The dual role of LSD1 and HDAC3 in STAT5-dependent transcription is determined by protein interactions, binding affinities, motifs and genomic positions

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STAT5 interacts with other factors to control transcription, and the mechanism of regulation is of interest as constitutive active STAT5 has been reported in malignancies. Here, LSD1 and HDAC3 were identified as novel STAT5a interacting partners in pro-B cells. Characterization of STAT5a, LSD1 and HDAC3 target genes by ChIP-seq and RNA-seq revealed gene subsets regulated by independent or combined action of the factors and LSD1/HDAC3 to play dual role in their activation or repression. Genes bound by STAT5a alone or in combination with weakly associated LSD1 or HDAC3 were enriched for the canonical STAT5a GAS motif, and such binding induced activation or repression. Strong STAT5 binding was seen more frequently in intergenic regions, which might function as distal enhancer elements. Groups of genes bound weaker by STAT5a and stronger by LSD1/HDAC3 showed an absence of the GAS motif, and were differentially regulated based on their genomic binding localization and binding affinities. These genes exhibited increased binding frequency in promoters, and in conjunction with the absence of GAS sites, the data indicate a requirement for stabilization by additional factors, which might recruit LSD1/HDAC3. Our study describes an interaction network of STAT5a/LSD1/HDAC3 and a dual function of LSD1/HDAC3 on STAT5-dependent transcription, defined by protein–protein interactions, genomic binding localization/affinity and motifs.

INTRODUCTION
Signal transducers and activators of transcription (STATs) modulate transcription through signal transduction from activated cell surface receptors to the nucleus and are key components of various signal-transduction pathways (1,2). The mammalian family of STATs includes seven members, STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6, which are all encoded by distinct genes. STAT5 includes two highly related proteins, STAT5a and STAT5b, sharing amino acid sequence similarity of more than 90%. They exhibit both redundant and distinct physiological functions, and they have been characterized mostly as transcriptional activators, although they have been reported to act also as transcriptional inhibitors (3,4).

Following stimulation, STAT5 proteins are rapidly tyrosine-phosphorylated allowing homo- or heterodimerization and translocation to the nucleus, where they exert their transcriptional regulation role through specific binding to DNA regulatory regions. STAT5 regulates a plethora of target genes involved in cellular responses to cytokines and growth factors, and its importance is highlighted by the devastating effects resulting from its aberrant constitutive activation in several forms of leukemia, as well as in other malignancies (5–8). Therefore, better understanding of the role and mechanism of action of STAT5 in activation and repression of target genes is of critical importance.

Studies in mouse models revealed a critical role for STAT5. Complete STAT5a and STAT5b deficiency in mice caused perinatal death and impaired lymphoid development and function (9,10). Interestingly, B cell maturation was abrogated at the pre-pro-B cell stage suggesting an important role for STAT5 in early B cell development. Furthermore, transgenic mice expressing a constitutively active form of STAT5b exhibit large increases in pro-B cells further underlining its role in B cell differentiation (11).

Since both STAT5a silencing or over-expression have a paramount effect on B cell development, it is important to understand how STAT5a exerts regulatory control on its target genes. The process of transcriptional programming
involves cooperative binding of various transcription factors (12), and as such it is important to identify STAT5a binding partners as co-regulators. Numerous STAT5 interacting proteins have been identified and the interactions can provide positive (13), negative (14) or no influence (15,16) on STAT5-dependent transcription. Despite previous studies of STAT5 target genes in various cell types, data on pro-B cells combining genome-wide binding and expression analyses to define how specific protein interactions influence STAT5 target genes transcription are not available.

Therefore, we combined proteomics and genomics approaches, to obtain insights into the STAT5 mediated transcriptional mechanisms leading to activation and repression of target genes. Such mechanisms might direct the physiological functions of pro-B cells, which pave the way to normal early B-cell development. Two novel STAT5a interacting partners (LSD1 - Lysine specific demethylase 1, and HDAC3 - Histone deacetylase 3) were identified in mouse IL-3-dependent pro-B cells (Ba/F3). Their role on STAT5a target genes regulation was investigated in a genome-wide manner by correlating ChIP-seq data for STAT5a, LSD1 and HDAC3 with RNA-seq data in wild type (WT) and knock-down (KD) Ba/F3 cells. Our results demonstrated that LSD1 and HDAC3 possess dual roles in determining transcriptional activation or repression of STAT5a target genes, providing novel insights into the mechanisms of activation and repression mediated by STAT5.

MATERIALS AND METHODS

Constructs, antibodies, primers and cell culture

The constructs and antibodies used can be found in Supplementary Data. All primer sequences are available upon request. Ba/F3 cells (17) maintenance and BirA/bio-STAT5a Ba/F3 cell clones generation and maintenance are previously described (18). The IL-3 deprivation condition of Ba/F3 cells is defined as culturing cells in the absence of IL-3 for 6 h, whereas the stimulation condition is defined as culturing cells in the presence of 10 ng/ml IL-3 for 30 min following the period of deprivation of IL-3 for 6 h.

Reverse RNA transcription and real time PCR

Total RNA was extracted and reverse transcribed as previously described (19,20). Real-time polymerase chain reaction (PCR) was performed with SYBR Green in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). The relative quantitation was performed using the ΔΔCt method (21) and the housekeeping gene Hprt for template normalization.

Nuclear and total cell extracts preparation

Nuclear extracts were prepared as previously described (22). Total cell extracts were prepared with whole cell lysis buffer (10 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 1 mM Na3VO4, 10 mM NaF, 5 mM EDTA, 1 mM PMSF and protease inhibitor cocktail (Roche)) for 30 min at 4°C. Gel filtration

Size fractionation of protein complexes was carried out on an AKTA FPLC apparatus with a Superose 6 10/30 column (Amersham). Fractions were precipitated with 100% trichloroacetic acid as previously described (23).

