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Title

Role of the histone demethylase LSD1 in Brain Tumor

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CHAPTER 1
INTRODUCTION

1.1 *Epigenetic and cancer*

Epigenetics studies hereditary changes in gene function that are not directly related to the underlying sequence(1).

Gene expression is strictly controlled by three-dimensional architecture of chromatin and the action of multiprotein complexes(2). In the nucleus, genomic DNA is wrapped around histones in nucleosomal subunits which are condensed into chromatin(3); highly condensed chromatin is called heterochromatin and contains mostly inactive genes(3,4). In contrast, euchromatin has a more open structure and contains active genes(5). The chromatin structure is dynamically regulated by histone modifications such as acetylation, methylation, ubiquitination, phosphorylation, sumoylation(6,7).

These modifications the accessibility to genome regions where proteins and enzymes bind, altering the gene expression.

Epigenetic regulation is a dynamic and reversible process. The proteins involved in epigenetic changes can be classified as writers, readers and erasers(8): epigenetic writers catalyze the addition of epigenetic marks on "tails" of histones that extend from the histone octamer structure; readers recognize or are recruited into a specific epigenetic sign; the erasers remove epigenetic marks(8).

Alterations of genes that regulate epigenetic processes are frequently found as carcinogenic factors and can cause widespread alterations of DNA methylation and modification of histone or chromatin that disrupt normal gene expression(5,9–11). The inhibitors that target these epigenetic processes are promising anticancer strategies(12).

The new generation sequencing has identified that mutations found in cancer are amplifications, deletions and rearrangements of genes, which influence the epigenetic regulation pathways(13,14). Driver mutations in epigenetic regulators can lead to alterations in DNA methylation or to modify the histone that disrupts the structure of chromatin and normal gene expression. DNA hypermethylation and/or repressive histone signature on a promoter can cause the loss of function mutations in tumor suppressor genes to become phenomena

by silencing gene expression(15,16). Conversely, loss of DNA methylation or activation signature, can significantly increase gene expression in a similar way to what would be observed after oncogenic chromosomal translocations or gene amplification(17,18).

Epigenetic changes are reversible. For this reason, deciphering how aberrant epigenetic mechanisms lead to malignant transformation can bring new ideas on how targeting these mechanisms could be used as therapy in cancer(9). Methylation inhibitors and histone deacetylase inhibitors are approved for certain hematological malignancies, including T-cell lymphoma (vorinostat (19), romidepsin (20)), multiple myeloma (panobinostat,(21)) and myelodysplastic syndrome (MDS) (decitabine or azacytidine)(22,23). To date, further clinical studies are underway on epigenetic therapies in solid tumors as single agents and in combination with other therapies.

1.2 ***LSD1***

Post-translational modifications of histones, including acetylation, phosphorylation, methylation, ubiquitination and sumoylation, occur for gene transcription and other processes that have chromatin as a substrate by directly modifying the chromatin structure and/or by recruiting effectors containing modules proteins that selectively bind to modified histones(1,7,10). Lysine methylation of histones can activate or repress transcription, depending on the site and modification degree (mono-, di-, and trimethylation)(24).

LSD1 was the first histone demethylase discovered and specifically demethylates H3 mono and dimethylated histones in lysine 4 and 9 (K4 3 K9)(25). The catalytic domain of LSD1 shares structural domain with amino oxidases and removes methyl groups through a FAD-dependent reaction(26–28). Histone methylation is a dynamic process regulated by enzymes with opposite activities. The percentage of demethylated histone H3-K4 is enriched in the promoter regions with actively transcribed genes(29).

1.2.1 Structure of LSD1

The LSD1 gene contains 19 exons, highly conserved in vertebrates, and there are 4 protein isoforms due to alternative splicing(3).

The human LSD1 protein is 852 aa long and consists of several domains (Figure1)(25,28,30,31):

• N-terminal domain, composed of the SWIRM domain consisting mainly of α-helices (commonly found in complexes responsible for chromatin remodeling) and of an unstructured region made of linear motifs that could represent functional sites responsible for the association LSD1 with different transcriptional protein complexes;

• A central tower domain consists of two long anti-parallel α-helix with a typical coiled-coil conformation, which offers a surface platform for interaction with partners. This domain is essential both for demethylase activity and for interaction with the CoREST co-repressor;

• A C-terminal domain includes the amino-oxidase domain (AO), which shares a homology with the FAD-dependent AO domains.

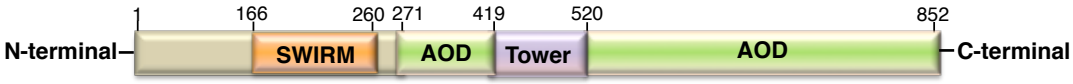

Figure 1 Lysine specific demethylase 1 (LSD1) protein domains and structure.

Based on the structural analysis of LSD1, the aspartic acid 375 and the glutamic acid 379 of the AO domain build an interaction with Arg8 of the histone H3, while the glutamic acids in position 553, 555, and 556 interact with Arg2 of histone H3 (32). Mutation of these critical residues within the catalytic domain of LSD1 abolish its catalytic activity on demethylation in H3K4. These observations indicate that histone H3 interacts in the catalytic cavity of LSD1 specifically with the AO domain of LSD1 itself(33).

1.2.2 LSD1 reaction mechanism

LSD1 demethylates H3K4 and H3K9, is classified as flavin-dependent monoamine oxidase (MAO) using the flavin dinucleotide (FAD) cofactor during demethylation catalysis(26,30). During the reaction, formaldehyde is formed in addition to methyl to H2O2 in the presence of O2. Molecular oxygen is used as an electron acceptor and the oxidation of the methyl group then proceeds with the transfer of the hydride (H-) from the N-methyl to the FAD forming amine. LSD1 alloy FAD accepting 2 electrons. The oxidation of the FAD is restored by molecular oxygen(25). (Figure 2)

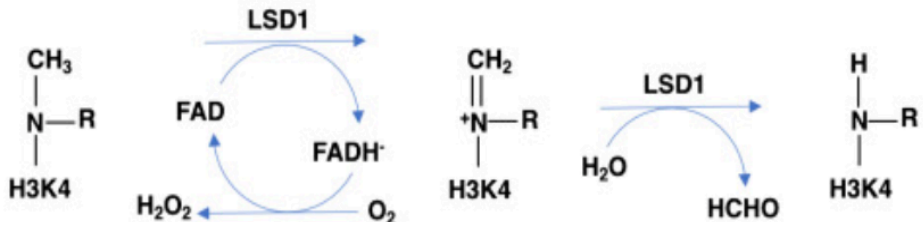

Figure 2. LSD1 reaction mechanism

1.2.3 Mechanism of transcriptional regulation of target genes

The function of LSD1 also depends on the association with specific transcriptional co-repressor complexes that often include histone deacetylase 1/2 (HDAC1 / 2)(34) and CoREST(35)(Figure3a).

The transcriptional corepressor complexes containing LSD1-CoREST are recruited on specific promoters from specific proteins that bind specific DNA sequences. For example, through its interaction with CoREST, the transcriptional repressor REST recruits LSD1-CoREST on promoters of specific

neuronal genes, thereby blocking their expression in non-neuronal tissues in vertebrates(35,36).

Global epigenetic alterations, including histone acetylation changes and methylation, can lead to the silencing of key tumor suppressor genes and collaborate with genetic mutations to promote tumorigenesis(9,10).

A further physical interaction between LSD1-MYCN has been studied, thus show that this last one leads to the repression of CDKN1A / P21. P21 protein is considered the universal inhibitor of the cycle progression and act inhibiting the Cyclin / CDK complexes and degrading pRB, leading to the activation of the control points G1 / S and inter-phase S, and therefore P21 is considered the mediator of the arrest of the cycle in phase G1(37,38).

LSD1 can also be an androgen (AR) and estrogen (ER) receptor dependent transcription activator. The interaction between the LSD1 and AR or ER nuclear receptors directly or indirectly modifies its substrate specificity through demethylation of the H3K9me1 / 2 marks associated with repression (36,39)(Figure 3b). Different roles and specificities of the tissues have been attributed to the junction variant of LSD1 (LSD1 + 8a), within the domain AO, containing four additional amino acids (exon 8a) (40), The presence of exon 8a generates a docking site for supervillain (SVIL) that converts LSD1 into an H3K9 demethylase in neuronal differentiation (41). Furthermore, LSD1 + 8a has also been described to promote transcription of genes regulated by neurons removing H4K20me2(Figure 3c)(42).

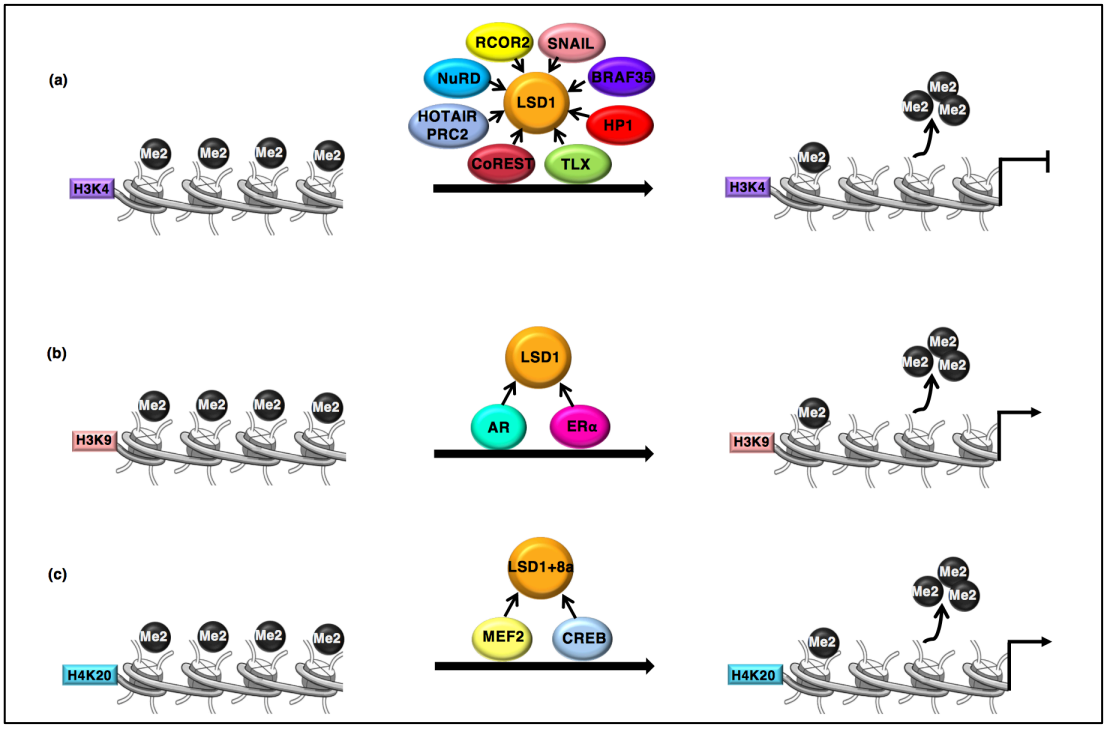

Figure 3 a) Demethylase 1 (LSD1) is recruited at the target gene by a wide number of transcription factors (indicated in figure). (b) LSD1 demethylates as coactivator with the androgen or estrogen receptor. (c) LSD1+8a interact with CREB and MEF2, catalyzes the demethylation of the repressive mark H4K20me2.

1.2.4 Non-Histone Substrates of LSD1

Although LSD1 was originally identified as histone lysine demethylase, several reports highlight the role of LSD1 to regulate the activity of non-histone proteins through LSD1-dependent demethylation mechanisms. Indeed, LSD1 participates to the methylation/demethylation dynamics on specific lysine residues of several non-histone proteins, including, p53, DNMT1, STAT3, E2F1, RB1, MEFD2, MTA1, ERα, HSP90, HIF-1α, and more recently AGO2. The demethylase activity of LSD1 alters both the function and stability of these proteins.

The first discovered target of LSD1 demethylation was p53(43), one of the most important tumor suppressor proteins. p53 controls the response to different cellular stress signals, in particular, it activates transcription targets involved in the cell cycle control and apoptosis.

The demethylation activity of LSD1 promotes DNA damage-induced cell death by stabilizing E2F1(44,45).

Cell-cycle phosphorylation dynamics of RB1 is required for proper control of cell cycle progression. RB1 is dephosphorylated by the phosphatase PPP1R12A, however, PPP1R12A lysine 442 is methylated by the histone lysine methyltransferase SETD7 and demethylated by LSD1(46)(Figure 4a).

LSD1, through HIF-1α stabilization, induces genes involved in glycolysis in cancer cells. LSD1 directly represses genes involved in mitochondrial metabolism demethylating histones H3K4 (47,48)(Figure 4b).

LSD1 plays a key role in the reprogramming of cancer metabolism inducing the shift from oxidative to glycolytic metabolism, maintenance of redox homeostasis, and cell survival.

Recent studies discovered a novel function of LSD1 in modulating the tumor immunogenicity. LSD1, through demethylation of AGO2 (Protein Argonaute 2) has a crucial role in the suppression of anti-tumor immunity and tumor immunogenicity(49).

LSD1 ablation in these tumor cells results in the upregulation of PD-(L)1, the programmed death-ligand 1, which might compromise the anti-tumor effect of T cells infiltration (Figure 4c).

In conclusion, LSD1 inhibition could be instrumental to convert “cold” tumors (resistant to PD-1 blockade) in “hot” tumors (responsive to PD-1 therapy) and provides a means to target LSD1 increasing the efficacy of immunotherapy of poor immunogenic cancers(49).

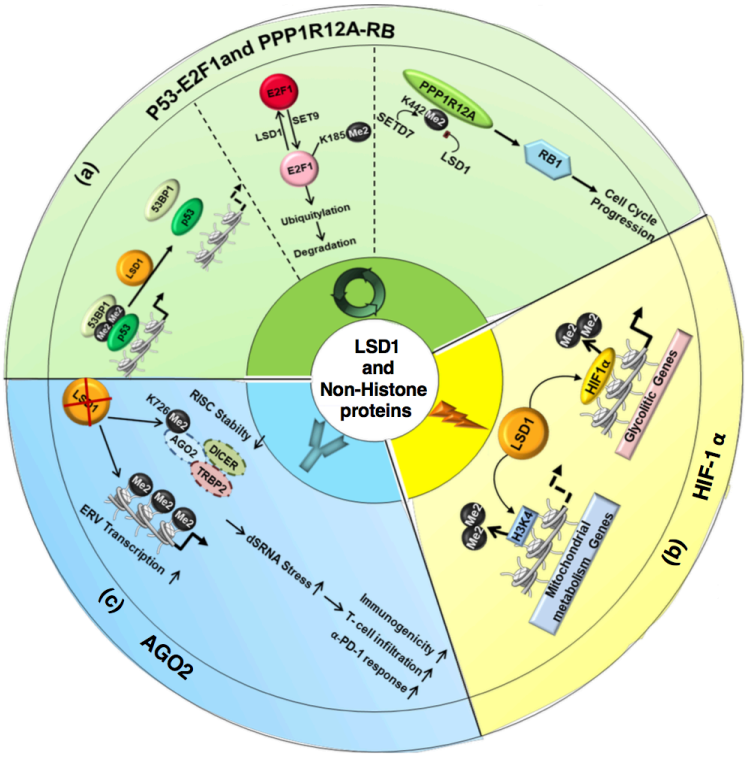

Figure 4 LSD1 regulates activity of non-histone proteins. Effects of LSD1-mediated demethylation of non-histone proteins p53-E2F1 and PPP1R12A (**a**), HIF-1α (**b**), and AGO2 (**c**).

1.2.5 LSD1 and lncRNA

Long non-coding RNAs (lncRNAs) are regulators that act as modulators in various molecular processes such as gene regulation, genome packing maintenance, chromatin dynamics and cell differentiation. Mainly, they contribute to the phenotypes of cancer cells through interaction with chromatin-modifying agents and proteins, such as the repressive poliform 2 complex, LSD1, CoREST and SMCX(36). Recently, it has been shown that LSD1 collaborate with many lncRNAs for different role, as the interaction between LSD1 and lncTERRA controls the telomere length with a strong impact in aging and cancer (50)(Figure5b). LSD1, in complex with HBXIP-HOTAIR, regulate c-Myc target genes and determines their activation. In particular, the oncoprotein HBXIP interacts directly with c-Myc leading to the recruitment of HBXIP-HOTAIR-LSD1 complex(51). Different studies highlight the scaffolding role of lncRNAs for epigenetic enzymes such as LSD1 and EZH2 to form

ribonucleoprotein complexes, which repress the transcription of tumor suppressor gene (Figure5b).

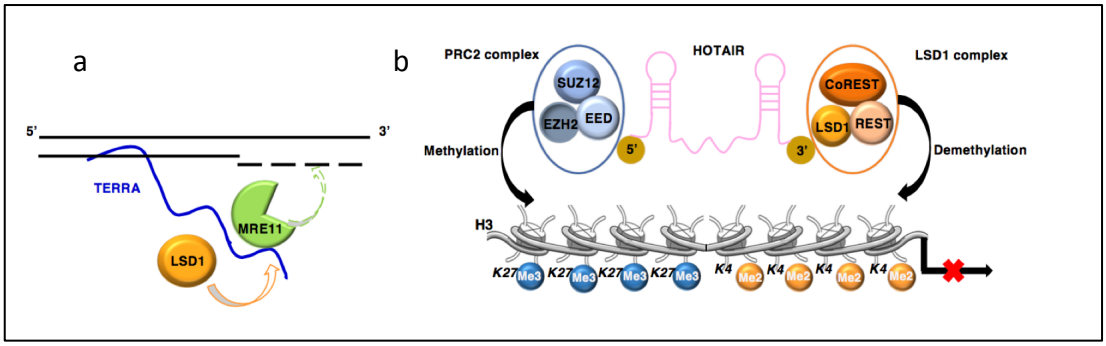

Figure 5. (**a**) (TERRA RNA modulates telomeric gene silencing through recruitment of LSD1 on uncapped telomeres, reinforcing the interaction between LSD1 and MRE11. (**b**) HOTAIR mediates target gene silencing; it binds to PRC2 complex (EZH2, SUZ12, EEDs) and LSD1 complex (CoREST/REST) through binding at 5′ and 3′ ends, respectively, regulating trimethylation of H3K27me3 and demethylation of H3K4me2.

1.2.6 LSD1 as a therapeutic target in cancer

The altered expression of LSD1 also plays a key role in other neoplasms, for example, in prostate cancer the inhibition of the demethylase activity of LSD1 leads to a drastic decrease in the growth of tumor cells or in breast cancer where has been verified that LSD1 is involved in the invasion and metastasis processes, in fact its levels are inversely correlated with TGF-β(38,52).

LSD1 is also involved in processes other than those described so far, as in the development process. It has been shown that in mice without LSD1, the viability rate did not exceed 6 days, this is due to disorders in meiotic processes, to their once due to the lack of methylase activity on histones H3K4-K9(53).

Recent studies have underlined the crucial role of LSD1 in the repression of differentiation genes, and its inhibition leads to a regression of the tumor phenotype promoting the differentiation process. Cell differentiation is orchestrated both spatially and temporally by a transcriptional regulation including histone modification; LSD1 plays an important role in stem cell biology, in particular, with its demethylase activity keeps the genes of cell

differentiation silent. It has been shown that in neuronal development, LSD1 can be ubiquitinated by Jade-2 and brought to degradation through the proteasome, in this way a stem cell can differentiate into multiple or multi-powerful cell(54).
Furthermore, it has been reported that a specific LSD1 isoform is able to regulate neuronal differentiation by specifically demethylating H3K9 demethylate, i.e. this isoform obtained by alternative splicing is able to interact with different promoters.
Clearly, LSD1 is up-regulated in a several tumor and its high expression correlate with more aggressive cancers with poor prognosis.
Various studies highlights that LSD1 is involved in maintaining the undifferentiated, malignant phenotype of neuroblastoma (NB) cells and that its overexpression correlates with aggressive disease, poor differentiation and infaust outcome(37,38,55).
In Glioblastoma (GBM), LSD1 and HDAC inhibitors, might be used as a potential combination therapy, in fact their inhibition regulates pathways of cell death. GBM is aggressive brain tumor and remains a clinically devastating disease, but epigenetic therapy may have effective therapeutic relevance in GBM(56).
Recently, in medulloblastoma, another most prevalent malignant brain tumors in children, Lsd1 interact with Gfi1, and that these proteins cooperate to inhibit genes involved in neuronal commitment and differentiation. We also show that Lsd1 is essential for Gfi1-mediated transformation(57).
It would seem quite clear that LSD1 inhibition might be to represent a strategy for Brain Tumor.

1.2.7 LSD1 inhibitors

Aberrant epigenetic modifications, including DNA methylation and histone changes, are well known tumor characteristics. The molecular machinery that governs these changes has become a major therapeutic target.

There are several pharmaceutical LSD1 inhibitors, but most derive from the inhibitors used for the proteins of the MAO(Monoamine oxidases) family, among the best known we find the 2-PCPA from which most of the inhibitors currently used have been synthesized.

1.2.7.1 TCP

LSD1, as already mentioned, belongs to the family of FAD-dependent amino oxidases. Two other components of this family, MAOs A and B, are responsible for the oxidative deamination of neurotransmitters, including serotonin and dopamine(58). Small molecules containing cyclopropylamine or propargylamine fractions function as irreversible MAO inhibitors. An inhibitor of this species, trans-2-phenylcyclopro-pylamine (hereinafter referred to as TCP, also known as tranylcypromine or Parnate, PCPA), is clinically used for this ability as an antidepressant.

Recent studies have shown that TCP also effectively inhibits LSD1, albeit with less potency than its inhibition on MAOs.

TCP covalently changes the LSD1 FAD cofactor and the FAD-TCP adduct is different from that with the MAO B. It is interesting to note that despite the similarity between LSD1 and MAO in their catalytic mechanisms, the inactivation of LSD1 and MAO B with TCP results in the formation of distinct covalent FAD-TCP adducts.

1.2.7.2 GSK2879552

GSK2879552 acts by inhibiting the complex LSD1/CoREST. The inhibition of LSD1 through GSK2879552 has been shown to improve the methylation of H3K4 and to increase the expression of tumor suppressor genes. GSK2879552 demonstrates growth effects anti-proliferative in AML cell lines and is currently undergoing clinical evaluation for the treatment of cancer(59)

1.2.7.3 OG-L002

It is a highly specific inhibitor of LSD1, with antiproliferative growth effects (IC50, ~0.02 μM). OG-L002 potently represses herpes simplex virus (HSV) IE gene expression, genome replication, and reactivation from latency(60).

1.2.7.4 SP2509

SP2509 is a reversible inhibitor of LSD1 activity, i.e. it inhibits the binding between the protein and the CoREST corepressor, acting as an LSD1 antagonist. SP2509 represents a new inhibitor, which promotes the competitive block for the binding with CoREST through the interface with the binding site for the FAD on LSD1. Therefore, SP2509 is able to inhibit LSD1 without affecting MAO proteins(61).

In the literature it appears that the SP2509 inhibitor reduces proliferation by increasing apoptosis in AML cells. This proliferative block may be due to an increase in methylation in H3K4 of the promoters specific for the genes p53 and p21, known to be involved in proliferative control. Furthermore, in the same cell line it has been shown to induce cell differentiation(62).

CHAPTER 2

LSD1 mediates MYCN control of epithelial-mesenchymal transition through silencing of metastatic suppressor NDRG1 gene

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Research Paper

LSD1 mediates MYCN control of epithelial-mesenchymal transition through silencing of metastatic suppressor NDRG1 gene

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ABSTRACT

Neuroblastoma (NB) with MYCN amplification is a highly aggressive and metastatic tumor in children. The high recurrence rate and resistance of NB cells to drugs urgently demands a better therapy for this disease. We have recently found that MYCN interacts with the lysine-specific demethylase 1 (LSD1), a histone modifier that participates in key aspects of gene transcription. In cancer cells, LSD1 contributes to the genetic reprogramming that underlies to Epithelial-Mesenchymal Transition (EMT) and tumor metastasis. Here, we show that LSD1 affects motility and invasiveness of NB cells by modulating the transcription of the metastasis suppressor NDRG1 (N-Myc Downstream-Regulated Gene 1). At mechanistic level, we found that LSD1 co-localizes with MYCN at the promoter region of the NDRG1 gene and inhibits its expression. Pharmacological inhibition of LSD1 relieves repression of NDRG1 by MYCN and affects motility and invasiveness of NB cells. These effects were reversed by overexpressing NDRG1. In NB tissues, high levels of LSD1 correlate with low levels of NDRG1 and reduced patients survival. Collectively, our findings elucidate a mechanism of how MYCN/LSD1 control motility and invasiveness of NB cells through transcription regulation of NDRG1 expression and suggest that pharmacological targeting of LSD1 represents a valuable approach for NB therapy.

INTRODUCTION

Neuroblastoma (NB), a disease of the sympathetic nervous system, is the most common solid tumor of infancy. Despite significant advances in the treatment of pediatric cancer over the past two decades, NB remains a highly refractory malignancy, with less than 50% 5-year survival rates for the majority of patients who are diagnosed with high-risk disease. One of the most powerful independent prognostic indicators for this disease is the amplification of the MYCN oncogene, which occurs at high levels in approximately 25% of NBs [1-3]. High-risk NBs often present hematogenous metastasis indicating that MYCN amplification control epithelial-mesenchymal transition (EMT) through which NB cells lose homotypic adhesion and acquire migratory capacity [4]. High level of MYCN expression has a great impact on global gene expression. [5]. Despite this richness of information, the entire and precise network of interactions that MYCN establishes within cancer cells remains elusive. Recently, we have demonstrated that MYCN interacts with LSD1/KDM1A, a monoamine oxidase that function as master epigenetic regulator in NB cell lines and that the MYCN/LSD1 complex is involved either in activation or repression of MYCN target genes in NB cell lines [6]. Importantly, the inhibition of LSD1 activity reduces neuroblastoma cell viability and induces differentiation. These findings suggest that LSD1 inhibition may have strong therapeutic relevance to counteract MYCN-driven oncogenesis.

LSD1 is an amine oxidase that catalyzes lysine demethylation in a flavin adenine dinucleotide (FAD)-dependent oxidative reaction. LSD1 removes mono- and dimethyl groups from lysine 4 (H3K4) and lysine 9 (H3K9) of histone H3, and can also targets non-histone proteins such as p53, E2F1, and DNMT1 [7-9]. LSD1 was initially described as a cofactor of the REST/CoREST complex. Although LSD1 can function as a co-repressor of transcription factors as REST, it also has been reported to function as a coactivator of specific transcription factors by removing H3K9 methylation, suggesting that its substrate specificity defines its biological outcome [10-12]. LSD1 is overexpressed in a variety of cancers and tends to correlate with more aggressive cancers with poor prognosis. There is a large body of evidence that LSD1 is involved in maintaining the undifferentiated, malignant phenotype of neuroblastoma cells and that its overexpression correlates with aggressive disease, poor differentiation and infaust outcome [13, 14].

