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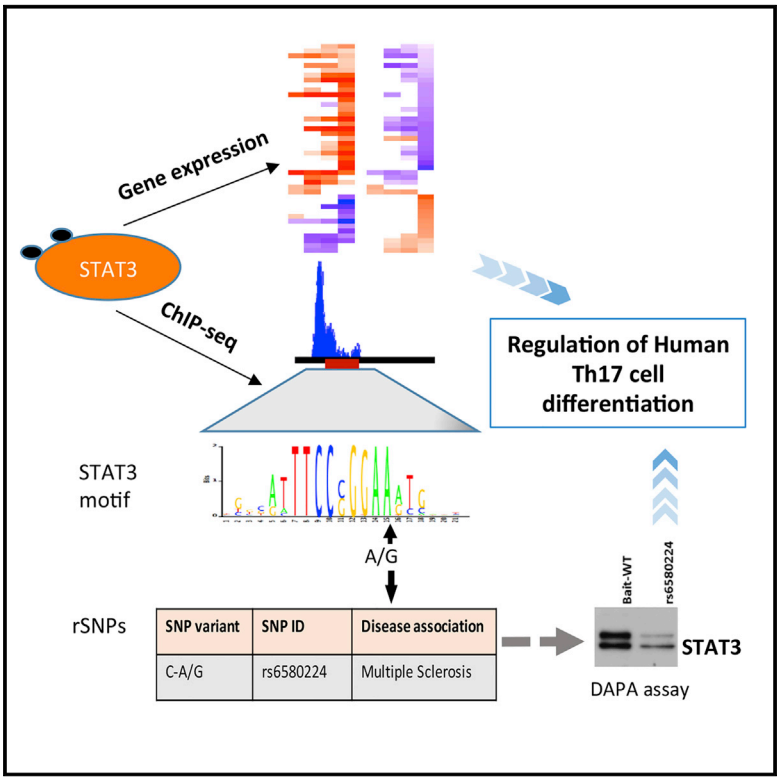
Graphical Abstract



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In Brief

Tripathi et al. show that STAT3 is critical for transcriptional regulation of early human Th17 cell differentiation. A number of SNPs from loci associated with immune-mediated disorders occur at STAT3-binding sites. Introduction of such SNPs alters STAT3 binding in DNA affinity precipitation assays.

Highlights

- Identification of early STAT3 targets in polarizing human Th17 cells
- SNPs associated with immune diseases localize in STAT3-binding sites
- Introduction of such SNPs alters STAT3 binding

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Genome-wide Analysis of STAT3-Mediated Transcription during Early Human Th17 Cell Differentiation

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SUMMARY

The development of therapeutic strategies to combat immune-associated diseases requires the molecular mechanisms of human Th17 cell differentiation to be fully identified and understood. To investigate transcriptional control of Th17 cell differentiation, we used primary human CD4$^+$ T cells in small interfering RNA (siRNA)-mediated gene silencing and chromatin immunoprecipitation followed by massive parallel sequencing (ChIP-seq) to identify both the early direct and indirect targets of STAT3. The integrated dataset presented in this study confirms that STAT3 is critical for transcriptional regulation of early human Th17 cell differentiation. Additionally, we found that a number of SNPs from loci associated with immune-mediated disorders were located at sites where STAT3 binds to induce Th17 cell specification. Importantly, introduction of such SNPs alters STAT3 binding in DNA affinity precipitation assays. Overall, our study provides important insights for modulating Th17-mediated pathogenic immune responses in humans.

INTRODUCTION

Th17 cell differentiation is selectively induced by a combination of the cytokines transforming growth factor β-1 (TGFβ-1), interleukin 6 (IL-6), and/or IL1-β (Acosta-Rodriguez et al., 2007; O'Garra et al., 2008; Veldhoen et al., 2006). Stimulation by IL-6 leads to the activation of the signal transducer and activator of transcription 3 (STAT3). Upon phosphorylation, STAT3 regulates the transcription of its target genes, including the transcription factors RORγt and RORα and the signature cytokines IL-17A, IL-17F, IL-21, and IL-23 (Chen and O'Shea, 2008; Durant et al., 2010; Murphy and Stockinger, 2010; O'Garra et al., 2008).

Th17 cell differentiation is significantly impaired in STAT3-deficient T cells, whereas overexpression of active STAT3 results in increased production of IL-17 (Durant et al., 2010; Laurence et al., 2007; Mathur et al., 2007; Yang et al., 2007). In spite of its importance, current understanding on STAT3 and its role in Th17 cell differentiation is primarily based on studies done in mouse cells, whereas very little is known from human studies. The importance of STAT3 in human diseases is highlighted by reports indicating that mutations directly influencing the DNA-binding and SRC homology 2 domains of STAT3 cause a complex disease, hyper-immunoglobulin E (hyper-IgE) syndrome (also called Job's syndrome) (Holland et al., 2007; Ma et al., 2008; Milner et al., 2008). On the other hand, recent studies show that gain-of-function mutations of STAT3 contribute to multi-organ autoimmunity, autoimmune cytopenia, lymphoproliferation, and immune deficiency (Flanagan et al., 2014; Haapaniemi et al., 2015; Milner et al., 2015). Identification of downstream targets of STAT3 in human cells is of particular interest, because STAT3 is a key upstream mediator of cytokine-receptor-induced initiation of Th17 cell differentiation. Therefore, genes regulated by STAT3 are responsible for eliciting the early stages of the Th17 cell differentiation program.

In this study, a combination of genome-wide analysis methods and computational data integration enabled us to identify direct and indirect STAT3 targets during the initiation of human Th17 cell differentiation. Within the STAT3-binding sites and STAT3-regulated genes, we detected a number of SNPs from loci that have been associated with immune disorders, including type 1 diabetes (T1D), Crohn's disease (CD), and psoriasis (PS). Introduction of such SNPs can influence transcriptional regulation and thereby cellular differentiation and immunopathogenesis.

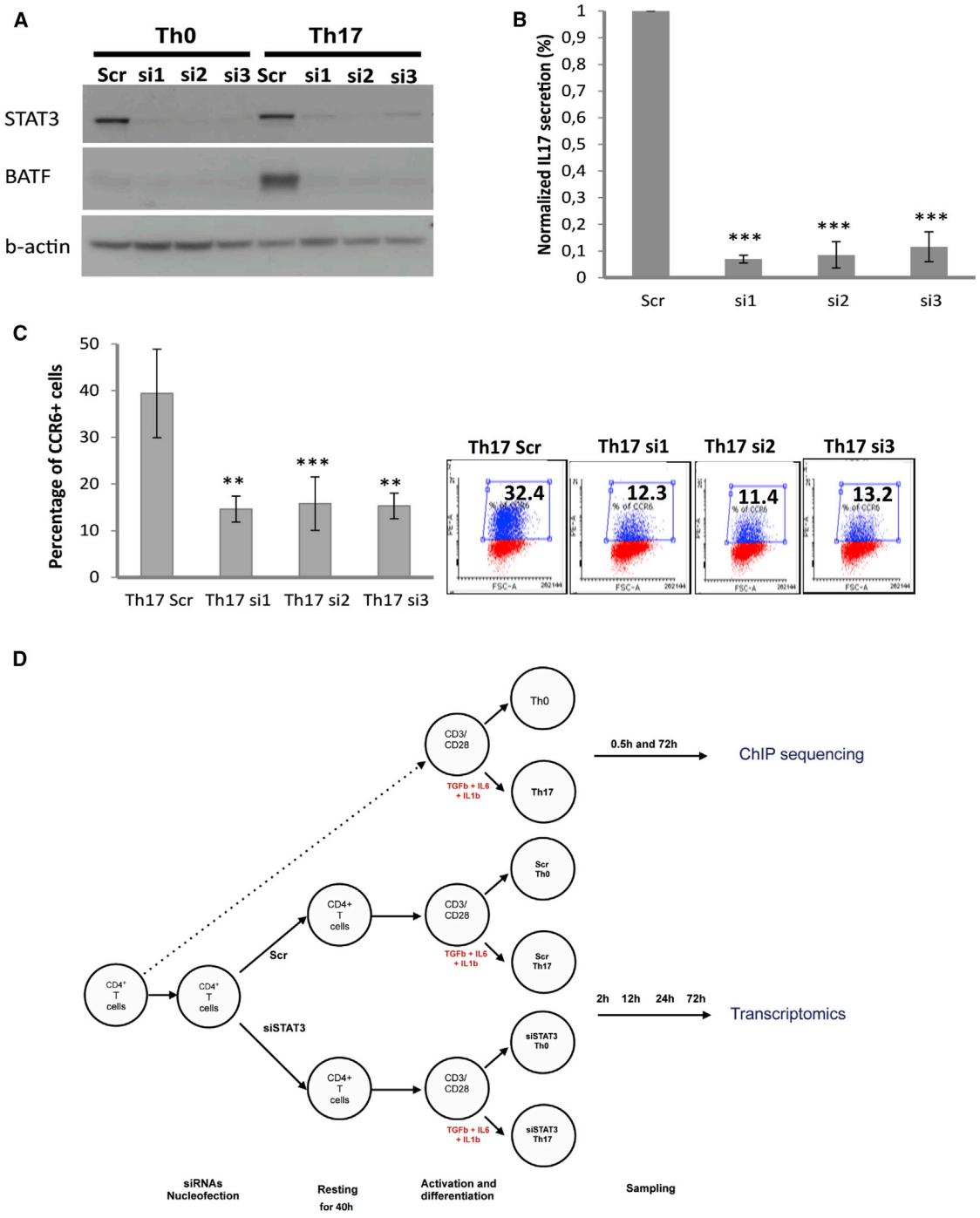

Figure 1. Downregulation of STAT3 Influences Human Th17 Cell Differentiation

(A) Western blot analysis showing the expression level of STAT3 and BATF proteins after introduction of three different individual STAT3 siRNAs (si1, si2, and si3) into cord blood $CD4^+$ T cells and culturing the cells for 24 hr in Th0 or Th17 cell polarizing conditions. Non-targeting control-siRNA (Scr) was used as a control and β-actin as a protein loading control. Representative results from three independent experiments are shown.

