particular, we noticed that LSD1 binds 30% (34/112 genes) of the all up regulated genes and only the 2.5% (2/80 genes) of the down regulated ones. Moreover, when we restricted our analysis to the LSD1 bound genes we found that almost all LSD1 regulated genes (97.5%) show up-regulation and only a modest portion appeared down regulated (34 up regulated, 2 down regulated FDR $\leq$0.05, $\geq$2 fold change) (Figure 8). This analysis was also repeated with other filtering conditions and the ratio between LSD1 bound and unbound remained comparable.

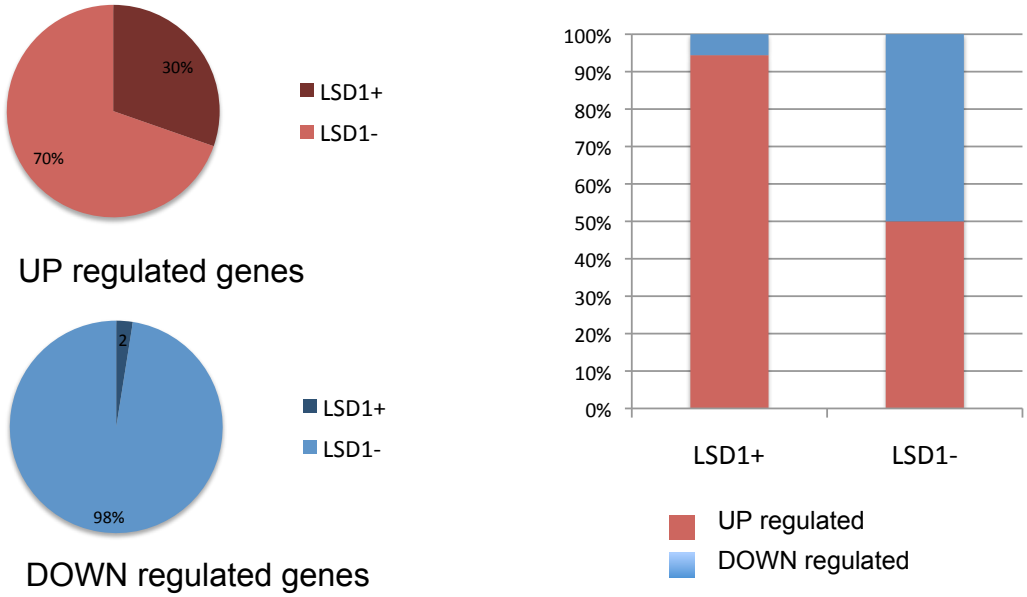


Figure 8: LSD1 represses transcription of its target genes. On the left, pies representing all the genes regulated upon 24h of MC treatment: LSD1 binds 30% of all induced genes and only 2.5% of down-regulated genes. On the right are shown bars representing the percentage of the regulated LSD1 positive and negative genes. Most of the LSD1 bound genes do not show modulation upon LSD1

inhibition but if regulated they result as being almost always induced. The genes considered dynamic (up or down) presented FDR $\leq$0.05, fold change $\geq$ or $\leq$ 2.

Then we moved our attention to the modulation of the expression in all the considered treatments (see table 1). We first checked the expression profiling of several known RA induced genes in NB4 cells, such as *Tgm2* and *Rarb* (Figure 9 and not shown). *Tgm2* was observed to be almost not expressed in control cells, while LSD1 inhibition alone did not induce any significant regulation, upon RA LOW treatment *Tgm2* exhibits a slight activation, further enhanced in the presence of LSD1 inhibition. Moreover, *Tgm2* expression increases in a RA concentration-dependent manner (Figure 9). The same regulation was also observed for the *RarB* gene (not shown).

Categories vs DMSO	Number of genes
MC 2 fold.up	112
RA LOW 2 fold.up	542
MC/ RA LOW 2 fold.up	989
RA HIGH 2 fold.up	671
MC.2 fold.down	80
RA LOW.2 fold.down	358
MC/RA LOW 2 fold.down	326
RA HIGH 2fold.down	346

Table 1: Number of regulated genes. Number of genes regulated at 24h in all the treatments versus the DMSO, showing FDR $\leq$0.05, fold change $\geq$ or $\leq$ 2.

LSD1 controls the expression of hematopoietic related genes

In order to have a better understanding on the global modulation of gene expression, we plotted a heatmap displaying all the genes regulated in at least one condition (Figure 9). MC has a modest impact on the global gene regulation, while RA LOW alone induces an appreciable increase of the expression levels of several genes. The co-treatment with MC/RA LOW showed a stronger effect on the induction of most of the regulated genes. It was also evident that LSD1 is associated with almost all the regulated genes (yellow flag on the top of the heatmap) (Figure 9). This led us consider LSD1 as a fine-tuner of transcription rather than a mere co-repressor.

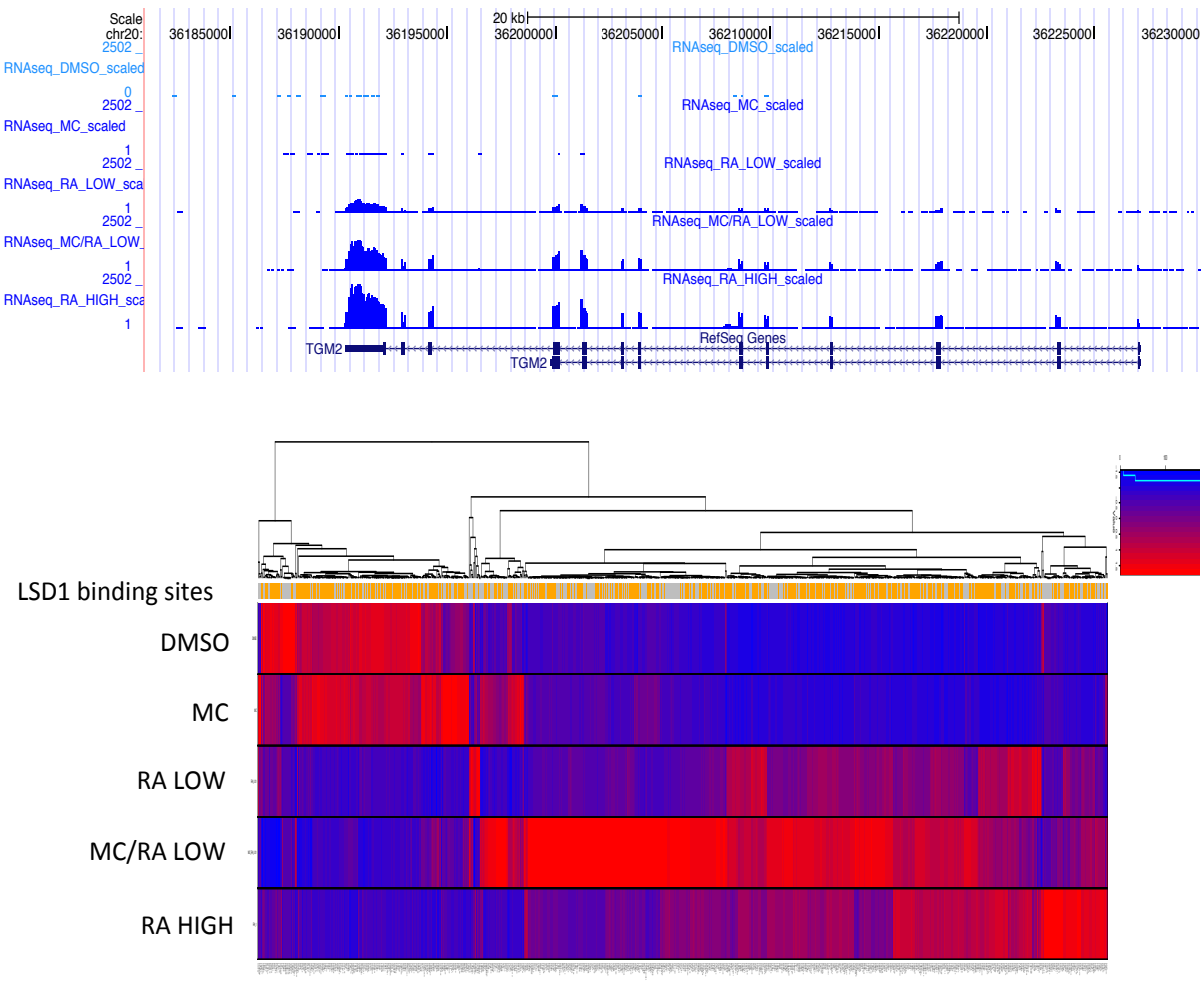


Figure 9: LSD1 binds almost all the genes regulated upon differentiating treatments in APL cells. Snapshot of the *Tgm2* gene and its expression (RNA-Seq tracks in blu) in all the experimental conditions. Tracks were obtained using MACS and scaled to the same sequencing depth using custom scripts. The heatmap shows clusterization of genes regulated in at least one of the 4 treatments against the DMSO. The expression levels range from blue (less induced), to red (more induced), while the yellow flag represents the LSD1 bindings.

We next assessed if LSD1 regulated genes could be relevant for the differentiation and the growth arrest observed in APL cells. Since RA LOW seems to have an impact on the transcription, and in order to dissect the genes more likely directly regulated by LSD1, we selected LSD1 bound genes specifically up regulated in the co-treatment versus both RA LOW and DMSO. Thus, we performed a gene ontology analysis by

using the Ingenuity software with standard settings (Figure 10). “Hematopoietic system development and function” and “cellular growth and proliferation” resulted among the top scoring networks enriched in the dataset. These data suggest that bona fide LSD1 dependent gene regulation gives a relevant contribution to APL cell differentiation and growth integrating results obtained in other AML systems (Harris, Huang et al. 2012); (Schenk, Chen et al. 2012).

