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The oncogenic role of class IIa HDACs in leiomyosarcomagenesis: HDAC9, a possible therapeutic target in leiomyosarcoma tumorigenesis.

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ABSTRACT

Uterine leiomyosarcoma (LMS) is a rare malignant smooth muscle connective tumor, that represents the second most frequent uterine sarcoma after carcinosarcoma and it is associated with high rates of recurrence and a poor clinical outcome. Studies evidenced that sarcomas present a strong repression of MEF2 and the highest activation of class IIa HDACs: approximately 22% of leiomyosarcoma patients presents high levels of HDAC4, HDAC5 and HDAC9. SK-UT-1, a high grade uterine leiomyosarcoma cell line, reflects these features, as mirrored by the high protein levels of HDAC9 and HDAC4. We have recently demonstrated that high levels of HDAC9 arise from an increased transcriptional activity, in fact a chromatin immunoprecipitation on HDAC9 promoter of SK-UT-1 cells, revealed an impressive increase of most common modifications of histon 3, that positively support active gene transcription. The generation of SK-UT-1 cells knock-out for HDAC4 or HDAC9 lead to different outcomes: surprisingly, only the KO of HDAC9 increases the expression of some MEF2 target genes, as indicated at mRNA level and by the detection of incresed H3K27 acetylation and H3K4me3. Ablation of HDAC9, obtained through the CRISPR/Cas9 technology, is able to significantly suppress the transformed phenotype of SK-UT-1 cells and our studies evidenced that the oncogenic activity of HDAC9 in high grade LMSs is mainly due to its capability to repress MEF2 transcription factors, consequently converting MEF2 proteins into transcriptional repressors. Further investigations highlighted that HDAC9 KO LMS cells are characterized by an increased expression of FAS receptor and a subsequent high susceptibility to cell death. Our purpose is to propose a new therapeutic approach consisting in targeting the tumor with a combination of drugs: class IIa HDACs inhibitors, to displace the repressive complex between HDAC9 and MEF2 on the promoter of some MEF2 target genes, in combination with other drugs actually used in clinical to treat LMSs, such as Doxorubicin. Overall, the strategy aims to reduce tumor aggressiveness and concurrently, to induce apoptosis of cancer cells. Preliminary data *in vitro* show strong synergistic effects of the novel compound NKL-54, suggesting its possible future translation into *in vivo* tumoral models.
Overall, our study underlines how new epigenetic drugs can reprogram the tumor cells and potentiate the standard treatment approaches.

AIM OF THE STUDY

This PhD project starts from the observation that high grade uterine leiomyosarcomas are characterized by a frequent co-expression of MEF2 and the histone deacetylase 9. The aim of this work is to investigate the potential oncogenic role of HDAC9 in the tumorigenesis of LMSs and to clarify the transcriptional role of MEF2. To demonstrate the key role of HDAC9 in the aggressivity of these tumors, we plan to knock-out HDAC9 using the CRISPR/Cas9 technology in a high grade LMS cell line, SK-UT-1, and to describe the cell phenotype derived from gene ablation. Furthermore, we aim to selectively target the MEF2-HDAC9 complex by using selective class IIa HDACs inhibitors. For this purpose, we selected a novel compound, NKL-54, which presents a backbone derived from BML-210. The high sensitivity to cell death of the SK-UT-1 cells HDAC9$^{-/-}$, suggests to try a novel therapeutical approach consisting in the combination of the class IIa HDAC inhibitor NKL-54 with traditional mutagenic and cytotoxic agents, actually used in clinic. The potential enrichment of the apoptotic cell death derived from the combined strategy would be determined through the induction of caspases activity. In future, we hope that positive *in vitro* results would be validated also *in vivo,* in a xenograft model.

1. INTRODUCTION

1.1 Epigenetics: the meta-level of genetic regulation

For a long time, it has been speculated that cancer is the result of genomic and genetic alterations, such as amplifications, deletions, translocations and point mutations. The dramatic consequence of these modifications is the activation of onco-genes and the inactivation of tumor-suppressor genes. However, cancer development is not only linked to genetic changes but may also involve epigenetic modifications (Weinhold B., 2006). The term "epigenetics" indicates the inheritance of informations based on gene-expression levels, as opposed to genetics, which consists in the transmission on the basis of gene sequence. The most significative epigenetic modifications in mammals, and particularly in humans, are the methylation of DNA and the modifications of the histones (phosphorylation, acetylation, methylation, ubiquitylation, sumoylation and others) that occur at post-translational level (Fig.1) (Ropero S. and Esteller M., 2007). These events are necessary for the regulation of various cellular functions, but if they occur improperly, there could be negative consequences on homeostasis. Until now, the best known epigenetic mechanism is the DNA methylation, that consists in the addition or removal of a methyl group (CH3) to the DNA, principally at cytosine rich motifs (Weinhold B., 2006). DNA methylation on the promoter of a gene is translated into transcriptional repression and this event may regulate several processes, including genomic imprinting, X-chromosome inactivation, aging and also carcinogenesis (Hackett JA. and Surani MA., 2013). The first evidences of methylated DNA in cancer cells have been detected in 1983 and further studies demonstrated its association also to various genetic disorders (Weinhold B., 2006).

Chromatin is a tight complex of histone proteins and DNA localized inside the nucleus, (Annunziato A., 2008) and its modifications are also considered an epigenetic process. Several enzymes, RNAs and the addiction of acetyl groups (acetylation) can modify the histone-DNA complex, leading to chromatin structure and gene expression alterations. Amino groups of conserved lysine residues are often subjected to acetylation, which influences the activity of several proteins, such as transcription factors, histones and cytoskeletal proteins. Acetylation is a post-translational modification under the supervision of two antagonistic families of enzymes, the histone acetyl-transferases (HATs) and the histone deacetylases (HDACs), whose action should be strictly balanced (Ropero S. and Esteller M., 2007). The presence of acetylated lysines on histone tails is associated with a more relaxed chromatin state, favouring the recruitment of transcription factors on the promoter of specific genes, while the deacetylation of lysine residues is associated with a more condensed chromatin state and, consequently, to transcriptional gene silencing (Iizuka M. and Smith MM., 2003).

Moreover, a relaxed chromatin state is associated with other genome functions, such as chromatin assembly, DNA repair and recombination (Polo SE. and Almouzni G., 2015). The removal of acetyl groups from lysine residues by HDACs increases the ionic interactions between the positively charged histone proteins and the negatively charged DNA, promoting the compacting of the chromatin and the repression of gene transcription. The levels of histone acetylation play a crucial role in chromatin remodeling and in the control of gene transcription, in particular the acetylation of lysine residues of histone 3 (H3) and histone 4 (H4). In addition, histone deacetylases are also involved in the deacetylation of non-histone proteins, which are essential in the regulation of cell-cycle progression, differentiation and apoptosis.

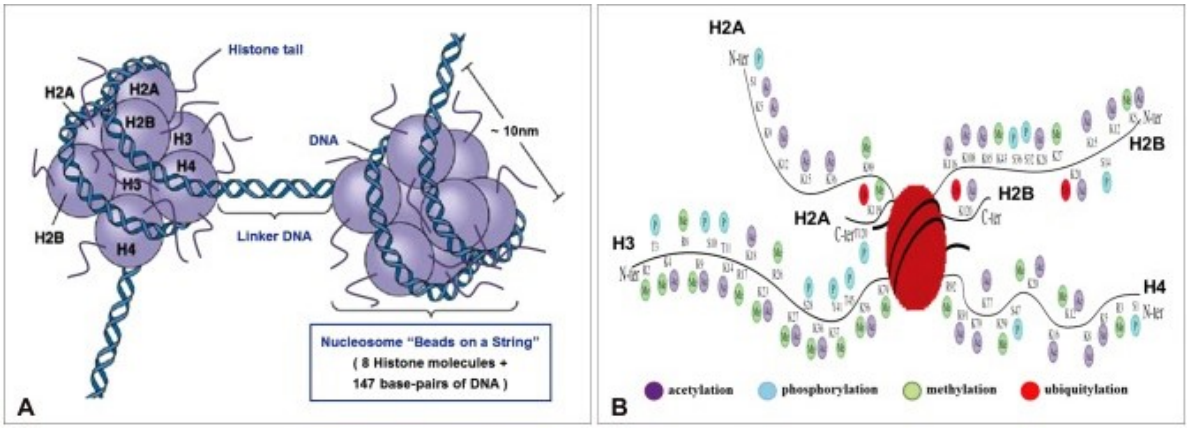


Fig.1: Representation of the nucleosome and major histone modifications: acetylation, methylation, phosphorylation and ubiquitylation (Kim WY., 2014).

1.2 General concepts on class IIa HDACs

1.2.1 Histone deacetylases family (HDACs)

In humans, eighteen histone deacetylases can be grouped into four different classes, according to their homology with gene orthologues in yeast (Rpd3, Hda1 and Sir2). Class I comprises HDAC1, -2, -3 and -8, that show homologies with Rpd3 gene, while class II includes HDAC4, -5, -6, -7, -9, -10 that present homologies with Hda1 gene. Class II HDACs are subdivided into classes IIa and IIb: histone deacetylase 4, -5, -7 and -9 belong to class IIa HDACs, whereas HDAC6 and 10 to class IIb. Class III includes Sirt1, 2, 3, 4, 5, 6 and 7 that are homologous to Sir2 in yeast and are also known as silent information regulators (SIR). HDAC11 represents the Class IV and its sequence shares similarities with both classes I and II (Fig.2) (Clocchiatti A. *et al*, 2013). Classes I, II and IV are characterized by a zinc-

dependent catalytic mechanism, whereas the sirtuins present a NAD^+-dependent catalytic mechanism (Clocchiatti A. *et al*, 2013).

Classes I and II share homologies in the catalytic domain, but present some differencies in the N-terminal region, extended only in class II (Yang XJ. and Seto E., 2008). The peculiar N-terminus domain of class IIa HDACs is involved in the interaction with relevant partners (Clocchiatti A. *et al*, 2013). Class I HDACs show preferentially a nuclear localization, whereas class II HDACs are present both in the nucleus and in the cytoplasm. Sirtuins are preferentially localized at subcellular level, such as in mitochondria (Clocchiatti A. *et al*, 2011).

The silencing of gene transcription exerted by class IIa HDACs occurs only in complex with different co-repressors; HDACs are involved also in non chromatin-related functions, for example in the deacetylation of non-histone proteins, including transcription factors involved in the cell-cycle transition, differentiation and apoptosis (Minucci S. and Pelicci PG., 2006).

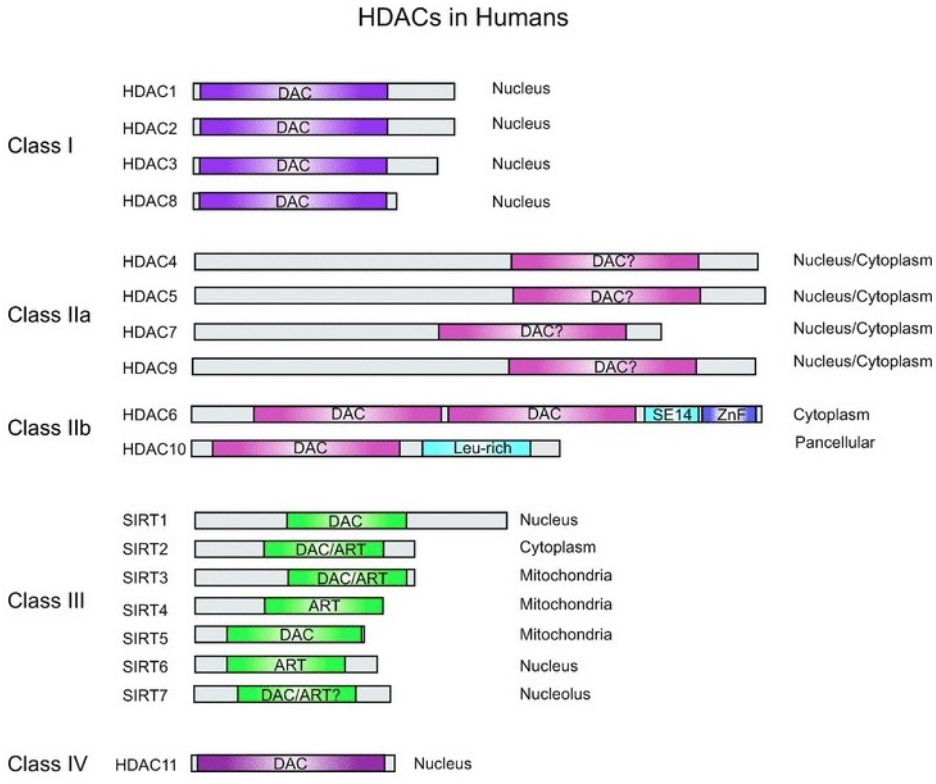


Fig.2: Classification of histone deacetylase family and subfamilies in humans (Clocchiatti A. *et al,* 2011).

1.2.2 Structure of class IIa HDACs and regulation of subcellular localization

The class IIa includes HDAC4, HDAC5, HDAC7 and HDAC9. These proteins share similar features: an amino-terminal region, which binds to several transcription factors necessary for the targeting of DNA; a carboxy-terminal deacetylase domain that is enzymatically 'inactive' in vertebrates (or at least inactive for acetyl-lysines), but able to acquire a deacetylase activity through the assembly of multiprotein complexes; a nuclear localization sequence (NLS) and a nuclear export sequence (NES), which regulate the nuclear/cytoplasmic shuttling (Fig.3) (Di Giorgio E. and Brancolini C., 2016).

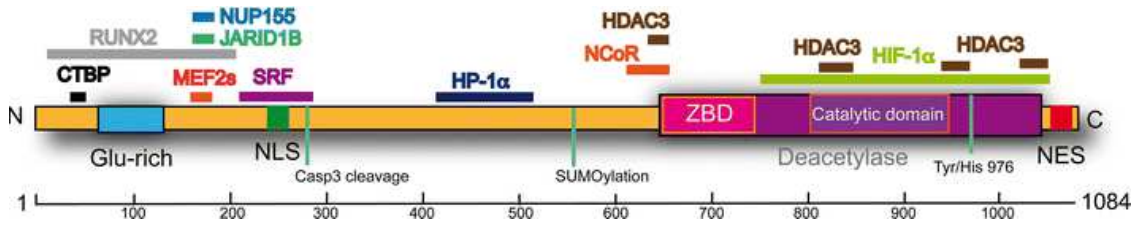


Fig.3: Schematic representation of class IIa HDACs structure (HDAC4) (Di Giorgio E. *et al,* 2015).

Several post-translational modifications, such as sumoylation, phosphorylation and caspase cleavages can modify the amino-terminal domain (Martin M. *et al*, 2007). Regulation of class IIa HDACs subcellular localization occurs in response to specific extracellular signals that activate different serine/threonine kinases. CaMK I and CaMK IV, members of the calcium/calmodulin-dependent protein kinase family (CaMK) are the main proteins that perform this role (Martin M. *et al*, 2007). In muscle tissue, these kinases promote the export of class IIa HDACs from the nucleus, consequently promoting the activation of MEF2 target genes and the myogenic differentiation (Backs J. *et al,* 2008). To the CaMK superfamily also belong PKD, MARK1 and MARK2, which are involved in the regulation of class IIa HDACs shuttling (Hanks SK., 2003; McKinsey TA., 2007). PKD is a regulator of class IIa HDACs during angiogenesis, muscle remodelling and also in B and T cells and it is a downstream effector of the PKC pathway (Parra M. *et al,* 2005). MARK1 and MARK2 phosphorylate the first serine of the 14-3-3 binding site localized in the N-terminal region (Dequiedt F. *et al,* 2006). Regulation of class IIa HDACs is obtained also by further kinases, such as the Salt inducible kinase 1 and Mirk/dyrk1B kinases (Berdeaux R. *et al,* 2007; Deng X. *et al,* 2005). PKA and GSK3-β are instead responsible of class IIa HDACs nuclear retention and protein degradation, respectively (Clocchiatti A. *et al*, 2011). HDACs localization is influenced also by phosphatases, which catalyze the removal of the phosphate groups. PP1 or PP2A stimulate

the nuclear accumulation of class IIa HDACs, in fact the treatment with some phosphatase inhibitors, such as the calyculin A and the okadaic acid, causes their cytoplasmic export (Paroni G. *et al,* 2008). The interaction with 14-3-3 proteins influences class IIa sub-cellular localization through different mechanisms: binding of 14-3-3 proteins can mask the NLS sequence, preventing the interaction with importin-alfa; alternatively, these interactions could promote a conformational change with the consequent exposition of the NES in the carboxy-terminal region, favouring the nuclear export (Fig.4) (Grozinger CM. and Schreiber SL., 2000). Thanks to the assembly of a multiprotein complex composed by HDAC3 and SMRT/N-CoR at the C-terminal domain, class IIa HDACs can exert their enzymatic activity, promoting lysine deacetylation (Fishle W. *et al*, 2002).

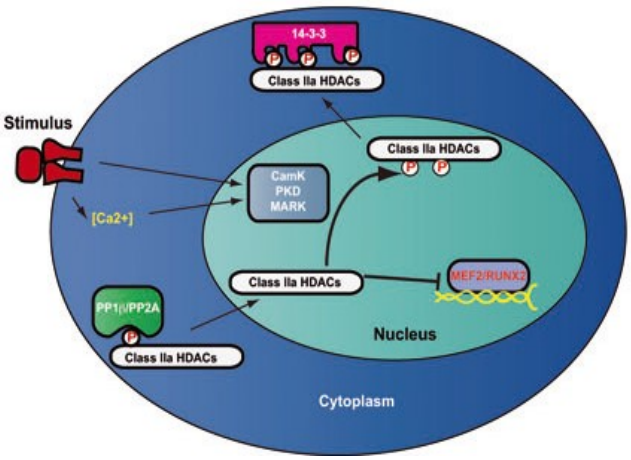


Fig.4: Shuttling of class IIa HDACs between the nucleus and cytoplasm. Phosphorylation of class IIa HDACs promotes their binding to 14-3-3 proteins and their nuclear export. Dephosphorilation stimulates class IIa HDACs nuclear retention and the repression of MEF2 targets (Clocchiatti A. *et al,* 2011).

1.2.3 Genomic organization of class IIa HDACs: an overview of HDAC9

Class IIa HDACs subfamily is complexly regulated at gene transcription level and this is mainly due to the existence of several transcriptional variants. Up to now, only a single isoform of HDAC4 has been described and this is composed by 26 coding exons and 1 non-coding exon (mRNA 8980 bp and ORF 3252 bp long) (Table 1) (Di Giorgio E. and Brancolini C., 2016). Two transcription variants are known for HDAC5: the longest RNA is 5324 bp long and produces a 3369 bp ORF. The second transcription variant lacks the exons 14 and 15 and encodes for a protein of 75 amino acids shorter, characterized by a deficient catalytic domain (Di Giorgio E. and Brancolini C., 2016). Two different splicing isoforms of HDAC7 have been characterized: the most common spliced isoform, which is composed by

25 exons, and the unspliced isoform with the first intron the follows the first ATG. The unspliced mRNA gives rise to two products: a small peptide of 7 amino acids and a protein of 22 amino acids (Zhou B. *et al*, 2011). Zhou B. *et al.* (2011) describe how the splicing of histone deacetylase 7 modulates smooth muscle cell proliferation and how their generation is supervised during vascular smooth muscle cells (VSMCs) differentiation.

Table 1. Class IIa histone deacetylases genomic organization.

Histone deacetylases	Localization	Extension	TSSs	SV	mRNA	Exons	Longer ORF
HDAC4	Chr 2q37.3 minus strand	353479	7	16 s 6 uns	1	27 (26c/1nc)	3256
HDAC5	Chr 17q21 minus strand	46893	8	17 s 6 uns	2	27 (26c/1nc)	3369
HDAC7	Chr 12q13.1 minus strand	37890	17	31 s 5 uns	2	25	2856
HDAC9	Chr 7p21.1 plus strand	912579	9	17 s 6 uns	6	23	3033

Extension: Genomic bp; mRNA: Described mature mRNAs; ORF: Open reading frame; S: Spliced; SV: Different splicing variant; TSS: Different transcription start site; Uns: Unspliced.
Data were taken from [6].

Table 1: Summary of the genomic localization of the different class IIa HDACs (Di Giorgio E. and Brancolini C., 2016).

In this study we mainly focused on histone deacetylase 9. Multiple protein isoforms of HDAC9 derived from alternative splicing processes and all of them may have different biological functions. The full-length sequence of HDAC9 contains an open reading frame of 3210 bp, which encodes for a protein of 1069 amino acids (exons 2–26, exon 1 is untranslated) (Petrie K. *et al,* 2003). MITR or HDAC9ΔCD (HDRP/MITR) is a truncated variant of HDAC9 (593 aa long), which lacks the deacetylase domain, therefore it is catalytically inactive (Petrie K. *et al,* 2003) (Fig.5).

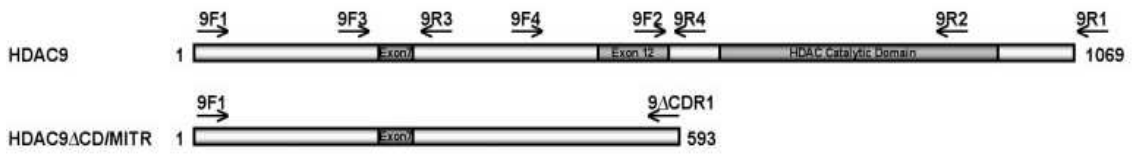


Fig.5: Representation of the full length isoform of HDAC9 and MITR (Petrie K. *et al,* 2003).

Evidences indicate that MITR accumulates in the nucleus and it is able to strongly repress the MEF2-dependent transcription, probably through the recruitment of co-repressors, such as CtBP (Zhang CL. *et al*, 2001) or the other class IIa HDACs (Zhang CL. *et al*, 2002).

1.2.4 Phylogenetic analysis of HDAC9

By evaluating the similarities (in terms of amino acid sequences) of the deacetylase domain of HDAC9 with the ones of other class II histone deacetylases, it is emerged a high phylogenetic correspondence with HDAC5 (Petrie K. *et al,* 2003). Based on the analysis of the HDAC catalytic domain, it is possible to subdivide the class II histone deacetylase in a further group, consisting of HDACs -6, -10, and -11 (Fig.6).

