Global Chromatin State Analysis Reveals Lineage-Specific Enhancers During the Initiation of Human T helper-1 and T helper-2 Cell Polarisation

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Inventory of Supplemental Information

Inventory for Supplemental Tables

Table S1. (A) Enhancers in Th1 and Th2 as compared with TCR activated CD4+ T cells (Th0) are identified by Heintzman et al. method. Shown are chromosomes, start and end locations, and strand. (Related to Figure 1B-D). **(B)** Enhancers identified comparing Th1 to control (Th0) and Th2 to control using the He et al. method. (Related to Figure 1E and F)

Table S2. (A) Lineage-specific enhancers identified as overlap of those found by Heintzman et al. and He et al. methods. **(B)** Lineage-specific enhancers from table S2 that have one or more genes within +-125kb around them. Column “H3K27ac correlation” shows the correlation between amounts of H3K27ac in 5kb windows centered on the enhancer and promoter. Column “H3K27ac-geneexpr correlation” shows the correlation between amount of H3K27ac on 5kb window centered on the enhancer and the gene expression value. Column “H3K27ac” has ‘a’ if the amount of H3K27ac on enhancer is higher than the 0.95-quantile of a set of 10000 randomly sampled locations on genome. (Related to Figure 2A-D)

Table S3. Enrichments of enhancer-bound TFs with respect to binding to random locations in genome. (Related to Figure 3)

Table S4. (A) Lineage-specifically differentially expressed genes (p-value<0.01, minimum fold-change 1.5). **(B)** Differentially expressed (p-value<0.01, minimum fold-change 1.5) Th1 and Th2 genes when comparing to TCR activated CD4+ T cells (Th0). (Related to Figure 1G and H)

Table S5. Digital gene expression log2 fold changes for all the genes.

Table S6. Enhancer target gene predictions: 1) upregulated genes within same CTCF blocks as enhancers, 2) genes that are closest to enhancers and upregulated, 3) genes within +-125kb of the enhancers and high enough correlation with the H3K27ac value of the 5kb-block centered on enhancer. (Related to Figure 5E)

Table S7. Enrichments of disease categories of SNPs from NHGRI GWAS catalog overlapping with enhancers. (Related to Figure 5 and 6)

Table S8. (A) Overlaps of associated (p<0.01) SNPs from selected autoimmune diseases with Heintzman et al. predicted enhancers. **(B)** Overlaps of associated (p<0.01) SNPs from selected autoimmune diseases with the lineage-specific predicted enhancers. (Related to Figure 5 and 6)

Table S9. Disease-associated SNPs overlapping with lineage-specific enhancers (Th0+Th1+Th2). (Related to Figure 5 and 6)

Inventory for Supplemental Figures:

Figure S1. (A) Immunoblotting data showing the expression of GATA3 and TBX21. **(B and C)** Flow cytometric analysis of surface (CRTH2) and intracellular cytokine staining (IL4 and IFNγ). (Related to Figure 1)

Figure S2. Lineage-specific nucleosome free regions (NFR) shown in a high-density plots. (Related to Figure 1)

Figure S3. Enhancer-based regulatory interactions. (Related to Figure 3 and 5)

Figure S4. Circos plots for lineage-specific enhancer targets harboring SNPs. (Related to Figure 6)

Figure S5. DAPA (DNA Affinity Precipitation Assay) experiments assessing the transcription factors bound to the enhancers and effect of SNP mutation on transcription factor binding. Related to Figure 6D)

SUPPLEMENTAL METHODS

Human Cord Blood $CD4^+$ T Cell Isolation

$CD4^+$ T cells were isolated (Dynal CD4 Positive Isolation Kit, Invitrogen) from umbilical cord blood of healthy neonates (Turku University Central Hospital, Turku, Finland) by Ficoll-Paque gradient centrifugation (Amersham Pharmacia Biotech, Uppsala, Sweden) and cultured in Yssel's medium (22) supplemented with 1% human AB serum (Red Cross Finland Blood Service). Cord blood $CD4^+$ cells from several individuals were pooled after the isolation. All the data included in this manuscript has been acquired under the permission from the Ethics Committee of the Hospital District of Southwest Finland.

