ARTICLE - Acute Lymphoblastic Leukemia

STAT5 does not drive steroid resistance in T-cell acute lymphoblastic leukemia despite the activation of *BCL2* and *BCLXL* following glucocorticoid treatment

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SUPPLEMENTAL MATERIALS AND METHODS

Generation of cell lines

Gateway cloning (Invitrogen) and lentiviral transduction of SUPT-1 and P12-ICHIKAWA cell lines with wild type or mutant IL7R or STAT5 constructs were performed as previously described (22). SUPT-1 and CCRF-CEM cells were cultured in RPMI-1640 medium (Gibco) supplemented with 1x Glutamax, 10% heat-inactivated fetal bovine serum (Gibco), 2% penicillin/streptomycin (Gibco) and 0,4% Fungizone (Gibco). Media for SUPT-1 cells was supplemented with 1-2 µg/mL puromycin for purification of doxycycline-inducible cells.

Antibodies

phospho-ERK (#4370; Cell Signaling Technologies (CST)), phospho-MEK (#9121; CST), BCLXL (#2764; CST), MCL1 (#4572; CST, and #sc-12756; Santa Cruz Biotechnology (sc)), BIM (#ab32158; Abcam (ab)), β-Actin (#ab6276; ab); IL7R alpha/CD127 (#MAB306; R&D), BCL2 (#sc-130308; sc), RAS (#05-516; Merck Millipore), AKT (#9272; CST), pAKT-T308 (#9275; CST), pAKT-473 (#9271; CST), NR3C1 (#12041; CST), NR3C1 (#sc-1003; sc), STAT5 (#94205; CST), STAT5 (#sc-835; sc) and pSTAT5 (#9351; CST). IRDye fluorescent secondary antibodies (LI-COR) were used for fluorescent signal detection on the Odyssey-CLx Imaging System (LI-COR). For extracellular IL7R expression the CD127 (IL7R) antibody (R&D Systems #MAB306) was used.

Primers

GAPDH Fw primer 5'-GTCGGAGTCAACGGATT-3', *GAPDH* Rev primer 5'-AAGCTTCCCGTTCTCAG-3'; *BIM* Fw primer 5'-GCGCCAGAGATATGGAT-3', *BIM* Rev primer 5'-CGCAAAGAACCTGTCAAT-3'; *IL7R* Fw primer 5'-AGTAAATGCAAAGCACCCTGAG-3', *IL7R* Rev primer 5'- TAAATGGGGCTTAAGCTCTGAC-3'; *SOCS2* Fw primer 5'- GGGAGCTCGGTCAGAC-3', *SOCS2* Rev primer 5'- CCAGCTGATGTTTTAACAGAT-3'; *PIM1* Fw primer 5'- GATCCTGCTGTATGATATGGT-3', *PIM1* Rev primer 5'- GAAGGTTGGCCTATCTGAT-3'; *CISH* Fw primer 5'- CCAGCCCAGACAGAGAG-3', *CISH* Rev primer 5'- TGGAGTCGGCATACTCA-3'; *OSM1* Fw primer 5'- AGCTGCTCGAAAGAGTACC-3', *OSM1* Rev primer 5'-AAGTCGGCCAGTCTGTG-3'; *BCL2* Fw primer 5'-TCGGTGGGGTCATGT-3', *BCL2* Rev primer 5'- GGGCCAAACTGAGCA-3'; *BC2L1* (BCLXL) Fw primer 5'-CCCAGGGACAGCATATC-3', *BCL2L1* (BCLXL) Rev primer 5'- GCTGCATTGTTCCCATAG-3'; *MCL1* Fw primer 5'- CGCCAAGGACACAAAG-3', *MCL1* Rev primer 5'- AAGGCACCAAAAGAAATG-3'.

Immunoprecipitation (IP)

Antibodies were linked to Dynabeads (Thermo Fisher Scientific) and crosslinked to BS^3 (2,5 mM). The total lysate protein eluate of each designated sample was mixed with the Dynabeads-antibody suspension and incubated overnight at 4 degrees. Afterwards, the antibody-bound proteins were eluted from the Dynabeads-antibody-antigen complexes and visualized by immunoblot analysis. Antibodies used for immunoprecipitation: BIM (C34C5 #2933, CST), NR3C1 (#12041; CST).

