Inhibition of the macrophage demethylase LSD1 reverses *Leishmania amazonensis*-induced transcriptomic changes and causes a decrease in parasite load

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ABSTRACT

Intracellular pathogens exploit host cell functions to favor their own survival. In recent years, the subversion of epigenetic regulation has emerged as a key microbial strategy to modify host cell gene expression and evade antimicrobial immune responses. Using the protozoan parasite *Leishmania* as a model system, we have recently demonstrated that infection causes histone H3 hypomethylation, which is associated with the establishment of an anti-inflammatory phenotype, suggesting that host cell demethylases may play a role in the intracellular survival of these parasites. In this study, we employed a combination of pharmacological, RNA sequencing and interaction studies to investigate the role of the macrophage lysine demethylase LSD1 (KDM1a) in *Leishmania* intracellular infection *in vitro*. Treatment of infected macrophages with two validated LSD1-specific inhibitors resulted in a significant reduction in parasite burden. We confirmed the impact of these inhibitors on LSD1 activity within macrophage nuclear extracts using an *in vitro* demethylase assay and established their LSD1 target engagement *in situ* by cellular thermal shift assay. RNA-seq analysis of infected and inhibitor-treated macrophages linked parasite killing to a partial reversion of infection-dependent expression changes, restoring the macrophage anti-microbial response and limiting cholesterol biosynthesis. While we ruled out any impact of *Leishmania* on LSD1 expression or localization, we uncovered significant alterations in LSD1 complex formation within infected macrophages, involving unique interactions with host proteins as well as interactions with *Leishmania* proteins that appear to be secreted into the macrophage nucleus. Our study sheds important new light on the epigenetic mechanisms of macrophage immuno-metabolic subversion by intracellular *Leishmania* and identifies LSD1 as a potential candidate for host-directed, anti-leishmanial therapy.

INTRODUCTION

Many viral, bacterial and eukaryotic pathogens infect mammalian cells and have co-evolved strategies to modulate the gene expression profiles of their host cells and promote their intracellular survival (Benoit et al., 2008; Herbein and Varin, 2010; Herbert et al., 2004; Kamhawi and Serafim, 2020; Noel et al., 2004; Pearce and MacDonald, 2002; Raes et al., 2007). This co-evolution is well illustrated for the protozoan parasites of the genus *Leishmania* that proliferate inside the sand fly insect vector as motile promastigotes and as non-motile amastigotes inside fully acidified phagolysosomes of mammalian macrophages. *Leishmania* subverts the immunological and metabolic functions of these important and highly toxic immune cells by interfering with cell signaling (Gregory and Olivier, 2005), metabolism (Rabhi et al., 2012) or epigenetic control (Afrin et al., 2019; Kamhawi and Serafim, 2020). The host consequences of *Leishmania* immune subversion are often devastating causing severe immune pathologies termed 'leishmaniases' that range from self-healing cutaneous to lethal visceral forms (Conceicao-Silva and Morgado, 2019; McGwire and Satoskar, 2014).

Despite the severe consequences to human health, the mechanisms underlying the subversion of macrophage functions by intracellular *Leishmania* remain poorly understood. Epigenetic control of host immuno-metabolic processes in infected macrophages and dendritic cells has recently emerged as a new parasite strategy for host cell subversion (Kamhawi and Serafim, 2020; Lecoeur et al., 2022). First, *Leishmania* has been shown to manipulate host cell miRNAs to limit pro-inflammatory gene expression (Ganguly et al., 2022; Lemaire et al., 2013; Mukherjee et al., 2015; Muxel et al., 2018; Nandan et al., 2021; Tiwari et al., 2017). Second, intracellular *Leishmania* infection has been correlated to changes in the macrophage DNA methylation pattern, influencing host cell signalling pathways, oxidative phosphorylation, and cell adhesion (Marr et al., 2014). Finally, we and others have shown that *Leishmania* infections

alters host cell histone modification, such as acetylation or methylation, whose levels are dynamically regulated by writer enzymes (e.g. acetyltransferases, methyltransferases) and eraser enzymes (i.e. deacetylases, demethylases). Our recent work has established a first link between a macrophage epigenetic eraser activity and *Leishmania* infection: applying a ChIPqPCR approach we provided first evidence that *L. amazonensis* infection causes hypo-methylation at pro-inflammatory gene promoters at activating histone H3K4 and repressing H3K9 marks, which correlated with a corresponding decrease in transcript output (Kamhawi and Serafim, 2020; Lecoeur et al., 2020). These H3 modifications are both removed by the Lysine-Specific Demethylase 1 (LSD1, a.k.a. KDM1a) (Shi et al., 2004), which in accordance to a given lysine mark can increase or decrease gene expression.

LSD1 is composed of three domains: the catalytic AO domain, the Swi3/Rcs8/Moira (SWIRM) domain and the Tower domain (Burg et al., 2015). Aside its enzymatic function, it carries important scaffolding functions via the Tower domain that serves as binding site for the CoREST transcription repressor complex (essential for LSD1 to associate with the nucleosome and exert its demethylase activity) and the SWIRM domain that functions as a platform for protein-protein interactions (Burg et al., 2015; Laurent and Shi, 2016). LSD1 is involved in diverse biological processes including differentiation (Whyte et al., 2012), autophagy (Ambrosio et al., 2019) or metabolic processes (Sakamoto et al., 2015). Conceivably, deregulation of LSD1 functions cause severe human diseases, including cancer (Majello et al., 2019) and, as a consequence, represents one of the prime therapeutic targets, with various compounds undergoing clinical testing for treating small lung cancer cells (SCLC) or acute myeloid leukaemia (Fang et al., 2019). Surprisingly, despite the availability of LSD1-specific inhibitors and the known role of role of LSD1 to control macrophage polarization, its potential as a host-directed target for anti-microbial intervention has not been explored yet, except for viral infections (Mazzarella et al., 2021; Zwergel et al., 2018). We previously proposed an

experimental framework for the discovery of host-directed inhibitors targeting macrophage histone-modifying enzymes (Lamotte et al., 2019; Lamotte et al., 2017) for which large compound libraries are available given current efforts to cure non-communicable human diseases such as cancer, obesity, auto-immunity by restoring a healthy epigenetic profile (Hogg et al., 2020; Nebbioso et al., 2018).

Here, we established a first proof-of-principle analysis for host-directed therapy by exploring the possibility to target LSD1 for anti-leishmanial drug discovery. Screening a diverse epigenetic inhibitor library against intracellular and extracellular *L. amazonensis* parasites identified a series of anti-parasitic compounds designed to target mammalian LSD1 (https://cordis.europa.eu/project/id/602080/fr). We demonstrate that LSD1 inhibition partially reverts infection-induced expression changes that are likely incompatible with robust parasite survival and growth, and provide first evidence that *L. amazonensis* subverts the LSD1 scaffolding functions to establish a macrophage phenotype permissive for intracellular infection.

MATERIAL AND METHODS

Ethics statement

Six-week-old female C57BL/6 and Swiss *nu/nu* mice were purchased from Janvier (Saint Germain-sur-l'Arbresle, France). All animals were housed in A3 animal facilities according to the guidelines of Institut Pasteur and the "Comité d'Ethique pour l'Expérimentation Animale" (CEEA) and protocols were approved by the "Ministère de l'Enseignement Supérieur, Direction Générale pour la Recherche et l'Innovation" under number 2013-0047. EP is authorized to perform experiments on vertebrate animals (license 75–1265) issued by the ''Direction Départementale de la Protection des Populations de Paris'' and is responsible for all the experiments conducted personally or under his supervision as governed by the laws and regulations relating to the protection of animals.

Bone marrow-derived macrophages and cell line cultures

Bone marrow cell suspensions were recovered from tibias and femurs of female C57Bl/6JRj mice in Dulbecco's phosphate buffered solution (PBS), and cultured in complete medium containing 4 g/L glucose, 1 mM pyruvate and 3.97 mM L-Alanyl-L-Glutamine and supplemented with 10% heat-inactivated fetal calf serum (FCS), streptomycin (50 µg/mL), and penicillin (50 IU/mL) (de La Llave et al., 2011). Briefly, one million cells per ml of complete medium supplemented with 50 ng/ml of mouse recombinant colony-stimulating factor 1 (rm-CSF1, ImmunoTools) were incubated in bacteriological Petri dishes (Greiner bio-one 664161) at 37°C in a 7.5% CO_2 atmosphere. After 6 days of culture, the medium was removed and adherent cells were incubated with pre-warmed PBS pH 7.4 containing 25 mM EDTA for 30 min at 37°C. Bone marrow-derived macrophages (BMDMs) were detached by gentle flushing,

collected, and resuspended in complete medium supplemented with 30 ng/ml of rmCSF-1. BMDMs were seeded in 100 mm tissue culture dishes (Petri BD Falcon, 353003) for nuclear or total protein extraction, and RNA isolation, and in 384-well tissue culture-treated flat-optically clear microplates (CellCarrier™ plate, PerkinElmer) for High Content Assay (HCA).

Parasite isolation and promastigote differentiation

mCherry-transgenic, tissue-derived amastigotes of *Leishmania amazonensis* strain LV79 (WHO reference number MPRO/BR/72/M1841) and *Leishmania donovani* strain 1S2D (WHO reference number MHOM/SD/62/1S-CL2D) were isolated from infected footpads of Swiss nude mice (Lecoeur et al., 2020) and infected spleen of golden Syrian hamsters, respectively. Promastigotes were differentiated from animal-derived amastigotes and maintained at 27°C in M199 medium supplemented with 10% FCS, 20 mM HEPES pH 6.9, 4 mM NaHCO3, 2 mM glutamine, 20 μM folic acid, 100 μM adenine, 13.7 μM hemin, 8 μM 6-biopterin, 100 U ml^{-1} of penicillin, and 100 μg ml^{-1} of streptomycin. Promastigotes were then maintained in culture by serial dilution in fresh medium once they reached stationary phase and used in Log growth phase for Dye Reduction Assay (DRA).

