Methods

Immuno-precipitation, Western blot analysis, and EMSA. Whole cell extracts were prepared in RIPA buffer (50 mM Tris-HCl pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTT, with 1 mM PMSF, 1 µg/ml each of Aprotinin, leupeptin and pepstatin, 1 mM Na3VO4, and 1 mM NaF) according to the manufacturer's recommendation (Santa Cruz Biotechnology). The Bio-Rad (Hercules, CA) protein assay was used to measure protein concentrations and 0.5-1 mg of whole cell extracts was for immunoprecipitation. For electrophoretic mobility shift assay (EMSA), whole extracts were incubated for 30 min with oligonucleotide probes that were labeled with γ-32P-ATP at the 5’end using T4 kinase. Unlabeled competitor oligonucleotides (50-fold molar excess) or anti-Stat3 antibody was incubated with the extracts on ice for 30 min prior to the addition of labeled probe.

In vitro deacetylation assay. Immuno-precipitated Flag-HDACs were stored in HD buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 10% glycerol) and used in the deacetylase reactions according to the published protocol with modifications (16). In brief, acetyl-Stat3 proteins immunoprecipitated (using antibodies to cMyc) from 293T cells transfected with cMyc-Stat3 and HA-p300 were incubated with immuno-precipitated Flag-Hdac1, 2, or 3 in 50 µl of HD buffer at room temperature overnight with mild shaking. To stop the reactions, 10 µl of stop solution (0.16 M acetic acid, 1.0 M HCl) was added into each reaction and tubes vortexed. Fractions of the reactions were subjected to Western blot analysis with antibodies to acetyl lysine.

FACS analysis and ³H-thymidine incorporation analysis. FACS analysis: After harvesting and washing, PC3 cells were fix in ice-cold 70% ethanol while vortexing, centrifuged at 2000 rpm for 5 min then washed twice in PBS. The cells were suspended 1 ml of propidium iodide (50µg/ml) staining solution and treated with 1mg/ml ribonuclease for 5 min at room temperature. Samples were analyzed by flow cytometry using 488 nm excitation wavelength, gating out doublets and clumps using pulse processing and collecting fluorescence above 620 nm. ³H-thymidine incorporation: PC3 cells were seeded into 6-well plates at a density of 3x10⁵ cells per dish and cultured in F-12K containing 10% FBS at 37°C under humidified air containing 5% CO₂. Six replicates were used for each sample. After 24 hr, the cells were washed three times with pre-warmed F-12K and serum-starved in F-12K for 18 hr. The culture medium was removed, and the cells were incubated in the presence or absence of OSM and containing F-12K 1 µCi/ml ³H-thymidine for 20 hr. At the end of
the incubation, the cells were washed three times with PBS, precipitated with 10% trichloroacetic acid (TCA) at room temperature for 30 min, washed 3 times with TCA, and solubilized with 1 M NaOH for 30 min and neutralized with 1N HCl and the radioactivity was determined by liquid scintillation counting.

**Mass spectrometry analysis of acetylated Stat3.** Stat3 proteins, immunoprecipitated from 293T cells cotransfected with Stat3 and p300, were resolved by SDS-PAGE and visualized using Coomassie Blue stain. The single Stat3 band (1.2 µg) was excised and digested with trypsin. The resulting tryptic peptides were analyzed by mass spectrometry to determine their individual mass values (Taplin Biological Mass Spectrometry Facility at Harvard Medical School).

In brief, protein bands excised from gels were subjected to a modified in-gel chymotrypsin digestion procedure. Peptides were then extracted by removing the ammonium bicarbonate solution, followed by two washes with a solution containing 50% acetonitrile and 5% formic acid. The samples were then dried and reconstituted in 5 µl of HPLC solvent A (5% acetonitrile, 0.005% heptafluorobutyric acid, 0.4% acetic acid). A nano-scale reverse-phase HPLC capillary column was created by packing 5 µm C18 spherical silica beads into a fused silica capillary (75 µm inner diameter x 12 cm length). After equilibrating the column, each sample was pressure-loaded off-line onto the column, which was then reattached to the HPLC system. A gradient was formed and peptides were eluted with increasing concentrations of solvent B (95% acetonitrile, 0.005% heptafluorobutyric acid, 0.4% acetic acid). By comparing the measured mass values of the peptide sequences that contain putative acetylation site(s) with the mass values of all possible trypsin-digest peaks of Stat3, a mass shift with a differential mass \( m = 42 \) Daltons of the chymotryptic peptide fragment LYPDIPK_{ac}EEAFGKY of Stat3 was observed in the spectrum. An LCQ linear ion trap mass spectrometer (Thermo Finnigan, San Jose, CA) was configured to continually perform directed MS/MS events for the chymotryptic peptide LYPDIPK_{EEAFGKY} (m/z 835.5 with a mass width of 2 Daltons) and the acetylated version of the same peptide (m/z 856.5 with a mass width of 2 Daltons). The peptides were identified by matching protein databases with the acquired fragmentation pattern by the software program, Sequest (ThermoFinnigan, San Jose, CA).
Fig. S1. Cytoplasmic and nuclear fractions were prepared from MCF-7 cells followed by OSM treatment for the indicated times. Anti-Stat3 immunoprecipitates (IP) from these fractioned extracts were analyzed with antibodies to acetyl lysine or pY$^{705}$-Stat3.