(B) IL-17A secretion after 72 hr of culture. The cytokine was detected directly from the culture supernatant with Luminex fluorescent-coded beads. The values were first normalized with the number of living cells determined based on forward (FSC) and side scattering (SSC) detected with a flow cytometer. IL-17A secretion was compared between cells transfected with non-targeting scramble siRNA and STAT3 siRNA and cultured under Th17 cell polarizing conditions for 72 hr. The data shown are the mean ± standard error of the mean (SEM) of three cultures. Statistical significance was determined for one sample using two-tailed Student's t test (**$p < 0.05$; ***$p < 0.01$).

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RESULTS

STAT3 Is Required for Cytokine-Induced Human Th17 Cell Differentiation

To characterize the role of STAT3 in human Th17 cell differentiation, RNAi was used to downregulate its expression in human $CD4^+$ T cells (see Figure S1 for information regarding cell purity) and investigate the effect of this treatment on Th17 cell differentiation. Three different small interfering RNAs (siRNAs) targeting *STAT3* mRNA were used to control reproducibility of the findings. Efficacy of RNAi-mediated knockdown was demonstrated by decreased expression on STAT3 and its known target, BATF (Figure 1A). The results showed that the nucleofected cells were able to polarize as expected indicated by IL-17 secretion (Figure 1B). Moreover, depletion of STAT3 resulted in decreased secretion of IL-17 and reduced expression of CCR6, a chemokine receptor highly expressed in Th17 cells (Figures 1B and 1C). These results demonstrate that STAT3 is also an important regulator of Th17 cell differentiation in human T cells. Thus, we were able to use these three siRNAs to downregulate STAT3 expression in order to identify STAT3-regulated genes (Figure 1D).

Identification of STAT3-Regulated Genes

Gene expression changes in response to Th17 cell polarizing signals were detected by comparing results on T cells transfected with non-targeting scramble (Scr) siRNA to those obtained in cells activated in the absence of Th17 cell polarizing signals (Th0 cell; Figure 2A). At 2 hr, expression of 2,194 genes was changed (false discovery rate [FDR] < 0.1). Culturing the cells for 72 hr in Th17 cell polarizing conditions resulted in changes in 1,446 genes (Table S1).

To identify STAT3-regulated genes during the early stage of human Th17 cell differentiation, gene expression profiles from cells nucleofected with STAT3 siRNAs were compared to profiles of cells nucleofected with non-targeting siRNA. Downregulation of STAT3 resulted in maximal effects in differentiating Th17 cells at the 72-hr time point, where it influenced the expression of 691 genes (Figure 2B). Even at 2 hr, the expression of 157 genes was reduced in response to STAT3-siRNA, indicating that STAT3 positively regulates the expression of these genes. Conversely, STAT3 suppresses the expression of 89 genes, including *STAT1*, *STAT2*, and *SMAD7* (Figure 2B; Table S1). Throughout all the time points studied, STAT3 positively and negatively regulated the expression of a similar number of genes (691 versus 576), which is consistent with the idea that STAT3 is important for both promoting Th17 cell polarization and preventing the ability of the cells to differentiate to alternative lineages.

To investigate which of the siSTAT3-regulated genes were changed during the process of Th17 cell differentiation, genes differentially expressed in Th17 cells (Figure 2A) were overlaid with siSTAT3-regulated genes (Figure 2B; Table S1). At 2 hr, siSTAT3 regulated 6.1% of the genes whose expression was changed in Th17 polarizing cells (Figure 2C). As time of differentiation increased, the proportion of siSTAT3-regulated genes increased, peaking at 72 hr, where STAT3 was found to regulate 31.6% of the differentially expressed genes (Figure 2C). These results indicate that STAT3 is an important transcriptional regulator of Th17 cell differentiation.

The expression of *NTRK3*, *IL16*, *DUSP10*, and *CASP1* was found to be regulated by STAT3 at all of the time points studied. The expression profile of these genes (together with *FOSL2*, which is regulated by STAT3 at 12, 24, and 72 hr time points) was also changed during the Th17 cell differentiation process (Figure 2D). Expression of 43 genes was changed at least 2.8-fold (signal log ratio [SLR] of 1.5) in response to both Th17 cell differentiating stimuli as well as to STAT3 siRNAs (Figure 2E). They responded to STAT3 regulation with different dynamics; for example, *GBP4*, *COL6A3*, and *CXCR5* responded quickly (at 2 hr) to Th17 cell polarizing stimulation as well as to STAT3 knockdown, whereas *CCR6* and *CSF2* responded later (at 72 hr).

CXCR5 has been reported to be highly expressed in follicular helper T (TFH) cells, and both IL-6 and IL-21 induce its expression in a STAT3-dependent manner (Nurieva et al., 2008). Interestingly, expression of CXCR5 was induced by Th17 cell polarizing signals, and this induction was reduced at both the 24 hr and 72 hr time points in STAT3-depleted cells, confirming that CXCR5 is regulated by STAT3 (Figure 2F).

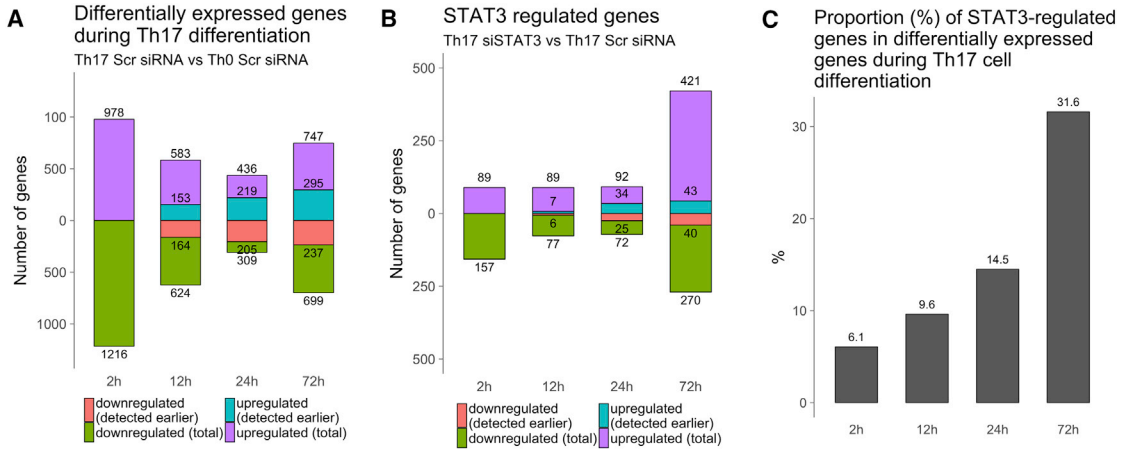
Immediate Target Genes of STAT3

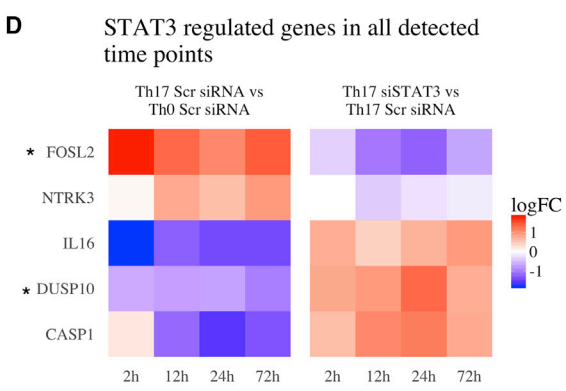
Having identified genes regulated by STAT3 during the early stages of Th17 cell differentiation, a STAT3 chromatin immunoprecipitation sequencing (ChIP-seq) study was carried out to further distinguish the direct and indirect targets of STAT3. To define an optimal early time point for ChIP-seq, the kinetics of STAT3 phosphorylation was studied (Figures 3A, S2A, and S2B). We selected a 0.5 hr time point post-induction of Th17 cell polarization for ChIP-seq, where maximum phospho-STAT3 was detected in an average of 67% of cells. In total, 2981 STAT3-binding sites were identified (Table S2; Figure S3B), approximately two-thirds of which were located in introns or intergenic regions, suggesting that STAT3 may regulate gene expression via binding to distal regulatory elements (Figure 3B). Further analysis revealed that 48% of the STAT3 binding sites were localized within 10 kb up- or down-stream of transcription start sites (TSS) (Figure 3C). Consistent with the importance of STAT3 in regulating gene expression via binding to core promoters of its targets, 20% of the binding sites were located in the immediate promoter region. DNA motif analysis further confirmed the STAT3 motif as being the strongest motif among the 2,981 binding sites ($p = 10^{-113}$).

