Supporting Information

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SI Materials and Methods

Cells and Tissue Samples. The SUDHL-1, JB6, SUP-M2, L82, and SR786 cell lines were derived from ALK$^+$ TCL tissue samples. PB-1, -2A, and -2B cell lines were established from a patient with a progressive CD30$^+$ cutaneous lymphoproliferative disorder. MyLa2059, MyLa3675, and IL-2–dependent Sez-4 and SeAx cell lines were derived from cutaneous T-cell lymphomas. Their IL-2–independent sublines were generated in vitro. PBMC harvested from healthy adults were isolated by Ficoll/Paque centrifugation and stimulated in vitro for 72 h with a mitogen (PHA; Sigma). Reactive and ALK$^+$ TCL-containing tissues were harvested from excisional biopsies of lymph nodes or extranodal tumors obtained for diagnostic purposes. The diagnosis was established by standard morphological and immunohistochemical criteria including expression of the CD30 and ALK proteins. For DNA isolation, cells were culled from tissue sections deposited on glass slides and then snap-frozen, enriched for lymphoma cells by collecting areas within the sections containing predominantly malignant cells as determined by microscopic evaluation of the H&E-stained control slides.

Treatment of ALK$^+$ TCL Cells with DNMT, ALK, mTORC1, and Proteosome Inhibitors and Cytokines. SUDHL-1 cells were treated with 0.75 μM of DNMT inhibitor 5-ADC (Sigma) or ALK inhibitor-labeled compound 13 (1) for up to 3–4 d at several dosages and harvested at 24-h intervals for DNA, RNA, and protein extraction. Treatment with 10 nM of mTORC1 inhibitor rapamycin (LC Laboratories) was carried out for up to 82 h, typically 14 h after cell transfection with STAT3 siRNA (Dharmacon). In the selected *IL-2Rγ* gene-transfection experiments (Fig. 6*E*), SUDHL-1 cells were treated for the last 16 h before harvest with a proteosome inhibitor MG132 (Calbiochem) at 35 μM. In another subset of the *IL-2Rγ* gene-transfection experiments, SUDHL-1 cells were treated for up to 68 h with human recombinant IL-2, IL-4, IL-9, and IL-15 (R&D) before processing.

DNA Oligonucleotide Array. Total RNA isolated from triplicate cultures of cells treated with 5-ADC was reverse transcribed, biotin labeled, and hybridized to U133 plus 2.0 array chips (Affymetrix) constructed with the 54,000 DNA oligonucleotide probe set. Hybridization results were normalized using Gene Spring (Agilent Technologies) and further analyzed using Partek GS, Spotfire Software, and Gene Spring programs.

RT-PCR and RT-qPCR. Total RNA was extracted (RNeasy kit; Qiagen) and reverse transcribed (SuperScript II reverse transcriptase; Invitrogen). In conventional RT-PCR experiments, the reverse-transcribed product was amplified in duplicate by PCR using primers specific for IL-2Rγ (5′-AATTCCCACCCTGAAGAACC-3′ and 5′-ACGAGGCAGAGTCGTTCACT-3′) and standard reagents and conditions for 30 cycles. Expression levels of DNMT1, DNMT3a, DNMT3b, NPM-ALK mRNA, and miR-21 were quantified in RT-qPCR by using an ABI/PRISM 7700 sequence detection system with TaqMan Gene Expression Assay and MicroRNA Assay kits (DNMT1, Hs00945899; DNMT3a, Hs01027166; DNMT3b, Hs01003410; NPM-ALK, Hs03024829; and miR-21, 4427975; all from ABI). Actin (Hs03023880) and RNU48 (Hs4427975) were used as endogenous controls. All assays were performed in duplicate. The fold difference in RNA levels was calculated on the basis of the difference between *Ct* values obtained for control and individual mRNA or miR (*dCt*).

Western Blotting. Western blotting experiments were performed using antibodies against NPM-ALK, NPM1, p-ALK, p-STAT3, and p-p70S6K (BD Pharmingen Cell Signaling Technology); STAT3, DNMT1, and actin (Santa Cruz); and DNMT3a and DNMT3b (Active Motif) according to the standard protocols.