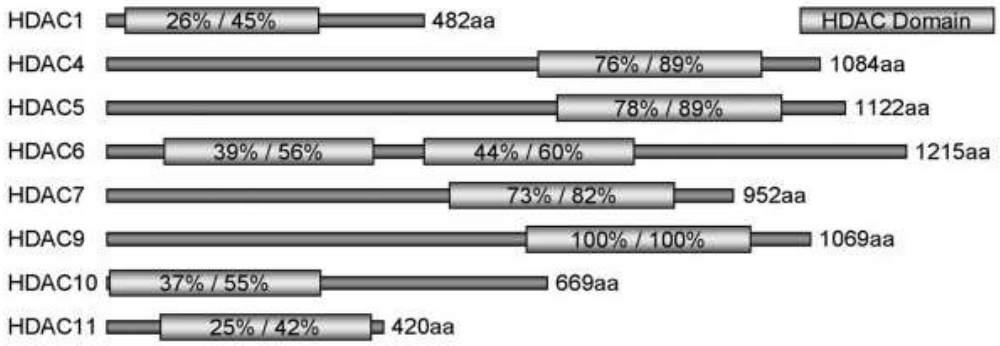


Fig.6: Phylogenetic analysis of HDAC9. In light gray are indicated the histone deacetylase domains and the percentage of amino acid identities (Petrie K. *et al,* 2003).

1.2.5 Distribution of HDAC9 transcripts in normal and cancer cell lines

Expression analysis of HDAC9 and its spliced isoforms, performed in various human tissues and cell lines, reveal that the full-lenght transcript and HDAC9ΔCD are more expressed in lung, skeletal muscle and in adult and fetal brain, although they are considerably more abundant in fetal tissue (Petrie K. *et al,* 2003). In normal hematopoietic tissues, the highest levels of the full-lenght HDAC9 were observed in cells expressing CD14 (monocyte/macrophage) and, to a lesser extent, CD19 (B cell) surface markers. Low levels of HDAC9 isoforms presenting the catalytic domain are expressed in the bone marrow, thymus and spleen. Further inspections in hematopoietic cell lines show that HDAC9 isoforms present a differential expression in the B cells and cell lines derived from B cell tumors. HDAC9 is generally expressed in pre-B cell acute lymphoblastic leukemia cell lines, B cell lymphoma cell lines and also in the plasma cell line U-266. HDAC9 expression has been also detected in some T cells. In particular, the T cell leukemia cell line MOLT-3 preferentially expresses HDAC9ΔCD. HDAC9 is not expressed in any acute myeloid leukemia cell lines with the exception of KG1 (a multi-lineage lymphomyelocytic cell line), which highly expresses HDAC9ΔCD. Up to now, data suggest that within the hematopoietic system, lymphoid and monocytic cells lack a detectable HDAC9 expression (Fig.7).

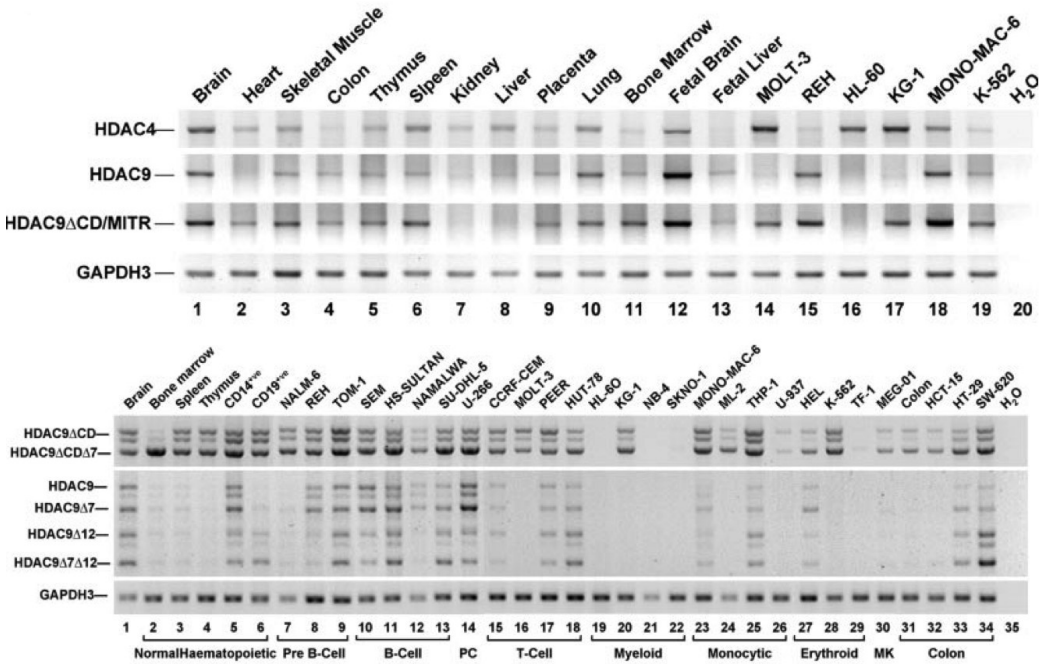


Fig.7: HDAC9 transcripts in different tissues. Upper panel shows the differential expression of HDAC4, HDAC9 and MITR in normal tissues and in leukemic cell lines. Lower panel indicates the HDAC9 isoforms in the B cell lineage and cell lines derived from B cell tumors (Petrie K. *et al,* 2003).

1.2.6 Class IIa HDACs binding partners

To negatively influence the transcription, class IIa HDACs need to associate into macromolecular complexes inside the nucleus (Clocchiatti A. *et al*, 2011). To selectively bind the DNA, class IIa HDACs need to interact with specific transcription factors. Different interactors associate with the N-terminal domain of class IIa HDACs, such as MEF2 (Myocyte Enhancer Factor-2), SRF (Serum Response Factor), RUNX2 (Runt Related Transcription Factor 2), CtBP (C-Terminal Binding Protein 1), or HP1 (Heterochromatin Protein 1). Actually, the MEF2 transcription factors family represents the most delineated class of HDAC binding partners (Arnold MA. *et al,* 2007; Li L. *et al,* 2018). Twelve amino acids localized in the N-terminal region of the deacetylase are involved in the binding to MEF2 (Di Giorgio E. *et al*, 2013). A mutant of HDAC4 deprived of these residues loses most of its functions (Di Giorgio E. *et al*, 2013). SRF is another member of the MADS box family of transcription factors and it is required for cell differentiation in diverse contexts (Owens GK. *et al,* 2004). RUNX2 belongs to the RUNX family of transcription factors and encodes a nuclear protein fundamental during the skeletal morphogenesis and the differentiation of osteoblasts (Vega RB. *et al,* 2004). Other transcriptional partners of class IIa HDACs are: GATA (Ozawa Y. *et al*, 2001), Forkhead (Liu R. *et al,* 2009), Ying and Yang 1 (Sucharov

CC. *et al*, 2008) and Hypoxia-inducible factors (HIFs) (Seo HW. *et al*, 2009). The recruitment of class IIa HDACs to DNA can be also driven by the association with hormone receptors inside the nucleus: estrogen receptor (ER) can interact with HDAC4, HDAC5 and HDAC9 (Leong H. *et al,* 2005) and androgen receptor (AR) can be bound by HDAC4 and HDAC7 (Karvonen U. *et al,* 2006). Several other potential partners of class IIa HDACs are still under investigation.

1.3 The Myocyte Enhancer Factor 2 (MEF2) family of transcription factors

1.3.1 The MEF2 family and gene mapping

MEF2 proteins are members of the MADS-box (Minichromosome maintenance genes agamous deficiens and serum response factor) family of transcription factors (TFs). *Drosophila*, *Caenorhabditis elegans* and *Saccharomyces cerevisiae* present a unique MEF2 gene, while vertebrates present four members: MEF2A (15q26), MEF2B (19q12), MEF2C (5q14) and MEF2D (1q12-q23) (Potthoff MJ. and Olson EN., 2007). MEF2 proteins of different species are composed by a similar N-terminal domain (composed by a MADS-box domain and an adjacent MEF2 domain), while the structure of the C-terminal transactivation domain is more heterogeneous (Potthoff MJ. and Olson EN., 2007). The sequences of MEF2A and MEF2C show high similarities, probably due to a duplication event, while MEF2B diverged from a single ancestral MEF2 gene (Potthoff MJ. and Olson EN., 2007). Differently from the transactivation domain, which presents a low amino acid identity (6%), the MADS and the MEF2 domains are well conserved among MEF2A and MEF2B, respectively for the 91% and 68% (Potthoff MJ. and Olson EN., 2007) (Fig.8).

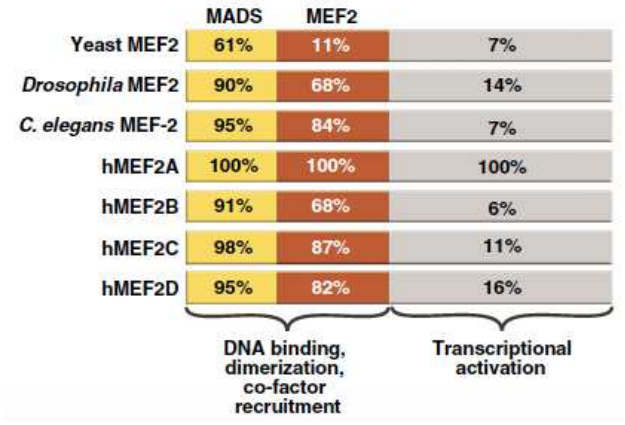


Fig.8: Conservation of the MADS-box, MEF2 and the transcriptional activation domains of MEF2 proteins between different organisms and human paralogues MEF2A, -B, -C, -D (Potthoff MJ. and Olson EN., 2007).

1.3.2 Structure of MEF2 proteins

MEF2 proteins contain a N-terminal MADS-box domain, a central MEF2 domain and a C-terminal transactivation domain. The MADS-box domain is a DNA-binding region (55 amino acids) essential for the recognition of the target sequences. MEF2 proteins also present an adjacent region of 29 amino acids, called central MEF2 domain, which, together with the MADS-box domain, allows the homo- and hetero-dimerization of MEF2 proteins, the binding to a conserved A-T rich DNA consensus sequence, YTA(A/T)$_4$TAR (Anders V. *et al*, 1995) and the interactions with transcriptional co-activators and co-repressors (Chen X. *et al,* 2017). The carboxyl-terminal region of MEF2 proteins is a transcriptional activation domain (TAD). It is different across the family members and it is often subjected to alternative splicing (Potthoff MJ. and Olson EN., 2007). The C-terminal domain of MEF2 proteins also presents a nuclear localization signal (NLS) (Wang AH and Yang XJ., 2001). MEF2 TFs are weak transcription activators and for this reason they exert their functions by creating molecular complexes with other TFs or co-activators (Ma K. *et al*, 2005; Kong NR. *et al*, 2016). Moreover, MEF2 activities are usually regulated by transcriptional, translational and post-translational modifications, such as phosphorylation, sumoylation, methylation, ubiquitylation and acetylation (Pon JR. and Marra MA., 2015)) (Fig.9).

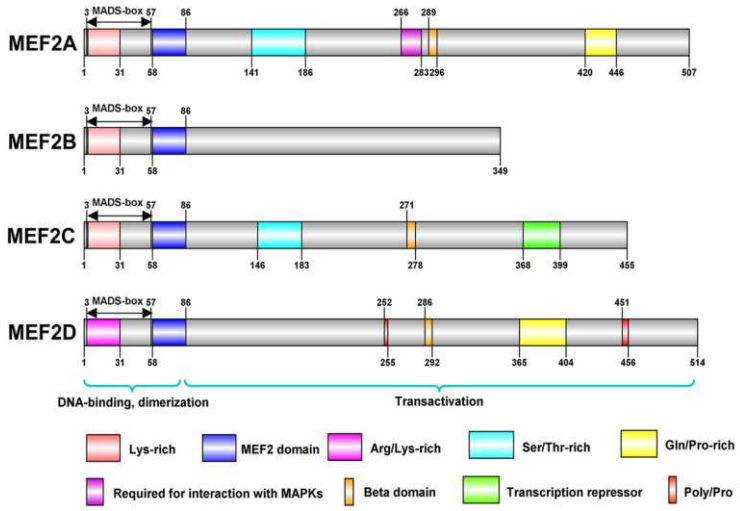


Fig.9: Structures of MEF2 proteins in humans. MEF2 family includes MEF2A, B, C, and D. (Chen X. *et al,* 2017).

1.3.3 MEF2 proteins regulate diverse developmental programs

MEF2s are defined as pleiotropic transcription factors (TFs), which supervise genetic and epigenetic programs that control cell differentiation, proliferation, morphogenesis, survival and apoptosis (Potthoff MJ. and Olson EN., 2007). Their expression is higher in skeletal, cardiac and smooth muscle cells, but also in neurons, chondrocytes, lymphocytes and bones (Potthoff MJ. and Olson EN., 2007). MEF2A, MEF2B, and MEF2D proteins are ubiquitously expressed (Molkentin JD. *et al*, 1995), while MEF2C is more expressed in skeletal muscle, heart and brain (Edmondson DG. *et al*, 1994).

Recent studies reveal the central role of MEF2 as regulator of several developmental programs, such as myogenesis, brain and lymphoid lineage development, endochondral bone ossification and vasculogenesis (Potthoff MJ. and Olson EN., 2007).

In adult tissues, MEF2 proteins regulate remodeling programs, cell survival and proliferation (Potthoff MJ. and Olson EN., 2007). Activation of MEF2 proteins at the G0/G1 transition of the cell cycle is necessary to mediate the expression of the early genes in response to growth factor stimulation (Di Giorgio E. *et al*, 2015). MEF2 also triggers the CDK inhibitor p21/CDKN1A exerting and anti-proliferative effect (Di Giorgio E. *et al*, 2015). MEF2 exerts a crucial role in stress responses, such as cardiac hypertrophy (Zhang CL. *et al*, 2002) and it actively stimulates apoptosis of T-cells by inducing the expression of *Nur77* (Youn HD. and Liu JO., 2000).

Post-translational modifications and the interaction with transcriptional co-factors are mechanisms that drive MEF2 proteins towards opposing cellular decisions, such as differentiation, proliferation and apoptosis (Potthoff MJ. and Olson EN., 2007).

1.3.4 Regulation of MEF2 activity by post-translational modifications

MEF2 activity is under a complex regulation that occurs through different transcriptional, translational and post-translational mechanisms. Many post-translational modifications (PTMs), including phosphorylation, sumoylation, methylation and acetylation may enhance or suppress their activities during different cellular programs, such as cell differentiation and cell growth (Pon JR. and Marra MA., 2015). Phosphorylation of MEF2 occurs at different sites: MEF2 proteins contain a single serine phosphorylation residue in the MEF2 domain and multiple phosphorylation sites in the transactivation domain. Studies demonstrate that the phosphorylation of Ser-59 in the MEF2 domain of MEF2C enhances the DNA binding and the transcriptional activity of MEF2C. This particular site is conserved in all members of the MEF2 family, highlighting its importance for the MEF2 functions (Molkentin JD. *et al,*

1996). Casein kinase II (CKII) is the enzyme involved in the phosphorylation of Ser-59 on MEF2. It is well known that the transactivation activity of many transcription factors is regulated by phosphorylation. The mitogen-activated protein (MAP) kinase family of serine/threonine kinases has been shown to play important roles in regulating MEF2 proteins: it has been previously described that the transactivation activity of MEF2A and MEF2C is stimulated by p38 mitogen-activated protein (MAP) kinase in response to growth or stress stimuli (Zhao M. *et al,* 1999). Kasler HG. *et al.* (2000) reported that also other MAPKs, such as JNK or ERK5, enhance the transcriptional activity of MEF2s, for example, in response to calcium flux in T cells. In contrast, by phosphorylating MEF2 in the transactivation domain, Cdk5 inhibits its activity and consequently, its protective effects in neurotoxicity-induced apoptosis (Gong X. *et al,* 2003). MEF2 proteins can also operate as transcriptional repressors, when they form complexes with co-repressors, through the binding with their N-terminal regulatory region. Phosphorylation of MEF2Dα1 by PKA provokes its association with class IIa HDACs during myogenesis (Sebastian S. *et al,* 2013). MEF2 activity can be also regulated by sumoylation: human MEF2D is sumoylated on Lys-439 and MEF2C Lys-391. Surprisingly, Cdk5 stimulates the sumoylation by phosphorylating Ser-444 that was also found to be essential for HDAC4 to stimulate the sumoylation (Grégoire S. *et al,* 2006). Studies evidence that also epigenetic modifiers regulate MEF2-dependent transcription via modifications of histones, in fact MEF2D can be methylated and demethylated at lysine 267 by G9a and LSD1, respectively, regulating its target genes during skeletal muscle differentiation (Choi J. *et al,* 2014). The acetylation of MEF2 is also important for MEF2 to enhance its DNA binding and transcriptional activity: the histone acetyltransferases p300, PCAF and CBP acetylate MEF2 proteins on conserved lysine residues. The transactivation domain of MEF2C is, for example, a target of p300 in differentiating myocytes (Ma K. *et al,* 2005).

1.3.5 Roles of MEF2 in human diseases

Many pathological conditions, including neurological disorders, cardiac dysfunctions and cancer are characterized by alterations of MEF2 activity (Pon JR. and Marra MA., 2015).
Decreased levels of MEF2C produces the MEF2C haploinsufficiency syndrome (Paciorkowski AR. *et al,* 2013), while increased MEF2C expression has been associated with congenital heart defects (Friedrich FW. *et al,* 2013). Only recent studies demonstrated the involvement of MEF2 proteins in cancer development. Specifically, MEF2 proteins have pro-oncogenic roles in hepatocarcinomas and in some hematological malignancies, which are

associated to increased expressions, mutations or genetic rearrangements of these transcription factors. By contrast, it has been described their actions as tumor suppressors in the case of soft-tissue sarcomas and in non-Hodgking lymphomas (Di Giorgio E. *et al*, 2017).

1.3.6 Alterations of MEF2 in cancer

1.3.6.1. Oncogenic activity of MEF2 family genes

MEF2 proteins mainly act as oncogenes in hematological cancers. Increased MEF2C expression is characteristic of immature T-cell acute lymphoblastic leukemia (Homminga I. *et al,* 2011). In this kind of tumor, increased expression of MEF2C is frequently associated to rearrengements or alterations affecting interaction proteins and consequently, leading to the inhibition of cell differentiation. B-cell acute lymphoblastic leukemias also exhibit recurrent rearrangements producing oncogenic fusion proteins (Prima V. and Hunger SP., 2007). Studies demonstrate that MEF2C expression is increased in myeloid leukemia patients, promoting cell migration, invasion and high ability of colony formation. Its normal expression in myeloid progenitor cells suggests the possible involvement of MEF2C in conferring stem-cell like properties (Canté-Barrett K. *et al,* 2014). In contrast to MEF2C and MEF2D, up to now there are not implications of MEF2A and MEF2B in leukemia development (Pon JR. and Marra MA., 2015).

MEF2 proteins are also involved in solid tumors: MEF2A, MEF2C and MEF2D mRNA and protein levels are increased in hepatocellular carcinoma cells and are associated to a poor prognosis (HCC) (Bai X. *et al,* 2008; Ma L. *et al,* 2014). It is reported that MEF2A, MEF2C and MEF2D promote epithelial-mesenchymal transition (EMT), favouring the acquisition of HCC cell invasiveness (Pon JR. and Marra MA., 2015). MEF2 proteins may have pro-tumorigenic activities in other types of carcinoma, as in pancreatic ductal adenocarcinoma (Zhang JJ. *et al*, 2014). Reduced expression of YY1, a negative regulator of MEF2C expression, increases the invasiveness of pancreatic adenocarcinoma cells by the indirect activation of the MMP10 (Zhang JJ. *et al,* 2014). Although still not completely characterized, oncogenic roles of MEF2 proteins in other types of carcinoma may be predicted from the recurrence of MEF2 gene alterations. Data from the cBioPortal database report that 6-21% of ovarian serous cystadenocarcinomas, uterine endometriod carcinomas, lung squamous cell and adenocarcinomas, pancreatic adenocarcinomas, stomach adenocarcinomas, esophageal carcinomas, adrenocortical carcinomas and bladder urothelial carcinomas present an amplification of MEF2, whereas up to 2.6% of these cancers contain a deletion of MEF2 gene (Pon JR. and Marra MA., 2015) (Fig.10).

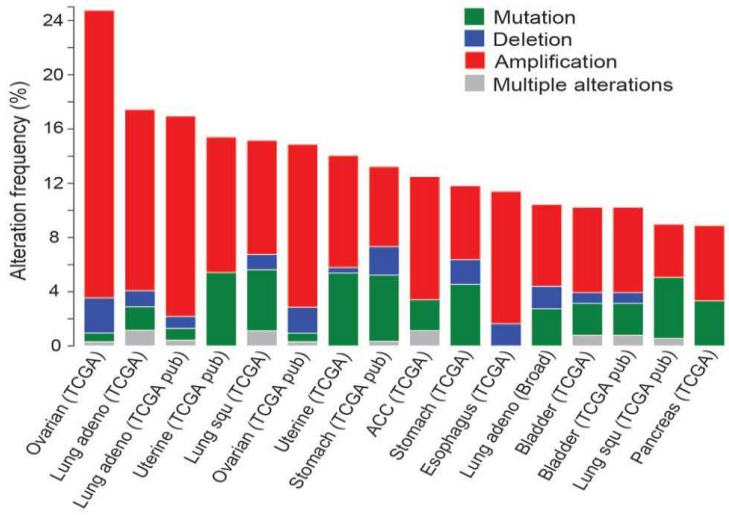


Fig.10: Carcinomas in which MEF2 proteins mainly act as oncogenes. Alterations affecting MEF2 genes are: gene amplifications (red), mutations (green), deletions (blue) and multiple alterations (grey) (Pon JR. and Marra MA., 2015).

