Transcriptional silencing of the *ETS1* oncogene contributes to human granulocytic differentiation

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Supplementary Design and Methods

Cell surface marker detection by flow cytometry

Cellular differentiation was monitored evaluating PE-conjugated anti-CD11b, -CD11a, -Cd11c, -CD18, -CD54, CD15, CD34, or isotype control (BD-Pharmingen). Cells were then washed and analyzed on a FACSCanto (Becton Dickinson). Non-viable cells were excluded by the addition of 10 μg/mL 7-amino-actinomycin D (7-AAD) (Sigma Aldrich, St. Louis, MO, USA). Data were acquired and analyzed using FACS Diva software (Becton Dickinson).

Morphological analysis

Cells were smeared on glass slides by cytospin centrifugation, stained with May-Grünwald-Giemsa and analyzed at 400x or 630x magnification under a microscope equipped with a digital camera (Eclipse 1000, Nikon, Tokio, Japan).

Leukemic blasts

Leukemias were classified by morphological criteria according to the French-American-British (FAB) classification (i.e. 2 M0, 5 M1, 3 M2, 3 M4, 1 M5, 1 M6) and contained, in all cases, more than 80% leukemic blasts. Leukemic blasts stained with PE-labeled anti-CD34 antibody (BD-Pharmingen, San Diego, CA, USA) were sorted using a FACSAria (Becton Dickinson). Leukemic blasts isolated from an AML patient with t(15;17) translocation were treated with 10^{-6} M ATRA.

Analyses were performed after informed consent from the patients, obtained in accordance with the Declaration of Helsinki with ethical approval of the University Tor Vergata of Rome (Italy).

RNA extraction and reverse transcriptase polymerase chain reaction

RNA was reverse transcribed by Moloney murine Leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA, USA) with oligo(dT). A commercial ready-to-use Taqman primers/probe mix was used for Ets-1 (Hs00428287 Applied Biosystems, Foster City, CA, USA) and expression levels normalized to those of a human *GAPDH* endogenous control. p42 Ets-1 mRNA was detected by SYBR Green system using the following primers for p42: 5'-GAGGACCAGTCGTAGTGGAC-CAATCCAG-3', rev 5'-CCACCTCATCTGGGTCAGAAAG-3' and for GAPDH : for-5' ACCTGACCTGCCGTCTAGAAAA-3', rev 5'-CCT-GCTTCACCACCTTCTTGA-3'.

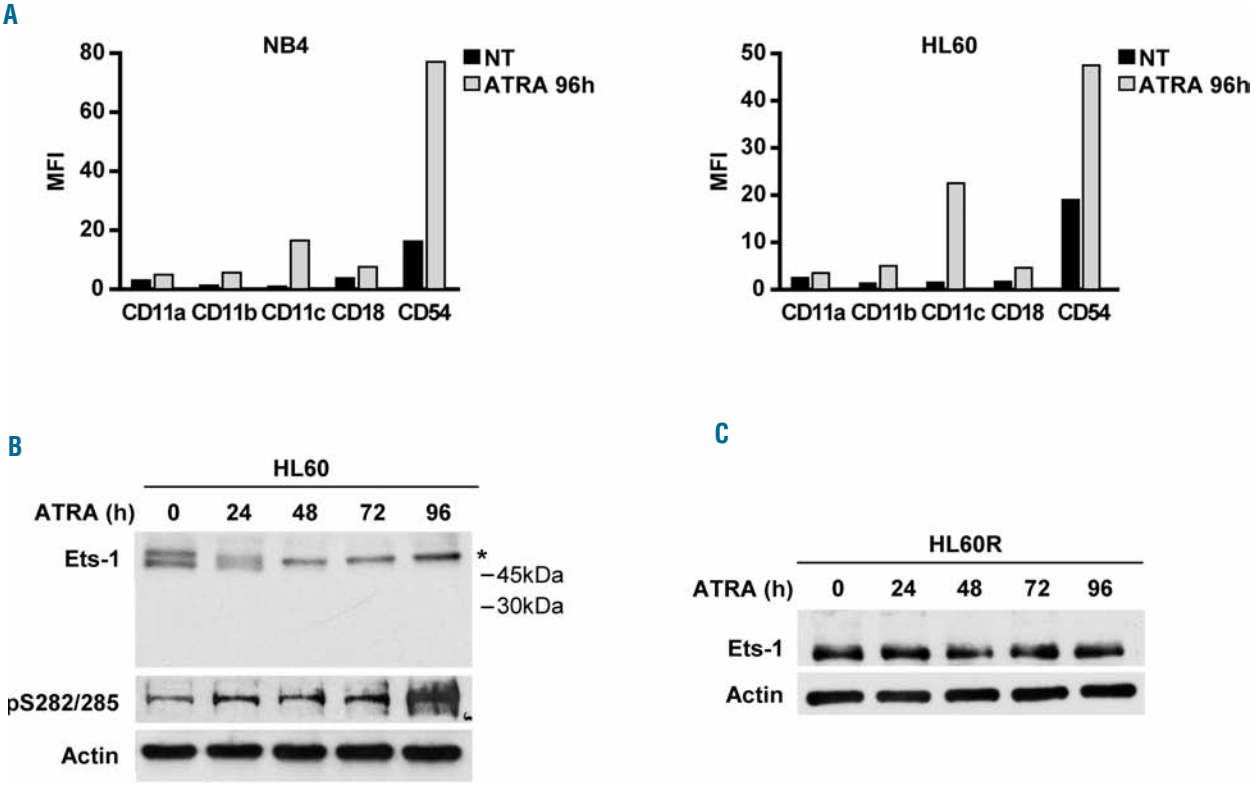
For semi-quantitative RT-PCR the reverse transcribed RNA was normalized to S26 values: amplification was achieved by 35 PCR cycles (94°C for 30 s, 58°C for 30 s, 72°C for 45 s). Samples were electrophoresed in 1.5% agarose gel, transferred onto Hybond-N filter (Amersham Biosciences, Little Chalfont, UK) and hybridized with an internal oligomer probe.