DNA Methylation Analysis. Total cellular DNA was isolated using the DNeasy Tissue kit (Qiagen), then bisulfite modified using the CpGenome DNA Modification kit (Intergen), and then amplified by PCR with Platinum Taq DNA polymerase (Invitrogen) for 40 cycles under standard conditions using biotinylated primer pairs specific for the *IL-2Rγ* gene promoter (5′-GGGAAGTTTGAAGTTAGTATTGTTGTT-3′ and 5′-AACCACCTTCTCCTCTAAATCATTACC-3′; and 5′-GAGAATGGTGTTAGTGGTAGTGAATAGA-3′ and 5′CCACTCCCAACAAAAACAACTACA-3′). PCR amplification products were purified, rendered single stranded on a Pyrosequencing workstation (Pyrosequencing AB), and annealed with the sequencing primers (S1, 5′-AATATTTTTGTATTATAAGTTATATT-3′; S2, 5′-GGAAAGATAGTTGTATATGTGTT-3′; S3, 5′-GGTGATTAAGTTAAGGAAGA-3′; S4, 5′-ATTTTATAGTAGTATTTAATTTTTTAGA-3′; S5, 5′-TGTAAAGTTTTGGTTTATAAG-3′; S6, 5′-GATTATATTTAGGGAATGAAGA-3′). Quantitative analysis of DNA methylation was performed on a PSQ 96MA system using the PyroGold SQA reagent kit (Pyrosequencing); results were analyzed using Q-CpG software (V1.0.9; Pyrosequencing). All samples were tested in duplicate.

Luciferase Reporter Assay. The IL-2Rγ promoter DNA sequence was amplified by PCR with primers 5′-*GGTAC*CCATTGACTGAGGTGGGGAAG-3′ and 5′-*CTCGA*GATGGCGCTTGCTCTTCATT-3′ (the italicized sequences represent nucleotides added to the complementary sequences to generate KpnI- and XhoI-specific restriction digest sites). The 695-bp PCR product was gel purified and cloned into the pGL3-Basic Luciferase Reporter Vector (Promega), placing the IL-2Rγ promoter upstream of the firefly luciferase gene. Integrity of the construct was verified by sequence analysis. To create derivatives of this construct methylated at specific CpG sites within the IL-2Rγ promoter, promoter fragments were excised using appropriate restriction enzyme pairs. The fragments were methylated separately and then ligated into the vector. For a construct in which all eight CpG sites in the IL-2Rγ promoter were methylated, KpnI and XhoI were used for digestion, and the IL-2Rγ promoter insert was methylated using SssI methylase. For a construct methylated at IL-2Rγ promoter sites 3 and 4, the plasmid was digested with CviAII and BbcCI, and the promoter fragment was methylated using SssI methylase. For constructs methylated at site 6 and site 8 in the IL-2Rγ promoter, the plasmid was digested with Kpn1 and XhoI, and the promoter fragment was methylated using HpaII (site 6) or HpaI (site 8). HEK293 cells were transfected in duplicate with each construct in combination with the pRL-TK plasmid (Promega), included to provide a measure of transfection efficiency, using Lipofectamine 2000 (Invitrogen) and SuperFect Transfection Reagent (Qiagen) according to the manufacturers' instructions. Twenty-four hours after transfection, cells were washed, lysed, and evaluated sequentially for firefly luciferase and Renilla luciferase activities (from the IL-2Rγ promoter construct and pRL-TK, respectively), using the Dual-Luciferase Reporter Assay System (Promega) and a BD Monolight 3010 luminometer (BD Biosciences). Results were normalized for transfection efficiency by dividing the firefly

luciferase activity by the Renilla luciferase activity in each sample. Promoter activity was reported as the mean ± 1 SD.

siRNA Assay. A mixture of four siRNAs specific for ALK, STAT3, or DNMT1 or control, nontargeting siRNAs (all from Dharmacon) or mimic-miR-21 (ABI) was introduced into cells using Lipofectamine 2000 as described previously (2).