Fig. S2. (A) Schematic illustration of Stat3 domains and their truncations. ND: N-terminal domain, CC: coiled-coil domain, DBD: DNA binding domain, and LD: linker domain. (B) Cytoplasmic (Cyt) and nuclear (Nuc) fractions were prepared from HeLa cells treated with IFN-α (1000 U/ml) for 30 min. Type I HDAC immunoprecipitates (IP) prepared by using antibodies to Hdac1 (cat # sc-7872, Santa Cruz Biotechnology), Hdac2 (cat # sc-7879, Santa Cruz Biotechnology), and Hdac3 (cat # sc-11417, Santa Cruz Biotechnology) were analyzed with antibodies to Stat3, Hdac1, Hdac2, or Hdac3. IκBα localization in these fractions was determined by Western blot analysis (bottom panel).

Fig. S3. (A) Analysis of lysine sites of Stat3 N-terminal domain and DBD in acetylation. cMyc-tagged Stat3 with single or double K→R substitution were transfected with HA-p300 in 293T cells. Anti-cMyc immunoprecipitates were analyzed by Western blot with antibodies to acetyl lysine and Stat3. (B) One µg each of unrelated peptide, Stat3 K$^{685}$-peptide (EEAFGK$^{685}$YCRPESQ), and Stat3 acetyl-K$^{685}$ peptide were spotted onto a nitrocellulose membrane. The peptide-immobilized membrane was immunoblotted with antibodies to acetylated lysine (1:1000). (C) Alignment of the dimerization domain among STAT family members. Blue letters indicate charged amino acids and the red color indicates the lysine, tyrosine, and serine sites that are post-translationally modified. Yellow shading indicates the α-helix predicted by a protein secondary structural analysis program.

Fig. S4. PC3 cells expressing wild-type Stat3 or Stat3-K$^{685}$R mutant were grown on a glass slide fixed and immuno-stained with an antibody to Stat3. Treatment of PC3 cells with OSM for 30 min resulted in an exclusive nuclear pattern of Stat3 distribution. Results are derived from inspection of at least 300 transfected cells present in multiple microscopic fields from two independent experiments.
Fig. S5. PC3 cells expressing empty vector (null), wild-type Stat3 (WT), or Stat3$^{K685R}$ (K685R) were treated with OSM (10ng/ml), TSA (0.2 µM), or OSM (10ng/ml) plus TSA (0.2 µM) for 12 hrs. Lysates were analyzed in Western blot with antibodies to cyclin D1 or β-tubulin.

Fig. S6. Schematic model for the role of post-translational modifications involved in Stat3 activation in gene regulation. Together with tyrosine phosphorylation, lysine acetylation in the dimerization domain allows Stat3 to form stable dimers required for maximal transcriptional activation.
Fig. S1

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Fig. S2

(A)

(B)

IP: Stat3

IFN-α: - + - +

Cyt | Nuc

Hdac3

Hdac2

IFN-α: - + - +

Cyt | Nuc

Hdac3

Hdac2

IFN-α: - + - +

Cyt | Nuc
Fig. S3

(A) N-terminal Domain and DBD

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(B) peptide: Unrelated K685 Ac-685 IB: anti-Ac-Lysine AB

(C) (A) Hs-Stat3: LVLYPDIPKTFAGKYCRPESQ--EHPEDPGSAP YLTKKFICVTPTTSNID----------LPMSPRA
Hs-Stat1: LKYLYPDIDKFAKYYSRKEAPEMELDGPKTGY TIKTELISVESVHSRLQTTTDN----------LPMSPEE
Hs-Stat4: LKYLYPDIPKFAKGHYSQFC--VSRPTERGDKTVPSVFPISTIRSDSTPHPSD----------LPMSPSV
Hs-Stat5a: LIYVFDPDKDEVSFLYTPVL----------AKAVDGTVKPQIKQVFPEFNASADAGGSSATMDQAPSPAV
Hs-Stat5b: LIYVFDPDKDEVSFLYTPVL----------AKAVDGTVKPQIKQVFPEFNASADAGGSSATMDQAPSPAV
Hs-Stat6: LKNLPKPSDEAFSSHKYEQ----------MGKDGRVTATIKMTERDQPLTPELQMPMVPSYLDGMAP
Hs-Stat2: LRFLYPRIIPDPDEFGCYEDEKV----------NLQERRKYLKHLIVVSNQRQDELQQPLELKPELESELEEL

DmD TAD
Fig. S4

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Fig. S5

Cytoplasm

Fig. S6