The sequences of the oligonucleotide primers and probes used for semi-quantitative RT-PCR were: Ets-1: for 5'-ACTACCCCTCGGT-CATTCTCCG-3', rev 5'-CCACCTCATCTGGGTCAGAAAG-3', probe 5'-CAGACAACATGTGCATGGG-3';

S26: for 5'-GCCTCCAAGATGACAAAG-3', rev 5'-CCAGA-GAATAGCCTGTCT-3', probe 5'-GAGCGTCTTCGATGCCTATGT-GCTTCCCAA-3'.

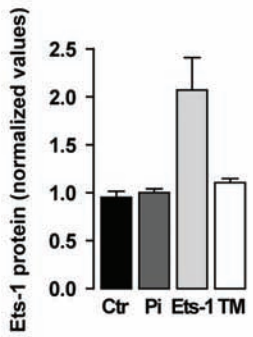
DNA electrophoretic mobility shift assay

The binding reaction (20 μL final volume) contained end-labeled double-stranded oligonucleotide probes (30,000 cpm) in binding buffer (75 mM KCl, 20 mM Tris [hydroxymethyl-aminomethane]-HCl pH 7.5, 1 mM DTT [dithiothreitol]), 1 μg bovine serum albumin, 14% (v/v) glycerol, 0.5 μg poly(dI)-poly(dC). Cell extracts (15 μg) were added and the reaction mixture was incubated for 20 min at room temperature. Samples were electrophoresed in a 5% polyacrylamide gel in 0.5 x Tris-borate/EDTA (TBE) buffer for 2 h at 200 V and 16°C. For supershift analysis, extracts were pre-incubated for 10 min at room temperature with anti-Ets-1 (C-20, Santa Cruz Biotechnology) antibody before the addition of the binding reaction. For competitions, a 200-fold molar excess of cold oligonucleotide was added to the reaction. The DNA sequence of the oligonucleotide used was: 5'- GATCTC-GAGCAGGAAGTTCGA -3' (Santa Cruz Biotechnology) and 5'-GATCTCGAGCAatctGTTCGA -3' for the mutated oligonucletide.