Western Blotting

At day 3, cells were harvested and lyzed in Triton-X sample buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 0.5% Triton-X-100; 5% glycerol; 1% SDS), containing proteinase (#4693159001, Roche) and phosphatase inhibitors (#8906738001, Roche). After adding 6x loading dye (330 mM Tris-HCl, pH 6.8; 330 mM SDS; 6% β-ME; 170 μMBromophenol blue; 30% glycerol), equal amounts of samples were loaded on 10-12% SDS-PAGE gels. The separated proteins were transferred to nitrocellulose membranes (Hybond ECL, Amersham Biosciences, Buckinghamshire, U.K.) and blocked with 5% nonfat dried milk and 0.1% Tween 20/TBS. All Ab incubations were performed in blocking solution and the following Abs/antisera were used: 1:5000 mouse anti-GATA3, 1:200 mouse anti-T-bet (4B10) (SC-21749) and 1:10,000 goat anti-mouse IgG-HRP (Santa Cruz Biotechnology). The ECL detection system (Amersham Biosciences) was used for visualization of proteins. β-actin was detected to control for equal loading with 1:10,000 anti–β-actin (AC-15; Sigma-Aldrich, St. Louis, MO).

Flow Cytometric Analysis

Sample preparation for flow cytometric analysis of surface and intracellular cytokine staining was performed as previously described (Filén S. et al., 2010). Briefly, cells harvested 7 d after culture were stimulated with 5 ng/ml PMA (Calbiochem) and 0.5 pg/ml ionomycin (Sigma-Aldrich) in Yssel's medium. A portion of the cells was incubated in Yssel's medium only and used as an unstimulated control. After 2 h of stimulation, 10 µg/ml brefeldin A (Alexis Biochemicals, Lausanne, Switzerland) was added to all samples and incubation was continued for 3 h. Cells were washed and fixed with 4% (w/v) paraformaldehyde/PBS and permeabilized with 0.5% (w/v) saponin/PBS. Anti-human–IFN-γ-FITC (B27; Caltag Laboratories, Burlingame, CA) and anti-human-IL4 (BD Pharmingen, USA) was used for staining of the intracellular IFN-γ and IL4. Mouse IgG1-FITC (Caltag Laboratories) and mouse IgG2k-PE were used as isotypic controls. For the surface staining, CRTH2-PE staining (#130-091-238, Miltenyi Biotech) was performed after one week of polarization. Cells were analyzed with LSR II flow cytometer (BD Biosciences) and CyflogicTM software (CyFlo Ltd, Finland).

ChIP and ChIP-seq

Human umbilical cord blood $CD4^+$ T cells were differentiated toward Th1 and Th2 until day 3 as described above. To fix the DNA-protein complex, cells were cross-linked with 1/10 volume of cross-linking solution (50 mM HEPES-KOH [pH 7.5], 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, and 11% formaldehyde) for 10 min followed by quenching with 1/20 volume of 2.5 mM glycine solution for 10 min. Cells were harvested and washed twice with ice-cold PBS and pelleted, and nuclear fractions were prepared. The cell pellet was dissolved in 20 ml lysis buffer 1 (50 mM HEPES KOH [pH 7.5], 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% Nonidet P-40, and 0.25% Triton X-100 with protease inhibitor Complete Mini [Roche]) and incubated for 10 min at 4°C followed by centrifugation at 2000 × *g* for 10 min at 4°C. The cell pellet was resuspended in 10 ml lysis buffer 2 (200 mM NaCl, 1 mM EDTA [pH 8], 0.5 mM EGTA, and 10 mM Tris [pH 8], with protease inhibitor) at room temperature for 10 min. Nuclei were pelleted at 2000 × *g* for 10 min at 4°C and resuspended in 1ml lysis buffer 3 (1 mM EDTA [pH 8], 0.5 mM EGTA [pH 8], and 10 mM Tris [pH 8], with protease inhibitor). The chromatin was sonicated for DNA fragmentation and analyzed with agarose gel electrophoresis. The majority of the DNA fragments were in the 500–100 bps range. Cell debris was pelleted by spinning at 10,000 × *g* for 10 min, and the supernatant was used for chromatin immunoprecipitation (ChIP). 20 µl chromatin supernatant was retained as the DNA input control. Sheep anti-rabbit IgG-conjugated Dynabeads (Dynal) were mixed with antibodies from previously validated (Egelhofer et al., 2010) commercial sources (α-H3K27ac, Abcam ab4729; α-H3K4me1, Abcam ab8895; α-H3K4me3, Upstate 07-473;) in blocking solution (PBS

