TRANSLATIONAL SCIENCE

Histone modifications underlie monocyte dysregulation in patients with systemic sclerosis, underlining the treatment potential of epigenetic targeting

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ABSTRACT

Background and objective Systemic sclerosis (SSc) is a severe autoimmune disease, in which the pathogenesis is dependent on both genetic and epigenetic factors. Altered gene expression in SSc monocytes, particularly of interferon (IFN)-responsive genes, suggests their involvement in SSc development. We investigated the correlation between epigenetic histone marks and gene expression in SSc monocytes.

Methods Chromatin immunoprecipitation followed by sequencing (ChIPseq) for histone marks H3K4me3 and H3K27ac was performed on monocytes of nine healthy controls and 14 patients with SSc. RNA sequencing was performed in parallel to identify aberrantly expressed genes and their correlation with the levels of H3K4me3 and H3K27ac located nearby their transcription start sites. ChIP-qPCR assays were used to verify the role of bromodomain proteins, H3K27ac and STATs on IFN-responsive gene expression.

Results 1046 and 534 genomic loci showed aberrant H3K4me3 and H3K27ac marks, respectively, in SSc monocytes. The expression of 381 genes was directly and significantly proportional to the levels of such chromatin marks present near their transcription start site. Genes correlated to altered histone marks were enriched for immune, IFN and antiviral pathways and presented with recurrent binding sites for IRF and STAT transcription factors at their promoters. IFNα induced the binding of STAT1 and STAT2 at the promoter of two of these genes, while blocking acetylation readers using the bromodomain BET family inhibitor JQ1 suppressed their expression.

Conclusion SSc monocytes have altered chromatin marks correlating with their IFN signature. Enzymes modulating these reversible marks may provide interesting therapeutic targets to restore monocyte homeostasis to treat or even prevent SSc.

Key messages

What is already known about this subject?

- Monocytes isolated from patients with systemic sclerosis (SSc) present with an altered transcriptome profile, particularly in interferon (IFN)-responsive genes, however the underlying pathways responsible of these aberrances are unclear.
- Chromatin remodelling driven by histone modifications entails an important epigenetic factor regulating gene expression. Previous studies in SSc have only looked at single promoter or total changes of histone modifications.

What does this study add?

- Simultaneous analysis of genome-wide histone alterations and transcriptomics data in SSc identified that altered H3K4me3 and H3K27ac, epigenetically imprinting the activation of promoters and enhancers, are associated with aberrant gene expression in SSc monocytes.
- Alterations in histone acetylation and methylation are mostly associated to IFN-related genes and are similar in all SSc subsets, including patients with early SSc, suggesting that these aberrant epigenetic marks may contribute to disease onset by initiating or sustaining this pathway.

How might this impact on clinical practice or future developments?

- Enzymes responsible for the deposition and maintenance of histone acetylation or methylation, or their binding partners, may provide interesting therapeutic targets to restore monocyte homeostasis to treat or even prevent SSc.

INTRODUCTION

Systemic sclerosis (SSc) is a severe autoimmune disease characterised by progressive and uncontrollable fibrosis of the skin and visceral organs including the lungs, heart and kidneys causing morbidity and increased mortality.[1] The aetiology of SSc is poorly understood and no preventive measures or curing therapy exists. Research on the onset and progression

of this deforming disease points at an early activation of circulating immune cells and their subsequent infiltration into the affected organs.[2–5] The release of cytokines and chemokines by these cells can cause activation of endothelial cells and fibroblasts, resulting in deposition of excessive amounts of extracellular matrix.[6–9] Later stages of SSc are often marked by irreversible skin fibrosis affecting the reticular dermis and subjacent adipose layer resulting in a largely acellular and atrophic skin.[10] The mechanisms triggering this fibrotic response are still poorly understood. Preventing the activation and migration of immune cells could have beneficial effects on the disease prognosis or halt fibrosis onset.

Monocyte numbers are higher in patients with SSc in both the skin and circulation.[5] Furthermore, SSc monocytes present with an increased expression of interferon (IFN)-dependent genes,[11 12] an enhanced profibrotic phenotype[13] and release larger amounts of proinflammatory mediators upon stimulation.[14] Importantly, the expression of IFN-responsive genes in circulating immune cells and in the skin of patients with SSc correlates with the degree of skin fibrosis and the presence of severe clinical phenotypes.[15–18] Although the differences between healthy and SSc monocytes are well established, the underlying pathways causing their aberrant gene expression and activated phenotype as well as their importance in the onset of fibrosis and perpetuation of SSc are unknown. Unravelling these pathways could lead to the identification of novel therapeutic targets.

Genome-wide association studies (GWAS) have demonstrated that SSc susceptibility loci are linked to immune-related genes, indicating that immune system dysregulation is likely a driving force of SSc development.[19 20] However, considering the low concordance rate of SSc in monozygotic twins (4.2%)[21] it is clear that genetic factors account for a small percentage of the risk to develop SSc. Environmental influences altering epigenetic factors such as histone modifications, non-coding RNA and DNA methylation are thought to play a major role in SSc development.[20]

Histones are fundamental proteins that stabilise and store DNA in chromatin form. Post-transcriptional modifications of histones lead to structural changes of the chromatin, either allowing or repressing gene transcription. Trimethylation of lysine 4 of histone 3 (H3K4me3) indicates active promoters while acetylation on lysine 27 of histone 3 (H3K27ac) is found in active enhancer and promoter elements.[22 23] Histone modifications in fibroblasts and B cells of patients with SSc have been implicated in their altered phenotype.[20] However, the importance of epigenetic mechanisms underlying immune system dysregulation in SSc and their role in the development of fibrosis are unknown for most immune cell subsets including monocytes.

With the aim of investigating the potential for epigenetic targeting in SSc, we examined the association of altered histone modifications at gene promoters and enhancers with differential gene expression in SSc monocytes, using ChIPseq of H3K4me3 and H3K27ac in parallel with transcriptome analysis.

METHODS AND MATERIALS

Study participants

Peripheral blood was drawn from patients with SSc as well as age and gender-matched healthy control (HC). Informed consent was obtained from all patients and healthy donors enrolled in the study at the University Medical Center Utrecht, the Maasstad Medical Center Rotterdam and the IRCCS Policlinico of Milan. All samples and clinical information were treated anonymously right after they were obtained. All patients with SSc fulfilled the American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) 2013 classification criteria and were divided into a discovery and a validation cohort (table 1). All individuals in the discovery cohort were of European ancestry (self-reported and confirmed by principal component analysis (PCA) of genotyping data (not shown)). The discovery cohort was analysed by RNAseq, while ChIPseq experiments were conducted on a subset of patients

Table 1 Demographics and clinical characteristics of patients included in the study

