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**FUNCTIONAL AND MECHANISTIC ANALYSES OF THE ROLE OF
HISTONE DEACETYLASES (HDAC3) IN INFLAMMATORY GENE
CONTROL**

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Acronyms and Abbreviations

AP-1	Activator protein 1
ATCC	American type culture collection
BCH110	BRAF-HDAC component 110
bp	Basepairs
BSA	Bovine serum albumin
CBP	CREB-binding protein
Chd1	Chromodomain-helicase-DNA-binding protein 1
ChIP	Chromatin immunoprecipitation
CoREST	Corepressor of REST
CtBP	Carboxyl-terminal binding protein
cDNA	Complementary DNA
DNA	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
Eaf3	Esa1-associated factor 3
E. coli	Escherichia coli
EDTA	Ethylene diamine tetra-acetate
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
GAL	Galactosidase
GCN	Glucose control negative
GO	Gene ontology
Hda1	Histone deacetylase-A
Hos2	High osmolarity sensitivity two
Hst1	Homolog of Sir2p, 1
ING	Inhibitor of Growth

IRF	Interferon regulatory factor
Ku70	Lupus Ku autoantigen protein p70
LPS	Lipopolysaccharides
LSD1	Lysine-specific demethylase 1
Mad/Max	MAX dimerisation protein 1/MYC associated factor X
MAPK	Mitogen activated protein kinase
MBD	Methy-CpG binding domain
MeCP2	Methy-CpG binding protein 2 gene
MEF2	Myocyte enhancer factor 2
Mi2	Dermatomyositis-specific autoantigen
MLL-5	Myeloid/lymphoid leukaemia 5
MTA2	Metastasis-associated protein 2
mRNA	Messenger RNA
MEF	Mouse embryonic fibroblast
Mxi1	Max interactor 1
NCoR	Nuclear receptor corepressor
NF- κ B	Nuclear factor κ B
NLS	Nuclear localisation signal
Nop1	Nuclear protein one
NP40	Nonidet P-40 detergent
N-terminus	NT amino terminus
NuRD	Nucleosome remodelling and histone deacetylase
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCAF	p300/CBP associated factor
PCR	Polymerase chain reaction
PHD	Plant homeodomain

PNPP	P-NitroPhenyl Phosphate
PPAR-g	Peroxisome proliferators activated receptor, gamma
Q-PCR	Quantitative PCR
Rap1	Repressor/activator protein 1
RbAp	Retinoblastoma-associated protein
RBP-1	Retinoblastoma binding protein 1
REST/NRSF	RE1-silencing transcription factor/Neuronal restricted silencing factor
Rpd3	Reduced potassium dependency three
RT-PCR	Reverse transcription-polymerase chain reaction
SA1/SA2	Stromal antigen 1/2
SAGA	Spt-Ada-Gcn5-Acetyltransferase
SANT	SWI3, ADA2, NCoR, and TFIIIB B
Sap	Sin3 associated protein
SBE	Smad-binding element
SET	SU(VAR)3-9, enhancer of Zeste, Trithorax
shRNA	Short-hairpin RNA
SID	Sin3 interacting domain
Sif2p	SIR4 interacting factor 2
Sin3	Switch-independent three
Sir2p	Silent information regulator 2
SirT1	Sir2-like (Sirtuin) 1
SIRT	Sirtuin
SLIK	SAGA-like
SMRT	Silencing mediator for retinoid and thyroid hormone receptors
STAT1	Signal transducer and activator of transcription 1-alpha/beta
STAT3	Signal transducer and activator of transcription 3

Sp1	Specificity protein-1
Srg3	SWI3-related gene product 3
Sum1p	Suppressor of uncontrolled mitosis
Swi/Snf	Switch/sucrose nonfermenting
SWI3	Matting-type switching defective mutant 3
TBL1	Transducin b-like protein 1
TBLR1	Transducin b-like related protein 1
TBP	TATA binding protein
TEMEB	N,N,N',N'-Tetramethyl-1-,2-diaminomethane
TSA	Trichostatin A
WT (wt)	Wild type

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Abstract

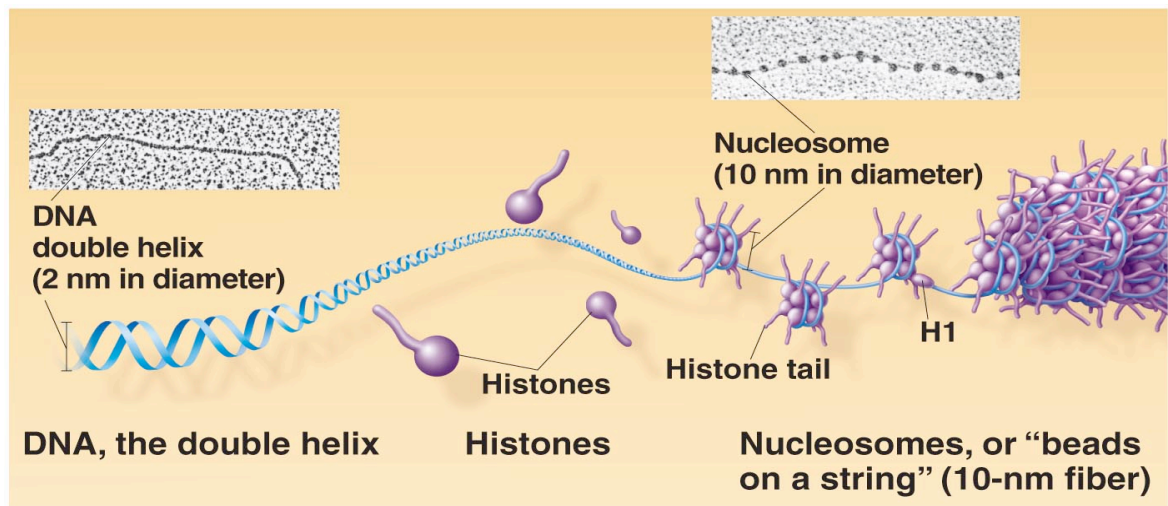
In this study, we investigated the role of individual class I histone deacetylases (HDACs) namely Hdac1, -2 and -3 using retroviral RNA interference to define their specific contribution to control an inducible gene expression program, namely inflammatory gene expression in 3T3 fibroblasts and primary macrophages. In addition to genes showing the expected transcriptional de-repression, we observed 8/20 genes in a test set being down-regulated following individual HDAC depletion. The requirement for HDACs function in gene induction as opposed to the more commonly observed role as transcriptional repressors may either underlie an indirect consequence of impaired HDAC mediated repression or a direct involvement of Hdac1 and Hdac3 in inducible gene activity. Therefore, we extended both the *in vitro* and *in vivo* analyses using conditional knockout (KO) mice. Genetic deletion of Hdac3 indicates that Hdac3 is required for the activation of 45% of the lipopolysaccharides (LPS)-induced genes. Global analysis of histone H4 acetylation showed that transcriptional down-regulation in *Hdac3*^{-/-} cells did not correlate with increased histone acetylation, suggesting the possible involvement of indirect or secondary effects. We found that the LPS-inducible, Hdac3-dependent genes include a large group of interferon- β (IFN β)-inducible genes (eg. IP10, Irf1) and another group (eg. Il-6) that may be regulated by AP-1 family proteins. In addition, gene expression analyses identified interferon-signalling pathway as being impaired in *Hdac3*^{-/-} cells. Basal and inducible Ifn β transcriptions require cJun/AP-1 and the decreased amount of AP-1 family proteins in *Hdac3*^{-/-} cells may explain the lack of Ifn β activation and the increased acetylation in genomic regions.

Introduction

1. Transcriptional regulation and the “histone code”

1.1 Chromatin structure

Work in the last decades has tremendously increased the awareness that chromatin doesn't simply contribute to packaging of DNA in a restricted physical space: conversely, chromatin integrates multiple layers of control over the accessibility and usage of the underlying genetic material. In eukaryotes, the fundamental unit of genome organisation is the nucleosome, which consists of 147 base pairs (bp) of DNA wrapped 1.7 times around an octamer of core histones composed of an H3-H4 histone tetramer and two H2A-H2B histone dimers [1, 2, 17, 18]. A stretch of linker DNA of variable length (between 20 to 60 bp, depending on species, cell type, and chromosomal region) connects each nucleosome to form a “beads-on-a-string” fibre with a diameter of 11 nm [3]. The incorporation of linker histones such as H1 or H5 (a histone H1 variant) subsequently organises the nucleosome arrays into a 30 nm condensed chromatin fibre (Figure 1).



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Figure 1. The organisation of chromatin structure.

Early work revealed that the combination of tight histone-DNA contacts and organisation of nucleosomes into higher order structure imposed an inhibitory effect upon transcription

[4, 5]. On the contrary, disruption of nucleosomal structure allows binding of the transcriptional machinery and other coactivators to their regulatory regions on DNA of genes [6, 7]. It is now apparent that chromatin can exist in an open, relaxed state or a closed, compacted conformation and that the state of the structure can regulate a diverse array of processes such as gene transcription, DNA replication and repair [14].

1.2 Covalent modifications of histones

Changes in the state of chromatin are governed in part by the post-translational modification (PTM) of histones. H2A, H2B, H3 and H4 are four canonical core histone proteins, which are composed of a structured globular domain that is in close contact with the DNA and an N-terminal tail that protrudes from the nucleosome. The conserved histone tails are subjected to a vast array of PTMs, including acetylation, methylation, ubiquitination, phosphorylation and sumoylation (Table 1) [8]. Among them, the most extensively characterised PTMs are lysine acetylation, catalysed by histone acetyltransferase (HAT) enzymes and to a lesser extent, methylation, catalysed by histone methyltransferase (HMT) enzymes. While acetylation of lysine in most cases correlates with increased gene expression [9, 10], lysine methylation of histones has different correlates depending on the modified residue and the extent of its modification (mono- vs. di- vs. tri-methylation) [11-13, 16]. Methylation on Lys4, Lys36 and Lys79 of histone H3 correlates with transcriptional activity, whereas methylation of histone H3 Lys9 and Lys27 is usually detected at repressed genes as well as at heterochromatic genomic regions, including repeats [13, 15]. It is implicit that some PTMs are mutually exclusive, in that the addition of one modification to a lysine residue will block other additional modification on the same amino acid. Methylation of histone H3 Lys9 (H3K9) has a well-established role in repressing transcription, which is believed to be mainly mediated by recruitment of the HP1 (Heterochromatin Protein 1) family of proteins, and particularly HP1a and HP1b. Conversely, H3K9 acetylation is associated with gene regulation, suggesting that the

mutually exclusive nature of these marks may help to reinforce the distinct transcription activity states. Taken together, depending on the type of chemical modification and its location on the histone octamer, these modifications can act to either activate or repress transcription.

Modifications	Position	Enzymes				Recognition Module(s) ^a
		<i>S. cerevisiae</i>	<i>S. pombe</i>	<i>Drosophila</i>	Mammals	
Methylation	H3 K4	Set1	Set1	Trx, Ash1	MLL, ALL-1, Set9/7, ALR-1/2, ALR, Set1	PHD, Chromo, WD-40
	H3 K9	n/a	Clr4	Su(var)3-9, Ash1	Suv39h, G9a, Eu-HMTase I, ESET, SETBD1	Chromo (HP1)
	H3 K27				E(Z)	Ezh2, G9a
	H3 K36	Set2			HYPB, Smyd2, NSD1	Chromo(Eaf3), JMJD
Arg Methylation	H3 K79	Dot1			Dot1L	Tudor
	H4 K20		Set9	PR-Set7, Ash1	PR-Set7, SET8	Tudor
	H3 R2				CARM1	
	H3 R17				CARM1	
Phosphorylation	H4 R26				CARM1	
	H3 S10	Snf1			PRMT1	(p300)
Ubiquitination	H2B K120/123	Rad6, Bre1	Rad6		UbcH6, RNF20/40	(Gcn5)
	H2A K119				hPRC1L	(COMPASS)
Acetylation	H3 K56					(Swi/Snf)
	H4 K16	Sas2, NuA4		dMOF	hMOF	Bromodomain
	Htz1K14	NuA4, SAGA				

^a The proteins that are indicated within the parentheses are shown to recognize the corresponding modifications but specific domains have yet to be determined.

Table 1. Histone modifications associated with transcription. (Li *et al*, *Cell*, 2007) [18]

1.3 Histone acetylation

Studies over the past 15 years, inspired from the initial discovery of histone acetylation by Vincent Alfrey (more than 40 years ago) and then fostered by the identification of a HAT activity in the transcription coactivator Gcn5 [19], have suggested a general correlation between histone acetylation and transcriptional activity. Acetylation involves the HAT-mediated catalytic transfer of an acetyl-group from an acetyl-coenzyme A (acetyl-CoA) molecule to the ϵ -amino group of lysine residues on the histones N-terminal tails (Figure 2a). In the context of a nucleosome, the N-terminal tails of histone strongly associate with DNA through charge interactions, i.e., positively charged histone tails with negatively charged DNA (Figure 2b).

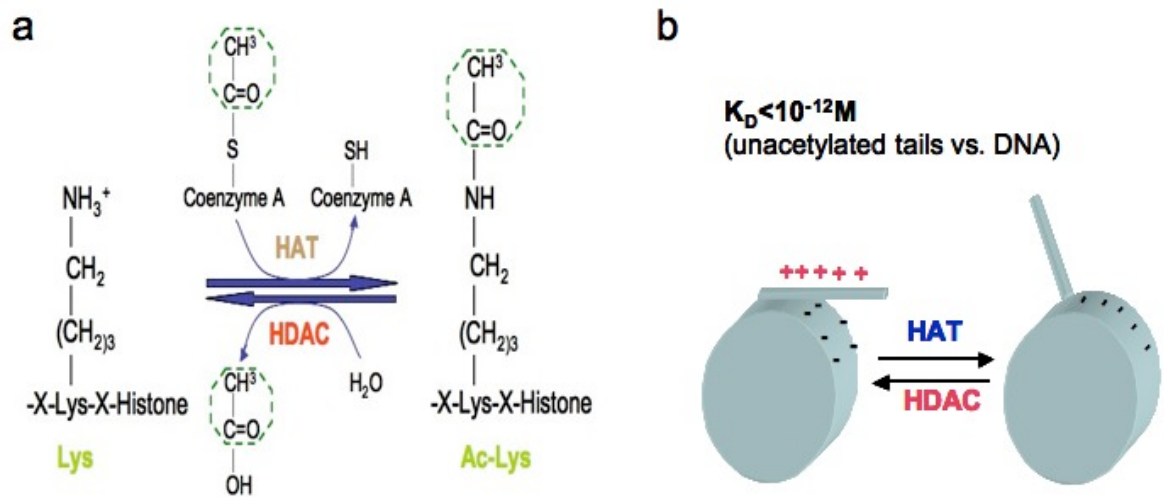


Figure 2. Acetylation is a reversible process.

(a) Equilibrium of steady-state histone acetylation is maintained by opposite activities of histone acetyltransferases and deacetylases. (b) Neutralisation of the lysine residues' positive charge by acetylation lowers the affinity of histone octamers for the negative charged DNA.

Acetylation of lysine residues neutralises the positive charge of histone tails, thus reducing attractive interactions with DNA and increasing its accessibility [26]. The notion that this charge neutralisation effect is sufficient to explain the link between acetylation and gene activation has dominated the field for several years. More recently, this simple electrostatic model has been challenged by the observation that some domains (e.g. bromodomain) are able to specifically recognise acetylated histones, thus suggesting that these modified residues act as platforms for recruitment of coregulator complexes [29]. For example, the bromodomain of Gcn5, the catalytic component of the SAGA complex, preferentially binds histone H4 acetylated at lysine 16 (H4K16) [20] and this is necessary to retain SAGA onto acetylated, open chromatin, after the activator that initially recruited is released [21]. Furthermore, Chd1, a subunit of both the SAGA and SLIK complexes, recognises methylated H3K4 through a chromodomain and deletion of Chd1 disrupts acetylation by SAGA and SLIK both in vitro and in vivo. This has led to the hypothesis that apart from influencing the structure of higher order chromatin via charge effects, combinations of different modifications of histone tails generate specific surfaces that are

recognised by specific coactivators or corepressors, with different combinations of modifications being specifically recognised by alternative interactors (the ‘histone code’ hypothesis) [22] (Figure 3). However, whether histone acetylation has a causal role in gene regulation or simply represents a mark of HATs recruitment to gene promoters and transcription start sites (TSS), is an open issue, since genetic evidence that HATs activity are mainly directed to histones rather than to other substrates (like chromatin remodelers and transcription factors) is still lacking.

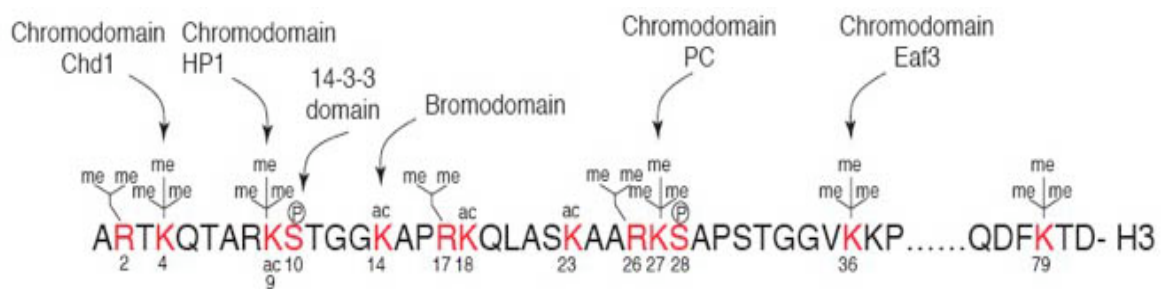


Figure 3. Part of the protein sequence of histone H3, together with factors that are known to bind to modifications.

Residues on histone H3 that can be subject to modifications are in red. Methylation (me) exists in different states: at arginine (R) there can be one or two me groups arranged symmetrically, or asymmetrically, whereas at lysine (K) there can be one, two or three me groups. A single lysine residue (e.g. K9) can be subject to either methylation or acetylation (ac). Serine residues can be phosphorylated (circled P). The factors that bind to modifications are: Chd1 and Eaf3 in budding yeast; HP1 in fission yeast and mammals; and PC and 14-3-3 domains in mammals [115, 116]. Factors with chromodomains are associated with Kme, those with bromodomains with Kac [117] and the 14-3-3 domain with phosphoserine. (Mellor J., *Trends in Genet.*, 2006 [118])

2. Histone Deacetylases

Histone acetylation is a dynamic modification erased by histone deacetylases (HDACs). In mammals, at least eleven distinct HDACs have been described to date that are grouped into three classes based on their sequence homology to the yeast founding members. Class I HDACs (HDAC1, 2, 3 and 8) are homologous in their catalytic sites and are related to

yRpd3 (Figure 4a). Class II HDACs (HDAC4, 5, 6, 7, 9 and 10) are similar to yHda1. Due to the differences in regulatory domains that influence subcellular distribution, class II HDACs are further divided into two subgroups: class IIa (HDAC4, 5, 7 and 9) and class IIb (HDAC6 and 10) (Figure 4b). HDAC11, although sometimes referred to as a class I member, shows a similar degree of homology to both yRpd3 and yHda1 and is thus considered the only member of class IV (Figure 4c). A separate class of HDACs consisting of the Sirtuin (Sir2-related) family members (class III) have histone deacetylase activity but are structurally and evolutionally unrelated to class I, II and IV. All class I, II and IV HDACs are zinc-dependent enzymes, whereas Sirtuins are nicotinamide adenine dinucleotide (NAD⁺) dependent [23].

2.1 Domains and Motifs of Histone Deacetylases

The class I HDACs are around 350-500 amino acids in length, generally localise exclusively to the nucleus (with the exception of HDAC3, which is also cytoplasmic) and comprise of an N-terminal deacetylase domain and a C-terminal tail. HDAC1 and HDAC2 are almost identical, and in addition to the homologous catalytic domains, they share homology in the C-terminal tails, which harbours tandem casein kinase-2 (CK2) phosphorylation sites. Differently from HDAC1 and 2, HDAC3 has only 1 CK2 phosphorylation site. The C-terminal tail of HDAC8 has a conserved motif for protein kinase A phosphorylation in place of the CK2 sites. The enzymatic activity of class I HDACs is regulated by the phosphorylation of the Serine (Ser421/423 on HDAC1; Ser422/424 on HDAC2; Ser424 on HDAC3 and Ser39 on HDAC8 residue(s) of the protein.

Class II HDACs span about 1000 amino acids and their catalytic domain contains several conserved motifs that are significantly different from those of class I HDACs. Besides their deacetylase domain, mammalian class IIa HDACs have sequence similarity in the extended long N-terminal domains and the C-terminal tails. The N-terminal extensions

contain binding sites for some substrates (like myocyte enhancer factor-2, MEF2) and 14-3-3 proteins, which are important for function and regulation of class IIa members. The activities of class IIa HDACs as transcriptional repressors are regulated in part by nucleocytoplasmic shuttling mediated by 14-3-3 proteins. Phosphorylation-dependent binding of 14-3-3 proteins to the N-terminal region masks the nuclear import signal and prevents nuclear import. HDAC6 (class IIb) differs from class IIa HDACs in that it contains a second deacetylase domain and a C-terminal zinc finger. The other member of class IIb, HDAC10 has a Leucine-rich C-terminal domain and an N-terminal half that is highly similar to the first deacetylase domain of HDAC6 [24].

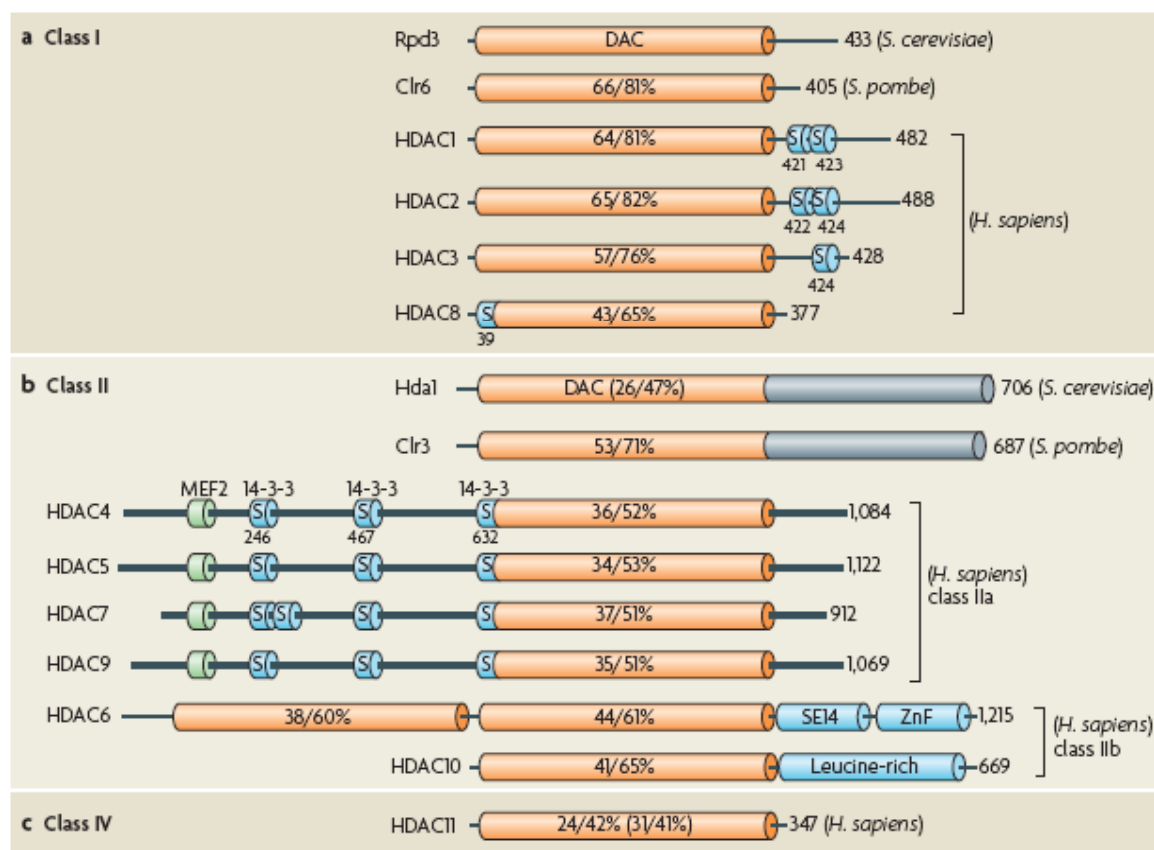


Figure 4. Domain organisation of classical HDACs from yeast and humans.

The histone deacetylases (HDACs) are grouped into different classes according to sequence similarity to yeast prototypes. In mammals, class I (Rpd3-like) members include HDAC1, -2, -3 and -8 (a); class II members are HDAC4, -5, -6, -7, -9 and -10 (b), which are related to yeast Hda1. Class IV consists of HDAC11 (c). Class II is further divided into two subclasses: IIa (HDAC4, -5, -7 and -9) and IIb (HDAC6 and -10). The total number of amino acid residues in each deacetylase is

shown on the right. Many of the deacetylases have isoforms that result from alternative splicing; for simplicity, the number of amino acids refers to the longest isoform. The deacetylase (DAC) domain is depicted as an orange cylinder, and the percentage amino acid sequence identity/similarity to that of Rpd3 (for class I) or Hda1 (class II/IV) is shown. The sequence identity/similarity of Hda1 and HDAC11 to Rpd3 is given in brackets. The C-terminal domains (grey) of Hda1 and Clr3 are homologous (identity/similarity: 26/57%). Thick black lines represent similar N-terminal domains and C-terminal tails of class IIa HDACs. Myocyte enhancer factor-2 (MEF2)-binding motifs are depicted as short green cylinders, whereas 14-3-3 binding motifs are shown as short blue cylinders labelled with 'S' (for Ser). Clr3, cryptic locus regulator-3; Hda1, histone deacetylase-1; *H. sapiens*, *Homo sapiens*; Rpd3, reduced potassium dependency-3; *S. cerevisiae*, *Saccharomyces cerevisiae*; SE14, Ser Glucontaining tetradecapeptide repeats; *S. pombe*, *Schizosaccharomyces pombe*; ZnF, ubiquitin-binding zinc finger. (Yang and Seto, *Nat. Rev. Mol. Cell Bio.*, 2008 [24])

2.2 Multiple Class I HDAC(s)-containing complexes

Within cells, HDACs exist in complexes with a number of additional transcriptional repressors and corepressors. With the exception of HDAC8, all class I members can function as the catalytic subunits of several multi-protein complexes. HDAC1 and HDAC2 are typically found in the three ubiquitously expressed corepressor complexes, namely the mSin3A complex, containing RbAp46, RbAp48, RBP1, SAP18, SAP30 and ING1/2; the nucleosome remodeling deacetylase (NuRD) complex, containing the ATP-dependent chromatin remodelling enzymes Mi-2a/b, RbAp46, RbAp48, MTA1-3, MBD2, MBD3; and the corepressor of RE1-silencing transcription factor (CoREST) complex, which includes the histone demethylase LSD1, BHC80 and CtBP1 (Figure 5a). While Sin3 complexes are conserved from yeast to mammals, the other complexes are only present in metazoans or plants. Mammals have two Sin3 homologues, Sin3A and Sin3B, that form the Sin3A and Sin3B complexes, which correspond to the yeast Rpd3L and Rpd3S complexes, respectively (Figure 5c). In Sin3 complex, mSin3A serves as the scaffold for

the assembly of the complex while SAP18/30 contributes to stabilise protein interactions [25]. Conversely, HDAC3 associates to, and is activated by, two homologous transcriptional corepressors, silencing mediator of retinoic acid and thyroid hormone receptors (SMRT) and nuclear receptor corepressor (NCoR) [27, 28, 30] (Figure 5b).

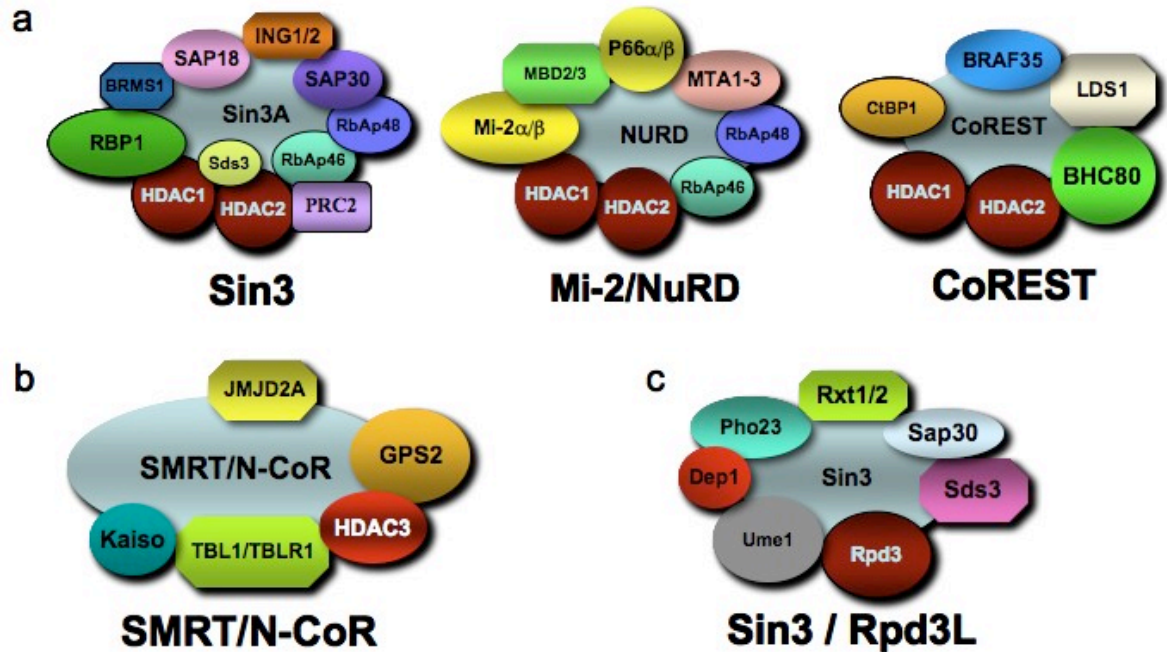


Figure 5. Multiple Class I HDACs complexes.