1.3.6.2 MEF2 family proteins as tumor suppressors

Several studies highlight also tumor suppressor activities of MEF2 proteins. MEF2 target genes are repressed in lipo- and leiomyosarcomas (Di Giorgio E. *et al*, 2013; Di Giorgio E. *et al*, 2017) and this repression often correlates with decreased levels of MEF2C and MEF2D and increased expression of negative regulators of MEF2, such as HDAC4 and PI3K/AKT signaling (Di Giorgio E. *et al*, 2013). In rhabdomyosarcoma cells, MEF2D expression is usually lost (Zhang M. *et al,* 2013) and a less active isoform of MEF2C (α1) tends to be expressed compared to MEF2Cα2 (Zhang M. *et al,* 2015). These alterations are reflected into the inhibition of cell differentiation and the induction of cell proliferation, anchorage independent-growth and cell migration (Zhang M. *et al*, 2013; Zhang M. *et al*, 2015).

Tremblay AM. *et al*. (2014) described a significant repression of MEF2C and MEF2D in a human embryonal rhabdomyosarcoma model, where YAP1 activity is elevated. The study reports how sustained YAP1 functions are responsible for the expression of two MEF2 repressors, Twist1 and Cabin1, able to displace MEF2 proteins from the promoters of myogenic differentiation genes (Tremblay AM. *et al*, 2014).

Muations of MEF2B have a crucial role in non-Hodgkin lymphomas: heterozygous somatic non-synonymous and indel mutations are reported in 8-18% of diffuse large B-cell lymphoma (DLBCL), 13% of follicular lymphoma (FL) and 3-7% of mantle cell lymphoma (MCL). MEF2B mutations at some residues (7% at K4, 8% at Y69, 33% at D83 in DLBCL and FL, and K23R mutations in MCL) give rise to nonsense, frameshift and stop codon read-through mutations in the transactivation domain (Morin RD. *et al,* 2011). Studies speculate that loss of

MEF2B activity may repress DLBCL cell chemotaxis, favouring the cell migration outside of germinal centers and therefore contributing to DLBCL and FL development. In HEK-293 cells, alterations of MEF2B are correlated to increased expression of the oncogene MYC and reduced levels of the tumor suppressor, TGFB1 (Pon JR. *et al,* 2015). Although the impact of the MEF2 on tumorigenesis is still undefined, tumor-associated alterations have been recently observed also in estrogen receptor-positive (ER) breast cancer (Clocchiatti A. *et al*, 2013). Studies evidenced a correlation between the down-regulation of some MEF2 target genes and the aggressiveness of ER^+ breast tumors. Moreover, data revealed an association between increased class IIa HDACs expression and reduced patient's survival (Clocchiatti A. *et al*, 2013). In particular, class IIa HDACs display a pro-survival effect by repressing the pro-apoptotic NR4A1/Nur77 (Clocchiatti A. *et al*, 2013).

Breast cancers overexpressing the tyrosine kinase receptor HER2 (also known as ERBB2) present significant dysregulation of the MEF2–HDAC axis: HDAC7 is over-expressed, while some MEF2-target genes are strongly repressed (Clocchiatti A. *et al*, 2015).

The investigation of the epigenetic status of the CDKN1A promoter leads to evaluate the involvement of HDAC7 in the regulation of CDKN1A transcription; the recruitment of HDAC7 on CDKN1A promoter occurs at a specific region (+1.5 kb), where HDAC7 highly influences epigenetic changes and consequently, the recruitment of MEF2 proteins (Clocchiatti A. *et al*, 2015). Overall, the study suggests that the MEF2–HDAC axis controls cell proliferation, principally by modulating the CDK inhibitor p21/ CDKN1A.

1.3.7 Tumorigenic potential of the MEF2-HDACs axis

Gene expression can be influenced by class IIa HDACs, exclusively through the co-operation with some DNA-binding factors (Di Giorgio E. *et al*, 2013). The transcription factors MEF2 are actually known as the main partners of the class IIa HDACs. The role of the MEF2-class IIa HDACs axis in tumorigenesis has been extensively studied by Di Giorgio E. *et al.* (2013), which propose a model that highlights the oncogenic potential of axis alterations. To determine the contribution of HDAC4 in cell growth and proliferation, a nucleus-localized version (TM) of HDAC4, defective in the 14-3-3 binding site, was expressed into murine fibroblasts (NIH 3T3 cells). HDAC4 TM expressing cells exhibit some morphological changes, overcome the contact inhibition and increase the motility and proliferation, revealing a transformed phenotype similar to that of HRasV12 transformed cells. NIH 3T3 cells, expressing HDAC4 TM, reveal their transforming properties also in immunosuppressed nude mice.

1.3.8 Dysregulation of MEF2-HDAC9 axis in cancer

Immunological studies report the involvement of MEF2D in the tumorigenesis of childhood pre-B acute lymphoblastic leukemia (ALL). Genomic analysis evidence that in these kind of tumors, the introns of MEF2D and DAZAP1 are often subjected to chromosomal translocations t(1;19)(q23;p1), that give rise to MEF2D-DAZAP1 and DAZAP1-MEF2D chimeric proteins. When ectopically expressed, they are able to promote cell proliferation. Interestingly, the fusion of DAZAP1 to the C-terminal domain of MEF2D increases the transcriptional capabilities of MEF2D, while DAZAP1/MEF2D is no more able to bind the genomic DNA (Prima V. *et al*, 2005; Prima V. *et al*, 2007).

Next generation studies allow the identification of further fusion proteins of MEF2D affecting pediatric patients. HDAC9, as consequence, is up-regulated by the chimeric MEF2D-BCL9 and MEF2D-HNRNPUL1, contributing to the repression of genes essential for B-lineage differentiation, such as *RAG1* (Liu YF. *et al*, 2016).

Alterations of MEF2D and HDAC9 are documented also in solid tumors, such as in lung cancer and in rhabdoid cancer cell lines. In these contexts, it is frequent the overexpression of HDAC9 in combination with high levels of MEF2D, which lead to *BRM* silencing (Liu G. *et al*, 2017). HDAC9 and MEF2D bind to the promoter of *BRM*, a member of the multiproteins complex SWI/SNF, close to two insertional polymorphisms, consequently creating two MEF2 binding sites (BRM-741: TTAAA and BRM-1321: TATTTTT). BRM expression may be restored by the specific HDAC9 inhibition that permits to regulate MEF2D transcriptional activity. Thus, this suggests how the epigenetic mechanisms drive the alteration of important regulators of chromatin status and gene expression in several contexts (Suvà ML. *et al*, 2013).

From genetic dysregulations observed in tumorigenic contexts, it may be possible to reconstruct the missing pieces of a highly functional framework existing between MEF2D and HDAC9; physiologically, HDAC9 is a MEF2-target gene and MEF2D, as a transcriptional activator, binds to HDAC9 promoter favouring its transcription. Moreover, this interaction is also governed by a strong negative feed-back: the HDAC9 repression leads to the down-regulation of MEF2D activities, maintaining the physiological levels of both proteins. Therefore, high levels of HDAC9 and MEF2D in tumorigenical context may be explained by an alteration of this circuitry (Fig.11).

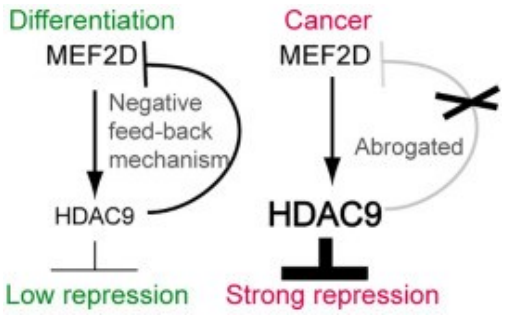


Fig.11: Schematic representation of the negative feedback mechanism between HDAC9 and MEF2D in normal and in tumorigenic contexts (Di Giorgio E. *et al*, 2018).

1.3.9 Sarcomas as a model to study the MEF2-HDACs axis in cancer

A deep investigation of several cancer types, done by scrutinizing the Oncomine database, revealed that soft-tissue sarcomas are characterized by the strongest alteration of the MEF2-HDAC axis (Di Giorgio E. *et al*, 2013).

In details, 25 genes containing a MEF2-binding site in the proximal promoters were found to be significantly repressed by HDAC4 in soft tissue sarcomas and in particular in leiomyosarcomas (Fig.12) (Di Giorgio E. *et al*, 2013).

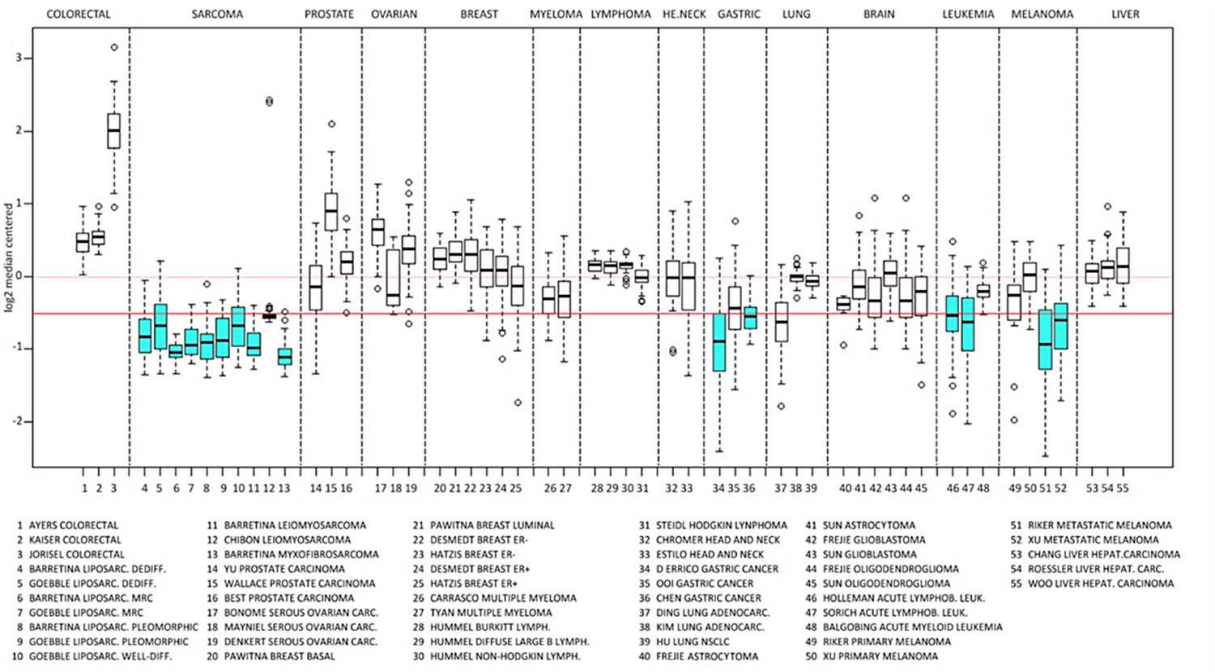


Fig.12: Expression of the MEF2 target genes in human tumors. In light-blue are indicated tumors where the MEF2 signature is significantly below zero (Di Giorgio E. *et al*, 2013).

1.4 Soft tissue sarcomas: uterine leiomyosarcoma (LMS)

Sarcomas are a group of tumors of mesenchymal origin (Xiao W. *et al*, 2013). These tumors are most common in the bones, muscles, cartilages, tendons, nerves, fat and blood vessels or any tissue that protects or supports the organs of human body (Singh Z., 2017). The most recent epidemiological data report that sarcomas represent about 20% of all solid tumors that arise in pediatric age and less than 1% of all solid malignant tumors in adults. Soft tissue sarcomas are the most diagnosed tumors, while malignant bone cancers represent just over 10% of sarcomas (Burningham Z. *et al*, 2012). Soft tissue sarcomas (STS) can originate from adipocytes (liposarcoma), vascular tissues (angiosarcoma), peripheral nerve tissues (malignant peripheral nerve sheath tumor), smooth muscle (leiomyosarcoma) or striated muscle (rhabdomyosarcoma) and from others (such as undifferentiated pleomorphic sarcoma). Among soft tissue sarcomas, leiomyosarcoma (LMS) represents an extremely rare form of malignant smooth muscle tumor, which represents the 5-10% of soft tissue sarcomas (Fig.13) (Singh Z. *et al*, 2018). This type of cancer can often remain dormant for long periods of time, as well as recur after years. It is not very responsive to chemotherapy or radiation, thus it is considered a resistant cancer type (Reed D. and Altiok S., 2011).

Actually, the main available treatments are based on surgery and radiotherapy, but unfortunately, 40% of treated patients develop local recurrences or metastasis. Because of these high percentages, therapeutic strategies in use are mainly palliative (Ducie JA. and Leitao MM., 2016).

Recently, genomic studies classified more than 70 heterogeneous types of adult soft tissue sarcomas in two broad classes: sarcomas with a simple karyotype and those with a complex karyotype (Cancer Genome Atlas Research Network, 2017).

Adult soft tissue sarcomas develop frequent copy-number alterations that mainly affect *MDM2*, *TP53*, *CDKN2A*, *RB1* and *ATRX* genes. *MDM2* gene is often subjected to amplifications in dedifferentiated liposarcomas (DDLPS), while deletions of *TP53* are frequently detected in 9% of LMSs, 16% of undifferentiated pleomorphic sarcomas (UPS) and 12% of myxofibrosarcomas (MFS). *RB1* deletions are found in 14% of LMSs, 16% of UPS, and 24% of MFS and deletions of *CDKN2A* are mostly found in 8% of LMS, 20% of UPS, and 18% of MFS (Cancer Genome Atlas Research Network, 2017).

Studies on leiomyosarcomas highlight the high recurrence of deletions and mutations of *PTEN*, that affect the PI3K-AKT-MTOR signaling pathway; also frequent amplifications and up-regulations of *IGF1R*, *AKT*, *RICTOR* and *MTOR* have been described (Cancer Genome Atlas Research Network, 2017). Thanks to the recent progress in clinical studies, more

effective drugs for LMS patients have been proposed, in particular TORC1/TORC2 inhibitors and dual PI3K/MTOR inhibitors, that may overcome traditional MTOR inhibitors (e.g. everolimus and temsirolimus), due to their indirect effect on AKT up-regulation (Cancer Genome Atlas Research Network, 2017).

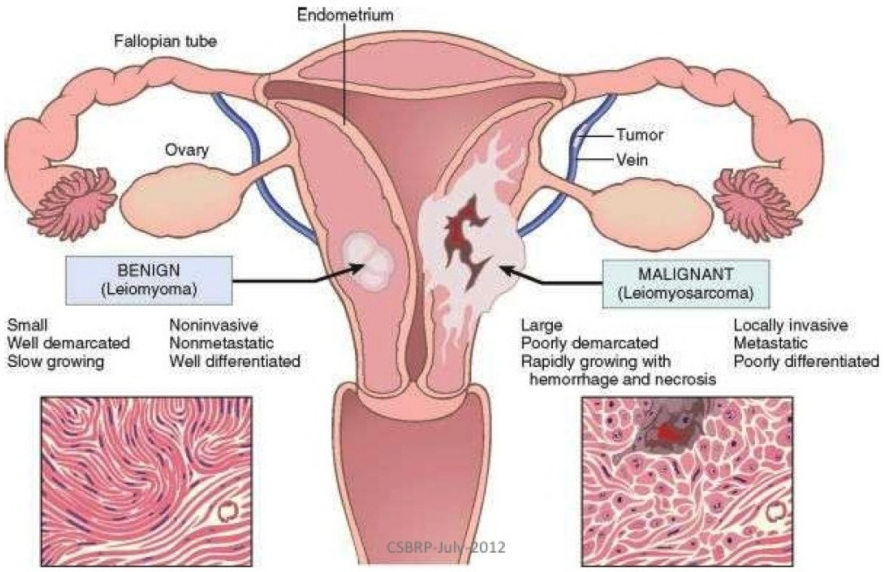


Fig.13: Representation of a benign tumor of the myometrium (leiomyoma) and a malignant tumor of the same origin (leiomyosarcoma) (http://aftercervicalcancer.blogspot.com/2012).

1.5 Inhibition of histone deacetylases in cancer therapy: a hope for the treatment of sarcoma

Given their involvement in approximately all cellular functions and their indispensable roles, HDACs may not be considered at a first sight as attractive targets for therapy, due to the possible interference of HDAC inhibitors with several physiological processes and therefore high risk of side effects (Ceccacci E. and Minucci S., 2016). Indeed, at the biginning, the first HDAC inhibitors were characterized for their antitumoral properties *in vitro,* before the discovery that they were able to inhibit histone deacetylase proteins: their use in preclinical models, showed a significant therapeutic window, with a selective cytotoxicity against tumoral cells and weak effects on normal ones (Ceccacci E. and Minucci S., 2016). Histone deacetylase inhibitors (HDIs) represent nowadays a considerable class of epigenetic drugs under study, which represent an opportunity for cancer therapy for several aspects: the most important might be the possibility to revert drug resistance and secondly, to prevent non-

responsiveness to anti-neoplastic drugs (Helin K. and Dhanak D., 2013). The applicability of HDIs in oncology may be useful for two main reasons:

a) Cancer cells are characterized by an enhanced degree of heterochromatinization compared to normal cells, making cancer genomes inaccessible to DNA-damage response enzymes (Carrier F., 2013).

b) Several tumor suppressor genes, including some pro-apoptotic genes, are inactivated in cancer cells because of ipo-acetylated or hyper-acetylated promoters (Hatziapostolou M. and Iliopoulos D., 2011).

These observations have incited the treatment of cancer cells with small molecules that target HDACs to relax the chromatin, consequently allowing the activation of DNA-damage responses, and to restore acetylation state at promoters of key genes. Histone deacetylase inhibitors represent a new class of anti-cancer agents. They display cytostatic effects, especially through the induction of p21 and the blockage of the cell cycle (Richon VM. *et al,* 2000) and the activation of apoptosis (Henderson C. and Brancolini C., 2003). Some HDIs stimulate *in vivo* the clearance of tumoral cells from the immune system (Maeda T. *et al*, 2000; Magner WJ. *et al,* 2000) or block angiogenesis (Rossig L. *et al*, 2002; Deroanne CF. *et al*, 2002). Despite these promising anti-neoplastic properties, the use of HDIs in clinic is slower than expected, because of efficacy (most are observed in selected cancer types, especially in haematological diseases) and safety (typical side effects are fatigue, diarrhoea, bone marrow toxicity, thrombocytopenia) issues. In fact, up to now the US FDA (the United States Food and Drug Administration) approved only few HDAC inhibitors for the treatment of T-cell lymphoma: Vorinostat (2006), Romidepsin (2009), Belinostat (2014) and Panobinostat (2015) (Goey A. *et al*, 2016) (Table 2).

Table 1. HDAC inhibitors classified according to: (a) status of clinical advancement; (b) HDAC(s) targeted and (c) chemical class

Compounds	Target	Class	Highest phase trial
Panobinostat (LBH-589)	Pan-HDAC inhibitor	Hydroxamic acids	Approved in 2015 for multiple myeloma
Belinostat (PXD101)	Pan-HDAC inhibitor	Hydroxamic acids	Approved in 2014 for PTCL
Romidepsin (desipeptide-FK228)	Pan-HDAC inhibitor	Cyclic tetrapeptides	Approved in 2009 for CTCL
SAHA(Vorinistat, Zolinza)	Pan-HDAC inhibitor	Hydroxamic acids	Approved in 2006 for CTCL
Valproic acid	Pan-HDAC inhibitor	Short-chain fatty acids	Phase III
Tacedinaline (CI994)	Subclass I-selective inhibitor (HDACs 1, 2 and 3)	Benzamides	Phase III
Givinostat (ITF2357)	Pan-HDAC inhibitor	Hydroxamic acids	Phase II
Resminostat (4SC201)	Pan-HDAC inhibitor	Hydroxamic acids	Phase II
Abexinostat (PCI24781)	Pan-HDAC inhibitor	Hydroxamic acids	Phase II
Rocilinostat (ACY1215)	Selective class II HDAC inhibitor	Hydroxamic acids	Phase II
Quisinostat (JNJ-26481585)	Pan-HDAC inhibitor	Hydroxamic acids	Phase II
Practinostat (SB939)	Inhibit class I, II and IV HDACs	Hydroxamic acids	Phase II
Mocetinostat (MGCD0103)	Specific against class I and IV HDACs	Benzamides	Phase II
Entinostat (MS275-SNDX-275)	Class I HDAC inhibitor	Benzamides	Phase II
Sodium phenylbutyrate	Inhibit class I and II HDACs	Short-chain fatty acids	Phase II
AR42	Pan-HDAC inhibitor	Hydroxamic acids	Phase I
4SC202	Selective class I HDAC inhibitor	Benzamides	Phase I
Pyroxamide (NSC696085)	Inhibitor of affinity-purified HDAC1	Hydroxamic acids	Phase I
CHR-3996	Selective class I HDAC inhibitor	Hydroxamic acids	Phase I
CHR-2845		Hydroxamic acids	Phase I

Abbreviations: CTCL = cutaneous T-cell lymphoma; HDAC = histone deacetylase; PTCL = peripheral T-cell lymphoma. Adapted from Valente and Mai (2014), http://www.fda.gov/default.htm.

Table 2: Classification of HDAC inhibitors: chemical class, targeted HDACs and status of phase trial (Ceccacci E. and Minucci S., 2016).

Considering the recent evidences about the pro-oncogenic properties of class IIa HDACs (Wilson AJ. *et al*, 2008; Clocchiatti A. *et al*, 2011) and their impact on epigenetics (Hohl M. *et al*, 2013), a way to bypass the side effects of current HDACs inhibitors might consist in targeting class IIa HDACs.

1.5.1 Selective class IIa HDACs inhibitors

Three different peculiarities of class IIa HDACs have been exploited to design specific inhibitors (Di Giorgio E. *et al*, 2015):

a) Targeting the catalytic site (Zn^{2+} binding domain).
b) Targeting the shuttling between nucleus and cytoplasm.
c) Targeting the amino-terminal domain.

a) Targeting the catalytic site (Zn^{2+} binding domain)

The study and the careful characterization of the crystal structure of the class II deacetylase domain has encouraged the development and the synthesis of many inhibitors, able to selectively target class II histone deacetylases. Most of inhibitors are hydroxamates compounds, stemmed from SAHA (Vorinostat) structure, targeting the catalytic site of HDACs (Fig.14) (Henkes LM. *et al*, 2012). The realization of SAHA derivatives, with higher specificity against class II HDACs, required small modifications of the CAP, the hydrophobic domain that interacts with aminoacids delimiting the deacetylase catalytic site, and of the ZBG, the zinc binding group, capable of chelating the Zn^{2+} in the catalytic sites of HDACs (Marek L. *et al*, 2013). Selective targeting of class II HDACs also required some changes in the connecting unit (CU), generally a linker hydrophobic region that mymics the acetyl-lysin, to better fit to the peculiar hydrophobic catalytic site of class II deacetylases (Di Giorgio E. *et al*, 2015; Zwergel C. *et al,* 2016).

