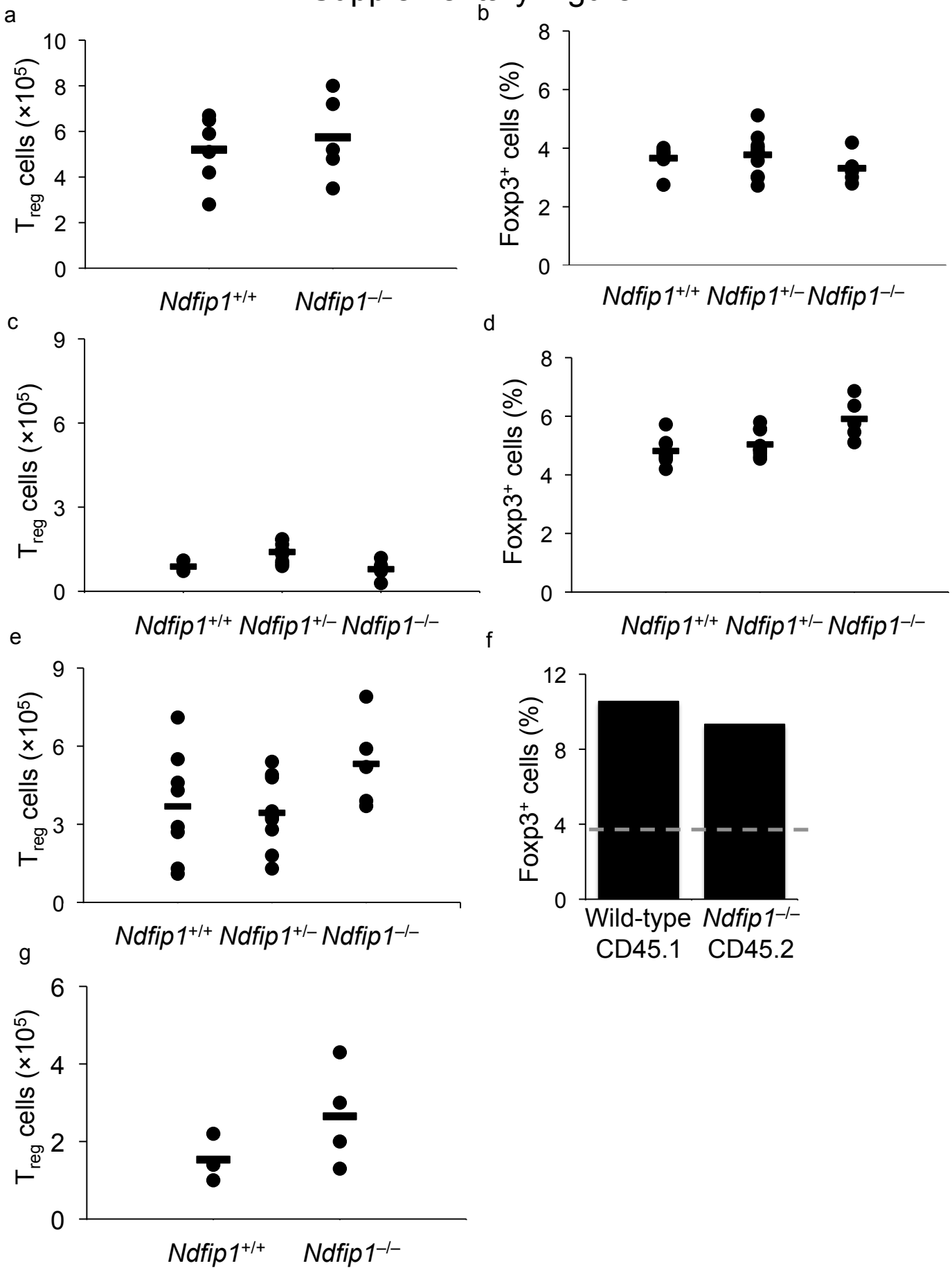
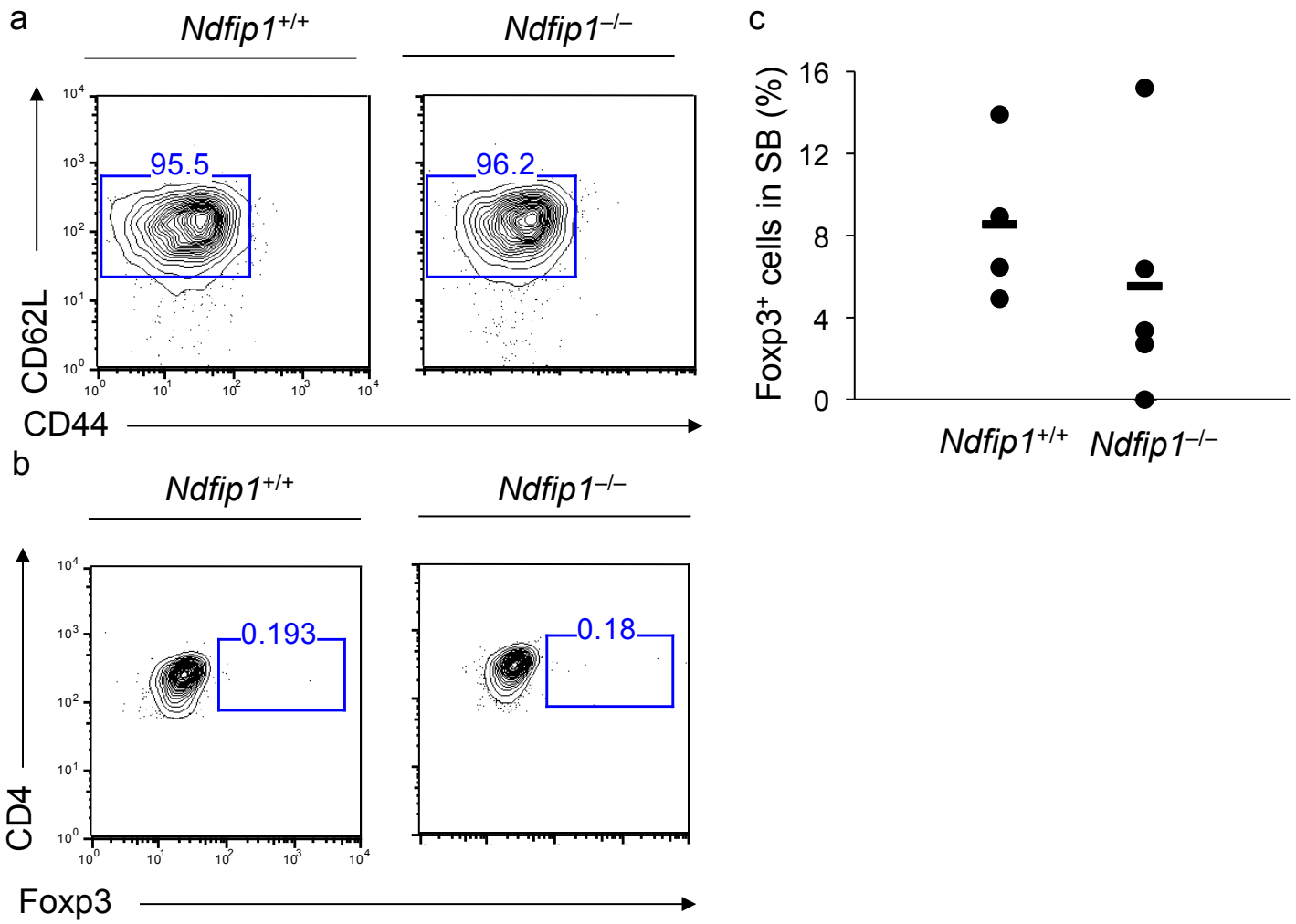
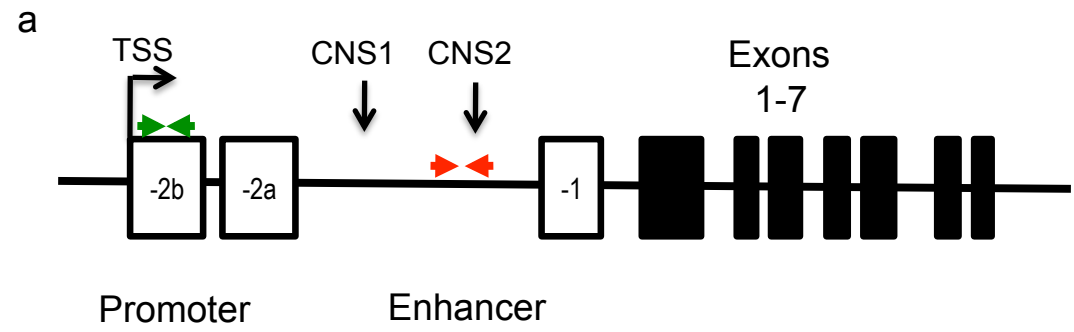
Supplementary Figure 1