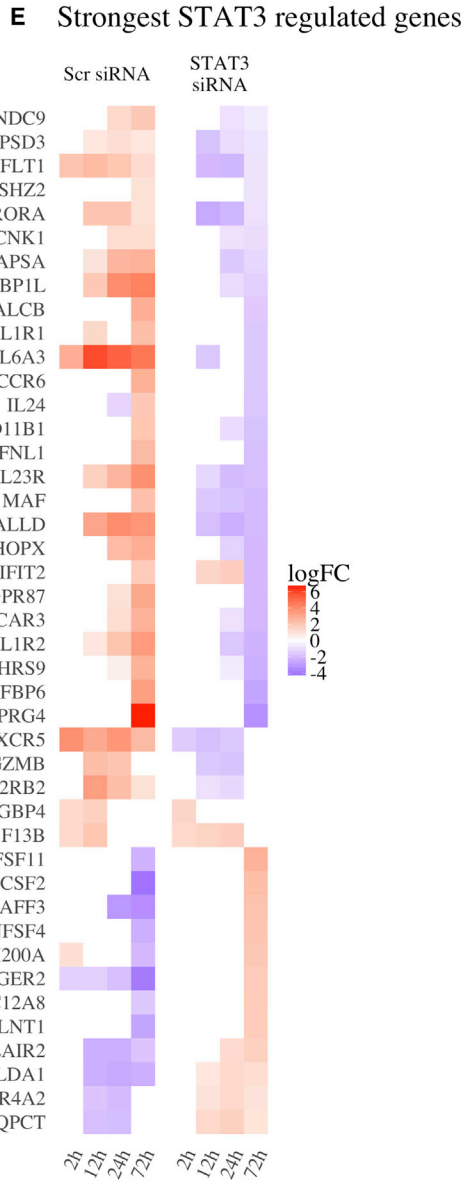
Identification of direct STAT3 target genes was carried out by combining ChIP-seq results with the list of genes whose expression was changed during Th17 cell polarization and regulated by

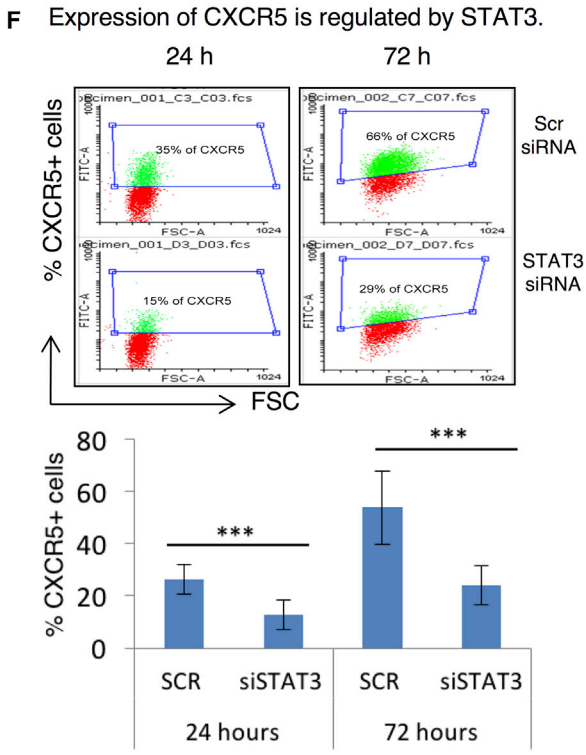
(C) The percentage of cells expressing CCR6 after 72 hr of polarization. Before induction of polarization, the cells were treated with STAT3-siRNAs or scramble siRNA. Living cells were gated for the analysis based on FSC and SSC. The data shown on the left are the mean ± SEM of three cultures, and statistical significance was determined using a two-tailed Student's t test (**$p < 0.05$; ***$p < 0.01$). Representative results from three independent experiments are shown on the right.

(D) Experimental design for gene expression profiling.









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STAT3 siRNAs. At 2 hr, 18.8% of the STAT3-regulated and differentially expressed genes in polarizing Th17 cells were found to be direct targets of STAT3. At later time points, the overlap was 28.4% and 25.9% at 12 hr and 24 hr, respectively (Figure 3D; Table S3). To further study how STAT3 binding to TSS and promoter regions influences gene expression dynamics, STAT3-bound genes at TSS and promoter regions were overlaid with siSTAT3-regulated differentially expressed genes. Interestingly, among the siSTAT3-regulated genes, the proportion of STAT3-bound genes at the TSS-promoter region was similar in all of the detected time points (Figure S3C; Table S3), even though STAT3 regulated the expression of many more genes at 72 hr (Figures 2B and 2C). This suggests that in addition to influencing gene expression by binding to TSS-promoter regions, STAT3 also influences gene expression by either binding to distal regulatory elements or through regulating expression of other transcription factors (TFs). These additional mechanisms amplify the effect of STAT3 on gene expression as the differentiation progresses.

In this study, several STATs, including STAT3 itself, were found to be among the direct STAT3-regulated targets. To compare the results of this human study with earlier mouse studies, we overlaid the panel of STAT3-bound genes identified here and previously published in murine polarizing Th17 cells (Ciofani et al., 2012; Durant et al., 2010). This revealed that ~20% of the STAT3-bound genes identified in this study overlapped with those found in the two previous studies, regardless of the species difference and experimental settings (Figure S4; Table S4). In addition, our work identified at the 0.5 hr time point 1,211 STAT3-bound genes that were not reported in the other two studies (Figure S4; Table S4).

To explore whether the dynamic changes in STAT3 phosphorylation (Figure 3A) correlate with the occupancy of STAT3 on its target genes, STAT3 ChIP was performed at 0, 0.5, 2, 12, and 72 hr after induction of Th17 cell differentiation. To validate results from ChIP-seq experiments with qPCR, based on the literature, 15 genes were selected (Figures S5 and S6); some of these have been previously reported as STAT3-bound genes, and others, including *MED16*, *ZNF460*, *TMEM235*, and *MALAT1*, are among the 1,211 new STAT3-bound genes in human T cells identified in this study. To demonstrate the specificity of STAT3 binding, based on our previous study (Elo et al., 2010), we selected two STAT6-regulated sites as negative controls and performed ChIP-qPCR detection in three independent cultures (Figure S6B). The results showed that STAT3 bound to the selected regions of the chosen genes only in cells cultured for 0.5 hr under Th17 polarizing conditions, confirming the ChIP-seq results at this time point (Figures 4A and S6A). However, consistent with the kinetics of STAT3 phosphorylation, which peaks at 0.5 hr in response to Th17 cell-inducing cytokines, STAT3 binding to these loci was either decreased or not detected (Figures 4A and S6A). The results indicate that the binding of STAT3 to its targets changes with time. To further study the dynamic binding of STAT3, we performed STAT3 ChIP-seq from cells cultured under Th17 polarizing conditions for 72 hr in three independent experiments. We identified 4,317 STAT3-bound sites, 94% of which are located in introns or intergenic regions and only 3.6% of which resided in TSS and promoter regions (Figure 4B; Table S2). This suggests that at later stages of Th17 differentiation, STAT3 influences gene expression mostly through mechanisms that do not involve direct binding to promoters. To determine whether STAT3 regulates gene expression by binding to enhancers, we compared our STAT3 ChIP-seq data obtained from both 0.5 hr and 72 hr to published human Th17 cell enhancer data (Farh et al., 2015). The results show that 74.8% and 35% of STAT3 peaks at 0.5 hr and 72 hr, respectively, overlap with the enhancer mark H3K4me1, suggesting that STAT3 is involved in shaping enhancers during Th17 differentiation at a very early stage. Comparing 0.5 hr and 72 hr ChIP-seq data, we found that STAT3 bound to 213 sites at both time points, representing 203 genes, including STAT3, BATF, TBX21, and SOCS3 (Table S2). We also overlaid STAT3-bound genes with differentially expressed genes and siSTAT3-regulated genes during Th17 cell differentiation. For 16.5% of STAT3-bound genes at 0.5 hr, their expression was changed after 2 hr of Th17 polarization; at 72 hr, expression levels of 325 (12%) STAT3-bound genes were changed, among which 118 genes(36%) were influenced by siSTAT3, including *IL-17F*, *IL-21*, *IL-23R*, *RORA*, *STAT4*, *GATA3*, *IL-4R*, *GFI1* (Figure 4C; Table S2). Our study was thus successful in identifying the very early direct STAT3 targets involved in the initiation of Th17 cell polarization.

