Supplemental Methods

Materials. Anti-tescalcin antibody was described earlier (33). Monoclonal FITC-conjugated antibodies to the human CD41, CD49d, CD49e, as well as antibodies to Erk1/Erk2, phospho-Erk1/Erk2, Cyclin D3, αIIb integrin, β-actin, Ets-1 and Fli-1 were obtained commercially. Recombinant mouse TPO was from Peprotech, Inc. Bryostatin, bovine plasma fibronectin, hemin and propidium iodide were purchased from Sigma. The MEK-specific inhibitor U0126, Cell Proliferation, and Dual-Luciferase Reporter assays were obtained from Promega.

Western blot analysis. For Western blot analysis cells were harvested by centrifugation and washed twice with PBS. Cells were suspended in lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1mM EDTA, 1% Triton X-100, 2.5mM Sodium pyrophosphate, 1mM β-Glycerolphosphate, 1mM Sodium orthovanadate, and Complete© mini protease inhibitor cocktail [Roche]) on ice for 30 min and then centrifuged to remove cell debris. Protein concentration in lysates was determined with Bradford reagent (BioRad). Cell lysates were subjected to SDS-PAGE using 12% or 8% gels and transferred to Protran nitrocellulose membrane (Schleicher & Schuell) in 25 mM Sodium bicarbonate buffer. Transfer of proteins was assessed by ponceau-red staining. The membranes were blocked in TBST (150 mM NaCl, 50 mM Tris/HCl [pH 7.5], and 0.1% Tween 20) containing 5% fat-

free milk for 1 hour at room temperature. Incubations with primary antibodies were carried out at room temperature in TBST-milk. Following 1 hour of incubation with goat anti-rabbit-HRP antibody (Promega) at room temperature, proteins were detected by ECL+plus (Amersham) according to the manufacturer's instructions.

Induction of differentiation. To induce megakaryocytic or erythroid differentiation, cells were washed with PBS, resuspended in growth media containing PMA (10 nM, final) or hemin (30 μM, final), respectively, and cultured for 72 h. Megakaryocytic maturation was monitored by staining cells with FITC-labeled antibody to CD41 and by DNA ploidy analysis with propidium iodide. Induction of hemoglobin during erythroid differentiation was analyzed by benzidine staining of cells and examination under light microscope.

Flow cytometry. For the analysis of surface receptors, non-adherent cells were collected by centrifugation. Adherent cells were detached by incubation with 1 mM EDTA and 1 mM EGTA in PBS. Cells were washed two times in cold PBS with 0.1% of Sodium azide. $1x10^6$ of non-induced and PMA-induced cells were blocked for 30 min with 1% BSA in PBS and incubated with FITC-conjugated monoclonal antibodies to different surface receptors. After two more washes, cells were analyzed by flow cytometry. For the negative control, we incubated cells with FITC-conjugated matching isotype antibody. To analyze the DNA content, cells were collected and fixed with ice-cold 70% ethanol overnight at

4^0C. The DNA was stained with propidium iodide solution in the presence of RNAse A (Sigma). All flow cytometry experiments were performed using Becton Dickinson FACScan with a minimum of 10,000 events acquired per sample. CellQuest (Becton Dickinson) and WinList 3D (Verity) software packages were used for data analysis.

Plasmid construction. To obtain human tescalcin cDNA, a RT-PCR reaction was carried out using total RNA from K562 cells as the template along with forward (5'-CCATGGGCGCTGCCCACTCC-3') and antisense (5'-GTCCGCCGGGCCTGGGCTGC-3') primers. The PCR product was inserted into pBluescript II SK+ vector and sequenced. A coding sequence for tescalcin was then excised and subcloned into pcDNA3 expression vector to yield pTSC. For luciferase reporter plasmid, region -597 to +32 of the human αIIb promoter (64) was amplified by PCR using genomic DNA from K562 cells as template and primers. PCR product was subcloned into pGL3-Basic vector to yield pGPIIb-luc and verified by sequencing.