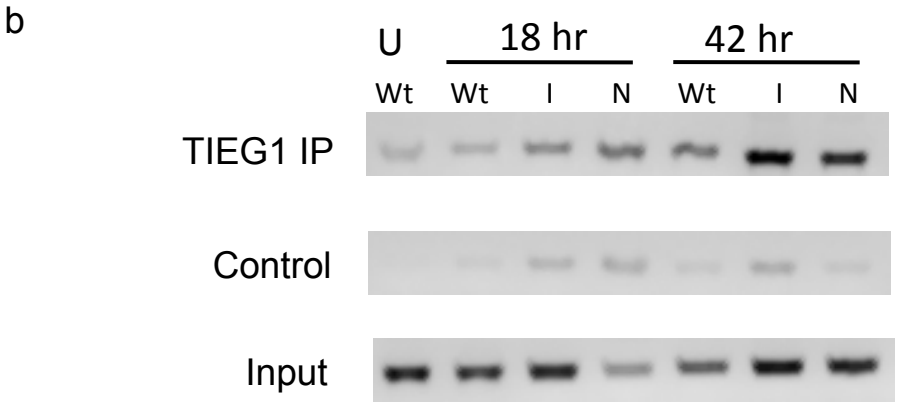


Supplementary Figure 2



Supplementary Figure 3





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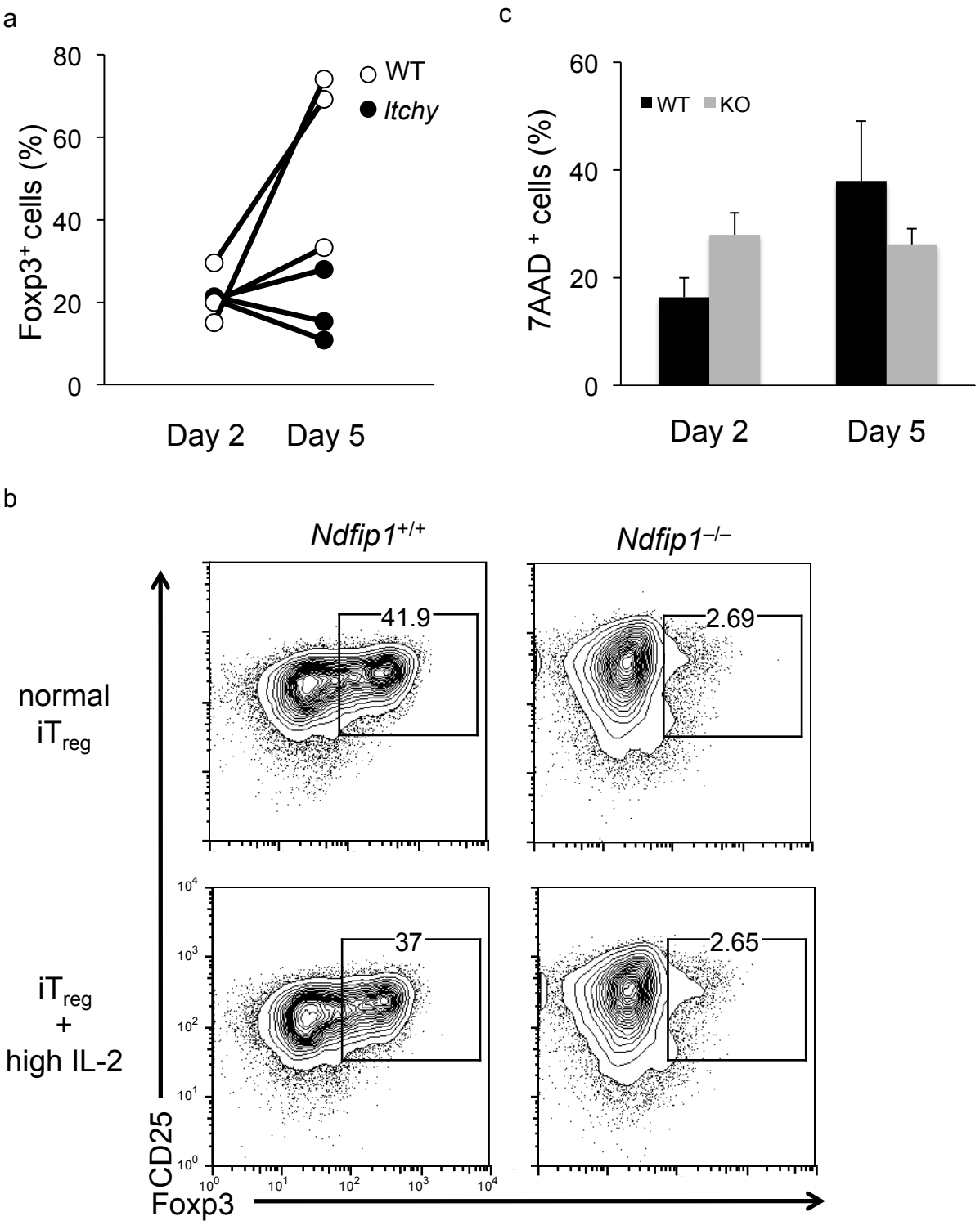

