Identification of STAT5A and STAT5B Target Genes in Human T Cells

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Abstract

Signal transducer and activator of transcription (STAT) comprises a family of universal transcription factors that help cells sense and respond to environmental signals. STAT5 refers to two highly related proteins, STAT5A and STAT5B, with critical function: their complete deficiency is lethal in mice; in humans, STAT5B deficiency alone leads to endocrine and immunological problems, while STAT5A deficiency has not been reported. STAT5A and STAT5B show peptide sequence similarities greater than 90%, but subtle structural differences suggest possible non-redundant roles in gene regulation. However, these roles remain unclear in humans. We applied chromatin immunoprecipitation followed by DNA sequencing using human CD4+ T cells to detect candidate genes regulated by STAT5A and/or STAT5B, and quantitative-PCR in STAT5A or STAT5B knock-down (KD) human CD4+ T cells to validate the findings. Our data show STAT5A and STAT5B play redundant roles in cell proliferation and apoptosis via SGK1 interaction. Interestingly, we found a novel, unique role for STAT5A in binding to genes involved in neural development and function (NDRG1, DNAJC6, and SSH2), while STAT5B appears to play a distinct role in T cell development and function via DOCK8, SNX9, FOXP3 and IL2RA binding. Our results also suggest that one or more co-activators for STAT5A and/or STAT5B may play important roles in establishing different binding abilities and gene regulation behaviors. The new identification of these genes regulated by STAT5A and/or STAT5B has major implications for understanding the pathophysiology of cancer progression, neural disorders, and immune abnormalities.

Introduction

Signal transducer and activator of transcription (STAT) comprises a family of universal transcription factors, playing important roles in regulating gene expression in multiple cell types. STAT1 through 6 are essential for transduction of extracellular signals into the cells. STAT5, in particular, plays critical roles in the cellular response to various cytokines and hormones and therefore is crucial to regulation of immune and nervous system functions, as well as cell proliferation and growth, in both humans and rodents [1,2]. Following cytokine stimulation, the STAT5 protein is rapidly tyrosine phosphorylated, allowing dimerization and translocation to the nucleus, where it binds regulatory regions of target genes [3].

STAT5 encompasses two highly related proteins, STAT5A and STAT5B in humans (Stat5a and Stat5b in rodents). STAT5A and STAT5B show peptide sequence similarities of more than 90%, differing only by 6 amino acids in their DNA binding domains, 20 amino acids in their C-termini [4], and 18 amino acids in their N-termini [5]. These structural differences may result in non-redundant roles for each protein, resulting in unique gene regulation profiles [4,6]; however this has yet to be clarified in humans.

Previous studies in mice have demonstrated both redundant and non-redundant roles for Stat5a and Stat5b in immune regulation and development. Both Stat5a and Stat5b were essential for normal lymphoid development, and function as critical signal mediators for CD8+ T cell homeostasis [7,8]. Deficiency of only Stat5a resulted in impaired prolactin-dependent mammary cell differentiation [9], whereas deficiency of Stat5b alone resulted in impaired growth [10]. At the same time, human studies suggest differences between human and mouse STAT5-mediated gene regulation that must be taken into consideration. In humans, both male and female patients carrying mutated STAT5B, but with normal levels of STAT5A, have similar growth defects (i.e., there is no sexual dimorphism of body growth rates as has been observed in Stat5b-/- mice). Moreover, deficiency in both Stat5a and Stat5b murine proteins is required to generate the growth defect observed in human STAT5B-/- patients [11]. In addition, another study demonstrated different binding abilities for human IL4RA between STAT5A and STAT5B, with chromatin immunoprecipitation (ChIP) followed by sequencing (ChIP-seq), although no such difference was observed between Stat5a and Stat5b in mice [12]. Therefore, the data collected thus far on human STAT5B-/- do not completely recapitulate the immune data reported in Stat5b-/- mouse models, and suggest there are

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unique roles for STAT5A and STAT5B in human immune modulation. It is therefore crucial to analyze human samples to elucidate the redundant and non-redundant roles of STAT5A and STAT5B in human gene regulation [13].

We reported that STAT5B deficient patients show severe growth hormone-resistant growth failure despite the presence of normal growth hormone receptor [14], reduced number of natural killer cells and T cells [14,15], impairment of IL-2 signaling, and decreased regulatory T cell [Treg] number [11]; all these features exist in the presence of normal STAT5A expression. Additionally, we have reported that in humans, the anti-apoptotic factor BCL2L1 is specifically regulated by STAT5A, whereas FOXP3 and IL-2RA expression are specifically regulated by STAT5B [16].

To identify STAT5A and STAT5B target genes, we performed genome-wide ChIP-seq in human CD4+ T cells, which are known to express STAT5 upon activation and can be easily expanded [17]. Genes detected by STAT5A and/or STAT5B ChIP-seq were further validated via quantitative RT-PCR (qRT-PCR) using siRNA-mediated STAT5A or STAT5B KD human CD4+ T cells.

To the best of our knowledge, this is the first report to show redundant and non-redundant roles of STAT5A and STAT5B in gene regulation in human CD4+ T cells.

**Methods**

This study was approved by the Stanford Administrative Panel on Human Subjects in Medical Research Institutional Review Board.

**Samples and Cell Isolation**

Whole blood samples from healthy adults were obtained from the Stanford Blood Center. CD4+ T cells from healthy adults were isolated using RosetteSep® Human CD4+ T Cell Enrichment Cocktail (StemCell Technologies, Canada) according to the manufacturer’s instructions. The purity of the sorted cells, as assessed by flow cytometry, was found to be greater than 92%.