Chemical compounds

All small chemical compounds used in screening assays were part of the EU FP7 A-ParaDDisE project (https://cordis.europa.eu/project/id/602080/fr) and represent potential or validated epigenetic inhibitors. Inhibitors of lysine specific demethylase 1, including MC2652, MC4030 and Tranylcypromine (TCP) were synthesized by the laboratory of Antonello Mai (Facolta di Farmacia e Medicina, Sapienza Universita, Roma, Italy). All compounds were solubilized in DMSO at a 10 mM concentration and stored as aliquots at -20°C in 1.5 mL Eppendorf tubes.

Compounds targeting LSD1 (LSD1*i*) were used at different concentrations depending on the assay as indicated in Results.

Cell infection and treatment

After seeding at day 6 of differentiation, BMDMs were left for 5 hours at 37°C to attach to their new culture support. BMDMs were infected with lesion-derived *L. amazonensis* amastigotes at a multiplicity of infection (MOI) of 4 and then cultured at 34°C. LSD1*i* were added to uninfected and *Leishmania*- Infected Macrophages (LIMs) 24 hours after infection, at a final concentration of 10 µM (HCA, epifluorescence microscopy) or 15 µM (WB, CETSA).

High Content Assay

The High Content Assay (HCA) was used to assess the anti-leishmanial activity against intramacrophagic amastigotes of *L. amazonensis* (Lamotte et al., 2019). 425 compounds (A-ParaDDisE libraryhttps://cordis.europa.eu/project/id/602080/fr) were added one day after macrophage infection at 10 µM final concentration (1% DMSO final concentration). Controls included a vehicle control (DMSO), a leishmanicidal control (amphotericin B), and a macrophage cytotoxic control (cycloheximide). Each compound was tested in quadruplicates. Briefly, after 3 days of co-incubation with compounds, BMDM nuclei and parasitophorous vacuoles (PVs) were stained with Hoechst 33342 (Invitrogen Molecular Probes, 10 µM) and LysoTracker Green (Life Technologies, DND-26, 1 µM), respectively. Acquisition of images was performed on live cell cultures using the OPERA QEHS device with a 10x air objective (NA 0.4) using the green (488 nm), the blue (405 nm) and the red (561 nm) channels for the detection of PVs, nuclei and amastigotes, respectively (Lamotte et al., 2019). Images were transferred to the Columbus Conductor™ Database (Perkin Elmer Technologies) and analysed

using the integrated Image analysis building blocks (Lamotte et al., 2019). Quantitative values obtained for host cell and parasite numbers were exported to Excel and SigmaPlot for further analysis and graphical representations.

Nuclear and cytoplasmic protein extraction

For western blotting, LSD1 Activity Assay and LSD1 co-immunoprecipitation (co-IP) assay, nuclear and cytoplasmic extracts were obtained after 2 days of infection. The medium was removed from each dish, and cells were gently washed with PBS at room temperature (RT). PBS was then replaced by 1 ml of Lysis Buffer (LBI or LBII, depending on the subsequent procedure, Table S2).

After 10 min of incubation on ice, cells were detached with a cell scraper and collected in 2 ml Eppendorf tubes. The cell homogenate was passed 5 times through a 27 G needle to isolate nuclei. The quality of isolated nuclei was assessed by the EVOS Cell Imaging System and by Western blotting (Figure S6). Samples were centrifuged for 10 min at 4°C and i) 250 g for activity assay, western blot, or ii) 500 g for LSD1 co-IP assay. Supernatants containing cytoplasmic proteins were removed and kept for further analysis, whereas 150 µl of Extraction Buffer (EBI or EBII, depending on the subsequent procedure, supplementary table 2) were added to pellets. Tubes were vortexed and incubated for 10 min at 4°C. Sonication was performed using the Diagenode's Bioruptor® Standard for 5 min at high power (30" on/30" off sonication cycles). Extracts were cleared by centrifugation at 15,000g for 10 min at 4°C.

Cellular Thermal Shift Assay (CETSA)

Uninfected and infected BMDMs were treated or not at 1 day after infection with 10 µM LSD1*i* (MC2652, MC4030, and TCP) or 0.1% DMSO as control. After one day of treatment, cells

were washed with the pre-warmed medium before adding 1.5 ml of cold Extraction Buffer (1x PBS – 1x PIC – 1 mM PMSF). Then, cells were collected with a cell scraper in two 1.5 ml Eppendorf tubes and subjected to three cycles of freezing in liquid nitrogen and thawing in a thermomixer at 25°C with a 400 rpm agitation step. Lysates were submitted to 3 sonication cycles (30" on/30" off) at high power, then pooled in one Eppendorf tube per condition and centrifuged at 20,000 g for 20 min at 4°C.

Supernatants containing soluble proteins were transferred to 1.5 ml tubes, then distributed into twelve 0.2 ml PCR tubes and heated to different temperatures (from 37°C to 70°C, ΔT°=+3°C) in a Thermocycler (Applied ProFlex ™ 96 Well PCR System) for 3 min followed by cooling at 4°C. Afterwards, protein solutions were transferred to 1.5 ml Eppendorf tubes and cleared at 20,000 g for 20 min at 4°C. Carefully, 75 µL of supernatant containing the soluble protein fraction was collected in a new LoBind® Eppendorf tube.

The detection of the remaining soluble proteins was performed by Western Blot, where 11 µL of each cleared sample were mixed with 2 µL of NuPAGE® Reducing Agent and 5 µL of NuPAGE® LDS Sample Buffer. The samples for each CETSA melt curve were loaded on the same gel. The quantification was performed by measuring the fluorescence intensity emitted by the ECL Plex fluorescent secondary antibodies with the Amersham™ ImageQuant™ 800. Normalization was carried out with the Amersham ImageQuant TL analysis software considering the band intensity of the sample heated to 37°C as 100%. Melting curves were generated with GraphPad Prism software package.

Western blotting

Experiments were performed on 5-10 µg proteins. Proteins were resolved by SDS–PAGE (4–12% Bis-Tris NuPAGE gels) in MOPS buffer and electroblotted onto polyvinylidene difluoride

(PVDF) membranes, that were blocked with 2% Amersham ECL Prime blocking agent in 1x PBS containing 0.25% Tween 20. Membranes were then probed overnight at 4°C with the following primary antibodies: rabbit anti-LSD1 (C69G12, Cell Signaling Technology), rabbit anti-MAO-A (10539-1-AP, Proteintech), mouse anti-GAPDH (60004-1-Ig, Proteintech), rabbit anti-RCOR1 (27686-1-AP), mouse anti-YY1 (66281-1-Ig, Proteintech), rabbit anti-Histone-H3 (17168-1-AP, Proteintech).

Following incubation, either with Amersham ECL Plex Cy3 or Cy5 conjugated antibodies or with the appropriate peroxydase conjugate secondary antibodies (Thermo Scientific), fluorescence or luminescence (SuperSignal West Pico reagent, ThermoFisher Scientific) were measured in an Amersham ImageQuant 800 Imaging System (Cytiva). Relative protein expression was calculated by densitometric analysis using the Amersham ImageQuant analysis software. For every band, the ratio between the values obtained for the target protein and the YY1 (nuclear extracts) and the GAPDH (cytoplasmic extracts) normalization controls were calculated. Fold changes were calculated using the control sample as calibrator, control values of uninfected and unstimulated samples being set to 1. Modulations of the LSD1 protein content were statistically determined by the nonparametric Wilcoxon rank-sum test using the GraphPad Prism 7.03 software.

Determination of LSD1 localization by epifluorescence microscopy analysis

For intracellular LSD1 localisation, $2x10^5$ macrophages were seeded in 500 µL of complete medium per well of 24-well plates containing sterile glass coverslips, infected or not with *L. amazonensis* amastigotes with a MOI of 4 for one day, and treated with 10 µM LSD1*i* or DMSO. After 3 days of treatment (4 days of infection), cells were fixed in a 4% PFA solution for 30 min at RT. After a PBS wash (1 ml/well, 10 min, RT), quenching solution (50 mM ammonium chloride) was added to capture free aldehyde groups (1 ml/well, 15 min, RT).

Cells were rinsed twice for 5 min in PBS and incubated with the blocking and permeabilization solution (5% donkey normal serum, 5% goat normal serum, 0.3% Triton X-100 in PBS 1X) for 1 hour at RT. Primary antibody solution (α-LSD1 antibody, Cell Signaling, 1/100; Mouse immune serum for parasite detection, 1/1000; 1% Bovine Serum Albumin, 0.3% Triton X-100, PBS 1X) was added for an overnight incubation at 4°C. The next day, cells were rinsed 3 times for 5 min in PBS 1X and subsequently incubated for 1h30 at RT in the dark with the secondary antibody solution (10 µg/ml Donkey α-mouse, 10 µg/ml Goat α-rabbit, 1% BSA, 0.3% Triton X-100 in PBS 1X). Afterwards, cells were rinsed once in PBS 1X for 5 min, incubated for 15 min in a PBS 1X solution containing 5 µM Hoechst 33342 and rinsed one last time with PBS 1X for 5 min.

Finally, coverslips were rinsed in Milli-Q water, dried and mounted using SlowFade™ Gold Antifade Mountant on clean glass slides. The slides were stored overnight in the dark at 4°C and sealed with transparent varnish. The slides were observed on an Axiophot ZEISS epifluorescence microscope for counting of uninfected and infected cells, and intracellular parasites and for identifying LSD1 sub-cellular localization. Four fields per culture condition were acquired at 20X magnification allowing the analysis of at least 100 cells.

Determination of parasite load in live macrophages by epifluorescence imaging

Parasite load was estimated by the level of the mCherry fluorescence of live hosting macrophages. Nuclei from macrophages were stained by 5 µM Hoechst 33342 (ThermoFisher Scientific) for 15 min at room temperature. Image acquisition was done with the EVOS M5000 Imaging System on live cells after 4 days of infection and 3 days of treatment, using the DAPI light cube filter for detection of Hoechst-stained nuclei (Excitation: 357/44 nm; Emission: 447/60 nm) and the Texas red light cube for parasite mCherry detection (Excitation: 585/29 nm; Emission: 624/40 nm).