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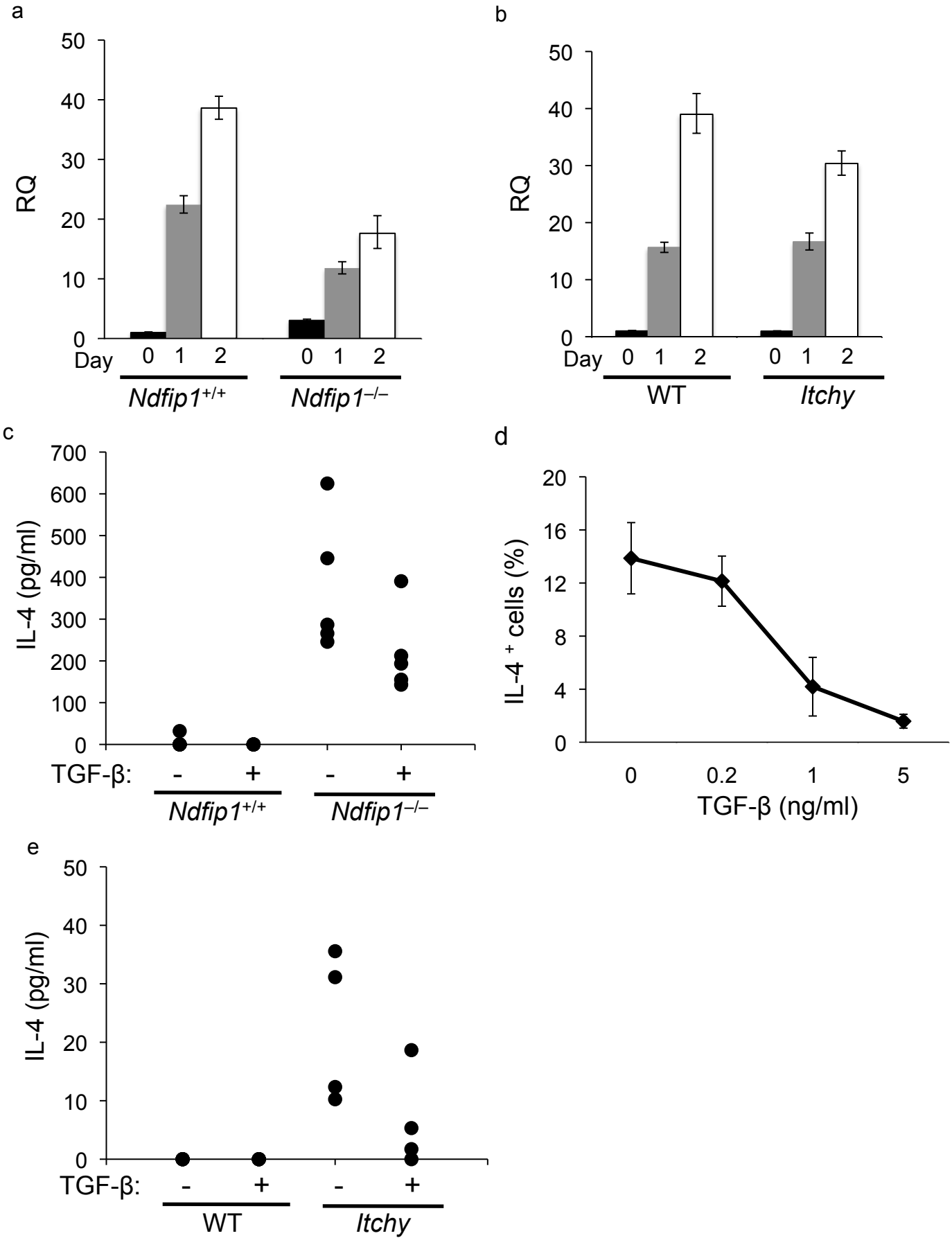