(a) In mammals, HDAC1 and HDAC2 interact with each other to form the catalytic core of several multi-subunit complexes, namely: Sin3, Mi-2/NuRD and CoREST. (b) HDAC3 is found different complexes from HDAC1/2, namely: SMRT and N-CoR. (c) The Sin3 complexes are conserved from yeast to mammals and the mammalian Sin3a complex correspond to the yeast Rpd3L-containing Sin3 complex.

Class I HDACs exhibit very weak activity in isolation, and interaction with additional complex components is required for their function. Studies over the last 10 years have demonstrated that HDACs interaction with SANT domain-containing proteins within a complex is crucial for both complex integrity and deacetylase activity [31-34]. The first clue that underscores the importance of associated proteins in modulating HDACs enzymatic activity in complexes came from the purification of the NuRD complex. Study by Zhang *et al* [35] showed that the HDACs activity of NuRD complex containing only

RbAp46/48 and HDAC1/2 (core complex) was severely compromised when compared to that of the native purified complex. The addition of MTA2 to the core complex re-established deacetylase activity of the complex and MBD3 was found to mediate the association of MTA2 to the NuRD core complex. CoREST, another SANT domain-containing protein, together with the histone demethylase LSD1 are important for deacetylase activity of CoREST complex [36]. Similarly, HDAC3 deacetylase activity is stimulated through its association with the SANT domains of N-CoR and SMRT corepressors [31].

While class I HDACs are believed to be recruited to selected genomic locations, they lack DNA binding activity and therefore specificity. Inclusion of HDACs in complexes is also relevant for targeting to specific genomic loci. Both Sin3A and NuRD complexes contain the histone-binding proteins RbAp46 and RbAp48. Furthermore, some subunits of the HDAC1/2 complexes have been identified that contain domains such as the PHD finger that bind specific histone marks and therefore contribute to selective recruitment. For example, ING2, which recognises tri-methylated lysine 4 of histone H3 (H3K4me3) targets the Sin3A complex to deacetylate nucleosomes at active TSSs in a methylation-dependent manner [37, 38]. In yeast, the chromodomain of Eaf3 and the PHD domain of Rco1 in the Rpd3S complex cooperate to bind tri-methylated lysine 36 of histone H3 (H3K36me3) [40]. More recently, BHC80, a PHD finger-containing protein of the CoREST corepressor complex, was reported to bind unmethylated lysine 4 at histone H3 (H3Kme0) and this interaction was abrogated by methylation of H3K4. Using chromatin immunoprecipitation (ChIP), it was demonstrated that BHC80 and LSD1, a demethylase for H3K4me and H3K9me, depend reciprocally on one another to associate with chromatin [39]. Interestingly, histone demethylation has also been shown to stimulate deacetylase activity, which in turn is required for optimal demethylation [36].

2.3 New roles for (H)DACs

As discussed above, addition of an acetyl group to a lysine residue neutralises the positive charge, resulting in a significant impact on the electrostatic properties of the protein. The initial discovery that p300 and CBP acetylate the tumor suppressor p53 [41], first demonstrated that some HATs can also acetylate non-histone substrates. Since then, hundreds of non-histone proteins have been identified as substrates for HATs (Table 2). Acetylation of a protein can bring about many different effects, such as changes in protein stability, protein-protein interactions, protein localisation and DNA binding. For instance, *in vitro* and *in vivo* experiments have shown that acetylation of multiple lysine residues on the p53 C-terminal DNA binding regulatory domain, activates its sequence-specific DNA binding activity, thus increasing transcription of its target genes [41-43]. Both acetylation and ubiquitination often occur on the same lysine residue and competition between these two modifications can influence the stability of the substrates [44]. As the sites of acetylated lysines in p53 overlap with those that are ubiquitinated, acetylation of p53 reduces its ubiquitin-mediated targeting to the proteasome and promotes protein stability. In line with this model, it was shown that MDM2 promotes the degradation of p53 by recruiting the HDAC1 complex to acetylated-p53 molecules [45]. Signal transducer and activator of transcription 3 (STAT3), which is activated by cytokine signalling, is acetylated by p300/CBP at lysine 685 upon treatment. Cytoplasmic acetylation of STAT3 triggers dimerisation and subsequent nuclear translocation [47, 48]. Mutation of this acetylation site impairs STAT3 dimerisation, and consequently reduces cytokine-stimulated DNA binding and transcriptional activity.

Biological implication	Proteins affected by acetylation	
Protein stability	Acetylation increases stability p53, p73, Smad7, c-Myc, Runx3, AR, H2A.z, E2F1, NF-E4, ER81, SREBP1a, HNF6, BACE1	Acetylation decreases stability GATA1, HIF-1 α , pRb, SV40 T-Ag
DNA binding	Increased DNA binding p53, SRY, STAT3, GATA transcription factors, E2F1, p50 (NF κ B), ER α , p65 (NF κ B), c-Myb, MyoD, HNF-4, AML1, BETA2, NF-E2, KLF13, TAL1/SCL, TAF(1)68, AP endonuclease	Decreased DNA binding YY1, IIMG-A1, IIMG-N2, p65 (NF κ B), DEK, KLF13, Fen-1
Gene expression	Transcriptional activation p53, HMG-A1, STAT3, AR, ER α (basal), GATA transcription factors, EKLf, MyoD, E2F1, p65(NF κ B), GR, p73, PGC1 α , MEF2D, GCMA, PLAG1, PLAGL2, Bcl-6, β -Catenin, KLF5, Sp1, BETA2, Cart1, RIP140, TAF(1)68	Transcriptional inactivation ER α (ligand-bound), HIF-1 α , STAT1, FOXO1, FOXO4, RIP140
Protein interactions	Enhanced STAT3, AR, EKLF, Importin A, STAT1, TFIIB, α -Tubulin, actin, cortactin	Decreased p65(ReIA), Ku70, Hsp90
Localisation	Ac $\rightarrow$ nucleus PCAF, SRY, CtBP2, POP-1, HNF-4, PCNA Sub-nuclear WRN, PCNA	Ac $\rightarrow$ cytosol c-Abl, p300, PAP
mRNA stability	Increased p21, Brm	Decreased Tyrosinhydrolase (Th), eNOS
Enzymatic activity	Enhanced p300, ATM	Decreased PTEN, HDAC1, Mdm2, ACS, Neil2, Pol β
Mitochondrial proteins	ACS (Ac-CoA-Synthetase), Sod1/2, Profilin I, Thioredoxin; multiple components of metabolic and oxidative phosphorylation machinery	
Viral proteins	E1A, S-HDAg, L-HDAg, HIV Tat, SV40 T-Ag	

Table 2. Selected acetylated non-histone proteins (Spange et al, *J. Biochem. Cell Bio* 2009)

[119]

3. Histone Deacetylases and transcriptional activation

3.1 Genome-wide functional analysis in yeast

The general paradigm is that acetylation is associated with gene activation, whereas lack of acetylation tends to correlate with repression, with HATs and HDACs acting in concert to establish balanced levels of transcription [54-56]. Genome-wide analyses of HDACs distribution, histone acetylation and gene expression has provided a comprehensive view of how each individual HDAC targets and regulates yeast genes (Table 3 [49, 50-52, 140]). An important conclusion of such genome-wide analyses is that HDACs are involved in both transcriptional activation and repression [57, 59, 140]. A similar conclusion was reached in studies of Hos2, the *Saccharomyces Cerevisiae* Hdac3 ortholog, which appears to be mainly involved in transcriptional activation. Upon galactose induction, Hos2 is recruited to intragenic regions of GAL and ERG11, where it is required for full transcriptional activation [50]. Removal of Hos2 leads to hyperacetylation of histone H3 and H4 in the coding region of active genes, which is believed to cause defects in

transcriptional activation, although the underlying mechanisms linking hyperacetylation to transcriptional impairment are unknown [50]. Similarly, by directly comparing nucleosome density, histone acetylation patterns and HDACs binding in fission yeast

(*Schizosaccharomyces pombe*), Wiren *et al* [52] reported that Hos2 promotes high-level expression of growth-related genes upon intragenic deacetylation of histone H3 and H4, mainly at lysine 16 of histone H4 (H4K16Ac).

Other deacetylases have also been demonstrated to play a role at active genes in yeast. In the case of Rpd3, a HDAC1/2 ortholog, osmotic stress induces the recruitment of the Rpd3-Sin3 deacetylase complex to the promoter region of some osmo-responsive genes and positively regulate their expression [49]. Although genome-wide expression analysis show that RPD3 deletion (*Rpd3Δ*) results in more genes being down-regulated (264 genes) than up-regulated (170 genes) [59], it remains a question of how deletion of HDACs results in the down-regulation of gene activity. A global analysis of histone acetylation by Robyr *et al* [58], showed that transcriptional down-regulation in *Rpd3Δ* strain did not correlate with increased histone acetylation, suggesting the possible involvement of indirect or secondary effects. It is important to notice that this conclusion was based on the assumption that the *direct* effects of HDACs on gene activity must be accounted for exclusively by histone deacetylation, even though the cellular acetylome that is under HDACs control is extremely broad [60]. Moreover, a direct role of Rpd3 in gene activation is supported by other observations. Genes down-regulated by TSA overlap those down-regulated in *Rpd3Δ* and some of these genes were rapidly down-regulated within a 15 minutes exposure to the compound, suggesting that Rpd3 may function as direct transcriptional activator [59].

There is some evidence that the ability of HDACs to activate or repress transcription is dependent on their lysine specificity. ChIP to acetylated lysine residues antibodies has shown that Rpd3 is required for deacetylation of all lysines in the core histones H3, H4, H2A, and H2B except for H4K16Ac [61]. Conversely, Hos2 is required for the preferential

deacetylation of histone H3 and H4 sites including H4K16Ac [50]. Interestingly, histone H4K16 hypoacetylation is linked to the binding of Bdf1, a bromodomain-containing transcription factor, to active genes, illustrating that global activity of HATs and HDACs mediates acetylation turnover at active genes [51, 141].

yHDAC	Functional category (affected)	Binding map
Rpd3	Ribosomal proteins, Cell cycles	Promoter, Intergenic regions
Hos2	Major metabolic pathways, Protein synthesis (Preferentially recruited to highly transcribed genes)	Open reading frames
HDA1	Carbon metabolism	Intergenic regions, preferentially recruited to subtelomeric (HAST) containing a high proportion of genes induced by nutritional stress

Table 3. Genome-wide analyses of *S. Cerevisiae* HDACs-binding sites and gene expression profiling.

3.2 Functional roles of class I HDACs in mammals

The ubiquitous expression and high homology among class I HDACs suggest functional redundancy. However, genetic deletion studies of individual members of class I HDACs in mice resulted in lethality in all cases, demonstrating a division of labour among the different enzymes of this class. Targeted deletion of *Hdac1* led to embryonic lethality at embryonic day 10.5 (E10.5), due to a general growth retardation and severe proliferation defects that are associated with an increased expression of the cyclin-dependent kinase inhibitors p21 and p27 [63]. Total cellular HDAC activity is significantly reduced in *Hdac1*-null embryonic stem (ES) cells despite an increase in the level of HDAC2 protein, indicating that HDAC1 is a major contributor to bulk histone deacetylation in ES cells. Microarray analysis of gene expression revealed that in addition to the 4% of the genes being up-regulated, 3% of the genes were down-regulated following *Hdac1* deletion [64]. Although transcriptomic studies do not allow discriminating direct vs. indirect effects,

these data suggest that HDAC1 may exert both positive and negative effects on gene expression.

The function of HDAC2 in vivo remains controversial. Germ-line deletion of *Hdac2* is reported to exhibit embryonic lethality in one case [63] while other studies have found that postnatal lethality is accompanied with a transient decrease in body size and severe cardiac malformations, owing to excessive cardiomyocyte proliferation [67, 69, 70].

Disruption of HDAC3 results in embryonic lethality at around E9.5 or earlier owing to gastrulation defects [71]. The target genes responsible for this phenotype have yet to be defined. *Hdac8*-null mice were recently reported to die within 4-6 h after birth with haemorrhages in the brain [68]. Microarray profiling of *Hdac8*-null neural crest cells revealed the up-regulation of multiple homeobox genes. This deregulation resulted in the ectopic expression of Hox genes in the anterior parts of the skull, which presumably leads to mis-patterning (Table 4).

Class I	Cellular Localisation	Time of lethality	Phenotype
HDAC1	Nucleus	E10.5	Proliferation defects in ES cells
HDAC2	Nucleus	P1	Perinatal death from cardiac malformation, Cardiomyopathy from heart-specific deletion of both HDAC1 and HDAC 2
HDAC3	Cytoplasmic and Nucleus	E9.5	Gastrulation defects, Liver hypertrophy
HDAC8	Nucleus	P1	Craniofacial defects

Table 4. Main features of class I HDAC knockout mice.

Because of embryonic or prenatal lethality, mice carrying conditional (floxed) null alleles for class I HDACs were generated. Conditional deletion of either *Hdac1* or *Hdac2* did not yield any obvious phenotype in a variety of tissues, including the heart, brain, endothelial cells, smooth muscle, and neural crest cells, whereas deletion of both genes together resulted in neonatal lethality, suggesting that there is a functional redundancy of HDAC1 and HDAC2 proteins in vivo [65-67]. Liver-specific *Hdac3* deletion resulted in hepatomegaly owing to the disruption of lipid and cholesterol homeostasis. Deletion of

Hdac3 apparently caused the loss of repression mediated by nuclear hormone receptors such as PPARs, THR, liver X receptor and retinoid X receptor, which was associated with modest increases in the acetylation of histone H4K5, H4K8 and H4K12 [74]. These data were interpreted as evidence of a role of HDAC3-NCoR/SMRT complexes in nuclear hormone-dependent gene repression. In addition, using *Hdac3*-null mouse embryonic fibroblasts (MEFs) generated from *Hdac3* conditional knockout mice, Bhaskara *et al* [71] reported that loss of *Hdac3* results in major cell cycle defects, ultimately leading to apoptosis, a result consistent with prior RNAi-mediated knockdown studies [72, 73].

3.3 Inhibiting HDACs

In recent years, inhibition of HDACs has emerged as potential therapeutic approach for cancer and inflammatory diseases. A large number of HDAC inhibitors (HDACi) have been purified from natural sources or synthetic peptides and some of them, including sodium butyrate (NaBu), valproic acid (SAHA), AN-9, MS-275 and depsipeptide have progressed to clinical trials [75, 76]. Depending on the chemical class to which it belongs, the dosage (ranging from nM to mM) used (Table 5), and time of exposure to the cells, HDACi may selectively inhibit different HDACs and induce, to a variable extent, differentiation, growth arrest or apoptosis *in vitro* and *in vivo* [77, 78].

HDAC	IC ₅₀ (μM)					
	Apicidin	MS275	MGCD-0103	SAHA	TSA	NaBu
Hdac1	> 10	0.368	0.05	0.04	0.005	351
Hdac2	0.12	0.3	0.2	0.065	-	-
Hdac3	0.04	1.2	2	0.04	0.0052	916
Hdac8	0.57	82.4	>20	0.077	0.486	284
Hdac6	no	>100	nd	0.017	0.016	-
Hdac10	-	94.7	>20	0.057	0.024	>3000

Table 5. Different HDACi and their IC₅₀ (μM) for HDAC1, 2, 3, 8, 6 and 10.

Remarkably, when treated with HDACi, normal cells are nearly always more resistant to apoptosis than tumour cells, making HDACs an attractive target for anti-cancer therapy

[77]. The general assumption is that inhibition of HDACs will lead to a widespread up-regulation in genes activity, resulting for instance in increased expression of tumour suppressor genes. However, blocking deacetylation has in fact complex outcomes for several reasons, including the interplay between acetylation and other modifications, as well as the presence of non-histone substrates that may greatly contribute to the final transcriptional phenotype. In fact, gene expression profiling of mammalian cells treated with HDACi has shown that of the large amount of genes affected by the treatment (5%-10% of all genes) similar proportions of them were up- and down-regulated [79]. The study by Rada-Iglesias *et al* showed that treatment of hepatocarcinoma HepG2 cells and colon adenocarcinoma HT-29 cells with either NaBu or TSA for up to 12h lead to a decrease in expression of some genes despite global increases of histone acetylation [80]. The patterns of alterations in genes expression induced by different HDACi in various transformed cell lines are usually similar [81-83], although there are some definite differences plausibly due to a different degree in the inhibition of individual HDAC or other secondary and downstream effects.

4. Inflammation and its transcriptional control

Inflammation is a process by which the body reacts to remove detrimental stimuli caused by tissue injury, irritation and microbial infection. The early phases of the host response to infection depend on innate immunity, which recognises invading pathogens and rapidly induces a number of antimicrobial and inflammatory responses. Depending on the type of inflammatory stimulus, it elicits specific signal transduction pathway in host cells, resulting in the activation of proinflammatory genes via both transcriptional and post-transcriptional mechanisms.

4.1 Inflammatory response to LPS

The Toll-like receptors (TLR) are germline-encoded pattern recognition receptors (PRRs) that are responsible for detecting components of foreign pathogens collectively indicated as pathogen-associated molecular patterns (PAMPs) [84]. TLRs are type I transmembrane proteins, characterised by N-terminal leucine-rich repeats (LRRs) and a transmembrane region followed by a cytoplasmic Toll/IL-1R homology (TIR) domain. Ten and twelve TLRs have been identified in humans and mice respectively, and each receptor appears to be involved in specific PAMPs recognition (Table 6).

PRRs	Localization	Ligand	Origin of the Ligand
TLR			
TLR1	Plasma membrane	Triacyl lipoprotein	Bacteria
TLR2	Plasma membrane	Lipoprotein	Bacteria, viruses, parasites, self
TLR3	Endolysosome	dsRNA	Virus
TLR4	Plasma membrane	LPS	Bacteria, viruses, self
TLR5	Plasma membrane	Flagellin	Bacteria
TLR6	Plasma membrane	Diacyl lipoprotein	Bacteria, viruses
TLR7 (human TLR8)	Endolysosome	ssRNA	Virus, bacteria, self
TLR9	Endolysosome	CpG-DNA	Virus, bacteria, protozoa, self
TLR10	Endolysosome	Unknown	Unknown
TLR11	Plasma membrane	Profilin-like molecule	Protozoa

Table 6. TLRs and their ligands (Takeuchi and Akira, *Cell* 2010) [84]

TLR4, the founding member of the TRL family, interacts with myeloid differentiation factor 2 (MD2) to form a TLR4-MD2 complex on the cell surface that in turn, serves as the main binding component for bacterial lipopolysaccharide (LPS). In response to stimulation with LPS, TLR4 can activate both the MyD88 and TRIF -dependent signalling pathway (Figure 6). In the MyD88-dependent signalling pathway, interaction of TLR4 with the adaptor molecule MyD88 initiates a common signalling cascade that leads to the nuclear translocation of the transcription factors NF- κ B and AP-1. Conversely, activation of TRIF -dependent signalling pathway relies on the interaction of TLR4 with the adaptor protein Toll-interleukin 1 receptor (TIR) domain containing adaptor protein (TIRAP) that leads to the activation of transcriptional factors IFN regulatory factor 3 (IRF3) and late-phase NF-

κ B, that control the induction of inflammatory cytokines and type I interferon (IFN) [84, 85].

IRF3 is primary responsible for the activation of type I IFN genes downstream of TLR4 activation. IRF3 resides in the cytosol in a latent form in unstimulated cells. Upon bacteria infection, specific serine residues in the C-terminal (regulatory) regions are phosphorylated, which allows phospho-IRF3 (pIRF3) dimerisation and its subsequent nuclear translocation. Dimerised pIRF3 binds specific target sequences known as IFN-stimulated response elements (ISRE, A/GNGAAANNGAAACT) localised in the promoter of type I IFN genes (and IFN- β in particular) and certain chemokine genes like Ccl5 [86, 87]. In the case of IFN- β gene promoter, other transcription factors such as NF- κ B and activator protein 1 (AP1) are also recruited to form a multi-molecular assembly (enhancesome) required to efficiently initiate transcription.

Activation of the IFN- β gene and the rapid release in the extracellular medium of IFN- β triggers a paracrine loop resulting in the activation of the Janus kinase-signal transducer and activator of transcription (JAK/STAT) signalling pathway. IFN- β acts via a ubiquitously expressed heterodimeric receptor composed of IFNAR1 and IFNAR2 subunits. IFN- β binding induces the dimerisation of receptor subunits, and then to auto- and trans-phosphorylation of the two receptor-associated JAK tyrosine kinases (Tyk2 on IFNAR1 and Jak1 on IFNAR2). Phosphorylation of JAKs results in phosphorylation of the intracellular domain of IFNAR1, creating the docking sites for STAT2, which then undergoes phosphorylation and serves as a platform to recruit and phosphorylate STAT1. The phosphorylated STAT1/STAT2 heterodimers subsequently dissociate from the receptor and get rapidly translocated from the cytoplasm into the nucleus, where they complex with IRF9 to form a heterotrimeric complex known as IFN-stimulated gene factor 3 (ISGF3). ISGF3 binds to the upstream regulatory consensus sequences (ISRE, AGTTT(N)₃TTTC) of type I IFN inducible genes and initiates their transcription. IFN- β also induces to a lower extent the formation of phosphorylated STAT1 homodimers which

activate transcription by binding to the IFN- γ -activated sequence (GAS, TTC (N)₂₋₄GAA) [88-91].

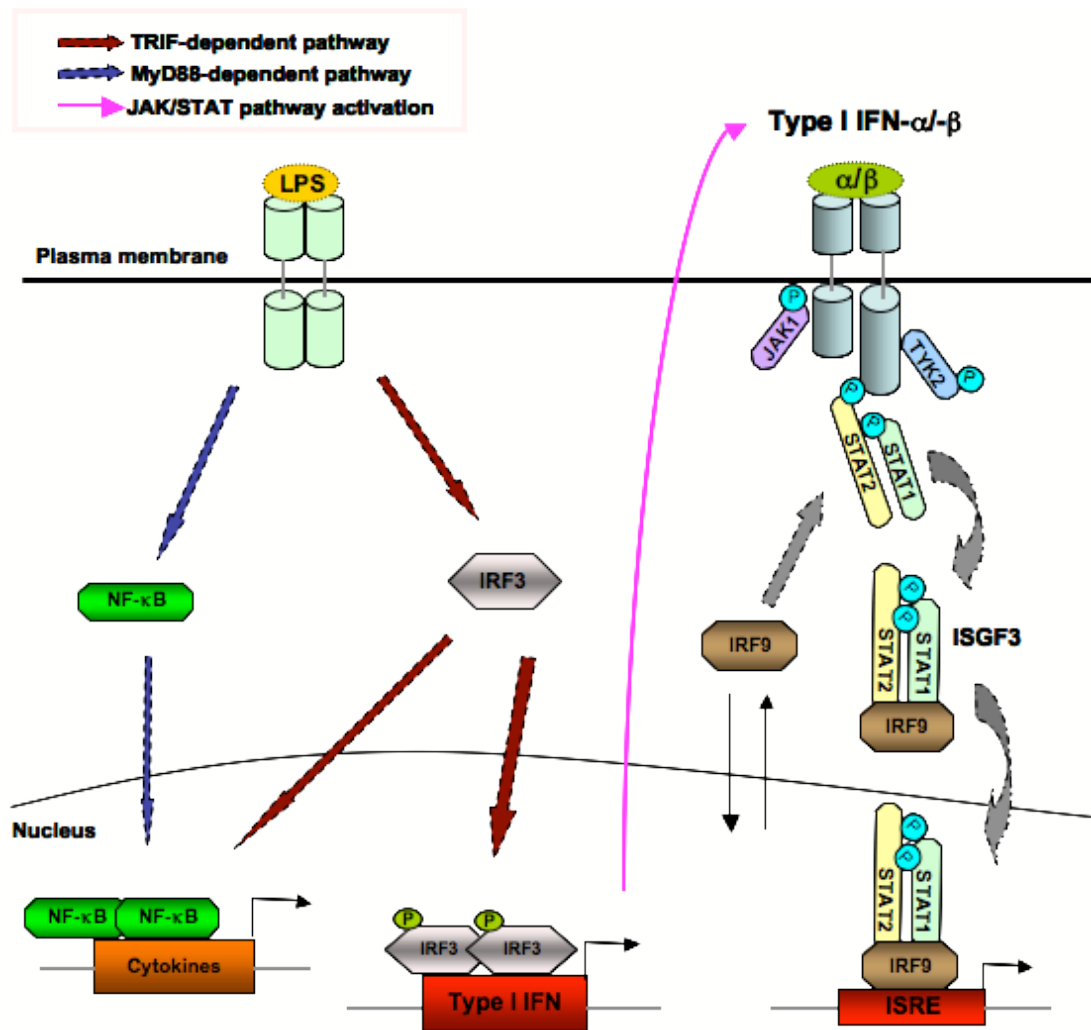


Figure 6. TLR4 Signalling pathways crosstalk with JAK/STAT Signalling pathways.

TLR4 in complex with MD2 engages LPS. The formation of a receptor multimer composed of two copies of the TLR-MD2-LPS complex initially transmits signals for the early-phase NF- κ B by activating the MyD88-dependent pathway. TLR-also recruits TRAM and TRIF (TRIF-dependent pathway), activating both late-phase NF- κ B and IRF3 for the induction of type I IFN genes. Accumulation of type I IFNs (IFN- α 4 or IFN- β) stimulate the activity of Jak1 and Tyk2 proteins, leading to STAT2 tyrosine phosphorylation. The STAT2 phospho-tyrosine is a docking site for latent STAT1. The activated ISGF3 is a heterodimer of pSTAT1 and pSTAT2 in association with IRF9, which alone can enter the nuclear, but is retained in the cytoplasm by interactions with STAT2.

4.2 HDACs and inflammatory gene expression

During inflammation, innate immune cells, such as dendritic cells (DCs) and macrophages (MΦ) initiate host immune responses against infectious agents by engaging PRRs, which activate the diverse mechanisms that have evolved to regulate distinct sets of inducible proinflammatory genes. It is now known that the differential regulation in proinflammatory genes activation, is not only governed by the molecular events orchestrated in nucleus by transcriptional factors, but that inducible chromatin modifications also act as an additional restriction point [92, 93, 100]. As a general rule, condensed chromatin results in transcriptional repression, whereas transcriptional activation requires open chromatin. Inhibition of HDACs activity would therefore, results in relaxation of heterchromatin and increase transcriptional activity. Studies in the last years have tried to assess the contribution of HDACs activity to TLR-regulated gene induction and the use of HDACi has emerged as potential therapeutic drug in immunotherapy. While some studies have lead to the identification of some TLR-inducible genes in which HDACs act as a classical transcriptional repressor [94, 95], others have also identified genes that require HDAC activity for efficient induction in response to LPS. HDACi have been shown to exert anti-inflammatory effects in a range of disease models in mouse, including septic shock [96], asthma [97] and experimental autoimmune encephalomyelitis [98], by generally reducing the production of proinflammatory cytokines. For example, administration of SAHA (10-50 mg/kg, *per os*) reduces greater than 50% decreased in circulating cytokines (TNF- α , IL-1- β , IFN- γ and IL6) during experimental endotoxemia, as well as to attenuate Con A-induced hepatic injury [96]. In addition, TSA and FR901228 have also been shown to have an immunosuppressive effect on CD4 T-cell proliferation by inhibiting the IL-2 production and subsequent CD154 expression on effector T-cells in a dose-dependence manner. *In vivo* studies also showed that FR901228 treatment could inhibit hyperglycemia and reverse the development of diabetes in NOD mice [99].

The transcription-activating role of HDACs is perhaps best exemplified by *in vitro* studies of cytokine-inducible regulation. General inhibition of HDAC activity with HDACi in mammalian cells has results in a decrease rather than increase in expression of IFN- γ -stimulated genes [57, 111, 112]. Genes expression profiling of cells treated with both IFN γ and TSA resulted in an almost universally impairment of IFN-stimulated expression; transcriptional induction of ISG54 and ISG15 reduced by 60% and 75% respectively. Similarly, TSA-treated cells have failed to induce IFN- γ -stimulated genes mediated by IRF5 in response to viral infection [112], preventing the cells from establishing a cellular antiviral state. Using siRNAs specific for HDAC1, HDAC2 and HDAC3, Klampfert *et al* [112] shows that IFN γ -driven gene activation requires HDACs activity.

4.3 Mechanistic regulation of HDACs and inflammatory transcriptional factors

Numerous transcription factors including NF- κ B, AP-1, IRFs and STATs that are key regulators of both the primary and secondary responses to inflammatory stimuli have been identified to date. Transcription factors, together with chromatin contribute to the selective regulation of inducible proinflammatory genes. In addition to histone acetylation, which facilitates the opening of chromatin structure, acetylation of transcription factor itself can also contribute to the state of gene activity.