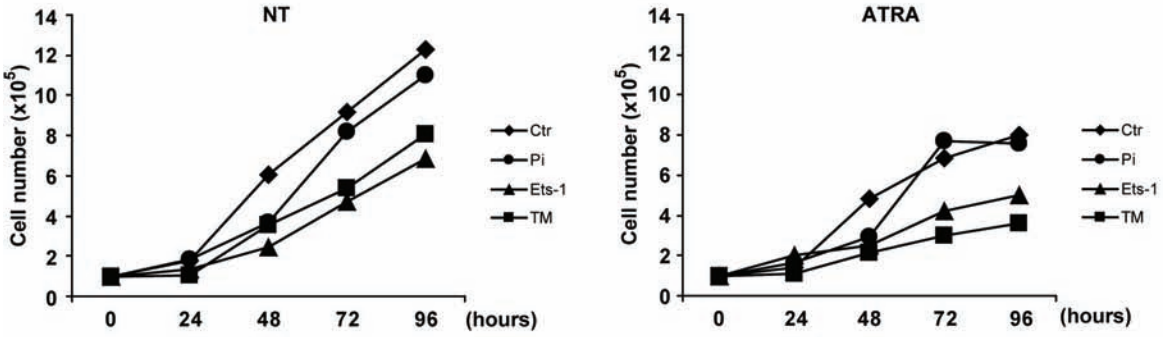
Online Supplementary Figure S1. ATRA-induced differentiation in NB4 and HL60 cells. Both cell lines were treated with differentiation-inducing doses of ATRA (10^{-6} M) and collected at the indicated time points. (A) FACS analysis of cell surface antigens, in untreated and 96 h ATRA-treated NB4 (left) and HL60 (right) cells. A representative experiment is shown. (B) ATRA reduced p51 Ets-1 protein expression in HL60 cells but not in their ATRA-resistant counterpart. Western blot analysis of whole cell extracts prepared from HL60 cells incubated with anti-Ets-1 or phospho-Ets-1 S282/285 antibodies. (C) Western blot analysis of Ets-1 expression in ATRA- treated HL60R cells.

A



Online Supplementary Figure S2. Enforced expression of full-length p51 Ets-1 protein and of a dominant negative mutant of Ets-1 in NB4 cells. (A) Actin-normalized Ets-1 protein values in NB4 transduced cells are reported. Mean ± SD values from three independent transduction experiments. (B) Growth curves of untreated and ATRA-treated NB4 transduced cells. (C) May-Grünwald-Giemsa staining analysis of NB4, NB4/Pi, NB4/Ets-1 or NB4/TM cells untreated and treated with ATRA for 96 h (original magnification 400x; original magnification 630x for ATRA-treated NB4/Pi and NB4/Ets-1 cells). A representative experiment out of three is presented.

B



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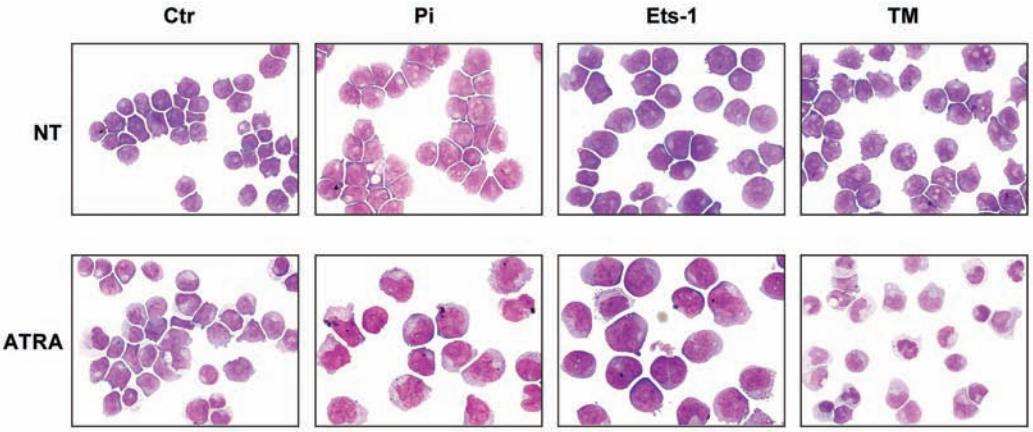