STAT3-Mediated Transcriptional Regulatory Networks at an Early Stage of Human Th17 Cell Differentiation

Global gene expression profiling and ChIP-seq allowed the identification of the TFs whose expression was changed during early

Figure 2. Dynamics of STAT3-Mediated Gene Expression

(A and B) The number of differentially expressed genes (A) and siSTAT3-regulated genes (B) during Th17 cell differentiation is shown at each time point from three independent biological replicates from different donors (FDR < 0.1; see Table S1 for lists of genes). The height of a bar shows the total number of genes detected at a particular time point, with the height of the darker inner part of the bar representing those genes that were not detected at earlier time points.

(C) Proportion of genes regulated by siSTAT3 from all the differentially expressed genes at each time point during Th17 cell differentiation (see Table S1 for lists of genes).

(D) Heatmap of STAT3-regulated genes throughout the time course. *FOSL2* is regulated by STAT3 only at the 12, 24 and 72 hr time points.

(E) Heatmap of genes that were the most differentially expressed in response to Th17cell differentiation and the most strongly regulated by STAT3 signal log ratio (SLR) between SCR Th17 cell versus Th0 cell >1.5 or <−1.5 and between STAT3 siRNA versus Scr Th17 cell >1.5 or <−1.5). Asterisks indicate that STAT3 bound to these genes in ChIP-seq experiments. Statistically significant changes in gene expression in response to STAT3 depletion by RNAi are based on three independent biological replicates from different donors (FDR < 0.1).

(F) Expression of CXCR5 is regulated by STAT3. After treating the cells with STAT3 siRNAs or control siRNA, cells were polarized to Th17 cell phenotype. The expression of CXCR5 was detected by flow cytometry at 24 and 72 hr after the induction of Th17 cell polarization. The Bar chart shows the percentage of cells expressing CXCR5 upon treatment with STAT3-siRNAs or control-siRNA in the cells polarized to Th17 cell direction. Error bars represent SD of the data derived from four biological replicates. p values were calculated based using a two-tailed Student's t test (***$p < 0.01$).

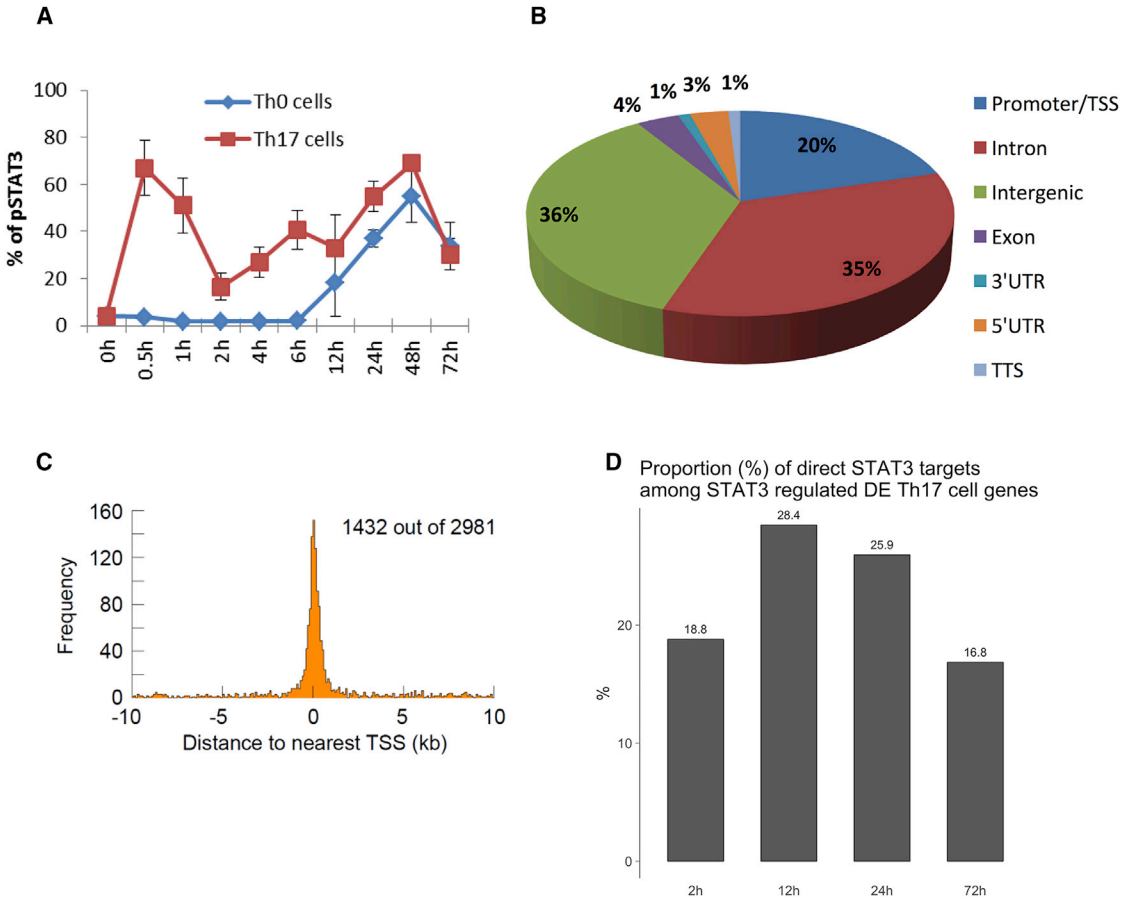


Figure 3. STAT3-Bound Genes

(A) The kinetics of STAT3 phosphorylation during early polarization of Th17 cells. The line chart summarizes the average percentage of phosphorylated STAT3 (pSTAT3)-positive cells at the indicated time points (0–72 hr) in three biological replicates. The error bars show the SD over the replicates. Umbilical cord blood $CD4^+$ T cells were activated with α-CD3 and α-CD28, with or without the cytokines IL-6, TGF-β, and IL1-β.

(B) The genomic distribution (%) of the identified STAT3-binding sites after 0.5 hr of Th17 cell polarization. STAT3-binding sites were detected by ChIP-seq and determined by using the MACS tool. See Table S2 for a complete list of STAT3-binding sites.

(C) Distribution of the STAT3 binding sites relative to the position of the closest transcription start site (TSS). TSS is defined to be at position zero in the graph. Out of all the 2,981 detected binding sites, 1,432 are within ±10 kb from the TSS.

(D) The proportion of STAT3 regulated differentially expressed genes during Th17 cell differentiation that were bound by STAT3 in the ChIP-seq experiment. These direct STAT3 targets are listed in Table S3.

Th17 cell differentiation and directly or indirectly regulated by STAT3. Maximal changes in the expression of TFs were observed at 2 hr, where expression of 131 or 113 TFs were increased or decreased, respectively (Figure 5A). Among them, 29 were regulated by STAT3 (Figure 5B). The expression of *NPAT, ZNF211*, and *NKAP* was downregulated and their expression levels increased when STAT3 was downregulated; conversely, the expression of *SMAD7*, *STAT1*, and *STAT2* was upregulated and their expression further enhanced when STAT3 was downregulated, suggesting that their expression is repressed by STAT3 (Figure 5C; Table S5). After 2 hr, the number of differentially expressed TFs was first reduced and then increased again, and the maximal number of STAT3-regulated TFs was observed at 72 hr (Figure 5B). A panel of TFs, including *BATF*, *RORA*, *FOSL2*, *IKZF2*, *IKZF3*, *HOPX*, and *AFF3,* were found to be differentially expressed throughout several time points (Figure 5D; Table S5), suggesting that these TFs play an important role in determining the fate of Th17 cell polarization.

The TFs were classified into four groups by integrating the results. As shown in Figure 5E, the first group, including *STAT1*, *STAT3*, *BATF*, *GATA3*, *RBPJ*, *FOSL2*, and *RORA*, contained direct STAT3 targets, whose expression was changed during Th17 cell differentiation (Th17 versus Th0 cells) and which were regulated (STAT3 siRNA versus non-targeting siRNA) and bound by STAT3 (STAT3 ChIP-seq). The second group contained indirect STAT3 targets whose expression was changed during Th17 cell differentiation and that were regulated, but not bound, by STAT3, suggesting that STAT3 influences their expression through other factors or mechanisms. This group of genes contained, for example, *ATF3* and *ELL2*. The third group consisted of putative STAT3 targets differentially expressed during Th17 cell differentiation that are bound, but not regulated, by

STAT3. Several TFs previously reported to be involved in differentiation of Th17 or regulatory T cells as well as in TGF-β signaling were part of this group, including *IRF4*, *NOTCH1*, *RUNX3*, *IKZF4*, and *JUN* (Brüstle et al., 2007; Fu et al., 2012; Keerthivasan et al., 2011; Klunker et al., 2009; Li et al., 2012; Sharma et al., 2013). The fourth group contained TFs differentially expressed in polarizing Th17 cells, but not regulated or bound by STAT3 (Table S5). This group of genes may act independently of STAT3 or interact or cooperate with STAT3 for Th17 cell lineage specification via the combinatorial transcription regulation network.