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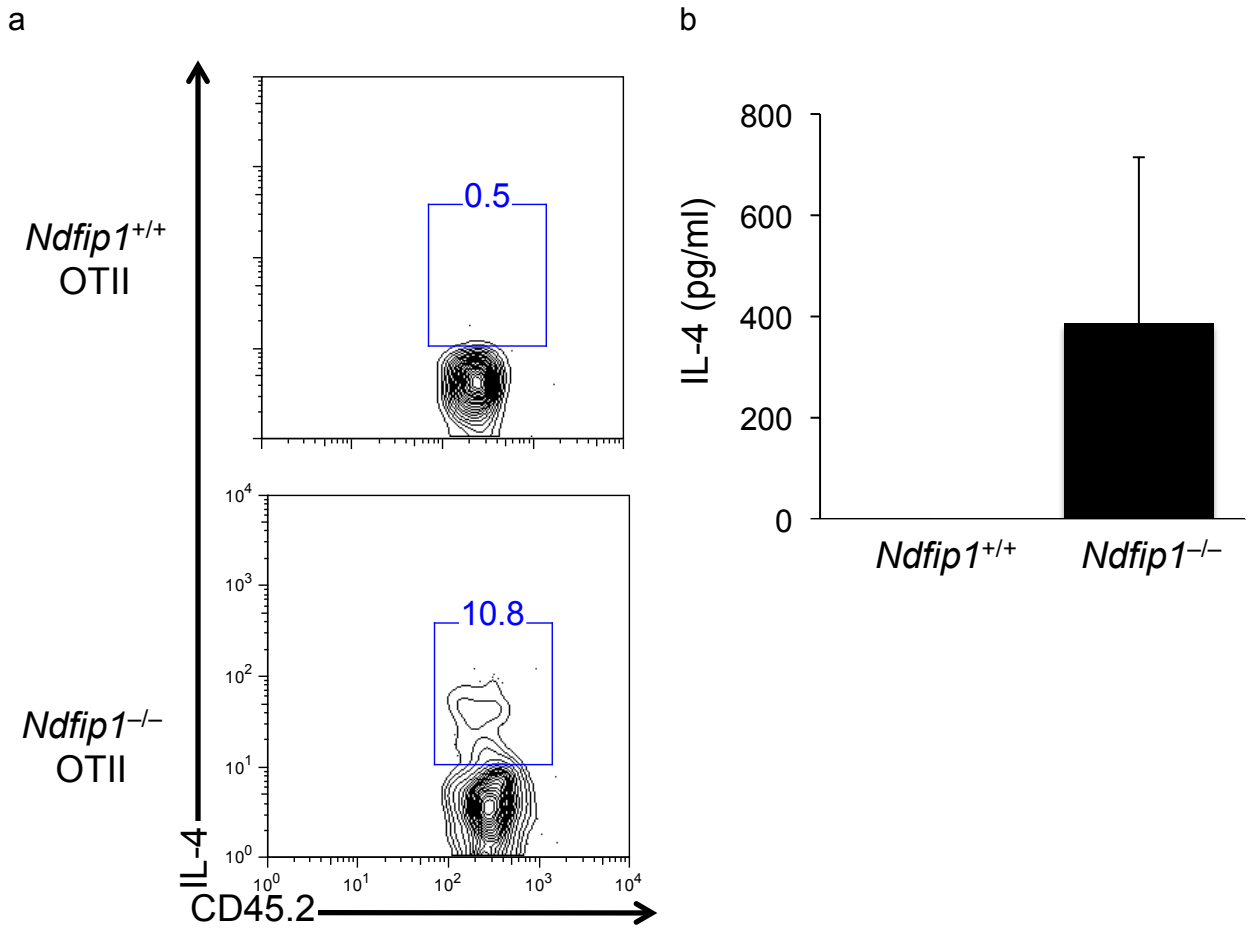
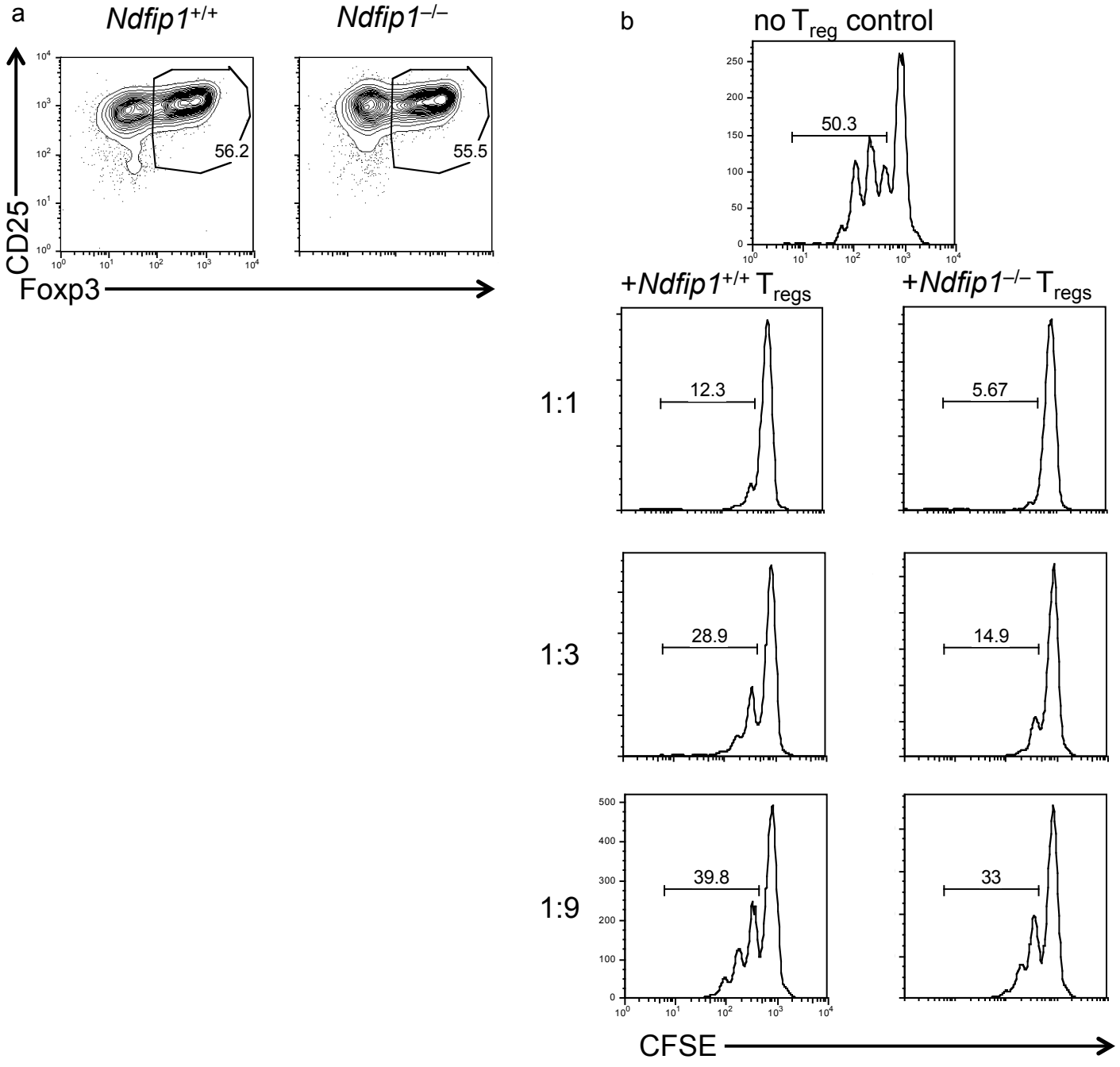
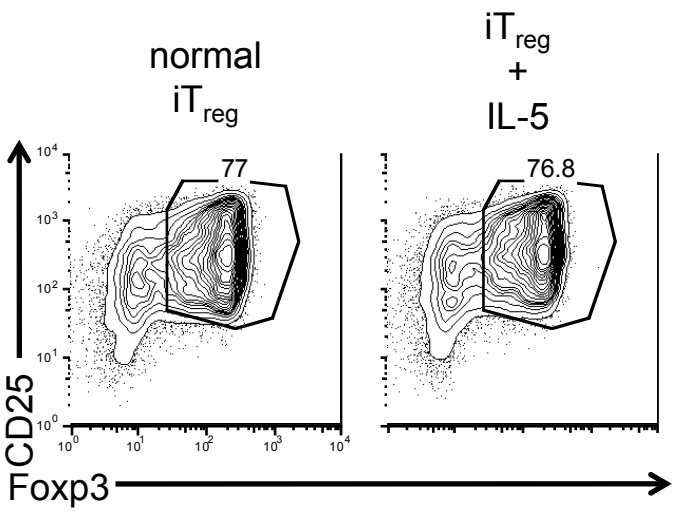