containing 5 mg/ml BSA) o/n at 4°C. Beads were collectedand washed three times with PBS/BSA. Chromatin was equally divided into sample and control, adjusted with Triton-X-solution (0.1% Triton X-100, 0.1% sodium deoxycholate, and 1 mM PMSF) and mixed with magnetic beads pre-coated antibodies and incubated at 4°C o/n. Next day, magnetic beads were collected, put on magnetic stand and the supernatant was removed. Then beads were washed with 1 ml RIPA buffer (50 mM HEPES [pH 7.6], 1 mM EDTA, 0.7% sodium deoxycholate, 1% Nonidet P-40, and 0.5 M LiCl, with protease inhibitor mixture) at 4°C to remove nonspecific binding. The beads were collected and washed with RIPA buffer a total of 8-10 times. After washing once with 1 ml TE buffer, the beads were collected by centrifugation at 2000 × *g* for 2 min and resuspended in 50 μl elution buffer (50 mM Tris [pH 8], 10 mM EDTA, and 1% SDS). Chromatin was eluted from the beads by incubation at 65° for 30 min in a thermo-mixer followed by centrifugation for 1 min at 2000 × *g*. A total of 50 μl eluted supernatants were removed and mixed with 120 μl TE buffer. Similarly, 20 μl chromatin input control was mixed with 150 μl TE buffer. Decrosslinking was performed by incubation at 65°C overnight. Proteins in the DNA sample were digested in 120 μl proteinase K solution (2% glycogen, 5% proteinase K stock solution, and TE) for 2 h at 37°C. The DNA was then extracted twice with phenol and once with 24:1 chloroform/isoamyl alcohol. The sample was adjusted to 200 mM NaCl. After ethanol precipitation, the DNA was dissolved in 30 μl TE containing 10 μg DNase-free RNase A and incubated for 2 h at 37°C. The DNA was further purified with a Qiagen PCR kit (Qiagen). Libraries for sequencing were generated as previously described (Hawkins et al., 2010).

DNA Affinity Precipitation assay

DNA Affinity Precipitation Assays (DAPA) were carried out as described earlier [Pan MR et al., 2009, Lo YH et al., 2008, Cesi, V., 2005] with the following modifications. Biotinylated and antisense bait oligonucleotides were ordered from Oligomer (Helsinki, Finland). Sequences for STAT6-specific and mutated sequence baits were 5´-Biotin-GGATCCGAGAGGTT**TC**CGGT**GA**ATGTTAGA-3´, and 5´-Biotin-GGATCCGAGAGGTT**AT**CGGT**CT**ATGTTAGA-3´, respectively used as positive control for DAPA (Tapio Lönnberg). Probes containing the predicted PPARG, CREB, and STAT1 binding sites on enhancers and with respective SNP mutations introduced were used. The detailed sequence information is given below with imposed mutations marked in bold.