Chromatin-immunoprecipitation sequencing (ChIP-seq)

Doxycycline-induced $STAT5^{WT}$ and $STAT5B^{N642H}$ SUPT-1 cells were cultured with or without 250 µg/ml prednisolone for 16 hours. A detailed description of ChiP-sequencing, data processing and visualization After incubation, $20x10^6$ cells were fixed and chromatin-immunoprecipitation (ChIP) was performed according to the manufacturer's instructions (Simple ChIP Enzymatic Chromatin IP Kit, #9003, CST). Water bath sonification was performed for seven cycles (30 seconds on, 30 seconds off), and DNA fragment size was validated by electrophoresis. Antibodies used for ChIP: STAT5 (CST, D206U) and NR3C1 (#sc-1003; sc). DNA library preparation was performed using the NEBNext Ultra II DNA Library Prep kit (New England Biolabs #E7103) with an additional 0,52X beads purification step.

Microarray data

From the raw (microarray) expression data, the following STAT5 target genes were selected: *BCL2*, *BCL2L1 (BCLXL)*, *PIM1*, *CISH*, *OSM1* and *SOCS2*. These genes were previously marked as target genes and were visualized using the R package pheatmap (pheatmap: Pretty Heatmaps, R package version 1.0.12). IL7R mutations, as detected in Li et al (6), are denoted at the top. An unsupervised clustering was performed using Euclidean distances. Clustering and visualization were done using the R-package "pheatmap". Based on Euclidean distances, multiple branches were formed. For analysis, we cut the tree in the most upper branches, resulting in branch A (and subsequentially A1 & A2) and branch B. Based on expression values from the target genes, a STAT5 'high' and 'low' group were formed. Besides the unsupervised clustering, no other criteria were used to include/exclude patients from a cluster.

SUPPLEMENTAL FIGURES

Supplemental Figure 1. (A) RNAseq data from 264 patients were downloaded from Liu et al (Nature Genetics, 2017) and log2 transformed. The following STAT5 target genes were selected and used for clustering: *BCL2*, *BCL2L1 (BCLXL)*, *PIM1*, *CISH*, *OSM1* and *SOCS2*. The heatmap was made using the pheatmap package, using an unsupervised clustering with Euclidean distances. This analysis resulted in 3 major clusters, were cluster 2 and 3 presented higher expression of STAT5 target genes. Genetic aberrations were downloaded from protein paint (https://viz.stjude.cloud/st-jude-childrens-research-hospital/visualization/the-genomic-landscape-of-pediatric-and-young-adult-t-lineage-acute-lymphoblastic-leukemia~6) and marked within the heatmap. **(B)** From this RNA-seq analysis, we marked reported oncogenic rearrangements (Liu et al., Nature Genetics 2017), representative for specific T-ALL subtypes (Homminga et al., Cancer Cell 2011; Zuurbier et al., Haematologica 2014). Subsequently, we performed cluster analysis to study the percentage of T-ALL subtypes and the presence of *IL7R, JAK1, JAK3*- and/or *STAT5* mutations in these patient clusters.

Supplemental Figure 2. **(A)** Doxycycline-induced expression of extracellular CD127 (IL7R) of $IL7R^{WT}$, $IL7R^{RFCPH}$ and $IL7R^{QSPSC}$ SUPT-1 cells. **(B)** Immunoblot analysis of wild type and mutant IL7R overexpressing cells (i.e., doxycycline-induced) treated with prednisolone, ruxolitinib (1 µM, JAK1/2-inhibitor), selumetinib (1 µM, MEK-inhibitor) and/or MK2206 (0,5 µM AKT-inhibitor). Cells were treated with targeted inhibitors 30 minutes before doxycycline-induction. Steroid exposed cells were treated with prednisolone (250 µg/ml) for 16 hours.

Supplemental Figure 3. (A) Immunoblot analysis of CCRF-CEM cells co-cultured with IL7. Cells were treated with targeted inhibitors (ruxolitinib (1 µM, JAK1/2-inhibitor), selumetinib (1 µM, MEK-inhibitor) and/or MK2206 (0,5 µM AKT-inhibitor)) 30 minutes before doxycycline-induction. Steroid exposed cells were treated with prednisolone (250 µg/ml) for 16 hours. **(B)** Corresponding expression of STAT5B target genes of these CCRF-CEM cells. Representative data of biological duplicate. **(C)** Steroid sensitivity of CCRF-CEM cells in the absence or presence of targeted inhibitors. Steroid sensitivity was determined by a 4-day MTT read-out. Representative data of biological duplicates.