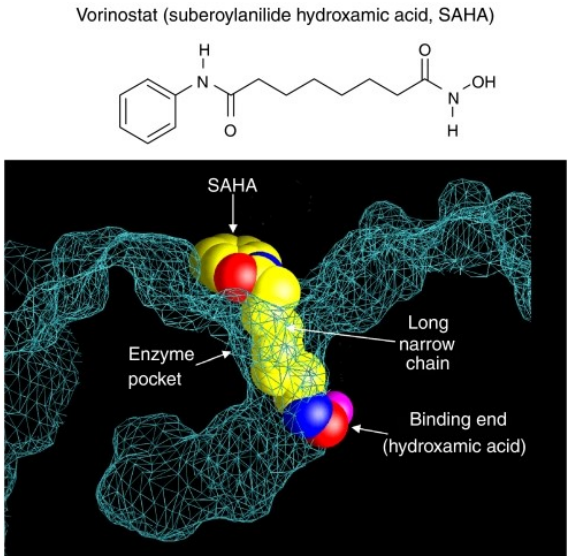


Fig.14: Crystallographic structure of SAHA bound to the pocket of the catalytic site of HDAC4 (Richon VM., 2006).

The most characterized hydroxamates-like drugs (SAHA derivatives) are:

- MC1568 and MC1575 that are debated selective class IIa histone deacetylase inhibitors specific for HDAC4 (Fleming CL. *et al*, 2014; Mai A. *et al*, 2005; Duong V. *et al*, 2008) and HDAC6 (Saito S. *et al*, 2011; Lemon DD. *et al*, 2015). They derived from class I HDACs inhibitors aroyl-pyrrolyl-hydroxyamides (APHAs) modified in the linker hydrophobic region. These two synthetic compounds seem to be selective for class IIa HDACs and evidences indicate their anti-proliferative effects *in vivo* and *in vitro*. Suppression of IL-8 expression in human melanoma cells (Venza I. *et al*, 2013) and the strong induction in $p21^{waf1}$ gene and protein expression in breast cancer cells, confirm their efficacy. However, the effective inhibition of class IIa HDACs has been claimed (Lemon DD. *et al*, 2015).
- Recently was found that a series of diphenylmethylene hydroxamic acids can act as novel and selective class IIa HDACs inhibitors. The original compound, N-hydroxy-2,2-diphenylacetamide (compound 6 in the original manuscript) has sub-micromolar class IIa HDAC inhibitory activity and the rigidified oxygen analogue N-hydroxy-9H-xanthene-9-carboxamide (compound 13 in the original manuscript) presents a structure that makes it more selective for HDAC7 (Tessier P. *et al*, 2009).

  TMP-195 and TMP-269 are class IIa HDACs specific inhibitors in which the classical hydroxamic Zn^{2+} binding domain, typical of class I HDIs, is substituted by a trifluoromethyloxadiazolyl group (TFMO) (Lobera M. *et al*, 2013). The ring structure of TFMO group increases their stability and, differently from hydroxamate, it acts as a non-chelating metal binding group, which interacts with the "catalytic" Zn^{2+} through weak

electrostatic interactions. As a consequence, the TFMO molecules have fewer off-targets compared to hydroxamates. Bonelli S. *et al*. (2018) proposes the novel inhibitor TMP-195 as a promising candidate for immunological diseases, given its ability to modify the tumor microenvironment by favouring the recruitment and differentiation of highly phagocytic and immunostimulatory macrophages within tumors (Bonelli S. *et al*, 2018). Evidences of anti-neoplastic properties of TMP-195 are reported also by Guerriero JL. *et al.* (2017) with a transgenic mouse model of luminal B-type mammary carcinoma, in which this novel drug is able to promote tumor suppression by enhancing the antitumor activity of macrophages that boost activation of cytotoxic T lymphocytes (Guerriero JL. *et al*, 2017). *In vivo* treatment induces changes in monocyte gene expression without affecting lymphocyte gene expression and promotes a type 1 proinflammatory monocyte phenotype.

- LMK235 ((N-((6-(hydroxyamino)-6-oxohexyl)oxy)-3,5-dimethylbenzamide) (compound 19i in the original manuscript) represents an innovative evolution of hydroxamate-based HDAC inhibitors. The chemical modification that improved its selectivity toward human HDAC4 and HDAC5, is the novel alkoxyamide connecting unit linker region (Hansen FK. *et al*, 2014). When compared to Vorinostat, the compound LMK235 significantly enhances the cytotoxic effect on human ovarian carcinoma cells (A2780) and breast cancer cells (MDA-MB-231). The combination of LMK235 with cisplatin also contributes in enhancing the chemosensitivity of the sublines under investigation (Marek L. *et al*, 2013).

Unconventional inhibitors (not SAHA derivatives) are:

- Tasquinimod, that was not rationally designed or screened to target HDACs. Nevertheless, this carboxamide is able to enter the zinc-binding domain of HDAC4 keeping it in the inactive form and thus reorganizing the catalytic site of HDAC4 (Isaac JT. *et al,* 2013). The peculiarity of this drug is the selective targeting of the "structural" Zn^{2+} that represents the "core" of the Zinc Binding Domain, distinctive feature of class II HDAC catalytic site. Some authors proposed Tasquinimod as an anti-angiogenetic drug, since it suppresses the angiogenic switch induced by tumor hypoxia, resulting in an enhanced therapeutic feedback of prostate cancer cells to radiation (Dalrymple SL. *et al,* 2012). Anti-cancer efficacy is being evaluated in pre-clinical models and recently, Tasquinimod has entered registration Phase III evaluation for the treatment of castration resistant prostate cancer.
- Ethyl 5-(trifluoroacetyl) thiophene-2-carboxylate is the founder of a class of compounds, the trifluoroacetylthiophenes, which targets with a discrete specificity class II HDACs. From a chemical point of view, this molecule is composed by a trifluoromethyl ketone group, which

chelates the active site zinc in a bidentate manner, the central thiophene ring, that fits perfectly to class IIa active site, and the amide group that interacts with the surrounding residues (Jones P. *et al,* 2008).

b) Targeting the shuttling between nucleus and cytoplasm

Since are demonstrated the oncogenic properties exerted by class II HDACs when localized into the nucleus (Di Giorgio E. *et al*, 2013), the strategy that it has been adopted is to interfere with class II HDACs nuclear accumulation. Some studies demonstrate the possibility to block class II HDACs in the cytoplasm by generating molecular compounds starting from the structure of SAHA, through the substitution of the amino-phenyl group with a fluorescent dansyl group (Kong Y. *et al,* 2011). This modification increases the specificity for class II HDACs, reducing the reactivity against class I HDACs. The consequence is that HDAC4 is bound to the inhibitor, thus impeding the interaction with importin-1a. Nevertheless, some drawbacks are still under investigation, in particular the fact that class IIa HDACs also possess cytoplasmic functions and, moreover, that the cytoplasmic accumulation could be an indirect effect of class I HDACs inhibition. Panobinostat (LBH589) is a representative drug belonging to this category and it derives from SAHA structure. It has been reported its ability to restrict HDAC4 localization in the cytoplasm in irradiated non-small cell lung cancer (Geng L. *et al*, 2006).

c) Targeting the amino-terminal domain

Class IIa HDACs are characterized by an exclusive N-terminal regulatory domain, which allows the homo and hetero-dimerization among the different class IIa members. This regulatory domain, which contains a glutamine-rich sequence (with the exception of HDAC7), is involved in the interaction with several partners, among which MEF2 proteins. In fact, several of the biological functions attributed to class IIa HDACs are due to the repression of MEF2 transcription. The design of an inhibitor that selectively displaces class IIa HDACs from binding MEF2 is extremely hard, since this aminoacid sequence is also involved in the interaction with other important proteins, one of which is p300. Starting from this issue, several groups decided to approach with an alternative strategy, which consists into targeting the region of MEF2 that interacts with class IIa HDACs. Crystallographic analyses and *in vitro* biochemical studies reveal that a short amphipathic helix conserved in the N-terminal regulatory domain of class IIa HDACs binds to a highly conserved hydrophobic groove on the MADS-box/MEF2 domain of MEF2. These studies suggest that the recruitment of class IIa

HDACs to DNA could be blocked by some small molecules binding to the hydrophobic pocket of MEF2, thereby inhibiting the sequence-specific genomic targeting (Lu J. *et al*, 2000). Jayathilaka N. *et al.* (2012) used X-ray crystallography and NMR to identify and characterize a group of small molecules that target class IIa HDACs by blocking their binding with MEF2 (Fig.15). The result is an exclusive indirect strategy to prevent HDACs activity on gene transcription. The screening identified small molecules from the pimeloylanilide o-aminoanilide (PAOA) class and further analyses focused on the most promising member of this family, N-(2-aminophenyl)-N0-phenyloctanediamide, which is commercially available as BML-210. This compound provides the first example of subtype-selective inhibition of HDACs by targeting the protein complex between class IIa HDACs and MEF2 and disrupting the co-localization of a class IIa HDACs and MEF2 *in vivo* (Jayathilaka N. *et al*, 2012). As BML-210 is a pan HDAC inhibitor (it inhibits also HDAC3 and Sirtuins in the micromolar range), its crystal structure has been used to guide the synthesis of more powerful BML-210 analogs, highly specific for targeting the MEF2-class IIa HDACs interaction. Preliminary results obtained in sarcoma cell lines demonstrated that BML-210 and its analogues could be a promising treatment for this type of tumor (Di Giorgio E. *et al*, 2013).

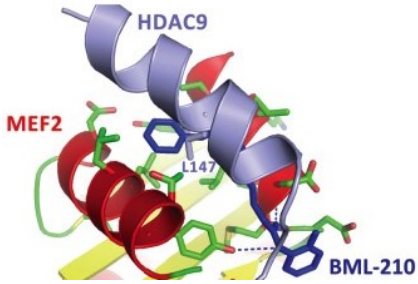


Fig.15: Graphic representation of the binding of BML-210 (phenyl group) to the hydrophobic pocket of HDAC9 (Jayathilaka N. *et al,* 2012).

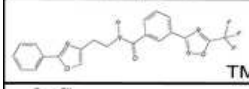
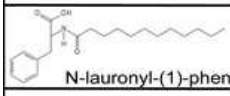
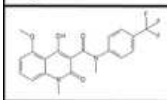
Compound	Cells	Treatment	Up-reg. MEF2-targets	Experiment
 TMP195	PBMCs PHA-stimulated	3μM 60 hrs	*ASB2,CCL1,ATP1B2*	DNA Microarray
 N-lauronyl-(1)-phenylalanine	MCF7	100μM 48 hrs	*KLF2, RHOB, NR4A1 KLF3, MARK1, GADD45γ*	qRT PCR
 BML-210	SK-UT-1 SK-LMA-1 DMR	10μM 36 hrs	*KLF2, RHOB, NR4A1*	qRT PCR
 Tasquinimod	LNCap	50μM 24 hrs	*IRS1, DTNA, ARRDC3 LHX4, KCNG1, ISL2*	DNA Microarray

Table 3: Class IIa HDACs inhibitors and their effects on MEF2 targets (Di Giorgio E. *et al*, 2015).

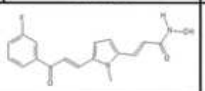
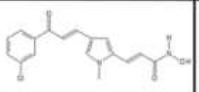
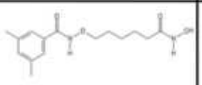
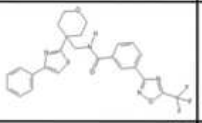
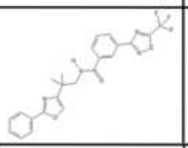
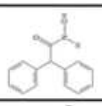
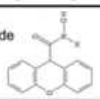
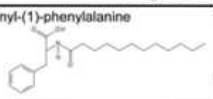
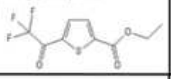
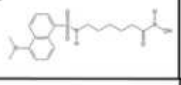
		IC50 (μM)											
		CLASS I				CLASS IIa				CLASS IIb		CLASS IV	
Name	Structure	HDAC1	HDAC2	HDAC3	HDAC8	HDAC4	HDAC5	HDAC7	HDAC9	HDAC6	HDAC10	HDAC11	Substrate
MC1568		38.72	NA	NA	NA	0.22	0.22	0.22	0.22	0.22	NA	NA	Acetyl pep
MC1575		>20	>20	>20	>20	5	NA	NA	NA	NA	NA	NA	Class I Acetyl-H3 Class IIa Trifluoroacetyl Lys
LMK235		0.320	0.881	NA	1.278	0.0119	0.00422	NA	NA	0.0557	NA	0.852	Class I ac-H3 Class IIa Boc-Lys trifluoro-acetyl-AMC Class IIb and IV ac-p53
TMP269		>100	>100	>100	4.2	0.157	0.097	0.043	0.023	8,2	>100	>100	Arg-His-Lys -Lys(Ac) HDAC1, 2,3,6,10,11 Arg-His-Lys(Ac) -Lys(Ac) HDAC8 Boc-Lys (trifluoro-acetyl)-AMC HDAC4,5,7,9
TMP195		>100	>100	>100	11.7	0.111	0.106	0.046	0.009	47,8	>100	>100	Arg-His-Lys -Lys(Ac) HDAC1, 2,3,6,10,11 Arg-His-Lys(Ac) -Lys(Ac) HDAC8 Boc-Lys (trifluoro-acetyl)-AMC HDAC4,5,7,9
N-hydroxy-2,2 -diphenylacetamide		>10	6.06	NA	66	0.75	0.14	0.39	NA	>10	NA	NA	Class I Acetyl-Boc-Lys Class IIa Boc-Lys-(e-trifluor methylacetyl-AMC HDAC6/8 ^{3}H-histone H4 peptide
N-hydroxy-9H -xanthene-9-carboxamide		NA	NA	NA	NA	0.25	0.11	0.05	NA	NA	NA	NA	Class I Acetyl-Boc-Lys Class IIa Boc-Lys-(ε-trifluor methylacetyl-AMC HDAC6/8 ^{3}H-histone H4 peptide
N-lauronyl-(1)-phenylalanine		>100	NA	NA	NA	NA	NA	21	NA	>100	NA	NA	Boc-Lys-(Ac)-AMC
Ethyl 5-(trifluoracetyl) thiopene-2-carboxylate		5.7	NA	3.5	NA	0.32	NA	NA	NA	0.55	NA	NA	Fluor de Lys HDAC1,3 Trfluoroacetamide -Lys HDAC4,6
Compound 2		0.95	1.38	1.12	3.98	0.33	0.40	2.56	NA	0.13	0.42	0.48	Fluor de Lys
BML-210		37.06	22.76	5.09	>300	NA	NA	>300	NA	>300	>300	NA	Competition binding
SAHA		0.22	0.56	1.79	2.74	>10	1.3	>10	>10	0.027	0.11	0.082	Class I, IIb, IV Fluor de Lys Class IIa Boc-Lys-(ε-trifluor methylacetyl-AMC

Table 4: Some class IIa HDACs inhibitors and their structure (Di Giorgio E. *et al*, 2015).

2. MATERIALS AND METHODS

Cell cultures and reagents

The human leio-myosarcoma cell lines SK-UT-1 and DMR were cultivated in Dulbecco modified Eagle medium (DMEM), supplemented with 10% fetal bovine serum (FBS) (Euroclone), L-glutamine (2mM) (Euroclone), penicillin (100U/ml) and streptomycin (100g/ml) (Euroclone). SK-UT-1 HDAC4 KO cells were previously generated (Di Giorgio E. *et al*, 2017). Primary antibodies used are: anti-GFP and anti-HDAC4 (Paroni G. *et al*, 2004), anti-HDAC5 (Clocchiatti A. *et al*, 2015); anti-MEF2A (C-21 sc-313 Santa Cruz Biotechnology), anti-MEF2D (BD Transduction Laboratories); anti-Actin (Sigma-Adrich), anti-H3K27ac (ab4729) and anti-H3K27me3 (ab6002) (Abcam); anti-H3K4me3 (GTX128954, GeneTex), anti-Caspase 3 (Santa Cruz) and anti-Caspase 8 (Santa Cruz).

Antibody production

Anti-HDAC9 antibody was produced by rabbit immunization with a His-tagged peptide of HDAC9 (aa 275-600) expressed in E.coli and elettroeluted from the unsoluble fraction. The same procedure was used to produce a GST-tagged fragment of HDAC9 that was irreversible fused and crosslinked to Affygel in order to affinity purify the policlyonal antobody from rabbit serum.

RNA extraction, reverse-transcription and quantitative qRT-PCR

Cell were lysed and homogenized directly on the culture dish by using TRI-REAGENT (Sigma-Aldrich). 1.0µg of the total isolated RNA was retro-transcribed by using 100 units of M-MLV (Moloney Murine Leukemia Virus) Reverse transcriptase (Life Technologies). qRT-PCRs were performed using the Bio-Rad CFX96 real-time PCR detection system and SYBR green (KAPA Biosystems) technology. Data were analysed by comparative threshold cycle using the expression levels of two housekeeping genes, HPRT (hypoxanthine phosphoribosyltransferase) and GAPDH as normalizer genes. All reactions were done in duplicate.

Generation of HDAC9 knock-out SK-UT-1 cells and lentiviral CRISPR/Cas9 infection

To obtain HDAC9 knock-out clones, the CRISPR/Cas9 technology was applied. In particular, to improve the specificity of Cas9-mediated genome editing, it was used the strategy that combines the D10A mutant nickase Cas9 (Cas9n) with a pair of offset sgRNAs complementary to opposite strands of the target site (Ran FA. *et al,* 2013). Firstly, it was

necessary a step of mutagenesis of Cas9 enzyme at the catalytic residue in the RuvC domain (D10A). Two complementary oligonucleotides containing the desired mutation, flanked by unmodified nucleotide sequences, were synthesized. The mutant strand synthesis reaction required 100ng of dsDNA template and 125ng of oligonucleotide primers. *DpnI* restriction enzyme digested the non-mutated DNA. Secondly, two guides against HDAC9 (exon 1) were synthetized in order to produce two single strand breaks simultaneously, respectively at nucleotide 60 and 74 of the genomic sequence:

GUIDE 1: GAA|GCAGCTTCTGATAGCA
GUIDE 2: GAACTTGACACGGCAGC|ACC

The distance beetween the two guides is 19nt. Each guide was cloned into a different plasmid: the GUIDE 1 associated to Cas9n (D10A) was cloned into a lentiCRISPRv2 vector, while the GUIDE 2 into a pLXSN vector.
HEK-293 cells (Human Embrionic Kidney) packaging cells were transfected with the relative plasmids. In particular, the cells were seeded $2.7*10^5$/ml and transfected at 50% of confluence using the Calcium Phosphate method. 8.0µg of each vector carring the guide were tranfected in combination with 5µg of Δ8.9 plasmid (the packaging plasmid that brings gag, pol, env genes) and 1.8µg of VSV-G (envelope protein with CMV promoter). After 36h of incubation at 37°C, virus supernatant was collected, filtered through a 0.45 µm syringe filter unit, diluted in fresh growth medium (1:1) and finally completed with polybrene (8µg/ul). The mixture was used to infect SK-UT-1 recipient cells (seeded $0.8*10^5$/ml), at 60% of confluence. To produce SK-UT-1 cells stably expressing both GUIDE 1 and GUIDE 2 against HDAC9, the virus surnatant of HEK-293 cells expressing lentiCRISPRv2 carring Cas9n and the GUIDE 1 was used to infect SK-UT-1 cells stably expressing pLXSN GUIDE 2. Cells infected were selected with puromycin antibiotic. To obtain HDAC9 knock-out clones, the infected SK-UT-1 cells were seeded in 96 multiwells in order to grow clones from one cell per well. The putative KO clones were screened by PCR, immunoblot and validated by Sanger sequencing. Two HDAC4 and HDAC9 KO clones were selected for the analysis (Fig. 16).

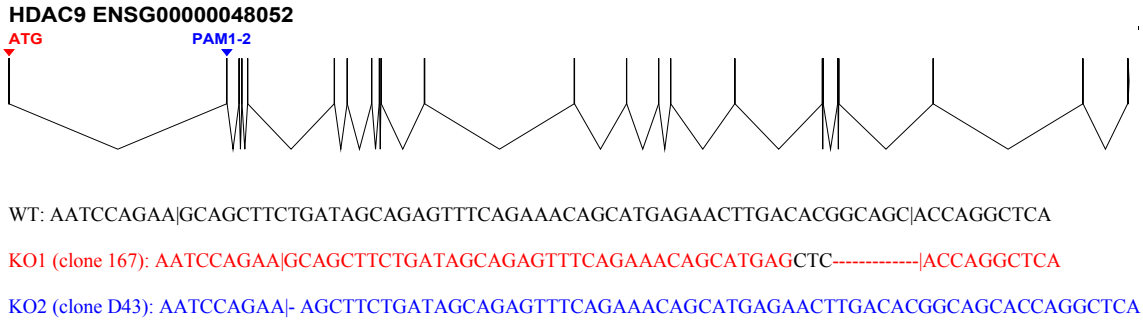


Fig.16: Schematic representation of HDAC9 genomic sequence. Vertical lines represent the introns and horizontal lines indicate the exons. The wild-type sequence (black) is compared to the two HDAC9 knock-out clones generated by nCas9. KO1 (red) presents a deletion of 16nt and an insertion of 3nt, while KO2 (blue) is characterized by a deletion of 2nt.