NF- κ B family members namely RelA/p65, c-REL, RELB, p50 and p52 are crucial transcription factors that control the expression of hundreds of inflammatory genes in response to both acute and chronic inflammatory stimulation. Using chromatin immunoprecipitation (ChIP), it has been demonstrated that different target genes can recruit NF- κ B with different kinetics, depending on constitutive or inducible histone H4 modifications in their chromatin structure upon cell stimulation. The ability of NF- κ B to drive gene expression is also dependent on the specific combinations of NF- κ B complex, capable to bind a given I κ B sites [102]. Over the last years, NF- κ B –driven gene

expression is found to involve various co-activators including histone acetylase (HATs) family members, p300/CEBP, PCAF and p160 proteins which possess intrinsic histone acetylase activity that induces chromatin changes necessary for efficient DNA binding. NF- κ B was reported to repress gene expression through the formation of co-repressor complex with SMRT, NCoR and histone deacetylase family members (HDAC1, -2 and -3). However, published data describing the regulation of NF- κ B by acetylation are very contradicting and do not provide a very clear picture. In one study, deacetylation of p65 at either K²²¹ or K³¹⁰ by HDAC1 and HDAC3 was shown to inhibit NF- κ B activity [102]. Deacetylation of p65 by HDAC3 allows binding to re-synthesized I κ B α , thus promoting nuclear export of NF- κ B and terminating response [101]. Kiernan *et al* [103] has however, reported that acetylation at K¹²² and K¹²³ of p65 reduces its DNA binding affinity. Microarray analysis comparing the level of genes expression in wild type and K³¹⁴ and K³¹⁵ mutant p65 after TNF- α treatment also identified a specific set of genes that is differently regulated, suggesting the modulation of genes expression by lysine-specific acetylation of p65 [104].

The activities of STAT (STAT1, STAT 2, STAT3 and STAT6) family members have also been shown to be regulated by acetylation. STAT6 was the first STAT protein shown to undergo acetylation and its acetylation correlates with the transcription of reticulocyte-type 15-lipoxygenase-1. By contrast, nuclear CBP- mediated acetylation of STAT1, negatively regulate its function by inducing the binding of T-cell protein tyrosine phosphatase (TCP45), which subsequently leads to signalling termination [105]. Depletion of HDAC3 led to a reduction in level of phospho-STAT1 and attenuated IFN-dependent transcriptional activation, suggesting that deacetylation by HDAC3 may provide a molecular switch to restore the level of STAT1 in the cytoplasm. In the case of STAT3, HDAC1 is required to ensure appropriate nucleo-cytoplasmic distribution of STAT3 during its activation by IL-6 [105].

4.4 Rationale

Although much has been learnt about the recognition of inflammation stimuli by host cells, with a number of pathogen-activated signal transduction pathways being defined and characterised, not much is known about the contributions of transcription factors and chromatin to the selective regulation of inducible proinflammatory genes. It is becoming clear that the selective activation of an inducible gene is not simply dictated by a combination of specific set of transcriptional factors, but that there are several other regulatory layers. Lysine acetylation is the best-characterised mechanisms among the class of histone modifications that affect chromatin structure and has generally been associated with transcriptional activation. Histone acetylation is a dynamic modification erased by histone deacetylases (HDACs). Even though studies on acetylation in the past decades, have mainly been focus on its role in transcriptional regulation, there is mounting evidence that acetylation is a general mechanism for controlling a variety of cellular processes. In fact, a single acetylation event may even directly govern transcription or alternatively be integrated within the overall chromatin context.

The class I HDACs (HDAC1, HDAC2 and HDAC3) are best known for their ability to participate in transcriptional regulation and are often described as transcriptional repressors. However, many studies indicate that gene regulation by acetylation is more dynamic and complex, and that HDACs can also function as activators. For instant, deletion of yeast Rpd3 (HDAC1) results in a more genes being down-regulation rather than an increase in gene activity. Hos2 (HDAC3) has also been shown to be required for GAL genes expression. The importance of HDACs as transcription activators is further underscored by gene expression profiling of mammalian cells treated with HDACi, in which similar proportions of genes being activated or repressed are observed.

Despite the accumulating evidences suggesting that HDACs can also function as gene activator, the contribution of individual HDACs to transcriptional activation and the mechanisms involved in HDAC-mediated gene expression in higher eukaryotes are

unknown. It is clear that the simple model suggesting that HDACs act as transcriptional repressors in virtue of their ability to deacetylate histones cannot account for all available observations. Moreover, thousands of proteins are acetylated or deacetylated in addition to histones, thus raising the issue of the relevance of histone versus non-histone substrate in HDAC activity. This brings to our attention that current pre-established models for the actions of these inhibitors need to be revised. Understanding HDACs activity from a mechanistic point of view is essential not only to provide an explanation for the commonly observed down-regulation of genes in HDACi treatment, but also to improve their use as anti-inflammatory and anti-cancer drugs. The design of efficient therapeutic drugs requires in-depth mechanistic knowledge of transcription regulation and this requires the characterisation of both functional and structural regulatory interactions at contract surfaces.

Given the premises above, the main objective of this project was to deconvolve these effects observed and identify the specific HDACs involved. In specifically, to define the function, role and mode of action of transcriptional co-regulators of the class I HDACs namely: HDAC1, HDAC2 and HDAC3 in inflammatory gene induction.

To understand the biological function of individual HDACs (redundancy among members), we initially carried out small-scale shRNA analysis of individual HDACs in inflammatory gene activation. Based on the effects observed in HDAC(s) knock-down experiments, we then use the relevant HDAC(s) knockout mice to carry out genome-wide analysis on genes affected by HDAC deletion via gene expression microarrays. We also generated genome-wide histone acetylation maps by ChIP-Seq, so as to discriminate direct versus indirect effects of HDACs deletion and to understand the correlation between recruitment, transcriptional activity and changes in histone acetylation.

Material and methods

1. Molecular Biology

1.1. Transformation of competent cells

A vial containing 50 µl of DH5α competent cells (Invitrogen) was thawed on ice prior to the addition of 5 ng of DNA from ligation mix. A negative control using mock ligation (cut plasmid alone without ligase enzyme) was included in the transformation. Competent cells were then incubated on ice for 30 minutes, followed by a heat shock of 45 seconds at 42°C, before returning to ice for 2 minutes. Subsequently, 500 µl of LB (Luria Bertani medium: 10 g/l casein hydrolysate peptone, 5 g/l yeast extract, pH 7.5) was added to the cells and incubated at 37°C for 1 hour with shaking. The cells were then plated onto LB plate (LB plus Bacto agar 15g/l) containing appropriate antibiotics (usually 100 µg/ml Ampicillin or 20 µg/ml Kanamycin) and incubated at 37°C overnight.

1.2. Small-scale DNA preparation (mini-prep)

Transformed colonies were individually picked and cultured in 2.5 ml of LB containing appropriate antibiotics aliquot, at 37°C overnight with shaking at 225 rpm. The bacterial cells were then harvested by centrifuging the overnight cultures at 4000 rpm for 10 minutes at 4°C. Next, plasmid DNA was isolated from these pellets using Plasmin mini-prep spin kit (Genomed), according to manufacturer's instructions.

1.3. Large-scale DNA preparation (maxi-prep)

Transformed colonies were individually picked and cultured in 2.5 ml of LB containing appropriate antibiotics aliquot for 6h at 37°C with shaking at 225 rpm. This starter culture was used to inoculate 250 ml selective LB medium and grown overnight at 37°C with shaking at 225 rpm. The bacterial cells were then harvested by centrifuging the overnight

cultures at 6000 x g for 15 minutes at 4°C. Next, plasmid DNA was isolated from these pellets using Qiagen Maxi-prep kit, according to manufacturer's instructions.

1.4. Glycerol stock of transformed bacteria

0.5 ml of 80% sterile glycerol was added to 0.5 ml of the overnight culture and mixed by brief vortexing. Glycerol stock was stored at -80°C.

1.5. Enzymatic modification of DNA

- Restriction analyses: plasmid DNA was digested with 10 U of the required restriction enzyme and buffer (Neb) for 1h at 37°C. The volume of each digestion was adjusted to a final volume of 50 µl with distilled H₂O and appropriate buffer.
- Gel extraction: DNA bands (digested vectors or inserts) were gel purified from 1-2% agarose gels using QIAquick Gel Extraction kit, according to manufacturer's instructions.
- Ligation: A 20 µl of ligation mixture containing a molar ratio of 1:3 vector to insert, ligation buffer, T4-DNA ligase (Boheringer) and distilled H₂O was prepared and left to ligate at room temperature, overnight.

1.6. Agarose gel electrophoresis

Gels were prepared by dissolving agarose (Euroclone) in 1x TAE buffer (4.8 g/l Tris base, 1.14% glacial Acetic acid, 0.74 g/l EDTA (1 M at pH 8.0) and contained 0.3 µg/ml ethidium bromide. DNA samples were loaded onto the 1-2% agarose gels along side DNA marker and electrophoreses at 80-100V until desired separation was achieved.

1.7. Polymerase chain reaction (PCR)

A 20 µl of reaction mix containing 0.5 U Phusion High-Fidelity DNA Polymerase (Finnzymes), the supplied 5x Phusion HF Buffer, 2.5mM dNTPs, 100 ng of each primer

and 10-50 ng of the DNA template was prepared and a general amplification protocol of the following PCR steps with 25-30 cycles was used:

1. Initialisation: 98°C, 2 minutes
2. Denaturation: 98°C, 30 seconds
3. Annealing: 55-65°C (depending on the T_m of primers pair used), 1 minute
4. Elongation: 72°C, 2 minutes/kb of desired fragment (return to step 2)
5. Final Elongation: 72°C, 10 minutes
6. Final hold: 4°C ad infinitum

PCR machine: T3000 Thermcycler (Biometra)

1.8. Plasmid construct

pLMP vector expressing shRNA against class I (HDAC1, -2, and -3):

HDAC 1

- Construct: pLMP-P2.6

22_mer: AGCCAGTCATGTCCAAAGTAAT

NM_008228 Start position: 744

TGCTGTTGACAGTGAGCGCGCCAGTCATGTCCAAAGTAATTAGTGAAGCCA
CAGATGTAATTACTTTGGACATGACTGGCTTGCCTACTGCCTCGGA

- Construct: pLMP-P3.2

22_mer: GGGATGTTGGAAACTACTATTA

NM_008228 Start position: 69

TGCTGTTGACAGTGAGCGAGGATGTTGGAAACTACTATTATAGTGAAGCCA
CAGATGTATAATAGTAGTTTCCAACATCCCTGCCTACTGCCTCGGA

HDAC 2

- Construct: pLMP-P6.2

22_mer: TGGTGATATTGGCAATTATTAT

NM_008229 Start position: 261

TGCTGTTGACAGTGAGCGCGGTGATATTGGCAATTATTATTAGTGAAGCCA
CAGATGTAATAATAATTGCCAATATCACCATGCCTACTGCCTCGGA

- Construct: pLMP-P8.9

22_mer: AGGCTTGGTTGTTTCAATCTAA

NM_008229 Start position: 1021

TGCTGTTGACAGTGAGCGCGGCTTGGTTGTTTCAATCTAATAGTGAAGCCA
CAGATGTATTAGATTGAAACAACCAAGCCTTGCCTACTGCCTCGGA

HDAC 3

- Construct: pLMP-P253

22_mer: CTGGCATTGACTCATAGCCTAG

NM_010411.2 Start position: 159

TGCTGTTGACAGTGAGCGATGGCATTGACTCATAGCCTAGTAGTGAAGCCA
CAGATGTACTAGGCTATGAGTCAATGCCAGTGCCTACTGCCTCGGA

- Construct: pLMP-P337

22_mer: GCGTGGCTCTCTGAAACCTTAA

AF074881.1 Start position: 1720 (**in 3' UTR*)

TGCTGTTGACAGTGAGCGACGTGGCTCTCTGAAACCTTAATAGTGAAGCCA
CAGATGTATTAAGGTTTCAGAGAGCCACGCTGCCTACTGCCTCGGA

Invitrogen chemically synthesized the shRNA oligos.

To generate pLMP-shRNA clones, all shRNA oligos were PCR amplified with primers to introduce EcoRI and XhoI sites into the shRNA. PCR products were purified with QIAquick PCR Purification Kit (Qiagen), according to manufacturer instructions and digested with EcoRI and XhoI restriction enzymes (Neb).

Double digestion reaction:

PCR products	40 µl
EcoRI	10 unit

XhoI	10 unit
EcoRI buffer (10x)	5 µl
BSA (100x)	0.5 µl

Top up to 50 µl with distilled water and incubated at 37°C for 1h.

Next, the EcoRI/XhoI cut shRNA fragments were ligated with opened pLMP (cut with EcoI and XhoI) puromycin vector, transformed and clones were checked for insert by sequencing with primers.

1.9. Fluorescence activated cell sorting (FACS)

Bone marrow from Cre-Hdac3 mice (*Hdac3*^{+/+} and *Hdac3*^{-/-}) was isolated and 2X10⁵ cells/well were seeded on 6-well plates in BM medium. After differentiation into macrophages, cells were stimulated with 100ng/ml LPS (0 and 14 h) and stained with appropriate antibody; differentiation marker (CD11b, F4/80) and activation marker (CD40, MHCII, CD86) for 1hr at 4°C. Cells were then fixed in PBS containing 1% paraformaldehyde, transferred to FAC tubes containing 150ul of FACs buffer (PBS+2%FCS+0.02% Sodium Azide+1mM EDTA) and then analysed with FACSCalibur (CellQuest software; BD Biosciences).

1.10. Cytokine measurements

Culture supernatant from LPS-treated (14h) WT and Hdac3 null bone marrow-derived macrophages was collected and IL-6, IL-10, KC and TNF-α concentrations were determined by commercially available ELISA (R&D systems). Optical densities were measured on a Bio-Rad Dynatech Laboratories ELISA reader at a wavelength of 450 nm (Hercules, CA, USA).

1.11. RNA extraction

- Homogenisation: Cells were lysed directly in culture dish by adding 0.5 ml of TRIzol (Invitrogen) to a 3.5 cm diameter dish. Lysate was passed through a pipette several times before transferring to a 1.5 ml centrifuge tube.
- Phase separation: 100 µl chloroform (BDH) was added to each sample, shaken vigorously by hand for 15 seconds and incubated at room temperature for 2 minutes before centrifuging for 15 minutes at 10 500 rpm, 4°C.
- RNA Precipitation: The upper aqueous phase was transferred to a fresh tube. In order to precipitate RNA, 250 µl of isopropyl alcohol was added to the transferred samples, mixed and incubated room temperature for 10 minutes. Samples were centrifuged for 15 minutes at 10 500 rpm, 4°C.
- RNA Wash: Supernatant was discarded and pellet washed with 300 µl of 70% ethanol. Samples were centrifuged for 5 minutes at 13 000 rpm, 4°C.
- Re-dissolving the RNA: Supernatant was discarded and pellet was air dry for 5-10 minutes before resuspending with 50 µl of RNase free water and incubated at 65°C for 10 minutes. Samples were stored in 10 µl aliquots at -80°C.
- Determination of RNA concentration and purity: RNA concentration was measured using NanoDrop ND-1000 and a ratio of A260/A280 between 1.8 to 2 was obtained.

For genes expression microarray (GENE 1.0 ST MOUSE Array, Affymetrix) analysis, RNA was further purified with RNeasy Kit (Qiagen), according to manufacturer's instructions.

1.12. Real-time polymerase chain reaction (RT-PCR)

0.5 µg of RNA was used for cDNA synthesis. A final reaction mix of 10 µl containing RNA, 0.5 µl of 50 µM random hexamers (Invitrogen), M-MuLV Reverse Transcriptase (Finnzymes), the supplied 10x reaction buffer and 0.5 µl of 10 mM dNTPs.

PCR Reaction steps were as followed:

25°C, 10 minute

42°C, 45 minute

95°C, 5 minute

4°C, ad infinitum

cDNAs was diluted with distilled water to 100µl.

1.13. Quantitative RT-PCR

Quantitative PCR was performed by running a 96 well reaction plate containing 20 µl of reaction mix per well, i.e. 4µl of diluted cDNA, 0.2 µl of a 10 µM primers mix and 10 µl Fast SYBR® Green Master Mix (Applied Biosystems) on 7900HT Fast Real-Time PCR System (Applied Biosystems). All reactions were performed in duplicate. To minimise the risk of “pipetting errors”, two master mix were prepared; one containing single cDNAs and the other containing Fast SYBR® Green Master Mix and gene specific primers.

Reaction steps were as followed:

50°C, 2 minutes

95°C, 10 minutes

95°C, 15 seconds (x40 cycles)

60°C, 1 minute

Quantification was performed using comparative CT method as described in the manufacturer procedures manual. TBP was used as both control gene and normalisation control. Primers were designed using MIT Primer3 software (<http://frodo.wi.mit.edu/primer3/>) with genomic sequences obtained from UCSC mouse genome browser (<http://genome.ucsc.edu/>).

Gene Expression Primers:

Gene symbol	RefSeq	Sense	Antisense
Nfkbia	NM_010907	CTTGCTGTGATCACCAACCAG	CGAAACCAGGTCAGGATTCTGC
Fas	NM_007987	TATCAAGGAGGCCCATTTTG	GGGTGCAGTTTGTTCCTACT
Tnfa	NM_013693	TCTTCTCATTCCTGCTTGTGG	CACCTGGTGGTTTGCTACGA
Il-12b	NM_008352	TCTGAGCCACTCACATCTGC	TCAGGGGAACCTGCTACTGCT
Il-6	NM_031168	CCATAGCTACCTGGAGTACATG	TGGAAATTGGGGTAGGAAGGAC
MIP-2 (Cxcl2)	NM_009140	AATGCCTGAAGACCCTGCCAAG	CTCCTTTCCAGGTCAGTTAGCC
Il-1a	NM_010554	GAGAGCCGGGTGACAGTATC	ACTTCTGCCTGACGAGCTTC
M-Csf (Csf1)	NM_007778	AGCATCCAGGCAGAGACTGAC	AGAGTGGCTTTTGGGAAGCAG
Gm-Csf (Csf2)	NM_009969	TGGTCTACAGCCTCTCAGCA	CCGTAGACCCTGCTCGAATA
iNos	NM_010927	CCATCATGAACCCCAAGAGT	CATCCAGAGTGAGCTGGTAGG
Il-1b	NM_008361	GACCTTCCAGGATGAGGACA	TCCATTGAGGTGGAGAGCTT
Ccl9	NM_011338	TGGGTCTGCCACTAAGAAG	TAGGACAGGCAGCAATCTGA
Icam1	NM_010493	AGGGCTGGCATTGTTCTCTA	CTTCAGAGGCAGGAAACAGG
IP-10 (Cxcl10)	NM_021274	CGCTGCAACTGCATCCATATCG	CCGGATTGAGACATCTCTGCTC
Ptgs2	NM_011198	CCACTTCAAGGGAGTCTGGA	AGTCATCTGCTACGGGAGGA
MCP-1 (Ccl2)	NM_011333	CACGTGTTGGCTCAGCCAGATGC	CCTTCTTGGGGTCAGCACAGACC
Rantes (Ccl5)	NM_013653	ACCATATGGCTCGGACACCACT	ACCCACTTCTTCTCTGGGTTGG
MCP-3 (Ccl7)	NM_013654	TCTGTGCCTGCTGCTCATAG	GACTTCCATGCCCTTCTTTG
Cxcl1	NM_008176	GCTGGGATTCACCTCAAGAA	TCTCCGTTACTTGGGGACAC
E-Selectin	NM_011345	GCATGTGGAATGACGAGAGA	GTCAGGGTGTTCCTGTGGTT
Cxcl9	NM_008599	ATCTTCTGGAGCAGTGTGG	AGTCCGGATCTAGGCAGGTT
Lif	NM_008501	GGCAACCTCATGAACCAGAT	ACCATCCGATACAGCTCCAC
JunB	NM_008416	CTTTGCGGACGGTTTTGT	TGCTGAGGTTGGTGTAGACG
JNK1	NM_016700	AGAAACTGTTCCCGATGTG	TGATGTATGGGTGCTGGAGA
JNK2	NM_207692	TGGAAGTGGACCATGAGAGA	CAGGCCAAAGTCAAGGATCT
Gcle	NM_010295	CGGAGGAACGATGTCTGAGT	CTCTGGGTGGGTCTGTGTT
Gsta3	NM_001077353	GGTAGAGATCGACGGGATGA	AGGCTTGCCTCTTCTCCTC
Bhlhe41	NM_024469	CTGAGCAGCAGCATCAGAAG	CTGGGTGTCCAGCTCTCAA
Layn	NM_001033534	AGCTCGTCAGCATTGAGACA	ATGGTACATGACCACGCAGA
Hdac 1	NM_008228	GACGGGGATGTTGGAAACTA	TCTCTGCATCTGCTTGTGT
Hdac 2	NM_008229	TATTATGGCCAGGGTCATCC	GGACAATCTTCTCCGACGTT
Hdac 3	NM_010411	AGCTGGACACCCAATGAAAC	TATTGGTGGGGCTGACTCTC
Hdac 8	NM_027382	ACA GAG GAG CAG GAA CTG GA	TTA TGC AGG GCA TAG GCT TC
Hdac11	NM_144919	CCC TTT GAT GCT GGG AAA T	GCA CAA GGA AGT TGG GAA GA
Hdac 4	NM_207225	CTG ACG CTG CTA GCA ATG AG	TTC CAA GGG CAG TGA GAA CT
Hdac5	NM_001077696	GGGAACCATCCTTGGAAATC	CTGCTGCAGTTGCTGCTC
Hdac 7	NM_019572	TCT CTT CCT GGC AGG CTT AC	GGA TCA CTT CAG CCA GCT TC
Hdac 9	NM_02412	TTT GAC AAG GCA GCA TCA AG	ACT GCC CTT TCT CGT CCT CT
Hdac6	NM_010413	TGG TGG AAA GAA GTG TGC TG	CCA AAG GCA ATG GAA GTG AT
Hdac10	NM_199198	CAG AGG AAG AGT TGG GCT TG	CAT TGT GCA CAG CTC CTG TT

1.14. Immunoprecipitation (IP)

- Pre-block beads: 1 ml of Protein-A or Protein-G sepharose beads was aliquot to a 1.5 ml centrifuge tube (1 tube per IP) and washed three times with 1 ml of block solution (2 % BSA in PBS), centrifuging at 8000 rpm, 1min. Beads were then resuspended in 800 µl of blocking solution (for 500 µl of beads) and incubated on a rotator for 1h in 4°C room. After blocking, the blocked sepharose beads were washed twice in 1 ml of RIPA buffer (50 mM Hepes-KOH at pH 7.5, 500 mM LiCl, 1 mM EDTA, 1% Nonidet P-40 (NP-40) and 0.7% Na-Deoxycholate) and kept on ice as ready-to-use beads.

- Lysate preparation: Cells stimulated with 100 ng/ml of LPS (Sigma) at 0-, 1- and 2-hour were washed twice with 5 ml of cold 1x PBS supplemented with inhibitors (50 ng/ml TSA, 10 µg Aprotinin, 10 µg Leupetin, 1 mM NaF, 10 mM PNPP, 10 mM Na₃VO₄, 10 mM β-glycerophosphate and 1 mM PMSF), collected and centrifuged at 2000 rpm for 5 minutes, 4°C. After centrifugation, cell pellets were lysed in 300 µl of ice-cold RIPA buffer (50 mM Hepes-KOH at pH 7.5, 500 mM LiCl, 1 mM EDTA, 1% Nonidet P-40 (NP-40) and 0.7% Na-Deoxycholate) plus inhibitors (composition as above) and kept on ice.
- IP cleaning: 100 µl of blocked sepharose beads was added to each of the cell lysate and incubated on a rotator for 3h in 4°C room. Lysates were then centrifuged at 10 000 rpm for 2 minutes, 4°C. Supernatant was transferred to a new 1.5 ml centrifuge tube and protein concentration was measured with Bio-Rad Bradford assay (Invitrogen). 20 µl of the supernatant was kept at -20°C as input.
- IP: After determination of protein concentration, appropriate amount of lysates (corresponding to 1 mg of protein) was aliquot to a new 1.5 ml centrifuge tube. 4 µg of appropriate antibody (anti-HA was set up as negative control) and 30 µl of blocked sepharose beads were subsequently added to each sample lysates and incubated overnight on a rotator at 4°C.
- Elution: Immune complexes bound beads were collected by centrifuging at 10 000 rpm for 5 minutes, 4°C and washed three times (2 minutes each) with 1 ml of RIPA buffer supplemented with inhibitors (composition as above). 5000 rpm for 5 minutes at 4°C was used to centrifuge the immune complexes bound beads during washes. After washes, immune complexes were extracted by resuspending the bound-beads in 35 µl of 2x Laemmli buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.125 M Tris HCl and 0.004% bromphenol blue) and incubated at 95°C for 10 minutes on a thermo-mixer. 30 µl of the elutant was loaded onto a 1.5

mm thick polyacrylamide gel and resolved by SDS-PAGE as described below.

1.15. SDS poly-acrylamide gel electrophoresis (SDS-PAGE)

Gels for resolution of proteins were cast using 30%, 30:08 mix of acrylamide:bisacrylamide (Fisher Scientific). 10% ammonium persulphate (APS) and TEMED (BDH) were added as polymerisation catalysts.

<u>Resolving gel mix:</u>	1 0 %	1 2 %	1 5 %
Distilled water (ml)	4 . 1	3 . 4	2 . 4
4x separating buffer (ml)	2 . 5	2 . 5	2 . 5
30% Acrylamide (ml)	3 . 3	4	5
10% APS (μl)	1 0 0	1 0 0	1 0 0
TEMED (μl)	4	4	1 0
Final volume (ml)	1 0	1 0	1 0

<u>Stacking gel mix</u>	
Distilled water (ml)	2 . 9
4x stacking buffer (ml)	1 . 2 5
30% Acrylamide (ml)	0 . 8 5
10% APS (μl)	5 0
TEMED (μl)	5
Final volume (ml)	5

1.16. Western blot analysis

- Whole cell lysate: Cells were washed once with 5ml of PBS, harvested and centrifuged at 1200 rpm, 5min. Cells were then lysed and homogenised in RIPA buffer (50 mM Tris-Cl at pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% Sodium deoxycholate and 0.1% SDS) containing inhibitors (10 μg Aprotinin, 10 μg Leupetin, 1 mM NaF and 1 mM PMSF).
- Cytosol and Nucleus Fraction Lysate: Cells were washed once with 5ml of PBS, harvested and centrifuged at 1200 rpm, 5min. The cell pellet was then lysed in 300 μl of L1 buffer (50 mM Hepes-KOH at pH 7.5, 140 mM NaCl, 1 mM EDTA, 0.25% Triton X-100, 0.5% Nonidet P-40 (NP-40) and 10% glycerol) containing inhibitors (10 μg Aprotinin, 10 μg Leupetin, 1 mM NaF, 0.05 ng/ml TSA (Sigma),

10 mM PNPP (Sigma), 10 mM Na₃VO₄, 10 mM β-glycerophosphate and 1 mM PMSF) with 5 minutes incubation on ice and centrifuged at 3000 rpm, 5 minutes, 4°C. The supernatant obtained was transferred to a new tube and kept as cytosol fraction. Nuclei pellet was washed once with 300 µl of L1 buffer, centrifuged at 3000rpm, 5min at 4°C and supernatant was discarded. Next, nuclei was disrupted in 150 µ of RIPA buffer (50 mM Hepes-KOH at pH 7.5, 500 mM LiCl, 1 mM EDTA, 1% Nonidet P-40 (NP-40) and 0.7% Na-Deoxycholate) containing the above inhibitors, kept on ice for 10 minutes, centrifuged at 13 000 rpm for 15 minutes and transferred supernatant to new tube (nuclei fraction).

All proteins concentration was measured with Bradford assay (Biorad). Proteins were denatured by adding Protein sample buffer (2% SDS, 31.25 mM Tris-Cl (1 M at pH 6.8), 0.5% glycerol, 5% β-metcapto-ethanol, 20 µ/ml of saturated Bromophenol Blue) to each lysate and heated at 95° C for 10 minutes. Equal amount of denatured proteins was then loaded onto 1.5 mm thick polyacrylamide gel (% of SDS-PAGE gel used depends on the molecular weight of proteins to be detected) assembled in Biorad apparatus and electrophoreses in 1x SDS-PAGE running buffer (25 mM Tris-base (pH 8.8), 192 mM Glycine and 0.1% SDS) initially at 80V for 15 minutes (or till proteins enter the resolving gel layer) before increasing the voltage to 140V and run for another 75 minutes. The protein gel was then blotted onto a nitrocellulose membrane (Whatman), stacked in the following order:

- Case (clear side)
- Sponge
- Filter paper
- Nitrocellulose membrane
- Gel
- Filter paper
- Sponge

- Case (black side)

(All materials were equilibrated in 1x Transfer buffer for 5 minutes prior to assembly)

The assembled cassette was placed in the transfer apparatus with black side facing the black and electro-transferred in 1x Transfer buffer (25 mM Tris-base, 192 mM Glycine (pH 8.3)) at 70V for 90 minutes, on ice or using ice-pack to cool down the apparatus.