SNPs Associated with Autoimmune Diseases Identified in STAT3-Binding Sites Alter STAT3 Binding and Are Enriched among STAT3-Regulated Genes

The essential role of Th17 cells in the pathogenesis of autoimmune diseases is well known (Chen and O'Shea, 2008; Korn et al., 2009). Genome-wide association studies (GWASs) have identified a large number of disease associated SNPs at different loci of the genome that can influence the regulation of a gene several kilobases away from the TSS. We therefore investigated whether SNPs associated with human diseases are enriched in regions where STAT3 target genes or STAT3-binding sites were identified in differentiating Th17 cells in this study. The STAT3-regulated genes detected at one or more time points were found to be enriched in many autoimmune diseases, such as primary biliary cirrhosis (PBC), Behçet's disease, rheumatoid arthritis (RA), CD, and ulcerative colitis (UC; Figure S7A). A list of SNPs with reported associations with 11 different autoimmune diseases in the GWAS catalog (Hindorff et al., 2009) was then generated, and their proxies ($r^2 > 0.8$) were calculated based on the EUR population from the 1000 Genomes Project. We then extracted the lead SNPs associated with 11 different autoimmune diseases and their proxies and intersected with STAT3-binding sites detected early after induction of Th17 cell differentiation. We found several SNPs to be localized within STAT3-binding sites (Figure 6A). To test whether STAT3-binding sites are enriched within each of the autoimmune diseases, we extracted 1,000 random sets of SNPs using the SNPsnap tool (see Experimental Procedures) and intersected with STAT3-binding sites. The enrichment analysis then indicated that although the individual diseases were not significant after multiple testing correction (Figure 6A), autoimmune-disease-associated SNPs, when considered as a single trait, overlapped more often with STAT3-binding sites than a random set of SNPs ($p = 0.01$, FDR = 0.11).

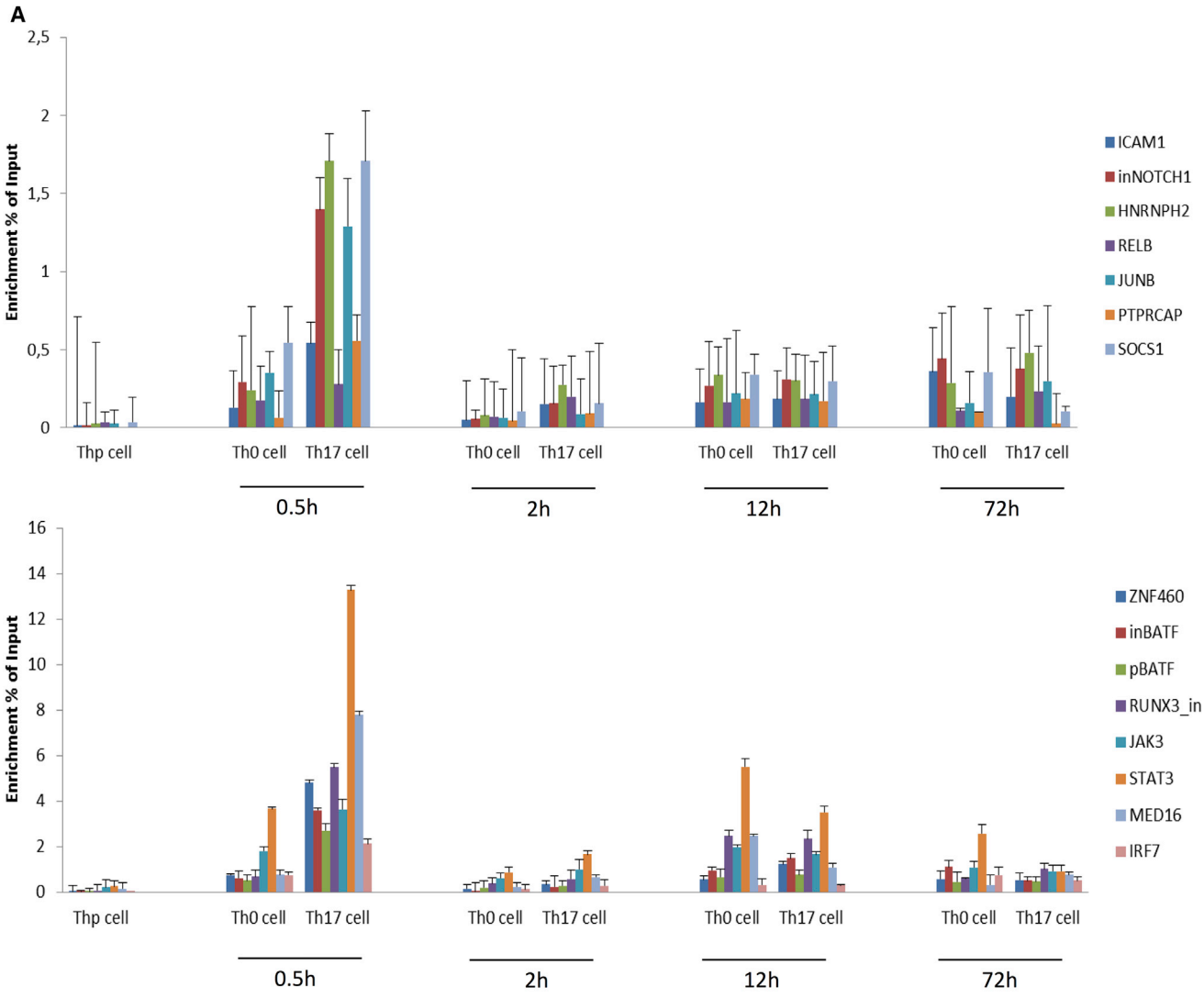
Next, searching STAT3 motifs shown in Figure 6B, several SNPs (or their proxies in the Utah residents with northern and western European ancestry [CEU] or European [EUR] population) associated with autoimmune diseases were found (Table 1). For example, a T1D-associated SNP (rs947474) overlapping with the STAT3 motif was identified upstream of PFKFB3 (6-phosphofructo-2-kinase/fructose-2, 6-bisphosphatase 3) and PRKCQ (protein kinase C, theta). The STAT3 motif was found in the plus strand, and the mutation was on the 12th nucleotide, which was "strong" in the motif, meaning that over 80% of the bound sequences from which this motif was constructed had this nucleotide on the same position. In this case, STAT3 was predicted to bind to the nonmutated sequence, but not to the mutated one (Table S6). Furthermore, after searching the blood expression quantitative trait loci (eQTL) browser (Westra et al., 2013), STAT3-binding sites were found to overlap with two SNPs that have a *cis* eQTL effect (FDR < 0.1). The target gene of one of them (rs842625) is REL, and the other SNP (rs947474) has no clear target gene (the probe is located between LOC399715 and PRKCQ).

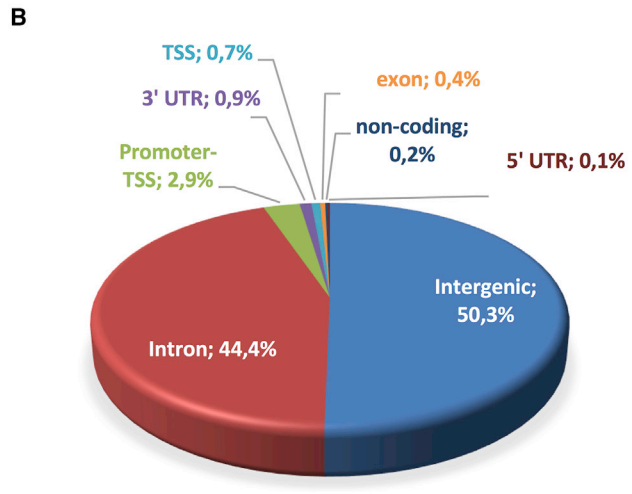
A SNP at the STAT3-binding motif could result in either gain or loss of STAT3 binding. This in turn may alter target gene expression and Th17 cell differentiation and eventually contribute to disease. Hence, to examine whether disease-associated SNPs (or their proxies in the CEU or EUR population) at the STAT3 motif could alter STAT3 binding and thereby have a functional effect, DNA affinity precipitation assays (DAPAs) were carried out using oligonucleotides containing a STAT3 motif or single base pair variants (associated SNP) as bait. Six SNPs that overlap STAT3-binding sites (Tables S7 and 1) were selected. The DAPA results showed that STAT3 bound to the oligonucleotides when the reference genome was used; however the binding was reduced when mutant oligonucleotides corresponding to disease associated SNPs rs3024505, rs947474, rs6580224, and rs2293607 were used instead (Figures 6C and S7B). Conversely, STAT3 binding was increased when mutant oligonucleotide corresponding to the PS-associated SNP rs12443808 was used. Mutant oligonucleotide corresponding to PBC-associated rs17313508 did not alter the STAT3 binding compared with the reference bait sequence (Figure 6C; Experimental Procedures). This indicates that introduction of an SNP in the STAT3 motif alters STAT3 binding and suggests that SNPs may regulate STAT3 mediated transcription by affecting the expression of targets that are important for early human Th17 cell differentiation.