	Discovery cohort				qPCR/ChIP-qPCR validation cohort				
RNAseq/qPCR	HC	ncSSc	lcSSc	dcSSc	HC	eaSSc	ncSSc	lcSSc	dcSSc
ChIPseq/ChIP-qPCR	HC		lcSSc	dcSSc	HC	eaSSc	ncSSc	lcSSc	dcSSc
n	9	6	11	7	19	13	27	22	17
	7		9	6	5	4	4	3	3
Age (years)	53 (45–64)	43 (34–55)	58 (51–67)	63 (43–69)	50 (43–55)	74 (43–78)	59 (50–68)	63 (52–71)	49 (44–61)
	53 (39–64)		58 (53–68)	64 (41–70)	44 (38–50)	54 (27–79)	55 (40–59)	76 (65–77)	46 (36–50)
Female, n (%)	5 (56)	5 (83)	8 (72)	3 (43)	17 (89)	13 (100)	27 (100)	21 (95)	13 (76)
	4 (57)		6 (67)	2 (33)	5 (100)	4 (100)	4 (100)	3 (100)	3 (100)
ANA, n pos (%)	–	6 (100)	10 (91)	7 (100)	–	13 (100)	26 (96)	21 (95)	16 (94)
	–		8 (89)	6 (100)	–	4 (100)	4 (100)	3 (100)	3 (100)
ACA, n pos (%)	–	3 (50)	6 (55)	1 (14)	–	11 (59)	20 (74)	12 (55)	0 (0)
	–		5 (56)	1 (17)	–	4 (100)	3 (75)	3 (100)	0 (0.0)
Scl70, n pos (%)	–	2 (33)	2 (18)	4 (57)	–	1 (18)	1 (4)	8 (36)	10 (59)
	–		1 (11)	3 (50)	–	0 (0.0)	0 (0.0)	0 (0.0)	3 (100)
mRSS	–	0	7 (2–9)	11 (7–18)	–	0	0	4 (3–5)	9 (5–14)
	–		7 (2–9)	11 (7–18)	–	0	0	4 (2–8)	6 (2–26)
ILD, n (%)	–	1 (17)	2 (18)	5 (71)	–	0	2 (7)	6 (27)	12 (71)
	–		2 (22)	4 (67)	–	0	0 (15)	0 (29)	3 (100)
Disease duration	–	3 (2–5)	10 (2–17)	8 (2–16)	–	–	8 (3–17)	21 (8–26)	8 (2–16)
	–		4 (2–15)	9 (2–19)	–	–	7 (2–15)	17 (11–23)	13 (2–18)

Values reported indicate the number (n) of patients and the median for each parameter (IQR), if not otherwise indicated. Blue and White fields indicate features of patients of the same cohort analysed either with RNAseq/qPCR or ChIPseq/ChIP-qPCR, respectively.
ACA, anticentromere antibodies; ANA, antinuclear antibodies; HC, healthy controls; ILD, interstitial lung disease; Scl70, antitopoisomerase antibodies; dcSSc, diffuse cutaneous SSc; eaSSc, early SSc; lcSSc, limited cutaneous SSc; mRSS, modified Rodnan skin score; ncSSc, non-cutaneous SSc; pos, positivity.

of the same cohort, according to sample availability. Patients with SSc were subdivided into limited cutaneous SSc (lcSSc) and diffuse cutaneous SSc (dcSSc) subsets on the basis of the extent of skin fibrosis (Leroy, *J Rheumatol*, 1988). Patients with non-cutaneous SSc met the 2013 ACR/EULAR criteria of SSc without having fibrosis at the time of recruitment. Patients with early SSc (eaSSc) presented with Raynaud's phenomenon in combination with either typical nailfold videocapillaroscopy abnormalities or SSc-specific autoantibodies. Ongoing treatment regimen of the discovery cohort is reflected in the online supplementary table S4.

A detailed description of the methods and materials used for experiments can be found in the online supplementary data.

RESULTS

The transcriptome profile and distribution of the histone marks H3K4me3 and H3K27ac are altered in monocytes of patients with SSc

Consistent with previous literature based on microarray analysis,[24] RNAseq of monocytes from lcSSc (n=11), dcSSc (n=7) and HC (n=9) (table 1) demonstrated that the transcriptome of SSc monocytes is markedly different from that of their healthy counterparts (online supplementary figure S1A). Upregulated genes were particularly enriched in pathways relevant for monocyte activation and pathways associated with SSc such as IFN response, cytokine/chemokine signalling and intracellular immune activation pathways (online supplementary figure S1B). PCA revealed no strong transcriptomic differences between treated and treatment naïve patients (not shown).

To gain insights into factors underlying alterations in the transcriptome profile, genome-wide screening of the level of H3K4me3 and H3K27ac histone modifications was performed using ChIPseq analysis on a subset of subjects from the same cohort (lcSSc, n=9; dcSSc, n=6; HC, n=7) (table 1). Out of 13 860 H3K4me3 and 13 364 H3K27ac peaks identified and localised within 20 kbp from the transcription start site (TSS) of a gene, 1046 and 534, respectively, were differentially abundant in at least one group of patients with SSc compared with HC (figure 1A). 55.6% of the H3K4me3 and 61.1% of the H3K27ac peaks were upregulated in patients with SSc.

Significant changes in the levels of H3K4me3 and H3K27ac mainly occurred in genomic regions containing promoters and promoter-flanking regions (figure 1B), indicating that altered histone marks in patients with SSc may affect the expression of neighbouring genes. To assess this hypothesis, variations in H3K4me3 and H3K27ac were paralleled with expression changes of genes in proximity of the peaks. Consistent with previous literature demonstrating the presence of these marks at active promoters and enhancers,[25] increased levels of H3K4me3 and H3K27ac were significantly associated with the upregulation of nearby genes, while a decrease in these marks correlated with downregulated gene expression (figure 1C). These findings demonstrated that SSc monocytes carry alterations of the chromatin landscape in regions relevant for gene transcription that are associated with changes in gene expression

Variations in H3K4me3 and H3K27ac are associated with the increased expression of immune, IFN and antiviral response-related genes

To identify genes whose expression is most strongly influenced by altered histone marks in SSc monocytes, the abundance of H3K4me3 and H3K27ac was directly correlated with the mRNA level of nearby genes. The expression of 302 and 114 genes was directly and significantly proportional to the amount of H3K4me3 and H3K27ac near their TSS, respectively (figure 2A). For 35 genes, the expression was significantly correlated with both chromatin marks (figure 2A). Pathway enrichment analysis revealed that genes whose expression is correlated with increased histone marks are involved in immune regulatory processes identified as dysregulated by means of RNAseq, including IFN and antiviral-related pathways and signalling implicated in cell-to-cell communication (figure 2B). Genes correlating with decreased H3K4me3 or H3K27ac marks were not enriched in any pathways. Demonstrating the reproducibility of these findings, the association between the expression of two IFN-responsive genes (figure 2C), myxoma resistance protein 1 (*MX1*) and cytidine/uridine monophosphate kinase 2 (*CMPK2*), and the altered levels of H3K27ac was validated in an additional cohort of patients with SSc (n=14) and healthy donors (n=5) by targeted ChIP-qPCR analysis (figure 2D,E). Most importantly, the upregulation of both the histone mark and gene expression was similar for all SSc subsets analysed (online supplementary figure S2), was not correlated to disease duration (not shown) and was also observed in patients with eaSSc (figure 2E). In summary, hyperactive promoters were reproducibly associated with genes involved in pathways altered in SSc monocytes and previously linked to SSc pathogenesis and severity, even before the onset of fibrosis.[15 16]

Genes with altered promoters in SSc monocytes are regulated by the STAT and IRF transcription factor families

The presence of H3K4me3 and H3K27ac near the TSS strongly correlates with transcription factor (TF) binding and gene expression.[26 27] Prediction analysis for TF binding sites was performed on genomic sequences characterised by altered histone marks in SSc. As TF predominantly binds in DNAse-hypersensitive sites (DHS)[28] the intersection of ChIPseq data with DHS tracks specific for CD14+ monocytes was used to conduct the analysis (figure 3A).