Immunoblotting

The protein sample preparation required a first SDS denaturating cell lysis in presence of protease and phosphatase inhibitors and of β-mercaptoethanol to reduce the intra and inter-molecular disulfide bonds. Protein samples were sonicated and heated in boiling water for 5 min before the electrophoresis. Proteins were then transferred to a 0.2µm pore-sized nitrocellulose membrane (Protran, Amersham) by an electroblotting device. Blocking of non-specific binding was achieved through incubation with non-fat dry milk in tris-buffered saline (TBS) with the 0.1% of Tween 20. Blots were incubated with primary antibodies (1h at RT or overnight at 4°C). After washes, blots were incubated with peroxidase-conjugated goat anti-rabbit or goat anti-mouse antibodies (Sigma-Aldrich) for 1h at RT. Secondary antibodies (Sigma-Aldrich) were developed with Super Signal West Dura (Pierce Waltham). For primary antibody stripping, blots were incubated for 30 min at 60°C in stripping solution (62.5mM Tris-HCl pH 6.8, 2% SDS) containing 100mM β-mercaptoethanol (Sigma-Aldrich).

Immunofluorescence

SK-UT-1 cells, SK-UT-1 HDAC9$^{-/-}$ (clone 167) and HDAC4$^{-/-}$ (clone 26) were seeded at 0.8*10^5/ml. Cells were fixed with 3% paraformaldehyde and permeabilized with 0.1% Triton X-100. Secondary antibodies used were Alexa Fluor conjugated (Molecular Probes). Actin was labeled with Phalloidin-AF546 (Molecular Probes). Nuclei were stained with Hoechst 33258 (Sigma). Imaged were performed with a Leica confocal scanner SP equipped with a 488 λ Ar laser and a 543 to 633 λ HeNe laser.

Invasion assay, soft agar assay and random cell motility measurement

All the approaches were performed to test the tumorigenic properties of SK-UT-1 cells and

SK-UT-1 HDAC9$^{-/-}$ cells. For soft agar assay, equal volumes of 1.2% agar and DMEM were mixed to generate 0.6% base agar. A total of 0.5*10^5/ml sarcoma cells were seeded in 0.3% top agar and incubated at 37°C. The cells were grown for 15 days changing the culture medium twice per week. Foci were evidenced with MTT staining and counted by using ImageJ. For invasion assay, each well of the invasion chamber (CLS3428, Corning) was coated with 200µl of Matrigel matrix coating solution (Cultrex, Trevigen). Next, a cell suspension of 0.5*10^5/ml cells in 0.1% FBS-DMEM was added. As chemoattractant, 20% FBS-DMEM was added in each lower chamber. As a control 0.1% FBS-DMEM was used to evaluate random invasion. After 16h, cells were fixed and stained with Hoechst. 5 random fields were counted.
Random motility was analysed by time-lapse video microscopy, as previously described (Cernotta N. *et al*, 2011).

Chromatin immunoprecipitation

For ChIP experiements, DNA-protein complexes were cross-linked with 1% fresh formaldehyde (Sigma-Aldrich) in DMEM for 15 min at RT. Cells were then collected directly from culture dishes with rubber scraper and lysed (5mM Pipes, 85 mM KCl, 0.5% NP40) in presence of protease inhibitors. The nuclear fraction was resuspended in RIPA-100 buffer and sonicated (Bioruptor UCD-200 (Diagenode) with pulses of 30 sec for 15 min to fragment DNA (average size of ~500bp). Samples were precleared and immunoprecipitated O/N at 4°C with: 1.5µg of anti-MEF2D and anti-MEF2A, 2µg of anti-HDAC4, 4µg of anti-HDAC9, 1µg of anti-H3K27ac, 2.5µg of anti-H3K4me3 and H3K27me3 antibodies or the same amount of control antibodies (FLAG M2 and USP33 serum). The IgG-DNA complexes were collected after incubation with protein A beads (GE Healthcare Bio-Sciences) blocked with BSA and salmon sperm DNA (1µg/µl) at 4°C for 90 min. Beads and inputs were treated with proteinase K at 56°C for 3h to degrade proteins and the cross-linking was reversed for 8-12h at 68°C. RNA was removed with RNAse A (10µg, Fisher Scientific). Genomic DNA was finally purified with Qiagen Qiaquick PCR purification kit and eluted in 100µl of water.

BrdU (Bromodeoxyuridine) assay

Cells were grown for 3h with 100µM BrdU and then fixed with 3% paraformaldehyde and permeabilized with 0.1% Triton X-100. Incubation with HCl (1N) for 10 min on ice permits to break open the DNA structure of the labelled cells. This is followed by HCl treatment (2N) for 10 min at RT and then the cells are incubated at 37°C for 20 min. After acid washes,

borate buffer (0.2M) is added to buffer the cells before the incubation of the mouse anti-BrdU antibody (Sigma-Aldrich). Secondary antibodies (AlexaFluor 488 or 533) were used for the detection. Nuclei were stained with Hoechst 33258 (Sigma-Aldrich).

Proliferation assay

SK-UT-1 Cas9, SK-UT-1 HDAC4$^{-/-}$ (clones 125 and 26) and HDAC9$^{-/-}$ (167 and D43) cells were seeded 0.3*10^5/ml in 12 multiwell plates. Cells were trypsinized and counted with a solution 0.1% Tripan blue. All experiments were done in triplicate.

Resazurin reduction assay

Cell proliferation/survival was measured for SK-UT-1 Cas9, SK-UT-1 HDAC4$^{-/-}$ (clones 125 and 26) and HDAC9$^{-/-}$ (167 and D43) cells through the resazurin cell viability assay. Cells were treated for 36h with several compunds at two increasing concentrations: Doxorubicin (50nM, 100nM), SAHA (2.5µM, 5µM), G5 (2.5µM, 5µM), Imatinib (10µM, 20µM), Lapatinib (10µM, 20µM), Metformin (10mM, 20mM) and MKK-2206 (10µM, 20µM). Initial cell seeding was at 0.8*10^5/ml.

IC50 of BML-210, MKK-2206, SAHA, NKL-54 and TMP-195 was calculated in SK-UT-1 and DMR seeded, respectively, at a confluence of 0.8*10^5/ml and DMR of 1.2*10^5/ml in 96-well plate. Cells were treated for 36h with 4 increasing concentrations of drugs: BML-210 (2.5µM, 5µM, 10µM, 20µM), MKK-2206 (5µM, 10µM, 20µM, 40µM), SAHA (0.5µM, 1µM, 2µM, 4µM), NKL-54 (2.5µM, 5µM,10µM, 20µM), TMP-195 (5µM, 10µM, 20µM, 40µM). Each well contained a final volume of 100µl (medium and compound). Resazurin dissolved in PBS to 0.15 mg/ml was added 1:1 to each well and the plate incubated at 37°C for 2h. Viable cells with an active metabolism are able to reduce resazurin (violet) into resorufin (pink). Quantification was done using a microplate fluorometer (PerkinElmer EnSpire 2300 Multilabel Reader) at 560 nm excitation/590 nm emission.

Generation of SK-UT-1 and DMR cells over-expressing FLIPs (Neo) or FLIPs (Puro)

To obtain SK-UT-1, SK-UT-1 HDAC9$^{-/-}$ and DMR cells with a stable expression of FLIPs, a protocol that provides a transfection followed by a retroviral infection was used. Ampho cells (cells of packaging) were transfected with calcium phosphate: a solution containing HBS2X (Hepes buffered saline solution) and a solution containing the plasmid DNA (12µg Pwzl Neo/pLPC Puro or 12µg Pwzl Neo FLIPs/pLPC puro FLIPs) and calcium chloride ($CaCl_2$)

were prepared. The solution containing DNA was added to HBS2X and the creation of precipitates was encouraged by the formation of bubbles. The mix was incubated at RT for 20 min, then added dropwise to Ampho cells layer and incubated at 30°C. Virus generated from packaging cells was used to stably infect recipient cells, SK-UT-1 (Pwzl Neo/pLPC Puro or Pwzl Neo FLIPs/pLPC puro FLIPs), DMR (Pwzl Neo/Pwzl Neo FLIPs) and SK-UT-1 HDAC9$^{-/-}$ clones 167 and D43 (Pwzl Neo or Pwzl Neo FLIPs).

Drug treatment

A drug treatment was done for SK-UT-1 Puro and SK-UT-1 Puro/FLIPs, SK-UT-1 Neo and SK-UT-1 Neo/FLIPs, DMR Neo and DMR Neo/FLIPs and finally, SK-UT-1 HDAC9$^{-/-}$ clone 167 Neo and 167 Neo/FLIPs and SK-UT-1 HDAC9$^{-/-}$ clone D43 Neo and D43 Neo/FLIPs. SK-UT-1 cells were seeded at a confluence of $0.8*10^5$/ml and DMR of $1.2*10^5$/ml. Cells were treated for 48h with different concentrations of several compounds, in single or in combination. SK-UT-1 Puro and SK-UT-1 Puro/FLIPs, DMR Neo and DMR Neo/FLIPs were treated with increasing amount of NKL-54 (1.25μM, 2.5μM, 5μM, 10μM). SK-UT-1 Puro and SK-UT-1 Puro/FLIPs were tested also for Doxorubicin (50nM), ABT-263 (100nM) and MKK-2206 (10μM) and for a combined therapy of NKL-54 (5μM) and Doxorubicin (25nM), NKL-54 (5μM) and ABT-263 (100nM), NKL-54 (5μM) and MKK-2206 (10μM). DMR Neo and DMR Neo/FLIPs were treated with NKL-54 (5μM), Doxorubicin (50nM) and a combination of NKL-54 (5μM) and Doxorubicin (25nM). SK-UT-1 Neo, SK-UT-1 Neo/FLIPs, SK-UT-1 HDAC9$^{-/-}$ clone 167 Neo and 167 Neo/FLIPs, clone D43 Neo and D43 Neo/FLIPs were single treated with MKK-2206 (20μM), Doxorubicin (50nM) and G5 (5μM). All the treated cells were tested at flow cytofluorometry and for the caspases activity.

Flow cytofluorometric analysis by propidium iodide staining

SK-UT-1 and DMR cell lines were seeded in 35mm plates at a confluence of $0.8*10^5$/ml and $1.2*10^5$/ml, respectively. After 48h of drug treatment, cells were collected and centrifuged at 2000 rpm for 5 min. After one wash with PBS 1X, cells were centrifuged and the pellet resuspended in 100μl of PBS 1X together with 10μg/ml of propidium iodide (Sigma-Aldrich). After 10 min of incubation at RT, protected from the light, cells were collected, washed and resuspended in 800μl of PBS 1X containing 1% formaldehyde (Sigma-Aldrich). After a fixation of 10 min on ice, cells were treated with RNase A (50μg/ml) (Sigma-Aldrich) for 15 min at 37°C. FACS analysis were performed by the BD FACSCalibur.

Caspase assay

The assay for the measurement of the activities of caspase-3 and -7 (Apo-ONE Homogeneous Caspase-3/7 Assay by Promega) was performed in SK-UT-1, in the two SK-UT-1 HDAC9$^{-/-}$ clones and in DMR cell line. Cells were seeded at a confluence of $0.8*10^5$/ml for SK-UT-1 and of $1.2*10^5$/ml for DMR into a 96-well plate, in duplicate. The test required to mix a lysis buffer with a profluorescent substrate; upon sequential cleavage and removal of the DEVD peptides by caspase-3/7 and excitation at 499nm, the rhodamine 110 is released, emitting green fluorescence. For each well, the mixed solution was aliquoted (1:1 with the medium) and the multi-well was incubated at 37°C for 30 min. Fluorescence reading and quantification was performed using the PerkinElmer EnSpire 2300 Multilabel Reader.

DNA Microarray

Total RNA was extracted using RNeasy columns (Qiagen). Aliquots of RNAs were amplified according to the specifications of the Illumina TotalPrep RNA Amplification Kit (Ambion). Hybridization on Illumina whole-genome HumanHT-12 v 4.0 chip (Illumina), scanning and background subtraction were done according to the manufacturer's specification. Fold-change and p-values for each probe set were calculated using a moderated t-statistic in the limma package, with the variance estimate being adjusted by incorporating global variation measures for the complete set of probes on the array. The p-value data were then corrected for multiple hypotheses testing using the Benjamini and Hochberg.

Statistics

For experimental data Student t-test was employed. Mann-Whitney test was applied when normality could not be assumed. $p<0.05$ was chosen as statistical limit of significance. For comparisons between samples >2 Anova test was applied, coupled to Krustal-Wallis and Dunn's Multiple Comparison Test. We marked with * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Unless otherwise indicated, all the data in the figures were represented as arithmetic means + the standard deviations from at least three independent experiments.

3. RESULTS

3.1 Summary of the published works

For many years, the role of class IIa HDACs in cancer has been intensely investigated. Nevertheless, these studies have not yet led to a conclusive and definitive role of these enzymes during the neoplastic progression. The oncogenic potential of class IIa HDACs has been firstly demonstrated in NIH-3T3 mouse immortalized fibroblasts (Di Giorgio E. *et al*, 2013; Barneda-Zahonero B. *et al*, 2015; Witt AE. *et al*, 2017) and only recently, in primary human fibroblasts (Paluvai H. *et al*, 2018).

Cancer-associated metabolic adaptations represent a new interestingly line of research and our aim was to better understand the impact of class IIa HDACs on the main metabolic pathways. *In vitro* transformation of NIH-3T3 cells with the nuclear version of HDAC4, failed to switch towards an anaerobic glycolytic phenotype, which mainly characterizes RAS-transformed fibroblasts. Instead, HDAC4 seems to be strongly involved in the regulation of the lipid metabolism and in the consumption of mitochondrial oxygen. In the oncogenic models under study, the acquisition of different metabolic phenotypes required the repression of a common group of MEF2 target genes (Peruzzo P. *et al*, 2016).

Further investigations revealed that the repression of MEF2 transcription factors is higher in soft tissue sarcomas and in particular, in uterine leiomyosarcomas. In low grade LMSs (SKL-LMS-1), the repression of a MEF2 signature is principally due to the higher degradation of MEF2 proteins, while in high grade tumors (SK-UT-1) this may be due to the envolvment of different MEF2 repressors. The silencing of MEF2D in the two cell lines triggers opposite responses, significantly reducing cell aggressiveness only in SK-UT-1 cells. This observation suggested a role for MEF2 also as transcriptional repressor, probably through the engagement of other co-factors, hence contributing to the cell malignancy (Di Giorgio E. *et al*, 2016). All these data have been published and included as additional material to this thesis. For this reason I'll restrict the result section to the still unpublished results.

3.2 HDAC9, HDAC5 and MEF2 are increased in high grade leiomyosarcomas

Leiomyosarcomas represent an excellent model to study the contribution of MEF2 to tumorigenesis, as their levels are high and frequently correlated with the overexpression of a class IIa HDACs member. By analyzing The Cancer Genome Atlas (TCGA) data of leiomyosarcomas we observed a frequent co-expression between HDAC4 and MEF2C, HDAC5 and MEF2A, and HDAC9 with MEF2D. In particular, it was evident the highest expression of MEF2D and HDAC9 in these aggressive types of tumors (Fig.17A). Based on these data, we hypothesized a key role of the MEF2D-HDAC9 axis in LMSs and the possible contribution to tumorigenesis derived from their inter-regulation.

We further confirmed the up-regulation of HDAC9 in our cohort of 26 LMS patients by means of RNAseq analysis (Fig.17B).

To study the molecular mechanisms behind the MEF2D-HDAC9 axis in LMSs, we validated the protein expression levels of MEF2 and class IIa HDACs in different uterine sarcoma cell lines: a grade II LMS cell line (SK-LMS-1), two grade III LMS cell lines (SK-UT-1 and DMR) and a carcinosarcoma cell line (MES-SA). SK-UT-1 cells are characterized by high levels of HDAC9, its splicing variant MITR, MEF2A and MEF2D; DMR have high expression levels of HDAC5, MITR and MEF2A. SK-LMS-1 cells are characterized by the lowest levels of the proteins under analysis (Fig.17C). Definitively, SK-UT-1 and DMR cell lines were selected for the further analysis, as their concurrent high levels of HDAC9, HDAC5 and MEF2 mirror their expression in human leiomyosarcomas.

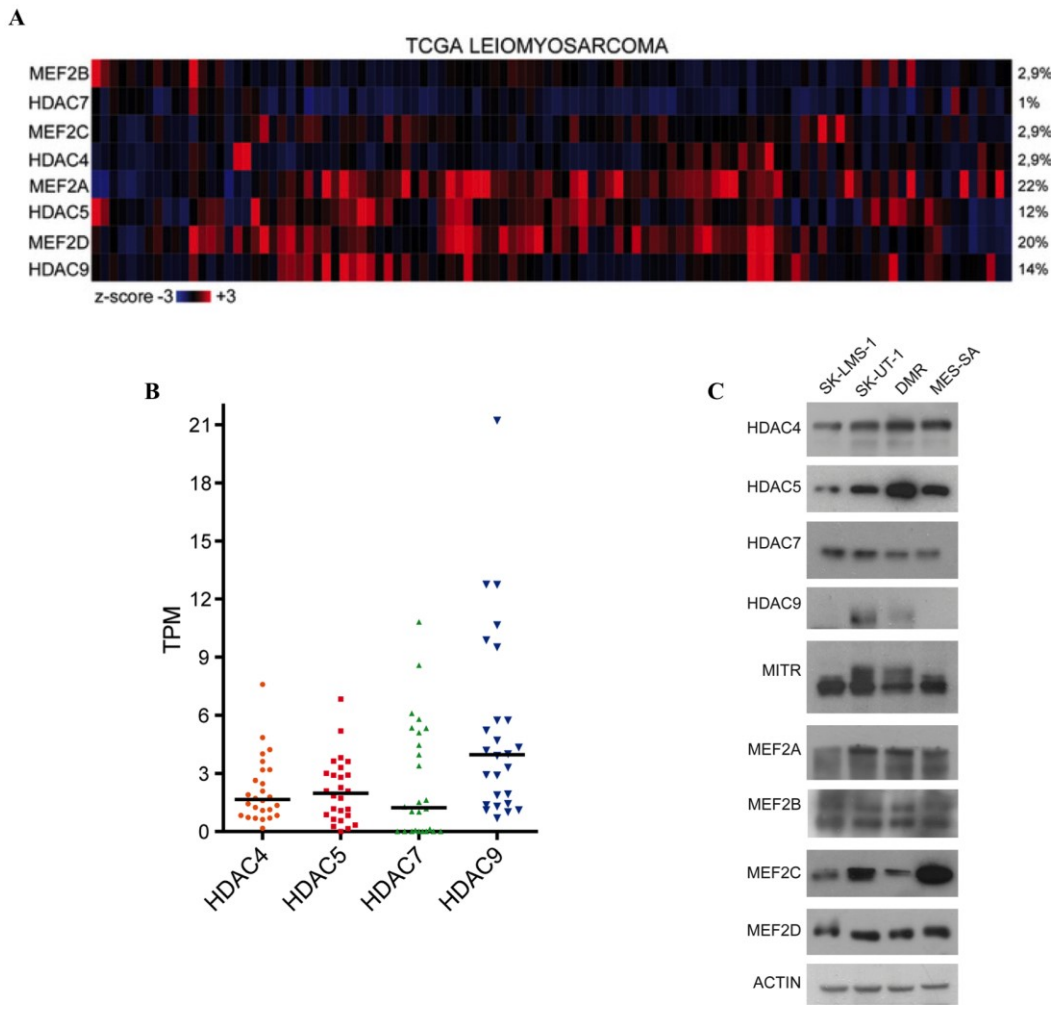


Fig.17: A) Oncoprint of TCGA leiomyosarcoma data for the z-scores of MEF2A, MEF2B, MEF2C, MEF2D and for class IIa HDACs in 100 LMS samples. B) Scatter dot plot representing the coding mRNA TPM levels of the four class IIa HDACs in a cohort of 26 LMS samples. C) Immunoblot analysis of class IIa HDACs family members and MEF2 proteins in low grade (SK-LMS-1), high grade (SK-UT-1 and DMR) LMS cells and a poorly differentiated uterine sarcoma cell line MES-SA. Actin was used as loading control.

3.3 The MEF2-HDAC9 axis

MEF2 transcribes HDAC9 as part of a feed-back mechanism well known in literature (Haberland M. *et al*, 2007). Considering the frequent co-expression between MEF2D and HDAC9 in LMS, we wonder to know if a similar mechanism is operating in LMS cells.

A luciferase assay revealed that HDAC9 expression could arise from the transcriptional regulation exerted by MEF2D, since its binding significantly increases the transcriptional activity of the HDAC9 promoter. The promoter activity was tested in HEK-293 cells transfected with the genomic region of HDAC9 promoter taken from primary fibroblasts IMR-90, a low grade LMS cell line, SK-LMS-1, and a high grade LMS cell line, SK-UT-1. In all the cases, MEF2 triggers HDAC9 promoter-driven luciferase activity at comparable levels, thus excluding any putative effect arising from the presence of any SNP in the proximal

promoter of HDAC9 (Fig.18B). Therefore, the high levels of HDAC9 observed in SK-UT-1 cells could originate from an increased transcriptional activity exerted at its promoter. In fact, when SK-LMS-1 and SK-UT-1 were transfected with the promoter of HDAC9 isolated from SK-LMS-1 cells, the luciferase activity indicated an higher transcription of HDAC9 in SK-UT-1 cells compared to SK-LMS-1 (Fig.18C). Next, it was investigated the consequences of MEF2D silencing on HDAC9 gene expression. The silencing of MEF2D in SK-UT-1 cells significantly reduces HDAC9 expression both at mRNA (Fig.18D) and protein levels (Fig.18E). ChIP analysis demonstrates the binding of MEF2 to the HDAC9 promoter region (Fig.18F) and that the binding event on this site dramatically enriches an epigenetic signature (an increase of H3K27ac and H3K4me3 and reduction of H3K27me3), typical of open chromatin/active transcription in SK-UT-1 cells (Fig.18G).

All these data demonstrate that the expression of HDAC9 is, effectively, under the control of MEF2 proteins.