**Cell culture**

Cells were cultured in RPMI 1640 medium (Gibco®, USA) supplemented with 10% fetal bovine serum and 100 U/mL of antimicrobial agent (Antibiotic-Antimycotic, Gibco®, USA) with PHA-P (5 μg/mL) in a 75 mL flask, and incubated at 37°C in a humidified 5% CO2 atmosphere for 3 days. Recombinant human IL-2 (rhIL-2, 100 U/mL) was then added to cultures for either 30 min or 3 days.

**Immunofluorescence staining**

CD4+ T cells were fixed on a glass slide with 100 μL cold Phosflow Fix Buffer 1 (Becton Dickinson, USA). The cells were permeabilized with 100 μL of Perm/Wash Buffer (Becton Dickinson), blocked with 10% normal donkey serum and 0.3 M glycine in phosphate buffer saline with 0.1% Tween 20, and incubated with anti-STAT5A Ab (sc-1081, Santa Cruz Biotechnology) or Alexa Fluor 594 donkey anti-rabbit IgG (Invitrogen) or Alexa Fluor® 488 donkey anti-mouse IgG (Invitrogen) was used at dilution of 1:1,000 for secondary Ab. The specificity of each Ab were shown in Fig. S1. Hoechst 33342, trihydrochloride, trihydrate (Invitrogen) was used at dilution of 1:10,000 for the nuclear stain.

**Immunoblotting and immunoprecipitation**

Cytoplasmic or nuclear proteins from CD4+ T cells were extracted using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, USA). Lysates were run on a 10% polyacrylamide gel (Bio-Rad, USA) for 30 min at 200 V, transferred to a nitrocellulose membrane (Bio-Rad) for 1 h at 100 V, blocked in 5% milk for 30 min. Proteins were detected by a 1:1,000 dilution of anti-STAT5A Ab, anti-STAT5B Ab or anti-Phospho STAT5 Ab (Cell Signaling Technology, USA) administered for 18 hours, followed by incubation with anti-rabbit or mouse IgG, HRP-linked Ab (Cell Signaling Technology) at 1:5,000 for 30 min at room temperature. An enhanced chemiluminescent substrate for detection of HRP (Thermo Scientific) was used for visualization.

Nuclear proteins separated from CD4+ T cells were also used for immunoprecipitation using anti-STAT5A Ab or anti-STAT5B Ab with the Protein A/G PLUS-agarose (Santa Cruz Biotechnology, USA) according to manufacturer’s instructions.

**ChIP-seq**

CD4+ T cells (2.0×10^7 cells) stimulated by PHA-P for 3 days followed by incubation with rhIL-2 for either 30 min or 3 days were cross-linked with formaldehyde (final concentration, 1%) for 10 min. The reaction was quenched with glycine and cell lysates were sonicated (12 rounds of 20 sec, Sonifier® S-250A, Branson Ultrasonic) and immunoprecipitated with anti-STAT5A Ab (12 μg), anti-STAT5B Ab (12 μg) or anti-IgG Ab (12 μg, sc-2025, Santa Cruz Biotechnology) for 18 hours. After washing with tris-ethylene diamine tetra acetic acid including of 1% of sulfuric acid dodecyl buffer, to reverse the cross-link, the immunoprecipitated DNA and control DNA samples were prepared using previously published methods [16]. Adaptor-ligand DNA fragments were size-fractionated in 2% agarose gel (E-Gel, Invitrogen), and the 150–350 bp fraction was recovered. Each DNA fragment obtained was amplified by 15 cycles of PCR (PCR primer 1.1 and 2.1, Illumina®). Each ChIP DNA library (3.5 pM) was denatured and loaded onto the Illumina Flow Cell using the single-read cluster plate kit (v2, Illumina®) on the cBot (Illumina® for cluster generation and sequenced using the Illumina Genome Analyzer (GAIIx, Illumina®) over 36 cycles with related SBS sequencing Reagents (v5, Illumina®). FASTQ files were generated from readings passing quality filters for further ChIP-data analysis.

**Data analysis for ChIP-seq, motif analysis, and the detection of candidate genes**

Short-read sequences were aligned to human genome sequences (hg19 from UCSC Genome Browser; http://genome.ucsc.edu/) using the DNAexos program (https://dnanexus.com/). Peak-calling was based on the QuEST algorithm. We set a strict threshold for the analysis to improve data quality. Peaks with more than a 3-fold increase in signal intensity versus input DNA and with more than 30-fold increase in read count in sample compared to a uniform distribution of the same sample reads across the genome were detected. Within these, peaks with a q-value<10^{-10} (i.e. the q-score in −log_{10}(q-score) is greater than 10) were accepted as statistically significant.

For motif analysis, binding site sequences were confirmed against consensus motif sequences using TRANSFAC® (BIOBASE, Germany).

Genes were identified as candidate genes for regulation by STAT5A and/or STAT5B if the gene existed within 10,000 bp from each detected binding site. Candidate genes were defined as “specifically detected genes” if they were detected by only anti-
STAT5A Ab ChIP-seq (STAT5A ChIP-seq) or only anti-STAT5B Ab ChIP-seq (STAT5B ChIP-seq). Genes were defined as “dominantly detected genes” if there was more than a 2-fold difference in q-score between STAT5A ChIP-seq results and STAT5B ChIP-seq results. Genes were regarded as “equally detected genes” if q-scores differed by less than 2-fold between STAT5A ChIP-seq results and STAT5B ChIP-seq results, or were equivalent.