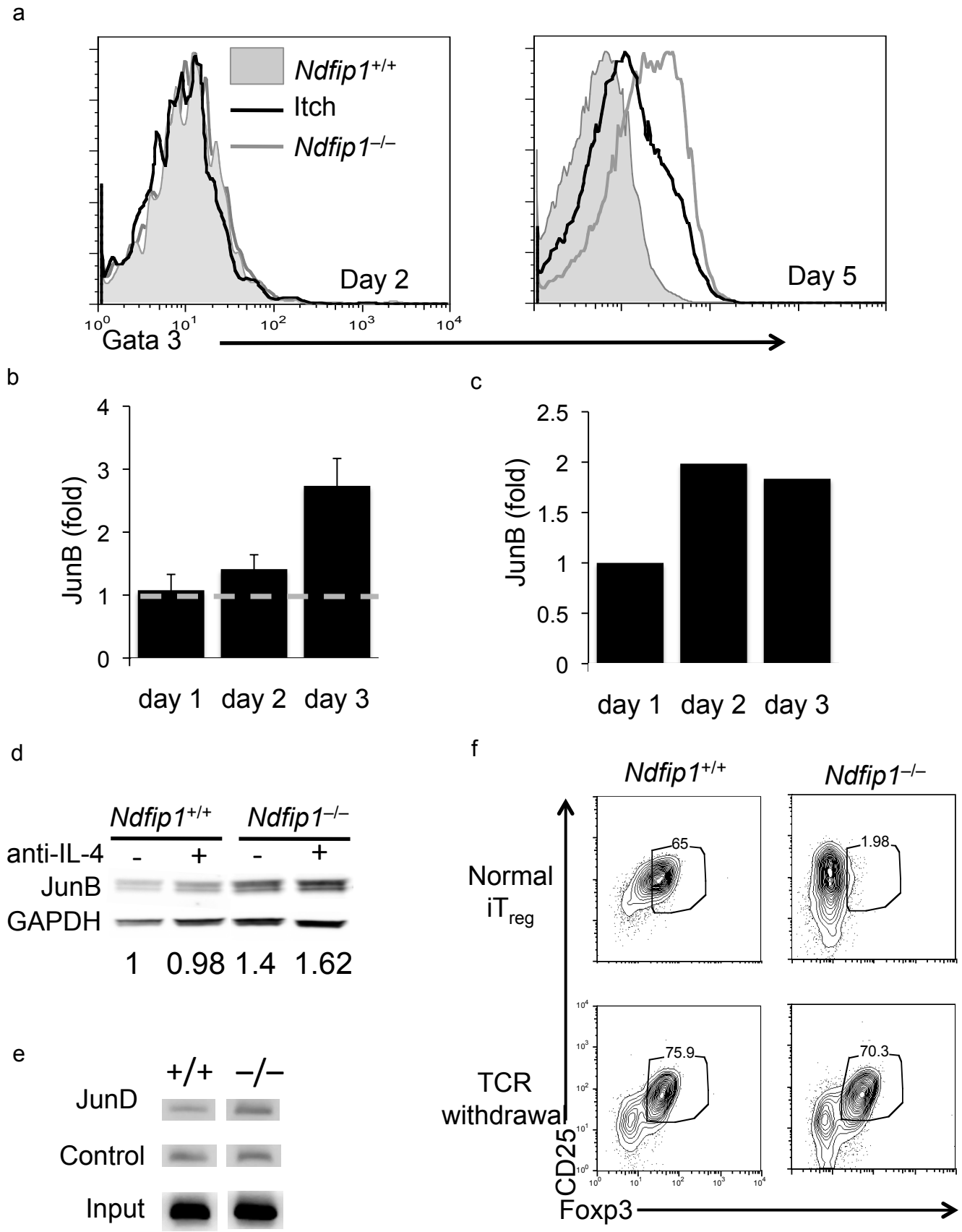
Supplementary Figure 7



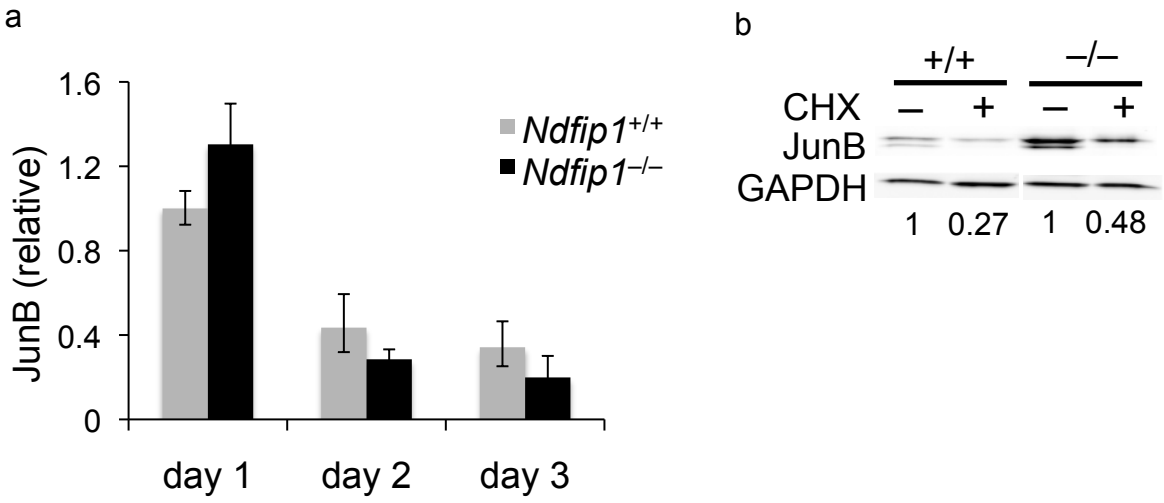
Supplementary Figure 8



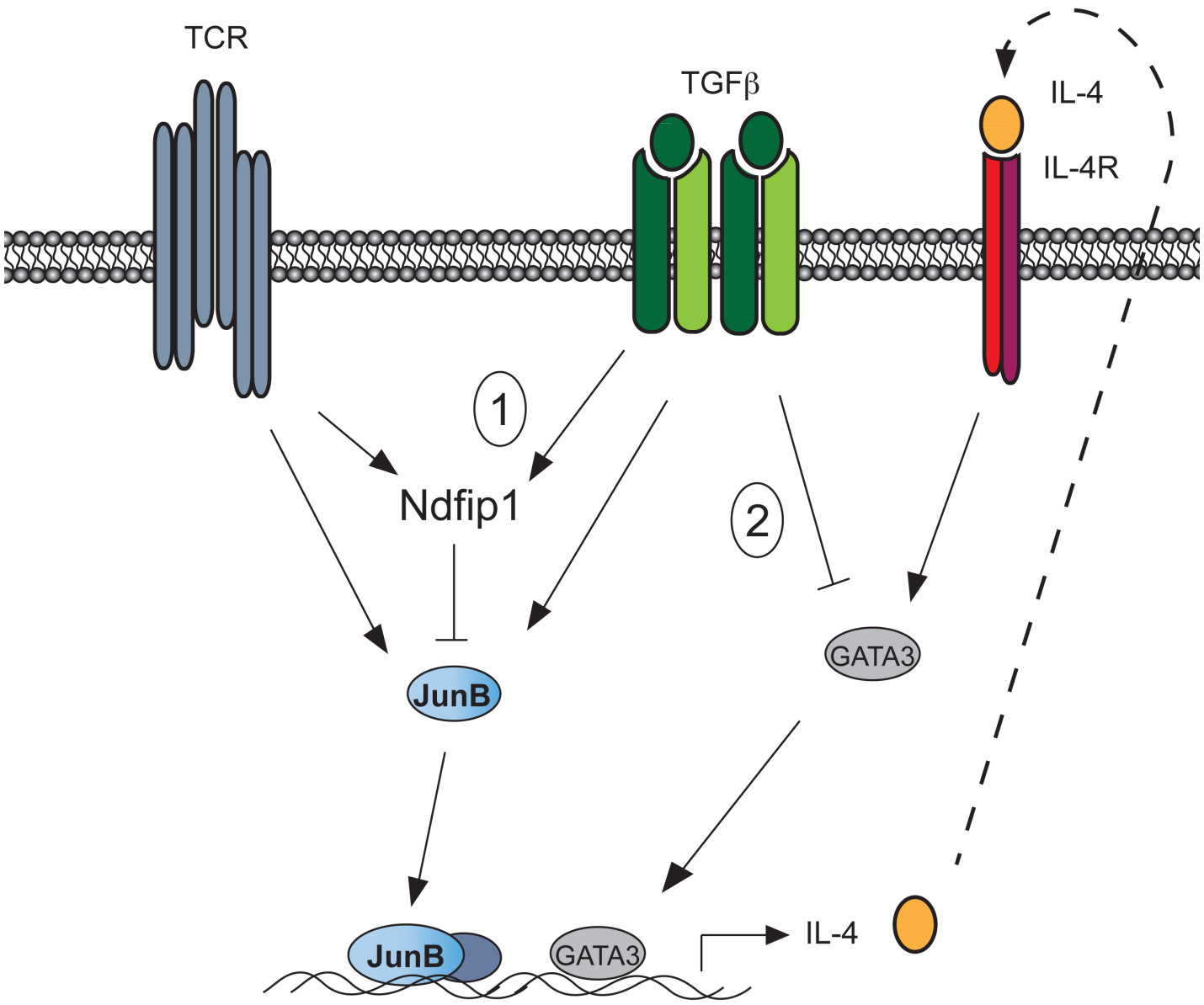
Supplementary Figure 9



Supplementary Figure 10



Supplementary Figure 11



Supplemental Material

Supplementary Figure Legends

Supplementary Figure 1. Foxp3$^+$ T_{reg} cell numbers and percentages in mice lacking Ndfip1.

(a-e) The number **(a, c and e)** and percentage **(b and d)** of CD4 SP Foxp3$^+$ T cells from the thymi of either 4-6 week old **(a)**, 9 day old **(b and c)**, or 2.5 week old **(d and e)** mice were analyzed by flow cytometry and the results are shown by the graphs. Each dot represents a single mouse and the bar represents the mean, n≥5. **(f)** Lethally irradiated CD45.1$^+$ recipient mice were reconstituted with either *Ndfip1*$^{+/+}$ or *Ndfip1*$^{-/-}$ CD45.2$^+$ bone marrow that was mixed at a 50:50 ratio with wild-type CD45.1$^+$ bone marrow. After reconstitution, the thymi of the chimeras were analyzed for the percentages of Foxp3$^+$ cells among the CD4 SP population, and the results are shown in the bar graph. The dashed line represents the percentage of CD45.2$^+$ *Ndfip1*$^{+/+}$ T_{reg} cells in the thymi of control bone marrow chimeras. **(g)** The total number of CD4$^+$ Foxp3$^+$ T_{reg} cells in the spleens of 4-8 week old *Ndfip1*$^{+/+}$ or *Ndfip1*$^{-/-}$ mice.

Supplementary Figure 2. OTII T cells are not activated effectors or T_{reg} cells prior to transfer and Ova feeding.

(a and b) Analysis of CD62L by CD44 **(a)** and Foxp3 **(b)** expression of purified OT-II T cells from *Ndfp1*$^{+/+}$ and *Ndfip1*$^{-/-}$ OT-II *Rag1*$^{-/-}$ mice prior to transfer and Ova feeding. Plots are representative of 3 mice. **(c)** CD4$^+$ T cells (CD45.2$^+$) from either *Ndfip1*$^{-/-}$*Rag1*$^{-/-}$OTII$^+$ or *Ndfip1*$^{+/+}$*Rag1*$^{-/-}$OTII$^+$ mice were transferred

intravenously into CD45.1$^+$ recipient mice as described in Figure 2. Recipient mice were then fed Ova for 5 days and the total number of CD45.2$^+$ T cells that converted into Foxp3$^+$ iT$_{reg}$ cells in the small bowel was assessed by flow cytometry and the results are depicted in the graph.