To address the functional significance of LSD1 inhibition in NB we performed global transcriptome analysis (RNA-seq) in LSD1-deficient NB cells. Analysis of differentially expressed gene (DEG) highlighted the biological relevance of co-target genes indicating that epithelial-mesenchymal transition pathway was significantly affected. Among genes positively affected by LSD1 inhibition we focused our attention on the metastatic tumor suppressor gene N-myc downstream regulated1, NDRG1. In fact, we find that NDRG1 is inhibited by LSD1. NDRG1 is one of the four members of the human NDRG family, and its designation comes from its expression being repressed by MYC and MYCN [15, 16] and its expression is negatively correlated with tumor progression in multiple neoplasms. NDRG1 is a potent metastatic suppressor that has been shown to restrain TGF-ß-induced EMT in prostate and colon cancer cells, while its reduction induces EMT [17-22]. Collectively these studies demonstrated that NDRG1 functions as a metastatic suppressor that inhibits EMT in human cancer a key initial step in metastasis.

We found that LSD1 inhibition suffices to de-repress NDRG1 expression even in the presence of MYCN amplification. Expression of NDRG1 suppresses motility and invasiveness of NB cells. In silico studies of neuroblastoma tumor samples revealed that low expression of NDRG1 was associated with poor survival. Low NDRG1 and high LSD1 levels were mutually exclusive in MYCN-amplified NB samples, corroborating the *in vitro* results. Taken together, our findings provide a previously unidentified model to control of EMT in NB, suggesting that LSD1 represents a novel and promising target for selective inhibition of cell migration and invasiveness in neuroblastoma cells.

RESULTS

LSD1 depletion selectively affects EMT pathway

LSD1 is highly expressed in undifferentiated Neuroblastoma and its high expression correlates with adverse outcome [13, 14]. We recently showed that MYCN interacts with LSD1 and that the LSD1/MYCN complex controls transcription of tumor suppressor genes such as p21 and CLU [6]. Moreover LSD1 inhibition results in cell growth arrest of cultured NB cells. To address in more details the role of LSD1 function in NB cells, we performed global transcriptome analysis (RNA-seq) of Tet-21/N cells treated with tranylcypromine (TCP) a potent inhibitor of LSD1. In parallel, we performed RNA-seq from Tet-21/N cells treated with siRNA targeting LSD1 (LSD1-KD). RNA-seq data from duplicate biological replicas were then analyzed for differentially expressed gene (DEG). Statistical analysis allows us to screen out 661 DEGs in TCP sample ($\log2FC \geq 1$; $FDR \leq 0.1$) and 526 DEGs in LSD1-KD ($\log2FC \geq 1$; $FDR \leq 0.1$). 125 were commonly present in both treatments (Figure 1A, B and Supplementary Table 3). To clarify the biological relevance of co-target genes we used Gene set enrichment analysis. GSEA revealed that among top scoring pathways the gene set of Epithelial-Mesenchymal Transition, EMT, was ranked as significantly affected in both TCP and LSD1-KD samples (Figure 1C). We quantified expression levels of EMT marker genes in TCP treated or LSD1-KD Tet-21/N cells *versus* control cells by qRT-PCR. As shown in Figure Supplementary 1, LSD1 inhibition increased the levels of the epithelial markers, E-cadherin, occludin and desmoplakin, and reduced the expression mesenchymal markers, Vimentin and α-SMA, whereas no significant differences were detected in N-cadherin expression.

Previous studies have shown that LSD1 is indeed involved in the control of EMT, through interaction with the SNAG domain of SNAI1, a master EMT regulator [23, 24]. Among the several genes that were affected in TCP-treated and LSD1-KD cells related to EMT (SAT1, PLAUR, TNFRSF12A, RGS4, BDNF, MPP3, NDRG1 and SGK1) we focused our attention on the MYCN regulated gene, the metastasis suppressor gene NDRG1 (N-myc downstream regulated gene 1). NDRG1 was first isolated as a gene up-regulated in N-Myc knockout mouse embryos [25] and directly repressed by MYCN and c-MYC through binding to the NDRG1 core promoter [26]. The metastasis suppressor NDRG1 is negatively correlated with tumor progression of several types of cancer, and most importantly down-regulation of NDRG1 expression enhances cell proliferation and invasiveness. In contrast, its up-regulation reduces cell proliferation and invasiveness [27-29].

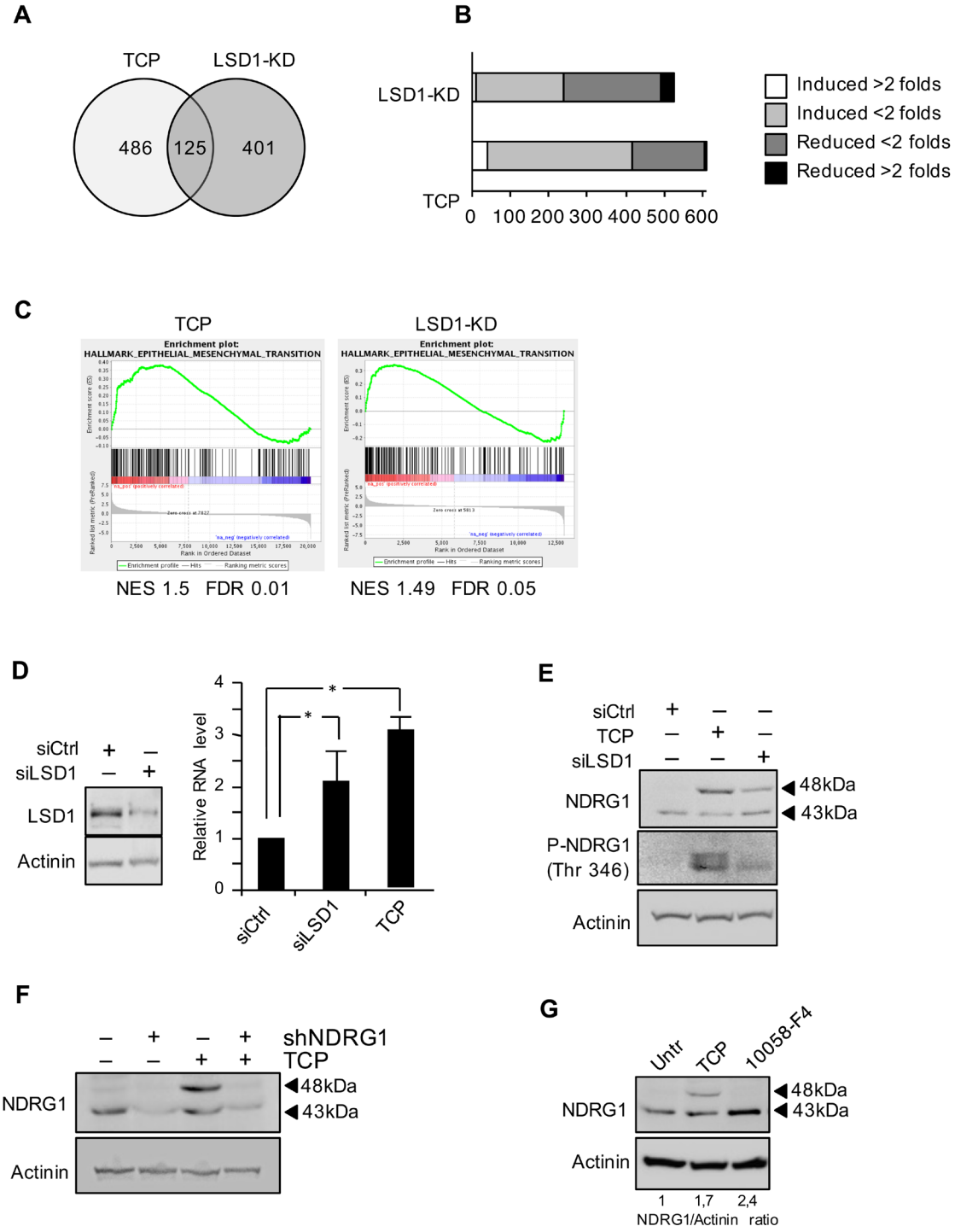

Figure 1: A. Venn diagram of the DEG present in both LSD1-knockdown (LSD1-KD) and TCP treatment. B. Gene set of regulated genes by TCP treatment and LSD1-KD. **C.** Gene set enrichment analysis (GESA) plots show enrichment of gene sets regulated by LSD1-KD and TCP treatment. In each panel, nominal NES and false discovery rates (FDRs) are indicated. **D.** NDRG1 gene expression was analyzed by qRT-PCR, using samples prepared from Tet-21/N cells and treated with TCP or siRNA-LSD1 and siRNA-control as indicated. LSD1 protein level in Tet-21/N cells transfected with siRNA-LSD1 or control was determined by western blot. *, statistical significance ($P < 0.01$; Student t test). **E.** Western blotting of protein extracts from Tet-21/N cells prepared as described in D, using NDRG1 and phospho-NDRG1 (Thr 346) antibodies. **F.** NDRG1 silencing using sh-NDRG1 in Tet-21/N cells treated with TCP or vehicle, was assayed by western blot. **G.** Western blotting of protein extract from Tet-21/N treated with vehicle, TCP or 10058-F4 for 48 hrs, using NDRG1 antibody. Actinin has been probed as loading control.

To validate the role of LSD1 in NDRG1 expression we inhibited LSD1 in Tet-21/N cells with TCP or siRNA-targeted knockdown and measured NDRG1 mRNA and protein expression levels. We found that TCP treatment or LSD1 silencing stimulates NDRG1 expression (Figure 1D and 1E). Previously immunoblotting studies revealed that NDRG1 might appear as multiple protein bands depending from the cellular context likely due to different isoforms and/or post-translational modifications such as phosphorylation and glycosylation [27, 30, 31]. It has been shown that the signal cascade mTORC2/serum glucocorticoid induced protein kinase1 (SGK1) phosphorylates NDRG1 at T346 and this modification is essential to suppress tumor growth [20, 32]. Tet-21/N cells treated with TCP or siLSD1 were probed with an antibody that specifically recognize NDRG1 phosphorylated at T346 demonstrating that LSD1 inhibition induces NDRG1 phosphorylation, Figure 1E. Finally shRNA-targeted NDRG1 knockdown demonstrates specificity of NDRG1 bands (Figure 1F). To address the contribution of MYC and LSD1 to NDRG1 expression, Tet-21/N cells were treated with 10058-F4, a small molecule inhibitor of MYC/MAX dimerization [6] that has effect on either cMYC then MYCN. Following 10058-F4 treatment we found an increase of the 43kDa NDRG1 band, while TCP activates the 48kDa (Figure 1G). These findings suggest that inhibition of either MYCN or LSD1 de-repress NDRG1 expression. However, while MYCN inhibition activates NDRG1, LSD1-KD also induces NDRG1 phosphorylation.

MYCN and LSD1 co-localize at NDRG1 promoter and repress its expression

To determine whether LSD1 is directly involved in transcriptional control of NDRG1 we inhibited LSD1 in Tet-21/N cells with TCP or with siRNA against LSD1 and assessed the relative binding of MYCN and LSD1 to the NDRG1 gene by chromatin immune-precipitation (ChIP) assays. The immunoprecipitated chromatin samples were subjected to qPCR using primers corresponding to the transcriptional start site (TSS) of the NDRG1 gene, Figure 2A. As shown in Figure 2B and 2C, MYCN and LSD1 were both recruited selectively at the transcriptional start site (TSS) of the NDRG1 gene but not at distal sites (-10kb), indicating that the MYCN/LSD1 complex binds to the NDRG1 promoter. We find also that MYCN binding was unaffected by TCP or LSD1 depletion implying that MYCN binding does not require LSD1 while, in contrast, LSD1 binding was reduced in TCP-treated and LSD1-KD samples, suggesting that the binding of LSD1 require the catalytic activity of the enzyme. Next, we monitored the histone modifications occurring at NDRG1 promoter (Figure 2D, 2E). Depletion of LSD1 enhances H3-acetylation whereas it reduces the repressive mark H3K27me3, consistent with the induction of NDRG1 expression in these cells. Overall, our findings demonstrate that: 1) both LSD1 and MYCN bare recruited to the NDRG1 promoter chromatin to repress NDRG1 expression; 2) LSD1 inhibition is sufficient to relieve MYCN-driven NDRG1 repression.

Effects of TCP and SP2509 inhibitors on LSD1/MYCN-mediated regulation of NDRG1

During last years several small molecular inhibitors of LSD1 based on different molecular mechanisms have been developed [33]. SP2509 is a reversible inhibitor of LSD1 and differently from TCP does not target the catalytic activity of the enzyme. SP2509 attenuates the binding of LSD1 to CoREST and it has been found to be effective in inhibition of cultured and primary AML blasts [34]. To further substantiate the role of LSD1 in the suppression of NDRG1 we analyzed the effects of treatment of NB cells on NDRG1 expression by treatment with this different LSD1 inhibitor. As shown in Figure 3A, SP2509 treatment enhances NDRG1 mRNA expression and increases the NDRG1 48kDa protein levels in a dose dependent manner. Thus, both TCP and SP2509 enhance NDRG1 expression albeit these drugs inhibit LSD1 through different mechanisms. Because LSD1/MYCN negatively controls NDRG1 transcription we assessed whether TCP or SP2509 may interfere with the LSD1/MYCN interaction. To this end, HEK293T cells co-transfected with expression vectors encoding LSD1 and MYCN were exposed to TCP and the complex between MYCN and LSD1 was analyzed by immunoprecipitation. As shown in Figure 3B, LSD1 and MYCN readily interact in the absence of TCP but their association was impaired in presence of the drug. This inhibitory effect of TCP is specific to LSD1-MYCN complex since it did not interfere with the interaction of MYCN with its endogenous partner MAX (Figure 3B). In contrast, SP2509 did not inhibit the interaction between LSD1 and MYCN. Also LSD1/CoREST association was inhibited by SP2509, not by TCP (Figure 3C). Collectively these results demonstrate that LSD1 activity is necessary for the interaction with MYCN, not with CoREST. Thus, inhibition by TCP or SP2509, de-represses NDRG1 expression, albeit the two drugs have a marked different mode of action.

Since SP2509 is a reversible inhibitor of LSD1, we tested whether re-activation of NDRG1 by SP2509 treatment was reversible. Tet-21/N cells were treated with SP2509 for 48 hrs and then cells were washed, fed with normal medium and collected at 12, 24 and 48 hrs thereafter SP2509 wash out. Results in Figure 3D shows that NDRG1 expression decreases in a time dependent manner following removal of the SP2509, demonstrating that NDRG1 activation is directly dependent upon LSD1 inhibition.

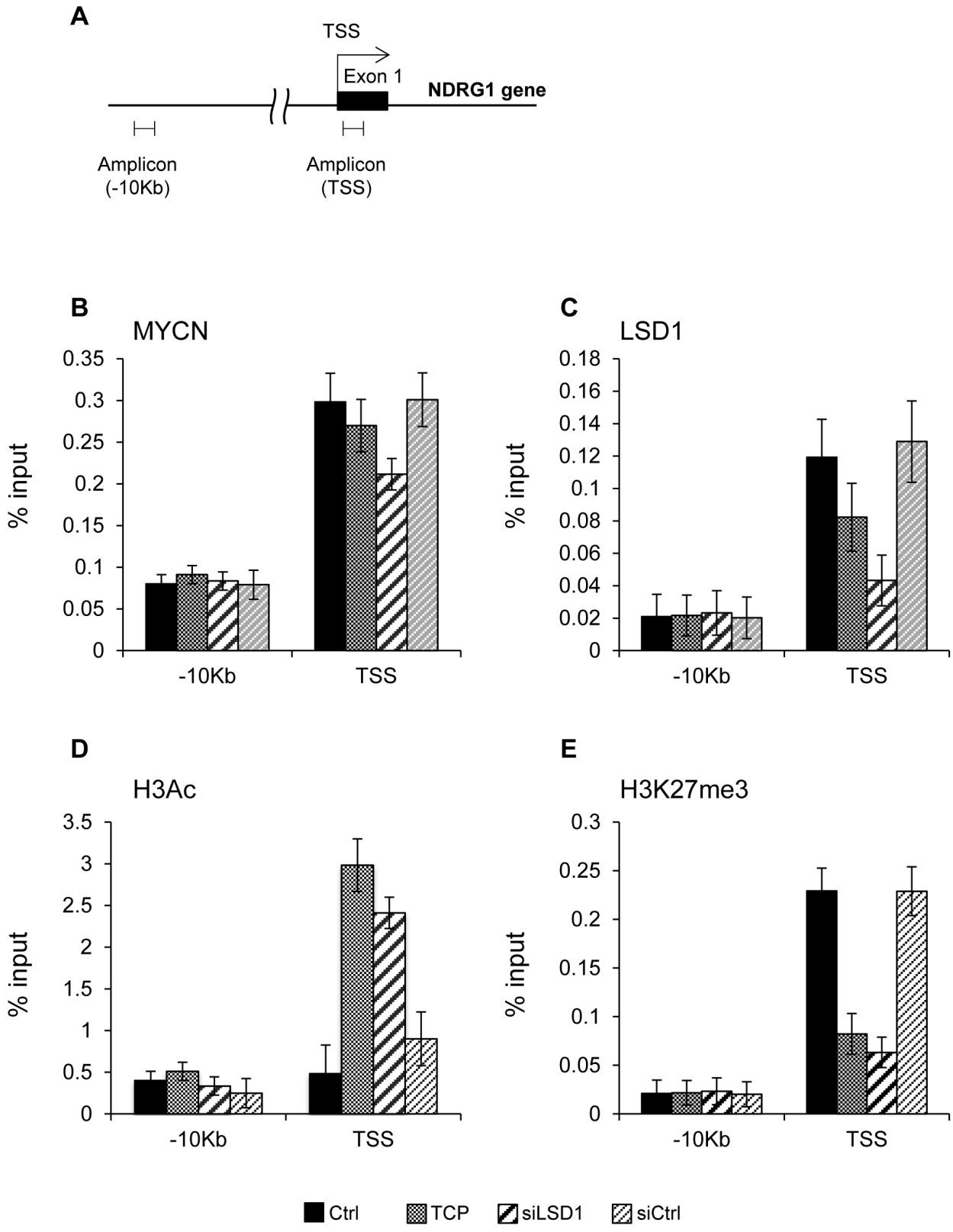

Figure 2: A. Schematic representation of the NDRG1 promoter. B. and **C.** MYCN and LSD1 binding to NDRG1 chromatin. Cell treatments are indicated at the bottom of the figure. qPCR was performed with primers for NDRG1 TSS, and -10kb. **D.** and **E.** Histone modifications at NDRG1 chromatin; ChIPs were carried out using the indicated antibodies and analyzed with primers encompassing the TSS region and -10kb from TSS. Values from three independent ChIP assays are presented along with standard deviations, $n = 3$. Changes in % input are shown normalized over IgG controls and are all statistically significant ($P < 0,05$; Student t test).

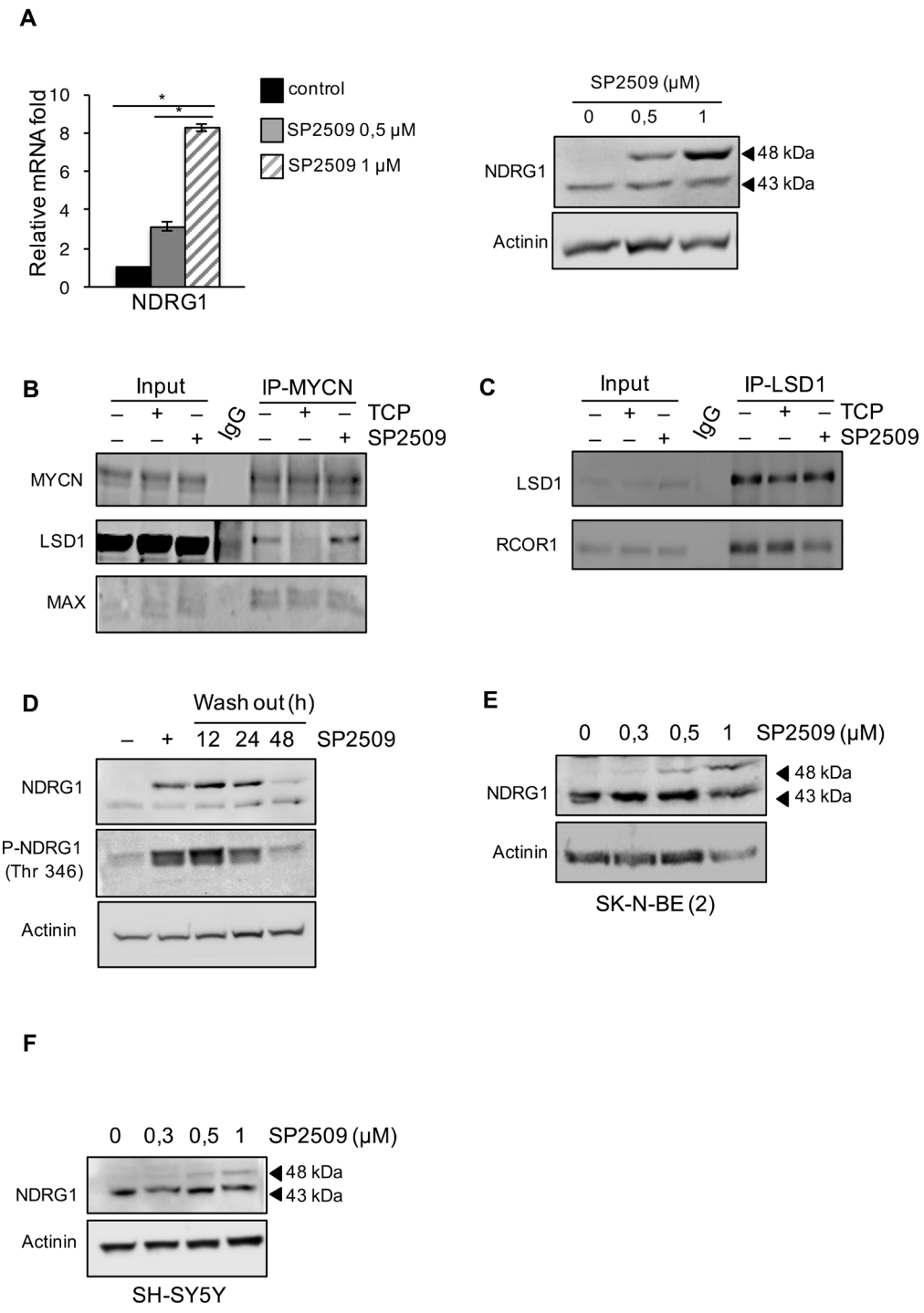

Figure 3: A. NDRG1 gene expression was determined by qRT-PCR or by western blot in Tet-21/N cells treated with SP2509 at different concentrations, as indicated. *, statistical significance (*P* < 0.01; Student t test). **B.** Co-Immunoprecipitation with MYCN antibody was performed in 293T cells co-transfected with LSD1 and MYCN expression vectors and treated with TCP, SP2509 or vehicle. Extract were analyzed by western blotting with MYCN, LSD1 and MAX antibodies as indicated. **C.** Interaction between endogenous LSD1 and MYCN in Tet-21/N cells, treated with TCP, SP2509 or vehicle, was assessed by co-Immunoprecipitation. Cell lysates were immune-precipitated with a LSD1 antibody Western blot analysis was performed on immuno-precipitated extracts with LSD1 and RCOR1 antibodies. IgG-sample was used as negative control. **D.** Tet-21/N cells were treated for 48 h with SP2509 or vehicle and then released into fresh medium for the indicated times. Cellular extracts were prepared and stained with anti-NDRG1 and phospho-NDRG1 (Thr 346). **E.** and **F.** Cell extracts from SK-N-BE (2) and SH-SY5Y cells treated with SP2509 at the indicated concentrations were prepared and probed with NDRG1 antibody. Actinin was probed as loading control.

To address if LSD1 inhibition affects NDRG1 expression in the context of MYCN amplification, we analyzed the effect of SP2509 in a non-amplified MYCN SH-SY5Y cell line. Moreover, since activation of NDRG1 may also occurs as result of p53 binding in colon cancer cell lines [35] we also used the p53 mutated, MYCN-amplified NB cell line SK-N-BE (2) to address the relative contribution of p53 in NDRG1 activation. The SH-SY5Y (MYCN non-amplified) and MYCN-amplified p53 mutated SK-N-BE (2) cells were treated with SP2509 at different concentration for 48 hrs and western blot was performed using the NDRG1 antibody. Results reported in Figure 3E, 3F show that up regulation of the 48 kDa NDRG1 band is observed in both cell lines demonstrating that NDRG1 activation by LSD1 inhibition is not due to p53 activity and is not cell specific.

Collectively our results demonstrate that NDRG1 expression is modulated by LSD1 and that pharmacological LSD1 inhibition in NB cells up-regulates NDRG1 expression.

Effect of LSD1 inhibition on migration and invasion of NB cells

NDRG1 over-expression promotes formation of adherent junctions and inhibits cell migration and invasion in several types of tumors cells indicating that NDRG1 inhibits the establishment of the epithelial-mesenchymal transition (EMT) program [18, 36]. Our findings suggest that LSD1 pharmacological silencing might control EMT in NB tumor cell lines by upregulating NDRG1 expression.

LSD1 was demonstrated to activate the Wnt/β-catenin signaling pathway by down-regulating the pathway antagonist DKK1 in colorectal cancer cells [37]. In different studies NDRG1 overexpression has been shown to inhibit β-catenin phosphorylation inducing its accumulation at cell membranes [21]. We examined if NDRG1 activation mediated by pharmacological inhibition of LSD1 affected β-catenin subcellular localization. To this end we performed immunofluorescence to detect β-catenin in Tet-21/N cells untreated (Ctrl) or treated with SP2509. As shown in Figure 4A, SP2509 enhanced β-catenin accumulation on cellular membrane. A modest increase of β-catenin protein levels was detected in Tet-21N and SH-SY5Y cells by immuno-blotting, Figure 4B, suggesting that SP2509 treatment enhanced β-catenin accumulation on cellular membrane. Consistent with such effect, expression of the β-catenin downstream target, Cyclin D1 was down-regulated in LSD1 inhibited cells. These results indicate that pharmacological treatment of NB cells with LSD1 inhibitor results in NDRG1 activation and suggest that the anti-metastatic activity of NDRG1 in NB occurs at least in part through accumulation of β-catenin at cell membrane.