Quantitative PCR
RNA was isolated from 2.0×10^5 CD4^+ T cells transfected with STAT5A, STAT5B, or control siRNA via RNaseq kits (Qiagen, Germany) according to the manufacturer’s protocol. KD cells were prepared using previously published methods [16]. For cDNA synthesis, 500 ng of total RNA were transcribed with cDNA transcription reagents (Applied Biosystems, USA) using random hexamers, according to the manufacturer’s protocol. Gene expression was measured in real time using primers

(Invitrogen, Applied Biosystems). The expression levels of each gene were adjusted with β-glucuronidase as an internal control, and were compared with expression levels detected in the control siRNA samples. In addition, ΔΔCq (Cq for quantification cycle) method was used as a normalized determination of genes that were knocked down in the siRNA experimental controls. The data are shown as a ratio relative to each control sample; the levels of each control sample are indicated as 1. All PCR assays were performed with Sybr-Green-based technology (Sigma-Aldrich). QT-PCR results were analyzed via paired t-test using GraphPad Prism 6 (GraphPad Software, Inc.). P<0.05 was set as the threshold for statistical significance. “Specifically regulated gene” is biologically defined in this article as a gene that is transcriptionally-controlled by STAT5A but not STAT5B, and by STAT5B but not STAT5A. “Specifically regulated gene” is statistically defined here as if P<0.01 in only STAT5A KD or STAT5B KD CD4^+ T cells compared to the control CD4^+ T cells.

Figure 1. Localization of STAT5A and STAT5B, and monomers and dimers of STAT5A and STAT5B. A demonstrates translocation of STAT5A and STAT5B into cell nuclei after 30 min stimulation with rhl-2 (40× confocal). Yellow, STAT5A; purple, STAT5B; blue, nucleus. B and C. Detection of STAT5A and STAT5B proteins in cytoplasmic or nuclear proteins fractionated from CD4^+ T cells after PHA-P stimulation for 3 days followed by incubation with rhl-2 for 30 min. B. STAT5A monomer (91 kDa, arrow 1) and STAT5A dimer (arrow 2) in native cytoplasmic or nuclear proteins, detected using anti-STAT5A Ab. C. STAT5B monomer (90 kDa, arrow 3) and STAT5B dimer (arrow 4) in native cytoplasmic or nuclear proteins, detected using anti-STAT5B Ab. D. Detection of phosphorylated STAT5 proteins in STAT5A- or STAT5B- immunoprecipitated nuclear proteins fractionated from CD4^+ T cells after PHA-P stimulation for 3 days followed by incubation with rhl-2 for 0 min, 30 min or 3 days. E. Detection of phosphorylated STAT5 proteins in cytoplasmic or nuclear proteins fractionated from CD4^+ T cells after PHA-P stimulation for 3 days followed by incubation with rhl-2 for 3 days, and control (unstimulated condition).

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Figure 2. Binding ability, motif sequences and binding sites. A shows binding ability of STAT5A after 3 days of exposure to rhIL-2 versus binding ability of STAT5A after 30 min of exposure to rhIL-2. It shows results of STAT5A ChIP-seq on chromosome 18 performed in CD4⁺ T cells incubated with rhIL-2 for 3 days (top); in CD4⁺ cells incubated with rhIL-2 for 30 min (middle); compared with control ChIP-seq in CD4⁺ T cells.
Results

Localization of STAT5A and STAT5B, and their dimerization

We first determined the kinetics of intracellular localization of STAT5A and STAT5B in CD4\(^+\) T cells at different time points after addition of rhIL-2. We found that nuclear translocation of both STAT5A and STAT5B was detectable as early as 30 min after addition of rhIL-2 (Fig. 1A). STAT5A and STAT5B monomers or dimers were detected in native cytoplasmic or nuclear proteins extracted from CD4\(^+\) T cells after 30 minutes in the presence of rhIL-2 (Fig. 1B, 1C). This time point was therefore chosen for basic condition of ChIP-seq experiment. Additionally, the phosphorylated STAT5 proteins were detected in nuclear or cytoplasmic proteins extracted from CD4\(^+\) T cells after 30 min or 3 days in the presence of rhIL-2 (Fig. 1D, 1E).

ChIP-seq analysis of human CD4\(^+\) T cells detected candidate genes potentially regulated by STAT5A and/or STAT5B

To detect candidate genes potentially regulated by STAT5A and/or STAT5B, we assessed DNA binding patterns via ChIP-seq analysis in human CD4\(^+\) T cells. Following Liao et al’s report of different binding abilities for Il4ra by Stat5a and Stat5b in mouse CD4\(^+\) T cells following different lengths of exposure to rhIL-2 [12], we tested whether different binding abilities of STAT5A and STAT5B in human CD4\(^+\) T cells were dependent on duration of rhIL-2 exposure. We found that when PHA-P activated CD4\(^+\) T cells were cultured in the presence of rhIL-2 for 30 min, STAT5A ChIP-seq detected 245 binding sites, and STAT5B ChIP-seq detected 248 binding sites. When PHA-P activated CD4\(^+\) T cells were exposed to rhIL-2 for 3 days, however, STAT5A ChIP-seq detected 908 binding sites, and STAT5B ChIP-seq detected 1286 binding sites (Fig. 2A). This indicates that longer exposure to rhIL-2 leads to increased changes in gene regulation. We therefore decided to focus our studies on results obtained after 3 days of rhIL-2 exposure.

Candidate genes were divided into 5 groups: genes detected by both STAT5A and STAT5B ChIP-seq (common), 273; genes detected only by STAT5A ChIP-seq (STAT5A-specific), 33; genes detected only by STAT5B ChIP-seq with a greater than 2-fold difference in q-score (STAT5B-dominant), 41; genes detected only by STAT5B ChIP-seq (STAT5B-specific), 187; genes detected by STAT5B ChIP-seq, with a greater than 2-fold difference in q-score (STAT5B-dominant), 146. The genes in each group are listed in Table S1.