Five fields were acquired with the 20x objective. Image analysis was performed using the ImageJ software package on at least 100 cells. Parasite loads were determined based on the Raw Integrated Density of the mCherry fluorescence of macrophages in the absence or presence of LSD1*i*. Statistical analyses applying the Wilcoxon-Mann-Whitney test and graphical representations were performed with the GraphPad Prism software package.

Viability assay for *Leishmania* promastigotes and amastigotes

Anti-leishmanial activity of compounds on free parasites was evaluated using an adapted resazurin-based dye reduction (DRA) assay with cell-cycling promastigotes from logarithmic growth phase maintained in M199 supplemented medium.

Fixed concentrations or serial dilutions of the inhibitors were done in triplicate or quadruplicate, at 27°C for promastigotes and 34°C for amastigotes in 96-well plates or 384-well plates. DMSO vehicle and AmB were used as controls. Two days later, resazurin was added (10 µL per well for a final concentration of 5 µg/ml) and resorufin-derived fluorescence intensity was measured 24 h later using a Tecan Safire 2 reader (excitation 558 ± 4.5 nm; emission 585 ± 10 nm). Following background subtraction (complete parasite culture medium with resazurin in absence of parasites), data were expressed as percentages of growth compared to DMSO-treated controls (POC).

RNA extraction

Large RNA above 200 nucleotides were isolated from macrophage samples using the NucleoSpin miRNA kit (Macherey-Nagel) according to the manufacturer's instructions. Evaluation of RNA quality was carried out by optical density measurement using a Nanodrop device (Kisker, http://www.kisker-biotech.com). RNAs were isolated from 3 independent

biological replicates of uninfected and *Leishmania*-infected macrophages treated or not with LSD1*i* MC4030.

RNA-Seq analysis

Library preparation and sequencing was performed at the Biomics platform of Institut Pasteur. Briefly, DNAse-treated RNA extracts were processed for library preparation using the Truseq Stranded mRNA sample preparation kit (Illumina, San Diego, California) according to the manufacturer's instructions. An initial poly (A)+ RNA isolation step (included in the Illumina protocol) was performed with 1 µg of large RNA to isolate the mRNA fraction and remove ribosomal RNA. The mRNA-enriched fraction was fragmented by divalent ions at high temperatures. The fragmented samples were randomly primed for reverse transcription followed by second-strand synthesis to create double-stranded cDNA fragments. No end repair step was necessary. An adenine was added to the 3'-end and specific Illumina adapters were ligated. Ligation products were submitted to PCR amplification. The quality of the obtained libraries was controlled using a Bioanalyzer DNA1000 Chips (Agilent, # 5067-1504) and quantified by spectrofluorimetric analysis (Quant-iT™ High-Sensitivity DNA Assay Kit, #Q33120, Invitrogen).

Sequencing was performed on the Illumina Hiseq2500 platform to generate single-end 65 bp reads bearing strand specificity. Reads were cleaned using cutadapt version 1.11 and only sequences at least 25 nt in length were considered for further analysis. STAR version 2.5.0a (Dobin et al., 2013), with default parameters, was used for alignment on the reference genome (GRCm38 from Ensembl database 94). Genes were counted using featureCounts version 1.4.6-p3 (Liao et al., 2014) from the Subreads package (parameters: -t gene -s 0). Transcriptomic data are made publicly available at the NCBI's Gene Expression Omnibus repository (Superseries ongoing submission).

LSD1 target genes

A list of LSD1 target genes was compiled from bibliographic analyses and presented in Table S3. Target genes were evidenced after LSD1 pharmacological inhibition (Augert et al., 2019; Barth et al., 2019; Egolf et al., 2019; Hoshino et al., 2019; Maiques-Diaz et al., 2018), LSD1 Knocking Out (Barth et al., 2019; Jin et al., 2013), and siRNA LSD1 knockdown (Chen et al., 2017; Jin et al., 2017) using transcriptomic (Augert et al., 2019; Barth et al., 2019; Chen et al., 2017; Egolf et al., 2019; Hoshino et al., 2019; Jin et al., 2013), proteomic (Jin et al., 2017) and Chip Seq (Augert et al., 2019; Chen et al., 2017; Egolf et al., 2019; Jin et al., 2013; Jin et al., 2017; Maiques-Diaz et al., 2018) analyses.

Gene ontology analysis

Ensembl gene identifiers were translated into ENTREZ gene identifiers prior to the enrichment analysis. ENTREZ genes linked to several Ensembl IDs were associated with the one having the highest expression level. Functional gene-set enrichment analysis was performed using the Fisher statistical test for the over-representation of differentially expressed genes (gene-level adjusted P-value lower than 5%). Several types of gene-sets have been tested: (i) Hallmark (https://doi.org/10.1016%2Fj.cels.2015.12.004), (ii) reactome (https://doi.org/10.1093/nar/gkab1028), (iii) KEGG pathways (https://doi.org/10.1093/nar/gkac963), (iv) GO terms (https://doi.org/10.1093/genetics/iyad031) and (v) Wikipathways (https://doi.org/10.1093/NAR/gkaa1024). Only gene-sets with a false discovery rate (FDR) lower than 0.05 were considered significantly enriched in differentially expressed gene sets.

For the of analysis epigenetic regulatory factors, the differential expression values, expressed as log2 fold changes (log2 FC), were computed for each gene, and were visually depicted as a network using the Cytoscape software package (version 3.10.0). Each node within this network projects the log2FC information. Protein-protein interactions were extracted from the STRING Database (version 5.0) (https://string-db.org/). These interactions were filtered based on confidence scores, with a minimum interaction score of 0.700, thus generating an interaction network grounded in known or predicted associations among these genes. Gene Ontology (GO) terms we also incorporated for each network node, facilitating functional annotations pertaining to biological processes, molecular functions, and cellular components.

LSD1 co-immunoprecipitation assay

LSD1 co-IP was performed using nuclear protein samples from four biological replicates of uninfected and infected BMDMs. Dynabeads™ Protein G (Invitrogen) were resuspended, transferred to Eppendorf tubes (50 µl for sample pre-clearing and 25 µl for immunoprecipitation) and washed three times with 200 µl of PBS-T pH 7.4 (1x PBS, 0.02% Tween-20, 1x PIC).

For sample pre-clearing, beads were incubated for 30 min at RT with 5 µg of negative control antibody (C-, IgG from healthy rabbits, Diagenode). For immunoprecipitation, 3 µg of antibody Ab1 or C- were incubated with the beads (Ab1, anti-LSD1 20752, kindly provided and validated by Eric Metzger, from Roland Schüle team at the University of Freiburg, Germany) (Duteil et al., 2014). Following three washes with 200 µl PBS-T, pH 7.4, Dynabeads-Ab complexes were washed with 250 µl of Conjugation Buffer (20 mM Sodium phosphate pH 7.4, 150 mM NaCl) and subsequently cross-linked by incubating for 30 min at RT with a 5 mM solution of Bissulfosuccinimidyl suberate (BS^3 diluted in Conjugation Buffer). The reaction was quenched by adding 12.5 µl of 1M Tris pH 7.4 for 15 min at RT. Cross-linked Dynabeads-Ab complexes

were washed three times with 200 µl of Beads buffer (50 mM Tris pH 7.4, 250 mM NaCl, 5 mM EDTA, 5 mM EGTA, 5 mM NaF, 0.1% NP-40).

Immunoprecipitation was performed by suspending the cross-linked Dynabeads-Ab complexes with 300 µg of pre-cleared samples and incubating overnight at 4°C. The following day, supernatants were recovered and transferred to a new tube (for further analysis) and Dynabeads-Ab-Antigen (Ag) complexes were washed 3 times with 1 ml of Wash Buffer (50 mM Tris pH 7.4, 250 mM NaCl, 5 mM EDTA, 5 mM NaF, 0.1% NP-40) and four times with 1 ml of 100 mM NH_4HCO_3. Finally, Dynabeads-Ab-Ag complexes were resuspended in 100 µl NH_4HCO_3 and digested by adding 0.2 µg of trypsin-LysC (Promega) for 1 h at 37°C. Samples were then loaded into custom-made C18 StageTips packed by stacking three AttractSPE® disk (#SPE-Disks-Bio-C18-100.47.20 Affinisep) into a 200 µL micropipette tip for desalting. Peptides were eluted using a ratio of 40:60 CH_3CN:H_2O + 0.1% formic acid and vacuum concentrated to dryness with a SpeedVac device. Peptides were reconstituted in 10 µL of injection buffer in 0.3% trifluoroacetic acid (TFA) before liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis.

LC-MS/MS Analysis

Online chromatography was performed with an RSLCnano system (Ultimate 3000, Thermo Scientific) coupled to an Orbitrap Eclipse mass spectrometer (Thermo Scientific). Peptides were trapped on a 2 cm nanoviper Precolumn (i.d. 75 µm, C18 Acclaim PepMap^{TM} 100, Thermo Scientific) at a flow rate of 3.0 µL/min in buffer A (2/98 MeCN/H2O in 0.1% formic acid) for 4 min to desalt and concentrate the samples. Separation was performed on a 50 cm nanoviper column (i.d. 75 µm, C18, Acclaim PepMap^{TM} RSLC, 2 µm, 100Å, Thermo Scientific) regulated to a temperature of 50°C with a linear gradient from 2% to 25% buffer B (100% MeCN in 0.1% formic acid) at a flow rate of 300 nL/min over 91 min. MS1 data were collected in the Orbitrap

(120,000 resolution; maximum injection time 60 ms; AGC 4 x 10^5). Charges states between 2 and 7 were required for MS2 analysis, and a 60 sec dynamic exclusion window was used. MS2 scan were performed in the ion trap in rapid mode with HCD fragmentation (isolation window 1.2 Da; NCE 30%; maximum injection time 35 ms; AGC 10^4)

Proteomics data processing

For identification, the data were searched against the *Mus musculus* (UP000000589_10090) and the *Leishmania amazonensis* MHOMBR71973M2269 in the TriTrypDB (release 60) databases using Sequest HT through proteome discoverer (version 2.4). Enzyme specificity was set to trypsin and a maximum of two miss cleavages sites were allowed. Oxidized methionine, Met-loss, Met-loss-Acetyl and N-terminal acetylation were set as variable modifications. Maximum allowed mass deviation was set to 10 ppm for monoisotopic precursor ions and 0.6 Da for MS/MS peaks. The resulting files were further processed using myProMS v3.10.0 (https://github.com/bioinfo-pf-curie/myproms) (Poullet et al., 2007). FDR calculation used Percolator (The et al., 2016) and was set to 1% at the peptide level for the whole study. The label free quantification was performed by peptide Extracted Ion Chromatograms (XICs), reextracted across all conditions and computed with MassChroQ version 2.2.21 (Valot et al., 2011). For protein quantification, XICs from proteotypic and non-proteotypic peptides were used by assigning to the best protein, peptides shared by multiple match groups, and missed cleavages, charge states and sources were allowed. Label-free quantification (LFQ) was performed following the algorithm as described (Cox et al., 2014).