We then asked whether treatment with LSD1 inhibitors and over-expression of NDRG1 might impair the migration and invasion of tumor NB cell lines. Untreated Tet-21/N (High MYCN), tetracycline-treated (Low MYCN) and the SH-SY5Y cells were used in wound-healing assays in presence or absence of TCP or SP2509. Both Tet-21/N (High MYCN) and SH-SY5Y cells filled almost completely the wounded area 24hrs after scratching the cell monolayer, while Tet-21/N (Low MYCN) showed impaired migration efficiency (Figure 5A and 5B). TCP or SP2509 treatment markedly suppressed repair of the wound area. Such inhibitory effect was enhanced in Low-MYCN cells suggesting that reduction of MYCN levels cooperates with LSD1 in blocking the migration of LSD1-KD cells. Next, we tested the effect of NDRG1 over-expression on cell invasiveness of Tet-21/N and SH-SY5Y cells. Both cell lines were transfected with a human expression vector for NDRG1, whose expression

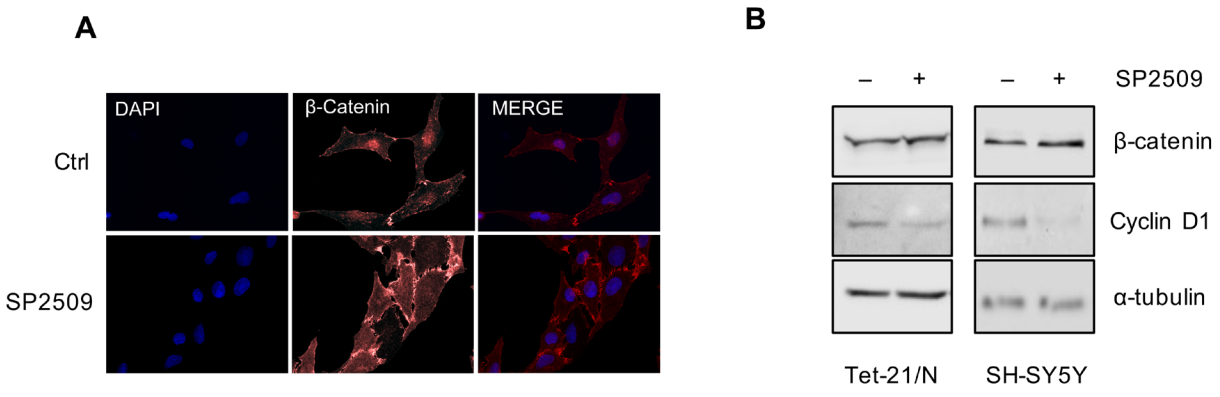

Figure 4: A. Tet-21/N cells were treated with SP2509 or vehicle, fixed and processed for anti-β-catenin immunofluorescence and DAPI staining. **B.** Western blot assay of protein extracts of Tet-21/N and SH-SY5Y cells treated as indicated using β-catenin and Cyclin D1 antibodies. α-tubulin has been probed as loading control. *, $P < 0,01$.

was assayed by Western blots, Figure 5C. We determined that overexpression of NDRG1 recapitulates the inhibitory effects exerted by LSD1 inhibitors. Next, we determined the effect of LSD1 inhibition and NDRG1 over-expression on cell invasion (Figure 6). Using the trans-well migration assay, we showed that NDRG1 overexpression as well as LSD1 pharmacological inhibition in both Tet-21/N and SH-SY5Y cells resulted in a significant reduction (≥ 25%) of migratory capacity compared with control cells.

These findings demonstrated that pharmacological inhibition of LSD1 blocks migration and invasion of neuroblastoma cells and most importantly overexpression

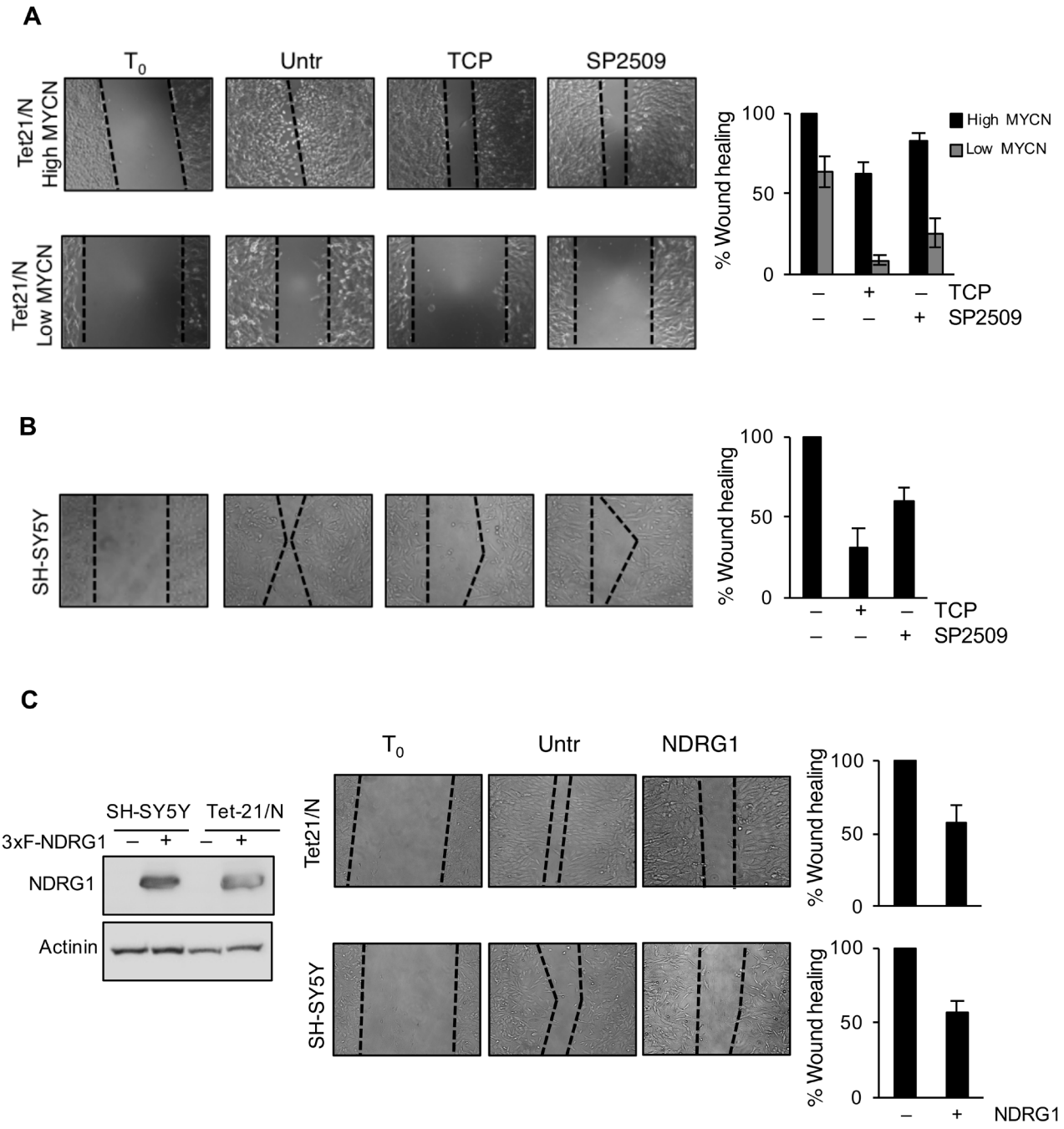

Figure 5: LSD1 inhibition reduces migration of Neurobastoma cells. A. Wound healing of Tet-21/N (High MYCN), Tetracycline-treated Tet-21/N (Low MYCN) and **B.** SH-SY5Y cells treated with vehicle, TCP or SP2509. **C.** Wound healing was performed in Tet-21/N and SH-SY5Y cells 3XFlag-NDRG1 or mock transfected. Migration was assessed under treatment conditions at several time points using a scratch wound assay. Representative phase contrast images were shown acquired at 24hrs after scratch. Western blot shows NDRG1 protein levels in 3xFlag-NDRG1 or mock transfected Tet-21/N and SH-SY5Y cells. Actinin was used as loading control. Graphs showing the results represent the mean ± SD of three independent experiments carried out in duplicate. Statistical significance $P < 0,01$.

of NDRG1 recapitulate these effects. Collectively, these findings demonstrated that pharmacological inhibition of LSD1 suppresses the mobility and invasiveness of cancer cells through up-regulation of NDRG1.

NDRG1 expression during differentiation and in NB tumors

It had been shown that LSD1 expression is reduced following *in vitro* induced differentiation of neuroblastoma cells [14, 38]. The findings reported above indicated that high levels of LSD1 inversely correlate to NDRG1 expression. To address the relative expression levels of MYCN, LSD1 and NDRG1 during differentiation, SK-N-BE(2) cells were induced to differentiate by treatment with RA. Cell samples were collected at different time points after treatment and analyzed for LSD1 and NDRG1 and MYCN expression levels. As shown in Figure 7A, *in vitro* induced differentiation results in reduction of LSD1 and MYCN expression along to a concomitant up-regulation of NDRG1 levels. These results further confirm the role of LSD1 on NDRG1 expression and highlight their antagonism during differentiation of NB cells. Moreover these data strongly suggest that NDRG1 can be used as marker of neuroblastoma differentiation *in vivo*.

To further corroborate the mutually exclusive expression of NDRG1 and LSD1 we examined the relevance of NDRG1 in neuroblastoma patients. Independent studies have shown that low NDRG1 levels

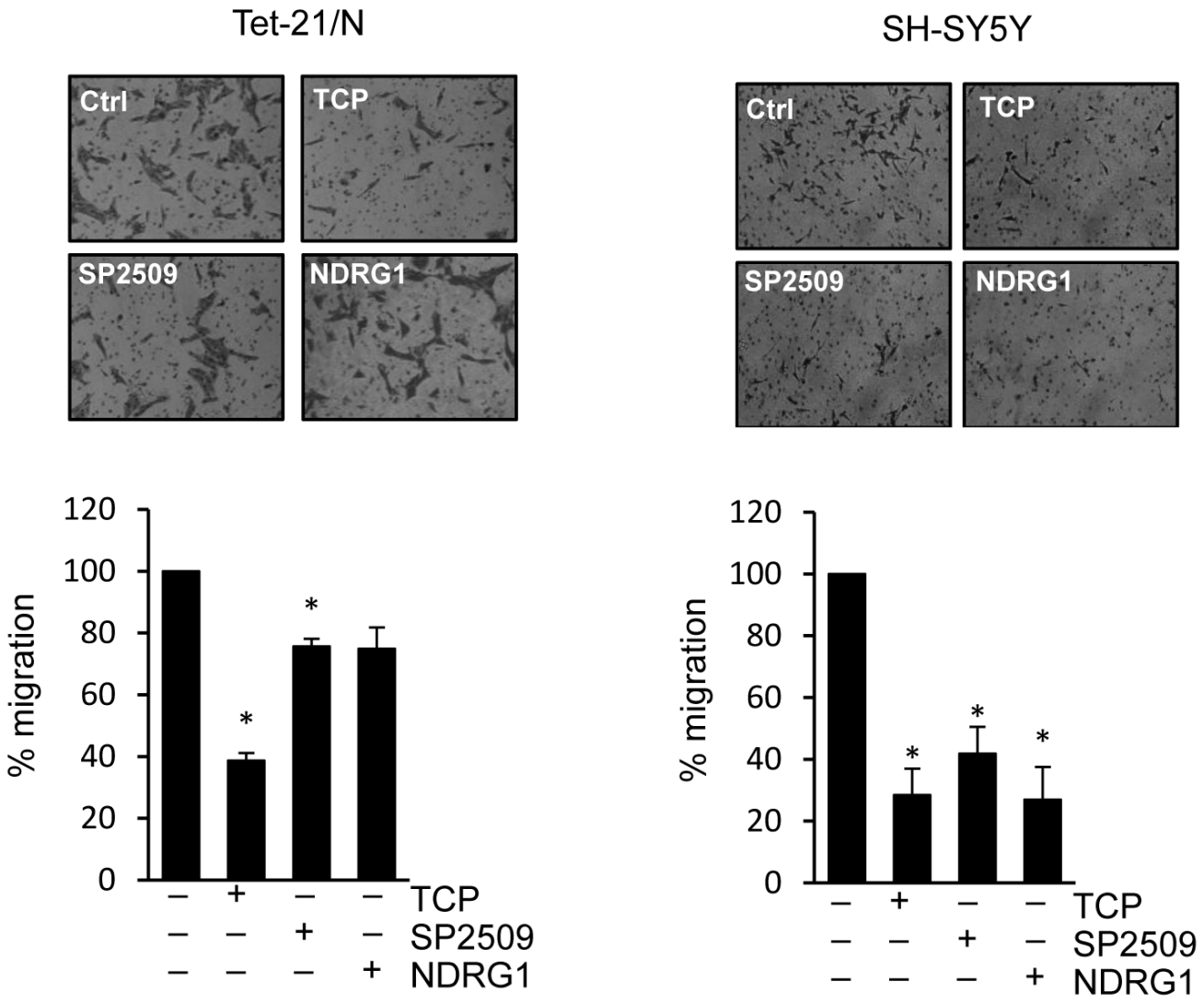

Figure 6: Trans-membrane migration assay of Tet-21/N and SH-SY5Y cells treated with vehicle, TCP or SP2509, or NDRG1-transfected. Graphs showing the results represent the mean ± SD of three independent experiments carried out in duplicate. *, $P < 0,05$.

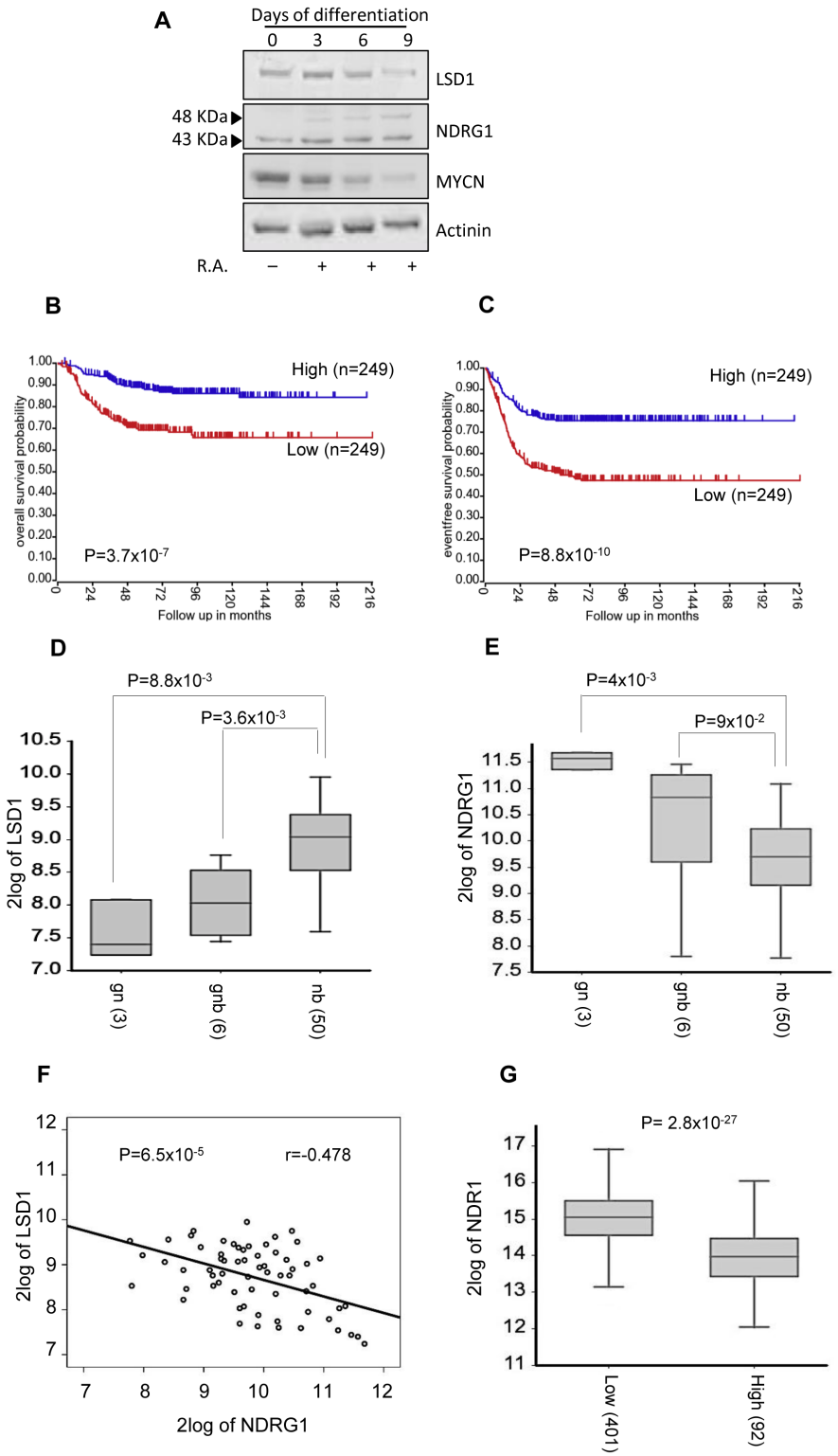

Figure 7: A. SK-N-BE (2) cells were treated with RA up to 9 days. LSD1, NDRG1 and MYCN protein levels were detected in differentiated SK-N-BE (2) cells at the indicated days by western blotting. NDRG1 expression is associated with good outcome and differentiated tumors. **B.** and **C.** Low NDRG1 expression is associated with negative prognosis. The number of tumors is indicated in parentheses. Kaplan-Meier analysis is shown, with individuals grouped by median of expression of NDRG1. Log-rank P values are shown. Changes in expression for LSD1 **D.** and NDRG1 **E.** in ganglioneuroblastoma (GNB), ganglioneuroma (GN) and neuroblastoma (NB). **F.** Inverse correlation between the expression values of NDRG1 and LSD1 in NB tumors (Pearson's correlation coefficient is shown). **G.** Box plot showing differential NDRG1 expression in NB tumors without (Low) or with (High) MYCN amplification.

are associated with worse prognosis for patients with breast, glioma, colorectal, esophageal squamous cell carcinoma, and prostate cancer [19]. More recently, it has been reported that low levels of NDRG1 is associated with poor prognosis in neuroblastoma patients [39]. In sharp contrast, LSD1 expression inversely correlates with differentiation and adverse outcome [14, 38] of neuroblastoma. Our *in vitro* findings imply that also in patients high LSD1 and low NDRG1 levels should be inversely correlated in metastatic Neuroblastomas. To this end we analyzed available RNAseq data of 498 NBs and we found that high NDRG1 expression correlates with better overall and event-free survival (Figure 7B and 7C, Mann-Whitney test, $P = 3.7x10^{-7}$ and $P = 8.8x10^{-10}$). Next, we analyzed LSD1 and NDRG1 expression in a microarray gene expression data of 59 NBs, of which 50 were neuroblastoma and 9 were ganglioblastoma and ganglioneuromas. LSD1 expression was considerably higher in neuroblastoma than in ganglioblastomas and ganglioneuromas (Figure 7D). In contrast, NDRG1 expression was higher in well-differentiated tumors (Figure 7E). Thus, LSD1 and NDRG1 appear to be expressed in opposite fashion in NB. Accordingly, we found that the expression of NDRG1 is inversely correlated with the expression of LSD1 (Figure 7F, $P = 6.5x10^{-5}$). Finally, we determined that NDRG1 expression levels were appreciably lower in MYCN-amplified NB samples (Figure 7G). Collectively, these findings demonstrated that high levels of LSD1 and NDRG1 expression are mutually exclusive in neuroblastoma, and the expression levels of NDRG1 are significantly lower in MYCN-amplified tumors.

DISCUSSION

In the current study, we demonstrated that LSD1 in cooperation with MYCN controls cell migration and invasiveness of neuroblastoma cells through transcription regulation of the metastatic suppressor NDRG1. Our findings support a previously unidentified model to control EMT in neuroblastoma, proposing that epigenetics changes caused by LSD1 inhibition lead to up-regulation of NDRG1 thereby inducing an NDRG1-dependent inhibitory effect on cell migration and invasiveness of neuroblastoma cells

We found that in neuroblastoma cells the MYCN/LSD1 complex binds and represses NDRG1 expression. Following LSD1 inhibition epigenetics changes occur on the chromatin region surrounding the transcriptional start site of NDRG1 leading to transcription activation of NDRG1 gene expression. In a recent study it has been shown that the signal cascade mTORC2/serum glucocorticoid induced protein kinase1 (SGK1) phosphorylates NDRG1 [20]. It is likely that LSD1-KD may also affect mTORC2/GSK1 pathway, clearly further investigations are required to clarify the role of LSD1 in the phosphorylation of NDRG1.

LSD1 inhibition suppresses motility and invasiveness of NB cells and ectopic over expression of NDRG1 phenocopy the pharmacological treatments with LSD1 inhibitors, suggesting that de-repression of NDRG1 expression plays a causative role in blocking cell migration and invasiveness. Moreover, lowering MYCN expression we observed a cooperative inhibition with TCP to restrain cell mobility, suggesting that MYCN and LSD1 cooperatively control EMT.

High-risk neuroblastoma (NB) with MYCN amplification is a highly metastatic tumor in children. NB presenting with hematogenesis metastasis is one of the most difficult cancers to cure [1, 40]. EMT is an important process that contributes to tumor invasion and dissemination [41]. How MYC control EMT is largely unknown [42]. EMT process requires extensive reorganization of the epigenetic information of the cells. Previous works showed that SNAIL represses transcription of epithelial genes such as E-cadherin, by recruiting repressive chromatin-modifying factors including Polycomb repressive complex 2 and LSD1-CoREST complex [41]. Our findings of targeting NDRG1 expression through LSD1 inhibitors add new insight on how MYCN may control EMT. Thus, LSD1 controls EMT through at least two different mechanisms, as co-factors of SNAI1 function and in association with MYCN as a direct epigenetic regulator of NDRG1 expression. Previous work showed that blocking interactions of LSD1 with SNAI1 blocks NB cell invasion [43]. The findings reported here add further support to the critical role of LSD1 in EMT and most importantly highlight an additional mechanism through which LSD1 inhibition affects cell migration and invasiveness of NB cancer cells. Clearly multiple signaling pathways cooperate in the initiation and progression of EMT and cooperation between different pathways likely occurs in a synergistic manner and in a cell-type specific fashion.

Therapy for high-risk patients includes differentiating agents. Previous studies showed that NDRG1 expression is regulated by differentiation-related environments [19]. We determined that during RA-mediated *in vitro* differentiation of NB cells the NDRG1 protein increases during time and inversely correlates with LSD1 and MYCN protein expression. Thus, these data address that NDRG1 is a biologically important MYCN/LSD1 target, and it is inversely expressed in relation to MYCN and LSD1 during NB differentiation.

The relative expression levels of NDRG1, LSD1 and MYCN were further analyzed in neuroblastoma patients. Analysis of publicly available expression data of large number of NBs highlighted that high NDRG1 expression correlates with better overall and event-free survival. Interestingly, high levels of LSD1 and NDRG1

expression are mutually exclusive in neuroblastoma tumors and NDRG1 expression levels are significantly lower in MYCN-amplified NB samples. Collectively, these findings support and corroborate the broad significance of our *in vitro* results, and suggest that NDRG1 and LSD1 expressions can be considered as valuable biomarkers to monitor NB development in humans.

In summary, our findings uncover a previously unidentified model in the control of EMT, suggesting that MYCN/LSD1 inhibition de-represses NDRG1 expression, thereby inducing an NDRG1-dependent inhibitory effect on cell migration and invasiveness of neuroblastoma cells. These findings raise the possibility that improved approaches aimed to target the epigenetic control of NDRG1 expression may lead to development of novel strategies to inhibit the invasive potential of neuroblastoma cells.

MATERIALS AND METHODS

Cell culture and treatments

Human HEK 293T, SH-SY5Y and SHEP Tet-21/N cells were cultured in Dulbecco's modified Eagle's Medium (DMEM) supplemented with antibiotics, 10% fetal calf serum. SK-N-BE (2) was cultured in 1:1 mixture DMEM/F-12 containing 10% FBS. All cell lines were incubated at 37°C in humidified atmosphere with 5% CO_2. Tet-21/N cells are cultivated with (Low MYCN) or without (High MYCN) tetracycline (6 days). When indicated, cells were treated with TCP (1mM, Enzo Life Sciences), SP2509 (0,3/0,5/1 μM, Cayman Chemical Company) or 10054-F4 (75 μM, Sigma) for 24 or 48 hrs. To induce differentiation in SK-N-BE (2) cells were exposed to 10 μM all-*trans* Retinoic Acid for 9 days.

LSD1 Knock-Down

100 nM siRNA targeting LSD1 (GE Dharmacon) or scramble were transfected in Tet-21/N cells using a MicroPorator Digital Bio Technology, according to the recently described protocol [6]. Briefly, $2x10^6$ cells were collected by trypsin/EDTA digestion, washed once with calcium and magnesium-free PBS and resuspended in 100μl of resuspension buffer, mixed with siRNA or scramble and electroporatated according to the manufacturer's protocol. Transfected cells were seeded in a 100 mm dish in antibiotic-free DMEM supplemented with 10% FBS. The efficiency of siRNA to knockdown LSD1 protein expression was assayed 48h after transfection by western blot.

RNA sequencing

RNA was prepared from Tet-21N cells treated with TCP or with siLSD1 and control untreated cells. RNA-seq libraries (two biological replicas for each sample) were generated using TruSeq RNA Sample Prep Kit v2 (Illumina) according to the manufacturer's recommendation. All the high-throughput sequencing experiments were run on a NexSeq 500 (Illumina) sequencer at the Genomix4life S.R.L., Baronissi, Salerno, Italy, according to standard operating procedures. Raw sequences files (-fastaq files) were aligned to the human genome (h19 version), gene-level quantification was performed using R-SEM and UCSC annotation [44]. Subsequently, data were normalized with VOOM method [45] and differential expression evaluated with limma Bioconductor packages. Differential expressed genes were detected applying the following cutoff: log2 Fold Change ≥ 1 and FDR ≤ 0.1. RNA-seq data were deposited to NCBI GEO and are available under accession number GSE80753.

RNA extraction and qRT-PCR

RNA was extracted from NB cells using EuroGold Trifast (EuroClone). cDNA was generated using Quantitec Reverse Transcription Kit (Qiagen), according to manufacturer's protocol. Quantitative analysis was performed using SYBR Green 2X PCR Master Mix (Applied Biosystem). Each sample was run in triplicate and normalized to the expression of housekeeping beta-glucoronidase (GUSb) gene as previously described [6]. Primers are presented in Supplementary Table S1.