1) STAT1 – Target enhancer Gene – IL2RA (Th0)

<u>Bait sequences:</u>

STAT1-specific prediction: 5’-Biot- TGGACAAACGGTTTAC**G**GAAGGTGAGGCTG-3’

STAT1- SNP mutated sequence: 5’-Biot- TGGACAAACGGTTTAC**A**GAAGGTGAGGCTG-3’

2) CREB – Target enhancer Gene – SIRPG (Th0)

Bait sequences:

CREB-specific prediction: 5'-Biot-GTTATAAGGACTAA**C**TTGTTCCATGGCTAT-3'

CREB- SNP mutated sequence: 5'-Biot-GTTATAAGGACTAA**T**TTGTTCCATGGCTAT-3'

3) PPARG – Target enhancer Gene – GATA3 (Th2)

Bait sequences:

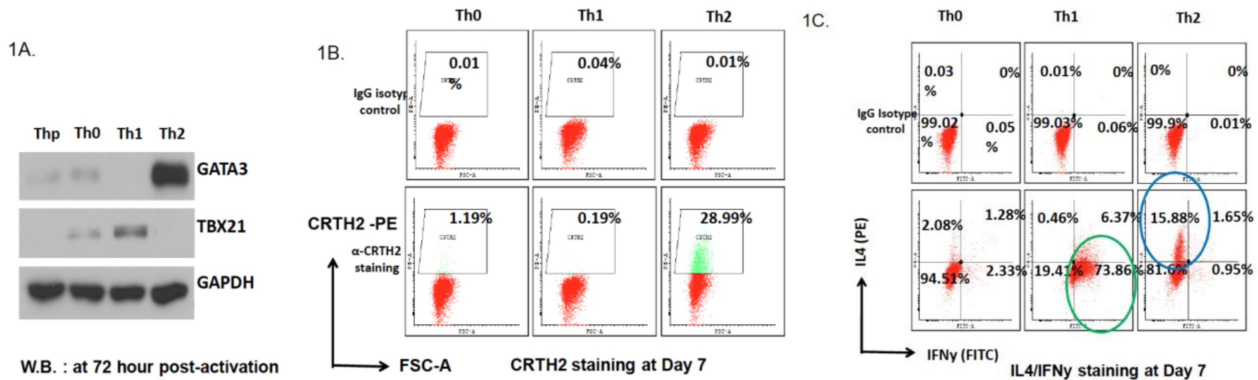
PPARG-specific prediction: 5'-Biot-ACCAGCAAACAG**C**GTCACCACCACCCTCTC-3'

PPARG-SNP mutated sequence: 5'-Biot-ACCAGCAAACAG**T**GTCACCACCACCCTCTC-3'

Antisense strands (complementary sequence) were not labelled with biotin. Annealing of oligonucleotides was performed by incubating at 95°C for 5 minutes and letting cool down slowly over night. Beads (Ultralink immobilized neutravidin protein, Pierce, Rockford Illinois, USA) were washed three times with buffer A (10mM HEPES pH 7.9, 60mM KCl, 2mM EDTA, 1mM EGTA, 0.1% Triton X-100, 1mM DTT, and protease and phosphatase inhibitors from Roche, Basel, Switzerland). 250 pmol of annealed oligos were conjugated with 25µl beads in 200µl buffer A for 2h in a 360° rotator at +4°C. After conjugation reaction, beads were washed four times with buffer A. Nuclear fractions were diluted to 60mM KCl using buffer B (10mM HEPES pH 7.9, 2mM EDTA, 1mM EGTA, 0.1% Triton X-100, 1mM DTT, and protease and phosphatase inhibitors from Roche) and pre-cleared with 100µl of uncoated beads by incubating for 90 minutes in a 360° rotator at +4°C. Pre-cleared nuclear fractions were incubated with beads coated with oligonucleotides for 4-6h in a 360° rotator at +4°C. After the binding reaction, beads were washed four times with buffer A, and precipitated proteins were eluted by incubating for 5 minutes at 95°C in 2xSDS buffer (125mM Tris-HCl pH 6.8, 4% w/v SDS, 20% glycerol, 100mM DTT). Eluted proteins were analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting using the following antibodies (catalog numbers are in parentheses): from Cell Signaling Technology, anti-phospho-Y641-STAT6 (#9361L), anti-STAT1 (#9172L), and anti-CREB (#9192); and from Perseus Proteomics, anti-PPARG (#PP-K 8713-00). Visualization was achieved by incubation with the appropriate secondary antibody conjugated to horseradish peroxidase and the Immobilon Western Chemiluminscent HRP substrate detection reagents (Cat. No. WBKLS0500) from Millipore (Billerica, Massachusetts, USA).