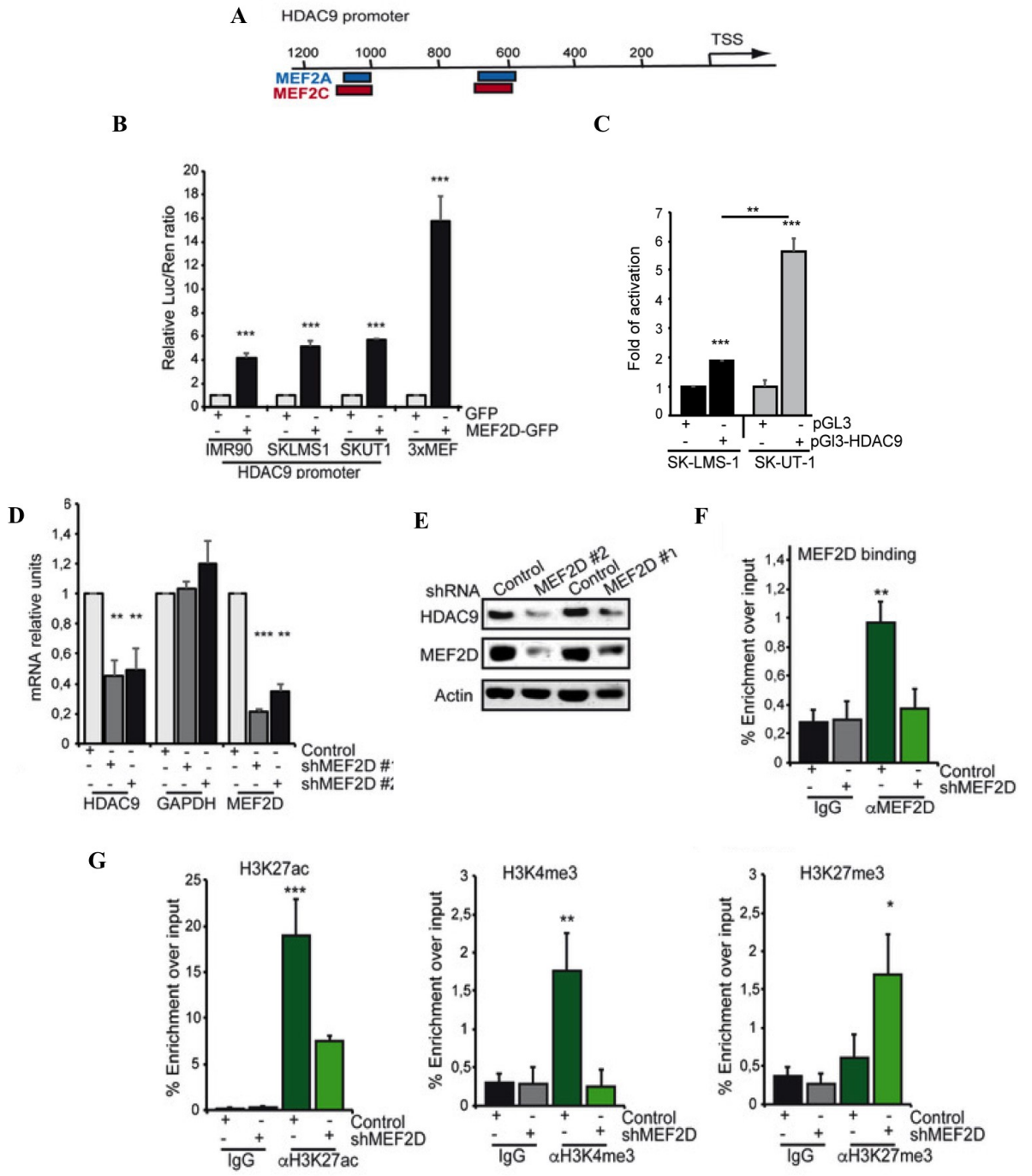


Fig.18: A) Characterization of HDAC9 promoter. In blue are indicated the binding sites of MEF2A and in red the binding sites of MEF2C in the proximal promoter of HDAC9 (previously validated by ENCODE). B) Luciferase assay for HDAC9 promoter activity in HEK-293 cells transfected with MEF2D-GFP or GFP and the promoter region amplified from IMR-90, SK-LMS-1 and SK-UT-1 cells. 3xMEF construct, presenting three binding sites for MEF2, was used as positive control. Data are presented as mean and st.dev. n=3. C) SK-LMS-1 and SK-UT-1 transfected with a pGL3 plasmid with the luciferase gene under the control of HDAC9 promoter isolated from SK-LMS-1 cells. Luciferase activity reveals a higher transcription of HDAC9 in SK-UT-1 cells compared to SK-LMS-1. Data are normalized by co-transfecting pRenilla. D) HDAC9 and MEF2D mRNA levels after the silencing of MEF2D. Silencing of MEF2D was obtained with two distinct shRNAs in SK-UT-1 cells, as indicated. Data are presented as mean and st.dev. relative to control not silenced cells. n=3. E) Immunoblot analysis indicates reduced protein levels of MEF2D induced by both shRNAs and reduced HDAC9 protein after MEF2D silencing. Actin was used as loading control. F) Chromatin was immunoprecipitated using the anti-MEF2D antibody (1.5µg) from SK-UT-1

cells WT or silenced for MEF2D by a shRNA. Mouse IgGs were used as control. Data are presented as mean and standard errors. n=4. G) ChIP analysis of the chromatin status in the MEF2D binding region. Chromatin was immunoprecipitated using the anti-H3K4me3, anti-H3K27ac and anti-H3K27me3 antibodies (1μg) from SK-UT-1 cells and SK-UT-1 silenced for MEF2D. Data are presented as mean and standard error. n=3.

3.4 The binding of HDAC9 to MEF2 gives rise to a repressive complex that contributes to tumor aggressiveness

Principal features of high grade LMSs are the concomitant up-regulation of class IIa HDACs and the consequent switch of MEF2 into repressors, which direct the cells towards an aggressive tumorigenic phenotype. Previous experiments demonstrated that the knockdown of MEF2 in SK-UT-1 cells induced the up-regulation of a group of genes (Di Giorgio E. *et al*, 2017). This peculiar gene response has been hypothesized to be due to a possible role of MEF2 as transcriptional repressors on these promoters. HDAC4 and HDAC9 were considered the most likely candidates for this role.

The best way to test whether class IIa histone deacetylases were effectively the critical players to switch MEF2 into repressive transcriptional factors, was to generate SK-UT-1 cells knock-out (KO) for HDAC4 or HDAC9. For this purpose, the CRISPR/Cas9 technology was applied. For each knock-out, two different clones were used for the further analysis (Fig.19A). Immunoblot assay shows the total ablation of HDAC9 protein and its splicing variant MITR in HDAC9 KO clones, in combination with increased levels of HDAC5 and MEF2D. Differently from HDAC9 KO clones, cells knocked-out for HDAC4 do not show a compensation for the reduced HDAC levels by increasing expression of HDAC9, while HDAC5 and MEF2D are slightly up-regulated.

HDAC9 localizes mainly in the nucleus of SK-UT-1 cells (Fig.19B), while HDAC4 is pancellular and cytoplasmic, as a consequence of a strong nucleo/cytoplasmic shuttling (Fig.19C, compare HDAC4 distribution between untreated and leptomycin B treated cells).

The immunofluorescence analysis of SK-UT-1 cells knocked-out for HDAC9 or HDAC4 revealed that the morphology of the KO clones is different. In particular, HDAC9 KO cells exhibit a larger spread area in comparison to HDAC4 KO cells (Fig.19D-E). On this ground, we hypothesized that the KO of HDAC9 determines a re-differentiation of LMS cells and a lowering of their tumorigenic grade. As a matter of fact, the KO of both HDAC4 and HDAC9 negatively impacts on the random motility of SK-UT-1 cells (Fig.19F), but only the KO of HDAC9 impairs the invasiveness and the malignancy of SK-UT-1 cells (Fig.19G-H).

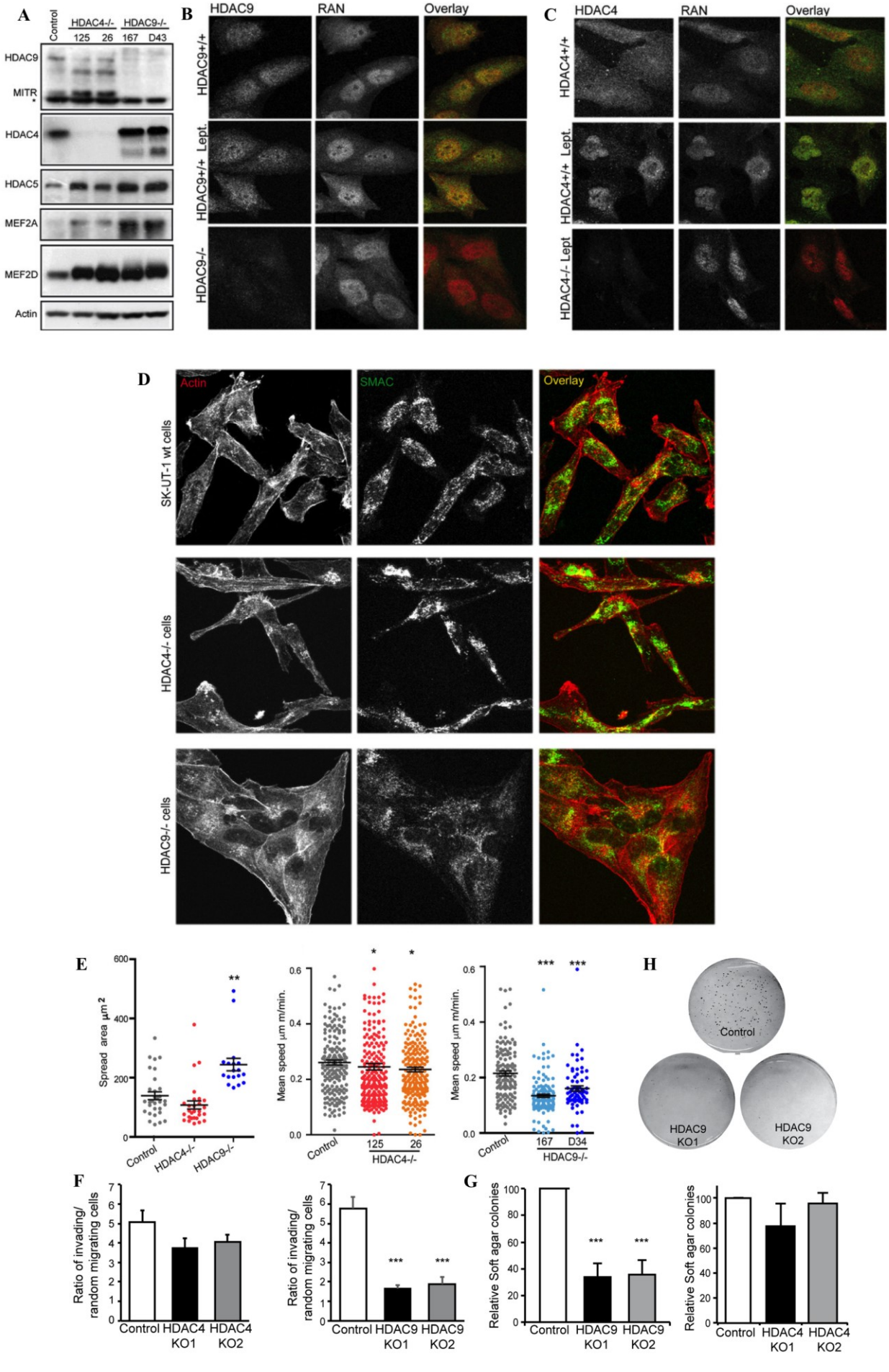

A
Control
HDAC4-/-
125
26
HDAC9-/-
167
D43
HDAC9
MITR
HDAC4
HDAC5
MEF2A
MEF2D
Actin
B
HDAC9
RAN
Overlay
HDAC9+/+
HDAC9+/+ Lept.
HDAC9-/-
C
HDAC4
RAN
Overlay
HDAC4+/+
HDAC4+/+ Lept.
HDAC4-/- Lept
D
Actin
SMAC
Overlay
SK-UT-1 wt cells
HDAC4+/- cells
HDAC9-/- cells
E
Spread area µm 2
Control
HDAC4-/-
HDAC9-/-
Mean speed µm m/min.
125
26
167
D34
H
Control
HDAC9 KO1
HDAC9 KO2
F
Ratio of invading/ random migrating cells
Control
HDAC4 KO1
HDAC4 KO2
HDAC9 KO1
HDAC9 KO2
G
Relative Soft agar colonies
Control
HDAC9 KO1
HDAC9 KO2
HDAC4 KO1
HDAC4 KO2

Fig.19: A) Immunoblot analysis of HDAC9, MITR, HDAC4, HDAC5, MEF2A and MEF2D in SK-UT-1 cells WT and in two KO clones for HDAC4 (125 and 26) and HDAC9 (167 and D43). Actin was used as loading control. B) Immunofluorescence analysis performed in SK-UT-1 cells WT and HDAC9$^{-/-}$ stained for HDAC9 with an anti-HDAC9 antibody. Leptomycin B (50μg/ml), an inhibitor of nuclear export machinery, was used for 2 hours to confine the protein inside the nucleus. Anti-RAN antibody was used to stain nucleus. C) Immunofluorescence analysis performed in SK-UT-1 cells WT and HDAC4$^{-/-}$ stained for HDAC4 by using an anti-HDAC4 antibody. Leptomycin B was used to confine the protein inside the nucleus. Anti-RAN antibody was used to stain the nucleus. D) Immunofluorescence analysis performed in SK-UT-1 cells WT, HDAC4$^{-/-}$, HDAC9 -/-stained for SMAC. Phalloidin AF546 was used to stain actin filaments. E) Dot plot representing the spread area of HDAC4$^{-/-}$ and HDAC9$^{-/-}$ cells quantified using Metamorph software. The median and the first and third quartiles are indicated. Dot plot representing the mean speed of HDAC4$^{-/-}$ and HDAC9$^{-/-}$ cells quantified using Metamorph software. The median and the first and third quartiles are indicated. F) Histogram representing the invasion rate of SK-UT-1 cells WT, HDAC4$^{-/-}$ and HDAC9$^{-/-}$. Data are presented as relative to random migrating cells. Mean and st.dev. are indicated n=3. G) Quantitative results of colony formation assay for SK-UT-1 cells WT, HDAC4$^{-/-}$ and HDAC9$^{-/-}$. Data are presented as mean and st.dev. n=4. H) Representative images of the foci stained with MTT of SK-UT-1 cells WT and HDAC9$^{-/-}$grown in soft agar for 18 days.

3.5 A different gene expression profile characterizes SK-UT-1 cells HDAC4$^{-/-}$ or HDAC9$^{-/-}$

To understand the different roles played by HDAC4 or HDAC9 in SK-UT-1 cells, we compared the gene expression profiles of SK-UT-1 HDAC4$^{-/-}$ and HDAC9$^{-/-}$ cells.

A DNA microarray analysis confirmed a different gene expression profile in SK-UT-1 cells knocked-out for HDAC4 or HDAC9. Genes up-regulated in SK-UT-1 cells HDAC4$^{-/-}$ were found to be poorly regulated in HDAC9$^{-/-}$ cells. Surprisingly, some of the genes induced in HDAC9$^{-/-}$ SK-UT-1 cells were even repressed in the HDAC4$^{-/-}$ cells (Fig.20A). This paradox could be explained as a consequence of the increased HDAC5 and MITR levels in HDAC4 KO cells (Fig.19A).

A Gene Onthology (GO) analysis indicated that the knock-out of HDAC4 in SK-UT-1 cells mainly induces the up-regulation of genes involved in the response to oxygen-containing compounds, in the programmed cell death and in the regulation of cell proliferation. The same analysis revealed a significant up-regulation of genes essential for the negative regulation of cell migration and locomotion and in the organization of actomyosin structures in SK-UT-1 cell knocked-out for HDAC9.

Reactome pathway analysis further highlighted the induction of genes involved in axon guidance, cellular response to external stimuli and in the signaling of Tyrosine Kinase receptor, when the expression of HDAC4 was abrogated. Wiki Pathways analysis identified in HDAC9$^{-/-}$ cells the up-regulation of genes related to lung fibrosis, TNF-alpha and FAS Ligand-induced apoptosis and stress induction of heat shock proteins. SK-UT-1 HDAC4$^{-/-}$ and HDAC9$^{-/-}$ cells were found to commonly up-regulate genes related to the mineral absorption (Fig.20B).

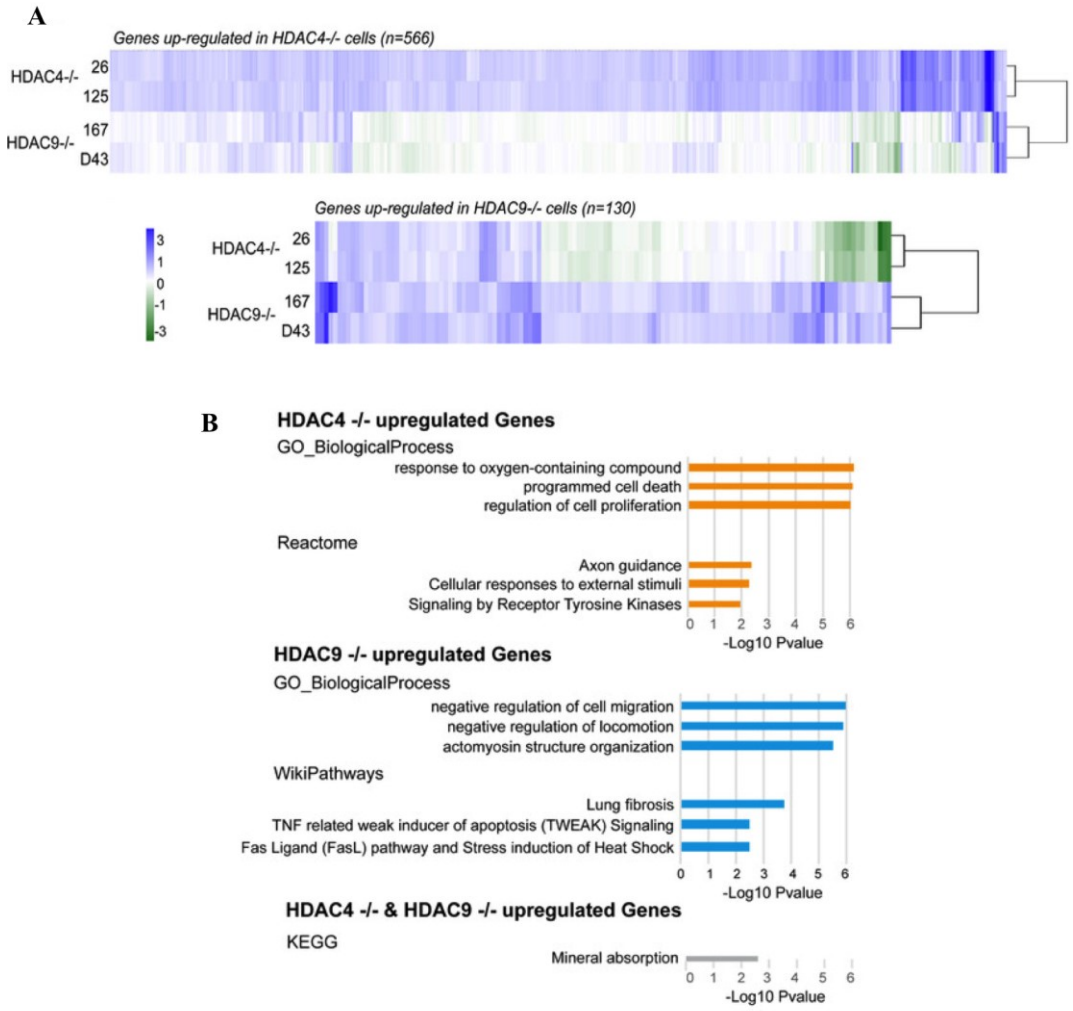
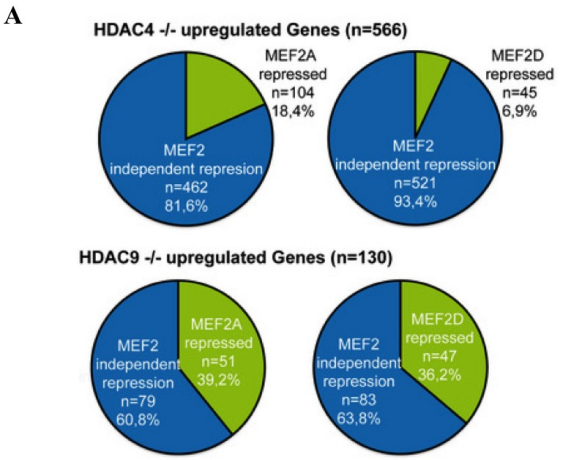


Fig.20: A) Heat map of the significant regulated genes in SK-UT-1 cells HDAC4$^{-/-}$ (clones 26 and 125) or HDAC9$^{-/-}$ (D43 and 167). The deepest blue indicates the up-regulated genes, repressed genes are indicated by intense green. B) Gene Onthology (GO), Reactome and Wiki Pathways analysis identified most common genes and related pathways up-regulated in SK-UT-1 HDAC4$^{-/-}$ or HDAC9$^{-/-}$.

3.6 The knock-out of HDAC4 and HDAC9 have a different impact on MEF2 proteins

Genes found to be significantly up-regulated in SK-UT-1 cells HDAC4$^{-/-}$ and HDAC9$^{-/-}$ were further correlated to MEF2A and MEF2D activities by analyzing the gene expression profile of SK-UT-1 cells knocked-down for MEF2A or MEF2D (Di Giorgio E. *et al*, 2017). Results revealed that just a small group of genes repressed by HDAC4 were up-regulated by MEF2A/D KD (Fig.21A). When the same analysis was performed for the HDAC9$^{-/-}$ signature, a different result was obtained. Most of the genes repressed by HDAC9 were under the control of MEF2A and MEF2D (Fig.21A). Moreover, most of genes up-regulated in SK-UT-1 cells HDAC9$^{-/-}$ correlate with a group of genes up-regulated in the same cells silenced for MEF2D/A (Fig.21B). We indicated as "atypical" this cluster of genes (Di Giorgio E. *et al*, 2017), as on these loci MEF2 should normally act as a transcriptional repressor.

A Gene Onthology (GO) analysis indicated that in SK-UT-1 cells KO for HDAC9, most of genes involved in the negative regulation of cell migration and locomotion and in the organization of actomyosin structures, depend on MEF2. Interestingly, it has emerged that the two main inductors of cell death, TNF and FAS Ligand were strongly dependent on MEF2 regulation (Fig.21C).