Table 1. Detected binding site sequences on candidate genes detected equally by both STAT5A and STAT5B ChIP-seq.

| Gene     | Sequence of Binding Site 1 | Sequence of Binding Site 2 | Sequence of Binding Site 3 |
|----------|---------------------------|---------------------------|---------------------------|
| SGK1     | TTCCAGGAA                 | TTCCCCAGAA                |                           |
| GTF2H5   | TTCCAAAGA                 |                           |                           |
| BCL2L1   | TTCTAAGA                  |                           |                           |
| SLC22A5  | TTCTTAA                   |                           |                           |
| CDKAL1   | TTCTTGGAA                 |                           |                           |
| DNM2     | TTCTCAGAA                 |                           |                           |
| MBP      | TTCTAGGAA                 |                           |                           |
| DUSP5    | TTCTGAAA                  |                           |                           |
| ARL4C    | 235,399,028–235,399,036   |                           |                           |

Additional binding site sequences are listed in Table S1. The genes listed in Table 1 are of greatest interest.

Non-Redundant Roles between STAT5A and 5B

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DNA binding sequences within candidate genes detected via ChIP-seq

To verify the binding sequences detected with ChIP-seq, we compared them to previously established consensus motif sequences for STAT5A and/or STAT5B. 30 of the 36 binding sequences detected by STAT5A and/or STAT5B ChIP-seq were in accordance with consensus motif sequences (Table 1, 2 and 3; Fig. 2B). The remaining 6 binding sequences, despite not aligning with consensus motif sequences, contained the core STAT5 consensus motif sequence “TTC-GAA” [19], TTC- or -GAA.

Several DNA binding sequences were shared between candidate genes (Tables 1, 2 and 3). The sequence “TTCCAGGAA” was detected equally by both STAT5A and STAT5B ChIP-seq in SGK1, as well as dominantly by STAT5A ChIP-seq in ST3GAL1 (Table 1, 2 and 4; Fig. 2C). The sequence “TTCCAGGAA” was detected equally by both STAT5A and STAT5B ChIP-seq in GTF2H5, but dominantly by STAT5B ChIP-seq in TNFSF10 (Table 1, 3 and 4). The sequence “TTCTTGGAA” was detected equally by both STAT5A and STAT5B ChIP-seq in BCL2L1, and detected dominantly by STAT5B ChIP-seq in FOXp3 and IL2RA (Table 1, 3 and 4). The sequence “TTCTTGGAA” was detected equally by both STAT5A and STAT5B ChIP-seq in DNM2 and detected specifically by STAT5A ChIP-seq in NDRG1 (Table 2, 3 and 4). The sequence “TTCTTGGAA” was detected equally by both STAT5A and STAT5B ChIP-seq in MBP, but detected dominantly by STAT5A ChIP-seq in SSH2 and specifically by

**Table 2.** Detected binding site sequences on candidate genes detected specifically or dominantly by STAT5A ChIP-seq.

| Gene     | Sequence of Binding Site 1 | Gene     | Sequence of Binding Site 1 | Sequence of Binding Site 2 (or 4) | Sequence of Binding Site 3 |
|----------|-----------------------------|----------|-----------------------------|----------------------------------|-----------------------------|
| NDRG1    | TTCCTGGAA                   | DNAJC6   | TTCCTGGAA                   | TTCTCTAGAA                       | TTCTCTAGAA                  |
| CBS      | Chr 21: 44,665,709–44,665,717|          |                             |                                  |                              |
| PPP2R2B  |                         | ST3GAL1  |                         |                                  |                              |
| SAMD4A   |                         | SSH2     |                         |                                  |                              |
| MAP3K5   |                         |          |                         |                                  |                              |

**Abbreviation:** sp, specifically detected; dom, dominantly detected.

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**Table 3.** Detected binding site sequences on candidate genes detected specifically or dominantly with STAT5B ChIP-seq.

| Gene     | Sequence of Binding Site 1 (or 4) | Sequence of Binding Site 2 (or 5) | Sequence of Binding Site 3 |
|----------|----------------------------------|----------------------------------|-----------------------------|
| DOKX8    | TTCCTAGAA                        |                                  | TTCTCTAGAA                  |
| SNX9     |                                 |                                  | TTCTCTAGAA                  |
| LNPEP    |                                 |                                  | TTCTCTAGAA                  |
| SKAP1    |                                 |                                  | TTCTCTAGAA                  |
| PTGER1   |                                 |                                  | TTCTCTAGAA                  |
| DIDO1    |                                 |                                  | TTCTCTAGAA                  |
| TNFSF10  |                                 |                                  | TTCTCTAGAA                  |
| FOXp3    |                                 |                                  | TTCTCTAGAA                  |
| IL2RA    |                                 |                                  | TTCTCTAGAA                  |
| UGCC     |                                 |                                  | TTCTCTAGAA                  |

**Abbreviation:** sp, specifically detected; dom, dominantly detected.

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STAT5B ChIP-seq in LNPEP. The sequence “TTCCTAGAA” was detected specifically by STAT5A ChIP-seq on DNAJC6, and was also detected specifically by STAT5B ChIP-seq on DOCK8 (Table 2 and 3; Fig. 2D and 2E).

Because ChIP-seq detected multiple binding sites for SGK1, MBP, and DUSP5, we evaluated whether each binding site was equally detected by STAT5A and STAT5B ChIP-seq, and found this to be the case (Table 4).