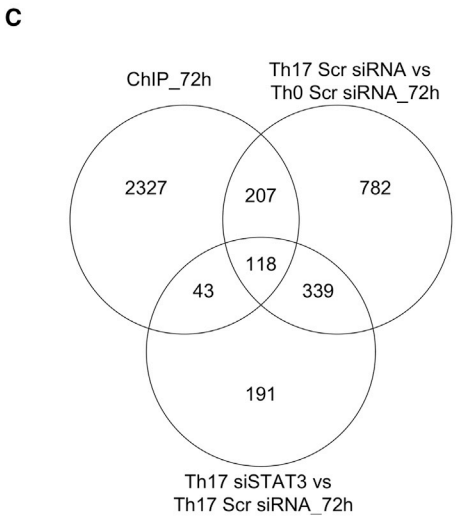
DISCUSSION

STAT3 is one of the key TFs needed for Th17 cell differentiation. The molecular mechanisms by which STAT3 influences downstream genes in Th17 cell differentiation have been identified on a genome-wide level, but only in murine T cells (Ciofani et al., 2012; Durant et al., 2010). It is therefore essential to examine in human T cells the targets of STAT3 that may affect differentiation or function of Th17 cells. Here, we identified the primary targets of STAT3 that are likely to play important roles in the initiation of Th17 cell differentiation and orchestrate the downstream process by influencing other TFs. STAT3 ChIP-seq at 0.5 hr after Th17 cell stimulation resulted in the mapping of 2,981 STAT3-binding sites in human T cells at this very early time point. Moreover, expression of over 1,000 genes was found to be influenced by STAT3 RNAi. Among the 25 primary STAT3 targets are 4 TFs, including STAT3 itself, suggesting that STAT3 is able to specify Th17 cell phenotype through a positive regulatory loop.

Our results show that over half of STAT3 binding sites are mapped to introns and intergenic regions. By mapping active enhancer landscapes in differentiated Th1 and Th2 cells, we (Hawkins et al., 2013) and Vahedi et al. (2012) have shown that binding of these STAT proteins can activate lineage-specific enhancers and suppress enhancers associated with alternative cell







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fates (Hawkins et al., 2010, 2013; Vahedi et al., 2012) and regulate transcription. It is therefore of interest to find out how STAT3 binding to intergenic regions contributes to epigenetic regulation or shaping chromatin structures at the early stages of human Th17 cell differentiation.

Here, we focused on determining the STAT3 targets in primary human T cells, whereas previous studies have identified STAT3 targets in murine T cells (Ciofani et al., 2012; Durant et al., 2010). In order to capture the earliest events to determine which molecules might be critical for the initiation of Th17 cell differentiation, ChIP-seq was performed at 0.5 hr and 72 hr in the present study, whereas in murine T cell studies, the 48 hr and 72 hr time points were used for STAT3 ChIP-seq experiments. Another difference compared with previous studies is that in addition to IL-6 and TGF-β1, IL1-β was used in our human T cell cultures. As a consequence, the signaling pathways and downstream transcriptional regulatory networks revealed by the present study differ from those described in the previous ones. Despite these differences, it was found that among the 2,981 STAT3-binding sites that were mapped to 2,519 annotated genes at 0.5 hr in our study, 1,232 genes (48.9%) were also bound by STAT3 in murine Th17 polarizing cells at 48 hr, and 570 genes (22.6%) were also bound by STAT3 in murine T cells cultured under Th17 cell stimulation for 72 hr and restimulated by IL-6 for 1 hr. Further analysis indicated that STAT3 bound to 493 genes in all of these three studies (Figure S2; Table S4). Importantly, 203 genes were already bound by STAT3 only 0.5 hr after induction of human Th17 cell differentiation (Figure S4). A group of TFs, including *BATF*, *BCL6*, *FOSL2*, *IRF4*, *IRF9*, *IKZF1*, *IKZF4*, *NOTCH1*, *RORA*, *SBNO2*, *RBPJ*, and *STAT3* were among the common genes in which STAT3 resided both in human and mouse Th17 differentiating cells. Some of these genes (Table S4), such as *IKZF4* and *SBNO2*, have not been previously reported to influence Th17 cell differentiation.

To enhance our understanding of the basis of Th7 cell-mediated autoimmune diseases such as RA, T1D, CD, UC, and PS, we investigated how STAT3-binding sites overlap with SNPs associated with immune-mediated diseases. Our results suggest that SNPs associated with several autoimmune diseases co-localize within STAT3-binding sites, and some SNPs localized within STAT3 motifs. An in vitro STAT3-binding assay of a panel of these STAT3-binding sites indicated that disease-associated SNPs altered STAT3 binding and hence may have downstream effects on gene expression. This in turn may alter Th17 cell differentiation and eventually contribute to disease. Our study provides insights into the molecular mechanism of human Th17 cell development and how it can be regulated, and it provides a basis for understanding and modulating Th17-mediated pathogenic immune responses in humans.

EXPERIMENTAL PROCEDURES

Human CD4$^+$ T Cell Isolation and Culturing

CD4$^+$ T cells were isolated from umbilical cord blood collected from healthy neonates born in Turku University Hospital, Hospital District of Southwest Finland, with approval from the Finnish Ethics Committee. CD4$^+$ T cell isolation and culture were done as previously described (Tuomela et al., 2012). Detailed information can be found in Supplemental Experimental Procedures.

STAT3 Knockdown through RNAi

For siRNA-mediated gene silencing, cells were transfected with 5 μg siRNA/4 × 10^6 cells targeting STAT3 or a non-targeting control siRNA (Sigma; sequences are listed in Supplemental Experimental Procedures) using a nucleofector device (Amaxa Biosystems, Lonza), after which cells were rested for 40 hr before culturing.

For STAT3 target gene identification, cells were harvested at 2, 12, 24, and 72 hr. Three biological replicates were prepared, each time including all three different individual STAT3 siRNAs and non-targeting control siRNA. Total RNA was processed and hybridized on Affymetrix GeneChip Human Genome U219 arrays.

STAT3 ChIP-Seq Studies

CD4$^+$ T cells were cultured in Th17 cell polarizing conditions for 0.5 hr and 72 hr. ChIP was performed as previously described (Elo et al., 2010). Cells were subjected to sonication using a Bioruptor sonicator (Diagenode) to obtain chromatin fragments of 100–500 bp. 200 μg fragmented chromatin was incubated with 10 μg STAT3 antibody (9132L, Cell Signaling Technology) and incubated for crosslinking with the magnetic beads (no. 112.04 Dynal Biotech, Invitrogen). After crosslinking, crosslinks were reversed (65°C for 12–16 hr), and precipitated DNA was treated with Proteinase K and RNase A and then purified (QIAquick PCR purification kit, QIAGEN). The DNA libraries were prepared by Fasteris Life Sciences. Sequencing was performed on Illumina HiSeq2000, producing 18.5–27 million reads per sample.

Kinetic qPCR Analysis of Selected ChIP-Seq Targets

Primers were designed for the representative STAT3-binding regions identified by the MACS program (Table S7). Two STAT6-binding regions based on our previous publication were selected as negative controls (Elo et al., 2010). Immunoprecipitated DNA was analyzed by real-time qPCR (7900HT Fast Real-Time PCR System, Applied Biosystems) as described earlier (Elo et al., 2010).

DNA Affinity Precipitation Assay

DAPA experiments were conducted as described in our recent study (Hawkins et al., 2013), with minor modifications. The procedure is described in detail in Supplemental Experimental Procedures. Oligonucleotides containing STAT3-binding sites from ChIP-seq data and STAT3 DNA-binding motif, with or without SNP mutation, were used. Detailed information regarding the sequences is presented in Table S7, where mutations introduced to the oligonucleotides are highlighted in bold.