Protein extraction and western blot

Whole-cell extracts were obtained using buffer F (10 mM TrisHCl pH 7.5, 150 mM NaCl, 30 mM Na4O7P2, 50 mM NaF, 5 mM ZnCl2, 0.1 mM Na3VO4, 1% Triton, 0.1mM PMSF). 50 μg of protein extracts were loaded and separated by SDS-PAGE and WB was performed with indicated antibodies. For NDRG1 silencing in Tet-21/N cells, 3 μg/10^6 cells of shRNA plasmid (Santa Cruz) targeting NDRG1 was used with the protocol described above.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation assays were performed as recently described [6]. Briefly $1x10^7$ cells were cross-linked using formaldehyde to a final concentration of 1% and reaction was stopped using 0.125M Glycine. Cell pellet was resuspended in Cell Lysis Buffer and after 6000 rpm centrifugation RIPA buffer

were added to perform nuclei lysis. DNA shearing was conducted by sonication using Bioruptor (Diagenode). A small aliquot of sonicated material was put aside and remaining sample immunoprecipitated using 5 micrograms of ChIP-grade antibodies. Rec-sepharose Protein A or G beads (Invitrogen) were used to immobilize immuno-complexes and after RNAse-A treatment (37°C 1 hour) reverse cross-linking were performed using Proteinase K (Roche) for 6 hours at 65°C. Immunoprecipitated DNA was purified using Phenol/Chloroform and Ethanol precipitation techniques. The antibodies used are listed in Table S2. The immunoprecipitated DNA was quantified by qPCR with the primer sets described in Table S1.

Migration assays

In migration experiments, 2,5 µg/10^6 cells of 3xFLAG-NDRG1 or empty vector were transiently transfected into Tet-21/N by electroporation, by protocol as described previously. For transient transfections of SH-SY5Y, cells cultured on 100 mm dishes were transfected with 3xFLAG-NDRG1 plasmid or empty vector using Lipofectamine 2000 according to manufacturer's protocol. The expression of protein was determined by western blot. For the wound-healing assay, NDRG1-trasfected or control cells were plated to confluence in a 12-well plate and scraped with a p200 pipet tip to create a scratch of the cell monolayer; when indicated cells were treated with TCP or SP2509 for an overnight before scratch and during the whole experiment. Cells were then allowed to fill the wounded area for 2 days and images were acquired using a Nikon Eclipse TE 2000-U microscope. Percentage of wound healing was measured as following: [(empty area at T_0)-(empty area at 24hrs)]/(empty area at T_0) x 100. For trans-membrane migration assay, cells were NDRG1-transfected or pre-treated with TCP or SP2509 for an overnight, before plating (150000 cells/chamber) in free serum medium in the upper side of chambers (BD Falcon Cell Culture Inserts). In the wells 20% of FBS was used as chemo-attractant. After 24 h, non-migrating cells were scraped-off, whereas migrating cells were stained with a 20% ethanol-1% crystal violet solution for 10', washed thrice with water and counted at least in ten fields with a 10x objective. For each assay three independent experiments were carried out in duplicate.

Co-immunoprecipitation

Co-immunoprecipitation assays were performed using Tet-21/N and HEK 293T cells. 293T cells were transiently co-transfected with 3xFLAG-LSD1, 3xFLAG-MYCN or scramble by the polyethylenimine (PEI 25 K) method. 1 mg of protein extract from Tet-21/N cells or 0,3 mg from HEK 293T cells, treated with TCP, SP2509 or vehicle, were incubated respectively with LSD1 or MYCN antibody and processed as previously described [6, 46]. Protein interactions were assessed by immunoblotting using the indicated antibodies.

Immunofluorence

For immunofluorescences assay Tet-21/N were seeded on coverslips and treated as indicated. Cells were than fixed in 4% paraformaldehyde in PBS, permeabilized in 0.1% Triton X-100 in PBS, pre-blocked in 2% BSA-3%NS-PBS for 30 min at room temperature, and then incubated for 1 h at 37° C with mouse anti-β-catenin. Primary antibodies were detected by incubation with Cy3-coniugated anti-mouse. Images were acquired using a Nikon Eclipse TE 2000-U microscope.

Gene expression data for survival analysis and association with neuroblastoma stages

Normalized gene expression data from RNA sequencing of 498 tumors were downloaded from "R2: Genomics Analysis and Visualization Platform" (GEO ID: GSE62564). To test association of gene expression levels with overall survival and event free survival, individual gene expression profiles were dichotomized by median split into 'high' or 'low' expression groups, and Kaplan-Meier survival curves were plotted for each group. Long rank test was used to evaluate the significant difference between the two groups. Another set of gene expression data of 64 tumors (GEO ID: GSE12460) including 50 NB, 6 ganglioneuroblastoma and 3 ganglioneuroma was downloaded. Mann-Whitney test was used to test the significant different gene expression among groups. The correlation between the gene expression between NDRG1 and KDM1A was evaluated by Pearson correlation in 64 NBs. The gene expression data for Low and High MYCN expression (493 samples) were generate by customized 4x44K oligonucleotide microarrays produced by Agilent Technologies and analyzed as previously reported [47].

Statistical analysis

All experiments were repeated two or three times. Graphs representing data express mean ± SD. Statistical significance was obtained by unpaired, two-tailed Student *t* test. $P < 0,05$ was considered statistically significant.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Editorial note

This paper has been accepted based in part on peer-review conducted by another journal and the authors' response and revisions as well as expedited peer-review in Oncotarget.

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CHAPTER 3

Lysine-specific demethylase LSD1 regulates autophagy in neuroblastoma through SESN2-dependent pathway

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ORIGINAL ARTICLE

Lysine-specific demethylase LSD1 regulates autophagy in neuroblastoma through SESN2-dependent pathway

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Autophagy is a physiological process, important for recycling of macromolecules and maintenance of cellular homeostasis. Defective autophagy is associated with tumorigenesis and has a causative role in chemotherapy resistance in leukemia and in solid cancers. Here, we report that autophagy is regulated by the lysine-specific demethylase LSD1/KDM1A, an epigenetic marker whose overexpression is a feature of malignant neoplasia with an instrumental role in cancer development. In the present study, we determine that two different LSD1 inhibitors (TCP and SP2509) as well as selective ablation of LSD1 expression promote autophagy in neuroblastoma cells. At a mechanistic level, we show that LSD1 binds to the promoter region of Sestrin2 (SESN2), a critical regulator of mTORC1 activity. Pharmacological inhibition of LSD1 triggers SESN2 expression that hampers mTORC1 activity, leading to enhanced autophagy. SESN2 overexpression suffices to promote autophagy in neuroblastoma cells, while loss of SESN2 expression reduces autophagy induced by LSD1 inhibition. Our findings elucidate a mechanism whereby LSD1 controls autophagy in neuroblastoma cells through SESN2 transcription regulation, and we suggest that pharmacological targeting of LSD1 may have effective therapeutic relevance in the control of autophagy in neuroblastoma.

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INTRODUCTION

Cancerous cells must deal with effective mechanisms of cell death, thereby reducing activation of defense pathways in response to oncogenic insults.[1,2] The induction of apoptosis is the major cause route of cell death yet multiple cellular processes, including autophagy, antagonize it.

Autophagy is a conserved intracellular process in which cytoplasmic components are degraded within lysosomes having a central role in cell metabolism and homeostasis. There are different types of autophagy: micro-autophagy, selective autophagy, macro-autophagy and chaperone-mediated autophagy.[3] Macro-autophagy is the main autophagic pathway and consists in the formation of double-membrane autophagosomes that sequester cellular components and then fuse with lysosomes for degradation and recycling of macromolecules and organelles. Autophagy normally operates at low, basal levels in cells but can be strongly induced by cellular stress. Defective autophagy is associated with human pathologies such as bacterial and viral infections, neurodegenerative diseases and cancer.[4–6]

Autophagy has dual roles in cancer; it can function as a tumor suppressor, by preventing the accumulation of damaged proteins and organelles, or a survival pathway, by impairing apoptosis and promoting the growth of tumor growth.[7–9] Recent studies showed that autophagy has a causative role in chemotherapy resistance in leukemia[10] and in solid cancers.[7,10] Nonetheless, the molecular mechanisms underlying the autophagy on tumorigenesis must be further investigated.

Mammalian target of rapamycin complex 1 (mTORC1) is the major regulator of autophagy. In the presence of nutrients, mTORC1 is activated, resulting in inhibition of the Ulk1 complex and repression of autophagy.[11] Following nutrient deprivation, mTORC1 is inhibited, and Ulk1 complexes can lead autophagosome formation. Given its pivotal role in autophagy regulation, mTORC1 is the main target for drug development to modulate the autophagic pathway.[12,13]

Recently, several reports demonstrate that autophagy is regulated by epigenetic alterations, as histone methylation and acetylation.[14–16] The mechanisms through which cancer-associated epigenetic alterations modulate autophagy have not yet been elucidated. An epigenetic enzyme that has been target of drug discovery is the lysine-specific demethylase 1, LSD1. LSD1 (also known as KDM1A and AOF2) is an amine oxidase that catalyzes lysine demethylation in a flavin adenine dinucleotide-dependent oxidative reaction[17] and removes mono- and dimethyl groups from lysine K4 and, in specific circumstances, K9 on histone H3.[17–19] More recently, it has been shown that the neuron-specific isoform LSD1n has a new substrate specificity, targeting histone H4 Lys 20.[20] Finally, LSD1 can also target non-histone proteins such as p53, E2F1 and DNMT.[21–23] LSD1 has been demonstrated to have important roles in many important aspects of cell biology, such as cell proliferation, cell mobility and differentiation.[24–26] Most importantly, LSD1 is overexpressed in a variety of tumors and its high expression correlate with more aggressive cancers with poor prognosis. There is a large body of evidence that LSD1 is involved in maintaining the undifferentiated, malignant phenotype of neuroblastoma (NB) cells and that its overexpression correlates with aggressive disease, poor differentiation and infaust outcome.[24,27]

In the present study, we define a novel role of the epigenetic regulator LSD1 in the modulation of autophagy. We found that selective ablation of LSD1, or pharmacological inactivation of its catalytic function, inhibits mTORC1 activity enabling enhanced

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autophagy. Mechanistically, we found that LSD1 binds to the promoter region of Sestrin2 (SESN2) and represses its expression. LSD1 inhibition triggers SESN2 expression that hampers mTORC1 activity leading to Transcription Factor EB (TFEB) nuclear translocation driving the expression of the Coordinated Lysosomal Expression and Regulation regulatory pathway. Taken together, our findings indicate that LSD1 regulates autophagy in NB cells via transcriptional regulation of SESN2 that serves as a key positive regulator of the mTORC1-dependent autophagy pathway.

RESULTS

LSD1 inhibition represses the mTORC1 pathway

LSD1 is highly expressed in undifferentiated NBs and its expression correlates with adverse outcome; however, the molecular mechanism underlying LSD1 effects is largely unknown. We initially undertook an unbiased approach to uncover how cells respond to the loss of LSD1 function looking at signaling alterations caused by treatment with tranylcypromine (TCP), a potent inhibitor of LSD1 in Tet-21/N NB cells. PathScan array was used to determine pathways involved in TCP response. In this assay mTORC1 pathway was the most responsive, evidenced by ribosomal protein S6 (Ser235/236), p70S6 Kinase (Thr389) and PRAS40 (Thr246) phosphorylation reduction (Figure 1a). To verify PathScan array results, we performed western blot analysis of mTORC1 downstream substrates, p70S6K and rpS6. Tet-21/N cells were also treated with SP2509, a reversible inhibitor of LSD1 that, differently from TCP, does not target the catalytic activity of the enzyme, but attenuates the binding of LSD1 to CoREST.[25,28] In addition, to address the specific role of LSD1 in mTORC1 activity, we inhibited LSD1 by short interfering RNA (siRNA)-targeted knockdown and measured expression of mTORC1 downstream targets. Protein extracts were prepared at the indicated times and probed with antibodies recognizing phosphorylated and total protein forms of mTORC1 substrates. In agreement with the array data, phosphorylation levels of p70S6K, and consequently of its target rpS6, were downregulated by either TCP or SP2509 treatment as well as in LSD1-KD cells (Figures 1b and c). In addition, similar results were observed in SH-SY5Y NB cells (Figure 1d). Collectively, these findings demonstrated that LSD1 inhibition downregulates mTORC1 signaling.

mTORC1 is known as a critical regulator of autophagy. In response to nutrient deprivation, mTORC1 is inactivated and it dissociates from the Ulk complex, inducing autophagy activation. In addition, mTORC1 has been shown to control autophagy through the functional regulation of the TFEB, a master regulator of lysosomal and autophagic functions. Active mTORC1 phosphorylates and sequestrates TFEB to the cytoplasm; on the contrary, mTORC1 inactivation leads to de-phosphorylation of TFEB, which translocates into the nucleus and drives the expression of lysosomal and autophagy genes that are part of the Coordinated Lysosomal Expression and Regulation regulatory network.[12,29]

We sought to investigate the impact of LSD1 inhibition on TFEB subcellular localization. Tet-21/N and SH-SY5Y cells were treated with TCP for the indicated times and analyzed by immunofluorescence using a TFEB antibody. In untreated cells TFEB is localized mainly in the cytoplasm. Consistently with mTORC1 repression, pharmacological inhibition of LSD1 leads to a significant increase of TFEB nuclear levels along with a decreased cytosolic localization (Figures 2a and b). This finding was further confirmed using specific siRNA against LSD1 (Figure 2c).

Because SP2509 is a reversible inhibitor of LSD1, we used this drug to test whether TFEB nuclear shuttling was directly dependent upon LSD1 inhibition. We monitored whether nuclear localization of TFEB was reversed in time after SP2509 wash out. Tet-21/N cells were treated with SP2509 for 48 h and then cells were washed and cultivated in fresh medium for additional 24 and 48 h after drug removal. The immunofluorescence results in Figure 2d show that SP2509 removal decreased the TFEB nuclear/cytoplasmic ratio. Concomitantly, we observed a significant reduction in TFEB levels, suggesting that addition of fresh medium results in degradation of nuclear TFEB.

Similar results have been described using the mTOR inhibitor Torin 1 in MCF7 cells.[30] These findings demonstrated that TFEB nuclear localization is directly dependent upon LSD1 inhibition.

LSD1 depletion induces autophagy

Autophagy begins with the sequestration of cytosolic proteins in a double-membrane structure called autophagosome. Following fusion with a lysosome, it becomes an autophagolysosome, then lysosomal hydrolases degrade the content of the phagosome that is released in the cytosol for the recycling of macromolecules. During autophagosome formation, the cytosolic form of the microtubule-associated protein 1A/1B-light chain 3 (LC3-I) is conjugated to phosphatidylethanolamine to form lipidated-LC3 (LC3-II), which becomes associated with autophagosomal membranes. Tet-21/N cells were treated with TCP (Figure 3a) or siLSD1-transfected (Figure 3b) and then processed for western blot analysis to monitor the conversion from LC3-I to lower-migrating form LC3-II, as a well-established marker of autophagosome formation.[31] TCP treatment induced LC3-II form accumulation over time compared with control cells (Figure 3a). Moreover, LSD1-KD also increases the LC3-II form level in a dose-dependent manner (Figure 3b). Similar findings were observed in SH-SY5Y cells (Supplementary Figure 1). Because LC3-II increment might also be interpreted as autophagosome accumulation due to the block of autophagosome–lysosome fusion, we evaluated the autophagic flux following LSD1 inhibition using a green fluorescent protein (GFP)-mRFP-tandem-tagged LC3. Indeed, by taking advantage of the properties of these two fluorescent proteins (GFP signal is quenched inside the acidic lysosomal lumen, but not mRFP) we can discriminate between autophagic compartments before and after fusion with lysosomes.[31,32]

Tet-21N cells were transiently transfected with the GFP-mRFP-LC3 tandem construct and treated with TCP alone or in combination with NH_4Cl to block autophagolysosomal degradation by preventing its acidification. In untreated cells, GFP and mRFP signals appear mainly diffused in the cytosol and with few puncta (Figure 3c). Conversely, the number of green and red puncta was higher following TCP treatment, indicating that LSD1 inhibition enhanced the autophagic flux (Figure 3c, upper graph). Moreover, a significant increase in autolysosomes (mRFP-positive, but GFP-negative puncta) was observed upon TCP treatment (Figure 3c, lower graph), indicating that the autophagosome maturation is occurring. Consistently, upon combined treatment of TCP and NH_4Cl green dots were augmented as expected by the fact that NH_4Cl, increasing the intralysosomal pH, prevents GFP quenching (Figure 3c).

Taken together, these results clearly indicate that pharmacological inhibition of LSD1 activity triggers a functional autophagic flux induction, as demonstrated by the nuclear localization of autophagy master regulator TFEB and the mature autophagolysosome formation.

LSD1 inhibition promotes autophagy by increasing SESN2 expression

To understand the mechanisms by which LSD1 inhibition induces autophagy, we analyzed data from our recent published RNA sequencing (RNA-seq) from Tet-21/N cells treated with TCP or siLSD1.[25] Among the common upregulated genes we identify *SESN2* as an LSD1-repressed target gene. SESN2 is a member of an evolutionarily conserved stress-inducible Sestrin gene family, and it has been shown that SESN2 directly inhibits mTORC1 activity via GATOR2 with a consequent inhibition of mTORC1 recruitment to

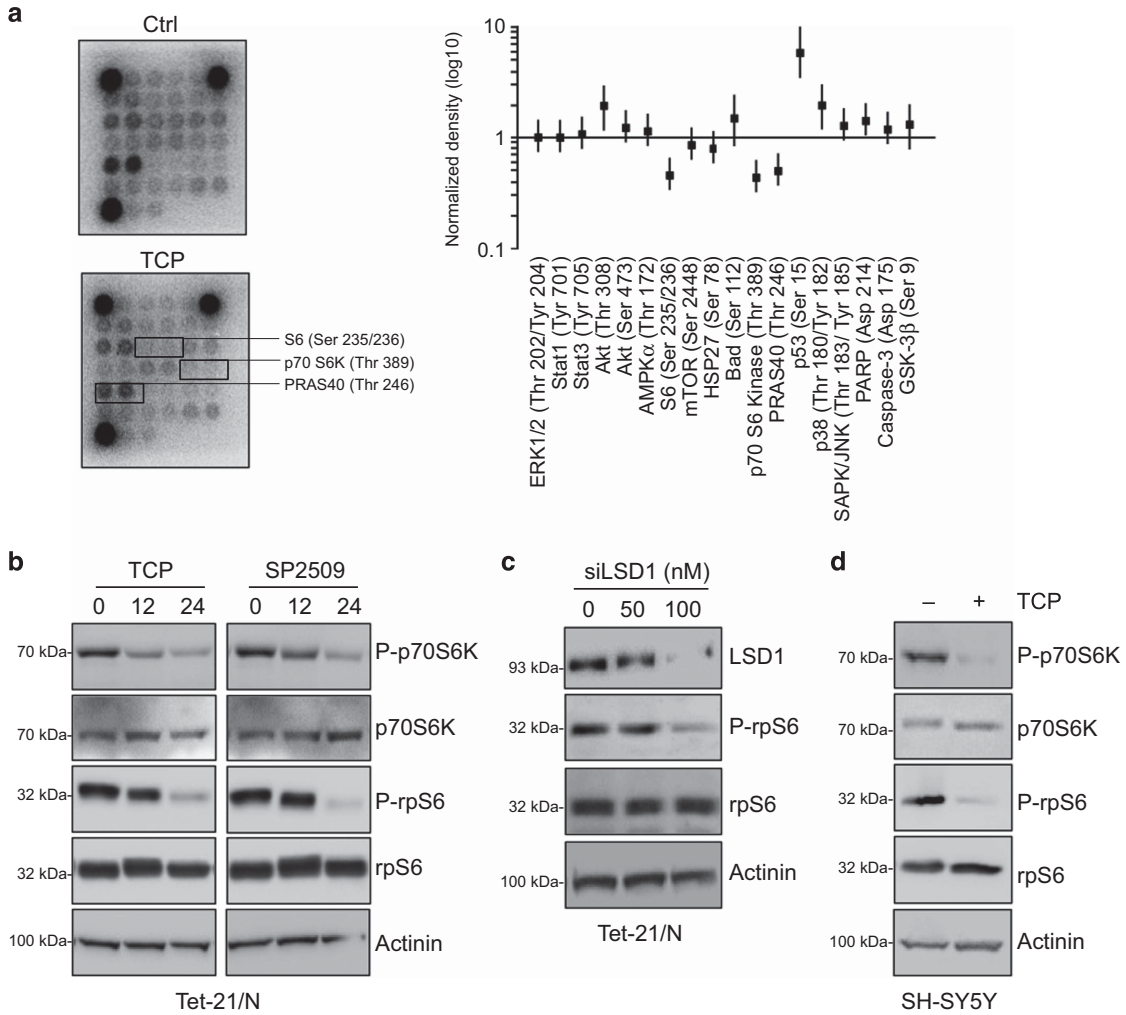

Figure 1. LSD1 inhibition represses mTORC1 activity. (**a**) Protein extract from Tet-21/N treated with 1 mM TCP or vehicle (Ctrl) for 24 h were analyzed for the signaling activation using the PathScan antibody arrays. Graph shows the pixel density ratio of signaling molecule dots. Values represent the means of two independent experiments (± s.d.). (**b**) Western blotting of protein extracts from Tet-21/N cells treated for 0, 12 or 24 h with TCP or SP2509 (1 μM). (**c**) Tet-21/N cells were transfected with siRNA against LSD1 at different concentrations, or scramble (0). Cells were collected 48h after transfection. (**d**) Western blotting of protein extracts from SH-SY5Y cells treated for 24 h with TCP using the indicated antibodies. Actinin was probed as loading control.

the lysosomal membrane.[33,34] Through these functions, SESN2 serves as a key positive regulator of the autophagic pathway.

Although our RNA-seq showed that the relative expression of the other two members of Sestrins, that is, SESN1 and SEN3, were unaffected by treatment with TCP or by siLSD1, we validated RNA-seq data by investigating the consequences of LSD1 inhibition on the relative expression levels of all three members of Sestrins family. Accordingly, with our RNA-seq data, we found by qRT–PCR (quantitative reverse transcriptase polymerase chain reaction) analyses that LSD1 inhibition or silencing enhances SESN2 expression (Figures 4a and b) and only marginally affects SESN1 and SESN3 (Figure 4a), suggesting that SESN2 is predominantly regulated by LSD1 in Tet-21/N cells. However, we cannot exclude that LSD1 might regulate SESN1 and 3 in different cellular backgrounds.

In addition to LSD1, the LSD2 (KDM1B) mammalian paralog also demethylates mono- and di-methylated H3K4 in an FAD-dependent manner. Thus, TCP treatment may also affect LSD2 function. To determine the relative contribution of LSD2 in Sestrins expression, Tet-21/N cells were treated with siRNA against LSD2 and Sestrins mRNA expression was assayed by qRT–PCR. As shown in Figures 4a and b, we found that siLSD2 treatment did not affect SESN2 expression. We conclude that LSD1 specifically affects SESN2 expression.

Next, we sought to determine whether LSD1 is directly involved in control of *SESN2* gene expression. Public available chromatin immunoprecipitation (ChIP)-seq LSD1 data from SH-SY5Y cells as well as from mouse ES cells indicate a putative LSD1 binding to the promoter region of SESN2 (Supplementary Figure 2). We then carried out ChIP assays to determine binding of LSD1 to SESN2 chromatin. LSD1 binding was analyzed in Tet-21/N cells treated with TCP or silenced for LSD1 expression. Chromatin isolated from Tet-21/N cells was immunoprecipitated with an anti-LSD1 antibody and qPCR analysis performed using primers corresponding to the 5' regulatory regions surrounding the transcription start site (TSS) of the *SESN2* gene. As shown in Figure 5a, LSD1 is recruited selectively at TSS of the *SESN2* gene but not at distal sites (−10 kb), indicating that LSD1 binds to the SESN2 promoter. LSD1 binding was reduced in TCP-treated and siLSD1 samples, suggesting that binding of LSD1 required catalytic activity; moreover, TCP treatment did not alter the relative LSD1 expression levels (Figures 4a and b).

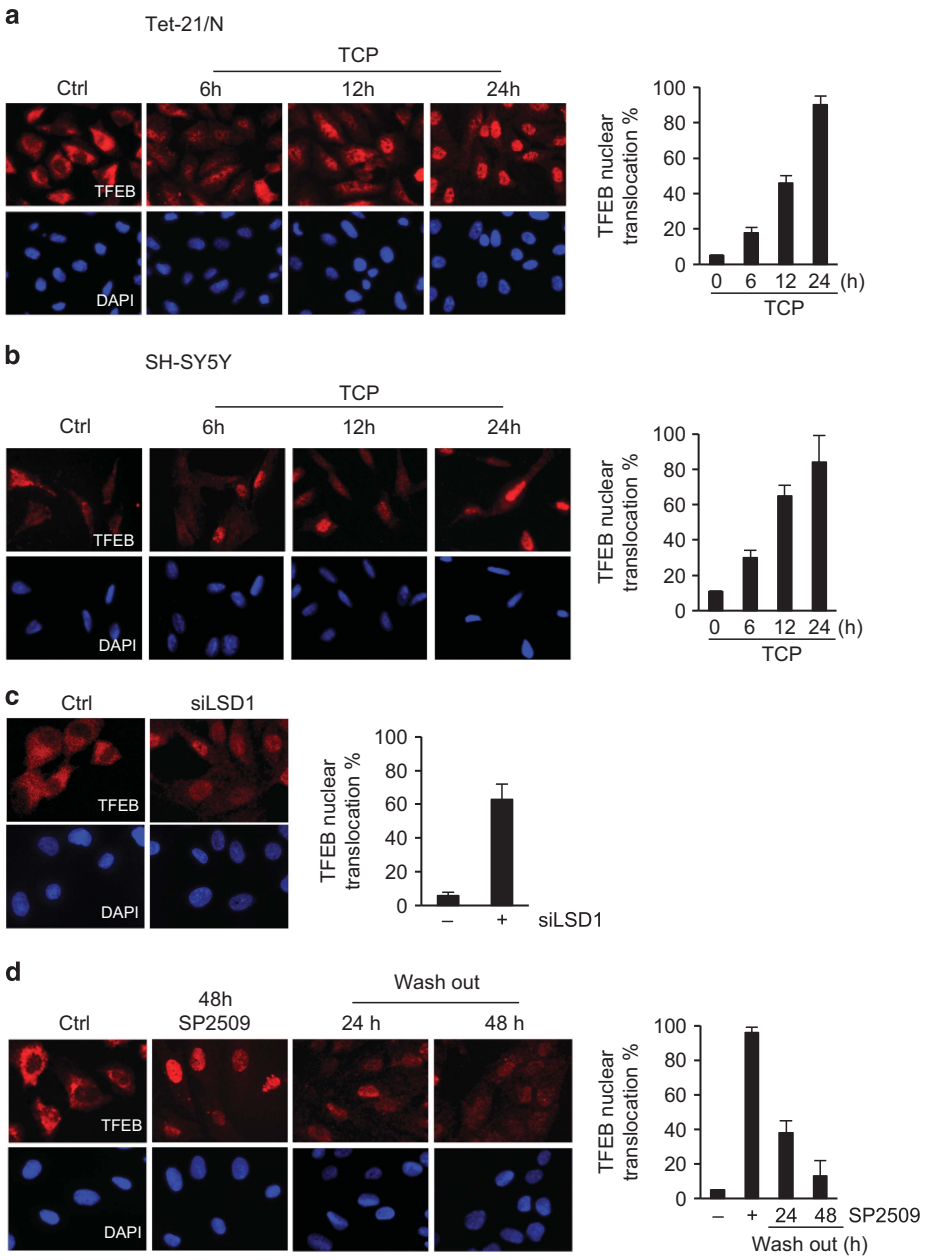

Figure 2. LSD1 inhibition leads to TFEB nuclear sublocalization. Tet-21/N (**a**) and SH-SY5Y (**b**) cells were treated with TCP or vehicle for the indicated times fixed and processed for anti-TFEB immunofluorescence and DAPI staining. (**c**) Tet-21/N cells were transfected with scramble (Ctrl) or siRNA against LSD1 (100 nM), fixed and stained with anti-TFEB and DAPI. (**d**) Tet-21/N cells were treated for 48 h with SP2509 or vehicle and then released into fresh medium for the indicated times before immunostaining with TFEB antibody and DAPI. Graphs show the ratio of TFEB nuclear/cytosolic signal intensity values average, normalized on negative and positive control samples (mean ± s.d., $n = 200$ cells). DAPI, 4',6-diamino-2-phenylindole.