Similarly, because there were multiple binding sites for both FOXP3 and IL2RA, we evaluated whether each binding site was detected specifically or dominantly by STAT5A ChIP-seq, and found this to be the case (Table 4).

Validation of candidate genes by QT-PCR in STAT5A or STAT5B KD human CD4+ T cells

To confirm whether the candidate genes were regulated by STAT5A and/or STAT5B, we measured expression levels of each gene (listed in Table 1, 2 and 3) via QT-PCR in STAT5A or STAT5B KD human CD4+ T cells. When compared with control siRNA-transfected CD4+ T cells, STAT5A siRNA-transfected human CD4+ T cells showed a 45% reduction in STAT5A gene expression as compared to control after 3 days (P<0.001); STAT5B siRNA-transfected human CD4+ T cells showed a 58% reduction in STAT5B gene expression as compared to control after 3 days (P<0.001) [16]. There was no significant difference in KD ratio between STAT5A and STAT5B (P = 0.20).
Expression levels of SGK1, GTF2H5 and SLC22A5 decreased similarly in STAT5A and STAT5B KD CD4+ T cells versus control CD4+ T cells, whereas no significant changes in expression levels of CDKAL1, DNME2, DUSP5, MBP and ARL4C were seen between KDs and control CD4+ T cells (Fig. 3). Expression levels of NDRG1, DNACJ6, ST3GAL1, SAMD4A, SSH2, MAP2K5 and BCL2L1 were specifically decreased in STAT5A KD CD4+ T cells versus control CD4+ T cells (i.e., P<0.01 in only STAT5A KD CD4+ T cells, Fig. 3). There were no significant differences in the expression levels of these genes between STAT5B KD CD4+ T cells and control CD4+ T cells. Expression levels of DOCK8, SYNX9, SKAP1, TNFSF10, FOXP3, IL2RA, and UGCG were specifically decreased in STAT5B KD CD4+ T cells versus control CD4+ T cells (i.e., P<0.01 in only STAT5B KD CD4+ T cells, Fig. 3). There were no significant differences in the expression levels of these genes between STAT5A KD CD4+ T cells and control CD4+ T cells. Finally, no significant changes were seen in the expression levels of CBS, PPP2R2B, LNPEP, PTGER1 and DIDO1 in either STAT5A or STAT5B KD CD4+ T cells versus control CD4+ T cells.

As was seen with ChIP-seq, genes sharing binding sequences did not always display identical patterns of expression in STAT5A and STAT5B KD CD4+ T cells. Genes sharing binding sequences but with differing behaviors were: SGK1 (decreased similarly in both KDs) and ST3GAL1 (decreased specifically in STAT5A KD, no change in STAT5B KD); GTF2H5 (decreased similarly in both KDs) and TNFSF10 (decreased specifically in STAT5B KD, no change in STAT5A KD); BCL2L1 (decreased specifically in STAT5A KD, no change in STAT5B KD); FOXP3 and IL2RA (decreased specifically in STAT5B KD, no change in STAT5A KD); CDKAL1 (no change in both KDs) and SAMD4A (decreased specifically in STAT5A KD, no change in STAT5B KD); DNME2

Table 5. Genes associated with regulation by STAT5A and STAT5B by QT-PCR.

| Gene       | Full name                                      | Location | Roles                                                                 |
|------------|-----------------------------------------------|----------|-----------------------------------------------------------------------|
| SGK1       | serum/glucocorticoid regulated kinase 1       | 6q23     | Activation of certain potassium, sodium and chloride channels and regulation of inflammatory cell proliferation and apoptosis [39]. |
| GTF2H5     | general transcription factor IIH, polypeptide 5 | 6q25.3   | Encodes a subunit of transcription/repair factor TFIIH, which functions in gene transcription and DNA repair. Mutations in this gene cause DNA repair-deficient trichothiodystrophy [40]. |
| SLC22A5    | solute carrier family 22 (organic cation/carnitine transporter), member 5 | 5q23.3   | Involved in the active cellular uptake of carnitine. Mutations of this gene cause systemic primary carnitine deficiency [41]. |

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(no change in both KDs) and NDRG1 (decreased specifically in STAT5A KD, no change in STAT5B KD); MBP and LNPEP (no change in both KDs) and SSH2 (decreased specifically in STAT5A KD, no change in STAT5B KD); and DNAJC6 (decreased specifically in STAT5A KD, no change in STAT5B KD) and DOCK8 (decreased specifically in STAT5B KD, no change in STAT5A KD).

Known roles of the genes are summarized in Table 5, 6 and 7.

**Discussion**

This study is the first to demonstrate, to our knowledge, redundant and non-redundant roles for STAT5A and STAT5B in human gene regulation. Genome-wide identification of STAT5A and STAT5B target genes in human CD4+ T cells yielded a number of candidate genes, some of which appeared to be regulated by both STAT5A and STAT5B, while others were associated with regulation by either STAT5A or STAT5B. ChIP-seq results were further tested for validity by QT-PCR in STAT5A or STAT5B KD CD4+ T cells, which identified several genes whose expression was regulated by STAT5A and/or STAT5B. Both STAT5A and STAT5B regulated genes implicated in cell proliferation and apoptosis, gene transcription and DNA repair, and the active cellular uptake of carnitine. STAT5A was associated with non-redundant (i.e., not shared with STAT5B) roles in regulating genes relevant to neurite extension, synaptic vesicle recycling, anti-apoptosis, hypoxic stress, and androgen response; STAT5B was linked to non-redundant (i.e., not shared with STAT5A) roles in regulating genes important to IgE production, peripheral CD8+ T cell survival and function, Treg development and function, and T cell activation and proliferation. Overall, STAT5B was therefore highly associated with regulation of genes associated with T cell-specific functions, while STAT5A appeared to interact with genes known to play major roles in neural development and function.