Top Networks

ID	Associated Network Functions	Score
1	Cell-To-Cell Signaling and Interaction, Hematological System Development and Function, Immune Cell Trafficking	44
2	Cellular Movement, Hematological System Development and Function, Immune Cell Trafficking	17
3	Lipid Metabolism, Small Molecule Biochemistry, Inflammatory Response	15
4	Cellular Growth and Proliferation, Cell-To-Cell Signaling and Interaction, Hematological System Development and Function	11
5	Inflammatory Response, Cell-To-Cell Signaling and Interaction, Cellular Function and Maintenance	8

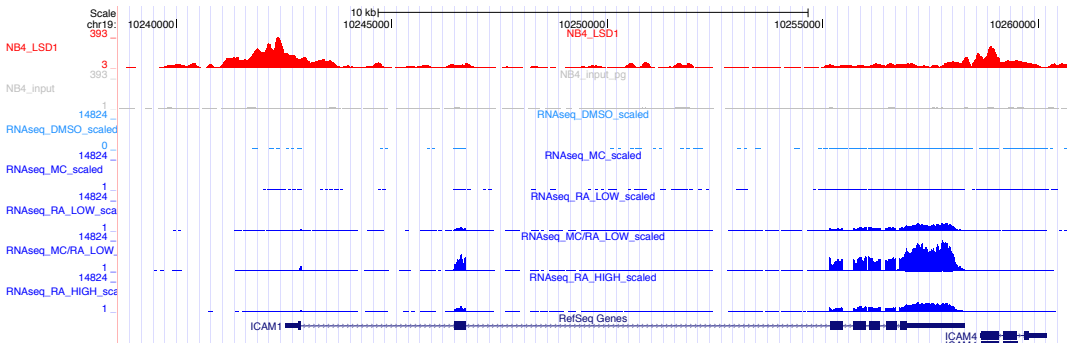


Figure 10: LSD1 regulates genes involved in hematopoietic development and cell growth. Top Networks from Ingenuity analysis, performed on LSD1 bound genes (LSD1 peak within 22 kb upstream or in all gene body) specifically up regulated (≥1.4 fold) in the co-treatment versus both RA LOW and DMSO. The analysis was performed with standard parameters. Below,a screenshot representing LSD1 binding (LSD1 ChIP-Seq in red) and RNA-Seq tracks (in blue).

LSD1 dependent epigenetic modulation is associated with MC primed APL cell differentiation

Since LSD1 has been shown to demethylate lysine 4 of histone H3, (Shi, Lan et al. 2004) we wanted to analyze a possible role in the modulation of this histone mark. H3K4me2 is a PTM involved in promoter and enhancer regulation and could be crucial to influence gene poising and activation (Barski, Cuddapah et al. 2007) (Ernst, Kheradpour et al. 2011). Thus we performed ChIP-Seq for H3K4me2 in NB4 cells upon LSD1 inhibition and all the considered conditions (see table 2).

Sample	Number of H3K4me2 enriched regions
NB4_DMSO	40585
NB4_MC	50.721
NB4_RA_LOW	39286
NB4_MC RA LOW	45440
NB4_RA_HIGH	45995

Table 2: **H3K4me2 regions enriched**. The raw signal was normalized versus input (p-value $\leq$0.05). Comparable number of regions was obtained in all samples

From 40 to 50 thousand regions were enriched for H3K4me2 in each condition and, as expected, about 40% of them were assigned to gene promoters (Figure 11). We also found H3K4me2 spreading in intronic and intergenic regions, likely corresponding to enhancer regions as suggested by Ernst and colleague (Ernst, Kheradpour et al. 2011)

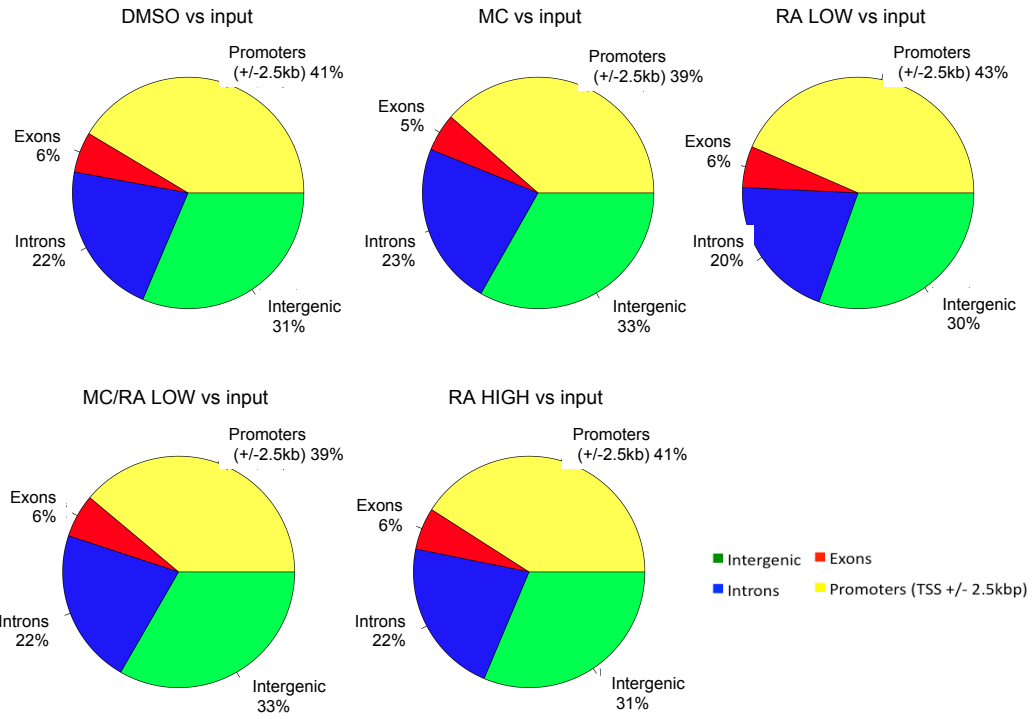


Figure 11: H3K4me2 genomic distribution in APL cells. H3Kme2 enriched regions were determined by normalizing ChIP-Seq signals versus input. The genomic distribution of the peaks remains constant in all treatments. A significant portion of regions is enriched around the TSS of annotated genes.

LSD1 localizes at H3K4me2-enriched regions

Our results suggest that LSD1 acts in our system mainly as a transcriptional repressor. LSD1 mediated transcriptional repression activity depends mainly on specific interactions with several cofactors (Shi, Matson et al. 2005). BHC80 belongs to the LSD1 complex and has been shown *in vitro* to recruit LSD1 on the unmethylated form of histone H3K4 (H3K4me0), linking LSD1 to gene repression (Lan, Collins et al. 2007). We consequently wondered whether LSD1 was recruited in regions

depleted of H3K4me2. As a first step, we assessed if the LSD1 binding was associated with H3K4me2 enriched/not enriched regions. We crossed H3K4me2 and LSD1 peaks and we found that 84% of LSD1 peaks fall within regions harboring detectable H3K4me2. Therefore, our data suggests that LSD1 preferentially binds H3K4me2 positive regions. Moreover, LSD1 and H3K4me2 overlapping sites occur both in TSS proximal (56%) and TSS distal (44%) elements. Since LSD1 functions as a lysine 4 demethylase (Shi et al., 2004) we reasoned that these LSD1/H3K4me2 double positive regions should show low levels of H3K4me2. So, we measured the level of the H3K4me2 enrichment in the LSD1 positive and the LSD1 negative regions. The box plot in Figure 12 clearly demonstrates that LSD1 bound regions show higher enrichment in H3K4me2 than the negative ones. This result is in accordance with the fact that LSD1 binds relatively more expressed genes and suggests that LSD1 activity is needed to modulate H3K4me2 levels, rather than to completely remove this histone mark at its binding loci.

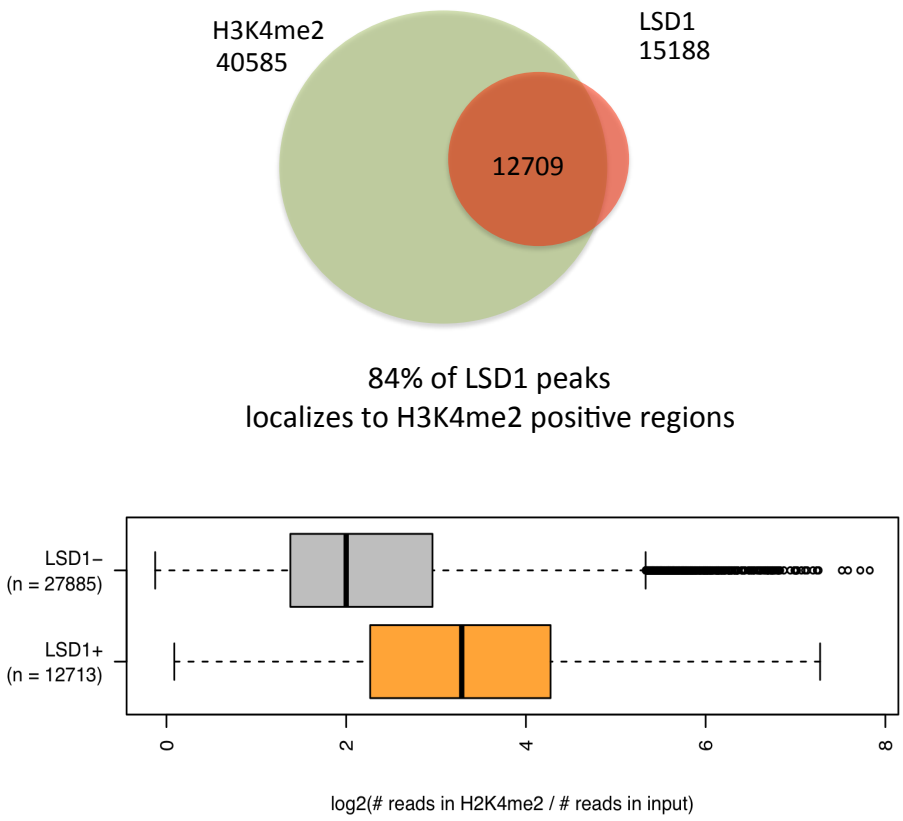


Figure 12: LSD1 bound regions are enriched for H3K4me2. The Venn diagram shows LSD1/H3K4me2 overlapping regions: H3K4me2 peaks in DMSO were crossed with the LSD1 peaks: 84% of LSD1 binding occurs at H3K4me2 positive regions. The box plot shows LSD1 positive regions (defined as regions which contain at least one LSD1 peak) and LSD1 negative regions. The medians of log2 ratio of H3K4me2 tags normalized against input are plotted. LSD1 positive regions exhibit stronger H3K4me2 enrichment than the negative ones.