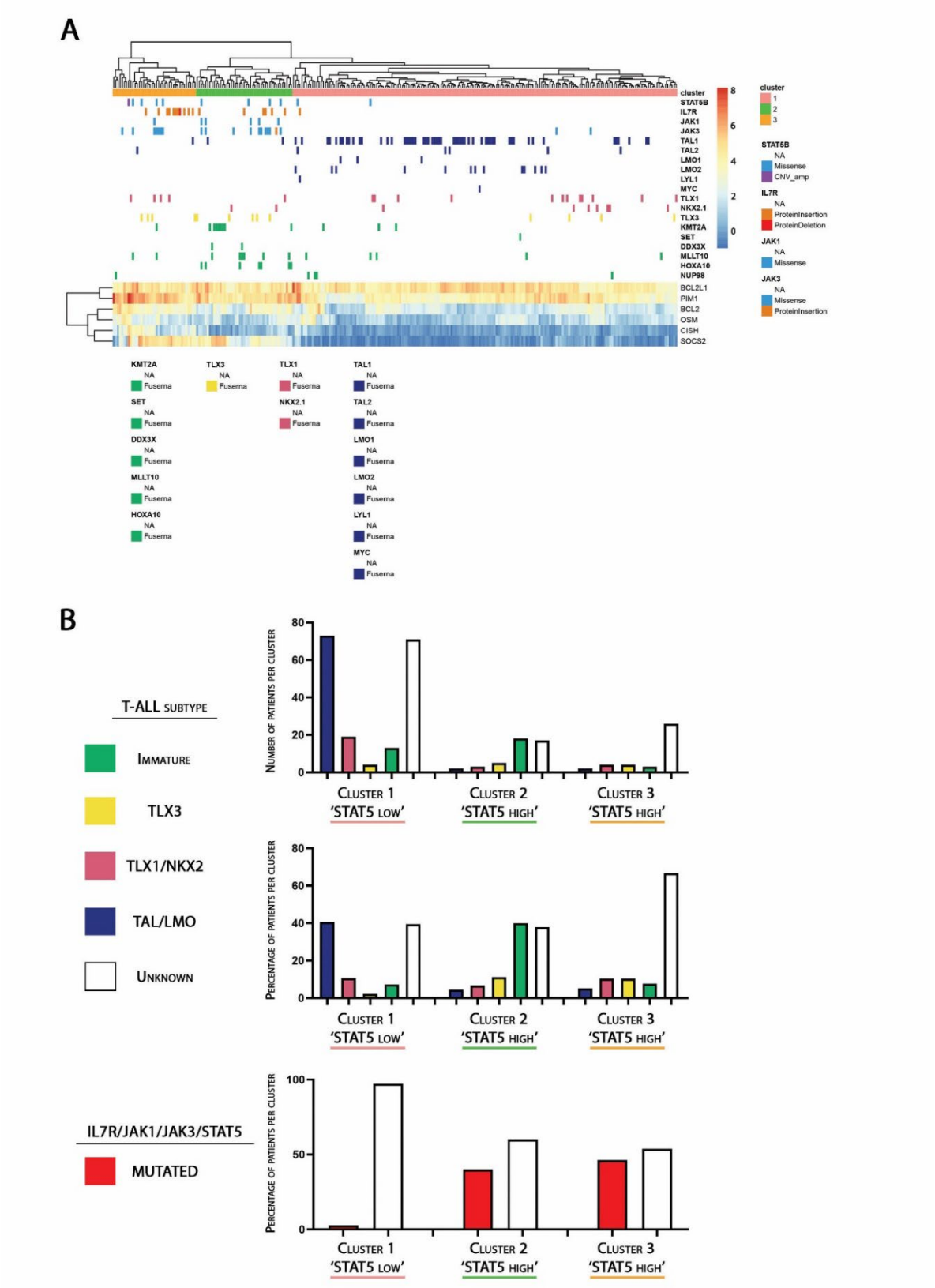
Supplemental Figure 4. (A) Activation of STAT5B signaling and expression of anti-apoptotic Bcl2 family proteins in STAT5B wild-type and mutant overexpressing P12 ICHIKAWA cells. Protein band intensity for

BCL2 and BCLXL represented relative to -dox-pred condition. **(B)** Expression of STAT5 target genes in $STAT5B^{WT}$ and $STAT5B^{N642H}$ overexpressing P12 ICHIKAWA cells in the absence or presence of prednisolone treatment (250 μg/ml) for 16 hours. Data of biological triplicate with SD indicated.