An enrichment of binding sites for IRF and STAT TF family members was found in both hypermethylated and hyperacetylated regions in SSc monocytes (figure 3B). Confirming this prediction, 166 out of the 236 genes whose expression is correlated with increased histone mark deposition are reported to have a STAT or IRF binding site at their genomic locus (figure 3C). Ingenuity pathway analysis provided further evidence that the most relevant upstream TFs driving the expression of upregulated genes in SSc monocytes are STAT and IRF family members (figure 3D). Consistently, 82% of the genes associated with increased histone marks significantly correlated with the expression of at least one *IRF* or *STAT* gene (online supplementary table S1) suggesting that these TFs can underlie the altered activation of gene expression observed in SSc monocytes, in association with variations in chromatin marks.

The significant correlation of *MX1* expression with *STAT1* and *STAT2* identified in the sequencing was replicated by qPCR analysis in a validation cohort comprising 79 patients with SSc and 19 HCs (figure 3E). A similar result was found for *CMPK2* (online supplementary figure S3A). Most importantly, both STAT1 and STAT2 were enriched on the promoter of *MX1* and *CMPK2* in a proof-of-concept experiment where monocytes were stimulated with IFNα (figure 3F; online supplementary figure S3B). STAT1/2 recruitment was paralleled by a similar level of induction in *MX1*/*CMPK2* expression (online supplementary figure S3C) demonstrating the capability of STAT1 and STAT2 in regulating these IFN-responsive genes.

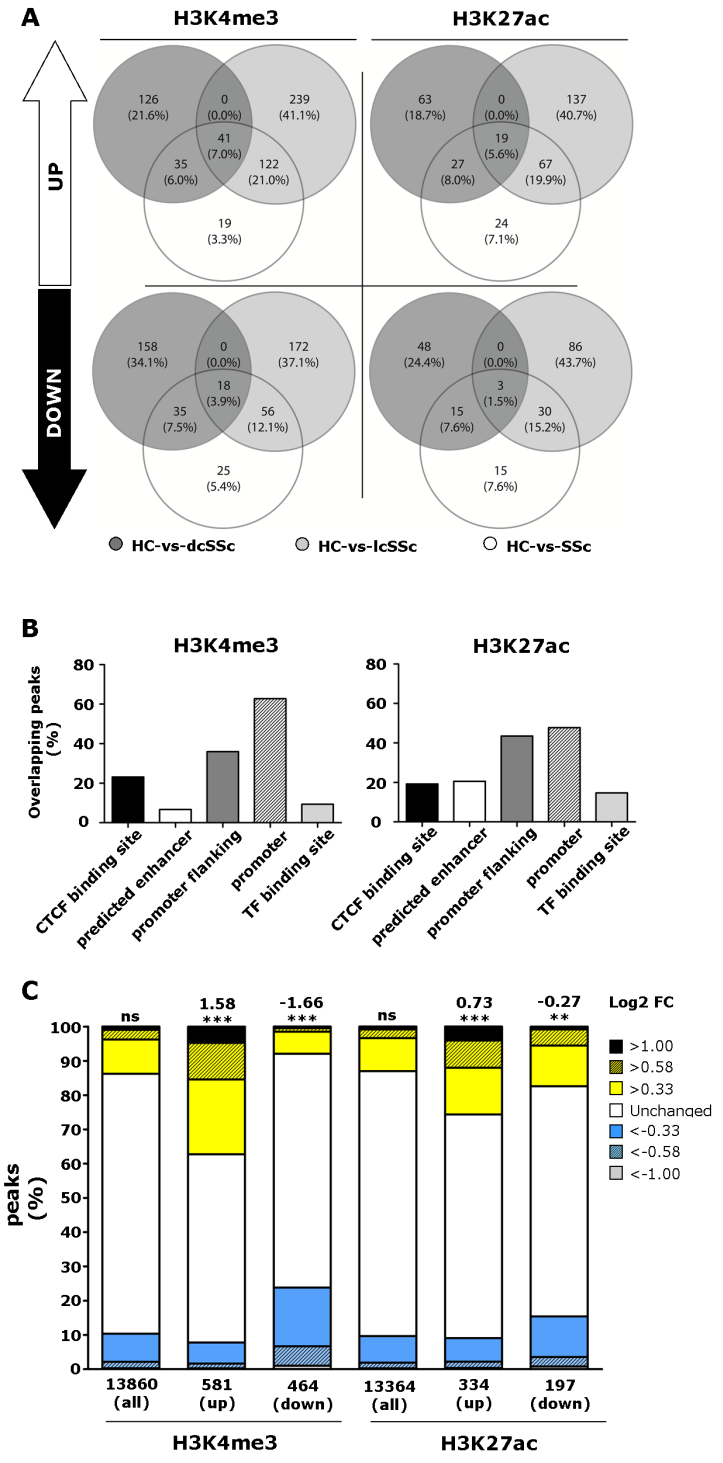


Figure 1 H3K4me3 and H3K27ac marks are altered in monocytes of patients with SSc in genomic locations important for the regulation of gene expression. (A) The abundance of H3K4me3 and H3K27ac was quantified by means of ChIPseq at the genome-wide level using monoclonal antibodies recognising these specific histone modifications. The number (and percentage, %) of differentially expressed peaks ($p<0.05$) in each SSc group or between all SSc considered together versus healthy controls (HC) was identified as described in the Methods and materials section and represented by Venn diagrams. Upregulated (UP) and downregulated (DOWN) genes are represented in distinct groups. (B) Differentially expressed H3K4me3 and H3K27ac peaks were intersected with regulatory elements annotated by ENCODE. Bar graphs indicate the percentage of peaks overlapping with previously annotated regulatory regions. (C) Genes were categorised into seven ranks according to the fold change in mRNA expression, from log2FC higher than 1 to lower than −1. The total amount of H3K4me3/H3K27ac peaks identified (all) or only those significantly higher (up) or lower (down) in patients with SSc compared with healthy donors was associated to the aforementioned categories of genes for all genes having their TSS within 20 kbp from the peak. The ratio of upregulated genes over the downregulated genes for each group of peaks was normalised for the ratio observed in the (all) group and reported on the top of each bar. The χ^2 test was used to assess significant differences in the distribution of up/downregulated genes when associated to differentially abundant peaks as compared with the total distribution (all). FC, fold change. **P<0.01; ***P<0.001. dcSSc, diffuse cutaneous SSc; FC, fold change; ns, not significant; SSc, systemic sclerosis; TF, transcription factor; TSS, transcription start site.