B

C

Genes		MEF2A	MEF2D	MEF2A & MEF2D	MEF2A + MEF2D
HDAC4 -/- upregulated	**n**	**%**	**%**	**%**	**%**
GO_BiologicalProcess					
response to oxygen-containing compound	95	30.5	15.8	11.6	35.8
programmed cell death	101	33.7	15.8	10.9	38.6
regulation of cell proliferation	74	24.3	13.5	8.1	29.7
Reactome					
Axon guidance	24	20.8	8,3	4.2	25.0
Cellular responses to external stimuli	22	27.3	4.5	4.5	27.2
Signaling by Receptor Tyrosine Kinases	21	19.0	14.3	4.8	28.6

Genes		MEF2A	MEF2D	MEF2A & MEF2D	MEF2A + MEF2D
HDAC9 -/- upregulated	**n**	**%**	**%**	**%**	**%**
GO_BiologicalProcess					
negative regulation of cell migration	33	42.4	54.5	36.4	60.6
negative regulation of locomotion	41	36.6	53.7	36.6	53.7
actomyosin structure organization	19	26.3	42.1	36.4	60.6
WikiPathways					
Lung fibrosis	10	30.0	30,0	20.0	40.0
TNF related weak inducer of apoptosis (TWEAK) Signaling	3	100.0	66.7	66.7	100.0
Fas Ligand (FasL) pathway and Stress induction of Heat Shock	5	80.0	60.0	28.6	80.0

Fig.21: A) Pie chart of most common up-regulated genes in HDAC4$^{-/-}$ and HDAC9$^{-/-}$ cells. Green and blue slices indicate, respectively, gene expression related to MEF2 repression and MEF2-independent repression. B) Gene set enrichment analysis (GSEA) obtained by overlapping the DNA microarray of shMEF2A/shMEF2D and shControl in SK-UT-1 cells with DNA microarrays relative to HDAC4$^{-/-}$ or HDAC9$^{-/-}$. The NES and the significance of the enrichment are indicated. C) Table representing the Gene Onthology (GO) and WikiPathways analysis in SK-UT-1 HDAC4$^{-/-}$ or HDAC9$^{-/-}$. The dependency from MEF2 was expressed as percentage.

3.7 HDAC9 KO cells are characterized by growth impairment and by a progressive reduction of survival

The predicted activation of death pathways mediated by TNF and FAS-Ligand in SK-UT-1 HDAC9$^{-/-}$ cells, led us to focus on the sensitivity of these cells to apoptosis. First of all, we tested the proliferative ability of SK-UT-1 HDAC9$^{-/-}$ cells in comparison to SK-UT-1 HDAC4$^{-/-}$ and SK-UT-1 WT cells. BrdU assay indicated that HDAC9 KO cells are characterized by a significative proliferative deficit compared to SK-UT-1 HDAC4 KO cells (Fig.22A). Cell growth was instead measured by counting both living cells (Fig.22B) and the fraction of death cells (Fig.22C), after 5 days in culture. Cell growth was impaired both in HDAC4 KO and HDAC9 KO cells in comparison to SK-UT-1 WT cells, even if HDAC9$^{-/-}$ cells showed an impressive accumulation of cell death at day 5. Sensitivity of HDAC9$^{-/-}$ cells to apoptosis was also demonstrated by immunoblot analysis, which evidenced the higher expression of FAS receptor on the surface of the cells (Fig.22D). Moreover, cleavages of proCaspase-3 and proCaspase-9 indicated the activation of apoptosis in cells KO for HDAC9 (Fig.22E).

To further prove the increased sensitivity of HDAC9 KO cells to cell death, we challenged these LMS cells with different drugs that can have a therapeutic relevance such as: SAHA, G5, MKK-2206, Metformin, Lapatinib and Imatinib. Two increasing concentrations in control cells (SK-UT-1 Cas9), SK-UT-1 HDAC4$^{-/-}$ (clones 125 and 26) and HDAC9$^{-/-}$ (167 and D43) cells were tested. HDAC9 KO clones showed a reduced survival at highest concentrations of SAHA, G5, MKK-2206, Metformin and Lapatinib. Instead, the proliferation and survival of SK-UT-1 HDAC4$^{-/-}$ cells were affected by G5, Metformin and MKK-2206 (Fig.22F).

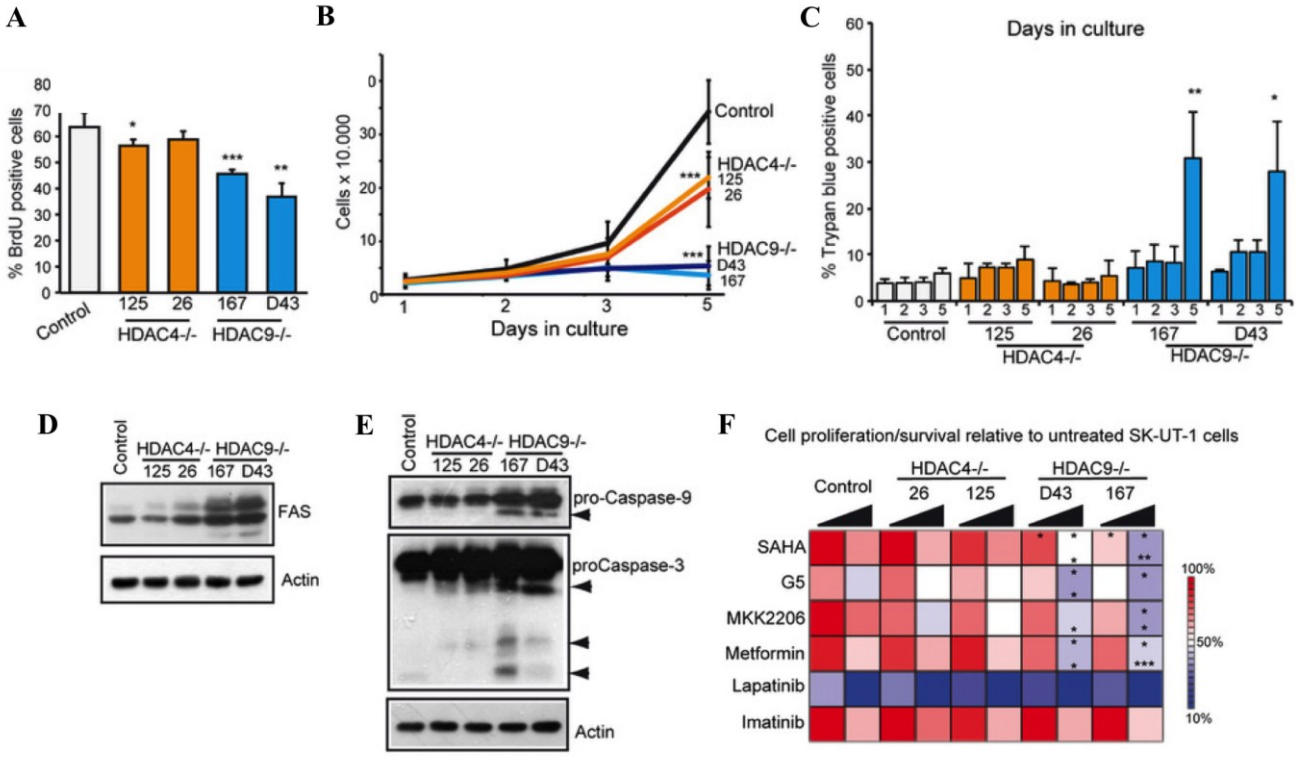


Fig.22: A) Histogram representing BrdU-positivity in SK-UT-1 Cas9 (control), SK-UT-1 HDAC4$^{-/-}$ (clones 125 and 26) and HDAC9$^{-/-}$ (167 and D43) cells. Data are presented as mean and st.dev. n=4. B) Counting of living SK-UT-1 Cas9 (control), SK-UT-1 HDAC4$^{-/-}$ and HDAC9$^{-/-}$ clones, at day 1, day 2, day 3 and day 5 from seeding. Data are presented as mean and st.dev. n=3. C) Trypan blue staining of SK-UT-1 Cas9 (control), SK-UT-1 HDAC4$^{-/-}$ (clones 125 and 26) and HDAC9$^{-/-}$ (167 and D43) cells at day 1, day 2, day 3 and day 5 from seeding. Data are presented as mean and st.dev. n=4. D) Immunoblotting analysis in SK-UT-1 Cas9 (control), SK-UT-1 HDAC4$^{-/-}$ (clones 125 and 26) and HDAC9$^{-/-}$ (167 and D43) cells of the protein levels of FAS-R. Actin was used as loading control. E) Immunoblotting analysis in SK-UT-1 Cas9 (control), SK-UT-1 HDAC4$^{-/-}$ (clones 125 and 26) and HDAC9$^{-/-}$ (167 and D43) cells of the protein levels of Caspase 9 and Caspase 3. Cleavages of pro-Caspase-9 and -3 are indicated. Actin was used as loading control. F) Heat map indicating cell proliferation/survival of SK-UT-1 HDAC4$^{-/-}$ (clones 125 and 26) and HDAC9$^{-/-}$ (167 and D43) cells relative to untreated SK-UT-1 Cas9 cells after drug treatment at two increasing concentrations of each drug.

3.8 The KO of HDAC9 induces activation of apoptotic cell death through the engagement of Fas receptor

Experimental data indicated two main features of SK-UT-1 HDAC9$^{-/-}$ cells: the reduced proliferative ability and their high sensitivity to cell death. This increased susceptibility to cell death could be due to the increased expression of FAS receptor. To prove this we treated the cells with Fas Ligand (FasL). As expected, HDAC9 KO cells were more sensitive to FasL treatment in respect to WT cells (Fig.23A). To further confirm this point, we expressed FLIPs in both HDAC9$^{+/+}$ and HDAC9$^{-/-}$ cells (Fig.23B). The generated SK-UT-1 Neo (as control) and Neo/FLIPs, SK-UT-1 HDAC9$^{-/-}$ D43 Neo and D43 Neo/FLIPs, and SK-UT-1 HDAC9$^{-/-}$

167 Neo and 167 Neo/FLIPs cells were firstly treated with 25ng/ml of FasL (Fig.23C-D). HDAC9 KO cells appeared more sensitive to FasL treatment, while FLIPs over-expression induced only a partial reduction of cell death (Fig.23C-D). It is possible that the limited impact of FLIPs depends on the low levels of transgene expression. However, FLIPs over-expression blunted to basal levels the apoptotic rate observed in HDAC9 KO cells (Fig.23E).

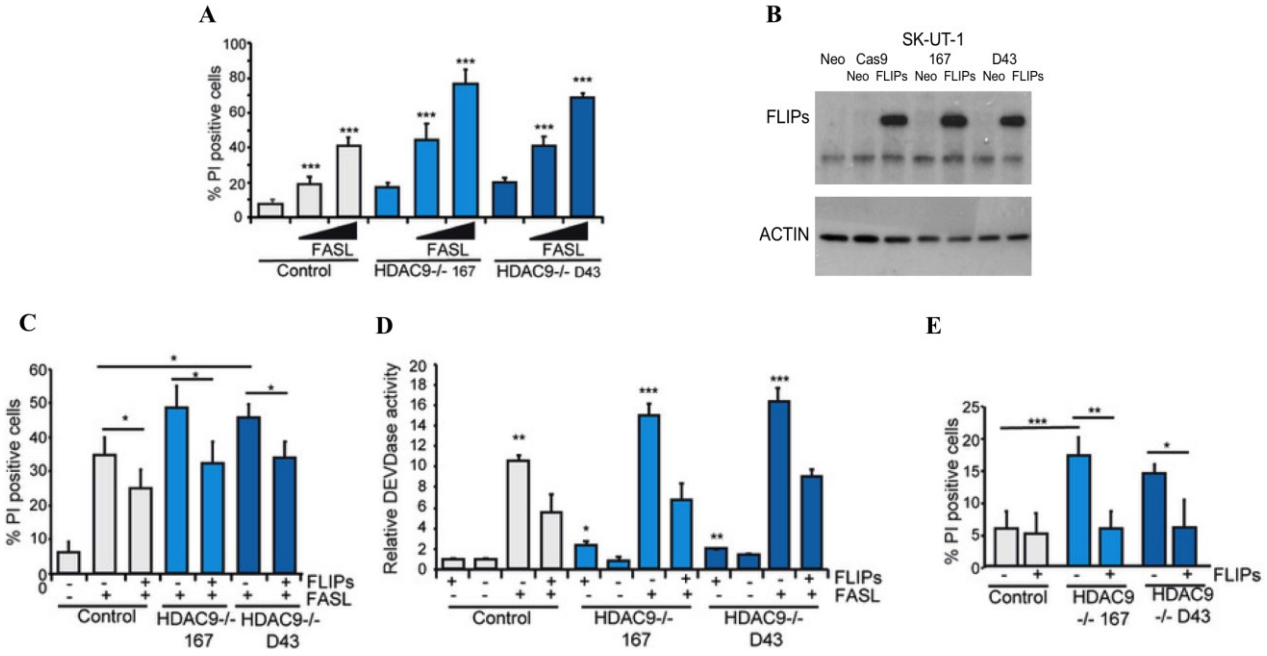


Fig.23: A) Cytofluorimetric analysis indicating the percentage of positive PI SK-UT-1 Cas9, HDAC9$^{-/-}$ 167 and HDAC9$^{-/-}$ D43 treated with increasing amount of FasL (25ng/ml and 50ng/ml). B) Immunoblot analysis of the protein levels of FLIPs in SK-UT-1 Cas9 Neo and Neo/FLIPs, HDAC9$^{-/-}$ 167 Neo and 167 Neo/FLIPs and HDAC9$^{-/-}$ D43 Neo and D43 Neo/FLIPs. Actin was used as loading control. C) Cytofluorimetric analysis indicating the percentage of positive PI SK-UT-1 Neo and Neo/FLIPs, HDAC9$^{-/-}$ 167 Neo and 167 Neo/FLIPs and HDAC9$^{-/-}$ D43 Neo and D43 Neo/FLIPs treated with 25ng/ml of FasL. Data are presented as mean and st. dev. n=3. D) Caspase assay of SK-UT-1 Neo and Neo/FLIPs, HDAC9$^{-/-}$ 167 Neo and 167 Neo/FLIPs and HDAC9$^{-/-}$ D43 Neo and D43 Neo/FLIPs treated with 25ng/ml of FasL. Data are presented as mean and st. dev. n=3. E) Cytofluorimetric analysis indicating the percentage of positive PI SK-UT-1 Neo and Neo/FLIPs, HDAC9$^{-/-}$ 167 Neo and 167 Neo/FLIPs and HDAC9$^{-/-}$ D43 Neo and D43 Neo/FLIPs.

3.9 The KO of HDAC9 increases the sensitivity to conventional chemotherapeutic drugs

The physiological activation of apoptosis observed in HDAC9$^{-/-}$ cells opened a window on the possibility to exploit this feature in a clinic perspective. In fact, the ablation of HDAC9 could be combined to conventional chemotherapeutic drugs, currently used in the treatment of leiomyosarcomas, potentially enhancing their therapeutic effects. To test this hypothesis, we treated the SK-UT-1 cells HDAC9$^{-/-}$ and HDAC9$^{+/+}$, expressing or not FLIPs, with standard concentrations of the genotoxic Doxorubicin (50nM), the AKT inhibitor MKK-2206 (20µM) and G5 (5µM). Cell death was quantified by means of incorporation of propidium iodide (PI) and scored in flow cytofluorimetry. In comparison to the untreated cells, it was appreciable

the increased cell death fraction after the treatment with the anti-neoplastic molecules (Fig.24A) Furthermore, the percentage of cell death was significantly enriched in SK-UT-1 HDAC9$^{-/-}$ cells in comparison to the SK-UT-1 control, demonstrating the additive effects of HDAC9 KO and Doxorubicin or MKK-2206 treatments (Fig.24A). Curiously, the anti-apoptotic protein FLIPs significantly attenuated cell death in the cells treated with Doxorubicin, while it was much less effective with MKK-2206 compound. The same cells treated with Doxorubicin were screened for caspases activation, thus confirming the PI analysis and the activation of an apoptotic response (Fig.24B).

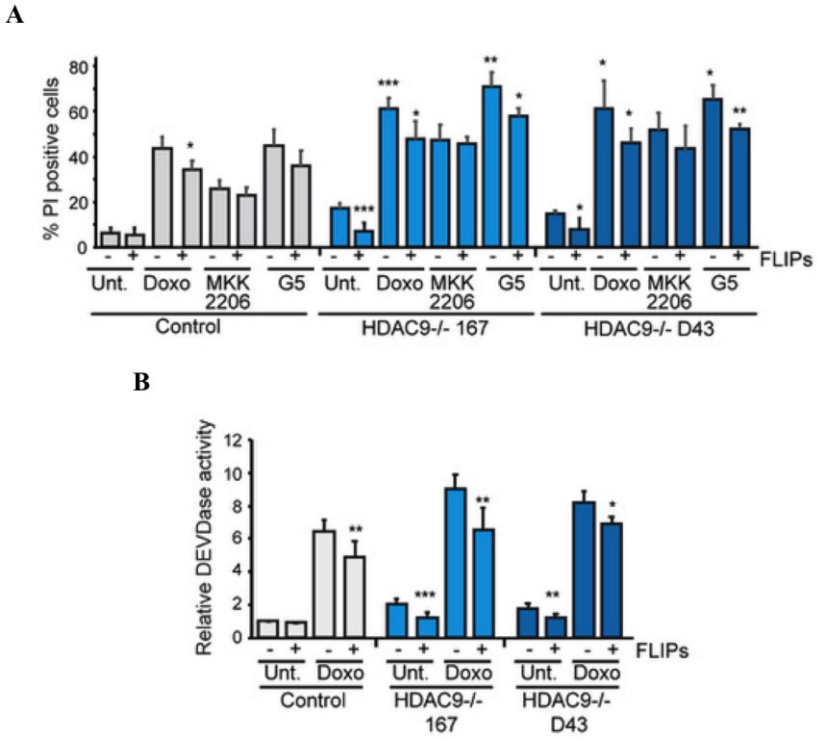


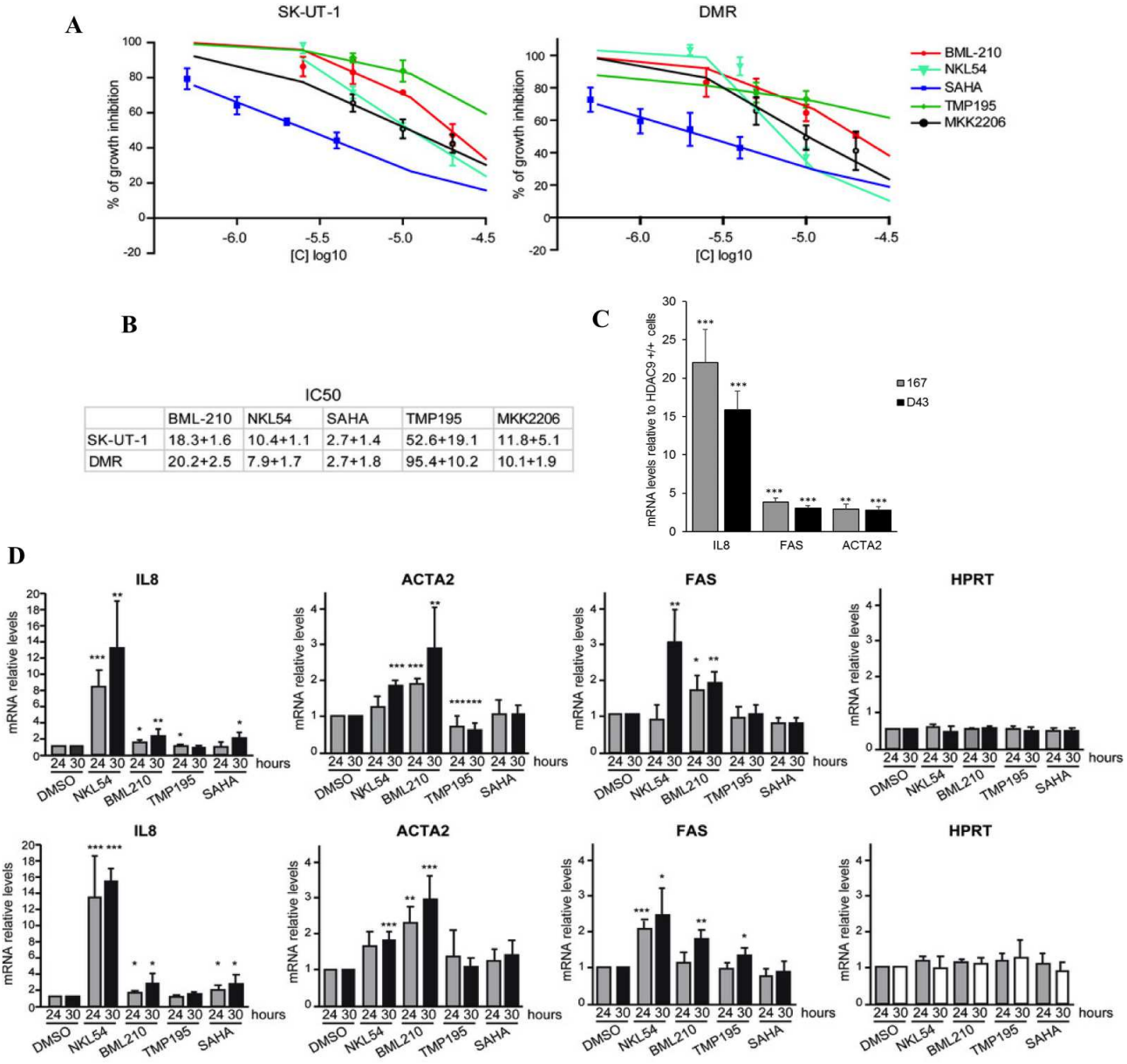
Fig.24: A) Cytofluorimetric analysis indicating the percentage of positive PI SK-UT-1, HDAC9$^{-/-}$ 167 and HDAC9$^{-/-}$ D43 Neo and FLIPs cells, treated with Doxorubicin (50nM), MKK-2206 (20μM) and G5 (5μM). Data are reported as mean and st. dev. in the histogram. n=3. B) Caspase assay of SK-UT-1, HDAC9$^{-/-}$ 167 and HDAC9$^{-/-}$ D43 Neo and FLIPs cells treated with Doxorubicin (50nM). Data are presented as mean and st. dev. n=3.