To better understand how LSD1 can affect chromatin organization at the SESN2 promoter, we analyzed four different histone modifications, H3 pan-acetyl (H3Ac), H3K27Me3, H3K4Me2 and H3K9Me2, around the TSS promoter region. Figure 5b shows that both LSD1 silencing (siLSD1) and inhibition by TCP determine a significant increase in H3 acetylation. As a marker of transcriptional repression, we analyzed lysine 27 tri-methylation of Histone H3. Data presented in Figure 5c demonstrate that both LSD1 silencing or its inhibition (TCP) determine an almost 2.5-fold decrease of the marker. ChIP assays were also performed on di-methylated Lysine 4 of histone H3 (Figure 5d), and we found a significant increase in H3K4me2 at SESN2 TSS following LSD1 inhibition. Conversely, both inhibition and repression of LSD1 do not affect H3K9Me2 signature at TSS level of SESN2 (Figure 5e). These findings highlight the critical role of LSD1 in the transcriptional regulation of the *SESN2* gene through direct binding to SESN2 promoter.

It has been recently shown that LSD1 depletion, synergistically with UBE4B inhibition, increases proteasomal and autophagy clearance activating the p53-mediated transcriptional program.[35] TP53/p53 is involved in the regulation of autophagy through two distinct mechanisms, according to its subcellular localization: cytoplasmic p53 inhibits autophagy through the inhibition of AMPK and the subsequent mTOR activation; nuclear p53 induces the transcription of pro-autophagic genes, including *SESN2*.[36–38] To verify that SESN2 upregulation mediated by LSD1 inhibition

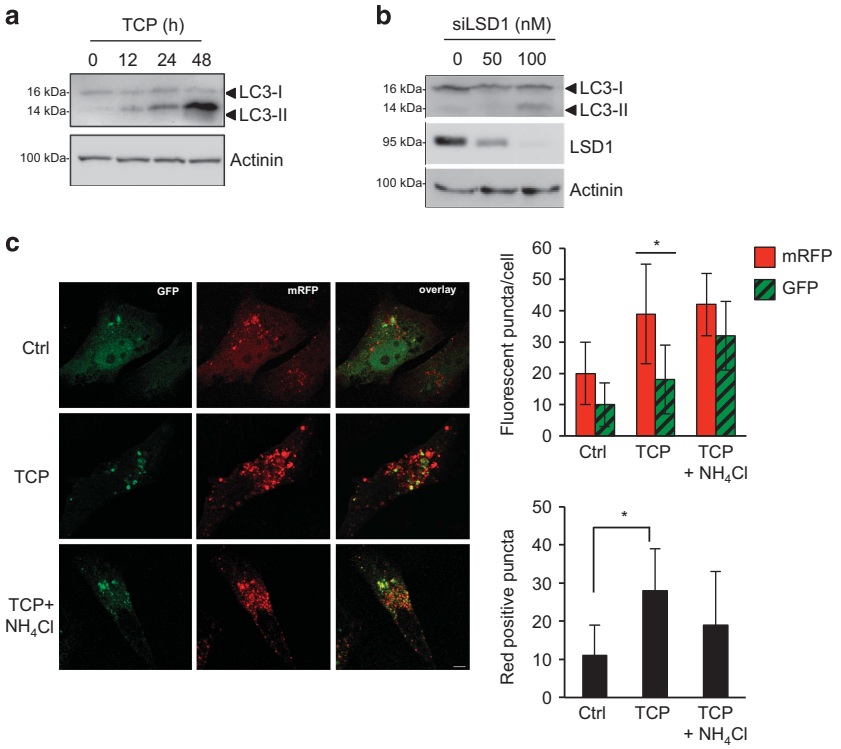

Figure 3. LSD1 inhibition induces autophagy. Tet-21/N cells were treated with TCP for the indicated times (**a**), or siLSD1 at different concentrations as indicated (**b**), and protein extract was prepared and probed using anti-LC3 antibody. LSD1 protein level in cells transfected with siLSD1 or control (0) was shown. Actinin was probed as loading control. (**c**) Tet-21/N was transiently transfected with GFP-mRFP-LC3 tandem construct and treated with TCP alone or in combination with 20 mM NH_4Cl for 24 h. After treatment, cells were fixed and analyzed by confocal microscopy. Bar, 10μm. Histograms show the number of GFP or mRFP puncta (upper graph) or the number of only-red positive (GFP-negative) puncta (lower graph). The values are expressed as means ± s.d. ($n > 100$ cells). *$P < 0.00001$.

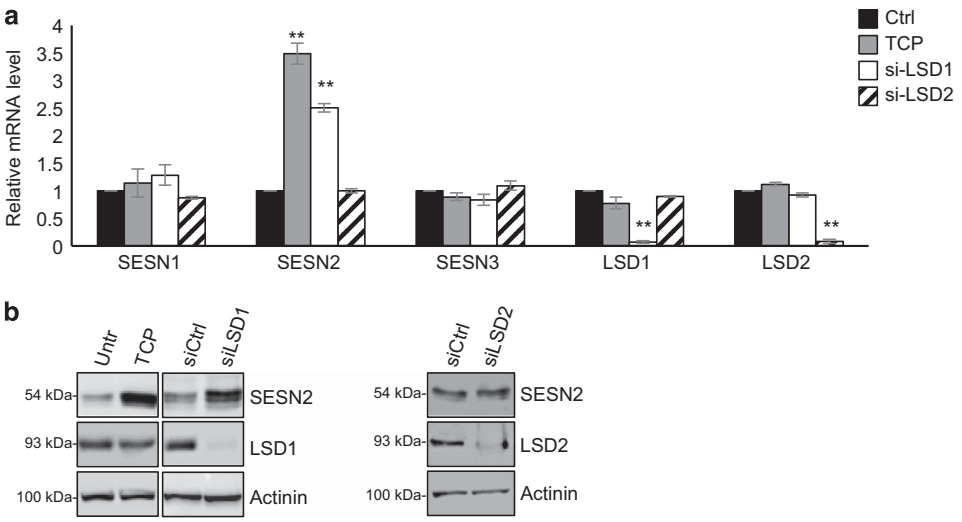

Figure 4. LSD1 represses SESN2 expression. SESN1–3 expression was analyzed by qRT–PCR (**a**) using samples prepared from Tet-21/N cells treated with TCP or siRNA against LSD1 or LSD2 (100 nM). The efficiency of siRNAs to knock down LSD1 and LSD2 expression was shown. (**b**) SESN2 expression was assayed by western blotting, using protein extracts from Tet-21/N cells treated with vehicle, TCP, siRNA-LSD1, siRNA-LSD2 or siRNA-control as indicated. LSD1 and LSD2 protein levels in Tet-21/N cells transfected with specific siRNA or control was analyzed by western blot. Actinin was probed as loading control. **$P < 0.01$.

occurs in a p53-independent manner, we employed SK-N-BE (2) NB cell line, which expresses a non-functional p53.[39] Cell extracts from SK-N-BE (2) cells treated with TCP, SP2509 or vehicle were processed and assayed for SESN2 expression. As shown in Figure 6a, both inhibitors induce significant increase of SESN2 protein levels, together with a strong induction of LC3-I/LC3-II conversion. Furthermore, we found a SP2509 dose-dependent reduction in phosphorylation of mTORC1 targets, S6p70K and rpS6, confirming that LSD1 inhibition specifically impairs mTORC1 activity (Figure 6b). These findings strongly suggest that p53 function was not essential for SESN2 transcriptional activation and mTORC1 inhibition triggered by pharmacological LSD1 depletion.

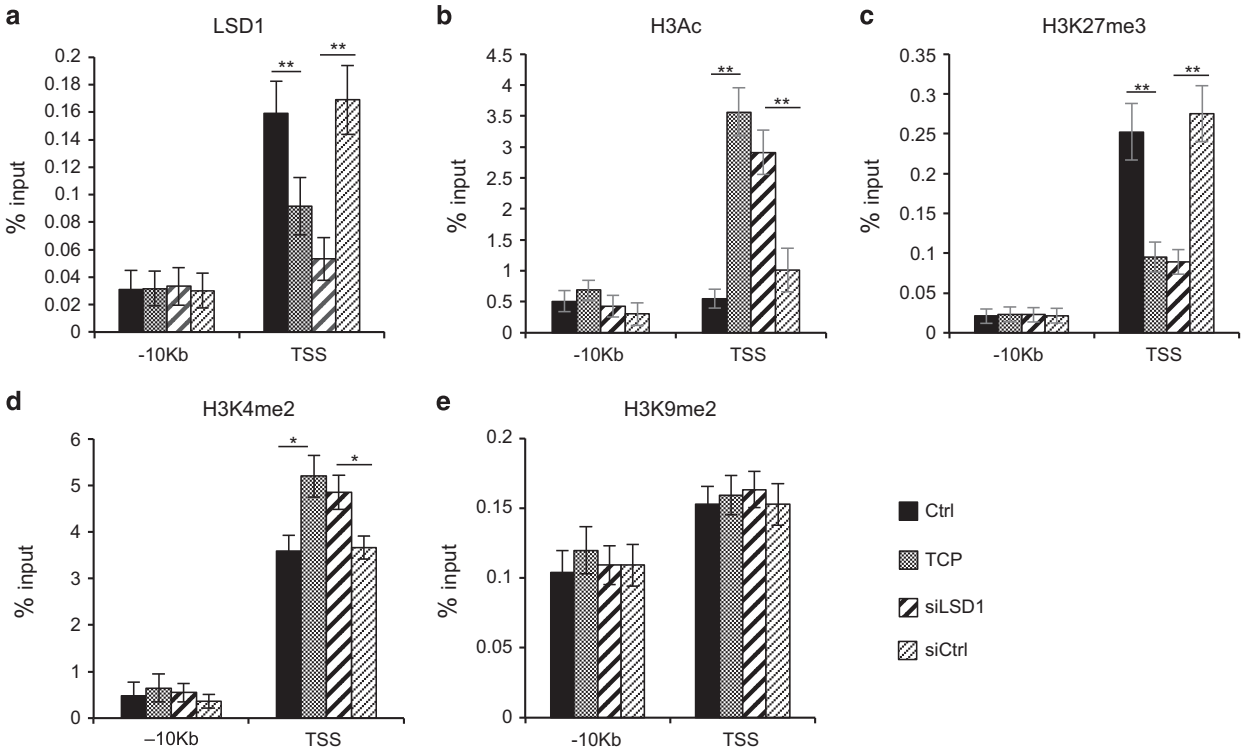

Figure 5. LSD1 binds *SESN2* gene promoter. (**a**) LSD1 binding to SESN2 chromatin. qPCR was performed with primers for SESN2 TSS and −10 kb. (**b**–**e**) Histone modifications at SESN2 chromatin; ChIPs were carried out using the indicated antibodies and analyzed with primers encompassing the TSS region and − 10 kb from TSS. Values from three independent ChIP assays are presented with s.d.'s, $n = 3$. Changes in % input are shown normalized over IgG controls (*$P < 0.05$; **$P < 0.01$; Student's *t*-test).

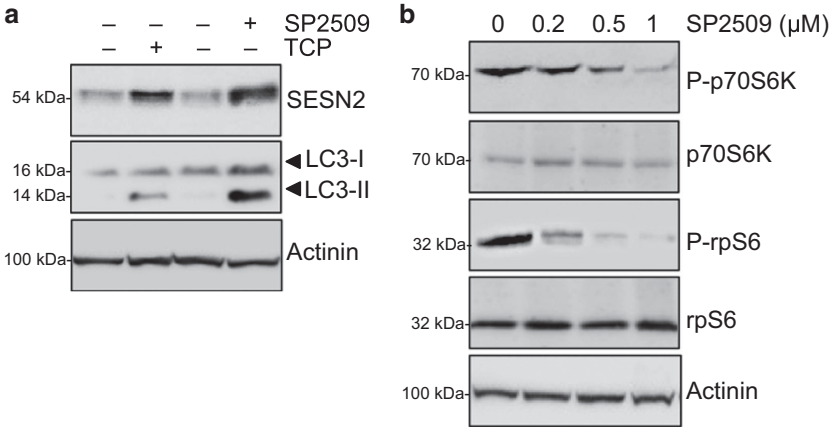

Figure 6. LSD1 depletion-mediated autophagy does not rely on p53 activity. (**a**) Cell extracts from SK-N-BE (2) treated with TCP (1 mM) or SP2509 (1 μM) for 48 h were prepared and probed with specific SESN2 and LC3 antibodies. (**b**) SK-N-BE (2) were treated with different concentrations of SP2509 (0, 0.2, 0.5 and 1 μM) for 48 h and protein extract were prepared and probed with indicated antibody. Actinin was probed as loading control.

SESN2 is required for autophagy induced by LSD1 inhibition

The findings reported above demonstrated that LSD1 binds and represses *SESN2* gene expression; thus, LSD1 inhibition triggers SESN2 expression. Because SESN2 serves as important regulator of mTORC1 activity, we hypothesized that its upregulation may have a causative role in LSD1-mediated mTORC1 activity modulation and autophagy induction.

To substantiate the relationship between SESN2 and autophagy induction following LSD1 inhibition, we ectopically overexpressed SESN2 in SH-SY5Y cells, and assessed the effect of increased expression of SESN2 on autophagy. We found that overexpression of SESN2 reduced rpS6 phosphorylation, along with a concomitant increase in TFEB nuclear localization. These findings provide functional evidence that enhanced expression of SESN2 induces autophagy through mTORC1 inhibition and recapitulates the effects of TCP treatment in NB cells (Figures 7a and b). To further define SESN2 role in autophagy induced by LSD1 inhibition, we performed SESN2 knockdown experiments, using a specific siRNA in SH-SY5Y cells, and investigated the functional consequences on LSD1 inhibition in SESN2-silenced cells. Lack of appreciable

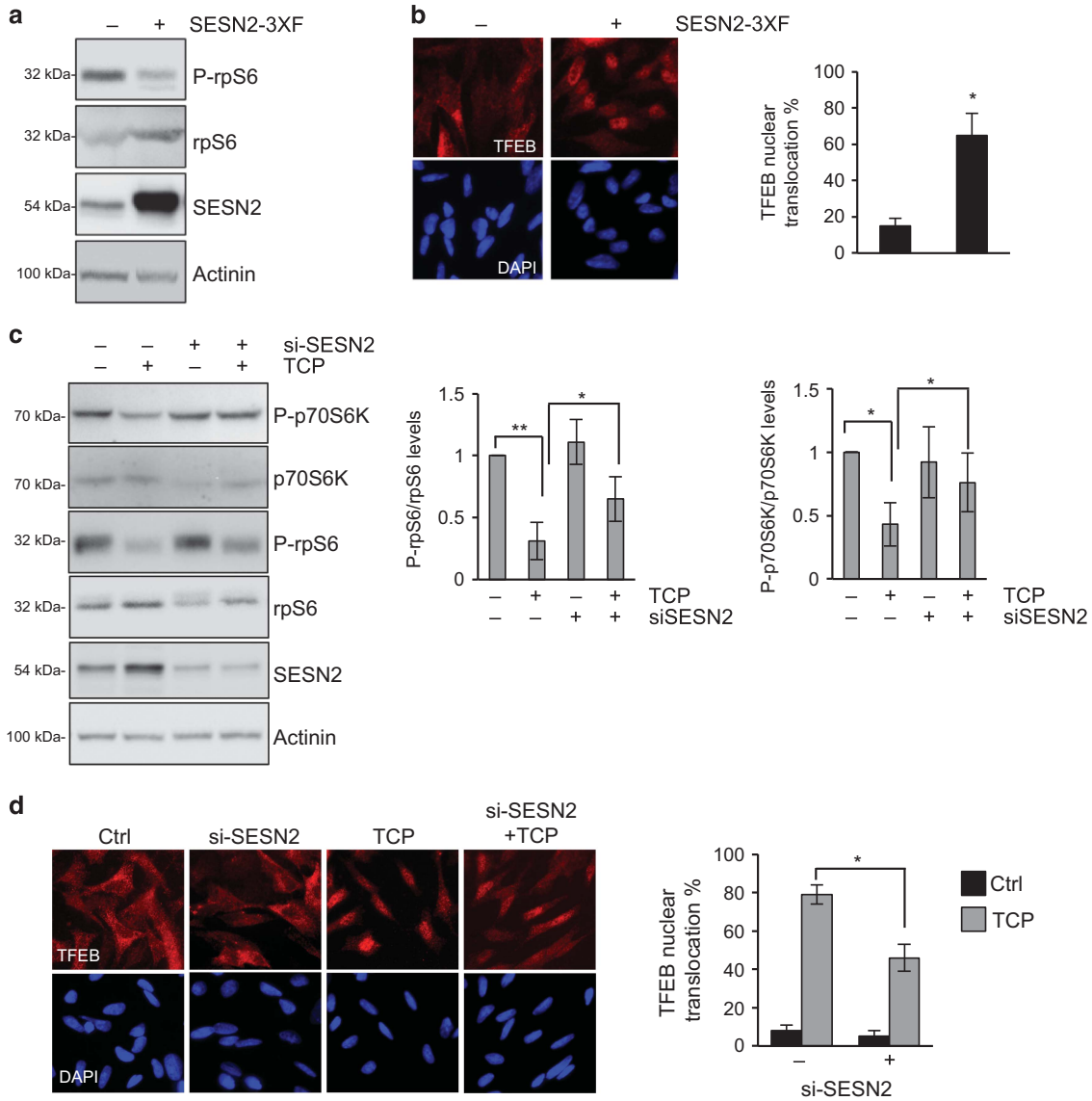

Figure 7. LSD1 regulates autophagy through SESN2 expression. (**a**) SH-SY5Ys were transfected with a SESN2 expression plasmid or empty vector, and protein extracts were prepared and stained with indicated antibody. (**b**) Localization of TFEB was analyzed in cells treated as described in A by immunostaining with TFEB antibody. (**c**) SH-SY5Y cells were transfected with siRNA against SESN2 or scramble. Twenty-four hours after transfection, cells were treated with TCP or vehicle for 24 h. Actinin was probed as loading control. Graphs show quantitative analysis of western blot experiments. A mean value ± s.d. of four independent experiments is shown (*$P < 0.05$; **$P < 0.005$). (**d**) SH-SY5Y treated as described in **c** were fixed and stained with anti-TFEB and DAPI. Histograms show the percentage of TFEB nuclear translocation (mean ± s.d., $n = 200$ cells). *$P < 0.0001$. DAPI, 4',6-diamino-2-phenylindole.

changes in the phosphorylation level of mTORC1 downstream targets was seen in siRNA-SESN2 cells (Figure 7c). In contrast, reduction of phosphorylation levels of p70S6K and rpS6 induced by TCP treatment is significantly weakened in SESN2-silenced cells; these data suggest that TCP requires SESN2 expression to decrease mTORC1 activity. Moreover, in SESN2 knockdown cells we found a reduced nuclear translocation of TFEB following TCP treatment (Figure 7d), indicating that SESN2 knockdown prevents the LSD1 inhibition-mediated autophagy. Taken together, these results identified SESN2 as a key factor in the autophagy activation mediated by LSD1 inhibition.

Association of SESN2 expression with clinical outcome in NB patients

The findings reported above indicated an inverse relationship between the expression of LSD1 and SESN2. It has been shown that high levels of LSD1 expression correlate with undifferentiated NB and adverse outcome.[27] Our *in vitro* findings imply that in NB patients LSD1 and SESN2 expression levels should be inversely correlated. If so, low expression of SESN2 in NB cancers would be predicted to correlate with poor prognosis. We queried public NB gene expression data repositories (the Oberthuer data set)[40] for the relative expression levels of LSD1 and SESN2. Accordingly, with previous studies, patients with high levels of LSD1 have poorer prognosis compared with those with low levels. In sharp contrast, high SESN2 expression correlates with better overall and event-free survival (Figure 8a).

Next, we analyzed LSD1 and SESN2 expression in a microarray gene expression data of 61 NBs (GSE12460), of which 50 were NB, 9 were ganglioblastoma and ganglioneuromas, and 2 were NB post chemotherapy. We have recently shown that LSD1 expression is considerably higher in differentiated NBs than in

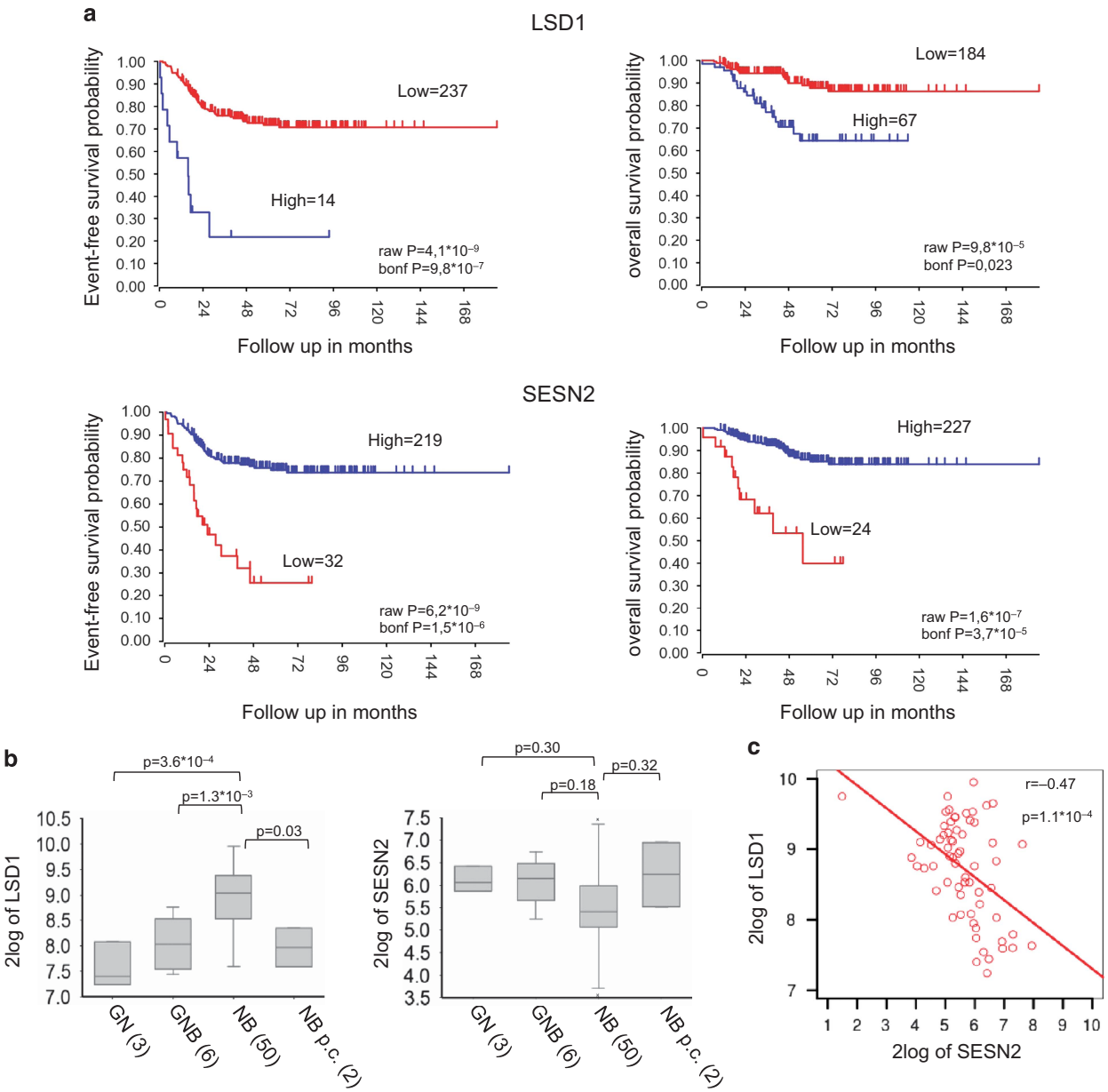

Figure 8. High levels of LSD1 and SESN2 expression are mutually exclusive in NB. (**a**) Kaplan–Meier analysis shows that high *SESN2* gene expression is associated with better survival in NB patients. Patients are grouped by the optimal cutoff of LSD1 and *SESN2* expression. The 'raw P' indicates the uncorrected *P*-value, and the 'bonf P' indicates the *P*-value corrected for multiple tests according to the Bonferroni method. (**b**) Changes in expression for LSD1 and Sestrin2 in ganglioneuroblastoma (GNB), ganglioneuroma (GN), NB and NB post chemotherapy (NB p.c.). (**c**) Correlation between the expression values of SESN2 and LSD1 in NB tumors (Pearson's correlation coefficient is shown).

ganglioblastomas and ganglioneuromas.[25] In contrast, we found that SESN2 expression was lower in NBs (Figure 8b), although the correlation between SESN2 expression in different tumors is only marginally significant. Furthermore, we found an inverse correlation between the expression values of LSD1 and SESN2 in NB tumors (Pearson's coefficient 0.47; Figure 8c). Collectively, these findings demonstrated that low levels of Sestrins expression correlate with poor prognosis of NB patients, and high levels of LSD1 and SESN2 expression are mutually exclusive in NB.

DISCUSSION

LSD1 is overexpressed in several types of cancers, and its enhanced expression correlates with more aggressive cancers and poor prognosis. LSD1 is implicated in several biologic processes, such as cell proliferation, epithelial–mesenchymal transition, stem cell pluripotency and differentiation.[24–27,41] However, its involvement in autophagy regulation is still poorly characterized. Here we show that pharmacological and genetic inhibition of LSD1 induces autophagy via the mTORC1-dependent pathway.