Electrophoretic Mobility Shift Assay (EMSA). Proteins extracted from nuclei isolated from various cell lines were incubated with biotin-labeled oligonucleotide probes corresponding to the *IL-2Rγ* gene promoter region that contains the STAT3 binding site (5′-CCACTCCTTTGTTAATACCAAGG-3′ and 5′-GAACATGATTGCTGAAAGAGTATGT-3′). Protein–DNA complexes were separated by gel electrophoresis and transferred to membranes for Western blotting as described previously (3). Blots were developed using the HPR system (Pierce).

Chromatin Immunoprecipitation (ChIP) Assays. These experiments were performed as described previously. In brief, cells were fixed with formaldehyde and lysed, and the lysates were sonicated. Samples of sonicated lysates were incubated with antibodies against DNMT1, DNMT3a, DNMT3b, STAT3, or murine IgG (Abcam, Active Motif, and Santa Cruz), after which DNA–protein immunocomplexes were collected using protein A–agarose beads and treated with RNase A and proteinase K. DNA was purified from the digested samples by phenol-chloroform extraction and ethanol precipitation and then assayed for the presence of the *IL-2Rγ* gene promoter or miR-21 promoter by quantitative PCR, using IL-2Rγ promoter-specific primers (5′-CATTGACTGAGGTGGGGAAG-3′ and 5′-ACTGGCGAGGAAGTGTGACT-3′) or miR-21 promoter-specific primers (5′-GCATACTGCTAAATGGCACCT-3′ and 5′-AACACAGATACGACAGAGTGTGG-3′). For two-step "re-ChIP" assays, anti-STAT3 immunoprecipitates were reimmunoprecipitated with antibodies against DNMT1, DNMT3a, or DNMT3b before further processing.

Plasmid Construction and Transient Transfections. The complete coding region of the human IL-2Rγ cDNA was cloned into the pcDNA3 vector (Invitrogen). Briefly, the IL-2Rγ cDNA was obtained by reverse transcription of total RNA from a healthy donor using an oligo(dT) primer and then amplified by PCR, using primers for the IL-2Rγ coding region 5′-GGTACCATGTTGAAGCCATCATTACC-3′ and 5′-CTCGAGTCAGGTTTCAGGCTTTAGGG-3′ designed with "tails" corresponding to specific restriction enzyme sites (KpnI in the 5′ primer and XhoI in the 3′ primer of IL-2Rγ). A 1,122-bp IL-2Rγ fragment was cloned using KpnI (upstream) and XhoI (downstream) sites within the vector. cDNA integrity was confirmed by sequence analysis of the insert. For transient transfection assays, final vectors were transfected into cells using the Lipofectamine 2000 Transfection kit (Invitrogen).

Flow Cytometry. Cells were incubated with medium, PE-conjugated anti-IL-2Rγ, or control anti-IgG antibody (BD Biosciences) for 30 min at room temperature and then washed in PBS and analyzed by flow cytometry (FACSCalibur; BD Biosciences). Data acquisition and analysis were performed using CellQuest Pro software.

Cell-Cycle Analysis. Cells were washed and suspended in 100 μL of cold PBS. Then 900 μL of cold methanol was added to the cells and incubated on ice for 1 h. The cells were washed twice and suspended in hypotonic propidium iodide solution (10 μg propidium iodide, 10 μg RNase A, 0.5% Tween 20) for 0.5 h at 37 °C before analysis by flow cytometry.

MTT Enzymatic Conversion Assay. Cells at 2×10^4/well were incubated at 37 °C in microtiter plates for up to 3 d and then incubated with MTT to 10 μL (Promega) for 4 h. Well contents were solubilized overnight in 10% SDS in 0.01 M HCI. Absorbance at 570 nm in each well was measured using a Titertek Multiskan reader.

Apoptosis Assay. Quantitative analysis of apoptosis in cells transfected with pcDNA3 or the pcDNA3–IL-2Rγ construct was performed at 72 h posttransfection using the Annexin-V-FLUOS Staining Kit (Roche) according to the manufacturer's instructions, with detection of staining by flow cytometry.

1. Lu L, et al. (2009) ALK mutants in the kinase domain exhibit altered kinase activity and differential sensitivity to small molecule ALK inhibitors. *Biochemistry* 48:3600–3609.
2. Zhang Q, Wang HY, Liu X, Wasik MA (2007) STAT5A is epigenetically silenced by the tyrosine kinase NPM1-ALK and acts as a tumor suppressor by reciprocally inhibiting NPM1-ALK expression. *Nat Med* 13:1341–1348.
3. Zhang Q, et al. (2005) STAT3- and DNA methyltransferase 1-mediated epigenetic silencing of SHP-1 tyrosine phosphatase tumor suppressor gene in malignant T lymphocytes. *Proc Natl Acad Sci USA* 102:6948–6953.