Locus specific modulation of H3K4me2 during differentiation of NB4 cells

We wanted to assess the dynamicity and the regulation of H3K4me2 levels upon NB4 differentiation due to the LSD1 inhibition and/or RA treatments. We identified H3K4me2 increasing regions, normalizing ChIP-Seq signals obtained in all the

experimental conditions (see Table 2) against DMSO (see figure 2-3). We will refer to these regions as IMRs (increased methylated regions). The distribution of IMRs, in each sample, was similar to the basal H3K4me2 signal, indicating that the H3K4me2 increase involves to the same extent both promoters and distal elements (Figure 13). We ended up with 10392 IMRs upon LSD1 inhibition, 4006 in RA LOW, 10858 in MC/RA LOW and 15796 in RA high. Of note, the absolute number of IMRs suggested that RA low alone has a milder effect on H3K4me2 at genome wide levels in comparison with all the other treatments. Indeed, about 4006 IMRs are RA LOW dependent while about 10000 IMRs appear concomitantly with the LSD1 inhibition. To validate the region specific increase of H3K4me2 in the different samples, we independently validated by qPCR a number of regions that show variable enrichment profiles. For example, in Figure 14 we report two screenshots and the corresponding qPCR analysis of two H3K4me2 regions that are regulated in a very different way. The first one is clearly LSD1 inhibitor-dependent while the second one seems to be only sensitive to RA. The trend we saw in the ChIP-Seq signals were maintained in the qPCR enrichments, where slight differences among the treatments were reproducible.

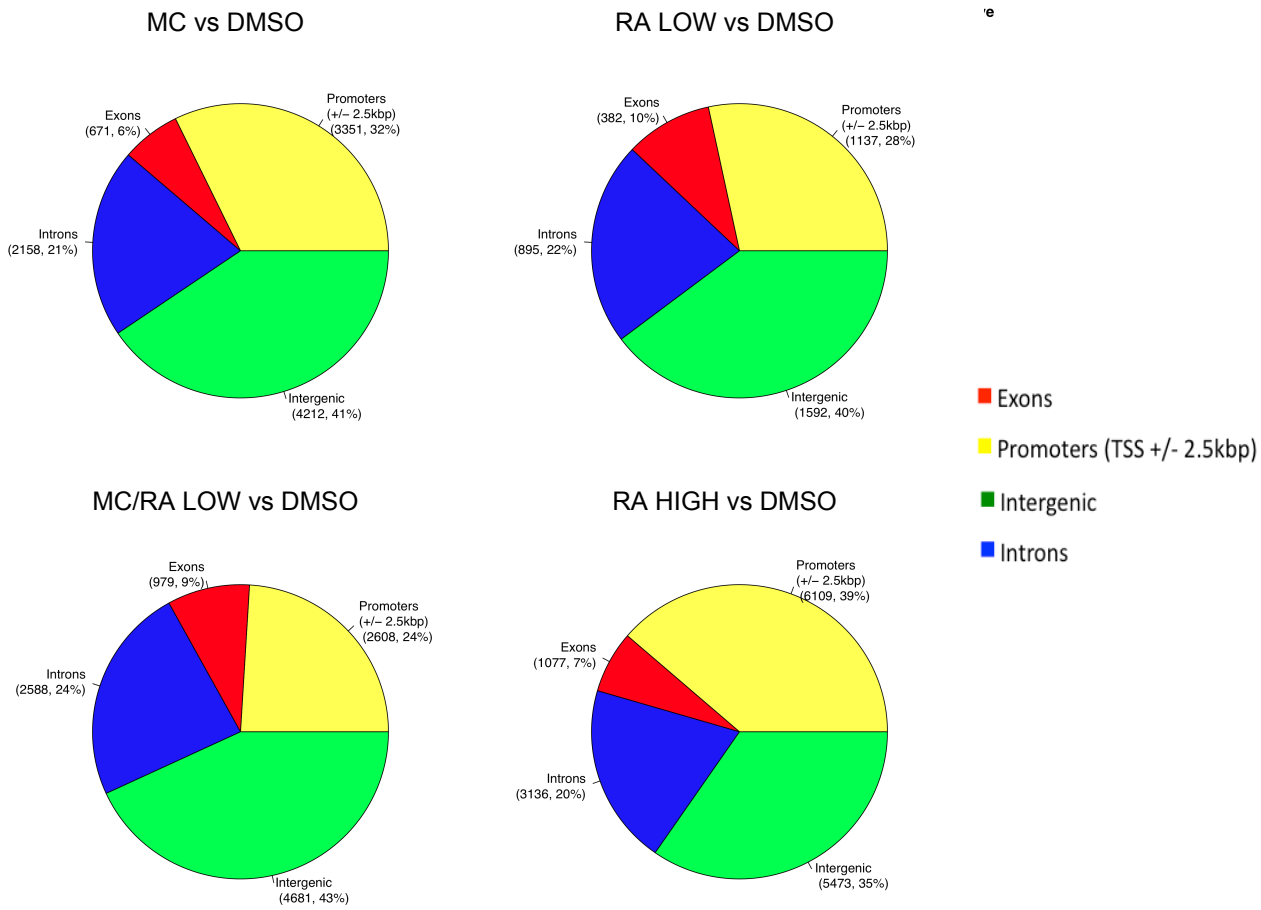


Figure 13: IMRs genomic distribution. We defined H3K4me2 increased regions (IMRs), normalizing ChIP-Seq signals first versus input and then versus DMSO signals (p-value $\leq$ 0.05).

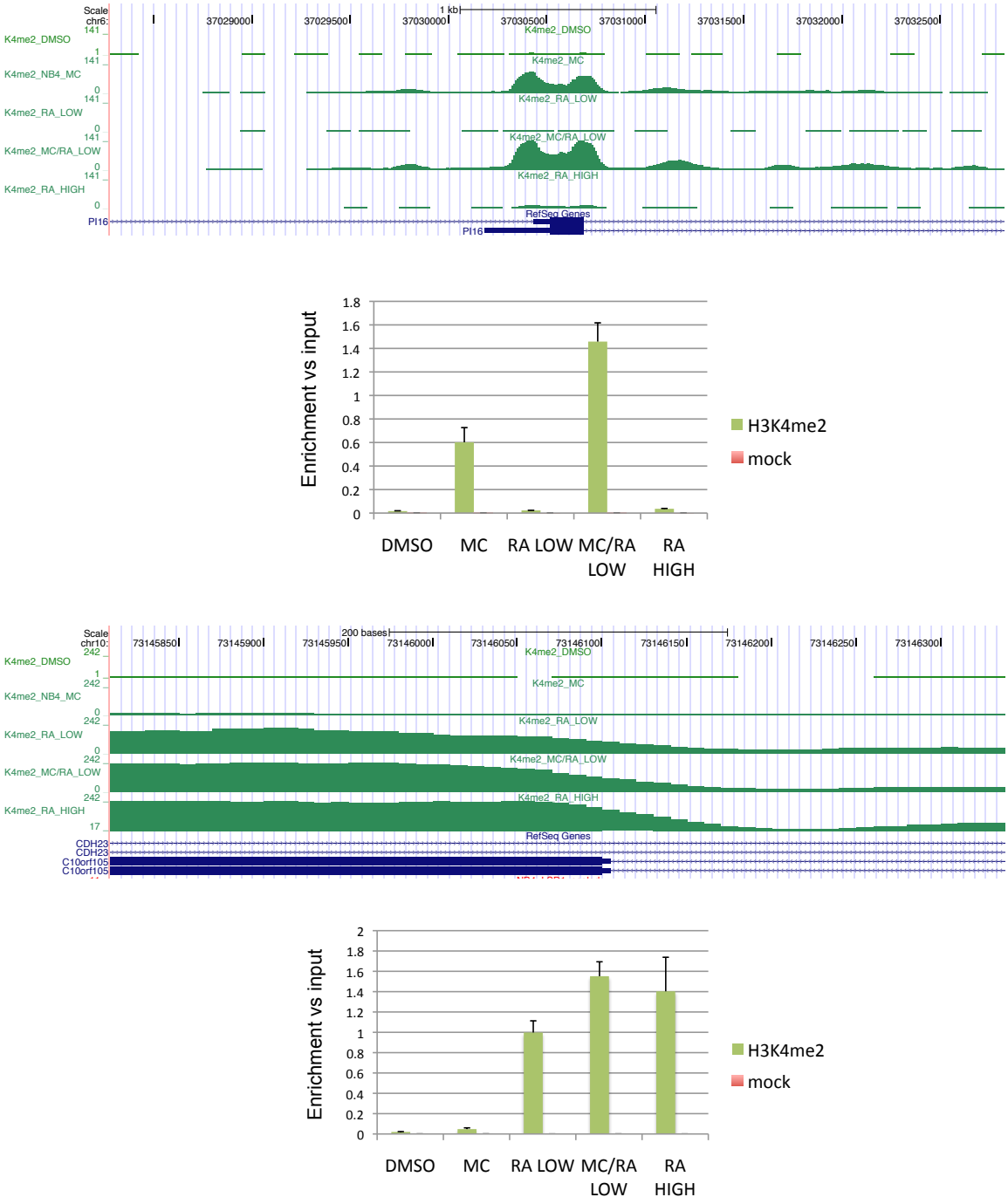


Figure 14: IMRs validation. A number of H3K4me2 regions that showed variable modulation among each treatment were validated by qPCR in biological replicates. Regions were selected with the aim to include different types of regulation. Enrichment is calculated by normalizing signals versus the input;.the mock correspond to IgG antibody.