The present work highlights a critical role of LSD1 in promoting autophagy in NB cells; we provide for the first time evidence supporting the role of LSD1 as an epigenetic regulator of the autophagic pathway through the modulation of SESN2 expression.

We demonstrated that LSD1 binds and represses the *SESN2* gene; alteration of chromatin structures following LSD1 inhibition leads to the de-repression of the *SESN2* gene, which resulted in a

decreased mTORC1 activity. Thus, LSD1 inhibition triggers autophagy, as demonstrated by mTORC1 inhibition, nuclear translocation of TFEB, accumulation of LC3-II and finally the formation of autophagosomes, suggesting a direct link between LSD1-specific transcriptional regulation, mTORC1 cascade and autophagy.

Recent lines of work highlight the involvement of LSD1 in the autophagic machinery. Indeed, in prostate[42] and ovarian cancer cells[43] LSD1 inhibition triggers LC3-II accumulation and autophagosome formation; however, the molecular mechanism underlying these effects was not described.

Here, we identify *SESN2* as an LSD1-repressed target gene involved in mTORC1 pathway control. We found that SESN2 promoter is directly bound and repressed by LSD1; pharmacological inhibition of LSD1 triggers a structural modeling in the chromatin surrounding the TSS of the *SESN2* gene, leading to transcriptional activation of SESN2 expression. Moreover, we demonstrated that SESN2-enhanced expression suffices to promote autophagy in NB cells and SESN2 silencing attenuates mTORC1 suppression and autophagy induction by LSD1 inhibition, providing evidence that SESN2 has a critical role in the autophagy activation mediated by LSD1 depletion. SESN2 is a member of the Sestrin family of PA26-related proteins, which has an important role in regulating the cellular response to oxidative stress. TP53/p53 is the master transcriptional regulator of SESN2 under DNA damage and oxidative stress.[38,44] Interestingly, it has been recently shown that LSD1 depletion, synergistically with UBE4B inhibition, increases proteasomal and autophagic clearance activating the p53-mediated transcriptional program.[35] We find that depletion of LSD1 induces activation of SESN2 expression in the SK-N-BE (2) cell line, which express non-functional p53. Thus, LSD1 appears to regulate transcription of SESN2 in both p53-dependent and p53-independent ways.

We recently reported that the MYCN/LSD1 complex inhibits the transcription of the molecular chaperone Clusterin,[41] which is involved in the autophagic process through the stabilization of the LC3–Atg3 heterodimer, increasing the autophagosome biogenesis and autophagy progression;[45] we suggest that LSD1 orchestrates a broad-spectrum regulation of the autophagic pathway via transcriptional regulation of several autophagy-related genes.

Autophagy is a catabolic process that, at basal levels, represents the major mechanism for the turnover of cytoplasm components and selective removal of unfolded proteins and damaged organelles. However, autophagy could be activated in response to several stimuli such as oxidative and nutrient stresses, and the mTOR pathway is the main regulator.[12,36] Recent studies suggest that the mTORC pathway may be associated with cancer-related epigenetic alterations,[1,46,47] unveiling a key role of the epigenetic network in the autophagy control. HDACI inhibitors have been shown to induce autophagy via FOXO1-dependent pathways;[48] the methyltransferase EZH2 has been demonstrated to repress several negative regulators of the mTOR pathway and inhibits autophagy.[49] However, epigenetic role in autophagy regulation and its association with tumorigenesis continue to be uncovered.

The relationship between autophagy and cancer remains controversial. Autophagy seems to have a dual effect in cancer, depending on stage and cell type, and it could act as tumor suppressor or driver of cancer progression.[7,9] Some tumors are sensitive to hyperactivation of autophagy; in other circumstances, inhibition of mTORC1 increases cell survival and prevents apoptosis, inducing chemoresistance.[50] Although relationship between autophagy and tumor progression is disputed, during early stages of carcinogenesis autophagy seems to suppress tumor initiation and cancer development is often associated with defective autophagy.[7,9] We cannot exclude that LSD1 overexpression may contribute to tumor initiation by suppressing the expression of key regulators of autophagy induction, although further studies are required to clarify this issue. Interestingly, analysis of publicly available expression data of large number of NBs highlighted that high SESN2 expression correlates with better overall and event-free survival. Interestingly, high levels of LSD1 and SESN2 expression are mutually exclusive in NB tumors. Collectively, these findings support and corroborate the broad significance of our *in vitro* results.

In conclusion, data reported here establish the critical role of LSD1 in autophagy and indicate that, in NB cells, LSD1 knockdown induces autophagy through the SESN2–mTORC1 pathway. Our results strongly support the concept that LSD1-dependent epigenetic alterations may influence the expression of autophagy-related genes and provide a novel link among epigenetic regulation, mTOR pathway and tumorigenesis.

MATERIALS AND METHODS

Cell culture and treatments

SH-SY5Y and SHEP Tet-21/N cells were cultured in Dulbecco's Modified Eagle Medium supplemented in 10% fetal bovine serum. SK-N-BE (2) was cultured in 1:1 mixture dulbecco's modified eagle medium/F-12 containing 10% fetal bovine serum. When indicated, cells were treated with TCP (1 mM, Enzo Life Sciences, Farmingdale, NY, USA) or SP2509 (0.2/0.5/1 μM, Cayman Chemical Company, Ann Arbor, MI, USA) for 6, 12, 24 or 48 h. An amount of 20 mM of NH_4Cl was administrated for 24 h to block autophagic flux.

Transfection and silencing

For transient transfections of Tet-21/N and SH-SY5Y, cells cultured on 100-mm dishes were transfected with 12 μg of SESN2 plasmid, GFP-mRFP-LC3 (kind gift from Dr A Fraldi, Tigem Institute) construct or empty vector using Lipofectamine 2000. For LSD1 knockdown, 50 or 100 nM siRNA targeting LSD1 or LSD2 (GE Dharmacon, Lafayette, CO, USA) or scramble were transfected in Tet-21/N cells using a MicroPorator Digital Bio Technology (Waltham, MA, USA), according to the recently described protocol.[41] Briefly, $2x10^6$ cells were collected by trypsin/EDTA digestion, washed once with calcium and magnesium-free phosphate-buffered saline (PBS) and resuspended in 100 μl of resuspension buffer, mixed with siRNA or scramble and electroporated as described.[25] The efficiency of LSD1 siRNA knockdown was assayed 48 h after transfection by western blotting. For silencing assays, 45 nM siRNA targeting SESN2 (GE Dharmacon) or scramble were transfected in SH-SY5Y cells using Viromer Green; Cells were collected for analysis 48 h after transfection.

PathScan assay

For PathScan assay (PathScan Intracellular Signaling Array Kit, Cell Signaling, Danvers, MA, USA), cell lysates from Tet-21/N cells, treated with TCP or vehicle for 24 h, were prepared according to the manufacturer's protocol; 75 μl of lysates was added to each well previously prepared with Blocking Buffer and the slide was incubated for 2 h at room temperature. After incubation, the slide was washed four times with Wash buffer and each well was incubated with 75 μl of Detection Antibody Cocktail for 1 h at room temperature. Then, the slide was washed three times and incubated for 30 min with 75 μl of horseradish peroxidase-linked Streptavidin reagent. Next, the slide was washed, incubated with Lumi Glo/peroxide solution and displayed by biochemiluminescence acquisition. Protein expression levels were quantified and normalized by positive controls using ImageJ32 software (National Institutes of Health, Bethesda, MD, USA).

Protein extraction and western blot

Whole-cell extracts were prepared with buffer F (10 mM Tris HCl pH 7.5, 150 mM NaCl, 30 mM $Na_4O_7P_2$, 50 mM NaF, 5 mM $ZnCl_2$, 0.1 mM Na_3VO_4, 1% Triton, 0.1 mM PMSF). Fifty micrograms of protein extracts were separated by SDS–PAGE and indicated antibodies are listed in Supplementary Table S1. Full scans of western blot data are in Supplementary Figure 3.

Immunofluorescence and microscopy

Immunofluorescence assay was performed as previously described.[25] Overall, 2×10^4 cells were plated in a 12-well plate, in which a coverslip had

been placed and treated as indicated. Cells were then washed, fixed in 4% paraformaldehyde in PBS, permeabilized in 0.1% Triton X-100 in PBS for 5 min, pre-blocked in 2% bovine serum albumin for 30 min and incubated at 37 °C for 1 h with rabbit anti-TFEB. Cells were then incubated for 30 min at room temperature with Cy3-coniugated secondary antibody and nuclei were stained with 4',6-diamino-2-phenylindole (DAPI). Nikon Eclipse TE 2000-U microscope (Nikon, Shinjuku, Japan) was used for image acquisition. Quantitative TFEB nuclear translocation analyses were performed by ImageJ32 software, calculating the ratio value resulting from the average of nuclear TFEB signal intensity divided by the average of the cytosolic TFEB signal intensity, normalized on negative and positive control samples. In case of GFP-mRFP-LC3 experiments, cells were just fixed in 4% paraformaldehyde and images were collected using a laser-scanning microscope (LSM 510 META, Carl Zeiss Microimaging Inc., Oberkochen, Germany) equipped with a Plan Apo ×63 oil immersion (numerical aperture 1.4) objective lens. Moreover, we acquired the images with the same setting (laser power and detector gain) as well as we kept the same threshold of fluorescence intensity in all experimental conditions. Quantification analyses were carried out using LSM 510 software. We evaluated the autophagic flux counting the number of green and red puncta per cell (number of cells >100). Student's *t*-test was used to determine the significance and error bars show the s.d. of the average.

RNA extraction and qRT–PCR

Total RNA was extracted from Tet-21/N cells and reverse transcription reaction was performed using Quantitec Reverse Transcription Kit (Qiagen, Hilden, Germany). cDNA thus obtained was analyzed by qPCR using SYBR Green 2X PCR Master Mix (Applied Biosystem, Waltham, MA, USA). Each sample was run in triplicate and the expression of housekeeping beta-glucoronidase (GUSb) gene used for normalization as described.[41] Primers are presented in Supplementary Table S2.

Chromatin immunoprecipitation assay

ChIP assays were performed as described.[25] In all, 1×10^7 cells treated as indicated were crosslinked using 1% formaldehyde. Cell pellet was lysed and sonicated into 200-bp fragments by using Bioruptor (Diagenode, Liege, Belgium). An aliquot of sonicated material was used as input. Remaining samples were incubated overnight with antibodies listed in Supplementary Table S1; immunoprecipitated DNA was purified and quantified by qPCR with the primer sets described in Supplementary Table S2 and normalized to input DNA.

Survival analysis and association with NB stages by gene expression studies

Kaplan–Meier curves were calculated for Oberthuer public gene expression data[40] using the R2 web tool (http://r2.amc.nl) as described.[51] Briefly, the optimal cutoff for each gene was determined by R2 package to generate the Kaplan–Meier curves, and significance (raw P) and the *P*-value (Bonferroni-corrected) were calculated. Another set of gene expression data of 61 tumors (GEO ID: GSE12460) including 50 NB, 6 ganglioneuroblastoma, 3 ganglioneuroma and 2 NB post chemotherapy was downloaded, and the Mann–Whitney test was used to test statistical significance of differences in gene expression among groups. The correlation between gene expression between Sestrins and LSD1 was evaluated by Pearson correlation in 64 NBs.

Statistical analysis

All experiments were repeated at least two times. Graphs representing data express mean ± s.d. Statistical significance was calculated by unpaired, two-tailed Student's *t*-test. $P<0.05$ was considered statistically significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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CHAPTER 4

Inhibition of lysine-specific demethylase LSD1 induces senescence in Glioblastoma cells through a HIF-1α-dependent pathway

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Inhibition of lysine-specific demethylase LSD1 induces senescence in Glioblastoma cells through a HIF-1α-dependent pathway

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ABSTRACT

Senescence is a stress-responsive cellular program that leads to cell cycle arrest. In cancer cells, senescence has profound implications for tumor aggressiveness and clinical outcome, but the molecular events that provoke cancer cells to undergo senescence remain unclear. Herein, we provide evidence that the histone demethylase LSD1/KDM1A supports the growth of Glioblastoma tumor cells and its inhibition triggers senescence response. LSD1 is a histone modifier that participates in key aspects of gene transcription as well as in the regulation of methylation dynamics of non-histone proteins. We found that down-regulation of LSD1 inhibits Glioblastoma cell growth, impairs mTOR pathway and cell migration and induces senescence. At mechanistic level, we found that LSD1 regulates HIF-1α protein stability. Pharmacological inhibition or siRNA-mediated silencing of LSD1 expression effectively reduces HIF-1α protein levels, which suffices for the induction of senescence. Our findings elucidate a mechanism whereby LSD1 controls senescence in Glioblastoma tumor cells through the regulation of HIF-1α, and we propose the novel defined LSD1/HIF-1α axis as a new target for the therapy of Glioblastoma tumors.

1. Introduction

Glioblastoma Multiforme (GBM), an aggressive tumor of the adult central nervous system, is the most malignant of glial neoplasm representing up to 50% of all primary brain gliomas [1]. GBMs tumors are characterized by intratumoral genetic heterogeneity and remarkable ability to invade surrounding normal brain tissues, thus evading total surgical resection as well as radiation treatments and chemotherapy [1,2]. Despite continuous and significant advances in clinical therapies for the treatment of GBM, the patient prognosis is poor and after initial diagnosis the medial survival duration is about 9–12 months, suggesting urgently the need for the development of novel therapeutic strategies [3].

GBMs have alterations in cell-cycle checkpoints, senescence and apoptosis pathways, giving rise to uncontrolled cell proliferation [2,4]. An important mechanism for preventing proliferation in tumor cells is the stress-responsive senescent cellular program, a state in which the cell is no longer able to proliferate [5]. Senescent cells have irreversibly lost their capacity for cell division, although senescent cells are vital and metabolically active [5–7]. Senescence process is characterized by several non-exclusive markers, such as the absence of proliferative signals, induction of growth arrest markers, β-galactosidase activity associated with senescence (SA-βgal), expression of tumor suppressors and cell cycle inhibitors and often induction of DNA damage markers [8–10].

The causative role of epigenetic enzymes, as histone deacetylases and demethylases, in the senescence process has been recently documented. It has been shown that Sirtuins regulate premature cellular senescent and accelerate aging [11,12]. Sirtuin proteins constitute class III histone deacetylases (HDACs) with important roles in cellular and biological processes, as well as in metabolic homeostasis and genomic integrity [13]. Loss of Lysine-specific demethylase 1 (LSD1) demethylase activity provokes senescence in trophoblast stem cells [14] and prevents age-programmed loss of beige adipocytes [15]. LSD1/KDM1A is an epigenetic eraser that catalyses lysine demethylation in a flavin adenine dinucleotide (FAD)-dependent oxidative reaction. LSD1 demethylates both Lys-4 (H3K4me/me2) and Lys-9 (H3K9me/me2) of histone H3, thereby acting as a coactivator or a corepressor, depending on the context [16–19]. LSD1 is overexpressed in a variety of human cancers and tends to correlate with more aggressive tumors with poor

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prognosis [20,21]. In addition, LSD1 can also target several non-histone proteins such as p53 [22], E2F [23], DNMT1 [24] and HIF-1α [25].

Hypoxia-inducible factor 1-alpha (HIF-1α), together with the homonym subunit beta, form a heterodimeric transcription factor hypoxia-inducible factor 1 (HIF-1) [26]. HIF-1α is a basic helix-loop-helix PAS domain containing protein and together with subunit beta binds hypoxia-responsive elements (HREs) that contain a conserved RCGTG core sequence. HIF-1α, under normoxic conditions, undergoes negative regulation via ODD domain [27]. This domain contains a number of prolyl residues that are recognized and hydroxylated by specific prolyl hydroxylase domain (PHD) enzymes; this results in the binding of a key negative regulator of HIF-1α, the von Hippel – Lindau protein (VHL) E3 ligase, which targets the HIF-1α protein for rapid degradation via the proteasome pathway [28].

The stability and function of the HIF-1α protein are affected by many post-translational modifications (PTMs), including hydroxylation, acetylation, ubiquitination and SUMOylation [29–31]. It has been shown that HIF-1α stability is regulated by LSD1 [25]; in particular, the Set9 histone methyltransferase induces HIF-1α methylation promoting HIF-1α protein degradation, while LSD1 reverses this process [32]. Furthermore, LSD1 upregulates hypoxia responses by demethylating RACK1 protein, a component of hypoxia-inducible factor (HIF) ubiquitination machinery, and consequently suppressing the oxygen-independent degradation of HIF-1α [33].

It has been reported that HIF-1α plays a role in cellular senescent state. Welford et al. highlighted a novel role for HIF-1α to delay premature senescence through the activation of macrophage migration inhibitory factor (MIF) [34]. Others studies propose that inhibition of HIF-1α in combination with ATRA treatments enhances senescent cells in RA-responsive cells and silencing of HIF-1α suffices to increase the number of senescent cells independently to the ATRA responsiveness [35].

In the present study, we define the role of the LSD1 demethylase in cell growth and senescence programs in GBM cells. At the molecular levels, we found that LSD1 regulates HIF-1α protein stability; pharmacological inhibition, as well as LSD1 silencing, effectively reduces HIF-1α protein levels and determine senescence induction. We propose that HIF-1α/LSD1 targeting may provide a new approach for the therapy of GBM tumors.

2. Materials and methods

2.1. Cell cultures and treatments

GBM cells lines (U87MG, U251, T98G), were cultured in Dulbecco's modified Eagle's Medium (DMEM) supplemented with antibiotics 1% penicillin/streptomycin and 10% fetal calf serum. When indicated, cells were treated with:

TCP (0,5/1/1,5 mM, Enzo Life Sciences);
OG-L002(50 nM Sigma-Aldrich);
GSK2879552 (2,5 μM Active Biochem);
SP2509 (1 μM Cayman Chemical Company);
MG132 (1 μM Sigma-Aldrich);
$CoCl_2$ (100 μM Sigma-Aldrich).

For hypoxia experiments, U87MG cells untreated or treated overnight with TCP 1 mM and then exposed to hypoxic culture conditions 6 h, using a hypoxic incubator (STEMCELL Technologies) in atmosphere containing 95% N_2 and 5% CO_2.

2.2. Cell viability, migration assays and Colony formation

Trypan blue exclusion test was utilized for cell viability. Wound healing assay, was performed as previously described [36]. Cells were treated with TCP for an overnight or silenced with siRNA before scratch. The scratch was monitored using the Nikon Eclipse TE 2000-U microscope. Percentage of wound healing was calculated as following: [(empty area at T_0) − (empty area at 2 days)] / (empty area at T_0) × 100. For Trans-membrane migration assay, cells were treated for 12 h with treatment as indicated and then plated on the upper side of chambers in the presence of 2% FBS, while on the other side of the chamber 20% FBS was used as attractive. After 16 h, cells were fixed and stained with 0,1% crystal violet in Et-OH 20%. Then, cells were counted with 10× objective. For colony formation assay cells were pretreated as described for 16 h, then seeded in six-well at different density (150, 300 and 500 cells). A week later, cells were stained with 1% crystal violet in Et-OH 20% and lysed in 10% acetic acid. The optical density of each well at 450 nm (OD450) was measured for quantification.

2.3. Immunofluorescence, BrdU and FACS analysis

For Immunofluorescence, cells were plated on coverslips and treated as indicated. Cells were than fixed in 4% paraformaldehyde in PBS, permeabilized in 0.1% Triton X-100 in PBS, pre-blocked in 2% BSA– 3% NS-PBS for 30 min at room temperature and then incubated for 1 h at 37 °C with primary antibody (γ-H2AX, Abcam, ab81299; Ki67, Santa Cruz, sc-7846). Cells were then incubated for 30 min at room temperature with Cy3-coniugated secondary antibody and nuclei were stained with DAPI. Three independent experiments were performed and for each three independent counts of 100 cells were obtained and data analyzed compared.

For BrdU analysis, cells were treated with TCP (24 h) or siRNA target LSD1 and then labelled with BrdU for 3 h. After fixation with 4% paraformaldehyde, cells were permeabilized with NP-40 0.1%, DNA was denatured with 50 mM NaOH and BrdU detected with mouse monoclonal antibodies (G3G4, Hybridoma Banck) and anti-mouse Alexa FluorR 594 (Invitrogen). DNA was counterstained with DAPI (100 ng/mL) and proliferation rate was quantified using ImageJ software. Fluorescence cells were imaged by Nikon Eclipse TE 2000-U microscope with 40× objective. For flow cytometry analysis (FACS), cells, treated as described, were pelleted by centrifugation and resuspended at 1×10^6 cells/mL in Ethanol 70% in PBS at 4 °C for one overnight for fixation. 2×10^6 cells were permeabilized with 0,1% Triton X-100/PBS for 15 min, blocked in 5% Bovine Serum Albumin/PBS and stained with 2,5 μg/mL Propidium Iodide for 1 h. Cells were analyses by a FACS Calibur (BD) and data analyzed by Cell Quest and Cyflogic Softwares.

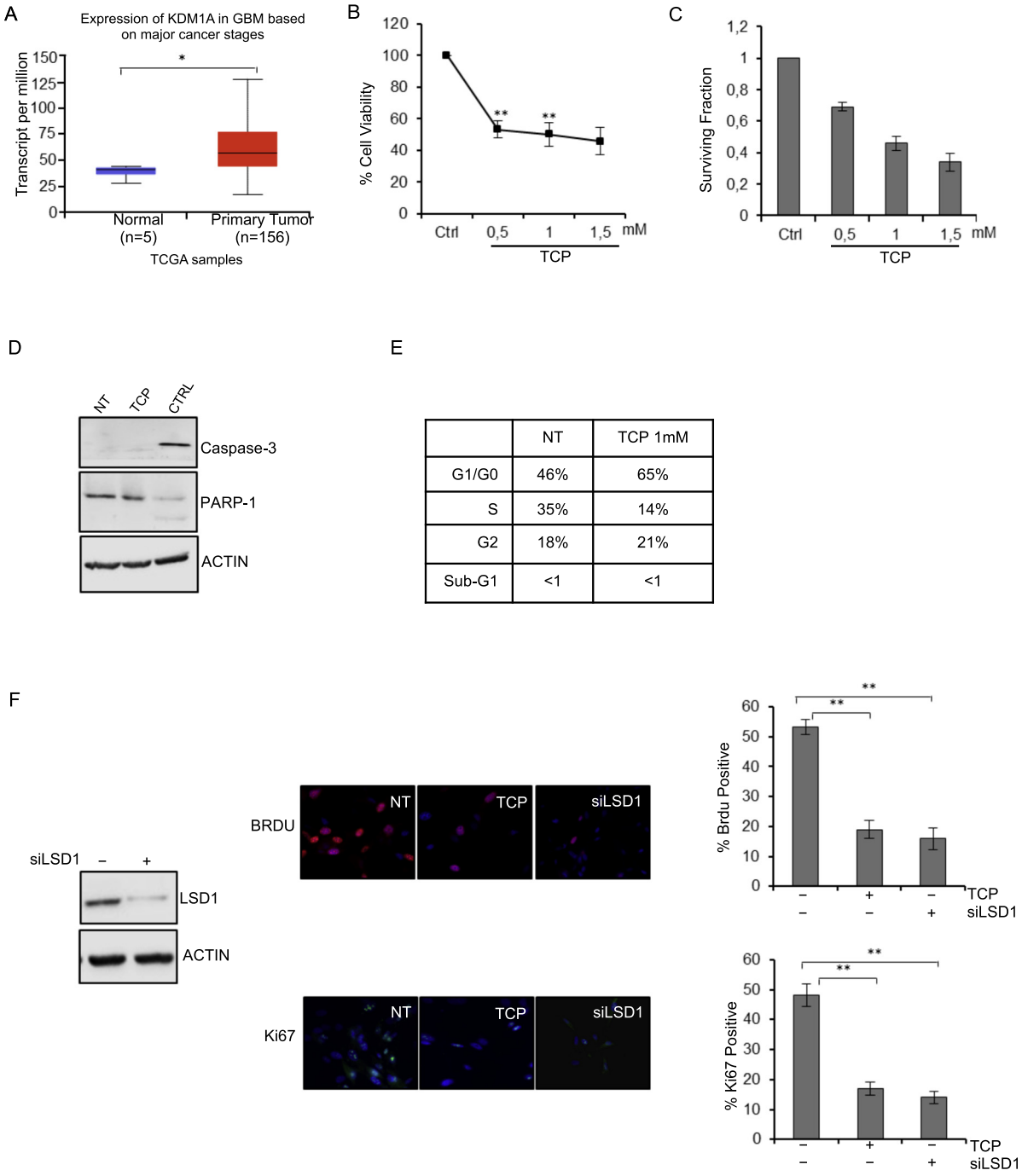
2.4. Sa-βgal assay

Sa-βgal activity was assayed in cells treated with TCP for 24 h and transfected with siRNA (48 h). Cells were washed with PBS, fixed with (2% formaldeide and 0,2% glutaraldeide) for 5 min at RT and then washed in PBS. Staining X-gal solution (30 mM citric acid/disodium phosphate pH 6,5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, 150 mM NaCl, 2 mM $MgCl_2$, 1 mg X-GAL) was added to cells for overnight at 37° as described. Then, cells were washed with PBS and the.

staining was monitored using the Nikon Eclipse TE 2000-U microscope. Cells were counted with a 10× objective.