Supplementary Figure 3. TIEG1 binding to the Foxp3 promoter in Ndfip1- and Itch-deficient mice.

(a) Schematic representation of part of the Foxp3 locus with the non-coding exons in white and the coding exons (up to 7) in black. The TSS indicates the transcription start site. CNS1 and CNS2 (conserved non-coding sequences) from Zheng et al.[1] are indicated in the 5' UTR. The approximate location of the primers used is shown by green and red arrows. The proximal promoter primers are green and the enhancer regions primers from Venuprasad et al.[2] are in red. Note that this schematic is not drawn to scale. **(b)** Naïve CD4$^+$ T cells from *Ndfip1*$^{+/+}$ (Wt), *Ndfip1*$^{-/-}$ (N), and *Itchy* mutant (I) mice were and stimulated in the presence of 1ng/ml TGF-β. After the indicated time of incubation, cells were fixed and chromatin immunoprecipitation (ChIP) assay was perfomed. Unstimulated (U) wild-type naïve T cells were used as a control. Chromatin DNA obtained before (Input) and after immunoprecipitation (IP) with anti-TIEG1 or control IP (control serum or beads alone) was analyzed by PCR with primers specific for the *FoxP3* promoter (green arrows in **a**). Data are representative of 2 independent experiments for each of the two timepoints.

Supplementary Figure 4. Increased cell death or insufficient amount of IL-2 does not explain abrogated Foxp3 expression.

(a) Foxp3 expression was assessed in WT (open circles) and Itchy mutant (closed circles) T cells at day 2 and day 5 during iT_{reg} cell differentiation. Each dot represents a single mouse and samples from the same mouse are connected by a line. n=3 from 3 independent experiments. **(b)** Representative plots of T cells from the indicated genotypes were differentiated into iT_{reg} cells either with 1ng/ml TGF-β plus 50 U/ml IL-2 (normal iT_{reg}) or 1ng/ml TGF-β plus 100 U/ml IL-2 (high IL-2). Cells were analyzed for Foxp3 expression after 5 days of culture. **(c)** Naïve T cells were cultured under iT_{reg} cell conditions with 1ng/ml TGF-β and harvested on the indicated day. The graph depicts 7AAD staining of cells from individual mice, n≥3.