Immunoprecipitation, streptavidin precipitation and mass spectrometry

Immunoprecipitation and isolation of the biotinylated STAT5a protein with paramagnetic streptavidin beads was performed as described previously (22). Bound material was analyzed by immunoblotting. A total of 10 μg of crude nuclear extracts from BirA and BirA/bio-STAT5a Ba/F3 cells (stimulated with IL-3) were used for binding to streptavidin beads under moderate stringency conditions (150 mM NaCl/0.3% Nonidet P-40/200 ng/μl chicken serum albumin). Proteins eluted from the streptavidin beads were separated by SDS/PAGE electrophoresis on an 8% polyacrylamide gel, processed for trypsinization and mass spectrometric analysis, as previously described (22).

Immunoblot analysis

Extracts (≥5 μg/lane) or eluates from immuno- or streptavidin precipitations were resolved by SDS/PAGE and blotted onto Immobilon-P membrane (Millipore). Membranes were blocked for 1 h in 5% milk/1x TBS/0.05% NP40 (or 5% BSA/1x TBS/0.05% NP40 for p-STAT5 antibody), incubated overnight at 4°C with primary antibodies, washed in 1x TBS/0.5 M NaCl/0.3% Triton X-100 and then incubated with secondary HRP conjugated antibodies. Bands were detected using enhanced chemiluminescence (Amersham Pharmacia). Densitometric quantitation of the signals was performed with Quantity One Software (Biorad) and ImageJ.

In vitro transcription/translation and GST-pull down experiments

HDAC3 and LSD1 were in vitro transcribed and translated using the TNT T7 Coupled Reticulocyte Lysate System (Promega). GST fusion proteins were expressed in and purified from Escherichia coli BL21 cells according to (24). Equal amounts of each fusion protein, immobilized on glutathione-agarose beads, were incubated with 35S-HDAC3 or 35S-LSD1. After washes of the beads, bound proteins were resolved by SDS-PAGE and visualized by autoradiography.

Short hairpin RNA-mediated knock-down experiments

The lentiviral particles were produced as previously described (18) using pLKO.1 TRC control non-targeting vector (gift of Dr Papanikolaou), STAT5a (TRCN0000012549, TRCN0000012550), STAT5b (TRCN0000012553), LSD1 (TRCN0000071376) and HDAC3 (TRCN0000039389) clones of the TRC1 Library (Sigma, St. Louis, MO, USA). Transductions of Ba/F3 cells were performed as described (18).
ChIPs, ChIP-seq and RNA-seq library construction

Chromatin streptavidin precipitation and ChIP experiments were performed as previously described (18). The ChIP-seq libraries (from two independent ChIP assays) were constructed as previously described (25, 26). The RNA-seq libraries (3–5 replicates) were constructed using the TrueSeq RNA Sample Preparation kit v2 (Illumina RS-122-2001) according to manufacturer’s instructions. All libraries were sequenced on the Illumina HiSeq 2000 high-throughput sequencer.

NGS data analysis

Sequencing reads from ChIP-seq experiments were aligned to the mouse genome (mm10) using Bowtie2 (27) and PCR duplicates were removed. Peak calling and gene annotation was performed using the Homer suite (28). Peak heatmaps were produced with the use of Homer, cluster3 and TreeView for enrichment calculations, clustering and visualization, respectively. MEME was used for de novo motif analysis (MEME-ChIP and CentriMo) (29). Sequencing reads from RNA-seq experiments were aligned to the mouse transcriptome (mm10), allowing for split reads, using TopHat2 (30). HTSeq was used for expression quantification (31) and DESeq2 for differential expression analysis with normalization steps for eliminating batch effects (32). Gene set enrichment analysis (GSEA) was performed using the pre-ranked analysis option, and analyzing the gene-set identified by the clustering of STAT5a peaks against the RNA-seq data (33). More information regarding the NGS data quality and analysis is presented at Supplementary Data, and the code used for the NGS analysis is available upon request. Data from NGS experiments have been deposited in GEO (accession number GSE79520).

Statistical analysis

Student’s t-test was performed for the statistical analysis of ChIP enrichment data, unless stated otherwise.

RESULTS

Identification of novel STAT5a interacting proteins: LSD1 and HDAC3

To identify with high efficiency novel STAT5a interacting partners in Ba/F3 cells in vivo biotinylation was applied (Figure 1A). A bio-tag (22) was fused to STAT5a and co-expressed with bacterial biotin transferase (BirA), which specifically biotinylates the tagged protein. The biotin provided a very high affinity tag for the purification of interacting partners, by virtue of its binding to streptavidin (18, 22). After verifying that the addition of the bio-tag does not affect the functional properties of the STAT5a protein (Supplementary Figure S1), bio-STAT5a containing nuclear complexes were isolated in a single-step purification scheme by direct binding to streptavidin beads. Following liquid chromatography and tandem MS, the proteins identified specifically in BirA/bio-STAT5a cells included, among others, factors involved in transcriptional regulation and chromatin modification. In contrast, analysis of the background binding proteins (from BirA control cells) showed that the most abundant background proteins include the naturally biotinylated carboxylases and associated enzymes, nuclear proteins involved in protein synthesis and RNA post-transcriptional modification, mRNA processing and ribosomal proteins. The full STAT5a interactors map will be presented elsewhere (manuscript in preparation). It is noteworthy that known STAT5 interacting partners (HDAC1, BRG1, NfκB and CBP) were included among the identified proteins, validating our method. Here, the physical and functional interactions of two novel STAT5a interacting partners were characterized; HDAC3 (histone deacetylase 3 (gi6840851, gi12643653, gi89257352), score 104, unique/total peptides: 2/2) and LSD1 (Lysine-specific histone demethylase 1 (gi51315882) score 224, unique/total peptides: 5/6).