It has been suggested that structural differences between STAT5A and STAT5B DNA-binding domains, C-termini (which contain transactivation domains), and N-termini (which mediate oligomerization) may result in non-redundant roles in each gene’s regulation. Our results supported this hypothesis, showing different binding behaviors between STAT5A and STAT5B to genes with differing binding sites: for example, MAP3K5 (STAT5A ChIP-seq, STAT5A-dominant; QT-PCR, regulated specifically by STAT5A), AXIN2 (STAT5B ChIP-seq, STAT5B-specific; QT-PCR, regulated specifically by STAT5B), and UGCG (STAT5B

| **Table 6.** Genes associated with regulation by STAT5A by QT-PCR. |
| Gene | Full name | Location | Roles |
|------|-----------|----------|-------|
| NDRG1 | N-myc downstream regulated 1 | 8q24.3 | Involved in hypoxic stress and androgen hormone response, cell growth and differentiation, and apoptosis [42]. NDRG1 deficiency causes Schwann cell dysfunction and is a cause of Charcot-Marie-Tooth disease type 4D, which is characterized by motor and sensory nerve dysfunction [43]. |
| DNAJC6 | DNAJ (Hsp40) homolog, subfamily C, member 6 | 1p31.3 | Involved in recycling of synaptic vesicles in neurons [44]. |
| ST3GAL1 | ST3 beta-galactoside alpha-2,3- sialyltransferase 1 | 8q24.22 | When inactivated, renders CD8+, but not CD4+, T cells susceptible to apoptosis [45]. |
| SAMD4A | sterile alpha motif domain containing 4A | 1q22.2 | Expressed during synaptogenensis; modulates synapse formation [46]. |
| SSH2 | slingshot protein phosphatase 2 | 17q11.2 | Critical for neurite extension through actin dynamics [47]. |
| MAP3K5 | mitogen-activated protein kinase kinase 5 | 6q22.33 | Contributes to apoptosis of plasma cells [48]. |
| BCL2L1 | BCL2-like 1 | 20q11.21 | Prevents apoptosis [49]. |

| **Table 7.** Genes associated with regulation by STAT5B by QT-PCR. |
| Gene | Full name | Location | Roles |
|------|-----------|----------|-------|
| DOCK8 | dedicator of cytokinesis 8 | 9p24.3 | Critical and intrinsic to peripheral CD8+ T cell survival and function [32]. Mutations in this gene result in the autosomal recessive form of hyper-IgE syndrome [33]. |
| SNX9 | sorting nexin 9 | 6q25.1-q26 | Subunit of WASPs (Wiskott-Aldrich syndrome protein)/SNX9/p85/CD28, which enables signal transduction pathway required for CD28-mediated T cell costimulation [35]. |
| SKAP1 | src kinase associated phosphoprotein 1 | 17q21.32 | SKAP-55 regulates integrin-mediated adhesion and conjugate formation between T cells and antigen-presenting cells [50]. |
| TNFSF10 | tumor necrosis factor (ligand) superfamily, member 10 | 3q26 | This protein is a member of TNF family of cytokines, which are structurally related proteins playing important roles in regulating cell death, immune response, and inflammation [51]. |
| FOXP3 | forkhead box P3 | Xp11.23 | Crucial for Treg development and function. Defects in this gene cause immunodeficiency polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) [52]. |
| IL2RA | interleukin 2 receptor, alpha | 10p15-p14 | Constitutes the alpha chain of the high-affinity IL2 receptor [53]. |
| UGCG | UDP-glucose ceramide glucosyltransferase | 9q31 | When silenced, leads to p53-dependent apoptosis [54]. Prolonged overabundance of glucosylceramide is detrimental, as is seen in Gaucher disease [55]. |

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ChIP-seq, STAT5B-dominant; QT-PCR, regulated specifically by STAT5B.

At the same time, we found that binding site sequences alone could not predict STAT5A versus STAT5B binding behavior, observing several gene-specific, but not sequence-specific, differences in STAT5A and STAT5B binding. For example, the same binding site sequence was detected within the gene SGK1 by both STAT5A and STAT5B ChIP-seq, but was dominantly detected by STAT5A ChIP-seq in ST3GAL1. After eliminating experiment-specific limitations such as differential affinity between anti-STAT5A and anti-STAT5B Abs, this discrepancy suggests that co-activators may also be important in establishing differential binding and transcriptional activities between STAT5A and STAT5B. This is supported by previous findings that Crkl, a STAT5B- and Stat5b-binding co-activator, significantly increases STAT5B and Stat5b binding to their target genes in humans and mice, respectively [20,21], and that centrosomal P4.1-associated protein augments STAT5 mediated transcriptional activity in humans [22], though this has not been reported in rodents.

Alternatively, the following mechanisms have been proposed for STAT protein regulation of target genes: (1) binding to DNA binding sites to directly drive transcription; (2) forming transcriptional complexes with non-STAT transcription factors to trigger transcription through a STAT; (3) interaction with non-STAT DNA binding sites; and (4) cooperation between STATs and non-STAT transcription factors to activate transcription via binding to clustered independent DNA binding sites [23].

It is also possible that the identified binding sites are not linked to the assigned candidate genes but are regulating further distant genes, as a binding site for STAT5 was found over 10,000 bp away from the target gene [6]. Additionally, the magnitude of opportunistic genomic STAT5 binding does not necessarily translate into transcriptional activation of neighboring genes. For example, Zhu et al. demonstrated that STAT5 binding to promoter upstream sequences does not automatically convey STAT5 control over those genes [20,21], and that centrosomal P4.1-associated protein augments STAT5-mediated transcriptional activity in humans [22], though this has not been reported in rodents.