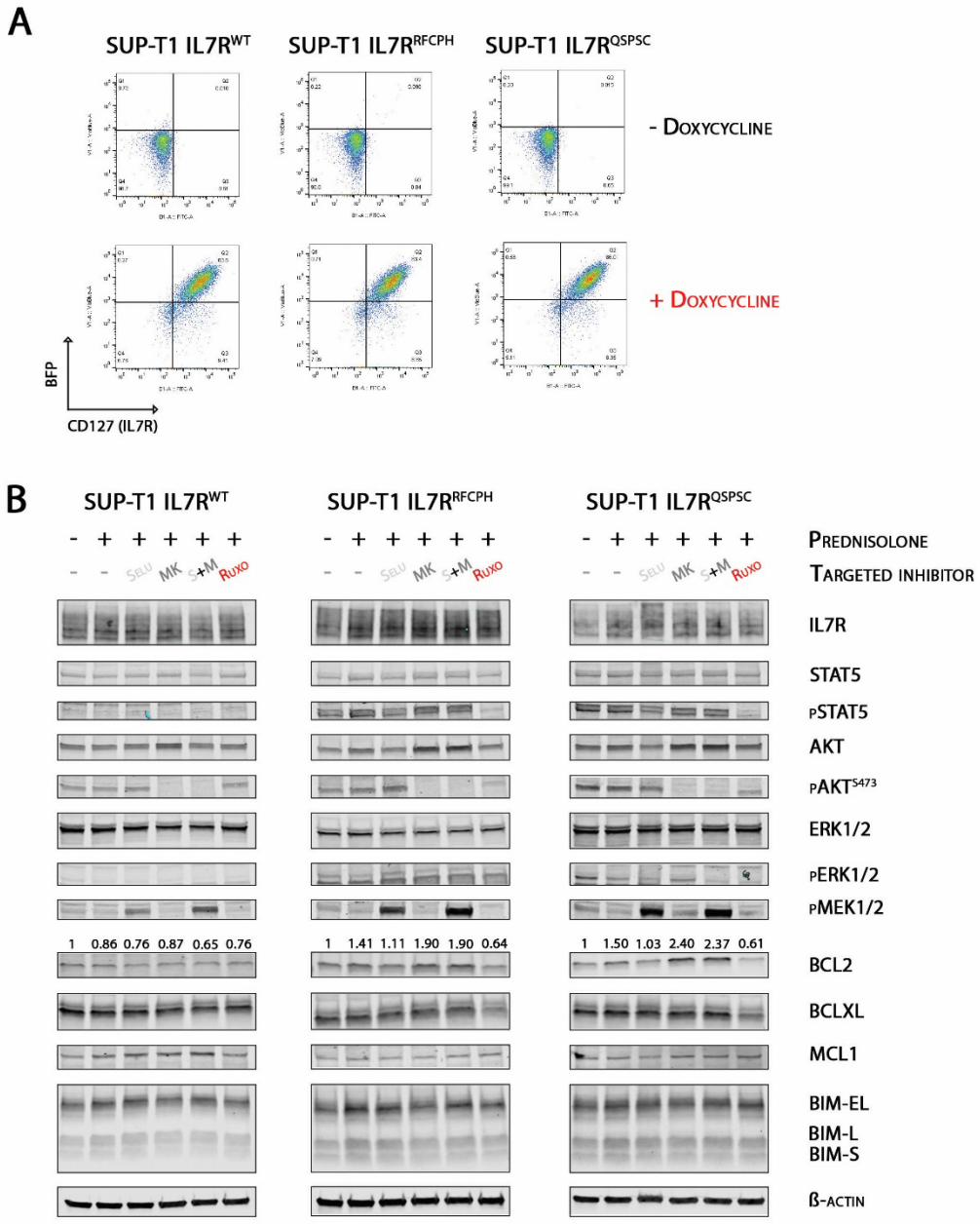
Supplemental Figure 5. (A) Expression of *IL7R* in CCRF-CEM cells exposed to IL7. Cells were treated with targeted inhibitors 30 minutes before IL7 exposure. Steroid exposed cells were treated with prednisolone (250 μg/ml) for 16 hours. Data of biological triplicate with SD indicated. **(B)** Immunoblot analysis of STAT5-immunoprecipitation in $STAT5B^{N642H}$ SUPT-1 cells. Steroid exposed cells were treated with prednisolone (250 μg/ml) for 16 hours. IgG representing IgG control, Ab representing antibody control. **(C)** ChIP-seq identified binding of NR3C1 and STAT5 transcription factors at STAT5 target genes *OSM, CISH* and *SOCS2*.