The final LFQ intensities were used as protein abundance and further analyzed using Prostar v1.32.1 following the guidelines outlined in the software manual (Tardif et al., 2023). Briefly, we addressed missing data in all conditions by first removing Partially Observed Values (POV) and Missing in Entire Condition (MEC). Subsequently, we applied mean centering to normalize

the filtered data. For the imputation of POV and MEC values, we employed the Structured Least Square Adaptive (SLSA) regression and the 2.5% quantile algorithm. Finally, we conducted a differential analysis using the Welch test.

RESULTS

L. amazonensis subverts the macrophage epigenetic landscape during infection.

We apply a multidisciplinary approach to (i) identify potential epigenetic pathways affected by *Leishmania* infection, (ii) reveal their relevance for intracellular parasite survival, and (iii) assess the mechanisms underlying their subversion. We first conducted an RNA-Seq analysis of Bone Marrow- Derived Macrophages (BMDMs) infected or not by *Leishmania amazonensis* virulent amastigotes (day 2 PI) (Figure 1A), which revealed significant expression changes for 5742 genes (Figure S1A, adjusted p value < 0.05) affecting the macrophage innate immune response- including antigen presentation and toll receptor signaling cascade- as well as metabolic processes such as cholesterol biosynthesis (Figure S2). To gain insight into epigenetic subversion strategies, we more specifically investigated the impact of infection on the expression of macrophage Epigenetic Regulatory Factors (EpiRFs), including histone / DNA modifying enzymes, associated transcription factors, and members of epigenetic complexes. From 787 EpiRFs obtained from manual curation of publicly available databases including EpiFactors (http://epifactors.autosome.org., Table S1), 315 showed significant changes in transcript abundance during infection, with increased expression observed for 154 (e.g. *Dnmt*, FC = +1.39; *Dot1l*, FC = +2.78), and decreased expression affecting 161 (e.g. *Sirt3*, FC = -1.46; *Ehmt,*: FC = -1.38) (Figure 1B). Overall, 39.7 % of EpiRFs were modulated by the

parasite, revealing epigenetic regulation as a key target for parasite subversion of host cell immune-metabolomic functions.

Pharmacological interference with macrophage epigenetic regulation reduces burden of intracellular *L. amazonensis*.

We next used a pharmacological approach to assess the relevance of this epigenetic subversion strategy for intracellular *L. amazonensis* survival. We assessed 425 epigenetic inhibitors provided by the FP7 A-PARADDISE project, which were designed to target various classes of mammalian epigenetic regulators, including sirtuins, Histone Acetyl Transferases (HATs), Histone Deacetylases (HDACs), Histone Methyl Transferases (HMT), Histone DeMethylases (HDMs) and DNA Methyl Transferases (DNMTs) (see https://cordis.europa.eu/project/id/602080). We screened this library by monitoring the intracellular amastigote survival in *L. amazonensis*-infected BMDMs using a high content assay, and extracellular parasite survival using a resazurin-based viability assay applied on promastigotes in culture (Lamotte et al., 2019). This dual strategy allowed us to distinguish anti-leishmanial compounds that affect parasite viability directly (killing both extracellular and intracellular parasites) from compounds that affect parasite viability indirectly via host-dependent mechanisms (killing exclusively intracellular parasites) (Lower right quadrant, figure 2A).

Of particular interest were the 18 compounds that caused significant decrease in intracellular but not extracellular parasite survival. Such putative host-dependent mode of action has been observed for different classes of targets including HMTs, HDMTs and DNMTs, suggesting that interference with macrophage epigenetic regulation both at DNA and histone levels can interfere with intracellular parasite development or trigger parasite death. Most effective were

a series of inhibitors directed against the Lysine-Specific Demethylase 1 (LSD1, a.k.a. KDM1A). This enzyme demethylates histone H3 at K4 and K9 residues, which has been linked to macrophage polarization and the transcription of pro-inflammatory cytokines (Janzer et al., 2012; Tan et al., 2019). The anti-leishmanial effect of these LSD1 inhibitors resonates well with our previous data that correlated *L. amazonensis* infection with macrophage H3K4 and H3K9 hypomethylation, suggesting that this demethylase may be key for parasite-mediated, epigenetic host cell subversion.

We next focused our attention on two derivatives of the canonical flavoenzyme monoamine oxidase A inhibitor Tranylcypromine (TCP), i.e. the compounds MC2652 and MC4030. These inhibitors were synthesized by replacing the 3-phenyl ring of TCP with distinct heterocyclic thiophene moieties, and display an increased LSD1 inhibitory effect in cancer cell lines (Fioravanti et al., 2022). While TCP did not affect intracellular parasite survival (data not shown), both MC2652 and MC4030 strongly reduced burden of both intracellular *L. amazonensis* and *L. donovani* as judged by epifluorescence microscopy (Figures 2B and S3).

We next documented the effects of these TCP derivatives on murine macrophage LSD1, first evaluating their inhibitory potential on the enzymatic activity of LSD1 in BMDM nuclear extracts. Both inhibitors demonstrated a more effective inhibition of H3K4me2 demethylation compared to TCP (Figure 2C). To determine *in cellula* whether this inhibition could be attributed to target engagement - i.e. direct binding to LSD1, we performed a Cellular Thermal Shift Assay (CETSA). The binding of the LSD1*i* to the demethylase was confirmed by the LSD1 thermal stabilization in the presence of the inhibitors (Figure 2D and 2E and Figure S4). Importantly, this interaction was specific to LSD1, as no thermal shift was observed for MAO-A or GAPDH in either uninfected (Figure 2D, and 2E) or *L. amazonensis*-infected BMDMs (Figure 2D1 and 2D2, and Figure S4A and S4B).

***L. amazonensis* affects LSD1 complex formation.**

Surprisingly, even though our screening data suggest an important role of LSD1 in supporting intracellular *Leishmania* survival, infection itself neither modulated LSD1 protein levels during the first three days of infection (Western blot analysis, Figure 3A), nor affect LSD1 nuclear localization (immunofluorescence analysis, Figure 3B).

Since LSD1 serves as an important scaffolding protein and its activity relies on its interaction with key partners in regulatory complexes (Burg et al., 2015), we next analyzed expression changes of 90 known LSD1-associated proteins during *L. amazonensis* infection mining our RNA-Seq data (Figure 3C). While no significant transcriptional modulation was observed for LSD1 itself, 35 of the LSD1-associated proteins (35.4%) were modulated (Figure 3C), including 19 showing decreased mRNA abundance (e.g. *Zfp217*, FC = -1.87; *Suv39h1*, FC = +1.40), and 16 showing increased mRNA abundance (e.g. *Mbd3*, FC = +1.48; *Gatad2a*, FC = +1.43). Changes in transcript abundance of known LSD1 interaction partners subverted the composition of the main LSD1 complex (Table 1).

Using CETSA we observed an important shift in LSD1 thermal stability in infected compared to uninfected BMDMs, providing first, indirect evidence that LSD1 complexes undergo infection-dependent changes (Figures 4A and 2E). We next performed a co-immunoprecipitation (co-IP) assay using nuclear extracts from uninfected and infected BMDMs to directly assess changes in LSD1 complex formation at day 2 post-infection. We employed two previously described, custom-made antibodies (termed Ab1 and Ab2, Biogenes) (Duteil et al., 2016). Notably, the antibody targeting the C-terminus of LSD1 (Ab1) exhibited a higher efficacy in precipitating LSD1 interactors (Figure S5) and was therefore considered for further assessment.

In comparison to co-IPs performed with non-specific IgGs, we identified a total of 489 and 330 proteins as LSD1-specific interaction partners in uninfected compared to infected BMDMs. Of these, 259 were common to both conditions (Figure S5), including well-known LSD1 interactors, such as RCOR1, ARID1A, POLR2A (Figure 4B). Notably, 230 LSD1 interactors were exclusively identified in uninfected BMDMs, while 71 were unique to infected BMDMs. Surprisingly, it was found that 24 out of these 71 interaction partners corresponded to *Leishmania* proteins (Table 2). Whether this is the result of specific protein transfer or parasite contaminating material remains to be established. STRING analysis performed on the remaining 47 macrophage proteins identified as unique LSD1 partners in infected BMDMs (Figure 4C) revealed notably three interaction networks that were enriched in GO terms linked to (i) ribosomal biogenesis, (ii) mRNA processing, binding, and splicing, and (iii) nucleosomal DNA binding and linker histones H1/H5 (Figure 4C). Collectively, these findings demonstrate that *Leishmania* infection has profound consequences on the formation of LSD1 complexes, which affects the protein's thermal stability and potentially down-stream regulated targets relevant for epigenetic, post-transcriptional and translational regulatory processes.

LSD1 inhibition counteracts parasite-driven expression changes in LIMs.