We found some genes to be specifically associated with regulation by STAT5B, and not STAT5A (i.e. DOCK8, SNX9, FOXP3 and IL2RA). These genes may provide explanations for the phenotype of STAT5B-deficient patients (Table 8). For example, DOCK8 is critical to IgE production and to the survival and function of peripheral CD8+ T cells in humans and mice [32]. DOCK8 deficiency results in autosomal recessive hyper-IgE syndrome with severe allergic manifestations, characterized by severe eczema, recurrent skin infection, mucocutaneous candidiasis, elevated serum IgE levels, and eosinophilia [33]. These characteristics align with previous reports from our group of increased serum IgE levels, decreased CD8+ T cell number, and severe eczema in STAT5B-deficient patients [11,34], and suggest these characteristics of STAT5B deficiency may be related to downregulation of DOCK8 in the absence of STAT5B. SNX9 (sorting nexin 9) encodes subunits of WASp (Wiskott-Aldrich syndrome protein)/SNX9/p85/CD28, a complex involved in signal transduction of CD28-mediated T cell co-stimulation [35]. SNX9 suppression may be a potential cause of impaired function and decreased number of T cells in STAT5B-deficient humans and mice.

Our results also suggest differential gene expression of STAT5A and STAT5B occurs in human vs. mouse. For example, in humans, IL2RA is controlled only by STAT5B, not STAT5A; however, murine IL-2γ is regulated by both Stat5a and Stat5b [36]. Furthermore, STAT5B-deficient patients show normal STAT5A expression and the phenotype similar to that of IL2RA-deficient patients [14,15,37,38].

In conclusion, our data demonstrate potential redundant and non-redundant roles for STAT5A and STAT5B in human gene regulation, highlighting novel specific interactions that could not have been deduced merely from available data in the murine system. Differences between STAT5A and STAT5B DNA binding domains, C-termini, and N-termini were not always

**Table 8. Phenotypic characteristics of STAT5B-deficient patients and potentially related genes, as detected via ChIP-seq and QT-PCR.**

| Phenotypic characteristic | Potentially related gene(s) |
|--------------------------|-----------------------------|
| Serum IgE level elevation | DOCK8                       |
| Normal CD4+/CD8+ T cell ratio (Low CD4+ and CD8+ T cell number) | IL2RA, DOCK8, SNX9 |
| Decreased Treg number    | FOXP3, IL2RA                |
| Low T cell number        | IL2RA, SNX9                 |

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associated with differences in ability of STAT5A and STAT5B to bind their targets, suggesting one or more additional mechanisms may be important for establishing differential binding and transcription behaviors of STAT5A and STAT5B. We found redundant roles for STAT5A and STAT5B with genes associated with cell proliferation and apoptosis, and non-redundant roles with genes associated with neural development and function (STAT3A), and genes implicated in T cell development and function (STAT3B).

The elucidation of the roles of STAT5A and STAT5B furthers our understanding of potential mechanisms in cancer pathophysiology, neurological disorders, and abnormal T cell immune function providing potential new targets to study in these diseases.

References

1. Lin JX, Leonard WJ (2000) The role of Stat5a and Stat5b in signaling by IL-2 family cytokines. Oncogene 19: 2566–2576.
2. Kochman Y, Spokis R, Leonard WJ (2009) New insights into the regulation of T cells by gamma(c) family cytokines. Nat Rev Immunol 9: 480–490.
3. Leonard WJ, O’Shea JJ (1998) Jak and Stats: biological implications. Ann Rev Immunol 16: 293–322.
4. Soldan E, John S, Moro S, Bollenbacher J, Schindler U, et al. (2000) DNA binding site selection of dimeric and tetrameric Stat5 proteins reveals a large repertoire of divergent tetrameric Stat5a binding sites. Mol Cell Biol 20: 389–401.
5. Lin JX, Mier J, Moqi WS, John S, Leonard WJ (1996) Cloning of human Stat3b. Recombination of interleukin-2-induced Stat5a and Stat5b DNA binding activity in COS-7 cells. J Biol Chem 271: 10738–10744.
6. Nelson EA, Walker SR, Li W, Liu XS, Frank DA (2006) Identification of human STAT5-dependent gene regulatory elements based on interspecies homology. J Biol Chem 281: 26216–26224.
7. Yao Z, Cui Y, Watford WT, Bream JH, Yamaoka K, et al. (2006) Stat5a/b are essential for normal lymphoid development and differentiation. Proc Natl Acad Sci U S A 103: 1000–1005.
8. Kelly J, Spokis R, Inadla K, Bollenbacher J, Lee S, et al. (2003) A role for Stat5 in CD8+ T cell homeostasis. J Immunol 170: 210–217.
9. Liu X, Robinson GW, Wagner KU, Garrett L, Wynshaw-Boris A, et al. (1997) Stat3a is mandatory for adult mammary gland development and lactogenesis. Genes Dev 11: 179–186.
10. Udy GB, Towers RP, Snell RG, Wilkins RJ, Park SH, et al. (1997) Requirement for Stat5a and Stat5b in T cell homeostasis and function. J Exp Med 186: 2275–2286.
11. Pathipati P, Gorba T, Berezowska S, Furse GM, Hamagami A, et al. (2011) Growth hormone insensitivity syndrome associated with STAT5b mutation. J Clin Endocrinol Metab 96: 2718–2724.
12. Craddock PS, Cheng WH, Liao D, Zhou J, Cardenas CA, et al. (2006) Impaired cytokine signaling in STAT5A-deficient mice. J Immunol 176: 5021–5026.
13. Kanai T, Jenks JA, Nadeau KC (2012) The STAT5b Pathway Defect and Impaired Crkl expression contributes to the defective DNA binding of Stat5b in nonobese diabetic mice. Metab 55: 734–741.
14. Schulze H, Ballmaier M, Welte K, Gernshemann M (2000) Thrombopoietin induces the generation of distinct Stat1, Stat3, Stat5a and Stat5b homo- and heterodimeric complexes with different kinetics in human platelets. Exp Hematol 28: 294–304.
15. Peng B, Sutherland KD, Sun EY, Oloyajoie M, Wiltin S, et al. (2002) CPAP is a novel stat5-interacting cofactor that augments stat5-mediated transcriptional activity. Mol Endocrinol 16: 2019–2033.
16. Jenks JA, Seki S, Kanai T, Huang J, Morgan AA, et al. (2013) Differentiating the roles of STAT5A and STAT5B Ab and anti-STAT5B Ab by Western blot. (TIF)
17. Kofoed EM, Hwa V, Little B, Woods KA, Buckway CK, et al. (2003) Growth hormone insensitivity associated with a STAT5b mutation. N Engl J Med 349: 1313–1323.
18. Liu X, Robinson GW, Wagner KU, Garrett L, Wynshaw-Boris A, et al. (1997) Stat5b is essential for normal lymphoid development and differentiation. Proc Natl Acad Sci U S A 103: 1288–1296.
19. Grimley PM, Dong F, Rui H (1999) Stat5a and Stat5b: fraternal twins of signal transduction behaviors of STAT5A and STAT5B. We found redundant roles for STAT5A and STAT5B with genes associated with cell proliferation and apoptosis, and non-redundant roles with genes associated with neural development and function (STAT3A), and genes implicated in T cell development and function (STAT3B).