Adhesion assay. Plastic 96-wells plates (Corning) were coated overnight at 4^0C with fibronectin solution (10 μg/ml). On the next day wells were thoroughly rinsed with PBS and free binding sites were blocked with 3% heat-denatured BSA. Assayed cells were suspended in culture media with or without 10 nM PMA. Cells ($1x10^5$) were added in each well in triplicates and allowed to attach for 30 min at 37^0C. Non-adherent cells were removed by three PBS washes. The

number of attached cells was quantified using the CellTiter 96 Aqueous One Solution Assay (Promega) according to manufacturer's instruction. After color development, the absorbance in each well was recorded at 490 nm with a 96-well microplate reader EL800 (BIO-TEK). A reference reading at 630 nm was used to eliminate background contributed by cell debris and nonspecific absorbance. Results were compared to the standard curve build with known cell concentrations.

Cell proliferation assay. Exponentially growing K562-Ctrl(+), K562-Tsc(+), HEL-Ctrl(-), HEL-Tsc(-) cells were washed three times in PBS and resuspended at concentration of $1x10^5$ cells/ml in fresh RPMI with 1% FBS and 1 mg/ml of G418. Aliquots of cultures were taken at 24 hours intervals after initiation of experiment, and number of viable cells was calculated using the CellTiter 96 Aqueous One Solution Assay as described above.

Luciferase assay. For luciferase assay, cells were transiently transfected by electroporation and plated in 10cm culture dishes. Lysates were processed and assayed using the Dual-Luciferase Reporter assay system (Promega). Briefly, cells from each dish were collected, washed two times with PBS by centrifugation and lysed in 500 μl of passive lysis buffer. After clearing the lysates by centrifugation, 20 μl was mixed with 100 μl of firefly luciferase substrate, and luminescence was quantified on TD20/20 luminometer (Turner Designs). Renilla

luciferase activity used for normalization was determined by adding 100 ml of Stop-and-Glow solution and measurement of luminescence.

Supplemental Figure Legends

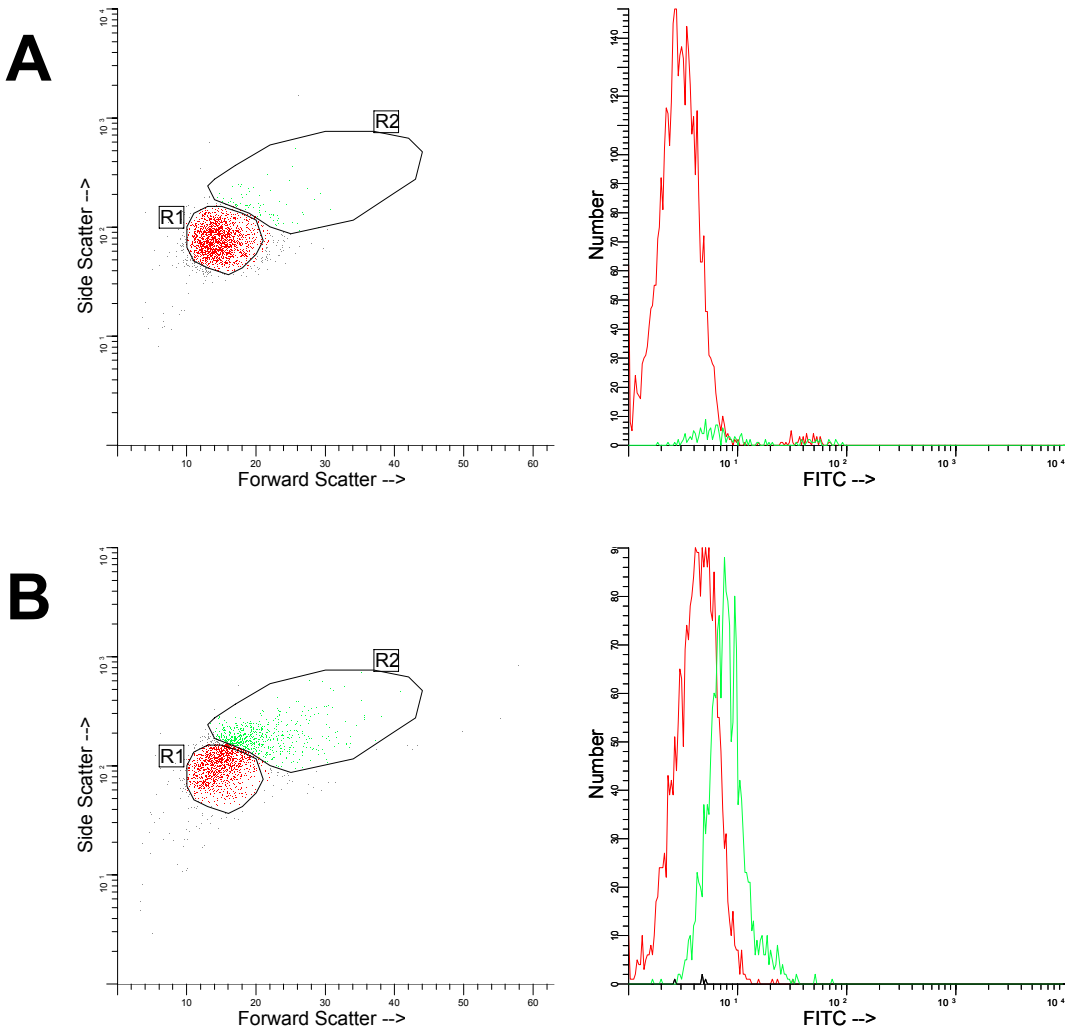
Figure S1. Overexpression of tescalcin in K562-Tsc(+) cells leads to an increased levels of GPIIb. (**A**) Control K562-Ctrl(+) and (**B**) tescalcin overexpressing K562-Tsc(+) cells were stained with FITC-conjugated CD41-specific antibody and analyzed by FACS. Forward scatter vs. side scatter plot shows a subpopulation of larger cells in K562-Tsc(+) that express higher levels of CD41. Expression profiles of surface CD41 for each gated population are shown as histogram plots (gate R1 – red; gate R2 – green).