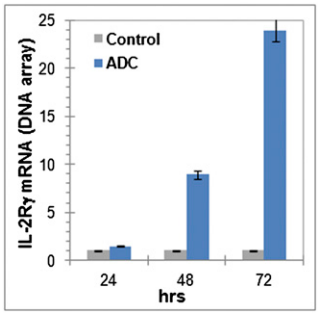

Fig. S1. Expression of the *IL-2Rγ* gene determined by the DNA oligonucleotide array in SUDHL-1 cells cultured in the presence of medium or DNMT inhibitor ADC for 24, 48, or 72 h. The data are depicted as fold increase in the IL-2Rγ mRNA expression in the ADC-treated cells compared with the cells exposed to medium containing the drug vehicle.

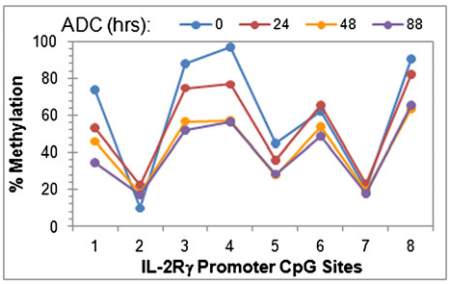

Fig. S2. Changes in IL-2Rγ promoter methylation induced in SUDHL-1 cells by treatment with ADC for 0, 24, 48, and 88 h.

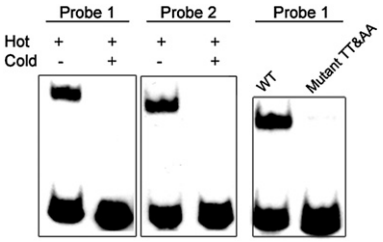

Fig. S3. Conformation of binding specificity of STAT3 to the *IL-2Rγ* gene promoter in vitro detected by EMSA using unlabeled (cold) and mutated DNA probes, using SUDHL-1 cell lysates.

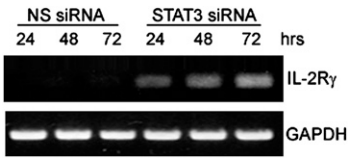

Fig. S4. Kinetics of the STAT3 siRNA-induced expression of IL-2Rγ mRNA detected by standard RT-PCR in SUDHL-1 cells.