In order to elucidate host cell pathways that may govern the host-directed, anti-leishmanial effect of compound MC4030, we analyzed the impact of drug treatment on uninfected and infected BMDMs. As noted above, *L. amazonensis* infection alone had a profound effect on the macrophage transcriptome (see Figures 1B and S1), affecting many cellular pathways, including protein kinase activity, sterol biosynthesis, and responses to lipopolysaccharide, hypoxia, and oxidative stress (Figure S2). Among the 5742 modulated transcripts, 1101 (19.17%) were previously reported to be regulated by LSD1 (Figure 5A1), which further

supports our hypothesis that *Leishmania* targets LSD1 to establish permissive conditions for intracellular macrophage infection.

MC4030 treatment of uninfected BMDMs resulted in expression changes of 1097 genes, 287 of which are regulated by LSD1 (26.1%) (Figure 5A2). These results further support the specificity of MC4030 to target macrophage LSD1 and sustains a host-directed mechanism for its anti-leishmanial activity. Finally, MC4030 treatment of infected BMDMs affected the abundance of 456 transcripts, 117 (25.7%) of which are regulated by LSD1 (Figure 5A3). MC4030 treatment seems to counteract the infection-induced phenotype as judged by PCA analysis of the three experimental groups, which shows a shorter distance between MC4030-treated infected and uninfected BMDMs compared to the distance between infected and uninfected BMDMs in absence of the inhibitor (Figure 5B). This is further confirmed by the double ratio plot shown in Figure 5C, providing direct evidence that 95% of expression changes caused by the treatment do counteract infection-dependent expression changes observed in infected BMDMs. For example, many genes implicated in the macrophage response to lipopolysaccharide and hypoxia showed reduced expression during infection, which was restored after drug treatment (Figure 5D1 and D2). Conversely, the increased expression of genes implicated in signal transduction and steroid biosynthesis observed in infected BMDMs was significantly reduced upon treatment (Figure 5D3 and D4). Importantly, this reversion was not just the consequence of parasite elimination but a direct effect of LSD1 inhibition given that the treatment had only a slight effect on parasite burden at the time point chosen for the RNA-Seq analysis (i.e. 48h of treatment).

In conclusion, our data provide evidence that MC4030 kills intracellular *Leishmania* by restoring a macrophage expression profile that interferes with the parasite survival by rescuing the host cell immune response and limiting the parasite's access to nutrients essential for its proliferation.

DISCUSSION

Leishmania infection of macrophages is associated with significant changes in gene expression that contribute to the creation of a favorable environment for parasite survival and replication within host cells. Here we provide pharmacological evidence that *Leishmania* exploits the host demethylase LSD1, raising questions on how *Leishmania* engages with LSD1, how this engagement changes the host cell phenotype, and how interfering with this engagement leads to parasite killing.

Leishmania can engage with LSD1 to modulate one or both of its biological functions, i.e. its enzymatic activity and / or its scaffolding properties. The absence of LSD1 modulation with respect to transcript abundance, protein abundance or subcellular localization levels suggested that LSD1 subversion relies on other mechanisms independent of its expression, such as complex formation. This hypothesis was confirmed by CETSA analyses that provided first evidence that the parasite increases LSD1 thermal stability. This increase might reflect changes in the nature of LSD1 host complexes, or new interactions with one or more of the *Leishmania* proteins we co-purified with nuclear LSD1, which opens the exiting possibility that *Leishmania* – similar to pathogenic bacteria (Bierne and Cossart, 2012)- may have co-evolved 'nucleomodulins" similar that can directly engage with nuclear host cells components to subvert their functions.

Infection-dependent LSD1 interactions directly inform on possible mechanisms of immune subversion. For example, LSD1 specifically interacts with the heterogeneous nuclear ribonucleoprotein K (HnRNPK), a non-histone target of LSD1 (Natarajan et al., 2022) that been previously implicated in the stabilization of the mRNA of CXCL10, a chemokine associated with resistance to *Leishmania* infection (Antonia et al., 2019). Likewise, other infection-specific LSD1 interactions may favor parasite survival, such as the splicing factor proline- and

glutamine-rich (SFPQ), the ATP-dependent RNA helicase A (DHX9), and Matrin 3 (MATR3), and the non-POU domain-containing octamer-binding protein (NONO). These molecules are respectively involved in i) viral RNA amplification (Labeau et al., 2022), ii) nuclear retention of RNAs that are defective or immature (Zhang and Carmichael, 2001), iii) DNA non-homologous end joining (NHEJ) recombination required for double-strand break repair and V(D)J recombination (Salton et al., 2010) and iv) modulation of transcription and translation processes (Li et al., 2022). LSD1 interaction partners within infected macrophages were also enriched in ribosomal proteins, whose methylation state may be affected by LSD1 (Hamidi et al., 2018). Altogether, these infection-specific changes in LSD1 interactions may significantly remodel the transcriptomic landscape of the host macrophage for the benefit of intracellular *Leishmania* survival via inhibition of immune-related gene expression (e.g. down regulation of pro-inflammatory response) or mobilizing of nutrients required for parasite growth (e.g. increase of the biosynthetic cholesterol pathway). Indeed, reversion of these expression changes in presence of the LSD1 inhibitor likely resorted the host cells innate defense mechanisms (Tan et al., 2019) and limited cholesterol availability and its master regulator, SREBP2 (Horton et al., 1998), both of which translated into a host-directed, anti-leishmanial activity, confirming that these pathways might be essential for parasite survival (Mukherjee et al., 2014).

In conclusion, our results shed new light on a *Leishmania*-specific epigenetic subversion strategy that targets the LSD1 scaffolding function of the host cell to change transcriptional complexes and establish an infection-permissive phenotype. This strategy was demonstrated in acute myeloid leukemia where the interaction between LSD1 and RCOR1 with the SNAG-domain transcription repressor GFI1 can be dispersed by TCP-derived inhibitors allowing myeloid differentiation (Maiques-Diaz, 2018). The identification of two LSD1-specific inhibitors with host-directed, anti-leishmanial activity pharmacologically validates host cell epigenetic regulation and downstream-regulated pathways as a fertile ground for the discovery

of macrophage drug targets that impact parasite survival, which may be broadly applicable to other intracellular pathogens known to target host cell epigenetic regulation for their survival, such as *Mycobacterium tuberculosis* (Pennini et al., 2006), *Listeria monocytogenes* (Hamon and Cossart, 2008) or *Toxoplasma gondii* (Schneider et al., 2013).

FIGURE LEGENDS

Figure 1: Experimental pipeline (A) and differential expression analysis (B) of Epigenetic Regulatory Factors (EpiRFs) in LIMs.

BMDMs were infected with lesion-derived, mCherry-transgenic amastigotes of *L. amazonensis* and compared to uninfected BMDMs by RNA-Seq analysis two days after infection. Out of 787 EpiRFs, 315 were modulated in LIMs compared to uninfected BMDMs. Constitutive (white), downregulated (blue) and upregulated (red) genes are visualized as STRING interaction networks using the the Cytoscape V3.10.0 software package. Log_2 Fold Change (Log_2 FC) for differentially expressed EpiRF genes with an adjusted p Value cutoff of 0.05 are shown.

Figure 2: Identification and validation of LSD1 inhibitors with host-dependent, anti-leishmanial activity.

(A) Biparametric dot plot showing the survival of *L. amazonensis* parasites after three days of treatment at 4 µM for cultured extracellular promastigotes (DRA) and at 10 µM for intracellular amastigotes in *Leishmania*-infected macrophages (LIMs) (HCA). Data were manually curated to remove toxic compounds for macrophages. Results are expressed as percentages of promastigote survival (X-axis) and of infected macrophages (Y-axis) compared to the DMSO control. Quadrant lines divide the high-low range antileishmanial activity into four sections. Potential host-directed compounds are located in the right lower quadrant. Different classes of inhibitors are represented by different colors.

(B) Violin plot of anti-leishmanial activity for two selected LSD1*i* (MC2652 and MC4030). Inhibitors were added at a concentration of 10 µM on BMDMs one day post-infection with lesion-derived mCherry-transgenic *L. amazonensis* amastigotes. Epifluorescence microscopy

was performed after three days of treatment. Violin plots display the quantification of red fluorescence per cell for each sample in uninfected BMDMs (white), untreated LIMs (grey) and compound-treated LIMs (MC2652, green; MC4030, orange). Raw Integrated Density per cell was determined by ImageJ on 100-180 cells. The p-value is indicated (*: $p<0.0001$).

(C) LSD1 activity assay. LSD1 demethylase activity was assessed in 1 µg of nuclear protein extracts from uninfected BMDMs in the absence or presence of increasing concentrations of the LSD1*i* MC2652, MC4030 and TCP. The percentage of inhibition of LSD1 activity was calculated for the two indicated inhibitor doses compared to untreated BMDMs set at 100%.

(D) Cellular thermal shift assay (CETSA). Uninfected BMDMs (upper panel) and LIMs at day 1 post-infection (lower panel) were treated for 24 hours with 1% DMSO as control (grey area), and with 15 µM MC2652 (green line), MC4030 (orange line), and TCP (black line). The results are presented as a percentage of the fluorescent signal detected at the lowest temperature in each melting curve, with values representing the mean and SEM of 2-3 biological replicates.

(E) Note that the thermal shifts detected in the presence of LSD1 inhibitors are specific for LSD1. Thermal shift values were calculated by subtracting the melting temperature (Tm) of the UI sample from the Tm of the sample of interest.

Figure 3. Impact of *L. amazonensis* infection on LSD1 expression and localization and on expression of LSD1 interaction partners.

(A) Western Blot analysis. Nuclear proteins were extracted from uninfected (UI) BMDMs and LIMS at day 2 post-infection. One representative WB (n = 2-5) with LSD1 and YY1 proteins in 2 days PI samples is shown (left panel). Signals were quantified (right panel) by normalizing LSD1 against the YY1 loading control and setting the value for UI BMDMs to 1. Values

obtained with samples from 24 to 72 hours after infection were collected for at least 2 biological replicates.