Bona fide LSD1 targets exhibit the largest increase of H3K4me2

Since we had characterized the LSD1 binding sites (Figure 4), we could then investigate the direct contribution of LSD1 to the observed K4me2 modulation. First, we wanted to characterize whether LSD1 is enriched in IMRs. We merged IMRs specifically induced upon LSD1 inhibition with the LSD1 binding dataset. As previously shown (Figure 14), LSD1 inhibition induces up-regulation of about 10000 H3K4me2 regions (vs DMSO) and LSD1 binds almost 15000 genomic loci (Figure 4). We found about 3000 regions corresponding to 30% of all MC dependent IMRs to be bound by LSD1. Moreover, 70% of MC dependent IMRs do not overlap with LSD1, suggesting a possible indirect effect of the LSD1 inhibition (Figure 15). We expanded the analysis supposing that LSD1 bound regions could be the ones showing the stronger H3K4me2 increase. To this end, we divided all the MC dependent IMRs in 4 quartiles depending on the relative increase of H3K4me2. The fraction of LSD1 positive regions increases together with the gain of H3K4me2 upon LSD1 inhibition (Figure 15). If we consider the top IMRs (corresponding to the 4^{th} quartile), LSD1 co-occupancy reaches 45%. Thus, the initial binding of LSD1 is preferentially distributed on regions that exhibit the largest regulation of this histone mark.

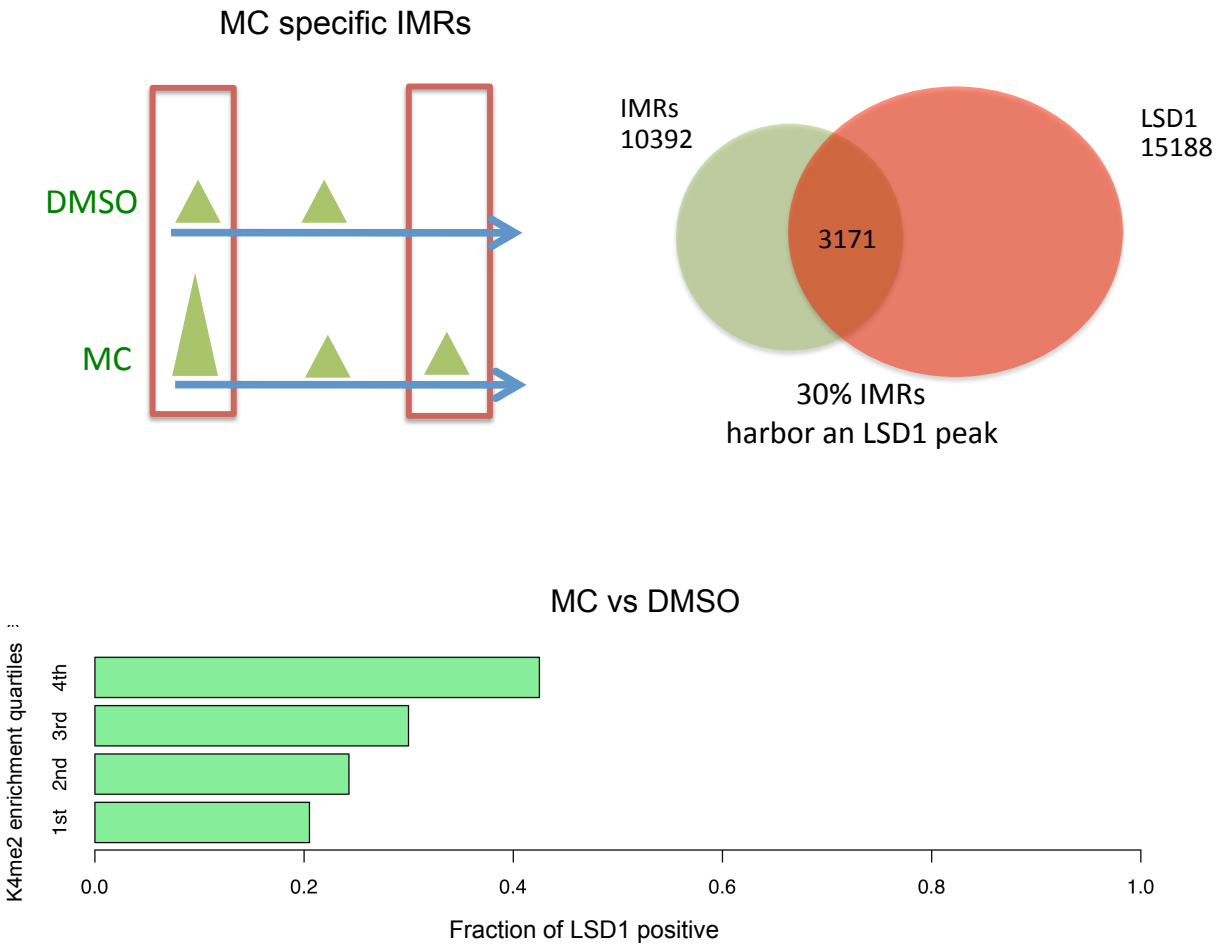
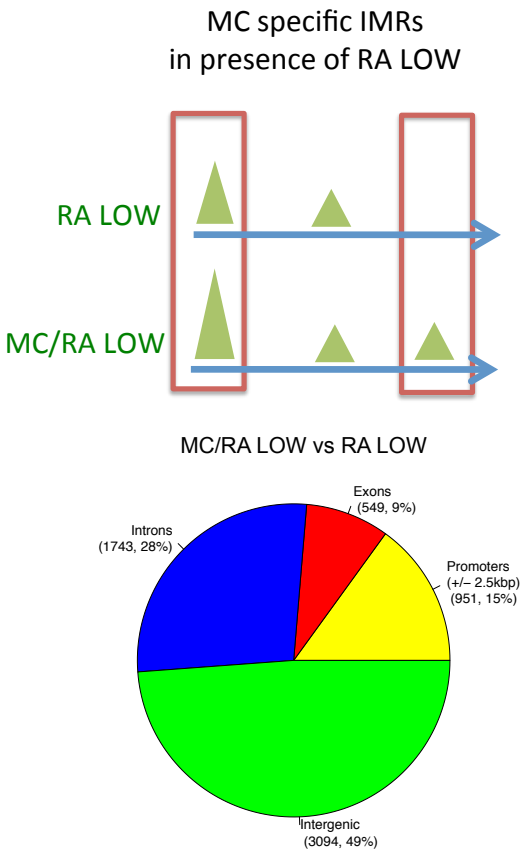


Figure 15: LSD1 binds and regulates IMRs showing the largest increase. A representative schematic of MC/LSD1 dependent IMRs is shown on the left. The Venn diagram shows the overlap between LSD1 peaks and MC specific IMRs. 3171 regions were LSD1-premarked. Below, the bars represent the 4 quartiles in which MC specific IMRs were divided. The division in quartile has been done on the base of H3K4me2 enrichment levels. LSD1 binds more than 45% of IMRs belonging to the 4th quartile.

Finally, since MC/RA LOW treatment induces differentiation and growth arrest (Figure 2-3 and Binda et al., 2010) a clear goal was to dissect the specific impact of LSD1 in the regulation of H3K4me2 in this condition. In particular, in order to distinguish among the RA LOW dependent IMRs and the contribution of LSD1 inhibition, we normalized K4me2 ChIP-Seq signals coming from co-treated cells

versus the K4me2 RA LOW ChIP-Seq data. The resulting MC specific IMRs corresponded to 6338 genomic loci (Figure 16). These regions were distributed mainly in TSS-distal portions of the genome. In fact only 15% of IMRs localize around TSS (+/- 2500bp) while the remaining 85% occur in introns, exons and intergenic regions. These IMRs may represent regulatory regions important for differentiation. If that is the case, they should be regulated by specific transcription factors and enriched for their binding motifs. In order to investigate which TF could have a role in the regulation of MC specific IMRs during differentiation, we performed a CLOVER analysis on the regions showing highest enrichment (top 1000) (Figure 16). The SPI1 matrix came out among the best scoring ones. This is particularly interesting because this TF has been shown to bind and regulate enhancers in the myeloid lineage (Ghisletti et al., 2010; Heinz et al., 2010; Pham et al., Blood 2012). Other TF binding motifs involved in the myeloid development also resulted as being enriched, such as RUNX1, MZF1 and GFI1. Of note, the latter was previously demonstrated to be a direct LSD1 recruiter (Saleque et al., 2007). These results sustain the idea that upon LSD1-inhibition specific IMRs could correspond to relevant regulatory regions and highlighted a possible interplay between LSD1 and master regulators.



Clover analysis

PWM	score
MA0080.2 SPI1	inf
ETS0025 h-SPIB	596
MA0055.1 Myf	586
ETS0026 h-SPIC	563
ETS0027 h-SPI1	563
MA0002.2 RUNX1	542
MA0081.1 SPIB	537
MA0056.1 MZF1_1-4	517
BU0003 Ascl2_primary	507
BU0119 Foxa2_secondary	472
BU0058 Sfpi1_primary	466
MA0136.1 ELF5	405
MA0065.2 PPARG::RXRA	396
MA0080.1 SPI1	392
MA0154.1 EBF1	387
MA0156.1 FEV	355
BMC_Inr	301
BU0012 Elf3_primary	293
BU0162 Sfpi1_secondary	243
MA0098.1 ETS1	238
MA0003.1 TFAP2A	238
MA0137.1 STAT1	196
MA0038.1 Gfi	193
ETS0004 h-ELF3	192

Figure 16: MC specific IMRs associated with differentiation correspond mainly to TSS distal regions and are enriched for TFs important for myeloid development. Schematic representation of MC specific IMRs in the MC/RA LOW co-treatment. MC/RA LOW ChIP-Seq signal was normalized on RA LOW reads. Shown below is the genomic distribution of the MC specific IMRs: IMRs occur preferentially at promoter distal regions. On the right TFs binding motif enrichment was performed on top the 1000 MC specific IMRs: top 25 enriched matrixes are reported.

