Supplement

Materials and methods

Reagents

The following antibodies were used for these studies: c-Jun from Cell Signaling Technology (Beverly, MA); HA.11 (Covance Research Products, Berkeley, CA); Grb2 (sc-255), Stat5b (sc-835X; sc-1656), Stat3 (sc-7179), c-Fos (sc-52X) and mouse IgG from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); phospho-Stat5a/b Tyr$^{694/699}$ (71-6900), and PRLR (35-9200) from Zymed (S. San Francisco, CA). Recombinant hPRL (Lot AFP795) was obtained through the National Hormone and Pituitary Program, NIDDK, and Dr. Parlow. All other reagents were obtained from Sigma-Aldrich Corp. unless otherwise noted.

Plasmids

The AP-1-luciferase construct (4XAP-1-luc) contains four GCN4 consensus AP-1 responsive elements located upstream of a luciferase reporter (Dong et al., 1996). The GAS-luc construct contains 3 copies of the γ-interferon activating sequences (GAS) from the β-casein gene upstream from a luciferase reporter (Schroeder et al., 2002). Expression constructs employed were: long PRLR isoform (lPRLR) from C. Clevenger (Northwestern University), wild type rat Stat5a and b (WT Stat5a/b) and mutant Stat5Y699F from E.B. Kabotyanski and J. Rosen (Baylor College of Medicine), and murine Stat5a with a truncated C-terminal transactivation domain (Stat5Δ53C) from R.L. Ilaria (Ilaria, Jr. et al., 1999).

Cell culture

PRL-deficient MCF-7 cells were maintained as previously described (Schroeder et al., 2002). These cells were grown in phenol red free RPMI-1640 containing 5% charcoal stripped FBS (3XCSS), penicillin, and streptomycin 4-5 days prior to experiments. MCF-10A cells were maintained in DMEM/F12 supplemented with 5% equine serum, penicillin/streptomycin, cholera toxin, hydrocortisone, insulin, and EGF. T47D cells were maintained in RPMI-1640 supplemented with 10% Fetal Bovine Serum (FBS) and penicillin/streptomycin. MDA-MB-231 cells were maintained in DMEM/F12 supplemented with 10% FBS and penicillin/streptomycin. CHO cells were maintained in DMEM/F12 supplemented with 5% FBS and penicillin/streptomycin.

Transient transfections

MCF-7, MCF-10A, T47D, MDA-MB-231 and CHO cells were plated into 12 well tissue culture plates at $3x10^{5,}$ $3x10^{5}$, $2x10^{5}$, $4x10^{5}$, $1x10^{5}$ cells/well, respectively, to achieve about 70% confluence prior to experiments. All cells were serum starved for 24 h prior to transfection. MCF-7, MCF-10A, and CHO cells were transiently transfected using SuperFect (Qiagen Inc., Valencia, CA) as previously described (Brockman et al., 2002). After 4 h, the transfection complex was replaced with serum free media containing the indicated treatments. MDA-MB-231 cells were transfected using TransIT-LT1 (Mirus, Madison, WI) according to manufacturer's protocol and treated as indicated in the presence of the transfection complex. T47D cells were transfected with Lipofectamine2000 (Invitrogen, Carlsbad, CA). DNA (1.6μg/well) and transfection and reagent (3.2μL/well) in Opti-MEM (Invitrogen, Carlsbad, CA) in serum free RPMI-1640 were incubated with cells for 24h at 37°C, and treated as indicated in the presence of the transfection complex. Within each experiment, the total amount of transfected DNA was equalized with vector DNA. Cells lysates were harvested following hormone treatment at the times indicated and analyzed for luciferase and β-galactosidase (β-gal) activities. Luciferase values were corrected for transfection efficiency using β-gal, as described (Brockman et al., 2002). "Relative activity" is the mean of at least three independent experiments represented as fold change relative to the vehicle control.

siRNA

In order to insure that we reduced both Stat5 isoforms, which can equally reduce AP-1 activity (Fig. 1), we designed Stat5 specific siRNA (5'-CUACAGUCCUGGUGUGAGA-3') with the si*Design* center (Dharmacon, Lafayette, CO) using a region homologous to both the Stat5a and Stat5b isoforms. A predesigned non-targeting siRNA (Dharmacon, Lafayette, CO) was used as a control. All siRNA duplexes were used at 100nM, and were transfected with Lipofectamine2000 for 24-48h as indicated using 2μL transfection reagent/20pmol of siRNA. When cotransfected with plasmid DNA, siRNA was incubated with the plasmid DNA, and the total volume of transfection reagent was adjusted accordingly.

Co-immunoprecipitation

Cells were serum starved for 24h and treated -/+4nM PRL for 1hr. Lysates were harvested in buffer containing 10mM Tris (pH 8), 150mM NaCl, 1mM EDTA, 10%(v/v) glycerol, 0.5% (v/v) NP-40, 1mM Na_3VO_4, 1μM PMSF and 1μg/mL aprotinin. Approximately 900μg of protein was

precleared by incubation with Protein-G-agarose beads (Upstate Biotechnology, Charlottesville, VA), and precleared lysate, along with 10% BSA, was added to anti-Stat5 conjugated agarose beads and incubated for 2-3h at 4°C. Agarose beads were washed with cold PBS and resuspended in 50µL of electrophoresis sample buffer.

Immunoblotting

Cells grown in complete media (above) were analyzed by Western blot as previously described (Schroeder et al., 2002). Primary antibody concentrations were as follows: c-Jun, 1:1000; c-Fos, 1:1000; hPRLR, 1:1000; Stat5 (sc-835X) 1:500 000; phospho-Stat5, 1:1000; and Stat3, 1:500.