Supplemental Figure 6. (A) ChIP-seq identified binding of NR3C1 and STAT5 transcription factors at NR3C1 target genes *BMF*, *GILZ* and *MCL1*. **(B)** Gene expression of NR3C1 target genes (*MCL1*, *KLF13*, *FKBP5*, *BIM*, *BMF* and *GILZ*) in wild type STAT5 overexpressing SUPT-1 cells in the absence or presence of overnight steroid treatment (250 μg/ml). Data of biological triplicate with SD indicated.

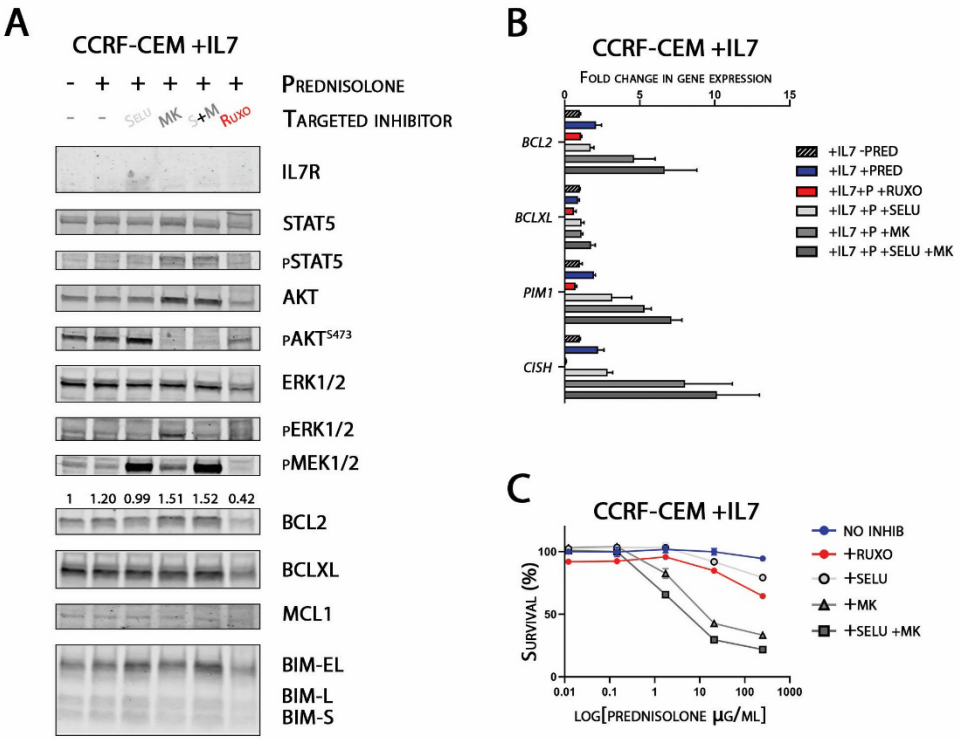
Supplemental Table 1. Peak details of ChIP motif analysis of NR3C1 and STAT5 motifs significantly enriched in NR3C1/STAT5 unique or overlapping peak sets.(excel.file)