The interactions of STAT5a with the novel partners were confirmed by streptavidin precipitations in BirA/bio-STAT5a and control BirA Ba/F3 nuclear extracts, followed by immunoblots of the purified material with antibodies specific for each identified partner (Figure 1B). The interactions of the endogenous factors were verified by immunoprecipitations of Ba/F3 extracts with anti-STAT5a antibody followed by immunoblot with antibodies specific for the interacting partners, and confirmed with the reverse experiment as well (Figure 1B). Furthermore, the DNA binding-linker and the SH2 domains of STAT5a interact with both partners, while the coiled-coil domain of STAT5a only with HDAC3, as demonstrated by GST-pull down experiments (Figure 1C). Additionally, high molecular weight complexes containing STAT5a and the interacting partners were identified by gel filtration chromatography suggesting that all proteins could be part of the same high molecular weight complex or different (probably interacting) complexes of the same size (Figure 1D).

Taken together, the above results demonstrate that HDAC3 and LSD1 interact with STAT5a both in vivo and in vitro, and these factors participate in high molecular weight complexes.

STAT5a interactions with LSD1 and HDAC3 influence transcription of target genes

ChIP-seq and RNA-seq experiments were then performed and cross-correlated to investigate at a genome-wide level the mechanisms of transcriptional regulation mediated by STAT5a and HDAC3 or LSD1 (Figure 2, Supplementary Figure S2; Tables S1, S2). In particular, STAT5a-regulated genes that were also bound by LSD1 and/or HDAC3 were identified and studied to decipher whether the interactions influence STAT5-dependent transcription. ChIP-seq experiments yielded a list of genomic locations bound by STAT5a, LSD1 and/or HDAC3 under two conditions; deprivation of IL-3 for 6 h or deprivation followed by IL-3 stimulation for 30 min, which activates STAT5 (Figure 2, Supplementary Figure S3). To visualize these targets, heatmaps were generated depicting the binding events (decreasing binding intensity values) for each specified factor per condition (Figure 2A1, B2, C3, D4, E5, F6). Subsequently, the genomic locations for each factor per condition were obtained and the binding profiles for all the other
Figure 1. Validation of STAT5a interactions with HDAC3 or LSD1. (A) Scheme for the specific biotinylation of bio-tagged STAT5a by the E.coli BirA biotin ligase. The bio-tag is fused to the N-terminal of STAT5a. bio-tagged STAT5a and BirA, cloned separately under the control of EF1a promoter, were co-expressed in Ba/F3 cells for protein complex purification. (B) Nuclear extracts from BirA/bio-STAT5a or BirA Ba/F3 control cells (deprived of IL-3 for 6 h and then stimulated with IL-3 for 30 min) were precipitated with streptavidin (Strept) beads and immunoblotted with antibodies against HDAC3 or LSD1 (top left), and immunoprecipitated with anti-STAT5a antibody or IgG control and immunoblotted with antibodies against HDAC3 or LSD1 (top right). The reverse immunoprecipitation experiment with antibodies against HDAC3, LSD1 or IgG control is also shown, immunoblotted with anti-STAT5a antibody (bottom). (C) Graphical structure of GST-fused full length or sub-fragments of STAT5a are schematically shown (top). Autoradiographies of 35S-LSD1 (left), 35S-HDAC3 (right) pull-downs with GST-STAT5a full-length or sub-fragments and respective coomassie-stained gels of purified GST-domains (bottom) are shown (DBD-L: DNA Binding-Linker, SH2: Src Homology 2, TAD: transactivation domain). (D) Gel filtration size-fractionation profiles. Nuclear extracts from Ba/F3 cells (deprived of IL-3 for 6 h and stimulated with IL-3 for 30 min) were run on a Superose 6 column. The collected fractions following SDS/PAGE electrophoresis were immunoblotted with antibodies against HDAC3, LSD1 or STAT5a. Overlapping complexes are indicated on the bottom by solid bars. Molecular mass markers are indicated on the top (Vo: Void volume).
Figure 2. Heatmaps of STAT5a, LSD1 or HDAC3 bound regions and expression levels of target genes. ChIP-seq experiments (N = 2) were performed of regions bound by STAT5a, LSD1 or HDAC3 (columns 1–6). Ba/F3 cells were cross-linked after either 6 h of IL-3 deprivation (–), or 6 h of IL-3 deprivation followed by 30 min IL-3 stimulation (+). (A–F) The heatmaps depict the enrichment of each factor (in red) at the peak summit, as well as at the genomic region surrounding the peak summit (2 kb upstream and downstream the peak center) for all the significant peaks per condition, as mentioned at the far left column. The enrichment values for each peak list were extracted from each library (indicated at the top row) with the significant peak list per ChIP-seq library being at the diagonal (in black frames). The binding regions are sorted according to decreasing intensity from top to bottom for each heatmap, being in the diagonal. RNA-seq experiments were performed in WT Ba/F3 cells (N = 5), as well as in STAT5a, STAT5b, LSD1, HDAC3 KD cells (N = 3) and control shRNA-treated cells (N = 5) (column 7). All expression heatmaps represent log2FC values of the associated gene containing or in proximity to each peak, as computed by DESeq2 (red depicts up-regulation and green down-regulation). Differential gene expression was analyzed against IL-3 deprived cells in the case of WT Ba/F3 cells, and against cells treated with control no-target shRNA at the same condition for all the KD cells (IL-3 stimulation). (G) Venn diagrams depict the direct overlap of the genomic peak coordinates between the two conditions for all factors.
selected factors in both conditions at the same loci were determined. For example in Figure 2, A1 panel demonstrates the genomic locations bound by STAT5a in IL-3 deprived cells. In A2, the same genomic locations are depicted, but the binding intensity of STAT5a in IL-3 stimulated cells is shown, in order to see the changes in binding between the two conditions at the exact same binding sites. Similarly, in panels A3 and A4, LSD1 binding under IL-3 deprivation or post IL-3 stimulation, respectively, is visualized at the same genomic locations, whereas A5 and A6 panels demonstrate HDAC3 binding at these sites and so on.