SUPPLEMENTARY FIGURES S1-S5.

Figure S1.



(A) Immunoblotting data showing the expression of GATA3 (BD Pharmingen, USA) and TBX21(BD Pharmingen, USA) after culturing cells for 72 hr towards Th1 or Th2 cells under polarizing conditions. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a loading control. A representative result from 3 independent experiments is shown. **(B and C)** Flow cytometric analysis of surface and intracellular cytokine staining was performed as previously described (Filén S. et al., 2010). For the surface staining, CRTH2-PE staining (#130-091-238, Miltenyi Biotech) was performed after one week of polarization and rat IgG2a-PE was used as isotypic control (C). For intracellular staining, cells harvested 7 d after culture were stimulated with 5 ng/ml PMA (Calbiochem) and 0.5 pg/ml ionomycin (Sigma-Aldrich) in Yssel's medium. A portion of the cells was incubated in Yssel's medium only and used as an unstimulated control. After 2 h of stimulation, 10 µg/ml brefeldin A (Alexis Biochemicals, Lausanne, Switzerland) was added to all samples and incubation was continued for 3h. Cells were washed and fixed with 4% (w/v) paraformaldehyde/PBS and permeabilized with 0.5% (w/v) saponin/PBS. Anti-human–IFN-γ-FITC (B27; Caltag Laboratories, Burlingame, CA) and anti-human-IL4 (BD Pharmingen, USA) were used for staining of the intracellular IFN-γ and IL4. Mouse IgG1-FITC (Caltag Laboratories) and mouse IgG2k-PE were used as isotypic controls. The representative result from 3 independent experiments is shown.