Statistical Methods

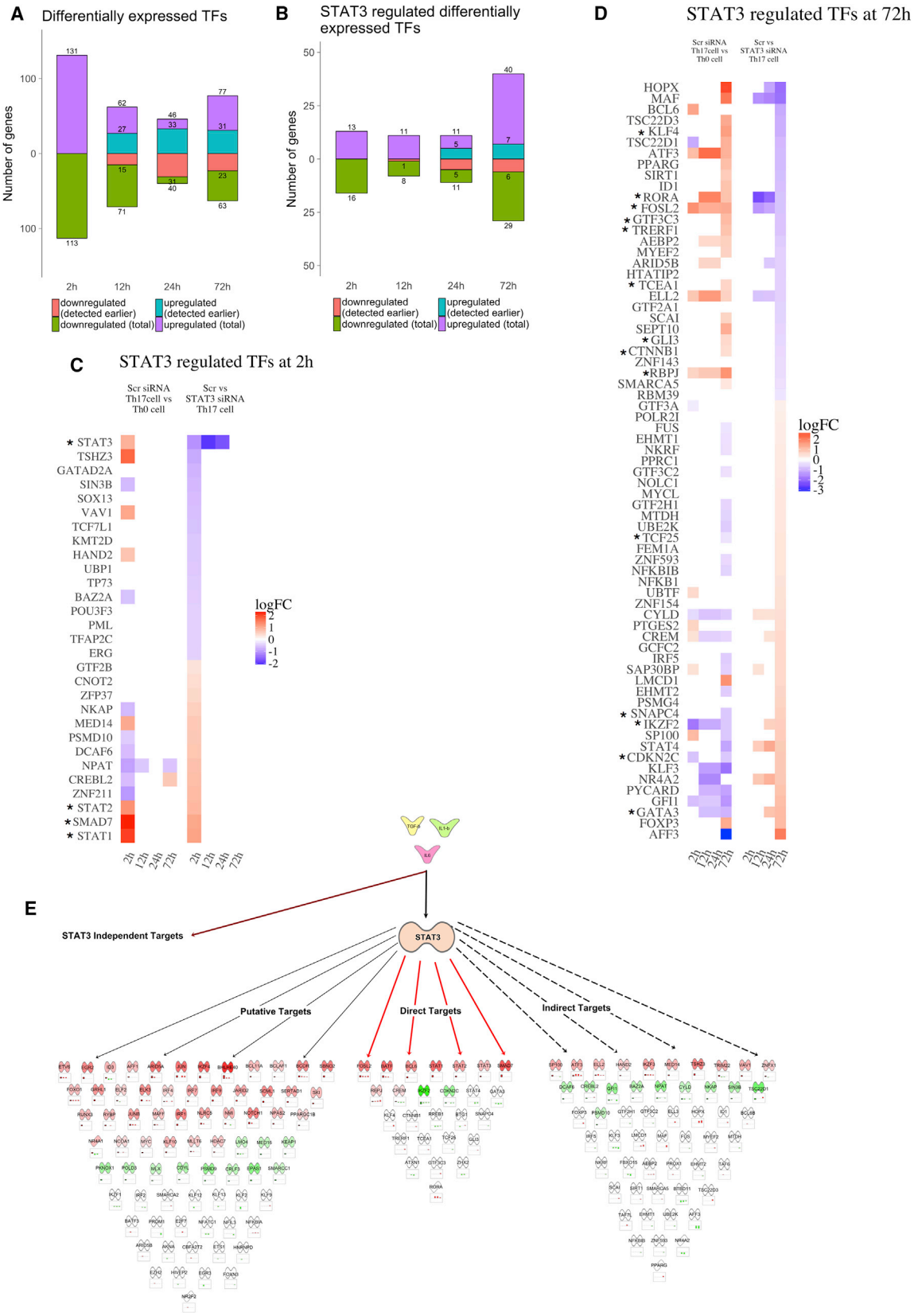
Statistical significance of IL17A secretion after 72 hr of culture was determined from three independent cultures for one sample using a two-tailed Student's t test. The statistical significance of the percentage of cells expressing CCR6 or

Figure 4. Kinetics of STAT3 Occupancy on Its Target Genes

(A) qPCR results of the selected STAT3 ChIP-seq sites measured at 0, 0.5, 2, 12, and 72 hr after induction of Th17 cell polarization. The input value percentage is an average of two to three cultures showing enrichment, with the bars representing the corresponding SEM. The primers and probes used are listed in Table S7. See also Figure S3 for UCSC snapshots.

(B) The genomic distribution (%) of the identified STAT3-binding sites after 72 hr of Th17 cell polarization. STAT3-binding sites were detected by ChIP-seq and determined by using the MACS tool. See Table S2 for complete list of STAT3-binding sites.

(C) Overlay of STAT3-bound genes at 72 hr detected by ChIP-seq with differentially expressed genes and siSTAT3 regulated genes during Th17 cell differentiation detected by microarrays.



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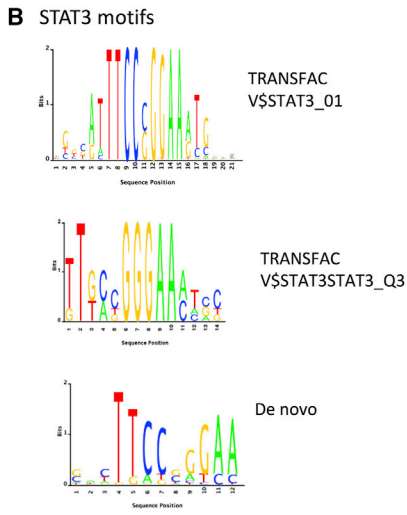
A

Trait	stat3 overlap	pvalue	corrected pvalue
CD	10	0.05	0.20
MS	8	0.09	0.22
PS	6	0.03	0.16
T1D	5	0.07	0.20
UC	3	0.26	0.46
PBC	2	0.27	0.46
SLE	1	0.41	0.62
RA	1	0.57	0.70
CeD	1	0.59	0.70
AS	0	1.00	1.00
IgA	0	1.00	1.00
all	30	0.01	0.11



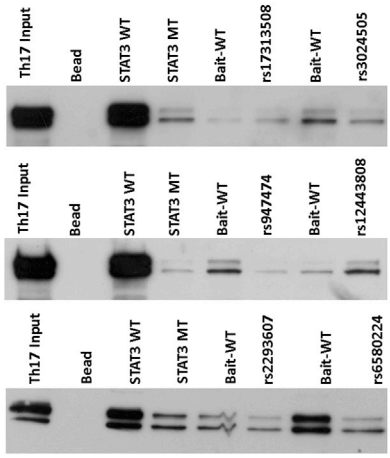


Figure 6. SNPs Associated with Autoimmune Diseases Overlap with STAT3 Targets and Binding Sites

(A) Enrichment of disease-associated SNPs at STAT3 binding sites relative to random sets of background SNPs.

(B) Published and de-novo-derived STAT3 DNA-binding motifs. Known binding motifs were taken from TRANSFAC (Wingender et al., 2000); de novo motifs were identified from the STAT3 ChIP-seq binding sites using Homer software (Heinz et al., 2010).

(C) DAPA experiments were carried out to assess whether disease-associated SNPs can alter STAT3 binding to the binding sites identified with STAT3 ChIP-seq. Double-stranded oligonucleotides containing the predicted STAT3-binding sites at different genomic locations were used as baits. STAT3 binding to the oligonucleotide bait sequences was examined using nuclear extracts from polarized Th17 cells at the 48 hr time point. The oligonucleotide baits containing the SNPs at STAT3-binding sites are shown in Table S7. A DNA sequence with a STAT3-binding site (STAT3 WT) and a negative control DNA sequence (STAT3 MT and an oligonucleotide where 4 nt of the conserved STAT3-binding site has been mutated) were used as controls. STAT3 binding to oligonucleotides was detected by western blotting using STAT3-specific antibodies. Data are representative of two biological replicates (Figure S7B).

CXCR5 after 72 hr of polarization was determined using a two-tailed Student's t test. Statistical analysis for microarray, ChIP-seq, and SNPs in association with STAT3-binding sites is described below.

Identification of STAT3 Targets

Affymetrix microarray data were processed using the robust multi-array average (RMA) algorithm (Irizarry et al., 2003), quantile normalized (Bioconductor Affy package v.1.52), and log2 transformed. Th17 cell-specific genes were identified between the matched Th17 cell and Th0 cell measurements (same time point and culture) in the control-siRNA data using paired and moderated t-statistic (Bioconductor limma package v.3.30.4) by using the batch as a covariate. Genes with an FDR < 0.1 were defined as "changed" for each time point (Benjamini, 1995). The effect of STAT3 knockdown on gene expression was assessed using the Th17 cell measurements from the matched STAT3 siRNA and control siRNA samples using the analysis methods described above.

STAT3 ChIP-Seq Data Analysis

The 50-nt reads were aligned to the human reference genome (hg19) using Bowtie software (Langmead et al., 2009). Only uniquely mapped reads were

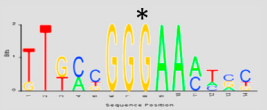
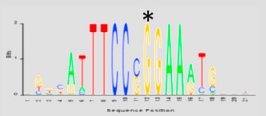
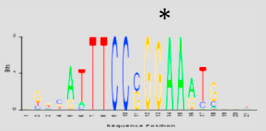
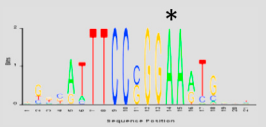
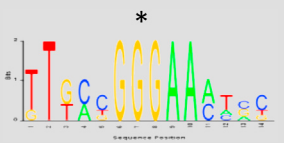
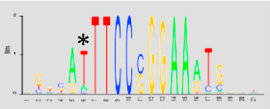
Figure 5. STAT3-Mediated Dynamic Transcription Network

(A and B) Bar charts showing total numbers of (A) differentially expressed TFs and (B) STAT3 regulated TFs during the early stage of Th17 cell polarization. The height of a bar shows the total number of differentially expressed genes detected at a particular time point. The height of the lighter inner part represents those TFs whose differential expression was already detected earlier, whereas the height of the darker outer part represents those TFs whose differential expression is now detected for the first time at a given time point. The expression profiles of these transcriptional regulators are listed in Table S5.