This analysis confirmed the co-localization of STAT5a with LSD1 and/or HDAC3 at many genomic sites, in particular for STAT5a binding events gained after IL-3 stimulation (Figure 2, B4, B6 and corroboratively D2, F2). Lower enrichment levels were seen in many STAT5a targets when considering HDAC3 binding and in particular when that binding was compared to LSD1 binding at the same genomic locations. The lower HDAC3 ChIP-seq signal, as seen in heatmaps, might indicate either weak binding or most probably indirect DNA binding through other transcription factor hindering efficient detection. Nevertheless, the majority of HDAC3 targets (post IL-3 stimulation) showed both strong STAT5a and LSD1 binding suggesting either the existence of a complex containing all these factors or their sequential occupancy on specific genomic locations as a means of regulating expression.

Next, RNA-seq experiments were performed to correlate the binding events of the different factors to the actual expression levels of their target genes. The expression levels of WT Ba/F3 cells upon IL-3 stimulation were first determined, revealing all genes that become activated or repressed in response to IL-3. In addition, Ba/F3 cells were also transduced with lentiviruses encoding shRNAs to knock-down either STAT5a, STAT5b, HDAC3 or LSD1 (Supplementary Figure S4) for assessment of the functional contribution of each factor's binding to the regulation of target genes expression. Hence, this allowed evaluation of the loss-of-function effect for each factor by calculating the fold change in target genes expression after its KD, when compared to no-target shRNA control, 30 min post IL-3 stimulation. For each gene exhibiting binding events from either of the selected factors under study, its expression levels were extracted from all the RNA-seq experiments and presented in differential expression heatmaps sorted by the ChIP-seq binding intensity (Figure 2, A7–F7). Genes were classified as activated or repressed in WT Ba/F3 cells (Figure 2, A7–F7, left column and Supplementary Table S3) and were further characterized by their change in expression at the stimulation state upon KDs (Figure 2, A7–F7, right columns and Supplementary Table S3).

Examination of the expression levels of STAT5a target genes revealed a correlation between strongly bound targets and gene activation upon IL-3 stimulation (Figure 2, A1, A7 versus B2, B7). As expected, KD of STAT5a or STAT5b led to inhibition of the genes activated by IL-3, as compared to controls. Interestingly, KD of LSD1 resulted in a widespread gene activation of STAT5a targets after IL-3 stimulation (Figure 2, B7). This widespread activation is seen in both STAT5a and LSD1 direct targets, but also, to a much lesser extent, HDAC3 targets (Figure 2, D4, D7 and F6, F7, respectively). This suggests that LSD1 is involved in repression of specific genes in WT cells. Nonetheless, a sub-population of the STAT5a targets exhibited reduced expression in the absence of LSD1 suggesting that LSD1 can also function as an activator. Finally, HDAC3 KD appears to promote gene activation of STAT5a and LSD1 targets to a small degree (Figure 2, B7 and D7), whereas more substantial gene up-regulation is observed for genes that also exhibit HDAC3 binding (Figure 2, E5, E7 and F6, F7).

Apart from identifying genomic locations that were bound by one, combination of or all factors, interesting information about the changes of genomic targets occupancy due to IL-3 stimulation was revealed (Figure 2G, Supplementary Figure S3). In particular, IL-3 stimulation induced de novo recruitment of STAT5a and only a small portion of genomic targets was retained from the IL-3-deprived state. As expected, the expression levels of the IL-3-induced de novo genomic targets increased due to IL-3 and decreased in the absence of STAT5a with the stronger binding correlating to the greater differential expression (Supplementary Figure S3A; STAT5a binding decreasing from top to bottom of the heatmap). These targets presented increased intergenic and intronic STAT5a binding and decreased promoter binding compared to all other STAT5a targets (i.e. targets with binding either in both conditions or during IL-3 deprivation) suggesting binding in distant regulatory elements (Supplementary Figure S3B). Similar trend in terms of expression was seen for the targets that exhibited STAT5a binding in both conditions, albeit less pronounced, whereas targets that exhibited STAT5a binding only post IL-3 deprivation showed more diverse expression profiles. When considering LSD1 recruitment, it is evident that more than half of its genomic targets were retained in both conditions indicating that LSD1 genomic interactions are only in part influenced by IL-3 (Figure 2G, Supplementary Figure S3C and D). The de novo LSD1 recruitment induced by IL-3 stimulation showed mainly increased expression in WT Ba/F3 cells and, interestingly, weaker LSD1 genomic binding appeared to promote activation in LSD1 KD cells possibly indicating indirect LSD1 binding through a repressor complex. On the other hand, HDAC3 recruitment was decreased after IL-3 stimulation with only a few targets retained in both conditions and a few more de novo targets induced by IL-3 (Figure 2G, Supplementary Figure S3E and F). Although differential expression of HDAC3 targets is seen, no clear preference in the expression profiles of HDAC3 targets is detected suggesting that other factors might be needed to predict the transcriptional outcome.