Supplementary Figure 5. Foxp3 expression is induced at day 2 of iT_{reg} culture in T cells lacking either Ndfip1 or Itch.

(a and b) qRT-PCR analysis of Foxp3 expression at days 0, 1, and 2 of iT_{reg} cell differentiation in *Ndfip1*$^{+/+}$ (WT), *Ndfip1*$^{-/-}$ **(a)** and *Itchy* mutant **(b)** T cells. Naïve T cells were stimulated in the presence of 1ng/ml TGF-β for the indicated time and then harvested as described in Figure 7a. Levels of FoxP3 in control cells (WT) at day 0 were set to 1 and results presented are relative to these levels. Data show one representative graph from 4 mice of each genotype (mean ± s.d. of triplicate samples). **(c and e)** ELISA analysis of IL-4 concentrations in the supernatants of T cells from the indicated mice stimulated for 2 days in the

presence (+) or absence (-) of 1ng/ml TGF-β. (**d)** The percentage of IL-4$^+$ cells detected by intracellular cytokine analysis of *Ndfip1*$^{-/-}$ T cells stimulated (anti-CD3/CD28, IL-2) in the presence of the indicated concentrations of TGF-β is shown in the graph. (mean ± s.d of 6 mice from 4 experiments)

Supplementary Figure 6. IL-4 production by transferred OT-II T cells lacking Ndfip1 from mice fed Ova for 5 days.

CD45.1$^+$ mice were intravenously injected with OTII CD45.2$^+$ *Ndfip1*$^{-/-}$ or *Ndfip1*$^{+/+}$ T cells and fed Ova for 5 days to induce Foxp3$^+$ iT$_{reg}$ cells as described in figure 2. IL-4 production was assessed after cells from the mLNs were stimulated overnight with Ova peptide (amino acids 323–339). **(a)** Cells were analyzed for IL-4 production the next day after restimulation for 4 hours with PMA and ionomycin followed by intracellular cytokine staining for IL-4. **(b)** IL-4 was measured by ELISA in supernatants harvested after overnight culture with Ova peptide, n=3.

Supplementary Figure 7. Once differentiated, iT$_{reg}$ cells that lack Ndfip1 can suppress WT T cell proliferation.

Ndfip1$^{+/+}$ and *Ndfip1*$^{-/-}$ T cells were differentiated under iT$_{reg}$ cell conditions with 1ng/ml TGF-β and anti-IL-4 antibodies. After 5 days, the cells were harvested and used in a T$_{reg}$ cell suppression assay. (a) Some of the cells were stained for Foxp3 to determine the efficiency of iT$_{reg}$ cell conversion. Representative plots are shown. (b) Representative histograms of CFSE dilution after 3 days are

shown for the T_{reg} cell suppression assay. Briefly, the indicated T_{reg} cells were added at the indicated ratios to WT T cells that were labeled with CFSE. WT T cells that did not receive T_{reg} cells are shown as a control.

Supplementary Figure 8. IL-5 does not inhibit iT_{reg} cell differentiation.
Since IL-5 is detected in the supernatants of *Ndfip1*$^{-/-}$ T cells undergoing iT_{reg} cell differentiation (data not shown), IL-5 was added to WT T cells undergoing iT_{reg} cell differentiation to test whether IL-5 blocks Foxp3 expression. WT cells were either stimulated under normal iT_{reg} cell conditions (1ng/ml TGF-β and 50 U/ml IL-2) or under iT_{reg} cell conditions in the presence of 1ng/ml IL-5. Foxp3 expression was analyzed by flow cytometry on day 5, n=2.

Supplementary Figure 9. JunB and Gata3 expression during iT_{reg} cell differentiation.
(a) Gata3 protein expression was assessed by flow cytometry in wild-type (gray shaded), *Itchy* mutant (black) and *Ndfip1*$^{-/-}$ (gray) T cells after 2 or 5 days of iT_{reg} cell differentiation. Plot is representative of two independent experiments. **(b)** Graph of JunB levels (normalized to GAPDH levels) in *Ndfip1*$^{-/-}$ T cells stimulated for the indicated days under iT_{reg} cell conditions. Levels are expressed as a fold expression over WT levels (set to one and indicated by the dashed gray line). Graph shows the mean + s.e.m. of at least 3 mice. **(c)** Graph of JunB levels (normalized to GAPDH levels) in WT cells stimulated for the indicated days under iT_{reg} cell conditions. Results are expressed as a fold expression over day 1 WT

levels (set to one). Graph shows the mean of 2 mice. (**d)** JunB protein expression was assessed by western blot in *Ndfip1*$^{+/+}$ and *Ndfip1*$^{-/-}$ T cells after 3 days of iT_{reg} cell differentiation in the presence (+) or absence (-) of anti-IL-4 antibodies, n=3. **(e)** Naïve $CD4^+$ T cells of the indicated genotypes were stimulated in the presence of 1ng/ml TGF-β for 2 days. Cells were then fixed and ChIP assays were performed as described above. Chromatin DNA obtained before (Input) and after immunoprecipitation (IP) with anti-JunD or control IP (beads alone) was analyzed by PCR with primers specific for the IL-4 promoter. Data are representative of 2 independent experiments, n≥4. **(f)** Foxp3 expression was assessed on day 4 after normal iT_{reg} cell differentiation or after TCR withdrawal. For TCR withdrawal conditions, naïve T cells were stimulated with 1ng/ml TGF-β and IL-2 and after 20-24 hours cells were removed from TCR stimulation and seeded into new wells with TGF-β and IL-2, n=4.

Note: T cells from both *Ndfip1*$^{-/-}$ and *Cd4-Cre Ndfip1*$^{fl/fl}$ mice and their respective controls were used for experiments in **b-f** (as well as **Fig. 10b** below). T cells from *Cd4-Cre Ndfip1*$^{fl/fl}$ mice responded the same as T cells from *Ndfip1*$^{-/-}$ mice.

Supplementary Figure 10. Decreased degradation of JunB in *Ndfip1*$^{-/-}$ T cells during iT_{reg} cell differentiation.