(**B)** Epifluorescence fluorescence analysis of LSD1 localization in fixed cell samples. Macrophage nuclei were stained with Hoechst 33342, parasites were visualized by their mCherry fluorescence (red channel), and LSD1 was detected by immunostaining (green channel).

(C) Expression changes observed for LSD1 interaction partners. BMDMs were infected with lesion-derived mCherry-transgenic *L. amazonensis* amastigotes and compared to UI BMDMs. RNA-Seq analysis of infected and uninfected BMDMS was performed as indicated in legend of Figure 1. Changes in RNA abundance for LSD1 and its known interaction partners are visualized as STRING interaction networks. Differentially expressed genes are defined by a cutoff of log2 FC and $p<0.05$). Constitutive transcript abundance, white; decreased transcript abundance, blue; increased transcript abundance, red.

Figure 4: Analyses of LSD1 interactions.

(A) Evidence of a LSD1 thermal stabilization in LIMs by CETSA analysis. Representative Western blots comparing uninfected (UI) BMDMs and LIMs two days post-infection in presence of DMSO (upper panel). Melting curves for both samples are shown in the lower panel with the temperature shift indicated.

(B) Volcano plots showing identified LSD1 interactors in uninfected (UI) BMDMs (left panel) and LIMs (right panel). Nuclear protein extracts from UI BMDMs and LIMs at day 2 PI were co-immunoprecipitated (co-IP) with the anti-LSD1 Ab1 (n = 4 biological replicates). Nonspecific binding events were revealed by performing co-IP experiments with rabbit IgGs

(grey dots). Only statistically significant interactions (cut-off $p < 0.01$) were retained (Prostar software tool).

(C) Gene ontology enrichment analysis of STRING networks identified between LSD1 interaction partners in LIMs using the Cytoscape V3.10.0 software package.

Figure 5: Transcript profiling of uninfected (UI) BMDMs and LIMs at day 2 post-infection treated or not for 1 day with MC4030.

(A) Volcano plots showing differential transcript abundance (adj. p values < 0.05) for the comparisons LIMs vs UI BMDMs (left), MC4030-treated vs UI BMDMs (middle), and MC4030-treated LIMs vs untreated LIMs (right). Colored dots correspond to transcripts whose expression is controlled by LSD1. Green, decreased mRNA abundance in a given comparisons; red, increases mRNA abundance in a given comparison.

(B) PCA analysis of the different replicates for each condition. Arrows indicate the distance between experimental groups.

(C) Double ratio plot displaying the log2 FC ratio of transcript expression in LIMs vs UI BMDMs (X-axis) and MC4030-treated LIMs vs LIMs (Y-axis).

(D) Visual representation of reversible gene expression changes for genes that share the Gene Ontology terms 'Response to lipopolysaccharide' (1), 'Response to hypoxia' (2), 'Regulation of protein kinase activity' (3), and 'Steroid biosynthetic process' (4). The color of the node border and node filling respectively shows expression changes for the comparisons LIMs *vs* UI BMDMs and M4030-treated LIMs vs LIMs, with the border thickness and the node size corresponding to the p-value as indicated in the legend.

SUPPLEMENTARY FIGURE LEGENDS

S1. Heat map of mRNA expression values in BMDM samples.

BMDMs were infected with lesion-derived mCherry-transgenic amastigotes of *L. amazonensis* (LIMs) and compared to uninfected BMDMs (UI BMDMs) (A). One day after infection, MC4030 was added or not to the samples (B). RNA-Seq was performed one day after this treatment using three independent biological replicates. A Z-score normalization is performed on the normalized read counts across samples for each gene. Z-scores are computed on a gene-by-gene (row-by-row) basis and used to plot heatmap (add the color legend with values).

S2. Gene Set Enrichment Analysis (GSEA) of transcriptionally modulated genes in LIMs. Downregulated and upregulated processes are indicated in blue and red colors, respectively.

S3: Analysis of the impact of LSD1*i* on parasite load in *Leishmania-infected* BMDMs. BMDMs were infected with mCherry-transgenic amastigotes of *L. amazonensis* isolated from infected mouse footpads (A) or *L. donovani* isolated from infected hamster spleens (B). After one day of infection, MC2652 and MC4030 were added at a concentration of 10 μM for 3 days. Merged fluorescence and bright field microscopy images of each sample are shown. Macrophage nuclei and parasites were visualized using Hoechst 33342 staining (blue fluorescence) and mCherry expression (red fluorescence), respectively. Green fluorescence corresponds to dead macrophages as revealed by YOPRO-1 incorporation. **(C)** Violin plots showing the level the mCherry fluorescence as a readout for parasite burden. Grey, BMDMs treated with DMSO treated; violet, MC2652-treated, green; MC4030-treated. Raw Integrated Density per cell was determined by ImageJ. The p-value is indicated (*: $p<0.0001$).

S4: CETSA analysis of LSD1, MAO-A, and GAPDH in protein lysates from uninfected (UI) BMDM and in LIMs samples in presence of the LSD1 inhibitors.

UI BMDMs (A) and LIMs at day 1 post-infection (B) were treated for 24 hours with 1% DMSO as control or with 15 µM MC2652 or MC4030. Extracts were resolved by SDS-PAGE and proteins of interest revealed by Western blot analysis using specific antibodies. Representative Western blots are shown out of 2-3 independent experiments performed.

S5: Venn diagrams representing the proteins immune-precipitated with LSD1.

(A) Number of identified LSD1 interacting proteins comparing the LSD1-specific antibodies Ab1 and Ab2 in uninfected (UI, left panel) and infected (LIMs, right panel) BMDMs.

(B) Number of identified LSD1 interacting proteins in uninfected (UI) and infected (LIMs) when using antibody Ab1 (left panel) and Ab2 (right panel).

S6. Quality control of nuclear and cytoplasmic extracts isolated from uninfected (UI) BMDMs and LIMs.

(A) Microscopic observations of live BMDMs (upper panel) and isolated nuclei (lower panel).

(B) Western blot analysis of GAPDH in nuclear and cytoplasmic extracts of 5 biological replicates. The absence of GAPDH indicates the absence of cytoplasmic contamination in nuclear extracts.

(C) Western Blot analysis of uninfected BMDMs for LSD1 in nuclear extracts of untreated cells and LPS+IFN-treated cells, a condition known to decrease the LSD1 expression (right

panel), and cytoplasmic extracts of 5 independent extractions showing the presence of cytoplasmic LSD1 and/or nuclear LSD1 contamination. The nuclear marker YY1 and cytoplasmic marker GAPDH were revealed for normalization purposes.

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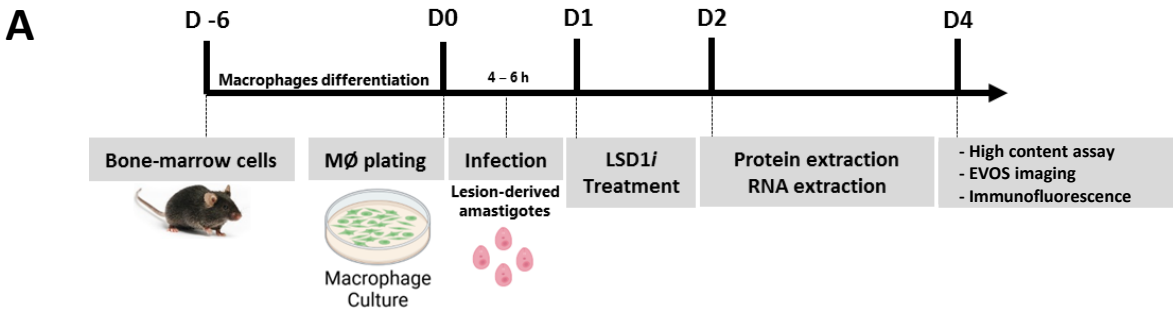
Table 1 Gutierrez Sanchez et al. : Transcriptional modulation of genes involved in the composition of the main LSD1 complexes

LSD1 CoREST complex																	
unmodulated	Cbx5	Gfi1	Gfi1b	Hdac1	Hmg20b	Hspa1a	Hspa1b	Kdm1a	MeCP2	Phf21b	Rcor2	Rest	Rreb1	Sin3b	SIX3	Tal1	Zmym2
down-modulated	Gtf2i	Phf21a	Sin3a	Zfp217													
up regulated	Rcor1	Rcor3															
NurD complex																	
unmodulated	Chd3	Chd4	Chd5	Hdac1	Kdm1a	Mbd2	Mbd3	Mta2	Rbbp4	Rbbp7							
down-modulated	Gatad2b	Kdm5b	Mta1	Mta3													
up regulated	Gatad2a	Hdac2															
CtBP complex																	
unmodulated	Ctbp1	Ctbp2	Ehmt1	Ehmt2	Hdac1	Kdm1a	Lcor	Rreb1	Zeb1	Znf217	Znf516						
down-modulated	Ehmt2	Cbx4															
up regulated	Cdyl	Hdac2	Rcor1	Rcor3													
TLX Orphan Nuclear Receptor																	
unmodulated	Nr2E1	Hdac1	Kdm1a	miR-137	Zmym2												
down-modulated	Hdac5	Zmym3															
up regulated	Gse1	Rcor1															
SNAI1 Complex																	
unmodulated	Hmg20b	Phf21a	Sfmbt1	Snai1	Snai2	Znf516											
down-modulated	Hmg20a																
up regulated	Gse1	Rcor1															
Hdac1-Ino80-Kdm1a-Phb2-Rbp1-Taf5 complex																	
unmodulated	Hdac1	Ino80	Kdm1a	Phb2	Rbp1												
Kdm1a-Plod2 complex																	
unmodulated	Kdm1a	Plod2															
BRAF53-BRCA2 complex																	
unmodulated	Brca2	Hdac1	Hmg20b	Kdm1a	Phf21a												
up regulated	Hdac2	Rcor1															
ALL1 super complex																	
unmodulated	Chd3	Cpsf2	Eftud2	Hdac1	Kdm1a	Mta2	Ran	Rbbp4	Rbbp5	Rbbp7	Sap30	Sin3A	Smarca5	Smarcb1	Smarcc2	Sympk	Taf1
	Taf6	Taf12	Tbp														
down-modulated	Smarca2																
up regulated	Hdac2	Kmt2a	Mbd3	Sap18	Smarcc1	Taf9	Wdr5										
AR complex																	
unmodulated	Ar	Kdm4c	Pkcb1														
ERα complex																	
unmodulated	Esr1	Cac1	Pelp1														
down-modulated	Set9	Suv39h1															

Table 2, Gutierrez Sanchez et al. *Leishmania* LSD1 proteins interactors, co-immunoprecipitated with the specific anti-LSD1 antibody Ab1 using LIMs nuclear protein extracts.