3.10 Class IIa HDACs inhibitors sensitize SK-UT-1 cells to cell death and differentiation

Considered the increased activation of apoptosis and the sensitivity of SK-UT-1 HDAC9$^{-/-}$ cells to some conventional chemotherapeutic drugs, our aim was to mimic the effect of the knock-out of HDAC9 in the neoplastic cells under study. The approach that we propose is to treat SK-UT-1 cells with some HDACs inhibitors: for the analysis, we selected new potential class IIa HDACs specific compounds: NKL-54, a BML-210 derivative (Jayathilaka N. *et al*, 2012) and TMP-195 (Guerriero JL. *et al*, 2017). Experiments compared the effectiveness of NKL-54 and TMP-195 with pan deacetylase inhibitors, such as SAHA (Richon VM., 2006)

and BML-210 (Jayathilaka N. *et al*, 2012). MKK-2206 was used as reference of cell death. IC50 of the selected drugs was calculated both in SK-UT-1 and DMR cell. SAHA showed the strongest effect on cell viability, but its low specificity for class IIa HDACs leads it to be the less beneficial therapeutic drug in a context of class IIa HDACs dysfunctions. BML-210 and NKL-54 showed a significant negative effect on cell growth. Less impact was obtained with TMP-195 (Fig.25A-B).

A group of genes, all up-regulated by HDAC9 KO (Fig.25C), were selected to determine the transcription effects and specificity of the tested compounds. *IL-8*, a marker of inflammation and cell stress, *ACTA2*, a marker of cell differentiation and *FAS* receptor, a marker of cell death. Transcriptional analysis were performed by using the IC25 of each molecule and their effects were evaluated at 24 and 30 hours of treatment. The results indicated that both in SK-UT-1 and DMR cells, NKL-54 and BML-210 induce *IL8, FAS* and *ACTA2* at levels comparable to HDAC9 KO (compare Fig.25C with Fig.25D). SAHA regulates significantly only *IL8*, while TMP-195 showed not relevant effects.



IC50	BML-210	NKL54	SAHA	TMP195	MKK2206
SK-UT-1	18.3+1.6	10.4+1.1	2.7+1.4	52.6+19.1	11.8+5.1
DMR	20.2+2.5	7.9+1.7	2.7+1.8	95.4+10.2	10.1+1.9

Fig.25: A) Resazurin assay was applied to calculate the IC50 of BML-210, MKK-2206, SAHA, NKL-54 and TMP-195 in SK-UT-1 and DMR. Cells were treated for 40h with serial dilutions of the compounds: BML-210 (2.5µM, 5µM, 10µM, 20µM), MKK-2206 (5µM, 10µM, 20µM, 40µM), and TMP-195 (5µM, 10µM, 20µM, 40µM), SAHA (0.5µM, 1µM, 2µM, 4µM) and NKL-54 (2.5µM, 5µM, 10µM, 20µM). B) Table indicating the IC50 and st.dev. of each drug in SK-UT-1 and DMR cells. C) mRNA expression levels of *IL8*, *ACTA2*, *FAS* were measured by qRT-PCR in HDAC9 KO clones 167 and D43. D) mRNA expression levels of *IL8*, *ACTA2*, *FAS* were measured by qRT-PCR after 24h and 30h of drug treatment in SK-UT-1 (upper panel) and DMR cells (lower panel). HPRT was used as housekeeping gene.

3.11 NKL-54 sensitizes SK-UT-1 cells to apoptosis and synergizes with other drugs to trigger cell death

NKL-54 was selected for further studies because of its capability to recapitulate the phenotype of the knock-out of HDAC9, in terms of genes activated and for its increased activity in comparison to BML-210, its derivative. SK-UT-1/Puro and SK-UT-1 Puro/FLIPs cells were generated and treated with increased concentrations of NKL-54 (1.25μM, 2.5μM, 5μM and 10μM). Cell death was tested by propidium iodide (PI) assay. As reported in figure 26A, the percentage of PI positive SK-UT-1/Puro cells increased by increasing the concentrations of NKL-54 and the results indicated that FLIPs expressing cells were less affected by NKL-54 treatment (Fig.26A).

To demonstrate that the increased cell death after the treatment with NKL-54 was related to the activation of the apoptotic process, caspase-3 and -7 activities were measured. As shown in figure 26B, in SK-UT-1/Puro cells, the levels of caspases activation is proportional to the concentrations of NKL-54. The same trend was visible for SK-UT-1 Puro/FLIPs cells, despite at lower levels in comparison to control cells (Fig.26B). The experiment confirmed that the NKL-54 was toxic for the cells and the toxicity was attenuated by the inhibition of the extrinsic apoptotic pathway. This could be basically due to the expression of the anti-apoptotic protein FLIPs, which significantly protected the cells from Caspase-8-dependent apoptosis (Kataoka T., 2005; Hughes MA. *et al*, 2016). Overall, this experiment demonstrated that cell death occurred principally as a result of caspases activation.

Similar results were obtained in DMR/Neo cells and Neo/FLIPs (Fig.26C-D). Interestingly, the over-expression of FLIPs strongly decreased the pro-apoptotic activity of NKL-54 in DMR cells (Fig.26D).

Cytofluorimetric analysis and caspase activation assay suggested that NKL-54 could be a promising drug to sensitize cancer cells to apoptosis, recapitulating the effect of the knock-out of HDAC9. Therefore, we evaluated whether NKL-54 could increase the cytotoxic effect of other compounds, such as Doxorubicin, MKK-2206 and ABT-263. For this purpose, SK-UT-1/Puro and SK-UT-1 Puro/FLIPs cells were treated with NKL-54, ABT-263, a Bcl-2/xL inhibitor (Wang B. *et al*, 2014), Doxorubicin and MKK-2206, individually and in combination with NKL-54. As reported in Figure 26E, the single treatment of NKL-54 in SK-UT-1/Puro cells triggers 20% of cell death, while Doxorubicin induces approximately 30% of cell death, as indicated by the percentage of PI positive cells. MKK-2206 and ABT-263 alone show a weaker impact on cell death. The co-treatment of the cells with NKL-54 and Doxorubicin displayed an additive effect that is partially blunted in FLIPs over-expressing

cells. A weak synergistic effect of NKL-54 can be appreciated when associated to MKK-2206 and to ABT-263. Moreover, the experiment showed how, in the case of NKL-54 associated to MKK-2206, the effect is only partially recovered by FLIPs (Fig.26E).

To confirm that the observed cell death phenotype was the consequence of the apoptotic process, the activation of caspases-3 and -7 was tested. The analysis was done for SK-UT-1/Puro and SK-UT-1 Puro/FLIPs cells treated with MKK-2206, Doxorubicin, ABT-263 and NKL-54 individually and with a combination of NKL-54 and MKK-2206, NKL-54 and ABT-263 and NKL-54 and Doxorubicin. The histogram (Fig.26F) shows that cell death was marked by caspase activation and the highest levels of activation occurred when cells were co-treated with Doxorubicin and NKL-54. This test also evidenced that cells treated with MKK-2206 and NKL-54 present a limited caspase activation, suggesting the co-occurence of caspase-independent cell death. The additive effects between Doxorubicin and NKL-54 was confirmed also in DMR cells (Fig.G-H).

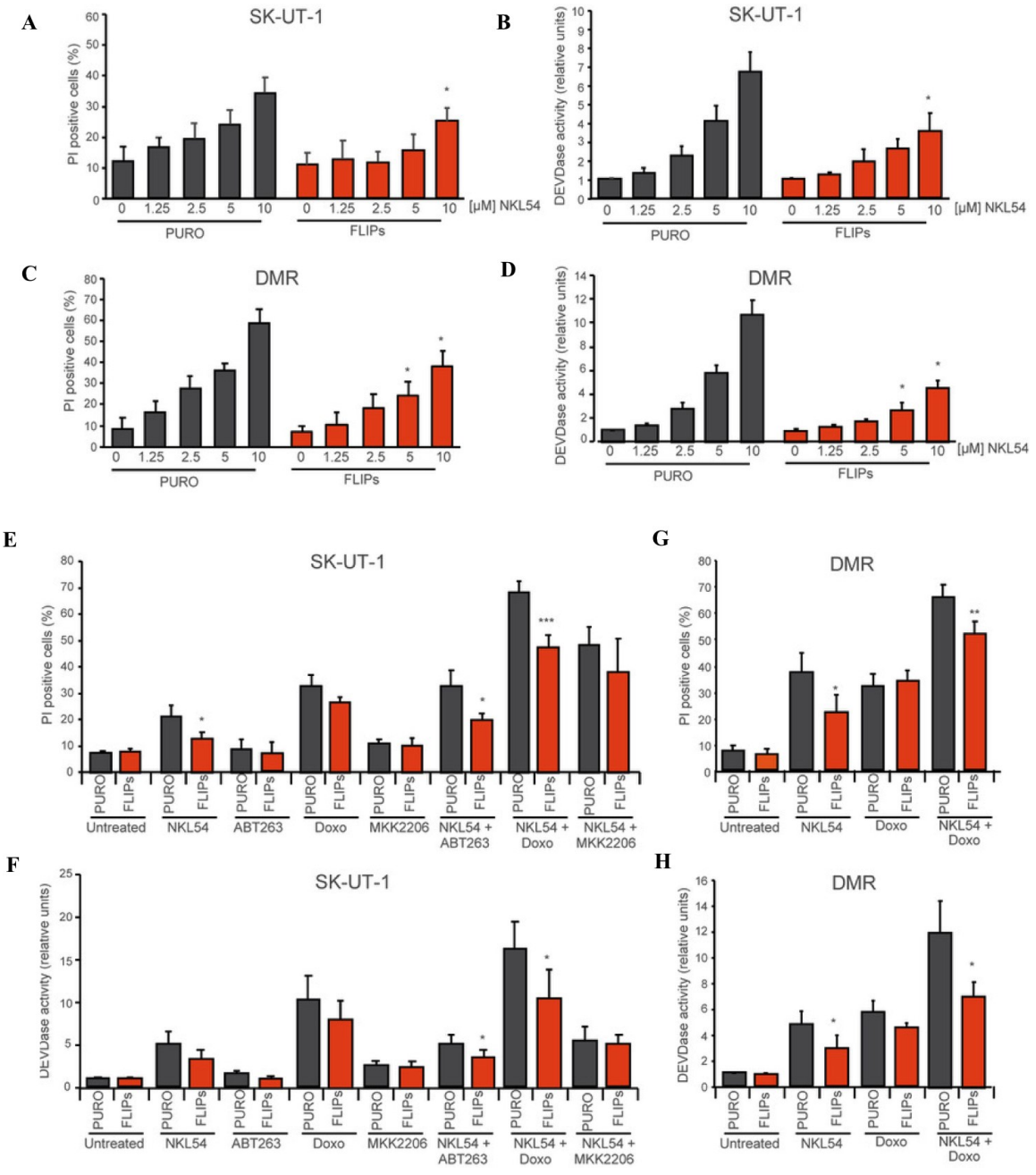


Fig.26: A) Cytofluorimetric analysis indicating the percentage of positive PI SK-UT-1/Puro and SK-UT-1 Puro/FLIPs cells treated with NKL-54 (1.25μM, 2.5μM, 5μM, 10μM). Data are presented as mean and st. dev. n=4. B) Caspase assay of SK-UT-1/Puro and SK-UT-1 Puro/FLIPs cells treated with NKL-54 (1.25μM, 2.5μM, 5μM, 10μM). Data are presented as mean and st. dev. n=3. C) Cytofluorimetric analysis indicating the percentage of positive PI DMR/Neo and DMR Neo/FLIPs cells treated with NKL-54 (1.25μM, 2.5μM, 5μM, 10μM). Data are presented as mean and st. dev. n=4. D) Caspase assay of DMR/Neo and DMR Neo/FLIPs cells treated with NKL-54 (1.25μM, 2.5μM, 5μM, 10μM). Data are presented as mean and st. dev. n=3. E) Cytofluorimetric analysis showing the percentage values of positive PI SK-UT-1/Puro and Puro/FLIPs cells, following individual treatment with NKL-54 (5μM), Doxorubicin (50nM), ABT-263 (100nM) and MKK-2206 (10μM) and after treatment with NKL-54 (5μM) in association with Doxorubicin (25nM), ABT-263 (100nM) and MKK-2206 (10μM). Data are presented as mean and standard deviation. n=3. F) Caspase assay of SK-UT-1/Puro and SK-UT-1 Puro/FLIPs cells treated with NKL-54 (5μM), Doxorubicin (50nM), ABT-263 (100nM) and after treatment with NKL-54 (5μM) in association with Doxorubicin (25nM), ABT-263 (100nM) and MKK-2206 (10μM). Data are presented as mean and st. dev. n=3. G) Cytofluorimetric analysis indicating the percentage of positive PI DMR/Neo and DMR Neo/FLIPs cells after treatment with NKL-54 (5μM), Doxorubicin (50nM) and with NKL-54 (5μM) in association with Doxorubicin (25nM). Data are presented as mean and st. dev. n=3. H) Caspase assay of DMR/Neo and DMR Neo/FLIPs treated with NKL-54 (5μM), Doxorubicin (50nM) and with NKL-54 (5μM) plus Doxorubicin (25nM). Data are presented as mean and st. dev. n=3.

4. DISCUSSION

Transcriptional dysregulation could be considered an emerging hallmark of cancer. Extensive studies on tumor pathogenesis have unveiled the impact of genetic alterations affecting proteins involved in the transcriptional control (Bradner J.E. *et al*, 2017). The tumorigenic process is characterized by alterations of the main transcriptional regulators that make normal cells more prone to proliferate, migrate and less sensitive to cell death. Thus, an approach to enrich cancer knowledges and find important therapeutic targets would be to discover the components involved in the deregulation of transcriptional programs in neoplastic cells on which the growth and survival of cancer cells depend on (Martin M.N. *et al*, 2018).

In recent decades, the treatment of neoplasm has obtained promising results by targeting the oncogenic addictions (Weinstein IB. and Joe A., 2008). For example, EGFR is identified as a driver oncogene in non–small cell lung carcinoma, as well as c-kit in gastrointestinal stromal tumors and KRAS in pancreatic adenocarcinoma. Even if fascinating, the genomic instability, as well as the tumor heterogeneity, have reduced the promising results of hitting a single molecular target and a rational combination therapy is still required (Weinstein IB. and Joe A., 2008).

In this project we investigated a new form of cancer addiction: the acquisition by cancer cells of an epigenetic mechanisms of addiction (Robison AJ. and Nestler E., 2011). In particular, our research focused on LMS for three main reasons: 1) LMSs are tumors with a not a completely defined panel of driver oncogenes (Tawbi HA. *et al*, 2017); 2) no advanced therapies have been developed yet; 3) we have shown, also thanks to the work of this thesis, that class IIa HDACs are highly active in high grade LMSs (Di Giorgio E. *et al*, 2013; Di Giorgio E. *et al*, 2017). Our previous studies led to the discovery that during the leiomyosarcomagenesis MEF2 family members are highly repressed and this is associated with increased tumor aggressiveness. In low grade LMS cells, this repression could be mainly due to decreased MEF2 protein half-life (Di Giorgio E. *et al*, 2013; Di Giorgio E. *et al,* 2017), while in high grade LMSs, MEF2 expression is stable and they are converted into transcriptional repressors through the engagement of class IIa HDACs.

Among the four HDACs, a relevant role is played by HDAC9, that is over-expressed in one quarter of LMSs. The KO of HDAC9 in high grade LMS cells (SK-UT-1 cell line) removes its repressive effect on MEF2 and significantly reduces the transformed phenotype of SK-UT-1 cells. These features are reflected by an accumulation of cell death and by a cell cycle arrest in G1 phase (manuscript in preparation), as previously reported for the knock-down of MEF2D and MEF2A in SK-UT-1 cells (Di Giorgio E. *et al*, 2017). Whether the arrest in G1 could be due to a mitotic crisis is still under investigation.

The key role of HDAC9 in the malignancy of LMS suggests the crucial role of this protein in the tumorigenic process of uterine LMSs.

However, the co-expression of MEF2 and HDAC9 is not limited to LMSs; evidences of their frequent correlation have been found also in other cancer types, such as in breast cancer and in pediatric leukaemias, reviewed by Di Giorgio E. *et al*. (2018). HDAC9 is also over-expressed in basal breast cancer cells; here it is described to be required to sustain malignancy, survival and resistance to HDIs (Lapierre M. *et al*, 2016).

High levels of HDAC9 have been reported also in B-ALL (B progenitor acute lymphoblastic leukaemias) cases, as a consequence of chromosomal rearrangements that give rise to chimeric fusion proteins (Gil VS. *et al*, 2016). In the last years, new frequent rearrangements between MEF2D and other genes (*BCL9*, *CSF1R*, *DAZAP1*, *HNRNPUL1* and *SS18*) have been detected; specifically, the MEF2D-BCL9 fusion is responsible of an increased transcription of *HDAC9* and at the same time, of a negative regulation of other MEF2 targets, such as *RAG-1* (Gu Z. *et al*, 2016).

In physiological contexts, the relationship between HDAC9 and MEF2D is strongly regulated by a negative feed-back loop, as demonstrated during the skeletal muscle development. MEF2 is active in the transcription of genes fundamental for muscle differentiation, such as myogenin (*MYOG*), but also in the activation of HDAC9, which in turn regulates MEF2 by suppressing its transcriptional activity (Haberland M. *et al*, 2007). This sophisticated system serves to keep their expression levels balanced, thus supervising muscle differentiation programs.

The co-existence of high levels of HDAC9 and MEF2D in cancer may be explained by an alteration of this circuitry (Di Giorgio E. *et al*, 2018). Curiously, we observed that in high grade LMSs, MEF2 are converted into transcriptional repressors through the binding of HDAC9 on certain genomic loci, but they maintain the role of transcriptional activators on other genes and also on the HDAC9 promoter. It this way, we speculate that in tumoral cells, MEF2 is entangled in promoting HDAC9 transcription in an altered mechanism enriched by the refractoriness of HDAC9 in binding and repressing its own transcription. We do not exclude that the altered feed-back established between HDAC9 and MEF2 in leiomyosarcomas could be responsible of the malignancy and aggressiveness of other cancer types.

The epigenetic addiction derived from the co-expression of MEF2 and class IIa HDACs, which occurs in 25% of LMS, could be exploited to selectively target the complex between MEF2 and class IIa HDACs. By scrutinizing a panel of molecules, we selected a

pharmacological approach with the BML-210 analogue, NKL-54 (Jayathilaka N. *et al*, 2012), which predisposes them to cell death. In details, we found that this agent sensitizes the cancer cells to apoptosis, recapitulating the effect of HDAC9 knocking-out. Moreover, it increases the cytotoxic effects of other compounds, in particular of Doxorubicin.

The anti-proliferative effects of synthetic benzamides on cancer cells, such as the BML-210 and its derivatives, were recently reported. Borutinskaitė V. and Navakauskienė R., in 2015, described how BML-210 is able in promoting a significant growth inhibition, by provoking increased levels of p21 expression, and apoptotic cell death of a promyelocytic leukemia cell line (NB4).

Several clinical and preclinical studies suggest the potential of the HDAC inhibitors for the treatment of different types of cancers, alone or in combination with standard doses of other cytostatic drugs (Marek L. *et al*, 2013). The majority of the data report the synergistic effects of the HDACi when combined with DNA-damaging chemotherapeutic agents or radiotherapy (Wagner JM. *et al*, 2010; Thurn KT. *et al*, 2011). For example, combination therapies of the deacetylase inhibitor Vorinostat with the DNA-methyltransferase inhibitor Decitabine, or with the proteasome inhibitor Bortezomib, have proved encouraging results in different haematological malignancies (Wagner JM. *et al*, 2010). Also patients affected by advanced prostate cancer show positive effects when treated with a combination of Panobinostat and the antimitotic chemotherapeutic agent Docetaxel (Rathkopf D. *et al*, 2010).

Combination therapies with more selective member-specific inhibitors are currently investigated, including also class IIa HDACs specific inhibitors. The discovery of these inhibitors may allow the development of new therapeutic drugs that could circumvent the side effects obtained with traditional hydroxamate-based compounds. Novel molecules are still under identification through increasingly sophisticated screening methods, as recently published by Hsu KC. *et al.* (2017). Little is still known from literature about the therapeutic effects of these innovative compounds. Recently, a new potent HDAC inhibitor, LMK235, which is characterized by a selective preference for HDAC4 and HDAC5, has been developed (Marek L. *et al,* 2013). Interestingly, the combination of this compound with cisplatin significantly enhances the cisplatin sensitivity of ovarian and breast cancer cisplatin-resistant cell lines (Marek L. *et al*, 2013; Stronach EA. *et al*, 2011). The effects of BML-210 are similarly studied for its properties to sensitize malignant cells to other drugs, for example when associated with retinoic acid on HeLa cervical carcinoma cells (Borutinskaitė V. *et al*, 2006).

Regarding advanced or metastatic uterine leiomyosarcomas, the standard first-line chemotherapy is represented by Doxorubicin, eventually combined with the alkylating agent Ifosfamide (Akin S. *et al*, 2018), with Gemcitabine, an inhibitor of DNA synthesis and repair (Momtahen S. *et al*, 2016), or with Olaratumab, a human monoclonal antibody against the platelet derived growth factor receptor alpha (PDGFR-α) (Okuno SH. *et al*, 2017). Moreover, Trabectedin (Recine F. *et al*, 2017) and the anti-PD-1 antibody Pembrolizumab (Tawbi HA. *et al*, 2017) have been recently approved in advanced soft-tissue sarcomas.

Unfortunately, all these approaches, even if more innovative and specific for various molecular targets, are characterized by a limited effectiveness due to the high molecular heterogeneity of the tumor.

Our study introduces an innovative approach for the treatment of high grade leiomyosarcomas, through a transcriptional re-setting. Our approach describes an alternative strategy to personalize the therapies, on the base of the expression levels of the two main actors of this tumor: HDAC9 and MEF2A/D. This transcriptional re-setting can potentiate the standard treatment approaches. In details, we propone the adoption of new epigenetic drugs, such as the novel NKL-54, to reprogram the tumor cells and to reduce the chemoresistance.

5. PUBLICATIONS

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