We merged this IMRs dataset with LSD1 peaks. We observed that the 27% of IMRs harbour an LSD1 peak (Figure 17). We again divided IMRs according to the intensity of H3K4me2 enrichment and assessed the correlation with the LSD1 binding. Interestingly, LSD1 preferentially occupies IMRs presenting the largest increase of

H3K4me2. Moreover, all the regions that were analyzed for the TF binding sites enrichment (Figure 16) belong to the 4th quartile, and correspond to the ones showing the strongest enrichment in H3K4me2 and the highest overlap with LSD1.

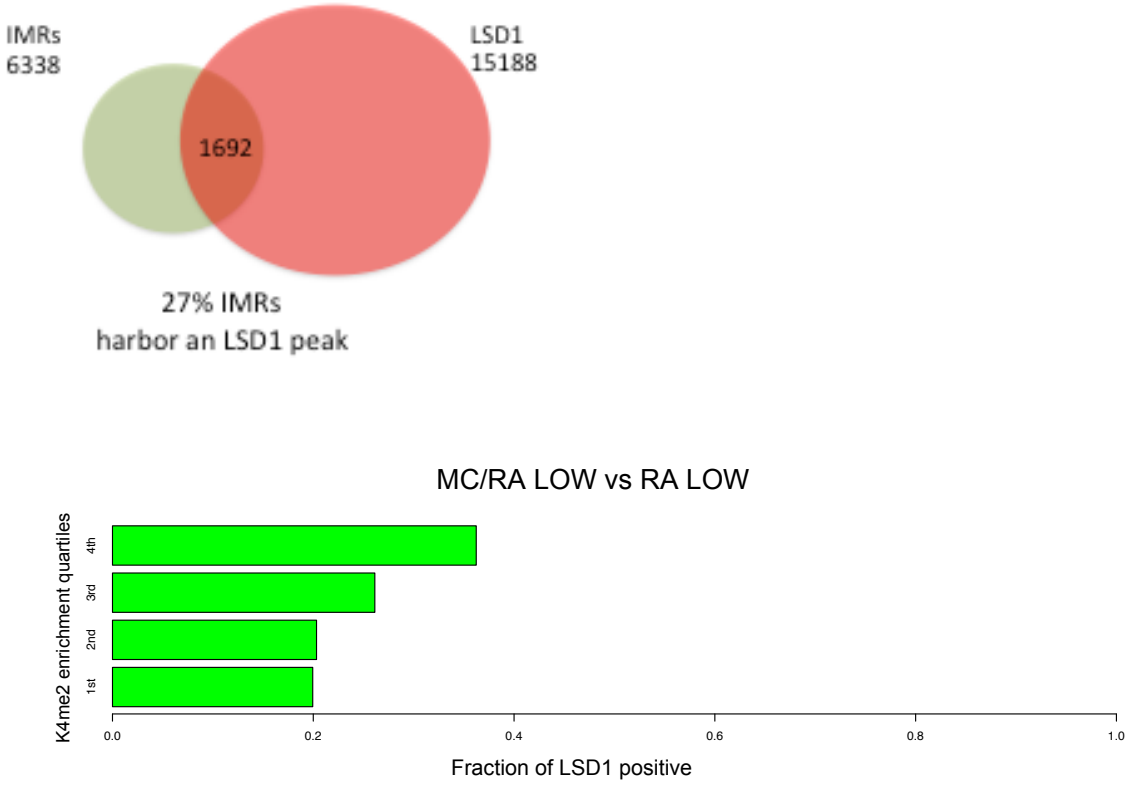


Figure 17: LSD1 regulates differentiation associated IMRs showing the largest increase in H3K4me2. A representative schema of MC/LSD1 dependent IMRs is shown on the left. The Venn diagram shows the overlap between LSD1 peaks in DMSO, and MC specific IMRs in the MC/RA LOW treated cells: Among the 6338 IMRs, 1692 are LSD1 positive. The bars represent IMRs divided in 4 quartiles, the first include regions with less H3K4me2 enrichment while the fourth quartile represents IMRs showing the largest increase and the largest fraction of LSD1 peaks.

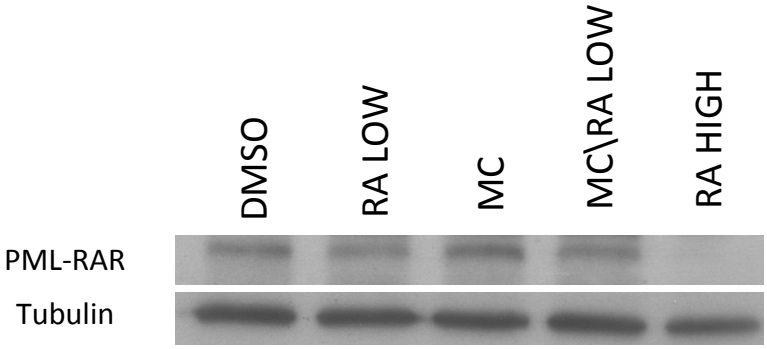
Characterization of the LSD1 – PML-RAR interplay

APL is characterized in 95% of the cases by PML-RAR (PR) expression and this translocation is the initiating event in APL as shown by murine models of the disease. PR determines the differentiation block and the aberrant proliferation of myeloid progenitors. Molecularly, RA activates PR-repressed target genes and mediates its proteasome-mediated degradation. While, pharmacological doses of RA (RA HIGH) trigger PR degradation, physiological concentrations of RA (RA LOW) do not (Nasr, Guillemin et al. 2008). We demonstrated that LSD1 inhibition induces a strong differentiation and a growth arrest in the presence of RA at a physiological concentration (see Figure 2-3). For this reason, we wanted to understand if the LSD1 primed differentiation/growth arrest of APL cells was associated with PR degradation. A possible explanation may be the synergistic activity of the LSD1 inhibitor and RA LOW treatment in the degradation of PR.

LSD1 priming of differentiation occurs in the presence of PR

We analyzed PR protein levels by western blot. We found that PR is almost completely degraded by RA HIGH and only started to be degraded by RA LOW treatment, while PR remains stable upon LSD1 inhibition alone. Interestingly, PR is not degraded in the co-treated cell extracts, presenting level comparable to the ones seen in RA LOW alone treated cells. Hence, we were in the condition to assay the NB4 differentiation/growth arrest in the presence of the oncogene (Figure 18). Since most of the PR oncogenic potential occurs via its function as an aberrant transcription factor (Saeed, Logie et al. 2011), we wanted to control whether PR still binds its

target genes upon MC/RA LOW treatment. For this reason, we tried to perform a PR ChIP-Seq in all our experimental conditions, but probably because of the low quality of the commercial antibody available we were unable to do so. We used an anti-PML antibody to specifically distinguish PR from the RARalpha receptor and performed ChIP-qPCR analysis. We saw that RA causes a concentration-dependent decrease in the PR recruitment on both of the regions tested, while LSD1 enzymatic activity does not impact upon the PR recruitment to these two binding sites. These results demonstrated that LSD1-inhibition triggered differentiation and growth arrest occur without PR degradation.



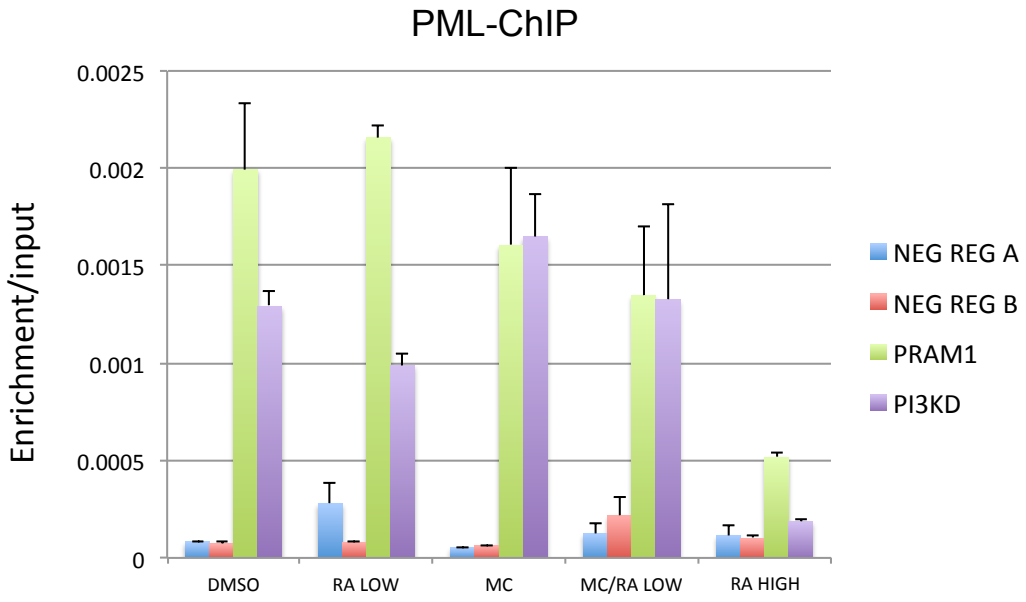


Figure 18: LSD1 inhibition does not trigger PML-RAR degradation. Western blot showing PR levels in NB4 cells after 24 hours of each treatment. Immunoblotting with an anti-RAR antibody was performed o.n.. and a band was deteced at about 120 KD. Tubulin was used as a loading control. Below, the histogram represents the ChIP qPCR for PML in NB4 cells. Enrichment was calculated by normalizing signal versus input. Two negative regions were used to distinguish aspecific signals. NEG REG A corresponds to a PR-negative intergenic region while NEG REG B is an intronic PR-negative region, occurring close to a PR peak.