LAMA_000014200.1	Tubulin, putative
LAMA_000030000.1	Ribosomal protein S7e, putative
LAMA_000050800.1	Hypothetical protein, conserved
LAMA_000069400.1	Hypothetical protein, conserved
LAMA_000111100.1	Histone H2A, putative
LAMA_000154400.1	Core histone H2A/H2B/H3/H4, putative – Annotated as Histone H2B in *L. mexicana*
LAMA_000206700.1	T-complex protein 10 C-terminus, putative
LAMA_000224800.1	C2 domain containing protein, putative
LAMA_000261800.1	Hypothetical protein, conserved
LAMA_000365000.1	Hypothetical protein, conserved - Annotated as KHARON in *L. mexicana*
LAMA_000388500.1	Hypothetical protein, conserved
LAMA_000424900.1	Hypothetical protein, conserved
LAMA_000472900.1	Hypothetical protein, conserved
LAMA_000475000.1	Ribosomal proteins L26 eukaryotic, putative
LAMA_000489100.1	Hypothetical protein, conserved
LAMA_000541300.1	Hypothetical protein, conserved
LAMA_000574600.1	Leucine Rich repeat, putative
LAMA_000582300.1	Hypothetical protein, conserved
LAMA_000615100.1	Glyceraldehyde 3-phosphate dehydrogenase, putative
LAMA_000622200.1	Hypothetical protein, conserved
LAMA_000657900.1	Hypothetical protein, conserved
LAMA_000685500.1	Leucine-rich repeat/Glycogen recognition site of AMP activated protein kinase, putative
LAMA_000721100.1	Hypothetical protein, conserved - Annotated as KHassociated protein 1 in *L. mexicana*
LAMA_000780200.1	TATA box binding protein associated factor (TAF) putative - Annotated as Histone H4 in *L. mexicana*



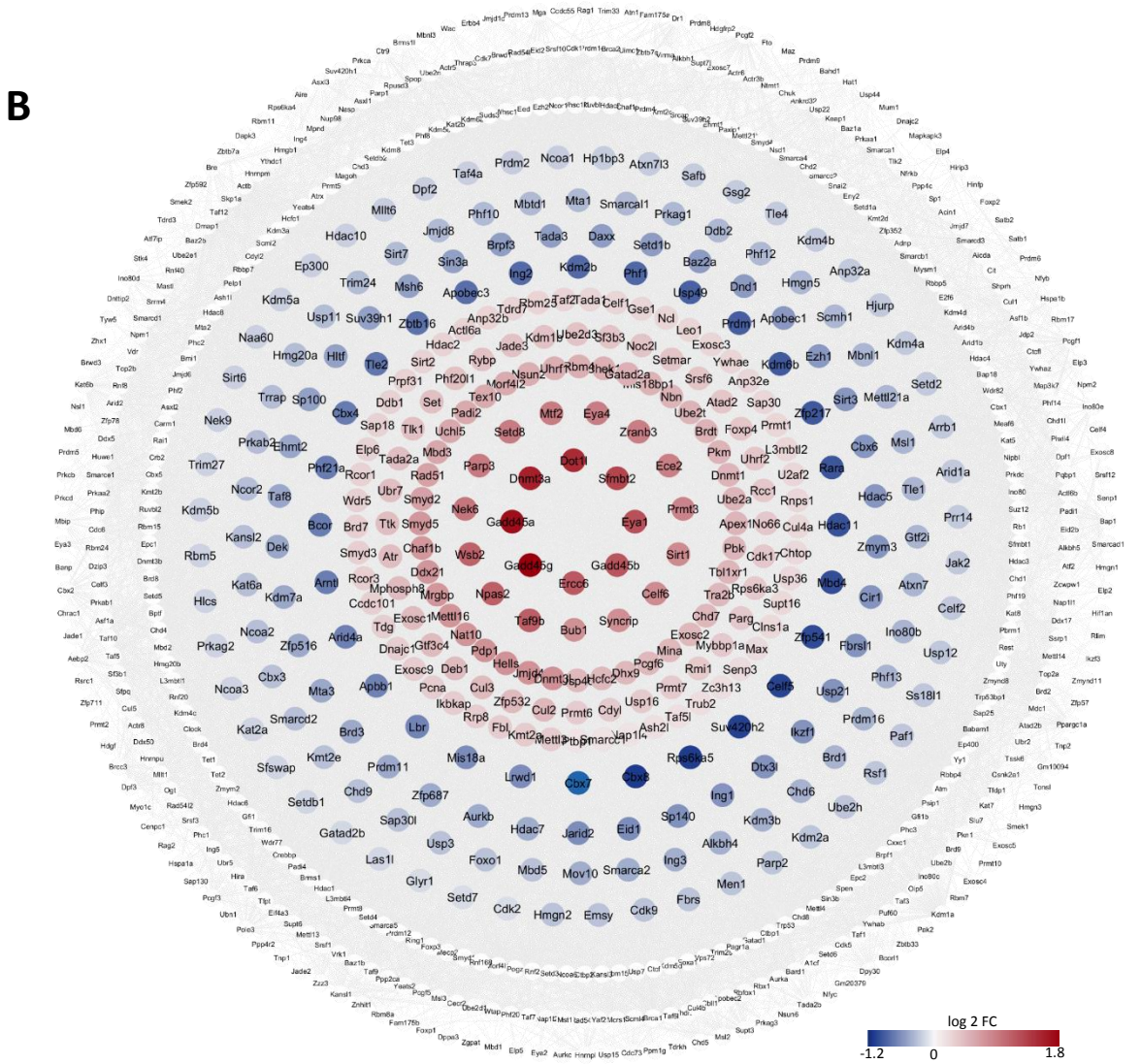
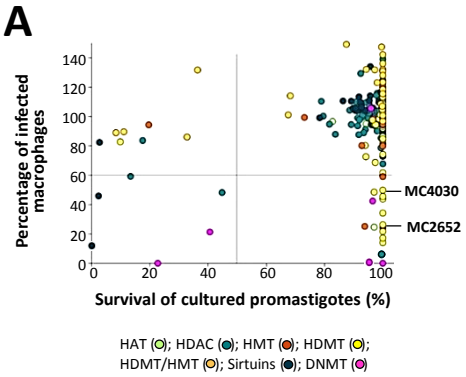
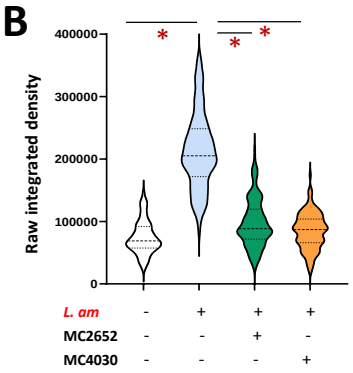
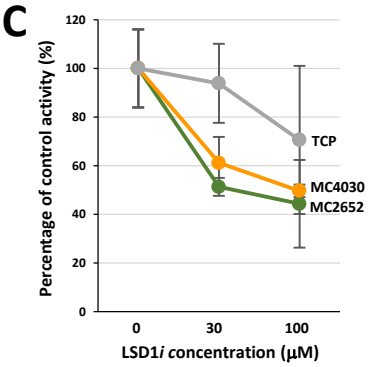
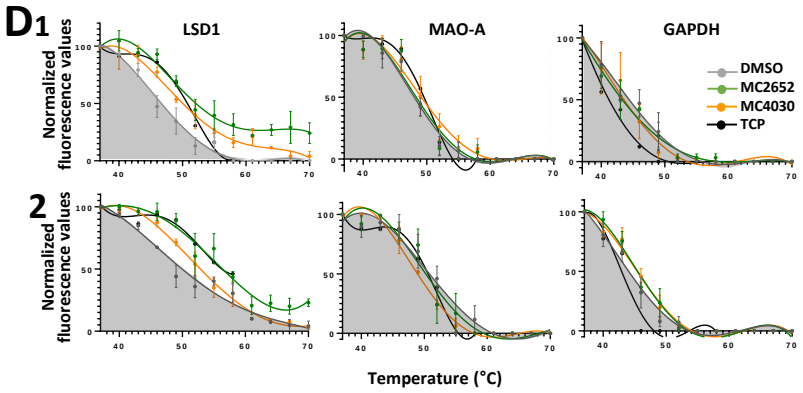


Figure 1, Gutiérrez-Sanchez M. *et al.*









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1) ΔTm	LSD1	MOA-A	GAPDH
	UI / **LIMs**	UI / **LIMs**	UI / **LIMs**
DMSO	N/A	N/A	N/A
MC2652	5.5 / **6.5**	0.6 / **-0.7**	-1.3 / **1.4**
MC4030	3.3 / **2.8**	1.4 / **-2.1**	-1.0 / **1.4**
TCP	4.1 / **6.3**	2.1 / **-0.3**	-3.4 / **-1.3**

2) ΔTm		MC2652	MC4030	TCP
	LIMs	UI / **LIMs**	UI / **LIMs**	UI / **LIMs**
LSD1	3.3	5.5 / **9.8**	3.3 / **6.1**	4.1 / **9.6**
MOA-A	2.9	3.3 / **2.3**	1.4 / **0.9**	2.0 / **2.7**
GAPDH	-0.5	-1.3 / **0.9**	-1.0 / **0.9**	-3.4 / **-1.8**