Figure S2. Enlargement of HEL cells after transfection with tescalcin expression construct. To generate stable constitutive overexpression of tescalcin, HEL cells were transfected with pTSC plasmid as described in Methods. Placing transfectants under neomycin selection with 1.5 mg/ml of G418 promoted growth arrest, rapid increase in cell size and cell death within 3 weeks. Scale bar, 25 μm

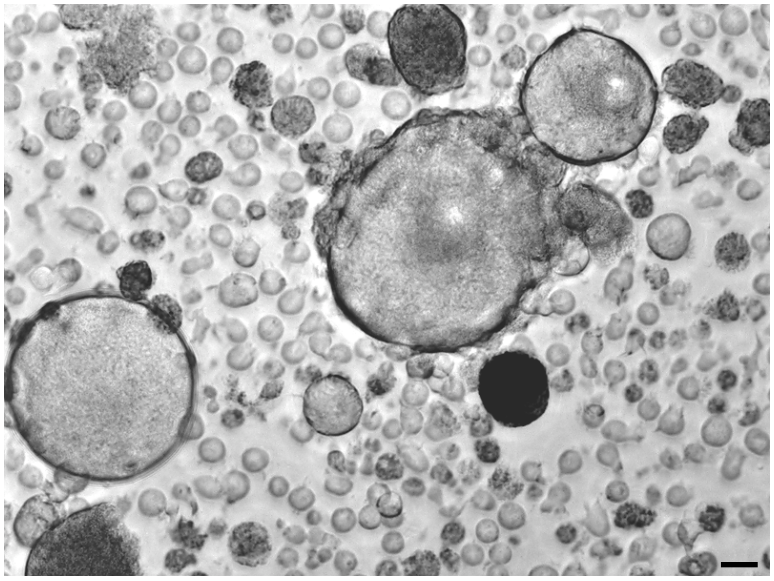
Figure S3. Tescalcin regulates transcription of GPIIb. Control Ctrl(-) and tescalcin knockdown Tsc(-) cells were transfected by electroporation with pGPIIb-luc (10 μg), pRL-null vector (5 μg), and carrier DNA (25 μg) as described in Methods. For the negative control, cells were transfected with empty pGL3

vector. After a 24 h recovery period, transfected cells were cultured in the absence or presence of 10 nM PMA for 72 h and harvested. Luciferase activities were determined as described in Methods. Data represent four independent experiments (mean±sd).

Levay and Slepak, Figure S1



Levay and Slepak, Figure S2



Levay and Slepak, Figure S3