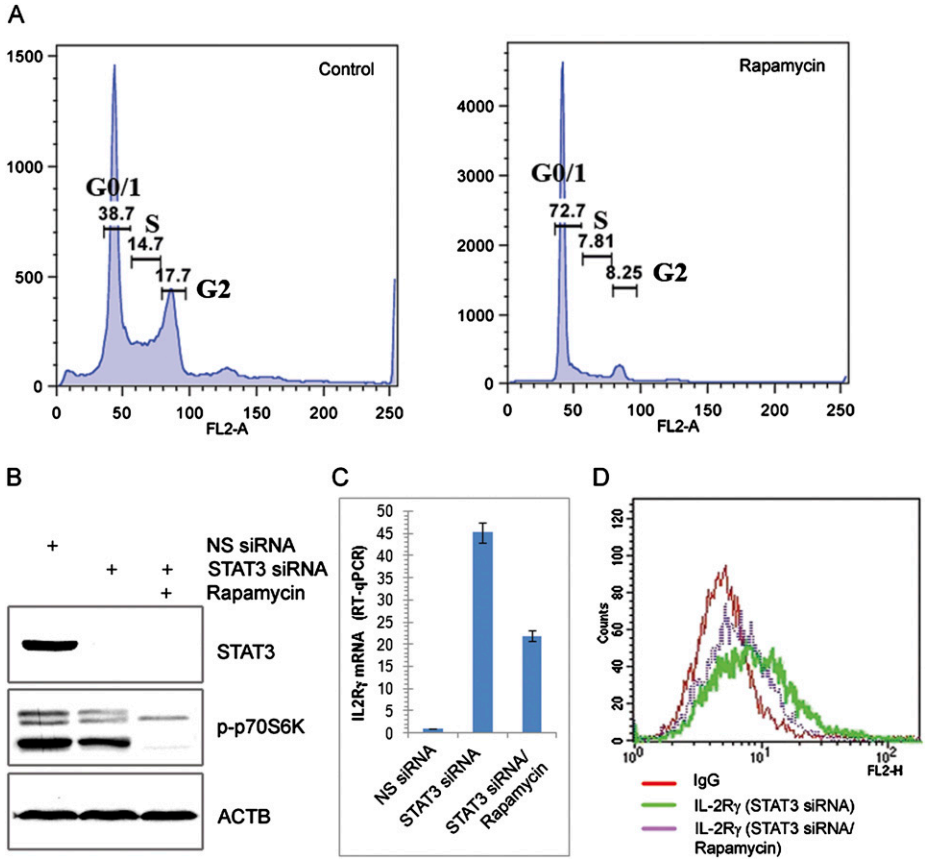

Fig. S5. Effect of cell replication inhibition on STAT3 depletion-induced expression of the *IL-2Rγ* gene. (*A*) Impact of the mTORC1 inhibitor rapamycin on cell-cycle progression in SUDHL-1 cells. (*B–D*) Impact of STAT3 siRNA and the mTORC1 inhibitor rapamycin on STAT3 expression and p70S6K phoshorylation (*B*) and IL-2Rγ expression at the mRNA (*C*) and protein (*D*) levels.

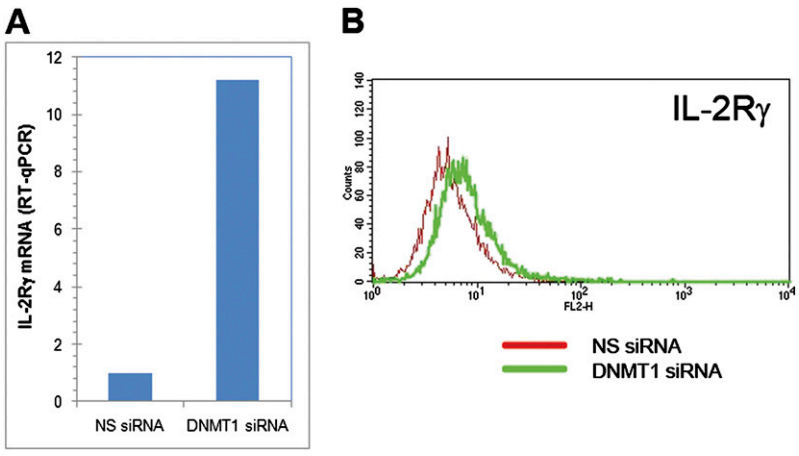

Fig. S6. Effect of siRNA-mediated DNMT1 depletion on expression of IL-2Rγ mRNA (*A*) and protein (*B*) in SUDH-L1 cells.

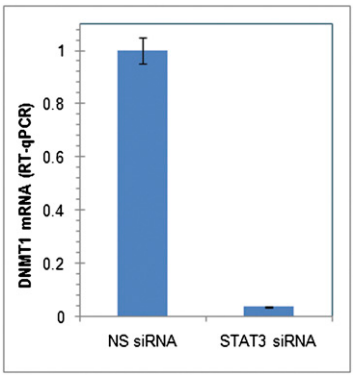

Fig. S7. Effect of siRNA-mediated STAT3 depletion on expression of DNMT1 mRNA by SUDH-L1 cells.

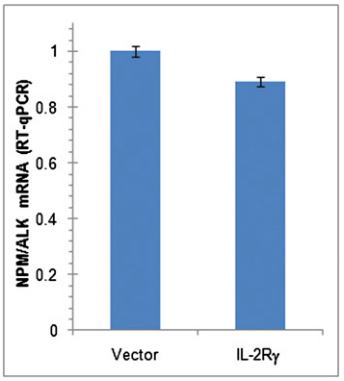

Fig. S8. Effect of enforced IL-2Rγ expression on expression of NPM-ALK mRNA in SR786 cells.