Figure 2, Gutiérrez-Sanchez M. *et al.*

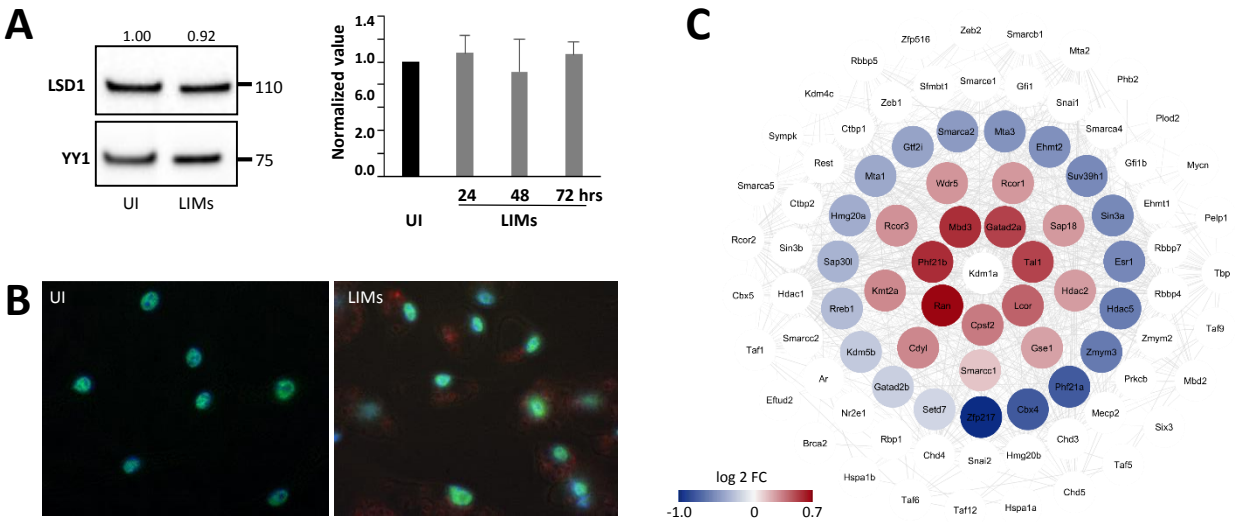

Figure 3, Gutiérrez-Sanchez M. ***et al.***

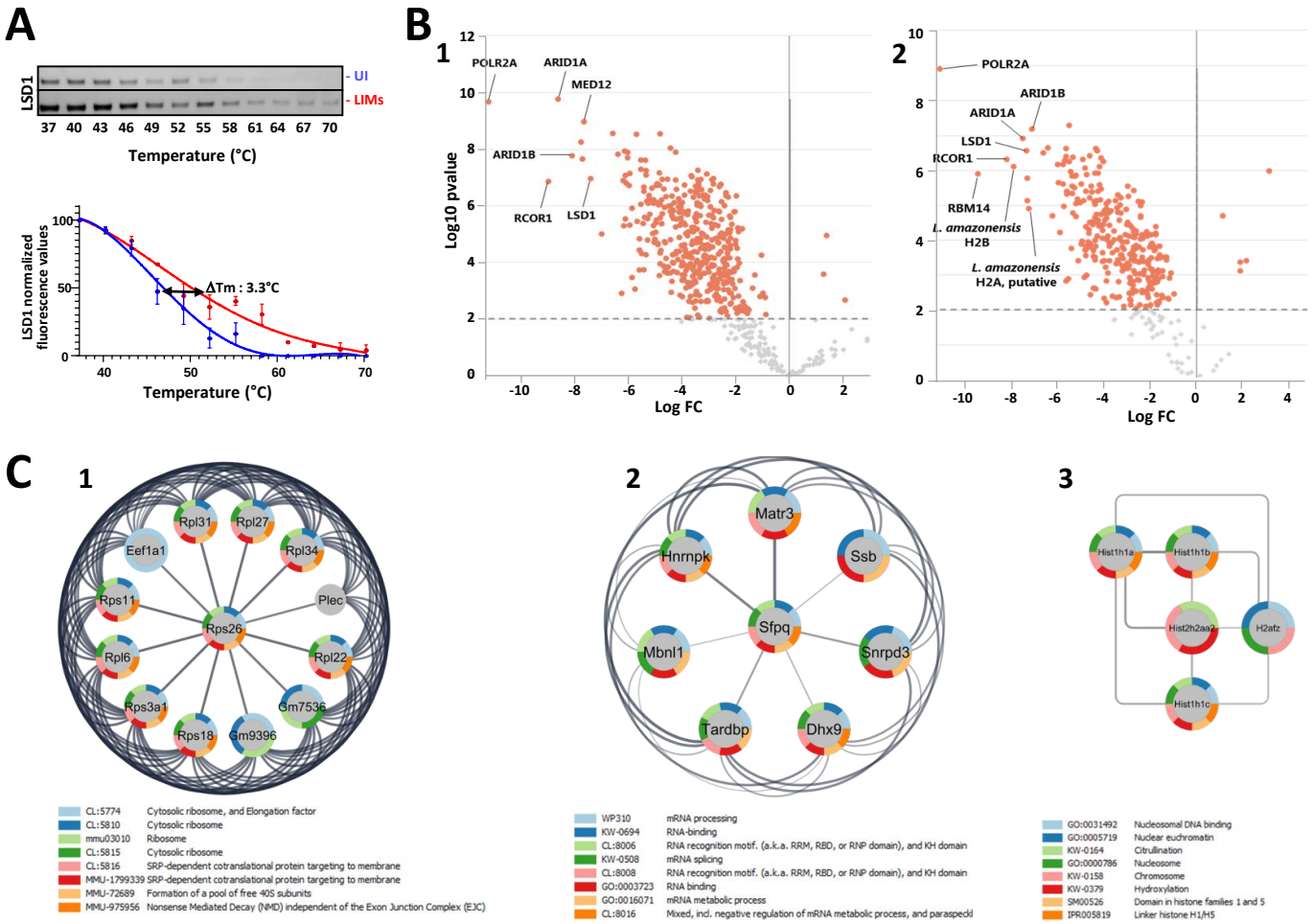

Figure 4, Gutiérrez-Sanchez M. *et al.*

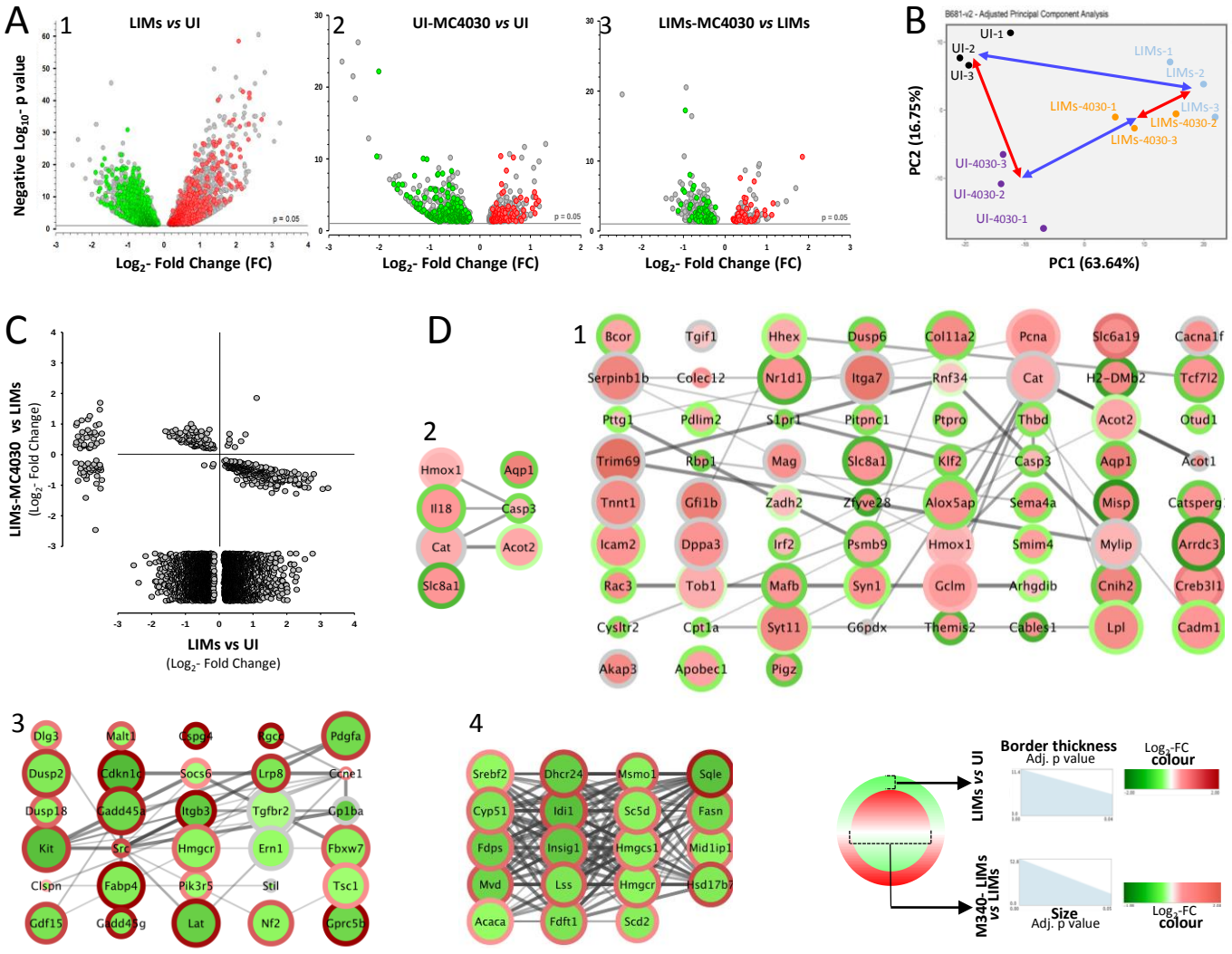

Figure 5, Gutiérrez-Sanchez M. *et al.*