2.5. Protein extraction and Western blot

Proteins were extracted with buffer F (10 mM TrisHCl pH 7.5, 150 mM NaCl, 30 mM $Na_4O_7P_2$, 50 mM NaF, 5 mM $ZnCl_2$, 0.1 mM Na_3VO_4, 1% Triton, 0.1 mM PMSF) and western blot was performed with specific antibodies as indicated follow: mouse anti-Actinin (Santa Cruz, Cat#sc-17,829), goat anti-ACTIN (Santa Cruz, Cat#sc-1616), rabbit anti-LSD1 (Abcam, Cat#ab17721), rabbit anti-Pp70S6K (Cell Signaling Technology, Cat#9205), rabbit anti-p70S6K (Cell Signaling Technology, Cat#2708), rabbit anti- PrpS6 (Cell Signaling Technology, Cat#2215), rabbit anti- rpS6 (Cell Signaling Technology, Cat#2217), rabbit anti-P-4EBP1 (Cell Signaling Technology, Cat#9456), rabbit



	NT	TCP 1mM
G1/G0	46%	65%
S	35%	14%
G2	18%	21%
Sub-G1	<1	<1

Fig. 1. LSD1 inhibition reduces proliferation in GBM cells. (A) Boxplot showing relative expression of KDM1A in normal and GBM Primary tumors samples. UALCAN Database and Statistical analysis student's *t*-test were used ($*p = 1.16 \times 10^{-10}$). (B) Trypan blue exclusion assay in U87MG cells treated with TCP for 24 h, at different concentrations as indicated. (C) Colony formation assay was performed on U87MG cells treated for 7 days as indicated. (D) Western blotting of protein extract from U87MG cells, treated with TCP, for 24 h, CTRL positive sample is U87MG irradiated with 254-nm UV light at 40 J/m^2, using antibodies as indicated. Actin antibody was utilized as loading control. (E) Percentage of cell-cycle distribution of U87MG cells before and after TCP treatment for 24 h, was measured by Flow cytometry analysis. The average values from three independent experiments are reported in the table; all standard deviations are < 15%. (F) BrdU incorporation assay and Ki67 immunofluorescence in U87MG cells treated with TCP 1 mM for 24 h or silenced for LSD1. DAPI was used to counterstain nuclei. Graphs represent number of positive cells. Data represents the mean and standard deviation of 3 independent experiments. ($**p < 0.001$, student's t-test.)

anti-4EBP1 (Cell Signaling Technology, Cat#9644), rabbit anti-pRB (Cell Signaling Technology, Cat#9307), rabbit anti-RB (Santa Cruz, Cat#sc-050),rabbit anti-HIF-1α (Elabscience, Cat#*E*-AB-16751), mouse anti-PARP-1 (Santa Cruz, Cat#sc-53,643), rabbit anti-p21 (Santa Cruz, Cat#sc-397), rabbit anti-PDH (Cell Signaling Technology, Cat#3205), rabbit anti-SDHA (Cell Signaling Technology, Cat#5839), rabbit anti-Aldolase A (Cell Signaling Technology, Cat#3188S), rabbit anti-Hexokinase 1 (Cell Signaling Technology, Cat#2024S), rabbit anti-Enolase 1 (Cell Signaling Technology, Cat#3810S), rabbit anti-PDHK1 (Cell Signaling Technology, Cat#3820S), rabbit anti-TOM20 (Proteintech, Cat#11802–1-AP), anti-CAIX.

2.6. RNA extraction and qRT-PCR and siRNA treatments

RNA was extracted from U87MG cells using EuroGold Trifast (EuroClone). Quantitec Reverse Transcription Kit (Qiagen), was used to generate cDNA according to manufacturer's protocol. Quantitative analysis was performed using SYBR Green 2× PCR Master Mix (Applied Biosystem). Samples were run in triplicate and normalized to the expression of housekeeping beta- glucoronidase (GUSb) gene as previously described [37]. 100 nM of siRNA targeting LSD1 (Dharmacon), HIF-1α (SIGMA), TSC2 (GenePharma Co.) or scramble were transfected in U87MG cells using a MicroPorator Digital Bio Technology, according to the described protocol [37]. The efficiency of siRNA knockdown was monitored at 48 h after transfection by western blot and qRT-PCR.

Primers sequences were listed as follows:

HIF-1α forward: 5′-CCCATAGGAAGCACTAGACAAAGT-3′
HIF-1α –reverse: 5′-TGACCATATCACTATCCACATAAA-3′
LSD1 –forward: 5′- AGACGACAGTTCTGGAGGGTA-3′
LSD1 –reverse: 5′- TCTTGAGAAGTCATCCGGTCA-3′
VEGFα –forward: 5′- CAGAATCATCACGAAGTGGTGAA-3′
VEGFα–reverse:5′-CTCGATTGGATGGCAGTAGCT-3′
CAIX –forward: 5′-CGGAAGAAAACAGTGCCTATGA-3′
CAIX –reverse: 5′-CTTCCTCAGCGATTTCTTCCA-3′
PAI-1–forward: 5′-GAGTGCCCAGCTCATCAGCCACTGG-3′
PAI-1 –reverse: 5′-CCTGAAACTGTCTGAACATGTCGGTCA-3′
TSC2 –forward: 5′-CCGCAGCATCAGTGTGTC-3′
TSC2 –reverse: 5′-CACTGGTGAGGGACGTCTG-3′
GUSb–forward: 5′-GTGGGCATTGTGCTACCTC-3′
GUSb–reverse: 5′-ATTTTTGTCCCGGCGAAC-3′

2.7. Metabolic studies

Real-time measurements of extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) were measured using an XFe-96 Extracellular Flux Analyzer (Seahorse Bioscience, Billerica, MA, USA). Cells were counted using an automated Cell counter (Countess from Life Technologies), seeded in XFe-96 plates (Seahorse Bioscience) at the density of $2{,}5 \times 10^4$ cells/well and incubated overnight at 37 °C in 5% CO_2 atmosphere in the presence or absence of TCP (1 mM). ECAR was measured in XF^e media in basal condition and in response to 10 mM glucose, 4 μM oligomycin and 100 mM of 2-Deoxy-D-glucose (2-DG) (all from Sigma-Aldrich). Basal glycolysis was calculated after glucose injection (subtracting the ECAR rate inhibited by 2-DG). Maximal glycolysis was measured after oligomycin injection, and glycolytic capacity, as the difference of oligomycin-induced ECAR and 2-DG-induced ECAR.

OCR was measured in XF^e media (non-buffered DMEM medium containing 10 mM glucose, 2 mM L-glutamine and 1 mM sodium pyruvate) under basal conditions and in response to 4 μM Oligomycin, 1,5 μM of carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP) and 1 μM of Antimycin-A and Rotenone (all from Sigma-Aldrich). The key parameters of mitochondrial function were determined as follows: Basal OCR was calculated as the difference between baseline measurements and antimycin-A/rotenone-induced OCR, the amount of OCR related to ATP production (ATP-linked OCR) was calculated as the difference between baseline measurements and oligomycin-induced OCR, and finally, the maximal respiratory capacity was calculated as the difference between the FCCP-stimulated OCR and the OCR after antimycin-A and rotenone injection. Each sample was plated at least in triplicate. Experiments with the Seahorse system were done with the following assay conditions: 3 min mixture; 3 min wait; and 3 min measurement. Data are expressed as mean and s.e.m. from five independent experiments. Statistical differences were evaluated using the Wilcoxon matched-pairs test using 0.01 as significant threshold.

3. Results

3.1. LSD1 sustains Glioblastoma cell viability

Inspection of primary tumor samples, categorized using GBM clinical patients' data, generated expression levels of KMD1A boxplots, shown in Fig. 1A. KDM1A gene is expressed at higher levels in primary GBM compared to normal samples.

To investigate whether LSD1 could be a valid therapeutic target for GBM, we explored the biological impact of LSD1 inhibition on cells viability of GBM cell line U87MG. Cells were treated with the LSD1 inhibitor, tranylcypromine (TCP), and assayed for cell viability. As shown in Fig. 1B, the suppression of viability occurred in a dose-dependent manner upon TCP treatment. Colony formation assay also revealed that TCP elicited significant anti-proliferative effects in U87MG cells (Fig. 1C).

To determine whether TCP induces apoptosis in GBM cells, we evaluated the apoptotic rate monitoring caspase3-depedent PARP1 cleavage, an indicator of apoptosis activation; Western blot analysis showed that up-regulation of cleaved-PARP1 and cleaved caspase-3 were only detected in positive control but not in TCP treated cells (Fig. 1D). To further determine whether TCP decreased cell viability by inducing cell death or inhibiting cell proliferation, we analyze the DNA profile using flow cytometry assays. As shown in Fig. 1E, TCP treatment for 24 h showed no obvious apoptosis (sub-G1 phase), while a significantly accumulation of cells in the G0/G1 phase was observed in TCP-treated U87MG cells. Consistently, lower percentage of BrdU-positive cells and labeling index of Ki-67, widely used markers of cell proliferation, were observed in TCP-treated cells, as well as in LSD1-KD cells (siLSD1) (Fig. 1F).

Overall, these results suggest that LSD1 depletion, by TCP treatment or silencing, reduces GBM cancer cells viability exerting a cytostatic function, rather than activating apoptosis.

3.2. LSD1 inhibition induces senescence in Glioblastoma cells

We explored whether LSD1 inhibition activates the senescence program in GBM cells. To this end, we monitored the expression of senescence-associated β-galactosidase activity, a well-defined marker of senescence [38,39]. As positive control, U87MG cells were treated with Camptothecin (CPT), a potent inducer of DNA damage-mediated senescence [40]. As shown in Fig. 2A, both TCP treatment and LSD1 knockdown cause a strong increment of the senescence marker SA-βgal in U87MG cells. As LSD1 has been shown associated with DNA damage response (DDR) [10], we sought to determine whether LSD1-knockdown triggers DDR-associated senescence. Phosphorylation at serine-139 of histone H2AX (γ-H2AX) constitutes the most sensitive marker of DNA double-strand breaks (DSBs) and telomere shortening. U87MG cells were treated with TCP and CPT and monitored for DDR activation by γ-H2AX immunofluorescence. Data shown in Fig. 2B demonstrate that LSD1 inhibition, as well as it's silencing, induces γ-H2AX foci formation at levels comparable to CPT-treated cells.

Induction and maintenance of senescence affects two critical tumor suppression pathways governed by RB/p16INK4a and p53/p21. To confirm the establishment of a cellular senescence state, we evaluated the phosphorylation level of RB and the expression of the cyclin-dependent kinase inhibitor p21. Following LSD1 inhibition, we found, as expected in senescent cells, that p21 protein levels increase in U87MG cells upon TCP treatment or LSD1-knockdown with a concomitant decrease of phosphorylated RB (Fig. 2C).

To confirm these results, we also used different LSD1 inhibitors. OG-L002 (50 nM), SP2509 (1 μM) and GSK2879552 (2,5 μM) were used to treat U87MG cells and senescence-associated β-galactosidase activity and RB phosphorylation were investigated. Results showed in Fig. 3A and B demonstrate that inhibitors effects on cell senescence are similar to those observed in LSD1 silenced cells. Considering that TCP

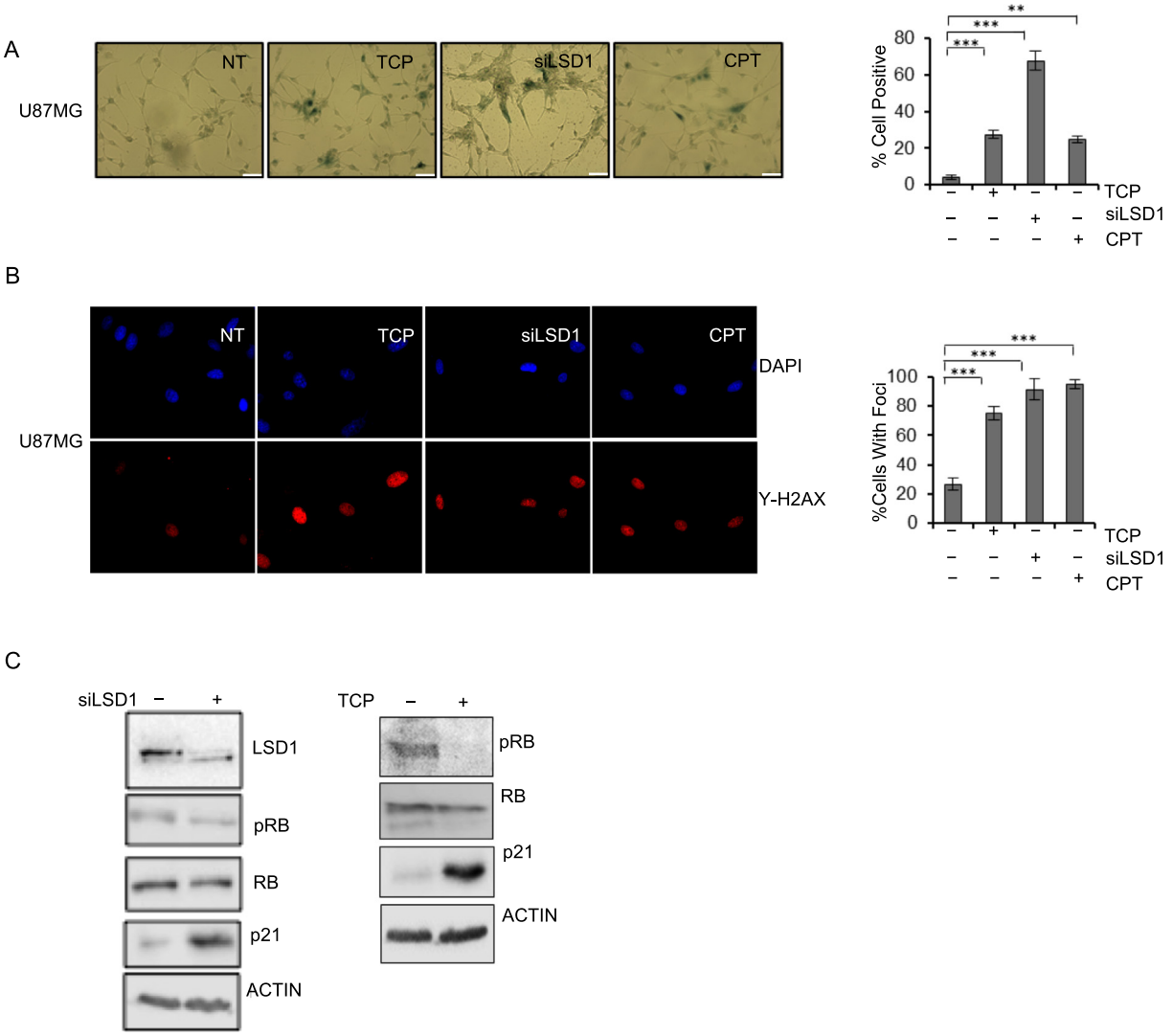


Fig. 2. LSD1 inhibition activates cell senescence. (A) U87MG cells were treated with 1 mM TCP for 24 h or Camptothecin 12 μM for 3 h, or transfected with specific siRNA (siLSD1) against LSD1 (100 nM), and analyzed for SA-βgal assay. Graphs represent number of SA-βgal positive cells. Data were analyzed from three independent counts each with 100 cells and represented as mean ± s.d., ***$p < 0.0001$ using the student's t-test. Scale bars 75 μm (B) Immunofluorescence analysis for γ-H2AX foci formation in U87MG cells treated as indicated. DAPI was used to counterstain nuclei. Histogram indicates the number of cells containing 5–10 γ-H2AX foci. Data were analyzed from three independent counts each with 100 cells and represented as mean ± s.d., ***$p < 0.0001$ using the student's t-test. (C) Representative western blot analysis of whole cell lysates obtained from U87MG cells silenced for LSD1 (left panel) and treated with 1 mM TCP for 24 h (right panel). Actinin antibody was used as loading control.

treatment may also affect LSD2 (KDM1B-AOF1, the mammalian homolog of LSD1) function, we performed silencing of LSD2 to evaluate its relative contribution on TCP-induced senescence. U87MG cells were silenced using a specific siRNA against LSD2 (Fig. 3C) and results shown in Fig. 3D show a very low change in β-galactosidase activity, confirming the specificity of LSD1 inhibition in inducing senescence. To further confirm our findings, we assayed two other GBM cell lines, T98G (PTEN +/+, p53 mutant) and U251 (PTEN −/−). We found that in both cell lines inhibition of LSD1 induces senescence (Supplementary Fig. 1A) and DDR (Supplementary Fig. 1B) at levels comparable to CPT treatment. Collectively, these findings demonstrated that either LSD1 pharmacological inhibition or knockdown trigger cellular senescence in GBM cells.

3.3. LSD1 regulates mTORC1 activity and mitochondria oxidative capacity in Glioblastoma cells

Senescent cells are metabolically active. In contrast to tumor cells, which primarily rely on glycolysis to produce energy, even in normoxic condition, senescent cells can exhibit hyperactive mitochondrial respiration [6]. Thus, we analyzed the extracellular acidification rate (ECAR), an indicator of glycolysis, and oxygen consumption rate (OCR), an indicator of oxidative phosphorylation in GBM cells following LSD1 inhibition. Surprisingly, we observed that TCP-treatment did not affect glycolysis in terms of basal ECAR, maximal ECAR and glycolytic capacity, (Fig. 4A,B). On the contrary, LSD1 inhibition severely affected mitochondrial oxidative capacity. Indeed we observed a significant reduction of mitochondrial respiration in terms of basal OCR, maximal OCR and ATP-linked OCR compared to untreated controls (Fig. 4C,D). To confirm results obtained with ECAR and OCR, we analyzed a subset of proteins involved in both glycolysis and mitochondrial respiration. As shown in Supplementary Fig. 2, protein levels of enzymes implicated in glycolysis remained unchanged in presence of TCP, conversely the actors of mitochondrial respiration strongly decreased upon treatment.

mTORC1 has been associated to mitochondrial activity and biogenesis as a key regulator of synthesis of nucleus-encoded mitochondrial proteins via 4E-BPs [41]. mTOR inhibition has been demonstrated to suppress respiration and to down-regulate TCA cycle activity and

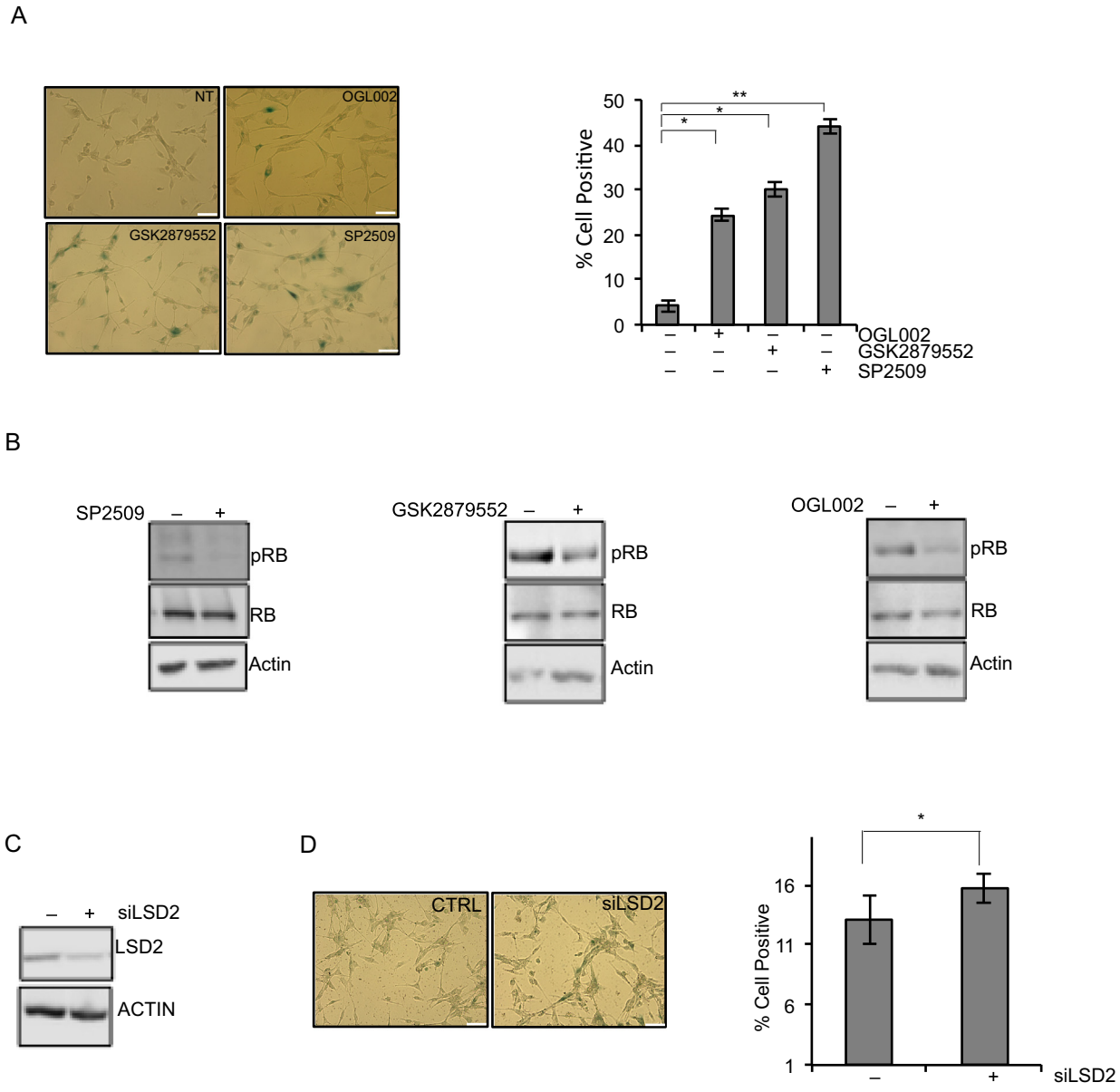


Fig. 3. LSD1, but not LSD2, inhibition activates cell senescence. (A,D) U87MG cells were treated with OG-L002(50 nM), SP2509 (1 μM), GSK2879552 (2,5 μM), or transfected with specific siLSD2 (100 nM) for 48 h, then fixed and analyzed for SA-βgal assay. Graphs represent number of SA-βgal positive cells. Data were analyzed from three independent counts each with 100 cells and represented as mean ± s.d., $^{**}p < 0.001$ using the student's t-test. (B,C) U87MG cells treated with OG-L002, SP2509, GSK2879552 (B) and silenced for LSD2 (C) were analyzed by Western blot. Actin antibody was used as loading control. Scale bars 75 μm.

ATP production capacity in proliferating cells [41]. In view of recent studies showing that LSD1 is associated with mTORC1 activity regulation [42], we sought to determine mTORC1 activity following LSD1 inhibition in U87MG cells. As readout of mTORC1 activity, we monitored phosphorylation of its protein targets in response to LSD1 inhibition. Protein extracts were prepared at the indicated times and probed with antibodies recognizing phosphorylated and total protein forms of mTORC1 substrates. In agreement with recent findings showing that LSD1 is associated with the regulation of mTORC1 activity, phosphorylation levels of 4-EBP1, p70S6K and consequently of its target rpS6, were down regulated in TCP-treated U87MG cells (Fig. 4E). Our result highlighted that LSD1 inhibition impairs mitochondrial respiration and a deregulation of mTOR signaling. To investigate the direct impact on mitochondrial respiration of mTOR in these conditions, we performed siRNA-mediated TSC2 silencing to constitutively activate mTORC1 (Supplementary Fig. 3A). As shown in Supplementary Fig. 3B our results indicate that TSC2 silencing partially rescues the impact on mitochondrial respiration of TCP treatment, suggesting that such effect depends, in part, on the Rheb/TSC2/mTORC1 axis. However, we cannot exclude other molecular mechanisms acting in this process.

All together our results indicate that TCP treatment results in mTORC1 inhibition and profoundly impacts the bio-energetic profile of GBM cells.

3.4. LSD1-HIF-1α pathway regulates senescence in Glioblastoma cells

In order to identify a potential mechanism that may explain how LSD1 inhibition induces senescence, we considered senescence-related genes that were previously identified as LSD1 targets. Among them, HIF-1α has been described to correlate with resistance to premature senescence and recent studies suggest that HIF-1α silencing suffices to promote cell senescent [35]. HIF-1α protein stability is increased by LSD1-mediated demethylation [25,32,33]. For these reasons, HIF-1α

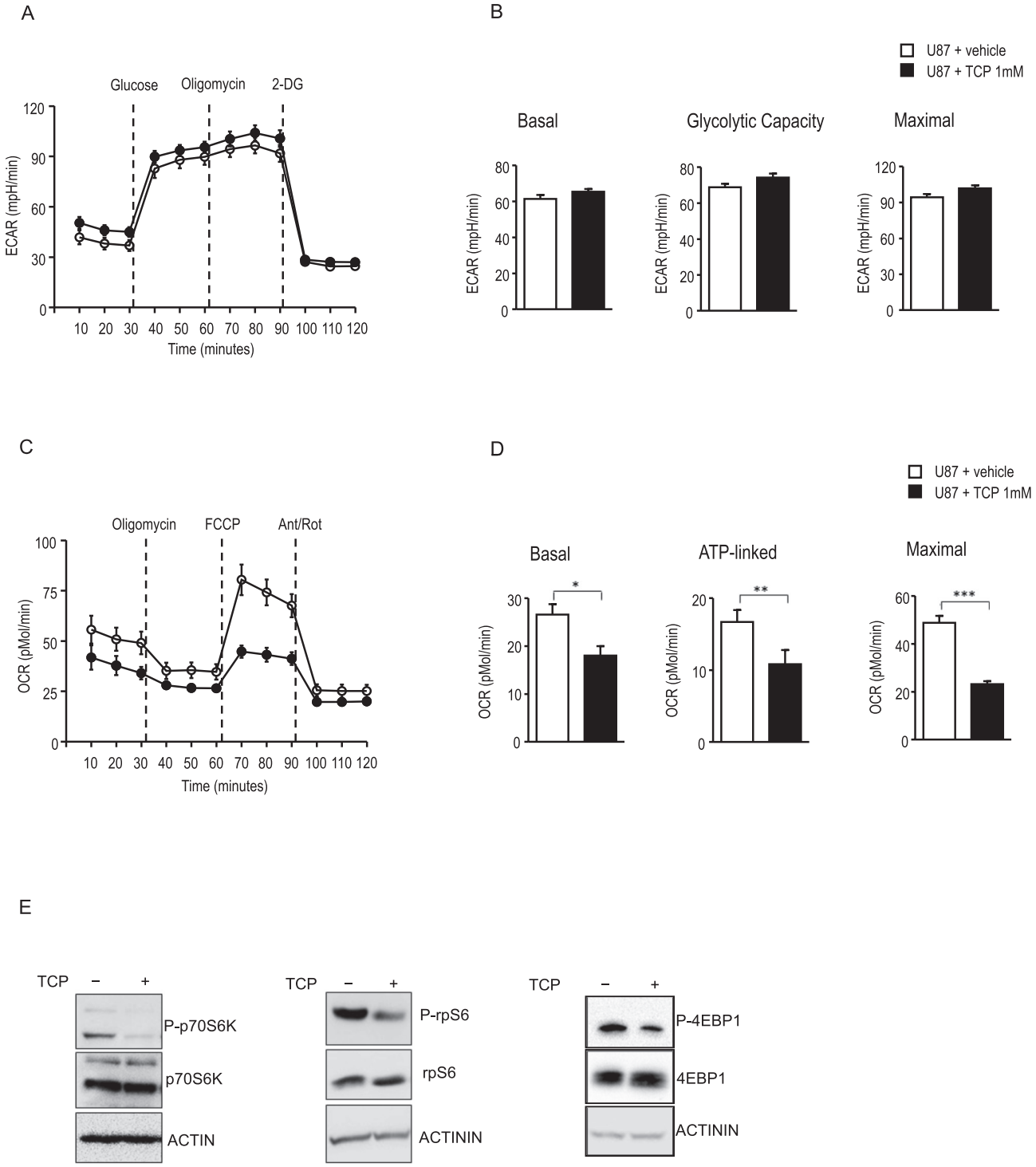


Fig. 4. Exposure to TCP modified GBM cells energetic metabolism. (A) Kinetic profile of ECAR in GBM cells treated or not with TCP 1 mM for 12 h. The data are shown as mean ± S.E.M. of five independent experiments. ECAR was measured in real time, under basal conditions and in response to glucose, oligomycin and 2-DG; (B) Parameters of glycolysis in GBM cells were calculated as detailed in materials and methods. Data are expressed as mean ± S.E.M. of three measurements, from five independent experiments; Statistical differences were evaluated using the Wilcoxon matched-pairs test ($p > 0.01$). (C) Kinetic profile of OCR in GBM cells treated or not with TCP 1 mM for 12 h. The data are shown as mean ± S.E.M. of five independent experiments. OCR was measured in real time, under basal conditions and in response to oligomycin, FCCP and Antimycin A + Rotenone. (D) Parameters of mitochondrial respiration in GBM cells were calculated as detailed in materials and methods. Data are expressed as mean ± S.E.M. of three measurements, from five independent experiments. Statistical differences were evaluated using the Wilcoxon matched-pairs test. ($*p < 0.01$, $**p < 0.001$, $***p < 0.0001$). (E) Western blotting of protein extracts from cells treated with TCP and probed with the indicated antibodies. Actinin or actin was used as loading control.