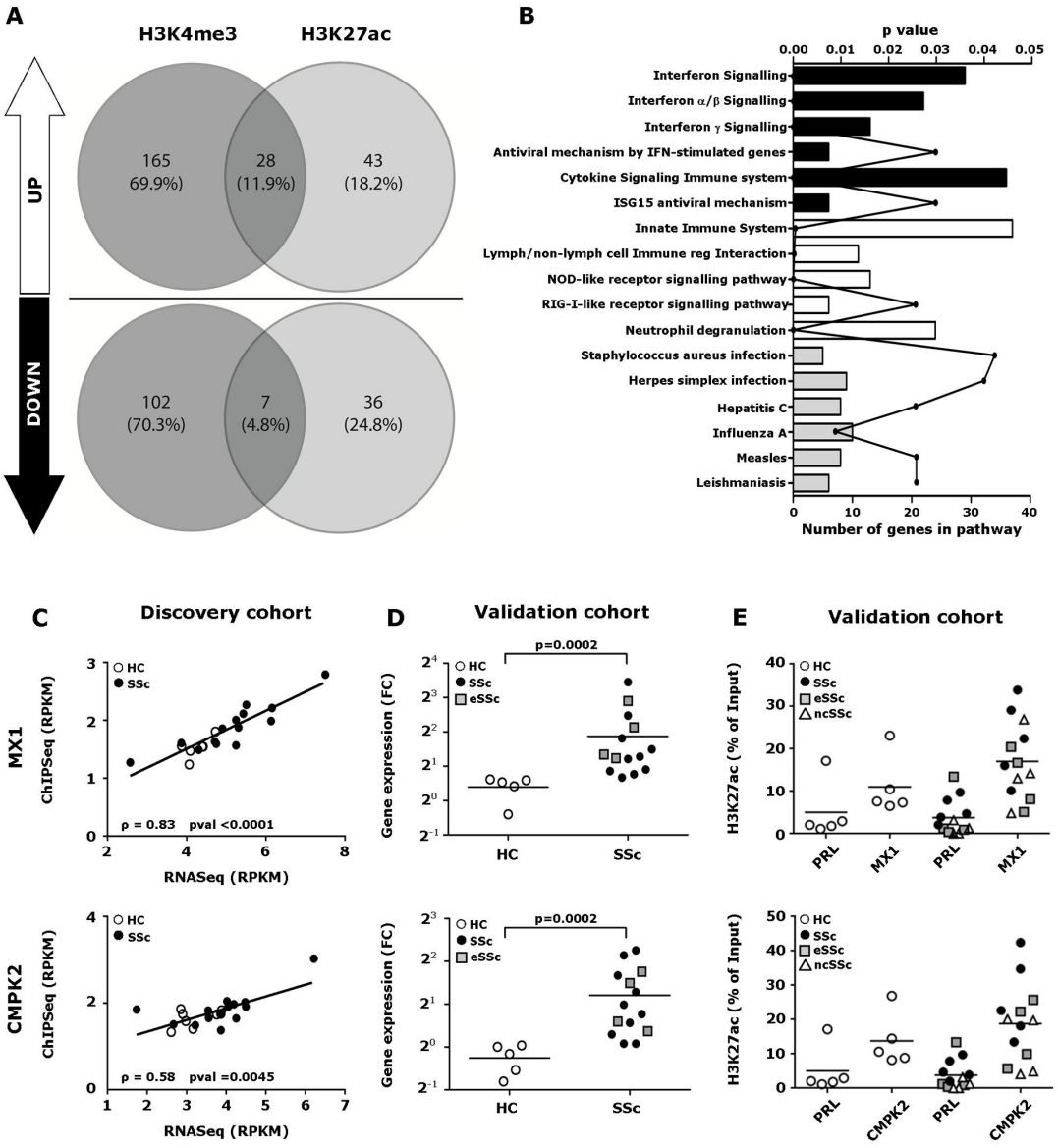
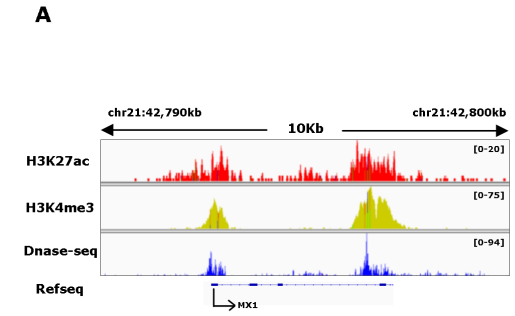


Figure 2 Altered H3K4me3 and H3K27ac levels correlate with gene expression in SSc monocytes. (A) Venn diagram indicating the amount (and percentage, %) of genes whose expression is directly and significantly correlated to changes in the level of H3K27ac or H3K4me3 within 20 kbp of their transcription start site (TSS) (p<0.05). Only genes associated to peaks that were significantly altered (either 'up' or 'down') in patients with SSc versus healthy donors were considered. (B) Pathways significantly enriched on the combined list of genes correlated to significantly upregulated H3K27ac and/or H3K4me3 peaks within 20 kbp from their TSS. Pathways were grouped in those IFN related (black), leading to immune cell activation (white) and implicated in response to infection (grey). Bar graph represents the number of genes in each pathway, while dots indicate the corresponding enrichment p value after controlling for the false discovery rate (FDR) using the *Benjamini–Hochberg* (B&H) procedure. (C) Correlation of the expression of myxoma resistance protein 1 (*MX1*) and cytidine/uridine monophosphate kinase 2 (*CMPK2*) with the level of H3K27ac within 20 kbp from the TSS in the discovery cohort. (D) RT-qPCR quantification of *MX1* and *CMPK2* expression in the ChIP-qPCR validation cohort. (E) ChIP-qPCR analysis of H3K27ac at the promoter of *MX1* and *CMPK2* in the ChIP-qPCR validation cohort. Detection of H3k27ac at the promoter of prolactin (*PRL*) was used as a negative control. Data are expressed as enrichment percentage over input DNA, horizontal bars represent means. eSSc, early SSc; FC, fold change; HC, healthy controls; IFN, interferon; ncSSc, non-cutaneous SSc; SSc, systemic sclerosis.

Histone-modifying enzymes and bromodomain-containing proteins are associated with aberrant gene expression in patients with SSc

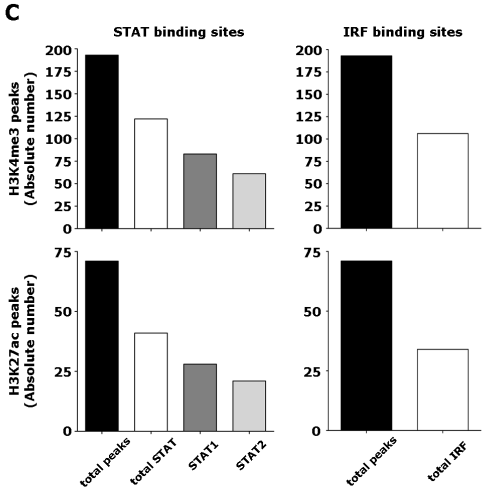
To further unravel those factors leading to altered histone marks and thus influencing gene expression in SSc monocytes, we used RNAseq data to retrieve the expression of 164 enzymes involved in the deposition of methylation and acetylation marks, such as histone methyltransferases, demethylases, histone acetyltransferase or deacetylases and acetylation readers such as bromodomain-containing proteins (online supplementary table S2).