The above data defined a correlation of strong STAT5a binding with gene activation. Furthermore, common STAT5a, LSD1 and/or HDAC3 targets were defined. LSD1 and HDAC3 binding sites overlapped the STAT5a binding site and KDs of these factors suggested that LSD1 and HDAC3 play a dual role in the regulation of targets' expression. LSD1 and HDAC3 can act both as transcriptional co-repressors and co-activators.
Protein interactions and genomic binding influence STAT5a-dependent transcription

To further examine the effect of LSD1 and HDAC3 in regulation of STAT5a targets, a more thorough investigation of the STAT5a targets was pursued by clustering the STAT5a peaks according to targets enrichment values and expression levels in Ba/F3 cells (WT and KD cells). Using the k-medians unsupervised clustering algorithm, groups of genes with similar characteristics (ChIP-seq or RNA-seq data) were identified in an attempt to find links between the binding pattern and expression profiles of the selected factors. Furthermore, GSEA was performed to determine any preference for activation or repression (Supplementary Figure S5). For the GSEA, all gene lists organized in clusters were tested against the expression levels generated from WT and KD Ba/F3 cells (pre-ranked data) to identify any potential enrichment in up- or down-regulated genes.

We identified 10 clusters of STAT5a targets, of which clusters 1–7 displayed LSD1 and HDAC3 binding at the same genomic location where STAT5a was bound. LSD1 appeared to bind more STAT5a targets than HDAC3, albeit weakly in some of the cases. Nonetheless, a similar pattern was seen for both factors in terms of target binding and global expression levels after their KD (Figure 3, Supplementary Figures S5 and S6). Clusters 1–3 included strong bound STAT5a targets (strongest binding was seen in cluster 1, then cluster 2 and then cluster 3) and induced by STAT5 (down-regulated in STAT5a and STAT5b KDs) (Figure 3, Supplementary Figure S6). These genes were weakly bound by LSD1 and HDAC3. Clusters 4–5 included STAT5a-bound targets with lower enrichment values compared to clusters 1–3, but displayed strong LSD1 and HDAC3 binding. Importantly, KD of STAT5a, LSD1 and HDAC3 positively affected expression levels, whereas STAT5b KD caused gene repression. Weaker LSD1 and HDAC3 binding in combination to weaker STAT5a binding (clusters 6 and 7) was interesting, as LSD1 appeared to act both as a repressor (cluster 6) and an activator (cluster 7) depending on the groups of genes, unlike HDAC3 that seemed to exert little or no regulatory role (Supplementary Figure S5). STAT5a (and STAT5b) had a similar effect on these genes, as in KD cells significant enrichment was seen in activated and repressed genes in clusters 6 and 7, respectively. Overall, when considering all clusters exhibiting commonly bound targets by all factors (clusters 1–7), STAT5a KD promoted gene repression with the exception of clusters 4–5 and 6, and LSD1/HDAC3 KD promoted gene activation with noticeable exception being cluster 7. Finally, clusters 8–10 included genes bound predominantly by STAT5a, without any significant LSD1 or HDAC3 binding, which were differentially affected by STAT5a KD and grouped accordingly. More specifically, in clusters 8 and 10 STAT5a appeared to function as an activator, with the genes being activated after IL-3 stimulation and repressed in the absence of STAT5. Furthermore, both LSD1 and HDAC3 showed differential regulation depending on the gene set, although in most cases they did not appear to significantly control the transcriptional status of the genes. In cluster 9 on the other hand, where activation in both LSD1 and HDAC3 KD was seen, an indirect mechanism of regulation most probably dictated transcription, as there was no LSD1 or HDAC3 binding.

Together, these data strongly suggest that STAT5a has both a positive and negative role in IL-3 stimulated gene expression. Detailed characterization of STAT5a targets revealed genes that are regulated by the combined action of STAT5a, LSD1 and HDAC3. It appears that varying affinity and combination of STAT5a, LSD1 and HDAC3 binding can differentially affect amplitude and direction of gene expression of distinct set of genes.

Genomic binding positions and motifs influence STAT5a-dependent transcription

To further understand the mechanisms of combinatorial regulation of target genes by STAT5a, LSD1 and HDAC3, the gene clusters obtained in Figure 3 were subjected to DNA motif analysis, and identification of the genomic distribution of the peaks (Figure 3C, D and E). All clusters showed a high enrichment of the gamma-activated sequence (GAS), which STAT5a is known to bind to (TTC-NNNGAA), except for clusters 4–5, 6 and 7 (Supplementary Table S4). Clusters 1–3 containing targets strongly bound by STAT5a, weakly by HDAC3 and LSD1 and highly activated by STAT5a show a clear enrichment for the GAS motif (E-value = $1.3 \times 10^{-708}$) indicating that these genes were bound directly by STAT5a dimers to induce their expression. Enrichment for the GAS motif was also seen in all the clusters exhibiting binding by STAT5a alone (clusters 8–10). Interestingly, a group of genes that appeared to be repressed by STAT5a (cluster 9) also exhibited enrichment for the GAS motif confirming that STAT5a binding can be also associated with gene repression. On the other hand, clusters 4–5 and 6, characterized by lower targets enrichment for STAT5a and at the same time binding from LSD1 and HDAC3, did not present any enrichment for the GAS motif, but for other motifs (the TTATC, the TGA(G/C)TCA and the GGA(A/T)GAA that resembles the STAT5 monomer motif). Similarly, cluster 7 showed only a modest enrichment for the GAS motif (E-value = $3.8 \times 10^{-46}$) suggesting that the binding events occurring in clusters 4–7 might not be driven by STAT5a/LSD1/HDAC3 complexes alone. Different mechanisms seem to dictate gene regulation, as KD of STAT5a or LSD1 in clusters 4–6 activated transcription, in contrast to cluster 7, where KD of STAT5a or LSD1 repressed transcription, albeit not significantly in the case of LSD1 (Figure 3, Supplementary Figure S5).