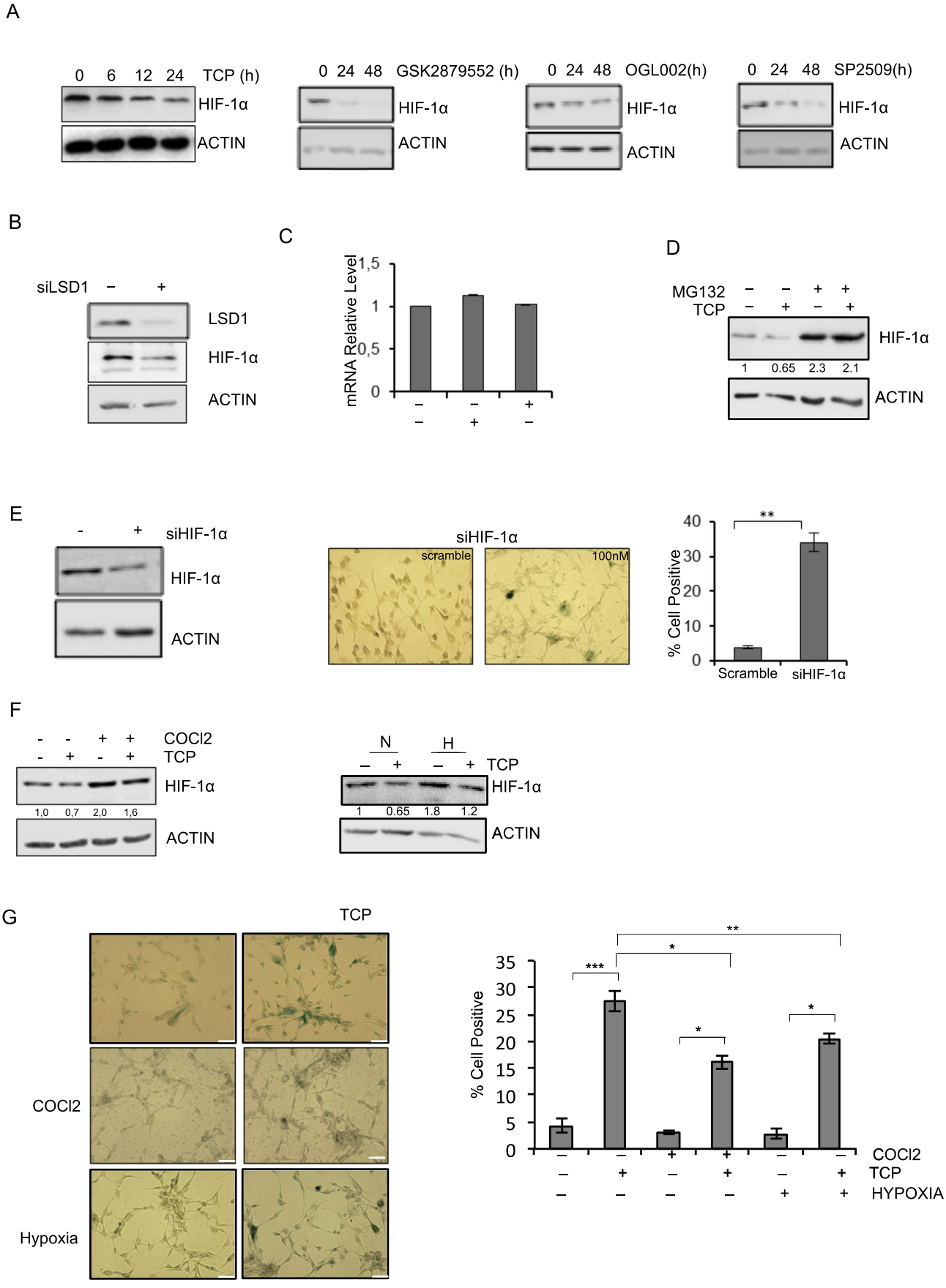


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Fig. 5. LSD1-mediated HIF-1α inhibition leads to senescence activation. (A) U87MG treated with 1 mM TCP, OG-L002(50 nM), SP2509 (1 μM),GSK2879552 (2,5 μM), and (B) silenced for LSD1, were analyzed for HIF-1α protein level by western blotting. (C) HIF-1α mRNA, in presence or absence of TCP and LSD1 silencing, were evaluated by qPCR. Bars represent the average of three independent experiments (*$p < 0.01$). (D) U87MG cells were treated with TCP (1 mM) and MG132 (1 μM) for 24 h and HIF-1α protein expression was monitored by western blotting. (E) U87MG cells were transfected with siHIF-1α (100 nM) and collected 48 h upon transfection. HIF-1α expression was analyzed by western blot. U87MG cells silenced for HIF-1α or scramble were fixed and incubated with SA-βgal solution for 24 h. Graphs represent the number of β-gal positive cells. Data were analyzed from three independent counts each with 100 cells and are represented as mean ± s.d., ***$p < 0.0001$ using the student's t-test. (F) U87MG cells were treated with TCP 1 mM in presence or absence of $COCl_2$, and in hypoxic condition, HIF-1α expression was analyzed by western blot while senescence was analyzed for SA-βgal assay (G), as indicated. Graphs represent the number of βgal positive cells. Data were analyzed from three independent counts each with 100 cells and are represented as mean ± s.d., ***$p < 0.0001$ using the student's t-test. Scale bars 75 μm.

was considered to be a potential link between LSD1 inhibition and senescence activation in GBM cells.

To verify our hypothesis, we examined HIF-1α expression level in response to LSD1 silencing. As shown in Fig. 5A, HIF-1α reduction over time was observed in TCP, GSK2879552, OG-L002, SP2509 treated cells; similarly, LSD1 silencing effectively reduces HIF-1α protein levels (Fig. 5B), while no obvious effect on HIF-1α mRNA level is observed (Fig. 5C), suggesting that transcription regulation might not account for the reduced HIF-1α expression following LSD1 inhibition.

To gain insights into the mechanism underlying the regulation of HIF-1α expression by LSD1, we next examined whether TCP-induced HIF-1α reduction in GBM cells is due to proteasome-mediated degradation. We found that treatment with the proteasome inhibitor MG132 stabilizes HIF-1α protein in U87MG cells, suggesting that the proteasome/ubiquitination pathway degrades HIF-1α in response to LSD1 inhibition (Fig. 5D).

To address if senescence was affected after HIF-1α silencing we used a SA-βgal assay. U87MG cells were transfected with a specific siRNA against HIF-1α, and results compared to those obtained in scramble-siRNA transfected cells (Fig. 5E). As shown, senescence is activated following HIF-1α silencing in U87MG cells.

To further confirm that LSD1 inhibition affects senescence through HIF-1α degradation also in hypoxic conditions, experiments were done incubating U87MG cells in a humidified hypoxic workstation or using the hypoxia-mimetic agent, $CoCl_2$. As shown in Fig. 5F, HIF-1α was stabilized during hypoxia in presence or absence of TCP. HIF-1α stabilization reduced SA-βgal accumulation in U87MG cells upon TCP treatment (Fig. 5G), indicating that the induction of senescence caused by LSD1-inhibiition was via HIF-1α degradation.

Collectively, our findings demonstrated that LSD1-mediated decrease of HIF-1α protein levels activates senescence in GBM cells.

3.5. TCP treatment inhibits the HIF-1α mediated adaptation to hypoxia in Glioblastoma cells

In the hypoxic microenvironment of necrotic areas of the solid tumor, HIF-1α accumulates and activates transcription of genes involved in hypoxic adaptation, promoting angiogenesis and tumor survival [43]. Consistently, a boxplot of HIF-1α expression in GBM clinical patients indicate that the HIF-1α gene is expressed at higher levels in primary GBM compared to normal samples (Fig. 6A). Hypoxia has been reported to enhance mesenchymal transition, facilitating the invasive behavior; recent reports indicated that HIF-1α mediates the hypoxia-mediated mesenchymal shift in GBM [44–46], suggesting that HIFs represent a potential therapeutic target for mesenchymal GBM cells. Then, we examined the effect of TCP on the HIF-1α-dependent increase of migration/invasion in U87MG cells. To verify whether LSD1 inhibition would decrease HIF-1α-mediated invasive potential, we performed migration assays in presence or absence of $CoCl_2$, to induce HIF-1α accumulation. As expected, $CoCl_2$ treatment (Fig. 6B,C) significantly enhanced the migration capabilities of U87MG, while TCP treated cells displayed a significant decrease in invasion and migration of U87MG cells, in both normal and hypoxia condition, as shown by wound healing and trans-well assays. Similar results were obtained in hypoxic condition (Supplementary Fig. 4A and B).

To examine whether LSD1 inhibition could effectively inhibit HIF-1α functions, we analyze expression level of three well-characterized HIF-1α target genes, the Vascular endothelial growth factor A (VEGFα), Plasminogen activator inhibitor-1 (PAI-1) and Carbonic Anhydrase 9 (CAIX) in presence or absence of TCP. Expression of these genes is stimulated by $CoCl_2$ administration (Fig. 6D) and hypoxia (Supplementary Fig. 4C). In contrast, TCP treatment compromised their induction in response to hypoxia, indicating that LSD1 inhibition prevents the hypoxia-mediated HIF-1α transcription program, while, TCP does not have strong effect in normoxia condition. Collectively these results indicate that TCP treatment effectively inhibits the HIF-1α-driven adaptation to hypoxia in GBM cells.

4. Discussion

LSD1 is involved in several biological processes, such as cell proliferation [47], epithelial-mesenchymal transition [42,48], pluripotency and stem cell differentiation [49]. Here we report that pharmacological or genetic inhibition of LSD1 induces senescence and reduces proliferation and migration through the regulation HIF-1α protein level.

HIF-1α regulates genes that play key roles in cancer-related process, such as proliferation, angiogenesis, apoptosis/autophagy, metabolism, cell migration and invasion [50–52]. Several studies reported a significant relationship between poor prognosis and HIF-1α overexpression in glioma patients and HIF-1α is considered an attractive target for GBM therapy [53,54]. Indeed HIF-1α targeting has been proposed in combination with radiation therapy for GBM treatment [55].

Our results suggest that, in GBM cell lines, high levels of LSD1 participate to the tumorigenic aggressive phenotype through HIF-1α stabilization; LSD1 inhibition negatively regulates HIF-1α protein levels, induces senescence and impairs cell migration capabilities under normoxic and hypoxia conditions. Thus, LSD1 ablation inhibits HIF-1α-driven adaptation to hypoxia in GBM cells.

GBM is frequently accompanied by the activation of the phosphatidylinositol 3-kinase (PI3K)/Akt/rapamycin-sensitive mTOR-complex (mTOR) pathway, with the majority of tumors displaying over-expression of the EGFRvIII variant and loss of PTEN [56]. Given the key role of mTORC1 in proliferation and metabolism [57], its aberrant activation contributes to tumor growth, angiogenesis and metastasis [57,58]. We found that LSD1 inhibition hampers the mTORC1 activity in GBM cells and such effect is associated with mitochondrial respiration impairment. Although high glycolysis is a hallmark of cancer, glycolytic cells also rely on mitochondrial intermediates to generate molecules required for tumor growth [59]. Moreover, it has been reported that cancer cells can use OXPHOS during tumor progression or under limiting glucose conditions [60,61]; thus, against Warburg's proposal, an active OXPHOS could be more advantageous for tumors than a completely glycolytic type of metabolism, which suggests the possibility of targeting mitochondria to alter tumor metabolic adaptation and progression [62,63].

HIF-1α regulation is not the only mechanism that connects inhibition of LSD1 and senescence. Telomere shortening and DNA damage lead to cellular senescent [10,64,65] and both these processes are regulated by LSD1 [66,67]. Moreover, Yu et al. show that two different types of H3K9 demethylases, LSD1 and JMJD2C, disable oncogenic-

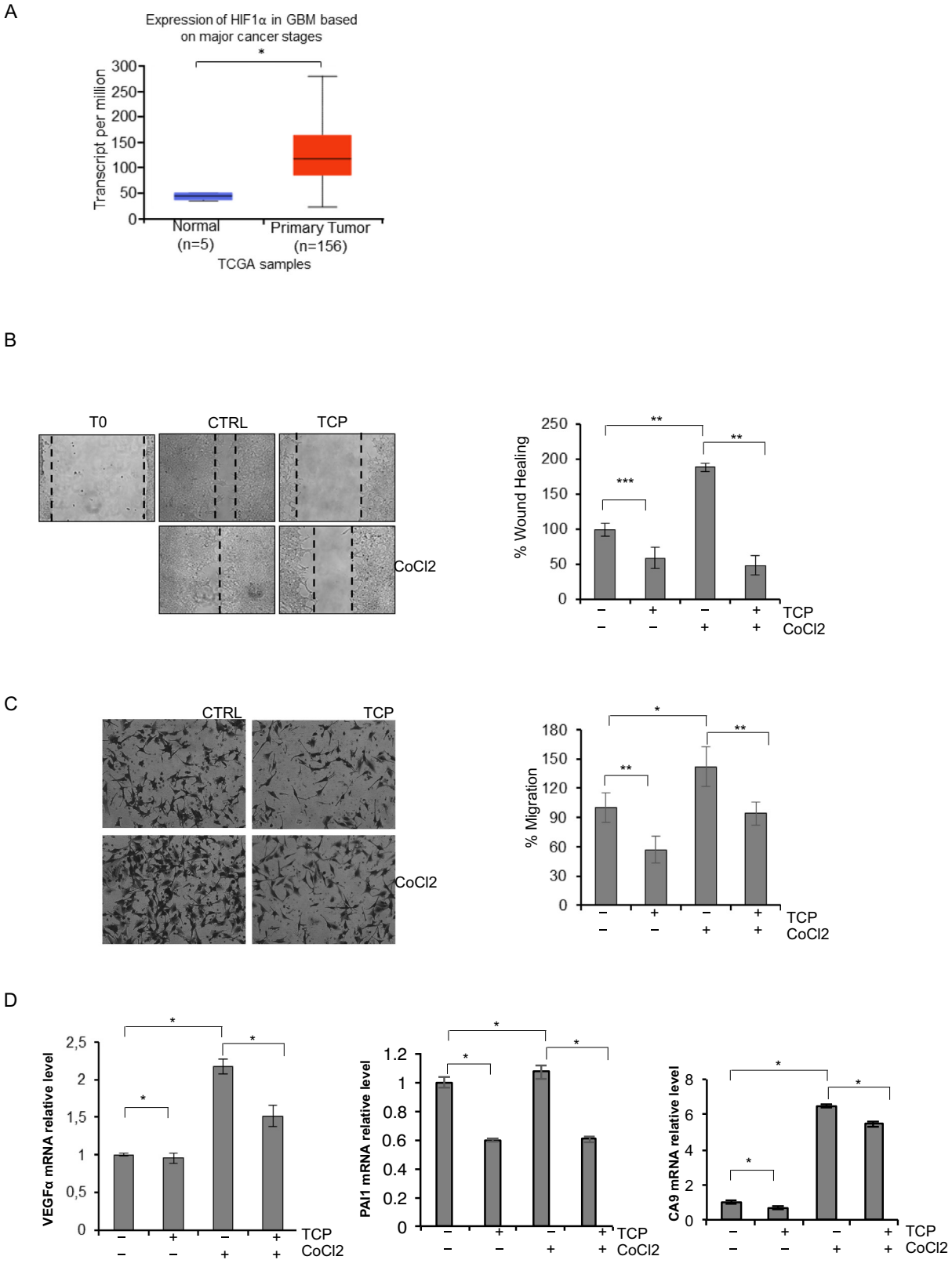

Fig. 6. LSD1-mediated inhibition of HIF-1α reduces migration of GBM cells. (A) Boxplot showing relative expression of HIF-1α in normal and GBM patients. Database utilized is UALCAN, statistical analysis student's t-test. $p = 1.78 \times 10^{-3}$ (B). U87MG cells were treated with 1 mM TCP and $COCl_2$ for 24 h before scratch wound assay (C). Cells was assessed using Trans-membrane migration assay. Representative phase contrast images were shown. Graphs in (B) and (C) show results representing means ± SD of three independent experiments carried out in duplicate. (D) qPCR for VEGF-α, PAI-1 and CAIX expression in U87MG cells treated for 24 h as indicated. Bars represent the average of three independent experiments (*$p < 0.01$).

induced senescence by enabling the expression of E2F target genes [68]. Finally, inactivation of LSD1 has been shown to boost senescence in trophoblast stem cells by induction of Sirt4 [14]. Thus, LSD1 appears to be a regulatory hub that controls different aspects of cellular senescent, metabolic pathways and cancer.

Senescence induction in cancers may function as a powerful weapon for eradicating tumorigenesis [69]. Therapies that enhance senescence not only promote a stable arrest of cell growth, but also act as a strong stimulus for the activation of the antitumor immune response [70].

Collectively our results have important implications for the use of drugs that target chromatin and epigenetic regulators for GBM cancer therapy and inhibition of LSD1 can be exploited in the future as adjuvant for GBM therapy.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbagrm.2019.03.004.

Transparency document

The Transparency document associated this article can be found, in online version.

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Conflict of interest

The authors declare no conflict of interest.

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CHAPTER 5

Conclusion

Brain tumors still remain a clinical challenge despite the progresses in tumor therapy(63,64). They are classified in primary tumors, which originate from native cells, and secondary, which arise from metastatic cells(65). Primary tumors can be classified based on the their original tissue.(64). The tumors arising inside the brain are called glioma; when they affect the membranes, they are called meningiomas; in case they strike the nerves, they take the name of "nerve sheath". Neuroblastoma is a solid extra-cranical tumor where the localization changes according to the patient's age(66).

Epigenetic enzymes are promising targets for cancer therapy due to their involvement in cellular processes leading to oncogenesis; accordingly, a number of epi-drugs are currently under investigation(67,68).

Lysine-specific demethylase 1 (LSD1) removes mono- and di-methylated groups from lysines 4 or 9 on histone H3, as well as non-histone protein targets, via a flavin adenine dinucleotide (FAD)-dependent oxidative reaction(69). Notably, high-levels of LSD1 expression are hallmarks of several human solid cancers and leukemia(32,52,61,70–74).

LSD1 overexpression is involved in different aspects of tumour cell biology including cellular proliferation and growth(69,73), altered cellular metabolism(75–77), cellular senescence (78–80)and differentiation blockage.(33,61,78) Additionally, LSD1 orchestrates modifications taking place in the tumour microenvironment through regulation of gene targets, activation of angiogenesis and suppression of the host immune response(52,81).

In brain tumours, LSD1 is over-expressed and it is correlated with aggressive disease, suggesting that its inhibition might be considered as therapeutic target. For these reasons, our work has been focused on understanding the role of LSD1, and on uncovering its potential as a new potential therapeutic agent for neuroblastoma (NB) and glioblastoma (GBM).

We have demonstrated by investigation on different brain tumors cellular models that LSD1 depletion is involved in three tumour-associated pathways: epithelial-mesenchymal-transition(38,52,61), autophagy(55) and senescence(78).

1) We found a novel role of LSD1 in cellular migration and invasiveness. In particular, LSD1 contributes to the genetic reprogramming that underlies to Epithelial-Mesenchymal Transition (EMT) and tumor metastasis. EMT consists in the loss of epithelial polarity and the achievement of a mesenchymal morphology(82). We demonstrated that LSD1 affects motility and invasiveness of NB cells by modulating the transcription of the metastasis suppressor NDRG1 (N-Myc Downstream-Regulated Gene 1). We shown that LSD1 co-localizes with MYCN at the promoter region of the NDRG1 gene and inhibits its expression. Mcdonald et al. demonstrated that epigenetic modifications largely depend on LSD1, and that loss of LSD1 functions affects EMT-driven cell migration and chemo-resistance(83). Taking in consideration different studies, it is clear that LSD1 may function as enhancer or inhibitor of EMT in a cell type-specific fashion, based on the cells' genetic background and depending on its interacting partners(84). Two independent reports demonstrated that LSD1 physically associates with SNAIL1 (snail family transcriptional repressor 1) in breast cancer cells (74,85,86). The members of the SNAIL family of zinc finger transcription factors (Snail, Slug and Smuc) control the invasive phenotype and metastastatic potential of several types of cancers. In the EMT pathway, SNAIL family proteins repress the expression of epithelial genes, such as E-cadherin (CDH1), through an LSD1-dependent molecular mechanism. It has been shown that LSD1, interacting with Snail, leads to CDH1 repression (74,86). Although the majority of work indicates that LSD1, in cooperation with other proteins such as the NuRD complex, is a key positive regulator of the EMT program, it could also promote opposite effects. It has been reported that the LSD1/NuRD complex inhibits TGF-β

signaling pathway and reduces breast cancer metastatic potential(87). Thus results obtained and published in manuscript described in chapter 2, demonstrate that pharmacological inhibition of LSD1 relieves repression of NDRG1 by MYCN and affects motility and invasiveness of NB cells.

2) We also demonstrated that LSD1 regulates autophagy through the impairment of the mammalian target of rapamycin complex 1 (mTORC1) pathway. Autophagy is a conserved intracellular process important for recycling of macromolecules and defective autophagy is associated with tumorigenesis(88). mTORC1 has different roles and is involved in translational control, metabolism, proliferation and tumorigenesis(89). Proteins involved in mTORC1 signaling are frequently altered in cancers. In detail, we found that LSD1 is a negative regulator of Sestrin2 (SESN2), whose high levels negatively regulate mTORC1 through the GATOR complex via interaction with GATOR2, thus activating autophagy(90). These results underline the key role of SESN2 in the autophagy dependent LSD1 inhibition. Different studies reinforced this result. For example, knockdown of LSD1 ameliorated Ox-LDL-stimulated NLRP3 activation and inflammation by promoting autophagy via SESN2-mediated PI3K/Akt/mTOR pathway in Atherosclerosis(91). Also, Wei and colleagues indicated that LSD1 may function as a driving factor of ovarian cancer progression via deregulating autophagy(92). Thus results obtained and published in manuscript described in chapter 3, demonstrate that LSD1 controls autophagy in neuroblastoma cells through SESN2 transcription regulation, and we suggest that pharmacological targeting of LSD1 may have effective therapeutic relevance in the control of autophagy in Neuroblastoma.

3) Recently in GBM cells, we also demonstrated that LSD1 inhibition reduces proliferation and migration, and activates senescence through the regulation of HIF-1α protein levels, under normoxic and hypoxia

conditions. Different studies reported a connection between poor prognosis and high level of HIF-1α in glioma patients and for this reason HIF-1α is considered a possible target in GBM therapy(93,94). HIF-1α regulation is not the only mechanism connecting the inhibition of LSD1 with senescence. Other processes regulated by LSD1 are Telomere shortening(50) and DNA damage (95) and have both been linked with cellular senescence. In cancer, Senescence activation may function as a powerful weapon for tumorigenesis eradication (96). Therapies that induce senescence do not only promote a stable arrest of cell growth, but also act as a stimulus for the activation of antitumor immune response. Moreover, Yu et al. showed that two different types of H3K9 demethylases, LSD1 and JMJD2C, are Abe to disable oncogenic induced senescence by enabling the expression of E2F target genes (80). Finally, inactivation of LSD1 has been shown to boost senescence in trophoblast stem cells by induction of Sirt4(79). Therefore, LSD1 appears to be a regulatory hub that controls different aspects of cellular senescence, metabolic pathways and cancer. Thus results obtained and published in manuscript chapter 4, elucidate a mechanism whereby LSD1 controls senescence in Glioblastoma tumor cells through the regulation of HIF-1α, and we propose the novel defined LSD1/HIF-1α axis as a new target for the therapy of Glioblastoma tumors.

Collectively LSD1 has an important role in the regulation of processes underlying cancer progression, such as senescence, autophagy and EMT, that are known to be linked but interconnection is not fully elucidated.

Autophagy appears to act as an anti-senescence mechanism by maintaining homeostasis in normal or stress-induced conditions. However, its activation timings could be a differential factor for "selective autophagy", i.e. autophagic processes where only some substrate are degradated(97–99).For example, GATA4's p62-dependent selective autophagy acts as an anti-senescence mechanism (100), however the LC3-lamin B1 selective autophagy of the nuclear lamina acts as a pro-senescence mechanism (101).

On the other hand the effects of autophagy on EMT seems to be closely dependent on the cell type and the stimulus used to activate or inhibit autophagy(102). Precious studies on this topic, revealed, an articulated dialogue between autophagy and EMT processes(103). In particular, in the early stages of metastatization, autophagy acts as a onco-suppressive signal, tending to inhibit the EMT program by destabilizing its main actors. Subsequently, metastatic cells may require prolonged autophagy to survive the encountered environmental and metabolic stress conditions (102,103).

LSD1 has high expression levels in different tumors with poor prognosis such as prostate, lung, brain, breast, hematological neoplasia and it has been proposed as therapeutic target in cancer(32,52,61,70–74). Therefore, different LSD1 inhibitors are actually in clinical trials(104). It is plausible that LSD1, through interaction with different factors, can exert distinct molecular mechanisms in several types of cancer. Since autophagy(55), cellular senescence(78) and EMT(38,52,61) play critical roles in cancer, our efforts will help define a potential new therapeutic tool to fight cancers and eventually enhance human health. The link between autophagy, EMT and senescence needs to be further clarified, but our results on the central role of LSD1 in these three processes are promising. In conclusion, we propose that pharmacological targeting of LSD1 by small molecules could modulate autophagy, senescence, migration capability and invasiveness of cancer cells through target protein derepression, and thus impairing the ability of cancers to metastasize. Thus, in a context in which drugs directed against chromatin and epigenetic regulators are increasingly used in tumor therapy, our results show that the inhibition of LSD1 in the future can be used as promising adjuvant in cancer therapies.

CHAPTER 6
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