Figure S2

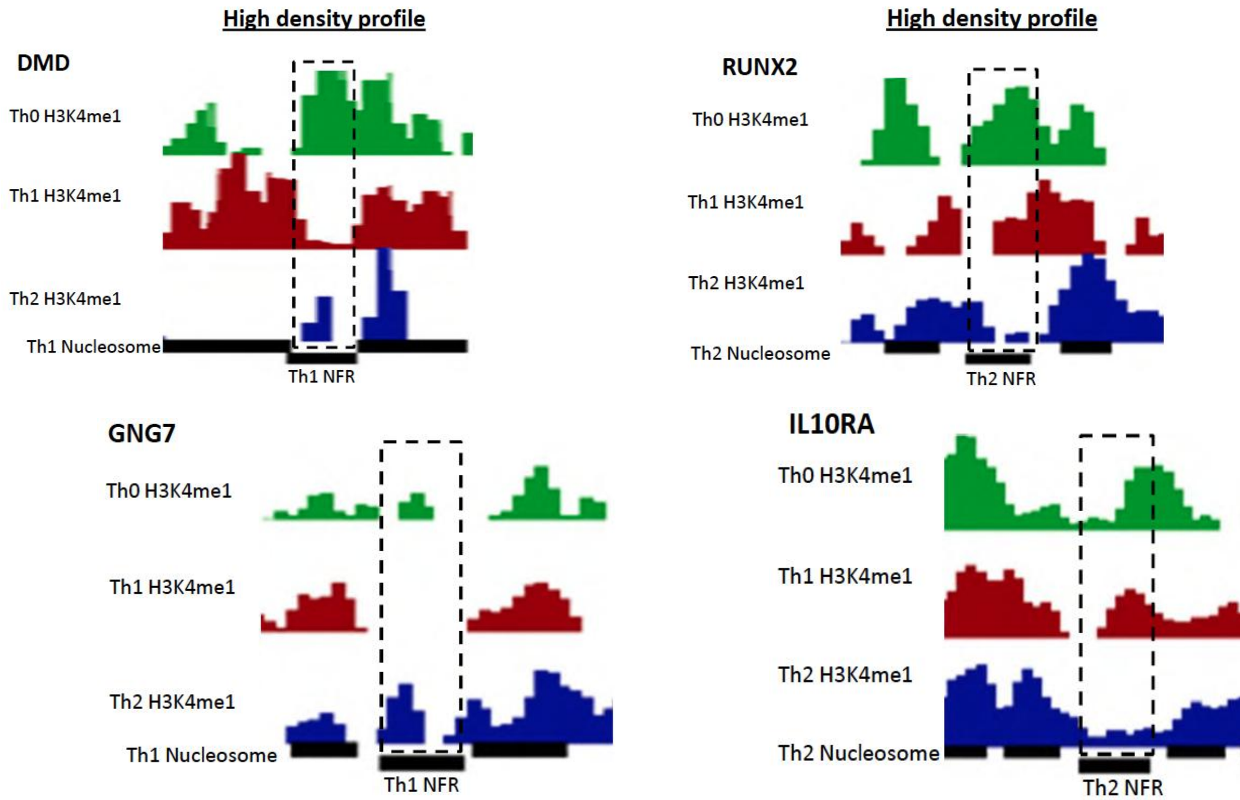


Figure S2. Lineage-specific nucleosome free regions (NFR) shown in a high density plot, as in Figure 1. The lineage-specific enhancer is determined by nucleosome spacing and overlapping nucleosome in the other two cell types (dashedrectangle). Examples are nearby or within genes listed. **(left)** Th1-specific. **(right)** Th2-specific.

Figure S3

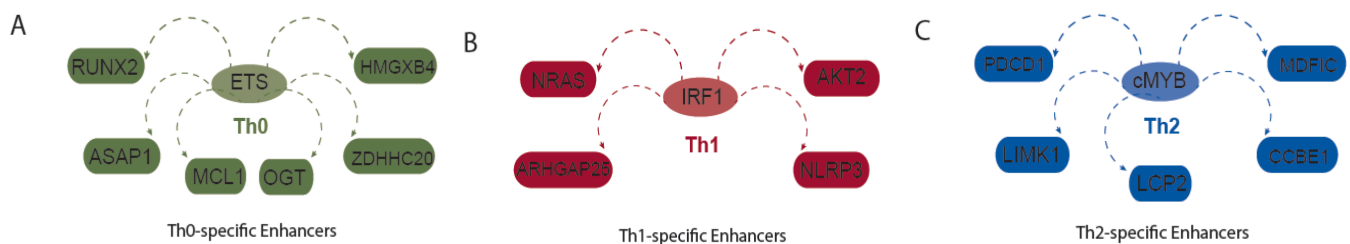


Figure S3. Enhancer-based regulatory interactions. **(A-C)** Micro-networks with enhancer containing motifs as hubs and predicted target genes as connecting nodes. The TF motif is found in the predicted interacting enhancer for the connected genes.