Membrane was stained with Ponceau S for a minute to check for successful transfer, washed once with PBS for 5 minutes and blocked in 10 ml of blocking buffer (5% non-fat dry milk, 0.1% Tween 20 and 1x PBS) for 1h at room temperature with gentle rocking. The blocked membrane was then incubated with appropriate primary antibody (diluted in 10 ml 1x TBS, 0.1% Tween 20, 5% BSA and 0.01% NaN₃) for 1h at room temperature or overnight at 4°C. Next, the membrane was washed six times (10 minutes each) in 10 ml wash buffer (1x PBS and 0.1% Tween 20) at room temperature with shaking, before incubating with appropriate horseradish peroxidase conjugated secondary antibody (diluted in blocking buffer) for 1h at room temperature. Membrane was washed five times (10 minutes each) in 10 ml wash buffer and once with 10 ml of PBS (last wash). Proteins were detected by incubating the membrane with 2 ml of ECL solutions (Amersham) for 3 minutes. Subsequently, ECL solution was drained off, membrane wrapped in plastic and exposed to film.

1.17. Chromatin immunoprecipitation (ChIP)

- Fixation: Culture medium was aspirated and cells were fixed directly in 10 cm plate by adding 3 ml of PBS containing 1% formaldehyde (Sigma) and incubated at room temperature for 10 minutes. 374 µl of Tris-Cl (1M at pH7.6) was added to each plate to stop the fixation. Cells were then washed three times (10 minutes each) with 10 ml of ice-cold PBS, collected and centrifuged at 1500 rpm for 5 minutes, 4°C.

- Cell lysis and sonication: Cells were lysed on ice for 10 minutes in 2.5 ml of L1 buffer (50 mM Hepes-KOH at pH 7.5, 140 mM NaCl, 1 mM EDTA, 0.25% Triton X-100, 0.5% Nonidet P-40 (NP-40) and 10% glycerol) supplemented with protease inhibitors (Roche). Nuclei were pellet at 1500 rpm for 5 minutes, 4° and washed with 2.5 ml of L2 buffer (10 mM Tris-HCl at pH 8.0, 200 mM NaCl, 0.5 mM EGTA and 1 mM EDTA) supplemented with protease inhibitors (Roche) for 10 minutes at room temperature. Suspension was then centrifuged at 1500 rpm for 5 minutes, 4°C before disrupting the nuclei pellet with 3 ml of L3 buffer (10 mM Tris-HCl at pH 8.0, 100 mM NaCl, 0.5 mM EGTA, 1 mM EDTA, 0.1% Na-Deoxycholate and 20% N-lauroylsarcosine) plus protease inhibitors (Roche). Chromatin was sheared by sonication, five times for 30 s (1 minute pause) at 30% ampere on ice. Next, 300 µl of TritonX-100 was added and centrifuged 13 000 rpm for 10 minutes, 4°C to pellet debris. Extracts were transferred to a new 15 ml conical tube for immunoprecipitation. 50 µl of the extracts from each sample was stored at -20°C as genomic DNA (input).
- To confirm a good sonication (250-500bp DNA fragments), 55 µl of the extracts were incubated at 52°C for 1h with 100 µl of reversed crosslink buffer (1% sodium dodecyl sulfate (SDS), 0.1 M NaCl, 5 µl of proteinase K (20 mg/ml) and 10 µl of RNase inhibitor). 8 µl of 4 M LiCl and 1 ml of 100% ethanol were then added to the extracts, centrifuged at 13 000 rpm for 15 minutes, 4°C and washed once with 70% ethanol. DNA pellet was suspended in 15 µl of distilled water plus 2 µl of 10x loading buffer (25mg bromophenol blue, 25mg xylene cyano and 2.5 g Ficoll 400 in 10ml distilled water) and loaded onto a 1.5% agarose gel.
- Pre-block and binding of antibody to magnetic beads: 100 µl of Dynabeads Protein G magnetic beads (Invitrogen) was aliquot to a 1.5 ml centrifuge tube (1 tube per IP) and washed three times with 1 ml of block solution (0.5 % BSA in PBS). Beads

were then resuspended in 250 µl of block solution, adding 10 µg of appropriate antibody (anti-HA was set up as negative control) and incubated overnight on a rotating platform at 4°C. Next day, beads were washed three times with 1 ml of block solution prior to resuspending in 100 µl of block solution (ready-to-use beads).

- Chromatin immunoprecipitation: 100 µl of the ready-to-use beads was added to each extract and gently mix overnight on rotator at 4°C.
- Reversed cross-link: Next day, IP beads were collected, washed six times (5 minutes each) with 1.2 ml of ice cold RIPA buffer (50 mM Hepes-KOH at pH 7.5, 500 mM LiCl, 1 mM EDTA, 1% Nonidet P-40 (NP-40) and 0.7% Na-Deoxycholate) in a magnetic stand followed by one wash with 1.2 ml of TE containing 50 mM NaCl and centrifuged at 2000 rpm for 2 minutes to remove any residual TE buffer. Subsequently, immune complexes were extracted in 250 µl of elution buffer (1x TE containing 2% sodium dodecyl sulfate) and protein: DNA crosslinks were reverted by incubating at 65°C overnight. 50 µl of the WCE extracts reserved after sonication was thawed, mixed with 150 µl of the elution buffer and incubated together with the above IP samples.
- Purification of DNA: Extracted DNA was divided into two tubes, diluted five fold with 600 µl of PB buffer (Qiagen) and incubated for 30 minutes at 37°C in a thermo-mixer. Next, extracted DNA was loaded onto Qiaquick column and washed according to manufacturer's protocol. Immunoprecipitated DNA was eluted in 70 µl of 1x TE while genomic DNA was eluted in 100 µl of 1x TE. DNA was quantified using PicoGreen (Invitrogen).

Design of ChIP Validation Primers:

To confirm that the data of histone H4 acetylation generated using ChIP-seq is reliable, eighteen different genome regions acetylated upon deletion of H dac3 were picked randomly accordingly to the p-value. Primer pairs targeting these hyperacetylated regions were then designed and checked by standard PCR.

chromosome	start	end	length	summit	tags	$-10 \times \log_{10}(\text{pvalue})$	fold enrichment	RefSeq	Symbol	txStart	txEnd	Strand	Exons number	Position	TSS distance
chr4	140978337	140984979	6643	3693	625	2591.96	22.06	NM_013868	Hspb7	140976693	140981225	+	3	exonintron last	4965
chr10	24627799	24631243	3445	744	213	1311.1	32.93	NM_027347	Med23	24389791	24633335	+	29	exonintron last	39730
chr18	60876396	60879329	2934	2372	145	1120.39	63.53	NM_008306	Ndst1	60845629	60873043	-	14	promoter	-4819
chr9	40327127	40328841	1715	629	150	1007.82	25.36	NM_172768	Grand1b	40105491	40263349	-	20	promoter	-64635
chr14	26278790	26292181	13392	5744	790	980.94	9.65	NM_183208	Zmiz1	26278777	26484195	+	24	exonintron 1	6708
chr12	8834032	8836825	2794	683	198	869.49	13.01	NM_008640	Laptn4a	8928112	8945547	+	7	promoter	-92683
chr3	97808658	97810413	1756	1022	117	730.35	28.69	NM_010928	Notch2	97817460	97954290	+	34	promoter	-7924
chr18	60892109	60893796	1688	549	105	619.93	24.71	NM_008306	Ndst1	60845629	60873043	-	14	promoter	-19909
chr7	132765486	132769842	4357	1887	391	556.91	6.54	NM_021887	Il21r	132746942	132777084	+	9	exonintron 3	20722
chr1	175962091	175963362	1272	423	83	479.03	21.01	NM_172648	Ifi205	175942128	175961886	-	7	promoter	-840
chr6	54897425	54899834	2410	879	147	396.61	9.8	NM_172729	Nod1	54873942	54922405	-	12	exonintron 1	23775
chr1	158596762	158598402	1641	638	60	356.04	21.24	NM_023141	Tor3a	158583747	158604470	-	6	exonintron 3	6888
chr3	35663948	35664914	967	198	48	286.83	31.29	NM_029570	Ap11b	35653059	35755198	+	30	intron 1	11372
chr11	120611439	120613420	1982	1027	94	237.81	9.33	NM_007621	Cbr2	120590798	120593335	-	8	promoter	-19094
chr8	84875923	84876549	627	293	14	101.35	22.88	NM_008357	Il15	84855522	84926444	-	8	intron 2	50208
chr12	53048564	53049090	527	187	18	97.97	13.24	NM_177171	Hear5a	52976859	53072308	-	36	exonintron 9	23481
chr6	134701770	134702248	479	122	24	90.11	11.03	NM_130447	Duspl6	134665490	134742646	-	7	intron 3	40637
chr1	175960593	175961244	652	323	18	79.78	16.81	NM_172648	Ifi205	175942128	175961886	-	7	intron 1	967

ChIP Validation Primers:

Chromosome region	Gene symbol	RefSeq	Primers
chr4:140979647-140979966	Hspb7	NM_013868	TGGCACAGTCATGAACACCT GCTGGACATGTTCTGTGTGG
chr10:24628319-24628628	Med23	NM_027347	CTTCAGTGGATGCAAGAGCA CACAATCCAGTACGGCTCCT
chr18:60878683-60878891	Ndst1	NM_008306	GCAAAGCTCTGTACAAACG TCCTGAGCACTGGGATTACA
chr18:60892511-60892750	Ndst1	NM_008306	CAGAAGTGGGCACAGAGACA ACTGAGCAGGCCACAGTAT
chr9:40327648-40327836	Gramd1b	NM_172768	TCTGGTGATCCACCTGCTCT TTTGAGGAGCTGGGAAGATG
chr14:26284425-26284705	Zmiz1	NM_183208	TCCCAGGAAGTCTGACCTGT CCCCAGCACAACTCTGTAGT
chr12:8834591-8834938	Laptm4a	NM_008640	CCCTTTTCTGTCCCAGGTCT CACTCCTTGTGCAGCTTGAG
chr3:97809627-97809809	Notch2	NM_010928	ATGGGTAAAGGTGCTTGCTG GTGTGGTGTGTGTATGCTGATG
chr7:132767659-132767809	Il21r	NM_021887	GTCCCTCCAGCCACTTGTA TAGAATGTGTGTGCCCCATC
chr1:175962340-175962590	Ifi205	NM_172648	ACAAGCACCAAACCTTCAG CCCAGAATCACAAAGGCACT
chr6:54898183-54898523	Nod1	NM_172729	TCTCTGCCTTTCCTGCTAA TTGACGGGAGTCCTGTTACC
chr1:158597317-158597539	Tor3a	NM_023141	AGCCAGCCTGCTCTATCTTG CTCATGTTCCGGTGGAGACCT
chr3:35664084-35664226	Atp11b	NM_029570	TAGTGTTCCGGGAGTGGCTTC AAATGCAGTGGTCCACACCT
chr11:120612369-120612542	Cbr2	NM_007621	GGACATGCCTTCCTCAGATG AAGAGCTGGTGGAGTCTGGA
chr8:84876105-84876338	Il15	NM_008357	AGCCCTTCAGTCACTCAGGA CCCAACAACAGAGGCATTTC
chr12:53048697-53048852	Heatr5a	NM_177171	TACCTTGCCCTTTCATGAG CAAGTCTTCACCAGAAGCAGTG
chr6:134701827-134702017	Dusp16	NM_130447	CCACAGTGAAAAGGCCAAAT TGGACTCTGGAGCCAACTAAA
chr1:175960769-175960985	Ifi205	NM_172648	CAGGGACATGGTAGTGGATG TGGAAAGTGGTGCATAGCTG
relative to TSS: 373-166	Gapdh	NM_008084	CACTCCAAGGACTCCTCGTC CCTCTCTCATTTCCCCTCCT
	Hoxa9	NM_010456	GCTGTGGCCTTCTCACTTTT CCTGCCTTGGTTATCCTTGA
	Hoxa10	NM_001122950	AAACTCTGGCTCGGGATTG GTCGACCAAAAAGGAGTTG
	Hoxa11	NM_010456	CCCCAGCCAACATGAGTTAC TCAGGGGAAGCAACAGATTG

2. Bioinformatics

2.1. Microarrays analyses

Raw data from Affymetrix Mouse Gene ST 1.0 plates were pulled together and analysed using R. Data that have been background corrected and RMA normalised. Probe sets showing at least a 2-fold change in expression in either positive or negative direction as well as a p-value lower than 0.05 (unpaired two-tailed Welch t-test) between conditions were considered as differentially expressed. Probe sets annotation was performed using Python custom scripts.

2.2. Literature microarrays analyses

Microarrays data generated using bone marrow derived macrophages that were either untreated or treated with Ifn β , was downloaded from GEO (GSE20403, [123]). Data was already RMA normalised; fold changes and t-tests (unpaired two-tailed Welch t-test) between each time point (1h, 2h, 4h from Ifn β treatment) and the basal condition were performed through R. Probe sets showing at least a 1.5-fold induction ($p < 0.05$) in at least one of the three comparisons were pulled together. This constitutes our Ifn β inducible dataset.

2.3. Overlaps and Statistics

For the follow-up analyses, overlaps among genomic intervals were computed using C++ custom scripts. Probe sets were collapsed into either RefSeqs or Gene Symbols as necessary. Overlaps among identifiers were performed using Python custom scripts. R was used to draw plots and to perform statistical tests.

2.4. ChIP-Seq analyses

Bowtie [124] has been used to map the 36-bp short reads onto the mm9 release of the mouse genome. We performed the alignments with a maximum of two mismatches and

keeping only the reads that align to unique positions in the genome. To define the ChIP enriched genomic regions (peaks), the Model-based Analysis for Chip-Seq (MACS) [125] was used. We ran MACS with default parameters and with a p-value threshold that was set to 1e-5. Reads obtained from inputs were used as a reference control.

For further characterisations, we decided to filter our peaks lists for a more stringent p-value of 1e-10. Final regions were annotated to the nearest UCSC known gene using GIN [126]. As parameters for GIN, we set the priority to gene and used a definition of promoter till -20 kbp from the nearest TSS.

2.5. Analysis of TFBS over-representation: RefSeq promoters analyses

For differentially expressed probe sets, the corresponding RefSeqs were considered. For each one of them, TSS coordinates were extracted from mm9 UCSC RefSeq table. In case of redundant TSS, they were considered just once. Corresponding genomic sequences were fetched and stored in FASTA format.

In order to identify TFBS (represented by position weight matrices, PWMs) over-represented in our datasets of promoters (with respect to what is the expectancy considering all the promoter regions in the mouse genome) we used Pscan [127]. It was ran for each one of the interested datasets against a reference set of sequences made up with all the non-redundant RefSeq TSS in the mouse genome (mm9). A custom dataset of 382 PWMs was built by merging different sources [120-122, 128].

For each PWMs, Pscan outputs a p-value representing the probability that a PWM is over-represented in the input dataset (compared to the reference) by chance. We set a threshold of $p < 0.01$ in order to consider a PWM that is significantly enriched.

2.6. Analysis of TFBS over-representation: Promoters and distal elements analyses

To overcome any constraints imposed by Pscan on the input data (every region must be of the same length in order to perform exact Statistics), we switched to using CLOVER [129]. In this way we were able to split ChIP-seq enriched regions (that are heterogeneous in length) in proximal and distal and to perform comparable TFBS over-representation analysis.

Given a set of PWMs and a coherent background set of sequences, CLOVER is able to assess which PWMs are significantly over- and under- represented in a set of input sequences. CLOVER was run with a p-value threshold of 0.01. We used the entire chromosome 19, and all of the 5 kbp upstream of the TSSs of all the annotated RefSeqs as background datasets. We considered only the PWMs that CLOVER reported significant with a p-value lower than 0.01 as ‘over-represented’ for both of the independent backgrounds. We used the same set of PWMs dataset as with Pscan [127] analyses.

3. Description of mice

Homozygous C57BL/6 *Hdac3* floxed (*Hdac3*^{FL/FL}) mice harbouring the conditional floxed (FL) allele of *Hdac3* obtained from Hiebert SW [71] were initially crossbred with either transgenic C57BL/6 Mx-Cre or LysM-Cre mice. The offspring from these mice were then bred to yield mice that are either wild type *Hdac3*^{FL/+} or null Mx-*Hdac3*^{FL/FL}, and Cre. Mice carrying a floxed exon 7, wild-type exon 7 and a deleted exon 7 were identified by PCR screening.

PCR Reaction steps were 94°C, 5min, 30 cycles of 94°C, 45 sec, 55°C, 25 sec, 72°C, 1 min 15 sec, followed by 72°C, 10 min.

1.2% agarose gel was used to resolved the resulting products floxed exon (935bp), wild-type exon 7 (895bp) and a deleted exon 7 (211bp).

HDAC3 Genotyping primers:

Hdac3 1263T	5'-CCA CTG GCT TCT CCT AAG TTC-3'
Hdac3 1258B	5'-CCC AGG TTA GCT TTG AAC TCT-3'

To induce Cre expression in heterozygous Mx- *Hdac3*^{FL/+} or null Mx-*Hdac3*^{FL/FL} mice, 12-week-old mice received 2 rounds of intraperitoneal injection of 250 µg Poly-IC (Amersham) in PBS every 2 days. Mice were sacrificed 2 days after the last injection for bone marrow cells extraction. For LysM- *Hdac3*, induction in the level of Cre expression occurs over the course of macrophages differentiation.

4. Mammalian cell culture**4.1. Preparation of 3T3 fibroblasts, bone marrow-derived macrophages and their culture conditions**

- 3T3 fibroblasts: Culture medium from a plate of 80% confluent mouse 3T3 fibroblasts was removed, briefly rinsed once with 0.25% (w/v) Trypsin-0.53 mM EDTA (Fisher Scientific) and incubated at 37°C for 5 minutes upon additional of 1 ml Trypsin-EDTA. Plate was gently tapped to detach cells before adding 9 ml of complete growth medium (Dulbecco's Modified Eagle's Medium, 10% fetal calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine). Cells were then aspirated by gently pipetting and 1 ml of the cell suspension (1:10 dilution) was transferred to a new 10cm² plate containing 7 ml of fresh complete growth medium and incubated in an incubator with humidified atmosphere containing 5% CO₂ at 37 °C. Cells were replated every three days and kept in culture for up to a maximum of 24 passages. For Retroviral transduction, 3T3 cells were plated in 6 well plate at the density of 5x10⁴ cells per well and used for infection on the following day.
- Bone marrow-derived macrophages: Femur and pelvic bones were isolated from mice (female FVB, Cre-*Hdac3*^{FL/+} or Cre-*Hdac3*^{FL/FL} strain). Excess muscle tissues were removed and cleaned bones were crushed in 10 ml of 1x PBS supplemented

with 1% Pen/Step. Suspension was filtered with a 70 μ M filter membrane. To isolate cells completely from bone, the above steps were repeated twice with fresh 5 ml of 1x PBS supplemented with antibiotics (100 units/ml penicillin, 100 μ g/ml streptomycin). Filter membrane was rinsed once with 5 ml of 1x PBS supplemented with antibiotics (100 units/ml penicillin, 100 μ g/ml streptomycin) before centrifuging at 1500 rpm for 5 minutes, 4°C. Next, red blood cells were lysed by resuspending the cell pellet in 2 ml of 0.2% NaCl₂, incubated at room temperature for 1 minute before adding 2 ml of 1.6% NaCl₂ and centrifuged immediately at 1500 rpm for 5 minutes, 4°C. Cells were washed with 5 ml of 1x PBS supplemented with antibiotics (100 units/ml penicillin, 100 μ g/ml streptomycin) by gentle aspiration, taking care only to resuspend the upper and middle phase of the pellet. The suspension was then transferred to a new 15 ml falcon tube and centrifuged at 1500 rpm for 5 minutes, 4°C. Cells were washed for another two times before resuspending in 10 ml of BM-medium (DMEM supplemented with 30% L929-cell conditional media, 100 units/ml penicillin, 100 μ g/ml streptomycin, 20% FCS, 2 mM L-glutamine, 0.5% Na pyruvate and 0.2% 2- β -mercapthanol). After counting the number of cells, bone marrow cells were plated at the density of one million per 10 cm plate containing 5ml of BM-medium and left over the course of 7 days in an incubator with humidified atmosphere containing 5% CO₂ at 37 °C to obtain fully differentiated macrophages. Cultures were fed with 2.5 ml of fresh medium every 2 days. Stimulations were carried out at day 7. For Retroviral transduction, bone marrow cells were plated in 6 well plate at the density of three million per well and used for infection on the following day.

4.2. Transfection

Transfection was carried out according to Lipofectamine Plus (invitrogen) protocol. In general, two solutions were prepared as below:

- Solution A: 5 µg pLMP-shRNA + 1.4 µg pCL-eco (Total DNA: 6.4 µg) in 10 µl of Plus reagent (invitrogen), making up to 100 µl (Final vol.) with Dulbecco's Modified Eagle's Medium (Fisher Scientific)
- Solution B: 16 µl of Lipofectamine (invitrogen) in 96 µl of Dulbecco's Modified Eagle's Medium (Fisher Scientific)

Solution A and B were mixed by gentle pipetting and incubated at room temperature for 15 minutes (This will be the ready-to-use transfection solution).

While solution was incubating, culture medium of Phoenix-ECO cells (plated at the density of 2×10^6 cells per 10cm plate the day before) was removed, briefly rinsed once with Dulbecco's Modified Eagle's Medium before adding 5 ml of Dulbecco's Modified Eagle's Medium. Transfection solution was then added to the Phoenix-ECO cells plate in a dropwise manner (gently swirl the plate) and incubated at 37°C with 5% CO₂. Medium was removed from plates after 7 hours and replaced with 13 ml of complete growth medium (Dulbecco's Modified Eagle's Medium, 10% fetal calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine).

4.3. Retroviral transduction

Supernatants containing retroviruses from transfected Phoenix-ECO cells were collected at 48h post transfection and filtered with 0.45 µm filter membrane. 8 µg/ml polybrene and 10 µl/ml of 1M HEPES (pH7.5) were then added to every 2 ml of the viral supernatant collected. Next, 2 ml of this viral supernatant (per well) was added to the 6 well plate containing cells to be infected upon removal of culture medium.

For bone marrow cells, plate was centrifuged at 2500 rpm for 5 minutes before removal of 1.5 ml medium from each well. After addition of viral supernatant, plate was centrifuged at 2500rpm for 1.5h, 30°C (spin infection) before returning the plate to an incubator with humidified atmosphere containing 5% CO₂ at 37 °C for 1.5h. Infection was conducted for two days and two spin infections were carried out per day. Cells were selected for 3 days

with 3 µg/ml puromycin, added 12h after the last spin infection and selected cells were used for further experiments as required.

4.4. Drug treatment

- HDAC inhibition: Wild-type bone marrow-derived macrophages were treated with a pan-HDACs inhibitor; Trichostatin A (TSA, Sigma) at 100 ng/ml for 1h prior to stimulation with either LPS or LPS/IFN γ for 1, 2, 4h. Cells were then harvested and used for further experiments.
- LPS from *Escherichia coli* serotype 055:B5 (Sigma) was used at 10 ng/ml; γ IFN (R&D) was used at 10 UI/ml.

5. Antibodies

The following antibodies were used for immuno-detection, ChIP and FACS analyses.

- Santa Cruz Biotechnology:
Hdac1 (sc-7872), Hdac2 (H-54), Hdac3 (sc-11417), c-Jun (H-79), HA-probe (Y-11)
- Upstate/ Millipore:
Anti-acetyl-Histone H3 (Lys9), Anti-acetyl-Histone H3 (Lys14), Acetyl-Histone H3 (06-599) and Acetyl-Histone H4 (06-866)
- cell signaling:
Stat1 (9172L), Phospho-Stat1 (Tyr701), IRF-3 (D83B9), Phospho-IRF-3 (Ser396) (4D4G), SAPK/JNK (56G8), Phospho-SAPK/JNK (Thr183/Tyr185) (81E11), Phospho-c-Jun (Ser63) II, Stat3 (9132), Phospho-Stat3 (Tyr705) (D3A7) XP™, Phospho-Tyk2 (Tyr1054/1055)
- BD Pharmingen:
CD11b/Mac-1 (clone ICRF44), CD40 (clone 3/23), CD86 (clone GL1), IA/IE
- Caltag Laboratories:
Rat anti-mouse F4/80

Results

1. Effects of global inhibition on inflammatory gene activation in macrophages

TSA is a hydroxamic acid that specifically and reversibly inhibits class I, II and IV HDACs, while being devoid of effects on sirtuins (class III HDACs). TSA has an IC_{50} in the nanomolar range and a similar activity towards all sensitive HDACs [107, 108, 110]. To initially define the impact of HDACs on inflammatory gene transcription, we first optimised the TSA concentration to be used in subsequent studies in primary bone marrow-derived macrophages (hereafter referred to primary macrophages). Primary macrophages were treated with TSA at 10, 15, 100 or 250 ng/ml for 1 hr, prior to stimulation with LPS+IFN γ for one or four hours as indicated (Figure 1a). Cells were then harvested for analysis of both histone acetylation (by western blot with either an acetyl-H3 or -H4 antibody) and inducible genes expression (by quantitative RT-PCR). TSA provoked a clear overall increase in bulk acetylation of both histone H3 and H4 at concentrations as low as 15 ng/ml (Figure 1b). Conversely, it showed an inhibitory effect on the transcriptional activity of *Nos2* and *Il-12b* only at the concentration of 100 ng/ml or higher (Figure 1c). To ensure that the enzymatic activity of all class I and II HDACs are being inhibited by TSA and given that the IC_{50} of TSA for HDAC8 is 0.486 μ M, therefore, unless otherwise stated, all subsequent experiments involving TSA-treated primary macrophages were done using 100 ng/ml TSA.

We next investigated TSA effects on the expression of a broader panel of LPS-inducible genes (including many NF- κ B target genes). Three types of responses were observed for the 20 genes analysed (Figure 1d). Induction of some NF- κ B target genes like RANTES (*Ccl5*), Fas and MCP-1 (*Ccl2*) remained unaffected over the time window investigated. Induced levels of some others, such as Gm-CSF (*Csf2*), IP-10 (*Cxcl10*), *Il-1 β* and E-selectin were modestly increased. Finally, induction of several genes was strongly

inhibited by TSA with the most significant reduction observed for Il-12 β , Il-6, MIP-2 (Cxcl2) and Il-1 α . Thus, HDAC inhibition affects the LPS-inducible transcriptional response in a restricted and gene-specific fashion and, as far as it can be concluded from the small panel analysed, the most obvious consequence of TSA treatment is the inhibition of target gene induction.

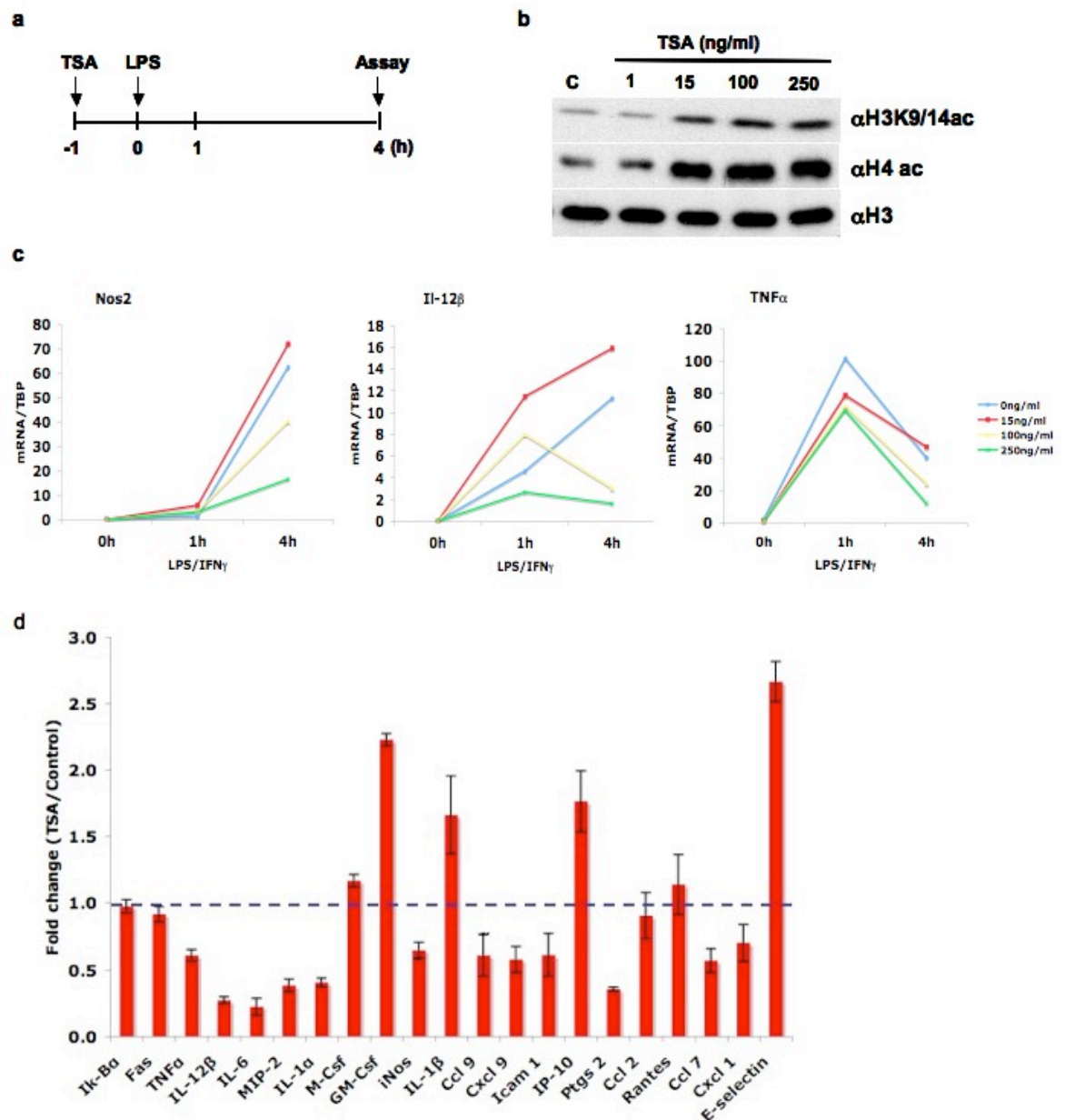


Figure 1. The effect of HDACs inhibition by TSA is gene specific.