(C and D) Heatmaps of STAT3-regulated TFs at 2 hr (C) or 72 hr (D). STAT3-regulated TFs were identified by comparing gene expression profiles from cells nucleofected by STAT3 siRNA versus Scr, cultured under Th17 cell polarizing conditions (FDR < 0.1). Statistical analysis was performed based on three independent experiments. Statistically significantly changed signal log ratios (SLRs) are shown. The asterisks on gene names indicate that STAT3 bound to these genes by ChIP-seq experiment in this study.

(E) STAT3-dependent and independent transcription circuitries. STAT3-dependent genes were chosen based on their regulation by Th17 cell polarizing cytokines, when compared to the Th0 cell sample (FDR < 0.1), regulation by STAT3 siRNA (FDR < 0.1), or by detected STAT3 binding. STAT3 direct target genes were induced by Th17 polarizing cytokines and regulated by direct DNA binding as well as in STAT3 RNAi experiments. Importantly, STAT3 indirect targets are regulated by STAT3 siRNA, but not bound by STAT3. Putative STAT3 target genes were regulated by Th17 cell polarizing cytokines in the kinetic data (FDR < 0.1) and were bound by STAT3 in ChIP-seq experiments but were not regulated by STAT3 in the RNAi experiments. Although the STAT3-independent genes were not regulated by STAT3-specific siRNA, they were regulated by Th17 cell polarizing cytokines in the control-siRNA experiments (FDR < 0.1). STAT3-independent genes were also expressed differentially when compared between Th17 and Th0 cell sample. The expression of Th17 cell-regulated genes (FDR < 0.1) from 2, 12, 24, and 72 hr are represented as bar charts next to the nodes.

Table 1. List of SNPs Associated with MS, PS, T1D, UC, CeD, and PBC that Overlap Directly within the STAT3 Motif of STAT3-Binding Sites

SNPs	Chromosome	SNP Coordinate	Variant Position	Disease Association	SNP Position at STAT3 Motif (strand/nt)	STAT3 Motif Sequence Logo[a]	Nearest Gene(s)	Gene Function[b]
rs3024505	1	206939904	C-C/T	CD, T1D, UC	+/8*, m+/8*		*IL10*	IL10 is an immunoregulatory cytokine with pleotropic functions. It plays a key role in the control of Th1, and pathogenic function of Th17 cell.
rs947474	10	6390450	G+A/G	T1D	+/12*, –/10*		*PFKFB3, LOC399715, PRKCQ*	PFKFB3 is required for the stimulation of glycolysis in T cells and T helper cell differentiation. PRKCQ enhances Th17 differentiation in a STAT3-dependent manner.
rs17313508	5	35953949	G+A/G	PBC	m+/14*, m–/8*		*IL7R, CAPSL, UGT3A1*	IL-7R is involved in long-term survival of T cells and has been associated with several inflammatory autoimmune diseases as well in cancer. The CASPL gene is located in IL-7R loci, and its function is unknown. UGT3A1 may play an important role in metabolism, and its role in the context of the immune system is understudied.
rs6580224	5	141489306	A+A/G	MS	+/14*, –/8*		*NDFIP1, GNPDA1, SPRY4*	NDFIP1 is induced by TGF-β to induce iTreg differentiation and limit Th2 and Th17 differentiation. The function of GNPDA1 is unknown. SPRY4 is inhibits the receptor-transduced mitogen-activated protein kinase (MAPK) signaling pathway.
rs12443808	16	30996871	C+C/G	PS	m+/6*		*SETD1A, HSD3B7, STX1B*	SETD1A is histone methylatransferase that specifically methylates Lys 4 residue of H3. Thus, it plays a key role in chromatin organization and gene regulation. HSD3B7 is an enzyme with a role in the biosynthesis of bile acid. STX1B has a role in synaptic transmission and has been implicated in several neurologic diseases.
rs2293607	3	169482335	T-A/G	CeD, MS	+/6, m+/6		*TERC, ACTRT3*	TERC is a telomerase RNA component associated with colitis and malignancies. ACTRT3 is a gene of unknown function

Several SNPs (or their proxies in CEU or EUR population) associated with autoimmune diseases overlapping with STAT3 motifs were selected. References for gene function are given in Supplemental Information. CeD, celiac disease.

[a]The STAT3 motif sequence logo derived from TRANSFAC motifs: V$STAT3_Q1 and V$STAT3STAT3_Q3.

[b]References for gene functions are provided in the Supplemental Information.

retained (~16–23 million reads per sample). STAT3-binding sites in Th17 cells relative to input DNA were identified using the MACS tool (Zhang et al., 2008) with default parameter settings. To obtain a consensus set of peaks from replicate experiments, peaks were called for each individual dataset using MACS2 (https://github.com/taoliu/MACS/) and then combined using irreproducible discovery rate (IDR) (Li et al., 2011) following instructions on https://sites.google.com/site/anshulkundaje/projects/idr and selecting the conservative peak set. De novo motifs from the STAT3 ChIP-seq peaks were detected using Homer software (Heinz et al., 2010). The binding sites were annotated in terms of genomic annotations using the "annotatePeaks.pl" script supplied by Homer. For overlapping features, the following priorities were applied: (1) TSS (TSS − 1 kb, TSS + 100 bp), (2) TTS (TTS − 100 bp, TSS + 1kb), (3) coding DNA sequence (CDS) exons, (4) 5′ UTR exons, (5) 3′ UTR exons, (6) introns, and (7) intergenic.

SNP Enrichment Analysis

All GWAS SNPs from 11 autoimmune diseases were extracted from the National Human Genome Research Institute (NHGRI) GWAS catalog as per January 2013 (Hindorff et al., 2009). SNPs associated with the white population were selected, and SNPs associated by meta-analysis of two diseases were excluded. Proxies within a distance limit of 500 kb and with $r^2 > 0.8$ were identified based on the CEU population from the 1000 Genomes Project using SNAP (Johnson et al., 2008) (Figure 6A; Table S6) and then searched for STAT3-binding sites containing these SNPs.

In order to test if there is enrichment of STAT3 sites, all traits were analyzed separately. We first excluded the lead SNPs located in the HLA region (chromosome 6, 25–35 Mb). If any SNPs were correlated (physical distance = 1,000 kb and LD $r^2 = 0.8$), we kept only one of the correlated SNPs. We then used SNPsnap (Pers et al., 2015) with the default parameters (except physical distance = 1,000 kb and LD buddies ±20% with $r^2 = 0.8$) to randomly sample 1,000 SNP sets with the same size as the original filtered SNP set. For both disease and background lead SNPs, proxies were generated from the 1000 Genomes EUR population biallelic variants using plink 1.9 (Chang et al., 2015) by selecting SNPs in 1,000-kbp window with $r^2 = 0.8$. The extended proxy SNPs (including the lead SNPs) were intersected with the STAT3 sites. p values were calculated based on these empirical background distributions and corrected for multiple testing using the Benjamini-Hochberg method (Benjamini, 1995).

To study enrichments with a larger set of diseases, genes were associated with a disease if an SNP associated with the disease as reported in the NHGRI GWAS catalog was within ±100 kbp of the gene. The focus was on genes that were regulated by STAT3 (absolute log2 fold-change >1.2 in at least one time point) during Th17 cell differentiation. Only diseases with more than one associated gene were included. Enrichment of diseases among STAT3 regulated genes were obtained by the binomial test and p values were corrected for multiple testing using the Benjamini-Hochberg method.

STAT3 motif locations within ChIP-seq peaks were detected by motif scanning using a uniform 0^{th} order background model and p value threshold of 0.001 (estimated by sampling 10,000 random sequences from the genome with a length equal to the mean of STAT3-binding site lengths). If more than one peak was found, the strongest peak was selected.

ACCESSION NUMBERS

The accession numbers for the microarray and ChIP-seq data reported in this paper are GEO: GSE67183, GSE67185, and GSE96912.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and seven tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2017.05.013.

AUTHOR CONTRIBUTIONS

S.K.T. designed, performed the experiments, analyzed data, prepared figures, and wrote the manuscript. Z.C. designed and performed the experiments, provided expertise, analyzed data, prepared figures, and wrote the manuscript. A.L., K.K., K.N., and T.Ä. analyzed data, prepared figures, and wrote parts of the manuscript. I.R.-P. analyzed data for the SNP study. B.H. contributed to SNP analysis. S.T. provided expertise. E.L. performed eQTL analysis. V.S. performed cell cultures. C.W. and V.K. provided expertise and guidance. H.L. provided expertise and guidance and supervised A.L., K.K., K.N., T.Ä., and E.L. R.L. designed and supervised the study and wrote the manuscript.

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