To provide further insight into the mechanisms of synergistic regulation exerted by STAT5a, LSD1 and HDAC3, the genomic location of binding was defined per cluster (Figure 3E). Interestingly, the gene sets of clusters 4–5, 6 and 7 exhibited increased occurrences of promoter binding compared to the other clusters, with significantly decreased occurrences of intergenic binding. By contrast, clusters with strong STAT5 binding and weak LSD1/HDAC3 binding (1–3) or STAT5 binding only (8–10) were characterized with increased frequencies of intergenic and intronic binding, suggesting that such regions may function as distal enhancer elements. Furthermore, to ensure that the effect seen in the clusters commonly regulated by STAT5a, LSD1 and HDAC3 was not due to low-level ChIP-seq promoter
Figure 3. Clustering of STAT5a bound regions according to enrichment values and expression levels of target genes. Clustering of STAT5a bound regions was performed using the k-medians algorithm according to both ChIP-seq and RNA-seq data and 10 clusters were produced. (A) ChIP-seq experiments of regions bound by STAT5a, LSD1 or HDAC3 in Ba/F3 cells after 6 h IL-3 deprivation, or 6 h IL-3 deprivation followed by 30 min IL-3 stimulation (N = 2 per factor per condition). The enrichment values of the significant STAT5a peaks identified in Ba/F3 cells post stimulation (15546 peaks) were extracted from each ChIP-seq library, as well as of the surrounding area of the peak window (2 kb upstream and downstream of the peak summit). STAT5a peaks that either show no binding of LSD1 or HDAC3, or not specific binding in relation to the STAT5a genomic localization are depicted in clusters 8–10. (B) RNA-seq experiments in WT Ba/F3 cells (N = 5), and cells with KD of STAT5a, STAT5b, LSD1 or HDAC3 (N = 3) or cells treated with control no-target shRNA (N = 5) presented in heatmaps. All expression heatmaps represent log2FC values of the gene containing or in proximity to each peak, as computed by DESeq2 (red depicts up-regulation, green depicts down-regulation). Differential gene expression was analyzed against IL-3 deprived cells in the case of WT Ba/F3 cells, and against cells treated with control no-target shRNA at the same condition for all the KD cells (IL-3 stimulation). (C) Motif analysis using MEME was performed of all the peak regions per cluster or similar clusters. The best motifs are shown along with the E-value produced by MEME that defines the significance of each motif. (D) Motif distribution at the peak window (174 bp) is shown, whenever the motif is locally enriched. The E-value and the motif distribution curve are color-coded, and the absence of a curve (or E-value without a color) indicates that the motif was not centrally enriched. (E) The genomic distribution of peaks per cluster (or similar clusters) was determined through HOMER.
binding, and as such an artefact, the motif analysis was repeated in only the intronic and intergenic regions per cluster yielding almost identical results (Supplementary Figure S7).

Overall, genes regulated by the combined action of STAT5a, LSD1 and HDAC3, appear to be differentially regulated based on targets enrichment and the genomic localization of the STAT5a (and its interacting partners') binding. Importantly, the combined action of STAT5a, LSD1 and HDAC3 was distinguished by the absence of the canonical GAS motif and was found more frequently in the promoter regions, indicating that the overall STAT5a binding may be stabilized by additional transcription factors, chromatin remodelers bound nearby or nucleosomes directing promoter structure dynamics. These factors might also contribute directly or indirectly to the recruitment of LSD1 and HDAC3. By contrast, strong STAT5 binding was observed in intergenic and intronic regions, which may function as enhancer elements from a distance.

Concomitant binding and co-regulation of novel targets by STAT5 and LSD1

Next, we identified 5187 common peaks, corresponding to 3465 genes by direct STAT5a and LSD1 peak overlaps (i.e. all overlapping genomic regions of the peak windows, Figure 4A and B). Motif analysis performed on these common targets (5187 sequences) detected the GAS motif in 121 sequences (E-value = 2.3 × 10^-62) and the GGAA motif in 2012 sequences (E-value = 5.7 × 10^-145), whereas when motif analysis was performed in the regions with promoter only binding (1418 promoter sequences) 63 sequences contained the GAS motif (E-value = 9.6 × 10^-33) and 500 sequences contained the GGAA motif (E-value = 9.8 × 10^-24).

The overlapping binding sites of STAT5a and LSD1 as shown by the ChIP-seq data, in conjunction with the RNA-seq expression profiles in the KD cells, suggest that LSD1 and STAT5a synergistically co-regulate a sub-population of STAT5 target genes. To investigate the potential co-regulation of genes bound by STAT5a and LSD1, a few individual genes exhibiting binding from both factors were selected and further characterized. All selected genes (Interleukin 4 receptor alpha chain – Il4ra, Growth factor independent 1B transcription repressor – Gfi1b and Cannabinoid receptor 2 – Cnr2) displayed binding from both STAT5a and LSD1 at the same genomic location (direct overlap), on intronic sequences or upstream the TSS (Figure 4C).

Gfi1b and Cnr2 genes were repressed by IL-3, whereas Il4ra gene was activated by IL-3 stimulation (Figure 4D). To test whether co-regulation depended on the concomitant or sequential binding of STAT5a and LSD1, re-ChIP assays were performed in WT Ba/F3 cells post IL-3 stimulation. The eluate obtained from the ChIP against STAT5a was used as input for a second ChIP against LSD1. The reverse experiment was also performed. The simultaneous co-occupancy of STAT5 and LSD1 on Il4ra, Gfi1b and Cnr2 peak sequences was confirmed (Figure 4E). Furthermore, ChIP assays in STAT5a KD and LSD1 KD cells demonstrated that KD of STAT5a or LSD1 decreases the recruitment of the complex containing STAT5a and LSD1 to the Il4ra loci (Supplementary Figure S8), suggesting that this gene is co-regulated by both factors.