(a) Time scheme for cells treatment. Control bone marrow-derived macrophages (primary macrophages) were treated with LPS+IFN γ for either one or four hours. For TSA-treated cells, primary macrophages were pre-treated with TSA for one hour and then co-stimulated with LPS+IFN γ as indicated. (b) Primary macrophages were treated with increasing concentrations of

TSA (1, 15, 100 or 250 ng/ml; 5 hr). “C”: control (unstimulated cells). Western blots were carried out with anti-acetyl-H3K9/K14, anti-tetra-acetyl-H4 or loading control anti-H3. Representative blots of three independent experiments. (c) mRNA for the indicated genes were measured by quantitative RT-PCR and expressed as ratio to a housekeeping gene (TBP). Data are representative of a broader analysis from three independent experiments. (d) Total amount of gene-specific mRNA for both control (without TSA pre-treatment) and TSA pre-treatment were calculated as ratio to a housekeeping gene (TBP) and expressed as fold change of TSA pre-treated over control, where wild type induction is 1. Values shown are the mean $\pm$ SD of pooled data from two independent experiments.

2. Regulation of inflammatory gene expression by individual class I HDACs

TSA has comparable inhibitory activity on all sensitive HDACs. To determine if the transcriptional effects of TSA in the LPS response could be ascribed to specific HDACs, we generated short hairpin RNAs (shRNAs) targeting individual HDACs. In mouse 3T3 fibroblasts as well as in primary macrophages, only Hdac1, Hdac2 and Hdac3 were highly expressed, while expression of all other HDACs (Hdac 8, 11, 4, 5, 7, 9, 6 and 10) was extremely low and almost undetectable (Figure 2).

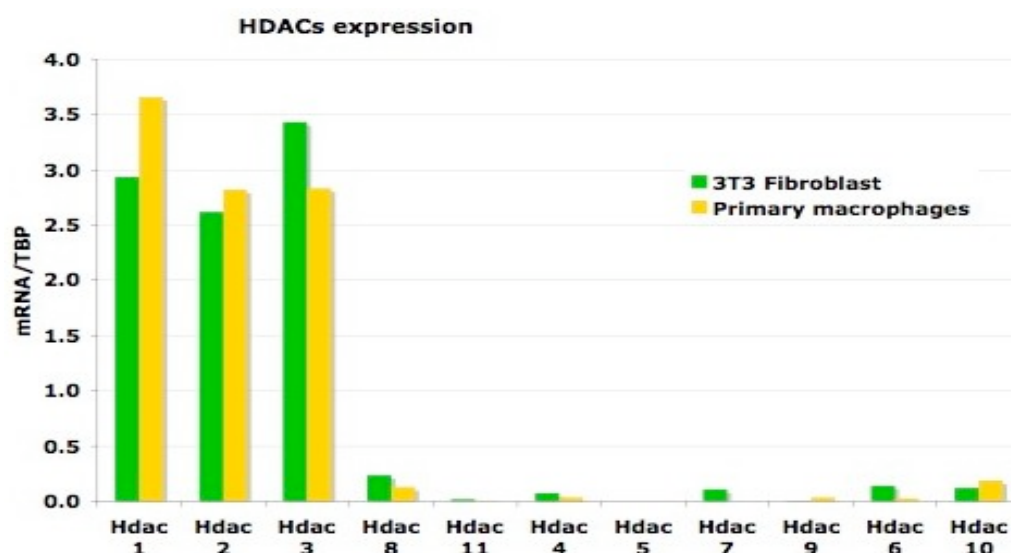


Figure 2. mRNA expression profile of class I, II and IV HDACs.

Total amount of gene-specific mRNA in 3T3 fibroblasts (green) and primary macrophages (yellow) were quantified by quantitative RT-PCR and expressed as ratio to a housekeeping gene

(TBP). Data are indicative of one experiment.

We set out to knock-down individual class I HDACs (Hdac 1, 2 and 3) by retroviral delivery of shRNAs. Efficient suppression (more than 85% relative to basal level; Figure 4b) of HDACs mRNA was observed by western blot, following infection of both 3T3 fibroblasts (Figure 3b) and primary macrophages (Figure 4c). Two shRNAs were used for each HDACs in order to rule out off-target effects in gene expression analyses. The loss of one HDAC did not affect either the protein or mRNA expression levels of other HDAC family members (Fig. 3 and data not shown).

Consistent with the current models of Hdac1 function as transcriptional repressor, down-regulation of Hdac1 in mouse 3T3 fibroblasts resulted in a modest but reproducible up-regulation of most genes tested (Figure 3a, left panel), Hdac2 knock-down showed variable and low-magnitude effects. However, no obvious inhibition of the tested inducible genes was observed following Hdac2 knock-down (data not shown). Conversely, induction of a subset of tested genes was impaired following Hdac3 knock-down, with the most significant effects for the mRNA of Il-6, Ccl5 and Ccl9 (3-fold or more reduction; Figure 3a, right panel).

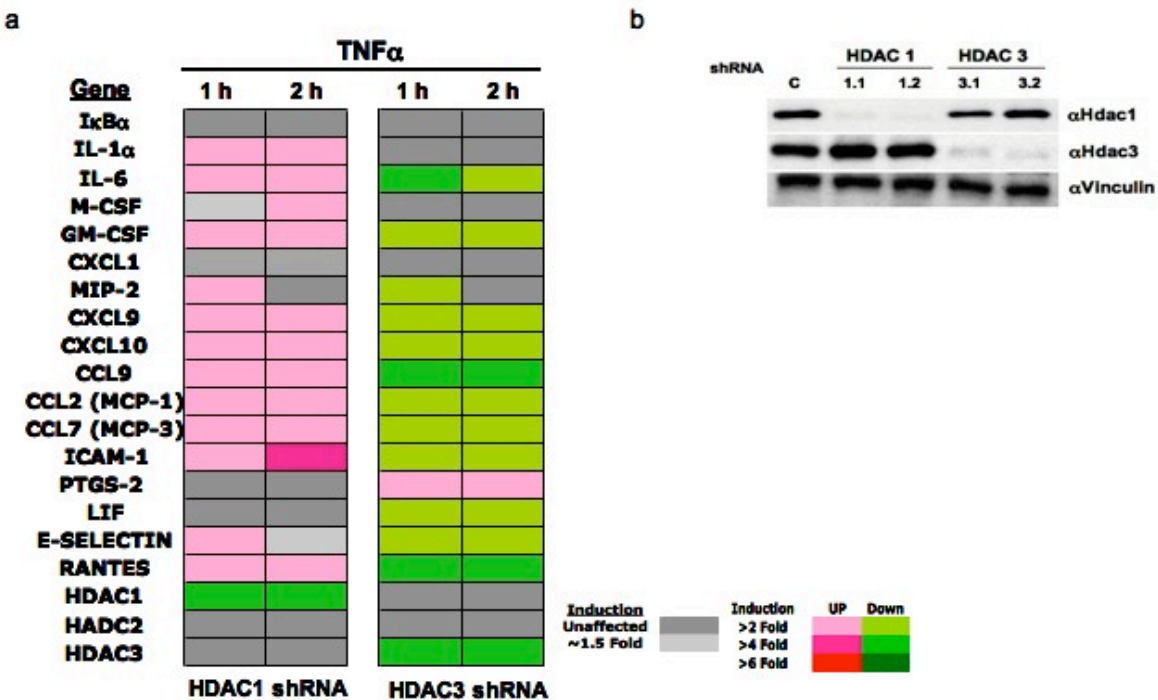


Figure 3. Regulation of inflammatory gene expression by individual class I HDACs in 3T3 fibroblasts.

(a) 3T3 fibroblasts (control and cells in which Hdac1 or Hdac3 were knocked-down) were treated with Tnf- α for one or two hours prior to RNA extraction. mRNA for the indicated genes following either Hdac1 or Hdac3 knockdown (KD) in 3T3 fibroblasts were quantified by quantitative RT-PCR and calculated as ratio to a housekeeping gene (TBP) before expressing as fold change of KD over wild type control, where wild type induction is 1. Data are representative of three independent experiments. Expression changes are colour-coded; colour towards red signifies increased in gene expression while colour towards green signify decreased in gene expression. Gray means no statistically significant difference. (b) Efficiency of individual class I HDACs KD in 3T3 fibroblasts (two shRNA clones, Hdac1: 1.1, 1.2; Hdac3: 3.1, 3.2). “C”: indicates control (mock infection). Western blots were carried out with anti-Hdac1, anti-Hdac3 or loading control anti-vinculin. Representative blots of three independent experiments.

We next investigated the effects of HDACs knock-down in primary macrophages. Only 8 out of the 20 genes surveyed were found to be deregulated following individual HDACs depletion. In contrast to Hdac1 knock-down in 3T3 fibroblasts, no general up-regulation of inflammatory genes expression was found, and down-regulation of Hdac3 levels resulted in the same number of genes being up- and down-regulated (Figure 4a). Of note, reduction in the level of Hdac3 by at least 80% in cells is required for the observed impairment at some genes (eg. Il-6, Gm-Csf and Ccl-9) and even a small increase in the residual amount of Hdac3 is sufficient to dramatically reduce the impact of its depletion on inflammatory gene expression (data not shown). Western blot with total protein lysates prepared from Hdac1 and Hdac3 knock-down in primary macrophages did not show any obvious change in acetylation of either histone H3 or H4 following HDACs depletion (Figure 4d).

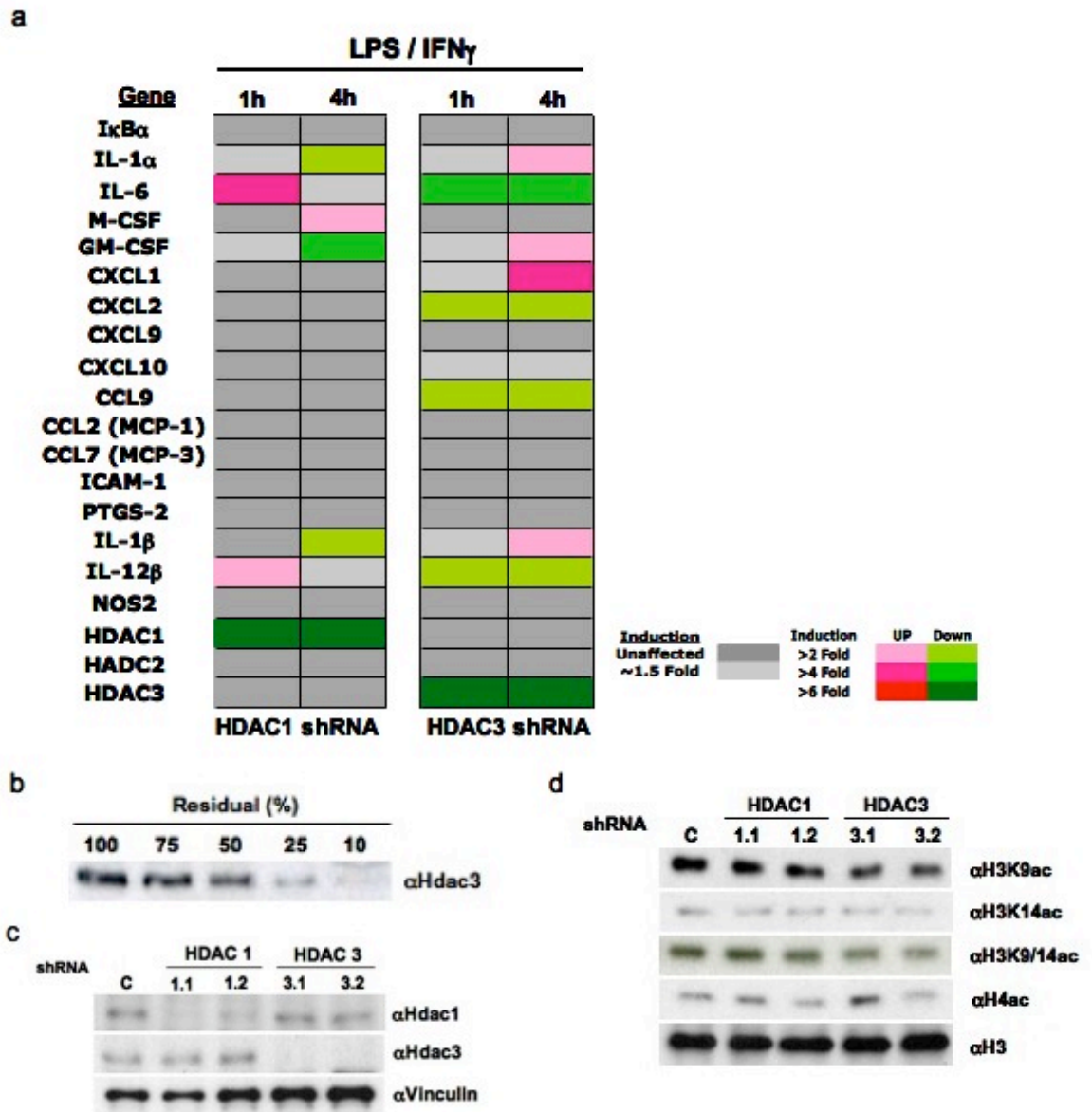


Figure 4. Regulation of inflammatory gene expression by individual class I HDACs in primary macrophages.

(a) Primary macrophages (control and cells in which Hdac1 or Hdac3 were knocked-down) were treated with LPS/IFN γ for either one hour or four hours prior to RNA extraction. mRNA for the indicated genes following either Hdac1 or Hdac3 knockdown (KD) in primary macrophages were quantified by quantitative RT-PCR and calculated as ratio to a housekeeping gene (TBP) before expressing as fold change of KD over wild type control, where wild type induction is 1. Data are representative of three independent experiments. Fold expression change are shown in colour code; colour towards red signify increased in gene expression while colour towards green signify decreased in gene expression. Gray means no statistically significant difference. (b) Titration of residual amount of knockdown protein using total lysate prepared from primary macrophages. (c)

Efficiency of individual class I HDACs KD in primary macrophages (two shRNA clones, Hdac1: 1.1, 1.2; Hdac3: 3.1, 3.2). “C” indicates control (mock infection). Western blots were carried out with anti-Hdac1, anti-Hdac3 or loading control anti-vinculin. (d) Effects on acetylation of histone H3 and H4 following Hdac1 and Hdac3 KD. Western blots were carried out with anti-acetyl-H3K9, anti-acetyl-H3K14, anti-acetyl-H3K9/K14 anti-tetra-acetyl-H4 or loading control anti-H3. Representative blots of three independent experiments.

The differences in regulation of genes expression by individual HDACs knock-down in two different cell types suggest that there is no redundancy between the enzymatic activities of Hdac1 and Hdac3 and that they exert both gene- and cell-specific effects. Interestingly, the effects on expression of some genes (namely Il-6, Il-12, Il-1 α , Il-1 β and Gm-Csf) were antagonistically regulated by Hdac1 and Hdac3 suggesting that Hdac1 and Hdac3 might act as an antagonistic pair. Overall, these data indicate that Hdac1 behaves according to the expectations in that it more often represses inflammatory gene expression; Hdac3 is often required for the activation of inflammatory genes. Given the *in vitro* evidences for the involvement of Hdac3 in inflammatory gene activation generated using Hdac3 knockdown experiments, we decided to extend both the *in vitro* and *in vivo* analyses using Hdac3 knockout (KO) mice.

3. Characterisation of conditional *Hdac3* knockout primary macrophages

Germline deletion of *Hdac3* has been reported to cause early embryonic lethality [36, 37]. Therefore, to gain mechanistic insights into the effect of *Hdac3* deletion on inflammatory genes regulation in primary macrophages, we used mice with a ‘floxed’ allele of *Hdac3*. Homozygous *Hdac3* floxed mice (*Hdac3*^{FL/FL}), in which exon 7 (encoding the deacetylase domain) of *Hdac3* was flanked by *loxP* sites (Figure 5a), were obtained from the laboratory of Scott Hiebert (Vanderbilt University, USA) and crossbred with either *Mx-Cre* or *LysM-Cre* mice, which express the Cre recombinase in response to inducible

activation of the interferon system (*Mx-Cre*) or constitutively in the myeloid compartment (*LysM-Cre*) (Figure 5b). We first analysed the efficiency of *Hdac3* deletion at protein and mRNA levels in both *Mx-Cre* and *LysM-Cre* mice.

In the case of *Mx-Cre*, deletion of *Hdac3* was carried out *in vivo*. In brief, *Mx-Cre*; *Hdac3^{FL/FL}* mice were subjected to two rounds of intraperitoneal injection with synthetic double-stranded RNA (Poly-IC), which induced the endogenous interferon system and the expression of *Mx-Cre*. Two days after the second injection of Poly-IC, bone-marrow cells were isolated from these mice and differentiated *in vitro* using M-Csf-containing medium (Figure 5c, upper panel). PCR analysis of the genomic DNA isolated from *Mx-Cre*; *Hdac3^{FL/+}* and *Mx-Cre*; *Hdac3^{FL/FL}* primary macrophages showed the excision of the floxed exon 7 (Figure 5c, lower panel) upon Poly-IC injection. In addition, western blot analysis with whole cell lysate prepared from either *Mx-Cre*; *Hdac3^{FL/+}* or *Mx-Cre*; *Hdac3^{FL/FL}* macrophages showed a complete loss of *Hdac3* expression (Figure 5e, upper panel). RT-PCR also confirmed the inactivation of *Hdac3* (Figure 5e, lower panel). For the *LysM-Cre* system, induction of *LysM-Cre* expression occurs over the course of macrophages differentiation (Figure 5d). Deletion with the *LysM-Cre* system though efficient, still results in approximately 10% of residual *Hdac3* protein (Figure 5e) and 15% of residual *Hdac3* mRNA (Figure 5f). Neither *Hdac1* nor *Hdac2* protein level was increased following *Hdac3* deletion in both *Cre*-systems (Figure 5e, upper panel).

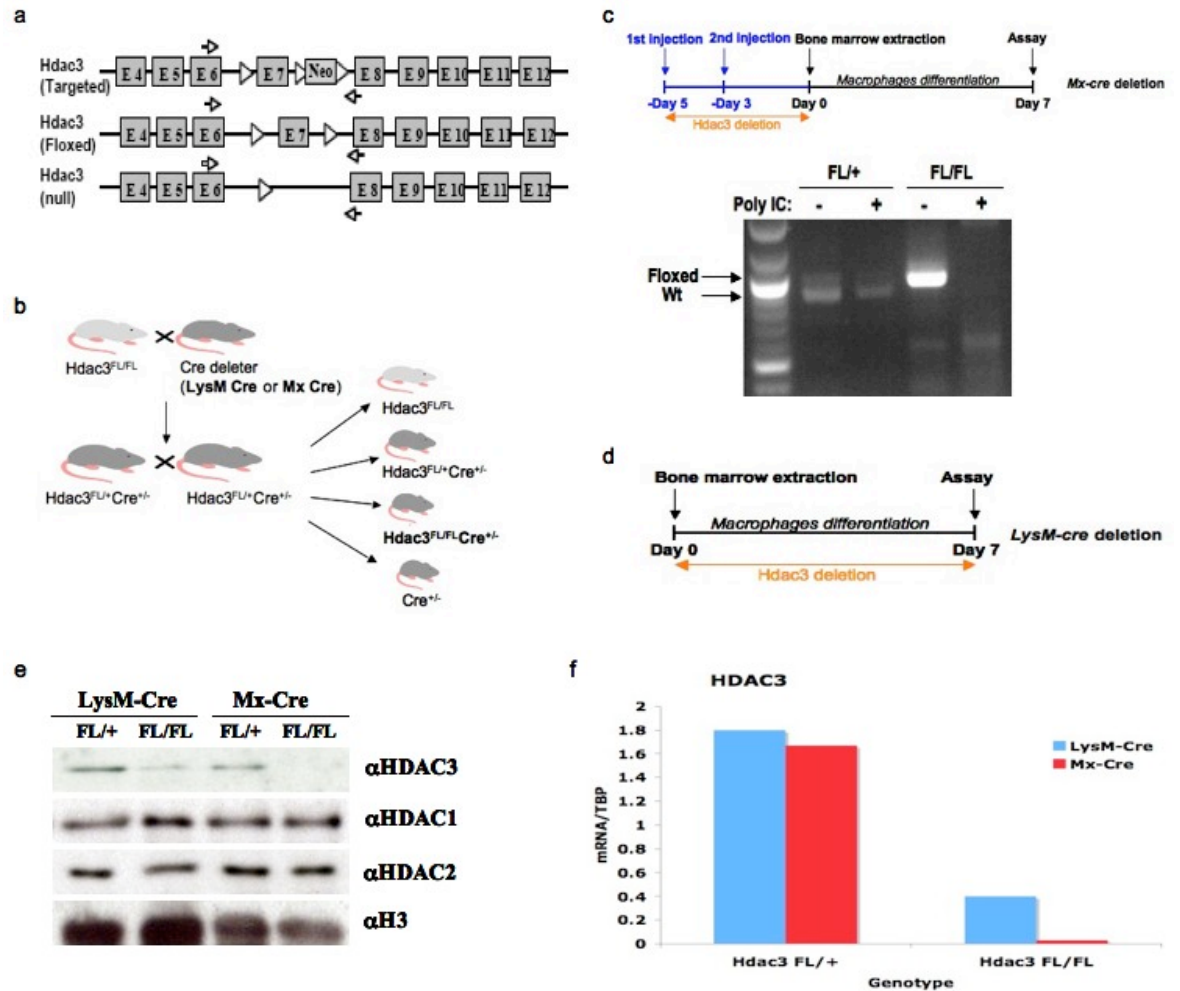


Figure 5. Inducible deletion of Hdac3 using *Mx-Cre* and *LysM-Cre* system.

(a) Schematic representation of the floxed (*Hdac3*^{FL}) and null (*Hdac3*⁻) alleles of *Hdac3*. Arrows indicate PCR primers used for PCR analysis and arrowheads indicate LoxP sites (Bhaskara et al, *Mol. Cell* 2008). (b) Schematic representation of *Hdac3*^{FL}-*Cre* transgenic mice crossbreeding. (c) Time scheme for generation of *Mx-Cre*; *Hdac3*^{FL/+} and null *Mx-Cre*; *Hdac3*^{FL/FL} macrophages ([c], upper panel). PCR analysis of the genomic DNA isolated from *Mx-Cre*; *Hdac3*^{FL/+} and *Mx-Cre*; *Hdac3*^{FL/FL} macrophages generated from mice injected with Poly-IC. PCR performed with these primers (arrows indication in [a]) that flank exon 7 to detect the floxed (935bp), Wt (895bp) and null (211bp) bands. “M”: indicates 100 bp ladder ([c], lower panel). (d) Time scheme for generation of *LysM-Hdac3*^{FL/+} and null *LysM-Hdac3*^{FL/FL} macrophages. (e) Western blot analysis for Hdac3 with protein lysates (wt, ko) prepared from either the *Mx-Cre* or *LysM-Cre* system. Anti-histone H3 is a loading control. Representative blots of three independent experiments using primary macrophages derived from a different *Hdac3*^{+/-} or *Hdac3*^{-/-} mouse. (f) mRNAs for the

Hdac3 were quantified by quantitative RT-PCR and expressed as ratio to a housekeeping gene (TBP).

Several studies have shown that *Hdac3* is involved in regulation of cell proliferation and differentiation [71, 74]. We noted that the complete loss of *Hdac3* in null *Mx-Cre*; *Hdac3*^{FL/FL} bone marrow cells resulted in a reduced yield of primary macrophages (usually 50% less than primary macrophages derived from *Mx-Cre*; *Hdac3*^{FL/+} mice). Conversely, no difference between wild type and null *Hdac3* was observed for primary macrophages generated with the LysM-Cre system. To ensure that the cells obtained with Mx-Cre system are truly bone marrow-derived macrophages, we analysed the primary macrophages generated from both the Mx-Cre and LysM-Cre system by flow cytometry (FACS) for expression of differentiated macrophage markers CD11b and F4/80. Percentage of F4/80⁺ CD11b⁺ cells obtained for both the null *LysM-Cre Hdac3*^{FL/FL} (Figure 6, upper panel) and *Mx-Cre; Hdac3*^{FL/FL} (Figure 6, lower panel) populations were the same as that of wild type, indicating that loss of *Hdac3* does not affect macrophages differentiation.

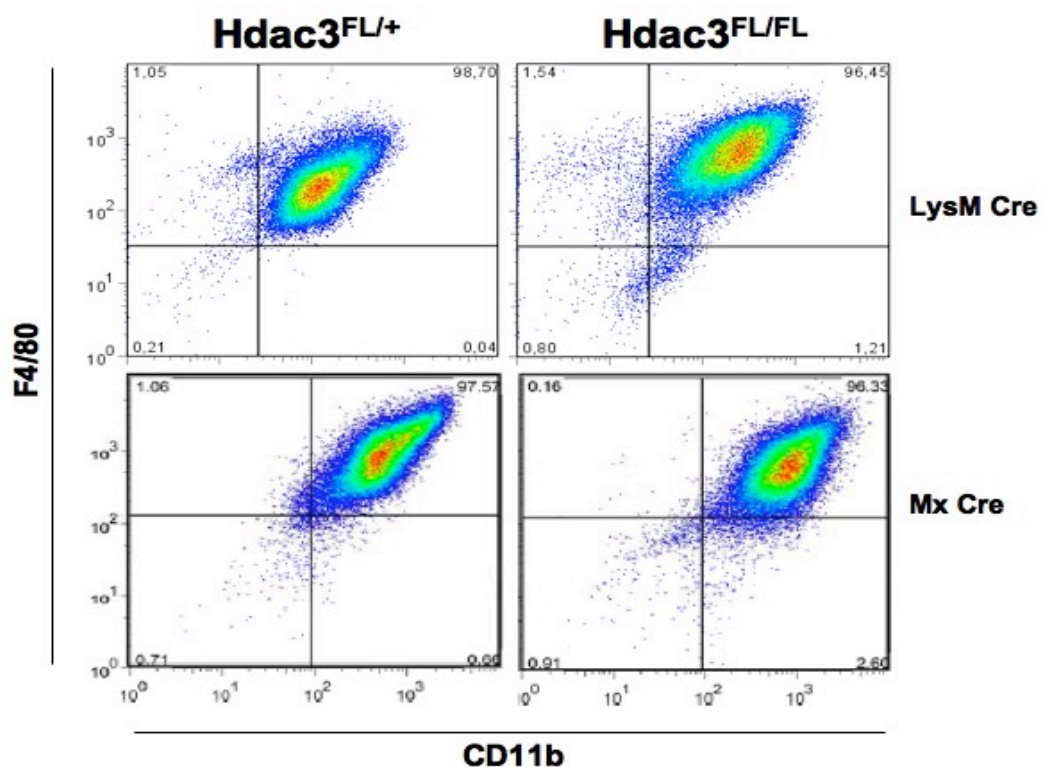


Figure 6. Loss of *Hdac3* does not affect macrophage differentiation.

Primary macrophages generated from either the LysM-cre system (*LysM-Hdac3*^{FL/+} and *LysM-Hdac3*^{FL/FL}; upper panel) or Mx-Cre system (*Mx-Hdac3*^{FL/+} and *Mx-Hdac3*^{FL/FL}; lower panel) were analysed by flow cytometry using antibodies specific for F4/80 and CD11b. Data are representative of three independent experiments each using primary macrophages derived from a different *Hdac3*^{+/-} or *Hdac3*^{-/-} mouse.

Because of the higher (and near-complete) deletion efficiency, we decided to carry out all experiments using primary macrophages from *Mx-Cre; Hdac3*^{FL/FL} mice. For simplicity, primary macrophages generated from *Mx-Cre; Hdac3*^{FL/+} and *Mx-Cre; Hdac3*^{FL/FL} mice will hereafter be referred to as *Hdac3*^{+/-} (heterozygous) and *Hdac3*^{-/-} (knockout).

4. Functional changes in *Hdac3*^{-/-} primary macrophages

We next determined the functional consequences of *Hdac3* deletion on the expression of LPS-induced macrophage surface molecules and cytokines. *Hdac3*^{+/-} and *Hdac3*^{-/-} primary macrophages were either untreated or treated with LPS overnight (14 h) and analysed by FACS for expression of the activation markers CD40 and CD86. Compared to *Hdac3*^{+/-} primary macrophages, the number of cells expressing CD40 (Figure 7a, left panel) was reduced by more than 3 -fold while CD86 up-regulation (Figure 7a, right panel) was completely abolished in LPS-treated *Hdac3*^{-/-} primary macrophages. Culture supernatants also revealed that *Hdac3*^{-/-} primary macrophages were impaired in the production of Il-6 and Il-10 but not keratinocyte-derived chemokine (KC) and Tnf- α (Figure 7b) upon LPS stimulation.

In addition, unlike cells treated with TSA, in which we found an increase in acetylation of histone H3K9 and H3K14 (Figure 7c, right panel), deletion of *Hdac3* in macrophages resulted in a small but reproducible decrease in acetylation of histone H3K9 and H3K14 (Figure 7c).