PML-RAR shares most of its binding sites with LSD1

PML-RAR has been shown to induce the differentiation block, in part, by repressing its target genes through the cooperation with several histone-modifiers, such as HDAC1 (Minucci and Pelicci 2006). Interestingly, HDAC is also found to contribute to the LSD1-dependent transcriptional regulation (Shi et al Mol Cell 2005), so we asked whether, in turn, LSD1 also cooperates with PR. We assessed if the two proteins show common binding sites by intersecting PML-RAR binding coordinates (previously established in NB4 by ChIP-Seq, performed with an antibody that was not accessible to us) (Martens et al., Cancer Cell 2010) and LSD1 ChIP-Seq data in NB4 cells. Strikingly, we found that more than 65% of PML-RAR positive regions overlap with LSD1 peaks (Figure 19). Despite that, we encountered a percentage of LSD1 binding sites not overlapping with PR, thus suggesting the existence of complementary LSD1 PR-independent functions (in accordance with previous reports in PR deficient AML cells). The overlapping peaks do not show any preferential genomic distribution, having a comparable percentage of promoter associated and distal peaks (not shown). Two screenshots of some PR/LSD1 overlapping sites are shown in Figure 19, representing both TSS proximal and distal common binding

regions. The common target genes include genes already shown to be important for the NB4 cell differentiation such as *Spi1*, *Tgm2* (Mueller et al., Blood 2006; Csomos et al., Blood 2010) and other well known PR targets, such as *Pram1*. Interestingly, one of the most studied PR targets *RarB* was not bound by LSD1 (not shown).

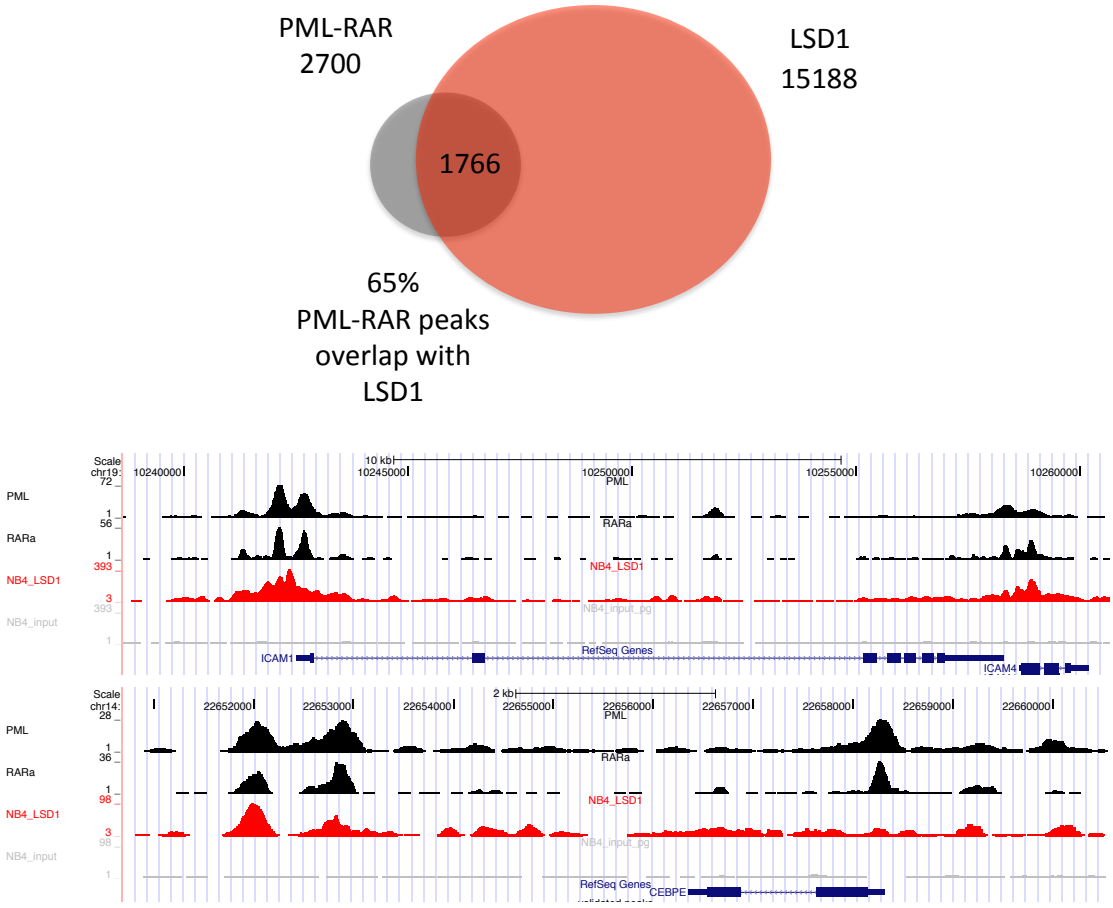
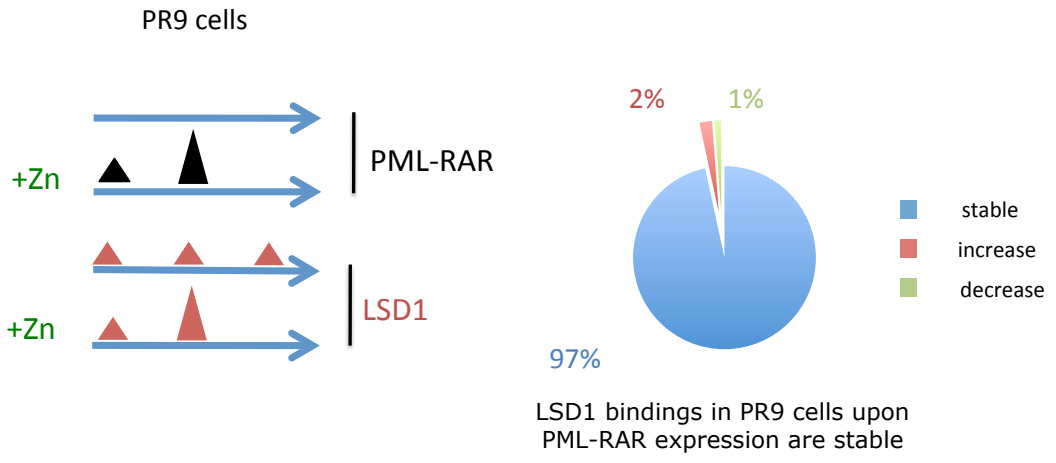


Figure 19: LSD1 and PR show a large portion of common binding sites. The 2700 PR binding regions were retrieved from the literature (Martens et al., 2010) and were merged with LSD1 peaks in NB4 cells. About 1800 regions show occupancy by both PR and LSD1. Among the common target genes there are also genes important for the hematopoietic development. Below two screenshots representing several PR/LSD1 double positive genes, among which are *Cebpe* and *Icam1*.

PML-RAR expression does not alter LSD1 recruitment

The existence of a large number of common binding sites prompted us to determine whether PR recruits LSD1 onto its target sites. In order to address this point we choose as a model system the PR9 clone. PR9 cells were obtained from the monoblastic U937 cell line by transfection of the PR cDNA under the control of the zinc (Zn)-inducible metallothionein promoter (Grignani et al., Cancer Res 1994). In this system we can induce PR expression via zinc treatment (Figure 20). We performed PML and LSD1 ChIP-Seq both prior to- and after 8h of Zn-dependent induction. We observed that the pattern of LSD1 binding in PR9 cells remains largely (97% of cases) unchanged by PML-RAR expression, suggesting that PML-RAR is not recruiting LSD1 in the early phases of the disease (Figure 20) and that PML-RAR expression does not induce the LSD1 displacement from its originals target sites. However, we cannot exclude recruitment/displacement occurring in the later phases of APL development.



LSD1 bindings in PR9 cells upon PML-RAR expression are stable

Figure 20: LSD1 occupancy does not change upon PR expression. A schematic representation of PR9 cells, a PR-inducible system (see methods). In brief, PR expression is under the control of a Zn promoter. Upon 8h of 100 μM Zn treatment a portion of cells was saved to verify the overexpression of the PR protein and the rest of the cells were fixed. LSD1 occupancy variation was gauged by normalizing ChIP-Seq signal coming from PR9 cells upon 8h of PR induction on the signal obtained from untreated cells. Only a modest portion of LSD1 occupancy is altered: only 2% increased and 1% decreased their enrichment.

A significant portion of PR binding events occurs at LSD1 pre-marked regions

It has been recently demonstrated that PR binding occurs at genomic region endowed with distinct features, such as chromatin accessibility, p300 binding and low acetylation levels (Martens et al., Cancer Cell 2010; Saeed et al., Blood 2012). For this reason, we asked whether in PR9 cells PR shows a preferential distribution relative to the LSD1 binding. We merged LSD1 peaks obtained in un-induced PR9 cells with the ones of PR. We saw that in about 70% of the cases PR binding occurs in an LSD1 pre-marked region (Figure 21). This evidence further confirmed the existence of a possible interplay between the two proteins and suggested that PR recruitment benefits from LSD1's presence at a majority of its target regions. Moreover, as well as in NB4 cells, some of the commonly bound genes are involved in the hematopietic compartment, such as *Runx1* (Figure 21). We observed that the LSD1 peaks number (48321) was higher in PR9 cells than the ones obtained in NB4 cells (15188). For this reason, we decided to repeat the analysis by cutting the LSD1 peaks dataset to the p-value considered reliable in NB4 cells. Despite this higher

stringency, the overlap remains highly significant, and PR targets LSD1 positive regions in more than 40% of the cases.