In conclusion, our data provide evidence for concomitant binding of STAT5a and LSD1 in the selected activated and repressed target genes suggesting a synergistic STAT5a and LSD1 action. As a proof-of-principle, functional data were presented for a specific STAT5a target gene (Il4ra) highlighting that regulation of Il4ra gene depends on both STAT5a and LSD1 factors and their interaction. These findings lay the ground for future studies on more complex transcription factors and chromatin interaction networks that require and maintain such tight synergistic regulation and define transcription amplitude and variability.

DISCUSSION

STAT proteins rarely act alone in transcriptional regulation and often depend on direct interactions with other partners to exert their regulatory function, as in the case of STAT5 and glucocorticoid receptor interaction (34). Here, by applying proteomics and genomics approaches, insights in STAT5-dependent activation and repression of target genes in IL-3 stimulated pro-B cells were provided. Many genomic targets were identified, which were correlated to the expression levels of both WT and KD cells. This approach, apart from STAT5a induced genes, revealed various target genes that were repressed by STAT5a. Motif analysis showed a significant enrichment of the canonical STAT5a dimer (GAS) motif, not only in activated, but also in repressed genes (Figure 3, cluster 9) suggesting that binding of STAT5a on the GAS motif can induce activation or repression. Thus, additional STAT5a interacting partners could direct transcription through recruitment of particular factors.

To identify the mechanisms by which STAT5a regulates transcription, novel interacting partners obtained from the proteomics data (LSD1 and HDAC3) were investigated as potential STAT5a co-regulators. The effect of these interactions on target genes transcription was determined at a genome-wide level. Both LSD1 and HDAC3 exert transcriptional regulation at STAT5a targets and show dual roles, being involved in their activation or repression. LSD1 is the first identified histone demethylase (35) capable of either repressing or activating target genes by interacting with various co-factors and catalyzing demethylation of mono- (M1) and di-methylated (M2) H3-K4 or K9 (36). LSD1 drives target activation by removing the repressive H3-K9/M2 mark (37,38), whereas it leads to gene repression by demethylating H3-K4 (M1) and (M2) (35,39,40). HDAC3 is responsible for the deacetylation of lysine residues on the N-terminal tail of core histones and, through multi-protein complexes, regulates gene transcription being a locus-specific co-repressor recruited to promoters to repress genes regulated by nuclear hormone receptors and other transcription factors (41). In terms of protein interactions, HDAC3 forms multi-protein complexes with the co-repressors SMRT and N-CoR (42,43), and STAT5 is known to interact with SMRT (44). LSD1 forms a complex with CoREST (45) and is reported to interact with HDAC3 (46) or N-CoR (40). Considering all these reported interactions and our findings, it is possible that STAT5 exists
Figure 4. Co-regulation of novel targets by STAT5 and LSD1. (A) Venn diagram showing common binding peaks of the STAT5a and LSD1 ChIP-seq libraries (from two independent ChIP assays per factor) in Ba/F3 cells after 6 h IL-3 deprivation followed by 30 min IL-3 stimulation. Only direct overlap of the peak sequence window was considered. (B) Genomic distribution of common STAT5a and LSD1 peaks (direct overlapping peaks only), as reported by HOMER. (C) UCSC representation of three genes (Il4ra, Gfi1b and Cnr2) that display binding of both STAT5a and LSD1 protein at the same genomic location. The common STAT5a and LSD1 binding is highlighted with a black box. The axes on the left indicate peak scores as produced by Homer (position-adjusted reads from initial peak region). (D) RNA-seq experiments were performed in WT Ba/F3 cells (N = 5), and cells with KD of STAT5a or LSD1 (N = 3) or cells treated with control no-target shRNA (N = 5) and are presented in heatmaps. All expression heatmaps represent log2FC values, as computed by DESeq2 (red depicts up-regulation as 2.0 ≥ log2FC ≥ 0; green depicts down-regulation as 0 ≥ log2FC ≥ -1.4). Differential gene expression was analyzed against IL-3 deprived cells in the case of WT Ba/F3 cells, and against cells treated with control no-target shRNA at the same condition for all the KD cells (IL-3 stimulation). The differential expression levels of Il4ra, Gfi1b and Cnr2 are depicted. (E) ChIP-re-ChIP assays were performed in Ba/F3 cells after IL-3 stimulation using antibodies against STAT5a and LSD1 proteins. The first ChIP was performed using the anti-STAT5a antibody, and the eluate was used at a second ChIP with the anti-LSD1 antibody. Relative enrichment values versus input were determined by real time PCR and depict binding from both STAT5a and LSD1 proteins. Error bars display ± SEM (three measurements) and stars show statistical significance (Student’s t-test, *P ≤ 0.05).

in complexes containing SMRT/N-CoR-HDAC3 and/or LSD1. Furthermore, interactions of HDAC1 or HDAC3 with RUNX1 (47), which inhibits STAT5 transcriptional activity and can itself be inhibited by STAT5 (14), open the way for future investigations on the potential involvement of RUNX1 in the complexes identified.

Genomic targets of both LSD1 and HDAC3 were identified though ChIP-seq experiments in IL-3 deprived and stimulated Ba/F3 cells. More than half of LSD1 targets were retained in both cell states indicating that these were IL-3-independent. However, differential LSD1 recruitment was also seen with de novo genomic targets between the two conditions suggesting that LSD1 plays an important role in regulating transcription. The distinct LSD1 genomic targets between the two states corroborate its dual role in gene repression and activation depending on the substrate for demethylation. On the other hand, HDAC3, which is linked closely to gene repression, appeared to have a marked reduction in recruitment to its targets upon IL-3 stimulation. This suggests that during IL-3 stimulation, the need for gene repression is greatly reduced and only a few de novo HDAC3 genomic targets are introduced.
Furthermore, LSD1 and HDAC3 exhibited many unique and common targets with STAT5a. We found both LSD1 and HDAC3 to have a repressive role in all common STAT5a, LSD1 and HDAC3 targets (Figure 3, clusters 1–6), with the exception of a fraction of the common targets that appears to be induced by both STAT5a and LSD1 (cluster 7). Such a dual function in transcription for LSD1 has been previously described, as for example in the regulation of androgen receptor transcriptional activity (37,48).