The expression of 12 histone-modifying enzymes was altered in SSc monocytes compared with HC monocytes (online supplementary figure S4A), including a signature possibly leading to an overall increased acetylation, namely the decreased expression of chromobox 4 (*CBX4*), Msx2-interacting protein (*SPEN*) and suppressor of variegation 3-9 homologue 1 (*SUV39H1*) that can result in a reduced histone deacetylation activity,[29–31] and the increased expression of inhibitor of growth family member 4 (*ING4*) that can enhance H3K27ac deposition[32] (figure 4A). Importantly, the altered expression of these genes was also



B

H3K4me3 peaks			H3K27ac peaks		
Name	*p-val*	*Adj. p-val*	*Name*	*p-val*	*Adj. p-val*
ETS2	6.16E-11	2.62E-08	**IRF3**	6.68E-07	2.84E-04
IRF8	2.85E-10	1.21E-07	**IRF8**	8.06E-07	3.43E-04
SP1	3.01E-10	1.28E-07	**STAT2**	1.12E-06	4.75E-04
SP1	4.70E-10	2.00E-07	**IRF4**	1.42E-06	6.05E-04
KLF4	6.76E-10	2.88E-07	EGR2	2.09E-06	8.88E-04
STAT2	2.39E-09	1.02E-06	**IRF1**	2.28E-06	9.70E-04
SP3	3.24E-09	1.38E-06	IRF2	4.16E-06	1.77E-03
WT1	1.11E-08	4.71E-06	SP1	4.58E-06	1.95E-03
SP2	3.54E-08	1.51E-05	WT1	7.13E-06	3.03E-03
GABP	4.36E-08	1.86E-05	SP1	8.32E-06	3.54E-03



D

H3K4me3	
Upstream Regulator	*p-value*
IRF7	1.17E-19
IRF3	3.99E-11
STAT3	8.63E-10
IRF5	8.71E-10
STAT1	1.83E-08
IRF1	3.30E-08
STAT2	5.47E-08
TRIM24	5.59E-08
TP53	1.45E-06
IRF9	1.32E-05

H3K27ac	
Upstream Regulator	*p-value*
IRF7	6.67E-10
STAT3	1.87E-08
STAT1	2.28E-06
IRF3	3.99E-05
IRF5	4.96E-05
STAT2	8.74E-05
TRIM24	3.55E-04
HOXD3	8.90E-04
SMAD4	1.08E-03
CREBBP	1.23E-03

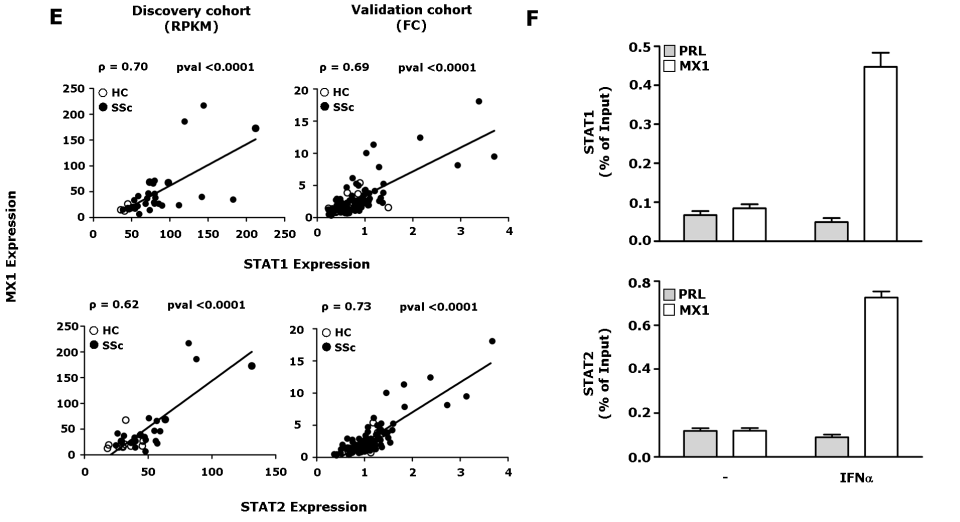


Figure 3 H3K4me3 and H3K27ac peaks associated with upregulated genes overlap binding sites for transcription factors of the IRF and STAT family and overlap with their expression. (A) Example of overlap of the tracks for H3K4me3, H3K27ac as detected by ChIPseq and the DNAse hypersensitive sites (DHS) signal from the ENCODE/OpenChrom database (GSE32970). (B) List of the top 10 transcription factor binding sites enriched under DHS overlapping with H3K27ac or H3K4me3 peaks significantly altered in patients with SSc, versus a set of 10% randomly selected peaks from the DHS data set. The adjusted p values were obtained using Bonferroni correction. (C) Number of H3K27ac and H3K4me3 peaks correlated to upregulated genes in SSc monocytes that contain a STAT or IRF binding site annotated in the ENSEMBL regulatory build 76.[56] STAT1 and STAT2 are also represented individually. (D) Upstream regulator analysis performed with ingenuity pathway analysis. Top 10 transcription factors predicted to regulate the genes whose expression correlated to altered levels of H3K4me3 and H3K27ac ($p<0.1$) within 50 kb from their transcription start site (TSS). (E) Correlation between the expression of *MX1* with *STAT1* and *STAT2* in the discovery cohort based on RNAseq data (left panels) and the validation cohort according to qPCR analysis (right panels). (F) ChIP-qPCR quantification of STAT1 (top) and STAT2 (bottom) bound at the promoter of *MX1* in monocytes cultured for 2 hours either with or without IFNα. Analysis of STAT binding at the promoter of *PRL* was used as a negative control. Data shown as a percentage of the input DNA, assayed in triplicate, error bars indicate SEM. FC, fold change; HC, healthy controls; IFN, interferon; MX1, myxoma resistance protein 1; PRL, prolactin; SSc, systemic sclerosis.