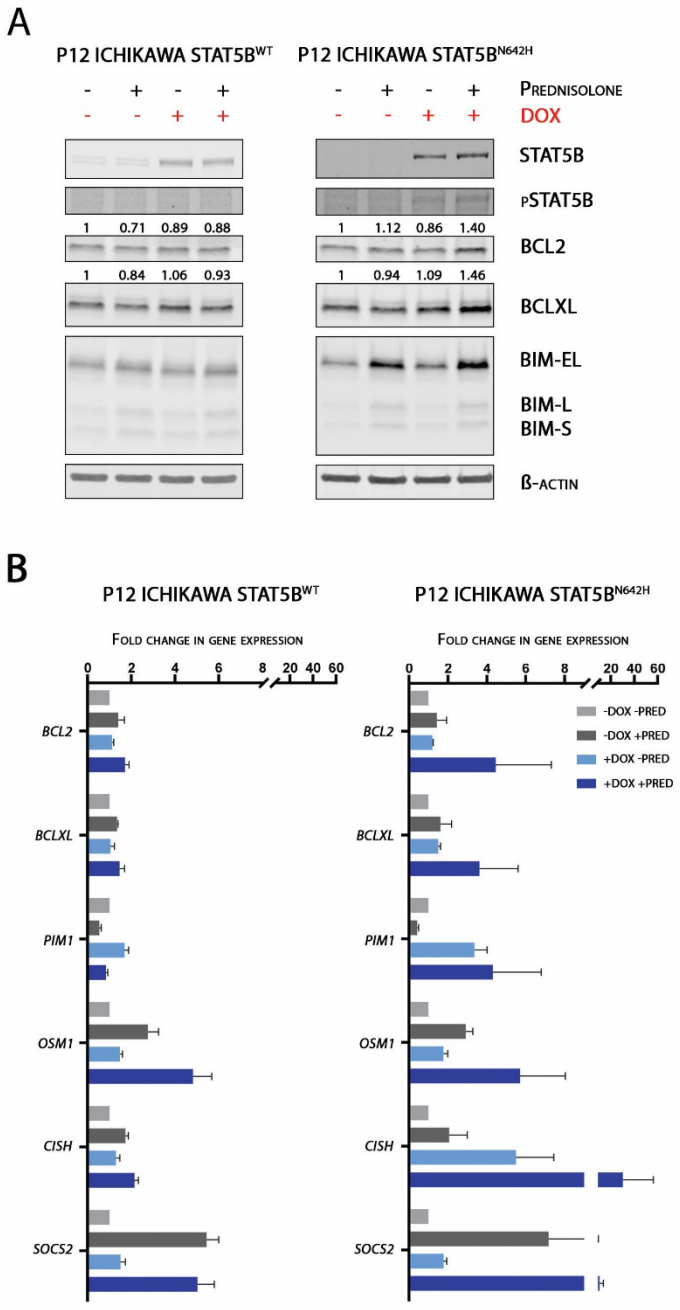
SUPPLEMENTAL FIGURE 1



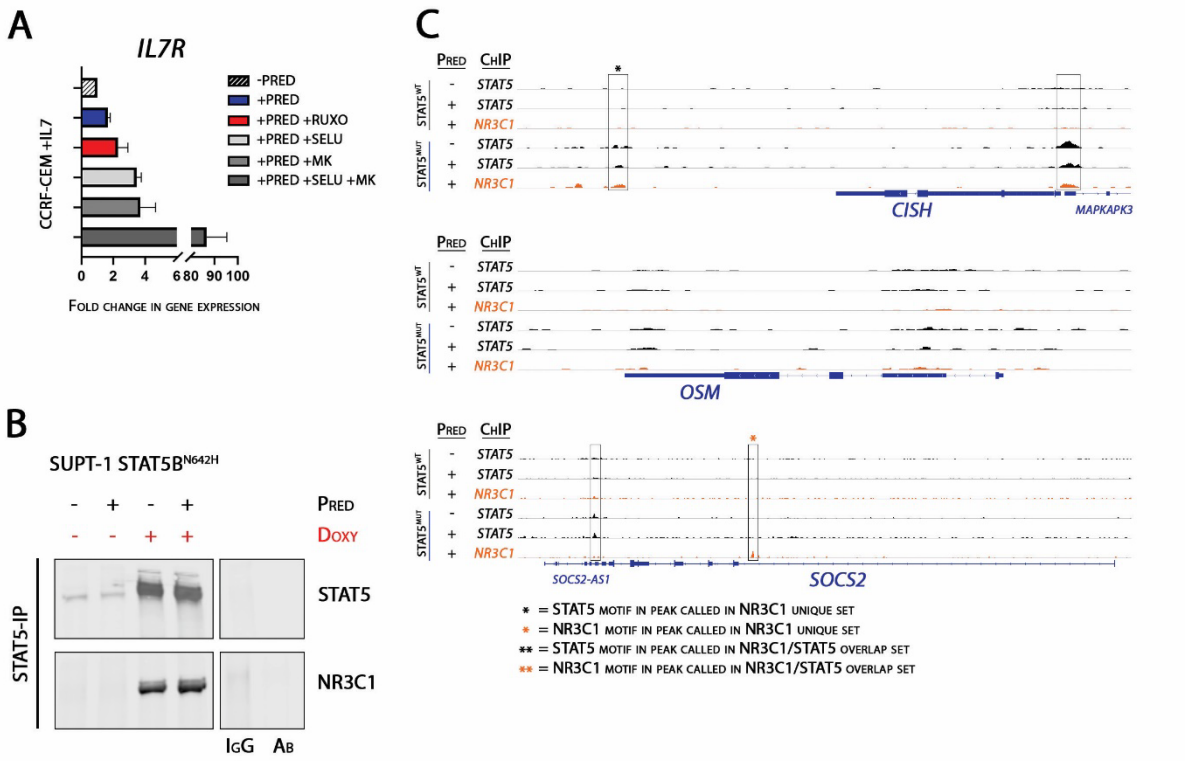
SUPPLEMENTAL FIGURE 2