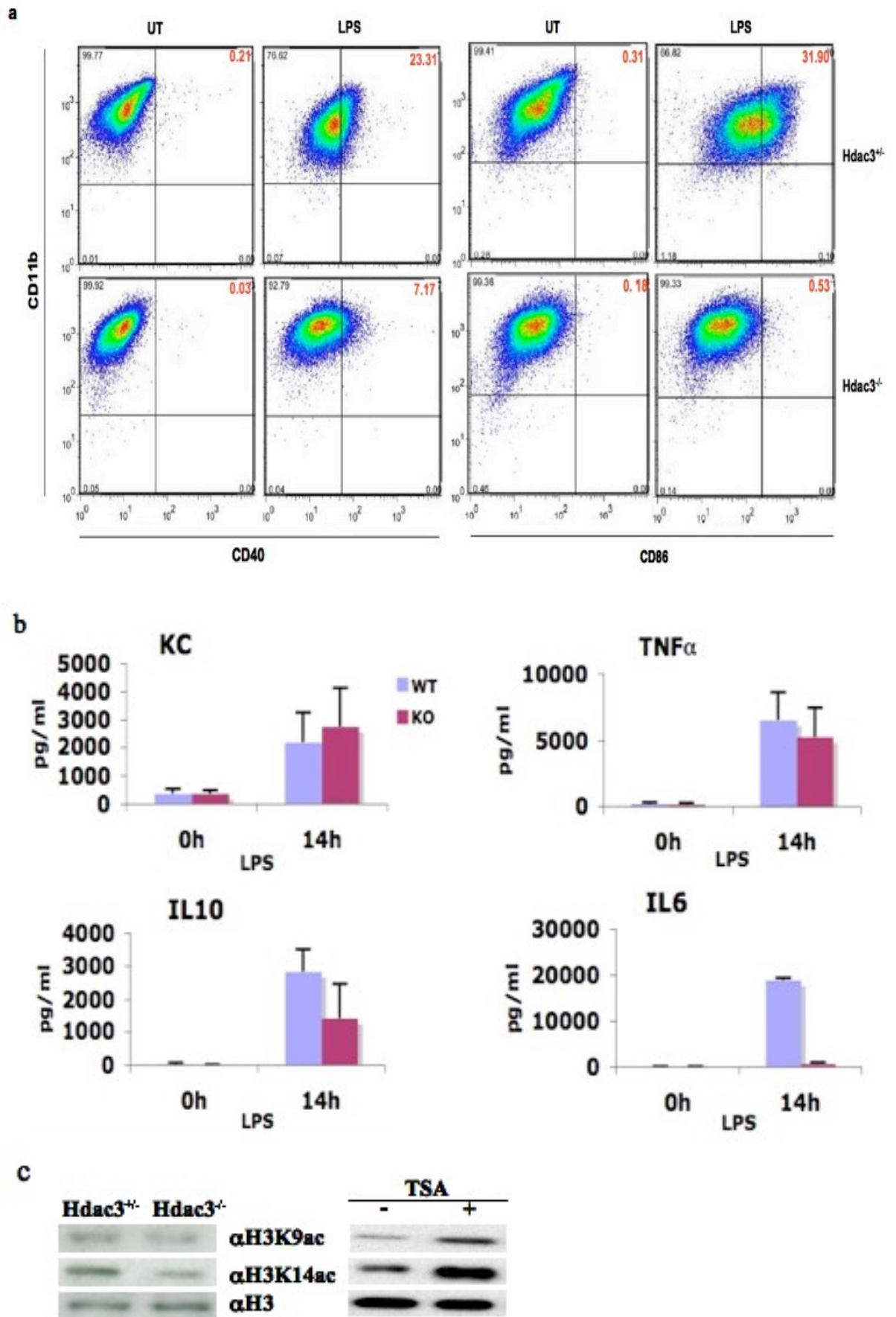


Figure 7. Loss of *Hdac3* impairs LPS-induced macrophage activation.

(a) Primary *Hdac3*^{+/+} and *Hdac3*^{-/-} macrophages were treated with 100 ng/ml LPS for fourteen

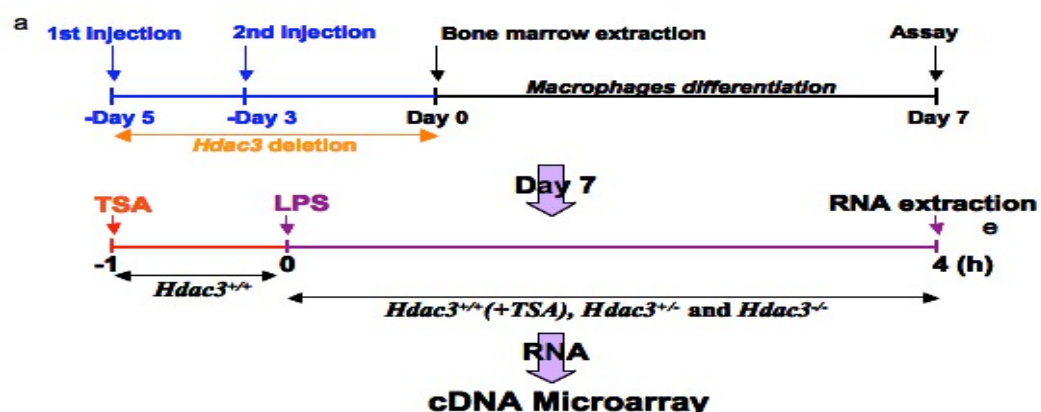
hours before harvesting for analysis by flow cytometry using antibody specific for CD40 and CD86. Data are representative of three independent experiments each using primary macrophages derived from a different *Hdac3*^{+/-} or *Hdac3*^{-/-} mouse. (b) ELISA of KC, Tnf- α , Il-10 and Il-6 in supernatants of wild type and *Hdac3*^{-/-} untreated or treated with LPS for fourteen hours. Data shown are the means $\pm$ SD and are representative of two independent experiments each using primary macrophages derived from a different *Hdac3*^{+/-} or *Hdac3*^{-/-} mouse. (c) Histone acetylation in *Hdac3*^{+/-}, *Hdac3*^{-/-} primary macrophages and wild type treated with 100 ng/ml TSA. Western blots were carried out with the indicated antibodies. Representative blots of two independent experiments using primary macrophages derived from a different *Hdac3*^{+/-} or *Hdac3*^{-/-} mouse.

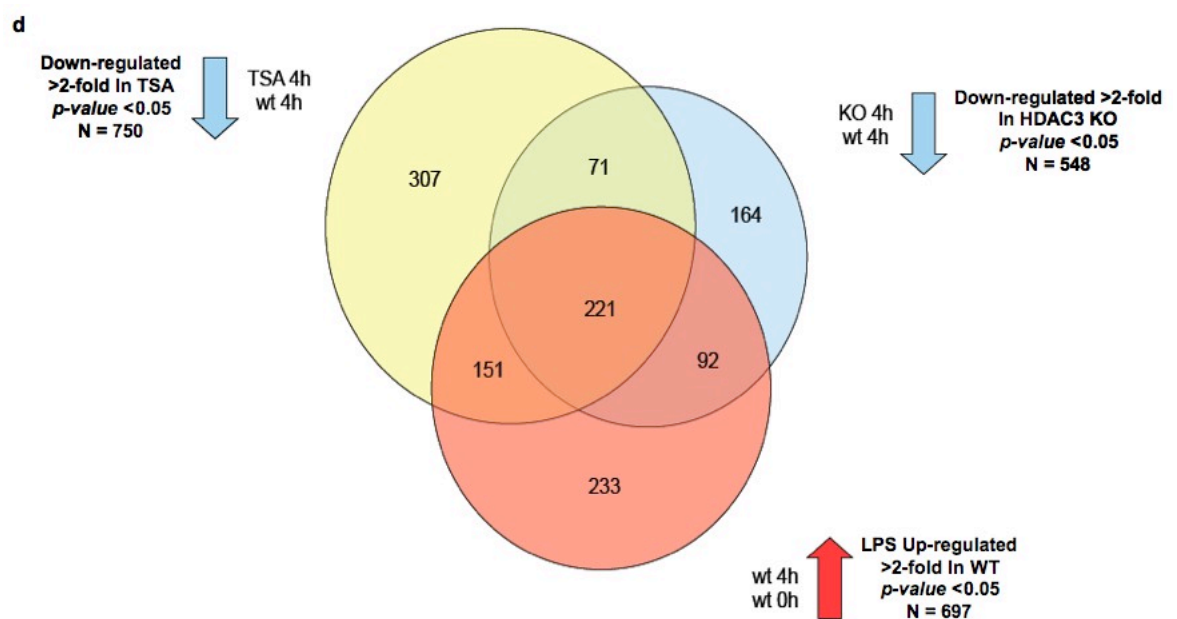
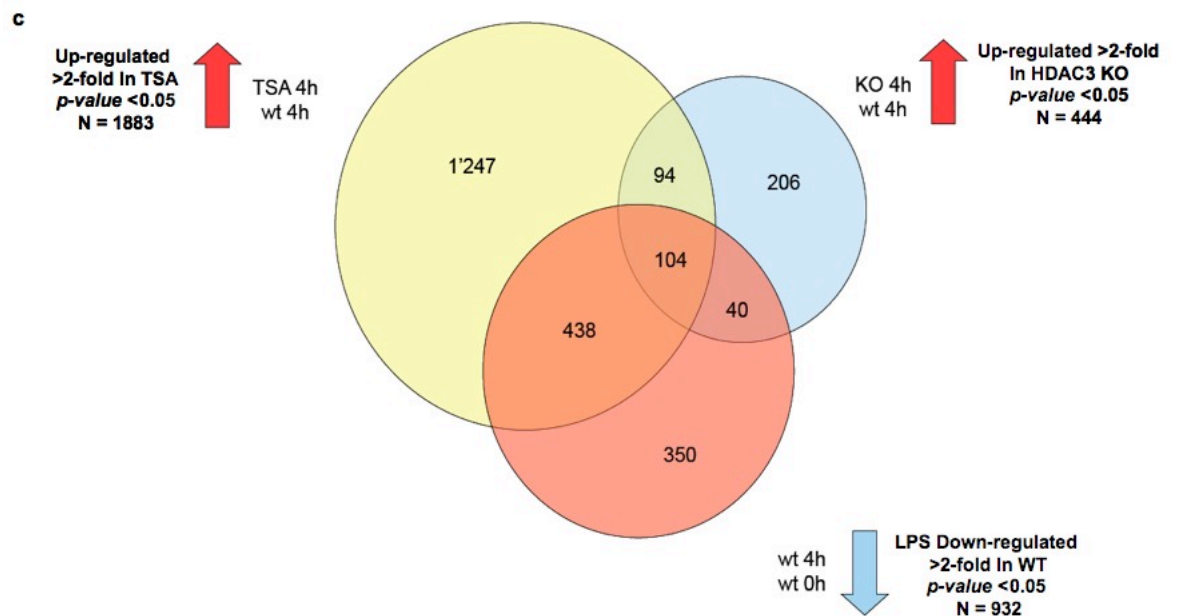
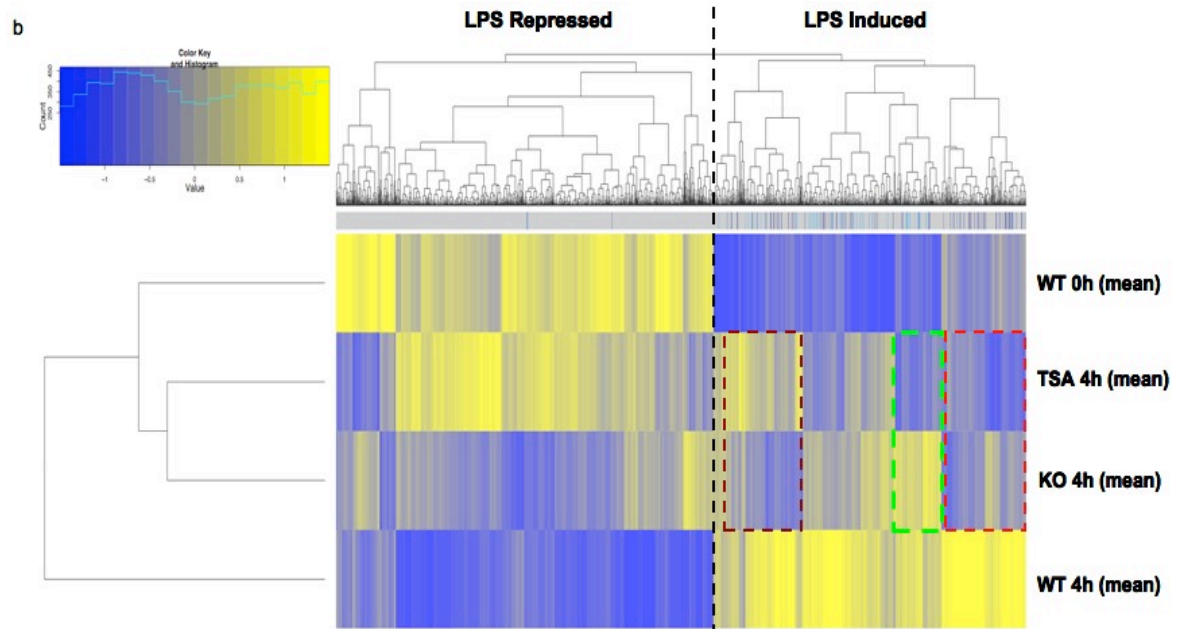
5. HDAC3 is extensively required for LPS-induced gene expression.

Given the requirement for *Hdac3* in the induction of selected genes in macrophages, we set out to obtain a more global view of gene expression changes using gene expression microarrays. *Hdac3*^{-/-} primary macrophages were compared to *Hdac3*^{+/-} controls under unstimulated or LPS-stimulated (4h) conditions. In order to better define the subset of down-regulated genes that are direct targets of HDAC3-mediated activation, we also determined the expression profile of TSA pre-treated *Hdac3*^{+/-} primary macrophages. Bone marrow cells were extracted from *Mx-Cre; Hdac3*^{FL/+} (*Hdac3*^{+/-}) and *Mx-Cre; Hdac3*^{FL/FL} (*Hdac3*^{-/-}) mice that had been subjected to two rounds of intraperitoneal injection with Poly-IC and left to differentiate into primary macrophages *in vitro*. At day 7 of differentiation, *Hdac3*^{+/-} and *Hdac3*^{-/-} primary macrophages were treated with LPS (4 h) or left untreated. In the case of TSA-treated cells, *Hdac3*^{+/-} primary macrophages were pre-treated with TSA (1 h) prior to stimulation with LPS or left unstimulated (Figure 8a). For each expression profile, RNA samples from three biologically independent experiments were analysed.

We first classified genes into two groups according to their expression changes in LPS-treated macrophages, namely the LPS-repressed and LPS-induced groups, which include 932 and 697 genes, respectively (using as cut-off a fold-change of 2 and a $p < 0.05$) (Figure

8b). 58.1% (n=542/932) of the genes that were repressed by LPS in wild type macrophages were de-repressed upon TSA treatment, suggesting that HDACs are crucial for LPS-induced gene repression. Conversely, loss of *Hdac3* rescued only 15.4% (n=144/932) of the LPS-repressed genes, thus suggesting that *Hdac3* provides a relatively marginal contribution to this response (Figure 8c). Importantly, genes rescued by *Hdac3* deletion overlapped by 44.6% with those rescued by TSA treatment. As for the LPS-inducible genes, 44.9% of them (n= 313/697) were down-regulated in *Hdac3*^{-/-} primary macrophages and 53.3 % were down-regulated upon TSA treatment, with an extensive overlap between the two groups (Figure 8d). Therefore, almost half of the LPS-inducible gene expression program specifically requires *Hdac3* and most of the effects of TSA on LPS-induced transcription can be recapitulated by the deletion of this single HDAC. Hierarchical clustering (Figure 8b) allows identifying groups of genes that are concordantly and discordantly regulated by *Hdac3* deletion and TSA treatment. Validation of the gene-expression analysis by quantitative RT-PCR using different biological samples confirmed the changes observed in the microarray assays (Figure 8e).





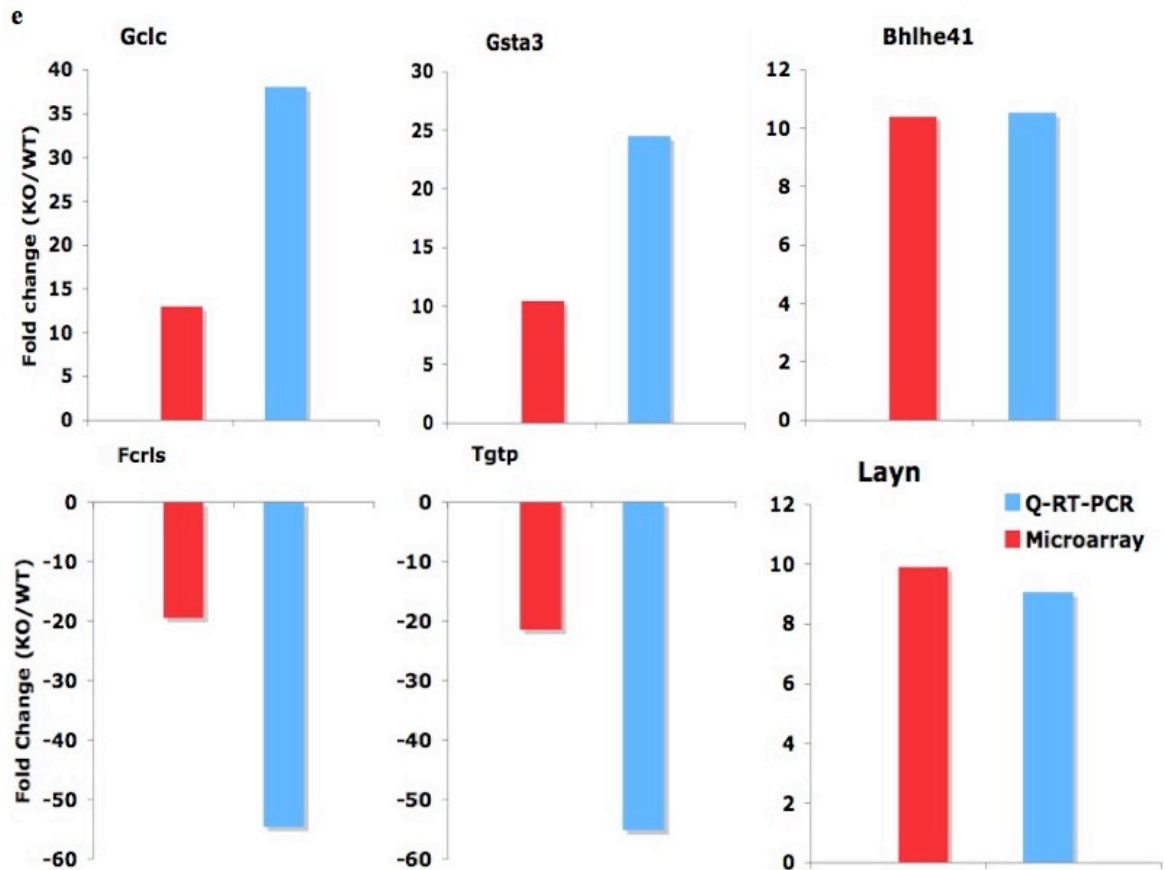


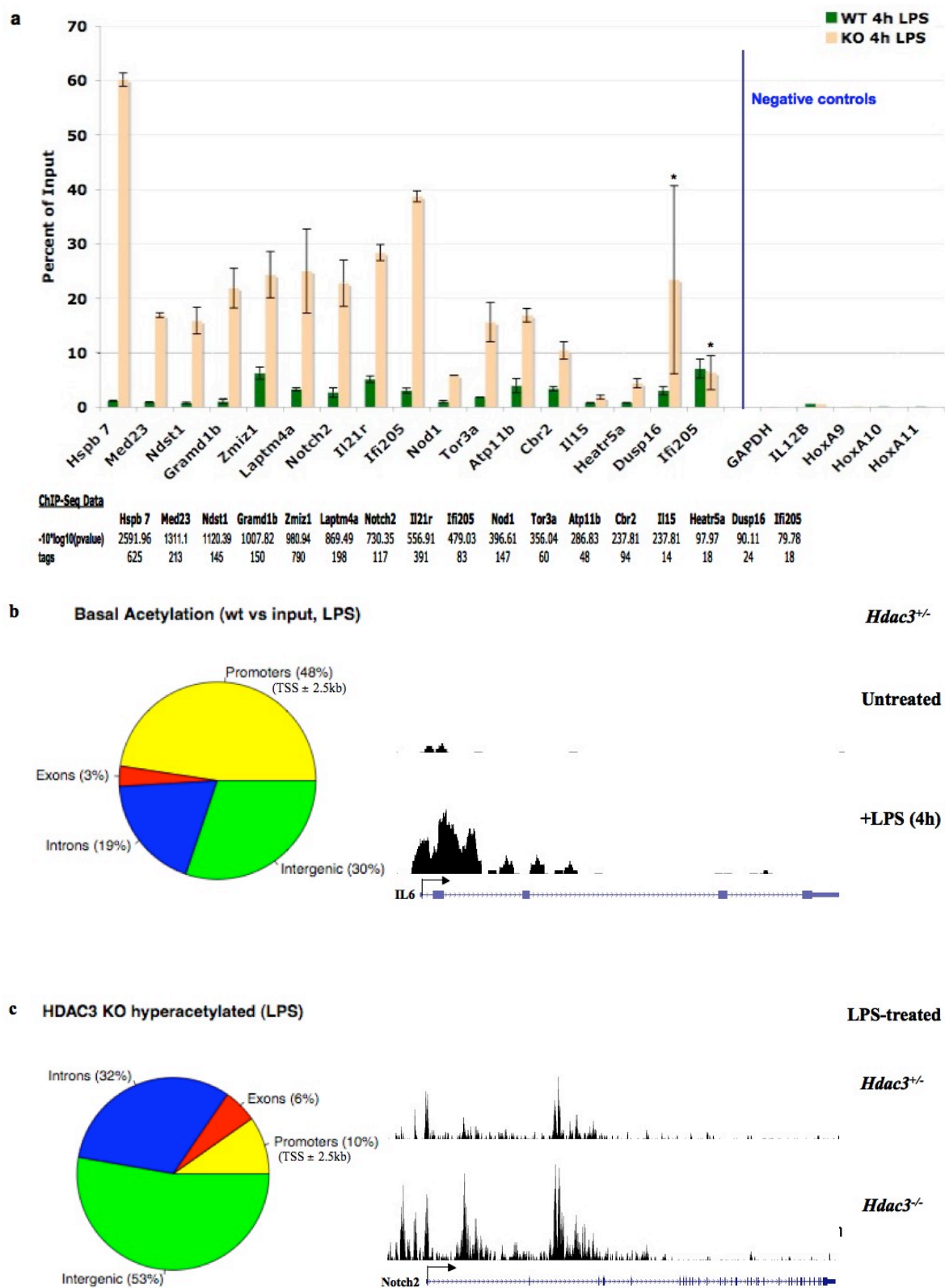
Figure 8. HDAC3 is required for the LPS-induced genes expression program.

(a) Time scheme for generation of *Hdac3*^{-/+} and null *Mx-Hdac3*^{-/-} primary macrophages and LPS treatment. Cells were treated with 100 ng/ml LPS for either one hour or four hour. For TSA-treated cells, primary macrophages were pre-treated with TSA for one hour and then co-stimulated with LPS as indicated. (b) Gene expression microarray data sets were divided into two groups: LPS-repressed and LPS-induced. The expression of genes from *Hdac3*^{-/+} (WT), TSA-treated *Hdac3*^{-/+} (TSA) and *Hdac3*^{-/-} (KO) is shown as heat plot after hierarchical clustering. Colour gradient indicates the fold-induction (yellow) and -repression (blue). Values shown are the mean of pooled data (fold change ≥ 2 , $p < 0.05$) from three biological replicates. (c) Venn diagram showing the overlap between genes down-regulated by LPS and up-regulated (rescued) by TSA or Hdac3 deletion (fold change ≥ 2 , $p < 0.05$). (d) Venn diagram showing the overlap between LPS-inducible genes and genes that are down-regulated by TSA treatment and/or *Hdac3* deletion (fold change ≥ 2 , $p < 0.05$). (e) Validation of up-regulated ([e], upper panel) and down-regulated ([e], lower panel) genes in *Hdac3*^{-/-} from microarray analysis by quantitative RT-PCR. Each graph represents fold change increase or decrease from microarray (blue) and fold change verified by quantitative RT-PCR (red).

6. Genome-wide histone acetylation patterns in *Hdac3*^{-/-} primary macrophages

Hdac3 inactivation has been shown to result in modest increases in the acetylation of histone H4K5, H4K8 and H4K12 in *Hdac3*-null liver. We used chromatin immunoprecipitation coupled to multi-parallel sequencing (ChIP-seq) to evaluate the distribution of histone H4 acetylation across the genome before and after LPS stimulation in wild type and *Hdac3*^{-/-} primary macrophages. DNA from formaldehyde cross-linked chromatin obtained from untreated and LPS-treated (4 h) *Hdac3*^{+/+} or *Hdac3*^{-/-} primary macrophages was purified by immunoprecipitation with an antibody to tetra-AcH4 (recognising: acetyl-lysine 5, 8, 12, and/or 16 on histone H4). Immunoprecipitated DNA was ligated to oligonucleotide adapters, amplified and subjected to massively parallel sequencing using a Solexa GAII platform. We obtained 12 008 792 reads, 4 639 850 (PCR bias purged) of which could be unambiguously aligned to the mouse genome. A group of peaks representative of a broad range of *p*-values was randomly picked and checked by standard ChIP-QPCR (Figure 9a). We found for *p*-values below 97.97×10^{-10} to be similar in both ChIP-seq and ChIP-PCR. Next, we divided the mouse genome into four regions (promoters (transcription start site [TSS] +/- 2.5 kb); exonic, intronic and intergenic regions) and analysed the distribution of hyper- and hypo-acetylated sites (using a cut-off of $p < 10^{-10}$). As shown in Figure 9b, LPS treatment of control cells induced histone hyperacetylation primarily at promoters (48%; *n* = 11742/24672) and to a lesser extent at intergenic (30%; *n* = 7448/24672) and intronic (19%; *n* = 4652/24672) regions. However, genomic regions that were hyperacetylated in LPS-treated *Hdac3*^{-/-} as compared to wild type macrophages were mainly distal (intergenic, 53% (*n* = 1366/2592); intronic 32% (*n* = 825/2592)), being associated to promoters only in 10% (*n* = 252/2592) of cases (Figure 9c). A marked hypoacetylation was also observed in *Hdac3*^{-/-} cells at a large number of regions, both at promoters (21%; *n* = 231/1095) and at distal locations (intergenic regions, 43% (*n* =

474/1095); introns 30% (n= 330/1095)) (Figure 9d).



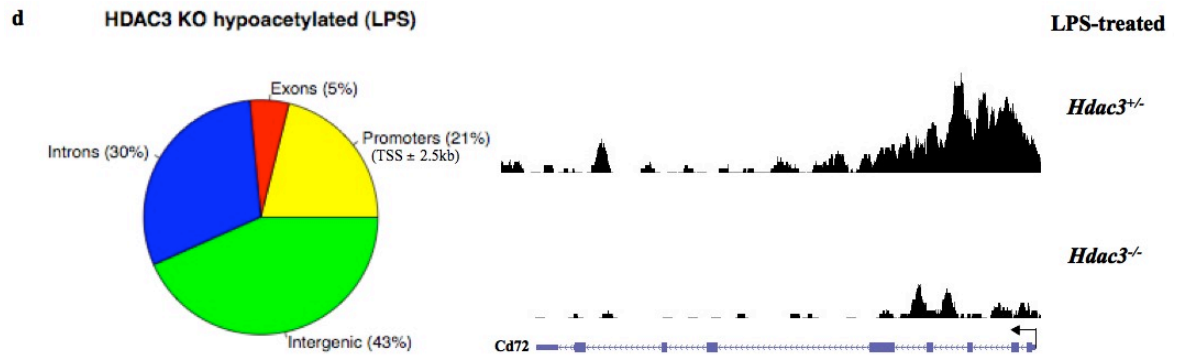


Figure 9. Distribution of hyper- and hypo-acetylated regions in *Hdac3*^{-/-} primary macrophages.

(a) Validation of acetyl-H4 ChIP-seq data set in LPS-treated *Hdac3*^{+/+} (WT) or *Hdac3*^{-/-} (KO) primary macrophages with standard ChIP-PCR. Data are expressed as percent of input and asterisk indicates peak that did not pass the criteria for validation ([a] upper panel). Regions are randomly selected based on the p-value of hyperacetylated regions in LPS-treated *Hdac3*^{-/-} ([a], lower panel).

(b) Genomic distribution of histone H4 hyperacetylation (relative to UCSC known genes) in LPS-treated wild type control, scored against input with the stringency of $p < 1e-10$ ([b], left panel). Representative screenshot showing hyperacetylation at promoter region upon LPS treatment ([b], right pane).

(c) Genomic distribution of histone H4 hyperacetylation (relative to UCSC known genes) in LPS-treated *Hdac3*^{-/-}, scored against LPS-treated wild type with the stringency of $p < 1e-10$ ([c], left panel). Representative screenshot showing hyperacetylation on the gene upon LPS treatment ([c], right pane).