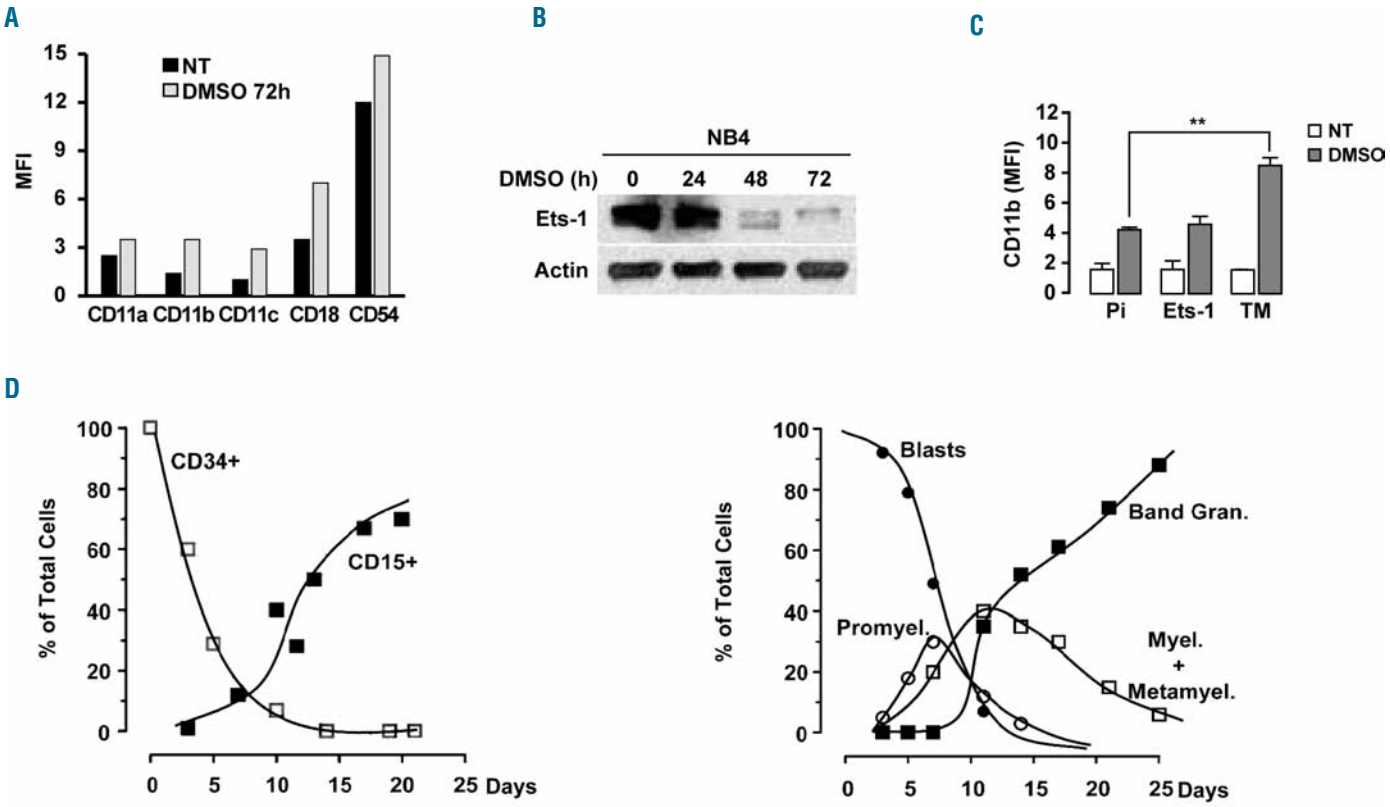

Online Supplementary Figure S3. Decreased expression of Ets-1 protein generalized to granulocytic differentiation. (A) FACS analysis of cellular surface antigens, in untreated and 72 h DMSO-treated NB4 cells. A representative experiment is shown. (B) NB4 cells, treated with 1.4% DMSO, were collected at the indicated time points for western blot analysis of Ets-1 expression. Actin was used as a loading control. (C) CD11b MFI in NB4/Pi, NB4/Ets-1 and NB4/TM cells analyzed 72 h after 1.4% DMSO treatment. The error bars represent mean ± SD (n=3). (D) Unilineage granulocytic cultures derived from $CD34^+$ hematopoietic progenitor cells. (left) Percentage of $CD15^+$ cells in unilineage granulocytic differentiation and maturation. (right) Schematic representation of May-Grünwald-Giemsa staining of unilineage granulocytic cultures at various differentiation stages. The percentages of blasts, differentiating and mature cells with respect to total cells are indicated. Data from representative experiments are presented.