Cellular fractionation

Cells were transfected with either HA-Stat5Y699F or HA-Stat5Δ53C, serum starved for 24h, and treated -/+4nM PRL for 30min. Cells were lysed with buffer containing 50mM HEPES (pH 7.5), 150mM NaCl, 10mM EGTA (pH 7.5), 1mM PMSF, 10% (v/v) glycerol, 0.1mM Na_3VO_4, and 2µg/mL aprotinin. Nuclei were spun down at 650xg at 4°C, and the supernatant containing the cytosolic fraction was removed. Nuclei were rinsed with cold PBS and lysed with buffer containing 20mM HEPES (pH 7.5), 0.5M NaCl, 0.1mM EDTA, 5mM $MgCl_2$, 20% (v/v) glycerol, 1% (v/v) NP-40, 0.1mM Na_3VO_4, 1mM PMSF, and 2µg/mL aprotinin. Fractions were analyzed by Western blotting as described above. Primary antibodies used were HA.11 (1:1000), Grb2 (1:5000), and c-Jun.

Real Time PCR

Cells were transfected with Stat5 or non-targeting siRNA for 24h as described above, and treated -/+4nM PRL for 48hr. RNA was isolated using RNeasy Mini Kit (Qiagen, Valencia, CA) following the manufacturer's protocol. cDNA was synthesized using random hexamers (Amersham Biosciences, Piscataway, NJ) and M-MLV Reverse Transcriptase (Promega, Madison, WI) from 1µg RNA in a total volume of 250µL. Quantitative RT-PCR reactions were performed in a total volume of 25µL using 3µL cDNA, 1X concentration of SYBR Green Master Mix (Applied Biosystems, Foster City, CA), and primers for MMP-2 (forward 5'- GAG AAC CAA AGT CTG AAG AG-3'; reverse 5'-GGA GTG AGA ATG CTG ATT AG-3' T_a=60°C) or Stat5 (forward 5'-TCA CGC AGG ACA CAG AGA ATG-3'; reverse 5'-CTG TGC CTC ACG CTG CAA-3' T_a=60°C) at a final concentration of 600nM or primers for 18S RNA (forward 5'-CGC CGC TAG AGG TGA AAT TCT-3'; reverse 5'-CGA ACC TCC GAC TTT CGT TCT-3' T_a=60°C) at a final concentration of 240nM. Samples, in duplicate, were amplified using the

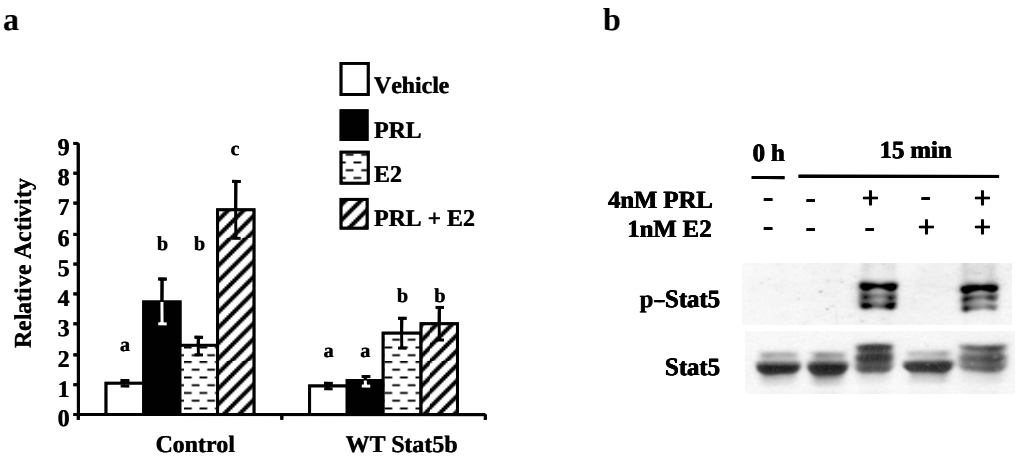
7300 Real-Time PCR System and analyzed with the Sequence Detection Software Version 1.3 (Applied Biosystems, Foster City, CA) and fold changes were calculated using the ΔΔCt formula as described in the 7300 Real-Time PCR literature, normalizing MMP-2 and Stat5 levels to 18S internal controls.

Invasion assays

Cells were transfected with non-targeting or Stat5-specific siRNA as above, and the ability to invade collagen I over 30h was assessed exactly as described (Keely, 2001).

Additional reference:

Dong Z, Xu RH, Kim J, Zhan SN, Ma WY, Colburn NH and Kung H. (1996). AP-1/jun is required for early Xenopus development and mediates mesoderm induction by fibroblast growth factor but not by activin. *J. Biol. Chem.* **271,** 9942-9946.



Supplemental Figure 1. Stat5 has no effect on E2-induced AP-1 activity. **(a)** MCF-7 cells were cotransfected with 4XAP-1-luc, lPRLR, β-gal, and either vector DNA or WT Stat5b. Following transfection, cells were treated -/+ 4nM PRL and/or 1nM E2 for 24 h. Luciferase activity was determined, and normalized as described in the Materials and Methods. Relative Activity indicates the mean of four independent experiments, shown as mean fold change relative to the vehicle treated control -/+ S.E.M. Different letters denote significant differences among groups ($P<0.05$), determined by one-way ANOVA followed by Mann-Whitney post-tests. **(b)** Cells were treated -/+ 4nM PRL and/or 1nM E2 for 15 min. Cell lysates were examined by Western analysis. Representative experiment.