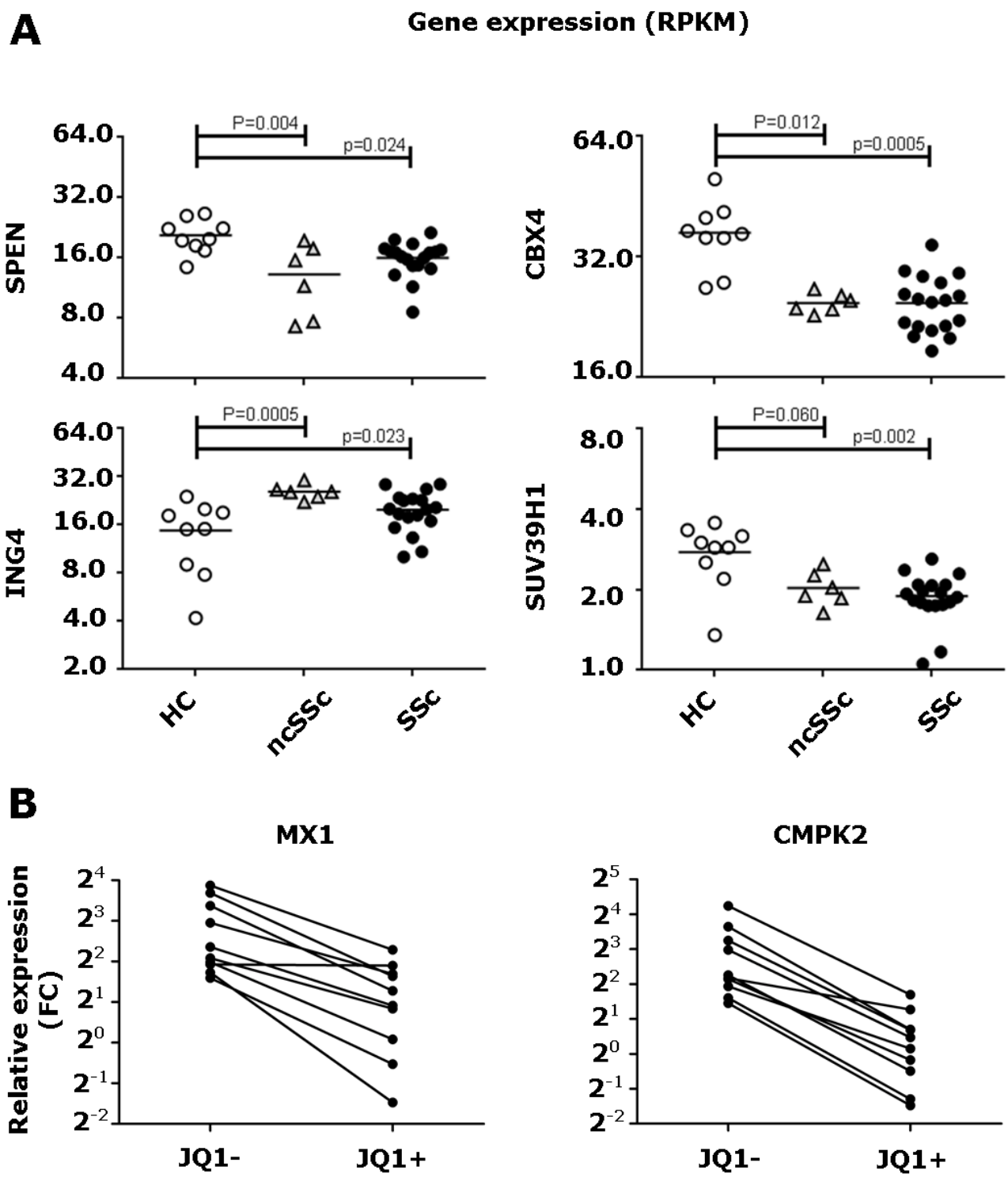


Figure 4 Aberrant histone acetylation deposition and reading may be implicated in the altered transcriptome of SSc monocytes. (A) Expression levels of chromobox 4 (*CBX4*), Msx2-interacting protein (*SPEN*), suppressor of variegation 3-9 homologue 1 (*SUV39H1*) and inhibitor of growth family member 4 (*ING4*) quantified in the discovery cohort by RNAseq. (B) Induction of *MX1* and *CMPK2* gene expression upon stimulation of healthy monocytes with interferon-α (IFNα) for 2 hours in the absence or presence of JQ1, as quantified by means of qPCR analysis. CMPK2, cytidine/uridine monophosphate kinase 2; FC, fold change; HC, healthy controls; MX1, myxoma resistance protein 1; ncSSc, non-cutaneous SSc; SSc, systemic sclerosis.

detected in patients with SSc not yet presenting with skin fibrosis (figure 4A). Interestingly, treatment of healthy monocytes with IFNα led to a similar regulation pattern for the majority of histone-modifying enzymes altered in SSc monocytes (8 out of 12, online supplementary figure S4B,C), while after stimulation with ligands for TLR7 (3 M-055) or TLR8 (VTX-2337) this pattern was not observed (online supplementary figure S5). Additionally, IFNα induced the deposition of acetylation on the promoter

of MX1 and CMPK2, two IFN-dependent genes found to be associated with increased acetylation in SSc monocytes (online supplementary figure S6). Using JQ1, a potent inhibitor of the BET family of bromodomain proteins that work as acetylation readers, we could inhibit the induction of both acetylation and expression of MX1 and CMPK2, validating the role of acetylation in the expression of genes found to be altered in SSc monocytes (figure 4B). These results suggest that a disrupted control of histone acetylation in SSc monocytes may determine, at least partially, the variations observed in gene expression, including at the early disease stage. Additionally, modulation of acetylation reading could restore gene expression back to basal levels.

DISCUSSION

SSc is a complex multifactorial autoimmune disease whose pathogenesis is suggested to be orchestrated by genetic and epigenetic aberrances.[20 33] As accumulating studies indicate the therapeutic potential of epigenetic targeting, we sought evidence for an epigenetic distortion underlying the transcriptome alterations of SSc monocytes, including the well-known type I IFN signature. This study reports for the first time in the field of SSc research a profile of two specific histone modifications at the genome-wide level. Combining ChIPseq data with publicly available data from ENCODE, we defined the presence of active enhancers and promoters, marked respectively by H3K27ac and H3K27ac plus H3K4me3, and verified that SSc monocytes are characterised by hypoactive and hyperactive regulatory elements. Consistent with the knowledge that histone modifications are key epigenetic factors in immune cells both in differentiation and activation processes,[34 35] we demonstrated that altered chromatin marks, epigenetically imprinting the activation of promoters and enhancers, are associated with modified gene expression in SSc monocytes.

We demonstrated that, in SSc monocytes, alterations in the expression of genes, in particular those involved in pathways related to the IFN response and cytokine signalling, are associated with altered promoter and enhancer activity. Among these, two of the most significantly correlated genes were *MX1* and *CMPK2*. The expression of *MX1* and other IFN-dependent genes in the lesional skin of patients with SSc was reported to correlate with clinical features, such as the skin thickness,[18] and coincides with the development of digital ulcers,[16] a decreased lung function[15] and a more severe phenotype associated with increased mortality.[36] The altered expression of IFN-responsive genes is evident from the earliest stages of the disease and is suggested to contribute to the pathogenesis and progression of SSc.[11 12 37] Consistently, we observed that alterations in histone acetylation and methylation associated to IFN-related genes are similar in all SSc subsets and are also found in patients with eaSSc, suggesting they may contribute to disease onset by initiating or sustaining this pathway. As the IFN signature is not limited to monocytes, it is possible that the altered deposition of histone modifications at the promoters of IFN-responsive genes is a common feature of multiple cell types in SSc. Considering that increased histone acetylation and methylation levels have also been previously reported in other systemic autoimmune diseases, such as systemic lupus erythematosus,[38–40] along with the presence of IFN signature, aberrant activation of promoters and enhancers may be a common occurrence in systemic autoimmunity.

Besides the association with IFN-responsive genes, increased H3K4me3 levels were correlated with the expression of interleukin-8 (IL8) receptors *CXCR1* and *CXCR2* and the C-C chemokine receptor type 1 (*CCR1*) (online supplementary table S3). These receptors were directly linked to the transendothelial migration of monocytes[41 42] and their arrest to activated endothelium.[43] These observations, along with the notion that monocytes/macrophages infiltrate the SSc skin, suggest that the enhanced migratory phenotype of SSc monocytes[5] may be supported by the altered activity of the promoters of chemokine receptor genes. Considering the role of monocyte infiltration as a potential trigger of fibrosis,[44] these findings can be of utmost importance from the therapeutic perspective. Interestingly, also five members of the activating-type Fc receptors for IgG family (*FCGR1B, FCGR1C, FCGR2A, FCGR3A* and *FCGR3B*) are hypermethylated near their TSS in SSc monocytes (online supplementary table S3). Though the role of Fc receptors in autoimmune diseases has not been fully elucidated, studies suggest that the uptake of immune complexes via Fc receptors leads to activation of toll-like receptor 7 or 9 and the production of cytokines such as IFN and IL8.[45 46] Overall, these observations suggest that the altered histone acetylation and methylation levels observed in SSc monocytes have a broad impact on their transcriptome profile, particularly on genes linked to pathways known to impact monocyte biology and likely linked to disease pathogenesis.