Supporting Information

Figure S1 Validation of anti-STAT5A Ab and anti-STAT5B Ab by Western blot. (TIF)

Table S1 The list of candidate genes detected by STAT5A and/or STAT5B, equally, dominantly and specifically. (DOCX)

Author Contributions

Conceived and designed the experiments: MS RB KN. Performed the experiments: T. Kanai SS JJ. Analyzed the data: T. Kanai. Contributed reagents/materials/analysis tools: T. Kawli DM. Wrote the paper: T. Kanai RB KN AK.
41. Nezu J, Tamai I, Oka A, Ohashi R, Yabuuchi H, et al. (1999) Primary systemic carnitine deficiency is caused by mutations in a gene encoding sodium ion-dependent carnitine transporter. Nat Genet 21: 91–94.

42. Melotte V, Qiu X, Ongenae M, van Criekinge W, de Bruine AP, et al. (2010) The N-myc downstream regulated gene (NDRG) family: diverse functions, multiple applications. FASEB J 24: 4155–4166.

43. Kalaydjieva L, Gresham D, Gooding R, Heather L, Baas F, et al. (2000) N-myc downstream-regulated gene 1 is mutated in hereditary motor and sensory neuropathy-Lom. Am J Hum Genet 67: 47–50.

44. Vauthier V, Jaillard S, Journel H, Dubourg C, Jockers R, et al. (2012) Homozygous deletion of an 80 kb region comprising part of DNAJC6 and LEPR genes on chromosome 1P31.3 is associated with early onset obesity, mental retardation and epilepsy. Mol Genet Metab 106: 345–350.

45. Van Dyken SJ, Green RS, Marth JD (2007) Structural and mechanistic features of protein O glycosylation linked to CD8+ T-cell apoptosis. Mol Cell Biol 27: 1096–1111.

46. Baez MV, Luchelli L, Maschi D, Habib M, Pascual M, et al. (2011) Smaug1 mRNA-silencing foci respond to NMDA and modulate synapse formation. J Cell Biol 195: 1141–1157.

47. Endo M, Ohashi K, Mizuno K (2007) LIM kinase and slingshot are critical for neurite extension. J Biol Chem 282: 13692–13702.

48. Lin FR, Huang SY, Hung KH, Su ST, Chung CH, et al. (2012) ASK1 promotes apoptosis of normal and malignant plasma cells. Blood 120: 1039–1047.

49. Nunez G, Merino R, Grillot D, Gonzalez-Garcia M (1994) Bcl-2 and Bcl-x regulatory switches for lymphoid death and survival. Immunol Today 15: 502–509.

50. Wang H, Moon EY, Azouz A, Wu X, Smith A, et al. (2003) SKAP-55 regulates integrin adhesion and formation of T cell-APC conjugates. Nat Immunol 4: 366–374.

51. Nomura S, Ishii K, Inami N, Uoshima N, Ishida H, et al. (2007) Role of soluble tumor necrosis factor-related apoptosis-inducing ligand concentrations after stem cell transplantation. Transpl Immunol 18: 115–121.

52. Sakaguchi S, Yamaguchi T, Nomura T, Ono M (2006) Regulatory T cells and immune tolerance. Cell 133: 775–787.

53. Sharfe N, Dadi HK, Shahar M, Roffman CM (1997) Human immune disorder arising from mutation of the alpha chain of the interleukin-2 receptor. Proc Natl Acad Sci U S A 94: 3168–3171.

54. Liu YY, Patwardhan GA, Bhinge K, Gupta V, Gu X, et al. (2011) Suppression of glucosylceramide synthase restores p53-dependent apoptosis in mutant p53 cancer cells. Cancer Res 71: 2276–2285.

55. Messner MC, Cabot MC (2010) Glucosylceramide in humans. Adv Exp Med Biol 688: 156–164.