(d) Genomic distribution of histone H4 hypoacetylation (relative to UCSC known genes) in LPS-treated *Hdac3*^{-/-}, scored against LPS-treated wild type with the stringency of $p < 1e-10$ ([d], left panel). Representative screenshot showing hypoacetylation on the gene upon LPS treatment ([d], right pane).

Iros Barozzi carried out computational analysis.

In yeast, deletion of *Hos2* (the HDAC3 ortholog) led to an increased acetylation at the 5' of some coding units, which correlated with impaired transcription of *GAL* genes under inducing conditions [62]. These data were taken as evidence that excessive histone acetylation downstream of the TSS may be detrimental to transcription elongation. To test

the hypothesis that the impaired activation of LPS-induced genes in *Hdac3*^{-/-} primary macrophages was due to an increase in histone acetylation in the body of the genes, we examined the level of acetylation inside the coding region of LPS-induced and Hdac3-dependent genes. Only 8.6% (27/313) out of all the LPS-inducible, Hdac3-dependent genes showed detectable histone hyperacetylation in their coding unit. Selected examples of regions selectively hypo- or hyper-acetylated in *Hdac3*^{-/-} cells are shown in Figure 10a and 10b. Therefore, that the activating role of Hdac3 in this system may occur mainly through mechanisms distinct from those suggested to operate in yeast and independent of intragenic histone hyperacetylation.

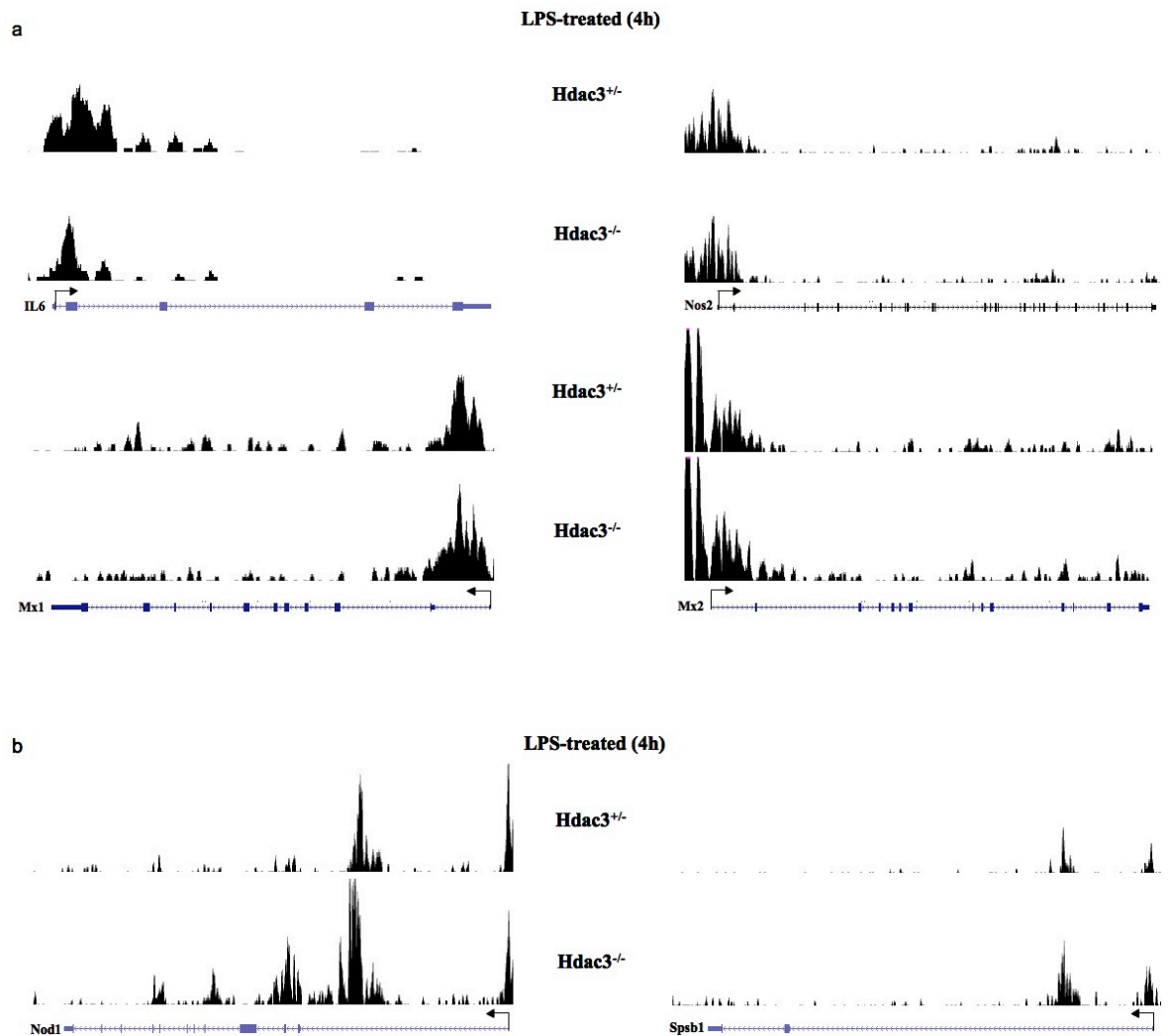


Figure 10. Acetylation changes at LPS-inducible, Hdac3-dependent genes in primary macrophages.

(a) Four representative screenshots showing no change in acetylation in the coding region of down-regulated, LPS-inducible genes in *Hdac3*^{-/-}. (b) Two representative screenshots showing hyperacetylation in Hdac3-dependent genes.

We next set out to determine if the regions that are differentially acetylated in *Hdac3*^{-/-} macrophages are preferentially bound by specific transcription factors. Therefore we searched transcription factor binding sites (TFBSs) that are over- and under- represented in the differentially acetylated regions. TFBS overrepresentation analysis was carried out using CLOVER [129]. Given a set of position weight matrices (PWMs) - representing known transcription factor DNA binding sites- and a coherent background set of sequences, CLOVER is able to assess which PWMs are significantly over- and under- represented in a set of input sequences. Position-specific score matrices (PSSM) were used to calculate the possibility of a specific transcription factor binding at one genomic locus, as described by Frith *et al.* [129] (see Methods).

Differentially acetylated regions were divided into four groups: hyper-acetylated proximal regions (spanning TSS ± 2.5 kb), hypo-acetylated proximal regions, and hyper- or hypo-acetylated regions distal to the promoters. As references for statistical over-representation analyses we used sets of sequences having the same genomic distribution (proximal or distal) and detectable levels of acetylation that did not change in *Hdac3*^{-/-} as compared to wt macrophages. We found that hyperacetylated and hypoacetylated regions show obvious differences in terms of TFBS composition. Specifically, both proximal and distal hyperacetylated sites were highly enriched for PMWs for AP-1 transcription factors (highlighted in yellow in Table 1a) and E-box binding proteins (Myf6, Tcf2a, Myc-Max) (highlighted in green in Table 1a). Conversely, hypoacetylated sites were enriched for binding sites specific for Interferon Regulatory Factor (Irf) and Signal Transducers and Activators of Transcription (Stat) family members (highlighted in tan in Table 1b). Regional hyperacetylation may be due to loss of TF-associated Hdac3 and to the

consequent prevalence of HATs over HDACs. Alternatively, AP-1 and E-box proteins may be hyper-activated in *Hdac3*^{-/-} macrophages. Conversely, hypoacetylation may be simply explained by a reduced activity of the TFs binding to the corresponding genomic regions.

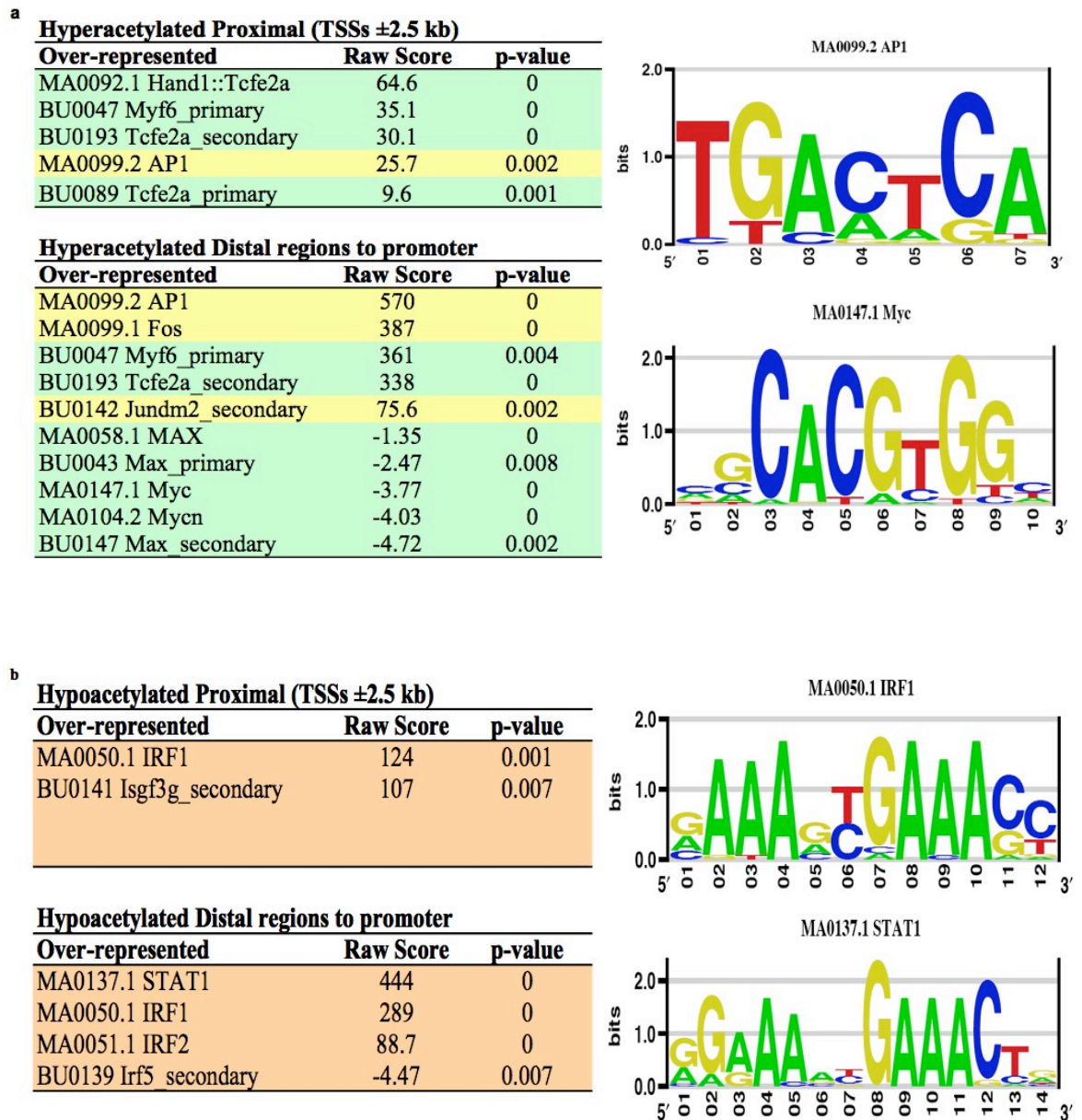


Table 1. Enrichment for TFBSs in the differentially acetylated regions of *Hdac3*^{-/-} macrophages.

(a) TFBSs (PWMs) that are over-represented in hyperacetylated regions of the promoters, TSSs (TSS ± 2.5 kb) and regions distal to TSSs. (b) TFBSs that are over-represented in hypoacetylated regions of the promoters, TSSs (TSS ± 2.5 kb) and regions distal to TSSs. Light yellow indicates PWMs for AP-1 transcription factors; light green indicates E-box-type PWMs and tan indicates Irf/Stat. Representative PWMs are shown in the right panel.

7. Network and Pathway analysis in *Hdac3*^{-/-} primary macrophages

To identify the biological pathways most affected in *Hdac3*^{-/-} primary macrophages, we used the Ingenuity Pathway Analysis (IPA), which identifies the biological processes, networks and canonical pathways within a gene expression dataset. The underlying concept is that genes with similar function as well as expression profiles are more likely to be under the same regulatory control than genes within either of the categories.

Among the five most altered pathways identified, “Interferon signalling” was the most significant one ($p=3.54E-9$), with 12 out of 26 (46%) molecules being affected (Table 2).

Top Canonical Pathways		
Name	p-value	Ratio
Interferon Signaling	3.54E-09	12/26 (0.462)
Retinoic acid Mediated Apoptosis Signaling	2.86E-07	11/40 (0.275)
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	6.42E-07	16/75 (0.213)
NRF2-mediated Oxidative Stress Response	8.66E-07	25/176 (0.142)
Communication between Innate and Adaptive Immune Cells	1.47E-06	14/75 (0.187)

Table 2. The top canonical pathways altered in *Hdac3*^{-/-} primary macrophages.

Iros Barozzi carried out computational analysis.

Transcription factors Stat1 and Stat2, which are essential for responsiveness to $\text{Ifn}\alpha$ and $\text{Ifn}\gamma$, as well as many of the Stats downstream targets, including Irf1 (which mediates some of the STAT1-dependent effects), *Ifitm1*, *Mx1*, *Psmb8*, *Ifi35*, *Ifit1*, *Ifit3* and *Ifi35* were shown to be down-regulated (Figure 11). Importantly, Irf- and Stat-type PWMs were found to be over-represented in both the TSS-proximal and -distal regions shown to be hypoacetylated in *Hdac3*^{-/-} cells, likely indicating that *Hdac3* deletion has a strong impact on the functionality of the Stat/Irf system. Differently from the Interferon signalling pathway, that was extensively down-regulated, the “NRF2-mediated oxidative stress

response” pathway was the one most induced in *Hdac3*^{-/-} macrophages. Nrf2 (Nuclear Factor E2-related Factor 2) controls the expression of a large number of enzymes involved in anti-oxidant and environmental stress responses, including the enzymes involved in glutathione synthesis and glutathione-dependent peroxidases. Most of the crucial anti-oxidant genes, such as Gclc and Gclm (encoding the catalytic and modifier subunit of the rate-limiting enzyme in glutathione synthesis, glutamate-cysteine ligase), Nqo1 (encoding the NAD(P)H:quinone oxidoreductase 1), Gst (Glutathione S-transferase) in the Nrf2-dependent pathway were significantly up-regulated in *Hdac3*^{-/-} macrophages, both at the basal level and after LPS stimulation (Figure 12). Nrf2 activation is involved in negative regulation of inflammation and prevents the excessive production of inflammatory mediators. Lack of Nrf2 in macrophages and mice was shown to increase TLR pathway activation, leading to increased expression of inflammatory genes such as Il-6, Il-1 β , iNos and Cox2 [139]. These data indirectly suggest that a dysregulation of oxidative control may underlie signalling problems that in the end impair inflammatory gene expression in *Hdac3*^{-/-} macrophages.

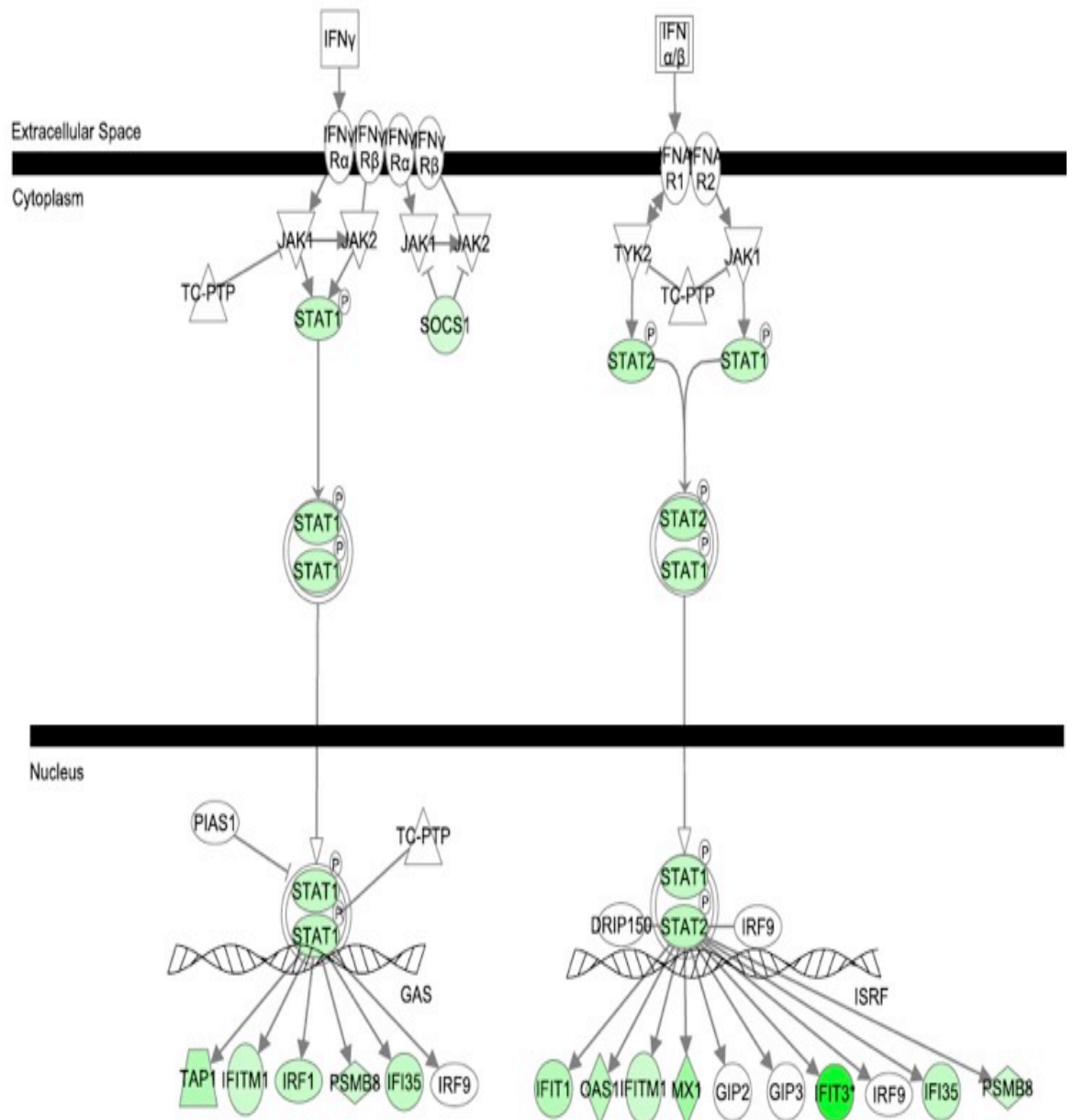


Figure 11. “Interferon signaling pathway” identified as the top canonical pathway altered in *Hdac3*^{-/-} primary macrophages.

De-regulated genes in *Hdac3*^{-/-} that fall in this pathway are shown in green (down-regulated).

Intensity of the colour indicates the fold change.

Iros Barozzi carried out computational analysis.

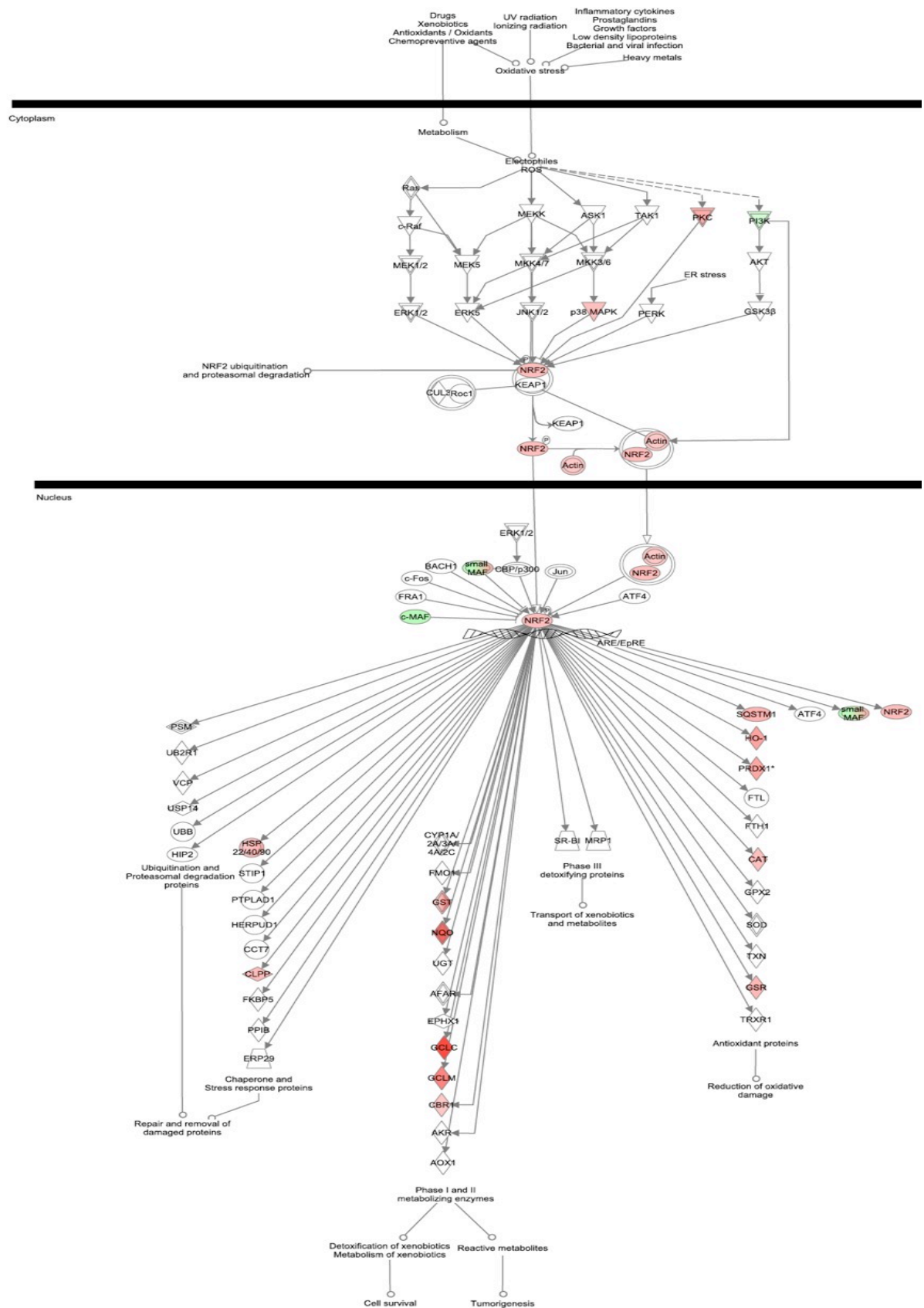


Figure 12. “NRF2-mediated oxidative stress response” identified as one of the top canonical pathway altered in *Hdac3*^{-/-} primary macrophages.

De-regulated genes in *Hdac3*^{-/-} that fall in this pathway are shown in either red (up-regulated) or green (down-regulated). Intensity of the colour indicates the fold change.

8. Promoter sequence analysis of LPS-induced genes that require Hdac3

Both histone acetylation changes in *Hdac3*^{-/-} cells and Ingenuity Pathway Analysis point to a prominent dysregulation of the Interferon/Stat1/Irf system in cells lacking Hdac3. To better characterise the sources of gene expression changes in *Hdac3*^{-/-} cells, we analysed the promoters of differentially expressed genes to identify the TFs that may be responsible for changes in expression. Sequences from -500 bp to +250 bp relative to the TSSs of the differentially expressed genes in LPS-treated *Hdac3*^{-/-} primary macrophages were scanned for TFBS motifs that were significantly over- or under-represented. When compared to Hdac3-independent genes, LPS-inducible Hdac3-dependent genes were enriched for Stat- and Irf-type PWMs, while E-box-type PWMs (e.g. Arnt, Myc/Max, Usf) were the most under-represented (Table 3). Conversely, PWMs for E-boxes are the most enriched, and those for Stats and Irf s the least enriched, in Hdac3-independent genes (Figure 13). It is interesting to note that the counter-correlation between Irf/Stat matrixes and E-box matrixes was also detected in the ChIP-Seq experiment, in which hyperacetylated regions are enriched for E-box (and AP-1) matrixes and depleted of Irf/Stat matrixes, while hypoacetylated regions show the opposite behaviour. Finally, it should be noticed that matrixes for some TFs that are crucial for the LPS-inducible gene expression program (e.g. NF-κB and AP-1) are similarly represented in the Hdac3-dependent and -independent groups.

(a) Most enriched in HDAC3 dependent genes

HDAC3 dependent vs independent			HDAC3 independent vs dependent		
TF_NAME	Z_SCORE	P_VALUE	TF_NAME	Z_SCORE	P_VALUE
BU0037 Isgf3g_primary	8.21	2.54E-39	MA0146.1 Zfx	5.42	1.79E-17
MA0137.1 STAT1	7.52	2.47E-31	MA0259.1 HIF1A::ARNT	5.42	7.40E-15
BU0036 Irf6_primary	7.37	1.39E-24	MA0004.1 Arnt	5.28	5.55E-18
BU0035 Irf5_primary	7.14	1.22E-28	BU0199 Zfp161_secondary	5.16	1.64E-15
MA0050.1 IRF1	6.79	3.69E-24	BU0147 Max_secondary	5.15	1.39E-17
BU0033 Irf3_primary	6.68	1.48E-23	MA0104.2 Mycn	4.65	1.29E-13
MA0051.1 IRF2	6.27	1.26E-21	MA0104.1 Mycn	4.59	1.09E-13

(b) Least enriched in HDAC3 dependent genes

HDAC3 dependent vs independent			HDAC3 independent vs dependent		
TF_NAME	Z_SCORE	P_VALUE	TF_NAME	Z_SCORE	P_VALUE
MA0146.1 Zfx	-5.42	1	BU0037 Isgf3g_primary	-8.21	1
MA0259.1 HIF1A::ARNT	-5.42	1	MA0137.1 STAT1	-7.52	1
MA0004.1 Arnt	-5.28	1	BU0036 Irf6_primary	-7.37	1
BU0199 Zfp161_secondary	-5.16	1	BU0035 Irf5_primary	-7.14	1
BU0147 Max_secondary	-5.15	1	MA0050.1 IRF1	-6.79	1
MA0104.2 Mycn	-4.65	1	BU0033 Irf3_primary	-6.68	1
MA0104.1 Mycn	-4.59	1	MA0051.1 IRF2	-6.27	1
MA0093.1 USF1	-4.48	1	BU0071 Sox4_primary	-4.00	1

Table 3. Over- and under-represented TFBSs in LPS-inducible Hdac3-dependent and -independent genes.

(a) TFBSs that are over-represented in Hdac3-dependent and -independent genes promoters (from -500 bp to +250 bp relative to TSSs). (b) TFBSs that are under-represented in Hdac3-dependent and -independent genes.

Iros Barozzi carried out computational analysis.

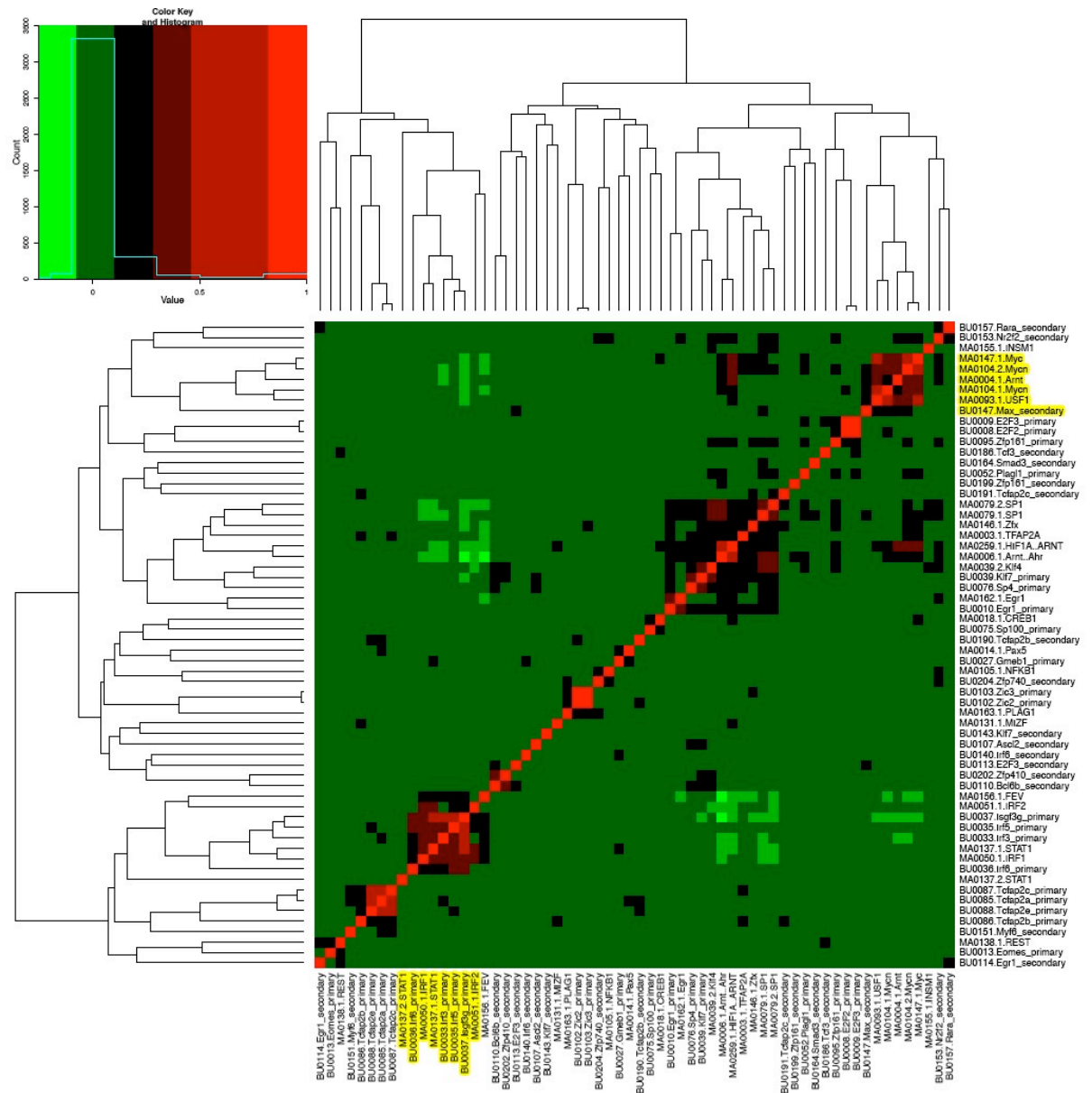


Figure 13. Correlations and anti-correlations between TFBSs enriched in LPS-inducible Hdac3 -dependent and –independent genes.