(a) JunB mRNA expression on days 1, 2, and 3 following iT_{reg} cell differentiation was analyzed by qRT-PCR. Naïve T cells were stimulated in the presence of 1ng/ml TGF-β for the indicated time and then harvested for RNA extraction and qRT-PCR analysis. Results presented are relative to the expression of JunB in

Ndfip1$^{+/+}$ T cells stimulated for 1 day and show one representative plot from 3-4 mice (mean + s.d. of triplicate samples). **(b)** Naïve *Ndfip1*$^{+/+}$ and *Ndfip1*$^{-/-}$ T cells were stimulated under iT_{reg} cell conditions for 2.5 days. At day 2.5, half of the cells were lysed while the remaining cells were treated with 20 ug/ml cyclohexamide for approximately 2 hours (+) prior to lysis. Western blot analysis of JunB and GAPDH is shown. Cyclohexamide-treated samples were normalized to their untreated counterparts from the same genotype and the normalized values are shown below the blots, n=3.

Supplementary Figure 11. Model: TGF-β induces Ndfip1 to degrade JunB, thus limiting IL-4 production during iT_{reg} cell differentiation.
TGF-β-stimulation induces an early peak of Ndfip1 mRNA expression at 24 hours (1). TGF-β-stimulation also causes an increase in JunB expression. Ndfip1 expression facilitates JunB degradation and this dampens IL-4 producton. Previously, TGF-β signaling was shown to inhibit GATA3 (2), which would also dampen IL-4 production. Thus, TGF-β limits IL-4 production during iT_{reg} cell differentiation through two distinct mechanisms.

Supplementary Methods

Mixed Bone Marrow Chimeras

Bone marrow was flushed from the femurs of various mice and depleted of T cells using complement and anti-Thy1.2 (AT83.A) antibodies. After depletion, cells were washed in sterile PBS, filtered and counted. A total of $5x10^6$ cells were

injected into the tail vein of lethally irradiated recipient mice (single dose of 1000 rads).

ELISA

Naïve T cells were stimulated as described above under iT_{reg} cell conditions with 1 ng/ml TGF-β and cultured for two days. Supernatants were collected on day two and kept at -80°C until used for ELISA. ELISAs were performed using the BD Biosciences Kit for detection of IL-4, according to the manufacturer's instructions. ELISA plates were analyzed using a Synergy HT Microplate Reader.

Intracellular IL-4 staining

Cells were cultured under iT_{reg} cell inducing conditions as described above and were restimulated on day five with 24 ng/ml of PMA, 1μM ionomycin, and gologistop (BD Biosiences) for 4 hr. Cells were collected and stained with anti-CD25. Cells were fixed and analyzed for intracellular IL-4 (11B11, BD Biosciences) and Foxp3 using the eBioscience Foxp3 staining buffer set.

ChIP for TIEG1 binding to Foxp3 gene

4.5×10^6 naïve T cells were stimulated as described for in vitro iTreg cell cultures in the presence of 1 ng/ml TGF-β for the indicated times. Cells were then harvested and washed two times with PBS for ChIP. After the last wash cells were then fixed as described in the Fast ChIP method[3]. After fixation cell pellets were frozen in -80^0C until all of the samples were ready for processing. We

followed the fast ChIP protocol with minor modifications to the immunoprecipiation protocol. Samples were precleared by incubating the lysates with protein-A beads (Millipore) blocked with sheared salmon sperm DNA for 45 minutes at 4^0C. Then precleared lysates were incubated overnight with either antibodies to TIEG1 (H-190, Santa Cruz) or rabbit IgG at 4^0C with end over end rotation followed by 45 minutes incubation with blocked protein-A beads at 4^0C with end over end rotation. Once the DNA was isolated, PCR was performed using either Foxp3 proximal promoter primers- forward, 5'-ACC TTT TAC CTC TGT GGT GAG GGG A-3' and reverse 5'-CGT GGA AGC CGC AGA CCT CG-3' or Foxp3 enhancer region primers- forward 5'-GAC TCA AGG GGG TCT CA-3' and reverse 5' TTG GGC TTC ATC GGC AA-3' (reverse)[2].

TCR withdrawal for iT_{reg} cell cultures

Naïve T cells were stimulated with 1ng/ml TGF-β anti-CD3, anti-CD28 and IL-2 and after 20-24 hours cells were removed from TCR stimulation and seeded into new wells with TGF-β and IL-2. Cells for cultured for either an additional 1 day or 3 days and then harvested for either western blot or FACS analysis for Foxp3 expression, respectively.

Q PCR for Foxp3 and JunB

Total RNA was extracted from cell lysates using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to manufacturers instructions. Since JunB is a single exon gene, isolated RNA was treated with DNase for 15 minutes at room

temperature followed by heating for 5 minutes at 70^0C to remove any possible contaminating genomic DNA. Following DNase treatment, cDNA was generated by reverse transcription using the High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA). Equal rates of conversion were assumed between the RNA template and the cDNA product when calculating the amount of cDNA needed for a 25 ng Q PCR reaction. For Q PCR, 25 ng of cDNA was added to TaqMan Gene Expression Master Mix and TaqMan Gene Expression primer/probe mix specific for Ndfip1 (Applied Biosystems, Foster City, CA) for a final reaction volume of 20 μl. Q PCR was performed using an Applied Biosystems 7500 Real-Time PCR system. The PCR protocol consisted of initiation of 1 cycle at 50°C for 2 minutes and 1 cycle at 95°C for 10 minutes. Following initiation, amplification occurred for 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. Ct data were collected using 7500 System Software (Applied Biosystems, Foster City, CA) during the 60°C hold time during amplification. Each sample was assayed in triplicate along with the endogenous control (either actin or 18s rRNA). Gene expression was calculated using the ΔΔCt method. The inventoried taqman gene expression assays Mm00475162.m1 and Mm01251660_s1 were from Applied Biosystems and were used to assess Foxp3 and JunB expression, respectively.

Western Blot

Cells were harvested at the indicated times and lysed for 15-20 minutes on ice with RIPA buffer (50mM Tris, pH7.5, 1% NP-40, 0.5% NaDeoxycholate, 0.1%

SDS + complete inhibitor tablets from Roche). Lysates were then either boiled for 5 minutes followed by freezing to be analyzed later or were then prepared for SDS-PAGE gel. The lysate was then centrifuged for 13000 rpm for 20 minutes. 5x laemmli sample buffer was added to cleared sample lysates and boiled for 5 minutes. Lysates were loaded by cell number equivalents and subjected to SDS-PAGE and transferred to PVDF. Blotting was performed as described in the materials and methods.

Supplementary References

1. Zheng, Y. et al. Role of conserved non-coding DNA elements in the Foxp3 gene in regulatory T-cell fate. *Nature*. **463**, 808-812 (2010).
2. Venuprasad, K. et al. The E3 ubiquitin ligase Itch regulates expression of transcription factor Foxp3 and airway inflammation by enhancing the function of transcription factor TIEG1. *Nat. Immunol*. **9**, 245-253 (2008).
3. Nelson, J. D, Denisenko, O. and Bomsztyk, K. Protocol for the fast chromatin immunoprecipitation (ChIP) method. *Nat. Prot*. **1**, 179-185 (2006).