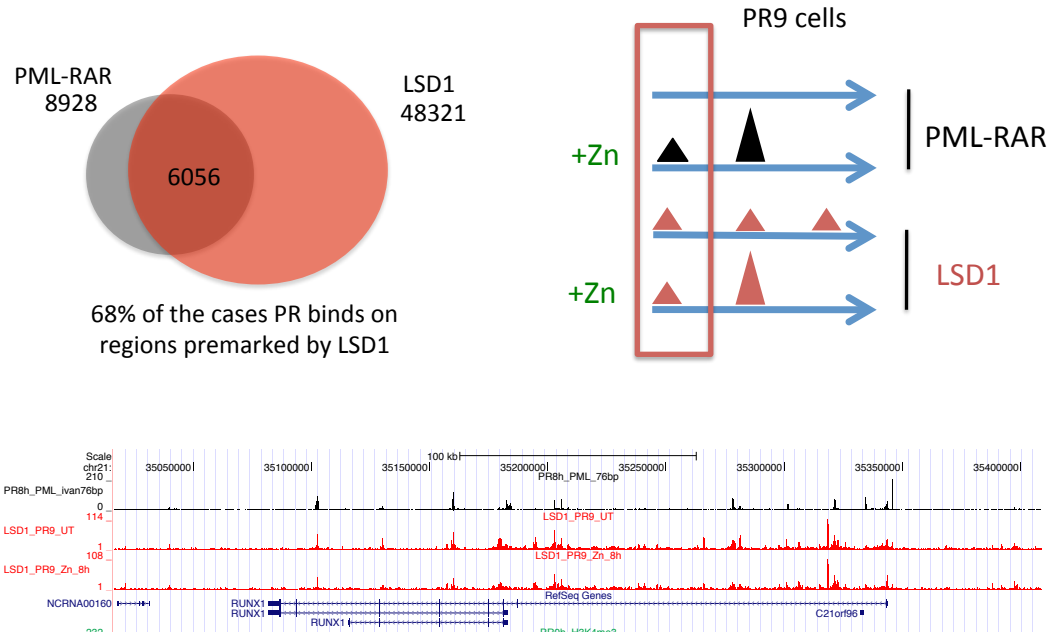


Figure 21: PR binds LSD1 pre-marked regions. Venn diagram showing PR and LSD1 common binding regions. LSD1 coordinates were obtained from un-induced PR9. A schematic representation of the PR binding in PR9 cells. PR is recruited to LSD1 binding sites and LSD1 does not increase nor decrease its enrichment upon PR expression. A representative screenshot of LSD1 and PR overlapping peaks on the *RUNX1 gene*.

LSD1 and PR cooperate to regulate regions enriched in H3K4me2

Disruption of the epigenetic landscape has been suggested as a crucial mechanism by which oncofusion proteins induce and maintain the leukemogenic status. (Chen, Odenike et al. 2010). To characterize the possible functional outcome of the interplay

between LSD1 and PR we investigated their correlation with H3K4me2, the main epigenetic mark controlled by LSD1. We analyzed the corresponding H3K4me2 levels within PR- or LSD1- exclusive regions and for the PR/LSD1 double positive regions in NB4 cells. We observed that PR-exclusive regions display comparable H3K4me2 enrichment in comparison with the double negative ones. Instead, looking at the LSD1 binding sites we noticed that the ones shared with PR (in red) display lower levels of H3K4me2 in comparison with the LSD1+/PR- (in yellow) (Figure 22). This result reveals that LSD1/PR double positive peaks are found in less enriched H3K4me2 regions in comparison with the LSD1 exclusive ones.

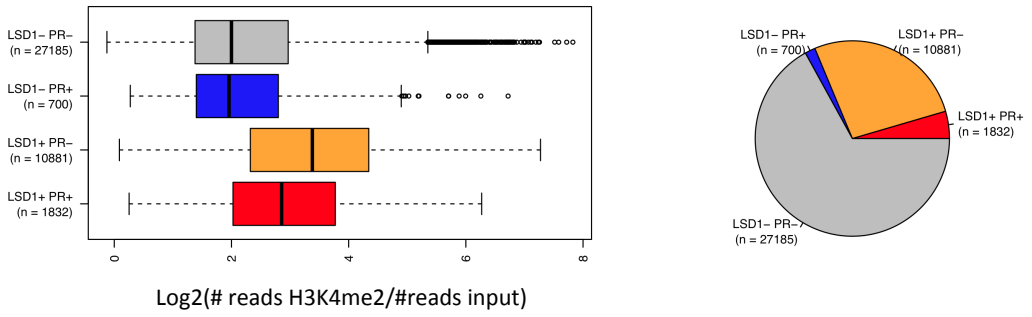


Figure 22: LSD1 and PR share regions owing to a specific H3K4me2 enrichment. The box plot shows LSD1/PR double negative regions (grey box), LSD1 negative PR positive regions (blue box), LSD1 positive/PR negative regions (yellow box), and LSD1/PR double positive regions (red box). The medians of log2 ratio of H3K4me2 reads normalized against input are plotted. On the right is shown the portion of regions assigned to each category.

We wanted to assess if one aspect of the PR/LSD1 interplay can involve the modulation of H3K4me2 levels at commonly regulated sites. To do so, we analyzed the H3K4me2 dynamic first in general and then specifically in double positive

regions, upon MC/RA LOW treatment (differentiation in presence of PR) and upon RA HIGH treatment (differentiation in absence of PR). We initially performed a global analysis considering all the IMRs occurring in these two conditions. We found that 70% of all IMRs in MC/RA LOW were also induced upon RA HIGH treatment, suggesting an overall similarity in their regulation (not shown). Then, we focused our attention on PR/LSD1 common target regions. We found that RA HIGH treatment causes H3K4me2 increase in 857 (50%) out of 1766 LSD1/PR common peaks, while upon MC/RA LOW the increasing regions were observed to be 516. Interestingly 444 LSD1/PR bound regions, corresponding to 86% of MC/RA LOW induced loci (and 50% of the RA HIGH induced), were regulated in both conditions. We then wanted to gain more insight into the contribution of each treatment (RA LOW and MC) to the H3K4me2 modulation. We analyzed PR/LSD1 commonly regulated regions and organized them into 4 quartiles according to the extent of H3K4me2 induction upon RA HIGH treatment. We plotted the enrichment of all the treatments versus the DMSO of all the regulated regions (Figure 23). We saw that in the majority of the cases RA LOW and MC given alone have a poor effect on the H3K4me2 regulation. Instead, co-treatment triggers an induction of H3K4me2 comparable to the one observed in the absence of PR (RA HIGH). This is true also for regions showing lower induction (2^{nd} and 1^{st} quartile). This indicates that the LSD1 inhibition in these regions can mimic PR depletion and that LSD1 is actually contributing to keep H3K4me2 levels under a certain threshold also in a subset of PR positive regions.

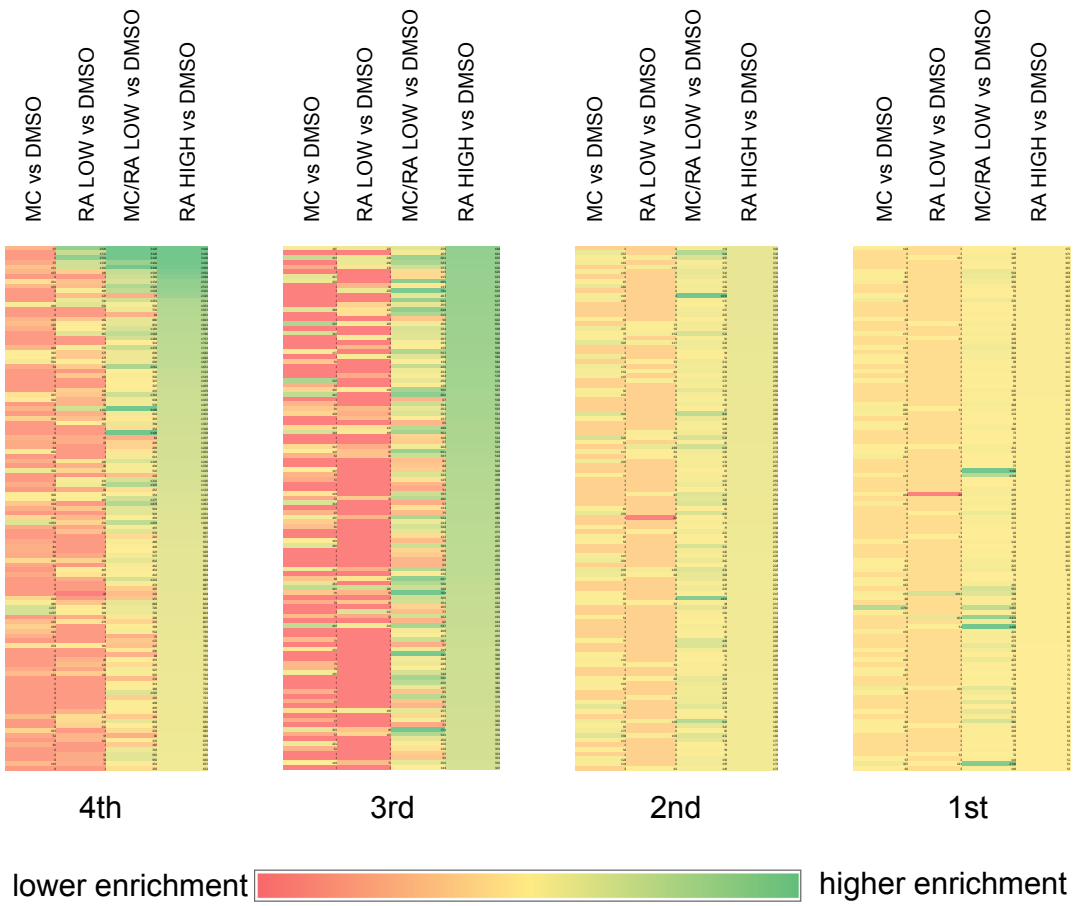


Figure 23: LSD1/PR commonly regulated IMRs show comparable H3K4me2 dynamics. The regions were divided in 4 quartiles according to the extent of H3K4me2 (corresponding to –log2 p-value of each region) induction upon RA HIGH treatment. The 4th and the 3rd quartiles correspond to regions with a higher enrichment.