Figure S4

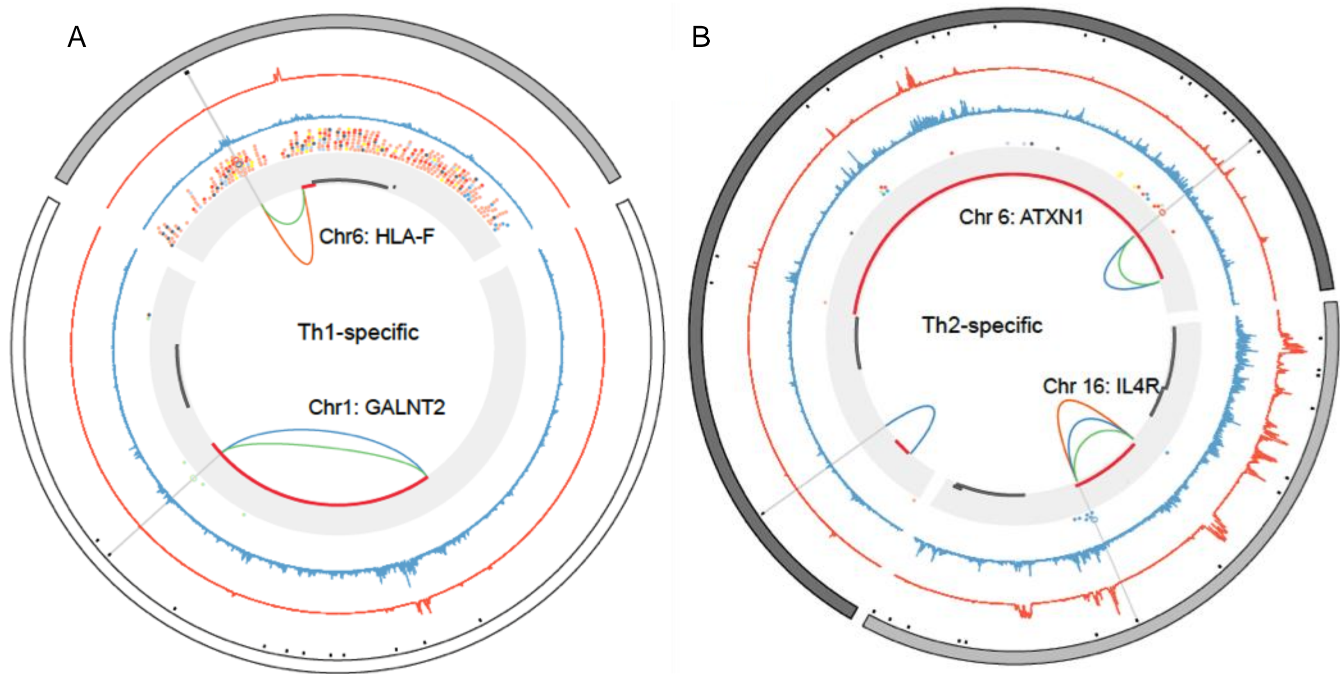


Figure S4. Circos plots for lineage-specific enhancer targets harboring SNPs. The figure legend is as in Figure 6. **(A)** Th1-specific examples for HLA-F and GALNT2. **(B)** Th2-specific examples for ATXN1 and IL4R. The outermost track shows the chromosome bands. Black boxes note enriched H3K4me1 peaks. Lineage-specific enhancers are displayed with light grey radial lines. The profiles show H3K27ac (orange) and H3K4me1 (blue). Disease SNPs are shown with color-coded circle as indicated. The SNPs overlapping a lineage-specific enhancer are highlighted as empty circles. Innermost track (with light grey background) shows the genes (upregulated in red, rest are grey). Arcs connect enhancers to genes based on the target prediction method (green: NN, blue: CB, orange: KAc).