This particular cluster (cluster 7), characterized by high frequency of promoter binding of LSD1 and STAT5a, included genes that are activated after IL-3 stimulation and they show decreased enrichment of the canonical GAS motif, known to mediate STAT5a binding to DNA. Thus, it is possible that induction of these genes by the synergistic action of STAT5a and LSD1 might not be facilitated by STAT5a, but either by LSD1 or by some other cofactor stabilizing the protein complex on the DNA.

Focusing on the mechanisms of combinatorial regulation of target genes by STAT5a, LSD1 and HDAC3, motif analysis revealed a high enrichment of the GAS motif in all gene sets, except the ones exhibiting LSD1 and HDAC3 binding, but only with weaker STAT5a binding (clusters 4–5, 6 and 7). Strong STAT5 binding in GAS motifs was observed in intergenic regions or introns, which may have putative enhancer activity. However, in the case of weaker STAT5a binding, the absence of GAS motif in the targets exhibiting common STAT5a, LSD1 and HDAC3 binding is of particular interest, as it suggests that STAT5a regulatory control is not mediated by the genomic interaction of STAT5a itself. In these clusters (clusters 4–7), the binding events occur mainly in promoter regions, whereas in the rest of the clusters in intergenic and intronic regions. The targets belonging to clusters 4–5, which exhibited increased LSD1 and HDAC3 binding, were repressed by STAT5a, LSD1 and HDAC3. The absence of a GAS motif could signify that the interaction is not dependent on the binding of a dimer STAT5 molecule. The other enriched motifs found in this group of genes include the recognition sequences for GATA factors (TTATC or GATAA), for factors belonging to the ETS family (GGAA or TTCC) and for factors belonging to leucine zipper family, such as c-fos, c-jun, myc family and others (TGA(G/C)TCA). Based on these findings, it is not unlikely for the DNA STAT5a binding to be mediated through binding of a GATA member or of factors belonging to leucine zipper family, such as c-fos, c-jun, myc family and others (TGA(G/C)TCA). Based on these findings, it is not unlikely for the DNA STAT5a binding to be mediated through binding of a GATA member or of factors belonging to leucine zipper family. Binding of an ETS family member, as part of protein complex containing STAT5a is also possible, as it is known that in response to IL-2 in human T lymphocytes a STAT5-ETS protein interaction is seen (49).

In addition, a recent study suggested that EWS/ETS fusion transcription factor could recruit LSD1, which in turn could recruit either the repressive NuRD complex or co-activator complex to direct transcription (50).

When considering, however, all targets exhibiting weaker LSD1 and HDAC3 binding (clusters 6–7), then two different transcriptional profiles are seen; repressed genes by IL-3, STAT5a and LSD1 and activated genes by IL-3, STAT5a and LSD1. The difference between the two groups is higher enrichment of the GGAA motif (ETS family recognition sequence) for the repressed genes by STAT5a and LSD1 (cluster 6), and the presence of GAS motif, albeit of very low enrichment compared to rest of the clusters, for the activated genes by STAT5a and LSD1 (cluster 7). Since the GGAA motif resembles the monomer STAT5a motif, one hypothesis could be that genomic interaction could occur through either binding of a free STAT5a monomer or binding of one of the two monomers building a dimer to a specific position and of the second one to a distant genomic location (e.g. via chromatin looping), potentially stabilized by co-binding of the other factors nearby. Monomer STAT5 has been reported to possess the ability of transcriptional regulation, as tyrosine-unphosphorylated STAT5 monomer colocalized with CTCF was found to repress a megakaryocytic transcriptional program (51). Similarly, unphosphorylated STAT3 molecules have also been reported to bind to the GAS DNA-binding site as monomers and dimers functioning as transcriptional activators and chromatin/genomic organizers (52).

One could argue that low level ChIP-seq signal could be an artefact explaining the absence of GAS motif. However, it is not uncommon to observe genomic binding in the absence of specific recognition sequence. For example, in GH stimulated mouse embryonic fibroblasts isolated from WT or overexpressing STAT5a mice, only 60% of the binding sites contained GAS motif (53) and similar findings were obtained in mammary tissue with the GAS motif being present only in 50% of the binding events, even in genes defined as regulated by STAT5 based on RNA-seq data (54). Moreover, in liver homogenates from WT mice, when high or low STAT5 activity was observed, many of the STAT5 binding events detected did not contain GAS motifs, possibly reflecting either STAT5 binding through non-consensus sequences or indirect STAT5 binding via other proteins (55). In addition, out of all the GAS sites in the mouse genome (more than 1 million), only up to 10% of these are occupied by STATs at any given moment (56). Thus, based on our findings and these published observations the presence of GAS motif does not necessarily correlate with STAT5 binding and the lack of GAS motifs cannot exclude binding of STAT5.

Although further studies are still necessary to completely characterize the STAT5a interaction network, this work importantly has identified multiple parameters influencing the outcome of STAT5a-dependent transcription. In particular, the STAT5a interacting partners, the binding motif governing the STAT5a genomic interaction (alone or in multi-protein complexes), the binding affinity (as indicated by targets enrichment), as well as the genomic location of the peak in relation to the TSS of target genes could dictate both transcription direction and amplitude. Finally, the underlying landscape of chromatin/histone targets for LSD1 and HDAC3 (H3K4 versus H3K9) might play a critical role in ruling the observed dual functions via locus specific enrichment for one rather than the contrasting mark. The novel insights on STAT5a-dependent transcriptional mechanisms provided here form a platform for delineation of the complete STAT5 interactions network in pro-B cell functions and B-cell development.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online.
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