Chromatin alterations are not directly responsible for the induced gene expression. The deposition of H3K27ac and H3K4me3 leads to chromatin unwinding, increasing accessibility to TFs driving the expression of target genes.[25 28] In hyperacetylated and hypermethylated regions in SSc, we identified a strong enrichment of binding sites for various STAT and IRF TFs. Additionally, the expression of *STAT1* and *STAT2* was significantly increased in SSc monocytes and their expression strongly correlated with genes containing their binding site near the TSS (online supplementary table S1), including *MX1* and *CMPK2*. Furthermore, the binding of STAT1 and STAT2 to the promoters of *MX1* and *CMPK2* increased upon stimulation with IFNα. This demonstrated that STATs are, at least partially, responsible for the altered expression of these genes in SSc monocytes and suggests that this causal relationship may be extended to other factors whose binding site is enriched under the altered chromatin marks. In line with these findings, the presence of IFNα and phosphorylated STAT1 in the affected skin of patients with SSc was previously shown[47] and GWAS identified SSc-risk loci in regions encoding for various STAT and IRF TFs.[48–52]

Considering the importance of H3K27ac and H3K4me3 chromatin marks in sustaining gene expression, proteins responsible for the deposition and maintenance of these histone modifications and their binding partners may entail novel therapeutic targets in SSc. Twelve of such enzymes were differentially expressed in patients with SSc, potentially leading to an increased H3K27ac and/or H3K4me3 status, through different mechanisms.[29–32] A consistent subset of such genes was also modulated, in the same direction, upon stimulation of healthy monocytes with IFNα. Consistently, IFNα induced the deposition of H3K27ac at the promoter of genes found altered in SSc monocytes. In the light of these results, we can speculate that exposure of monocytes to IFNα, possibly released by activated plasmacytoid DCs,[45 53] represents one of the factors affecting chromatin modifiers/readers and H3K27ac deposition in SSc monocytes, thus ultimately establishing the type I IFN signature. Further investigations should be directed to unravel the pathogenetic pathways contributing to increased H3K4me3, which we did not find to be modulated near the selected genes upon IFNα stimulation (not shown). In addition to altered soluble factors, as previously demonstrated in healthy condition,[54] genetic

differences may also underlie the altered epigenetic landscape in patients with SSc.

Considering that the deposition of chromatin marks is reversible, targeting histone-modifying enzymes could reprogramme the epigenetic imprinting of SSc monocytes and constitute a potential therapeutic strategy to revert their activated phenotype and block the development of fibrosis. This is particularly interesting considering that these enzymes and the H3K27ac levels are already altered in patients before the onset of fibrosis. In a proof-of-concept experiment using IFNα-stimulated monocytes we demonstrated that interfering with acetylation readers, that is, by inhibiting the activation of the transcriptional machinery using JQ1, modulates both the acetylation and the expression of genes that are also dysregulated in SSc monocytes. The inhibition of bromodomain proteins with compounds similar to JQ1 has previously shown clinical efficacy in in vitro and animal models for various autoimmune disorders.[55]

Taken together, as epigenetic targeting of the immune system holds great promise for the treatment of immune-mediated inflammatory diseases, our data justify further research on the role of chromatin modifiers as therapeutic targets in SSc. At the early stages of disease onset, such targeting could aim at disease interception.

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REFERENCES

1 Gabrielli A, Avvedimento EV, Krieg T. Scleroderma. *N Engl J Med Overseas Ed* 2009;360:1989–2003.
2 Whitfield ML, Finlay DR, Murray JI, *et al*. Systemic and cell type-specific gene expression patterns in scleroderma skin. *Proc Natl Acad Sci U S A* 2003;100:12319–24.
3 Hussein MR, Hassan HI, Hofny ER, *et al*. Alterations of mononuclear inflammatory cells, CD4/CD8+ T cells, interleukin 1beta, and tumour necrosis factor alpha in the bronchoalveolar lavage fluid, peripheral blood, and skin of patients with systemic sclerosis. *J Clin Pathol* 2005;58:178–84.
4 Kalogerou A, Gelou E, Mountantonakis S, *et al*. Early T cell activation in the Skin from patients with systemic sclerosis. *Ann Rheum Dis* 2005;64:1233–5.
5 Higashi-Kuwata N, Jinnin M, Makino T, *et al*. Characterization of monocyte/macrophage subsets in the skin and peripheral blood derived from patients with systemic sclerosis. *Arthritis Res Ther* 2010;12:R128.
6 Parel Y, Aurrand-Lions M, Scheja A, *et al*. Presence of CD4+CD8+ double-positive T cells with very high interleukin-4 production potential in lesional skin of patients with systemic sclerosis. *Arthritis Rheum* 2007;56:3459–67.
7 Crestani B, Seta N, Palazzo E, *et al*. Interleukin-8 and neutrophils in systemic sclerosis with lung involvement. *Am J Respir Crit Care Med* 1994;150:1363–7.
8 Khan K, Xu S, Nihtyanova S, *et al*. Clinical and pathological significance of interleukin 6 overexpression in systemic sclerosis. *Ann Rheum Dis* 2012;71:1235–42.
9 Gruschwitz M, Müller PU, Sepp N, *et al*. Transcription and expression of transforming growth factor type beta in the skin of progressive systemic sclerosis: a mediator of fibrosis? *J Invest Dermatol* 1990;94:197–203.
10 Wei J, Bhattacharyya S, Tourtellotte WG, *et al*. Fibrosis in systemic sclerosis: emerging concepts and implications for targeted therapy. *Autoimmun Rev* 2011;10:267–75.
11 Brkic Z, van Bon L, Cossu M, *et al*. The interferon type I signature is present in systemic sclerosis before overt fibrosis and might contribute to its pathogenesis through high BAFF gene expression and high collagen synthesis. *Ann Rheum Dis* 2016;75:1567–73.
12 York MR, Nagai T, Mangini AJ, *et al*. A macrophage marker, Siglec-1, is increased on circulating monocytes in patients with systemic sclerosis and induced by type I interferons and Toll-like receptor agonists. *Arthritis Rheum* 2007;56:1010–20.
13 Mathai SK, Gulati M, Peng X, *et al*. Circulating monocytes from systemic sclerosis patients with interstitial lung disease show an enhanced profibrotic phenotype. *Lab Invest* 2010;90:812–23.
14 van Bon L, Popa C, Huijbens R, *et al*. Distinct evolution of TLR-mediated dendritic cell cytokine secretion in patients with limited and diffuse cutaneous systemic sclerosis. *Ann Rheum Dis* 2010;69:1539–47.
15 Liu X, Mayes MD, Tan FK, *et al*. Correlation of interferon-inducible chemokine plasma levels with disease severity in systemic sclerosis. *Arthritis Rheum* 2013;65:226–35.
16 Bos CL, van Baarsen LG, Timmer TC, *et al*. Molecular subtypes of systemic sclerosis in association with anti-centromere antibodies and digital ulcers. *Genes Immun* 2009;10:210–8.
17 Higgs BW, Liu Z, White B, *et al*. Patients with systemic lupus erythematosus, myositis, rheumatoid arthritis and scleroderma share activation of a common type I interferon pathway. *Ann Rheum Dis* 2011;70:2029–36.
18 Farina G, Lafyatis D, Lemaire R, *et al*. A four-gene biomarker predicts skin disease in patients with diffuse cutaneous systemic sclerosis. *Arthritis Rheum* 2010;62:580–8.
19 Broen JC, Coenen MJ, Radstake TR. Deciphering the genetic background of systemic sclerosis. *Expert Rev Clin Immunol* 2011;7:449–62.
20 Broen JC, Radstake TR, Rossato M. The role of genetics and epigenetics in the pathogenesis of systemic sclerosis. *Nat Rev Rheumatol* 2014;10:671–81.
21 Feghali-Bostwick C, Medsger TA, Wright TM. Analysis of systemic sclerosis in twins reveals low concordance for disease and high concordance for the presence of antinuclear antibodies. *Arthritis Rheum* 2003;48:1956–63.
22 Ernst J, Kheradpour P, Mikkelsen TS, *et al*. Mapping and analysis of chromatin state dynamics in nine human cell types. *Nature* 2011;473:43–9.
23 Kouzarides T. Chromatin modifications and their function. *Cell* 2007;128:693–705.
24 Duan H, Fleming J, Pritchard DK, *et al*. Combined analysis of monocyte and lymphocyte messenger RNA expression with serum protein profiles in patients with scleroderma. *Arthritis Rheum* 2008;58:1465–74.
25 Shlyueva D, Stampfel G, Stark A. Transcriptional enhancers: from properties to genome-wide predictions. *Nat Rev Genet* 2014;15:272–86.
26 Liu L, Jin G, Zhou X. Modeling the relationship of epigenetic modifications to transcription factor binding. *Nucleic Acids Res* 2015;43:3873–85.
27 Benveniste D, Sonntag HJ, Sanguinetti G, *et al*. Transcription factor binding predicts histone modifications in human cell lines. *Proc Natl Acad Sci U S A* 2014;111:13367–72.
28 Thurman RE, Rynes E, Humbert R, *et al*. The accessible chromatin landscape of the human genome. *Nature* 2012;489:75–82.
29 Shi Y, Downes M, Xie W. SHARP, an inducible cofactor that integrates nuclear receptor repression and activation. *Genes Dev* 2001;15:1140–51.
30 Tie F, Banerjee R, Fu C, *et al*. Polycomb inhibits histone acetylation by CBP by binding directly to its catalytic domain. *Proc Natl Acad Sci U S A* 2016;113:E744–53.
31 Robin P, Fritsch L, Philipot O, *et al*. Post-translational modifications of histones H3 and H4 associated with the histone methyltransferases SUV39H1 and G9a. *Genome Biol* 2007;8:R270.