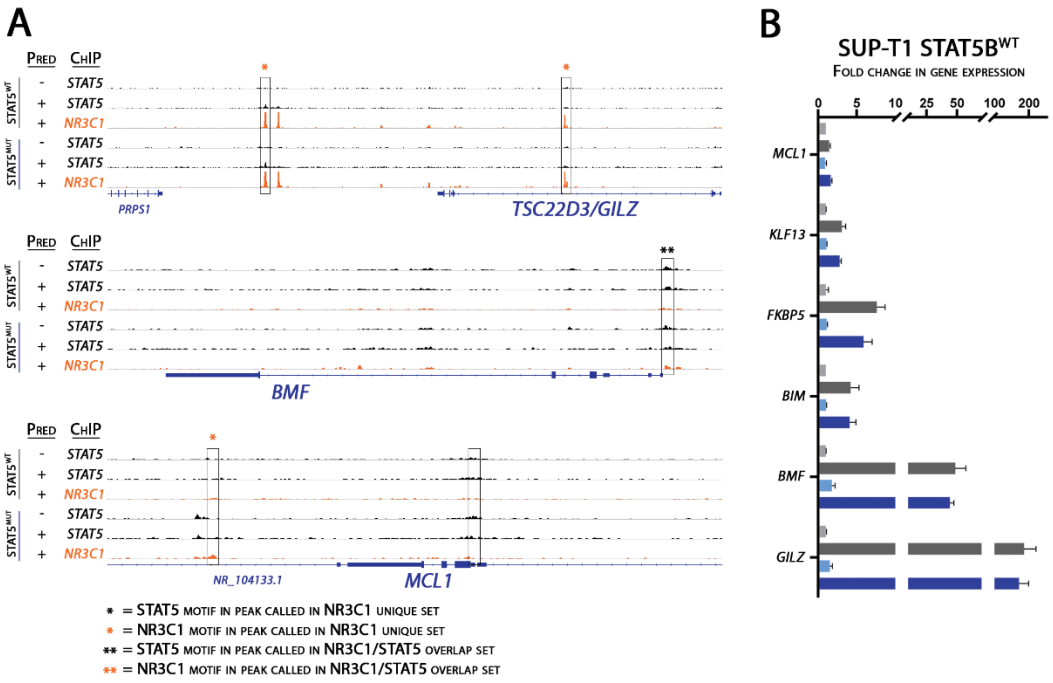
SUPPLEMENTAL FIGURE 3



SUPPLEMENTAL FIGURE 4



SUPPLEMENTAL FIGURE 5



SUPPLEMENTAL FIGURE 6