Figure S5

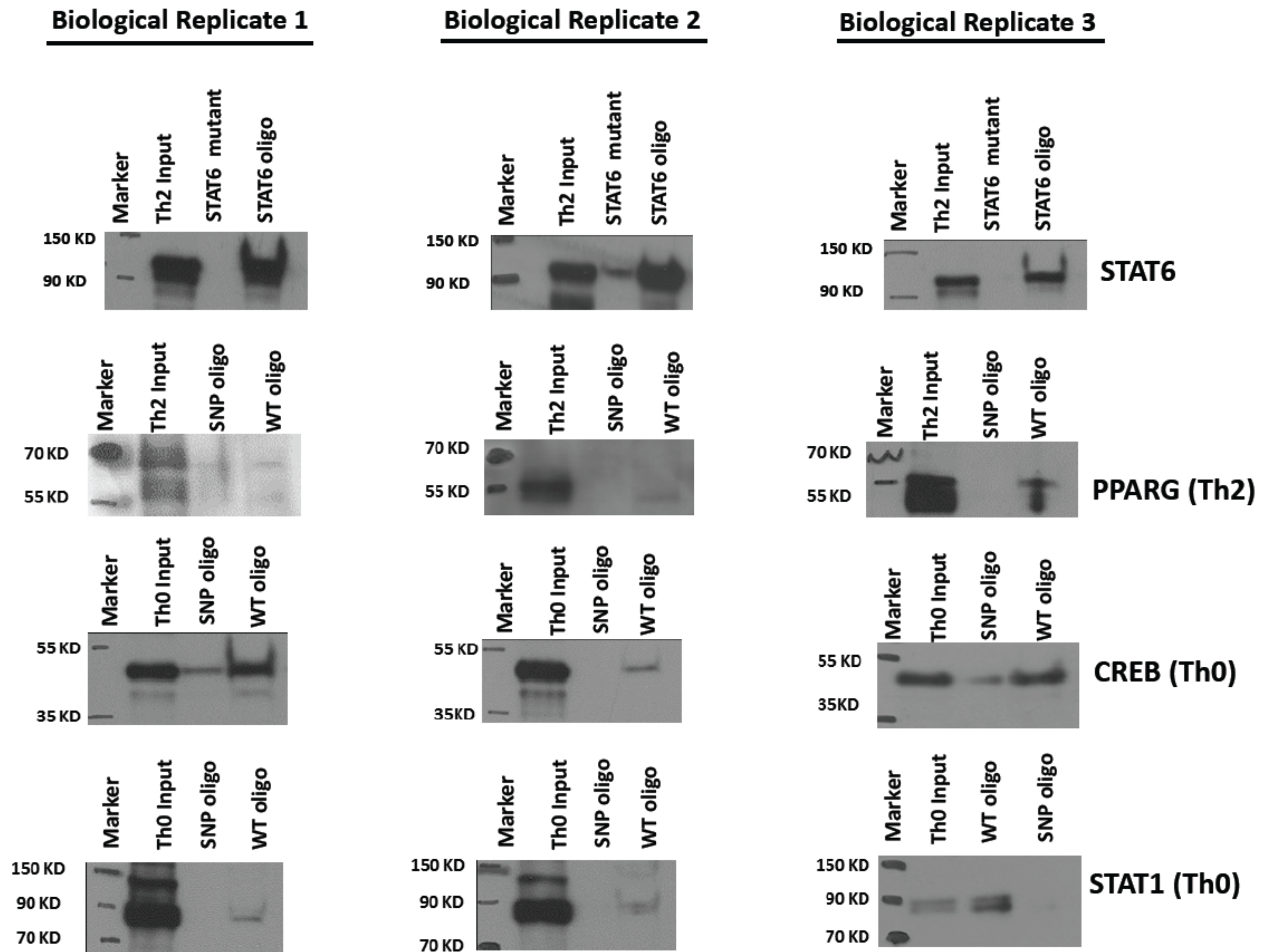


Figure S5. DAPA (DNA Affinity Precipitation Assay) experiments assessing the transcription factors bound to the enhancers and effect of SNP mutation on transcription factor binding, as in Figure 6D. Data shown here is for three biological replicates. The legend is the same as in Figure 6D. See Supplemental Materials and Methods above for complete probe sequences. See Supplemental Materials and Methods above for antibodies used for detection experimental conditions.

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