TFBSs enriched in at least one of the two groups (Hdac3-dependent, horizontal; and Hdac3-independent, vertical), are compared to each other. PWMs are shown as a two-dimensional heat plot after hierarchical clustering. Colour gradient indicates the likelihood ratio (from Pscan analysis) describing the probability that each gene contains a given PWM. Counter-correlated matrices are indicated by the light green colour away from the diagonal.

Iros Barozzi carried out computational analysis.

The high representation of PWMs for Irfs and Stats in Hdac3-dependent genes suggests that the IFN signalling pathway is the most affected in *Hdac3*^{-/-} primary macrophages.

Therefore, we used two publicly available microarray datasets obtained in the same cellular model, namely LPS-induced Irf3-dependent genes [138] and Ifn β inducible genes [123], to initially understand to what extent a malfunctioning of the Irf3/ Ifn β axis can account for Hdac3 effects. It is important to note that LPS-inducible genes that require Irf3 for expression include both direct Irf3 targets (e.g. Ccl5) and genes downstream of Ifn β , whose induction requires Irf3. To this aim, we first examined how many of these LPS-induced, Irf3-dependent genes and Ifn β -inducible genes overlapped with the genes differentially expressed in *Hdac3*^{-/-} macrophages. We found that 190 out of the 313 Hdac3-dependent genes (61%), overlapped Ifn β -inducible genes, 64 genes (20%) were Irf3-dependent and 51 were both Irf3-dependent and Ifn β inducible (Figure 14).

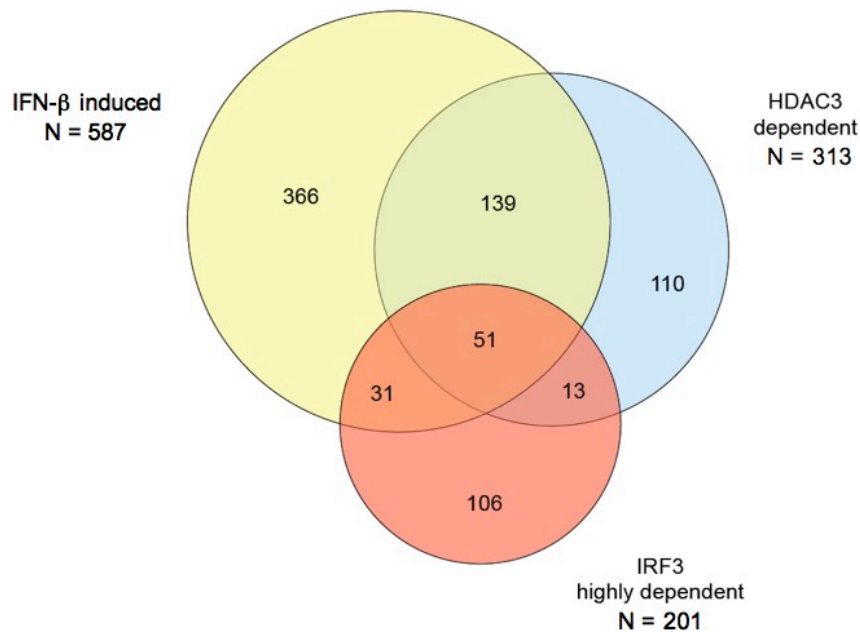


Figure 14. Number of Hdac3-dependent genes that overlap with either Ifn β inducible genes or Irf3-dependent genes.

A Venn diagram showing the number of Ifn β inducible genes (p-value 1.67E-25) and Irf3 dependent genes (p-value 1.68E-11) that overlaps with Hdac3 dependent genes (fold change ≥ 1.5 , p-value of 0.05).

Iros Barozzi carried out computational analysis.

It should be noticed that most Irf3-dependent genes that require Hdac3 for induction are

Ifn β regulated, and therefore likely downstream of the Ifn β autocrine loop. Moreover, the vast majority of the LPS-inducible Irf3-dependent genes are unaffected by *Hdac3* deletion, suggesting that Irf3 function is largely preserved. Therefore, the Hdac3-dependent subgroup of LPS-inducible genes includes genes that are obviously Ifn β -regulated and genes that are apparently not linked to the Irf3/ Ifn β axis. When compared to Hdac3-independent genes, the first group (Hdac3-dependent, Ifn β -inducible genes) showed a strong over-representation of Irf/Stat PWMs (Table 4), which is fully consistent with the requirement for the Stat1/Stat2/Irf9 complex (activated by autocrine Ifn β) for the activation of genes in this group. Unexpectedly, also Hdac3-dependent genes that did not overlap with the Ifn β dataset were enriched (albeit at a much lower statistical significance) in Irf/Stat PWMs. The simplest possibility accounting for this observation is that our set of Ifn β -regulated genes is incomplete, so that some genes regulated by Ifn β through the Stat1/Stat2/Irf9 complex are incorrectly classified as Ifn β -independent. Alternatively, these genes may include bona-fide Ifn β -independent genes that are regulated through alternative Irf or Stat proteins. For instance, impaired activation of Il-6 (which is an Ifn β -independent gene) will result in the loss of an autocrine loop leading to Stat3 activation [137]. Overall, the involvement of Irf and Stats dysregulation in *Hdac3*^{-/-} cells appears to be extremely broad.

(a) Most enriched in IFN genes

IFN genes vs HDAC3 independent			non-IFN genes vs HDAC3 independent		
TF_NAME	Z SCORE	P VALUE	TF_NAME	Z SCORE	P VALUE
BU0037 Isgf3g_primary	9.07267	1.27E-38	BU0036 Irf6_primary	4.86965	2.35E-08
MA0137.1 STAT1	8.16109	9.41E-30	BU0037 Isgf3g_primary	4.40902	4.77E-08
MA0050.1 IRF1	7.89575	5.97E-26	BU0035 Irf5_primary	4.35294	1.84E-07
BU0035 Irf5_primary	7.59309	2.39E-27	MA0137.1 STAT1	4.16423	2.66E-07
MA0051.1 IRF2	7.36462	5.12E-23	BU0033 Irf3_primary	3.80444	4.13E-06
BU0036 Irf6_primary	7.28727	6.27E-21	MA0099.2 API	3.17634	0.000126012
BU0033 Irf3_primary	7.0176	8.99E-22	MA0050.1 IRF1	3.08143	0.000122839
BU0071 Sox4_primary	3.73023	5.74E-06	MA0072.1 RORA_2	2.97074	0.000353673
MA0156.1 FEV	3.6784	2.34E-06	MA0051.1 IRF2	2.95622	0.000140488
BU0061 Sox11_primary	3.67321	7.71E-06	BU0144 Lef1_secondary	2.79679	0.000725093

(b) Least enriched in IFN genes

IFN genes vs HDAC3 independent			non-IFN genes vs HDAC3 independent		
TF NAME	Z SCORE	P VALUE	TF NAME	Z SCORE	P VALUE
MA0259.1 HIF1A::ARNT	-5.36064	1	MA0004.1 Arnt	-4.37872	0.999999
MA0146.1 Zfx	-5.17734	1	BU0199 Zfp161_secondary	-3.73314	0.999999
BU0199 Zfp161_secondary	-4.67435	1	MA0024.1 E2F1	-3.68738	0.999984
BU0147 Max_secondary	-4.3671	1	MA0104.1 Mycn	-3.66748	0.999979
MA0003.1 TFAP2A	-4.30349	1	BU0147 Max_secondary	-3.65895	0.999972
MA0104.2 Mycn	-4.00403	0.999999	BU0202 Zfp410_secondary	-3.46099	0.999966
MA0014.1 Pax5	-3.92624	0.999999	MA0006.1 Arnt::Ahr	-3.43633	0.999946
MA0004.1 Arnt	-3.9037	0.999998	MA0104.2 Mycn	-3.3839	0.999923
BU0088 Tcfap2e_primary	-3.8613	0.999999	MA0093.1 USF1	-3.32295	0.999878
BU0052 Plagl1_primary	-3.84681	0.999998	MA0146.1 Zfx	-3.27756	0.999893

Table 4. Enrichment for TFBSs in *Ifnβ*-regulated genes.

(a) TFBSs that are over-represented in *Ifnβ*-inducible, *Hdac3*-dependent genes (indicates as light green) and non- *Ifnβ* -inducible but *Hdac3*-dependent genes (indicates as light turquoise) at region -500 bp to +250 bp relative to TSSs. (b) TFBSs that are under-represented IFN genes (indicates as light green) and non-IFN genes (indicates as light turquoise) at region -500 bp to +250 bp relative to TSSs.

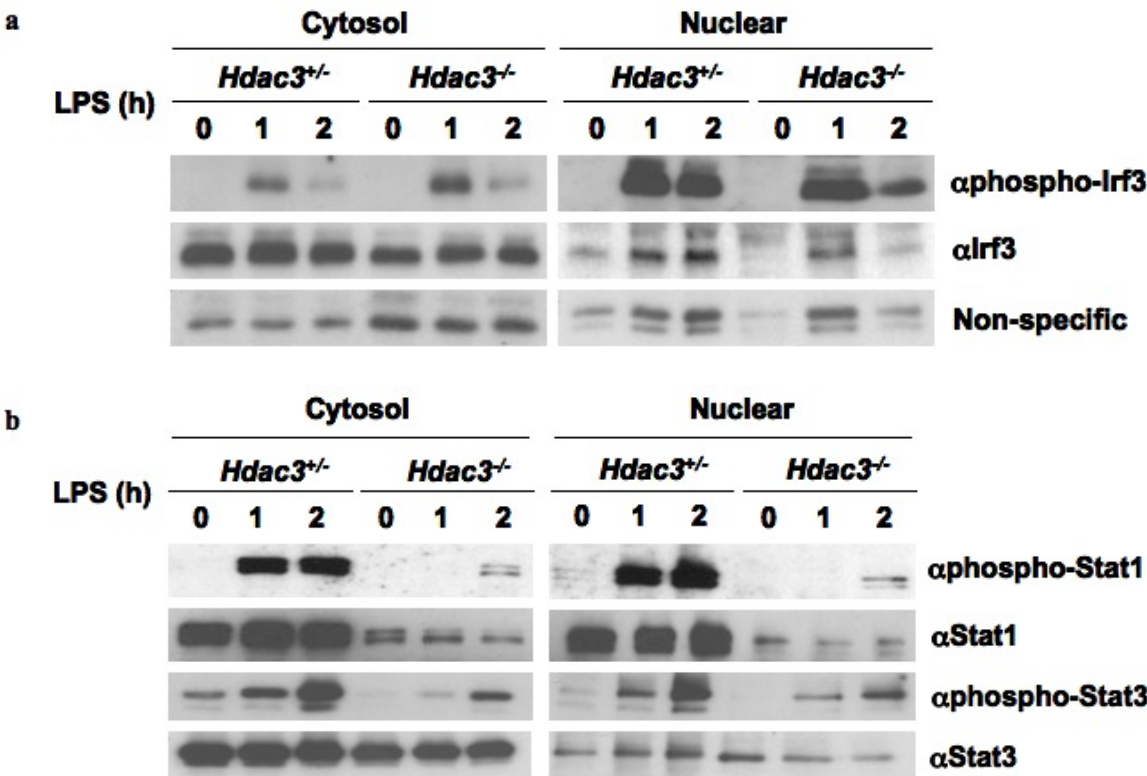
Iros Barozzi carried out computational analysis.

9. STATs are affected by *Hdac3* loss.

The results above indicate that *Irf*- and/or *Stat*-dependent transcriptional responses are affected in *Hdac3*^{-/-} primary macrophages. To understand the mechanistic basis of this *Hdac3*^{-/-} phenotype, we carried out a biochemical dissection of the signal transduction processes involved in response to LPS. Binding of LPS to TLR4 leads to TRAM-TRIF-dependent transcription of interferon-inducible chemokines and cytokine genes (eg. *Ccl5*/*RANTES*, *Cxcl10*, *Ccl2*, *Ifnβ*, *Il-15*) through the direct activation of the transcription factor *Irf3* and *Ifnβ*-mediated activation of *Stat1* [130-133]. We first investigated the activation of both *Irf3* and *Stat1* and their total protein levels in *Hdac3*^{-/-} primary macrophages. Western blot analysis with lysates prepared from both wild type and *Hdac3*^{-/-} primary macrophages (either unstimulated or stimulated with LPS) showed no differences in the amount of total *Irf3*. Moreover, kinetics of *Irf3* activation as measured

with an anti-phospho-Irf3 antibody revealed no differences in wt and knockout cells (Figure 15a). Loss of Hdac3, however, led to a significant reduction of total Stat1, associated with an almost complete absence of phosphorylated Stat1 (Figure 15b, upper panel). We also checked for the levels of Stat3 and observed a mild reduction in its total level and a slight reduction of its phospho-form in *Hdac3*^{-/-} primary macrophages (Figure 15b, lower panel).

In macrophages, activation of LPS-induced Stat1 signalling requires *Inf*β production, which in turn leads to the activation of an autocrine/paracrine loop [135, 136]. Using macrophages derived from *Inf*β^{-/-} mice, Thomas *et al.* demonstrated that phosphorylation of Stat1 on Tyr-701 was blocked following the loss of *Inf*β. In addition, expression profiling of *Inf*β^{-/-} macrophages also identified as Stat1 as one of its target genes (136). As we were unable to measure the level of *Inf*β due to its low expression levels, we checked for the level of phospho-Tyk2 whose activation is dependence on the engagement of *Inf*β to IFNAR. Undetectable Tyk2 phosphorylation indicates an impaired in both basal and induced *Inf*β production (Figure 15c).



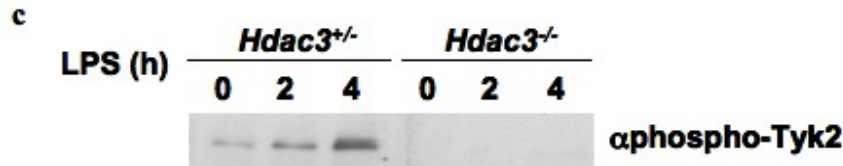


Figure 15. Reduction of Stat1 proteins in *Hdac3^{-/-}*.

Hdac3^{+/-} and *Hdac3^{-/-}* primary macrophages were either untreated or treated with 100 ng/ml LPS for one or two hour. Cells were then harvested and fractionated into cytosol and nuclear extracts.

(a) Western blots of cytosol and nuclear extracts were carried out with anti-phospho-Irf3 and anti-Irf3 (non-specific band is used as loading control). (b) Western blots of cytosol and nuclear extracts were first detected with anti-phospho-Stat1, anti-phospho-Stat3 and then reblotted with anti-Stat1, anti-Stat3. (c) Western blot of cytosol extracts harvest from *Hdac3^{+/-}* and *Hdac3^{-/-}* primary macrophages either untreated or treated with 100 ng/ml LPS for two or four hour was carried out with anti-phospho-Tyk2. Representative blots of three independent experiments using primary macrophages derived from a *Hdac3^{+/-}* or *Hdac3^{-/-}* mouse.

10. AP-1 is affected by *Hdac3* loss.

Recently, analysis of *c-Jun^{-/-}* MEFs showed that cJun is required for the maintenance of basal Ifn β level in cells [134]. To understand if a defect in Ifn β production is due to a dysregulation of c-Jun, we evaluated the mRNA and protein levels of Jun family members (JunB, JunB and c-Jun) in LPS-induced *Hdac3^{+/-}* and *Hdac3^{-/-}* primary macrophages. mRNA levels of Jun family members showed no differences in the *Hdac3^{-/-}* primary macrophages (Figure 16c). However, we could not detect JunB, JunD and c-Jun proteins in Hdac3-deleted cells (Figure 16a) indicating a post-transcriptional defect. Next, we checked if the MAPKs pathway is also affected by loss of Hdac3. Western blot detection for JNK1/2 showed no reduction in the protein levels of either the total- or phospho-JNK (Figure 16b).

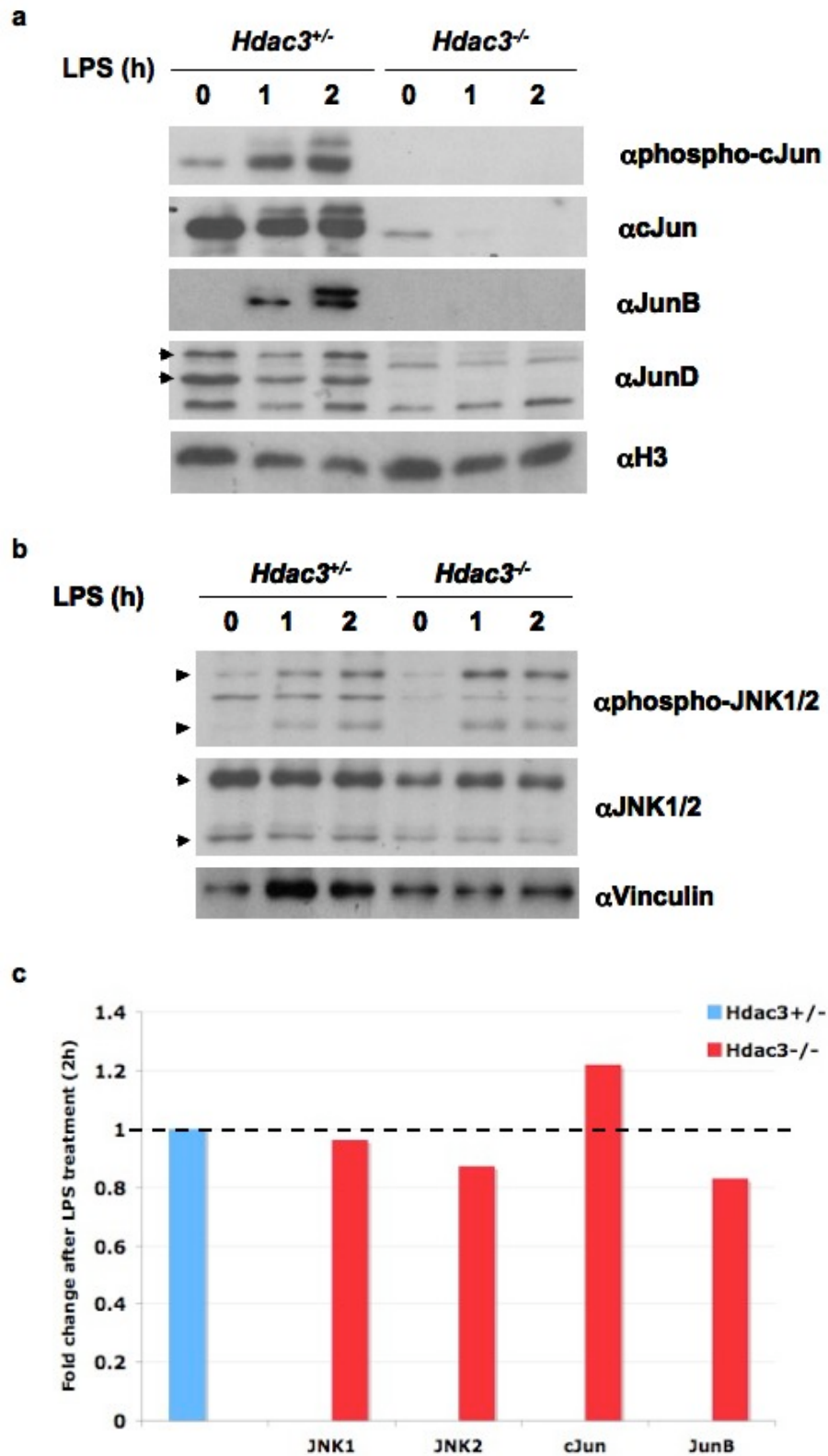


Figure 16. Reduction of AP-1 proteins in *Hdac3^{-/-}*.

Hdac3^{+/-} and *Hdac3^{-/-}* primary macrophages were either untreated or treated with 100 ng/ml LPS for one or two hour. Cells were then harvested and fractionated into cytosol and nuclear extracts.

(a) Western blots of nuclear fraction extracts were carried out with anti-phospho-cJun and anti-cJun, anti-JunB or anti-JunD. (b) Western blots of cytosol extract were first detected with anti-

phospho-JNK1/2 and reblotted with anti-JNK1/2. Anti-vinculin is used as loading control for cytoplasmic extract and anti-histone H3 is used as loading control for nuclear extract. Representative blots of three independent experiments using primary macrophages derived from a *Hdac3*^{-/+} or *Hdac3*^{-/-} mouse. (c) mRNA for the indicated genes were measured by quantitative RT-PCR and expressed as ratio to a housekeeping gene (TBP). Total amount of gene-specific mRNA for both *Hdac3*^{-/+} (control) and *Hdac3*^{-/-} were calculated as ratio to a housekeeping gene (TBP) and expressed as fold change of *Hdac3*^{-/-} over control, where wild type induction is 1. Data are representative of three independent experiments.

Discussion

HDACs have been commonly associated with transcriptional repression, by acting as the catalytic subunits of corepressor complexes that negatively regulate transcription of target genes [14, 54-56]. It is widely accepted that HDAC-mediated repression involves the removal of acetyl groups from histones, resulting in a closed chromatin that renders DNA less accessible to transcription factors. More recently, it has been clarified that hundreds of additional non-histone proteins that regulate diverse biological processes, and are located both in the cytoplasm and in the nucleus, are dynamically acetylated and deacetylated, suggesting that the regulatory functions of HDACs are much broader than initially believed (Table 2, [41-45, 47, 119]).

The requirement for HDACs as positive regulators of transcription, both in yeast and mammalian cells suggests that HDAC activity in some cases can stimulate gene expression [9, 57, 59, 79-83, 140]. Consistent with these observations [79-83], we observed the same number of LPS-regulated genes to be either activated or repressed by TSA in primary macrophages in spite of an overall increase in histone H3/H4 acetylation. Possible mechanisms whereby HDACs function to positively regulate transcription include both intragenic deacetylation of histones, which has been suggested to promote RNA Pol II progression in yeast cells, and modification of non-histone proteins directly or indirectly involved in transcriptional control. Despite the increasing evidences supporting HDACs role in transcriptional activation, mechanisms by which how individual HDAC activity would mediate transcription induction in mammalian system remain largely unknown. To address this question, we made use of *Hdac3*^{-/-} mice to delineate the contribution of Hdac3 function in the LPS-inducible macrophage gene expression programme.

In the present study, we demonstrated that Hdac3 activity plays an important role in LPS-induced gene expression as indicated by the observation that loss of Hdac3 resulted in impaired activation of about 45% of LPS-induced genes. Computational analyses of

macrophage gene expression profiles and comparative promoter analysis of genes identified as Hdac3-dependent revealed that loss of Hdac3 interferes with the activation of IFN β -regulated genes are activated through IRF/STAT sites. In addition to this large group of Ifn β -inducible genes (eg. Cxcl10 and Irf1), we have identified another group of genes whose activation is impaired upon Hdac3 deletion (eg. Il-6) and that may be regulated by AP-1 family proteins.

A study by Kramer *et al* showed that depletion of Hdac3 by shRNA resulted in reduced level of phosphorylated Stat1 and impaired IFN-dependent transcriptional activation [105]. It has been proposed that deacetylation of Stat1 by Hdac3 promotes Stat1 phosphorylation, which in turn allows the translocation of phospho-Stat1 into the nucleus. However in our system, we found a strong reduction of Stat1 in *Hdac3*^{-/-} macrophages at both the mRNA and protein level indicating that the impairment is unlikely to be due to the changes in steady-state of Stat1 phosphorylation. Stat1 has been identified as a direct Ifn β target gene and it has been reported that both mRNA and protein levels of Stat1 are significantly lower in Ifn β ^{-/-} macrophages [135, 136]. In addition to the reduction of Stat1 levels and its activity, the undetectable level of Tyk2 phosphorylation (Figure 15c) also indicates a defect in the basal production of Ifn β protein in *Hdac3*^{-/-} macrophages. The LPS-TLR4 response is mediated by both MyD88-dependent and MyD88-independent pathways that lead to activation of MAPKs, NF- κ B and Irf3 [132]. During microbial infection, maximal Ifn β production depends mainly on Irf3. We found neither an alteration in the levels of Irf3 protein nor any impairment in LPS-stimulated Irf3 phosphorylation and nuclear translocation in *Hdac3*^{-/-} macrophages. Consistent with these observations, many classic Irf3-dependent genes (e.g. Ccl5) are normally activated in *Hdac3* mutant macrophages. Overall, the simplest scenario compatible with this model is that *Hdac3*^{-/-} macrophages have a defect in basal production of IFN β , which reduces cellular Stat1 levels and impairs IFN β responsiveness (which is normally mediated by the IFN β autocrine loop induced by LPS). A recent study by Gough *et al* [134] showed that cJun is essential for the constitutive

production of low level of $\text{Ifn}\beta$, which activates the autocrine and paracrine feedback loops that are required for Stat1 expression. Since the constitutive $\text{IFN}\beta$ autocrine loop is mediated by cJun, and since all Jun family members are strongly reduced in $\text{Hdac3}^{-/-}$ cells, it is likely that the primary defect in $\text{Hdac3}^{-/-}$ macrophages lies in the reduced Jun/AP-1 protein levels. The molecular bases of this defect remain to be determined.

Activation of c-Jun through phosphorylation by cJun NH_2 -terminal kinases (JNK1/2) reduced ubiquitination and subsequently stabilised c-Jun (142-144). The observation that loss of Hdac3 results in the absence of Jun proteins while JNK1/2 are not affected at either the mRNA or the protein levels (Figure 15d, left panel), suggest that the reduction in JunB, JunD and c-Jun proteins is not a consequence of an alteration in their phosphorylation status. Acetylation of proteins (eg. HIF-1 α , p53) has been shown to enhanced degradation [44, 45]. Our data suggests a possible functional phospho-acetyl switch, which is regulated by an acetylation/deacetylation balance that modulates the levels of Jun family proteins.

Earlier study by Weiss *et al* [146] showed that phosphorylation of c-Jun by JNK dissociates Hdac3 -containing complex and activates c-Jun transcriptional activity by derepression in reporter gene assays. Wolter *et al* [145] later confirmed the interaction between endogenous c-Jun and Hdac3 in coimmunoprecipitation experiments using both c-Jun and phospho-c-Jun specific antibodies. Although we still do not have direct data in this regard, an interesting possibility is that Hdac3 is required to maintain c-Jun deacetylated and that deacetylation is in turn required to maintain the cellular pool of Jun proteins. Finally, Wolter *et al* [145] also demonstrated that c-Jun is essential for recruitment of Hdac3 to genes, and reduced Hdac3 binding in $\text{c-Jun}^{-/-}$ cells is associated with increased levels of acetylated histone H4. This is in consistent with our data that AP-1 sites are over-represented in the hyperacetylated regions of $\text{Hdac3}^{-/-}$ macrophages.

In summary, our data demonstrate the requirement of Hdac3 deacetylase activity in LPS-induced gene expression programme. In this system, Hdac3 functions as an activator by exerting its effects though modulating the deacetylation of non-histone proteins

(transcription factors), which in turn leads to the failure of $\text{Ifn}\beta$ production and the lack of subsequent activation of the $\text{Ifn}\beta$ -inducible, Stat1-dependent genes activation. The possibility that hyperacetylation of Jun family proteins may lead to their down-regulation in $\text{Hdac3}^{-/-}$ macrophages will require further investigation.

Future plan

- 1) To understand if the diminishing levels of AP-1 proteins are due to an increase in proteosomal degradation, *Hdac3*^{-/-} macrophages will be pre-treated with a potent proteasome inhibitor MG132. Pretreatment with MG132 should subsequently increase the amount of AP-1 proteins, which we could then co-immunoprecipitate with JunB, c-Jun or junD specific antibody and analysed for the level of acetylation.
- 2) To understand if AP-1 proteins are required for the recruitment of Hdac3 complexes to genomic regions containing AP-1 sites, thus modulating the turnover of histone acetylation, we will ChIP wild-type primary macrophages with AP-1 (more specifically c-Jun) antibody. Putative AP-1 binding sites, which have been identified in the hyperacetylated regions of *Hdac3*^{-/-} primary macrophages will be analysed.
- 3) Restoring the levels of Stat1 in *Hdac3*^{-/-} primary macrophages. Conditioning of *Hdac3*^{-/-} primary macrophages with Ifn β -containing medium should restore the levels of both Stat1 mRNA and protein to that observed in wild-type primary macrophages.

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