32 Hung T, Binda O, Champagne KS, *et al*. ING4 mediates crosstalk between histone H3 K4 trimethylation and H3 acetylation to attenuate cellular transformation. *Mol Cell* 2009;33:248–56.
33 Luo Y, Wang Y, Wang Q, *et al*. Systemic sclerosis: genetics and epigenetics. *J Autoimmun* 2013;41:161–7.
34 Lim PS, Li J, Holloway AF, *et al*. Epigenetic regulation of inducible gene expression in the immune system. *Immunology* 2013;139:285–93.
35 Shakespear MR, Halili MA, Irvine KM, *et al*. Histone deacetylases as regulators of inflammation and immunity. *Trends Immunol* 2011;32:335–43.
36 Ioannidis JP, Vlachoyiannopoulos PG, Haidich AB, *et al*. Mortality in systemic sclerosis: an international meta-analysis of individual patient data. *Am J Med* 2005;118:2–10.
37 Pendergrass SA, Lemaire R, Francis IP, *et al*. Intrinsic gene expression subsets of diffuse cutaneous systemic sclerosis are stable in serial skin biopsies. *J Invest Dermatol* 2012;132:1363–73.
38 Zhang Z, Song L, Maurer K, *et al*. Global H4 acetylation analysis by ChIP-chip in systemic lupus erythematosus monocytes. *Genes Immun* 2010;11:124–33.
39 Leung YT, Shi L, Maurer K, *et al*. Interferon regulatory factor 1 and histone H4 acetylation in systemic lupus erythematosus. *Epigenetics* 2015;10:191–9.
40 Dai Y, Zhang L, Hu C, *et al*. Genome-wide analysis of histone H3 lysine 4 trimethylation by ChIP-chip in peripheral blood mononuclear cells of systemic lupus erythematosus patients. *Clin Exp Rheumatol* 2010;28:158–68.
41 Gerszten RE, Garcia-Zepeda EA, Lim YC, *et al*. MCP-1 and IL-8 trigger firm adhesion of monocytes to vascular endothelium under flow conditions. *Nature* 1999;398:718–23.
42 Bonecchi R, Facchetti F, Dusi S, *et al*. Induction of functional IL-8 receptors by IL-4 and IL-13 in human monocytes. *J Immunol* 2000;164:3862–9.
43 Weber C, Weber KS, Klier C, *et al*. Specialized roles of the chemokine receptors CCR1 and CCR5 in the recruitment of monocytes and T(H)1-like/CD45RO(+) T cells. *Blood* 2001;97:1144–6.
44 Kräling BM, Maul GG, Jimenez SA. Mononuclear cellular infiltrates in clinically involved skin from patients with systemic sclerosis of recent onset predominantly consist of monocytes/macrophages. *Pathobiology* 1995;63:48–56.
45 Kim D, Peck A, Santer D, *et al*. Induction of interferon-alpha by scleroderma sera containing autoantibodies to topoisomerase I: association of higher interferon-alpha activity with lung fibrosis. *Arthritis Rheum* 2008;58:2163–73.
46 Means TK, Latz E, Hayashi F, *et al*. Human lupus autoantibody-DNA complexes activate DCs through cooperation of CD32 and TLR9. *J Clin Invest* 2005;115:407–17.
47 Fleming JN, Schwartz SM. The pathology of scleroderma vascular disease. *Rheum Dis Clin North Am* 2008;34:41–55.
48 Radstake TR, Gorlova O, Rueda B, *et al*. Genome-wide association study of systemic sclerosis identifies CD247 as a new susceptibility locus. *Nat Genet* 2010;42:426–9.
49 Rueda B, Broen J, Simeon C, *et al*. The STAT4 gene influences the genetic predisposition to systemic sclerosis phenotype. *Hum Mol Genet* 2009;18:2071–7.
50 Sharif R, Mayes MD, Tan FK, *et al*. IRF5 polymorphism predicts prognosis in patients with systemic sclerosis. *Ann Rheum Dis* 2012;71:1197–202.
51 Carmona FD, Gutala R, Simeón CP, *et al*. Novel identification of the IRF7 region as an anticentromere autoantibody propensity locus in systemic sclerosis. *Ann Rheum Dis* 2012;71:114–9.
52 Martin JE, Broen JC, Carmona FD, *et al*. Identification of CSK as a systemic sclerosis genetic risk factor through genome wide association study follow-up. *Hum Mol Genet* 2012;21:2825–35.
53 van Bon L, Affandi AJ, Broen J, *et al*. Proteome-wide analysis and CXCL4 as a biomarker in systemic sclerosis. *N Engl J Med* 2014;370:433–43.
54 Chen L, Ge B, Casale FP, *et al*. Genetic drivers of epigenetic and transcriptional variation in human immune cells. *Cell* 2016;167:1398–414.
55 Mele DA, Salmeron A, Ghosh S, *et al*. BET bromodomain inhibition suppresses TH17-mediated pathology. *J Exp Med* 2013;210:2181–90.
56 Zerbino DR, Wilder SP, Johnson N, *et al*. The ensembl regulatory build. *Genome Biol* 2015;16:56.