DISCUSSION

Differentiation accompanies LSD1 depletion and inhibition-primed growth arrest and differentiation

Our lab, in collaboration with Antonello Mai and Andrea Mattevi, previously developed a new compound working as an LSD1 specific inhibitor, MC (Binda et al., 2010). Taking advantage of its high specificity we aimed to characterize the role of LSD1 in APL. We found, by morphological characterization and expression analysis of a differentiation-associated marker, that LSD1 KD and pharmacological inhibition sensitize a PML-RAR expressing APL cell line to RA-induced differentiation. We showed that LSD1 inhibition in the presence of physiological concentrations of RA has an even stronger effect than the RA alone given at a concentration 100-fold higher. These results confirm the importance of LSD1 in the maintenance of APL and were in accordance with LSD1's role in hematopoietic differentiation as originally proposed by the Orkin lab (Saleque et al., Mol Cell 2007). Moreover, LSD1 inhibition and protein reduction were demonstrated to induce differentiation of RA insensitive cells in AML systems (Shenk et al., Nat Med 2012; Harris et al., Cancer Cell 2012). We also demonstrated that a pulse of LSD1 inhibition was sufficient to prime APL cells to differentiate suggesting that LSD1 dependent activities play a role in the initial phases of RA induced differentiation.

LSD1 genomic distribution

We provide for the first time genome wide information about the LSD1 genomic distribution in an acute myeloid leukemia cell line and specifically in NB4 cells that

express the PML-RAR fusion protein. We found that LSD1 occupancy in APL cells is mainly promoter-associated as its tag density is higher around the TSS of the annotated genes. We also found the presence of a significant part of LSD1 peaks in promoter distal regions, in accordance with prior results obtained in mES and an immature murine granulocytic cell line (Whyte et al., Nature 2012; Kereniy et al., eLIFE 2013).

We also found that promoters bound by LSD1 were enriched for binding matrixes of TFs important for the hematopoietic differentiation process, among which PU.1, EGR-1 and KL4 are specifically involved in the function of the myeloid compartment, thus supporting the idea of LSD1 as a direct regulator of genes relevant for the differentiation. In the literature other TFs have been show to recruit LSD1 in the hematopoietic development on specific target genes such as GFI1/1B, TAL1 and SALL4, (Saleque et al., Mol Cell 2007; Li et al., Oncogene 2012; Hu et al., PNAS 2009) and our result further increase the pattern of possible LSD1 recruiter/interactor suggesting a lineage specific LSD1 dependent regulation consistent with the model proposed by Orkin (Saleque et al., Mol Cell 2007).

LSD1 dependent transcriptional control during differentiation

LSD1 has always been supposed to have a dual role in regulating transcription, both as a co-repressor and a co-activator (Metzger et al., Nature 2005; Wissman et al., Nat cell Bio 2007). In our system, we found LSD1 overlapping with PolII occupancy and binding relatively more expressed genes. This may indicate that LSD1 is cooperating to keep transcription of target genes under a certain threshold rather than repressing it

completely. In accordance with this idea, we found that upon LSD1 inhibition the number of all genes both up regulated and down regulated were almost the same percentage-wise (112 upregulated, 80 down regulated upon MC treatment: FDR $\leq$0.05, $\geq$2 fold change). When we restricted this analysis to the LSD1-bound fraction we found that almost all LSD1 regulated genes showed up-regulation and only a modest portion appeared as down-regulated (34 up regulated, 2 down regulated FDR $\leq$0.05, $\geq$2 fold change). These results were in accordance with a general repressive role of LSD1 in the majority of its regulated regions suggested in mES (Whyte et al., 2012) We also observed that the majority of LSD1 bound genes were not regulated upon inhibition alone and undergo only mild activation upon RA LOW treatment while their expression strongly increases during co-treatment with LSD1 inhibition in the presence of a physiological RA concentration. It is possible that LSD1 activity is regulated by RA LOW dependent interactions with specific co-activators according to the model proposed *in vitro* by Shi and colleagues (Shi, Matson et al. 2005) and that in this specific chromatin context LSD1 contributes to the modulation of RA LOW induced transcription. Another hypothesis can be that such genes need RA LOW dependent activators to be transcribed and LSD1 inhibition instead has a poising function.

LSD1 dependent epigenetic modulation associated with MC primed APL cell differentiation

Two works on other subtypes of myeloid leukemia have analyzed genome wide variation of H3K4me2 upon LSD1 depletion or inhibition in the presence and absence of RA. In human AML non PML-RAR expressing cell lines, a specific modulation of

K4me2 in LSD1 inhibitor/RA co-treated cells around the TSS of hematopoietic related genes was shown (Schenk, Chen et al. 2012). Instead, in a mouse model of MLL-AF9 of leukemia, the H3K4me2 modulation was specifically associated with the oncogene bound genes (Harris, Huang et al. 2012).

We characterized H3K4me2 modulation in PML-RAR expressing cells. We found that H3K4me2 distributes within promoters and TSS distal regions in accordance with previous works (Barski et al., 2007; Heintzman et al., 2007). We have shown that almost all LSD1 peaks co-localize with H3K4me2 regions in control cells and that these regions have higher enrichment than the LSD1 negative ones. This reveals that LSD1 does not completely deplete H3K4me2 at its binding regions but could contribute to the balance of the methylation together with a methyltransferase on these specific loci. A similar model was proposed for HDACs that share acetylated genomic region with the acetyltransferases (Wang, Zang et al. 2009).

We also found many regions to be regulated that increase the enrichment of K4me2 (IMRs). Interestingly, the ones showing the greatest increase are more likely to be the ones bound by LSD1 both in the absence and in the presence of RA LOW.

IMRs are distributed in both distal and proximal TSS regions and upon LSD1 inhibition, but upon co-treatment with RA LOW, an LSD1 inhibition specific 3K4me2 increase occurs preferentially in TSS distal regions (85% of the cases). Clover analysis highlighted several myeloid TF binding motifs enriched in the top scoring IMRs among which was PU.1, which has been show to regulate enhancers in the hematopoietic lineage and specifically in the monocytic compartment (Ghisletti et al., Immunity 2010; Pham et al., Blood 2012), thereby supporting the hypothesis that these IMRs can be important regulatory regions. The subset of regions which showed

H3K4me2 modulation and are not LSD1 bound probably reflect indirect effects of LSD1 inhibition. On the other hand, we also encountered LSD1 regions that do not show a measurable H3K4me2 increase in our experimental settings. It is possible that LSD1's enzymatic activity *in vivo* depends on the surrounding chromatin status and that in those particular regions LSD1 is not capable of exploiting all of its enzymatic potential. This would be consistent with *in vitro* evidence regarding the influence of a number of histone PTMs on the ability of LSD1 to effectively demethylate the lysine 4 of the histone H3 (Forneris et al., J Biol Chem 2006). Another possibility could be that in those sites, LSD1 is acting on non-histone substrates (Nicholson and Chen., Epigenetics 2009).

Collectively our data for the first time dissect the bona fide LSD1 directly controlled regions merging the LSD1 binding regions with the H3K4me2 data in APL cells.

Characterization of the LSD1 – PML-RAR interplay

In 95% of cases, APL patients present the expression of PML-RAR (PR) due to the translocation (15;17). PML-RAR exploits its oncogenic potential by influencing both the PML pathway and the retinoic acid receptor functions. The standard therapeutic approach consists in supplying pharmacological doses of RA in order to reactivate the downstream RARa pathway and to induce differentiation of APL blasts. Pharmacological doses of RA also induce PR degradation, which has been proposed as a crucial goal in order to eradicate APL. We observed that LSD1 inhibition and RA LOW treatment induces cell differentiation without inducing PR degradation and

displacement. Thus LSD1 inhibition can overcome the complete PR degradation to reach differentiation and growth arrest of APL cells.

The mechanism by which PR induces the block of the normal myelopoiesis at the promyelocytic stage includes the repression of its target genes by interacting with several histone modifiers. This model is based on evidence obtained from only a few PR target genes. By merging documented PR bindings sites (Martens, Brinkman et al. 2010) with our LSD1 targets we noticed that the majority of PR targets are shared with LSD1. We also determined that in the initial phases of the disease, LSD1 binding is not recruited by PR and also that its physiological binding remains substantially unchanged upon PR expression. Moreover, we also discovered that PR binding occurs, in the majority of the cases, in regions where LSD1 is already present suggesting that PR may benefit from the LSD1 binding. A question that remains open is whether LSD1 facilitates the recruitment of PR. It could be possible that LSD1 works to create a chromatin environment that favours PR localization at its binding sites. An analogous correlation has been demonstrated for ERG and AML1-ETO. In fact, AML1-ETO binds regions pre-marked by ERG and ERG is able to facilitate the binding of the oncofusion protein (Martens et al., Blood 2012). Genomic regions characterized by the presence of both proteins, revealed a typical chromatin conformation showing a marked H3K4me2 enrichment. PML-RAR and AML1-ETO show a preferential pattern of chromatin features within their bindings sites comprising histone acethylation, p300 binding and chromatin accessibility (Saeed S et al., 2012). Our data provide further evidence of a distinctive chromatin environment surrounding PR binding and specifically in the presence of LSD1. We also found that at LSD1-PR double positive regulated regions, LSD1 inhibition cooperates with physiological concentrations of RA to reach a level of H3K4me2 comparable to the

one reached in RA high treated cells. Consequently, LSD1 inhibition can overcome the incomplete PR degradation to induce an increase in H3K4me2 levels. The K4me2 increase at LSD1/PR double positive regions is also accompanied by a synergistic effect on the expression of the corresponding gene for a subset of targets at this time point, including *cdkn1a (p21)* and *itgb2* (CD18). This may mean that the majority of these regions become poised for later activation.

Collectively, our experiments characterized the role of LSD1 in APL suggesting a mechanistic interpretation of its action. LSD1 controls most of the genes regulated in this system upon differentiating conditions. Its action seems to be mediated by its H3K4me2 demethylase activity. LSD1 directly demethylates putative regulatory regions and represses genes important for the differentiation of APL. Together with PR, LSD1 modulates H3K4me2 levels in a subset of shared PR target genes possibly in order to control poising and/or induction in later phases of RA LOW induced differentiation in the presence of the fusion protein.

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